Diese Dissertation haben begutachtet

Univ. Prof. Dr. Günter Allmaier

Ao. Univ. Prof. Dr. Rainer Schuhmacher

DISSERTATION

Intact cell mass spectrometry of mycotoxin-producing *Fusarium* fungi for differentiation and protein identification

Ausgeführt zum Zwecke der Erlangung des akademischen

Grades eines Doktors der technischen Wissenschaften

unter der Leitung von

Univ. Prof. Dr. Günter Allmaier

E 164

Institute für Chemische Technologie und Analytik

Eingereicht an der Technischen Universität Wien

Fakultät für Technische Chemie

von

Mag. Hongjuan Dong

Matr. Nr. 0627656

Webgasse 7/2

1060 Wien, Österreich

Wien, im Februar 2010

Acknowledgements

My research and thesis would have not materialized if I did not have love, support, and encouragement from a number of people.

First of all I would like to express gratitude to Univ. Prof. Dr. Günter Allmaier, who gave me the opportunity to join his excellent research group and led me through the interesting and challenging project with exciting discussion. He contributed to my education with interesting lectures and suggestions on scientific writing. I am honored to present my thesis under his supervision.

My sincere thanks shall be addressed to Univ. Ass. Dr. Martina Marchetti-Deschmann, Dr. Ernst Pittenauer and Dr. Wolfgang Winkler. It is due to their technical support, the available fruitful discussion and the polishing of my written English, that the core of this thesis could be successfully performed.

It is my pleasure to extend my thanks to my colleagues Mag. Birgit Seyfried, M.Sc. Pelin Candarliouglu, Mag. Alexander Kassler, M.Sc. Ela Herwig, Mag. Stefan Bugovsky, Dipl.ing. Marlene Havlik, who provide me friendly helps and a great research environment. Special thanks shall be given to Dipl.ing. Marlene Havlik for her help of the scientific part written in German.

I would also like to thank secretaries of Institute of Chemical Technologies and Analytics, Miss. Romana Bischof and Miss Anna Satzinger, for their friendly helping and supportive attitude.

Thanks to numerous friends who endured this long process with me. Thanks to my parents, my young sister and brother for their encouragement, love and continuous support of my studies. Finally, I want to express greatest thanks to my husband, Yang Tian, for always offering supports and endless love.

Name der Kandidatin: Mag. Hongjuan Dong

Name der Prüferinnen: Univ. Prof. Dr. Günter Allmaier, Ao. Univ. Prof. Dr. Rainer Schuhmacher

Title der Dissertation: Intact cell mass spectrometry of mycotoxin-producing *Fusarium* fungi for differentiation and protein identification

Zusammenfassung

Schnelle und zuverlässige Identifizierung von Mikroorganismen wie Bakterien oder Pilzen ohne umfangreiche Probenvorbereitung ist von großer bedeutung im Bereich der Industrie, der Landwirtschaft und der Gesundheit. Die konventionellen Techniken wie Polymerase-Kettenreaktion (PCR) oder klassische mikrobiologische Methoden sind sehr zeitaufwendig und arbeitsintensiv. Des Weiteren führen sie oft nicht zu eindeutigen Resultaten. Die direkt Oberflächenproteinmusterbestimmung von Mikroorganismen durch „Intact Cell/Spore Massenspektrometrie (ICMS/ISMS) basierend auf matrix-unterstützter Laserdesorption/Ionisation und linearer Flugzeitmassenspektrometrie (MALDI LTOF MS) stellt eine alternative Methode dar, die für die Erfüllung dieser Aufgabe geeignet erscheint. Der Schwerpunkt der vorliegenden Arbeit liegt in der Weiterentwicklung eines Probenvorbereitungsprotokolls für die Zwecke der Differenzierung und Identifizierung von *Fusarium* Spezies und Isolaten, die Mykotoxine produzieren, mit Hilfe der MALDI LTOF MS (IC-MS).

Wegen der Komplexität und Vielfalt von Mikroorganismen, gibt es keine standardisierten ICMS oder ISMS Protokolle, die auf ein breites Spektrum von Mikroorganismen angewendet werden können. So ist die Evaluierung der Probenvorbereitung, d.h. die Ernte und Reinigung der *Fusarium* Konidiensporen aus dem Kulturmedium und die Entwicklung einer geeigneten Probenpräparation für die MALDI massenspektrometrische Analyse notwendig und unverzichtbar für die Generierung reproduzierbarer massenspektrometrischer Peptid/Protein-Muster. Auf der Grundlage dieser Untersuchungen wurde eine optimierte MALDI MS Methode entwickelt, die für die erfolgreiche Differenzierung und Identifizierung von fünf *Fusarium* Stämmen und zahlreichen Isolaten geführt hat. Ein ernsthaftes Problem zusammen mit dem zugehörigen Kultivierung und Sammlung von *Fusarium* Sporen ist, dass die gewonnenen und gewaschenen Sporen of gefärbt sind, nämlich von leicht orange bis rot-braun. Die Anwendung der bereits optimierten MALDI MS Probenvorbereitungsmethode auf die stark gefärbten *Fusarium* Sporen, führte zu sehr wenigen massenspektrometrischen

Peaks oder überhaupt zu keinen brauchbaren Massenspektren. Um dieses Problem zu minimieren wurde die MALDI Probenvorbereitung weiter optimiert und schließlich wurde eine geeignete Methode für die Probenvorbereitung für die IC-MS Analyse von schwach und stark gefärbten *Fusarium* Sporen für die Differenzierung der verschiedenen *Fusarium* Spezies mit hoher Zuverlässigkeit entwickelt.

Für die Überprüfung des Vorhandenseins von Peptiden/Proteinen in den IC Massenspektren wurde eine Strategie entwickelt, in der die Spaltung der Oberflächenproteine der *Fusarium* Sporen mit dem Enzym Trypsin am Target durchgeführt wurde, gefolgt von „Post Source Decay“ Fragmentionenanalyse mit einem MALDI Reflektron Flugzeitmassenspektrometer. Diese Strategie ist schnell, einfach in der Durchführung und die erzielten PSD Spektren waren zwar sehr komplex, aber dennoch interprätierbar, sodaß eine manuelle *de novo* Peptidsequenzierung möglich war. Damit konnten dann Proteine von *Fusarium* Konidiensporen mit BLAST Suche eindeutig identifiziert werden. Darüberhinaus wurde die Wirksamkeit von immobilisierten und nicht-immobilisierten Trypsin in dieser Strategie verglichen. *On-target* Trypsinverdau von *Fusarium* Sporen vermeidet zeitaufwendige Isolierungs- und Fraktionierungsverfahren von Proteinen und bietet sich ideal als Methode zur Identifizierung von Sporenproteinen an. Diese Strategie ist geeignet zur Verifizierung der Anwesenheit von Proteinen in den IC Massenspektren und zur Identifizierung der Oberflächenproteine von Sporen.

Name der Kandidatin: Mag. Hongjuan Dong

Name der Prüferinnen: Univ. Prof. Dr. Günter Allmaier, Ao. Univ. Prof. Dr. Rainer Schuhmacher

Title der Dissertation: Intact cell mass spectrometry of mycotoxin-producing *Fusarium* fungi for differentiation and protein identification

Abstract

Rapid and reliable differentiation and identification of microorganisms such as bacteria or fungi without extensive sample handling is of great importance in the field of industry, agriculture and public health. However, the conventional technique like polymerase chain reaction (PCR) or polymorphisms normally are time consuming and labor intensive. Direct profiling of microorganisms by intact cell/spore mass spectrometry (ICMS/ISMS) based on matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometry (MALDI LTOF MS) provided an alternative research tool which is capable of fulfilling the mentioned tasks. The main focus of the present work is the further development of a sample preparation protocol on the purpose of identifying and differentiating mycotoxin-producing fungi with the focus on *Fusarium* species by intact cell MALDI LTOF MS (IC-MS).

Due to the complexity of microorganisms, there are no standardized ICMS or ISMS protocols which can be applied to a broad range of microorganisms. So the evaluation of sample pretreatment, i.e. the collection and washing of *Fusarium* conidia spores from the culture medium and the development of a proper sample preparation technique for MALDI mass spectrometric analysis are necessary and crucial for acquiring reproducible peptide/protein mass spectrometric profiles. Based on these investigations an optimized MALDI MS method was first developed which resulted in the successful identification and differentiation of *Fusarium* conidia spores of five reference species. However, a serious problem accompanying along with the culturing and collection of *Fusarium* conidia spores is that the extracted and washed *Fusarium* spores showed colored layers of various types and intensities from slightly orange to deep red-brown. Application of the previous optimized MALDI sample preparation methods to the deep-colored *Fusarium* spores resulted in very few mass spectrometric peaks or no useful mass spectrum at all. To minimize this problem the MALDI sample preparation was further optimized and finally a suitable sample preparation method for IC-MS analysis of weakly and strongly colored *Fusarium* conidia spores has been developed allowing the differentiation of various of *Fusarium* species and strains with high reliability.

Furthermore for verification of the presence of peptides/proteins detected in the IC mass spectra, we proposed a strategy in which the cleavage of the *Fusarium* spore surface proteins with the enzyme trypsin was carried out by using *on-target* spore digestion, followed by MALDI reflectron TOF post source decay fragment ion analysis. This strategy turned out to be rapid, easy to operate and the obtained PSD spectra were complex but still interpretable, which allowed us to tentatively assignment of peptide sequences using manual *de novo* sequencing. Therefore the identification of *Fusarium* conidia spore surface proteins by BLAST database search could be accomplished. Additionally the effectiveness of immobilized and non-immobilized trypsin for on-target conidia spore digestion has been compared and evaluated, resulting in a maximum of peptides if applying the non-immobilized enzyme. *On-target* spore tryptic digestion method avoids the previously used time-consuming isolation or fractionation procedure and provided further information on the proteins of *Fusarium* spores. This strategy is a proper way to verify and identify the presence of peptides/proteins on the surface of intact and very resistant fungal spores.

Table of content

1. Characterization of microorganisms by intact cell mass spectrometry (ICMS)

1.1 Introduction

Mass spectrometry has been proved to be a powerful analytical tool for rapid classification and identification of microorganisms. In 1975, pyrolysis-combined electron ionization mass spectrometry was first demonstrated for characterization of certain pathogenic bacteria species [1]. Since the late 1980s, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) has found many applications in many areas, mainly in the proteomics field [2, 3]. MALDI is considered to be a soft desorption/ionization technique generating ions from intact molecules without fragmentation of the analyte. It also offers relative high tolerance against contaminants such as salts or detergents. Researchers recognized the capability of this instrumentation and were quick to adapt methodologies to analyze cell extracts as well as intact cells. In many aspects it is superior to classical microbiological techniques and avoids laborious as well as time consuming sample preparation. Intact cell MALDI TOF MS (IC-MS) was brought to analysis of microorganisms in 1996 based on their specific biomarkers [4] or spectral patterns [5]. It provides large amounts of information in a simple straightforward protocol. The term "intact cell" here refers to the microbial cells suspend in a solution without any sample fractionation but should not be considered that the cells are architecturally intact or whole. In fact, the exposure to water, organic solvent or the strong organic acid in the MALDI matrix solution made cell wall ablation to a certain extant possible [6]. IC-MS has only small amount of sample volume requirements, minimal sample preparation and can be automated. It can yield very specific peptide/protein profiles for different species/strains with fast speed. All this advantages have made IC-MS itself the most suitable and popular technique for rapid analysis of microorganisms [7-12]. Through computer-assisted comparison of spectral fingerprints obtained directly from intact cells or the identification of specific biomarkers by searching internet-accessible protein database, microorganisms on genus, species level, even on strain level can be identified. Now, practically all rapid MS characterization of microorganisms falls under the umbrella of IC-MS. Many other applications of this technique have also been described [13, 14].

The application of IC-MS to achieve direct peptide/protein profiles of microorganisms especially for bacteria is now well established [15-18]. By far a number of bacterial cells have been analyzed by IC-MS such as *Enterobacteriaceae* [19], *Haemophilus* [20], *Cyanobacteria* [21], *Bacillus* [9, 22-24], *Eschericia coli* [7, 19], *Staphylococcus aureus* [25-27], *Helicobacter* [11, 12], *Camplyobacter* [11], *Arthrobacter* [28] *Brukholderia* [29] and so on.

This method is extremely quick. Bacteria culture on an agar plate, sample harvest and mixed with MALDI MS matrix, mixture deposition onto the MALDI MS target, air-dried before transferred into the mass spectrometer. Even though only a fraction of proteins are detected, each bacterial strain can generate characteristic spectral pattern and provide unambiguous identification. In addition to identification of microorganisms from pure bacterial strains, IC-MS has been extended to analyze mixtures of bacteria known as blind study [30] successfully with computer-supported algorithm. With modification of the sample preparation technique, IC-MS has also been successfully applied to the identification of bacterial spores of gram-positive strains of *Bacillus* [23]. *Bacillus* species identification based on whole bacterial spores has also been developed by Ullom and his coworkers by utilizing matrix-free infrared laser desorption and ionization mass spectrometry [31].

The application of IC-MS for typing various intact microorganisms and the use of different mass spectral protocols has extensively reviewed [14], mostly focuses on the analysis of bacteria. Comparatively, mass spectral characterization of intact fungal cells [32-34] or fungal spores [6, 35-38] are not yet well described in the literature. Fungi are physically differentiated from bacteria by their larger size. They have rigid walls which architecturally, but not chemically, resemble plant cells. Fungal cell walls are generally 80-90% polysaccharide, including the presence of the long chain carbohydrate polymer chitin which adds rigidity and structural support to the thin cells. Proteins, lipids and polyphosphates together with inorganic ions make up the cell wall cementing matrix. The IC-MS technique was first applied to fungal species analysis in the year of 2000 by Welham and his coworkers [35]. Nine different matrices were comparative evaluated for the purpose of biomarker selection and pattern recognition. In the same year four different strains of *Aspergillus falvus* have been analyzed by IC-MS [6]. The unique characteristic peak pattern representing *Aspergillus* species was obtained and the differentiation between aflatoxigenic and non-aflatoxigenic strain was successfully illustrated. By far most studies of intact fungal species analysis by MALDI TOF MS focus their work on the sample preparation method evaluation [37, 38] (matrix selection, pretreatment method such as sonication and so on) and biomarker detection [39]. More recently Two-dimensional canonical discriminant plot for 12 different *Aspergillus* species as well as their cluster analysis dendrogram based on MALDI MS data of cellular extracts but not intact cells was successfully constructed. The 12 species of fungi of genus *Aspergillus* (100% accuracy) and 5 strains of *Aspergillus flavus* (95-100% accuracy) have been classified by discriminant analysis of the reproducible MALDI MS data [40].

In summary, IC-MS as a phenotyping method offers an attractive alternative to well established methods for microorganisms characterization, for example, biochemical analysis, polymerase chain reaction or gas/liquid chromatography. The successful use of comparison of spectral patterns for strain differentiation has to be dependent upon the reproducibility of the spectra. Some of the difficulties involved in the IC-MS analysis have been the complexity of the spectra and their reproducibility.

1.2 Sample preparation for intact cell MS analysis

As it is well-known that sample preparation is critical to the successful MALDI analysis, a number of protocol parameters that affect spectral signal and reproducibility have also been published [27, 41]. Several parameters are important for IC-MS analysis of microorganisms. The first parameter is the concentration of the sample. While only small volumes (μL) of sample are needed for IC-MS analysis, approximately 10^6 intact cells per microliter are routine use. Removal of growth media as well as other contaminants from the microorganisms is also important for successful IC-MS analysis.

The simplest sample collection in earliest stage is the removal of the microorganism from the growth media (e.g. agar) and deposit the sample directly onto the sample support before addition of MALDI MS matrix and MALDI analysis. However, material from culture media will be unavoidably included in samples which resulted in somewhat changes of mass spectral pattern. It is interesting that Valentine and his coworkers [33] slant fungal cultures using a double-stick tape and applied directly to MALDI plate. Although it is possible to get better result with some pretreatment method, if a rapid screening is needed, this double-stick tape sampling method provides a viable option. One effective way to get rid of liquid growth media is to use centrifugation and wash the cell pellets with different solvents such as pure water [32, 38] or 2% ammonium chloride [33, 41].

A procedure for disrupt the cell walls of microorganisms especially fungal cells is often needed or considered to be essential in order to get or improve the signal-to-noise ratio. Several techniques for cell wall disruption such as ultrasonication, glass beads, and corona plasma discharge has been investigated [32]. Most often addition of a strong organic acid such as trifluoroacetic acid (TFA) or formic acid (FA) is helpful to disrupt the cell walls. In our laboratory we also proved that a better quality of mass spectra can be obtained by washing our e.g. deep-colored *Fusarium* spores with the addition of FA or TFA [37]. It was reported that addition of methanol or ethanol has been effective in enhancing the signals of high molecular weight biomarkers from intact cells [37, 42]. It was also reported that dilution of

the cultures with methanol can improve the sample stability [11, 15]. The methanol-water mixture may fix the bacterial cell and preserve its structure [11].

Once the proper sample solutions are prepared well, the next step is to deposit the sample onto the MALDI MS target along with a MALDI MS matrix solution. MALDI MS matrix is used to mix with intact microorganisms to form co-crystals. The MALDI matrix absorbs the laser light and the transferred energy is used for the purpose of desorption and ionization of intact proteins from the surface of the intact cells without fragmentation. Due to the complexity of the microorganisms, the researchers currently do not use consistent MALDI MS matrices and deposition techniques for IC-MS analysis. Numerous matrix compounds have been tested by different laboratories for performing the IC-MS analysis of microorganisms. It was reported that 2, 4-dihydroxybenzoic acid (DHB) was more efficient for the detection of lower mass secondary metabolites [43]. Sinapinic acid (SA) [11, 20] and ferulic acid (FA) [38, 41] are more suited for use with intact cells as well as intact spores better suited for higher molecular mass analytes.

The solvent system for dissolving the MALDI MS matrix compounds is also a crucial factor. It is not only has an impact on co-crystal structures but on the degree of the analyte incorporated into them, too. Usually the MALDI MS matrix was dissolved in a saturated condition in certain ratio of water and different organic solvents. Most often a strong organic acid such as TFA or FA are added to the matrix solution, which also provided dissolving ability to extract the biomolecules from the cell wall or bring them to the surface of the cell walls of intact microorganisms when it was mixed with intact cells or spores [6]. This works especially for the intact bacterial cells with small acid-soluble proteins as key components, because normally the solvent systems are kept at a low pH value by means of the addition of an organic acid [44].

Different deposition techniques (e.g. dried-droplet, thin layer, sandwich, etc.) have been developed by various laboratories based on their specific applications [37, 38]. Normally the dried-droplet technique is favored, but mainly just due to the simplicity. Several modified deposition techniques have also been developed for different microorganisms. For example the two-layer volume technique [37] can obtain mass spectra with a higher number of peaks and intensities compared to the application of the dried droplet technique.

1.3 Instrumentation

The intact microorganism analysis is commonly performed on an MALDI TOF instrument. This technique is of benefit to routine microbiology because of its speed of analysis and its relative low cost per sample. Instrument-related parameters have significant impact on the

successful IC-MS analysis. For most intact cell studies it was operated in the linear, delayed extraction and positive ion mode. Typically an acceleration voltage of 20 kV is placed on the sample plate. In intact cell analysis mostly UV-MALDI equipped with a nitrogen laser at 337 nm but sometimes Nd-YAG laser at 335 nm [5] are used. However, the using of infrared (IR) laser on a Fourier transform mass spectrometer for the analysis of intact cells has also been reported [45]. In most TOF analyser, the intact microorganism analysis can be performed on both positive and negative ion mode. However, studies showed that there are more positive ions detected in IC-MS than negative ions [16]. Hence positive ion mode of acquisition is preferred. The majority of proteins extracted from intact cells fall in the m/z range of 2000 - 20000. Commonly external calibration was used but calibration with internal standards [6, 36] has also been reported. Internal calibration is useful for accurate mass determinations. However, the problem accompanying with internal calibration is competitive desorption/ionization between internal standards and the components from the intact cells. As a result a suppression of some analyte peaks would be occurred.

Other types of mass analyser have successfully been coupled with matrix-assisted laser desorption/ionization for the analysis of microorganisms, such as quadrupole-RTOF [46], and quadrupole ion trap [47].

1.4 Interpretation of intact cell mass spectra

One of the key difficulties as well as the most important step for IC-MS analysis is the interpretation of the data. Initial efforts focus on tabulating [10, 12] or comparing of biomarkers [24, 32, 48] which are specific to different genera, species or strains. Each microorganism species or strain has at least one or possibly more unique mass spectral peaks that can be used to unambiguously identify that species or strains. However, most work has used qualitative identification and this qualitative difference determination between different mass spectra is subject to personal bias. And in these cases the high reproducibility of the observed peaks rather than the peak intensities is emphasized. But many peaks appeared in all of the mass spectra at the same m/z value with only intensity variations. So the comparison of two mass spectra would like to be done in a quantitative and objective way based on peak locations as well as peak intensities. With the assistance of computers several algorithms for quantitatively compare and estimate the similarity between two mass spectra have been developed. In 1998 Arnold [7] developed a modified cross-correlation method to compare 25 *E. coli* entire mass spectra in which each spectrum is divided into different number of intervals. The program calculates the normalized correlation index which is varied from 0 to 1 (1 refers as perfect match) between two entire mass spectra based on both m/z value and ion

intensities. This quantitative method allowed the unambiguous differentiation of 25 *E. coli* pure strains as well as the identification of organisms by comparison with reference fingerprinting in the library. In 1999 Jarman [49] developed an automated and statistical approach for quantitative construction and visualization of MALDI MS fingerprinting. They extracted the essential biomarkers described by peak heights, locations, their standard deviation and the frequency of their occurrence from the replicate mass spectrum and used them to construct MALDI MS fingerprinting for identification rather than comparing the entire raw MALDI mass spectra. One benefit of this approach is the allowance of analysis of very dirty samples or samples containing mixtures of more than one species which is further validated in another paper [50]. Recently, differentiation of microorganisms on species or on strain level with cluster analysis and derived dendrograms based on it are becoming popular. Normally "clusters" of peaks were generated by selecting all peaks with certain S/N ratio (e.g. S/N > 3) using an automatic software tool or manually from the whole m/z range of interest.
The successful use of comparison of mass spectral patterns for strain differentiation is at least somewhat dependent upon the reproducibility of the mass spectra. It is to be noted that, for classification and identification of microorganisms, the reproducibility of the mass spectra is always an important issue as well as a problem especially for complex sample mixtures. Inconsistent appearance of peak patterns or m/z shifts can result in error assignments. Several reproducibility studies based on IC-MS has been reported [15, 41]. The factors that may influence the appearance of MALDI MS fingerprints include culture conditions (growth media and phase), MALDI MS sample preparation (matrix, deposition technique, matrix/sample ratio), and mass spectrometric instrument parameters. So the specific conditions for obtaining reproducible spectra should be carefully selected and executed.

1.5 Characterization of proteins from microorganisms

Today MALDI TOF MS of intact cells without pre-separation is one of the most popular approaches based on the characteristic biological molecules contained in the spectra. Since it was reported that the ions detected above m/z 4000 with IC-MS represent intact proteins from microorganisms [14], one way to produce additional information is deducing the total or at least partial amino acid sequence of intact proteins contained in IC-MS spectra after fragmentation in a tandem MS experiment. A major advantage of this method is that biomarker MS/MS spectra are obtained without the need for biomarker pre-fractionation, digestion, separation and cleanup. By far several studies utilized this approach for microorganism proteome characterization [51-53]. The unambiguous identification of one or more intact protein biomarkers by top-down proteomics allows successful microorganism

identification if the proteome database contains the protein sequences and respective organism. It is reported that in top-down proteomics the probability for positive matching of a protein but false matching of a microorganism is lower as compared to bottom-up shotgun proteomics [54]. Another way is through proteolysis, which results in a systematic production of polypeptide fragments by cleavage of proteins using enzymes referred as bottom-up proteomics. Traditional bottom-up proteolysis of complex microorganisms typically involves extensive separation of proteolytic peptides by high-performance liquid chromatography. In order to avoid time-consuming separation and isolation steps, *on-target* enzymatic digestion of intact cells and lyses combined with MALDI MS analysis has been developed by many research groups [55-58]. It is not necessary to employ any prior complicated separation or extraction procedure but only minimal sample washing treatment is sufficient. It is especially attractive when rapid analysis of proteins in microorganisms is needed.

1.6 Conclusion

Success has been demonstrated in the application of IC-MS for differentiation and identification of microorganisms. So far there is no unique or standardized sample preparation method/protocol for IC-MS analysis due to the huge number of complex microorganisms. The identification of microorganisms based on IC-MS need to be constructed on the requirements that all experimental variables such as sample purity or sample to matrix ratios and so on should be controlled in a reproducible way. However, it provided a very rapid method for analyzing the proteins desorbed directly from the intact cells without tedious separation procedure and only small volume sample solutions was needed. So in the future it can be expected that the IC-MS technique will continue to be used for such demanding applications.

1.7 Reference

1. Anhalt JP, Fenselau C. Identification of bacteria using mass spectrometry. Anal. Chem. 1975. 47: 219-225.
2. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 1988. 2: 151–153.
3. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10000 daltons. Anal. Chem. 1988. 60: 2299-2301.

4. Krishnamurthy T, Ross PL, Rajamani U. Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 1996. 10: 883-888.

5. Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees Kj, Lay JO Jr. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 1996. 10: 1227-1232.

6. Li TY, Liu BH, Chen YC. Characteritation of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 2000. 14: 2393-2400.

7. Arnold RJ, Reilly JP. Fingerprint matching of *E. coli* strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correction approach. Rapid. Commun. Mass. Spectrom. 1998. 12: 630-636.

8. Welham KJ, Domin MA, Scannell DE, Cohen E, Aston DS. The characterization of microorganisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 1998. 12: 176-180.

9. Leenders F, Stein TH, Kablitz B, Franke P, Vater J. Rapid typing of *Bacillus subtilis* strains by their secondary metabolites using matrix-assisted laser desorption/ionization mass spectrometry of intact cells. Rapid. Commun. Mass. Spectrom. 1999. 13: 943-949.

10. Demirev PA, Ho YP, Ryzhov V, Fenselau C. Microorganism identification by mass spectrometry and protein database search. Anal. Chem. 1999. 71: 2732-2738.

11. Winkler MA, Uher J, Cepa S. Direct analysis and identification of *Helicobacter* and *Campylobacter* species by MALDI-TOF mass spectrometry. Anal. Chem. 1999. 71: 3416-3419.

12. Nilsson CL. Fingerprinting of *Helicobacter pylori* strains by matrix-assisted laser desroption/ionization mass spectrometric analysis. Rapid. Commun. Mass. Spectrom. 1999. 13: 1067-1071.

13. Lay JO Jr. MALDI-TOF mass spectrometry of bacteria. Mass. Spectrom. Rev. 2002. 20: 172-194.

14. Fenselau C, Demirev PA. Characterization of intact microorganisms by MALDI mass spectrometry. Mass. Spcetrom. Rev. 2001. 20: 157-171.

15. Wang Z, Russon L, Li L, Roser DC, Long SR. Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 1998. 12: 456-464.

16. Evason DJ, Claydon MA, Gordon DB. Effects of ion mode and matrix additives in the identification of bacteria by intact cell mass spectrometry. Rapid. Commun. Mass. Spectrom. 2000. 14: 669-672.

17. Liu H, Du Z, Wang J, Yang R. Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Appl. Environ. Microbiol. 2007. 73: 1899-1907.

18. Dieckmann R, Graeber I, Kaesler I, Szewzyk U, von Döhren H. Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by intact-cell-MALDI-TOF mass spectrometry (ICM-MS). Appl. Microbiol. Biotechnol. 2005. 67:539-548.

19. Conway GC, Smole SC, Sarracino DA, Arbeit RD, Leopold PE. Phyloproteomics: species identification of *Enterobacteriaceae* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Mol. Microbiol. Biotechnol. 2001. 3: 103-112.

20. Haag AM, Taylor SN, Johnston KH, Cole RB. Rapid identification and speciation of *Haemophilus* bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Mass. Spectrom. 1998. 33: 750–756.

21. Erhard M, von Döhren H, Jungblut P. Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI-TOF mass spectrometry. Nat. biotechnol. 1997. 15: 906-909.

22. Ruelle V, EI Moualij B, Zorzi W, Ledent P, Pauw ED. Rapid identification of environmental bacteria strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 2004. 18: 2013-2019.

23. Hathout Y, Demirev PA, Ho YP, Bundy JL, Ryzhov V, Sapp L, Stutler J, Jackman J, Fenselau C. Identification of *Bacillus* spores by matrix-assisted laser desorption ionization – mass spectrometry. Appl. Environ. Microbiol. 1999. 65: 4313-4319.

24. Ryzhov V, Hathout Y, Fenselau C. Rapid characterization of spores of *Bacillus cereus* group bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Appl. Environ. Microbiol. 2000. 66: 3828-3834.

25. Rajakaruna L, Hallas G, Molenaar L, Dare D, Sutton H, Encheva V, Culak R, Innes I, Ball G, Sefton AM, Eydmann M, Kearns AM, Shah HN. High throughput identification of clinical isolates of *Staphylococcus aureus* using MALDI-TOF-MS of intact cells. Infect. Genet. Evol. 2009. 9: 507-513.

26. Moura H,Woolfitt AR, Carvalho MG, Pavlopoulos A, Teixeira LM, Satten GA, Barr JR. MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive *Streptococcus pyogenes* isolates. FEMS. Immunol. Med. Microbiol. 2008. 53: 333-342.

27. Jackson KA, Edwards-Jones V, Sutton CW, Fox AJ. Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. J. Microbiol. Methods. 2005. 62: 273-284.

28. Vargha M, Takáts Z, Konopka A, Nakatsu CH. Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. J. Microbiol. Methods. 2006. 66: 399-409.

29. Miñán A, Bosch A, Lasch P, Stämmler M, Serra DO, Degrossi J, Gatti B, Vay C, D'aquino M, Yantorno O, Naumann D. Rapid identification of *Brukholderia cepacia* complex species including strains of the novel Taxon K, recovered from cystic fibrosis patients by intact cell MALDI-TOF mass spectrometry. Anaylst. 2009. 134: 1138-1148.

30. Wahl KL, Wunschel SC, Jarman KH, Valentine NB, Petersen CE, Kingsley MT, Zartolas KA, Saenz AJ. Analysis of microbial mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 2002. 74: 6191-6199.

31. Ullom JN, Frank M, Gard EE, Horn JM, Labov SE, Langry K, Magnotta F, Stanion KA, Hack CA, Benner WH. Discrimination between bacterial spore types using time-of-flight mass spectrometry and matrix free infrared laser desorption and ionization. Anal. Chem. 2001. 73: 2331-2337.

32. Amiri-Eliasi B, Fenselau C. Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. Anal. Chem. 2001. 73: 5228-5231.

33. Valentine NB, Wahl JH, Kingsely MT, Wahl KL. Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry. Rapid. Commun. Mass. Spectrom. 2002. 16: 1352-1357.

34. Schmidt O, Kallow W. Differentiation of indoor wood decay fungi with MALDI-TOF mass spectrometry. Horzforschung. 2005. 59: 374-377.

35. Welham KJ, Domin MA, Johnson K, Jones L, Ashton DS. Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 2000. 14: 307-310.

36. Chen HY, Chen YC. Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry. Rapid. Commun. Mass. Spectrom. 2005. 19: 3564-3568.

37. Dong H, Kemptner J, Marchetti-Deschmann M, Kubicek CP, Allmaier G. Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry. Anal. Bioanal. Chem. 2009. 395: 1373-1383.

38. Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinina IS, Kubicek CP, Allmaier G. Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 2009. 23: 877-884.

39. Sulc M, Peslova K, Zabka M, Hajduch M, Vladimir H. Biomarkers of *Aspergillus* spores: strain typing and protein identification. Int. J. Mass. Spectrom. 2009. 280: 162-168.

40. Hettick JM, Green BJ, Buskirk AD, Kashon ML, Slaven JE, Janotka E, Blachere FM, schmechel D, Beezhold DH. Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Anal. Biochem. 2008. 308: 276-281.

41. Saenz AJ, Petersen CE, Valentine NB, Gantt SL, Jarman KH, Kingsley MT, Wahl KL. Reproducibility of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for replicate bacterial culture analysis. Rapid. Commun. Mass. Spectrom. 1999. 13: 1580-1585.

42. Madonna AJ, Basile F, Ferrer I, Meetani MA, Rees JC, Voorhees KJ. On-probe sample pretreatment for detection of proteins above 15 kDa from whole cell bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 2000. 14: 2220-2229.

43. Hathout Y, Ho YP, Ryzhov V, Demirev P, Fenselau C. Kurstakins: a new class of lipopeptides isolated from *Bacillus thuringiensis*. J. Nat. Prod. 2000. 63: 1492-1496.

44. Hathout Y, Setlow B, Cabrera-Martinez RM, Fenselau C, Setlow P. Small, acid-soluble proteins as biomarkers in mass spectrometry analysis of *Bacillus* spores. Appl. Environ. Microbiol. 2003. 69: 1100-1107.

45. Ho YP, Fenselau C. Applications of 1.06-μm IR laser desorption on a Fourier transform mass spectrometer. Anal. Chem. 1998. 70: 4890-4895.

46. Shevchenko A, Sunyaev S, Loboda A, Shevchenko A, Bork P, Ens W, Standing KG. Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. Anal. Chem. 2001. 73: 1917-1926.

47. Gieray RA, Reilly PTA, Yang M, Whitten WB, Ramsey JM. Real-time detection of individual airborne bacteria. J. Microbiol. Methods. 1997. 29: 191-199.

48. Shaw EI, Moura H, Woolfitt AR, Ospina M, Thompson HA, Barr JR. Identification of biomarkers of whole *Coxiella burnetii* phase I by MALDI-TOF mass spectrometry. Anal. Chem. 2004. 76: 4017-4022.

49. Jarman KH, Daly DS, Petersen CE, Saenz AJ, Valentine NB, Wahl KL. Extracting and visualizing matrix-assisted laser desorption/ionization time-of-flight mass spectral fingerprints. Rapid. Commun. Mass. Spectrom. 1999. 13: 1586-1594.

50. Jarman KH, Cebula ST, Saenz AJ, Petersen CE, Valentine NB, Kingsley MT, Wahl KL. An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 2000. 72: 1217-1223.

51. Demirev PA, Ramirez J, Fenselau C. Tandem mass spectrometry of intact proteins for characterization of biomarkers from *Bacillus cereus* T spores. Anal. Chem. 2001. 73: 5725-5731.

52. Demirev PA, Feldman AB, Kowalski P, Lin JS. Top-down proteomics for rapid identification of intact microorganisms. Anal. Chem. 2005. 77: 7455-7461.

53. Cargile BJ, McLuckey SA; Stephenson JL. Identification of bacteriophage MS2 coat protein from *E. coli* lysates via ion trap collisional activation of intact protein ions. Anal. Chem. 2001. 73: 1277-1285.

54. Demirev PA, Fenselau C. Mass spectrometry in biodefense. J. Mass. Spectrom. 2008. 43: 1441-1457.

55. Yao ZP, Afonso C, Fenselau C. Rapid microorganism identification with on-slide proteolytic digestion followed by matrix-assisted laser desorption/ionization tandem mass spectrometry and database searching. Rapid. Commun. Mass. Spectrom. 2002. 16: 1953-1956.

56. Warsheid B, Jackson Kathryn, Sutton C, Fenselau C. MALDI analysis of bacilli in spore mixtures by applying a quadrupole ion trap time-of-flight tandem mass spectrometer. Anal. Chem. 2003. 75: 5608-5617.

57. Warsheid B, Fenselau C. Characterization of *Bacillus* spore species and their mixtures using postsource decay with a curved-field reflectron. Anal. Chem. 2003. 75: 5618-5627.

58. Dugas AJ Jr, Murray KK. On-target digestion of collected bacteria for MALDI mass spectrometry. Anal. Chim. Acta. 2008. 627: 154-161.

2. Matrix-Assisted laser desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF MS)

2.1 Operating principle

2.1.1 The MALDI MS matrix

In the MALDI process, the sample is mixed with an organic compound – matrix in a convenient solvent for co-crystallization. The matrix is typically a weak acid and the actual choice depends on the type of analyte. The compounds must meet a number of requirements simultaneously to be able to act as matrix:

- Matrix should be soluble in solvents compatible with analyte
- Matrix should be able to embed and isolate analyte, for example, by co-crystallization
- Matrix should stay stable under vacuum conditions
- Matrix should absorb the laser wavelength and transfer the energy to analyte
- Matrix should promote the ionization of analyte and
- Matrix should not be reactive with analyte.

The MALDI matrix provides several essential functions. One role of matrix is to separate the analyte molecules (by dilution) to prevent analyte-analyte molecular (or ionic) interactions during desorption/ionization process. The most important role of the matrix is to absorb the laser radiation, thereby protecting the analyte from radiation damage and transfer efficient energy to the analyte and thus promote analyte ionization. It is the crucial factor for the success of this desorption/ionization method.

Nicotinic acid was the first organic compound that was successfully employed as a solid matrix in UV-MALDI [1-3]. Afterwards several “better” matrix compounds have been experimentally tested for their character and utility [4]. Table 2.1 collected some now commonly used MALDI matrices and their main properties. Different matrices of first choice depend on different classes of analyte and analytical problems. For example, CHCA is used in the majority of proteomics applications for analysis of peptides generated by protein enzymatic digests with trypsin [5], while FA is preferred to be used for analysis of intact proteins [6]. In this Ph. D thesis, two selected MALDI matrices, namely FA and CHCA were frequently used.

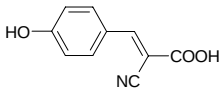
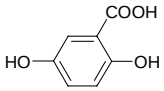
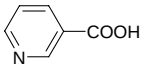
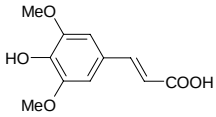
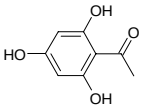
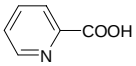
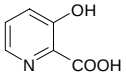
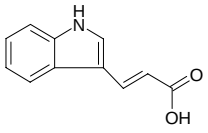
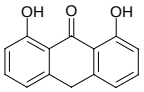
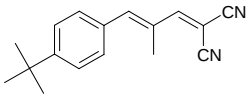
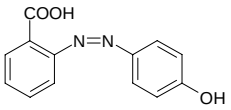
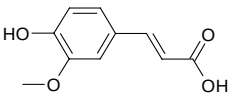
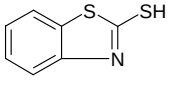
Name	Abbrev.	Chemical structure	Wave-length[nm]	Application
α-Cyano-4-hydroxycinnamic acid [5]	CHCA		337 355	Peptides Carbohydrates Nucleotides
2,5-Dihydroxybenzoic acid [6]	DHB		337 355 266	Peptides nucleotides Oligonucleotides oligosacchrides
Nicotinic acid [1-3]	NA		266	Peptides Proteins
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic) [7, 8]	SA		337 355 266	Peptides Proteins Lipids
Trihydroxyacetophenone [9]	THAP		337 355	Lipophilic compounds Oligonuleotides Carbohydrates
Picolinic acid [10]	PA		266	Oligonucleotides
3-Hydroxypicolinic acid [11]	HPA		337 355	Oligonucleotides
Trans-3-indolylacrylic acid [12]	IAA		337	Synthetic polymers
Dithranol [13]	DIT		355 337	Synthetic polymers Lipids
Trans-2-(3-(4-tert-butylphenyl)-2-methyl-2-propenyliedene) malononitrile [14]	DCTB		337	Polymers Carbohydrates
2-(4-Hydroxyphenylazo) benzoic acid [15, 16]	HABA		337	Peptides Proteins Glycoproteins Polystyrene
4-Hydroxy-3-methoxycinnamic acid (Ferulic acid) [7, 8]	FA		337 355 266	Proteins Glycoproteins
2-Mercapto-benzothiazole [17]	MBT		327	Peptides Proteins Synthetic polymers

Table 2.1 Some commonly used MALDI MS matrices

2.1.2 The MALDI MS laser

A variety of gas and solid-state lasers have been used successfully for MALDI MS. The MALDI lasers are characterized by their emission (wavelength, pulse width, and pulse energy) and beam (beam diameter and divergence) parameters. The most important parameter to produce significant ion current depends on the total energy in the laser pulse at a given wavelength [18]. For ultraviolet-MALDI (UV-MALDI), nitrogen (N_2) gas laser at 337nm wavelength are by far the most commonly used ones because of their ease of operation, small size and relatively low cost. But for high throughput applications such as MALDI imaging (around 100 Hz) and the limited life span ($2 \times 10^7 - 6 \times 10^7$ shots), the solid state frequency-tripled Nd:YAG laser with the wavelength at 335 nm are getting popular owning to their high repetition rate (up to 1 kHz) and extended lifetimes (1×10^9 shots), offer an alternative to nitrogen lasers [19]. In parallel infrared (IR) light have been used in MALDI MS. The Er:YAG laser (2.79 μm) and CO_2 laser (10 μm) have been used successfully in MALDI. MALDI mass spectra obtained with UV or far IR lasers are similar for most analyzed samples. Only small difference like less fragmentation is observed with an IR laser which indicated that IR-MALDI is somewhat softer than UV-MALDI [19]. But a relative low sensitivity is also observed due to the shorter life time of the sample induced by a large depth of vaporization per shot with IR lasers. A summary of laser wavelengths and pulse widths usually used for MALDI MS is listed in Table 2.2.

Laser	Wavelength	Photon energy (eV)	Pulse width
Nitrogen	337 nm	3.68	< 1ns to a few ns
Nd:YAG triplicated	355 nm	3.49	typ. 5 ns
Nd:YAG Quadruplicated	266 nm	4.66	typ. 5 ns
Er:YAG	2.94 μm	0.42	85 ns
CO_2	10.6 μm	0.12	100 ns + 1 μs tail
Excimer (XeCI)	308 nm	4.02	typ. 25 ns
Excimer (KrF)	248 nm	5.00	typ. 25 ns
Excimer (ArF)	193 nm	6.42	typ. 15 ns

Table 2.2 Some commonly used laser for MALDI MS

2.1.3 The MALDI MS sample preparation method

Proper sample preparation is always known to be critical to the success of the MALDI experiment. Unfortunately, there is no standardized sample preparation protocol which is suited to all analytical problems and analytes in MALDI MS. Many parameters included in sample preparation such as choice of matrix and organic solvent in which the matrix has been dissolved, concentration of sample and matrix, matrix/sample ratio and sample deposition procedure are most time-consuming and has to be optimized to achieve high sensitivity, reproducibility and mass accuracy. So far a variety of MALDI sample preparation methods have been developed [20]. Next some representative MALDI sample preparation methods are briefly described below:

- Dried droplet technique [20]

The matrix and sample solution were mixed directly on the MALDI target and allowed to dry in the ambient air. This method is surprisingly simple and provides good results for many different types of samples. The dried droplet method was one of the first reported sample/matrix preparation procedures and is still practiced by many unexperienced MALDI MS users. The major drawback of this method is that it produces uneven sample/matrix crystals and thus requires the search for “sweet” spots to generate good quality spectra

- Thin layer technique [21]

The matrix was dissolved in a volatile solvent such as acetone and was applied onto the MALDI target. Fast evaporation of the solvents results in a thin layer of matrix crystals. A droplet of sample solution is then applied to the solidified matrix layer.

- Sandwich technique [20, 22]

The sandwich technique was reported for the first time by Liang Li and collaborators in 1996 [22]. This method comprised of first applying a matrix layer onto the MALDI target and sample solution was applied onto the dried matrix bed which is analogue to the thin layer technique. But the application of another matrix layer onto the surface of thin layer preparation. The sample is basically sandwiched between the two matrix layers.

With the development of robust and sensitive MALDI mass spectrometers, the real challenge for the analysis of complex peptide or protein mixtures is the sample preparation step prior to mass spectrometric analysis. Many different MALDI sample preparation methods and strategies have been developed to obtain the best possible mass spectra from complex microorganisms [21, 23, 24]. In this thesis, a two-layer volume technique for intact *Fusarium* spores was developed and optimized.

- two-layer volume technique [24]

Pre-mix the matrix and sample solution at a certain ratio in a tube and then apply an aliquot of the mixture onto the MALDI target to form co-crystal, which is referred to as volume technique. Additional a second matrix layer then was applied on top of this dried co-crystal layer and allowed to dry again at room temperature.

2.2 MALDI TOF mass spectrometry instrumentation

2.2.1 Matrix-assisted laser desorption ionization

In early applications of lasers in mass spectrometry, Laser desorption ionization (LDI) turned out to be an efficient method, where the neat analyte without matrix was irradiated directly with intense pulses of laser light for short durations. However, in this process energy transfer uptake upon UV or IR laser irradiation is difficult to control and only high light absorbing molecules absorb radiation at the laser wavelength while the others were accompanied with extensive fragmentation. Furthermore, the detected ions are almost always fragment ions of the larger original molecule due to a thermal spike created by laser irradiation. So only those compounds that have molecular mass between 1 and 2 kDa can be analyzed. This situation was dramatically improved by the appearance of matrix-assisted laser desorption ionization (MALDI) in the late 1980s.

MALDI is one of the two “soft” ionization technique which revolutionized mass spectrometric analysis beside electrospray ionization (ESI). It was introduced in 1988 principally by Karas and Hillenkamp [25-27] and was used in mass spectrometry, allowing the analysis of large, non-volatile and thermally labile molecules. The compound to be analyzed is dissolved in a solvent containing in solution small organic molecules, called the matrix which has strong a strong absorption at the laser wavelength and acts as a means of facilitating desorption and enhancing ion yield (The role of matrix has been described in the chapter 2.1.1 already). This mixture is dried to form solid analyte-doped matrix crystals before analysis and the analyte molecules are embedded in a very large excess of a matrix compound deposited on a MALDI target so that they are completely isolated from each other.

Ion formation step occurs under vacuum conditions inside the source of the mass spectrometer. Despite the rapid acceptance of this technique after its introduction, the exact mechanism of the MALDI process is still not completely elucidated. A variety of possible mechanisms are under consideration. Current model of UV-MALDI is a two-step framework, where the primary ion formation and separation in the primary ionization step and ion molecular reactions take place in desorption/ablation plume resulting in the secondary ions in

the secondary ionization step. Over these years the ideas of various models for MALDI technique have been reviewed [28-31].

2.2.2 Time-of-flight analyser

MALDI ions can be mass analyzed by various different types of mass analyzers. By far the most commonly used mass spectrometer combined with MALDI is the time-of-flight (TOF) mass spectrometer. On the one hand, MALDI usually generate a pulse of ions. The TOF mass analyser requires the ions to be produced in bundles and it thus especially well suited for pulsed laser source. On the other hand, MALDI can generate ions of very large masses of greater than 1 MDa. Again the TOF analyser has in principle no limitation of m/z range. The concept of TOF MS was proposed by W. E. Stephens in 1946 at the University of Pennsylvania [32]. The operating principle of the TOF mass spectrometer is measuring the time required for an ion to fly from an ion source to a detector. All the ions received the same kinetic energy during acceleration, but they have different velocities due to their different m/z values. So in the field-free region between the ion source and detector, they are separated into groups or packets according to their velocities. Finally they are recorded by the ion detector at different time to form mass spectroscopic spectra.

After desorption/ionization of the analyte by the MALDI process ions are produced. These ions with mass m and total charge $q = ze$ are accelerated by the applied electric field with potential difference U. Z is the charge state and e is the elementary charge. The electric potential energy E_{el} is converted into kinetic energy E_k, Thus:

$$E_k = \frac{1}{2}mv^2 = qU = zeU = E_{el}$$

Ions with the same kinetic energy will have different velocities v based on their different mass:

$$v = \sqrt{\frac{2zeU}{m}}$$

After the acceleration the ions enter into the field-free region (of the TOF analyzer) with a length L at a constant velocity. The time needed to reach the detector can be obtained:

$$t = \frac{L}{v}$$

This can be inverted to:

$$\frac{m}{z} = 2eU\left(\frac{t}{L}\right)^2$$

This equation shows that m/z values can be determined as well as a TOF mass spectrum can be recorded by its time-of-flight to the detector. This equation also shows that, all other factors being equal, the ions with lower mass reached the detector faster than higher mass ions. There is no theoretical limit to the upper m/z value, which make it especially suitable for so-called "soft ionization" techniques. This is one of the significant advantages of TOF mass spectrometers when combined with MALDI.

2.2.2.1 Linear TOF mass spectrometer

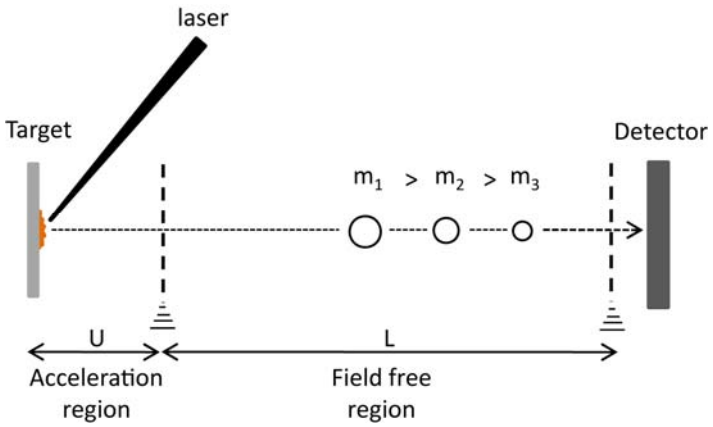


Figure 2.1 Principle of an LTOF mass analyser

Figure 2.1 displays the scheme of a linear TOF (LTOF) instrument. Such an instrumental setup where the ions are traveling on a straight line from the location of their creation to the detector is called linear TOF.

The analyte is supplied as a thin layer on a target upon which a pulsed laser is focused. The acceleration voltage U is applied between this target and a grounded counter electrode (grid or lens). Ions desorbed and formed during or shortly after the laser pulse are continuously extracted and accelerated as they emerge from the target into the gas phase. When leaving the

acceleration region the ions should possess equal kinetic energies. They drift down a field-free flight path in the order of 0.2 – 2 m and finally hit the detector.

The drawback of linear TOF analyzers was their poor mass resolution. In theory, the ions with identical mass and charge are detected at the same time. But actually, the ions are extracted from their desorption plume with an initial time, velocity and spatial distribution which cause ions of the same m/z value to arrive at slightly different times, thus resulting in signal broadening. The TOF mass analyser has been improved significantly with the development of numerous techniques, such as delayed extraction and the reflectron.

2.2.2.2 Reflectron TOF mass spectrometer

One way to compensate the difference of initial kinetic energy of the same m/z ions is to use an electrostatic reflector also called a reflectron, proposed for the first time by Mamyrin [33] in 1973. The basic geometry of a reflectron TOF (RTOF) analyzer is shown in Fig. 2.2. It was located at the end of the flight tube, creating a retarding field which reverse the direction of travel of ions entering it and send them back to the detector.

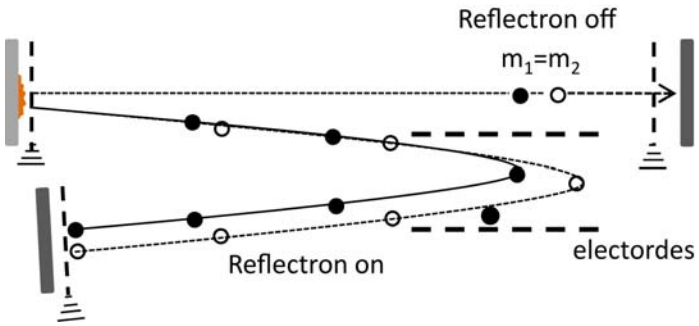


Figure 2.2 Principle of an RTOF mass analyser

The reflectron improves the mass resolution by the increase of flight path without enlarging the dimension of the flight tube but mostly also by reducing the initial kinetic energy dispersion. Imagine there are two ions with the same m/z but of slightly different initial kinetic energy. When reflection mode is turned on, the ion with more kinetic energy will enter first (because it has higher velocity) and penetrate the reflectron deeper than the ion with less kinetic energy. Both ions are decelerated in reflectron until their kinetic energy reach zero. At this moment they begin to be accelerated by the reflectron in the opposite direction. The ion which penetrates deeper into the reflectron will obtain more kinetic energy during reaccelerating. So when they come out from the reflection, they have the same distribution of

kinetic energies and velocities as when they entered the reflectron. However the lengths of their flight paths are different. The ion with more initial kinetic energy moves deeper hence its flight path is longer than the ion with less initial kinetic energy. By properly chosen voltages, path lengths and fields, both kinds of ions can reach the detector at the same time, leading to an improvement of mass resolution.

The simplest type of reflectron is single-stage reflectron, a single electric field region composed by a series of equally distributed grid electrodes or preferably ring electrodes. The performance of the reflectron is improved by dual-stage reflection or even multistage reflector designs. Dual-stage reflection consists of two successive homogeneous electric field regions where the field strength in the first region is significantly larger than in the second region so as to reduce the size of device. The ions are subjected to a strong deceleration in the first region, but suffered from a soft transmission due to the less intense of the second electric field region. Another variation of reflectron TOF analyzer is the so-called curved-field reflectron, mainly designed for seamless post source decay fragment ion analysis (please see the discussion in 2.2.2.4).

2.2.2.3 Delayed extraction (DE) technique

The ideal model is that all ions of the same mass and charge state are detected at the same time resulting in a high mass resolution. However, in reality ions with the same m/z ratio possibly acquire different kinetic energy when they leave the acceleration region due to a number of effects influence. Thus they reach the detector at slightly different time, resulting in peak broadening. This situation is improved with the development of the technique called delayed extraction [34].

The fundamental idea of delayed detraction technique is to give all ions of the same m/z the same kinetic energy. In delayed extraction mode, the ions are initially allowed to separate according to their kinetic energy in the field-free region by applying the same potential to the sample holder and the grid. Those ions with more initial kinetic energy travel farther than the ions having a lesser kinetic energy. So that after some time (typically tens of nanoseconds) the extraction field is turned on, the ions having the lesser initial kinetic energy remain for a longer time in the source thereby picking up more kinetic energy in a compensatory effort. Through adjusting the delayed potential and timing, the initially less kinetic energy ions can catch up with the faster ions and reach the detector at the same time. So the delayed extraction technique corrects the energy dispersion of the ions with the same m/z ratio and thus improves the mass resolution of the TOF analyser.

2.2.2.4 Post-source decay with RTOF mass analyzer

"Post-source decay" (PSD) is a name to remind you that the ions of interest are those that their fragmentation occurs in the flight tube after they leave the ion source of a TOF mass spectrometer. Those ions are stable enough to leave the ion source but still contain enough excess internal energy to undergo fragmentation in the flight tube before they reach the detector. PSD fragment ions have the same velocity as their precursors. So in a linear TOF mass spectrometer, both precursors and fragment ions will reach the detector at the same time and thus can not be differentiated. However, they have different kinetic energies as a function of their different masses. The fragment ions have lower kinetic energies than their precursors. Consequently, in a reflectron TOF mass analyzer, the lighter fragment ions which have lower kinetic energies penetrate the reflectron less deeply and spend a less time in the reflectron. Thus both precursor and fragment ions can be detected. So PSD is a technique specific to reflectron TOF mass spectrometer.

It is impossible to record fragments of all ions decayed in flight tube in a single spectrum. So it is necessary to select a single precursor ion or at least set up a mass window for a few ions. Generally precursor ion selection was performed with a deflection gate which consists of a pair of electrodes. Only the ions within the mass window can pass through and be recorded by the ion detector. However, it is important to note that the optimum reflectron potential for observing the precursor ion is not optimum for observing all the fragment ions. If they are detected at all, they generate very poorly resolved peaks. Therefore, the voltage on the reflectron should be adjusted to suit the mass of each fragment to obtain an optimum mass spectrum. This requires the reflectron voltage to be sequentially stepped thereby obtain the segments of the PSD spectrum. By combining these individual sections of PSD spectrum together, the entire PSD spectrum can be observed. But the time of acquisition of a whole PSD spectrum is therefore quite long and the main part of sample is lost without contributing to the analysis.

The solution to makeup this drawback is using a curved field reflectron, which was proposed by Robert Cotter [35]. The curved field reflectron has many more grids than the conventional design to provide an increased nonlinear (curved) electric field to focus a wide range of m/z values at one time. It decreases the penetration distance for heavy ion and thus results in a small dispersion of all fragment ions with different m/z values. Thus a complete PSD spectrum can be obtained in a single step without stepping the reflectron potential, i.e. seamless.

2.3 References

1. Karas M, Hillenkamp F, Laser desorption ionization of proteins with molecular mass exceeding 10,000 daltons. Anal. Chem. 1988. 60: 2299-2301.
2. Karas M, Bahr U, Ingendoh A, Hillenkamp F. Laser-desorption-MS of 10000-250000-dalton proteins. Angew. Chem. 1989. 101: 805-806.
3. Karas M, Ingendoh A, Bahr U, Hillenkamp F. Ultraviolet-laser desorption/ionization mass spectrometry of femtomolar amounts of large proteins. Biomed. Environ .Mass. Spectrom. 1989. 18: 841-843.
4. Fitzgerald MC, Parr GR, Smith LM. Basic matrices for the matrix-assisted laser desorption/ionization mass spectrometry of proteins and oligonucleotides. Anal. Chem. 1993. 65: 3204-3211.
5. Beavis RC, Chaudhary T, Chait BT. α-Cyano-4-hydroxycinnamic acid as a matrix for matrix-assisted laser desorption-MS. Org. Mass. Spectrom. 1992. 27: 156-158.
6. Strupat K, Karas M, Hillenkamp F. 2, 5-Dihidroxybenzoic acid: a new matrix for laser desorption-ionization mass spectrometry. Int. J. Mass. Spectrom. Ion. Process. 72: 89-102.
7. Beavis RC, Chait BT. Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of proteins. Rapid. Commun. Mass. Spectrom. 1989. 3: 432-435.
8. Beavis RC, Chait BT. Matrix-assisted laser desorption mass spectrometry using 355 nm radiation. Rapid. Commun. Mass. Spcetrom. 1989. 3: 436-439.
9. Meyer A, Spinelli N, Imbach JL, Vasseur JJ. Analysis of solid-supported oligonucleotides by MALDI-TOF-MS. Rpaid. Commun. Mass. Spectrom. 2000. 14: 234-242.
10. Tang K, Taranenko NI, Allman SL, Chen CH, Chang LY, Jacobson KB. Picolinic acid as a matrix for laser-MS of nucleic acids and proteins. Rapid. Commun. Mass. Spectrom. 1994. 8: 673-677.
11. Wu KJ, Steding A, Becker CH, Matrix-assisted laser desorption-TOF-MS of oligonucleotides using 3-hydroxy-picolinic acid as an ultraviolet-sensitive matrix. Rapid. Commun. Mass. Spectrom. 1993. 7: 142-146.
12. Kassis CM, Desimone JM, Linton RW, Lange GW, Friedman RM. An investigation into the importance of polymer-matrix miscibility using surfactant modified MALDI-MS. Rapid. Commun. Mass. Spectrom. 1997. 11: 1462-1466.

13. Brown T, Clipston NL, Simjee N, Luftmann H, Hungebühler H, Drewello T. Matrix-assisted laser desorption/ionization of amphiphilic fullerene derivatives. Int. J. Mass. Spectrom. 2001. 210/211: 249-263.
14. Montaudo G, Montaudo MS, Puglisi C, Samperi F. 2-(4-Hydroxyphenylazo) benzoic acid: a solid matrix for MALDI of polystyrene. Rapid. Commun. Mass. Spectrom. 1994. 8: 1011-1015.
15. Juhasz P, Costello CE, Biemann K. MALDI-MS with 2-(4-Hydroxyphenylazo)benzoic acid matrix. J. Am. Soc. Mass. Spectrom. 1993. 4: 399-409.
16. Xu N, Huang ZH, Watson JT, Gage DA. Mercaptobenzothiazoles: a new class of matrixes for laser desorption ionization-MS. J. Am. Soc. Mass. Spectrom. 1997. 8: 116-124.
17. Demirev P, Westman A, Reimann CT, Håkansson P, Barofsky D, Sundqvist BUR, Cheng YD, Seibt W, Siegbahn K. Matrix-assisted laser desorption with ultra-short laser pulses. Rapid. Commun. Mass. Spectrom. 1992. 6: 187-191.
18. Hillenkamp F, Peter-Katalinic J. MALDI MS - A practical guide to instrumentation, methods and applications. Wiley-VCH, 2007.
19. Hoffmann ED, Stroobant V. Mass spectrometry - principles and applications. Third edition, Wiley, 2007.
20. Kussmann M, Nordhoff E, Rahbek-Nielsen H, Haebel S, Rossel-Larsen M, Jakobsen L, Goborn J, Mirgorodskaya E, Kroll-Krestensen A, Palm L, Roepstorff P. Matrix-assisted laser desorption/ionization mass spectrometry preparation techniques designed for various peptide and protein analysis. J. Mass. Spectrom. 1997. 32: 593-601.
21. Vorm O, Roepstorff P, Mann M. Improved resolution and very high sensitivity in MALDI-TOF of matrix surfaces made by fast evaporation. Anal. Chem. 1994. 66: 3281.
22. Li L, Golding RE, Whittal RM. Analysis of single mammalian cell lysates by mass spectrometry. J. Am. Chem. Soc. 1996. 118: 11662-11663.
23. Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinina IS, Kubicek CP, Allmaier G. Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 2009. 23: 877-884.

24. Dong H, Kemptner J, Marchetti-Deschmann M, Kubicek CP, Allmaier G. Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry. Anal. Bioanal. Chem. 2009. 395: 1373-1383.
25. Karas M, Bachmann D, Bahr U, Hillenkamp F. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. Int. J. Mass. Spectrom. Ion. Process. 1987. 78: 53-68.
26. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal. Chem. 1988. 60: 2299-2301.
27. Hillenkamp F, Karas M, Ingendoh A, Stahl B, Matrix assisted UV-laser desorption/ionization: A new approach to mass spectrometry of large biomolecules. Bio. Mass. Spectrom. (Burlingame A and McClosky JA, eds.), Elsevier, Amsterdam, NL, 1990. pp. 49–60.
28. Karas M, Krüger R. Ion formation in MALDI: the cluster ionization mechanism. Chem. Rev. 2003. 103: 427-440.
29. Dreisewerd K. The desorption process in MALDI. Chem. Rev. 2003. 103: 395-425.
30. Knochenmuss R, Zenobi R. MALDI ionization: the role of in-plume process. Chem. Rev. 2003. 103: 441-452.
31. Knochenmuss R. Ion formation mechanisms in UV-MALDI. Analyst. 2006. 131: 966-986.
32. Stephens W. A pulsed mass spectrometer with time dispersion. Phys. Rev. 1946. 69: 691.
33. Mamyrin BA, Karataev VI, schmikk DV, Zagulin VA. The mass-reflectron, a new nonmagnetic time-of-flight mass spectrometer with high resolution. Sov. Phys. JETP. 1973. 37: 45-48.
34. Spengler B, Cotter RJ. Ultraviolet laser desorption/ionization mass spectrometry of proteins above 100,000 daltons by pulsed ion extraction time-of-flight analysis. Anal. Chem. 1990. 62: 793-796.
35. Cornish TJ, Cotter RJ. A curved-field reflectron for improved energy focusing of product ions in time-of-flight mass spectrometry. Rpaid. Commun. Mass. Spectrom. 1993. 7: 1037-1040.

3. Aim of this thesis

Filamentous fungi of the genus *Fusarium* are widespread cereal contaminants and well-known plant, notably causing cereal diseases with important economic repercussions. Furthermore, the infection of cereal plants is accompanied by the production of mycotoxins which leads to hazardous to human and animal health when entering the food chain. Therefore rapid detection and identification of fungal species related to this health threat at an early stage is of great importance.

This work was conducted with the following objectives:

- Rapid differentiation and identification of *Fusarium* species using MALDI LTOF MS

The aim is to develop a suitable sample preparation method for rapid identification and differentiation of *Fusarium* species by intact cell MALDI LTOF MS (IC-MS). Different solvent systems for washing colored intact *Fusarium* conidia spores and various of MALDI MS sample deposition techniques were evaluated with aim to generate reproducible and characteristic MALDI TOF mass spectra. Finally this optimized sample preparation for the first time should provided mass spectrometric fingerprints of *Fusarium* spores with both weak and strong colored layers, resulting in the establishment of an appropriate database for peptide/protein patterns of *Fusarium* species and strains as well as the possibility of differentiation of such spores at least at the species level.

- Verification and identification of *Fusarium* spore surface peptides/proteins presented in IC mass spectra.

The purpose is to verify the presence of peptides/proteins in the peak patterns of IC mass spectra. Based on this we proposed a strategy in which the *Fusarium* spores were subjected to a brief *on-target* tryptic digestion. Subsequently the matrix-covered tryptic digest should be analyzed by MALDI reflectron TOF MS and PSD fragment ion analysis should be performed on selected abundant peptides. In combination with *de novo* sequencing and BLAST search, identification of proteins from *Fusarium* spore should be achievable. Due to the widespread use of IC-MS and IS-MS for identification and characterization of different species or strains of spores, this strategy should provide a way to verify and identify the presence of peptides/proteins on the surface of spores.

4. Publication and manuscripts

4.1 Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry

Analytical and Bioanalytical Chemistry. 2009. 395 (5): 1373-1783

Anal Bioanal Chem (2009) 395:1373–1383
DOI 10.1007/s00216-009-3067-3

ORIGINAL PAPER

Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry

Hongjuan Dong · Jasmin Kemptner · Martina Marchetti-Deschmann · Christian Peter Kubicek · Günter Allmaier

Received: 30 April 2009 / Revised: 10 August 2009 / Accepted: 11 August 2009 / Published online: 30 August 2009

Abstract Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) has been proved to be a powerful tool for the identification and characterization of microorganisms based on their surface peptide/protein pattern. Because of the complexity of microorganisms, there are no standardized protocols to acquire reproducible peptide/protein profiles for a broad range of microorganisms and for fungi in particular. Small variations during MALDI MS sample preparation affect the quality of mass spectra quite often. In this study, we were aiming to develop a sample preparation method for the analysis of colored, a quite often observed phenomenon, and mycotoxin-producing *Fusarium* conidia spores using MALDI–TOF MS. Different washing solvent systems for light- and deep-colored (from slightly orange to red-brown) conidia spores and connected sample deposition techniques were evaluated based on MS reproducibility and number and intensities of peaks. As a method of choice for generation of reproducible and characteristic MALDI–TOF mass spectra, the use of a washing process for colored *Fusarium* conidia spores with acetonitrile/0.5% formic acid (7/3) was found and subsequently combined with two-layer volume technique (spores/matrix (ferulic acid) solution was deposited onto a MALDI target, and after solvent evaporation, a second matrix layer was deposited). With the application of this sample preparation method, for deep-colored *Fusarium* species, 19 abundant molecular ions in the *m/z* range 2,000–10,000 were always detected with an S/N ratio of 3:1 or better. Finally this optimized sample preparation for the first time provided mass spectrometric fingerprints of strongly colored *Fusarium* conidia spores resulting in the possibility of differentiation of such spores at the species level.

Keywords *Fusarium* · Conidia spores · Sample preparation · ICMS · MALDI–TOF MS · Mass spectrometry

H. Dong · J. Kemptner · M. Marchetti-Deschmann · G. Allmaier (✉)
Institute of Chemical Technologies and Analytics,
Vienna University of Technology,
Getreidemarkt 9/164-IAC,
1060 Vienna, Austria
e-mail: guenter.allmaier@tuwien.ac.at

C. P. Kubicek
Institute of Chemical Engineering,
Vienna University of Technology,
Getreidemarkt 9/166,
1060 Vienna, Austria

Introduction

Fungi form the fifth important kingdom of eukaryotes [1, 2]. Fungal cells are surrounded by rigid cell walls, which in ascomycetes and basidiomycetes are composed of up to 90% polysaccharides, dominated by the long-chain carbohydrate polymers chitin and β-glucan. Proteins, lipids, and polyphosphates together with inorganic ions are also present in fungal cells [3, 4]. Members of the ascomycetous genus *Fusarium* are frequently cereal contaminants and well-known plant pathogens, but also widely distributed in soil. The infection of cereal plants is accompanied by the production of mycotoxins that can affect human and animal health when entering the food chain [5, 6]. *Fusarium* toxins are therefore currently a serious agricultural and public health problem in several parts of the world [7]. Rapid detection and identification

of fungal species related to this health threat at an early stage is of great importance.

Intact cell (ICMS) or intact spore (ISMS) mass spectrometry based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS), a rapid and sensitive method, has been widely used for identification and classification of microorganisms [8–11]. MALDI–TOF MS as a so-called soft ionization technique enables desorption and ionization of peptides as well as proteins as molecular ions from the surface of intact microorganisms without fragmentation or pyrolysis [12]. It is not necessary to employ any prior separation or extraction procedure and the sample (i.e., the bacterium or fungus) can be analyzed "as a whole" with only minimal sample pretreatment [13]. MALDI–TOF MS is also relatively insensitive compared to other "soft" ionization techniques (e.g., fast atom bombardment or electrospray ionization) to the presence of salts and detergents, making it suitable for direct and automated analysis of microorganisms. So far, it has been successfully used to differentiate a variety of Gram-negative and Gram-positive bacteria and fungi [3, 4, 8, 10, 11, 14]. The simplicity in sample preparation and speed of the data acquisition described in a number of papers [10, 15–18] indicates that ICMS or ISMS has great potential for routine use in intact fungal spore characterization and identification.

The quality of MALDI–TOF mass spectra of microorganism produced is highly dependent on the applied sample preparation methods as well as how detailed the optimization was performed and what the final aim of the analysis was. Especially because of the complexity of microorganisms, there are no standardized ICMS or ISMS protocols, which can be applied to a broad range of bacterial and fungal species to acquire reproducible peptide/protein mass spectrometric profiles. Small variations during sample preparation affect the quality of MALDI–TOF mass spectra often significantly [10, 14]. A serious problem is that in *Fusarium* and other fungi in cultures, they often form colored layers of various types and intensities during growth [19–21]. In particular, our fungal species of interest formed slightly orange to red-brown colored *Fusarium* conidia spores after removal of mycelium and growth media [22]. When *Fusarium* conidia spores with deep brown color were washed with water and subsequently prepared with the dried droplet technique for MALDI–TOF MS analysis (the method described in Ref. [10]), there was almost no crystals formed, and the whole sample spot was showing a deep brown color. Finally it resulted in very few peaks with quite low intensities in the positive ion MALDI mass spectrum or no useful mass spectrum at all. Due to this reason and on the basis of our previous work [10], different washing solvent systems and sample deposition techniques were evaluated to find an improved method in terms of mass spectrometric reproducibility, peak numbers, and peak intensities. Finally a suitable sample preparation method for weakly and strongly colored *Fusarium* conidia

Table 1 Evaluated solvent systems for washing intact *Fusarium* conidia spores

Solvent systems	Ratio (*v*/*v*)	Volume/washing cycle (μL)	Washing times using centrifugation
Water	–	100	3
0.1% aqueous trifluoroacetic acid	–	100	3
0.5% aqueous formic acid	–	100	3
Acetonitrile	–	100	3
Acetonitrile/0.1% aqueous trifluoroacetic acid	7/3	100	3
Acetonitrile/0.5% aqueous formic acid	7/3	100	3
Acetonitrile/water	1/1	100	3
Methanol/water	1/1	100	3
Ethanol/water	4/6	100	3
0.1% aqueous *n*-octylglycosid	–	100	3
Isopropanol/water	6/4	100	2
0.1% aqueous formic acid	–		1
Water	–		2
0.1% aqueous *n*-octylglycosid	–	100	Shaking 15 min at 40 °C
Isopropanol/water	6/4		2
0.1% aqueous formic acid	–		1
Water	–		2

spore analysis using MALDI–TOF MS should be provided, which allows to generate mass spectrometric peptide/protein fingerprints for differentiation of *Fusarium* conidia spores at the species level with high reliability.

Experimental

Chemicals

Ferulic acid was obtained from Fluka (Buchs, Germany). Acetonitrile analytical grade (p.a., pro analysis, ACN), methanol p.a. (MeOH), ethanol 96% (EtOH), isopropanol p.a. (iPrOH), formic acid (FA) 98–100%, and water p.a. were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Riedel-de-Haën (Seelze, Germany). *N*-Octylglycosid was purchased from Sigma-Aldrich (Vienna, Austria).

Strains and cultivation conditions

Fusarium poae CPK 2786 and *Fusarium graminearum* CPK 2765 were used throughout this work and are maintained in the strain collection of the Institute of Chemical Engineering (Vienna University of Technology, Vienna, Austria).

They were cultivated on fully grown SNA plates (Synthetischer Nährstoffarme Agar). Mungbean soup (25 mL; 20 g mungbean in 1 L water were heated for 20 min and directly used after filtration) was inoculated with one fully grown SNA plate for spore generation. After 4 days of inoculation in shaking flasks (28 °C, 160 rpm), the fungal biomass has been formed for MALDI analysis. Then the spores were filtered through a sterile glass funnel containing glass wool and centrifuged at 7,200×*g*, 4 °C for 10 min, in order to remove mycelium and mungbean soup residues. Then the so obtained conidia spores were mixed with an aqueous solution containing 20% glycerol (*w*/*v*) and finally stored at −20 °C. The concentration of the conidia spore solution was determined by counting in a light-optical microscope (Nikon Instruments Europe, Amstelveen, The Netherlands).

Intact conidia spore purification

Before MALDI MS analysis, *Fusarium* conidia spores were usually washed to get rid of glycerol and contaminants [10]. During optimization of washing solvent systems, the colored *Fusarium* conidia spores were washed three times with 100 μL of different solvent systems (see Table 1) at 19,500×*g* for 10 min using Nanosep^{TM} (Pall, Ann Arbor, MI, USA) centrifugal devices (MWCO 10 kDa). Then the *Fusarium* conidia spores were resuspended in water resulting to a final concentration of 3 million spores/μL.

MALDI MS sample preparation

According to our previous experiment [10], 10 mg ferulic acid dissolved in 1 mL ACN/aqueous 0.1% TFA (7/3, *v*/*v*) was used as matrix solution. Three sample deposition techniques, including dried droplet technique [23], volume technique (i.e., premixing before deposition) [24], and two-layer volume technique [25], were evaluated. Dried droplet technique (DD): First, 0.5 μL conidia spore solution was applied onto the target, and 0.5 μL matrix solution was added immediately by direct mixing on the target using the pipette tip. Afterward, the solvent was evaporating at room temperature (RT). Volume technique (V): The intact spores and matrix solution were placed into an Eppendorf tube and mixed well by vortexing. Then 1 μL of the mixture was deposited onto the MALDI target, and the solvent was evaporated at RT. Two-layer volume technique (2LV): The

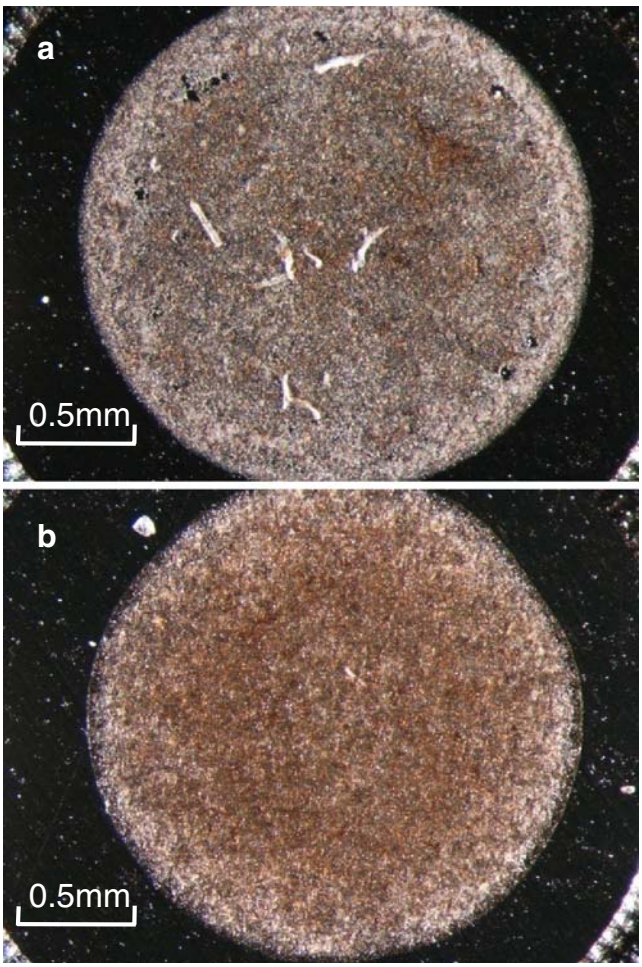


Fig. 1 Microscopic images of *Fusarium* conidia spores **a** light-colored *F. poae* strain 2786 and **b** deep-colored *F. graminearum* strain 2765. *Fusarium* conidia spores were washed with pure water and suspended in water. Conidia spore solution (1 μL) was deposited onto the target (without MALDI Matrix), and the *Fusarium* spots were photographed after solvent evaporation at RT

same preparation was used as described for the volume technique, but 0.5 μL of additional matrix solution was deposited onto the already dried sample/matrix spot (a kind of reverse form of the two-layer technique developed peptides and proteins [26]).

MALDI–TOF mass spectrometry

MALDI–TOF mass spectra were obtained on Axima-CFR^{plus} instrument (Shimadzu Biotech Kratos Analytical, Manchester, UK), a curved field reflectron mass spectrometer equipped with a nitrogen laser (wavelength 337 nm, 3 ns pulse width) and an integrated 1-GHz recorder. The instrument was operated at an acceleration potential of 20 kV. All mass spectra were recorded in positive ion linear mode with delayed extraction (optimized for *m*/*z* 5,000) by accumulating up to 2,500 single unselected laser shots in the *m*/*z* range of 1,000 to 15,000. Baseline subtraction (baseline filter width 60) and smoothing (company supplied Savitzky–Golay algorithm, smoothing filter width 20) were applied for all the mass spectra. The blanking gate was set at *m*/*z* 1,500 to remove the ions below this *m*/*z* value arising from the matrix and their clusters, small peptides, and other unknown contaminants. An external three-point calibration was performed with the protein cytochrome *c* (protonated molecule at *m*/*z* 12,361.2 and double protonated molecule

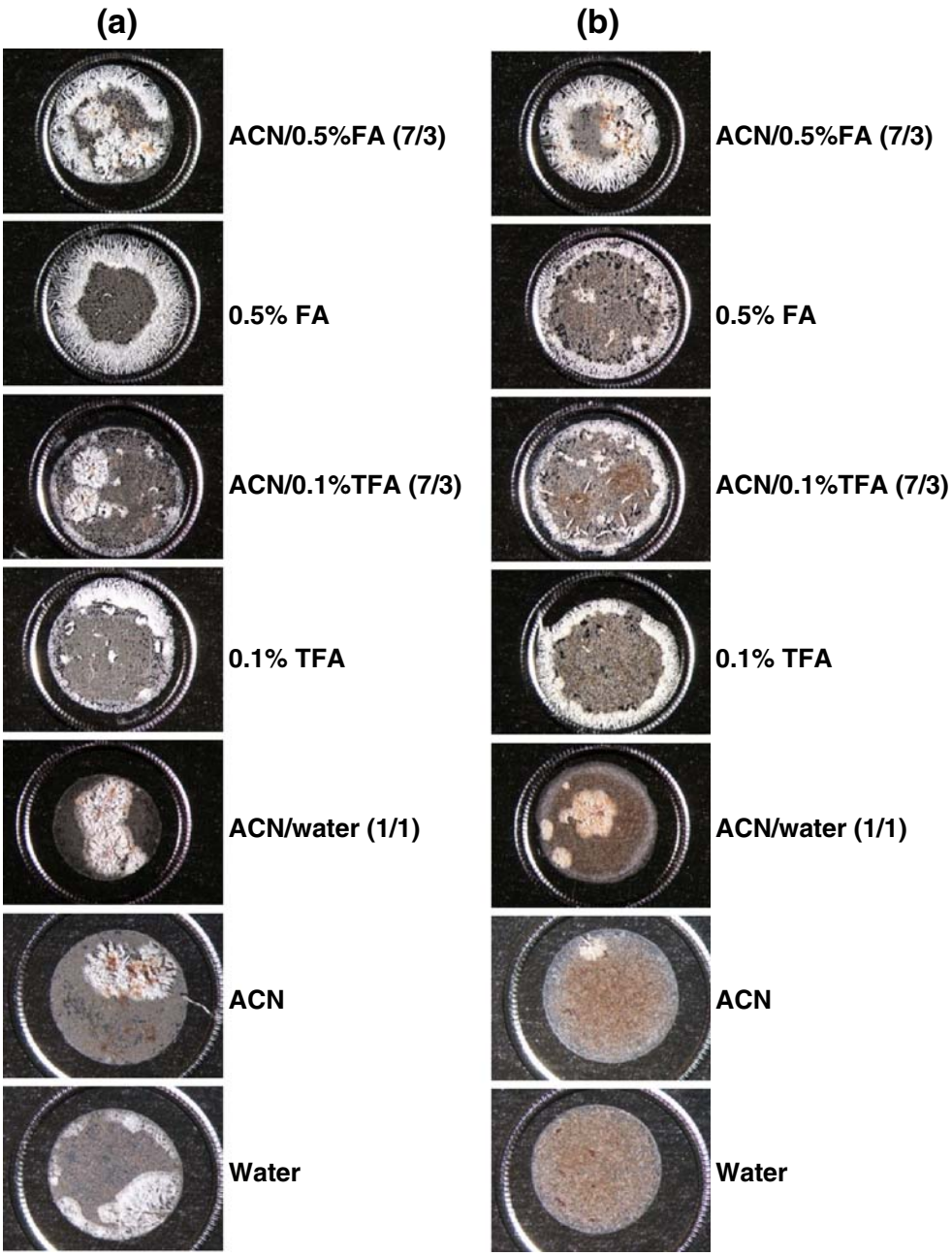


Fig. 2 Microscopic images of matrix/sample spots applied with two-layer volume technique with *Fusarium* conidia spores washed by different solvent systems **a** *F. poae* strain 2786 (*light-colored*) and **b** *F. graminearum* strain 2765 (*deep-colored*)

at *m*/*z* 6,181.1) and the peptide ACTH 7-38 (protonated molecule at *m*/*z* 3,657.9).

Results and discussion

In our previous work [10], *Fusarium* conidia spore preparations were washed with water before MALDI–TOF MS and subsequently applied with dried droplet technique, which works fine for non-colored or slightly colored preparations (Fig. 1a). But for some *Fusarium* species or strains exhibiting a deep color (Fig. 1b), it resulted in very few peaks with quite low intensities in their positive ion MALDI mass spectra or no useful mass spectra at all (containing practical no ions in the *m*/*z* range of interest). Due to this fact, we further developed the ISMS sample preparation method, making it suitable for both light- and deep-colored *Fusarium* species.

Comparison of different solvent systems for washing colored and intact *Fusarium* conidia spores

After *F. graminearum* CPK 2786 conidia spores (we define it as light-colored species) were washed with plain water and deposited onto the target using the two-layer volume technique with ferulic acid as MALDI matrix, it yielded small white crystals with a little bit light orange color, which resulted in a useful and distinct mass spectrum. But for *F. poae* CPK 2765 (we define it as deep-colored species), there was almost no crystal formation, and the whole spot was covered with very deep brown color. Finally it yielded a positive ion MALDI mass spectrum with quite low quality. Figure 1 shows the light-optical microscope images of spots (without MALDI matrix) of the light-colored *F. graminearum* CPK 2786 and the deep-colored *F. poae* CPK 2765. In order to get high-quality MALDI–TOF mass spectra of both light- and deep-colored *Fusarium* species, several solvent systems composed of water, acetonitrile, methanol, ethanol, isopropanol, organic acids such as formic acid and TFA, and the detergent *n*-octylglycosid were used to wash *Fusarium* conidia spores for removal of the colorizing surface components (Table 1) and were evaluated in terms of mass spectrometric performance. They were either used in pure form or were mixed in certain ratios (*v*/*v*). The using of some of solvent systems such as methanol/water (1/1), ethanol/water (1/1), acetonitrile/water (1/1), and the additive *n*-octylglycosid and so on did not give any improvement of the mass spectrometric quality. But when some solvent systems containing an organic acid were used, big advancement was obtained. Figure 2 presents light-optical microscope images of matrix/sample crystal layers from *Fusarium* conidia spores treated with various solvent systems, and Fig. 3 exhibits their corresponding positive ion MALDI–TOF mass spectra. From the images of deep-colored

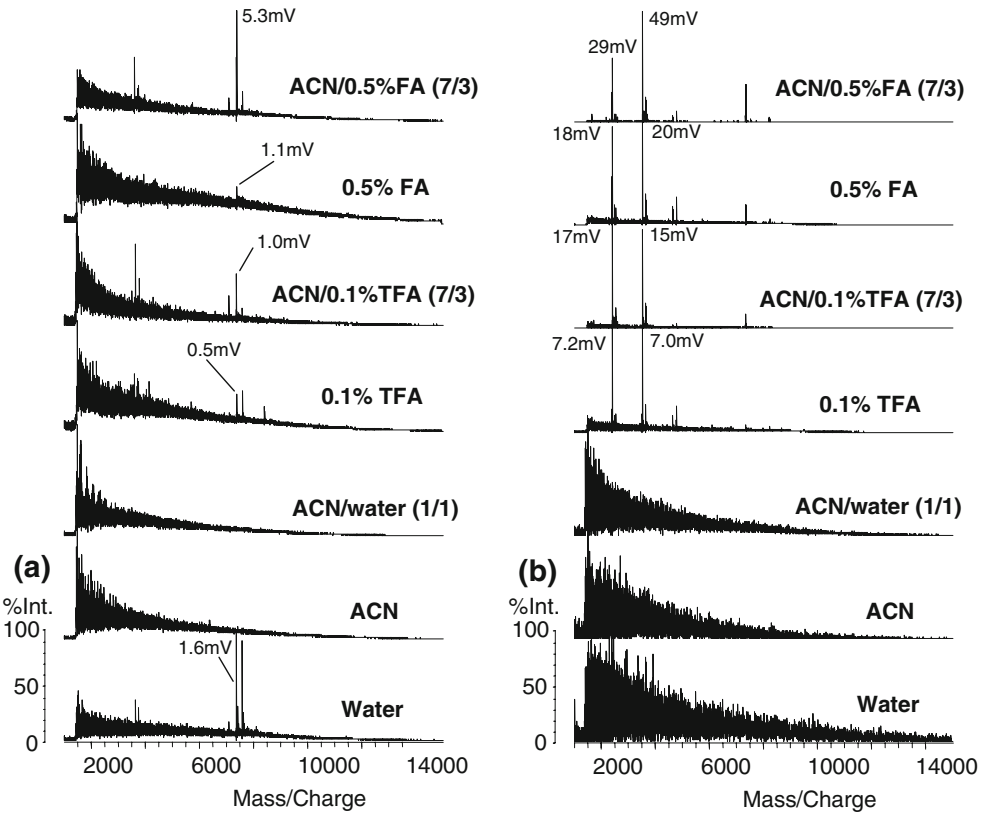


Fig. 3 Comparison of MALDI mass spectra of intact *Fusarium* conidia spores washed by different solvent systems applied with two-layer volume technique (**a**, *left column*) *F. poae* strain 2786 (*light-colored*) and (**b**, right column) *F. graminearum* strain 2765 (*deep-colored*). Matrix solution, 10 mg ferulic acid dissolved in 1 mL ACN/0.1% TFA (7/3, *v*/*v*); matrix solution/conidia spore solution ratio, 1/1 (*v*/*v*); matrix solution/conidia spore solution mixing time, 3 min

samples, we could see that brown color still exists after washing by various solvent systems. It could not be washed off only by centrifugation with organic solvents. This indicates that the colored compounds are covalently linked to the surface of *Fusarium* conidia spores or are present in the deeper cell wall layers of the spores. But when both selected *Fusarium* species were treated with some solvent systems containing an organic acid such as formic acid or trifluoroacetic acid, the color of the formed crystals became lighter and mass spectra with high quality were generated. It gave rise to a high number of peaks with higher intensity as well as quite good reproducibility (19 abundant ion species for deep-colored species and 10 ion species for light-colored species were always detected with an S/N ratio of 3:1 or better in the *m*/*z* range 2,000–10,000). From this result, it could be concluded that, to obtain informative MALDI–TOF mass spectra of intact *Fusarium* conidia spores with deep color, acid treatment of *Fusarium* conidia spores play an important role and might change the cell wall architecture (better extraction of peptide/proteins) and/or the change of pH value might be a reason for the lighter color. Especially when *Fusarium* conidia spores were washed with ACN/0.5% formic acid (7/3, *v*/*v*), both light- and deep-colored species showed mass spectra with good quality and reproducibility. So the optimized solvent system containing formic acid turned out to be the more proper washing

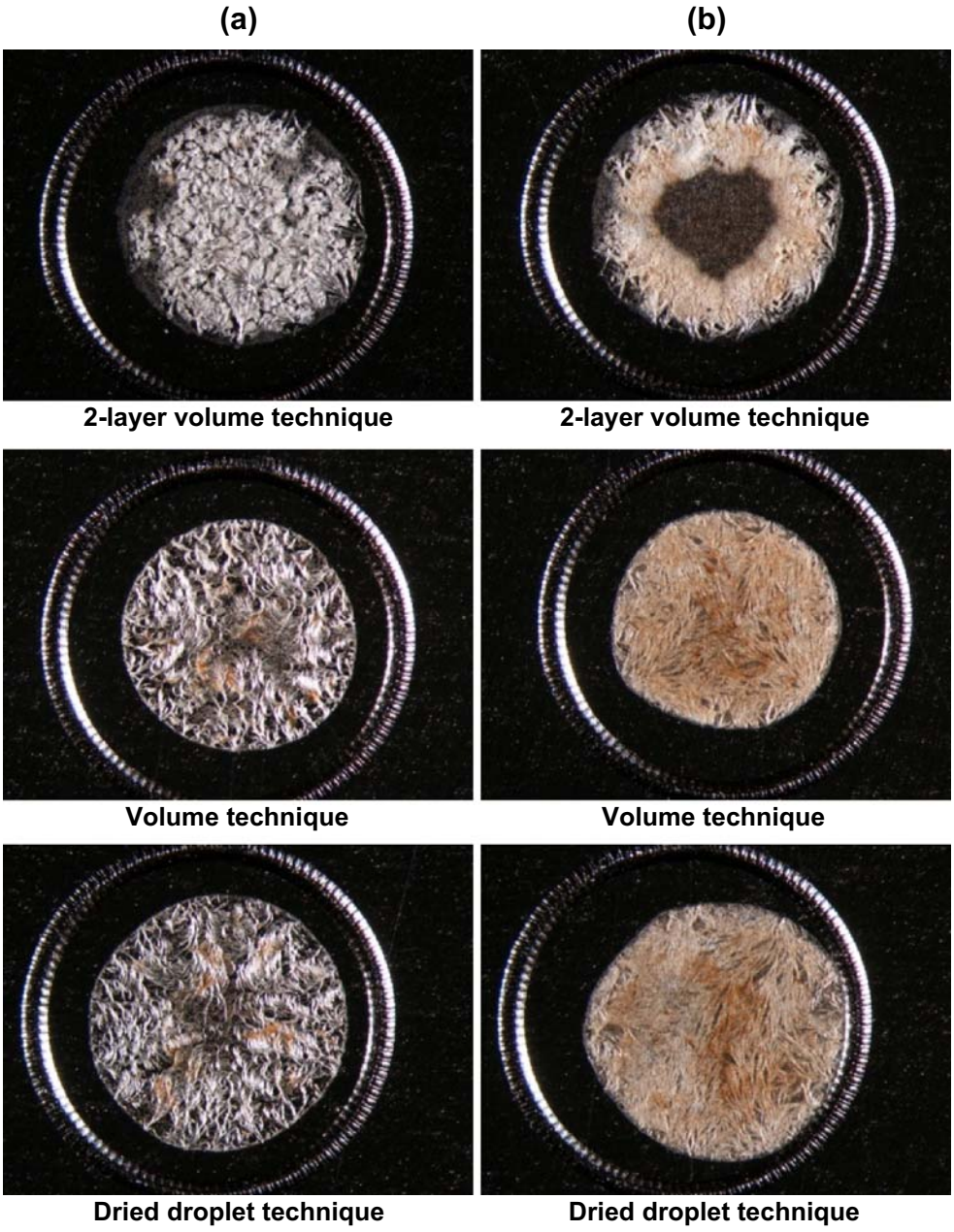


Fig. 4 Microscopic images of matrix/sample spots generated with different MALDI preparation techniques of (**a**, *left column*) *F. poae* strain 2786 (*light-colored*) and (**b**, *right column*) *F. graminearum* strain 2765 (*deep-colored*)

solution for colored as well as non-colored conidia spores (no improvement for non-colored samples through the addition FA) of *Fusarium* particularly for differentiation of fungus species/strains in ISMS.

Comparison of sample deposition procedures after application of the optimized washing procedure

It is common knowledge that the right matrix/analyte preparation constitutes one of the key requirements for a successful MALDI MS analysis in terms of peak reproducibility, peak numbers, and intensities [27]. In this study, dried droplet technique, volume technique, and two-layer volume technique resulted in similar peptide/protein profiles, but the latter technique generated higher number of peaks (ion species) and intensities as well as a mass spectrum with less noise for deep-colored species (6 (DD), 7 (V), and 19 (2LV) abundant ion species were detected with an S/N ratio of 3:1 or better, respectively, using the three different techniques in the *m*/*z* range 2,000–10,000). Particular improvement was found in the *m*/*z* range 6,000–9,000. In Fig. 4, light-optical microscope images of matrix/sample crystal preparations from *Fusarium* conidia spores applied with the mentioned three different sample deposition techniques with the same MALDI matrix are shown, and in Fig. 5, their corresponding MALDI–TOF mass spectra are presented. The first layer that formed from the mixture of matrix and conidia spore solutions consists of small bundles of branched crystals radiating out from distinct spots and cover the whole preparation area. With the application of the second matrix layer on top of the dried matrix/sample spot, a dissolving and re-crystallization step occurred, which resulted in a better incorporation of the spores into the matrix crystals and finally the formation of smaller as well as thicker crystals covering the surface. Although a consistent crystal layer was not always formed throughout the whole spot area, reproducible peptide/protein profiles with high intensities and very low noise were obtained using automated MALDI MS data

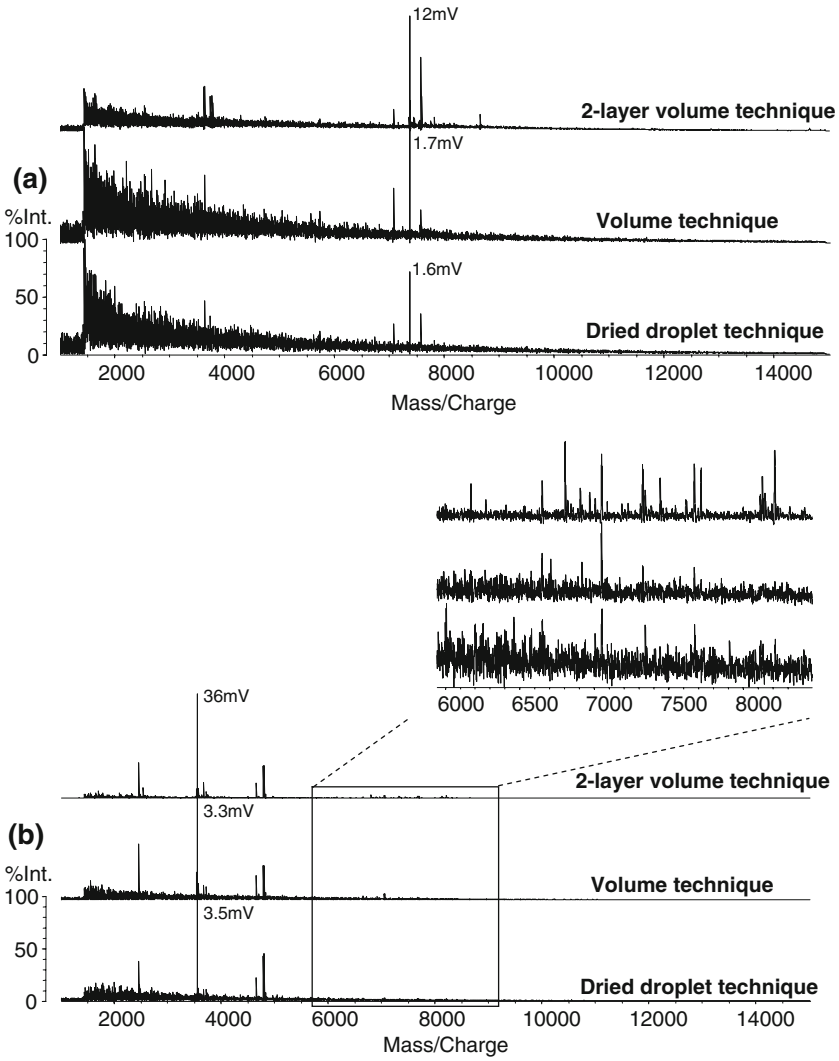


Fig. 5 Comparison of MALDI mass spectra of intact *Fusarium* conidia spores applied with different MALDI preparation techniques **a** *F. poae* strain 2786 (*light-colored*) and **b** *F. graminearum* strain 2765 (*deep-colored*). Matrix solution, 10 mg ferulic acid dissolved in 1 mL ACN/0.1% TFA (7/3, *v*/*v*); washing solvent system: ACN/0.5% FA (7/3, *v*/*v*); matrix solution/conidia spore solution ratio, 1/1 (*v*/*v*); matrix solution/conidia spore solution mixing time, 3 min

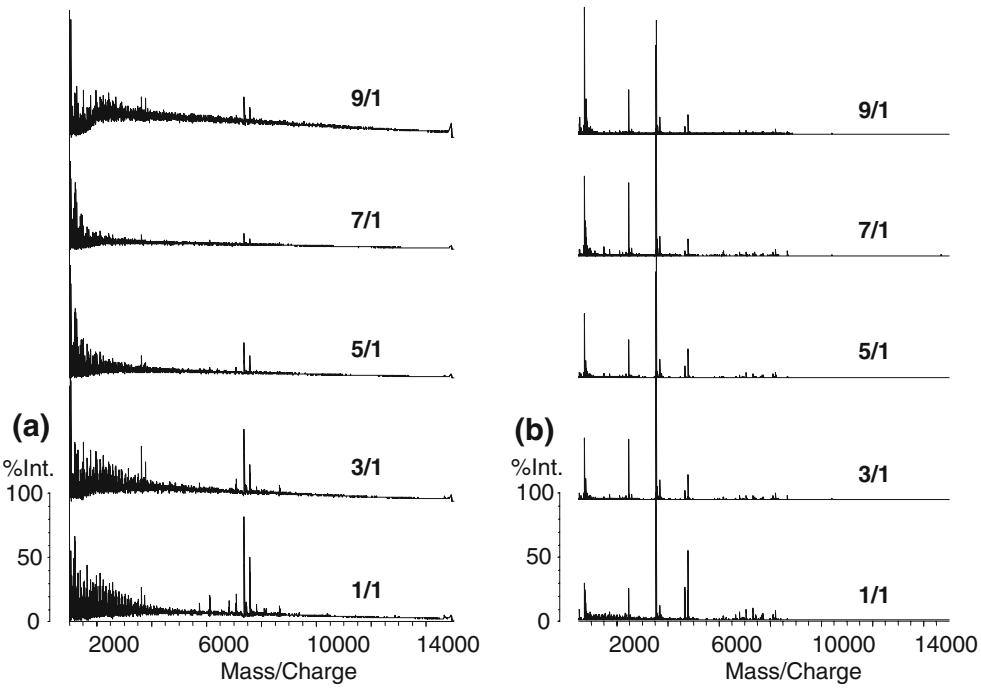


Fig. 6 Comparison of MALDI mass spectra of intact *Fusarium* conidia pores using different matrix solution/conidia spore solution ratios applied with two-layer volume technique (**a**, *left column*) *F. poae* strain 2786 (*light-colored*) and (**b**, *right column*) *F. graminearum* strain 2765 (*deep-colored*). Matrix solution, 10 mg ferulic acid dissolved in 1 mL ACN/0.1% TFA (7/3, *v*/*v*); washing solvent system: ACN/0.5% FA (7/3, *v*/*v*); matrix solution/conidia spore solution mixing time, 3 min

acquisition ("rastering across large areas"). Apparently, a crucial factor in this MALDI sample preparation is the "rewetting" process, allowing possibly the release of surface-located analytes from spores into the matrix solution and finally its co-crystallization.

Next, the influence of ratio of volumes (conidia spores and matrix solution) and time of matrix/conidia spore solution interaction on the subsequent generated MALDI mass spectra were investigated (Figs. 6 and 7). After the washing process of *Fusarium* conidia spores with ACN/0.5% FA (7/3, *v*/*v*), ferulic acid matrix solution and purified *Fusarium* conidia spore solution were mixed in an Eppendorf tube at different matrix/conidia spore solution ratios of 1/1, 3/1, 5/1, 7/1, and 9/1 (*v*/*v*) for different times

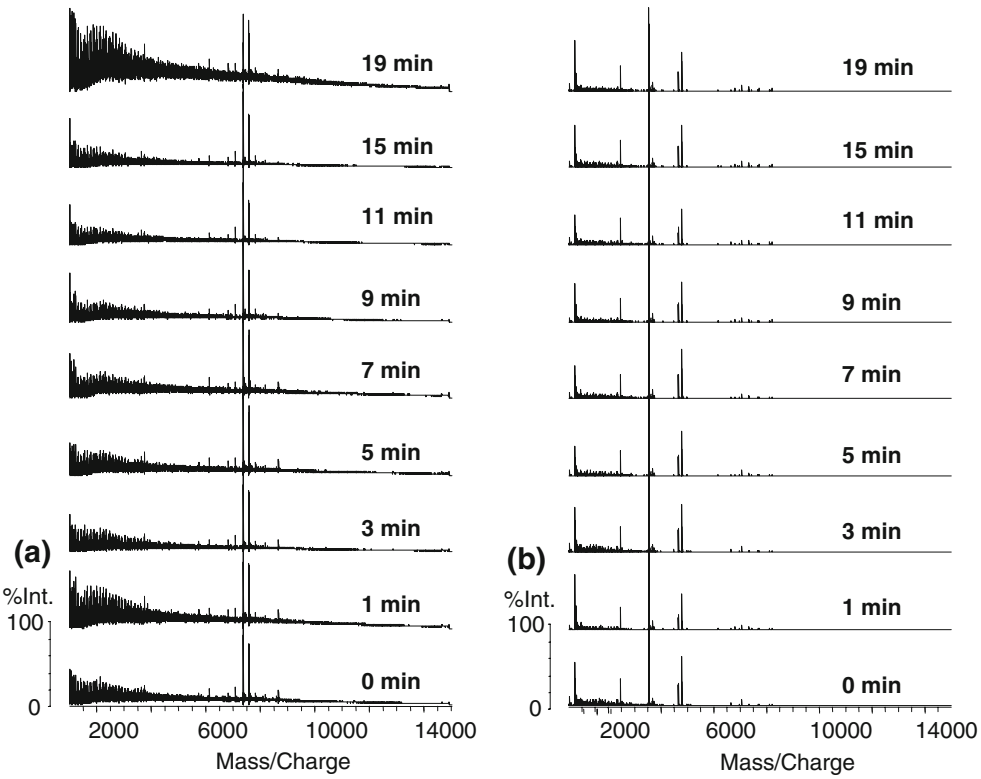


Fig. 7 Comparison of MALDI mass spectra of intact *Fusarium* conidia spores applied with two-layer volume technique at different matrix solution/conidia spore solution mixing time (**a**, *left column*) *F. poae* strain 2786 (*light-colored*) and (**b**, *right column*) *F. graminearum* strain 2765 (*deep-colored*). Matrix solution, 10 mg ferulic acid dissolved in 1 mL ACN/0.1% TFA (7/3, *v*/*v*); washing solvent systems, ACN/0.5% FA (7/3, *v*/*v*); matrix solution/conidia spore solution ratio at 1/1 (*v*/*v*)

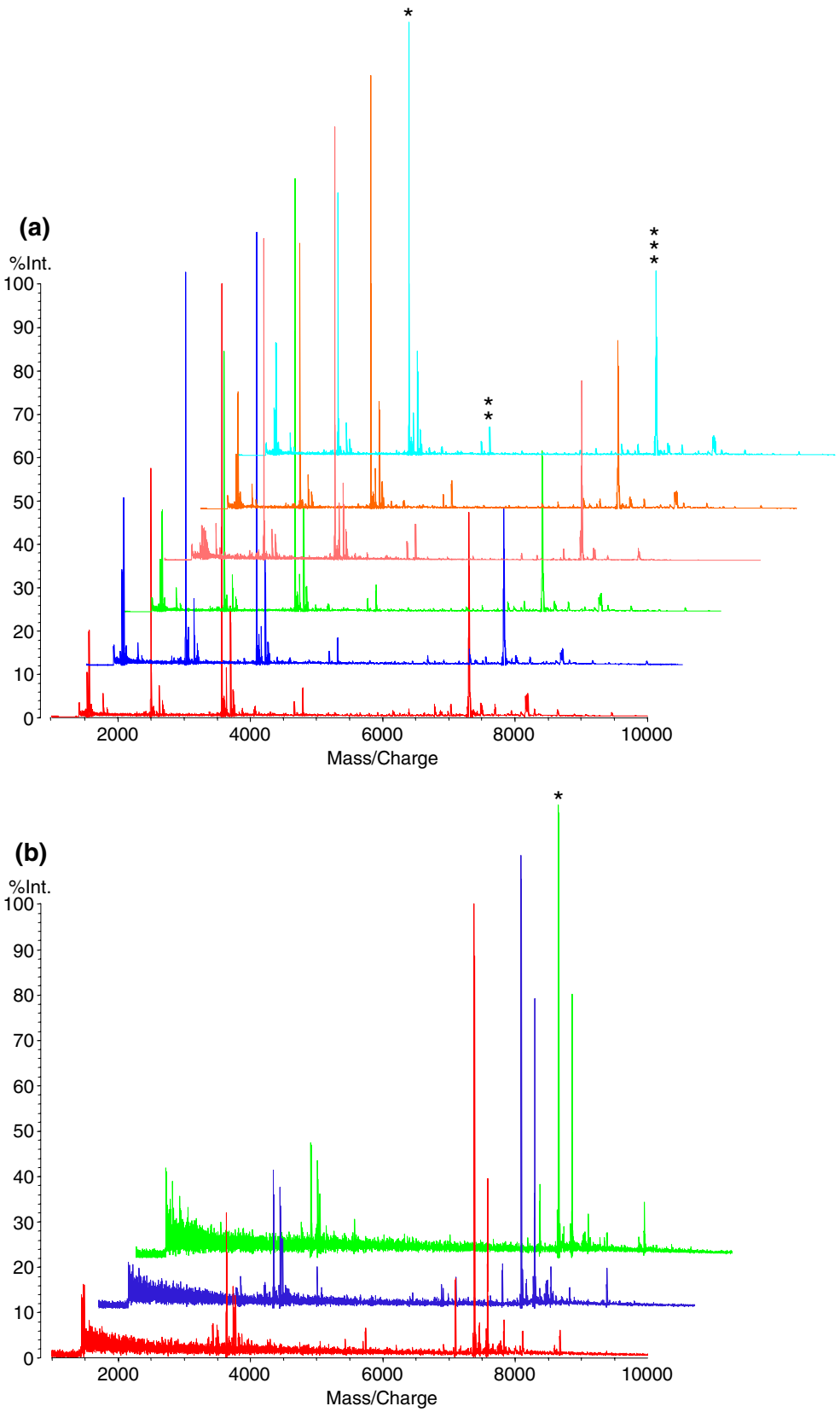


Fig. 8 Repeatability of MALDI mass spectrometric analysis by the optimal sample preparation method developed in this study. **a** Six replicate MALDI mass spectra obtained from different spots of the same sample of *F. graminearum* strain 2765; **b** MALDI mass spectra derived from three different sample batches of *F. poae* strain 2786

of 0, 1, 3, 5, 7, 9, 11, 15, and 19 min before deposition of the mixture onto the target applied with the two-layer volume technique. For the MALDI mass spectra of deep-colored species, it seems that ratio and time of matrix/conidia spore solution interaction did not affect the quality of MALDI mass spectra too much. But for light-colored species, the ratio of matrix to *Fusarium* conidia spore solution inversely correlated with the quality of MALDI mass spectra. So for further comparison of MALDI mass spectra of different *Fusarium* species and strains, we suggest to use matrix/spore solution ratio of 1/1 (*v*/*v*) at a given conidia spore concentration (see Experimental) and the duration of mixing time between both solutions should be at least 3 min for a sufficient matrix/conidia spore interaction.

Repeatability of the developed ISMS sample preparation method

MALDI–TOF mass spectra of the same *Fusarium* conidia spore preparation were reproducibly generated when samples were treated in the same well-defined way and were recorded with the same instrumental operating conditions (Fig. 8). The positive ion mass spectra in Fig. 8a were obtained from the different matrix/sample spots of the same *F. graminearum* strain 2765 preparation on the same target plate. Six replicate MALDI mass spectra were compared for reproducibility. The base peak (marked with one asterisk) in Fig. 8a was selected as an example: The mean and standard deviation (SD) for *m*/*z* value was 3,571.18 and ±0.34 Da, and the mean and the SD for its intensity was 110.5 and ±13.4 mV. Further two other peaks, which are marked with two and three asterisks, respectively, in Fig. 8a were evaluated. The peak with two asterisks (peak of low abundance) exhibited the following values: The mean and the SD for *m*/*z* value were 4,794.55 and ±0.28 Da, and the mean and the SD for its intensity were 9.5 and ±1.4 mV. For the peak (high *m*/*z* value ion) with three asterisks, the following data could be obtained: The mean and the SD for *m*/*z* value were 7,308.90 and ±0.46 Da, and the mean and the SD for its intensity were 37.2 and ±6.8 mV.

The MALDI mass spectra in Fig. 8b are the results of mass spectrometric analysis of *F. poae* strain 2786 derived from three different batches, which were independently cultivated and treated with the optimized sample preparation method. Again the base peak (marked with an asterisk) in Fig. 8b was selected as an example: The mean and the SD for *m*/*z* value was 7,384.23 and ±0.18 Da, and the mean and the SD for its intensity was 25 and ±5.28 mV. From these data, it is clear that reproducible mass spectra independent of area of desorption/ionization could be generated with the presented sample preparation method.

Conclusions

The aim of this study was to develop an ISMS sample preparation method for MALDI–TOF MS analysis of both light and strongly colored conidia spores of *Fusarium* spp. During evaluation of washing solvent systems, acid treatment turned out to be crucial to acquire useful MALDI–TOF mass spectrometric peptide/protein profiles of *Fusarium* conidia spores. The reverse two-layer volume technique was found to be the most suitable preparation technique due to its essential character of rewetting by the second matrix solution deposition and the subsequent co-crystallization process. The optimal colored intact *Fusarium* conidia spore preparation method for generating highly qualitative MALDI–TOF mass spectra can be summarized as follows: *Fusarium* conidia spores were washed three times with ACN/0.5% FA (7/3, *v*/*v*) using centrifugal devices at 19,500×*g* for 10 min. The pellet was resuspended in water with the final concentration of 3 million conidia spores/μL. Then the matrix (10 mg ferulic acid dissolved in 1 mL ACN/0.1% TFA (7/3, *v*/*v*)) and *Fusarium* conidia spore solutions were premixed in an Eppendorf tube at ratio 1/1 (*v*/*v*) for at least 3 min and subsequently applied with the reverse two-layer volume technique. By means of this sample preparation method, an automated ISMS-based analysis for differentiation of colored *Fusarium* conidia spores was for the first time possible and opens the way of a more general applicability.

Acknowledgments This investigation was supported financially in part by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management. Furthermore the authors thank Mariella Pauls for the preparation of the conidia spores.

References

1. Burnett J (2003) Fungal populations and species. Oxford University Press, NY
2. Kavanagh K (2005) Fungi—biology and applications. Wiley, Chichester
3. Welham KJ, Domin MA, Johnson K, Jones L, Ashton DS (2000) Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 14:307–310
4. Vanlaere E, Sergeant K, Dawyndt P, Kallow W, Erhard M, Sutton H, Dare D, Devreese B, Samyn B, Vandamme P (2008) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of intact cells allows rapid identification of *Burkholderia cepacia* complex. J Microbiol Methods 75:279–286
5. Phalip V, Delalande F, Carapito C, Goubet F, Hatsh D, Leize-Wagner E, Dupree P, Dorsselaer AV, Jeltsch JM (2005) Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. Curr Genet 48:366–379
6. Varga M, Bartók T, Mesterházy Á (2006) Determination of ergosterol in *Fusarium*-infected wheat by liquid chromatography-atmospheric

pressure photoionization mass spectrometry. J Chromatogr A 1103:278–283
7. Carapito R, Hatsch D, Vorwerk S, Petkovski E, Jeltsch JM, Phalip V (2008) Gene expression in *Fusarium* graminearum grown on plant cell wall. Fungal Genet Biol 45:738–748
8. Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO (1996) Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10:1227–1232
9. Chen HY, Chen YC (2005) Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 19:3564–3568
10. Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinina IS, Kubicek CP, Allmaier G (2009) Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 23:877–884
11. Fenselau C, Demirev PA (2001) Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 20:157–171
12. Lay JO (2000) MALDI-TOF mass spectrometry and bacterial taxonomy. TrAC-Trends Anal Chem 19:507–516
13. Lasch P, Nattermann H, Erhard M, Stämmler M, Grunow R, Bannert N, Appel B, Naumann D (2008) MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. Anal Chem 80:2026–2034
14. Hettick JM, Green BJ, Buskirk AD, Kashon ML, Slaven JE, Janotka E, Blachere FM, Schmechel D, Beezhold DH (2008) Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Anal Biochem 380:276–281
15. Liu H, Du Z, Wang J, Yang R (2007) Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 73:1899–1907
16. Vargha M, Takáts Z, Konopka A, Nakatsu CH (2006) Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. J Microbiol Methods 66:399–409
17. Li TY, Liu BH, Chen YC (2000) Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 14:2393–2400
18. Valentine NB, Wahl JH, Kingsley MT, Wahl KL (2002) Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 16:1352–1357
19. Malz S, Grell MN, Thrane C, Maier FJ, Rosager P, Felk A, Albertsen KS, Salomon S, Bohn L, Schafer W, Giese H (2005) Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. Fungal Genet Biol 42:420–433
20. Bhardwaj S, Shukla A, Mukherjee S, Sharma S, Guptasarma P, Chakraborti AK, Chakrabarti A (2007) Putative structure and characteristics of a red water-soluble pigment secreted by *Penicillium marneffei*. Med Mycol 45:416–427
21. Medentsev AG, Arinbasarova AY, Akimenko VK (2005) Biosynthesis of naphthoquinonne pigments by fungi of the genus *Fusarium*. Appl Biochem Microbiol 41:503–507
22. Sasanya JJ, Hall C, Wolf-Hall C (2008) Analysis of deoxynivalenol, masked deoxynivalenol, and Fusarium graminearum pigment in wheat samples, using liquid chromatography-UV-mass spectrometry. J Food Protection 71:1205–1213
23. Karas M, Hillenkamp F (1988) Laser desorption ionization of proteins with molecular masses exceeding 10, 000 daltons. Analytical chemistry 60:2299–2301
24. Rechthaler J, Allmaier G (2002) One-way hydrophobic surface foil for UV matrix-assisted laser desorption/ionization mass spectrometry of peptides. Rapid Commun Mass Spectrom 16:899–902
25. Jarman KH, Daly DS, Petersen CE, Saenz AJ, Valentine NB, Wahl KL (1999) Extracting and visualizing matrix-assisted laser desorption/ionization time-of-flight mass spectral fingerprints. Rapid Commun Mass Spectrom 13:1586–1594
26. Dai Y, Whittal RM, Li L (1996) Confocal fluorescence microscopic imaging for investigating the analyte distribution in MALDI matrices. Anal Chem 68:2494–2500
27. Kussmann M, Nordhoff E, Rahbek-Nielsen H, Haebel S, Rossel-Larsen M, Jakobsen L, Gobom J, Mirgorodskaya E, Kroll-Kristensen A, Palm L, Roepstorff P (1997) Matrix-assisted laser desorption/ionization mass spectrometry sample preparation techniques designed for various peptide and protein analytes. J Mass Spectrom 32:593–601

Journal of Microbiological Methods, *to be submitted* (2010).

4.2 Characterization of *on-target* generated tryptic peptides from *Giberella zeae* conidia spore proteins by means of matrix-assisted laser desorption/ionization post source decay fragment ion analysis

Hongjuan Dong [a], Martina Marchetti-Deschmann [a], Christian Peter Kubicek [b] and Günter Allmaier [a,*]

[a] *Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164-IAC, A-1060 Vienna, Austria*

[b] *Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/166, A-1060 Vienna, Austria*

Corresponding author:

G. Allmaier
Institute of Chemical Technologies and Analytics
Vienna University of Technology
Getreidemarkt 9/164-IAC, A-1060 Vienna, Austria
Phone: +43 1 58801 15160
Fax: +43 1 58801 15199
E-mail: guenter.allmaier@tuwien.ac.at

ABSTRACT

Traditionally characterization of microbial proteins is performed by a complex sequence of steps with the final step to be either Edman sequencing or mass spectrometry, which generally takes several weeks or months to be complete. In this work, we proposed a strategy for the characterization of tryptic peptides derived from *Giberella zeae* (anamorph: *Fusarium graminearum*) proteins in parallel to intact cell mass spectrometry (ICMS) in which no complicated and time-consuming steps were needed. Experimentally, after a simple washing treatment of the spores, the aliquots of the intact *G. zeae* macroconidia spores solution, were deposited two times onto the MALDI (matrix-assisted laser desorption ionization) mass spectrometry (MS) target (two spots). One spot was used for ICMS and the second spot was subject to a brief *on-target* digestion with bead-immobilized or non-immobilized trypsin. Subsequently, one spot was analyzed immediately by MALDI MS in the linear mode (ICMS) whereas the second spot containing the digested material was investigated by MALDI MS in the reflectron mode ("peptide mass fingerprint") followed by protonated peptide selection for post source decay fragment ion analysis. Based on the formed fragment ions of selected tryptic peptides a complete or partial amino acid sequence was generated by manual *de novo* sequencing. These sequence data were used for homology search for protein identification. Finally four different peptides of varying abundances have been identified successfully allowing the verification that our desorbed/ionized surface compounds were indeed derived from proteins. The presence of three different proteins could be found ambiguously. Interestingly, one of these proteins is belonging to the ribosomal superfamily which indicates that not only surface-associated proteins were digested. This strategy minimized the amount of time and labor required for obtaining deeper information on spore preparations within the nowadays widely used ICMS approach.

Keywords: Conidia spores, *Giberella, Fusarium,* MALDI TOF mass spectrometry, ICMS, *on-target* tryptic digestion, post source decay fragment ion analysis

1. Introduction

Giberella zeae (anamorph form: *Fusarium graminearum*) is a common cereal contaminant and a well-known plant pathogen causing head blight in wheat and an important cause of crown/foot rots in maize as well as other cereals (Sutton, 1982). Since the end of the last century, fungal pathogens are causing more and more infections in human beings, too (Martin et al., 2003). *G. zeae* belongs to the large genus of filamentous fungi which are geographical widely distributed (Burnett, 2003; Kavanagh, 2005). It produces asexual spores (macro conidia) and sexual spores (ascospores) which allow different ways of distribution and subsequently infections. Under certain conditions it can produce mycotoxins on important cereal crops which can affect human and animal health if they are entering the food chain (Phalip et al., 2005; Varga et al., 2006). Therefore it is currently a serious agricultural and public health problem in several parts of the world (Carapito et al., 2008). Rapid detection and characterization is of great importance and in demand. In accordance to previous results reported by our laboratory, intact cell (ICMS) or better termed intact spore (ISMS) mass spectrometry, based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) has been proven to be a powerful tool for differentiation of this kind of fungal microorganisms (Dong et al., 2009; Kemptner et al., 2009a; Kemptner et al., 2009b). However, during these investigations the following two main questions came up: (1) How do we know that the peaks in intact cell/spore MALDI mass spectra are peptides or proteins? (2) What proteins are they? Based on these questions we tried to develop a simple and straightforward strategy to answer them without a tedious isolation/purification.

In proteomics, the strategies of identification of proteins from microorganisms can be broadly divided into two categories “bottom-up” and “top-down” (Bogdanov and Smith, 2005; Wehr, 2006). Bottom-up methods, the most widely used approach for protein identification and characterization, in which the analytes that are introduced into the mass spectrometer are enzymatically generated peptides from many proteins, which means the crude protein extract was digested and afterwards separated by mostly multi-dimensional liquid chromatography or capillary electrophoresis, or alternatively the proteins from the extract were separated by 1D or 2D planar gel electrophoresis and the isolated protein spots *in-gel* digested (Ahn et al., 2007; Bogdanov and Smith, 2005; Chait, 2006; Wehr, 2006). Despite bottom-up approach is a mature technique and has broad proteome coverage and good throughput, it provides limited information about the intact proteins, as for example molecular mass and heterogeneity (Choudhary et al., 2003; Gao et al., 2008). Top-down proteomics, relies on intact protein level analysis which starts with separation of proteins and is followed by high-end mass

spectrometric analysis (Bogdanov and Smith, 2005; Demirev et al., 2005; Getie-Kebtie et al., 2008; Wehr, 2006). It is relatively young field compared to bottom-up proteomics and is still under development. It suffers from lower coverage, relative low sensitivity and can not be considered as high throughput technology at the moment. Furthermore its favored instrumentation (Wehr, 2006), Fourier transform ion cyclotron resonance mass spectrometry is expensive to purchase and also to operate.

Traditionally characterization of microbial proteins (not applying an proteomic approach) are performed by a complex sequence of steps applying mostly detergent-supported extraction/isolation, chromatography and/or electrophoresis, eventually enzymatic digestion and finally Edman sequencing or mass spectrometry, which generally takes several weeks or months to complete (Yu et al., 2006; Padliya et al., 2007). These steps, in addition to being time-consuming, also maximize the risk of sample contamination and loss of protein particular in case of handling surface proteins which are often hydrophobic in nature. *On-target* spore digestion technique, however, is an alternative method that reduces the required time efficiently (Lin et al., 2004; Warscheid and Fenselau, 2003; Warscheid et al., 2003; Yao et al., 2002). It is not necessary to employ any prior complicated extraction/isolation or separation procedure but only a minimal sample washing treatment is required. Furthermore, IC/ISMS has been widely used for characterize different species or strains of microorganisms (Amiri-Eliasi and Fenselau, 2001; Chen and Chen, 2005; Fenselau and Demirev, 2001; Lasch et al., 2008; Li et al., 2000; Ryzhov et al., 2000; Shaw et al., 2004; Welham et al., 2000). So the combination of *on-target* spore digestion with MS analysis could provide information for the identification of proteins and the connected microorganisms.

In this study, determination of peptide sequence tags from spores of *G. zeae* has been developed using *on-target* tryptic digestion followed by MALDI mass spectrometric analysis for answering the two questions mentioned earlier. It means, after a simple washing procedure, the *Giberella* spores were deposited onto the MALDI target. One aliquot was just prepared for IC/ISMS analysis and the second aliquot was *on-target* digested directly by means of a bead-immobilized or a non-immobilized trypsin. Subsequently one preparation was analyzed by IC/ISMS and the second preparation, namely the digest was analyzed by MALDI MS and several formed tryptic peptides were selected for post source decay (PSD) fragment ion analysis to generate sequence data. The strategy of this entire process, which is outlined in Figure 1, was rapid and straightforward to perform. The challenges faced here were to obtain precursor (tryptic peptide) ions of sufficient abundance for PSD experiments after *on-target* spore digestion in a relative short digestion time (to try to cleave mainly the surface proteins) as well as to do the manual *de novo* sequencing.

2. Materials and methods

2.1. Materials and reagents

Acetonitrile analytical grade (p.a., pro analysis, ACN), formic acid 98-100% (FA) and water p.a. were obtained from Merck (Darmstadt, Germany). Trifluoroacetic aicd (TFA) was purchased from Riedel-de Häen (Seelze, Germany). Ammonium bicarbonate and ferulic acid (FA) was obtained from Fluka (Buchs, Germany) and α-cyano-4-hydroxy cinnamic acid (CHCA) from Sigma-Aldrich (St. Lois, MO, USA). Non-immobilized trypsin isolated from bovine pancreas was from Roche Diagnostics (Cat. No. 11418025001, Mannheim, Germany). TPCK-treated trypsin immobilized on agarose beads, also isolated from bovine pancreas, was purchased from Pierce Biotechnology (No. 20230, Rockford, IL, USA).

2.2. Strains and cultivation conditions

Giberelle zeae (*Fusarium graminearum* CPK No. 2985) was used in this work and it is maintained in the strain collection at the Institute of Chemical Engineering of the Vienna University of Technology (Vienna, Austria). It was at first cultivated on SNA-plates (Synthetischer Nährstoffarmer Agar) to get fully vitalization. Afterwards one fully grown SNA plate was inoculated with 25 mL of mungbean soup (20 g mungbean are cooked with 1 L of tab water for half an hour. Before use the filtered broth was autoclaved at 121 °C for 20 min) for 4 days at 28 °C in shaking flasks (160 rpm) to get sufficient amount of conidia spores. Then the conidia spores were filtered through sterile glass wool to remove mycelium and subsequently the obtained filtrate was centrifuged at 8000 rpm, at 4 °C for 10 min. The obtained conidia spores were pipetted into sterile Eppendorf tubes and mixed with 20% aqueous glycerol (w/v) for long term storage at -20 °C. The concentration of the spores suspension was determined via a light-optical microscope (Nikon Instruments Europe, Amstelveen, The Netherlands) using a counting chamber.

2.3. Conidia spore purification

Before MALDI MS investigations conidia spores were washed to get rid of glycerol and contaminants from the media (Dong et al., 2009). The spores were washed 3 times with 100 µL ACN/0.5% aqueous FA (7/3, v/v) at 13200 rpm for 10 min using Nanosep™ (Pall, Ann Arbor, MI, USA) centrifugal devices (molecular weight cut-off 10 kDa). Then the spores were resuspended in pure water with the concentration at 3 million spores/µL for IC/ISMS analysis and in case of *on-target* digest in 25 mM ammonium bicarbonate solution with a spore concentration of 1.5 million/µL, respectively.

2.4. Sample preparation for MALDI MS (IC/ISMS)

MALDI MS sample preparation was just prepared following the optimized method described in our previous paper (Dong et al., 2009). Briefly, 10 mg FA were dissolved in 1 mL ACN/aqueous 0.1% TFA (7/3, v/v) as matrix solution and the two-layer volume deposition technique (conidia spores/matrix mixture was deposited onto a stainless steel MALDI target and after solvent evaporation a second matrix layer was deposited) was used for intact spore MALDI MS analysis.

2.5. On-target digestion for MALDI post source decay fragment ion analysis

Before use for *on-target* spore digestion immobilized TPCK trypsin was washed following the instruction provided by the company and finally 4 times diluted (in terms of volume) with 25 mM ammonium bicarbonate solution. An aliquot of 1 µL conidia spores suspension in 25 mM ammonium bicarbonate solution was placed onto the microliter format stainless-steel MALDI MS target. After completely drying the spot at room temperature (RT), 0.5 µL of the prepared bead-immobilized trypsin solution or 0.5 µL of 0.1 µg/µL non-immobilized trypsin in 25 mM ammonium bicarbonate solution were added onto each MALDI MS sample spot, respectively. Then the samples prepared on the MALDI MS target plate were incubated in a humidity chamber at RT to prevent spot drying. *On-target* digestion was stopped after 25 min by allowing the samples to dry at RT and addition of 0.5 µL of 10 mg/mL CHCA in ACN/0.1% aqueous TFA (70/30, v/v) for MS analysis.

2.6. MALDI mass spectrometry

All MALDI mass spectra were obtained on Axmia-CFR^{plus} instrument (Shimadzu Biotech Kratos Analytical, Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width) and a curved field reflector. The instrument was operated at an acceleration voltage of 20 kV. Intact cell/spore MALDI mass spectra were recorded in positive ion linear mode with delayed extraction (optimized for m/z 5000) by accumulating up to 2500 single unselected laser shots in the m/z range of 500 to 15000 using the above mentioned optimized sample preparation protocol (Dong et al., 2009). The blanking gate was set at m/z 500 to remove the ions below this m/z value arising from the intact matrix, their clusters as well as fragments and other unknown contaminants. Baseline substraction (baseline filter width 60) and smoothing (Shimadzu Biotech supplied Savitzky-Golay algorithm, smoothing filter width 20) were applied to all mass spectra. An external three-point calibration was performed with the protein cytochrome C (protonated molecule at m/z 12361.2 and double protonated molecule at m/z 6181.1) and the standard peptide ACTH 7-38 (protonated molecule at m/z 3657.9).

MALDI mass spectra of the tryptic digests were collected in positive ion reflectron mode with delayed extraction (optimized for m/z 2000) by accumulating up to 2500 single unselected laser shots in the total m/z range of 10 - 5000. Internal calibration was performed using the singly charged peptides from tryptic autodigestion products at m/z 659.38, 805.42, 2163.06 and 2273.16 (monoisotopic m/z values). The monoisotopic m/z values of protonated peptides (see Table 1) were manually derived from smoothed (smoothing filter width 3) mass spectra without base-line subtraction. Typically mass spectra showing a resolution of 7000 to 9000 (full-width half-maximum at m/z 2258.11) and a monoisotopic mass accuracy of ± 30 mDa could be observed.

For peptide identification based on sequence data PSD fragment ion analysis was carried out on selected peptides under standard vacuum conditions (~ 1×10^{-7} mbar). PSD spectra were recorded in positive ion mode by accumulating up to 2500 single unselected laser shots. The lists of m/z value for *de novo* sequencing were automatically derived as average m/z values from smoothed spectra (smoothing filter width 20) with baseline subtraction (baseline filter width 60). Typically PSD spectra exhibited an average mass accuracy of ± 0.5 Da in the low m/z range (< m/z 1500) and up to ± 2 Da in the high m/z range (> m/z 2000).

2.7. Data analysis and database search

De novo peptide sequencing was performed manually in this paper. *De novo* sequencing is the term used for the process of deriving peptide sequences from PSD or CID spectra without using a sequence database (Johnson et al., 2005). In case of MALDI MS yielding relatively complex but interpretable fragmentation spectra, it is possible to generate complete or partial peptide sequences *de novo* from the spectra (Zhang et al., 2003; Zhang et al., 2008). In this study, four *de novo* determined peptide sequences were deduced manually and used for similarity searches using Protein Prospector search engine with MS-Homology, available on-line from the University of California, San Francisco (http://prospector.ucsf.edu/). The searches were performed against the NCBI non-redundant database with all taxonomy as well as BLOSUM 50 as search matrix.

3. Results and discussion

3.1. Intact cell / intact spore mass spectrometry

Initial studies were performed to do ISMS of intact *Giberella zeae* conidia spores. Fig. 2 showed the typical IC/IS mass spectrometric profiles for the strain 2985 and the corresponding microscopic image of the matrix/spores spot. The mass spectrum was measured in the m/z range of 500 - 15000 but the majority of the observed mass spectrometric peaks (about 24 peaks with a signal-to-noise ratio of at least 3:1 or better) were detected within the m/z range of 700 - 5000. The analysis showed quite high reproducibility (data not shown) and the base peak was all the times at m/z 3549.71 (average m/z value) in eight replicate MALDI mass spectra. Using the method of IC/ISMS, different species or strains could be distinguished based on peak patterns (Dong et al., 2009; Kemptner et al., 2009a), but it was not shown that the peaks observed are of peptide/protein nature and no protein identification was performed. Therefore the following *on-target* spore digestion combined with MALDI reflectron MS to generate a PMF and subsequent curved field PSD fragment analysis were performed for identification of surface-associated proteins from conidia spores.

3.2. On-target digestion of conidia spores using both non-immobilized and bead-immobilized trypsin

First the feasibility of *on-target* tryptic digestion from conidia spores in combination with MALDI reflectron MS has been investigated. After the preparation of *on-target* spore digestion with both non-immobilized and immobilized trypsin, the generated tryptic digests from the surface of spores were directly subject to MALDI reflectron mass spectrometric analysis without any spot cleanup procedure (Fig. 3). In order to evaluate (in terms of m/z values and abundances) the generated background ions in the MALDI mass spectra the *on-target* spore tryptic digestion method was performed without enzyme presence. The corresponding MALDI mass spectrum and microscopic image of sample/matrix spot is shown in Fig. 3 (C). From the Fig. 3 (C) we can see that the mass spectrum exhibits only a few peaks or peak groups below m/z 1100. In contrast, the samples digested with both non-immobilized and immobilized trypsin yielded quite informative MALDI mass spectra in the m/z range of 500 - 2500 (Fig. 3 (A) and Fig. 3 (B)). Low abundant peaks between m/z 2500 and 5000 could also be detected, but these were usually only present at a low S/N ratio and therefore were not abundant enough to be selected afterwards for PSD fragment ion analysis. The m/z values given in Fig. 3(A) and Fig. 3(B) are based on internal calibration with autotryptic peptides. In general, MALDI mass spectra based on samples generated with non-immobilized

trypsin exhibit higher peak intensities than the mass spectra derived from samples generated with bead-immobilized trypsin especially in the important m/z range of 1500 - 2500. Both sample types (generated by the two kinds of trypsins) yielded distinctive tryptic peptides of their own, but there were still quite a lot of peaks with the same m/z value but different abundances. For example, three of selected four precursor ions in the following section for PSD fragment ion analysis, namely m/z 673.43, 1888.90, 2258.11 could be obtained from both kinds of typsin digests. The MALDI MS matrix/sample morphology (see insets in Fig. 3 (A) and (B)) is also slightly different, which might influence the absolute and relative ion abundances too despite sample rastering was used. *On-target* digestion with agarose bead-immobilized trypsin (relative large particle size) has been applied to *Bacillus* spores before in combination with the MALDI matrix CHCA (Warscheid and Fenselau, 2003). However unfortunately, unlike the results provided by Warscheid and Fenselau, peptide peaks generated from immobilized trypsin digests of our macroconidia spores were not in general abundant enough to do PSD fragment ion analysis. There are two reasons from our viewpoint, why this difference was observed: (1) Possibly the presence of agarose beads affect the desorption/ionization of the tryptic peptides when rastering across the whole spot is applied and not the "sweet spot" approach is used and/or (2) the surface character of *Giberella* macoconidia spores is different to *Bacillus* spores (i.e. less surface proteins are present due to fact that our organism is a fungus). So finally all PSD fragment ion analyses were performed on the tryptic peptides derived from *on-target* digestion using non-immobilized trypsin.

3.3. De novo peptide sequencing based on MALDI PSD fragment ion mass spectra

Based on the generated tryptic peptide pattern PSD experiments were performed on selected (based on their precursor ion abundance) peptides. Because of the short digestion time (to generate only tryptic peptides from the surface proteins), limited amount of accessible cleavage sites and the sensitivity of the mass spectrometer, only a few protonated peptide molecules of the tryptic digests were abundant enough for PSD fragmentation. In the end four peptides with determined monoisotopic m/z values at 673.43, 924.56, 1888.90 and 2258.11 were selected to perform PSD fragment ion analysis and exhibit sufficient fragment ions. In Fig. 4 examples of successful (in terms of interpretation) PSD spectra are shown after smoothing and baseline subtraction. Sequence-specific information was obtained using manual *de novo* peptide sequencing from these PSD spectra. A considerable number of PSD spectra were obtained furthermore, but the number and S/N ratio of the fragment ions was too low for reliable manual *de novo* sequence or sequence tag determination.

Fig. 4 (A) showed the PSD spectrum of the precursor ion at m/z 673.7 (the precursor ion selection gate was set on the value of the average protonated molecules for all four peptides, av.). The y_1 value of m/z 175.45 allowed C-terminal residue Arg determination to be made. Then the corresponding penultimate b ion was $[M+H]^+ - y_1 + 1$ at m/z 499.2. The b ion series extended all of the way down into the low m/z region of the PSD spectrum. The a-type ion fragments did accompany some of the b-type ions down the PSD spectrum giving us some confidence that we were following the correct sequence. By simple calculation of the differences either the adjacent y or b type ion fragments, the same contiguous full sequence [L|I]TGN[L|I]R could be obtained (shown in Fig. 4 (A)).

Fig. 4 (B) exhibits the fragment ion spectrum of the peptide with an $[M+H]^+$ ion at m/z 1890.6 (av.). We found that the C-terminal residue matched for Arg residue with y_1 value at m/z 175.87. As expected, the y-type ion series were evident in the PSD spectrum, and all y-type ions were accompanied by y-17 ions which is known to be the loss of ammonia (-17 Da). Once the y-ion series was marked, a sequence tag of 16 amino acid residues length, namely TT[Q|K]SSATWG[L|I]GTVSHR, was easily assigned based on the mass difference of adjacent y-type ions.

In the same way we deduced the peptide sequence of the precursor ion at m/z 924.6 (av.), for which the spectrum is shown in Fig. 4 (C). From the selected precursor ion both a series of y- and b-type ions was formed. By working in the C-terminal to N-terminal direction, we found y_1 at m/z 175.19 again which indicates Arg residue at C-terminal end. Then a sequence tag VHGS[L|I]AR according the difference of adjacent y-type ions was deduced. By analyzing the spectrum from the N-terminal to C-terminal end, a sequence tag HGS[L|I]AR according to the difference of adjacent b-type ions was deduced. The same section of the two sequence tags gave us high confidence that we assigned the peaks in a correct way.

Fig. 4 (D) shows the PSD fragment ion spectrum with the sequence annotation derived from the peptide with the protonated molecule at m/z 2261.3 (av.). We could not observe the y_1 value indicating an Arg residue at the C-terminus in the spectrum as before. If the difference between the $[M+H]^+$ m/z value and a lower mass fragment is equal to an amino acid residue, then there is a good probability that this ion is the N-terminal residue of the peptide sequence. In the present case, we could observe a mass difference of 86.9 Da between the protonated molecule (m/z 2261.3) and the fragment ion at m/z 2174.4corresponding to a Ser residue at the N-terminal end. From this point y-type ions could be assigned down into the low m/z range of the PSD spectrum. Based on these y-type ions a peptide sequence tag STTE[Q|K]E[L|I][Q|K]E[L|I]A[Q|K]DA[L|I]S[Q|K] could be deduced.

3.4. Database Search and protein identification

Due to fact that the proteins of *Giberella zeae* are only available in a very limited number in the public database, protein identification in the present study relied on homology search. Using Protein Prospector search engine with MS-Homology algorithm and the NCBI non-redundant database, a search with the sequence tags obtained from the PSD spectra was performed. With sequence tags [L|I]TGN[L|I]R and TT[Q|K]SSATWG[L|I]GTVSHR input, two protonated tryptic peptides (K)ITGNLR(S) and (R)AETTQSSATWGLGTVSHR(S), located in the same hypothetical protein FG00806.1, were determined in the present work, and comprise the amino acids 394-399 and 129-146, respectively. From the amino acid sequence of the protein FG00806.1 a tryptic peptide with theoretical monoisotopic ion at m/z 2844.37 (average m/z 2845.37 in Fig. 3 (A)) could be calculated, which was detected in the IC/IS mass spectrum of the *on-target* digested sample. Unfortunately this peptide ion was not intense enough to generate a PSD spectrum with sufficient informative product ions. But all these results in combination gave us a high confidence that the protein FG00806.1 was unambiguously identified and is located on the spore surface of *Giberella zeae* strain 2985. In the same way sequence tags VHGS[L|I]AR and STTE[Q|K]E[L|I][Q|K]E[L|I]A[Q|K]DA[L|I]S[Q|K] were used for homology search and the tryptic peptide sequences (M)GKVHGSLAR(A) and (R)STTEQEIQELAQDAISKPGGR(A) were matched, belonging to hypothetical protein FG04915.1 and FG07774.4, respectively.

An overview of the individual proteins identified in this work from macroconidia spores is given in Tab. 1, which may provide useful information for future studies on fungal spores. Through homology search of the identified proteins it was found that protein FG00806.1 and FG04915.1 were belonging to protein superfamily peptidase S8 and ribosomal S30, respectively. For protein FG07774.4 there is no functional annotation available to our knowledge. By the observation that one protein is considered as ribosomal protein it can be tentatively concluded that, proteins identified in conidia spores with *on-target* digestion are not always surface-associated proteins. There is a high probability that the ribosomal protein is deriving from inner parts of the spores. This result is corroborated by the fact that the tryptic peptide at m/z 924.6 indicating the presence of a ribosomal protein can only be obtained from samples with the digestion by non-immobilized trypsin, but not by agarose bead-immobilized trypsin. One possible reason may be that the digestion with trypsin immobilized on agarose-beads could not move forward into the inner layers of the spores.

4. Conclusion

On-target tryptic digestion of fungal spores combined with MALDI reflectron MS and PSD fragment ion analysis has been successfully applied to verify the presence of peptides/proteins in the IC/IS mass spectra and to identify by means of sequence tags *Giberella zeae* macroconidia spore proteins. PSD fragmentation produced complex but still interpretable fragment ion spectra which allowed us to tentatively assign peptide sequences or sequence tags using a manual *de novo* sequencing approach (no good software for PSD fragmentation interpretation is available). The identification of spore proteins by MS-homology search could be accomplished. Furthermore, the effectiveness of immobilized and non-immobilized trypsin has been compared in the present work and the results proved that the latter is more proper (in terms of signal abundance and number of tryptic peptides) for the MALDI PSD fragment ion analysis of tryptic peptides from fungal spores.

Acknowledgements

This investigation was supported partly by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (grant number 100053). Furthermore the authors thank Pelin Candarlioglu for the preparation of the macroconidia spores.

References

Ahn, N.G., Shabb, J.B., Old, W.M., Resing, K.A., 2007. Achieving in-depth proteomics profiling by mass spectrometry. ACS. Chem. Biol. 2, 39-52.

Amiri-Eliasi, B., Fenselau, C., 2001. Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. Anal. Chem. 73, 5228-5231.

Bogdanov, B., Smith, R.D., 2005. Proteomics by FTICR mass spectrometry: top down and bottom up. Mass. Spectrom. Rev. 24, 168-200.

Burnett, J., 2003. Fungal populations and species. Oxford University Press, N.Y., USA.

Carapito, R., Hatsch, D., Vorwerk, S., Petkovski, E., Jeltsch, J.M., Phalip, V., 2008. Gene expression in *Fusarium graminearum* grown on plant cell wall. Fungal. Genet. Biol. 45, 738-748.

Chait, B.T., 2006. Mass spectrometry: bottom-up or top-down?. Science. 314, 65-66.

Chen, H.Y., Chen, Y.C., 2005. Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry. Rapid. Commun. Mass. Spectrom. 19, 3564-3568.

Choudhary, G., Wu, S.L., Shieh, P., Hancock, W.S., 2003. Multiple enzymatic digestion for enhanced sequence coverage of proteins in complex proteomic mixtures using capillary LC with ion trap MS/MS. J. Proteome. Res. 2, 59-67.

Demirev, P.A., Feldman, A.B., Kowalski, P., Lin, J.S., 2005. Top-down proteomics for rapid identification of intact microorganisms. Anal. Chem. 77, 7455-7461.

Dong, H., Kemptner, J., Marchetti-Deschmann, M., Kubicek, C.P., Allmaier, G., 2009. Development of a MALDI two-layer volume sample preparation technique for colored intact *Fusarium* spore analysis by MALDI linear TOF mass spectrometry. Anal. Bioanal. Chem. 395, 1373-1383.

Fenselau, C., Demirev, P.A., 2001. Characterization of intact microorganisms by MALDI mass spectrometry. Mass. Spectrom. Rev. 20, 157-171.

Gao, M., Yu, W., Zhang, Y., Yan, G., Deng, C., Yang, P., Zhang, X., 2008. Integrated strong cation exchange/capillary reversed-phase liquid chromatography/on-target digestion coupled with mass spectrometry for identification of intact human liver tissue proteins. Analyst. 133, 1261-1267.

Getie-Kebtie, M., Franke, P., Aksamit, R., Alterman, M.A., 2008. Experimental evaluation of protein identification by an LC/MALDI/on-target digestion approach. J. Proteome. Res. 7, 3697-3707.

Johnson, R.S., Davis, M.T., Taylor, J.A., Patterson, S.D., 2005. Informatics for protein identification by mass spectrometry. Methods. 35, 223-236.

Kavanagh, K., 2005. Fungi - Biology and Applications. John Wiley and Sons, Chichester, UK.

Kemptner, J., Marchetti-Deschmann, M., Mach, R., Druzhinina, I.S., Kubicke, C.P., Allmaier, G., 2009a. Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 23, 877-884.

Kemptner, J., Marchetti-Deschmann, M., Kubicek, C.P., Allmaier, G., 2009b. Mixed volume sample preparation method for intact cell mass spectrometry of Fusarium spores. J. Mass. Spectrom. 44, 1622-1624.

Lasch, P., Nattermann, H., Erhard, M., Stämmler, M., Grunow, R., Bannert, N., Appel, B., Naumann, D., 2008. MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. Anal. Chem. 80, 2026-2034.

Li, T.Y., Liu, B.H., Chen, Y.C., 2000. Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 14, 2393-2400.

Lin, Y.S., Yang, C.H., Chen, Y.C., 2004. Glass-chip-based sample preparation and on-chip trypic digestion for matrix-assisted laser desorption/ionization mass spectrometric analysis using a sol-gel/2,5-dihydroxybenzoic acid hybrid matrix. Rapid. Commun. Mass. Spectrom. 18, 313-318.

Martin, G.S., Mannino, D.M., Eaton, S., Moss. M, 2003. The epidemology of sepsis in the United States from 1979 through 2000. N. Engl. J. Med. 348, 1546-1554.

Padliya, N.D., Garrett, W.M., Campbell, K.B., Tabb, D.L., Cooper, B., 2007. Tandem mass spectrometry for the detection of plant pathogenic fungi and the effects of database composition on protein inferences. Proteomics. 7, 3932-3942.

Phalip, V., Delalande F., Carapito, C., Goubet, F., Hatsch, D., Leize-Wagner, E., Dupree, P., Dorsselaer, A.V., Jeltsch, J.M., 2005. Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall. Curr. Genet. 48, 366-379.

Ryzhov, V., Hathout, Y., Fenselau, C., 2000. Rapid characterization of spores of *Bacillus cereus* group bacteria by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Appl. Environ. Microbiol. 66, 3828-3834.

Shaw, E.I., Moura, H., Woolfitt, A.R., Ospina, M., Thompson, H.A., Barr, J.R., 2004. Identification of biomarkers of whole *Coxiella burnetii* phase I by MALDI-TOF mass spectrometry. Anal. Chem. 76, 4017-4022.

Sutton, J.C., 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. Can. J. Plant. Pathol. 4, 195-209.

Varga, M., Bartók, T., Mesterházy, Á., 2006. Determination of ergosterol in *Fusarium*-infected wheat by liquid chromatography-atmospheric pressure photoionization mass spectrometry. J. Chromatogr. A. 1103, 278-283.

Warscheid, B., Fenselau, C., 2003. Characterization of *Bacillus* spore species and their mixtures using postsource decay with a curved-field reflectron. Anal. Chem. 75, 5618-5627.

Warscheid, B., Jackson, K., Sutton, C., Fenselau, C., 2003. MALDI analysis of *Bacilli* in spore mixture by applying a quadrupole ion trap time-of-flight tandem mass spectrometer. Anal. Chem. 75, 5608-5617.

Wehr, T., 2006. Top-down versus bottom-up approaches in proteomics. LCGC. North. America. 24, 1004, 1006-1008, 1010.

Welham, K.J., Domin, M.A., Johnson, K., Jones, L., Ashton, D.S., 2000. Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry. Rapid. Commun. Mass. Spectrom. 14, 307-310.

Yao, Z.P., Afonso, C., Fenselau, C., 2002. Rapid microorganism identification with on-slide proteolytic digestion followed by matrix-assisted laser desorption/ionization tandem mass spectrometry and database searching. Rapid. Commun. Mass. Spectrom. 16, 1953-1956.

Yu, W., Li, Y., Deng, C., Zhang, X., 2006. Comprehensive two-dimensional separation in coupling of reversed-phase chormatography with capillary isoelectric focusing followed by MALDI-MS identification using on-target digestion for intact protein analysis. Electrophoresis. 27, 2100-2110.

Zhang, W., Krutchinsky, A.N., Chait, B.T., 2003. "*De novo*" peptide sequencing by MALDI-quadrupole-ion trap mass spectrometry: a preliminary study. J. Am. Soc. Mass. Spectrom. 14, 1012-1021.

Zhang, X., Rogowska-Wrzesinska, A., Roepstorff, P., 2008. On-target sample preparation of 4-sulfophenyl isothiocyanate-derivatized peptides using AnchorChip Targets. J. Mass. Spectrom. 43, 346-359.

Legends to table and figures

Fig. 1. Strategy for generation and identification of peptide from *Giberella zeae.*

Fig. 2. Intact spore MALDI mass spectrum of *Giberella zeae* strain CPK NO. 2985.

Fig. 3. MALDI TOF mass peptide mass spectra of *Giberella* strain 2985 with *on-target* digestion using (A) non-immobilized trypsin and (B) immobilized trypsin as well as (C) without enzyme (the applied MALDI MS sample preparation in this case is not useful for ICMS). Trypsin autodigestion peaks were marked with * and the selected peaks for PSD fragment ion analysis were marked with ▼.

Fig. 4. MALDI PSD (MS/MS) spectra obtained from the precursor ions at (A) m/z 673.7 (B) m/z 924.6 (C) m/z 1890.6 (D) m/z 2261.3 (average values of protonated molecules)**.**

Table 1 Proteins identified by *de novo* MS sequencing of selected tryptic peptides and subsequent BLAST searching.

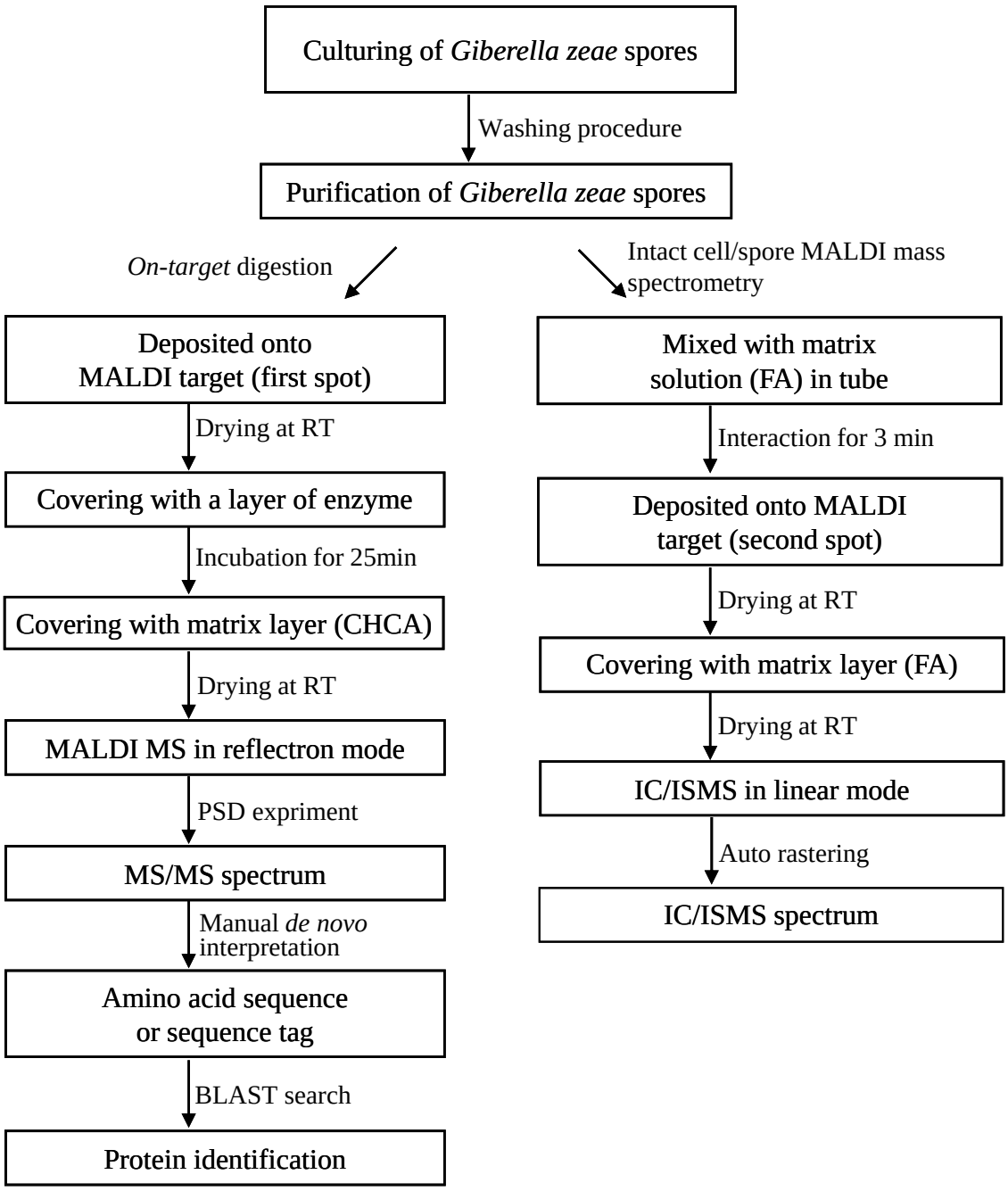


Fig. 1. Strategy for generation and identification of peptide from *Giberella zeae*.

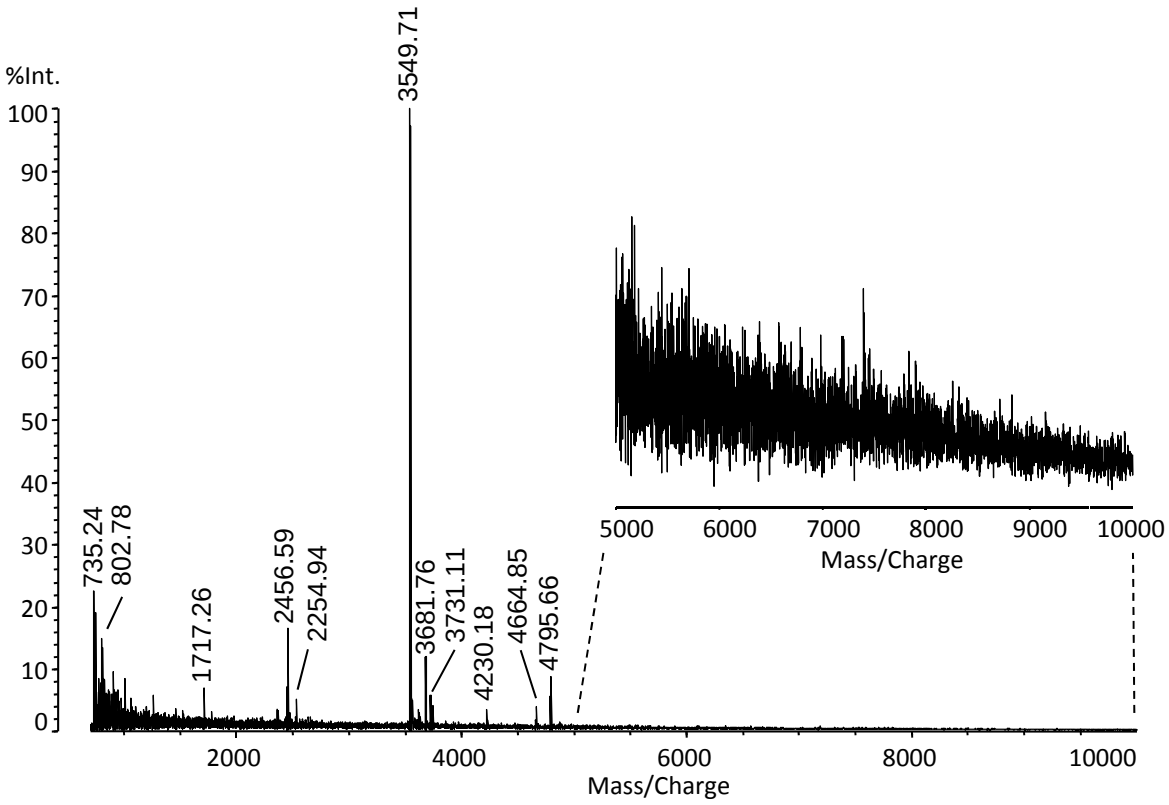


Fig. 2. Intact spore MALDI mass spectrum of *Giberella zeae* strain CPK NO. 2985.

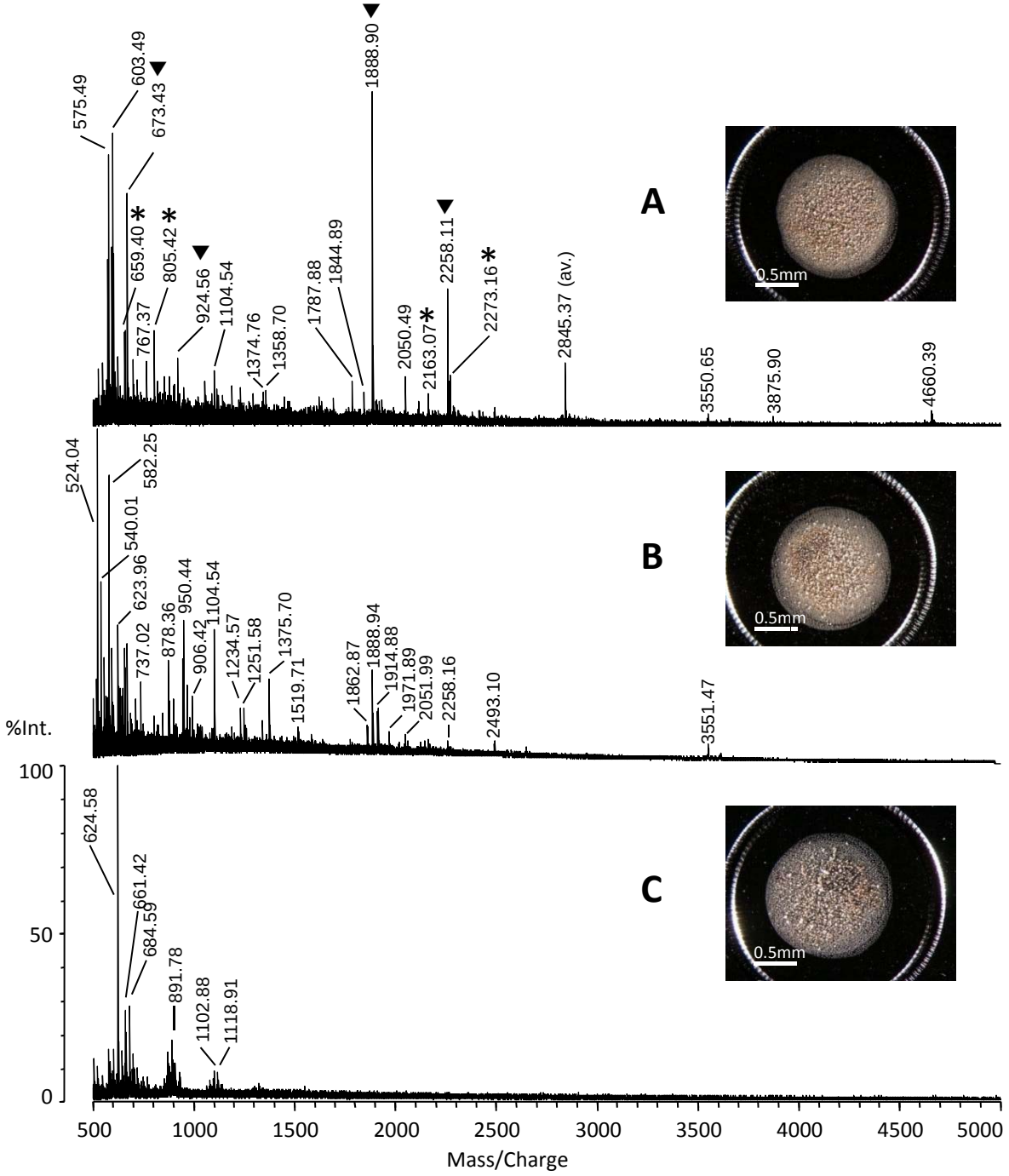


Fig. 3. MALDI TOF mass peptide mass spectra of *Giberella* strain 2985 with *on-target* digestion using (A) non-immobilized trypsin and (B) immobilized trypsin as well as (C) without enzyme (the applied MALDI MS sample preparation in this case is not useful for ICMS). Trypsin autodigestion peaks were marked with * and the selected peaks for PSD fragment ion analysis were marked with ▶.

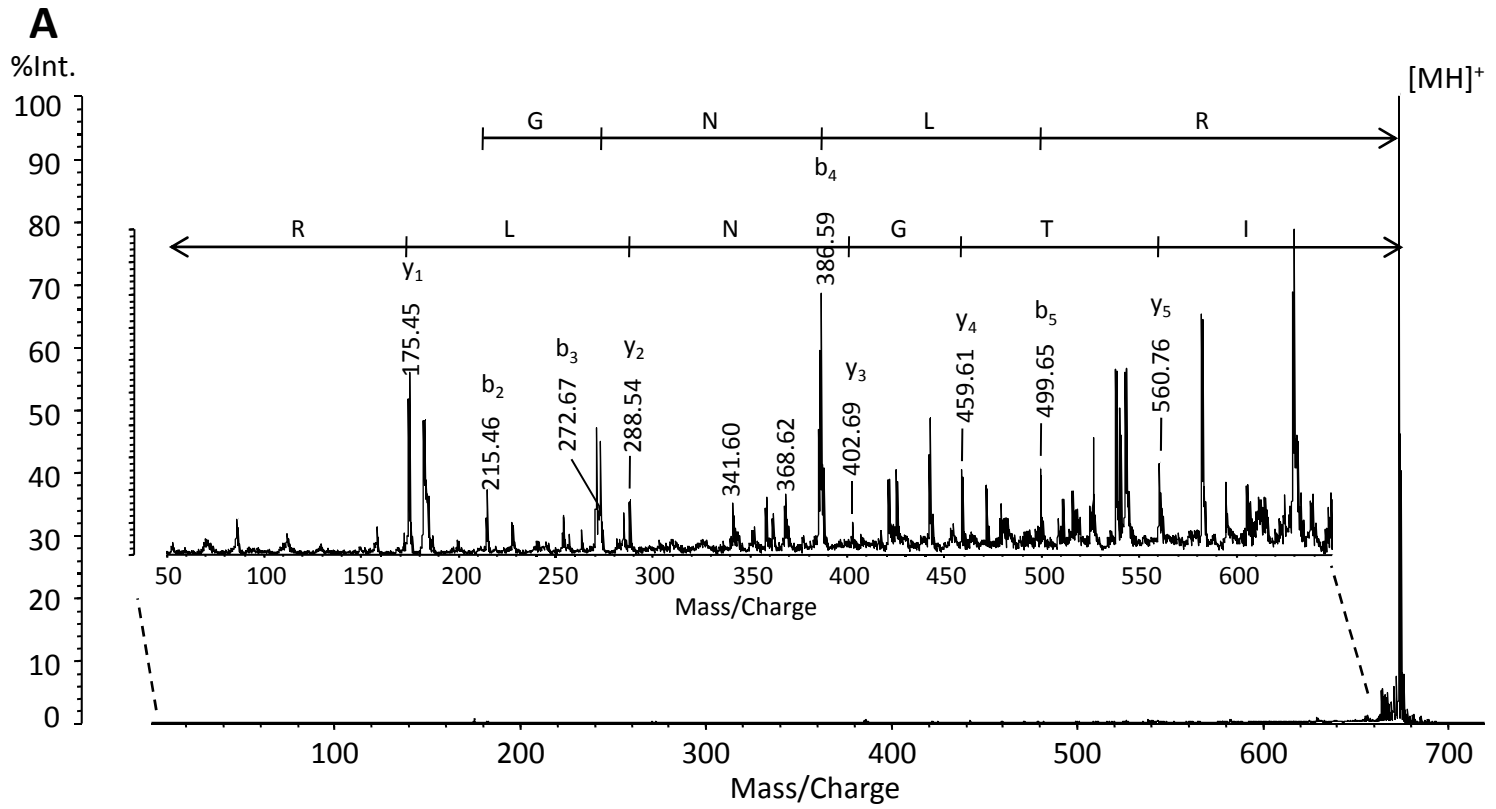


Fig. 4 (A). MALDI PSD (MS/MS) spectra obtained from the precursor ions at m/z 673.7.

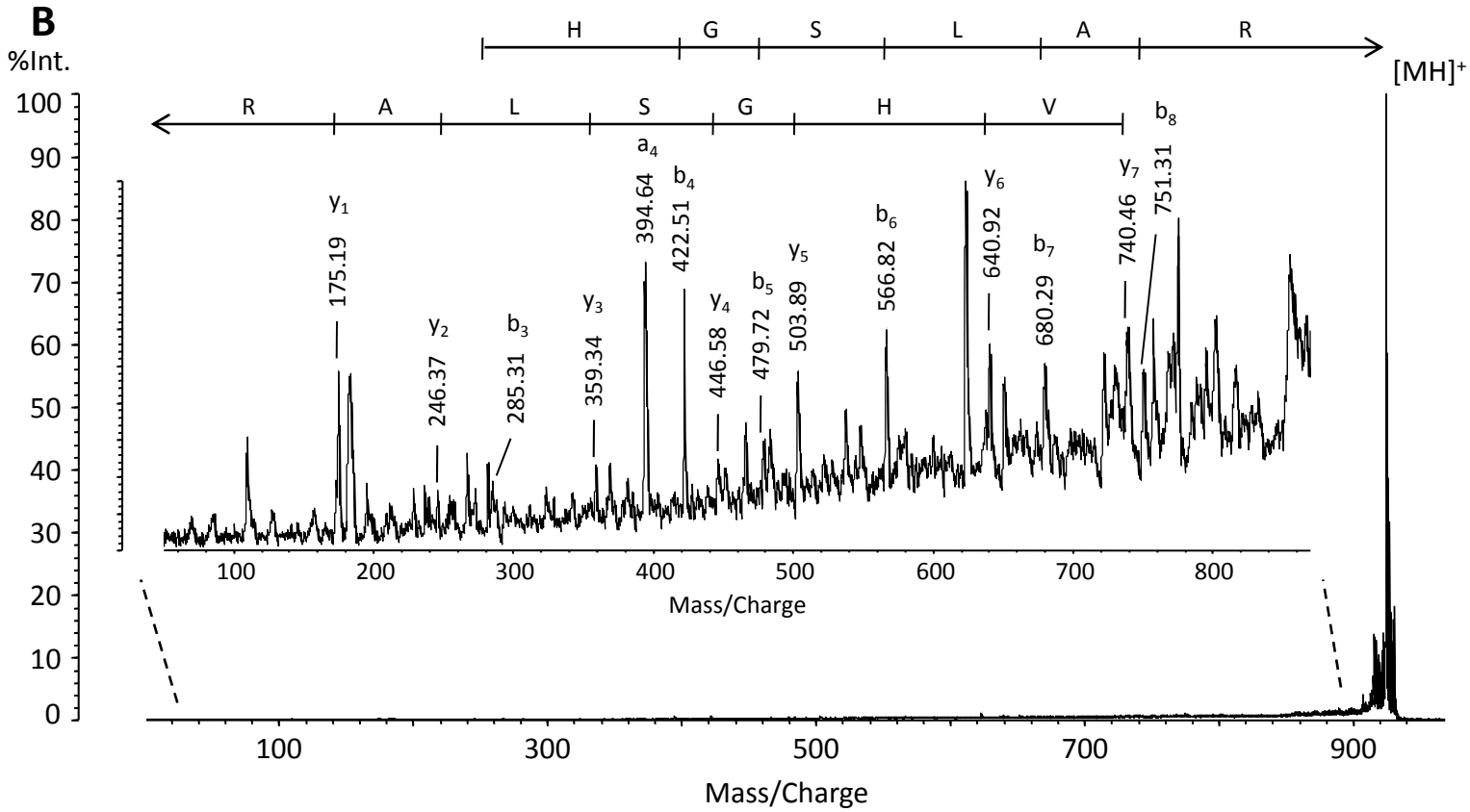


Fig. 4 (B). MALDI PSD (MS/MS) spectra obtained from the precursor ions at m/z 924.6.

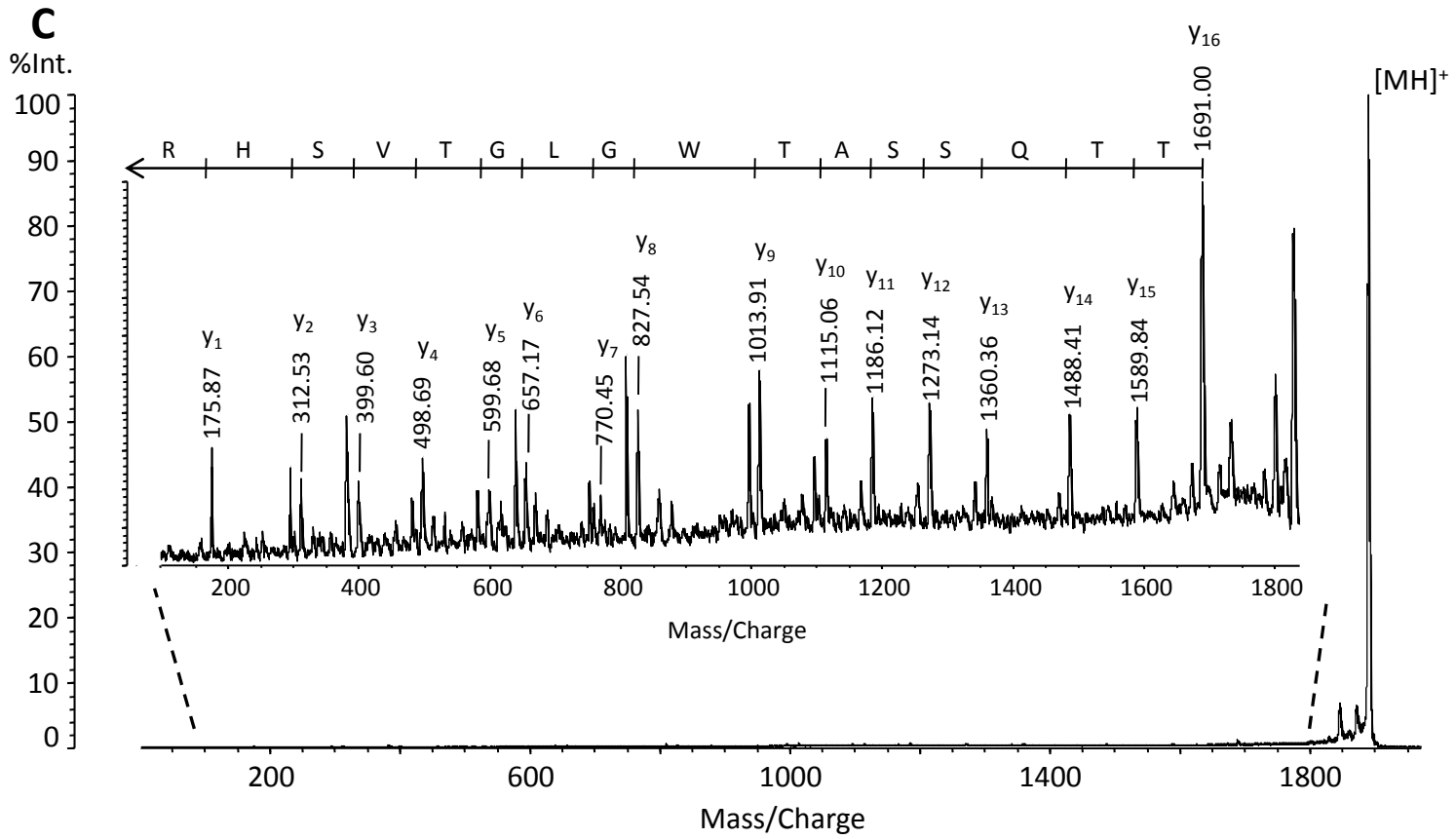


Fig. 4 (C). MALDI PSD (MS/MS) spectra obtained from the precursor ions at average m/z 1890.6.

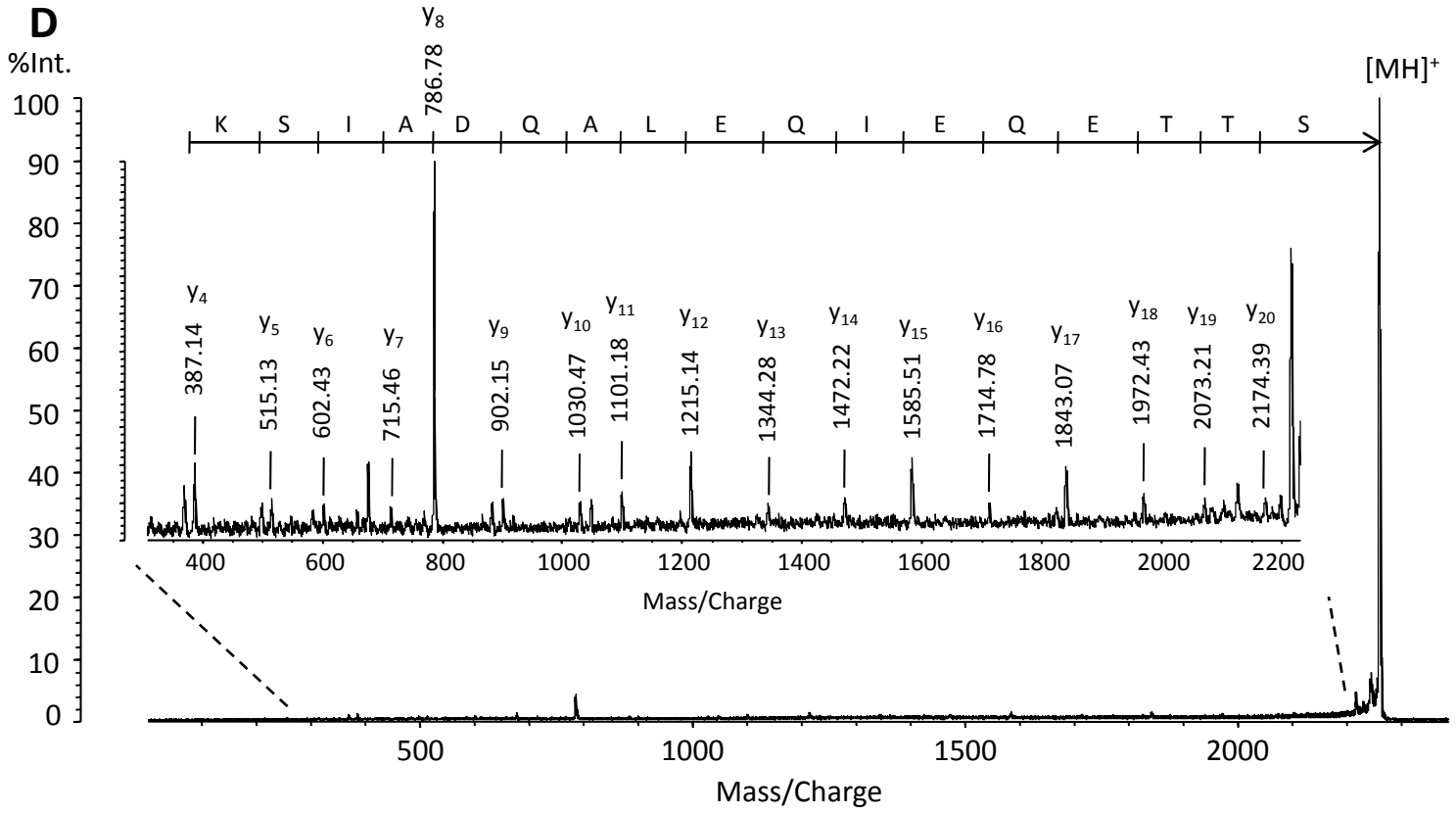


Fig. 4 (D). MALDI PSD (MS/MS) spectra obtained from the precursor ions at average m/z 2261.3.

Peptide	Obsd in PMF (mi)	Calcd (mi)	Missed cleavages	*De novo* sequencing	Homology searching result	Protein description	Conserved data Id. by BLAST serarch	Digestion by
1	673.43	673.40	0	[L|I]TGN[L|I]R	(K)ITGNLR(S)	Hypothetical protein FG00806.1 (*Gibberella zeae PH-1*)	Peptidase S8 superfamily	immobilized and non-immobilized trypsin
2	1888.90	1888.91	0	TT[Q|K]SSATW G[L|I]GTVSHR	(R)AETTQSSATW GLGTVSHR(S)			
3	924.56	924.54	1	VHGS[L|I]AR	(M)GKVHGS LAR(A)	Hypothetical protein FG04915.1 (*Gibberella zeae PH-1*)	Ribosomal S30 superfamily	non-immobilized trypsin
4	2258.11	2258.13	1	STTE[Q|K]E[L|I][Q|K]E[L|I]A[Q |K]DA[L|I]S[Q| K]	(R)STTEQEIQ ELAQDAIS KPGGR(A)	Hypothetical protein FG07774.1 (*Gibberella zeae PH-1*)	No conserved domain, no functional annotation available yet	immobilized and non-immobilized trypsin

Table 1 Proteins identified by *de novo* MS sequencing of selected tryptic peptides and subsequent BLAST searching

Series: NATO Science for Peace and Security Series – A: Chemistry and Biology

Detection of Biological Agents for the Prevention of Bioterrorism

Editor: Joseph Banoub

Book chapter, submitted (2010)

4.3 INTACT CELL/SPORE MASS SPECTROMETRY OF *FUSARIUM* MACRO CONIDIA FOR FAST ISOLATE AND SPECIES DIFFERENTIATION

SHORT TITLE: ICMS OF FUSARIUM MACRO CONIDIA

HONGJUAN DONG, MARTINA MARCHETTI-DESCHMANN, WOLFGANG WINKLER, HANS LOHNINGER, and GUENTER ALLMAIER*

Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164-IAC, A-1060 Vienna, Austria

*To whom correspondence should be addressed. Guenter Allmaier, Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164-IAC, A-1060 Vienna, Austria; e-mail: guenter.allmaier@tuwien.ac.at

Abstract

The focus of this paper is the development of an approach called intact cell mass spectrometry (ICMS) or intact spore mass spectrometry (ISMS) based on the technique matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) for the rapid differentiation and identification of *Fusarium* species. Several parameters, which are known to affect the quality of IC mass spectra, have been investigated in detail by varying the MALDI matrix as well as the solvent system, in which the matrix has been dissolved, the solvent system for sample purification and the type of sample/MALDI matrix deposition technique. In the end characteristic as well as highly reproducible IC or IS mass spectra or peptide/protein fingerprints of three *Fusarium* species (*F. cerealis*, *F. graminearum* and *F. poae*) including 16 *Fusarium* isolates derived from different hosts and geographical locations have been obtained. Unscaled hierarchical cluster analysis based on ICMS data of eight selected *Fusairum* isolates of 2 species *F. graminearum* and *F. poae* revealed significant difference among the peptide/protein pattern of them. The results of the applied cluster analysis proved that, ICMS is a powerful approach for the rapid differentiation of *Fusarium* species. In addition, an *on-target* tryptic digestion was applied to *Fusarium* macro conidia spores to identify proteins using MALDI post source decay (PSD) fragment ion analysis. Two kinds of trypsin, namely bead-immobilized – to favour cleavage of surface-associated proteins – and non-immobilized trypsin were applied and compared. The results showed that the latter is more suitable for generating sequence tags by PSD fragment ion analysis.

Keywords: MALDI; mass spectrometry; ICMS; Species differentiation; Fungi; *Fusarium*; *On-target* digestion

1. Introduction

To many microbiologists in medicine, homeland security and food sciences, rapid and reliable differentiation as well as identification of microorganisms without extensive manipulation has become of major interest as well as represent an analytical challenge. Direct profiling/imaging of the surface of intact microorganisms such as bacteria or fungi by intact cell mass spectrometry (ICMS) or intact spore mass spectrometry (ISMS) based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) has emerged as a valuable research tool which is capable of fulfilling several tasks.[1-4] MALDI TOF MS is the technique of choice providing high tolerance against the presence of salts and detergents as well as the possibility of automation, making itself suitable for direct and rapid analysis of microorganisms. The sample preparation, after often time-consuming development, in ICMS/ISMS can be carried out within minutes in a straightforward manner, because the samples are analyzed "as a whole" with minimal direct sample pretreatment after collection and purification from a cell culture suspension.[5] Briefly, intact vegetative cells or spores are mixed with MALDI MS matrix solution and deposited directly onto the MALDI MS target to co-crystallize before transferred into the high vacuum ion source of the MALDI mass spectrometer for analysis. The matrix absorbs the laser energy and releases it into the solid preparation of intact cells/matrix molecules. Then a surface-associated peptides as well as small proteins derived from intact cells or spores are desorbed and ionized to form mostly singly charged molecules which can then be mass analyzed by the linear TOF mass analyzer. The mass spectra obtained by ICMS/ISMS are usually considered as mass spectral fingerprints (even without understanding which specific components were desorbed/ionized from the intact microorganisms) and the identification as well as differentiation was to be based on comparison with those from the others or mass spectrometric database.[6-9] Several algorithms[10,11] based on m/z and intensity values as well as numerical analysis such as cluster analysis[12,13] have also been developed to compare and estimate the similarity between two or more mass spectra. All these techniques allow accurate typing (differentiation and identification, latter if a reference database is available) of microorganisms at genus, species, and even at the strain level.

The relative simplicity in sample preparation, sensitivity, broad capability, tolerance to contaminants and speed of the method indicates that ICMS/ISMS has great potential for routine use in differentiation, identification and classification of microorganisms. Most research groups have focused their research on identification of intact bacterial cells[1,8,14-16] or bacterial spores[17-19] allowing the use of well-established experimental methods and to a

certain extent of mass spectral databases. So far the ICMS/ISMS technique has also applied successfully to only few intact fungal cells[20,21] and fungal spores[2,22-26]. Fungi are the fifth important kingdom of eukaryotes[27,28] and compared to bacterial cells, fungi are typically larger and are surrounded by relative rigid cell walls which are generally composed by up to 90% polysaccharides. Proteins, lipids, polyphosphates and inorganic ions are also present in fungal cells[12,22] (Fig. 1 showed the electron microscopic image of a macroconidia spore of the fungus *Fusarium*). Members of the ascomycetous genus *Fusarium* is a large genus of filamentous fungi which are distributed on plants and in soil, representing an important group of fungal plant pathogens and may cause various infections in humans, too.[29,30] Due to the latter facts it is of great importance to identify and differentiate them rapidly, for example by ICMS/ISMS in an early stage. In one recent paper, [31] 62 *Fusarium* isolates were identified by MALDI TOF MS based on the extracts of hyphae/spores, however, not with intact spores. In previous studies on ICMS of fungal spores[2,22-26], various parameters has been reported to influence mass spectra data such as the purification of intact spores, choice of matrix compounds as well as their dissolved solvents and the MALDI sample deposition techniques. Small variations during sample preparation will affect the quality and reproducibility of mass spectrum significantly. Another quite common problem is that, during the culturing of *Fusarium* as well as many other filamentous fungi, they often produce colored pigments of different types and intensities.[32-34] Especially for the *Fusarium* species of our interest, slightly orange to red-brown colored macroconidia spores were produced.[24] The presence of this colored components resulted in quite poor quality of IC/IS mass spectra. Therefore the deep-colored spores need to be pre-treated properly.

In this paper we reported on the differentiation of intact *Fusarium* macroconidia spores by MALDI TOF mass spectrometry. We optimized the experimental parameters for ISMS, showed that differentiation is feasible by cluster analysis and started to build a mass spectral database on *Fusarium* species as well as strains. In addition, identification of proteins from *Fusarium* spores preparation (*F. graminearum* CPK 2985) has been achieved by *on-target* digestion technique in combination with MALDI post source decay (PSD) fragment ion analysis.

2. Experimental

2.1 Sample generation of macroconidia spores

All *Fusarium* isolates (collection of the Institute of Chemical Engineering (Vienna University of Technology, Vienna, Austria)) were first vitalized on SNA-Plates (Synthetischer Nährstoffarme Agar) and the subsequently macroconidia spore generation was carried out at 28 °C in a shaker incubator at 160 rpm with mungbean soup (20 g mungbean in 1 L of water were heated for 20 min and directly used after filtration) as nutritional medium. The spores were collected by filtration through a sterile glass funnel containing glass wool and centrifugation of the filtered liquid suspension containing spores at 8000 rpm and 4 °C for 10 min. The obtained supernatant was discarded while the pellet was mixed with an aqueous solution containing 20% glycerol (w/v) and stored at -20°C if the MALDI MS analysis was not performed immediately. The concentration of the spore solution was determined by spore counting in a light-optical microscope (Nikon Instruments Europe, Amstelveen, The Netherlands).

2.2 Sample preparation

The major amount of glycerol and other contaminants in spore suspension has to be removed prior to MALDI MS analysis. Several solvent systems were used to wash both colorless, light- and deep-colored *Fusarium* spores for removal of the colorizing surface components and were evaluated in terms of mass spectrometric performance (detailed description see reference 24). The spore suspension was washed 3 times with various solvent systems at 19,500 × g for 10 min using Nanosep^{TM} (Pall, Ann Arbor, MI, USA) centrifugal devices (MWCO 10 kDa). Then the spore pellet was resuspended in pure water with a final concentration of 3 million spores/µL and in case of *on-target* digest in 25 mM ammonium bicarbonate solution with a conidia spore concentration of 1.5 million/µL, respectively.

12 MALDI MS matrix compounds in different concentrations and 15 different matrix solvent mixtures were evaluated (detailed description see reference 25). Five different sample deposition techniques comprise of dried droplet, mixed volume, thin layer, sandwich, two-layer volume technique were evaluated based on mass spectrometric reproducibility, peaks numbers and intensities[24-26].

For *on-target* digestion, 1µL *Fusarium* conidia spores suspension in 25 mM ammonium bicarbonate solution was placed onto stainless steel MALDI MS target. After drying the spores solution at room temperature (RT), 0.5 µL of bead-immobilized TPCK trypsin

solution[35, 36] isolated from bovine pancreas (Pierce Biotechnology, No. 20230, Rockford, IL; Before using 20 µL bead-immobilized TPCK trypsin was washed following the instruction provided by the company and finally 4 times diluted (in terms of volume) with 25mM ammonium bicarbonate solution) or 0.5 µL of 0.1 µg/µL non-immobilized trypsin isolated from bovine pancreas (Roche Diagnostics, Mannheim, Germany, Cat. No. 11418025001) in 25mM ammonium bicarbonate solution were added onto each sample spot, respectively. Then conidia spore samples prepared on the MALDI MS target plate were incubated in a humidity-controlled chamber at RT to prevent spot drying. *On-target* digestion was stopped after 25 min by allowing the samples to dry at RT. Finally, 0.5 µL of 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/0.1% aqueous trifluoroacetic acid (70/30, v/v) was added for MALDI MS analysis and dried at RT.

2.3 MALDI TOF MS

Positive ion MALDI mass spectra were acquired on Axmia-CFR^{plus} instrument (Shimadzu Biotech Kratos Analytical, Manchester, UK) equipped with a nitrogen laser (337 nm, 3ns pulse width) and a curved field reflector. The instrument was operated at an acceleration voltage of 20 kV. All the mass spectra were accumulated (up to 2500 single unselected laser shots) across the whole matrix/analyte spot automatically in the rastering mode.

For IC/ISMS analysis, the instrument was operated in linear mode with delayed extraction (optimized for m/z 5000) in the m/z range of 1000 to 15000. The blanking gate was set at m/z 1000 to remove the ions below this m/z value arising from matrix (their clusters as well as fragments) and other unknown contaminants exhibiting low molecular mass. An external three-point calibration was performed prior to every automatic measurement of each *Fusarium* species/strain with the protein cytochrome c (protonated molecule at m/z 12361.2 and double protonated molecule at m/z 6181.1) and the standard peptide ACTH 7-38 (protonated molecule at m/z 3657.9). The singly charged peptides from tryptic autodigestion products at monoisotopic m/z 659.38, 805.42, 2163.06 and 2273.16 (mass accuracy of ± 0.03 Da) were used for internal calibration of the digested samples. The PSD mode has been calibrated with the synthetic peptide $(P)_{14}K$. For PSD fragment ion analysis 2500 single unselected laser shots were accumulated. The average m/z values of fragment ions, which were used for manual *de novo* sequencing, were automatically derived from smoothed PSD spectra (Shimadzu Biotech supplied Savitzky-Golay algorithm, smoothing filter width 20) with baseline subtraction (baseline filter width 60). Typically PSD spectra showing an

average mass accuracy of ± 0.5 Da in the low m/z range (< m/z 1500) and in the m/z range > m/z 1500 of ± 2 Da were acquired.

2.4 Data handling

The first step of data analysis consisted of spectral preprocessing using the software supplied by the instrument manufacturer (Launchpad 2.7.3) by applying baseline subtraction (filter width 200) and smoothing (Savitzky-Golay algorithm, 20 channels). The processed mass spectra were exported in mzXML[37] format and re-imported into mMass[38] software (version 2.4) for further analysis. Peaks were manually picked in the m/z range of 2400-15000 omitting those peaks known to be caused by the matrix as well as sodium or potassium adducts of peptide/protein analytes. For each sample (i.e. isolate), peak lists containing m/z and intensity (normalized by the most intense peak in the m/z range 2400-15000) values of those peaks, which could be detected in at least 50% of all replicate measurements were generated. An alignment of all peak lists was generated considering peaks from different mass spectra to be identical if their m/z difference was less than 1 + m/z / 3000 (variable threshold of 1.8 Da at m/z = 2400 extending to 6 Da at m/z = 15000). Peaks, which were not detected in one mass spectrum, but were present in others were added with an intensity of zero. This procedure resulted in a data matrix consisting of a total of 270 features (i.e. peaks) defined for 95 mass spectra, which was used for hierarchical clustering (distance measure: euklidean distance, linkage type: Ward's method) using Datalab (version 2.4, http://www.lohninger.com/datalab/de_home.html, Epina, Austria).

3. Results and Discussion

3.1 Short description of the applied strategy

In this study, we demonstrated a rapid screening approach that employs MALDI TOF MS of intact conidia spores (Fig. 1) for differentiation of *Fusarium* species. Fig. 2 showed the strategy of IC/ISMS of *Fusarium* macroconidia. Experimentally, after spore generation and a simple washing procedure with organic/water solvent mixture by centrifugation, the intact *Fusarium* spores and matrix solution were pre-mixed in an polypropylen tube (Fig. 3) or mixed on MALDI MS target directly (based on different sample deposition technique) for sufficient interaction which indicates an intimate contact between *Fusarium* conidia spores, MALDI MS matrix compound and solvents. Then the prepared sample/matrix spot was allowed to dry by evaporating of solvents at RT and subsequently was used for MALDI TOF MS analysis. The generated mass spectral profiles of macroconidia spores turned out to be sufficient "different" to differentiate various *Fusarium* species.

3.2 Different types of MALDI MS sample preparations

Sample preparation of *Fusarium* conidia spores for IC/ISMS includes purification and concentrating of spores as well as subsequently transfer of the spores onto the MALDI target and embedding into the MALDI matrix. During these steps several parameters have been evaluated for the optimization of IC/ISMS based on mass spectrometric reproducibility, in terms of m/z values, as well as number and intensities of peaks. The washing solvent systems for purification, matrix compounds as well as solvents in which the matrix has been dissolved and sample deposition technique were included in the evaluation process.

A previous study[24] proved that the solvent system for washing *Fusarium* macroconidia spores especially for washing the deep-colored *Fusarium* spores is of vital importance for successful analysis. In fact, due to the rigid cell walls of fungal spores, the purpose of this procedure is not only the purification of spores but also for extraction of proteins/peptides (i.e. bringing them to the surface) in order to improve the signal-to-noise ratio. Several solvents composed of water, acetonitrile, methanol, ethanol, isopropanol, organic acids such as formic acid or trifluoroacetic acid have been investigated. They were either used in pure form or were mixed in a certain ratios (*v/v*). The result showed that the organic acid play an important role in this washing step. For example, the use of solvent system acetonitrile/water (7/3) for washing the *Fusarium* isolate CPK NO.2765 deep-colored spores generated a poor mass spectrum without any useful peaks. However, when aqueous 0.1% trifluoroacetic acid or aqueous 0.5% formic

acid replaced pure water as washing system, the color of the intact spores became lighter and the quality of obtained MALDI mass spectra improved significantly (and for some spore preparations for the first time useful mass spectra were obtained). Many other researchers have also noted the utility of acid pre-treatment of other microorganisms for enhanced mass spectral data.[14,18,21,39] The use of organic acid changes the pH of the spore suspension and might modify the architecture of the spore cell wall resulting in a better release of proteins and peptides. Furthermore, the change of pH value of the solution might be an effective way of color fading (although not totally) of *Fusarium* macroconidia spores.

The MALDI mass spectra varied with the MALDI MS matrix used significantly. For IC/ISMS analysis of *Fusarium* conidia spores, different matrix compounds in different concentrations and solvents, in which matrix has been dissolved, have been investigated in our previous study.[25] The commonly used acidic matrix compounds including CHCA, 2,5-dihydroxybenzoic acid (DHB), ferulic acid (FA), sinapinic acid (SA) and neutral ionic liquid matrix system composed of DHB/butylamine and CHCA/butylamine were tested. The solvent system for dissolving the matrix compounds is also a crucial factor. It has not only an impact on crystal structure but also on the degree of the spores (analyte) incorporated into the matrix layer. Various solvents namely acetonitrile, methanol, ethanol, isopropanol in pure form or combinations with pure water or TFA-acidified water were used for dissolving matrices. Through comparison based on the mass spectrometric performance (e.g. reproducibility or signal intensity) of the preparation resulted in the selection of the matrix FA dissolved in the traditional acetonitrile/0.1% aqueous trifluoroacetic acid (7/3, *v/v*) solvent system at a concentration of 10mg/mL as most suitable for ISMS analysis of *Fusarium* conidia spores. In a study of ICMS of the other fungal genus *Aspergillus*, it was reported that the addition of organic acids in the MALDI MS matrix solution provided sufficient dissolving ability to release bioactive compounds (e.g. small proteins) from the cell walls of intact spores.[23] Furthermore the organic acid in matrix/spore crystals might also provide a source of protons thus facilitating the protein/peptide ionization resulting in high quality MALDI mass spectra.

The optimal matrix/analyte deposition technique is one further key requirement for a successful MALDI MS analysis. The morphology of the crystal layer of matrix and analyte varied with the change of deposition technique. Hence five types of sample deposition techniques in total were evaluated for IC/ISMS of *Fusarium* spores, namely thin layer, sandwich, dried droplet, mixed volume, two-layer volume technique.[24-26] At the beginning the first three deposition technique including thin layer, sandwich, and dried droplet were

compared by Kemptner *et al*..[25] As a result, no significant peptide/protein profiles could be achieved with thin layer technique. The sandwich technique generated homogeneous crystal layers and in most cases a well-defined peptide/protein profile. However, the sample preparation reproducibility was not satisfactory. Direct mixing of *Fusarium* conidia spores and MALDI MS matrix solution on the MALDI MS steel target, called dried droplet technique, yielded highly reproducible as well as abundant peptide/protein profiles compared to the first two techniques. The reproducibility can be further improved by pre-mixing matrix solution and *Fusarium* conidia spore suspension in a tube prior to application onto the MALDI target, referred to as mixed volumes method.[26] But in later investigations, both dried droplet and mixed volume techniques were found not to work properly for the deep-colored *Fusarium* conidia spores, which is a serious drawback. So dried droplet, mixed volume and two-layer volume technique were compared in parallel for the development of a preparation technique applicable to deep-colored *Fusarium* conidia spores.[24] Comparing with dried droplet technique, the plain mixed volume technique resulted in a homogeneous matrix/spore crystal layer and generated an improvement of the MALDI mass spectrometric reproducibility. But the two-layer volume technique was found to be the most suitable technique with the highest number and abundances of peaks. The essential character of the two-layer volume technique is the application of second MALDI MS matrix layer, which is connected to a dissolving and re-crystallization step, and resulted in a better incorporation of the spores into the matrix layer and maybe kind of purification. The flow chart of steps of the two-layer volume technique for IC/ISMS of *Fusarium* macroconidia spores is outlined in Fig. 3. Because two-layer volume technique is associated with the exposure of the spores to organic as well as acidic solvents in the MALDI MS matrix solution, the volume ratio and time of matrix/spore solutions interaction were also investigated.[24, 26] Finally for both, light-colored and deep-colored *Fusarium* conidia spores, a matrix/spore solution ratio of 1/1 (v/v) and the duration of mixing time of at least 3 min were recommended to use.

3.3 Verification of peptides/proteins desorbed/ionized from conidia spore surface

In this work, MALDI PSD fragment ion analysis of protonated tryptic peptides generated via *on-target* tryptic digestion from the surface of *F. graminearum* macroconidia spores was tested for its applicability. Generally after spore purification, i.e. washing with acetonitrile/aqueous 0.5 % formic acid, the intact *Fusarium* spores were suspended in 25 mM ammonium bicarbonate and applied onto the MALDI target for 25 min tryptic *on-target* digestion (details please see experimental part 2.2). The generated tryptic peptides were analyzed directly by MALDI RTOF MS without any further sample cleanup generating a

complex peptide mass fingerprint (PMF). Then the MALDI PSD fragment ion analysis was applied to the selected tryptic peptides with high abundance. In addition, the effectiveness of *on-target* digestion using both bead-immobilized and non-immobilized trypsin as enzyme has been compared and the results proved that the latter is more efficient for generating a good PMF. This strategy is rapid, easy to operate and the obtained positive ion PSD spectra were complex but still interpretable allowing partial peptide sequencing followed by database search. Fig. 4 presents a typically a PSD spectrum of the abundant precursor peptide ion at m/z 1888.90. With manual *de novo* peptide sequencing from such PSD spectra and combined with database homology search, protein identification based on the shown PSD spectrum and another PSD spectrum from the precursor peptide ion at m/z 673.43 (data not shown) could be accomplished. Two other hypothetical proteins FG04915.1 and FG07774.1 that contribute precursor peptides at m/z 924.56 and m/z 2258.11 separately are also identified (data were not shown). However, due to the limitation of *Fusarium* database, the proteins identified in this study are all hypothetical proteins. But they can be tentatively explained based on their superfamily annotation.

3.4 Differentiation of IC/IS mass spectra of different *Fusarium* species and isolates

Two previous studies[25, 26] performed in our laboratory presented that the different reference *Fusarium* species can be differentiated based on their evident difference in their protein/peptide peak patterns. Here in this study three *Fusarium* species including 16 unique isolates which were derived from various hosts and geographical locations were included (Table 1) in such a differentiation study. Fig. 5 (A) –(C) exhibit the IC/IS mass spectra of the species *F. cerealis*, *F. graminearum* and *F. poae* as well as from different isolates. The IC/IS mass spectra of *Fusarium* conidia spores were measured in the mass range from m/z 1000 to 15000. But most of the protein/peptide signals observed in the mass spectra occurred between the m/z values 2000 - 10000. We found significant differences in the peak pattern of the three different *Fusarium* species. Furthermore the protein/peptide pattern of B isolates of the same species show common peaks in terms of m/z values but some peaks could be found with quite different relative abundance to each other (e.g. see Fig. 5 (A) a-d). To evaluate quantitatively whether IC/ISMS could distinguish isolates of different species, we performed a hierarchical cluster analysis of selected 8 isolates of 2 *Fusarium* species from various hosts and geographical locations (*F. graminearum* isolates CPK No. 2763, 2764, 2765, 1122 and *F. poae* isolates CPK NO. 2772, 2774, 2775, 2781) as well as analyzed them several times. To exclude peaks potentially derived from the MALDI MS matrix as well as other contaminants, only peaks with m/z values between 2400 and 15000 in the IC/IS mass spectra were used for

the cluster analysis. The dendrogram is constructed using the degree of association and unscaled hierarchical clustering techniques that help visualize similarities between different protein/peptide fingerprints. Conceptually, the two fingerprints with the highest degree of association are joined together first, followed by those with the next highest degree of association, and so on. Fig. 6 shows the derived dendrogram indicating clearly the differences. In the dendrogram, the two species *F. graminearium* and *F. poae* grouped separately from each other were strikingly dissimilar indicating that differentiation of isolates at species level is possible. Replicate analyses of single isolates showed a very high degree of association and therefore grouped tightly together. Although the isolates within a species are closely related in terms of their IC/IS mass spectra, some differences between each of them other were also observed. Until now it is difficult or impossible to differentiate the isolates derived from different hosts (e.g. corn, barley and unknowns) and geographical locations within eastern regions of Austria. The number of well-defined isolates which must be analyzed by IC/ISMS has to be increased significantly to present a final statement to the last topic.

4. Conclusions

We have described successfully the use of MALDI TOF MS approach for differentiation of macroconidia spores of *Fusarium* species. Sample preparation of intact *Fusarium* spores (stored for example at -80° C) from light- to deep-colored samples for IC/ISMS analysis do not need to employ any prior tedious separation or extraction procedure and can be finished in a few minutes. MALDI mass spectrometric measurement can be made quickly, with a relative high tolerance to contaminants, and can be automated. The described method is specific, reproducible and accurately identifies differences in large sets of closely related isolates of *Fusarium* species. It represents a fast approach for *Fusarium* macroconidia species differentiation and has the potential for other fungal microorganisms differentiation and meets the requirements for high-throughput screenings. Thus in the future it is easy to expect that IC/ISMS will continue to be widespread used for such demanding applications and the development as well as growth of IC/IS mass spectra databases will further foster the described method. In addition, *on-target* tryptic digestion of *Fusarium* spores is readily accomplished and has the capability to identify the proteins found in the IC/IS mass spectra of fungal spores by PSD fragment ion analysis TOF/RTOF or QqRTOF experiments in case of available databases.

Acknowledgements

The investigation was supported financially in part by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management (grant number 100053). Furthermore the authors thank Christian P. Kubicek (Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria) for the supply of macroconidia spores during all the investigations.

References

1. R. D. Holland, J. G. Wilkes, F. Rafii, J. B. Sutherland, C. C. Persons, K. J. Voorhees and J. O. Lay, Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry, *Rapid Commun. Mass. Spectrom* 10(10), 1227-1232 (1996).
2. H. Y. Chen and Y. C. Chen, Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry, *Rapid. Commun. Mass. Spectrom* 19(23), 3564-3568 (2005).
3. R. Dieckmann, I. Graeber, I. Kaesler, U. Szewzyk and H. von Döhren, Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by intact-cell-MALDI-TOF mass spectrometry (ICM-MS), *Appl. Microbiol. Biotechnol* 67(4), 539-548 (2005).
4. C. Fenselau and P. A. Demirev, Characterization of intact microorganisms by MALDI mass spectrometry, *Mass. Spectrom. Rev* 20(4), 157-171(2001).
5. P. Lasch, H. Nattermann, M. Erhard, M. Stämmler, R. Grunow, N. Bannert, B. Appel and D. Naumann, MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores, *Anal. Chem* 80(6), 2026-2034 (2008).
6. R. J. Arnold and J. P. Reilly, Fingerprint matching of *E.coli* strains with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells using a modified correction approach, *Rapid. Commun. Mass. Spectrom* 12(10), 630-636 (1998).
7. C. L. Nilsson, Fingerprinting of *Helicobacter pylori* strains by matrix-assisted laser desroption/ionization mass spectrometric analysis, *Rapid. Commun. Mass. Spectrom* 13(11), 1067-1071 (1999).
8. K. A. Jackson, V. Edwards-Jones, C. W. Sutton and A. J. Fox, Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. *J. Microbiol. Methods* 62(3), 273-284 (2005).
9. M. Vargha, Z. Takáts, A. Konopka and C. H. Nakatsu, Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates, *J. Microbiol. Methods* 66(3), 399-409 (2006).
10. K. H. Jarman, D. S. Daly, C. E. Petersen, A. J. Saezn, N. B. Valentine and K. L. Wahl, Extracting and visualizing matrix-assisted laser desorption/ionization time-of-flight mass spectral fingerprints, *Rapid. Commun. Mass. Spectrom* 13(15), 1586-1594 (1999).
11. K. H. Jarman, S. T. Cebula, A. J. Saenz, C. E. Petersen, N. B. Valentine, M. T. Kingsley and K. L. Wahl, An algorithm for automated bacterial identification using

matrix-assisted laser desorption/ionization mass spectrometry, *Anal. Chem* 72(6), 1217-1223 (2000).

12. E. Vanlaere, K. Sergeant, P. Dawyndt, W. Kallow, M. Erhard, H. Sutton, D. Dare, B. Devreese, Samyn B and P. Vandamme, Matrix-assisted laser desorption ionisation-time-of of-flight mass spectrometry of intact cells allows rapid identification of *Brukholderia cepacia* complex. *J. Microbiol. Methods* 75(2): 279-286 (2008).
13. J. M. Hettick, B. J. Green, A. D. Buskirk, M. L. Kashon, J. E. Slaven, E. Janotka, F. M. Blachere, D. Schmechel and D. H. Beezhold, Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting, *Anal. Biochem* 380(2), 276-281 (2008).
14. Z. Wang, L. Russon, L. Li, D. C. Roser and S. R. Long, Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid. Commun. Mass. Spectrom* 12(8), 456-464 (1998).
15. D. J. Evason, M. A. Claydon and D. B. Gordon, Effects of ion mode and matrix additives in the identification of bacteria by intact cell mass spectrometry, *Rapid. Commun. Mass. Spectrom* 14(8), 669-672 (2000).
16. V. Ryzhov and C. Fenselau, Characterization of the protein subset desorbed by MALDI from whole bacterial cells, *Anal. Chem* 73(4), 746-750 (2001).
17. Y. Hathout, P. A. Demirev, Y. P. Ho, J. L. Bundy, V. Ryzhov, L. Sapp, J. Stutler, J. Jackman and C. Fenselau. Identification of *Bacillus* spores by matrix-assisted laser desorption ionization – mass specttometry, *Appl. Environ. Microbiol* 65(10), 4313-4319 (1999).
18. V. Ryzhov, Y. Hathout and C. Fenselau, Rapid characterization of spores of *Bacillus cereus* group bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Appl. Environ. Microbiol* 66(9), 3828-3834 (2000).
19. J. N. Ullom, M. Frank, E. E. Gard, J. M. Horn, S. E. Labov, K. Langry, F. Magnotta, K. A. Stanion, C. A. Hack and W. H. Benner, Discrimination between bacterial spore types using time-of-flight mass spectrometry and matrix free infrared laser desorption and ionization, *Anal. Chem* 73(10), 2331-2337 (2001).
20. N. B. Valentine, J. H. Wahl, M. T. Kingsely and K. L. Wahl, Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry, *Rapid. Commun. Mass. Spectrom* 16(14), 1352-1357 (2002).
21. B. Amiri-Eliasi and C. Fenselau, Characterization of protein biomarkers desorbed by MALDI from whole fungal cells, *Anal. Chem* 73(21), 5228-5231 (2001).

22. K. J. Welham, M. A. Domin, K. Johnson, L. Jones and D. S. Ashton, Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry, *Rapid. Commun. Mass. Spectrom* 14(5), 307-310 (2000).
23. T. Y. Li, B. H. Liu and Y. C. Chen, Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid. Commun. Mass. Spectrom* 14(24), 2393-2400 (2000).
24. H. Dong, J. Kemptner, M. Marchetti-Deschmann, C. P. Kubicek and G. Allmaier. Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry, *Anal. Bioanal. Chem* 395 (5), 1373-1383 (2009).
25. J. Kemptner, M. Marchetti-Deschmann, R. Mach, I. S. Druzhinina, C. P. Kubicek and G. Allmaier, Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of-flight mass spectrometry, *Rapid. Commun. Mass. Spectrom* 23(6), 877-884 (2009).
26. J. Kemptner, M. Marchetti-Deschmann, C. P. Kubicek and G. Allmaier, Mixed volume sample preparation method for intact cell mass spectrometry of *Fusarium* spores, *J. Mass. Spectrom* 44(11), 1622-1624 (2009).
27. J. Burnett, Populations and species. (Oxford University Press, N.Y., USA, 2003)
28. K. Kavanagh, Fungi - Biology and Applications (John Wiley and Sons, Chichester, UK, 2005)
29. V. Phalip, F. Delalande, C. Carapito, F. Goubet, D. Hatsh, E. Leize-Wagner, P. Dupree, A. V. Dorsselaer and J. M. Jeltsch, Diversity of the exoproteome of *Fusarium graminearum* grown on plant cell wall, *Curr. Genet* 48(6), 366-379 (2005).
30. M. Varga, T. Bartók and Á. Mesterházy, Determination of ergosterol in *Fusarium*-infected wheat by liquid chromatography-atmospheric pressure photoionization mass spectrometry, *J. Chromatogr. A* 1103(2), 278-283 (2006).
31. C. Marinach-Patrice, A. Lethuillier, A. Marly, J. Y. Brossas, J. Gené, F. Symoens, A. Datry, J. Guarro, D. Mazier and C. Hennequin, Use of mass spectrometry to identify clinical *Fusarium* isolates, *Clin. Microbiol. Infect* 15(7), 634-642 (2009).
32. S. Malz, M. N. Grell, C. Thrane, F. J. Maier, P. Rosager, A. Felk, K. S. Albertsen, S. Salomon, L. Bohn, W. Schäfer and H. Giese, Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex, *Fungal. Genet. Biol* 42(5), 420-433 (2005).

33. S. Bhardwaj, A. Shukla, S. Mukherjee, S. Sharma, P. Guptasarma, A. K. Chakraborti and A. Chakrabarti, Putative structure and characteristics of a red water-soluble pigment secreted by *Penicillium marneffei*, *Med. Mycol* 45(5), 419-427 (2007).
34. A. G. Medentsev, A. Y. Arinbasarova and V. K. Akimenko, Biosynthesis of naphthoquinone pigments by fungi of the genus *Fusarium*, *Appl. Biochem. Microbiol* 41(5), 503-507 (2005).
35. B. Warscheid, K. Jackson, C. Sutton and C. Fenselau, MALDI analysis of *Bacilli* in spore mixtures by applying a quadrupole ion trap time-of-flight tandem mass spectrometry, *Anal. Chem* 75(20), 5608-5617 (2003)
36. B. Warscheid and C. Fenselau, Characterization of *Bacillus* spore species and their mixtures using postsource decay with a curved-field reflectron, *Anal. Chem* 75(20), 5618-5627 (2003)
37. P. G. Pedrioli, J. K. Eng, R. Hubley, M. Vogelzang, E. W. Deutsch, B. Raught, B. Pratt, E. Nilsson, R. H. Angeletti, R. Apweiler, K. Cheung, C. E. Costello, H. Hermjakob, S. Huang, R. K. Julian, E. Kapp, M. E. McComb, S. G. Oliver, G. Omenn, N. W. Paton, R. Simpson, R. Smith, C. F. Taylor, W. Zhu and R. Aebersold, A common open representation of mass spectrometry data and its application to proteomics research, *Nat. Biotechnol* 22(11),1459-1466 (2004).
38. M. Strohalm, M. Hassman, B. Kosata and M. Kodicek, mMass data miner: an open source alternative for mass spectrometric data analysis, *Rapid. Commun. Mass. Spectrom* 22(6), 905-908 (2008).
39. A.J. Madonna, F. Basile, I. Ferrer, M. A. Meetani, J. C. Rees and K. J. Voorhees, On-probe sample pretreatment for the detection of proteins above 15 kDa from whole cell bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *Rapid. Commun. Mass. Spectrom* 14(23), 2220-2229 (2000).

Legends to Table 1 and Figures

TABLE 1. Investigated *Fusarium* species and strains grown on different hosts in different locations of Austria.

Figure 1. Electron microscope image of *Fusarium* macroconidia spore (white bar corresponds to 10 μm).

Figure 2. Strategy of intact cell/spore mass spectrometry (IC/ISMS) of *Fusarium* macroconidia.

Figure 3. IC/ISMS sample preparation with two-layer volume technique for *Fusarium* macroconidia.

Figure 4. MALDI PSD spectrum obtained from the precursor ion at m/z 1888.9 after on-target tryptic digestion of *Fusarium* macroconidia spores.

Figure 5 (A). IC/IS mass spectra of four (a-d) strains of *F. cerealis.*

Figure 5 (B). IC/IS mass spectra of six (e-j) strains of *F. graminearum*.

Figure 5 (C). IC/IS mass spectra of six (k-p) strains of *F. poae*.

Figure 6. Dendrogram representing the result of unscaled hierarchical cluster analysis of IC/ISMS data of *Fusarium* species/strains from different hosts (maize, barley and unknown) and locations.

Species	Strains CPK No.	Host	Geographic origin	Label
F. cerealis	2739	maize	Wieselsdorf	a
F. cerealis	2740	maize	Wieselsdorf	b
F. cerealis	2741	maize	Mogersdorf	c
F. cerealis	2743	barley	Lambach	d
F. graminearum	2761	maize	Wieselsdorf	e
F. graminearum	2763	maize	Wieselsdorf	f
F. graminearum	2764	maize	Wieselsdorf	g
F. graminearum	2765	barley	Probstdorf	h
F. graminearum	2766	wheat	unknown	i
F. graminearum	1122	unknown	unknown	j
F. poae	2772	maize	Tulln	k
F. poae	2774	maize	Tulln	l
F. poae	2775	barley	Probstdorf	m
F. poae	2781	barley	Probstdorf	n
F. poae	2793	barley	Lambach	o
F. poae	2794	barley	Lambach	p

TABLE 1. Investigated *Fusarium* species and strains grown on different hosts in different locations of Austria.

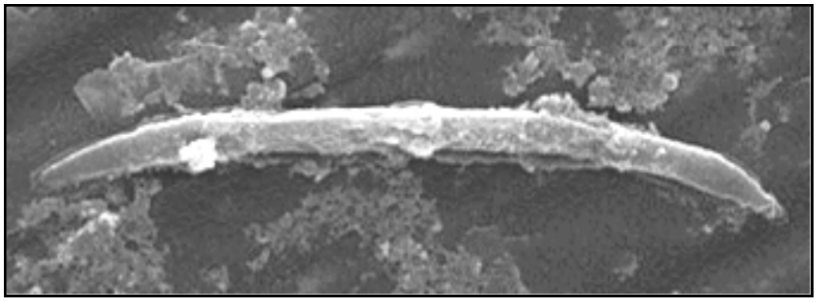


Figure 1. Electron microscope image of *Fusarium* macro conidia spore (white bar corresponds to 10 μm).

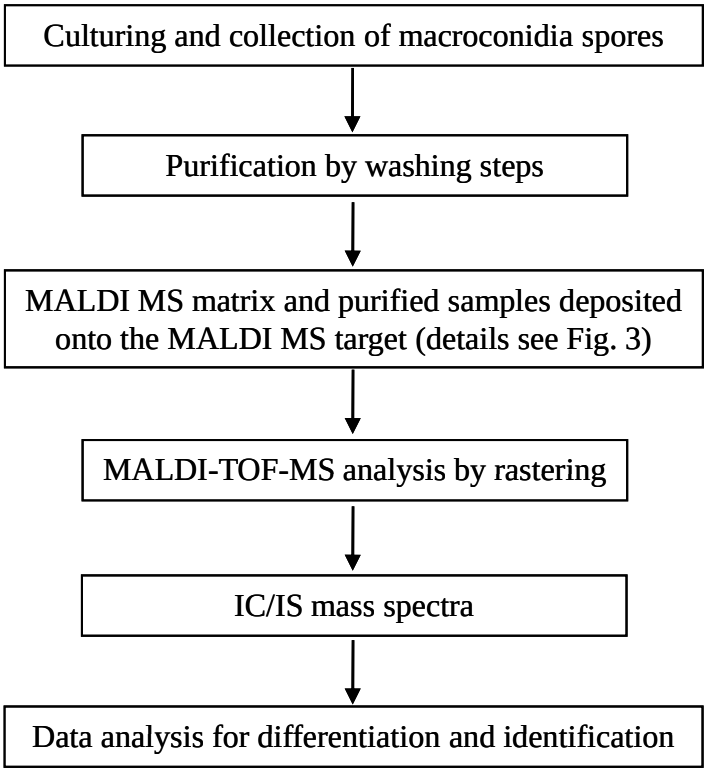


Figure 2. Strategy of intace cell/spore mass spectrometry (IC/ISMS) of *Fusarium* macroconidia.

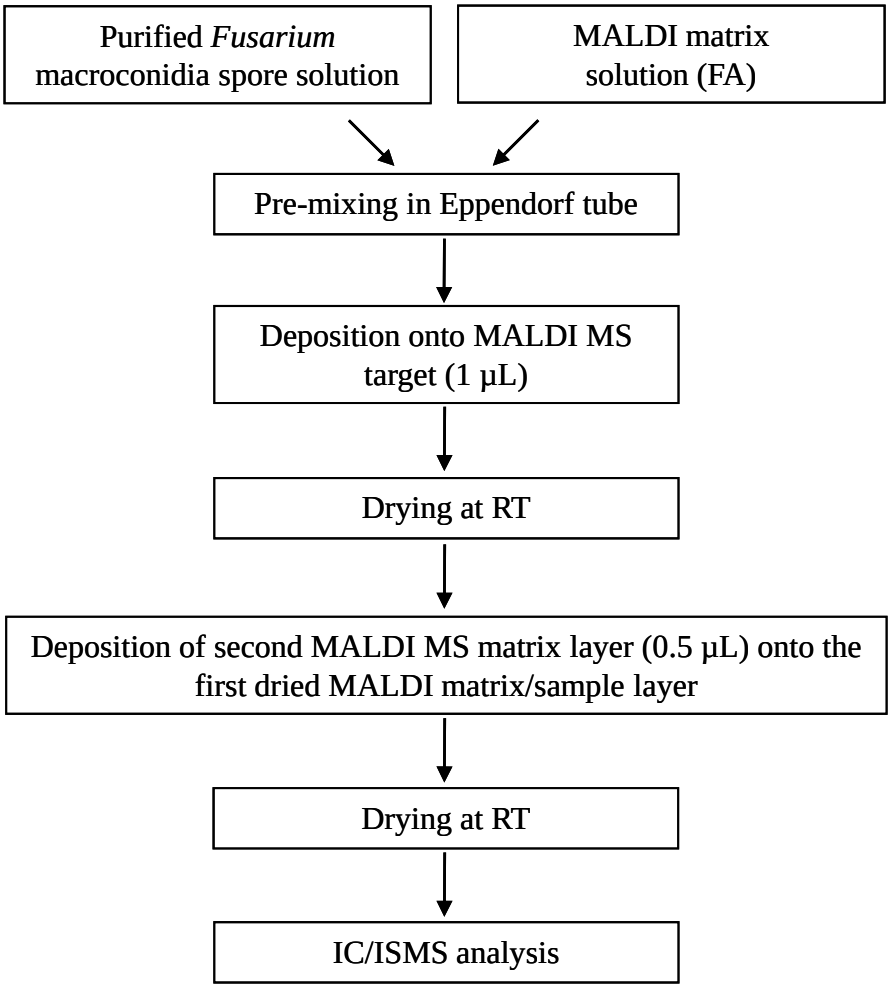


Figure 3. IC/ISMS sample preparation with two-layer volume technique for *Fusarium* macroconidia.

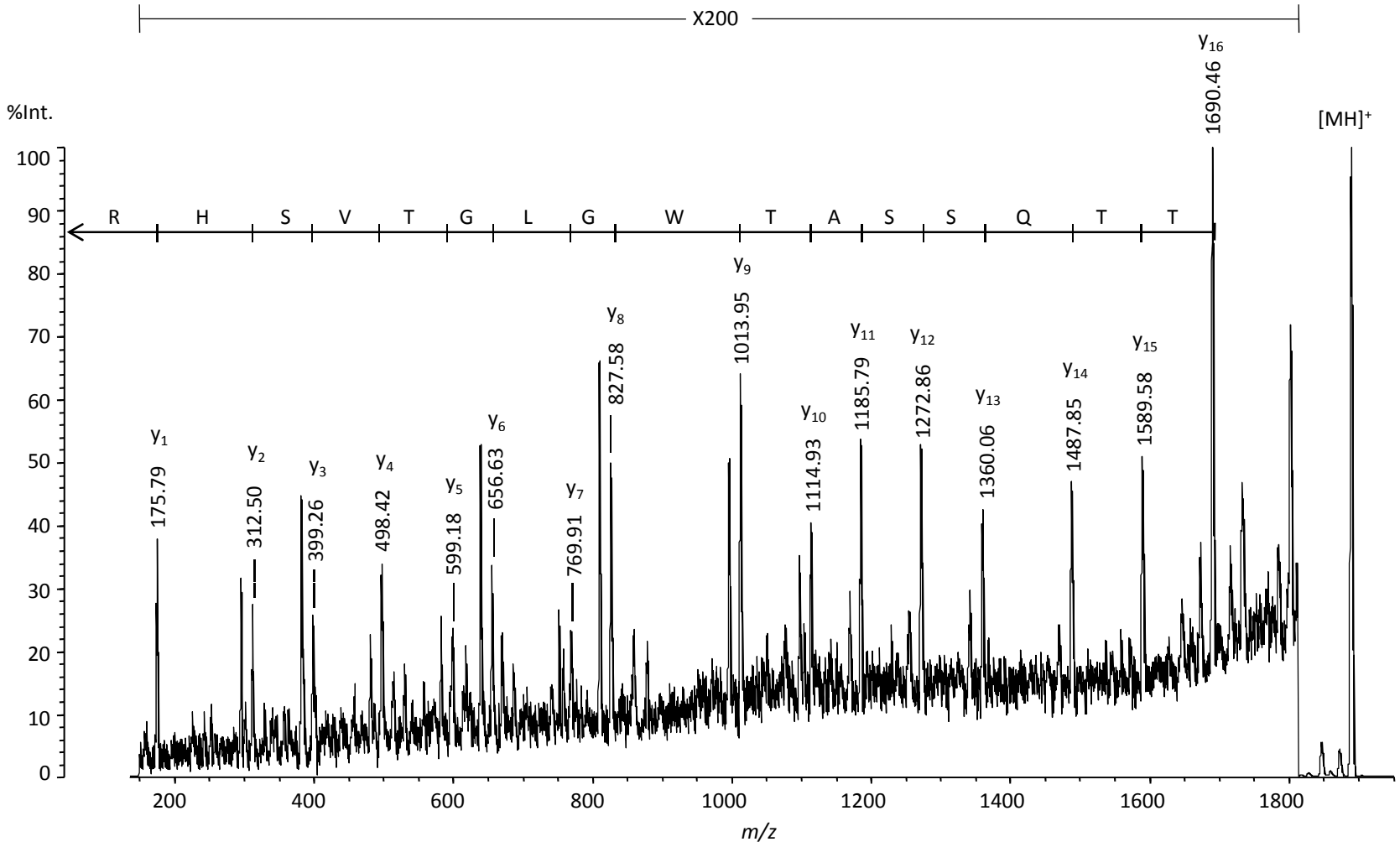


Figure 4. MALDI PSD spectrum obtained from the precursor ion at m/z 1888.9 after *on-target* tryptic digestion of *Fusarium* macroconidia spores.

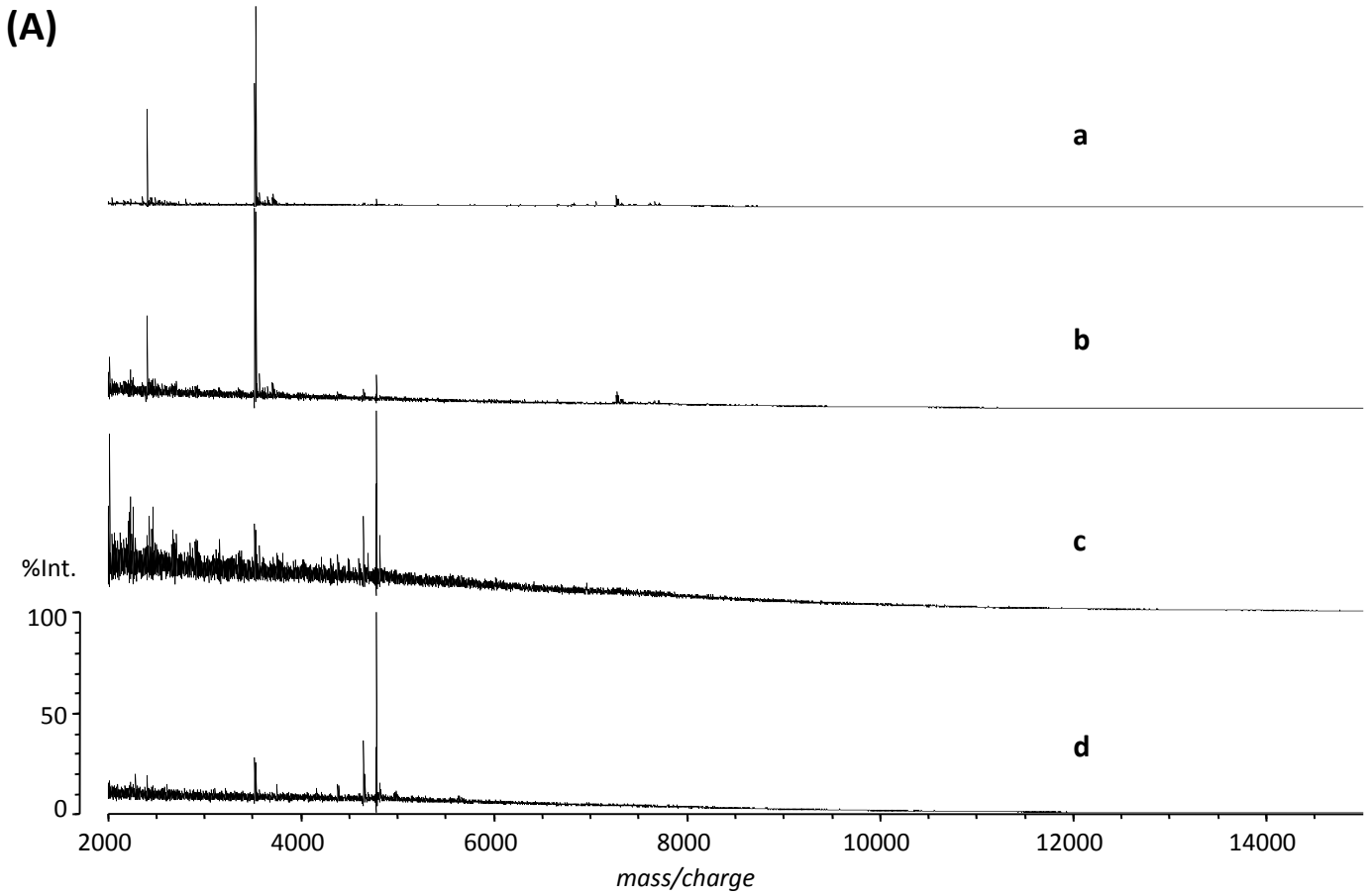


Figure 5 (A). IC/IS mass spectra of four (a-d) strains of *F. Cerealis.*

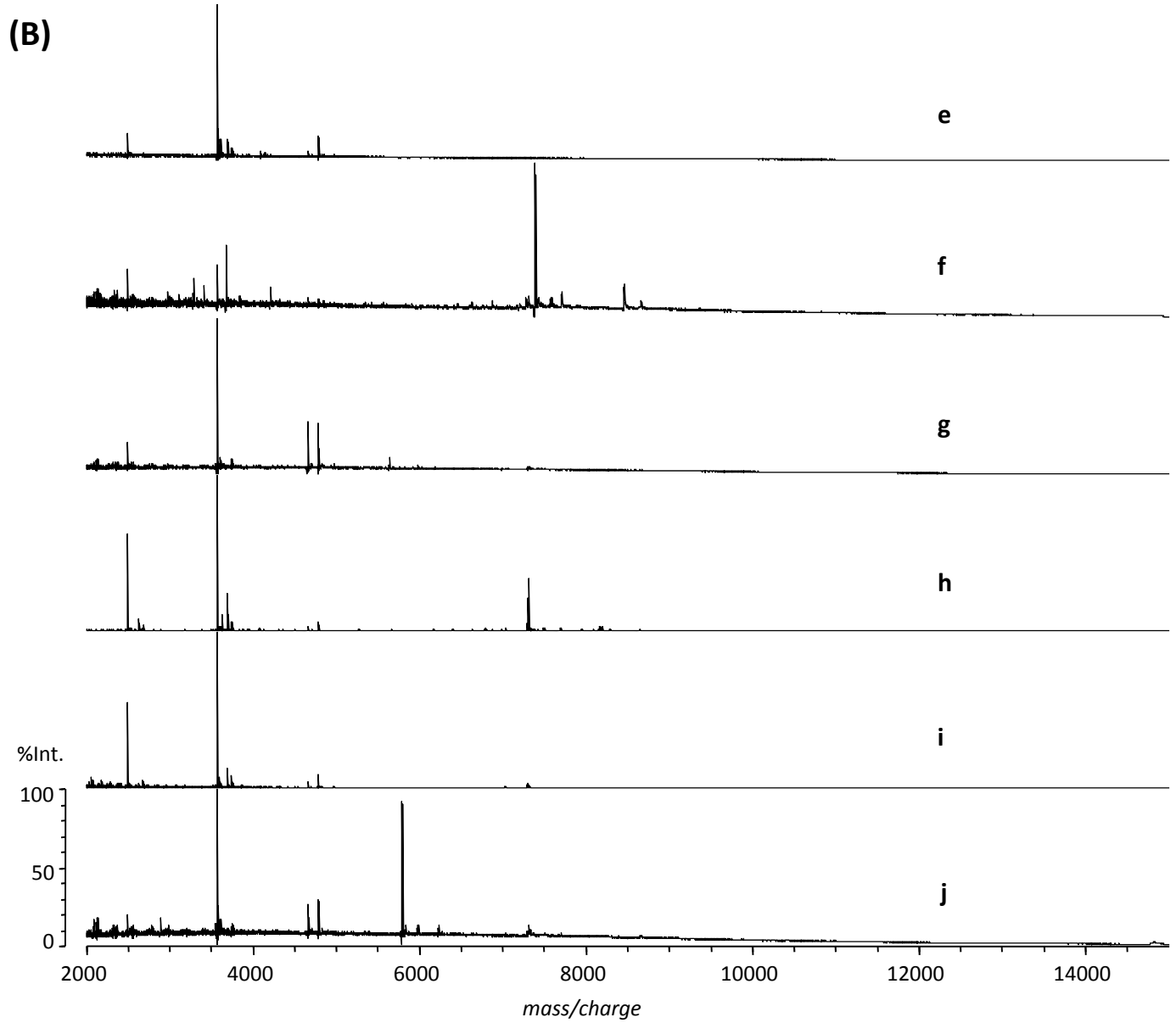


Figure 5 (B). IC/IS mass spectra of six (e-j) strains of *F. Graminearum.*

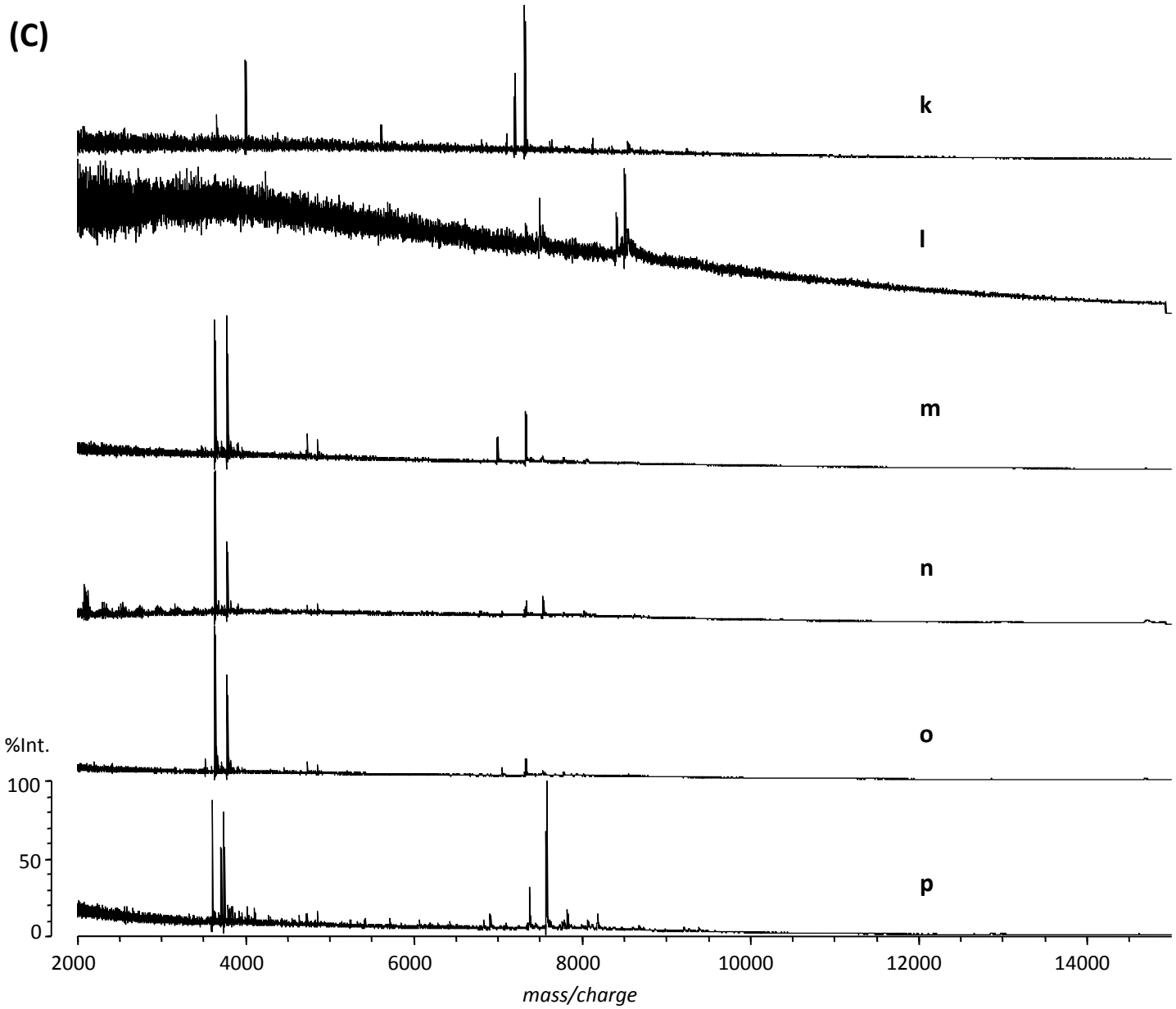


Figure 5 (C). IC/IS mass spectra of six (k-p) strains of *F. poae*

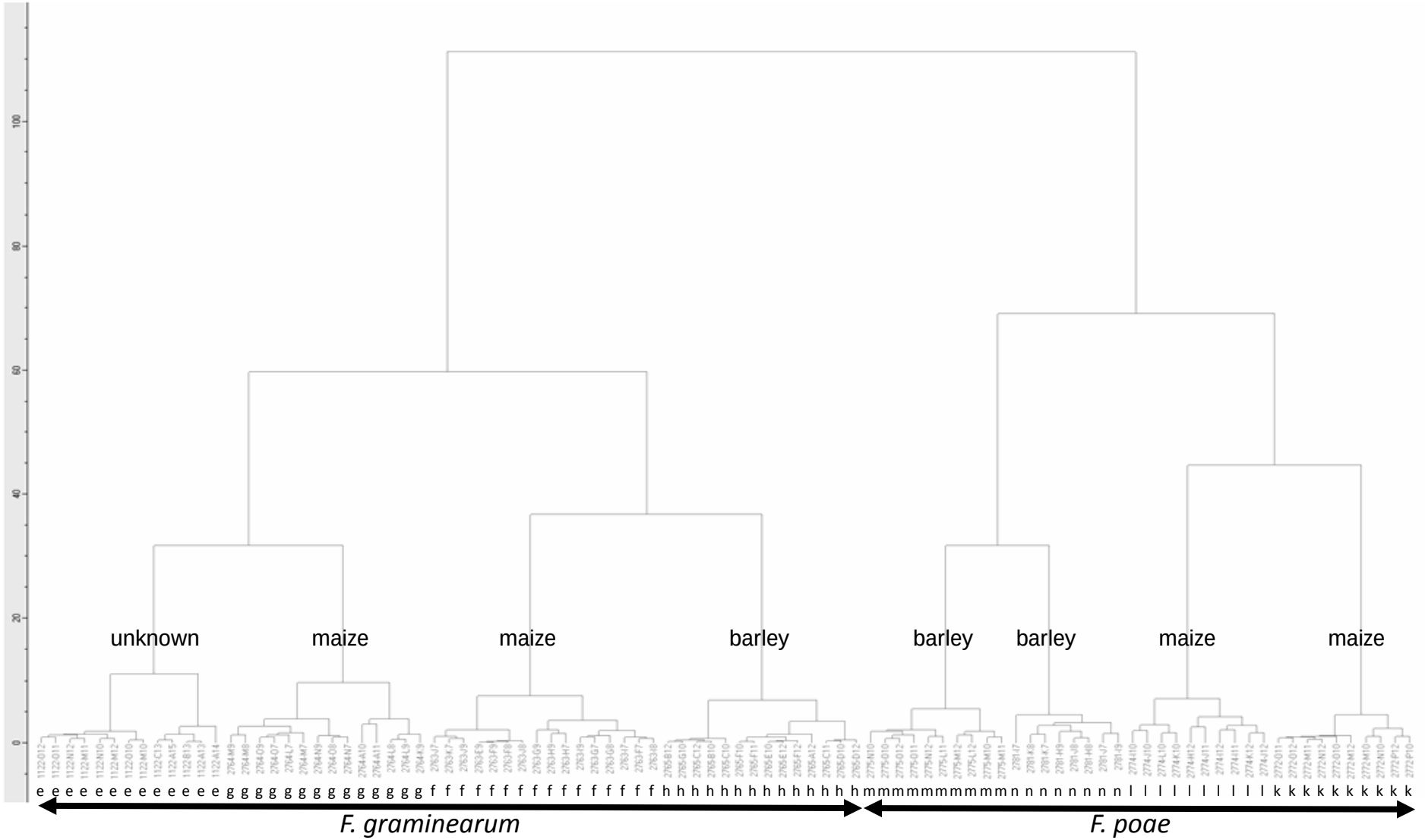


Figure 6. Dendrogram representing the result of unscaled hierarchical cluster analysis of IC/ISMS data of *Fusarium* species/strains from different hosts (maize, barley and unknown) and locations.

5. Curriculum Vitae

Personal Information

Name: Hongjuan Dong
Current address: Webgasse 7/2, 1060, Vienna, Austria
Mobile phone: +43 699 11800906
Email: hongjuan.dong@yahoo.de
Gender: Female
Date of Birth: 05.02.1982
Nationality: Chinese

Education

- 03/2007 – Now Ph. D thesis under supervision of Univ. Prof. Dr. Günter Allmaier
  Institute of Chemical Technologies and Analytics
  Vienna University of Technology

  Intact cell mass spectrometry of mycotoxin-producing Fusarium fungi for differentiation and protein identification

- 09/2003 – 07/2006 Master thesis under supervision of Prof. Zhiqiang Liu
  Changchun Institute of Applied Chemistry,
  Chinese Academy of Sciences

  Chemical research of preparation and prescription compatibility of Paenoia lactiflora Pall. and Scutellaria baicalensis Georgi.

- 09/1999 – 07/2003 Study of chemistry
  Shandong Normal University

Publications

- Hongjuan Dong , Z. Liu, F. Song, Z. Yu, H. Li, S. Liu
  Studies of Paeoniflorin by Electrospray Ionization Tandem Mass Spectrometry
  Chemical Journal of Chinese Universities. 2006. 27 (11): 2066 - 2069

- Hongjuan Dong, Z. Liu, F. Song, Z. Yu, H. Li, S. Liu
  Structural analysis of monoterpene glycosides extracted from *Paeonia lactiflora Pall.* using electrospray ionization fourier transform ion cyclotron resonance mass spectrometry and high performance liquid chromatography – electrospray ionization tandem mass spectrometry
  Rapid Communications in Mass Spectrometry. 2007. 21(19): 3193-3199

- Hongjuan Dong, J. Kemptner, M. Marchetti-Deschmann, C. P. Kubicek, G. Allmaier
  Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry
  Analytical and Bioanalytical Chemistry. 2009. 395 (5): 1373-1383

- Z. Liu, Hongjuan Dong, S. Wang, S. Liu
  Method optimization of concoction of Chinese Traditional Medicine *Paeonia Lactiflora Pall.*
  Chinese Patent No. 200610016678.4

- Hongjuan Dong, M. Marchetti-Deschmann, W. Winkkler, H. Lohninger, G. Allmaier
  Intact cell/spore mass spectrometry of *Fusarium* macroconidia for fast isolate and species differentiation
  Book chapter: NATO Science for Peace and Security Series A: Chemistry and Biology, Detection of Biological Agents for the Prevention of Bioterrorism. Submitted.

- Hongjuan Dong, M. Marchetti-Deschmann, C. P. Kubicek, G. Allmaier
  Characterization of *on-target* generated tryptic peptides from *Giberella zeae* conidia spore surface proteins by means of matrix-assisted laser desorption/ionization post source decay fragment ion analysis
  Journal of Microbiological Methods. To be submitted.

Poster

- Hongjuan Dong, J. Kemptner, M. Marchetti-Deschmann, C. P. Kubicek, G. Allmaier
  Optimization of MALDI TOF MS sample preparation for the differentiation of intact spores derived from the fugus *Fusarium spp*.
  27th informal meeting on mass spectrometry. Retz, Austria, 3-7 May 2009

- Hongjuan Dong, M. Marchetti-Deschmann, C. P. Kubicek, G. Allmaier
  On-target tryptic digestion of intact *Fusarium* conidia spores combined with MALDI PSD fragment ion analysis for verification of surface-associated proteins
  3rd CEEPC, 3rd Annual Meeting of the Hungarian Proteomics Society, 4th Czech Proteomic conference, 7th AuPA Proteome Research Symposium. Budapest, Hungary, 6-9 Oct 2009