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MALDI-TOF MS Application Note

From Sample to Species

Automated MALDI-TOF MS Workflows for Streamlined Bacterial Identification

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Abstract

This application note demonstrates the successful automation of two sample preparation workflows, Formic Acid (FA) and Beads method, for bacterial identification using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Both methods were successfully automated and identified various bacteria at the genus level, including those relevant to clinical diagnostics (e.g., *Escherichia coli, Staphylococcus argenteus*), food safety (e.g., *Listeria* species), and environmental monitoring (e.g., *Acinetobacter radioresistens, Acinetobacter venetianus*). Notably, the FA method, employing chemical lysis, proved more effective for species-level identification, particularly for Listeria species. The Beads method identified fewer Listeria species than the FA method but may be more universal due to the mechanical disruption as the mechanism behind the extraction. Following the sample preparation, the resulting samples were spotted onto the MALDI target plate (also automated) and analyzed using MALDI-TOF MS, generating unique spectral fingerprints for each bacterial sample. These spectra are then compared to an extensive online database, MabritecCentral, to facilitate accurate bacterial identification at the species level. Both automated sample preparation approaches significantly reduce processing time, minimize human error, and improve the consistency and reliability of results, offering a potential solution to streamline bacterial identification in clinical diagnostics, food safety, and environmental monitoring.

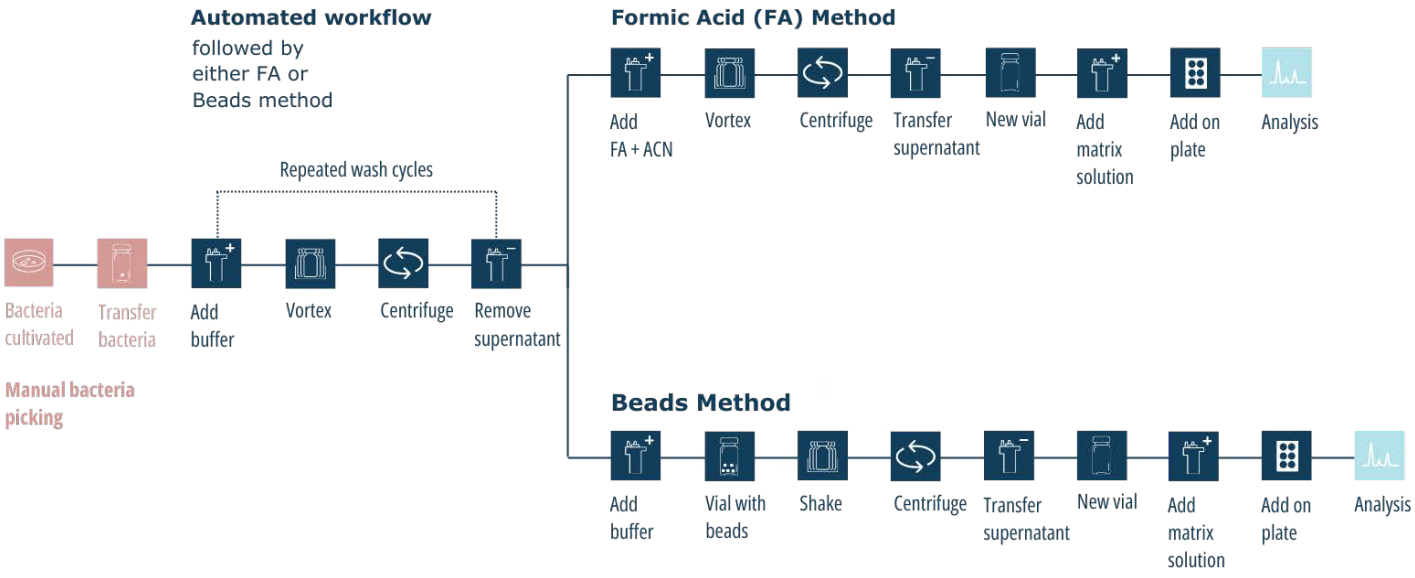


Figure 1. Workflow overview: The initial sample preparation is followed by either the Formic Acid (FA) method or the Beads methods. Dark blue indicates automated aspects, light blue the analysis and, light red manual aspects.

Introduction

The ability to quickly and accurately identify bacteria is essential in numerous fields, from diagnosing patient infections to ensuring food safety and monitoring environmental health. While traditional methods for identifying bacteria exist, they often lack specificity and require significant time and manual labor.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a powerful technique for bacterial identification in clinical and food safety microbiology laboratories (Elbehiry et al., 2022; Kassim et al., 2017; Thouvenot et al., 2018).

This technology analyzes bacteria's unique protein "fingerprint", i.e., ribosomal subunits, generating spectral profiles that are compared to extensive databases for rapid species-level identification.

However, preparing samples for MALDI-TOF MS bacterial identification can be a bottleneck. Manual sample processing is not only time-consuming but also introduces variability and the risk of human error, impacting the accuracy and reproducibility of results. Additionally, the sample preparation of choice also influences the quality of the results (Cuénod et al., 2023). This application note investigates automating bacterial sample preparation for MALDI-TOF MS using the PAL System robotic platform, aiming to streamline workflows and boost reliability (Figure 1 and 2).

MALDI-TOF MS - Some of its key advantages

Speed and Cost-Effectiveness

It provides significantly faster results than conventional methods, enabling timely decision-making. Additionally, its cost-effectiveness makes it a sustainable choice for routine diagnostics.

Broad Applicability

MALDI-TOF MS can identify a wide range of bacteria, including fastidious organisms and those challenging to identify by traditional methods, such as Gram-negative rods and anaerobes.

Accuracy and Resolution

Ongoing advancements in sample preparation protocols and database refinement have further enhanced the accuracy of MALDI-TOF MS, allowing for the differentiation of closely related species.

Figure 2. Automating the path to bacterial identification: The PAL System handles sample preparation, from mixing (3) and centrifugation (6) to liquid handling (4) and washing (5). Other modules: (1) Park Station, (2) Tray Holder, (7) Thermal Mixer (no heat).

Two distinct bacterial sample preparation methods were automated and evaluated: the Formic Acid (FA) method and the Beads method (Figure 1).

The FA method, needing less sample material, involves washing, formic acid-mediated cell lysis, and mixing with a sinapinic acid/acetonitrile/water/TFA matrix solution (Cuénod et al., 2021; Šedo et al., 2011).

The Beads method, suitable for a broader range of bacteria, follows a similar wash step but employs mechanical lysis using glass beads (Šedo et al., 2011). While the Beads method offers broader applicability due to its mechanical lysis approach, it entails additional steps compared to the chemically-driven FA method. Similar to the FA methods, the resulting samples are mixed with the sinapinic acid/acetonitrile/water/TFA matrix solution.

Following the automated preparation, samples were directly spotted onto the MALDI target plate by the PAL System and analyzed via MALDI-TOF MS. The PAL System and its modular and flexible design allow for customization to specific applications. Here, a 3D-printed MALDI-TOF plate holder was integrated for direct, online spotting onto the MALDI target plate (Figure 2).

The resulting spectral fingerprints were then compared against the MabritecCentral database for species-level bacterial identification. This automated workflow promises significant improvements in efficiency and data quality for bacterial identification.

In this application note, we will:

1. Compare the two automated methods (FA and Beads) by analyzing their limitations and benefits.

2. Assess the performance of these methods in identifying different types of bacteria, including closely related species.

3. Explore how spectral data is matched against the MabritecCentral database for bacterial identification.

4. Conclude the broader implications of this automated approach for improving bacterial identification in various applications and future directions.

Results and Discussion

This application note evaluated the performance of two automated sample preparation methods, the Formic Acid (FA) method and the Beads method, for the identification of various bacterial species using MALDI-TOF MS. The resulting spectra were then uploaded to the MabritecCentral database for species-level identification based on ribosomal protein markers. The FA method, which uses a minimal amount of bacterial material, relies on chemical lysis with formic acid to release proteins. The Beads method, in contrast, employs physical disruption using glass beads to break open bacterial cells.

To compare the two workflows, we analyzed a diverse range of bacterial species, including *Escherichia coli, Bacillus thuringiensis, Staphylococcus argenteus, Acinetobacter radioresistens, Acinetobacter venetianus, and various Listeria species (L. innocua, L. faberi, L. cossartiae subsp. Cayugensis and cossartiae, L. immobilis, and L. portnoyi)*.

The primary goal was to assess the effectiveness of these automated workflows in accurately identifying bacteria, with a particular focus on differentiating between closely related species.

Evaluation of Automated Workflows

To assess the performance of the automated FA and Beads methods, we first compared their effectiveness in identifying a diverse set of bacterial species (Table 1).

This analysis focused on both genus- and species-level identification, highlighting the strengths and limitations of each approach. Following this general comparison, we focused on the detailed analysis of closely related Listeria species, further demonstrating the capabilities of the automated workflows in resolving taxonomic ambiguities.

Table 1 summarizes the results obtained for the various bacterial species as well as strains within a species using both the FA and Beads methods on the PAL System.

Both methods successfully identified various bacteria at the genus level, including those relevant to clinical diagnostics (e.g., *Escherichia coli, Staphylococcus argenteus*), food safety (e.g., Listeria species), and environmental monitoring (e.g., *Acinetobacter radioresistens, Acinetobacter venetianus*).

Table 1. Comparison of Formic Acid (FA) and Beads methods for bacterial identification.
The table summarizes the performance of FA and Bead method for identifying various bacteria at the genus and species level. Checkmarks indicate successful identification. The repeated names indicate strains within a species.

	Formic acid (FA)		Beads	
	Genus	Species	Genus	Species
E.coli	✓	✓	✓	✓
B.thuringiensis	✓	✓	✓	✓
L.innocua	✓	✓	✓	✓
S.argenteus	✓	✓	✓	✓
A.radioresistens	✓	✓	✓	✓
A.venetianus	✓	✓	✓	✓
L.faberi	✓	✗	✓	✗
L.cossartiae subsp. cayugensis	✓	✓	✓	✓
L.immobilis	✓	✓	✓	✗
L.portnoyi	✓	✓	✓	✓
L.portnoyi	✓	✓	✓	✗
L.cossartiae subsp. cossartiae	✓	✓	✓	✗
L.cossartiae subsp. cossartiae	✓	✓	✓	✓
L.cossartiae subsp. cossartiae	✓	✓	✓	✓
L.faberi	✓	✓	✓	✓
L.faberi	✓	✗	✓	✗

FA Method Identifies more Species

However, differences in performance became apparent when considering species-level identification. The FA method demonstrated superior performance for several species, including *L. cossartiae subsp. cossartiae , L. immobilis and L. portnoyi*. Notably, neither method could reliably differentiate certain strains of L. faberi at the species level.

The FA method appears to be generally more versatile for species-level identification, particularly for the Listeria species examined in this application note (Table 1).

The Beads method, while less broadly applicable for species-level differentiation, as it identified fewer species compared to the FA method, may be better suited for bacteria with tougher cell walls, where physical disruption is necessary for efficient protein extraction. It also may be considered more universal as it does not rely on chemical lysis that may introduce additional unspecific signals downstream (Figure 3).

Ribosomal Subunits - Decoding Bacterial Identity

Ribosome Structure

Ribosomes consist of two subunits, a large subunit (LSU) and a small subunit (SSU), each composed of ribosomal RNA (rRNA) and ribosomal proteins.

Evolutionary Clues

The composition and sequence of ribosomal proteins are highly conserved across bacterial species, providing valuable information for phylogenetic analysis.

MALDI-TOF MS Target

MALDI-TOF MS-based bacterial identification relies on the unique mass spectrum of ribosomal proteins, which serves as a fingerprint for each species.

Detailed Analysis of Closely Related Listeria Species

To further illustrate the effectiveness of the automated workflows in identifying closely related bacterial species, a comparison between *Listeria innocua and Listeria faberi* was performed. These two species are known to be difficult to distinguish using traditional methods due to their highly similar ribosomal protein profiles.

Ribosomal proteins are essential components of the cellular machinery responsible for protein synthesis. Their specific amino acid sequences can vary slightly between different bacterial species, making them useful markers for identification. Here, the analysis focused on identifying differences in the mass-to-charge ratios (*m/z*) of specific ribosomal proteins across L. faberi and L. innocua strains.

As shown in Figure 4, several ribosomal proteins exhibit mass differences between *L. faberi* (light blue) and *L. innocua* (dark blue). For instance, ribosomal proteins L25, L6, and L10 show distinct mass shifts that can be used to differentiate between these species. However, many of these distinguishing proteins produce signals in the higher mass range, above m/z 12000.

Figure 3. Comparison of the FA and Beads method. Depicted are the key advantages and disadvantages of the two methods, highlighting their impact on sample processing and analysis. In lighter colors are the benefits of each method, in darker colors the negative aspects.

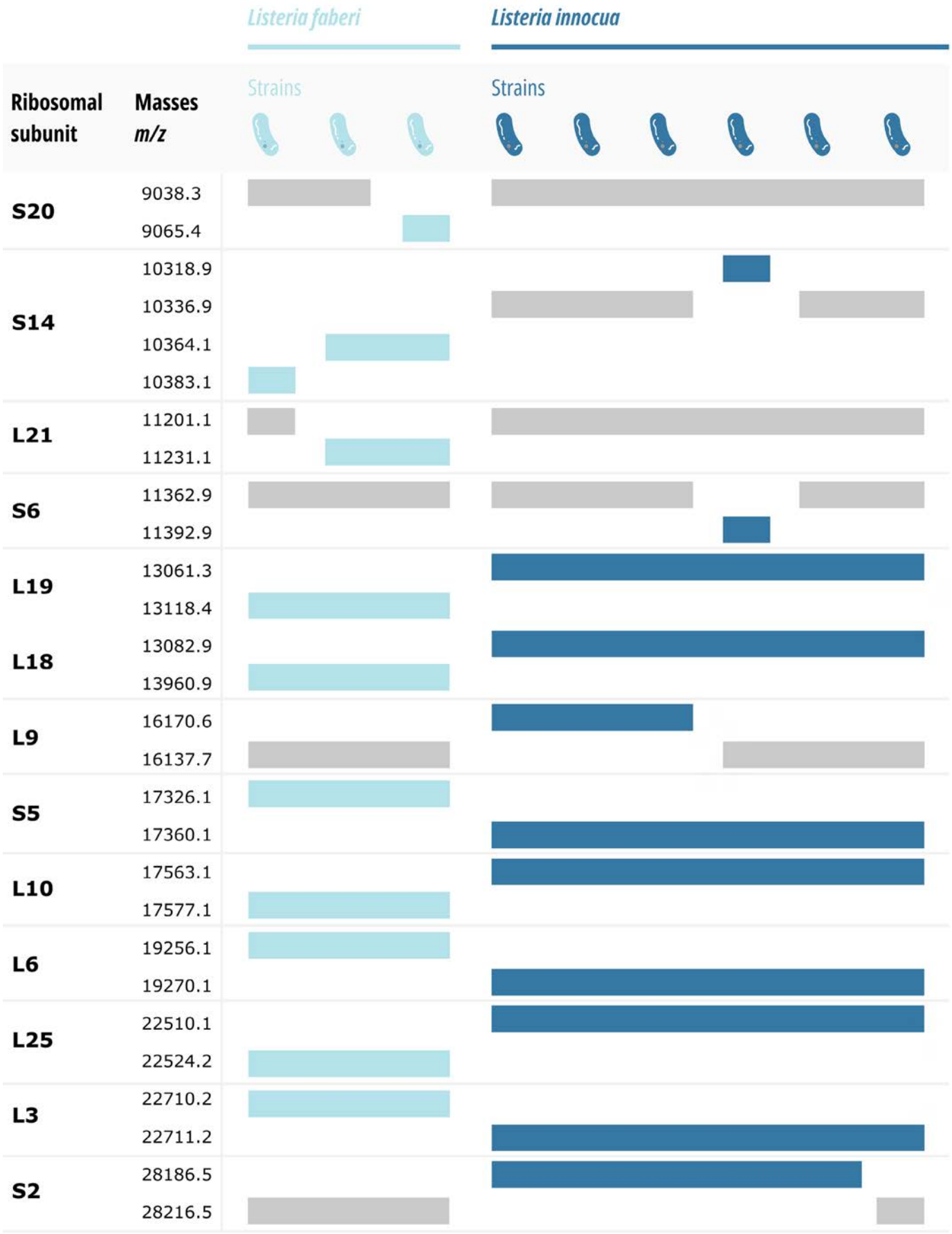


Figure 4. Ribosomal protein mass differences between *Listeria faberi and Listeria innocua* strains. This figure shows variations in ribosomal protein masses (*m/z*) between *L. faberi* and *L. innocua* strains, highlighting potential markers for species differentiation.

Choosing the Right Peaks

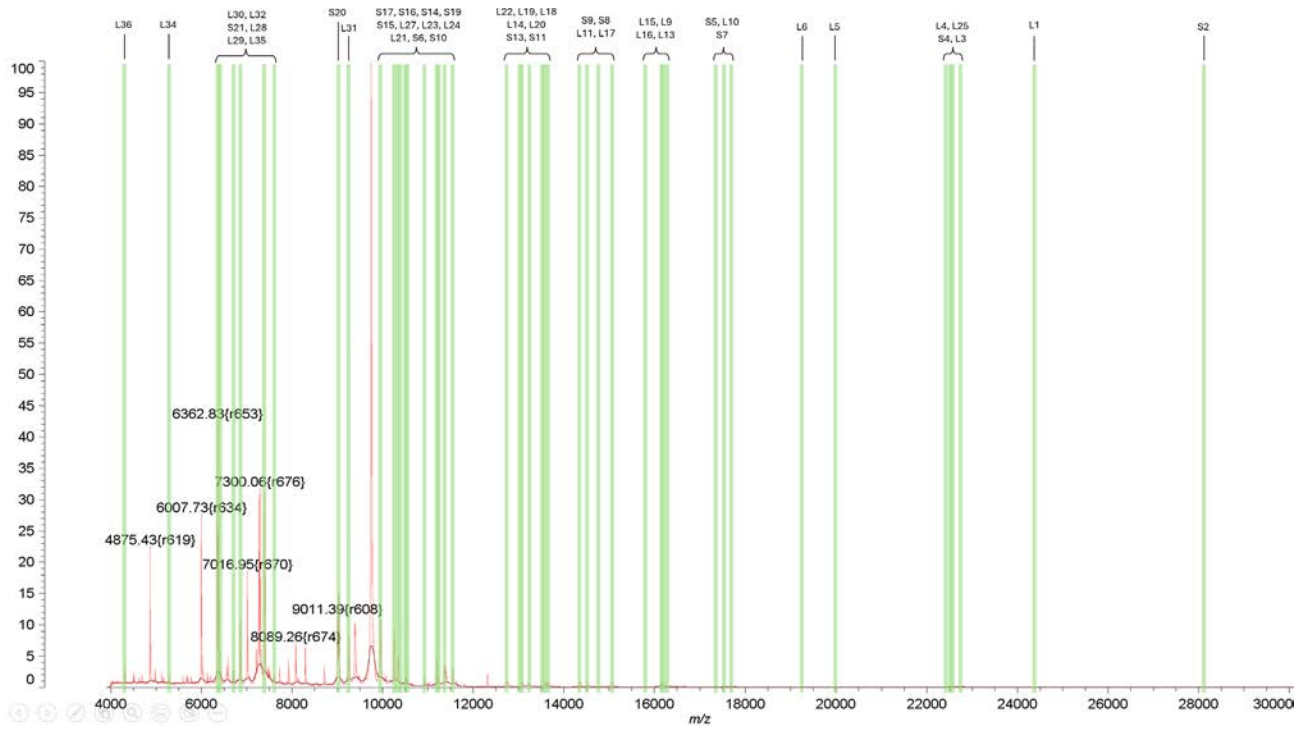
One significant challenge in analyzing Listeria species using MALDI-TOF MS is the presence of prominent, non-specific metabolic peaks, e.g., Figure 5A at approximately *m/z* 9754.8.

As illustrated in Figure 5A, this peak can be so intense that it overshadows the smaller, yet crucial, peaks from ribosomal proteins needed for accurate species identification.

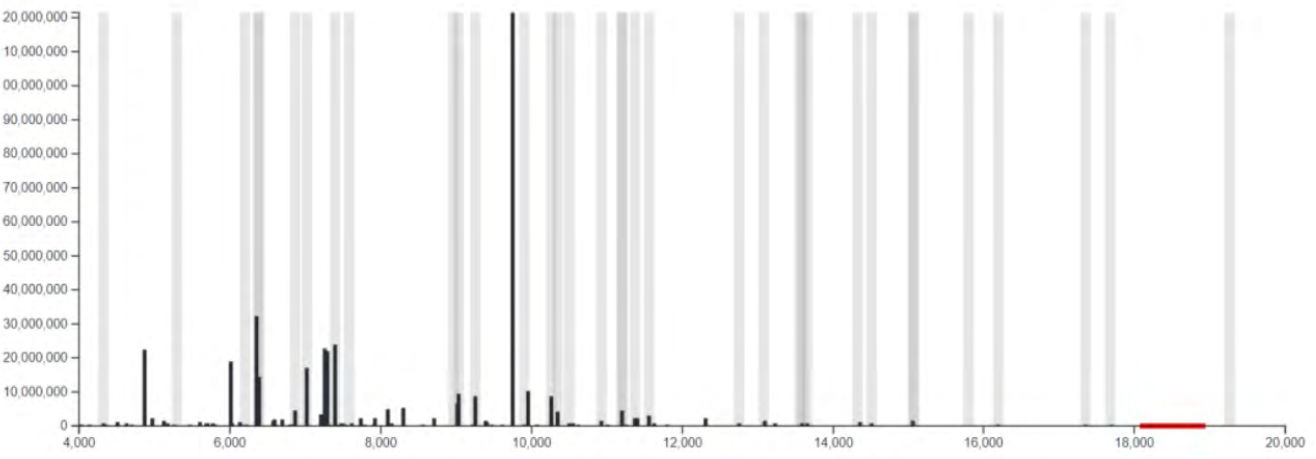
This is particularly problematic when those distinguishing proteins are in the lower mass range, where the *m/z* 9754.8 peak dominates the spectrum.

This further increases the challenge of differentiating these species, especially as the error range increases at higher masses. With a tolerance of 800 ppm for this MALDI-TOF MS workflow, subunits such as L3 (44 ppm) cannot be distinguished (Figure 4).

A



B



Filename	Sample Name	Datacount	Score	Qualified result	Score	Non-Qualified result
24_1245-2L1.txt	L.innocua Washed FA2	191	98.23%	Listeria innocua	---	---

Figure 5. MALDI-TOF Mass Spectrum of *Listeria innocua*. (A) Spectrum of *L. innocua* with key ribosomal protein and metabolic peaks labeled, including a major (unspecific) peak at m/z 9754.8. (B) Matched profile for *L. innocua* in the MabritecCentral Database. Note that the x and y axes of the two spectra are not identical.

Subunits L25 (626 ppm), L6 (727 ppm), and L10 (796 ppm) fall within this 800 ppm tolerance, ultimately leaving only nine units with differentiable masses, that are above *m/z* 9000.

Therefore, despite several ribosomal markers that are available in theory (Figure 4), only a few can be utilized due to technical limitations. These peaks, highlighted in Figure 5 and B, are used for the identification of *L. innocua*.

To accurately identify the bacteria despite these challenges, all spectra obtained were subsequently evaluated using the MabritecCentral database. This database employs a unique "marker mass" approach, focusing on specific ribosomal proteins as biomarkers for identification that are independent of the sample preparation method used. It offers a comprehensive and reliable platform for bacterial identification, especially in cases with closely related species or non-specific peaks.

A closer look at the identification – MabritecCentral Database

For bacterial identification, MALDI-TOF MS spectra (saved as ASCII files) were uploaded to the MabritecCentral database. This database leverages a combined genomics and MALDI-TOF approach: marker masses are predicted in silico from publicly available whole genome data, quality controlled and taxonomically classified, and then validated by MALDI-TOF MS. The database compares the uploaded spectrum to its library of theoretical reference spectra.

Figure 5B visualizes the results, with black peaks representing experimental data and grey bars indicating expected peak locations for *L. innocua* generating a score. MabritecCentral's "marker mass" approach, based on specific ribosomal protein biomarkers, addresses the challenges of non-specific peaks and high similarity between Listeria species. Matches that do not meet the statistical threshold are listed as non-qualified results at the genus or species level.

Addressing Variability in MALDI-TOF MS Measurements

While MALDI-TOF MS is a powerful tool for bacterial identification, the measurements can exhibit variability. This variability may stem from factors such as sample heterogeneity, the crystallization process of the matrix on the MALDI plate, and inconsistencies in the measurement process itself. To mitigate this variability, a bacterial sample was prepared, and the resulting matrix solution was applied 8-fold and analyzed. In some cases, a second bacterial sample was prepared and analyzed in the same way, ensuring robust and reliable results.

Conclusion

We demonstrated the successful automation of a MALDI-TOF MS sample preparation and online plate spotting workflow for bacterial identification using the PAL System. The platform's flexibility was showcased through the integration of a custom 3D-printed MALDI plate holder, to facilitate the automated plate spotting.

Two distinct sample preparation methods, the Formic Acid (FA) method and the Beads method, were automated and evaluated for their efficacy in identifying diverse bacterial species, including the challenging differentiation of closely related Listeria species. The FA method, requiring minimal sample and employing chemical lysis, generally proved superior for species-level identification, particularly for the tested Listeria strains. This aligns with the findings of Cuénod et al., (2021), who demonstrated that the use of formic acid in sample preparation improves mass spectral quality, leading to more accurate species identification. The Beads method, while exhibiting lower species-level resolution for the bacteria investigated and involving a more complex protocol, may offer advantages for bacteria with robust cell structures, where mechanical disruption is essential for efficient protein extraction.

This automated approach to MALDI-TOF MS sample preparation offers significant advantages over traditional manual methods. It reduces processing time and labor, minimizes human error, and enhances the consistency and specificity of results. By streamlining the workflow and improving accuracy, this automated platform has the potential to improve bacterial identification across diverse applications, from clinical diagnostics and food safety to environmental monitoring. Future research could explore further optimization of MALDI-TOF MS parameters, including laser settings and data analysis algorithms, to maximize the information obtained from each sample and potentially improve the speed of identification.

Materials and Methods

This section details the materials, instruments, and methods used in this application note, including bacterial cultivation, 3D printing of a custom MALDI plate holder, automated sample preparation workflows, and MALDI-TOF MS analysis.

List of Chemicals

- Acetonitrile (Supelco)
- Ammonium chloride (Sigma-Aldrich)
- Dichloromethane (Sigma-Aldrich)
- Ethanol (Merck)
- Formic acid (FA) (Sigma-Aldrich)
- Glass Mill Beads 0.1 to 0.15 mm (BioSpec)
- Magnesium chloride (Sigma-Aldrich)
- Natriumlaurylsulfat (Carl Roth)
- Perfom disinfection (Schülke)
- Sinapic acid (SA) (Sigma-Aldrich)
- Sudan III (Sigma-Aldrich)
- TRIS-HCI (Sigma-Aldrich)
- Trifluoroacetic acid (TFA) (Sigma-Aldrich)
- Triton X-100 (Sigma-Aldrich)

Materials

- 2mL Autosampler Vials (WICOM)
- Columbia Agar with Sheep Blood Plus (Thermo Scientific)
- Micro insert (WICOM)
- Plastic Loop (Greiner)
- Vμ-Vials (Infochroma)

SA-Matrix Solution

The SA-matrix solution was prepared by dissolving 40 ± 2 mg of sinapinic acid (SA) in a mixture of 600 µL acetonitrile (ACN), 400 µl H2O, and 3 µl trifluoroacetic acid (TFA). The solution was then sonicated for 3 minutes to ensure complete dissolution.

Buffer Solution

The buffer solution was prepared by dissolving 157 mg TRIS-HCI, 160 mg NH4Cl, and 94 mg MgCl2 in 100 ml H2O. The pH of the solution was adjusted to 7.8 using NH3.

Reference Standard

A fresh culture of E. coli was used as the reference standard. A small amount of bacteria was smeared onto positions G3 and G4 of the MALDI plate and overlaid with 1 µl of the matrix solution.

3D Printing of MALDI Plate Holder

A custom MALDI plate holder was designed using Autodesk Fusion software and printed using a Bambu Lab X1 Carbon Combo 3D printer. The holder was printed using polylactic acid (PLA) and featured a slightly overhanging edge to secure the MALDI plate during vibration. The resulting plateholder was integrated with the support of application specialists at CTC Analytics AG.

Bacterial Strains and Culture Conditions

The bacterial strains used in this study included Escherichia coli, Bacillus thuringiensis, Staphylococcus argenteus, Acinetobacter radioresistens, Acinetobacter venetianus, and various Listeria species (*L. innocua, L. faberi, L. cossartiae subsp. Cayugensis and cossartiae, L. immobilis, and L. portnoyi*). The bacteria were cultivated on either normal agar or blood agar plates at 37°C for 16 hours. Blood agar plates were prepared using Columbia Agar with Sheep Blood Plus (Thermo Scientific). The "four-quadrant" streaking method was used to inoculate the agar plates.

After plating, the bacteria were incubated overnight (approximately 16 hours) at 37°C and used only on the day of cultivation to ensure optimal results.

Automated Sample Preparation

Automated sample preparation was performed using a PAL RTC system equipped with a custom 3D-printed MALDI plate holder. Two different automated workflows were implemented: the Formic Acid (FA) method and the Beads method.

Formic Acid (FA) Method

The FA method uses a minimal amount of bacterial material and relies on chemical lysis with formic acid to release proteins for analysis. The automated FA method involves several steps: First, a small amount of bacteria (comparable to what can be picked up with a toothpick tip) is added to 100 µl of buffer solution in a vial with an inlet (manually). The bacteria are then washed in a wash cycle, which involves vortexing the vial at 2000 rpm and centrifuging it at 7200 rpm. The supernatant is removed, and the wash cycle is repeated if necessary. After the wash cycles, FA and ACN are added to the vial and mixed well using the syringe. The vial is then centrifuged again, and 5 µl of the supernatant is mixed with 20 µL of matrix solution in a new vial. This mixture is then spotted onto the MALDI metal plate, with 1 µl per spot. The syringe is washed with 70% ethanol throughout the procedure to prevent contamination.

Beads Method

The Beads method employs physical disruption using glass beads to lyse bacterial cells. In this method, one to two loops of bacteria are added to 1 ml of buffer solution in a Vμ-Vial (manually). The wash cycle is the same as in the FA method, but the volume of buffer used is 1 ml due to the larger vial size. After washing, the bacteria pellet is resuspended in 200 µl of buffer and transferred to a new Vμ-Vial containing 150 mg of glass beads. This vial is then shaken at 2000 rpm in the thermal mixer (without heating). After shaking, the vial is centrifuged, and 5 µl of supernatant is mixed with 20 µl of matrix solution in a new vial with an inlet. This mixture is then spotted onto the MALDI metal plate, with 1 µl per spot.

PAL RTC Automation Platform

The PAL RTC autosampler used in this application has a modular setup (Figure 2), allowing for the implementation of different components as needed.

The robotic system includes the following modules:

- PAL Park Station: This is a place to store tools that are not in use. (1)
- PAL Head: This is a robotic head that moves the samples and reagents between the different modules. It can be equipped with different tools. (4)
- PAL Liquid Tool: This is a liquid handling tool that dispenses and aspirates liquids. (4)
- PAL Tray Holder: This holds the sample trays and reagent vials. (2)
- PAL Vortex Mixer: This mixes the samples and reagents. (3)
- PAL Fast Wash Module: This washes the Liquid Tool to prevent contamination between samples. (5)
- PAL Centrifuge: This centrifuges the samples. (6)
- PAL Thermal Mixer: This plate mixer mixes and heats the samples (not heated in this application). (7)

In this study, a custom tray holder was also used to accommodate the direct handling of the MALDI plate including sample loading.

MALDI-TOF MS Analysis

The MALDI-TOF MS analysis was performed using a Shimadzu AXIMA Performance instrument with settings adopted from MabritecCentral. The specific settings included a regular circular raster type, circular well shape with a diameter of 1300 µm and 130 µm spacing, and a mass range of 4000-30000 Da. The laser power limits were set to 80, with a starting power of 75, and the minimum intensity and signal-to-noise ratio were set to 25 mV and 5, respectively. Each spot was analyzed with 50 profiles, each containing 5 laser shots at a repetition rate of 50.0 Hz, and the pulsed extraction was optimized for 15000 Da. The ion gate blank was set to 3900 Da, and the start noise was set to 17000 with a noise width of 50. The maximum intensity was 800, and the maximum number of rejects was 2.

Data Analysis

Spectra were evaluated based on their quality, including baseline noise and the presence of specific ribosomal protein peaks. The MabritecCentral database, version V2 (updated 2023.11.21), was used for bacterial identification. This version contains 16312 valid species and 236000 entries.

The spectra were uploaded to the MabritecCentral database, and the instrument used for analysis was specified. The database then compares the uploaded spectrum to its library of reference spectra. The database's "marker mass" approach, which relies on specific ribosomal proteins as biomarkers, was used to identify the bacteria. If the statistical comparison between the experimental and reference spectra did not meet the threshold for a qualified match, the database listed potential results at the genus level as non-qualified results.

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