Mass spectrometry in the diagnostic laboratory

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Mass spectrometry (MS) was developed at the beginning of the 20th century and is generally used to acquire knowledge of molecular structure based on a mass spectral pattern consisting of a number of structurally related mass spectral peaks. Since the first report showing the ability of MS to detect biomarkers unique for selected microorganisms, knowledge and the volume of reports on this topic have dramatically increased, especially in the last five years. MALDI-TOF MS (matrix assisted laser desorption ionisation – time of flight mass spectrometry) is now increasingly used in the diagnostic laboratory as a cost effective, rapid and reliable technique for the identification and typing of microorganisms. This article aims to provide an overview of this technology, which was to have a major impact on microorganism diagnostics.

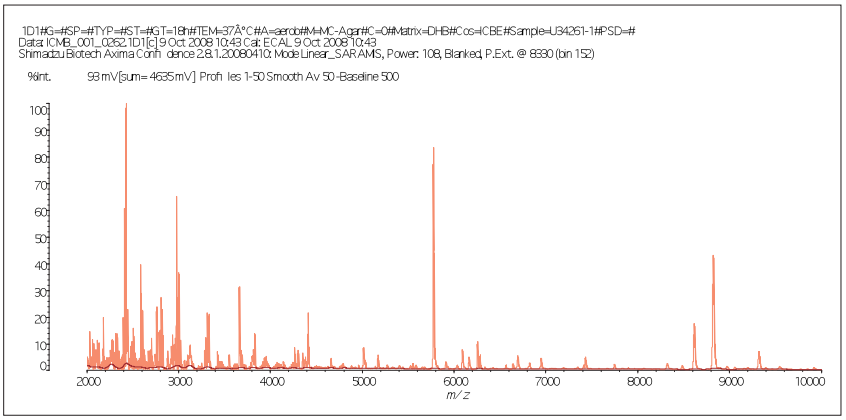

Figure 1
MS spectrum of an *Acinetobacter calcoaceticus var. lwoffii* strain.

Introduction

Microbiologists working in the diagnostic laboratory have always striven to identify microorganisms as quickly and accurately as possible as to genus, species and possibly the intraspecific level (subspecies, serogroup, genotype or other subtypes).

The classical methods in use so far include the establishment of a pure culture from which the germ can be characterised and identified using morphological, biochemical and chemotaxonomic characters. Pure cultures, however, are time-consuming and often difficult to obtain, in particular when dealing with fastidious organisms, e.g. intracellular pathogenic bacteria.

Rapid identification of microorganisms is important in several areas of microbiology, but may be crucial in clinical settings. More rapid techniques have been introduced in routine analysis. Serology (ELISA, immunofluorescence-based detection kits), molecular biology (e.g. nucleic acid hybridisation, PCR, sequencing), as well as other methods such as flow cytometry, are now saving identification time and costs. Each method, however, has advantages and disadvantages and is often confined to the identification of only selected organisms. At present none is fully satisfactory as far as speed, accuracy and precision and total absence of drawbacks are concerned [1].

Recently mass spectrometry (MS) has been used for the identification of microorganisms, mainly bacteria. The method, based on the characterisation of biomarker molecules, is rapid and accurate and constitutes a valid alternative to conventional methods of identification and classification in microbiology [1, 2].

Mass spectrometry (MS)

Mass spectrometry is an extremely sensitive technique that has been known for almost a century. Analysis of biomolecules by MS dates back to the 60s, but its application was limited by the fact that it could not be used to detect molecules of low volatility and heavy weight (over 20–30 kDa) as well as complex mixtures. These limitations were overcome in the late 80s with the introduction of new ionisation techniques for non-volatile molecules, including proteins and peptides [1].

MS is based on the ionisation of molecules. The formed ions run in a magnetic or electric field and are then separated and detected in a mass spectrometer according to their mass-to-charge ratio (m/z). The spectra originating from the analysis of complex mixtures, such as a bacterium colony, are characterised by several peaks, among which some are typical and representative of the taxon to which the analysed microbe belongs (fig. 1).

Identification of peaks by MS include transfer of the analyte in the gas phase, ionisation (and possibly fragmentation) of the molecules, acceleration of charged ions, separation of ions according to their m/z, detection of ions and interpretation of the mass spectrum obtained. The peak pattern is then used to characterise and eventually to identify the microorganisms studied.

Various devices are marketed for this type of analysis and, depending on the technique used, different mass spectra are obtained.

The mass spectrometer

A mass spectrometer is composed of five main parts (Fig. 2):

- ***A system for the introduction or injection*** of micro- or nanograms of a solid, liquid or gaseous sample in the mass spectrometer.
- ***The source of ions*** by which the sample is irradiated. Electron impact (EI), chemical ionisation (CI), fast atom bombardment (FAB), plasma desorption (PD), electrospray (ESI), atmospheric pressure chemical ionisation (APCI), and matrix-assisted laser desorption/ionisation (MALDI) are available; the most appropriate is chosen depending on the nature of the sample and the type of analysis envisaged. ESI and MALDI can result in vaporisation of biomolecules with high molecular mass by transforming them into the gas phase from a liquid (ESI) or solid phase (MALDI).
- ***An analyser*** that separates ions according to m/z by applying an electric or magnetic field. Magnetic field, time of flight (TOF), quadrupole (Q), ion trap (IT), and cyclotron resonance ion (ICR) are the most common analysers used in MS.
- ***A sensor*** that detects separated ions and amplifies the originated signal (gain sensitivity up to about 10^6).
- ***A computer*** for data processing able to transform the information received by the detector to a mass spectrum.

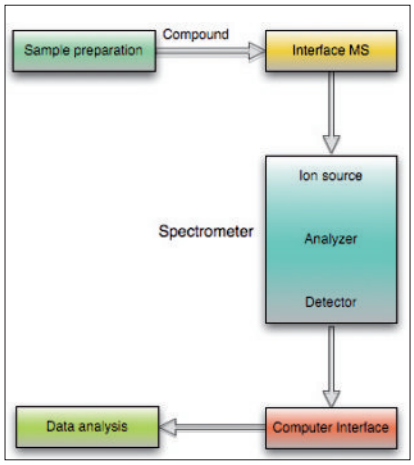

Figure 2
Mass spectrometer: components and workflow (modified after [3]).

The performance of a mass spectrometer is characterised by:

- ***The mass range***, recognised as the ratio m/z value.
- ***The resolving power (R)***, which denotes the ability of an analyser to distinguish adjacent fragments. For two ions neighbouring masses, M and M + M, R = M / M. MS devices may have low (R <300), average (300 >R >10 000) and high (R >10 000) resolution. If the resolution is high enough, it is possible to determine the exact mass of each individual ion.
- ***The scanning speed***, the number of scans per second required to obtain a full spectrum.

We shall now describe in more detail the matrix-assisted laser desorption ionisation scanner and time of flight (MALDI-TOF) in view of its usefulness in microbiological analysis.

Ionisation by matrix-assisted laser desorption

The classic ion sources use sample evaporation by heating and ionisation through a high energy beam of electrons (approx. 70 eV). This technique may lead to a breakdown of the analyte, which is of course undesirable for biological molecules. For this reason, «soft» methods for evaporation and irradiation of the sample have been developed. In fact, the application of mass spectrometry to fragile biomolecules has been the basis of part of the work that enabled Koichi Tanaka to win the Nobel Award for Chemistry in 2002.

MALDI meets this requirement by spraying (desorbing) and ionising sample molecules through non-destructive mechanisms which limit fragmentation. The two processes take place at the same time.

Sample preparation for MALDI is carried out by mixing the sample with a matrix present in large excess (matrix/sample ratio: about 10 000: 1). The composition of the matrix varies depending on the analyte and the type of laser used. It is usually a weak organic, non-volatile acid, with a chromophore absorbing light at the same wavelength of the laser used. The five most frequently used compounds are sinapinic acid, 2,5-dihydroxybenzoic acid, 2,4-hydroxyphenyl benzoic acid (DHB), α-cyano-4-hydroxycinnamic acid and ferulic acid [7] (Lay 2001). The mixture is usually deposited on a metal support called target. After crystallisation of matrix and compound, the target is introduced into the ionisation spectrometer. The surface of the target is then bombarded with high-energy photons from a pulsed laser beam, normally a nitrogen laser with an emission wavelength of 337 nm. The laser energy is absorbed primarily by the matrix present on the surface of the target plate. This leads to the desorption of the analyte into the gaseous state with minimal warming. This process causes vaporisation of both ionic and neutral species (Fig. 3). Polar compounds such as proteins attract protons from the matrix, which is a weak acid and becomes charged positively. This may take place both in the solid and in the gaseous phase above the target desorption surface. In both cases an analyte-proton gas phase is formed. Neutral species are not detected by the spectrometer and charged matrix particles are ignored because of the very different m/z as compared with that of the sample.

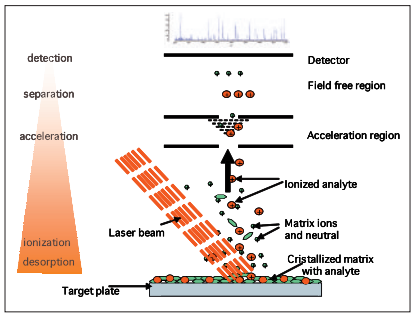

Figure 3
The MALDI-TOF process. Courtesy of M. Welker, Anagnostec GmbH, modified.

MALDI mass spectrometry (Fig. 4) can be used to detect non-volatile and thermally unstable molecules from a few to several hundred kDa; the usual interval is 2 to 20 kDa [2, 3, 4].

This technique, usually coupled with a TOF (time of flight) analyser, is used not only for the study of peptides and proteins, but also to analyse polymer sugars or nucleic acids. In particular, proteins are an ideal target for analysis because they are good acceptors of

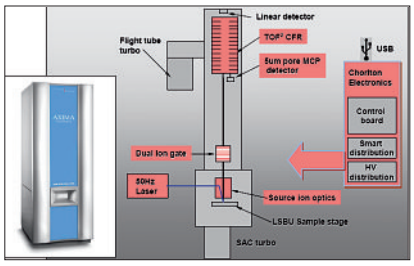

Figure 4
Model of a mass spectrometer. Courtesy of Shimadzu Ltd.

protons and have masses much higher than those of the protonated matrix compounds. The technology also produces a single component in the spectrum for each charged molecule. Fragmentation may occur, but results in particles of such magnitude that they can easily be distinguished in the spectrum. Hence each component in a mixture may be associated with a significant signal.

MALDI has also been applied to the analysis of proteins isolated from bacterial extracts, including those that can be used in the taxonomic classification of bacteria. The great advantage of this method lies, however, in the possibility of detecting proteins with taxonomically relevant characteristics, and its ability to identify microorganisms directly from whole cells without prior stages of separation, fractionation or washing [1].

The time of flight analyser (TOF)

The TOF analyser is often coupled with a MALDI ionisation system (known as MALDI-TOF MS) for the analysis of biomolecules.

The device is simple and consists of a metal tube subjected to a vacuum. A high voltage (ca. 20 000 V during some µs) is generated in the ionisation chamber and creates an electric field that transmits the kinetic energy to the formed ions. The ions are accelerated, move from the target to the detector and during this trip are separated according to their speed. Thus, the time spent to reach the detector (flight time) is dependent on the mass and charge of each ion.

Often the value of the charge is 1 and m/z corresponds to the numerical value of the mass. Small ions move faster than larger ions.

The detector placed at a fixed distance from the source of ions allows correlation of the time employed by the ions to reach it in relation to the m/z.

A TOF analyser is characterised by a fast scanning speed (flight times are usually in the order of milliseconds), a large mass range (few kDa to over 300 kDa) [1], which is essential for analysis of biomolecules with high molecular weight and high sensitivity. Its main limitation is low resolution power, which is in the range of 300 to 400 and does not distinguish between peptides with masses differing by only a few Da. The resolution of TOF systems could be improved by introducing reflectron systems (Fig. 5) making it possible to reach resolutions close to 5000, though this reduces the quantity of ions detected.

Identification of microorganisms by MALDI-TOF mass spectrometry

The versatility and speed of this method have rapidly conquered several fields related to the characterisation of microorganisms. A Medline search carried out using the keywords «microbiology taxonomy MALDI-TOF» has given 61 hits between 1998 and 2008, of which 49 were published after 2003. In fact this technique now plays an important role in water, air and food quality monitoring, in medical and veterinary diagnostics, forensic investigations (including cases of bioterrorism attacks), and environmental microbiology [1, 2, 3, 5 , 6].

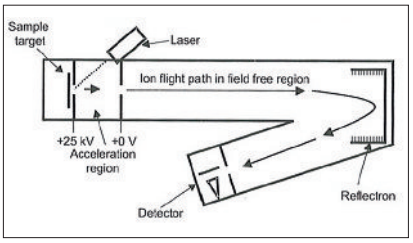

Figure 5
Diagram of a TOF analyser: reflectron mode (high resolution and low sensitivity for masses ≤5 kDa).

Principle

MALDI-TOF MS can be used for the identification of bacteria, viruses, fungi and spores [2, 3, 7, 8, 9].

Sample preparation is straightforward and spares the researcher time-consuming separation steps. The turnaround analysis time is very short (a few minutes), thanks inter alia to the possibility of using whole, untreated cells for the analysis. These are transferred to support plates as suspension and/or directly by picking the colony from a solid medium. A small volume (e.g. 0.5 microlitres) of matrix solution (DHB or α-cyano-4-hydroxycinnamic acid) is then poured over the spotted microorganisms, thus causing the disruption and death of cells and the formation of matrix crystals harbouring the molecules needed for analysis. In-

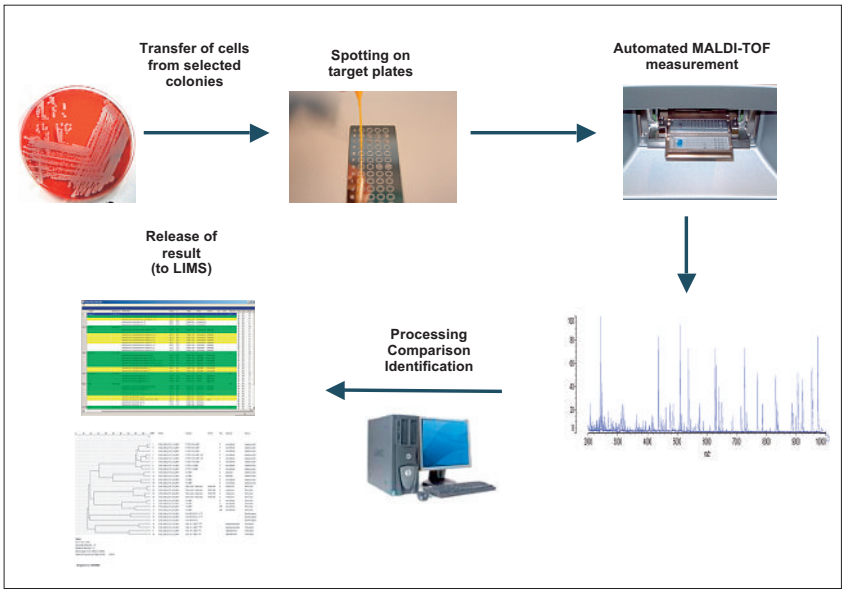

Figure 6
General workflow for microorganism identification/classification.

activation with trifluoric acid prior to the analysis is performed only for highly pathogenic microorganisms as a precautionary biosafety measure [1, 2, 3]. In general, colonies or a centrifuged portion of a liquid culture are directly used, while other MS techniques linked with chromatography or electrophoresis call for prior treatments for separation of the components of interest [2].

The resolution power of MALDI-TOF MS is very large since characterisation of whole cells involves analysis of a very complex mixture of molecules. A good resolution is the starting point for obtaining reproducible and taxonomically valid spectral profiles [1]. The sensitivity of the method is such that the detection limit lies around 10^4–10^5 cells, depending on the species of microorganism investigated [3, 7].

Identification of microorganisms

The identification of microorganisms by MALDI-TOF mass spectrometry is based on the detection of mass signals from biomarkers that are specific at genus, species or sub-group level [1]. Protein biomarkers are the most characteristic and accessible molecules in the analysis of whole cells, because they provide good signals without having to go through extraction, separation or amplification steps. Moreover they constitute some 50% of the dry weight of vegetative bacterial cells, compared with 5–8% of polar lipids and 0.01% of RNA. DNA, on the contrary, is present only in one copy per cell and is therefore inaccessible [2].

A MALDI-TOF spectrum from whole bacterial cells typically contains 10–30 peaks with masses ranging from 2 to about 20 kDa. Their ions after desorption are considered intact proteins, because most prokaryotic proteins belong in this interval [2], as shown in Fig. 7.

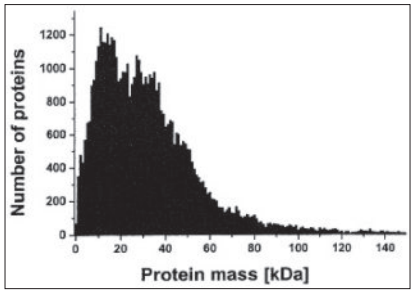

Figure 7
Mass dependent distribution of prokaryotic proteins (from the Swiss-Prot database).

Prerequisites for correct identification of microorganisms with MALDI-TOF are the use of type isolates and inclusion of the results of their characterisation in a database consisting either of a library of spectra of known samples or of proteins against which new identifications can be compared (Fig. 8). The quality of the database to be used to compare the spectrum of the unknown microorganisms is crucial. At present libraries of known spectra that permit identification of more than 90% of clinically relevant bacteria are commercially available (e.g. SARAMIS™ Anagnostec, http://www.anagnostec.eu/; Maldi Biotyper™, Bruker, http://www.bdal.com/; MicrobeLynx™, http://www.waters.com/). In the case of protein samples, the information accessible through genomic databases can be used, but these are not standardised for taxonomic use [1].

Identification by comparison of unknown to reference spectra involves analysis of the total spectrum and comparison of only distinctive peaks. Comparing specific peaks is more selective, because it "weighs" peaks specific for a given microorganism, excluding those originating from background noise or exogenous factors.

Comparing total spectra is also possible and provides a fingerprint of the whole reference organism. On the other hand, all peaks are used for the analysis even if they are not specific for the microorganism.

For the definition of reference spectra it is important to use rigorous algorithms that take into account the experimental variables affecting the spectra. The reproducibility of a spectrum, which is primarily related to resolution power, may be affected by different factors such as growing conditions, growth media, the type of matrix, solvents and instruments (fig. 8) [7, 10, 11, 12].

In general, organisms belonging to different genera have very different spectra with few or no shared peaks. Conversely, at the species and subgroup level, spectra are increasingly similar and differences may be limited to only a few (3–4) peaks [1, 13] that correspond to specific biomarkers for the organism analysed and are often expressed under all culture conditions, because in many cases they represent highly expressed housekeeping proteins such as ribosomal proteins [11, 14]. On the other hand, the abundance of signals is dependent on cultural conditions. Thus, the comparison and therefore the characterisation of spectra must be carried out with software that considers in particular the m/z ratio rather than the abundance of ions [1].

The specificity of MALDI-TOF identification is very good. In our lab, 1021 bacterial isolates from our routine diagnostic work were analysed using the Axima Confidence™ MALDI-TOF (Shimatzu Ltd.) coupled with the Saramis™ database (Anagnostec GmbH) and the identification results were compared to those obtained with the biochemical testing system Phoenix™ (Becton Dickinson).

For 967 strains (95%) the results of MALDI-TOF MS corresponded to those obtained with the Phoenix system. In 63% of the discordant results the MALDI-TOF MS identification was confirmed by additional biochemical methods (API) or PCR/sequencing. Thus, MALDI-TOF was able to identify correctly more than 98% of the isolates tested [15].

Taxonomic research

MALDI-TOF has been widely used to identify and characterise microorganisms isolated from clinical and environmental samples.

Adenovirus serotype 5, intact spores of *Bacillus cereus* and the yeast *Saccharomyces cerevisiae* are among the organisms that have been studied and identified using MALDI-TOF [2].

Species in the fungal genera *Serpula*, *Coniophora* and *Antrodia* have also been identified using MALDI-TOF spectra [16]. In our lab, MALDI-TOF MS has proven to be a powerful, reliable, rapid and relatively inexpensive method of identifying *Trichoderma* strains at the species level. Further studies are ongoing to evaluate the usefulness of the method for intraspecific characterisation [15].

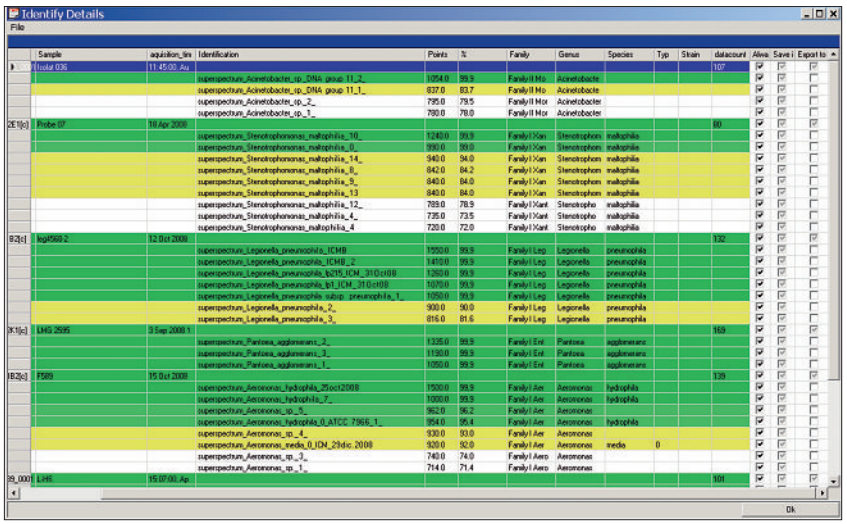

Figure 8
Result of a typical identification by comparison of spectra with SARAMIS™. Green: perfectly matching unknown organism and reference; Yellow: identification with a wider error interval; White: correspondence lower than 80%.

Slow growing, fastidious bacteria such as *Campylobacter coli*, *C. jejuni*, and other species in this genus [18], as well as some species of mycobacteria, have also been successfully identified by MALDI-TOF analysis [19]. In 1999, Hathout and co-workers characterised biomarkers useful for the identification of spores of *Bacillus* species (*B. subtilis*, *B. thuringensis*, and *B. anthracis*) [20]. Another group compared the phylogenetic trees of the genera *Myxococcoccus* and *Corallococcus* obtained by MALDI-TOF MS and by sequence analysis of the genes 16S rRNA and *gyrB*, and showed a high correlation between both methods [21].

Other groups have focused their efforts on the development of standard protocols, in particular to improve the reproducibility of the analyses. Reproducible and reliable profiles suitable for the discrimination of 24 species of bacterial pathogens and non-pathogenic *Salmonella*, *Listeria* and *Escherichia* were produced by Mazzeo and co-workers [22]. *Salmonella* and *Acinetobacter* were studied by Ruelle et al. [10], who developed a standardised protocol that has been applied to 65 other bacterial species. Libraries of reproducible spectra have been obtained for several geno species of *Aeromonas* and *Legionella* [23, 24, 25], both of clinical and environmental origin. Arnold & Reilly [26] developed a mathematical approach with which to compare mass spectra and evaluate similarity, eliminating the subjective component of visual analysis.

The work of many research groups is now directed towards the production and collection of spectra from a variety of species in different growing conditions [4, 12, 27, 28]. Valentine et al. [4] compared the spectra of three bacterial species (*E. coli*, *B. subtilis* and *Y. enterocolitica*) grown on four different culture media and observed variations in spectra from one medium to another; they also observed the presence of some peaks that can be considered characteristic of the species and used for identification.

Another factor affecting the spectra is the age of the bacterial culture [7, 29]. The authors reported that the spectra of cultures grown in the same conditions vary considerably for number and intensity of the peaks depending on the incubation time [29]. Parisi and co-workers [30] demonstrated cyclical daily protein expression for *E. coli*, whereas Welham et al. [8] highlighted changes in spectra over time, with specific peaks, however, persisting even after three months. Conversely, *Bacillus atrophaeus* did not show any change in spectrum composition over time [31].

Wahl and co-workers [32] analysed mixtures of up to 9 bacterial species, each with known spectra. All but one mixture had their bacterial components properly identified.

Preliminary work indicates that MALDI-TOF may also be useful in genetic research to detect PCR products [7, 33, 34]. Several studies suggest that MALDI-TOF is a versatile tool for DNA and RNA fragment analysis after a preliminary PCR step [34–35]. Ikryannikova and coworkers [36] validated a MALDI-TOF MS minisequencing method for rapid detection of TEM-type beta-lactamases in *Enterobacteriaceae*.

Conclusions

MALDI-TOF is a well-established method for direct characterisation of bacteria, fungi, and even viruses. Several studies have shown that the results of MALDI-TOF analysis are similar to those obtained by phylogenetic analysis (PCR, sequencing technologies). In the diagnostic laboratory, the outcome of MALDI-TOF identifications is comparable to and as reliable as that originating from classic and established methods. The method is already in use in public health laboratories (e.g. National Institute of Health, Gaithersburg, Maryland, and Robert Koch Institute, Berlin, Germany) to identify prokaryotes of clinical importance to genus, species and subgroup level and certifiy strains. In Europe, in March 2009, the AXIMA@SARAMIS™ (Shimadzu Anagnostec) system for identification of microorganisms obtained the official accreditation by the DACH (German Accreditation Council – Chemistry), responsible for the accreditation of medical laboratories following DIN EN ISO 15189 in Germany.

MALDI-TOF is technically straightforward, rapid and sensitive. It requires minimal sample preparation and its costs are low after a steep initial acquisition price for the spectrometer. Reproducible mass spectra are crucial for the establishment of a validated database, but the quality of the spectra needs to be assessed depending on the influence of instrumental factors as well as the growth conditions of the microorganisms. Despite the variety of MALDI-TOF methods proposed for taxonomic analysis using whole cells,

no standard conditions for the production of characteristic spectra have yet been determined, and case-by-case validation is still needed before routine use of this method in diagnostic laboratories.

Current and future research must therefore be directed towards investigating the factors influencing reproducibility, the detection of biomarkers expressed in any condition and the development of standard protocols and systems aimed at facilitating data interpretation, comparison of spectra and research databases.

Unfortunately, speed of analysis is counteracted by the need to grow cells in culture media before analysis. The emergence of new technologies and strategies based on affinity capture of specific cells could make it possible to perform MALDI analysis with a smaller number of cells, thus omitting the preliminary stages of culture. This approach would considerably increase the already high potential of the method.

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