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

Catherine Hennechart-Collette, Océane Dehan, Michel Laurentie, Audrey Fraisse, Sandra Martin-Latil, Sylvie Perelle

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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 LOD₉₅
35 was 10² genome copies/g for HAV, HEV and norovirus GII and 10³ genome copies/g for
36 norovirus GI. The LOQ was 2.90x10⁴, 1.40x10³, 1.60x10⁴ and 1.30x10⁴ 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.

43

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

46

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.

80

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[®] CRL-1688[™]) (LGC standards SARL, Illkirch, France). These
86 epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco[™])
87 supplemented with non-essential amino acids (NEAA, Gibco[™]) and 10% of heat-inactivated
88 foetal bovine serum (FBS, Gibco[™]) (Thermo Fisher Scientific, Waltham, MA, 209 USA). Cells
89 were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

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 (Fraisse et al., 2017). The HAV stock
96 had a titre of 2.90×10^9 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 1.40×10^7 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 1.60×10^7 and 1.30×10^7 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 CO₂ 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 2.15×10^7 TCID₅₀/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
134 (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 2.90×10^3 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 TCID₅₀ 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 ± 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% (LOD₅₀) and 95%**
239 **(LOD₉₅)** 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 β expectation tolerance interval (β -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 (λ) at ± 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% (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

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 LOD₅₀ and LOD₉₅ values for HAV,
289 HEV, norovirus GI and norovirus GII are shown in Table 4.

290 The LOD₉₅ values were 10² genome copies/g for HAV, HEV and norovirus GII and 10³ genome
291 copies/g for norovirus GI. The LOD₅₀ 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 2.90x10⁴, 1.40x10³, 1.60x10⁴, 1.30x10⁴ 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 0.97x10⁴, 0.47x10⁴, 0.53x10⁴ and 0.43x10⁴ 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 2.90×10^3
311 HAV genome copies, 1.40×10^2 HEV genome copies and 1.6×10^3 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 LOD₉₅ varied from 10² to 10³ genome copies per g regardless of the virus
344 analysed. Because of the complexity of the food matrices used in our study, the LOD₉₅ values
345 obtained for norovirus and HAV in composite foods and multicomponent foodstuffs were
346 higher than the LOD₉₅ 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 4.33×10^3 and 9.67×10^3 genome copies per g. These values are slightly different than the
349 estimated LOD₉₅ but the difference, which is around one log, can be explained by the
350 definition of these parameters. LOD₉₅ 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 LOD₉₅ 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

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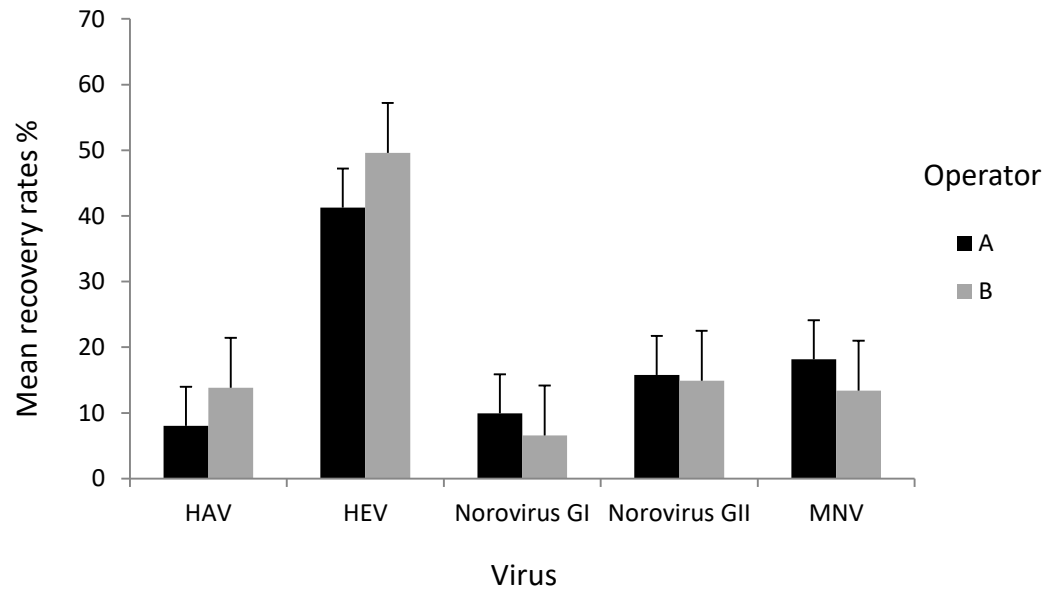


Figure 1: Comparison of mean recovery rates for HAV, HEV, norovirus GI, norovirus GII and MNV according to the operator factor (operator A and operator B). For each virus, 16 analyses were performed by each operator (four foodstuffs for all settings with all levels of inoculation) and RNA extracts were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

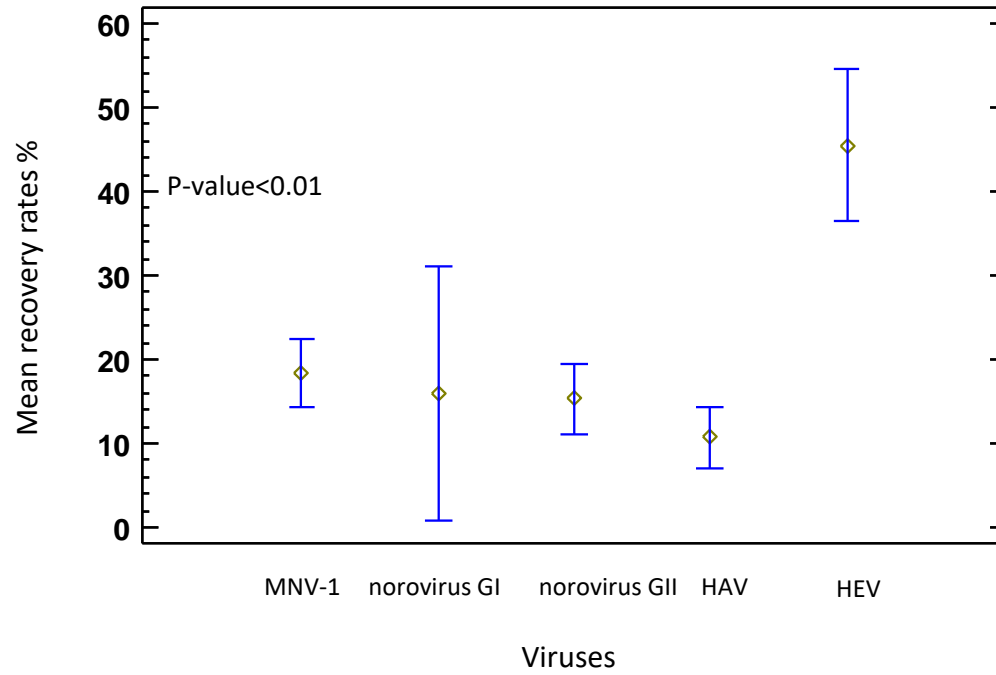


Figure 2: Comparison of mean virus recovery rates from spiked samples for all settings

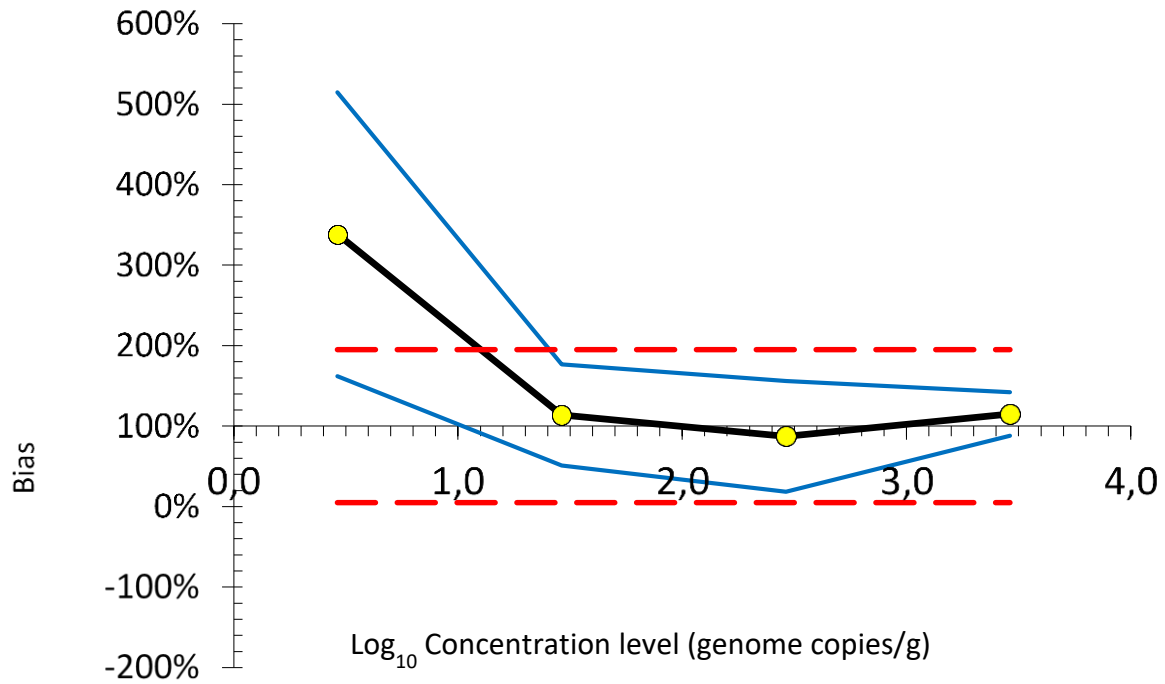


Figure 3: Example of accuracy profile for HAV. The black line is the bias and blue lines are the tolerance limits that define the 80% tolerance interval around the bias. Yellow points are the concentration levels tested. Dotted lines are the acceptance limit.

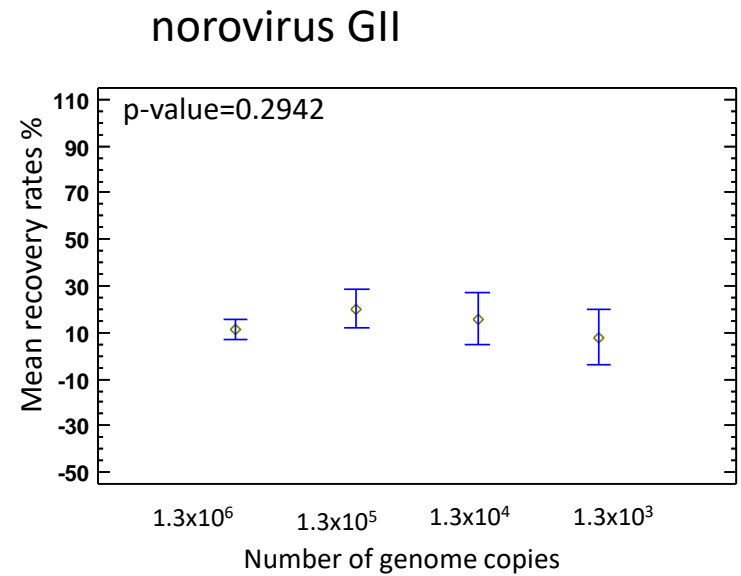
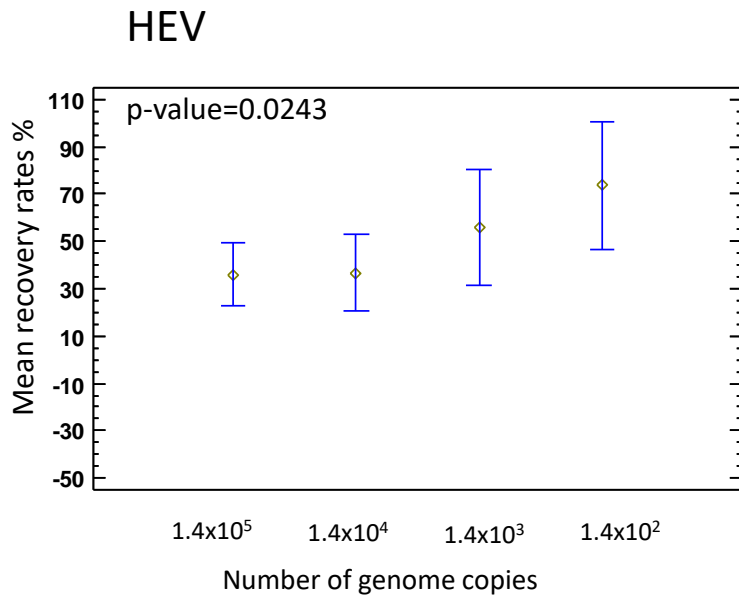
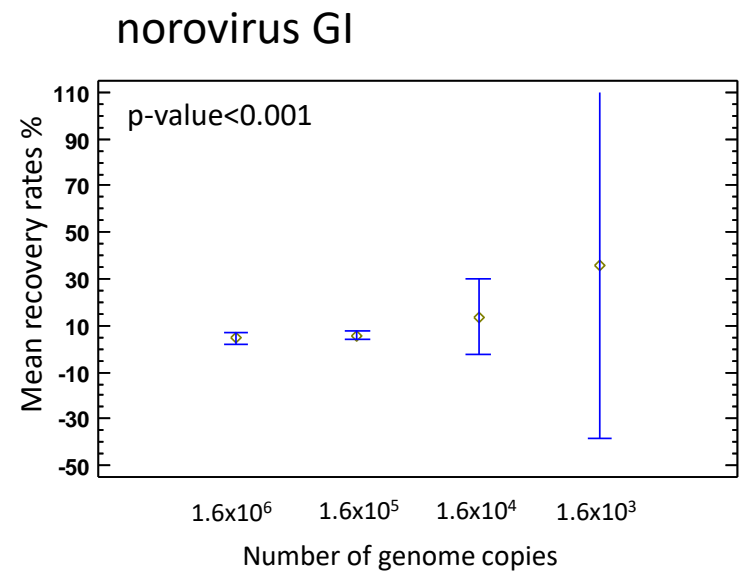
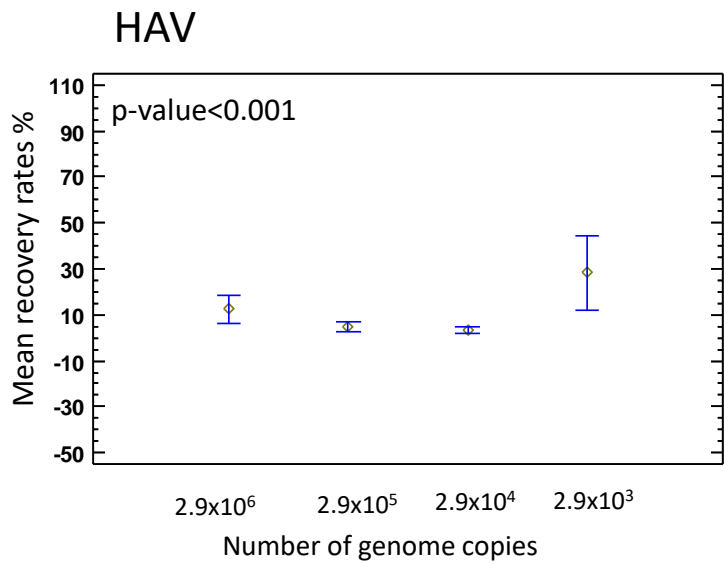
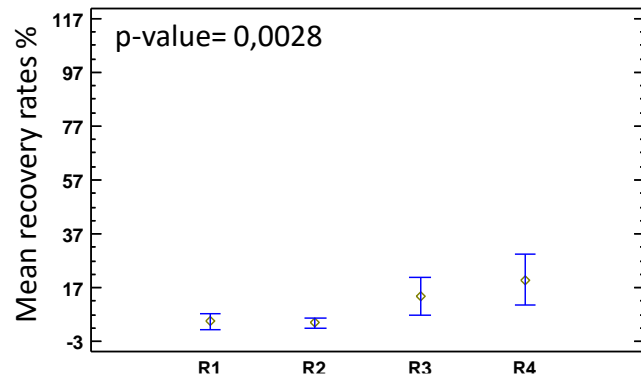
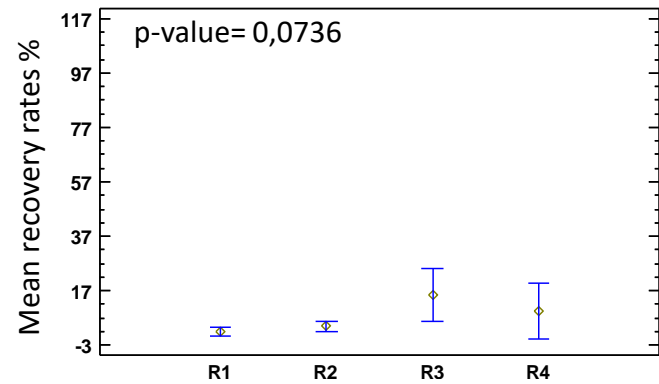


Figure 4: Recovery rates for HAV, HEV and norovirus with respect to artificial inoculation levels

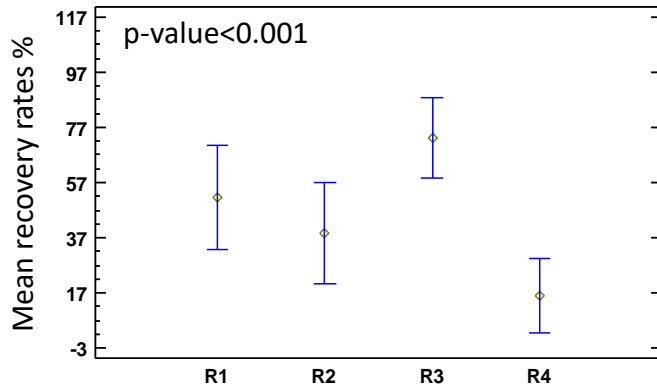
HAV



norovirus GI

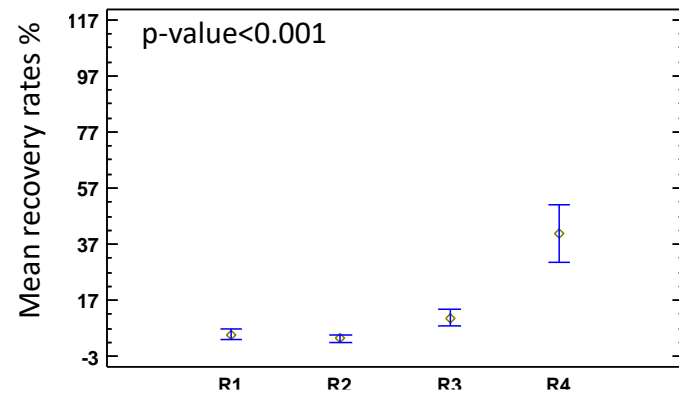


HEV



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 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		
	2.90x10⁴	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	1.40x10⁵	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)	
	1.60x10⁵	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)	
	1.30x10⁴	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: LOD₅₀ and LOD₉₅ 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

(*LOD₅₀ and LOD₉₅ 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 ⁴	1.40x10 ³	1.60x10 ⁴	1.30x10 ⁴
LOD (genome copies/g)	0.97x10 ⁴	0.47x10 ⁴	0.53x10 ⁴	0.43x10 ⁴
Validated range level (genome copies/g)	2.90x10 ⁴ to 2.90x10 ⁶	1.40x10 ³ to 1.40x10 ⁵	1.60x10 ⁴ to 1.60x10 ⁶	1.30x10 ⁴ to 1.30x10 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)