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# Evaluation of porcine gastric mucin assay for detection and quantification of human norovirus in fresh herbs and leafy vegetables

Menka Suresh, Jennifer Harlow, Neda Nasheri*

*National Food Virology Reference Centre, Bureau of Microbial Hazards, Food Directorate, Health Canada, 251 Sir Frederick Banting Driveway, Ottawa, ON, K1A 0K9, Canada*

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ABSTRACT

Leafy vegetables and fresh herbs are important parts of a healthy diet, however, they can be common vehicles of norovirus (NoV) infection and lead to serious health and economic concerns globally. NoV is highly infectious and persistent in the food and the environment, while being resistant to conventional food decontamination practices. Herbs and leafy greens are often consumed raw, and if contaminated with NoV, they may cause illness. Consequently, for outbreak prevention and surveillance purposes, sensitive and rapid methods are required to detect the presence of infectious NoV in naturally contaminated produce during its shelf life. Herein, we compared the extraction efficiency of the ISO/TS 15216-1:2017 method with the porcine gastric mucin coated magnetic beads (PGM-MB) assay, combined with heat-denaturation for RNA extraction, for detection of human NoV in artificially contaminated fresh green seaweed, basil, mint, and baby spinach. Droplet-digital RT-PCR was used to quantify the extracted genome by both methods. Our data demonstrated that while the PGM-MB assay takes considerably less time, it yields significantly higher recovery rates compared with the ISO/TS 15216-1:2017. Furthermore, since this method has the ability to be adapted in high-throughput and automated systems, it can be further modified to be employed by the food industry to reduce the number of NoV illnesses and outbreaks at the source of distribution.

## 1. Introduction

In developed countries, NoV is the leading cause of gastroenteritis, which is often associated with consumption of contaminated fresh produce such as leafy greens and culinary herbs (Li and Uyttendaele. 2018; Yeargin and Gibson. 2019; Thomas et al., 2015; Deng and Gibson. 2017; Mattison et al., 2010; Hall et al., 2012). Contamination of leafy greens and herbs with NoV can occur at any stage, including production, processing, and distribution, and can persist for months (Yeargin and Gibson. 2019; Chatziprodromidou et al., 2018). Importantly, these commodities are often consumed raw or undercooked and decontamination methods are limited and not completely effective in removing NoV (Cook et al., 2016). Under these circumstances, improved surveillance and source tracking is the key for outbreak prevention and intervention (Rivera et al., 2018).

Due to high infectivity and low infectious dose of NoV (Atmar et al., 2014), small numbers of viral particles can cause illness, therefore sensitive methodologies are required to detect and characterize the virus in naturally contaminated foods (Stals et al., 2013). In the absence of a robust and readily available cell culture system for NoV, current detection methods are based on virus extraction and identification of the viral genome by conventional or quantitative RT-PCR assays (Stals et al., 2013). However, these methods are sensitive to the PCR inhibitors that are often present in foods and cannot discriminate between infectious and non-infectious virus (Manuel et al., 2018). For these reasons, NoV testing is not routinely performed in regulatory food laboratories worldwide (Bosch et al., 2018; Bosch et al., 2011). In 2013 the International Organisation for Standardization (ISO) published a two-part technical method for the detection and quantification of hepatitis A virus (HAV) and NoV in food matrices (ISO, 2013a; ISO, 2013b), which was later revised in 2017 as ISO/TS 15216-1:2017 (ISO, 2017). While they were validated by many laboratories for virus detection and quantification, the ISO/TS 15216 methods are laborious and do not discriminate between infectious viruses and non-infectious remnants including damaged virus particles and naked genomic RNA.

NoV capture by the porcine gastric mucin conjugated magnetic beads (PGM-MB) was developed as an alternative virus recovery method, which relies on the integrity of the viral capsid to bind to PGM (Dancho et al., 2012). PGM has been shown to be chemically similar to histo-blood group antigens (HBGA) in the human intestine (Tian et al.,

* Corresponding author.
*E-mail address:* neda.nasheri@canada.ca (N. Nasheri).

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2007) that acts as the ligand to NoV. Multiple lines of evidence suggest that the PGM-MB capture followed by qRT-PCR assay can predict loss of NoV infectivity (Dancho et al., 2012; Kingsley et al., 2018; Wang et al., 2014; Kingsley et al., 2014; Li and Chen. 2015; Lou et al., 2015). On-bead viral RNA extraction and qRT-PCR subsequent to the PGM-MB NoV capture, also allows for quantification of (likely) infectious virus without the intervention of potential PCR inhibitors, as it has been shown that the PGM-MB method can remove potential RT-PCR inhibitors (Tian et al., 2008). Thus, the extracted RNA might be more suitable for downstream genomic analyses required for source attribution and outbreak delineation (Ronholm et al., 2016; Nasheri et al., 2017).

In this study, we have optimized the PGM-MB method and compared its efficacy in extraction of highly prevalent NoV GII.4 (Centers for Disease Control and Prevention (CDC). 2018, Petronella et al., 2018), from fresh green seaweed (*Enteromorpha Spp*), baby spinach (*Spinacia oleracea*), sweet basil (*Ocimum basilicum*), and mint (*Mentha spicata*) with ISO/TS 15216-1:2017 method using droplet-digital RT-PCR (ddRT-PCR) for absolute quantification of the recovered viral genome. ddRT-PCR is a promising technology for viral load quantification in food virology that enables the determination of target copy numbers without external quantitative standards and offers greater precision in NoV genome quantification compared with conventional qRT-PCR (Coudray-Meunier et al., 2015; Fraisse et al., 2017; Persson et al., 2018). Herein we chose GII.4 Sydney 2012 strain as it is recognized as the predominant strain in global foodborne NoV outbreaks (Hasing et al., 2014). Our results revealed that more efficient virus recovery by the PGM-MB method can be achieved in shorter time compared to the ISO/TS 15216-1 method.

## 2. Method

### 2.1. PGM bead preparation

PGM beads were prepared as described previously (Dancho et al., 2012). Briefly, 10 mg of type III mucin from porcine stomach (PGM) (Sigma Aldrich) were dissolved in 1 mL of conjugation buffer ((0.1 M MES (2-(N-morpholino) ethanesulfonic acid), 0.9% NaCl, pH 4.7) (Fluka). 1 mL of MagnaBind carboxyl-derivatized beads (Thermofisher) was placed into a fresh tube and washed three times with 1 × phosphate-buffered saline (PBS). The beads were next resuspended with the PGM solution. Ten milligrams of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) were dissolved in conjugation buffer and 100 µl of this solution was added the beads solution. The bead solution was incubated on a rotary for 30 min at room temperature. The supernatant was discarded and the beads were washed three times with 1 × PBS. The PGM beads were resuspended in 1 mL of 1 × PBS and stored at 4 °C (Supplementary Fig. 1).

### 2.2. Inoculation of basil, mint, baby spinach and seaweed

Fresh basil, mint, seaweed and spinach were obtained from a local market from September to December of 2018. Five grams of each leafy green were evenly spiked with $1.8 \times 10^8$ genome copies (in 100 µl volume) of aliquoted fecal filtrate containing NoV GII.4 Sydney 2012 (SRA# SRR6743875) (Petronella et al., 2018) in triplicates and allowed to dry inside a biosafety cabinet for 20 min (Supplementary Fig. 2). Fecal filtrate preparation, and viral load calculation was performed as described previously (Nasheri et al., 2017).

### 2.3. Virus recovery

Virus recovery following the ISO/TS 15216-1:2017 method was performed according to the instructions (ISO, 2017). Briefly, once the inoculum dried, the samples were placed in mesh filter bags (VWR, #11216-904, PA) and 40 mL of Tris/glycine/beef extract (TBGE) buffer were added to each sample and incubated on a rocking plate at room temperature for 30 min. The eluates from the mesh filter bags (35 mL) were transferred to centrifuge tubes and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was transferred to a new tube and the pH of the sample was brought to 7 using 12N HCl. 5 × polyethylene glycol 6000 (PEG)/NaCl of ¼ volumes of the weight of each sample was added to each tube, followed by incubation on ice, on a rocking plate for 1 h. Samples were centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 500 µl of 1 × PBS and stored at −80 °C prior to RNA extraction.

For virus recovery using the PGM-MB method, samples were placed in mesh filter bags, 40 mL of 1 × PBS were added and the samples were incubated at room temperature for 30 min on a rocking plate. The eluate (35 mL) was then removed, transferred to a new tube and weighed for future calculations. The samples were stored at −80 °C until the next step. One milliliter of the viral eluate was placed in a microcentrifuge tube and 100 µl of PGM beads were added following the incubation on the rotary for 30 min at room temperature. The supernatant was discarded and the beads were washed three times with 1 × PBS prior to RNA extraction.

### 2.4. Viral RNA extraction and quantification

Viral RNA Mini Kit (Qiagen, Mississauga, Ontario, Canada) was used to extract RNA subsequent to virus isolation by the ISO/TS 15216-1 method. All steps were followed according to the manufacturer's protocol. For RNA extraction from the viruses captured by the PGM-MB, the beads were resuspended in 50 µl ultrapure water and heated at 100 °C for 10 min. The supernatant (containing RNA) was stored at −80 °C.

RNA recovered by both the ISO/TS 15216-1 and the PGM-MB methods was quantified using the Bio-Rad droplet digital PCR technology as described previously (Nasheri et al., 2017; Mykytczuk et al., 2017).

### 2.5. Recovery rate calculation and statistical analysis

The recovery rates for both methods were calculated by comparison of the absolute genome copy number of the virus recovered from the leafy greens with the absolute genome copy number of the virus aliquot used to inoculate the leafy greens:

$$\text{Recovery rate}(\%): \frac{\text{obtained genome copy number}}{\text{inoculated genome copy number}} \times 100$$

Statistical analysis was performed by Microsoft Excel 2016 to determine significant differences between the recovery rates obtained by both methods using the paired *t*-test, and the *p* values are provided.

## 3. Results

### 3.1. Comparison of the PGM-MB method with the ISO/TS 15216-1:2017 method in recovery of human NoV from leafy vegetables

Virus extraction efficiency of the PGM-MB method was compared with the ISO/TS 15216-1 using 5g of inoculated basil, mint, seaweed, and baby spinach as demonstrated in Fig. 1, in replicated samples over three independent experiments. RNA extraction using heat-denaturation was adopted for the PGM-MB method, because it is quick, and higher concentrations of RNA can be achieved compared to conventional RNA extraction methods (Tian et al., 2010). The extracted RNA was then quantified by ddRT-PCR in duplicates. Virus recovery and RNA extraction by the PGM-MB method on average take 80 min, while the same procedures by the ISO/TS 15216-1 method take 180 min (Fig. 1).

All un-inoculated samples (negative controls) yielded no genome

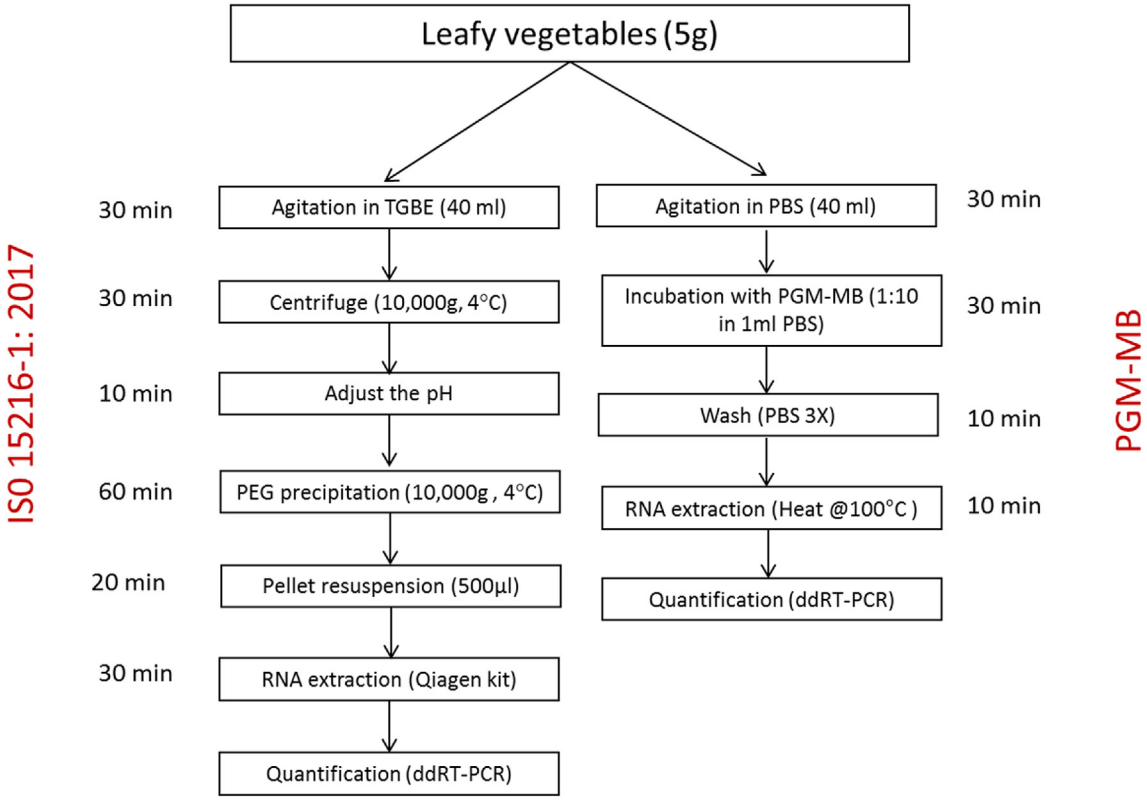


**Fig. 1.** An overview of the virus extraction methods used in this study. The average time that each step takes is shown.

copy count, demonstrating no natural contamination with NoV GII in the tested leafy vegetables. The extraction efficiency for each leafy vegetable obtained by the PGM-MB and the ISO/TS 15216-1 is shown as the mean recovery rate in Fig. 2 A. The highest recovery rate by the PGM-MB method was obtained for Seaweed (47 ± 7.8%), followed by mint (33.7 ± 3.35%), basil (6.6 ± 3.6%), and spinach (5.5 ± 2.2%). While the highest recovery with the ISO/TS 15216-1 method was obtained from basil (10.7 ± 2.6%) followed by mint (3.0 ± 2.2%), seaweed (2.86 ± 1.7%), and spinach (1.46 ± 0.65%). Except for basil, the recovery rate was significantly higher (ranging from 3.8 to 16.4-fold, Table 1) with the PGM-MB method compared with the ISO/TS 15216-1 (*P* values are provided in Table 1).

In order to investigate the effect of RT-PCR inhibitors on each of the examined methods, we compared the recovery yields by testing 10-fold diluted RNA extracts. As shown in Fig. 2 B, the recovery rates for the ISO/TS 15216-1 method improved for all of the leafy greens except for basil. The recovery rate for basil did not change significantly between undiluted and 1:10 diluted RNA extract (10.7 ± 2.6% and 11.4 ± 4.2%, respectively). The recovery rate in 1:10 diluted RNA extract for the PGM-MB method for all of the studied leafy greens improved significantly (Fig. 2B), especially for basil, and the extraction efficiency increased by approximately 7-fold (6.5 ± 3.6% versus 42.4 ± 10.1%), indicating the presence of inhibitory components in extracted nucleic acids. The overall extraction efficiency for the PGM-MB method using the 1:10 diluted RNA was significantly higher than the ISO/TS 15216-1 method, as the recovery ratio was ranging from 3.2 to 6.7-fold (Table 1).

In general, the highest recovery rates by the PGM-MB method were achieved from seaweed with 47 ± 7.8% and 52.3 ± 12.9% for undiluted and 1:10 diluted RNA, respectively. The lowest recovery was achieved from baby spinach with 5.5 ± 2.2% and 9.2 ± 1.3% for undiluted and 1:10 diluted RNA, respectively. On the other hand, the ISO/TS 15216-1 method produced the highest yield in basil with 10.7 ± 2.6% and 11.4 ± 4.2%, recovery rates for undiluted and 1:10 diluted RNA, respectively, and lowest yield in baby spinach with 1.45 ± 0.65% and 2.9 ± 2% recovery rates for undiluted and 1:10 diluted RNA, respectively. Therefore, baby spinach yielded the lowest recovery rates by both methods (Figs. 1 and 2).

## 4. Discussion

Fresh produce such as herbs and leafy vegetables are implicated in the majority of foodborne NoV outbreaks in the USA (Hall et al., 2012). Importantly, once contaminated, complete removal or inactivation of NoV from these commodities is unlikely (Cook et al., 2016; FDA, 2017). Due to the short shelf life of fresh vegetables and herbs, fast, highly sensitive and reliable methods are required to detect the infectious virus and allow further genomic characterization for outbreak delineation and intervention.

Specificity and efficacy of the PGM-MB method in capturing a wide range of NoV genotypes and strains has already been demonstrated (Tian et al., 2010). For this study, we did not use the cultivable surrogates of human NoV, as recent data suggested that the surrogates behaviour can be different from human NoV (Knight et al., 2016), therefore we used NoV GII.4 Sydney 2012 strain, which is still the most prevalent strain in North America (CDC, 2018; Petronella et al., 2018) and thus more relevant for this work.

We chose fresh herbs and leafy vegetables for method comparison since they are considered high-risk foods for virus contamination (Chatziprodromidou et al., 2018, WHO, 2019). For example, 5.3% of leafy vegetables marketed in the U.K (Cook et al., 2019), and 28.2% of the leafy greens marketed in Canada (Mattison et al., 2010) have been shown to be positive for the NoV genome. Green seaweed has been implicated in multiple NoV outbreaks in Japan and South Korea, affecting more than 2,000 individuals (Kusumi et al., 2017; Park et al., 2015; Sakon et al., 2018), and contaminated seawater and infected food-handlers have been identified as the source of contamination. Furthermore, the herbs and leafy vegetables have relatively large

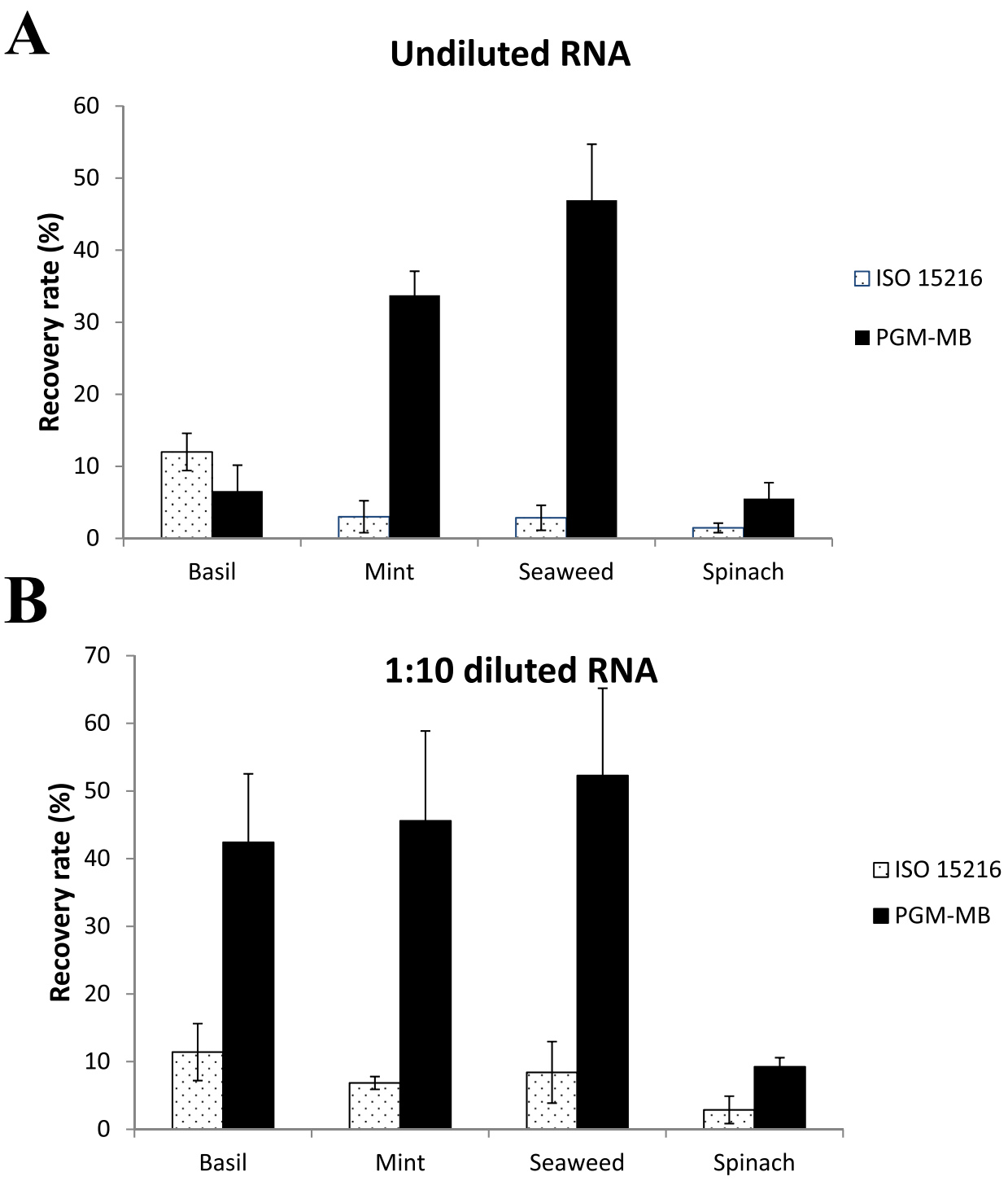


**Fig. 2.** A) The mean extraction efficiency (%) obtained from the undiluted RNA extract. Error bars represent the standard deviation. B) The mean extraction efficiency (%) obtained from the 1:10 diluted RNA extract. Error bars represent the standard deviation.

surface area for potential NoV binding, plus they are considered ready-to-eat foods and consumed with minimal processing. However, there is a lack of rapid and up-to-date standard methods for virus recovery, detection and quantification in these food matrices. For instance, several recent studies have demonstrated that dd-RT-PCR performs better in the quantification of viral genomes in different food commodities (Coudray-Meunier et al., 2015; Fraisse et al., 2017; Persson et al., 2018, Mykytczuk, et al. 2017). Another advantage of using dd-RT-PCR is that the viral enumeration is performed without using quantification standards, which can introduce bias and uncertainty to method validation studies (Lowther et al., 2019), but this technology has not been adopted in any of the standard methods.

Combination of the PGM-MB method with heat-denaturation for RNA extraction offers several advantages; a) it is fast and takes less than half the amount of time compared to other methods (Fig. 1) and thus can be employed on foods with short shelf-life; b) allows for detection of the viruses with intact capsids, therefore floating (uncapsidated) viral genomes, which may lead to false-positive results, will be excluded (Kingsley et al., 2018; Kingsley et al., 2014); c) potential RT-PCR inhibitors that may cause false-negative results will be removed (Tian et al., 2008).

Another advantage of using the PGM-MB assay for NoV detection is

**Table 1**
Recovery ratio of PGM-MB method over ISO 15216-1 method and *P* value of higher recovery rates obtained by the PGM-MB versus ISO 15216-1 method (Paired *t*-Test: Two-Sample assuming unequal variances).

| | Recovery ratio | | *P* value | |
|---|---|---|---|---|
| | Undiluted | 1:10 diluted | Undiluted | 1:10 diluted |
| Basil | 0.6 | 3.7 | 2.29E-01 | 8.16E-03 |
| Mint | 11.2 | 6.7 | 4.64E-04 | 2.50E-02 |
| Seaweed | 16.4 | 6.2 | 5.34E-03 | 1.54E-02 |
| Spinach | 3.8 | 3.2 | 4.67E-02 | 9.98E-03 |

its adaptability to the automated and high-throughput systems. These systems have already been employed for capturing and detecting other viruses (Wang et al., 2018; Hofler et al., 2014; Farhat et al., 2015; Chen et al., 2016), therefore, if developed and implemented for food virology testing, they allow for rapid, sensitive and reliable detection of potential contaminating viruses.

Some surrogates of NoV such as murine norovirus (MNV) and Tulane virus (TV) are shown to bind to PGM (Li and Chen. 2015; Le Pendu et al., 2014), these viruses can then be used as process control virus in the PGM-MB assay to determine the virus recovery rate and to monitor the quality of the extraction procedure (Hennechart-Collette et al., 2015). Consequently, the PGM-MB method can be suitable for food virology testing for surveillance and outbreak investigations.

The data obtained in this study reveals that the PGM-MB method is highly efficient in capturing NoV in the examined herbs and leafy vegetables in a considerably shorter time compared to the ISO/TS 15216-1 method. However, the results from basil show that the PGM-MB method is not completely efficient in removing the RT-PCR inhibitors as the recovery rate obtained from the 1:10 diluted RNA extract from basil is 7-fold higher than the undiluted RNA extract (Figs. 1 and 2). High levels of RT-PCR inhibition have already been reported for ISO/TS 15216 method (Lowther et al., 2019). RT-PCR inhibitors are a very heterogeneous group of chemical substances, and plants carry many of them (Schrader et al., 2012). QiaAmp Viral RNA kit may efficiently remove a wide range of PCR inhibitors, however, according to our data, it failed to completely remove all the inhibitors present in mint and seaweed, as the recovery rate is higher for these commodities in the 1:10 diluted RNA compares with the undiluted RNA.

The higher recovery rates obtained by the PGM-MB method can be partially explained by the fact that there are more steps in the ISO/TS 15216 method compared with the PGM-MB method and the loss of virus can occur at each step (Fig. 1). A fraction of viruses might not be released to TGBE buffer from the examined food commodity. It is also possible that a proportion of viral particles do not precipitate, or are not completely resuspended. Loss of viral genome can also happen during RNA extraction using columns. All of these might contribute to lower efficiency of the ISO/TS-15216 method compared to the PGM-MB method. On the other hand, the concentration step in the ISO/TS-15216 method allows for analysis of the whole 5g of the sample, while due to lack of a concentration step in the PGM-MB method, only a proportion of the sample is analysed. This can affect the method efficacy when the viral load in the analyzed sample is low, and analysis of a portion of the sample by the PGM-MB method can lead to false-negative results.

The attachment of human NoV to the carbohydrates present in the cell wall material of leafy greens has been demonstrated previously (Esseili et al., 2012). Therefore, relatively low virus recovery by the PGM-MB method in baby spinach may be related to the carbohydrate composition of baby spinach cell wall material, which might support strong NoV binding and compete with PGM.

In this study, we did not compare the limit of detection (LOD) and the limit of quantification (LOQ) of the two methods; however, we applied a relatively high quantity of NoV genome copies to compare the efficiency of the two methods in virus recovery. Based on two different dose response studies (Atmar et al., 2014; Teunis et al., 2008), the infectious dose for NoV ranges between 18 and 1320 genome equivalent and it can vary with different genotypes and strains. The PGM-MB method is expected to capture the intact viral particles, but whether the captured viral particles are infectious, cannot be addressed by this method. Without having a robust infectivity assay, we are not able to measure the efficacy of the PGM-MB method in capturing the infectious particles within the inoculated population.

In summary, our results demonstrate that the PGM-MB assay provides a rapid and effective virus recovery method, and release of viral RNA from the captured NoV by heat-denaturation is an efficient method for obtaining RNA for use in quantitative RT-PCR assays.

## Declarations of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103254.

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