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1 **Validation of ISO Method 15216 Part 1 – Quantification of Hepatitis A Virus and Norovirus in**
2 **Food Matrices**

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26

27 **Abstract**

28 Hepatitis A virus (HAV) and norovirus are important agents of food-borne human viral illness, with
29 common vehicles including bivalve molluscan shellfish, soft fruit and various vegetables. Outbreaks of
30 viral illness due to contamination of the surfaces of foods, or food preparation surfaces by for example
31 infected food handlers are also common. Virus analysis of food matrices can contribute towards risk
32 management for these hazards and a two-part technical specification for determination of Hepatitis A
33 virus and norovirus in food matrices (ISO/TS 15216:2013) was published jointly by the European
34 Committee for Standardisation and the International Organization for Standardization in 2013.

35 As part of the European Mandate No. M381 to validate 15 standards in the field of food microbiology,
36 an international validation study involving 18 laboratories from 11 countries in Europe was conducted
37 between 2012-2014. This study aimed to generate method characteristics including limit of detection,
38 limit of quantification, repeatability and reproducibility for ISO 15216 – Part 1, the method for
39 quantification, in seven food matrices.

40 The organisation and results of this study, including observations that led to improvements in the
41 standard method are presented here. After its conclusion, the method characteristics generated were
42 added to the revised international standard, ISO 15216-1:2017, published in March 2017.

43 **Keywords**

44 norovirus; hepatitis A virus; real-time RT-PCR; validation; standardisation; bivalve molluscan shellfish;
45 soft fruit; vegetables; bottled water; surfaces

46 **1. Introduction**

47 Hepatitis A virus (HAV) and norovirus are important agents of food-borne human viral illness. The
48 foodstuffs most commonly linked to illnesses caused by these viruses include bivalve molluscan
49 shellfish (reviewed in Bellou et al, 2013), soft fruit (Bernard et al., 2014; Severi et al., 2015) and a variety
50 of leaf, stem or bulb vegetables including lettuce (Ethelberg et al, 2010) and green onions (Dentinger
51 et al, 2001). Separately the presence of norovirus RNA in bottled water has been variously reported
52 and debated (Beuret et al., 2000; Blanco et al., 2017; Sanchez et al., 2005). Outbreaks of viral illness
53 due to contamination of the surfaces of foods, or food preparation surfaces by for example infected food
54 handlers have also been documented (Chen et al., 2016; Thornley et al., 2016). Virus analysis can
55 contribute towards risk management for these hazards. However, until recently no standard method
56 has existed for virus analysis in foods and it is documented that different methods can give divergent
57 results (Lees and CEN WG6 TAG4, 2010). For these reasons a European project to develop a
58 standardised method to detect these viruses in a variety of food matrices was launched in 2004 by
59 CEN/TC275/WG6, the European Committee for Standardisation (CEN) Working Group on the
60 Microbiology of the Food Chain, with a Technical Advisory Group convened specifically for this purpose
61 (CEN/TC275/WG6/TAG4). Method development within this group proceeded by a combination of
62 consensus, methodological ring-trials within the group and methodological investigations by individual
63 group members. This project culminated in the publication in 2013 of a two-part technical specification
64 for determination of the viruses in food matrices in 2013 (ISO/TS 15216:2013; Anonymous, 2013a and
65 Anonymous, 2013b).

66 As part of the European Mandate No. M381 to validate 15 standards in the field of food microbiology,
67 this study aimed to validate ISO 15216 – Part 1, the method for quantification, in seven food matrices;
68 bottled water, food surfaces (bell pepper pieces), Pacific oysters (*Crassostrea gigas*), common mussels
69 (*Mytilus edulis*), raspberries, lettuce and green onions, in order to replace the technical specification
70 with a full, validated EN/ISO standard.

71

72 **2. Materials and methods**

73 *2.1 The method evaluated*

74 As no routine methods exist for culture of norovirus, and HAV culture methods (Flehmgig, 1980) are not
75 appropriate for routine application to food matrices, detection is reliant on molecular methods using the

76 reverse-transcriptase polymerase chain reaction (RT-PCR). In this study, testing of all samples for
77 norovirus GI, norovirus GII and HAV followed matrix-specific test protocols compliant with ISO 15216-
78 1, using methods for RNA extraction and real-time RT-PCR, real-time RT-PCR primers and probes
79 (Costafreda et al., 2006; da Silva et al., 2007; Hoehne and Schreier, 2006; Loisy et al., 2005; Svraka et
80 al., 2007) and the process control virus (mengo virus strain MC₀; Costafreda et al., 2006), detailed in
81 the informative annexes. For each matrix, the method consisted of a matrix-specific virus extraction
82 followed by common RNA extraction and real-time RT-PCR detection elements. Briefly, for food
83 surfaces, virus extraction used swabbing of the surface with a sterile cotton swab, followed by elution
84 into lysis buffer. For fruit and vegetable matrices, virus extraction was by elution with agitation followed
85 by precipitation with PEG/NaCl (with additional extraction steps for pectin rich fruit). For bottled water,
86 adsorption and elution using positively charged membranes followed by concentration by ultrafiltration
87 was used and for bivalve molluscan shellfish, viruses were extracted from the tissues of the digestive
88 glands using treatment with a proteinase K solution. Virus extracts from all matrices were subjected to
89 a common RNA extraction method based on virus capsid disruption with chaotropic reagents followed
90 by adsorption of RNA to silica particles. Detection of virus sequences within the sample RNA utilised
91 real-time RT-PCR with hydrolysis probes in duplicate reactions for each sample RNA and target virus
92 combination. Undiluted and 1/10 diluted RNA was tested for each sample; in accordance with ISO
93 15216-1, the results for undiluted RNA were used unless this demonstrated significant RT-PCR
94 inhibition, in which case results for 1/10 diluted RNA would be checked. Due to the complexity of the
95 method, a comprehensive suite of controls was included, including negative process, extraction and
96 RT-PCR controls, and controls for RT-PCR inhibition and extraction efficiency. Quantification of target
97 copies per microliter of sample RNA was by reference to a standard curve generated from a dilution
98 series of dsDNA carrying the relevant target sequence as described in Annex G of ISO 15216-1:2017
99 (Anonymous, 2017b). In accordance with ISO 15216-1, quantities were not corrected according to
100 extraction efficiency and RT-PCR inhibition results.

101 *2.2 Design of the study*

102 For each matrix, the study comprised two parts; part 1; method characterisation and part 2;
103 interlaboratory study. For each matrix part 1 was carried out by a single expert laboratory (Table 1)
104 testing 60 samples of matrix contaminated with a dilution series of the three target viruses in order to
105 determine method characteristics including limit of detection and limit of quantification. Part 2 was

106 carried out by one organising expert laboratory and 10 participating laboratories per matrix. Each
107 participating laboratory tested duplicate samples, prepared and distributed by the organising laboratory,
108 designed to represent 4 levels of contamination (high, medium, low, and negative) in order to determine
109 method characteristics including reproducibility and repeatability. In total, 18 laboratories from 11
110 countries in Europe participated in one or both parts of the study for one or more matrices.

111 In its role as project leader, the European Union Reference Laboratory for Monitoring Bacteriological
112 and Viral Contamination of Bivalve Molluscs, based at the Centre for Environment, Fisheries and
113 Aquaculture Science, Weymouth, United Kingdom, was responsible for management of the validation
114 project, development of study protocols, generation and distribution of control materials and virus
115 stocks, and collation and analysis of study data.

116 *2.3 Part 1; method characterisation – general considerations*

117 For part 1 of the study, test sample sizes were 2g (bivalve shellfish digestive tissues), 25g (fruit and
118 vegetables), 330ml (bottled water) and 25cm² (bell pepper pieces to represent the food surfaces matrix).

119 All samples were tested using the relevant matrix-specific test protocols (compliant with ISO 15216-1)
120 then both calculated quantities and raw data were forwarded to the project leader for quality checking
121 according to ISO 15216-1 and analysis as described under Generation of Method Characteristics below.

122 *2.4 Part 1; method characterisation – preparation of the virus dilution series*

123 The viruses used for contamination were genotypes GI.4 and GII.4 norovirus from faecal suspensions
124 and HM175/43c strain HAV derived from tissue culture. The limited availability of norovirus stocks
125 (particularly GI) meant that a simple log₁₀ dilution series with sufficiently high starting levels to allow for
126 contamination across a wide range of dilutions was not practical; for this reason a 0.5 log₁₀ dilution
127 series (~3.16x) was used instead. For each matrix 0.5 log₁₀ dilution series of contaminated matrix
128 samples at 9 separate levels were prepared as below (different strategies reflect the modes of natural
129 contamination of the different food matrices). In each case virus dosing was calculated to provide
130 detectable levels of 100 - 1,000 copies/μl in the RNA extract at the highest contamination level
131 (equivalent to ~20,000 - 200,000 copies/g for bivalve shellfish samples, 400 - 4,000 copies/g for fruit
132 and vegetable samples, 400 - 4,000 copies/cm² for food surface samples and 30 – 300 copies/ml for
133 bottled water samples), based on the results of a trial contamination:-

- 134 a) Bivalve shellfish matrices; contaminated matrix was prepared by bioaccumulation of
135 shellfish with the 3 target viruses. Food containing the viruses was added to a tank containing

136 shellfish undergoing filter feeding behaviour, then shellfish were harvested after 18hrs
137 (oysters) or 24hrs (mussels). Contaminated digestive tissues were dissected and
138 homogenised by blending, then a $\approx 0.5 \log_{10}$ dilution series of this contaminated matrix in
139 homogenised uncontaminated matrix (digestive tissues from clean shellfish) was prepared by
140 serial blending of 3.8 g of contaminated matrix in 8.2 g of uncontaminated matrix.

141 b) Non-shellfish matrices; a mixture of the 3 target viruses at high initial levels was
142 prepared in buffer, then a $\approx 0.5 \log_{10}$ dilution series of this was prepared by serial dilution of
143 e.g. 100 μl contaminated virus mix in 216 μl buffer. Clean matrix samples were then
144 contaminated with the different dilutions. For fruit, vegetable and food surface samples, the
145 virus solution was pipetted across the surface of the samples, then left to air dry for 20 minutes
146 in a laminar flow cabinet. For bottled water samples, the virus solution was added directly to
147 the sample.

148 *2.5 Part 1; method characterisation – structure of the study*

149 For each matrix a total of 54 contaminated samples were prepared as follows (the different strategies
150 reflect the time practicalities of virus extraction methods for the different food matrices; strategies a)
151 and b) with triplicate subsamples were used where practicable, strategies c) or d) were used where it
152 was not practical to perform virus extraction on 27 samples simultaneously):-

153 a) Bivalve shellfish matrices; triplicate subsamples were prepared for each level of the 0.5
154 \log_{10} dilution series as described above. This entire procedure was carried out on two
155 separate occasions (9 dilution levels x 3 subsamples per level x 2 occasions = 54 samples).

156 b) Food surface matrix; for each level of the 0.5 \log_{10} dilution series of virus mix triplicate
157 subsamples of clean matrix (bell pepper pieces) were prepared. In each case, the virus
158 solution was pipetted across the exterior surface of the samples, then left to air dry for 20
159 minutes in a laminar flow cabinet. This entire procedure was carried out on two separate
160 occasions (9 dilution levels x 3 subsamples per level x 2 occasions = 54 samples).

161 c) Bottled water matrix; single samples were prepared for each level of the 0.5 \log_{10}
162 dilution series of virus mix. In each case, the virus solution was added directly to the sample.
163 This entire procedure was carried out on six separate occasions (9 dilution levels x 6
164 occasions = 54 samples).

165 d) Fruit and vegetable matrices; single samples were prepared for each level of the 0.5
166 \log_{10} dilution series of virus mix. In each case, the virus solution was pipetted across the
167 exterior surface of the samples, then left to air dry for 20 minutes in a laminar flow cabinet.
168 This entire procedure was carried out on six separate occasions (9 dilution levels x 6
169 occasions = 54 samples).

170 In addition to 54 contaminated samples, 6 samples of uncontaminated matrix were tested in parallel to
171 give a total of 60 test samples per matrix.

172 *2.6 Part 2; interlaboratory study – general considerations*

173 For part 2 of the study, test sample sizes were 2g (bivalve shellfish digestive tissues), 25g (fruit and
174 vegetables), 300ml (bottled water) and 50cm² (bell pepper pieces to represent the food surfaces matrix).
175 For each matrix interlaboratory study all samples were tested using the relevant matrix-specific test
176 protocols (compliant with ISO 15216-1) then both calculated quantities and raw data were forwarded to
177 the project leader for quality checking according to ISO 15216-1 and analysis as described under
178 Generation of Method Characteristics below.

179 *2.7 Part 2; interlaboratory study – preparation of test samples*

180 The viruses used for contamination were the same as for part 1. For each of the 7 matrices the relevant
181 expert laboratory prepared four batches of test samples each comprising multiple samples containing
182 high, medium, low or negative levels of each of the three target viruses. In each case virus dosing was
183 calculated to provide detectable levels of 20 - 200 copies/ μ l in the RNA extract at the highest
184 contamination level (equivalent to ~4,000 - 40,000 copies/g for bivalve shellfish samples, 80 - 800
185 copies/g for fruit and vegetable samples, 40 - 400 copies/cm for food surface samples and 6.7 – 67
186 copies/ml for bottled water samples), based on the results of a trial contamination. For medium and low
187 levels respectively, intended levels were 1/5th and 1/25th those in the high levels samples respectively.
188 For Pacific oysters a single bioaccumulation to produce highly contaminated digestive tissues was
189 carried out. This material was homogenised then high, medium and low level batches were prepared
190 by diluting this contaminated matrix in uncontaminated matrix (digestive tissues from uncontaminated
191 Pacific oysters) to the appropriate levels. Through this approach it was aimed to produce homogenous
192 starting materials with a clearly defined proportional difference between the different contamination
193 levels. For common mussels due to the practical difficulties of dissection of sufficient quantities of
194 digestive tissues in a single laboratory (the digestive tissues of individual mussels are smaller than for

195 oysters), three separate bioaccumulations to produce high, medium and low levels were carried out.
196 For other matrices, multiple portions of matrix were directly contaminated with virus stocks containing
197 a mix of all three target viruses at high, medium or low levels using contamination methods as described
198 for part 1.

199 To demonstrate adequate homogeneity and stability a minimum of ten samples were tested for each
200 contamination level by the expert laboratory using the relevant matrix-specific protocol. The number of
201 samples and testing schedule was dependent on the storage temperature and the required lifetime of
202 test samples as described below.

203 *2.8 Part 2; interlaboratory study – testing by participant laboratories*

204 For each matrix eight anonymised test samples (duplicate samples for each contamination level) were
205 sent to each of ten participating laboratories (expert laboratories did not participate in the interlaboratory
206 study for matrices where they prepared test samples). Each participating laboratory tested the eight
207 samples using the relevant matrix-specific protocol within a specified timescale. Depending on whether
208 it was practical to freeze samples (due to considerations including damage to the samples from freeze-
209 thaw cycles) test samples were either distributed frozen or chilled. For matrices where test samples
210 were distributed frozen (bivalve molluscs, green onions, raspberries) laboratories were permitted to
211 store samples frozen prior to testing, but were instructed to return data to the project leader by a
212 specified date 2 months from the receipt of samples. For matrices where test samples were distributed
213 chilled (lettuce, bottled water, food surfaces) laboratories were instructed to carry out virus and RNA
214 extractions within 48hrs of receipt, and were further instructed to return data to the project leader by a
215 specified date 1 month from the receipt of samples. In all cases protocols required participating
216 laboratories to respect maximum storage times and temperatures of intermediate test materials (virus
217 extract and RNA).

218 *2.9 Generation of method characteristics – general considerations*

219 Results for test samples that were invalid according to the protocols due to unacceptable extraction
220 efficiency or RT-PCR inhibition levels were excluded from the analysis, as were false negative results.
221 Obtained results below the theoretical limit of detection (tLOD; the concentration equivalent to the
222 detection of a single target copy across the two real-time RT-PCR reactions) were adjusted upwards to
223 the tLOD.

224 *2.10 Generation of method characteristics – limit of detection and limit of quantification*

225 Limit of detection (LOD) and limit of quantification (LOQ) characteristics were determined using the data
226 generated in part 1; method characterisation. For these data sets, “anticipated results” (the designated
227 correct result) for each contaminated test sample were calculated (separately for each dilution series)
228 as follows:-

229 a) Bivalve shellfish matrices and food surfaces (matrices where triplicate subsamples at
230 each level of the dilution series were used); the anticipated result at each level for each dilution
231 series was calculated as the geometric mean of the obtained results for the 3 subsamples at
232 the highest concentration within the series (\equiv anticipated result at the highest concentration)
233 multiplied by the dilution factor. For example, for oysters, norovirus GI, occasion 1, obtained
234 results for the 3 subsamples at the highest concentration were 18036 copies/g, 21220
235 copies/g and 9415 copies/g respectively (geometric mean = 15331 copies/g). The anticipated
236 results at the different dilutions of the series were therefore calculated at 15331, 4848 (15331
237 $\times 10^{-0.5}$), 1533, 484, 153, 48, 15, 5 and 2 copies/g respectively.

238 b) Bottled water, fruit and vegetable matrices (matrices where single samples at each
239 level of the dilution series were used); the anticipated result at each level for each dilution
240 series was calculated as the antilog of the intercept of the line of best fit for the plot of \log_{10}
241 positive obtained results vs. \log_{10} dilution factor (\equiv anticipated result at the highest
242 concentration within the series), multiplied by the dilution factor. For example, for bottled
243 water, norovirus GI, occasion 1, results for the 9 samples within the dilution series were 141.84
244 copies/ml, 56.86 copies/ml, 19.72 copies/ml, 4.35 copies/ml, 0.75 copies/ml, 0.24 copies/ml,
245 0.08 copies/ml, 0.04 copies/ml and not detected. The intercept of the line of best fit of the \log_{10}
246 positive obtained results vs. \log_{10} dilution factor was 2.23, corresponding to an antilog of
247 170.01 copies/ml. The anticipated results at the different dilutions of the series were therefore
248 calculated at 170.01, 53.76 ($170.01 \times 10^{-0.5}$), 17.00, 5.37, 1.70, 0.54, 0.17, 0.05 and 0.02
249 copies/ml respectively.

250 The LOD and LOQ were then calculated for each matrix/target virus combination using the datasets of
251 anticipated vs. obtained results as follows:-

252 a) the LOD_{95} (the lowest concentration of target virus that can be consistently detected in 95%
253 of samples tested under routine laboratory conditions) was calculated using an online
254 Microsoft EXCEL program for the estimation of the POD (probability of detection) function and

255 the LOD of a qualitative microbiological measurement method according to Wilrich and Wilrich
256 (2009) using the number of positive and negative results at each anticipated level
257 (http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/PODLOD_ver9.xls
258 accessed 10th November 2017).

259 b) the LOQ was calculated for each matrix/target virus combination using a method adapted
260 from Armbruster and Pry (2008). This characteristic was determined by looking at the \log_{10}
261 transformed data from the half \log_{10} immediately below the LOD and higher (e.g. if LOD was
262 $1.85 \log_{10}$ then results from an anticipated level of $1.5 \log_{10}$ and upwards were used). A
263 regression line was fitted to these selected data and the residuals calculated (differences
264 between observed value and fitted line). The standard deviation of these residuals was
265 calculated in one \log_{10} intervals of the data (moving up in half \log_{10} steps) and the LOQ was
266 determined as the level above which the standard deviation was always below $0.5 \log_{10}$. The
267 LOQ was set no lower than the LOD.

268 *2.11 Generation of method characteristics – repeatability and reproducibility*

269 Repeatability and reproducibility characteristics were determined using the data generated in part 2;
270 interlaboratory study. All data returned by the participant laboratories was \log_{10} transformed then
271 outlying results for each matrix/target virus/contamination level combination were identified using
272 Mandel's h and k statistic (test for graphical consistency; Mandel, 1985), Cochran's test for within-
273 laboratory variability (Cochran, 1941) and Grubbs' test for between-laboratory variability (Grubbs,
274 1950). Pairs of results identified by these tests as outliers were removed from the data set provided this
275 did not result in fewer than 16 valid, positive data points remaining in the set (out of 20 total). Where
276 individual laboratories recorded a high proportion (>50%) of invalid, false negative or outlying results
277 across a given matrix, the entire data set for that laboratory/matrix combination (for all target viruses
278 and all contamination levels) was excluded from the analysis. Repeatability and between-laboratory
279 variances were calculated according to the formulae provided in ISO 5725-2:1994 (Anonymous, 1994).
280 The reproducibility variance was then calculated as the sum of the repeatability and between-laboratory
281 variances, and the repeatability and reproducibility standard deviations determined as the square root
282 of the respective variances. Repeatability and reproducibility limits were calculated as the respective
283 standard deviations multiplied by a fixed factor of 2.8 ($\sim 2\sqrt{2}$). These limits are defined as the absolute
284 difference between two independent single (\log_{10} -transformed) test results or the ratio of the higher to

285 the lower of the two test results on the normal scale, obtained under repeatability or reproducibility
286 conditions respectively, that will not be exceeded in more than 5% of cases.

287 **3. Results and Discussion**

288 *3.1 Recalibration of norovirus GII results*

289 After completion of all practical work it became apparent through unconnected sequencing analysis
290 carried out by a CEN/TC275/WG6/TAG4 group member that the dsDNA quantification standard for
291 norovirus GII used in both parts of the validation comprised a mix of two distinct sequences (sequence
292 y and sequence z – see Figure 1), rather than the intended sequence (sequence x). Sequences y and
293 z each included mismatches relative to the primer and probe set used (major for sequence y, including
294 several missing nucleotides in the probe target region, minor for sequence z). As a result, the mixed
295 dsDNA standard exhibited reduced amplification efficiency and increased Cq values. This caused an
296 upward bias in quantitative results obtained for the test samples relative to those that would have been
297 obtained using the intended sequence.

298 To rectify this issue a calibration factor was used to adjust GII test sample results to compensate for
299 the quantification bias introduced by the incorrect sequences. To establish the appropriate calibration
300 factor, the GII dsDNA quantification standard used in the validation (adjusted to 1×10^5 copies/ μ l
301 following quantification by spectrophotometry) was subjected to qPCR alongside a dilution series
302 prepared using newly synthesised dsDNA quantification control with no mismatches relative to the
303 primer and probe sequences (sequence x). This established that the validation standards exhibited an
304 effective 3.157x consistent reduction in amplification efficiency (≈ 1.66 Cq values), resulting in a
305 corresponding 3.157x overestimation in quantitative results for test samples in the validation (results
306 not shown). All GII results in both parts of the validation were therefore adjusted downwards by this
307 same factor. This issue affected absolute quantification but not relative quantification of samples within
308 and between laboratories. Therefore, of the performance characteristics determined, only LOD and
309 LOQ were affected by this adjustment. Repeatability and reproducibility characteristics were not
310 impacted.

311 Specific investigations to identify the root cause of the mixed sequences in the GII dsDNA quantification
312 standard were not carried out. However, it seems likely that sequence errors were introduced either
313 during the synthesis of the insert sequence, or the cloning of the insert into the plasmid vector. The
314 presence of two sequences in the purified plasmid preparation could be explained if during the cloning

315 procedure a mixed colony of transformed *E. coli* host cells containing two different clonal populations
316 (due to two different transformant cells colonising the selection media in close proximity) was inoculated
317 into the growth medium used for plasmid preparation.

318 As a result of this issue a mandatory sequence verification for quantification standards (either by the
319 user laboratory or the supplier) was introduced to the text of ISO 15216-1:2017 (Anonymous, 2017b)
320 to ensure quantification standards contain the intended sequences.

321 *3.2 Dilution of quantification standards using water only*

322 Quantification standards prepared for part 1 of the validation study were diluted to working
323 concentration, and further diluted to produce the standard curve, using molecular biology grade water
324 as the diluent, consistent with the text of ISO/TS 15216-1:2013 (Anonymous, 2013a). During the course
325 of part 1 it became apparent that sporadic problems with stability and homogeneity of standards, and
326 the production of the standard curve, had occurred. Analysis of quantification standard curve data from
327 part 1 of the validation study (data not shown) indicated that significant issues with the performance of
328 the quantification standards were experienced in the studies on the food surfaces and green onions
329 matrices, with problems including high intercept values, significant variability in intercept values
330 between real-time RT-PCR runs and poor PCR efficiency values (slope <-3.6). For other matrices no
331 such issues were apparent. For these reasons LOD and LOQ values generated for the affected matrices
332 in part 1 were considered unreliable and unrepresentative of the affected matrices and are not included
333 here.

334 Prior to the start of part 2 of the validation a series of experiments to investigate this issue were carried
335 out (data not shown). It was determined that the issues experienced had been caused by the use of
336 water as diluent in the dilution of quantification standards to working concentration and for the
337 production of the standard curve, and that these issues could be eliminated by using an appropriate
338 buffer (e.g. TE buffer) as diluent. Accordingly, this practise was adopted for part 2 of the validation study
339 and no problematic results were noted. As a result of these findings, the text of ISO 15216-1:2017
340 (Anonymous, 2017b) specifically mandates the use of an appropriate buffer for such dilutions, rather
341 than water only.

342 *3.3 RT-PCR inhibition in the common mussel matrix*

343 During part 1 of the validation study for the common mussel matrix a very high proportion (64.4%) of
344 test samples provided results above the acceptable RT-PCR inhibition threshold (75% as detailed in

345 ISO 15216-1) when undiluted RNA was tested. A smaller proportion (11.1%) also provided results
346 above the acceptable threshold when 1/10 diluted RNA was tested. Accordingly, the majority of test
347 sample results for the mussel matrix were determined using diluted RNA, as per the text of ISO 15216-
348 1. The protocol for bivalve mollusc matrices for part 1 of the validation study specified the preparation
349 of a dilution series from a single stock of contaminated digestive tissues diluted in a single stock of
350 uncontaminated digestive tissues. It was not possible within the study protocol to take account of
351 possible sample-to-sample matrix variations affecting parameters such as RT-PCR inhibition. From this
352 study it is not clear whether high levels of RT-PCR inhibition are commonly found in common mussel
353 samples, or whether the issue was specific to the particular sample of mussels selected for use in the
354 study. Of note however in part 2 of the validation study only 1.8% of results for common mussel samples
355 were affected by high RT-PCR inhibition levels. It can reasonably be assumed that the use of results
356 from diluted RNA for the majority of test samples in part 1 will have impacted the LOD and LOQ values
357 calculated for common mussels, and that these method characteristics may be unrepresentative of the
358 matrix as a result. For this reason, they are not included here. Further data would therefore be required
359 using non-inhibitory mussels to establish representative LOD and LOQ values for this matrix.

360 *3.4 Results of part 1; method characterisation*

361 As an example of the data obtained in part 1; method characterisation, the results for norovirus GI in
362 the bottled water are shown as a plot of obtained versus anticipated results in Figure 2. The probability
363 of detection/limit of detection plot derived from this data according to Wilrich and Wilrich (2009) is shown
364 in Figure 3. For this dataset the determined LOD was 0.18 copies/ml. For determination of LOQ a line-
365 of-best-fit was prepared for all data points corresponding to \log_{10} transformed anticipated levels above
366 0.1 copies/ml (shown in Figure 2). The standard deviations of the residuals from the line-of-best fit of
367 all data points in one \log_{10} intervals are shown in Table 2. In all cases these were below 0.5; the LOQ
368 for norovirus GI in bottled water was therefore set at the same level as the LOD. Results were generated
369 for the other study matrices using the same approach (calculations not shown).

370 *3.5 Results of part 2; interlaboratory study*

371 As an example of the data obtained in part 2; interlaboratory study, the results for norovirus GI in the
372 bottled water for the high, medium, low and negative levels are shown in Figure 4. Test samples for this
373 matrix were distributed chilled; the expert laboratory tested 5 subsamples at each level prior to the
374 distribution ("post-preparation") and five subsamples at the end of the period for testing allotted to the

375 participating laboratories (“post distribution”). Following quality control checking, one negative result
376 (Lab 08, low, subsample 1) was removed from the data set. The method characteristics derived from
377 the dataset for this matrix/target virus combination are shown in Table 3. Results were generated for
378 the other study matrices using the same general approach (calculations not shown).

379 *3.6 Method characteristics for all matrices*

380 The LOD, LOQ, repeatability standard deviations and reproducibility standard deviations calculated for
381 the seven matrices under examination are given in Tables 4 to 10. The repeatability and reproducibility
382 standard deviations shown are the averages of those obtained at high, medium and low contamination
383 levels; separate repeatability and reproducibility standard deviations and limits for all contamination
384 levels are provided in Annex J of ISO 15216-1:2017 (Anonymous, 2017b).

385

386 **4. Conclusion**

387 This paper details the validation study carried out on ISO 15216-1, the standard method for
388 quantification of hepatitis A virus and norovirus in foods. Detailed information on the performance of the
389 method in seven different matrices was generated and has been included in ISO 15216-1:2017
390 (Anonymous, 2017b), the newly published edition of the standard. Acceptability criteria for the method
391 characteristics have not been determined. The values obtained for repeatability and reproducibility in
392 this study are comparable to other quantitative (enumeration) methods for bacterial pathogens validated
393 at the same time under European Mandate No. M38 however. For example, ISO 10272-2, the colony
394 count method for *Campylobacter* spp. (Anonymous, 2017a) produced average repeatability and
395 reproducibility standard deviations of 0.20 and 0.40 log₁₀ respectively, compared with 0.23 and 0.50 for
396 the virus method. For LOD and LOQ characteristics it was notable that in the majority of cases the
397 values determined for HAV were higher than for norovirus GI and GII. The primer set used in this study
398 amplifies a relatively long product (157-188 bp depending on the strain of HAV), while for norovirus
399 shorter, more optimal products are amplified (86 bp and 89 bp for GI and GII respectively; Anonymous,
400 2017b). This difference may have accounted for the different relative sensitivities of the methods for the
401 different target viruses. Design of a broadly-reactive real-time RT-PCR primer probe set for HAV that
402 amplifies a shorter product is complicated by the sequence diversity across strains however.

403 A number of technical issues were encountered through the validation study. It was necessary to
404 recalibrate results for norovirus GII due to an issue with the sequence of the quantification standard. A

405 mandatory sequence check has been added to the text of the new ISO, however the potential issue of
406 variability between plasmids prepared in different laboratories remains. In this context, the availability
407 of verified standard materials would be desirable. The European Union Reference Laboratory for
408 Monitoring Bacteriological and Viral Contamination of Bivalve Molluscs supports laboratories within its
409 network through the provision of quantification standards, however introduction of commercially
410 available standards would be beneficial, to reduce one possible source of variability in results between
411 laboratories.

412 For the green onions and food surfaces matrices it was not possible to determine LOD and LOQ
413 characteristics due to problems with the performance of the quantification standards. These problems
414 were completely alleviated through the use of TE buffer to dilute the standards; on this matter the
415 literature is diverse with many contradictory examples of the use of either water or a variety of different
416 buffers as a diluent, and no clear recommendations within journal publications in either direction. This
417 presumably reflects the sporadic and unpredictable nature of the problems encountered, as in many
418 cases the use of water will not result in problems (and indeed did not always in this validation study).
419 However, the introduction of a mandatory requirement for dilution of standards with a suitable buffer
420 should ensure more stable performance.

421 Finally, problems were encountered during part 1 of the validation for mussels, due to the inhibitory
422 nature of the matrix. This raises questions of the universal applicability of the method described in the
423 ISO to different types of foods within the broad categories of bivalve molluscs, leaf, stem and bulb
424 vegetables etc. In the case of mussels it seems likely that the problems encountered were due to an
425 unusually inhibitory batch; many TAG4 members have successfully applied the ISO method to the
426 analysis of mussels. However, the possibility of certain species of bivalve or types of vegetable
427 providing consistently poor results remains; laboratories encountering such issues should consider
428 whether the method in ISO-15216 is appropriate in these cases.

429 In addition to ISO 15216-1, a second part to the standard, ISO 15216-2, detailing a method for
430 qualitative detection (not quantification) was published as a technical specification in 2013 (Anonymous,
431 2013b). Although the validation study was not designed to examine this part of the method, the data
432 generated is sufficient to determine relevant method characteristics such as LOD₅₀, specificity and
433 sensitivity, and CEN/TC275/WG6 and its ISO sister group, ISO/TC34/SC9 have resolved that a new

434 revision of ISO 15216-2, harmonised with ISO 15216-1:2017, and including method characteristics
435 determined in this way, should be developed.

436 European Union legislation foreshadows the adoption of virus controls for bivalve shellfish when the
437 methods are sufficiently developed (Anonymous, 2005) while emergency legislation mandating virus
438 testing on imports to the European Union of strawberries from China (Anonymous, 2012) and
439 raspberries from Serbia (Anonymous, 2015) has also been passed in recent years. The availability of a
440 validated EN/ISO standard should enable the introduction of more robust and quality assured food
441 hygiene controls for high risk foods both in Europe and more widely, and will contribute to improved
442 food safety and a reduced burden of food-borne viral illness.

443

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479

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564 microbiological measurement method. *AOAC International.* 92, 1763 - 1772.
565

566 Table 1

567 Expert laboratories involved in the validation

Laboratory	Matrix or matrices
State Office for Consumer Protection Saxony-Anhalt	Food surfaces
National Institute for Public Health and the Environment	Raspberries and Lettuce
Enteric Virus Laboratory, University of Barcelona	Green onions
Nestlé Research Centre	Bottled water
Centre for Environment, Fisheries and Aquaculture Science	Pacific oysters
Institut Français de Recherche pour l'Exploitation de la Mer	Common mussels

568

569 Table 2

570 Calculation of the LOQ for norovirus GI in bottled water

Range (\log_{10} copies/ml)	Number of data points in range	Standard deviation of residuals ^a
-1.0 to 0.0	12	0.29
-0.5 to 0.5	12	0.31
0.0 to 1.0	12	0.29
0.5 to 1.5	12	0.27
1.0 to 2.0	12	0.17
1.5 to 2.5	11	0.14

571 ^a residuals calculated for each data point in the range against a line-of-best-fit for all observations (-1.0

572 to 2.5 \log_{10} copies/ml)

573

574

575 Table 3

576 Repeatability and reproducibility characteristics for norovirus GI in bottled water

	contamination level		
	low	medium	high
Number of samples tested	20	20	20
Number of samples retained after evaluation of the data	19	20	20
Mean value Σa (\log_{10} copies/ml)	-0.36	0.32	0.97
Repeatability standard deviation s_r (\log_{10} copies /ml)	0.19	0.27	0.10
Repeatability limit r as difference on \log_{10} scale (\log_{10} copies /ml)	0.53	0.74	0.28
Reproducibility standard deviation s_R (\log_{10} copies /ml)	0.50	0.44	0.40
Reproducibility limit R as difference on \log_{10} scale (\log_{10} copies /ml)	1.39	1.24	1.13

577

578 Table 4

579 Method performance characteristics for food surfaces

	Target virus		
	HAV	GI	GII
Limit of detection (copies/cm ²)	nd ^a	nd	nd
Limit of quantification (copies/cm ²)	nd	nd	nd
Repeatability standard deviation (\log_{10} copies/cm ²)	0.20	0.24	0.21
Reproducibility standard deviation (\log_{10} copies/cm ²)	0.39	0.34	0.46

580 ^a nd = not determined

581

582 Table 5

583 Method performance characteristics for raspberries

	Target virus		
	HAV	GI	GII
Limit of detection (copies/g)	3.97	0.65	0.79
Limit of quantification (copies/g)	10.0	10.0	31.6
Repeatability standard deviation (\log_{10} copies/g)	0.19	0.29	0.36
Reproducibility standard deviation (\log_{10} copies/g)	0.38	0.39	0.50

584

585 Table 6

586 Method performance characteristics for lettuce

	Target virus		
	HAV	GI	GII
Limit of detection (copies/g)	3.18	0.46	0.88
Limit of quantification (copies/g)	31.6	0.46	0.88
Repeatability standard deviation (log ₁₀ copies/g)	0.23	0.23	0.25
Reproducibility standard deviation (log ₁₀ copies/g)	0.50	0.42	0.52

587

588

589 Table 7

590 Method performance characteristics for green onions

	Target virus		
	HAV	GI	GII
Limit of detection (copies/g)	nd ^a	nd	nd
Limit of quantification (copies/g)	nd	nd	nd
Repeatability standard deviation (log ₁₀ copies/g)	0.22	0.27	0.24
Reproducibility standard deviation (log ₁₀ copies/g)	0.40	0.59	0.67

591 ^a nd = not determined

592

593 Table 8

594 Method performance characteristics for bottled water

	Target virus		
	HAV	GI	GII
Limit of detection (copies/ml)	0.40	0.18	0.07
Limit of quantification (copies/ml)	1.00	0.18	0.10
Repeatability standard deviation (log ₁₀ copies/ml)	0.16	0.19	0.18
Reproducibility standard deviation (log ₁₀ copies/ml)	0.54	0.45	0.62

595

596

597 Table 9

598 Method performance characteristics for Pacific oysters

	Target virus		
	HAV	GI	GII
Limit of detection (copies/g)	198	34	53
Limit of quantification (copies/g)	198	34	53
Repeatability standard deviation (log ₁₀ copies/g)	0.19	0.18	0.22
Reproducibility standard deviation (log ₁₀ copies/g)	0.57	0.53	0.51

599

600

601 Table 10

602 Method performance characteristics for common mussels

	Target virus		
	HAV	GI	GII
Limit of detection (copies/g)	nd ^a	nd	nd
Limit of quantification (copies/g)	nd	nd	nd
Repeatability standard deviation (log ₁₀ copies/g)	0.27	0.21	0.25
Reproducibility standard deviation (log ₁₀ copies/g)	0.60	0.54	0.52

603 ^a nd = not determined

604

605

606 **Figure 1** Alignment of GII quantification standard sequences with primer and probes used in the
 607 validation study. Matching nucleotides are highlighted in black, mismatches are not highlighted.

```

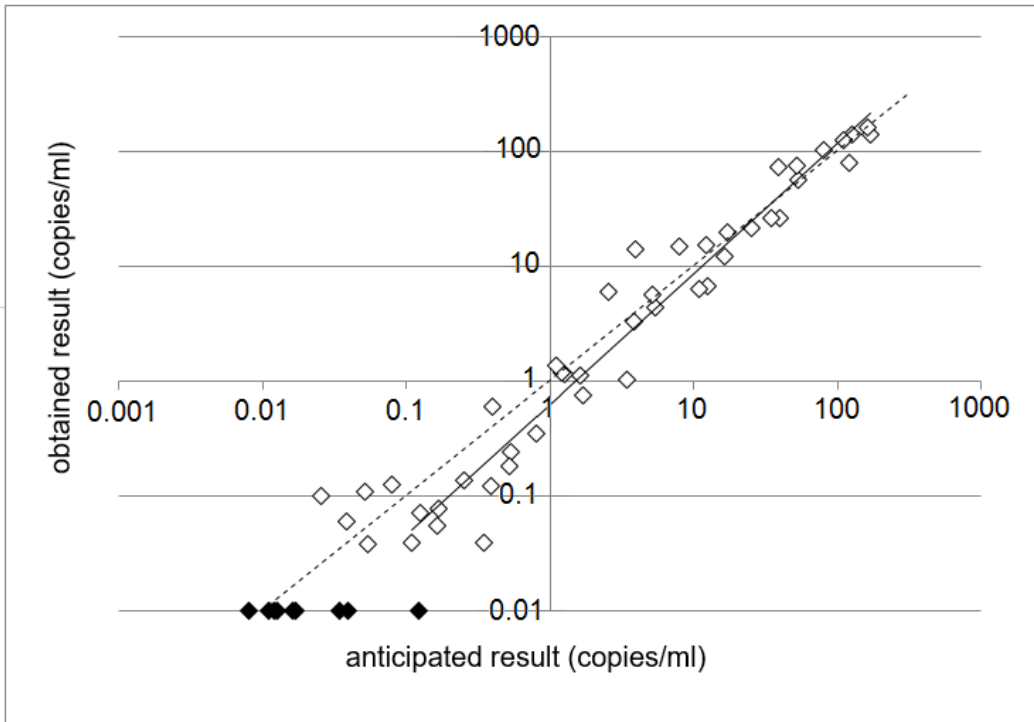
sequence x : ATGTT* 20 * 40 * 60 * 80 *
              CAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCCCAATCTGGCTCGGATCCCCAGCTTTGTGAATGAAGATGGCGTCGA : 95
sequence y : ATGTT* 20 * 40 * 60 * 80 *
              CAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGG-CG-CG-AATCTGG-TCGGATCCCCAGCTTTGTGAATGAAGATGGCGTCGG : 90
sequence z : ATGTT* 20 * 40 * 60 * 80 *
              CAGATGGATGAGATTCTCAGATCTGAGCACGTGGGAGGGCGATCCCAATCTGGCTCGGATCCCCAGCTTTGTGAATGAAGATGGCGTCGG : 95
primers    : ATGTT* 20 * 40 * 60 * 80 *
              CAGATGGATGAGATTCTCWGA-----AGCACGTGGGAGGGCGATCC-----TGTGAATGAAGATGGCGTCGA
  
```

608

609

610

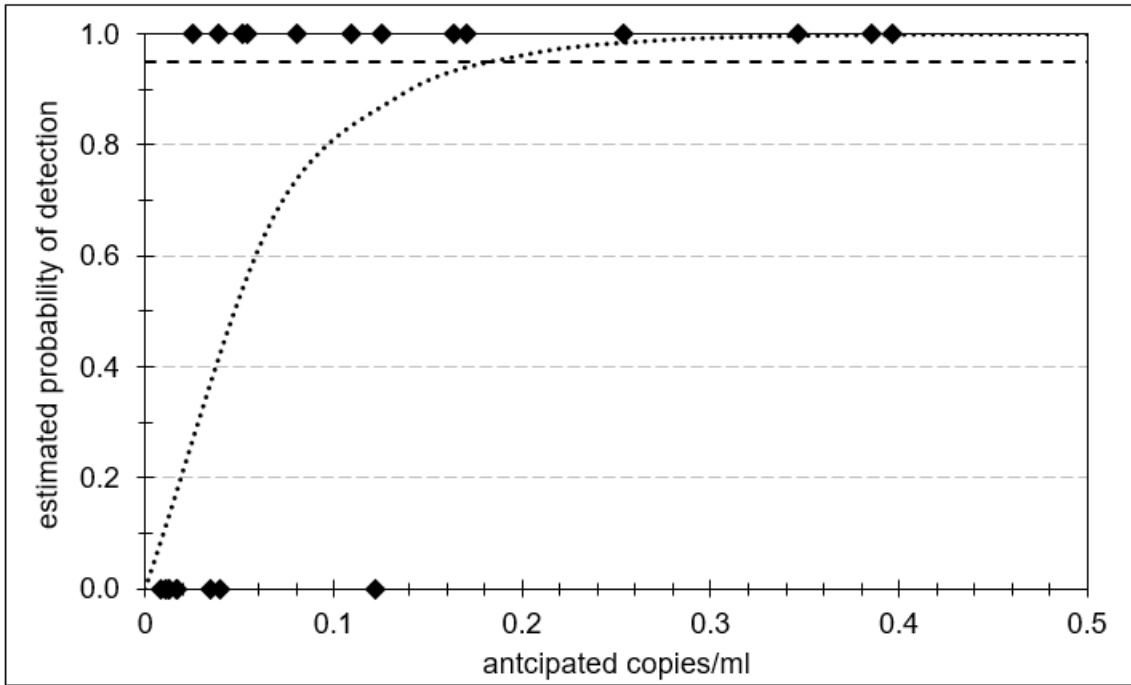
611 **Figure 2** Data for part 1; method characterisation for norovirus GI in bottled water. Data points for
612 negative results are shown at 0.01 copies/ml and shaded black. A line of equivalence is shown as a
613 dashed line, and the line of best-fit used to calculate residuals for determination of the LOQ is shown
614 as a solid line.



615

616

617 **Figure 3** Probability of detection/limit of detection for norovirus GI in bottled water. The probability of
618 detection function derived from the data is shown as a dotted line. The dashed line marks the 95%
619 probability of detection used to determine the LOD.

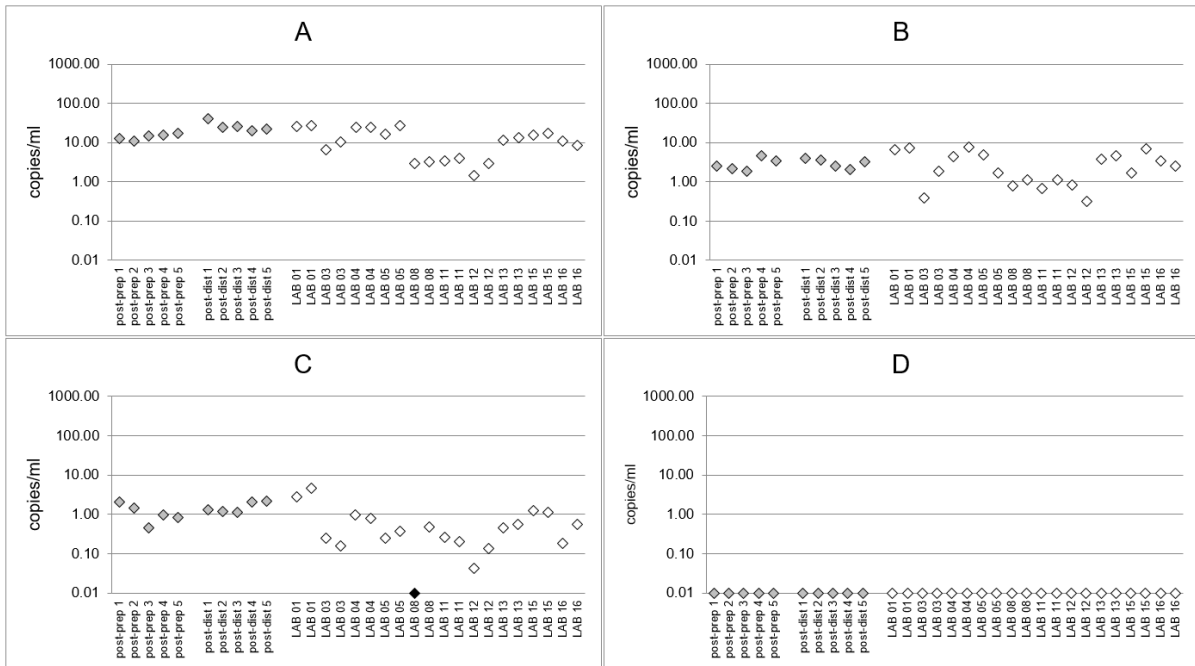


620

621

622

623 **Figure 4** Part 2; interlaboratory study results for norovirus GI in bottled water. A, high contamination
 624 level; B, medium contamination level; C, low contamination level; D, negative. Quality control results
 625 obtained by the expert laboratory are shown to the left of each sub-plot (data points shaded grey).
 626 Results for duplicate subsamples as obtained by the 10 participating laboratories are shown to the right
 627 (data points unshaded). Data points that were removed from the dataset after quality checking are
 628 shaded black. Negative results are shown at 0.01 copies/ml.



629

630