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Many carcinogens and their electrophilic metabolites react readily with DNA, and form different types of DNA adducts. Clinically, DNA adducts have been linked to cancer diseases. Also, different DNA adducts have been used as biomarkers to monitor the exposure of individuals to specific carcinogens. In this study, we have explored the use of high throughput matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) technique to quantitate carcinogen-DNA adducts. A pure synthetic aromatic amine-DNA adduct, namely N-(2'-deoxyguanosin-8yl)-4-aminobiphenyl (dG-ABP) that has been clinically associated with bladder cancer, was selected as a representing carcinogen-DNA adduct in this study. Among the four natural nucleobases, guanine is the most frequent site for DNA adduction. Another reason for choosing a dG adduct is due to the N-glycosidic bond between guanine and ribose is the weakest when comparing to the other deoxyribonucleotides. This intrinsic property of dG adducts has led to the dissociation of their corresponding aglycon ions when mass spectroscopic techniques were used to perform their qualitative measurements, including MALDI-TOF MS. In our initial study, a novel approach of using G-ABP aglycon ion instead of the dG-ABP ion to perform the quantitation of dG-ABP has been examined. In an alternative approach to perform the dG-ABP quantitation, the effects of different MALDI matrices, sample preparation methods, and various instrumental parameters were

studied. Using the optimal conditions and dG-ABP ion, a calibration graph for the quantitation of dG-ABP was constructed with using 2'-deoxyguanisine monohydrate as internal standard. The linearity of the calibration graph had a R-squared value of 0.9897. The limit of quantitation for dG-ABP was at 2.23μM with a signal-to-noise ratio of 32.2, and the linear dynamic range for quantitation was extended to 1,000μM. The results of this study have demonstrated for the first time MALDI-TOF MS is a viable technique for carrying out quantitative measurements of carcinogen-DNA adducts.

QUANTITATIVE MEASUREMENTS OF CARCINOGEN-DNA ADDUCT USING MALDI TIME-OF-FLIGHT MASS SPECTROMETRY

By

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Approved by

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APPROVAL PAGE

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- acknowledge The University of North Carolina at Greensboro, The Department of Chemistry and Biochemistry, and Faculty as supporters of my research in future publication.
- provide The Department of Chemistry and Biochemistry a copy of research.
- notify The Department of Chemistry and Biochemistry when the results of our research are published.

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CHAPTER I

INTRODUCTION

1.1 Carcinogen and carcinogen-DNA adduct

The term carcinogen refers to any substance, radionuclide or radiation that is an agent directly involved in the promotion of cancer or in the facilitation of its propagation. Carcinogens can be classified as genotoxic and nongenotoxic. The term "genotoxic carcinogen" indicates a chemical capable of producing cancer by directly altering the genetic material of target cells. 4-aminobiphenyl (4-ABP) has shown to be a major etiological factor in human bladder cancer [1,2] and is also considered as a significant factor of human breast cancer [3] . In addition, lung and colon cancers have been also associated with 4-ABP. 4-ABP is considered to be a tobacco smoke constituent, an environmental contaminant, and a well-established carcinogen in humans.[4]

The occurrence of cancer disease can be induced by carcinogen. However, different carcinogens may have different levels of cancer-causing potential. Some may cause cancer only after prolonged, high levels of exposure. For each individual, the risk of developing cancer also depends on the person's genetic makeup. In order to study the impact of cancer-causing effect of a specific

carcinogen, researcher's started to put efforts on investigating the carcinogen-DNA adducts. Carcinoge-DNA adducts are the results of various in vivo reactions between cancer-causing chemicals and DNA. For instance, 4-ABP can bind covalently to DNA bases at various positions of guanosine and adenosine. 4-ABP has showed a preference to adduct deoxyguanosine at C8 position forming dG-C8-ABP (Figure 1). In rodents and humans, 4-ABP is first N-hydroxylated, and forms adducts with DNA. Carcinogen-DNA adducts could be used as biomarkers and reflects the status of cancer disease in the subject.

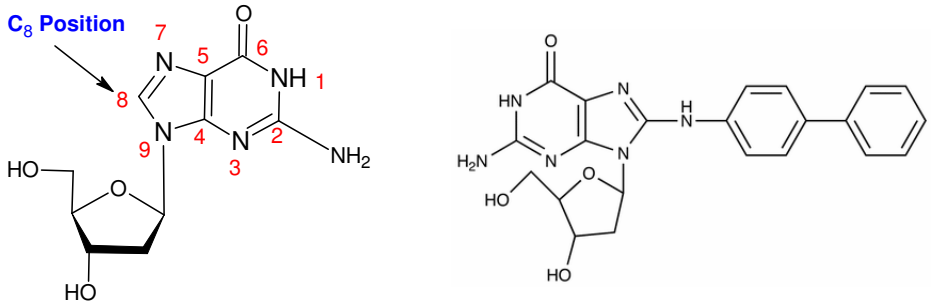

Figure.1. The chemical structure of 2'-deoxyguanosine monohydrate and dG-ABP carcinogen DNA adducts.

1.2 The relationship between cancer disease and carcinogen-DNA adducts

Cancer occurs when cells in part of the body begin to grow out of control. They continue to grow and crowd out normal cells. Although there are many kinds of cancer, they all have in common this out-of-control growth of cells. Different kinds of cancer can behave very differently and grow at different rates and

respond to different treatments. [5] Generally, the carcinogens in the environment react with DNA molecules when the human body has exposed to certain levels of genotoxic carcinogens for a certain time period which resulted in the formation of carcinogen-DNA adducts. Most of these carcinogen-DNA adducts can return to normal function by repairing DNA. However, a small amount of these modified carcinogen-DNA adducts can potentially DNA mutations which may lead to the initial stage of cancer disease.(Figure 2) Carcinogens do not only react with DNA but also react with other bio-molecules which however will not be described in the thesis.

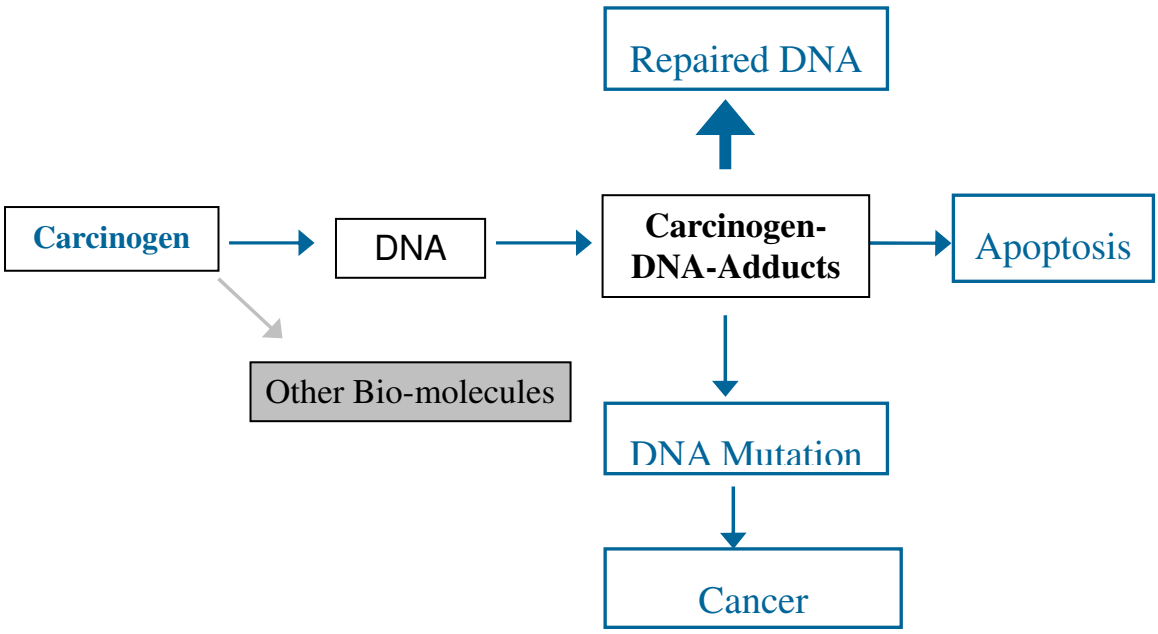

Figure 2. Schematic of biological consequences of carcinogen-DNA adducts to cancer in human body

According to our current understanding to the origination of cancer, it is initiated by these substances by their biochemical activation to reactive moieties which then attack the DNA bases (A, G, C, T). Once the carcinogens bound to the bases, they can alter the DNA conformation. This changing can profoundly impact on the normal functioning of DNA during replication. In this process the replication machinery unwinds the double helix and synthesizes a daughter strand with bases that are the complement of the parent template-A and T are paired, and G and C are paired. Therefore, the machinery places a G in the daughter where the parent has a C. When the DNA is damaged by a carcinogen, this process can fail in accuracy. For example, mismatch occurs at the modified base, or the modified base may be skipped altogether, or an extra base may be added at the lesion site. All these errors constitute genetic mutations which may cause the synthesis of aberrant proteins that lead to cancer. [6]

1.3 Selection of N- (2'–Deoxyguanosin-8-yl)-4-aminobiphenyl (dG-ABP) as target analyte

In this study, the experimental model should be focused on pure carcinogen-DNA adduct because of the purpose of this study is to lay down the foundation of carcinogen DNA adduct quantitation using MALDI-TOF MS in order to achieve lower limit of detection and therefore higher sensitivity in the mass

spectroscopic detection. One of the criteria to choose experimental model in our research project is the represented nucleotide which considered as the most often target of adduction with the class of carcinogens our research group. The initiation of many genotoxic cancers is thought to involve binding of a xenobiotic to cellular biomolecules, in particular nucleic acids. It is well known that there is base sequence selectivity in the binding of certain carcinogens to DNA. Under this circumstance, deoxyguanosine (dG) is considered as the one which has the highest chance for adduction occurrence with the various carcinogens which normally represented as polycyclic aromatic hydrocarbons (PAHs) comparing to the other nucleoside, i.e. deoxyadenosine(dA), deoxyuridine(dU), deoxycytidine(dC) and deoxythymidine(dT).

The mechanism of dG-ABP adduction in the specific nucleotide was due to 4-ABP which associate with urinary bladder cancer. Since 4-ABP is metabolized in vivo to a reactive N-hydroxy arylamine that binds covalently to DNA; the major adduct results from binding at the C8 position of guanine. Recently, it has been reported that 4-Aminobiphenyl-DNA adduct standard has been successfully synthesized by reacting calf thymus DNA with [2,2'-^{3}H]-N-hydroxy-4-aminobiphenyl. HPLC analyses following enzymatic hydrolysis to nucleosides indicated one major adduct, N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-ABP). The adduct identity was confirmed by HPLC/electrospray ionization mass spectrometry. The tandem mass

spectrometry technique was used since it was necessary to distinguish dG-ABP from the other possible isomers[7]. When biochemically activated it binds to DNA, it is primarily to the base guanine at a position known as carbon-8. All of the literature information could be obtained from the National Center for Biotechnology Information (NCBI) literature database, i.e. PubMed. The detection of dG-ABP becomes feasible when the pure standard of dG-ABP nucleoside become available. Our research projects need the qualitative and quantitative measurement particularly for calibration curve for selected target. Thus the testing sample for dG-ABP standard should be in high purity after synthesis procedure. The target analyte of this study we have chosen was supplied by Toronto Research Chemicals, Inc.

1.4 Detection techniques for carcinogen DNA-adduct

There are several detection and quantitation methods for carcinogen-DNA adducts. These include radioactive labeling[8,9], fluorescence spectroscopy[10], electrochemical methods[11], immunoassay [12]and Mass Spectrometry[12].

1.4.1 Radioactive Labeling

The ^{32}P-postlabeling assay is an extremely sensitive technique for detecting carcinogen DNA adducts. The method appears applicable to the ultrasensitive detection of a large number of carcinogen - DNA adducts of diverse structure without requiring radioactive labeled carcinogens or specific antibodies. Generally, the DNA-adducts are postlabelled after they have been extracted from

the cells. However, radioactivity may be problematic, even though radioactive labeling technique is a standard method.

1.4.2 Fluorescence Spectroscopy.

Fluorescence spectroscopy is a electromagnetic spectrosphotometry technique which analyzes fluorescence from a sample following a excitation. Fluorescence labels may or may not give an accurate signal since not all biomolecules fluoresce and fluorescent signal from other biomolecules may overlap with the intended signal and thus reduces the reliability of fluorescence measurements. Therefore, fluorescence spectroscopy technique requires that carcinogen-DNA adduct is intrinsically fluorescent which may be not applicable to certain carcinogen-DNA adducts.

1.4.3 Electrochemical methods

Electrochemical methods have also been used for the detection of DNA adducts. Van Zeeland et al reported the development of a sensitive, non-radioactive method for the quantification of deoxyguanine-8-yl)-aminofluorene (dG-C8-AF) and N-(deoxyguanine-8-yl)-acetylaminofluorene (dG-C8-AAF) in 1997.[13] Essentially, the modified DNA bases are separated by high-performance liquid chromatography (HPLC) and quantified by electrochemical detection. In addition,they established that both modified bases guanine-C8- aminofluorene and guanine-C8-acetylaminofluorene are electrochemically active. Subsequently, a procedure was developed to quantify dG-C8-AF and dG-C8- AAF in genomic

DNA. The quantification of relatively low dG-C8-AF and dG-C8-AAF adduct levels in mouse liver DNA demonstrated the sensitivity of this electrochemical detection procedure. The detection limit of the method is 1 adduct per 10^6 nucleotides for both adducts using 20 mg of DNA and 4 adducts per 10^8 nucleotides using 500 mg DNA.

1.4.4 Immunoassays

Immunoassays have high sensitivity in the range of 1 adduct in 10^8 nucleotides but lack good specificity because the antisera usually recognize multiple adducts from the same chemical as well as adducts of other carcinogens in the same chemical class. A unique advantage of this approach is the ability to perform cost-effective long-term dosing and determine the amount of DNA adducts over time in animal or cell culture models. The experimental procedure of immunoassay is relatively easy to perform with low cost. However, the requirement to immunize rabbits and necessity to characterize the antiserum and validate the assays are known disadvantages and limitation also. It must be possible to obtain a DNA modified in the range of 1% to use as immunogen. In the mid-1960s and early 1970s, researcher demonstrated that antibodies could be generated against the normal nucleosides which suggested that antibodies to modified nucleosides were also feasible. Antibodies have been developed against a wide array of carcinogen-DNA adducts as well as UV-damaged or oxidized bases. The methods of antibody development and their application to DNA adduct

detection were introduced. In addition, a couple of studies demonstrated the reliability of the assays at high doses and thus relatively high adduct levels. However, unlike the postlabeling assay that can detect multiple hydrophobic carcinogen adducts in a single experiment, specific antibodies must be developed to each adduct or class of adducts of interest which may be a major drawback of this technique.

1.4.5 Mass Spectrometry

Mass spectrometry has been considered to have the greatest potential because of its high chemical specificity that allows for unequivocal characterization of DNA binding products. Furthermore, another advantage of mass spectroscopic techniques is that very low analytical backgrounds is achieved by using high resolution chromatography coupled with selected ion recording or with multiple reaction monitoring in ESI-MS. High mass spectrometric mass resolution has also been used to enhance signal-to-noise ratios of some adducts. Elimination of background noise from the analysis is not easily achievable with the other non mass spectrometric techniques. In current literature, mass spectrometry has been used for DNA adduct detection, mostly by GC-MS. Examples include the detection of adducts with 4-aminobiphenyl, benzo[a]pyrene, tobacco-specific nitrosamines, etc..

1.4.5.1 Electrospray Ionization Mass Spectrometry.

Over the past several years, more research studies have been focused on the

utilization of liquid chromatography-mass spectrometry for the analysis of DNA adducts. It was reported that the micro-electrospray mass spectrometric (ESI-MS) isotope dilution technique was used for the detection and quantification of dG-ABP in human pancreatic tissue.[14] The results presented in the report provided evidence of the applicability of LC/MS for both the detection and the quantitation of 4-aminobiphenyl adducts. A reverse phase capillary column was connected to a triple quadrupole mass spectrometer via a commercially available micro-ESI source. ESI-MS technique was widely used to perform detection and quantification of dG-ABP adducts in analytical community. Even though ESI-MS technique was commonly used in DNA adducts analysis. However, the drawbacks in dG-ABP analysis using ESI-MS techniques was the fragment ions at m/z ~ 319 might be generated after loss of the deoxyribose moiety (116 Da). Among the chemical reactions of PAH with the four natural nucleotides, 2'-deoxyguanosine PAH adducts are the major products. The labile N-glycosidic bond between 2'-deoxyribose and guanine has, however, led to a characteristic dissociation of aglycon from its corresponding 2'-deoxyguanosine PAH adduct. Both adduct and its aglycon ions could be easily detected when ESI-MS was used to measure a DNA adduct sample. Theoretically, the aglycon has carried the PAH identity, and could be used to identify the DNA adduct. The reason for breaking N-glycosidic bond and produce fragment at ~ 319 may be related to high energy in ionization. The fragmentation, produced from N-glycosidic bond breakage and dissociattion

from the dG-ABP molecular ion, will expectedly weaken the target analyte signal. The quantitation of dG-ABP adduct may be affected due to the fact that determination for limit of detection will be unexpectedly brought to higher concentration comparing to the case without any N-glycosidic bond breakage and no fragmentation. With the rapid recent developments of liquid chromatography-mass spectrometry (LC-MS) techniques, it implies the methodology for the future DNA adduct detection will most likely be mass spectrometry. Beatriz Zayas et al. analyzed bladder DNA from 27 cancer patients for dG-C8-ABP adducts and reported the detection and quantification of 4-ABP adducts in DNA from bladder cancer patients using LC-ESI-MS/MS in 2007.[15] The objective of this study was to develop and optimize a sensitive liquid chromatography mass spectrometry (LC/MS) method for the quantitative analysis of 4-ABP DNA adducts in human bladder tissue. Level of DNA adducts expected from exposure to 4-ABP in tissues of humans range from 1 per 10^9 to 1 per 10^6 nucleotides, implying the need for highly sensitive methods for detection.

1.4.5.2 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.

Mass spectrometry represents another approach to label-free detection of biological analytes and has the benefit of providing molecular information on the analyte.[16] Mass spectrometry identifies each species due to different

mass-to-charge ratio (m/z). Sowers et al reported the successful characterization of synthetic oligonucleotides containing biologically important modified bases by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [17]. They described the application of MALDI–TOF MS to the characterization of a series of modified synthetic oligonucleotides.

The potential for obtaining high mass accuracy for confirming oligonucleotide composition were discussed in the article. The article also indicated that proper characterization of modified oligonucleotides is essential for the correct interpretation of experiments performed with these substrates, and MALDI–TOF MS analysis provides a simple yet extensive method of characterization that can be used at multiple stages of oligonucleotide production and use.

Indeed, plenty of research works have been done with MALDI-TOF MS to examine oligonucleotides and peptides. However, less work have been done in the analytical area of carcinogen DNA adduct. Indeed, Mass spectrometric methods have been an important approach to analyze DNA adducts. And it was estimated as a better-suited to distinguish adducts that have a minimal shift in mass, to analyzing mixtures, and to resolving intended analytes from background.

To facilitate future clinical studies on DNA adduction, Dr. Norman Chiu's research group has recently reported the use of Applied Biosystems 4700 Proteomic Analyzer (Figure 3) to increase sample throughput and achieve higher

accuracy on characterizing the structure of carcinogen DNA adducts. Among the chemical reactions of carcinogen with the four natural nucleotides, 2'-deoxyguanosine carcinogen adducts are the major products.

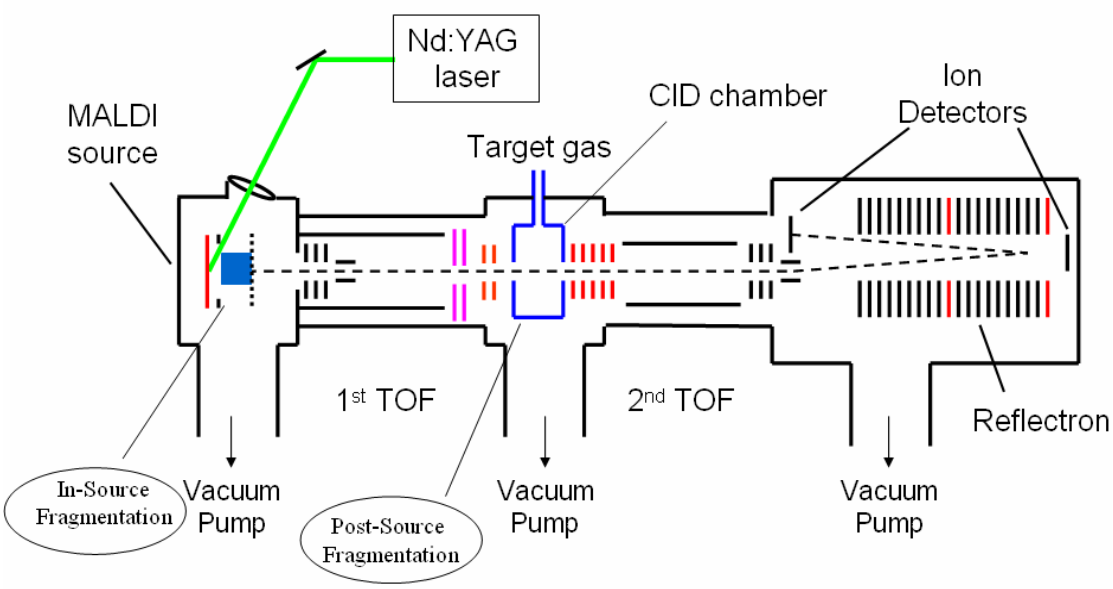

Figure 3. Schematic diagram of the components in an Applied Biosystems 4700 Proteomics Analyzer.

1.5 Research Objectives

According to the list of human carcinogens that was published by *Carcinogenesis*, there are over two hundred different carcinogens. Most of these carcinogens have the potential to become cancerous DNA adducts. In order to identify and validate each DNA adduct as a biomarker for association studies or

other clinical investigations, multiple healthy and patient samples ought to be analyzed. All these studies require high accuracy on determining the adduct identity and its quantity. Equally important, the sample throughput has to be fast enough to handle the large volume of samples while keeping the costs affordable. Owing to the high accuracy and sensitivity that can be achieved by electrospray ionization mass spectrometry, it has become the choice of method for measuring DNA adducts as demonstrated in the current literature. However, with the advances on high repetitive solid-state UV laser (≥200Hz) and automation for acquiring mass spectra, MALDI-TOF mass spectrometry has been adopted by many high throughput laboratories. To further improve the sample throughput for studying DNA adduction, our research group has recently demonstrated a higher accuracy for determining the identity of DNA adducts could be achieved by using the high-energy collision induced dissociation in MALDI tandem time-of-flight mass spectrometry. **The goal of this research project is to explore the use of MALDI-TOF mass spectrometry to perform the quantitation of DNA adducts.** Since the frequency of DNA adduction can be as low as ~1 adduct per 10^6 nucleotides, high sensitivity for the detection of DNA adducts is desirable. **For this reason, the quantitative study in this project also includes the improvement on the limit of detection.** A pure synthetic aromatic amine-DNA adduct, namely N-(2'-deoxyguanosin-8yl)-4-aminobiphenyl (dG-ABP) that has been clinically associated with bladder cancer,

was selected as a representing carcinogen-DNA adduct in this study. Among the four natural nucleobases, guanine is the most frequent site for DNA adduction. Another reason for choosing a dG adduct is due to the N-glycosidic bond between guanine and ribose is the weakest when comparing to the other deoxyribonucleotides.

CHAPTER II

EXPERIMENTAL PROCEDURES

2.1 Materials and Chemicals.

N-(2'- Deoxyguanosine-8-yl)- 4-aminobiphenyl (dG-ABP) was bought from Toronto Research Chemicals Inc. (Ontario, Canada). 3-hydroxypicolinic acid (3-HPA) , 2',4',6'- trihydroxyacetophenone monohydrate (THAP), diammonium citrate dibasic 2'-deoxyguanosine monohydrate (dG) (99-100%) and N,N-Dimethylformamide (anhydrous,99.8%) were obtained from Sigma-Alrich Cooperation (Saint Louis, USA). Various pipettes and eppendorf tips (0.1μL - 1000 μL) were obtained from Eppendorf North America. 15 mL disposable centrifuge tube and 1.5 mL flat top micro-centrifuge tubes were obtained from Fisher Scientific Corporation.

2.2 Preparation of dG-ABP and 2'-deoxyguanosine monohydrate solutions

Deoxyguanosine (dG) was used as internal standard in Matrix Assisted Laser/Desorption Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS) to analyze the dG-ABP adduct. Based on our own observations, dG-ABP was not dissolved in water but in N,N-dimethylformamide (DMF) completely. The speculation was due to the fact that the hydrophobicity of water is much lower than those of

DMF and methanol[18].The preparation method specifically the solvent for dissolving the target analyte should be comparable to those of dG. The appropriate approach to dissolve dG and dG- ABP was dissolving both of them into the same solvent, anhydrous DMF. Therefore, 1 mg target analyte dG- ABP, shipped with a small glass vial, was diluted to concentrated stock solution at 20 mM in anhydrous DMF. In addition, further attention should be pay on a container carrying anhydrous DMF. When extracting DMF from the container, it was important that dry DMF should be removed to a small container using a plastic syringe with a disposable syringe needle. The purpose of this execution was to prevent the dry DMF from any moisture. High concentration of dG-ABP was prepared in order to maintain stability performance of the analyte. The dG was used as internal standard in mass spectrometry measurement and was also prepared in 20 mM in DMF which stored at – 20 C..

2.3 Preparation of MALDI matrix solution

Standard 3-hydroxypicolinic acid (3-HPA) matrix solutions were prepared by dissolving 35.0 mg 3-HPA and 7.14 mg diammonium citrate dibasic into 1.0 mL 10% acetonitrile and deionized autoclaved water.[19] Matrix solutions were filtered with syringe filters (0.22 um; Millipore Corporation, Bedford, MA) prior to dispensing in order to remove any insoluble particles. Standard 10mg/ mL THAP matrix solutions were prepared by dissolving 10.0 mg THAP and 7 .0 mg diammonium citrate dibasic

in 1.0 mL deionized autoclaved water/acetonitrile (50:50, v/v) [20]. Since different concentration of THAP matrices were needed in the research, different amount of THAP should be dissolved with appropriate solvent with the same amount of ammonium citrate dibasic, respectively. To prepared higher concentration of THAP matrix, the same procedure was followed unless the concentration was too high and not all THAP particles could be dissolved completely. However, the THAP matrix was filtered with syringe filter to remove excessive particles. Thus, homogeneous matrix solution was obtained. Ammonium citrate dibasic was used to suppress the undesirable cationization in this case.[21] These matrix solutions were used for MALDI-TOF mass spectrometric analysis.

2.4 MALDI sample preparation methods

There have been several innovative methods to enhance MALDI sample spot homogeneity. The analyte mixtures were prepared by 50.0 μM dG-ABP and 50.0 μM dG solution in 1.5 mL centrifuge tube and vortex it well. The most common method was thin-layer method and dried-droplet method [22,23]. For thin layer method, 0.300 μL of matrix solution were applied on each spot of metal sample plate then apply another 0.300 μL 25 μM dG-ABP with dG solution on top of the matrix as a thin layer after the matrix layer was dried completely. It was not allowed to move the metal plate before the samples were dry. For dried droplet method, equal volume of MALDI matrix solution and 25 μM dG-ABP analytes were mixed even before

dispense on metal plate.0.600 μL pre-mixed matrix-analyte solution was applied on plate with a total of 12 replicates (Figure 4).The MALDI plate was placed into the MALDI plate holder after the samples were dry completely. These two methods were speculated to have different co-crystal structures when the matrix and analyte were dry. In summary, two common matrices known for DNA measurements,i.e. 3-HPA and THAP with two different matrix-analyte preparation methods provided four different sample preparation methods in this study.

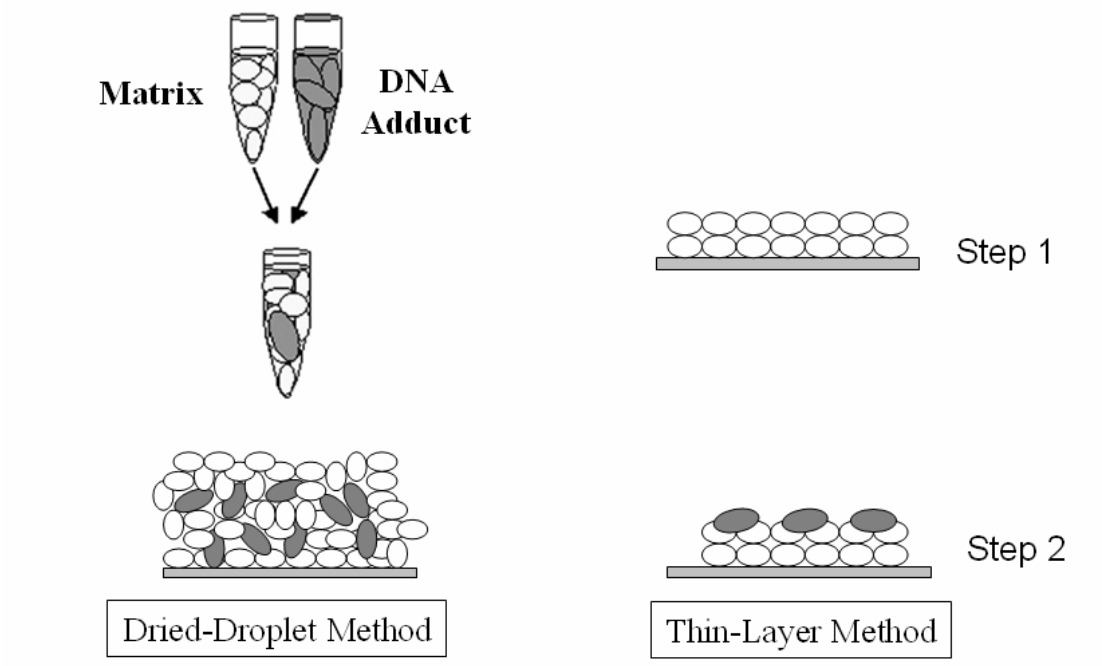

Figure 4. The schematic diagram of MALDI matrix dry-droplet and thin-layer sample preparation methods

2.5 MALDI-TOF Mass Spectrometry Measurements

Applied Biosystems 4700 Proteomic Analyzer was used through the whole study. After choosing the MALDI matrix and preparing the samples with appropriate methods, the next steps should be focused on MALDI-TOF mass spectrometry settings in order to lower the limits of detection and establish a high sensitivity technique for DNA adduct analysis using MALDI-TOF MS, which can assist high throughput clinical analysis for cancer disease research with relatively low concentration of biomarker carcinogen DNA-adducts.

2.5.1 Laser Intensity Study

In the laser intensity study, the 50.0 µM dG-ABP and 50.0 µM were prepared from 20.0 mM concentrated stock solution. Specifically, 2.00 µL 20.0 mM dG-ABP solution was added into 78.0 µL anhydrous DMF and vortexed. At this point, 500 µM dG-ABP solution was prepared. 500 µM dG solutions were prepared in the same approach as mentioned before. Equal volume of 10 µL 500 µM dG -ABP and 10 µL dG solution were mixed and 250 uM dG -ABP and dG homogeneous mixture were prepared. 10 µL 250 µM mixture was added into 90 µL anhydrous DMF and the solution was diluted to 25 µM solution. The final concentration of dG-ABP was 25 µM. The dG solution was used as internal standard in mass spectrometric measurements. Once MALDI -TOF MS instrument was ready, equal volume of 25 µM prepared solution was mixed with 10.0 mg /mL THAP matrix using dried-droplet method in 1.5 mL centrifuge tube. The MALDI matrix samples were dispensed on

metal plate with 0.600 µL in each spot total 12 replicates. The mass spectrometric measurements were executed after the sample were dry and the co-crystals were formed. Batch mode was used in this study with linear low mass positive acquisition method and linear low mass internal processing method. The laser intensity range was from 3000 to 7900 arb.units and the other parameters were 210 nanoseconds delayed time and 1000 laser shots per spectrum. Laser beam was adjusted in the random uniform mode.

2.5.2 The Study Of Total Number of Laser Shot Per Spectrum

In additions to laser intensity, the next step is the number of laser shots. The sample preparation method was the same as those of laser intensity study in previous statement. The analyte dG -ABP should be freshly prepared from the most concentrated stock solution in 20.0 mM in order to prevent hydrolysis of the target analyte. After 25 µM dG- ABP and dG solutions were prepared, equal volumes of dG and dG -ABP mixtures were mixed with 10.0 mg/mL THAP MALDI matrix with well-mixed. Dried-droplet method was used to perform the number of laser shots study. Before starting the experiment, the total number of laser shots that consumed all the matrix-analyte co-crystals was necessary to be determined as the threshold value before the measurements. Based on the observation under the metal plate screen in MALDI-TOF MS measurement, the matrix-analyte co-crystals would be consumed completely at the number of laser shots around 13,000.Specifically, the range of laser

shots were established from 1,000 to 13,000 total shots/spectrum with 2000 total shots/spectrum interval between two data points. The setting of shots/sub-spectrum is used normally to set a threshold to exclude the invalid sub-spectrum. However, the acceptance criteria were set to accept every sub-spectrum in this study. Therefore, the variation of shots/sub-spectrum and the numbers of sub-spectrum would not influence the results as long as the total shots per spectrum were determined.(Figure 5) The other parameters were adjusted to 210 nanoseconds delayed time, 6000 laser intensity. The laser beam was adjusted in the random uniform mode.

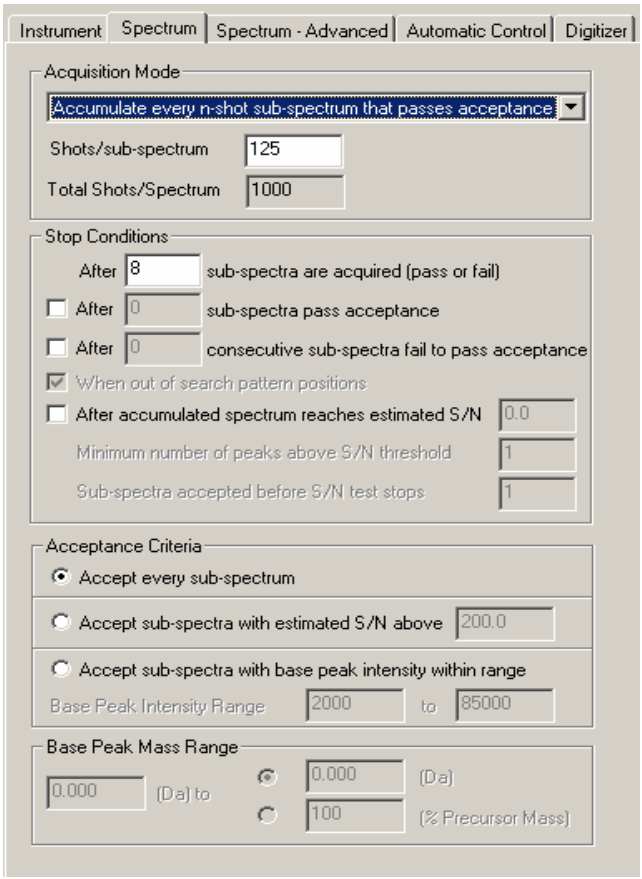

Figure 5. The number of laser shot parameter in linear positive mode acquisition method in applied biosystems 4700 proteomics analyzer

2.5.3 Delayed Extraction Study

The same sample preparation method was proceeded as previous section. The delayed extraction is normally used to increase the homogeneity of the created molecular ions. However, since the N-glycosidic bond was easily broken following by dissociation from dG- ABP molecules to G-ABP and other smaller fragments, decreasing delayed extraction was used to reduce the fragmentation of the created dG-ABP ions. We speculated that the total amount of detected G-ABP fragments would decrease because of the decreasing delayed time. The dG-ABP would not have sufficient time to break apart before reaching to the detector. The default setting was 1000 laser shots (Figure 6).The sample preparation method was the same as those in laser intensity and laser shots study.

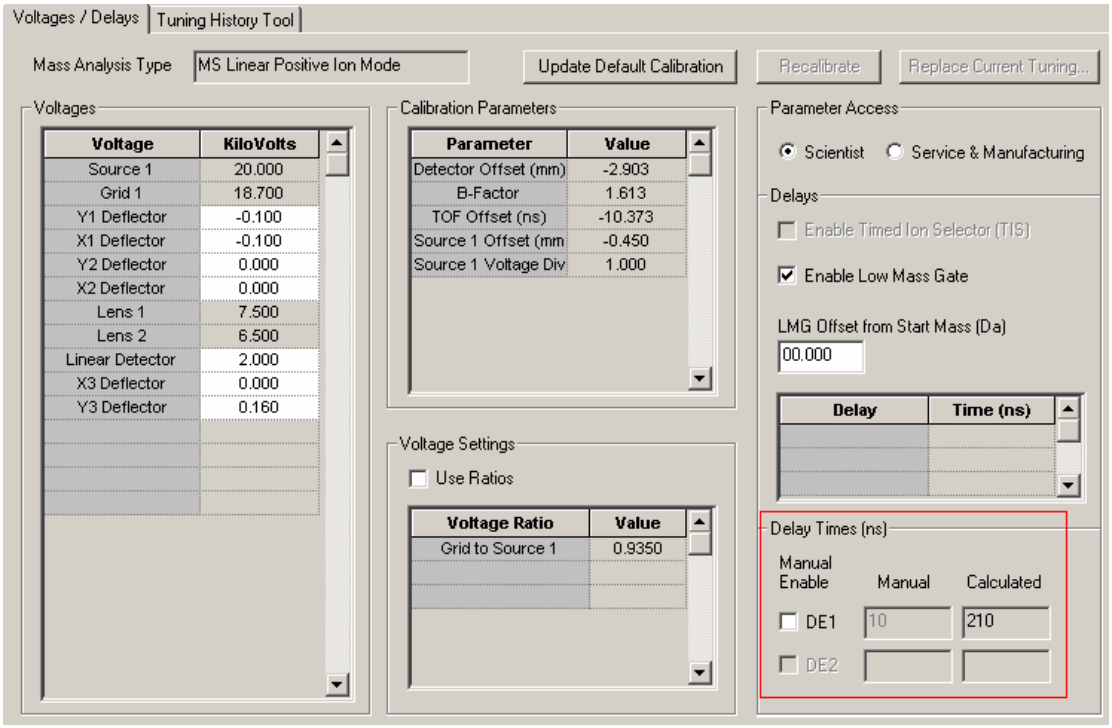

Figure 6. The delayed time parameter in linear positive mode acquisition method in applied biosystems 4700 proteomics analyzer.

2.6 Quantitation of dG-ABP adduct

For the quantitation approach of mass spectrometric technique, optimized parameters of the instruments play a key role in the measurements. For this specific carcinogen DNA-adducts dG-ABP, the optimized acquisition methods were focused on 6000 laser intensity, 1000 total laser shots and 210 nanoseconds delayed time in Applied Biosystems 4700 Proteomic Analyzer. In addition, 10.0 mg/mL THAP MALDI matrix was selected in this study and the THAP had the good matrix performance in the 10.0 mg/mL concentration when dried-droplet preparation was used. In order to obtain the linear dynamic range, limit of detection and limit of

quantitation, a serial dilution of the dG-C8-ABP analytes were designed at the concentration range of 2000 µM to 0.0997 µM in the span of six order magnitudes. Specifically, the dilution was starting from 20.0 mM dG-ABP stock solution. 3.0 uL 20.0 mM dG-ABP was dissolved in 27.0 µL anhydrous DMF to prepare 10-fold dilution and obtain 2000 µM diluted analyte. Then the diluted analyte from 2000 µM concentration, 4.6 fold serial dilution were performed in each step and 434.78 µM, 94.52 µM, 20.55 µM, 4.47 uM , 0.971 µM, 0.211 µM ,0.0459 µM and 0.00997µM dG-ABP were obtained respectively. dG was used as the internal standard and should be added into in analyte and measured together in the calibration curve study. The concentration of dG was 40 µM which was diluted from 20 mM stock solution. Equal volume of 40 µM dG and different concentration dG-ABP analyte were well-mixed in the 1.5mL centrifuge tube so the final concentration of dG solution was 20 µM and the concentration of dG- ABP solutions were from 1000 µM, 217.39 µM, 47.26 µM, 10.27µM, 2.23 µM, 0.49µM, 0.11 µM, 0.023µM,0.050 µM in 9 different concentration analytes. Equal volume of the sample and 10.0 mg/mL THAP matrix were well-mixed respectively. Before using MALDI-TOF MS for the quantitation, acquisition method and processing method were adjusted to appropriate parameters as mentioned in previous procedures. 0.60 µL of matrix-analyte sample was applied on the spots from the low concentration to high concentration of dG-ABP using dried-droplet method. Each data set had 12 replicates with total 9 data sets. 10.0 mg/mL THAP matrix solutions were mixed with anhydrous DMF in equal volume for

negative control in this calibration curve study.12 replicates for negative control were necessary.

2.7 Background elimination in mass spectrometry measurement

In order to eliminate the background noise originated from THAP cluster ions induced by DMF solvent, the negative control that was prepared by equal volume of 10.0 mg/mL THAP matrix and DMF solution were measured simultaneously when the analyte were measured. All the mass spectrometric measurements for all studies included negative control measurements in order to effectively eliminate the non-analyte background noise. After the MALDI-TOF MS measurement, signal of negative control should be deducted from the obtained signal of analyte that is represented as average peak area. The purpose of this execution was to eliminate the effect of THAP cluster induced by anhydrous solvent DMF, which produced the specific peak overlapping the target analyte peak. Smoothing the target analyte peaks when adjusting the parameter of processing method, i.e. Gaussian smoothing was necessary. (Figure 7)

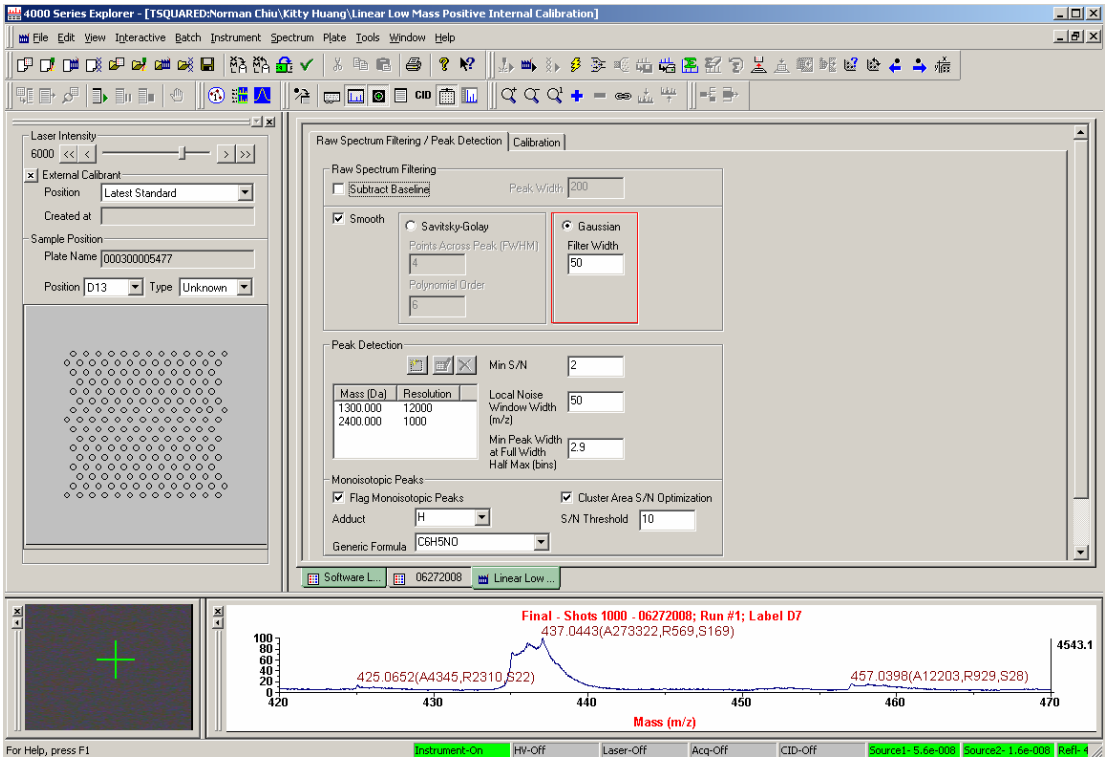

Figure 7. The Gaussian smoothing parameter of linear positive mode processing method in applied biosystems 4700 proteomic analyzer.

In summary, 3-hyroxypiconinic acid (3-HPA) and 2',4',6'-trihydroxyacetophenone monohydrate (THAP) MALDI matrices were examined in this study. In different MALDI sample preparation method, i.e. thin-layer preparation and dried-droplet preparation method were studied. During the quantitation procedure, since dG-ABP is more soluble in DMF, the effects of DMF on MALDI-TOF MS measurements have also been examined and the result indicated that THAP matrix clusters induced by DMF solvent will formed which overlaps the peak of dG-ABP in the spectra. In addition, the concentration of THAP matrix which help to maximize the signal was determined before the quantitation measurements. Since the carcinogen-DNA adducts

have been used as biomarkers to monitor the exposure of individuals to specific carcinogens in the environment, the high sensitivity is the critical part in the mass spectrometry measurements which suggest that the selected parameters should be optimized so the unique approach to lower the limit of detection of carcinogen-DNA adducts could be achievable. In this study, laser intensity, laser shots and delay time were investigate in order to generate the calibration curve with high sensitivity and also for quantiation measurements.

2.8 Batch Mode and Manual Mode.

Applied Biosystems 4700 Proteomics analyzer can provide batch mode (Figure 8) and manual mode for MALDI TOF Mass Spectrometry detection. In this research project, batch mode that can automatically follow the instruction of acquisition method and processing method to analyze the sample on the MALDI metal plate. The advantage of batch mode, besides time saving, is to assure all the data acquiring from each spot is comparable since the laser beam can hit the sample randomly following the required acquisition and processing method.

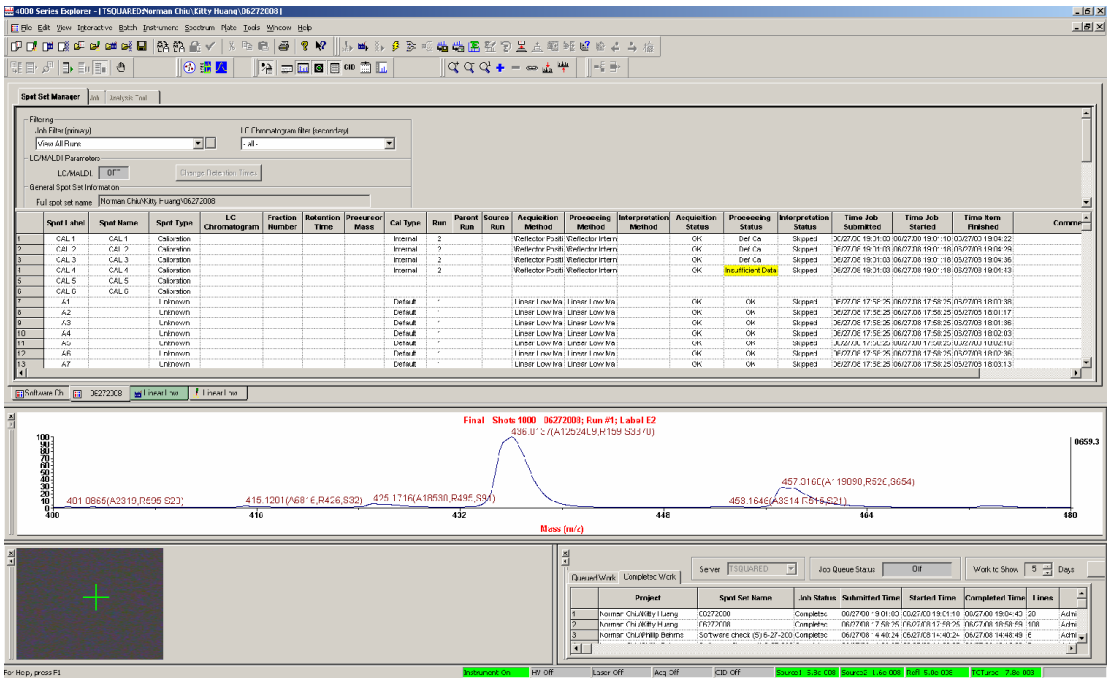

Figure 8. Batch Mode was used to run the sample analysis automatically after setting specific acquisition and processing method.

2.9 Acquisition Method and Processing Method.

With the increase in interdisciplinary research in recent years, the need for accurate and sensitive methods for the analysis of biomolecules has been increasingly important. Mass spectrometry technique has emerged as an important tool for detecting and characterizing large biomolecules in complexity. The MALDI mass spectrometric technique have gradually increased the high end of mass limit for biomolecules to over 300,000 Da and has enabled the analyses of large biomolecules by mass spectrometry to become easier and more sensitive. The principle of time of flight mass spectrometers is based on that the molecular ions will traverse this region in a time depending on their mass to charge ratios, when a well defined group of ions

of differ m/z ratios are subjected to the same applied electric field and allowed to drift in a region of constant electric field.

Compare to linear TOF-MS, the reflectron mode can improve the mass resolution by the utilization of a single-stage or a dual-stage reflectron. Even though reflectron mode in the MALDI TOF Measurement can provide better resolution for the target analyze, however, linear mode can provide higher sensitivity for the analysis that is a significant part for the determination of limit of detection in the procedure to generate calibration curve.

In order to keep a balance between obtaining higher sensitivity from the measurement and solve the problem with lower resolution from linear positive mode for the MALDI MS measurement, the appropriate approach is to use linear mode to maintain higher sensitivity. However, for the target molecular ions with different energy, the spectrum showed the peaks envelope or peak shoulder instead of a sharp peak which can be identified clearly. Under this circumstance, the only approach is to consider the peaks without higher resolution but belongs to the same kind of molecular ions as only one peak. Therefore, the correct value of signal can be obtained. For example, during the target analyte measurement, the focusing m/z ratio of dG-ABP was 435.1 with one addition proton. However, another peak showed up with one Da more than the theoretical m/z due to the different isotope for the compound where there is the possibility for the origination, i.e. carbon-13 (^{13}C). For example, the spectra in figure.3 showed two peaks in m/z 435.1 and 436.1 which

indicated that, in most of the case, they are generated from the same kind of molecular ions, i.e. dG-ABP but contain different isotope C-12 and C-13. Since quanitatively the total amount of same kind of analyte should be included in the measurement and plus they were estimated to be same kind of molecular ions due to 1 Da or less than 1 Da difference, smoothing the peaks should be reasonable in quantitation measurements.

CHAPTER III

RESULTS AND DISCUSSION

In an initial evaluation of using MALDI-TOF MS to measure carcinogen-DNA adducts, dG-ABP was selected as representing model. The dG-ABP sample was prepared by following a protocol that has been commonly used for preparing DNA samples prior to the MALDI-TOF MS measurements. Briefly, using 3-HPA as the MALDI matrix in the protocol, the dG-ABP sample was prepared by using the thin-layer method. [24] When the default setting were used in either positive or negative linear mode of MALDI-TOF MS, peaks that corresponded to dG-ABP and G-ABP aglycon ions were observed as shown in Figure 9. Interestingly, similar aglycon ions have been reported when electrospray ionization MS was used to measure dG-ABP adducts.[25] The presence of the aglycon ions were attributed to the fragmentation of dG adducts during the process of electrospray ionization, i.e. in-source fragmentation. In the case of MALDI-TOF MS of dG-ABP, the in-source fragmentation of dG-ABP could result from an excess amount of energy being transferred to dG-ABP from the laser pulses that were used during the process of MALDI. Also, when preparing the dG-ABP sample prior to the MALDI measurements, dG-ABP was mixed with the acidic 3-HPA MALDI matrix (pH = 3) which could hydrolyze the N-glycosidic bond in dG-ABP and resulted in the

formation of G-ABP.[26] (Figure 10)

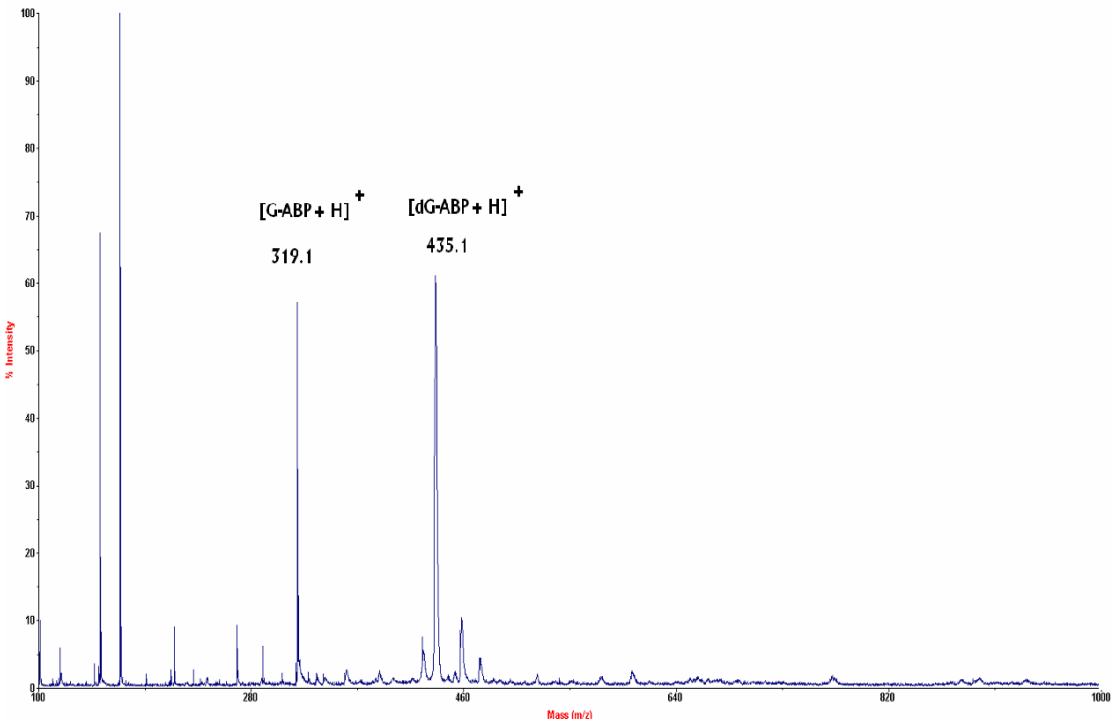

Figure 9. Mass spectrum of dG-ABP and G-ABP aglycon ions by using MALDI-TOF MS

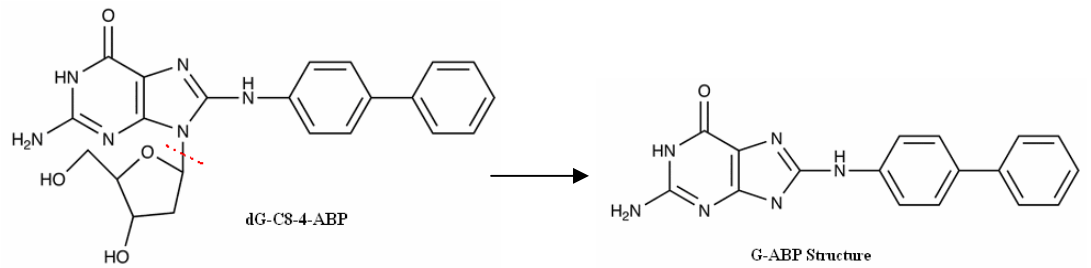

Figure 10. Laser induced fragmentation or acidic hydrolysis of dG-ABP

To take advantage of the unavoidable in-source fragmentation and acidic

hydrolysis of dG-ABP, a novel approach of using only G-ABP signal to perform the quantiation of dG-ABP was explored.The characteristic G-ABP aglycon ion does carry the most significant chemical identity of selected DNA adduct. Providing the presence of the dG-ABP signal can be confirmed, the use of the G-ABP signal to perform the quantitation of dG-ABP should be as accurate as using the dG-ABP signal. In order to ensure the highest sensitivity would be achieved, this approach required a complete conversion of dG-ABP to G-ABP. For increasing the extent of laser induced fragmentation of dG-ABP, laser intensities that were about two times higher than the threshold intensity for acquiring the mass spectrum of dG-ABP was used. The results indicated the higher-energy laser induced fragmentation was unable to achieve 100% in-source fragmentation of dG-ABP.(Figure 11) In addition, the graph at 5000 laser intensity onward, the fragmentation for G-ABP happened further. The fragmentation percentage was defined as the following equation. (Figure 12).

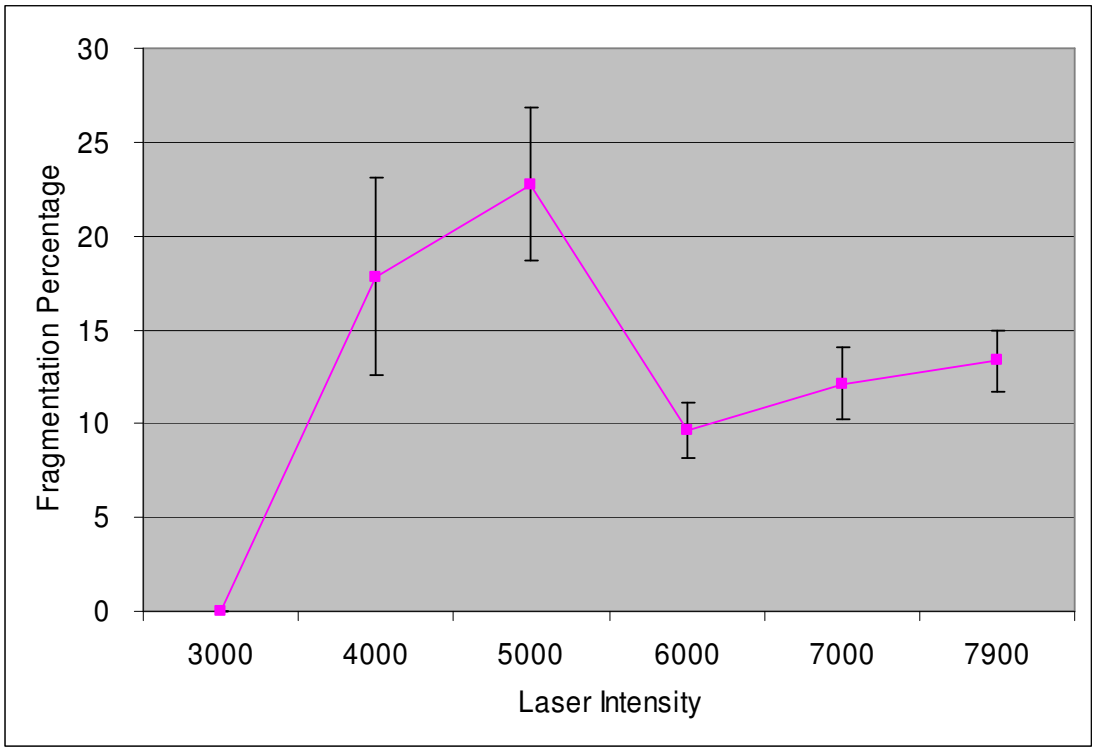

Figure 11. Fragmentation percentage of dG-ABP in default setting MALDI-TOF MS measurement. On the X-axis , it represents laser intensity ranged from 3000 to 7900. The Y-axis represents the average peak area from twelve spectra.

$$\text{Fragmentation Percentage} = \frac{\text{Signal intensity of G-ABP}}{\text{Signal intensity of (G-ABP + dG-ABP)}} \times 100\ \%$$

Figure 12. Formula of fragmentation percentage

For performing the acidic hydrolysis of the N-glycosidic bond between guanine and ribose in deoxyribonucleoside, dG monohydrate was used instead of dG-ABP. To facilitate the MALDI sample preparation, the acidic 3-HPA matrix solution (pH = 3) without any ammonium citrate additive was used to hydrolyze an equal volume of 100 μM dG.. The mixture was incubated at 37 ° C for as many as 18 hours in the

darkness. Following the incubation, the mixture was spotted on a MALDI sample plate and measured by MALDI-TOF MS in linear positive mode. In the mass spectrum of the treated dG sample, the peaks that corresponded to guanine and dG were found (data not shown),which indicated an incomplete hydrolysis of dG.. Owing to the fact that the solubility of dG-ABP in aqueous solutions is rather poor, the use of other acids to completely hydrolyze dG was not considered in this study. For this reason, the rest of this study focuses on using the signal of dG-ABP instead of G-ABP to perform the quantitation.The error bars were added in each data set which represented one standard deviation (n=12) of the fragmentation percentage. In probability and statistics, the standard deviation is a measure of the dispersion of a set of values. It was defined as the root-mean-square (RMS) deviation of the values from their mean, or as the square root of the variance. The error bars were used onward in the whole thesis. To ensure the highest sensitivity would be achieved, different experimental approaches, which aimed at increasing the signal intensity of dG-ABP, were explored and described below.

3.1 MALDI Matrix and Sample Preparation

The matrix consists of crystallized molecules, of which the three most commonly used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix) and 2,5-dihydroxybenzoic acid (DHB). However, for DNA analysis, 3-hydroxypicolinic

acid (3-HPA) [27] and THAP[28] matrix were better options. The rationale of 3-HPA and THAP could be compared as the follows. 3-HPA is normally used for oligonucleotide with more than 3500 Da mass-to-charge ratio. Instead, THAP MALDI matrix performs better for smaller molecules comparing to 3-HPA MALDI matrix. In addiction, amount all the sample preparation method of MALDI sample, for both protein and nucleic acid, the easiest and most commonly method is dried-droplet according to the literature [29]. Thin-layer preparation in the MALDI sample preparation is defined as apply the matrix on the plate to spread and dry then place the sample on top of the matrix layer and leave to dry in air. Instead, dried-droplet preparation which is the most common method used with all matrices is defined as premix sample and matrix solution by following spotting them on sample plate and air dry.

It is known that the use of different MALDI matrices and/or sample preparation methods would have different signal intensities and/or mass resolution in MALDI-TOF MS measurements, especially for measuring DNA samples.[30] The first approach to increase the signal intensity of dG-ABP focused on the choice of MALDI matrix and the sample preparation method for dG-ABP sample. Since there is no report on using MALDI-TOF MS to measure any modified monodeoxyribonucleosides in the literature, the two most commonly used MALDI matrices for measuring oligonucleotides, namely 3-hydroxypicolinic acid 3-HPA THAP, were therefore chosen in this study. In comparison to THAP, the benefits of

3-HPA matrix to measure oligonucleotides include less depurination, and higher signals and mass resolution could be achieved. Whereas, THAP has been more commonly used for measuring other molecules that were smaller than an oligonucleotide.[31]

In order to determine the most appropriate matrix for MALDI-TOF MS measurements , two typical MALDI matrices for DNA were chosen. These MALDI matrices were 3-hydropicolinic acid (3-HPA) and 2',4',6'-trihydroxyacetophenone (THAP). The selection of MALDOI matrix first depends on the type of analyte, and is essential to the formation of the analyte molecular ions. For the same analyte, different crystal morphologies were observed when different matrices were used. Also, it was known that the sample preparation method played a role in MALDI-TOPF MS measurements since the use of different sample preparation methods might formed different co-crystals between the matrix molecules and the analyte molecules. Therefore, the effects of using either one of the two selected MALDI matrices with two different sample preparation methods were evaluated. The results for measuring 250 M dG-ABP were shown in Figure 13. It was obvious that signals with THAP matrix were at least 10 times higher than those obtained with 3-HPA no matter which preparation method was used, despite the background noise of THAP matrix clusters. Apparently, THAP with dried-droplet gave a stronger signal and was approximately three times higher than the thin-layer method with THAP. In summary, it turned out that 10 mg/ mL THAP matrix presented the best

matrix if the dG-ABP sample was prepared by dried droplet method, even though hihger fragmentation percentage of THAP than 3-HPA were present.

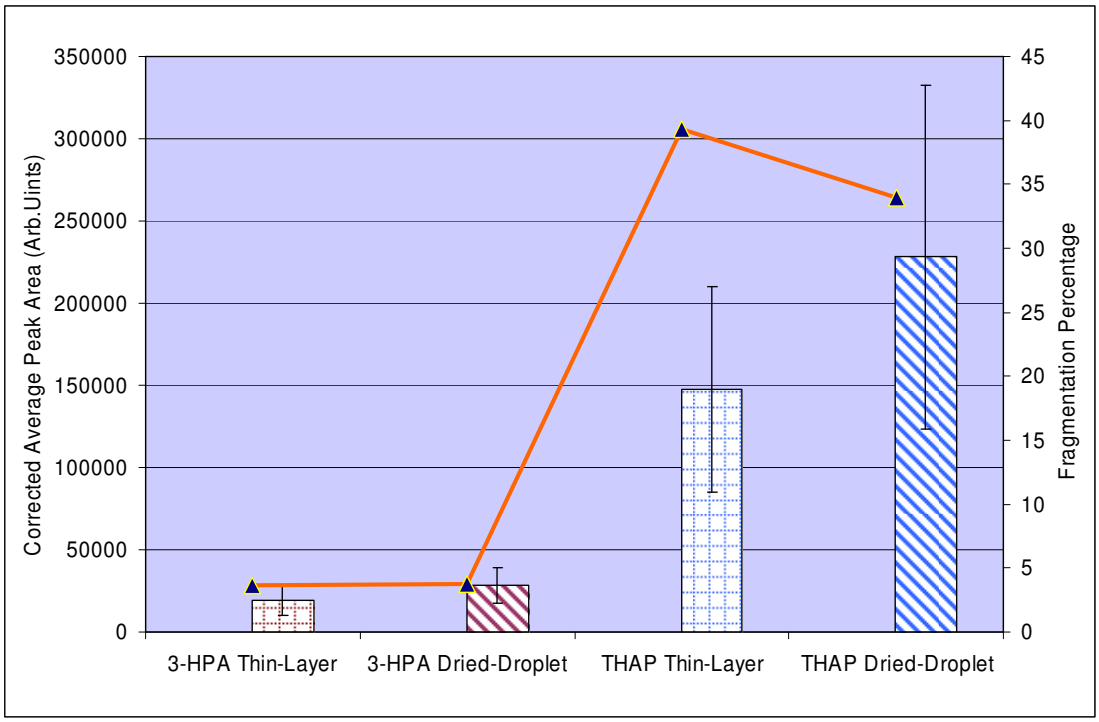

Figure 13. A figure to compare of THAP matrix and 3-HPA matrix using thin-layer preparation and dried-droplet preparation was shown. On the X-axis, it represents different sample preparation methods with either 3- HPA or THAP. The primary Y-axis on the left-hand side represented the corrected average peak area from twelve spectra. The signal correspond to the 250 μM dG-ABP. The secondary Y-axis on the right-hand side represents the percentage of in-source fragmentation of dG-ABP into its aglycon G-ABP ion. The MALDI-TOF MS parameter settings were 5000 laser intensity, 210 nanosecond delayed time, 1000 laser shots and random uniform in laser beam. The error bars represent one standard deviation (n=12) of the average peak area.

As demonstrated in our earlier publication, both MALDI matrices did not generate any interfering matrix cluster ion that would affect the dG-ABP measurement in the positive mode of MALDI-TOF MS. (Figure 14, 15 and 16)

However, when measuring THAP matrix that was mixed with an equal volume of DMF, the diluent for preparing dG-ABP standard solutions, a signal at 436.9 m/z was detected, which overlapped with the dG-ABP signal.(Figure 17 and 18) In an attempt to identify the unknown signal at 436.9 m/z, DMF was mixed with an equal volume of 3-HPA matrix. No signal at 436±5 m/z was detected. Hence the unknown signal was not identified as an impurity in DMF. Since many MALDI matrices, including 3-HPA and THAP, are known to form different matrix cluster ions during the MALDI process, a possible explanation for the unknown peak at 436.9 m/z could be the formation of a new cluster ion of THAP in the presence of DMF or a complex cluster ion between THAP and DMF. Despite this drawback of using the THAP matrix for measuring dG-ABP, the corrected signal of dG-ABP (i.e. the background signal of THAP cluster ion was subtracted from the dG-ABP signal) was still ten times higher than using 3-HPA matrix (Figure 13). Also, due to the fact that dG-ABP is not completely soluble in other organic solvents, the approach of subtracting the background signal of THAP cluster ion was then adopted in the rest of this study. Also, the use of different sample preparation methods would lead to different morphologies in the sample-matrix co-crystals.

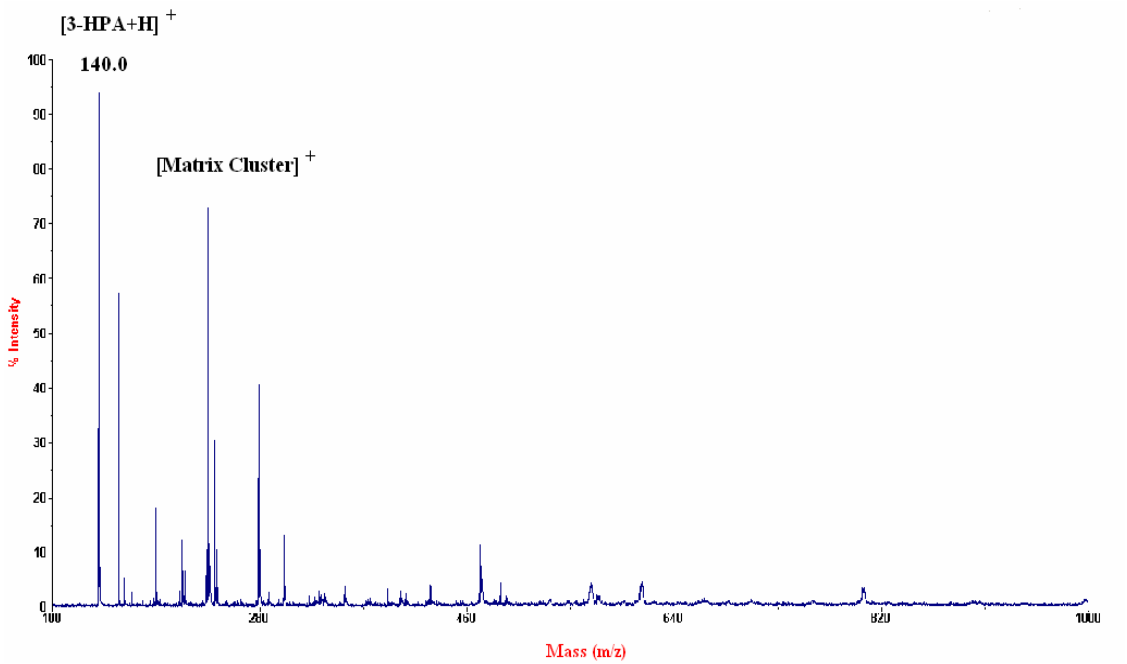

Figure 14. Mass spectrum of 3-HPA matrix that was examined by using linear positive mode in MALDI-TOF MS.

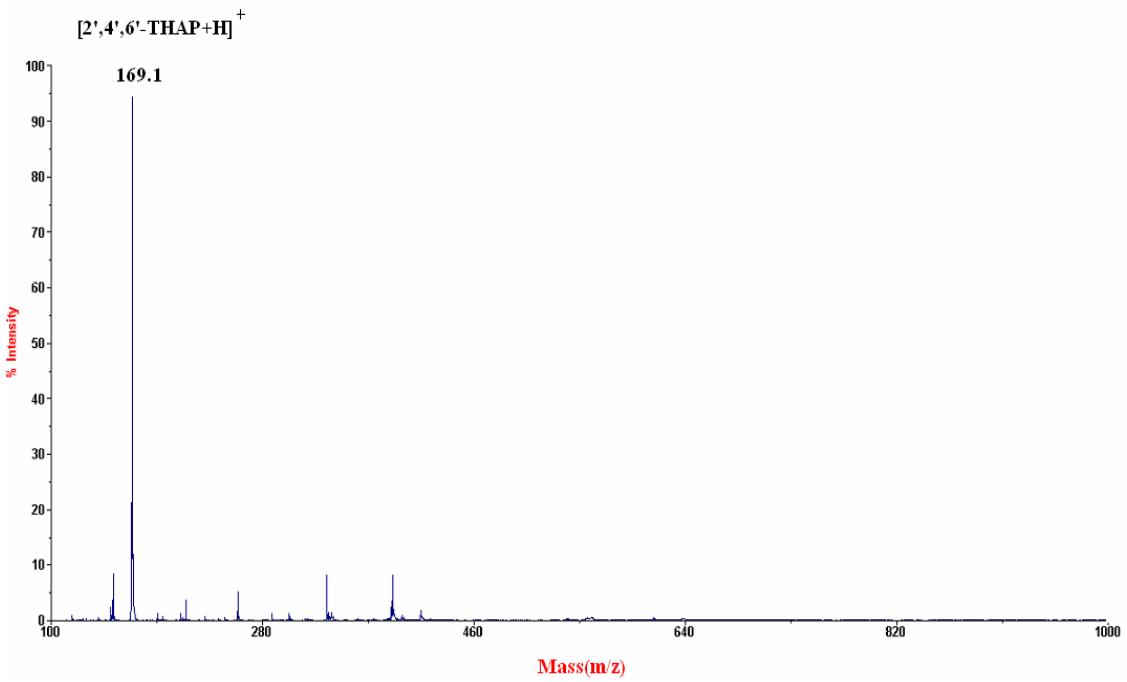

Figure 15. Mass spectrum of THAP matrix that was examined by using linear positive mode in MALDI-TOF MS.

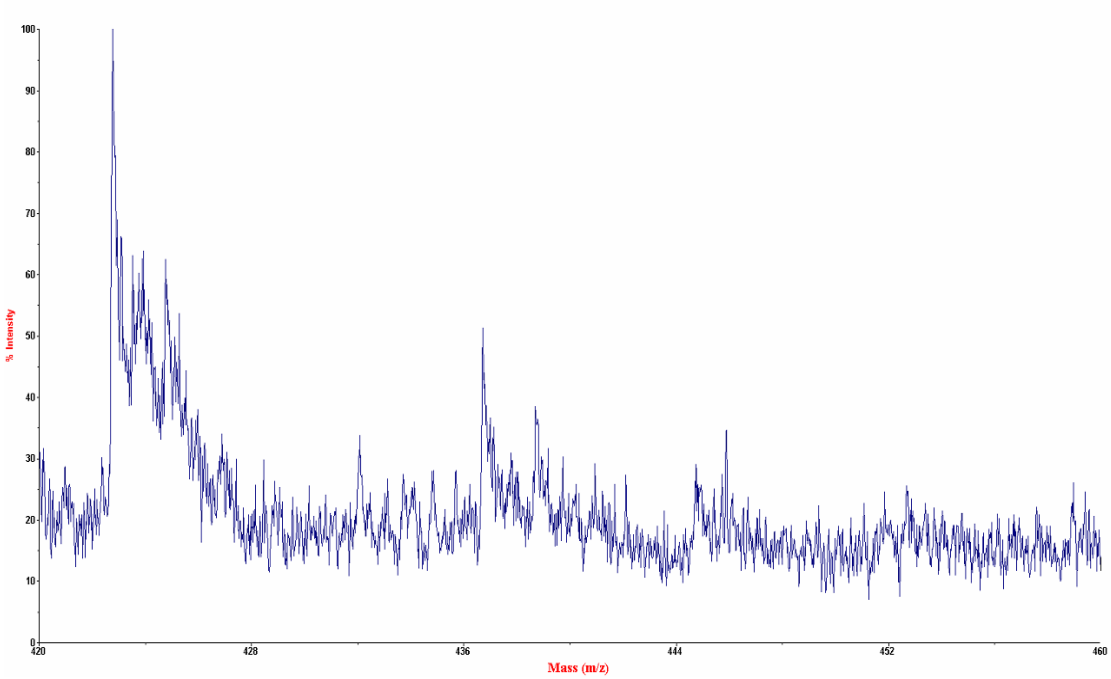

Figure 16. Zoom-in mass spectrum of THAP matrix.

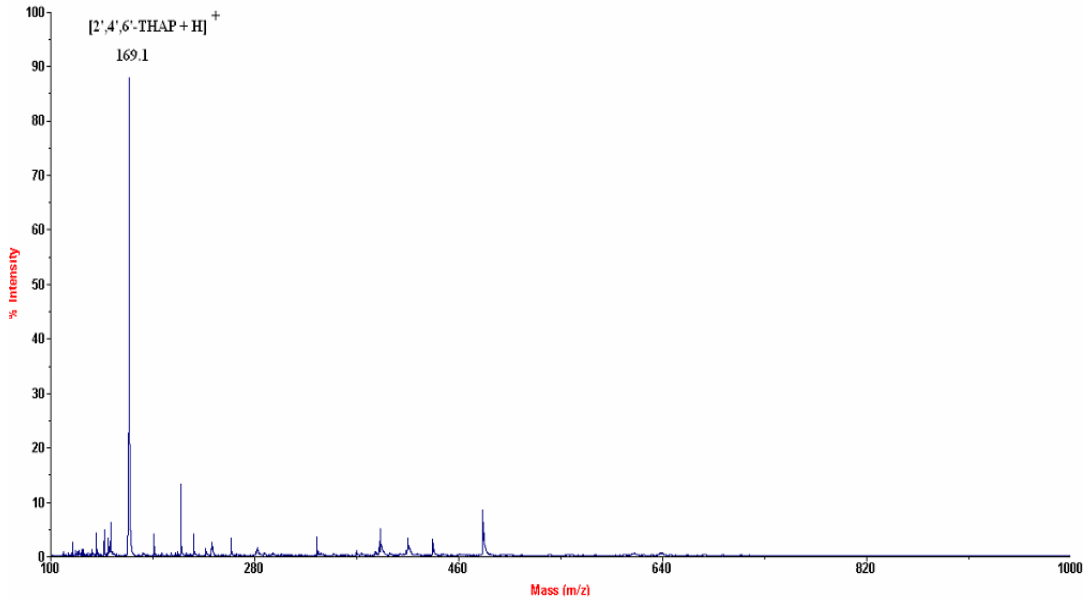

Figure 17. Mass spectrum of DMF-induced THAP matrix that was examined by using linear positive mode in MALFI-TOF MS

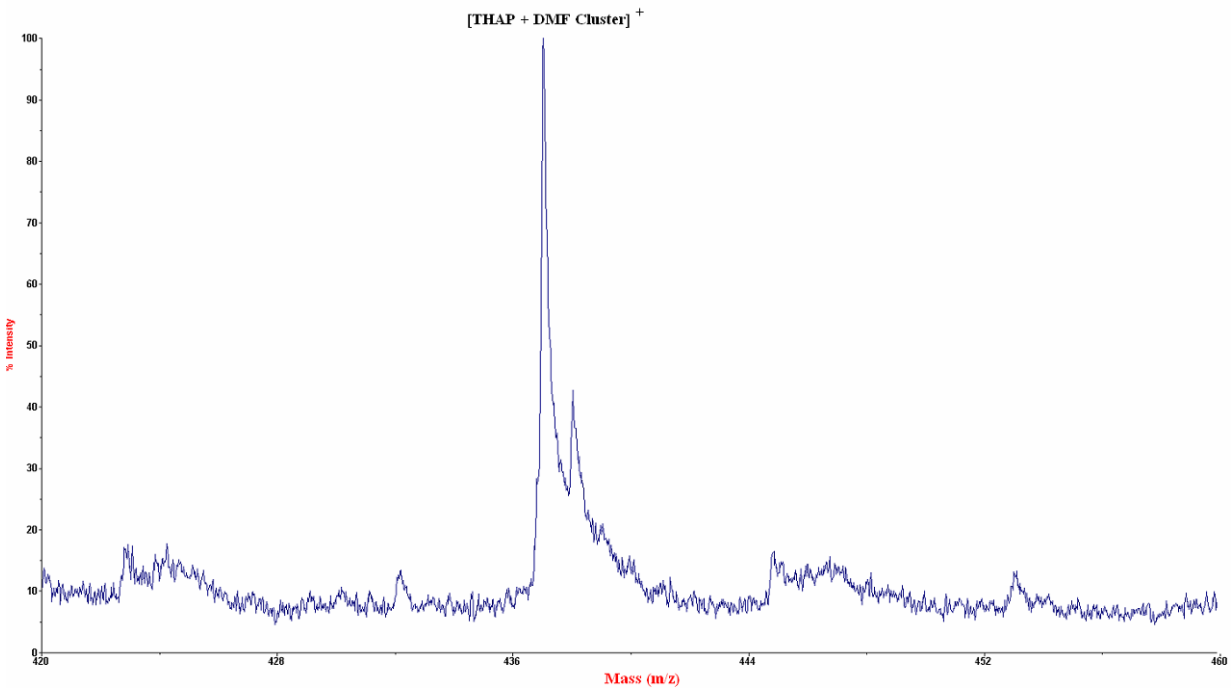

Figure 18. Zoom-in mass spectrum of DMF-induced THAP matrix that was examined by using linear positive mode in MALFI-TOF MS

3.2 Optimization of concentration of standard matrix

THAP was determined to be the most suitable matrix based on the previous study. According to the information in the literature, both 10 mg/mL and 30 mg/ mL THAP were used for performing MALDI-TOF MS analysis. Different matrix concentration would apparently change the matrix-analyte ratio. After the laser pulses hit the sample, the efficiency of protons transferring to the analytes may change due to different concentration. As a result, the vaporization and ionization effect may be influenced and the part of the analyte molecules may not be turned into molecular ions with positive charge. In that case, the created ions reaching the detector will less than the detector supposed to catch. Based on above demonstration, further study need to continue to optimize the concentration of the matrix so the best setting with

strongest signal from the mass spectrum could be used to the following study and eventually improve to sensitivity of the dG-ABP measurement.

In order to figure out which concentration was the most friendly for dG-ABP DNA adducts in MALDI MS measure, a experiment model established for both of 25 μM and 250 μM dG-ABP were illustrated in figure 2. In this model, each of the concentration dG-ABP, i.e. 25 μM and 250 μM was mixed with 10 mg/mL,30 mg/mL or 50mg/mL of THAP using the dried-droplet method. The result in Figure 19 showed that use of THAP concentration higher than 10 mg/mL were not helpful to improve the signal of dG-ABP. Also, based on the observation, different ratios of THAP and matrix solvent may lead to different crystal morphologies. In addition, more THAP dissolved might not favor the desorption or ionization for dG-ABP.

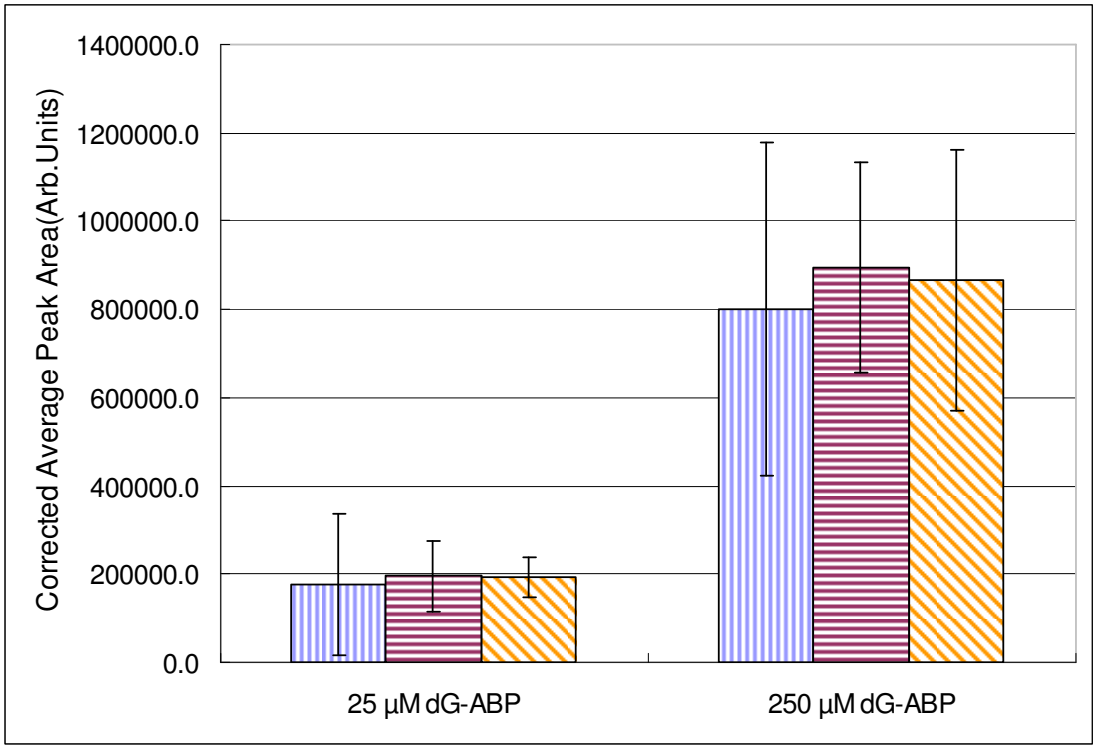

Figure 19.Optimization of THAP matrix concentration. The average peak area obtained from 25 μM

and 25 0 µM dG-ABP with 10 mg/ mL (),30 mg / mL () or 50mg/mL () 2',4',6 –trihydroxyacetophenone (THAP) were shown. The parameter settings were 5000 laser intensity, delayed time 210 nanosecond, 1000 laser shots and random uniform in laser beam. The error bars represent one standard deviation (n=12) of the average peak area.

One of the most significant issues in any analytical practice is optimization. Optimization and calibration are key factors in quantitation. In matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), one of the limitations restricting quantitation is instrument optimization. Understanding which parameters are most influential and the effects of these parameters on the mass spectrum is required for optimization.

After investigating the concentration of THAP matrix, instrumental approach was taken into account. parameter that would increase the signal without sacrificing the ability to obtain an adequate mass resolution. The first parameter , i.e. laser intensity was considered. After the concentration of the THAP matrix was optimized, the next step should be focused on the parameter setting in Applied Biosystem Instrument 4700 Proteomic Analyzer. They were a couple settings can be adjusted to apply to different samples in different conditions. In order to obtain good mass resolution for addressing the low concentration of the DNA adducts, optimized parameter setting i.e. laser intensity, number of laser shots, delay extraction played a significant role in MALDI MS measurement.

3.3 Optimization of Laser Intensity Parameter in MALDI measurement

Based on the above information, the optimization of the laser intensity was put forward since the optimized value of laser intensity indicated the laser pulse was intensive enough to create the appropriate amount of molecular. In theory, the laser energy increase as the laser intensity increase. Then more molecular ions are created with the laser pulse hits the matrix-analyte co-crystal. The signal therefore can be maximized. As shown in the Figure 20, the laser intensity was ranged from a broad range 3000 to 7900 arb. Units (maximum setting). As the laser intensity started increasing from 3000 to 4000 arb. units, the signal remained level off and did not show any increasing trend. Then the signal reached maximum value at 6000 laser intensity. Then the signal slightly decreased in 7000 and 7900 laser intensity. This was because more G-ABP fragments dissociated from dG-ABP were present due to higher energy from the laser beams. As for the fragmentation percentage, it kept going up as the laser intensity go up and reach the summit in 5000 laser intensity then decrease in higher laser intensity. Based on results, the reason of lower percentage fragmentation when laser intensity was higher than 5000 laser intensity could be due to further fragmentation of G-ABP moiety.

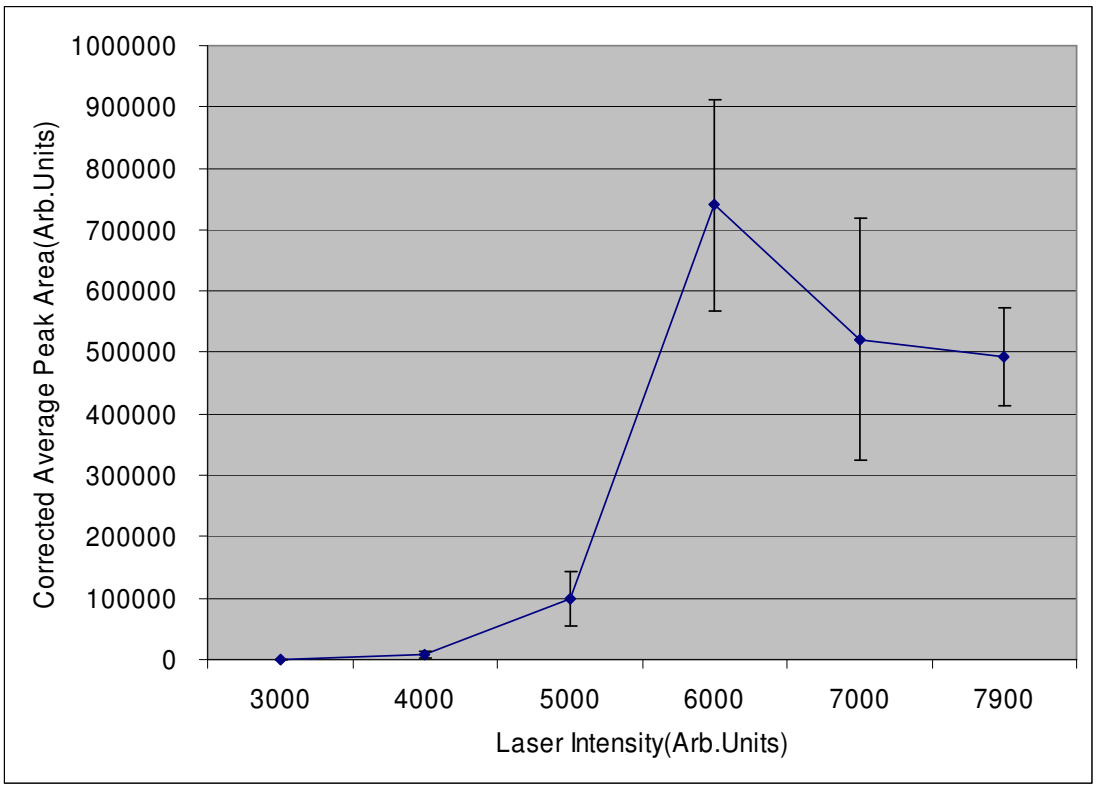

Figure 20. Optimization of laser intensity. The laser intensity used to measured 25 μM dG-ABP are plotted against the connected average peal area of dG-ABP. 10 mg /mL THAP dried-droplet preparation was used for measurement in linear positive mode. The parameter settings were 6000 laser intensity, delayed time 210 nanoseconds, 1000 laser shots and random uniform in laser beam. The error bars represent one standard deviation (n=12) of the average peak area.

A further investigation was executed to confirm THAP MALDI matrix was better than 3-HPA MALDI matrix in this specific dG-ABP adduct. For the sample using 3-HPA as the matrix, the usual thin-layer preparation method was employed and for the sample using THAP as the matrix, the usual dried-droplet method was employed. The reasons of this execution was due to the literature information indicated that 3-HPA perford better ionization effect in thin-layer methods while THAP performed better in dried-droplet methods. Thus ,two dG-ABP samples prepared by 3-HPA thin-layer method and THAP dried-droplet method were detected by MALDI

MS instrument in linear low mass positive acquisition method. The signals from these two methods were compared in the same range of laser intensity 3000 to 7000 which were typical range to detect biological sample. Plus the parameter settings were 210 nanosecond delayed time, 1000 laser shots and random uniform in laser beam. The results indicated that the signal presented in the average peak in THAP-dried droplet method had much higher responds than 3-HPA thin-layer method. It shown that 3000 to 4000 laser intensity were the threshold for detectable signal. As the laser intensity increased gradually, both of the responds of mass spectrometry from the two different matrices samples increased. At 3000 and 4000 laser intensity, analytes prepared by 3-HPA and THAP matrices were almost at the same level and magnitude. After increasing from 4000, the increasing rate of the analytes with THAP matrix is much higher than analytes with 3-HPA. It was obvious that the higher the laser intensity, the lager the difference of the signal which were produced by the detector were bigger. At 7000 laser intensity, the signal of analytes with THAP matrix was approximately 10 times higher than analytes with 3-HPA matrix. Based on above illustration, the conclusion was drawn that, for dG-ABP carcinogen DNA adduct, THAP matrix prepared in dried-droplet method was more appropriate than 3-HPA when using MALDI TOF Mass spectrometer to perform qualitative and quantitative measurement. Obviously, THAP matrix had better effect to promote ionization of analytes for the dG-ABP carcinogen DNA adducts and these results comply with theory that THAP matrix performs better for small molecules than 3-HPA matrix.

3.4 Optimization of numbers of laser shots per spectrum in MALDI-TOF MS measurements

Another important parameter related to laser intensity is the number of laser shots per spectrum. In this study, mass spectra were obtained between 100 and 2000 m/z with 6000 laser shots intensity by linear positive mode on an Applied Biosystems 4700 Proteomics Analyzer mass spectrometer. Apparently the total number of laser shots was generated from accumulative sub-spectra which lower the noise level and thus increase the signal to noise ratio. In this study, the default setting for the number of laser shot was 1000 laser shot per spectrum. Specifically, 1000 laser shots was the generated by 125 shots per sub-spectra with 8 sub-spectra were acquired. The increasing laser shots was used to improve the signal intensity [32] In order to establish a range of total laser shots, the experiment required the total shots value for consuming all of the analyte-matrix co-crystals. And the result indicated that the sample in each spot were often consumed after 13000 laser shots in the optimized 6000 laser intensity. Therefore, the range of 1000 to 13000 laser shots was the appropriate range to perform this experiment.Totally, 7 sample sets were prepared with 12 replicates in each set. 1000 total laser shots represented the minimum value and was achieved by 125 shots/sub-spectrum and a total of 8 sub-spectra were acquired. According to Figure 21, the spectrum showed a highest signal with 1000 total laser shots. When the laser shots increased to 3000, the signal drop down to half then kept fluctuating but generally level off from 3000 to 13000 laser shots. The reasons to explain stronger signals in 1000 laser shots but weaker signal when laser shots increased might be due to the uneven distribution of the target analyte and the THAP matrix in the spot of MALDI metal plate. We speculate that the

analyte dG-ABP were pushed to the top in the upper layer of co-crystals, while the THAP matrix remained under the upper thin layer of the analyte. For the rest of the co-crystals, it was more homogeneous and the averaged signal maintained at the same level with higher laser shots.

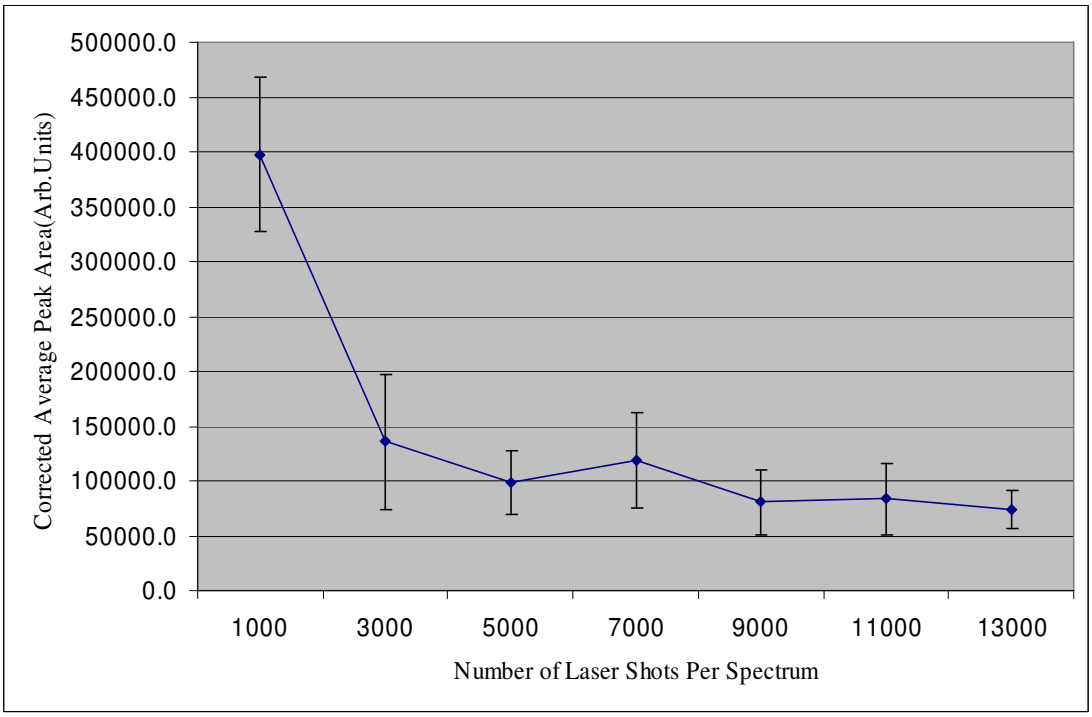

Figure 21. Optimization pf number of laser shots per spectrum. Results obtained from the optimization of laser shots in MALDI Mass Spectrometry measurement. X-axis represented the laser intensity values. Y-axis represented average peak area from the spectrum. The measurement was performed by MALDI MS using 25 uM dG-ABP and 10 mg /mL THAP as the sample and standard matrix. The parameter settings were 6000 laser intensity, delayed time 210 nanoseconds, 1000 to 13000 laser shots and random uniform in laser beam.

3.5 Optimization of delayed extraction in MALDI MS measurement

Besides the laser intensity and laser shot, time length of delayed extraction is another parameter in MALDI TOF MS measurement. Delayed extraction refers to delaying the onset of the extraction potential by some short time after the ionization

event. Delayed extraction is often used with MALDI ion sources or other situations where the ions to be analyzed are produced in an expanding plume or sphere. Since spatial homogeneity is important to mass resolution, on first inspection it can appear counter-intuitive to allow the ion plume or sphere to further expand before extraction.

Since the created molecular ions will be discharged due to the limited half life or collision between charged molecular ions. In addiction, In the DE setting, ions created from ion source underwent fast metastable decay prior to acceleration during the acceleration pulse delay time lasting for a few hundred nanoseconds.Therefore the significance of minimizing the delay time is obvious and reduce delayed time from default setting at 210 nanoseconds might preserve more dG-ABP ions. And the signal could be increased eventually. Under this circumstance, the created ions for target analyte dG-ABP may not have sufficient time to break into G-ABP aglycon ions if decreasing the delayed time. A time delay between laser excitation and application of the extracting field of 10 (lowest possible setting) to 210 nanoseconds were used. Based on the results, the average peak area fluctuated and did not reflect the trend of increasing signal as delay time decrease.(Figure 22) We speculated that certain amount of the target analyte dissociated into G-ABP fragment once accepting laser irradiation. It took no time to beak the N-glycosidic bond before extracting the created ions. Thus, no matter how low the delayed time setting, the detected target ions still showed the similar fragmentation percentage. Another possibility was that

the certain amount of analyte dG-ABP had dissociated into G-ABP even before matrix-analyte co-crystals were formed. Since dried-droplet method was used and the analyte was premixed with THAP matrix which was acidic. The N-glycosidic bond linking the ribose and the guanine base was broken due to the acidic hydrolysis in the mixing procedure. In summary, the delayed time targeted on default set 210 nanoseconds could satisfy the quantitation measurement.

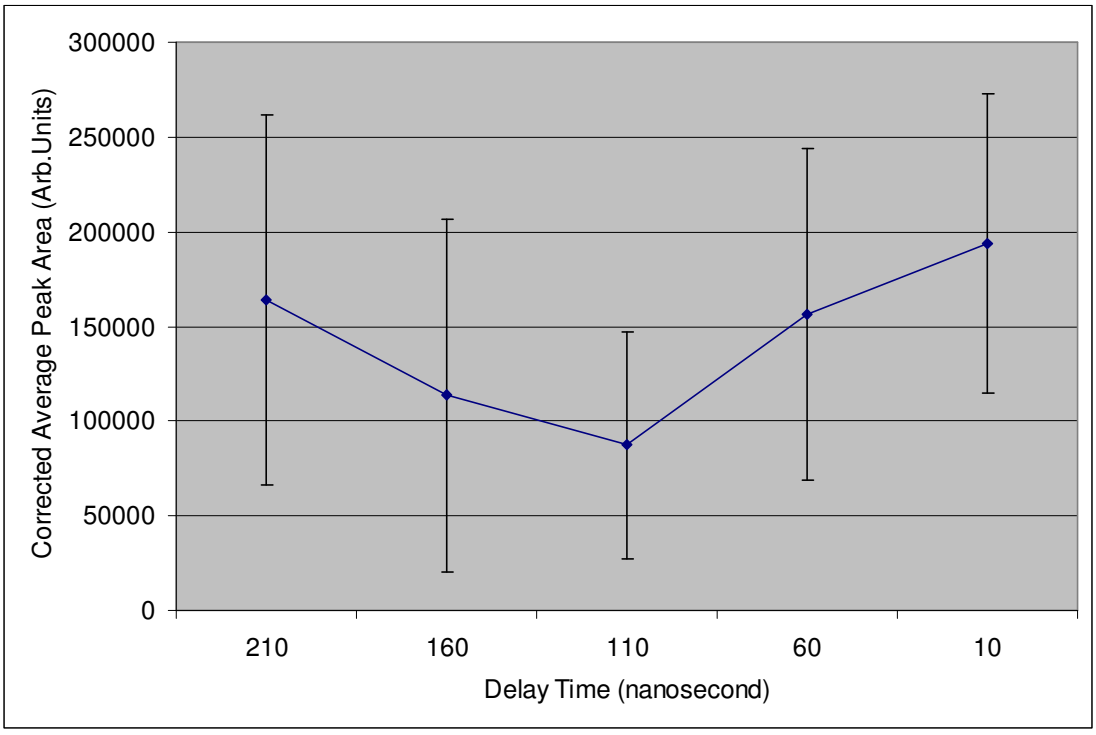

Figure 22. Optimized delayed extraction study. Results obtained from the optimization of delay time in MALDI Mass Spectrometry measurement. X-axis represented delay time. Y-axis represented the corrected average peak area from the spectrum. The measurement was performed by MALDI MS using 25 μM dG-ABP and 10 mg / mL THAP as the sample and standard matrix. The parameter settings were laser intensity 6000, 1000 laser shots and decreasing delay time from 210 nanoseconds to 10 nanoseconds and random uniform in laser beam.

3.6 Calibration curve and determination of limit of detection, limit of quantitation and linear dynamic range

3.6.1 Selection of 2'-deoxyguanisine monohydrate as internal standard

Since the necessity of this calibration curve in this project, an appropriate internal standard should be chosen. In analytical chemistry, internal standard is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis. This substance can then be used for calibration by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. The purpose of this execution is to correct the loss of analyte during sample preparation or sample inlet. The principle of selecting internal standard is the internal standard should match as closely, but not completely, the chemical species of interest in the samples, as the effects of sample preparation should be the same for the signal from the internal standard as for the signal(s) from the species of interest in the ideal case. The internal standard used needs to provide a signal that is similar to the analyte signal in most ways but sufficiently different so that the two signals are readily distinguishable by the instrument.

The best internal standard is an isotopically labeled version of the molecule you want to quantify. An isotopically labeled internal standard will have a similar extraction recovery, ionization response in ESI mass spectrometry, and a similar chromatographic retention time. Paul Vouros et al. reported the usage of

dG-C8-ABP-d9 internal standard in the quantitation of dG-ABP DNA adducts in year 2006[33]. However, since the deuterium-labeled dG-C8-ABP-d9 was not easy to obtained, further consideration should be taken on choosing another appropriate internal standard, initial focusing on 2'-deoxyguanosine monohydrate (dG) . The reason of choosing dG is it has the same guanine base and deoxyribose as dG-ABP and can provide the signal which can sufficiently distinguish the analyte while similar to analyte.

First of all an internal standard should be added at the beginning of the sample work-up. The internal standard should be added at the same level in every sample including the standards. An internal standard should give a reliable MS response. The amount of the internal standard is well above the limit of quantitation but not so high as to suppress the ionization of the analyte. A reasonable range for the internal standard is to target the lower 1/3 of the working standard curve. This is a range that will give a comfortable response without interfering with the ionization of the analyte. Normally dimethylformamide (DMF) is used to dissolve 2'-deoxyguanosine in the reaction of dG and other chemicals, i.e. B[a]P-7,8-quinone[34] . DMF should be chosen to be the solvent of dG-ABP in order to be comparable to dG internal standard that is dissolved in DMF also.

3.6.2 Selection of analytical techniques MALDI TOF mass spectrometry

Applied Biosystems 4700 Proteomic Analyzer was used through out the whole

project. According to the literature information, it was reported that the MALDI-TOF MS analysis determined the mutations induced by a single DNA adduct and characterized the deoxyoligocucleotides. The MALDI-TOF MS took advantage of difference in molecular weight between bases to identify any induced mutation. Therefore, this analysis method therefore provides qualitative and quantitative information regarding the type and frequency of mutations induced.[35] In addition, researchers developed a rapid, accurate, high sensitive and high-throughput approach to identify mt DNA SNPs using MALDI-TOF MS and the sensitivity and specificity of MALDI-TOF MS instrument were evaluated.[36] The instrument can process samples in high-throughput with more than 400 sample per hour depending on application and sample plate loading: Automated single-plate sample-loading system is installed. The Laser beam is used Nd:YAG at 255 nm wavelength, 3 to 7 nanoseconds pulse, 200 Hz firing rate.

As mentioned in previous chapter, optimized parameters play an important role in the calibration curve. After performing a series of studies on the laser intensity, the number of laser shot and delayed time, the optimized settings in MALDI TOF MS measurement for dG-ABP adducts were determined. In addition, THAP matrix was showed to be the appropriate MALDI matrix in comparison to 3-HPA and dried-droplet method should be used in calibration curve since the configuration of matrix and analyte co-crystals in this preparation was laser-friendly based on the results.

3.6.3 Calibration curve and determination of limit of detection, limit of quantitation and linear dynamic range

In order to determine the limit of quantitaion and linear dynamic range, a series of increasing concentration dG-ABP analyte was prepared spanning 5 order of magnitudes. The reason to use internal standard, as mentioned before, was because the signal variation of dG-ABP. In order to have better solution, the ratio of target analyte dG-ABP and internal standard dG was used as illustrated in Figure 23, the linear dynamic range was from 2 μM to 1000 μM and limit of quantitation and limit of detection were 2.23μM with signal to noise (S/N) 32.2. It was due to the background noise of DMF-induced THAP cluster affected the LOD to be higher than expected.

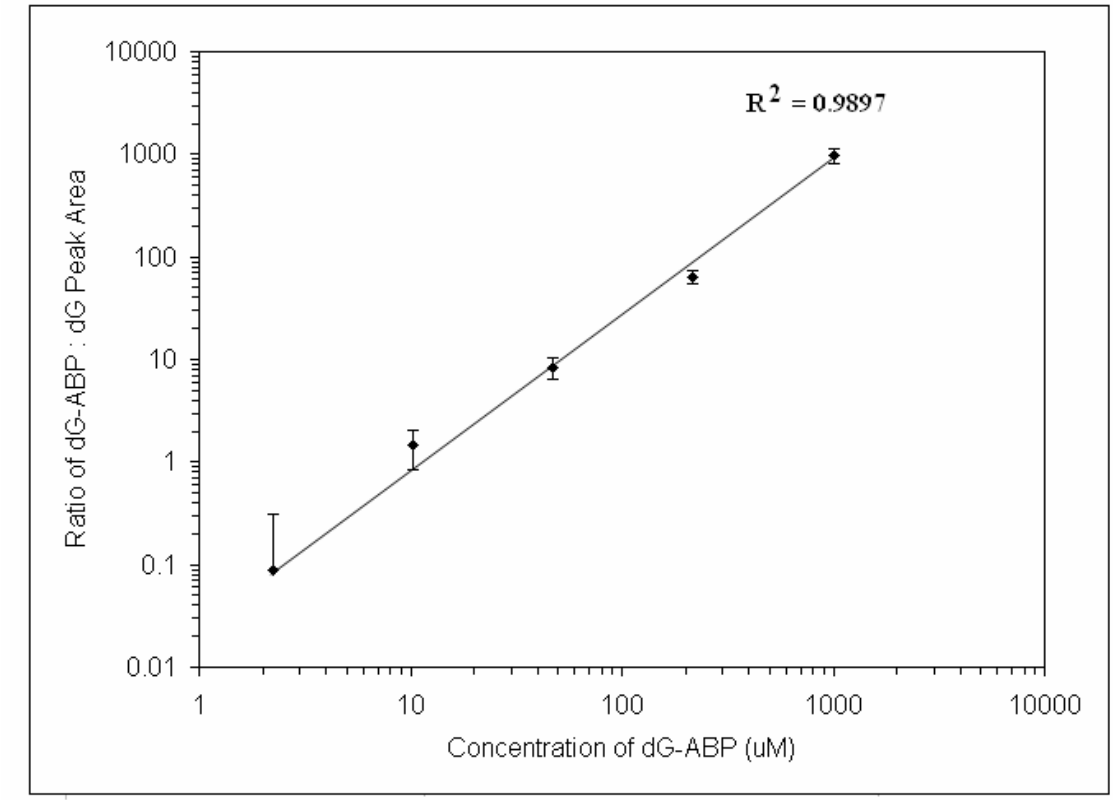

Figure 23. Calibration curve and determination of Limit Of Quantitation and Linear Dynamic Range

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

Following the use of MALDI-TOF MS to characterize the selected dG-ABP standard in another project within our research group, we have successfully established the analytical performance of this technique for the quantitation of dG-ABP standard. Initially, for the purpose to confirm the integrity of our selected dG-ABP sample, acidic 3-HPA MALDI matrix was used as the MALDI matrix and the sample was prepared by following the conventional thin-layer method. However, peaks that corresponded to dG-ABP and G-ABP aglycon ions were observed. The presence of the latter ion would theoretically lower the sensitivity of MALDI-TOF MS measurements of dG-ABP. Since the softer ESI-MS technique could not avoid the presence of G-ABP aglycon ions when dG-ABP was measured. Instead of avoiding the G-ABP aglycon ions, a investigation to completely convert dG-ABP to G-ABP was performed. Specifically, increasing the extent of laser induced fragmentation of dG-ABP was examined by using two times higher than the threshold laser intensity. However, the results indicated the presence of dG-ABP ion even though higher laser energy was applied on the sample. In addition, acidic hydrolysis of the N-glycosidic bond was chosen as an alternative approach to obtain a complete conversion of

dG-ABP to G-ABP, which carried the identity of dG-ABP. The acidic hydrolysis of dG-ABP turned out to be partially completed. Due to the concern on the solubility of dG-ABP, the approach on hydrolyzing the dG-ABP was not considered. Therefore, appropriate approaches to increase the signal intensity of dG-ABP were studied to ensure the highest sensitivity would be achieved.

Firstly, aimed to improve the signal intensity of dG-ABP, the sample preparation method was investigated. When using two different MALDI matrices, namely 3-HPA and THAP, to analyze the dG-ABP adduct, THAP MALDI matrix prepared in dried-droplet method turned out to be the best sample preparation method. Secondly, the concentration of THAP matrix was also studied, and 10 mg/mL of THAP was the optimized concentration.Thirdly, it was necessary to optimize the parameters in MALDI-TOF MS measurement when performing quantitation in order to establish the highest sensitivity. The results indicated that 6000 arb. units laser intensity, 1000 laser shots per spectrum, 210 nanosecond delayed time were the optimized settings for measuring dG-ABP adduct. The calibration curve of dG-ABP indicated the linear dynamic range was from 2 μM to 1,000 μM which spanned more than 2 order magnitudes. The limit of detection and limit of quantitation was 2.23 μM dG-ABP adduct with a signal-to-noise ratio of 32.2. This study suffered from background noise which resulted from DMF-induced peaks. While 0.02 μM limit of detection was achieved while in another project when measuring oligonucleotides. The study should have at least the same level limit of detection. With solid phase extraction technique

to concentrate sample and appropriate sample preparation method, we foresee the limit of detection can be improved to the level that needed for clinical sample analysis. The results of this study are the first step to demonstrate that MALDI-TOF MS is a viable technique for carrying out quantitative measurements of carcinogen-DNA adducts.

The future work of this project includes the discovery of other possibilities to achieve higher sensitivity for measuring carcinogen-DNA adducts. One of the possibilities will be using sugar-doped matrix to minimize dissociation of aglycon ion through the absorption of excessive laser energy by the sugar additive. For DNA molecules, using MALDI MS to obtain high quality mass spectra is a critical part since the rate of matrix-analyte crystallization would be influenced by a couple factors such as sample impurity, solvent content, environment conditions. Thus, the laser fluence threshold for the metastable decay caused by the excess energy transferred to dG-ABP molecules would result in deteriorated mass resolution assuming that laser-fluence is higher than the threshold value. Therefore, lower limits of detection might not be achieved and the sensitivity would remain low.

REFERENCES

1. Vineis,P. Epidemiology of cancer from exposure to arylamines. *Environ. Health.Prospect*.**1994**.102(Suppl.6),7-10

2. Feng,Z.,Hu,W.,Rom,W.W..Beland,F.A.,And Tang,M.4-Aminobiphenyl is a major etiological agent of human bladder cancer: evidence from its DNA binding spectrum in human p53 gene.*Carcinogenesis*.**2002**.23,1721-1727

3. Faraglia,B.,Chem,S.Y.,Gammon,M.D.,Zhang,Y.L.,Teitelhaum,S.L.,Neugut,A. L.,Ahsan,H.,Garbowski,G.C.,Hibshoosh,H.,Lin,D.,Kadlubar,F.F.,and Santella,R.M. Evaluation of 4-aminobiphenyl-DNA adducts in human breast cancer: the influence of tobacco smoke. *Carcinogenesis.***2003**. 24,719-725.

4. Saletta,F., Matullo,G., Manuguerra,M., Arena,S., Bardelli,A., Vineis,P. Exposure to the tobacco smoke constituent 4-aminobiphenyl induces chromosomal instability in human cancer cells. *Cancer Res*. **2007**.67(15),7088-94

5. http://www.cancer.org/docroot/CRI/content/CRI_2_4_1x_What_Is_Cancer.asp ?siteareas

6. Hingerty,B.E., Broyde,S. Molecular Structures of Carcinogen-DNA Adducts. NERSC 3 Greenbook.

7. Beland,F.A., Doerge,D.R., Churchwell,M.I., Poirier,M.C., Schoket,B., Marques,M.M.. Synthesis, Characterization, and Quantitation of a 4-Aminobiphenyl-DNA Adduct Standard Chem. *Res. Toxicol*. 1999, 12, 68-77

8. Hatcher,J.F.,Swaminathan,S..Detection of deoxyadenosine-4-aminobiphenyl adduct in DNA of human uroepithelial cells treated with N-hydroxy-4-aminobiphenyl following nuclease P1 enrichment and 32P-postlabeling analysis. *Carcinogenesis.* **1995**.16(2),295-301

9. Weston,A.,Bowman,E.D. Fluorescence detection of benzo[*a*]pyrene-DNA adducts in human lung. *Carcinogenesis.***1991,**12(8):1445-1449

10. Phillips,D.H,Farmer,P.B.,Beland,F.A.,Nath,R.G.,Poirier,M.C.,Reddy,M.V.,Turt eltaub,K.W. *Environmental and Molecular Mutagenesis* **2000**,35,222-233

11. Santella, R.M. Immunological Methods for Detection of Carcinogen-DNA Damage in Humans. *Cancer Epidemiol.Biomarkers Prev.*,**1999**. 8, 733–739.

12. Bol,S.A.;de Groot,A.J.;Tijdens, R.B.,Meerman,J.H.; Mullenders,L.H.;van Zeeland,A.A., Electrochemical detection and quantitation of acetylated and deacetylated C8-deoxyguanosine DNA adducts induced by 2-acetylaminofluorene.*Anal.Biochem* **1997**,251,(1),24-31

13. Bol,S., Groot,A., Tijdens,R.B., Meerman,J., Mullenders,L., van Zeeland,A.. Electrochemical Detection and Quantification of the Acetylated and Deacetylated C8-Deoxyguanosine DNA Adducts Induced by 2-Acetylaminofluorene Anal Biochem. **1997.** 251(1),24-31.

14. Elaine M. Ricicki, John R. Soglia, Candee Teitel, Robert Kane,Fred Kadlubar, and Paul Vouros. Detection and Quantification of N-(Deoxyguanosin-8-yl)-4-aminobiphenyl Adducts in Human Pancreas Tissue Using Capillary Liquid Chromatography-Microelectrospray Mass Spectrometry. *Chem. Res. Toxicol.* **2005**, 18, 692-699

15. Zayas,B., Stillwell, S.W., Wishnok,J.S., Trudel,L.J., Skipper, P., Yu,M.C., Tannenbaum,S.R. and Gerald N. Wogan. *Carcinogenesis*. **2007**,28(2),342-349

16. Cui,Z, Theruvathu,J.A., Farrel ,A., Burdzy,A., Sowers,L.C. Characterization of synthetic oligonucleotides containing biologically important modified bases by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Biochem.***2008**, 379,196–207

17. Environ.Sci.Technol.,Vol.26,No.11,1992

18. Kumon,S., Nakanishi, K., Hirao,K. Macroporous Silicate Films by Dip -Coating.*Journal of Sol-Gel Science and Technology*. **2000**,(19),553–557

19. Fux,C., Moser,S., Schlatter,S., Rimann,M., Bailey,J.E., Fussenegger,M.. *Nucleic Acids Research*,**2001**, 29, No.19e1

20. Yang,X., Wu,H., Kobayashi,T., Solaro,R.J., van Breemen,R.B.. Enhanced Ionization of Phosphorylated Peptides during MALDI TOF Mass Spectrometry.*Anal. Chem*. **2004**, 76, 1532-1536

21. Sudha,R., Zenobi,R. The Detection and Stability of DNA Duplexes Probed by MALDI Mass Spectrometry**.***Helvetica Chimica Acta.***2002**,85,3136-3143

22. Zhang,N., Doucette, A., Li.L.. Two-Layer Sample Preparation Method for MALDI Mass Spectrometric Analysis of Protein and Peptide Samples Containing Sodium Dodecyl Sulfate .*Anal. Chem.* **2001**, 73, 2968-2975

23. Horneffer, V., Forsmann,A., Strupat,K., Hillenkamp,F., Kubitscheck,U. Localization of Analyte Molecules in MALDI Preparations by Confocal Laser Scanning Microscopy. *Anal. Chem*. **2001**, 73, 1016-1022

24. Capillary isoelectric focusing hyphenated to single- and multistage matrix-assisted laser desorption/ionization-mass spectrometry using automated sheath-flow assisted sample deposition. *Electrophoresis*. **2008**, 29, 1974–1984.

25. Chan,W., Zheng,Y., Cai,Z. Liquid Chromatography–Tandem Mass Spectrometry Analysis of the DNA Adducts of Aristolochic Acids. *J Am Soc Mass Spectrom.***2007**, 18, 642–650

26. Tang,W., Krause,J., Zhu,L.,Smith,L.M.. Factors influencing oligonucleotide stability in matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy. *International Journal of Mass Spectrometry and Ion Processes.***1997**,169, 301-311

27. Wu,K.J., Steding, A., Becker, C.H. Matrix-assisted laser desorption time-of-flight mass spectrometry of oligonucleotides using 3-hydroxypicolinic acid as an ultraviolet-sensitive matrix". *Rapid Commun. Mass Spectrom.* **1993**, 7 (2),142–146

28. Kuhn-Holsken, E., CLenz,C., Sander, B., LÜHRMANN,R., and Urlaub,H. Complete MALDI-TOF MS analysis of cross-linked peptide–RNA oligonucleotides derived from nonlabeled UV-irradiated ribonucleoprotein particles. *RNA***. 2005.**11(12),1915-1930

29. Vaidyanathan, S;Winder, C.L.;Wade, S.,C.; Kell, D.,B.; Goodacre, R. Sample preparation in matrix -assisted laser desorption/ionization mass spectrometry of whole bacterial cells and the detection of high mass (>20 kDa) proteins.*Rapid Communications in Mass Spectrometry*.**2002**, 16(13), 1276-1286

30. Chen, C; Walker, A.,K.,Wu, Y.,Timmons, R.B., Kinsel, G.R.. Influence of sample preparation methodology on the reduction of peptide matrix-assisted laser desorption/ionization ion signals by surface-peptide binding. *Journal of Mass Spectrometry*.**1999**, 34(11), 1205-1207

31. Detection and quantitation of curcumin in mouse lung cell cultures by matrix-assisted laser desorption ionization time of Fight mass spectrometry. Maya,L.A.; Tourkinab, E.; HoVmanb,S.R., Dixa,T.A.; Anal. Biochem. **2005**,337, 62–69

32. Caldwell,K.L., Murray,K.K. Mid-infrared matrix assisted laser desorption ionization with a water/glycerol matrix. Applied Surface Science **1998**, (127–129),242–247

33. Ricicki,E.M., Luo,W., Fan, W., Zhao,L.P., Zarbl,H., Vouros,P. Quantification of N-(Deoxyguanosi-8-yl)-4-aminobiphenyl Adducts in Human Lymphoblastoid TK6 Cells Dosed with N-hydroxy-4-acetylaminobiphenyl and Their Relationship to Mutation, Toxicology, and Gene Expression Profiling.*Anal.Chem*.**2006**,78, 6422-6432.

34. Balu,N., Padgett,W.T., Lambert,G.R., Swank,A.E., Richard, A.E., Nesnow, S.Identification and Characterization of Novel Stable Deoxyguanosine and Deoxyadenosine Adducts of Benzo[a] pyrene-7,8-quinone from Reactions at Physiological pH.Chem. *Res. Toxicol*. **2004**, 17, 827-838

35. McLuckie,K.I., John H. Lamb, Sandhu,J.K. Pearson,H.L, Brown, B., Farmer,P.B. and Jones,D.,J. Development of a novel site-specific mutagenesis assay using MALDI-ToF MS (SSMA-MS). *Nucleic Acids Res.* **2006**.34(22),e150

36. Fan,X., Garritsen, Henk S. P., Tarhouny, Shereen E. L., Morris, Michael, Hahn, Sinuhe, Holzgreve, Wolfgang, Zhong,X. *Clin Chem Lab Med.* **2008**.46(3),299-305