Standing Committee of Analysts

The Identification of Microorganisms using MALDI-TOF Mass Spectrometry (2020)

Methods for the Examination of Waters and Associated Materials

Methods for the Examination of Waters and Associated Materials

This booklet contains a method for the identification of microorganisms by Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as an illustrative example of the type of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used. It is left to users to evaluate methods in their own laboratories.

Within this series there are separate booklets, each dealing with different topics concerning the microbiology of drinking water. Booklets include

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

The Microbiology of Drinking Water (2006)
Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

The Microbiology of Drinking Water (2007)
Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

The Microbiology of Drinking Water (2010)
Part 2 - Practices and procedures for sampling
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

The Microbiology of Drinking Water (2012)
Part 5 - The isolation and enumeration of enterococci by membrane filtration

The Microbiology of Drinking Water (2014)
Part 11 – The determination of Taste and Odour in drinking water

Microbiology of Water and Associated Materials (2017)
Practices and Procedures for Laboratories

Microbiology of Water and Associated Materials (2019)
Part 2 - The determination of Legionella bacteria in waters culture methods
Part 3 - The determination of Legionella bacteria in waters qPCR

The Microbiology of Drinking Water (2020)
Part 7 - Methods for the enumeration of heterotrophic bacteria

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, wastewater and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts (established 1972 by the Department of the Environment). At present, there are seven working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (http://www.gov.uk/environment-agency) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Rob Carter
Secretary
October 2020

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc. Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

A Method

A1 Introduction

In the context of microbiological analysis MALDI-TOF is a diagnostic mass spectrometer (MS) for the rapid identification of microorganisms.

MALDI-TOF identifies microorganisms using mass spectrometry to measure highly abundant proteins that are found in all microorganisms. The characteristic fingerprint like patterns of these proteins are used to reliably and accurately identify microbial species by comparison to a digital reference library of spectra.

It can be used to confirm the identity of microorganisms isolated through the application of other blue book methods as detailed in the Microbiology of Drinking Water series.

A2 Scope

The method is suitable for identifying bacteria cultured in such a way to achieve discreet colonies and can be employed as the principal means of confirmation, or as part of a confirmatory process in conjunction with a selective isolation method for example.

Users wishing to employ this method should verify its performance under their own conditions over the range of variables encountered in the course of routine testing (for example, sample matrices and growth media) to identify any adverse impacts on the identifications achieved. Guidance on appropriate verification of this technique can be found in Part B and Appendix 1 and 3.

A3 Definitions

MALDI-TOF MS is Matrix Assisted Laser Desorption/Ionisation Time of Flight, Mass Spectrometer. MALDI is an ionisation technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. Time of flight mass spectrometry is a method of mass spectrometry in which an ion's mass-to-charge ratio is determined via a time of flight measurement.

Mass Spectrum. Mass spectrum is the two-dimensional representation of signal intensity (peak) on the vertical axis versus the mass to charge (m/z) ratio on the horizontal axis.

Protein fingerprint. The highly abundant ribosomal proteins of a microorganisms result in a species-specific characteristic fingerprint like mass and intensity spectra.

Library. A digital library containing reference mass spectrum peak lists for a range of microorganisms used to compare and match with the mass spectrum peaks of the analysed microorganism to provide an identification and corresponding confidence level. Also known as a spectral database.

Matrix. This is a Cyano-4-hydroxycinnamic acid, also named as alpha-cyano-4-hydroxycinnamic acid and abbreviated to CHCA or HCCA, which is a cinnamic acid derivative and is a member of the phenylpropanoid family. The matrix facilitates the ionisation of peptides and proteins in MALDI-TOF analysis.

Standard Solvent. A standard solvent comprised of 2.5% trifluoroacetic acid (TFA), 47.5% LC-MS grade water and 50% acetonitrile used for MALDI mass spectrometry. The solvent is used to prepare the matrix solution.

Liquid Chromatography Mass Spectrometry (LC-MS) Grade. 0.2 micron filtered ultrapure water with low UV-absorptivity.

Target Plate. A plate consisting of numerous wells upon which samples are fixed before they are loaded in to the MALDI-TOF instrument. Disposable and reusable target plates are available.

In the context of this method, the user should define how the identifications generated constitute a positive or negative confirmation outcome. This is relatively straight-forward for a selective method for a single species or genus where it is clear if the colony has identified as the target organism(s) or not. For methods where the parameter is not a defined species or genus, but a group of bacteria usually defined by common biochemical characteristics such as presence of enzymes e.g. coliform bacteria; the definition will need to be based on comparison to the confirmation outcomes from the traditional confirmation method. Comparison exercises between the traditional confirmation techniques and the MALDI-TOF identification technique should be carried out as part of the verification exercise and are described in Part B.

A4 Principle

Isolates are cultured in such a way to achieve discreet colonies which are then transferred to the target plate. The matrix is added which extracts ribosomal proteins from the microorganism. These are then analysed on the MALDI-TOF instrument which bombards the fixed colony with a focused, intense burst of ultraviolet light (UV). This causes rapid evaporation of the matrix and proteins resulting in the release of intact, charged proteins and peptides. The charged molecules are propelled through the MS tube to a detector. The mass of each protein molecule produced influences its “time of flight” along the MS tube. The make up of protein molecules within the sample is then analysed by the instrument software, using the measured “times of flight”, and expressed as a spectrum.

This spectrum for many microorganisms is species specific and can be used as a ‘protein fingerprint’ to identify the microorganism. A proprietary biostatistical algorithm is used to match the ‘protein fingerprint’ to a spectral database which determines an identity and corresponding confidence level for the match.

A5 Limitations

The ability to identify test microorganisms using the MALDI-TOF instrument relies on the diversity of the spectral records stored within the library. The library must therefore be suitable for the intended use and an assessment of this should form part of the verification exercise.

Identification of closely related species is not possible using MALDI-TOF. For example, Shigella spp. and *Escherichia coli* (*E. coli)* share many common phenotypic characteristics and genotypically could be considered the same species. Further biochemical testing would be needed.

Depending on the MALDI-TOF system, microorganisms such as Salmonella may only be

identified at genus level. Determination of identity to species, sub-species or serotype would require further biochemical testing or strain-typing if necessary.

Legionella pneumophila is typically identified to the serogroup, which cannot currently be reliably achieved on standard MALDI-TOF instruments. Further biochemical testing to determine the serogroup maybe necessary.

The colony being tested must be a pure culture to successfully attain a mass spectrum.

Colonies should be freshly cultured and guidance on the time between enumeration and MALDI-TOF analysis is detailed by the manufacturer. Guidance in the Microbiology of Drinking Water – Part 4 states that colonies should be set up for confirmatory analysis, preferably within 60 minutes, as colony colours can fade after removal from the incubator. Petri dishes should therefore be stored in the appropriate incubator prior to analysis, if confirmation is not undertaken immediately after enumeration. It is not recommended to use colonies that have been refrigerated - re-culturing should be undertaken unless otherwise validated.

Excess colony deposition may result in poor identification discrimination. While gaining experience in the technique, it can be worthwhile preparing more than one target spot without changing the loop or stick.

For best results, the sample preparation should be performed at room temperature.

Culture media, and other variables may also limit the identification of test colonies. A list of culture media that have been verified for use are listed in Appendix 2.

A6 Health and Safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations [1] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed, and guidance is given elsewhere [2] in this series.

When using the reagents required for MALDI-TOF analysis, good chemical handling practice should always be adhered to along with using chemical proof PPE and fume cupboards. Specifically, Trifluoroacetic acid (TFA), Guanidine hydrochloride (GdnHCl) and Formic Acid. TFA is corrosive and a serious health hazard, Guanidine hydrochloride (GdnHCl) is harmful and Formic Acid is corrosive. All local legislation should be adhered to for the use of TFA.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [2] in this series.

Principally, an appropriate MALDI-TOF system is required which comprises of MALDI-TOF, PC with instrument software containing an appropriate spectral database. Suitable target plates and inoculating loops or wooden cocktail sticks.

If using reusable target plates the cleaning procedure is detailed in Appendix 5.

The MALDI-TOF system must be stored in an appropriate environment of 16 – 33°C with

the range for optimal performance being between 18 – 25ºC, or as detailed by the manufacturer.

A8 Media and reagents

All reagents should be of highest purity and suitable for MALDI or HPLC analysis, distilled or deionised water should be used throughout. For best results, preparation of all solutions should be performed at room temperature.

Commercial formulations of these reagents are available but may possess minor variations to their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method [(2)]. Variations in the preparation and storage of media or reagents should also be verified.

A8.1 Water - LCMS Grade.

A8.2 Standard Solvent

Acetonitrile	125ml
Water	118.75ml
Trifluoroacetic acid	6.25ml

Combine the Acetonitrile, Water and Trifluoroacetic acid to create a standard solvent. Larger volumes can be prepared and stored at room temperature for up to 6 months from preparation. The standard solvent should be stored with minimum headspace. It is important to ensure that the storage vessel and lid does not react with the solvent or release plasticisers.

A8.2 Matrix

alpha-cyano-4-hydroxycinnamic acid	2.5mg
Standard Solvent (A8.2)	250µl

Dissolve the alpha-cyano-4-hydroxycinnamic acid in the solvent, vortexing thoroughly to ensure that the alpha-cyano-4-hydroxycinnamic acid is fully dissolved. It can then be stored at room temperature for up to one week.

A8.3 Suitable calibration test standard provided by the manufacturer

A8.4 70% Formic Acid

Required if following the extended sample preparation outlined in A9.1.3.

A8.5 Absolute Ethanol

Required if following the extraction sample preparation outlined in A9.1.4.

A8.6 Acetonitrile

Required if following the extraction sample preparation outlined in A9.1.4.

A8.7 Cleaning reagents (re-useable target plates only – see Appendix 5).

70% Ethanol

80% Trifluoroacetic acid (TFA).

4M Guanidine Hydrochloride

A9 Analytical Procedure

Depending on the intended purpose of the analysis and the required accuracy, select a suitable number of target colonies for analysis.

If the aim of the examination is to estimate the identity of the microorganisms present, then for the greatest accuracy, all colonies should be chosen if fewer than ten are present. At least ten colonies should be chosen if more than ten are present.

Colonies should be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be analysed. Where several colonies of different appearance are clearly distinguishable, a note of the number of each morphological type should also be made. Where one type of colonial species greatly exceeds another type, random choosing of colonies may result in the failure to analyse the less frequently occurring species. In these cases, additional considerations should be given to choosing all colonial species. The data and information from the analysed isolates are then used to calculate the identity for the microorganisms present.

Each colony to be tested can be prepared via the following detailed in 9.1. Minor optimisations of these preparation methods are acceptable if recommended by the manufacturer or suitably validated following the guidance detailed in Part B.

A9.1 Target Plate Preparation

A9.1.1 General

Three options for the preparation of biological material undergoing MALDI-TOF analysis are described in this section which the laboratory may elect to use.

The direct sample preparation (A9.1.2) is the quickest, requiring no additional reagents such as formic acid for the preparation. Most isolates will provide acceptable identification results with this technique.

The extended sample preparation (A9.1.3) utilises solvent extraction through the addition of formic acid. This preparation stage is recommended when the direct sample preparation procedure is unsuccessful. This sample preparation is also advantageous when analysing microorganisms with stronger cell walls such as yeast together with other types as maybe recommended by the manufacturer.

The full extraction sample preparation (A9.1.4) is recommended for use when the first

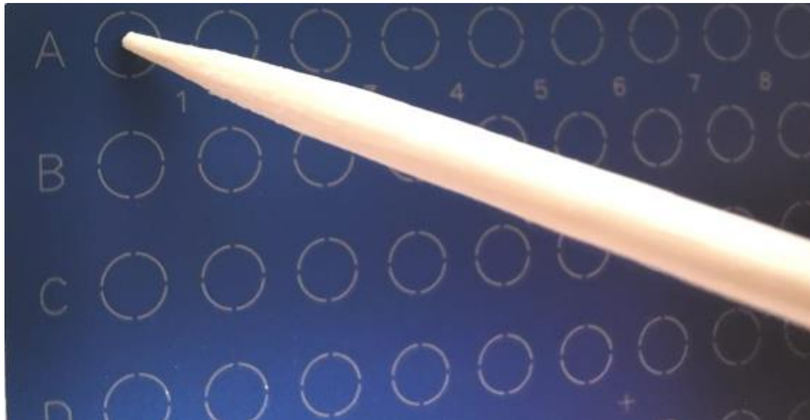
two sample preparation methods (9.1.2 and 9.1.3) are unsuccessful or where manufacturers guidance suggests its use for certain microorganisms. This technique inactivates the test sample and is therefore advantageous for Class 3 microorganisms. This preparation stage must be validated by the user to ensure it will not inactivate sporulating organisms such as a *Bacillus*.

Laboratories should assess which of these techniques is most appropriate for their intended use of the MALDI-TOF method. The assessment and selection of these techniques should form part of the verification exercise (Part B).

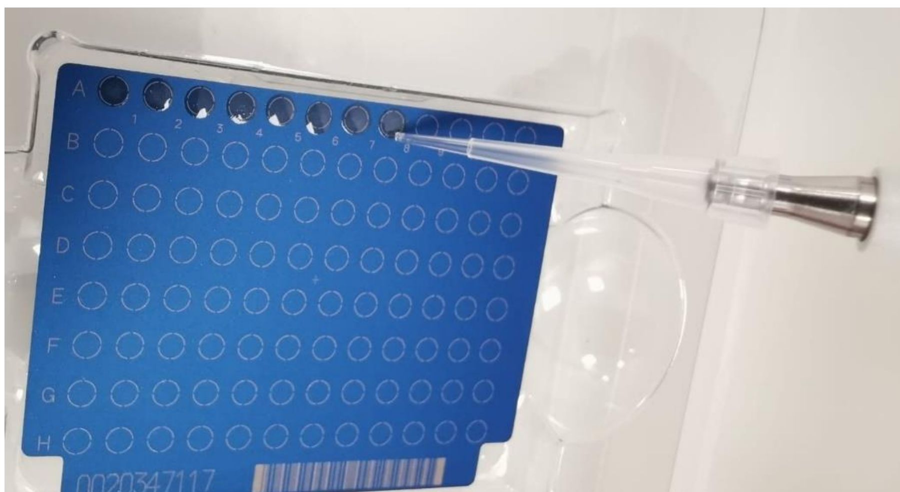
NOTE: If at first a suitable identification is not achieved, this may be due to inconsistent colony preparation on the target spot. The target spot may be manually re-selected and re-analysed. Depending on the instrument used, the target spot may be viewable with a camera to aid target selection. If a suitable identification is still not achieved, re-spotting of colony should be considered or re-preparation using a different technique as described above. If this is still unsuccessful (particularly if testing direct from isolation media or if further work is known to be needed) it is recommended to subculture residual colony material on to an appropriate, validated, non-selective medium for subsequent incubation and retention.

A9.1.2 Direct Sample Preparation

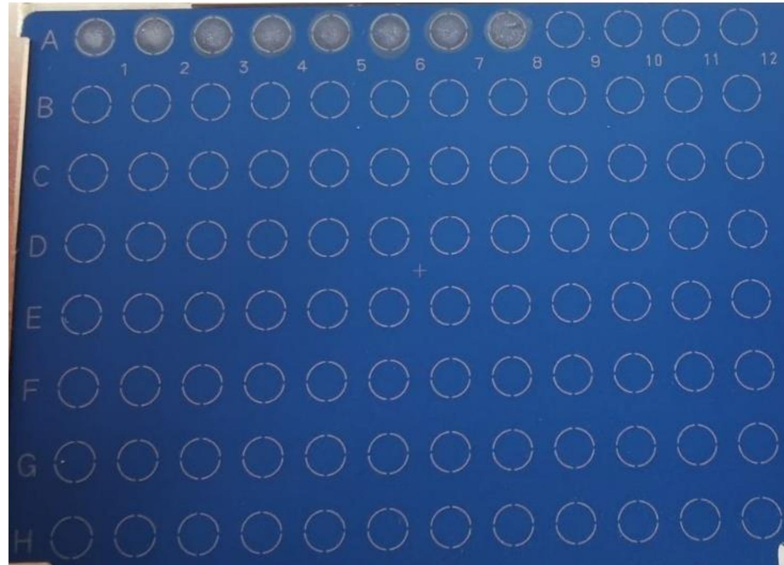
Transfer sufficient sample from a discrete colony using a wooden cocktail stick or 1µl inoculating loop as a thin film directly onto an unoccupied MALDI target plate well.



Overlay each of the occupied wells with 1µL of matrix solution. This must be done within the time detailed in the manufacturer's guidance or an appropriately validated time limit.



Allow the wells to dry at room temperature. A homogeneous preparation should be observed.



Once samples are prepared on target plates, they must be measured within the manufacturer recommendations or an appropriately validated time limit.

A9.1.3 Extended Sample Preparation

Transfer sufficient sample from a discrete colony as a thin film directly onto an unoccupied MALDI target plate well.

Overlay occupied wells with formic acid according to manufacturer’s instructions.

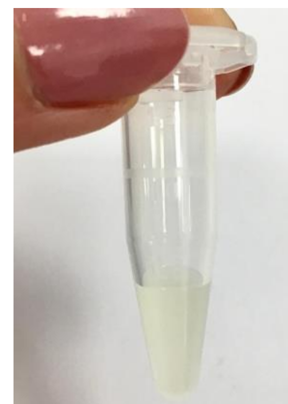
Overlay each of the wells with 1µL of matrix solution. This must be done within the time detailed in the manufacturer’s guidance or an appropriately validated time limit.

Allow the wells to dry at room temperature. A homogeneous preparation should be observed.

Once samples are prepared on target plates, they must be measured within the manufacturer recommendations or an appropriately validated time limit.

A9.1.4 Extraction Sample Preparation

Transfer 300µl of DI water into a microcentrifuge tube then using a 1µl inoculating loop transfer discrete colonies from the culture plate into the water. Mix thoroughly using a vortex until the material is completely in suspension.

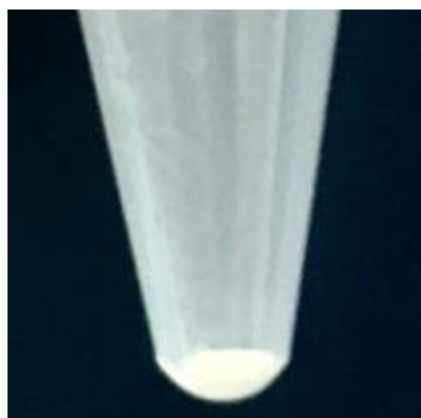


Add 900µl of absolute Ethanol to the microcentrifuge and mix thoroughly using a vortex.

Once vortexed, centrifuge for a minimum of 2 minutes at 14,000 ± 1000 rpm.

Remove the supernatant by pipette leaving the pellet intact.

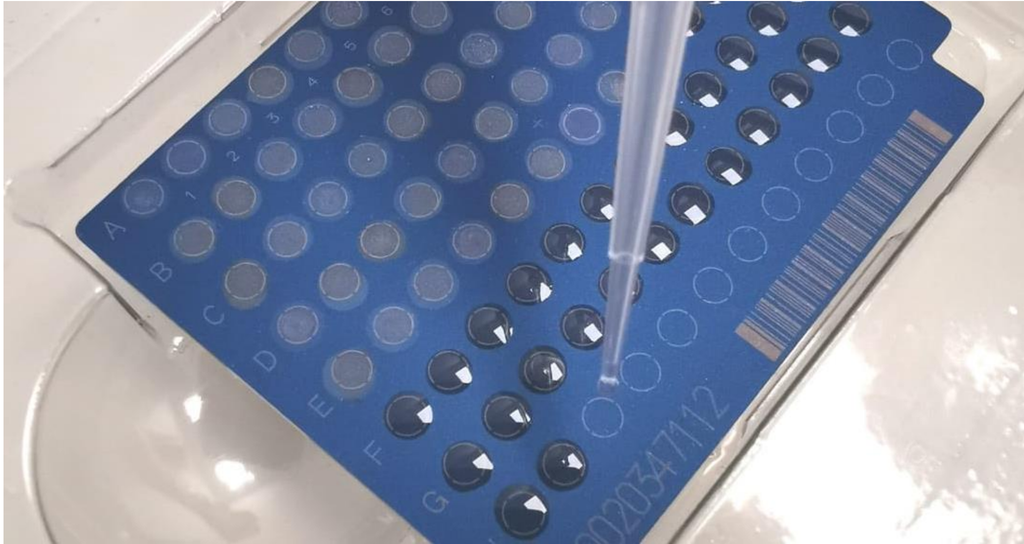
Repeat the centrifugation step and remove the supernatant as detailed above.



Air-dry the pellet for a minimum of 5 minutes at room temperature.

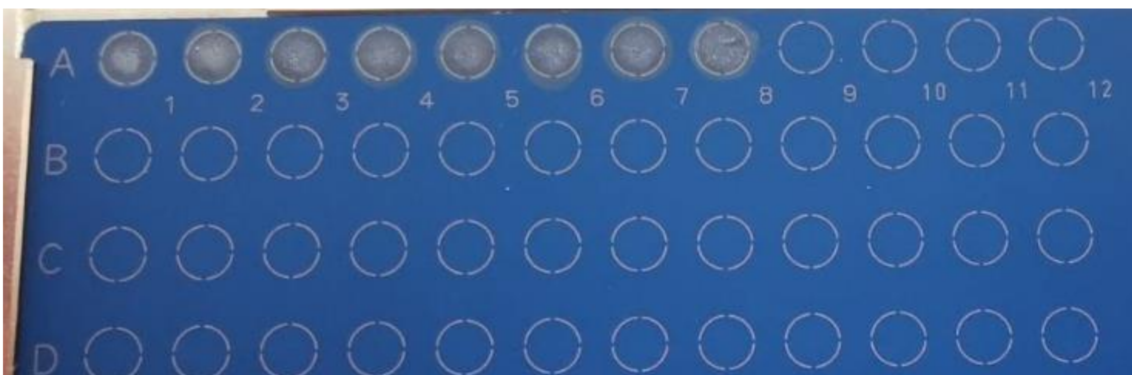
Add 25µl of 70% Formic Acid and resuspend the pellet before further adding 25µl of 100% Acetonitrile. Mix pipetting the solution up and down two to three times before centrifuging for 2 minutes at 14,000 ± 1000 rpm

Pipette 1µl of the supernatant onto unoccupied well on a MALDI target plate and allow to dry at room temperature.



Overlay each occupied well with 1µL of matrix solution. This must be done within the time detailed in the manufacturer's guidance or an appropriately validated time limit.

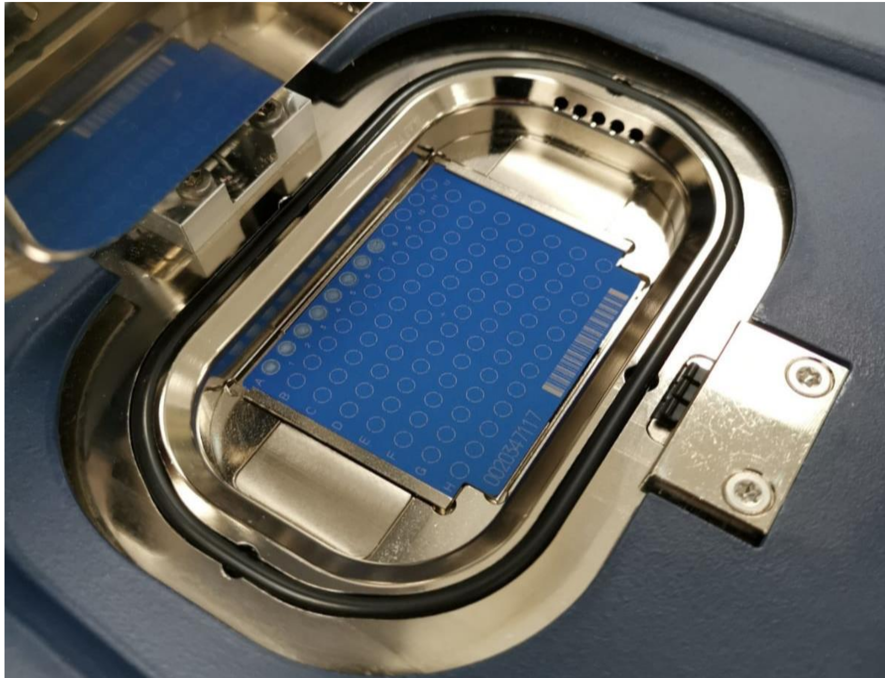
Allow the spots to dry at room temperature. A homogeneous preparation should be observed.



Once samples are prepared on target plates, they must be measured within the manufacturer recommendations or an appropriately validated time limit.

A9.2 Sample Analysis

Once the target plate has been prepared load the target plate into the MALDI-TOF. The acquisition and analysis can then be carried out.



A10 Interpretation of Results

Once the acquisition has been carried out an identification result will be available along with a corresponding value indicating the confidence of probability of the match with the library. An acceptable confidence level is specified in the manufacturer's guidance. Alternative acceptable confidence levels must be appropriate for use and verified by the laboratory.

An example of the result obtained by MALDI analysis is detailed below.

Position	ID	Detected Species	Score	Comment	Consistency		
E4	E.f	Enterococcus faecalis	2.31		high	low	none
E5	P.a	Pseudomonas aeruginosa	2.39		high	low	none
E6	E.a	Klebsiella aerogenes	2.52	synonym of Enterobacter aerogenes	high	low	none
E7	S.w	Staphylococcus warneri	2.22		high	low	none
E8	B.c	Bacillus cereus	2.39	Bacillus anthracis, cereus, mycoides, pseudomycoides, thuringiensis and weihenstephanensis are closely related and members of the Bacillus cer...	high	low	none
E9	A.v	no peaks found			high	low	none

This method can be used as the principal means of confirmation, or as part of a confirmatory process in conjunction with a selective isolation method for example.

The user must define how the identifications generated constitute a positive or negative confirmation outcome. This is relatively straight-forward for a selective method for a single species or genus where it is clear if the colony has identified as the target organism(s) or not. For methods where the parameter is not a defined species or genus, but a group of bacteria usually defined by common biochemical characteristics such as presence of enzymes e.g.

coliform bacteria; the definition will need to be based on comparison to the confirmation outcomes from the traditional confirmation method.

A11 Calculations

If the identification is to be used as a confirmatory technique the number of confirmed colonies is calculated by multiplying the presumptive count by the proportion of the isolates that have been identified as the target microorganism i.e. coliform bacteria.

A12 Expression of results

Presumptive and confirmed counts are expressed in colony forming units per volume of sample. For drinking water, the volume is typically 100 ml.

Identification results are expressed as genus or species name.

A13 Maintenance

Manufacturer guidance should be followed for routine maintenance of the MALDI-TOF system. Routine maintenance may consist of daily maintenance and the steps outlined in 13.1 and 13.2.

Daily maintenance should ensure that exposure of the instrument to dust is minimised. This should be done without exposing the components of the vacuum seals to water as this can negatively impact the vacuum performance.

A13.1 Source cleaning

During MALDI-TOF analysis some of the matrix material and analyte is not ionised and this neutral plume continues to expand from the sample well until it is deposited on surfaces near the ion source. Over time this contamination can build up forming an insulating layer which adversely affects the operation of the ion source.

Depending on the MALDI-TOF system, routine cleaning of the ion source as per the manufacturer's instructions ensures optimum performance of the MALDI-TOF instrument.

A13.2 Detector Check

The detector is an ion multiplier which generates an electrical current when positive protein ions strike the surface, these are registered as electrical pulses from which the peaks of the spectrum are generated.

The detector is constantly bombarded with positive ions which means that over time it will degrade and require more energy to generate the same pulses. A check should be carried out to ensure the detector is functioning within manufacturer's specifications and providing optimal performance.

The detector check can be carried out by using a target plate spotted with matrix. As the matrix components are at specific concentration the pulses they generate should fall within a specific range and these can be monitored over time to determine the detectors performance.

A13.3 Software library updates

The manufacturer may update their microorganism libraries periodically or make other updates which impact the operation of the instrument. Whilst it is important that manufacturers updates are installed in a timely fashion to ensure optimal instrument operation, all updates should be assessed to determine if there is any impact on identifications and any subsequent use of these as part of a confirmation technique. All updates must be reviewed and approved by an appropriate person before they are installed.

A13.4 Customised Library

Although extensive organism libraries are typically supplied with the instruments, it is usually possible to create custom libraries, either by adding microorganism protein mass spectrum or by copying existing entries from the supplied libraries.

NOTE: it is usually not possible to modify the libraries supplied by the manufacturer, other than updates supplied by and/or performed by the manufacturer.

Should the library provided by the manufacturer be deemed not suitable or have clear gaps or omissions that would impact the intended usage, additional microorganism mass spectrum can be added to extend the range of organisms within the library. Following additions by the laboratory to custom libraries, the new spectra should be validated to ensure correct operation.

If creating or adding to custom libraries, a known pure culture of the microorganism should be used such as from a traceable reference material, or if an environmental strain is to be used, its identity should be confirmed by for example genotyping or by specific biochemical tests relevant for the organism. Where there is any doubt over an identity, it should not be added.

The process typically involves protein extraction, then multiple replicates are analysed by the instrument. The resulting mass spectrum are scrutinised to ensure consistency within the replicates. The mass spectrum is then compared to existing library entries to ensure there is no interference with existing organism entries, as well as being tested with a culture to ensure the correct identification is given. The procedure is relatively complex and should only be carried out by trained individuals. Further guidance and training on the method for this is typically given by the manufacturer.

A14 Quality assurance

New batches of reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli* and *Klebsiella aerogenes*) and non-target bacteria (for example, *Pseudomonas aeruginosa*).

Routine calibration or system suitability of the instrument by the laboratory is recommended and specific manufactures may have unique requirements.

Routine calibration of the instrument is recommended in order to ensure optimum performance. Specific manufacturers may have unique requirements for the frequency of calibration. Calibration allows the instrument to determine where proteins of known mass are appearing in the spectrum and adjust the mass calculations to align to pre-set values thus ensuring optimum performance and accounting for any changes that can impact the

flight distance such as ambient temperature. Manufacturer's instructions should be followed. Calibration typically involves the analysis of a designated test standard or reference strain organism.

This calibration may also be used for on-going monitoring of instrument performance by monitoring for possible drift in specific peaks on the mass spectrum. For example, on-going monitoring of the lowest and highest peaks in the mass range.

A suitable control sample should be analysed at regular intervals for the target organisms being sought, in order to demonstrate acceptable performance of the MALDI-TOF system. Such control microorganisms should be relevant to the target group/genus/species under investigation, be traceable and prepared in a consistent manner (in terms of passages and media/incubation conditions/age). The performance of control microorganisms can be monitored for drift in identification confidence value to enable early intervention should a trend be detected regardless of the correct identification being returned.

Blank controls should be carried out which typically consist of any reagents used and the matrix placed on an un-inoculated position on the target plate. This is particularly important where re-usable plates are used in order to demonstrate that cleaning protocols remain fully effective and that inevitable deterioration in the surface finish are not influencing identification results.

A15 References

1. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

2. Standing Committee of Analysts, The Microbiology of Water and Associated Materials (2017). Water Quality and Public Health. Practices and Procedures for Laboratories, in this series, Environment Agency.

3. Standing Committee of Analysts, The Microbiology of Drinking Water (2016) – Part 4 – Methods for the isolation and enumeration of coliform bacteria and Escherichia coli (including E. coli O157:H7). Methods for the Examination of Waters and Associated Materials, Environment Agency.

4. Standing Committee of Analysts, The Microbiology of Drinking Water (2012) - Part 5 – Methods for the isolation and enumeration of enterococci. Methods for the Examination of Waters and Associated Materials, Environment Agency.

5. Standing Committee of Analysts, The Microbiology of Drinking Water (2012) - Part 6 – Methods for the isolation and enumeration of sulphite-reducing clostridia and Clostridium perfringens by membrane filtration. Methods for the Examination of Waters and Associated Materials, Environment Agency.

6. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. Methods for the Examination of Waters and Associated Materials, in this series, Environment Agency.

7. Molecular Microbial Diagnostic Methods - Pathways to Implementation for the Food and Water Industries – Chapter 9: MALDI-TOF: A rapid microbiological confirmation technique for food and water analysis. Elsevier ISBN 9780124169999, Capocefalo M., Ridley E. V., Tranfield E. Y. & Thompson K. C 2015.

B Technical protocol for the characterisation and verification of MALDI-TOF method and use as a confirmatory technique

B1 General

Technical criteria and requirements described in this part of the booklet may be used for the characterisation and verification of the MALDI-TOF method for the identification of bacteria and use as a confirmatory technique.

For verification, all requirements stated in this part of the booklet are applicable. For method comparison studies, simplified requirements can be used for verification e.g. a comparison exercise assessing a second instrument to a previously verified instrument.

B2 Characterisation of the Method

The MALDI-TOF method must first be characterised in terms of its intended use i.e. bacterial identifications and/or confirmatory analysis.

Prior to purchasing the instrument an assessment of the most appropriate library for the intended application of the MALDI-TOF should be made.

Once purchased the library should be reviewed using the instrument software and assessed to determine if it is suitable for all target microorganisms for the intended use. The library review should also include reviewing the known limitations of the library i.e. distinguishing between certain species and these limitations should be taken into consideration when carrying out the method validation.

MALDI-TOF identifications are provided with a confidence level which relates to the match between the analysed microorganism spectrum and the library spectrum. The manufacturer provides recommended guidance on the use of these confidence levels. A suitable acceptable confidence level should be determined and taken into consideration when carrying out the method validation.

B3 Assessment of Variables

There are a range of variables which may be assessed prior to carrying out the larger library assessment and method comparison study.

B3.1 Agar Assessment

A range of microorganisms should be isolated using the range of specific and non-specific agar routinely used within the laboratory.

The range of microorganisms will depend on the intended use of the MALDI-TOF instrument. It is recommended to validate all agar types that will be used for both primary isolation and sub-culturing colonies. An example is detailed below using coliforms as an example:

A range of coliform species and non-target organisms e.g. *Pseudomonas* tested primary isolation agar, MLGA.

A coliform species e.g. *K. aerogenes* analysed using confirmatory agar e.g. Tryptone Nutrient Agar and non-specific agar e.g. Yeast Extract Agar that may be used for routine isolation within the laboratory carrying out the assessment.

The MALDI-TOF confirmation method should only be used on an agar that was assessed and shown to be acceptable within the validation study.

B3.2 Colony Age

The manufacturer provides guidance on the time limit between the sample being isolated and the sample preparation. Some methods such a Microbiology of Drinking Water – Part 4 [1] may state specific time constraints for carrying out confirmatory analysis due to morphology changes. It is recommended that colonies should be analysed as soon as practicable. If cultures are to be stored prior to analysis, then suitable storage conditions and time limits can be established through suitable validation.

The assessment of colony age is also important where a test requires multiple reads or preliminary examination/pre-reads e.g. *Pseudomonas aeruginosa*.

Prepare a range of target and non-target organism replicates isolated from the primary agar of the intended application of the MALDI-TOF. It is recommended that a minimum of 10 replicates of both target and non-target microorganisms are analysed. These microorganisms should then be prepared at appropriate time limits after isolation for assessment i.e. 4 hours, 8 hours and 12 hours, or over a representative period for tests with multiple reads.

Time limits are considered acceptable if all replicates generate the correct identification results with an acceptable confidence level value. Significant degradation in confidence level values over the time period should be assessed and the suitability of that storage time assessed.

B3.3 Prepared Sample Stability

The manufacturer provides guidance on the time limit between sample preparation and sample analysis via the MALDI-TOF. The manufacturer's guidance should be adhered to or a time limit and storage conditions can be established through suitable validation.

Prepare a range of target and non-target microorganism replicates isolated from the primary agar of the intended application of the MALDI-TOF. It is recommended that a minimum of 10 replicates of both target and non-target microorganisms are analysed. These microorganisms should then be analysed at appropriate time limits under storage conditions to be used for assessment i.e. 24 hours, 48 hours and 72 hours or longer as required.

Time limits and storage conditions are considered acceptable if all replicates generate the correct identification results with an acceptable confidence level value.

B3.4 Cleaning of Target Plate – Re-usable target plates only.

The manufacturer provides guidance on cleaning reusable target plates.

To validate the cleaning process, prepare a reusable target plate using suitable microorganisms. Once it has been analysed it should be cleaned as per the recommended manufacturer's guidance. A number of randomly chosen spots should be tested using Matrix only to ensure that the cleaning procedure is effective.

B3.5 Water Matrices

During the library assessment and method comparison exercise (B4) all water types analysed by the laboratory should be used in order to demonstrate that there is no difference in confirmatory and/or identification results for each water type typically analysed by the laboratory.

B3.6 Repeatability and Reproducibility

The repeatability of results from individual analysts and the reproducibility or results between analysts should be assessed. In addition, the ability of the instrument to achieve repeatable identifications should be assessed

B3.7 Any Other Variables

Any other variables such as analyst technique or types/manufacturers of membrane filters

should be assessed using the same approach as detailed in the above sections using replicates of target and non-target microorganisms[2].

B4 Library Assessment and Method Comparison

B4.1 Library Assessment

As a first step a desktop review should be carried out of the library contents, including the microorganisms it contains and any relevant documentation provided by the manufacturer. This should assess if the library is appropriate for the intended use by the laboratory. If the libraries available are incomplete, not suitable or require additional organisms this should be noted. Any subsequent work required on the library should form part of the validation and guidance on how to supplement the library is detailed in A13.4.

B4.2 Practical Library Assessment

For practical examination of the library a series of known traceable reference material and wild type strains of target and non-target microorganisms should be analysed via the MALDI-TOF to determine if the library is suitable for the intended application.

The number of target and non-target microorganisms to be assessed is dependent on the intended application of the MALDI-TOF. It is important to include suspect and interfering colonies that may grow on the typical media used within the laboratory for the reference method e.g. *Clostridium bifermentans* on TSC agar.

The library is considered acceptable for use if it can achieve a correct identification in line with the manufacturer's guidance or published data [3, 4] to the appropriate phylogenetic level for the intended use of the MALDI-TOF instrument. The limitations of the MALDI-TOF system should be taken into consideration when assessing the identification results.

B4.3 Method Comparison

The method comparison study consists of the analysis of target and non-target organisms using both the MALDI-TOF confirmation method and the reference confirmation method.

A range of target and non-target strains shall be analysed from both reference cultures and naturally derived samples e.g. raw water. A guide on the number of strains and colonies to be analysed is given in Appendix 1. Method comparison and library assessment can be combined into one study where reference material and natural samples are analysed using the MALDI-TOF and the reference comparison method e.g. reference strain species of coliforms analysed via both MALDI-TOF and TNA confirmatory methods[1]. This provides data for both library assessment and method comparison.

The method comparison study results should be assessed by the two-sided evaluations of comparison data approach (section 6.2.4.1, ISO 17994[5]).

The method comparison study results may also be assessed by the determination and expression of performance characteristics approach detailed in ISO 13843[6], whereby the confirmation outcomes achieved using the reference method and using the MALDI-TOF method are directly compared.

The performance characteristics calculated for each method should be compared and the significance of any difference determined. It may be required to review individual differences in confirmation outcomes achieved if deemed to be significant.

The MALDI-TOF method is deemed acceptable for use if it is demonstrated to perform equally or better than the reference method used for comparison.

It is also recommended to participate in a minimum of two proficiency testing rounds to further assess the suitability of the MALDI-TOF method for the intended application.

B5 Uncertainty of Measurement

The concept of uncertainty cannot be applied directly to qualitative test results such as those from the determination of attributes for identification[7]. For the MALDI-TOF method all individual sources of variability, should be identified and demonstrated to be under control. Examples are detailed below:

Analytical Procedure – The proportion of target colonies to be confirmed is controlled through documented procedures and training. Only analysts fully trained in confirmation testing are permitted to analyse samples. External proficiency testing is used to demonstrate the ability of the analyst to correctly carry out the method.

Instrument performance – In order to control the instrument performance, the instrument should be calibrated prior to use along with regular maintenance and routine servicing.

B6 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2016) – Part 4 – Methods for the isolation and enumeration of coliform bacteria and Escherichia coli (including E. coli O157:H7). Methods for the Examination of Waters and Associated Materials, Environment Agency.
2. Molecular Microbial Diagnostic Methods - Pathways to Implementation for the Food and Water Industries – Chapter 9: MALDI-TOF: A rapid microbiological confirmation technique for food and water analysis. Elsevier ISBN 9780124169999, Capocefalo M., Ridley E. V., Tranfield E. Y. & Thompson K. C 2015.
3. Performance of a matrix-assisted laser desorption ionization time-of-flight mass spectrometry system for the identification of bacterial isolates in the clinical routine laboratory (2009). Eigner U, Holfelder M, Oberdorfer K, Betz-Wild U, Bertsch D, Fahr AM. Clin Lab. 55:289–296
4. Evaluation of the Vitek MS® MALDI-TOF mass spectrometry system in a routine clinical laboratory (2012). N. Reading, A.Dadrah, A.Symonds, H.M. Kilgariff, N Ratnaraja. Department of Microbiology, Sandwell & West Birmingham Hospitals NHS Trust.
5. Water quality - Requirements for establishing performance characteristics of quantitative microbiological methods, ISO13843:2017, International Organisation for Standardisation, Geneva.
6. Water Quality - Guidance on Validation of Microbiological Methods, Technical Report ISO/TR 13843:2000, International Organisation for Standardisation, Geneva.
7. Eurachem: Accreditation for Microbiological Laboratories (2013) https://www.eurachem.org/index.php/publications/guides/microbiol.

Appendix 1 Guidance on Microorganism Selection

Guidance on selecting microorganisms to carry out validation study for MALDI-TOF identification and use as confirmatory analysis.

Confirmatory or Identification Level	Reference Strains						Natural Bacteria
	Target Strains			Non-target strains*			
	Number of species	Number of strains	Number of colonies tested	Number of species	Number of strains	Number of colonies tested	Number of colonies tested
Group (e.g. Coliform)	11	11	260	3	3	40	300
Genus (e.g. Enterococci)	7	7	260	2	2	40	300
Species (e.g. *Clostridium perfringens*)	1	3	260	2	2	40	300

*Non-target organisms should be organisms that grow on the primary isolation media for the target organism e.g. *Aeromonas* species for as a Coliform non-target species.

Appendix 2 Culture media

The following isolation culture media have been validated as per the guidance detailed in part B for culturing samples to be identified using the MALDI-TOF methodology:

Membrane lactose glucuronide agar (MLGA)

Slanetz and Bartley

Tryptose Sulphite Cycloserine (TSC)

Glycine Vancomycin Polymyxin Cycloheximide agar

Pseudomonas CN agar

Xylose Lysine Desoxycholate (X.L.D) agar

Tryptone nutrient agar

Columbia blood agar base

Nutrient agar

Yeast extract agar

MacConkey agar

Legionella BCYE and GVPC agar

The manufacturer will also have a list of culture agars that have been validated for their instrument.

Appendix 3 A single laboratory method validation study of Coliform, Enterococci and *Clostridium perfringens* confirmatory analysis via MALDI-TOF.

1 Introduction

The current conventional tests for confirming presumptive bacteria from water samples, described previously in this series [1-3] involves sub-culture and biochemical testing. An alternative method for confirming bacteria based upon identification via ribosomal protein fingerprints using MALDI-TOF technology has been proposed by a member of the Standing Committee of Analysts (SCA) for Microbiology.

A colony is transferred to the target plate and a standard solvent matrix added which extracts ribosomal proteins from the microorganism. These are then analysed on the MALDI-TOF instrument which bombards the fixed colony with a burst of laser light. This causes rapid evaporation of the matrix and proteins resulting in the release of intact, charged proteins and peptides. The charged molecules are propelled through the MS tube to a detector where the mass of the protein molecules influences its 'time of flight' along the MS tube. The protein molecules within the sample are then analysed by the instrument software, using the measured 'time of flight', and expressed as a spectrum. This spectrum for many microorganisms is species specific and this is used as a 'protein fingerprint' to identify the microorganism and thus provide a confirmatory result.

This study was carried out by a single laboratory to assess the MALDI-TOF technique for the confirmation of coliform bacteria, *E. coli*, Enterococci and *C. perfringens* and establish whether the technique can be regarded as equivalent to the conventional tests detailed in this series [1-3].

2 Materials and Methods

Samples of a range of drinking waters covering surface and ground water were analysed by procedures previously described[1-3] involving membrane filtration and enumeration on membrane lactose glucuronide (MLGA), Slanetz and Bartley (S&B) and Tryptose Sulphite Cycloserine (TSC) agars.

Following incubation, colonies were counted in accordance with previously published procedures [1-3], presumptive counts recorded, and colonies selected for confirmation.

The reference methods used for confirmatory analysis were the TNA confirmation method for Coliforms and *E.coli*, Acid Phosphatase for *C.perfringens* and KAAA for Enterococci [1-3].

2.1 Assessment of Variables

A range of target and non-target microorganisms were analysed in both the library assessment and method comparison exercises using the primary isolation media of MLGA, TSC and S&B.

Reference material organisms were isolated onto Tryptone Nutrient Agar (TNA), Nutrient Agar (NA), Columbia Blood Agar Base (CBA), Yeast Extract Agar (YEA) and MacConkey (MAC). The identification results and confidence level generated were assessed to determine if the culture agar had an impact on microbial identification via MALDI-TOF.

2.2 Library Assessment

Reference material bacteria was obtained from a range of sources including NCTC, ATCC strains along with wild type bacteria isolated, cultured and identified by reference methods.

Either all presumptive or a maximum of 10 colonies were selected for confirmation. Colonies were confirmed via reference methods [1-3] and via MALDI-TOF analysis to provide both library assessment and method comparison data. A range of drinking waters were examined covering ground and surface water samples from customer tap, service reservoir and treatment works samples.

2.3 Method Comparison

Either all presumptive or a maximum of 10 colonies were selected for confirmation. Colonies were confirmed via reference methods [1-3] and via MALDI-TOF. A range of drinking waters were examined covering ground and surface water samples from customer tap, service reservoir and treatment works samples. Environmental samples (raw water) were also analysed ranging from surface freshwaters (for example river and reservoir waters) and ground waters.

2.4 Reading of Analysis

Coliform bacteria, *E. coli*, Enterococci and *C. perfringens* confirmatory analysis was read as described elsewhere in this series [1-3].

For the MALDI-TOF analysis, identification results were reviewed and deemed acceptable for either genera or species level where the confidence level was above the manufacturer's recommended value. Where identifications of certain species could not be distinguished due to known limitations of the MALDI-TOF system these were factored into the validation statistics.

2.5 Quality Control

All analysts involved in the validation trial were fully trained or supervised by a competent analyst.

Daily and routine instrument maintenance was carried out following the manufacturer's instructions for the MALDI-TOF.

All the media used is prepared and quality controlled in accordance with the Microbiology of Drinking Water and Associated Materials (MWAM) 2017.

Analytical Quality Controls (AQCs) were set up with each set of samples analysed.

3 Results and Discussion

3.1 Assessment of Variables

Analysis of a range of bacteria using both selective and non-selective agar demonstrated that there is no significant impact of culture agar on the confirmation and identification of bacteria using MALDI-TOF. The following agars were verified and deemed acceptable for use; MLGA, TSC, Slanetz and Bartley, TNA, Nutrient Agar, Columbia Blood Agar, Yeast Extract Agar and MacConkey.

All identifications carried out using bacteria isolated using non-specific agars were correct and generated acceptable confidence level values.

No other variables were assessed as the manufacturer's recommendations will be adhered to for sample preparation.

3.2 Library Assessment

The library contained on MALDI-TOF system was reviewed to determine if it was appropriate for use for the confirmation and identification of coliforms, *E. coli*, Enterococci and *Clostridium perfringens*. The library contains 7014 strains of bacteria covering 8 phyla. For coliforms there are 596 strains covering 40 genera including those most identified in drinking water samples. For Enterococci there are 109 strains covering 34 species including those most identified in drinking water samples. For *Clostridium perfringens* there are 9 strains.

A range of target and non-target reference microorganisms were spiked into a variety of drinking water samples to assess the suitability of the library within the Bruker MALDI-TOF system

(Table 1).

Microorganism Group	Number of Colonies Analysed	Number of correct identifications	Number of incorrect identifications
Coliform	1235	1125 (91.09%)	110 (8.91%)
Enterococci	882	859 (97.39%)	23 (2.61%)
C.perfringens	399	395 (99.00%)	4 (1.00%)
Non-target	360	272 (75.56%)	88 (24.44%)
Total	2876	2651 (92.18%)	225 (7.82%)

Table 1: Identification of spiked samples for a range of organisms typically isolated and identified in drinking water.

The misidentified organisms were *Shigella sonnei* and *Clostridium beijerinckii* which the MALDI-TOF gave an identification of *E. coli* and *Clostridium diolis* respectively. These are known limitations of the MALDI-TOF system as these species are difficult to distinguish due to their similar mass spectrum. Removing these organisms from the statistical calculations the percentage of correct identifications would be 98.59%.

3.3 Method Comparison

Treated water samples spiked with known reference strains of coliforms, Enterococci and *Clostridium perfringens* demonstrated that the MALDI-TOF confirmatory method performed better or equivalent to the reference methods for each organism (Table 2).

Microorganism Group	Reference Method		MALDI-TOF Method	
	Number of Colonies Analysed	Number of correctly confirmed colonies	Number of Colonies Analysed	Number of correctly confirmed colonies
Coliform	1235	1135 (91.90%)	1235	1224 (99.11%)
Enterococci	882	776 (87.98%)	882	859 (97.39%)
C. perfringens	399	310 (77.69%)	399	395 (99.00%)
Non-target	360	360 (100%)	360	360 (100%)

Table 2: Comparison of MALDI-TOF confirmation method and reference methods in the confirmation on spiked treated water samples.

The total confirmation rate for coliform, Enterococci and *C. perfringens* confirmation by the reference methods was 88.28% compared to the MALDI-TOF confirmation rate of 98.49%.

All non-target colonies were correctly confirmed as non-target colonies i.e. gave non confirmatory reactions or identified as non-confirmed species.

In total 2876 colonies were analysed with 395 colonies having disagreeing results between the reference confirmation methods and the MALDI-TOF confirmation method. This is a percentage agreement of 86.27% between the two confirmation methods.

The reason for this discrepancy is due to 100 colonies of *Hafnia alvei* failing to produce β-galactosidase on TNA, 89 colonies of Acid Phosphatase negative *C. perfringens* and 105 colonies of *E. avium* and *E. casseliflavus* failing to hydrolyse aesculin.

Environmental water sample analysis demonstrated that the MALDI-TOF confirmatory

method performed better or equivalent to the reference methods for each organism (Table 3). For the coliform colony numbers a total of 304 colonies were setup for confirmation with 44 colonies being oxidase positive, these colonies were removed from the statistics detailed in Table 3.

Microorganism Group	**Number of Colonies Analysed**	**Reference Method**	**MALDI-TOF Method**	**Number of Colonies with Disagreeing Results**	**Percentage Agreement**
		Number of confirmed colonies	**Number of confirmed colonies**		
Coliforms	260	257 (98.85%)	259 (99.62%)	3	98.85%
Enterococci	303	289 (95.38%)	293 (96.70%)	12	96.04%
C.perfringens	310	258 (83.23%)	259 (83.55%)	15	95.16%

Table 3: Comparison of MALDI-TOF confirmation method and reference methods in the confirmation on raw water samples.

In total 873 colonies were analysed with 30 colonies having disagreeing results between the reference confirmation methods and the MALDI-TOF confirmation method. This is a percentage agreement of 96.56%.

A summary of the disagreeing colonies is given in Table 4.

Presumptive Organism	**Reference Method**	**MALDI-TOF**
Coliform	No growth ONPG -ve *E. coli*	*B. gaviniae* *B. gaviniae* *C. freundii*
Enterococci	Aesculin -ve Aesculin -ve Aesculin -ve Aesculin -ve Aesculin +ve Aesculin +ve Aesculin +ve Aesculin +ve	*E. moraviensis* (5) *E. haemoperoxidus* *E. saccharolyticus* *E. malodoratus* *S. gallolyticus* *P. pentosaceus* No ID possible Low confidence level (*E. gilvus*)
C. perfringens	Acid Phosphatase -ve Acid Phosphatase +ve Acid Phosphatase +ve Acid Phosphatase +ve Acid Phosphatase +ve Acid Phosphatase +ve	*C. perfringens* (7) *C. sordellii* (4) *C. sardiniense* *C. baratii* *C. bifermentans* *Clostridium* species

Table 4: Summary of colonies with disagreeing results for raw water samples. Number of occurrences in brackets.

The reference confirmatory methods and the MALDI-TOF method were compared using the comparison data approach detailed in ISO 17994[(4)] for the environmental water sample analysis and the results are detailed in table 5.

Microorganism Group	**Number of paired counts**	**Mean Relative Difference**	**Standard Deviation**	**W** *	**XL** **	**XU** ***	**Outcome**
Coliform	34	-0.78	7.08	2.43	-3.20	1.65	Not

							different
Enterococci	31	-2.52	15.74	5.65	-8.18	3.13	Not different
C. perfringens	32	-0.26	10.76	3.81	-4.07	3.54	Not different
Total	97	-1.17	11.53	2.34	-3.51	1.18	Not different

*Table 5: Outcome of mean relative difference analysis of MALDI-TOF vs Reference method results according to ISO 17994 analysis. *Half width of the 'confidence interval' around the mean relative difference. **Value of the relative difference at the lower 'confidence limit.' ***Value of the relative difference at the upper 'confidence limit.'*

The ISO 17994 comparison data approach demonstrated that there was no significant difference between the two methods for confirmatory analysis of coliform bacteria, Enterococci and *C. perfringens*.

4 Conclusion

This study compared the efficacy of a MALDI-TOF method, with the traditional agar and biochemical based tests for the confirmation of coliform, *E. coli*, Enterococci and *C. perfringens* bacteria from water samples. The principal conclusions of the study are:

i. There is a confirmation agreement rate of 88.66 % between the MALDI-TOF method and the reference confirmation methods. There was an agreement rate of 86.27% when comparing the methods using reference strain organisms and an agreement rate of 96.56% when comparing the methods using naturally derived bacteria from environmental water samples.

ii. Based on the reference material comparison analysis, the data indicates that the MALDI-TOF method is superior (98.49% confirmation rate) to the reference methods (88.28% confirmation rate) for the confirmation of coliform, *E. coli*, Enterococci and *C. perfringens* bacteria. This is probably reflective of the MALDI-TOF method not relying on biochemical reactions and ribosomal proteins being a specific and effective method for the identification of bacterial organisms.

iii. The MALDI-TOF method has known limitations of not being able to distinguish between certain species. The most relevant of these is that the MALDI-TOF method cannot distinguish between Shigella species and *E. coli* as they are closely related and are not currently distinguishable via their ribosomal protein mass spectrum. It is advised that laboratories determine their approach for dealing with these limitations prior to implementing MALDI-TOF identification and subsequent confirmation analysis.

iv. The MALDI-TOF confirmatory method has been shown to produce equivalent or better confirmation rates of coliform, *E. coli*, Enterococci and *C. perfringens* bacteria from a range of water samples compared to the traditional confirmation methods.

5 References

1. The Microbiology of Drinking Water (2016) - Part 4 – Methods for the Isolation and enumeration of coliform bacteria and Escherichia coli (including E. coli O157 H7). Standing Committee of Analysts, Environment Agency, Nottingham, UK.
2. The Microbiology of Drinking Water (2012) - Part 5 – Methods for the isolation

and enumeration of enterococci. Standing Committee of Analysts, Environment Agency, Nottingham, UK.

3. The Microbiology of Drinking Water (2012) - Part 6 – Methods for the isolation and enumeration of sulphite-reducing clostridia and Clostridium perfringens by membrane filtration. Standing Committee of Analysts, Environment Agency, Nottingham, UK.

4. ISO 17994:2014, Water quality – Requirements for the comparison of the relative recovery of microorganisms by two quantitative methods. International Organization for Standardization, Geneva, Switzerland.

Appendix 4 A summary of a single laboratory method validation study of confirmatory analysis via MALDI-TOF in drinking water.

The validation exercise comprised several aspects as detailed below.

- Direct comparison to the traditional confirmation methods. Results from comparison testing summarised in a 2 x 2 matrix.

		Current confirmation method		
		+	-	
Biotyper confirmation	+	a	b	a+b
	-	c	d	c+d
		a+c	b+d (%)	n

The following are represented; n = no. colonies tested; a = no. colonies confirmed by both methods; b = no. colonies confirmed by Biotyper, but not by current method; c = no. colonies not confirmed by Biotyper, but confirmed by current method; d = no. colonies not confirmed by both methods.

Statistical evaluation carried out by calculating the efficiency, and using a test based on the Poisson index of dispersion, χ^2

- Repeatability and reproducibility (Recorded as a percentage – 95% agreement is accepted):

  - Analyst repeatability – colonies analysed in duplicate.
  - Instrument repeatability – assessed by repeat analysis on the same prepared colonies.
  - Reproducibility – analysis of the same colonies by multiple analysts.

- Prepared colony stability and transport/storage conditions.

The colonies used for the MALDI-TOF confirmation were "presumptive" colonies observed on the primary isolation media stated in the relevant reference methods (Microbiology of Drinking Water /BS/ISO as appropriate). The colonies were mainly "wild" strains typically isolated by the laboratory from accredited matrices, and reference strains.

Exercises comparing colonies grown on the primary isolation media and "non-selective" media to identify potential media interference, plus exercises on the effects of colony age were also carried out. The standard manufacturer's library of spectra was bolstered where necessary with in-house libraries, created using reference strains purchased from recognised culture collections. Comparison of the identifications achieved using the MALDI-TOF were also compared to identifications using alternative commercially available identification test kits (API and BBL Crystal).

Results

***Salmonella* species**		Current confirmation method		
		+	-	
Biotyper Confirmation	+	112	2	114
	-	5	13	18
		117	15	132
***Legionella* species**		Current confirmation method		
		+	-	
Biotyper Confirmation	+	378	0	318
	-	0	6	6
		318	6	384
Enterococci		Current confirmation method		
		+	-	
Biotyper Confirmation	+	190	6	196
	-	0	18	18
		190	21	214
Clostridium perfringens		Current confirmation method		
		+	-	
Biotyper Confirmation	+	179	9	188
	-	0	80	80
		179	89	268
Total Coliforms		Current confirmation method		
		+	-	
Biotyper Confirmation	+	348	10	358
	-	4	156	160
		352	166	518
E. coli		Current confirmation method		
		+	-	
Biotyper Confirmation	+	175	5	180
	-	6	11	17
		181	16	197
Pseudomonas aeruginosa		Current confirmation method		
		+	-	
Biotyper Confirmation	+	66	0	66
	-	0	166	232
		66	166	229

Parameter	Efficiency (target >90%)	X^2 Result (target ≥ 4)	X^2 Outcome
Salmonella species	94.7%	1.29	Not Different
Legionella species	100%	0	Not Different
Enterococci	97%	6	Different*
Clostridium perfringens	96%	9	Different*[1]
Total Coliforms	97%	2.57	Not Different
E. coli	94%	0.09	Not Different
Pseudomonas aeruginosa	100%	0	Not Different

*6 colonies from raw water sources identified as *Enterococcus malodoratus* but did not confirm by the traditional method. This is an organism associated with faecal sources and the identification was confirmed by a third-party laboratory. This indicated better performance by the MALDI-TOF method in this instance

*[1] 6 colonies identified as *Clostridium perfringens* but showed a weak acid phosphatase reaction which developed after the time limit of 3 minutes stated in the reference method, indicating better performance using MALDI-TOF identification

Parameter	Analyst repeatability	Instrument Repeatability	Reproducibility
Salmonella species	30 (100%)	54 (100%)	48 (100%)
Legionella species	124 (100%)	71 (100%)	97 (100%)
Enterococci	30 (100%)	103 (100%)	30 (100%)
Clostridium perfringens	33 (100%)	98 (100%)	33 (100%)
Total Coliforms	71 (100%)	81 (100%)	32 (100%)
E. coli	57 (100%)	100 (100%)	31 (100%)
Pseudomonas aeruginosa	40 (100%)	60 (100%)	32 (100%)
Total	385 (100%)	567 (100%)	303 (100%)

Values stated are number of colonies tested and the number is parentheses is the % agreement.

Discussion

It is accepted that *Salmonella* can only reliably be identified to genus level. Further identification if required would therefore need subculture and serology tests. The 7 non-matching results were all from heavily contaminated sewage sludge samples indicating that suitable purity plates may be required before identification. Suitable for confirmation of *Salmonella* colonies from XLD agar, by identification to genus level. It is not suitable for reporting species identification.

The instrument is unable to separate the serogroups of *Legionella pneumophila* (*L. pneumophila* is typically reported as serogroup 1 or serogroups 2-15), therefore serology must be carried out on all colonies confirming as *L. pneumophila*. Suitable for the confirmation of *Legionella* species (including *Legionella pneumophila*) colonies from GVPC

agar by the identification of species from the genus Legionella.

The discrepancy noted above for Enterococci indicated that the MALDI-TOF gave slightly better performance than the traditional confirmation method when compared using positive sample colonies. Suitable for the confirmation of Enterococci colonies from Slanetz and Bartley agar, by identification of the *Enterococcus* genus.

The discrepancy noted above for *Clostridium perfringens* indicated that the MALDI-TOF gave slightly better performance than the traditional confirmation method. Suitable for the confirmation of *Clostridium perfringens* colonies from TSC agar, by identification of the *Clostridium perfringens* species.

Suitable for the confirmation of *E. coli* colonies from MLGA, by identification of the *E. coli* species. For the total coliform test, the definition of a positive coliform is based on the validation against the outcomes of the traditional confirmation and on published, recognised coliform genera. A coliform genus reference list is therefore required. Suitable for the confirmation of coliform colonies from MLGA, by identification of approved species and genera.

Suitable for the confirmation of *Pseudomonas aeruginosa* colonies from CN agar, by identification of the *Pseudomonas aeruginosa* species.

Appendix 5 Cleaning procedure for reusable target plates.

TFA is corrosive and a serious health hazard. Good chemical handling practice should always be adhered to along with using a fume hood and chemical proof PPE. All local legislation should be adhered to for the use of TFA.

If using disposable targets these do not require a cleaning/washing procedure.

For reusable target the following cleaning/washing process should be followed:

- In a crystalizing dish overlay the surface of the target with 70% aqueous Ethanol.
- Incubate for 5 minutes at room temperature.
- Remove the target and rinse it under flowing tap water.
- Rinse the target with tap water and wipe with a lint free cleaning wipe e.g. lens tissue.
- In a fume hood using 100µl of 80% aqueous TFA cover the target. Wearing chemical protective gloves wipe all target positions with a lint free cleaning wipe or a wooden cotton end swab.
- Rinse the target with deionised water and wipe it dry with an optic cleaning wipe.
- Let the target completely dry for a minimum of 15 minutes at room temperature.

An alternative cleaning procedure is available where TFA is either unavailable or not permitted by site rules or local legislation. The alternative cleaning process is:

Guanidine hydrochloride (GdnHCl).

GdnHCl is harmful and good chemical handling practice should always be adhered to, along with using a fume hood and chemical proof PPE.

- In a crystallising dish overlay the surface of the target with 70% aqueous Ethanol.
- Incubate for 5 minutes at room temperature.
- Remove the target and rinse it under flowing tap water.
- Rinse the target with tap water and wipe with an optic cleaning wipe.
- Overlay the target with 4M aqueous GdnHCl and incubate at 10 minutes at room temperature.
- Rinse the target with plenty of tap water and wipe it intensively with an optic cleaning wipe.
- Repeat the GdnHCl and rinsing with tap water steps twice.
- Rinse the target with deionised water and wipe dry with an optic cleaning wipe.
- Let the target completely dry for a minimum of 15 minutes at room temperature.

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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