Master Thesis

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MSc Chemistry
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Master Thesis

Evaluation of new matrices for the detection of intact proteins by MALDI-time-of-flight mass spectrometry

by

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Chapter I

1. Introduction

In this project a series of MALDI matrices for the detection of intact proteins by MALDI-time-of-flight mass spectrometry has been evaluated. Moreover, a series of new matrices, named ionic liquids, which have not been used for the evaluation of protein analysis by MALDI have been compared with conventional matrices. Additionally, the initial ion velocity which is based on the desorption processes of the matrices and it is a unique intrinsic property for each one of them, has been investigated. The initial ion velocity can be measured using delayed ion extraction within the ion source of the mass spectrometer. Furthermore, the visualization of the ions impact on the detector surface has been employed using Timepix detector. This serves as a way to investigate the effect of the initial ion velocity on the spatial focusing of the ion cloud.

1.1 Mass Spectrometry

Mass spectrometry is a technique which is used for the detection, identification and quantification of molecules according to their mass to charge ratio (m/z) [1]. Historically, the origin of the technique is approximately 100 years ago and it was mainly used for the measurement of the atomic weights of elements and also for the natural abundance of particular isotopes [2]. The first application of mass spectrometry in the field of biological sciences was for tracing heavy isotopes via biological systems. Later the technique enabled the sequencing of oligonucleotides and peptides and also the analysis of the structure of nucleotides [3].

Nowadays, applications of mass spectrometry are used for a wide variety of disciplines and settings in a number of different fields of research. Analytically, it can be used for academic research reasons, such as for the determination of protein structure, function, folding and interactions, identification of a protein from the mass of its peptide fragments, detection of post-translational modifications, quantification of proteins, monitoring enzyme reactions or chemical modifications and protein digestion, and sequencing of oligonucleotides. More specifically, its applications are used for clinical and pharmaceutical testing, such as for the determination of structures for drugs or metabolites, screening for metabolites, forensic analysis, or detection of disease biomarkers. Furthermore, mass spectrometry can be used for environmental analysis and geological testing, such as for testing water quality or food contamination, measurements for petroleum composition, carbon dating performance [4].

A mass spectrometer device consists of an ion source, a mass analyzer and an ion detector therefore sometimes there are variations mainly based on the type of mass
spectrometer, the type of data required and the physical properties of the sample. The procedure involves as a first step the loading of the sample into the mass spectrometer in liquid or dried state and then the vaporization and ionization by the ion source like for example ESI or MALDI. Ionization of the analyte molecule is performed by reception or loss of an electron. The charge received by the molecules enables the mass spectrometer for the acceleration of the ions throughout the system. Electrical and magnetic fields are implemented from mass analyzers with object to avert the routes of individual ions which face them according to their mass to charge ratio (m/z). Mass analyzers that are used widely in various applications are time-of-flight [TOF], quadrupoles, ion traps and ion cyclotrons. Each one of them is combined with the appropriate ionization process and has particular characteristics. They can be used either for the separation of all analytes in a sample for general analyses, or for the specific deflection of desired ions in the direction of the detector [4].

These preferred ions hit the ion detector, which can be either a secondary electron multiplier (SEM) or a micro channel plate (MCP), and gives out numerous electrons after every ion hit to the detector plate. The large number of electrons emitted amplifies each ion hit and subsequently the sensitivity is improved [3].

Extreme vacuum conditions (10^-6 to 10^-8 torr) are used for the removal of non-sample ions which are responsible for any possible contamination and more specifically for the collision with sample ions and the change of their routes or the production of non-specific reaction products [5], [6].

Another significant tool for scientists and researchers is the connection between the devices and computers with software, a fact that provides extremely significant results concerning the analysis of the ion detector data and the production of graphs organized according to their individual m/z ratio and relative abundance. The process of these ions with the help of databases for the prediction of the exact form of the molecule based on the m/z ratio is also possible [4].

1.2 MALDI

Mass spectrometric analysis has been employed since the 1970’s, but the laser desorption/ionization mass spectrometry (LDI-MS) was of less practical use for the analysis of bio molecules since only ions of a molecular weight below 1000 Da could have been detected.

In 1988, Hillenkamp and Karas developed an analytical method named Matrix-assisted laser desorption/ionization mass spectrometry (MALDIMS) [7]. This method is highly useful analytical technique which has been used in a wide range of analytical applications for peptides, proteins and the vast majority of biomolecules, such as oligonucleotides, carbohydrates, natural products, and lipids, as a tool which can provide extremely significant results. This technique is relied on the direct and
efficient energy transfer that happens during the matrix-assisted laser-induced desorption event and it leads to high abundance of intact analyte ions. Subsequently, the measurement of compounds consists of high accuracy and subpicomole sensitivity.

The first step of the MALDI technique involves the co-crystallization of the analyte with a large molar excess of a matrix compound, most of the times an organic acid with weak ultraviolet (UV)-absorbance. Later, this analyte-matrix mixture is exposed to an intensive, short waved, laser pulse that leads a part of the solid-state surface to vaporize. The role of the matrix in this step is crucial as it is responsible not only for the strong absorption of the laser light, but also for the indirect vaporization of the analyte. Apart from this, it has a role either of proton donor or receptor, which is responsible for the ionization of the analyte in both positive and negative modes [7].

After the co-crystallization step, irradiation of a nanosecond laser beam, such as ultraviolet laser (UV) with a range of wavelength between 266 and 337 nm, follows. Laser energies of $1 \times 10^7 - 5 \times 10^7$ W/cm$^2$, are responsible not only for the decomposition in the structure of the irradiated crystal but also for the generation of the “plume”, a particle cloud where the extraction of the ions takes place using an electric field. The desorption mechanism has not been examined totally in depth. One relatively easy explanation of this is that the laser energy of the crystal molecules is converted to vibrational oscillation and as a result, that leads to the breakdown of the crystal [1].

After being accelerated because of the electric field, the ions pass a field-free drift region in order to reach the detector. TOF measurements, in the range of several microseconds, are used for the typical calculations of ion masses (mass-to-charge ratios [m/z]). According to the data, larger molecules show longer time of flight compared to smaller ones, as long as their initial energies are identical. The determination of parent ion masses from the spectrums which are created is relatively easy, without the processing of complex data to be necessary, because mainly the generation of single-charged, non fragmented ions, occurs. Scientists have full access to the numerical data of different masses, hence they are able to process them directly and also analyze them [1].

Matrix-assisted laser-induced desorption events offer high ion abundance of the intact analyte due to the direct and efficient energy transfer. As a consequence, this type of events can be used for highly accurate measurements and also with a high degree of sensitivity (class of subpicomole). This significant accuracy in measurements can provide extremely important information for scientists in order to identify and characterize proteins. More specifically, the identification of a protein in some cases can be clearly conducted by analysis of the mass of its constituent peptides accurately. These peptides can be the result of treating the sample either in a chemical or an enzymatic way [7].
According to reports and reviews concerning MALDI MS, in most of the studies there are combinations with TOF mass analyzers. One drawback of these combinations is their low resolution which affects the accuracy of the method (0.2% to 0.005% using internal calibrant). Moreover, the sample preparation step is extremely important for MALDI MS applications. Another important parameter in order to obtain successful MALDI data is the selection of the matrices and solvents that are going to be used in a study. Furthermore, properties concerning the structure and the function of the analyte, the sample purity and the preparation of the MALDI plate also play a crucial role to the success of the data [7].

MALDI applications in the field of protein and peptide analysis provide extremely useful results and especially really high accuracy as far as the molecular weight of the compounds is concerned. This is the main characteristic which enables scientists and researchers during their studies to identify and characterize proteins. One characteristic example is the identification of a protein by analyzing accurately the mass of its peptides. All these peptides are produced either in a chemical or an enzymatic way. Furthermore, in order to identify proteins, scientists can analyze the protein’s proteolytic peptide fragments in the gas phase. Inside MALDI mass spectrometers, collision-induced dissociation (CID) leads to the generation of fragment ions that can provide extremely significant information concerning the primary structure of the protein and any modifications that may happen. Tandem mass spectrometry (nth series) (MSn) experiments can also be conducted by MALDI sources and they are known as post source decay or PSD [7].

1.3 TOF and Delayed Extraction

In the vast majority of applications a MALDI ionization source is combined with a linear time-of-flight (TOF), which is the simplest one, a TOF reflectron, and a Fourier transform mass analyzer. TOF analysis’s main principle concerns the acceleration of a set of ions with the same amount of energy to the detector. As the ions have the same energy but different mass, they approach the detector at different times. Large ions with high m/z values strike the detector later than small ions, which develop greater velocity. The analyzer, hence, is called TOF as the ions’ time of flight determines the mass. The TOF reflectron is a combination of TOF principle with the reflectron, an electrostatic analyzer. The reflectron is responsible for the rise in the amount of time that is necessary for ions to strike the detector by decreasing their kinetic energy distribution and therefore the temporal distribution [7].

In most of the MALDI TOF applications for the analysis of ions, a relatively poor mass resolution is achieved, leading to a new method being implemented, namely delayed extraction (DE). Delayed ion extraction (DE) or else time-lag focusing or space-velocity correlation, is responsible for the improvement of mass resolution on linear and reflector TOF instruments [8]. The main principle of DE (delayed
extraction) method is based on “time lag energy focusing” as it was described in the study of Wiley and McLaren in 1955.

The main difference of DE method compared to traditional MALDI instruments is that after the MALDI ionization process, ions “cool down”, for 150 ns before being accelerated to the analyzer, therefore, focusing on them is also possible. Normally the acceleration step is taking place after the formation of the ions, out of the ionization source. This progress as far as mass resolution is concerned has also significant effect on the mass accuracy of the technique, which is also a crucial parameter in order to evaluate a mass spectrometric technique [8].

More specifically, the generation of ions during delayed extraction is happening when the extraction filed is switched off to zero or disappeared electric field, and after a pre-decided under the best possible conditions delay time extraction is achieved. During this delay time, diffusion of the ions due to their initial velocities is taking place, therefore they are spreading out in space and the acceleration by the delayed extraction is the main reason for obtaining different total kinetic energies [8].

The application of delayed extraction method has a significant effect on the compensation of the initial ion velocity distribution in a two-stage acceleration ion source, with an adequate set-up of the first and second acceleration potential gaps. The measurements of this method have shown better resolution results in comparison with the static ion extraction method. According to studies dealing with a wide range of samples like peptides, proteins, synthetic polymers and oligonucleotides, the application of delayed ion extraction (DE) has shown significant progress in applications of MALDI-TOF mass spectrometry [8].

1.4 Initial Ion Velocity

The matrix-assisted laser desorption ionization (MALDI) technique enabled the development of an innovative scientific method for measuring the initial velocity of ions. The initial velocity of the MALDI ions has been considered as a powerful tool to characterize the desorption mechanism of the MALDI process [9].

According to many reports and studies both in a theoretical and an experimental degree, the application of a delayed extraction method leads to a linear correlation between the flight time and the extraction delay. The examination of the slope of this linear curve assists to the determination of the initial velocity of the ion, which can be defined as a consequence of the desorption process. A significant number of reports and studies are interested in initial velocity measurements and they examine if this intrinsic property depends on different parameters such as the matrix substance, the molecular weight of the analyte, the ion polarity and also the wavelength of irradiation [10].
According to the study of Whittal and Li [11], the mass accuracy of peptides and small proteins with external and internal calibration is improved. Despite that, the application of internal calibration, which is supposed to be the most accurate method to mixtures of DNA, provided significant results as far as the linear curve between t versus (m/z) 1/2 is concerned. It was also assumed that the systematic deviations which were observed, are connected to the initial velocity of the sample ions obtained during the desorption process.

The effects of the initial velocity are not obvious in conventional MALDI-TOF applications, because of energy loss based on collisions developed in the first stages of the ion extraction, during the acceleration of the ions into the dense plume of a desorbed material. Delayed extraction applications are responsible for this decrease in the energy loss due to the delay of ion extraction until the dispersion of the plume. The examination of the initial velocity of the process is allowed because of the experimental decoupling of desorption and the ion extraction steps [10].

According to a large number of studies and reports, a variation in the values of average initial velocity, between 300 and 1000 m/s for sample ions and between 300 and 1700 m/s for matrix ions, has been recorded. Research on initial velocity (or else ejection or desorption velocity) is needed not only in order to describe totally the desorption process, but also to improve the accuracy of the calibration by taking into consideration the effects of this intrinsic property. Furthermore, attention must be paid on the experimental conditions which take place when initial velocity is being investigated [10].

1.5 Timepix

In a theoretical point of view, the combination of MALDI-TOF MS applications is based on the unlimited mass range of this type of analyzers. Nonetheless, the efficient detection of singly charged ions with high m/z ratios is limited during these methods. In a number of studies concerning ion detection in TOF mass spectrometry, it is reported that the latter can be achieved by using the micro channel plates’ ability (MCPs) to convert an ion to electron. The main problem is that this type of conversion of MCPs presents a decline when the ion momentum decreases, like for example at higher mass. As a result, the generation of high mass ions is possible, although their detection might not be efficient and the inspected ions may not be abundant. Moreover, there is also the possibility that these quantities of ions might not be detected at all [12].

Scientists and researchers have developed alternative detection formats, mainly not based on MCPs, in order to find a solution especially for m/z values up to 1 MDa. One alternative detection system that is going to be used during this study is the Timepix active pixel detector which is applied to a commercial MALDI linear TOF instrument and it can provide ion acceleration voltages up to 25 kV. It can be used in
order to detect ions up 400 kDa with high m/z ratios based on the high acceleration voltages which are combined with a pixelated detector with high sensitivity. One advantage of this detection system compared to the conventional ones is the improvement in signal-to-noise ratios [13]. The detector consists of a 512x512 detector array and thus provides 262,144 parallel detectors where each pixel is a single-stop time-to-digital converter (TDC). During Timepix operations in time of flight mode the arrival time measurements are conducted by taking into consideration an external trigger [14].

Another significant advantage of this detection system is the acquisition not only of the arrival time, but also of the position of incoming ions at the same time. The direct visualization of the spatial distribution of incoming ions with a particular m/z ratio offers insights into the basic procedures that take place as the ions are formed and extracted through the ion source region. Moreover, scientists and researchers are able to extract rapidly under optimal conditions and to focus on parameters in order to augment ion signals for the desired mass range, by using this visualization of the ion density that strikes the detector at real time [15].

A Timepix detection system applied to a commercial linear time-of-flight mass spectrometer is used in order to detect efficiently high-mass ions. This combination of systems permits the high (25 kV) ion acceleration voltages to be used, hence, useful results are provided in comparison with conventional ADC and TDC detection technologies. This detection system is not extremely popular when used for slower ions (higher m/z ratios) due to the decreased ion-to-electron conversion. However, when an appropriate ionization technique is implemented, with the permission of more efficient MCP electron quantities, Timepix detection offers advanced analysis for large protein complexes as the accessible mass range of MCPs is extended [15].

1.6 Matrices

The most common used matrices in MALDI applications for peptides and proteins analysis are a-cyano-4-hydroxycinnamic acid (CHCA) mainly used for peptides, glycopeptides and small proteins, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid or SA) used for both peptide and protein analysis, and 2,5-dihydroxybenzoic acid (DHB) used for used for glycopeptides, glycoproteins, small proteins, and oligonucleotides (<10 bases).

During peptide mapping namely the analysis of protein digests, the selection of the appropriate matrix plays a crucial role especially in the examination of the production of peptides. In some reports of protein sequences, scientists are able to use matrices that complement each other for even better results. One characteristic example is the peptide mapping process either with CHCA, which is a really good choice for the yield of low-mass peptide ions (<2500 Da), or with SA that can provide better results for the higher-mass peptides (>2500 Da).
As far as the sample-matrix preparation method is concerned, there is a wide range of procedures, but in most of the cases the dried-droplet method is used. Analytically, the matrix solution after being saturated is mixed with the analyte solution in a ratio of matrix-to-sample approximately 5000: 1. The next step contains the application of a portion of the total amount of the mixture usually (0.5–2.0 mL), to the sample target, followed by the drying step.

The conventional matrices which have been used during this study are the following:

- a-cyano-4-hydroxycinnamic acid (CHCA)
- 2,5 Dihydroxy benzoic acid (2,5-DHB)
- sinapinic acid (SA)
- ferulic acid (FA)
- 2,6-dihydroxyacetophenone (DHAP)
- 9-amino acridine (9-A.A)
- 1,5-diamino naphthalene (1,5-DAN)
- 3-nitrobenzonitrile (3-NBN)
- 2′,4′,6′-triiodoacetophenone (THAP)
- trans-3-indoleacrylic acid (IAA)
- trans-3-indoleacrylic acid (IAA) with silver trifluoroacetate (AgTFA)
- picolinic acid (PA)

Moreover, a series of new matrices, named ionic liquids, which have not been used for the evaluation of protein analysis by MALDI are going to be used. These are called Ionic liquid matrices (ILMs) and they are organic salts formed by equimolar mixtures of crystalline MALDI matrices with organic bases. In this study four of them have been used, as follows:

- CHCA Butylamine salt (CHCA BA)
- CHCA Diethylammonium salt (CHCA DEA)
- CHCA N-tert-butyl-N-isopropyl-N-methylammonium salt (CHCA IL)
- Ferrulic acid N-ethyl-N,N-diisopropylammonium salt (FA IL)

Ionic liquid matrices are easily prepared and require no co-crystallization with the analyte, which prevents “hot spots” and thus provides better shot-to-shot and spot-to-spot reproducibility [16]. Their main properties are their higher signal intensity, the creation of more homogeneous sample spots during sample preparation, and also the
more reproducible signals without great losses of hydrolyzable samples. In many occasions, ionic liquid matrices exceed the crystalline matrices as far as homogeneity of crystallization, tolerance to salts, vacuum stability and resistance to laser irradiation, are concerned. In addition these matrices present better mass resolution and sensitivity [17].

**Influence of additives on matrices**

Improvements on the performance of different matrices can be held using additives. In general, they are used in order samples to become more homogeneous, to minimize spot-to-spot fluctuations, to reduce cationization and also in order to be cleaned-up of salt contamination [18].

Additives significantly influence the initial ion velocity measurements. To be more specific, according to the nature of the additive they can either increase or decrease the initial ion velocity measured in comparison with the pure matrix [18].

**1.7 Microscopy**

Microscopy has become an extremely significant research tool during the last decades, with a major role in analysis and studies of a wide variety of samples. Developments in the field of microscope optics have enabled researchers to examine in depth the morphology and the function of cells, tissues etc [19].

A conventional light microscope consists of a condenser lens, an objective lens, and oculars. The role of the condenser lens is to focus the light of a tungsten filament bulb illumination onto the specimen. After passing through it, there is an objective lens that works out the fine detail that is present within the specimen. The magnified image is projected to a fixed position behind the lens and specific eyepieces, named oculars are responsible for their enlargement. Finally, focus of the light, which is emerged from the oculars to a particular point where the eye can see the magnified image, is taking place [19].

The main difference of fluorescent light microscopy compared to light microscopy is the replacement of the tungsten light source by a high pressure mercury vapor or xenon bulb. As a consequence, a very intense beam is emitted. After the passing of this high energy light through the optical excitation filters, only one particular wavelength range of light is allowed to reach the specimen. The fluorochromes in the tissue on the microscope stage are excited by this light and as a response they emit light energy of higher wavelength. An optical filter is used in order to permit only the pass of a wavelength range of light specific for the fluorochrome that is used. Nonetheless, according to the demands of specific studies, there are different types of microscopes which can be used [19].
Chapter II

2. Experimental methods

2.1 Chemicals and reagents

The following solvents, matrices, additives and trypsinogen (the precursor form or zymogen of the pancreatic enzyme trypsin) were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). The protein trypsinogen 1mg.mL\(^{-1}\) was purchased from Bruker GmbH (Bremen, Germany).

Sinapinic acid (SA), 2,5 Dihydroxy benzoic acid (2,5-DHB), ferulic acid (FA), 2,6-dihydroxyacetophenone (DHAP), 3-nitrobenzonitrile (3-NBN), picolinic acid (PA) were prepared as 20 mg.mL\(^{-1}\) solutions in 1:1 acetonitrile:water (v/v) + 0.1% trifluoroacetic acid (TFA).

a-cyano-4-hydroxycinnamic acid (CHCA), 9-amino acridine (9-A,A), 1,5-diamino naphthalene (1,5-DAN), 2',4',6'-tri hydroxyacetophenone (THAP), trans-3-indoleacrylic acid (IAA) and also the two ionic liquids CHCA N-tert-butyl-N-isopropyl-N-methylammonium salt, Ferrulic acid N-ethyl-N,N-diisopropylammonium salt were prepared in the same way as a 10mg.mL\(^{-1}\).

Moreover, trans-3-indoleacrylic acid (IAA) with silver trifluoroacetate (AgTFA) was prepared as a 7 mg.mL\(^{-1}\) solution. More specifically, silver TFA was prepared as a 5 mg.mL\(^{-1}\) aqueous solution and then applied to 1:1 acetonitrile:water (v/v). After this, trans-3-indoleacrylic acid (IAA) was added for the final solution.

As far as the additives are concerned, sodium chloride and potassium chloride were prepared as 10mM solutions, while D-fructose was prepared both in 10mM and 100mM concentrations.

2.1.1 Sample preparation for MALDI-TOF MS analysis

For the standard dried droplet preparation, samples were prepared by mixing 2μl of trypsinogen solution with 2μl of the matrix solution and, when mentioned, by additionally applying 2 μl of additive solution 1:1 (v/v). 1 μl aliquots of analysis solutions were then deposited onto a stainless-steel target plate holding up to 384 samples (16 x 24 spots). Samples were dried subsequently by cold air before the MALDI analysis.

2.2 Instrumentation - Ion detection system

All mass spectrometry experiments were performed on an Ultraflex III MALDI TOF-MS (Bruker Daltonik GmbH, Bremen, Germany) equipped with a Smartbeam system and a 355 nm Nd:YAG laser. Experiments were performed with a 25 kV acceleration
voltage and a laser frequency of 10 Hz. Control over extraction and Einzel lens voltages, in addition to the pulsed-ion extraction (PIE) delay time, was achieved via the standard FlexControl 3.0 software (Bruker Daltonik GmbH, Bremen, Germany).

![Image 1](image1.png)

**Image 1:** Photograph of an Ultraflex III MALDI TOF-MS instrument

Instead of the conventional linear detector, the Timepix-based detection system (Omics2Image BV, Amsterdam, The Netherlands) is used. The detector introduces additional length of 20 cm to the ion flight path. Each one of the Timepix chips has dimensions of 1.4x1.6 cm² and contains an array of 256x256 pixels (55 μm pitch), as well as it behaves as an independent single stop time-to-digital converter (TDC).

Because I want the trypsinogen ions to hit first the Timepix detector and they give a peak at approximately 15000 seconds, channel A in the delay generator is adjusted to 142 μs. The maximum number of clock ticks for each pixel is 11810. Subsequently, the maximum width is applied to channel B of the delay generator at the value of 1181 μs.

A pulse and delay generator (DG535, Stanford Research Systems, CA, USA) is used to generate the trigger signals for the determination of not only the start time of the Timepix acquisition windows based on the laser pulse, but also the length of the acquisition window.

The Timepix chip array was mounted 2mm behind a chevron MCP stack (active area of 4cm, 12 μm pores and 15 μm pitch). The front MCP was held at -1.8kV and the back held at -400V (1.4 kV bias). In general, each pixel records the arrival time of an event according to the laser pulse which is determined as t=0 (time-of-arrival mode).
The “Pixelman” software package was used for chip control and data acquisition. MATLAB (Matlab; Mathworks, Natick, MA, USA; ver7.13.0.564, R2010b) software and Microsoft Excel (ver.2010) software were used for data analysis and time-to-mass calibration. For the collection of images and spectra, combinations of 500-1000 laser shots were utilized.

Figure 1: Schematic of the ion optics and detection system for the linear-TOF instrument used. The coordinate system is defined with the z-axis along the flight axis and the detector plane lying in the x-y plane [20].

As it is depicted in figure 1, the Einzel lens (P4) is set apart for special use where the holes in P2 and P3 create electrostatic aperture lenses. As a result, an electrostatic ion optical lens system is formed.

Chapter III

3. Results

3.1 Crystal properties

During MALDI techniques, both sample and matrix preparation methods play significant role in order for an optimal analysis to be accomplished. Characteristic properties such as selectivity, mass resolution and sensitivity are strongly related to these preparation protocols [21]. It is also extremely important the documentation and measurement of the crystal size distribution on the different samples under well documented conditions. Investigation of the crystal size distribution in matrices is of high importance in order to totally understand their role during the whole process. For instance, better signal reproducibility and improved quantitation can be achieved by uniform analyte distribution.

The first part of this study includes the inspection of the crystal morphology of different matrices and additives. Therefore, I am going to present photographs of
preparations and also microscope images of the crystalline and ionic liquid matrices that I used in my experiments. Additionally, photographs and microscope images of the additives, which I utilized for the investigation of the effect of those to the initial ion velocity measurements that I conducted, will be included.

3.1.1 Photographs of preparations of crystalline matrices and ILMs

As described in the experimental chapter of this report, samples were prepared using the standard dried droplet method. 1 μl aliquots of analysis solutions were deposited onto a stainless-steel target plate for the photographs of preparations. The latter were obtained using Ultraflex III MALDI TOF-MS and they are shown in figure 2.
Figure 2: Photographs of preparations of crystalline matrices, ionic liquid matrices and additives. The diameter of all spots shown is about 2mm.

Photograph of the solid CHCA matrix shows cracks, crystals, and numerous incongruities, and photographs of the ionic liquid matrices show a transparent film or droplet. Sometimes imperfections of the steel plate can be seen through the latter.
3.1.2 Microscope images of crystalline matrices, ILMs and additives

Initially, in order to check the homogeneity of the different crystals produced from the different matrices, I used the CETI optical light microscope, shown in the image below.

![Image 2: Photograph of the CETI optical light microscope](image)

After this, for the documentation and measurement of crystal size distribution, I used the optical microscope Leica DMRX. This is an upright microscope equipped with a wide range of objective lenses and hence, it is extremely helpful for various studies concerning bright field, dark field and fluorescence.

It contains two filter blocks available for fluorescence and a digital colour camera which is attached to the system. I used objective lens x10 dry, one filter block and a scale of 200 μm for the images that I obtained. The microscope is equipped with a Nikon DXM1200 digital camera. Therefore, the software that I used in order to save the pictures was Nikon ACT-1 Software. Its main advantage is the high resolution interference contrast that can be achieved.
After adding 1μl aliquots of sample solutions in glass microscope slides, the latter were placed in the specimen holder of the microscope. Following, I obtained the microscope images, shown in figure 3, with a scale bar of 200μm.

Image 3: Photograph of the optical microscope Leica DMRX
In these images characteristic crystals produced from different matrices are observed. To be more specific, the 2,5-DHB single crystals are parallelepipeds with several pm in thickness. The main characteristic of DHB matrix solutions during sample preparation is the production of a coarse-crystalline outer rim and so different signal intensities in different spot areas are noticed. The spot areas with really high signal are called “sweet-spots” (figure 2) [22].

Furthermore, ferulic acid forms long thin crystals that rise up off the microscope slide. Picolinic acid forms really beautiful dendritic, needle like crystals. Sinapinic acid gives a characteristic region with small crystals in the center of the spot, while 9A.A shows super molecular architectures. Finally, microscope images of ionic liquid matrices form transparent film or droplet.
3.1.3 Crystal size measurements

In this section I measured the longest dimension of the crystals of five different matrices, namely CHCA IL, DHB, PA, SA and THAP as shown in figure 3. This selection of matrices was based on the fact that they presented more clear crystal size distribution.

<table>
<thead>
<tr>
<th>Matrices</th>
<th>Number of Crystals Measured</th>
<th>Average of Longest Dimension (μm)</th>
<th>Average of Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHB</td>
<td>10</td>
<td>414.68</td>
<td>159.19</td>
</tr>
<tr>
<td>PA</td>
<td>76</td>
<td>54.37</td>
<td>23.83</td>
</tr>
<tr>
<td>THAP</td>
<td>57</td>
<td>53.21</td>
<td>20.64</td>
</tr>
<tr>
<td>SA</td>
<td>124</td>
<td>50.72</td>
<td>19.96</td>
</tr>
<tr>
<td>CHCA IL</td>
<td>55</td>
<td>42.07</td>
<td>9.67</td>
</tr>
</tbody>
</table>

Table 1: Average of measurements of the longest dimension and the standard deviation for five different matrices.

Figure 4: Correlation of the matrices with the average of the longest dimension measurements. The errorbars show the standard deviation.
According to the average of measurements of the longest dimension and the standard deviation using Image J programme (Image processing and analysis in Java), crystals of the DHB matrix are the largest ones, PA crystals are second in the order of size, THAP and SA crystals follow without big difference between them and the smaller ones are the crystals of the CHCA IL matrix, as shown in table 1. The errorbars show the standard deviation.

3.2 Initial Ion Velocity

3.2.1 Measurements of initial ion velocity using the delayed extraction method

The initial ion velocity $v_0$ was determined by measuring the dependence of flight times on the applied extraction delay $t$, which is linear to a good approximation.

Measurements were performed in eleven steps separated by a time of 100 ns. Each data point and its error bar represent the average of five measurements for different spots on one sample. The vast majority of them include up to 500 laser shots. For the DHB and DHAP matrices which evaporate really fast, I used up to 1000 laser shots. The standard deviations ($\sigma$) associated with the average values of the initial velocity result from these different experiments. In order to draw the error bars I used the LINEST excel function. Only the slope of the linear dependence time of flight versus the extraction delay was used to determine the initial ion velocity, as depicted in the following linear curves.
Figure 5: Determination of initial ion velocities of trypsinogen ions produced from the fifteen different matrices. The laser powers used for each matrix solution are also included. The slope of the linear fit function is proportional to the initial ion velocity. Error bars represent a standard deviation of five replicate measurements at each PIE delay.

The main reason for the relatively poor linearity and large errors for the spectrums of FA IL, PA, DAN, CHCA IL and CHCADEA, is the very broad peaks observed for these spectrums during this study. This is mainly based on their large variety of different spots during the preparation procedure, while standard matrices such as SA and CHCA are characterized as reproducible enough.

As it is depicted in the figure, the calibration curves of the ionic liquid matrices show good linearity and reproducibility. Their correlation coefficients ($R^2$) are 0.99, apart from FA IL which is 0.98. More specifically, for the FA IL matrix at 800, 900, 1000ns of extraction delay its peak width was really broad. Therefore, it was difficult to find exactly the peak in the spectrum and this is the reason why there are less data points in the linear curve.

Concerning the errors which are present in the initial ion velocity measurements, the main reason is the inhomogeneities in the sample preparation procedure of each matrix. Inhomogeneous surfaces which are created during mixing the matrix with the trypsinogen protein might change the position which the ions begin from. In addition,
the exact position and the flatness of the sample holder may have an effect in changes occurring during initial ion velocity measurements.

Generally, variations in the determination of the initial ion velocity of different matrices with the help of the slope are less significant in the case of matrices having a more homogeneous surface such as the CHCA than in the case of the ones which have an inhomogeneous surface [10]. For instance, matrices like DHB with rough sample surfaces have a strong effect on the starting position of ions with increased desorption velocities. One possible reason for these variations is the penetration of the accelerating field (field penetration) into the field-free zone, which results in overestimations of the initial velocity.

3.2.1.1 Matrix dependence on the initial ion velocity measurements

In order to examine if the initial ion velocity measurements depend on the matrix used, I concluded in the following bar graphs.

Figure 6: Comparison of the slopes acquired from the different matrices. The ionic liquid matrices are depicted in yellow color, the standard crystalline matrices in green and the rest of the matrices in blue.
According to the initial ion velocity measurements, the matrix with the biggest slope is the fastest one. Figure 6 provides evidence that three of the four ionic liquid matrices have the highest initial ion velocity.

If we compare the slopes acquired from the five standard crystalline matrices (figure 7), DHB has the biggest one, CHCA the lowest one and FA with SA are really close to each other. I used 1000 laser shots for the measurements of DHB and DHAP because these two matrices have the tendency to sublime in hours, contrary to stable crystalline matrices such as CHCA with lower velocities, where 500 laser shots were used.

Results for different matrices indicate that low values of the initial ion velocity are found in the case of microcrystalline homogeneous matrix-analyte preparations, e.g. CHCA. Thus, low values of the initial ion velocity correlate with surface or near-surface deposition of the analyte.

### 3.2.2 Effect of additives on the initial ion velocity measurements

In my study, I used carbohydrates such as D-fructose (in two different concentrations, 10mM and 100mM), potassium chloride and sodium chloride and by adding them to a solution of trypsinogen protein with a matrix of SA, I tried to investigate the effect of these three additives on the initial velocity measurements.
Figure 8: Determination of initial ion velocities of trypsinogen ions produced from the fifteen different matrices. The laser powers used for each additive in the matrix solution are also included. The error bars represent a standard deviation of five replicate measurements at each PIE delay.
Figure 9: Comparison of the slopes acquired from SA with several additives.

The effect of additives on the slopes which determine the initial ion velocity is not uniform. Addition of KCl resulted in a high increase of the slope. Significant rise has been also noticed with the addition of 100mM Fructose. On the other hand, after adding 10mM Fructose and NaCl the slope decreased.

Increase in the slope can be a result of better homogeneity of the sample, as indicated by the small error bars for the initial ion velocity and the trapping of salt contamination. The latter is the main reason, together with a very inhomogeneous distribution of matrix and additive, for the large effect of NaCl.

3.2.3 Effect of laser intensity on the initial ion velocity measurements

I also tried to determine the initial ion velocity through the linear curves (figure 10) produced from trypsinogen with the CHCA matrix using three different laser powers, 37%, 42% and 50%. I used CHCA as the standard matrix for this set of measurements, due to its large signal to noise ratio and subsequently its really good quality as a spectrum.
Figure 10: Determination of initial ion velocities of trypsinogen ions produced from CHCA with three different laser powers: A) 37%, B) 42%, C) 50%.

Figure 11: Comparison of the slopes acquired from the CHCA matrix with three different laser powers. The blue line represents the laser power, while the red one the slope.
The above figure provides evidence that for the first increase of the laser intensity, the slope also increases, but a further increase of it results in a sudden drop of the slope. Hence, there is no correlation between laser power and initial ion velocity. Initial ion velocity depends only on the matrix of the compound analysed. And this was proved in general, for all the matrices that I have studied. Therefore, there is no strong effect of laser intensity on the initial ion velocity.

3.2.4 Relation between initial ion velocity and crystal size measurements

In addition, I tried to connect the crystal size measurements that I performed in the previous part of my study with the values of the slopes acquired from the initial ion velocity measurements. The figure below provides evidence that there is no correlation between them.

![Figure 12: Correlation of the slopes acquired from five different matrices with their crystal size. The blue line represents the longest dimension of the crystals for five matrices, while the red one the slope.](image)

3.3 Timepix

The image of the spatial distribution of a particular component in the MALDI ion cloud is possible using the Timepix detector. This is mainly based on its time-resolved imaging capabilities after the projection of the ion cloud onto the detector surface. Apart from the ion-optical effects which are taking place during the extraction and acceleration steps of the process, the initial ion velocity distribution has also effect on this spatial orientation of the ion cloud.
There are many parameters which affect the combined focal length of the ion-optical system, such as the design and the voltages that are applied to each electrode element, in addition to ion characteristic properties like mass, charge and velocity.

### 3.3.1 MALDI imaging - Timepix images

In order to decide which is the appropriate extraction voltage and potential of the Einzel lens that I would use in my study, I compared the figures of the ion cloud distribution of trypsinogen ions produced from seven different matrices, for five different extraction voltages and four different potentials of Einzel lens.

The images below show the incorporated (500 laser shots, 1000 for DHB and DHAP matrices) ion cloud distribution of trypsinogen protein (m/z 23982) the instant it arrives at the detector, as a function of extraction voltage (Ev).
Figure 13: Ion cloud distribution of trypsinogen ions produced from seven different matrices the instant it arrives at the detector in a range of extraction voltage from 23 to 23.20 kV.

DHB is considered a suitable matrix to obtain reliable molecular mass values of intact glycoproteins by MALDI-MS because it prevents sugar fragmentation [16]. For this reason, I checked where the best focus for the DHB matrix solution was observed. As it is obvious from the Figure 13 the best focus for it, is given for the extraction voltage of 23.20 kV.

Following, I repeated the measurements for this solution in order to double check the measurements and the result was the same, as it is presented in figure 14.
Figure 14: Ion cloud distribution of trypsinogen ions produced from DHB, the instant it arrives at the detector in a range of extraction voltage from 23.25 to 22.80 kV.
Furthermore, it was also noticed that both for higher and lower laser intensities, defocusing was taking place. To be more specific, at high kV values a dispersion of the ions which reach the detector over most of its surface, is observed. On the contrary, when the voltage is decreased more ions are focused on the detector. Moreover, ultimately the impact positions are brought into a well-defined focused spot on the detector surface. After an even higher decrease of the kV values, it is observed that defocusing of the ion cloud is taking place. In general, it was shown that defocused images are obtained both with higher and lower extraction voltages, as evidenced by the cross-like shape of the ion clouds. The latter can be explained by the design of the extraction electrode, as depicted in the following image.

![Image 4: Extraction electrode of the Timepix detector [20].](image)

During the travel of the ions through the small central bore, the larger holes enable the delivery of the laser beam and the lighting. In addition, they work as a camera port. Both the electric field structure and the geometry of the electrode around it, affect the ion optical properties of the central aperture. Hence, the shape of the ion cloud looks like the one of the electrode geometry, due to the distortion of the extraction field when larger holes are present in the electrode.

Additionally, in order to select the appropriate potential for the Einzel lens that I would use, I applied four different potentials, namely 0, 2, 4 and 6 kV for the five standard crystalline matrices.
Figure 15: Ion cloud distribution of trypsinogen ions produced from five standard crystalline matrices the instant it arrives at the detector, for an extraction voltage of 23.20 kV in a range of four different potentials of Einzel lens.

From the figure above, it is observed that with the potential of 6 kV, the focusing-defocusing differences between the matrices are clearer. Hence, I decided to use 6 kV as a potential of the Einzel lens.

Figure 16 shows the ion cloud images of trypsinogen protein (m/z 23982) acquired from fifteen different matrices with identical ion source conditions of 23.20 kV extraction voltage, 0 ns PIE delay and a potential of 6 kV for the Einzel lens.
Figure 16: Ion cloud images of trypsinogen ions acquired from fifteen different matrices with identical ion source conditions.

Under these conditions ions generated from matrices such as FA IL, PA, CHCA BA and DHB are observed to be well space focused on the detector. On the other hand, ions generated for instance from CHCA IL, SA and CHCA provided defocused images on the detector surface, as evidenced by the cross-like shape in the ion cloud distributions.

The additional blurriness observed for example in the cross region of CHCA, suggests that the ion cloud image obtained from this matrix is more defocused than others. The higher ion intensities observed in the cross-region of CHCA BA compared to DHB do not necessarily suggest one is more focused the other. This can be easily explained as the outcome of the higher ion counts observed with CHCA BA.

Other conclusions of the above set of images include the hole of the ion cloud image obtained from the FA IL matrix, which can be easily explained because there are ions which arrive at or just before the Timepix window and they give a spike peak. In general, these cover the positions of the ions of ionic liquid matrices and this is the reason for the hole created in the middle of the image. Furthermore, using a lower extraction voltage for the image obtained from SA (23 kV), the ion cloud distribution looks very similar to that of FA (23.20 kV). This can be easily comprehended in figure 13.

Finally, concerning the FA matrix solution, the nature of crystals plays significant role in the defocusing. There are characteristic in plume collisions, so the loss of energy is related to these processes.

3.3.2 Correlation between focusing order and initial ion velocity measurements for different matrices
As it was mentioned in a previous chapter, the initial ion velocity of desorbed ions, which are produced during the MALDI process, is strongly dependent on the matrix that is used.

For the detection of the ions of interest, all pixels are available, due to their rapid arrival inside the acquisition window. This is mainly based on their intrinsic property that every pixel is a single-stop time-to-digital converter.

In this step, I tried to compare the ion cloud images of trypsinogen protein acquired from the fifteen different matrices that I use, under the ion source conditions described before, with the focusing-defocusing order of their ion-cloud distribution images.

**Figure 17**: Comparison of the slopes, acquired from the trypsinogen ions produced from different matrices, which determine the initial ion velocities of them, with the focusing order of their ion cloud distribution images, using Timepix (figure 16).

From this diagram it is observed that the focusing order becomes consistent with the values acquired from the relative initial ion velocities of the analyzed matrices. The only deflection that is noticed concerns the matrix solution of DAN.

More specifically, the focusing-defocusing order of the images obtained from the five standard crystalline matrices is correlated with the values of the slopes that determine the initial ion velocity measurements, as it is shown in the figure below.
Figure 18: Comparison of the slopes, produced from the five standard crystalline matrices with trypsinogen ions, which determine the initial ion velocities of them, with the focusing order of their ion cloud distribution images, using Timepix (figure 16).

3.4 Evaluation of different matrices

This chapter is centered on the evaluation of the spectrums obtained from the fifteen different matrices for an extraction delay of 100 ns. This choice was due to the fact that these had the best resolution compared to the other spectrums acquired with different extraction delays, during initial ion velocity measurements.

3.4.1 Comparison between spectrums of crystalline matrices and ILMs

First of all, I compared the spectrums of the five standard crystalline matrices.
Figure 19: Spectrums of standard crystalline MALDI matrices.

As it is illustrated, higher laser pulses were used for the measurements where DHB and DHAP were used as matrices. This can be explained as these two matrices evaporate really fast and especially for DHB matrix solutions a crystal rim is created during sample preparation and it is characterized for different intensities in different areas, as we are looking for high signal areas, named the “sweet” spots. Subsequently, significant fragmentation is taking place.

In addition, if the effect of laser intensity on the five standard crystalline matrices is investigated, it is noticed that for the matrix of DHB, where the highest laser intensity
(LP 55) is applied, the average molecular mass (m/z 24018.002) is the lowest. Finally, concerning the FA matrix solution, the nature of crystals is important and the loss of energy is related to characteristic in plume collisions.

Following, I compared the spectrum obtained from the CHCA matrix solution with the spectrums of the four ionic liquid matrices that I use.

![Comparison of spectra](image)

**Figure 20:** Comparison between the spectrum of CHCA and ionic liquid matrices’ spectrums.
It is observed that the standard crystalline matrices, such as CHCA, produce adequate mass spectrum responses. On the other hand, more intense spectrums are created under well documented conditions by the ionic liquid matrices. The average molecular mass value of the standard crystalline matrix CHCA (avg m/z 24030.113) was lower than the ones of the ionic liquid matrices.

It was also noticed here that the CHCA IL and CHCA BA matrices give cleaner spectrums with trypsinogen ions compared to the CHCA DEA and FA IL matrices. Generally, ionic liquid matrices provide extensive adduct formation and peak broadening because of the desorption processes, leading to a reduced resolution. Moreover, the ionic liquid matrices have a more homogeneous surface, and as a result, more laser shots per spot are needed because of diffusion and sample flow [23].

Finally, I compared the rest six matrices out of the fifteen that I use throughout my research (figure 21).
Figure 21: Spectrums of six crystalline MALDI matrices.

This figure provides evidence that the 9A,A matrix solution has the lowest signal to noise ratio, hence the worst quality as a spectrum. It is also noticeable that the THAP and DAN matrix solutions present characteristic peak broadening. In addition, the highest laser intensities were used for the 9A,A (78%) and PA (82%) matrix solutions.

3.4.2 MALDI-TOF mass spectra and S/N ratios

Figure 22: MALDI-TOF mass spectra and S/N ratios.

As it is shown in the above figure, crystalline matrices such as CHCA and SA give better signal to noise ratio in comparison with ionic liquid matrices. This occurs due
to the co-crystallization and consequently, higher ion production is taking place as a result of a higher transfer of energy. Increased signal to noise ratios results in better quality of spectrums.

3.4.3 Measurements of resolution for different matrices

In the last part of this chapter, using MATLAB software I tried to measure the resolution of the fifteen different matrices that I used, as well as to determine their correlation with the total ion intensity.

![Graph showing measurements of resolution for different matrices.](image)

**Figure 23:** Measurements of resolution for different matrices.

As it is widely known, resolution in mass spectrometry is a significant property which enables researchers to distinguish two peaks of slightly different m/z ratios in mass spectrums. In general, higher resolution means smaller peak width.

Ionic liquid matrices present reduced resolution due to extensive adduct formation and peak broadening, as it is shown in the spectrums in figure 20. It is also observed that the lower the laser intensities are, the higher the resolutions (figures 19 and 20). The same is also noticed in figure 21, apart from the PA and DAN spectrums.

For the matrices of CHCA and SA, there are more than one measurements as they were used for the normalization of the data. Moreover, for the PA and 9A,A matrix solutions there are again more measurements, as I tried to obtain spectrums with better quality.
Figure 24: Resolution and total ion intensity for different matrices. The blue line represents the resolution, while the red one the total ion intensity.

As it is found from the figure above, there is no correlation between the total ion intensity and resolution. Therefore, as the spectrums were obtained according to the initial ion velocity measurements, we can conclude that the latter only depend on the matrix used.

Chapter IV

4. Conclusions

This study was centered on the evaluation of a series of MALDI matrices for the detection of the trypsinogen protein by MALDI-time-of-flight mass spectrometry. In order for a MALDI analysis to have successful results, the selection of the appropriate matrix that is going to be used, as well as the sample preparation procedure is extremely significant. These two factors have a great effect on the ionization process, the stability of the analytes which is related to the fragmentation step, the formation of adducts and also, they are strongly related to the general performance of the experiments [24].

For the evaluation of the series of matrices, initially I examined the crystal properties of sixteen different matrices, including five standard crystalline ones as well as a series of new ones, named ionic liquid matrices, by presenting photographs of their preparations and also microscope images. It is really important the sample to be pure
without any possible contamination which can affect the quality of the images. The concentration of the sample is also important in order to achieve appropriate measurements. Concerning the most characteristic created crystals, a distinct photograph was the one of the 2,5-DHB single crystals, which were the largest ones (parallelepipeds). The FA matrix solution formed long thin crystals rising up off the microscope slide, while microscope images of ionic liquid matrices formed transparent film or droplet.

From the experimental results it was shown that the NBN matrix did not work properly with the trypsinogen protein. Concerning the rest of the matrices, all of them seemed to work, with some providing better results than the others. Moreover, it was observed that the standard crystalline matrices, such as the CHCA, produced adequate mass spectrum responses, while the ionic liquids created more intense spectrums under well documented conditions. Furthermore, for the DHB and DHAP matrix solutions high laser pulses were used as these two matrices evaporate extremely fast. The worst quality as a spectrum was obtained by the 9A,A matrix solution, as this had the lowest signal to noise ration, while generally crystalline matrices such as the CHCA and SA give better signal to noise ratio compared to ionic liquid matrices.

Important characteristics of ionic liquid matrices are their good spot homogeneity, shot-to-shot reproducibility, low vapor pressure and also the fact that even in high temperatures (above 200 °C) they stay liquids. Based on these properties, investigation of "sweet spots" is decreased. Many ionic liquid matrices are solid at room temperature with visible crystals [25]. One drawback however, which has significant effect on applications of ionic liquid matrices for MALDI-MS quantitations and spatial resolved imaging, is their inhomogeneous crystallization.

In order to proceed with my study, I investigated the initial ion velocity using delayed ion extraction. It has been widely accepted that the initial ion velocity is a superior tool which is used for the characterization of the "desorption step" of the MALDI process [9]. Inhomogeneous surfaces which are created during mixing the matrix with the trypsinogen solution are the main reason for any potential error in the measurements of the initial ion velocity. In addition, the exact position and the flatness of the sample holder may have an effect on changes occurring during initial ion velocity measurements [18]. Improved preparation protocols may need to be implemented for the achievement of more reproducible results. In general, initial ion velocity depends only on the matrix used. In my study, the laser fluence did not show any noticeable influence and moreover, the effect of additives was not uniform.

Finally, using the Timepix detector I acquired the MALDI-linear-TOF ion cloud image which is shown on the detector surface. The greatest advantage of this detector is that it enables the simultaneous acquirement not only of the arrival time but also of the position of the incoming ions. As the position and information concerning the time-of-flight are acquired at the same time, there is a direct relationship between the characteristics of the mass spectrum and the effect of the ion
impact position. The optimization of the application of interest is also possible. The good spatial focusing is the main reason for the achievement of higher ion counts throughout the mass range of interest.

The use of Timepix detector has enabled researchers to improve and enable TOF-MS in order to detect high m/z (>20,000) species mainly because of its sensitivity as a detector. The imaging of these species is also possible. Furthermore, it can be also used in the diagnosis field, displaying the necessary ion optical processes in order to perform studies with TOF instruments [20].

Generally, this series of experiments is not only influenced by the initial ion velocity effect. The acceleration of the ions is taking place over a dense plume, where in-plume collisions are the main reason for any possible loss of energy and therefore velocity, by pulling the ions through this dense particle.

For the ion focusing effects which are based on the choice of the appropriate matrix, apart from the initial ion velocity, combinations of the latter with other plume-related methods are considered. This enables initial ion velocity to be used in many applications of imaging mass spectrometry with great results.


Chapter V

5. References


