Doctoral Thesis

Ion formation and detection in matrix-assisted laser desorption/ionization mass spectrometry

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Ion Formation and Detection in Matrix-Assisted Laser Desorption/
Ionization Mass Spectrometry

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

For the degree of
Doctor of Natural Sciences

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Table of contents

1. Introduction .................................................................................................................. 13

2. MS: How it works .......................................................................................................... 19
   2.1 The ion source:.......................................................................................................... 19
      2.1.1 Brief presentation of some ionization methods: ............................................ 19
      2.1.2 MALDI .............................................................................................................. 20
         2.1.2.1 Principle ..................................................................................................... 20
         2.1.2.2 MALDI matrices ....................................................................................... 22
         2.1.2.3 MALDI sample preparation ..................................................................... 23
         2.1.2.4 MALDI matrix properties ......................................................................... 23
         2.1.2.5 Two-phase MALDI .................................................................................... 24
         2.1.2.6 Types of lasers ......................................................................................... 24
         2.1.2.7 MALDI ionization/Desorption models ....................................................... 25
   2.2 Mass separation methods .......................................................................................... 25
      2.2.1 Principle ............................................................................................................ 25
      2.2.2 Theoretical description of TOF measurements .............................................. 27
      2.2.3 Delayed extraction ......................................................................................... 30
   2.3 Ion detection in TOF ................................................................................................ 31
      2.3.1 Principle ............................................................................................................ 31
      2.3.2 Secondary electron multipliers ........................................................................ 32
         2.3.2.1 SEM with discrete dynodes ..................................................................... 33
         2.3.2.2 SEM with continuous dynodes (Channeltron and MCP) ....................... 33
         2.3.2.3 The secondary electron emission yield .................................................... 35
         2.3.2.4 Advantages and Limitations of SEMs ...................................................... 36
      2.3.3 Inductive detectors ........................................................................................... 38
      2.3.4 Faraday cup detectors ....................................................................................... 38
      2.3.5 Cryogenic particle detectors .......................................................................... 39
      2.3.6 Ion-to-photon detectors ................................................................................... 39
   2.4 Data acquisition ........................................................................................................ 41
      2.4.1 Transient recording ......................................................................................... 41
      2.4.2 Single event counting mode ............................................................................ 42
   2.5 References ............................................................................................................... 45
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>18C6</td>
<td>18-Crown-6</td>
</tr>
<tr>
<td>BBOT</td>
<td>2,5-bis(5'-t-butyl-2-benzoxazolyl)thiophene</td>
</tr>
<tr>
<td>BC-404</td>
<td>Bicron 404</td>
</tr>
<tr>
<td>Bu-PBD</td>
<td>2-(4-t-butyl-phenyl)-5-(4-biphenylyl)-1,3,4-oxidiazole</td>
</tr>
<tr>
<td>CFD</td>
<td>Constant Fraction Discriminator</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-DiHydroxyBenzoic Acid</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>Er:YAG</td>
<td>Erbium Yttrium Aluminum Garnet</td>
</tr>
<tr>
<td>ESI</td>
<td>ElectroSpray Ionization</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast Atom Bombardment</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier Transform-Ion Cyclotron Resonance</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full Width at Half Maximum</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively-Coupled Plasma Atomic Emission Spectrometry</td>
</tr>
<tr>
<td>IEPD</td>
<td>Ion-to-Electron-to-Photon Detector</td>
</tr>
<tr>
<td>IPD</td>
<td>Ion-to-Photon Detector</td>
</tr>
<tr>
<td>IR</td>
<td>Infra Red</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>MCP</td>
<td>MicroChannel Plates</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NBA</td>
<td>3-NitroBenzyl Alcohol</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium Yttrium Aluminum Garnet</td>
</tr>
<tr>
<td>NE102A</td>
<td>Nuclear Enterprise 102A</td>
</tr>
<tr>
<td>NPOE</td>
<td>2-NitroPhenyl Octyl Ether</td>
</tr>
<tr>
<td>PD</td>
<td>Plasma Desorption</td>
</tr>
<tr>
<td>PMT</td>
<td>PhotoMultiplier Tube</td>
</tr>
<tr>
<td>SEM</td>
<td>Secondary Electron Multiplier</td>
</tr>
<tr>
<td>SIMS</td>
<td>Secondary Ion Mass Spectrometry</td>
</tr>
<tr>
<td>TFA</td>
<td>TriFluoroacetic Acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>TDC</td>
<td>Time-to-Digital Converter</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-Of-Flight</td>
</tr>
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The discovery of "soft" ionization methods that allow the desorption and ionization of large, involatile, and labile compounds, highly increased the interest for mass spectrometry. A range of different classes of molecules, such as proteins, oligonucleotides, and polymers, can now be mass analyzed. One of these ionization techniques, matrix-assisted laser desorption/ionization (MALDI), was introduced only 12 years ago. It relies on mixing the sample with a large excess of a small organic molecule, the matrix, and to illuminate the preparation with a laser pulse. Coupled with a time-of-flight mass spectrometer that in principle has no upper mass limit, the system is utilized by many laboratories for routine analysis.

Very large ions are, however, difficult to detect in time-of-flight mass spectrometers. Most of the detectors function by amplifying electrons that are created upon impact of the primary ions with a conversion surface. Unfortunately, the conversion efficiency drops with decreasing impact velocity. In time-of-flight mass spectrometers, large ions reach the detector with low velocities and the detection efficiency is highly reduced.

In this work, an alternative technology for ion detection is presented. Ion-to-photon detectors (IPD) make use of photons emitted when ions impinge on a scintillating surface. Two configurations were tested. In the first study, the primary ion beam was directly collected on a quartz plate covered with a scintillator. Outside the vacuum, a photomultiplier detected the photons. Different types of scintillators were tested. Organic materials, such as BBOT (2,5-bis (5'-t-butyl-2-benzoazolyl) thiophene), were the most efficient, with short fluorescence lifetimes in the order of 1-2 ns. The total response time of the detector was, in our case, limited by the speed of the photomultiplier and was about 4 ns, which is sufficient for many time-of-flight applications. Using single event counting techniques, the device was compared to a standard detector, the microchannel plate. It was found that the efficiency of the IPD relative to the microchannel plate, decreased as the molecular mass of the ion increased, for a given ion energy. Small ions (in our case smaller than 150 Da at 22 keV) produced more photons than secondary electrons. We also found that the conversion of ions into photons depends on the ion energy. Signal intensities can be increased by post-accelerating the ions just in front of the scintillator. In contrast to microchannel plates, that can only use limited post-acceleration voltages, the IPD can be floated at much larger voltages.

Using the fact that the IPD is more sensitive to small particles, a second detector
configuration was tested, the ion-to-electron-to-photon detector (IEPD). In this device, the primary ion beam is first converted into smaller particles -ions or electrons- by impact onto a conversion dynode. These are then accelerated to the scintillator. It was found that a large fraction of the light signal was due to secondary electrons. In contrast to the IPD, a plastic scintillator was used that was mostly sensitive to electrons. This detector gave large signal intensities and suitable signal-to-noise ratios. A time response similar to the one estimated for the IPD was found. Test measurements showed that a mass resolution comparable to standard secondary electron detectors could be obtained. Compounds with very high molecular weights (> 500'000 Da) were also readily detected.

In the second part of this work, the ion formation in MALDI was investigated. Ions in MALDI originate from different sources. We focus our work on ions that are already present in the condensed phase in the form of a salt. Upon laser illumination, the salt is liberated and the charges separated. This ion category is termed “preformed ions”. It was shown that MALDI mass spectra are influenced by the sample preparation. The relative amount of preformed ions can to some extent be controlled by the solution pH, the amount of salt, or by the solvent used in the sample preparation. The sample preparation also influences the quality of the mass spectra. Better MALDI mass spectra were obtained with polar solvents and acidic conditions.

In an attempt to use MALDI in a quantitative way, we propose a procedure to correct for the different ionization/desorption and transmission efficiencies of the molecules. It was found that very similar compounds can have quite different efficiencies. Using two-phase MALDI, a modified sample preparation in which the sample stays in a vacuum-stable solvent, relative stability constants were determined for a crown ether complexed with metal cations. The values found were in good agreement with literature. It was therefore concluded that, for this system, the MALDI mass spectra reflected the equilibrium chemistry that existed in the solution.
**RÉSUMÉ**

L'intérêt pour la spectrométrie de masse a beaucoup augmenté, en particulier depuis la découverte de nouvelles méthodes d'ionisation douce permettant la désorption et l'ionisation de composés labiles et peu volatiles. Différents types de molécules, tels que protéines, oligonucléotides ou polymères, peuvent de nos jours être analysées. L'une de ces techniques, la désorption et ionisation par laser assistée par une matrice (MALDI en anglais), fut introduite il y a une douzaine d'année. Son principe consiste à mélanger aux composés à analyser un large excès d'une petite molécule organique –la matrice- et à irradier l'échantillon avec une impulsion laser. Couplé à un spectromètre de masse à temps de vol, qui n'a en principe pas de limite supérieure en masse, ce système est utilisé par beaucoup de laboratoires pour des analyses de routine.

En spectrométrie de masse à temps de vol, les ions de masse élevée sont cependant difficiles à détecter. La plupart des détecteurs amplifient les électrons secondaires qui sont créés lors de l'impact des ions primaires avec une surface de conversion. Malheureusement, en temps de vol, les gros ions atteignent le détecteur avec de faibles vitesses et l'efficacité de détection est réduite.

Dans ce travail nous présentons une approche alternative de détection de ions. Le détecteur “ion-en-photons” (IPD en anglais) utilise les photons produits lors de l'impact des ions avec une surface scintillatrice. Deux configurations ont été testées. Dans la première, le faisceau d'ions primaires est directement collecté sur un disque de quartz recouvert d'un scintillateur. A l'extérieur du système sous-vide, un photomultiplicateur détecte les photons. Différents types de scintillateurs ont été utilisés. Les composés organiques tel le BBOT (2,5-bis(5'-t-butyl-2-benzoazolyl)thiophène) ont la plus grande efficacité ainsi que des fluorescences avec de courtes durées de vie de l'ordre de 1 à 2 ns. Le temps de réponse total du détecteur était, dans notre cas, limité par le photomultiplicateur et se situe aux alentours de 4 ns, ce qui est suffisant pour de nombreuses applications. Des mesures événement par événement nous ont permis de comparer ce dispositif avec un détecteur standard: la galette micro-canaux. À énergie donnée, l'efficacité de l'IPD relative à celle des micro-canaux diminue lorsque la masse de l'ion primaire augmente. Cependant, les ions de faibles masses (dans notre cas plus petit que 150 Da à 22 keV) produisent plus de photons que d'électrons secondaires. D'autre part, les intensités des signaux peuvent être augmentés en post-accelérant les ions.
directement en face du scintillateur. Au contraire des galettes qui ne peuvent utiliser que des voltages limités, l'IPD peut être amené à de bien plus grands voltages. Prenant avantage du fait que l'IPD est plus sensible aux ions légers, nous avons testé un deuxième dispositif : le détecteur “ion - électrons - photons” (IEPD). Le faisceau d'ions primaires est dans ce cas d'abord fragmenté en plus petites particules lors de l'impact sur une dynode de conversion. Ces petites particules sont ensuite accélérées en direction du scintillateur. Il a été observé qu'un grande partie du signal était due aux électrons secondaires. Contrairement à l'IPD, le scintillateur utilisé avec l'IEPD est essentiellement efficace pour la détection d'électrons. Ce détecteur émet des signaux de hautes intensités avec de bons rapports signal sur bruit. Le temps de réponse de l'IEPD est proche de celui de l'IPD. Des mesures ont démontré que des résolutions en masse au moins comparables à celles obtenues avec des détecteurs à multiplication d'électrons secondaires peuvent être obtenues. Des composés de haute masse moléculaire (> 500'000 Da) ont également pu être aisément détectés.

Dans la deuxième partie de ce travail, les mécanismes de formation des ions en MALDI ont été étudiés. Les ions en MALDI proviennent de différentes sources. Nous nous sommes concentrés sur les ions déjà présents dans la phase condensée sous forme de sel. Lors de l'impulsion laser, les molécules de sel sont libérées et les charges séparées. Cette catégorie d'ions est appelée couramment “ions préformés”. Il a été montré que les spectres de masse MALDI sont influencés par la façon de préparer l'échantillon. La quantité de ions préformés peut être, jusqu'à un certain point, contrôlée par le pH de la solution, la quantité de sel dissoute et par le solvent utilisé pour la préparation. Cette préparation influence également la qualité des spectres de masse puisqu'il a été montré que de meilleurs résultats sont obtenus avec des solvants polaires et sous des conditions acides.

Afin de pouvoir utiliser la MALDI quantitativement, nous avons utilisé une méthode pour corriger les efficacités différentes de désorption/ ionisation et transmission pour chacune des molécules, des molécules de compositions chimiques proches pouvant avoir des efficacités assez différentes. Les constantes de stabilité relatives entre un ether couronne et des cations métalliques ont été ensuite déterminées à l'aide de MALDI à deux phases (la MALDI à deux phases est une modification de la préparation d'échantillon de MALDI. Au lieu d'obtenir un échantillon cristallin, l'analyte est dissout dans un solvant résistant au vide). Les valeurs de stabilités relatives trouvées correspondaient bien aux valeurs de la littérature. Nous en avons conclu que, pour ce système, les spectres MALDI reflétaient les équilibres chimiques qui existaient au sein de la solution.
Chapter 1.

1. Introduction

The chemical industry has enormously expanded in domains such as polymers, organic synthesis, and biochemistry. The complexity and size of the investigated molecules has steadily continued to grow. In parallel to these developments, analytical chemistry has also undergone a noticeable evolution to adapt its methods to the challenges posed by the researchers and the industry. Obviously, one of their demands was to develop methods with the ability to identify and analyze large and complex compounds.

One of the candidates is mass spectrometry. Mass spectrometry is an analytical technique that separates molecules based on molecular weight. The general principle, already known at the beginning of the century, is to transform the molecules into ions. Ions can then easily be manipulated by electrical, or magnetic fields. Early ionization methods such as electron impact or chemical ionization could not be applied to large, involatile species. Moreover, if ionization was achieved, then strong fragmentation was often observed. The utilization of mass spectrometry was therefore limited to classes of smaller molecules. In the '80s and '90s the development of "soft" ionization techniques capable of ionizing large involatile and labile molecules with a much lower fragmentation rate increased the interest for mass spectrometry.

Currently proteins as large as 1 MDa are readily ionized. The molecular mass is not the only information that can be obtained: molecular structure, 3D-geometry information, complexation energies, weak protein-protein interactions, and study of chemical reactions can all be investigated by mass spectrometry. Its sensitivity to very small sample concentrations is another valuable asset particularly appreciated in biochemistry. The number of new instruments and users therefore exploded. Today many laboratories are equipped with several complementary ionization techniques, for example, chemical ionization, electron impact, electrospray,
or matrix-assisted laser desorption/ionization (MALDI), and use them for routine analyses. Besides the utilization of these techniques as tools, it is also important to understand their fundamentals. It will often result in the development of new applications and method optimization.

The focus of this research was on MALDI coupled with a time-of-flight (TOF) mass spectrometer. In MALDI, the analyte is mixed with an additional compound (the matrix), whose role is to assist in sample desorption and ionization upon illumination with a laser pulse. This method is very sensitive and is able to produce ions from many different compounds such as proteins, oligonucleotides, and polymers. When coupled with a TOF mass spectrometer, it has, in principle, no upper molecular mass limit. In contrast to other methods such as sector and quadrupole instruments, TOFs also do not require the instrument to be scanned but rather the information over the whole mass range is obtained quasi simultaneously.

Despite all their advantages, MALDI-TOF instruments also have some limitations. New matrices, for instance, are still only found by trial and error, after testing different types of molecules. This is because their exact roles and requirements, as well as the ionization mechanisms, are not sufficiently known. Understanding the ionization mechanisms can also help to optimize the sample preparation and increase the spectral quality.

Another limitation of MALDI-TOF is its limited capability to give quantitative information. To illustrate this, a MALDI mass spectrum of an equimolar mixture of different polyethylene glycol (PEG) samples is shown in Fig. 1.1. The polymer samples only differ by their average molecular weights. The different PEGs do not appear in the spectrum with the same maximum intensity, as expected. There are two main reasons for this. First of all, even if all the PEGs are at the same concentration in the sample, not all the molecules are desorbed, ionized, and transmitted through the mass spectrometer with the same probability. However, the chemical composition of the molecules in Fig. 1.1 is very similar but the number of sites where each molecule can be ionized (i.e. by protonation or cationization), as well as the desorption efficiency, will depend on the oligomer size. The second reason that explains the different signal intensities is that, in TOF mass spectrometers, the standard ion detectors have a very strong mass bias. Large masses are detected with a much lower probability than smaller ions. Today, very heavy compounds can, thanks to the new “soft” ionization methods, be transferred into the gas phase and ionized. Unfortunately, in some cases, the detection mass bias is so problematic that these large ions are very poorly, if at all, detected.
In this work, we concentrate on two main questions:
1 Is it possible to design an ion detector with improved efficiency for large ions?
2 Despite the different ionization and detection efficiencies, can MALDI be used in a quantitative way? Furthermore, can MALDI mass spectra reflect the concentrations of compounds in the condensed phase?

In Chapter 4, we address the first question and will present alternative ion detectors. Rather than amplifying secondary electrons created during the impact of the ions on a surface, these new detectors, called ion-to-photon detectors (IPD), are sensitive to light produced by the ion impact. The effects of the scintillating surface material on the IPD efficiency will be investigated. Their efficiency to see very large ions is compared to standard ion detectors.

To answer the second question, we tested the influence of the sample preparation on the MALDI mass spectrum. It will be show in Chapter 5 that ions in MALDI spectra originate from different sources. Some ions are already present in the condensed phase in a salt form (so-called preformed ions). Ions are also created by
different mechanisms during the desorption event. The relative contribution of
the preformed ions can be to some extent controlled. From a practical point of
view, it will be shown that the spectral quality can be improved by using the right
sample preparation. A procedure to correct for different ionization and detection
efficiencies will then be described and applied to a test system. Under conditions
where the MALDI ions mostly consist of preformed ions, we will test whether
MALDI mass spectra can reflect the equilibrium chemistry that existed in the
sample solution.
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Chapter 2.

2. MS: How it works

Mass spectrometry is an analytical method that separates the different compounds contained in a sample according to their molecular masses. To do so, the sample is brought into the gas phase and ionized. The ions are then mass analyzed and detected. Although many different types of mass spectrometers exist, they all consist of three important parts:
1. The ion source
2. The mass separation
3. The ion detection

In the next paragraphs, a short review of the different existing systems will be given. We will then focus on the techniques that were utilized in this work, in particular on matrix-assisted laser desorption/ionization (MALDI) and time-of-flight (TOF) mass spectrometry. The ion detection is an important subject of this thesis and will be discussed in Chapter 4.

2.1 The ion source:

2.1.1 Brief presentation of some ionization methods:

In the ion source, the sample is vaporized and ionized. Electron impact \(^1\), \(^2\) was one of the first ionization methods used. It consists of a heated filament that produces electrons. These electrons are accelerated and collide with the gaseous molecules injected into the source. Usually strong ion fragmentation is observed with this method and the molecular ion is sometimes not detected. In a chemical ionization source, the ions are produced through gas phase reactions between the sample molecule and a reagent gas present in the source \(^3\). Two similar methods, secondary ion mass spectrometry (SIMS) \(^4\) and fast atom bombardment (FAB) \(^5\),
focus a particle beam (ions and neutrals, respectively) on the sample. With FAB and SIMS, the upper molecular mass limit reached with the "older" techniques described above was greatly improved. Ions of peptides as large as 10,000 Da and their sequences were observed. In plasma desorption, described in more detail in Chapter 4.2.9, molecules are ionized by the impact of fission fragments of $^{252}$Cf decay on the sample. In field desorption, the sample is deposited on a whisker, a filament covered with carbon needles. The high electrical field that is applied between this whisker and an electrode ionizes the molecules. A very successful variation of this method is the electrospray. The sample is dissolved and introduced into the ion source through a capillary. On the exterior of this capillary, a strong electrical field is applied and, as in the case of field desorption, ionizes the sample. This method usually produces multiply charged ions and is able to ionize very high molecular weight molecules. Matrix-assisted laser desorption/ionization (MALDI), introduced 12 years ago, is a powerful laser volatilization technique.

Mainly two ionization techniques have been used in this work. The first one, MALDI will be the subject of Chapter 2.1.2. Secondly, plasma desorption was used to produce a low ion rate necessary for counting techniques and will be discussed in Chapter 4.2.9.

2.1.2 MALDI

2.1.2.1 Principle

Matrix-assisted laser desorption/ionization (MALDI) is a newly developed method that has proven to be very efficient for many applications. It was successfully utilized to detect very heavy molecules (for example, IgM ~1MDa, 1.5 MDa polymers) and to analyze polymers, proteins, and oligonucleotides. In this technique, the analyte is mixed with a large excess of a matrix. The matrix is a liquid or solid compound of low molecular weight that absorbs the laser wavelength used (UV or IR).

A drop of the analyte/matrix mixture is deposited on the metal target and the solvent is vaporized. The sample is then illuminated with a short laser pulse and ions are created. A schematic of the desorption/ionization process is shown in Fig. 2.1.1.
Fig. 2.1.1: Principle of MALDI. The sample, mixed with a large excess of a matrix, is deposited on the "repeller" held at high voltage (HV1). A short laser pulse irradiates the surface (A), creating a dense plume of ions and neutrals (B). The ions with the corresponding polarity are accelerated by the electrical field (\(|HV_1| > |HV_2|\)). The electrode held at HV2 is called the "extractor." The ions are separated according to their masses (C).

After laser irradiation, a dense plume is created containing neutral and charged particles. Only a small fraction of the desorbed molecules are effectively ionized during this process. The ionization efficiency of MALDI was found by Mowry and Johnston to be around 1 ion per 10,000 neutral particles for laser irradiances just above threshold (the threshold is the minimum laser irradiance necessary to "see" ions).

The fundamentals of MALDI ionization are still a subject of discussion. In Chapter 2.1.2.7, we will describe some of the main ionization models proposed. Then
in Chapter 5, a contribution to better understand ion formation in MALDI will be presented. We will also discuss on the importance of preformed ions in MALDI.

2.1.2.2 MALDI matrices

As matrices, Hillenkamp and Karas\(^{14}\) used small organic molecules whereas Tanaka et al.\(^{15}\) introduced the method with fine metal particulate. The solid organic molecule preparation is nowadays the most used method. Some commonly used UV-matrices are shown in Fig. 2.1.2.

![Fig. 2.1.2: Some commonly used MALDI matrices with their chemical structures and names.](image)

Due to the lack of a clear understanding of the real matrix properties necessary for efficient ionization yields, the search for optimized matrices is not yet possible by using defined rules (composition, acidity/basicity, functionality, etc...) but is rather found by trial-and-error. Many researches have focused on finding new matrices with increased efficiencies\(^{16, 17, 18}\) for each class of analyte compounds. DHB is a very common matrix that is mostly used for mid-sized proteins (< 50kDa) with UV and IR lasers. 4-HCCA is essentially a UV matrix (337 nm and 355 nm) used to obtain high resolution positive peptide signals. Sinapinic acid and ferulic
acid are useful for high mass protein ionization.

### 2.1.2.3 MALDI sample preparation

The mixing molar ratio of sample/matrix is typically between 1/100 to 1/10,000. Although many sample preparations have been tried and optimized for such applications, a standard method can be described as follows: The analyte and matrix are dissolved and mixed in the desired concentrations. Proteins and peptides are generally dissolved in a mixture of an organic solvent (i.e. 2-propanol or acetonitrile) mixed with water acidified with 0.1% trifluoroacetic acid (TFA). Acidic conditions were found to enhance the signal intensities, probably due to increased sample solubility. A drop of the mixture is then deposited on the probe-tip and allowed to dry. Different drying methods have been proposed for solid preparations. The most common ones are: vacuum or cold/hot air sample drying, dried-droplet method and sample spinning. The main goal of all of these methods is to obtain homogenous samples. MALDI signal intensities often fluctuate quite significantly from laser shot to laser shot, mainly due to surface inhomogeneity. This fact has motivated the search for better sample preparations.

### 2.1.2.4 MALDI matrix properties

As previously mentioned, the real conditions necessary for a good MALDI solid matrix are still not fully understood. Although, some general properties can be described as follows:

1. The matrix must have a strong absorption band for the laser wavelength. A good laser absorption is a prerequisite.
2. The sample, which is at much lower concentration than the matrix, must be correctly dispersed and embedded in the solid. For that purpose, the matrix should act as a solid solvent for the analyte and should have some chemical affinity for it. The solvents for the matrix and the sample should be compatible to obtain a well mixed solid crystal and thereby more constant MALDI signal intensities.
3. A low matrix ionization potential is believed to be necessary. Karbach et al. measured the ionization potential of typical UV-MALDI matrices in the gas phase. Most of the matrices had values around 8 eV. These relatively small energies are nevertheless not sufficient for a direct two nitrogen laser photon ionization process (337 nm = 3.7 eV) of the matrix molecules in the gas phase.
2.1.2.5 Two-phase MALDI

In a two-phase MALDI preparation\textsuperscript{24, 25}, the analyte is dissolved in a slurry containing a low vapor pressure solvent and fine particulates. The particulates, usually graphite powders, absorb the laser pulse. Several vacuum-stable liquid solvents have been successfully utilized: glycerol, o-NPOE (2-nitrophenyl octyl ether), NBA (3-nitrobenzyl alcohol), and tetraglyme. This sample preparation can be used for light and mid-sized ions (20kDa). Compared to the standard preparation, a much better laser shot-to-shot reproducibility is observed\textsuperscript{25}. The sample crystallization that can be problematic with the standard preparation is not necessary with two-phase MALDI.

2.1.2.6 Types of lasers

Many different laser types have been used in MALDI as well as many different wavelengths. Historically, Hillenkamp et al. produced the first MALDI spectra by using a frequency quadrupled Nd:YAG (266 nm). Since then the following wavelengths have been successfully used:

<table>
<thead>
<tr>
<th>Laser</th>
<th>Wavelength</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nd:YAG</td>
<td>266, 355 nm</td>
<td>Historically the first laser used for MALDI.</td>
</tr>
<tr>
<td>N\textsubscript{2}</td>
<td>337 nm</td>
<td>Cheap and easy-to-use laser. One of the most used lasers for UV-MALDI.</td>
</tr>
<tr>
<td>Excimer</td>
<td>193-308 nm</td>
<td>Not very frequently used, mostly due to its poor laser beam profile and divergence. It also utilizes dangerous halogen gases.</td>
</tr>
<tr>
<td>Er:YAG</td>
<td>2.9 (\mu)m</td>
<td>The most used laser for IR-MALDI.</td>
</tr>
<tr>
<td>CO\textsubscript{2}</td>
<td>9.3-10.6 (\mu)m</td>
<td>Used for IR-MALDI although Er-YAG is preferred. Is produced at low price and very easy to use.</td>
</tr>
</tbody>
</table>

The N\textsubscript{2}-laser, emitting at 337 nm, is the typical laser type for UV-MALDI mainly due to its reasonable price and ease of use. Such a UV-laser was utilized for most of the experiments contained in this work. IR lasers were introduced in MALDI later than UV-lasers. However, it is believed that IR ionization is softer than with UV-photons. This results in a mass spectrum containing less fragments and the possibility to distinguish larger molecules\textsuperscript{26}. Another advantage of IR-MALDI is the possibility to use a rich variety of solid and also liquid matrices that cannot be
utilized with UV-lasers. Er-YAG lasers are used most often for IR-MALDI. They are however expensive compared to N2-lasers. Experiments are performed in other research groups to optimize the use of CO2 lasers. These lasers can be produced with a price comparable to that of N2-lasers.

2.1.2.7 MALDI Ionization/Desorption models

Several models were proposed to explain the MALDI mechanisms that create the initial ions, including multi-photon ionization, energy pooling, disproportionation reactions, excited-state proton transfer, thermal ionization, and desorption of preformed ions.

i) Multi-photon ionization: radical matrix ions are created by the absorption of one or several photons. With a N2-laser (3.7 eV), it is usually believed that more than two photons are necessary to reach the matrix ionization potential.

ii) Energy pooling: the energy necessary for the ion creation results in the sharing of several excited-state matrix molecule energies.

iii) Disproportionation model: explains the formation of positive and negative matrix ions by light induced disproportionation of matrix clusters.

iv) Excited-state proton transfer: if an excited matrix molecule becomes more acidic than in its ground state, then the proton transfer to an analyte molecule becomes easier.

v) Thermal ionization: this model explains the ion formation with the rapid heating of the substrate due to the laser shot absorption.

vi) Desorption of preformed ions: some molecules are present in the sample in a salt form. Due to the laser shot, the salts and their counter-ions are liberated and separated.

All these models are described and discussed in more detail in the cited review 27.

2.2 Mass separation methods

2.2.1 Principle

In this work, the ions were mass separated with a linear time-of-flight (TOF) mass spectrometer. This type of mass spectrometer works on the following principle: the ions are accelerated with an electrical field and gain velocities that are directly linked to their mass and charge. After the acceleration, they enter the so-called
“field-free region” where they fly through the tube at their own speed. The small masses gain more velocity than the heavier during acceleration and will therefore reach the end of the “field-free region” first, where they are detected. Fig. 2.2.1 illustrates the time-of-flight mass separation principle.

![Diagram of boats and fan](image)

Fig. 2.2.1: "Scientific" illustration of the time-of-flight principle. One boat is occupied by several persons, the second one only by one person. Both boats are on the starting line when the fan is switched on (a). The acceleration of the heavier boat is smaller compared to the one of the light boat. The fan is then switched off (b). The boats have gained different speeds: the light one is faster than the heavier. They will navigate at the same speed even if the fan is off (the frictions are neglected). As a result, the lighter boat will reach the finish-line first (c).

The detector records the particle current reaching its active surface as a function of time. Using calibration methods, molecular masses can be deduced from the ion flight times. This method is very practical since there is no theoretical upper limitation in the ion mass and no need for complicated instruments. However, the
quality of the mass spectra in terms of mass resolution (see Chapter 2.2.2) can be insufficient for some applications. To improve the resolution, a technique called delayed-extraction can be used. With this set-up, the ions are accelerated only after a certain time delay. It results in increased ion focusing on the detector and the possibility to distinguish between ions with similar masses. Mass separation devices that do not rely on the flight-time principle include quadrupole mass spectrometers, ion traps, sector instruments, and Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. The reader is invited to consult the review article from Burlingame et al. for a further description of these methods.

2.2.2 Theoretical description of TOF measurements

As mentioned previously, the ions in TOF are accelerated by an electrical field. This is achieved by using electrodes held at different potentials, the final one usually held at ground. The simplest description of a time-of-flight (TOF) mass spectrometer is shown in Fig. 2.2.2 below. It consists of a linear TOF with single stage acceleration optics. The ions are accelerated with a single homogenous electrical field created with two electrodes: A at high voltage and B at ground, separated by a distance "s". In the region between the electrode B and the detector, the electrical field is zero.

![Diagram of a simplified time-of-flight mass spectrometer](image)

*Fig. 2.2.2: Description of a simplified time-of-flight. The ions are created on "A". In the ion source, the ions are accelerated with an electrical field. The ions then enter the field-free-region. Its length "D" is usually much larger than "s". The ion detector is placed at the end of the field-free-region.*
Due to energy conservation, the potential energy of an ion with mass "m" and number of charges "z" is, when it leaves the acceleration region, transformed into kinetic energy. It can be written:

\[ \frac{1}{2} z \cdot e \cdot U = m \cdot \frac{v^2}{2} \]

where "v" is the ion velocity as it leaves the acceleration region, "U" the potential difference between the electrodes, and "e" the elementary charge constant. For a given potential energy, the velocity of an ion is inversely proportional to the square root of its mass.

\[ v = \sqrt{\frac{2 \cdot z \cdot e \cdot U}{m}} \]

Outside of the ion optics, every ion will have gained a velocity that univocally characterizes its mass to charge ratio (m/z).

The flight times, in the case where "s" is much smaller than "D" are:

\[ t = D \sqrt{\frac{m}{2 \cdot z \cdot e \cdot U}} \]

This equation can also be written as follows:

\[ \frac{m}{z} = \frac{2 \cdot z \cdot e \cdot U}{D^2} \cdot t^2 \]

The term \(2 \cdot z \cdot e \cdot U/D^2\) is constant for a given instrument and voltages. The m/z ratio can therefore be calculated with a second order calibration equation. In practice however, an equation containing at least a constant term "c" (time-offset) is often used. Sometimes a linear term is introduced to improve the quality of the fit:

\[ \frac{m}{z} = a \cdot t^2 + b \cdot t + c \]

The parameters a, b and c are found by using calibrant samples with known m/z.
ratios. For very precise mass determinations, calibrants with molecular masses close to the unknown substance are added to the sample. This method is called "internal calibration" as compared to "external calibration" where the "a", "b", and "c" parameters are calculated in a first measurement with calibrants. The parameters are assumed to stay constant for the following measurements and are used for the unknown sample. It can be particularly difficult to prepare samples with internal calibrants. The mass spectrum peaks of both calibrant and sample must be of comparable intensities and attention must be paid that they do not interact with each other. The external calibration technique was therefore preferred in this work as long as the exactness of the molecular mass was not an important factor.

Ideally, every ion with the same m/z ratio should arrive exactly at the same time on the detector. In practice however, this is not true. Some of the reasons for this time-spread in a TOF are listed below:

1- the ions have slightly different initial kinetic energies. Their final velocities, and consequently their flight times, are therefore different.

2- the ions are created at different spatial positions. The ions therefore feel slightly different electrical fields. It results in a velocity distribution at the output of the acceleration optics and a peak broadening on the detector. In the case of MALDI, this is less problematic because the target is a geometrically well-defined surface (the probe tip) in contrast to other ionization methods such as chemical ionization or electron ionization where the sample is first vaporized and, in a second step, ionized. Resolution loss due to the points 1 and 2 can be partially compensated by using delayed extraction. This was discussed in 1955 by Wiley and McLaren\textsuperscript{29} and will be presented in Chapter 2.2.3.

3- the electrical fields are not perfect. It results in slightly different ion trajectory lengths or in different ion energies.

4- the residual gas molecules in the TOF interact statistically with the ion packages and slow them down.

5- the response time of the ion detector as well as the sampling rate of the recorder are finite.

All these factors and many others negatively influence the mass spectral resolution. The mass resolution (Fig. 2.2.3) is defined as the ratio between the mean m/z ratio of a peak and its full width at half its maximum (FWHM). The larger the value, the better the ions with very close m/z can be separated.
Fig. 2.2.3: Definition of the mass resolution \( R \). It is defined as the \((m/z)\) value at the peak maximum divided by the “full-width-at-half-maximum” \((\Delta m)\).

2.2.3 Delayed extraction

In a time-of-flight mass spectrometer with continuous extraction, the electrode voltages are switched on before the measurements and remain constant during the whole experiment. Due to the effects mentioned above, the mass resolution is decreased. The initial kinetic energy distribution can be partially focused by using delayed extraction. In this technique, a time lag is introduced between the ion creation and their acceleration. By doing so, the ions with the higher kinetic energy will fly faster in the extractor direction. When the electrical field is applied, these ions will be closer to the extractor and will therefore feel a less intense electrical potential than ions with lower initial velocities. This results in a refocusing of the ions on the detector. The Fig. 2.2.4 describes the process.

Fig. 2.2.4: Principle of the delayed extraction. The ions are created during (or shortly after) the laser pulse. With delayed extraction, the ions are not immediately accelerated because the repeller and the extractor are at the same voltage. Only after a variable delay (usually in the order of several hundred of nanoseconds), the electrodes are set at different voltages and the ions accelerated. In our set-up, the repeller was switched to a higher voltage.
With this set-up, a much higher mass resolution can be obtained. As an example, a mass resolution with continuous extraction of ca. 300 was observed on our instrument, for substance-P, a small 1300 Da protein. With delayed extraction, this value approached 1000.

One disadvantage of delayed extraction is its inability to re-focus the ions independently of their masses. In other words, only a certain mass range is well resolved. Larger and smaller ions show a mass resolution that is sometimes worse than with normal continuous extraction.

2.3 Ion detection in TOF

2.3.1 Principle

An ion detector in a TOF is a device that senses the ions arriving on its surface. The times that the ions took to cross the instrument are then calculated. These flight times can then be transformed into (m/z) values using the calibration procedure described in Chapter 2.2.2. Many different detection methods exist but their final goal is to transform the primary ion current into an electrical current. An ion detector should have the following features to be efficiently used in TOF applications:

i) Sensitivity. Even though MALDI produces a significant amount of ions, the number of charges are small and the detector needs to be sensitive. An ideal detector would have a sensitivity independent of the ion velocity and the ion chemical composition.

ii) Speed. Ions are accelerated in TOF with energies usually on the order of some tens of kilovolts. Each different ion packet (or single ions in the case of ion counting) is therefore flying in the TOF with large velocities (usually km/s). To be able to distinguish between ion packets that have very close velocities, the detector must have a short time response (usually ns).

iii) Low noise. Noise is a statistical signal that interferes with the ion signals. It is common to calculate the signal-to-noise ratio of a signal to quantify the importance of the noise. This signal-to-noise ratio can be improved by summing experiments. However, single event counting experiments will not be successful if noise signals are larger than ion signals.

iv) Good electron amplification. As already mentioned, the ion number is too small to be used without amplification. This amplification is achieved by transforming the ion current into an electron current that is then amplified.
A typical electron amplification factor (gain) is between $10^6$ and $10^8$.

v) Short recovery time. After receiving the first signal, many ion detectors have a period where they need to recover and are therefore not able to detect another ion packet arriving during this time. This effect can be crucial since, in TOF measurements, it is often the case that ion packets arrive in very small intervals.

vi) High dynamic range. A detector is linear if its output electron current is proportional to the amount of ions that hit its surface. It depends on the ion current and on the detector working conditions. This linearity simplifies quantitative measurements. When the primary ion current is too large, the output electron current of the detector is no longer proportional to the ion current. The detector is then saturated. In some cases, this effect can damage the detector.

vii) Large working area. In TOF applications, it is advantageous to have a detector with a large working area since the ion beam dimensions are relatively broad. It would be possible to focus this beam. In practice, however, this is not often used because the ion density on the detector is then increased, increasing the risks of saturation.

viii) Other considerations: Low price, robustness, ease of implementation and use are important in the design of detectors.

The main types of ion detectors used in TOF applications are reviewed in the following chapters with attention given to their respective advantages and disadvantages. We will see that the need for an optimized ion detector, which is capable of detecting efficiently light and heavy molecules, inexpensive and easy to use is still a necessity. In Chapter 4, an alternative detector will be described and tested.

### 2.3.2 Secondary electron multipliers

In most TOF mass spectrometers, the ion signal is amplified using a secondary electron multiplier (SEM), e.g. microchannel plates (MCP) or Channeltron® detectors. The principle of such detectors is the following: when a particle with kinetic energy impinges a solid surface, secondary particles (ions, neutrals and electrons) may be created. The created electrons are then amplified to reach a readable current.
2.3.2.1 SEM with discrete dynodes

In a SEM detector with discrete dynodes, the electrons created on the so-called "conversion dynode" are accelerated with an electrical field, to a second surface (dynode) where the impact will create more electrons. This conversion/amplification phenomenon is repeated several times on further dynodes. Typically these detectors consist of 8 to 12 discrete dynodes. After this type of avalanche amplification, a charge is created on the last dynode (collector) that can be electronically amplified and recorded. This system is schematically described in Fig. 2.3.1.

![Secondary electron multiplier detector principle.](image)

*Fig. 2.3.1: Secondary electron multiplier detector principle. The particle enters the detector and hits the first dynode. The electrons created during the impact are accelerated to a second dynode and the process is repeated. The last dynode collects the charges. With virtually grounded collector, the high voltages (HV) are negative and |HV1| > |HV2| > |HV3| > ...*

2.3.2.2 SEM with continuous dynodes (Channeltron and MCP)

SEM with continuous dynodes are based on the same principle as that of discrete dynodes but the dynodes are no longer discrete, rather they are transformed into tubes. The electrons are accelerated by an electrical field through the tube. The size of the amplification channel is variable: microscale for microchannel plates (MCP) and up to macroscale for Channeltron type detectors.

The shape of a Channeltron detector can vary. Two examples are shown in Fig. 2.3.2. The first detector is a "horn-shaped" and the second "spiral-shaped". Primary ions enter the cone and hit the inner side of the detector. The produced electrons are accelerated to the inside of the tube by the electrical field applied between the input of the detector and the collector. They are amplified by the same avalanche reaction as described above. For some TOF applications, the intrinsic time-response of these devices is too slow.
Fig. 2.3.2: Two examples of Channeltron detectors. The impact of the ions on the cone creates secondary electrons that are then accelerated inside the tube by an electrical field. Subsequent impacts on the walls amplify the electron amount.

An MCP is an array of miniature electron multiplier tubes oriented parallel to one another (see Fig. 2.3.3). Their diameter varies typically between 5μm and 25μm for mass spectrometry applications. The inside of the glass tube is treated to optimize secondary electron emission and all the channel-ends are electrically connected together by a metallic deposition. An electrical field is applied through the plate to accelerate the electrons. The channel axes are usually biased to the MCP surface (<10°) to favor the contact between the primary particles and the inside of the channels. For higher gain, MCPs can be mounted in pairs being rotated 180° from the one other. This configuration, called "Chevron" allows electron multiplication factors as high as 10^8.

Fig. 2.3.3: Microchannel plate detector. The secondary electron multiplication principle is the same as that of discrete dynodes. The dynodes consist of micro-tubes stacked together. An electrical field is applied through the plate.
2.3.2.3 The secondary electron emission yield

The production of electrons caused by the impact of ions with a surface is a complex phenomenon. As discussed in more detail in Ref. 30, two independent mechanisms have been proposed to explain this phenomenon: potential emission and kinetic emission. The total electron production is in fact the sum of these two contributions.

The kinetic electron emission is caused by the direct interaction between the impinging particles and the surface. This becomes particularly important for large ion velocities (>10^5 m/s). Different models have been proposed to describe this mechanism for simple atomic systems. These models include the notion of a velocity threshold, below which the kinetic electron emission is absent. Due to a lack of experimental evidences, velocity thresholds are difficult to estimate.

The potential electron emission is preponderant for slower particles. The potential energy of the projectiles is dissipated through Auger effects. This mechanism requires that the impinging particle is charged. The interaction starts already before the ion reaches the target and, for atoms, its effect increases with ion charge.

These mechanisms are well studied for large velocities and simple atomic systems. Slower molecular ions showed more complicated behavior.

At constant ion energy, Chaurand et al. showed that the electron emission yield is strongly dependent on the molecular ion mass (and therefore on the ion velocity). Their result is depicted in Fig. 2.3.4. The global conclusion that can be drawn from these experiments is that the detection efficiency of an ion, at constant initial energy, decreases with its mass.

In a more general manner, the dependence of the electron emission yield (γ) was found to be a function of the projectile mass and of its velocity. The same research group found a general dependence of the form:

\[ \gamma_e = M^{0.6} f(V) \]

where \( \gamma_e \) is the electron emission yield, \( M \) the ion mass, and \( V \) the ion velocity. The function was close to \( V^4 \) for small values and linear with larger velocities. Geno and MacFarlane found a different relationship where \( A, B \) are constants:

\[ \gamma_e = A M^B V \]

where \( A \) and \( B \) are constants.
Fig. 2.3.4: Secondary electron emission yield as a function of the ion molecular mass. All the ions have 18 keV energy and are impacted on a CsI surface. This graph is reprinted from Ref. 30 with permission.

2.3.2.4 Advantages and Limitations of SEMs

Some of the advantages and limitations of SEMs are listed below:

i) As previously discussed, we saw that the primary ion-to-electron conversion step is strongly velocity dependent\(^ {37, 38, 36} \), making detection of heavy ions in TOF difficult since \( v = \sqrt{2E/m} \), where \( v \) is the velocity, \( E \) the energy, and \( m \) the mass.

To improve the conversion yield, higher acceleration potentials have been used (up to 50kV). In addition to the fact that high voltages often lead to undesired electric discharges, flight times are also reduced requiring fast and expensive oscilloscopes and electronics. Another closely related approach is to post-accelerate the ions with an electrical field in front of the
detector to increase the ion velocity and thereby the sensitivity of the detection.

ii) Heavy ions, such as those produced by MALDI, were also shown to preferentially create secondary ions instead of electrons when impacting on a surface. For this reason, large molecules should be broken into smaller pieces prior the detection to enhance secondary electron production. A grid placed in front of the detector is often used for this purpose. One of its functions is to multiply the number of ions arriving at the detector surface by secondary ion emission. These secondary ions are also accelerated onto the detector and the signal intensity is increased. Unfortunately, their flight times are all slightly different from the primary ion's flight time. The consequence is often a loss in mass resolution.

iii) These detectors are usually operated at high voltages, which increases the risk of electric discharges. This is particularly true when post-acceleration is used. MCPs are very sensitive to electrical sparks and can easily be destroyed by one discharge. Extremely high post-acceleration voltages (50kV) are therefore not used frequently in such set-ups.

To avoid this problem, a modified ion-to-electron detector was developed by Daly. In this detector, the secondary electrons created by the impact of the primary ions onto an aluminum target are accelerated onto a plastic scintillator, giving rise to a light signal detected by a photomultiplier (PMT). In this set-up, the conversion and the signal amplification are physically separated and the problem of electric discharges notably reduced. A commercial implementation of this principle is used by Micromass (previously Fisons).

iv) The maximal working pressure is limited as well as their robustness to resist against chemicals. Although some MCP factories have started to develop devices capable of working under higher pressures, pressures higher than 10^-5 mbar remains unusual because of increased risks of electrical discharge, higher background noise and shorter life-time. Exposure to water, oil or other chemical compounds must be absolutely avoided as the detector can be destroyed or the active conversion surface rendered inefficient. As a consequence, MCPs are usually kept under vacuum when not in use.
2.3.3 Inductive detectors

Ion detection methods, which do not rely on ion-to-electron conversion, have also been reported. Park and Callahan developed a non-destructive inductive detector working on the same principle that is used in FT-ICR-MS (Fourier transformation ion cyclotron resonant mass spectrometry). As described in Fig. 2.3.5, it senses the image charges induced as an ion flies past an electrode. The resulting signal shape looks like depicted in Fig. 2.3.5. The time resolution of this design is still not satisfactory for many applications.

Fig. 2.3.5: Inductive detector. It consists of three grids. The external grids are held at a fixed potential and act as energy filters. The central grid is connected to the amplification and acquisition system. It senses the charges approaching it. This is a non-destructive detector.

2.3.4 Faraday cup detectors

Direct charge measurements (Faraday cup detectors) have also been successfully used in TOF-MS. This type of detector has no inherent gain and signals have to be amplified externally. Bahr et al. estimated that the minimum number of singly charged ions detectable by their device was about 18,000.

In a Faraday cup detector, the charges are collected on a metal surface. To avoid sputtering, the detector usually contains an additional ring that repulses the sputtered particles back to the dynode. The geometry of this kind of device must be precisely controlled to minimize the artifacts.
2.3.5 Cryogenic particle detectors

These types of detectors are operated at cryogenic temperatures, typically below 1K, and are sensitive to the energy deposited by a single particle. Twerenbold et al. 46 and Benner et al. 47 are developing cryogenic particle detectors. They measure the total deposited kinetic energy and therefore have a mass independent detection efficiency. Although many different types exist, they all work on the principle that, at very low temperature the thermal energy is small enough so that the perturbations of a system, caused by a particle, become measurable. For example, phonon-mediated detectors sense the temperature increase induced by the impact of the projectile on the detector. Superconducting tunnel junction detectors consist of two superconducting films separated by a thin insulating barrier. The impact of the projectile breaks the charge equilibrium within one of the superconductor film and a tunneling current is created through the insulator. This current is proportional to the energy deposited and therefore to the amount of particles that impinged the surface. D. Twerenbold reviewed in detail the different types of cryogenic detectors in Ref. 48. Up to now, their small active areas (typically 0.04 mm² in Ref. 47, 0.0016 mm² in Ref. 46, and more recently 0.15 mm² (3mm/50 µm) [personal communication from D. Twerenbold]), as well as the costs and difficulty of ultra low temperature techniques, are the principal barriers to wider utilization. However this approach is still promising. The time responses of such detectors are larger than 100 ns. Unless very small ion acceleration energies are used, this is not sufficient to resolve light ions.

2.3.6 Ion-to-photon detectors

When ions with kinetic energy impinge on a surface, it may result in the production of light sparks. We will describe in Chapter 4 the possibility of using this phenomenon to build a useful ion detector. The general phenomenon is not new: in 1950, Richards and Hays 49 used light flashes produced by impacts of particles onto a scintillator to detect small ions, such as potassium and rubidium, in a mass spectrometer. In their study, silver-activated zinc sulfide, thallium-activated sodium iodide, and anthracene were used as ion-to-photon conversion materials. Ionoluminescence has also been reported for the impact of small ions onto metallic surfaces 50, 51. Low energy (10-30 keV) H⁺ and He⁺ ions incident on polycrystalline aluminum were shown to give rise to broadband emission, mostly in the visible region. Daly 40 made a significant contribution to the utilization of photons to detect ions. A schematic of his detector is shown in Fig. 2.3.6. In this device, the ions are first transformed into electrons by the impact of the beam with a metallic dynode. The electrons are then accelerated to a scintillator and the light produced is detected by a photomultiplier.
Fig. 2.3.6: The Daly detector, reprinted from Ref. 40. The positive ion beam is accelerated on a conversion dynode. This conversion dynode is held at a negative voltage. The electrons created are accelerated to the scintillator held at ground and coated with an aluminum layer. The photo emission is detected by a photo-multiplier.

Kaercher et al. 52 demonstrated the possibility to simultaneously detect secondary ions and photons produced by the impact of keV Csl clusters with masses up to ca. 1000 Da on a Csl conversion dynode. A PMT, operating under vacuum, as well as a MCP, were placed in front of the Csl target to collect two types of signals. Considering the solid angle of photon collection and the quantum efficiency of the PMT, a poor overall quantum efficiency of ca. 0.3% was estimated. In comparison, the same estimation for the MCP gave ca. 50%. Secondary ions and photons were found to be produced independently of each other through different mechanisms. The authors observed a velocity dependence of the photon production. Moreover, for the clusters studied, they found that unlike the secondary ion yield, the photon yield was not dependent on the primary ion's complexity. The excitation energy deposited on the target electronic system could be correlated with the photon emission yield.
More recently, light emission was used, in parallel with secondary ion detection, in an electrospray quadrupole mass spectrometer. A detection efficiency on the same order as the one reported by Kaercher et al. was calculated for multiply charged ions. These authors estimated a photon emission yield on the order of 0.5 photons per ion for 55 km/s apo-myoglobin and lysozyme ions (16'951 Da and 14'306 Da, respectively) impacting on different target materials such as CsI, quartz, stainless steel, Si, Au, or Ta. The emission yield was not found to be strongly dependent on the target material.

We developed an ion-to-photon detector (IPD) that was designed to collect a large fraction of the light produced. This detector will be the subject of the Chapter 4. It is based on the detector used by Knochenmuss et al. In that design, the surface of a photomultiplier was coated with a thin layer of a scintillator compound, and placed directly in the ion beam.

### 2.4 Data acquisition

Detector signals can be processed by using a transient recording or single event counting system. Both methods have been used in this work. To understand the fundamental differences between them, they will be briefly described.

#### 2.4.1 Transient recording

The electron current coming out of the detector is transformed into a voltage signal by passing through a resistance. In a transient recorder, this voltage signal is monitored as a function of time. The amplitude of the signals in the recorder therefore reflects the amplitude of the detector current.

Final signal intensities for TOF measurements using a transient recorder are the result of different processes:

\[
\text{Signal current} = N \cdot Y_{\text{conversion}} \cdot Y_{\text{collection}} \cdot \text{QE} \cdot \text{Gain}_{\text{electrons}}
\]

\(N\) is the number of impinging ions per time, \(Y_{\text{conversion}}\) the conversion yield of ions into either photons or electrons (for the IPD and MCP, respectively). \(Y_{\text{collection}}\) is the collection efficiency and \(\text{QE}\) the quantum efficiency describing the probability to detect the secondary particles (photons or electrons). \(\text{Gain}_{\text{electrons}}\) is the electron amplification factor of the SEM chain. All these processes are shown in Fig. 2.4.1.
2.4.1 The transformation of ions into a current (I); the case of the IPD. The ion packet contains “N” ions. The impact of these ions on the scintillator creates a certain amount of photons (other secondary particles are also created but they do not contribute to the final electron signal). 

\( Y_{\text{conversion}} \) describes the number of photons created per incident ion. Only a certain fraction \( Y_{\text{collection}} \) of the photons are emitted in the photomultiplier direction. The photomultiplier itself only sees part of the photons and transforms them into electrons. This yield is called the quantum efficiency (QE). The electrons are then amplified in the secondary electron multiplier chain contained in the photomultiplier. The amplification factor is the gain of the detector. It strongly depends on the voltage at which the photomultiplier is set.

In this mode, the amplification or reduction in the signal intensity is strongly influenced by the detector specifications (Gain, QE, \( Y_{\text{collection}} \)), by the conversion process \( Y_{\text{conversion}} \) and ion number (N). It is therefore difficult to separate the effects due to each process. To study more carefully the conversion process, the single event counting technique described below is preferred.

2.4.2 Single event counting mode

For single event counting experiments, a low rate of arriving ions is necessary so that only up to one ion (event) reaches the detector at a time. The output signal of the detector is introduced into a constant fraction discriminator (CFD). A CFD is an electronic device that gives a logical signal. This logical signal is equal to “1” if the detector output intensity (transformed into a voltage by a resistance) is larger than the CFD threshold. In the opposite case, it returns “0”.

\[ Y_{\text{conversion}} \] describes the number of photons created per incident ion. Only a certain fraction \( Y_{\text{collection}} \) of the photons are emitted in the photomultiplier direction. The photomultiplier itself only sees part of the photons and transforms them into electrons. This yield is called the quantum efficiency (QE). The electrons are then amplified in the secondary electron multiplier chain contained in the photomultiplier. The amplification factor is the gain of the detector. It strongly depends on the voltage at which the photomultiplier is set.

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Chapter 2

CFDThreshold time

Fig. 2.4.2: Functioning of a constant fraction discriminator (CFD). If the signal intensity is lower than the CFD threshold value then the CFD returns no count. In the opposite case, an additional count is added. The CFD threshold can be manually varied.

In TOF applications, one detector/CDF system detects the creation of the ion in the source (start signal) and a second system detects their arrival at the end of the flight tube (stop signal). The time difference between the start signal and the stop signal is recorded and an additional count is added at the corresponding time. Signal is accumulated to obtain good peak-to-background ratios. Similar to transient recording, the signal can be described as follows:

\[
\text{Count rate} = \frac{dN}{dt} \cdot Y_{\text{conversion}} \cdot Y_{\text{collection}} \cdot \text{QE}
\]

The electron gain no longer influences the count rate so strongly. As soon as the signal intensity from a single event is above the CFD threshold, the gain of the electron amplification chain does not influence the count rate. With a constant CFD threshold, the technique gives no information about the effective value of the signal current, or the number of secondary charges generated per input ion. It is however possible to study the pulse height distribution by varying the CFD threshold. An example of the pulse height distribution for Cs⁺ and Cs₂I⁺ measured with the IPD is shown in Fig. 2.4.3. The ion energies were kept constant (22 keV) and the threshold value was varied. The count rates were reported as a function of the threshold value.
A more convenient way to display the data is obtained by deriving the curve to give the pulse height distribution for each mass (Fig. 2.4.4). The maximum of this distribution was found at the same value (ca. 16 mV) and the distributions had very similar shapes. Only one peak in the distribution was observed, no further peaks were found at half or double of the first peak value. The CFD threshold in our experiments was set at its minimum (7 mV). This value is suitable because it is lower than most of the one-event pulses and is above the noise.
Fig. 2.4.4: Pulse height distribution for 22 keV Cs$^+$ and Cs$_2$I$^+$ ions detected with the IPD. The curves shown in this figure were obtained by derivating the curves count rate vs. threshold of Fig. 2.4.3.

### 2.5 References


42. B. N. Laprade and R. Cochran, in Operation of Microchannel Plate Based Detectors at Elevated Pressure, Galileo, Corp. (1998).


Chapter 3.

3. Experimental

3.1 Introduction

The MALDI TOF experiments were performed mainly with an instrument in our laboratory at ETH Zurich. This home-built mass spectrometer as well as the pumping systems, the laser, and the data acquisition will be described below. Some experiments were performed in Orsay (University of Paris-Sud, France) in the group of Prof. Y. Le Beyec and in Münster (University of Münster, Germany) in the group of Prof. F. Hillenkamp. The corresponding instruments will be described briefly in Chapter 4.2.9 and 4.3.9, respectively. The ion detectors developed during this work are also depicted in detail.

3.2 The MALDI time-of-flight

3.2.1 The TOF and the pumping systems

The linear MALDI time-of-flight that was used for the large majority of the experiments is described below in Fig. 3.2.2. It consisted of a home-built instrument with a flight path of 2 meters. In its middle a valve was mounted to separate the optics chamber from the detection side. The optics chamber was evacuated with a diffusion pump (Balzers, Model DIF200) equipped with a cold water trap. The fore-vacuum was obtained by a two-stage mechanical pump (Leybold; Model Trivac 16B). The detection chamber was pumped with a turbo-molecular pump (Leybold, Model Turbovax 151) connected with a two-stage mechanical pump (Alcatel, Model Pascal 2015). The working pressure inside the tube was typically $10^{-6}$ mbar or lower. Two ion gages situated in both chambers allowed monitoring the pressures.
3.2.2 The ion optics

The optics was a home-designed two-stage acceleration arrangement (Wiley-McLaren extraction type). Two pairs of plates could be added to the system to deflect the ions horizontally and vertically. The first electrode (repeller) was designed to receive the probe-tip and was set to the highest voltage (typically 25kV). The middle electrode (extractor) was set at 20kV when continuous extraction mode was used and at 21kV for delayed extraction. Both voltages were generated by two reversing power supplies from Bertan (Model 2341). The third electrode was grounded.

Two sets of ion-optics were used during this work. The electrode holes of the first one were covered with gold high transmission micro-grids (87% transmission, from Buckbee Mears, St. Paul, MN, USA) to flatten the electrical field lines. The newest optics was built without grids to avoid surface induced fragmentation of the primary ions on the grids. A schematic of it is shown on Fig. 3.2.1. Dimensions are proportional to the real set-up. If necessary, deflection plates could be mounted for both vertical and horizontal directions. In that case, “E” was removed and replaced by two parallel electrodes: one grounded and the other one connected to the high voltage. The second pair of plates was placed after “F”.

Fig. 3.2.1: Second ion optics utilized in our TOF. The electrodes were gridless. (A) probe tip; (B) repeller electrode, set at the highest voltage; (C) extractor electrode, set at intermediate voltage; (D), (E), (F) electrodes set at ground; (G), (H) isolating spacers, made out Macor®; (I) plastic isolating washers.
The sample holder “A” was introduced in a pre-vacuum chamber where the air was evacuated with a two-stage mechanical pump and then pushed to contact the repeller inside the flight-tube. The surface where the sample was deposited was a disc of about 3mm diameter.

3.2.3 The laser and the data acquisition

The UV-laser (LSI, MA, USA, Model VSL-337ND) had the following specifications: emission at 337.1 nm, 3 ns pulse duration, maximum pulse energy of 250 µJ and a repetition rate up to 10 Hz. The laser was directed into the vacuum chamber with three dichroic mirrors. The beam was focused with a lens (f=50 cm) and the energy density could be reduced with an iris. Part of the non-reflected beam was detected behind the first mirror with a photodiode (model 210/579-7227, Thorlabs Inc., NJ, USA) and used to trigger the digital oscilloscope (Lecroy, Model 9420, 350 MHz, 10 ns per sample). Recently we purchased a faster oscilloscope (Lecroy, Model 9350C, 500 MHz, 1 ns per sample), necessary when using delayed extraction.

The signal coming out of the detector (microchannel plate or else) was generally amplified with a pre-amplifier (model 322-1-B-50, Analog modules Inc., FLA, USA) and fed into the scope.

The laser itself was triggered from the PC (486) or from an external trigger pulse generator. The computer was connected with GPIB (IEEE Standard) cables to the oscilloscope to download the signal. Data signal averaging, smoothing and treatment were performed directly on the computer with the tools contained in the Hewlett-Packard software (G2025A Software A.02.01-IEEE).
Fig. 3.2.2: MALDI TOF instrument of Zurich. The different parts are described below

1) 2 m time-of-flight tube equipped with a central valve
2) Front pumping system: diffusion pump/mechanical pump
2') Back pumping system: turbomolecular pump/mechanical pump
3) Two-stage ion-optics: the "Repeller" is at the highest voltage and contains the probe holder. The "Extractor" is at intermediate voltage, and is followed by a grounded electrode
4) Reversible high voltage power supplies used for ion acceleration
5) Microchannel plates detector
6) High voltage power supply
7) UV-Nitrogen-laser (337 nm)
8) Dichroic UV-mirrors
9) Photodiode
10) Focusing lens
11) Low voltage power supplies (12, 24 V) and laser trigger generator
12) Digital oscilloscope
13) Personal computer
3.3.4 Delayed extraction

Delayed extraction was used for some experiments. It consisted of a time delay generator and of a fast high voltage switch that changed the repeller voltage from the intermediate voltage (equal to the extractor voltage) to the highest voltage (typically from 21 kV to 25 kV). The electronics were built in our laboratories by A.L. Weissberg. It was designed for positive voltage polarity. It contained a fast transistor switch (model HTS 81, Behlke, Germany). The total switch rise time was 9 ns. The schematics of the two parts are shown in Fig. 3.2.3 and 3.2.4.
Fig. 3.2.4: Time delay generator used for delayed extraction. The time delay could be adjusted between 5 ns and 1 µs. Adapted with permission [A.L. Weissberg]
3.3 The IPD and the IEPD

The IPD (Ion-to-Photon Detector) was developed first. The IEPD (Ion-to-Electrons-to-Photons) is a modification of the IPD. They both used the same head-on photomultiplier encapsulated in an anodized aluminum box. This box isolated the detector from the outside light.

The photomultiplier and its shielding box were mounted on a flange (CF-150) containing a 4 cm diameter hole. In this hole, an acrylic transparent window was introduced. The vacuum tightness was obtained with an O-ring.

The voltage distribution on the photomultiplier was as proposed by the manufacturer (Hamamatsu) and is depicted in Fig. 3.3.1. With this resistance and capacitor chain, a suitable detector time response is obtained (3-4 ns). A modified set-up is also proposed by Hamamatsu for a better resistance to signal saturation, to the detriment of the detector time response.

![Voltage division of the photomultiplier](image)

Fig. 3.3.1: Voltage division of the photomultiplier. We used $R_1=200 \, k\Omega$, $R_2-R_{11}=100 \, k\Omega$, $C_1-C_4=0.1 \, \mu F$

On the IPD and IEPD, the organic scintillators were deposited on a transparent substrate (quartz or plastic window). CsI or the commercial scintillators were simply mounted as bulk materials. The scintillator and post-acceleration system were fixed on the detector flange in front of the photomultiplier as shown in Fig. 3.3.2. A schematic view of the ensemble is shown on Fig. 4.3.1 of Chapter 4.
The IEPD consisted of a plastic scintillator plate (1 mm thick) held by a metallic ring, as shown in Fig. 3.3.2 (1). In front of it, two metallic rings (2 and 3) were used to delimit a field-free-region in front of the scintillator. Ring (4) was made out of plastic (Polyvinyl chloride, PVC) and was electrically isolating. The conversion dynode (5) was screwed on the metal ring (6). In some cases, an additional ring, covered with a 95% transmission copper grid (7) was fixed in front of the conversion ring. In between was a plastic ring (8). Voltages were brought into the chamber by two high voltage feedthroughs (9) (Lemo, model FFA.3S). The transparent Plexiglas® window (10) separated the vacuum chamber from the atmospheric pressure. The head-on photomultiplier was mounted behind it.
Chapter 4.

4. **ION-TO-PHOTON DETECTORS**

With the development of modern ionization methods such as MALDI, very large molecules can now be transferred from the condensed phase to the gas phase and ionized for mass spectrometric analysis. As mentioned in Chapter 2.3.2, the detection probabilities in TOF mass spectrometers equipped with SEM detectors drops for large ions. This is due to the fact that the ion velocities become too small to efficiently produce the secondary electrons that are eventually detected. Thus, the search for a detector with improved efficiency for slow moving particles is still a challenging task.

In an attempt to contribute to that search, we have developed an ion-to-photon detector. It detects the light produced by ions impinging on a scintillator surface. In this chapter, two devices will be presented and tested to verify if they could be of interest for the common MALDI user in terms of usability, mass resolution, and high mass detection. In the first device, the Ion-to-Photon (IPD), the ions impact directly on the scintillator and create photons that are detected by an external photomultiplier. The construction is very simple and, as will be shown, sufficient for many applications. The second set-up includes an additional conversion step: the primary ions are transformed into secondary particles (electrons and ions) that are then accelerated to the scintillator. This second construction will be called the Ion-to-Electron-to-Photon-Detector (IEPD). It needs an additional high voltage generator but proved to be very efficient in detecting bio-molecules.

4.1 The scintillation effect

Scintillation is a general term to describe the processes by which a molecule, excited by highly energetic particles or quanta, emits light during its de-excitation. In organic scintillators, this is mainly due to fluorescence. The incident particle energy is consumed by ionization and excitation of the scintillator material. Only a small fraction is effectively transformed into light emission, the rest being liber-
ated by heat and other non-radiative processes. Scintillators are used in crystalline form, liquid form (dissolved in a solvent), plastic form (embedded in a solid matrix), and even in gas form (see Ref. 2 for an introduction to organic scintillators). They are not always present at large concentrations. For example, some liquid scintillators are prepared with only $10^{-4}$ M of the active material. Even at such low concentrations, the technique is efficient. Why? Ageno et al. 3, 4 showed that the energy in organic and liquid scintillators is transferred over distances of up to a few millimeters in the bulk of the scintillator. To explain this energy transfer, two main models have been proposed: the nonradiative transfer and the radiative transfer. In the nonradiative transfer model, the energy of an excited molecule is given to a neighboring molecule. The process is repeated. This model is also known as the “exciton migration” model. According to the radiative model the energy transfer occurs by the re-absorption of a photon emitted by a first molecule further in the bulk. Also called the “photo-cascade” theory, this model was introduced by Birks 5.

The scintillation efficiency of a material is usually given by its efficiency relative to a reference material. An anthracene crystal is frequently chosen as the reference. In Chapter 4.2.2, the scintillator efficiencies relative to anthracene will be given.

Scintillators are used in many applications. They are, for instance, frequently utilized to measure radioactivity 6. Liquid and solid preparations are normally used for this purpose. The liquid preparation is more sensitive and is often preferred to measure, for instance, the low energy $\beta^-$ emitted by $^3$H or $^{14}$C. In a typical experiment, the sample and the dissolved scintillator are mixed together. Surrounding the container, a photomultiplier system detects the light emission, which intensity is proportional to the radioactivity. Other radiations, such as $\alpha$, $\gamma$, and X-rays, can also be detected with similar techniques. Solid scintillators are, for instance, used in X-rays diffraction experiments (in physics and in medicine).

Scintillators are also used in scanning electron microscopes to monitor the image of the sample or to adjust the electron beam position and intensity. In nuclear physics, giant scintillation detectors are used to detect neutrino and other particles.
4.2 The IPD

4.2.1 The IPD set-up

The set-up of the IPD was designed to collect a large fraction of emitted photons (ca. 20%, see Chapter 4.2.2), by placing the photomultiplier tube (PMT) directly behind a large area conversion surface. The commercial head-on PMT (Hamamatsu, model R2154-02, 2” diameter, 26% max. quantum efficiency at 380 nm) was mounted, as shown in Fig. 4.2.1, in the center of the end flange, on the ion beam axis. A transparent acrylic vacuum window enabled the PMT to be operated at atmospheric pressure. The conversion of ions to photons was achieved at the end of the time-of-flight tube using a piece of window glass coated with the conversion material.

![Schematic of the IPD](image)

**Figure 4.2.1: Schematic of the IPD. The head-on photomultiplier is mounted in the center of the end flange of the TOF and separated from the vacuum by an acrylic window. The scintillator is deposited on a plate in front of the photomultiplier.**

4.2.2 Scintillators

Several ion-to-photon conversion materials were investigated in these experiments, and large differences in photon yields were observed. In contrast to many of the applications in physics that often use the dissolved materials, solid films of the pure compound were used.
The following compounds were tested: 2-(4-t-Butylphenyl)-5-(4-biphenylyl)-1,3,4-oxidiazole (Bu-PBD), 2,5-Bis(5'-tert-Butyl-2-benzoxazolyl) thiophene (BBOT), 2-(4'-Biphenyl,1-6-phenylbenzoxazole) (PBBO), and Rhodamine B. The chemical structures of some of those molecules are shown in Fig. 4.2.2.

Non-organic materials were also tested including plain glass, acrylic plastic, CsI, and sapphire. These samples were mounted as bulk materials. Commercial inorganic phosphor materials were also investigated. We tested in particular two samples from the Levy Hill Laboratories (20 mm diameter, Standard and Aluminized discs, coating: P47). A piece of NE102A and BC-404, two commercial plastic scintillators, were also tested.

The fraction of light collected by the photomultiplier was estimated by exciting the scintillator with laser pulses and measuring with a pyroelectric detector the light emitted in the front and in the back of the scintillator at symmetrical positions (Fig. 4.2.3). A UV cutoff filter was placed directly in front of the detector to remove the reflected laser light. Assuming spherically symmetric emission, we found transmission values ranging between 20 and 85%.
Figure 4.2.3: Set-up used to estimate the fraction of the scintillator light transmitted vs. reflected. The sample was irradiated with a \( N_2 \)-laser. The amount of emitted light (usually blue) was measured with pyroelectric detectors (detector A and B) at symmetrical positions. The \( N_2 \)-laser wavelength was removed with a UV-cutoff filter.

The light transmissions for some scintillators, as well as their specifications, are shown in Table 4.2.1 (From Refs. 2, 7, 8).

Table 4.2.1: Specifications of some scintillators

<table>
<thead>
<tr>
<th>Scintillator</th>
<th>Light output (% anthracene)</th>
<th>Decay time [ns]</th>
<th>Wavelength of max. emission [nm]</th>
<th>% Transmitted to the photomultiplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBOT</td>
<td>30</td>
<td>1.6</td>
<td>432</td>
<td>20</td>
</tr>
<tr>
<td>NE102A</td>
<td>65</td>
<td>2.4</td>
<td>423</td>
<td>85*</td>
</tr>
<tr>
<td>BC-404</td>
<td>68</td>
<td>1.8</td>
<td>408</td>
<td>45</td>
</tr>
<tr>
<td>Bu-PBD</td>
<td>51</td>
<td>1.2</td>
<td>368</td>
<td>-</td>
</tr>
<tr>
<td>CsI @ 70 K</td>
<td>500</td>
<td>600</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>Anthracene</td>
<td>100</td>
<td>30</td>
<td>448</td>
<td>-</td>
</tr>
</tbody>
</table>

*The NE102A was coated with a 40 nm thick layer of aluminum to reflect photons emitted in the direction opposite to the photomultiplier. Only electrons could penetrate the aluminum at the applied energies. All heavier particles were stopped in the metal layer.
Unlike Sullivan et al. 9, substantial variation in the ion-to-photon conversion efficiency was observed due to the different materials. Although CsI is known to be a good ion-to-photon converter, some organic scintillators were found to be more efficient. Bu-PBD 10 was qualitatively found to give the highest photon yield of all tested materials in these experiments. A quantitative approach to comparing the photon yields will be presented in Chapter 4.2.9.

4.2.3 Scintillator layer preparation

Organic scintillators, such as BBOT and Bu-PBD, were obtained as powders. To prepare the scintillator film, we usually sprayed a solution of the material onto the glass plate using an air brush (Fig. 4.2.4).

![Image of air nebulizer](image)

*Figure 4.2.4: Air-nebulizer utilized to deposit a fine and homogenous layer of the organic scintillator onto a glass plate.*

High macroscopic surface homogeneity could be obtained with this technique so that the signal intensity will not be affected by the position where the ion package hits the glass. The homogeneity of the sprayed layer was dependent on the scintillator material and on the solvent used to dissolve it. Bu-PBD was, for instance, an efficient compound but the surface layer was difficult to prepare. Instead of flat surface, it formed long and fine needles.

The solvent also affected the quality of the surface. In addition to dissolving the scintillator, the air-spraying technique requires that the solvent evaporates quickly when deposited on the glass plate. Otherwise droplets are formed resulting in inhomogeneous samples. The optimum solvent for many scintillators, such as Bu-PBD and BBOT, was chloroform. If the glass on which the material is deposited is heated (~60-80°C), other solvents such as methanol were successfully used. The surfaces that resulted were very similar.
4.2.4 Surface charging on the scintillator

Surface charging on the scintillator was suspected to be one reason for signal loss: the charge accumulated on the conversion surface may repel and deflect the ion packages away from the active detection area. In other words, the large ions arriving on the converter after the matrix ions would be detected less efficiently. To investigate this possibility, two identical scintillator samples (Levy Hill Laboratories) were used, differing only in their surface. Both were deposited on glass, but one was coated with a 5 nm layer of aluminum on the top of the film. This latter sample was connected to ground. The surface resistance was measured to ensure sufficient electrical conductivity of the aluminized surface, because aluminum may become oxidized, forming non-conductive Al₂O₃. A resistance of 40Ω was found across the 2 cm diameter of the aluminized disc. The non-aluminized sample had a resistance higher than 10 MΩ. MALDI time-of-flight measurements showed that no significant improvement was obtained when grounding the scintillator, indicating that surface charging was not affecting our measurements negatively.

4.2.5 MALDI instrument & sample preparation

All experiments were carried out in Zurich with our 2m linear TOF previously described in the experimental part of this work (Chapter 3). Both MCP and IPD detectors were mounted on interchangeable flanges. The change of detectors could be achieved relatively rapidly (1-2 hours) due to an independent pumping system for the detector region. The same amplification and data processing electronics were used in both cases.

Substance P, ubiquitin (from bovine red blood cells), cytochrome-c, Thyrosine-6 ((Tyr)₆), and human insulin were purchased from Sigma (Buchs, Switzerland). Bovine serum albumin (BSA), ferulic acid, 2,5-dihydroxybenzoic acid (DHB), and trifluoroacetic acid (TFA) were obtained from Fluka (Buchs, Switzerland). Chemicals were used without further purification.

Peptides and proteins were dissolved in i-propanol/water/TFA (50/50/0.1 by volume). Ferulic acid (in i-propanol/water/TFA) was used as a matrix. Matrix and analytes were mixed in 1000:1 to 10'000:1 ratios, and 1.5 µl of the solution were deposited on the probe tip and allowed to air dry. (Tyr)₆ samples were dissolved in water with DHB as a matrix.
4.2.6 Mass resolution, comparison with MCPs

In this qualitative comparison, the mass resolution of the IPD will be compared to that obtained with a pair of MCPs.

Resolution is an important criterion for detector performance. Fig. 4.2.5 shows MALDI mass spectra of the (Tyr)$_6$ (poly-thyrosine) sample, measured with the IPD and with the MCP. The highest possible resolution in both cases was achieved by adjusting the laser power. The calculated mass resolution ($M/\Delta M$, $\Delta M =$ full width at half the maximum of the peak) is about 370 when measured with the MCP and 310 with the IPD. This small difference can originate from many parameters. In particular, a small angular inclination of the MCP or the IPD from the ion beam axis is enough to degrade the resolution dramatically (for a 1000 Da ion with 24 keV energy, a difference of 0.5 mm in flight distance corresponds to a flight time difference of ca. 7 ns and to a mass shift of 0.5 Da).

![MALDI mass spectra](image)

Figure 4.2.5: MALDI mass spectra (molecular region) of the (Tyr)$_6$ (matrix: DHB, 100 shots). The lower panel spectrum was measured with the IPD (Bu-PBD) and the upper with the MCP. Resolution obtained with the IPD: 310; with the MCP: 370.
Despite this limitation, the fundamental time resolution of the IPD can be estimated. For this purpose, we assumed that the lifetime of the ion-induced luminescence is on the same order as the fluorescence lifetime of the material in solution. Fluorescence lifetimes of Bu-PBD (in toluene solution) were measured by Anderson to be around 1 ns. Due to the roughness of the surface, differences in ion flight times up to 1 ns can be estimated. The intrinsic response time of the PMT used was 3.4 ns. The fundamental time resolution of the whole system is the convolution of all these parameters and was calculated to be less than 4 ns, sufficient for many linear TOF measurements. For comparison, MCPs can achieve, under optimum conditions (impedence matched anode, preamplifier and oscilloscope inputs), a time resolution on the order of 100 ps. The electron amplification with discrete dynodes in our PMT is therefore the limiting factor for the time resolution of the whole photon detection system. If a faster detector is needed, then a MCP-PMT (rise time of 150 ps), where the electron amplification is achieved with the MCP, could be used instead. These detectors are however expensive.

4.2.7 Signal intensities and signal-to-noise ratios

MALDI mass spectra of substance P with ferulic acid as a matrix are shown in Fig. 4.2.6. The spectrum in the upper panel was measured with the standard MCPs whereas the lower one was taken with the IPD. It can be seen that both spectra look very similar, proving that the IPD system is fast and sensitive. The same external amplification system was used in both cases. The areas of the two detectors were equal. All experimental parameters were kept as constant as possible, the laser intensity, ion acceleration potentials as well as the chamber pressure were identical. The same sample was used for both experiments.

In Fig. 4.2.6, the IPD spectrum had to be multiplied by 4 to obtain comparable absolute peak heights. As already mentioned, the absolute intensities obtained with a transient recorder system strongly depend on the secondary electron amplification and therefore on the voltage at which the detector is operated. In many cases, the signal-to-noise ratio is a good way to compare detectors. In Fig. 4.2.6, a signal-to-noise ratio of 600 was found for the upper trace (MCP) and 100 for the lower trace (IPD).

Larger ions can also be successfully detected with the IPD. In Fig. 4.2.7, a MALDI spectrum of a high molecular mass sample is shown, measured with the MCP and the IPD. Bovine serum albumin (BSA, 67'000 Da), was readily detected with the IPD. Conditions were adjusted in both cases to reach the highest mass resolution. The laser power and thus the number of ions were found to influence the
noise intensity, especially with the IPD. From these findings, we suggest that part of the noise may be due to slower unfocused ions created after the initial desorption event and arriving at the detector with a very large time distribution. Such a phenomenon was also observed by D. Twerenbold [personal communication]. The signal-to-noise ratio was 50 and 17 for the MCP and the IPD, respectively. Absolute signal intensities are, however, comparable.

![Graph](image)

**Figure 4.2.6: MALDI mass spectra of substance P (matrix: ferulic acid, 100 shots).** The upper spectrum was measured with the MCP and the lower with the IPD (Bu-PBD). The same laser power was used in both cases.

From these findings, we concluded that the IPD is a rapid device that can detect, with large absolute intensities, molecules as large as BSA. By comparison with the MCP, the signal-to-noise ratio was found to be decreased. In an attempt to increase this ratio, ion post-acceleration was investigated.
Figure 4.2.7: MALDI mass spectra of BSA (matrix: ferulic acid; 100 shots). The lower panel was taken with the IPD (Bu-PBD) and the upper with the MCP of the same sample. $M^+$ represents the singly charged molecular peak and $M^{2+}$, the doubly charged.

4.2.8 Ion post-acceleration

As in the case of secondary electron detectors, the IPD would benefit from larger ion energies. This can be obtained by increasing the voltage in the ion acceleration optics of the TOF. However, this method has several disadvantages: the flight times are reduced, necessitating faster sampling recorders, detectors and electronics. The risk of electrical discharges in the ion source is also increased. The second alternative is to post-accelerate the ions just in front of the detector. This is frequently done in MCP assemblies. A voltage difference of a few kilovolts is applied between the front grounded grid and the surface of the first MCP. The increased ion energy improves the signal intensities. An additional effect is the fact that secondary particles sputtered from the grid are also accelerated to the detector and in turn create additional secondary electrons. The post-acceleration is, however, limited for MCPs. In fact, MCPs are fragile devices and any electrical
discharge must absolutely be avoided. In practice, they are typically floated at 5 kV in relation to the ground. The IPD, in contrast, is more robust and does not suffer if the scintillator is subjected to minor arcing.

Figure 4.2.8: Effect of ion post-acceleration. The +22 keV substance P ions were post-accelerated in the IPD onto the BBOT scintillator. The normalized substance P intensities are shown as a function of the additional post-acceleration voltage and the total ion energy. The error bars correspond to the standard deviation calculated from 150 shots.

A BBOT layer deposited on a transparent plate was used as scintillator for the following investigations. It was covered with a 95% optical transmission copper grid that was held at high voltage. A grounded metal ring was placed in front of this system. Fig. 4.2.8 shows the effect of additional ion energies on substance P signal intensities (mass 1347 Da). The initial ion energy was 22 keV. As it will be described in more detail in Chapter 4.3, all detector signals were normalized to the values obtained with an additional detector (a Faraday collector) to minimize the fluctuations in the amount of ions created at each laser pulse. Each experiment was integrated for 150 shots to improve the signal-to-noise ratio. Clearly the increase in intensity with post-acceleration is large. 15 keV additional energy, which
corresponds to an augmentation of 70% compared to the initial 22 keV, improves the peak intensities by about a factor of 18. The mass resolution change was only minor.

It is known that the photon yields for electrons or small ions impinging on scintillators increase quasi-linearly with the ion energy (see for instance Refs. 13, 14). The increase with ion energy in our data is surprisingly high. This behavior may be due to the fact that the electrical field in the post-acceleration region repels secondary sputtered ions back to the scintillator and may increase the ion signal intensity. To test this, we compared the normalized IPD signals obtained for the same ion total energy but distributed differently between the initial- and the post-acceleration. We found that IPD signals of ions with 20 keV initial kinetic energy and 2 kV post-acceleration were about the double that of signals obtained with 22 keV and no post-acceleration. A precise comparison of the intensities was, however, difficult due to the fact that the Faraday signal might also be dependent on the initial ion kinetic energy. Post-acceleration of the ions and the subsequent secondary particles were still found to be more efficient than increasing the initial kinetic energy. A further advantage of this technique is that the ion initial energy can be reduced and compensated at the end of the time-of-flight. Slower transient recorders can therefore be used.

4.2.9 Quantitative comparison of IPD vs. MCP

In this chapter, the efficiencies of both detectors were quantitatively compared. As discussed in Chapter 2.4, transient recording techniques are not the method of choice for such measurements, principally due to the fact that the signal outputs are strongly dependent on the amplification power of the SEM. To study the conversion of ion into photons more carefully, single event counting methods were therefore used.

4.2.9.1 The plasma desorption source

Although single ions could, in principle, be produced with MALDI by reducing the beam size with skimmers, this is impractical since the laser has a repetition rate of <10 Hz. In addition the signal is not sufficiently stable, due to sample depletion and shot-to-shot intensity fluctuations. Plasma desorption/ionization (PD-MS) was preferred and used to provide a low, constant ion production rate so that single event counting techniques could be utilized (Fig. 4.2.9).
In PD-MS, ions were produced by the impact of fission fragments of $^{252}$Cf decay onto the sample, while the oppositely directed fragments from the same decay process were detected by a MCP, and used to start the time measurements. The start rates were approximately 1000 s$^{-1}$. The sample was deposited on an aluminized Mylar foil that was held close to the $^{252}$Cf source at up to 24 kV with respect to the grounded grid of the one-stage acceleration optics. PD-MS is not a very soft ionization method so that fragments are often observed in the mass spectra. The upper ion mass limit is in the order of 10'000 Da. Due to poor ionization efficiencies, large molecule signals have to be integrated for many hours to reach an acceptable signal-to-noise ratio.

4.2.9.2 PD mass spectrometer and data acquisition

The experiments were carried out in Paris on a linear time-of-flight (TOF) with a field-free region of 72 cm and one-stage acceleration optics. As seen in Fig. 4.2.10, ions were produced by a $^{252}$Cf plasma desorption source (PD-MS).
The ion-to-photon detector (IPD) was placed on the end flange of the flight tube. The two MCPs (Galileo Corp., MA, USA, Standard MCP Set 40 mm, type S40-10-D-SET) were mounted in a Chevron configuration on a mobile holder that could be moved directly in front of the IPD without breaking the vacuum. In contrast to the experiments carried out in Zurich, the MCP assembly in Paris was gridless. The surface of the MCP exposed to the ion beam was grounded whereas the collector was held at a high positive voltage. The output signal was capacitively coupled to the electronics. The total voltage applied to the MCPs was divided equally between the first, second plates and the collector. The maximum voltage recommended by the manufacturer is 1kV per plate. The difference in flight distance between the IPD and MCP detector was about 5 cm. The working area of both detectors was the same, with a diameter of 4 cm.

The single event counting experiments were performed with a time-to-digital converter (TDC, Model: CTN2, IPN Orsay, France). The system is described in more detail in Refs. 17, 19. The start signals were obtained by using the complementary fission fragments of $^{252}\text{Cf}$ impacting on an electron conversion foil. These electrons were accelerated and detected by a second pair of MCPs (MCP start on Fig. 4.2.10). 

Figure 4.2.10: $^{252}\text{Cf}$ PD linear time-of-flight mass spectrometer equipped with MCP and IPD detectors.

The former can be moved in front of the IPD. Data acquisition was performed in single ion counting mode with a time to digital converter.
The output signals went through a constant fraction discriminator (CFD, Model: Enertec-Schlumberger 7174) to give the start pulse. The detector output signal, either coming from the MCP or the IPD, was also fed into a second CFD and used as the stop signal. The CFD delay was optimized separately for both detectors by comparing the count rates obtained with the same sample and with the same rate of starts. The CFD delay for the IPD was found to be greater than that for the MCP. This is expected due to the larger intrinsic pulse width of this device compared to the MCP (nominally 3 ns for the PMT and <1 ns for the MCP). The CFD thresholds for both CFDs were set at their minimum value of 7 mV in order to obtain the maximum ion count rate. The start/stop time difference was digitized at 500 ps/channel with a time-to-digital converter and transferred to a direct memory increment card installed in a personal computer. Acquisition times were adjusted to reach a reasonable peak-to-background ratio in the TOF mass spectra and were typically between some minutes for CsI to several hours for insulin.

4.2.9.3 Sample preparation

The following chemicals were purchased and used without further purification: luteinizing hormone releasing hormone (LHRH, mass 1182 Da), human insulin (5734 Da), penta-L-phenylalaline (Phe₅, 754 Da) from Sigma (Buchs, Switzerland), nitrocellulose from Bio-Rad Laboratories (Richmond, CA, USA), and CsI from Fluka (Buchs, Switzerland). The PD-MS samples were prepared as follows: CsI samples were electrosprayed directly on the Mylar foil, whereas heavier molecules such as Phe₅, LHRH or insulin were deposited from solution onto Mylar which had been previously coated with electrosprayed nitrocellulose.

4.2.9.4 Setting the detector working conditions

The amplitude of the signals delivered by both detectors depends on the voltages at which they are operated. The gain of MCPs increases exponentially with the applied voltage. Following photoelectric emission at the cathode, photomultipliers are also secondary electron multipliers and show similar dependence on the voltage. It was therefore important to determine the optimum operating conditions for both detectors. To be sure that the maximum number of events is detected, the optimum electron
amplification conditions for both detectors were researched. This optimum is reached when every detector output signal has an intensity above the discriminator level. The count rate vs. voltage applied on the IPD's photomultiplier is shown in Fig. 4.2.11, for Cs\(^+\) and Cs\(_2\)I\(^+\) ions emitted from CsI sample. The CFD threshold was held constant. The curve reaches a plateau at about -1.3 to -1.4 kV for both ions. Above this detector voltage the ion detection efficiency does not increase further. Therefore, for 22 keV ions, the maximum photomultiplier detection efficiency is reached. Other impinging ions showed the same behavior. As a result, the photomultiplier voltage was set at -1.55 kV for the rest of the experiments. This value was found to give the best signal-to-noise ratio.

![Figure 4.2.11: Count rates obtained with the IPD in the single ion counting mode for 22 keV Cs\(^+\) and Cs\(_2\)I\(^+\) ions as a function of the photomultiplier voltage. The vertical line indicates the voltage used during the subsequent investigations.](image)

Similar experiments were performed with the MCP to find the detector voltage at which the output signals of the MCP are higher than the discriminator voltage (detection plateau). The same procedure was followed as for the IPD. The optimum voltage was found at 900 V across each MCP. This value was applied during the remaining single event experiments. The MCPs were new. It is known that the gain of MCPs rapidly drops during the initial period of utilization. Therefore, this
test was made several times during the measurements to verify that the MCP voltage still corresponded to the initially measured detection plateau.

4.2.9.5 *Quantitative comparison of the scintillator efficiencies*

In our previous work, described in Chapter 4.2.2, direct comparison of the scintillator efficiencies was difficult. The difference in photon yields could not be easily distinguished from MALDI signal fluctuations. A qualitative observation led to the conclusion that Bu-PBD was one of the best materials tested when prepared with our methods. With single ion counting technique, more quantitative measurements are possible.

In these experiments, the various scintillators were bombarded with the same number of ions. The measured count rates showed little dependence on the identity of the impinging ion and the projectile mass dependence was the same for all scintillator materials studied. The count rates obtained from two glass plates covered with the same scintillator were identical. In Table 4.2.2, the efficiencies of the scintillators investigated are shown, relative to the BBOT efficiency. Bu-PBD has the highest efficiency (120%). An even larger relative efficiency is expected from Table 4.2.1. This difference is likely due to the Bu-PBD emission wavelength. At around 370 nm, the maximum emission wavelength of Bu-PBD, some light was absorbed by the plastic window in front of the photomultiplier and the detection efficiency is decreased. BBOT has also a rather high light emission efficiency. In addition, it exhibits a high vacuum stability and films can be easily fabricated. Therefore BBOT scintillators were used for the remaining investigations. Surprisingly, anthracene was found to be less sensitive. It also has the additional disadvantage of a high vapor pressure and therefore a short lifetime of few hours in vacuum. A piece of NE102A, a standard commercial plastic scintillator, was cut from a sheet of 1 mm thickness and placed directly in front of the photomultiplier. The resulting ion sensitivity is very poor. It is thought that this is mainly due to the large dead layer of the material. Box et al. estimated that the first 5-15 μm layer is oxidized and does not fluoresce efficiently. The penetration depths of large 24 keV ions are in the 10 nm range, explaining the low emission yield. For this same reason and also because of the large dilution of the active compound into an inert material, all the attempts to embed organic scintillators in a polymer matrix were not successful.
Table 4.2.2: Relative efficiencies of some scintillators used in the IPD

<table>
<thead>
<tr>
<th>Scintillators</th>
<th>Relative efficiencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BuPBD</td>
<td>120 %</td>
</tr>
<tr>
<td>BBOT</td>
<td>100 %</td>
</tr>
<tr>
<td>POPOP</td>
<td>70 %</td>
</tr>
<tr>
<td>Anthracene</td>
<td>30 %</td>
</tr>
<tr>
<td>NE102A</td>
<td>5 %</td>
</tr>
</tbody>
</table>

4.2.9.6 Projectile energy dependence

One important parameter determining the efficiencies of the detectors is the ion kinetic energy. This is shown in Fig. 4.2.12 which displays count rates observed with both detectors for various impinging ions as a function of the ion energy (the ion energy can be changed by changing the acceleration voltage).

\[\text{Figure 4.2.12: Count rates of } H^+, \text{ Cs}_2 F^+ \text{ and LHRH}^+ \text{ ions as a function of the ion energy as obtained with the MCP (solid symbols) and IPD (open symbols) detectors operating in the single ion counting mode. Cs}_2 F^+ \text{ and LHRH}^+ \text{ IPD signals have been multiplied by a factor 10 for clarity.}\]
The count rates increase with increasing energy and, in the case of the MCP, reach a plateau, indicating that the maximum possible efficiency was achieved. For \( \text{H}^+ \) ions the maximum efficiency is reached at energies below 10 keV with a MCP detector. This means that \( \text{H}^+ \) ions with \( E_{\text{kin}} \geq 10 \text{ keV} \) produce at least one electron when entering one of the MCP channels. For the IPD, a plateau is not reached, except in case of \( \text{H}^+ \) ions with \( E_{\text{kin}} > 20 \text{ keV} \). Higher acceleration energies could not be used in this instrument due to risks of electrical discharges.

The maximum efficiency was not obtained with the IPD for heavier molecules, even for the highest ion energies. Consider the \( \text{Cs}_2 \text{I}^+ \) cluster in Fig. 4.2.12b. The MCP count rate reaches a plateau at ca. 12 keV whereas the IPD count rate increases over the whole range of ion energies. Similar results were found with heavier molecular ions. Fig. 4.2.12c shows the results obtained with LHRH. It can be concluded that the MCP's detection efficiency for 20 keV ions is at its maximum for masses up to 1000 Da (LHRH).

### 4.2.9.7 Secondary photon and electron yield dependence on mass

To study the photon production as a function of the primary ion mass at a given energy, ions with various masses (LHRH, Phe, insulin and some of their fragments) were measured with both detectors in single ion counting mode. The samples were prepared on Mylar-nitrocellulose substrates as described above, and the total acceleration voltage of the positive ions was set to 22kV. After mass calibration, the integrals of the parent and a number of fragment ions were determined. The ratio of the count rates for the IPD and the MCP is reported, with no further treatment in Fig. 4.2.13.

Figure 4.2.13a is a comparison of the detector counting efficiencies. It is seen that the IPD/MCP count rate ratio drops rapidly with mass. The very light ions (\( \text{H}^+, \text{H}_2^+, \) etc.) are detected with similar efficiencies for both systems, whereas the IPD insulin count rate is only 4% of that found with the MCP. Insulin is the largest ion that was produced with PD in this investigation. Due to the poor PD ionization efficiency and the poor response of the IPD for large molecules, the signal was integrated over 14 hours. The change in the IPD over MCP count rates is more pronounced between 1 and ca. 1000 Da than for molecules ranging from 1000 to 6000 Da. As observed in Chapter 4.2.7 for similar ion energies, the IPD detector gives good signals for molecules as large as 70000 Da, confirming that the IPD/MCP ratio probably does not change much for larger ions, at least up to ca. 30000 Da.
Figure 4.2.13: a) Relative count rate ratios for 22 keV ions with various masses obtained with the IPD and the MCP detectors, operating in the single ion counting mode. The solid line is a guide for the eye. Data plotted with the same symbols refer to ions desorbed from the same sample. The right hand ordinate gives the number of photons relative to the number of secondary electrons generated per ion impact. b) Same data as in a), but plotted as a function of the ion velocity.
It is also interesting to view the same data when plotted as a function of ion velocity. As shown in Fig. 4.2.13b (linear scales) for a given energy, the IPD/MCP ratio increases linearly with the velocity of the ions, except for very low velocities. It can be noted from Fig. 4.2.13 that the value of the IPD/MCP ratio depends little on impinging ion species but strongly on their mass and therefore velocity. The results shown in Fig. 4.2.13 represent the relative responses of both detectors. Another interesting point is to compare the initial conversion of ions into photons vs. secondary electrons. This can be estimated by using the data from Fig. 4.2.13 and correct for various detector characteristics. As shown in Chapter 2.4.2, the observed count rates are the product of the ion flux (dN/dt) and the conversion yield of ions into either photons or electrons ($Y_{\text{conversion}}$), the collection efficiency ($Y_{\text{collection}}$) and the quantum efficiency of the detectors (QE).

The quantum efficiency describes the detection probability of the detector itself. At its maximum, this value is, according to the manufacturer specifications, 26% at 380 nm for the photomultiplier. In other words, 26% of the photons that reach the photomultiplier are detected. This value can approach 100% for an MCP (100% of the secondary electrons entering a microchannel are detected). We used both detectors at their maximum efficiencies (see above) and can therefore use these values in our estimation. Due to the inactive area between the channels, the MCP can only "see" about 60% of the ions reaching the detector. The collection efficiency for this device is therefore taken to be 60%. For the IPD, we estimate that about 20% of the photons created on the scintillator are emitted in the photomultiplier direction and are collected. The products ($Y_{\text{collection}}$QE) are 0.05 and 0.6 for the IPD and MCP, respectively. The ion flux was kept constant during the measurements and therefore cancels when the IPD/MCP ratio is calculated. The ratio of ion conversion into photons and the conversion into electrons ($Y_{\text{conversion photons}} / Y_{\text{conversion electrons}}$) is found by multiplying the count rate ratios of Fig. 4 by a factor of 12 (=0.6/0.05). The results are found in Fig. 4.2.13a on the right hand scale. For m/z up to ca. 150 and for 22 keV energy, more photons are produced than secondary electrons, whereas the opposite is observed for larger masses. Insulin, the largest molecule measured in these experiments, produces ca. 2 times more secondary electrons than photons. The absolute number of photons could be calculated if the secondary electron yield on the MCP were known. Using the values for ions impacting on CsI found in Refs. 17 and 24 one can roughly estimate that 22 keV LHRH creates 1 photon per ion.
**4.2.10 Has the IPD an ion number threshold?**

From the curve shown in Fig. 4.2.13, it is surprising that the IDP used with MALDI give such strong signals and is able to detect molecules as large as BSA (ca. 67,000 Da). As an example, cytochrome-c (ca. 12,300 Da) was not seen at all with the IPD using PD-MS, whereas its detection was easy with MALDI. MALDI produces ions in packets contrary to plasma desorption that creates mostly one ion at a time. One hypothesis is that the IPD is more efficient to detect ions when they are created in packets, or, in other words, that the IPD has an ion number threshold necessary for improved detection. To study whether the number of ions reaching the detectors affects the signal intensities differently for the IPD and the MCP, we compared their sensitivity to ion numbers. This is difficult because the signal intensity fluctuation with MALDI is large. To partially compensate for this, measurements were alternatively taken with both detectors. After a few laser shots, the signal was integrated and the detector changed. This was repeated for several cycles.

![Graph showing signal intensities for different laser powers and detection methods.](image)

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Fig. 4.2.14: MALDI experiments with insulin/CCA. Insulin signal intensities were monitored with the MCP and IPD alternately. The laser energy was varied with a variable density filter. At low laser attenuation values, the laser energy is large, and at large laser attenuation values the laser energy is small. Each point corresponds to a new spot on the sample. Up to 4 shots were summed per measurements. Curve a) was obtained with the MCP at 850 V per plate, b) with the IPD at -1.55 kV, and curve c) with the MCP at 850 V per plate and two grids mounted in front of it to reduce the transmission down to 30%.
In Fig. 4.2.14, insulin was monitored with both detectors alternately and the laser energy changed with a variable density filter. In MALDI the number of analyte ions increases with the laser energy so that the curves shown in Fig. 4.2.14 can be viewed as the detector signal amplitude vs. the number of ions impacting the detector at the same time. This is obviously a qualitative plot since the relationship between the laser attenuation and the number of ions is not known.

The MCP output signal (b) increases with the number of primary ions in a regular way and reaches a nearly constant intensity value for a large amount of ions (small laser attenuation). The MCP was operated at a relatively high voltage, unfortunately increasing the probability of saturation. This plateau could therefore be due to detector saturation or there could be a maximum in the number of emitted ions vs. laser fluence. To verify that the MCP was not saturated at large laser fluences, additional grids were placed in front of the MCP to transmit only 30% of the initial beam. The resulting intensities are represented in Fig. 4.2.14 c). It can be observed that the amplitudes obtained with the 30% transmission grids are also about 30% of the MCP amplitudes without grids. It can be concluded that the MCP is still in its linear regime. We therefore believe that the MCP was not saturated even at the highest laser fluence.

The IPD signal (a) behaves differently. Hardly any signal is seen for low laser fluences. Above a certain pulse energy, the signal increases dramatically, as if there would be an ion number threshold for efficient conversion.

The threshold hypothesis was supported by the following experiment. Molecules with various molecular weights (from ca. 1000 to 67'000 Da) were measured alternately with both detectors. The laser beam position was kept the same for each sample. Again, after few shots with one detector (typically 2-4 shots) molecular signal were integrated and the detector exchanged. This procedure was repeated until the signal disappeared. It was observed that the MCP signal decreased much less rapidly with the number of laser shots than that of the IPD. With cytochrome-c, during the first 45 shots the MCP signal dropped 40% whereas for the IPD the decrease was as large as 85% (Fig. 4.2.15).

The MALDI IPD/MCP ratio as a function of shot number was estimated, and found to depend very strongly on how many shots had occurred. For example, the ratio for cytochrome-c varies between 1.5 after 10 shots to 0.3 after 35 shots, for a factor 5 difference. This phenomenon can also be explained in terms of the number of ions reaching the detector. It is well known by MALDI users that the signal from the first shots on a new spot is much more intense than afterwards. More experiments would be necessary to confirm or refute this hypothesis. In particular, similar experiments should be repeated at lower detector voltages to
exclude all risks of detector saturation. Further experiments, where the number of incoming particles is known, are also necessary. For that purpose, an ion gun could be used (for instance a cesium source used for SIMS).

![Graph](image)

**Fig. 4.2.15: MALDI experiments with cytochrome-c/CCA.** Cytochrome-c intensities were monitored with the IPD and MCP alternately. The laser was kept on the same sample position during the whole experiment. The laser shot number corresponds to the total amount of shots that already illuminated the spot.

### 4.2.11 Conclusions on the IPD

Detection of photons created by the impact of ions on a luminescent organic conversion surface was shown to be a viable alternative to the usual secondary electron multipliers for modern mass spectrometric applications. The set-up proposed here is inexpensive, easy to handle, and robust. Moreover, its operation is possible under higher working pressures as the secondary electron amplification system is located outside the vacuum chamber. The resolution of small peptides (ca. 1000 Da) was found to be slightly worse than with standard MCPs. A fundamental time resolution for the whole IPD system of less than 4 ns was estimated, limited by the PMT, which is sufficient for standard linear TOF experiments. Analytes
up to 70'000 Da, ionized with MALDI, were detected successfully. The signal intensities were similar, the signal-to-noise ratios factors of 6 and 3 smaller with the IPD compared to the MCPs for substance P and BSA, respectively. At higher mass the absolute efficiency of the IPD appears to decrease. The implementation of ion post-acceleration in front of the scintillator increased the ion signal intensities drastically.

An appreciable advantage of the IPD is that the electron amplification process is achieved in the photomultiplier via a discrete dynode amplification chain. This construction is known to be less susceptible to signal saturation than MCPs. This is of importance for MALDI measurements, where the number of ions, particularly in the low mass range, can saturate the detector and noticeably reduce signal intensities at longer flight times. A comparison between photomultiplier and MCP saturation limits will be demonstrated in Chapter 4.3.6.

A quantitative comparison between the IPD and MCPs was achieved with single event counting techniques. Ions were created using plasma desorption. With constant ion energy, the relative efficiency of the IPD for single ions compared to that of the MCP was shown to decrease as the mass of the projectile increases. A linear relationship was observed between this relative efficiency and the velocity of the ions. The conversion of ions into photons relative to the conversion into electrons was studied. It was found that 22 keV ions up to ca. 150 Da produced up to tenfold more photons than secondary electrons. For larger molecules ranging between 1000 and 6000 Da, 2 times more electrons are produced than photons. At those masses, the amount of photons relative to the electrons is much less influenced by the sample molecular mass as it is for smaller masses.

We found some indications that the IPD may have an ion number threshold to be efficient but this needs to be verified.

### 4.3 The IEPD

In Chapter 4.2, it was observed that the conversion of ions into photons was more efficient for small ions than for larger ions. Signals of large ions would therefore be enhanced if they were transformed into smaller particles, through sputtering or fragmentation. For that purpose, a conversion dynode, placed in the ion beam, was added to the IPD. The secondary particles created were accelerated to the scintillator. The new configuration of the IPD, the IEPD, is essentially a modified "Daly detector". It has the additional possibility to detect not only electrons, but also secondary ions of both polarities if necessary. This could be of impor-
tance for heavy ions where the amount of secondary ions becomes more important compared to the secondary electrons 24, 27.

4.3.1 The IEPD set-up

The IEPD consisted of the same head-on photomultiplier (Hamamatsu, type R2154-02) placed on the terminating flange and separated from the vacuum system with a transparent window. The post-acceleration system was mounted inside the time-of-flight tube. An exploded view of the set-up is shown in Fig. 4.3.1. The scintillator was fixed on the metal ring (5). In front of it, rings (4) and (3) were held at the same voltage and defined a short field-free region. The front ring (1), on which the conversion dynode could be attached, was isolated from the high voltage by a plastic ring (2) and was grounded. The inside of the whole system was well shielded from the rest of the flight tube.

A venetian blind copper/beryllium conversion dynode was mounted in front of the scintillator. It was taken from a secondary electron multiplier detector (EMI-Thorn, Model 9643/4A, optical transmission ~0%).

![Figure 4.3.1: Schematic of the IEPD and the Faraday collector. The scintillator was mounted on ring (5). Rings (3), (4), and (5) were held at the same potential (HV2). The plastic ring (2) electrically isolated ring (1) where the conversion dynode was affixed. The photomultiplier remained placed outside the vacuum. In front of the detector, a Faraday collector was introduced, consisting of two grounded shielding rings and, in between, a ring partially covered by a metal plate. The middle ring was connected to a high impedance scope input.](image-url)
4.3.2 The scintillators

Several scintillators were used. BBOT was dissolved in chloroform and air-sprayed on a transparent support. The resulting BBOT preparation transmitted about 20% of the total photons produced in the photomultiplier direction. Plastic scintillators can be machined to any desired size. Two of these, known to be very fast and efficient, were also used: a 1-mm thick piece of NE102A produced by Nuclear Enterprise (Edinburgh, Scotland) and a 1-mm thick BC-404 from Bicron (Soest, Netherlands). The NE102A was coated with a 40nm thick layer of aluminum to reflect photons emitted in the direction opposite to the photomultiplier. Only electrons penetrate the aluminum, at the energies used, whereas all heavier particles are stopped in the metal layer. Their specifications as well as the percentage of light transmitted to the photomultiplier (as estimated with the laser-induced fluorescence technique) are shown in Table 4.2.1. In the literature, it is usual to describe the efficiency of a scintillator by its relative efficiency compared to anthracene (in percent).

4.3.3 The Faraday collector for detector normalization

Direct measurements of the output amplitudes are difficult in MALDI, due to the fact that the signals usually fluctuate strongly from laser shot to laser shot. To overcome this problem, an additional ion detector was introduced in the system to normalize the signals. As shown in Fig. 4.3.1, a Faraday collector was placed in front of the detectors to intercept part of the ion beam. It consisted of a metal plate with a "half moon" shape that intercepted ca. 50% of the ion beam. The plate was connected to the 1 MΩ impedance input of a digital oscilloscope. The other half of the ions continued their trajectories to the IEPD or MCP. To geometrically delimit the size of the beam, a grounded ring was placed just in front of the Faraday collector. To avoid interferences created by charged particles coming back from the IEPD or MCP detectors, the backside of the Faraday collector was protected by an additional grounded plate. This set-up was mounted 10 cm in front of the detector.

With no internal conversion or further gain, this device directly senses the ion current. Unfortunately, it is not guaranteed that the signals are only due to the primary ions because sputtered ions created during the impact can leave the detector. Furthermore this sputtering is mass dependent. It is therefore incorrect to equate the Faraday signal with the number of ions that impinged on its surface, but it should be an excellent way of normalizing MALDI signals.
A typical single shot Faraday signal is shown in Fig. 4.3.2a for substance P. Because of its large time constant, this device is a current integrator and ion packets appear as steps. Fig. 4.3.2b shows the IEPD signal (50 Ω impedance, photomultiplier at -1.5kV) for the same laser shot. It can be seen that the mass spectrum mostly consists of the molecular ion peak. The matrix peaks have minor intensities, which simplifies the integration procedure (flat baseline) and prevents matrix signals from saturating the detector. The quasi-absence of matrix peaks (matrix suppression effect) was obtained by using a sample-to-matrix molecular ratio of 1:50, higher than that typically used in MALDI 29. The normalized detector signals were obtained by dividing the signal peak integrals by the height of the Faraday step signals.

Figure 4.3.2: Example of MALDI spectrum measured with the IEPD (b) and the Faraday collector (a) for the same single laser shot. The sample was substance P mixed with DH parallel to the MCP. Both signals were smoothed (11 points, binomial algorithm). *: artifact signals caused by the pumps.
Under typical MALDI conditions (laser power just above threshold), we verified that the IEPD and MCP detector signals were directly proportional to the Faraday collector signals, showing that neither the IEPD nor the MCP were saturated. Because of this linear relation, it was possible to improve the signal-to-noise ratio of the signals by accumulating laser shots and dividing the sum of the detector signals with the sum of the Faraday signals. The normalization is therefore still valid. It will be shown further that, at higher laser irradiance, saturation can occur and the detector signals are no longer proportional to the Faraday signals. Normalization in cases where saturation may occur was only carried out for single shots.

### 4.3.4 Samples

Substance P, cytochrome-c, bovine insulin, and γ-globulin were purchased from Sigma (Buchs, Switzerland). Cesium iodide, calcium ionophore II (ETH-129), 2,5-dihydroxybenzoic acid (DHIB), and sinapinic acid were bought from Fluka (Buchs, Switzerland). Samples from Münster: substance P, cytochrome-c, and bovine serum albumin were obtained from Sigma (Deisenhofen, Germany). Gramicidin S synthetase was prepared by Dr. Vater, Max Vollmer Institute for Biophysical Chemistry and Molecular Biology, Technical University Berlin.

### 4.3.5 Effect of secondary particle post-acceleration

The conversion dynode used is a venetian blind dynode with an optical transmission close to 0%. An insignificant percentage of the primary ions can pass through it. Signals observed with the IEPD are therefore only due to secondary particles impinging on the scintillator. Two scintillators are compared in Fig. 4.3.3. The sample was substance P with 22 keV kinetic energy. In separate experiments, either the positive or the negative secondary particles were accelerated to the scintillator with various voltages. For substance P, the negative particles gave much greater peak intensities than the positive ones. The difference is most likely due to secondary electrons, which are also accelerated to the scintillator. In the upper part of Fig. 4.3.3, the minimum obtainable full width at half maximum (FWHM) for substance P is shown as a function of the post-acceleration voltage. The scintillator was BBOT but very similar results were found with BC-404. Other scintillators were not tested.
Figure 4.3.3: Effect of secondary particle post-acceleration. Either positive or negative particles were accelerated to the scintillator. The photomultiplier was operated at $-1.2 \text{kV}$. The results for BBOT and BC-404 scintillators are shown. The normalized intensities of 22 keV substance P signals are shown as a function of the potential applied to the scintillator. In the upper part, the full width at half maximum of the substance P peak is shown as a function of the post-acceleration voltage. The scintillator was BBOT.

The maximum mass resolution that could be obtained with $+5 \text{kV}$ post-acceleration was about double than that with $+20 \text{kV}$. The dependence of the mass resolution with the post-acceleration energy may be caused by ions that are sputtered from the scintillator during the ion impact and accelerated back to it. If the signal intensities are large enough, it is therefore preferable to reduce the post-acceleration voltage to increase the resolution.

In Fig. 4.3.3, signals obtained with BC-404 were between 1.4 and 2.3 times larger than those obtained with BBOT. The error bars correspond to the standard deviation calculated with 500 laser shots. The light collection with the BC-404 scintillator, measured above, was about 2.5 times more efficient (Table 4.2.1) than with BBOT. Although the absolute signal intensities were higher with the BC-404, the
electron-to-photon conversion yield of the BC-404 was slightly smaller than that of BBOT.

An additional experiment was performed to show that electrons contribute a large part of the signal intensity. A piece of plastic scintillator NE102A was coated with a fine layer of aluminum (40 nm thick). At the energies used in this work (up to 20 keV), only electrons can penetrate through the aluminum and reach the scintillator. Very intense signals were observed with this set-up when negative particles were accelerated but no signals were seen with positive particles. The signals due to electrons are therefore predominant and must be true in the case of an uncoated scintillator as well. From a comparison of the intensities, we estimate that the signals due to secondary ions are only around 3% of the electron signals at 20 keV. With the aluminized NE102A, only electrons were used. This preparation had the additional advantage of reflecting the light emitted in the wrong direction back to the photomultiplier. The collection efficiency was therefore improved (about 85% compared to 45% transmission for an uncoated scintillator, see Table 4.2.1). Unfortunately, the aluminum strongly degraded the mass resolution. When secondary particles hit the aluminum, additional sputtering of ions took place (most probably aluminum ions). The positive ions are accelerated to the conversion dynode and the detection process repeats itself. The result is that one IEPD signal consists of many “waves”, created at slightly different times. Furthermore, the coated NE102A does not allow detection of particles other than electrons.

4.3.6 Linearity, saturation and signal intensities

The final output intensities are highly dependent on the electron amplification and therefore on the voltage used for the photomultiplier or for the MCP. The linearity of a secondary electron multiplier (MCP or photomultiplier) is limited and depends on the detector working conditions. If too many primary particles (electrons or photons) arrive at the detector at the same time, the output current is so large that the potential difference across the dynodes cannot be maintained. This results in signal intensities smaller than expected. In extreme cases, saturation due to small masses arriving first on the detector can totally suppress the heavier ion signals. We therefore used the IEPD and MCP under different working conditions to check for the linearity and compare the signal intensities. The sample peak integrated from the detector (IEPD or MCP) and the corresponding step height coming from the Faraday detector were recorded and the laser power varied to cover a large range in the number of primary ions. This proce-
The procedure was executed with both detectors, and the values transformed into number of charges. The number of charges that was calculated from the Faraday signal is proportional to the number of ions hitting the collector but may not be considered to be exactly the number of ions. Secondary particles sputtered from the Faraday plate also influence the signal height. Furthermore, the areas of the collector and of the IEPD and MCP are different. Thus, the Faraday charge is not the number of ions that reached the IEPD or MCP but is proportional to it. As these effects are independent of the kind of detectors placed behind the Faraday collector, it can still be used to normalize the signals.

![Figure 4.3.4: Faraday-normalized IEPD and MCP signals of CsI under different operating conditions. Every point corresponds to a single laser shot. The amount of ions was varied by changing the laser power. In b) and c), the secondary ions generated on the venetian blind dynode were accelerated with +15 kV onto BC-404. The photomultiplier was operated at -1.2 kV in b) and -1.5 kV in c). Curves a) and d) were obtained with the MCPs operating at -700 V and -900 V per plate, respectively. The dashed curves are guides for the eye.](image-url)
In Fig. 4.3.4, 22 keV Cs\(^+\) ions were used as test particles. CsI is a very practical sample because it ionizes readily and does not necessitate a matrix that may saturate the detector. The number of charges coming from either the IEPD or the MCP after electron amplification is shown as a function of the number of charges measured with the Faraday collector. In Fig. 4.3.4, each point corresponds to a single laser shot.

Different detector configurations were compared. Curves b) and c) were obtained with the IEPD. Negative secondary ions and electrons were accelerated with +15 keV. In b) the photomultiplier was operated at -1.2 kV (ca. 67\% of the maximum voltage recommended by the manufacturer) and in c) at -1.5 kV (ca. 83\% of max.). Curves a) and d) result from the MCP at -700 V per plate and -900 V per plate, respectively. Those voltages correspond to 70\% and 90\% of the maximum recommended voltages.

Only curve a) does not show any signs of saturation. The detector signal and the Faraday signal show a linear relationship over the whole x-axis range. The slope is about 2\times10^7 for the MCP at -700 V (a). IEPD saturation is clearly observed in curves b) and c). The curves in the cases where saturation occurs consist of three regions: a first linear region, an intermediate region, and a final flatter region at high ion current. The slopes of the linear regions are 6\times10^7 and 2\times10^8 for curves b) and c), respectively. Using the same photomultiplier voltage, the conversion dynode and 15 kV post-acceleration therefore increases the Cs\(^+\) signals by a factor of around 70 (2\times10^8/3\times10^6). The saturation points were estimated by evaluating the intercept between the extrapolated initial linear region and the linearly extrapolated plateau-shaped saturation region. The Faraday signals, where these saturation points were observed, were ~3.6\times10^{15} and ~1.2\times10^{15} for curves b) and c), respectively. The MCP has to be operated at a very high voltage to reach similar signal intensities: curve d) has, in its linear region, a slope of 4\times10^8. However, saturation happens even earlier than with the IEPD (saturation point at ~0.9\times10^{15}). Here it should be noted that laser powers much larger than the threshold values were used to produce more ions. Under normal MALDI conditions, none of the detectors would have been saturated for Cs\(^+\). However, larger molecules necessitate the use of a matrix. The matrix signals can then, as mentioned above, suppress the sample signals. This can, for example, be observed for substance P with DHB for matrix (data not shown). We found a strong correlation between the total amount of charges (from DHB and substance P) and the substance P IEPD or MCP signals. This finding emphasizes the utility of diminishing the amount of matrix molecules arriving at the detector. One simple way to accomplish this is to deflect the matrix ions with a transverse electrical field.
Fig. 4.3.4 also gives some information about the intrinsic gain of the two different secondary electron multipliers. In the IEPD, the gain of the photomultiplier secondary electron multiplier was increased by a factor of -3.6 when the voltage was changed from -1.2 to -1.5 kV. The observed change in intensity with increased voltage is more pronounced with the MCP: signals were 30 times larger when the MCP was run at -900 V compared to -700 V (this also includes the signal improvement due to the post-acceleration). Using the manufacturer's data, we estimated that the internal electron multiplication gain of the MCP at -900 V is at least an order of magnitude larger than the gain of the photomultiplier at -1.5 kV (see Chapter 4.3.7). In Fig. 4.3.4, the MCP intensities (curve d) were about double than those with the IEPD (curve c), despite the fact that the electron amplification gain of the photomultiplier was more than a factor of 10 lower than the MCP gain. The detection of electrons through conversion into photons therefore seems to be very efficient. This emphasizes the findings of Chapter 4.2.9 where it was shown that ions with low molecular weights produce more photons than secondary electrons. Possibly, this remains true for electrons impinging on the detector.

4.3.7 How to estimate SEM gains?

Electron amplification gains in secondary electron multipliers (SEM) depend on the potential difference applied through the chain. For a SEM consisting of discrete dynodes, the following general dependence is found:

\[ G = K \cdot V^{\alpha/n} \]

where "G" is the electron gain, "K" a pre-exponential factor, "V" the potential difference, "α" a coefficient determined by the dynode material, and "n" the number of dynodes (10 in our case). According to the manufacturer, "α" has a value of 0.7 to 0.8.

MCPs, on the other hand, have continuous dynodes. The gain dependence with the potential difference is as follows:

\[ G = K \cdot e^{A \cdot V} \]

where "A" is a coefficient.

"K" and "A" are easily estimated for our MCP because the manufacturer (Galileo Corp.) provided us with values of the gains at two different voltages. As an example, we found for an MCP (extended dynamic range):
\[ G_{MCP} = 0.39 \cdot e^{1.1 \cdot 10^{-2} \cdot V[V]} \]

In case where two MCPs are used together, one behind the other (in the Chevron configuration), the total gain is simply the multiplication of the respective gains. Only one gain is known at one voltage for our photomultiplier. The procedure was therefore more complicated. From Chapter 4.3.6, it is known that the gain is increased by a factor \( \sim 3.3 \) \( (2 \cdot 10^8 / 6 \cdot 10^7) \) when the potential difference is changed from 1200 V to 1500 V. This allows us to estimate the \( \alpha \) factor. We found \( \sim 0.55 \), a value which is much lower than that claimed by the manufacturer. The pre-exponential factor \( K \) was calculated using the known gain \( (10^6) \) at 1250 V and 0.55 for \( \alpha \). The final expression of the gain reads:

\[ G_{pmt} = 9.3 \cdot 10^{12} \cdot V^{5.5} \]

Fig. 4.3.5 and 4.3.6 show the electron gain dependences with the voltage for the MCP and photomultiplier, respectively. The curve in Fig. 4.3.5 corresponds to the MCP pair used for the experiments done in Chapter 4.3.

![Graph showing electron gain dependence](image-url)
4.3.8 Detection yield as a function of the ion molecular mass

To study the dependence of the signal intensities of both detectors on the mass of the primary ion, we compared normalized MCP signal intensities to IEPD normalized values and estimated the IEPD/MCP ratio for different molecules. Care was taken to use laser energies just above threshold so that the detectors did not saturate. All samples were prepared with low matrix/sample ratios to suppress the matrix signals. With the MCP at -900 V and the IEPD at -1.2 kV plus 12 kV post-acceleration, the IEPD/MCP ratios were, for example: 0.5±0.2 for DHB-H₂O (mass 137 Da), 1.2±0.2 for CaII-ionphore (461 Da), 1.2±0.3 for substance P (1347 Da), 0.6±0.2 for bovine insulin (5734 Da) and 1±0.3 for cytochrome-c (12300 Da). Obviously, this ratio is not clearly correlated with the parent ion mass. In the mass range studied here, the IEPD's and the MCP's detection efficiencies depended on mass in a similar fashion. We therefore suspect that the ion-to-electron conversion process is the limiting factor in both detectors for the detection of those ions at 22 keV.
4.3.9 IEPD mass resolution

As discussed in Chapter 4.2.6, an intrinsic detector rise-time in the order of 4 ns was estimated for the IPD system. Due to the additional conversion step, a slightly larger value is expected for the IEPD (10 keV electrons take less than 1 ns to cross from the venetian blind to the scintillator).

The IEPD was tested in the group of Prof. Hillenkamp (Münster, Germany). Their instrument is a reflectron TOF containing delayed extraction technology. According to their results, a maximum mass resolution of around 10'000 to 11'000 is obtainable for a small peptide when a fast MCP is used. In their laboratory, UV and IR MALDI can be utilized by using either a N2-laser (337 nm) or an Er:YAG laser (2.9 μm).

Substance P (MW: 1347 Da) and insulin (MW: 5737 Da) were measured with UV-MALDI. In Fig. 4.3.7, substance P was mixed with DHB as the matrix. The IEPD post-acceleration was set at relatively low voltage: 6 kV.

![UV-MALDI mass spectrum of substance P](image)

Figure 4.3.7: UV-MALDI mass spectrum of substance P (molecular ion region; matrix: DHB, 23 shots), measured in Münster with the IEPD (6 kV post-acceleration). The IEPD was on the reflectron side and delayed extraction was used. Substance P is isotopically resolved (mass resolution about 3000).
The peptide is isotopically resolved with a mass resolution of around 3000. The corresponding rise time is about 6 ns. This was found to be limited by the IEPD and not by the instrument. We arrived at this conclusion because of the following reason: for each molecule, the optimum instrument conditions are found by adjusting, for instance, the time delay for the delayed extraction or the lens voltages. With a very fast detector (MCP), the mass resolution seems to be limited by the instrument. The quality of the spectrum reached a maximum only at the optimum parameters and then decreases rapidly. In our case, the quality reaches a maximum for a much larger range of parameters. This concept is described in Fig. 4.3.8. It was therefore concluded that the substance P mass resolution in Fig. 4.3.7 was limited by the detector rapidness.

![Graph](image)

**Figure 4.3.8: Description of a mass spectrum limited by the TOF (a) and by the detector (b). In (a), the mass resolution maximum is reached for very specific parameters whereas in (b) the same mass resolution can be observed for different parameters.**

In addition to MCPs, a spectral resolution as good as the spectrum shown in Fig. 4.3.7 is only obtained with the fastest (and most expensive) SEM. Similarly, the mass resolution reached with insulin (Fig. 4.3.9) is comparable to the results obtained with the best SEM. In this case, the mass resolution is about 1000, which corresponds to a FWHM of 7.7 Da. In this spectrum, the protonated molecular ion is the dominant peak. The water loss and the K⁺-cationized peaks are also observed.
4.3.10 IEPD to detect very large molecules

Heavier molecules are efficiently detected with the IEPD. If desired, the post-acceleration voltage can be increased to improve the signal. As discussed previously, large post-acceleration energies must be balanced against mass resolution. In the IEPD, the post-acceleration and the photomultiplier gain can be separately adjusted. This supplementary flexibility allows optimization for signal intensity or for mass resolution. In general, we used high post-acceleration energies in the IEPD to adjust the experimental parameters and to find good spots on the sample with the laser. The post-acceleration voltage was then reduced during the measurement. However, for very large ions, the loss in mass resolution observed when the post-acceleration is increased is no longer significant because the time-spread of the ion packet itself is greater. As it will be described below, mid-sized molecules can show peak splitting.

In Fig. 4.3.10, cytochrome-c was measured with UV-MALDI. The detector post-acceleration was varied from 6 kV to 20 kV. Two peaks are clearly observed. The
sharper peak corresponds to the pulse of electrons created on the venetian dynode and accelerated towards the scintillator.

![UV-MALDI mass spectra of cytochrom-c (matrix:DHB), measured in Münster with the IEPD and different post-acceleration voltages: 6, 10, 12, and 20 kV. Large post-acceleration voltages increase the second broader peak intensity. Its position is also shifted relatively to the first peak.](image)

Figure 4.3.10: UV-MALDI mass spectra of cytochrom-c (matrix:DHB), measured in Münster with the IEPD and different post-acceleration voltages: 6, 10, 12, and 20 kV. Large post-acceleration voltages increase the second broader peak intensity. Its position is also shifted relatively to the first peak.

The second peak position shifts with the energy. Due to its large intensity, we think that this signal is also due to electrons. But their origin is still unclear. The following hypotheses have been made but refuted:

1. During the impact of the electron on the scintillator, secondary ions are created. They are accelerated back to the dynode where additional electron emission happens. Assuming that the secondary sputtered particles weight approximately 100 Da \(^3\), it takes them only around 150 ns to return to the dynode (with 12 kV) and less than 1 ns for the electrons to return to the
scintillator. In Fig. 4.3.10, we observed much longer time differences between the two peaks (μs range).

2- The same process than described in 1) is repeated several times. However, it seems illogical that the broad signal has a maximum after about 1 μs. Rather, a decay function would be expected (max. at time=0).

3- The initial positive parent ion (cytochrome-c) undergoes a polarity change on the conversion dynode and is accelerated to the scintillator where process 1) takes place. The time differences calculated were too large compared to the observations if the initial velocity was taken as equal to zero, and too small if the initial velocity was taken as the velocity of cytochrome-c in the TOF.

Possibly, ions sputtered from the conversion dynode may be different from the initial primary ion (case 3) and may be larger than 100 Da (case 1). Their initial velocities may also be different from zero or from the velocity of the primary ions in the TOF. In Fig. 4.3.10, the time differences between the two peak maximums can be estimated for each post-acceleration. Using these values, we solved the equation with two unknown parameters: the initial sputtered ion velocity and the mass of the sputtered ion. The best fit gave the following values:

- Mean mass of the sputtered ions: 8900 Da
- Mean initial velocity: 6700 m/s

For comparison, a 8900 Da, singly charged ion with 20 keV has a velocity of 20 km/s. The mean initial sputtered ion energy calculated with the values above is 2000 eV. Double peaks were also observed with the insulin dimer, which has a very similar mass. The problem did not appear with smaller and larger molecular masses.

Bovine serum albumin was dissolved in a glycerol preparation and ionized/desorbed with an Er:YAG laser. In Fig. 4.3.11, strong clustering is observed. The mass resolution is comparable to that obtained with an SEM detector (including a Venetian dynode), as well as the clustering range (the clustering can be strongly influenced by the instrument parameters). Molecules as large as the 11*BSA (ca. 740'000 Da) are readily detected.

In this case, a post-acceleration of 20 keV was used. The set-up was slightly modified to post-accelerate the primary ions on the Venetian dynode as well. For that purpose, the Venetian dynode was set at high negative voltage (for positive ions) and an additional copper grid (95% transmission) was placed in front of the dynode (at 1 cm). The advantage of this set-up is that the ions impact with more energy on the Venetian dynode. The secondary electron sputtering is therefore increased. The electron acceleration on the scintillator can also be adjusted as de-
sired. The spectrum in Fig. 4.3.11 was obtained with the scintillator set to ground. The electron energy was therefore 20 keV. Larger or smaller electron energies can be obtained by varying the scintillator voltage. We tested an electron total energy as large as 36 keV with our set-up. Although the absolute signal intensities increased dramatically, the quality of the spectra was not much improved. As a matter of fact, very large electron acceleration energies resulted in larger noise. We attributed this effect to the spontaneous electron emission that can occur under high electrical fields. A way to improve this would be to increase the distance between the scintillator and the venetian dynode. It was also suggested that the shielding between the dynode and the scintillator helped to diminish the rate of "sparks".

*Figure 4.3.11: IR-MALDI mass spectrum of BSA (36 shots) dissolved in a glycerol preparation and measured in Munster with the IEPD (20 kV post-acceleration). Strong clustering is observed.*
Molecules much larger than BSA were also detected with the IEPD. In Fig. 4.3.12, the IR-MALDI spectrum of gramicidin S synthetase with a molecular mass above 500'000 Da is shown. This sample was prepared in glycerol as well and irradiated with 2.9 μm from the Er:YAG laser. The mass resolution and signal-to-noise ratio are once more similar to what was obtained with a SEM and a venetian dynode. Here we would like to point out that for all the large ions, the SEM requires the use of a conversion dynode. As in the case of the IEPD, the dynode is floated at high negative voltage. The positive primary ions are therefore post-accelerated and the secondary electrons are pushed in the SEM direction. It is however more difficult to detect negative primary ions. If the dynode remains, as before, at negative voltage, the primary ions are decelerated and the secondary electron production is decreased. An alternative technique is to set the dynode at ground and to float the SEM at a positive voltage. Floating a device like a SEM is however limited. The output signal must be capacitively coupled to the data acquisition and the risks of electrical sparking that can damage the detector are increased. In the case of the IEPD, all of these problems are eliminated. The scintillator can be floated at any desired potential without risking damage to the detector. Negative ion post-acceleration can also be used.

![Figure 4.3.12: IR-MALDI mass spectrum of gramicidin S synthetase (63 shots) dissolved in a glycerol preparation and measured in Münster with the IEPD (20 kV post-acceleration). The molecular peak is noted $M^+$ and the doubly charged molecular ion $M^{2+}$.

Other samples were also successfully detected with the IEPD, including oligonucleotides, gama-globulin, and polymers (polyethylene glycols).
4.3.11 Conclusions on the IEPD

Compared to the IDP, the construction of the IEPD is slightly more complicated and necessitates the use of one (or two) supplementary power supplies. However, much larger signal intensities are observed. This device is able to isotopically resolve peptides with molecular weights around 1000 Da. The speed of the detector was the limiting factor to obtain even better time-responses. The photomultiplier time-rise (around 3 ns) contributes a large fraction to the total IEPD time response (around 4-5 ns). If necessary, faster photomultipliers can be used. Compared to SEMs, the IEPD time response is at least comparable to the fastest SEM obtainable on the market.

We showed that a large part of the signal was due to secondary electrons created on the conversion dynode and accelerated onto the scintillator. With the optimum scintillator, a plastic commercial compound, the secondary ions contributed only to a minor extent to the total signal intensities.

Very large ions above 500'000 Da were readily detected. Primary ions and secondary electrons can be separately post-accelerated to increase signal intensities. Relatively small electron acceleration values are preferred for mid-sized ions to avoid sputtering on the scintillator and a degradation of the mass resolution. Larger voltages can be used for heavier ions because the change in mass resolution is negligible compared to the initial ion packet spread. During our test measurements, it was observed that ions around 10'000 Da (with 20 keV initial energy) appeared as two peaks on the mass spectrum. We attributed the first one to the electrons created directly on the dynode. The second signal was much broader and is probably due to secondary ions sputtered and accelerated on the scintillator. We are convinced that the signal must be due to electrons. The sputtered ions therefore undergo several transformations to finally generate the electrons. This double peak was only observed for molecules around 10'000 Da. For the reasons described above, larger ions or lighter ions were detected as single peaks.

Positive and negative ions can also be detected with the IEPD. Ions of both polarities can be easily post-accelerated with similar energies, which is a real advantage compared to SEM detectors. The electron acceleration energy can also be independently adjusted. At too large values, however, an increase in noise was seen. This noise may be due to spontaneous electron emission caused by the high electrical field.
4.4 On the utility of the IPD & IEPD

The devices described above each have advantages and disadvantages. After analysis of the detectors available in the market or utilized by other research groups, we will try to give an objective point of view on the utility of the IPD and IEPD.

4.4.1 Market analysis

We noted that many companies sell their TOF instruments with MCPs (e.g. "Micromass", "Bruker", "Comstock", and "Kaesdorf"). After contacting some of them, we were told that MCPs are also sold for detecting very large masses. Usually post-acceleration is already mounted in the commercial instruments. The group of Liang Li in Canada uses MCPs or MSPs (microsphere plates, which have a larger dynamic range) to measure large ions such as 1.5 MDa polymers. The speed of the MCPs is probably the most interesting advantage of these devices. Some companies sell discrete secondary electron multipliers, especially in small TOFs (e.g. "Comstock"). In a large MALDI TOF, secondary electron multipliers plus a conversion dynode are used by the group of F. Hillenkamp for the detection of large masses. For these masses, this group found superior efficiencies with their system than with MCPs. Unfortunately, the time response of their best SEM is around 5 ns and costs significantly more than a "standard" SEM (around 2 times more) or than an MCP kit.

To avoid MCP saturation, "Cameca" sells a detector that consists of a single MCP followed by a scintillator. "Physical Electronics" proposes two MCPs followed by a scintillator. The two MCPs are floated at 10 kV to post-accelerate the electrons onto the scintillator. The "Himass" detector was originally fabricated by "Bruker". In this set-up, the ions impacted onto a venetian blind dynode and the secondary particles were accelerated with 6 kV on a single MCP. Behind it, a Csl disc transformed the electrons into photons that were then detected with a photomultiplier. This detector is not delivered anymore, mainly due to the fact that its time response was too slow. Only masses larger than 20 kDa could be detected with reasonable mass resolution in their TOF. Montaudo et al. used the "Himass" detector to estimate large polymer molecular weight distributions. They wrote:

The conversion dynode enables the detection of high mass ions. The scintillator avoids detector saturation by providing a very wide dynamic range. The Himass detector has, however, low temporal resolution. This is probably due to the production of secondary ions.
A very similar detector is sold by Galileo Corp. In their device, the ions hit an MCP. Secondary electrons are then accelerated with up to ±10 kV on a scintillator. "Micromass" sells a modified Daly detector. The ions are accelerated on a metal converting dynode. The electrons are then accelerated to a scintillator and the photons produced during the impact are detected with a photomultiplier. This detector is nevertheless only used for sector instruments and the technicians contacted did not know the fundamental reason why it was not used in TOF instruments. One of them mentioned the fact that a good ion focus is necessary to obtain a good mass resolution; in a TOF this would not be sufficient.

Some research groups use hybrid detectors. P. Williams 36, 37 combined a MCP and a discrete dynode secondary electron multiplier to improve the dynamic range of the detector. This device was successfully utilized to detect IgM molecular mass (~1 MDa).

### 4.4.2 Advantages/disadvantages of the detectors

Some of the advantages and disadvantages of the IPD, IEPD, MCP, and SEM are listed below:

*Table 4.4.1: Detector comparison*

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<th>IPD</th>
<th>IEPD</th>
<th>MCP</th>
<th>SEM + dynode</th>
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<tbody>
<tr>
<td>1 Time-response</td>
<td>+/-</td>
<td>+/-</td>
<td>++</td>
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<tr>
<td>2 Sensitivity for large masses</td>
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<td>3 Sensitivity for small masses</td>
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<td>4 Signal-to-noise ratio</td>
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<td>5 Saturation</td>
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<td>6 Easiness to mount</td>
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<td>7 Robustness</td>
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<td>8 Price per year of use</td>
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<tr>
<td>9 Detector life-time</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10 Possibility to work under higher pressures</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11 Possibility to measure (+) and (−) ions</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12 Possibility to post-accelerate the ions</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>13 Possibility to use in miniaturized TOF</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(−−) : very poor; (−) : poor; (+/-) : fair; (+) : good; (++) : very good.
Remarks:

2- the MCP sensitivity for large ions is considered sufficient by some research groups and not by others (including our group).

4- the signal-to-noise ratio of the IEPD depends on the electron acceleration voltage. The higher this value, the larger the noise. This was attributed to spontaneous field emission.

8/9- IPD, IEPD, and the SEM have quite long lifetimes. A good SEM is however much more expensive than an IPD and IEPD.

10- The electron amplification chain is outside the TOF chamber for the IPD and IEPD. It is therefore less sensitive to possible electrical sparks.

11- Measurements of negative ions with MCPs and SEM is often obtained by decreasing the ion energy (see text for more details).

4.5 Conclusions

Undeniably MCPs are very efficient detectors for light and mid-sized ions. For larger ions, their sensitivity is questionable. Some groups use it for studying large ions while others claim that its sensitivity is highly reduced. From our experience, the MCP is not the detector of choice for ions above BSA (67'000 Da). It is however one of the fastest devices. Unfortunately, it is a very fragile detector and people who have had to replace MCPs would certainly agree that this is a painstaking work. Its lifetime is also quite limited and incidents that frequently happen in a TOF (i.e. sudden air-leak, chemical poisoning with oil, water or other aggressive compounds, and electrical discharges) can easily break it.

The IPD is a cheap device that does not suffer from air exposure. Its construction is optimal for an implementation in miniaturized TOFs. Its sensitivity is sufficient for mid-size ions. In our instrument, 22 keV BSA (67'000) was detected with the IPD. Its signal-to-noise ratio was poorer than with an MCP. It was shown that signal intensities could be increased by adding an ion post-acceleration in front of the scintillator. We would, however, not recommend this alternative because the simplicity of the construction is then reduced. In cases where more signal is required, the IEPD seems to be more convenient and does not necessitate many transformations.

The IEPD is, to our opinion, a very powerful detector. One of its main advantages is its ability to detect small ions with adequate time resolution and, at the same
time, its ability to measure very large compounds. Its versatility is such that it can be used for most experiments in a TOF with no need to change the detector. The IEPD time-response was found to be comparable or better than the fastest SEM detectors. Also based on the conversion of ions into secondary particles in the first step, its mass response seems to be similar to the SEM. However, the IEPD has some additional advantages. For example, it has the potential to post-accelerate the ions as much as desired and to increase the secondary particle energy. Moreover, secondary electrons can not only be utilized to produce the signal; secondary ions, preferentially produced for large ions, can also be used. Last but not least, the implementation of IEPDs is economically compelling, due to its low price, robustness and user-friendliness.

4.6 References

Chapter 5.

5. PREFORMED IONS: UNDERSTANDING AND APPLICATIONS.

The ion formation pathways in MALDI are still not fully understood. A common statement is that ions are not formed by a single pathway but rather by several different mechanisms. As discussed in detail in Ref. 1, ionization mechanisms in MALDI can be divided into primary and secondary ion formation processes. Primary ions are formed directly from neutral molecules whereas secondary ions result from subsequent chemical reactions between primary ions themselves or between primary ions and neutrals or other secondary ions.

One possible mechanism is the desorption of preformed ions. By preformed ions, we refer to ions that were already present in the sample solution and, once the solvent is evaporated, in the MALDI crystals. Upon laser ablation, the ions are brought into the gas phase and separated from their counter-ions. Although no ionization happens during desorption (ions are already present in the form of salts), this mechanism can be considered as a primary ion formation process in the sense that no other initial ions are necessary for the creation of preformed ions. Other primary ion formation mechanisms include excited-state proton transfer, energy pooling, disproportionation, multi-photon ionization, and thermal ionization (see Ref. 1).

In this chapter, we will focus on the preformed ions. Very often, this pathway is in competition with other primary and secondary ion formation processes. The relative contribution of each mechanism varies depending on the sample and on the sample preparation. Investigation of the parameters influencing their relative contribution is necessary to efficiently control and optimize ion yields and spectral quality. For example, the MALDI signal of some samples is sometimes strongly enhanced by adding a cationizing agent (often a salt). This methodology is often applied to polymer analysis. On the other hand, an excess of salt can also lead to poor ionization or loss in mass resolution. In this chapter, the effect of salts in bio-
samples will be discussed in terms of mass spectral quality but also in terms of ion formation mechanisms.

It is also important to understand the parameters influencing the relative amount of preformed ions in order to study the chemistry of complexes in solution such as ligand/metal and protein/protein interactions. In this field, one of the important questions to answer is to what extent the MALDI spectrum reflects the solution phase chemistry. In other words, is it reasonable to use the mass spectral information to determine the sample composition in solution?

In Chapter 5.2, we investigate the complexes formed between crown ethers and metal cations. In Chapter 1, we already discussed the importance of taking into account the fact that different ions, even with very similar chemical structures, may be ionized and detected with different probabilities. A procedure to correct for these effects and to use MALDI in a quantitative way will be presented. Relative stability constants will be estimated and compared to literature values.

5.1 On the mechanism and control of salt-induced resolution loss in MALDI

5.1.1 Introduction

MALDI has the advantage of relatively high salt tolerance. Many groups have applied MALDI to the analysis of biological molecules such as DNA fragments or other very large molecules. However, such samples often contain significant amounts of salts, which affects the quality of the spectra noticeably. Therefore, it is important to understand their role on the efficiency and precision of MALDI-TOF MS.

Good sample preparation is a prerequisite for obtaining a good mass spectrum. The choice of the matrix is maybe the most important factor and many different molecules have been tested. Another less studied factor that can affect the quality of a mass spectrum is the composition of the matrix-analyte solution. It was observed, for example, that addition of acid can enhance the signal intensity of high-mass proteins. According to Cohen and Chait, this effect may be related to analyte solubility, which is increased in acidic conditions. Trifluoroacetic acid (TFA) is commonly chosen by many researchers for acidification; it is rapidly eliminated under vacuum due to its volatility.

Natural or added salts in the preparation solution are also known to have a significant effect on MALDI spectra. Some compounds, such as polyglycol polymers,
are detected primarily as adducts with alkali metal cations. For that reason, some workers add salts to try to enhance cationized analyte signals. For example, O’Malley et al. and Mowat and Donovan have studied the attachment of different metal ions to polystyrene, polyglycols and polybutadiene. They found that cationization of non-polar polymers could significantly increase the ion yield.

On the other hand, salt removal via liquid chromatography, extraction, or ion exchange is often attempted in an effort to improve the mass resolution. Shaler et al. recently studied the effects of impurities on MALDI spectra of oligodeoxynucleotides. The decrease of mass resolution was explained by adduct formation with cations. Instead of a single molecular peak, many peaks can be observed, corresponding to alkali metal cation-analyte adducts. Most frequently, adducts are formed with sodium, potassium and calcium ions. At low mass, the resolution in MALDI is high enough to distinguish between the different cluster peaks, but at higher mass these signals will be unresolved. With our apparatus, the limit where this becomes problematic is around m/z 8000 but this can vary for other TOF spectrometers. Both mass accuracy and resolution are lost, and mixtures will be more difficult to analyze.

These different sample preparation strategies, either adding or removing salts, are somewhat contradictory. Clearly, understanding the effect of salt ions on MALDI spectra can lead to better sample preparation strategies, especially for bio-samples which are often prepared in buffer solutions or naturally contain large amounts of salts.

5.1.2 Samples

The MALDI experiments were performed on our 2 m linear TOF described in Chapter 3. The data shown in this chapter were obtained by averaging 100 single laser shots and were not smoothed. The FTICR consisted of a 4.7 Tesla superconducting magnet, an “Infinity” cell (Bruker, Fällanden, Switzerland) and a workstation-based data acquisition system (Odyssey, Finnigan/Extrel FTMS, Madison, WI). The sample was irradiated with 355 nm from a frequency-tripled Nd:YAG laser (Continuum, Model Surelite II, 5 ns pulsewidth). The working pressure was below 1*10^-8 mbar.

The chemicals were obtained from Fluka (Buchs, Switzerland) and used without further purification, except for the 2,5-dihydroxybenzoic acid (DHB) which was purified by recrystallization from ethyl acetate (puriss. p.a.) and sublimation. Cation contamination of the DHB was checked by ICP-AES. No contaminants were found at the detection limit (<.0.1 ppm). The solvents were distilled water and methanol (puriss. p.a., >99.8%), also from Fluka. All compounds were dissolved in the solvent just before the measurement.

DHB was prepared at a concentration of 0.1 M. For some experiments, dilute TFA,
ammonia or calcium chloride was added to the solution. Valinomycin was prepared separately in water or methanol at a concentration of 0.1 M. Matrix and analyte were mixed immediately prior to use to obtain a molar ratio of 100 to 1. 2 μl of this solution was deposited on the 2 mm diameter probe tip and dried under vacuum for about 2 minutes.

Milk samples were prepared as follows: 300 μl of whole milk were first dried under a flow of nitrogen gas, then re-dissolved in 1 ml of 0.1% TFA in water or methanol. 10 μl of this mixture were mixed with 1 ml 0.1 M DHB (+0.1% TFA). 2 μl of this solution were deposited on the sample holder.

5.1.3 Effects of salts on matrix spectra

In Fig. 5.1.1, a MALDI-TOF spectrum of DHB, prepared as a 0.1 M methanol solution, is shown. The solution was spiked with different concentrations of CaCl₂. The different spectra were normalized in intensity using the peak at m/z 137, which corresponds to [DHB-OH]+. This fragment was chosen because it is not cationized and its concentration is not reduced by complexation chemistry. Clusters were clearly observed even at a relatively low salt concentration and become more prevalent with increased amounts of added CaCl₂. The same clustering behavior and overall spectral patterns were found in the MALDI-FTMS. We therefore assumed that the FTMS measurements gave the exact m/z values of the clusters observed at low resolution in the TOF. The most intense peaks of the cluster series are labeled in Fig. 5.1.1. All these signals, except the one at 137 Da, appear only when calcium is added to the sample, suggesting that they contain Ca²⁺. Probable singly charged cluster compositions are as follows:

- 137 Da: [DHB-OH]+
- 347 Da: [DHB·DHB·Ca²⁺]+
- 539 Da: [DHB·DHB·Ca²⁺]+ + [DHB²·Ca²⁺]
- 731 Da: [DHB·DHB·Ca²⁺]+ + 2[DHB²·Ca²⁺]
- 923 Da: [DHB·DHB·Ca²⁺]+ + 3[DHB²·Ca²⁺]

...  

[DHB·DHB·Ca²⁺]+ + n[DHB²·Ca²⁺], n=1, 2, ...

where DHB⁺ represents singly deprotonated 2,5-dihydroxybenzoic acid and DHB²⁻ doubly deprotonated DHB.

The repeat unit has a mass of 192 Da. To maintain the net +1 charge, each increment in cluster size requires addition of a neutral adduct, formed by a DHB molecule attached to a calcium ion. From the ICR measurements, we concluded that this adduct
must contain a doubly deprotonated DHB: $[\text{DHB}^2^-\bullet\text{Ca}^{2+}]^0$ (152+40=192), instead of $[\text{DHB}^-\bullet\text{Ca}^+]^0$ (153+40=193). The net charge is thus reduced by proton ejection rather than electron capture. The preference for a single net charge is not surprising, since multiple charges on such small clusters would entail a large Coulomb repulsion energy.

![Fig. 5.1.1: Positive ion MALDI-TOF mass spectra of DHB in methanol. Calcium chloride was added at the indicated concentrations (in mol/mol). Clusters appear when Ca$^{2+}$ ions were present in the solution. Labeled peaks are: 137 $\text{[DHB-OH]}^+$, 347 $\text{[DHB$\bullet$DHB$\bullet$Ca$^{2+}$]}^+$, 539 $\text{[DHB$\bullet$DHB$\bullet$Ca$^{2+}$]}^+ \text{+ [DHB$^{2-}\bullet$Ca$^{2+}$]}$, 731 $\text{[DHB$\bullet$DHB$\bullet$Ca$^{2+}$]}^+ \text{+ 2[DHB$^{2-}\bullet$Ca$^{2+}$]}$, 923 $\text{[DHB$\bullet$DHB$\bullet$Ca$^{2+}$]}^+ \text{+ 3[DHB$^{2-}\bullet$Ca$^{2+}$]}$](image-url)
It is thought that this proton ejection occurs in two steps: one proton, located on the carboxylic group, can easily be released (pKₐ~3) in solution so that part of the dried sample is composed of the DHB calcium salt, as described below in Fig. 5.1.2.

![Diagram](image)

*Fig. 5.1.2: Possible structure of two deprotonated DHB molecules around a calcium ion. The complex may exist in the crystal, depending on the sample solution conditions.*

The solution pKₐ values of the other protons are much higher (pKₐ>10) and the second proton is probably removed in the desorption/ionization step. Sumner et al. calculated the acidity of matrices in the gas-phase and found that the hydroxy protons of the DHB were highly acidic in the excited state.

### 5.1.4 Effects of pH

As a further indication of the importance of solution conditions, the relative intensity of the clusters can be varied, at constant salt concentration, by changing the pH of the solution with volatile acid and base (acid: trifluoroacetic acid (TFA); base: ammonia (NH₃)) (see Fig. 5.1.3). Because of their high vapor pressures, non-reacted TFA and NH₃ are removed from the sample by the vacuum and do not interfere with the MALDI desorption/ionization process.
Chapter 5

Fig. 5.1.3: Positive ion MALDI-TOF mass spectra of DHB in methanol, with 1 mol% of CaCl₂. TFA or ammonia were used to control the solution pH. The intensity of cluster peaks is reduced under acidic conditions and increased by base.

5.1.5 Effects of the solvent

Ion-matrix cluster formation is also strongly influenced by the solvent as is shown in Fig. 5.1.4. We compared two DHB samples, both spiked with 1% calcium and 50% ammonia. As discussed above, the ammonia enhances adduct formation, thereby simulating a particularly unfavorable sample. In the top panel (b) of Fig. 5.1.4, the solvent was methanol. In the bottom panel (a), water was used. The relative amount of clusters is much lower in water. This effect is not limited to
matrix; it was also observed that valinomycin does not form adducts when the sample is prepared in water. In contrast to low polarity solvents, water apparently does not favor the formation of preformed clusters and therefore suppresses analyte adduct formation. It is interesting to note that even mixtures of water with lower polarity solvents (like the commonly used water/acetonitrile mix\textsuperscript{17, 8}) give poorer results and should be avoided as much as possible when high salt concentrations are present.

Fig. 5.1.4: Positive ion MALDI mass spectra of DHB spiked with 1% of calcium. In the lower spectrum (a), DHB was dissolved in water to which 50 mol% ammonia was added. In the upper spectrum (b), the same conditions were used except that the sample was dissolved in methanol.
5.1.6 Controlling the amount of preformed ions

From the results shown above, a picture emerges for salt/solvent pH effects. The fraction of preformed ions will vary depending on the sample preparation conditions. In the case of DHB matrix, this fraction can be significant because of its acidity: addition of acid (base) into the solution forces the equilibrium between protonated and deprotonated DHB toward the protonated (deprotonated) form. We assume that the acid/base kinetics are fast and that the equilibrium is established at all times during sample drying. After drying, the acid (base) is removed, but a fraction of DHB can crystallize in the deprotonated form with salt cations (see Eq. below).

$$\text{RCOOH (l)} + \text{Base} \rightleftharpoons \text{RCOO}^- (l) + \text{H}^+ (l) \rightleftharpoons (\text{RCOO}^-) \text{Ca}^{2+} (s) + \text{HCl (g)}$$

As mentioned above, another proton is lost during the desorption/ionization event. Under acidic conditions, adduct formation with metal ions will be reduced because of the lower concentration of DHB$^-$ in the solution and, after evaporation, in the solid. As observed, clusters were mostly eliminated under acidic conditions (see Fig. 5.1.3, lower spectrum). On the other hand, a base such as ammonia deprotonates a large part of the matrix leading to much more calcium salt (Fig. 5.1.3 upper spectrum).

The solvent also influenced the clustering. We observed that polar liquids, such as water, usually decrease the cluster intensities whereas less polar solvents have the opposite effect.

5.1.7 Consequences of clustering

To demonstrate that these ion-mediated matrix clusters are also relevant to analyte peak broadening and shifting mechanisms, we show a mass spectrum of valinomycin (see Fig. 5.1.5). In MALDI mass spectra, this molecule is normally found to be cationized with sodium and other alkali metal ions, as in Fig. 5.1.5 (a). The solution was prepared in methanol with 0.1 M DHB and $10^{-3}$ M valinomycin concentrations. In Fig. 5.1.5 (b), the sample was spiked with 5 mol% of CaCl$_2$ (relative to DHB). As was observed with DHB alone, salt addition leads to extra peaks. The clusters formed with valinomycin and [DHB$^{2+}$•Ca$^{2+}$]$^0$ are, in this case, even stronger than the normal cationized peak.
This behavior may not be problematic for small analytes and simple mixtures where all the peaks and their adducts are resolved, but it is likely to be an important contribution to peak broadening for larger masses. At masses $>8$ kDa with our instrument, the "normal" molecular peak and the clusters overlap and are observed as a single unresolved, broad peak. Shift of the peak centroid to higher masses is another consequence of adduct formation.

These effects were observed with the MALDI mass spectrum of whole bovine milk. This sample naturally contains salts. Similar spectra were previously reported by Beavis and Chait\textsuperscript{17} in one of the early demonstrations of the capabili-
ties of MALDI. No special purification or treatment was performed before analysis. The concentration of mineral salts in whole milk is quite high, typically 8-10 g l⁻¹. Calcium salts are present at ca. 1.25 g l⁻¹.¹⁹ The amount of calcium in the 2 µl of milk solution is about 60 nmol. Fig. 5.1.6 shows the milk spectra after preparation in different solvents. Peak assignments were taken from Refs. 5 and 17 and are noted in the figure caption.

Fig. 5.1.6: Positive ion MALDI-TOF mass spectra of bovine whole milk in DHB matrix. (a) The sample solution was prepared in water + 0.1% TFA. (b) The same sample was recrystallized with methanol. (c) The same sample was again recrystallized with methanol and spiked with calcium chloride salt. The following peaks could be assigned: Cas: casein; Lg: lactoglobulin; La: lactoalbumin; Pp: proteoso-peptin.⁵,¹⁷ The mass centroid of the Pp peaks are indicated in each spectrum.
Using water as the solvent, the peak at ca. 8600 Da had a full width at half maximum (FWHM) of ca. 90 Da. The quality of the spectrum changes significantly with solvent. Fig. 5.1.6 (b) was obtained by redissolving the same sample in 2 µl of methanol, while the laser intensity was kept the same. The FWHM of the largest peak has more than doubled (220 Da) and the two resolved peaks in Fig. 5.1.6 (a) at ca. 24000 Da were observed as a broad single peak. Similar effects were observed with other lower polarity solvents such as tetrahydrofuran or acetonitrile. To study the effects of different salt concentrations, more salt was added to the milk. For the spectrum of Fig. 5.1.6 (c), the sample was dissolved again in 2 µl of a 1 mM CaCl₂ solution in methanol (2 nmol of Ca²⁺). The amount of total calcium salt was thus increased by 3 mol% compared to whole milk. The FWHM increased further to 370 Da.

Peak positions were also affected by salts, they shifted to larger apparent masses by salt adduct formation. In Fig. 5.1.6, the position of the largest peak moves from 8592 Da (Fig. 5.1.6 a) to 8631 Da (Fig. 5.1.6 b) to finally 8715 Da in Fig. 5.1.6 c. This shift of 123 Da between a) and c) corresponds to an error of about 1.5%. This suggests that salts can play a major role in both loss of resolution and accuracy in mass determination.

5.1.8 Conclusions

We have investigated the influence of sample preparation on MALDI mass spectra of salt-containing samples. The results are particularly applicable to analysis of natural materials containing high mass analytes and high salt concentrations. Salt impurities lead to a loss of both mass resolution and accuracy. This can be attributed to adducts of analyte with matrix, mediated by salt cations. Both pH and solvent polarity strongly affect the extent of adduct formation, suggesting that these adducts are formed in solution prior to desorption.

Optimum conditions for obtaining better MALDI mass spectra of large molecules in the presence of salts are:

- Polar solvents (such as water) should be used as much as possible for sample dissolution. Lower polarity solvents (such as acetonitrile, methanol, tetrahydrofuran...) or even mixtures with water were found to give much broader analyte peaks.
- Acidic conditions decrease the amount of clusters and therefore samples should be prepared at low pH (while keeping in mind that it could induce hydrolysis or denaturation). For protein and peptide analysis, low pH is typically used for sample preparation, as signals are strongly enhanced. For other classes of analytes the advantages of low preparation pH has not yet been recognized, and should be explored further.
Chapter 5

5.2 MALDI for quantification of complexation constants

5.2.1 Introduction

Many experiments have attempted to correlate condensed phase chemistry with MALDI mass spectra. This is valid only if the mass spectrum reflects the conditions that existed in the sample solution. This is by no means certain since the original sample is usually dissolved, then mixed with a matrix, and a drop of the solution is crystallized on the MALDI target. The environment of the analyte in the matrix is therefore much different than in solution and the chemistry is possibly changed. One way to confirm that a MALDI mass spectrum reflects the initial solution phase chemistry is to monitor the different stages of the sample preparation step-by-step. Lehmann et al. 20, for instance, followed the complexation between ligands and metal ions in solution, in the solid form, and in the gas phase. They found that MALDI mass spectra of their systems qualitatively reflected the amount of preformed ions. Other studies 21, 22, 23 have shown that, under certain conditions (choice of the “correct” matrix, solution pH, etc), MALDI spectra can give qualitative information about the condensed phase system.

5.2.2 Equilibrium constants by MS: a way to study solution chemistry

Another way to verify to what extent MALDI reflects the solution chemistry is to determine chemical equilibrium constants using MALDI mass spectra and to compare them with values found with other techniques. Similar equilibrium constants will show that MALDI and this second technique give the same information. It is therefore no longer necessary to follow the chemistry of the complexes from the solution to the MALDI sample. Recently, ionization methods such as field desorption 24, fast atom bombardment 25, 25 Cf plasma desorption 26, or electrospray ionization (ESI) 27, 28, 29 have been used to directly measure stability constants by mass spectrometry (MS). Young et al. 28 used ESI and internal standards to eliminate signal intensity fluctuations and determined solution stability constants of substituted crown ethers with small metal cations in methanol. Another promising approach is being developed by Brutschy and coworkers 30. They built a so-called “laser-induced liquid beam ionization/desorption mass spectrometer”
(LILBID-MS) capable of ionizing and mass analyzing molecules injected into a time-of-flight mass spectrometer in a liquid jet. Stability constants of some crown ether-cation complexes in glycerol were evaluated by Man et al. \(^{31}\) using electrohydrodynamic mass spectrometry and internal standards. In their method, the preformed ions are extracted from the bulk by an electrostatic field. The results were believed to reflect the solution chemistry.

### 5.2.2 Advantages of the two-phase MALDI sample preparation

Quantitative information is difficult to obtain with a standard MALDI sample preparation. Despite its advantages (high tolerance to salt contamination, simplicity of the resulting mass spectrum, capability for studying high molecular weight compounds), conventional MALDI often results in unstable signal intensities. Ralph et al. \(^{32}\) studied crown ether \(^{33}\) and antibiotic complexes with metal ions and concluded that standard MALDI sample preparation was not suitable for measuring relative ion concentrations in solution. For this reason, two-phase MALDI \(^{34, 35}\) was used here, a sample preparation method in which the analyte remains in solution. In two-phase UV-MALDI, the solid matrices used in conventional MALDI preparations are replaced by vacuum-stable liquids mixed with particulates that absorb the laser light. Liquid solvents such as glycerol, nitro-benzyl alcohol, or nitrophenyl octyl ether have been successfully used as matrices. In addition, two-phase MALDI was shown to be much more reproducible and stable than solid sample preparations. Due to the improved sample homogeneity the search for "hot-spots" on the samples is also eliminated. Another advantage of using two-phase MALDI is that conventional MALDI involves crystalline samples while most of the other MS techniques described above use liquid samples. Ionization from the solution phase may better reflect the behavior of the complex in solution and the possible problems occurring during the MALDI sample crystallization disappear.
5.2.3 Theory

The thermodynamic stability constants $K$ of a ligand $L$ binding with metal ions $A$ and $B$ are defined as follows:

$$L + A^+ \rightleftharpoons L \cdot A^+ \quad K_A = \frac{a(L \cdot A^+)}{a(L) \cdot a(A^+)}$$

$$L + B^+ \rightleftharpoons L \cdot B^+ \quad K_B = \frac{a(L \cdot B^+)}{a(L) \cdot a(B^+)}$$

Where $a(\cdot)$ is the activity of the compound in the solution. The relative stability $K_{A,B}$ of the ligand $L$ with the ion $A$ towards $B$ is defined as the ratio of the stability constants. Using Eq. (1) and (2):

$$K_{A,B} = \frac{K_A}{K_B} = \frac{a(L \cdot A^+)}{a(L \cdot B^+)} \frac{a(L) \cdot a(A^+)}{a(L) \cdot a(B^+)}$$

Activities are not easily measured, we therefore used diluted solutions in glycerol to have activity coefficients as close as possible to unity and replaced the activities with concentrations.

As already proposed by Johnstone et al. \(^{36}\) and Young et al. \(^{28}\) we assume a proportionality between the concentration of a compound in the MALDI sample preparation and its peak integral in the mass spectrum. For this to hold, some factors must be verified, in particular that the ion detector is operated in its linear regime. The risk of saturation can be significantly reduced by operating the detector at a reduced voltage. In addition, the number of ions brought into the gas phase must be proportional to the ion concentration in the sample and the transmission through the instrument must be independent of the number of ions. Under optimal MALDI conditions (laser intensity just above threshold), we believe that these conditions are fulfilled:

$$[X] = t \frac{I_x}{x}$$

where $I_x$ is the mass spectral peak integral of the signal and $[X]$ its concentration in solution. The constant $t$, called the “instrument response” or “transfer coeffi-
cient”, includes factors such as ionization efficiency, detector mass bias, transmission of the TOF, etc. By substitution of (4) into Eq. (3), the relationship between concentration and peak intensity gives access to selectivity measurements using mass spectrometry.

Two special cases are typically considered:

1) The ligand is in large excess compared to the ions. With a large $K$, the complex concentration is close to the salt concentration added initially, $[A^+]_0$ or $[B^+]_0$, respectively. Using Eq. (4), the following expression can be derived:

$$\frac{[A^+]_0}{[B^+]_0} = \frac{I_A}{I_B} \cdot \frac{t_A}{t_B} \cdot \frac{1}{K_{AB}} \cdot \frac{I}{I_A} \cdot \frac{1}{1 - K_{AB}}$$

The ratio $t_A / t_B$, termed “relative instrument sensitivity”, will be called $S_{AB}$. This value is experimentally found by plotting the relative amounts of the salts A and B versus the ratio of their corresponding complex mass peak integrals.

2) When concentrations of the metal ions (A, B) are in large excess compared to the ligand L, the concentrations of the free salts in solution, $[A^+]$ and $[B^+]$, are not much different from the initial values, $[A^+]_0$ and $[B^+]_0$. Using Eq. (5) and (4) in Eq. (3) the following relationship is found:

$$K_{AB} = S_{AB} \cdot \frac{I}{A_B} \cdot \frac{1}{1 - K_{AB}} \cdot \frac{[B^+]}{[A^+]_0}$$

If $S_{AB}$ is found by using an excess of ligand (cf. case 1), this allows one to directly determine relative ion stabilities from a single mass spectrum.

### 5.2.4 Sample & sample preparation

The ion selective molecule used in this work is 18-Crown-6 (18C6), purchased from Fluka (Buchs, Switzerland). Glycerol and 2-nitrophenyl octyl ether (NPOE) were also obtained from Fluka. 3-nitrobenzyl alcohol 98% (NBA) was purchased from Aldrich (Buchs, Switzerland). The graphite particulates (2 μm diameter powder) were purchased from Aldrich, and methanol (HPLC Grade) from Baker (Deventer, Holland). Sodium chloride (NaCl) and acetate ($\text{CH}_3\text{COONa}$) were obtained from Aldrich and potassium, rubidium, cesium chloride and acetate from Fluka.

The 18C6 ether was dissolved in distilled water in $10^{-2}$ and $10^{-3}$ M concentrations. The salts were prepared in distilled water as 0.1 M and $10^{-3}$ M stock solutions.
Two-phase matrices were prepared as described by Dale et al. The liquid matrix was diluted in methanol (30/70 by vol.) and an equal volume of 2 μm diameter graphite powder was added to the solution. The resulting mixture was shaken for 30 min.

Samples with an excess of ligand were prepared as follows: ca. 1μl of the matrix solution was deposited on the sample holder. The crown ether was added to the matrix (ca. 0.5 μl of the 10⁻² M solution). A solution containing two salts was prepared using the low concentration stock solutions. Volumes were adjusted to obtain the desired relative salt concentration. Approximately 0.5 μl of the aqueous solution was added on top of the tip. Following solvent evaporation (ca. 10 min., dried in the air) the probe was introduced into the vacuum chamber and the mass spectrum measured.

Samples with excess of salts were prepared as follows: the matrix solution was deposited in the same way as described above. 0.5 μl of a 10⁻³ M crown solution and 0.5 μl of a mixture of 0.1 M salt solution were then added.

Although the samples were prepared from aqueous solutions, most of the water evaporated. During the mass spectrometric measurements only the MALDI liquid matrix remained.

### 5.2.5 Influence of the laser fluence

Before trying to use MALDI in a quantitative way, the parameters influencing the relative peak intensities for the various metal ion-crown complexes were carefully studied. In particular, the laser fluence can strongly influence the relative intensities. With two-phase MALDI, this was found to have a minor effect. The laser intensity was varied by adjusting the aperture of an iris placed in the beam, and measured with a calibrated pyroelectric detector. Samples containing 18C6 and salts were measured and the peak intensities of the complexes were evaluated for various laser energies. Although the laser fluence determines the absolute signal heights, the relative complex intensities were not found to vary strongly. In Fig. 5.2.1 the potassium vs. cesium complex intensities stay within 30%, which is much less than found in Ref. 32 for a solid UV-MALDI preparation. A linear trend is, however, observed. The potassium complexes are, relative to the cesium complexes, of smaller intensities at higher laser pulse energies.
Fig. 5.2.1: Relative peak intensities of 18C6 ether complexed with potassium vs. cesium as a function of the laser pulse energy. The sample was prepared in a two-phase MALDI matrix using glycerol and graphite.

To avoid an additional error source the laser power was therefore kept as constant as possible during the following experiments. The shot-to-shot reproducibility of samples dissolved in liquid matrices has previously been shown to be considerably enhanced compared to conventional MALDI preparations. This was also observed here. The sample-to-sample reproducibility was enhanced as well.

5.2.6 Determination of the relative sensitivities

As described above, relative sensitivities $S_{AB}$ were determined using an excess of ligand. All salts were measured against the cesium reference salt. The salts were dissolved in glycerol/graphite (30/70% v/v) and mixed with the 18C6 ether. Relative concentrations were varied typically from 0.1 to 10. For each ratio the mass spectrum was acquired several times and the relative integrals were calculated for each salt/crown ether complex. These relative integrals were then plotted as a function of the initial relative salt concentrations. Results are shown in Fig. 5.2.2 for NaCl, KCl and RbCl vs. CsCl. Divalent or trivalent metal ions were not found to form simple complexes with the ionophore. We were therefore unsuccessful in
applying the same procedure to complexes formed with these ions. The error bars correspond to a standard deviation calculated from 150 laser shots. In each case, a linear relationship was found. The slope corresponds to the inverse value of the relative instrument sensitivity $S_{X,Cs}$ between the two salts (see above).

![Graph showing relative peak intensities of $^{18}$C6 ether complexed with Na', K', Rb' vs. the Cs' concentration ratios in glycerol. The relative efficiencies ($S_{AB}$) were obtained by taking the inverse of the linear-fit slopes as described in the text. The error bars represent one standard deviation as calculated from 150 shots.]

Fig. 5.2.2: Relative peak intensities of $^{18}$C6 ether complexed with Na', K', Rb' vs. the Cs' concentration ratios in glycerol. The relative efficiencies ($S_{AB}$) were obtained by taking the inverse of the linear-fit slopes as described in the text. The error bars represent one standard deviation as calculated from 150 shots.

This value accounts for differences in ionization and desorption efficiencies, fragmentation, ion transmission and detection efficiencies of the complexes. In Fig. 5.2.2 all the slopes are different from 1. A slope of 1 would have been expected if all the complexes would have been ionized and detected with the same efficiencies. Similar experiments performed with the conventional solid MALDI preparation were not sufficiently reproducible to give well-defined $S_{AB}$ values.
5.2.7 Determination of the relative stability constants

Once the relative sensitivities were known, the procedure described above was used to obtain the relative stabilities $K$. The metal ion adduct peak integrals and their respective relative sensitivities, as well as their concentrations, were introduced into Eq. 6 to obtain their relative stabilities. In Table 5.2.1 the relative stability constants are listed for Na, K, and Rb relative to Cs. The counter-ion was in each case chloride and the solvent was glycerol/graphite. In the same table, the values obtained by Man et al. \(^{31}\) with electrohydrodynamic mass spectrometry (EHMS) are presented, as well as the stability constants in water and methanol measured by calorimetric titrations.

The experiments were repeated with various graphite concentrations to check its influence on the stability constants. This was not found to have a significant effect. The influence of the salt counter-ions was also investigated by replacing chloride with acetate, again with no effects on the results. Values were obtained from different samples containing various relative salt concentrations; in each case the same stability constants were obtained.

Table 5.2.1: Relative stability constants of sodium, potassium, rubidium, and cesium ions with $^{18}$C$_6$ ether normalized to the stability constant of $^{18}$C$_6$/Cs$^+$. The values found in water, methanol, glycerol with the EHMS method\(^{31}\), and glycerol/graphite with two-phase MALDI method are shown. * from Refs. 31, 37.

<table>
<thead>
<tr>
<th>MALDI Glycerol/Graphite $K_{\text{rel Cs}}$</th>
<th>EHMS Glycerol (Ref. 31) $K_{\text{rel Cs}}$</th>
<th>Potentiometry (Ref. 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanol, 25 °C</strong></td>
<td><strong>Water, 25 °C</strong></td>
<td></td>
</tr>
<tr>
<td>Log $K_1$ [l/mol]</td>
<td>$K_{\text{rel Cs}}$</td>
<td>Log $K_1$ [l/mol]</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>K$^+$</td>
<td>4.7 ± 0.5</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>Rb$^+$</td>
<td>1.6 ± 0.3</td>
<td>1.3 ± 0.07</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
5.2.8 Effects of the metal ion size and of the solvent

In water, methanol, and glycerol, 18C6 ether forms more stable complexes with potassium cations than with sodium, rubidium, or cesium ions. The diameter of the 18C6's cavity is between 0.26 nm and 0.32 nm, an optimum size for potassium (ionic diameter: 0.266 nm). The cesium ionic diameter is 0.334 nm, which is too large to fit entirely into the cavity. The cesium adducts, which have lower stability constants compared to potassium, show the highest instrument sensitivity. Instrument sensitivity and stability constants are therefore clearly not correlated: the detection efficiency for sodium adducts was found to be six times lower, and that for potassium two times lower compared to the cesium complex. This is remarkable since, in MALDI, many samples are detected as cationized adducts with sodium and potassium, even if these ions are not added to the preparation and only present in trace amounts. In glycerol, the larger the metal diameter, the smaller its relative instrument sensitivity.

![Diagram](attachment:diagram.png)

**Fig. 5.2.3**: Two-phase MALDI mass spectra of three samples containing the 18C6 ether and the same amount of sodium, potassium, and cesium chloride salts (excess of salts compared to the ligand). The liquid matrices were: (a) glycerol, (b) NPOE, (c) NBA. "L" represents the ligand. In the spectra (a) and (b) the cluster ion (Cs₂Cl⁺) is also observed.
A very different behavior is observed if the solvent is changed to a less polar liquid matrix. Fig. 5.2.3 shows spectra for a mixture of 18C6 and a solution of Na, K and Cs chloride salts (1:1:1 molar conc.) with different liquid matrices: glycerol, NPOE and NBA (dielectric constants 46.5 39, 23.9 40, 22 41 at 20 °C, respectively). All were mixed with 70% (v/v) graphite particulates. For NPOE and NBA, both less polar solvents, the cesium complex is not seen, whereas its intensity is high with glycerol. Two general effects may explain these differences: (i) the relative instrument sensitivities are affected by the liquid medium. Relative sensitivities between sodium and potassium were estimated in the three liquids. We found $S_{\text{Na}^+\text{K}^+} = 2.6, 7.4$ and 4.2 in glycerol, NBA and NPOE, respectively. This large spread of relative sensitivities shows the importance of taking this phenomenon into account. (ii) The stability constants are also influenced by the solvent 42. As can be seen, for instance, in Table 5.2.1, the metal ion/crown ether stability constants are quite different in glycerol, water, and methanol.

5.2.9 Comparison of the stability constants obtained by MALDI and by other methods

The EHMS values have typical errors between 5% and 15%. The MALDI errors were calculated from different samples containing different relative salt amounts. Including the uncertainty in $S_{\text{AB}}$, an error between 10% and 30% was found with MALDI. The values measured with MALDI and EHMS in glycerol are very close. This strongly suggests that both experiments give the same stability constants for the same interacting species measured under similar conditions. Because of this similarity and because the EHMS technique measures preformed ions contained in the sample solution, we believe that two-phase MALDI is also able to quantitatively reproduce the solution chemistry. As in aqueous solution, the Na$^+$ /18C6 complex in glycerol is the weakest, followed by Cs$^+$, Rb$^+$, and K$^+$. In the gas phase, the Cs$^+$ selectivity is the smallest, followed by Rb$^+$, K$^+$, and Na$^+$. This difference is a strong indication that the MALDI signals do not result from gas phase complexation. Gas phase reactions cannot be totally excluded by these experiments, but if they happen then they do so with the same probability for all ions present. It is therefore likely that, under the conditions described in this work, the complex ions present in the glycerol are directly desorbed into the gas phase by the laser pulse.

The model system (metal ion/crown ether) that was chosen here is simple and the complexes are very similar; they only differ in the metal ion. However, the
differences in relative instrument efficiencies were large. In more complicated systems, it is therefore vital to take into account possible differences in these relative efficiencies even for similar compounds. Once these sensitivities are known, two-phase MALDI could be of great interest for the investigation of non-covalently bound molecules.

5.2.10 Conclusions

To verify whether MALDI mass spectra can, under well-chosen conditions, quantitatively reflect solution chemistry, stability constants were determined by mass spectrometry and compared with literature values obtained with a different method. To eliminate problems due to sample crystallization in a standard solid MALDI preparation and to improve reproducibility, we used two-phase MALDI. As a model system, the complexes between a crown ether (18C6) and alkali metal ions (Na\textsuperscript{+}, K\textsuperscript{+}, Rb\textsuperscript{+}, Cs\textsuperscript{+}) were investigated. In a first step the relative instrument sensitivities of the different complexes were determined. In glycerol, these were found to be inversely proportional to the ionic diameter of the monovalent metal ion. A totally different behavior was observed in other liquid MALDI matrices. This large spread in relative sensitivities shows the importance of taking this parameter into consideration for quantitative measurements.

The relative stability constants of the 18C6 ether complexes with sodium, potassium, rubidium, and cesium were then determined in glycerol. They were found to be similar to literature values. It was therefore concluded that, under the conditions used in this work, the MALDI results quantitatively describe the relative concentrations of the complexes in the initial solution. If differences in ionization efficiencies – which can be significant - are taken into account, two-phase MALDI is a useful tool for quantitative investigations.

5.3 Summary of chapter 5

We showed that MALDI mass spectra are influenced by the sample preparation. The pH of the solution and the composition of the solvent affected the mass spectrum patterns. From a practical point of view, sample preparations, made with polar solvent and low pH values, were found to give the best results.

The picture that emerged from these experiments is that the relative amount of preformed ions can be varied by changing the solution conditions. Two-phase MALDI was found to be particularly suitable to control the ion origin. For the test
model used in this work, the MALDI mass spectra correlated with the conditions in the liquid. Consequentially, MALDI can, under certain conditions, reflect solution chemistry and can be utilized, for instance, to investigate the interactions between metal cations and crown ether. However, as the relative amount of preformed ions may change for each new chemical system, care must be taken to check whether this correlation still exists or not. In other words, raw MALDI information should always be interpreted with caution and test experiments should be done to verify the relevance of the peaks.

Another major finding of Chapter 5 is that quantitative measurements are possible with MALDI. A method using internal references was described. We found that, even for chemically similar ions, the ionization and detection efficiency (so-called sensitivity) can be very different. Estimation of these sensitivities should therefore always be considered first.

The standard solid MALDI sample preparation was also found not to be optimal, mainly due to the poor resulting shot-to-shot reproducibility of the signal intensities. Instead, two-phase MALDI showed a less dramatic signal fluctuation.

5.4 References

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Chapter 6.

6. SUMMARY AND OUTLOOK

Two new ion detectors were presented in this thesis that were based on a different principle than the standard secondary electron multipliers. Energetic ions impacting on scintillating surfaces create photons that can be detected with a photomultiplier. In the first device, called ion-to-photon detector (IPD), the primary ions directly hit the scintillator. Its construction is very simple and robust. 24 keV ions as large as 67'000 Da were readily detected with the IPD and a total detector response time on the order of 4 ns was estimated. The signal-to-noise ratio was however poorer than the one obtained with a microchannel plate (MCP) detector. Using single event counting techniques, it was shown that, at a given ion energy, the efficiency of the IPD compared to the MCP decreased as the molecular mass of the projectile increased. We estimated that small ions (<150 Da with 22 keV) produced more photons than secondary electrons.

This finding was utilized to develop a second device based on the same principle but that included an additional element: the primary ion beam first hit a conversion dynode that fragmented the ions into smaller particles. These particles were then accelerated onto the scintillator. This device, the ion-to-electron-to-photon detector (IEPD), requires only an additional high voltage generator. A detector response time similar to the one found with the IPD was observed. The signal-to-noise was much improved and molecules as large as 500'000 Da were detected. Clusters with even larger masses (800'000 Da) were also measured.

Both detectors have promise for future applications. The IPD is a simple and cheap device that is fully adapted to detect light and mid size ions. Due to its robustness, it can be operated at higher pressure. Easily miniaturized, it could be the detector of choice for small portable mass spectrometers. The IEPD construction is slightly more complicated but has the ability to detect very large ions. In our experience, its sensitivity for large masses is better than that of MCPs. It is a very versatile and fast detector that can be utilized for small and large ions. This is in
contrast to many secondary electron multipliers that have, despite their sensitivity for large masses, a rise time too long to be utilized for small ions. Combined with its other advantages, the IEPD could be utilized in time-of-flight mass spectrometers instead of secondary electron multipliers or MCPs. Some directions for possible future developments and experiments to better understand the fundamentals of these detectors are:

- A large fraction of the photons are emitted in the opposite direction to the photomultiplier. Two possibilities for improvement are proposed: the scintillator could be covered with a metal layer. The layer has to be thin enough to allow the electrons to pass but thick enough to form a mirror for photons. We tested this method and deposited 40 nm of aluminum. Unfortunately the aluminum strongly degraded the mass spectral quality, probably due to sputtering. Other metals need to be tested. A second possibility is to place a reflector with a central hole in front of the scintillator that allows the secondary particles to go through.

- Scintillation is a phenomenon that utilizes molecular fluorescence. It may be possible to instead use vibrational de-excitation emissions (IR). As the associated energies are much lower, the ion detection efficiencies may be increased. Special scintillators having a strong emission in the IR range should then be used (for example IR laser dyes). IR light is however more difficult to detect. Some of the possibilities are: special photomultipliers, diodes or wavelength shifters.

- It is still unclear whether the IPD has an ion number threshold for improved detection. The experiments described in this work demonstrate that the efficiency of the IPD when used to detect ion packets may be larger than when for single ions. Further experiments may be done to study this effect, for instance, by projecting with an ion gun a variable amount of particles onto the scintillator and comparing the detector count rates.

- Very large molecules preferentially fragment instead of emitting secondary electrons upon impact onto a surface. We showed that 20 keV ions up to 800'000 Da could be detected with secondary electrons. However, primary ion detection via secondary ions may be, for these large compounds, an interesting alternative for increased signal intensities. For that purpose, scintillators that can detect ions efficiently may be tested.

In the second part of this thesis, it was shown that mass spectral quality can be improved by using polar solvents and acidic pH. The amount of preformed ions can be to some extent controlled by the sample conditions. Signals observed in
some mass spectra were mainly produced by preformed ions. We showed, for example, that two-phase MALDI mass spectra of a solution of a crown ether and metal cations reflected the equilibrium chemistry that existed in the solution. Quantitative MALDI measurements are possible under the condition that the ionization/desorption and transmission efficiencies of each compound contained in the sample are first determined. The methodology was applied to the system crown ether - metal cations described above but can be utilized for many other systems. In particular, we would like to come back to the figure depicted in the introduction (Chap. 1). A MALDI mass spectrum of an equimolar mixture of PEGs was shown to illustrate the difficulties in doing quantitative measurements with this method. From the spectrum, it seemed that direct quantitative measurements were not possible. However, the relative efficiencies of each polymer can also be estimated by varying their relative concentrations. After correcting with these efficiencies, quantitative information should be possible to obtain. Similarly, other chemical systems may also benefit from this procedure enabling MALDI to add quantitative information to its powerful ionization capabilities.
Publications

The following publications based on the work described in this thesis have already appeared or have been submitted:


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1969 born on October 30, in Lausanne, Switzerland
1975-1980 primary school, Le Mont
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1995 diploma thesis in electrochemistry with Prof. H. Girault, Swiss Federal Institute of Technology Lausanne. Title: "Etude des transferts d'électrons à travers l'interface liquide-liquide".
1995-1999 Ph. D. thesis in analytical chemistry, Swiss Federal Institute of Technology Zurich, under the direction of Prof. Dr. R. Zenobi. Title: "Ion formation and detection in MALDI mass spectrometry".
1998 4 month project at the University of Paris-Sud, group of Prof. Dr. Y. Le Beyec.

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