# MALDI-TOF MS for identification of food spoilage filamentous fungi

Nelson Lima[1] and Cledir Santos[2]

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Filamentous fungi (ff) are a diverse group of unique eukaryotic organisms currently accepted to belong to the Eumycota kingdom. They are ubiquitous in nature with an extraordinary ability to decompose plant wastes while also causing much spoilage of food commodities. Information about each ff, namely, morphological and molecular descriptions, including modern spectral data—MALDI-TOF MS, physiological and biochemical features, ecological roles, and societal risks or benefits is the key element in fungal identification. In order to attain a rapid ff identification in food the genomic and proteomic approaches are currently used. The present paper focus on the main contribution of MALDI-TOF MS to identification spoilage ff in food.

**Addresses**
[1] CEB-Biological Engineering Centre, University of Minho, Campus de Gualtar, Braga, Portugal
[2] Universidad de La Frontera, Facultad de Ingeniería y Ciencias, Departamento de Ciencias Químicas y Recursos Naturales, Temuco, Chile

Corresponding authors: Lima, Nelson (nelson@iec.uminho.pt), Santos, Cledir (cledir.santos@ufrontera.cl)

## Introduction

Fungi are eukaryotic organisms with a cell wall rich in glucans and chitin that survive by absorption of organic materials as source of energy and carbon (organochemoheterotrophs). They range in size from species with massive mycelia to microscopic single-cell yeasts. The large group characterised by hyphal growth which supports sexual reproductive structures (e.g., mushrooms) or asexual reproductive structures that produce conidia (spores) are referred as moulds or filamentous fungi (ff). Fungi are ubiquitous microorganisms known to produce a wide variety of secondary metabolites, including mycotoxins, which play important ecological role in diversification and adaptation of these microorganisms to plants cultivated for food and feed production. They are also a very diverse biological group referred as the kingdom of Fungi or, sometimes, as the 'Eumycota kingdom'.

Fungi as one of six kingdoms of life was introduced in 1949 [1] and a five kingdom system was advanced later by Whittaker [2,3•]. However, from the formal point of view, neither of these works included a Latin diagnosis as required by the International Code of Botanical Nomenclature and the name of this kingdom was therefore invalid until 1980 [4]. This kingdom comprises seven currently recognised phyla: namely, Basidiomycota (mushrooms, rusts, smuts, etc.), Ascomycota (sac fungi, yeasts, etc.) and the basal fungi Glomeromycota (arbuscular mycorrhizal fungi and relatives), Microsporidia (microscopic parasitic group), Blastocladiomycota (zoosporic fungi found in soil and fresh water habitats) and its 'sister' Neocallimastigomycota (microscopic anaerobic fungi), and the most ancient phyla, Chytridiomycota (microscopic and zoosporic fungi) [5]. Currently, this kingdom accepts the subkingdom Dikarya, which include the phyla Basidiomycota and Ascomycota, 10 subphyla, 35 classes, 12 subclasses, and 129 orders. However, fungal classification is very dynamic and the recent discovery of the new proposed phyla Cryptomycota (fungi isolated from aquatic environments) shows that even today we have only a limited understanding about fungi [6].

The fungal taxonomy progresses continuously with uninterrupted redefinitions of the fungal tree of life. The constant changes on fungal taxonomic schemes as well as the new proposed concept of 'one species one name', to avoid different names for the anamorphic and teleomorphic fungal states [7,8,9••], show that the fungal identification and classification remain difficult and for specialists only. To overwhelm this, some proposals have been published in order to make the fungal identification more practical, like for example, for mycotoxigenic ff in food industry, which consists of identifying a strain at genus level and then undertaking mycotoxin analysis avoiding the minutia of the species identification [10]. In addition, recently, Hawksworth [9••] published a list of the 100 fungi known to cause spoilage problems with the current names and selected synonyms. In Table 1 is shown, as example, a much more reduced list of relevant ff involved in spoilage of different food commodities.

Rapid and reliable identification of ff in food able to contaminate with mycotoxins or spoilage raw materials or

**Table 1**

**Food spoilage by filamentous fungi**

| Food spoiled | Species name |
|---|---|
| Cereals and dried fruits | *Aspergillus flavus*; *A. parasiticus*; *Fusarium graminearum*; *Fusarium* spp.; *Neurospora sitophila*; *Penicillium citrinum*; *P. verrucosum* |
| Fruits | *Botritys cinerea*; *Gloesporium* spp.; *Monilia* |
| Vegetables | *Alternaria* spp.; *Botritys cinerea*; *Cladosporium* spp.; *Fusarium* spp.; *Sclerotinia* |
| Meat, fish and eggs | *Aspergillus* spp.; *Eurotium*; *Penicillium* spp.; *Scopulariopsis* sp. |
| Raw milk | *Aspergillus glaucus*; *Aspergillus versicolor*; *Scopulariopsis* sp. |
| Jam and jellies | *Aspergillus glaucus*; *Penicillium corylophilum*; *Wellemia sebi*; *Xeromyces bisporus* |

processed food is of the extremely important. Nowadays, to address this desiderate, in contrast to classic methods of isolation, cultivation and ff identification using dichotomous keys or books with detailed diagnosis descriptions [11••,12••], more modern genomic and proteomic methods can be applied. The former involves the extraction of ff nucleic acids (DNA or RNA) and amplification using PCR approach. The subsequent step of house-keeping gene(s) (ITS, IGS, calmodulin, β-tubulin, etc.) sequencing is important to accomplish the ff identification. The latter methods is based on the proteome extraction and mass spectrum fingerprinting generation using mass spectrometry (MS) by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) [13••].

The present paper aims to summarise the main contribution of MALDI-TOF MS to the modern, fast and reliable identification spoilage ff in food.

## Identification of filamentous fungi

Fungal polyphasic identification aims at the integration of different taxonomic characters [14]. By using numerous techniques, it is assumed the level of variation in the technique can be reduced, although variation in the fungal intraspecies level remains. There is also the time and resources required to undertake a particular identification. What can be done in a fully equipped mycological centre is different to that in a laboratory with limited resources or in an industrial food laboratory where the practical approach based on fungal hygiene or mycotoxin characters can provide answers to specific problems. These factors intensify the need for the scientific community to find new and complementary fungal identification tools.

## MALDI-TOF MS

In the innovative paper published by Cain *et al*. [15], a new methodology for the identification of bacteria by MALDI-TOF MS was presented, where sample preparation involved minimal purification of cells. Two years later, it was described for the first time an improved method for the rapid identification of whole bacterial cells by MALDI-TOF MS, establishing the basis of the current methodology [16]. This stimulated the use of MALDI-TOF MS in fungal identifications where the *Aspergillus* section *Nigri* was used as a proof of concept. The results for the close related species of *A. niger*, *A. ibericus* and *A. carbonarius* of MALDI-TOF MS analysis, using mass range from 5000–20 000 Da, were similar to those of phylogenetic analysis giving a sound input for fungi identification and showing the potentialities of the method for taxonomic purposes [17]. The application of this technique for the identification of fungal samples is currently well-established based on the remarkable reproducibility for the measurement of constantly expressed and highly abundant proteins, such as ribosomal proteins, that are used to generate a fingerprint profile [18••,19–25].

### MALDI-TOF MS general principles of operation

MALDI-TOF MS runs through the soft ionisation of the molecules, resulting in minimum fragmentation [26]. MALDI-TOF MS involves subjecting a sample covered with an UV-absorbing matrix that functions as an energy mediator, to a pulsed nitrogen laser. When the laser shuts the matrix and analyte mixture generates a gas-phase analyte ions that they will be separated according to their mass-to-charge ratio ($m/z$) and, eventually, these ions are detected in detector (Figure 1).

The nitrogen laser operates at a wavelength of 337 nm, with an energy pulse of 170 mJ and with a pulse width of less than 3.5 ns. Matrices are chemical compounds generally containing aromatic moieties that transfer the absorbed photoenergy from the irradiation source to the surrounding sample molecules, resulting in minimum fragmentation [18••,27•]. Several matrices are commercially available and the choice depends on the laser wavelength used in each MALDI-TOF MS instrument. Choosing the appropriate matrix for the identification of filamentous fungi is crucial. Matrices are used as liquid solution and its final composition is constituted by the organic chemical compound that is the matrix properly dissolved in an organic solvent, generally ethanol and/or acetonitrile, and water. In order to help analyte ionisation a strong acid such as trifluoroacetic acid (TFA) is normally employed during matrix liquid solution preparation. The use of the appropriate matrix leads to an optimal signal/noise ratio

**Figure 1**
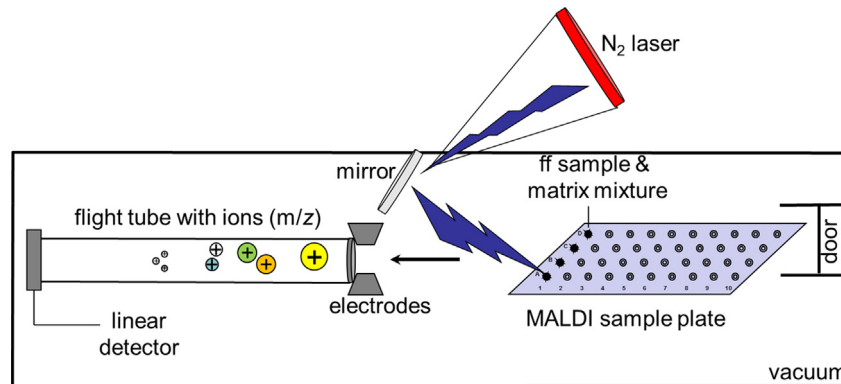


MALDI-TOF MS general principles of operation. After a gentile laser ionization of the sample mixed with matrix the ions fly, in the flight time tube (drift zone) in function of their mass-to-charge ratio (*m/z*), towards the detector.

with narrowest analyte peaks and little signal suppression. Currently, the two most commonly used matrices for filamentous fungi identification are 2,5-dihydroxybenzoic acid (DHB) and $\alpha$-cyano-4-hydroxycinnamic acid (CHCA). Both DHB and CHCA matrices are appropriate for the analysis of molecules, with a mass range between 5 000–20 000 Da, constantly expressed and highly abundant proteins, such as ribosomal proteins that appear in this specific mass range can be used as biomarkers.

## MALDI-TOS MS bench workflow

A general workflow is presented in Figure 2 which comprise the ff grown using standardised methods. Briefly, this can be obtained by selective media plates or tubes containing liquid media inoculated with the sample and incubated until enough biological material is observed. A tiny sample from the fresh colony is taken and directly transfer to the MALDI-TOF sample plate where CHCA matrix solution in 50% acetonitrile and 1.5% TFA is added. In liquid culture tubes the sample is taken from

**Figure 2**



**(step 1)**

**(step 2)**

**(step 3)**

**(step 4)**
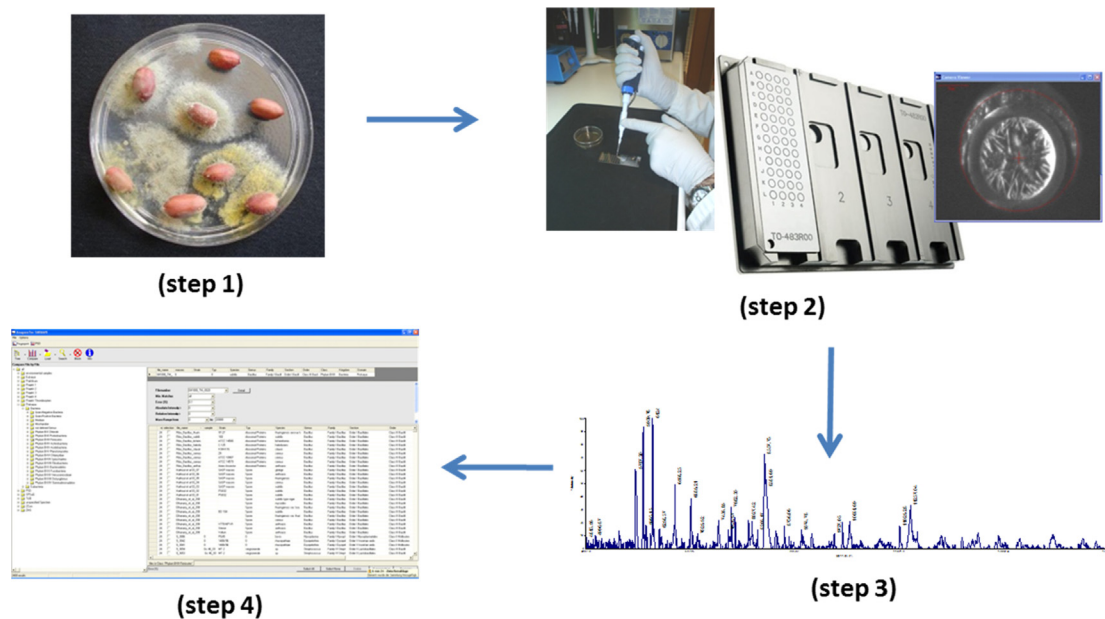
Workflow for direct fungal identification by MALDI-TOF MS. The fungal isolate (step 1) is directly transfer to the MALDI sample plate and mixed with CHCA matrix to extract the proteins (step 2) which are ionised by the laser shuts to generate the mass fingerprinting (step 3). Finally, the mass peak list is analysed and compared with the data in the library to obtain an identification (step 4).

the sediment and centrifuged for 2 min at full speed (e.g., 10 000 g). The supernatant is carefully removed and 1 mL distilled water is added to the pellet. The sample is vortexed for 1 min with washing, and re-vortexed. An extraction with ethanol is performed prior to sample transfer to the MALDI-TOF sample plate where CHCA matrix solution in 50% acetonitrile and 1.5% TFA is added. In both approaches the samples are then air dried at room temperature and analysed. Based on this knowledge, different studies have demonstrated the high potential of this technique for species and strain identification of ff [18$^{••}$,28].

### MALDI-TOF MS external calibration
External MALDI-TOF MS calibration has been performed by use of well characterised proteins from *Escherichia coli* strain K-12 [29]. From tens of ribosomal proteins of intact *E. coli* K-12 cells 13 well defined proteins are used as MALDI-TOF MS standard (4365.4, 5096.8, 5381.4, 6241.4, 6255.4, 6316.2, 6411.6, 6856.1, 7158.8, 7274.5, 7872.1, 9742 and 12 227.3 Da). The low costs, simple growth conditions, and reliability found in the *E. coli* biomarkers make them the first choice to be use in microbial identification.

### MALDI-TOF MS databases and identification
MALDI-TOF MS for the identification and classification of spoilage ff needs dedicated software and database [e.g., BioTyper$^{TM}$ (Bruker Daltonics Inc., Bremen, Germany) or VITEK$^{®}$ MS (bioMérieux, Craponne, France)] to enable comparisons of the unknown proteins with reference molecular masses. Ribosomal proteins are used normally as reference molecular masses as they are the most abundant ones in the cells, as mentioned above. However, commercial data bases of MALDI-TOF MS spectra have also limitations in their coverage of taxa, although this has expanded recently [30,31].

Commercial databases are based on the different protocols used by the main MALDI-TOF MS manufacturers and are widely available. In some cases, sample preparation protocols have been changed over the time even for the same database, as MALDI-TOF MS evolved and sample preparation to produce databases may have been developed in a generalised manner without using food spoilage ff samples. For example, sample optimisation may have occurred when comparing small differences in spectra from the same taxon. This evolution of sample preparation was not always accomplished with a data cleaning process or data storage conversion. Commercial databases for ff are less well-established and comprehensive than for bacteria.

For ff, and other, identifications by MALDI-TOF MS, commercial databases are built with software that uses a point system based on the peak list with mass signals weighted according to their specificity. Similarity

between individual spectra is expressed as the relative or absolute number of mass signals matching after subjecting the data to a single link agglomerative clustering algorithm analysis. In general, the protocols used in different laboratories are not standardised. A large number of publications contain different (1) protocols for protein extraction, (2) matrices for MALDI-TOF MS analysis and (3) growth conditions for fungi are available in the scientific literature. These can affect negatively the spectra quality and consequently fungal identification with potentially serious shortfalls in food risk assessment. The sources of variation are protein extraction, matrices, growth conditions and databases without even considering the natural variation of fungal strains.

## Conclusions and future prospects
MALDI-TOF MS is an accurate and rapid technique for identification of spoilage ff. It is inexpensive in terms of labour and consumables. However, general acceptance as the only procedure required for an identification will be realised only when more diverse taxa are studied employing extensive databases. The method adds an important additional range of characters for the generally-recommended polyphasic approach essential for food spoilage ff. The limitations of databases with limited coverage of representative fungal taxa and diverse sample preparation protocols demonstrate the need to standardise results and extend research and development. The intrinsic variation in characters of different strains of the same fungal taxa also needs addressing, although this is a general requirement addressed by the polyphasic approach identification.

## Acknowledgements

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