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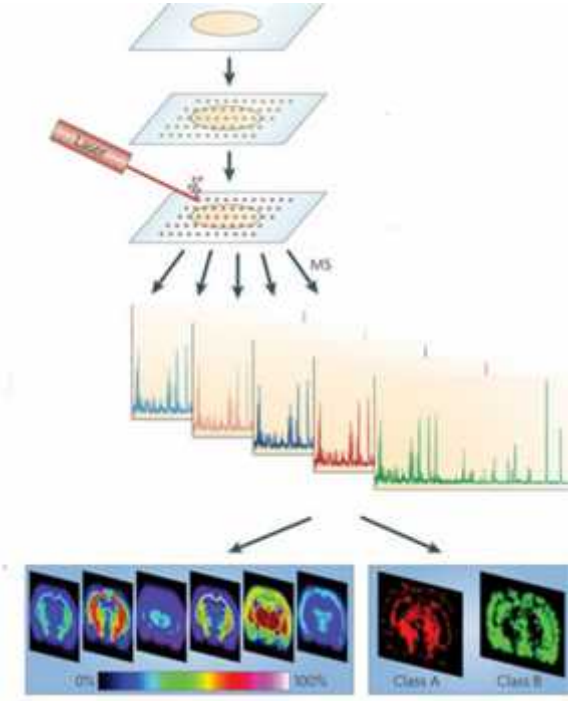

Optimizing Sample Preparation Protocols for Multi-Modal Imaging Mass Spectrometry (IMS)

Master of Science Thesis in the Master Degree Programme, Chemistry and Bioscience

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Keywords: Matrix Deposition, Matrix Sublimation, ImagePrep, MeSIMS, MALDI Imaging.

Abstract

Matrix application is one of the most crucial steps in sample preparation of Imaging Mass Spectrometry techniques such as MALDI and MeSIMS. Essentially an efficient and reproducible matrix deposition method needs to be applied in order to obtain high quality, trust worthy and reproducible images in IMS. Several strategies have been developed to apply matrix in both manual and automatic procedures in recent years.

In this workflow two matrix application techniques of ImagePrep and sublimation were utilized to apply DHB (2, 5-dihydroxybenzoic acid) used as the matrix on tissue samples. In the first step, size, distribution and coverage of produced matrix crystals through use of ImagePrep and sublimation were investigated. ImagePrep creates inhomogeneous matrix deposition and relatively big matrix crystals with poor coverage, whereas sublimation is able successfully to generate very small crystals and homogeneous films.

In next step of this project, deposition temperature and time were optimized for sublimation of DHB which were found to be 125°C and 8 min, respectively. Eventually, in order to obtain a proper thickness of deposited DHB for MALDI and SIMS imaging, sublimation of DHB was performed at125°C in 10, 30 and 60 min.

SIMS imaging results showed that none of deposition generates an appropriate thickness for SIMS imaging. In conclusion, times of 5, 15 and 20 min should be performed to obtain an optimized time.

For MALDI imaging, 10 min sublimation produced insufficient thickness of the deposited DHB, whereas the thickness produced after 60 min deposition was too thick. Ultimately, 30 min deposition time was found to produce the most intense signals.

Keywords: Matrix Deposition, Matrix Sublimation, ImagePrep, MeSIMS, MALDI Imaging.

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1. INTRODUCTION

Imaging mass spectrometry (IMS) is a powerful technique for determining, mapping and visualizing the distribution of endogenous biological molecules such as lipids or neuropeptides across biological materials. The IMS technique combines the chemical specificity and spatial analysis of surfaces. As a result, the analyst is able to obtain spatial localization of several different compounds, complex biomolecules and parallel detection of multiple analytes [1-2].

These species are recorded directly without the need of target-specific reagent and radioactive or fluorescence labels in contrast to commonly used in other imaging techniques such as optical imaging, positron tomography, electron microscopy, atomic force microscopy and scanning tunnel microscopy [3-4].

Recently, research efforts have been applied to a more comprehensive study of compounds in tissue via IMS. For instance, secondary ion mass spectrometry (SIMS) imaging is frequently utilized to investigate the distribution of atoms and small molecules with spatial resolution below one micron. Another advance in the field of IMS is matrix-assisted laser desorption/ionization (MALDI). This well-known technique is a prominent imaging method used to study lipids, peptides and proteins at a spatial resolution ranging from 3 to 250 micro meters [5-6].

For MALDI imaging the following steps are preformed to prepare the sample, 1) the deposition of matrix, 2) data acquisition, and 3) image re-construction [7]. For protein and peptide targeted analyses, the samples are washed to remove lipid contamination before the matrix application step [8].

Although it seems obvious that each of these steps are needed to be fulfilled accurately to achieve a high quality image, it is important to focus on the deposition of matrix step to find an optimized protocol as a main aim in this workflow. Typically, several application strategies are utilized for matrix application. Some key aspects such as homogeneity of the matrix layer, reproducibility of the matrix application, deposition of sufficient amount of matrix, and spatial integrity of the sample have to be considered during this process in order to obtain desired resolution and high quality results [2] [8-9].

In this project two matrix application techniques have been compared, a spray technique using an automated commercial system, the ImagePrep from Bruker, Gmb, and sublimation, a non-commercial technique. Matrix thickness, incubation time and wetness have been optimized in ImagePrep. For sublimation-based matrix application, matrix deposition time and the sublimation temperature have been optimized.

2. THEORY

This section provides some of the theoretical framework for the MALDI matrix deposition methods used in this thesis as well as their testing and application.

2.1 What is Mass Spectrometry?

Mass spectrometry is an analytical method used to obtain the molecular mass of sample by measuring the mass-to-charge ratio (m/z) of charged particles to determine elemental composition. It is used to characterize the chemical composition of different compounds. Typically all MS instruments consist of three fundamental parts; an ion source, mass analyzer and detector (Figure 2.1). At the first step, molecules of sample in the gas phase are ionized. These ions are then introduced into mass analyzer where they are extracted and sorted according to their mass (m) to charge (z) ratio (m/z). The separated ions hit the detector, the signal is sent to a data system which records the event and forms a mass spectrum [10-11].

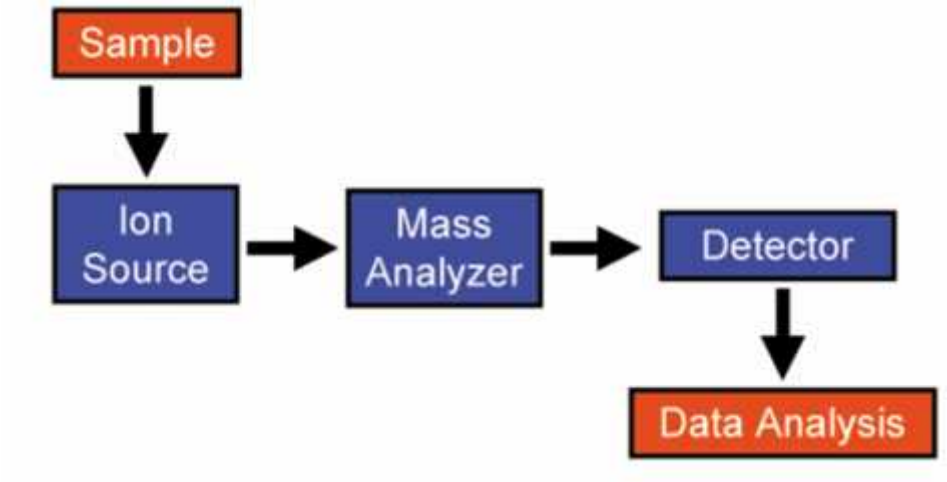


Figure 2.1.Schematic representation of mass spectrometry (from Wikipedia).

2.2 What is Imaging Mass Spectrometry (IMS)?

Generally imaging mass spectrometry is used to directly map the distribution of different compounds that range in size from atoms to intact proteins in biological tissues. Typically in mass imaging techniques, molecular images are generated by rasterizing across the sample to collect a series of mass spectra from an ordered array of positions on the surface based on the size of the probe beam. Following data acquisition, the intensity of the signal from analytes of interest are plotted and represented by a color scale leading to a localization pattern and ion images or distribution map for individual molecular species which exist on the sample (Figure 2.2) [6] [12].

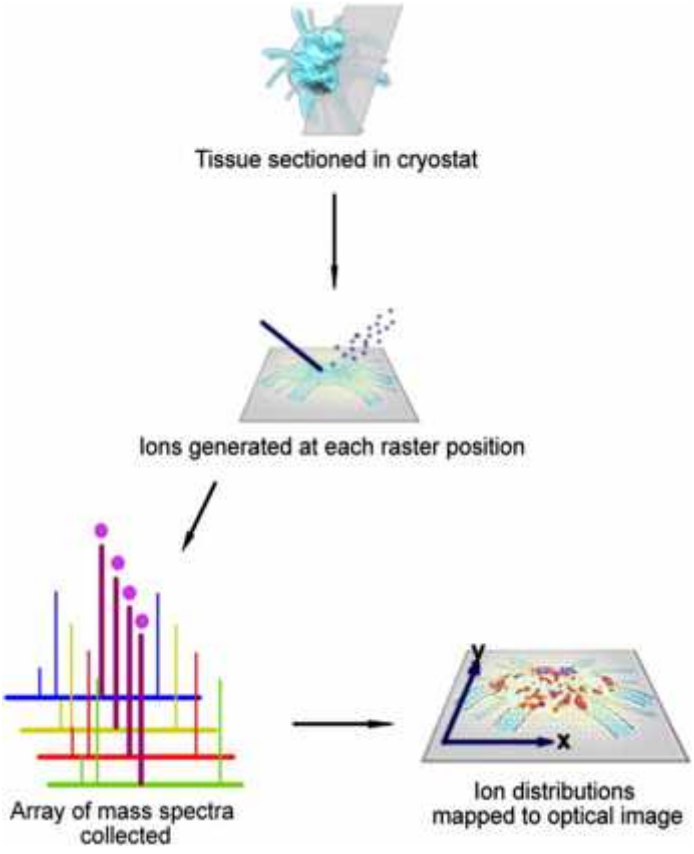


Figure 2.2.Overview of creating the ion images in IMS [13].

2.2.1 Techniques

In general, the following steps are commonly used for mass spectrometry methods: analyte must be ionized, separated in a vacuum, and then detected. Consequently based on the ionization method used, mass spectrometry has been divided into three powerful techniques including secondary ion mass spectrometry (SIMS), matrix-assisted laser desorption (MALDI), and desorption electrospray ionization (DESI) [11].

2.1.1.1 SIMS

SIMS is a widely used ionization technique in IMS has been well known as a sensitive surface analysis method in which a solid sample is bombarded by a focused primary ion beam. The bombardment event leads to the emission of charged particles, named secondary ions, from the surface of target. After the collection and analysis of these secondary ions in mass spectrometry, information about the elemental and molecular composition of the surface is obtained (Figure 2.3). This technique can be applied to any kind of substances that can be placed in a vacuum. Owing to sputtering of materials, SIMS is generally considered a destructive technique [14].

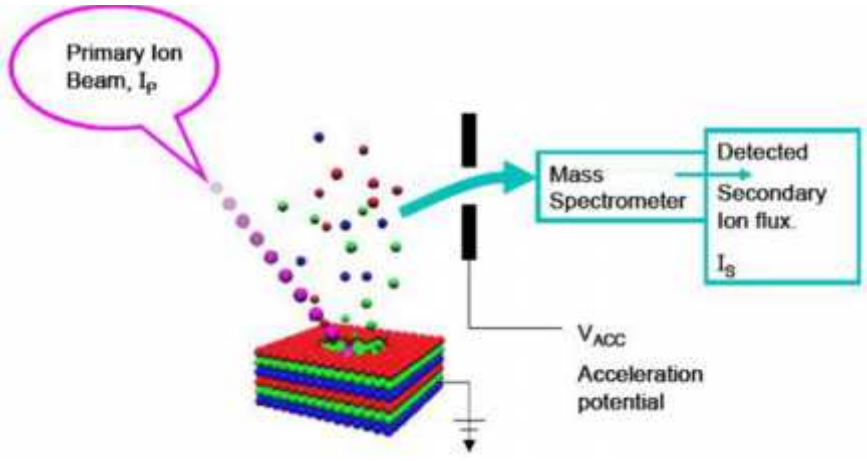


Figure 2.3. A brief illustration of SIMS [15].

2.1.1.2 MALDI

Matrix-assisted laser desorption ionization (MALDI) is a laser based soft ionization method utilized in mass spectrometry which has been known as one of the most successful methods for investigation of large molecules. In MALDI a matrix that consists of crystallized molecules is mixed with the analyte (or placed on the analyte). Following vaporization of the solvent, molecules of analyte are extracted and cocrystallized with matrix leading to formation of analyte-doped matrix crystals. The sample-matrix crystals are bombarded with a UV laser beam to excite the matrix and subsequently transfer energy to the analytes, causing them to be ejected from the surface. This process gives rise to ablation, desorption and ionization of the analytes mainly as charged particles [1, 11]. Figure 2.4 shows the process by which ions are formed in MALDI.

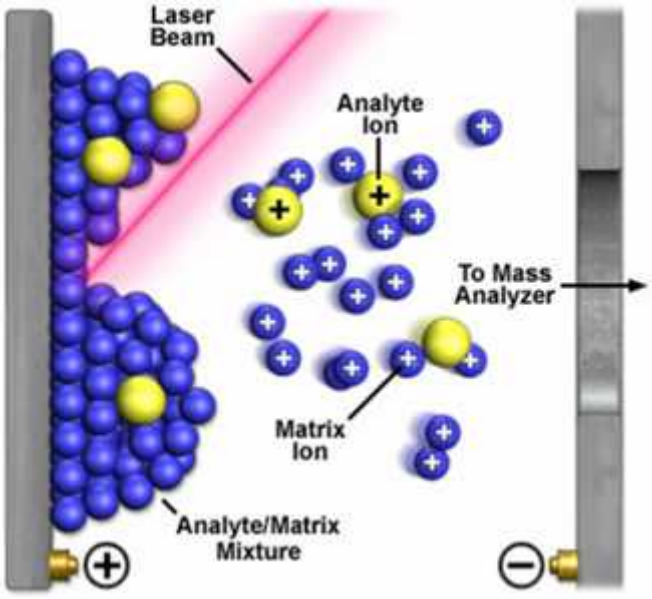


Figure 2.4. The formation of ions during MALDI [16].

2.1.1.3 DESI

Desorption electrospray ionization (DESI) is a combination of electrospray (ESI) and desorption (DI) techniques and is unique as it is carried out at atmospheric pressure. DESI is accomplished by directing an electrosprayed mist of charged droplets onto a surface of the sample from a few millimeters away. By applying an electrical potential, the stream of the electrospray impacts the sample surface leading to the generation of ions from the chemical species present. Consequently, these ions move through the air to the mass spectrometer inlet under the influence of an applied potential field. Figure 2.5depicts a simple illustration of procedure to produce ions by the DESI technique [6] [17].

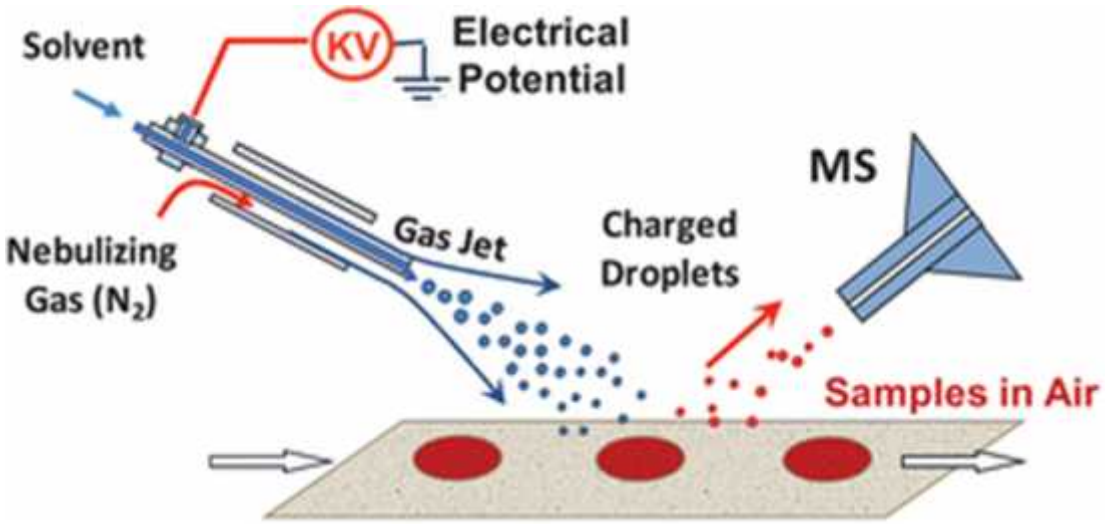


Figure 2.5. Schematic diagram of DESI desorption and ionization process [17].

2.3 Pitfalls of Imaging Mass Spectrometry (IMS)

Compatibility of the samples in high vacuum, which is a typical condition to work in IMS instruments, is important. At room temperature, there is the possibility of molecular diffusion in all the steps of sample preparation, such as cutting, washing, matrix application, which influence negatively on data reproducibility and the quality of the images.

Biological samples are too complex to analyze by IMS directly. The multiple molecules existing in tissue sections including proteins, lipids, oligonucleotides, carbohydrates, matrix ions, and salts which adversely impress efficiency of desorption and ionization and also lead to inhibition of optimal detection. This phenomenon, called ion suppression, in which the ionization process is less efficient causes reduction the quality of the IMS analysis.

When the size of the matrix crystals exceeds 10 µm, this can negatively effect on the spatial resolution in MALDI imaging. In SIMS, a matrix is not required for ionization and desorption [16] [18-19].

2.3.1 Resolution and Sensitivity

Generally there is a trade-off between lateral resolution and the number of ions produced. This is considered the sensitivity of analysis leading to a limitation in instrumental work at cellular level. In fact, at high lateral resolution there is an obvious reduction in the amount of available molecules to be ionized and detected. In other words, lateral resolution is increased at the cost of sensitivity. To improve sensitivity, surface treatment methods, including adding a thin layer of matrix-metal - named metal assisted (MetA) and adding a matrix to enhance(Me) SIMS have been reported to increase the desorption/ionization of analytes [20].

2.4 Secondary Ion Mass Spectrometry (SIMS)

In SIMS, a primary beam of either positive or negative ions focused on the surface of sample to produce secondary ions, which are then transferred into a mass spectrometer to achieve quantitative and qualitative chemical information [6].

2.4.1 Principles of SIMS

As the primary ion beam impacts the sample surface a sufficient energy is added to ionize many molecules and elements. Typically the range of this energy is about 5-25 keV, which is much bigger than the bond energies of surface molecules leading to a huge fragmentation of molecules and emission of atomic particles especially near the interaction site. In other words, less fragmentation is generated in the crater region around the impact site [16]. When the surface binding energy is overcome, a small amount of intact molecules are produced from the first layer of the surface. The majority of ejected molecules are neutral and only around 1% of them are charged.

These resultant ions that are freed from the surface are accelerated, separated, and analyzed in a mass spectrometer. SIMS is widely used in two modes; static and dynamic. In static SIMS a low energy primary ion beam is sputtered on the surface extremely slowly, and this is used for analysis of atomic or molecular mono layers on the sample surface. Static SIMS is run with ion beams such that less than 1% of the surface is sputtered – the so called static limit.

In contrast, in the dynamic SIMS mode, a higher energy primary ion beam is utilized to produce a high yield of secondary ions. This technique has been well known for bulk analysis of elements, depth profiling, and as the only approach that can provide truly quantitative information [16] [21].

2.4.1.1Cluster Ion Sources

The overall concept of desorption of molecules by ion bombardment of a surface began around 30 years ago and was successfully utilized to detect organic molecules [22]. Although, unfortunately, this earliest production of ToF-SIMS instruments did not work for organic molecules analysis owing to low sensitivity, high molecular fragmentation [20] and too much damage of the sample surface.

Many of the weaknesses of SIMS promise to be overcome with the advent of modern of primary ion beam sources formed from molecular clusters such as C_{60}, SF_5, Bi_3, Au_n, Cs_n, and Ar_{4000} rather than single atomic particles [22]. Typically, the use of these polyatomic primary beams is now leading to extensive development and change in direction of SIMS approaches, particularly in the areas of bioanalysis.

Cluster sources offer several advantages over atomic ion sources, including a reduction in the damage of the sample surface physically and chemically and also the enhancement of secondary ion yields mainly for high-mass organic molecular ions. These advantages lead to a larger mass detection range and a wider range of biomolecules that can be studied. In addition, the amount of molecules detected per image pixel is increased resulting in brighter signals and better contrast between neighboring pixels. This ultimately improves the sensitivity for chemical mapping of samples like single cells. The bombardment pattern is significantly different when compared to atomic ion sources owing to an energy distribution between all of individual atoms in the clusters that gives rise to higher molecular ion sensitivity, this results partly from less fragmentation.

The ability of cluster ion sources to desorb secondary ions from the sample surface without too much fragmentation makes it a more appropriate technique for the analysis of intact biomolecules from tissue surfaces. For instance cluster SIMS has been used a lot recently in the analysis of lipids from cells and tissue as a direct result of its ability to detect intact molecular species particularly in two or three dimensions [16] [20] [22].

2.4.1.2 MeSIMS (matrix enhanced SIMS)

Standard SIMS essentially suffers from low sensitivity for higher mass molecules and high fragmentation for smaller molecules. Recently some surface modification methods have been developed to enhance sensitivity of larger molecules and the production of intact molecular ions. One of them is MeSIMS in which a normal SIMS apparatus and a protocol for MALDI sample preparation can be utilized in parallel. In fact, MeSIMS can be regarded as a combination of MALDI and SIMS in which a thin layer of small molecule organic matrices are applied to the surface sample in a similar way used for MALDI IMS. The main advantages of MeSIMS is that it is easy to work with and inexpensive [8] [16].

In MeSIMS the coating of organic acid on tissue leading to incorporation of analyte into the matrix layer results in enhancement the ionization efficiency of larger molecules such as proteins as large as lysozyme at 14 kDa [6]. Besides a significant improvement of the maximum mass range for SIMS, matrix enhancement also decreases the fragmentation of lower mass analytes giving rise to increased sensitivity [16].

2.4.2 Applications of SIMS

SIMS as an imaging technique has numerous applications in biotechnology and biological studies particularly in medicine and pathology fields. There have been some reports using this technique successfully in imaging of unicellular organisms and single cells, chromosomes and some tissues in organs like the kidney, brain, prostate, cockroach and snail tissue and also to provide 3D imaging of tumors and oocytes [16].

2.5 Matrix Assisted Laser Desorption Ionization (MALDI)

MALDI is a powerful technique to study intact biomolecules such as proteins directly from tissue sections. This is inherently a soft ionization technique using a laser combined with a chemical matrix, usually an organic acid, to produce ions from an analyte sample mixed with the matrix molecules [16].

2.5.1 Principles of MALDI

MALDI is a two-step process; in the first step a matrix is applied having a strong absorption of at the wavelength of the ablation laser. Analyte molecules are embedded in the matrix. Application of the laser causes ablation of upper layer of the matrix material and the analyte molecules with it. The majority of the laser energy is absorbed by the matrix causing desorption of material with analyte into the gas phase [10-11].

The MALDI matrix is also thought to result in ionization of molecules in the gas phase. There have been some chemical and physical ionization mechanisms suggested for MALDI including gas-phase photoionization, excited state proton transfer, ion-molecule reactions, desorption of preformed ions. Among these mechanisms, ion formation from proton transfer in the solid phase before desorption or gas-phase proton transfer from photoionized matrix molecules are the most generally recognized mechanisms [10].

Typical MALDI spectra contain the singly charged compounds produced by protonation in the positive ion mode. While deprotonated species are generally identified in the negative mode. Furthermore some multiply charged ions and a lot of fragments are also detected.

In MALDI, the analyte is usually incorporated into the matrix crystals which results in a less sensitive method to contaminants such as salts, buffers and detergents compared to other ionization techniques.

A high concentration of contaminants such as buffer salts can affect negatively the desorption and ionization steps. Generally prior purification is helpful to eliminate this sort of contamination and subsequently to obtain higher quality mass spectra [16].

2.5.2 Choice of Matrix

Typically the matrix is a small organic acid which has a strong absorbance at wavelength of used laser. In general, these compounds applied to tissue samples, play a major role in desorption and ionization of analyte molecules. Hence, selecting the proper matrix is a critical step. Basically the matrix crystals absorbing energy of laser light are evaporated leading to release of the trapped analyte molecules into the gas phase [1] [8].

The matrix serves another fundamental function containing the ionization of analytes. The ionized molecules, typically detected by MALDI-MS, are generally singly charged species $[M+H]^+$ [16].

However, the choice of a proper matrix does not follow any general rules. There are some considerations such as solubility of matrix and analyte in solvents used in matrix solution, the ability of the matrix in absorbance at the laser wavelength, the stability of matrix in vacuum conditions, and also the chemical properties and mass range of analytes with a minimal overlap between analyte and applied matrix.

Many different kinds of matrices are derived from benzoic acid, cinamic acid, and picolinic acid are commonly used in MALDI. There are some matrices normally used in MALDI such as alpha-cyano-4-hydroxycinnamic acid (CHCA) are routinely used and recommended for lower molecular weight peptides whereas sinapic acid (SA) is more suitable for higher molecular weight proteins. For phospholipids2, 5-dihydroxybenzoic acid (DHB) is usually used [8] [16].

2.5.2.1DHB (2, 5-Dihydroxybenzoic acid)

DHB, a derivative of benzoic acid with molar mass 154.12 g/mol and molecular formula $C_7H_6O_4$, is a well-known matrix used in MALDI. It absorbs laser energy at 337 and 355 nm [23]. DHB is soluble in both water and organic solvents. In comparison to the other matrices, DHB is less sensitive to contaminants, such as salts and/or detergents.

The main disadvantage of DHB is its large crystal size. Large crystals with varying geometries cause spot to spot variation within the samples and result in the decrease of resolution significantly [24].

DHB is frequently used for peptides, glycopeptides, nucleotides, glycoproteins and small proteins [8]. This matrix has been also properly used for the negative ion MALDI-MS glycolipids [23].

2.5.2.2 CHCA (α- cyano-4-hydroxycinnamic acid)

Alpha-cyano (α-cyanois) is a yellow powder and a derivative of cinnamic acid with molecular formula $C_{10}H_7NO_3$ and molar mass 189.17 g/mol. It is frequently used as a suitable matrix for peptides particularly in low mass range for MALDI analysis. This matrix is insoluble in water while it has very solubility in organic solvents [24].

The most important advantage of α-cyanois is its ability to produce small homogeneous crystals leading to good resolution especially in measurement of peptides. Also, since this matrix is not soluble in water, it can be used to wash samples on target. The main disadvantage of α-cyanois is an extreme amount of fragmentation. Generally, α-cyano is commonly used for peptides, small proteins, and glycopeptides [8] [24].

2.5.3 Applications of MALDI

MALDI provides mass data for lipids, peptides, proteins and metabolites directly from tissue sections and is well known as a sufficiently developed technique to analyze biomolecules and investigate tissue properties at the molecular level. Significantly, MALDI imaging is finding more and more application in the life sciences. One such example is cancer classification and diagnosis [25].

2.6 Mass Analyzers

In general, gas-phase ions that are produced are separated based on their masses, which must be identified in any of the mass spectrometry techniques. Typically a mass analyzer has the ability to measure mass to charge ratio (m/z). There are a great variety of mass analyzers available which have been developed for IMS. There are some reasons leading to more focus on time-of-flight (TOF) among other types of mass analyzers in this workflow. Firstly, the majority of IMS studies are successfully carried out with TOF. Secondly, TOF mass analyzers are commonly coupled with IMS techniques and ultimately a TOF mass analyzer provides parallel detection of several species from each single pixel [6] [10] [16].

2.6.1 Time of Flight

The TOF mass analyzer separates accelerated ions according to their molecular mass and works based on the fact that the velocities of two ions with different mass is different with lighter ions having a higher speed arriving at the detector faster. Thus, ions reach the detector at times that are inversely proportional to ratio of their mass to charge. According to equation 2.1 the kinetic energy of an accelerated ion within an electrical field with potential V will be

$$zV = \frac{mv^2}{2} \qquad \text{Equation 2.1}$$

where z, m and v are charge, mass and velocity of the ion respectively.

Equation 2.2 demonstrates the velocity of the ion in which L is length of the flight path and t shows the time.

$$v = \frac{L}{t} \qquad \text{Equation 2.2}$$

Replacing the term v in equation 2.1 gives equation 2.3.

$$zV = \frac{mL^2}{2t^2} \qquad \text{Equation 2.3}$$

Rearranging leads to equation 2.4.

$$\frac{m}{z} = \frac{2Vt^2}{L^2}$$

Equation 2.4

There are three main types of TOF analyzer based on their defined geometries including linear, reflectron, and orthogonal to improve sensitivity, mass accuracy, and mass resolving power. The linear geometry shown in figure 2.6 is generally utilized in TOF imaging mass spectrometry and supply the highest sensitivity and mass resolution [11] [16].

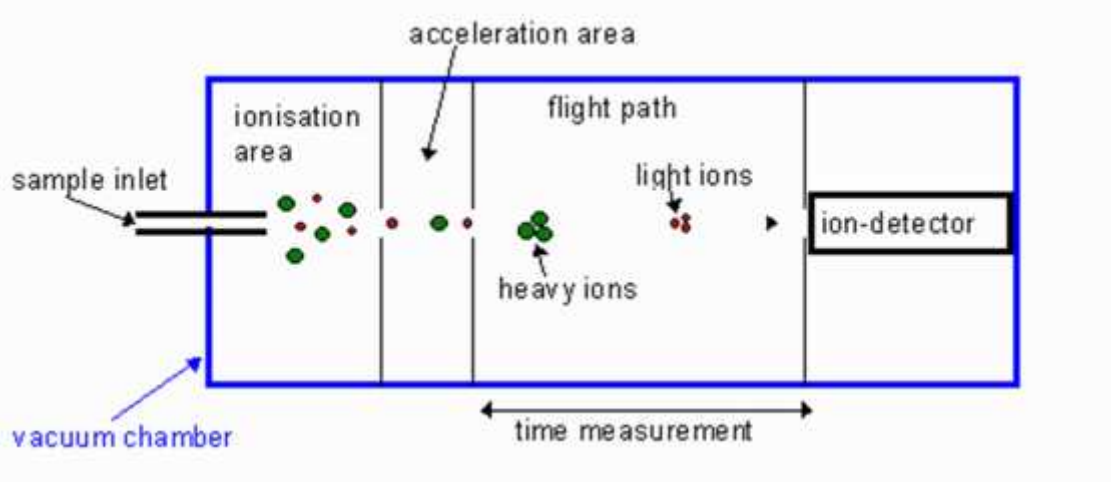


Figure 2.6. Schematic representation of a TOF mass spectrometer [26].

2.7 Sample Preparation

The sample preparation step plays a key role in order to obtain high quality, trust worthy, and reproducible images [9]. A large variety of samples that remain stable under high vacuum conditions can be used in IMS techniques. Samples that can be analyzed include pressed powders, bulk films, fibers, particles, and thin films. In particular for SIMS analysis the thickness and morphology of the sample can have effects on the possibility of producing secondary ions and consequently the stability of the spectrum. The accuracy of the analysis is negatively affected by contaminants deposited on the sample surface, thus cleanliness of sample surface is important during sample preparation process.

Surface diffusion of analytes is another issue that is important. To prevent molecular reorientation and surface contamination, analysis of samples must be started immediately after preparation. Yet another important consideration in sample preparation is spatial integrity, which is required to remain constant to obtain high spatial resolution during analysis. Experimentally, spatial resolution is sacrificed due to any failure in spatial integrity larger than pixel size [1] [14].

The sample preparation protocol in the case of tissue sections consists of different steps including preserving, sectioning, and washing the tissue and application of the matrix. However, each step has a significant impact on the spectrum and image quality, thus this workflow is typically focused on optimization of the protocol for the matrix application [7] [8] [27].

2.7.1 Deposition of Matrix

Deposition of matrix on the sample surface, which must be performed before MALDI and Me-SIMS, is one of the crucial steps in sample preparation. There are some features that need to be considered during matrix application including reproducibility of the process and homogeneity of the matrix layer, which plays a key role in obtaining similarity in the extraction and desorption of analytes. The matrix crystal size, which is another important parameter in deposition of matrix, has to be equal to or smaller than the size of single pixel in order to obtain high-resolution imaging.

For instance in MeSIMS small crystals lead to higher spatial resolution while crystals that are larger (10 µm) and that cover a huge area of the tissue cause extraction of molecules from many cells at the same time and reduction of spatial resolution. In addition, the size of crystals has significant impact on the sensitivity of IMS detection. Typically, lower sensitivity for intact bimolecules is achieved when the crystals are too small in MALDI. Hence, the desired crystal size depends on the combination of several parameters such as the desorption and ionization technique, required sensitivity, and spatial resolution.

The concentration of the matrix in solution is another important parameter and this needs to be carefully measured, since concentration that is too low leads to diffusion of analyte from its original position before crystallization and also proper crystals will not be formed owing to the lack of enough organic acid. In contrast, a concentration of matrix that is too high causes rapid formation of crystals and this reduces the time to extract and incorporate of analyte.

The thickness of the deposited matrix layer is another fundamental consideration since a matrix thickness that is not optimal can give rise to unstable analyte signals and too much wetting results in analyte migration. However, the desired thickness of matrix in the two methods of MeSIMS and MALDI are different. In fact, Me-SIMS is a surface analysis method with the primary ions impacting just several nanometers into the sample. Thus, in MeSIMS, an extremely thin layer of any traditional MALDI matrix needs to be coated on sample surface. In MALDI, the laser has a higher penetration depth and much more material is expelled [6] [8] [16].

Several strategies have been developed to apply matrix on tissue sections for SIMS and MALDI in recent years. These methods, which are used based on the required spatial resolution of the analysis, can be carried out either manually or automatically.

Manual procedures suffer from poor reproducibility, whereas automated devices provide better reproducibility and control during the extraction process. This leads to the possibility for comparison between different samples. In both cases a particular issue is the formation of possible homogenous crystals trapping most of the analytes without any diffusion of molecules [16] [28-29].

There are not any available commercial matrix deposition instruments for MeSIMS-based sample preparation. However, an automated device named ImagePrep is commercially available and is commonly used for MALDI sample preparation. This can be applied for deposition of matrix in MeSIMS. In addition, a non-commercial and a solvent-free method, called sublimation, can be used for matrix deposition in both MALDI and SIMS. Therefore between all of the current strategies of matrix deposition, these have been used and the following sections provide some of theoretical framework for ImagePrep and sublimation individually.

2. 7. 1. 1 ImagePrep Commercial Matrix Application

Recently, Bruker Daltonics has developed an automated sample preparation instrument for MALDI imaging called ImagePrep (Figure 2.7). This device provides high reproducibility with excellent spectral quality at high speed. The ImagePrep device, under controlled conditions, uses a vibrational vaporization of the matrix with a piezo-electric spray head. A thin sheet with pinholes next to the matrix reservoir is moved by the spray head leading to ejection of small droplets with an average diameter of 20 µm which are deposited on the tissue section or other sample [8] [28].

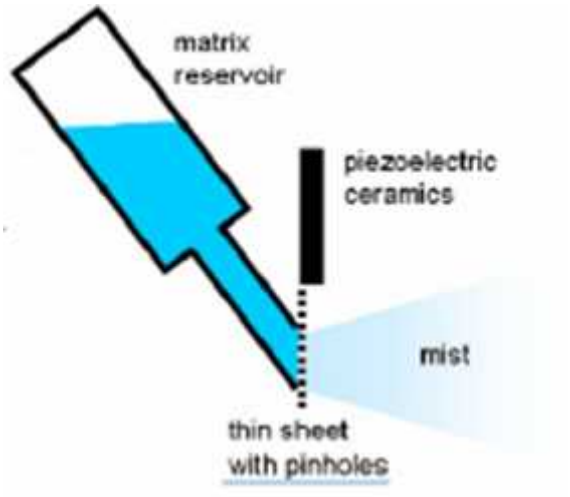


Figure 2.7. Schematic creation of matrix aerosol by vibrational vaporization [30].

The ImagePrep station has an optical sensor to monitor the scattered light from the matrix layer deposited on the tissue section to control via direct feedback the deposition. This makes the device fully automated and controls all the relevant preparation parameters including the matrix thickness, incubation time, and wetness [28] [30].

The matrix thickness, incubation time and wetness affect the lateral resolution and chemical homogeneity of the measurement and sample, respectively. The matrix thickness is determined by the number of sprays and the spray intensity.

To achieve increased matrix thickness, multiple passes of matrix application are used, which results in greater homogeneity of the coating, but over-coating can suppress analyte signal. The incubation time is defined as the waiting time after each spray cycle. During this time the sample is allowed to dry under a stream of nitrogen. More incubation time generates better analyte incorporation into matrix crystals leading to more sensitivity but the time of matrix application is increased.

The wetness parameter controls the residual wetness of the sample before starting of the next spray cycle. For instance, more wetness gives rise to more covered analyte molecules, greater homogeneity, and better spectra, whereas excessive wetness can cause more analyte migration and delocalization. Therefore these parameters must be experimentally optimized to obtain a desired resolution and quality of IMS analysis [16] [30].

2. 7. 1. 2 Matrix sublimation

Sublimation is a physical phenomenon that refers to the direct transition of solid to the gas phase without passing through an intermediate liquid phase. Essentially, sublimation is an endothermic phase transition that occurs at temperatures and pressures below the triple point of a substance in its phase diagram.

Figure 2.8 depicts a diagram phase showing the effect of temperature and pressure on a system. The diagram with three different areas containing the solid, liquid, and gaseous phases of the substance show what phases exist at any given temperature and pressure [31-32].

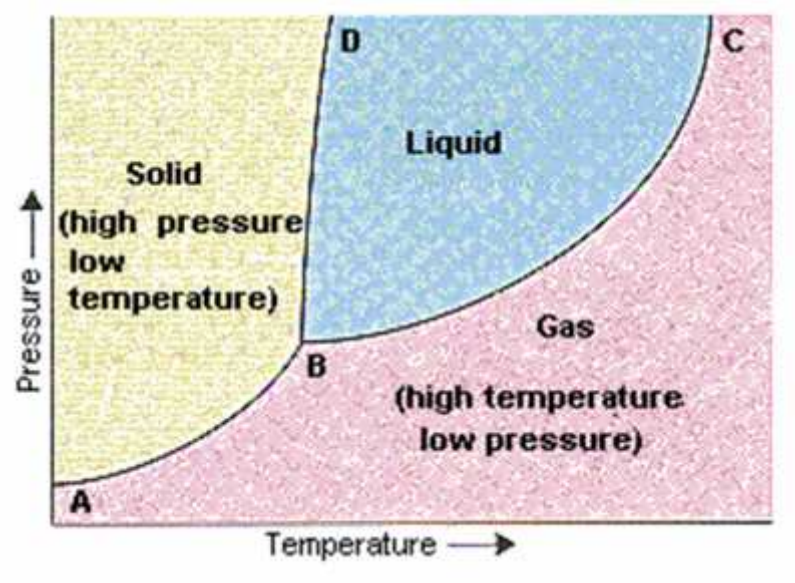


Figure 2.8. An example of a diagram phase [32].

As illustrated in Figure 2.8 the line between A and B show all combinations of temperature and pressure at which the solid is in equilibrium with the gas phase. At the pressure of this region, by increasing the temperature the gas phase is obtained and by decreasing of the temperature gas molecules condense to form a solid [32].

Recently, Hankin et al have introduced sublimation as a matrix deposition method. They successfully generated a homogenous coating of matrix with very small crystals leading to high resolution images [31].

This method is a solvent free technique to deposit of matrix and eliminates the possibility of analyte diffusion and spreading in the tissue and also provides a high purity matrix directly deposited on the sample. The apparatus of sublimation is relatively simple and inexpensive. Moreover, because of the small crystals achieved, this method is suitable for MeSIMS as well as MALDI. The time and temperature of sublimation need to be systematically optimized to reach optimal protocol resulting in a homogenous coverage, high lateral resolution, and the best detection efficiency [8] [16].

3. MATERIALS AND METHODS

3.1 Materials

All solvents used in this project were of liquid chromatography grade and purchased from Sigma-Aldrich. Deionized water used throughout the experiments was purified using a Millipore purification system to resistance of 18 mega ohms. The matrices used in this project were 2, 5-dihyroxybenzoicacid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA), purchased from Sigma-Aldrich. Sections of mouse brain (20 micrometers thick) were mounted on conductive ITO coated glass slides obtained from Sahlgrenska University Hospital. The tissue was stored at -80 ºC. Before matrix deposition, the tissue sections were brought to room temperature in a desiccator (30 min) to avoid condensation of humidity on the sample surface.

3.2 Methods

3.2.1 Deposition of matrix

In this project matrix deposition was carried out by two technique ImagePrep and sublimation apparatus described as below.

3.2.1.1 Imageprep

An automated Imageprep device purchased from Bruker Daltonik GmbH was used to deposit the matrix solution on tissue conductive ITO coated glass slide (75x25 mm). Fresh matrix solution was prepared consisting of 30 mg/mL DHB in 50% methanol, 50% water and 0.2% trifluoroacetic acid in water. To start the deposition of matrix on the sample through ImagePrep the following steps were carried out. First the 10mL bottle was filled with approximately 5mL of the fresh matrix solution and remounted into to the ImagePrep instrument.

Afterward the glass including sample was placed on the elevated rectangular area on the bottom of the ImagePrep spray chamber so that the sensor window was not covered by sample material.

Three sliders including parameters such as matrix thickness, incubation time, and wetness were set in different positions. After pressing the **Start** button, automated sample preparation was started.

3.2.1.2 Sublimation

The sublimation apparatus was purchased from Sigma Aldrich. The apparatus was coupled to a pump to supply appropriate vacuum for sublimation and was placed on a sand bath heated by a hot plate. The temperature was monitored by a digital thermometer. Sublimation was performed by following these steps.

First, ITO coated glass slides including tissue used as a target for deposited matrix were cut to 2 mm dimensions to fit in the sublimation chamber and affixed to the condenser using double sided tape. Then 300 mg of DHB powder was added to the bottom section of the apparatus. The two pieces were attached together using O-ring seal.

Vacuum was applied by the pump connected to the apparatus and after 15 min at reduced pressure, the condenser was filled with a flow of cold water (5 °C). After 5 additional min, heat was applied to the base of sublimation chamber using a sand bath placed on the heating plate. Figure 3.1 shows the sublimation device used in this project to apply matrix on tissue samples.

Figure 3.1. A photograph of the sublimation apparatus utilized in this project.

Sublimation of DHB was performed at 50, 75, 100, 125, 150 °C for a period from 1 to 15 min for each temperature. The heat was then removed and the system which was still under vacuum was allowed to slowly adjust to room temperature.

All above steps was carried out for sublimation of CHCA at 125, 150, 175 and 200 °C over the period from 1 to 30 min for each temperature and 225 °C from 1 to 20 min. To obtain the average amount of deposited DHB on per square centimeter, the slide was weighed before and after DHB sublimation using the analytical balance. This procedure was repeated 3 times at each time point, 10, 30 and 60 min at 125 °C.

3.2.2 Mass spectrometry imaging

Profiling and imaging MS of tissue sections were performed using the two methods of SIMS and MALDI imaging in this project. The images shown in the Results and Discussion were obtained in positive ion mode using an Ion-ToF ^{5} GmbH and Bruker UltrafleXtreme^{TM} MALDI-TOF/TOF spectrometer using FlexControl 3.3 software.

4. RESULTS AND DISCUSSION

The results obtained during this project are presented and discussed in this section of the thesis. This includes results related to crystal size measured by microscopy and the thickness of deposited matrix as carried out by the two methods of ImagePrep and sublimation. Ultimately, in order to evaluate the proper thickness of deposited matrix on tissue, SIMS and MALDI imaging data are depicted, investigated, and comprehensively discussed.

4.1 ImagePrep

4.1.1 Coverage of matrix (DHB) crystals on the sample surface

ImagePrep has typically been designed to work in two modes manually and automatically. Deposition of the DHB solution was performed on the glass slides through both of these modes to achieve the percentage of matrix coverage. Based on the procedure followed in this part of project, a gradient from 10 to 60 cycles of DHB solution, which was sprayed in the manual mode of the device, was obtained on one glass slide. To achieve this gradient, one part of the slide was exposed to a specific spray cycle while the rest of slide was covered as shown in Figure 4.1. Afterwards, to apply the DHB solution on another glass slide in the automatic mode of ImagePrep, the first three parameters of matrix thickness, incubation time, and wetness were set in the middle position and then the device was run in the automatic mode.

Figure 4.1. A scheme of the gradient of different sprayed cycle on one glass slide.

In order to obtain the percentage of DHB coverage, distribution, and size of the deposited matrix crystals in both modes of ImagePrep, the matrix deposited slides were observed visualized through the optical microscope (Figure 4.2). As shown in Figures 4.2 ImagePrep produces large crystals that distribute unevenly on the sample surface.

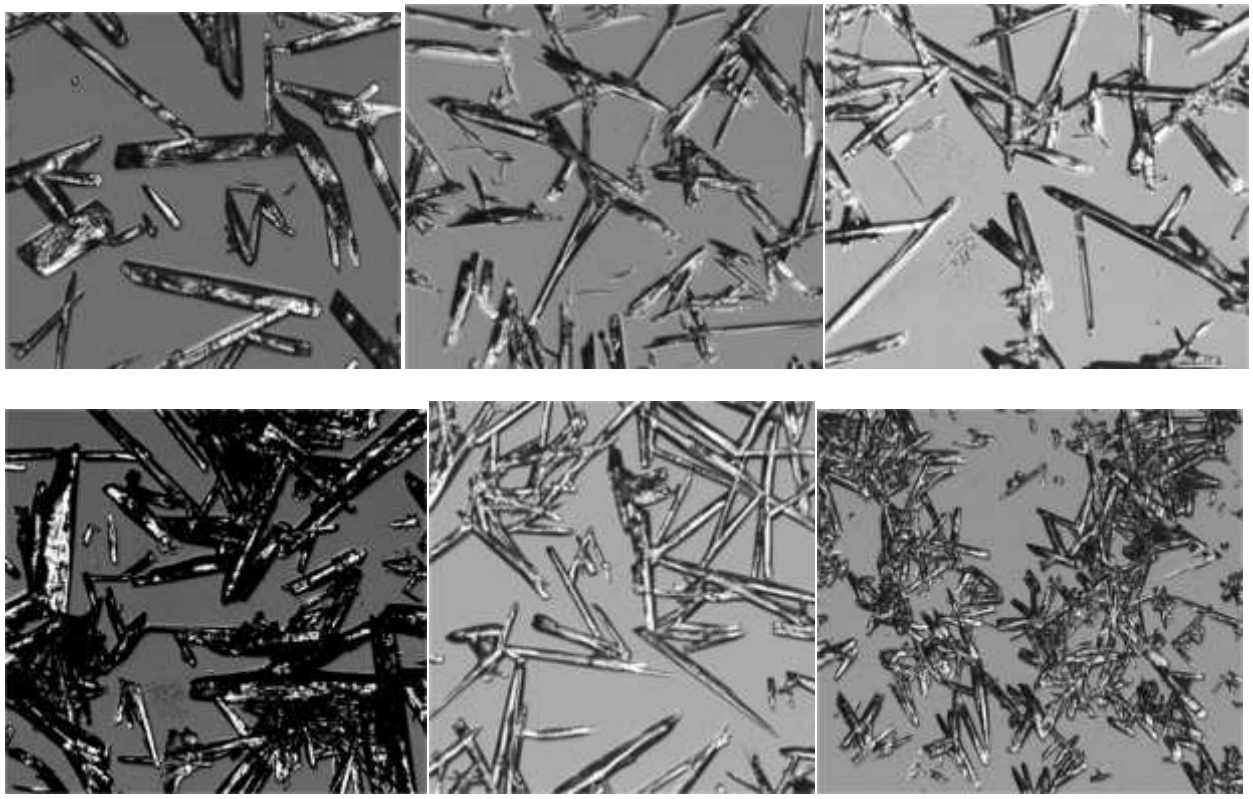


Figure 4.2. Images of DHB crystals deposited on the glass surfaces obtained in the optical microscope.

The percentage of DHB coverage was obtained through the microscope pictures and equation 4.1 then plotted for each spray cycle as shown in Figure 4.3.

$$\text{Crystal coverage}(\%) = \frac{\#\text{ pixels of covered area by crystals}}{\#\text{ pixels of total image}} \times 100 \qquad \text{Equation 4.1}$$

Figure 4.3 depicts clearly that the percentage of DHB coverage produced in both modes of manual and automatic is very poor and roughly reaches 52% coverage.

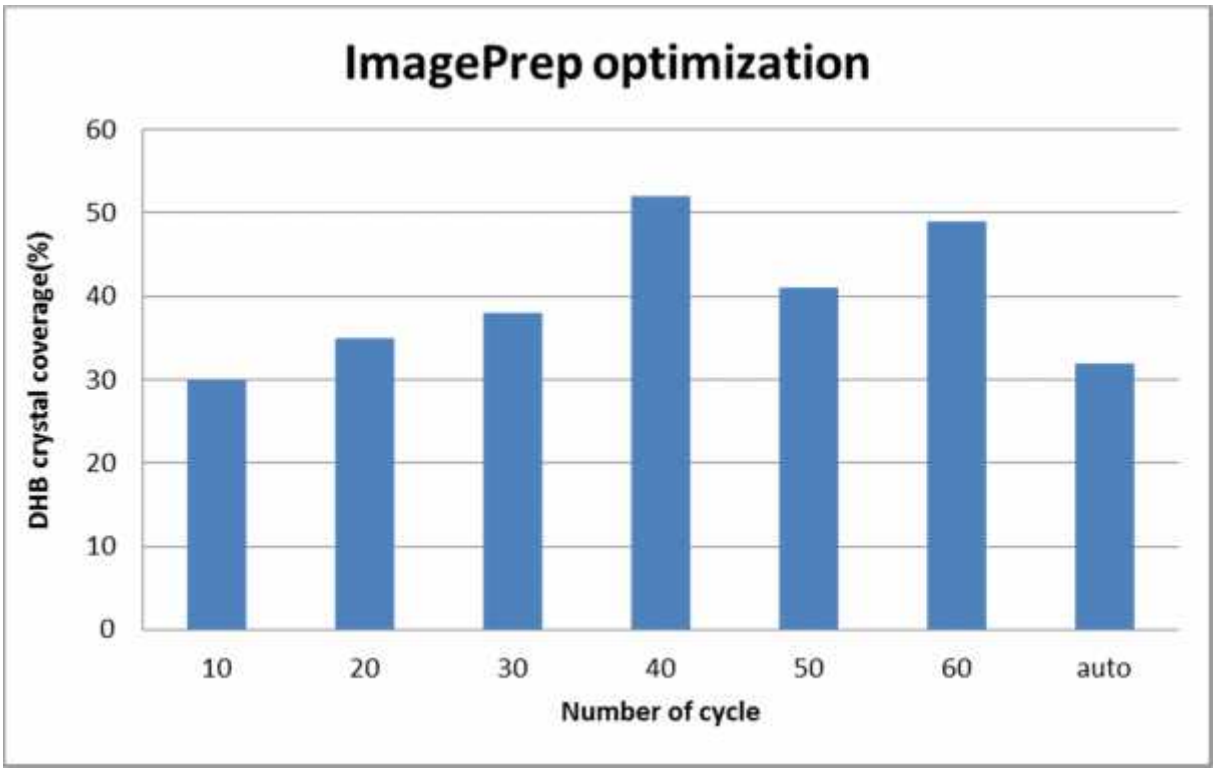


Figure 4.3. Coverage of DHB crystals for different cycles of DHB solution which were sprayed in the two modes - manually and automatically.

4.2 Sublimation

4.2.1 Optimization of time and temperature for sublimation of DHB

In order to find the proper temperature and time at which DHB is sublimated and coated on the sample surface homogenously, sublimation of DHB was performed at 50, 75, 100, 125, 150 °C over the period from 1 to 15 min for each temperature through the procedure described in the method section. As shown in Table 4.1, DHB was not sublimed at 50 and 75 °C during the period of times evaluated. Eventually DHB started to be sublimed at 125 °C after 4 min poorly and after 4 min more a uniform coating layer of DHB increasing with time was formed on the sample surface perfectly, which was simply distinguishable by eye as shown in Figure 4.4.

Temperature °C / Time (min)	50	75	100	125	150
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					

Table 4.1.Deposition of DHB at 50, 75, 100, 125, 150 °C from 1 to 15 min for each temperature.

	No deposition
	Poor deposition
	Too much deposition
	Perfect deposition

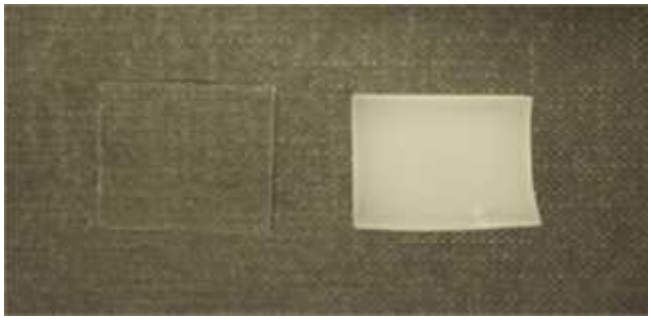


Figure 4.4.A uniform coating layer of DHB formed on the sample surface at 125 °C after 8 min.

As shown in Table 4.1, at 150 °C the layer of deposited DHB was too thick, and thus covered the tissue densely but not very evenly. Therefore the proper temperature for sublimation of DHB using the procedure carried out in this project appears to be 125 °C and the optimal time is 8 min.

4.2.2 Optical microscope images

In order to compare matrix deposition by sublimation and ImagePrep, crystal size and matrix coverage were visualized using the optical microscope. As shown in Figure 4.5 the size of DHB crystals formed on the glass surface through sublimation are significantly less than the crystals deposited using the ImagePrep technique.

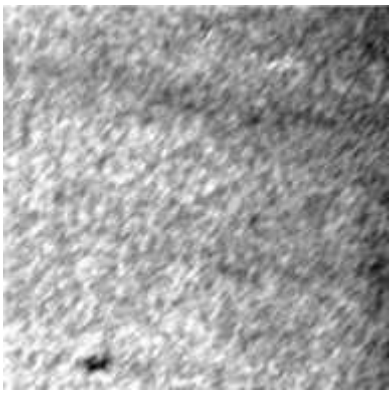
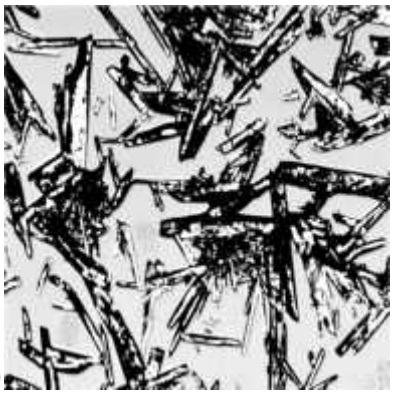
(A)  *(B)* 

Figure 4.5. The microscopy images of DHB crystals using (A) sublimation and (B) Imageprep.

4.2.3 Optimization of time and temperature for sublimation of CHCA

Sublimation of CHCA was performed at 125, 150, 175 and 200 °C from 1 to 30 min and at 225 °C over the period from 1 to 20 min. Sublimation and deposition of CHCA did not occur at temperatures of 125, 150, 175 and 200 °C even after 30 min.

Ultimately CHCA started to deposit on the target glass surface very poorly at 225°C and after 8 min. A thin and uneven layer of CHCA was coated on surface after 12 min and increased with time, but not uniformly.

Sublimation of CHCA based on the procedure used in this project did not create a homogeneous layer, whereas DHB was simply sublimated and deposited as an even layer on the glass surface. Therefore DHB appears to be preferable as a matrix in this work.

4.3 Instrumental measurements

As explained in theoretical part, the thicknesses of deposited matrix required for the two techniques of SIMS and MALDI are different. In order to achieve an appropriate thickness in each one, sublimation of DHB was performed at 10, 30 and 60 min on tissue and repeated 3 times. To obtain the average amount of deposited DHB per square centimeter, the weight of each slide with a tissue sample was measured before and after sublimation using the analytical balance. The dimensions of the slides were obtained using a ruler to calculate surface area. According to equation 4.2, the height of DHB deposited on each slide was obtained and then listed in table 4.2.

$$\text{Height} = \frac{\text{m}}{\text{d.area}} \qquad \text{Equation 4.2}$$

Here, m and d are the weight and density of DHB respectively.

	Height of deposited DHB on glass surface (um)				
Timeof deposition (min)	**run 1**	**run 2**	**run 3**	**average**	**Std. dev.**
10	1,197	1,50	1,857	1,52	0,33
30	11,54	11,28	10,06	10,96	0,79
60	24,44	24,82	22,68	23,98	1,14

Table 4.2. The thickness of deposited DHB after 10, 30 and 60 min deposition time.

Figure 4.6 shows the data from Table 4.2 as a plot of the height of deposited DHB on glass versus of time. As expected, the thickness of the deposited layer increases with time of deposition. In addition, the small standard deviation achieved in this experiment indicates reproducibility of the deposition.

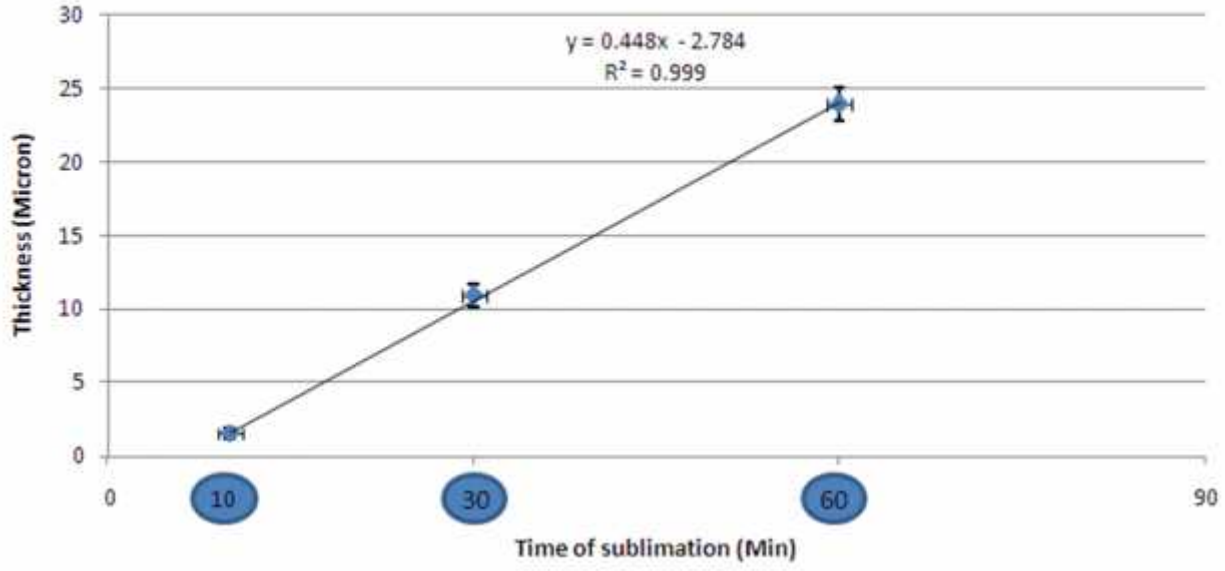


Figure 4.6. Height of deposited DHB on glass versus time of deposition.

4.3.1 SIMS imaging

In order to optimize of matrix thickness for SIMS imaging using sublimation, DHB was deposited on mouse brain tissue slices for 10, 30 and 60 min. These samples and one without matrix (control) were imaged separately using SIMS. With the aim of examine how the DHB matrix affects the ion intensity of endogenous lipids, two lipids of phosphocholine (PC) head group at m/z 184 and cholesterol at m/z 369 were selected for imaging.

High-resolution TOF-SIMS images of the PC head group at m/z 184 are shown in Figure 4.7 for the control sample (A) and with sublimation of DHB after 10 min (B), 30 min (C) and 60 min (D).

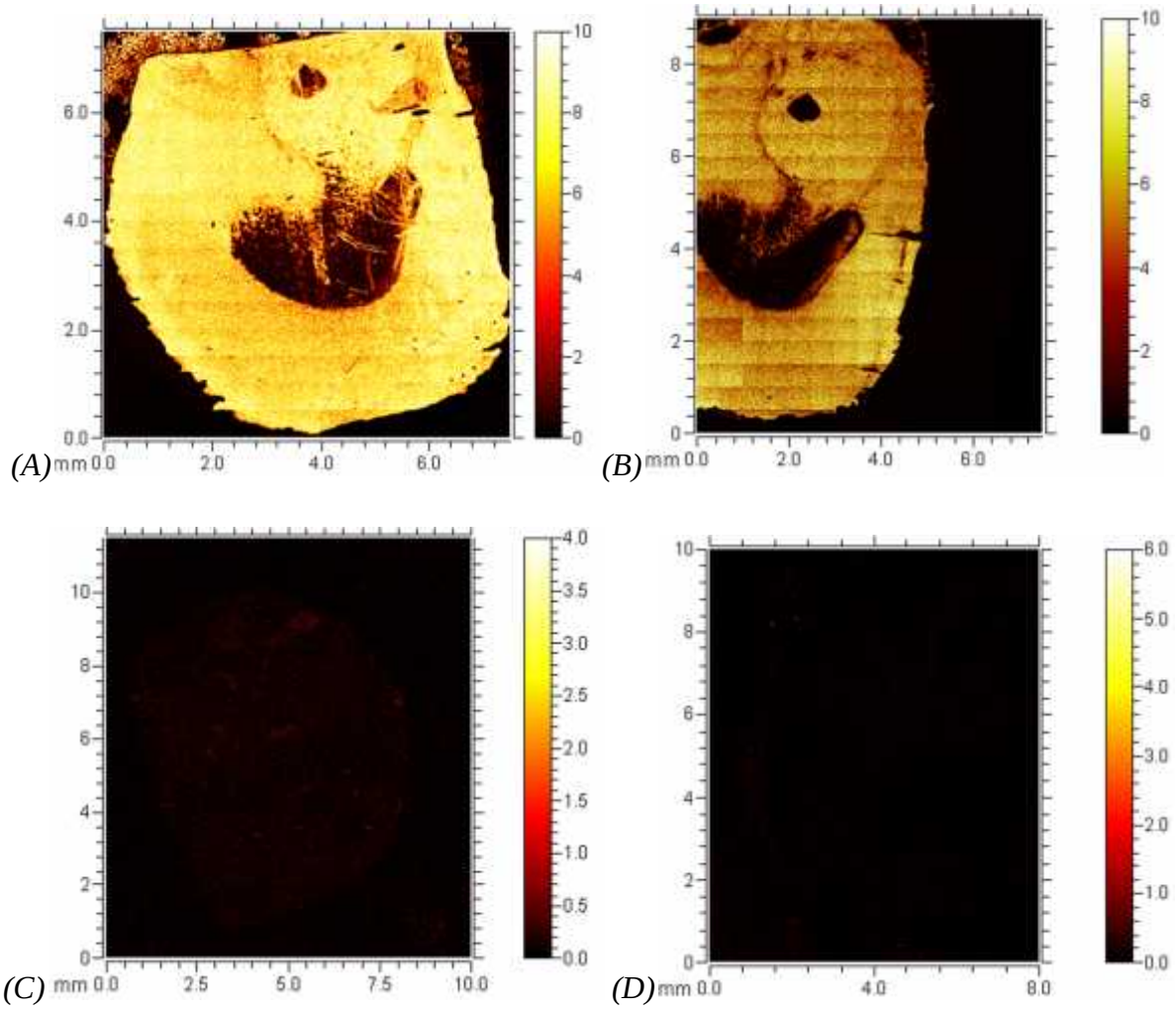


Figure 4.7. Overlay of SIMS images for PC head group at m/z 184 for

(A) Control, (B) 10 min, (C) 30 min and (D) 60 min of sublimation.

As shown in the overlaid images, it is clear that increasing the time of matrix deposition diminished the intensity of the PC ion fragment image.

With regard to images in Figure 4.7, the scanned areas of SIMS measurement are not the same for different sublimation times which can affect on ion intensity. In order to have a more accurate comparison in peak area and intensity, the same size of area (1.1 x 1.1 mm^2) in both gray and white matter of each brain tissue slice were selected and imaged as shown in Appendix. The integrated peak areas of the PC fragment obtained from mentioned regions were plotted versus different sublimation times as illustrated in Figure 4.8.

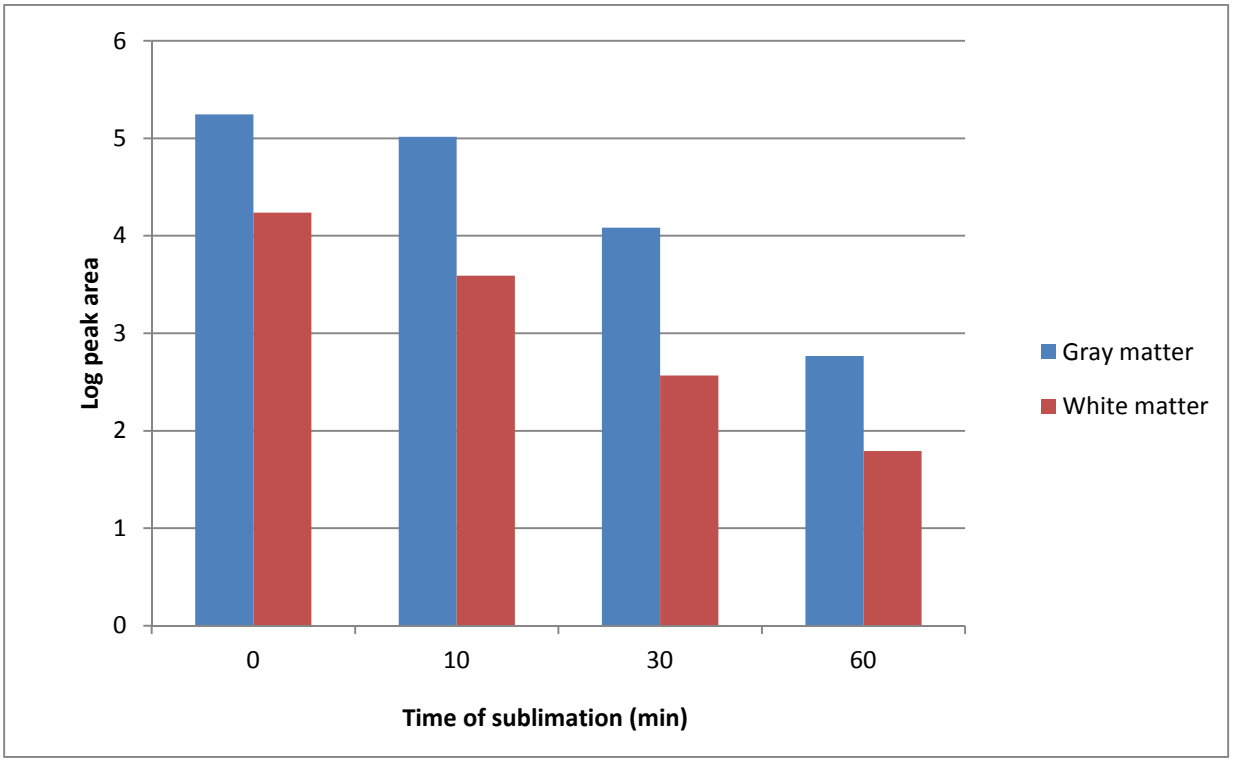


Figure 4.8. Log peak areas of phosphocholine with m/z 184 for different time of sublimation in gray- (blue bars) and white (red bars) matters of brain tissue slices (number of trails=1).

As shown in Figure 4.8, the peak areas of the PC fragment in both gray and white matters decreases with increasing matrix thickness.

Cholesterol with m/z of 369 was probed as another fragment to see the effect of sublimation time on ionization in SIMS imaging. SIMS images of the cholesterol fragments are shown in Figure 4.9 without deposition of DHB as a control sample (A) and with sublimation of DHB after 10 min (B), 30 min (C) and 60 min (D).

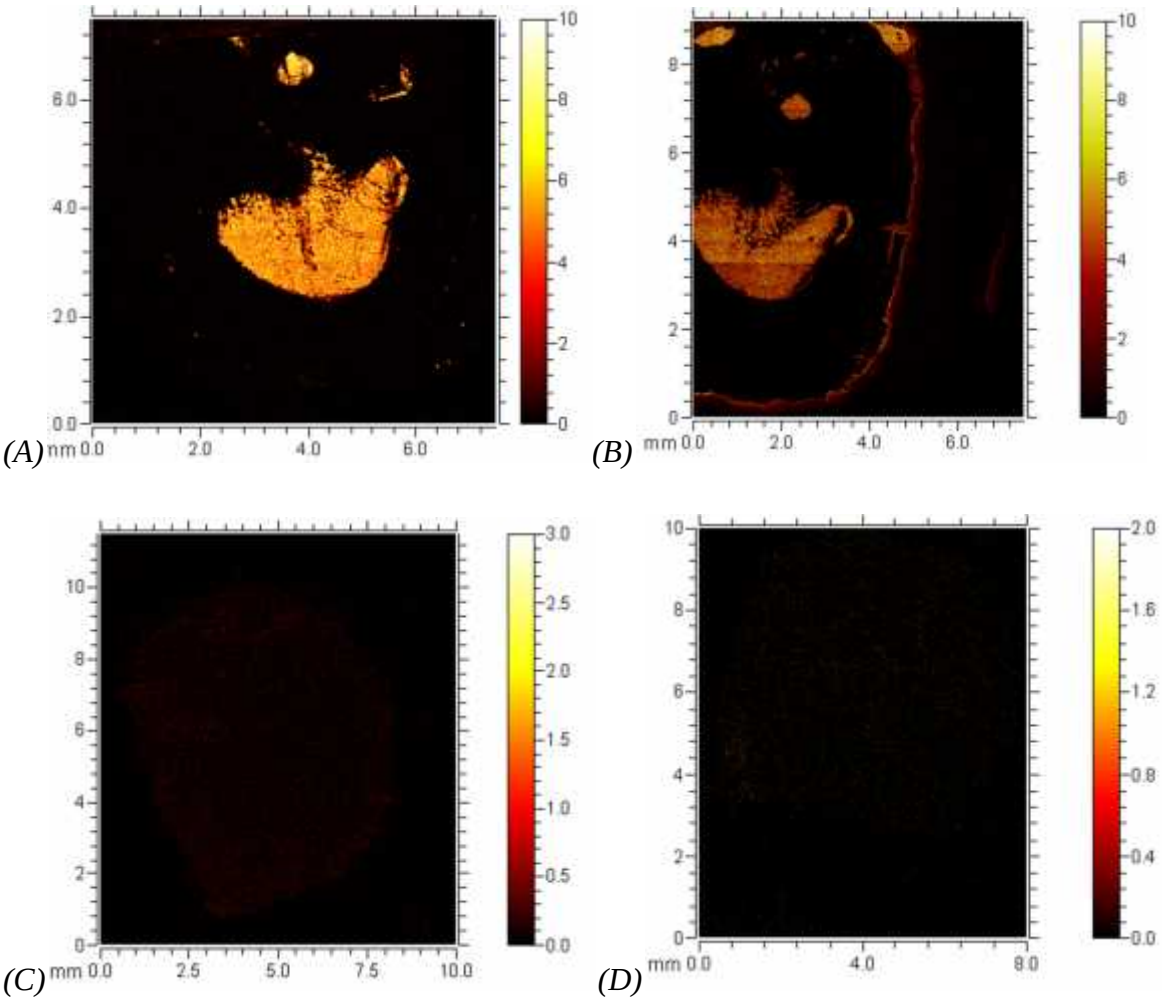


Figure 4.9. SIMS images of cholesterol (A) control, (B) 10 min, (C) 30 min and (D) 60 min sublimation.

It is clear in the TOF-SIMS images shown in Figure 4.9 that increasing the sublimation time leads to a significant decline of intensity for cholesterol ions.

The integrated peak areas of the cholesterol fragment acquired from SIMS measurements of the same size regions (1.1 x 1.1 mm^2) in both gray and white matter of each brain tissue slice were plotted versus different sublimation times as illustrated in Figure 4.10.

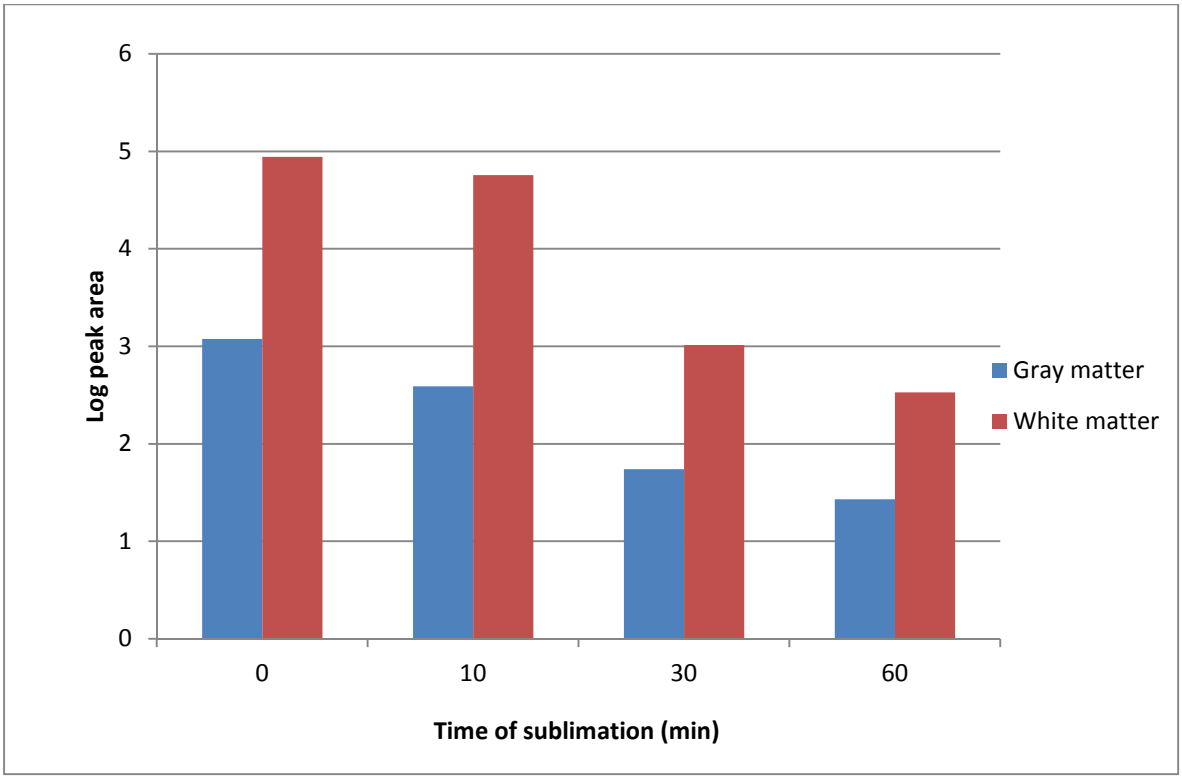


Figure 4.10. Log peak areas of cholesterol fragment with m/z 369 for different sublimation times in gray - (blue bars) and white (red bars) matters of brain tissue slices (number of trails=1).

As shown in Figure 4.10 clearly, increasing the time of sublimation decreases the ionization observed for this fragment in both gray and white matters.

In conclusion, for both the PC and cholesterol fragments the matrix enhancement effects are not observed at the different deposition times for sublimation of DHB. This can be explained by the fact that SIMS is highly surface sensitive and can only probe a few nanometers in to the sample surface. Thus, thicknesses obtained from 30 and 60 min sublimation times as illustrated in Table 4.2 are too thick for SIMS detection and the analyte is buried under the matrix.

Since none of times 10, 30 and 60 min could generate a proper thickness of deposited DHB for SIMS imaging, times of 5, 15 and 20 min should be performed to obtain an optimized time.

In order to see the effect of time on ionization of the matrix itself, distribution of the detected DHB ions at m/z 154 was imaged by SIMS imaging and illustrated in Figure 4.11 after 10 min (A), 30 min (B) and 60 min (C) deposition time.

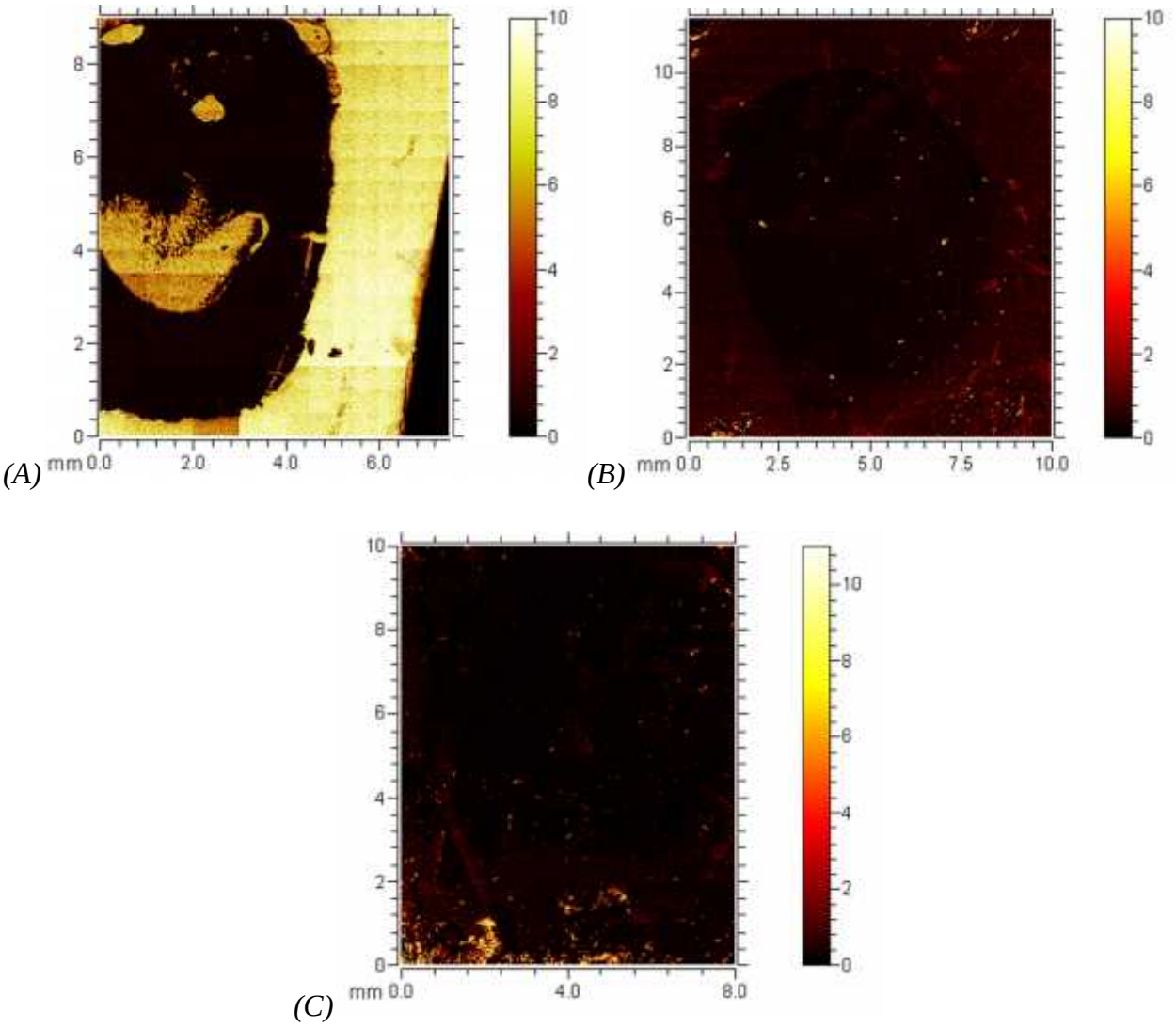


Figure 4.11. SIMS images of DHB after (A) 10 min, (B) 30 min and (C) 60 min sublimation.

Surprisingly, as depicted in Figure 4.11, the intensity of DHB ions significantly diminishes by increasing of the deposition time.

Furthermore, the logarithm peak areas obtained from SIMS measurements of the same size regions (1.1 x 1.1 mm^2) in both matters of gray and white for DHB at m/z 154 were measured and these are shown in Figure 4.12.

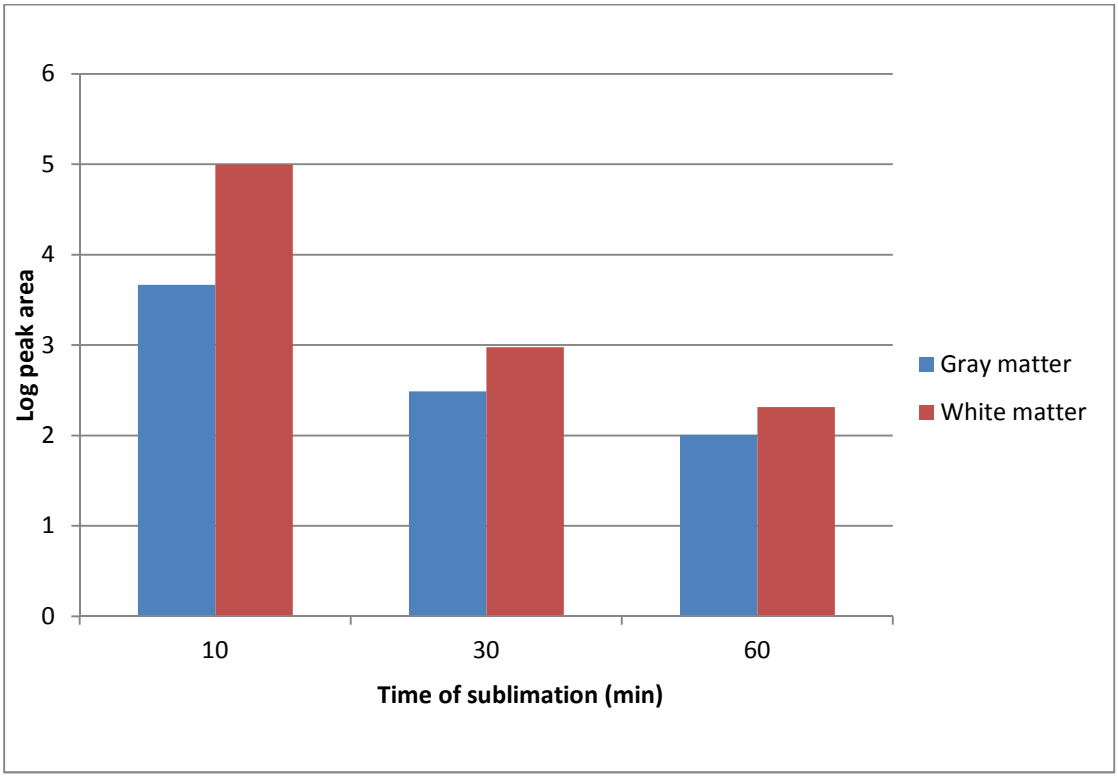


Figure 4.12.Log peak areas of DHB with m/z 154 for different times of sublimation in gray (blue bars) and white (red bars) matters of brain tissue slices (number of trails=1).

As demonstrated in Figure 4.12, ionization of DHB decreases with increasing amounts of deposited DHB. This can be explained by charging effects in which the matrix acts as an insulating layer resulting in significant decrease in the ionization yield.

4.3.2 MALDI imaging

The samples containing mouse brain with DHB deposited using sublimation for times of 10, 30 and 60 min were imaged with MALDI and the relative ion intensities were compared. Also we attempted to optimize the matrix layer thickness for DHB in MALDI using the sublimation-based matrix deposition procedure. Figure 4.13 shows the mass spectrum obtained from MALDI imaging of a sample coated with DHB for 10 min. The lack of signal indicates that the thickness of the DHB layer after only a 10 min deposition time is not sufficient for MALDI.

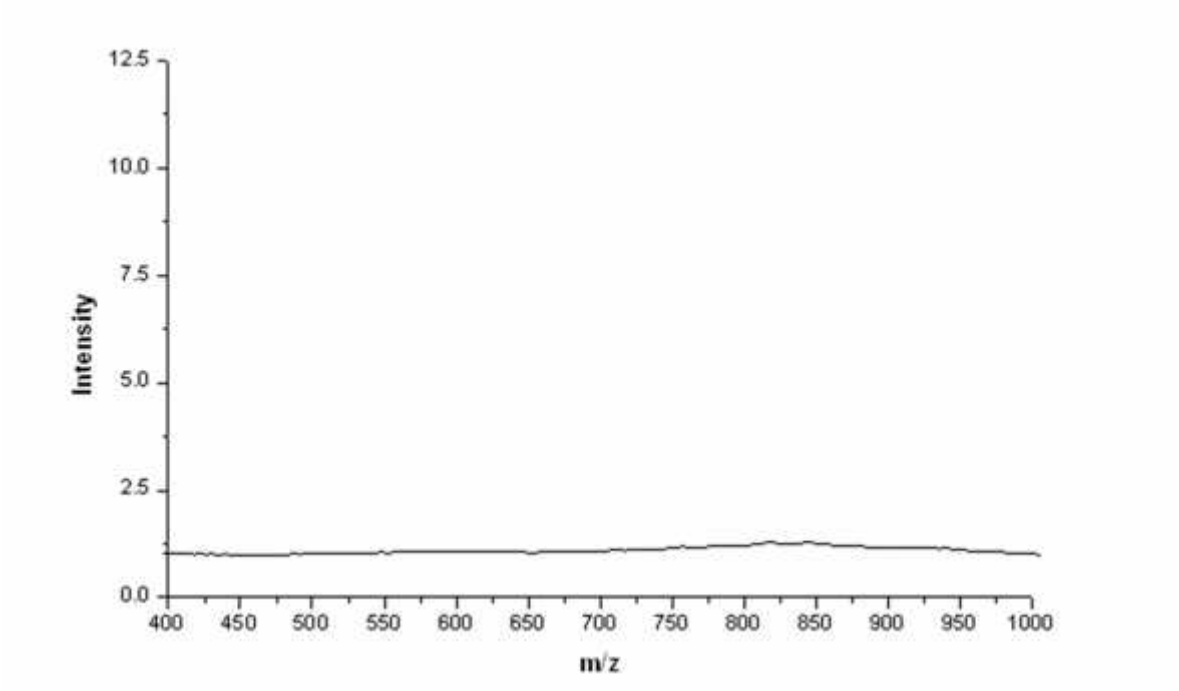


Figure 4.13. Mass spectrum of a sample deposited with DHB during a10 min sublimation.

In contrast, the mass spectrum following a 30 min deposition of DHB, shown in Figure 4.14, has good signal at several m/z values. This time of deposition time for the matrix produces much more intense signals.

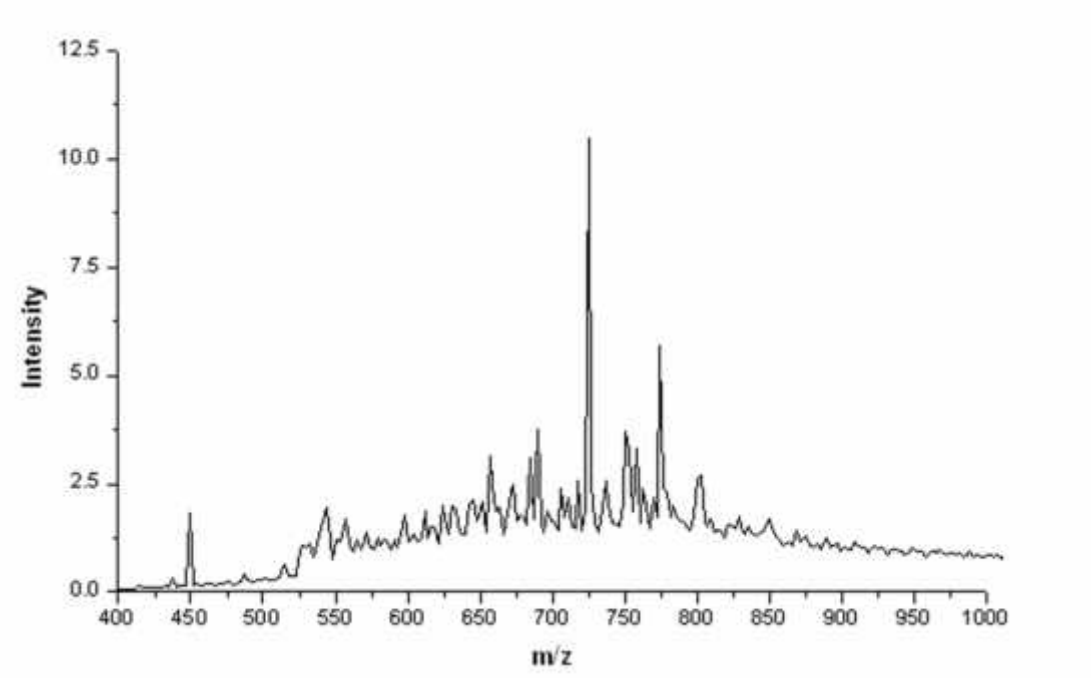


Figure 4.14. Mass spectrum of a sample deposited with DHB during a 30 min sublimation.

The mass spectrum obtained after a 60 min deposition (Figure 4.15) provided poorer ionization yields again meaning that the deposited thickness of the matrix after 60 min is now too thick for MALDI imaging.

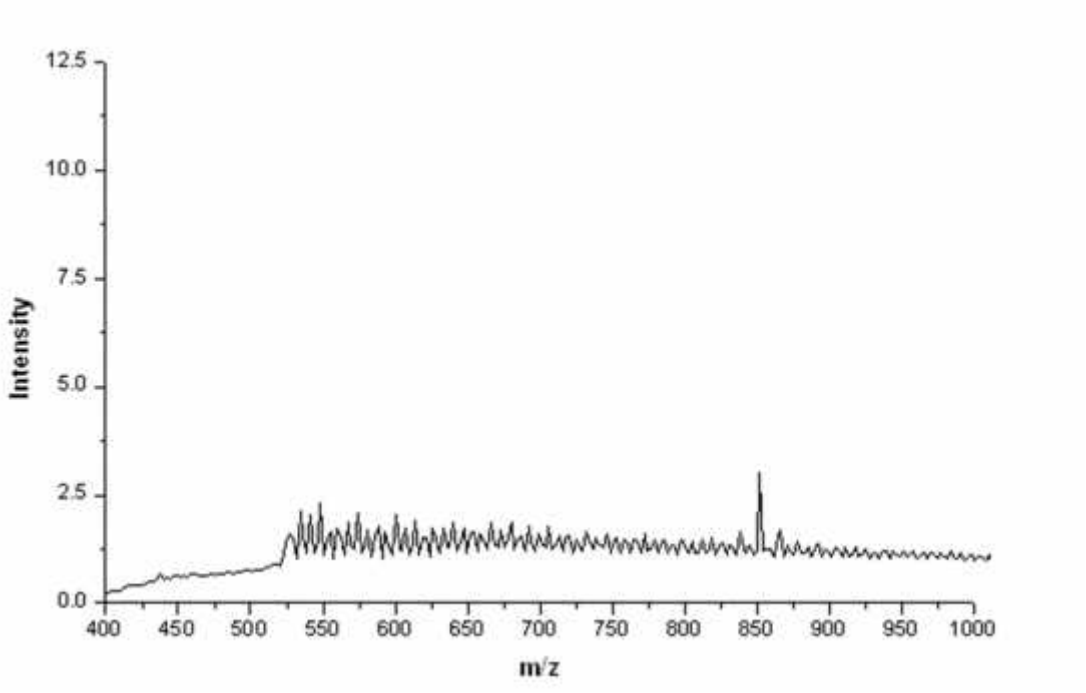


Figure 4.15. Mass spectrum of a sample deposited with DHB during a 60 min sublimation.

Looking at Figure 4.14, the main peak obtained from 30 min deposition is related to an ion with m/z 723.5. This matches phosphocholine ($[M+H]^+$ = m/z 760.5) plus sodium (Na^+ = 23 Da) minus tri-methyl-amine (TMA=59 Da). The structure of POPC shown in Figure 4.16 is a major glycerophosphocoline lipid component in tissue and this peak is commonly observed in IMS measurements of the mouse brain [16]. This fragment with m/z 723.5 was observed in each MALDI image for each time of sublimation as shown in figures from 4.17 to 4.19.

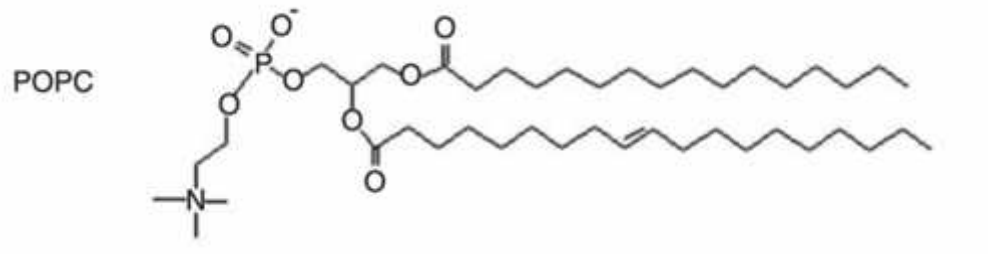


Figure 4.16. The structure of POPC (16:0/18:1) [from Wikipedia].

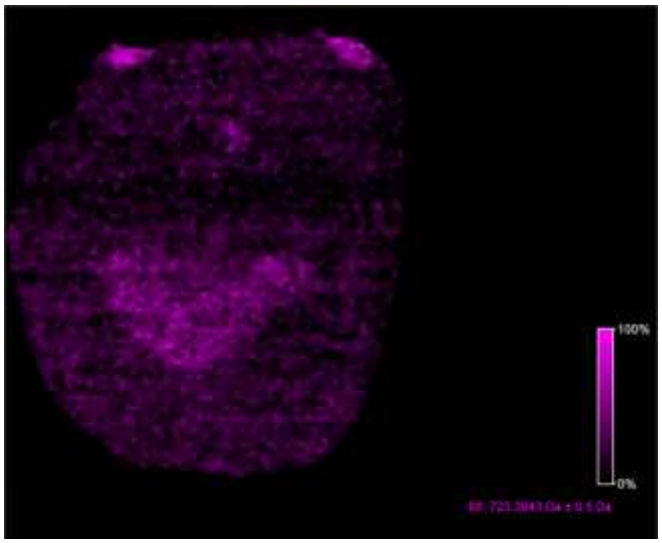


Figure 4.17. MALDI image showing the distribution of m/z 723.5 after deposition with a 10 min sublimation with 50 µm raster size.

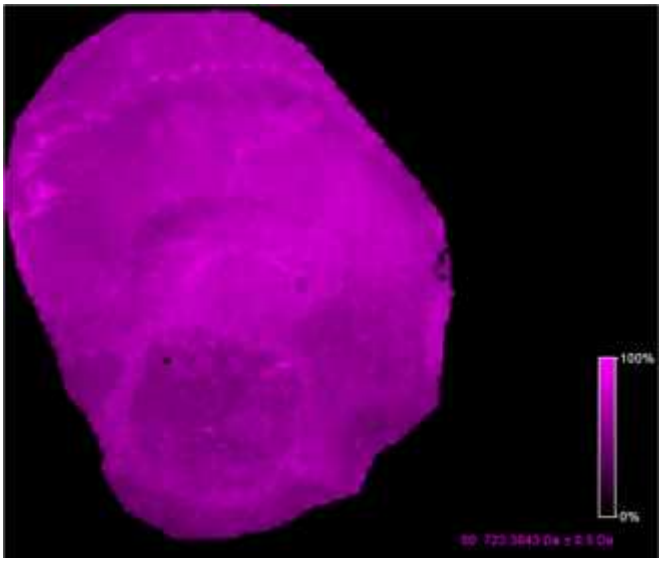


Figure 4.18. MALDI image showing the distribution of m/z 723.5 across the tissue after deposition with a 30 min sublimation with 50 µm raster size.

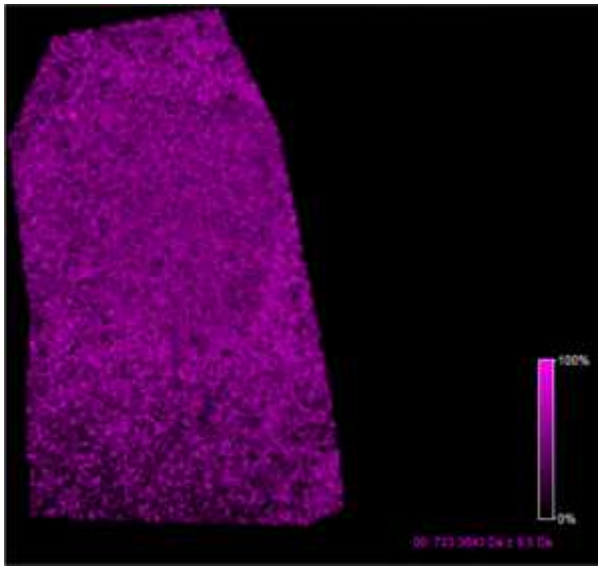


Figure 4.19. MALDI image showing the distribution of m/z 723.5 across a tissue sample after deposition with a 60 min sublimation with 50 µm raster size.

From the MALDI images, the highest intensity of the POPC ion is observed after a 30 min deposition of DHB. Although the 30 min deposition is optimal for those times evaluated, it might be useful to evaluate the signals after 20 and 40 min deposition times.

5. CONCLUSIONS

In this project, two matrix application techniques, ImagePrep and sublimation, have been compared in terms of coverage and crystal size. Based on microscopy images, it was obvious that ImagePrep suffers from inhomogeneous matrix deposition and also produces relatively large matrix crystals. On the other hand, sublimation is capable of generating very small crystals and homogeneous films.

In another part of the project, DHB and CHCA were compared to find an optimal time and temperature for each sublimation-based matrix. The optimal temperature and time for DHB sublimation was found to be 125 °C with a deposition time of 8 min that is very close to the optimized conditions found by Hankin et al. (120°C and 10 min); however, the vacuum pump and sublimation chamber used were different in those experiments than those used here. Sublimation of CHCA with the procedure used here did not produce successful images. Hence, DHB deposited by sublimation as an even layer on the sample was then used as the preferable matrix in this work.

SIMS imaging was performed on tissue samples after DHB was deposited for10, 30 or 60 min by sublimation at 125 °C. None of these deposition times produced a thickness suitable for optimal SIMS imaging. In conclusion, deposition times of 5, 15 and 20 min should be investigated to obtain an optimized time producing an appropriate thickness of the deposited DHB for SIMS imaging.

For MALDI imaging, it was the 10 min sublimation time did not produce a thick enough layer of the DHB, whereas the thickness generated after a 60 min deposition was too thick for MALDI imaging. However, it was found that deposition of DHB for only 30 min produced more intense signals. For more accurate conclusions, depositions of 20 and 40 min should be examined.

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8. APPENDIX

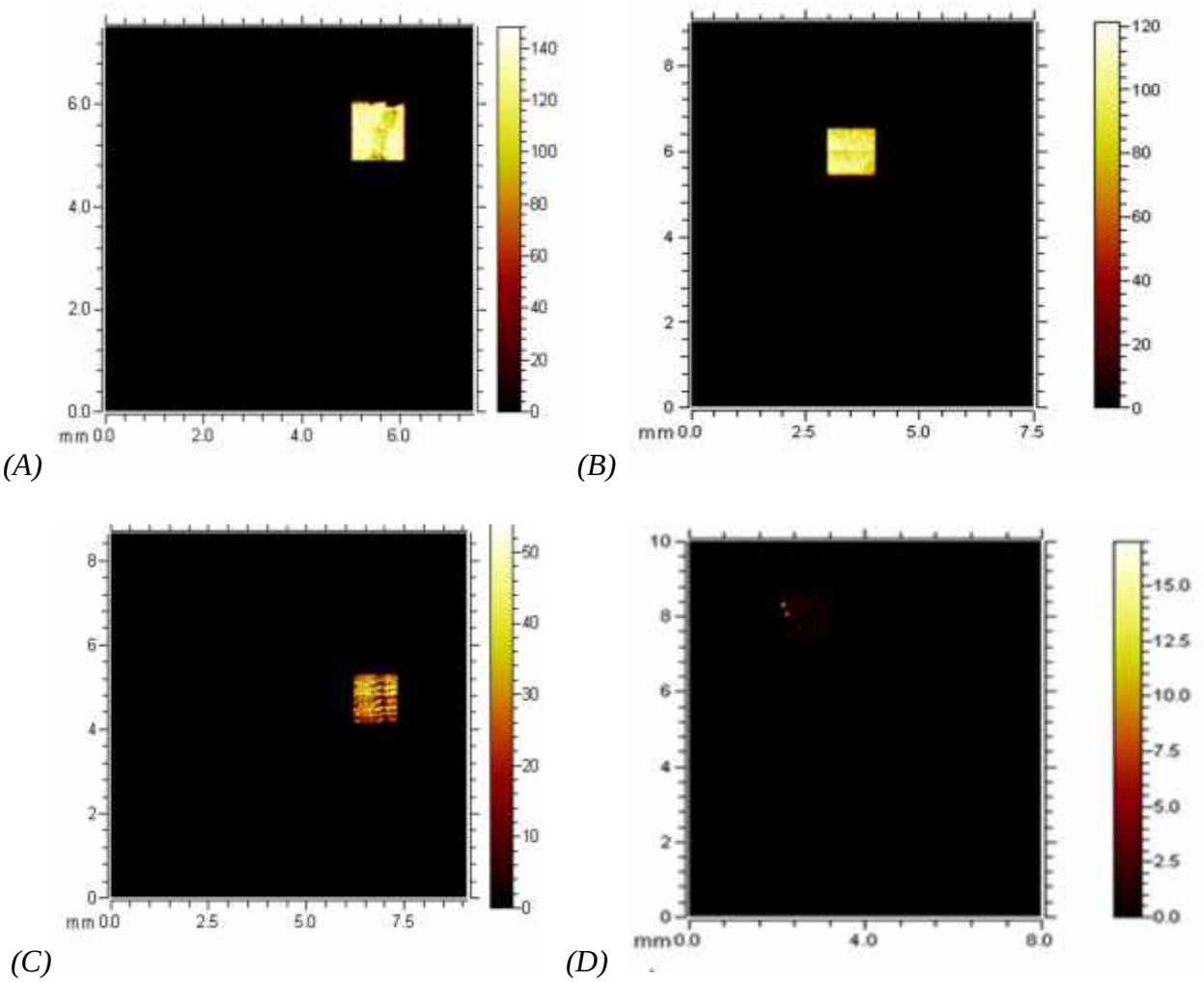

Figure 8.1. Regions of interest (1.1 mm x 1.1 mm) for PC head group at m/z 184 in gray matter of brain tissue for (A) Control, (B) 10 min, (C) 30 min and (D) 60 min of sublimation.

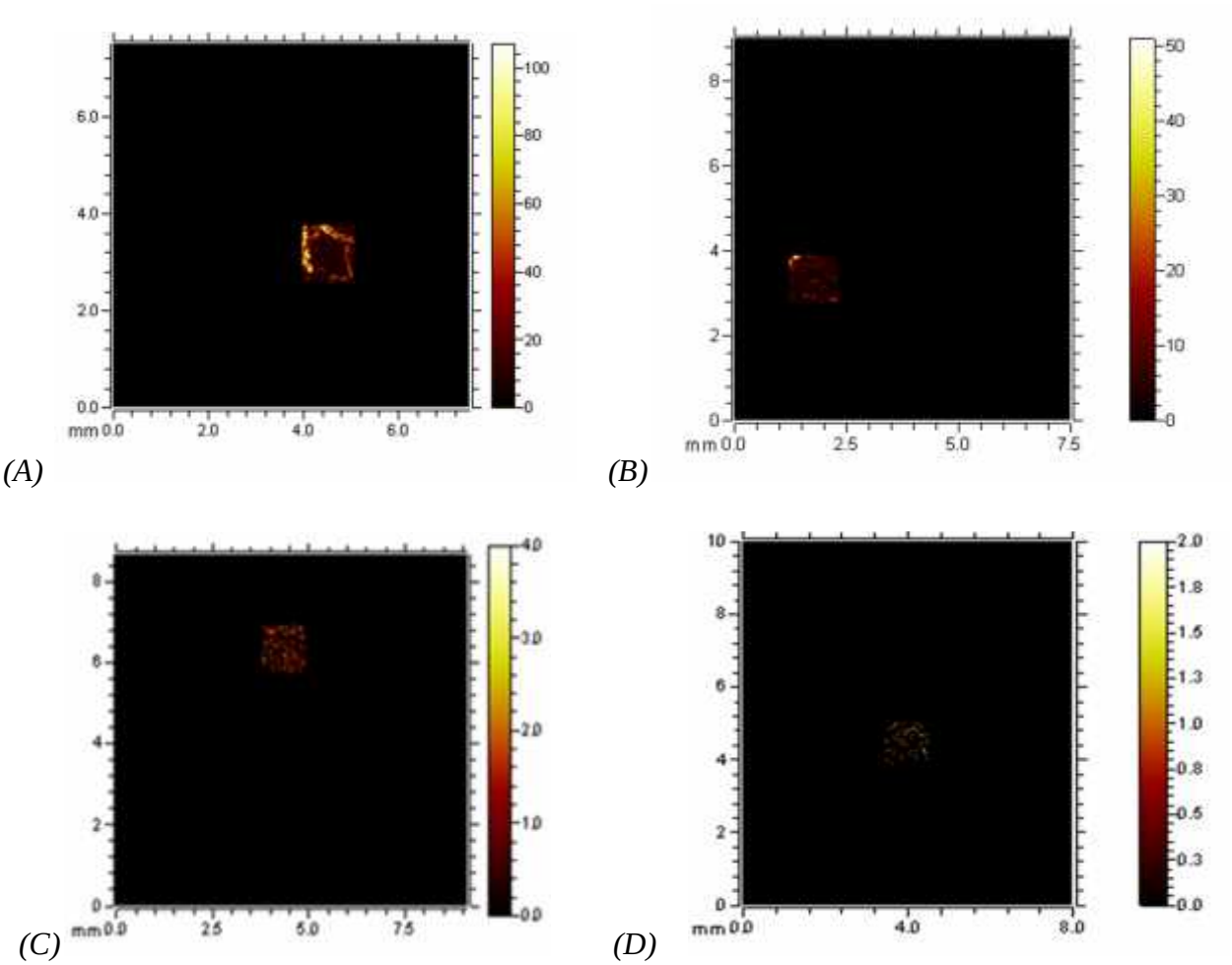

Figure 8.2. Regions of interest (1.1 mm x 1.1 mm) for PC head group at m/z 184 in white matter of brain tissue for (A) Control, (B) 10 min, (C) 30 min and (D) 60 min of sublimation.

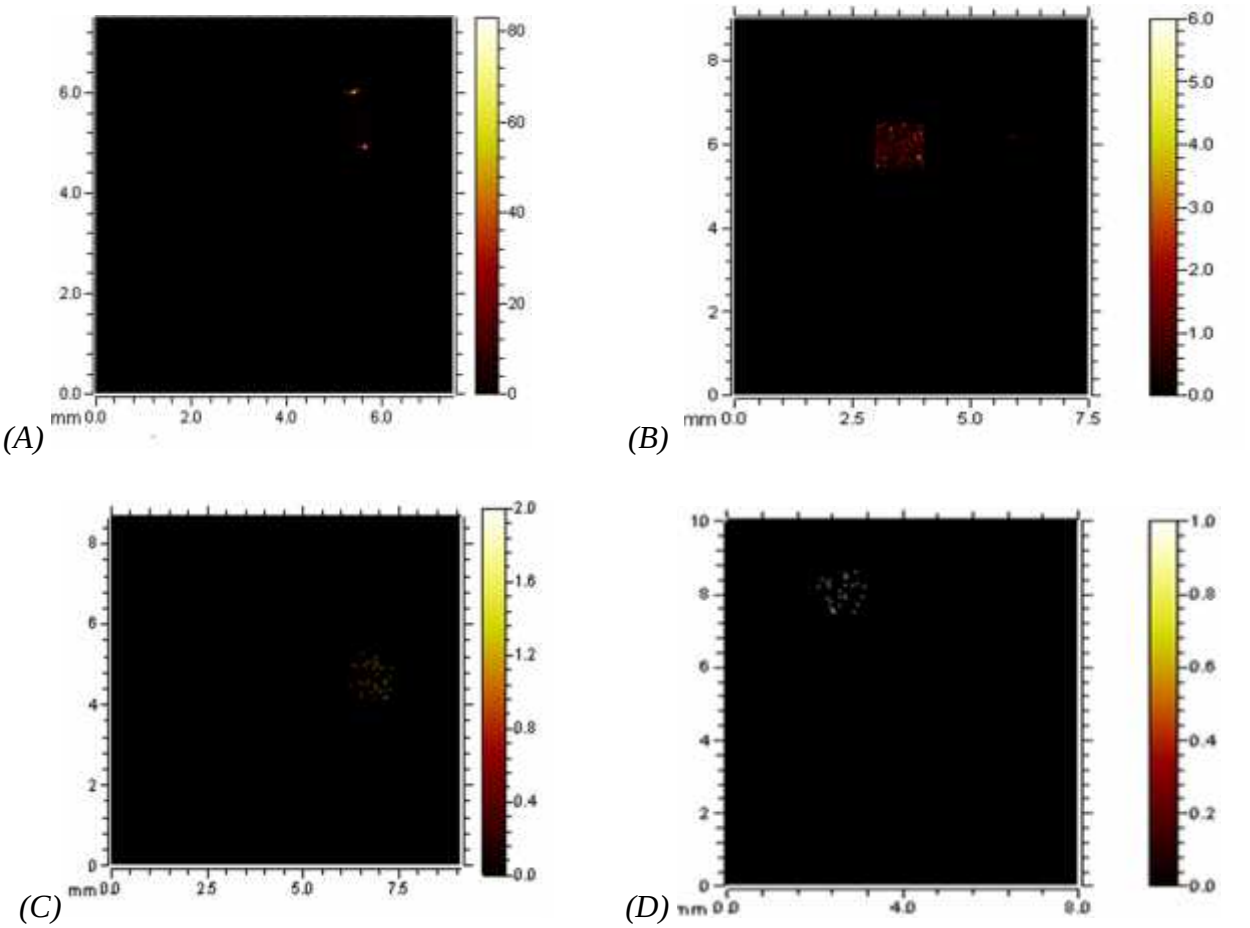

Figure 8.3. Regions of interest (1.1 mm x 1.1 mm) for cholesterol at m/z 369 in gray matter of brain tissue for (A) Control, (B) 10 min, (C) 30 min and (D) 60 min of sublimation.

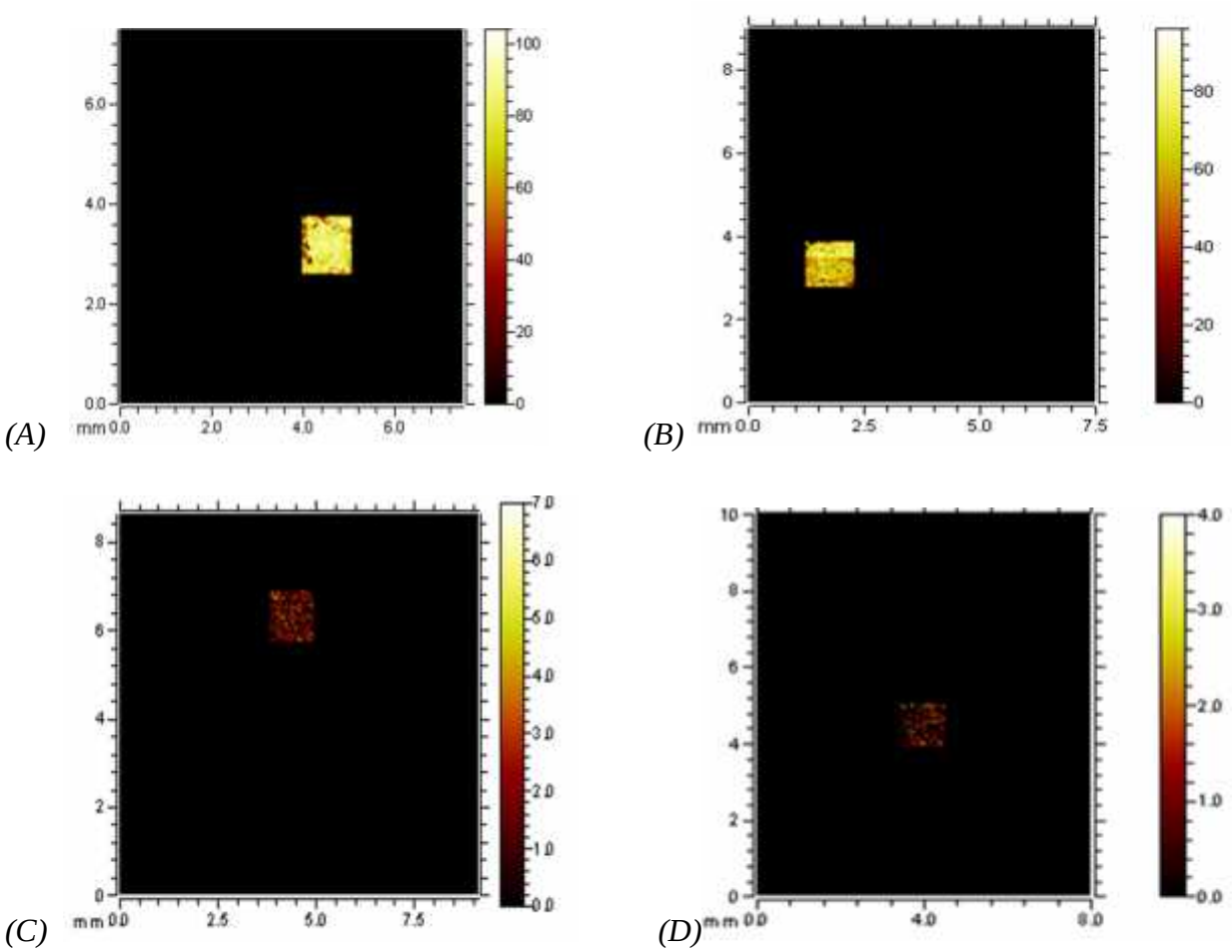

Figure 8.4. Regions of interest (1.1 mm x 1.1 mm) for cholesterol at m/z 369 in white matter of brain tissue for (A) Control, (B) 10 min, (C) 30 min and (D) 60 min of sublimation.

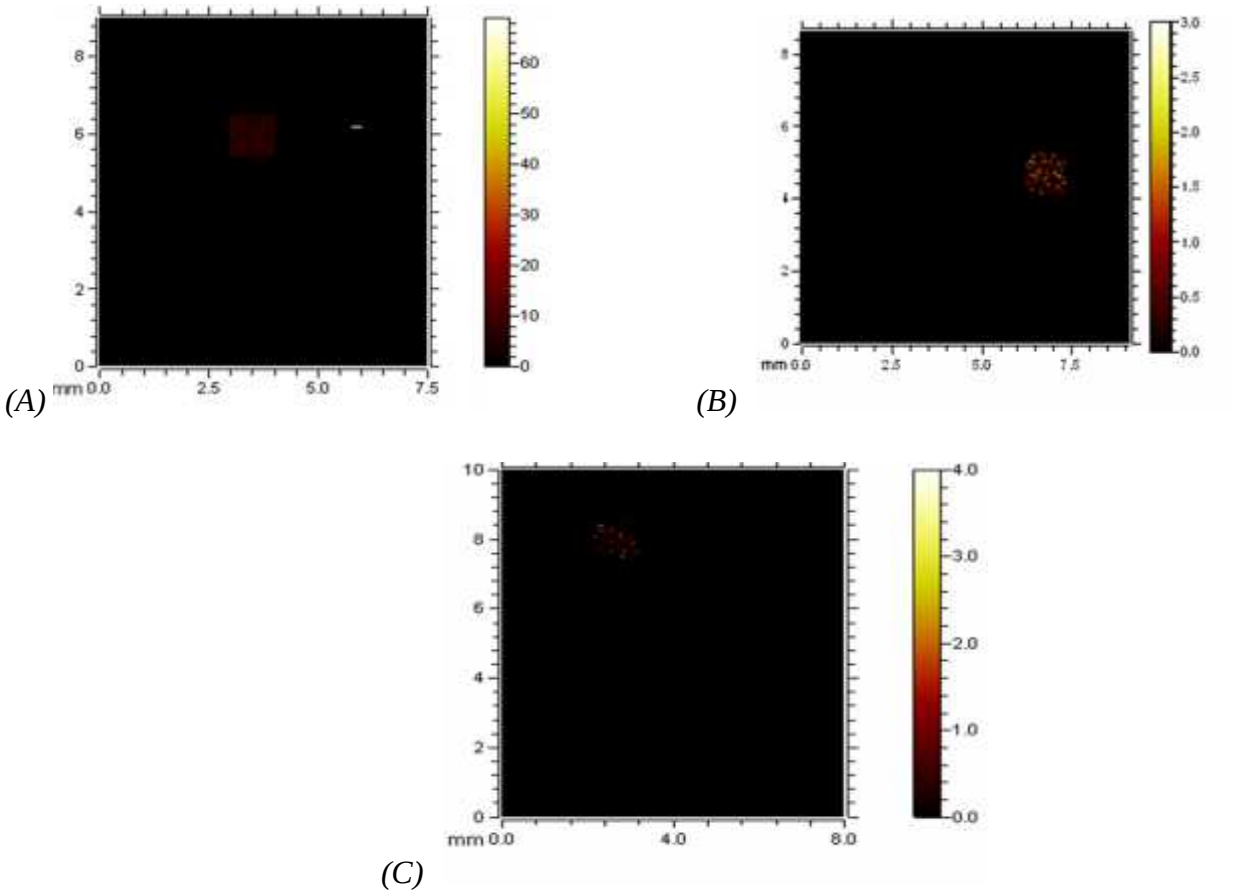

Figure 8.5. Regions of interest (1.1 mm x 1.1 mm)for DHB at m/z 154 in gray matter of brain tissue for (A) 10 min, (B) 30 min and (C) 60 min of sublimation.

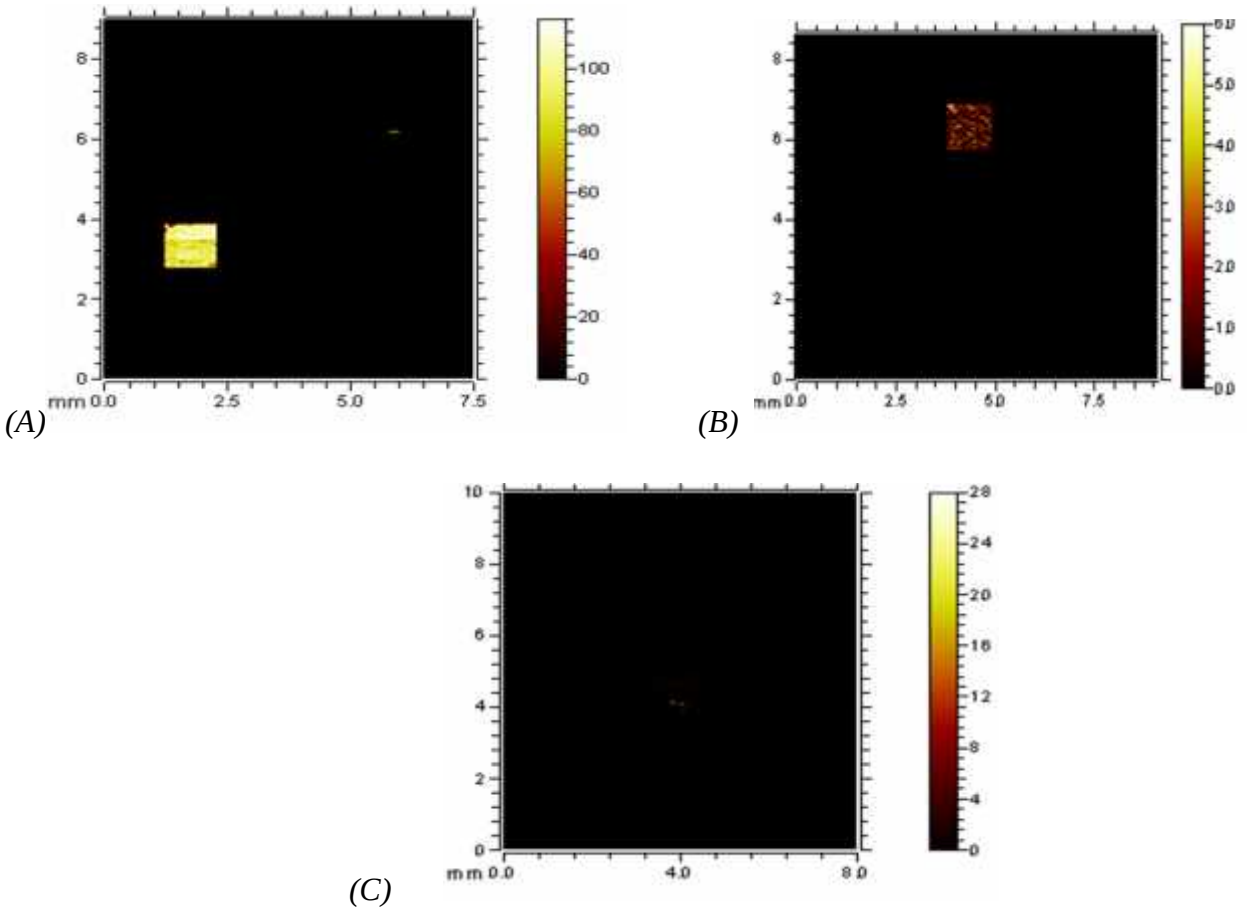

Figure 8.6. Regions of interest (1.1 mm x 1.1 mm)for DHB at m/z 154 in white matter of brain tissue for (A) 10 min, (B) 30 min and (C) 60 min of sublimation.