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## Original Article

# Identification of bacteria in juice/lettuce using magnetic nanoparticles and selected reaction monitoring mass spectrometry



Cheng-Tung Chen, Je-Wei Yu, Yen-Peng Ho\*

Department of Chemistry, National Dong Hwa University, Hualien, Taiwan

## ARTICLE INFO

## Article history:

Received 31 May 2018

Received in revised form

20 September 2018

Accepted 26 September 2018

Available online 26 October 2018

## Keywords:

Food matrixes

Mass spectrometry

Magnetic nanoparticles

Pathogen

Selected reaction monitoring

## ABSTRACT

Ensuring food safety requires a rapid and reliable method for detecting food-borne pathogens. Mass spectrometry has been demonstrated as a powerful tool to classify pure bacterial species. However, matrix interference from food backgrounds may lead to false results because of the suppression of microbial signals. It is useful to develop a method for bacterial enrichment and marker identification in food samples. Magnetic zirconia nanoparticles were used to concentrate spiked microorganisms from apple juice/lettuce under specific conditions (pH 4.5). Bacterial identification was achieved using nanoLC–MS. Selected reaction monitoring of bacteria-related peptides was applied for the first time to identify bacteria including *Staphylococcus aureus* and *Escherichia coli*. This study presents an accurate means for bacterial identification in food matrixes using MS. The analysis time is less than 90 min and the minimum concentration of *E. coli* detected was  $5 \times 10^3$  CFU/mL. The interaction between bacteria and the magnetic nanoparticles was electrostatic and nonspecific, in contrast to immunoassays which require specific antibodies. The targeted peptide analysis focuses on the bacterial markers, thus significantly simplifying the analysis and leading to an accurate identification of bacteria.

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## 1. Introduction

Accurate detection of bacteria in food and produce may prevent illness caused by foodborne pathogens. Traditional identification methods include cultivation [1], polymerase chain reaction (PCR) [2], immunological assays [3], and fluorescence-based techniques using chemical dyes [4]. Some of these well-known phenotypic and genetic means are labor

intensive and complicated while others lack adequate target specificity. Improved techniques, such as the use of real-time PCR [5], DNA microarray-based chips [6,7], and biosensors [8] have revolutionized conventional methods for identifying microorganisms in clinical diagnostics and food manufacturing, offering quick, highly-sensitive, high-throughput screening. Nevertheless, certain bacterial species are still difficult to identify using nucleic acid-based

\* Corresponding author. Department of Chemistry, National Dong Hwa University, Hualien 974, Taiwan. Fax: +886 3 863 3570.

E-mail address: [ypho@mail.ndhu.edu.tw](mailto:ypho@mail.ndhu.edu.tw) (Y.-P. Ho).

<https://doi.org/10.1016/j.jfda.2018.09.006>

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procedures due to high sequence similarities [9]. Moreover, even one false-positive amplicon can generate a noteworthy signal and result in an incorrect outcome [10].

Mass spectrometry (MS) is considered to be a useful tool for species classification [11–14] because of its rapidness, minimal sample preparation, and high dynamic range. Most importantly, MS allows for the detection of a wide bacterial spectrum without the need for specific DNA primers or antibodies. Currently, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS are two key ionization techniques for analysis of biomolecules. High-mass spectral profiles could be obtained from MALDI and ESI analysis of the proteins extracted from bacteria. These protein patterns could be constructed as bacteria “fingerprints” for species-classification [15–17]. Intact cell mass spectrometry was also developed to discriminate bacteria without any sample pretreatment [18,19]. Currently, most published studies of the direct mass spectrometric analysis of microorganisms are based on MALDI techniques because of its speed and simplicity. Alternatively, methods have been developed based on a bottom-up strategy, in which extracted proteins are digested into peptides with specific proteases. Digests are separated by liquid chromatography (LC) prior to ESI-MS analysis. Improved instrumental efficacy allows thousands of peptides to be sequenced in a single run, resulting in protein identification and the subsequent characterization of the microorganisms expressing those proteins [14,20,21]. Selected reaction monitoring has been used in proteomic studies such as discovery of disease markers. The method may identify a specific peptide by monitoring multiple transitions for a given peptide obtained even from chromatographic coelution. This method should be very useful for identifying bacterial markers in food samples.

Convenient and highly-sensitive nanoparticle-based methods have been used for pathogen detection in food without using MS [8,22]. Given their high surface area-to-volume ratios and increased suitable binding sites, functionalized particles with diameters of 10–100 nm could efficiently conjugate with the target cells [23]. Coating antibodies on the nanoparticle is a promising development for pathogen-specific detection in foods. Zhao et al. added organometallic compound-doped silica conjugated with anti-*Escherichia coli* antibodies to ground beef to label the target cells [24]. The cell-particle complexes were detected using a flow cytometer, reducing total analysis time to within 20 min, while the detection limit could reach 1 CFU/g. Kim et al. used an antibody-coated impedimetric biosensor to detect *Salmonella enteritidis* [25]. Spiking quantum dots into a buffer containing *S. enteritidis* cells improved the detection limit from  $10^6$  CFU/mL to  $10^4$  CFU/mL. Moreover, the biosensor could detect  $10^5$  CFU/mL of cells in milk.

Despite the applicability of MS approaches to differentiate bacteria, most studies have aimed toward clinical applications, and relatively few have focused on analyzing pathogens in foods [26–28]. Abundant food matrixes (e.g., salts, lipids, proteins etc.) may reduce sensitivity in the MS identification of microorganisms. Therefore, the enrichment step is required to reduce interference and provide a sufficient number of bacteria. Nanoparticles with magnetic properties could be used to concentrate the bacteria, simplify the purification

procedures, and remove interference from complicated matrixes for further analysis. Coating oligonucleotides on magnetic beads to select the target pDNA of pathogens from milk for PCR detection resulted in the successful detection of *Listeria monocytogenes* at concentrations as low as 10 CFU/mL [29]. Furthermore, Yang et al. combined immunomagnetic separation with real time-PCR to estimate bacteria detection limits [30]. Using magnetic nanoparticles, they captured and detected *L. monocytogenes* at a concentration of 226 CFU/0.5 mL in a milk sample. However, these strategies require specific antibodies and pose difficulties in determining whether the cells are alive or dead. Chen et al. used non-toxic magnetized zirconium hydroxide to capture bacteria in pudding/milk [31]. After cultivation for 5 h, many characteristic signals of extracted proteins could be observed in MALDI-TOF MS spectra. The approach had a detection threshold of 33 CFU/mL for *Enterococcus faecalis* in nonfat dry milk (NFDM). Chen et al. investigated the trapping affinities between  $\text{Fe}_3\text{O}_4@\text{TiO}_2$  and bacteria [32]. Five bacterial strains were differentiated based on potential biomarkers.

The present study aims to develop a nonspecific bacterial probe to detect bacteria in food samples without specific recognition materials. ESI-MS instead of MALDI-MS was used to characterize bacteria. Two common food-borne bacteria which result in frequent global outbreaks were chosen as model species: *E. coli* and *Staphylococcus aureus*. Magnetic zirconia particles were used to concentrate the bacterial cells. Proteotypic peptides were analyzed by nanoLC-ESI MS and database searching. The bacterial species were identified through the detection of proteotypic peptides as bacterial markers.

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## 2. Methods

### 2.1. Materials

Zirconium butoxide solution (80%) was purchased from Aldrich (Steinheim, Germany). Iron (II) chloride tetrahydrate was purchased from Acros (New Jersey, USA). Ammonia solution and trifluoroacetic acid were purchased from Reidel-de Haën (Seelze, Germany). Hydrochloric acid and methanol were obtained from J. T. Baker (Phillipsburg, NJ, USA). Nitric acid was purchased from Shimakyu's Pure Chemicals (Osaka, Japan). Ammonium bicarbonate and iron (III) chloride hexahydrate were obtained from Sigma Chemical Co. (St. Louis, MO). LC-MS grade formic acid and tetraethyl orthosilicate (TEOS) were obtained from Fluka (Buchs, Switzerland). LC-MS grade acetonitrile and coomassie blue G-250 were obtained from Fluka (Buchs, Switzerland). Porcine trypsin (sequence grade) was obtained from Promega (Madison, WI). Urea was obtained from Wako Pure Chemical Industries (Osaka, Japan). Water was purified using a Milli-Q system from Millipore (Bedford, MA). Apple juice, lettuce, and coffee filtering paper were obtained locally in Hualien, Taiwan. All samples were stored at 4 °C before use. The bacteria used in this study, including *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), were cultivated in a BSL-2 safety laboratory at Tzu Chi University. The bacteria were harvested, washed three times with water, boiled in water for 10 min, lyophilized to dryness, and then stored at –20 °C.

## 2.2. Preparation of nanomagnetic-zirconia

The preparation of magnetic zirconia particles was performed as described previously [33,34].  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  (2 g) and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (5.4 g) were dissolved in 25 mL of 2 M HCl with continuous stirring, and nitrogen gas was consistently introduced to expel oxygen. Ammonium solution (25%, 30 mL) was slowly added to the solution, producing deep brown particles in the suspension. After 30 min of continuous stirring, the solution was washed three times with 50 mL of distilled water to remove excess ammonium ions. The  $\text{Fe}_3\text{O}_4$  particles were further washed twice with 20 mL ethanol and re-suspended in 80 mL ethanol. After sonication for 1 h, 7.5 mL of 25% ammonium solution, 6.25 mL DI water, and 0.125 mL tetraethyl orthosilicate were sequentially added to the solution at 40 °C and the reaction was performed for 2 h. A magnetic field was applied to concentrate the  $\text{SiO}_2$ -coated  $\text{Fe}_3\text{O}_4$  particles and the self-assembled silica was removed. To increase the bonding strength,  $\text{SiO}_2$ -coated magnetic nanoparticles were heated under reflux to 60 °C overnight. The resulting particles were further washed by ethanol and re-suspended in 50 mL ethanol. The zirconia-coated magnetic nanoparticles were prepared by the sol–gel method. A mixture of zirconium butoxide (1.22 mL) and nitric acid (2%, 17 mL) was continuously stirred at room temperature in the dark for 24 h, until the white precipitates disappeared and the solution became colorless. After adding 20 mL magnetic silica suspension into the solution, the mixture was sonicated for 1 h. The well-dispersed suspension was stirred continuously at 30 °C for 24 h. Synthesized particles were washed using distilled water. To enhance the bonding between the zirconia and silica, the particles were heated to 60 °C for 6 h. Finally, the magnetic zirconia nanoparticles were washed with distilled water and stored at 4 °C before use. The particles have an average diameter of ca. 15 nm according to the measurement in our previous research [33].

## 2.3. Zeta potential of zirconia nanoparticles

Magnetic zirconia nanoparticles (4.4 µg) were spiked in a 0.9% NaCl solution (1 mL) at various pH values (pH = 3, 5, 7, 9 and 11) and the suspension was transferred into a clear cuvette. Zeta potential measurements of nanoparticles were made using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Each measurement was accomplished with a minimum of 100 runs. Data were processed automatically using Dispersion Technology Software version 4.2.

## 2.4. Bacterial capture efficiency of zirconia particles

Bacteria (*S. aureus* and *E. coli*,  $5 \times 10^7$  CFU/mL each) were spiked in 0.9% NaCl solution (1 mL) at various pH values (pH = 3, 5, 7, 9 and 11) and the optical density (OD) value of the suspension was measured at a wavelength of 600 nm. The correlation between OD and CFU/mL is  $1 \text{ OD} = 1 \times 10^8$  CFU/mL for *E. coli* and  $0.5 \text{ OD} = 1.5 \times 10^8$  CFU/mL for *S. aureus*. The saline solution (ca. 0.9%) was used to simulate a physiological condition for living cells. An appropriate amount of zirconia nanoparticles (22 µg) was added to the solution and the mixture was gently mixed at room temperature for 5 min. The bacteria

captured by the magnetic nanoparticles were separated from the solution by applying a magnet to the side of an eppendorf tube. The supernatant containing the non-immobilized bacteria was carefully removed by pipetting. The capture efficiencies of Gram-positive and -negative bacteria were estimated by calculating the reduced OD value. The OD measurement of the supernatant was corrected against a blank obtained from a parallel experiment performed on a nanoparticle-containing suspension without the bacteria.

## 2.5. Tryptic digestion

One milliliter of *S. aureus*/*E. coli*-spiked solution ( $10^8$  CFU/mL, 0.9% NaCl) was mixed with 88 µg of magnetic zirconia nanoparticles for 5 min. The bacteria-particle complexes were concentrated with a magnet and washed with 0.9% NaCl. The complexes were mixed in 50 µL of a buffer solution (25 mM  $\text{NH}_4\text{HCO}_3$ , pH = 8.5) and vortexed for 5 min. A 50 µL protease solution (1 µg sequence grade trypsin, 25 mM  $\text{NH}_4\text{HCO}_3$ , pH = 8.5) was added to the mixture. Different strategies were used to digest the proteins from the bacteria: (1) conventional digestion: at 37 °C for 12 h and (2) microwave-assisted digestion at 595 and 700 W for 1, 3, 5 and 10 min. A domestic microwave (Sanyo EM-17P, Japan) was used. The power settings were high (700 W) and medium high (595 W). The settings were converted to power output according to the manufacturer's manual. The final temperature in solution was measured using a thermocouple.

## 2.6. Immobilization of bacteria from juice/lettuce samples

Apple juice and lettuce were used as sample matrixes. Apple juice (0.1 g) was mixed with a 0.9 mL of 0.9% NaCl solution. For lettuce, 0.1 g of the sample in 0.9 mL of 0.9% NaCl solution was homogenized in a blender. The bacteria at concentrations of  $5 \times 10^4$ – $10^7$  CFU/mL were spiked into the sample matrixes. The suspension was agitated by 360° rotational mixing for 10 min to disperse the bacterial cells. Before capturing the target cells with the magnetic nanoparticles, a paper coffee filter soaked with a 0.9% NaCl solution was used to exclude large solid particles. The bacterial recoveries were evaluated by measuring the OD (wavelength, 600 nm) of the pure bacterial suspensions before and after filtering. The bacteria were then mixed with magnetic zirconia particles for 10 min. After incubation, the zirconia particles were collected and washed by applying a magnetic field. After 5 min of vortexing in a 25 µL of buffer (25 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.5), 1 µg of trypsin in the same buffer (25 µL) was added to the sample. Following digestion, the magnetic particles were removed using a magnet and the solution was filtered (through a Millipore filter, Durapore-PVDF 0.45 µm) for LC–MS analysis. Microwave-assisted digestion and nanoLC–MS analysis were performed at this time.

## 2.7. NanoLC–ESI-MS

Tryptic digests were analyzed with an Agilent 1100 HPLC system coupled online with a HCT ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The fused silica

capillary was manually pulled to a tip and was packed with C18 resin (with a particle size of 5  $\mu\text{m}$ ). The flow rate on the separation column was 200 nL/min. Gradient elution from 5 to 80% acetonitrile (0.1% formic acid) in 45 min was applied for each sample. Mass spectrometric measurements were performed using the following parameters: auto MS(n) mode, scan range: MS, 350–2000 m/z, MS/MS, 100–3000 m/z; scan speed: MS, 8100 m/z/s, MS/MS, 26,000 m/z/s; ion polarity: positive ion; isolation width, 3 Da; MS/MS fragmentation amplitude, 1.7 V; ICC target, 80,000; maximum accumulation time: 100 ms; precursor ions auto MS(n), 3; threshold MS/MS, 2,50,000. In the selected reaction monitoring (SRM) mode, the ion selection was based upon the data-dependent runs. The SRM was acquired in the UltraScan mode with a scan range between 100 and 3000 m/z. The isolation width and fragmentation amplitude was set to 3 Da and 1.7 V, respectively. The detection of each bacterium was realized using several SRM signatures (2 markers for *S. aureus* and 4 markers for *E. coli*). The establishment of a SRM signature was based on the two transitions of the most abundant ions. The peak lists were generated into a mascot generic format (MGF) file using DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Proteins were identified by searching against the NCBI nr database (bacteria (eubacteria)) using the MASCOT 2.2.04 search engine (Matrix Science, London, UK). Searching parameters were set as follows: enzyme selected as trypsin with two maximum missed cleavage sites, a mass tolerance of 1.5 Da for peptide tolerance, and 0.5 Da for MS/MS tolerance. Oxidized methionine was searched as a variable modification. All identified peptides for each bacterium were analyzed using blastp software from the Basic Local Alignment Search Tool (BLAST).

### 3. Results and discussion

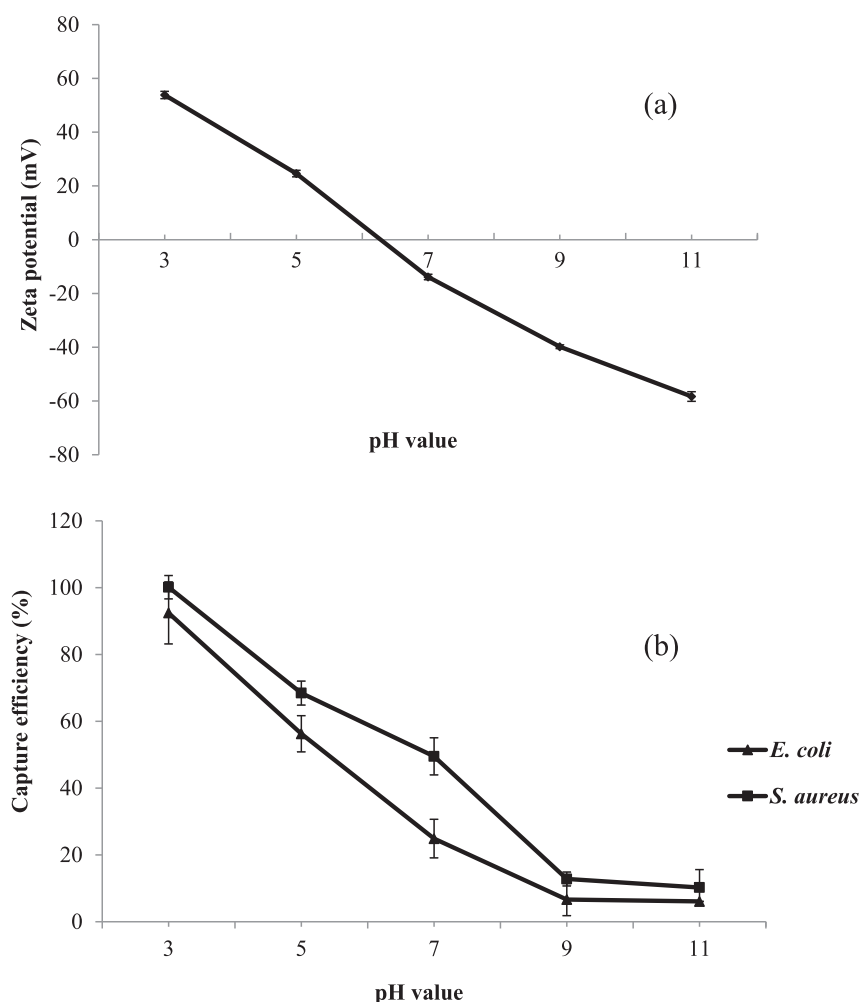
The isoelectric points (IP) of Gram-positive and Gram-negative bacteria respectively ranged from 1.75 to 4.15 and from 2.07 to 3.65 according to the literature [35]. Hence, the surface charge of the bacteria should be negative under physiological conditions. Fig. 1a shows the zeta potential of magnetic zirconia particles in the solutions under various pH conditions. The zeta potential of the zirconia particles decreased as the pH increased. The zeta potential indicated that the particle charge changed from positive to negative when the pH value was increased from 5 to 7. If the pH of a sample solution is kept between 4 and 7, the positively charged particles should combine with the negatively charged bacterial cells. Fig. 1b represents the capture efficiencies of the zirconia particles for two foodborne bacteria at different pH values. When the pH value increased, the capture efficiencies rapidly decreased because of the repulsive force developed between the particles and bacteria. The capture efficiency of the zirconia particles for *S. aureus* is better than that for *E. coli*, potentially due to the teichoic acids on the surface of the Gram-positive bacteria [36]. The phosphate groups on the teichoic acid could chelate with the zirconia, thus increasing the capture efficiency of *S. aureus* because both the electrostatic and covalent (chelating) interactions were involved. The bacterial cells can be cultivated if the captured cells stay alive. The cultivation may be

useful if the number of captured cells is small. Since most foodborne pathogens can survive at a pH above 4.4, pH 4.5 was used for bacterial capture. The capture efficiency for bacteria at pH 4.5 was acceptable at 70–80%. The difference in the capture efficiency between the two bacterial species at pH 4.5 is not significant. Therefore the bias between the species should be insignificant in the qualitative identification of bacteria. Although magnetic  $\text{Fe}_3\text{O}_4$  particles may capture bacteria, the capture efficiency is not as good as that of zirconia particles. It is likely because that the  $\text{Fe}_3\text{O}_4$  nanoparticles do not carry as many charges as zirconia particles do [33].

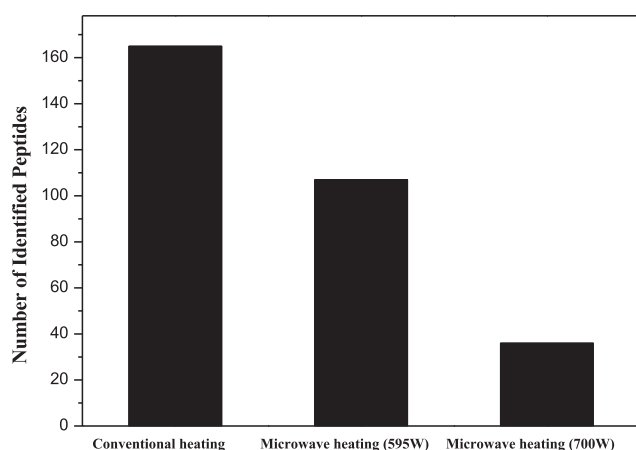
Bacterial identification was carried out by analyzing the proteins of each species using MS. The peptides, which are derived from proteins associated with the target bacterium, were used as markers. If the peptide is associated with only one bacterium, it is a unique marker for the bacterium. Some unique biomarkers were used in SRM analysis for *S. aureus*. The biomarkers for the target bacteria were chosen only if the MS signals were generated consistently and had ion count greater than  $1 \times 10^5$  in the centroid mode. These detected peptides should come from the abundant proteins in bacteria.

Given the use of microwave-assisted enzymatic digestion, the results under several conditions were compared with those taken under conventional conditions. In general, *S. aureus* is relatively reluctant to release proteins for digestion due to its rigid cell wall. It is reasonable to assume that the chosen condition for *S. aureus* is also applicable to other bacteria. *S. aureus* ( $10^8$  CFU) in 0.9% NaCl (1 mL) solution was trapped by the zirconia particles and the proteins were digested under microwave and conventional heating (Fig. 2). Because we did not intend to investigate the whole bacterial proteome, a short LC gradient (45 min) was used. The conventional method identified most peptides (165 matched peptides) under the short gradient condition, but it was still time-consuming because of the digestion time (12 h). When the microwave irradiation (595 W, 5 min) was applied to the digestion, 107 peptides were identified. When a higher power (700 W) was used, only 36 tryptic peptides were identified. Although conventional conditions resulted in the identification of more peptides, analysis time can be greatly reduced using microwave (595 W) irradiation, and was thus used in the present study. To shorten sample preparation time, microbial proteins were only extracted for 5 min. Thus, the identification of only a limited number of peptides was expected and the corresponding proteins were likely to be the most abundant ones.

We further examined digestion at microwave power of 595 W for several time periods ranging from 1 to 10 min. The MS/MS spectra of the tryptic digests from each pathogen were acquired in a data-dependent mode. All tandem spectra were searched against an NCBI nr database using the MASCOT application. The number of peptides obtained from digestion at 1, 3, and 5 min was 9, 10, and 120, respectively (Fig. 3). Five-minute digestion time yielded the most identified peptides. The peptide number (120) is different from that (107) shown in Fig. 2 because of the experimental deviation. The number of identified peptides was very sensitive to the microwave irradiation time. When the irradiation time was less than 3 min, the reaction temperature was not high enough to speed up the



**Fig. 1 – (a) Effect of pH on the zeta potential of the magnetic zirconia nanoparticles. (b) Capture efficiencies of the magnetic zirconia for *S. aureus* and *E. coli* under different pH values. The error bars associated with the data points represent the standard deviation based on three replicates.**



**Fig. 2 – Number of digested peptides detected from the LC–MS/MS analysis of *S. aureus*. Proteins were digested under conventional heating for 12 h, microwave heating at 595 W for 5 min or microwave heating at 700 W for 5 min.**

digestion. When the irradiation time is longer than 6 min the temperature is too high (>75 °C) to be useful for digestion. Therefore, microwave irradiation for 5 min (60 °C) was used in the experiments. The identification of peptides was carried out in triplicate. The numbers of peptides identified for *S. aureus* and *E. coli* are listed in [Table S1 of Supplementary information](#).

[Fig. 4](#) illustrates a tandem mass spectrum of doubly-charged ions at  $m/z$  774.9, which identified a peptide sequence NFDVLDEATGLAQR corresponding to the protein alkyl hydroperoxide reductase subunit c from *S. aureus*. Among many bacterial species we had investigated, unique peptides were easily found in most species [37,38] except for *E. coli*. It proved difficult to identify a unique peptide for *E. coli* as many proteins were derived from several microorganisms in the database. Further, we often found that certain proteins identified from pure *E. coli* were also associated with *Shigella flexneri*, which has been reported to be more appropriately treated as a subgenus of *Escherichia* due to a phenomenon

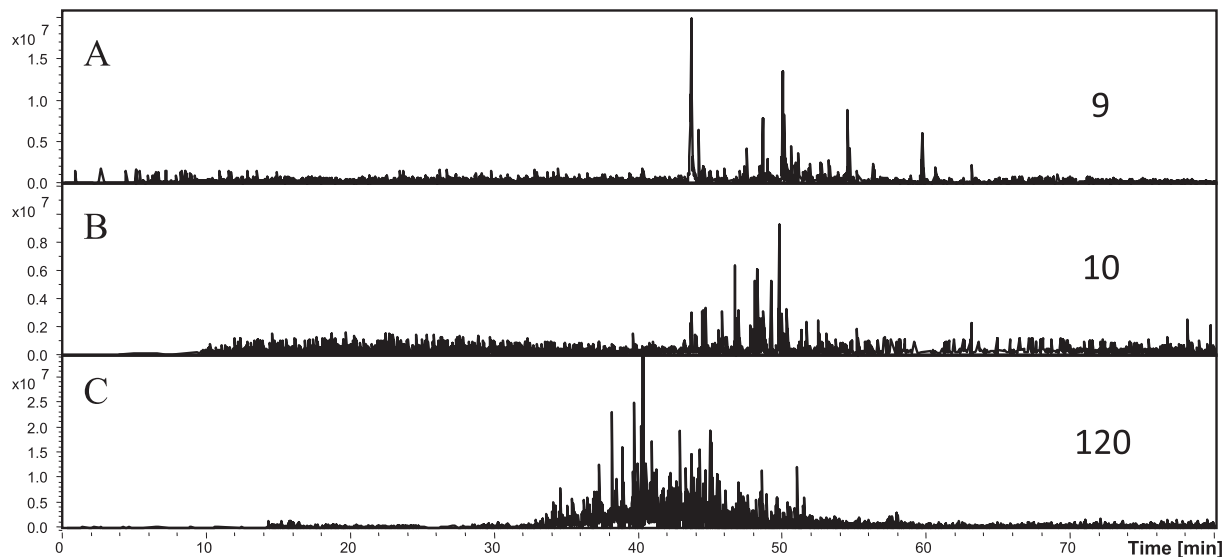


Fig. 3 – Base peak chromatogram obtained from the LC–MS/MS analysis of *S. aureus*. The proteins were digested under microwave heating at 595 W for (A) 1; (B) 3; (C) 5 min. The embedded values indicate the numbers of identified peptides.

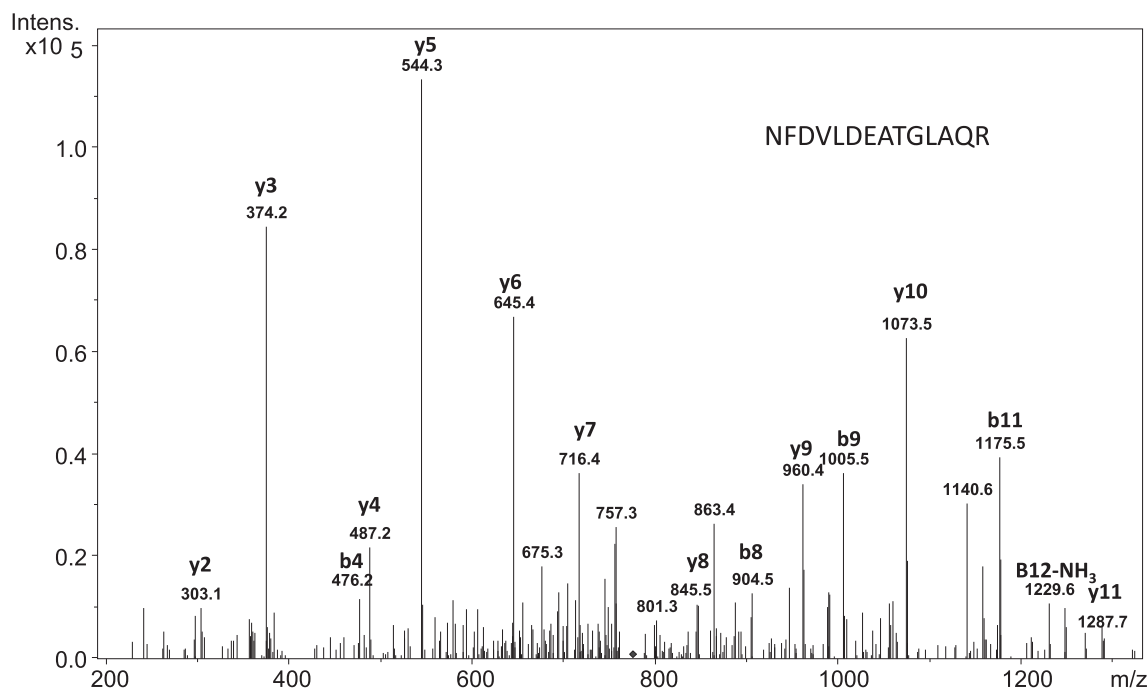


Fig. 4 – Tandem mass spectrum of monitored ion at  $m/z$  774.9, corresponding to the sequence NFDVLDEATGLAQR from alkyl hydroperoxide reductase subunit C acquired by LC–MS analysis. Singly charged b and y ions are labeled in the spectrum.

termed taxa in disguise [39]. For example, two *E. coli* peptides, EVPADAYYGVHTLR and GITINTSHVEYDTPTR, are also associated with *Shigella flexneri*. Therefore, we used four peptide markers to increase the reliability of *E. coli* identification. Although these four peptides are not unique, all their corresponding sources of the identified proteins contain *E. coli*. Further, these four peptides were chosen such that they were

simultaneously present only in *E. coli*. Thus, if these four markers are identified together, the identification should be very reliable. The database searching approach is very accurate although the increasing size of database might change the uniqueness. Table 1 lists identified biomarkers for each microorganism, which we chose from data-dependent experiments. The marker peptides could be identified in each

**Table 1 – Selected biomarker ions and the corresponding SRM transitions for *S. aureus* and *E. coli*.**

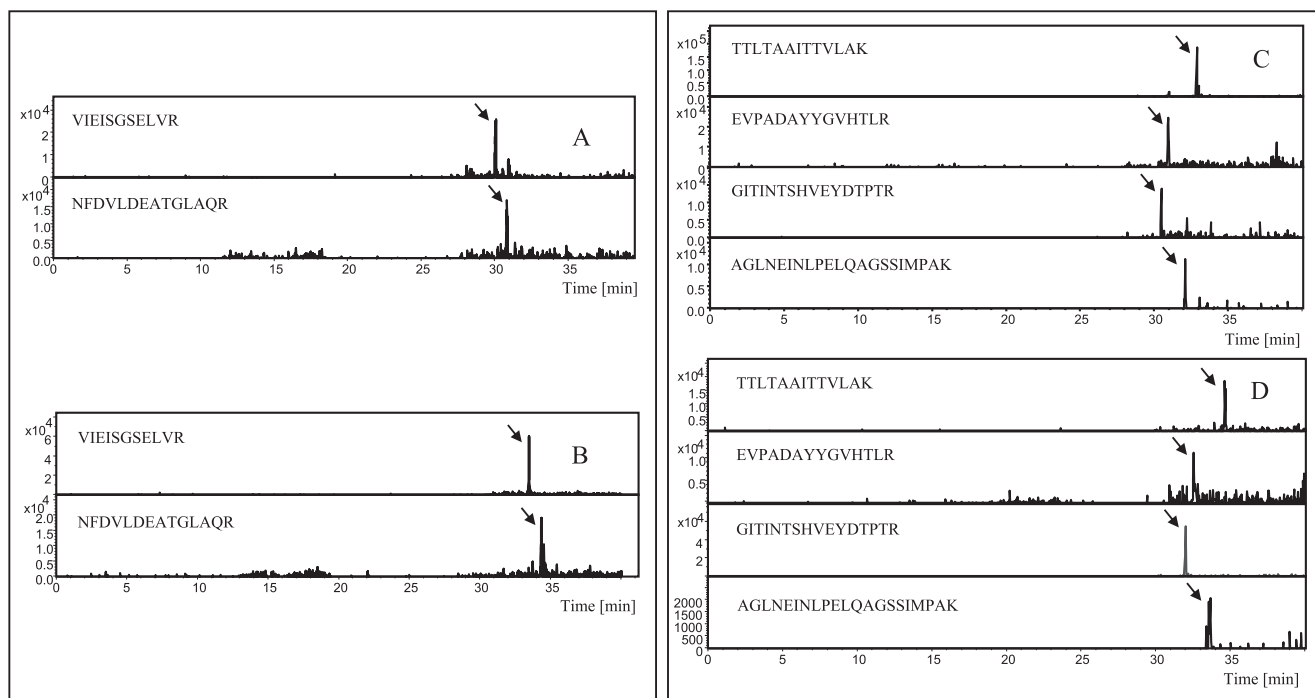
Bacteria	m/z (charge)	Peptide	Protein	Product ion m/z (b/y ion)
<i>S. aureus</i> (ATCC 25923)	601.3 (2+)	K.VIEISGSELVR.G	Arginine deiminase	747.4 (y7); 989.5 (y9)
	774.9 (2+)	R.NFDVLDEATGLAQR.G	Alkyl hydroperoxide-reductase subunit C	374.3 (y3); 544.3 (y5)
<i>E. coli</i> (ATCC 25922)	652.4 (2+)	K.TTLTAAITTVLAK.T	Elongation factor Tu	745.5 (y7); 816.5 (y8)
	795.9 (2+)	R.EVPADAYYGVHTLR.A	Aspartate ammonia-lyase	682.4 (y6); 845.5 (y7)
	902.4 (2+)	R.GITINTSHVEYDTPTR.H	Elongation factor Tu	474.3 (y4); 1330.6 (b12)
	1077.1 (2+)	R.AGLNEINLPELQAGSSIMPAK.V	Aspartate ammonia-lyase	1328.7 (y13); 1555.8 (y15)

replicate (three in total). The search results for the proteins associated with the chosen peptides are listed in [Tables S2 and S3](#).

In the SRM mode, we monitored two ions at m/z 601.3 (2+) and 774.9 (2+) for *S. aureus* and four ions at 652.4 (2+), 795.9 (2+), 902.4 (2+) and 1077.1 (2+) for *E. coli*. The two most abundant MS/MS product ions from each monitored peptide were selected for SRM transitions. [Table 1](#) also lists the product ions that were monitored in the SRM experiments. The SRM approach of combining zirconia enrichment was applied to analyze bacteria in two food matrixes including apple juice and lettuce. The SRM method continuously monitors selected precursor to fragment ion transitions, and is a well-established technique to detect the presence of specific organic compounds within samples. The method has gained popularity in proteomic studies such as discovery of disease markers and quantification of protein expression [29]. Although full-scan MS/MS spectra are not acquired in SRM, the method provides high selectivity and sensitivity by monitoring multiple transitions for a given peptide obtained from chromatographic

coelution. The present work applies the SRM concept to detect the marker peptides representing specific bacteria. The proposed method is aimed to qualitatively analyze bacterial species in food matrix. Quantitation of bacteria may require peptide standards and is beyond the scope of this research.

The food matrixes may suppress the binding between the zirconia and bacterial surface. Various groups have used filtration methods to concentrate bacteria for biological studies [40,41]. The bacteria-containing samples in this study were passed through a paper filter to exclude the major matrix components prior to enrichment with the functionalized particles, with 94% of the *E. coli* and 77% of *S. aureus* cells recovered from the filtrate. After the samples were filtered and the extracted proteins were digested, the tryptic peptides were analyzed using nanoLC–MS in the SRM mode. To evaluate the applicability of the proposed strategy, samples containing various concentrations ( $10^7$ ,  $10^6$ ,  $5 \times 10^5$ , and  $10^5$  CFU/mL) of bacteria in apple juice and lettuce were prepared (total volume of 1 mL). The minimum concentration of *S. aureus* detected in both apple juice ([Fig. 5A](#)) and lettuce ([Fig. 5B](#)) was



**Fig. 5 – SRM of *S. aureus* biomarkers obtained from *S. aureus*-spiked (A) apple juice and (B) lettuce at a concentration of  $10^6$  CFU/mL by magnetic zirconia capturing. SRM of *E. coli* biomarkers obtained from *E. coli*-spiked (C) apple juice and (D) lettuce at a concentration of  $5 \times 10^5$  CFU/mL by magnetic zirconia capturing. The arrows indicate the signals corresponding to the target sequences. Trypsin digestion was conducted under microwave irradiation of 595 W for 5 min.**

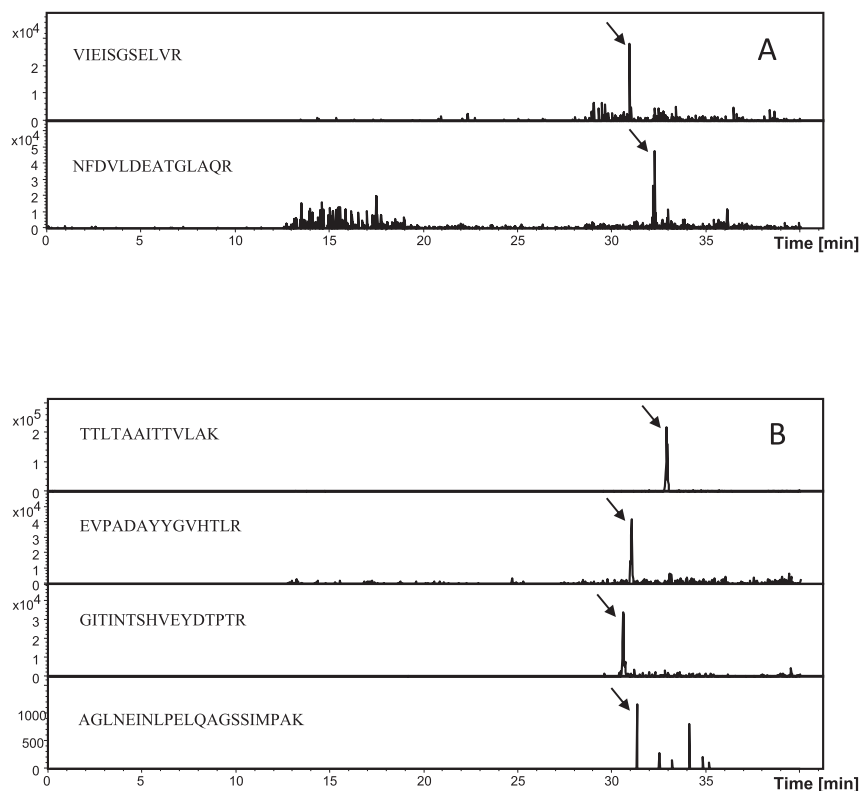
$10^6$  CFU/mL. The minimum concentration of *E. coli* detected in both apple juice (Fig. 5C) and lettuce (Fig. 5D) was  $5 \times 10^5$  CFU/mL. The chosen marker peptides were detected consistently. The reproducibility of the SRM experiments for *S. aureus* ( $10^6$  CFU/mL) and *E. coli* ( $5 \times 10^5$  CFU/mL) in apple juice is shown in Figs. S1 and S2 (Supplementary information).

The minimum concentration of *S. aureus* and *E. coli* detected in the absence of food matrixes was  $5 \times 10^3$  CFU/mL and  $5 \times 10^2$ , respectively (data not shown). When the SRM approach was applied to the blank juice/lettuce samples, no significant marker signals were observed. The results indicate the sample matrixes did not interfere with the SRM analysis. The SRM analysis of bacteria-specific peptides has several advantages. In addition to improved selectivity, the major advantage for bacterial identification was the significant simplification of the data analysis. For the identification method based on database searching, tandem mass spectra are searched against a protein database, a process which may last several hours. For the SRM approach, the target peptides were selected in advance and only the specific transitions were analyzed. Therefore, the analysis time was reduced to seconds. We note that the success of the database searching approach relies on the availability of the protein database associated with the investigated species. The present approach is focused on identifying species. Identification of bacteria at the strain level would require that the protein database at the strain level and unique peptides are available. Simple protein databases can be constructed from experimental results, which has been applied to

analyze antibiotic-resistance and -susceptible strains of *Acinetobacter baumannii* [42].

Adding more zirconia nanoparticles to the samples did not further improve the detection limit, indicating that there were sufficient nanoparticles to trap the spiked bacteria in the food samples. Since the sample matrixes significantly influence the capture of bacteria, we further diluted the sample ten-fold to improve the detection limit. *S. aureus* and *E. coli* in apple juice/lettuce could be detected easily at a concentration of  $10^4$  CFU/mL. *E. coli* cells at a concentration as low as  $5 \times 10^3$  CFU/mL could be detected in 10 mL of diluted apple juice (Fig. 6). Despite the ten-fold dilution of the samples, the minimum concentration detected was lowered by two orders of magnitude. This clearly indicates the effect of the matrix on the bacterial capturing process. The food matrix might interact with the particles and interfere with the capture of bacteria. Therefore, appropriate dilution and filtering is crucial to the success of bacterial detection using the proposed approach.

In summary, detection of targeted peptide markers should be a very efficient means for bacterial identification in real samples. Because interference from sample matrixes may increase the difficulty of analysis, targeted peptide analysis focuses on the bacterial markers, thus significantly simplifying the analysis. Many targeted peptides can be monitored through the SRM mode. Because LC is used for peptide separation, theoretically, there is no limit to maximum number of peptide markers monitored if the retention times for the peptide



**Fig. 6** – SRM of specific biomarkers from (A) *S. aureus* ( $10^4$  CFU/mL); (B) *E. coli* ( $5 \times 10^3$  CFU/mL). The bacteria in apple juice were concentrated with magnetic zirconia and the extracted proteins were digested under microwave heating at 595 W for 5 min.



markers are known. This is useful for identifying multiple bacterial species in samples. This approach presents significant potential for use in food safety area. Future studies will explore the use of different types of nanoparticles in concentrating bacterial cells and the applicability of the proposed method to other food samples such as meat and poultry.

### Conflicts of interest statement

The authors declare that there are no conflicts of interest.

### Acknowledgment

We thank the Ministry of Science and Technology of the Republic of China (grant number: MOST-106-2113-M-259 -006) for supporting this research financially.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jfda.2018.09.006>.

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