Development of MALDI-TOF MS methods for quantitative small molecules analysis

Thesis Submitted to AcSIR For the Award of the Degree of
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In Chemical Science

By
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CERTIFICATE

Certified that the work incorporated in the thesis entitled: "**Development of MALDI-TOF MS methods for quantitative small molecules analysis**", submitted by Mr. Ajeet Singh, for the degree of Doctor of Philosophy, was carried out by the candidate under my supervision at the Chemical Engineering and Process Development Division, National Chemical Laboratory, Pune, India. Materials acquired from other sources have been duly acknowledged. To the best of my knowledge, the present work or any part thereof has not been submitted to any other University for the award of any other degree or diploma.

Date: Dr. Venkateswarlu Panchagnula

Place: NCL, Pune (Research guide)

DECLARATION

I hereby declare that the work incorporated in this thesis entitled "**Development of MALDI-TOF MS methods for quantitative small molecules analysis**" submitted for the degree of Doctor of Philosophy in Chemical science has been carried out by me at the Chemical Engineering and Process Development Division, National Chemical Laboratory, Pune, India under the supervision of Dr. Venkateswarlu Panchagnula. Materials acquired from other sources have been duly acknowledged in this thesis. The work is original and has not been submitted in part or full by me for award of any other degree or diploma in any other University.

Date: Ajeet Singh

Place: NCL, Pune (Research student)

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Introduction

1.1 Mass Spectrometry

In 19th century, cathode rays experiment gave birth to mass spectrometry.[1] In principle mass spectrometry is a technique to determine the mass of the molecule based on its mass to charge ratio (*m/z*) and with the timely development it has become an indispensable analytical tool in chemical, biological, geological, environmental and forensic sciences. In 1912, with the help of Francis Aston, Sir J.J. Thomson built the first raw mass spectrometer to determine the masses of charged atoms.[2] Few years later in 1918 Dempster invented the first ion source based on electron impact.[1,3] Initially for few decades mass spectrometry had been an area of interest only to the Physicists. It was Alfred Niel who invented the magnetic sector mass analyzer and introduced the practical mass spectrometer to the world.[1]

Milestones in mass spectrometry

***1897**: Discovery of electron*
***1912**: First mass spectrometer by Thomson*
***1918**: EI and Magnetic focusing*
***1946:** TOF*
***1953:** Quadrupole*
***1956:** GC-MS*
***1965:** Ion cyclotron*
***1968:** ESI*
***1974:** FTICR*
***1975:** APCI*
***1981:** FAB*
***1985:** MALDI*
***2000:** Orbitrap*
***2004:** DESI*
***2005:** DART*

1.1.1 Mass Spectrometer

A typical mass spectrometer consists of three basic components: ion source, mass analyzer and ion detector. Ion source converts the molecules into charged or ionized species and introduces them into the mass spectrometer. There are various types of ion sources available based on the ionization mechanism used and configuration of

the instrument(s). Currently available ion sources can operate both at atmospheric pressure and in vacuum. Mass analyzer resolves the ions based on mass-to-charge ratio using several possible physical principles prior to the detection typically using an ion counter that converts the ion current to simple *m/z* versus relative abundance spectrum. Figure 1.1 shows the typical block diagram of a mass spectrometer with both the ion source and the analyzer operating under vacuum.

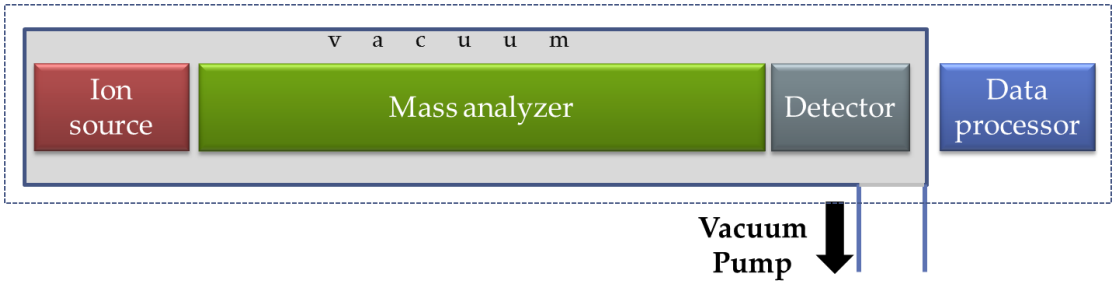


Figure 1.1: *Schematic of a mass spectrometer showing the basic components. The mass analyzer operates under very high vacuum (10^{-4}-10^{-8} torr).*

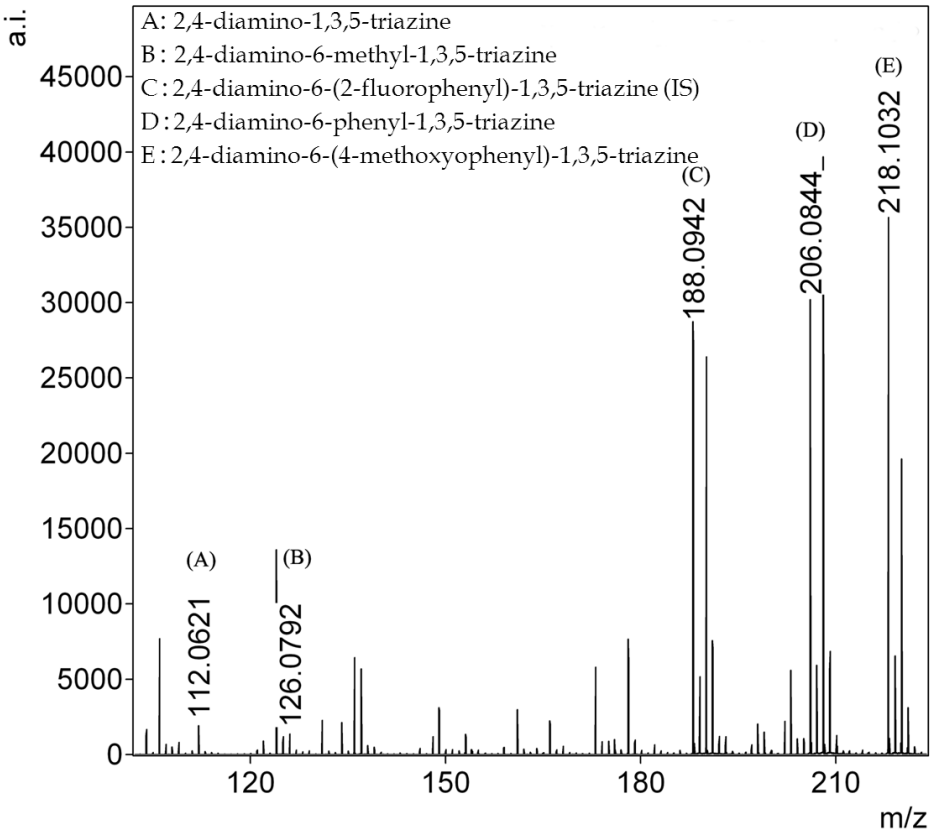


Figure 1.2: *A typical mass spectrum of triazine molecules. X-axis and Y-axis represent the m/z and ion abundance respectively.*

1.1.2 Mass spectrum

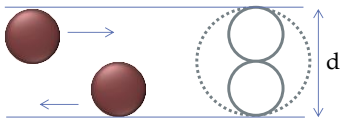
A mass spectrum represents the plot of signal intensity versus the *m/z* value of the molecules in the sample. The intensity of signal reflects the total abundance of an ionic species at particular *m/z* recorded at detector (Figure 1.2).

Vacuum in MS

At very low pressure or high vacuum mean free path of ions is increased to avoid collision between ions during their movement in analyzer.

$$\lambda = kT / \sqrt{2}p\sigma$$

λ = Mean free path (m)
k = Boltzman constant
T = Temperature (K)
P = pressure (Pa)
$\sigma = \pi d^2$ Collision cross section (m^2)



1.1.3 Ionization techniques and ion source

As described earlier ion source generates the gas phase ions and introduces them into a mass analyzer. Many ionization processes have been developed and each has its own advantages and disadvantages.[4] Selection of ionization method depends upon the nature of sample and analyzer. The ionization process can be categorized mainly in three types.[5]

I. Ionization of neutral molecule through charge transfer (e.g. protonation deprotonation, cationization)

II. Transition of a charged molecule from a condensed phase to the gas phase

III. Electron capture and electron ejection

The table 1.1 shows the various ionization sources with their method of ion formation. Electron ionization (EI) is the classical ionization method in which gaseous molecules are ionized by bombardment with 70 eV electron beam and mainly produce M^+ ions.[6] It has been used mainly with gas chromatography for structure elucidation of volatiles. Being a hard ionization technique, EI produces excessive fragmentation of the molecule. Chemical ionization (CI) is a comparatively softer ionization process than EI. In this process, gaseous molecules interact with a reagent gas (e.g. methane)

and charge transfer takes place. CI mainly produces protonated adduct $[M+H]^+$ with relatively less fragmentation and unlike EI is suitable for less volatile molecules as well. Hence CI is also considered as complementary to EI.[6-9] Field desorption (FD) and plasma desorption (PD) ionization methods were developed for the ionization of non-volatile molecules of molecular weight up to 10,000 Da. In both ionization methods, sample is deposited on a metallic surface. FD ionization occurs by applying very strong electric field around the deposited sample whereas in PD, desorption of ion happens when fission particles generated from a radioactive material are deposited on the sample surface.[6, 10, 11] Due to technical difficulties and safety hazards both ionization methods could not supersede the other conventional ionization methods.[12,13] Fast atom bombardment (FAB) is also a desorption ionization method where ions are generated from solid/liquid by bombardment with beam of energetic atoms. In this process sample is dissolved in glycerol and energetic beam is generated from one of inert gas (Xe, Ar and Ne) by an atom gun.[14-16]

Apart from above ionization methods, electrospray ionization (ESI) and laser desorption ionization (LDI) are two widely used methods of ionization. In this paragraph ESI is discussed and LDI is described separately. ESI is a very versatile ionization method, capable of ionization from small molecules to high molecular weight biomolecules. It was discovered by Malcolm Dole in late 1960s and later it was further explored to large extent by John B. Fenn who was awarded Nobel Prize in Chemistry in 2002 [Cite]. ESI is leading atmospheric pressure and soft ionization method and mainly used with liquid chromatography. The ionization process in ESI involves the formation of charged droplet in Taylor cone by nebulizer and applied potential. As these charged droplets approach to the inlet of mass spectrometer, drying

gas evaporates the solvent followed by Rayleigh disintegration until they convert into gas phase ions.[17-20]

Table 1.1: *Description of various ionization sources*

Ionization source	**Method**	**Type**
Electron ionization	Electron ejection	Under vacuum
Chemical ionization	Protonation, deprotonation, cationization, transfer or charge	Under vacuum
Field and plasma desorption	Electron ejection	Under vacuum
Fast atom bombardment	Electron capture	Under vacuum
Electrospray ionization	Protonation, deprotonation, cationization, transfer or charge	Atm. pressure
Matrix assisted laser desorption ionization	Protonation, deprotonation, cationization, transfer or charge	Under vacuum, atm. pressure
Ambient pressure ionization (DESI, DART, ASAP etc.)	Protonation, deprotonation, cationization	Ambient pressure

1.1.4 Mass analyzers

After the ionization, ions enter into the mass analyzer where they get separated on their mass-to-charge ratio (*m/z*). There are several mass analyzers based on different principles utilized in the separation of the gas phase ions.[21, 22] These mass analyzers can be used solely or can be coupled with another mass analyzer to form a hybrid mass analyzer. Selection of mass analyzer depends upon a variety of factors such as mass range, mass resolution, scan rate, sensitivity and detection limit, which are different and characteristic of each mass analyzer.[22-24] Mass analyzers can be divided in two categories; pulsed or continuous.[25] In continuous mass analyzers, ions of selected mass range are transmitted to the detector. It is comparable to the filtering of

light of a particular wavelength through filter in optical spectroscopy. In this process certain ions get detected and remaining ions are lost. Quadrupole and magnetic sector mass analyzers are the example of continuous mass analyzer.[23] In case of pulsed mass analyzers, all the ions in entire mass range are analyzed in single pulses increasing the sensitivity.[6] Time-of-flight and ion cyclotron are examples of pulsed mass analyzers.[26]

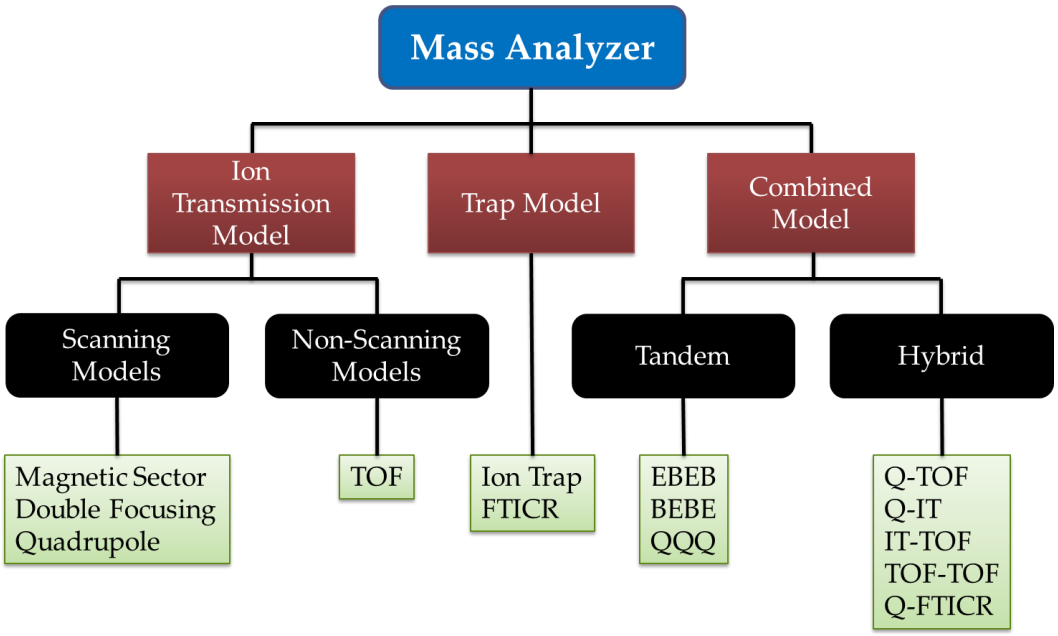


Figure 1.3: *Types of mass analyzers used in mass spectrometry. All the mass analyzers can be divided broadly into three categories; transmission, trap and combined models.*

Table 1.2: *Description of various mass analyzers* [25]

Mass analyzer	**Quantity measured**	**Maximum mass range (*m/z*)**	**Resolution**
Quadrupole	Ratio of RF to DC	~3000	10^3
Magnetic sector	Momentum and charge	~20000	10^5
Ion trap	Frequency	~2000	10^4-10^5
Ion cyclotron	Angular frequency	~2000	10^9
Time-of-flight	Time of flight	No upper limit	10^3-10^4

1.1.5 Characteristics of a mass analyzer

Resolution and mass accuracy are two important characteristics to describe a mass analyzer. The principle function of a mass analyzer is to separate and resolve the ions by their *m/z* ratio. The resolution (R) is described as the power of a mass analyzer to distinguish between two peaks. It is defined as ratio of a peak (m_1) to the difference in mass between m_1 and adjacent peak of mass m_2 or ratio of peak m_1 and its FWHM (full width at half maximum).

$$R = \frac{m_1}{\Delta m} = \frac{m_1}{(m_2 - m_1)}$$

$$R = \frac{m_1}{FWHM}$$

For example if m_1 is 127.0521 and m_2 is 127.0732 than the resolution required for the separation would be ~6000. Higher value of R indicates the high separation ability of the analyzer.

Mass accuracy or accurate mass measurement is an essential criterion for identification of a molecule. Poor mass accuracy often leads to wrong identification. Mass accuracy of an analyzer is defined in parts-per-million (ppm). Magnetic sector and ion trap analyzers provide the highest mass accuracy followed by TOF and quadrupole provide lowest mass accuracy.

$$\text{Mass accuracy (ppm)} = \frac{\text{Observed mass}}{(\text{theoretical mass} - \text{observed mass})} \times 10^6$$

1.1.6 Detectors

Ultimately ions reach the detector once they leave mass analyzer except in the case of an ion cyclotron mass analyzer. Many types of detectors are available but now days most commonly used detectors are faraday cup and electron multiplier. Table 1.2 lists commonly used detector in mass spectrometer.

Table 1.3: *Description of various detectors used in mass spectrometry*

Detector	**Mechanism**
Faraday cup	Captured ion transfer charge to cup and emitted electron are captured by cup
Electron multiplier	Secondary ion generated by ion beam amplified by series of dynode
Photomulitplier	Ion beam generate secondary electrons, which generate photons and photons get multiplied
Multichannel plate electron multiplier	Multiple channel (~10 μm) in the plate multiply the electron emitted by incoming ions
Cryogenic detector	Excitation of photons at superconducting film

1.2 Matrix assisted laser desorption ionization (MALDI)

For a very long period ionization in mass spectrometry was dominant by hard ionization techniques such as electron ionization (EI) and chemical ionization (CI). Their applications were limited to the analysis of volatile and low molecular weight compounds until the development of soft ionization techniques. Soft ionization implies the transfer of little internal energy to the analyte during ionization. Development of soft ionization techniques made possible the ionization of non-volatile and molecules of molecular weight up to 10,000 Da.[27, 28] Plasma desorption and field desorption were preliminary soft ionization but very soon plasma desorption was replaced by fast atom bombardment because of use of radioactive material whereas field desorption was limited to analysis of mainly non-polar compounds such as hydrocarbons.[13] Fast atom bombardment showed the capability of ionization of peptide and other non-volatile molecules.[29,30] Furthermore coupling of chromatographic platform such as HPLC was possible with FAB.[31] Later on development of electrospray ionization and laser desorption ionization became more

popular technique over FAB.[32] ESI and MALDI both have revolutionized the application of mass spectrometry in biomolecule analysis.[33, 34]

In last decade MALDI has been successfully used for the ionization of biomolecules.[35, 36] With continuous development in electronics and hardware, now MALDI has become one of the leading ionization methods for biomolecule as well as small molecules and metabolomics.[37-39] Started in late 1960, laser desorption ionization (LDI) techniques were not very popular until late 1980's. It was the efforts of Koichi Tanaka (Shiamdzu corp, Japan), who invented the LDI by using admixture of ultrafine cobalt powder to the analytes dissolved in glycerol.[28] Koichi Tanaka was awarded Noble prize in year 2002 with John Fenn (Virginia Commonwealth University, Richnond) who invented the ESI.[17, 40] But independently Michael Karas and Franz Hillenkamp (Germany) developed the LDI using UV absorbing organic molecules which was the far superior and versatile for the analysis of biomolecule.[27, 41, 42] They termed it as 'matrix assisted laser desorption ionization' (MALDI).

Laser used in MALDI

Gas laser	
N_2	337 nm
CO_2	10.6 µm
Excimer laser	
XeCl	308 nm
KrF	248 nm
ArF	193 nm
Solid state laser	
Nd:YAG µ3	355 nm
Nd:YAG µ4	266nm
Er:YAG	2.94 µm

1.2.1 Principle of matrix assisted laser desorption ionization mass spectrometry

In MALDI, analyte of interest is mixed with molar excess of matrix which are mainly organic acids such as 2,5-dihydroxy benzoic acid (DHB). This mixture is spotted on the MALDI target plate, which is primarily made of stainless steel or gold and dried under air. The samples are then introduced into the ionization chamber where a beam of laser strikes the dried spot. Matrix absorbs the UV radiation upon irradiation of laser (e.g. λ= 337 or 355) and dissipate the energy to the analyte which help in

desorption and ionization of molecule. The entire process involves the transition of analytes from solid phase to gas phase.[4, 6, 43]

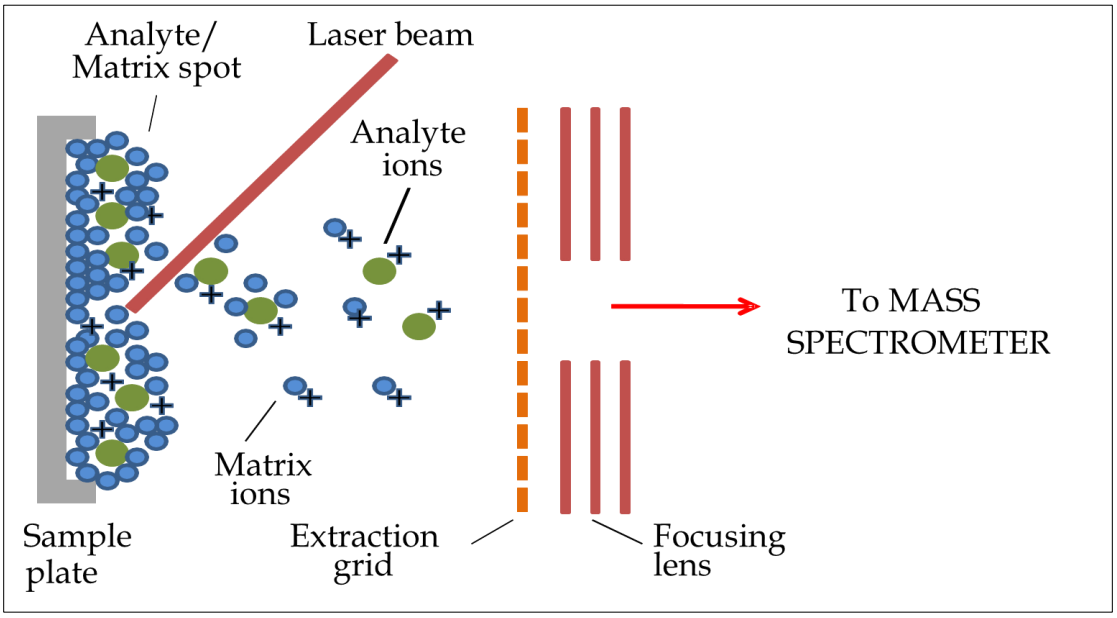


Figure 1.4: *Ionization process in MALDI*

According to the article on ion formation in MALDI by Knochenmuss, ionization in MALDI is two-step process. [44] First step or primary step is ion formation and second step is desorption/ablation of secondary ions. Two different explanations of the mechanism of primary ion formation in LDI have been proposed: (1) cluster model,[45] which states the matrix only behaves as desorption/ablation vehicle whereas (2) photoexcitation model[46] mentions the pooling of excitation states of two neighboring molecules giving rise to primary ions. Other than these two popular approaches a few other mechanism theories also have been discussed. Direct multi-photon ionization of matrix-analyte complex model, excited state proton transfer model, polar fluid model, pneumatic assistance model were also suggested as possible mechanisms for primary ion formation.[44] Mechanism for the second step (desorption) is widely understood due to the contribution of local thermal equilibrium in the plume for the generation of secondary ions.[47]

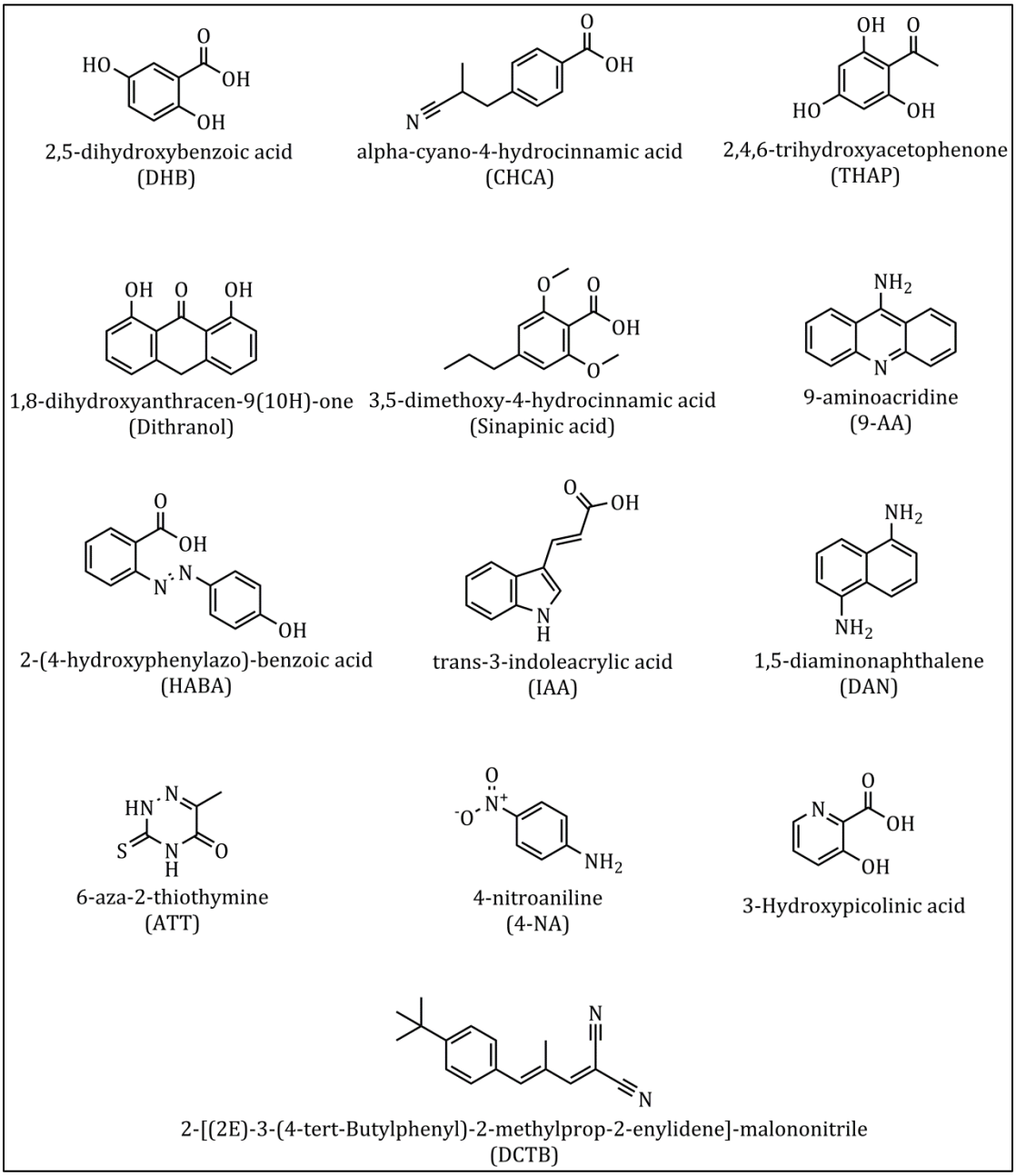


Figure 1.5: *Commonly uses organic matrices in MALDI MS.*

1.2.2 Matrix for ionization

Matrices in MALDI play the crucial role in ionization and desorption. Commonly used MALDI matrices are crystalline solids of low vapor pressure. Selection of MALDI matrix depends upon the type of laser, chemical structure of molecule and the mass range.[43,48-50] For UV-MALDI, matrix must contain the chromophore for the strong absorption of UV radiation and co-crystallize with sample. Organic acid with

aromatic ring are the most preferred matrices. In IR-MALDI laser radiation is absorbed by stretch vibration of O-H, N-H, C-O and O-H bending vibration.[51-55] Figure 1.5 shows some of the commonly used MALDI matrices.

1.3 Time-of-flight (TOF) mass analyzer

The ions in the MALDI process are formed in pulse and time-of-flight mass analyzers are best suited for the separation of such ions. TOF analyzer was developed in late 1946 by William Stephens but first commercial TOF analyzer was produced in 1957 after the significant improvement in the resolution.[56] But its popularity waned in 1960's due to the versatile magnetic sector and quadrupole mass analyzers. Development of laser desorption ionization in late 1990's and technology improvement brought in renaissance of TOF analyzer.[57-62]

1.3.1 Principle of time-of-flight mass analyzer

Time-of-flight mass analyzer differs from other mass analyzers as TOF separates the ions as a function of time as compared to spatial separation in magnetic sector and the quadrupole. TOF analyzer measures the *m/z* based on the time taken by an ion to reach the detector from the ion source. Ions pass through a flight tube of approximately 1-2 meters in length, which is kept under very high vacuum and has no electric or magnetic field. This region is called as 'field-free region'. According to the principle of time-of-flight, the time taken by an ion from one end to another end of flight tube is directly proportional to the mass-to-charge ratio of the ion. If the ions of different *m/z* are dispersed in flight tube, provided they have same kinetic energy, the lighter ion (small *m/z*) will arrive at detector first; heavier ions (larger *m/z*) arrive later.[59, 63, 64]

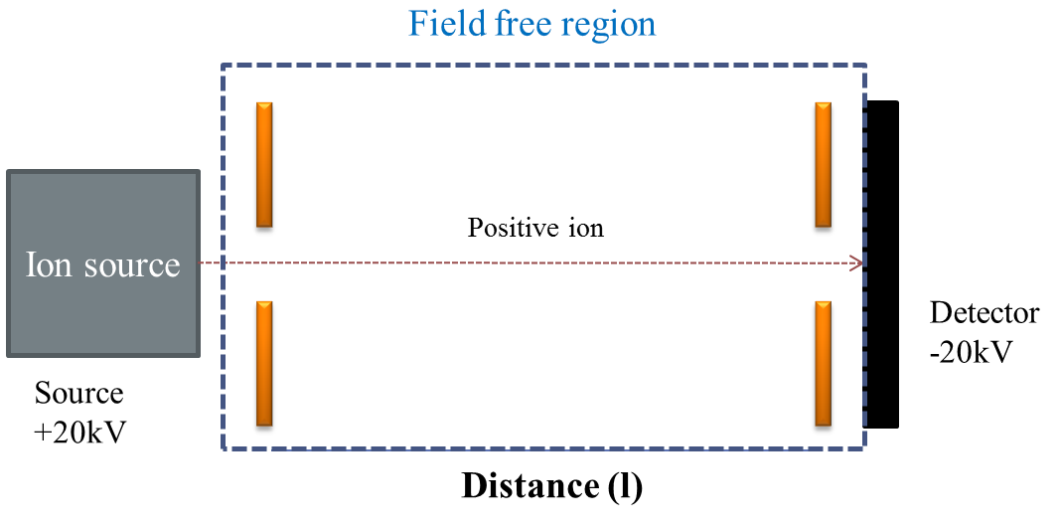


Figure 1.6: *Schematic of linear TOF analyzer.*

Ions formed in the ion source are pushed into flight tube as a pulse using accelerating voltage (V) applied on the grid. Considering the ions have mass *m* and charge *z* and linear velocity *v* then their kinetic energy is given by eq. 1.

$$\text{K. E.} = \frac{1}{2}mv^2 = zeV \qquad \text{--- eq. 1}$$

If the length of flight tube is *l* and *t* is the time taken by ions to pass the flight tube then drift time can be calculated as

$$t = l/v \qquad \text{--- eq. 2}$$

Substituting eq. 1 into eq. 2

$$t^2 = \frac{m}{z}\left(\frac{l^2}{2eV}\right) \qquad \text{--- eq. 3}$$

Here in the eq. 3 the length of flight tube and accelerating voltage are fixed hence the time taken by an ion to reach the detector is directly dependent on the mass-to-charge ratio.

$$t^2 = \frac{m}{z}\left(\frac{l^2}{2eV}\right)$$

$$t^2 = \frac{(1.5\text{m})^2}{2(15000\text{V})} \frac{(1000\text{Da})(1.672\text{X}10-27\ \text{kg Da}^{-1})}{1.602\text{X}10-19\text{C}}$$

m = 1000 Da
e= 1
V= 20000V
l= 1.5m

$$t = 2.792\text{X}10^{-5}\ \text{s} \quad (28\mu\text{s})$$

Figure 1.7: *Example of calculation of drift time in TOF analyzer using equation 3.*

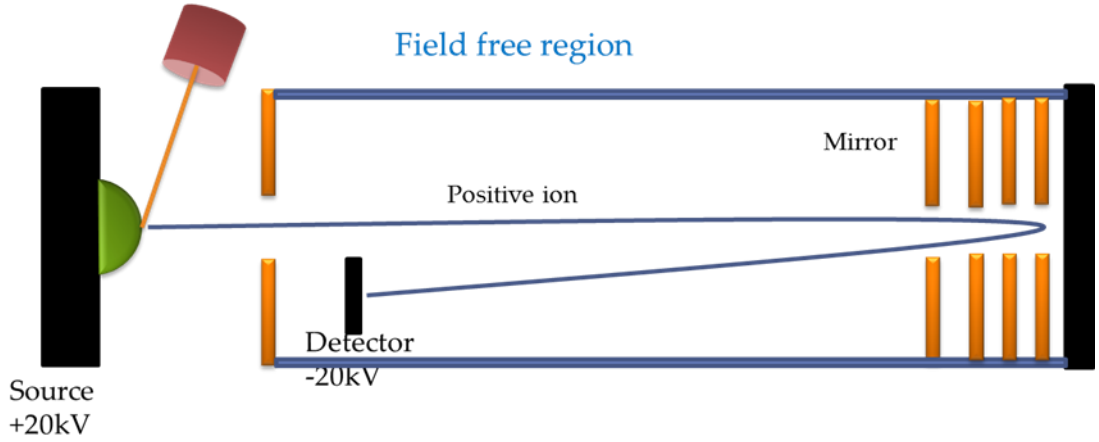


Figure 1.8: *Schematic of reflector TOF analyzer.*

A simple TOF analyzer can be a single flight tube with approximate length of 1 m. This simple configuration is called linear TOF. In linear TOF, at one end voltage grid is fixed to accelerate the ion and the detector is kept at opposite end of the flight tube. The drift path is equal to the length of flight tube. Due to the short distance, high molecular weight molecule can be easily detected without much loss. But the drift path in linear TOF is not suitable for differentiating ions of very close *m/z*. Practically not all the ions generated in ion source receive same initial kinetic energy, which results in poor resolution and inaccuracy of drift time. To overcome this problem, the length of drift path was increased using an additional flight tube and reflector mirror at one end.[65] The reflector mirror is the set of grids in increasing potential. As the ion

hits the potential mirror, it gets reflected due to repulsion in opposite direction of end of second flight tube and finally arrives at the detector (figure 1.8). This provides the extra drift path, accounts for any surface inhomogenity and also compensates the difference in kinetic energy resulting in an improvement in the resolution and mass accuracy.[66,67]

1.3.2 Mass calibration in TOF analyzer

TOF analyzer records the flight time or drift time of an ion, which is converted to *m/z* using the equation mentioned above. For a given set of parameters the drift time of a particular *m/z* remains constant but it gets changed as soon as there is any deviation in the parameter(s). These parameters include acceleration voltage, spatial distribution of ions, temperature and other electronic parameters. Accurate mass value can be obtained by accurate flight time. To get the reproducible mass accuracies, TOF needs to be calibrated after certain time duration. In the calibration, a solution of known compounds whose masses are known is analyzed and flight time is recorded for each mass. For these values a linear relationship between mass and flight time is established and slope and intercept are calculated. The slope of the equation is then used for the determining the mass of unknown compound. The parameters can be adjusted if necessary to obtain the desired value calibrating the TOF

1.3.3 Detector for TOF anlayzer

TOF analyzer transmits the ions at very high speed and several ions arrive together at the detector in a single event. Also in TOF, ions can be from a very small *m/z* to very high *m/z* thus it require the detector with capability of receiving large number of ions together with high dynamic range.[68] Micro-channel plates (MCP) and electron multiplier are the two detectors used with TOF analyzer.[69-71] Both the detectors are very sensitive as they can detect even single ion because of achieving gains in the

range of 10^5-10^7. One of the limitations for these detectors is saturation at very high ion flux. In MALDI often certain spots (sweet spot) give very high flux of ions saturate the detector, which results in peak broadening, low resolution and low mass accuracy.[68,72]

1.3.4 Quadrupole orthogonal TOF (Q-TOF) with MALDI

First hybrid TOF was built by combining a quadrupole and TOF analyzer which had capability of MS and tandem MS. [73] In this configuration, a quadrupole is placed after the ion source and beam of ion enters in a direction perpendicular to TOF axis. In Synapt QTOF G1 used in this work (Fig 1.9), three quadarupoles q0, Q1 and q2 are aligned with ion source followed by orthogonal TOF tube. During single MS stage, quadrupole q0, Q1 and q2 operate in rf-only mode and transmit the ions in TOF whereas in MS/MS mode Q1 act as quadrupole mass filter and q2 act as collisional cell.[62,73,74] Ions generated in MALDI source enters into quadrupole q0 which converts the pulsed ion beam in quasi-continuous ion beam by collisional cooling. The collisional cooling reduces the radial and axial velocity distribution for quadrupole mass filter Q1. Unlike axial TOF, ions in orthogonal TOF do not enter directly. Instead a pusher directs the beam of incoming ions in TOF tube. A voltage pulse of 800-1000V applied to a pusher plate and with combination of energy imparted by pusher give extra kinetic energy to ions to reach detector.[58]

There are several advantages of Q-TOF over conventional TOF analyzer. Since the pulse of ion can be directed into TOF at the rate of several kHz, thousands of spectra can be recorded per second. On summation of large number of spectra, improved signal-to-noise ratio is obtained. Low spread in initial velocity in the TOF direction improves the mass resolving power thus orthogonal TOF show higher resolution and mass accuracy compare to conventional TOF.[58,62,74,75]

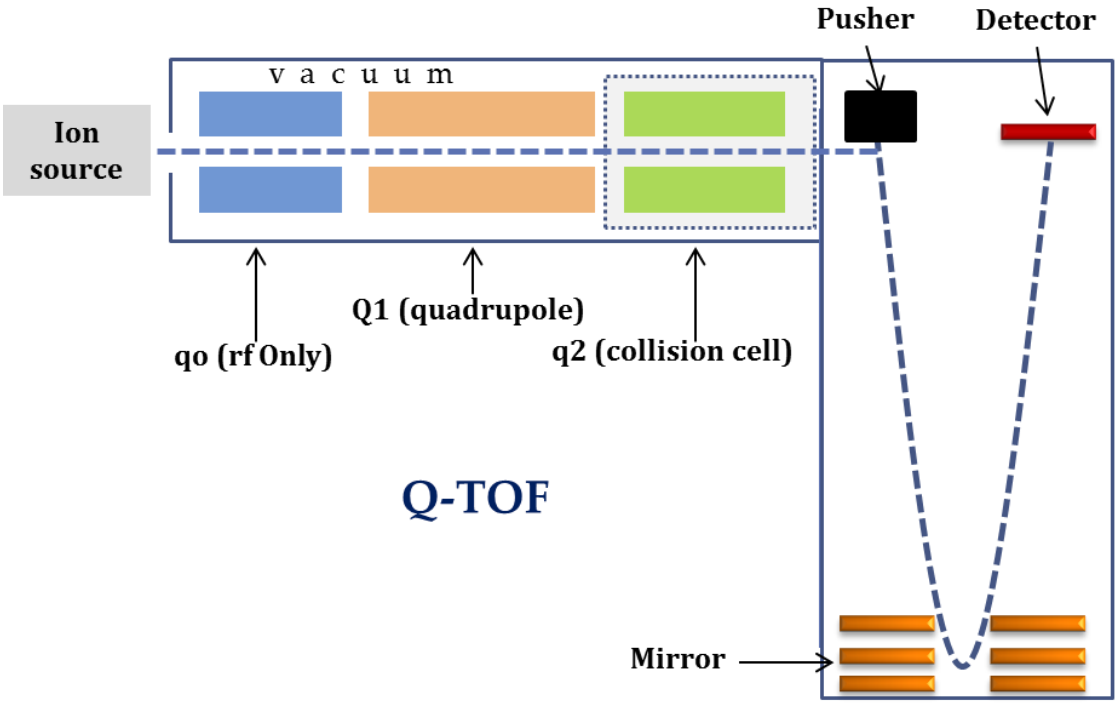


Figure 1.9: *Schematic of orthogonal Q-TOF analyzer.*

1.3.5 Advantages and disadvantages of TOF

TOF has several advantages over other mass analyzer.[24,59,60,63,68,76,77] One of the biggest advantages of TOF is that it records entire mass spectrum in single event of microsecond order. Thus several mass spectra can be recorded in very short time. Ions generated in very short pulses from pulsed ion sources such as MALDI can be easily analyzed with TOF. This high speed acquisition reduces the time of analysis compared to quadrupole mass filter where it take typically few seconds to record a mass spectrum. Since all the ions produced during ionization are recorded for each mass spectrum, the sensitivity for TOF analyzer is very high compared to scanning mass analyzer except ion traps.[78,79] Also the high transmission of ion because of no slit at both end, make it suitable for high throughput analysis. Another advantage of TOF analyzer is the theoretically unlimited mass range for ion detection although there are some practical limitation such as low resolution and detection sensitivity at

very high mass range. Other scanning instruments have fundamental limitations mass range and transmission due to the electric, magnetic and rf fields.[6,24,68]

Several characteristics of TOF make it a superior technology for the ion separation in mass spectrometry but it has several limitations. Variation in mass accuracy over the certain time period is one of the challenges in TOF analyzer. The ion velocity in the flight tube is directly affected by the surrounding temperature. A little variation in surrounding temperature of flight tube shows a large deviation in drift time. Hence TOF analyzer requires very stable temperature and frequent mass calibration. Furthermore in TOF all the ions generated in a pulse reach the detector in very short duration; high abundance of ions saturates the detector which results in loss of mass resolution and mass accuracy [22,26,63,80]

1.4 Advantages and disadvantages of MALDI-TOF MS

After incorporation in main stream mass spectrometry, initially application of MALDI was limited to analysis of only large molecular weight molecules but later on advancement in electronics and computation, its application has spawned to metabolomics and small molecule analysis [55, 81-83] Its simplicity and ease of operation definitely make it ionization technique of choice from small molecule to large molecular weight biomolecules. In all the available ionization technique in mass spectrometry, MALDI is most versatile ionization technique and has unmatched advantages over other contemporary techniques especially electrospray ionization (ESI). No chromatographic separation, production of mainly singly charged ion, low fragmentation, minimal effect of salts on ionization efficiency, simultaneous ionization of molecules of different chemical nature, very low sample requirement, rapid acquisition and high throughput analysis are the major advantages of MALDI-TOF MS.[84-88]

Despite many advantages of MALDI-TOF MS in few areas, it also has limitations. TOF transmit all the ions simultaneously and they reach the detector in very short time. Sometime very high abundance of few ions saturates the detectors which affect detection of very low abundance ions.[4,6,89]

1.5 Applications of MALDI-TOF MS

From small molecule to large molecules MALDI-TOF MS has shown its utility. As a qualitative technique it has been widely used in proteomics. However due to advantageous high throughput nature and minimal sample preparation, MALDI-TOF MS finds use in other areas as well. Table 1.4 summarizes several applications of MALDI-TOF MS.

Category	Application	Description	References
Large molecules	Proteomics	Protein sequencing, biomarker discovery, post translational modification (PTM), non-covalent protein interaction,	35, 36, 90-102
	Genomics	DNA, RNA analysis	103-112
	Glycomics	Analysis of glycans isolated from tissues, cells and organs	113-116
	Intact cell analysis	Biotyping and fingerprinting of a cells	117-121
	Histology	imaging of protein, peptides in the cells	92, 122-128
	Polymer analysis	Molecular weight determination, structural analysis of polymers, degradation studies	129, 130
	Biopharmaceuticals	Characterization of biosimilar drugs	131-134
	Clinical microbiology	Identification of	135-141

		microorganism	
Small molecule	Metabolomics	Comprehensive characterization of the small molecule metabolites in biological systems	142-147
	Food analysis	Analysis of contaminants, adulterants, ingredient,	87, 148-166
	Carbohydrate analysis	Oligosaccharide and polysaccharide analysis	167-175
	Lipid analysis	Analysis of fatty acids, triglyceride etc.	176-186
	Toxins detection	Analysis of endogenous and exogenous toxic molecules	187-190
	Drug analysis	Drug molecule characterization, quantitative analysis	191-200
	Environmental analysis	Detection of pollutants, contaminants in water and soil	201-205
	Forensic analysis	Detection of possible evidences, prohibited substance in sports etc.	206-210
	Inorganic complex analysis	Complex formation analysis	211-215
	Imaging	Profiling of small molecules in the cells/tissue	216-221
	Adhesive and surfactant analysis	Characterization of constituents	222, 223

1.6 Method development in MALDI-TOF MS

MALDI-TOF MS is very user friendly and easy to understand mass spectrometric technique. Compared to LC-ESI-MS where several parameters need to be optimized

for different classes of molecules, very few parameters are required to operate and record the mass spectrum in MALDI-TOF MS. Crucial parameters for obtaining best results using MALDI includes optimization of laser energy and acquisition time.[224, 225] Laser energy depends upon type of MALDI matrix used. For example, 2,5-dihydroxybenzoic acid (DHB) requires higher laser energy compared to alpha-cyano-4-hydroxycinnamic acid (CHCA) provided that both the matrices are mixed with same analyte. Similarly acquisition time depends upon sensitivity of analyte towards ionization and co-crystallization with matrix. Every MALDI matrix shows a distinct crystallization pattern after drying. Crystallization behavior varies with type of matrix.[226]

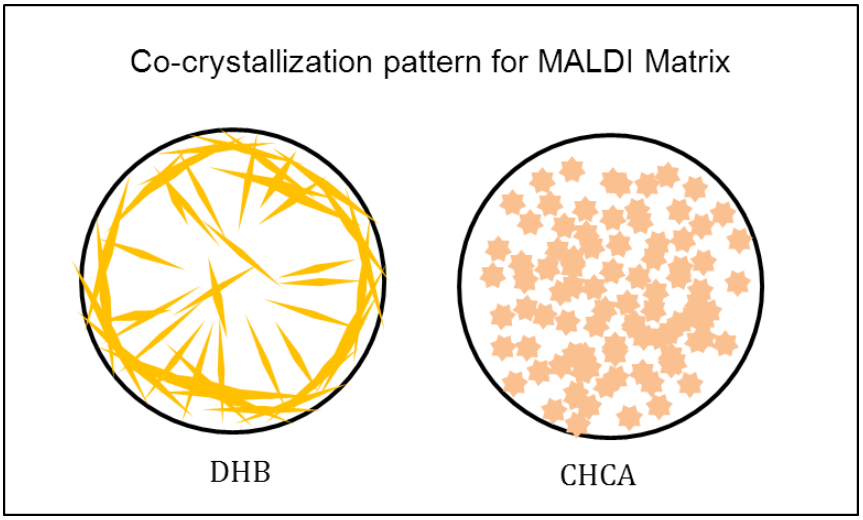


Figure 1.10: *Schematic of co-crystallization pattern for MALDI matrix after drying.*

1.7 Quantitative analysis of small molecules by MALDI-TOF MS

Because of inherent irreproducibility in data from spot-to-spot, it has been a topic of debate whether MALDI-TOF MS is suitable for small molecule analysis especially in the quantitative analysis or not. In classical mass spectrometry where sample is introduced in mass spectrometer through direct injection or spray mechanism, the reproducibility of peak is very high considering that the sample volume is completely

ionized. The concentration of compound can be known directly from the peak area or peak intensity. Whereas in MALDI-TOF MS spot-to-spot variation in co-crystallization lead to irreproducibility in signal responses which limits the use of MALDI-TOF MS as quantitative analysis without normalization of data.[227-229]

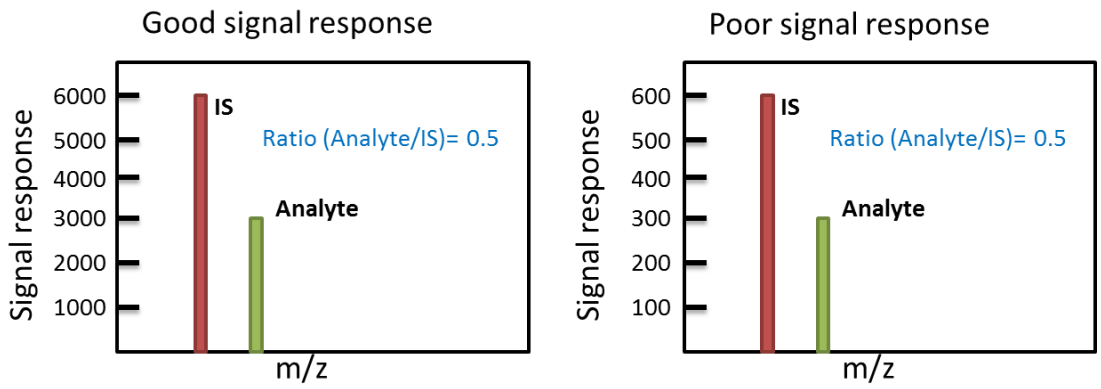


Figure 1.11: *diagram showing importance of internal standard in two cases of signal response in MALDI-TOF MS.*

To overcome the problem of irreproducibility and performing the quantitative analysis by MALDI-TOF MS, Internal standards have been used successfully.[226, 228, 230, 231] Internal standard are the molecules of known quantity which are added to sample. During the ionization process the internal standards undergoes similar experience along with the sample and compensate the variation in signal responses. The ratio of peak area of internal standard and analyte is calculated which remain constant for particular concentration of analyte and instrumental parameter. The example is illustrated with the figure, where two cases are demonstrated; in one case the signal response is very good but in another case due to poor crystallization there is poor signal response, but the ratio of responses from analyte and internal standard remain same in both cases (figure 1.11). If the concentration of internal standard is kept constant then the ratio of signal responses of internal standard and analytes changes with change in concentration of analyte. This concept of internal standard is used

during quantitative analysis of analyte using MALDI-TOF MS. A concentration-response curve or calibration plot is generated by plotting the ratio of signal responses on y-axis and varying concentration of analyte on x-axis.[226, 228, 230-233]

1.8 References

(1) Griffiths, J. *Analytical Chemistry* **2008**, *80*, 5678-5683.

(2) Svec, H. J. *International Journal of Mass Spectrometry and Ion Processes* **1985**, *66*, 3-29.

(3) Beynon, J. H.; Morgan, R. P. *International Journal of Mass Spectrometry and Ion Physics* **1978**, *27*, 1-30.

(4) Vestal, M. L. *Chemical Reviews* **2001**, *101*, 361-375.

(5) *http://masspec.scripps.edu/mshistory/whatisms_details.php#references* **2014**.

(6) Gross, J. H. *Mass Spectrometry - A Textbook 2nd ed*, 2 ed.; Springer, 2011.

(7) Field, F. H. *Accounts of Chemical Research* **1968**, *1*, 42-49.

(8) Munson, M. S. B.; Field, F. H. *Journal of the American Chemical Society* **1966**, *88*, 2621-2630.

(9) Munson, B. *International Journal of Mass Spectrometry* **2000**, *200*, 243-251.

(10) Beckey, H. D. *Journal of Physics E-Scientific Instruments* **1979**, *12*, 72-83.

(11) Reynolds, W. D. *Analytical Chemistry* **1979**, *51*, A283-&.

(12) Przybylski, M. *Fresenius Zeitschrift Fur Analytische Chemie* **1983**, *315*, 402-421.

(13) Vandergreef, J.; Debrauw, M. C. T. *International Journal of Mass Spectrometry and Ion Processes* **1983**, *46*, 379-382.

(14) Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. *Journal of the Chemical Society-Chemical Communications* **1981**, 325-327.

(15) Macfarlane, R. D. *Biomedical Mass Spectrometry* **1981**, *8*, 449-453.

(16) Sundqvist, B.; Macfarlane, R. D. *Mass Spectrometry Reviews* **1985**, *4*, 421-460.

(17) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Mass Spectrometry Reviews* **1990**, *9*, 37-70.

(18) Gaskell, S. J. *Journal of Mass Spectrometry* **1997**, *32*, 677-688.

(19) Delamora, J. F. *Mass Spectrometry Reviews* **1992**, *11*, 431-434.

(20) Fenn, J. B. *Journal of the American Society for Mass Spectrometry* **1993**, *4*, 524-535.

(21) McLuckey, S. A.; Wells, J. M. *Chemical Reviews* **2001**, *101*, 571-606.

(22) Jennings, K. R.; Dolnikowski, G. G. *Methods in Enzymology* **1990**, *193*, 37-61.

(23) Dawson, P. H. *Mass Spectrometry Reviews* **1986**, *5*, 1-37.

(24) El-Aneed, A.; Cohen, A.; Banoub, J. *Applied Spectroscopy Reviews* **2009**, *44*, 210-230.

(25) Siuzdak, G. In *Mass Spectrometry for Biotechnology*; Academic Press: San Diego, 1996, pp 4-31.

(26) Wollnik, H. *Mass Spectrometry Reviews* **1993**, *12*, 89-114.

(27) Karas, M.; Hillenkamp, F. *Analytical Chemistry* **1988**, *60*, 2299-2301.

(28) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T.; Matsuo, T. *Rapid Communications in Mass Spectrometry* **1988**, *2*, 151-153.

(29) Bell, D. J.; Brightwell, M. D.; Haran, M.; Neville, W. A.; West, A. *Organic Mass Spectrometry* **1991**, *26*, 454-457.

(30) Compagnini, A.; Fisichella, S.; Foti, S.; Saletti, R.; Sardo, L. *Rapid Communications in Mass Spectrometry* **1994**, *8*, 459-464.

(31) Sato, K.; Kumazawa, T.; Katsumata, Y. *Journal of Chromatography A* **1994**, *674*, 127-145.

(32) Briand, G.; Fontaine, M.; Schubert, R.; Ricart, G.; Degand, P.; Vamecq, J. *Journal of Mass Spectrometry* **1995**, *30*, 1731-1741.

(33) Doerr, A. *Nat Meth* **2007**, *4*, 781-781.

(34) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198-207.

(35) Hillenkamp, F. *Molecular & Cellular Proteomics* **2005**, *4*, S7-S7.

(36) Vestal, M.; Hayden, K. *International Journal of Mass Spectrometry* **2007**, *268*, 83-92.

(37) Doroshenko, V.; Cotter, R. J. *Abstracts of Papers of the American Chemical Society* **1994**, *208*, 135-ANYL.

(38) Bornsen, K. O. *Analytical Methods and Instrumentation* **1995**, *2*, 202-205.

(39) van Kampen, J. J. A.; Burgers, P. C.; de Groot, R.; Gruters, R. A.; Luider, T. M. *Mass Spectrometry Reviews* **2011**, *30*, 101-120.

(40) Robinson, C. V. *Nature*, *469*, 300-300.

(41) Karas, M.; Bahr, U.; Hillenkamp, F. *International Journal of Mass Spectrometry and Ion Processes* **1989**, *92*, 231-242.

(42) Hillenkamp, F.; Karas, M. *International Journal of Mass Spectrometry* **2000**, *200*, 71-77.

(43) Karas, M.; Bahr, U.; Giessmann, U. *Mass Spectrometry Reviews* **1991**, *10*, 335-357.

(44) Knochenmuss, R. *Analyst* **2006**, *131*, 966-986.

(45) Karas, M.; Gluckmann, M.; Schafer, J. *Journal of Mass Spectrometry* **2000**, *35*, 1-12.

(46) Ehring, H.; Karas, M.; Hillenkamp, F. *Organic Mass Spectrometry* **1992**, *27*, 472-480.

(47) Zenobi, R.; Knochenmuss, R. *Mass Spectrometry Reviews* **1998**, *17*, 337-366.

(48) Strupat, K.; Karas, M.; Hillenkamp, F. *International Journal of Mass Spectrometry and Ion Processes* **1991**, *111*, 89-102.

(49) Bourcier, S.; Bouchonnet, S.; Hoppilliard, Y. *International Journal of Mass Spectrometry* **2001**, *210*, 59-69.

(50) Knochenmuss, R. *Analyst* **2014**, *139*, 147-156.

(51) Berkenkamp, S.; Kirpekar, F.; Hillenkamp, F. *Science* **1998**, *281*, 260-262.

(52) Niu, S.; Zhang, W.; Chait, B. T. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1-7.

(53) Zhang, W.; Niu, S.; Chait, B. T. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 879-884.

(54) Macha, S. F.; Limbach, P. A.; Savickas, P. J. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 731-737.

(55) Bergman, N.; Shevchenko, D.; Bergquist, J. *Analytical and Bioanalytical Chemistry* **2014**, *406*, 49-61.

(56) Wiley, W. C.; McLaren, I. H. *Review of Scientific Instruments* **1955**, *26*, 1150-1157.

(57) Price, D.; Milnes, G. J. *International Journal of Mass Spectrometry and Ion Processes* **1990**, *99*, 1-39.

(58) Coles, J.; Guilhaus, M. *Trac-Trends in Analytical Chemistry* **1993**, *12*, 203-213.

(59) Guilhaus, M. *Journal of Mass Spectrometry* **1995**, *30*, 1519-1532.

(60) Guilhaus, M. In *Advances in Mass Spectrometry, Vol 13*, 1995; Vol. 13, pp 213-226.

(61) Guilhaus, M.; Mlynski, V.; Selby, D. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 951-962.

(62) Guilhaus, M.; Selby, D.; Mlynski, V. *Mass Spectrometry Reviews* **2000**, *19*, 65-107.

(63) Weickhardt, C.; Moritz, F.; Grotemeyer, J. *Mass Spectrometry Reviews* **1996**, *15*, 139-162.

(64) Cotter, R. J. In *Time-of-Flight Mass Spectrometry*, 1994; Vol. 549, pp 16-48.

(65) Mamyrin, B. A.; Karataev, V. I.; Shmikk, D. V.; Zagulin, V. A. *Journal of Experimental and Theoretical Physics* **1973**, *37*, 45-48.

(66) Boesl, U.; Weinkauf, R.; Schlag, E. W. *International Journal of Mass Spectrometry and Ion Processes* **1992**, *112*, 121-166.

(67) Chen, C. C.; Su, C. S. *International Journal of Mass Spectrometry and Ion Processes* **1993**, *124*, 37-43.

(68) Siuzdak, G. In *Mass Spectrometry for Biotechnology*; Academic Press: San Diego, 1996, pp 32-55.

(69) Laprade, B. N.; Labich, R. J. *Spectroscopy* **1994**, *9*, 26-&.

(70) Meier, K.; Seibl, J. *Journal of Physics E-Scientific Instruments* **1973**, *6*, 133-135.

(71) Wiza, J. L. *Nuclear Instruments & Methods* **1979**, *162*, 587-601.

(72) Westman, A.; Brinkmalm, G.; Barofsky, D. F. *International Journal of Mass Spectrometry* **1997**, *169*, 79-87.

(73) Chernushevich, I. V.; Loboda, A. V.; Thomson, B. A. *Journal of Mass Spectrometry* **2001**, *36*, 849-865.

(74) Selby, D. S.; Mlynski, V.; Guilhaus, M. *International Journal of Mass Spectrometry* **2001**, *210*, 89-100.

(75) Ens, W.; Standing, K. G. In *Biological Mass Spectrometry*, 2005; Vol. 402, pp 49-78.

(76) Shevchenko, A.; Loboda, A.; Ens, W.; Standing, K. G. *Analytical Chemistry* **2000**, *72*, 2132-2141.

(77) Person, M. D.; Lo, H. H.; Towndrow, K. M.; Jia, Z.; Monks, T. J.; Lau, S. S. *Chemical Research in Toxicology* **2003**, *16*, 757-767.

(78) Rousu, T.; Herttuainen, J.; Tolonen, A. *Rapid Communications in Mass Spectrometry* **2010**, *24*, 939-957.

(79) Thurman, E. M.; Ferrer, I. In *Liquid Chromatography/Mass Spectrometry, Ms/Ms and Time-of-Flight Ms*, 2003; Vol. 850, pp 14-31.

(80) Laures, A. M. F.; Wolff, J. C.; Eckers, C.; Borman, P. J.; Chatfield, M. J. *Rapid Communications in Mass Spectrometry* **2007**, *21*, 529-535.

(81) Zubritsky, E. *Analytical Chemistry* **1998**, *70*, 733A-737A.

(82) Macfarlane, R. D. *Brazilian Journal of Physics* **1999**, *29*, 415-421.

(83) Shrestha, B.; Li, Y.; Vertes, A. *Metabolomics* **2008**, *4*, 297-311.

(84) Volmer, D. A.; Sleno, L.; Bateman, K.; Sturino, C.; Oballa, R.; Mauriala, T.; Corr, J. *Analytical Chemistry* **2007**, *79*, 9000-9006.

(85) Markus, P.; Michael, K. *Rapid Communications in Mass Spectrometry* **2009**, *23*, 3555-3562.

(86) Yukihira, D.; Miura, D.; Saito, K.; Takahashi, K.; Wariishi, H. *Analytical Chemistry* **2010**, *82*, 4278-4282.

(87) Singh, A.; Panchagnula, V. *Analytical Methods* **2011**, *3*, 2360-2366.

(88) Singh, A.; Bhattacharya, N.; Ghanate, G.; Panchagnula, P. *Lc Gc North America, Current Trends in Mass Spectrometry* **2013**, *march 2013*, 24-29.

(89) Vorm, O.; Roepstorff, P.; Mann, M. *Analytical Chemistry* **1994**, *66*, 3281-3287.

(90) Albalat, A.; Stalmach, A.; Bitsika, V.; Siwy, J.; Schanstra, J. P.; Petropoulos, A. D.; Vlahou, A.; Jankowski, J.; Persson, F.; Rossing, P.; Jaskolla, T. W.; Mischak, H.; Husi, H. *Proteomics* **2013**, *13*, 2967-2975.

(91) Albrethsen, J. *Journal of Proteomics* **2011**, *74*, 765-773.

(92) Minerva, L.; Ceulemans, A.; Baggerman, G.; Arckens, L. *Proteomics Clinical Applications* **2012**, *6*, 581-595.

(93) Zhang, Y.; Li, L. L.; Yang, P. Y.; Lu, H. J. *Analytical Methods* **2012**, *4*, 2622-2631.

(94) She, Y. M.; Ji, Y. P. *Chemical Journal of Chinese Universities-Chinese* **1999**, *20*, 852-855.

(95) Acklool, S.; Ziegert, T.; Lobodal, A.; Sapp, L.; Lynch, C.; Kuzdzal, S. *Molecular & Cellular Proteomics* **2004**, *3*, S133-S133.

(96) Liu, Z. Y.; Schey, K. L. *Journal of the American Society for Mass Spectrometry* **2005**, *16*, 482-490.

(97) Openshaw, M.; Connolly, J. *Molecular & Cellular Proteomics* **2005**, *4*, S355-S355.

(98) Hajivandi, M.; Liang, X.; Leite, J.; Pope, M. *Molecular & Cellular Proteomics* **2006**, *5*, S298-S298.

(99) Yanes, O.; Villanueva, J.; Querol, E.; Aviles, F. X. *Nature Protocols* **2007**, *2*, 119-130.

(100) Liu, Z. L.; Schey, K. L. *Journal of the American Society for Mass Spectrometry* **2008**, *19*, 231-238.

(101) Ebhardt, H. A.; Xu, Z. Z.; Fung, A. W.; Fahlman, R. P. *Analytical Chemistry* **2009**, *81*, 1937-1943.

(102) Lee, J.; Soper, S. A.; Murray, K. K. *Analytica Chimica Acta* **2009**, *649*, 180-190.

(103) Josten, M.; Oedenkoven, M.; Szekat, C.; Alsabti, N.; Roemer, T.; Kostrzewa, M.; Rhode, H.; Sahl, H. G.; Bierbaum, G. *International Journal of Medical Microbiology* **2012**, *302*, 141-141.

(104) Zhong, X. Y.; Holzgreve, W. *Transfusion Medicine and Hemotherapy* **2009**, *36*, 263-272.

(105) Buchan, B. W.; Riebe, K. M.; Timke, M.; Kostrzewa, M.; Ledeboer, N. A. *American Journal of Clinical Pathology* **2014**, *141*, 25-34.

(106) Gao, X.; Tan, B. H.; Sugrue, R. J.; Tang, K. *Applications of Maldi-Tof Spectroscopy* **2013**, *331*, 55-77.

(107) Lecchi, P.; Pannell, L. K. In *New Methods for the Study of Biomolecular Complexes*, 1998; Vol. 510, pp 217-224.

(108) Gut, I. G. *Human Mutation* **2004**, *23*, 437-441.

(109) Jurinke, C.; Oeth, P.; van den Boom, D. *Molecular Biotechnology* **2004**, *26*, 147-163.

(110) Tost, J.; Gut, I. G. *Clinical Biochemistry* **2005**, *38*, 335-350.

(111) Douthwaite, S.; Kirpekar, F. In *Rna Modification*, 2007; Vol. 425, pp 3-20.

(112) Vogel, N.; Schiebel, K.; Humeny, A. *Transfusion Medicine and Hemotherapy* **2009**, *36*, 253-262.

(113) Huang, H. H. *Australian Journal of Chemistry* **2003**, *56*, 65-65.

(114) Nishimura, S. K.; Kurogochi, M. *Abstracts of Papers of the American Chemical Society* **2004**, *228*, U230-U230.

(115) Nishimura, S. I.; Niikura, K.; Kurogochi, M.; Matsushita, T.; Fumoto, M.; Hinou, H.; Kamitani, R.; Nakagawa, H.; Deguchi, K.; Miura, N.; Monde, K.; Kondo, H. *Angewandte Chemie-International Edition* **2005**, *44*, 91-96.

(116) Tissot, B.; Gasiunas, N.; Powell, A. K.; Ahmed, Y.; Zhi, Z. L.; Haslam, S. M.; Morris, H. R.; Turnbull, J. E.; Gallagher, J. T.; Dell, A. *Glycobiology* **2007**, *17*, 972-982.

(117) Dong, H. J.; Shen, W.; Cheung, M. T. W.; Liang, Y. M.; Cheung, H. Y.; Allmaier, G.; Au, O. K. C.; Lam, Y. W. *Analyst* **2011**, *136*, 5181-5189.

(118) Marchetti-Deschmann, M.; Winkler, W.; Dong, H. J.; Lohninger, H.; Kubicek, C. P.; Allmaier, G. *Food Technology and Biotechnology* **2012**, *50*, 334-342.

(119) Munteanu, B.; von Reitzenstein, C.; Hansch, G. M.; Meyer, B.; Hopf, C. *Analytical and Bioanalytical Chemistry* **2012**, *404*, 2277-2286.

(120) Pathak, K. V.; Keharia, H. *Journal of Applied Microbiology* **2013**, *114*, 1300-1310.

(121) Kulkarni, M.; Vinod, V. P.; Umashankar, P. K.; Patole, M.; Rao, M. *Molecular & Cellular Proteomics* **2006**, *5*, S62-S62.

(122) Gemoll, T.; Roblick, U. J.; Habermann, J. K. *Molecular Medicine Reports* **2011**, *4*, 1045-1051.

(123) Neubert, P.; Walch, A. *Expert Review of Proteomics* **2013**, *10*, 259-273.

(124) Schone, C.; Hofler, H.; Walch, A. *Clinical Biochemistry* **2013**, *46*, 539-545.

(125) Cornett, D. S.; Mobley, J. A.; Dias, E. C.; Andersson, M.; Arteaga, C. L.; Sanders, M. E.; Caprioli, R. M. *Molecular & Cellular Proteomics* **2006**, *5*, 1975-1983.

(126) Wisztorski, M.; Lemaire, R.; Stauber, J.; Menguellet, S. A.; Jardin-Mathe, O.; Day, R.; Salzet, M.; Fournier, I. *M S-Medecine Sciences* **2007**, *23*, 31-36.

(127) Fournier, I.; Wisztorski, M.; Salzet, M. *Expert Review of Proteomics* **2008**, *5*, 413-424.

(128) Franck, J.; Arafah, K.; Elayed, M.; Bonnel, D.; Vergara, D.; Jacquet, A.; Vinatier, D.; Wisztorski, M.; Day, R.; Fournier, I.; Salzet, M. *Molecular & Cellular Proteomics* **2009**, *8*, 2023-2033.

(129) Macha, S. F.; Limbach, P. A. *Current Opinion in Solid State & Materials Science* **2002**, *6*, 213-220.

(130) Montaudo, G.; Samperi, F.; Montaudo, M. S. *Progress in Polymer Science* **2006**, *31*, 277-357.

(131) Kroon, D. J.; Freedy, J.; Burinsky, D. J.; Sharma, B. *Journal of Pharmaceutical and Biomedical Analysis* **1995**, *13*, 1049-1054.

(132) Hansen, R.; Dickson, A. J.; Goodacre, R.; Stephens, G. M.; Sellick, C. A. *Biotechnology and Bioengineering* **2010**, *107*, 902-908.

(133) Kafka, A. P.; Kleffmann, T.; Rades, T.; McDowell, A. *International Journal of Pharmaceutics* **2011**, *417*, 70-82.

(134) Thuy, T. T.; Thorsen, G. *Journal of the American Society for Mass Spectrometry* **2013**, *24*, 1053-1063.

(135) Buyse, I. M.; Pace, R. P.; Lurix, P.; Roa, B. B. *American Journal of Human Genetics* **2002**, *71*, 532-532.

(136) Carbonnelle, E.; Mesquita, C.; Bille, E.; Day, N.; Dauphin, B.; Beretti, J. L.; Ferroni, A.; Gutmann, L.; Nassif, X. *Clinical Biochemistry* **2011**, *44*, 104-109.

(137) Croxatto, A.; Prod'hom, G.; Greub, G. *Fems Microbiology Reviews* **2012**, *36*, 380-407.

(138) DeMarco, M. L.; Ford, B. A. *Clinics in Laboratory Medicine* **2013**, *33*, 611-+.

(139) Legarraga, P.; Moraga, M.; Lam, M.; Geoffroy, E.; Zumaran, C.; Garcia, P. *Revista Chilena De Infectologia* **2013**, *30*, 140-146.

(140) Ng, E. W. Y.; Wong, M. Y. M.; Poon, T. C. W. In *Chemical Diagnostics: From Bench to Bedside*, 2013; Vol. 336, pp 139-175.

(141) Seng, P.; Rolain, J. M.; Fournier, P. E.; La Scola, B.; Drancourt, M.; Raoult, D. *Future Microbiology* **2010**, *5*, 1733-1754.

(142) Musshoff, F.; Arrey, T.; Strupat, K. *Drug Testing and Analysis* **2013**, *5*, 361-365.

(143) Naidu, N.; Munyu, S.; Yakubu, M. A. *Faseb Journal* **2013**, *27*.

(144) Shanta, S. R.; Kim, T. Y.; Hong, J. H.; Lee, J. H.; Shin, C. Y.; Kim, K. H.; Kim, Y. H.; Kim, S. K.; Kim, K. P. *Analyst* **2012**, *137*, 5757-5762.

(145) Yukihira, D.; Miura, D.; Saito, K.; Takahashi, K.; Wariishi, H. *Analytical Chemistry* **2010**, *82*, 4278-4282.

(146) Cornett, D. S.; Frappier, S. L.; Caprioli, R. M. *Analytical Chemistry* **2008**, *80*, 5648-5653.

(147) Shroff, R.; Svatos, A. *Analytical Chemistry* **2009**, *81*, 7954-7959.

(148) Sporns, P.; Abell, D. C. *Trends in Food Science & Technology* **1996**, *7*, 187-190.

(149) Wingerath, T.; Stahl, W.; Kirsch, D.; Kaufmann, R.; Sies, H. *Journal of Agricultural and Food Chemistry* **1996**, *44*, 2006-2013.

(150) Sporns, P.; Wang, J. *Food Research International* **1998**, *31*, 181-189.

(151) Weiss, K. C.; Yip, T. T.; Hutchens, T. W.; Bisson, L. F. *American Journal of Enology and Viticulture* **1998**, *49*, 231-239.

(152) Wang, J.; Sporns, P. *Journal of Agricultural and Food Chemistry* **1999**, *47*, 2009-2015.

(153) Wang, J.; Sporns, P.; Low, N. H. *Journal of Agricultural and Food Chemistry* **1999**, *47*, 1549-1557.

(154) Wang, J.; Sporns, P. *Journal of Agricultural and Food Chemistry* **2000**, *48*, 1657-1662.

(155) Reed, J. D.; Krueger, C. G.; Vestling, M. M. *Phytochemistry* **2005**, *66*, 2248-2263.

(156) Carpentieri, A.; Marino, G.; Amoresano, A. *Analytical and Bioanalytical Chemistry* **2007**, *389*, 969-982.

(157) Hager, T. J.; Howard, L. R.; Liyanage, R.; Lay, J. O.; Prior, R. L. *Journal of Agricultural and Food Chemistry* **2008**, *56*, 661-669.

(158) Williamson, R. A.; Dubay, G. R. *Abstracts of Papers of the American Chemical Society* **2009**, *237*.

(159) Fernandez-No, I. C.; Bohme, K.; Gallardo, J. M.; Barros-Velazquez, J.; Canas, B.; Calo-Mata, P. *Electrophoresis* **2010**, *31*, 1116-1127.

(160) Ivanova, V.; Dornyei, A.; Stefova, M.; Stafilov, T.; Vojnoski, B.; Kilar, F.; Mark, L. *Food Analytical Methods* **2011**, *4*, 108-115.

(161) Garcia, J. S.; Sanvido, G. B.; Saraiva, S. A.; Zacca, J. J.; Cosso, R. G.; Eberlin, M. N. *Food Chemistry* **2012**, *131*, 722-726.

(162) Tsilia, V.; Devreese, B.; de Baenst, I.; Mesuere, B.; Rajkovic, A.; Uyttendaele, M.; Van de Wiele, T.; Heyndrickx, M. *Analytical and Bioanalytical Chemistry* **2012**, *404*, 1691-1702.

(163) Calvano, C. D.; De Ceglie, C.; Aresta, A.; Facchini, L. A.; Zambonin, C. G. *Analytical and Bioanalytical Chemistry* **2013**, *405*, 1641-1649.

(164) Su, X.; Zhou, H. Y.; Chen, F. C.; Gao, B. X.; Liu, Z. W.; Zhang, Y. H.; Liu, F.; Li, Z. R.; Gao, Z. X. *International Journal of Mass Spectrometry* **2013**, *338*, 39-44.

(165) Wang, X.; Wang, S. J.; Cai, Z. W. *Trac-Trends in Analytical Chemistry* **2013**, *52*, 170-185.

(166) Ivanova, B.; Spiteller, M. *Ecotoxicology and Environmental Safety* **2014**, *100*, 233-241.

(167) Choi, S. S.; Lee, H. M.; Jang, S.; Shin, J. *International Journal of Mass Spectrometry* **2009**, *279*, 53-58.

(168) Fukuyama, Y.; Funakoshi, N.; Takeyama, K.; Hioki, Y.; Nishikaze, T.; Kaneshiro, K.; Kawabata, S.; Iwamoto, S.; Tanaka, K. *Analytical Chemistry* **2014**, *86*, 1937-1942.

(169) Garozzo, D. In *Selected Topics in Mass Spectrometry in the Biomolecular Sciences*, 1997; Vol. 504, pp 477-499.

(170) Liang, J. R.; Ai, X. X.; Gao, Y. H.; Chen, C. P. *Journal of Applied Phycology* **2013**, *25*, 477-484.

(171) Park, E.; Yang, H. J.; Kim, Y. S.; Kim, J. *Food Chemistry* **2012**, *134*, 1658-1664.

(172) Ren, S. F.; Zhang, L.; Cheng, Z. H.; Guo, Y. L. *Journal of the American Society for Mass Spectrometry* **2005**, *16*, 333-339.

(173) Rohmer, M.; Meyer, B.; Mank, M.; Stahl, B.; Bahr, U.; Karas, M. *Analytical Chemistry* **2010**, *82*, 3719-3726.

(174) Wang, H. M.; Zhang, P.; Huang, L. J.; Wang, Z. F. *Progress in Chemistry* **2009**, *21*, 1335-1343.

(175) Xia, B. Y.; Asif, G.; Arthur, L.; Pervaiz, M. A.; Li, X. L.; Liu, R. P.; Cummings, R. D.; He, M. *Clinical Chemistry* **2013**, *59*, 1357-1368.

(176) Angelini, R.; Vitale, R.; Patil, V. A.; Cocco, T.; Ludwig, B.; Greenberg, M. L.; Corcelli, A. *Journal of Lipid Research* **2012**, *53*, 1417-1425.

(177) Cerruti, C. D.; Benabdellah, F.; Laprevote, O.; Touboul, D.; Brunelle, A. *Analytical Chemistry* **2012**, *84*, 2164-2171.

(178) Fuchs, B.; Suss, R.; Schiller, J. *Progress in Lipid Research* **2010**, *49*, 450-475.

(179) Liang, M.; Zhang, P.; Shu, X.; Liu, C. G.; Shu, J. N. *International Journal of Mass Spectrometry* **2013**, *334*, 13-18.

(180) Shu, X.; Li, Y. Y.; Liang, M.; Yang, B.; Liu, C. G.; Wang, Y. F.; Shu, J. N. *International Journal of Mass Spectrometry* **2012**, *321*, 71-76.

(181) Valdes-Gonzalez, T.; Goto-Inoue, N.; Hirano, W.; Ishiyama, H.; Hayasaka, T.; Setou, M.; Taki, T. *Journal of Neurochemistry* **2011**, *116*, 678-683.

(182) Voorhees, K. J.; Jensen, K. R.; McAlpin, C. R.; Rees, J. C.; Cody, R.; Ubukata, M.; Cox, C. R. *Journal of Mass Spectrometry* **2013**, *48*, 850-855.

(183) Harvey, D. J. *Journal of Mass Spectrometry* **1995**, *30*, 1333-1346.

(184) Schiller, J.; Suss, R.; Fuchs, B.; Muller, M.; Zschornig, O.; Arnold, K. *Frontiers in Bioscience* **2007**, *12*, 2568-2579.

(185) Fuchs, B.; Schiller, J. *European Journal of Lipid Science and Technology* **2009**, *111*, 83-98.

(186) Jackson, S. N.; Woods, A. S. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2009**, *877*, 2822-2829.

(187) Hooff, G. P.; van Huizen, N. A.; Meesters, R. J. W.; Zijlstra, E. E.; Abdelraheem, M.; Abdelraheem, W.; Hamdouk, M.; Lindemans, J.; Luider, T. M. *Plos One* **2011**, *6*.

(188) Ranasinghe, C.; Akhurst, R. J. *Journal of Invertebrate Pathology* **2002**, *79*, 51-58.

(189) Sleno, L.; Volmer, D. A. *Analytical Chemistry* **2005**, *77*, 1509-1517.

(190) Welker, M.; Fastner, J.; Erhard, M.; von Dohren, H. *Environmental Toxicology* **2002**, *17*, 367-374.

(191) Su, A. K.; Liu, J. T.; Lin, C. H. *Talanta* **2005**, *67*, 718-724.

(192) Su, A. K.; Liu, J. T.; Lin, C. H. *Analytica Chimica Acta* **2005**, *546*, 193-198.

(193) Notari, S.; Mancone, C.; Tripodi, M.; Narciso, P.; Fasano, M.; Ascenzi, P. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2006**, *833*, 109-116.

(194) van Kampen, J. J. A.; Burgers, P. C.; de Groot, R.; Luider, T. M. *Analytical Chemistry* **2006**, *78*, 5403-5411.

(195) Hannewald, P.; Maunit, B.; Muller, J. F. *Analytical and Bioanalytical Chemistry* **2008**, *392*, 1335-1344.

(196) van Kampen, J. J. A.; Burgers, P. C.; Gruters, R. A.; Osterhaus, A.; de Groot, R.; Luider, T. M.; Volmer, D. A. *Analytical Chemistry* **2008**, *80*, 4969-4975.

(197) Notari, S.; Mancone, C.; Sergi, M.; Gullotta, F.; Bevilacqua, N.; Tempestilli, M.; Urso, R.; Lauria, F. N.; Pucillo, L. P.; Tripodi, M.; Ascenzi, P. *Iubmb Life* **2010**, *62*, 387-393.

(198) Meesters, R. J. W.; van Kampen, J. J. A.; Scheuer, R. D.; van der Ende, M. E.; Gruters, R. A.; Luider, T. M. *Journal of Mass Spectrometry* **2011**, *46*, 282-289.

(199) Iloro, I.; Gonzalez, E.; Gutierrez-de Juan, V.; Mato, J. M.; Falcon-Perez, J. M.; Elortza, F. *Analytical and Bioanalytical Chemistry* **2013**, *405*, 2311-2320.

(200) McEwen, A. B.; Henson, C. M.; Wood, S. G. *Bioanalysis* **2014**, *6*, 377-391.

(201) Cao, D.; Hu, M.; Han, C. G.; Yu, J. Y.; Cui, L.; Liu, Y. X.; Wang, H. L.; Cai, Y. Q.; Kang, Y. H.; Zhou, Y. Q. *Analyst* **2012**, *137*, 2218-2225.

(202) Ivanova, B.; Spiteller, M. *Ecotoxicology and Environmental Safety* **2013**, *91*, 86-95.

(203) Madla, S.; Miura, D.; Wariishi, H. *Analytical Sciences* **2012**, *28*, 301-303.

(204) Walton, S. L.; Mitchell, D. J. *Journal of Radioanalytical and Nuclear Chemistry* **2013**, *296*, 1113-1118.

(205) Zhang, J.; Dong, X. L.; Cheng, J. S.; Li, J. H.; Wang, Y. S. *Journal of the American Society for Mass Spectrometry* **2011**, *22*, 1294-1298.

(206) Galano, E.; Fidani, M.; Baia, F.; Palomba, L.; Marino, G.; Amoresano, A. *Journal of Pharmaceutical and Biomedical Analysis* **2012**, *71*, 193-197.

(207) Yonezawa, T.; Asano, T.; Fujino, T.; Nishihara, H. *Chemical Physics* **2013**, *419*, 17-22.

(208) Seraglia, R.; Teatino, A.; Traldi, P. *Forensic Science International* **2004**, *146*, S83-S85.

(209) Aurelie, P.; Khaled, B.; Louis, G.; Pierre, M. *Therapeutic Drug Monitoring* **2007**, *29*, 503-503.

(210) Kosanam, H.; Prakash, P. K. S.; Yates, C. R.; Miller, D. D.; Ramagiri, S. *Analytical Chemistry* **2007**, *79*, 6020-6026.

(211) Deacon, G. B.; Field, L. D.; Fisher, K.; Jaroschik, F.; Kay, D. L.; Maschmeyer, T.; Masters, A. F. *Journal of Organometallic Chemistry* **2014**, *751*, 482-492.

(212) Grishin, I. D. *Journal of Analytical Chemistry* **2013**, *68*, 1123-1129.

(213) Petkovic, M.; Petrovic, B.; Savic, J.; Bugarcic, Z. D.; Dimitric-Markovic, J.; Momic, T.; Vasic, V. *International Journal of Mass Spectrometry* **2010**, *290*, 39-46.

(214) Petroselli, G.; Mandal, M. K.; Chen, L. C.; Ruiz, G. T.; Wolcan, E.; Hiraoka, K.; Nonami, H.; Erra-Balsells, R. *Journal of Mass Spectrometry* **2013**, *47*, 313-321.

(215) Tomalova, I.; Foltynova, P.; Kanicky, V.; Preisler, J. *Analytical Chemistry* **2014**, *86*, 647-654.

(216) Berry, K. A. Z.; Hankin, J. A.; Barkley, R. M.; Spraggins, J. M.; Caprioli, R. M.; Murphy, R. C. *Chemical Reviews* **2011**, *111*, 6491-6512.

(217) Bonnel, D.; Legouffe, R.; Willand, N.; Baulard, A.; Hamm, G.; Deprez, B.; Stauber, J. *Bioanalysis* **2011**, *3*, 1399-1406.

(218) Cazares, L. H.; Troyer, D. A.; Wang, B. H.; Drake, R. R.; Semmes, O. J. *Analytical and Bioanalytical Chemistry* **2011**, *401*, 17-27.

(219) Kim, H. K.; Kim, I. H. In *Nanosystems in Engineering and Medicine*, 2012; Vol. 8548.

(220) Shanta, S. R.; Kim, Y.; Kim, Y. H.; Kim, K. P. *Biomolecules & Therapeutics* **2011**, *19*, 149-154.

(221) Agar, N. Y. R.; Yang, H. W.; Carroll, R. S.; Black, P. M.; Agar, J. N. *Analytical Chemistry* **2007**, *79*, 7416-7423.

(222) Thomson, B.; Wang, Z. Y.; Paine, A.; Rudin, A.; Lajoie, G. *Journal of the American Oil Chemists Society* **1995**, *72*, 11-15.

(223) Treverton, J. A.; Humphrey, P. *International Journal of Adhesion and Adhesives* **1995**, *15*, 249-256.

(224) Dreisewerd, K. *Chemical Reviews* **2003**, *103*, 395-425.

(225) Qiao, H.; Spicer, V.; Ens, W. *Rapid Communications in Mass Spectrometry* **2008**, *22*, 2779-2790.

(226) Lekha, S.; Dietrich, A. V. *Rapid Communications in Mass Spectrometry* **2005**, *19*, 1928-1936.

(227) Yong-Chien, L.; Lihnian, L.; Yi-Ting, C. *Rapid Communications in Mass Spectrometry* **1998**, *12*, 317-327.

(228) Bucknall, M.; Fung, K. Y. C.; Duncan, M. W. *Journal of the American Society for Mass Spectrometry* **2002**, *13*, 1015-1027.

(229) Masoud, Z.-M.; Elmar, H.; Andreas, T. *Rapid Communications in Mass Spectrometry* **2004**, *18*, 141-148.

(230) Lekha, S.; Dietrich, A. V. *Rapid Communications in Mass Spectrometry* **2006**, *20*, 1517-1524.

(231) Peter, K.; Chantal, G.; Emmanuel, B.; Gérard, H. *Rapid Communications in Mass Spectrometry* **2007**, *21*, 911-919.

(232) Notari, S.; Mancone, C.; Alonzi, T.; Tripodi, M.; Narciso, P.; Ascenzi, P. *Journal of Chromatography B* **2008**, *863*, 249-257.

(233) Szajli, E.; Feher, T.; Medzihradszky, K. F. *Mol. Cell. Proteomics* **2008**, *7*, 2410-2418.

High throughput quantitative MALDI-TOF MS analysis of triazines

2.1 Introduction

Triazines are N-heterocyclic structures that are biologically relevant and versatile chemical probes for biomolecular detection.[1, 2] Triazines also find use as dyes, herbicides and starting materials for the synthesis of resins.[2, 3] Maximum residual limit (MRL) of s-triazines such as atrazine in potable water is at low parts-per-billion level (3 ppb). However, at the sources of contamination such as ground water, these are present in much higher concentrations and can undergo chemical transformations including degradation that need quantitative monitoring to assess the toxicological impact on the environment.[4, 5] Adulteration of milk by melamine, an s- triazine with three amine group, has been implicated in serious health complications in children.[6] FDA mandates the amount of melamine (2, 4, 6-triamine-1, 3, 5-triazine) in milk products to be not more than 1 ppm.[7] Methods to detect melamine and s-triazines require a low limit of detection mandated by regulatory agencies in addition to providing a high throughput analysis.[8] Various analytical methods for melamine screening in milk were developed in the recent past. [9-14] Several atmospheric pressure desorption / ionization methods were reported for melamine and small molecules including some with a potential for high throughput analysis.[9, 11, 15-19] MALDI MS has also been used for the detection of atrazines as well as melamine and related analytes from solution.[20-22] Melamine has been previously detected using MALDI MS from urine samples with an intention of detecting kidney stones formed by the melamine-

cyanuric acid complex.[23] However, to our knowledge a high throughput quantitative method assessing the performance of MALDI-TOF MS vis-à-vis other methods such as LC-ESI-MS has not been reported for either melamine or s-triazines in general.

In this work, we demonstrate MALDI-TOF MS for the quantitative analysis of s-triazines from aqueous mixtures and for melamine detection from milk. A peak intensity-based MALDI-TOF MS quantitation is explored as an alternative to chromatographic peak areas generally used in LC- based quantitation. The robustness of the method for high throughput analysis has been compared with LC-ESI-MS and LC-PDA analysis. Additionally, a software utility tool that processes mass spectral files in ASCII format for peak intensity-based quantitation has also been developed to compliment the MALDI-TOF MS analysis.

2.2 Experimental

2.2.1 Materials

Melamine (2,4,6-triamino-1,3,5-triazine) was purchased from Loba Chemie (India), 2,5-dihydroxybenzoic acid (2,5-DHB), 2,4-diamino-1,3,5-triazine, 2,4-diamino-6-methyl-1,3,5-triazine, 2,4-diamino-6-(2-fluorophenyl)-1,3,5-triazine, 2,4-diamino-6-phenyl-1,3,5-triazine, 2,4-diamino-6-(4-methoxyphenyl)-1,3,5-triazine, methanol, acetonitrile and trifluoroacetic acid were from Sigma-Aldrich. Distilled and deionized water with specific resistivity 18.2 MΩ cm^{-1} was from SG ultrapure water unit (Germany). Packaged and commercially available cow's milk was used for melamine analysis.

2.2.2 Sample preparation

All the standards used are stable at room temperature and freshly prepared. Stock solution containing 1 mg/ml (1000 ppm) of melamine was prepared using methanol/water (1:1) with ultra-sonication for 5 minutes. For generating the calibration curve, dilution series of melamine was prepared in methanol/water (1:1) along with internal standard to obtain final volumes containing 0.1, 0.2, 0.3, 0.5, 0.8 and 1 ppm of the analyte respectively. 2,4-diamino-1,3,5-triazine was used as the internal standard in methanol/water (1:1) to obtain a concentration of 1 ppm.

For the multi component analysis, 2,4-diamino-6-phenyl-1,3,5-triazine (10 ppm final concentration), which was used as the internal standard (IS). Individual stock solutions containing 1000 ppm of the remaining four s-triazines were prepared and used in methanol/water (1:1) as described above for the single components. The final solution contained 1, 2, 5, 8 and 10 ppm of each of the four triazines used. A solution containing 6 ppm of individual component analytes were considered as unknown sample to test the method.

2.2.3 Melamine analysis in milk samples

Melamine was spiked in commercially available cow's milk to obtain a final concentration of 1 ppm. One liter of milk was taken and 1 mg of melamine was added to milk. The spiked sample was subjected to extraction using methanol/water (1:1). In 1.5 mL tube equal amounts of spiked milk and methanol/water (1:1) was vortexed and sonicated for 10 minutes. After sonication, 1 µL of Trifluoroacetic acid (TFA) was added to precipitate the milk solids. Thereafter, the tubes were kept on centrifugation for 15 min at 14000 rpm. Supernatant was removed into another tube, mixed with an equal amount of internal standard and used for the determination of melamine. This final solution for detection resulted in a fourfold dilution. For a milk sample spiked

with 1 ppm melamine, the final analyzed solution contained 0.25 ppm post extraction and IS addition, which falls within the linear range of 0.1 to 1 ppm as described above.

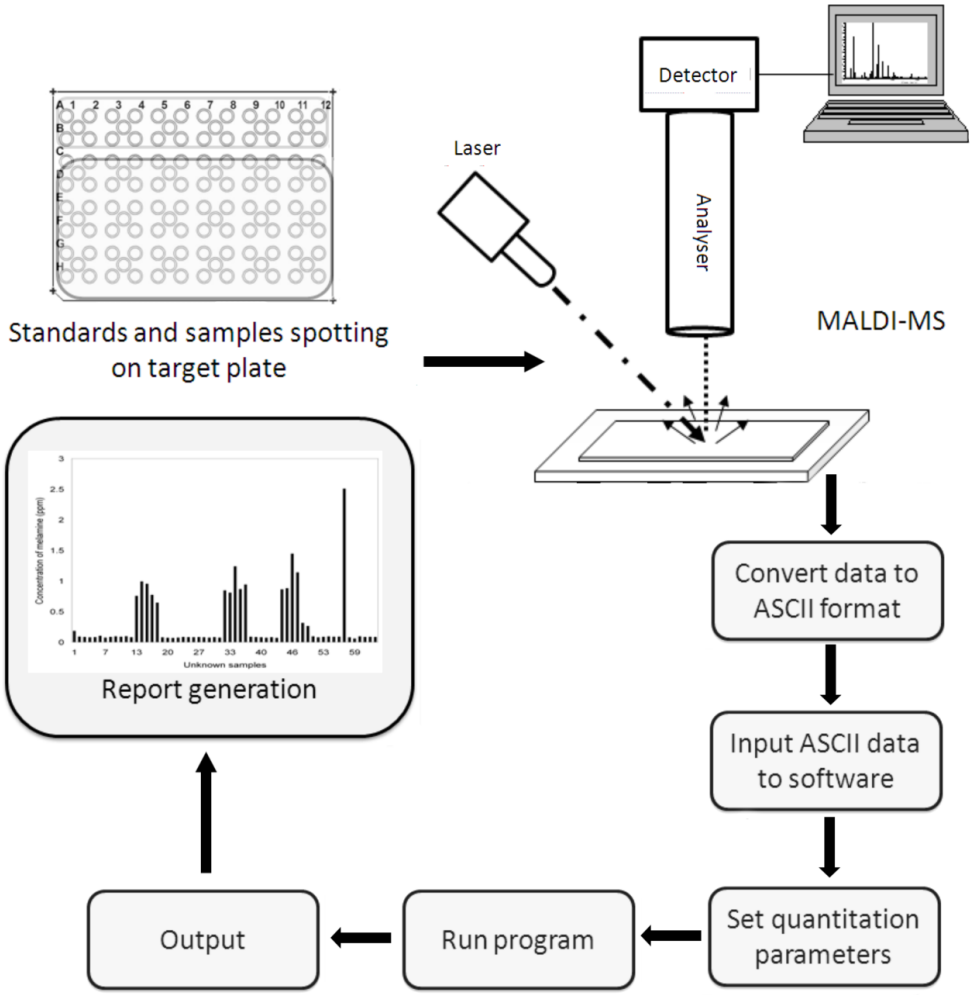


Figure 2.1: *Flow diagram showing the quantitative MALDI-TOF-MS analysis.*

2.2.4 MALDI-TOF-MS and UPLC-ESI-MS

MALDI-TOF-MS was performed on Waters Synapt HDMS (Manchester, UK) operated in V-positive mode or ABI Voyager DE-STR MALDI-TOF-MS in the positive ion mode. Detector voltage was set to 1700 V, quadrupole setting were set

according to mass range (1-600 *m/z*). Laser energy was optimized to get good signal to noise ratio. The data was processed with Masslynx 4.1 or Data explorer software. Standard 96-well stainless steel MALDI target plates were used after thorough cleaning10 mg/ml of 2,5-DHB was prepared and used by dissolving it in acetonitrile : 0.1% TFA (1:1, v/v). Sandwich model was adapted for spotting wherein 1 µL of 2,5-DHB was placed first on an individual spot, allowed to dry and 1 µL of working standards or samples were placed before being topped by yet another 1 µL of 2,5-DHB. The spot was thoroughly air dried before the analysis.

Quantitative analysis of the multi-component analyte solutions were also carried out on Waters UPLC-ESI-MS equipped with single quadrupole detector (SQD), ESCi source, an auto sampler and PDA detector (190 to 500 nm). 10 µL of the sample was injected into a Waters Acquity UPLC BEH C18 column (1.7 µM 2.1 x100 mm) using an auto sampler. All components in the sample were separated by isocratic binary solution of methanol/water (80:20) for 10 minutes at flow rate of 0.3 mL/min. After every sample run, a blank run containing only methanol/water (1:1) was carried out through column to clean any leftover analytes. Separated components were detected with the SQD in positive ion mode at capillary voltage of 3 kV and a capillary temperature of 120 ^{o}C.

2.2.5 High throughput analysis

For the melamine high throughput analysis, working standards (0.1 to 1 ppm) placed on a MALDI target plate for generating the calibration curve. A predetermined concentration of melamine, both from water: methanol (50:50) solution (0.8 ppm melamine) and extracts from melamine spiked milk (1 ppm and 2 ppm) were placed at different known locations of the target plate. The remaining spots contained negative

controls of either water: methanol (50:50) without melamine or extracts of milk without melamine. For the high throughput analysis of the mixture of four s-triazines, samples as described earlier have been used and placed on 96-well target plate(s) along with calibrants. 24 spots contained calibrants in quadruplicates and the remaining 72 spots had unknown test samples (17 spots) as well as blanks without the triazines. Finally, four 96-well target plates with 5 ppm s-triazine mixture (288 samples total) were also analyzed along with calibrants to check the reproducibility.

2.2.6 Algorithm for high throughput MALDI-TOF MS data processing and quantitation

For the mass spectral data analysis using 'MQ' software, all the spectra were converted into ASCII files containing *m/z* and absolute signal intensities using the manufacturer's software. An algorithm to process these files was coded in Perl script. Typically, the input file specifies the number of calibration points, corresponding concentrations, replicates, number of components along with a choice of adducts and the desired level of mass accuracy. Peaks are subsequently picked from data files based on the highest intensities for each of the components within the mass accuracy in ppm specified by the user. Following the selection of the analyte(s) as well as the internal standard peaks, the calibration curves are generated (analyte: IS ratio Vs concentration) and the unknown concentration determined. A linear regression using least square fitting is then enforced to output the linearity performance as represented by the mean slope, intercept and R-squared values for each of the components. Quantitation obtained using the MQ algorithm was thoroughly verified using manual quantitation for both the s-triazine and melamine analysis before implementing it for the high throughput workflows.

2.3 Results

2.3.1 Quantitative MALDI-TOF MS analysis of s-triazines and melamine from milk

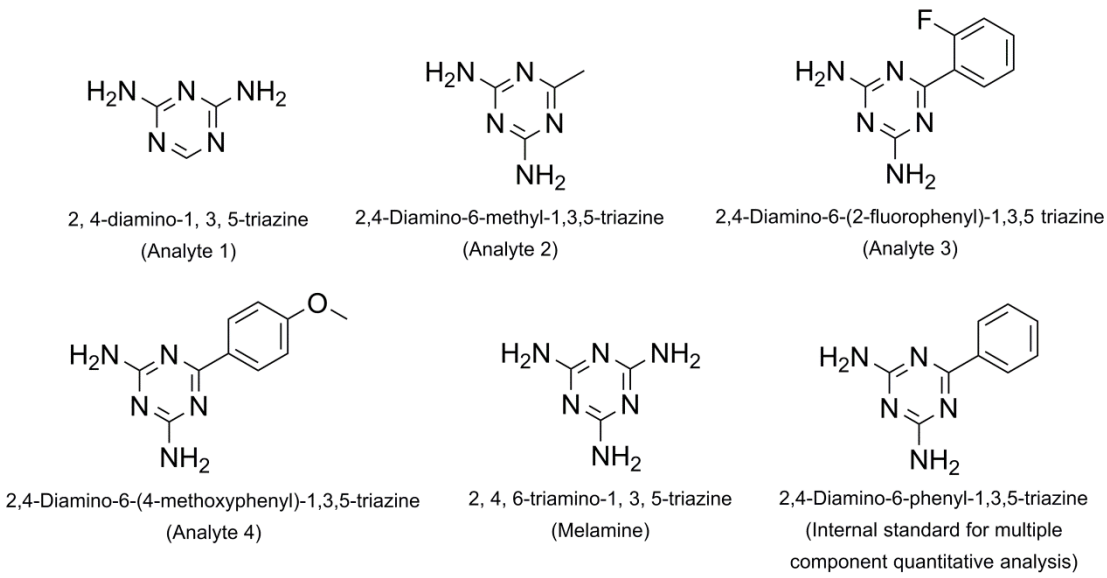


Figure 2.2: *Structures of s-triazines used for the quantitative analysis.*

2,4-diamino triazines with different substitutions on the heterocycle, analytes 1 through 4 having nominal masses between *m/z* 111 to 217, were chosen for the multicomponent analysis as shown in figure 2.2. 2,5-DHB used in this study is generally considered as a suitable matrix for analysis in the low molecular weight region. However, there are several peaks at *m/z* 137, 154, 155 and 273 from the matrix that can potentially interfere and care has to be taken to ensure that analyte peaks are resolved. MALDI-TOF MS of the mixture of the four s-triazines resolves the analyte peaks without any matrix interference as shown in figure 2.3 (a). All the masses indicated are for the respective $[M + H]^+$ adducts of the analytes 1 through 4. The masses were obtained with high accuracies and were within 3 ppm from their exact masses for analytes 1, 3, 4 and IS. Analyte 2 had a mass within 10 ppm from its

exact mass. Figure 2.3 (b) shows the MALDI-TOF MS of 0.25 ppm (~2 µM) melamine extract from spiked milk. Melamine (*m/z* 127.0748; mass accuracy 12 ppm) can clearly be resolved from the interfering peak of 5-hydroxymethylfurfural (*m/z* 127.0415). 5-Hydroxymethylfurfural is formed during dehydration of sugar present in milk and fruits on heating or via Maillard reaction.[16, 17, 24]

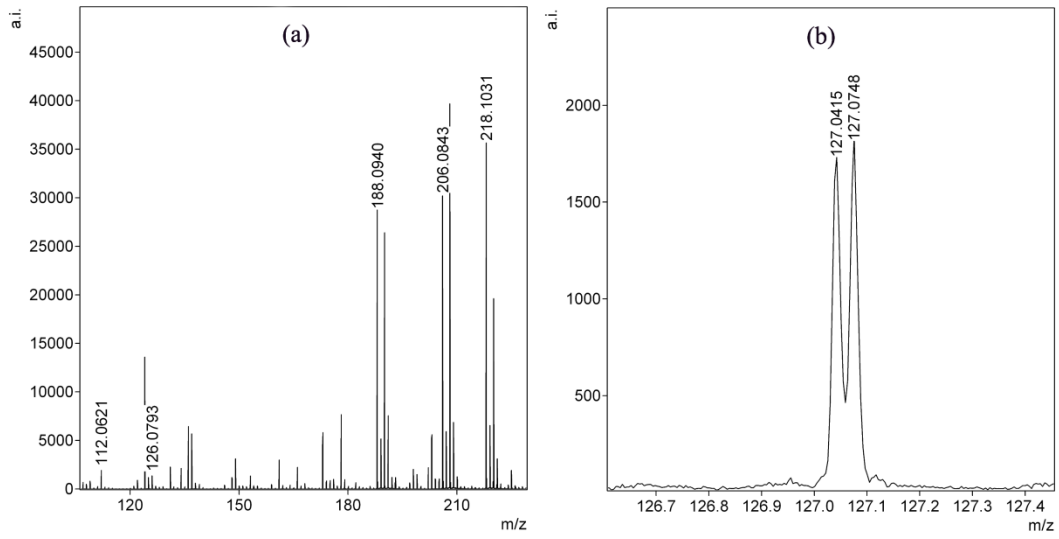


Figure 2.3: *MALDI-TOF MS of (a) s-triazine mixture and (b) melamine from an extract of 1 ppm melamine spiked liquid milk using 2,5-dihydroxybenzoic acid (2,5-DHB) as the matrix. . Melamine (at m/z 127.0748) and the peak from 5-hydroxymethylfurfural peak (at 127.0415) are well resolved.*

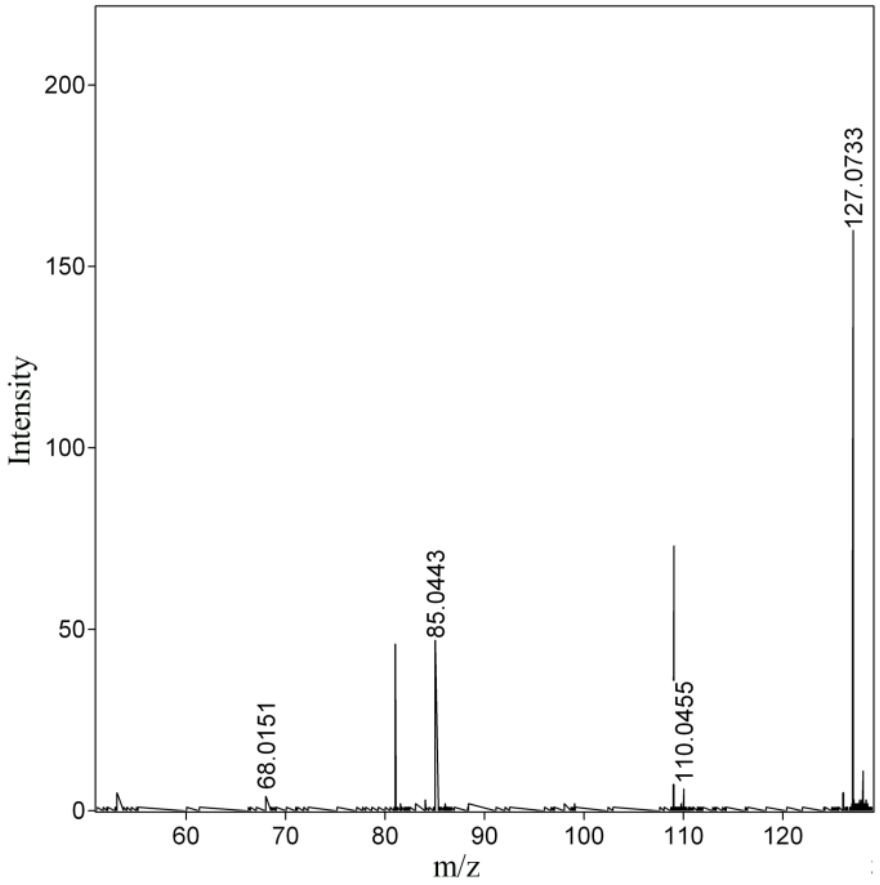


Figure 2.4: *CID fragments of melamine in 1ppm melamine spiked milk. Peaks at m/z 110, 85 & 68 are fragmented ions of melamine.*

Further confirmation of the melamine precursor ion ruling out any interference was obtained using collision induced dissociation (CID) fragmentation. The melamine precursor ion yielded daughter ions at *m/z* 110 accounting for a loss of ammonia (Δ, -17 Da), *m/z* 85 with a loss of CH_2N_2 (Δ, - 42 Da) and *m/z* 68 with a loss of CH_5N_3 (Δ, -59Da) (figure 2.4) and are in agreement with previously reported data.[25] Quantitation of the s-triazine mixture and melamine extract from milk was performed using MALDI-TOF MS and compared with UPLC-ESI-MS. Concentration response curves were calculated as a ratio of the absolute mass spectral peak intensities of the analytes versus those of the internal standard and plotted against the concentration in ppm. Linearity data for both the methods are tabulated in table 2.1.

Table 2.1: *Data for the concentration response curves obtained using peak intensities for the multiple triazine components indicating linearity in 1 to 10 ppm range. Mean percentage errors mentioned in parenthesis.*

Analytes	MALDI-TOF MS	LC-ESI-MS
1	$y = 0.0049x + 0.0009$, $R^2 = 0.99$ (3.52)	$y = 0.0303x + 0.1315$, $R^2 = 0.95$ (0.81)
2	$y = 0.0040x + 0.0025$, $R^2 = 0.99$ (0.22)	$y = 0.0861x + 0.1306$, $R^2 = 0.97$ (2.00)
3	$y = 0.1015x + 0.1308$, $R^2 = 0.97$ (1.16)	$y = 0.1403x + 0.0035$, $R^2 = 0.99$ (0.50)
4	$y = 0.1129x + 0.2038$, $R^2 = 0.97$ (1.13)	$y = 0.1995x - 0.0076$, $R^2 = 0.99$ (0.90)

The LC-ESI-MS curves yielded higher slopes than the corresponding MALDI-TOF MS curves. The sensitivities in MALDI-TOF MS and LC-ESI-MS found to be similar for analytes 3 and 4. The intercepts for the analytes in MALDI-TOF MS indicate responses in two broad mass distributions. Analytes 1 and 2 with lower masses (112 and 126 respectively) have lower corresponding intercepts as compared to analytes 3 and 4 with relatively higher masses (206 and 218 respectively). Thus, the intercepts correlate with the analyte masses and indicate a difference in their respective ionizations. Linearity for the MALDI-TOF MS was obtained with the squared correlation coefficients (R^2) in the range of 0.95 to 0.99. The relative standard deviations for all the analytes are very small. Linearity for LC-ESI-MS is in the range of 0.97 to 0.99 (R^2). It can be clearly seen that the linearity of the concentration response curves obtained by MALDI-TOF MS are comparable with those obtained using LC-ESI-MS validating the use of the former for quantitative analysis. A limit of detection (LOD) of around 20 ppb (femtomole/μL) was obtained for all the analytes from the s-triazine mixture (figure 2.5).

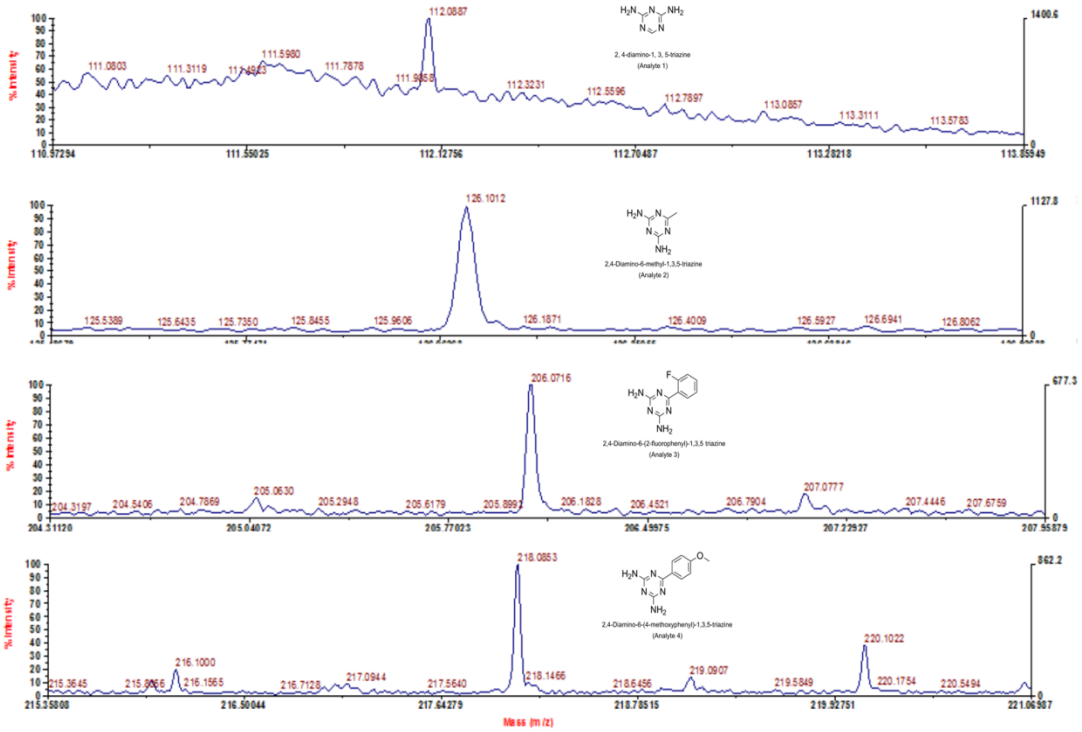


Figure 2.5: *MALDI-TOF MS of individual s-triazine molecule at 20 ppb level.*

Table 2.2: *Mean concentrations (in ppm) of a triazine test mixture containing 6 ppm of each individual analyte as determined using MALDI-TOF MS and LC-based methods. Relative standard deviations (RSD) are given in parenthesis based on three replicates each.*

Analytes*	**MALDI-TOF MS (Peak Intensity)**	**LC-ESI-MS (Peak Intensity)**	**LC–MS chromatogram (Peak area)**	**LC-PDA chromatogram (Peak area)**
1	5.7 (22)	6.2 (6.7)	NA#	NA#
2	5.8 (6.8)	5.9 (4.8)	6.1 (3.3)	7.0 (7.5)
3	5.9 (3.1)	5.6 (2.5)	5.7 (9.9)	6.5 (5.2)
4	6.1 (5.1)	6.1 (7.0)	6.0 (1.1)	6.4 (1.5)

To test the usefulness of this method for the analysis of known analytes within the linear concentration range considered, a test mixture containing 6 ppm of each of the four s-triazines was prepared and analyzed by MALDI-TOF MS. LC-ESI-MS and

LC-PDA analysis on the same set of samples were performed for comparison. Peak intensities in the case of MALDI-TOF MS and LC-ESI-MS were compared. Alongside, peak areas were also estimated from the LC-ESI-MS extracted ion chromatograms (XIC) and chromatographic peak areas (PDA). After the analysis, the peak-intensity data was processed using the MQ data processing software and verified manually and the amounts of each of the individual analytes placed in three replicates were quantified. The peak areas were estimated manually. The results thus obtained for all the three methods of analysis are given in table 2.2. All the three methods could detect the s-triazines from the test mixture within a close range of the expected 6 ppm concentration. Analyte 1 was not resolved with the chromatographic method used in the LC mode, and hence quantitation could not be performed using either the extracted ion chromatogram or the area under the PDA curve leaving the *m/z* peak as the only choice. The determined values using MALDI-TOF MS are indeed comparable with the LC-based methods with precision less than 15% RSD for most analytes. The only exception is MALDI-TOF MS peak-intensity based determination of analyte 1 with over 20% RSD. The mean error percentages from the theoretical concentrations were 14.9, 4.2, 2.1 and 4.3 for analytes 1 through 4 respectively. These are comparable to the mean error percentages that we obtained for LC-ESI-MS data with the exception of analyte 1, where the latter gave a mean percentage error of 4.9%. Thus, MALDI-TOF MS quantitation of multiple components from the test mixture is analytically robust with good accuracy and precision. Agreement with the LC-ESI-MS and LC-PDA results validates the MALDI-TOF MS quantitation. Calibration curve for melamine detection using MALDI-TOF MS peak intensity ratios was generated from standard dilutions as described earlier (figure 2.6). Reproducible melamine *m/z* signal intensities with good enough S/N ratios were

obtained even at the lowest concentration of 0.1 ppm used for the calibration curve. Excellent linearity with a correlation coefficient 0.99 was obtained within the range of 0.1 to 1 ppm melamine concentration. The robustness and linearity of the calibration plots and the detection method was ascertained over a period of three consecutive days. Calibration plots were generated on all three days and a test sample extract from liquid milk spiked with 1 ppm melamine was detected to check the repeatability of the method. Post extraction, the sample was subjected to a dilution step followed by MALDI-TOF MS detection and estimation of the concentration from the calibration curve.

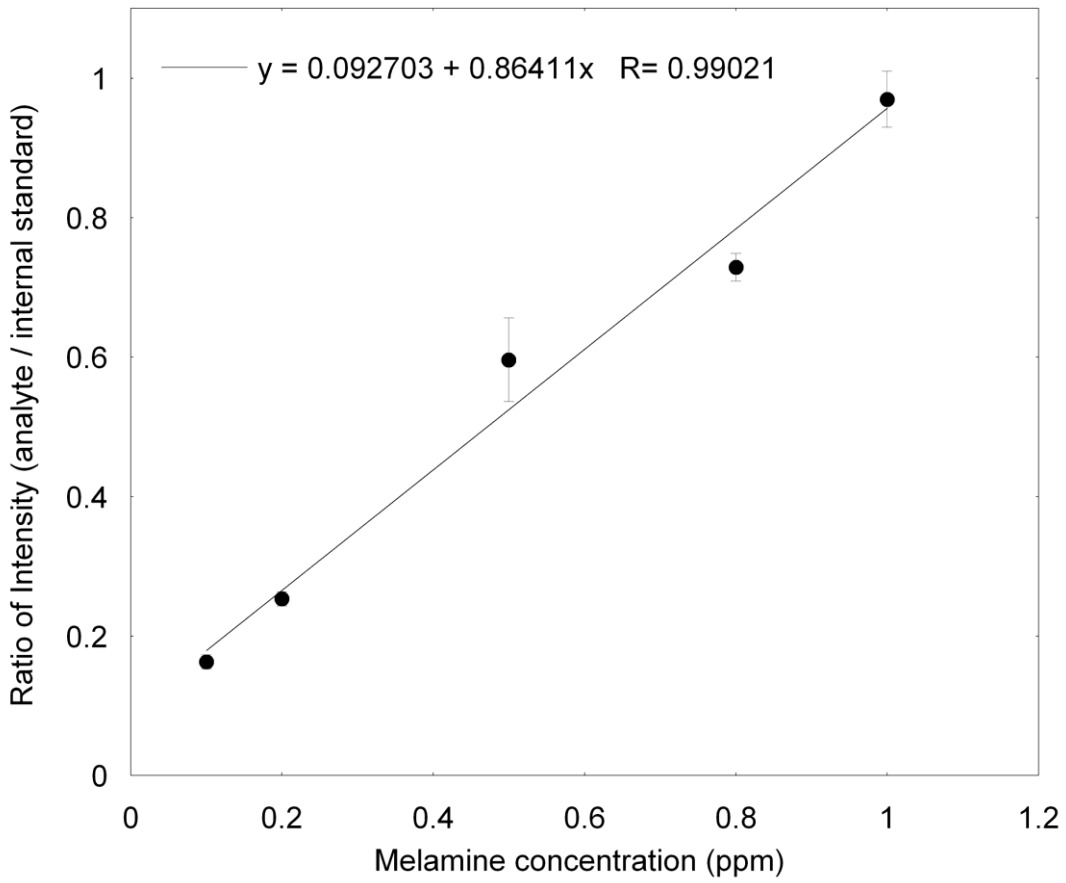


Figure 2.6: *Concentration response curve for melamine standard showing linearity (R 0.99) in the range of 0.1 ppm to 1 ppm obtained using MALDI-TOF MS.*

Table 2.3 summarizes the concentrations estimated for the spiked sample on all three days along with the slope, intercept and mean R^2 for the calibration curves. The concentrations for the extracted sample showed good precision on all three days. Linearity with R^2 of 0.96 - 0.98 and comparable slopes and intercepts were consistently obtained on all the different days. Hence the MALDI-TOF MS method for melamine detection shows a good reproducibility, accuracy and sensitivity.

Table 2.3: *Reproducibility of peak-intensity based MALDI-TOF MS quantitation of 1 ppm (7.9 µmole/L) melamine spiked in liquid milk. RSD values are indicated in the parenthesis for three replicates (n = 3).*

Days	Slope	Intercept	R^2	Recovery (ppm)
Day 1	0.00086	0.09270	0.98	0.9 (12.9)
Day 2	0.00071	0.08951	0.96	1.3 (1.4)
Day 3	0.00077	0.04358	0.97	1.2 (10.3)

2.3.2 High throughput quantitative analysis

A mock high throughput experiment was attempted with two sets of samples. The first set contained melamine extracts from spiked milk in various concentrations. Secondly, triazine mixture in various concentrations was placed on yet another MALDI target plate. In both instances calibrants and controls were placed on the same target plates, which were analyzed in an automated mode with the same set of acquisition conditions for all the spots. Melamine was detected in all the test spots in four labeled sample regions as shown in figure 2.8. Region A with 0.8 ppm, regions B and C with 1 ppm each and region D containing 2 ppm were detected with mean error percentages of 2.3, 6.05, 13.32 and 25.38 respectively. The average relative standard deviations for A, B and C are 16.3, 21.3 and 19.1 respectively. In the case of triazine mixture, they vary broadly with the minimum at 0.81 for analyte 1 at 2 ppm and a

maximum of 59.19 for analyte 2 at 8 ppm. About 80% of the determined concentrations in this high throughput analysis have mean error less than 25%.

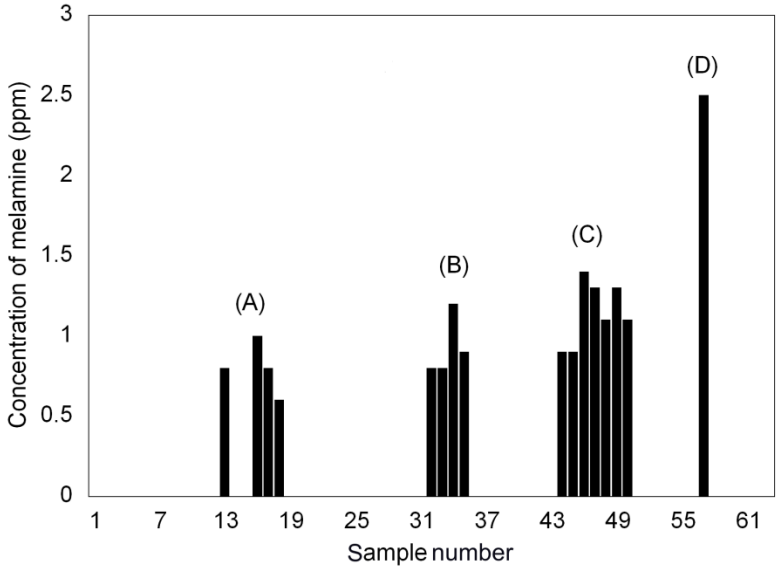


Figure 2.7*: Graphical presentation of high throughput MALDI-TOF MS quantitation output of melamine samples placed on the target plate in region (A) containing 0.8 ppm, regions (B) and (C) containing 1 ppm each, and region (D) containing 2 ppm respectively.*

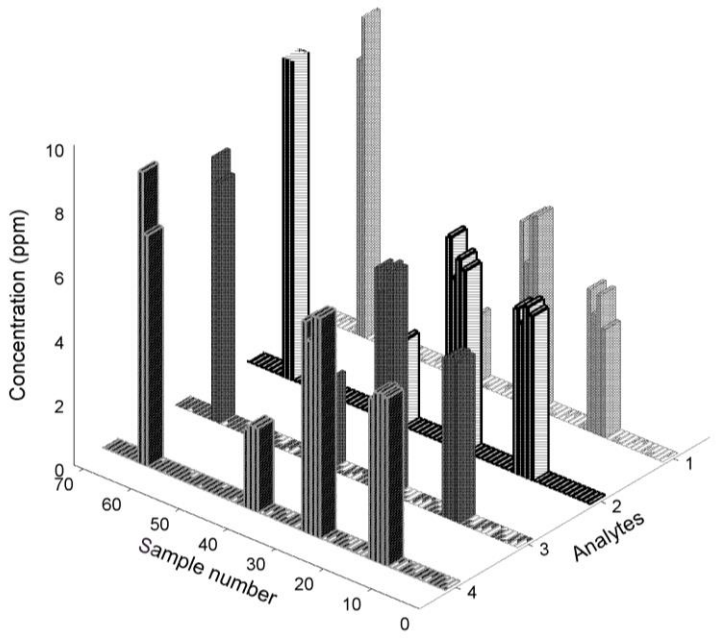


Figure 2.8: *Graphical presentation of high throughput MALDI-TOF MS quantitation output of s-triazine mixture having analytes 1 through 4 placed on a 96-well target plate in different regions alongside negative controls given by the sample number indicated on x-axis. The bars in the plot represent concentrations of 5, 6, 2 and 8 ppm respectively for the regions where s-triazines were detected.*

To further establish the statistics over a much larger sample set and to rule out any non-linearity in the desorption ionization spread over various spots, target plates and on different days, we had performed the multi component analysis on 288 samples of the triazine test mixture. Four different target plates containing individual test samples placed on 72 spots of each target plate and 24 spots having calibrants in multiplicates were analyzed. The sample spots containing triazine test mixture had 5 ppm of each individual component. Table 2.4 summarizes the results from this analysis. Analytes 3 and 4 having higher *m/z* than 1 and 2 respectively were detected with much better precision of 10% RSD or less. Analytes 1 and 2 were detected with a precision slightly higher than acceptable (21 and 17% respectively), but within what is acceptable at LLOQ. The high throughput data presented in table 2.4 also indicates excellent linearity over the four days and good accuracies as indicated by the low percentage mean error.

Table 2.4: *Linearity, reproducibility and accuracy of high throughput analysis of the triazine test mixture. 72 test samples containing 5 ppm of each individual analyte were quantified using MALDI-TOF MS on four different days with 72 test samples on each day amounting to a total of 288 samples.*

Analyte*	**Mean concentration (ppm) N=288**	**% Mean error**	**%RSD**	**Mean R^2 with %RSD**
1	4.7	5.1	21.3	0.989 (0.63)
2	5.2	3.1	17.7	0.990 (0.67)
3	5.1	2.8	7.1	0.998 (0.12)
4	5.1	2.7	10.1	0.997 (0.37)

**5 ppm translates to 45, 40, 24 and 23 µmole/L respectively for analytes 1, 2, 3 and 4 respectively.*

2.4 Discussion

s-Triazines used in this study (analytes 1, 2, 3 and 4 in figure 2.2) have proton, methyl, fluorophenyl and methoxyphenyl substitutions respectively at the position 6 of the heterocycle, while the internal standard chosen has a phenyl ring at the same position. In the case of melamine analysis from milk, 2,4-diamino- 1,3,5-triazine (analyte 1) has been used as the internal standard. Thus, the analytes as well as the internal standards belong to the triazine family and are most likely to exhibit similarity in their ionization patterns. More importantly, the chosen internal standards have high signal-to-noise ratio, are homogeneous, not reactive to analyte(s) of interest and generate reproducible signals.

LC-ESI-MS is widely accepted to be the gold standard for quantitation of small molecules. MALDI-TOF MS quantitation was previously shown to be comparable and better than LC-ESI-MS analysis in the case of pharmacokinetic analysis of drug candidates.[26] The authors had performed quantitation using LC and MALDI in the single reaction monitoring (SRM) mode by calculating the peak areas. In the cited reference, the mode of ionization (ESI Vs MALDI) was varied while the analyzer used, a triple quadrupole, was the same in both modes. In the present study, the analyzers used for the ESI and MALDI modes were different (single quadrupole and an orthogonal QTOF respectively), and hence the resolution as well as mass accuracies were different. Our approach assumes significance as we have explored quantitation based on the peak intensities, which were compared to quantitation based on LC chromatographic peak areas (PDA as well as XIC) and ESI-MS peak intensities while peak areas alone were used in the above mentioned study. MALDI quantitative analysis based on peak intensities alone has also been previously used

without comparison to LC methods.[27] The analytical figures of merit in this study place MALDI-TOF MS analysis on equal footing with the LC data. Also, the comparisons of precision and accuracy of MALDI-TOF MS quantitation with those of LC-ESI MS and LC-PDA measurements validates the methods across different analytical platforms and modes of quantitation. It is noteworthy to point out that this study highlights that the peak intensity-based MALDI-TOF MS quantitation is not only simple and sufficient, but could also be the method of choice considering the difficulties faced in method development for chromatographic peak resolution.

The ability to detect melamine below the prescribed FDA limit of 1 ppm concentration indicates the robustness of MALDI-TOF MS for routine analysis in a toxicology laboratory. Similarly, multiple triazines detected were efficiently quantified with the aid of MQ algorithm indicating the use of the MALDI-TOF MS method for simultaneous and quantitative detection of multiple components. Though the test mixture contains pure standards, the results can be extrapolated for more complicated mixtures, especially targeted analysis of biological and environmental samples considering the ability to reach a LOD of upto 20 ppb. Moreover, the average time spent per target plate containing 100 samples that includes manual spotting of the samples on the target plates, performing MALDI-TOF MS, data conversion and processing was about 3-4 hours. This can be further optimized and made less error-prone by using an automated sample spotter, which would result in a truly high throughput and rapid analysis that is much faster than most conventional LC-based methods. Multicomponent analysis of large numbers of samples within this short time would enable optimal usage of instrumental resources and drastically reduce the time taken from sample to report generation in most routine analytical applications. The high throughput is obtained without compromising on the precision and accuracy of

the method as demonstrated by the analysis of 288 samples containing 5 ppm each of the s-triazine performed over multiple days. Most importantly, this also indicates negligible nonlinear behaviors arising out of desorption / ionization process of MALDI-TOF MS quantitation. Any significant contribution from non-linearity would have resulted in a lot more variation in the precisions.

2.4.1 Comparison of MALDI-TOF MS with other high throughput ambient pressure ionization methods

MALDI-TOF MS has several advantages over other atmospheric / ambient pressure techniques such as desorption electrospray ionization (DESI) and desorption atmospheric pressure chemical ionization (DAPCI). Although melamine analysis directly from milk powder has been reported, accurate analysis is difficult as the vapor in DESI or DAPCI blows away the sample from the inlet unless the analyte is presented in a solid form.[11] Another recent report using direct analysis in real time (DART) could not achieve the necessary resolution of the melamine peak from an interfering ion at lower concentrations while higher mass accuracies obtained only at concentrations above LOQ, and in source fragmentation yielded only one daughter ion.[16] MALDI analysis does not have these limitations and CID induced fragmentation yielded all the known fragmentation patterns for melamine. MALDI, in vacuum or ambient pressure mode, is available from many commercial vendors and with various analyzers along with capability to perform tandem analysis. MALDI-TOF MS also has been extensively used in the last decade or so, for proteins and peptides albeit, and hence as a technique matured quite significantly. Quantitation using MALDI-TOF MS has been addressed, again in the larger molecule analysis. Thus, due to its versatility, accessibility, availability and farther reach, LDI MS and

MALDI-TOF MS hold the greatest promise of acceptability when it comes to high throughput small molecule analysis (melamine and s-triazines in this particular case).

2.5 Conclusions

Quantitative data from LC-ESI-MS compares well with the MALDI-TOF MS data and validates the latter method for trace analysis of s-triazines from aqueous mixtures and melamine contamination in milk. The peak-intensity based MALDI-TOF MS analysis is comparable to the corresponding values from both peak areas and peak intensity measurements in the LC-ESI or LC-PDA mode. MALDI-TOF MS quantitation of s-triazines is characterized by low LOD, high throughput, rapid analysis and excellent analytical efficiency. Tedious sample preparation, derivatization and time consuming chromatographic or method development steps can potentially be eliminated when using the MALDI-TOF MS making it attractive to a large number of users for high throughput analysis of low molecular weight analytes.

2.6 References

(1) Ahn, Y.-H.; Chang, Y.-T. *Accounts of Chemical Research* **2007**, *40*, 1025-1033.

(2) Shapir, N.; Mongodin, E. F.; Sadowsky, M. J.; Daugherty, S. C.; Nelson, K. E.; Wackett, L. P. *Journal of Bacteriology* **2007**, *189*, 674-682.

(3) Duliban, J. *Macromolecular Materials and Engineering* **2006**, *291*, 147.

(4) Jason Krutz, L.; Shaner, D. L.; Weaver, M. A.; Webb, R. M. T.; Zablotowicz, R. M.; Reddy, K. N.; Huang, Y.; Thomson, S. J. *Pest Management Science* **2010**, *66*, 481.

(5) Prosen, H. In *Herbicides - Properties, Synthesis and Control of Weeds*; Hasaneen, D. M. N., Ed.; InTech Europe, 2012.

(6) Gossner, C. M. E.; Schlundt, J.; Ben Embarek, P.; Hird, S.; Lo-Fo-Wong, D.; Beltran, J. J. O.; Teoh, K. N.; Tritscher, A. *Environmental Health Perspectives* **2009**, *117*, 1803-1808.

(7) Sherri Turnipseed; Christine Casey; Cristina Nochetto; Heller, D. N. *FDA LIB 4421 Melamine and Cyanuric Acid Residues in Infant Formula* **2008**, *24*.

(8) FDA *Guidance for Industry :Pharmaceutical Components at Risk for Melamine Contamination* **2009**.

(9) Huang, G. M.; Zheng, O. Y.; Cooks, R. G. *Chemical Communications* **2009**, 556-558.

(10) Ibanez, M.; Sancho, J. V.; Hernandez, F. *Analytica Chimica Acta* **2009**, *649*, 91-97.

(11) Yang, S. P.; Ding, J. H.; Zheng, J.; Hu, B.; Li, J. Q.; Chen, H. W.; Zhou, Z. Q.; Qiao, X. L. *Analytical Chemistry* **2009**, *81*, 2426-2436.

(12) Wei, R. C.; Wang, R.; Zeng, Q. F.; Chen, M.; Liu, T. Z. *Journal of Chromatographic Science* **2009**, *47*, 581-584.

(13) Xia, J. G.; Zhou, N. Y.; Liu, Y. J.; Chen, B.; Wu, Y. N.; Yao, S. Z. *Food Control* **2009**, *21*, 912-918.

(14) Zhu, L.; Gamez, G.; Chen, H.; Chingina, K.; Zenobi, R. *Chemical Communications* **2009**, 559-561.

(15) Jia, B.; Ouyang, Y. Z.; Sodhi, R. N. S.; Hu, B.; Zhang, T. T.; Li, J. Q.; Chen, H. W. *Journal of Mass Spectrometry* **2010**, *46*, 313-319.

(16) Vaclavik, L.; Rosmus, J.; Popping, B.; Hajslova, J. *Journal of Chromatography A* **2010**, *1217*, 4204-4211.

(17) Dane, A. J.; Cody, R. B. *Analyst* **2010**, *135*, 696-699.

(18) Van Berkel, G. J.; Pasilis, S. P.; Ovchinnikova, O. *Journal of Mass Spectrometry* **2008**, *43*, 1161-1180.

(19) Venter, A.; Nefliu, M.; Graham Cooks, R. *TrAC Trends in Analytical Chemistry* **2008**, *27*, 284-290.

(20) Cheng, Y.; Hercules, D. M. *Microchemical Journal* **2002**, *72*, 255-267.

(21) Hu, L.; Xu, S.; Pan, C.; Yuan, C.; Zou, H.; Jiang, G. *Environmental Science & Technology* **2005**, *39*, 8442-8447.

(22) Campbell, J. A.; Wunschel, D. S.; Petersen, C. E. *Analytical Letters* **2007**, *40*, 3107-3118.

(23) Tang, H.-W.; Ng, K.-M.; Chui, S. S.-Y.; Che, C.-M.; Lam, C.-W.; Yuen, K.-Y.; Siu, T.-S.; Lan, L. C.-L.; Che, X. *Analytical Chemistry* **2009**, *81*, 3676-3682.

(24) Murkovic, M.; Pichler, N. *Molecular Nutrition & Food Research* **2006**, *50*, 842-846.

(25) Desmarchelier, A.; Cuadra, M. G.; Delatour, T.; Mottier, P. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 7186-7193.

(26) Volmer, D. A.; Sleno, L.; Bateman, K.; Sturino, C.; Oballa, R.; Mauriala, T.; Corr, J. *Analytical Chemistry* **2007**, *79*, 9000-9006.

(27) Markus, P.; Michael, K. *Rapid Communications in Mass Spectrometry* **2009**, *23*, 3555-3562.

Direct quantitation of pharmaceutical drugs from urine

3.1 Introduction

Pharmacokinetics plays a very important role in drug development process for prioritizing lead compounds. It involves the investigation of adsorption, distribution, efficient metabolism and successful excretion from body of drug (ADME), and helps in screening of drug candidates. Once a drug is administrated into the blood stream orally or intravenously, it is distributed to the site of action followed by metabolism or biotransformation. Finally, the drug and its metabolites get excreted from body through kidneys in the form of urine. Slow or incomplete excretion can lead to accumulation of higher concentration of drug at the site of action. Quantitative estimation of excreted drug is important to determine the therapeutic dosage of drug, its toxicological effect, rate of excretion and the amount of drug absorbed by the body. Simultaneous analysis and quantitation of different drugs with diversity in their chemical nature, especially from biological fluids, is indeed a challenging task.

Mass spectrometry (MS) has established itself as a crucial bioanalytical tool in the pharmaceutical industry and in the drug development process.[1, 2] Versatility coupled with high sensitivity, resolution and dynamic range make MS a method of choice in pharmacokinetic studies. LC-MS based methods for quantitative determination have been used routinely in past decade and is now considered as benchmarks for bioanalytical analysis.[3-6] However, chromatographic techniques require a lot of

attention and can be time consuming during analytical method development. In drug development processes where time is a critical factor, any time consuming methods are undesirable. Often, there are also large sample sets in the analytical pipeline that demand high throughput analytical techniques.[7]

Direct mass analysis and quantitation is an efficient alternative that is not yet being used to its optimum potential. This is primarily due to concerns of introducing an impure sample without chromatographic separation. Major time consuming steps and tedious sample preparation can be avoided by introducing sample directly into the ionization source of a mass spectrometer. [8] However, most of the recently developed direct mass analytical techniques that can bypass the chromatographic step are yet to be fully explored for quantitative analysis. [9, 10] Among the direct mass analytical methods, matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) has several advantages.[11-15] Generation of mainly singly charged ions with high resolution provide uncomplicated spectra in MALDI-TOF MS[16] The interference of matrix ions in small molecule region of the mass spectra can be offset by choosing the matrices carefully and with MS/MS validation of the precursor ions. Particularly, the use of MALDI-TOF MS in imaging of drugs distributed in cells has also gained much interest. [17-19] Considering the limited time span for bioanalytical study during drug development processes, MALDI-TOF MS is an alternative for drug screening from biological fluids due to its inherent advantages.

Urine has been routinely used as biofluid for analytical studies because of its non-invasive sampling, lower protein content, and larger quantities that can be collected for multiple cycles of analysis. Urine excreted from human body also contains higher concentration of endogenous and exogenous metabolites. In this study, we explored direct and quantitative analysis of several drugs spiked in blank urine samples using

MALDI-TOF MS. Simple and rapid quantitative workflow of drugs from human urine without any further sample preparation, along with their characterization with comprehensive collision induced fragmentation, is being demonstrated.

3.2 Experimental

3.2.1 Material

Acetaminophen (N-(4-hydroxyphenyl)acetamide), griseofulvin ((1'S,6'R)-7-chloro-2',4,6-trimethoxy-6'-methyl-3H-spiro[benzofuran-2,1'-cyclohex[2]ene]-3,4'-dione), ampicillin ((2S, 5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid), verapamil (5-((3,4-dimethoxyphenethyl) (methyl)amino)-2-(3,4-dimethoxyphenyl)-2-isopropylpentanenitrile), 2,4-diamino-6-(2-fluorophenyl)-1,3,5-triazine, 2,5-dihydroxybenzoic acid, trifluoroacetic acid, methanol, acetonitrile, were purchased from Sigma-Aldrich. Purity of drug standards was meeting USP testing specification, i.e. 98-102%. Deionized water with specific resistivity 18.2 MΩ was obtained from SG ultrapure water unit (Germany).

3.2.2 Sample preparation

100 mM stock solution of each drug was prepared in methanol, diluted to desired concentration and mixed in equal volume. The final concentration of each drug molecule in the mixture was as follows: 2 mM acetaminophen, 40 µM griseovin, 1 mM ampicillin and 2 µM verapamil. 2,4-diamino-6-(2-fluorophenyl)-1,3,5-triazine was used as internal standards for quantitative analysis. 1000 ppm stock solution of internal standard was prepared in methanol: water (1:1 v/v) and diluted to 10 ppm. 2,5-dihydroxybenzoic acid (DHB) was used as MALDI matrix. 20 mg/mL DHB solution was prepared in acetonitrile: 0.1% TFA (1:1, v/v). Later a mixture of DHB and internal standard was prepared for quantitative MALDI-TOF MS.

Urine was collected from a non-patient healthy volunteer (during 2-3 PM) and immediately aliquoted and stored at -20 ^{o}C. To prepare urine sample spiked with pharmaceutical drugs, 100 µL of urine was mixed with 100 µL of the drug mixture and kept on centrifugation at 4^{o}C temperature for 1 hr at 13,200 rpm. The supernatant was collected in a separate vial. A series of calibrants was prepared from urine samples by spiking varying concentrations of the drug mixture.

3.2.3 MALDI TOF-MS analysis

Synapt MALDI TOF-MS (Waters, UK) was used in the positive ion mode for the entire mass spectrometric experiments. Prior to the experiments, the instrument was properly tuned and calibrated for mass accuracies with standards and protocols as prescribed. Detector voltage and quadrupole settings were adjusted to obtain maximum sensitivity. The laser energy (Nd:YAG laser, 355 nm) was optimized to achieve good signal to noise ratio above the threshold energy of MALDI matrix.

All the samples were analyzed on standard 96-wells stainless steel MALDI target plates that were pre cleaned thoroughly. Before spotting, the samples were premixed with MALDI matrix (mixture of DHB and internal standard). The samples (1µL per spot) were spotted manually in replicates using a micropipette. The quantitative data was acquired by using instrument in automatic acquisition mode. During automatic acquisition, the instrumental conditions and acquisition time was kept uniform across the MALDI target plate. For detection and identification of drugs molecules, precursor ion of each drug molecule was subjected to collision induced dissociation (CID) fragmentation.

3.2.4 Data analysis and method validation

The qualitative data analysis was performed with Masslynx 4.1 (Waters) and mMass (open source) software. For quantitative data analysis, in-house developed software,

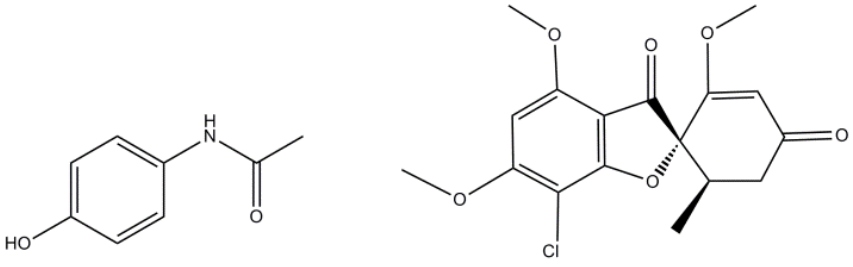
'MQ', was used to generate calibration curves based on ratios of peak intensities of analyte to internal standard. These calibration curves were used to determine concentration of drugs in quality control samples and drug spiked urine samples. Six replicates were used for each calibration point and average was calculated for generating the calibration curve. Linear regression equation was used in calculating correlation coefficient (R^2), slope, intercept, recoveries and relative standard deviations (RSD).

3.3 Results and discussion

3.3.1 Qualitative determination of drug molecules

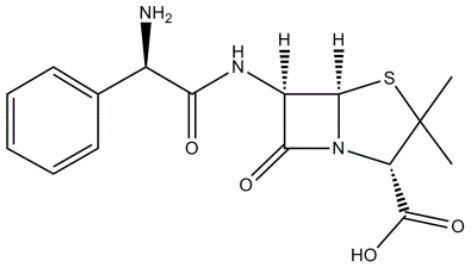
In this study, commonly used pharmaceutical drugs were taken from different classes of drugs. The drugs were selected according to their function, structure and molecular weight (Figure 3.1). Acetaminophen is a frequently used analgesic that has the lowest molecular weight among the drugs selected for this study. Gresiofulvin is a metabolic product of *P. grseiofulvum* and it has been used in dermatophytic infection as an antifungal agent. Ampicillin has been used extensively as an antibiotic in bacterial infections. It is a beta-lactam antibiotic and belongs to the penicillin family. Verapamil, a derivative of phenylalkylamine, is an effective anti-arrhythmic and vasodilator. It is also used as a calcium channel blocker. The qualitative study of drug molecules was carried out in two stages. Firstly, MALDI-TOF MS of the drug mixture premixed with DHB matrix was performed. All the drug molecules showed mainly protonated adduct of the respective molecular ion $[M+H]^+$ and had good mass accuracy (within 10 ppm). DHB matrix peaks were also present in the spectra, but the drug molecule peaks were clearly resolved. Subsequently, the precursor ions of drugs were allowed to fragment in the collision chamber. The fragmentation patterns

matched with those reported in previously published data and thus corroborating the identification of each drug molecule.[20-23]

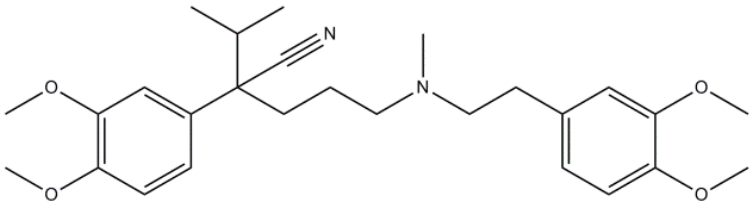


Acetamiophen

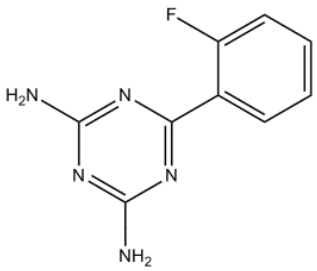
Griseofulvin



Ampicillin



Verapamil



2,4-diamino-6-(2-fluorophenyl)-1,3,5-triazine

Figure 3.1: *Chemical structures of pharmaceutical drugs spiked in urine and the internal standard used in this work.*

MALDI-TOF MS was subsequently used directly on urine samples spiked with drugs followed by MS/MS validation as described above for the pure standards. Figure 3.2 shows the representative MALDI-TOF MS of drugs from spiked urine samples. Zoomed-in portion of the spectra having protonated/sodiated adduct of drug

molecules are also shown in the figure. As expected, a very complex spectrum was obtained from spiked urine with many peaks seen across the entire mass range till *m/z* 600. This complexity can be attributed to the presence of various endogenous metabolites along with several possible adducts and fragments thereof. Accurate masses for protonated and sodiated adducts of all the spiked drugs were detected with good resolution and s/n ratio.

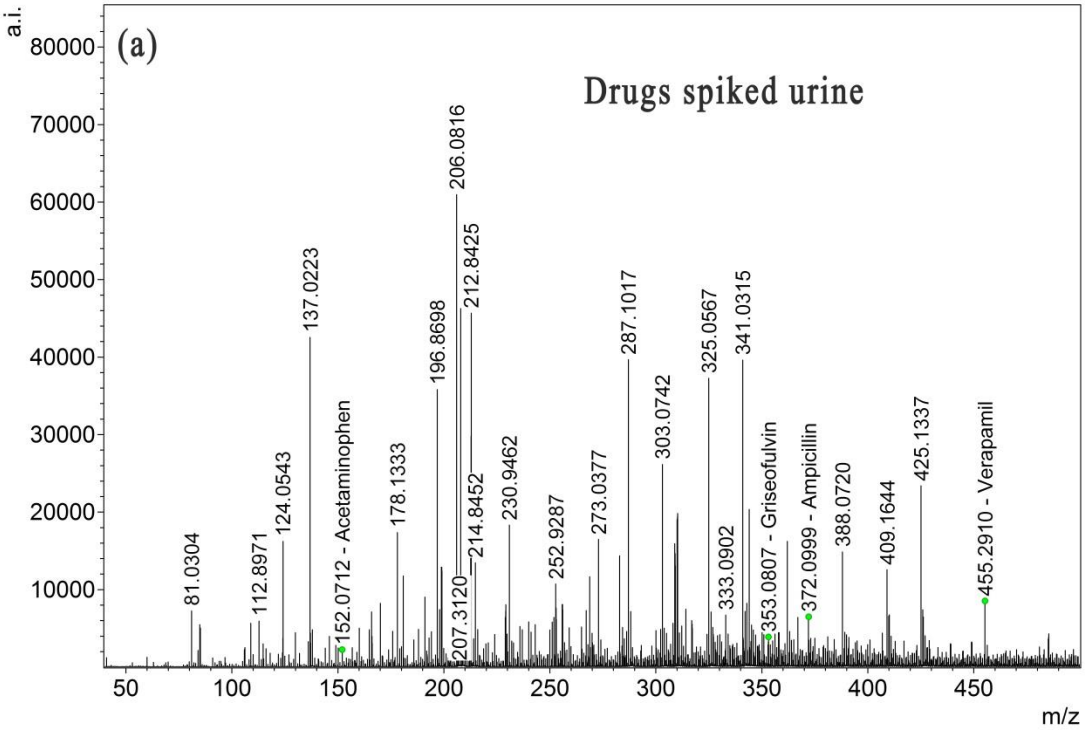


Figure 3.2a: *MALDI-TOF MS spectra of pharmaceutical drugs spiked in urine and analyzed directly. 2,5-dihydroxybenzoic acid (DHB) was used as matrix.*

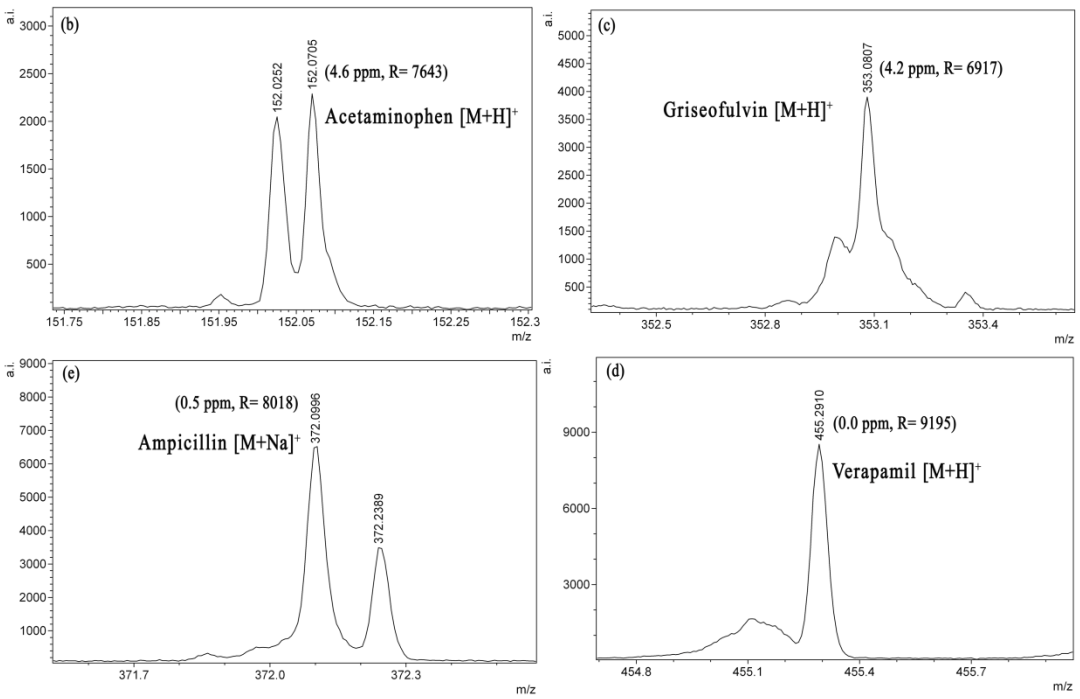


Figure 3.2b-e: *Enlarged MALDI-TOF MS spectra of individual pharmaceutical drugs showing mass accuracy and resolution.*

Table 3.1: *Comprehensive CID fragmentation of spiked drugs in urine. The expected masses of fragments were obtained from published reports.*[20-23]

Drug molecule	CID fragments	s/n ratio	Expected mass (*m/z*)	Observed mass (*m/z*)
Acetaminophen	$[M\text{-}H_2O+H]^+$	14	134.0606	134.0628
$C_8H_9NO_2$	$[M\text{-}COCH_2+H]^+$	185	110.0606	110.0606
$[M+H]^+$ 152.0712	$[M\text{-}COCH_2\text{-}NH_3+H]^+$	23	93.0341	93.0328
	$[M\text{-}COCH_2\text{-}H_2O+H]^+$	26	92.0500	92.0473
	$[M\text{-}COCH_2\text{-}CO+H]^+$	6	82.0657	82.0643
Ampicillin	$[M\text{-}NH_3+H]^+$	12	333.0910	333.0902
$C_{16}H_{19}N_3O_4S$	$[M\text{-}C_9H_8N_2O_2+H]^+$	49	174.0589	174.0590
$[M+H]^+$ 350.1175	$[M\text{-}C_6H_{10}N_2O_2+H]^+$	61	160.0427	160.0445
	$[M\text{-}C_{11}H_{12}N_2O_2S+H]^+$	41	114.0550	114.0384
	$[M\text{-}C_9H_{12}N_2O_4S+H]^+$	55	106.0651	106.0642
Griseofulvin	$[M\text{-}CH_4O+H]^+$	6	321.0530	321.0602
$C_{17}H_{18}ClO_6$	$[M\text{-}C_4H_4O+H]^+$	29	285.0530	285.0536
$[M+H]^+$ 353.0792	$[M\text{-}C_8H_{10}O_2+H]^+$	75	215.0111	215.0143
	$[M\text{-}C_8H_9O_3Cl+H]^+$	30	165.0553	165.0533
Verapamil	$[M\text{-}C_9H_{12}O_2+H]+$	58	303.2067	303.2090
$C_{27}H_{38}N_2O_4$	$[M\text{-}C_{11}H_{17}NO_2+H]^+$	12	260.1651	260.1663
$[M+H]^+$ 455.2910	$[M\text{-}C_{17}H_{26}N_2O_2+H]^+$	87	165.0916	165.0937
	$[M\text{-}C_{18}H_{29}N_2O_2+H]^+$	31	150.0681	150.0702

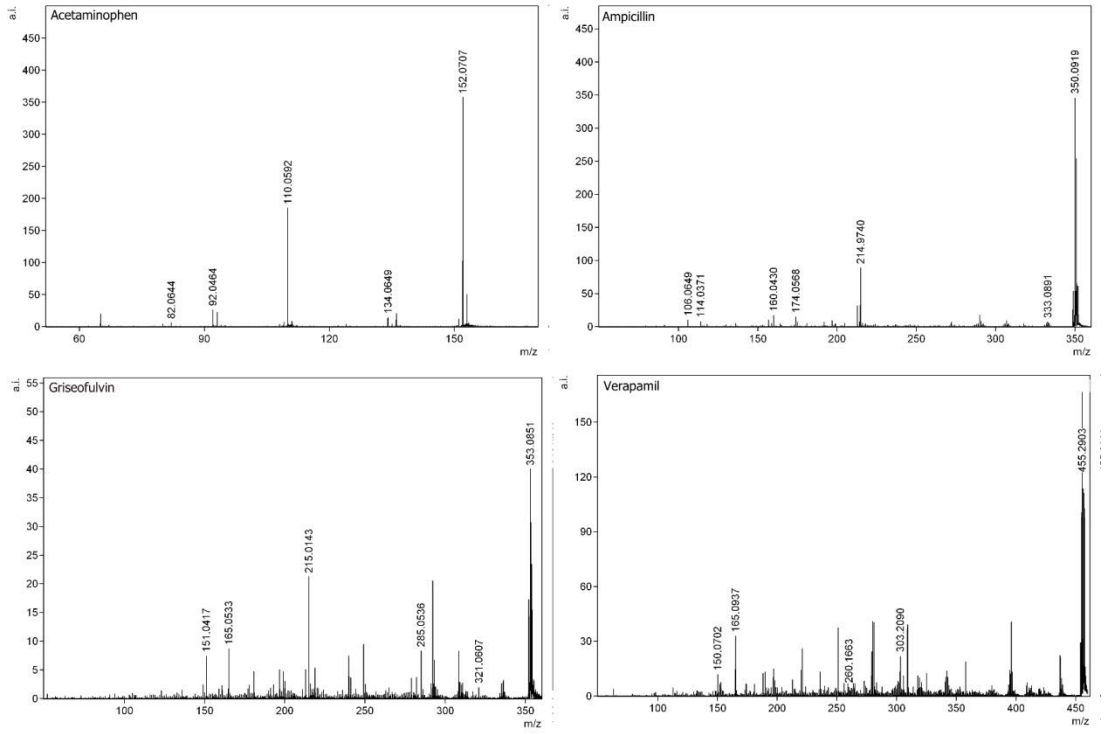


Figure 3.3: *MALDI-TOF CID MS/MS spectra of individual pharmaceutical drugs.*

MS/MS from precursor ions reveal the identity of analyte molecules unambiguously and is especially useful for analysis from complex samples such as urine. Table 3.1 summarizes the CID fragments of each drug molecules in MALDI-TOF MS/MS and figure 3.3 showing the fragmentation pattern. The overall identification of drugs spiked in urine was performed reproducibly in a rapid manner. It is noteworthy that all the drugs were simultaneously detected directly from the urine without any chromatographic separation or sample preprocessing.

3.3.2 Quantitative determination of drugs molecules

The quantitative trends of the drug molecules were studied by constructing calibration curves both from pure standards and subsequently from spiked urine samples.

Calibration responses were measured as peak height ratios of the sample normalized against an internal standard. Internal standard compensates for the variation in signal response which arises due to inhomogeneity in sample spotting and fluctuation in instrumental parameters over the time. The selection of internal standard was based on reproducibility of signals, miscibility, and non-interference with drug.[24, 25] Isotopically labeled internal standards have been a preferred choice of users but have disadvantages due to their cost and non-availability. Non labeled standards provide alternative to isotopic labeled internal standards. Previously published reports have successfully shown the usage of chemically non-analogous compounds as internal standards.[26, 27] Symmetric triazine derivative was used as internal standard, which is non-endogenous to urine metabolites. Table 3.2 represents the quantitative statistics of drug molecules directly quantified from mixture of drugs. All drug molecules showed good linearity with correlation coefficient (R^2) above 0.97. The calibration curve was prepared by taking average values of six replicates per dilution and the concentration range was started above the LOQ. The detection limit of standard solution of each drug molecule was estimated prior to quantitative analysis by taking those concentrations which showed signal-to-noise ratio (S/N) of peak above 3. Using these criteria, all the drug molecules were obtained with detection limits around low picomoles /µL. To verify the method, quality control samples containing drugs in low, mid and high concentration range of calibration curve were analyzed on the same target plate. Further recoveries of drugs from QC samples were calculated from the linear equation of calibration curve. All the drug molecules showed reproducible recoveries within 15% RSD in most cases, and extending up to 20% in a few, validating the linear relationship between concentrations of drugs and response from MALDI-TOF MS data.

Table 3.2: *Quantitation data for the mixture of drugs from standards.*

Drug	Conc. range	Calibration equation	R^2	QC conc.	% Recovery with %RSD
Acetaminophen	10-800 µM	y=0.001000x+0.052083	0.98	50 µM 100 µM 400 µM	127 (17) 99 (10) 117 (16)
Griseofulvin	0.2-12 µM	y=0.472238x+0.016586	0.99	1 µM 2 µM 8 µM	114 (4) 86 (16) 109 (7)
Ampicillin	5-300 µM	y=0.002830x+0.039271	0.98	25 µM 50 µM 100 µM	123 (19) 104 (18) 105 (11)
Verapamil	10-600 nM	y=0.003901+0.102985	0.97	50 nM 80 nM 200 nM	111 (15) 90 (14) 97 (15)

The quantitative analysis of drugs was further extended to samples present in urine to demonstrate the utility of the method for biological fluids. The blank urine was fortified with drugs, which simulate the presence of drug in excreted urine after administration of drug in human body. Acetaminophen, griseofulvin, ampicillin and verapamil were spiked in urine according to previously published reports (Table 3.3).[28-31] Serial dilutions of the drugs were added to urine samples with no further dilution. The LOD and LOQ of drug molecules were determined from spiked urine. Acetaminophen and ampicillin were detected up to 7.6 and 8.7 ng/µL whereas, the detection limit for griseofulvin and verapamil were found to be 0.35 and 0.036 ng/µL. The LOD and LOQ were higher for the urine spikes when compared with the pure standards. Calibration curves were generated by plotting concentration of drugs spiked and their ratios of peak height with internal standard. Table 3.4 summarizes concentration ranges, linearity equations, correlation coefficients and recoveries from spiked urine samples analyzed directly using MALDI-TOF MS with no sample pre-processing. Excellent linearity was observed for each drug molecule with high

correlation coefficients and reproducibility fulfilling 15/20 ± FDA criteria. The recoveries from QC samples were calculated as mean of 12 samples. 91-112% recoveries within 15% RSD except ampicillin where one of QC sample had RSD 19% were obtained.

Table 3.3: *Concentration range used in quantitation of drugs from urine samples.* [28-31]

Drug	Class	Conc. range in urine
Acetaminophen	analgesic	49-933 µM
Griseofulvin	antifungal	1.42-17 µM
Ampicillin	antibiotic	28-280 µM
Verapamil	antiarrhythmic	10-1000 nM

Once again, it is noteworthy that the entire analysis starting from sample preparation to data analysis was performed within a few hours and did not necessitate any chromatographic separation. The analysis time can further be reduced using automated spotting. Additionally, accuracy and reproducibility of data can also be improved with automation.

Until recently, MALDI was not considered as a suitable ionization method for small molecules analysis (< *m/z* 500) due to matrix interference in the low molecular region. However, several publications in the recent past have demonstrated the utility of MALDI-TOF MS in targeted small molecule analysis.[26, 27, 32] Newer generation of MALDI-TOF MS are equipped with high duty cycle analyzers and advanced electronics, which provide high resolution data and subsequently, mass accuracies less than 10 ppm. An accurate identification of a molecule can be obtained based on peaks with high mass accuracy and subsequent CID fragmentation. Peak area based quantitation can be challenging for metabolites and other small molecules from biological fluids in the absence of a chromatographic separation as often times the

contiguous peaks in mass spectrum is poorly resolved. Peak intensity based quantitation demonstrated on large sample sets previously has shown comparable efficiency as peak area based quantitation [26, 27], and can be advantageous. This approach is quite useful for MALDI-TOF MS where inconsistent desorption ionization processes may lead to poor quantitation.

Table 3.4: *Quantitation data for drugs spiked in urine followed by direct MALDI-TOF MS. Recovery was calculated for 12 replicates of each spiked concentration.*

Drug	Conc. range	Calibration equation	R^2	QC conc.	% Recovery with %RSD
Acetaminophen	200-2000 µM	y=0.000023x+0.009537	0.95	800 µM 1200 µM 1600 µM	96 (14) 91 (14) 98 (11)
Griseofulvin	4-40 µM	y=0.001243x+0.017721	0.98	16 µM 24 µM 32 µM	96 (14) 110 (14) 97 (12)
Ampicillin	100-1000 µM	y=0.000136x-0.001083	0.91	400 µM 600 µM 800 µM	94 (12) 103 (10) 93 (19)
Verapamil	200-2000 nM	y=0.000066x+0.003450	0.99	800 nM 1200 nM 1600 nM	112 (14) 111 (10) 98 (9)

3.4 Conclusions

The application of MALDI-TOF MS in qualitative and quantitative analysis of pharmaceutical drugs spiked in urine was demonstrated. Drug molecules were characterized by MALDI-TOF MS followed by CID fragmentation. High resolution and mass accuracies within 10 ppm along with MS/MS fragmentation patterns corresponding to the analytes of interest aid in unambiguous detection from urine. Peak height based quantitation performed using a chemically unrelated internal standard on both pure standards and spiked urine samples showed excellent sensitivity, linearity, accuracy and reproducibility. The method was validated with

quality control checks. The method reported establishes the versatility of MALDI-TOF MS in direct analysis from urine with minimal sample processing and without any chromatographic separations. Tolerance to sample impurities along with the higher throughput obtained using MALDI-TOF MS can increase the efficiency of ADME and similar bioanalytical studies.

3.5 References

(1) Want, E.; Compton, B. J.; Hollenbeck, T.; Siuzdak, G. *Spectroscopy-an International Journal* 2003, *17*, 681-691.

(2) Hopfgartner, G.; Bourgogne, E. *Mass Spectrometry Reviews* 2003, *22*, 195-214.

(3) Gillespie, T. A.; Winger, B. E. *Mass Spectrometry Reviews* 2011, *30*, 479-490.

(4) Tolonen, A.; Turpeinen, M.; Pelkonen, A. *Drug Discovery Today* 2009, *14*, 120-133.

(5) Plumb, R. S.; Potts, W. B.; Rainville, P. D.; Alden, P. G.; Shave, D. H.; Baynham, G.; Mazzeo, J. R. *Rapid Communications in Mass Spectrometry* 2008, *22*, 2139-2152.

(6) Lee, M. S.; Kerns, E. H. *Mass Spectrometry Reviews* 1999, *18*, 187-279.

(7) Shou, W. Z.; Zhang, J. *Expert Opinion on Drug Metabolism & Toxicology* 2010, *6*, 321-336.

(8) Yu, S. X.; Crawford, E.; Tice, J.; Musselman, B.; Wu, J. T. *Analytical Chemistry* 2009, *81*, 193-202.

(9) Chernetsova, E.; Morlock, G. *Bioanalytical Reviews* 2011, *3*, 1-9.

(10) Chernetsova, E. S.; Morlock, G. E. *Mass Spectrometry Reviews* 2010, *30*, 875-883.

(11) Bucknall, M.; Fung, K. Y. C.; Duncan, M. W. *Journal of the American Society for Mass Spectrometry* 2002, *13*, 1015-1027.

(12) Cohen, L. H.; Gusev, A. I. *Analytical and Bioanalytical Chemistry* 2002, *373*, 571-586.

(13) Masoud, Z.-M.; Elmar, H.; Andreas, T. *Rapid Communications in Mass Spectrometry* 2004, *18*, 141-148.

(14) Duncan, M. W.; Roder, H.; Hunsucker, S. W. *Briefings in Functional Genomics & Proteomics* 2008, *7*, 355-370.

(15) Szajli, E.; Feher, T.; Medzihradszky, K. F. *Molecular & Cellular Proteomics* 2008, *7*, 2410-2418.

(16) Karas, M.; Gluckmann, M.; Schafer, J. *Journal of Mass Spectrometry* 2000, *35*, 1-12.

(17) Bonnel, D.; Legouffe, R.; Willand, N.; Baulard, A.; Hamm, G.; Deprez, B.; Stauber, J. *Bioanalysis* 2011, *3*, 1399-1406.

(18) Takai, N.; Tanaka, Y.; Inazawa, K.; Saji, H. *Rapid Communications in Mass Spectrometry* 2012, *26*, 1549-1556.

(19) Pallua, J. D.; Schaefer, G.; Bittner, L. K.; Pezzei, C.; Huck-Pezzei, V.; Schoenbichler, S. A.; Meding, S.; Rauser, S.; Walch, A.; Handler, M.; Netzer, M.; Osl, M.; Seger, M.; Pfeifer, B.; Baumgartner, C.; Lindner, H.; Kremser, L.; Sarg, B.; Klocker, H.; Bartsch, G.; Bonn, G. K.; Huck, C. W. *Lc Gc North America* 2011, 21-28.

(20) Borlak, J.; Walles, M.; Elend, M.; Thum, T.; Preiss, A.; Levsen, K. *Xenobiotica* 2003, *33*, 655-676.

(21) Weinmann, W.; Stoertzel, M.; Vogt, S.; Svoboda, M.; Schreiber, A. *Journal of Mass Spectrometry* 2001, *36*, 1013-1023.

(22) Huang, C. R.; Gao, J.; Miao, L. Y. *Journal of Pharmaceutical and Biomedical Analysis* 2012, *59*, 157-161.

(23) Mistri, H. N.; Jangid, A. G.; Sanyal, M.; Shrivastav, P. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* 2007, *850*, 318-326.

(24) Wilkinson, W. R.; Gusev, A. I.; Proctor, A.; Houalla, M.; Hercules, D. M. *Fresenius Journal of Analytical Chemistry* 1997, *357*, 241-248.

(25) Lekha, S.; Dietrich, A. V. *Rapid Communications in Mass Spectrometry* 2005, *19*, 1928-1936.

(26) Markus, P.; Michael, K. *Rapid Communications in Mass Spectrometry* 2009, *23*, 3555-3562.

(27) Singh, A.; Panchagnula, V. *Analytical Methods* 2011, *3*, 2360-2366.

(28) Ibrahim, E.-S. A.; Abdel-hamid, M. E.; Abuirjeie, M. A.; Hurani, A. M. *Analytical Letters* 1988, *21*, 423-434.

(29) Zia, H.; Proveaux, W. J.; Odonnell, J. P.; Ma, J. K. H. *Journal of Chromatography* 1980, *181*, 77-84.

(30) Heitmeier, S.; Blaschke, G. *Journal of Chromatography B* 1999, *721*, 93-108.

(31) Kasim, E. A.; Ghandour, M. A.; El-Haty, M. T.; Ahmed, M. M. *Journal of Pharmaceutical and Biomedical Analysis* 2002, *30*, 921-929.

(32) Yukihira, D.; Miura, D.; Saito, K.; Takahashi, K.; Wariishi, H. *Analytical Chemistry* 2010, *82*, 4278-4282.

Quantitation of metabolites implicated in cardiovascular disease

4.1 Introduction

Metabolites play a vital role in different physiological and biochemical processes in all life forms. An array of small molecule metabolites from biofluids reflects the physiological snapshot of a dynamic system and their manifested measurable variations could potentially indicate disease.[1] Various biochemical assays, chromatographic, and mass spectrometry (MS) platforms have been used to measure metabolites.[2-7] Often, metabolites undergo biotransformations, degradations and in some cases systematic elimination. Thus, detection and quantitation of metabolites is challenging even in investigations that target a known set of metabolite markers. There continues to be a significant need for comprehensive, high throughput, and efficient analytical methods for metabolite detection from biological fluids.[8]

S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH), asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA) are known to regulate diverse biochemical functions inside the body. SAM is the prominent methyl donor in a cell and SAH is the end-product of this reaction. Thus, SAM and SAH ratio is crucial in determining the methylation status of a cell. ADMA is a known inhibitor of nitric oxide synthase (NOS) and is implicated directly to cardiovascular disease. SDMA on the other hand, is a chronic kidney disease marker. Incidentally, ADMA and SDMA are structural isomers. Analytical methods with chromatographic

separation as a front end coupled to either mass spectrometer (MS), UV-Vis and fluorescence as analyzer is widely reported in literature for these metabolites.[2-4, 9-11] However, separation and sample preparation are time-consuming as some samples require derivatization or different column chemistries. MALDI MS detection of ADMA and SDMA has been reported.[12, 13] MALDI MS quantitation of these metabolites has not been reported as yet.

In this study, we report peak area-based MALDI-TOF quantitation of these four metabolites that are clinically relevant and give rise to multiple fragment ions in the MS mode. The possibility of beneficially utilizing the apparent disadvantage of fragmentation to simultaneously quantify targeted metabolites was demonstrated.

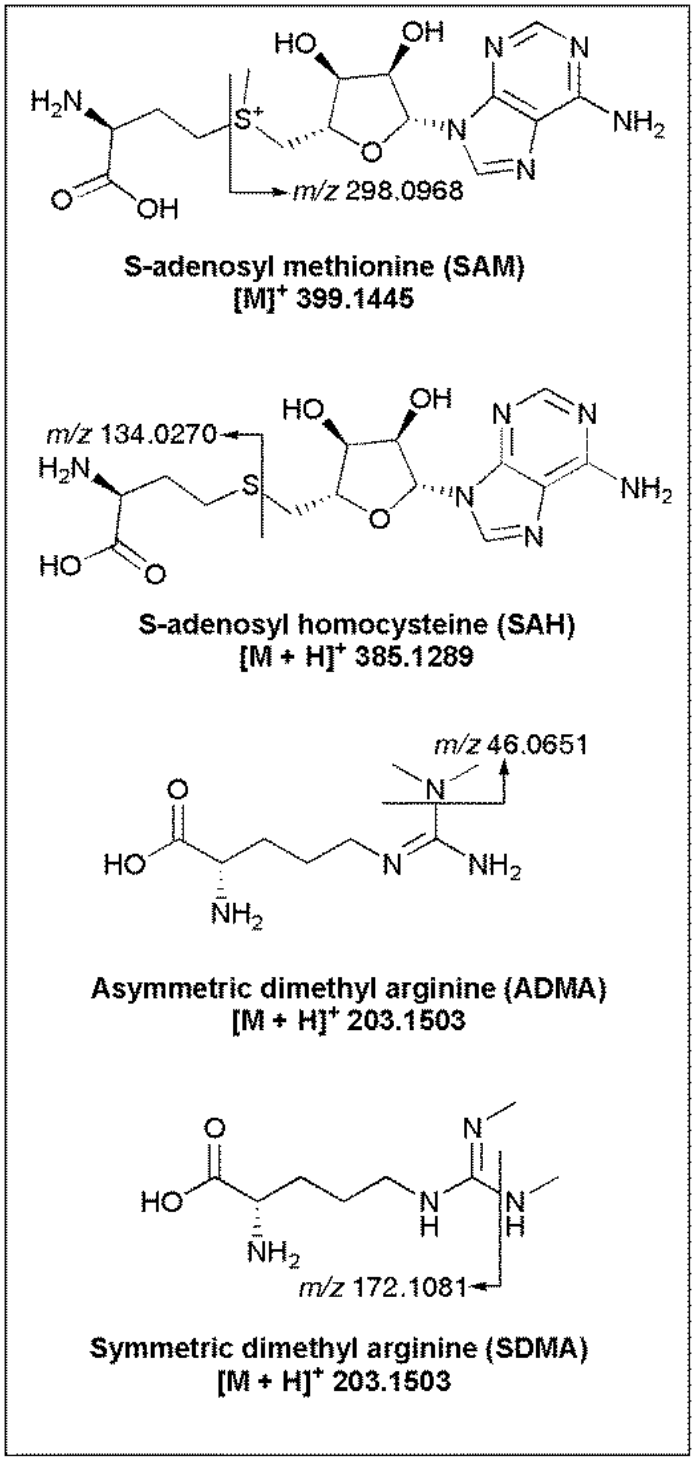


Figure 4.1: *Structures of SAM, SAH, ADMA and SDMA along with their respective unique fragment ions formed in the MS or MS/MS mode. The exact masses of molecular ions are indicated by* $[M]^+$ *or* $[M+H]^+$ *notations and exact masses of the fragment ions are indicated by m/z notation.*

4.2 Experimental

4.2.1 Materials

SAM (75%), SAH (98%), ADMA (99%), SDMA (99%), LC-MS grade acetonitrile, methanol, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. 2,5-dihydroxybenzoic acid (2,5-DHB) was purchased from Fluka. L-methoxytyrosine was purchased from LobaChemie (India). Deionised water with specific resistivity 18.2 $M\Omega\ cm^{-1}$was collected from SG ultrapure water unit (Germany).

4.2.2 Sample preparation

100 µM stock solutions of SAM, SAH, SDMA and ADMA were prepared in acetonitrile: 0.1% TFA (1:1, v/v) and stored at -20°C. 100 µL aliquots were dispensed in separate tubes. A maximum of three freeze thaw cycles were allowed. Dilutions series for the standard solution calibrators with final concentrations of 0.5, 0.75, 1, 2.5, 5, 7.5, 10 µM, and quality control samples were prepared separately from the stock solutions and used for quantitation.2,5-DHB was prepared in acetonitrile : 0.1%TFA (1:1, v/v) and used as MALDI matrix. 1µL of matrix was spotted on individual wells of MALDI target plate and dried in air. Analyte samples premixed with internal standard were spotted on to the matrix layer. L-methoxy tyrosine was used as internal standard in preparing calibration curve from analyte standards 0.5 mM. While preparing the samples, care was taken to avoid contamination.

4.2.3 MALDI-TOF MS analysis

Qualitative and quantitative analysis was performed on Waters Synapt HDMS with the MALDI ionization source operated in reflectron V-positive ion mode. The detector voltage was set at 1750 V after detector sensitivity test. Detector sensitivity was optimized by standardizing the ion counts of standard Glu-1-fibrinopeptide B

(Waters) as recommended by the manufacturer. Temperature of the room was maintained at 22°C. Quadrupole filters were set to pass ions between 40 - 600 *m/z*. All mass spectra were acquired with optimized laser energy (Nd:YAG, 355 nm for Waters Synapt HDMS. Prior to acquisition, the instrument was calibrated with PEG (mixture of PEG 200, 600 and 1000) to obtain rms mass accuracy within 5 ppm. For sample spotting, standard 96-well stainless steel MALDI target plate were used after rigorous cleaning as per manufacturers' instructions to avoid contamination from previous experiments. No harsh reagents were used for cleaning. A calibration in MS/MS mode was performed to achieve exact masses of all fragmented ions. To avoid interferences, the precursor ion selection window was adjusted to select precursor ion within a Dalton unit. The quadrupole was set according to precursor mass to achieve maximum sensitivity. For tandem MS of all analytes, nitrogen gas was used in the collision cell.

4.2.4 Data analysis

Data procedding was performed with Masslynx 4.0 (Waters) and mMass.[14] Quantitative interpretation of the MALDI MS data was performed using a a homebuilt software tool 'MQ' as reported earlier.[15, 16] MQ compliments the MALDI-TOF MS workflows and offers a user-friendly interface along with an ability to process large number of files within a short timeframe (Upto 100 files can be processed within 15 minutes). Raw instrumental data was first converted to ascii format using 'mzXML2txt', also developed in-house. For calibration curves, and for calculating recoveries from QC samples, the ratios of peak area or peak intensities of the analyte to internal standard were considered. Peak areas were calculated within an optimal mass extraction window. A ppm window of 20-35 was provided for entire analysis. Regression coefficients based on unweighted univariate linear regression fitting

method were estimated and the concentration response curves were plotted. QC samples as unknowns were subsequently estimated using these response curves.

4.3 Results and discussion

4.3.1 MALDI-TOF MS and MS/MS analysis of metabolite standards

Structure of SAM SAH ADMA and SDMA is given in figure 4.1. SAH is the precursor of homocystein in the body and demethylated product of SAM whereas the SDMA and ADMA are the structural isomers and isobars. To characterize all the analytes, MALDI-TOF MS was performed on standard solutions containing SAM, SAH, ADMA and SDMA. Figure 4.2 show the MALDI-TOF MS spectrum of mixture of SAM, SAH, ADMA and SDMA with comparison to 2,5-DHB. No interference from DHB peak was found when the mixture of SAM, SAH, ADMA and SDMA was analyzed. SAM showed very intense peak at *m/z* 399.1524 which correspond to $[M]^+$. Because of a positive charge on sulfur atom along with methyl group on SAM, further protonation or adduct formation is not feasible during ionization. Remaining other analyte showed mainly $[M+H]^+$ peak for SAH at *m/z* 385.1355, for ADMA and SDMA at *m/z* 203.1552 reproducibly from the standard solutions. In addition to the molecular ion peaks, fragment ions belonging to all the analytes were also observed in the MS mode itself. The structures of the analytes and the *m/z* values for the respective molecular and unique fragment ions are shown in figure 4.3. The fragment ions observed in the MS mode were: SAM fragment ion at *m/z* 298.0998; SAH fragment ion at *m/z* 134.0280; SDMA fragment ion at *m/z* 172.1114 SAM and SAH also yielded common fragment ions at *m/z* 136.0618 corresponding to the adenosine ring. The precursor ions and their corresponding fragments in the MS mode had mass accuracies within 10-20 ppm. At higher

concentrations, SDMA and ADMA could be differentiated in MS mode itself based on the unique fragment ions formed at *m/z* 172.1114 and *m/z* 46.0579 respectively. However, at lower micromolar concentrations, the unique fragment ion of ADMA was not observed or observed with higher mass error (180 ppm). This could be due to a lack of calibration method below *m/z* 50 for the MALDI-TOF analyzer used. The analyte adducts formed in the MS mode and *m/z* values observed were consistent with previously published literature.[17, 18]The targeted analyte peaks including the fragment peaks were observed with good S/N values. Interestingly, the unique fragment ions of SAM and SAH showed a better peak resolution as compared to the peak broadening of their respective molecular ions**.**

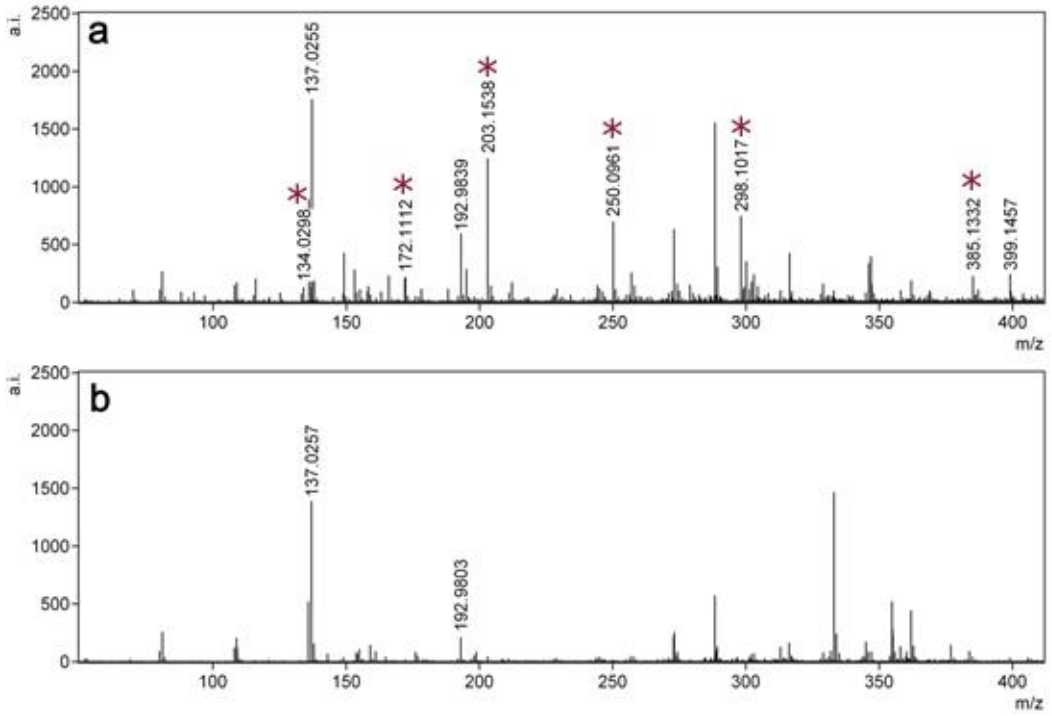


Figure 4.2: *MALDI-TOF MS spectra of (a) SAM, SAH, ADMA and SDMA (b)MALDI matrix 2,5-dihydroxybenzoic acid as control. Analytes are marked with asterisk.*

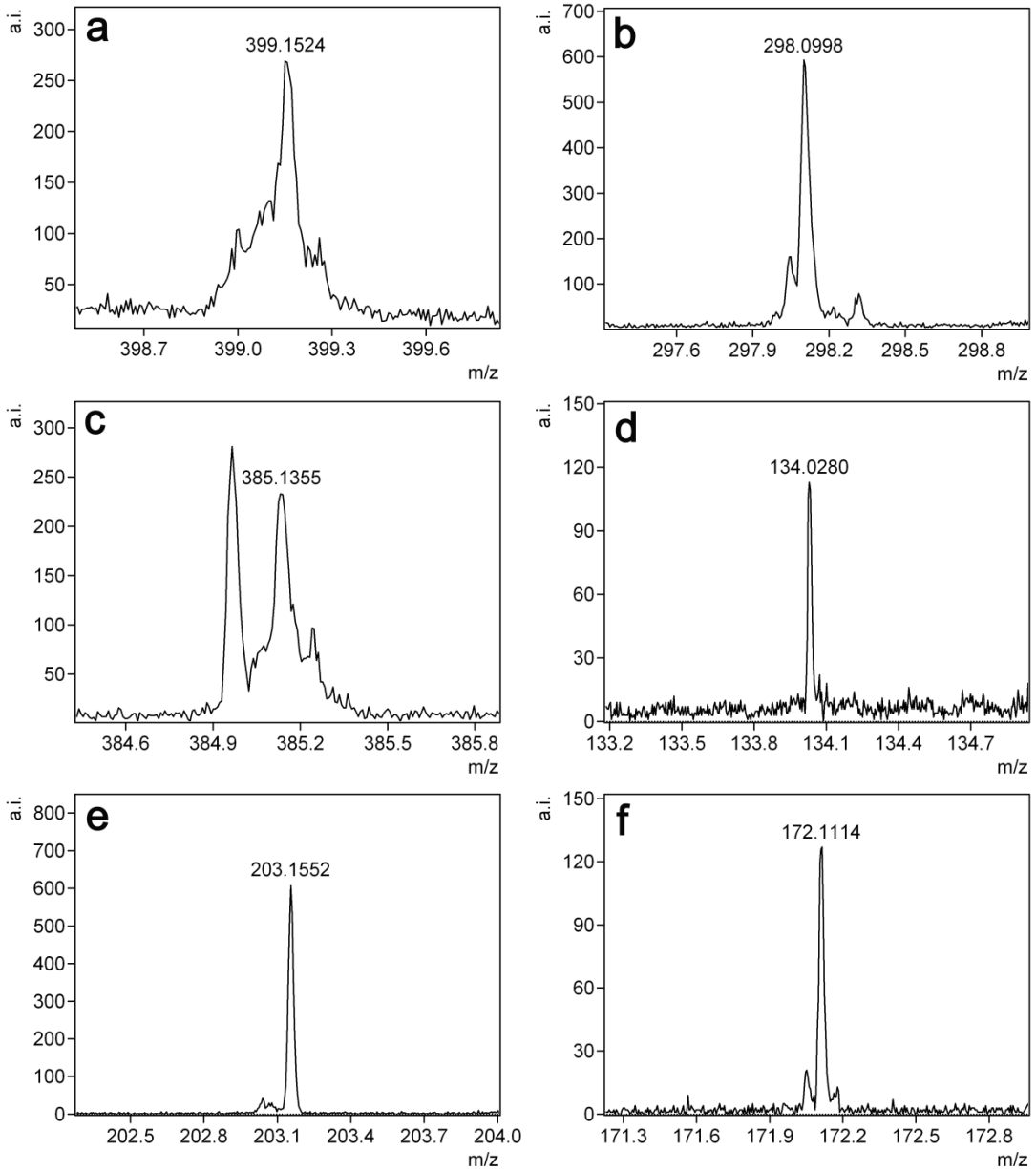


Figure 4.3: *MALDI-TOF MS of SAM, SAH and SDMA from a mixture of standards at 0.8 μM each showing the m/z peaks of both the precursor ions as well as their unique fragments visible in the MS mode. The peak annotations are: Precursor ion peaks (left panel, a, c and e respectively): $[M]^+$ peak for SAM at m/z 399.1524; $[M+H]^+$ peak for SAH at m/z 385.1355; $[M+H]^+$ peak representing SDMA at m/z 203.1552; Unique fragment ion peaks (right panel, b, d and f respectively): SAM fragment ion at m/z 298.0998; SAH fragment ion at m/z 134.0280; SDMA fragment ion at m/z 172.1114.*

The precursor ions were further subjected to collision induced dissociation (CID). MS/MS patterns of the individual analytes from standard solutions are shown in

Figure 4.4. The specific analyte fragment ions observed in the MS mode itself were unambiguously detected in the MS/MS mode along with all the previously reported fragments for SAM, SAH, ADMA and SDMA.[17-19] MS/MS fragments for ADMA and SDMA clearly indicate the presence of the unique fragment ions at *m/z* 46 and 172 respectively. Thus, MS/MS results conclusively ascertain the identities of the precursor ions. Further, the unique ions from the self-fragmentation of SAM, SAH, SDMA (*m/z* 298, 134 and 172 respectively) were also subjected to CID induced fragmentation. These matched with the fragments generated from the MS/MS of the parent ions validating the respective ions formed during MS mode self-fragmentation.

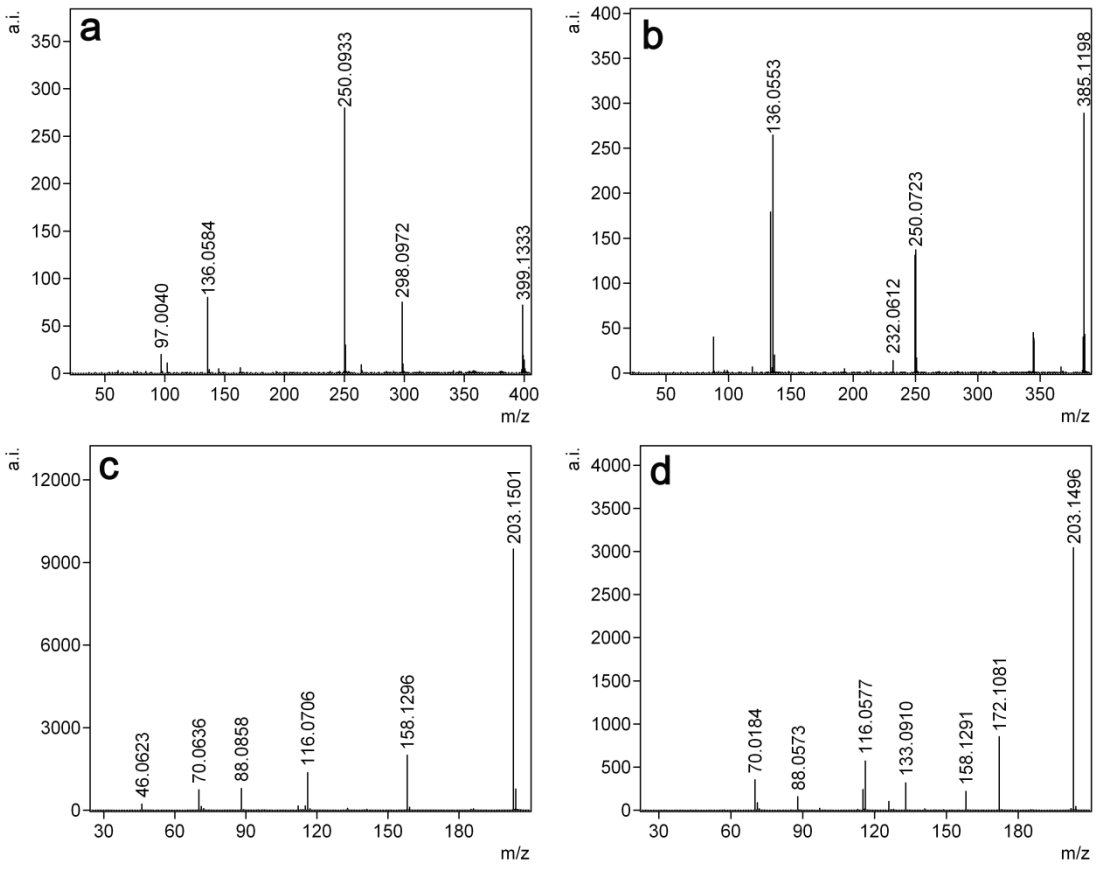


Figure 4.4: *MALDI-TOF MS/MS from standards of (A) SAM, (B) SAH, (C) ADMA and (D) SDMA. Fragment ion descriptions and the corresponding m/z values observed are as outlined in Table 1. MS/MS fragmentation pattern confirms the identity of the analyte precursor ions. Moreover, they also validate the analyte self-fragment ions observed in the MS mode itself.*

4.3.2 Quantitative MALDI-TOF MS analysis of metabolite standards

The quantitative study of analyte was studied by constructing calibration curves. Before the generation of calibration curve, limit of detection (LOD) and limit of quantitation (LOQ) was determined on the basis of signal-to-noise ratio (S/N) of peak intensity of individual ion in MALDI spectrum. Since due to self-fragmentation of analytes in MS mode, LOD and LOQ of molecular ion peak as well as fragment peak was determined (Table 4.1). Interestingly SAM and SAH have shown LOD 200 nM and LOQ 250 nM for fragments whereas, $[M]^+$ of SAM and $[M+H]^+$ of SAH exhibits higher LOD compared to their fragments. In case of SDMA, the LOD and LOQ for molecular ion and fragment were comparable (LOD - 100 nM and LOQ -200 nM). Thus, for quick distinction of SDMA from ADMA (especially when present in a mixture or complex biological sample), SDMA's fragment was considered for quantitation. Molecular ion was considered for quantitative analysis of ADMA (LOD – 100 nM and LOQ – 200 nM) since; ion at *m/z* 46.06 Da was not reproducible in MS mode. The lower LOD and LOQ of fragments of SAM and SAH clearly indicate the advantages of fragments when analysis is performed at very low concentration.

Table 4.1: *LOD and LOQ of molecular ion peak as well as fragment peak of SAM, SAH and DMA isomers.*

Analyte	Ion (*m/z*)	LOD (µM)	LOQ (µM)
SAM	399.1395	0.5	0.75
	298.0963	0.2	0.25
SAH	385.1286	0.5	0.75
	134.0282	0.2	0.25
SDMA	203.1508	0.1	0.2
	172.1097	0.1	0.2
ADMA	203.1508	0.1	0.2

After determining the LOD and LOQ of each analyte, calibration curves were generated. In the calibration curve, lowest concentration was well above the LOQ. Standard solution of each analyte was diluted to the concentration range which represents the physiological concentration in the human plasma. Instrumental conditions were optimized prior to quantitative analysis and all the spectra were obtained in automated mode. Further to validate the calibration curve, quality control samples of known quantity were placed on same target plate containing the dilution series of standards. Typically two QC samples were used, one in lower range of concentration and other in higher range of concentration.

The 'MQ' software provided all the quantitative results for SAM, SAH, ADMA and SDMA which are summarized in table 4.2. Linear regression equation was considered for the generation of calibration curve. Due to spot-to-spot irreproducibility in MALDI-TOF MS, internal standard was used to compensate the variation. Here methoxy tyrosine was used as an internal standard. The internal standard was selected on the basis of molecular mass and solubility with analytes. Peak area ratios of the analyte molecular ions or the fragments and the internal standard were used for quantitation. Calibration curves for SAM, SAH and SDMA standards were constructed using both the molecular ion peaks and fragments, while only the molecular ion peak was used for ADMA. The calibration curves exhibited good linearity with R^2 in the range of 0.92-0.99. Recoveries for QC samples were obtained in the range of 73-120 % with RSDs < 20%. The QCs for 2.5 µM using the SAM molecular ion peak and fragment showed RSDs higher than 20%. Slopes of the calibration curves increased marginally for the calibration curve based on fragments for SAM compared to the precursor ion. The corresponding fragment slopes for SAH and SDMA decreased.

Based on the above results, it is clearly shown that MALDI-TOF MS is appropriate for the quantitative analysis of metabolites which have tendency to fragment in ion source. Furthermore in the biological sample where concentration of these metabolites is already in very low concentration, fragment based quantitation is better way. The smaller LOD/LOQ and of fragment ion also support the quantitation based on fragment ion rather than molecular ion based quantitation.

The concentration ranges used for the metabolite quantitation are physiologically relevant. In the diseased state, SDMA and ADMA are present in the low micromolar range[20] in plasma. SAM and SAH are also present in the micromolar range in urine and RBCs.[21] Thus, the method can be further adapted for estimating these metabolite markers from biological fluids. Absolute quantitation using a non-endogenous, chemically similar, synthetic internal standard removed any inconsistencies in the signal generated as well as made comparison across spectra possible removing systematic errors. It is also interesting to note that the chemically similar internal standard also generated fragmentation similar to the metabolites. Although a reproducible fragment from the internal standard was used in this work, selection of a non endogenous chemically dissimilar standard would be preferable for a targeted metabolomics analysis from biological matrices.

Table 4.3: *MALDI-TOF MS quantitation of SAM, SAH and DMA isomers from standard solutions.*

Analyte	Ion (*m/z*)	Conc. range (µM)	Calibration Equation; R^2	QC samples (µM)	% Recovery (% RSD)
SAM	399.1395	0.75-10	y=0.054x+0.033; 0.97	2.5 8	78 (22.5) 110 (10.4)
	298.0963	0.5-10	y=0.086x+0.033; 0.97	2.5 8	81 (26.6) 104 (14.6)
SAH	385.1286	0.75-10	y=0.237x+0.062; 0.93	2.5 8	73 (10.9) 116 (12.2)
	134.0282	0.5-10	y=0.058x+0.020; 0.94	2.5 8	78 (9.6) 114 (14.2)
SDMA	203.1508	0.5-10	y=0.029x-0.012; 0.98	2 8	74 (10.2) 99 (19.0)
	172.1097	0.5-10	y=0.006x-0.002; 0.97	2 8	75 (10.8) 106 (19.8)
ADMA	203.1508	0.5-10	y=0.050x+0.032; 0.99	2 8	105 (10.4) 120 (16.7)

4.4 Conclusion

Fragmentation occurring from in source or post source decay (ISD or PSD) is a common phenomenon observed. The mechanisms involved in fragmentation with an aim of controlling fragmentation or post source decay have been investigated before.[22] In MALDI, fragmentation is generally regarded as unimolecular decomposition of metastable ions.[22] It has been broadly attributed to higher laser energies (and intensities, fluences), initial velocity of analytes, collisional effects in the plume, exothermic proton transfer reaction arising out of varying matrix (low PA) and analyte (higher PA than matrix) proton affinities. Fragmentation has been thought to

occur via either a radical-induced or a collision activated pathway.[23] Radical induced pathway rests on the formation of hydrogen bonds between analyte and matrix in condensed state that upon UV excitation gives rise to hydrogen radical transfer reactions ultimately leading to breakdown of analyte into ISD fragments. This mechanism was probed using hydrogen/deuterium exchange reactions[24] and 2,5-DHB was found to generate fragmentation. On the other hand, collisions in the plume are thought to lead to PSD. An understanding of the underlying mechanisms helps in designing experiments with different applications of ISD for characterizing biomolecules. The results from the current study demonstrate that MALDI-TOF MS quantitative analysis of clinically relevant metabolites can be achieved using the respective fragment ion peaks of the metabolites.[25] Simultaneous measurement of multiple metabolites using MALDI-TOF MS has the potential to facilitate large scale clinical investigations for biomarker discovery.[6] As a high resolution method, MALDI-TOF MS also has the potential for use in diagnostics once the disease markers are established.

4.5 References

(1) Patti, G. J.; Yanes, O.; Siuzdak, G. *Nature Reviews Molecular Cell Biology* **2012**, *13*, 263-269.

(2) Brown, C. M.; Becker, J. O.; Wise, P. M.; Hoofnagle, A. N. *Clin. Chem. (Washington, DC, U. S.)* **2011**, *57*, 701-709.

(3) El-Khoury, J. M.; Bunch, D. R.; Reineks, E.; Jackson, R.; Steinle, R.; Wang, S. H. *Anal. Bioanal. Chem.* **2012**, *402*, 771-779.

(4) Teerlink, T.; Nijveldt, R. J.; de Jong, S.; van Leeuwen, P. A. M. *Anal. Biochem.* **2002**, *303*, 131-137.

(5) Wang, D.; Strandgaard, S.; Borresen, M. L.; Luo, Z. M.; Connors, S. G.; Yan, Q.; Wilcox, C. S. *American Journal of Kidney Diseases* **2008**, *51*, 184-191.

(6) Griffiths, W. J.; Koal, T.; Wang, Y. Q.; Kohl, M.; Enot, D. P.; Deigner, H. P. *Angew. Chem. Int. Edit.* **2010**, *49*, 5426-5445.

(7) Zinellu, A.; Sotgia, S.; Usai, M. F.; Pintus, G.; Deiana, L.; Carru, C. *Analytical and Bioanalytical Chemistry* **2011**, *399*, 1815-1821.

(8) Meinitzer, A.; Kielstein, J. T.; Pilz, S.; Drechsler, C.; Ritz, E.; Boehm, B. O.; Winkelmann, B. R.; MÃ¤rz, W. *Clin. Chem. (Washington, DC)* **2011**, *57*, 112-121.

(9) Fleck, C.; Schweitzer, F.; Karge, E.; Busch, M.; Stein, G. *Clin. Chim. Acta* **2003**, *336*, 1-12.

(10) Gellekink, H.; Van Oppenraaij-Emmerzaal, D.; van Rooij, A.; Struys, E. A.; den Heijer, M.; Blom, H. J. *Clin. Chem. (Washington, DC, U. S.)* **2005**, *51*, 1487-1492.

(11) Martens-Lobenhoffer, J.; Bode-Boger, S. M. *Clin. Chem. (Washington, DC, U. S.)* **2006**, *52*, 488-493.

(12) Gehrig, P. M.; Hunziker, P. E.; Zahariev, S.; Pongor, S. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 142-149.

(13) Shirakawa, T.; Kako, K.; Shimada, T.; Nagashima, Y.; Nakamura, A.; Ishida, J.; Fukamizu, A. *Mol. Med. Rep.* **2011**, *4*, 615-620.

(14) Strohalm, M.; Kavan, D.; Novak, P.; Volny, M.; Havlicek, V. *Anal. Chem.* **2010**, *82*, 4648-4651.

(15) Singh, A.; Bhattacharya, N.; Ghanate, G.; Panchagnula, P. *Lc Gc North America, Current Trends in Mass Spectrometry* **2013**, *march 2013*, 24-29.

(16) Singh, A.; Panchagnula, V. *Analytical Methods* **2011**, *3*, 2360-2366.

(17) Cataldi, T. R. I.; Bianco, G.; Abate, S.; Mattia, D. *Rapid Communications in Mass Spectrometry* **2009**, *23*, 3465-3477.

(18) Gehrig, P. M.; Hunziker, P. E.; Zahariev, S.; Pongor, S. *J. Am. Soc. Mass. Spectrom.* **2004**, *15*, 142-149.

(19) Appolonova, S. A.; Dikunets, M. A.; Rodchenkov, G. M. *Russ. J. Phys. Chem. A* **2009**, *83*, 520-529.

(20) Kielstein, J. T.; Boger, R. H.; Bode-Boger, S. M.; Frolich, J. C.; Haller, H.; Ritz, E.; Fliser, D. *J. Am. Soc. Nephrol.* **2002**, *13*, 170-176.

(21) Stabler, S. P.; Allen, R. H. *Clin. Chem. (Washington, DC, U. S.)* **2004**, *50*, 365-372.

(22) Zenobi, R.; Knochenmuss, R. *Mass Spectrometry Reviews* **1998**, *17*, 337-366.

(23) Cai, Z.; Liu, S.; Debois, D.; Smargiasso, N.; Demeure, K.; Asakawa, D.; Zimmerman, T.; Quinton, L. c.; Pauw, E. In *Applications of MALDI-TOF Spectroscopy*; Springer Berlin Heidelberg, 2013; Vol. 331, pp 117-141.

(24) Kocher, T.; Engstrom, A.; Zubarev, R. A. *Anal. Chem.* **2005**, *77*, 172-177.

(25) Andrade, F.; Rodriguez-Soriano, J.; Prieto, J. A.; Aguirre, M.; Ariceta, G.; Lage, S.; Azcona, I.; Prado, C.; Sanjurjo, P.; Aldamiz-Echevarria, L. *Nephrol. Dial. Transpl.* **2011**, *26*, 328-336.

Quantitative analysis of atrazine and related herbicides

5.1 Introduction

Herbicides constitute almost half of the large number of chemicals encompassing all pesticide classes used in modern agricultural practices. Many of the herbicides have slow degradation process resulting in their transportation to the surface and ground water bodies through percolation and runoff. It is estimated that approximately 11261 tonnes of herbicides were used in India during the year 2009-2010.[1] Triazine compounds are an important class of herbicides with widespread use for containing growth of broad-leaf weed and annual grasses.[2] Atrazine, a triazine herbicide, constitutes a major part of the herbicide market, despite being banned entirely in the European Union. Atrazine and other related triazine compounds are commonly used during production of a diverse variety of crops, including rice, sugar cane, corn, pulses, and other fruits and vegetables.[2] These triazine pesticides are routinely monitored and controlled in most countries with strict limitations on permissible limits in foods, soil, ground water and industrial effluent. The established permissible limits themselves though are under intense scrutiny. Atrazine in particular has been studied extensively for its adverse effects on soil condition [3-5], microbial systems [5-8], organisms such as amphibians, rodents [9, 10] as well as the presence of its long-lasting residues in water systems.[11-13] It has also been implicated in defects at birth in humans.[14] Numerous studies have been conducted in the past to establish possible carcinogenicity of the triazine pesticides, in particular atrazine.[15-18] Though less

explored than atrazine, simazine and other triazine herbicides also prove to be harmful to the eco-systems useful in agriculture and leach into the water table effortlessly.[11] Despite the current debate over complete ban on the use of atrazine and other triazine herbicides, there is a continued need for monitoring and assessment of their presence in environment and potential harmful effects.

Currently defined maximum permissible levels are generally based on the limit of detection (LOD) and limit of quantitation (LOQ) of an analytical method which is widely accepted for analysis of the compounds in question. Several contemporary analytical techniques have been utilized for establishing analytical methods for Atrazine and related herbicides. Though methods using thin-layer chromatography (TLC) [19, 20], gas chromatography (GC) [21, 22], electrokinetic capillary chromatography (EKC) [23] have been established, the majority of quality control laboratories rely on the use of high pressure liquid chromatography coupled with diode array detector (HPLC-DAD) [23] or mass spectrometry [24-27] (HPLC-MS or LC-MS) analyzers for detection and quantification of these pesticides and their chemical modification in variety of sources. Several LC-MS methods have been published with successful application of sample preparation techniques using online solid phase extraction [28, 29], nano-tube based extraction [30], molecularly imprinted polymer beads.[31] Due to significant applications of pesticide analysis, several critical reviews of analytical methods have been published comparing multiple platforms for several classes of pesticides in a variety of matrices.[21, 32, 33] Mass spectrometry-based analytical techniques such as MALDI-TOF MS have been explored for quantitative analysis of various pesticides [34-36], but are - yet to be employed within the industry for routine analysis.

The application of MALDI-TOF MS method for the identification and quantitation of triazines and melamine contamination in milk has been published previously by our

group with emphasis on the high-throughput screening achieved and applicability of minimal sample preparation procedures.[36] Qualitative MALDI detection and characterization of triazine herbicides has been previously explored with different MALDI matrices.[37] One report of qualitative MALDI-TOF MS of representative herbicides and several classes of pesticides has also been published [34] where the emphasis was on the selection of appropriate matrices for the detection of pesticides. However a comprehensive quantitative MALDI-TOF MS analysis of atrazine and related herbicides is yet to be explored. In this work, we report MALDI-TOF MS quantitation of atrazine and related herbicides from water samples. Confirmation of analytes was based both on high resolution data (high mass accuracy) and MS/MS fragmentation. Overall the chromatography free method presented in this study showcases an analytical approach using high resolution mass spectrometry drastically improving throughput while achieving high sensitivity.

5.2 Experimental

5.2.1 Materials

1 mg/mL mixture of ametryn (*N*-ethyl-*N′*-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine), atrazine (1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine), prometon (*N,N′*-diisopropyl-6-methoxy-1,3,5-triazine-2,4-diamine), prometryn (*N,N′*-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine), propazine (6-chloro-*N,N′*-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine), simazine (6-chloro-*N,N′*-diethyl-1,3,5-triazine-2,4-diamine) and terbutryn (*N*-(1,1-dimethylethyl)-*N′*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine) (donated by AB Sciex), 2,4-Diamino-6-(2-fluorophenyl)-1,3,5 triazine and 2,5-dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA), methanol (MeOH) and acetonitrile (ACN) were purchased

from Sigma Aldrich [Bangalore, India]. Distilled and deionized water (DI water) with specific resistivity 18.2 MΩ cm^{-1} was from SG ultrapure water unit (Germany).

5.2.2 Sample preparation

Stock solution of herbicide mixture was prepared at 2 ppm concentration in 1:1 H_2O:MeOH solution. LOD and LOQ studies were performed with solutions of final concentration from 0.1 ppb to 75 ppb level (0.1, 0.2, 0.5, 0.75, 1, 2, 10, 20, 30, 50, 75 ppb levels). The 2,4-Diamino-6-(2-fluorophenyl)-1,3,5 triazine (IS) solution was prepared at concentration of 2 ppm level in 1:1 H_2O:MeOH diluent solution. A final calibration study solution for spotting were separately prepared by 1:1 addition of dilutions and internal standard solution at concentrations 1 ppm, 0.8 ppm, 0.6 ppm, 0.4 ppm, 0.2 ppm, 0.08 ppm, 0.06 ppm, 0.04 ppm, 0.02 ppm, 0.01 ppm, 0.008 ppm, 0.006 ppm, 0.004 ppm. DHB solution was prepared at 10 mg/mL in 1:1 ACN:0.1% TFA solution to be used as matrix. No additional concentration steps were used.

5.2.3 MALDI-TOF MS analysis of herbicides

MALDI-TOF MS was performed with AB Sciex 5800 TOF/TOF system. Analysis was carried out in the positive reflectron mode with laser energy (4800 units) optimized for DHB matrix. High number of laser shots (5000) was applied to maximise the sample desorption from the surface. Prior to analysis the instrument was calibrated with mixture of small synthetic molecules for mass accuracy in 50-300 *m/z* region. MALDI target plates were prepared by spotting 1 μL of the 1:1 mixture of matrix and sample solution followed by air drying. All samples for quantitative study were analysed in the automated batch mode. The typical time required to analyse all spots on 96-wells target plate was 15 minutes. Collision induced dissociation at 1kV was used for tandem MS.

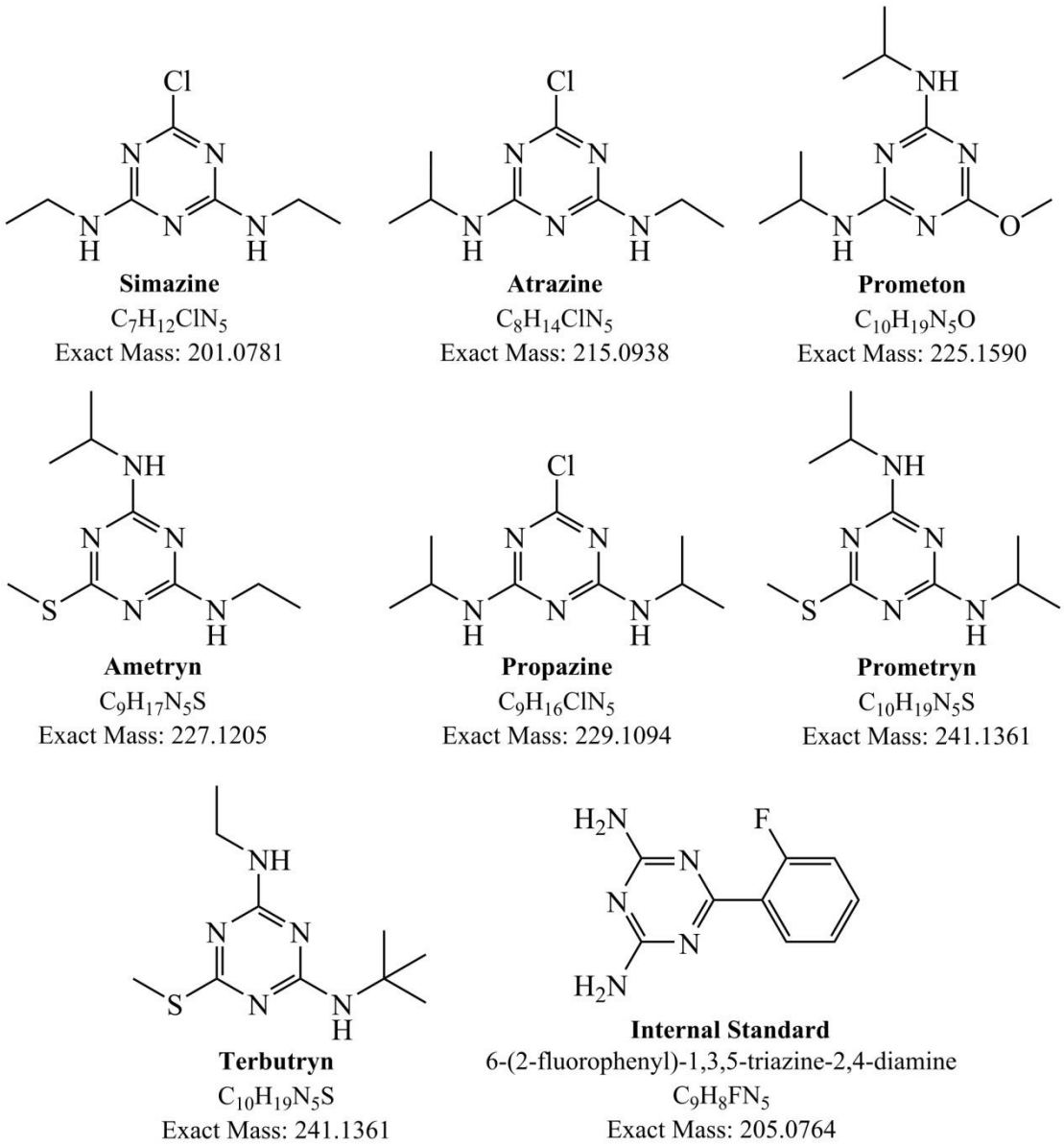


Figure 5.1: *Chemical structures of triazine herbicides used in the quantitative study.*

5.2.4 Spiked water sample analysis

DI water was spiked at 0.25 ppm, 0.55 ppm and 0.75 ppm levels with the herbicide mixture and the final solutions for spotting were prepared by 1:1 addition of the spiked solutions and separately prepared IS solution (2 ppm in 100% Methanol). No extraction protocol was followed while preparing the spiked water sample for mass spectrometry. These samples were also analyzed as unknown samples. Using

extraction and pre-concentration steps will possibly extend the analysis range below what is being reported.

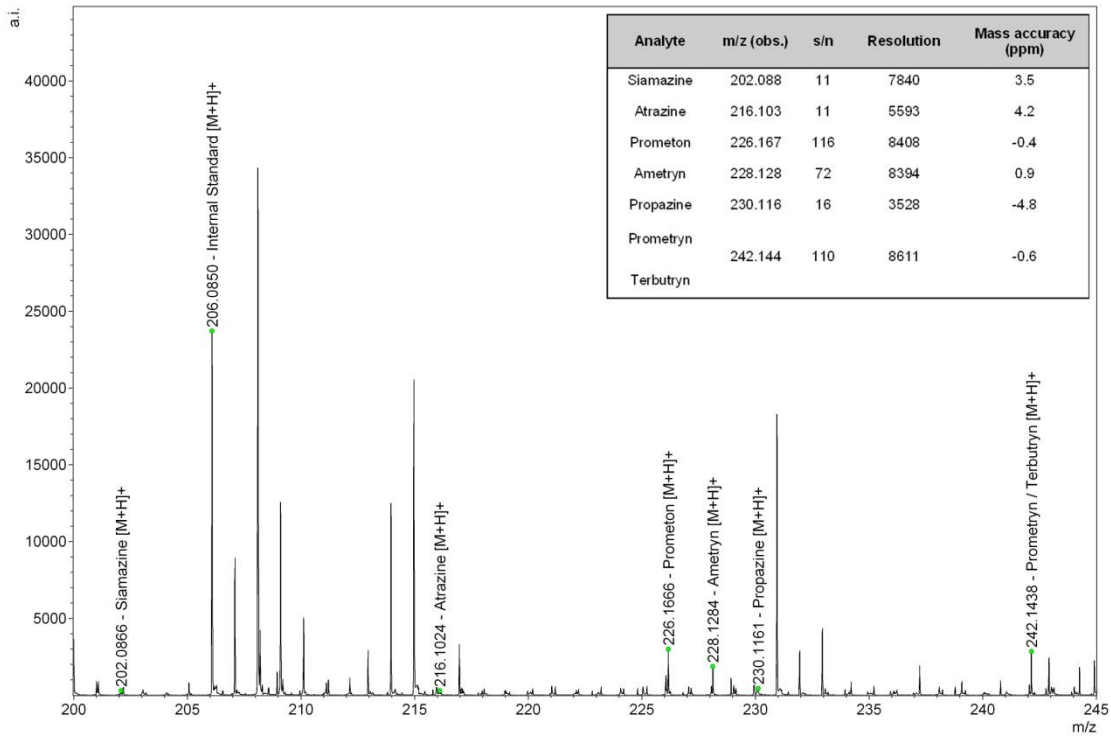
5.2.5 Quantitative data analysis

In-house quantitation software 'MQ' was used for quantitative analysis. All raw MS data files were converted to ASCII format. The calibration curves for all analytes were generated by plotting linear relationship between peak area ratios of analyte to internal standard versus the concentration. These calibration curves were used to determine the regression coefficient and recovery from the spiked water samples. Additional information about the software can be found at http://www.ldi-ms.com.

5.3 Results and discussion

5.3.1 Detection and characterization of the herbicides

Atrazine and related herbicides are triazine compounds containing heterocyclic 'azine' ring with substitutions, below molar mass of 500Da (Figure 5.1). MALDI-TOF spectrum of herbicide mixture shows predominantly protonated adduct peaks for the analytes (Figure 5.2). At 1ppb level detection, s/n for all components is higher than 10 with resolution greater than 3500 and less than 5 ppm error in mass accuracy (Figure 5.2). The peaks for prometon, ametryn, prometryn / terbutryn were found to be more intense than rest of the analytes, which may be due to the strongly basic heterogeneous atomic centres of oxygen and sulphur in these compounds, allowing higher ionization of these molecules in the MALDI ionization source. Propazine, simazine and atrazine show consistently lower signal response than other analytes, which may be partially attributed to the lower molecular mass and also the possibility of formation of degradation products in water. However, it is not unusual to encounter varying desorption ionizations for different analytes in MALDI-TOF MS.



Analyte	m/z (obs.)	s/n	Resolution	Mass accuracy (ppm)
Siamazine	202.088	11	7840	3.5
Atrazine	216.103	11	5593	4.2
Prometon	226.167	116	8408	-0.4
Ametryn	228.128	72	8394	0.9
Propazine	230.116	16	3528	-4.8
Prometryn Terbutryn	242.144	110	8611	-0.6

Figure 5.2*: MALDI-TOF MS spectrum for the selected herbicides and internal standard at 1 ppb concentration. Mass accuracy within 5 ppm was observed for all the analytes with resolution above 5000 except propazine.*

Nevertheless, the signal intensities showed consistent behavior. Presence of all the analytes has been confirmed by tandem MS experiments as summarized in table 5.1. The assigned fragments and the respective *m/z* match previously published MALDI-TOF MS data (Table 5.1). All the triazine analytes showed peaks at loss of CH_4, CH_3, C_2H_3, C_3H_7. In case of prometryn and terbutryn, the fragmentation of the *m/z* 242 peak shows presence of fragments from both prometryn and terbutryn (Figure 5.3), indicating that both the compounds are being detected.

5.3.2 Limit of Detection and Quantitation

Limit of Detection (LOD) and Limit of Quantitation (LOQ) for the analytes obtained using MALDI-TOF MS was observed to be significantly lower than the stringent maximum permissible levels in treated potable water (Table 5.2). LOD and LOQ were set at minimum signal-to-noise ratios of 3:1 and 10:1 respectively. Simazine and atrazine were found to have LOQs at higher concentration than the rest of the-

Table 5.1: *Comprehensive CID fragmentation of individual triazine herbicide in MALDI-TOF MS. Fragmentation pattern was matched with previously published report [38]. (Y = present, N = absent)*

	Fragment calculated		**Fragment observed**
	Ion	***m/z***	
Simazine $C_7H_{12}ClN_5$ $[M+H]^+$ 202	$[M-CH_3]^+$	186	N
	$[M-C_2H_5]^+$	172	N
	$[M-C_3H_7]^+$	158	Y
	$[M-Cl-C_2H_4]^+$	138	Y
	$[C_3H_7]^+$	43	Y
Atrazine $C_8H_{14}ClN_5$ $[M+H]^+$ 216	$[M-CH_3]^+$	200	N
	$[M-C_3H_6+H]+$	174	Y
	$[M-C_3H_7]^+$	172	Y
	$[M-NHC_3H_7+H]^+$	158	Y
	$[M-Cl-C_2H_4]^+$	138	Y
	$[C_3H_7]^+$	43	Y
Propazine $C_9H_{16}ClN_5$ $[M+H]^+$ 230	$[M-CH_3]^+$	214	N
	$[M-C_3H_6+H]+$	188	Y
	$[M-C_3H_7]^+$	186	Y
	$[M-NHC_3H_7+H]^+$	172	Y
	$[M-Cl-C_2H_4]^+$	152	Y
	$[C_3H_7]^+$	43	Y
Ametryn $C_9H_{17}N_5S$ $[M+H]^+$ 228	$[M-CH_3]^+$	212	N
	$[M-C_3H_6+H]^+$	186	Y
	$[M-C_3H_7]^+$	184	Y
	$[M-NHC_3H_7+H]^+$	170	Y
	$[M-NC_3H_8-CH_2]^+$	155	Y
	$[C_3H_7]^+$	43	Y
	$[C_2H_3]^+$	27	Y
Prometon $C_{10}H_{19}N_5O$ $[M+H]^+$ 226	$[M-CH_3]^+$	210	N
	$[M-C_3H_6+H]+$	184	Y
	$[M-C_3H_7]^+$	182	Y
	$[M-NHC_3H_7+H]^+$	168	Y
	$[C_3H_7]^+$	43	Y
Prometryn $C_{10}H_{19}N_5S$ $[M+H]^+$ 242	$[M-CH_3]^+$	226	N
	$[M-C_3H_6+H]+$	200	Y
	$[M-C_3H_7]^+$	198	N
	$[M-NHC_3H_7+H]^+$	184	Y
	$[C_3H_7]^+$	43	Y
Terbutryn $C_{10}H_{19}N_5S$ $[M+H]^+$ 242	$[M-CH_3]^+$	226	N
	$[M-C_4H_8+H]^+$	186	Y
	$[M-C_4H_8-CH_3]^+$	170	Y
	$[C_3H_7]^+$	43	Y

- pesticides, which concurs with the comparatively lower signal response observed for these analytes. It is interesting to note that triazines containing chlorine atom had

shown marginally higher LOD compared to non-chlorine containing triazine herbicides. As can be seen from table 5.2, the LOQs for simazine and atrazine are above the requirements for potable water as per the minimal permissible limits. This difference can be matched by employing any of the established sample preparation and pre-concentration protocols. The LOQ for the rest of the analytes is comfortably within the maximum permissible levels in water. MALDI-TOF MS can thus be utilized as a sensitive and quick screening method for the detection of the herbicides from water while a more rigorous quantitation can be performed if necessary.

Table 5.2: *Limits of detection and quantitation of triazine herbicides using MALDI-TOF MS compared with current permissible levels* [39, 40].

Analyte	Regulated levels in U.S.A.		MALDI-TOF MS	
	Treated Water (ppb)	Food (ppb)	LOD (ppb)	LOQ (ppb)
Simazine	4	200 (corn grain)	2	30
Atrazine	3	200 (corn grain)	1	20
Prometon	NR	NR	0.75	1
Ametryn	60	50 (corn grain)	0.75	1
Propazine	NR	250 (sorghum)	1	10
Prometryn	28 (Florida)	500 (celery)	0.1	0.75
Terbutryn	330 (Florida)	NR	0.1	0.75

(key: ppb = part per billion, NR = not regulated)

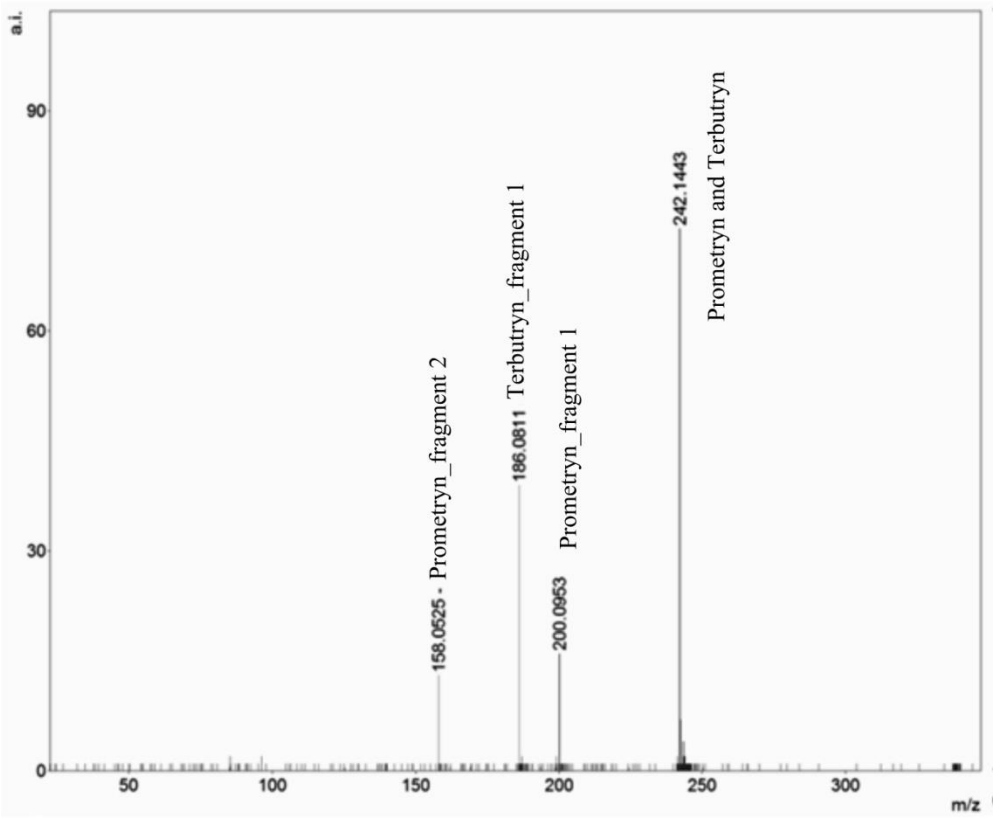


Figure 5.3: *MALDI-TOF MS/MS spectra for the mixture of prometryn and terbutryn. The presence of fragments from both Terbutryn and Prometryn indicates that both analytes are detected at 1 ppb level, but were not resolved due to their isomeric nature.*

5.3.3 Linearity

All the herbicides show linear response to increasing concentration in solution in the entire selected range of concentration for analysis (Table 5.3). The regression value R^2 was consistently observed to be greater than 0.99 in most cases, with low replicate variation. The QCs also were found to be within the acceptable error limits with relative error of less than 15% (RSD < 15%), establishing MALDI-TOF MS as a quantitative method for the analysis of the selected triazine herbicides. Linearity was observed over the concentration range of 3 orders of magnitude for the analytes. This enables quantitative MALDI analysis over a wide concentration range without further manipulations of the established sample extraction protocol, especially in a quality control laboratory where high throughput and accurate analysis are necessary.

***Table 5.3**: MALDI-TOF MS quantitative analysis of triazine herbicides. The water was spiked at low, mid and high concentration of mixture of herbicides and used as quality control samples.*

Analyte	**Conc. range (ppm)**	**Calibration equation**	**R^2**	**Spike level in water (ppm)**	**% Recovery (% RSD)**
Simazine	0.1-1.0	y=0.00603+0.12012x	0.99	0.25 0.55 0.75	88.9 (9) 90.7 (5) 77.3 (9)
Atrazine	0.1-1.0	y=0.00697+0.11524x	0.99	0.25 0.55 0.75	91.1 (6) 89.1 (5) 86.0 (10)
Prometon	0.1-1.0	y=0.01952+1.02141x	0.98	0.25 0.55 0.75	81.0 (8) 104.8 (10) 101.2 (12)
Ametryn	0.1-1.0	y=0.00949+0.79263x	0.99	0.25 0.55 0.75	84.5 (10) 105.6 (9) 99.3 (4)
Propazine	0.1-1.0	y=0.00877+0.13748x	0.99	0.25 0.55 0.75	84.8 (7) 89.5 (7) 85.6 (6)
Prometryn/ Terbutryn	0.1-1.0	y=0.02126+1.48099x	0.99	0.25 0.55 0.75	85.5 (8) 102.3 (6) 104.7 (8)

(key: ppm = part per million)

5.3.4 Unknown sample analysis

All the analytes were detected from spiked quality control (QC) water samples and were quantified with a high degree of confidence. The recoveries for all the analytes were within 15% of the levels spiked in water and the overall RSDs for multiple replicates were within 15% (Table 5.3). The two isomers, prometryn and terbutryn, were also detected simultaneously, as seen from the MS/MS data, but were not distinguishable individually from a mixture without considering MS/MS data. For simplicity of analysis, the signal response of the two analytes has been considered to be equal for analysis of QC samples. For in-field analysis though, a two-fold approach will be required for quantitation. The MALDI-TOF MS method may be used as high-throughput screening tool for prometryn and terbutryn. If the presence of a peak a *m/z* = 242 crosses the set limit, further analysis may be carried out to accurately quantify each of the isomers and thus pass or fail a quality control test for the submitted samples. A different analytical platform which is capable of separating the isomers efficiently such as chiral LC-MS or CE-MS may be employed for conformational analysis for these isomers.

5.4 Conclusions

A Quantitative MALDI-TOF MS method for the analysis of triazine herbicides from water sample was demonstrated with high accuracy and precision. Mixture of triazine herbicides were identified using exact mass criteria and characterized by tandem MS. Quantitation using a specified mass extraction window for high resolution data has been reported for LC-ESI MS analysis and broad guidelines have also been suggested for this approach.[41, 42] This work demonstrates a similar approach using MALDI-TOF MS and does not require any chromatography or elaborate sample pre-processing steps. Furthermore, qualitative and quantitative analysis can be performed in a single

experiment. Information regarding any further degradation products or additional analytes suspected in the sample can be readily verified as a full scan MS spectrum generated is used in this approach. Existing reaction monitoring schemes, although are sensitive and selective, lack this crucial additional feature and unit resolution could also pose limitations in complex samples. The total analysis time for MALDI-TOF MS analysis was significantly less compared to routinely followed HPLC-MS or GC-MS method showcasing the potential of MALDI-TOF MS for rapid, accurate and high throughput analysis of pesticides and other contaminants in water analysis.

5.5 References

(1) FAO *http://faostat3.fao.org/home/index.html#VISUALIZE* **2013**, last accessed on December 6, 2013.

(2) Farland, J. M.; Burnside, O.; LeBaron, H. M. *The Triazine Herbicides*, 1 ed.; Elsevier Science, 2005.

(3) Cheyns, K.; Martin-Laurent, F.; Bru, D.; Aamand, J.; Vanhaecke, L.; Diels, J.; Merckx, R.; Smolders, E.; Springael, D. *Chemosphere* **2012**, *86*, 1028-1034.

(4) Jablonowski, N. D.; Hamacher, G.; Martinazzo, R.; Langen, U.; Köppchen, S.; Hofmann, D.; Burauel, P. *Journal of Agricultural and Food Chemistry* **2010**, *58*, 12869-12877.

(5) Sebiomo, A.; Ogundero, V. W.; Bankole, S. A. *African Journal of Biotechnology* **2011**, *10*, 770-778.

(6) Cole, M. A. *Weed Science* **1976**, *24*, 473-476.

(7) Moreno, J. L.; Aliaga, A.; Navarro, S.; Hernándeza, T.; Garcíaa, C. *Applied Soil Ecology* **2007**, *35*, 120-127.

(8) Perucci, P.; Dumontet, S.; Bufo, S. A.; Mazzatura, A.; Casucci, C. *Biology and Fertility of Soils* **2000**, *32*, 17-23.

(9) Fan, W. Q.; Yanase, T.; Morinaga, H.; Ondo, S.; Okabe, T.; Nomura, M.; Komatsu, T.; Morohashi, K. I.; Hayes, T. B.; Takayanagi, R.; Nawata, H. *Environmental Health Perspectives* **2007**, *115*, 720-727.

(10) Rohr, J. R.; Sager, T.; Sesterhenn, T. M.; Palmer, B. D. *Environmental Health Perspectives* **2006**, *114*, 46-50.

(11) Faust, M.; Altenburger, R.; Backhaus, T.; Blanck, H.; Boedeker, W.; Gramatica, P.; Hamer, V.; Scholze, M.; Vighi, M.; Grimme, L. H. *Aquatic Toxicology* **2001**, *56*, 13-32.

(12) Serge A. Carpy, W. K. J. D. *Journal of Toxicology and Environmental Health, Part B* **2000**, *3*, 1-25.

(13) Solomon, K. R.; Baker, D. B.; Richards, R. P.; Dixon, K. R.; Klaine, S. J.; La Point, T. W.; Kendall, R. J.; Weisskopf, C. P.; Giddings, J. M.; Giesy, J. P.; Hall, L. W.; Williams, W. M. *Environmental Toxicology and Chemistry* **1996**, *15*, 31-76.

(14) Lim, S.; Ahn, S. Y.; Song, I. C.; Chung, M. H.; Jang, H. C.; Park, K. S.; Lee, K.-U.; Pak, Y. K.; Lee, H. K. *PLoS ONE* **2009**, *4*, e5186.

(15) Albanito, L.; Lappano, R.; Madeo, A.; Chimento, A.; Prossnitz, E. R.; Cappello, A. R.; Dolce, V.; Abonante, S.; Pezzi, V.; Maggiolini, M. *Environmental Health Perspectives* **2008**, *116*, 1648-1655.

(16) Basini, G.; Bianchi, F.; Bussolati, S.; Baioni, L.; Ramoni, R.; Grolli, S.; Conti, V.; Bianchi, F.; Grasselli, F. *Ecotoxicology and Environmental Safety* **2012**, *85*, 59-63.

(17) Freeman, L. E. B.; Rusiecki, J. A.; Hoppin, J. A.; Lubin, J. H.; Koutros, S.; Andreotti, G.; Zahm, S. H.; Hines, C. J.; Coble, J. B.; Barone-Adesi, F.; Sloan,

J.; Sandler, D. P.; Blair, A.; Alavanja, M. C. R. *Environmental Health Perspectives* **2011**, *119*, 1253-1259.

(18) Gammon, D. W.; Aldous, C. N.; Carr, W. C.; Sanborn, J. R.; Pfeifer, K. F. *Pest Management Science* **2005**, *61*, 331-355.

(19) Broszat, M.; Ernst, H.; Spangenberg, B. *Journal of Liquid Chromatography & Related Technologies* **2010**, *33*, 948-956.

(20) Butz, S.; Stan, H. J. *Analytical Chemistry* **1995**, *67*, 620-630.

(21) Cserháti, T.; Forgács, E.; Deyl, Z.; Miksik, I.; Eckhardt, A. *Biomedical Chromatography* **2004**, *18*, 350-359.

(22) Real, B. D.; Ortiz, M. C.; Sarabia, L. A. *Journal of Chromatography B* **2012**, *910*, 122-137.

(23) Frías, S.; Sánchez, M. J.; Rodríguez, M. A. *Analytica Chimica Acta* **2004**, *503*, 271-278.

(24) Mou, R.-X.; Chen, M.-X.; Cao, Z.-Y.; Zhu, Z.-W. *Analytica Chimica Acta* **2011**, *706*, 149-156.

(25) Benvenuto, F.; Marín, J. M.; Sancho, J.; Canobbio, S.; Mezzanotte, V.; Hernández, F. *Analytical and Bioanalytical Chemistry* **2010**, *397*, 2791-2805.

(26) Di Corcia, A.; Crescenzi, C.; Guerriero, E.; Samperi, R. *Environmental Science & Technology* **1997**, *31*, 1658-1663.

(27) Janfelt, C.; Græsbøll, R.; Lauritsen, F. R. *International Journal of Environmental Analytical Chemistry* **2012**, *92*, 397-404.

(28) Beltran, J.; López, F. J.; Hernández, F. *Journal of Chromatography A* **2000**, *885*, 389-404.

(29) Chiron, S.; Dupas, S.; Scribe, P.; Barceló, D. *Journal of Chromatography A* **1994**, *665*, 295-305.

(30) Yu, Z.-g.; Qin, Z.; Ji, H.-r.; Du, X.; Chen, Y.-h.; Pan, P.; Wang, H.; Liu, Y.-y. *Chromatographia* **2010**, *72*, 1073-1081.

(31) Ara, B.; Chen, Z.; Shah, J.; Rasul Jan, M.; Ye, L. *Journal of Applied Polymer Science* **2012**, *126*, 315-321.

(32) Dean, J. R.; Wade, G.; Barnabas, I. J. *Journal of Chromatography A* **1996**, *733*, 295-335.

(33) Hernández, F.; Sancho, J. V.; Pozo, O. J. *Analytical and Bioanalytical Chemistry* **2005**, *382*, 934-946.

(34) Madla, S.; Miura, D.; Wariishi, H. *Analytical Sciences* **2012**, *28*, 301-303.

(35) Anderson, D. M. G.; Carolan, V. A.; Crosland, S.; Sharples, K. R.; Clench, M. R. *Rapid Communications in Mass Spectrometry* **2010**, *24*, 3309-3319.

(36) Singh, A.; Panchagnula, V. *Analytical Methods* **2011**, *3*, 2360-2366.

(37) JEOL, *SpiralTOF Application note (MS-111020)*.

(38) JEOL, *Application note (MS-111020)*.

(39) Hamilton, D. J.; Ambrus, Á.; Dieterl, R. M.; Felsot, A. S.; Harris, C. A.; Holland, P. T.; Katayama, A.; Kurihara, N.; Linders, J.; Unsworth, J.; Wong, S. S. *Pure and Applied Chemistry* **2003**, *75*, 1123-1155.

(40) USDA **2013**, http://www.mrldatabase.com/

(41) Rochat, B.; Kottelat, E.; McMullen, J. *Bioanalysis* **2012**, *4*, 2939-2958.

(42) Zhou, J. H.; Zhao, J.; Xue, X. F.; Zhang, J. Z.; Chen, F.; Li, Y.; Wu, L. M.; Li, C. C.; Mi, J. Q. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2009**, *877*, 4164-4170.

(43) USDA *http://www.mrldatabase.com/* **2013**, last accessed on December 6, 2013.

Comprehensive detection of milk metabolites

6.1 Introduction

In human civilization, mammalian milk has been a sole source of feeding in newborns and essential constituent of nutrition from ancient time. Apart from the source of energy, milk has been a supplement to growth and development. Milk contains several important constituents mainly fat, protein, carbohydrates, vitamins and enzymes, which make milk a comprehensive source of nutrition.[1-3] Across the world milk and milk products obtained from animals are highest consumed foods thus represent one of the largest areas of commercial activity. Consistency in quality of milk has been a major challenge for dairy industry. Milk components play a major role in assessing the breeding scores, metabolic and health and diseases status of the cow. The biochemical composition of milk changes according to diet of animal, breed and geographic regions. [3, 4] It is indeed very difficult task for dairy services to have a control on the source of milk. Moreover the addition of adulterant or artificial constituent to falsely increase the quality of milk has become a problem for dairy industries. Every year tonnes of milk and milk products get rejected during export from one country to another country due the adulteration or poor quality of the milk.

Milk is a complex mixture of macromolecules (peptides/protein) and metabolites. On the average milk contain 87% water, 4% fat, 3% protein 4.5% carbohydrates and remaining other components including mineral substances.[1, 4-6] Milk can be further divided into groups of metabolites e.g. amino acids, vitamins, fatty acids etc. which

shows the diverse chemical nature from nonpolar to polar molecules. This complex mixture of metabolites in milk has been assessed by several analytical methods but chromatography has been a frontier analytical technique to profile the milk metabolites.[7-20] Hyphenation of chromatography with mass spectrometry had made the milk analysis very accurate, reliable and efficient analytical method. In previous chapters, applicability and limitations of chromatography based mass spectrometry and application of MALDI-TOF MS has been discussed for small molecule analysis and metabolites identification. MALDI-TOF MS has been used in milk analysis for the identification of various protein and biomolecules, adulteration of milk from one species to the milk of another species, adulteration of milk with vegetable oils and fat, antibiotic in milk.[21-32] In the present work, a targeted approach for metabolites identification in the whole milk obtained from cow has been applied using the MALDI-TOF MS.

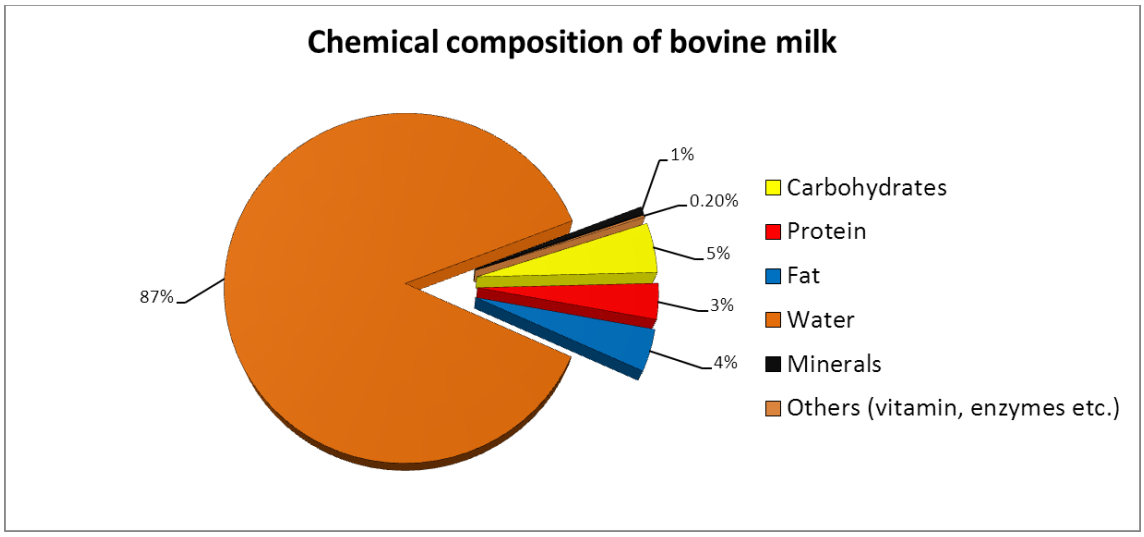


Figure 6.1: *Chemical composition of bovine milk*

6.2 Experimental

6.2.1 Materials

2,5-dihydroxybenzoic acid (2,5-DHB), 2,4-diamino-1,3,5-triazine, methanol, acetonitrile and trifluoroacetic acid were from Sigma-Aldrich. Distilled and deionized water with specific resistivity 18.2 MΩ cm^{-1} was from SG ultrapure water unit (Germany). Packaged and commercially available cow's milk was used for analysis.

6.2.2 Sample preparation

The milk sample was subjected to extraction using methanol/water (1:1). In 1.5 mL tube equal amounts of milk and methanol/water (1:1) was vortexed and sonicated for 10 minutes. After sonication, 1 µL of trifluoroacetic acid (TFA) was added to precipitate the milk solids. Thereafter, the tubes were kept on centrifugation for 15 min at 14000 rpm. Supernatant was removed into another tube, mixed with an equal amount of internal standard (1ppm 2,4-diamino-1,3,5-triazine) and used for the analysis. This final solution for detection resulted in a fourfold dilution.

6.2.3 MALDI-TOF MS

MALDI-TOF MS was performed on Waters Synapt HDMS (Manchester, UK) operated in V-positive mode or ABI Voyager DE-STR MALDI-TOF-MS in the positive ion mode. Detector voltage was set to 1700 V, quadrupole setting were set according to mass range (1-600 *m/z*). Laser energy was optimized to get good signal to noise ratio. The data was processed with Masslynx 4.1 or Data explorer software. Standard 96-well stainless steel MALDI target plates were used after thorough cleaning10 mg/ml of 2,5-DHB was prepared and used by dissolving it in acetonitrile : 0.1% TFA (1:1, v/v). Sandwich model was adapted for spotting wherein 1 µL of 2,5-DHB was placed first on an individual spot, allowed to dry and 1 µL of working standards or samples were placed before being topped by yet another 1 µL of 2,5-

DHB. The spot was thoroughly air dried before the analysis. All mass spectra were acquired with the same instrumental condition in automated mode as described above.

6.2.4 Data analysis

Raw data files were later converted into ASCII format. In house developed, MQ software was subsequently used to generate quantitative results from the MALDI-TOF MS experiment. Prior to the data analysis, a library of milk metabolites was prepared which contained the monoisotopic masses of protonated and cationized adducts of metabolites. In the given spectra metabolites were identified using exact mass matching and signal-to-noise ratio criteria (s/n =10).

Table 1: List of total metabolites present in bovine milk (source FooDB)

Category	Metabolite	Monoisotopic mass
Amino acids	Glycine	75.032
	L-Alanine	89.048
	L-Lactic acid	90.032
	L-Alpha-aminobutyric acid	103.063
	L-Serine	105.043
	L-Proline	115.063
	L-Valine	117.079
	L-Threonine	119.058
	L-Isoleucine	131.095
	Ornithine	132.090
	L-Aspartic acid	133.038
	L-Lysine	146.106
	L-Glutamic acid	147.053
	L-Methionine	149.051
	L-Histidine	155.069
	Orotic acid	156.017
	L-Carnitine	161.105
	L-Phenylalanine	165.079
	L-Arginine	174.112
	L-Tyrosine	181.074
	L-Tryptophan	204.090
	L-Cystine	240.024
Fatty acids	Fumaric acid	116.011
	Caprylic acid	144.115
	Capric acid	172.146
	Dodecanoic acid	200.178

	Myristoleic acid	226.193
	Myristic acid	228.209
	Pentadecanoic acid	242.225
	Palmitoleic acid	254.225
	Palmitic acid	256.240
	Heptadecanoic acid	270.256
	Alpha-Linolenic acid	278.225
	Bovinic acid	280.240
	Linoleic acid	280.240
	Stearic acid	284.272
	Arachidonic acid	304.240
	Eicosenoic acid	310.287
	Arachidic acid	312.303
	Docosapentaenoic acid	330.256
	Behenic acid	340.334
	Tetracosanoic acid	368.365
	Calcitriol	416.329
Steroids	Estrone	270.162
	Androstenedione	286.193
	Estriol	288.173
	Dehydroepiandrosterone	288.209
	Epitestosterone	288.209
	Testosterone	288.209
	Progesterone	314.225
	Estrone sulfate	350.119
	Cholesterol	386.355
	Estroneglucuronide	446.194
Lipids	Glycerophosphocholine	257.103
	Phosphatidylethanolamine	747.520
	Delta-Tocopherol	402.350
	24,25-Dihydroxyvitamin D	416.329
	25,26-dihydroxyvitamin D	416.329
	Gamma-Tocopherol	416.365
	B-Carotene	536.438
	Lutein	568.428
Nucleosides	1-Methyladenosine	281.112
	Guanine	151.049
	Cytidine	243.086
	Uridine	244.070
	Adenosine	267.097
	Inosine	268.081
	Guanosine	283.092
	Cytidine monophosphate	323.052
	Uridine 5'-monophosphate	324.036
	Adenosine monophosphate	347.063

	Guanosine monophosphate	363.058
	Adenosine diphosphate	427.029
	Adenosine triphosphate	506.996
	Uridinediphosphategalactose	566.055
	GDP-L-fucose	589.082
Vitamin	Niacinamide	122.048
	Nicotinic acid	123.032
	Pyridoxal	167.058
	Pyridoxine	169.074
	Dehydroascorbic acid	174.016
	Ascorbic acid	176.032
	Pantothenic acid	219.111
	Pyridoxamine 5'-phosphate	248.056
	Thiamine	265.112
	Vitamin A	286.230
	Riboflavin	376.138
	cholecalciferol	384.339
	Alpha-Tocopherol	430.381
	Folic acid	441.140
	Phylloquinone	450.350
Carbohydrate	Glycerol 3-phosphate	172.014
	D-Fructose	180.063
	D-Galactose	180.063
	D-Glucose	180.063
	Pseudouridine	244.070
	Glucose 6-phosphate	260.030
	N-Acetylneuraminic acid	309.106
	D-Maltose	342.116
	Sucrose	342.116
	Lactose	343.124
	3'-Sialyllactose	633.212
	6'-Sialyllactose	633.212
	Disialyllactose	924.307
Flavonoids	Equol	242.094
	Daidzein	254.058
	Formononetin	268.074
	Genistein	270.053
	Naringenin	272.068
	Biochanin A	284.068
	Glycitein	284.068
	Prunetin	284.068
Diethanolamine	N-octyl ethanolamine	174.186
	N-decyl ethanolamine	202.217
	N-octyldiethanolamine	218.212

	N-dodecylethanolamine	230.248
	N-decyldiethanolamine	246.243
	N-tetradecylethanolamine	258.280
	N-2-octyloxyethyldiethanolamine	262.238
	N-dodecyl diethanolamine	274.275
	N-2-decyloxyethyldiethanolamine	290.270
	N-tetradecyldiethanolamine	302.306
	N-2-dodecyloxyethyldiethanolamine	318.301
	N-2-tetradecyloxyethyldiethanolamine	346.332
	N-octyl ethanolamine	174.186
	N-decyl ethanolamine	202.217
	N-octyldiethanolamine	218.212
	N-dodecylethanolamine	230.248
	N-decyldiethanolamine	246.243
	N-tetradecylethanolamine	258.280
	N-2-octyloxyethyldiethanolamine	262.238
	N-dodecyl diethanolamine	274.275
	N-2-decyloxyethyldiethanolamine	290.270
Miscellaneous	Trimethylamine	59.073
	Urea	60.032
	Ethanolamine	61.053
	Trimethylamine N-oxide	75.068
	Pyruvic acid	88.016
	Acetoacetic acid	102.032
	3-Hydroxybutyric acid	104.047
	Choline	104.108
	Histamine	111.080
	Creatinine	113.059
	Betaine	117.079
	Succinic acid	118.027
	Taurine	125.015
	Malic acid	134.022
	Hypoxanthine	136.039
	Spermidine	145.158
	Xanthine	152.033
	Allantoin	158.044
	Aminoadipic acid	161.069
	Uric acid	168.028
	Pyridoxamine	168.090
	3-Methylhistidine	169.085
	Citric acid	192.027
	Isocitric acid	192.027
	Spermine	202.216
	Acetylcarnitine	204.124
	Melatonin	232.121
	Biotin	244.088
	Pyridoxal 5'-phosphate	247.025

Enterolactone	298.121
Enterodiol	302.152
Secoisolariciresinol	362.173

6.3 Results and discussion

6.3.1 Comprehensive detection of milk metabolites

One of the most powerful applications of MALDI-TOF MS is comprehensive and untargeted identification of analytes in given sample. In this analysis assessment of metabolites in bovine milk was performed on different days. Milk contains several metabolites and majority of them falls under the category of small molecules (m/z < 500 Da). According to FooDB, bovine milk contains approximately 150 metabolites from various categories.[33] Table 6.1 lists the category wise metabolites present in bovine milk. In this analysis all the metabolites listed in table# were studied using MALDI-TOF MS in untargeted manner.

The comprehensive analysis of metabolites using MALDI-TOF MS has several challenges. It includes the interferences of matrix peak in small molecules region, in-source fragmentation of molecules and mass accuracy. Mass accuracy can be obtained by using appropriate internal standard. Internal standard not only compensate the variation in mass accuracy but also compensate the spot-to-spot signal variation in MALDI-TOF MS. Small molecules have tendency of self-fragmentation in the ion source due to availability of extra energy from matrix and requirement of lesser energy for fragmentation. Self-fragmentation in the ion source would increase the complexity of mass spectra by introducing more number of peaks. Moreover due to the fragmentation, the probability of detection of molecular ion at very low concentration also gets lower. Though in-source fragmentation would increase the confidence of identifying the molecules but in case of detection of large number of

metabolites in single sample, it would be a very complex mass spectrum. There are few ways to reduce the complexity of mass spectra. Matrix free method is one of them. Various research publications have explained and showed the utility of matrix free LDI MS. But still these matrix free methods are at preliminary stages; require more development to demonstrate the capability in comprehensive detection of large set of metabolites. To reduce the complexity another method is to use matrix which provide low fragmentation or no fragmentation. These matrixes are called as 'cold' matrix. 2,5-dihydroxybenzoic acid (DHB) is a cold matrix which provides lower fragmentation compared to other MALDI matrices. Thus 2,5-DHB matrix is considered as suitable matrix in small molecule analysis. In this analysis 2,5-DHB was used as MALDI matrix for entire analysis. Paseturiesed cow's milk (Katraj Dairy, India) was brought form the market and each day, milk samples were treated and prepared for mass spectrometry. The MALDI-TOF MS analysis was performed on Synapt HDMS (Waters, UK). Synapt HDMS, along with quadrupole filters, has orthogonal time-of-flight mass (Oa-TOF) analyzer. Orthogonal geometry is known to provide better mass accuracy compare to axial TOF mass analyser.[34]

Table6.2 summarizes the MALDI-TOF MS detection of all metabolites in bovine milk over the period of five days. For each metabolite proton, sodium and potassium adduct were identified and adduct having highest occurrence was selected. Metabolites having mass above 500 Da was not considered. Approximately 40-58 % metabolites were detected using single MALDI-TOF MS method. Among all the five days, milk at day 4 showed highest number of metabolite detection. Compare to total number of metabolites present in bovine milk, this lower number of detected metabolites can be attributed to several factors. The total metabolites in bovine milk listed in FooDB is based on raw milk i.e. milk without any processing and treatment.

Whereas the in this study pasteurized milk was used which is the final product after many processing steps including ultra-heat treatment (UHT). Thus there is greater chance that many metabolites could be degraded or transformed into some other metabolites. Overall here the main purpose is to access the reproducibility of presence of possible metabolite in the milk on different days. For example in carbohydrate and flavonoids category all the metabolites were detected reproducibly for the five days. Similarly Amino acid composition was found to be same for all the five days with little difference. Overall 11 amino acids were identified based on s/n=10 criteria. Figure 6.3 shows the relative occurrence of individual amino acid in terms of probability. Most abundant amino acids were Ala, Arg, Car, Phe, Pro, Tyr and Val. The occurrence and reproducibility for other metabolites can be seen from table 6.4 to 6.11.

Table 6.2: *MALDI-TOF MS detection of metabolites in bovine milk over the period of five days.*

S. No.	**Category**	**Total numbers of metabolites**	**Days**				
			1	**2**	**3**	**4**	**5**
1.	Amino acids	22	7	7	9	10	9
2.	Fatty acids	21	2	5	4	11	7
3.	Steroids	10	2	1	2	8	2
4.	Lipids	8	2	2	2	4	2
5.	Nucleosides	15	10	9	10	12	11
6.	Vitamins	15	9	8	9	9	5
7.	Carbohydrates	8	8	8	8	8	8
8.	Flavonoids	8	8	8	8	8	8
9.	Diethanolamines	12	2	2	3	3	0
10.	miscellaneous	32	14	8	13	16	7
11.	Total	**151**	**64**	**58**	**68**	**89**	**59**

Table 6.3: *Detection of essential and non-essential amino acid in bovine milk.*

Amino acids	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Glycine	○	○	○	○	○
L-Alanine	●	●	●	●	●
L-Alpha-aminobutyric acid	○	○	○	○	○
L-Arginine	●	●	●	●	●
L-Aspartic acid	○	○	○	○	○
L-Carnitine	●	●	●	●	●
L-Cystine	○	○	○	○	○
L-Glutamic acid	○	○	○	●	●
L-Histidine	○	○	○	○	○
L-Isoleucine	○	○	○	○	○
L-Lactic acid	○	○	○	○	○
L-Lysine	○	○	●	○	○
L-Methionine	○	○	○	○	○
L-Phenylalanine	●	●	●	●	●
L-Proline	○	●	●	●	●
L-Serine	○	○	○	○	○
L-Threonine	○	○	○	●	○
L-Tryptophan	●	○	●	●	○
L-Tyrosine	●	●	●	●	●
L-Valine	○	●	●	●	●
Ornithine	●	○	○	○	○
Orotic acid	○	○	○	○	○
Legend	○ *not detected* ● *detected*				

Figure 6.2: *Relative abundance of individual amino acid found in all milk samples for 5 different days.*

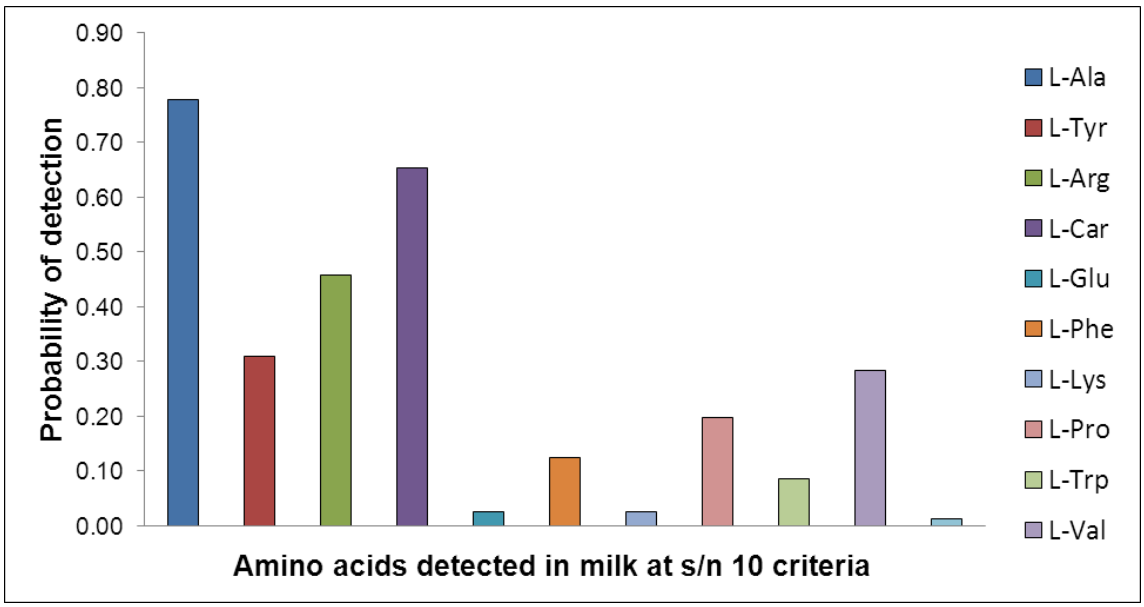


Table 6.4: *Detection of vitamin in bovine milk.*

Vitamins	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Dehydroascorbic acid	●	○	○	●	○
Pyridoxamine 5'-phosphate	●	●	●	●	●
Phylloquinone	○	●	○	○	○
Alpha-Tocopherol	○	○	○	●	○
Cholecalciferol	●	○	●	●	○
Ascorbic acid	●	●	●	○	○
Folic acid	○	○	○	○	○
Pyridoxine	●	●	●	●	○
Pyridoxal	○	○	○	○	○
Pantothenic acid	●	●	●	●	●
Nicotinic acid	●	●	●	●	●
Niacinamide	○	○	●	○	○
Riboflavin	●	●	●	●	●
Thiamine	●	●	●	●	●
Vitamin A	○	○	○	○	○

Legend ○ *not detected* ● *detected*

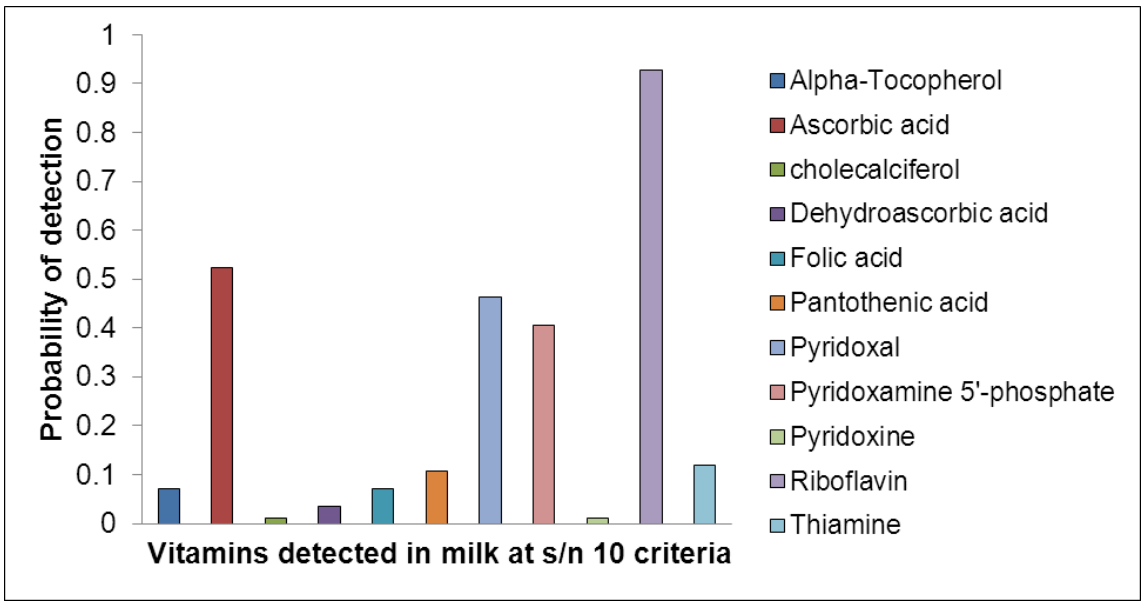


Figure 6.3: *Relative abundance of vitamins found in all milk samples for 5 different days.*

Table 6.5: *Detection of carbohydrates in bovine milk.*

Carbohydrates	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
D-Glucose	●	●	●	●	●
D-Maltose	●	●	●	●	●
Glucose 6-phosphate	●	●	●	●	●
Glycerol 3-phosphate	●	●	●	●	●
Lactose	●	●	●	●	●
N-Acetylneuraminic acid	●	●	●	●	●
Pseudouridine	●	●	●	●	●
Sucrose	●	●	●	●	●
Legend ○ *not detected* ● *detected*					

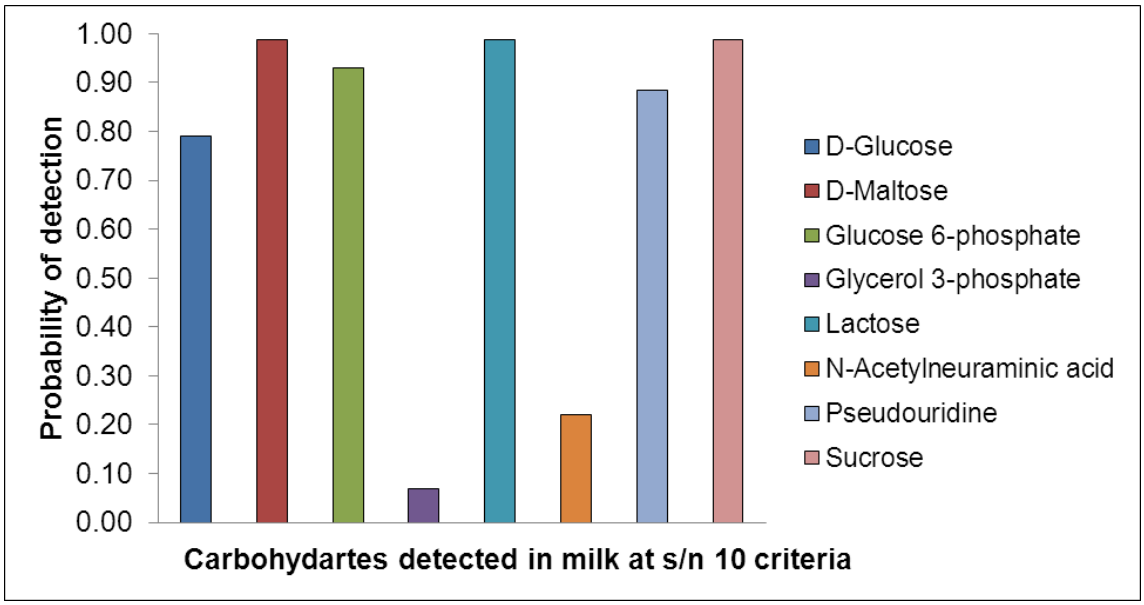


Figure 6.4: *Relative abundance of carbohydrates found in all milk samples for 5 different days.*

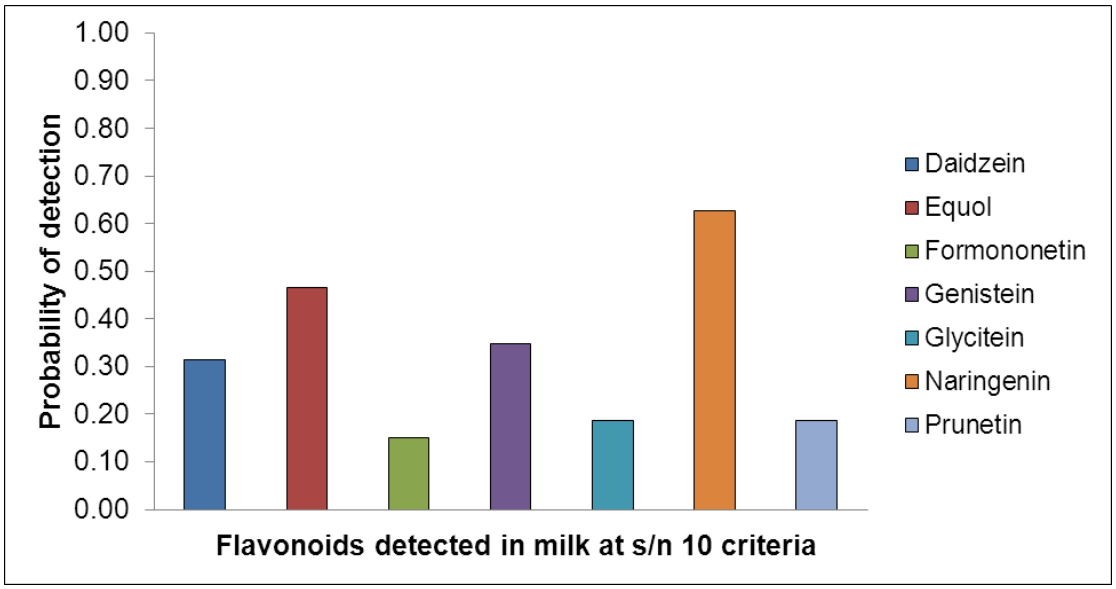


Figure 6.5: *Relative abundance of flavonoids found in all milk samples for 5 different days.*

Table 6.6: *Detection of flavonoids in bovine milk.*

Flavonoids	Day 1	Day 2	Day 3	Day 4	Day 5
Daidzein	●	●	●	●	●
Equol	●	●	●	●	●
Formononetin	●	●	●	●	●
Genistein	●	●	●	●	●
Glycitein	●	●	●	●	●
Naringenin	●	●	●	●	●
Prunetin	●	●	●	●	●

Legend ○ not detected ● detected

Table 6.7: *Detection of fatty acids in bovine milk.*

Fatty acids	Day 1	Day 2	Day 3	Day 4	Day 5
Alpha-Linolenic acid	○	○	○	●	●
Arachidic acid	○	●	●	○	○
Arachidonic acid	○	○	○	●	●
Behenic acid	○	○	●	○	○
Bovinic acid	○	○	○	○	○
Calcitriol	○	○	○	●	○
Capric acid	●	●	●	●	●
Caprylic acid	●	○	○	●	●
Docosapentaenoic acid	○	○	○	○	○
Dodecanoic acid	○	○	○	○	○
Eicosenoic acid	○	○	○	○	○
Fumaric acid	○	○	○	○	○
Heptadecanoic acid	○	●	●	●	●
Linoleic acid	○	○	○	○	○
Myristic acid	○	○	○	○	○
Myristoleic acid	○	●	○	●	○
Palmitic acid	○	○	○	●	●
Palmitoleic acid	○	○	○	●	●
Pentadecanoic acid	○	○	○	●	○
Stearic acid	○	○	○	●	○
Tetracosanoic acid	○	●	○	○	○

Legend ○ not detected ● detected

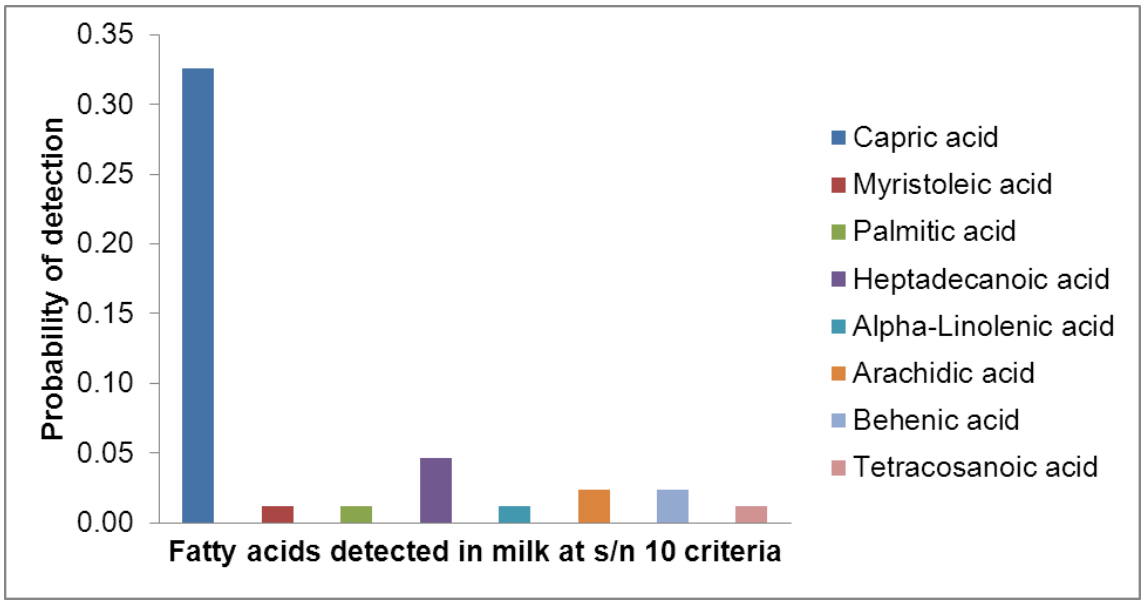


Figure 6.6: *Relative abundance of fatty acids found in all milk samples for 5 different days.*

Table 6.7: *Detection of nucleosides in bovine milk.*

Nucleosides	Day 1	Day 2	Day 3	Day 4	Day 5
1-Methyladenosine	●	○	○	●	●
Adenosine	●	●	●	●	●
Adenosine diphosphate	●	●	●	●	●
Adenosine monophosphate	●	●	●	●	●
Cytidine	○	○	●	●	○
Cytidine monophosphate	●	●	●	●	●
Guanine	○	●	○	●	●
Guanosine	●	●	●	●	●
Guanosine monophosphate	●	●	●	●	●
Inosine	●	○	●	●	●
Uridine	●	●	●	●	●
Uridine 5'-monophosphate	●	●	●	●	●

Legend ○ *not detected* ● *detected*

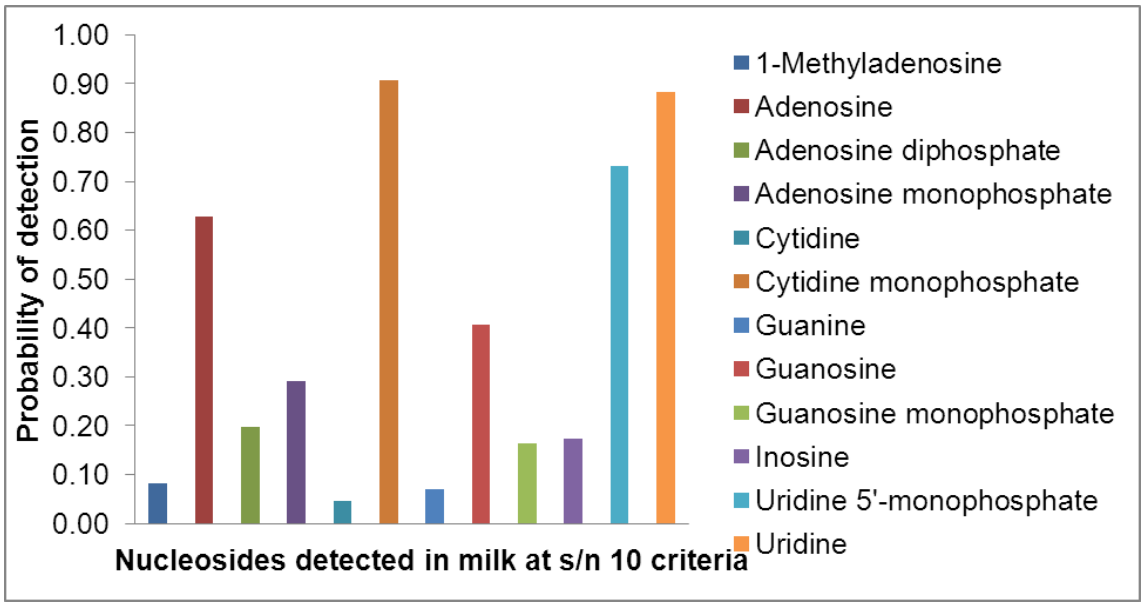


Figure 6.7: *Relative abundance of nucleosides found in all milk samples for 5 different days.*

Table 8:*Detection of steroids in bovine milk.*

Steroids	Day 1	Day 2	Day 3	Day 4	Day 5
Androstenedione	○	○	○	●	○
Cholesterol	○	●	●	●	●
Dehydroepiandrosterone	○	○	○	●	○
Epitestosterone	○	○	○	●	○
Estriol	○	○	○	○	○
Estrone	○	○	○	●	●
Estroneglucuronide	●	○	○	●	○
Estrone sulfate	●	○	●	●	○
Progesterone	○	○	○	○	○
Testosterone	○	○	○	●	○

Legend ○ *not detected* ● *detected*

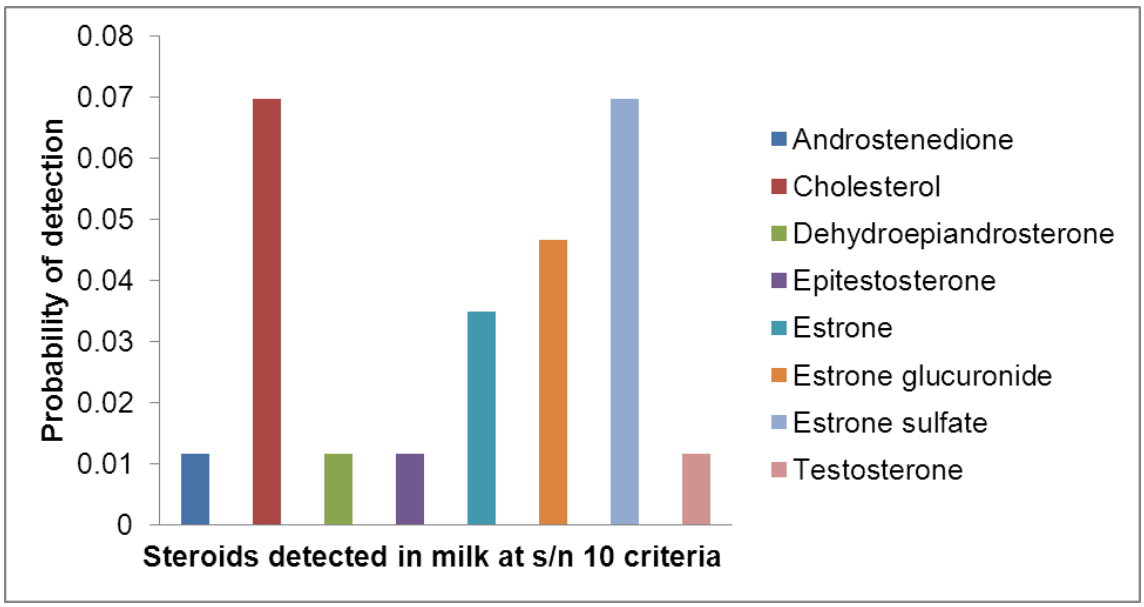


Figure 6.8: *Relative abundance of steroidsfound in all milk samples for 5 different days.*

Table 6.9: *Detection of lipids in bovine milk.*

Lipids	Day 1	Day 2	Day 3	Day 4	Day 5
Glycerophosphocholine	●	●	●	●	●
24,25-Dihydroxyvitamin D	○	○	○	●	○
25,26-dihydroxyvitamin D	○	○	○	●	○
Delta-Tocopherol	●	●	●	●	●
Gamma-Tocopherol	○	○	●	○	○

Legend ○ *not detected* ● *detected*

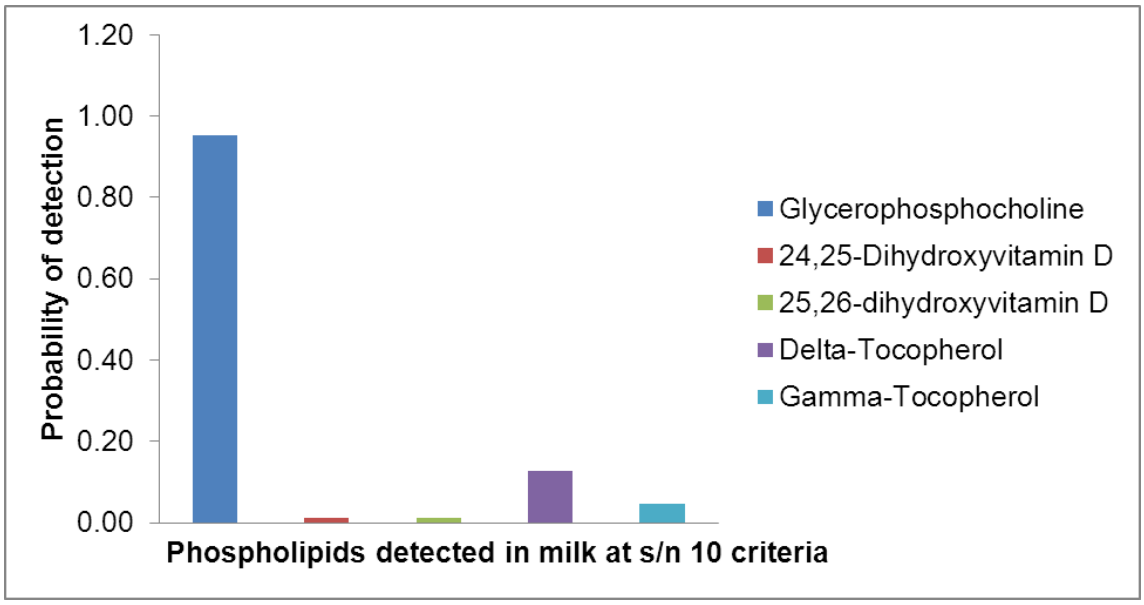


Figure 6.9: *Relative abundance of phospholipids found in all milk samples for 5 different days.*

Table 6.10: *Detection of diethanolamines in bovine milk.*

Diethanolamine	Day 1	Day 2	Day 3	Day 4	Day 5
N-octyldiethanolamine	○	○	○	●	○
N-decyldiethanolamine	○	○	○	○	○
N-dodecyl diethanolamine	○	●	●	●	○
N-tetradecyldiethanolamine	●	○	●	○	○
N-2-octyloxyethyldiethanolamine	○	○	○	○	○
N-2-decyloxyethyldiethanolamine	○	○	○	○	○
N-2-dodecyloxyethyldiethanolamine	●	●	●	●	○
N-2-tetradecyloxyethyldiethanolamine	○	○	○	○	○
N-octyl ethanolamine	○	○	○	○	○
N-decyl ethanolamine	○	○	○	○	○
N-dodecylethanolamine	○	○	○	○	○
N-tetradecylethanolamine	○	○	○	○	○

Legend ○ not detected ● detected

Table 6.11: *Detection of miscellaneous metabolites in bovine milk.*

Miscellaneous	Day 1	Day 2	Day 3	Day 4	Day 5
3-Hydroxybutyric acid	○	○	○	○	○
3-Methylhistidine	○	○	●	○	○
Acetoacetic acid	●	●	●	●	●
Acetylcarnitine	●	●	●	●	●
Allantoin	○	○	○	●	○
Aminoadipic acid	○	○	○	●	○
Betaine	●	●	●	●	●
Biotin	●	○	●	●	○
Choline	○	●	○	○	○
Citric acid	●	●	○	○	○
Creatinine	○	○	○	●	○
Enterodiol	●	○	●	●	●
Enterolactone	●	●	●	●	●
Ethanolamine	○	○	●	○	○
Histamine	●	○	○	●	○
Hypoxanthine	●	●	●	●	●
Isocitric acid	●	●	○	○	○
Malic acid	○	○	○	○	○
Melatonin	○	○	○	●	○
Pyridoxal 5'-phosphate	●	○	●	●	○
Pyridoxamine	●	○	●	○	○
Pyruvic acid	○	○	○	○	○
Secoisolariciresinol	●	○	●	●	●
Spermidine	○	○	○	○	○
Spermine	○	○	○	●	○
Succinic acid	○	○	○	○	○
Taurine	●	○	●	●	○
Trimethylamine	○	○	○	○	○
Trimethylamine N-oxide	○	○	○	○	○
Urea	○	○	○	○	○
Uric acid	○	○	○	○	○
Xanthine	○	○	○	○	○

Legend ○ not detected ● detected

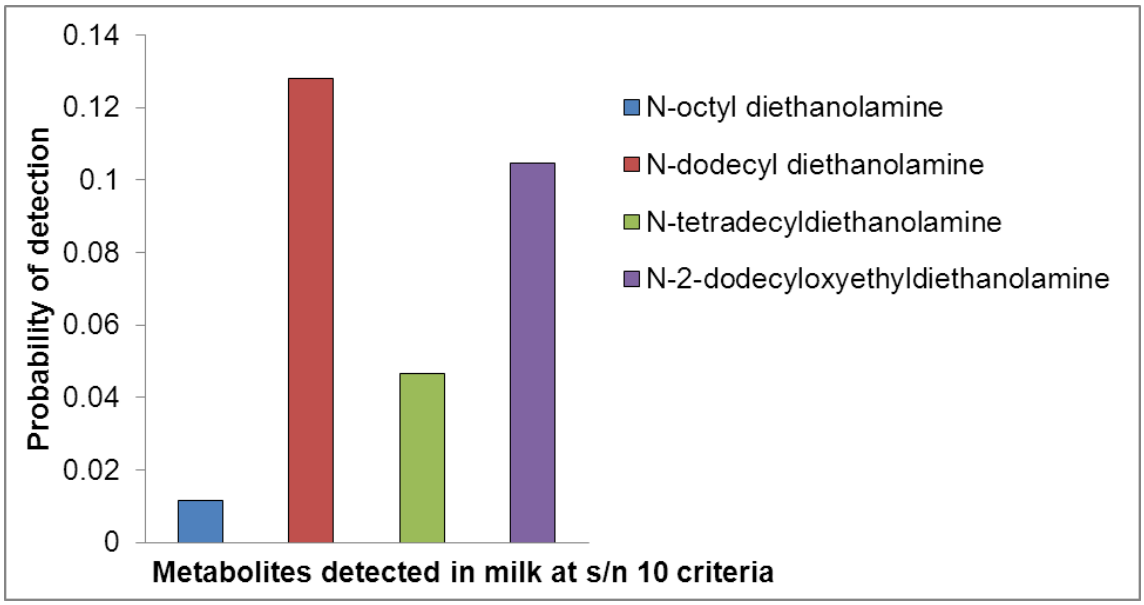


Figure 6.10: *Relative abundance of miscellaneous metabolites found in all milk samples for 5 different days.*

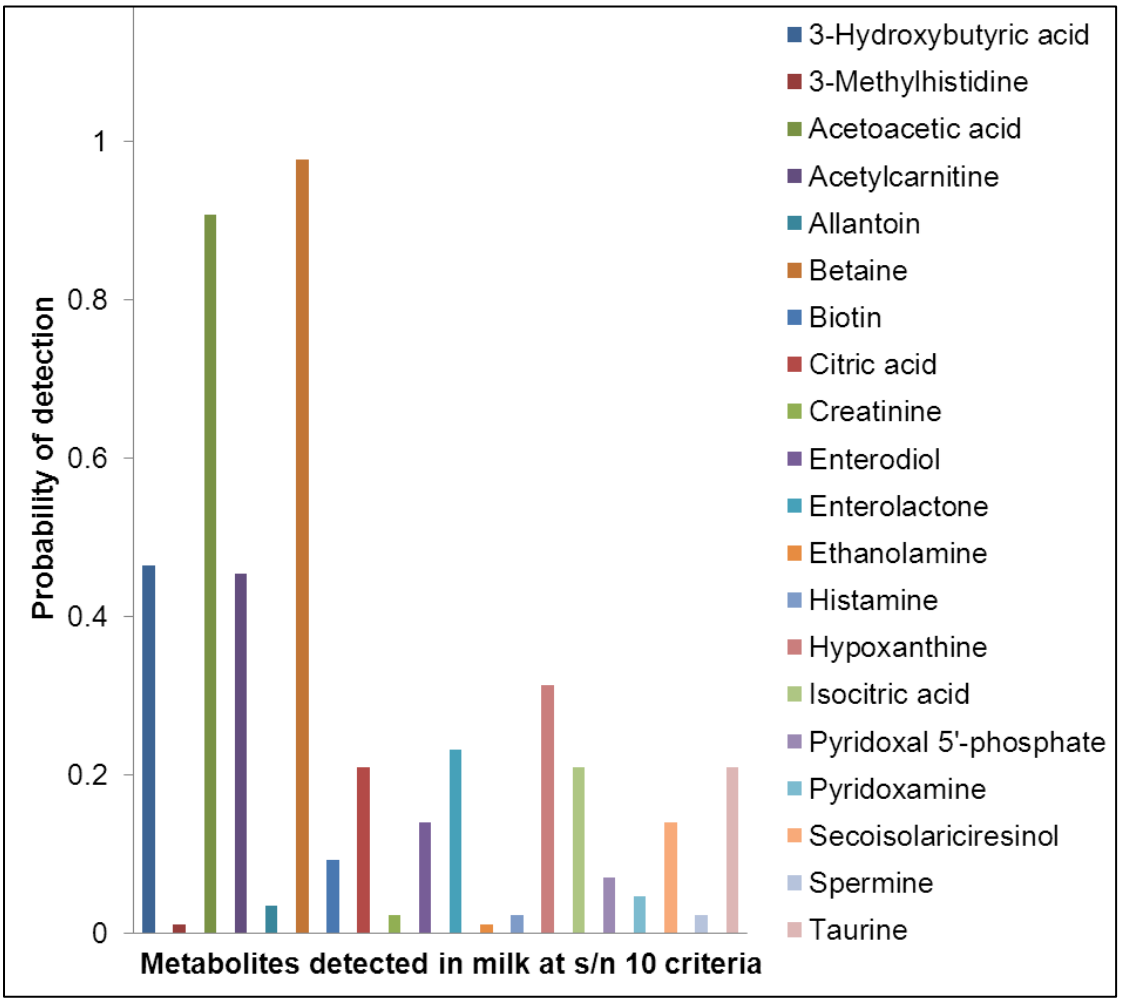


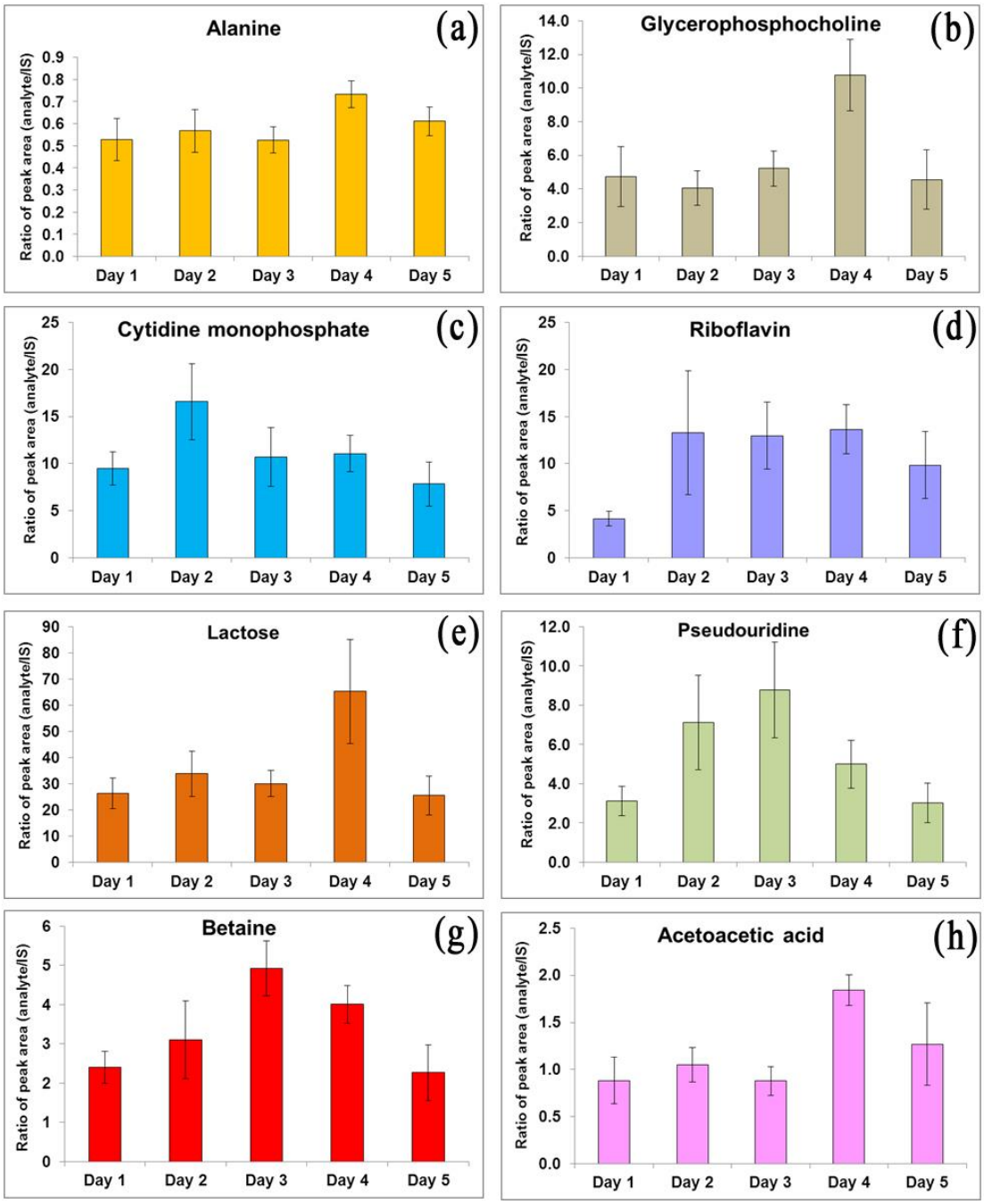
Figure 6.11: *Relative abundance of miscellaneous metabolites found in all milk samples for five different days.*

6.3.2 Relative quantitative analysis of milk metabolites

This section provides an overview of measurement of relative quantity of metabolites in the milk over the period of different days. To maintain the consistency in milk quality, dairy units require the profiling of all the constituents as well as their quantity. This quantity is variable in the milk which depends on the geographic location, breed, storage and method of transportation. Thus the relative quantitation is very useful in assessing the consistency of metabolites level in the milk. Here few metabolites were selected for the relative quantitation according to the category and their detection in milk sample in all the five days. To quantify these metabolites an internal standard of known quantity was added to milk prior to analysis. Similar to previous work, triazine molecule (2,4-diamino-1,3,5-triazine) was used as internal standard. The purpose of adding internal standard was to compensate the signal variability in MALDI-TOF MS and to relatively measure the amount of metabolites.

Initially the peak area of particular metabolites was normalized with peak area of internal standard. The ratio of area of metabolite to internal standard was plotted against the days. In the plot, higher peak area ratio indicates the higher amount of metabolite. Thus the plot clearly shows the quantitative profile of a metabolite on the different days. For example in relative quantitation of alanine in milk sample it can be conclude that the alanine was present in similar concentration except the day 4 milk sample, which had highest concentration. Similarly in other metabolites, variability in concentration can be seen for different days (figure 6.12). Overall this exercise demonstrates a method to keep an eye on the behavior of metabolites in the milk sample which further brings the attention of the user to know the other information like health of animal, storage and transportation.

Figure 6.12: *Relative concentration of (a) alanine (amino acid), (b) glycerophosphocholine (lipid), (c) cytidine monophosphate (nucleoside), (d) riboflavin (vitamin), (e) lactose (carbohydrate), (f) pseudouridine (flavonoid), (g) betaine and (h) acetoacetic acid in milk on five different days*



6.4 Conclusions

A simple and rapid method of analysis of milk metabolites using MALDI-TOF MS was demonstrated. 'MQ' algorithm was used for screening the mass spectral files generated from the MALDI TOF MS of 9 independent milk samples (10 replicates each). A a library containing a compilation of 151 metabolites was used for this screening. Out of these, a total of 89 metabolites were detected using MALDI-TOF MS. No chromatographic separation was performed prior to mass spectrometric analysis, thus major time consuming steps were eliminated. The entire identification was performed in the positive ion mode of the mass spectrometer. Further analysis using negative ion mode could increase the coverage. Using a chemically heterogenous internal standard, relative quantitation was performed on 8 of the metabolites that were consistently present in all the milk samples. Variations of these metabolites spread over several days were found. The potential ability of MALDI MS for use in monitoring metabolites of importance in assessing milk quality has been showcased.

6.5 References

(1) Meigs, E. B.; Marsh, H. L. *Journal of Biological Chemistry* **1913**, *16*, 147-168.

(2) Hollis, B. W.; Roos, B. A.; Draper, H. H.; Lambert, P. W. *Journal of Nutrition* **1981**, *111*, 1240-1248.

(3) Haug, A.; Hostmark, A. T.; Harstad, O. M. *Lipids in Health and Disease* **2007**, *6*.

(4) Boudonck, K. J.; Mitchell, M. W.; Wulff, J.; Ryals, J. A. *Metabolomics* **2009**, *5*, 375-386.

(5) German, J. B.; Dillard, C. J. *Critical Reviews in Food Science and Nutrition* **2006**, *46*, 57-92.

(6) Ceballos, L. S.; Morales, E. R.; Adarve, G. D. T.; Castro, J. D.; Martinez, L. P.; Sampelayo, M. R. S. *Journal of Food Composition and Analysis* **2009**, *22*, 322-329.

(7) Guy, P. A.; Fenaille, F. *Mass Spectrometry Reviews* **2006**, *25*, 290-326.

(8) Rodziewicz, L. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2008**, *864*, 156-160.

(9) Fong, B.; Ma, K.; McJarrow, P. *Journal of Agricultural and Food Chemistry* **2011**, *59*, 9788-9795.

(10) Fusch, G.; Choi, A.; Rochow, N.; Fusch, C. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2011**, *879*, 3759-3762.

(11) Reis, M. G.; dos Reis, M. M.; Leath, S.; Stelwagen, K. *Journal of Chromatography A* **2011**, *1218*, 316-323.

(12) Wang, H. X.; Zhou, Y.; Jiang, Q. W. *Chinese Journal of Analytical Chemistry* **2011**, *39*, 1323-1328.

(13) Hampel, D.; York, E. R.; Allen, L. H. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2012**, *903*, 7-13.

(14) Harzia, H.; Kilk, K.; Joudu, I.; Henno, M.; Kart, O.; Soomets, U. *Journal of Dairy Science* **2012**, *95*, 533-540.

(15) Deceuninck, Y.; Bichon, E.; Monteau, F.; Dervilly-Pinel, G.; Antignac, J. P.; Le Bizec, B. *Journal of Chromatography A* **2013**, *1294*, 76-86.

(16) Kaklamanos, G.; Theodoridis, G. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2013**, *930*, 22-29.

(17) Vadla, N. C.; Davalagar, V. D.; Sripadi, P. *Metabolomics* **2013**, *9*, 623-630.

(18) Junza, A.; Barbosa, S.; Codony, M. R.; Jubert, A.; Barbosa, J.; Barron, D. *Journal of Agricultural and Food Chemistry* **2014**, *62*, 2008-2021.

(19) Liu, D. Y.; Gu, J.; Ma, Y. J.; Ji, W. L.; Liu, H. L. *Chinese Journal of Analytical Chemistry* **2014**, *42*, 197-202.

(20) Zhou, Q.; Gao, B. Y.; Zhang, X.; Xu, Y. W.; Shi, H. M.; Yu, L. L. *Food Chemistry* **2014**, *143*, 199-204.

(21) Finke, B.; Stahl, B.; Pfenninger, A.; Karas, M.; Daniel, H.; Sawatzki, G. *Analytical Chemistry* **1999**, *71*, 3755-3762.

(22) Napoli, A.; Aiello, D.; Di Donna, L.; Prendushi, H.; Sindona, G. *Analytical Chemistry* **2007**, *79*, 5941-5948.

(23) Calvano, C. D.; De Ceglie, C.; Monopoli, A.; Zambonin, C. G. *Journal of Mass Spectrometry* **2012**, *47*, 1141-1149.

(24) Garcia, J. S.; Sanvido, G. B.; Saraiva, S. A.; Zacca, J. J.; Cosso, R. G.; Eberlin, M. N. *Food Chemistry* **2012**, *131*, 722-726.

(25) Gasilova, N.; Gassner, A. L.; Girault, H. H. *Electrophoresis* **2012**, *33*, 2390-2398.

(26) Liu, J. Y.; Liu, Y.; Gao, M. X.; Zhang, X. M. *Journal of the American Society for Mass Spectrometry* **2012**, *23*, 1424-1427.

(27) Calvano, C. D.; De Ceglie, C.; Aresta, A.; Facchini, L. A.; Zambonin, C. G. *Analytical and Bioanalytical Chemistry* **2013**, *405*, 1641-1649.

(28) Calvano, C. D.; Monopoli, A.; Loizzo, P.; Faccia, M.; Zambonin, C. *Journal of Agricultural and Food Chemistry* **2013**, *61*, 1609-1617.

(29) Cunsolo, V.; Muccilli, V.; Saletti, R.; Foti, S. *Journal of Mass Spectrometry* **2013**, *48*, 148-153.

(30) Barreiro, J. R.; Ferreira, C. R.; Sanvido, G. B.; Kostrzewa, M.; Maier, T.; Wegemann, B.; Bottcher, V.; Eberlin, M. N.; dos Santos, M. V. *Journal of Dairy Science* **2010**, *93*, 5661-5667.

(31) Xu, Z.; Wang, H. Y.; Huang, S. X.; Wei, Y. L.; Yao, S. J.; Guo, Y. L. *Analytical Chemistry* **2010**, *82*, 2113-2118.

(32) Lee, H.; An, H. J.; Lerno, L. A.; German, J. B.; Lebrilla, C. B. *International Journal of Mass Spectrometry* **2011**, *305*, 138-150.

(33) TMIC **2014**, http://foodb.ca.

(34) Guilhaus, M.; Selby, D.; Mlynski, V. *Mass Spectrometry Reviews* **2000**, *19*, 65-107.

Research Publications

- *High throughput quantitative analysis of melamine and triazines by MALDI-TOF MS*
  Singh, A.; Panchagnula, V. Analytical Methods **2011**, 3, 2360-2366.

- *Rapid and Direct Quantitation of Pharmaceutical Drugs from Urine Using MALDI-MS*
  Singh, A.; Bhattacharya, N.; Ghanate, G.; Panchagnula, P. Lc Gc North America, Current Trends in Mass Spectrometry **2013**, march 2013, 24-29.

- *Matrix-assisted laser desorption ionization mass spectrometry analysis of dimethyl arginines isomers from urine*
  Bhattacharya, N.; **Singh, A**.; Ghanate, A.; Phadke, G.; Parmar, D.; Dhaware, D.; Basak, T.; Sengupta, S.; Panchagnula, V. Analytical Methods **2014**, DOI:10.1039/c4ay00309h.

- *Quantitative MALDI-TOF MS analysis of atrazine and related herbicides*
  Phadke G. **Singh, A**.; Chinthanuri K.; Ghanate, G.; Panchagnula, V. manuscript under review Journal of AOAC International, 2014

- *Comprehensive detection of milk metabolites using MALDI-TOF MS - a rapid method to assess the milk quality*
  Singh, A.; I. Imamdar.; Ghanate, G.; Panchagnula, V. manuscript to be submitted in Journal of Agricultural and Food Chemistry