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Absolute Quantification of Norovirus Capsid Protein in Food, Water, and Soil Using Synthetic Peptides with Electrospray and MALDI Mass Spectrometry

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Abstract

Norovirus infections are one of the most prominent public health problems of microbial origin in the U.S. and other industrialized countries. Surveillance is necessary to prevent secondary infection, confirm successful cleanup after outbreaks, and track the causative agent. Quantitative mass spectrometry, based on absolute quantitation with stable-isotope labeled peptides, is a promising tool for norovirus monitoring because of its speed, sensitivity, and robustness in the face of environmental inhibitors. In the current study, we present two new methods for the detection of the norovirus genogroup I capsid protein using electrospray and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The peptide TLDPIEVPLEDVR was used to quantify norovirus-like particles down to 500 attomoles with electrospray and 100 attomoles with MALDI. With MALDI, we also demonstrate a detection limit of 1 femtomole and a quantitative dynamic range of 5 orders of magnitude in the presence of an environmental matrix effect. Due to the rapid processing time and applicability to a wide range of environmental sample types (bacterial lysate, produce, milk, soil, and groundwater), mass spectrometry-based absolute quantitation has a strong potential for use in public health and environmental sciences.

Keywords

Norovirus; Mass spectrometry; Absolute quantification; Environmental detection

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1. Introduction

Norovirus infections are one of the most prominent public health problems of microbial origin in the U.S. and other industrialized countries [1]. As members of the *caliciviridae* family, human noroviruses have been identified in three of the at least five norovirus genogroups (G) (I, II and IV). Norovirus outbreaks have occurred on cruise ships [2], in emergency rooms [3], in evacuation shelters following Hurricane Katrina [4], from water [5], and food products [6]. Typical symptoms of the disease include vomiting and diarrhea [7] lasting for 24 to 48 hours. Norovirus infections are usually non-lethal but occasionally result in fatalities, particularly in the elderly and immunosuppressed [8, 9]. Basic hygiene and management plans to analyze and control potential hazards are routinely used to prevent disease outbreaks. Nevertheless, outbreaks are still common. Disease surveillance is an important tool to prevent secondary infections, confirm successful cleanup after outbreaks, and track the routes and spread of the causative agent.

Due to its speed, mass spectrometry (MS) is one surveillance tool that is particularly attractive for the identification of virus particles in infected individuals and contaminated substances. One of the main challenges in the detection of norovirus is its recalcitrance to standard laboratory culturing techniques [10–12]. Because this frank human pathogen cannot easily be cultured *in vitro*, analytical approaches concentrate on the direct detection of norovirus particles or nucleic acids in complex biological matrices, such as human stool. Current, popular strategies include reverse transcription polymerase chain reaction (RT-PCR) [13], electron microscopy [14, 15], and enzyme-linked immunoassays [16, 17], all of which have potential issues that hinder reproducible and effective determination of viruses in the target matrix [1, 18]. In addition to the above approaches, we reported previously a novel method for virus detection by MS, using both purified, noninfectious virus-like particles (VLPs) and VLP-spiked stool samples [19]. However, one of the issues of this emerging avenue of research is the sensitivity of the method. When using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, detection of the 57 kD capsid protein is possible at a lower method detection limit of 75 femtomoles (fmol), which correlates to the upper level of virus mass present in clinical samples from symptomatic individuals (8.5×10^7 viruses/ml of diarrheal stool). Because much lower viral loads are still highly infective, the development of more sensitive methods is desirable.

Ideally analytical strategies should be both highly sensitive and quantitative. Quantitative methods employing RT-PCR are challenged by inhibitory components of stool [20]. A complementary quantitative assay would strengthen qualitative PCRbased measurements. To this end, MS is an attractive method, as it targets the capsid protein using an approach orthogonal and thus complementary to nucleic acid-based detection.

Absolute quantitation represents the ultimate goal of virus determination, as it allows for the comparison of results across studies, whereas relative quantitation is only valid between samples analyzed in the same study [21]. Quantitative detection in protein mass spectrometry has borrowed certain concepts from the environmental analytical chemistry field, where sensitive and reproducible detection methods have long been used to detect ubiquitous pollutants [22]. Isotope-labeled internal standards, e.g., commercially available

peptides for absolute quantification (AQUA, Sigma-Aldrich), can be used for the sensitive quantification of peptide biomarkers in complex matrices [23, 24]. In this method, synthetic isotope-labeled peptides identical in sequence to the target molecule are spiked at known amounts into a protein digest of samples assayed for presence of the authentic marker. Separation and detection using liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) results in simultaneous detection of the authentic target and its analog, which serves as an internal standard. Because the concentration of the internal standard is known, the absolute concentration of the target can be calculated by direct comparison [23]. This method monitors specific ion transitions produced by the target peptide, determined using *a priori* knowledge of its sequence, ionization behavior, and fragmentation [23]. The use of individual peptides to quantify proteins is analogous to that of small oligonucleotides in qPCR. In AQUA, the highly accurate detection of a parental mass and its unique product ions make this method very specific, similar to the fidelity of base-pairing in PCR. AQUA is also potentially more sensitive than previous MS approaches developed for the detection of viruses in complex matrices [19].

The influence of complex environmental matrices is an essential factor in method development, as interferences can adversely affect method performance. Upstream sample preparation must effectively remove such interferences without introducing biases or incompatibilities with downstream detection [1]. Many virus collection and concentration methods have been developed for nucleic acid-based detection, but it is unknown whether these methods will be effective for MS-based detection.

The aims of this study were to improve sensitivity of a previously developed MS-based method to detect norovirus biomarkers and to design an approach amenable to determining absolute quantities of viral peptides, and thus the abundance of virus particles, using both ESI and MALDI MS. An additional aim was to demonstrate the applicability of these approaches to virus detection in complex sample matrices.

2. Materials and methods

2.1. Target selection

Isotope-labeled internal standard peptides were selected for adequate chromatographic separation, ionization, and peptide fragmentation. Peptide sequences were compared to other proteins using the BLASTp tool (<http://www.ncbi.nlm.nih.gov/BLAST>) to ensure that they were unique to norovirus GI. Their location within the capsid protein was charted using Chimera [25]. Candidate standard peptides were synthesized by Sigma-Aldrich (St. Louis, MO). Additional peptides for MALDI analysis were ordered from AnaSpec (Fremont, CA). All peptides were >95% purity; lyophilized peptides were resuspended in 0.1% trifluoroacetic acid (TFA).

2.2. Virus-like particles (VLPs)

Samples of recombinant norovirus VLPs, kindly provided by the laboratory of Mary Estes, were stored in phosphate buffered saline at 4°C. The sequence of this synthetic construct was derived from the norovirus GI capsid protein [26]. The VLP stock contained

approximately 3.59 mg/ml of protein, which corresponds to 2×10^{11} VLPs (which at 180 capsid proteins/virion corresponds to 635 picomoles of VLP capsid protein) per microliter, as measured by the bicinchoninic acid (BCA) method following the manufacturer's instructions (Pierce, Rockford, IL). Stock purity was evaluated using gel electrophoresis followed by in-gel digestion and protein identification using MALDI-TOF MS.

2.3. ESI sample preparation

VLP stocks were serially diluted in 100 mM ammonium bicarbonate buffer (~pH 7.0). For samples to be spiked into a matrix, 15 µg of crude cell lysate from the cells of *Escherichia coli* strain K12 (Bio-Rad, Hercules, CA) were mixed with the VLP dilutions.

Samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Known amounts of VLPs in 1X Laemmli sample buffer with 5% β-mercaptoethanol were loaded onto a 4–12% Bis Tris gel (Invitrogen, Carlsbad, CA) and separated at 125 V for 1.5 hours in MES running buffer (Invitrogen). Gels were stained using Colloidal Coomassie G-250 stain as previously described [27]. Gel areas corresponding to the capsid protein were excised using a clean razor blade and destained twice in 9% acetonitrile/100 mM ammonium bicarbonate for 30 min at 37°C. Following drying in a SpeedVac (Savant) for 10–15 min, the samples were digested using 400 ng trypsin in 100 mM NH₄HCO₃ buffer (pH 7) overnight at 37°C in the presence of the internal peptide standards, which were spiked at levels from 1–20 fmol. The digested peptides were extracted using 50% acetonitrile/0.1% trifluoroacetic acid and subsequently dried in a SpeedVac and resuspended in 90% acetonitrile/1% formic acid.

2.4. MALDI sample preparation

VLP stocks were prepared as for ESI samples. Environmental sample matrices, comprising groundwater, produce, soil, and milk, were prepared following common practices outlined below.

Groundwater from a perchlorate-contaminated well in Mesa, AZ was collected using a bailer, transported in a cooler, and stored at –20°C until analysis. Aliquots of 5 ml were thawed and concentrated to <500 µl using a nanofilter with a cutoff value of 5,000 nominal molecular weight (Agilent, Santa Clara, CA).

Fresh produce samples, consisting of strawberries and green leaf lettuce, were obtained from a local retail outlet. Samples were processed based on methods described by Tahk et al. [28]. Strawberries and lettuce were cut to produce 5 g aliquots, which were washed with approximately 50 ml 0.25 M threonine-0.3 M NaCl on a rotary shaker for 5 h. The elution was then concentrated to <500 µl using Vivaspinn-20 filters featuring a 100,000 nominal molecular weight cutoff value (Sartorius Stedim North America, Bohemia, NY).

Agricultural soil samples from Baltimore, MD [29] were processed using the direct soil protein extraction method described by Chourey et al. [30]. Briefly, 5 g aliquots of frozen soil were mixed with 10 ml Alkaline-SDS buffer and heated in a boiling water bath for 10 min. The mixture was then cooled and centrifuged at $2,095 \times g$ for 10 min. Proteins were concentrated from the supernatant via a TCA-acetone precipitation and resuspended in 1 ml

of 50 mM ammonium bicarbonate. Non-homogenized, organic whole milk was obtained from a local retail outlet and stored at -80°C . Samples were processed as in Lippolis, et al. [31]: 5 ml aliquots of milk were thawed and centrifuged at $10,000 \times g$ for 20 min at 4°C . The resulting pellets were washed twice and resuspended in 25 ml chilled Dulbecco's Phosphate Buffered Saline (DPBS) without calcium or magnesium (0.2 g/l KCl, 0.2 g/l KH_2PO_4 , 0.8 g/l NaCl, 1.15 g/l NaH_2PO_4 ; pH = 7.4). Resuspensions were twice overlaid with 15 ml 45% sucrose and centrifuged at $10,000 \times g$ for 40 min at 4°C . Finally, pellets were washed 6 times with 45 ml DPBS and resuspended in 1 ml of 50 mM ammonium bicarbonate.

Aliquots of the above preparations were mixed 1+1 (v+v) with Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% β -mercaptoethanol and heated at 95°C for 5 min. After cooling to room temperature, samples were loaded onto 4- 20% TGX gels (Bio-Rad, Hercules, CA) and run at 200 V for 35 min. Gels were stained using the Flamingo fluorescent stain (Bio-Rad, Hercules, CA) and visualized using a UV light box. Bands at 57 kD, corresponding to the norovirus capsid protein, were excised using a razor blade and dried using a SpeedVac for in-gel digestion. Gel bands were digested overnight at 37°C in a solution containing 10 ng sequencing-grade modified trypsin (Promega, Madison, WI) in 20 μl of 50 mM ammonium bicarbonate. Norovirus capsid protein standards were digested in a solution containing 1:100 trypsin:VLP and 35 μl of 50 mM ammonium bicarbonate. Digests were cooled to room temperature and adjusted to 0.1% TFA. For standard curves, digests of target samples were diluted in 0.1% TFA. To evaluate the influence of environmental matrices, VLP digests were diluted in digests of environmental samples. Standard peptides were added to the acidified digests, and the mixtures were concentrated and desalted using Omix C_{18} ZipTips (Varian, Palo Alto, CA) and directly eluted with α -cyano-4-hydroxycinnamic acid (CHCA; LaserBio Labs, Sophia-Antipolis, France) onto a 384-well stainless steel target plate (AB/Sciex, Framingham, MA).

2.5. NanoLC-MS/MS analysis

Buffered stocks of VLPs were resuspended in approximately 10–50 μl of buffer B (0.1% formic acid, 90% Acetonitrile), and loaded onto a covered 96-well plate. Samples were analyzed using an LCQ DecaXP (ThermoFinnigan, San Jose, CA) with an Agilent 1100 autosampler connected to Eksigent 2D nanoflow pumps using standard instrument settings. Samples were injected at a volume of 1–5 μl and separated on a C_{18} 75 μm column packed with YMC ODS-AQ (5 μm particle, 120 pore size) in 0.1% formic acid on a gradient (5 – 40%) using 0.1% formic acid/90% acetonitrile in 30 min at a flow rate of 300 nl/min before being introduced into the mass spectrometer with a spray voltage of 2.2 kV. Mass spectrometry was performed using a single segment with six scan events in MS/MS selected reaction monitoring (SRM) mode (m/z 641 $\rightarrow$ m/z 402.2, m/z 646 $\rightarrow$ m/z 412.2, m/z 597 $\rightarrow$ m/z 949.4, m/z 602.4 $\rightarrow$ m/z 959.4, m/z 748 $\rightarrow$ m/z 728.3, m/z 753 $\rightarrow$ m/z 738.3) with isolation windows of 3 Da for the parent and 5 Da for the product ion. Clean column methods were conducted between runs, followed by a post run blank to confirm the lack of carry over between samples. Data were analyzed using Xcalibur software version 2.0.6 (Thermo, San Jose, CA). Peaks were integrated using the ICIS algorithm, and data were

exported to Excel for analysis. Target peptides were quantified by comparing their peak area to the corresponding labeled peptide after correction using the external calibration curve.

2.6. MALDI-TOF/TOF MS analysis

Samples were analyzed using a 4800 MALDI-TOF/TOF MS (AB/Sciex, Framingham, MA). For quantitation, spectra were acquired in positive reflector mode with a fixed laser intensity between 2800 and 3500 arbitrary units. To confirm the identity of the target peaks, MS/MS spectra were acquired in positive mode using post-source decay for fragmentation. Spectra were acquired using 4000 Explorer software and exported to Data Explorer (AB/Sciex, Framingham, MA). Mass calibration was performed using internal trypsin autolysis peaks or external calibrants (Sigma, St. Louis, MO). Quantitation on peaks with a signal-to-noise ratio greater than 3 or isotope clusters with a combined signal-to-noise ratio greater than 10 in the relevant mass ranges was performed in Excel software (Microsoft). Target peptides were quantified by comparing their peak area to the corresponding labeled peptide [32].

3. Results

3.1. Selection of peptides

In silico digestion of the norovirus GI capsid protein (PDB accession 1IHMA) using ProteinProspector's MSDigest tool (<http://prospector.ucsf.edu>) and data from previous studies [19] yielded a list of potential standard peptides. Preliminary MS scans using 1 pmol of VLPs determined the ionization behavior and chromatographic separation of the potential standards. Further selection criteria, including amino acid composition, selection of labeling amino acid, and production quality were applied following internal review of candidate peptides and consultation with Sigma-Aldrich technical support staff. Three peptides, all of which are located in the shell (S) domain of the norovirus GI capsid protein, were selected following this screening effort (Fig. 1): IMLAGNAF*TAGK, TLDPIEVPLEDVR*, and TGGGTGDSFVVAGR*. The underlined and asterisked amino acid is the ¹³C labeled residue.

3.2. Utility of selected peptides

During LC-MS/MS analysis, two of the peptides performed as anticipated and were used for quantification of norovirus VLPs. However, one peptide (IMLAGNAFTAGK, m/z 602, 2⁺) was found to undergo variable oxidation in the methionine residue during sample preparation, resulting in a mass shift by 16 Da in some peptides. The variability complicated quantification, so this peptide was excluded from subsequent quantitative data analysis and was used only to confirm the presence and detection of norovirus capsid protein.

3.3. Calibration results for norovirus peptides detected using LC-MS/MS

Standard curves were prepared in the range of 0.5–100 fmol; each chromatographic run contained the three internal standards at the same concentration (20 fmol). For the first peptide, TGGGTGDSFVVAGR (m/z 412.2, 2⁺), the calibration curve was linear ($R^2 = 1.00$) across the entire range (Fig. 2A). Calibration curves were less favorable for the other two internal standards: $R^2 = 0.89$ and 0.95 for m/z 959.4 (Fig. 2B) and 738.3 (Fig. 2C), respectively, corresponding to IMLAGNAFTAGK and TLDPIEVPLEDVR. Quantification

was thus based on the isotopically labeled peptides at m/z 412.2 and 738.3 using only internal calibration. Accurate peak integration was possible to as low as 500 attomoles (amol). A peak was observed at 125 amol, but quantification and reproducibility were poor.

3.4. Quantification of VLPs using LC-MS/MS

In-gel digests of VLPs at a range of concentrations (100–0.5 fmol) were spiked with known amounts of standard peptides and analyzed in triplicate. The VLPs were detected at concentrations as low as the lowest point for the standard calibration curve (500 amol). Calculated amounts of VLPs varied considerably within the range of measurements. Recovery was modest (10%) in the high-concentration range (250–1,000 fmol). However, VLPs were quantified with 100% recovery at 500 ± 100 amol in the 500-amol spikes, which are environmentally more relevant.

3.5. Calibration results for norovirus peptides detected using MALDI-MS/MS

The peptide TLDPIEVPLEDVR was chosen for further evaluation using MALDI MS because it performed well in ESI assays and its mass is suitable for MALDI analysis. To obtain an appropriate mass shift, the peptide was modified to contain one $^{13}\text{C}_5$, ^{15}N -valine residue: TLDPIEVPLEDV*R. Quantification was evaluated directly using VLP digests. VLPs were digested and a dilution series spanning 5 orders of magnitude was prepared. Dilutions in 0.1% TFA were spiked with 0.1 to 1,000 fmol standard peptide, corresponding to the dilution step. These mixtures were then desalted using micro C_{18} solid phase extraction columns and eluted in CHCA directly onto the MALDI target plate. All samples were prepared in triplicate.

The VLP targets were detected at concentrations as low as the lowest point that was assayed (100 amol). Amounts of VLPs were calculated based on the ratio of the peak areas (centroid masses) corresponding to the native and modified peptides. The calculated amount of VLP was correct to within a factor of 4 and was most accurate at 1 fmol; the coefficients of variation (CV) for the pure standard were between 5 and 19% with the least variation at 100 amol (Table 1). These CVs are similar to those determined in a previous evaluation of MALDI-based quantitation with heavy-labeled peptides [33]. The limit of quantitation for this kind of method has previously been estimated at 2–10 fmol [32].

Ideally, the heavy-isotope labeled standard and the native analyte are both present at relatively similar (preferably equimolar) amounts. The peak ratios of analytes with the same sequence deviate from the predicted value as the standard:analyte ratio deviates from 1 in both ESI- and MALDI-based systems [32, 34]. However, it may not be possible to determine the proper ratio of analyte to standard due to lack of time, limited sample availability, or other constraints. To test the tolerance of the present method to differences in VLP and standard peptide amounts, we analyzed 10-fold serial dilutions of the standard peptide mixed with a constant amount of VLP digest, corresponding to approximately 50 fmol norovirus capsid protein. As expected, the CV increased as the peak ratio deviated from 1 (Fig. 3). The increased variability could indicate peak suppression, which is an inherent limitation of MALDI-TOF MS, or competition for binding sites in the extraction process directly preceding the MS analysis. CVs were favorable (<20%) for native:labeled

peptide ratios from 0.05 to 1, indicating that it is still possible to quantify an unknown agent by targeting a specific peptide to within a 2-order of magnitude difference between the amounts of the analyte and the standard as long as the standard is in excess. This working range is in agreement with previous MALDI methods [32]. If the CV is found to be outside of the acceptable range of predetermined quality assurance/quality control (QA/QC) parameters, samples may be reanalyzed using an adjusted spike level to bring measurements into compliance.

3.6. Method performance for complex samples

The LC-MS/MS quantitation method was challenged using an environmental matrix consisting of *E. coli* cell lysate (15 µg) spiked with up to 100 fmol of VLP standard and 20 fmol of labeled peptides. The average amount of VLPs was measured to be 78.8 fmol using two peptides (79±15% recovery). When 10 fmol of VLPs were spiked into the *E. coli* lysate, the average amount measured was 5.4 fmol (54±63% recovery).

To test the MALDI-TOF/TOF MS method against challenging matrices, VLP digests and the TLDPIEVPLEDV*R peptide were spiked into preparations of groundwater, lettuce, strawberries, soil, or milk. Environmental extracts were analyzed prior to standard addition and were found to be free of norovirus particles (no norovirus-associated peptides detected; data not shown). In environmental matrices, the limit of detection of the standard peptide increased from 100 amol to 1 fmol. Serial dilutions from 100 to 1 fmol of VLP digests and standard peptide were mixed with parallel dilutions of standard peptides and analyzed (Fig. 4). Dilutions were performed in 0.1% TFA as a control or in a background of environmental extract. None of the environmental matrices produced ratios significantly different from those of the control (*t*-test, *p*<0.1); however, the CVs increased on average from 15% to 20–53% depending on the specific sample (Table 1).

4. Discussion

Quantitative protein measurement using isotopically labeled peptides is a relatively new procedure that has focused on quantifying phosphorylated proteins from cell cultures [23, 24, 35]. The applicability of this approach to the surveillance of microbial pathogens has not been previously explored. In this study, we quantified VLPs in environmental simulations using mass spectrometry, thereby demonstrating the feasibility of this approach for the qualitative and quantitative detection of this common and widespread viral pathogen in complex matrices. This proof-of-concept study should serve as the springboard for the development of similar methods for other norovirus genogroups and hybrid protein- and nucleic acid-based detection methods. Further method comparisons should also be performed on genuine clinical and environmental samples.

The AQUA approach was traditionally developed for LC-based ESI mass spectrometry; however MALDI instruments are generally more robust and therefore potentially better suited for detecting targets in complex environmental matrixes. For some types of samples, the analysis can even be performed without the elimination of environmental background, which is particularly attractive because sample preparation can be tedious [1]. For example, MALDI-TOF MS-based semiquantitative analysis of tissues has been successfully used to

measure peptide hormones [31]. In a comparison test using isobaric tags for relative and absolute quantification (iTRAQ) to measure *E. coli* tryptic digests, MALDI and ESI instruments performed comparably well, with MALDI offering the added benefit of sample archiving by freezing of the target plates [34]. Although less common, MALDI also performs on par with ESI for quantitation with heavy isotope-labeled standard peptides [32, 33]. For these practical reasons, MALDI-based quantitation deserves consideration alongside the more traditional and accepted ESI-based techniques.

Here, peptide biomarkers from the capsid protein from norovirus were detected at 500 amol using LC-MS/MS and 100 amol using MALDI-MS/MS, representing a target mass of 1.67×10^6 and 3.34×10^5 virions, respectively. These levels are well within the clinical range of the virus mass shed by infected patients per milliliter of diarrheal stool, which has been estimated to be as high as 10^{12} genome copies for infected individuals [36]. While these limits are still orders of magnitude above those of gene-based detection methods, they show a dramatic improvement over other protein-based detection methods [1]. For example, these limits of detection represent an increase in sensitivity by more than two orders of magnitude over previously reported MS-based methods for norovirus detection [19].

The LC system coupled to ESI MS adds another dimension that can be used to identify a target (retention time) and separate it from other matrix components, but it often represents the main bottleneck in rapid sample throughput due to the time required to run a gradient [32, 33]. Alternative methodological approaches include using immunocapture in lieu of LC [32, 37]. This approach is excellent for concentrating target peptides from complex samples; however, it relies on antibodies whose availability is limited and requires further method optimization for antibody binding, so it may not be practical or economical. However, immunocapture-based assays coupled with MALDI-TOF MS quantitation have been successfully employed for a variety of synthetic peptides of human origin [32], 11 diabetes-related proteins [33], and allelic variants specific to the sheep prion protein [38].

In situations where antibodies are not available, relatively simple sample preparation, such as filtration [19] or one-dimensional gel electrophoresis [39], may suffice. These minimal separation techniques do not require highly skilled operators or expensive and toxic solvents, nor do they require the time- and cost-intensive development of antibodies. Similar to LC, these separation methods, including immunoseparation, can provide some information to corroborate the identity of the target. In tandem MS (*e.g.*, TOF/TOF) systems, the identity of the target and standard peptides are confirmed in the second MS. Fragmentation patterns, along with particle size, molecular weight, or antibody-binding affinity, can compensate for information lost by excluding LC from the workflow (*e.g.*, retention time).

One of the challenges of measuring microbial pathogens in clinical samples is the presence of interferences from various potential environmental matrices, such as fresh produce. To explore the method robustness, we spiked a known amount of norovirus into 15 µg of a commercial *E. coli* lysate preparation for LC-MS/MS analysis as well as into groundwater, produce, soil, and milk for MALDI-MS/MS analysis. The addition of these interferences at a ratio of over 1:10,000,000 (w/w, target/interference) did not hinder the success of the LC-

MS/MS method; viral biomarkers were detectable at 10 fmol (3×10^7 virions) with this challenge. The MALDI-MS/MS method similarly withstood potential interferences from a wide variety of sample matrices, and biomarkers remained detectable down to 1 fmol (3×10^6 virions). This robust performance is largely thanks to the use of specific target ions that are unique to the capsid protein of the norovirus, as opposed to peptide mass fingerprinting or other “discovery-mode” data analysis methods that rely on the detection of multiple peptides. Furthermore, the sample preparation techniques employed effectively removed a significant level of the interference prior to injection into the mass spectrometer.

In addition to detection, mass spectrometry is capable of quantification of viral biomarkers at comparably low levels. While individual quantitative measurements of the target peptides varied by up to 25%, the average reported abundance of the targets varied by less than 10% for low abundance measurements (500 amol and 100 amol for LC-MS/MS and MALDI-TOF/TOF MS, respectively). In clinical samples, it is anticipated that norovirus protein levels will be in the low femtomolar range where quantification is more accurate. Furthermore, in many cases viral loads only need to be determined to an order of magnitude, as is usually obtained from the widely used microbial count and most probable number approaches in microbiology.

Several challenges remain in using quantitative MS for virus detection. Availability and capital cost of instrumentation historically has been an obstacle that is now fading in importance. A more critical present day challenge lies with the peptides themselves. In this study, we were forced to exclude one of the three candidate peptides due to a methionine residue that was partially oxidized during sample preparation. This conclusion was drawn from the observation of a 16 Da shift in the peptide during MALDI-TOF MS analysis (data not shown). This unwanted chemical alteration of the standard resulted in poor reproducibility in quantitative measurements during SRM, a limitation that potentially could be addressed by amending the sample preparation strategy such that any methionine residues are completely oxidized [40]. Another of the pitfalls of peptide-based quantification is that methods optimized for one type of instrumentation (*e.g.*, ESI) may not translate to another (*e.g.*, MALDI). In this work, the TLDPIEVPLEDVR peptide performed well for both ionization strategies; however, the same may not be true for all standard peptides, so methods will have to be evaluated on a case-by-case basis.

Peptide-based internal standards are accurate and reproducible in measuring the presence of a target as observed here, but similar to oligonucleotides targeted in PCR, they only represent a small part of the virus they are used to identify. Techniques for virus purification, enrichment, and concentration result in losses that may complicate accurate, absolute quantification. This important limitation applies to any method seeking to purify viruses from a complex matrix [1]. Internal standards should be integrated into a surrogate virus particle or process control having properties identical to those of the protein coat surrounding the pathogen of interest. One of the major challenges in environmental surveillance is that of determining infectivity. To be truly successful, only viable pathogens must be captured and measured. While this goal is still elusive, coupling MS-based protein measurements with RT-PCR detection of RNA fragments is an important step toward accomplishing this goal. Detection of both nucleic acids and protein in a given sample

would provide two lines of evidence of viable pathogens in the clinical or environmental sample. Using both PCR- and MS-based methods allows for the detection of both of the major components of virions, *i.e.*, their genetic information and the surrounding protein coat. Improvements in the methodology of the surveillance of microbial threats are possible, as shown in this work, and will help to address this major public health concern. To fully understand the relative benefits and limitations, a direct comparison between protein- and nucleic acid-based detection methods or a combination thereof should be performed, ideally with genuine clinical or environmental samples that can be linked to epidemiological data to infer infectivity.

5. Conclusions

The use of heavy-isotope labeled standards is rich with untapped potential for absolute quantitation of viral targets and related threats, such as prions, whose importance as public health threats continues to surge [41]. Using this technique, we have demonstrated detection limits as low as 1 fmol against a background of environmentally relevant sample matrices. Furthermore, the MALDI-based method has lenient requirements for the analyte-to-standard ratio, an important characteristic for applications where sample or standard availability are limited. Due to the rapid processing time and applicability to a wide range of environmental sample types (bacterial lysate, produce, milk, soil, and groundwater), this method of quantitation has a strong potential for use in public health and environmental sciences.

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Abbreviations

MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
ESI	electrospray ionization
LC	liquid chromatography
MS	mass spectrometry
VLP	virus-like particle
CV	coefficient of variance

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Highlights

- Mass spectrometry-based methods for norovirus quantification are developed.
- Absolute quantification is achieved using internal heavy isotope-labeled standards.
- A single labeled peptide serves in two distinct detection strategies.
- These methods are validated for food, water, and soil analysis.
- MS-based detection limits are lowered by two orders of magnitude.

Fig. 1. A digitally rendered 3-D image showing the Chain A of the virus capsid protein (Protein Databank ID 1IHM) with the shell (S) domain in black; the three target peptides are highlighted in red (TLDPIEDVDR) shown at the top, green (IMLAGNAFTAGK) shown in the middle and blue (TGGGTGDSFVVAGR) shown as the lower peptide.

Targets were selected for their compatibility with the method and their being unique to the virus. The image was created using Chimera, a software program developed at the University of California at San Francisco [25].

Fig. 2. Representative standard curves for the heavy isotope-labeled peptide utilized in the ESI method

Calibration curves ($n = 3$) were prepared routinely on the day of analysis, with representative results shown here. Linear responses were observed for MS/MS transition fragment masses m/z 412.2 (A), m/z 959.4 (B), and m/z 738.3 (C), corresponding to, respectively, the 2^+ parent m/z 646 (TGGGTGDSFVVAGR), 602 (IMLAGNAFTAGK), and 753 (TLDPIEDVDR).

Fig. 3. Tolerance of the MALDI method to deviations from equimolar ratios of native to labeled peptide

Coefficients of variance (CV) were calculated for measurements taken from 10-fold serial dilutions of the standard peptide mixed with a constant amount of VLP digest, corresponding to approximately 50 fmol norovirus capsid protein. The dashed line represents the average CV.

Fig. 4. Robustness of the MALDI-MS method in the presence of environmental interferences

To test the dynamic range of the method, serial 10-fold dilutions were prepared and analyzed of target analytes with parallel dilutions of standard peptides in a variety of environmental backgrounds. Controls were diluted in 0.1% trifluoroacetic acid. Values are presented as averaged ratios normalized to the 1 fmoles control ($n = 3$). Error bars represent the standard deviation; for clarity, only positive error bars are shown.

Table 1
Performance metrics for analysis of norovirus-like particles by MALDI based detection and quantitation

Ratios of native to heavy isotope-labeled standards represent the average of at least 3 technical replicates. Coefficients of variance (CVs) were calculated for pure standards from 0.1 to 1,000 fmol ($n=3$ each) and for environmental preparations fortified with norovirus to final concentrations ranging from 1 to 100 fmol ($n=9$ each).

Sample	Ratio	CV (%)
pure standard		
1,000 fmol	2.5	19
100 fmol	3.8	16
10 fmol	2.8	11
1 fmol	1.04	6
0.1 fmol	0.44	5
average	2	15
strawberry	3.2	53
lettuce	4.3	20
milk	3.6	36
groundwater	2.9	21
soil	3.1	42