Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI TOF MS)

MALDI TOF MS has matured into an indispensable tool in the field of modern bioanalysis. It allows rapid analysis and identification of a wide scale of biomolecules (proteins, peptides, nucleic acids, lipids, saccharides), as well as synthetic polymers, drugs, small organic molecules and inorganic compounds.

Theoretical part

Together with the electrospray ionization, MALDI is a soft ionization technique that allows analysis of various biomolecules, especially biopolymers, and synthetic polymers, which tend to fragment when ionized by classical ionization techniques. As a pulsed ionization technique MALDI it usually coupled with TOF mass analyzers.

A. Principle of MALDI

MALDI is a two-step process (Fig. 1). First, the sample, composed of an analyte (A) mixed with an excess of MALDI matrix (M), is irradiated by short (~ ns) laser pulses. The matrix molecules strongly absorb laser light leading to fast sample desorption. Second, the protonized matrix species transfer protons to the analyte molecules and charge them. For MALDI, a low fragmentation level and the formation of so called pseudomolecular ions $[A+H]^+$ are typical. .
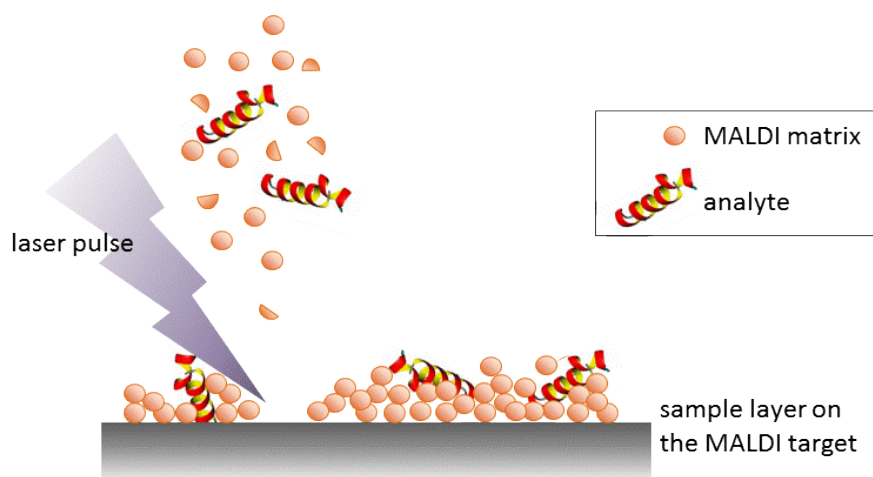

Figure 1: Principle of the MALDI process

The requirements for a suitable matrix selection result from the MALDI mechanism:
- **strong absorption** at the wavelength of the employed laser, usually a nitrogen laser (337 nm) or frequency-tripled Nd:YAG lasers (355 nm)
- the formation of **desirable „co-crystals"** with analyte molecules (usually based on previous experience, by trial and error to some extent)
- **acidic properties** of matrix (efficient proton source for analyte ionization)

The most commonly used MALDI matrices are small aromatic acids, such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) or α-cyano-4-hydroxycinnamic acid (CHCA) (Fig. 2).
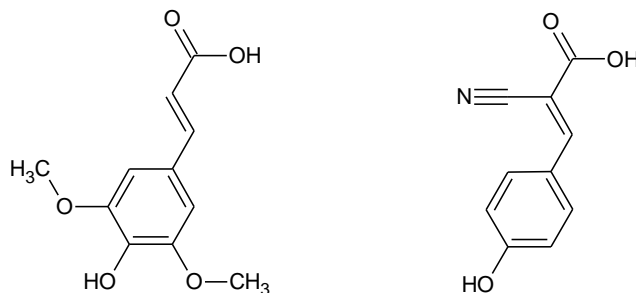

Figure 2: Commonly used MALDI matrices:
sinapinic acid and α-cyano-4-hydroxycinnamic acid

B. Principle of Time-of-flight (TOF)

Ions generated by MALDI are usually analyzed in a time-of-flight mass analyzer (Fig. 3). Ions are given a defined kinetic energy by an accelerating electric field and are allowed to drift through a field-free region. Their time of flight, *t* can be calculated as a function of the *m/z* ratio of the ion according to the following equation:

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

where *m* is mass, *z* is number of charges, *e* is the elemental charge, *U* is the acceleration voltage and *L* is the length of the drift zone.

The advantage of the TOF mass analyzer is the high ion transmission (and thus high sensitivity), short time of the analysis (~hundred μs for a single-shot laser pulse) and theoretically unlimited *m/z* range.
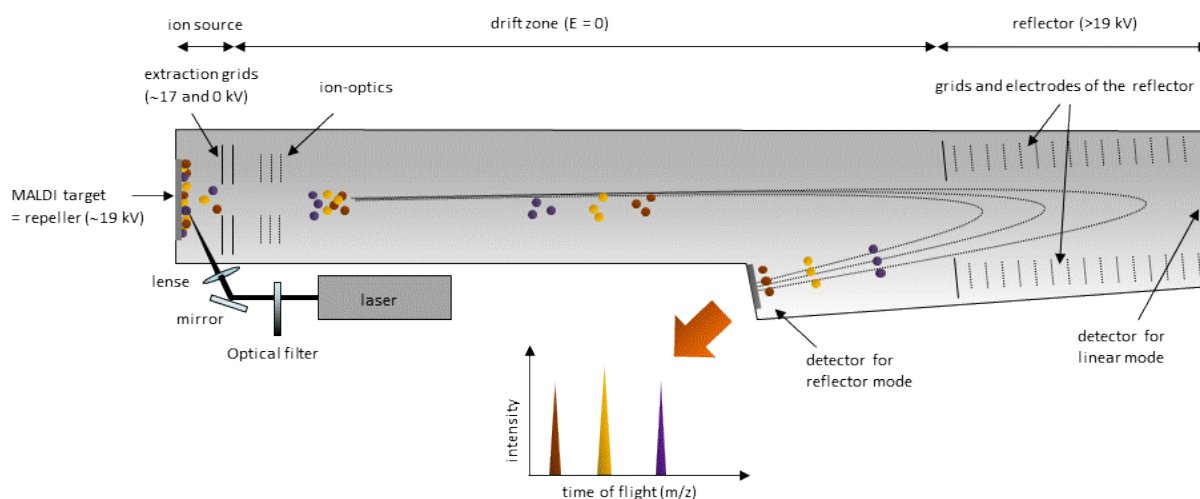

Figure 3: A schematic of the MALDI TOF mass spectrometer operating in the reflector mode

In MALDI TOF MS, mass resolution can be improved in two ways, which correct the negative influence of dispersion of the initial kinetic energy (E_{kin}) of the ions:

The use of ***delayed extraction*** (DE) refers to delaying the application of the extraction potential by a certain time of $100 - 1000$ ns after the laser pulse. Within the delay period, ions with a given *m/z* that have high E_{kin} travel further away from the sample target (repeller) than ions with lower E_{kin}. When the extraction voltage is applied, the slow ions, which are closer to the repeller, are accelerated more, leading to the simultaneous arrival of ions of a given *m/z* on the detector. The drawback of the DE is that the optimum delayed extraction time differs for various *m/z* and therefore this method works well only for the selected interval of *m/z*.

Another method for the correction of the ion initial kinetic energy distribution is the use of an **ion mirror (reflector, reflectron)**. The reflector uses an electrostatic field to reflect the ion beam toward the detector and it consists of a series of ring electrodes. The first electrode is held at a potential of the same polarity but slightly higher magnitude compared to the acceleration voltage, the voltage on the following electrodes drops gradually to zero on the last electrode. More energetic ions penetrate deeper into the reflector, travel a slightly longer path and spend a longer time in the reflector. Less energetic ions of the same *m/z* will penetrate a shorter distance into the reflector, thus spending a shorter time in the reflector. The detector is placed at the focal point, where ions of the same *m/z*, but with different energies strike the detector at the same time. The disadvantage of using the reflector is a certain decrease in the ion transmission and consequently sensitivity.

C. Selected applications of MALDI TOF MS

1. Molecular mass determination

Pseudo-molecular ions generated by MALDI in positive mode are usually single-charged as $[A+H]^+$. Other types of ions, such as double-, triple- or even multi-charged ions $[A+2H]^{2+}$, $[A+3H]^{3+}$ might also be observed. Analyte dimers, such as $[A_2+H]^+$ as well as various adducts of analyte/matrix/alkali metals $[A+Na]^+$, $[A+K]^+$, $[A+MH]^+$, $[A+MNa]^+$ may occur in the spectra. MALDI TOF MS is not usually applied for low molecular mass determination (below 500 Da) due to possible interferences of ions of matrix fragments, adducts and clusters.

MALDI TOF MS is mostly used for determination of molecular mass of peptides, proteins, nucleic acids and saccharides. It can also be powerful for determining molecular weight distribution and structures of synthetic polymers.

However, the molecular mass information itself is generally not sufficient for unambiguous identification of a compound.

2. Peptide Mass Fingerprinting (PMF)

One of the most significant applications of MALDI TOF MS is protein identification. Sequences of known proteins are stored in DNA and protein databases. As mere determination of protein mass is not sufficient for protein identification, other techniques based on analysis of peptides generated by specific cleavage of the analyzed protein are employed. Proteins can be identified based on more detailed information about a group of peptides (PMF) or using tandem mass spectrometry (MS/MS) of a single peptide.

Peptide Mass Fingerprinting (PMF) is a technique used to identify proteins by matching their constituent fragment masses (peptide masses) to the theoretical

peptide masses generated from a protein or DNA databases. The first step in PMF is that an intact, unknown protein is cleaved with a proteolytic enzyme to generate peptides. Trypsin is most commonly used (it cleaves peptide bonds X-K or X-R if X ≠ P). Masses of the peptides obtained from MALDI TOF mass analysis are compared with theoretical fingerprints generated from protein databases using programs, such as Protein Prospector, Mascot, Proteomics etc. The result of the database search is a list of proteins that might provide the measured peptides. The probability of protein identification is given by parameters, such as the ratio matched/entered peptides, so called expectation factor, or sequence coverage. Presumption of successful identification is high accuracy of *m/z* determination and low number of proteins in the original sample. Should more proteins be present in the sample, the protein of interest has to be isolated using a suitable separation technique prior to the enzymatic cleavage.

Gel electrophoresis (i.e. 1D or 2D SDS-PAGE) and liquid chromatography are used almost exclusively for protein separation, nowadays. Ideally, the output of separation is a single protein.

D. Solid Phase Extraction (SPE)– ZipTip™

ZipTip™ (Millipore, MA) is a 10 µl pipette tip with a 0.6 or 0.2 µl bed of chromatography media fixed at its end with no dead volume. It is ideal for concentrating and purifying samples for sensitive analyses by mass spectrometry, liquid chromatography, capillary electrophoresis and other analytical methods (Fig. 4). The chromatographic medium is placed in the very end of the tip assuring virtually zero dead volume.

Two types of resins are available in ZipTip™ pipette tips: C4 resin with 30 nm pores for larger peptides and proteins and C18 resin with 20 nm pores for peptides.
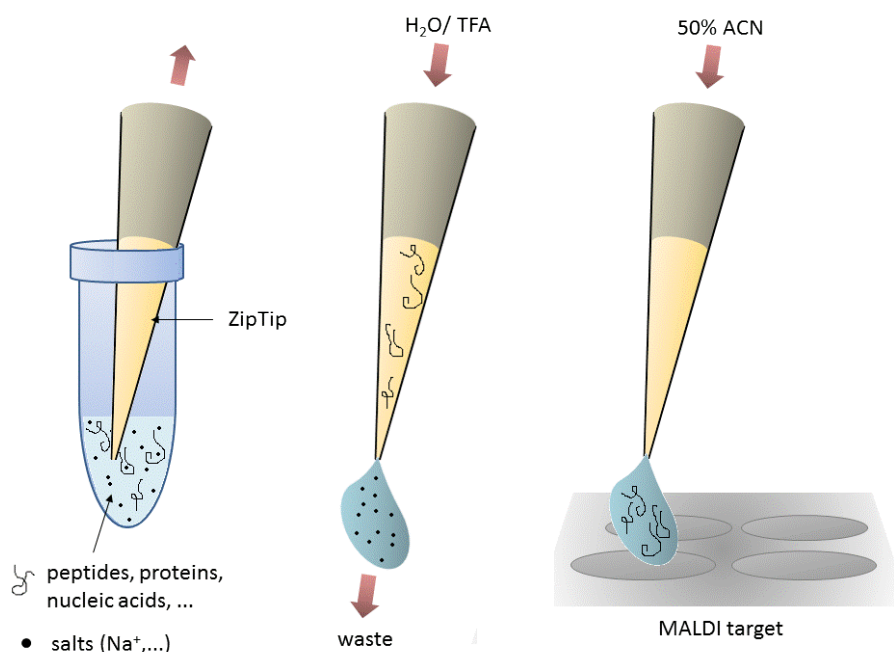

Figure 4: Principle of the ZipTip™ pipette tips. Application details are explained in the following chapter

Experimental part

Task: Identify two unknown proteins by MALDI TOF MS and peptide mass fingerprinting. Use SPE ZipTip™ pipette tips for purification of protein digests.

A. Solutions and materials

MALDI matrices

- **solution TA30** (30% acetonitrile, 0.1% TFA): mix 300 μl ACN, 600 μl H_2O and 100 μl 1% TFA
- **CHCA matrix solution**: dissolve ~ 2 mg of CHCA in 200 μl TA30, sonicate cca 1 min in an ultrasonic bath and centrifuge undissolved crystals
- **SA matrix solutions:**
  - **solution I:** dissolve cca 2 mg of SA in 200 μl 100% EtOH, sonicate cca 1 min in an ultrasonic bath and centrifuge undissolved crystals
  - **solution II:** dissolve cca 2 mg of SA in 200 μl TA30, sonicate cca 1 min in an ultrasonic bath and centrifuge undissolved crystals

ZipTip™ purification

- ZipTip™ C_{18} pipette tips
- **0.1% TFA**: mix 50 μl 1% TFA with 450 μl of H_2O
- **50% acetonitrile (ACN)**: mix 200 μl of ACN with 200 μl of H_2O

Proteins – unknown samples

- Protein solutions: 0.1 $mg.ml^{-1}$ in 50 $mmol.l^{-1}$ NH_4HCO_3
- Tryptic digests of unknown proteins (0.1 $mg.ml^{-1}$)

Peptide calibration mixture

- Mixture of peptides designed for mass spectrometer calibration. Composition of the mixture is given in the following table:

peptide	concentration (μM)	Relative molecular weight, M	m/z ([M+H]$^+$)	
			monoisotopic	dominant
Bradykinin	1	1059.5610	**1060.5692**	1060.5692
Angiotensin I	1	1295.6775	**1296.6853**	1296.6853
Renin	2	1758.9332	**1759.9396**	**1760.9474**
ACTH	5	2464.1911	2465.1989	**2466.2067**
Insulin	10	5733.6	5734.7 – average	

Protein calibration mixture

- Mixture of proteins designed for mass spectrometer calibration. Composition of the mixture is determined in the following table:

Protein	M	Ion	m/z (average)
Trypsinogen	23981	$[M+H]^+$	23982 Da
Protein A	44612	$[M+H]^+$	44613 Da
Protein A		$[M+2H]^{2+}$	22307 Da
Albumin-bovine (BSA)	66463	$[M+H]^+$	approx. 66.5 kDa
Albumin-bovine (BSA)		$[M+2H]^{2+}$	approx. 33.3 kDa

Protein digest desalinating with ZipTip™ (see Fig. 4)

1. Prepare $3n$ aliquots of 0.1% TFA (50 μl), where n is the number of the unknown samples. For each sample, 1 aliquote will be used for equilibration and 2 aliquotes will be used for washing.
2. Add 1 μl 1% TFA to the samples (10 μl in each).
3. Dispense 5 μl of 50% ACN into an empty microvial using a standard pipette tip and close it. This microvial will be used to elute the peptides in step 7.
4. **Equilibrate a ZipTip™ pipette tip:**
   a. Pre-wet the tip with 10 μl 50% ACN: aspirate the solution into the tip, dispense to waste. Repeat.
   b. Equilibrate the tip by aspirating and dispensing 10 μl of 0.1% TFA from the prepared aliquot. Repeat.
5. **Bind the peptides to ZipTip™**: Aspirate and dispense the sample (10 μl) carefully and slowly for 10 times, avoid introducing air.
6. **Wash the peptides:** Aspirate 10 μl of 0.1% TFA from a new aliquot and dispense to waste. Repeat with another aliquot.
7. **Elute the peptides:** Insert the tip into the microvial (from step 3) and dispense 5 μl of 50% ACN. Slowly aspirate and dispense the solution at least three times without introducing air bubbles.
8. The peptides are in 5 μl of 50% ACN.

B. Sample preparation for MALDI TOF MS

Deposit your samples on the MALDI target as described below. Choose two free columns and deposit each sample twice onto two neighbor wells (write down the exact well positions for all samples).

row	analyte	matrix
1	H_2O	SA (2)
2	H_2O	SA (1+2)
3	Protein calibration mixture	SA (1+2)
4	unknown protein 1	SA (1+2)
5	unknown protein 2	SA (1+2)
6	-	-
7	H_2O	CHCA
8	Protein digest 1	CHCA
9	Peptide calibration mixture	CHCA
10	Protein digest 2	CHCA
11	Protein digest 1 (desalted)	CHCA
12	Peptide calibration mixture	CHCA
13	Protein digest 2 (desalted)	CHCA

Sample preparation of proteins – „double layer" method

Deposit ~ 0.1 µl of the SA matrix solution I (in 100% EtOH) on each spot in the row 2-5. A thin layer of matrix crystals will be formed. Premix 2 µl of SA matrix solution (solution II) with 2 µl of water/protein calibration mixture/unknown protein in a cap of a small microvial. Apply 0.5 µl of this solution on the top of the thin layer of matrix in row 2-5. In the first row, apply 0.5 µl of the solution of the SA matrix with water directly on the MALDI target. Write down all sample well positions.

Sample preparation of peptides – „quick and dirty" method

For the samples in row 7-13, apply 0.5 µl of the sample solution and 0.5 µl of the CHCA matrix solution and mix carefully with the pipette tip directly on the MALDI target.

C. MALDI TOF MS of peptides and proteins

Mass spectra acquisition is performed using a MALDI TOF/TOF mass spectrometer AutoflexSpeed (Bruker). This instrument is equipped with a 1 kHz Nd:YAG laser (355 nm). Except for the insertion of the MALDI target into the instrument, the whole system is controlled by the software *FlexControl* (Fig. 5). Other programs, such as *FlexAnalysis* and *BioTools* are available for spectra processing and analysis.
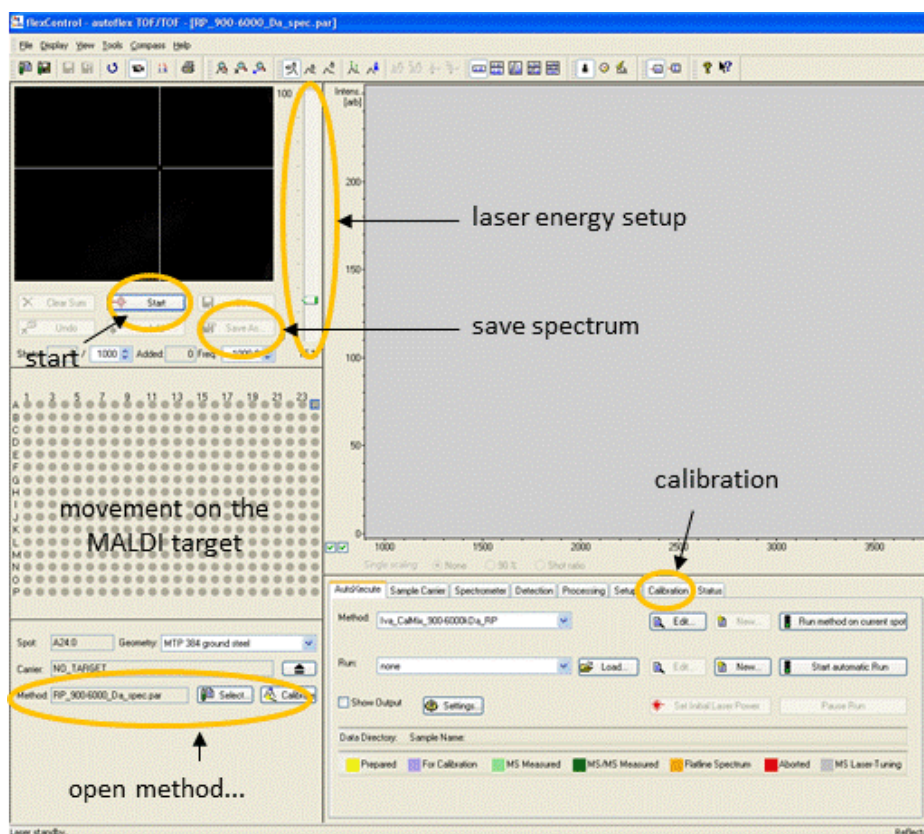

Figure 5: Screen of the FlexControl software

1. Determination of the molecular weight of unknown proteins

- Insert the MALDI target into the instrument and wait until the system is pumped down completely (p < 3x10^{-6} mbar).
- Load the method for measurement of proteins (Select Method -> file *vyuka* -> method *linear_proteins.par*). Parameters (extraction voltage, delay time etc.) of this method were set in advance for measurement of proteins with size of 10 – 100 kDa in the linear positive mode.
- Find the position with protein calibration mixture on the MALDI target.
- Start the spectra acquisition by clicking the *Start* button; the final spectrum is an average spectrum from 1000 accumulated mass spectra.

Optimal laser energy adjustment

Increase the laser energy gradually until the first analyte peaks appear (this energy is called threshold energy and it differs for various matrices, analytes and sample preparation), increase the energy by 10 – 30 % over the threshold energy; record this value and use it in further experiments.

Calibration:

In TOF mass spectrometry, only the x-axis is usually calibrated. The y-axis is often normalized, i.e. the most intense peak is assigned 100% intensity. Calibration is performed in a defined range of m/z, which a calibration mixture of selected compounds is prepared for. In the case of low m/z (<500) peaks of matrix, matrix clusters and fragments can be used; for m/z in the range of 500 - 5000 Da a mixture of peptides can be applied. Synthetic polymers or a mixture of selected proteins can be used for heavy molecules (<6000 Da).

After the spectra acquisition of the protein calibration mixture, open the bookmark *Calibration* and assign successively the *m/z* values to the relevant peaks. When all peaks are assigned, confirm the calibration with the *Apply* button. In the next measurement, the instrument will work with this calibration.

Save the spectrum of the protein calibration mixture (*Save as*).

Measurement of the mass spectra of the unknown proteins

Acquire the mass spectra of the unknown proteins in the same way as the spectra of the protein calibration mixture. Acquire also the spectra of the matrix. Save all spectra.

Questions:

1) *Compare the morphology of the sinapinic acid crystals using the „double layer" and the „dry droplet"(the spot on row 1) sample preparation. Try to explain what are advantages of the „double layer" sample preparation for analysis of proteins.*
2) *Explain what you observed in the mass spectra – number of peaks, their shape and resolution.*
3) *Calculate the approximate amount of substance (mol) and the number of molecules of the unknown proteins which were deposited on the MALDI target.*

1. Measurement of peptides/protein digests

Acquire spectra of the protein digests in the same way as in the case of proteins. Measure in the linear mode (method: *linear_peptides.par*) first and then in the reflector mode (*reflector_peptides.par*). In both cases, calibrate the instrument using the peptide calibration mixture before the measurement of the digests of the unknown proteins and blank samples (matrix only). Note the calibration error and save the spectra for further database search. While saving the spectra, choose the methods *PMF.FAMS* in the section *Processing* and check the box *Open in FlexAnalysis*.

Questions:

4) *Explain the peak isotope distribution. Characterize the monoisotopic peak.*
5) *Explain the presence of signals in the blank sample.*
6) *Explain the difference between the spectra acquired in linear and reflector mode (resolution, peak intensity). Summarize advantages and disadvantages of both modes.*

D. Protein Mass Fingerprinting (PMF)

Open the mass spectrum of the digest of an unknown protein in the *FlexAnalysis*. Send this spectrum (list of monoisotopic peaks) to *BioTools* by clicking on the traffic lights icon . This program will be used for data analysis. In *BioTools*, click on MS icon , which opens the dialog (Fig. 6) for the database search. Enter your name, email address, the database name (Swissprot), the digesting enzyme (trypsin), the maximal number of uncleaved sites (partials; 1), variable modification (oxidation (M)), mass tolerance (50 ppm) and start the search with the *Start* button.

The results of the search will appear in a new window; more details of the results can be displayed in the *BioTools* (button *Get Hits*).

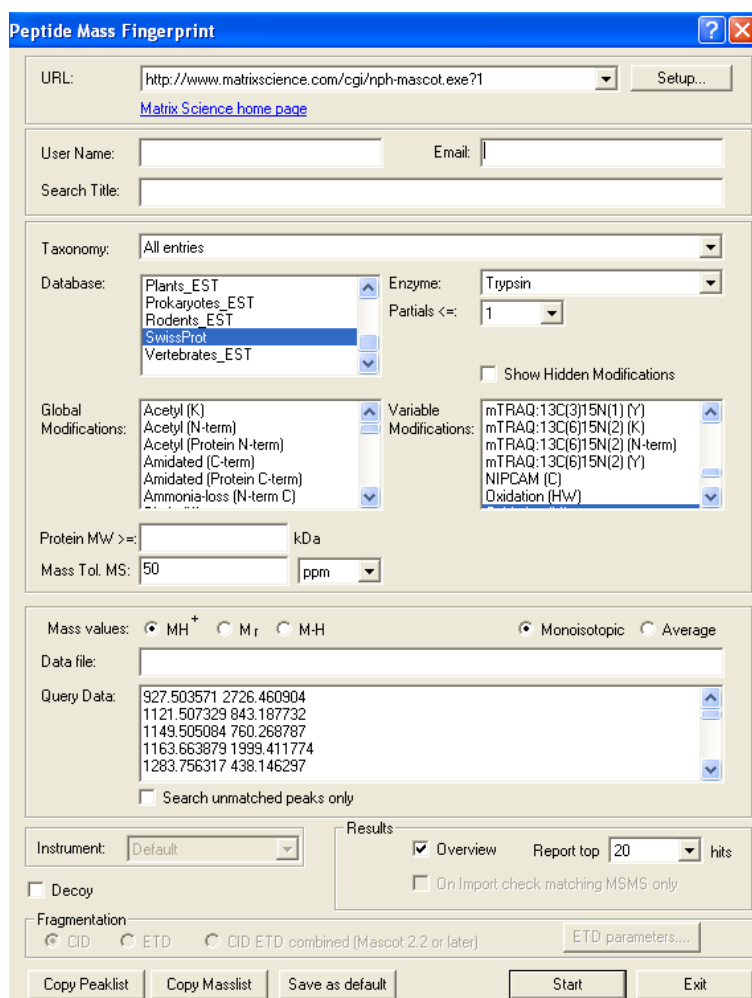Discuss the results with your supervisor.

Figure 6: Dialog for the PMF database search

E. Laboratory report

The report has to contain the date, all names of participants, and titles of the finished tasks. One report for the entire group is sufficient, preferably in electronic format. Please, enclose major experimental results, such as spectra and tables, answers to the questions and a brief discussion of the results. Save the results to the computer and turn them in to your supervisor.