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Inactivation of murine norovirus and hepatitis A virus on fresh raspberries by gaseous ozone treatment

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ABSTRACT

Raspberries are vulnerable products for which industrial treatment solutions ensuring both food safety and sensory quality are not easily applicable. Raspberries have been associated with numerous foodborne outbreaks in recent decades. Ozone has been proven effective as a drinking water treatment against pathogenic microorganisms. Nevertheless, to date, little information is available regarding the effect of gaseous ozone on viruses in food matrices. A comparison of the effect of gaseous ozone on murine norovirus (MNV-1) and hepatitis A virus (HAV) adsorbed on fresh raspberries was performed. Infectious MNV-1 was highly inactivated (>3.3 $\log_{10}$) by ozone (3 ppm, 1 min). The raspberry matrix seems to enhance inactivation by ozone compared to water. The same treatment was observed to have little effect on HAV even for the highest dose under the tested conditions (5 ppm, 3 min). Ozone treatment (5 ppm, 3 min) did not affect the appearance of raspberries even after three days post-treatment. No ozone effect was observed on the genomes detected by RT-PCR on both tested viruses, irrespective of the matrix or tested doses used. Gaseous ozone could therefore be a good candidate for human norovirus inactivation on raspberries but new conditions are needed for it to have significant effects on HAV inactivation.

1. Introduction

Enteric viruses are the main cause of foodborne outbreaks in the USA (36%) and in Europe (20.4%) (CDC, 2015; EFSA, 2016). Human norovirus (HNoV) is by far the most common viral cause (i.e. nearly 90% of the viral foodborne outbreaks). Although not as common, hepatitis A virus (HAV) also causes a large number of diseases that may require hospitalization. Fruits are described as the second main matrices involved in viral outbreaks after shellfish such as oysters or clams (EFSA, 2015; Painter et al., 2013). For example, in the first half of 2013, approximately 1500 cases of hepatitis A were linked to the consumption of frozen mixed berries in 12 European countries (EFSA, 2014). Raspberries in particular were demonstrated to be highly involved in outbreaks (EFSA, 2015; EFSA, 2014; Falkenhorst et al., 2005; Maunula et al., 2009; Painter et al., 2013).

Due to its low infectious dose, estimated at 10–100 viral particles (Carter, 2005; Caul, 1994; Hirneisen et al., 2010; Koopmans et al., 2002; Teunis et al., 2008), HNoV requires highly effective inactivation treatments. Heat and chlorine, commonly used as inactivating treatments in the food industry (Bozkurt et al., 2015; Hirneisen et al., 2010), have limitations on fragile matrices such as raspberries. Indeed, changes in appearance and sensory quality as well as formation of potentially carcinogenic chlorinated by-products are frequently observed when using chlorinated treatments (Guzel-Seydim et al., 2004). Ozone (O_3) could be used as an alternative treatment option. However, except for its long-standing use in water treatment (Guzel-Seydim et al., 2004; Kim et al., 2003; von Gunten, 2007), it is currently seldom or not often used in the industry. Yet, ozone has higher oxidizing potential than chlorine. It has a short half-life ($\approx$20 min) which allows food or food surfaces to be exposed very briefly (Guzel-Seydim et al., 2004; Kim et al., 2003).

The virucidal efficacy of ozone in water has been largely demonstrated (Finch and Fairbairn, 1991; Herbold et al., 1989;

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Hirneisen et al., 2011; Lim et al., 2010; Predmore et al., 2015; Roy et al., 1982; Shin and Sobsey, 2003; Thurston-Enriquez et al., 2005). Ozone inactivation of viruses is variable and depends on the (i) ozone concentration, (ii) contact time, (iii) matrix, (iv) experimental conditions (i.e. temperature and pH), and (v) viruses that are used (i.e. HNoV, HAV, or poliovirus). Comparisons between enteric viruses have been conducted (Herbold et al., 1989; Roy et al., 1981). HAV appeared sensitive to ozone in water but less than poliovirus (Herbold et al., 1989). Differences in inactivation were also observed between two subgroups of viruses (i.e. poliovirus 1 and poliovirus 2) (Roy et al., 1981). Other studies provided inactivation data of HAV in water (Khadre et al., 2001; Vaughn et al., 1990), where low ozone doses ($\leq$1 ppm) applied for 1 min led to a loss of infectivity of $\approx$4 $\log_{10}$ $TCID_{50}$. Ozone was also reported to inactivate surrogates of HNoV in water such as murine norovirus (MNV), feline calicivirus, or Tulane virus (Hirneisen et al., 2011; Lim et al., 2010; Predmore et al., 2015; Thurston-Enriquez et al., 2005). To date, the inactivation mechanisms of ozone are poorly understood. It has been shown to possibly affect the capsid (Predmore et al., 2015) or both the capsid and viral genome (Kim et al., 1980; Roy et al., 1981; Shin and Sobsey, 2003). Two studies have described the effect of gaseous ozone on viruses on food matrices (i.e. lettuce, strawberries, and onions) (Hirneisen et al., 2011; Predmore et al., 2015). They used MNV, feline calicivirus, or Tulane virus as viral surrogates of HNoV. Ozone inactivation of MNV was similar to that of Tulane virus on lettuce, but weaker on strawberries (Predmore et al., 2015). Comparable inactivation was also obtained for MNV and feline calicivirus on lettuce and onions (Hirneisen et al., 2011). Unlike pathogenic HNoV, these viruses can be cultivated routinely *in vitro*. Cell culture is the only method for evaluating inactivation (Gassilloud et al., 2003). To date, no data are available regarding inactivation of viruses on raspberries despite many reported cases of raspberry-related viral outbreaks. Moreover, no studies have assessed the effect of gaseous ozone on HAV in food matrices.

The main objective of this study was thus to evaluate the relevance of using gaseous ozone to inactivate viruses on fresh raspberries. Two surrogates were used: MNV-1 and non-pathogenic HAV (vaccine strain). We first compared the inactivation rates of MNV-1 by gaseous ozone in water and on fresh raspberries to assess the matrix effect. We then investigated the effect of the same treatment on the appearance of raspberries. Finally, we evaluated the inactivation of MNV-1 and HAV on raspberries by cell culture based infectivity assays and RT-PCR assays.

2. Materials and methods

2.1. Preparation of viruses

Hepatitis A virus strain HM175/18f (ATTC VR-1402) was propagated in Fetal Rhesus monkey Kidney cells (FRhK4, ATCC CRL-1688) using Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific), 1% non-essential amino acids (NEAA, ThermoFisher Scientific), 1% L-glutamine (ThermoFisher Scientific), and 1% antibiotics (penicillin G, streptomycin, amphotericin B, ThermoFisher Scientific).

Murine norovirus strain MNV-1 S99 was kindly provided by Friedrich Löeffler Institute (Germany). MNV-1 was propagated in the mouse macrophage cell line RAW 264.7 (ATCC TIB-71) using the cell culture method described by Cannon et al. (2006), which was similar to that used for FRhK4 cell culture. Enteric cytopathic bovine orphan (ECBO, ATCC VR-248) virus was propagated in Madin-Darby Bovine Kidney cells (MDBK, ATCC CCL-22).

FRhK4 cells and RAW 264.7 cells were infected with HAV and MNV-1, respectively, at a multiplicity of infection (MOI) of 0.1 for 2–3 days and for 3–4 days at 37 °C in a 5% CO_2 atmosphere. Three freeze/thaw cycles and centrifugation (400 g for 15 min) were then performed to remove cell debris from the virus lysate as described by Wobus et al. (2004). The supernatant was filtered through a 0.45-μm membrane filter, collected, aliquoted, and stored at - 80 °C. The final viral concentrations of the two viruses following this protocol were $\approx$7 $\log_{10}$ tissue culture infectious dose 50 per mL ($TCID_{50}$/mL) and 7 $\log_{10}$ plaque forming units per mL (PFU/mL) for MNV-1 and HAV, respectively. The ECBO virus propagation was done according to French standard recommendation (NF EN 14675, AFNOR) and Ley et al. (2002) with slight modifications. Briefly, virus propagation was performed with MDBK cells with ECBO virus at a MOI of 0.3 in Eagle's minimum essential medium (MEM) containing 2% FBS, 1% NEAA and 1% antibiotics. Supernatant containing viruses were harvested after 2 days. After three freeze/thaw cycles, supernatant was clarified by centrifugation (400 g for 15 min). Virus stocks were then filtered through a 0.45-μm membrane filter then stored at - 80 °C before used.

2.2. Preparation of food samples: artificial contamination of raspberries by spotting

Fresh raspberries were purchased from a local store and used directly without any treatment. Raspberries (25 g) were inoculated by spotting 100 μL of each virus (7 $\log_{10}$ $TCID_{50}$/mL and 7 $\log_{10}$ PFU/mL for MNV-1 and HAV, respectively) and stored for 20 h before treatment with ozone as described by Butot et al. (2007). Some contaminated raspberries, untreated by gaseous ozone, were used as positive controls in the infectivity assay and in the genome detection by RT-PCR.

2.3. Treatment of water and fresh raspberries with gaseous ozone

Gaseous ozone was generated by corona discharge from extra-dry compressed purified air using an ozone generator (LAB2B 5, Triogen Ltd). The pilot system for treatment by gaseous ozone for the decontamination of berries and water was designed and delivered by Alphatech (France). The amount of gaseous ozone in the chamber was displayed using a PortaSens II gas detector (ES, France). Ozone concentrations in water were determined using the Indigo colorimetric method (Bader and Hoigné, 1981). Prior to treatment with ozone, MNV-1 suspensions were dialyzed (Float-A-Lyzer G2 dialysis device 100 kDa 1 mL, Spectra/Por) overnight at 4 °C in 0.1X PBS (pH 7.4, Fisher Bioreagents). One mL of dialysed MNV-1 was then treated with different doses and contact times of bubbling gaseous ozone. At the end of each treatment time, the residual ozone was quenched with sodium thiosulfate (2 mol of sodium thiosulfate for 1 mol of ozone).

Contaminated raspberries were placed on 316 L stainless steel baskets inside the ozone chambers and treated with different doses and contact times. For each condition, C.t values (mg.min.L^{-1}) were determined as the ozone concentration (ppm) multiplied by the contact time (min). The relative humidity (%) and the temperature (°C) were also checked. The mean relative humidity and temperature were 52% ± 7% and 17.1 °C ± 0.4 °C, respectively.

2.4. Virus extraction from raspberries

After exposure to ozone, MNV-1 or HAV were recovered from raspberries using the elution/concentration method of the ISO/TS 15216 standard (2013). Each sample was transferred into a mesh filter bag with 40 mL of TGBE buffer (Tris–HCl 100 mM, glycine 50 mM, 1% beef extract, pH 9.5) and 30 units of pectinase (17389, Sigma-Aldrich, France), and kept constant at pH 9.5 ± 0.2 during incubation with shaking (400 rpm, 20 min, room temperature). The