

1           **Detection of norovirus, hepatitis A and hepatitis E viruses in**  
2                                   **multicomponent foodstuffs**

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20

21 **Abstract**

22 Among the enteric viruses implicated in foodborne outbreaks, the human norovirus and  
23 hepatitis viruses A and E (HAV and HEV) represent a serious public health concern.  
24 International standard ISO 15216 proposes methods for detecting HAV and norovirus  
25 (genogroups I and II) RNA from soft fruit, leaf, stem and bulb vegetables, bottled water or  
26 food surfaces. These methods had not previously been validated for detecting the targeted  
27 viruses in other foodstuffs such as multicomponent foods, nor for detecting other viruses in  
28 foodstuffs. The aim of this study was to characterise a method derived from the vegetable  
29 method described in ISO 15216 to detect HAV, HEV and norovirus in artificially-  
30 contaminated multicomponent foodstuffs according to the recent international standard ISO  
31 16140-4.

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

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

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## 47 1. Introduction

48 Viruses are a leading cause of foodborne disease worldwide. Human norovirus and hepatitis  
49 viruses (hepatitis A (HAV) and hepatitis E (HEV)) are recognised to be the main viruses of  
50 public health importance. Enteric viruses are primarily transmitted via the faecal-oral and  
51 vomit-oral routes, including direct person-to-person contact, consumption of contaminated  
52 food or water, contact with contaminated environmental surfaces (Kotwal and Cannon,  
53 2014; Matthews et al., 2012) and for hepatitis E virus direct contact with infected animals  
54 (Dalton et al., 2013; Meng, 2010; Pavio et al., 2006, 2010). Various food products, such as  
55 bivalve molluscs, fresh fruit and vegetables including different types of lettuce, onions or  
56 berries have been involved in foodborne disease outbreaks worldwide (Bernard et al., 2014;  
57 Donnan et al., 2012; Ethelberg et al., 2010; Fournet et al., 2012; Gallot et al., 2011; Herman  
58 et al., 2015; Le Guyader et al., 2010; Muller et al., 2016; Sarvikivi et al., 2012; Thebault et al.,  
59 2013; Wadl et al., 2010). A wide variety of foodstuffs have been implicated and mixed foods  
60 and ready to eat meals have frequently been considered as the carriers. In France, in  
61 addition to the high-risk food categories, meat, water, dishes with mixed foods or ready to  
62 eat meals have also been found to be responsible for 33% of foodborne illnesses and more  
63 specifically for 31% of viral foodborne disease outbreaks (*Santé publique France*, 2017). Virus  
64 outbreaks have been much more frequently reported in settings using catering services and  
65 in restaurants (*Santé publique France*, 2017). Food contamination mainly occurs in  
66 restaurants during food preparation by infected workers, and is most often associated with

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

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

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

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## 81 **2. Materials and methods**

### 82 ***2.1. Viruses and cells***

83

84 The FRhK-4 (foetal rhesus monkey kidney) cell line was purchased from the American Type  
85 Culture Collection (ATCC) (ATCC<sup>®</sup> CRL-1688<sup>™</sup>) (LGC standards SARL, Illkirch, France). These  
86 epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco<sup>™</sup>)  
87 supplemented with non-essential amino acids (NEAA, Gibco<sup>™</sup>) and 10% of heat-inactivated  
88 foetal bovine serum (FBS, Gibco<sup>™</sup>) (Thermo Fisher Scientific, Waltham, MA, 209 USA). Cells  
89 were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>.

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

97

98 A clarified HEV genotype 3e suspension was obtained from faecal samples of infected swine  
99 provided by the ANSES Maisons-Alfort Laboratory for Animal Health. The partial sequence of  
100 ORF2 had previously been deposited with GenBank (accession number JF718793). The faecal  
101 sample was suspended in 10 mM Phosphate Buffered Saline (PBS), pH 7.4, to obtain a final  
102 10% suspension (w/v), vortexed and centrifuged at 4000 g for 20 min at 4°C. Aliquots of  
103 supernatants containing viral particles were then stored at -80°C. The number of HEV RNA  
104 copies in the faecal suspension was quantified by using the standard curve obtained with  
105 the quantified *in vitro* RNA transcripts as previously described (Martin-Latil et al., 2012). The  
106 clarified suspension stock of HEV had a titre of  $1.40 \times 10^7$  genome copies/mL.

107

108 Stool samples of norovirus GI (E8050) and norovirus GII (E7022) from infected humans were  
109 provided by the French National Reference Center for Gastroenteritis Viruses in Dijon,  
110 France. The faecal samples were suspended in 10 mM **phosphate-buffered saline** (PBS), pH  
111 7.4 to obtain a final 10% suspension (w/v), and then vortexed and centrifuged at 4,000 x g  
112 for 20 min at 4°C. Aliquots of 100 µL were frozen and kept at -80°C for later use. The  
113 genomic titres of the clarified faecal suspensions were determined by **quantitative real-time**

114 RT-PCR using a quantitative real-time RT-PCR standard curve obtained with the 10-fold  
115 diluted *in vitro* RNA transcripts as previously described (Hennechart-Collette et al., 2014).  
116 The clarified suspension stocks of norovirus GI and norovirus GII had titres of approximately  
117  $1.60 \times 10^7$  and  $1.30 \times 10^7$  genome copies/mL respectively.

118  
119 The murine norovirus MNV-1 (CW1 strain) was provided to the ANSES Fougères Laboratory  
120 (Fougères, France) by Dr. H. Virgin from Washington University (Saint Louis, MO, USA), and  
121 was propagated in a mouse leukaemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell  
122 line (Cannon et al., 2006). RAW 264.7 was grown at 37°C in an atmosphere containing 5%  
123 CO<sub>2</sub> in DMEM supplemented with GlutaMAX™, 1% non-essential amino acids and 10% foetal  
124 bovine serum (Life Technologies, Saint Aubin, France). The production stock of MNV-1 had  
125 titres of approximately  $2.15 \times 10^7$  TCID<sub>50</sub>/mL.

126

## 127 **2.2. Experimental design and artificial contamination of multicomponent foodstuffs**

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

135 The experimental design described in ISO 16140-4:2017 was used. Multicomponent foods  
136 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  $2.90 \times 10^3$  to  
139  $2.90 \times 10^6$  genome copies for HAV,  $1.40 \times 10^2$  to  $1.40 \times 10^5$  genome copies for HEV,  $1.60 \times 10^3$  to  
140  $1.60 \times 10^6$  genome copies for norovirus GI and  $1.30 \times 10^3$  to  $1.30 \times 10^6$  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  $2.15 \times 10^4$  TCID<sub>50</sub> 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

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

171

#### 172 **2.4. Viral RNA extraction**

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

178

#### 179 **2.5. Primers and probes**

180 The primers and probes used to quantify HAV, norovirus GI and norovirus GII have already  
181 been described in the literature (Costafreda et al., 2006; da Silva et al., 2007; Kageyama et  
182 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

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

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

247 The accuracy profile is calculated by a two-sided  $\beta$  expectation tolerance interval ( $\beta$ -ETI) for  
248 each level from the results of the validation experiments expressed, such as recovery  
249 (trueness) and precision (repeatability and intermediate precision). Data are  $\log_{10}$   
250 transformed and we defined the acceptability limits ( $\lambda$ ) at  $\pm 95$  % of bias because the  
251 expected recovery is low. To calculate the accuracy profile a specified proportion has been  
252 defined. This proportion is fixed to 80% ( $\beta$ ) 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<sub>10</sub> concentration level (genome copies/g) and the vertical  
260 (y) axis shows at each level the bias, the acceptability limits ( $\lambda$ ), and the  $\beta$ -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

276 recoveries with pure RNA extracts ranged from 0.35% to 40.44% for HAV, from 5.19% to  
277 100% for HEV, from 0.1% to 40.61% for norovirus GI and 0.88% to 69.16% for norovirus GII.

278 The mean recovery rates obtained for HAV, HEV, norovirus GI and norovirus GII showed that  
279 the recovery rates from composite matrices vary according to the virus inoculated (one-way  
280 ANOVA; p-value<0.001) (Figure 2). More specifically, the multiple comparison tests showed  
281 that the recovery rates of HEV were higher and significantly different from other viruses.

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

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

287 The limits of detection (LOD) for the method were calculated by the Wilrich approach for  
288 each virus in each setting with the twelve samples. The LOD<sub>50</sub> and LOD<sub>95</sub> values for HAV,  
289 HEV, norovirus GI and norovirus GII are shown in Table 4.

290 The LOD<sub>95</sub> values were 10<sup>2</sup> genome copies/g for HAV, HEV and norovirus GII and 10<sup>3</sup> genome  
291 copies/g for norovirus GI. The LOD<sub>50</sub> values were between 80 and 92 genome copies/g for  
292 HAV, HEV and norovirus GII and 360 genome copies/g for norovirus GI.

293 The limit of quantification (LOQ) was assessed for different viruses using the accuracy  
294 profile. Figure 3 shows a typical accuracy profile for HAV. Table 5 summarizes the results for  
295 the LOQ and LOD and some performance characteristics (repeatability and intermediate  
296 precision). The LOQ was 2.90x10<sup>4</sup>, 1.40x10<sup>3</sup>, 1.60x10<sup>4</sup>, 1.30x10<sup>4</sup> genome copies/g for HAV,  
297 HEV, norovirus GI and norovirus GII respectively. The LOD for HAV, HEV, norovirus GI and  
298 norovirus GII was established at 0.97x10<sup>4</sup>, 0.47x10<sup>4</sup>, 0.53x10<sup>4</sup> and 0.43x10<sup>4</sup> genome copies/g  
299 respectively.

300

### 301 **3.3 Influence of experimental factors on virus extraction yield**

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

310 More specifically, the multiple-comparison tests showed that inoculations with  $2.90 \times 10^3$   
311 HAV genome copies,  $1.40 \times 10^2$  HEV genome copies and  $1.6 \times 10^3$  norovirus GI genome copies  
312 differed significantly from other **inoculation** levels of HAV, HEV and norovirus GI.

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

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

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

### 326 ***3.4 Recovery rates for external amplification control (EAC)***

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

333

## 334 **4. Discussion**

335 Enteric viruses are the leading cause of foodborne outbreaks. Their detection is challenging  
336 because many of them are either difficult or impossible **to replicate in cell culture**, and the  
337 number of viral particles present in the food may be very low. Today, **quantitative real-time**  
338 **RT-PCR** is widely used for virus detection because it is sensitive, specific, rapid and can  
339 deliver quantitative data. ISO standards 15216-1 and 15216-2 were published in 2017 and  
340 2019 respectively for detecting and quantifying norovirus and HAV in high-risk food  
341 categories such as shellfish, bottled water and vegetables. The method used in this study to  
342 recover viruses from composite foods is adapted from the ISO 15216 procedure described  
343 for vegetables. The LOD<sub>95</sub> varied from 10<sup>2</sup> to 10<sup>3</sup> genome copies per g regardless of the virus  
344 analysed. Because of the complexity of the food matrices used in our study, the LOD<sub>95</sub> values  
345 obtained for norovirus and HAV in composite foods and multicomponent foodstuffs were  
346 higher than the LOD<sub>95</sub> recently reported for lettuce or raspberries (Lowther et al., 2019).

347 Derived from the LOQ estimated by accuracy profile, the LOD in our study ranged between  
348  $4.33 \times 10^3$  and  $9.67 \times 10^3$  genome copies per g. These values are slightly different than the  
349 estimated LOD<sub>95</sub> but the difference, which is around one log, can be explained by the  
350 definition of these parameters. LOD<sub>95</sub> is the level at which the probability of detection is  
351 equal to 95 % (ISO standard 16140-1), whereas the LOD is estimated using a quantitative  
352 approach based on a chemical method. Classically, the LOD is the lowest level that differs  
353 from the background noise with an accepted confidence level. Armbruster et al. (2008) have  
354 previously explained that the LOD is a value as mean + k SD with k, the coverage factor,  
355 ranged between 2 and 10. The common value for k is 3 for LOD and if k = 10 it is the LOQ.  
356 However, the LOQ is the lowest level quantifiable with a defined trueness and precision (ISO  
357 standard 16140-1). In our case, we defined the LOQ using an accuracy profile with a defined  
358 trueness and precision, then derived the LOD from this LOQ. These two approaches are  
359 totally different because one is for a qualitative approach and the other for a quantitative  
360 approach. The results are nonetheless of the same magnitude and consequently the LOD  
361 confirms the LOD<sub>95</sub> values.

362

363 Various methods have been described for extracting viruses from vegetables, composite  
364 foods or dairy products (Baert et al., 2008; Blaise-Boisseau et al., 2010; Cheong et al., 2009;  
365 Coudray et al., 2013; Dubois et al., 2002; El Sanousy et al., 2013; Hennechart et al., 2017;  
366 Hida et al., 2013; Hyeon et al., 2011; Kim et al., 2008; Morillo et al., 2012; Pan et al., 2012;  
367 Sanchez et al., 2012; Scherer et al., 2010; Schwab et al., 2000; Stals et al., 2011a, 2011b).

368 The PEG concentration method was used in our study to concentrate viruses from  
369 multicomponent foods. Our results are in agreement with data reported in other studies on  
370 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 (*Crassostrea*  
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,

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

400 To calculate repeatability, foods were randomly allocated to four different settings (R1, R2,  
401 R3 and R4) and results show that the differences between repeated experiments (R1 to R4)  
402 were significant for the extraction rate of norovirus GII, HAV and HEV. Different studies have  
403 shown that the composition of food products can affect virus extraction (Blaise-Boisseau et  
404 al., 2010; Butot et al., 2007; Summa et al., 2012; Yavarmanesh et al., 2013, 2010). **Due to the**  
405 **presence of substances that can inhibit PCR amplification, the implementation of different**  
406 **controls such as the virus process control and EAC are necessary to validate results.**  
407 **According to the recommendations in ISO 15216, the inhibition rates for RNA extracted from**  
408 **food samples have to be lower than 75% and virus process control extraction yields higher**  
409 **than 1%.** In this study, the mean percentages of **quantitative real-time RT-PCR** inhibition  
410 were always lower than 75% and the recovery rate of MNV-1 was more than 1% in 93% of  
411 the RNA extracts analysed. In our study, MNV-1 was successfully tested as a process control  
412 virus for detecting HAV, HEV and norovirus in multicomponent foodstuffs. Its use had  
413 previously been described in the literature for different types of water, shellfish, milk  
414 products, meat such as sausages, soft red fruits, lettuces and ready-to-eat foods (Coudray et  
415 al., 2013; Hennechart et al., 2015; Martin-Latil et al., 2014; Sanchez et al., 2012; Stals et al.,  
416 2011a, 2011b).

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

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

423

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

428

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436

#### 437 **References**

438 Anonymous, 2016: NF EN ISO 16140-1: 2016: Microbiology of the food chain-Method validation-Part  
439 1: Vocabulary. International Organization for Standardization, Geneva.

440 Anonymous, 2018 : PR ISO/DIS 16140-4: 2018-01: Microbiology of the food chain-Method validation-  
441 Part 4 : Protocol for single-laboratory (in-house) method validation. International  
442 Organization for Standardization, Geneva.

443 Anonymous, 2017 EN ISO 15216-1:2017. Microbiology of the food chain—Horizontal method for  
444 determination of hepatitis A virus and norovirus using real-time RT-PCR- Part1: Method for  
445 quantification. International Organization for Standardization, Geneva.

446 Anonymous, 2019 EN ISO 15216-2:2019. Microbiology of the food chain-Horizontal method for  
447 determination of hepatitis A virus and norovirus using real-time RT-PCR- Part2: Method for  
448 detection. International Organization for Standardization, Geneva.

449 Armbruster, D.A., Pry, T. 2008. Limit of blank, limit of detection and limit of quantitation. *Clin.*  
450 *Biochem. Rev.* 29, Suppl (i) S49-S52.

451 Baert, L., Uyttendaele, M., Debevere, J. 2008. Evaluation of viral extraction methods on a broad  
452 range of ready-to-eat foods with conventional and real-time RT-PCR for Norovirus GII  
453 detection. *Int. J. Food Microbiol.* 123, 101-108.

454 Barrabeig, I., Rovira, A., Buesa, J., Bartolome, R., Pinto, R., Pallezo, H., Dominguez, A., 2010.  
455 Foodborne norovirus outbreak: The role of an asymptomatic food handler. *BMC Infect. Dis.*  
456 10, 269.

457 Bernard, H., Faber, M., Wilking, H., Haller, S., Hohle, M., Schielke, A., Ducomble, T., Siffczyk, C.,  
458 Merbecks, S.S., Fricke, G., Hamouda, O., Stark, K., Werber, D., Outbreak Investigation Team,  
459 2014. Large multistate outbreak of norovirus gastroenteritis associated with frozen  
460 strawberries, Germany, 2012. *Euro Surveill.* 19, 20719–20727.

461 Blaise-Boisseau, S., Hennechart-Collette, C., Guillier, L., Perelle, S., 2010. Duplex real-time qRT-PCR  
462 for the detection of hepatitis A virus in water and raspberries using the MS2 bacteriophage  
463 as a process control. *J. Virol. Methods* 166, 48-53.

464 Boubetra, A., Le Nestour, F., Allaert, C., Feinberg, M., 2011. Analysis of drinking water and their  
465 validation of alternative methods for their application to *Escherichia coli*. *Appl. Environ.*  
466 *Microbiol.* 77, 3360–3367.

467 Butot, S., Putallaz, T., Sanchez, G., 2007. Procedure for rapid concentration and detection of enteric  
468 viruses from berries and vegetables. *Appl. Environ. Microbiol.* 73, 186-92.

469 Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., Vinje, J., 2006. Surrogates for the  
470 study of norovirus stability and inactivation in the environment: comparison of murine  
471 norovirus and feline calicivirus. *J. Food Prot.* 69, 2761-2765.

472 Cheng, D., Zou, S., Liao, N., Shi, X., Chen, J., Zhang, Y., Sun, L., Zhang, R., 2017. Evaluation of an  
473 extraction method for the detection of GI and GII Noroviruses in fruit and vegetable salads.  
474 *Food Sci.* 83, 393-400.

475 Cheong, S., Lee, C., Choi, W.C., Lee, C.H., Kim, S.J., 2009. Concentration method for the detection of  
476 enteric viruses from large volumes of foods. *J. Food Prot.* 72, 2001–2005.

477 Costafreda, M. I., Bosch, A., Pinto, R. M., 2006. Development, evaluation, and standardization of a  
478 real-time taqman reverse transcription-Pcr assay for quantification of Hepatitis A virus in  
479 clinical and shellfish samples. *Appl. Environ. Microbiol.* 72, 3846-55.

480 Coudray, C., Merle, G., Martin-Latil, S., Guillier, L., Perelle, S., 2013. Comparison of two extraction  
481 methods for the detection of hepatitis A virus in lettuces using the murine norovirus as a  
482 process control. *J. Virol. Methods*, 193, 96-102.

483 Cromeans, T., Sobsey, M.D., Fields, H.A., 1987. Development of a plaque assay for a cytopathic,  
484 rapidly replicating isolate of hepatitis A virus. *J. Med. Virol.* 22, 45-56.

485 Dalton, H.R., Hunter, J.G., Bendall, R.P. 2013. Hepatitis E. *Current opinion in infectious diseases*, 26,  
486 471-478.

487 da Silva, A.K., Le Saux, J.C., Parnaudeau, S., Pommepuy, M., Elimelech, M., Le Guyader, F.S., 2007.  
488 Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse  
489 transcription-PCR: different behaviors of genogroups I and II. *Appl. Environ. Microbiol.* 73,  
490 7891-7897.

491 Donnan, E.J., Fielding, J.E., Gregory J.E., Lalor, K., Rowe, S., Goldsmith, P., Antoniou, M., Fullerton,  
492 K.E., Knope, K., Copland, J.G., Bowden, D.S., Tracy, S.L., Hogg, G.G., Tan, A., Adamopoulos, J.,  
493 Gaston, J., Vally, H., 2012. A multistate outbreak of hepatitis A associated with semidried  
494 tomatoes in Australia, 2009. *Clin. Infect. Dis.* 54.

495 Dubois, E., Agier, C., Traore, O., Hennechart, C., Merle, G., Cruciere, C., Laveran, H. 2002. Modified  
496 concentration method for the detection of enteric viruses on fruits and vegetables by reverse  
497 transcriptase-polymerase chain reaction or cell culture. *J. Food Prot.* 65, 12, 1962-19699.

498 El-Senousy, W.M., Costafreda, M.I., Pintó, R.M., Bosch, A. 2013. Method validation for norovirus  
499 detection in naturally contaminated irrigation water and fresh produce. *Int.J. Food Microbiol.*  
500 167, 74-79.

501 Ethelberg, S., Lisby, M., Bottiger, B., Schultz, A.C., Villif, A., Jensen, T., Olsen, K.E., Scheutz, F., Kjelse,  
502 C., Muller, L., 2010. Outbreaks of gastroenteritis linked to lettuce, Denmark, January 2010.  
503 *Euro Surveill.* Feb 11, 15, 6.

504 Feinberg, M., Sohler, D., David, J.-F., 2009. Validation of an alternative method for counting  
505 enterobacteriaceae in foods based on accuracy profile. *J. AOAC Int.* 92,527–537.

506 Fournet, N., Baas, D., van Pelt, W., Swaan, C., Ober, H., Isken, L., Cremer, J., Friesema, I., Vennema,  
507 H., Boxman, I., Koopmans, M., Verhoef, L., 2012. Another possible foodborne outbreak of  
508 hepatitis A in the Netherlands indicated by two closely related molecular sequences, July to  
509 October 2011. *Euro Surveill.* 17, 20079–20081.

510 Fraise, A., Coudray-Meunier, C., Martin-Latil, S., Hennechart-Collette, C., Delannoy, S., Fach, P.,  
511 Perelle, S., 2017. Digital RT-PCR method for hepatitis A virus and norovirus quantification in  
512 soft berries. *Int. J. Food Microbiol.* 21, 243, 36-45.

513 Franck, K.T., Lisby, M., Fonager, J., Schultz, A.C., Bottiger, B., Villif, A., Absalonsen, H., Ethelberg, S.  
514 2015. Sources of Calicivirus contamination in foodborne outbreaks in Denmark, 2005-2011-  
515 the role of the asymptomatic food handler. *J. I. Dis.* 211, 563-570.

516 Gallot, C., Grout, L., Roque-Afonso, A.M., Couturier, E., Carrillo-Santistevé, P., Pouey, J., Letort, M.J.,  
517 Hoppe, S., Capdepon, P., Saint-Martin, S., De Valk, H., Vaillant, V., 2011. Hepatitis A  
518 associated with semidried tomatoes, France, 2010. *Emerg. Infect. Dis.* 17, 566-567.

519 Hamza, I.A., Jurzik, L., Uberla, K., Wilhelm, M. (2011). Methods to detect infectious human enteric  
520 viruses in environmental water samples. *Int. J. Hyg. Environ. Health* 214, 424-436.

521 Hennechart-Collette, C., Niveau F., Martin-Latil S., Fraisse A., Perelle S., 2019. Development of an  
522 extraction method to detect enteric viruses in dressed vegetables. *Int. J. Food Microbiol.*  
523 311:108349.

524 Hennechart-Collette, C., Martin-Latil, S., Fraisse, A., Perelle, S., 2017. Comparison of three extraction  
525 methods to detect noroviruses in dairy products. *Food Microbiol.* 61, 113-119.

526 Hennechart-Collette, C., Martin-Latil, S., Guillier, L., Perelle, S., 2015. Determination of which virus to  
527 use as a process control when testing for the presence of hepatitis A virus and norovirus in  
528 food and water. *Int. J. Food Microbiol.* 202, 57-65.

529 Hennechart-Collette, C., Martin-Latil, S., Guillier, L., Perelle, S., 2014. Multiplex real-time RT-qPCR for  
530 the detection of Norovirus in bottled and tap water using murine norovirus as a process  
531 control. *J. Appl. Microbiol.* 116, 179-190.

532 Herman, K.M., Hall, A.J., Gould, L.H., 2015. Outbreaks attributed to fresh leafy vegetables, United  
533 States, 1973-2012. *Epidemiol. Infect.* 143, 14, 3011-3021.

534 Hida, K., Kulka, M., Papafragkou, E., 2013. Development of a rapid total nucleic acid extraction  
535 method for the isolation of hepatitis A virus from fresh produce. *Int. J. Food Microbiol.*, 161,  
536 143-150.

537 Hubert, P., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., Compagnon, P.A.,  
538 Dewé, W., Feinberg, M., Lallier, M., Laurentie, M., Mercier, N., Muzard, G., Nivet, C., Valat,  
539 L., & Rozet, E., 2007a. Harmonization of strategies for the validation of quantitative analytical  
540 procedures a SFSTP proposal – Part II. *J. Pharm. Biomed. Anal.* 45, 70–81.

541 Hubert, P., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Cohen, N., Compagnon, P.A., Dewé, W.,  
542 Feinberg, M., Laurentie, M., Mercier, N., Muzard, G., Valat, L., & Rozet, E., 2007b,  
543 Harmonization of strategies for the validation of quantitative analytical procedures a SFSTP proposal –  
544 Part III 2007. *J. Pharm. Biomed. Anal.* 45, 82–96.

545 Hubert, P., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Cohen, N., Compagnon, P.A., Dewé, W.,  
546 Feinberg, M., Laurentie, M., Mercier, N., Muzard, G., Valat, L., & Rozet, E., 2008.

547 Harmonization of strategies for the validation of quantitative analytical procedures: A SFSTP  
548 proposal. Part IV. Examples of application . J. Pharm. Biomed. Anal. 48, 760–771.

549 Hyeon, J.Y., Chon, J.W., Park, C., Lee, J.B., Choi, I.S., Kim, M.S., Seo, K.H., 2011. Rapid detection  
550 method for hepatitis A virus from lettuce by a combination of filtration and integrated cell  
551 culture-real-time reverse transcription PCR. J. Food Prot. 74, 1756-1761.

552 Kim, H.Y., Kwak, I.S., Hwang, I.G., Ko, G., 2008. Optimization of methods for detecting norovirus on  
553 various fruit. Journal of virological methods, 153, 104-110. Jothikumar, N., Lowther, J.A.,  
554 Henshilwood, K., Lees, D.N., Hill, V.R., Vinje, J., 2005. Rapid and sensitive detection of  
555 noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and  
556 application to naturally contaminated shellfish samples. Appl. Environ. Microbiol. 71, 1870-  
557 1875.

558 Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N., Katayama,  
559 K., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-  
560 time quantitative reverse transcription-PCR. J. Clin. Microbiol. 41, 1548-1557.

561 Kotwal, G. and Cannon, J.L., 2014. Environmental persistence and transfer of enteric viruses. Curr.  
562 Opin .Virol. 4, 37-43.

563 Le Guyader, F.S., Krol, J., Ambert-Balay, K., Ruvoen-Clouet, N., Desaubliaux, B., Parnaudeau, S., Le  
564 Saux, J.C., Ponge, A., Pothier, P., Atmar, R.L., Le Pendu, J., 2010. Comprehensive analysis of a  
565 norovirus-associated gastroenteritis outbreak, from the environment to the consumer. J.  
566 Clin. Microbiol. 48, 915–920.

567 Loisy, F., Atmar, R.L., Guillon, P., Le Cann, P., Pommepuy, M., Le Guyader, F.S., 2005. Real-time RT-  
568 PCR for norovirus screening in shellfish. J. Methods. 123, 1-7.

569 Lowther, J.A, Bosch, A., Butot, S., Ollivier, J., Mäde, D., Rutjes, S.A., Hardouin, G., Lombard, B., in't  
570 Veld, P., Leclercq, A., 2017. Validation of ISO 15216 part 1 –Quantification of hepatitis A virus  
571 and norovirus in food matrices. Int. J. Food Microbiol. 288, 82-90.



572 Martin-Latil, S., Hennechart-Collette, C., Guillier, L., Perelle, S., 2014. Method for HEV detection in  
573 raw pig liver products and its implementation for naturally contaminated food. *International*  
574 *J. Food Microbiol.* 176, 1-8.

575 Martin-Latil, S., Hennechart-Collette, C., Guillier, L., Perelle, S., 2012a. Comparison of two extraction  
576 methods for the detection of hepatitis A virus in semi-dried tomatoes and murine norovirus  
577 as a process control by duplex RT-qPCR. *Food Microbiol.* 31, 246-253.

578 Martin-Latil, S., Hennechart-Collette, C., Guillier, L., Perelle, S., 2012b. Duplex RT-qPCR for the  
579 detection of hepatitis E virus in water, using a process control. *International J. Food*  
580 *Microbiol.* 157, 167-173.

581 Matthews, J.E., Dickey, B.W., Miller, R.D., Felzer, J.R., Dawson, B.P., Lee, A.S., Rocks, J.J., Kiel, J.,  
582 Montes, J.S., Moe, C.L., et al., 2012. The epidemiology of published norovirus outbreaks: a  
583 review of risk factors associated with attack rate and genogroup. *Epidemiol Infect.* 140, 1161-  
584 1172.

585 Meng, X.J., 2010. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vete. Microbiol.* 140, 256-265.

586 Morillo, S.G., Luchs, A., Cilli, A., do Carmo Sampaio Tavares Timenetsky, M., 2012. Rapid detection of  
587 norovirus in naturally contaminated food: foodborne gastroenteritis outbreak on a cruise  
588 ship in Brazil, 2010. *Food Environ. Virol.* 4, 3, 124-129.

589 Müller, L., Rasmussen, L.D., Jensen, T., Schultz, A.C., Kjelsø, C., Barnadas, C., Sigsgaard, K., Larsen,  
590 A.R., Widstrup Jensen, C., Jeppesen, S., Uhrbrand, K., Hove, N., Mølbak, K., Ethelberg, S.,  
591 2016. Series of norovirus outbreaks caused by consumption of green coral lettuce, Denmark,  
592 April 2016. *PLoS Curr.* 4, 8.

593 Lemon, S.M., Murphy, P.C., Shields, P.A., Ping, L.H., Feinstone, S.M., Cromeans, T., Jansen, R.W.,  
594 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during  
595 persistent infection: evidence for genetic recombination. *J. Virol.* 65, 2056e2065.

596 Pan, L., Zhang, Q., Li, X., Tian, P., 2012. Detection of human norovirus in cherry tomatoes, blueberries  
597 and vegetable salad by using a receptor-binding capture and magnetic sequestration  
598 (RBCMS) method. *Food Microbiol.* 30, 420-426.

599 Pavio, N., Lunazzi, A., Barnaud, E., Bouquet, J., Rogée, S., 2010. Hépatite E: nouvelles connaissances.  
600 *Bull.Epid. N°38/Sécial zoonoses.*

601 Pavio, N., Renou, C., Boutrouille, A., Eloit, M., 2006. L'hépatite E : une zoonose méconnue. *Viol.* 10,  
602 341-351.

603 Pinto, R.M., Costafreda, M.I., Bosch, A., 2009. Risk assessment in shellfish-borne outbreaks of  
604 hepatitis A. *Appl. Environ. Microbiol.* 75, 7350-7355.

605 Rönqvist, M., Aho, E., Mikkela, A., Ranta, J., Tuominen, P., Ratto, M., Maunula, L., 2014. Norovirus  
606 transmission between hands, gloves, utensils, and fresh produce during simulated food  
607 handling. *Appl. Environ. Microbiol.* 80, 5403-5410.

608 Rutjes, S.A., van den Berg, H.H., Lodder, W.J., de Roda Husman, A.M., 2006. Real-time detection of  
609 noroviruses in surface water by use of a broadly reactive nucleic acid sequence-based  
610 amplification assay. *Appl. Environ. Microbiol.* 72, 8, 5349-5358.

611 Saito, H, Toho, M, Tanaka, T, Noda, M., 2015. Development of a practical method to detect  
612 Noroviruses contamination in composite meals food. *Environ. Virol.* 7, 3, 239-248.

613 Saint-Cyr, M.J., Perrin-Guyomard, A., Houée, P., Vasseur, M.V., Laurentie M., 2014. Use of accuracy  
614 profile procedure to validate a real-time PCR method to quantify bacteria in feces. *JAOAC*  
615 *Int.* 97, 2, 1-7.

616 Sanchez, G., Elizaquivel, P., Aznar, R., 2012. A single method for recovery and concentration of  
617 enteric viruses and bacteria from fresh-cut vegetables. *Int. J. Food Microbiol.* 152, 9-13.

618 Santé publique France, 2017. Données relatives aux toxi-infections alimentaires collectives déclarées  
619 en France en 2017 (Data on collective food-borne outbreaks reported in France in 2017).  
620 Santé Publique France. Available from: <https://www.santepubliquefrance.fr/maladies-et->

621 traumatismes/maladies-infectieuses-d-origine-alimentaire/toxi-infections-alimentaires-  
622 collectives/documents/bulletin-national/donnees-relatives-aux-toxi-infections-alimentaires-  
623 collectives-declarees-en-france-en-2017.

624 Sarvikivi, E., Roivainen, M., Maunula, L., Niskanen, T., Korhonen, T., Lappalainen, M., Kuusi, M., 2012.  
625 Multiple norovirus outbreaks linked to imported frozen raspberries. *Epidemiol. Infect.* 140,  
626 260–267.

627 Scherer, K., Johne, R., Schrader, C., Ellerbroek, L., Jörg Schulenburg, J., Klein, G., 2010. Comparison of  
628 two extraction methods for viruses in food and application in a norovirus gastroenteritis  
629 outbreak. *J. Virol. Methods.* 169 , 22-27.

630 Schwab, K.J., Neill, F.H., Fankhauser, R.L., Daniels, N.A., Monroe, S.S., Bergmire-Sweat, D.A., Estes,  
631 M.K., Atmar, R.L., 2000. Development of methods to detect "Norwalk-like viruses" (NLVs) and  
632 hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak. *Appl.*  
633 *Environ. Microbiol.* 66, 1, 213-218.

634 Stals, A., Uyttendaele, M., Baert, L., Van Coillie, E., 2013. Norovirus transfer between foods and food  
635 contact materials. *Journal of food protection*, 76, 1202-1209.

636 Stals, A., Baert, L., De Keuckelaere, A., Van Coillie, E., Uyttendaele, M., 2011a. Evaluation of a  
637 norovirus detection methodology for ready-to-eat foods. *Int. J.Food Microbiol.* 145, 420-425.

638 Stals, A., Baert, L., Van Coillie, E., Uyttendaele, M., 2011b. Evaluation of a norovirus detection  
639 methodology for soft red fruits. *Food Microbiol.* 28, 52-58.

640 Summa, M., von Bonsdorff, C.H., Maunula, L., 2012. Evaluation of four virus recovery methods for  
641 detecting noroviruses on fresh lettuce, sliced ham, and frozen raspberries. *J. Virol.Methods.*  
642 183, 154-160.

643 Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteyn, B., Koopmans, M.,  
644 2007. Etiological role of viruses in outbreaks of acute gastroenteritis in the Netherlands from  
645 1994 through 2005. *J. Clin.Microbiol.* 45, 1389-1394.

646 Thebault, A., Teunis, P.F., Le Pendu, J., Le Guyader, F.S., Denis, J.B., 2013. Infectivity of GI and GII  
647 noroviruses established from oyster related outbreaks. *Epidemics* 5, 98–110.

648 Yavarmanesh, M., Abbaszadegan, M., Alum, A., Mortazavi, A., Habibi Najafi, M. B., Bassami, M. R.,  
649 Nassiri, M. R., 2013. Impact of milk components on recovery of viral RNA From MS2  
650 bacteriophage. *Food and Env. Virol.* 5, 103-9.

651 Yavarmanesh, M., Abbaszadegan, M., Mortazavi, A., Najafi, M. B., Bassami, M. R., Nassiri, M. R.,  
652 2010. Impact of milk components in recovery of the MS2 bacteriophage as an indicator of  
653 enteric viruses. *J. Virol. Methods.* 168, 103-107.

654 Wadl, M., Scherer, K., Nielsen, S., Diedrich, S., Ellerbroek, L., Frank, C., Gatzer, R., Hoehne, M., Johne,  
655 R., Klein, G., et al., 2010. Food-borne norovirus-outbreak at a military base, Germany, 2009.  
656 *BMC Infect. Dis.* 10, 30.

657 Wilrich, C., Wilrich, P.T., 2009. Estimation of the POD function and the LOD of a qualitative  
658 microbiological measurement method. *J. AOAC Int.* 92, 1763-1772.

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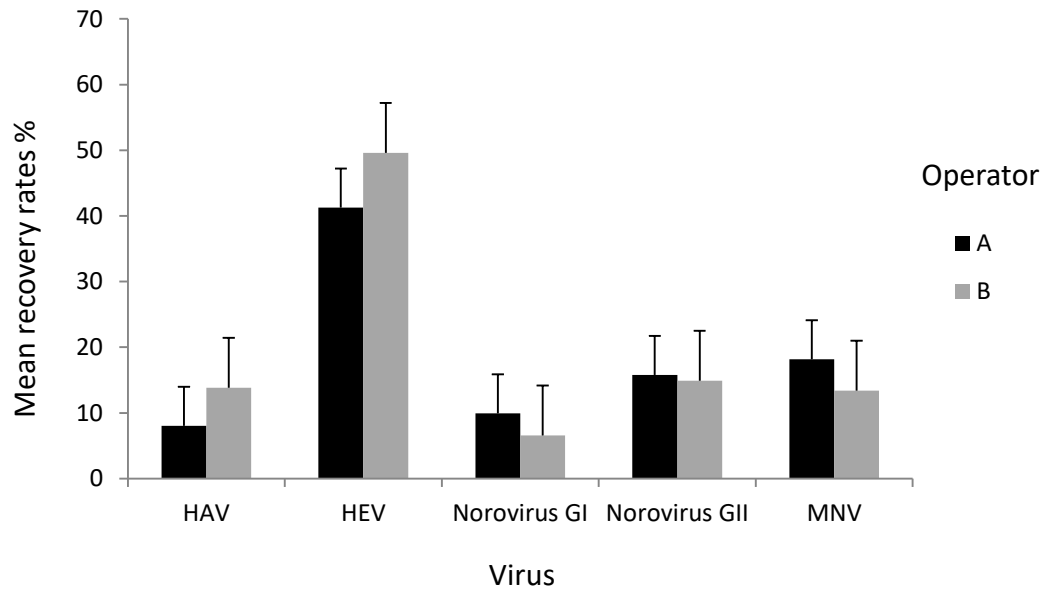


Figure 1: Comparison of mean recovery rates for HAV, HEV, norovirus GI, norovirus GII and MNV according to the operator factor (operator A and operator B). For each virus, 16 analyses were performed by each operator (four foodstuffs for all settings with all levels of inoculation) and RNA extracts were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

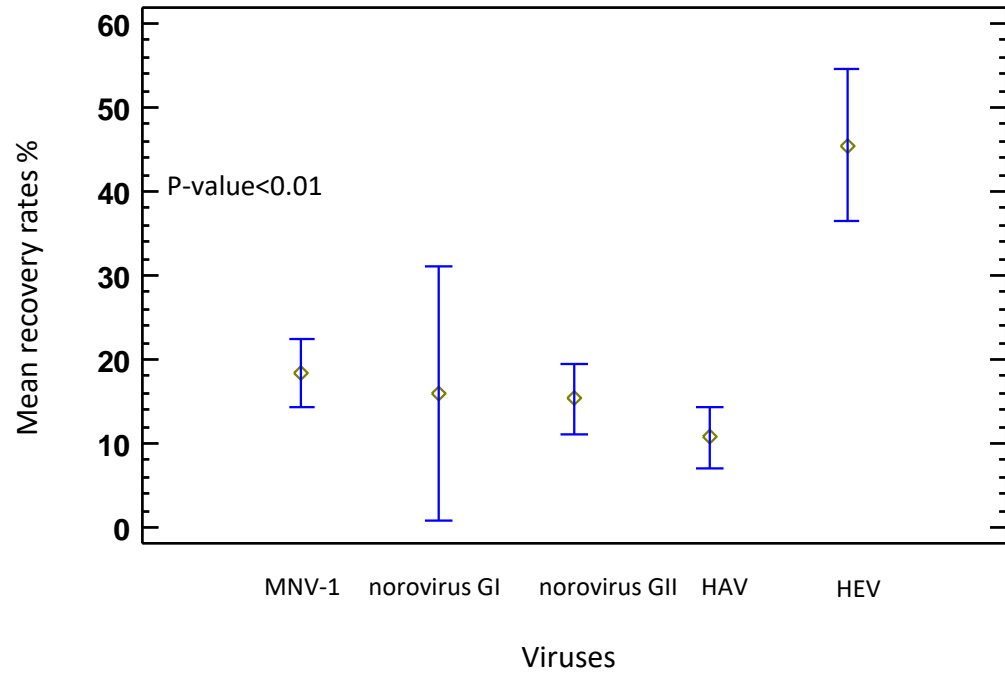


Figure 2: Comparison of mean virus recovery rates from spiked samples for all settings

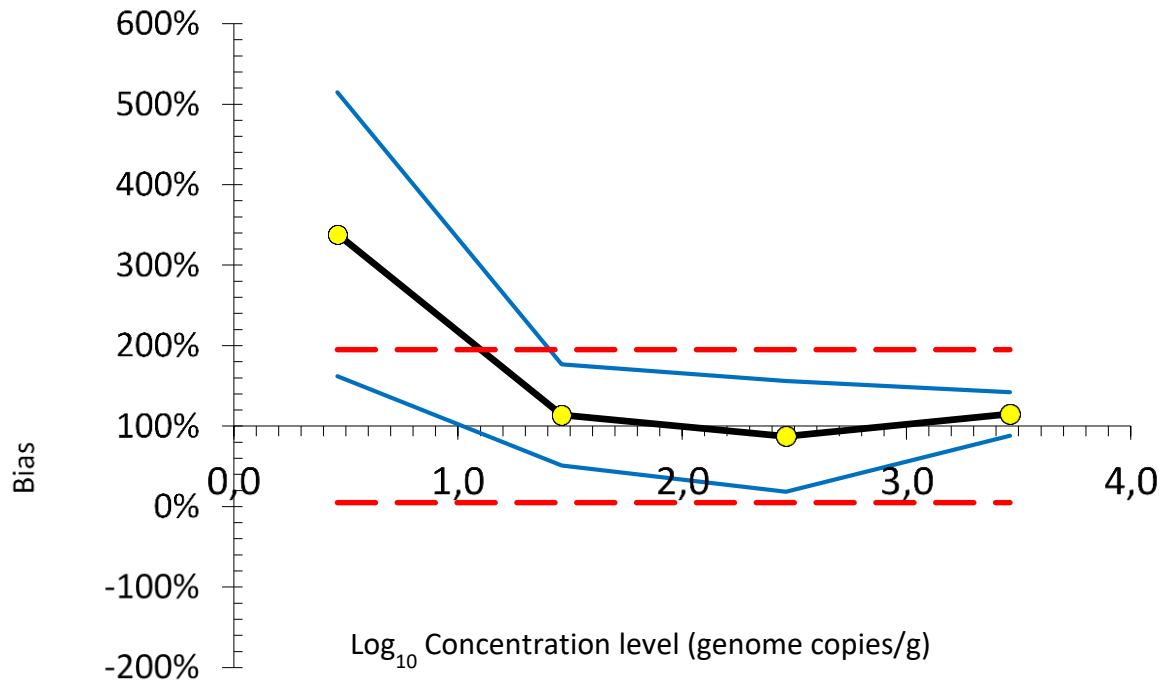


Figure 3: Example of accuracy profile for HAV. The black line is the bias and blue lines are the tolerance limits that define the 80% tolerance interval around the bias. Yellow points are the concentration levels tested. Dotted lines are the acceptance limit.

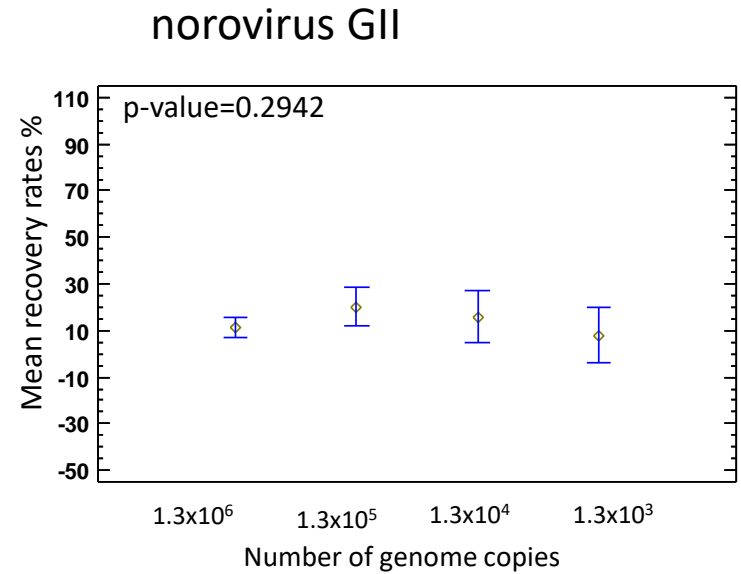
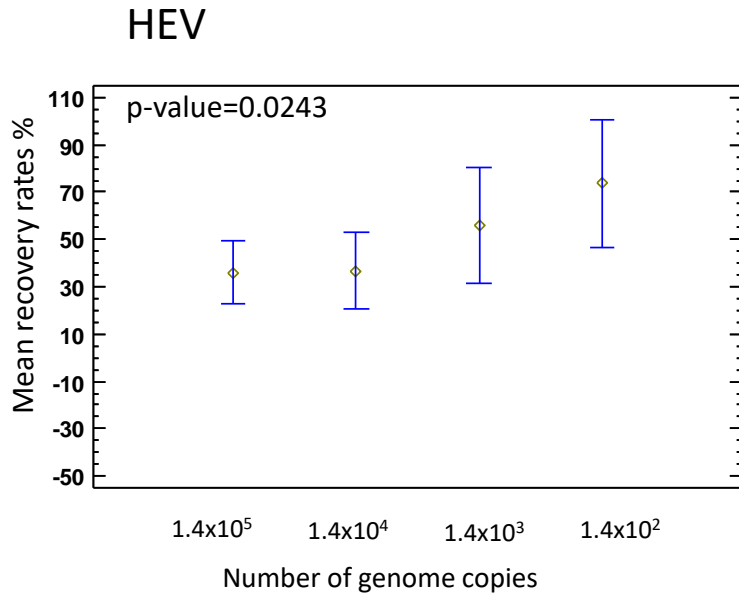
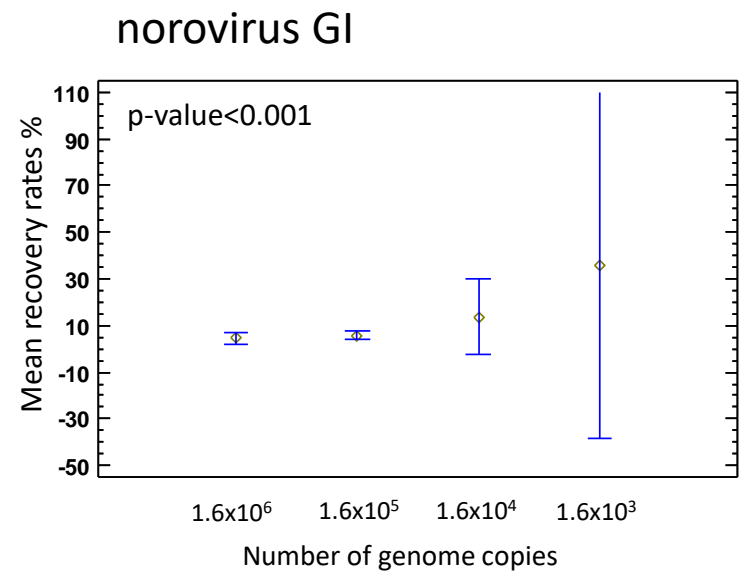
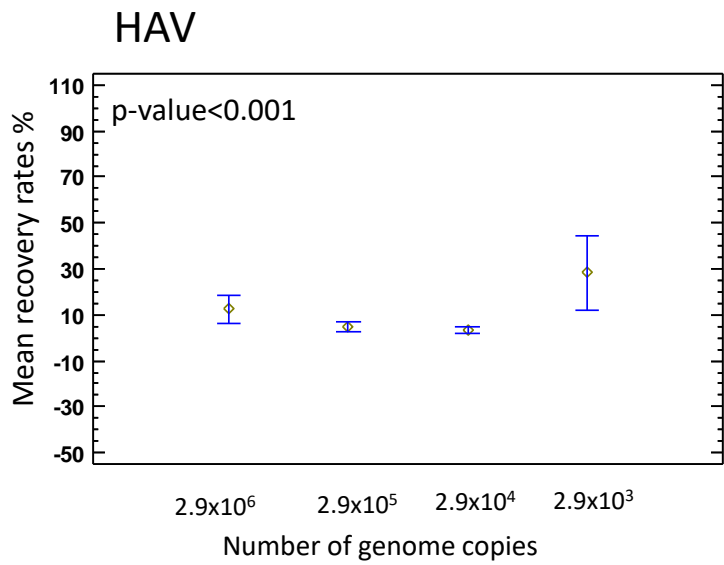
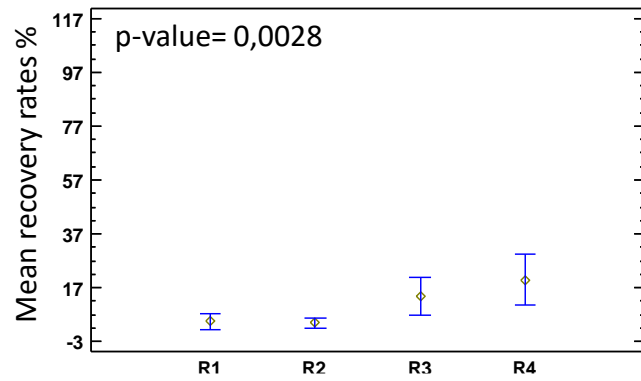


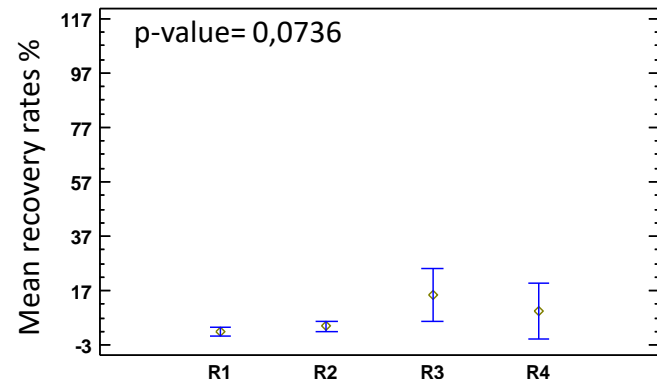
Figure 4: Recovery rates for HAV, HEV and norovirus with respect to artificial inoculation levels



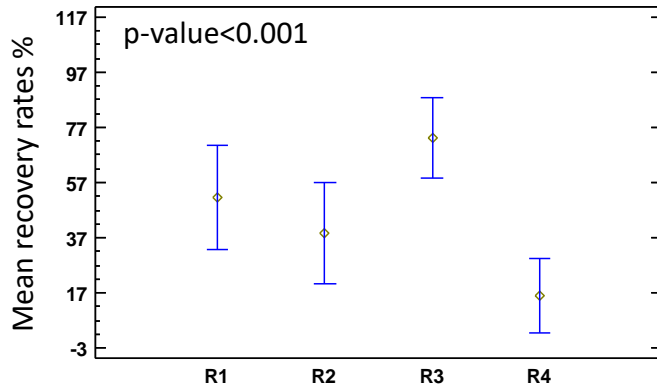
### HAV



### norovirus GI

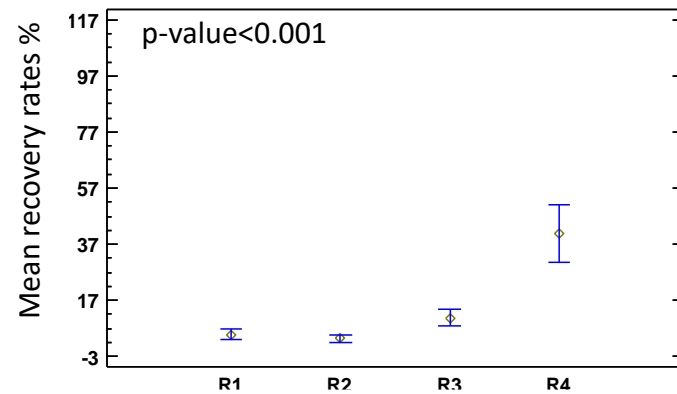


### HEV



Repeat experiments

### norovirus GII



Repeat experiments

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
	Composite dishes with meat	Meat stew (A03VY)	1
		Vol-au-vent (A040G)	2
Tin can	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 <sup>6</sup>	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 <sup>5</sup>	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		
	<b>2.90x10<sup>4</sup></b>	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 <sup>3</sup>	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	<b>1.40x10<sup>5</sup></b>	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 <sup>4</sup>	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 <sup>3</sup>	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 <sup>2</sup>	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 <sup>6</sup>	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 <sup>5</sup>	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 <sup>4</sup>	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 <sup>3</sup>	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 <sup>6</sup>	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 <sup>5</sup>	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 <sup>4</sup>	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 <sup>3</sup>	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<sub>50</sub> and LOD<sub>95</sub> calculated by Wilrich approach for HAV, HEV and norovirus

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

(\*LOD<sub>50</sub> and LOD<sub>95</sub> 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)	2.90x10 <sup>4</sup>	1.40x10 <sup>3</sup>	1.60x10 <sup>4</sup>	1.30x10 <sup>4</sup>
LOD (genome copies/g)	0.97x10 <sup>4</sup>	0.47x10 <sup>4</sup>	0.53x10 <sup>4</sup>	0.43x10 <sup>4</sup>
Validated range level (genome copies/g)	2.90x10 <sup>4</sup> to 2.90x10 <sup>6</sup>	1.40x10 <sup>3</sup> to 1.40x10 <sup>5</sup>	1.60x10 <sup>4</sup> to 1.60x10 <sup>6</sup>	1.30x10 <sup>4</sup> to 1.30x10 <sup>6</sup>
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)