20

# Principles and Applications of LC-MS/MS for the Quantitative Bioanalysis of Analytes in Various Biological Samples

Ju-Seop Kang
*Department of Pharmacology & Clinical Pharmacology Laboratory, College of Medicine; Division of Molecular Therapeutics Development, Hanyang Biomedical Research Institute, Hanyang University, Seoul, South Korea*

## 1. Introduction

Standard technology for analyte detection in clinical chemistry fields rely on indirect characteristics of an analyte, e.g. its absorption capability of light, chemical reactivity or physicochemical interaction with macro-molecules as like antigen-antibody interaction. Otherwise, analytes are detected directly from molecular characteristics as molecular mass and molecular disintegration patterns in mass spectrometric method. Thus, mass spectrometric technology are very attractive for the quantification of biomarkers or chemicals in the context of diagnostic procedures since those techniques can be provide higher quality of analysis of much higher specificity compared to standard technologies such as photometry or ligand-binding tests. Even if gas chromatography-mass spectrometry (GC-MS) is first introduced mass spectrometric methods to laboratory medicine about 40 years ago and provide highly specific and sensitive quantification of thermo-stable molecules below a molecular weight of about 500, application of GC-MS method remained restricted to few specialized institution in laboratory medicine such as mainly toxicology laboratories, medical centres, and reference laboratories. The handling and maintenance of GC-MS instruments is very demanding and time-consuming; sample preparation is very laborious and includes procedures of sample extraction and analyte derivatisations and long run time with a typical sample throughput of less than 50 samples per day. Over the past decade with introduction of atmospheric pressure ionization (API) techniques, liquid chromatography-mass spectrometry (LC-MS) has undergone tremendous technological improvement allowing for its applications to endogenous components such as proteins, peptides, carbohydrates, DNA, and drugs or metabolites. Furthermore, powerful new technologies of ion-analyses (tandem MS, time-of-flight MS, ion-trap MS) substantially increased the capabilities of MS analyzers with respect to specificity and to the extent of data read out. These developments suggest a more widespread use of MS techniques superior to other analytical methods in routine laboratory medicine.

The aims of this chapter is to comprehensively characterize the basic principles of mass spectrometric detection, recent development of MS, bottleneck of LC-MS method associate

with matrix effect, application fields of LC with tandem MS in basic and clinical laboratory fields such as drug discovery and development, drug metabolism and toxicology studies, quantification of biogenic amines, doping control, TDM and pharmacokinetic (PK) studies.

## 2. Basic principles of mass spectrometric detection

Mass spectrometry (MS) has been described as the smallest scale in the world, not because of its size of what it weighs a molecule and a microanalytical technique that can be used selectively to detect and determine the amount of a given analyte (Watson & Sparkman, 2007; Chiu & Muddiman, 2008). MS is also used to determine the elemental composition and some aspect of the molecular structure of an analyte. Unique features of MS include its capacity for direct determination of the nominal mass of an analyte, and to produce and detect fragments of the molecule that correspond to discrete groups of atoms of different elements that reveal structure features (Watson & Sparkman, 2007). The tools of MS are mass spectrometers, and data are called mass spectra that can be displayed in many different ways, which allow the desired information about the analyte to be easily extracted (Watson & Sparkman, 2007). A MS is an apparatus which produces a beam of gaseous ions from a sample, sorts out the resulting mixture of ions according to their mass-to-charge ratios, and provides output signals which are measures of relative abundance of each ionic species present. MS are usually classified on the basis of how the mass separation is accomplished, but they all can be described as ion optical devices which separate ions according to their mass-to-charge (*m/z*) ratios by utilizing electric and/or magnetic force fields (Figure. 1). The concept of MS is to form ions from a sample, to separate the ions based on their *m/z* ratio (this can be considered to be the same as the mass because the ion has only a single charge in most cases), and to measure the abundance of the ions. In modern MS instrumentation used in environmental analyses, all of the functions (ionization separation of the ions, rate of data acquisition, detection of the ions, and storage of the data) are under computer control. Gaseous molecules are ionized in the ion source to form molecular ions which some of that will fragment. By various processes, ions of differing *m/z* values pass through the mass analyzer one at a time to reach the detector. When the ions strike the detector, they are converted into an electrical signal which, in turn, is converted into a digital response that can be stored by the computer (Sparkman, 2000).

A mass specterometer does not directly determine mass but, determines the mass of a molecule by measuring the *m/z* of its ion. The knowledge of the *m/z* of the ions enables one to determine what is present, while the measured ion intensities answer the question of how much is present. In addition, systematic interpretation of the mass spectra provides a detailed picture of the ionization process which, in turn, may be utilized in the elucidation of molecular structures. This definition of the term *m/z* is important to understanding of MS. It should be noted that the *m/z* value is a dimensionless number that is always used as an adjective, e.g. the ions with *m/z* 256, or the ion has an *m/z* value of 256. A recording of the number of ions (abundance) of a given *m/z* value as a function of the *m/z* value is a mass spectrum (Watson & Sparkman, 2007). The mass component that makes up the dimensionless *m/z* unit is based on an atomic scale rather than the physical scale normally considered as mass. Only ions are detected in mass specterometer and any nonionic particles that have no charge are removed from the mass spectrometer by the continuous pumping that maintains the vacuum. The MS first must produce a collection of ions in the gas phase. These ions are separated according to their *m/z* values in a vacuum where the

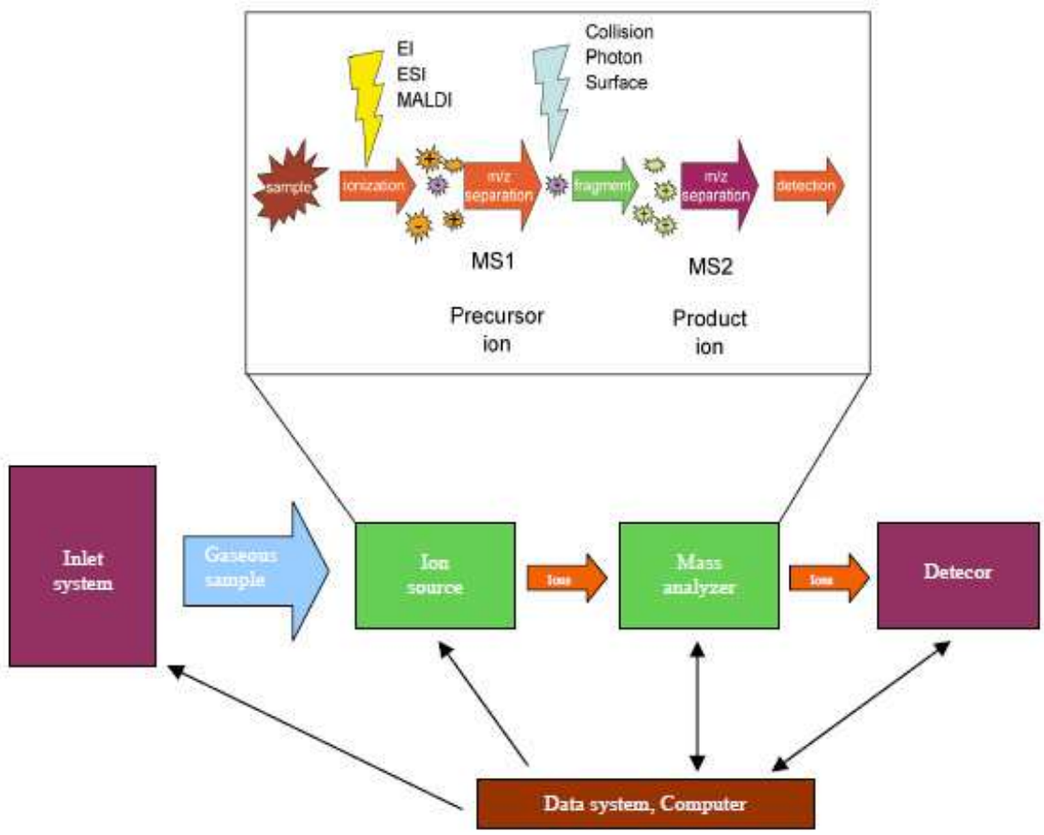



Fig. 1. Diagram of mass spectrometer system.

ions cannot collide with any other forms of matter during the separation process. Ions of individual *m/z* values are separated and detected in order to obtain the mass spectrum. Separation of ions in an evacuated environment is mandatory. If an ion collides with neutrals in an elastic collision during ion separation process, the ion's direction of travel could be altered and ion might not reach the detector. If an ion's collision with neutral is inelastic, sufficient energy transfer may cause it to decompose, meaning that the original ion will not be detected. Close encounters between ions of the same charge can be cause deflection in the path of each. Direct contact between ions of opposite charge sign will result in neutralization.

Ions are positively or negatively charged atoms, groups of atoms, or molecules. The process whereby an electrically neutral atom or molecule becomes electrically charged, due to losing or gaining one or more of its extra nuclear electrons, is called ionization (Chiu & Muddiman, 2008). Although both positive and negative ions can be analyzed by MS, the majority of instruments are used to investigate positive ions because in most ion sources they are produced in larger number than negative ions. (Chiu & Muddiman, 2008)

There is a minimum amount of energy, characterized by the "ionization potential," that must be provided in order for ion formation to occur. The first ionization potential of an atom or molecule is defined as the energy input required removing (to infinite distance) a valence electron from the highest occupied atomic or molecular orbital of the neutral particle to form the corresponding atomic or molecular ion, also in its ground state. When

only one electron is removed the ion is called an atomic or molecular ion; often the term "parent ion" is used. The formation of parent ions may be considered as ionization without cleavage. The numerical magnitude of the ionization potential is influenced by such factors as the charge upon the nucleus, the atomic or molecular radius, the shielding effect of the inner electronic shells, and the extent to which the most loosely bound electrons penetrate the cloud of electric charge of the inner shells (Figure 2).

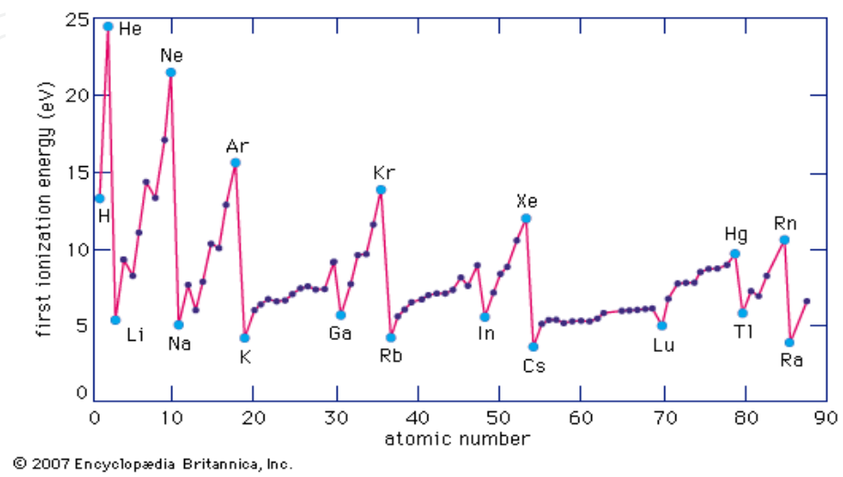



Fig. 2. Ionization potential (http://www.britannica.com/EBchecked/media/647/First-ionization-energies-of-the-elements).

Because only ions can be detected in MS, any particles that are not ionic (molecules or radicals) are removed from the MS by the continuous pumping that maintains the vacuum. When only individual ions are present, they can be grouped according to their unique properties (mass and number of charges) and moved freely from one point to another. In order to have individual ions free from any other forms of matter, it is necessary to analyze them in a vacuum, which means that the ions must be in the gas phase. It is a fundamental requirement of MS that ions be in the gas phase before they can be separated according to their individual *m/z* values and detected (Watson & Sparkman , 2007).

Due to ionization sources such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), MS has become an irreplaceable tool in the biological sciences. Over the past decade, MS has undergone tremendous technological improvements allowing for its application to proteins, peptides, carbohydrates, DNA, drugs, and many other biologically relevant molecules (Chiu & Muddiman, 2008).

### 2.1 Instrumentation of mass spectrometric detection

Mass spectrometry is a particularly powerful scientific technique because it can be successfully applied even if you have only a tiny quantity available for analysis—as little as $10^{-12}$ g, $10^{-15}$ moles for a compound of mass 1000 Daltons(Da). Compounds can be identified through mass spectrometry at very low concentrations (one part in $10^{12}$) in chemically complex mixtures. The basic mass spectrometry processes of instrumentation are consisted of (1) introduction of sample; a sample which can be a solid, liquid, or vapor is loaded onto a mass spectrometry device and is vaporized, (2) ionization; sample components are ionized by one of several available methods to create ions, (3) analyzer sorting; the ions are sorted in

an analyzer according to their *m/z* ratios through the use of electromagnetic fields, (4) detector; the ions then pass through a detector where the ion flux is converted into a propertional electrical current and (5) date conversion; the magnitude of the ion/electrical signals is converted into a mass spectrum (Watson & Sparkman , 2007).

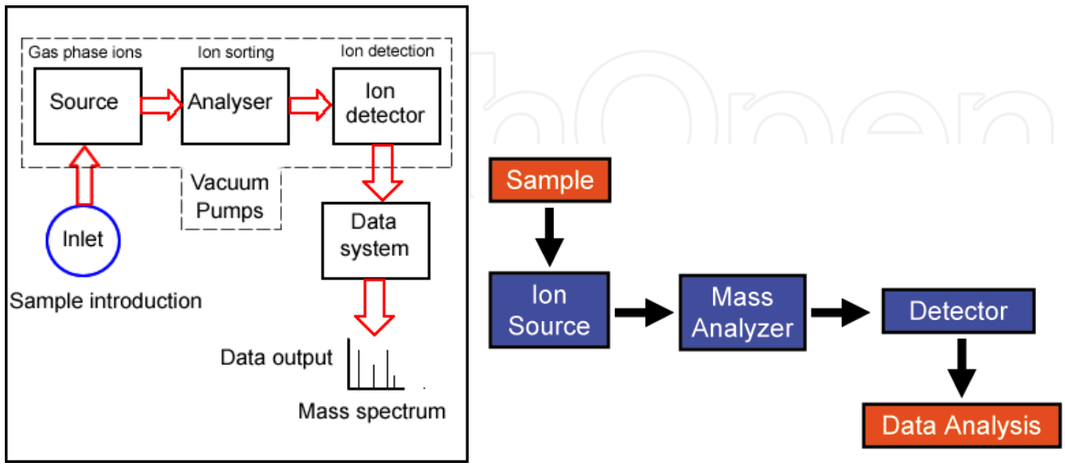



Fig. 3. Simplified diagram and function of instrumentation of typical mass spectromer (Image Source: The University of Hull, Mass Spectrometry Principles and Interpretation, Encyclopedia).

MS instruments consist of three modules: an ion source, which can convert gas phase sample molecules into ions (or, in the case of ESI, move ions that exist in solution into the gas phase); a mass analyzer, which sorts the ions by their masses by applying electromagnetic fields; and *a* detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present. The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. Other uses include quantifying the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in a vacuum). MS is now in very common use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.

## 2.2 Basic principles of mass spectrometric detection

### 2.2.1 Sample Introduction Techniques

Sample introduction was an early challenge in MS. In order to perform mass analysis on a sample, which is initially at atmospheric pressure (760 torr), it must be introduced into the instrument in such a way that the vacuum inside the instrument remains relatively unchanged (~$10^{-6}$ torr). The most common methods of sample introduction are direct insertion with a probe or plate commonly used with MALDI-MS, direct infusion or injection into the ionization source such as ESI-MS (Chiu & Muddiman, 2008).

1) Direct insertion: Using an insertion probe/plate is a very simple way to introduce a sample into an instrument. The sample is first placed onto a probe and then directly inserted into the ionization region of the mass spectrometer, typically through a vacuum interlock.

Vaccum interlock allows for the vacuum of the mass spectrometer to be maintained while the instrument is not in use. It also allows for the sample (at atomopheric pressure) to be introduced into high vacuum of the mass specterometer. The sample is then subjected to any number of desorption processes, such as laser desorption or direct heating, to facilitate vaporization and ionization.

2) Direct infusion: A simple capillary or a capillary column is used to introduce a sample as a gas or in solution. Direct infusion is also useful because it can efficiently introduce small quantities of sample into a mass spectrometer without compromising the vacuum. Capillary columns are routinely used to interface separation techniques with the ionization source of mass spectrometry. These techniques, including gas chromatography (GC) and liquid chromatography (LC), also serve to separate a solution's different components prior to mass analysis. In GC, separation of different components occurs within a glass capillary column. As the vaporized sample exits the GC, it is directly introduced into the MS.

In the 1980s the incapability of LC with MS was due largely to the ionization techniques being unable to handle the continuous flow of LC. However, ESI, APCI and atmospheric pressure photoionization (APPI) now allows LC/MS to be performed routinely. LC-MS ion chromatogram and the corresponding electrospray mass spectrum are typical technique. GC-MS produces results in much the same way as LC-MS, however, GC-MS uses an electron ionization source, which is limited by thermal vaporization (UV refers to ultraviolet and TIC is the total ion current) (Chiu & Muddiman, 2008).

### 2.2.2 Ionization method

Ionization method refers to the mechanism of ionization while the ion source is the mechanical device that allows ionization to occur. The different ionization methods work by either ionizing a neutral molecule through electron ejection, electron capture, protonation, cationization, or deprotonation, or by transferring a charged molecule from a condensed phase to the gas phase (Watson & Sparkman, 2007).

Protonation is a method of ionization by which a proton is added to a molecule, producing a net positive charge of $1^+$ for every proton added. Positive charges tend to reside on the more basic residues of the molecule, such as amines, to form stable cations. Peptides are often ionized via protonation. Pronation can be achieved via MALDI, ESI, and APCI.

Deprotonation is an ionization method by which the net negative charge of $1^-$ is achieved through the removal of a proton from a molecule. This mechanism of ion-ization, commonly achieved via MALDI, ESI, and APCI is very useful for acidic species including phenols, carboxylic acids, and sulfonic acids.

Cationization is a method of ionization that produces a charged complex by non-covalently adding a positively charged ion to a neutral molecule. While protonation could fall under this same definition, cationization is distinct for its addition of a cation adduct other than a proton (e.g. alkali, ammonium). Moreover, it is known to be useful with molecules unstable to protonation. The binding of cations other than protons to a molecule is naturally less covalent, therefore, the charge remains localized on the cation. This minimizes delocalization of the charge and fragmentation of the molecule. Cationization is commonly achieved via MALDI, ESI, and APCI. Carbohydrates are excellent candidates for this ionization mechanism, with $Na^+$ a common cation adduct.

The transfer of compounds already charged(=precharged) in solution is normally achieved through the desorption or ejection of the positive or negative charged species from the

condensed phase into the gas phase. This transfer is commonly achieved via ESI, APCI, FAB and MALDI.

Electron ejection achieves ionization through the ejection of an electron to produce a $1^+$ net positive charge, often forming radical cations. Observed most commonly with electron ionization (EI) sources, electron ejection is usually performed on relatively nonpolar compounds with low molecular weights and it is also known to generate significant fragment ions. It can provide observation of EI and can provide molecular mass as well as fragmentation information. However, it often generate too much fragmentation and can be unclear whether the highest mass ion is the molecular ion or a fragment.

With the electron capture ionization method, a net negative charge of -1 is achieved with the absorption or capture of an electron. It is a mechanism of ionization primarily observed for molecules with a high electron affinity, such as halogenated compounds. It can provide observation of electron ionization and can provide molecular mass as well as fragmentation information. However, it often generate too much fragmentation and can be unclear whether the highest mass ion is the molecular ion or a fragment (Chiu & Muddiman, 2008).

### 2.2.3 Ionization sources

The ion source is the part of the mass spectrometer that ionizes the material under analysis (the analyte). Ionization methods include the following; APCI, CI, EI, ESI, fast atom/ion bombardment (FAB), field desorption/field ionization (FD/FI), MALDI, thermospray ionization (TSP). With most ionisation methods there is the possibility of creating both positively and negatively charged sample ions, depending on the proton affinity of the sample. Before embarking on an analysis, the user must decide whether to detect the positively or negatively charged ions (Ashcroft, 2011). Prior to the 1980s, EI was the primary ionization source for mass analysis. However, EI limited chemists and biochemists to small molecules well below the mass range of common bio-organic compounds. These techniques have revolutionized biomolecular analyses, especially for large molecules. Among them, ESI and MALDI have clearly evolved to be the methods of choice when it comes to biomolecular analysis. ESI and MALDI are now the most common ionization sources for biomolecular mass spectrometry, offering excellent mass range and sensitivity.

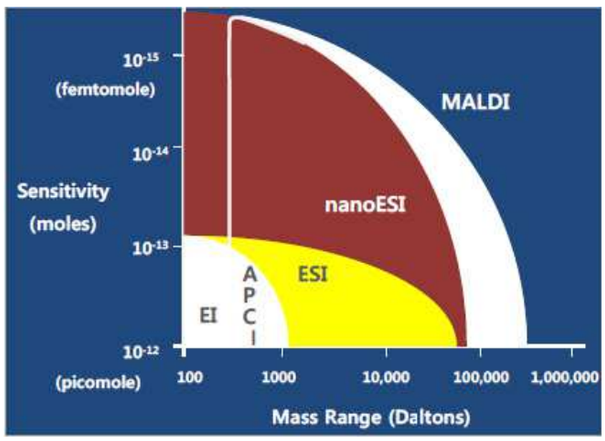



Fig. 4. A glance at the typical sensitivity and mass ranges allowed by different ionization techniques provides a clear answer to the question of which are most useful; EI, APCI and DIOS are somewhat limiting in terms of upper mass range, while ESI, nanoESI, and MALDI have a high practical mass range (Chiu & Muddiman, 2008).

Ion loses between major components of a mass spectrometer are inevitable and occur between the source and the analyzer, within the analyzer, and between the analyzer and the detector. Atmospheric pressure ion sources, such as encountered with ESI, APCI, APPI, etc., present a particular challenge with respect to efficient transfer of ions from the ion source into the *m/z* analyzer. Techniques for ionization have been key to determining what types of samples can be analyzed by MS. EI and chemical ionization(CI) are used for gases and vapors. Two techniques often used with liquid and solid biological samples include ESI and MALDI and these techniques have clearly evolved to be the methods of choice when it comes to biomolecular analysis. The idea of ESI, while not new, has been rejuvenated with its recent application to biomolecules.

| Ionization Source | Event |
|---|---|
| Electrosray ionization(ESI) | Evaporation of charged droplets |
| Nanoelectrospray ionization(nanoESI) | Evaporation of charged droplets |
| Atmospheric pressure chemical ionization (APCI) | Corona discharge and proton transfer |
| matrix-assisted laser desorption ionization (MALDI) | Photon absorption/proton transfer |
| Desorption/ionization on silicon(DIOS) | Photon absorption/proton transfer |
| Fast atom/ion bormbardment(FAB) | Ion desorption/proton transfer |
| Electron ionization(EI) | Electron beam/electron transfer |
| Chemical Ionization(CI) | Proton transfer |

Table X. Summary of event according to ionization sources (Chiu & Muddiman, 2008).

Inductively coupled plasma (ICP) sources are used primarily for cation analysis of a wide array of sample types. In the ion source technology, a 'flame' of plasma that is electrically neutral overall, but that has had a substantial fraction of its atoms ionized by high temperature, is used to atomize introduced sample molecules and to further strip the outer electrons from those atoms. The plasma is usually generated from argon gas, since the first ionization energy of argon atoms is higher than the first of any other elements except He, O, F and Ne, but lower than the second ionization energy of all except the most electropositive metals. The heating is achieved by a radio-frequency current passed through a coil surrounding the plasma. Others include glow discharge, field desorption (FD), FAB, thermospray, DIOS, Direct Analysis in Real Time (DART), APCI, secondary ion mass spectrometry (SIMS), spark ionization and thermal ionization (TIMS) (Bruins, 1991). Ion attachment ionization is a newer soft ionization technique that allows for fragmentation free analysis (Wikipedia, 2011).

### 2.2.4 Mass analyzers

The *m/z* analyzer (mass analyzer) is used to separate the ions according to their *m/z* ratio based on their characteristic behavior in electric and/or magnetic fields (Jennings & Dolnikowski, 1990; Farmer & McDowell, 1963). MS takes advantages of these different behaviors to separate the ions of different *m/z* values in space or time so that their abundances can be determined. Together with the particle's initial conditions, it completely determines the particle's motion in space and time in terms of *m/q*. When presenting data, it is common to use the dimensionless *m/z*, where *z* is the number of elementary charges (*e*) on

the ion ($z=q/e$). This quantity, although it is informally called the *m/z* ratio, more accurately speaking represents the ratio of the mass number, *m* and the charge number, *z*. Many MS use two or more mass analyzers for tandem MS (MS/MS).

With the advent of ionization sources that can vaporize and ionize biomolecules, it has become necessary to improve mass analyzer performance with respect to speed, accuracy, and resolution. More specifically, quadrupoles, quadrupole ion traps, time-to-flight (TOF), time-to-flight reflection (TOFR), and ion cyclotron resonance (ICR) mass analyzers have undergone numerous modifications and improvements over the past decade in order to be interfaced with MALDI and ESI. In the most general terms, a mass analyzer measures gas phase ions with respect to their *m/z*, where the charge is produced by the addition or loss of a proton(s), cation(s), anion(s) or electron(s). The addition of charge allows the molecule to be affected by electric fields thus allowing its mass measurement. This is important aspect to remember about mass analyzers that they measure the *m/z* ratio, not the mass itself. Multiple charging is especially common with ESI, yielding numerous peaks that correspond to the same species yet are observed at different *m/z*.

The performance of a mass analyzers can be typically be defined by the following characteristics such as accuracy, resolution, mass range, tandem analysis capabilities, and scan speed. Accuracy is the ability with which the analyzer can be accurately provide *m/z* information and is largely a function of an instrument's stability and resolution. For example, an instrument with 0.01% accuracy can provide information on a 1000 Da peptide to±0.1 Da or a 10,000 Da protein±1.0 Da. (1) The accuracy varies dramatically from analyzer to analyzer depending on the analyzer type and resolution. (2) Resolution so called resolving power is the ability of a mass spectrometer to distinguish between ions of different *m/z* ratios. Therefore, greater resolution corresponds directly to the increased ability to differentiate ions. (3) The mass range is the *m/z* range of the mass analyzer. For instance, quadrupole analyzers typically scan up to *m/z* 3000. A magnetic sector analyzer typically scans up to *m/z* 10,000 and TOF analyzers have virtually unlimited *m/z* range. (4) Tandem MS analysis is the ability of the analyzer to separate different molecular ions, generate fragment ions from a selected ion, and then mass measure the fragmented ions. The fragmented ions are used to for structural determination of original molecular ions. Typically, MS/MS experiments are performed by collision of a selected ion with inert gas molecules such as argon or helium, and the resulting fragments are mass analyzed. MS/MS analysis is used to sequence peptides, and structurally characterize carbohydrates, small oligonucleotides, and lipids. Tandem mass analysis in space is performed by consecutive analyzers whereas tandem mass in time is performed with the same analyzer, which isolates the ion of interest, fragments it, and analyzes the fragment ions. (5) Scan speed refers to the rate at which the analyzer scans over a particular mass range. Most instruments require performing a full scan; however this can vary widely depending on the analyzer. TOF analyzers, for example, complete analyses within milliseconds or less (Watson & Sparkman, 2007; Chiu & Muddiman, 2008).

#### 2.2.4.1 Sector

A **sector field mass analyzer** uses an electric and/or magnetic field to affect the path and/or velocity of the charged particles in some way. The sector instruments bend the trajectories of the ions as they pass through the mass analyzer, according to their *m/z* ratios, deflecting the more charged and faster-moving, lighter ions more. The analyzer can be used to select a narrow range of *m/z* or to scan through a range of *m/z* to catalog the ions present (Paul & Steinwedel, 1953).

#### 2.2.4.2 Time-of-flight (TOF)

The operating principles of the TOF-MS involve measuring the time required for an ion to travel from ion source to a detector usually located 1 to 2 meter from the source (Graff, 1995; Guilaus, 1995). The TOF analyzer uses an electric field to accelerate the ions through the same potential, and then measures the time they take to reach the detector. If the particles all have the same charge, the kinetic energies will be identical, and their velocities will depend only on their masses. As the ions traverse the "field-free" region between the ion source and detector, they separate into groups or packets according to velocity, which is a function of their *m/z* values, i.e. lighter ions will reach the detector first (March, 2000). As will be seen, all other types of *m/z* analyzers have upper limits for *m/z* values that can be transmitted and separated based on the way the electric and/or magnetic fields are used to separate ions of different *m/z* ratios.

#### 2.2.4.3 Quadrupole

Quadrupole mass analyzers use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field. Only a single *m/z* ratio is passed through the system at any time, but changes to the potentials on magnetic lenses allows a wide range of *m/z* values to be swept rapidly, either continuously or in a succession of discrete hops. A quadrupole mass spectrometer acts as a mass-selective filter and is closely related to the quadrupole ion trap, particularly the linear quadrupole ion trap except that it is designed to pass the untrapped ions rather than collect the trapped ones, and is for that reason referred to as a transmission quadrupole. A common variation of the quadrupole is the triple quadrupole. Quadrupoles offer three main advantages such as tolerance to relatively high pressures, significant mass range with the capability of analyzing up to an *m/z* 4000, which is useful because ESI of proteins and other biomolecules commonly produce charge distributions from *m/z* 1000 to 3500 and relatively low cost instruments. In order to perform tandem MS analysis with a quadrupole instrument, it is necessary to place three quadrupoles in series. Each quadrupole has a separate function; the first (Q1) is used to scan across a preset *m/z* range and select an ion of interest. The second (Q2), also known as the collison cell, focuses and transmits the ions while introducing a collison gas (argon or helium) into the flight path of the selected ion. The third (Q3) serves to analyze the fragment ions generated in the collison cell (Q2) (Watson & Sparkman, 2007).

#### 2.2.4.4 Quadrupole ion trap (QIT)

The name quadrupole is derived the fact that an electric field is created between four opposing electrical poles (Watson & Sparkman, 2007). The QIT works on the same physical principles as the quadrupole mass analyzer, but the ions are trapped and sequentially ejected. Ions are created and trapped in a mainly quadrupole RF potential and separated by *m/q*, non-destructively or destructively. There are many *m/z* separation and isolation methods but most commonly used is the mass instability mode in which the RF potential is ramped so that the orbit of ions with a mass $a > b$ are stable while ions with mass *b* become unstable and are ejected on the *z*-axis onto a detector. Ions may also be ejected by the resonance excitation method, whereby a supplemental oscillatory excitation voltage is applied to the endcap electrodes and the trapping voltage amplitude and/or excitation voltage frequency is varied to bring ions into a resonance condition in order of their *m/z* ratio (Schwartz et al., 2002; Lammert et al., 2006). Two method of using an ion trap for MS are involved to generate ions internally with EI, followed by mass analysis

and, more popular method involving in generation of ions externally with ESI or MALDI and using ion optics for sample injection into the trapping volume. The QIT typically consists of a ring electrode and two hyperbolic endcap electrodes. The isolated ions can subsequently be fragmented by collisional activation and the fragments detected. The primary advantage of QIT is that multiple collision induced dissociation experiments can be performed quickly without having multiple analyzers, such that real time LC-MS/MS is now routine. Other important advantages of QIT include their compact size, and thier ability to trap and accumulate ions to provide a better ion signal. QIT have been utilized in a number of applications ranging from electrospray ionization MS of biomolecules to their recent interface with MALDI. LC-MS/MS experiments are performed on proteolytic digests which provide both MS and MS/MS information. This information allows for protein identification and post-translational modification characterization. The mass range (~ *m/z* 4000) of commercial LC-traps is well matched to *m/z* values generated from the ESI of peptides and the resolution allows for charge state identification of multiple-charged peptide ions. QIT-MS can analyze peptides from a tryptic digest present at the 20~100 fmol level. Another asset of the ion trap technique for peptide analysis is the ability to perform multiple stages of MS, which can significantly increase the amount of structural information. The cylindrical ion trap MS is a derivative of the QIT mass spectrometer (Watson & Sparkman, 2007).

**2.2.4.5 Linear quadrupole ion trap (LIT)**

The linear ion trap (LIT), sometimes referred to as the 2-dimensional quadrupole ion trap (2D QIT), is one of the more recent additions to the single *m/z* analyzer group (Douglas et al, 2005; Hager, 2002). The LIT makes use of the basic structure of a transmission quadrupole; i.e., an array of four electrical surfaces; however, instead of being used to filter ions of all *m/z* values except for those of a desired value from an unresolved ion beam, they are used for trapping, manipulation of ion trajectories, and *m/z*-selective ion ejection (Londry & Hager, 2003). A LIT is similar to a QIT, but it traps ions in a 2D quadrupole field, instead of a 3-dimensional (3D) quadrupole field as in a 3D QIT. The LIT differs from the 3D ion trap as it confines ions along the axis of a quadrupole mass analyzer using a 2D radio frequency (RF) field with potentials applied to end electrodes. The primary advantage to the linear trap over the 3D trap is the larger analyzer volume lends itself to a greater dynamic ranges and an improved range of quantitative analysis (Chiu & Muddiman, 2008). This toroidal shaped trap is a configuration that allows the increased miniaturization of an ion trap mass analyzer. The LIT has higher injection efficiencies and storage capacities than conventional 3D QIT (or Paul trap). 2D multipole fields are well known for their capacity to trap and manipulate ions (Gerlich, 1992). Given the power of the ion trap the major limitations of this device that keep it from being the ultimate tool for pharmacokinetics and proteomics include the following: (1) the ability to perform high sensitivity triple quadrupole-type precursor ion scanning and neutral loss scanning experiments is not possible with ion traps. (2) The upper limit on the ratio between precursor *m/z* and the lowest trapped fragment ion is ~0.3 (also known as the "one third rules"). (3) The dynamic range of ion traps is limited because when too many ions are in the trap, space charge effects diminish the performance of the ion trap analyzer. To get around this, automated scans can rapidly count ions before they go into the trap, therefore limiting the number of ions getting in. Yet this approach presents a problem when an ion of interest is accompanied by a large background ion population (Chiu & Muddiman, 2008).

#### 2.2.4.6 Orbitrap

The orbitrap mass spectrometer is the latest development in trapping devices used as an *m/z* analyzer. The orbitrap ia a new mass analyzer (Hardman & Makarov, 2003; Hu et al., 2005; Makarov et al., 2006); however, it is useful to consider the orbitrap as a modified Knight-style Kingdon trap with specially shaped inner spindle-like (axial) electrode and a outer barrel-like (coaxial) electrodes that form an electrostatic field with quadro-logarithmic potential distribution (Makarov, 2000; Makarov et al., 2006a; Hu et al., 2005). The frequency of these harmonic oscillations is independent of the ion velocity and is inversely proportional to the square root of the mass-to-charge ratio (*m/z* or *m/q*). By sensing the ion oscillation in a manner similar to that used in Fourier transform ion cyclotron resonance (FTICR)-MS, the trap can be used as a mass analyzer. Orbitraps have a high mass accuracy (1–2 ppm), a high resolving power (up to 200,000) and a high dynamic range (around 5000) (Makarov et al, 2006a; Makarov et al., 2006b). Very similar nonmagnetic FTMS has been performed, where ions are electrostatically trapped in an orbit around a central, spindle shaped electrode. Mass spectra are obtained by Fourier transformation of the recorded image currents. Similar to Fourier transform ion cyclotron resonance mass spectrometers, Orbitraps have a high mass accuracy, and high sensitivity and a good dynamic range (Hu et al., 2005).

#### 2.2.4.7 Fourier transform ion cyclotron resonance

Fourier transform mass spectrometry (FT-MS), or more precisely Fourier transform ion cyclotron resonance MS, measures mass by detecting the image current produced by ions cyclotroning in the presence of a magnetic field. Instead of measuring the deflection of ions with a detector such as an electron multiplier, the ions are injected into a Penning trap (a static electric/magnetic ion trap) where they effectively form part of a circuit. Since the frequency of an ion's cycling is determined by its *m/z* ratio, this can be deconvoluted by performing a Fourier transform on the signal. FTMS has the advantage of high sensitivity (since each ion is "counted" more than once) and much higher resolution and thus precision (Comisarow & Marshall, 1974; Marshall et al., 1998). Ion cyclotron resonance (ICR) is an older mass analysis technique similar to FT-MS except that ions are detected with a traditional detector. Ions trapped in a Penning trap are excited by an RF electric field until they impact the wall of the trap, where the detector is located. Ions of different mass are resolved according to impact time.

### 2.2.5 Detectors

The final element of the mass spectrometer is the detector that records either the charge induced or the current produced when an ion passes by or hits a surface. In a scanning instrument, the signal produced in the detector during the course of the scan versus where the instrument is in the scan (at what *m/q*) will produce a mass spectrum, a record of ions as a function of *m/q* (Wikipedia). Typically, some type of electron multiplier is used, though other detectors including Faraday cups and ion-to-photon detectors are also used. Because the number of ions leaving the mass analyzer at a particular instant is typically quite small, considerable amplification is often necessary to get a signal. Microchannel plate detectors are commonly used in modern commercial instruments (Dubois et al., 1999). In FT-MS and Orbitraps, the detector consists of a pair of metal surfaces within the mass analyzer/ion trap region which the ions only pass near as they oscillate. No DC current is produced, only a

weak AC image current is produced in a circuit between the electrodes. Other inductive detectors have also been used (Park et al., 1994). Sensitivity, accuracy, and response time are importantparameters that distinguish different ion detection system (Koppenaal et al., 2005). High accuracy and fast-response times usually are mutually exclusive features, therefore, the fast-response electron multiplier required to follow rapid scans (e.g. *m/z* 50 to 500 in 0.1 sec) probably will not provide the accuracy desired (better than ±0.1 %) in isotope ratio measurements. Such isotope ratio measurements are usually made with the Faraday cup detector (Nygren et al., 2006; Harris et al., 1984; Mckinney et al., 1950). Ideally, a statement of sensitivity should explicity define the ion current received by detector for a specified rate of sample consumption. The definition should include (1) the name of the calibration compound, (2) the *m/z* value of the ion current that is measured, (3) the resolving power of the instrument, (4) the quantity of calibration compound consumed per second, (5) the intensity of the electron beam and pressure in the ion source for CI, and (6) the ion current arriving at the detector (e.g., that impinging the conversion dynode of an electron multiplier.

#### 2.2.5.1 Electron Multiplier (EM)

The EM uses the principle of secondary-electron emission to effect amplification (Harris et al., 1984). Perhaps the most common means of detecting ions involves an EM, which is made up of a series (12 to 24) of aluminum oxide ($Al_2O_3$) dynodes maintained at ever increasing potentials. Ions strike the first dynode surface causing an emission of electrons. These electrons are then attracted to the next dynode held at a higher potential and therefore more secondary electrons are generated. Ultimately, as numerous dynodes are involved, a cascade of electrons is formed that results in an overall current gain on the order of one million or higher. The high energy dynode (HED) uses an accelerating electrostatic field to increase the velocity of the ions. In the discrete-dynode version of an EM, The ion beam from the *m/z* analyzer is focused onto the conversion dynode, which emits electrons in direct proportion to the number of bombarding ions. The secondary electrons from the conversion dynode (ions to electrons) are accelerated and focused onto a second dynode, which itself emits secondary electrons (electrons to electrons). This means, amplification is accomplished through a ‘‘cascading effect“ of secondary electrons from dynode to dynode, because the number of electrons impinging on it (Fies, 1988). The efficiency of EM is dependent upon the velocity of the impinging particle (Westmacott et al., 2000), a feature responsible for the disappointing performance of channel EM arrays in MALDI-TOF/MS for high-mass ions (Chen et al., 2003). The secondary electrons are attracted along the positive electrical gradient farther on into the cornucopia. Each time these electrons collide with the wall, additional secondary electrons are expelled, thereby providing amplification. This form of the continuous dynode is curved so as to prevent positive ions from causing spurious signals or feedback signals due to seconadry ionization of residue gas molecule (Watson & Sparkman , 2007).

#### 2.2.5.2 Faraday cup

In this conventional electrical detector, positive ions impinging on the collector are neutralized by electrons dawn from groud after passage through a high-ohmic resistor. A Faraday cup involves an ion striking the dynode (BeO, GaP, or CsSb) surface which causes secondary electrons to be ejected. This temporary electron emission induces a positive charge on the detector and therefore a current of electrons flowing toward the detector. This

detector is not particularly sensitive, offering limited amplification of signal, yet it is tolerant of relatively high pressure (Chiu & Muddiman, 2008). Faraday cup detectors have high accuracy, constant sensitivity, and low electrical noise. The principal disadvantage of the classical Faraday is tis relatively long time constant inherent in the amplification system associated with the use of a high-ohmic resistor in the circuitry (Watson & Sparkman, 2007).

#### 2.2.5.3 Negative-ion detection

Modification of a magnetic mass spectrometer to detect negative ions instead of positive ions is relatively simple and the polarity of the accelerating voltage and the magnet current must be reversed. The negative ions have sufficient injection kinetic energy (typically 3~4 kV) to impinge on the EM even though it has a negative bias on its first dynode so that secondary electrons will experience an overall positive gradient toward the last stage(Watson & Sparkman, 2007). In mass spectrometer that uses quadrupole fields no change is required in the analyzer part of the instrument because the field acts on positive and negative ions equally. In the transmission quadrupole, the repeller and acceleration voltages have to be changed from positive to negative.

#### 2.2.5.4 Post-acceleration detector

With the advent of the desorption/inonization technique MALDI, it has been possible to generate ions of extraordinarily high *m/z* values, however, it is often difficult to detect such ions efficiently because their velocity is relatively low if it depends only on the accelerating voltage of the ion source (Beuhler et al., 1991; Matthew et al., 1986). One technique for improving the detection efficiency of high *m/z* value ions is to provide a "post-acceleration" electric field to increase their velocity prior to impacting the conversion dynode of the detector (Hedin et al., 1987). Detection of macro-ions (those heavier than hundreds of kDa having a single or double charge) has been challenge in modern MS. Conventional MS has relied on ionization-based detectors, namely those producing and multiplying secondary electrons associated with particle bombardment of a metal surface; such detectors are restricted to ions having a mass less than $10^6$ Da. An ion-to-proton conversion detector (IPD) shows promise for detecting macro-ions (Dubois et al., 1997; Dubois et al., 1999). Photosensitive detectors, relying on laser-indued fluorescence (LIF) or elastic light scattering (ELS), show promise in this area.

#### 2.2.5.5 Channel Electron Multiplier Array (CEMA)

CEMA consists of a honeycomb arrangement of many channels up to several hundred per square inch, each having a diameter on the order of 10 μm made possible with fiber optic technology using metal-doped glass. A potential difference applied to opposite ends of the channels with top and bottom of the honeycomb creates an electrical gradient along the resistive but conducting surface of each channel. The ions enter the individual channels slightly off-axis so that they impinge the wall of the channel. This primary event (an ion colliding with the surface to expel electrons) has very poor efficiency; the efficiency is directly proportional to the velocity of the impacting ion, which explains the exceptionally poor response of the CEMA to massive ions. The secondary electrons resulting from ions impinging the wall continue to ricochet down the channel, producing more secondary electrons upon each impact, thereby amplifying the original ion beam (Watson & Sparkman, 2007). The CEMA is also employed in TOF-MS because it presents a simple flat surface that can be arranged perpendicular to the ion beam to preserve velocity resolution of the ion packets (Price & Milnes, 1984).

#### 2.2.5.6 Photomultiplier conversion dynode

The photomultiplier conversion dynode detector is not as commonly used at the EM yet it is similar in design where the secondary electrons strike a phosphorus screen instead of a dynode. The phosphorus screen releases photons which are detected by the photomultiplier. One advantage of the conversion dynode is that the photomultiplier tube is sealed in a vacuum, unexposed to the environment of the mass spectrometer and thus the possibility of contamination is removed. This improves the lifetimes of these detectors over electron multipliers. A five-year or greater lifetime is typical, and they have a similar sensitivity to the EM (Chiu & Muddiman, 2008).

#### 2.2.5.7 The daly detector

The positive ion beam passing the detector slit is attracted toward an aluminized cathode (the Daly knob) held at a very large negative potential (e.g. -15,000 V) (Daly et al, 1968a; Daly et al, 1968b). Positive ions impacting the Daly knob, which essentially serves ac a conversion dynode, produces up to eight secondary electrons, which are attracted to scintillator unit held at ground potential. The Daly detector offers two significant advantages over other similar detectors. First, most of components (all except the Daly knob) are located outside the vacuum chamber, and thus can be served without distrupting the pressure regime of the mass spectrometer. Second, the large potential difference used between the Daly knob and the slit is particularly advantageous for detecting ions of high mass as a post-accelerator detector. In addition, a deceleration lens can be installed in front of Daly knob to distinguish between stable ions and those that are ionic products of metastable decay (Watson & Sparkman, 2007)

#### 2.2.5.8 Array detector

An array detector is a group of individual detectors aligned in an array format. The array detector, which spatially detects ions according to their different $m/z$, has been typically used on magnetic sector mass analyzers. Spatially differentiated ions can be detected simultaneously by an array detector. The primary advantage of this approach is that, over a small mass range, scanning is not necessary and therefore sensitivity is improved ( Chiu & Muddiman, 2008).

### 2.2.6 Vacuum techniques and systems

All mass spectrometer need a vacuum to allow ions to reach the detector without colliding with other gaseous molecules or atoms. If some collisions between molecules did occur, the instrument would suffer from reduced resolution and sensitivity. Low pressure (i.e. infrequent ion/molecule or molecule/molecule collisions) is also essential for preservation of resolving power in $m/z$ analyzers. Once the direction of an ion's path has been established, any interaction with other matter can cause that direction to change (Watson & Sparkman, 2007). One of the first obstacles faced by the originators of mass spectrometer was coupling the sample source to a mass spectrometer. The sample is initially at atmospheric pressure (760 torr) before being transferred into the mass spectrometer's vacuum (~$10^{-6}$ torr), which represents approximately a billion-fold difference in pressure. One approach is to introduce the sample through a capillary column (GC) or through a small orifice directly into the instrument. Another approach is to evacuate the sample chamber through a vacuum lock (MALDI) and once a reasonable vacuum is achieved (< $10^{-2}$ torr) the sample can be presented to the primary vacuum chamber (< $10^{-5}$ torr). The

mechanical pump serves as a general workhorse for most MS and allows for an initial vacuum of about $10^{-3}$ torr to be obtained. Once a $10^{-3}$ torr vacuum is achieved, the other pumping systems, such as diffusion, cryogenic and turbomolecular can be activated to obtain pressures as low as $10^{-11}$ torr (Figure 5) (Chiu & Muddiman, 2008).

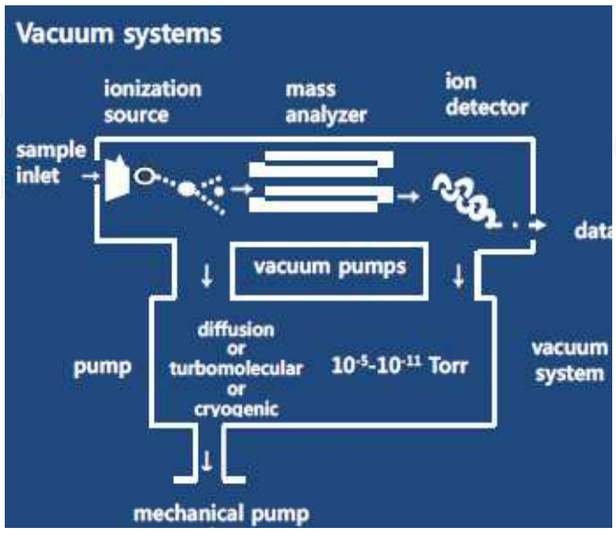



Fig. 5. A well-maintained vacuum is essential to the function of a mass spectrometer (Chiu & Muddiman, 2008).

### 2.2.7 Conclusion

The mass spectrometer as a whole can be separated into distinct sections that include the sample inlet, ion source, mass analyzer, and detector. A sample is introduced into the mass specterometer and is then ionized. The ion source produces ions either by electron ejection, electron capture, cationization, deprotonation or the transfer of a charged molecule from the condensed to the gas phase. MALDI and ESI have had a profound effect on mass spectrometry because they generate charged intact biomolecules into the gas phase. In comparison to other ionization sources such as APCI, EI, FAB, and CI, the techniques of MALDI and ESI have greatly extended the analysis capabilities of mass spexterometry to a wide range of compounds with detection capabilities ranging from the picomole to the zeptomole level. The mass analyzer is a critical component to the performance of any mass specterometry. Among the most commonly used are the quadrupole, quadrupole ion trap, TOF, TOFR, and FTMS. However, the list is growing as more specialized analyzers allow for more difficult questions to be addressed. For example, the development of the quad-TOF has demonstrated its superior capabilities in high accuracy tandem mass spectrometry experiments. Once the ions are separated by the mass analyzer they reach the ion detector, which is ultimately responsible for the signal we observe in the mass spectrum.

## 3. Recent development of in high-throughput quantitative bioanalysis by liquid chromatography (LC) coupled with mass spectrometry (MS or MS/MS)

Development of standard techniques of analytical detection in clinical chemistry relies on indirect characteristics of an analyte, e.g. its absorption of light, chemical reactivity or

physical interaction with macro-molecules (Vogeser & Kirchhoff, 2011). During the past decade, LC-MS/MS technologies have substantially extended the methodologic armamentarium of clinical laboratories (Vogeser & Seger, 2008). It has become one of the essential basic technologies used in laboratory medicine that shown in the analytical technology mindmap (Figure 6).

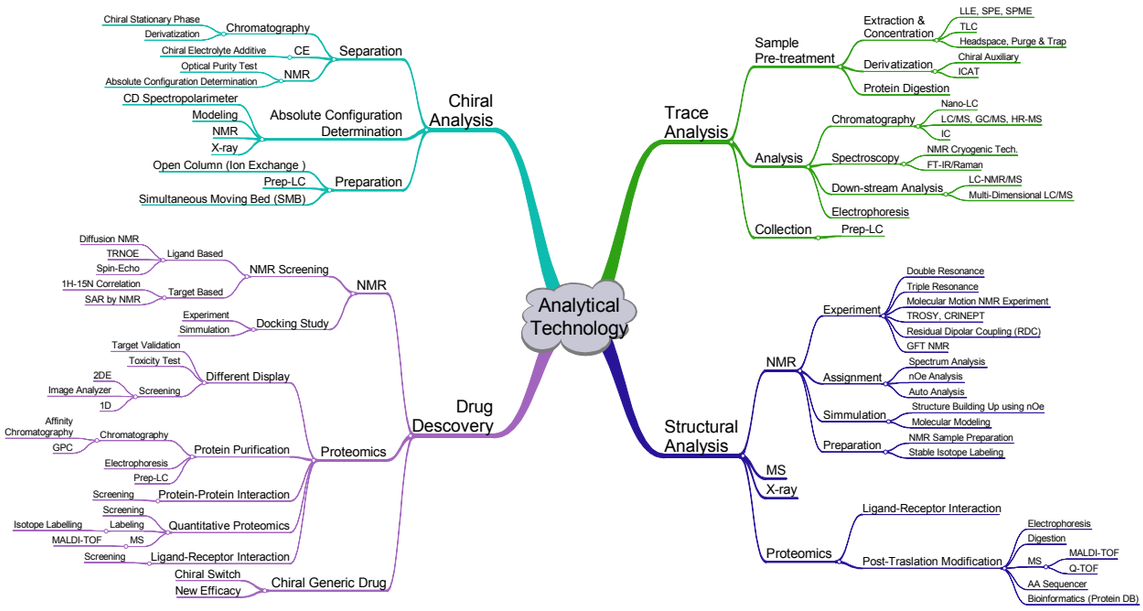



Fig. 6. Mindmap for description of analytical technology (www.bene-technology.com).

In the MS method, in contrast, analytes are detected directly from the molecular characteristics as molecular mass and molecular disintegration patterns. Thus, MS techniques are very attractive for quantification of biomarkers or xenobiotics in the context of diagnostic procedures, since those techniques can enable analyses of much higher specificity compared to standard technologies such as photometry or ligand binding tests. With GC-MS, first MS was introduced to laboratory medicine about 40 years ago. GC-MS allowed the highly specific and sensitive quantification of thermo-stable molecules below a molecular weight of about 500 and became a key method in the toxicology field. With the respect to standardisation and quality assurance of small molecule analytical routine methods the introduction of GC-MS as a reference method was an essential progress, in particular for endocrinology. However, for the several reasons the application of GC-MS remained restricts too few specialized institution in laboratory medicine including mainly toxicological laboratories, metabolism centre, and reference laboratories. The handling and maintenance of GC-MS instruments is very demanding and time-consuming; sample preparation is very laborious and includes sample extraction and analyte derivatization; the analytical run times are long with a typical sample throughput of less than 50 samples per day (Vogeser & Kirchhoff, 2011).

Introduction of API techniques about 20 years ago made practically all potential bio-medical analytes amenable for MS. Furthermore, powerful new technologies of ion-analyses (tandem MS, TOF-MS, ion-trap MS) substantially increased the capabilities of MS analyzers with respect to specificity and to the extent of data read-out in the 1990s. These developments

suggested a widespread use of MS methods in routine laboratory medicine. In particular tandem MS instruments hyphenated to LC systems used for sample introduction and pre-fractionation have been implemented in a constantly growing number of clinical laboratories worldwide now (Vogeser & Seger, 2008). LC-MS/MS is attractive for laboratory medicine for three main reasons. (1) The development of new methods is in general straightforward and independent from the diagnostic industry, without the need e.g. to develop analytical antibodies. (2) The highly multiplexed analyses are feasible with very low current costs; the range of potential analytes is practically unlimited; individual "metabolomic analyses" addressing hundreds of analytes from different biochemical pathways and from different chemical classes are possible, as well as a comprehensive and individual description of xenobiotics ("xenobiom"). (3) When applying the principle of isotope dilution internal standardisation, analyses on a reference method-level of accuracy can be performed in a routine laboratory setting (Vogeser & Kirchhoff, 2011). LC-MS/MS today holds enormous potentials for improvements in the pharmaceutical fields and laboratory medicine mainly including TDM, endocrinology, toxicology and metabolomic analyses, therefore, hyphenated techniques are examples of new tools that adoped for developing fast and cost-effective analytical methods. One of the most prevalent hyphenated techniques, LC-MS/MS, has led to major breakthroughs in the field of quantitative bioanalysis since the 1990s due to its inherent specificity, sensitivity, and speed. It is now generally accepted as the preferred technology for quantitating small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices as like plasma, blood, serum, urine, and tissue (Xu et al., 2007).

Because samples from biological matrices are usually not directly suitable with LC-MS/MS analysis, sample preparation is inevitable and has traditionally been done using protein precipitation (PPT), liquid-liquid extraction (LLE), or solid-phase extraction (SPE) processes. Manual operations associated with sample preparation are very laborious and time-consuming. Parallel sample processing in 96-well format using robotic liquid handlers and direct injection of plasma using an on-line extraction method have significantly shortened the time for analysis and generated a lot of interests in recent years. A major advantage of on-line SPE over off-line extraction techniques is that the sample preparation step is included into the chromatographic separation and thus eliminates most of the sample preparation time classically performed at the bench-side. Fast gradients and short columns were first utilized in early applications of high-throughput LC-MS/MS assays to reduce run times. Better understanding of how matrix effects can compromise the integrity of bioanalytical methods has reemphasized the need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis using LC-MS/MS method (Chiu & Muddiman, 2008).

New developments from chromatographic techniques such as ultra-performance LC with sub-2 mm particles and monolithic chromatography are showing promise in delivering higher speed, better resolution and sensitivity for high-throughput analysis while minimizing matrix effects. On the other hand, automation in LC-MS/MS is great advance in instrumentation field and has to address a number of different processes in laboratory medicine application of LC-MS/MS that includes management of primary samples, assay-specific work-up of samples prior to actual MS analysis, integrated control of the subunits of the LC-MS/MS systems, processing of primary read-outs, and further handling of result data.

### 3.1 Sample preparation

#### 3.1.1 Basic principles of sample preparation

Adequate sample preparation is a key aspect of quantitative bioanalysis and can often be the cause of bottlenecks during high-throughput analysis. Sample preparation techniques in 96-well format have been well adopted in high-thoughput quantitative bioanalysis. Four main principles of sample extraction are applied in LC: PPT by addition of organic solvents, inorganic acids and/or chaotropic salts, protein filtration, LLE, and SPE. Typically not a very high degree of protein removal is achieved. The increasing demand for high-throughput causes a unique situation of balancing cost versus analysis speed as each sample preparation technique offers unique advantages. Sample preparation with PPT is widely used in bioanalysis of plasma samples. The method has been extended to quantitation of drug and metabolites from whole blood. A sensitive and specific LC–MS/MS method for the simultaneous determination of cyclosporine A and its three main metabolites (AM1, AM4N, and AM9) in human blood have been developed (Koseki et al., 2006). Overall, PPT offers a generic and fast sample preparation technique that can be easily automated. However, when analyzing supernatant from a plasma sample using PPT, salts and endogenous material are still present and cause ion suppression or enhancement that will lead to higher variation from sample to sample. Solvent extraction and SPE typically result in very clean extracts; ion-suppression effects due to residual matrix components are reduced with solvent extraction and SPE compared to mere PPT (Annesley, 2003; Taylor, 2005). While LLE is predominantly based on differential polarity, a variety of different extraction surfaces is available for SPE such as carbohydrates (C2, C8 and C18), ion exchange materials (De Jong et al., 2007; De Jong et al., 2010a), phenylic groups, amino groups, co-polymer mixed mode materials (Dunér et al., 2007), immobilized on particles packed in cartridges. SPE materials can have added particular functions, as removing of phospholipids. Both LLE and SPE allow up to ten-fold concentration of analytes; however, these methods are technically far more demanding compared to PPT. The optimum choice of one of the four main principles of sample preparation is specific for the respective analyte, but also for the individual MS/MS system.

#### 3.1.2 Automated off-line sample preparation

The most widely used principle for automated sample preparation in LC-MS/MS is so far SPE. A wide range of SPE materials is available today and a highly efficient extraction protocol can be tailored for practically all analytes by optimizing the content of organic solvents, pH or ion strength. The use of SPE often gives superior results to those by a PPT method but may not be as cost-effective as PPT due to the labor and material costs associated with the process. A novel 96-well SPE plate that was designed to minimize the elution volume required for quantitative elution of analytes and the plate was packed with 2 mg of a high-capacity SPE sorbent that allows loading of up to 750 µL of plasma. The novel design permitted elution with as little as 25 µL solvent (Mallet et al., 2003). The evaporation and reconstitution step that is typically required in SPE is avoided due to the concentrating ability of the sorbent. A sensitive µElution-SPE-LC–MS/MS method were developed for the determination of $M^{+4}$ stable isotope labeled cortisone and cortisol in human plasma (Yang et al., 2006). In the method, analytes were extracted from 0.3 mL of human plasma samples using a Waters Oasis HLB 96-well µElution SPE plate with 70 µL methanol as the elution solvent. The lower limit of quantitation was 0.1 ng/mL and the linear calibration range was from 0.1 to 100 ng/mL for both analytes. A direct coupling of a pre-analytics module

directly with a LC-MS/MS instrument is feasible as well, either by a dedicated system (Alnouti et al., 2005; Koal et al., 2006) or by implementing a HPLC injection valve onto a generic liquid handling system (Daurel-Receveur et al., 2006). A 96-well LLE LC-MS/MS method for determination of a basic and polar drug candidate from plasma samples developed (Xu et al., 2004). A LLE procedure for sample preparation of dextromethaphan, an active ingredient in many OTC regimens, and dextrophan, an active metabolite of dextromethaphan in human plasma were reported (Bolden, 2002). Combination of sample preparation techniques have been developed to achieve desired sample extract purity with high throughput. A simplified PPT/SPE procedure was investiged (Xue et al., 2006). A mixture of acetonitrile and methanol along with formic acid was used to precipitate plasma proteins prior to selectively extracting the basic drug. After vortexing and centrifugation, supernatants were directly loaded onto an unconditioned Oasis MCX µElution 96-well extraction plate, where the protonated drug was retained on the negatively charged sorbant while interfering neutral lipids, steroids or other endogenous materials were eliminated. Additional wash steps were deemed unneccessary and not performed prior to sample elution (Xu et al., 2007). However, the system primarily aims to the analysis of research samples in micro-vials and is not yet useful for the direct application of standards sample containers as used in clinical laboratories.

### 3.1.3 On-line solid-phase extraction (SPE)

The on-line SPE technique offers speed, high sensitivity by the pre-concentration factor, and low extraction cost per sample, but typically requires the use of program controlled switch valves and column re-configuration. However, the on-line techniques can be fully automated and several generic approaches have recently been developed for on-line sample extraction coupled to LC-MS (Veuthey et al., 2004; Xu et al., 2005; Zang et al., 2005; Xu et al., 2006b). Various column demensions can be configured for the fast analysis of drug and their metabolites in biological matrix at the ng/mL level or lower. A generic method for the fast determination of a wide range of drugs in serum or plasma has been presented for the Spark Holland system (Schellen et al., 2003). The method comprises generic SPE with HySphere particles, on-line coupled to gradient HPLC-MS/MS detection. The optimized generic SPE-LC-MS/MS protocol was evaluated for 11 drugs with different physicochemical properties. An approach for on-line introduction of internal standard (IS) for quantitative analysis was developed on the Spark Holland system (Alnouti et al., 2006). This new technique was applied for direct analysis of model compounds in rat plasma using on-line SPE-LC-MS/MS quantification. On-line IS introduction allows for non-volumetric sample (plasma) collection and direct analysis without the need of measuring and aliquoting a fixed sample volume prior to the on-line SPE-LC-MS/MS that enables direct sample (plasma) analysis without any sample manipulation and preparation (Koal et al., 2006; Alnouti et al., 2005). On-line SPE with high flow rate (normally, 4~6 mL/min) has been achieved by using extraction columns packed with large diameter particles. Sample extraction occurs with very high solvent linear velocity without significant backpressure. Turbulent flow chromatography (TFC) columns marketed by Cohesive Technologies are widely used for this purpose. Minimum or no sample pre-treatment is required and significant sample preparation time is saved (Chassaing et al., 2005). Another commonly used on-line SPE sorbent material is restricted access material (RAM). With a small pore size, RAM works by eliminating the access of large molecules such as proteins to the inner surface of the particles, otherwise, small molecules can freely bind to the sorbent in the normal hydrophobic interaction mode.

Protein molecules quickly pass through the column and are washed out to waste. RAM columns have been used as the SPE and analytical column in the single column mode or coupled with another analytical column in column-switching mode (Vintiloiu et al., 2005; Kawano et al., 2005). A polar functionalized polymer(Strata-X, Phenomenex) has been explored as the extraction support in an on-line SPE LC-MS/MS assay that allows direct analysis of plasma samples containing multiple analytes (Zang et al., 2005). Beside rapid chromatographic seperation, new monolithic-phases have been investigated as extraction support for on-line SPE and an good examples of it was automated procedure using on-line extraction with monolithic sorbent for pharmaceutical component analysis in plasma by LC-MS/MS (Xu et al., 2006a). Endogenous materials from urine contains a great deal of amount of metabolic products that may present a significant challenge to assay developers and often require tedious sample preparation to remove the interfering small molecules. Method development for determining drug or metabolite concentrations from urine samples has been simplified with the implementation of on-line SPE. A sensitive method using on-line SPE and LC-MS/MS system for quantification of urinary cortisol metabolite and cortisol was developed and human urine sample were injected directly onto an on-line SPE apparatus followed by HPLC separation and LC-MS/MS detection (Barrett et al., 2005).

#### 3.1.4 New techniques for sample preparations

While SPE in a 96 position array, PPT in an array, and on-line SPE is used in many clinical MS laboratories now, several further methods of sample preparation may become useful for clinical LC-MS/MS applications in the future. Some of them are already in environmental and food analyses. SPE materials can be packed into pipetting tips; the steps of SPE can be performed within these tips by automated pipetting of sample, washing solution and finally elution medium with direct injection of the eluate into an injection port (Erve et al., 2009). A clear separation between sample preparation and chromatographic fractionation can indeed be overcome. A quadrupole TOF-MS was interfaced with a NanoMate system for immunosuppressant quantification is described with automated clean-up of the hemolysed samples by chromatographic zip-tips and direct injection to an 400 nozzle nano flow-ESI chip source without further chromatographic separation (Almeida et al, 2008). SPE materials can be packed in permanently used syringes of autosamplers as well and also referred to as solid phase micro extraction (SPME) or microextraction by packed sorbents (MEPS) (Blomberg et al., 2009; Vuckovic et al., 2010). Reliable methods can be applied in modern high-end autosampler devices (e.g., CTC PAL) and can be used an alternative to on-line SPE. Ferromagnetic micro-particles with modified surfaces (e.g., C-18 material) might be interesting for the automation of sample preparation for LC methods as well (Vogeser et al, 2008). Such particles represent a solid phase, however, can be handled in a suspension as a liquid. The use of such particles in mass spectrometry might parallel the achievements in the automation of heterogonous immunoassays which also require a convenient handling of solid phases. Ferromagnetic particles may also be handled within a HPLC system (Vogeser M., 2009). Another methods of sample preparation for mass spectrometry also include miniaturized "lab-on-the-chip" solutions for microfluidic applications (Koster & Verpoorte, 2007).

### 3.2. Separation

#### 3.2.1 Ultra-performance liquid chromatography(UPLC) with sub-2 μm particles

The use of smaller particles in packed-column LC is a well-known approach to shorten the diffusion path for a given analyte. Recent technology advances have made available

reverse phase chromatography media with sub-2 µm particle size along with liquid handling systems that can operate such columns at much higher pressures. This technology termed UPLC, offers significant theoretical advantages in resolution, speed, and sensitivity for analytical applications, particularly when coupled with mass spectrometers capable of high-speed acquisitions (Xu et al, 2007; Mazzeo et al, 2005). Today, two driving forces continue to test the limits of HPLC. One is the need for faster separations, such as analyses of either simple samples or a few constituents in a complex sample (Romanyshyn, 2001). The second is the desire to achieve greater separation power to quantify or identify all the constituents of a complex sample or to compare the contents of complex samples with each other (van der Horst & Schoenmakers, 2003; Liu et al. 2002; Plumb et al. 2003). The same driving forces resulted in the overwhelming breakthrough in the past decade of LC-MS techniques, which continue to spawn new approaches for faster or more powerful separations (Niessen, 2003; Tiller et al, 2003). The trend in LC has been the continued reduction in particle size. Harnessing the chromatographic potential of sub-2-µm particles leads to significant improvements in terms of resolution, analysis speed, and detection sensitivity. The van Deemter plots for hexylbenzene demonstrate the performance improvements that 1.7-µm particles offer over the currently used 5.0- and 3.5-µm sizes. The 1.7-µm particles give 2~3 x lower plate-height values. The particles also achieve the lower plate height at higher linear velocities and over a wider range of linear velocities. The result is better resolution and sensitivity as well as reduced analysis time (Niessen, 2003; Tiller et al, 2003). In isocratic separations, the resolution is proportional to the square root of efficiency. Particle size and efficiency at the optimum linear velocity are inversely proportional. Therefore, resolution is inversely proportional to the square root of particle size. With this knowledge, we can calculate that 1.7-µm particles will offer 1.7 x and 1.4 x greater resolution than 5.0-µm and 3.5-µm particles, respectively, at equal column lengths. Because analysis time in isocratic separations is inversely proportional to flow rate, 1.7-µm particles offer 1.7 x higher resolution than 5.0-µm particles in a third of the time, or 5 x higher productivity (resolution per unit time). The benefit of 1.7-µm particles over 3.5-µm ones is 1.4 x higher resolution in half of the time, or 3 x higher productivity. More efficient peaks translate to narrower and taller peaks. Peak width is inversely proportional to the square root of efficiency; the peak height is inversely proportional to peak width. Therefore, when smaller particles are used to make the peaks narrower, the peak height is also increased. If the detector is assumed to be concentration-sensitive (as is the case for UV detectors) and the detector noise remains constant, then sensitivity, as defined by S/N, will also increase. Specifically, in comparison with 5.0- µm particles, 1.7-µm particles offer 1.7 x higher sensitivity. When compared with 3.5-µm particles, 1.7-µm particles provide 1.4 x higher sensitivity for the same column length. Several practical issues must be addressed to achieve optimum performance. At constant $L$ and optimum flow rate, back pressure is inversely proportional to the third power of $d$p (Martin et al, 1974). Therefore, the 3-fold reduction in $d$p in UPLC translates to back pressures that are 27 x higher compared with HPLC separations when 5-µm particles are used. Therefore, UPLC is an exciting new area of LC. A natural extension of HPLC, this technique is easy to take full advantage of and requires minimal training. Although we have demonstrated the use of UPLC for reversed-phase separations, we expect that it will also be beneficial in the areas of normal-phase, hydrophilic interaction and ion-exchange chromatographies as well as chiral separation modes that applied for the determination of doxazosine in human plasma by UPLC–MS/MS (Al-Dirbashi et al, 2006) and investigated

UPLC as an alternative to HPLC for the analysis of pharmaceutical development compounds (Wren & Tchelitcheff, 2006). Data on three compounds were presented showing that significant reductions in separation time can be achieved without compromising the quality of separation. Using a poly-drug reference standard and whole blood extracts, the authors successfully separated and identified amphetamine, methamphetamine, ephedrine, pseudoephedrine, phentermine, MDA, MDMA, MDEA, and ketamine in less than 3 min by the Acquity UPLC-Micromass Quattro Micro API MS system (Waters Corporation, USA) (Apollonio et al, 2006). The approach of orthogonal extraction/chromatography and UPLC significantly improves assay performance while also increasing sample throughput for drug development studies (Shen et al, 2006). Other direct comparison experiments using UPLC–MS/MS and HPLC–MS/MS have shown that the UPLC–MS/MS improved cycle time by 50–100% with increased sensitivity In the study about the differences in LC–MS performance by conducting a side-by-side comparison of UPLC for several methods previously optimized for HPLC-based separation and quantification of multiple analytes with maximum throughput (Churchwell, 2005). Sensitivity increases with UPLC, which were found to be analyte-dependent, were as large as 10-fold and improvements in method speed were as large as 5-fold under conditions of comparable peak separations. Improvements in chromatographic resolution with UPLC were apparent from generally narrower peak widths and from a separation of diastereomers not possible using HPLC. A similar HPLC-MS/MS quantification protocol was developed for comparison purposes (Yu et al, 2006). Both UPLC-MS/MS and HPLC-MS/MS analyses were performed in both positive and negative ion modes during a single injection. Peak widths for most standards were 4.8 sec for the HPLC analysis and 2.4 sec for the UPLC analysis. Compared with the HPLC-MS/MS method, the UPLC-MS/MS method offered 3-fold decrease in retention time, up to 10-fold increase in detected peak height, with 2-fold decrease in peak width. Limits of quantification (LOQ) for both HPLC and UPLC methods were evaluated.

### 3.2.2 Monolithic chromatography

There is considerable interest to improve throughput by using monolithic columns because they exhibit higher seperation efficiency at high flow velocities when compared to conventional LC columns (Zhou et al, 2005; Ikegami & Tanaka, 2004; Cabrera, 2004; Li et al., 2005; Wang et al, 2006).The structural characteristics of the monoliths and those of the conventional beds of particular packing materials are very different that are their high external porosity resulting from the structure of the network of through macropores and the structure of the stationary phase skeleton that consists of a network of small, thin threads of porous silica. Two types of monolithic supports are currently available organic polymers such as polymethacrylates, polystyrenes, or polyacrylamide and inorganic polymers based on silica, carbon and zirconia (Xu et al, 2007). A high-throughput LC-MS/MS method using a Chromolith RP-18 monolithic column was developed for the determination of bupropion, an antidepressant drug, and its metabolites, hydrobupropion and threo-hydropupropion in human, mouse, and rat plasma (Borges et al, 2004). The monolithic column performance as a function of a column backpressure, peak asymmetry, and retention time reproducibility are adquately maintained over 864 extracted plasma injections like as a high-througput LC-MS/MS method for the determination of methylphenidate (MPH), a central nervous system stimulant, and its de-esterified metabolite, ritalinic acid (RA) in rat plasma samples (Barbarin et al, 2003). A monolithic

column was directly compared to a conventional C18 column as the analytical column in method validation of a drug and its epimer metabilite (Huang et al, 2006). Because the chosen drug and its epimer metabolite have same selected reaction monitoring (SRM) transitions, chromatographic baseline seperation of these two compounds was required. Sample preparation, mobile phases and MS conditions were kept the same in the column comparison experiment. The methods on the two systems were found to be equivalent in validation parameters and chromatographic seperation, but the monolithic column method increased the sample throughput by a factor of two (Xu et al, 2007). The significantly improved seperation speed by monolithic columns demanded higher throughput on sample extraction. An attractive approach using monolithic separation is to combine it with high-flow on-line extraction, which allowed for the fast extraction and separation of samples (Zeng et al, 2003; Zhou et al, 2005). A normal phase method on silica was adapted for the high-throughput analysis with separations and run times less than 1 min. Another high-throughput approach using a monolithic column showed tha analysis of over 1100 plasma samples preparaed by PPT in approximately 9 h (Mawwhinney & Henion, 2002). Some disadvanages for using monolithic columns for high-speed methods have been reported(Chen et al, 2002) that a lack of separation for very polar analytes (nicotinic acid and five metabolites) using a monolithic RP-18e column.

### 3.2.3 Hydrophilic interaction chromatography (HILIC)

HILIC-MS has been gaining recognition as a valuable technique for analyzing polar molecules in biological matrix in recent years (Eerkes et al, 2003; Song et al, 2006; Deng et al, 2005). Polar compounds typically have very limited retention on reverse-phase(RP)-columns. Reverse phased(RP)-HPLC mobile phase with a very low organic content must be used to seperate the anlayte from matrix interference. When using ESI-MS, the very high aqueous mobile phase can cause low ionization efficiency. The highly volatile organic mobile phases used in HILIC provide increased ESI-MS sensitivity. A bioanalytical method using automated sample transferring, automated LLE and HILIC-MS/MS for the determination of fluconazole in himan plasma are developed (Eerkes et al, 2003). After LLE, the extracts was evaporated to dryness, reconstituted, and injected onto a silica column using an acquous-organic mobile phase. The chromatographic run time was 2.0 min per injection. The use of HILIC could eliminate the evaporation and recostitution steps that hamper improvement of throughput and automation. In a validated a single-pot LLE with HILIC-MS/MS method for the determination of Muraglitazar, a hydrophobic diabetic drug, in human plasma, organic layer was then directly injected into an LC-MS/MS after extraction with acetonitrile and toluene. Incomparison with a reversed-phase LC-MS/MS, this single-pot LLE, HILIC-MS/MS imporved the detection sensitivity by greater than 4-fold based on the LLOQ signal to noise ratio.

### 3.2.4 Turbulent flow chromatography

Turbulent flow chromatography(TFC) is a high flow chromatographic technique, which allows high-throughput bioanalysis by requiring little sample preparation and potentially fast cycle times. The TFC system takes advantage of unique flow dynamics that allow us to analyze compounds in biological matrices with very little sample preparation (Ayton et al, 1998). High-throughput LC-MS/MS approaches using TFC employ small internal

diameter columns (typically 1 mm or 0.18 µm) with large particles (20~60 µm), which allow high flow rates to be utilized without impractical pressure increases. The large particles serve to trap the analytes and thus allow unretained compounds to be washed out. The resulting trapped analyte is typically eluted onto an analytical column (Ayrton et al, 1997). First reports of high-throughput bioanalysis using TFC coupled to MS/MS did not use an analytical column but a 50 mm x 1 mm, 50 µm column to perform cleanup and analysis that describes the direct analysis of plasma samples using a commercially available TFC system; the total run time was 2.5 min, and the method was validated from 5 to 1000 ng/mL(Ayrton et al, 1997) and later extended the use of "ultra-high flow" to a capillary LC column packed with large particles of 30 µm (Ayrton et al, 1999). Although there are advantages in using ultra-high flow on capillary (0.18 mm i.d.) versus narrow bore (1.0 mm i.d.) columns, trade offs must be in sample handling, column capacity, and robustness. Comparison of the methods showed the LLOQ of the TFC and SPE methods was 1 ng/mL; the TFC method used a much smaller plasma aliquot. It should be noted that drug B analyzed using a PPT method, which achieved an LLOQ of 0.5 ng/mL (Zimmer et al, 1999). In several reports about comparison of methods, these reports show that TFC is an attractive approach for high-throughput work involving metabolites (Ramos et al, 2000; Lim et al, 2001; Hopfgartner et al, 2002; Herman, 2002). Commercial systems are available which acommodate 1 to 4 seperate TFC systems and one mass spectrometer (Berna et al, 2004).

## 4. Bottlenecks of liquid chromatography with mass spectrometric method associate with matrix effect

### 4.1 What is the matrix effect?

HPLC-MS/MS detection has been demonstrated to be a powerful technique of choice for the quantitative determination of drugs and metabolites in biological fluids (Matuszewski et al, 2003; Taylor, 2005a; Kebarle & Tang, 1993). However, the common perception that utilization of HPLC–MS/MS guarantees selectivity has been challenged by a number of reported examples of lack of selectivity due to ion suppression or enhancement caused by the sample matrix (Clarke et al, 1996; Buhrman et al, 1996; Matuszewski et al, 2003) and interferences from metabolites (Constanzer et al, 1997; Jemal & Xia, 1999). The central issue is what experiments, in addition to the validation data usually provided for bioanalytical methods, need to be conducted to demonstrate the absence of a relative ("lot-to-lot") matrix effect and to confirm HPLC–MS/MS assays selectivity. Matrix effects occur when molecules coeluting with the compounds of interest alter the ionization efficiency of the electrospray interface. The matrix effects is probably originates from the competition between an analyte and the coeluting, undetected matrix components. Matrix effects are the result of competition between nonvolatile matrix components and analyte ions for access to the droplet surface for transfer to the gas phase (King et al, 2000). Better understanding of how matrix effects can compromise the integrity of bioanalytical methods has re-emphasized the need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis using LC–MS/MS (Xu et al, 2007). Inherent specificity of LC–MS/MS methods results in chromatograms that do not present any apparent interference, although relatively high concentrations of matrix components are sometimes present (Hernández et al, 2005). Matrix effects are also compound-dependent and the chemical nature of a compound has

a significant effect on the degree of matrix effects (Bonfiglio et al, 1999). In a study of 4 compounds of different polarities under the same mass spectrometric conditions, the most polar was found to have the largest ion suppression and the least polar was affected less by ion suppression. These findings of differential matrix effects have important ramifications particularly when selecting a suitable IS for quantification purposes. For example, if a drug and a glucuronide metabolite were quantified by IS against a close analogue of the parent drug and matrix effects were slightly different between samples, then the change in ionization of the more polar glucuronide metabolite would probably not be compensated by the IS. Thus if there are multiple analytes to be quantified, with varying degrees of polarity, there may be requirements for multiple IS (Lagerwerf et al, 2000). The importance of matrix effects on the reliability of HPLC–ESI–MS/MS has been shown in terms of accuracy and precision (Matuszewski et al, 1998), and when ion suppression occurs, the sensitivity and lower limit of quantification of a method may be adversely affected (Buhrman et al, 1996). Thus to develop a reliable HPLC–ESI–MS/MS method, experiments should be performed to understand these matrix effects by careful consideration to evaluate and eliminate matrix effects.

### 4.2 Evaluation of matrix effects

The two main techniques used to determine the degree of matrix effects on an HPLC–ESI–MS/MS method are postextraction addition and postcolumn infusion. The postextraction addition technique requires sample extracts with the analyte of interest added postextraction compared with pure solutions prepared in mobile phase containing equivalent amounts of the analyte of interest (Annesley, 2003; Matuszewski et al, 2003; Matuszewski, 1998). The difference in response between the postextraction sample and the pure solution divided by the pure solution response determines the degree of matrix effect occurring to the analyte in question under chromatographic conditions. The post-column infusion method provides a qualitative assessment of matrix effects, identifying chromatographic regions most likely to experience matrix effects (Bonfiglio et al, 1999). Any endogenous compound that elutes from the column and causes a variation in ESI response of the infused analyte is seen as a suppression or enhancement in the response of the infused analyte (Bakhtiar & Majumdar, 2007; Bonfiglio et al, 1999). This approach, however, does not provide a quantitative understanding of the level of matrix effect observed for specific analytes. In addition, if several compounds are determined in one method, all compounds should be infused separately to investigate possible matrix effects for every analyte. Therefore, matrix effects are not investigated for lower than LLOQ. In contrast, the post-extraction spike method quantitatively assesses matrix effects by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process (Matuszewski et al, 2003; Chambers et al, 2007). The absolute matrix effect was defined as the comparison of the signal response of a standard present in a sample extract from one single lot to the response of a standard in neat solution. However, even more important is the evaluation of the relative matrix effect, which is the comparison of matrix effect values between different lots of biofluids and therefore proposed that matrix effects should be investigated in biofluid samples from at least five different sources (Matuszewski et al, 2003). In a more recent paper, they have suggested to use the precision of the calibration line slopes in five different lots of a biofluid as an indicator of

relative matrix effects. The relative standard deviation should not exceed 3–4% for the method to be considered practically free from relative matrix effects (Matuszewski, 2006). Recently, Heller presented a new concept, namely matrix effect maps, for visualizing the impact of various parameters on matrix effects associated with a given method. In this approach matrix effects are studied as a function of the amount of co-injected matrix extract (Heller, 2007). In Heller's approach, two sets of mixtures were prepared in different formats and, to provide different matrix effect conditions for testing these mixtures, chromatographic conditions were altered as well. Furthermore, operational variables like desolvation gas flow and temperature could also be included in these matrix effect maps. However, these issues remain to be fully tested. In the future, this approach could help to determine the ruggedness of a developed method.

### 4.3 Methodology to overcome matrix effects

Different actions can be taken to overcome matrix effects. Matrix effects may be reduced by simply injecting smaller volumes or diluting the samples, which is useful as long as instrumental sensitivity remains adequate (Heller, 2007). Other possibilities to reduce or eliminate matrix effects are the optimization of sample preparation and/or chromatographic parameters (Xu et al, 2007; Hernández et al, 2005; Niessen et al, 2006). Another approach is the use of an internal standard (IS) to compensate for the alteration in signal. If sensitivity is not an issue, an alternative ionization source, less sensitive to matrix effects, can be used, e.g. APCI (Chambers et al, 2007) or electron ionization. In many cases, several approaches are combined to achieve adequate quantitative results (Niessen et al, 2006). Because the presence of coeluting compounds cause matrix effect, there is need to remove or minimize their presence to obtain a robust LC-MS/MS.

#### 4.3.1 Sample preparation techniques

In general, matrix effects may be reduced by simply injecting smaller volumes or diluting the sample. However, these solutions will clearly influence the sensitivity of the method and are therefore in many cases not appropriate (Antignac et al, 2005). Proper sample clean-up is therefore of primordial importance. Even if PPT is the simplest and fastest method for preparing samples, however, it does not result in a very clean extract and is most likely to cause ion suppression in ESI. Co-elution of these compounds with the compound of interest affects the ESI droplet desolvation process (Dams et al, 2003; Bakhtiar & Majumdar, 2007; Chambers et al, 2007). In comparison with PPT, the extracts obtained from SPE are relatively cleaner (Bakhtiar & Majumdar, 2007; Chambers et al, 2007). LLE often yields rather clean extracts, but the procedures are usually cumbersome and have many pitfalls. Multiple extraction steps are commonly needed to increase analyte recovery and to obtain cleaner extracts (Jessome & Volmer, 2006). Supported LLE can be used to decrease sample preparation time and improve analyte recovery. Rapid extraction of analyte occurs during this intimate contact between the two immiscible phases. The solvent moves through the packing by gravity flow or use of a gentle vacuum(Majors, 2006).

#### 4.3.2 Chromatographic sepreration

Improved chromatography is a straightforward way to separate interfering compounds from analytes. Gradient elution can help to wash the column after injection and prevent late-eluting compounds from the previous injection to interfere. If the analytes are ionizable, the

pH of the mobile phase can have a significant impact on the retention, selectivity and sensitivity of the separation (Chamber et al, 2007). It was also shown that fast gradient LC promotes matrix effects by reducing chromatographic separation between analytes and endogenous compounds. If high throughput is required, effective sample pretreatment becomes critical, since the chromatographic conditions are not able to reduce matrix effects. Another possibility is the use of a stationary phase with a different selectivity, for example hydrophilic-interaction liquid chromatography (HILIC). HILIC which combines the use of bare silica or polar bonded stationary phases and mobile phases with a high content of organic solvents, has been proven to be a valuable tool for the analysis of polar compounds in biological samples (Ji et al, 2008). UPLC retains the practicality and principles of classical LC, but increases the speed, resolution and sensitivity of the method by using columns with small diameter (1.7-µm) particles and high pressures. The improved resolution might provide a benefit with respect to matrix effects, through improved separation from endogenous components and obtained a statistically significant reduction in matrix effects under a variety of chromatographic conditions and with multiple basic analytes, using the UPLC technology (Chambers et al, 2007).

### 4.3.3 Mass spectrometric analysis

APCI-MS is less susceptible to matrix effects than ESI-MS and, however, the occurrence of matrix effects has also been shown with APCI (Dams et al, 2003; Sangster et al, 2004; Niessen et al, 2006). In addition, the APCI interface was more susceptible to matrix effects than the ESI interface for the same instrument. Even with this source, matrix effects were observed for most analytes and internal standards at all concentrations tested, with values ranging from 85.2 to 149.4% (Mei et al, 2003). Miniaturized ESI methods are proven to be more tolerant towards contaminations in the analyte solution. Dialysate matrix effects were estimated at different concentration levels of oxcarbazepine and its major metabolite, using a column switching microbore, capillary and nanoLC–MS/MS system (Lanckmans et al, 2006). Only at the lowest level of the microbore system, a significant matrix effect was observed. Since a lower flow rate reduces the size of the charged droplets, fewer droplet fission events and less solvent evaporation are required for ion release in the gas phase. This leads to a reduction in contaminant concentration(Lanckmans et al, 2006; Schmidt et al, 2003). The ionization suppression caused by matrix effects after plasma or urine injection have been observed in a conventional restricted-access media-LC system (Georgi & Boos, 2006), in contrast, assaying similar compounds with a capillary chromatographic setup, such matrix effects were not present and this attribute to the better characteristics of ESI-MS/MS under low flow rate (Santos-Neto et al, 2008). The extent of matrix effects also depends on the source design of the LC–MS system used. In some cases, problems observed with matrix effects can be solved by using a MS instrument from another manufacturer (Niessen et al, 2006; Mei et al, 2003). Regarding the ionization polarity, the negative mode is usually considered as more specific and consequently less subjected to ion suppression, but in practice, this is of course not possible for all analytes. (Antignac et al, 2005; Niessen et al, 2006).

### 4.3.4 Role of internal standard

As IS either a structural analogue or a stable isotope labeled-IS can be applied. However, the ionization of the analogue IS and the analyte may be differently affected by the matrix.

This can be solved by using a stable isotope labeled-IS which co-elutes with the drug, since matrix effects should not affect the relative efficiency of ionization of the drug and its stable isotope labeled-IS. The stable isotope labeled-IS are compounds in which several atoms in the analyte are replaced by their stable isotopes, such as $^{2}H$ (D), $^{13}C$, $^{15}N$ or $^{17}O$ with $^{2}H$ being the most frequently used isotope. It is important that the mass difference between the analyte and the stable isotope labeled-IS is at least 3 mass units (Stokvis et al, 2005), in order to avoid signal contribution of the abundance of the natural isotopes to the signal of the IS. If the compound and IS are not separated adequately by mass, this will result in quadratic standard curves (http://www.ionsource.com/tutorial/msquan). However, issues like isotopic purity of compounds, cross-contamination and cross-talk between MS/MS channels, isotopic integrity of the label in biological fluid and during sample processing, etc. should be carefully addressed (Matuszewski et al, 2003). The $^{13}C$, $^{15}N$ or $^{17}O$-labeled IS may be more ideal than the $^{2}H$- labeled ones, since deutrium and hydrogen have greater differences in their physical properties than for example $^{12}C$ and $^{13}C$ (Wang et al, 2007). It is of primordial importance to choose an appropriate IS during method development and to closely monitor the method performance in routine use, since only limited lots of biological matrix are tested during method validation (Wang et al, 2007). If ion suppression significantly reduces the signal of analyte and/or of the IS, the signal to noise may decrease to a poit where accuracy and precision may be negatively affected (Annesley, 2003). Even if this is not always practically feasible, a number of labeled IS identical to the number of compounds to be analyzed would be required (Hernandez et al, 2005).

#### 4.3.5 Matrix effect on the biological method validation

Studies about matrix effects in quantitative bioanalysis revealed that the ion suppression or ion enhancement is frequently accompanied by significantly deterioration of the precision and accuracy of the method. The precision, expressed as relative standard deviation, is plotted as a function of the analyte concentration. While for a single plasma lot the precision is acceptable, this is not the case when five different lots of plasma are taken into account (Matuszewski et al, 2003; Niessen et al, 2006). An absolute matrix effects on the other hand will primarily affect the accuracy of the method (Matuszewski et al, 2003). Even if matrix effects can be compensated by an appropriate IS, efforts should be made to eliminate these co-eluting compounds, since their presence will be reduce mathod sensitivity. When analyzing low concentrated samples, this can lead to false negative results. Biofluids such as plasma and especially urine represent highly complex matrices and its composition can be various significantly between individuals and species, but also within an individauls (Georgi & Boos, 2006). Most method validations are performed using calibration standards and QC samples prepared from the same pool of blank matrix. Using these homogenous samples for validation does not take into account the inter-and intrapatient matrix variability (Taylor, 2005a). Checking the quality of an assay using QC samples, which are prepared in the same matrix as calibration standards, will not reveal matrix effects observed in the incurred samples (Dewe et al, 2007). Repetitive analysis incurred samples is one of the best strategies to evaluate any hidden analytical effect in the method. While it is not practical to prepare calibration standards and QC standards for each individual matrix source, some assessment of patients variability must be undertaken (Taylor, 2005b).

## 5. Application of LC-MS/MS in basic and clinical laboratory fields

### 5.1 LC-MS/MS in drug metabolism & toxicology studies

Studies of the metabolic fate of drugs and other xenobiotics in living systems may be divided into three broad areas such as qualitative studies for elucidation of metabolic pathways through identification of circulatory and excretory metabolites, quantitative studies for determination of pharmacokinetics of the parent drug and/or its primary metabolites, and mechanistic studies for identification of chemically-reactive metabolites, which play a key role as mediators of drug-induced toxicities. The mass spectrometry has been regarded as one of the most important analytical tools in studies of drug metabolism, pharmacokinetics and biochemical toxicology. With the commercial introduction of new ionization methods such as API techniques and the combination of LC-MS or LC-MS/MS, it has now become a truly indispensable technique in pharmaceutical research. Triple stage quadrupole and ion trap mass spectrometers are presently used for this purpose, because of their sensitivity and selectivity. API-TOF mass spectrometry has also been very attractive due to its enhanced full-scan sensitivity, scan speed, improved resolution and ability to measure the accurate masses for protonated molecules and fragment ions (Kamel & Prakash, 2006). The study of the metabolic fate of drugs is an essential and important part of the drug development process. The analysis of metabolites is a challenging task and several different analytical methods have been used in these studies. However, after the introduction of the API technique, ESI and APCI, LC-MS has become an important and widely used method in the analysis of metabolites owing to its superior specificity, sensitivity and efficiency. Among the pharmacokinetic properties, metabolic characterization is a key issue and nowadays it is integrated into the early discovery phase. Biotransformation can lead also to some unwanted consequences, such as rapid clearance of the drug from the body, formation of active metabolites, drug–drug interactions due to enzyme induction or competition and formation of reactive or other toxic metabolites (Gibson & Skett, 1994). Metabolic pathways are divided into phase I and phase II reactions, and both classes of reaction often occur in parallel for particular compounds. The information required to determine the metabolic fate of an NCE includes detection of metabolites, structure characterization and quantitative analysis. Since the introduction of the ESI and APCI techniques, LC/MS has become an ideal and widely used method in the analysis of metabolites owing to its superior specificity, sensitivity and efficiency (Sinz & Podoll, 2002). API techniques are compatible with reversed-phase eluent systems, taking into account the use of volatile solvents and additives in chromatographic separation, thus preserving all the advantages of LC. Together, ESI and APCI provide efficient ionization for very different type of molecules including polar, labile, and high molecular mass drugs and metabolites (Kostiainen et al, 2003). Most work in metabolite analysis is carried out by using triple-quadrupole mass spectrometers (QQQ), as their MS/MS scan types (Schwartz et al, 1990) are highly helpful in the identification of metabolites and provide the required specificity and sensitivity (Clarke et al, 2001). Product ion scans are used for identification and MRM provides the high sensitivity required in quantitative analysis. The unique feature of QQQ is its capability to identify families ofmetabolites by using neutralloss and precursor ion scans.33 However, the sensitivity of identification of metabolites by the full-scan mode may not always suffice and instead of QQQ the use of IT and TOF-MS has increased. Both of these techniques provide high full-scan sensitivity. In addition, modern IT-MS technology with its MS*n* (Schwartz et al, 1990) capability is highly efficient in the structural analysis of

metabolites. The API-TOF mass spectrometry provides high-resolution analysis with a mass accuracy better than 10 ppm, and hence the possibility of the determination of the elemental compositions of metabolites and high specificity in their detection. (Zhang et al, 2000a; Zhang et al, 2000b). Furthermore, the quadrupole-TOF mass spectrometer (Q-TOF) provides high sensitivity for the determination of full-scan product ion spectra of metabolites (Zhang et al, 2000a). The first step in metabolite profiling for dertection of metabolitea and deferentiation of the site of biotransformation is to identify all the possible metabolites. The second step is their structural characterization and finally quantitation. The classical method in metabolic analysis is radioactive labeling ($^{14}C$, $^{3}H$) of a parent drug and detection of metabolites by LC with radioactivity detection (Veltkamp et al, 1987; Egnash & Ramanathan, 2002). Themethod is especially powerful when combinedwith on-line MS detection. Radioactivity detection provides localization of the metabolites in a chromatogram and MS ensures structure specific identification of the metabolites. However, this approach has several disadvantages. For example, synthesis and purification of radioactive compounds are expensive and time-consuming, radiation is a potential health risk for humans and the requirements for handling radioactive material and wastes make the use of radiolabeled compounds very costly. For these reasons and the fact that radioactively labeled compounds are only very rarely available in early discovery phase, different, simpler, MS techniques are increasingly used in metabolite identification (Kostiainen et al, 2003). MS/MS offers more specific detection of metabolites in complex matrices than unit resolution full-scan MS. MS/MS scanning methods include product ion, precursor ion and neutral loss scans (Schwartz et al, 1990). Metabolites are derivatives of the parent drug and as such it can be assumed that many of the metabolites show the same fragment ions or neutral losses as the parent drug. Therefore, the precursor ion and neutral loss scan modes with QQQ are especially useful in group-specific detection of metabolites (Clarke et al, 2001). To overcome the problems in metabolic analysis, new technologies are continuously being developed. The recently introduced LC-MS/NMR technology provides unambiguous structure characterization of metabolites. Although the sensitivity of NMR does not suffice for the analysis of metabolites in trace quantities, the sensitivity of the technology is continuously being improved. The use of standards or radiolabeled compounds in quantitative analysis and thus the time-consuming synthesis step of reference compounds can be avoided, when on-line coupling of LC-MS to detection techniques that provide equimolar responses, such as ICP-MS or chemiluminescent nitrogen detection, are used. Microfluidic systems offer possibilities to integrate all the experimental steps of metabolite analysis on one microchip, providing complete analysis steps (e.g. sample pretreatment, chemical reactions, analytical separation, and detection and data processing steps) on a single device with a high level of automation. Progress in microfluidics gives reason to assume that the metabolite analysis will be carried out by miniaturized lab-on-a-chip techniques integrated with miniaturized mass spectrometers in the near future (Kostiainen et al, 2003).

### 5.2 LC-MS/MS as quantification method for biogenic amines

The term biogenic amines refer to amine containing biogenic substrates such as catecholamines, serotonin and histamine. Biogenic amines function throughout the body, both in the central and peripheral nervous system. Disorders affecting their metabolism or action can have devastating effects on homeostasis of the human body (Hyland, 1999). In

clinical chemistry quantification of biogenic amines is mainly used for diagnosis of neuroendocrine tumors such as pheochromcytoma and carcinoids. HPLC coupled tandem MS is becoming an indispensible technique in the special chemistry laboratories in clinical chemistry as it greatly increases sensitivity and specificity of test results. Applying this technique will result in improved biochemical diagnosis of endocrine disorders, and opens new roads to gain insight in pathophysiological processes. For the measurement of biogenic amines in biological matrices, LC-MS/MS needs to compete with conventional HPLC with UV, fluorescence or ECD and to a lesser extend GC methods and immunoassay (Yi & Brown, 1991). In pharmaceutical industries and toxicology laboratories, LC-MS/MS is the method of choice for the development and the measurement of drugs (Maurer, 2007). For clinical chemical analyses LC-MS/MS is rapidly emerging and is applicable to a broad selection of compounds, especially for the diagnosis of aberrations within the endocrine system, such as biogenic amines (Chan et al, 2000; Kushnir et al, 2002; Lionetto et al, 2008; de Jong et al, 2008; de Jong et al, 2009a), but also steroids (Soldin and Soldin, 2009), thyroid hormones (Yue et al, 2008), and vitamin D (Higashi et al, 2008). Furthermore LC-MS/MS enable the use of sophisticated sample pretreatment techniques and automation of the whole process by on-line coupling of the separate techniques. To correct for losses during sample pretreatment, analyte separation and detection, stable isotopes of analytes are used as internal standard with mass spectrometry. LC-MS/MS combines the physical separation capabilities of HPLC with the high analytical sensitivity, specificity and accuracy of mass spectrometric detection. In recent years, LC-MS/MS equipment has been improved in performance. Due to its superior specificity, shorter runtimes and less laborious sample preparation, LC-MS/MS methods replace more and more of the conventional HPLC, GC-MS and immunoassay techniques (Taylor, 2005b; Vogeser & Seger, 2008b; Maurer, 2007). LC-MS/MS has, just as HPLC and GC-MS, the advantage that several compounds can be measures simultaneously (Holst et al, 2007).

Introduction of on-line solid-phase extraction coupled to LC-MS/MS shortened chromatographic run times and allowed automation of sample preparation (de Jong et al, 2007). Urinary deconjugated metanephrines have been analyzed with LC-MS/MS for many years. Since these markers occur in higher concentration ranges and require less sensitive assays. Catecholamines assist in the diagnosis of neuroendocrine catecholamine-producing tumors, such as pheochromocytoma and neuroblastoma, in addition to metanephrine and HVA (Lenders et al, 2002; Sawka et al, 2003). Recently, an on-line SPE LC-MS/MS method has been described for catecholamines in urine, especially improving specificity, sensitivity and run time compared to the conventional HPLC-ECD method used on the same laboratory (de Jong et al, 2010a).

Serotonin in blood is mainly stored in platelets. Free serotonin occurs in low concentrations in plasma because of active reuptake and fast metabolism which complicates detection with most conventional techniques (de Jong et al, 2011). Platelet serotonin is specifically measured for the detection of carcinoid tumors that secrete little serotonin. With LC-MS/MS, it is possible to measure accurate and reproducible serotonin both in platelet-rich and plasma-poor plasma (de Jong et al, 2009b; Monaghan et al, 2009; de Jong et al, 2010b). For this purpose, protein precipitation, using acetonitrile, combined with chromatography based on strong cation exchange and reversed-phase interaction, was used with a total run time of 6 min (Monaghan et al, 2009) and a detection limit of 5 nmol/L. Solid phase

extraction based on weak cation exchange and HILIC, comparable to the method described for plasma metanephrines, resulted in the same run time with detection limits even below 1 nmol/L (de Jong et al, 2010). Therefore, LC-MS/MS is becoming an indispensible tool for low-molecular weight biomarker quantification also in the field of special clinical chemistry. It overcomes drawbacks of conventional techniques, such as long analysis times and chance of interferences, has a broad analyte compatibility and high analytical performance. It enables more sensitive and specific measurement of biogenic amines and metabolites, and the routine quantification of biomarkers in low concentration ranges.

### 5.3 Usefulness of LC-MS/MS in doping control

LC-MS(/MS) has become an integral part of modern sports drug testing as it offers unique capabilities complementing immunological and GC-MS(/MS)-based detection methods for prohibited compounds. The improved options of fast and sensitive targeted analysis as well as untargeted screening procedures utilizing high resolution/high accuracy MS have considerably expanded the tools available to anti-doping laboratories for initial testing and confirmation methods. One approach is to focus on preselected target analytes that are measured with utmost specificity and sensitivity using diagnostic precursor–product ion pairs in low resolution tandem mass spectrometers. The other scenario is to measure and plot extracted ion chromatograms of protonated or deprotonated molecules as well as product ions as recorded in the full scan mode with high resolution/high accuracy MS (Thevis et al, 2011).

For modern doping control laboratories, the use of LC-MS(/MS) has become obligatory to meet the needs of fast, robust, sensitive, and specific detection methods in sports drug testing. GC with or without low or high resolution GC-MS held a superior position in routine doping controls until almost the end of the last century, and various analytical challenges such as those presented by heavy volatile or polar target analytes were successfully overcome, e.g., by means of sophisticated derivatization strategies; a few aspects, however, remained unsolved by mass spectrometry, particularly concerning high molecular weight analytes. In addition, the demands with regard to time and manpower as well as the necessity to use hazardous derivatizing reagents in some applications have further strengthened the position of LC-MS(/MS) in the sports drug testing arena (Hemmersbach, 2008; Thevis & Schänzer, 2007). Traditionally, most of test methods in doping control laboratories were established for urine samples as dictated by the class of analytes, e.g., one assay for stimulants, one for narcotics, one for anabolic-androgenic steroids, etc., because most of these compounds within one category share common physicochemical properties. Because derivatization and structure-specific pre-concentration and purification are not mandatory anymore for a considerable number of analytes that are detectable by LC-MS(/MS) at relevant concentration levels, screening procedures have become (at least partly) independent of such groupings and allow comprehensive analyses within significantly shortened analytical times. Nevertheless, besides its enormous value for doping control purposes, the complementary strategy of LC-MS(/MS) bears its own challenges such as the needs to account for positive and negative ionization, the generation of multiply charged ions (particularly with molecules of higher molecular mass) and, consequently, adequate identification criteria, as well as the higher susceptibility of LC-MS(/ MS) to ion suppression/matrix effects compared to GC-MS(/MS) methods.

With the constantly improving resolving power of modern LC systems and enhanced robustness and sensitivity of tandem mass spectrometers interfaced with atmospheric

pressure ionization, a considerable trend towards multianalyte testing procedures with or even without prior sample preparation and purification steps has been recognized. This trend is evidenced by a variety of earlier studies, the common goal of which was the increase of sample throughput, thus meeting the constantly growing demands concerning the implementation of new drugs, short reporting times, and cost-effectiveness (Thevis et al, 2011). Following an established extraction protocol based on alkaline (pH=9.5), LLE of urine with diethyl ether, ultrahigh-performance liquid chromatography (UHPLC) and MS/MS with scan-to-scan polarity switching was employed for the combined analysis of 34 target compounds, namely 25 diuretics, 5 stimulants, and 4 other substances (Ventura et al, 2008).

Using a comparable instrumental setup composed of a 2.1×50 mm BEH C-18 column (1.7-µm particle size), gradient elution with 10 mM ammonium acetate (solvent A) and methanol (solvent B), and scan-to-scan polarity switching, a total of 133 target analytes directly from diluted urine were measured (Thörngren et al, 2008). The target analytes were 37 diuretics and masking agents, 24 narcotics, and 72 stimulants, all of which were analyzed in a total runtime of 7.5 min with LODs between 1 and 50 ng/mL (i.e., well below the corresponding MRPLs) while a minimum of 10 data points/peak was maintained. Considering the rapid (and automated) sample preparation and very short runtimes, a high-throughput option for initial doping control tests is provided. As an alternative to targeted MRM analyses, the use of high resolution/high accuracy mass spectrometry as a screening tool for sports drug testing purposes has been thoroughly investigated and initial studies and approaches were successfully pursued. One of the major advantages of these procedures over purely targeted measurements with dedicated and fixed ion transitions is the complete collection of raw data, which provides the retrospective option to mine the analytical data for formerly unknown compounds as well as new drugs when they become relevant for doping controls. Because the International Standard for Laboratories (ISL) allows forre-processing and re-analysis of doping control samples (that were initially reported negative) in the case of new relevant information, the evaluation of electronic data is a fast and cost-effective means to filter the enormous amount of samples for those being suspicious in light of new intelligence (World Anti-Doping Agency, 2011). The combined use of considerably improved instrumental options has initiated the development of a variety of multi-analyte test methods in sports drug testing laboratories and these assays cover a great variety of compounds with highly diverse physicochemical properties.

Conventional HPLC was still in use in the cases of several low resolution mass spectrometry-based approaches, whereas procedures relying on high resolution/high accuracy mass spectrometry used either monolithic columns or UHPLC capable stationary phases to fully exploit the analytical power provided by these technologies. The strategy to investigate the metabolic fate of therapeutics as well as non-approved designer drugs in a doping control context deviates slightly from clinical settings.

Analytical goals of sports drug testing usually involve the long-term detection of drug administration, most often targeting inactive metabolites. However, temporal indications of drug abuse are supportive in order to distinguish between long-term and acute use, e.g., when only the in-competition use of the drug is prohibited. LC-MS(/MS) has been an integral part of sports drug testing efforts for more than a decade, and the continuously improving instrumentation with a gradually expanding number of supportive features has considerably enhanced the quality and speed of doping control analysis for low and high molecular weight compounds (Thevis et al, 2011).

### 5.4 LC-MS/MS in therapeutic drug monitoring for immunosuppressants

The outcome of post-transplantation patient care has been improved dramatically over the last decades, primarily due to the availability of appropriate immunosuppressive regimens (Yang et al., 2005). The immunosuppressants present toxicity and have narrow therapeutic ranges. For example, CsA showed numerous side effects including immunological, renal, hepatic and neurological complications, requiring dose adjustments or discontinuations in a significant percentage of patients (Rezzani, 2004). In addition, blood levels of the active drugs vary significantly in different individuals and ethnicities as well as different combinations of immunosuppressants (Holt, 2002; Yang et al, 2005). Therefore, the success of the post-transplant patient care largely depends on optimization of immunosuppressive therapy based on routine therapeutic drug monitoring (TDM). The available analytical methods for monitoring immunosuppressant levels in patient specimens can be divided into two categories: immunoassays, such as microparticle enzyme immunoassay (MEIA), enzyme multiplied immunoassay technique (EMIT), fluorescent polarization immunoassay (FPIA), cloned enzyme donor immunoassay (CEDIA), and liquid chromatography-based methods (Yang et al, 2005). Immunoassays are widely employed to measure mycophenolic acid (MPA, active metabolite of MMF), CsA, tacrolimus, and sirolimus in human blood or serum/plasma. Chromatography-based methods include high performance liquid chromatography (HPLC) with ultraviolet detection, HPLC-MS, and HPLC-MS/MS. HPLC-MS/MS has gained increasing popularity in clinical laboratories due to the advantages of the technology over other methods while the capital cost for instruments has been decreased. HPLC-MS/MS provides high specificity and sensitivity for the above mentioned immunosuppressants. In addition, HPLC-MS/MS is able to simultaneously measure several drugs and/or their major metabolites in one single analytical run. The combination of HPLC and MS has revolutionized the analytical society during the past decades (Wilson & Brinkman, 2003). MS measures the abundance of charged particles based on mass over charge ratio ($m/z$). MS/MS technique utilizes multiple (two or more) MS with collision cell in between resulting in improved specificity by providing characteristic molecular fragments (fingerprints) generated by collision-induced dissociation. Because of high specificity of MS/MS detection, the baseline separation of target compounds from their potential interferents by HPLC, which is a must for many HPLC-UV or HPLC-MS assays, becomes unnecessary leading to significant savings in analytical time, sample purification effort and chemical reagents. Usually a typical HPLC-MS/MS run can be accomplished within a few minutes in immunosuppressant monitoring (Yang et al., 2005; Ceglarek et al., 2006). In addition MS/MS can measure multiple ions or transitions to determine multiple drugs in a single analytical run. Like any other technique, however, HPLC-MS/MS has its own limitations. HPLC-MS/MS instruments require high initial capital investment and need highly trained analysts to appropriately operate and maintain the systems. Thus HPLC-MS/MS instruments are only available in a limited number of clinical laboratories. The most widely used ESI source for MS/MS is vulnerable to ion suppression (Annesley, 2003), which could lead to significant sensitivity loss and erroneous results. Ion suppression occurs when co-eluting compounds suppress the ionization of the target compounds in the ionization sources. One effective way to eliminate ion suppression is to remove extraneous matrix components through sample cleanup procedures. However, it adds extra time and effort that many clinical laboratories cannot afford. In addition, HPLC-MS/MS is not completely immune to interferences caused by isomers or isobaric molecules (Vogeser & Spohrer, 2005).

There have been a few new HPLC-MS/MS methods developed in recent years to measure single immunosuppressive drug in patient specimens, including sample types other than blood, plasma or serum. There are also recent development on application of new technologies in immunosuppressant TDM including sample preparation and ionization (Yang & Wang, 2008). Dried blood spot sampling is an alternative to venous blood sampling for immunosuppressant TDM due to the convenient sample collection from fingerprick and small blood volume collected. A preliminary investigation of an HPLC-MS/MS assay of tacrolimus in dried blood spot formed on sample paper by fingerprick blood sampling was reported (Hoogtanders et al., 2007a). There was no significant bias observed for dried blood sampling compared to venous blood sampling using 34 samples in 26 stable renal transplant outpatients measured by HPLC-MS/MS (Hoogtanders et al., 2007b). Because of various targets of action from different immunosuppressants, combinations of immunosuppressants are often used together to achieve synergistic therapeutic effects with reduced toxicity (Yang et al., 2005). Therefore, it is ideal to simultaneously measure these drugs in one analytical run. A rapid HPLC-MS/MS method was developed for determination of MPA and MPAG in plasma using the same sample preparation and HPLC-MS/MS conditions as used in simultaneous measurement of CsA, tacrolimus, sirolimus, and everolimus in whole blood (Ceglarek et al., 2006). For the practical issues with application of HPLC-MS/MS in immunosuppressant monitoring, Internal standards should be carefully selected when using HPLC-MS/MS to measure immunosuppressants, because they are critical to compensating for sample preparation variations, ionization efficiency differences, and matrix effects with MS/MS detection. The ideal internal standards are isotope-labeled target compounds, which have the exact same physical and chemical properties as the target compounds. In many cases, however, the isotope-labeled compounds are not always available and the alternative structural analogues should be considered and evaluated carefully before use including the consideration of inter-subject differences(Taylor, 2007). Nine commercial methanol used in HPLC eluting solvents were evaluated for signal suppression of sirolimus, tacrolimus, and MPA in MS/MS detection (Annesley, 2007). Product ion intensity was found to vary by 10 folds among the methanol tested. Though appropriate internal standards could compensate for the signal loss, performance of the assay (e.g., LLOQ) could be compromised. Therefore, HPLC-MS/MS continues being considered as the method of choice in TDM of immunosuppressants due to its high sensitivity and specificity while random access immunoassays, though may give significant different results compared to HPLC-MS/MS, also play an important role in this area. However, one should carefully select the internal standards and organic solvents used in HPLC-MS/MS method and evaluate their effects on the assay performance prior to implementation of an HPLC-MS/MS method.

## 6. Conclusion

In this chapter, we reviewed basic principles and most recent advances of LC-MS/MS methdology including sample preparation, seperation and MS/MS detection and appications in the several areas such as quantification of biogenic amines, pharmacokinetic and TDM for immunosuppresants and doping control. Until now, together with advancement including automation in the LC-MS/MS instrumentations along with pararell sample processing, column switching, and usage of more efficient supports for SPE, they drive the trend towards less sample clean-up times and total run times–high-throughput

methodology-in today's quantitative bioanalysis area. Newly introduced techniques such as ultra-performance liquid chromatography with small particles (sub-2μm) and monolithicchromatography offer improvements in speed, resolution and sensitivity compared to conventional chromatographic techniques. Hydrophilic interaction chromatography (HILIC) on silica columns with low aqueous/high organic mobile phase is emerging as a valuable supplement to the reversed-phase LC–MS/MS. Sample preparation formatted to 96-well plates has allowed for semi-automation of off-line sample preparation techniques, significantly impacting throughput. On-line SPE utilizing column-switching techniques is rapidly gaining acceptance in bioanalytical applications to reduce both time and labor required producing bioanalytical results. Extraction sorbents for on-line SPE extend to an array of media including large particles for turbulent flow chromatography, restricted access materials (RAM), monolithic materials, and disposable cartridges utilizing traditional packings such as those used in Spark Holland systems. Also this chapter also discusses recent studies of evaluation and overcome of matrix effect in LC–MS/MS analysis and how to reduce/eliminate matrix effect in method development and validation and clinical applications in the several areas as like biogenic amines analysis, therapeutic drug monitoring of immunosuppressant and doping control.

## 7. Acknowledgment

I thank Dr. Y.S. Park, Ms. S.Y. Kim and Ms. H.J. Kim for preparation of chapter.

## 8. References

Al-Dirbashi, O.Y., Aboul-Enein, H.Y., Jacob, M., Al-Qahtani, K. & Rashed, M.S. (2006) UPLC-MS/MS determination of doxazosine in human plasma. *Analytical and Bioanalytical Chemistry*, Vol.385, No.8, (August 2006), pp. (1439-1443), ISSN 1618-2642.

Almeida, R., Mosoarca, C., Chirita, M., Udrescu, V., Dinca, N., Vukelić, Z., Allen, M. & Zamfir, A.D.(2008). Coupling of fully automated chip-based electrospray ionization to high-capacity ion trap mass spectrometer for ganglioside analysis. *Analytical Biochemistry*, Vol. 378, No.1, ( July 2008), pp.( 43-52), ISSN 0003-2697.

Alnouti, Y., Li, M., Kavetskaia, O., Bi, H., Hop, C.E. & Gusev, A.I. (2006). Method for internal standard introduction for quantitative analysis using on-line solid-phase extraction LC-MS/MS. *Analytical Chemistry*, Vol.78, No.4, (Feburary 2006), pp. (1331-1336), ISSN 0003-2700.

Alnouti, Y., Srinivasan, K., Waddell, D., Bi, H., Kavetskaia, O. & Gusev, A.I.(2005). Development and application of a new on-line SPE system combined with LC-MS/MS detection for high throughput direct analysis of pharmaceutical compouns in plasma. *Journal of Chromatography. A*, Vol.1080, No.2, (July 2005), pp. (99-106), ISSN 0021-9673.

Annesley, T.M. (2003). Ion suppression in mass spectrometry. *Clinical Chemistry*, Vol.49, No.7, (July 2003), pp.( 1041–1044), ISSN 0009-9147.

Annesley, T.M. (2007). Methanol-associated matrix effects in electrospray ionization tandem mass spectrometry. *Clinical Chemistry*, Vol.53, No.10, (October 2007), pp. (1827-1834), ISSN 0009-9147.

Antignac, J., de Wasch, K., Monteau, F., De Brabander, H., Andre F. and Le Bizec. B. (2005). The ion suppression phenomenon in liquid chromatography–mass spectrometry and its consequences in the field of residue analysis. *Analytica Chimica Acta*, Vol. 529, No.1-2, pp. (129-136), ISSN 0003-2670.

Apollonio, L.G., Pianca, D.J., Whittall, I.R., Maher, W.A. & Kyd, J.M. (2006). A demonstration of the use of ultra-performance liquid chromatography–mass spectrometry [UPLC/MS] in the determinationof amphetamine-type substances and ketamine for forensic and toxicological analysis. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol. 836, No.1-2, (May 2006), pp. (111-115), ISSN 1570-0232.

Ashcroft, A.E. (2011). An Introduction to Mass Spectrometry, (http://www.astbury.leeds.ac.uk/facil/MStut/msturorial.htm.)

Ayrton, J., Dear, G.J., Leavens, W.J., Mallet, D.N. & Plumb, R.S. (1997). The use of turbulent flow chromatography/mass spectrometry for the rapid, direct analysis of a novel pharmaceutical compound in plasma. *Rapid Communication in the Mass Spectrometry*, Vol. 11, No.18, (December 1997), pp. (1953-1958), ISSN 0951-4198.

Ayrton, J., Dear, G.J., Leavens, W.J., Mallet, D.N. & Plumb, R.S. (1998). Optimisation and routine use of generic ultra-high flow-rate liquid chromatography with mass spectrometric detection for the direct on-line analysis of pharmaceuticals in plasma. *Journal of Chromatography A*, Vol. 828, No.1-2, (December 1998), pp.(199-207), ISSN 0021-9673.

Ayrton, J., Clare R.A., Dear, G.J., Mallet, D.N. & Plumb, R.S. (1999). Ultra-high flow rate capillary liquid chromatography with mass spectrometric detection for the direct analysis of pharmaceuticals in plasma at sub-nanogram per millilitre concentrations. *Rapid Communications in Mass Spectrometry*, Vol.13, No.16, (August 1999), pp. (1657-1662), ISSN 0951-4198.

Bakhtiar, R. and Majumdar, T. (2007). Tracking problems and possible solutions in the quantitative determination of small molecule drugs and metabolites in biological fluids using liquid chromatography-mass spectrometry. *Journal of Pharmacological and Toxicological Methods*, Vol. 55 , No.3, pp. (262-278), ISSN 1056-8719.

Barbarin, N., Mawhinney, D.B., Black, R., & Henion, J.(2003). High-throughput selected reaction monitoring liquid chromatography–mass spectrometry determination of methylphenidate and its major metabolite, ritalinic acid, in rat plasma employing monolithic columns. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*.Vol.783, No.1, (Januarl 2003), pp. (73-83), ISSN 1570-0232.

Barrett, Y.C., Akinsanya, B., Chang, S.Y. & Vesterqvist, O. (2005). Automated on-line SPE LC-MS/MS method to quantitate 6beta-hydroxycortisol and cortisol in human urine: use of the 6beta-hydroxycortisol to cortisol ratio as an indicator of CYP3A4 activity.*Journal of Chromatography. B: Analytical Technologies in the Biomedical and Life Sciences*, Vol.821, No.2, (July 2005), pp. (159-165), ISSN 1570-0232.

Berna, M.J., Ackermann, B.L. & Murphy, A.T. (2004). High-throughput chromatographic approaches to liquid chromatographic/tandem mass spectrometric analysis to support drug discovery and development. *Analytica Chimica Acta*, Vol.509, No.1,( April 2004), pp.(1-9), ISSN 0003-2670.

Beuhler, R.J., Chu, Y.Y., Friedlander, G., Friedman, L., Alessi, J.G., LoDestro, V. V. & Thomas, J.P.(1991). Cluster-impact fusion: time-to-flight experiments. *Physical Review Letters,* Vol.67, No.4, (July 1991) pp. (473-476), ISSN 0031-9007.

Blomberg, L.G. (2009). Two new techniques for sample preparation in bioanalysis: microextraction in packed sorbent (MEPS) and use of a bonded monolith as sorbent for sample preparation in polypropylene tips for 96-well plates. *Analytical and Bioanalytical Chemistry*, Vol.393, No.3, (February 2009), pp. (797-807), ISSN 1618-2642.

Bolden, R.D., Hoke 2nd, S.H., Eichhold, T.H., McCauley-Myers, D.L. & Wehmeyer, K.R. (2002). Semi-automated liquid–liquid back-extraction in a 96-well format to decrease sample preparation time for the determination of dextromethorphan and dextrorphan in human plasma. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. Vol.772, No.1, (May 2002), pp. (1-10), ISSN 1570-0232.

Bonfiglio, R., King, R.C., Olah T.V. & Merkle, K. (1999).The effects of sample preparation methods on variability of the electrospray ionization response for model drug compounds. *Rapid Communications in Mass Spectrometry*, Vol.13, No.12, (June 1999), pp. (1175–1185), ISSN 0951-4198.

Borges, V., Yang, E., Dunn, J. & Henion, J. (2004). High-throughput liquid chromatography-tandem mass spectrometry determination of bupropion and its metabolites in human, mouse and rat plasma using a monolithic column. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, Vol.804, No.4, (May 2004), pp. (277-287), ISSN 1570-0232.

Bruins, A.P. (1991). Mass spectrometry with ion sources operating at atmospheric pressure. *Mass Spectrometry Reviews,* Vol.10, No.1, (January 1991), pp. (53–77), ISSN 0277-7037.

Buhrman, D., Price, P.I. & Rudewicz, P. J. (1996). Quantitation of SR 27417 in human plasma using electrospray liquid chromatography–tandem mass spectrometry: a study of ion suppression. *Journal of the American Society for Mass Spectrometry,*Vol.7, No.11, (November 1996),pp. (1099–1105), ISSN 1044-0305.

Cabrera, K. (2004). Applications of silica-based monolithic HPLC columns. *Journal of Separation Science,* Vol.27, No.10-11, (July 2004),pp. (843-852), ISSN 1615-9306.

Ceglarek, U., Casetta, B., Lembeke, J., Baumann, S., Fiedler, G.M., Thiery, J. (2006). Inclusion of MPA and in a rapid multi-drug LC-tandem mass spectrometric method for simultaneous determination of immunosuppressants. *Clinica Chimica Acta*, Vol.373, No.1-2, (November 2006), pp. (168-171), ISSN 0009-8981.

Chambers, E., Wagrowski-Diehl, D., Lu Z. and Mazzeo, J. (2007). Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, Vol. 852, No.1-2, pp. (22-34), ISSN 1570-0232.

Chan, E.C. & Ho, P.C. (2000). High-performance liquid chromatograpy/atmospheric pressure chemical ionization mass spectrometric method for the analysis of catecholamines and metanephrines in human urine. *Rapid Commmunications in Mass Spectrometry*, Vol.14, No.21, pp.(1959-1964), ISSN 0951-4198.

Chassaing, C., Stafford, H., Luckwell, J., Wright, A. & Edgington, A. (2005). A Parallel Micro Turbulent Flow Chromatography-Tandem Mass Spectrometry Method for the

Analysis of a Pharmaceutical Compound in Plasma. *Chromatographia,* Vol. 62, No.1-2, (July 2005), pp. (17-24), ISSN 0009-5893.

Chen, X., Westphall, M.S. & Smith, L.M. (2003). MS of DNA mixtures: Instrumental effects responsible for decreased sensitivity with increasing mass. *Analytical Chemistry*, Vol.75, No.21, (November 2003), pp. (5944-5952), ISSN 0003-2700.

Chen, Y., Junga, H., Jiang, X., Weng, N. (2002). In: Presented at the 50th ASMS Conference, Orlendo, FL.

Chiu, C.M. & Muddiman, D.C. (2008). What is Mass Spectrometry?, American Society for Mass Spectrometry, http://www.asms.org/whatisms.

Churchwell, M.I., Twaddle, N.C., Meeker L.R. & Doerge. D.R. (2005). Improving LC–MS sensitivity through increases in chromatographic performance: Comparisons of UPLC–ES/MS/MS to HPLC–ES/MS/MS. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences,* Vol.825, No.2, (October 2005), pp. (134-143), ISSN 1570-0232.

Clarke, S.D., Hill, H.M., Noctor, T.A.G. & Thomas, D. (1996). Matrix-related Modification of Ionization in Bioanalytical Liquid Chromatography-Atmospheric Pressure Ionization Tandem Mass Spectrometry. *Pharmaceutical Sciences*, Vol.2, No.4, (April 1996), pp. (203-207), ISSN 2042-7158.

Clarke, N.J., Rindgen, D., Korfmacher,W.A., Cox, K.A. (2001). Systematic LC/MS metabolite dentification in drug discovery. *Analytical Chemistry, Vol.* 73, No.15, pp. (430A-439A), ISSN 0003-27000.

Comisarow, M.B. & Marshall, A.G. (1974). Fourier transform ion cyclotron resonance spectroscopy. *Chemical Physics Letters*, Vol.25, No.2, (March 1974), pp. 282–283, ISSN 0009-2614.

Constanzer, M.L., Chavez, C.M., Matuszewski, B.K., Carlin J. & Graham. D. (1997). Low level determination of a novel 4-azasteroid and its carboxylic acid metabolite in human plasma and semen using high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. *Journal of Chromatography B: Biomedical Sciences and Applications*, Vol. 693, No.1, (May 1997), pp. (117-129), ISSN 1387-2273.

Costello, C.E. (1997). Time, life…and mass spectrometry. New techniques to address biological questions. *Biophysical Chemistry*, Vol.68, No.1-3, (October 1997), pp. (173-188), ISSN 0301-4622.

Daly, N.R., McCormick, A. & Powell, R.E. (1968a) Detector for the metastable ions observed in the mass spectra of organic compounds. *Review of Scientific Instruments* Vol.39, No.8, (Feburary 1968), pp. (1163-1167), ISSN 0034-6748

Daly, N.R., McCormick, A. & Powell, R.E. (1968b) Metastable spectra of cis- and trans-butanes. *Organic Mass Spectrometry,* Vol. 1, No.1, pp. (167-168), ISSN 0030-493X.

Dams, R., Huestis, M.A., Lambert, W.E. & Murphy, C.M.(2003). Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *Journal of American Society for Mass Spectrometry*, Vol.14, No.11, (November 2003), pp. (1290-1294), ISSN 1044-0305.

Daurel-Receveur, M., Titier, K., Picard, S.P., Ducint, D., Moore. N, & Molimard, M. (2006). Fully automated analytical method for mycophenolic acid quantification in human plasma using on-line solid phase extraction and high performance liquid

chromatography with diode array detection. *Therapeutic Drug Monitoring,* Vol.28, No.4, (August 2006), pp. (505-511), ISSN 0163-4356.

de Jong, W.H., Graham, K.S., van der Molen, J.C., Links, T.P., Morris, M.R., Ross, H.A., De Vries, E.G. & Kema, I.P. (2007). Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry. *Clinical Chemistry*, Vol.53, No.9, (September 2007), pp. (1684-1693), ISSN 0009-9147.

de Jong, W.H., Graham, K.S., de Vries, E.G., Kema, I.P. (2008). Urinary 5-HIAA measurement using automated on-line solid extraction-high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, Vol.868, Vol.1-2, (June 2008) pp. (28-33), ISSN 1570-0232.

de Jong, W.H., de Vries E.G., Kema, I.P. (2009a). Serotonin rising, *The New England Journal of Medicine*, Vol.360, No.24, (June 2009), pp. (2580-2581), ISSN 0028-4793.

de Jong, W.H., Smit, R., Bakker, S.J., de Vries, E.G., Kema, I.P. (2009b). Plasma tryptophan, kynurenine and 3-hydroxykynurenine measurement using automated on-line solid-phase extraction HPLC-tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, Vol.877, Vol.7, (March 2008), pp. (603-609), ISSN 1570-0232.

de Jong, W.H., de Vries, E.G., Wolffenbuttel, B.H., Kema, I.P. (2010a). Automated mass spectrometric analysis of urinary free catecholamines using on-line solid phase extraction. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences.* Vol.878, No.19, (June 20010), pp. (1506-1512), ISSN 1570-0232.

de Jong, W.H., Wilkens, M.H., De Vries, E.G. & Kema, I.P. (2010b). Automated mass spectrometric analysis of urinary and plasma serotonin. *Analytical amd Bioanalytical Chemistry,* Vol.396, No.7, (April 2010), pp. (2609–2616), ISSN 1618-2642.

de Jong, W.H., de Vries, E.G., Kema,I.P. (2011). Current status and future developments of LC-MS/MS in clinical chemistry for quantification of biogenic amines, *Clinical Biochemistry*, Vol.44, No.1, (January 2010), pp. (95-103), ISSN 0009-9120.

Deng, Y., Zhang, H., Wu, J.T. & Olah, T.V. (2005). Tandem mass spectrometry with online high-flow reversed-phase extraction and normal-phase chromatography on silica columns with aqueous-organic mobile phase for quantitation of polar compounds in biological fluids. *Rapid Communications in Mass Spectrometry,* Vol.19, No.20, (October 2005), pp. (2929-2934), ISSN 1097-0231.

Dewe, W., De Smet, M., Evrard, N., Culot, B., Lastelle, M., Saucez, M., Ingels, D., Taillieu, X. (2007). Partial within-animal calibration: a new calibration approach in lead optimization high-throughput bioanalysis. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, Vol.854, No.1-2, (July 2007), pp.(183-191), ISSN 1570-0232.

Douglas, D., Frank, A. & Mao, D. (2005). Linear ion traps in mass spectrometry. Mass Spectrometry Reviews, Vol. 24, No.1, (January-Feburary 2005), pp.(1-29), ISSN 0277-7037.

Dubois, F., Knochenmuss, R. & Zenobi, R. (1997). An ion-to-photon conversion detector for mass spectrometry. *International Journal of Mass Spectrometry and Ion Processes,* Vol.169-170, (December 1997), pp. (89-98), ISSN : 1387-3806.

Dubois, F., Knochenmuss, R., Zenobi, R., Brunelle, A., Deprun, C. & Beyec, Y.L. (1999). A comparison between ion-to-photon and microchannel plate detectors. *Rapid Communications in Mass Spectrometry,* Vol.13, No.9, pp. 786–791, ISSN 1097-0231.

Dunér, K., Bäckström, J., Magnell, N., Svennberg, H., Ahnoff, M. & Logren, U. (2007). Determination of ximelagatran, melagatran and two intermediary metabolites in plasma by mixed-mode solid phase extraction and LC-MS/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, Vol.852, No.1-2, (June 2007), pp. (317–324), ISSN 1570-0232.

Eerkes, A., Shou, W.Z. & Naidong, W. (2003). Liquid/liquid extraction using 96-well plate format in conjunction with hydrophilic interaction liquid chromatography–tandem mass spectrometry method for the analysis of fluconazole in human plasma. *Journal of Pharmaceutical and Biomedical Analysis,* Vol.31, No.5, (April 2003), pp. (917-928), ISSN 0731-7085.

Egnash. L.A. & Ramanathan, R. (2002). Comparison of heterogeneous and homogeneous radioactivity flow detectors for simultaneous profiling and LC–MS/MS characterization of metabolites. *Journal of Pharmaceutical and Biomededical Analysis.* Vol.27, No.1-2, (January 2002), pp. (271-284), ISSN 0731-7085.

Erve, J.C. , Beyer, C.E., Manzino L. & Talaat, R.E. (2009). Metabolite identification in rat brain microdialysates by direct infusion nanoelectrospray ionization after desalting on a ZipTip and LTQ/Orbitrap mass spectrometry. *Rapid Communications in Mass Spectrometry,* Vol.23, No.24, (December 2009), pp. (4003-4012), ISSN 1097-0231.

Farmer, J. B. In Mass Spectrometry, McDowell, C. A., Ed.; McGraw-Hill: New York, 1963; pp 10-11

Fies, W. J. (1988). A Method for measuring the gain of an electron multiplier. *International Journal of Mass Spectrometry and Ion Processes,* Vol.82, No.1-2, (January 1988), pp. (111-129), ISSN 0168-1176.

Gerlich, D. (1992). *Inhomogenous RF fields: A versatile tool for the study of processes with slow ions, In Advances in chemical physices, Vol.82*, (eds C.-Y. Ng, M. Baer, I. Prigogine and S. A. Rice), pp. 1-176, John Wiley & Sons, Inc, ISBN 0-471-53258-3, Hoboken, NJ, USA.

Georgi, K. & Boos, K. (2006). Multidimensional on-line SPE for undisturbed LC-MS-MS analysis of basic drugs in biofluids. *Chromatographia*, Vol.63, No.11-12, (June 2006), pp. (523-531), ISSN 0009-5893.

Gibson, G.G. & Skett, P. Pathways of Drug Metabolism. In *Introduction to Drug Metabolism*, Gibson GG, Skett P (eds). Blackie Academic and Professional: London, 1994; 1–34.

Graff, D.K. (1995). Fourier and Hadamard: Transforms in Spectroscopy. *Journal of Chemical Education*, Vol.72, No.4, (April 1995) pp. 304-309, ISSN 0021-9584.

Guilaus, M. (1995). *Special feature: Tutorial.* Principles and instrumentation in time-of-flight mass spectrometry. Physical and instrumental concepts. *Journal of Mass Spectrometry*, Vol.30, No.11, (November 1995), pp. (1519-1532), ISSN 1076-5147.

Hager, J.W. (2002). A new linear ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry,* Vol.16, No.6, (March 2002), pp. (512-526), ISSN 1097-0231.

Hardman, M. & Makarov, A.A. (2003). Interfacing the orbitrap mass analyzer to an electrospray ion source. *Analytical Chemistry,* Vol.75, No.7, (April 2003), pp. (1699-1705), ISSN 0003-2700.

Harris, F.M., Trott, G.W., Morgan, T.G., Brenton, A.G., Kingston, E.E. & Beynon, J.H. (1984). Signal-to-noise ratios in the measurement of low ion currents using electron multipliers. *Mass Spectrometry Reviews,* Vol.3, No.2, (Summer 1984), pp. (209-229), ISSN 0277-7037.

Hedin, A., Haakansson, P. & Sundqvist, B.U.R. (1987). On the detection of the large organic ions by secondary electron production. *International Journal of Mass Spectrometry and Ion Processes*, Vol.75, No.3, (April 1987), pp. (275-289), ISSN 0168-1176.

Heller, D.N. (2007). Ruggedness testing of quantitative atmospheric pressure ionization mass spectrometry methods: the effect of co-injected matrix on matrix effects. *Rapid Communications in Mass Spectrometry,* Vol.21, No.5, (March 2007), pp. (644-652), ISSN:1097-0231.

Hemmersbach, P. (2008). History of mass spectrometry at the Olympic Games. *Journal of Mass Spectrometry*, Vol.43, No.7, (July 2008), pp. (839–853), ISSN 1076-5174.

Herman, J.L. (2002). Generic method for on-line extraction of drug substances in the presence of biological matrices using turbulent flow chromatography. *Rapid Communications in Mass Spectrometry,* Vol.16, No.5, (March 2002), pp. (421-426), ISSN 1097-0231.

Hernández, F., Sancho, J.V. & Pozo, O.J. (2005). Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples. *Analytical amd Bioanalytical Chemistry,* Vol.382, No.4, (June 2005), pp. (934-946), ISSN 1618-2642.

Higashi, T., Shibayama, Y., Fuji, M., Shimada, K. (2008). Liquid chromatography-tandem mass spectrometric method for the determination of salivary 25-hydroxyvitamin D3: a noninvasive tool for the assessment of vitamin D status. *Analytical and Bioanalytical Chemistry,* Vol.391, No.1, (May 2009), pp. (229-238), ISSN 1618-2642.

Hoogtanders, K., van der Heijden, J. , Christiaans, M., Edelbroek, P., van Hooff J.P., Stolk, L.M.L. (2007a). Therapeutic drug monitoring of tacrolimus with the dried blood spot method. *Journal of Pharmaceutical and Biomedical Analysis*, Vol.44, No.3, (July 2007), pp. (658-664), ISSN 0731-7085.

Hoogtanders, K., van der Heijden, J., Christiaans, M., van de Plas, A., van Hooff J., Stolk, L. 2007b). Dried blood spot measurement of tacrolimus is promising for patient monitoring. *Transplantation*, Vol. 83, No.2, (January 2007), pp. (237-238), ISSN 0041-1337.

Holst, J.P., Soldin, S.J., Tractenberg, R.E., Guo, T., Kundra, P., Verbalis, J.G., Jonklaas, J. (2007). Use of steroid profiles in determining the cause of adrenal insufficiency. *Steroids*, Vol.72, No.1, (January 2007), pp. (71-84), ISSN 0039-128X.

Holt, D.W. (2002). Therapeutic drug monitoring of immunosuppressive drugs in kidney transplantation. *Current Opinion in Nephrology and Hypertension*, Vol.11, No.6, (November 2002), pp. (657-663), ISSN 1062-4821.

Hopfgartner, G., Husser, C. & Zell, M. (2002). High-Throughput Quantification of Drugs and Their Metabolites in Biosamples by LC-MS/MS and CE-MS/MS: Possibilities and Limitations. *Therapeutic Drug Monitoring*, Vol. 24, No.1, (February 2002), pp.(134-143), ISSN 0163-4356.

Hu, Q., Noll, R.J., Li, H., Makarov, A., Hardman, M. & Cooks, R.G. (2005). The Orbitrap: a new mass spectrometer. *Journal of Mass Spectrometry*, Vol.40, No.4, (April 2005), pp. (430-443), ISSN 1076-5147.

Huang, M.Q., Mao, Y., Jemal, M. & Arnold, M. (2006). Increased productivity in quantitative bioanalysis using a monolithic column coupled with high-flow direct-injection liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry,* Vol.20, No.11, (June 2006), pp. (1709-1714), ISSN:1097-0231.

Hyland, K. (1999). Neurochemistry and defects of biogenic amine neurotransmitter metabolism. Journal of Inherited Metabolic Disease, Vol.22, No.4, (June 1999), pp. (353-363), ISSN 0141-8955.

Ikegami, T. & Tanaka, N. (2004).Monolithic columns for high-efficiency HPLC separations. *Current Opinion in Chemical Biology*, Vol.8, No.5, (October 2004), pp. (527-533), ISSN 1367-5931.

Jemal, M., Ouyang, Z. & Powell, M.L.(2002). A strategy for a post-method-validation use of incurred biological samples for establishing the acceptability of a liquid chromatography/tandem mass-spectrometric method for quantitation of drugs in biological samples.*Rapid Communications in Mass Spectrometry,* Vol.16, No.16, (August 2002), pp. (1538-1547), ISSN 1097-0231.

Jennings, K.R. & Dolnikowski, G.G.(1990). Mass analyzers. *Methods in Enzymology,* Vol.193, pp. (37-61), ISSN:0076-6879.

Jessome, L.L. and Volmer, D.A. (2006). Ion suppression: A major concern in Mass Spectrometry. *LCGC North America*, Vol. 24, No.5, (May 2006), pp. (498-506), ISSN 1527-5949.

Ji, H, Park, E., Lee, K. & Lee. H. (2008). Quantification of doxazosin in human plasma using hydrophilic interaction liquid chromatography with tandem mass spectrometry. *Journal of Seperation Science.* Vol. 31, No.9 (May 2008), pp. (1628-1633), ISSN 1615-9314.

Kamel, A. & Prakash, C. (2006). High performance liquid chromatography/atmospheric pressure ionization/tandem mass spectrometry (HPLC/API/MS/MS) in drug metabolism and toxicology. Current Drug Metabolism, Vol.7, No.8, (Dec 2006), pp. (837-852), ISSN 1389-2002.

Karas, M., Bachmann, D., Bahr, U. & Hillenkamp, F. (1987). Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *International Journal of Mass Spectrometry and Ion Processes,* Vol.78, (September 1987), pp. (53-68), ISSN 0168-1176.

Kawano, S., Takahashi, M., Hine, T., Yamamoto, E. & Asakawa, N. (2005). On-line pretreatment using methylcellulose-immobilized cation-exchange restricted access media for direct liquid chromatography/mass spectrometric determination of basic drugs in plasma.*Rapid Communications in Mass Spectrometry,* Vol.19, No.19, (October 2005), pp. (2827-2832), ISSN 1097-0231.

Kebarle, P. & Tang, L. (1993). From ions in solution to ions in the gas phase - the mechanism of electrospray mass spectrometry. *Analytical Chemistry*, Vol.65, No.22, (November 1993), pp. (972A-986A), ISSN 0003-2700.

King,R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C. & Olah, T. (2000), Mechanistic investigation of ionization suppression in electrospray ionization. *Journal of the American Society for Mass Spectrometry*, Vol.11, No.11, (November 2000), pp. (942–950), ISSN 1044-0305.

Koal, T., Sibum, M., Koster, E., Resch, K. & Kaever, V. (2006). Direct and fast determination of antiretroviral drugs by automated online solid-phase extraction-liquid chromatography-tandem mass spectrometry in human plasma. *Clinical Chemical Laboratory Medicine*,Vol.44, No.3,(July 2006), pp.( 299-305), ISSN 1434-6621.

Koppenaal, D.W., Barinaga, C.J., Denton, M.B., Sperline, R.P., Hieftje, G.M., Schilling, G.D., Andrade, F.J. & Barnes, J.H.IV. (2005). MS detectors. *Analytical Chemistry*, Vol.77, No.21, (November 2005), pp. (418A-427A), ISSN 0003-2700.

Koseki, N., Nakashima, A., Nagae, Y. & Masuda, N. (2006). Simultaneous quantitative determination of cyclosporine A and its three main metabolites (AM1, AM4N and AM9) in human blood by liquid chromatography/mass spectrometry using a rapid sample processing method. *Rapid Communications in Mass Spectrometry,* Vol.20, No.5, (March 2006), pp. (733-740), ISSN 1097-0231.

Koster, S. & Verpoorte, E. (2007). A decade of microfluidic analysis coupled with electrospray mass spectrometry: an overview. *Lab on a Chip*, Vol.7, No.11, (September 2007), pp. (1394-1412), ISSN: 1473-0197.

Kostiainen, R., Kotiaho, T., Kuuranne, T., Auriola, S. (2003). Liquid chromatography/atmospheric pressure ionization–mass spectrometry in drug metabolism studies. *Journal of Mass Spectrometry*, Vol.38, No.4, (March 2003), pp. (357-372), ISSN 1076-5174.

Kushnir, M.M., Urry, F.M., Frank, E.L., Roberts, W.L., Shushan, B. (2002). Analysis of catecholamines in urine by positive-ion electro-spray tandem mass spectrometry. *Clinical Chemistry,* Vol.48, No.2, (Feburary 2002), pp. (323-331), ISSN 0009-9147.

Lagerwerf, F.M., van Dongen, W.D., Steenvoorden, R.J.J.M., Honing, M. & Jonkman, J.H.G. (2000). Exploring the boundaries of bioanalytical quantitative LC–MS–MS. *TrAC Trends in Analytical Chemistry*, Vol.19, No.7, (July 2000), pp. (418-427), ISSN: 0165-9936.

Lanckmans, K., Van Eeckhaut, A., Sarre, S., Smolders, I. & Michotte. Y. (2006). Capillary and nano-liquid chromatography-tandem mass spectrometry for the quantification of small molecules in microdialysis samples: Comparison with microbore dimensions. *Journal of Chromatography A*, Vol. 1131, No.1-2, (October 2006), pp. (166-175), ISSN 0021-9673.

Lammert, S.A., Rockwood, A.A., Wang, M., Lee. M,L,, Lee. E,D,, Tolley. S,E., Oliphant, J.R., Jones, J.L. & Waite, R.W.(2006). Miniature Toroidal Radio Frequency Ion Trap Mass Analyzer. *Journal of the American Society for Mass Spectrometry,* Vol.17, No.7, (July 2006), pp. (916–922), ISSN 1044-0305.

Lenders, J.W., Pacak, K, Walther, M.M., Linehan, W.M., Mannelli, M., Friberg, P., Keiser, H.R., Goldstein, D.S., Eisenhofer, G. (2002). Biochemical diagnosis of pheochromocytoma: Which test is best? *JAMA*, Vol.287, No.11, pp. (1427-1434), ISSN 0098-7484.

Li, R., Dong, L. & Huang, J. (2005). Ultra performance liquid chromatography–tandem mass spectrometry for the determination of epirubicin in human plasma. *Analytica Chimica Acta*, Vol.546, No.2,(August 2005), pp. (167-173), ISSN:0003-2670.

Lim, H.K., Chan, K.W., Sisenwine, S. & Scatina, J.A., (2001). Simultaneous Screen for Microsomal Stability and Metabolite Profile by Direct Injection Turbulent-Laminar Flow LC−LC and Automated Tandem Mass Spectrometry. *Analytical Chemistry*, Vol.73, No.9, (May 2001), pp. (2140-2146), ISSN:0003-2700.

Lionetto, L., Lostia, A.M., Stigliano, A., Cardelli, P., Simmaco, M. (2008). HPLC-mass spectrometry method for quantitative detection of neuroendocrine tumor markers: vanillyl-mandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid. *Clinica Chimica Acta*, Vol.398, No.1-2, pp. (53-56), ISSN 0009-8981.

Liu, H., Berger, S.J., Chakraborty, A.B., Plumb, R.S. & Cohen, S.A.(2002). Multidimensional chromatography coupled to electrospray ionization time-of-flight mass spectrometry as an alternative to two-dimensional gels for the identification and analysis of complex mixtures of intact proteins. *Journal of Chromatography. B,*

*Analytical Technologies in the Biomedical and Life Sciences,* Vol.782, No.1-2, (December 2002), pp. (267-289), ISSN 1570-0232.

Londry, F.A. & Hager, J.W. (2003). Mass selective axial ion ejection from a linear quadrupole ion trap.*Journal of the American Society for Mass Spectrometry,* Vol.14, No.10,(Octorber 2003), pp. (1130-1147), ISSN 1044-0305.

Majors, R.E. (2006). Miniaturized approaches to conventional liquid-liquid extraction. *LC-GC North America*, Vol.24, No.2,(February 2006), pp.(118-130), ISSN 1527-5949.

Makarov, A. (2000). Electrostatic axially harmonic orbital trapping: A high-performance technique of mass analysis. *Analytical Chemistry*, Vol.72, No.6, (March 2000), pp. (1156-1162), ISSN 0003-2700.

Makarov, A., Denisov, E., Lange, O. & Horning, S. (2006a). Dynamic range of mass accuracy in LTQ Orbitrap hybrid mass spectrometer. *Journal of the American Society for Mass Spectrometry,* Vol.17, No.7,(July 2006), pp. (977-982), ISSN 1044-0305.

Makarov, A., Denisov, E., Kholomeev, A., Balschun, W., Lange, O., Strupat, K. & Horning, S. (2006b). Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer. *Analytical Chemistry*, Vol.78, No.7, (April 2006), pp. (2113-2120), ISSN:0003-2700.

Mallet, C.R., Lu, Z., Fisk, R., Mazzeo, J.R. & Neue, U.D. (2003). Performance of an ultra-low elution-volume 96-well plate: drug discovery and development applications. *Rapid Communications in Mass Spectrometry,* Vol.17, No.2, (January 2003), pp. (163-170), ISSN 1097-0231.

March, R.E. (2000). Quadrupole ion trap mass spectrometry: a view at the turn of the century. *International Journal of Mass Spectrometry*, Vol.200, No.1–3, (December 2000), pp. (285–312), ISSN 1387-3806.

Marshall, A.G., Hendrickson, C.L. & Jackson, G.S. (1998). Fourier transform ion cyclotron resonance mass spectrometry: a primer. *Mass Spectrometry Reviews,* Vol.17, No.1, (January-February 1998) pp. (1–34), ISSN:0277-7037.

Martin, M., Eon, C. & Guiochon, G. (1974). Study of the pertinency of pressure in liquid chromatography: I. theoretical analysis. *Journal of Chromatography. A*, Vol. 99, pp. (357-376), ISSN 0021-9673.

Matthew, M.W., Beuhler, R.J., Ledbetter, M. & Friedman, L. (1986). Large, energetic impacts on surface. *Nuclear Instruments & Methods in Physics Research, Section B: Beam interactions with Materials and Atoms B,* Vol.14, No.4-6, (April 1986), pp. (448-460), ISSN 0168-583X

Matuszewski, B.K. (2006). Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol.830, No.2, (January 2006), pp. (293-300), ISSN 1570-0232.

Matuszewski, B.K., Constanzer M.L. & Chavez-Eng, C.M. (1998). Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per millilitre concentrations. *Analytical Chemistry*, Vol.70, No.5, (March 1998), pp. (882-889), ISSN 0003-2700.

Matuszewski, B.K., Constanzer, M.L. & Chavez-Eng, C.M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Analytical Chemistry*, Vol.75, No.13, (July 2003), pp. (3019-3030), ISSN 0003-2700.

Maurer, H.H. (2007). Current role of liquid chromatography-mass spectrometry in clinical and forsenic toxicology. Analytical and Bioanalytical Chemistry Vol.388, No.7, (August 2007), pp. (1315-1325), ISSN 1618-2642.

Mawwhinney, D. & Henion, J. (2002), in: Presented at the 50th ASMS conference, Orlando, FL,

Mazzeo, J.R., Neue, U.D., Kele, M. & Plumb R.S. (2005).Advancing LC Performance with Smaller Particles and Higher Pressure. *Analytical Chemistry*, Vol.77, No.23, (December 2005), pp. (460A-467A), ISSN:0003-2700.

Mckinney, C.R., McCrea, J.M., Epstein, S., Allen, H.A. & Urey, H.C. (1950). Improvements in mass spectrometers for the measurement of small differences in isotope abundance ratios. *The Review of Scientific Instruments*, Vol.21, No.8, (August 1950), pp. (724-730), ISSN: 0034-6748.

Mei, H., Hsieh, Y., Nardo, C., Xu, X,. Wang, S., Ng K. & Korfmacher. W. (2003), Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: Application to drug discovery. *Rapid Communication in Mass Spectrometry*, Vol. 17, No.1, pp. (97-103), ISSN 0951-4198.

Monaghan, P.J., Brown, H.A., Houghton, L.A., Keevil, B.G. (2009). Measurement of serotonin in platelet depleted plasma by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol.877, No.22, pp. (2163-2167), ISSN 1570-0232.

Niessen, W. M. A. J. (2003). Progress in liquid chromatography–mass spectrometry instrumentation and its impact on high-throughput screening. *Journal of chromatography. A*, Vol.1000, No.1-2, (June 2003), pp. (413-436), ISSN:0021-9673.

Niessen, W., Manini P. & Andreoli. R. (2006), Matrix effects in quantitative pesticide analysis using liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*, Vol.25, No.6, (November-December 2006), pp. (881-899), ISSN 0277-7037.

Niessen, W. M. A. J. (2003). Progress in liquid chromatography–mass spectrometry instrumentation and its impact on high-throughput screening. *Journal of chromatography. A*, Vol.1000, No.1-2, (June 2003), pp. (413-436), ISSN 0021-9673.

Nygren, U., Ramebaeck, H., Berglund, M. & Baxter, D.C. (2006). The importance of a correct dead time setting in isotope ratio mass spectrometry: Implementation of an electronically determined dead time to reduce measurement uncertainty.*Journal of Mass Spectrometry*,Vol.257, No.1-3, (November 2006), pp. (12-15), ISSN:1387-3806.

Park, M.A., Callahan, J.H. & Vertes, A. (1994). An inductive detector for time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry,* Vol.8, No.4, (April 1994), pp. (317-322), ISSN 1097-0231.

Paul, W. & Steinwedel, H. (1953). Ein neues Massenspektrometer ohne Magnetfeld. *Zeitschrift für Naturforschung A,* Vol.8, No.7, (June 1953), pp. 448–450, ISSN : 0932-0784.

Plumb, R.S., Stumpf, C.L., Granger, J.H, Castro-Perez, J., Haselden, J.N. & Dear, G.J. (2003). Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. *Rapid Communications in Mass Spectrometry,* Vol.17, No.23, (December 2003), pp. (2632-2638), ISSN:1097-0231.

Price, D. & Milnes, G.J. (1984). Recent developments in techniques utilizing TOF MS. *International Journal of Mass Spectrometry and Ion Processes,* Vol.60, No.7, (September 1984), pp. (61-81), ISSN :1387-3806.

Ramos, L., Brignol, N., Bakhtiar, R., Ray, T., McMahon, L.M. & Tse, F.L.S. (2000). High-throughput approaches to the quantitative analysis of ketoconazole, a potent inhibitor of cytochrome P450 3A4, in human plasma. *Rapid Communications in Mass Spectrometry,* Vol.14, No.23, (December 2000), pp. (2282-2293), ISSN 1097-0231.

Rezzani, R. (2004). Cyclosporine A and adverse effects on organs: histochemical studies. *Progress in Histochemistry and Cytochemistry*, Vo.39, No.2, (August 2004), pp. (85-128), ISSN 0079-6336.

Romanyshyn, L., Tiller, P.R., Alvaro, R., Pereira, A., Hop, C.E. (2001). Ultra-fast gradient vs. fast isocratic chromatography in bioanalytical quantification by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, Vol.15, No.5, pp. (313–319), ISSN 0951-4198.

Sangster, T., Spence, M., Sinclair, P., Payne, R. & Smith. C. (2004). Unexpected observation of ion suppression in a liquid chromatography/atmospheric pressure chemical ionization mass spectrometric bioanalytical method. *Rapid Communications in Mass Spectrometery*, Vol. 18, No.12, pp. (1361-1364), ISSN 0951-4198.

Santos-Neto, A., Bergquist, J., Lanças F. & Sjöberg, P. (2008). Simultaneous analysis of five antidepressant drugs using direct injection of biofluids in a capillary restricted-access media-liquid chromatography-tandem mass spectrometry system. *Journal of Chromatography A*, Vol.1189, No.1-2, (May 2008), pp. (514-522), ISSN 0021-9673.

Sawka, A.M., Jaeschke, R., Singh, R.J., Young Jr, W.F. (2003). A comparison of biochemical teats for pheochromocytoma: measurement of fractionated plasma metanephrines compared with the combination of 24-hour urinary metanephrines and catecholamines. *The Journal of Clinical Endocrinology and Metabolism*. Vol.88, No.2, (Febrary 2003), pp. (553-558), ISSN 0021-972X.

Schellen, A., Ooms, B., Van de Lagemaart, D., Vreeken, R., van Dongen, W.D. (2003). Generic solid phase extraction-liquid chromatography-tandem mass spectrometry method for fast determination of drugs in biological fluids. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol.788, No.2, (May 2003), pp. (251-259), ISSN 1570-0232.

Schmidt, A., Karas, M. & Dülcks. T. (2003). Effect of different solution flow rates on analyte ion signals in nano-ESI MS, or: When does ESI turn into nano-ESI? *Journal of the American Society for Mass Spectrometry*, Vol. 14, No.5 (May 2003), pp. (492-500), ISSN 1044-0305.

Schwartz, J.C., Wade, A.P., Enke, C.G., Cooks, R.G. (1990). Systematic delineation of scan modes in multidimensional mass spectrometry. *Analytical Chemistry, Vol.* 62, No.17, (September 1990), pp. (1809-1818), ISSN 0003-2700.

Schwartz, J.C., Senko, M.W., Syka, J.E. (2002). A two-dimensional quadrupole ion trap mass spectrometer. *Journal of the American Society for Mass Spectrometry,* Vol.13, No.6, (June 2002), pp. (659–669), ISSN 1044-0305.

Shen J.X., Wang H., Tadros S., Hayes R.N. (2006). Orthogonal extraction/chromatography and UPLC, two powerful new techniques for bioanalytical quantification of desloratadine and 3-hydroxydesloratadine at 25 pg/mL, *Journal of Pharmaceutical and Biomedical Analysis*. Vol.40, No.3, (Feburary 2006), pp. (689-706), ISSN 0731-7085.

Sinz, M.W., Podoll, T. The mass spectrometer in drug metabolism. In *Mass Spectrometry in Drug Discovery*, RossiDT, SinzMW (eds). Marcel Dekker: New York, 2002; 271–335.

Soldin, S.J. & Soldin, O.P. (2009). Steroid hormone analysis by tandem mass spectrometry. *Clinical Chemistry*, Vol.55, No.6, (July 2009), pp. (1061-1066), ISSN 0009-9147.

Song, Q., Naidong, W. (2006). Analysis of omeprazole and 5-OH omeprazole in human plasma using hydrophilic interaction chromatography with tandem mass spectrometry (HILIC-MS/MS)--eliminating evaporation and reconstitution steps in 96-well liquid/liquid extraction. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol.830, No.1, (January 2006), pp. (135-142), ISSN 1570-0232.

Sparkman, O.D. (2000). Mass Spectrometry: Overview and History, Mass Spectrometry Section, In: *Encyclopedia of Analytical Chemistry*, R.A. Meyers, (Ed.), pp. 11501-11557, Wiley, ISBN 3-540-40739-1, Chichester, England.

Taylor, P.J. (2005a). High-performance liquid chromatography-mass spectrometry in the clinical laboratory, *Therpeutic Drug Monitoring*, Vol.27, No.6, (December 2005), pp. (689-693), ISSN 0163-4356.

Taylor, P.J. (2005b). Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clinical Biochemistry*, Vol.38, No.4, (April 2005), pp. (328–334), ISSN 0009-9120.

Taylor, P.J. (2007). Internal standard selection for immunosuppressant drugs measured by high-performance liquid chromatography tandem mass spectrometry. *Therapeutic Drug Monitoring*, Vol.29, No.1, (Faburary 2007), pp. (131-132), ISSN 0163-4356.

Thevis, M., Thomas, A., Schanzer, W. (2011). Current role of LC-MS(/MS) in doping control. *Anal Bioanl Chem* Vol.301, No.2, (August 2011), pp. (405-420), ISSN 1618-2642

Thevis, M., Schänzer, W. (2007). Current role of LC-MS (/MS) in doping control. *Analytical Bioanalytical Chememistry*, Vol.388, No.7. (August 2007), pp. (1351–1358), ISSN 1618-2642.

Thörngren, J.O., Östervall, F., Garle, M. (2008). A high-throughput multicomponent screening method for diuretics, masking agents, central nervous system (CNS) stimulants and opiates in human urine by UPLC-MS/MS. *Journal of Mass Spectrometry*, Vol.43, No.7, pp. (980–992), ISSN 1076-5174.

Tiller, P. R., Romanyshyn, L. A., Neue, U. D. (2003). Fast LC/MS in the analysis of small molecules. *Analytical and Bioanalytical Chemistry,* Vol.377, No.5, (November 2003), pp. (788-802), ISSN 1618-2642.

van der Horst, A. and Schoenmakers, P. J. (2003). Comprehensive two-dimensional liquid chromatography of polymers. Journal of Chromatography A, Vol.1000, No.1-2, (June 2003), pp. (693–709), ISSN 0021-9673.

Veltkamp, A.C., Das, H.A., Frei, R.W., Brinkman, U.A. (1987). On-line low-level radiometric detection of [$^{14}$C]remoxipride in liquid chromatographic effluents. Application to urine samples. *Journal of Chromatography. Vol.* 384, (January 1987), pp. (357-369), ISSN 0021-9673.

Ventura, R., Roig, M., Montfort, N., Saez, P., Berges, R., Segura, J. (2008). High-throughput and sensitive screening by ultraperformance liquid chromatography tandem mass spectrometry of diuretics and other doping agents. *European Journal of Mass Spectrometry* (*Chichester, Eng*) Vol.14, No.3, pp. (191–200), ISSN 1469-0667.

Veuthey, J.L., Souverain, S. & Rudaz, S. (2004). Column-switching procedures for the fast analysis of drugs in biologic samples. *Ther Drug Monit,* Vol.26, No.2, (April 2004), pp. (161-166), ISSN 0163-4356.

Vintiloiu, A., Mullett, W.M., Papp, R., Lubda, D., Kwong, E. (2005). Combining restricted access material (RAM) and turbulent flow for the rapid on-line extraction of the cyclooxygenase-2 inhibitor rofecoxib in plasma samples. *Journal of Chromatography A,* Vol.1082, No.2, (August 2005), pp. (2660-2668), ISSN 0021-9673.

Vogeser, M. & Kirchhoff, F. (2011). Progression in automation of LC-MS in laboratory medicine. *Clinical Biochemistry*, Vol.44, No.1, (January 2011), pp. (4-13), ISSN 0009-9120.

Vogeser, M. & Seger, C. (2008). A decade of HPLC-MS/MS in the routine clinical laboratory: goals for further developments. *Clinical Biochemistry,* Vol.41, No.9, (June 2008), pp. (649-662), ISSN 0009-9120.

Vogeser, M., Geiger, A., Herrmann R. And Kobold, U. (2008). Sample preparation for liquid chromatography-tandem mass spectrometry using functionalized ferromagnetic micro-particles. *Clinical Biochemistry*, Vol. 41, No.16-17, (November 2008), pp. (1417–1419), ISSN 0009-9120.

Vogeser, M. & Spohrer, U. (2005). Pitfall in the high-throughput quantification of whole blood cyclosporin A using liquid chromatography-tandem mass spectrometry. *Clinical Chemistry and Laboratory Medicine*, Vol.43, No.4, (Feburay 2005), pp. (400-402), ISSN 1434-6621.

Vuckovic, D., Zhang, X., Cudjoe, E., Pawliszyn J. (2010). . Solid-phase microextraction in bioanalysis: New devices and directions. *Journal of Chromatography A*. Vol.1217, No.25, (June 2010), pp. (4041–4060), ISSN 0021-9673.

Wang, G., Hsieh, Y., Cui, X., Cheng, K.C., Korfmacher, W.A., (2006). Ultra-performance liquid chromatography/tandem mass spectrometric determination of testosterone and its metabolites in in vitro samples. *Rapid Communicatios in Mass Spectrometry*, Vol.20, No.14, pp. (2215-2221), ISSN 0951-4198.

Wang, S., Cyronak, M, Yang, E. (2007). Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *Journal of Pharmaceutical and Biomedical Analysis,* Vol.43, No.2, (January 2007), pp. (701-707), ISSN 0731-7085.

Watson, J.T. & Sparkman, O.D. (2007). Introduction of Mass Spectrometry: Instrumentation, Applications and Strategies for Data Interpretation. (4th Eds), John Wiley & Sons Ltd, ISBN 978-0-470-51634-8, West Sussex, England

Westmacott, G., Frank, M., Labov, S.E. & Benner, W.H. (2000). Using a superconducting tunnel junction detector to measure the secondary electron emission efficiency for a microchannel plate detector bombarded by large molecular ions. *Rapid Communicatios in Mass Spectrometry*, Vol.14, No.19, pp. (1854-1861), ISSN 0951-4198.

Wilson, I.D. & Brinkman, U.A.T. (2003). Hyphenation and hypernation — the practice and prospects of multiple hyphenation. *Journal of Chromatography A*, Vol.1000, No.1-2, (June 2003), pp. (325-356), ISSN 0012-9673.

Wikipedia, http://en.wikipedia.org/wiki/Mass_spectrometry.

World Anti-Doping Agency (2009). The world anti-doping code -international standard for laboratories. http://www.wada-ama.org/rtecontent/document/International_ Standard_for_ laboratories_v6_0_January_2009.pdf. Accessed 24 Nov 2008.

Wren, S.A. & Tchelitcheff, P. (2006) Use of ultra-performance liquid chromatography in pharmaceutical development. *Journal of Chromatography A*, Vol.1119, No.1-2, (June 2006), pp. (140–146), ISSN 0021-9673.

Xu, R.N., Fan, L., Rieser, M.J. & El-Shourbagy, T.A. (2007). Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis,* Vol.44, No.2, (June 2007), pp. (342-355), ISSN 0731-7085.

Xu, R.N., Kim, G.E., Gregg, H., Wagdy, A., Swaine, B.A. & Chang, M.S., El-Shourbagy, T.A. (2004). Automated 96-well liquid-liquid back extraction liquid chromatography-tandem mass spectrometry method for the determination of ABT-202 in human plasma. *Journal of Pharmaceutical and Biomedical Analysis,* Vol.36, No.1, (September 2004), pp. (189-195), ISSN 0731-7085.

Xu, R.N., Fan, L., Rieser, M.J., El-Shourbagy, T.A. (2007). Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, Vol.44, No.2, (June 2007), pp. (342-355), ISSN 0731-7085.

Xu, X.S., Yan, K.X., Song, H. & Lo, M.W. (2005). Quantitative determination of a novel dual PPAR alpha/gamma agonist using on-line turbulent flow extraction with liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol.814, No.1, (January 2005), pp. (29-36), ISSN 1570-0232.

Xue, Y.J., Akinsanya, J.B., Liu, J. & Unger, S.E. (2006). A simplified protein precipitation/mixed-mode cation-exchange solid-phase extraction, followed by high-speed liquid chromatography/mass spectrometry, for the determination of a basic drug in human plasma, *Rapid Communications in Mass Spectrometry*, Vol.20, No.18, pp. (2660-2668), ISSN 0951-4198.

Yang, A.Y., Sun, L., Musson, D.G. & Zhao, J.J. (2006). Determination of M+4 stable isotope labeled cortisone and cortisol in human plasma by microElution solid-phase extraction and liquid chromatography/tandem mass spectrometry *Rapid Communications in Mass Spectrometry*, Vol.20, No.2, pp. 233–240, ISSN 0951-4198.

Yang, Z., Peng, Y., Wang, S. (2005). Immunosuppressants: pharmacokinetics, methods of monitoring and role of high performance liquid chromatography/mass spectrometry. *Clinical and Applied Immunology Reviews*, Vol.5, No.6, (December 2005), pp. (405-430), ISSN 1529-1049.

Yang, Z. & Wang, S. (2008). Recent development in application of high performance liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressants, *Journal of Immunological Methods*, Vol.331, No.2, (July 2008), pp. (98-103), ISSN 0022-1759.

Yi, Z. & Brown, P.R. (1991). Chromatographic methods for the analysis of basic neurotransmitters and their acidic metabolites. *Biomedical Chromatography*, Vol.5, No.3, (May 1991), pp.(101-107), ISSN 0269-3879.

Yu, K., Little, D., Plumb, R. & Smith, B. (2006) High-throughput quantification for a drug mixture in rat plasma – a comparison of Ultra Performance™ liquid chromatography/tandem mass spectrometry with high-performance liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, Vol.20, No.4, pp. (544–552), ISSN 0951-4198.

Yue, B., Rockwood, A.L., Sandrock, T., La'ulu, S.L., Kushnir, M.M., Meikle, A.W. (2008). Free thyroid hormones in serum by direct equilibrium dialysis and online solid-phase extraction-liquid chromatography/tandem mass spectrometry. *Clinical Chemistry*, Vol.54, No.4, (April 2008), pp. (642-651), ISSN 0009-9147.

Zang, X., Luo, R., Song, N., Chen, T.K. & Bozigian, H. (2005). A novel on-line solid-phase extraction approach integrated with a monolithic column and tandem mass

spectrometry for direct plasma analysis of multiple drugs and metabolites. *Rapid Communications in Mass Spectrometry,* Vol.19, No.22, pp. (3259-3268), ISSN 0951-4198.

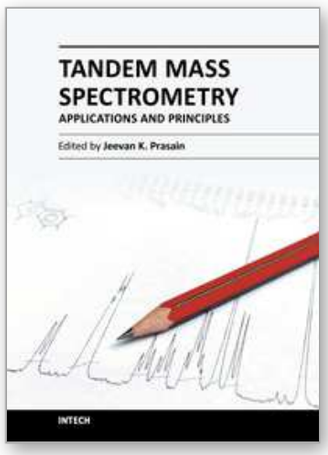
Zeng, H., Deng, Y., Wu, J.T. (2003). Fast analysis using monolithic columns coupled with high-flow on-line extraction and electrospray mass spectrometric detection for the direct and simultaneous quantitation of multiple components in plasma. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences,* Vol.788, No.2, (May 2003), pp. (331-337), ISSN 1570-0232.

Zhang, N., Fountain, S.T., Bi, H., Rossi, D.T. (2000a). Quantification and rapid metabolite identification in drug discovery using API time-of-flight LC/MS. *Analytical Chemistry,* Vol. 72, Vol.4, (Feburary 2000), pp. (800-806), ISSN 0003-2700.

Zhang, H., Henion, J., Yang, Y., Spooner, N. (2000b). Application of atmospheric pressure ionisation time-of-flight mass spectrometry coupled with liquid chromatography for the characterization of in vitro drug metabolites. *Analytical Chemistry.* Vol.72, No.14, (July 2000), pp. (3342-3348), ISSN 0003-2700.

Zhou, S., Zhou, H., Larson, M., Miller, D.L., Mao, D., Jiang, X., Naidong, W. (2005). High-throughput biological sample analysis using on-line turbulent flow extraction combined with monolithic column liquid chromatography/tandem mass spectrometry. *Rapid Communication in Mass Spectrometry,* Vol.19, No.15, (August 2005), pp. (2144-2150), ISSN 0951-4198.

Zimmer, D., Pickard, V., Czembor, W., Muller, C., (1999). Comparison of turbulent-flow chromatography with automated solid-phase extraction in 96-well plates and liquid-liquid extraction used as plasma sample preparation techniques for liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, Vol.854, No.1-2, (August 2007) pp. (23-35), ISSN 0021-9673.




**Tandem Mass Spectrometry - Applications and Principles**

Edited by Dr Jeevan Prasain

ISBN 978-953-51-0141-3
Hard cover, 794 pages
**Publisher** InTech
**Published online** 29, February, 2012
**Published in print edition** February, 2012

Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

**How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ju-Seop Kang (2012). Principles and Applications of LC-MS/MS for the Quantitative Bioanalysis of Analytes in Various Biological Samples, Tandem Mass Spectrometry - Applications and Principles, Dr Jeevan Prasain (Ed.), ISBN: 978-953-51-0141-3, InTech, Available from: http://www.intechopen.com/books/tandem-mass-spectrometry-applications-and-principles/principles-and-applications-of-lc-ms-ms-for-the-quantitative-bioanalysis-of-analytes-in-various-biol

INTECH
open science | open minds

**InTech Europe**
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

**InTech China**
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen