1 # Rapid Detection of Human Norovirus in Frozen Raspberries

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24 **ABSTRACT**

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26 Raspberries have lately caused several human norovirus (HuNoV) outbreaks in Europe.

27 In this study, we developed and evaluated for HuNoV reverse transcription (RT)-PCR

28 detection in frozen raspberries extraction methods that have equal sensitivity but are

29 less time-consuming than widely used methods based on polyethylene glycol (PEG)

30 precipitation and chloroform-butanol purification. One method was applied to stored

31 frozen raspberries linked to previous HuNoV outbreaks and berries on sale. In the virus

32 elution based Method 1, sparkling water eluted viruses most efficiently from the berries.

33 Method 2, based on direct nucleic acid extraction with minor PEG supplement, yielded

34 the highest number of positive findings (four out of nine) at low virus concentration

35 level of 100 genome copies HuNoV genogroup II per 25 g raspberries. Both methods

36 showed approximately equal sensitivity to a method including PEG precipitation and

37 chloroform-butanol purification. Two naturally contaminated berry samples linked to

38 HuNoV outbreaks in 2006 and 2009 were still positive for HuNoV genogroup I, but all

39 berry products purchased from a local store remained negative for HuNoV. In

40 conclusion, this study presents two efficient and rapid methods which can be used in

41 urgent HuNoV outbreak investigations, since the results of the virus analysis are

42 available in a few hours.

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1. INTRODUCTION

Human norovirus (HuNoV) has been one of the most significant causes of food-related illness in developed countries, inducing gastroenteritis outbreaks through the contamination of water and various foodstuffs (Mathijs et al. 2012). In recent years, frozen raspberries in particular have caused numerous HuNoV episodes in European countries (Cotterelle et al. 2005; European Commission 2014, 2015; Falkenhorst et al. 2005; Hjertqvist et al. 2006; Le Guyader et al. 2004; Maunula et al. 2009; Sarvikivi et al. 2012). Lately, several published studies have increased our knowledge of how HuNoVs came to contaminate food items during food handling (Mokhtari and Jaykus 2009, Rönnqvist et al. 2014; Stals et al. 2013; Tuladhar et al. 2013; Verhaelen et al. 2013), harvesting and irrigation (Kokkinos et al. 2012, Maunula et al. 2013).

HuNoVs, members of the *Caliciviridae* family, are currently classified into six genogroups (Gs) (White 2014), and the strains infectious to humans belong to GI, GII and GIV (Zheng et al. 2006). Recent reviews have proposed that water-related outbreaks are more often caused by GI noroviruses and food-related outbreaks as well as person-to-person infections by GII noroviruses (Bitler et al. 2013; Mathijs et al. 2012; Matthews et al. 2012). The authors explain this difference by GI noroviruses' greater tolerance of environmental stress in water. HuNoVs can survive cold temperatures for years but can also withstand a temperature of 60°C for 30 min (Carter 2005; Dolin et al. 1972). Consequently, the consumption of unheated foodstuffs such as berries, vegetables and shellfish poses the highest risk of food-related norovirus infection.

75 A literature review by Stals et al. (2012), covering the past decade, has identified several

76 methods for detecting noroviruses in various high-risk foodstuffs such as berries, salads

77 and cold cuts. The methods are based mainly on ultrafiltration, ultracentrifugation,

78 cationic separation and polyethylene glycol (PEG) precipitation, and they yield virus

79 recovery efficiencies ranging from 3% to 72% (Stals et al. 2012). High variability in

80 the recovery of HuNoV with commonly used detection methods was one of the issues

81 that led to the need for a standardized method. In 2013, ISO published the first technical

82 specification for HuNoV detection in foods (ISO/TS 15216) and the part of the

83 specification concerning quantitative detection was published as ISO standard 15216-

84 1:2017 in 2017. The method is the achievement of the efforts of several experts in the

85 field. The application for soft fruit, however, involves numerous steps such as time-

86 consuming virus concentration, and it often requires two working days to obtain results,

87 especially if the analysis involves several samples.

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89 Because viral contamination levels in foods are usually low and the presence of only a

90 few virus particles can lead to disease (Teunis et al. 2008), highly sensitive detection

91 methods are needed. Furthermore, to prevent the spread of an outbreak, the results of

92 virus analysis are needed quickly, which also emphasizes the need for a rapid method.

93 The objective of this study was to create and present simple and rapid methods for

94 detecting HuNoV in raspberries and to evaluate them using berries artificially

95 inoculated with known quantities of HuNoV GII. To determine the capability to

96 detect HuNoV also in naturally contaminated berries, we applied the fastest method,

97 which was used with minor modifications in an outbreak situation in 2009 (Maunula

98 et al. 2009), to a selection of naturally contaminated stored frozen berry samples

99 linked to suspected HuNoV outbreaks in 2006–2013. A small-scale screening study

100   was also implemented with this method to find out the incidence of HuNoV in frozen

101   raspberries on sale in local grocery shops.

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103   **2. MATERIALS AND METHODS**

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105   A total of 170 raspberry samples were used in the study according to the scheme

106   shown in Figure 1.

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108   **2.1 HuNoV and inoculation of berries for elution study and for method evaluation**

109   An anonymous faecal sample containing HuNoV strain GII.4-2006b and obtained from

110   Huslab, Helsinki, Finland was used in this study. Processing of the virus-containing

111   faecal sample and the inoculation procedure of raspberries are described by Summa et

112   al. (2012a). Briefly, the endpoint dilution polymerase chain reaction (PCR) method

113   defined the virus concentration for this strain as 1.7×10^8 genome copies (gc)/ml in a

114   10% stool suspension in phosphate buffered saline (PBS) as the stock solution. Spiking

115   solutions containing virus loads from 10^4 to 10^1 gc in tenfold dilutions were prepared

116   from the stock solution. Fresh Finnish raspberries, all belonging to the same batch, were

117   obtained from a wholesaler of berries. 25 g portions of raspberries were spiked by

118   spreading 100 μl of spiking solutions as small droplets onto the berries, which were

119   then kept in a fume chamber for up to 2 hr until the droplets had dried before freezing

120   the samples at −20°C. These berry portions were used in HuNoV elution study and for

121   method evaluation (Figure 1). 100 μl portions of spiking solution were frozen as a

122   spiking control for each virus load for the purpose of recovery efficiency evaluation.

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2.2 Description of the untreated samples

HuNoV analysis was also performed on archived frozen berries linked to nine suspected HuNoV outbreaks in Finland and one outbreak in Sweden in 2006–2013. The berries had been kept stored at −20°C (altogether 11 samples, later called 'epidemic samples'). Virus analysis made with an in-house PEG precipitation based method and real-time reverse transcription (RT)-PCR during the course of the outbreak investigation had revealed that seven of these samples contained HuNoV GI or GII genome or both, four had remained negative. In addition, 39 packages of frozen raspberries or mixed berries containing raspberries were purchased from several local grocery shops in southern Finland in 2010, 2014, and 2017 (later called 'screening samples') and analysed for HuNoV GI and GII. More detailed information about 'screening samples' is presented in Figure 1. All 'epidemic' and 'screening' samples were stored at −20°C and 25–30 g portions were analysed in 2014 with the exception of 20 samples purchased in 2017.

2.3 HuNoV elution study

Five elution fluids for berries were tested. The fluids were (1) untreated tap water, (2) salt solution (1.04 mM $NaHCO_3$, 0.59 mM K_2CO_3, 0.25 mM $CaCl_2$, 0.37 mM $MgCl_2$) prepared in the laboratory, (3) commercial sparkling mineral water (water, carbon dioxide and salts corresponding to the salt solution in (2), Vichy, Rainbow, Finland, stored at room temperature), (4) sparkling water prepared in the laboratory (untreated tap water was turned into sparkling water with SodaStream© equipment powered by a CO_2 cylinder according to the manufacturer' instructions, no other supplements were added to the water) and (5) TGBE buffer, (pH 9.5) (100 mM Tris, 50 mM glycine, 1% beef extract; beef extract, Becton Dickinson, USA). The elution test, which was repeated twice with each elution fluid, included three 25 g raspberry samples each

150 contaminated with 10^4 gc of HuNoV GII.4, i.e. six samples for every fluid. Briefly,

151 after melting at room temperature for 1 hr, the berries were washed in a Stomacher®

152 bag (Seward Ltd, UK) with 30 ml of one of the five elution fluids for 10 min in a shaker.

153 The elution fluid was collected and decanted into a 50 ml tube that contained 1 ml of

154 chloroform-butanol (CB) (1:1) solution. The tubes were then shaken vigorously by

155 hand for 1 min and centrifuged at $15\ 000 \times g$ for 30 min at room temperature. After

156 centrifugation, the water phase was moved to another 50 ml tube that contained 10 g of

157 guanidine thiocyanate (GITC, Sigma-Aldrich, USA). The procedure was completed as

158 described for Method 1 for RNA extraction.

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160 **2.4 Virus extraction**

161 Two of the methods presented in Figure 2 served to extract viruses in frozen raspberries

162 spiked with 10^4, 10^3, 10^2 or 10^1 gc of HuNoV GII.4. Three samples were analysed

163 simultaneously for each dilution, and analysis was repeated three times for each

164 triplicate. To scale the results of the two methods, a third method based on PEG

165 precipitation and CB purification (Butot et al. 2007; Dubois et al. 2002) described in

166 detail in Figure 2 and also by Summa et al. (2012a) was applied for each dilution in two

167 (10^4 and 10^3 gc) or nine (10^2 gc) samples. Briefly, the melted berries were washed with

168 TGBE buffer (pH 9.5), which was then centrifuged for 15 min. The pH of the

169 supernatant was adjusted to 7.2 and it was then incubated at room temperature with

170 1000 units of pectinase (≥ 3800 units/ml, Pectinex, Sigma, USA) for 30 min. Then the

171 supernatant was incubated at 4°C with PEG and NaCl solution (50% (w/v) PEG 8000,

172 Sigma-Aldrich, USA and 1.5 M NaCl) for 2 hr. After 30 min centrifugation, the

173 resuspended pellet was treated with CB mixture. Before the virus analyses, all berry

174 samples, including also 'epidemic' and 'screening samples', were allowed to melt in a

175 fume chamber at room temperature for 1 to 2 hr. A commonly used process control,

176 mengovirus (a kind gift from Dr A. Bosch, University of Barcelona, Spain) as a load of

177 10^5 PCR-units was added to all the berry samples immediately after melting. RNA

178 extraction took place directly after virus extraction.

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180 **2.5 Method 1**

181 Method 1 took about 2.5 hr to complete from a frozen berry sample to the nucleic acid

182 with 1 hr melting time. Melted berries were first quickly (1–5 min) washed in a

183 Stomacher® bag with 27 ml of sparkling water (prepared with SodaStream©

184 equipment) which was then supplemented with 3 ml of $10 \times$ TGBE buffer (pH 11) (1

185 M Tris, 500 mM glycine, 5 % beef extract; beef extract, Becton Dickinson, USA) for

186 the elution step. After centrifugation in a 50 ml tube, the supernatant was combined

187 with 10 g of GITC powder and 3 ml PEG/NaCl solution in another 50 ml tube. The tube

188 was then incubated in a water bath (at about 55°C) until the GITC had completely

189 dissolved before beginning the lysis step of RNA extraction.

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191 **2.6 Method 2**

192 Method 2 consisted of only three steps: melting the berries, combining the juice from

193 the berries with PEG/NaCl solution, and RNA extraction. Completion of the procedure

194 from melting the berries to extracted RNA took about 1.5 hr, including 1 hr melting

195 time. First, 1 ml of juice released from the defrosted berries was collected in a tube

196 where 250 µl of PEG/NaCl solution was added. After a 1 min manual shaking of the

197 tube, 2 ml lysis buffer was inserted into the tube to perform the lysis step of RNA

198 extraction. Method 2 was also tested without PEG/NaCl; in this case the RNA was

199 extracted directly from the juice from the melting raspberries. Method 2 with PEG/NaCl

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200   supplement was used in the HuNoV investigations of the 'epidemic' and 'screening'

201   berry samples.

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203   **2.7 RNA extraction**

204   For Method 2, the RNA extraction was performed using a miniMAG (MM) RNA

205   extraction system and a NucliSens Magnetic Extraction Reagents kit (BioMerieux,

206   France) according to the manufacturers' instructions. For Method 1 some modifications

207   were made. The lysis step began by adding 12 ml of ethanol (Etax A 96.1 vol-%, Altia

208   Oyj, Finland) and 100 µl of sodium dodecyl sulphate (SDS, solution of 10% (w/v), ICN

209   Biomedicals, Inc., USA) to the 50 ml tube containing the supernatant, PEG/NaCl and

210   GITC, and then incubation continued at room temperature for 10 min. After incubation,

211   100 µl of the MM magnetic beads were added to the tube, which was briefly manually

212   shaken and then incubated at 37°C in a rotator for 10–15 min. The beads were then

213   separated from the supernatant in a magnetic rack and moved to the MM tubes with

214   wash buffer 1. After this, the MM procedure was continued according to the

215   manufacturers' instructions. The viral RNA was eluted in 50 µl of elution buffer and

216   then frozen at −20°C. Each extraction group comprised, in addition to the berry

217   samples, a corresponding HuNoV spiking control (10^4 to 10^1 gc in 100 µl) and at least

218   one blank sample to control for cross-contamination.

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220   **2.8 Viral nucleic acid detection**

221   Virus detection targeting the polymerase-capsid junction was completed using one-step

222   TaqMan real-time RT-PCR as described by Summa et al. (2012a) and a QuantiTect

223   Probe RT-PCR kit (Qiagen, USA). All PCR reactions containing 15 µl of Master Mix

224   which included primers and a FAM-labelled probe, and 5 µl of extracted RNA from the

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225 berry samples were performed as duplicates for the neat samples and their tenfold

226 dilutions. In addition to the berry samples, each PCR run included the spiking control,

227 a standard and one to two blank samples. For each 25 g berry sample, the minimum

228 detectable quantity of viruses was 10 gc. All 'screening' and 'epidemic samples' were

229 analysed for HuNoV GI and GII separately as described by Summa et al. (2012b) using

230 primers and probe COG2R/QNIF2/QNIFS, respectively, for GII and

231 QNIF4/QNIF3/JJV1P, respectively, for GI.

232 Mengovirus served as an internal process control to estimate the success of the virus

233 extraction for each analysis during this study. The same protocol as for HuNoV GII,

234 but using primers and probe Mengo110/Mengo209/Mengo147, respectively, was used

235 to detect mengovirus in the master mix as described by Summa et al. (2012a).

236 Murine norovirus (MuNoV) (obtained from Herbert W. Virgin at the Washington

237 University School of Medicine, St. Luis, MO, USA) served as an external control in

238 evaluating the degree of PCR inhibition in each sample. The master mix formula for

239 MuNoV was the same as used for HuNoV GII with the primers (MNVfor and MNVrev)

240 and the probe (MNV) described by Hewitt et al. (2009). One MuNoV reaction

241 contained 15 µl of Master Mix, which included primers and probe, 5 µl of RNA from

242 the berry samples, and 1 µl (10^4 PCR-units) of MuNoV RNA. As a control, each run

243 included a reaction consisting of 15 µl of mastermix, 5 µl of water and 1 µl of MuNoV

244 RNA.

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246 **2.9 Determination of virus recovery efficiency and inhibition of the PCR reaction**

247 Qualitative virus analysis was used for the 'epidemic' and 'screening samples'.

248 Quantitative analysis of the samples of elution fluid tests and the evaluation of methods

249 were based on a standard curve generated by sequential tenfold dilutions of the viral

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250   RNA of the HuNoV GII.4 strain used in the study. The results of each PCR run were

251   quantified by plotting against the standard curve. The virus recovery efficiency of the

252   higher virus loads was explored. The recovery percentage was calculated by dividing

253   the number of gc in the sample by the number of gc in the spiking control and

254   multiplying by 100.

255   As noted in Section 2.7, MuNoV served as an external control to evaluate the degree of

256   inhibition of the berry samples in the PCR runs by comparing the quantification cycle

257   (Cq) value of the MuNoV RNA in the berry samples against the corresponding value

258   of the controls (for details, see Sections 2.7 and 3.2). In this study, the amplification

259   efficiencies of MuNoV and HuNoV GII.4 were sufficient to enable reliable estimations

260   of the effect of PCR inhibitors in HuNoV PCR assays.

261

262   **3. RESULTS**

263   **3.1 Selection of elution fluid for virus extraction**

264   Five fluids (untreated tap water, salt solution, sparkling water, sparkling mineral water

265   and TGBE buffer) were tested for their suitability to elute viruses from 25 g portions of

266   raspberries spiked with 10^4 gc HuNoV GII.4. HuNoV detection in all six samples was

267   possible only with the sparkling water elution (Table 1). Water, salt solution and TGBE

268   failed to detect HuNoV GII.4 in several raspberry samples, although mengovirus, the

269   process control, was detectable in all samples. MuNoV as an external control showed

270   less than one log difference ($\Delta Cq < 3$ cycles) in levels of PCR inhibitors between the

271   elution fluids. Based on these results, sparkling water without minerals was selected for

272   elution of the viruses from the berries in Method 1.

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3.2 Evaluation of the virus extraction methods

Two rapid methods were evaluated using frozen raspberries artificially contaminated with HuNoV loads of 10^4, 10^3, 10^2 and 10^1 gc per 25 g sample. We scaled the results of the two methods to results of a common method based on PEG precipitation and CB purification (for details, see Section 2.3).

As Table 2 shows, both rapid methods successfully detected HuNoV GII.4 in all nine berry samples with a virus load of 10^4 gc. Method 1 yielded positive results in nine samples and one sample (of nine) with virus loads of 10^3 gc and 10^2 gc, respectively. For Method 2, the PEG/NaCl supplement increased the positive results from six to nine (of nine) with a virus load of 10^3 gc and from zero to four (of nine) with a virus load of 10^2 gc. Thus, Methods 1 and 2 appeared to work at least as well as the PEG precipitation method (PEGP), which successfully detected HuNoV GII.4 in all berry samples spiked with virus loads of 10^4 and 10^3 gc, and one with 10^2 gc. Neither Method 2 nor PEGP was capable of detecting HuNoV GII.4 in berry samples with lower levels of contamination. The positive signal of mengovirus as a process control in the neat sample or its tenfold dilution was a condition for the acceptance of each result of the HuNoV analysis.

The highest virus recovery efficiencies for a virus load of 10^4 gc were obtained by Method 2 and they varied between 10% and 81% with an overall mean of 32% (standard deviation, SD, 0.16). The recovery efficiencies showed less variation with Method 1; however, these were slightly lower than those of Method 2, with a mean recovery of 9% (SD 0.05). Efficiencies of Methods 1 and 2 were comparable to that of PEGP, which had an efficiency of 24 % (SD 0.02).

300

301 The PCR inhibition level, revealed by MuNoV, in Method 2 was lower and had less

302 variability between the samples when the PEG/NaCl supplement was used in the virus

303 extraction protocol than when the supplement was not used (Table 2). PCR inhibitors

304 affected virus detection more severely (even over one log) when berries inoculated with

305 lower amounts of the virus were analysed. When the amplification was performed with

306 1:10 dilutions of RNA extracted from the berry samples only minor PCR inhibition was

307 seen for each method (Table 2).

308

309 **3.3 Screening of frozen berries**

310 Method 2, which proved to be the most sensitive and quickest method was used for

311 screening the frozen raspberries or berry mixes. The method was used to detect HuNoV

312 in 11 stored berry samples that were linked to suspected HuNoV outbreaks and of which

313 seven had earlier been verified as positive for HuNoV genome. In the current analysis,

314 two of the berry samples kept frozen for five and eight years (outbreaks in 2006 and

315 2009, respectively) still contained a detectable amount of HuNoV GI genome (Table

316 3). All 39 frozen berry packages purchased from local stores in 2010, 2014 and 2017

317 tested negative for HuNoVs GI and GII.

318

319 **4. DISCUSSION**

320 Numerous rapid methods for HuNoV detection have been introduced in recent years

321 for different kinds of foods (Baert et al. 2008; Fumian et al. 2009; Lee et al. 2013;

322 Morillo et al. 2012; Perrin et al. 2015; Rzezutka et al. 2005; Schwab et al. 2000).

323 However, some of them require special equipment such as ultracentrifugation or

324 filtration devices and only a few of them are suitable for frozen raspberries, which have

325   been recognized as an important source of HuNoV outbreaks in Europe (Cotterelle et

326   al. 2005; European Commission 2014, 2015; Falkenhorst et al. 2005; Hjertqvist et al.

327   2006; Le Guyader et al. 2004; Maunula et al. 2009; Sarvikivi et al. 2012). Raspberries

328   are a challenging food matrix for virus detection because the low-pH juice they release

329   contains compounds that inhibit the PCR reaction. This study demonstrated that

330   Method 2, which is suitable for food that releases liquid when melted, was found to be

331   more sensitive, quicker and more efficient with artificially contaminated frozen

332   raspberries than Method 1. It was capable of detecting HuNoV genomes in naturally

333   contaminated berries stored frozen for up to eight years. Sparkling water was shown to

334   elute viruses most efficiently and was therefore chosen to be used in Method 1, which

335   is suitable also for fresh foods and frozen non-juicy food matrices. Benefits of these

336   rapid methods are that most laboratories can perform them easily and the results of the

337   whole virus analysis are available within one working day.

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339   Butot et al. (2014) divided various virus extraction methods into two groups: elution-

340   concentration of virus particles and direct viral RNA extraction, which usually involves

341   washing the food sample directly with a GITC-based lysis buffer. Method 1 presented

342   in this study is a combination of these as it has an elution step but no concentration step.

343   Method 2, in contrast, involves direct RNA extraction, as does a method originally

344   presented by Schwab et al. (2000) for deli meat, which proved successful with foods

345   other than oysters or berries suspected of causing viral gastroenteritis outbreaks

346   (Anderson et al. 2001; Boxman et al. 2007). Baert et al. (2008) and, recently, Perrin et

347   al. (2015) have described applications of direct viral RNA extraction for artificially

348   contaminated frozen raspberries. These methods entail eluting the virus from berries by

349   washing them with GITC and phenol or with GITC-based buffers; in our Method 2,

350 melted juice from the berry sample is directly added to the GITC-based lysis buffer. To

351 our knowledge, no direct RNA extraction methods other than Method 2 have been

352 successfully used and published for naturally contaminated frozen raspberries.

353

354 Different buffers and equipment have been tested for the elution of viruses from a

355 variety of food matrices in recent studies (Cheong et al. 2009; de Abreu Corrêa and

356 Miagostovich 2013; Dubois et al. 2006; Park et al. 2010). Data in many of these studies

357 suggested that the best buffer was Tris base and glycine for the elution step, which is

358 one of the most critical points of virus extraction, as incomplete elution will

359 compromise the entire method. In this study, we wanted to test simple and easily

360 available fluids against the widely used TGBE buffer. In our previous study (data not

361 shown) we discovered that gas bubbles formed when carbonate-based buffers came into

362 contact with the surface of the raspberries. Reports indicate that the use of sonication,

363 which in our experiments (data not shown) also created bubbles on the surface of the

364 food, increases virus recoveries when combined with ultrafiltration (Jones et al. 2009).

365 Other studies have also examined the use of vacuum-induced bubbles in inactivation

366 tests for HuNoV surrogates in lettuce (Fraisse et al. 2011). All of these findings evoked

367 an idea about bubbles enhancing the release of virus particles from the surface of the

368 food, making the elution more efficient. In our tests, the two different elution fluids

369 inducing bubbling eluted more HuNoV GII.4 particles from the berries than did the

370 other liquids tested.

371

372 Certain components such as organic compounds, fats, sugars and Ca^{2+}, which are

373 known to inhibit the PCR reaction and to be released from food matrices during

374 processing, are a significant challenge to HuNoV detection in foods (Wilson 1997).

375   Raspberry analyses, especially, are easily harmed by PCR inhibitors which, however,

376   have rather efficiently been removed with CB treatment (Butot et al. 2007; Dubois et

377   al. 2002; Summa et al. 2012a). When developing the two methods presented in this

378   study, we tested Method 2 also with CB treatment. However, when analysing naturally

379   contaminated raspberries, Method 2 produced a weak positive HuNoV signal only

380   when performed without this treatment (data with CB treatment not shown), perhaps

381   partly because of a loss of virus particles when separating the water phase from the

382   organic phase when the treatment was used. In addition, concentrating viruses using

383   PEG/NaCl has proved an efficient way to extract viruses in various kinds of foods

384   (Baert et al. 2008; Boxman et al. 2007; Butot et al. 2007; Guévremont et al. 2006; Kim

385   et al. 2008; Rutjes et al. 2006; Stals et al. 2011a). PEG polymer is widely used in

386   numerous chemical, biological and industrial applications for various purposes. In this

387   study, we decided to use PEG as a supplement without precipitating the viruses, because

388   it was found to reduce the effect of PCR inhibitors released from raspberries.

389

390   Method 2, without the PEG/NaCl supplement, served to detect HuNoV in naturally

391   contaminated raspberries during an outbreak situation in 2009 (Maunula et al. 2009).

392   In this study, the capability of this method, supplemented with PEG/NaCl, was

393   demonstrated since it detected HuNoV genome in two archived naturally contaminated

394   berry samples from suspected HuNoV outbreaks in 2006 and 2009. The results also

395   show that the HuNoV genome is quite stable for several years at least in high

396   contaminations in frozen berries. The five originally HuNoV-positive berry samples

397   which now tested negative had produced only a weak positive signal in tests when

398   performed during the outbreak three to five years earlier, which may explain negative

399   results. All our retail frozen raspberries tested negative for HuNoV, whereas other

400 research groups in Europe (Baert et al. 2011; De Keuckelaere et al. 2015; Stals et al.

401 2011b) have screened berry batches from raspberry processing companies and found

402 HuNoV-positive signals in some of them. However, there were no reported HuNoV

403 outbreaks related to frozen raspberries in Finland during the sampling periods (personal

404 communication, Finnish Food Safety Authority Evira); thus the Finnish epidemic data

405 and other recent studies (Bouwknegt et al. 2015; Maunula et al. 2013) are in line with

406 our negative results. On the other hand, because the number of samples analysed was

407 not high, the conclusions that can be drawn based on these results are limited. Another

408 limitation of this study is, that the sensitivity of the methods has been tested only for

409 HuNoV GII and not for GI. However, as mentioned above, GI HuNoV could be

410 detected in naturally contaminated berries using Method 2, which is a promising result.

411

412 The rapid methods presented here would be most valuable when used to detect sources

413 of sudden bursts of outbreaks and when the results are needed quickly. The current

414 virus analysis, however, comprises the PCR step, which is often vulnerable to PCR

415 inhibitors. Further improvement is needed to increase the sensitivity of the genome

416 detection without the need to dilute the samples for PCR reaction. Some promising

417 results have already been achieved using an additional purification step for extracted

418 nucleic acid with the PCR Inhibitor Removal Kit and/or digital PCR, which is less

419 influenced by inhibitors (Coudray-Meunier et al. 2015; Fraisse et al. 2017). These

420 findings may offer solutions to overcome the current deficiencies in virus analysis.

421

424 **Conflict of Interest:** The authors declare that they have no conflict of interest.

425

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614

615   **Figure legends**

616   Figure 1. An overview of the raspberry sample preparation and sampling used in this

617   study

618

619   Figure 2. Flow charts of the virus extraction methods

Table 1. Detection of HuNoV GII.4 by real-time RT-PCR in artificially contaminated raspberries using different elution fluids and the direct elution-based virus extraction protocol described in Section 2.2

Elution fluid	Positive samples[a]		PCR inhibition[c] (ΔCq)
	HuNoV	Mengovirus[b]	
Water	2 / 6	6 / 6	<0.5–1
Salt solution	2 / 6	6 / 6	1–2
Sparkling mineral water	5 / 6	6 / 6	<0.5–1
Sparkling water	6 / 6	6 / 6	<0.5–1
TGBE	3 / 6	6 / 6	<0.5–1

a: Number of positive replicates / number of spiked samples tested
b: Process control, results were accepted only when positive signal obtained
c: PCR inhibition: the difference between the MuNoV Cq value in 1:10 dilutions of the samples and the MuNoV Cq value in H_2O (range between the six replicates)

Table 2. Detection of HuNoV GII.4 by real-time RT-PCR in frozen raspberries inoculated with virus at various concentrations by Methods 1 and 2, and PEG precipitation method (PEGP).

Virus load (gc)	HuNoV positive samples[a]			PCR inhibition (ΔCq)[b]		
	Method 1	Method 2	PEGP	Method 1	Method 2	PEGP
10^4	9 / 9	9 / 9	2 / 2	<0.5–1	<0.5	<0.5
10^3	9 / 9	9 / 9 (6 / 9)[c]	2 / 2	0.5–1	<0.5 (0.5–1)[c]	0.5–1
10^2	1 / 9	4 / 9 (0 / 6)[c]	1 / 9[d]	<0.5–2	<0.5 (0.5–2)[c]	<0.5–2
10^1	e	0 / 6	0 / 2		0.5–4	<0.5

a: Number of positive replicates / number of spiked samples tested (positive results were accepted only when positive signal was obtained by mengovirus used as a process control)

b: The difference between the MuNoV (10^4 PCR-units in 1 µl MuNoV RNA was added) Cq value in 1:10 dilution of the samples and the MuNoV Cq value in H_2O (range between the replicates)

c: In brackets, results for Method 2 without PEG supplement (details in Section 2.5)

d: Comparison with PEGP focused on critical virus detection level
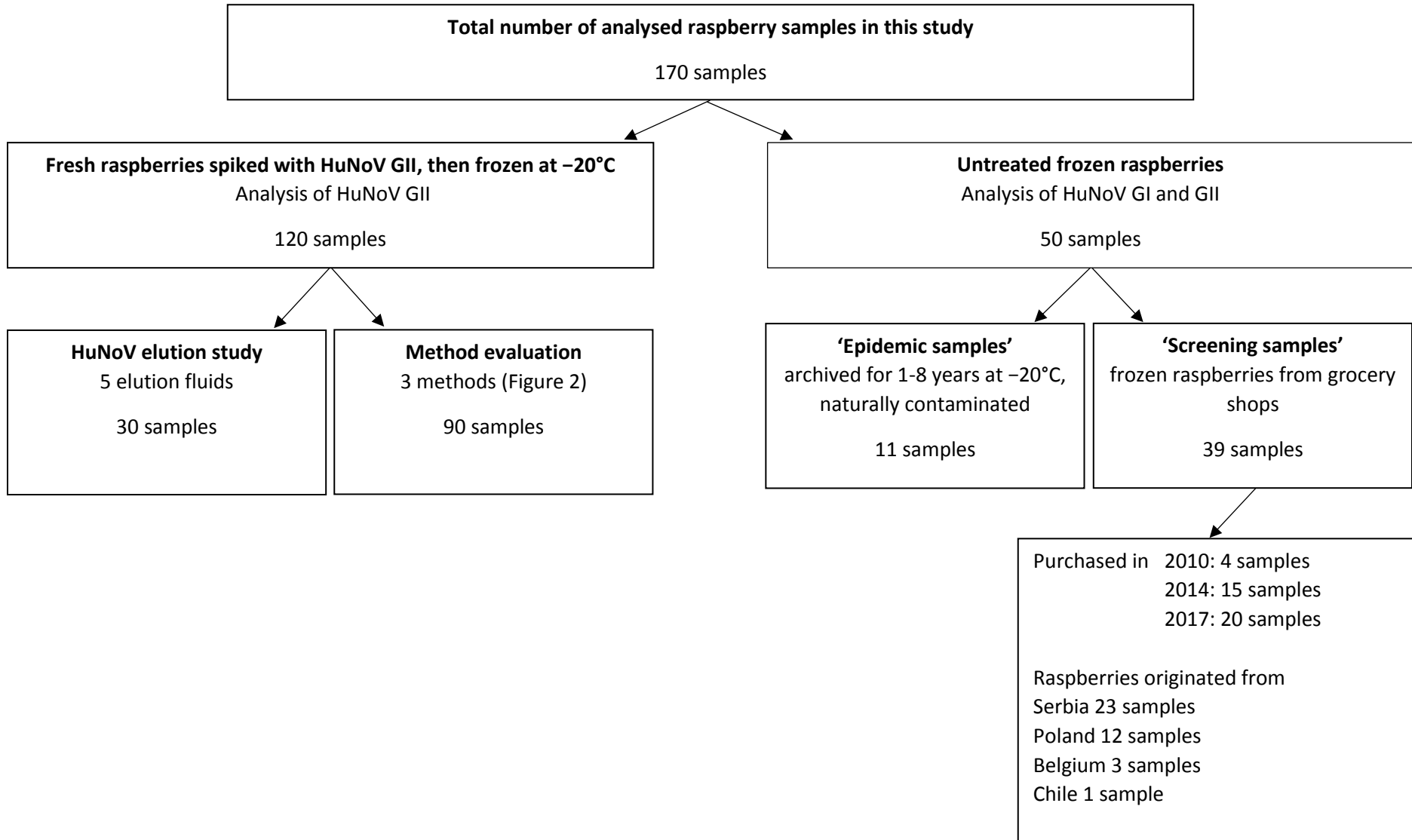
e: The lowest virus load was not tested with Method 1 because only one positive result with virus load of 10^2 was obtained

Table 3. Stored berry samples linked to ten previously suspected HuNoV outbreaks reanalysed for HuNoVs GI and GII using Method 2

Sample number	Sample type	Country of origin	HuNoV analysis during the outbreak (year)[a]	HuNoV analysis in 2014
1	Frozen raspberries	China	Positive GI and GII (2006)	Positive GI
2	Frozen raspberries	Poland	Positive GII (2009)	Negative
3	Frozen raspberries	Poland	Positive GII (2009)	Negative
4	Frozen raspberries	Poland	Positive GI (2009)	Positive GI
5	Frozen raspberries	Finland	Positive GI (2010)	Negative
6	Frozen raspberries	Finland	Positive GI (2010)	Negative
7	Frozen raspberries	Poland	Positive GI (2011)	Negative
8	Frozen raspberries	Estonia	Negative (2012)	Negative
9	Frozen blueberries, strawberries, redcurrants	Poland	Negative (2012)	Negative
10	Frozen raspberries, strawberries, blackcurrants, redcurrants	Poland	Negative (2013)	Negative
11	Frozen raspberries	Unknown	Negative (2013)	Negative

Samples 5 and 6 are from the same outbreak

a: Samples were analysed with in-house PEG precipitation based methods; some of the results have been published previously (Maunula et al. 2009)

Total number of analysed raspberry samples in this study

170 samples

Fresh raspberries spiked with HuNoV GII, then frozen at −20°C
Analysis of HuNoV GII

120 samples

Untreated frozen raspberries
Analysis of HuNoV GI and GII

50 samples

HuNoV elution study
5 elution fluids

30 samples

Method evaluation
3 methods (Figure 2)

90 samples

'Epidemic samples'
archived for 1-8 years at −20°C, naturally contaminated

11 samples

'Screening samples'
frozen raspberries from grocery shops

39 samples

Purchased in   2010: 4 samples
                      2014: 15 samples
                      2017: 20 samples

Raspberries originated from
Serbia 23 samples
Poland 12 samples
Belgium 3 samples
Chile 1 sample

PEG precipitation minimum 6.5 hr	Method 1 2.5 hr	Method 2 1.5 hr
(Total time to have the extracted viral RNA from the berries when 1 hr melting time is included)		

Virus extraction

Melting of 25 g berries for 1–2 hr at room temperature (addition of 10 µl mengovirus)

PEG precipitation	Method 1	Method 2
Elution of berries with 40 ml of TGBE buffer (pH 9.5) in a filter bag in a shaker for 20 min at room temperature (keeping pH > 9 with NaOH)	Elution of berries with 30 ml of buffer (details in Section 2.4) in a plastic bag (Stomacher® bag) in a shaker for 10 min at room temperature	Collection of 1 ml juice from defrosted berries in tube containing 250 µl PEG/NaCl (or without PEG/NaCl)
Centrifugation of recovered elution buffer at 10 000 × g for 15 min	Centrifugation of recovered elution buffer at 10 000 × g at 4°C for 15 min	Manual shaking for 1 min
Adjusting of pH of supernatant to 7.2 with HCl	Removal of supernatant to a 50-ml tube containing 10 g GITC and 3 ml PEG/NaCl	
Addition of 300 µl pectinase and incubation at room temperature for 30 min	Warming of the tube in a water bath at 55°C for 5 min	
Addition of PEG/NaCl and incubation at 4°C for 2 hr		
Centrifugation at 10 000 × g for 30 min		
Resuspension of pellet in 500 µl of PBS		
Mixing of the suspended pellet and 500 µl chloroform-butanol mixture by vortex and incubation for 5 min at room temperature		
Centrifugation at 10 000 × g for 15 min		

Nucleic acid extraction

PEG precipitation	Method 1	Method 2
Transfer of the aqueous phase to a tube containing 2 ml miniMAG (MM) lysis buffer and incubation at room temperature for 10 min	Addition of 12 ml ethanol with 100 µl 10% SDS solution and incubation at room temperature for 10 min	Addition of 2 ml MM lysis buffer to the tube and incubation at room temperature for 10 min
Addition of 50 µl MM beads and incubation at room temperature for 10 min	Addition of 100 µl MM beads and incubation at 37°C in a rotator for 10–15 min	Addition of 50 µl MM beads and incubation at room temperature for 10 min

Separation of magnetic beads in a magnetic rack. Nucleic acid extraction according to miniMAG procedure