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Comparison of MALDI-TOF/MS and LC-QTOF/MS methods for the identification of enteric bacteria

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ABSTRACT

Two complementary mass spectrometric techniques were evaluated for their applicability as methods for foodborne pathogen characterization, biomarker candidate discovery and identification. Ten clinical isolates of closely related organisms, *Escherichia coli*, *Shigella sonnei* and *Shigella flexneri*, were chosen for this study. Whole cell lysates were analyzed using both matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF/MS). Evaluation of isolate mass spectra revealed unique protein profile patterns and potential biomarkers capable of species and sub-species identification. Strengths of each MS method, in addition to their advantages over traditional methods, are highlighted and a brief discussion of their potential as standard tools in the laboratory is presented.

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1. Introduction

An estimated 76 million cases of foodborne illness occur each year in the United States causing over 325,000 hospitalizations and 5200 deaths [1]. Typical symptoms of infection include, but are not limited to, severe exhaustion, diarrhea, abdominal cramping, fever, headache, and vomiting [2]. In terms of pain and suffering, reduced productivity, and medical expenses, the estimated economic cost of foodborne illness is extensive, ranging between $10 and 83 billion each year [3]. Pathogenic *E. coli* and *Shigella* spp. are among the leading causative agents of foodborne illness [2] that are classified category B select agents. In addition, their ability to inflict severe illness and economic detriment make pathogenic *E. coli* and *Shigella* spp. attractive bioweapons [4–6]. Establishing fast, accurate, and sensitive methods of pathogen detection and identification is crucial for proper infection control measures and for timely, effective medical treatment of exposed individuals.

Traditionally, molecular methods such as polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE) and enzyme-linked immunosorbent assay (ELISA) are commonly utilized for pathogen identification. While these methods are laboratory standards, each assay possesses some unique drawbacks. The specificity and speed of PCR are advantageous; however, this target-based approach is limited to detecting pathogens for which primers are available, and organisms that have undergone genetic modifications (e.g., mutagenesis) may yield unreliable or insufficient information (i.e., false positive or negative results). Lastly, PCR identification of foodborne pathogens remains problematic due to the presence of amplification inhibitory factors in some food matrices [5,6]. ELISA is a fast, accurate target-based approach whose specificity comes from the interaction of antibody and its target antigen. Like PCR, ELISA is limited to detecting pathogens for which discriminate antibodies are available. In addition, cross reacting epitopes of non-target antigens and insufficient concentration or absence of target antigens can lead to false positive and negative results, respectively [7,8]. PFGE requires no primers and is capable of detecting subtle differences between DNA fragments that may be missed by target-based approaches. Despite these advantages, PFGE is a meticulous, time-consuming method that takes a minimum of 2-day post-culture to perform. In PFGE, optimal digestion requires pathogen-specific restriction enzymes; therefore, genus identification must be performed prior to PFGE analysis further increasing the turn-around time.

These inherent limitations in current molecular methods have researchers exploring new avenues such as mass spectrometry (MS) for rapid and accurate pathogen identification [9–23] where

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soft ionization techniques have proven optimal for whole protein analysis of bacteria. Most of the recent work evaluating bacterial proteins has utilized MALDI-TOF/MS where speed and ease of analysis dominate [9–16]. This research has largely focused on differentiation based on comparisons between mass spectra to obtain fingerprints of ions that are characteristic of the organism [12–14] or to develop a scoring system that is centered on the observed masses [15,16]. Of note is the work of Liang et al. where collected HPLC fractions from bacteria were analyzed by MALDI-TOF/MS demonstrating that the combination of separation followed by MS analysis proved useful in the differentiation of bacteria [9]. Liquid chromatography/mass spectrometry (LC/MS) has also been implemented, to a lesser extent, in the analysis of bacterial proteins [15–23]. Two research groups have previously compared the performance of LC/MS to MALDI-TOF/MS [15–17]. The work of Li and co-workers [15,16] favored the MALDI-TOF/MS approach for bacterial identification because the data processing was easier than with LC/MS. The work of Krishnamurthy et al. was focused upon development of a more rapid approach (i.e., commensurate with MALDI-TOF/MS); however, this work was limited by the complexity of the data generated from electrospray ionization [17]. A solution to this problem was reported by Williams and co-workers using an automated data handling algorithm that provided charge state deconvolution for eluting proteins from HPLC [18]. This approach has proven useful for identifying protein biomarkers [19], and identifying potential PCR targets for bacteria [20]. More recently, Everley et al. utilized ultra performance liquid chromatography with mass spectrometry to significantly reduce the analytical runtime without compromising the mass spectral data [21]. In addition research from this group has shown the ability of this LC/MS approach to differentiate bacteria at the species [22] and sub-strain [23] level.

For this study, several strains of pathogenic *E. coli*, *S. flexneri* and *S. sonnei* were analyzed by MALDI-TOF/MS and compared to the results previously observed for LC/MS analysis [22] while expanding the previous sample set to include new strains. For comparative purposes all samples were prepared concurrently and analyzed as soon as possible after sample preparation. The goals of this study were (1) to uncover reproducible protein biomarker candidates, (2) to challenge candidate validity in a blind study of unknowns previously characterized by molecular methods and separate from the initial 10 isolates analyzed, (3) to compare the ability of both MS methods to differentiate, characterize and identify closely related strains of pathogenic bacteria, and (4) to assess the potential of these MS methods, individually or in combination with traditional methods, to serve as standard tools of pathogen research and identification.

2. Experimental

2.1. Chemicals

3,5-Dimethoxy-4-hydroxycinnamic acid (Sinapinic Acid) was obtained from Sigma–Aldrich Inc. (St. Louis, MO). Acetonitrile (ACN), trifluoroacetic acid (TFA) and formic acid were purchased from ThermoFisher Scientific Inc. (Fairlawn, NH). Milli-Q water used for bacterial inactivation was purified using the RiOs 5 Water Purification System (Millipore Billerica, MA) and then autoclaved. The 2-propanol was purchased from Honeywell, Burdick and Jackson (Morristown, NJ).

2.2. Bacterial growth

Bacterial isolates analyzed in this study (Table 1) were obtained from in-house clinical samples and handled under Biosafety Level II (BSL 2) conditions. The unique sample number assigned to each isolate was maintained for all samples analyzed in the study. Isolates were grown on trypticase soy agar with 5% sheep's blood for 24 h at 37 °C.

2.3. Sample preparation

Using a disposable inoculating loop, colonies were collected and suspended in 500 μL of water to obtain a turbidity of 1.0. Each sample was washed three times with 500 μL of water and centrifuged at 6000 rpm for 5 min. After the final wash, the water was removed taking care not to disturb the bacterial pellet. The pellet was then suspended in 150 μL of 50% ACN (0.1% TFA) for bacterial cell lysis.

2.4. MALDI-TOF analysis

One microliter aliquots of whole cell lysate were mixed with 1 μL of sinapinic acid (30 mg/mL) matrix solution and spotted onto a stainless steel MALDI target plate. The mixture was allowed to air dry prior to analysis on a Bruker Daltonics Ultraflex II (Billerica, MA) instrument operating in linear, positive ion mode. Calibration was performed using a linear fit of protein 1 (5700–17,000 m/z) mix from Bruker Daltonics. Optimum mass spectra were acquired under the following instrument conditions: pulsed ion extraction delay of 300 ns, ion source voltage one 25 kV, ion source voltage two 23.25 kV, and ion source lens voltage 6.20 kV. Triplicate mass spectra were acquired by accumulating 200 laser shots at 54% laser power in the m/z range of 2000–25,000 for each sample.

Peak lists were obtained using FlexAnalysis (Bruker Daltonics) software after the mass spectra were baseline subtracted, Gaussian smoothed and peak centered. The peak lists (m/z values and corresponding peak intensities) were exported as text files to MS Manager (Advanced Chemistry Development Laboratories, Toronto, ON) for comparison.

2.5. LC/MS analysis

A Waters Acquity HPLC system (Milford, MA) equipped with a Prosphere P-HR 2.1 mm × 150 mm, 4 μm particle size, non-porous column (Alltech, Columbia, MD) was used to separate proteins from the whole cell bacterial extract. A 20 μL aliquot was injected onto the column, and separation was carried out at 50 °C with a flow rate of 0.225 mL/min using gradient elution where A = H_2O (1% formic acid) and B = 2-propanol (1% formic acid) (5–55% B in 60 min.). LC/MS analyses were performed using a QTOF Premier (Waters, Milford, MA) equipped with an electrospray ionization source. The instrument was operated in positive ion mode, performing full-scan analysis over the m/z range 620–2450 at 2 spectra/s. Capillary voltage and cone voltage were maintained at 3.9 kV and 40 V, respectively. The source temperature was set at 115 °C and nitrogen was used as the desolvation gas (900 L/h) at 500 °C.

Automated analysis of LC/MS data was performed using ProTrawler6 software (BioAnalyte, Portland, ME), which allowed the export of deconvoluted mass, intensity, and retention time information as a text file, prior to mass spectral comparison using MS Manager.

2.6. Data analysis

Mass spectra were compared at various taxonomic levels (i.e., genus, species, serotype and strain) using spectral mirroring, a function that displays two inverse mass spectra on the same abscissa. Text files containing protein mass and intensity were imported into MS Manager and reconstructed into mass spectra. For further evaluation and biomarker identification, all common masses were removed (i.e., spectral subtraction) with a window of ±5 Da and

Table 1
Isolates investigated in this study.

Family	Enterobacteriaceae					
Genus	*Escherichia*				*Shigella*	
Species	*E. coli*				*S. flexneri*	*S. sonnei*
Serotype	Non-pathogenic (Untyped)	O111:NM	O26:H11	O157:H7	(Untyped)	(Untyped)
Strain (Accession #)	06-0004 06-0006	06-1440	06-1418	06-1439 06-1464	04-0497 06-0967	06-1362 06-1364

± 2 Da for MALDI-TOF/MS and LC/MS, respectively. Proteins unique by mass, retention time and/or relative intensity and present in five replicate studies were deemed biomarker candidates since a number of peaks are not reproducible which are likely due to the heterogenic nature of the sample (MALDI-TOF/MS) and artifacts from the deconvolution process (LC/MS). To this end, uniqueness in LC/MS was defined as a protein being absent/present (qualitative markers) higher/lower abundant (quantitative markers) in all five biological replicates within the mass accuracy tolerance of +/−2 Da and a retention time window of +/−0.5 min. For MALDI-TOF/MS proteins present in five replicates within the mass accuracy of +/−5 Da were deemed biomarker candidates.

3. Results and discussion

3.1. Utilizing theoretical mass spectra for biomarker discovery

Mass spectral fingerprints were obtained for each isolate listed in Table 1 using both MALDI-TOF/MS and LC/MS methods of analysis. Mass spectra from each approach were evaluated in parallel for the presence of protein biomarkers. MS manager software only allows two mass spectra to be evaluated simultaneously; therefore, theoretical mass spectra were generated for ease of comparison (Fig. 1). As seen in the figure, the mass spectrum for *E. coli* O111:NM isolate 06-1440 (Fig. 1, top) was compared to the three shiga-toxin producing *E. coli* O157:H7 (STEC) isolates 06-1439, 06-1418 and 06-1464 by creating a composite mass spectrum of all observed masses from the three isolates (bottom). Combining mass spectra in this manner allows inclusion of low abundant proteins that may prove useful for the identification of unique proteins.

3.2. Species/genus specific biomarkers

To decipher the discriminative power of MS analysis, comparisons of *S. sonnei*, *S. flexneri* and *E. coli* mass spectra were made to uncover genus/species-specific biomarkers. Fig. 2 depicts a spectral comparison of *S. sonnei* (top) versus a theoretical mass spectrum of *S. flexneri* and *E. coli* (bottom) after spectral mirroring and subtraction relative to *S. sonnei*. As can be seen in the figure the mass spectra are compared along the *x*-axis (mass), i.e., mirrored. Subsequently, all common masses are removed, i.e., subtracted leaving the unique proteins. Peaks present in the top mass spectra of Fig. 2 were those unique to *S. sonnei* and represented potential biomarkers of identification. In multiple *E. coli* and *S. flexneri* comparison studies, data derived from both MALDI-TOF/MS and LC/MS analysis revealed species-specific biomarkers that were exclusive and only present in the mass spectra of *S. sonnei*, which were designated by the asterisks in Fig. 2. Biomarkers at masses of 3830 Da and 11,846 Da were discovered with MALDI-TOF/MS analysis (Fig. 2a) while LC/MS derived biomarkers were observed at masses of 11,795 Da and 12,235 Da (Fig. 2b). In some cases overlap between the proteins observed with each technique were detected; however, these proteins were not unique with respect to the mass spectrometric method employed. The resulting data from both approaches is summarized in Table 2.

3.3. Serotype-specific biomarkers

At the serotype level, the mass spectral data revealed biomarkers capable of identifying and differentiating between multiple serotypes. LC/MS comparison of *E. coli* serotypes O157:H7 versus O26:H11 and O111:NM, revealed a reproducible protein at 18,996 Da, a marker previously reported and sequenced by Williams et al. [20] (Fig. 3a). Although no additional biomarkers were discovered for individual serotype identification, MALDI-TOF/MS uncovered reproducible proteins at 15,438 Da and 15,645 Da (Fig. 3b), which allowed *E. coli* serotypes O26:H11 and O111:NM (top) to be distinguished as non-O157:H7. Subsequent serotype-specific biomarkers discovered are reported in Table 2.

3.4. Strain-specific biomarkers

Identification at the strain level using both MS approaches was evaluated by further inspection of isolate mass spectra. By comparing each isolate identified in Table 1 to the remaining nine, strain-specific biomarkers were discovered for all isolates with the exception of *S. sonnei* isolate 06-1364. Fig. 5 shows a spectral comparison of *E. coli* O157:H7 isolate 06-1464 (top) versus all other isolates (bottom) after spectral subtraction relative to *E. coli* O157:H7 isolate 06-1464. Strain-specific biomarkers discovered with MALDI-TOF/MS analysis (Fig. 4a) at 9554 Da, 12,435 Da and 14,890 Da. The LC/MS (Fig. 4b) derived biomarker has a mass value of 14,880 Da. Closer inspection of the data revealed a biomarker at 14,885 Da for *E. coli* O157:H7 isolate 06-1464 present in mass spectra derived from both approaches. Here again, it should be noted, that while overlap does occur between the two techniques, it is not universal. Strain-specific biomarkers of all isolates in this study are reported in Table 3.

3.5. Quantitative biomarkers

The homogeneity of the LC/MS samples allowed differences in protein abundance to be used as quantitative biomarkers for pathogen identification. When normalized to the base peak, comparison of non-O157:H7 shiga toxin producing *E. coli* (STEC) isolates 06-1418 and 06-1440 to the remaining eight isolates showed significant differences in the relative intensity of proteins at 15,406 ± 2 Da and 15,425 ± 2 Da (Fig. 5). As seen in the inset, the intensity of the protein at 15,425 ± 2 Da is significantly greater than the protein at 15,406 ± 2 Da for samples 06-1418 and 06-1440 (top). In five replicate studies the mean value of the relative peak intensity for the proteins at mass 15,406 and 15,425 were 5.70% (SD = 3.34) and 98.35% (SD = 2.94), respectively. However, this trend was reversed for the other eight isolates (bottom). Here, the mean value of relative peak intensity for proteins 15,406 and 15,425 was 93.31% (SD = 11.25) and 6.30% (SD = 3.17), respectively. Since this quantitative difference involved one of the most abundant proteins from the lysates, the change in protein expression was immediately obvious and required no spectral subtraction of common proteins.

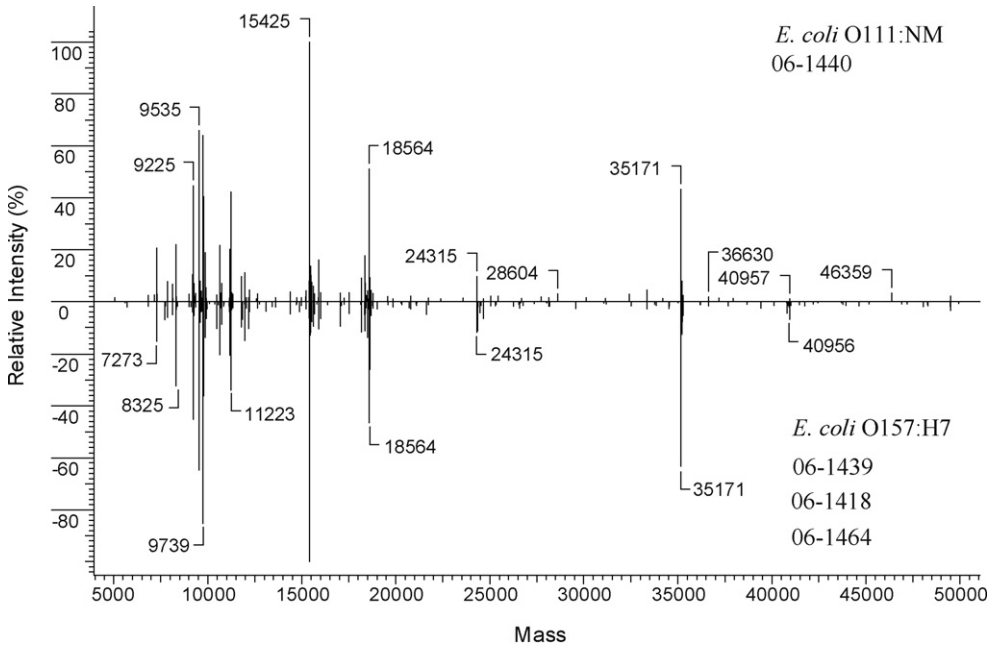

Fig. 1. Comparison of multiple isolates utilizing theoretical mass spectra. A mass spectrum for *E. coli* 06-1440 is compared to a theoretical mass spectrum of shiga toxin producing *E. coli* (STEC) isolates 06-1439, 06-1418 and 06-1464.

Table 2
Species- and serotype-specific biomarker candidates discovered by mass spectrometric analysis.

Species/serotype	*S. flexneri*	*S. sonnei*	*E. coli* O111:NM *E. coli* O26:H11	*E. coli* O157:H7
Unique proteins	7287 (L) 16,886 (L) 35,250 (L)	3830 (M) 11,850 (M) 11,795 (L) 12,235 (L)	15,437 (M) 15,641 (M) 15,478 (L) 24,315 (L)	18,996 (L)

(M) MALDI-TOF/MS derived biomarkers ±5 Da.
(L) LC/MS derived biomarker ±2 Da.

3.6. Unknown bacterial sample analysis

A blind study was performed to test the validity of the biomarkers discovered. Thirteen unknowns, isolates previously classified by molecular methods to the same taxonomic classification as the initial 10, were analyzed by both MS methods. Mass spectra were evaluated for presence of species and serotype-specific biomarkers, and LC/MS data were further evaluated for quantitative markers. Using method specific markers, LC/MS accurately identified and characterized all 13 isolates at the species level and *E. coli* at the serotype levels. MALDI-TOF/MS accurately identified eleven of the 13 isolates at the species level. Upon further review of the MALDI-TOF/MS mass spectra using LC/MS markers, all *E. coli* isolates were accurately characterized to their serotype levels.

Distinct protein profile patterns and biomarkers found here are restricted to identifying unknowns within the same classification as the *E. coli* and *Shigella* spp. analyzed. Since these MS methods required no further sample processing other than cell lysis, protein profiles from a multitude of pathogens could be complied into one comprehensive fingerprint library and utilized for pathogen identification. Protein profiles that served as the source for characteristic fingerprint pattern and biomarker discovery were generated five times on different days from pure isolates. Replicative analysis of pure pathogenic isolates was important to ensure reproducibility and accurate translation of pathogen-specific characteristics into protein profiles. After potential biomarkers are validated, they can be used as identifiers in a one-time analysis of unknowns in a pure or mixed culture.

Pathogen identification by PFGE analysis is based on the pattern of chromosomal bands regardless of their sequence. Similarly, these MS methods of unknown identification are based on the presence of established biomarkers despite their identity. Therefore, any further analysis on the biomarker itself (e.g., proteolytic digestion, MS/MS analysis, etc.), while important for other applications of this work, is not required for pathogen identification. Unlike PFGE and most conventional methods, species identification is not a prerequisite for MS analysis and subsequent identification. As previously mentioned, this protocol for sample processing

Table 3
Strain-specific biomarkers discovered by mass spectrometric analysis.

Species/serotype	*E. coli* O157:H7	*E. coli* O157:H7	*E. coli* O111:NM	*E. coli* O26:H11	*S. flexneri*	*S. flexneri*	*S. sonnei*
Accession #	06-1464	06-1439	06-1440	06-1418	04-0497	06-0967	06-1362
Unique proteins	9554 (M) 12,433 (M) 14,890 (M) 14,880 (L)	10,747 (M)	10,126 (M)	3701 (M)	9445 (M) 10,119 (L) 18,860 (L)	10,089 (L) 18,874 (L) 18,151 (L)	15,200 (M)

(M) MALDI-TOF/MS derived biomarkers ±5 Da.
(L) LC/MS derived biomarker ±2 Da.

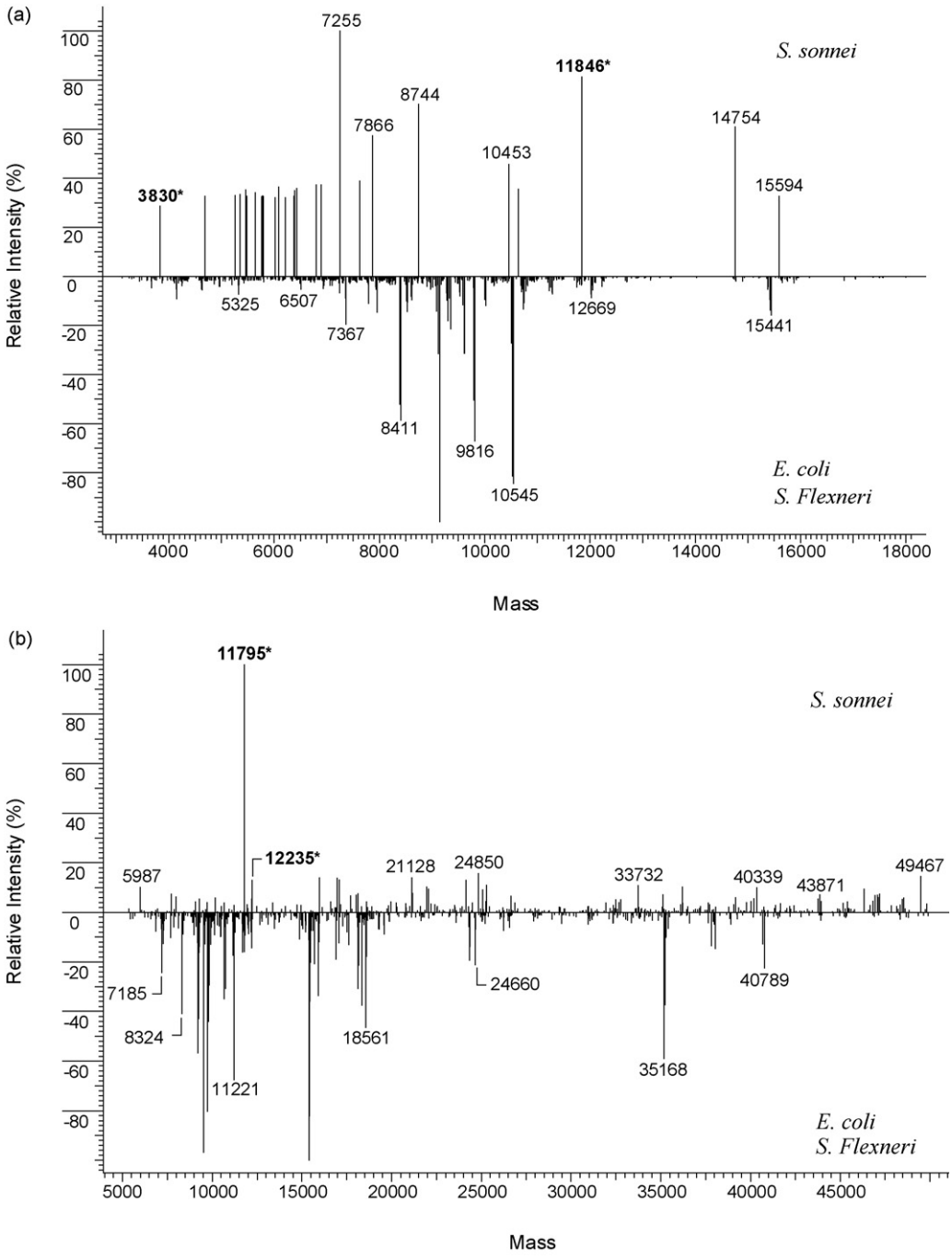

Fig. 2. MALDI-TOF/MS (a) and LC/MS (b) comparisons of mass spectra *S. sonnei* (top) and combined *S. flexneri* and *E. coli* (bottom). Masses of commonality within a window of ±5 Da for MALDI-TOF/MS and ±2 Da for LC/MS were subtracted relative to *S. sonnei*. Remaining masses unique to *S. sonnei* are displayed (top) and are shown mirrored to all *S. flexneri* and *E. coli* masses (bottom). Unique masses present in three replicate studies were deemed a *S. sonnei* biomarker and denoted with *.

is void of any species-specific reagents. This advantage allows for the circumvention of time-consuming species identification steps, such as differential plating and biochemical testing. In addition, pathogens with unsequenced genomes or for which primers, antibodies, restriction enzymes, etc., are not available can be analyzed and identified.

3.7. Comparison of the techniques

MALDI-TOF/MS offered a rapid approach to pathogen identification, and has the potential to expedite the progression of further subtyping by molecular methods in which prior knowledge of the pathogen's species is required. While the separation step in LC/MS increases turn-around time for sample analysis, it is important to note that the presented method is just as fast and in some cases faster than the aforementioned conventional methods of pathogen identification. In addition, current work has utilized ultra performance liquid chromatography to reduce the analytical time for this type of analysis [21]. Only LC/MS derived mass spectra revealed the genus/species-specific biomarkers reported in Table 2 for *S. flexneri*. Another advantage was that electrospray ionization (ESI) allowed detection of proteins in higher mass ranges than MALDI-TOF.

The ability of MS analysis to generate reproducible spectra is crucial for biomarker detection. MALDI-TOF/MS was shown to be

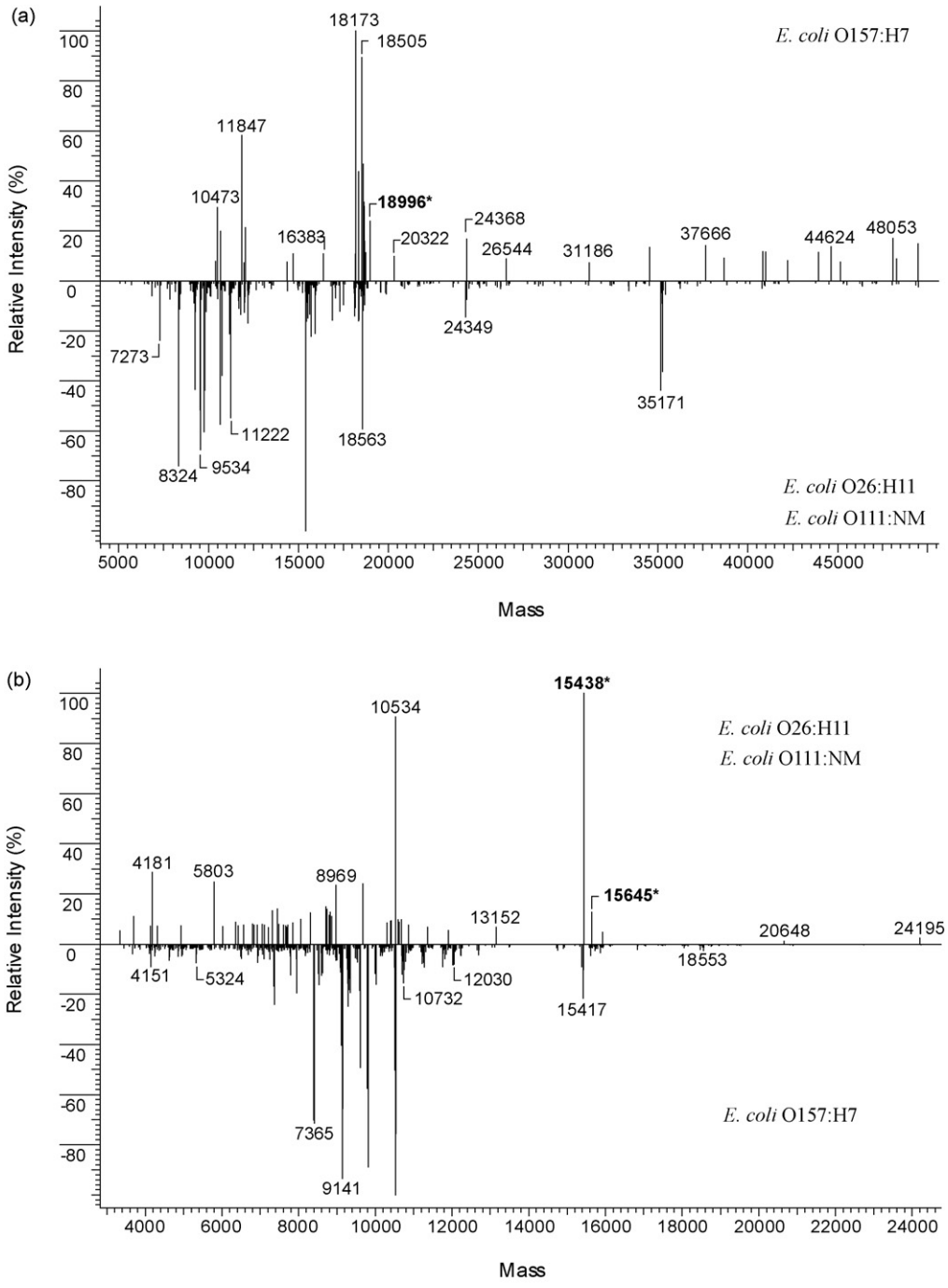

Fig. 3. LC/MS (a) and MALDI-TOF/MS (b) mass spectral comparison of *E. coli* serotypes. (a) Common masses (±2 Da) for LC/MS were subtracted relative to *E. coli* O157:H7 and the remaining masses unique to *E. coli* O157:H7 (top) are displayed mirrored with all combined masses of *E. coli* O26:H11 and *E. coli* O111:NM (bottom). (b) Common masses (±5 Da) for MALDI-TOF/MS were subtracted relative to *E. coli* O26:H11 and *E. coli* O111:NM. The remaining unique masses for both *E. coli* serotypes (top) and mirrored with all combined masses of *E. coli* O157:H7 (bottom). Unique masses present in five replicate studies were deemed biomarkers and denoted with *.

reproducible for biomarker discovery with a mass window of ±5 Da. LC/MS demonstrated the greater capability to deliver reproducible spectra, a smaller window (±2 Da), and more biomarkers reported including a biomarker previously reported by Williams et al. [20]. These features illustrate data collection to be instrument and laboratory independent. The intra- and inter-laboratory reproducibility of the MS approach further demonstrates the potential for the generation of a dynamic database compiled with this and data similar to the current CDC PulseNet program [24]. The employment of such a database would allow for real-time communication among local, state and national health departments as well as international partners, such as the World Health Organization. Additionally it would facilitate the detection of foodborne disease case clusters and identification of common source outbreaks.

Secondary applications of LC/MS allow the added advantage of detecting uniqueness in a pathogen's profile otherwise undetectable by either mass discrimination or DNA (i.e., PFGE and/or PCR) alone. The more amenable approach to quantitative analysis, LC/MS has demonstrated the potential to uncover discriminative differences in expression intensities of similar mass proteins. Such

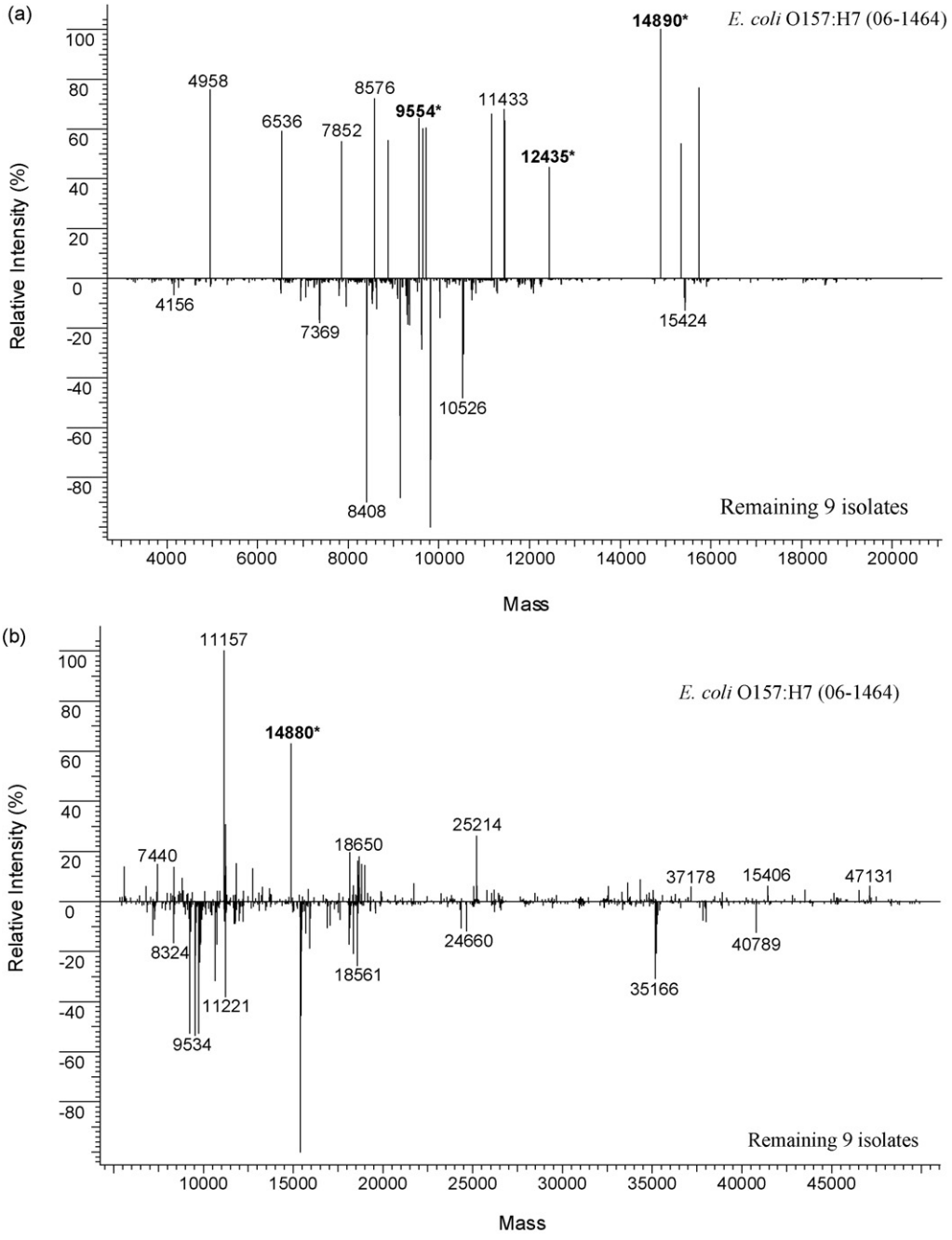

Fig. 4. MALDI-TOF/MS (a) and LC/MS (b) comparisons of mass spectra from *E. coli* O157:H7 (06-1464) (top) and the other 9 isolates (bottom) where common masses relative to *E. coli* O157:H7 (06-1464) were removed. Unique masses present in five replicate studies were deemed *E. coli* O157:H7 (06-1464) biomarkers and denoted by *.

information could prove useful in identifying pathogens that elicit a disease state not by their expression of unique proteins but by the uniqueness in which their proteins are expressed. Mass and retention time as identifying characteristics versus protein sequence is directly applicable to bacteria whose genomes either have not been sequenced or because the bacteria evolved phenotypic traits (e.g. drug or heat resistance) that were manifested through a change in genetic code of certain genes. For example, if drug resistance was manifested through a switch in a Serine for a Lysine in a protein and this change had not been noted in the database used to search bottom-up proteomic data, then this peptide, which bears the important mark of resistance, would not be identified. This approach is ideal in a case where the discriminating power of a traditional technique such as PFGE or PCR is insufficient for identification. The LC aspect of this approach can be utilized for pathogen distinction based on unique protein elution time in its hydrophobicity profile (i.e., chromatogram); thus, providing an additional factor that can be utilized for pathogen identification. Information from both quantitative and LC analysis can be used for confirmation of qualitative results and differentiation of strains with identical protein masses. Since elution time is known, any protein of interest, whether unique by mass, expression intensity, hydrophobicity, etc., can be concentrated and purified through fraction collection and further evaluated. Thus, not only can this MS approach be utilized as a method of pathogen identification, but also as a screening tool providing new targets for identification.

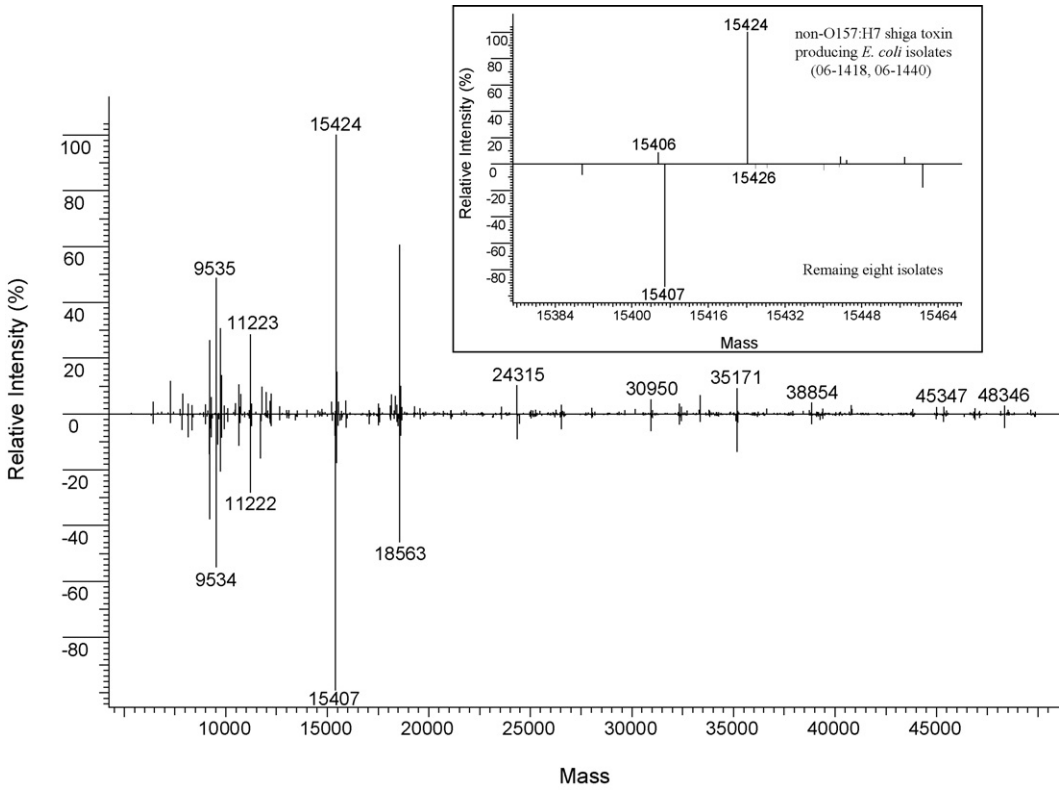

Fig. 5. LC/MS mass spectral comparison of non-O157:H7 shiga toxin producing isolates 06-1418 & 06-1440 (top) versus the remaining eight isolates (bottom). The mean value ($n = 5$) of relative peak intensity for proteins 15406 and 15425 were 5.70%, SD = 3.34, and 98.35%, SD = 2.94 (inset). In the remaining eight isolates this trend is reversed. Mean values of relative peak intensity for proteins 15,406 and 15,425 were 93.31%, SD = 11.25, and 6.30%, SD = 3.17.

For example, biomarkers uncovered from MS analysis could be used to direct the design of novel PCR primers [19,20]. In addition, the ability of LC/MS to detect hydrophobic differences for pathogen distinction and identification can be assessed through the comparison of isolate chromatograms. For example both *E. coli* O157:H7 isolates 06-1464 and 06-1439 and *S. flexneri* isolate 06-0967 expressed a single protein at 18,120 Da, while *S. flexneri* isolate 04-0497 expressed two proteins at this same mass. The extracted ion chromatograms of *S. flexneri* isolate 04-0497 showed a 15 min retention time difference between the two same mass proteins (data not presented). The first protein eluted at 21.1 min and is unique to *S. flexneri* isolate 04-0497. The second protein that eluted at approximately 37 min was common to both isolates of *E. coli* O157:H7 and *S. flexneri*. It should be noted here that this difference would not be observed by a mass spectrometric-only approach like MALDI-TOF/MS.

4. Conclusions

Using a proteomic-like approach, MALDI-TOF/MS and LC/MS were evaluated for their applicability as methods for pathogen identification and characterization. Both MS methods showed great capability in generating reproducible mass spectra in which discriminating factors such as protein biomarkers and spectral patterns were discovered. Favorable results from a blind study served to authenticate discriminating factors and validated both approaches, either singularly or combined, as powerful new tools for pathogen study and identification. When used in the manner outlined above, MALDI-TOF/MS and LC/MS provide the opportunity to gain a more comprehensive view of a pathogen's proteome, attain a better understanding of pathogen diversity and relatedness, discover new biomarkers, and elucidate the role of suspect proteins in disease.

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