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Rapid quantification of *Escherichia coli* in food and media using bacteriophage T7 amplification and liquid chromatography-multiple reaction monitoring tandem mass spectrometry

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ABSTRACT

Conventional microbiological assays have been a valuable tool for specific enumeration of indicative bacteria of relevance to food and public health, but these culture-based methods are time-consuming and require tedious biochemical and morphological identification. In this work, we exploit the ability of bacteriophage T7 to specifically infect *Escherichia coli* and amplify nearly a 100-fold in 1–2 h. Bacteriophage amplification is integrated with liquid chromatography-multiple reaction monitoring tandem mass spectrometry (LC-MRM–MS/MS) for quantitation of phage-specific peptides. Heavy isotopic ^{15}N labeled T7 is introduced as the inoculum phage and internal standard. Quantification is performed by determining the ratio of phage-specific peptides over the internal standard which value is proportional to *E. coli* numbers. A broad dynamic range of 6-log orders ranging from 3.0×10^3 to 3.0×10^9 CFU/ml is attained in LB, while between 4.1×10^4–2.7×10^9 CFU/ml and 1.9×10^3–3.0×10^7 CFU/ml was enumerated respectively in coconut water and apple juice. With this method, viable *E. coli* are quantified in 4 h with a detection limit of 3.0×10^3 CFU/ml, 4.1×10^4 CFU/ml and 1.9×10^3 CFU/ml in LB, coconut water and apple juice, respectively. This method has potential as a rapid tool for detection of fecal contamination during food bioprocessing and distribution to safeguard public health.

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1. Introduction

The detection and quantification of microorganisms is of primary importance for food safety. Food related diarrhoeal diseases are one of the top five leading causes of death worldwide with an estimated 4.7% of death in 2011 alone (WHO Fact Sheet N°310, 2014). Traditional microbiological culture methods for the detection of indicative bacteria of fecal contamination like *E. coli* require 2 days or more. Confirmation of species identity requires additional morphological testing or further subculturing in differential media (El-Hadedy and El-Nour, 2012). While the rapid detection of these cells is essential to prevent the risk of foodborne diarrheal disease outbreaks, the time constraint and inconveniency of traditional methods severely limits the ability to provide a rapid response to the presence of virulent bacteria (Manafi, 1996). Other than standard cultural and biochemical test methods, rapid culture-independent methods such as those based on polymerase chain reaction (PCR) are also used for identifying bacteria in water and food samples. These DNA-targeted PCR-based methods detect the presence of genes specific to bacterial species or strains and they offer good sensitivity and speed using minimal amount of sample (Cui et al., 2003; Odonkor and Ampofo, 2013). However, PCR is unable to discriminate dead from viable bacteria. They are also susceptible to ambiguous and false positive results that can only be verified through microbiological or other methods (McLain et al., 2011). Surface plasmon resonance (SPR)-based immunosensors is another rapid method offering sensitive and rapid quantification of bacteria (Dudak and Boyaci, 2009; Zhang et al., 2012). However, it involves a higher experimental setup cost, and it could be susceptible to false positives. More recently, Strömberg et al. (2014) developed a biplex volume-amplified magnetic nanobead detection assay for sensitive detection of bacterial DNA using a portable AC susceptometer. Although their method is fast, sensitive

Abbreviations: CFU, colony forming units; LB, Luria-Bertani broth; LC-MRM–MS/MS, liquid chromatography-multiple reaction monitoring tandem mass spectrometry; LC–MS/MS, liquid chromatography tandem mass spectrometry; MRM, multiple reaction monitoring; LOD, limit of detection; MOI, multiplicity of infection; OD, optical density; PFU, plaque forming units.

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and requires minimal sample amount preparation, it is also unable to discriminate dead bacteria from live, viable cells (Strömberg et al., 2014). Therefore, alternative rapid methods allowing sensitive detection and quantification of *E. coli* from food are needed.

Bacteriophages are obligate parasites of bacteria that rapidly multiply within their specific host cells upon infection, and they are widely used tools for bacteria detection (Hagens and Loessner, 2007; Smartt and Ripp, 2011). Bacteriophages have been used in assays for the detection of *Mycobacterium tuberculosis* in pulmonary tuberculosis diagnosis (McNerney et al., 2004), *Pseudomonas aeruginosa*, *Salmonella typhimurium* (Stewart et al., 1998) and *E. coli* (Tanji et al., 2004). Although these methods are sensitive and specific, they are not quantitative over a dynamic range of bacteria cell numbers. Bacteriophage T7 is a lytic phage which infects common strains of *E. coli* including the laboratory K12 strains and pathogenic strains of O157: H7 (Molineux, 2005; Heineman and Bull, 2007). The tail spikes of the bacteriophage recognize and bind to lipopolysaccharide on the cell surface of *E. coli* and enzymatic activity of the tail spikes rapidly degrades the O- or K-antigens on the cell surface (Steven et al., 1988). T7 self-replicates upon infecting the host bacteria and only infects metabolically active viable cells (Heineman and Bull, 2007; Ripp et al., 2008). Within minutes, hundreds of newly formed phages are released from each lysed cell. Bacteriophages have comparatively simple protein make-up and are less affected by factors that influence bacterial growth like sample preparation, temperature and nutrients. They are relatively inexpensive to produce in large quantities (Shabani et al., 2008) and have a strong resistance to heat, pH, solvents, acids, alkali and chemicals (Arya et al., 2011). Hence, as diagnostic agents, bacteriophages have the advantage of extensive shelf-lives and low production cost.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has emerged as a mainstream quantitation analytical tool in many environmental and food safety laboratories largely due to its superior sensitivity, molecular specificity and multiplexing capability. Such analyses are typically performed using the triple quadrupole mass spectrometers, which dates back to the development work first reported by Yost and Enke (1978). Today, such analytical instruments are capable of a wide range of data acquisition techniques but the primary function most frequently used for high sensitivity targeted quantitation is the multiple reaction monitoring (MRM) approach. Using the MRM approach, each MRM transition specifies the targeted molecular precursor mass (*m/z* value) and its most abundant fragment ion generated after a fragmentation process known as collision-induced dissociation. For our work, rapid quantitation of viable *E. coli* in samples (correlated to colony forming units per ml) was determined from the optimized MRM analyses of the selected peptide targets of bacteriophage T7 digested capsid proteins. Absolute quantitation was enabled by the use of isotopically labeled peptides by the internal standard methodology. The "best" peptide targets for MRM use were determined from the shotgun proteomic analysis of the digested capsid protein mixture using a separate hybrid high resolution mass spectrometer based on the orbitrap mass analyzer (Perry et al., 2008; Olsen et al., 2009).

In this work, we evaluated the use of bacteriophage T7 coupled with LC–MS/MS for the detection and quantification of *E. coli* in LB media, coconut water and apple juice. Coconut water is a common drink found in the warmer climates and it is a possible vehicle for foodborne diseases. Due to its nutritional quality and favorable pH for physiological growth, the drink has been shown to support propagation of *E. coli* to numbers as high as 2.0×10^8 CFU/ml (Awua et al., 2012). Apple juice is also a favorite drink with the highest consumption in the world being in Europe and North America (Franz et al., 2009). Apple juice has a relatively low pH (3.1–4.4) and was thought to be safe from pathogens. However, there has been several disease outbreaks caused by consumption of apple juice contaminated with *E. coli* O157:H7 (Choi et al., 2012; Williams et al., 2004). Of all recorded outbreaks, an outbreak of *E. coli* O157:H7 traced to apple juice in 1996 caused the most damage involving people from three states in the British Columbia. *E. coli* has since been shown to survive and propagate in damaged apple tissue (Dingman, 2000; Fatemi et al., 2006). Pierce et al. (2012) also made use of isotope-labeled lytic bacteriophage 53 to specifically detect *Staphylococcus aureus*. They coupled bacteriophage amplification to MRM to achieve a limit of detection (LOD) at $<5.0 \times 10^4$ colony forming units (CFU) per ml in Luria-Bertani (LB) media. Martelet et al. (2014) adopted the method to detect *E. coli* and *Bacillus subtilis* using unlabeled bacteriophage T4 and SPP1 respectively in LB media and orange juice, where they achieved a LOD of $<5.0 \times 10^5$ CFU/ml in food. In our study, we modified the methods of Pierce et al. (2012) and Martelet et al. (2014) to detect *E. coli* W3110, using the obligate-lytic bacteriophage T7 in LB media, coconut water and apple juice. We aimed to develop a specific, rapid and sensitive quantification LC-MRM–MS/MS method to detect viable *E. coli* over a wider dynamic range of cell count. We demonstrated that (MRM) tandem mass spectrometry coupled to isotope-labeled bacteriophage amplification could perform quantification of *E. coli* over a linear dynamic range of 6-log orders. The modified method was able to detect *E. coli* from 3.0×10^3 to 3.0×10^9 CFU/ml *E. coli* in LB media, 4.1×10^4 to 2.7×10^9 CFU/ml *E. coli* in coconut water and 1.9×10^3 to 3.0×10^7 CFU/ml *E. coli* in apple juice within 4 h. This method offers speed, sensitivity, specificity and dynamic quantification of bacterial cells across 6-log order of magnitude.

2. Materials and methods

2.1. Bacterial strains and preparation of heavy isotopic ^{15}N bacteriophage T7

E. coli K12 W3110 wildtype (ATCC 27325), *E. coli* BL21 (ATCC BAA-1025) and bacteriophage T7 (ATCC BAA-1025-B2) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). For propagation of bacteriophage T7, *E. coli* BL21 was grown in 25 ml LB media (BD Diagnostics, Franklin, NJ) at 37 °C under agitation (200 rpm) and 500 μl bacteriophage T7 (1.0×10^9 PFU/ml) added at OD_{600} 0.5–0.7. After 2 h, the suspension was centrifuged (15 min, 4600 rpm) and filtered through a 0.2 μm polyvinylidene difluoride filter (Sartorius AG, Germany). The bacteriophage T7 stock solution was kept at 4 °C until use. Plaque assay was carried out to determine the titer of bacteriophage T7 in plaque forming units (PFU) per ml. Overnight culture of *E. coli* BL21 (200 μl) and phage sample (10 μl) were mixed in 3 ml of 0.5% top-agar, and the suspension was poured across the LB agar plates. Plates with solidified soft top-agar were incubated overnight at 37 °C to determine phage titer.

E. coli BL21 were cultured in Bioexpress U–^{15}N 98% cell growth media (Cambridge Isotope Laboratories, Inc., Andover, MA) as described by the manufacturer with minor modifications. 1 ml ^{15}N media was diluted in 9 ml of PBS buffer (100 mM), and inoculated with 500 μl overnight *E. coli* BL21 (37 °C, 200 rpm). Following overnight culture, the same procedure was repeated by inoculating 500 μl ^{15}N labeled cells with 10 ml fresh ^{15}N cell growth media. This second round of culture in ^{15}N growth media was to ensure efficient labeling of *E. coli*. Following the second overnight incubation, 500 μl ^{14}N wildtype bacteriophage T7 from the stock solution (1.0×10^9 PFU/ml) was added to the ^{15}N labeled *E. coli* (OD_{600} 0.6–1.0) and incubated for 2 h at 37 °C, 200 rpm. The mixture was centrifuged (15 min, 4600 rpm) and filtered through an autovial 0.2 μm polyvinylidene difluoride filter (Sartorius AG, Germany). The supernatant containing ^{15}N phage was added to a ^{15}N labeled agar plate (1.5% agar), and incubated overnight (37 °C). The ^{15}N

phage was collected into a 50 ml conical tube by washing the top agar with 3 ml PBS, scraping with a sterile plastic loop, and aspirating with a pipette. The suspension was then centrifuged (15 min, 4600 rpm) to remove the debris, and the supernatant was filtered through another 0.2 μm polyvinylidene difluoride filter (Sartorius AG, Germany). The ^{15}N labeled bacteriophages were quantified using plaque assay and background levels of ^{14}N unlabeled bacteriophage peptides evaluated with LC-MRM–MS/MS. 5 μl of ^{15}N bacteriophage T7 stock sample was processed for MRM analysis. ^{15}N bacteriophage T7 stock suspensions (1.0×10^{10} PFU/ml) were stored at 4 °C.

2.2. Preparation and in-solution digestion of phage proteins

For the standard curve of MRM signal intensity of the $^{14}N/^{15}N$ SAQFPVLGR and AALTDQVALGK phage peptides over phage count. Ten millilitres LB broth was inoculated with *E. coli* W3110 and grown in a shake flask at 37 °C under agitation at 200 rpm. Optical density (OD_{600}) was recorded at regular intervals and when OD_{600} reached 0.5–0.6, 5 ml of bacteriophage T7 stock culture (1.0×10^9 PFU/ml) was added to the 10 ml of bacterial suspension with a multiplicity of infection (MOI) ratio of 5. Samples were taken at regular intervals upon infection and centrifuged (15 min, 4600 rpm). The supernatant containing the phage particles was used for plaque assay to quantify the bacteriophage concentration over time.

Media or food samples spiked with *E. coli* W3110 were infected with 100 μl of ^{15}N bacteriophage T7 (1.0×10^9 PFU/ml) and incubated for 2 h (37 °C, 200 rpm). Samples were centrifuged (15 min, 4600 rpm) and the supernatant containing phage particles was recovered and buffer-exchanged with four volumes of 50 mM ammonium bicarbonate (pH 9) by centrifuging at 4000 rpm (5 min), using 30 kDa filters (Amicon Ultra; Millipore, Billerica, MA). The concentrated filtrate (25 μl) was collected for subsequent enzymatic digestion, followed by LC–MS/MS analysis. For the spiking experiments, serial 10-fold dilutions of *E. coli* W3110 (OD_{600} 0.5) were performed. Coconut water and apple juice were diluted with five volumes LB and pH were adjusted to 6.8–7.0 if necessary before phage infection. Cells were spiked at a final concentration of 10^3–10^9 CFU/ml and three biological replicates of experiment were carried out.

In-solution digestion on phage filtrate (25 μl) was performed using a modified version of acid-labile surfactant digestion protocol. A 1% solution (3 μl) of sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate (RapiGest, Waters Corporation, Milford, MA) in 50 mM ammonium bicarbonate digestion buffer was added to 25 μl phage filtrate and incubated at 100 °C for 5 min and rapidly cooled to room temperature to solubilize proteins and aid protein digestion. Subsequently, 1.3 μl (1 μg/μl) sequence-grade trypsin (Promega, Madison, WI) was added and the mixture incubated at 37 °C for 5 min for proteolysis. Following digestion, the sample was acidified with 10% formic acid in 10% acetonitrile (6 μl) and incubated for 10 min at 37 °C to cleave the acid-labile surfactant. The digested samples were dried in a SpeedVac vacuum system (Savant Instruments, Hyderabad, India) and reconstituted in 10 μl of LC–MS buffer (1% formic acid, 2% methanol). The samples were transferred to autosampler vials for LC–MS/MS analysis.

2.3. Nano liquid chromotography–MS/MS and bacteriophage protein identification

Tryptic peptide (5 μl) of the digested bacteriophage T7 was analyzed by LTQ Orbitrap Velos Pro mass spectrometry (Thermo Fisher Scientific, Waltham, MA), which was coupled to a *nano*ACQUITY UPLC system (Waters Corporation, Milford, MA) fitted with identical trap (C18 symmetry, 5 μm, 180 μm × 20 mm, Waters) and nanoanalytical columns (BEH130C18 1.7 μm, 75 μm × 200 mm, Waters). The elution program used two mobile phases, A (deionized water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), where B was increased linearly from 2% to 40% over 60 min at a flow rate of 0.3 μl/min. For each sample run, the sample was trapped and desalted for 8 min at 8 μl/min, 1% B before applying the reversed-phase elution program. The LTQ Orbitrap Velos Pro was operated in a top 10 data dependent mode using survey scans at 60,000 resolution from 300 to 1800 *m/z*. Tandem MS scans were acquired with normalized collision energy set to 35.00 for CID, ion trap and orbitrap maximal injection times were set to 120 ms and 10 ms respectively. Identification of phage proteins was achieved by database searching using Sequest algorithm in Proteome Discoverer 1.3 software (Thermo Fisher Scientific) with the following parameters: fragment ion mass tolerance of 0.50 Da, parent ion tolerance of 5 ppm, two missed trypsin or nonspecific cleavages permitted. A database of 302,581 entries were generated by retrieving sequences from the National Center for Biotechnology (NCBI) protein database (downloaded on 01 October 2012) that contained at least one of the following strings in the entry description; "Enterobacteria phage", "bacteriophage", "phage".

2.4. Targeted bacteriophage peptides quantification analysis via LC MRM triple stage quodrupole mass spectrometry

The peptide sequences SAQFPVLGR and AALTDQVALGK were selected for quantification purposes. The ^{15}N labeled SAQFPVLGR and AALTDQVALGK were 13 Da heavier than the naturally occurring peptides because of the incorporation of serine, alanine, phenylalanine, proline, valine, leucine, glycine, threonine and aspartic acid each with a ^{15}N nitrogen count of one, and glutamine and lysine amino acids with a ^{15}N nitrogen count of two, and arginine with a ^{15}N nitrogen count of four.

The trypsin digested samples were injected to nanoLC-MRM–MS. Peptide separation was carried out with *nano*ACQUITY UPLC (Waters) fitted with C18 trap column (C18 symmetry, 5 μm, 180 μm × 20 mm, Waters) and an analytical column (BEH130C18 1.7 μm, 75 μm × 200 mm, Waters) using two mobile phases, A (deionized water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) similar to LTQ Orbitrap Velos Pro. The injection volume was 5 μl. Flow rate is maintained at 0.3 μl/min and a gradient of 2–40% B over 60 min was established to separate the peptides. Trapping was performed at 6 μl/min for 3 min at 0.5% B. The

Table 1
List of precursor and fragment ion transitions for MRM detection of ^{15}N labeled and unlabeled bacteriophage T7 major capsid protein. Two unique peptide sequences (SAQFPVLGR and AALTDQVALGK) were chosen to quantify the major capsid protein of bacteriophage T7.

Target peptide	Actual mass (Da)	Precursor ion (*m/z*)	Fragment ion (quantification)	Fragment ion (confirmation)	Fragment ion (confirmation)
SAQFPVLGR-^{14}N	973.5	487.8	541.4 (17)	688.4 (15)	816.5 (17)
SAQFPVLGR-^{15}N	986.5	494.3	549.3 (17)	697.4 (15)	827.4 (17)
AALTDQVALGK-^{14}N	1085.6	543.8	831.5 (17)	730.4 (17)	944.5 (17)
AALTDQVALGK-^{15}N	1098.6	550.3	841.4 (17)	739.4 (17)	955.5 (17)

The collision energy (volts) for fragmentation is indicated in brackets for each transition.

Table 2
Unique bacteriophage T7 proteins detected using shotgun proteomics. Samples were trypsin digested for 5 min before LC–MS/MS analysis on a LTQ Orbitrap Velos.

No.	Accession no.	Description	No. identified peptides
1	P03725	Internal virion protein C Enterobacteria bacteriophage T7	24
2	P03726	Internal virion protein D Enterobacteria bacteriophage T7	23
3	P19726	Major capsid protein 10 A Enterobacteria bacteriophage T7	18
4	P03728	Head-to-tail joining protein Enterobacteria bacteriophage T7	18
5	P03716	Capsid assembly protein Enterobacteria bacteriophage T7	14
6	P03748	Tail fiber protein Enterobacteria bacteriophage T7	14
7	P03747	Tail tubular protein B Enterobacteria bacteriophage T7	9
8	P03724	Internal virion protein B Enterobacteria bacteriophage T7	9
9	P03696	Helix-destabalizing protein Enterobacteria bacteriophage T7	6
10	P03751	Host specificity protein B Enterobacteria bacteriophage T7	2
11	P03746	Tail tubular protein A Enterobacteria bacteriophage T7	1
12	P03800	Uncharacterized 6.5 protein Enterobacteria bacteriophage T7	1

column eluent was introduced into a Thermo TSQ vantage triple stage quadrupole mass spectrometer (Thermo Scientific, Waltham, MA). The instrument was operated in positive ion multiple reaction monitoring mode. The precursor → fragment ion transitions were m/z 487.8 → 541.4 and m/z 543.8 → 831.5 for the native peptides and m/z 494.3 → 549.3 and m/z 550.3 → 841.4 for the ^{15}N corresponding labeled peptides. For each peptide, two additional transitions were monitored for confirmation purposes (Table 1). Dwell time per transition is set at 20 ms, collision gas pressure to 1.3 mTorr and individual collision energy was optimized for each transition. Mass spectrometry data obtained were analyzed on Pinpoint software (Thermo Scientific). Peak integrations were obtained after smoothing (7 points) and were reviewed manually. Transitions from analyte peptides were confirmed by having the same retention times of the heavy stable isotope-labeled peptides.

3. Results and discussion

3.1. Bacteriophage T7 infectivity on E. coli K12

The ability of a bacteriophage to specifically infect their target host cells and release close to hundreds of progeny phage copies in a short time is crucial to the objective of developing a rapid *E. coli* detection method. The consequential copy number amplification of bacteriophages from host infection will also lower the limit of detection using mass spectrometry. To evaluate the infectivity of bacteriophage T7 on wildtype *E. coli* W3110 strain cultured in LB, these cells were infected at an OD_{600} of 0.8 (Fig. 1a). Within an hour after infection with T7, OD_{600} decreased drastically indicating rapid lysis of the bacterial cells. Cell lysis was also visually confirmed using a microscope. Phage counts increased rapidly between 0.5 and 1 h and reached a maximum between 2 and 3 h. This correlates with a relative increase of phage copy number about 90-fold compared to the initial inoculum number per cell (Fig. 1b). Under the conditions tested, an infection time of 2 h was determined to be adequate for effective T7 phage amplification and lysis of *E. coli*.

3.2. Identification of bacteriophage T7 proteins and peptides for MRM quantification

In order to identify target phage proteins for quantification by LC-MRM–MS/MS, trypsin-digested T7 samples were first subjected to a global proteomic profiling on a LTQ Orbitrap Velos to identify phage proteins that can be consistently detected by LC–MS/MS. The target protein will be selected based on the following criterion: (1) it is a robust protein hit with high signal intensity peptides that can be monitored using MRM, (2) the protein is unique to bacteriophage T7, and typically, and this will be a structural protein which is more abundant than nonstructural proteins. Bacteriophage particles from cell lysate were digested in-solution using a rapid 5 min trypsin treatment, followed by quenching with acid and reconstituted in LC–MS buffer. The samples were then analyzed on a LTQ Orbitrap Velos Pro. Phage proteins were identified by matching acquired peptide tandem mass spectra to theoretical digests found in a customized protein database for bacteriophages from Uniprot. From the shot-gun proteomic analysis of three replicate experiments, 12 unique bacteriophage T7 protein hits were obtained (Table 2).

Out of the 12 short-listed proteins (Table 2), the major capsid protein 10A was selected for further quantification using LC-MRM–MS/MS. Major capsid protein 10A is a structural 36.5 kDa protein, which self-assembles to form an icosahedral capsid that holds the genomic DNA material (Cerritelli et al., 1997). Two peptides with high confidence scores from major capsid protein (SAQFPVLGR and AALTDQVALGK) were chosen for MRM quantification. The peptides were selected based on the following criterion: (1) high signal intensities, (2) between 5 and 25 amino acids in length, (3) unique for the target protein. In particular, the peptides should not be present in other organisms associated with water and food contamination. In order to reduce potential sources of variability, peptides for MRM are selected for optimal stability. Peptides that contain multiple adjacent cleavage sites such as arginine/lysine, lysine/lysine or arginine/proline, which are likely to give missed cleavage, and peptides containing cysteine and methionine are avoided as these residues are susceptible to modifications such as oxidation, which would affect the accurate peptide quantification and lead to inconsistent mass spectrometry analysis.

The LC-MRM–MS/MS response for the two peptides SAQFPVLGR and AALTDQVALGK was measured on a Thermo TSQ vantage triple stage quadrupole mass spectrometer using MRM. Serial dilution of bacteriophage T7 samples were prepared for MRM analysis in duplicates as before. The results showed that the MRM peak areas for both the peptides were proportional to the phage count (Fig. 2). The MRM transition signals of both peptides of the major capsid protein show a linear dynamic range which is proportional to the bacteriophage count. The lowest detection limit of bacteriophage was about 1.85×10^4 PFU/ml. The curves for the peptides were linear in 1.85×10^4–1.85×10^8 PFU/ml range. Peptide SAQFPVLGR showed good linearity with an R-value of 0.999, whereas peptide AALTDQVALGK gave an R-value of 0.867.

3.3. Heavy isotopic ^{15}N labeling of bacteriophage T7 as an internal standard for MRM

The use of heavy isotope-labeled bacteriophages allows for greater amounts of inoculum bacteriophage to be used, which reduces the time of analysis and improves detection limits with LC-MRM–MS/MS. Heavy isotopic ^{15}N bacteriophage T7 was generated by infecting ^{15}N labeled *E. coli* with T7 in ^{15}N-cell growth media. The process was repeated twice to ensure effective heavy isotopic labeling of the phages. To evaluate the background levels of any ^{14}N unlabeled bacteriophage peptides in the heavy ^{15}N T7

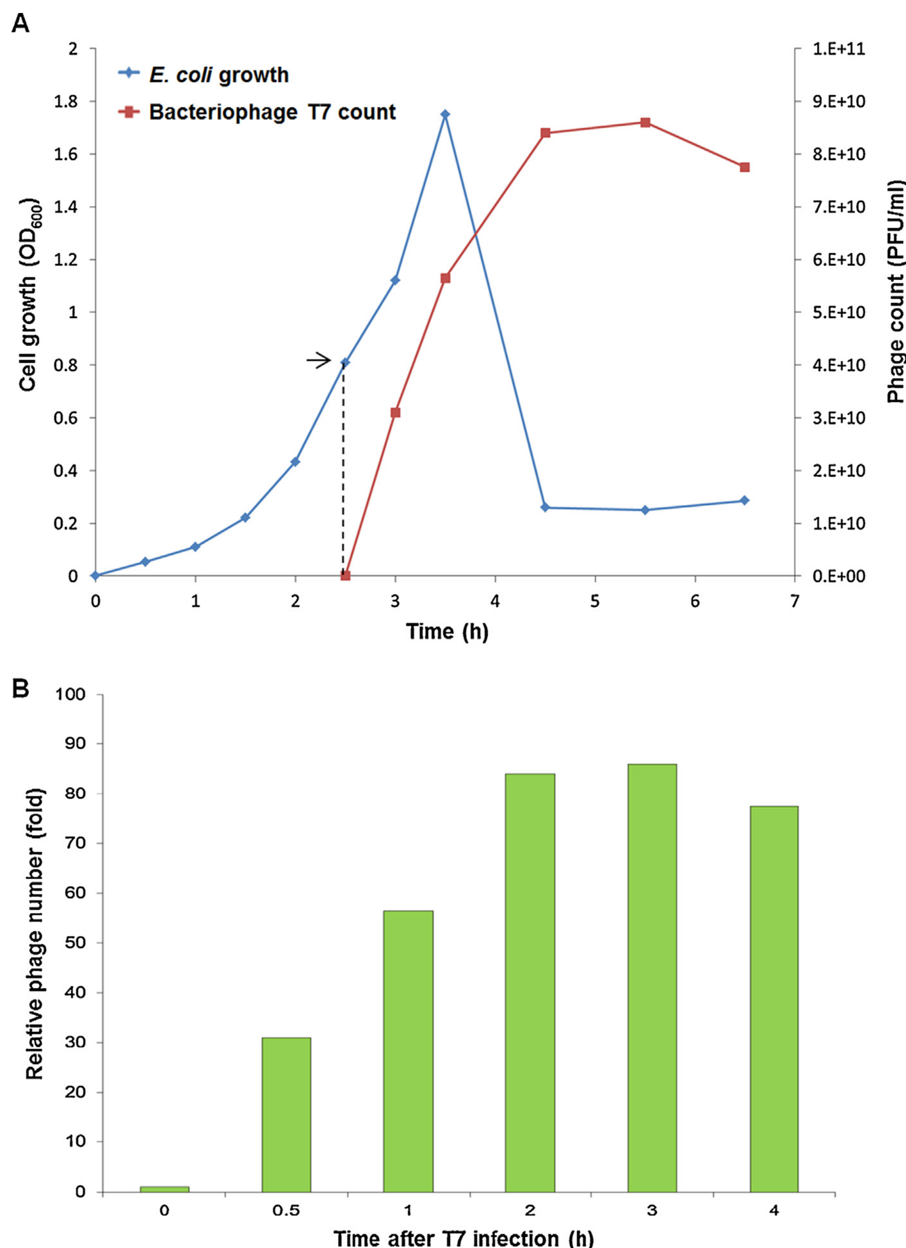

Fig. 1. Bacteriophage T7 amplification in *E. coli* over time. (A) Bacteriophage (1.0×10^9 PFU/ml) was added to bacteria culture suspension at OD_{600} 0.8 and was incubated at 37°C. Samples of culture suspension were taken at suitable intervals for OD_{600} measurement and plaque assay. The graph shows the OD_{600} of *E. coli* culture suspension (diamonds) and bacteriophage count (squares) with time. (B) Bacteriophage amplification was calculated in relative fold change by dividing the total bacteriophage count over the initial inoculum bacteriophage count per *E. coli* cell. The graph shows relative bacteriophage amplification over time.

stock and to test for interference, dilutions of the ^{15}N T7 stock solution (1.0×10^{10} PFU/ml) was analyzed by mass spectrometry. The ^{15}N labeled SAQFPVLGR and AALTDQVALGK peptides is expected to shift by 13 Da compared to the unlabeled peptides as the ^{15}N labeled SAQFPVLGR and AALTDQVALGK are heavier than naturally occurring ^{14}N peptides. Quantification of relative abundance was achieved by comparing peak areas of target peptides. Analysis showed negligible background levels of ^{14}N phage peptides ($<3.7 \times 10^4$) relative to ^{15}N phage peptides in the ^{15}N labeled bacteriophage T7, and there were no interference detected (Fig. 3). Transitions from the heavy stable isotope-labeled peptides were confirmed by having the same retention times of the ^{14}N unlabeled bacteriophage T7 peptides in the LC run. This confirms that bacteriophage T7 was efficiently labeled with the heavy isotope, ^{15}N. The purified ^{15}N bacteriophage T7 serves as an inoculum phage and an internal standard in samples. Upon trypsin digest, ^{15}N labeled peptides differ from the unlabeled peptides in terms of mass but display similar chemical properties such as LC retention times and ionic charge. The use of ^{15}N bacteriophage T7 as an internal standard will allow the number of bacteria can be quantified with greater accuracy.

3.4. Quantifying E. coli numbers in LB media, coconut water and apple juice using ^{15}N bacteriophage T7 as standard

Coconut water is a popular tropical drink that supports the survival and propagation of *E. coli* (Awua et al., 2012). *E. coli* has been detected in coconut water and it is a likely vehicle for foodborne diseases (Walter and Hoorfar, 2014). Due to its rich nutrients and physiological pH, it enables the growth of *E. coli* to high viable load up to 2.0×10^8 CFU/ml with a bacterial growth lag time of less than 2 h. *E. coli* is also able to withstand the low pH of apple juice to

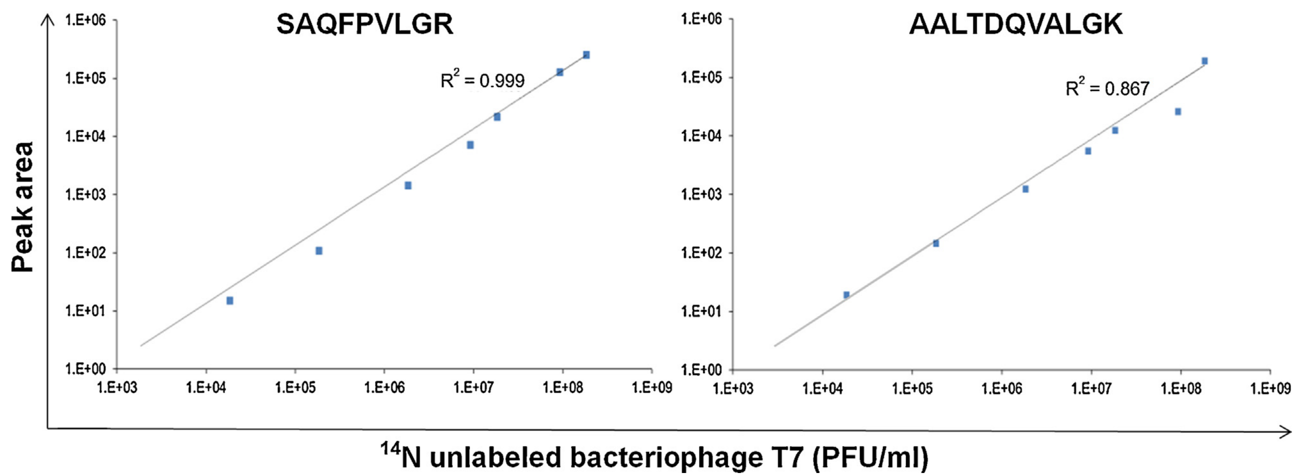

Fig. 2. MRM peptide signal intensity vs. unlabeled ^{14}N bacteriophage T7 concentration. The observed area counts for major capsid protein and peptides SAQFPVLGR and AALTDQVALGK were plotted against unlabeled ^{14}N bacteriophage concentration (PFU/ml). Bacteriophage count was determined by performing the plaque assay. The resulting curves for the quantitative peptides were linear in the 1.85×10^4 PFU/ml to 1.85×10^8 PFU/ml range.

propagate and survive, causing foodborne diseases to those who consume (Liao et al., 2007; Fatemi et al., 2006). In this work, coconut juice and apple juice were chosen to demonstrate the application of the method in food matrix. *E. coli* was spiked into samples at a concentration between 10^3 and 10^9 CFU/ml and a standard curve was constructed with decreasing *E. coli* numbers in LB media, coconut water and apple juice (Fig. 4). Following 2 h incubation with an inoculum of ^{15}N bacteriophage, the samples are subjected to a rapid 5 min trypsin digestion, clean-up and analysis by liquid chromatography tandem mass spectrometric detection targeting peptides of both the ^{15}N and ^{14}N bacteriophage major capsid proteins. Quantification was based on peak areas of target peptides from the ^{14}N peptides that were produced by phage amplification in *E. coli* over that of the heavy isotope-labeled ^{15}N phage peptides from the inoculum. A single *E. coli* cell infected by a bacteriophage releases around a hundred progeny phages (Häuser et al., 2012). This allows an amplification of ^{14}N peptide signal which lower the detection limits of the method (Edgar et al., 2006). The number of ^{14}N progeny bacteriophage will be proportionate to *E. coli* numbers. The more cells present in the sample, higher number of cells will be infected by ^{15}N inoculum bacteriophage. And the infected cells will release ^{14}N bacteriophage that is proportional to the number of infected cells. Therefore, bacteriophage amplification is expected to be proportional to bacteria numbers.

The samples were spiked with ^{15}N inoculum bacteriophage at a standard concentration of 1.0×10^9 PFU/ml with an estimated multiplicity of infection (MOI) ≥ 5. Multiplicity of infection is the ratio of infectious bacteriophage to bacteria. A higher MOI is needed to ensure that at least one infective ^{15}N bacteriophage to each *E. coli* bacterium even in the presence of a significantly high concentration

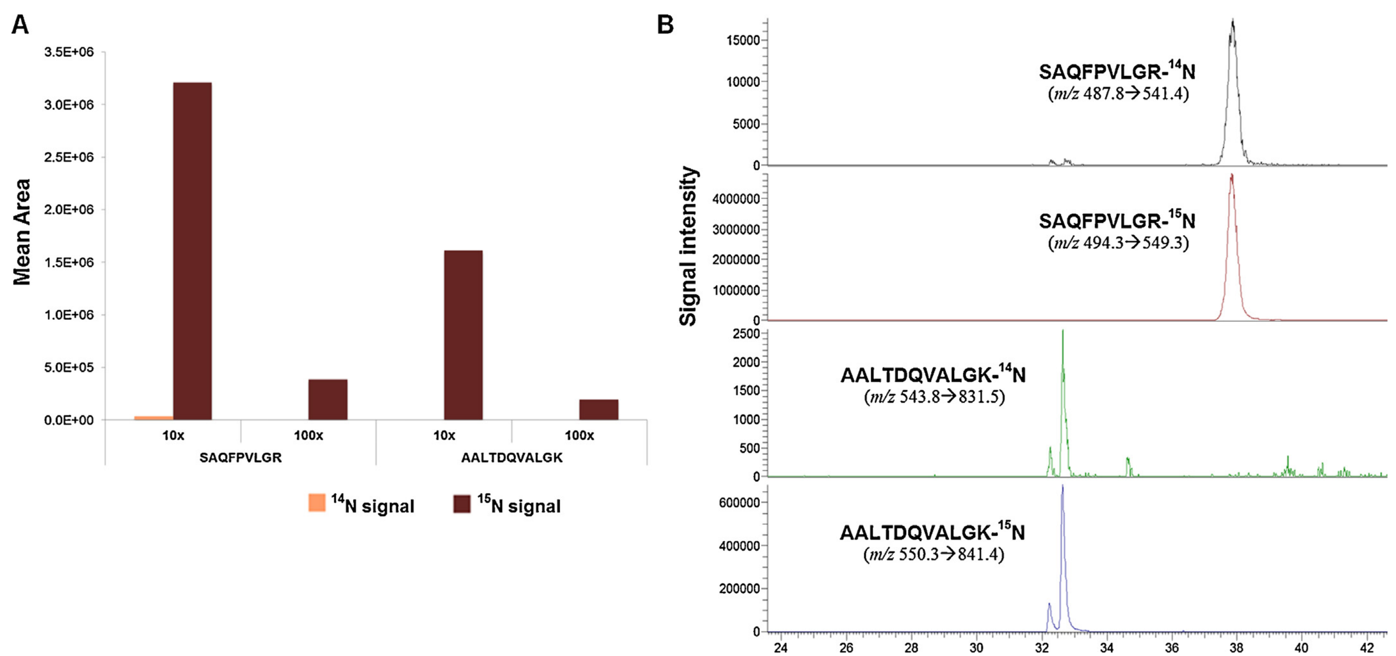

Fig. 3. Mean peak area of ^{14}N/^{15}N transitions and chromatogram of the quantitative MRM transitions in ^{15}N-labeled bacteriophage T7. (A) The peak area for the major capsid protein peptides. 10-fold and 100-fold dilution of the ^{15}N bacteriophage T7 stock solution (1.0×10^{10} PFU/ml) was analysed using the MRM transitions of *m/z* 487.8→541.4 (SAQFPVLGR) and *m/z* 543.8→831.5 (AALTDQVALGK) for the unlabeled native peptides and *m/z* 494.3→549.3 (SAQFPVLGR) and *m/z* 550.3→841.4 (AALTDQVALGK) for ^{15}N labeled peptides respectively (Table 1). (B) Extracted ion chromatograms from LC-MRM-MS/MS of SAQFPVLGR and AALTDQVALGK quantitative transitions and their corresponding heavy isotopically-labeled forms showing similar retention time on the instrument.

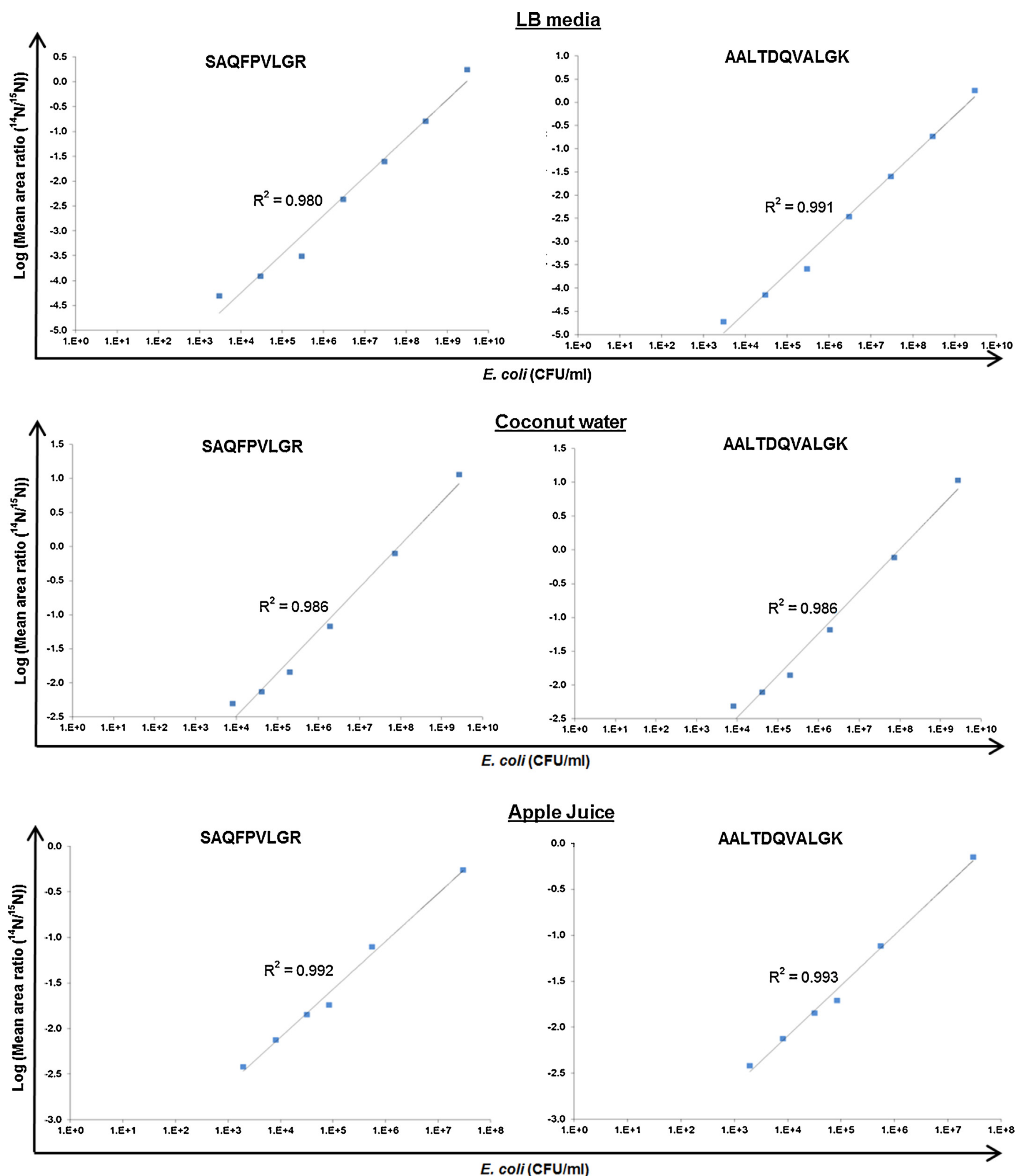

Fig. 4. Quantification of *E. coli* in LB media, coconut water and apple juice using LC-MRM-MS/MS. Samples were spiked with serial dilutions of *E. coli* in LB media (A), coconut water (B) and apple juice (C). Bacteriophage amplification was performed using an inoculum ^{15}N phage of MOI ≥ 5 ($\leq 10^9$ PFU/ml). Mass spectrometry analysis were performed for each sample 2 h after infection and the log mean area ratios (^{14}N unlabeled bacteriophage/^{15}N heavy isotope-labeled bacteriophage) for each quantitative peptide were plotted against *E. coli* numbers. The curves for SAQFPVLGR and AALTDQVALGK were approximately linear (R^2 values 0.980-0.993) between 3.0×10^3 to 3.0×10^9 CFU/ml range in LB, 4.1×10^4 to 2.7×10^9 CFU/ml in coconut water and 1.9×10^3 to 3.0×10^7 CFU/ml in apple juice.

of dead *E. coli* cells. Under high MOI, we can assume that all metabolically active and viable bacteria are bacteriophage-infected, leading to accurate quantification. The log of mean ^{14}N unlabeled and ^{15}N labeled mass spectrometry area ratios for each quantitative peptide were plotted against expected *E. coli* concentrations for each sample upon MRM analysis (Fig. 4). Results were reproducible over three biological replicates. Regression analysis for quantification in LB media showed good linearity ($R^2 = 0.980–0.991$) over 3.0×10^3–3.0×10^9 CFU/ml range for each quantitative peptide. The limit of detection was determined to be as low as 3.0×10^3 CFU/ml, which is an improvement over the results of Pierce et al. (2012) who reported a detection limit of 5.0×10^4 CFU/ml in LB for *S. aureus*. Martelet et al. (2014) reported a detection limit of around 5.0×10^5 CFU/ml *E. coli* in orange juice and bean stew using T4 phage. Our results show, that in apple juice using T7, the limit of detection for *E. coli* can be significantly lower at 1.9×10^3 CFU/ml. Linear regression analysis on the standard curve for coconut water and apple juice showed good linearity ($R^2 = 0.986–0.993$) over a broad dynamic quantification range (from 4.1×10^4 CFU/ml to 2.7×10^9 CFU/ml in coconut water and from 1.9×10^3 CFU/ml to 3.0×10^7 CFU/ml in apple juice) for both peptides.

4. Concluding remarks

In this paper, we demonstrated a robust quantitative method based on LC-MRM–MS/MS with improved LOD values of 3.0×10^3 CFU/ml, 4.1×10^4 CFU/ml and 1.9×10^3 *E. coli* in LB, coconut water and apple juice respectively. We achieved this by exploiting a biological amplification process based on the intrinsic lytic infection cycle of T7 phage for *E. coli* and using ^{15}N labeled T7 as an internal quantitative standard. The number of *E. coli* cells was accurately quantified by the increase in MRM peak areas of ^{14}N bacteriophage peptides of the capsid protein normalized to the ^{15}N labeled T7 standard. This method offers rapid analysis time (<4 h total analysis time), sensitivity (LOD: 3.0×10^3 CFU/ml) and specific quantification of *E. coli* cells with low probability of false-positive results. In addition, quantification of *E. coli* cells in samples can be achieved over a broad dynamic range over 6-log orders from 3.0×10^3 CFU/ml to 3.0×10^9 CFU/ml. This method can be a rapid tool for the diagnosis and control of fecal contamination for food safety and public health. Specific bacteriophages against other bacteria could be used as detection tools to quantify viable bacteria numbers in more complex matrixes like those found in the environment, hospital and food.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.10.017.

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