Validation of EN ISO method 15216 - Part 1 – Quantification of hepatitis A virus and norovirus in food matrices

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Abstract

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

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

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

Keywords

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

1. Introduction

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

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

2. Materials and methods

2.1 The method evaluated

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

reverse-transcriptase polymerase chain reaction (RT-PCR). In this study, testing of all samples for norovirus GI, norovirus GII and HAV followed matrix-specific test protocols compliant with ISO 15216-1, using methods for RNA extraction and real-time RT-PCR, real-time RT-PCR primers and probes (Costafreda et al., 2006; da Silva et al., 2007; Hoehne and Schreier, 2006; Loisy et al., 2005; Svraka et al., 2007) and the process control virus (mengo virus strain MC_0; Costafreda et al., 2006), detailed in the informative annexes. For each matrix, the method consisted of a matrix-specific virus extraction followed by common RNA extraction and real-time RT-PCR detection elements. Briefly, for food surfaces, virus extraction used swabbing of the surface with a sterile cotton swab, followed by elution into lysis buffer. For fruit and vegetable matrices, virus extraction was by elution with agitation followed by precipitation with PEG/NaCl (with additional extraction steps for pectin rich fruit). For bottled water, adsorption and elution using positively charged membranes followed by concentration by ultrafiltration was used and for bivalve molluscan shellfish, viruses were extracted from the tissues of the digestive glands using treatment with a proteinase K solution. Virus extracts from all matrices were subjected to a common RNA extraction method based on virus capsid disruption with chaotropic reagents followed by adsorption of RNA to silica particles. Detection of virus sequences within the sample RNA utilised real-time RT-PCR with hydrolysis probes in duplicate reactions for each sample RNA and target virus combination. Undiluted and 1/10 diluted RNA was tested for each sample; in accordance with ISO 15216-1, the results for undiluted RNA were used unless this demonstrated significant RT-PCR inhibition, in which case results for 1/10 diluted RNA would be checked. Due to the complexity of the method, a comprehensive suite of controls was included, including negative process, extraction and RT-PCR controls, and controls for RT-PCR inhibition and extraction efficiency. Quantification of target copies per microliter of sample RNA was by reference to a standard curve generated from a dilution series of dsDNA carrying the relevant target sequence as described in Annex G of ISO 15216-1:2017 (Anonymous, 2017b). In accordance with ISO 15216-1, quantities were not corrected according to extraction efficiency and RT-PCR inhibition results.

2.2 Design of the study

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

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

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

2.3 Part 1; method characterisation – general considerations

For part 1 of the study, test sample sizes were 2g (bivalve shellfish digestive tissues), 25g (fruit and vegetables), 330ml (bottled water) and 25cm^2 (bell pepper pieces to represent the food surfaces matrix). All samples were tested using the relevant matrix-specific test protocols (compliant with ISO 15216-1) then both calculated quantities and raw data were forwarded to the project leader for quality checking according to ISO 15216-1 and analysis as described under Generation of Method Characteristics below.

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

The viruses used for contamination were genotypes GI.4 and GII.4 norovirus from faecal suspensions and HM175/43c strain HAV derived from tissue culture. The limited availability of norovirus stocks (particularly GI) meant that a simple $\log_{10}$ dilution series with sufficiently high starting levels to allow for contamination across a wide range of dilutions was not practical; for this reason a 0.5 $\log_{10}$ dilution series (~3.16x) was used instead. For each matrix 0.5 $\log_{10}$ dilution series of contaminated matrix samples at 9 separate levels were prepared as below (different strategies reflect the modes of natural contamination of the different food matrices). In each case virus dosing was calculated to provide detectable levels of 100 - 1,000 copies/µl in the RNA extract at the highest contamination level (equivalent to ~20,000 - 200,000 copies/g for bivalve shellfish samples, 400 - 4,000 copies/g for fruit and vegetable samples, 400 - 4,000 copies/cm^2 for food surface samples and 30 – 300 copies/ml for bottled water samples), based on the results of a trial contamination:-

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

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

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

2.5 Part 1; method characterisation – structure of the study

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

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

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

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

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

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

2.6 Part 2; interlaboratory study – general considerations

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

2.7 Part 2; interlaboratory study – preparation of test samples

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

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

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

2.8 Part 2; interlaboratory study – testing by participant laboratories

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

2.9 Generation of method characteristics – general considerations

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

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

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

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

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

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

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

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

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

2.11 Generation of method characteristics – repeatability and reproducibility

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

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

3. Results and Discussion

3.1 Recalibration of norovirus GII results

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

To rectify this issue a calibration factor was used to adjust GII test sample results to compensate for the quantification bias introduced by the incorrect sequences. To establish the appropriate calibration factor, the GII dsDNA quantification standard used in the validation (adjusted to $1x10^5$ copies/µl following quantification by spectrophotometry) was subjected to qPCR alongside a dilution series prepared using newly synthesised dsDNA quantification control with no mismatches relative to the primer and probe sequences (sequence x). This established that the validation standards exhibited an effective 3.157x consistent reduction in amplification efficiency (≈1.66 Cq values), resulting in a corresponding 3.157x overestimation in quantitative results for test samples in the validation (results not shown). All GII results in both parts of the validation were therefore adjusted downwards by this same factor. This issue affected absolute quantification but not relative quantification of samples within and between laboratories. Therefore, of the performance characteristics determined, only LOD and LOQ were affected by this adjustment. Repeatability and reproducibility characteristics were not impacted.

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

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

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

3.2 Dilution of quantification standards using water only

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

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

3.3 RT-PCR inhibition in the common mussel matrix

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

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

3.4 Results of part 1; method characterisation

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

3.5 Results of part 2; interlaboratory study

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

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

3.6 Method characteristics for all matrices

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

4. Conclusion

This paper details the validation study carried out on ISO 15216-1, the standard method for quantification of hepatitis A virus and norovirus in foods. Detailed information on the performance of the method in seven different matrices was generated and has been included in ISO 15216-1:2017 (Anonymous, 2017b), the newly published edition of the standard. Acceptability criteria for the method characteristics have not been determined. The values obtained for repeatability and reproducibility in this study are comparable to other quantitative (enumeration) methods for bacterial pathogens validated at the same time under European Mandate No. M38 however. For example, ISO 10272-2, the colony count method for *Campylobacter* spp. (Anonymous, 2017a) produced average repeatability and reproducibility standard deviations of 0.20 and 0.40 $\log_{10}$ respectively, compared with 0.23 and 0.50 for the virus method. For LOD and LOQ characteristics it was notable that in the majority of cases the values determined for HAV were higher than for norovirus GI and GII. The primer set used in this study amplifies a relatively long product (157-188 bp depending on the strain of HAV), while for norovirus shorter, more optimal products are amplified (86 bp and 89 bp for GI and GII respectively; Anonymous, 2017b). This difference may have accounted for the different relative sensitivities of the methods for the different target viruses. Design of a broadly-reactive real-time RT-PCR primer probe set for HAV that amplifies a shorter product is complicated by the sequence diversity across strains however.

A number of technical issues were encountered through the validation study. It was necessary to 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 LOD_{50}, 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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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

Table 9

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

Table 10

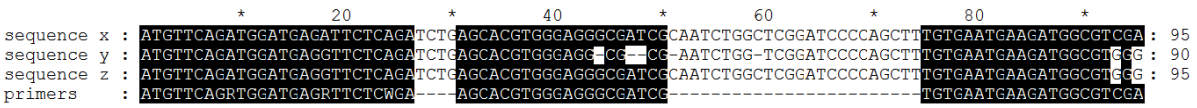
Method performance characteristics for common mussels

	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

[a] nd = not determined

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.



608

609

610

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

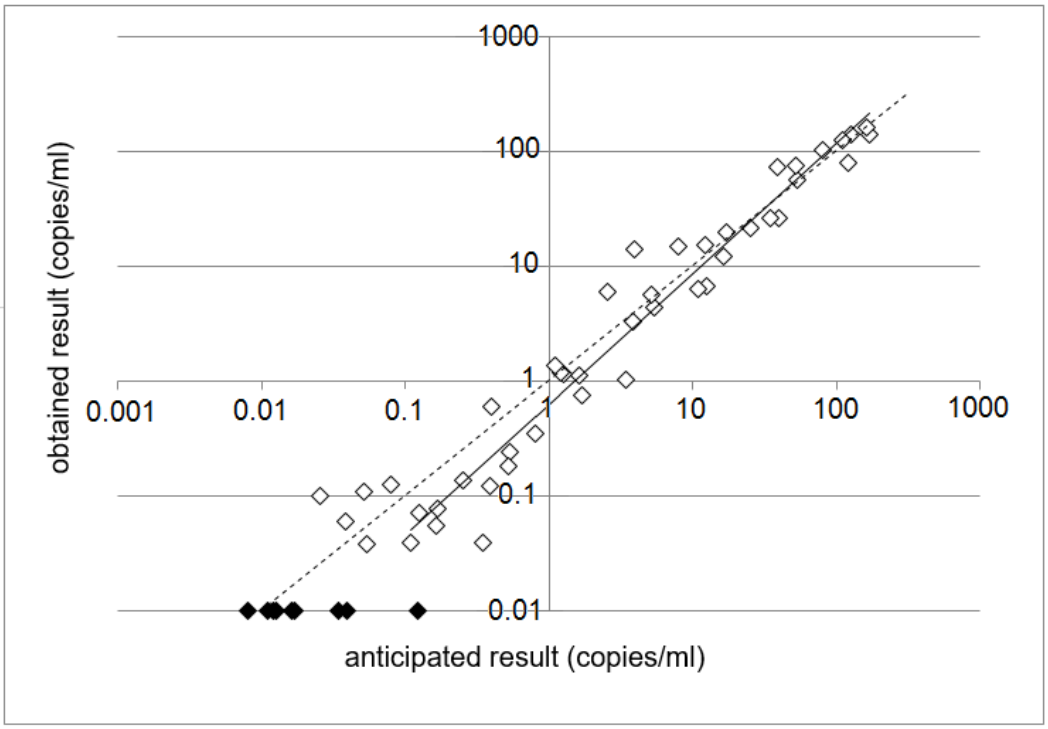


Figure 3 Probability of detection/limit of detection for norovirus GI in bottled water. The probability of detection function derived from the data is shown as a dotted line. The dashed line marks the 95% probability of detection used to determine the LOD.

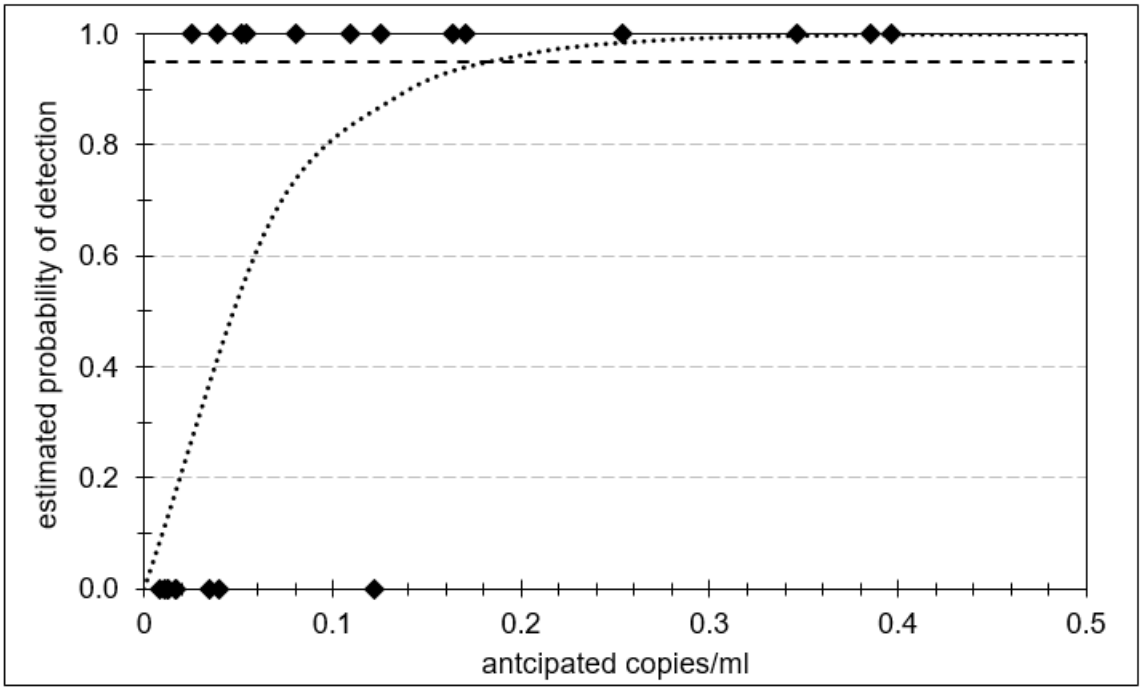


Figure 4 Part 2; interlaboratory study results for norovirus GI in bottled water. A, high contamination level; B, medium contamination level; C, low contamination level; D, negative. Quality control results obtained by the expert laboratory are shown to the left of each sub-plot (data points shaded grey).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