Evaluation of High Hydrostatic Pressure Inactivation of Human Norovirus on Strawberries,
Blueberries, Raspberries and in their purees

Runze Huang¹, Mu Ye¹, Xinhui Li¹, Lin Ji², Mukund Karwe², Haiqiang Chen^{2*}

¹Department of Animal and Food Sciences, University of Delaware, Newark, DE 19716, USA

²Department of Food Science, Rutgers, The State University of New Jersey, New Brunswick, NJ
08901, USA

*Corresponding author: Tel.: +1-302-831-1045; Fax: +1-302-831-2822.

Email address: haiqiang@udel.edu (H. Chen).

ABSTRACT

Human norovirus (HuNoV) has been an increasing concern of foodborne illness related to fresh and frozen berries. In this study, high hydrostatic pressure (HHP) inactivation of HuNoV on fresh strawberries, blueberries, and raspberries and in their purees was investigated. Porcine gastric mucin (PGM)-conjugated magnetic beads (PGM-MBs) and real-time reverse transcriptional polymerase chain reaction (RT-qPCR) were utilized for infectious HuNoV discrimination and quantification. Strawberry puree inoculated with HuNoV genogroup I.1 (GI.1) strain was HHP-treated at 450, 500 and 550 MPa for 2 min each at initial sample temperatures of 0, 4 and 20 °C. HuNoV GI.1 strain became more sensitive to HHP treatment as the temperature decreased from 20 to 0 °C. HuNoV GI.1 or genogroup II.4 (GII.4) strains were inoculated into three types of berries and their purees and treated at pressure levels from 250 to 650 MPa for 2 min at initial sample temperature of 0 °C. For the purees, the HHP condition needed to achieve > 2.9 log reduction of HuNoV GI.1 strain and > 4.0 log reduction of HuNoV GII.4 strain was found to be $\geq$ 550 MPa for 2 min at 0 °C. HHP treatment showed better inactivation effect of HuNoV on blueberries than on strawberry quarters and raspberries. HuNoV GI.1 strain was more resistant to HHP treatment than HuNoV GII.4 strain under different temperatures and environment. The physical properties and sensory qualities of HHP-treated and untreated blueberries and the three types of berry purees were evaluated. Color, pH and viscosity of blueberries and three berry purees showed no or slight changes after HHP treatment. Sensory evaluation demonstrated that HHP treatment of 550 MPa for 2 min at 0 °C did not significantly reduced the sensory qualities of three berry purees. The results demonstrated that the HHP treatment of 550 MPa for 2 min at 0 °C could be a potential nonthermal intervention for HuNoV in berry purees without adversely affecting their sensory qualities and physical properties.

Keywords:

High Hydrostatic Pressure; Human Norovirus; Berry; Berry Puree

1. Introduction

Human norovirus (HuNoV), a group of non-enveloped viruses belonging to the *Caliciviridae* family, is the most common cause of foodborne disease outbreaks and has been identified as the leading cause of acute nonbacterial gastrointestinal disease in USA (Hall et al., 2013; Scallan et al., 2011). Recently, a concern for microbial safety for fresh and frozen berries and berry products was raised due to the frequent foodborne illness outbreaks associated with these products. Strawberries, blueberries and raspberries have been reported to be involved with foodborne viral outbreaks (Calder et al., 2003; Cotterelle et al., 2005; Falkenhorst et al., 2005; Friedman et al., 2005; Hutin et al., 1999; Korsager et al., 2005; Le Guyader et al., 2004; Mäde et al., 2013; Niu et al., 1992). In Denmark, an outbreak associated with frozen raspberries, involving 180 cases was reported which was linked to HuNoV in 2005 (Korsager et al., 2005). In 2012, 11,000 people were affected by HuNoV contaminated frozen strawberries in Germany (Mäde et al., 2013). Many sources could lead to viral contamination of berries and berry products, such as irrigation water, fertilizers, pesticides and food handlers (Carter, 2005; Potera, 2013; Rodriguez-Lazaro et al., 2012). Thus, an intervention of viral contamination is needed for berries and berry products.

Few methods have been applied to prevent berries and berry products from viral contamination. Fresh berries are usually picked, packaged and sold without washing. As for berry products, such as frozen berries and berry purees, a washing step using a sanitizer such as chlorine is applied prior to further processing. Due to the negative impact on sensory quality,

berry products usually do not adopt thermal processing. High hydrostatic pressure (HHP) processing, a nonthermal processing technique, has been successfully commercialized for processing a variety of food products, such as fruit jams, orange juice, salsa, guacamole, ready-to-eat meats, oysters, and lobsters since it can greatly retain the original sensory attributes and nutritional qualities of food. Huang et al. (2013) showed that HHP treatment had little detrimental effect on the physical properties and visual appearance of strawberry puree. Beneficial compounds, such as anti-oxidative factors and carotenoids, in fruits and vegetables only decreased slightly by HHP treatment (Butz et al., 2003). Compared to thermal processing, HHP treatment also shows less impact on low molecular weight compounds, like vitamins and pigments (Butz et al., 2003; Sánchez-Moreno et al., 2003; Tauscher, 1995). In addition, it has been shown that HHP was able to inactivate viral pathogens. Li et al. (2013a) showed that HHP treatment of 600 MPa at 21 °C for 2 min could inactivate HuNoV GI.1 strain by more than 3 logs on blueberries with the presence of water during pressure treatment. Ye et al. (2014) demonstrated that HHP treatment of 450 MPa at 1 °C for 5 min could reduce HuNoV GI.1 strain by 4 logs in oyster and clam homogenates. Sánchez, et al. (2011) showed that a 15-min HHP treatment of 500 MPa at 45 °C reduced RNA of HuNoV by 0.5 log₁₀ units determined using the RT-qPCR technique.

Jones et al. (2015) reported an *in vitro* cell culture system for HuNoV with commensal bacteria as an infection cofactor, but the system needed further development due to the modest viral output (0.5 to 3.5 logs increase). A gnotobiotic pig model was also proposed to estimate the HuNoV inactivation effect by HHP. The drawback of this model is that it is difficult to provide accurate log reduction data (Lou et al., 2015). Current research work usually relies on surrogates, such as murine norovirus 1 (MNV-1), feline calicivirus (FCV) and Tulane virus (TV) (Cannon et

al., 2006; Chen et al., 2005; Li et al., 2013b). However, it is impossible to predict actual inactivation effect of HuNoV using surrogates due to variable responses of surrogates under different processing techniques (Li et al., 2013a). Inactivation of HuNoV has also been evaluated using molecular methods such as RT-qPCR (Ye et al., 2014). However, RT-qPCR lacks the ability to discriminate infectious virus particles from impaired virus that still have detectable RNA genome. Marionneau et al. (2002) demonstrated that histo-blood group antigens (HBGAs) in the human intestinal tract were the receptors of HuNoV. Later work showed that porcine gastric mucin (PGM), similar to HBGA chemically and antigenically, was able to bind HuNoV and norovirus-like particles (Tian et al., 2008; Tian et al., 2007; Tian et al., 2010). Dancho et al. (2012) used PGM conjugated magnetic beads (PGM-MB) to differentially bind and extract infectious HuNoV from a mixture of infectious and inactivated HuNoV and then quantify the bound virus particles using RT-qPCR (referred as PGM-MB/PCR assay in this study). Li et al. (2013a) later demonstrated that the PGM-MB/PCR assay was very likely able to estimate HHP inactivation of HuNoV GI.1 and GII.4 strains at ≤ 2 log-reduction levels. It would also likely to conservatively quantify HHP inactivation of GI.1 strain at 2-3 log-reduction levels and GII.4 strain at 2-3.5 log-reduction levels (Chen et al., 2005; Dancho et al., 2012; Leon et al., 2011; Li et al., 2013b).

The objectives of this research were to determine the effect of HHP on the inactivation of HuNoV GI.1 and GII.4 strains on strawberries, blueberries, raspberries and in their purees using the PGM-MB/PCR assay and to determine the selected HHP processing condition on the sensory and physical qualities of berries and purees.

2. Materials and Methods

2.1 HuNoV stock preparation

A HuNoV GII.4 strain in fecal suspensions was provided by Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA. The information of the GI.1 strain (8FIIb) used in this study was described by Leon et al. (2011). Fecal suspensions were centrifuged at $4000 \times g$ for 20 min, filtered through a 0.22- μm filter, aliquoted, and stored at -80°C until use.

2.2 Inoculation of strawberries, blueberries, raspberries and purees

Fresh strawberries, blueberries and raspberries were purchased from a local store the day before each experiment and stored at 4°C until use. To prepare berry purees, berries were washed with tap water and dried in a strainer. Calyxes of the berries were removed with a flame-sterilized stainless knife and berries were blended in a food blender at high speed for 1 min until no chunks of berries were visible. One gram of berry purees was inoculated with 10 μl of the HuNoV GI.1 stock or GII.4 stock and bagged in individual sterile stomacher bag, sealed and double-bagged. To prepare fresh berries for HHP treatment, they were cleaned using tap water. The calyxes of strawberries were removed and the whole strawberries were then cut into quarters (~ 5 g/quarter) from the top to the bottom with a flame-sterile knife since the pressure chamber of Avure PT-1 pressure unit (Avure Technologies, Kent, WA) could not hold a whole strawberry. One strawberry quarter (~ 5 g/quarter), three blueberries (~ 5 g in total) or one whole raspberry (~ 4 g) was spot-inoculated with 10 μl of the HuNoV GI.1 stock or GII.4 stock on the surface and dried for 1 h in a biosafety cabinet for virus attachment. Each berry sample was placed into a sterile stomacher bag containing 5 ml of sterile double distilled water (ddH_2O). The bags were sealed and double-bagged.

2.3 Effect of initial sample temperature on pressure inactivation of HuNoV GI.1 in strawberry puree

Pressure treatments were conducted using the Avure PT-1 pressure unit which used water as a pressurizing medium. A circulating water bath surrounded the pressure vessel to control the temperature $> 0\text{ }^{\circ}\text{C}$. For the $0\text{ }^{\circ}\text{C}$ treatment, the chamber was filled with ice and water mixture at equilibrium. The pressure come-up rate was approximately 22 MPa/s . The pressure-release was almost immediate ($<4\text{ s}$). Pressurization hold time reported in this study did not include the pressure come-up or release times. To determine the effect of initial sample temperature on pressure inactivation of HuNoV GI.1, inoculated strawberry puree samples were treated at 450, 500 and 550 MPa for 2 min at initial sample temperatures of 0, 4 and $20\text{ }^{\circ}\text{C}$. HuNoV were extracted from the pressure-treated and untreated (control) samples and quantified by the PGM-MB/PCR assay as described in Sections 2.5, 2.6 and 2.7.

2.4 HHP treatment of HuNoV GI.1 and GII.4 on strawberries, blueberries, raspberries and in purees

To determine HHP inactivation of HuNoV GI.1 and GII.4 strains, inoculated berry and puree samples were treated at 200 – 650 MPa for 2 min at an initial sample temperature of $0\text{ }^{\circ}\text{C}$. These treatment conditions as shown in Table 1 were chosen to identify HPP processing parameters that could achieve $\geq \sim 3\text{ log}_{10}$ reduction of HuNoV. After HHP treatments, HuNoV were extracted from the pressure-treated and untreated (control) samples and quantified by the PGM-MB/PCR assay. pH of water surrounding the berries were measured after the HHP treatment for the HHP-treated and untreated berry samples.

2.5 Extraction of HuNoV from strawberries, blueberries, raspberries and purees

HuNoV GI.1 and GII.4 were extracted from strawberries, blueberries and raspberries following the protocol described by Li et al. (2013a) with minor modifications. Briefly, each berry sample along with 5 ml of water in the bag was placed into a 50 ml sterile centrifuge tube and additional 5 ml of ddH₂O was added to the tube. To release the virus from the berry surface, each tube containing the berry sample was vortexed for 5 times and each pulse lasted for 10 s on a Vortex-Genie 2 vortexer (Scientific industries, Bohemia, NY) with speed set at “4”-“5”. The suspension was then transferred to a 15 ml sterile centrifuge tube and 10 × PBS (KeraFAST) was added to the tube to buffer the suspension to 1 × PBS.

HuNoV GI.1 and GII.4 were extracted from the three berry purees following the protocol described by Kingsley et al. (2002) with minor modifications. Each puree sample was transferred to a 50 ml sterile centrifuge tube and 9 volumes of vegetable extraction buffer (100 mM Tris (Thermo Fisher Scientific Inc.), 50 mM glycine (Promega Corporation), 3% (m/v) beef extract (Becton Dickinson Company), 50 mM MgCl₂ (Thermo Fisher Scientific Inc.), pH 9.5) was added to the tube. The sample was then homogenized by a vortexer (Scientific industries, Bohemia, NY) and incubated at 37 °C for 30 min. The homogenate was centrifuged at 10,000 × g for 15 min at 4 °C. One volume of 16% (m/v) PEG8000 (Thermo Fisher Scientific Inc.) and 0.525M NaCl (AMRESCO LLC) were added to the supernatant, followed by incubation on ice for 1 h. The mixture was then centrifuged at 10,000 × g for 15 min at 4 °C. The pellet was resuspended in 5 ml PBS.

2.6 Preparation of PGM-MBs and PGM-MB binding assay

PGM-MBs were prepared as described by Li et al. (2013a). One milliliter of MagnaBind carboxyl-derivatized beads (Thermo Scientific, Rockford, IL) was washed 3 times with 1 ml PBS for each wash and separated from the liquid using a bead attractor (EMD Millipore). One

milliliter of 10 mg/ml type III mucin from porcine stomach (Sigma, St. Louis, MO) and 0.1 ml of 10 mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), both in conjugation buffer (0.1 M MES (2-(N-morpholino) ethanesulfonic acid), 0.9% NaCl, pH 4.7), were added to the beads. The mixture was rotated for 30 min on a Labquake shaker rotisserie (Thermo Scientific, Waltham, MA). The beads were then washed 3 times using 1 ml PBS for each wash, suspended in 1 ml PBS containing 0.05% sodium azide, and stored at 4 °C.

Before PGM-MB binding assay, each sample was treated with 100 µg of RNase A (Life Technologies, Carlsbad, CA) at 37 °C for 30 min. The PGM-MB binding assay was conducted as described by Li et al. (2013a) with slight modifications. One hundred and 200 µl of PGM-MBs were added to samples inoculated with HuNoV GI.1 or GII.4, respectively. The samples were then incubated at room temperature for 15 min on the Labquake shaker rotisserie and PGM-MBs were separated from the liquid using the bead attractor. The PGM-MBs were then washed 3 times with 1 ml PBS for each wash, suspended in 140 µl ddH₂O and stored on ice.

2.7 RNA Extraction and RT-qPCR Detection

RNA of HuNoV was extracted using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol with slight modifications. For each sample, 560 µl buffer AVL containing 5.6 µl carrier RNA was added, pulse-vortexed for 15 s and incubated at room temperature for 10 min. PGM-MBs were then separated by the bead attractor. The liquid was transferred to a new 1.5 ml sterile centrifuge tube and 560 µl of ethanol was added followed by a 15 s pulse-vortex. The liquid was then transferred to the QIAamp Mini column and centrifuged at 6000 × g for 1 min. The column containing the RNA was then washed by 500 µl buffer AW1 and 500 µl buffer AW2. Finally, the RNA was eluted in 2 × 40 µl AVE elution buffer.

The RT-qPCR quantification, including the primers, the TaqMan probes and their concentration, followed the protocol described by Li et al. (2013a). For HuNoV GI.1, NIF4 (+) CGCTGGATGCGNTTCCAT (500 nM), NV1LCR (–)CCTTAGACGCCATCATCATTTAC (900 nM), and NVGG1p 6'-FAM- TGGACAGGAGAYCGCRATCT-BHQ (250 nM) were used. For HuNoV GII.4, QNIF2 (+) ATGTTTCAGRTGGATGAGRTTCTCWGA (500 nM), COG2R (–) TCGACGCCATCTTCATTCACA (900 nM) and QNIFS 6'-FAM- AGCACGTGGGAGGGCGATCG-BHQ (250 nM) were used. All primers were synthesized by Life technologies and all probes were synthesized by Integrated DNA Technologies. RT-qPCR was performed using the Fast Virus 1-Step Master Mix (Life Technologies). For each sample, 10 µl RT-qPCR system was adopted, containing 2.5 µl master mix, 0.5 µl forward primer, 0.5 reverse primer, 0.5 µl probe and 6 µl sample RNA template. The following condition was used for the PCR process: 50 °C for 5 min for reverse transcription, followed by 95 °C for 20 s for initial denaturation, 45 cycles of 95 °C for 3 s and 60 °C for 30 s. RNA standard template was prepared to determine the reduction of HuNoV after the HHP treatment. Viral RNA of HuNoV GI.1 or GII.4 virus stock (280 µl) was extracted as RNA standard template and eluted in 200 µl AVE buffer. The RNA standard template was aliquoted and store at – 80 °C until use. The RNA standard template was 10-fold diluted for RT-qPCR. Serial 10-fold dilutions of GI.1 RNA and GII.4 RNA were used as standard for RT-qPCR. Reduction of HuNoV was calculated using the difference between untreated samples and treated samples corresponding to the RNA standard. When quantity of HuNoV detected by RT-qPCR was below the most diluted RNA standard, the result was considered as below detection limit. The maximum reduction levels that could be detected was ~ 3.0 logs for GI.1 and ~ 4.0 logs for GII.4. The difference in maximum reduction levels between the two strains was due to the different virus titers in the fecal samples.

2.8 Effect of HHP on the physical properties and sensory qualities of berries and purees

Based on the HHP results on the inactivation of HuNoV, blueberries and the three types berry purees were chosen for sensory and physical properties tests. Fresh berries were bought from a local supermarket the day before HHP treatment and berry purees were prepared as described in Section 2.2. Un-inoculated blueberries (1000 g) were double-bagged and immersed in distilled water (1000 ml). Berry purees (1000 g) were double-bagged. HHP treatment of un-inoculated blueberries and purees were conducted at 550 MPa at 0 °C for 2 min using a 10-liter HHP unit (Elmhurst Research, Inc., Albany, NY) at Rutgers University. This unit was capable of reaching a maximum working pressure of 690 MPa in less than 3 min. The maximum depressurization time was 10 s. Filtered tap water was used as the pressure transmitting fluid. Before the HHP treatment, the sample bags were placed on ice to be equilibrated to 0 °C. Ice and the sample bags were placed into a plastic basket. The basket was then put into the pressure chamber pre-chilled to around 7 °C. The ice and water mixture in the basket maintained the berry samples at 0 °C before the pressure treatment. Since the entire stainless steel vessel was pre-chilled, it acted as a heat sink and the temperature of the water remained close to 7 °C during pressurization and pressure hold. After the pressure treatment, HHP-treated and untreated samples were kept frozen at -20 °C.

The physical properties of the un-treated and HHP-treated berry and berry puree samples were analyzed right after frozen (day 0) and after 7 and 14 days of frozen storage. The color of the frozen blueberries and the thawed purees was measured using a colorimeter (Konica Minolta's CR-10 Tristimulus Colorimeter, Konica Minolta Sensing Americas, Inc., NJ, USA) which utilizes the Hunter L , a , and b scale with L representing the lightness on a scale of 0 (dark) to 100 (white), $+a$ for redness, $-a$ for greenness, $+b$ for yellowness and $-b$ for blueness. Color of

243 berry purees (~ 1 cm height) was measured in a petri dish (100 x 15 mm stackable petri dish,
244 Kord Valmark, PA, U.S.A) on a white board. Three blueberries were randomly picked for color
245 measurement in a petri dish on a white board and each blueberry was measured from the stem,
246 calyx and skin. pH and apparent viscosity of thawed purees were measured using a pH meter
247 (Accumet AB15, Fisher Scientific, NH, U.S.A) and a digital spindle viscometer (DV-E,
248 Brookfield, MA, U.S.A), respectively. The sample temperature was maintained at 21 °C in a
249 water bath during viscosity and pH measurement. Apparent viscosity and pH of berry puree
250 samples were measured in a 600 ml low form griffin glass beaker following the manufacturers'
251 instructions. Color of blueberries was measured in frozen state and color, pH and viscosity of
252 purees were measured in the thawed state. All the instruments, colorimeter, pH meter, and
253 viscometer, were calibrated before use.

254 Sensory evaluation was conducted by 68 untrained volunteers recruited from the
255 University of Delaware campus. Two types of sensory evaluation methods, triangle and hedonic
256 tests, were used. For the triangle test, each volunteer was given 3 unknown samples labeled with
257 3-digit numbers. These samples contained the pressure treated sample(s) (treated at 550 MPa at
258 0 °C for 2 min and stored at – 20 °C for 7 days) and the control sample(s) (untreated and stored
259 at – 20 °C for 7 days) and presented to the volunteers in random order. The volunteers were
260 informed that two of the samples were identical and asked to smell and visually examine them
261 and determine the odd sample. Volunteers were also asked to rate the samples on a 1-5 hedonic
262 scale with 5 being “Like extremely”, 4 being “Like moderately”, 3 being “Neither like nor
263 dislike”, 2 being “Dislike moderately” and 1 being “Dislike extremely”. The evaluated
264 characteristics included appearance, aroma, color, and overall acceptability. Prior to conducting
265 the sensory evaluation, a study protocol was approved by the Institutional Review Board of

University of Delaware. Berry purees and blueberries were evaluated in the thawed state and frozen state, respectively.

2.9 Statistical Analysis

At least three independent trials (3 to 5 replicates) were conducted for all the experiments. Log reductions in virus population were calculated via the difference between untreated samples and treated samples. Statistical analyses were performed using JMP (SAS Cary, NC, USA). A p -value < 0.05 was considered significantly different among treatments using one-way analysis of variance and Tukey's multiple comparisons. For samples with HuNoV titer below the detection level of 10 genome copies/sample, the value of 10 was used to calculate the log reduction.

3. Results

3.1 Effect of initial sample temperature on pressure inactivation of HuNoV GI.1 in strawberry puree

As shown in Figure 1, HHP inactivation of HuNoV GI.1 in strawberry puree was significantly influenced by the initial sample temperature. As the initial temperature decreased from 20 to 0 °C, HuNoV GI.1 became more sensitive to HHP treatments. For example, HHP treatment at 500 MPa for 2 min at 0, 4, and 20 °C reduced HuNoV GI.1 in strawberry puree by 2.5, 2.2, and 1.0 log, respectively.

3.2 HHP inactivation of HuNoV GI.1 and GII.4 on berries and in purees

As for HuNoV GI.1 strain, the extraction rates were 11.2%, 16.8%, 13.4%, 11.2%, 10.8% and 10.8%, for strawberry quarters, blueberries, raspberries, strawberry puree, blueberry puree and raspberry puree, respectively. As for HuNoV GII.4 strain, the extraction rates were 11.2%,

13.4%, 13.4%, 16.8%, 18.75% and 16.8%, for strawberry quarters, blueberries, raspberries, strawberry puree, blueberry puree and raspberry puree, respectively. The detection limits for HuNoV GI.1 and GII.4 strains were around 10^{-3} and 10^{-4} dilution levels, respectively.

As shown in Table 1, HuNoV GII.4 strain was more sensitive to HHP treatment than GI.1 strain on the three types of berries and in their purees. HHP treatment at 550 MPa achieved > 2.9 log reduction of HuNoV GI.1 strain in three berry purees. GI.1 strain on blueberries was reduced by > 3.2 log reduction with a 550 MPa HHP treatment, while HHP treatment at 650 MPa only achieved 1.7 and 2.5 log reduction of GI.1 strain on strawberry quarters and raspberries, respectively. As for HuNoV GII.4 strain, HHP treatments at 400 and 350 MPa could reduce > 4.2 log of GII.4 strain in strawberry puree and raspberry puree, respectively. A 550 MPa HHP treatment was needed to achieve > 4.4 log reduction of GII.4 strain in blueberry puree. HHP treatments at 300 and 550 MPa could reduce > 4.1 log of GII.4 strain on blueberries and raspberries, while a HHP treatment of 650 MPa could only reduce GII.4 strain on strawberry quarters by 3.1 log.

The pH of water surrounding the HHP-treated and untreated strawberry quarters, blueberries and raspberries was tested. As shown in Table 2, water surrounding the untreated (control) and pressure treated blueberries samples had the highest pH values compared to the water surrounding the other two types of berry samples. HHP treatments reduced the pH of water surrounding the three types of berry samples.

3.3 Physical properties and sensory evaluation of blueberries and three types of purees

Since the treatment of 550 MPa for 2 min at 0 °C could achieve satisfactory inactivation effect of HuNoV GI.1 and GII.4 on blueberries and the three types of purees, this treatment was

applied on un-inoculated blueberries and the three types of purees and its effect on their physical properties and sensory quality was studied. Figure 2 shows the pictures of HHP-treated and untreated blueberries and purees after 7 days of frozen storage.

No significant differences ($P > 0.05$) in the L , a , and b values were observed between the control and HHP-treated samples right after freezing and after 7 and 14 days of frozen storage (Table 3). Also during the 14 days of frozen storage, the changes in L , a , and b values were minimal. No significant differences ($P > 0.05$) in the pH values were observed between the control and HHP-treated strawberry puree and raspberry puree samples right after freezing and after 7 and 14 days of frozen storage (Table 4). Significant differences in pH values between the control and HHP-treated blueberry puree samples on day 0 ($P = 0.003$) and day 14 ($P = 0.006$) were observed, but they were very small, ≤ 0.11 , and of no practical importance. During the 14 days of frozen storage, pH of HHP-treated and untreated strawberry puree decreased very lightly, while the pH of HHP-treated and untreated raspberry puree increased slightly. pH of untreated blueberry puree varied between 2.91 and 3.00 and HHP-treated blueberry puree varied between 3.00 and 3.02 during the frozen storage.

The effects of HHP treatment and frozen storage time on puree viscosity were shown in Figure 3. For all the purees, viscosity curves for the HHP-treated and control samples were almost identical. Viscosity of the purees decreased as the frozen storage time increased.

Sensory evaluation was conducted for the control and HHP-treated blueberries and three types of purees after 7 days of frozen storage. For the triangle test (Table 5), untrained volunteers could detect a significant difference between HHP-treated and untreated samples of blueberries ($P < 0.0001$) and strawberry puree ($P = 0.0005$). As for blueberry purees ($P = 0.5$) and raspberry purees ($P = 0.91$), volunteers could not detect a significant difference between the HHP-treated

and untreated samples. Sensory evaluation on 4 sensory attributes (appearance, aroma, color, and overall acceptability) using the hedonic test was also conducted to determine the acceptability of the blueberries and puree samples. The volunteers gave significantly higher scores ($P < 0.05$) of the untreated blueberries over the HHP-treated blueberries in the categories of appearance, color and overall acceptability (Table 6). However, no significant differences ($P > 0.05$) in appearance, aroma, color and overall acceptability were found between the HHP-treated purees and the untreated samples.

4. Discussion

In this study, RT-qPCR were used for RNA quantification and PGM-MB binding assay was used for infectious viral particles discrimination. The PGM-MB/PCR assay has been clearly demonstrated to be a practical mean of distinguishing between infectious and non-infectious HuNoV. In a human HuNoV challenge study, HHP could completely inactivate HuNoV GI.1 strain (10^4 genomic equivalent copies) in inoculated oyster and no HuNoV infection showed among the subjects (Leon et al., 2011). Using the PGM-MB/PCR assay, Ye et al. (2014) demonstrated that the same pressure treatment could achieve more than 4.1 log reduction of the same GI.1 strain. Li and Chen (2015) compared HHP inactivation results of MNV-1 and TV obtained through the PGM-MB/PCR assay and plaque assay. The two pressure inactivation curves obtained using the plaque and PGM-MB/PCR assays were almost identical at $\leq \sim 2$ -log reduction levels for both surrogates. A very recent study by Lou et al. (2015) also demonstrated that the HHP inactivation data of a HuNoV GII.4 strain obtained by the PGM-MB/PCR assay corresponded very well with the results obtained through a gnotobiotic pig model. In that study, PGM-MB/PCR assay showed 3.7 and 1.0 log reduction of HuNoV GII.4 after treatments of 350 MPa for 2 min at 0 and 35 °C, respectively. Correspondingly, newborn gnotobiotic piglets orally

fed with inoculated oyster homogenate treated at 350 MPa and 0 °C for 2 min showed no HuNoV infection symptoms while piglets fed with oyster homogenate treated at 350 MPa and 35 °C for 2 min showed HuNoV infection symptoms. The result in our study was based on an assumption that PGM-MB binding/RT-qPCR assay provided a good estimate of pressure inactivation effect of HuNoV. However, it should be noted that currently no method is available to validate the PGM-MB binding/RT-qPCR assay. Therefore, results from this study should be interpreted with caution.

In this study, the initial sample temperature played a significant role in the HHP inactivation of HuNoV GI.1. As the initial sample temperature decreased, HuNoV GI.1 strain became more sensitive to HHP treatment. This result is consistent with those reported by Li et al. (2013a) who found that as the initial sample temperature decreased from 35 °C to 1 °C, both HuNoV GI.1 and GII.4 strains became more sensitive to HHP treatment. Similar temperature effect was also observed for HHP inactivation of HuNoV GI.1 and GII.4 strains in oysters and clams (Ye et al., 2014). The clinical trial conducted by Leon et al. (2011) also showed that low temperature enhanced HHP inactivation of HuNoV GI.1 strain. In that study, human subjects were challenged with pressure-treated and un-treated oysters inoculated with HuNoV GI.1 via injection. The HuNoV infection rate of subjects challenged with oysters treated at 400 MPa for 5 min at 25°C was 60%. The same HHP treatment (400 MPa for 5 min) conducted at a lower temperature, 6°C, reduced the infection rate to 21%. Surrogate studies using MNV-1, FCV and TV also demonstrated that cold temperature enhanced their inactivation by HHP (Chen et al., 2005; Huang et al., 2013; Kingsley et al., 2007; Li et al., 2013b; Lou et al., 2011).

As shown in Table 1, HuNoV GI.1 strain was more resistant to HHP treatment than GII.4 strain on three types of berries and in their purees. These results are consistent with those

378 reported by Li et al. (2013a) and Ye et al. (2014). Li et al. (2013a) observed that a HHP
379 treatment of 350 MPa at 21 °C for 2 min reduced HuNoV GII.4 strain in PBS by > 4.0 log,
380 while a more severe HHP treatment, 500 MPa at 21 °C for 2 min, only reduced HuNoV GI.1
381 strain by 0.5 log. In oyster homogenates, a HHP treatment of 400 MPa at 25 °C for 5 min
382 reduced HuNoV GII.4 and GI.1 strain by 3.6 and 1.0 log, respectively (Ye et al., 2014).
383 Considering the different behavior of HuNoV strains towards HHP treatments, it is important to
384 select relevant strain for target food product. To be conservative, selecting the most HHP-
385 resistant strain might be a better alternative. HuNoV GII strains were the leading foodborne
386 outbreaks cause, while GI strains showed similar prevalence as GII strains in waterborne
387 outbreaks (Bitler et al., 2013; Vega et al., 2014). From 2009 to 2013 in USA, GII.4 was the most
388 prevalent strain among GII strains and GI.6 was the most prevalent strain among GI strains
389 (Vega et al., 2014).

390 One of the main objectives in this study was to identify HHP treatment conditions for
391 berries and berry puree processing as an intervention for HuNoV contamination. Hutson et al.
392 (2004) reported that infectious dose of HuNoV was relatively low (10 – 100 virions). Baert et al.
393 (2011) reported that concomitant titers of HuNoV ranged from 2 to 3.5 log genome copies per
394 gram were found in HuNoV-positive strawberries and raspberries from Belgium and France.
395 Based on these findings, a treatment that could achieve more than 3 log reduction of HuNoV was
396 probably adequate to ensure the safety of berries and purees. Using this criterion, the HHP
397 treatment of 550 MPa for 2 min at 0 °C could achieve the targeted HuNoV inactivation effect for
398 blueberries and the three types of purees. Unfortunately, HHP treatment up to 650 MP could not
399 achieve desirable inactivation of HuNoV on strawberries and raspberries. Berries in this study
400 were pressurized while being immersed in water since Li et al. (2013a) observed that the

presence of water could significantly enhance HHP inactivation of HuNoV GI.1 on blueberries compared to when dry blueberries were HHP treated. As HuNoV GI.1 and GII.4 were inoculated on the skin of the berries and were in direct contact with surrounding water, water pH could be the main cause of different inactivation effect for the two viruses. The pH values of water surrounding strawberry quarters and raspberries were lower than those for blueberries water. Li et al. (2013a) demonstrated that the HHP inactivation of both HuNoV GI.1 and GII.4 strains was pH dependent and increasing the pH of the virus suspending substrate enhanced HHP inactivation. MNV-1 and TV also showed similar response to pH during HHP treatment; becoming more resistant to HHP treatment under acidic environment (Li et al., 2013b; Lou et al., 2011). Considering that enteric viruses such as HuNoV, MNV-1 and TV need to survive the low pH environment of a human stomach to reach their target cells in the small intestine, they are expected to be acid-tolerant (Cannon et al., 2006). The detailed mechanism of HuNoV resistance to HHP treatment under different pH conditions is currently not clear and needs to be further researched.

Another aim of the study was to investigate the effect HHP processing on berry and puree sensory qualities. Based on the HHP inactivation results of HuNoV, blueberries and three types of purees were chosen to be tested for their sensory quality. As fruits become softer during pressurization, application of HHP on fresh blueberries is not feasible (Oey et al., 2008). HHP-treated blueberries can only target the frozen market since freezing causes similar texture damage. In this study, we compared the sensory quality of untreated frozen blueberries to that of HHP-treated frozen blueberries using the triangle and hedonic tests. For the triangle test, untrained volunteers could detect a significant difference ($P < 0.0001$) between the HHP-treated and untreated blueberries. In addition, the volunteers gave significant higher scores ($P < 0.05$) of

the untreated blueberries than the HHP-treated ones in the hedonic test, demonstrating that HHP was not a suitable technique for processing blueberries. Although HHP induced changes to the strawberry puree that was noticeable to the volunteers since untrained volunteers could detect significant difference between HHP-treated and untreated samples (Table 5), these changes were not perceived as undesirable since no significant differences in appearance, aroma, color and overall acceptability were found between the HHP-treated strawberry puree and the untreated one ($P > 0.05$) (Table 6). For blueberry and raspberry purees, HHP did not induce noticeable changes (Table 5) and did not reduce their acceptability (Table 6).

5. Conclusions

The overall goal of this study was to identify suitable and optimum HHP processing condition for HuNoV inactivation in three berry fruits and purees. It should be noted that conclusions made in this study were based on the assumption that the PGM-MB/PCR assay could provide a good estimate of HHP inactivation of HuNoV. The results on the effect of temperature advocate the use of cold temperature in HHP processing to enhance HuNoV inactivation. The results obtained also demonstrate that HHP treatment could serve as a good HuNoV inactivation method for the three berry purees. For strawberries and raspberries, HHP treatment seemed to be less effective for HuNoV inactivation while for blueberries, HHP treatment could still be a good inactivation method. It seems the mechanism underlying the differences in HHP inactivation effect among the three berries could be attributed, at least partially, to the substrate pH during HHP. Sensory evaluation showed that HHP had little negative impact to the sensory qualities of three berry purees. In all, HHP could be a practical processing technique to inactivate HuNoV in strawberry, blueberry and raspberry purees.

Acknowledgements

This project was supported by the USDA National Integrated Food Safety Initiative (Award No: 2010-01498) and Agriculture and Food Research Initiative Competitive Grants Program of the USDA National Institute of Food and Agriculture (NIFA Award No: 2011-68003-30005).

References

- Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., Stals, A., Van Coillie, E., Herman, L., Uyttendaele, M., 2011. Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health? *International Journal of Food Microbiology* 151, 261-269.
- Bitler, E., Matthews, J., Dickey, B., Eisenberg, J., Leon, J., 2013. Norovirus outbreaks: a systematic review of commonly implicated transmission routes and vehicles. *Epidemiology and Infection* 141, 1563-1571.
- Butz, P., García, A.F., Lindauer, R., Dieterich, S., Bogner, A., Tauscher, B., 2003. Influence of ultra high pressure processing on fruit and vegetable products. *Journal of Food Engineering* 56, 233-236.
- Calder, L., Simmons, G., Thornley, C., Taylor, P., Pritchard, K., Greening, G., Bishop, J., 2003. An outbreak of hepatitis A associated with consumption of raw blueberries. *Epidemiology and Infection* 131, 745-751.
- Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., Vinje, J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. *Journal of Food Protection* 69, 2761-2765.
- Carter, M., 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. *Journal of Applied Microbiology* 98, 1354-1380.

470 Chen, H., Hoover, D.G., Kingsley, D.H., 2005. Temperature and treatment time influence high
 471 hydrostatic pressure inactivation of feline calicivirus, a norovirus surrogate. *Journal of Food*
 472 *Protection* 68, 2389-2394.

473 Cotterelle, B., Drougard, C., Rolland, J., Becamel, M., Boudon, M., Pinede, S., Traoré, O., Balay,
 474 K., Pothier, P., Espié, E., 2005. Outbreak of norovirus infection associated with the consumption
 475 of frozen raspberries, France, March 2005. *Eurosurveillance* 10, 2690.

476 Dancho, B.A., Chen, H., Kingsley, D.H., 2012. Discrimination between infectious and non-
 477 infectious human norovirus using porcine gastric mucin. *International Journal of Food*
 478 *Microbiology* 155, 222-226.

479 Falkenhorst, G., Krusell, L., Lisby, M., Madsen, S.B., Böttiger, B., Mølbak, K., 2005. Imported
 480 frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. *Eurosurveillance* 10,
 481 2795.

482 Friedman, D., Heisey-Grove, D., Argyros, F., Berl, E., Nsubuga, J., Stiles, T., Fontana, J., Beard,
 483 R., Monroe, S., McGrath, M., 2005. An outbreak of norovirus gastroenteritis associated with
 484 wedding cakes. *Epidemiology and Infection* 133, 1057-1063.

485 Hall, A.J., Lopman, B.A., Payne, D.C., Patel, M.M., Gastañaduy, P.A., Vinjé, J., Parashar, U.D.,
 486 2013. Norovirus disease in the United States. *Emerging Infectious Diseases* 19, 1198-1205.

487 Huang, Y., Ye, M., Chen, H., 2013. Inactivation of *Escherichia coli* O157: H7 and *Salmonella*
 488 spp. in strawberry puree by high hydrostatic pressure with/without subsequent frozen storage.
 489 *International Journal of Food Microbiology* 160, 337-343.

490 Hutin, Y.J., Pool, V., Cramer, E.H., Nainan, O.V., Weth, J., Williams, I.T., Goldstein, S.T.,
 491 Gensheimer, K.F., Bell, B.P., Shapiro, C.N., 1999. A multistate, foodborne outbreak of hepatitis
 492 A. *New England Journal of Medicine* 340, 595-602.

493 Hutson, A.M., Atmar, R.L., Estes, M.K., 2004. Norovirus disease: changing epidemiology and
 494 host susceptibility factors. *Trends in Microbiology* 12, 279-287.

495 Jones, M.K., Grau, K.R., Costantini, V., Kolawole, A.O., de Graaf, M., Freiden, P., Graves, C.L.,
 496 Koopmans, M., Wallet, S.M., Tibbetts, S.A., 2015. Human norovirus culture in B cells. *Nature*
 497 *protocols* 10, 1939-1947.

498 Kingsley, D.H., Holliman, D.R., Calci, K.R., Chen, H., Flick, G.J., 2007. Inactivation of a
 499 norovirus by high-pressure processing. *Applied and Environmental Microbiology* 73, 581-585.

500 Kingsley, D.H., Hoover, D.G., Papafragkou, E., Richards, G.P., 2002. Inactivation of hepatitis A
 501 virus and a calicivirus by high hydrostatic pressure. *Journal of Food Protection* 65, 1605-1609.

502 Korsager, B., Hede, S., Boggild, H., Bottiger, B., Molbak, K., 2005. Two outbreaks of norovirus
 503 infections associated with the consumption of imported frozen raspberries, Denmark, May-June
 504 2005. *Eurosurveillance* 10, 2729.

505 Le Guyader, F.S., Mittelholzer, C., Haugarreau, L., Hedlund, K.-O., Alsterlund, R., Pommepuy,
 506 M., Svensson, L., 2004. Detection of noroviruses in raspberries associated with a gastroenteritis
 507 outbreak. *International Journal of Food Microbiology* 97, 179-186.

508 Leon, J.S., Kingsley, D.H., Montes, J.S., Richards, G.P., Lyon, G.M., Abdulhafid, G.M., Seitz,
 509 S.R., Fernandez, M.L., Teunis, P.F., Flick, G.J., 2011. Randomized, double-blinded clinical trial
 510 for human norovirus inactivation in oysters by high hydrostatic pressure processing. *Applied and*
 511 *Environmental Microbiology* 77, 5476-5482.

512 Li, X., Chen, H., 2015. Evaluation of the porcine gastric mucin binding assay for high-pressure-
 513 inactivation studies using murine norovirus and tulane virus. *Applied and Environmental*
 514 *Microbiology* 81, 515-521.

515 Li, X., Chen, H., Kingsley, D.H., 2013a. The influence of temperature, pH, and water immersion
 516 on the high hydrostatic pressure inactivation of GI. 1 and GII. 4 human noroviruses. *International*
 517 *Journal of Food Microbiology* 167, 138-143.

518 Li, X., Ye, M., Neetoo, H., Golovan, S., Chen, H., 2013b. Pressure inactivation of Tulane virus,
 519 a candidate surrogate for human norovirus and its potential application in food industry.
 520 *International Journal of Food Microbiology* 162, 37-42.

521 Lou, F., Neetoo, H., Chen, H., Li, J., 2011. Inactivation of a human norovirus surrogate by high-
 522 pressure processing: effectiveness, mechanism, and potential application in the fresh produce
 523 industry. *Applied and Environmental Microbiology* 77, 1862-1871.

524 Lou, F., Ye, M., Ma, Y., Li, X., DiCaprio, E., Chen, H., Krakowka, S., Hughes, J., Kingsley, D.,
 525 Li, J., 2015. A gnotobiotic pig model for determining human norovirus inactivation by high-
 526 pressure processing. *Applied and Environmental Microbiology* 81, 6679-6687.

527 Mäde, D., Trübner, K., Neubert, E., Höhne, M., Johne, R., 2013. Detection and typing of
 528 norovirus from frozen strawberries involved in a large-scale gastroenteritis outbreak in Germany.
 529 *Food and Environmental Virology* 5, 162-168.

530 Marionneau, S., Ruvoën, N., Le Moullac-Vaidye, B., Clement, M., Cailleau-Thomas, A., Ruiz-
 531 Palacois, G., Huang, P., Jiang, X., Le Pendu, J., 2002. Norwalk virus binds to histo-blood group
 532 antigens present on gastroduodenal epithelial cells of secretor individuals. *Gastroenterology* 122,
 533 1967-1977.

534 Niu, M.T., Polish, L.B., Robertson, B.H., Khanna, B.K., Woodruff, B.A., Shapiro, C.N., Miller,
 535 M.A., Smith, J.D., Gedrose, J.K., Alter, M.J., 1992. Multistate outbreak of hepatitis A associated
 536 with frozen strawberries. *Journal of Infectious Diseases* 166, 518-524.

537 Oey, I., Lille, M., Van Loey, A., Hendrickx, M., 2008. Effect of high-pressure processing on
 538 colour, texture and flavour of fruit-and vegetable-based food products: a review. *Trends in Food*
 539 *Science & Technology* 19, 320-328.

540 Potera, C., 2013. Pesticide spraying may spread norovirus. *Environmental Health Perspectives*
 541 121, A148.

542 Rodriguez-Lazaro, D., Cook, N., Ruggeri, F.M., Sellwood, J., Nasser, A., Nascimento, M.S.J.,
 543 D'Agostino, M., Santos, R., Saiz, J.C., Rzeżutka, A., 2012. Virus hazards from food, water and
 544 other contaminated environments. *FEMS Microbiology Reviews* 36, 786-814.

545 Sánchez-Moreno, C., Plaza, L., de Ancos, B., Cano, M.P., 2003. Vitamin C, provitamin A
 546 carotenoids, and other carotenoids in high-pressurized orange juice during refrigerated storage.
 547 *Journal of Agricultural and Food Chemistry* 51, 647-653.

548 Sánchez, G., Aznar, R., Martínez, A., Rodrigo, D., 2011. Inactivation of human and murine
 549 norovirus by high-pressure processing. *Foodborne Pathogens and Disease* 8, 249-253.

550 Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones,
 551 J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-major pathogens.
 552 *Emerging Infectious Diseases* 17, 7-15.

553 Tauscher, B., 1995. Pasteurization of food by hydrostatic high pressure: chemical aspects.
 554 *Zeitschrift für Lebensmittel-Untersuchung und Forschung* 200, 3-13.

555 Tian, P., Engelbrektson, A., Mandrell, R., 2008. Two-log increase in sensitivity for detection of
 556 norovirus in complex samples by concentration with porcine gastric mucin conjugated to
 557 magnetic beads. *Applied and Environmental Microbiology* 74, 4271-4276.

558 Tian, P., Jiang, X., Zhong, W., Jensen, H.M., Brandl, M., Bates, A.H., Engelbrektson, A.L.,
 559 Mandrell, R., 2007. Binding of recombinant norovirus like particle to histo-blood group antigen
 560 on cells in the lumen of pig duodenum. *Research in Veterinary Science* 83, 410-418.
 561 Tian, P., Yang, D., Jiang, X., Zhong, W., Cannon, J., Burkhardt Iii, W., Woods, J., Hartman, G.,
 562 Lindesmith, L., Baric, R., 2010. Specificity and kinetics of norovirus binding to magnetic bead-
 563 conjugated histo-blood group antigens. *Journal of Applied Microbiology* 109, 1753-1762.
 564 Vega, E., Barclay, L., Gregoricus, N., Shirley, S.H., Lee, D., Vinjé, J., 2014. Genotypic and
 565 epidemiologic trends of norovirus outbreaks in the United States, 2009 to 2013. *Journal of*
 566 *Clinical Microbiology* 52, 147-155.
 567 Ye, M., Li, X., Kingsley, D.H., Jiang, X., Chen, H., 2014. Inactivation of human norovirus in
 568 contaminated oysters and clams by high hydrostatic pressure. *Applied and Environmental*
 569 *Microbiology* 80, 2248-2253.

570

Figure 1. Effect of initial sample temperature on HHP inactivation of HuNoV GI.1 in strawberry puree. Puree samples were inoculated with HuNoV GI.1 and treated at 450 - 550 MPa for 2 min at initial sample temperatures of 0, 4 and 20 °C. Error bars represent one standard deviation. Bars with different letters at the same pressure level are significantly different ($P < 0.05$). Letters marked with an asterisk indicate the titer of HuNoV in the HHP-treated sample was below detection limit for some of the replicates. For the 550 MPa treatment group, 3 out of 3 replicates for the 0 °C treatment and 2 out of 3 replicates for the 4 °C treatment were below detection limit. The maximum reduction that could be detected was ~ 3.0 log.

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582

583 Figure 2. Pictures of HHP-treated and untreated blueberries and strawberry, blueberry and
584 raspberry purees after frozen storage. Un-inoculated blueberries and puree samples were treated
585 at 550 MPa for 2 min at an initial temperature of 0 °C. HHP-treated and untreated blueberries
586 and puree samples were stored frozen at - 20 °C for 7 days.

589 Figure 3. Viscosity change of strawberry, blueberry and raspberry purees after pressurization and
 590 subsequent frozen storage. Un-inoculated puree samples were treated at 550 MPa for 2 min at an
 591 initial temperature of 0 °C. HHP-treated and untreated puree samples were stored frozen at -
 592 20 °C for 0 (right after freezing), 7 and 14 days. The viscosity measurement of thawed purees
 593 was conducted at 21 °C and at different viscometer spindle rotating speed.

Table 1. HHP inactivation of HuNoV GI.1 and GII.4 on berries and purees. Berry and puree samples were inoculated with HuNoV GI.1 or GII.4 and HHP-treated at an initial sample temperature of 0 °C for 2 min. Data are the means of log₁₀ reductions of genome copies/g ± 1 standard deviation from at least 3 replicates.

HuNoV Strain	Pressure (MPa)	Strawberry Puree	Blueberry Puree	Raspberry Puree	Strawberry Quarters	Blueberries	Raspberries
GI.1	450	1.4 ± 0.1 ^{Aab}	1.6 ± 0.5 ^{Ab}	2.7 ± 0.1 ^{Ac}	0.6 ± 0.3 ^{Aa}	2.7 ± 0.2 ^{Ac}	1.5 ± 0.3 ^{Ab}
	500	2.5 ± 0.5 ^{Bbc}	2.4 ± 0.6 ^{ABbc}	2.7 ± 0.4 ^{Ac}	0.9 ± 0.4 ^{Aa}	> 3.2 ± 0.1 ^{Bc}	1.9 ± 0.4 ^{Ab}
	550	> 3.0 ± 0.1 ^{Bc}	> 2.9 ± 0.2 ^{Bc}	> 2.9 ± 0.4 ^{Ac}	1.4 ± 0.4 ^{Ba}	> 3.2 ± 0.1 ^{Bc}	2.1 ± 0.5 ^{Ab}
	600	ND	ND	ND	1.9 ± 0.5 ^{Ba}	ND	2.2 ± 0.4 ^{Aa}
	650	ND	ND	ND	1.7 ± 0.4 ^{Ba}	ND	2.5 ± 0.2 ^{Ab}
GII.4	250	0.1 ± 0.1 ^{Aa}	ND	ND	ND	3.2 ± 0.3 ^{Ab}	ND
	300	0.7 ± 0.1 ^{Ba}	1.1 ± 0.3 ^{Aa}	3.7 ± 0.2 ^{Ab}	ND	> 4.1 ± 0.2 ^{Ab}	ND
	350	2.4 ± 0.1 ^{Cb}	1.6 ± 0.1 ^{ABa}	> 4.2 ± 0.3 ^{Bc}	ND	ND	ND
	400	> 4.2 ± 0.2 ^{Db}	2.1 ± 0.4 ^{BCa}	> 4.2 ± 0.2 ^{Bb}	ND	ND	ND
	450	> 4.2 ± 0.1 ^{Db}	2.6 ± 0.2 ^{Ca}	ND	ND	ND	ND
	500	> 4.2 ± 0.4 ^{Da}	3.5 ± 0.2 ^{Da}	ND	ND	ND	3.5 ± 0.3 ^{Aa}
	550	ND	> 4.4 ± 0.1 ^{Eb}	ND	2.8 ± 0.4 ^{Aa}	ND	> 4.1 ± 0.3 ^{Ab}
	600	ND	> 4.4 ± 0.1 ^{Eb}	ND	3.0 ± 0.5 ^{Aa}	ND	> 4.1 ± 0.3 ^{Ab}
	650	ND	ND	ND	3.1 ± 0.3 ^A	ND	ND

Data with different lowercase letters in the same row are significantly different (P < 0.05). Data with different uppercase letters in the same column for the same virus are significantly different (P < 0.05). ND: not done.

599 Table 2. pH of water surrounding berries after HHP treatments. Un-inoculated berry samples
600 were immersed in water and HHP treated at 400 and 600 MPa for 2 min at initial sample
601 temperature of 0 °C or untreated (control). The surrounding water of HHP-treated and untreated
602 samples was tested for pH right after HHP treatments. Data are the means of pH values $\pm$ 1
603 standard deviation from at least 3 replicates.

	Strawberry quarters	Blueberries	Raspberries
Control	3.92 $\pm$ 0.02 ^{Ba}	5.62 $\pm$ 0.15 ^{Bb}	4.24 $\pm$ 0.23 ^{Ba}
400 MPa	3.81 $\pm$ 0.01 ^{Aa}	4.82 $\pm$ 0.08 ^{Ab}	3.72 $\pm$ 0.14 ^{Aa}
600 MPa	3.79 $\pm$ 0.02 ^{Aa}	4.88 $\pm$ 0.10 ^{Ac}	3.92 $\pm$ 0.06 ^{Ab}

604 Data with different lowercase letters in the same row are significantly different ($P < 0.05$). Data
605 with different uppercase letters in the same column are significantly different ($P < 0.05$).

606

Table 3. Effect of HHP treatment and frozen storage on Hunter *L*, *a* and *b* values of blueberries and strawberry, blueberry and raspberry purees. Un-inoculated blueberries and puree samples were treated at 550 MPa for 2 min at an initial sample temperature of 0 °C. HHP-treated and untreated blueberries and puree samples were stored frozen at - 20 °C for 0 (right after freezing), 7 and 14 days. Color was measured for frozen blueberries and thawed purees. Data are the means of at least 3 replicates $\pm$ 1 standard deviation.

Food	Treatment	<i>L</i>			<i>a</i>			<i>b</i>		
		Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Blueberries	Control	22.6 $\pm$ 1.7 ^a	25.0 $\pm$ 2.0 ^a	22.2 $\pm$ 1.2 ^a	1.5 $\pm$ 1.4 ^a	0.7 $\pm$ 0.9 ^a	-1.1 $\pm$ 0.9 ^a	1.7 $\pm$ 0.2 ^a	1.0 $\pm$ 0.2 ^a	1.3 $\pm$ 0.4 ^a
	HHP	24.0 $\pm$ 2.5 ^a	22.0 $\pm$ 1.0 ^a	21.8 $\pm$ 0.9 ^a	1.9 $\pm$ 0.2 ^a	0.1 $\pm$ 0.4 ^a	-0.9 $\pm$ 0.4 ^a	1.9 $\pm$ 0.2 ^a	1.3 $\pm$ 0.1 ^a	0.8 $\pm$ 0.3 ^a
Strawberry	Control	32.7 $\pm$ 0.7 ^a	30.4 $\pm$ 1.5 ^a	31.3 $\pm$ 1.0 ^a	18.5 $\pm$ 2.1 ^b	12.8 $\pm$ 1.3 ^a	13.2 $\pm$ 0.8 ^a	7.5 $\pm$ 0.8 ^b	6.1 $\pm$ 0.3 ^a	6.2 $\pm$ 0.3 ^{ab}
	Puree	31.4 $\pm$ 1.0 ^a	29.3 $\pm$ 0.9 ^a	29.5 $\pm$ 2.3 ^a	16.2 $\pm$ 2.1 ^a	12.6 $\pm$ 0.5 ^a	11.7 $\pm$ 2.8 ^a	6.3 $\pm$ 0.7 ^a	5.5 $\pm$ 0.3 ^a	5.4 $\pm$ 1.0 ^a
Blueberry	Control	22.6 $\pm$ 1.7 ^a	23.7 $\pm$ 1.5 ^a	23.6 $\pm$ 1.1 ^a	1.5 $\pm$ 1.4 ^a	0.9 $\pm$ 0.6 ^a	2.6 $\pm$ 0.5 ^a	1.7 $\pm$ 0.2 ^a	1.5 $\pm$ 0.3 ^a	2.4 $\pm$ 0.1 ^b
	Puree	24.0 $\pm$ 2.5 ^a	23.0 $\pm$ 1.6 ^a	24.2 $\pm$ 0.5 ^a	1.9 $\pm$ 0.2 ^a	1.6 $\pm$ 0.3 ^a	1.7 $\pm$ 0.3 ^a	1.9 $\pm$ 0.2 ^a	1.7 $\pm$ 0.3 ^a	2.1 $\pm$ 0.2 ^a
Raspberry	Control	30.2 $\pm$ 0.5 ^a	27.7 $\pm$ 2.0 ^a	29.2 $\pm$ 0.3 ^a	18.2 $\pm$ 2.3 ^a	16.4 $\pm$ 1.9 ^a	17.4 $\pm$ 0.4 ^a	6.2 $\pm$ 0.7 ^a	5.5 $\pm$ 1.5 ^a	6.0 $\pm$ 0.2 ^a
	Puree	29.7 $\pm$ 2.2 ^a	27.2 $\pm$ 1.3 ^a	29.5 $\pm$ 0.1 ^a	18.6 $\pm$ 2.6 ^b	11.7 $\pm$ 1.7 ^a	16.1 $\pm$ 0.9 ^{ab}	6.1 $\pm$ 0.8 ^b	4.3 $\pm$ 0.5 ^a	5.7 $\pm$ 0.4 ^{ab}

Data in the same row within the same color value labeled with different lowercase letters are significantly different ($P < 0.05$).

Table 4. Effect of HHP treatment and frozen storage on pH of strawberry, blueberry and raspberry purees. Un-inoculated puree samples were treated at 550 MPa for 2 min at an initial sample temperature of 0 °C. HHP-treated and untreated puree samples were stored frozen at -20 °C for 0 (right after freezing), 7 and 14 days. pH was measured for thawed purees. Data are the means of at least 3 replicates $\pm$ 1 standard deviation.

Food	Treatment	Day 0	Day 7	Day 14
Strawberry	Control	3.72 $\pm$ 0.03 ^{Ab}	3.71 $\pm$ 0.01 ^{Aab}	3.67 $\pm$ 0.01 ^{Aa}
	Puree HHP	3.75 $\pm$ 0.02 ^{Ab}	3.72 $\pm$ 0.01 ^{Ab}	3.66 $\pm$ 0.02 ^{Aa}
Blueberry	Control	2.91 $\pm$ 0.02 ^{Aa}	3.00 $\pm$ 0.02 ^{Ab}	2.97 $\pm$ 0.01 ^{Ab}
	Puree HHP	3.02 $\pm$ 0.02 ^{Ba}	3.00 $\pm$ 0.02 ^{Aa}	3.01 $\pm$ 0.01 ^{Ba}
Raspberry	Control	3.15 $\pm$ 0.03 ^{Aa}	3.23 $\pm$ 0.01 ^{Ab}	3.41 $\pm$ 0.01 ^{Ac}
	Puree HHP	3.20 $\pm$ 0.04 ^{Aa}	3.21 $\pm$ 0.02 ^{Aa}	3.41 $\pm$ 0.01 ^{Ab}

Data in the same column within the same food category labeled with different uppercase letters are significantly different ($P < 0.05$). Data in the same row labeled with different lowercase letters are significantly different ($P < 0.05$).

621 Table 5. Sensory comparison between HHP-treated and untreated blueberries and strawberry,
 622 blueberry and raspberry purees using the triangle test. Un-inoculated blueberries and puree
 623 samples were treated at 550 MPa for 2 min at an initial sample temperature of 0 °C. HHP-treated
 624 and untreated blueberries and puree samples were stored frozen at - 20 °C for 7 days. A total of
 625 68 volunteers participated in the sensory evaluation.

Food	# of people that correctly identified the odd sample	P - value
Blueberries	54 [*]	< 0.0001
Strawberry Puree	37 [*]	0.0005
Blueberry Puree	26	0.5
Raspberry Puree	30	0.09

626 Data labeled with an asterisk indicates untrained volunteers could significantly tell the difference
 627 between HHP-treated and untreated samples ($P < 0.05$ using Chi-square test).

628

Table 6. Scores of hedonic scale ratings (mean $\pm$ standard deviation) for sensory characteristics of HHP-treated and untreated blueberries and strawberry, blueberry and raspberry purees. Uninoculated blueberries and puree samples were treated at 550 MPa for 2 min at an initial sample temperature of 0 °C. HHP-treated and untreated blueberries and puree samples were stored frozen at - 20 °C for 7 days. A total of 68 volunteers participated in the sensory evaluation.

Food	Treatment	Appearance	Aroma	Color	Overall
					Acceptability
Blueberries	Control	4.12 ± 0.97 ^a	3.21 ± 0.68 ^a	3.91 ± 1.03 ^a	3.93 ± 0.85 ^a
	HHP	3.13 ± 1.13 ^b	3.28 ± 0.82 ^a	3.40 ± 1.20 ^b	3.34 ± 1.04 ^b
Strawberry	Control	4.16 ± 0.91 ^a	4.32 ± 0.82 ^a	4.34 ± 0.78 ^a	4.31 ± 0.74 ^a
	Puree	HHP	4.12 ± 0.97 ^a	4.24 ± 0.98 ^a	4.35 ± 0.73 ^a
Blueberry	Control	3.34 ± 1.06 ^a	3.47 ± 0.91 ^a	3.90 ± 0.90 ^a	3.63 ± 0.90 ^a
	Puree	HHP	3.62 ± 1.01 ^a	3.59 ± 0.95 ^a	4.09 ± 0.79 ^a
Raspberry	Control	4.07 ± 0.94 ^a	3.93 ± 1.07 ^a	4.22 ± 0.86 ^a	4.04 ± 0.89 ^a
	Puree	HHP	4.16 ± 0.89 ^a	4.06 ± 0.98 ^a	4.18 ± 0.91 ^a

Data in the same column within the same food category labeled with different lowercase letters are significantly different ($P < 0.05$).