

Method for detecting norovirus, hepatitis A and hepatitis E viruses in tap and bottled drinking water

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27 Abstract

Viruses are a leading cause of foodborne disease worldwide. Human norovirus and hepatitis 28 viruses (hepatitis A (HAV) and hepatitis E (HEV)) are recognised to be the main viruses of 29 30 importance to public health. The ISO 15216 procedure describes molecular methods for detecting HAV and norovirus in bottled water by using an electropositive filter to 31 concentrate viruses. The aim of this study was to validate the Zeta Plus 1MDS membrane 32 (1MDS) for detecting enteric viruses from tap and bottled water using the recent 33 international standard ISO/DIS/16140-4:2018, which describes the protocol for validating 34 35 methods for microbiology in the food chain. Method with direct lysis of viruses from the 1MDS filter, and RNA extraction was used for detecting noroviruses, HAV and HEV from 36 37 different tap and bottled drinking water. By taking into account virus's inoculation levels above the LOD, the recovery rates of noroviruses and HAV obtained from pure RNA extracts 38 ranged from 2.50% to 14.31% and for HEV from 27.87% to 53.54% according to the water 39 samples analysed. The virus recovery rates did not differ according to the operator or 40 drinking water analysed but did according to the virus inoculated. The LOD₉₅ values were 41 respectively 50 genome copies/mL for HAV and 2.8 genome copies/mL for HEV, 420 genome 42 copies/mL for norovirus GI and 134 genome copies/mL of water sample for norovirus GII. 43 LOQs were determined for HAV and HEV by the total error approach and were 15.8 genome 44 copies/mL for HAV and 2.8 genome copies/mL of water sample for HEV. The described 45 46 method could be used for detecting viruses from tap and bottled water for routine diagnosis needs. 47

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Keywords: Drinking water; Filtration; Human norovirus; Hepatitis virus (A, E); Quantitative
 RT-PCR; Detection

51 **1. Introduction**

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Among the enteric viruses implicated in waterborne outbreaks, human norovirus and 53 54 hepatitis viruses (hepatitis A and hepatitis E) are a serious public health issue. Outbreaks associated with drinking water are rare in developed countries, though norovirus has already 55 56 been detected in tap water (Kukkula et al., 1999; Kauppinen et al., 2019) and bottled water 57 (Blanco et al., 2017). Viral waterborne outbreaks have generally been the result of raw water 58 contamination by faeces, treatment deficiencies or a distribution network failure (Beer et al., 2015; Blanco et al., 2017; Craun et al., 2010; Giammanco et al., 2014; Riera-Montes et al., 59 2011; Whatley et al., 1968). In the case of hepatitis E virus, consumption of contaminated 60 drinking water is suspected as a primary cause of almost all the epidemics of hepatitis E, 61 62 while contaminated meats and direct contacts with infected animals have been linked to sporadic cases worldwide (Colson et al., 2010; Dalton et al., 2013; Meng, 2010; Pavio et al., 63 2006, 2010). 64

Due to the small amounts of viruses in naturally contaminated water samples, viral 65 66 concentration methods such as filtration, ultrafiltration, flocculation or precipitation are 67 usually used. The ISO 15216 procedure recommends an electropositive filter to concentrate viruses from bottled water. Viruses adsorbed on the filter are eluted, recovered using a basic 68 69 elution buffer and further concentrated by ultrafiltration before the final step: molecular 70 detection of viral genomes. A method adapted from the ISO procedure with direct extraction 71 of virus from a Zetapor filter or Zeta Plus 1MDS membrane (1MDS) filter with the filter 72 characteristics described in the ISO procedure has been previously described (Coudray-73 Meunier et al., 2015; Hennechart-Collette et al. 2020; Martin-Latil et al., 2012; Perelle et al., 74 2009; Schultz et al., 2011).

To monitor the efficiency of this virus extraction procedure and to validate analyses, the ISO method describes the use of a process control virus and external amplification controls (EACs) as an external control RNA to assess the inhibition of PCR amplification (Anonymous, ISO 15216-1, 2017). Due to the diverse mineral compositions of the drinking water tested, which could potentially inhibit PCR amplification of viral genomes, an additional extraction step could be necessary (Bartsch et al., 2016; Butot et al., 2007; Fraisse et al., 2017).

The process control used should have similar morphological and physicochemical properties and environmental persistence as the target viruses, thus providing comparable extraction efficiency. In addition, it should rarely be found in analysed samples. Murine norovirus (MNV-1) has already been successfully used as a process control for HAV, HEV and norovirus detection in water (Hennechart-Collette et al., 2015, 2014; Martin-Latil et al., 2012) and as a surrogate for enteric viruses (Wobus et al., 2006).

The ISO 16140 procedure establishes both the general principle and technical protocol for validating alternative methods in the field of microbiological analysis of food. The recent international standard ISO/DIS/16140-4:2018 (Microbiology of the food chain — Method validation — Part 4: Protocol for single-laboratory method validation) (Anonymous, 2018) describes experimental designs to take into account the effect of various factors and their interactions, and reflects the variation within a single laboratory under routine conditions.

93 The aim of this study was (i) to evaluate an additional purification step after RNA extraction 94 from a 1MDS membrane filter from two types of drinking water (ii) to validate the selected 95 method for the detection of HAV, HEV and norovirus in artificially-contaminated water 96 according to the recent ISO/DIS/16140-4 standard.

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98 2. Materials and methods

99 **2.1 Viruses**

HAV strain HM175/18f, clone B (VR-1402) was obtained from the American Type Culture 100 101 Collection (ATCC). This clone replicates rapidly and is able to show clear cytopathic effects in cell culture when infectious (Lemon et al., 1991). The HAV stock was produced by 102 propagation in the kidney cells (ATCC, CRL-1688) of foetal rhesus monkeys (FRhK-4) 103 104 (Cromeans et al., 1987). These epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco[™]) supplemented with GlutaMAX[™], non-essential amino acids 105 (NEAA, Gibco[™]) and 10% of heat-inactivated foetal bovine serum (FBS, Gibco[™]) (Thermo 106 Fisher Scientific, Waltham, MA, USA). Cells were maintained at 37°C in a humidified 107 atmosphere containing 95% air and 5% CO₂. HAV stock was produced by propagation in 108 109 FRhK-4 cells and titrated by a plaque assay. Results were obtained 12 days after HAV infection and expressed in plaque-forming units/ml (PFU/ml). The HAV stock titre was 110 1.10x10⁷ PFU/mL. The supernatant was aliquoted for storage at -80°C. The virus titre was 111 determined as HAV RNA genomic copies with a quantitative real-time RT-PCR standard 112 curve. HAV stock had a titre of 2.90x10⁹ genome copies/mL. 113

A Clarified HEV genotype 3f suspension was obtained from faecal samples of infected swine 114 115 provided by ANSES's Maisons-Alfort Laboratory for Animal Health. The GenBank accession number of the partial sequence of ORF2 is JF718793. The faecal sample was suspended in 10 116 117 mM phosphate buffered saline (PBS), pH 7.4, to obtain a final 10% suspension (w/v), vortexed and centrifuged at 4,000 g for 20 min at 4°C. Aliquots of supernatants containing 118 viral particles were then stored at -80°C. The number of HEV RNA copies in the faecal 119 120 suspension was quantified by quantitative real-time RT-PCR. Based on this approach, the HEV stock had a titre of 1.40x10⁷ genome copies/mL. 121

122 Stool samples of norovirus GI.3 (E8050) and norovirus GII.3 (E7022) from infected humans were provided by the French National Reference Centre for gastroenteritis viruses in Dijon, 123 France. The faecal samples were suspended in 10 mM PBS, pH 7.4, to obtain a final 10% 124 suspension (w/v), and then vortexed and centrifuged at 4,000 g for 20 min at 4°C. Aliquots 125 were stored at -80°C for later use. The genomic titres of the clarified faecal suspensions were 126 127 determined by quantitative real-time RT-PCR using a quantitative real-time RT-PCR standard curve. The clarified suspension stocks of norovirus GI and norovirus GII had titres of 1.60x10⁷ 128 and 1.30x10⁷ genome copies/mL respectively. 129

Dr. H. Virgin from Washington University (Saint Louis, MO, USA) provided the ANSES 130 Fougères Laboratory (Fougères, France) with murine norovirus MNV-1 (CW1 strain), which 131 was propagated on a mouse leukaemic monocyte macrophage (RAW 264.7, ATCC TIB-71) 132 133 cell line (Cannon et al., 2006). RAW 264.7 cells were grown at 37°C in an atmosphere containing 5% CO₂ in DMEM supplemented with GlutaMAX[™], 1% NEAA, and 5% heat-134 inactivated foetal bovine serum (Thermo Fisher Scientific). Aliquots were stored at -80°C for 135 136 later use. The stock of MNV-1 was titrated as previously described (Wobus et al., 2004) and had a titre of 2.15×10^7 TCID₅₀/mL. 137

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139 2.2 Sample processing

Figure 1 gives an overview of the methods used to extract viral genomes from water anddetails of these methods are described below.

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143 **2.2.1** Sample processing to select the best method for detecting viruses in drinking water

One sample of tap water (Maisons-Alfort, a French municipality in Val-de-Marne in the south-eastern suburbs of Paris) and one of bottled water (Ca^{2+} , 11.5 mg/L; Mg²⁺, 8 mg/L;

Na²⁺, 11.6 mg/L; K⁺, 6.2 mg/L; Cl⁻, 13.5 mg/L; NO₃⁻, 6.3 mg/l; SO₄ ²⁻, 8.1 mg/L; SiO₂, 31.7 146 mg/L; HCO₃⁻, 71.0 mg/L; F⁻, 0.2 mg/L) were used to select the method. Chlorine residues in 147 500 mL of tap water were neutralised with a 0.005% final concentration (weight/volume) of 148 sodium thiosulphate (Na₂S₂O₃) solution (Sigma-Aldrich, Saint-Quentin-Fallavier, France) 149 (Méndez et al., 2004). The two types of drinking water (500 mL sample) were contaminated 150 151 with 2.15x10⁴ TCID₅₀ of MNV-1 and homogenised by gentle shaking using manual inversion. Three repetitions of each experiment set were performed. One unspiked water sample was 152 153 used as a negative control for virus extraction. Briefly, virus particles were concentrated using one filter with 47 mm positively-charged membrane with a pore size of 0.45 µm: a Zeta 154 Plus 1MDS membrane filter (cellulose) (1MDS) (3M, Filtreri, Amberieu en Bugey, France). 155 156 The flow rate used during 1MDS filtration was approximatively 500 mL/3 min. The filter membrane with adsorbed viruses was then directly incubated for 10 min at room 157 temperature in a 60 mm diameter Petri dish containing 3 mL of NucliSens[®] easyMAG[™] lysis 158 buffer (bioMérieux, Marcy l'Etoile, France). The total nucleic acid was extracted using the 159 NucliSens[®] easyMAG[™] platform with the "off-board Specific A" protocol according to the 160 manufacturer's instructions (https://resourcecenter.biomerieux.com/). In this approach, the 161 162 commercially available NucliSens[®] easyMAG[™] bio-robot ensures viral RNA purification with silica magnetic beads that mediate purification of nucleic acids by binding to them and 163 164 allowing other substances to be washed away. The nucleic acids thus extracted were then eluted in 100 µL of elution buffer and an additional purification step was performed on 50 µL 165 of viral RNA with the OneStep[™] PCR Inhibitor Removal Kit (Zymo Research, Irvine, USA). 166 167 Briefly, samples were loaded onto columns and centrifuged at 8,000 g for 3 min. All RNA 168 samples, purified or not, were stored at -80°C prior to analysis.

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170 **2.2.2 Sample processing and experimental design to validate the filtration method**

Different samples of tap water and different varieties of bottled water purchased from a 171 local supermarket were used. Twelve different tap and bottled water samples (water nos. 1 172 to 12) were chosen to validate the filtration method using a 1MDS filter. Six types of mineral 173 water and six types of tap water were used. Supplementary Table 1 describes the bottled 174 175 mineral water samples taking into account the differences in their mineral composition. The six different tap water samples were collected in glass flasks. Chlorine residues in 500 mL of 176 177 tap water were neutralised with a 0.005% final concentration (weight/volume) of sodium thiosulphate (Na₂S₂O₃) solution (Sigma-Aldrich, Saint-Quentin-Fallavier, France) (Méndez et 178 al., 2004). These tap water samples came from different cities and different French 179 *départements*: Neuilly-Plaisance (a municipality in Seine Saint Denis, in the eastern suburbs 180 181 of Paris; water no. 2), Saint Valery sur Somme (a municipality in the Somme département in northern France; water no. 4), Villeneuve d'Ascq (a municipality near Lille in northern 182 France; water no. 6 and water no. 8), Maisons-Alfort (a municipality in Val-de-Marne, in the 183 184 south-eastern suburbs of Paris; water no. 10), and central Paris (water no. 12) were selected. The experimental design described in ISO/DIS/16140-4:2018 was used. Various water 185 186 samples were randomly allocated to four different settings (R1, R2, R3 and R4). For each setting, three different water samples per contamination level were analysed by two 187 188 operators (operator A and operator B). Table 1 describes the allocation of water samples to the settings along with the viruses targeted. Bottled mineral water and tap water samples 189 190 (500 mL) were spiked with 100 μ L of clarified virus suspensions at three contamination 191 levels. Four viruses were used: HAV, HEV, norovirus GI and norovirus GII.

HAV, HEV and norovirus inocula were serially diluted ten-fold in DEPC water from viral stocks. The contamination levels ranged from 2.90×10^3 to 2.90×10^5 genome copies per

194 sample for HAV, 1.40x10² to 1.40x10⁴ for HEV, 1.60x10³ to 1.60x10⁵ for norovirus GI and 195 1.30x10³ to 1.30x10⁵ for norovirus GII. Each sample was co-inoculated with 2.15x10⁴ TCID₅₀ 196 of MNV-1 (process control virus) just before filtration with the 1MDS filter. For each type of 197 water sample, one sample inoculated only with sterile water was used as a negative control 198 during the entire sample processing and viral detection procedure. Samples were 199 homogenised by gentle shaking using manual inversion.

Virus particles were concentrated from water samples by the previously selected method using the 1MDS filter followed by a direct virus lysis and RNA extraction. The RNA extracts (undiluted and 10-fold diluted) were analysed in duplicate with RT-qPCR assays. Tests were conducted on three different days to evaluate the reproducibility of the method. R1 and R2 settings were analysed on the same day, R3 and R4 on two different days (Table 1).

205

206 2.3 Primers and probes

The primers and probes used to quantify HAV, HEV, norovirus GI, norovirus GII and MNV-1 have already been presented in previous article (Hennechart-Collette et al., 2021). The HAV probe was labelled with the FAM reporter dye at the 5'-end and a MGB at the 3'-end.HEV, norovirus GI, norovirus GII and MNV-1 probes were labelled with the FAM reporter dye at the 5'-end and a BHQ1 at the 3'-end.

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213 2.4 Quantitative real-time RT-PCR conditions

One-step quantitative real-time RT-PCR amplifications were performed in duplicate on the
CFX96[™] real-time PCR detection system (Bio-Rad) for each viral target. The reactions were
performed in a 25 µL reaction mixture containing 1x of RNA UltraSense[™] master mix and
1.25 µL of RNA UltraSense[™] enzyme mix, which are components of the RNA UltraSense[™]

218 One-Step Quantitative RT-PCR System (Thermo Fisher Scientific), 2 U RNase inhibitor 219 (Thermo Fisher Scientific), 1.25 µg of bovine serum albumin (Thermo Fisher Scientific), 500 220 nM of forward primer, 900 nM of reverse primer, 250 nM of probe and 5 μ L of RNA extract. Positive controls containing RNA extracted from virus suspensions and a negative control 221 222 containing all the reagents except the RNA template were included in all real-time RT-PCR 223 experiments. The one-step quantitative real-time RT-PCR programme involved a 60 min reverse transcription of RNA at 55°C, followed by a 5 min denaturation step at 95°C, and 224 225 finally 45 cycles of 15 s at 95°C, 1 min at 60°C and 1 min at 65°C. Fluorescence was recorded by the apparatus at the end of the elongation steps (1 min at 65°C) for each amplification 226 cycle. All the samples were characterised by a corresponding cycle threshold (Cq) value. 227 Negative samples gave no Cq value. A standard curve for each viral target was generated 228 229 with 10-fold diluted RNA extracts resulting from the viral stock which was titrated using the 10-fold diluted in vitro RNA transcripts (Fraisse et al., 2017; Hennechart-Collette et al., 2014; 230 Martin-Latil et al., 2012). The slopes (S) of the regression lines were used to calculate the 231 232 amplification efficiency (E) of the quantitative real-time RT-PCR reactions, according to the formula $E=10^{|1/s|}-1$ to determine the performance of quantitative real-time RT-PCR assays. 233 234 Pure and 10-fold diluted sample RNA extracts were analysed in duplicate with the quantitative real-time RT-PCR assay. HAV, HEV, norovirus GI, norovirus GII and MNV-1 235 236 recovery rates in spiked samples were calculated using the standard curves and the following formula: quantity of virus recovered after spiking experiments/quantity of viral 237 inoculum x100. RNA transcripts of norovirus GI, norovirus GII, HEV or HAV were used as an 238 239 external amplification control (EAC) to monitor real-time RT-PCR inhibition in drinking water 240 samples. This approach is described in ISO 15216-1, where an external control RNA (i.e. an 241 RNA species carrying the target sequence of interest) is added to an aliquot of RNA sample.

The degree of the quantitative real-time RT-PCR inhibition in each tested sample is obtained by comparing these results with the results of the EAC RNA in the absence of sample RNA (i.e. in DEPC water). Inhibition rates in extracted RNA were calculated using following formula: 100 - (quantity of external control RNA detected in sample / quantity of external control RNA detected in water x100).

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248 2.5 Statistical analysis

All statistical analysis was performed by using the Statgraphics Centurion XVII software (Statgraphics Centurion Version 17.1.04).

The influence of the additional purification step on MNV-1 extraction yields after RNA extraction was first assessed by using a one-way analysis of variance (ANOVA). The result of the ANOVA is a *p* value associated with the hypothesis that the mean recovery rates of all groups were the same.

To validate the selected method, the influence of the operator factor on recovery rates of viruses (HAV, HEV, norovirus and MNV-1) was first assessed by using an ANOVA. Next, the influence of additional factors on extraction yields of pathogenic viruses were studied: (1) influence of operator, (2) the virus tested, (3) type of water (bottled vs. tap water), (4) dilution of RNA extracts (pure vs. 10-fold diluted), (5) experiment settings (R1 to R4) and (6) the inoculation level of viruses.

When the extraction yields were statistically different (ANOVA, *p*-value < 0.05), a multiple comparison procedure (Fisher's least-significant-differences (LSD)) was applied to determine which condition provided the highest extraction yields. Graphs plotting the mean and its standard error for each group illustrate the multiple comparison procedure. When the

265 confidence intervals of means do not overlap, the difference between two groups for a266 single factor is significant.

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268 **2.6 Limits of detection and accuracy profile**

The limits of detection (LOD), which correspond respectively to 50% (LOD₅₀) and 95% (LOD₉₅) 269 270 of the probability of virus detection in water, were calculated with the method for estimating POD (probability of detection) function and the LOD of a qualitative 271 microbiological measurement method as described by Wilrich et al. (2009). The POD-LOD 272 calculation software program was used (version 9, dated 2017-09-23) (Wilrich et al., 2009). 273 downloaded 274 This program can be freely from www.wiwiss.fu-275 berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html.

The total error approach based on an accuracy profile (Hubert et al, 2007a, 2007b) was used to determinate the lowest concentration that can be quantified with an acceptable level of precision and trueness (LOQ) for HAV and HEV. LOD is easily derived from the LOQ using the classical ration between LOD and LOQ described by Shrivastava et al. (2011). The ratio used is 3 and the LOD is calculated as LOQ/3.

281 The accuracy profile is calculated by a two-sided β expectation tolerance interval (β -ETI) for each level from the results of the validation experiments expressed, such as recovery 282 283 (trueness) and precision (repeatability and intermediate precision), and used to build the accuracy profile. Data are log_{10} transformed and we defined the acceptability limits (λ) at ± 284 285 95% of bias because the expected recovery is low. The risk was set at 20% supporting that 286 80% (β) of future measurements would fall within the acceptability limits. Accuracy profiles 287 were first described by Hubert et al. (Hubert et al., 2007a; Hubert et al., 2007b; Hubert et al., 288 2008) and their use in microbiological applications, in particular to validate methods, was described by Boubetra et al. (2011) and Feinberg et al. (2009). The procedure used here is
suited to molecular methods, such as those published by Saint-Cyr et al. (2014).

The graphical representation of the accuracy profile is a plot of the bias versus the log₁₀ concentration level.

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294 **3. Results**

3.1 Evaluation of an additional purification step after RNA extraction

Three independent adsorption methods with a 1MDS filter for concentrating MNV-1 were performed on artificially-contaminated waters (one tap water and one bottled water) and an additional purification step after RNA extraction was assessed for its contribution to reducing the impact of PCR inhibitors. The mean percentage recoveries of MNV-1 processed with or without an additional purification step are reported in Table 2.

The mean recoveries of MNV-1 calculated with pure and 10-fold diluted RNA extracts ranged from 44.02% to 57.00% without the one step TM PCR inhibitor removal Kit and from 30.77%

to 41.31% with the additional purification step.

Furthermore, testing 10-fold diluted RNA extracts showed that extraction yields for MNV-1 in tap and bottled water varied slightly by a factor that ranged from 1.0 to 1.2 without an additional purification step and 0.7 to 1.1 with an additional purification step after RNA extraction. The mean extraction yields obtained without the additional purification step were not significantly different between tap water or bottled water (one-way ANOVA; *p*value=0.0931) and for pure or ten-fold diluted samples (one-way ANOVA; *p*-value=0.3575).

The mean extraction yields obtained with the additional purification step were not significantly different between tap water or bottled water (one-way ANOVA; *p*value=0.7283) and for pure or ten-fold diluted samples (one-way ANOVA; *p*-value=0.2386).

After taking into account all the results, the 1MDS filtration without an additional purification step after RNA extraction was selected for characterising a method for detecting enteric viruses in different types of drinking water

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317 3.2 Characterisation of a method using a 1MDS filter for hepatitis viruses and norovirus
 318 detection in water

The adsorption method with a 1MDS filter for concentrating viruses was characterised on twelve different types of artificially-contaminated water analysed by two operators.

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322 **3.2.1** Mean virus recoveries per operator

To identify whether the operator factor influenced HAV, HEV, norovirus and MNV-1 recovery 323 324 rates, the mean recovery rates obtained for all settings were compared. For each operator (operator A and operator B), the mean recovery rates obtained for HAV, HEV and norovirus 325 are reported in Figure 2. Results showed that for operator A, the mean recovery rates for all 326 327 settings were 9.51% for HAV, 57.76% for HEV, 10.90% for norovirus GI and 5.08% for 328 norovirus GII. For operator B, the mean recovery rates were respectively 13.27%, 72.55%, 329 5.36% and 3.36% for HAV, HEV, norovirus GI and norovirus GII respectively. For MNV-1, the mean recovery rates were respectively 60.58% for operator A and 58.23% for operator B. 330 331 The statistical analysis revealed that the operator factor did not influence virus recovery from either tap or bottled drinking water (one-way ANOVA; p-value=0.4691). 332 Since the operator factor did not influence virus recovery, the mean recovery rate obtained 333

for each virus is the mean recovery result obtained by operator A and operator B.

335

336 **3.2.2** Mean virus recovery in drinking water and limit of detection

Table 3 gives the mean recovery yields obtained for HAV, HEV, norovirus GI and norovirus GII according to the inoculum level and the repeat experiments (R1 to R4). The average recoveries with pure RNA extracts ranged from 2.92% to 14.31% for HAV, 27.87% to 54.84% for HEV, 1.69% to 51.35% for norovirus GI and 0.79% to 8.02% for norovirus GII. As expected, no viral RNA was detected in the uninoculated samples.

The process control, MNV-1, was detected in all RNA extracts analysed and was recovered with an efficiency of between 37.70% and 83.29% (Table 3).

The LODs for the method were calculated for each virus and for each setting with the twelve samples. LOD₅₀ and LOD₉₅, which correspond respectively to a 50% and 95% probability of detection for HAV, HEV, norovirus GI and norovirus GII, are shown in Table 4.

The LOD₉₅ for all settings was 50 genome copies/mL for HAV, 2.8 genome copies /mL for HEV, 420 genome copies/mL for norovirus GI and 134 genome copies/mL for norovirus GII. The LOD₅₀ was between 0.6 and 11.8 genome copies/mL for HAV and HEV and between 30 and 94 genome copies/mL for norovirus GI and norovirus GII.

Limits of quantification were assessed from the accuracy profile for HAV and HEV. Supplementary Table 2 summarises the results for LOQ and LOD and some performance characteristics (repeatability and intermediate precision). The LOQ ranged from 2.8 genome copies/mL for HEV to 15.8 genome copies/mL for HAV. The LOD, derived from the LOQ estimated on the basis of the accuracy profile for HAV and HEV, was established at 5.27 genome copies/mL and 0.93 genome copies/mL for HEV. Figure 3 shows a typical accuracy profile for HAV and HEV.

No LOQs were calculated for noroviruses GI and GII because the variability was higher thanthe variability for HAV or HEV.

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361 **3.2.3** Influence of experimental factors on virus extraction yield

The mean recovery rates obtained for HAV, HEV, norovirus GI, norovirus GII and MNV-1 in drinking water showed that they vary according to the virus inoculated (one-way ANOVA; *p*value=0.0001) (Figure 4). More specifically, the multiple comparison tests showed that the recovery rates for HEV and MNV-1 were significantly different from the other three viruses.

To assess the influence of the type of water on HAV, HEV and norovirus extraction yields, we compared the mean recovery rates of viruses from bottled water with those obtained for tap water. They showed that the type of water has no influence on the recovery rates (one-way ANOVA; *p*-value=0.9702).

Furthermore, extraction yields obtained for norovirus GI, norovirus GII and HEV for undiluted and 10-fold diluted RNA extracts showed no significant amplification inhibition (one-way ANOVA; *p*-value=0.9080; *p*-value=0.2246; *p*-value=0.7336 respectively), but showed significant amplification inhibition for HAV (one-way ANOVA. *p*-value=0.0176).

To determine whether the repeated experiments (R1 to R4) or whether the inoculation 374 375 levels had an impact on HAV, HEV and norovirus recovery rates from food samples, statistical analyses were performed. The differences between R1 to R4 were not significant 376 377 for the extraction yield of HEV (one-way ANOVA; p-value=0.0656), norovirus GI (one-way ANOVA; p-value=0.9413) and norovirus GII (one-way ANOVA; p-value=0.3722) but were 378 379 significant for the extraction yield of HAV (one-way ANOVA; *p*-value=0.0004). More specifically, the multiple comparison tests showed that R4 was significantly different from 380 381 other repeat experiments. The statistical analysis showed that there was no statistically 382 significant difference in recovery rates for HAV with respect to the HAV inoculation level 383 (one-way ANOVA; *p*-value=0.4129), but the extraction yield for different inoculation levels 384 was significantly different for HEV (one-way ANOVA; p-value=0.0089), norovirus GI (one-way ANOVA; *p*-value=0.0095) and norovirus GII (one-way ANOVA; *p*-value=0.0001). More specifically, the multiple comparison tests showed that inoculation levels close to the LOD (1.40x10² genome copies of HEV, 1.0x10⁵ genome copies of norovirus GI and 1.30x10³ genome copies of norovirus GII) were significantly different from other inoculation levels of HEV, norovirus GI or norovirus GII.

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391 **3.2.4** Recovery rates for the external amplification control (EAC)

The EAC corresponding to all viral targets was used to examine quantitative real-time RT-PCR inhibition with pure RNA extracts. Supplementary Table 3 shows the mean inhibition of quantitative real-time RT-PCR for each of the twelve samples. The mean inhibition of quantitative real-time RT-PCR varied from 10.53% to 38.20%.

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397 **4. Discussion**

The general strategy for detecting enteric viruses in water consists of three steps: virus 398 399 extraction, purification of viral RNA then quantitative molecular detection of the purified RNA. The ISO 15216 procedure proposes molecular methods for detecting HAV and 400 401 norovirus in bottled water. This method includes concentrating the virus from water and 402 recommends using a microporous electropositive filter with a pore size of 0.45 µm, followed 403 by molecular detection. Electropositive filters are recommended because the electronegative charge of the viral particles is attracted by the filter's positive charge. The 404 Zetapor electropositive filter was widely used in the past for concentrating viruses found in 405 406 drinking water (Butot et al., 2007; Martin-Latil et al., 2012; Schultz et al., 2011). In this study, 407 the 1MDS filter — which has the same characteristics in terms of pore size and charge — 408 gave the same MNV-1 detection results as the Zetapor filter (data not shown).

409 Since past studies showed that the virus recovery rates were different in tap water and in 410 bottled water (Hennechart-Collette et al., 2014; Martin-latil et al., 2012), a purification step after RNA extraction was evaluated in this study. Our results showed that mean recoveries 411 for MNV-1 were not improved by using an additional purification step after RNA extraction. 412 413 In this case, the removal of inhibitors was not necessary and the use of columns could not 414 improve virus detection. For certain foods that generate strong PCR inhibition, such as lettuce, strawberries, raspberries or mixed red fruits, virus recovery may be improved by 415 416 adding a PCR inhibitor removal step after RNA extraction (Bartsch et al., 2016; Butot et al., 417 2007; Fraisse et al., 2017).

The filtration method using a 1MDS filter followed by the direct extraction of viral RNA from 418 419 drinking water was validated according to the recent international standard ISO/DIS/ 16140-420 4:2018. In addition to the factors studied in this standard, part 4 describes the calculation of repeatability and reproducibility. The ISO 15216 procedure describes a method to detect 421 viruses from bottled water while in this study, two types of different drinking waters 422 423 (various/different tap waters and bottled waters) were tested. The ISO 15216 recommends 424 volumes up to 2L and the validation of this procedure in bottled water was realized on 300 425 mL (Lowther et al., 2019). In our study, 500 mL of tap and bottled waters were filtered. In the presence of low virus concentration, a large starting volume can lower the detection limit. 426 427 According the ISO 16140 procedure, the validation of a method without a reference method is based on the number of various water samples and the number of assays. The 428 429 experimental design from 16140-4 procedure was applied in this study to characterize the 430 1MDS filtration method. The experimental design from 16140-4 procedure was also used to 431 characterise a method for detecting enteric viruses in multicomponent foodstuffs 432 (Hennechart-Collette et al., 2021). In this study, to investigate the filtration method under a

433 defined range of conditions commonly encountered in routine application, different tap and bottled water samples were used, and two operators performed the analyses. The operator 434 factor did not influence virus recovery from either tap or bottled drinking water. This result 435 should also be correlated with the calibration and maintenance of equipment, which should 436 also limit the difference between operators working in the same laboratory. Similar results 437 438 were also observed in another study (Hennechart-Collette et al., 2021). The numerous water samples analysed taking into account their different mineral compositions and potential 439 440 inhibitors provide information on the accuracy profile and relative trueness. After characterising the filtration method with a 1MDS filter, an interlaboratory assay should be 441 carried out so as to evaluate the quality of virus detection in tap and bottled drinking water 442 443 for other laboratories.

444 In this study, the impact of water variability when detecting enteric viruses was taken into account through the use of different water samples with different mineral compositions. The 445 HAV extraction yields of R4 setting were different from other repeat experiments. This result 446 447 could be explained by the experimental design of this study: each R had a different combination of samples with different inoculation levels. The virus recovery rates from 448 449 bottled water were similar to those obtained for tap water. The influence of the type of water when detecting enteric viruses has already been described (Blaise-Boisseau et al., 450 451 2010; Huguet et al., 2012; Martin-Latil et al., 2012). The HEV and noroviruses extraction yields in tap water were slightly lower than in bottled water (Hennechart-Collette et al., 452 453 2014; Martin-latil et al., 2012). These two studies have been conducted with the same 454 detection method but only with one tap water and one bottled water. Recently, one other 455 study which was carried out under the same conditions with one tap and one bottled water 456 has shown that the recovery rates obtained for norovirus and HAV were similar regardless of

the water tested (Hennechart-Collette et al., 2020). The different number of samples testedand the evolution of the water quality over the year could explained these results.

Ours results showed that mean recovery rates vary according to the virus inoculated. The 459 HEV recovery rates were significantly higher than HAV and noroviruses recovery rates. 460 461 According to the ISO 16140 method, without reference method, the characterization of the 462 method has to rely on known contamination levels from artificially inoculated samples. Different target viruses (HAV, norovirus genogroup I and II) were tested in this study. Stocks 463 464 of our viruses were produced by propagation in cell culture for HAV, obtained from clarified faecal samples of infected swine for HEV and obtained from stool samples of infected 465 humans for norovirus GI and norovirus GII. The viral family, the genotype of viruses, the 466 origin of the inoculum and the quality of the faecal samples used should play a role in the 467 468 virus recovery rates.

The LOD₉₅ values varied from 3 to 760 genome copies per mL of water sample regardless of the virus analysed. In our study, statistical analysis revealed that the inoculation levels close to the LOD₉₅ were significantly different from other inoculation levels. The extraction yield were higher for HEV, norovirus GI or norovirus GII around inoculation levels close to the LOD₉₅. These results highlight the importance of determining the LODs of the method for each virus which reflected the virus detection with an acceptable level of precision and trueness.

By taking into account virus's inoculation levels above the LOD, the recovery rates of noroviruses and HAV obtained from pure RNA extracts ranged from 2.50% to 14.31% and for HEV from 27.87% to 53.54% according to the water samples analysed. A previous study was conducted with 1MDS filter, under the same experimental conditions and with the same starting concentrations of norovirus GI and norovirus GII (Hennechart-Collette et al. 2020).

481 The virus recovery rates of norovirus GI and norovirus GII were 62.41% and 37.4% respectively while in this study they drop to a mean percentage of recovery of 6.16% and 482 5.5% respectively for the 4 settings. This difference of noroviruses recovery rates could be 483 explained by the difference of water used and the different number of water samples tested. 484 The evaluation of three different filters and two methods for recovering viruses from 485 486 drinking water were conducted with one tap water and one bottled water (Hennechart-Collette et al., 2020). In this study, four waters with different mineral composition (two tap 487 488 water and two bottled water) were evaluated according one level of virus inoculation. The evaluation of three different filters and two methods for recovering viruses from drinking 489 water had been conducted with one tap water and one bottled water (Hennechart-Collette 490 et al., 2020). In this study, four waters with different mineral composition (two tap water 491 492 and two bottled water) were evaluated according one level of virus inoculation.

Past studies using a Zetapor filter and direct extraction method found that norovirus and HAV recovery values for bottled water ranged between 32.50% and 102.00% (Coudray-Meunier et al., 2015; Hennechart-Collette et al., 2015). Similar results were also observed by Schultz et al. (2011) for HAV and FCV recovery rates. Another study demonstrated an HEV recovery rate from tap and bottled water that ranged from 3.00% to 129.00% and a similar LOD was also observed (Cuevas-Ferrando et al., 2020; Martin-Latil et al., 2012).

The LOD₉₅ values obtained for norovirus and HAV in both tap and bottled drinking water in our study were higher than the LOD₉₅ recently reported for bottled water (Lowther et al., 2019). The diversity of tap and bottled water used in our study should explain the difference in the LOD₉₅ value obtained compared with the ISO validation.

503 Derived from LOQ values estimated on the basis of an accuracy profile, the LOD ranged 504 between 5.27 and 0.93 genome copies per ml for HAV and HEV respectively. These values

505 are slightly different than the LOD₉₅ estimated. The difference is around one log, which can 506 be explained by the definition of these parameters. As mentioned, LOD₉₅ is the level at which 507 the probability of detection is equal to 95% (ISO 16140-1). The LOD is by convention the lowest level that can be differentiated from the background noise with an accepted 508 509 confidence level. As Armbruster et al. (2008) have previously explained, the LOD is a mean 510 value + k SD, where k lies between 2 and 10. The common value for k is 3 for the LOD, and if k = 10 it is the LOQ. However, the LOQ is the lowest level quantifiable when both trueness 511 512 and precision are defined (ISO 16140-1). In our case, we defined the LOQ based on an accuracy profile giving both the trueness and precision, and the LOD was then derived from 513 the LOQ. These two approaches are totally different, one being qualitative and the other 514 515 quantitative. However, the results are of the same magnitude so the LOD confirms the LOD₉₅ 516 values.

In order to monitor performance of the analysis, different controls were included in 517 accordance with the ISO procedure. It was necessary to implement the virus process control 518 519 (MNV-1) in order to validate results and monitor extraction efficiency. MNV-1 used as 520 process control in this study is an enteric virus of the same size than human norovirus, 521 negatively charged. Data obtained in our study showed that the recovery rates of MNV-1 was higher than for noroviruses or HAV recovery rates. According to the ISO 15216 522 523 procedure, the quantity of virus process control added to the sample under test was not definite. The amount of MNV-1 inoculated in our study was higher than noroviruses or HAV 524 525 target and could explained this result. The extraction of efficiency of MNV-1 was used as 526 quality assurance parameters only and was not used to adjust target virus results. According 527 to the recommendations in ISO 15216, virus process control extraction yields have to be 528 higher than 1% to validate the assay. MNV-1 was used as a process control virus in this study

529 for detecting HAV, HEV and norovirus in all 12 drinking water samples. It was successfully detected in all the RNA extracts. Inhibitory substances could be concentrated along with the 530 virus target during filtration, and the detection of viral genomes by quantitative real-time 531 RT-PCR could be sensitive to such inhibitors (Butot et al., 2007). The second control is 532 533 therefore an external amplification control (RNA carrying the target sequence of interest) 534 added prior to the RT-PCR detection step to monitor any real-time RT-PCR inhibition in samples. According to the recommendations in ISO 15216, the inhibition rates for RNA 535 536 extracted from food samples have to be lower than 75% to validate the assay. In this study, the inhibition of quantitative real-time RT-PCR varied from 10.53% to 38.20% according to 537 the different water samples analysed. Similar quantitative real-time RT-PCR inhibition in 538 539 bottled water was observed by Coudray-Meunier et al. (2015).

To conclude, the method validated according to the ISO 15216 and ISO 16140 procedures is a sensitive and robust molecular method for detecting norovirus and hepatitis viruses and could be used to detect viruses in drinking water for routine diagnostic needs.

543

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Figure 1: Flowchart of the methods evaluated with or without purification step for the recovery and detection of MNV-1.



Figure 2: Comparison of mean recovery rates of HAV, HEV, norovirus GI, norovirus GII and MNV-1 from water samples according to the operator factor (operator A and operator B). For each virus, the 12 samples were analysed by each operator (three samples for all settings with all levels of contamination). The RNA extracts (pure and ten-fold diluted) were analysed in duplicate with the quantitative real-time RT-PCR assay.



Figure 3: Examples of an accuracy profile for HAV (top) and HEV (bottom).

The results are presented through a graphical representation as follows: the horizontal (x) axis shows the reference level in log_{10} concentration level (genome copies/g) and the vertical (y) axis shows at each level the bias, the acceptability limits (λ), and the β -ETI limits.

The black line is the bias; the blue lines are the tolerance limits that define the 80% tolerance interval around the bias. Yellow points are the concentration levels tested. The dotted lines are the acceptance limit.



Figure 4: Comparison of mean virus recovery rates from spiked water for all settings.

Table 1: Experimental design for detecting HAV, HEV and norovirus in tap and bottled drinking water adapted from the ISO/DIS 16140-4:2018 procedure performed by two operators.

		Repeat experiments									
		R1		R2	R2			R4			
	Operator	А	В	А	В	А	В	А	В		
	High	Water no. 3 Bottled water		Water no. 6 Tap water		Water no. 9 Bottled water		Water no. 12			
Virus contamination								Tap water			
level	Medium	Water no. 2 Tap water		Water n	Water no. 5		Water no. 8		Water no. 11		
				Bottled	Bottled water		Tap water		Bottled water		
	Low	Water no. 1 Bottled water		Water n	Water no. 4		Water no. 7		Water no. 10		
				Tap wat	Tap water		Bottled water		Tap water		

Table 2: Mean percentage recovery of MNV-1 in tap and bottled drinking water processed with a 1MDS filter tested with or without the One Step[™] PCR Inhibitor Removal Kit.

		1MDS Filter								
Wator	PNA ovtracto	Additional purification step after RNA extraction								
water	RINA EXITACIS	Without		With						
		Recovery yield	F	Recovery yield	F					
		(%±SD)		(%±SD)						
Bottled water	pure	44.02±6.65	1.0	41.31±4.45	0.7					
bottled water	10-fold diluted	44.03±9.84		30.77±8.06						
Tap water	pure	47.67±13.97	1.2	36.38±6.06	1.1					
iap watei	10-fold diluted	57.00±14.24		38.28±12.86						

Results are expressed as the mean ± standard error (SD). Three experiments were performed. Pure and ten-fold-diluted RNA extracts were tested twice. The ratio (F) between the mean recovery yields obtained with pure RNA extracts and those obtained with 10-fold diluted RNA extracts was calculated to determine whether the dilution of RNA extracts improves mean extraction yield.

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

			Repeat experiments							
Virus	Quantity of genome copies/500mL	RNA extracts	R1 (%±SD)	(F)	R2 (%±SD)	(F)	R3 (%±SD)	(F)	R4 (%±SD)	(F)
			9.85±0.58		11.03±0.47		12.20±2.42		9.03±3.64	
	2.90x10 ⁵	Pure	(4/4)	1.4	(4/4)	1.4	(4/4)	1.3	(4/4)	1.2
		10-fold	14.17±4.74		15.24±1.31		15.89±3.86		10.79±3.12	
		diluted	(4/4)		(4/4)		(4/4)		(4/4)	
			14.31±5.36				10.18±2.16		6.03±2.42	
ΗΔΛ	2.90x10 ⁴	Pure	(4/4)	1.3	nd	-	(4/4)	2.0	(4/4)	0.2
		10-fold	18.32±10.44				21.42±17.49		1.45±1.40	
		diluted	(4/4)		nd		(4/4)		(4/4)	
			8.39±4.24		11.55±9.35		10.31±5.97		2.92±2.04	
	2.90x10 ³	Pure	(4/4)	0.6	(4/4)	-	(3/4)	3.8	(4/4)	0.3
		10-fold								
		diluted	4.60 (1/4)		nd		39.18 (1/4)		0.87 (1/4)	
MNV-1			68.11±10.25		52.84±37.98		83.29±31.48		44.48±31.69	
			(12/12)		(12/12)		(12/12)		(12/12)	

			Repeat experim	ents						
Virus	Quantity of genome copies/500mL	RNA extracts	R1 (%±SD)	(F)	R2 (%±SD)	(F)	R3 (%±SD)	(F)	R4 (%±SD)	(F)
			33.99±3.37		33.58±13.89		46.79±10.19		27.87±18.38	
	1.40x10 ⁴	Pure	(4/4)	1.4	(4/4)	1.2	(4/4)	>2.1	(4/4)	2.3
		10-fold	46.82±4.36		38.69±26.68				63.12±44.91	
		diluted	(3/4)		(4/4)		>100 (4/4)		(4/4)	
			36.82±7.78		29.96±12.18		45.61±34.56		53.54±25.50	
HEV/	1.40x10 ³	Pure	(3/4)	1.3	(2/4)	>3.3	(4/4)	>2.2	(4/4)	1.2
IIL V		10-fold	48.33±66.59						65.94±59.28	
		diluted	(3/4)		>100 (1/4)		>100 (2/4)		(4/4)	
			52.59±64.40						54.84±45.31	
	1.40x10 ²	Pure	(3/4)	>1.9	nd	-	>100 (3/4)	>1.0	(4/4)	>1.8
		10-fold								
		diluted	>100 (1/4)		nd		>100 (2/4)		>100 (2/4)	
N/INI\/_1			48.21±20.98		37.70±11.61		39.80±11.56		47.81±12.18	
			(12/12)		(12/12)		(12/12)		(12/12)	

			Repeat experime	ents						
Virus	Quantity of genome copies/500mL	RNA extracts	R1 (%±SD)	(F)	R2 (%±SD)	(F)	R3 (%±SD)	(F)	R4 (%±SD)	(F)
	1.60x10 ⁵	pure	5.39±3.44 (4/4)	-	3.40±0.81 (4/4)	-	12.86±2.62 (4/4)	0.1	2.99±1.00 (2/4)	0.93
		diluted	nd		nd		1.67±1.04 (2/4)		2.87 (1/4)	
norovirus	1.60x10 ⁴ pure		nd	-	nd	-	6.91 (1/4)	0.3	0.21 (1/4)	-
GI		10-fold diluted	nd		nd		1.81 (1/4)		nd	
	1.60x10 ³	pure	12.47 (1/4)	-	nd	-	51.35 (1/4)	-	1.69 (1/4)	-
		10-fold diluted	nd		nd		nd		nd	
MNV-1			69.05±15.81 (12/12)		53.934±39.02 (12/12)		77.35±26.26 (12/12)		57.47±15.10 (12/12)	

			Repeat experir	nents						
Virus	Quantity of genome copies/500mL	RNA extracts	R1 (%±SD)	(F)	R2 (%±SD)	(F)	R3 (%±SD)	(F)	R4 (%±SD)	(F)
			4.95±1.38		8.02±0.18		6.51±0.53		2.50±2.48	
	1.30x10 ⁵	pure	(4/4)	0.1	(4/4)	0.5	(4/4)	0.4	(4/4)	2.4
		10-fold	0.75±0.79		3.72±3.59		2.55±2.93		5.94±1.14	
		diluted	(4/4)		(4/4)		(4/4)		(2/4)	
			1.12±0.25				0.79±0.56			
norovirus Gli	1.30x10 ⁴	pure	(2/4)	-	nd	-	(4/4)	-	nd	-
GII		10-fold								
		diluted	nd		nd		nd		nd	
	1.30x10 ³	pure	nd	-	nd	-	31,95 (1/4)	-	2.99 (1/4)	-
		10-fold								
		diluted	nd		nd		nd		nd	
			58.68±18.18		52.46±31.47		62.50±33.07		56.87±26.45	
MNV-1			(12/12)		(12/12)		(12/12)		(12/12)	

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 cycle threshold (Ct) determinations is mentioned for HAV, HEV, noroviruses GI and GII. RNA extracts were tested twice for each operator, resulting in four Cq values for each sample. The ratio between mean values for extraction yields obtained with undiluted RNA extracts and those obtained with 10-fold diluted RNA extracts was calculated to determine whether the dilution of RNA extracts improved mean extraction yields (F). nd: not detected. The negative results some samples were not considered in any way while calculating the recoveries.

Virus target	Genome copies/mL of water sample	R1	R2	R3	R4	All settings
HAV	LOD ₅₀	<5.8*	22	3	<5.8*	11.8
	LOD ₉₅	<5.8*	182	12.6	<5.8*	50
HEV	LOD ₅₀	0.68	3.2	0.14	<0.28*	0.64
	LOD ₉₅	3	13.4	0.6	<0.28*	2.8
norovirus Gl	LOD ₅₀	66	96	40	176	94
	LOD ₉₅	280	42	174	760	420
norovirus GII	LOD ₅₀	28	78	4.6	54	30
	LOD ₉₅	126	340	20	220	134

Table 4: LOD₅₀ and LOD₉₅ calculated using the Wilrich approach for HAV, HEV and norovirus.

(*LOD₅₀ and LOD₉₅ were estimated. It was not possible to determine values because the LOD was not reached.)