UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

Visegrad Fund

MALDI-TOF mass spectrometry tools for microbial identification in archival document investigation

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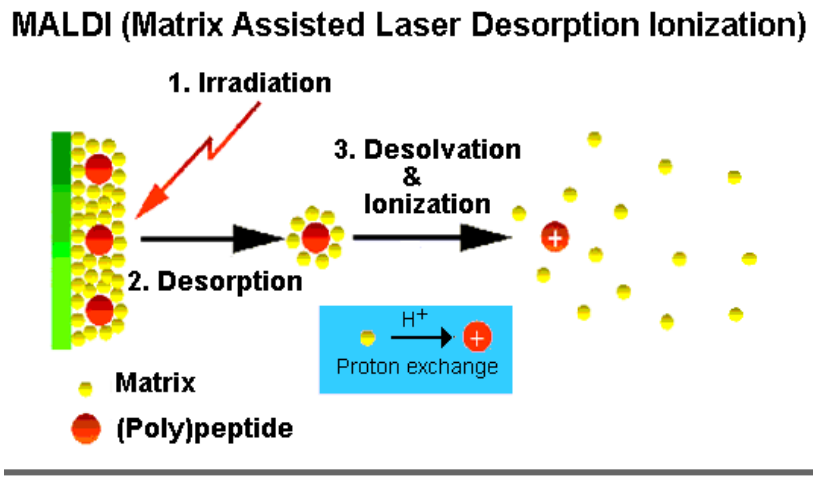
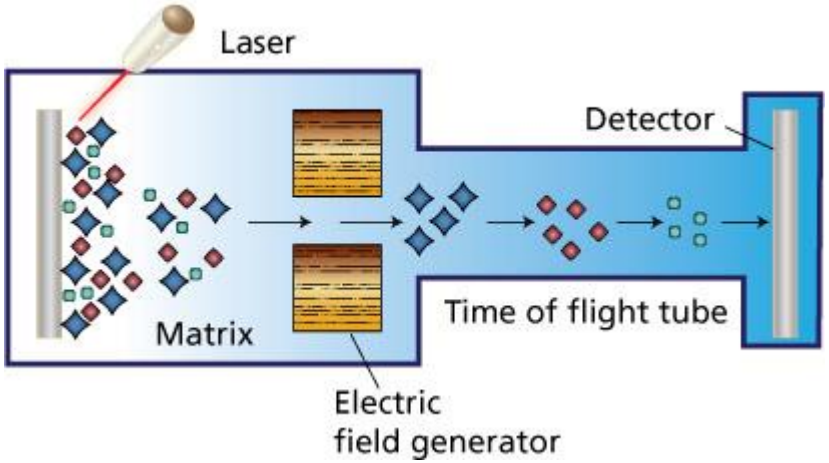
SMALL GRANT CO-FUNDED
BY INTERNATIONAL VISEGRAD FUND

MALDI - TOF MS

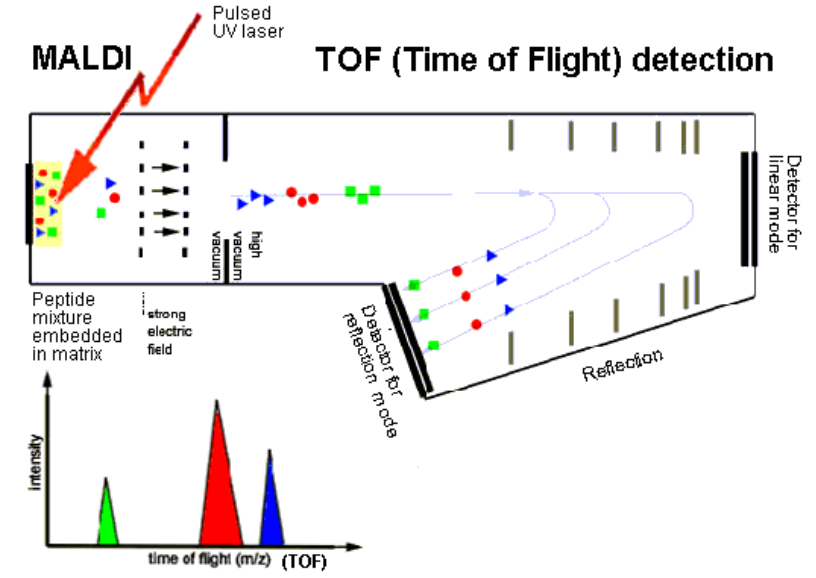
Matrix-**A**ssisted **L**aser **D**esorption **I**onization-**T**ime **o**f **F**light **M**ass **S**pectrometry

MALDI-TOF MS: PRINCIPLE





http://www.sigmaaldrich.com/technical-documents/articles/biology/custom-dna-oligos-qc-analysis-by-mass-spectrometry.html



Animation: http: /cmgm.stanford.edu/pan/section_html/MS/

<u>Soft ionisation method:</u>

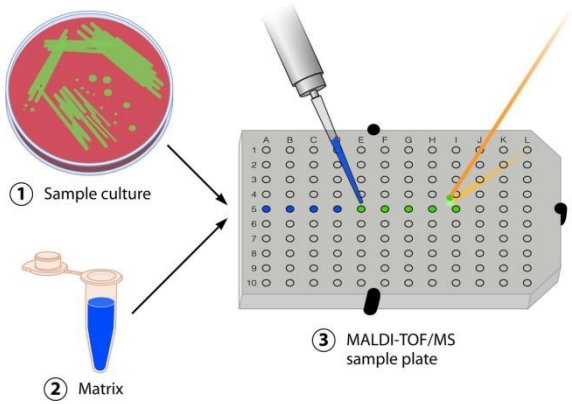
- low level of sample fragmentation

<u>Time of flight</u> is a function of the specific ion mass (m/z)

$$\frac{m}{z} = 2eU\frac{t^2}{L^2}$$

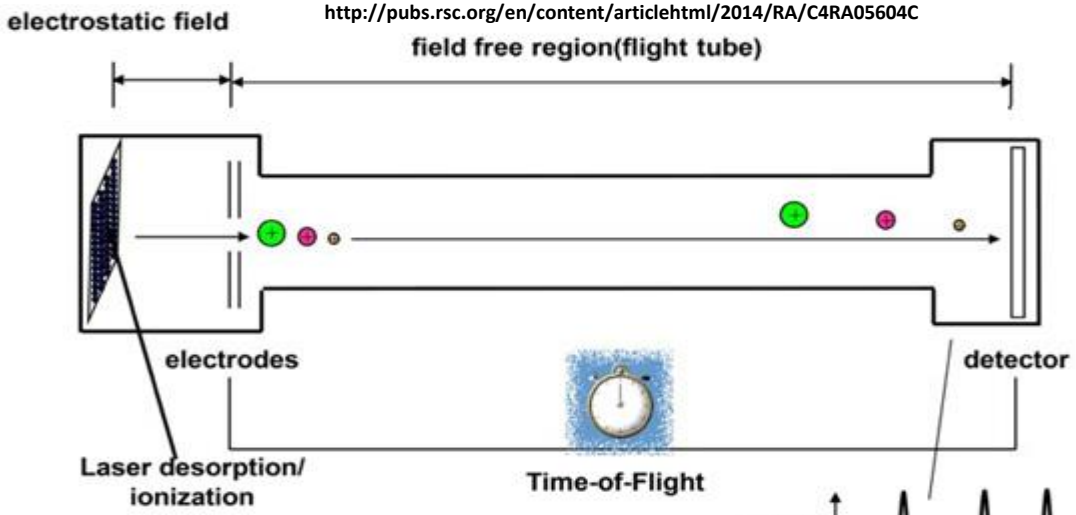
m mass,
z charge,
L length of drifting zone,
e elementary charge,
U speeding voltage

MALDI-TOF MS



http://cmr.asm.org/content/26/3/547.figures-only

http://pubs.rsc.org/en/content/articlehtml/2014/RA/C4RA05604C



Matrix-**A**ssisted **L**aser **D**esorption **I**onization-**T**ime of **F**light **M**ass **S**pectrometry

1) sample preparation

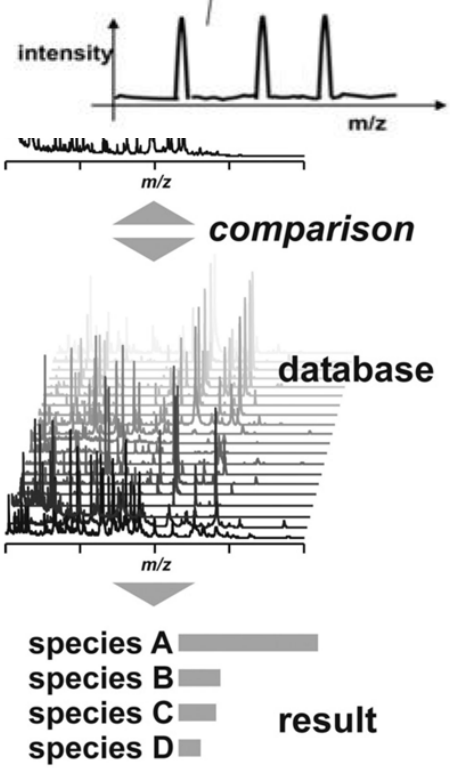
- microbial culture or its proteins extract is smearing onto a steel plate and covered over by matrix
- matrix enables the sample (A) to be desorped and ionised as pseudomolecule ionts $[A+H]^+$

2) MALDI-TOF MS analysis

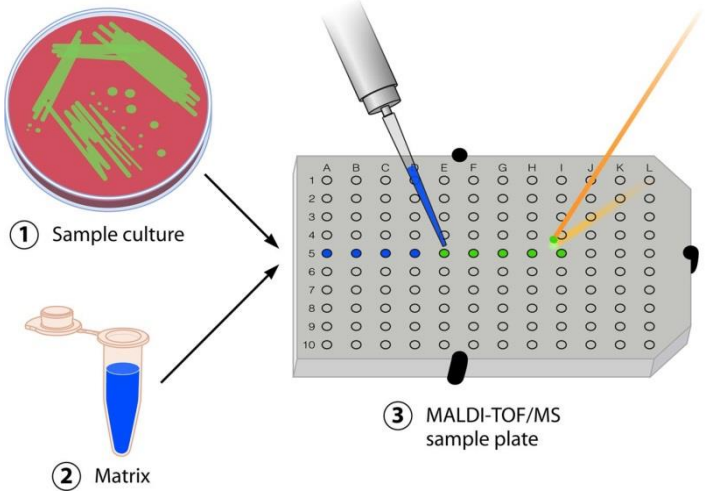
unique mass spectral fingerprint of desorbed microbial cell components (mainly intracellular proteins), different among genera, species or also some strains

3) identification:

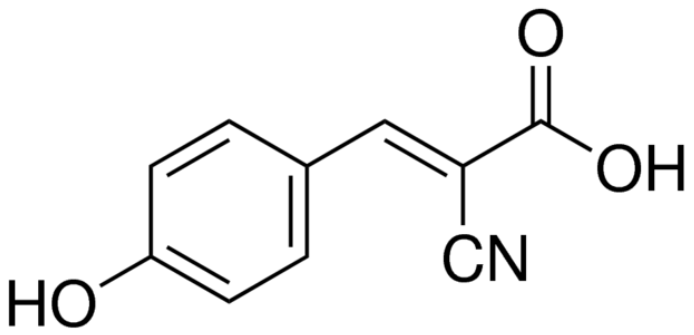
comparison of mass spectrum to those of reference strains in database



MALDI-TOF MS: SAMPLE AND MATRIX



http://cmr.asm.org/content/26/3/547.figures-only



http://www.sigmaaldrich.com/catalog/product/sigma/c8982?lang=en&region=CZ

<u>CHC: α-Cyano-4-hydroxycinnamic acid</u>
(organic solvent: 50% acetonitrile with 2,5 % trifluoracetic acid)

<u>Sample preparation</u>

Direct transfer

- spreading of intact **cells directly onto a steel plate**
- lysis of cells occurs during the contact with **acid matrix and by laser desorption**
- most bacteria

Proteins extraction

- previous extraction of proteins by organic acids and/or alcohol (**e.g. ethanol and 70% formic acid**)
- yeasts, moulds, some species of bacteria (depending on the cell wall composition)

<u>Matrix:</u>

- able to **absorb the energy of the laser** (usually 337 nm)
- able to **crystalise with samples** (empirically tested) – necessary for sample desorption
- usually **acid character** (proton ionisation of sample), **dissolved in organic solvent**

SA : 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)

DHB: **2,5-Dihydroxybenzoic acid**

MALDI-TOF MS: MICROORGANISMS

Mass spectrum protein profile

z equals usally to 1+ (so m/z usually corresponds to mass of molecula)

the range usually used for identification: **2000 -20 000 m/z**

the intensity of single peaks corresponds to the abundance of the protein

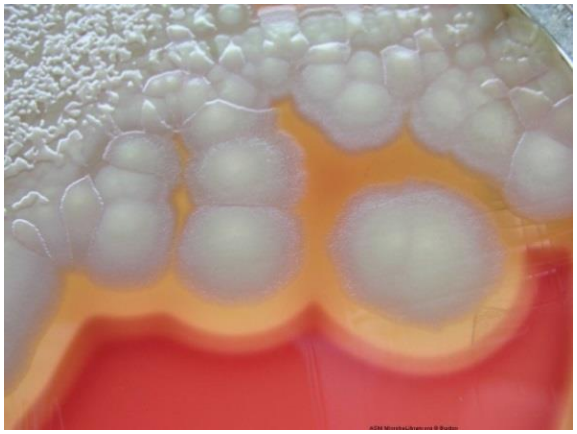
Which proteins dominates in the protein profile ?

abundant, basic and mediumly hydrophobic

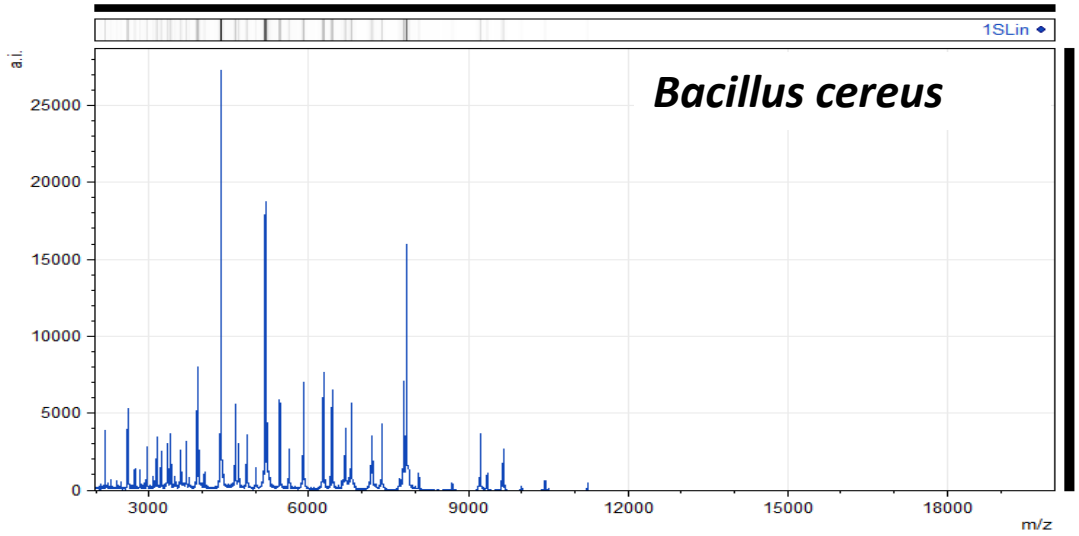
: **mainly ribosomal proteins**

: further cold-shock and heat-shock proteins, chaperons etc.

conserved house-keeping gene = conserved proteins = in acordance with identification based on DNA

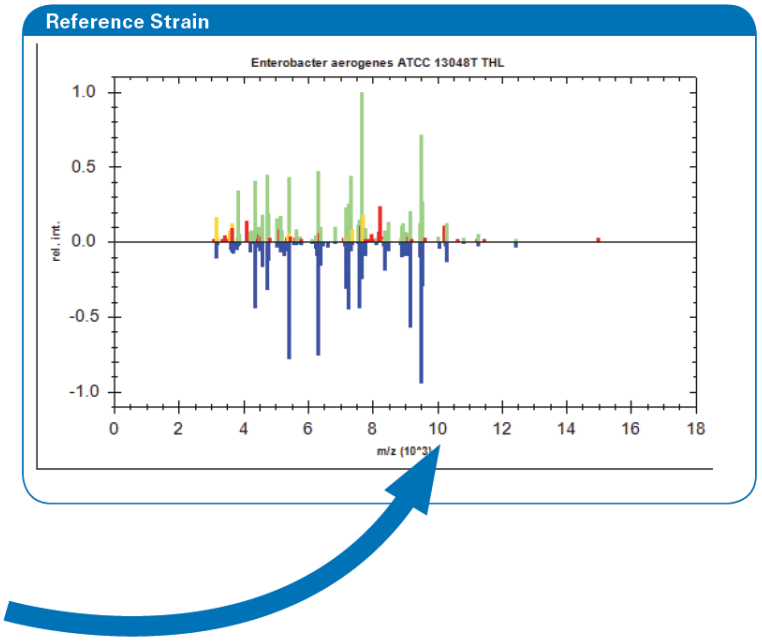


Analysis is recomended (and validated) to be performed from colonies grown onto **non-selective agar**



Visualisation of mass spectrum protein profile – (software mMass 5, Strohalm *et al.*, 2010)

MALDI-TOF MS: ANALYSIS



Comparison of mass spectrum protein profile of unknown sample with these of reference strains present in database by software

BioTyper:
The statistical analysis for correlation includes peak positions, intensities and frequencies across the complete range of microorganisms.
Score value:
0 (none similarity) - 1000 (absolute similarity)
But it is expressed in decadic logarithm
log(score value): 0-3

Commercial databases from different MALDI-TOF MS producers
Bruker Daltonics – MALDI BIOTYPER
Shimadzu - Shimadzu Launchpad software + SARAMIS database
Biomérieux - VITEK® MS
Other databases compatible with different hardware systems (e.g. Andromas)

Range	Description	Symbols	Color
2.300 ... 3.000	**highly probable species identification**	**(+++)**	green
2.000 ... 2.299	**secure genus identification, probable species identification**	**(++)**	green
1.700 ... 1.999	**probable genus identification**	**(+)**	yellow
0.000 ... 1.699	**not reliable identification**	**(-)**	red

MALDI-TOF MS - PROCEDURE

Direct method: smearing sample in four parallels in lower and higher cells concentrations – after drying to cover over by matrix (1-2 µl) and let to crystallise at room temperature

Matrix: solution of α-Cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% acetonitrile with 2,5 % trifluoroacetic acid (prepared with 10% TFA solution)

Protein standard (1 µl): Bruker Bacterial Test Standard (Bruker Daltonics, SRN) – proteins extracted from z *Escherichia coli* DH5alpha BRL + some others

Equipment

Bruker Autoflex Speed

Database MALDI Biotyper 3.1

MALDI-TOF MS:PROTEIN STANDARD

Bruker Bacterial Test Standard (referred to as 'Bruker BTS') contains a carefully manufactured extract of *Escherichia coli* DH5 alpha that shows a characteristic peptide and protein profile in MALDI-TOF mass spectra. The extract is spiked with two additional proteins that extend the upper boundary of the mass range covered by Bruker BTS. The overall mass range covered by Bruker BTS is 3.6 to 17 kDa.

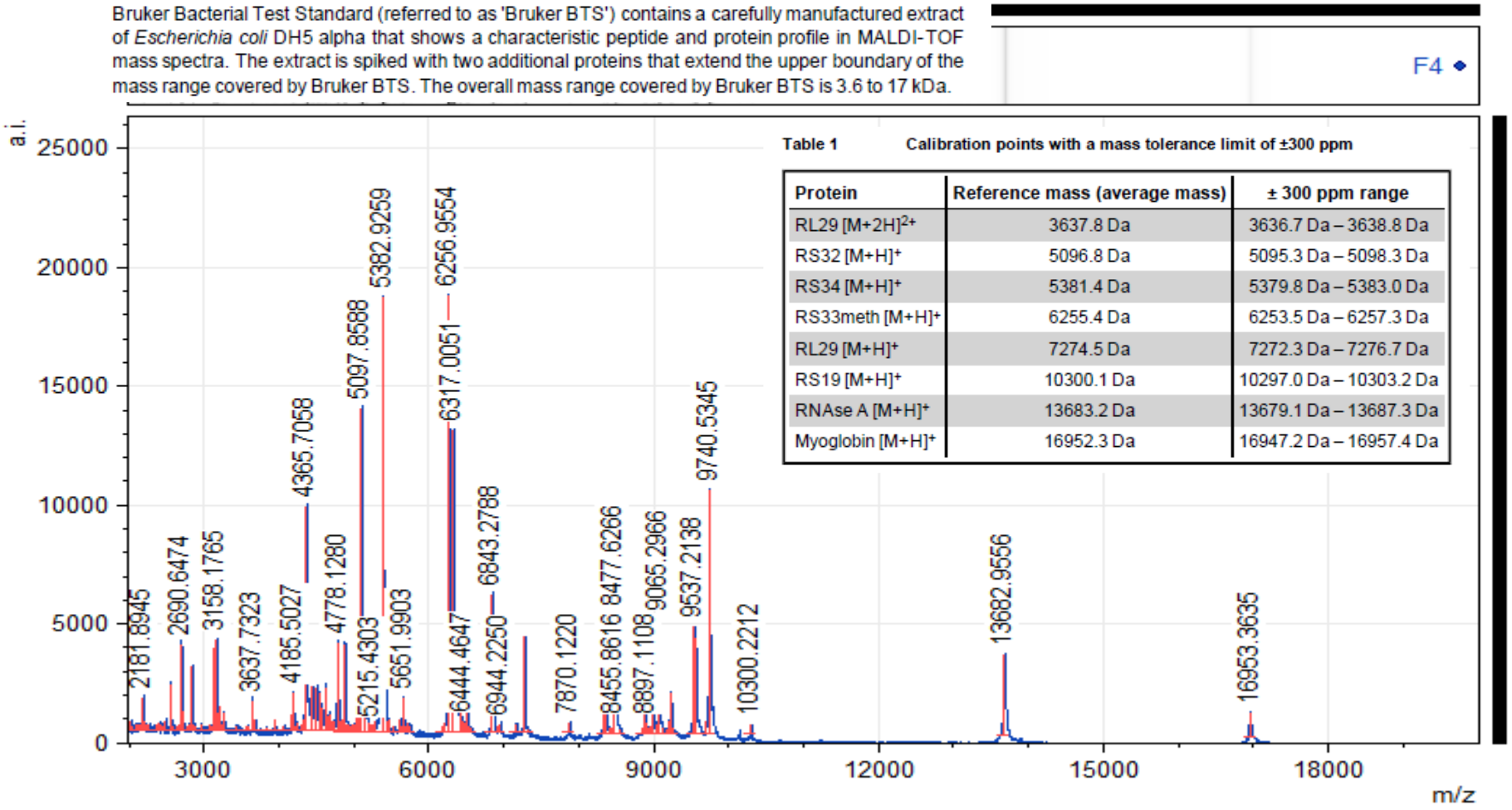


Table 1 Calibration points with a mass tolerance limit of ±300 ppm

Protein	Reference mass (average mass)	± 300 ppm range
RL29 $[M+2H]^{2+}$	3637.8 Da	3636.7 Da – 3638.8 Da
RS32 $[M+H]^{+}$	5096.8 Da	5095.3 Da – 5098.3 Da
RS34 $[M+H]^{+}$	5381.4 Da	5379.8 Da – 5383.0 Da
RS33meth $[M+H]^{+}$	6255.4 Da	6253.5 Da – 6257.3 Da
RL29 $[M+H]^{+}$	7274.5 Da	7272.3 Da – 7276.7 Da
RS19 $[M+H]^{+}$	10300.1 Da	10297.0 Da – 10303.2 Da
RNAse A $[M+H]^{+}$	13683.2 Da	13679.1 Da – 13687.3 Da
Myoglobin $[M+H]^{+}$	16952.3 Da	16947.2 Da – 16957.4 Da

Analyte name	Rank (Quality)	Matched Pattern	Score Value
F4	1 **(+++)**	*Escherichia coli* DH5alpha BRL	2.439

RESULTS

GR.	SAMPLE 1 (FOR BIOCHEMICAL TESTS)			SAMPLE 2 (OTHERS)		
	IDENT.	SCORE VALUE	SYMBOL	IDENT.	SCORE VALUE	SYMBOL
1*	*Chryseobacterium indologenes*	2.332	+++	*Pantoea septica*	2.17	++
2	*Erwinia sp*	2.236	++	*Staphylococcus aureus*	2.017	++
3	*Cronobacter sakazakii*	2.083	++	*Staphylococcus aureus*	2.365	+++
4	*Klebsiella oxytoca*	2.418	+++	*Pseudomonas monteilii*	2.29	++
5	*Leclercia adecarboxylata***	2.097	++	*Staphylococcus aureus*	2.36	+++
6	*Klebsiella oxytoca*	2.375	+++	*Bacillus cereus*	1.841	++
7	*Escherichia vulneris*	2.185	++	*Pseudomonas monteilii*	2.224	++
SP	*Leclercia adecarboxylata***	2.261	++	*Cronobacter sakazakii*	2.098	++

*Group 1: samples to be taken in an opposite rank ? (*Chrys. ind.* is not family *Enterobacteriaceae*)

** *Leclercia adecarboxylata* – previously (before 1986) known as *Escherichia adecarboxylata*

Range	Description	Symbols	Color
2.300 ... 3.000	**highly probable species identification**	**(+++)**	green
2.000 ... 2.299	**secure genus identification, probable species identification**	**(++)**	green

RESULTS

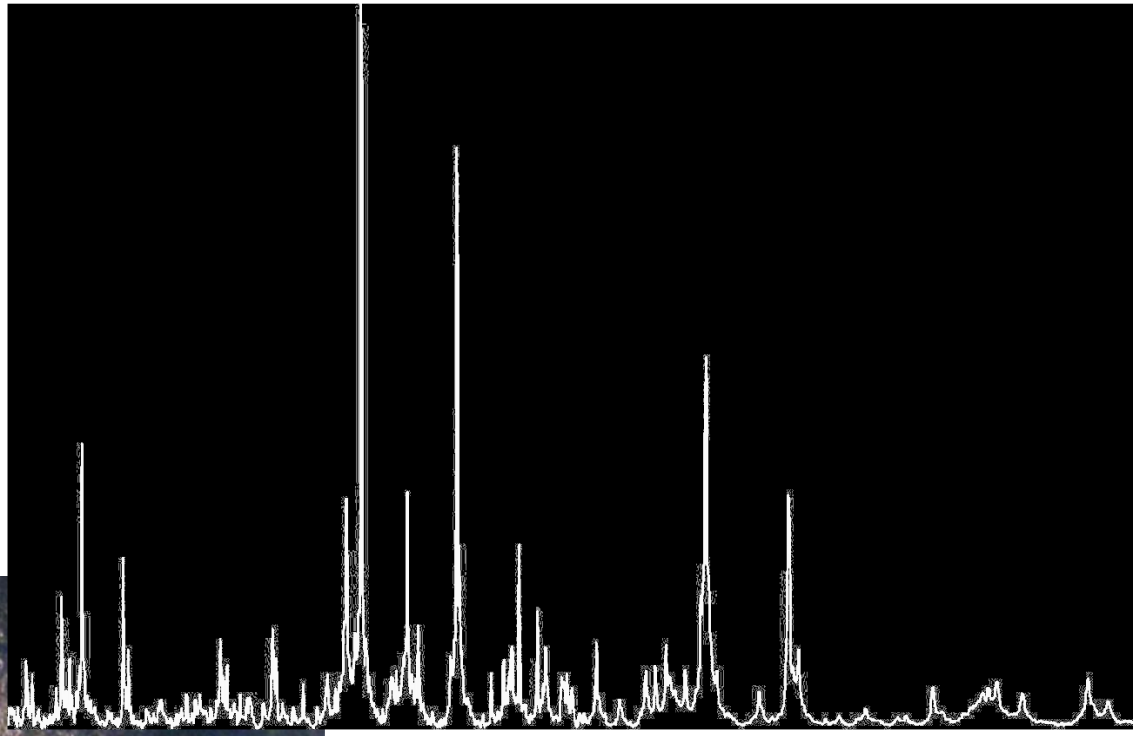
GROUP	SAMPLE 1 (FOR BIOCHEMICAL TESTS)			SAMPLE 2 (OTHERS)		
	IDENT.	SCORE VALUE	SYMBOL	IDENT.	SCORE VALUE	SYMBOL
4	*Klebsiella oxytoca*	2.418	+++	*Pseudomonas monteilii*	2.29	++
6	*Klebsiella oxytoca*	2.375	+++	*Bacillus cereus*	1.841	++
7	*Escherichia vulneris*	2.185	++	*Pseudomonas monteilii*	2.224	++

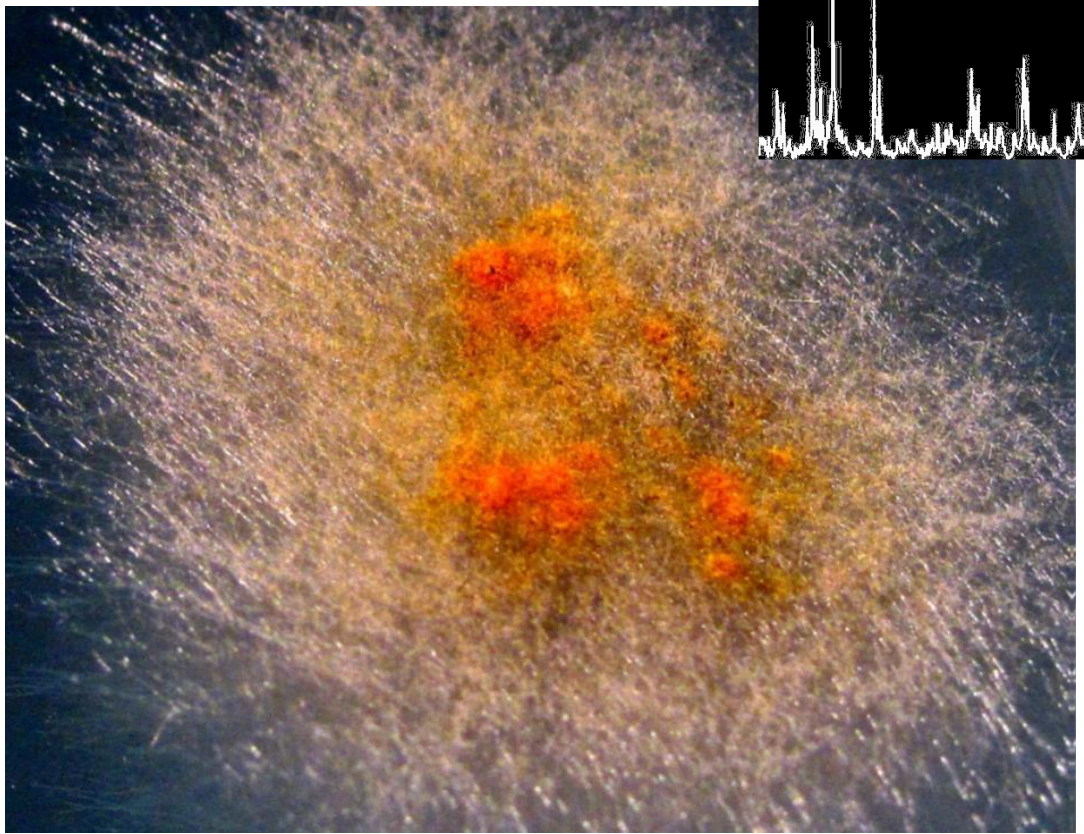
Klebsiella oxytoca and species *ornithinolytica* / *planticola* / *terrigena* of the genus *Raoultella* have very similar patterns: Therefore distinguishing their species is difficult.

Pseudomonas montelii is a member of *Pseudomonas putida* group

Bacillus anthracis, *cereus*, *mycoides*, *pseudomycoides*, *thuringiensis* and *weihenstephanensis* are closely related and members of the *Bacillus cereus* group. In particular *Bacillus cereus* spectra are very similar to spectra from *Bacillus anthracis*. *Bacillus anthracis* is not included in the IVD MALDI Biotyper database. For differentiation an adequate identification method has to be selected by an experienced professional. The quality of spectra (score) depends on the degree of sporulation: Use fresh material. **For *Bacillus* spp. the protein extraction is recommended.**

MOLDS





Alternaria alternata

Mass spectrometry has revolutionized the identification of microorganisms within the past several years, setting new standards in speed and reliability. But even in these advanced times of microbial mass spectrometry, the **identification of molds and multicellular fungi still persists as one of the most challenging aspects of microbiology.** This can be mainly attributed to the effects of culture conditions. Bruker´s method:

- **cultivation method**
- **standard preparation method**
- **Filamentous Fungi library to facilitate the identification of these microorganism groups.**

Standardized liquid cultivation. In order to reduce the effects of culture conditions and to aid in the production of a uniform mycelium, a **liquid based cultivation** method has been developed which standardizes the physiological status. This method has been used to create the Filamentous Fungi library and is recommended where quick identification from front mycelium is not possible.

In essence, tubes are inoculated with the fungi and placed on a rotator to incubate overnight or until enough biological material is observed. Using the standardized liquid cultivation method **prevents the germination process and the formation of spores.** This enables fast and reliable species identification of slow- or fast-sporulating filamentous fungi and many other difficult-to-handle organisms such as agar adhering filamentous fungi.

If a mycelium is clearly visible and can be harvested, then it is possible to **sample directly from the agar and**, using the simple ethanol extraction method, good results can usually be obtained for most of the samples **without the need** for liquid cultivation.

In cases were **direct harvesting is difficult**, the liquid cultivation method should be used.
Standardized liquid cultivation has been developed in order to reduce the effects of culture conditions and to aid in the production of a **uniform mycelium**.

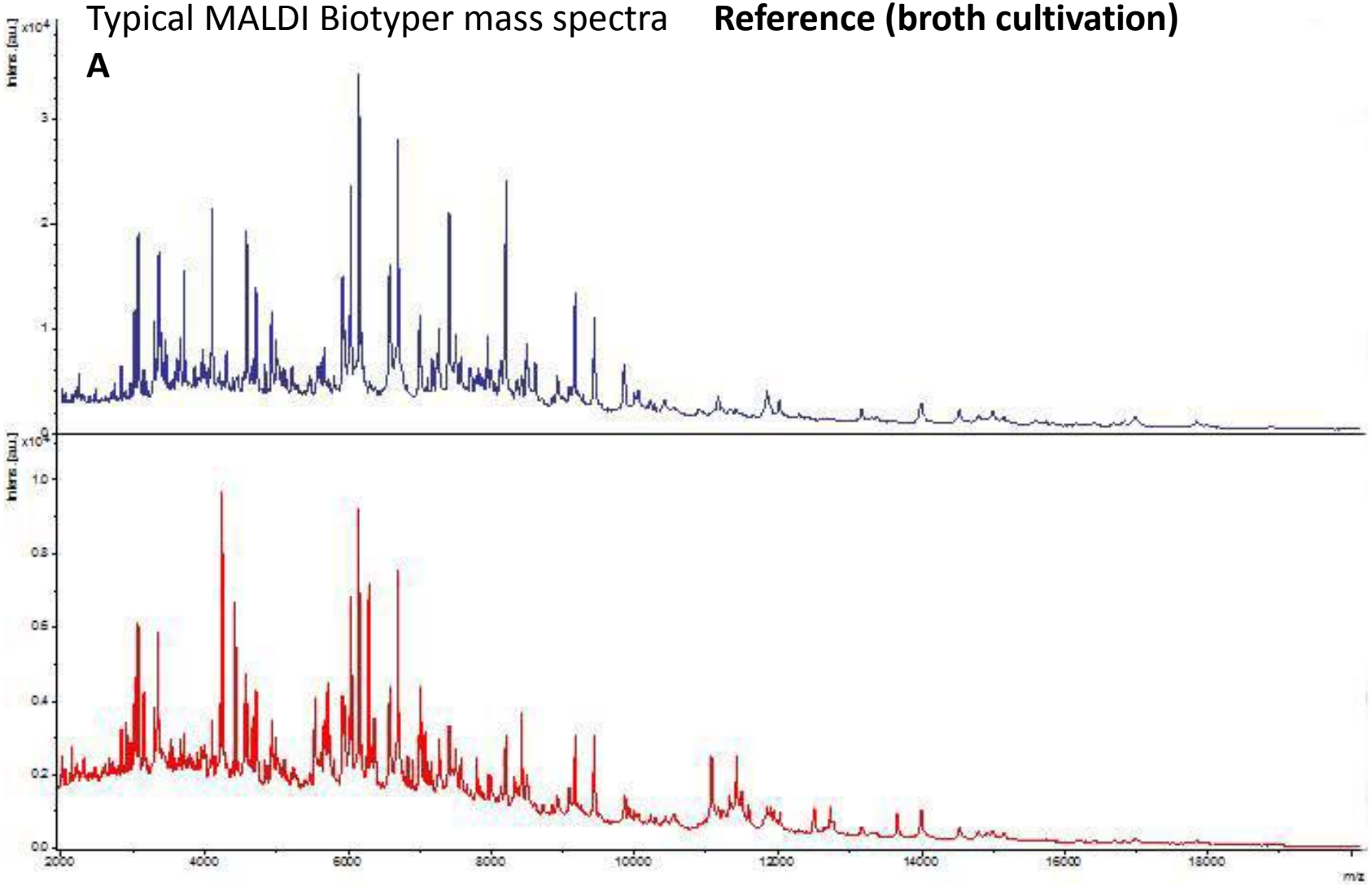
This method has been used to create the Filamentous Fungi library and is recommended where quick identification from front mycelium is not possible.

In essence, tubes are inoculated with the fungi and placed on a rotator to incubate overnight or until enough biological material is observed. Using the standardized liquid cultivation method prevents the germination process and the formation of spores.

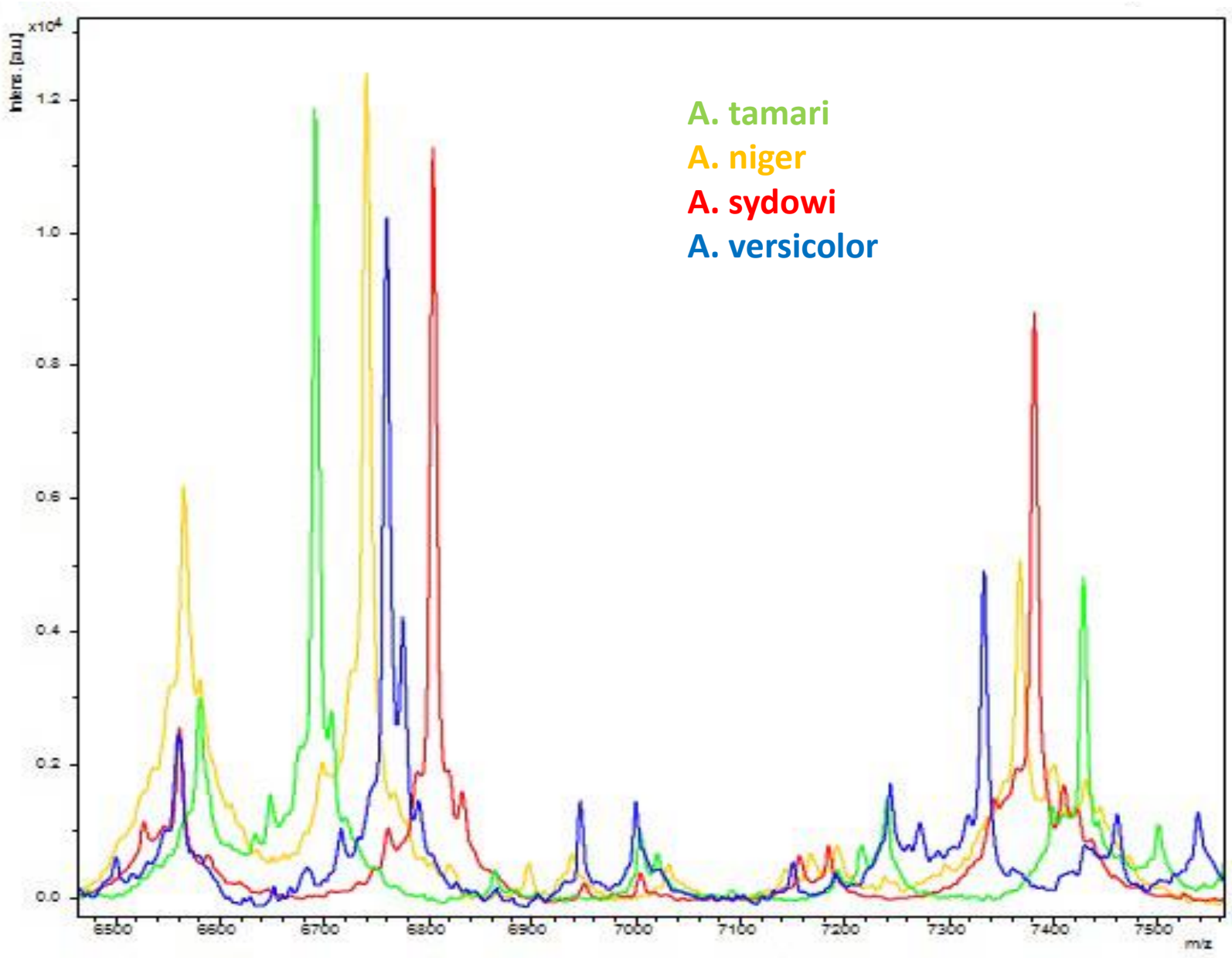
This enables fast and reliable species identification of slow- or fast-sporulating filamentous fungi and many other difficult-to-handle organisms such as agar adhering filamentous fungi.

Typical MALDI Biotyper mass spectra **Reference (broth cultivation)**

A

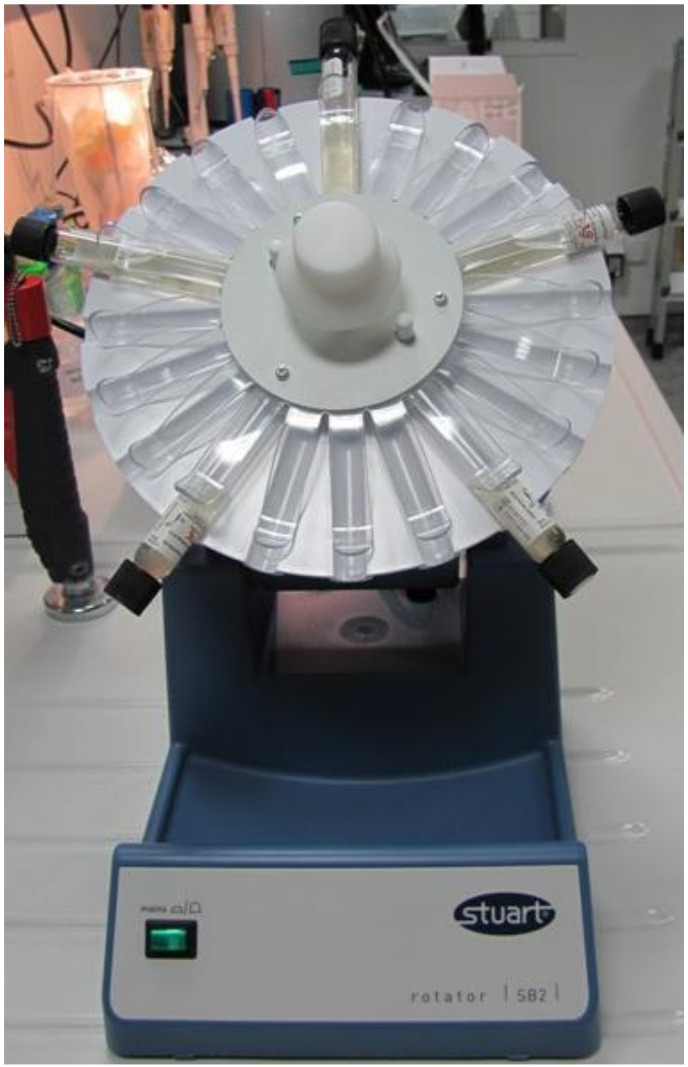


Sample direct harvesting **B**


A. tamari
A. niger
A. sydowi
A. versicolor
Inters. [a.u.]
x10^4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6500
6600
6700
6800
6900
7000
7100
7200
7300
7400
7500
m/z

Cultivation Procedure for Fungi

Cultivation Tube



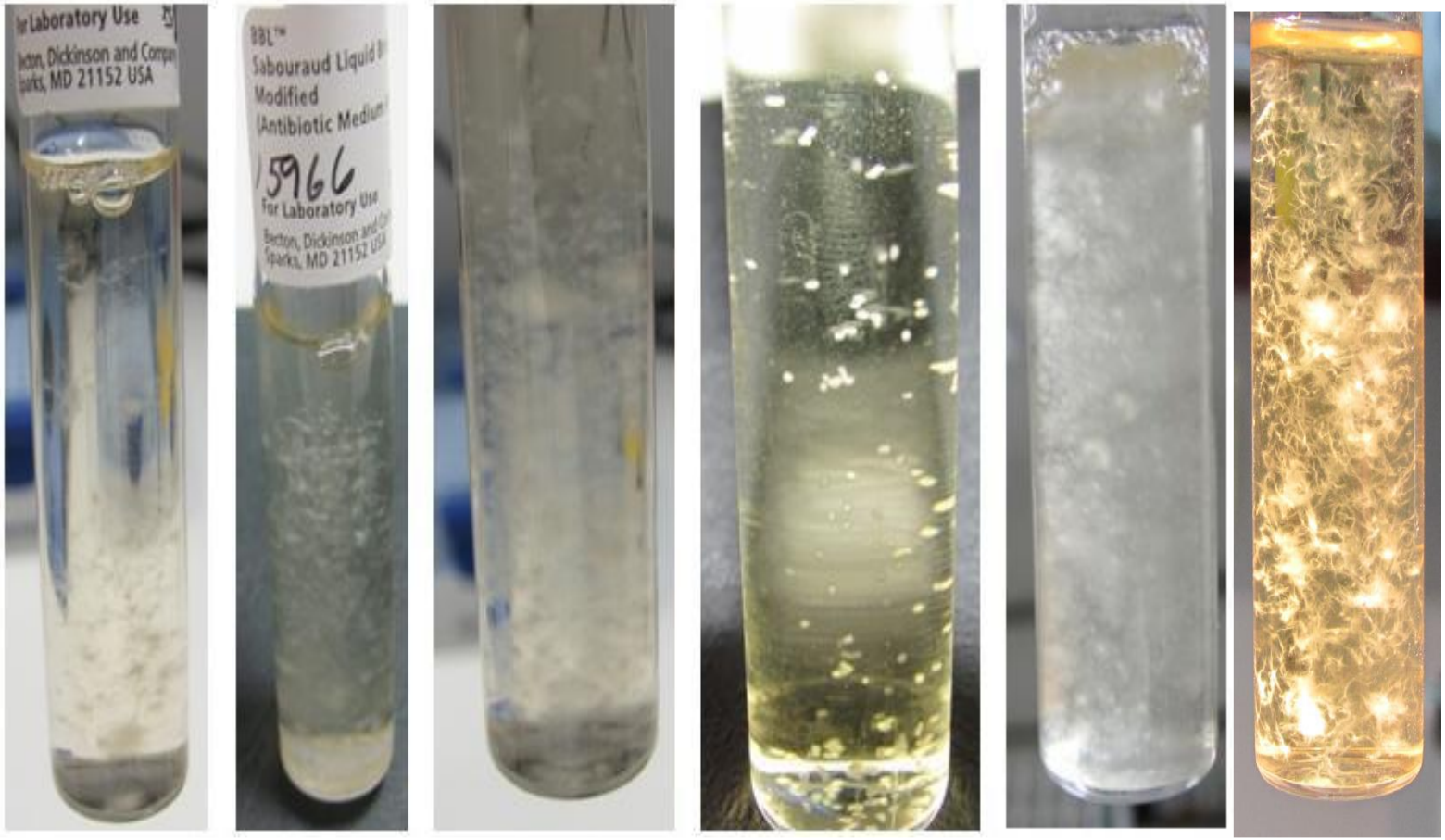
Rotator SB 2 and Rotary dish

Cultivation Method:

· Inoculate the tubes with few biological material and close the lid

· Rotate the rotator to shake “over head”.

· Incubate until enough biological material is observed



Sample Preparation

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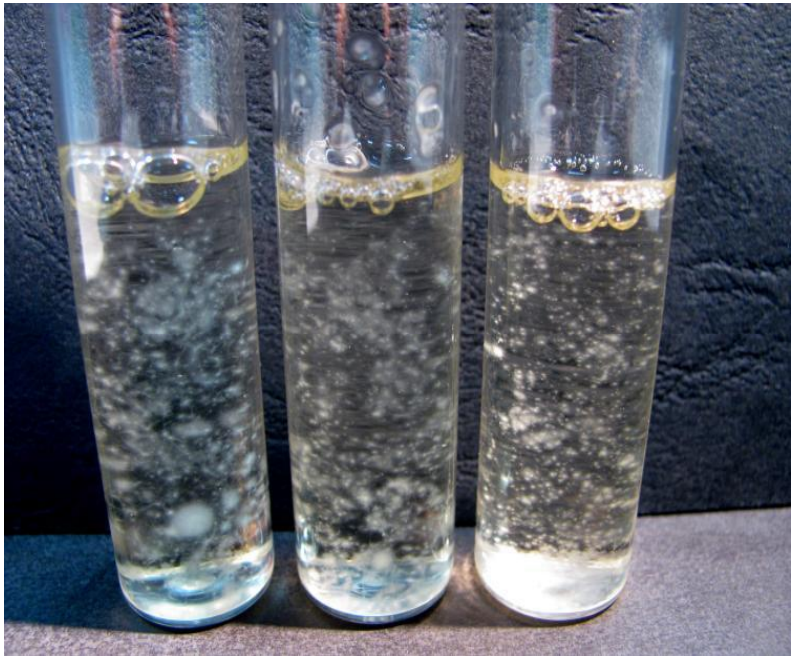
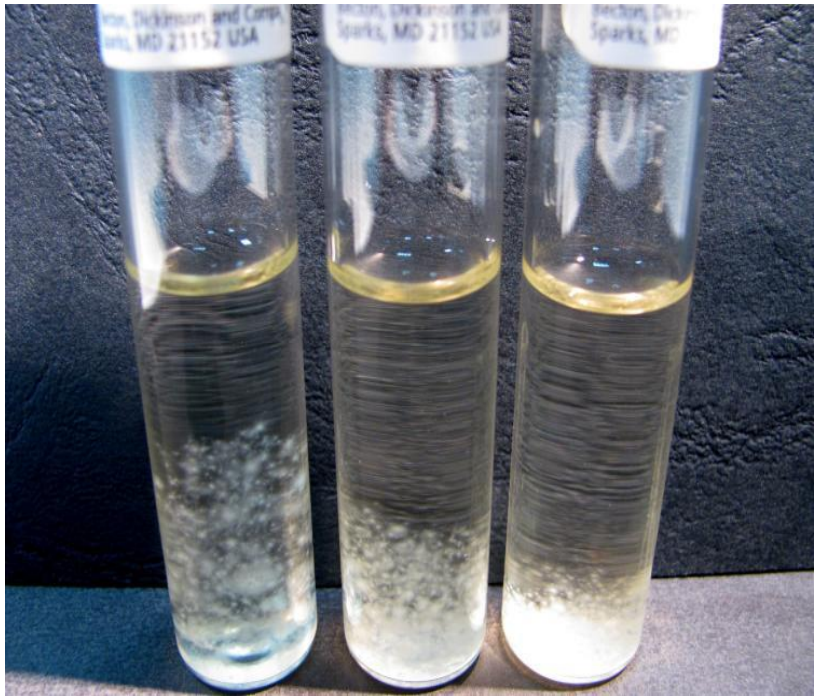
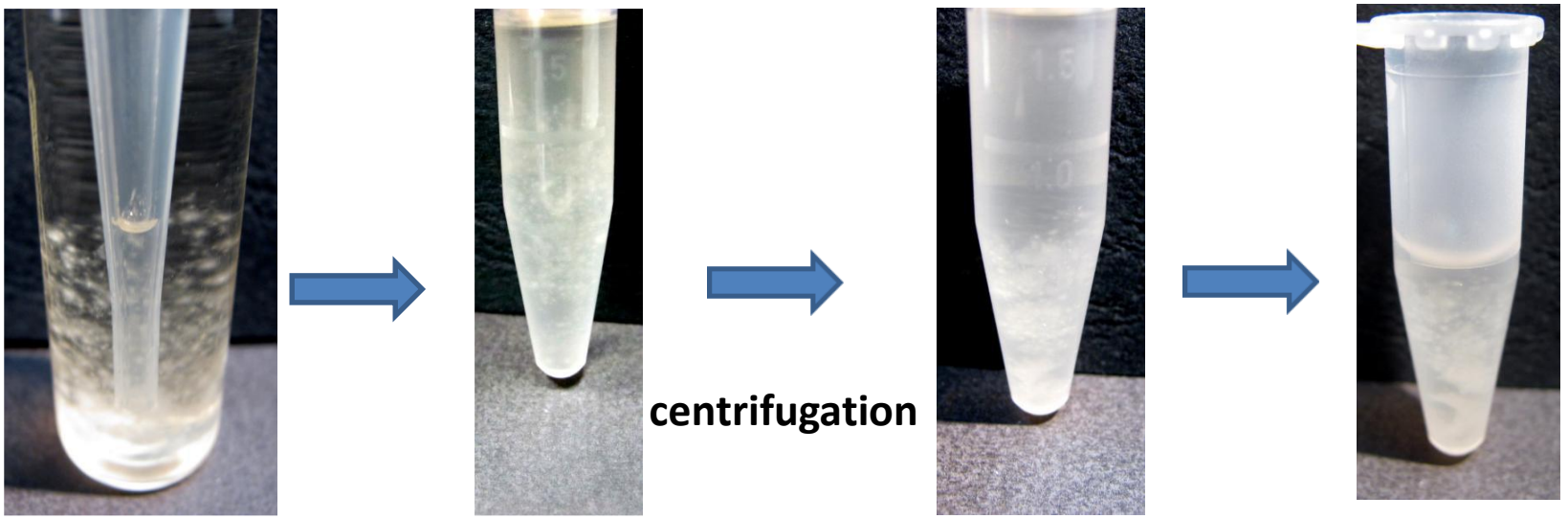
- Remove cultivation tubes from the rotator, place it on the working table and wait for 10 minutes
- ·Filamentous fungi sediment to the bottom of the tube.





- Harvest up to 1.5ml from the sediment and transfer it to an Eppendorf tube.
- Centrifuge for 2min at full speed (e.g. 13.000 upm).
- Carefully remove the supernatant.



- Add 1ml water to the pellet and vortex for one minute.
- Centrifuge for 2min at full speed (e.g. 13.000 upm).
- Carefully remove the supernatant again and repeat washing and vortexing once

- **Suspend the pellet in 300µl water, add 900µl ethanol, and vortex it.**
- **Centrifuge for 2min at full speed (e.g. 13.000 upm).**
- **Remove supernatant carefully by pipetting (avoid decanting), centrifuge shortely, and remove the residual ethanol completely.**
- **Dry the pellet completely (e.g. in a SpeedVac or drying for a while at 37°C).**

completely dried pellet

- **Add according to the pellet size a certain amount of formic acid. A very small pellet will require 10µl to 20µl and a big pellet could require up to 100µl formic acid. Please refer to the picture series below.**

too few formic acid

correct volume of formic acid

- **Add the same volume acetonitril to the tube and mix it carefully.**
- **Centrifuge for 2min at full speed (e.g. 13.000 upm).**
- **Add 1µl supernatant to the MALDI target and continue with standard target preparation.**

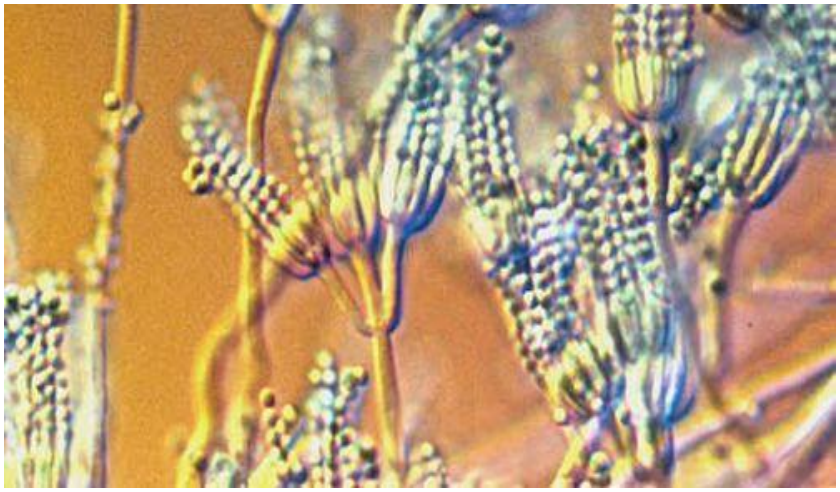
MALDI-TOF MS: PROBLEMS

PROBLEMS DUE TO THE SAMPLE PREPARATION

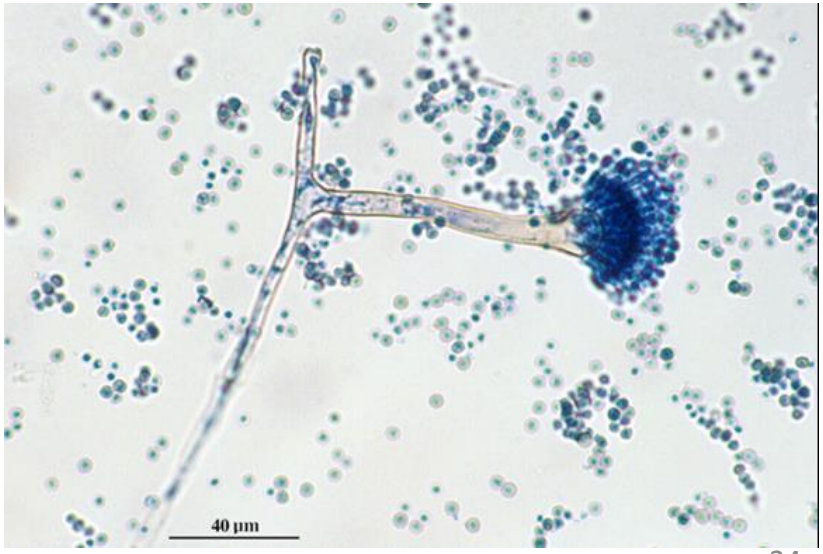
- **QUALITY OF MATRIX** – global problems in crystalisation, ionisation and desorption of samples – the quality of matrix to be checked in the samples of standard !!!
- **QUALITY OF SAMPLE**
  - **TOO LOW OR VERY IRREGULAR CONCENTRATION on the spot –** „NO PEAKS FOUND“ (only some places of spots are measured)
  - **TOO HIGH CONCENTRATION OR NOT TO BE SMEARED REGULARLY on the spot –** worse crystalisation, high level of noise
  - **THE PRESENCE OF OTHER CHEMICALS IN THE SAMPLE (AGAR, NaCl…) –** influences behavior of samples (crystalisation, noise, peaks shift etc.)
  - **THE PROCEDURE FOR SAMPLE PREPARATION IS NOT OPTIMAL –** e.g. for yeasts, moulds and Bacillus spp. the extraction procedure is recommended – higher quality of spectra
  - **PUR CULTURE** !!!

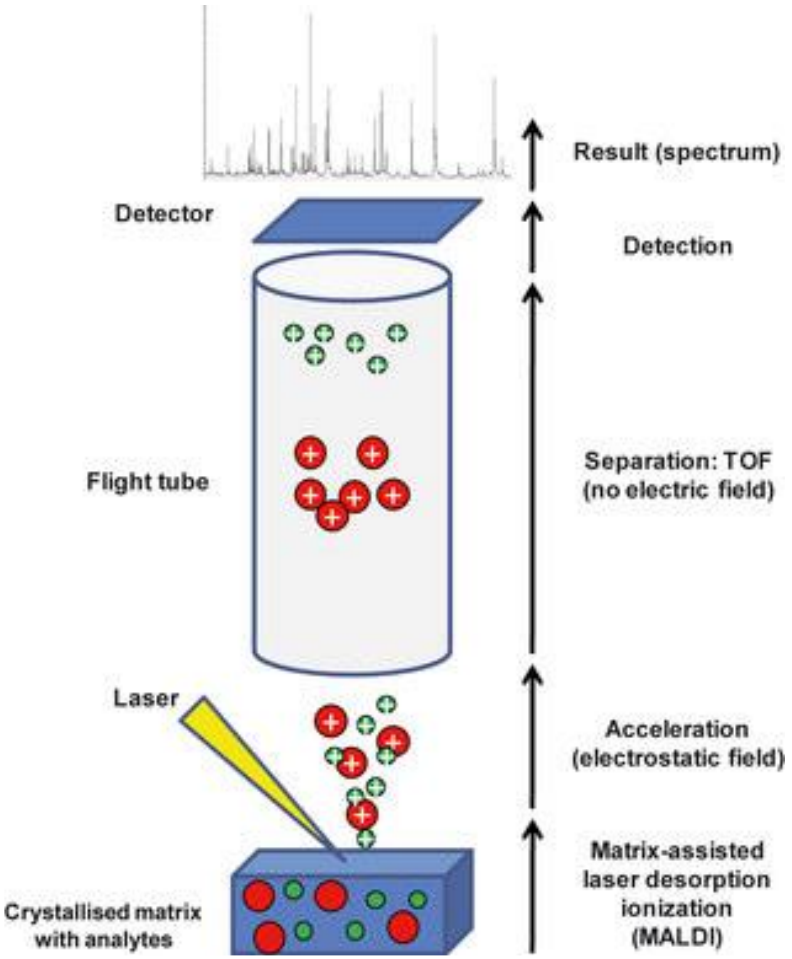
PROBLEMS DUE TO THE IDENTIFICATION

- **SOME SPECIES OR EVEN GENERA ARE DIFFICULT TO BE DISTINGUISHED as to be in very close** (or taxonomically changing) **relation**
  - *Escherichia coli* is not definitely distinguishable at the moment from *Shigella* (four *Shigella* species and *E. coli* are in reality one species)
- **THE GENUS OR SPECIES IS NOT PRESENT IN A DATABASE**
  - The spectrum is of a good quality, but the similarity with database spectra is low and/or unprobable (similarity on the same level to very different groups)



Thank you for your attention




Result (spectrum)
Detector
Detection
Flight tube
Separation: TOF
(no electric field)
Laser
Acceleration
(electrostatic field)
Crystallised matrix
with analytes
Matrix-assisted
laser desorption
ionization
(MALDI)