**REPUBLIC OF TURKEY**

**YILDIZ TECHNICAL UNIVERSITY**

**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

# DETERMINATION OF TRACE ANALYTES IN BIOLOGICAL FLUIDS BY Q-TOF LC-MS USING ISOTOPE DILUTION METHOD

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DOCTOR OF PHILOSOPHY THESIS

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A thesis submitted by Elif ÖZTÜRK ER in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY is approved by the committee on 21.12.2020 in Department of Chemical Engineering, Chemical Engineering Program.

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Elif ÖZTÜRK ER

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*Dedicated to my little girl and*

*the memory of my dad*

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To my father, I won't be this stronger without you. You have always guided me to be a better person for the 12 years that you have been in my life. You are the reason why I keep pushing, facing all the struggles and pains. To my baby girl, I will do my best to teach you all I know. Welcome to our life.

Elif ÖZTÜRK ER

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# LIST OF SYMBOLS

| | |
|---|---|
| $A$ | Absorbance |
| $\epsilon$ | Absorptivity Coefficient |
| $n_A$ | Amount of Analyte in the Sample |
| $n_B$ | Amount of Analyte in the Spike |
| $\beta_0$ | Constant Term |
| $R^2$ | Determination Coefficient |
| $I_0$ | Intensity of Incident Light |
| $i^x$ | Isotope Abundances of Natural Form of the Analyte |
| $i^y$ | Isotope Abundances of Isotopic Form of the Analyte |
| $r_A$ | Isotopic Composition of the Sample |
| $r_B$ | Isotopic Composition of the Spike |
| $R_{AB}$ | Isotopic Ratio of the Final Mixture |
| $\beta_i$ | Linear Effect |
| $K$ | Mass Bias Calibration Factor |
| $m_{A(AB)}$ | Masses of Aqueous Solution of Sample |
| $m_{B(AB)}$ | Masses of Aqueous Solution of Spike |
| $\omega_A$ | Mass Fractions of the Analyte in the Sample |
| $\omega_B$ | Mass Fractions of the Analyte in the Spike |
| $\mu$ | Mean of Repeated Measurements |
| $r$ | Measured Isotope Ratio |
| $c$ | Molar Concentration of the Sample |
| $R_m$ | Multiple Response |
| $N$ | Noise |
| $\sigma$ | Normalized Standard Deviation |
| $B$ | Number of Marked Species in the Second Capture |
| $l$ | Path Length |
| $A_{sample}$ | Peak Area of Analyte in the Sample |
| $A_{standard}$ | Peak Area of Analyte in the Standard Solution |
| %ME | Percent Matrix Effect |
| $\varepsilon$ | Residual Related to the Experiments or Random Error |
| $Y$ | Response |

| | |
|---|---|
| S | Signal |
| m | Slope of the Calibration Plot |
| SD | Standard Deviation |
| R | Theoretical Isotope Ratio |
| $N_B$ | Total Number of First Captured and Marked Species |
| W | Total Number of Second Captured Species |
| $N_W$ | Total Population of the Specified Environment |
| I | Transmitted Light Intensity |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5'-GMP | 5’-Guanosine Monophosphate |
| AED | Atomic Emission Detector |
| ATR | Attenuated Total Reflection |
| BBD | Box-Behnken Experimental Design |
| CAD | Charged Aerosol Detector |
| CA-MNP | Citric Acid Coated-Magnetic Nanoparticle |
| CC | Corpora Cavernosa |
| CE | Collision Energy |
| CGMP | Cyclic Guanosine Monophosphate |
| CI | Chemical Ionization |
| CID | Collision-Induced Dissociation |
| CNT | Carbon Nanotube |
| CRM | Certificated Reference Materials |
| CV | Coefficient of Variation |
| DAD | Diode Array Detector |
| DC | Direct Current |
| DHBMA | 3,4-dihydroxybutyl mercapturic acid |
| DLLME | Dispersive Liquid-Liquid Microextraction |
| DSPE | Dispersive Solid Phase Extraction |
| ECD | Electron Capture Detector |
| ED | Erectile Dysfunction |
| EI | Electron Impact Ionization |
| ELISA | Enzyme-Linked Immuno Sorbent Assay |
| ESI | Electrospray Ionization |
| FDA | Food and Drug Administration |
| FID | Flame Ionization Detector |
| FL | Fluorescence Spectrophotometry |
| FPD | Flame Photometric Detector |
| FTIR | Fourier-Transform Infrared Spectrophotometer |
| FT-ICR | Fourier Transform-Ion Cyclotron Resonance |
| GC | Gas Chromatography |

| | |
|---|---|
| GLC | Gas-Liquid Chromatography |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| GO | Graphene Oxide |
| GSC | Gas-Solid Chromatography |
| HF-LPME | Hollow-Fiber Liquid Phase Microextraction |
| HILIC | Hydrophilic Interaction Liquid Chromatography |
| HPLC | High Performance Liquid Chromatography |
| IDMS | Isotope Dilution Mass Spectrometry |
| $ID^2MS$ | DoubleIsotope Dilution Mass Spectrometry |
| $ID^3MS$ | Triple Isotope Dilution Mass Spectrometry |
| $ID^4MS$ | Quadruple Isotope Dilution Mass Spectrometry |
| IS | Internal Standard |
| IR | Infrared Spectrometry |
| IT | Ion-Trap |
| iTRAQ | Isobaric Tag for Relative and Absolute Quantitation |
| LBA | Ligand Binding Assay |
| LC | Liquid Chromatography |
| LC-MS | Liquid Chromatography-Mass Spectrometry |
| LC-MS/MS | Liquid Chromatography-Tandem Mass Spectrometry |
| LC-QTOF-MS | Liquid Chromatography-QuadrupoleTime-of-Flight-Mass Spectrometry |
| LLE | Liquid-Liquid Extraction |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| MALDI | Matrix-Associated Laser Desorption Ionization |
| MHBMA | Monohydroxy-3-butenyl mercapturic acid |
| MNP | Magnetic Nanoparticle |
| MRM | Multiple Reaction Monitoring |
| MS | Mass Spectrometry |
| MS/MS | Tandem Mass Spectrometry |
| MSPE | Magnetic Solid Phase Extraction |
| NMR | Nuclear Magnetic Resonance |
| NO | Nitric Oxide |
| NP | Nanoparticle |
| NPD | Nitrogen/Phosphorus Detector |

| | |
|---|---|
| PA | Polyacrylate |
| PDE-5 | Phosphodiesterase-5 |
| PDMS | Polydimethylsiloxane |
| PID | Photoionization Detector |
| PP | Protein Precipitation |
| Q | Quadrupole |
| QQQ | Triple Quadrupole |
| Q-TOF | Quadrupole Time-of-Flight |
| RAM | Restricted Access Materials |
| RF | Radio Frequency |
| RI | Refractive Index |
| RIA | Radio Immunoassay |
| RPLC | Reversed Phase Liquid Chromatography |
| RSD | Relative Standard Deviations |
| RT | Retention Time |
| SBSE | Stir-Bar Sorptive Extraction |
| SDME | Single Drop Microextraction |
| SEM | Scanning Electron Microscope |
| SIM | Selected Ion Monitoring |
| SPE | Solid Phase Extraction |
| SPME | Solid Phase Microextraction |
| SRM | Selected Reaction Monitoring |
| TCD | Thermal Conductivity Detector |
| TEM | Transmission Electron Microscope |
| TIMS | Thermal Ionization Mass Spectrometry |
| TLC | Thin Layer Chromatography |
| TOF | Time-of-Flight |
| UV-Vis | Ultraviolet-Visible |
| XRD | X-Ray Diffraction |

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# ABSTRACT

## Determination of Trace Analytes in Biological Fluids by Q-TOF LC-MS using Isotope Dilution Method

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Department of Chemical Engineering

Doctor of Philosophy Thesis

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Determination of trace analytes brought a series of questions related to the method performance for low concentration levels in diverse matrices. As one of the relatively new developments, the isotope dilution method is a highly sensitive and selective analytical method with high precision for trace analysis.

This thesis presents the determination of erectile drugs and amino acids using isotope dilution strategy. All determinations were performed with Quadrupole–Time–of–Flight Liquid Chromatography–Mass Spectrometry system. The chromatographic separation of erectile drugs including sildenafil, tadalafil, vardenafil and avanafil in urine and plasma was performed on a reversed phase column. To achieve higher sensitivity for analytes, magnetic solid phase extraction was employed and the detection limits for combined method were found to be between 0.16 – 0.94 ng $g^{-1}$.

In the second part of this thesis, it was focused on the selective and sensitive determination of 17 free amino acids in biological matrices. In this context, two approaches were employed including hydrophilic interaction chromatography for underivatized amino acids and reversed phase chromatography for derivatized amino acids. The detection limits were obtained between 0.03 – 2.26 μmol $kg^{-1}$ and 0.016 and 0.266 μmol $kg^{-1}$ for underivatized and derivatized amino acids, respectively.

Accuracy and precision of developed methods were evaluated by performing recovery experiments in urine, plasma and serum samples using matrix matching strategy. Despite obtained satisfactory limits in percent recoveries, isotope dilution strategy was employed for each study to obtain quantitative results with unprecedented analytical quality (accuracy levels close to 100%).

The analytical methods presented in this study provided simple and feasible routes for accurate and precise determinations of erectile drugs and amino acids in complex biological matrices. Therefore, these methods could be easily adapted for quantification of selected analytes in clinical applications which have crucial importance to diagnose and screen disorders linked to physiological levels of these analytes or to discover new biomarkers.

**Keywords:** Trace analytes, isotope dilution, erectile drugs, amino acids, biological fluids

# ÖZET

## Vücut Sıvılarında Eser Analitlerin İzotop Seyreltme Yöntemi Kullanılarak Q-TOF LC-MS ile Tayini

Elif ÖZTÜRK ER

Kimya Mühendisliği Bölümü

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Herhangi bir eser analitin tayini, farklı matrislerdeki analitlerin düşük konsantrasyon seviyeleri için yöntem performansıyla ilgili bir dizi soruyu beraberinde getirmektedir. Son zamanlarda geliştirilen yeni yöntemlerden biri olan izotop seyreltme yöntemi, eser analitlerin analizi için yüksek hassasiyette, oldukça duyarlı ve seçici bir analitik yöntemdir.

Bu çalışmada, erektil ilaçların ve aminoasitlerin izotopik seyreltme stratejisi kullanılarak tayin edilmeleri sunulmaktadır. Bu amaçla, analitik tayinler Kuadropol-Uçuş Zamanlı Sıvı Kromatografisi – Kütle Spektrometresi sistemi kullanılarak gerçekleştirilmiştir. Sildenafil, tadalafil, vardenafil ve avanafili kapsayan erektil ilaçlar idrarda ve plazmada ters faz yöntemi ile kromatografik olarak ayrılmıştır. Bu analitler için daha yüksek hassasiyet elde edebilmek için, Manyetik Katı Faz Ekstraksiyonu yöntemi uygulanmıştır ve kombine yöntem için tayin limitleri 0.16 – 0.94 ng $g^{-1}$ aralığında tespit edilmiştir.

Sunulan bu tezin ikinci bölümünde ise biyolojik matrislerdeki 17 serbest amino asidin seçici ve duyarlı bir yöntem ile tayin edilmesi üzerine yoğunlaşılmıştır. Bu

bağlamda, türevlendirilmemiş serbest amino asitlerin tayini için hidrofilik etkileşim kromatografisi, türevlendirilmiş amino asitlerin tayini için ise ters faz kromatografisini içeren iki farklı yaklaşım üzerine çalışılmıştır. Tayin limitleri türevlendirilmemiş ve türevlendirilmiş amino asitler için sırasıyla; 0.03 – 2.26 μmol $kg^{-1}$ ve 0.016 - 0.266 μmol $kg^{-1}$ aralıklarında tespit edilmiştir.

Geliştirilen yöntemlerin doğruluğu ve kesinliği, matris eşleştirme stratejisi kullanılarak idrar, plazma ve serum örneklerinde geri kazanım deneyleri ile değerlendirilmiştir. Yüzde geri kazanımlarda tatmin edici sınırlar içerisinde sonuçlar elde edilmesine rağmen, benzeri görülmemiş analitik kalitede kantitatif sonuçlar (%100'e yakın doğruluk) elde etmek için her çalışmada ayrıca izotop seyreltme stratejisi de uygulanmıştır.

Bu çalışmada sunulan analitik yöntemler, karmaşık biyolojik matrislerde erektil ilaçların ve amino asitlerin doğru ve kesin tespiti için basit ve uygulanabilir yollar göstermektedir. Geliştirilen bu yöntemlerin klinik uygulamalarda seçilen analitlerin miktarlandırılması için uygulanması, bu analitlerin fizyolojik seviyelerine bağlı olarak ortaya çıkabilen hastalıkları teşhis etmek ve taramak, ya da bazı hastalıklar için yeni biyobelirteç olma ihtimallerini doğru değerlendirmek adına oldukça önemli bir yer tutmaktadır.

**Anahtar kelimeler:** Eser analitler, izotop seyreltme, erektil ilaçlar, amino asitler, biyolojik sıvılar

# 1
# INTRODUCTION

## 1.1 Literature Review

As the biological systems consist of a variety of organic compounds, quantification methods for the determination of trace analytes in these complex matrices are one of the demanding experimental tasks facing modern science. The concentrations of biomolecules, metabolites and drugs in biological fluids present very significant information in diagnosis, medical treatments and scientific research. Therefore, the studies focused on the increase of accuracy and precision in such measurements and on the decrease of the detection limits for an ever-widening range of analytes [1].

The studies on the quantitative determination of organic compounds in complex matrices have been carried out in a wide range of applications of spectrometric and/or chromatographic methods until now. The recent techniques are based on instrumental applications including high performance liquid chromatography (HPLC) and gas chromatography (GC) coupled with sensitive universal detectors. The detectors are responsible to convert the physical or chemical attribute to a measurable signal corresponding to the concentration or identity [2]. As sensitivity and flexibility are the most important parameters for the efficiency of a detector, mass spectrometers have been applied in a wide range of applications including drug discovery and metabolism, pollution control, food control, forensic science and natural science [3]. Regarding the informative electrical signals generated from MS detectors, quantitative trace analysis using MS detectors takes place for endogenous and exogenous compounds in biological matrices with different analytical techniques.

Even though the detection methods are improved with high technologies, all quantitative analysis ultimately requires high purity standards of the related analyte and its highest possible accurate weight on an analytical balance [1]. Quantitative

determination always starts from the known amount of the analyte standard and gets to the results by comparing the signals of the analyte from the sample with the signals of the known amount of analytical standard. This is achieved by correlating the signal intensity with the analyte quantity present in the sample for a mass spectrometer. There have been several quantitative methods developed using mass spectrometry (MS) for many applications. All of these experimental procedures for mass spectrometers involve several steps resulting from a final error. The experimental errors which are originated from the mass spectrometer and sample handling affect the reproducibility and precision of the measurement [3].

To minimize the final errors, some approaches have been applied including external and internal standards. The external standard method is based on the comparison of the signal intensity of the analyte in the sample with the calibration standards. The responses for signal intensities are assumed to be the same for samples and calibration standards. On the other hand, the signal variations can be eliminated by a reference compound which is added at the same known quantity to all measurements. This method is called as internal standard method. In this method, the ratio of analyte signal to reference compound signal is used in the calibration plot [3]. However, the analyte losses during extraction steps and complex matrix interferences may not be compensated with these methodologies [4]. At this point, the use of a reference standard that exhibits the same physical and chemical properties with the analyte is required to fully compensate for the analytical preparation steps and complex matrix interferences. Herein, the reference standard refers to the isotope labeled species of the target analyte and this method is called as isotope dilution mass spectrometry (IDMS) [4].

Despite the difficulty of detection and identification of trace analytes in biological systems, IDMS provides accurate quantification of analyte(s) in complex biological matrices. In recent years, the IDMS technique has been applied for many organic compounds such as creatinine [5, 6], 3,4-dihydroxybutyl mercapturic acid (DHBMA) and monohydroxy-3-butenyl mercapturic acid (MHBMA) [7], homoarginine, guanidinoacetate and their common precursor arginine [8], 1-vinyl-2-pyrrolidone-mercapturic acid [9], triglycerides and total glycerides [10], β-hydroxyethoxyacetic

acid [11], oxysterols and oxyphytosterols [12] and total homocysteine [13] in different biological matrices including human plasma, human serum, human urine, brain cortex and brain tissue. The IDMS technique paves the way for highly precise, sensitive and accurate determination of trace analytes in biological systems [14].

## 1.2 Objective of the Thesis

The main aim of this research was to develop highly sensitive methods for accurate determination of trace organic analytes in biological fluid matrices. In this context, Quadruple Isotope Dilution Mass Spectrometry ($ID^4MS$) strategy has been applied for the accurate and precise determinations of two different classes of organic compounds including erectile drugs and free amino acids. In the first part of the study, it was aimed to develop an analytical method with superior analytical characteristics for the determination of erectile dysfunction (ED) drugs including sildenafil, tadalafil, vardenafil and avanafil in human urine and plasma samples.

- To obtain low detection limits, magnetic solid phase extraction (MSPE) was combined with liquid chromatography - quadrupole time of flight - mass spectrometry (LC-QTOF-MS) system.
- Herein, citric acid coated magnetic nanoparticles were synthesized, characterized and applied as a solid sorbent in the MSPE procedure.
- To obtain maximum extraction efficiency, all influential parameters was optimized using an experimental design called Box-Behnken.
- Under the optimum experimental conditions, the analytical performance parameters of the combined MSPE-LC-QTOF-MS method was evaluated and the proposed method was applied to human urine and plasma samples.
- Furthermore, $ID^4MS$ strategy was also combined with the proposed method to obtain high accuracy at trace levels.
- The accurate quantitation of ED drugs in clinical studies is expected to exhibit a bright future for monitoring the treatments that require erectile drug use and also in monitoring cardiovascular risks.

In the second part of the study, the analytical determination method was developed by using ID$^4$MS technique for the simultaneous determination of 17 different amino acids in biological matrices.

- Two different approaches were studied for chromatographic separation of amino acids; hydrophilic interaction and reversed phase chromatograph (HILIC and RPLC). HILIC system was utilized for the direct separation of free amino acids while the RPLC system was used after the derivatization of amino acids.
- The experimental variables of the derivatization procedure were optimized using the experimental design. The analytical parameters of both systems were evaluated in terms of linear range, sensitivity, accuracy and precision.

As a result, it was planned to perform two different analytical methods with high accuracy and sensitivity for the screening of amino acids in the early diagnosis of disorders in amino acid metabolism.

## 1.3 Hypothesis

Since the development and identification of new biologically active compounds and biomarkers strongly depend on the reliable determination of components or their metabolites in biological samples. It is very significant to develop new analytical strategies providing high accuracy and sensitivity. This becomes an extremely demanding and challenging task for samples with high complexity and low concentrations of analytes due to the matrix effect. Therefore, several strategies based on hyphenated systems and/or new analytical approaches have been employed to open-up new avenues for the development of quantitative methods with high metrological quality [15].

One of the new technologies used for the qualitative and quantitative determination of organic components in biological matrices is LC-QTOF-MS method. LC-QTOF-MS, on the one hand, enables the accurate identification of target analytes thanks to the mass spectroscopy component and the maximum error rate of 2.0 ppm, on the other hand, it is also possible to use the physicochemical separation feature of liquid

chromatography [16]. In this way, identification of the components is achieved by retention time and the relative abundance ratio of the characteristic analyte fragments in the mass spectrum. Although this method enables high-precision mass selectivity, it no longer guarantees the effective elimination of interferences from matrix components.

IDMS is considered as the most powerful analytical technique for the quantitative determination that provides both selectivity and sensitivity of the MS instruments. IDMS provides unchallenged accuracy and control of error sources. The method relies on the addition of a known amount of enriched isotope to sample as spike. After the equilibration of the enriched isotope with the natural analyte, the altered isotope ratios are measured by MS studies [17]. As the latest version of IDMS designs, ID$^4$MS enables the elimination of isotope ratios of natural and enriched materials, and the mass fraction of enriched material. It only includes the gravimetric data of isotopic compositions of four blends where one of the blends needs to match the isotopic composition of the sample blend. Three calibration blends preparation with the possibility of choosing the isotopic composition provides high-precision measurements with more sophisticated designs [18].

In the present study, superior accuracy and precision were aimed for the determination of four different ED drugs and 17 different amino acids. Therefore, ID$^4$MS combined LC-QTOF-MS method was studied for biological matrices including plasma, urine and serum samples. The matrix matching strategy was also applied and the improvement in accuracy and precision of the developed methods was compared to discuss the applicability of ID$^4$MS strategy.

# 2
# GENERAL INFORMATION

## 2.1 Bioanalytical Determination of Trace Analytes

The bioanalytical determination of trace analytes that present in living organisms and biological fluids is of primary importance for diagnosis, treatment and development in many research fields including biological, medical and pharmaceutical sciences. The analysis of trace analytes in biological samples is still a challenging task due to very demanding requirements in terms of the sensitivity, selectivity, reliability, speed of analysis method and sample throughput [19].

The biological samples involve urine, whole blood, serum, plasma, saliva, cerebrospinal fluid, breast milk, exhaled breath and tissue samples. The trace analytes include drugs, metabolites, peptides, lipids, carbohydrates and proteins. The determination of these analytes usually comprises the qualification of substances by identification or structural elucidation and quantification by determining the actual amount of substances within the real samples [19, 20].

The analysis of biological samples require very complicated process due to three main reasons:

1. The biological samples are very complex matrices. They contain significant amounts of endogenous compounds, salts, acids, cells, lipids and lipoproteins that cause a high degree of interferences on target analytes during the measurement.
2. The analytes are mostly not detectable by the analytical instrument for direct measurement due to the presence of these analytes at very small quantities.
3. Most of the biological samples are not compatible with most analytical instrumentations [20-22].

Depending on all the issues mentioned above, the sample preparation step is mandatory prior to analysis. The sample preparation is also called sample clean-up,

sample extraction or sample pretreatment, and this step is the most difficult and critical part of the bioanalytical method [23].

## 2.2 Sample Preparation Methods

Biological samples usually contain moderate-to-high level proteins and high amounts of salts. Hence, they are incompatible with the analyzing systems and cannot be injected into the instrument of choice directly. Sample preparation is a fundamental step for bioanalytical studies to make the biological samples suitable for the instrumentation [23].

In a bioanalytical method, as the weakest link of the chain, the sample preparation step affects all the other parts in the assay and hence, a poorly treated sample causes invalidate results. On the other hand, an ideally treated biological sample provides improved detection and separation efficiency, longer periods for instrument maintenance and reduced costs of the whole method [24].

The basic concept of a sample preparation process is to obtain the analyte of interest in an appropriate solution and this process also provides substantial benefits including [19, 25]:

- Removal of matrix interferences or undesired substances from the sample, thereby improving selectivity of the method.
- Stabilization of sample by reconstitution to an appropriate solvent.
- Preconcentration of analyte to enhance the sensitivity of the method.

The quality of the sample preparation step is directly related to the quality of the samples which encompasses the collection, transport and storage of the samples. Since approximately 80% of a bioanalytical method consists of sampling and sample preparation steps, the sample preservation during these steps is of the utmost importance for ensuring sample integrity, in parallel with the terms of accuracy and precision of the method [26, 27].

Recently, to cope up with the demands for improved accuracy, precision and robustness, several sample preparation protocols were developed and these protocols can be classified under three main strategies: precipitation, derivatization and extraction. These strategies present different approaches providing various

benefits and the combination of these strategies among each other is also a useful way to achieve more efficient sample preparation [28-30]. Efficient sample preparation is expected to show the following features [30]:

- High recovery with the minimal sample loss,
- Efficient removal of coexisting substances,
- Low cost of whole procedure,
- Simplicity of operation,
- High potential to be automatable for high throughput analysis,
- Compatibility with the analytical instruments.

In the following sections, the descriptions for the commonly used sample preparation methods were introduced.

### 2.2.1 Protein Precipitation

Protein Precipitation (PP) is one of the oldest and simplest sample preparation methods for the analysis of biological samples [31]. This method has been also known as the "dilute and shoot" method which refers to the dilution of the sample matrix by a precipitation solvent, acid, base or salt prior to analysis [31, 32].

The basic concept of PP relies on the direct or indirect interaction between the protein moieties and the reagent. Typically, a hydrophilic surface surrounds the hydrophobic core of the soluble proteins and the ionic groups involved in the hydrophilic surface do not participate in molecular bonding with any ionic moiety in the opposite charge. Acids and bases initiate precipitation by affecting the ionization states of protein moieties while salts affect the water content of protein structures [33, 34]. Similarly, organic solvents also affect the hydration level of protein by withdrawing water content from the structure and in all cases, the ionic attraction of proteins increases resulting in the formation of protein aggregates [35].

The most common precipitation reagents for biological fluids are organic solvents regarding their compatibility with chromatographic techniques. The procedure generally starts with the addition of organic solvent at least twice the volume of samples. After the addition of precipitation reagent, the mixing is applied to

accelerate the aggregation of proteins. Finally, the separation of supernatant is achieved by centrifugation and/or filtration [32, 33].

PP can be applied to extract the most of the organic analytes from the sample matrices. Despite its universality and simplicity, the whole process is time-consuming for high throughput analysis and the supernatant is not clean enough when compared to extraction methods [23, 31].

### 2.2.2 Derivatization

Derivatization is a promising method especially for chromatographic techniques and used to modify the structure of the target compound(s). Derivatization can be applied to a wide range of compounds and instrumentation. The integration of derivatization with instrumental techniques serves several advantages such as the improvement of chromatographic separation, the enhancement of detection and the removal of endogenous species [36].

Derivatization is performed by the chemical reaction between the functional group of analyte and the reaction group of derivatization reagent. The common chemical reactions comprise the replacement of hydrogen in polar groups such as OH, COOH, CONH, SH and NH resulting to the classification of main categories including alkylation, silylation, acylation and the formation of cyclic derivatives. Besides the replacement of active hydrogen, the derivatization can also be performed by the replacement of other functionalities with condensation reactions [37, 38].

The most important part of a derivatization reaction is to select the appropriate derivatization agent. Herein, the key factors are the type of functional group in the target compound, the chemical structure of the compound and the type of sample matrix [38]. The preferences for selecting an appropriate derivatization agent according to functional groups are summarized in Table 2.1.

**Table 2.1** The preferences for selecting an appropriate derivatization agent according to functional groups [38]

| Compound | First preference | Second preference |
|---|---|---|
| Amine | Acylation | Alkylation |
| Amide | Acylation | Alkylation |
| Alcohol | Silylation | Acylation |
| Phenol | Silylation | Acylation |
| Acid | Alkylation | Silylation |

Depending on the instrumental technique, there are several studies on analytical derivatization aiming to enhance volatilization, thermal stability, separation, detection and the overall quality of the method [39-41]. Nevertheless, the derivatization step is considered an undesirable process regarding the addition of an extra step to sample preparation which may increase the analyte loss and impurity content of the matrix [28]. Even though, this step is mostly imperative to determine the biological substances because of their polarity and low volatility. Therefore, great efforts have been made to develop key reactions for many biological substances to convert them into extractable and thermally stable forms prior to analysis [36].

### 2.2.3 Extraction

Extraction terminology in sample preparation refers to the isolation of a target compound from complex sample matrices to bring them into a suitable form for analysis [42]. The extraction method plays a vital role in biological sample preparation. Its development has grown exorbitantly and has exhibited astonishing vitality with the introduction of new technologies. In the face of this rapid growth, various modern extraction techniques have been developed such as solid phase microextraction (SPME), dispersive solid phase extraction (DSPE), stir-bar sorptive extraction (SBSE), single drop microextraction (SDME), dispersive liquid-liquid

microextraction (DLLME) and hollow-fiber liquid phase microextraction (HF-LPME) along with the conventional techniques including liquid-liquid extraction (LLE) and solid phase extraction (SPE) [19, 20, 43].

The ultimate goal of all mentioned extraction techniques is to provide high productivity and this can be achieved either through improving an existing method or developing a new method. The productivity of extraction can be assessed by some specific features including handling time, efficiency, automation, cost, sensitivity and greenness profile [44].

#### 2.2.3.1 Liquid-Liquid Extraction (LLE)

Separation of the analyte of interest from the sample matrix is the problem in sample preparation. In this regard, the oldest and most common solution is the introduction of a second phase which is immiscible or partially miscible with the sample matrix. The principle of LLE relies on the distribution coefficients of substances between two phases [45].

The selective separation of analyte from sample interferences is achieved by partitioning the sample between two phases. In the LLE procedure, the component mixture is firstly dissolved in a proper solvent and after that, a second solvent which is immiscible with the dissolving solvent is added to the mixture. The final solution is mixed and then, two immiscible solvents are separated into upper and lower layers depending on their densities [46].

In the application of LLE to biological samples, a water-immiscible organic solvent is used as the second solvent including hexane, diethyl ether, ethyl acetate, 1-chlorobutane or methyl-tert-buthyl ether [43]. After vigorous mixing, hydrophobic compounds are mainly transferred to the organic phase while ionized substances including phospholipids and salts are mainly seen in the aqueous layer. The final organic phase that contains the analyte of interest is evaporated and the residue is reconstituted with a suitable solvent for the analysis [46].

The main advantages of LLE comprise the easy operation and relatively low cost per sample. The procedure does not require any special equipment and the operational steps are very simple. These features also provide consistent results between

batches. On the other hand, LLE also suffers from several disadvantages for high-throughput considerations. First of all, the high consumption of organic solvent is required for the extraction that yields a huge amount of toxic waste. Second, the procedure is time-consuming when compared to modern extraction techniques. Third, emulsion formation may arise during the process and forth, LLE is not applicable for the extraction of polar compounds, limiting the determination of many compounds in biological samples [41, 43, 46].

#### 2.2.3.2 Liquid-Liquid Microextraction (LLME)

To overcome the drawbacks of conventional LLE including the use of large amounts of solvent and the formation of emulsions, researches have been studied on the new strategies for the miniaturization of LLE. For this purpose, microextraction techniques where the amount of extracting solvent is very small have been introduced as an alternative to LLE. The most common advanced LLME methods include single drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME) and hollow-fiber liquid phase microextraction (HF-LPME). There are many variations between these methods such as the nature of the solvent, the design of extraction device and types of targeted analytes [47, 48].

***Single Drop Microextraction:***

The first applications of SDME date back to the mid-1990s [49-51]. In the most common approach, a conventional microsyringe is used as the extraction unit and the extraction medium is formed as a single drop. The extraction process is carried out according to the concentration differences between two liquid phases and mass transfer takes place from the aqueous phase to the organic phase (single drop) until thermodynamic equilibrium is reached or the process is stopped. Since the diffusion rate is very small, agitation is applied to increase the transport of analyte molecules. The agitation period required for the equilibrium depends on the agitation degree, interfacial contact area, phase volumes and distribution constant of equilibrium. Therefore, to avoid longer operational periods, the extraction is completed under non-equilibrium conditions. The implementation of SDME to almost all laboratories is very easy due to the simplicity and low cost of the procedure. On the other hand,

the analytical performance parameters of SDME including precision and sensitivity still require further improvements [52, 53].

***Dispersive Liquid-Liquid Microextraction:***

Dispersive liquid-liquid microextraction has been attracted special attention since its first introduction as a microextraction technique in 2006, by Rezaee and coworkers [54]. It is an environmentally friendly sample preparation technique based on the use of organic solvents in very small quantities. In this method, the extraction of the analyte is conducted by the dispersion of extractant in the aqueous medium. Since the water-immiscible organic solvents are preferred as the extractant, a second organic phase is required to disperse the extracting solvent in the aqueous medium, which is called as disperser, dispersing solvent or dispersive solvent. The extraction process consists of two steps [55, 56]:

1. The rapid injection of an appropriate mixture of extraction and dispersive solvents into the aqueous medium containing the target analyte(s). In this step, the dispersion of extracting solvent through fine droplets facilitates the extraction of analyte and, the infinitely large surface area between the aqueous media and extracting solvent allows to reach the equilibrium state very quickly. This is the most remarkable feature of DLLME.
2. The dispersion is separated from the sample solution by centrifugation. The extracting solvent is taken from the bottom (when the density of extracting solvent is heavier than water) or top (when the density of extracting solvent is lighter than water) of the conical test tube and injected into the instrument for analysis.

The schematic representation of the DLLME procedure is presented in Figure 2.1. The extraction efficiency in DLLME is affected by several factors including the types of extracting and dispersive solvents, the amounts of extracting and dispersive solvents, pH of the sample solution, salt effect and the extraction period. Among these factors, the choice of suitable extracting solvent has a critical role in extraction efficiency. There are three essential features for extracting solvent: one is the low solubility with water, the other is the good extraction capability for the analyte of interest and the last one is the good compatibility with the analytical instrument.

The common extracting solvents used are halogenated hydrocarbons including chloroform, chlorobenzene, carbon disulphide, tetrachloroethylene, dichloromethane and carbon tetrachloride. Since these solvents are highly toxic, the amount of extracting solvent is required to be carefully optimized for optimal extraction output [55].

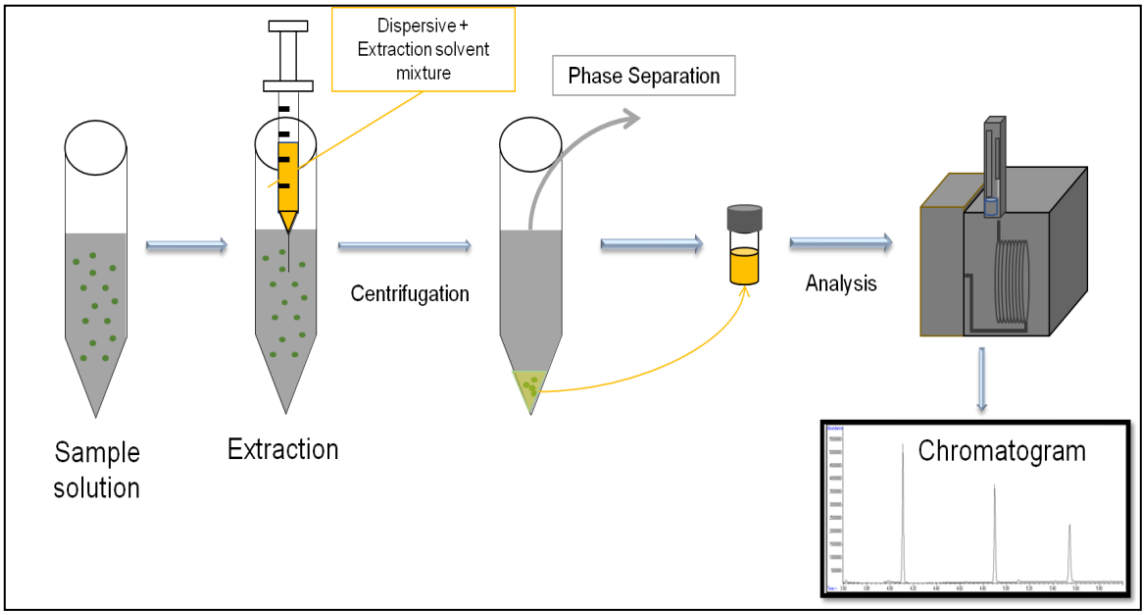



**Figure 2.1** Schematic representation of DLLME procedure

As the second most significant parameter of the DLLME process, the dispersive solvent must be soluble in both water and extracting solvent. It enables the formation of a cloudy solution for the interaction of aqueous and extracting phases, thereby, directly relates to the extraction efficiency. The transfer of analyte of interest from the sample solution to the extracting phase is greatly affected by the degree of dispersion which corresponds to the type and amount of dispersive solvent. The commonly preferred dispersive solvents are methanol, acetone, isopropyl alcohol, ethanol and acetonitrile regarding to their low toxicity and low cost [47, 57].

The other parameters including the salting effect and pH of the solution can also be optimized to enhance the extraction efficiency via decreasing the solubility of analyte in the aqueous medium. The extraction period does not have a significant

effect on the enrichment of the majority of analytes. Indeed, the longer periods may enhance the extraction efficiency for particular analytes [56, 58].

The advantages of DLLME include a very short extraction period, simple operation, environmental benignity and high enrichment factor. It has also some drawbacks including the limited applicability due to the restricted selection of extracting solvents and the difficulty of automation [57-59].

***Hollow-Fiber Liquid Phase Microextraction:***

Hollow-fiber liquid phase microextraction was reported as a new extraction technique by Pedersen-Bjergaard and Rasmussen using polypropylene hollow fiber as the extraction unit [60]. In HF-LPME, a porous hollow fiber is immobilized with an organic solvent and immersed in an aqueous sample solution containing the analyte of interest. The procedure begins with the direct immersion of hollow fiber in the organic solvent to make a thin layer within the wall of the hollow fiber. In this step, the organic solvent has to be immiscible with water in order to prevent any leakage to the aqueous solution. In the next step, the acceptor solution which is usually an acidic, basic or organic solution fills the lumen of the hollow fiber. In the case of the organic acceptor phase, the system results in a two-phase mode and in this mode, the collected phase can be directly analyzed by GC. On the other hand, acidic or basic acceptor phases result to the three-phase mode and the analyte is extracted by acceptor solution through the thin film of organic solvent. This system is more compatible with HPLC [61, 62].

In both modes, HF-LPME has led to remarkable advantages including excellent sample clean up, high potential for automation, high enrichment factor and low cost. Despite these advantages, there are also several limitations of this system such as the risk of memory effect and the requirement for preconditioning of membrane. Moreover, the pores of the membrane can be blocked by adsorption of other hydrophobic compounds which is usually the case for complex biological samples [47, 62].

#### 2.2.3.3 Solid Phase Extraction (SPE)

Solid phase extraction (SPE) has been widely used for more than 60 years and emerged as a powerful sample preparation technique for the isolation of compounds of interest from biological samples. In SPE, the isolation of compounds is conducted using disposable cartridges as the solid phase and the extraction is occurred according to the partitioning between the solid phase and liquid phase (sample matrix). The liquid sample is loaded to the cartridge and passed through the sorbent material in the cartridge. During this passage, analytes are retained on sorbent material and following the end of the passage, analytes are recovered by the elution with a small amount of suitable solvent [23, 30].

The typical operation procedure of SPE consists of four steps including conditioning, loading, washing and elution. In the first step, the sorbent material is conditioned by wetting using an appropriate solvent to enable the solvation of functional groups and remove the impurities coming from the sorbent material. Furthermore, the void volume of the column is filled with the solvent and the air inside the column is also removed. In the second step, the liquid sample is loaded onto the column and passed through the column by pumping, gravity, aspiration with the vacuum or an automated system. The analytes are retained on the column during this step. Hence, the flow rate of the sample strongly affects the retention of analytes. The matrix components may also be adsorbed on the packing material and they can be removed with the washing step. In this step, an eluent with low strength is used to wash the column without displacing the analytes of interest. Before the final elution step, drying may be also applied to prevent the presence of water in the extract. The final step includes the elution of analytes using a proper solvent and the amount of elution solvent needs to be adjusted to get quantitative recovery of analytes [63, 64].

The objectives of the SPE method are the preconcentration of trace analytes, reduction of the level of matrix interferences and exchange of medium by transferring to a solvent that is compatible with the instrument. The achievement of these objectives strongly depends on the selectivity of the sorbent material for the analyte(s) arising from the intermolecular interactions, the relative strength of this interaction and the physicochemical properties of analyte(s). Therefore, the proper

choice of sorbent material is very critical and related to the hydrophobic, inorganic and polar properties of the sorbent. The retention of analytes on sorbent material is mainly conducted by van-der Waals forces (nonpolar), dipole-dipole forces (polar), hydrogen bonding and cation-anion (ionic) interactions [46, 48].

SPE can be applied for the extraction of a huge variety of analytes from non-polar to very polar analytes with high extraction efficiency. Nowadays, various types of sorbent materials have been developed for the extraction of a wide range of analytes such as chemically bonded silica (C8, C18), polymer-based materials, carbon-based materials, ion-exchange materials, monolith sorbents and restricted access materials (RAM) [43, 46]. The other advantage of the SPE method that it generates less toxic waste when compared to LLE and, unlike LLE, there is no formation of emulsions and foaming. Furthermore, SPE can be automated with robotic systems, allowing high throughput analysis [48].

Although the SPE method is very popular now, it has several disadvantages including multi-operational and time-consuming steps, limited selectivity, analyte losses during the evaporation step and batch-to-batch variations. Moreover, the chemical background of packing material possesses a high level of contamination caused by the impurities and this can lead to serious interferences. Besides that, SPE columns have high costs and have been mostly designed for single use only. The operation costs may significantly increase when the sorbent pores in the column plugged by complex matrix components or sample overload. Taking these drawbacks into account, many modern approaches have been developed to improve the SPE performance [65, 66].

#### 2.2.3.4 Solid Phase Microextraction

Solid phase microextraction (SPME) has gained rapid and substantial progress since its introduction as a new microextraction strategy in 1990 by Arthur and Pawliszyn [67]. SPME possesses numerous attributes desirable for an effective analytical procedure, as it integrates several steps including sampling, extraction, enrichment and even sample introduction specifically for gas chromatography systems. In this technique, a syringe-like device consisted of a fiber holder is used for the extraction. This silica fiber is coated with an organic polymer and exposed to the sample

solution by moving the plunger of syringe backward and forwards. Herein, the extraction takes place based on the distribution constant between the solid fiber support and sample solution for the analyte of interest.

Depending on the contact time between the sample and the extraction unit, the two strategies can be applied for the sorption of analytes: equilibrium extraction and pre-equilibrium extraction. In equilibrium extraction, the fiber is withdrawn after the partition equilibrium is reached while in pre-equilibrium extraction, a well-defined time is used to complete the process. After the process ends, the analytes on the sorbent material are thermally desorbed or re-dissolved in a suitable solvent prior to analysis [22, 62, 66].

In a classical SPME procedure, the coating material is the key parameter for the partition of analyte. There are many commercial available coatings including polydimethylsiloxane (PDMS), polyacrylate (PA), carboxen/PDMS and divinylbenzene/PDMS. The proper coating for a given analyte is selected based on the "like-dissolves like" principle. Correspondingly, PDMS coating is more suitable for non-polar compounds whereas PA is useful for polar ones. The mixed phases are mostly applied for volatile compounds for higher extraction yields. The thickness of the coating material also affects the extraction yield. The thinner coatings yield to a faster equilibration period while the thicker coatings are suitable for the compounds with small molecular mass [62, 66].

The following parameters are needed to be examined for maximum extraction yield: pH, the concentration of salt, sample volume, temperature and extraction period. SPME offers cost-effectiveness, high detection limits and operational simplicity. Furthermore, the complete elimination of organic solvents from the extraction makes the method environmentally friendly. The main drawback of this technique is the narrow application range as its application is very limited for hydrophilic and nonvolatile compounds [68].

#### 2.2.3.5 Stir Bar Sorptive Extraction

Stir bar sorptive extraction (SBSE) was first proposed by Baltussen and coworkers in 1999 [69]. SBSE has been developed based on the SPME principle but the

extraction unit in SBSE differs from SPME. In SBSE, the surface of a glass-enveloped magnetic bar is coated with the extraction phase, instead of fiber. This extraction unit allows coating with 50-250 times larger sorbent volume than the fiber. The increased surface area of sorbent presumably results to greatly increased extraction efficiency [23].

The extraction in the SBSE process takes place during the stirring period and after plenty of time for optimal extraction, the spin bar is removed from the aqueous solution for desorption of analytes. Desorption can be achieved via thermal desorption (TD) or back extraction with a small volume of organic solvent (liquid desorption, LD). Comparing with SPME, the desorption step is relatively longer due to the slow mass transfer because of the thicker coating on the extraction unit [70, 71].

SBSE provides several advantages over SPME such as simplicity and higher sample capacity of sorptive beds resulting in higher enrichment factors and better recovery. However, there is a major limitation that restricts its application for compounds over a wide range of polarities. Presently, the extraction phase is only commercialized with PDMS and because of the non-polar nature of PDMS, this technique fails to extract polar compounds from the sample matrix. To overcome this limitation, a few attempts have been made to develop different extraction phases that present better affinities for polar compounds such as monolithic materials, molecularly imprinted polymers (MIPs) and dual stir bars [71-73].

#### 2.2.3.6 Dispersive Solid Phase Extraction

In recent years, as a very promising alternative to SPE, dispersive solid phase extraction (DSPE) has been developed to address several challenges, as it is applicable for a wide variety of analytes, cost-effective and environmentally friendly. DSPE procedure involves the extraction of analytes by the sorbent material that is added to the sample solution followed by the dispersion of sorbent within the aqueous solution. After adequate interaction of sorbent with the sample matrix within a period of mixing, the sorbent is separated from the solution using centrifugation. In the next step, the isolated analytes are eluted from the surface of sorbent material using a very small amount of organic solvent [23].

DSPE offers rapid extraction due to the direct contact between the analyte and sorbent material. Herein, the extraction period and capacity strongly depends on the type and surface area of the sorbent material. The analyte polarity, solubility and the nature of the sample matrix are the key parameters that should be considered for the proper choice of sorbent material. Unlike classical SPE, packed devices with sorbents are not needed and the conditioning step is removed. Furthermore, the enhanced interaction of sorbent and analytes improves the equilibrium rate and recovery of the analyte [74, 75].

The development of new materials has greatly influenced the spread of DSPE. Great attention has been paid especially to nanoparticles (NPs) and nanocomposites as solid sorbents for the extraction of a large variety of compounds [74]. Examples of sorbent materials are metallic NPs such as AgNPs, AuNPs, $TiO_2$, $Al_2O_3$ and $ZrO_2$; silica nanomaterials such as $SiO_2$ nanoparticles; carbon-based nanomaterials such as carbon nanotubes (CNTs), graphene and graphene oxide (GO); and polymer-based sorbents such as organic polymers, hybrid polymers and molecularly imprinted polymers (MIPs) [74, 76, 77].

***Magnetic Solid Phase Extraction*:**

As a special class of metallic NPs, magnetic nanoparticles (MNPs) offers the additional advantage of easy and fast phase separation with the help of an external magnetic field. The magnetic separation facilitates the separation step and reduces the required time especially for large scale operations. Moreover, MNPs can be easily functionalized with specific site groups and stabilizing materials including polymers, silica, noble metals, surfactants and carbon nanomaterials which provide a specific binding for target compounds and also a protective coating to prevent irreversible aggregations [78].

In general terms, MSPE process involves four steps [79]:

1. Addition of magnetic sorbent to aqueous sample solution,
2. Adsorption of target analyte(s) onto the magnetic sorbent material,
3. Separation of magnetic material using an external magnetic field and

4. Desorption of target analyte(s) from the surface of sorbent by elution with an appropriate organic solvent.

A general scheme of MSPE procedure is presented in Figure 2.2.

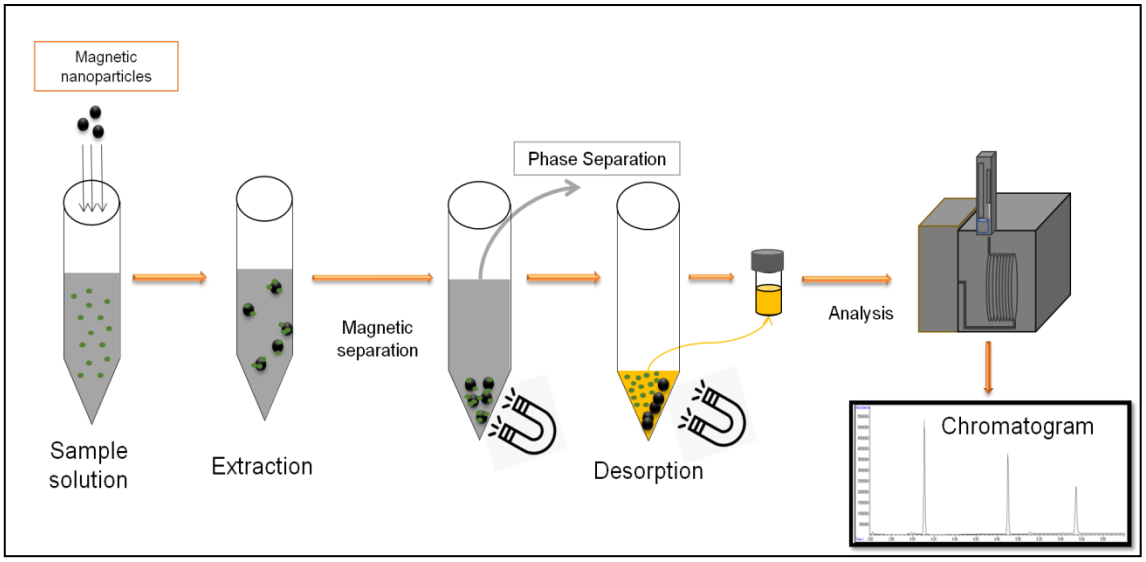



**Figure 2.2** The schematic representation of MSPE procedure

Magnetic separation and preconcentration of analytes have several advantageous features like multi-analyte capability, easy automation, low cost, high selectivity and high enrichment factors [80]. In particular, the most remarkable feature of this technique is the chemically modifiable surfaces of magnetic nanoparticles with different functional groups which makes them highly selective to a target molecule [81]. The preconcentration mechanism of a target analyte mostly depends on the interaction between the surface functional groups of sorbent and analyte molecules which is strongly related to the ionic, dipole-dipole, dipole-induced dipole, hydrogen bonding and dispersion forces [80]. Therefore, the choice of the sorbent material is the most significant step for high preconcentration factors and is directly related to the nature of analyte molecules such as polarity and solubility [80].

In recent years, several solid materials have been synthesized and employed as magnetic sorbent after coating the magnetic cores with carbon nanomaterials [82, 83], chitosan [84], alginate [85], alumina [86], molecularly imprinted polymers [87, 88] and organic acids [89, 90] for the preconcentration and determination of diverse types of organic molecules. The development of new magnetic sorbent materials is

a challenge to obtain reusability of sorbent material, high enrichment factors, high repeatability and high selectivity to target molecules even in complex matrices [91].

The well-known ferromagnetic elements including iron (Fe), nickel (Ni) and cobalt (Co), and the ferromagnetic element based oxides including magnetite ($Fe_3O_4$) and maghemite ($\gamma$-$Fe_2O_3$) can be used to impart magnetic features on the resulting material. Among them, $Fe_3O_4$ is the most prevalent form of MNPs owing to its exceptional properties such as superparamagnetism, good stability, low toxicity, simplicity of synthesis and controllable magnetism [75, 78]. There have been several synthesis routes developed for the production of shape-controlled and stable magnetite nanoparticles mainly including co-precipitation, hydrothermal and solvothermal synthesis, thermal decomposition, microemulsion and metal reduction [78, 79].

**Co-precipitation:** It is one of the simplest method for the synthesis of maghemite and magnetite nanoparticles. In this method, the synthesis is carried out through the growth of iron oxide nuclei by the hydrolysis of mixture of Fe(II) and Fe(III) in aqueous solution under alkaline conditions. The sulfate, chloride or nitrate salts of Fe(II) and Fe(III) are mixed with a molar ratio of 2 ($Fe^{3+}$):1 ($Fe^{2+}$) M and the reaction takes place as follows:

$$2Fe^{3+} + Fe^{2+} + 8OH^- \rightarrow 2Fe(OH)_3 + Fe(OH)_2 \rightarrow Fe_3O_4(s) + 4H_2O$$

Herein, the precipitation occurs via treatment with basic solution of NaOH, $NH_4OH$ or KOH. To avoid the oxidation of Fe(II), the reaction is carried out at the temperatures of 70-85˚C under an inert atmosphere. This method allows for the large-scale production of nanoparticles. However, the size distribution may occur in broad ranges unless reaction parameters are not carefully adjusted [78, 92].

**Hydrothermal and solvothermal synthesis:** The hydrothermal and solvothermal synthesis are carried out in autoclaves at elevated temperatures, e.g. 200˚C, and high-pressure levels (>2000 psi). The hydrothermal naming refers to that the reaction takes place in aqueous media whereas the solvothermal refers to reaction medium with solvents. The reaction is conducted via liquid-solid-solution phase transfer or the reduction of $FeCl_3$ in the presence of ethylene glycol. This route yields

narrow size distribution, small-sized particles and high homogeneity. The main drawback of this method is the low reaction yield and small scale production [75, 78].

**Thermal decomposition:** This method involves the thermal decomposition of organometallic iron precursors in the solvent media with the high-boiling point at high pressure and high temperature. The reaction takes place in the presence of additional stabilizing surfactants such as oleic acid, oleamine. This method offers good size control, narrow size distribution and monodispersity. However, the high toxicity of organic reagents used, high cost and the requirement for additional modification are the major drawbacks of this method [93, 94].

**Metal reduction:** Metal reduction involves the reduction of metal salts including nitrates, acetates, acetylacetonates, chlorides and oxides in the presence of reducing agents. This reaction is conducted by electrochemical reduction or using surfactants. Organic reducing agents such as polyols, dihydrogen gas or hydrazine and hydride-based reducing agents such as sodium borohydride can be used for the reduction of metal salts [94].

**Microemulsion:** Microemulsion synthesis relies on the chemical transformation reactions such as the co-precipitation or metal reduction reactions in a microemulsion system. The microemulsion can be described as the single-phase systems which are consisted of thermodynamically stable isotropic dispersion of two immiscible liquids. Herein, an interfacial film of surface-active molecules stabilizes the microdomain of one or both liquids in other [95]. The reaction is carried out by mixing and coalescence of two micellar systems or by thermally initiated reaction inside a single micelle. The controlled particle sizes can be achieved through the microemulsion method. On the other hand, low production yield and high consumption of solvent are the main disadvantages of this method [78, 96].

## 2.3 Instrumental Detection Techniques

The development of highly sensitive instrumental techniques for the analysis of biological matrices poses a particularly significant challenge for screening, identification and quantification of analytes to achieve reliable data in clinical and forensic toxicological studies. To fulfill ever-increasing demands for reliable qualification and quantification of analytes in biological samples, great efforts have been made for the innovation and improvement of conventional instrumental techniques. These efforts have been particularly focused on the simultaneous determination of multi-analytes as well as increasing the instrumental sensitivity to get lower detection limits [97].

In this regard, the most common instrumental techniques for the determination of analytes in biological samples comprise, but are not limited to, ligand binding assays such as enzyme-linked immuno sorbent assay (ELISA) and radio immunoassay (RIA); chromatographic based methods such as GC coupled to flame ionization (FID) and electron capture (ECD) detectors, and HPLC coupled to ultraviolet detection; and mass spectrometry-based methods such as GC- mass spectrometry (GC-MS), LC-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS/MS) [33, 98].

### 2.3.1 Ligand Binding Assays

Ligand binding assays (LBAs) can be defined as the bioanalytical methods that rely on the specific capture and detection of a target analyte in biological samples using assay reagents that have high binding affinities for the analyte [99]. The reaction between binding reagent and analyte is driven by the law of mass action and ended depending on the analyte concentration in the sample. The format of LBAs may exist competitive or noncompetitive, with solid or solution phase configurations. In LBAs, antibodies, oligonucleotides, receptors and transport proteins can be used as binding reagents. The detection and quantification of the single analyte can be employed using various technologies including enzyme activity or radioactivity of the labeled reagents generating UV absorbing, chemiluminescent, fluorescent or luminescent properties [100].

LBAs can be applied for a wide variety of molecules ranging from low molecular weights to large macromolecules. However, the major limitation of LBAs for small molecules on the lack of specificity has been restricted its application in the pharmaceutical industry. Therefore, nearly all quantification methods of small molecules today are performed using chromatographic techniques. Indeed, LBAs are still the most preferred methods for the determination of macromolecules and play a key role in the understanding of immunogenicity, tissue distribution and unexpected metabolism, and for supporting preclinical and clinical development of biotherapeutics [100, 101].

Since this study has been focused on the determination of small molecules in biological matrices, the following sections include a deep overview of chromatographic techniques, devoting a special concern to the LC-MS/MS system.

### 2.3.2 Basics of Chromatographic Techniques

Chromatographic techniques are increasingly employed in the analytical procedures for the separation, identification and quantification of a wide range of endogenous compounds in biological matrices. Chromatographic separations are located at the heart of earlier generations of instrumentation used to isolate and measure components of mixtures [102, 103].

The principle of chromatographic separation is based on the concentration equilibrium of components between stationary and mobile phases. Herein, the stationary phase refers to an immobilized solid or a liquid fixed on solid support while the mobile phase refers to a liquid or gaseous component. The separation is conducted according to molecular characteristics that relate to adsorption, affinity, partition or molecular weight differences. These differences lead to retention differences of components in the stationary phase. Some of the components move slowly and stay longer in the system while others leave the system rapidly. As the components move through the stationary phase at different rates, they are separated from each other. The separation process directly depends on the interaction between the mobile phase, stationary phase and components in the mixture [104]. After the proper separation is achieved, the detection of compounds

using special techniques is a prerequisite for qualitative and quantitative analysis [105].

Chromatography, from its very beginning, has been classified with different groups that are based on the separation mechanism, physical nature of the phases or basis of chromatographic bed shape. Among others, the consideration of the physical nature of phases elucidate the major distinction. Based on the different combinations of mobile and stationary phases, chromatographic techniques can be classified into the following groups: gas-solid chromatography (GSC), gas-liquid chromatography (GLC), LC and supercritical fluid chromatography (SFC) [106]. In addition to that, adsorption and/or partition chromatography take place according to the nature of the stationary phase.

In the adsorption chromatography, the separation is based on the affinity towards the solid stationary phase that shows adsorptive properties. Examples of adsorption chromatography include column chromatography, thin layer chromatography (TLC) and HPLC. In the partition chromatography, the stationary phase is a liquid adsorbed onto the solid matrix and the partition of solute between two immiscible liquids retards the separation. Of all the chromatographic techniques, these two mechanism have the broadest application [107, 108].

### 2.3.3 Gas Chromatography

In gas chromatography (GC), the stationary phase is a packed or capillary type column containing high boiling point liquids adsorbed onto the solid support, usually silica, and the mobile phase is an inert gas (carrier gas), usually helium, hydrogen and nitrogen. After the sample is injected and vaporized at the head of the column, it is transported through the column by carrier gas under high pressure. The carrier gas does not possess significant solvation interactions with the components in the sample mixture [102]. Under controlled gradient temperatures, the components are separated depending on many physicochemical properties including boiling points, polarities and molecular sizes. The carrier gas leaving the column enters a detector to create an electrical signal [109, 110].

The basic components of GC include an injector, column, oven and detector. The sample enters the GC system through the injector that provides vaporization and also, dilution and split (in some cases). The split is applied by sending a predetermined ratio of injected sample to the column to prevent the overload. To increase the sensitivity, the splitless injection can be preferred. In this mode, the split valve is closed and all of the injected samples enter to the column [111].

The column allows the separation of sample components through the stationary phase. There are two basic GC columns; packed and capillary. In the packed columns, a granular material containing a liquid coated on the solid support is used to pack the column. The dimensions of the packed column may vary between 1.6-2.7 mm of outer diameter and 0.50-5.0 m in length covered with a stainless steel or glass wall. Since the separation efficiency of packed columns fail for trace analysis, GC systems have evolved from packed to capillary columns. The capillary columns are made of hollow fused silica with high purity and tube length ranges from 5.0 to 100 m. Although such a long column requires long analysis periods, it also gives high resolution resulting in better separation. The inner diameter of the column ranges from 100 to 530 μm. The column is covered with a polyimide material to prevent breakage and improve the strength. This outer coating is thermally stable up to 370˚C and there are also columns on the market made up of nickel, steel or aluminum that thermally stable up to 450˚C [110].

There are various types of stationary phases developed to obtain high efficiency and the selection of stationary phase requires adequate knowledge concerning the chemical properties of analytes, efficiency and durability of stationary phases, the interaction between the stationary phase and analytes, and the nature of the sample matrices [109]. The high separation efficiency can be obtained by applying a proper temperature program. The temperature of the column is controlled with an oven allowing to heat the column at different temperature rates. The high-temperature rates provide sharp peaks with shorter analysis periods while the slow rates usually lead to better resolution within two peaks [110].

As the final major component of the GC system, the detector produces an electrical signal which is usually proportional to the concentration of the analyte in the

sample. The recorder displays the signal as a chromatogram which represents the signal intensity over retention time. In principle, any physical or physicochemical properties of components in the sample deviated from the carrier gas can produce the signal for the detection. Accordingly, there have been over 100 detectors suggested for GC systems, but now, only a few of them are used commercially. The commercially available GC detectors are thermal conductivity (TCD), FID, ECD, photoionization (PID), nitrogen/phosphorus (NPD), flame photometric (FPD), atomic emission (AED), electroconductivity, chemiluminescent and radioactivity detectors [111]. Among them, FID, TCD and ECD are the most common detectors in routine laboratories. TCD is a universal detector and operates by measuring the conductivity difference in sample cells over blank carrier gas. In FID, the detection is carried out through the current produced by the ions generated in an oxygen-hydrogen flame. Most of the carbon compounds can be detected by FID, but there are a few exceptions, e.g. ringed nitrogen compounds. ECD is a specific detector for halogenated compounds and those with nitro or some oxygen-containing groups. It provides very high sensitivity, especially for halogenated pesticides. The detection is conducted by recording the continuous current between two electrodes. A potential difference is supplied, and when electro-negative components enter the detector, they capture electrons and lead to the reduction in the background current, thereby producing a measurable signal. Unfortunately, this type of detectors is easily saturated and contaminated, thereby resulting in severe nonlinearity of detector response [102, 112].

### 2.3.4 Liquid Chromatography

Of all the chromatographic techniques, liquid chromatography (LC) has reached an outstanding stage with the rapid development of modern instruments. As the modern form of LC, HPLC was regarded as a complementary to GC at the first stages, but in recent years, it has overlapped a major application area of GC concerning its versatility. Unlike GC, HPLC does not have limitations due to component volatility, thermal stability and molecular weight. The advances made in column packing for specific applications, the better understanding of mobile phase combination and the introduction of more sensitive detectors make the HPLC method of choice for the

determination of agrochemicals, pharmaceuticals, biological fluids, environmental residues and many other substances [113].

An HPLC system can be either a module system consisting of individual components or a single apparatus. There are essential components for HPLC instruments including an injector, pump for the mobile phase, column and detector. Additional components are enclosed for specific purposes, such as a degasser for the removal of dissolved gasses from mobile phase, a column thermostat for temperature-controlled separations and a cooling system for the injector unit. A flow of mobile phase is delivered through the injector, column and detector. The liquid sample is introduced into the continuous flow of the mobile phase by an injector (manual injection with syringe or autosampler) for the introduction to the column. The sample introduction is separated from the high-pressure eluent system by the valve type injectors that have a fixed-volume loop. The injection of the sample is carried out by rotating the valve from load to injection position. Hence, the sample is transported through the column [109, 113].

The separation of sample components is achieved through the column which contains packing material as the stationary phase. It comprises a metal (stainless steel) or plastic (PEEK) body in a tube shape that is packed with a bed of spherical or irregular particles. The monolithic materials are also used as support for packing bed. The separation of components directly depends on the physical and chemical characteristics of particles in the stationary phase. These particles can be in three forms including porous, superficially porous (core-shell) or pellicular. The size of particles strongly influences the retention of components in the column. The particles usually consist of an active phase covered on an inert substrate, usually silica.

Silica provides a large surface area for reactive groups and also, it has high rigidity and resistance. Furthermore, the desired hydrocarbon chains or other organics can be attached onto the silica surface by the chemical reactions of silanol groups through covalent bonds. The widely used bonded phases are octyl groups (C8) or octadecyl groups (C18 or ODS) that are presented as reversed phase (RP) columns. Many other types also exist for a wide range of applications exhibiting different

interactions with components to be separated and mobile phases [114]. The common types of stationary phases and HPLC separation mechanisms are summarized in Table 2.2.

The elution in the chromatographic column can be performed as isocratic or gradient. Isocratic elution refers to the constant mobile phase composition while gradient elution refers to a predetermined change in the composition of the mobile phase during the analysis. For samples containing analytes with different polarities, it is not often possible to achieve an effective separation with isocratic elution. In these cases, the separation efficiency may be improved with suitable gradient programs [115].

**Table 2.2** The common types of separation mechanisms and stationary phases [114]

| HPLC mechanism | Stationary phase |
|---|---|
| **RP** | Inert solid material covered with nonpolar long hydrocarbon chains |
| **Normal phase** | Polar materials such as bare silica |
| **Hydrophilic interaction liquid chromatography (HILIC)** | Organic moieties containing polar groups on the solid support |
| **Hydrophobic interaction chromatography (HIC)** | Hydrophilic stationary phase covered with organic moieties with nonpolar groups |
| **Gel filtration and gel permeation** | Porous particles that have special pore sizes |
| **Ion exclusion** | Special support with ion exchange capability |
| **Chiral chromatography** | Chiral phases |

The column effluent passes through the detector to be monitored. The detector produces an electrical signal that is proportional to a specific property of the analyte. There are various types of HPLC detectors, and the most common ones include ultraviolet-visible (UV-Vis) and fluorescence spectrophotometry (FL),

electrochemical analysis and refractive index (RI). As the most popular HPLC detector today, UV-Vis has broad applicability for a wide range of compounds that adsorb light in the UV or visible region (190-600 nm) by a chromophore, e.g. aromatic rings, double bonds and some hetero-atoms [2]. The underlying principle of UV-Vis is based on Lambert-Beer Law that defines the absorption as proportional to sample concentration:

$$A = \log\left(\frac{I_0}{I}\right) = \epsilon c l \tag{2.1}$$

where A represents the absorbance, $I_0$ is the intensity of incident light, I is the transmitted light intensity, $\epsilon$ is the absorptivity coefficient, l is the path length and c is the molar concentration of the sample. The absorption of radiation occurs through the electron transitions from $\pi \rightarrow \pi^*$, $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ molecular orbitals [116].

UV-Vis detectors can be classified into three main types: fixed wavelength, variable-wavelength and diode array spectrophotometers. The fixed wavelength is the simplest and cheapest design amongst others and operates at a single wavelength. The single wavelength restricts its utility for compounds that have similar absorbance profiles. The variable wavelength is commonly used detector for general purposes. It is employed with an optical monochromator that allows changing wavelength by adjusting diffraction. The light from a broad spectrum is provided by tungsten and deuterium lamps. This type of detectors enable operation at the wavelength giving maximum absorbance for an analyte or at the wavelength presenting high selectivity. The third type of UV-Vis detector employs a photodiode array as the sensor. The light from full-spectrum is focused onto the flow cell and after passing the cell, it is dispersed into the spectrum across an array of photodiodes. In these detectors, the absorbance at different wavelengths can be recorded simultaneously. This feature extends the utility of detection through peak identification, peak homogeneity and purity [2, 109, 117].

As one of the most sensitive detectors in HPLC, FL detectors are commonly used as a strong alternative to UV-Vis detectors. They involve the following components: a light source for excitation, a filter for separation of emitted light from the excitation

and a detector to sense the emitted light [117]. The detector measures the light emission after excitation of electrons in sample molecules at a higher energy wavelength. The electrons are usually excited by ultraviolet light and emit light at longer wavelengths than the excitation light. To allow only the desired wavelength to pass through the sample cell, lamb/filter or lamb/monochromator pairs are used. Similarly, a second filter placed at 90˚ to the direction of excitation ensures the detection of emission signal that results only from the sample by the photomultiplier [118]. Many important biological compounds such as drugs, steroids and vitamins can be detected with high sensitivity because of the little interference occurring from co-eluting substances in biological samples. The main limitation of FL detectors is that they are only applicable for the compounds displaying fluorescent properties. The derivatization of non-fluorescent compounds with fluorogenic reagents is an effective way to extend the applicability range of this method. FL detection may provide up to 1000 times more sensitive analytical methods comparing with absorbance spectrophotometry [109, 118].

Electrochemical detectors are another popular detectors in HPLC systems offering high specificity, sensitivity and broad application areas such as clinical chemistry, drug analysis, biochemistry and neuroscience. The compounds with electrochemical activity are more common than fluorescent compounds. Electrochemical detection is conducted based on the measurement of electron flow on the surfaces of electrodes during oxidation or reduction reactions, or conductivity changes. Working, the auxiliary and reference electrodes are employed in electrochemical detectors. The electrochemical reaction is driven at the surface of the working electrode through the potential difference between working and reference electrodes. The auxiliary and reference electrode play a significant role in the compensation of changes in the conductivity of mobile phases. The most common materials of electrodes are glassy carbon for the working electrode, silver for the reference and stainless steel for the auxiliary electrode [117]. In this detection method, selectivity and sensitivity can be adjusted by changing the potential between working and reference electrodes. Although high potentials provide improved sensitivity, constraints exist in two modes: the oxidation of eluent in oxidative mode and the interference caused by dissolved oxygen in reductive mode.

Moreover, the products of the electrochemical reaction accumulate on the surface of the working electrode and cause the activity loss followed by the loss of detector response [119].

The RI detectors used in HPLC systems are based on the measurement of the RI changes between the sample and mobile phase. Since the refractive index is a physical bulk property, virtually any substances that have a different RI value from the mobile phase can be detected by RI detectors. The deflection refractometer is the most widely used design that consists of a tungsten lamb as the light source and, wedge-shaped reference and sample flow cells. Since RI is strongly affected by temperature changes, modern systems are designed with thermostatted flow cells. In addition to that, RI is also sensitive to the changes in the flow rate and pressure making the system incompatible to gradient elutions. Taking into consideration these limitations, RI detectors are commonly applied for compounds without chromophores such as carbohydrates and lipids [118, 120].

Unfortunately, there is no ideal detector with all the desired characteristics including high sensitivity, specificity, reproducibility, reliability, fast response and wide working range. To fulfill ever-growing demands for improved instrumentation, many attempts have been made to design hyphenated techniques. These techniques include infrared spectrometry (IR), nuclear magnetic resonance (NMR) and MS. The coupling of chromatographic techniques with MS detectors has been more extensively studied owing to their superior sensitivity [121, 122].

### 2.3.5 Mass Spectrometry

Advances in analytical instrumentation have witnessed the emergence of an interesting combination of separation methods with MS detectors. The remarkable improvements in MS systems have opened up its great potential in the identification, characterization and quantitation of a wide variety of small and large molecules in complex biological matrices. In this regard, the development of two designs has provided a step forward and gave rise to the popularity of MS instruments. The first one is the coupling of GC and LC systems with MS detectors that leads to increased confidence of specificity and significantly lowered detection limits. The second one

is the hybrid systems that act as a milestone for accurate and precise mass measurements [109, 123]. Today, these systems are indispensable for analytical challenges for complex biological samples such as screening biomarkers, trace determinations of toxicants, monitoring the use or abuse of drugs and detection of many endogenous substances for diagnosis and treatment purposes of diseases [122, 124].

Up to now, the majority of bioanalytical applications have been performed with LC-MS systems focusing on whole blood, plasma, urine, saliva and hair [125, 126]. Additionally, GC-MS systems have been used for breath analysis and cellular respiration measurements [127, 128]. Indeed, LC-MS systems are still keeping the ultimate advantages owing to the rapid implementation of hybrid configurations [129].

#### 2.3.5.1 Principles of Mass Spectrometry

Mass spectrometry consists of two separate processes including ionization and mass analysis. In the ionization process, the compounds in the sample are converted into ions by any suitable methods and then, the generated ions are separated and detected according to their mass-to-charge ratio (m/z) by applying radio frequency (RF) and electrostatic field in the mass analyzer. The signals are recorded upon the contact of ions with the detector and displayed as a mass spectrum representing the relative abundance of ions as a function of m/z ratio. These processes are carried out under a high vacuum to avoid colliding and interactions with other charged species [130]. The key elements of a mass spectrometer are represented in Figure 2.3 [130].

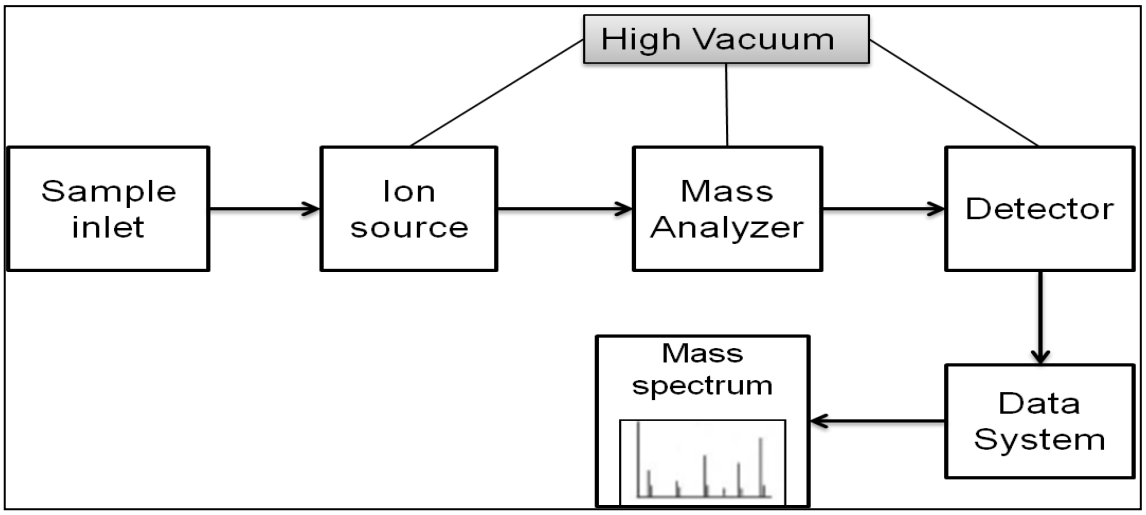



**Figure 2.3** A block diagram for the representation of key elements of a mass spectrometer

### 2.3.5.2 Ionization modes

The ionization process is the most significant step in the analysis of compounds by mass spectrometry. In this process, the motions of ions are easily directed and separated by controlling their velocities with the help of electric and magnetic forces. Thus, the key parameter of an ion source is the extent in the converting neutral molecules into the charged species. To date, several different designs have become available which differ from each other in terms of ionization mechanism and the nature of target analytes. The common types of ion sources for the generation of ions are electron impact ionization (EI), chemical ionization (CI), matrix-associated laser desorption ionization (MALDI) and electrospray ionization (ESI). These types can be also classified as hard and soft techniques according to the extent of fragmentation during ionization. EI is a hard ionization technique while others are typical examples of soft ionization [130].

- ***Electron impact ionization***

In the EI process, the sample molecules are subjected to a beam of energetic electrons emitted from a hot wire filament giving the adequate kinetic energy for ionization of the sample, typically 70 electron volts (eV). Upon interaction of the electron beam with analyte molecules, the positively charged species of analyte molecule is formed:

$$M + e^- \rightarrow M^{\cdot +} + 2e^- \qquad (2.2)$$

The resulting positive ion ($M^{\cdot+}$) is called as molecular ion or radical cation. The excess internal energy can cause the decomposition of molecular ion through a series of reactions resulting in the formation of fragment ions or neutral products. The specific fragments of a molecule act as fingerprint and allow the identification of the compound. On the other hand, extensive fragmentations may result in the complicated interpretation of EI mass spectra. Moreover, under EI conditions, many compounds do not have stable molecular ions making the determination of molecular mass difficult. Another limitation of EI is that, it is not suitable for nonvolatile and thermally labile compounds. Therefore, this mode is widely used in GC-MS systems but remained very limited for LC-MS [131, 132].

- ***Chemical ionization***

To complement the structure identification of analyte molecules, CI was introduced as soft ionization technique that relies on the molecule-ion reactions between the analyte molecules and reagent gas ions. The ionization process starts with the ionization of reagent gas at relatively high pressure by the electron bombardment. In the next step, stable reagent ions formed in the first step interact with analyte molecules with the following reactions:

$$R + e^- \rightarrow R^{\cdot+} + 2e^- \quad (2.3)$$

$$R + R^{\cdot+} \rightarrow R - H^+ + R_x^{\cdot} \quad (2.4)$$

$$R - H^+ + M \rightarrow M - H^+ + R^{\cdot} \quad (2.5)$$

Herein, the ionization of analyte molecules may be formed by charge exchange, proton transfer, anion abstraction and electrophilic addition. Since the formed molecular ions have low internal energy, the majority of them remain unfragmented resulting in simple mass spectrum patterns [114].

- ***Matrix-assisted laser desorption ionization***

MALDI is a soft ionization technique that involves the ionization of sample molecules by absorbing laser energy after embedded in a matrix. In this ionization technique, the matrix has two key roles: the first one is absorbing energy at the wavelength of

the laser and the second one is initiating charge transfer by acting as proton donor or acceptor. The commonly used weak organic acids as matrix are sinapinic acid, gentisic acid, hydroxycinnamic acid and 2,5-dihydroxy benzoic acid. Upon interaction of the matrix with the laser energy, the upper lattice layer of the matrix is expulsed followed by the evaporation arising from the increased molecular motion. Bearing in mind that, the ionization mechanism is not clear, it is believed that the charged analyte molecules are formed by the proton transfer from the matrix [107, 133].

Owing to the privileged formation of single charged molecular ions, MALDI represents a good approach for the identification of large molecules such as biopolymers, proteins, nucleic acids and carbohydrates [133].

- ***Electrospray ionization***

ESI is an atmospheric pressure ionization technique that has become the most widely used ionization mode for LC-MS systems regarding its high ionization efficiency and the applicability to a large variety of chemical substances. In a typical ESI device, the eluent is pumped through a small capillary needle where a high voltage is applied to create an aerosol of charged droplets. Then, the solvent is evaporated with the help of nitrogen gas while formed ions moving from the needle tip toward the metal plate. In the meantime, the surface tension forces keeping the spherical structure of microdroplets become imbalanced and the inside of microdroplets are charged by the repulsive forces. Finally, the generated ions at the droplet surface are emitted into the gaseous phase once the critical point is reached. The emitted ions are transferred to the mass analyzer by a skimmer cone to be analyzed in mass spectrometer [134, 135]. A photograph of the ESI source and the schematic representation of the ESI mechanism are presented in Figure 2.4 and Figure 2.5.

**Figure 2.4** A photograph of ESI source

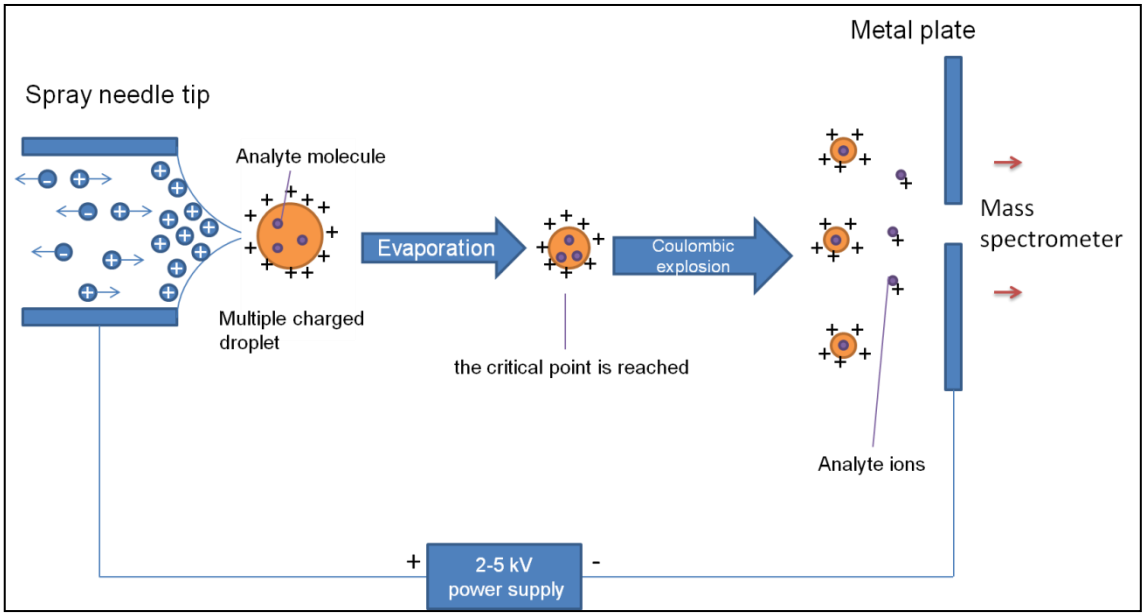



**Figure 2.5** The mechanism of ESI

The technical maturation of the ESI technique favored this method over other ionization modes to be used in most of the routine laboratories worldwide. The main advantage of ESI mode relies on its ability to inducing very low or no fragmentation which makes it very useful for the analysis of large molecules. Considering the multiple charge phenomenon, large structures can be identified with processing in powerful software programs. Moreover, it is compatible with compounds having

moderate to high polarity. They provide efficient ionization for the qualitative and quantitative determination of a large variety of compounds [109, 136].

However, as with all other techniques, the ESI method faces a certain limitation which is termed as matrix effect or ion suppression/enhancement phenomenon. Matrix effect can be defined as the variation in the signal intensity of analyte due to the presence of matrix components. This limitation can be overcome by matrix factor corrections using isotopically labeled internal standards or applying matrix matching strategy [137].

#### 2.3.5.3 Mass Analyzers

The main function of the mass analyzer is to separate ions of different m/z values according to their behavior in magnetic and electric fields. The quality of mass analysis is characterized by several technical specifications such as resolution, resolving power, m/z range and acquisition speed. The mass analyzers can be categorized according to resolution as low and high resolution mass spectrometers. Later on, another significant parameter has been also added that refers to mass accuracy which provides further information by determining the elemental formula of a certain analyte. There are many types of mass analyzers such as single mass spectrometers including quadrupole (Q) and time-of-flight (TOF), and tandem mass spectrometers including ion-trap (IT), triple quadrupole (QQQ), quadrupole time-of-flight (Q-TOF) and Fourier transform-ion cyclotron resonance (FT-ICR). The choice of proper mass analyzer depends on the performance, availability and cost-effectiveness of the instrument and the purpose of analysis [138].

- ***Single mass spectrometers***
  - ***Quadrupole type mass spectrometers:*** It include a set of quadrupole rods in which four cylindrical magnets are placed parallel and symmetrical to each other with the intent to separate ions based on m/z values. The separation is achieved by applying a fixed direct current (DC) and alternating radio frequency (RF) voltages. The potential difference can be varied to filter a specific mass range through the quadrupoles. This allows the continuous

detection of a specific m/z value which is defined as selected ion monitoring (SIM). The typical resolution of quadrupole mass analyzers is very low (generally one mass unit) and they have a mass range between 2.0 and 1100 m/z. These instruments have intrinsically lower cost and simple operation when compared to hybrid systems [114, 139].

- ***Time-of-flight instruments:*** This systems imply as high resolution mass spectrometers and they are very useful for the analysis of large molecules and biopolymers thanks to the unlimited mass range feature of this system. TOF instruments operate based on the acceleration of ions with the same kinetic energy that results in different velocities of ions of different m/z values. Thus, the accelerated ions travel a fixed distance from the ion source to the detector (usually 1.0 or 2.0 m) in different periods and thereby, strike the detector in order of increasing m/z values. TOF instruments provide the highest scanning speed, high mass accuracy (<3-5 ppm) and relatively low cost among other high resolution mass analyzers [138, 140].

➢ ***Tandem mass spectrometers***

- ***Ion trap mass spectrometers:*** This system uses a combination of RF and DC potentials that applied to the ring and end cap electrodes to trap the ions with a broad range of m/z. The trapped ions are ejected from the ion trap chamber according to their m/z values by increasing the amplitude of voltages. The ion trap analyzers allow sequential trapping and fragmentation of trapped ions with multiple stages of MS. The fragmentation is conducted by applying resonance excitation in the trapped chamber. In recent years, the development of linear ion trap systems has left the shortcomings of classical ion trap (3D) behind and improved the resolution, mass accuracy and the sensitivity of the system [141].
- ***Triple quadrupole mass spectrometers (QQQ):*** They serve as the most common mass spectrometers in routine laboratories regarding their great sensitivity in quantitative determinations of analytes. These instruments involve a linear series of three quadrupoles. The ions are filtered in the first

quadrupole, fragmented in the second quadrupole and the fragmented ions are filtered in the third quadrupole prior to reaching the detector. The first and third quadrupoles operate as mass filters while the second one as a collision cell. The collision of precursor ions is achieved by the application of a specific voltage and the introduction of argon, nitrogen or helium to the cell as the collision gas. This enables the operation of the instrument in Selected Reaction Monitoring (SRM) mode which reduces the noise and increase sensitivity. Moreover, the compounds can be identified using not only their masses but also by the identity of fragments [114].

- ***Fourier transform-ion cyclotron resonance mass spectrometers****:* They employ based on the cyclotron motion in a powerful electrical and magnetic field. A time-dependent current is produced by the ions followed by their excitation. The generated current is converted to m/z value by Fourier transform providing high resolution mass spectra. Despite the attractive features of this design including superior mass accuracy, high mass resolution and sensitivity, the high costs and low scan speed restrict its widespread use in analytical laboratories [142].
- ***Quadrupole time-of-flight mass spectrometers:*** They are hybrid instruments that combine the features of a QQQ with a TOF analyzer. This combination makes the hybrid system to exploit the advantages of both configurations which are high mass accuracy, high sensitivity and mass resolution in both molecular and fragment ions. The hybrid configuration includes the TOF analyzer instead of the third quadrupole in the QQQ systems. In its early times, it was considered as applicable for only qualitative purposes but the improvements made in recent years have proved its applicability in quantitative determinations with high resolution and fast acquisition [138, 142].

## 2.4 Quantitative Determinations

Routine clinical studies are mostly performed based on the immunoassay methods to obtain quantitative results for a variety of compounds in biological samples. Although many of immunoassay methods have been in-house developed methods, in the last years, automated analyzing systems and commercial kits have become more popular. However, the reliabilities of these methods in terms of accuracy and precision are under question in health care. Nowadays, LC-MS/MS has become an integral part of clinical research to achieve accurate quantitative results with the ability to prove the specificity. Therefore, the standard methods for LC-MS/MS are becoming valuable for the confirmation and quantitation of analytes in biological samples and the adoption of hyphenated systems to clinical research. Despite the superior advantage of LC-MS/MS systems, the widespread application of these systems seems to take some time regarding the need for a high level of expertise for data processing and method validation [143, 144].

### 2.4.1 Data Acquisition, Processing and Calibration

MS detection can be acquired by three different modes including full scan, SIM and SRM or multiple reaction monitoring (MRM). In full scan mode, a total ion chromatogram is obtained representing the total ions in each MS scan. This approach enables the analysis of almost all ionized compounds in an injection. The selectivity of full scan mode increases with narrowing the mass range. In SIM mode, the mass spectrometer scans a very narrow mass range (typically one mass unit) and only the ions in this mass range are detected enabling more specific data than full scan spectrum. As the last approach, MRM is the most sensitive transition and used for both quantification and confirmation purposes. MRM data is obtained by specifying the precursor (parent) and product (daughter) ions for the analyte of interest. This results in the monitoring of a characteristic fragment ion generated by a target ion and a very simple chromatogram with usually containing a single peak is obtained [145].

The data obtained from a typical mass spectrometer includes three dimensions: retention time, mass-to-charge ratio (m/z) and signal intensity. In the first part of

data processing, the intensity information of a compound is obtained by extracting the chromatogram for a given m/z value recorded as a function of retention time. Then, the analyte peak present in the extracted ion chromatogram is integrated to calculate the peak area for each measurement. The calculated peak areas are used for quantitative analysis. All the data obtained for quantitative analysis needs to be examined carefully for anomalies and checked for the following features [43, 144]:

- Repeatability and reproducibility of retention times
- Good peak symmetry and shape to check the detector saturation
- Clear blank injections and stable chromatographic baseline
- Consistent quantifier/qualifier value between injections
- Wide linearity of the calibration curve

There are several approaches for calibration in bioanalytical studies that result in different outputs on the levels of accuracy and precision. Therefore, the choice of proper calibration method is very crucial for reliable method development studies [43, 145].

#### 2.4.1.1 External calibration method

The external calibration is based on constructing a calibration curve by plotting the peak areas versus different concentrations of the analyte. This method is applied by preparing calibration solutions from pure analytical standards covering a proper range of concentration of the analyte. According to bioanalytical method guidelines, at least six calibration points including the blank measurement should be used to construct the calibration plot with at least three replicates for each point [146]. The calibration plot is specified within the linear range of the calibration curve giving a correlation coefficient ($R^2$) ≥ 0.99. When the detector reaches saturation, the calibration plot deviates from linearity. The concentration of the unknown sample is calculated using the linear equation generated from the calibration plot. This method is the simplest approach for the quantification of an analyte [147].

On the other hand, external standardization quite often results in poor accuracy and precision for mass spectrometry techniques. This is because that the instrumental response for a particular compound may vary within different time windows and

instrumental tune parameters. Moreover, the analyte losses caused by sample preparation, sample introduction and chromatograph are not compensated either. Nevertheless, the external standard method is still used to develop novel LC-MS/MS methods when a suitable internal standard is not available and acceptable validation results are obtained. In these situations, special care must be taken when analyzing different batches and biological samples [43, 148].

#### 2.4.1.2 Internal standard method

The internal standard (IS) method is the most common analytical method used for high throughput quantification of analytes and is known as a more effective method than the external standard method. This method relies on the addition of an internal material in a constant amount to the sample and calibration solutions at the earliest stage of an analytical procedure. The internal standard method is applied by constructing the calibration curve using the ratio of analyte response to internal standard response versus different concentrations of the analyte [147].

The internal standard approach aims to compensate instrumental and chromatographic variations, matrix effects and the analyte losses during sample processing. Herein, the selection of IS is very critical to achieve these goals. IS should exhibit similar properties to the analyte during the sample preparation and ionization process. However, in some circumstances, any slight difference in physical, chemical and chromatographic properties of the target compound may affect all processes and prevent the full compensation of experimental and instrumental errors. The other criterion for selecting proper IS is that it should not be present naturally in the sample matrix. Since different biological systems contain various endogenous substances at different levels, the utilization of the IS method to analyze biological samples may be very difficult. To prevent any potential overlap of endogenous substances with IS material in the analyzing process, non-endogenous molecules needs to be utilized as IS [149].

Apart from the above-mentioned criteria, the main problems appear when determining multiple compounds in one assay. In this case, the behavior of IS during the extraction and ionization process may not represent all target compounds and this may affect the accuracy and precision of analytical method adversely. This

problem can be overcome by the application of additional IS or by the analysis of compounds in separate assays [150].

#### 2.4.1.3 Matrix-matching and Standard Addition Methods

The matrix matching is a common practice for the analysis of very complex matrices like biological samples to obtain accurate results with LC-MS/MS. The concept of matrix matching is based on the preparation of calibration standards in the blank sample matrix. The blank matrices for biological samples are composed of a pooled source of the matrix (urine, plasma and etc.) and the real samples are assumed to have the same matrix effect. Unfortunately, the preparation of a sample matrix that resembles all real samples in a batch is not possible. In this case, the standard addition method can be applied by adding aliquots of working standard solution containing analyte proportionally increasing order [151].

The main purpose of matrix matching and standard addition strategies is to correct the matrix effect on the analyte originated from the competition of co-eluting molecules. Even if preventive actions are taken to reduce/minimize the matrix effect such as clean-up and extraction procedures, matrix effect may occur due to the remaining co-eluting species. The matrix effect can occur as either ion suppression or enhancement. In both situations, the accuracy and precision of the method are affected negatively. Thus, the matrix-matching and standard addition strategies are very useful to obtain good accuracy and precision [152].

### 2.4.2 Method Validation

Quantitative bioanalytical methods are developed and validated with the purpose of reliable clinical evaluation including diagnosis, new drug or biomarker investigations, clinical pharmacology, bioavailability, bioequivalence, toxicokinetic and pharmacokinetic studies. The validation studies are designed to declare the fitness of purpose and evaluated for the performance characteristics. The analytical performance parameters required for a method depend on the intended application of the method. In bioanalytical methods, the analytical parameters are required to define the design, operating conditions, limitations and applicability of the method

for the intended use. The key validation parameters for bioanalytical methods are given as follows [144, 146, 147]:

- Sensitivity
- Working Range
- Selectivity
- Accuracy
- Precision

Validation studies involve the documenting of analytical performance parameters for the use of laboratory investigations and ensure the reliability of the obtained quantitative data for the intended purposes [153].

#### 2.4.2.1 Precision

The precision of a method can be described as the variability observed within a group of determinations under defined conditions. The assessment of precision is performed by the repeated measurements of a sample. Precision can be expressed as the coefficient of variation (CV) or relative standard deviations (RSD) and defined as the normalized standard deviation (σ) to the mean of repeated measurements (μ), %CV, %RSD = (σ/μ) x100. There are two aspects of precision including repeatability and reproducibility. The repeatability serves to express the deviations under the same conditions (by a single analyst, same instrument and reagents) within a short time frame while reproducibility refers to the deviations under varying conditions such as inter-laboratory and inter-analysts [103, 154].

#### 2.4.2.2 Accuracy

Accuracy refers to the trueness of a method and describes the agreement between the experimental value and the true value of a measurement. The accuracy of a method can be assessed by the recovery studies, method comparison studies or analysis of certificated or standard reference materials (CRM/SRMs). Recovery experiments are performed by the analysis of spiked sample aliquots with known amounts of analyte.

The accuracy is evaluated by comparing the experimental quantitative value with the known amounts of analyte. As another approach, the method comparison

studies are conducted by comparing the experimental results of the new method with the results of a reference method. Lastly, the analysis of CRM/SRM is the most preferred way to assess the accuracy of a method. However, the production of CRM/SRMs is very limited now and so, it is not always possible to obtain a suitable one containing the target compounds in a certain matrix [154].

#### 2.4.2.3 Sensitivity

The definition of sensitivity by the IUPAC refers to the change of the response by the change of concentration of analyte [155]. According to this definition, the sensitivity of a method increases when a small change results in a big change in the response value. The sensitivity of a method is also described by the lowest concentration that can be measured with acceptable accuracy and precision levels.

The limit of detection (LOD) of a method can be determined by at least five replicate measurements of the lowest concentration that gives a reliably differentiated signal from the background noise (signal/noise, S/N>3.0). In the case of the calculation of limit of quantification (LOQ) for a method, S/N is expected to be greater than 10. The precision of these replicate measurements should be less than %20 [154, 156].

#### 2.4.2.4 Selectivity

The selectivity of a method is examined to show the ability of the method to determine the target analyte distinguishably from other interference substances in the sample matrix. The selectivity can be evaluated by the analysis of a blank sample of the biological matrix and the lack of any response at the retention time of analyte in sample matrix confirms the absence of interference on the analyte.

However, this approach is usually not applicable to endogenous compounds because of the absence of analyte-free biological matrices. In such cases, a second approach can be used that relies on the monitoring consistency of relative ratios of at least two transitions in tandem mass spectrometry. Even so, there is still a risk of interference caused by the closely-related endogenous compounds. As the final approach, the application of an accurate-mass spectrometer provides the ultimate proof of selectivity [103, 154].

### 2.4.3 Isotope Dilution Mass Spectrometry

In the hierarchy of analytical methods, isotope dilution mass spectrometry (IDMS) is regarded as a definitive method providing the most accurate results in quantitative analysis. The application of IDMS for organic analytes are carried out using mass spectrometry systems including GC-MS, LC-MS and LC-MS/MS. In these systems, many critical points that lead to quantitation errors including matrix effects, column deterioration, low recovery of the analyte, changes in detector response and analyst errors. IDMS provides a total compensation for all error sources caused by sample preparation through the instrumental analysis. All variables that result in a final experimental error can be assessed and controlled with considerable attention. This method is the most powerful approach for the analysis of biological samples to cope with the interferences caused by the complexity of the sample matrix and offers unchallenged precision and accuracy [3].

#### 2.4.3.1 History of IDMS

IDMS strategy has a history extending back over a hundred years which is older than the discovery of isotopes. As one of the earliest known applications, in 1896, zoologists have employed a technique that is similar to the basic principle of IDMS, to estimate plaice populations [157]. Later on, this technique has been called as "catch and release" method and used widely for the estimation of biological populations such as fish and bird populations. [158, 159]. An illustration of isotope dilution strategy in zoology is presented in Figure 2.6.

The catch and release method relies on the catching, marking and releasing of a group of individuals to a specified region. After waiting enough time to allow the dispersion of marked species in the specified environment, a second capture is carried out. The total population of the specified environment ($N_W$) can be estimated using the total number of first captured and marked species ($N_B$), the total number of second captured species (W) and the number of marked species in the second capture (B) ($N_W=N_B x W/B$). This approach enables to count the uncountable groups in a given area and more suitable for chemical applications than biological measurements [160].

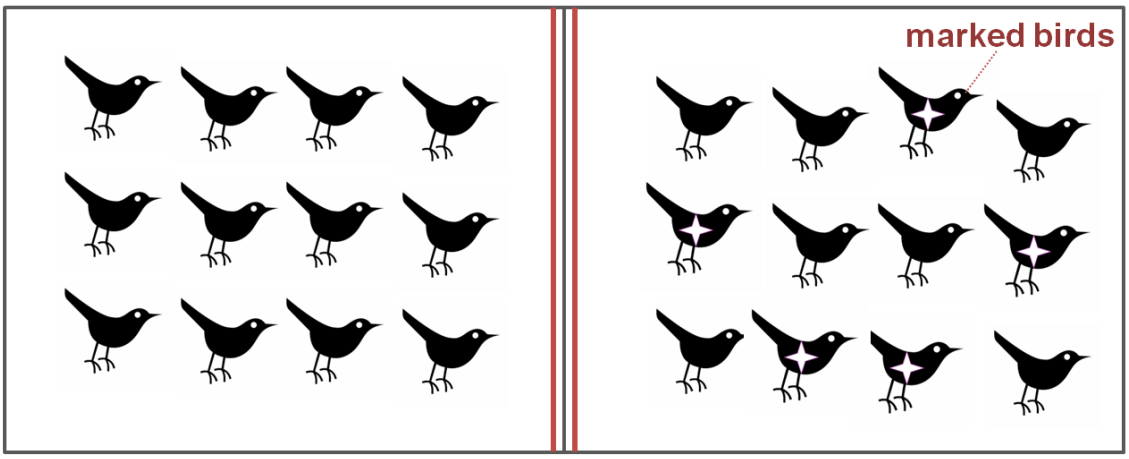



**Figure 2.6** An illustration of isotope dilution application in zoology

As the first chemical application, Hevesy and Paneth (1913) proposed the radiochemical isotope dilution technique to determine the solubility of lead sulphide using the radioactive isotope form of lead [161]. Several years later, with the development of mass spectrometry systems, inorganic and organic compounds were marked by enriched isotopes and the counting step was performed by adapting mass spectrometry to isotope dilution strategy. This adaption resulted in a combined technique called as isotope dilution mass spectrometry [160].

In 1939, Schoenheimer and Rittenberg were used IDMS technique to investigate the protein and amino acid metabolism in healthy animals using $^{15}N$-enriched amino acids [162]. In the late 1940s, the initial applications of IDMS for elemental analysis were introduced for determining carbon, oxygen, nitrogen and sulphur elements [163]. By the 1950s, IDMS was used to determine the isotope ratios with high accuracy and precision by applying thermal ionization mass spectrometry (TIMS) [164, 165]. The modern applications of IDMS started in the early 1990s with the development of hyphenated MS techniques and with the availability of isotopically enriched materials. Now, there are more than 8000 commercially available isotopically enriched material in the market [160, 166-168].

#### 2.4.3.2 Principle of IDMS

The principle of IDMS strategy is based on the use of an isotopically enriched (inorganic MS) or isotopically labeled (organic MS) form of analyte for the quantification of the natural form of the analyte. These isotopic materials are very useful to be used as internal standard as they have identical behaviors of natural

analytes during all analytical stages and they can be distinguished clearly by their mass difference in mass spectrometry.

IDMS procedure is performed by adding accurately known amount of isotopic material to the sample and measuring the consequent isotope amount ratio in the sample blends. This blend ratio is directly related to the amount of analyte in the sample [169]. The principle of IDMS is shown schematically in Figure 2.7 for a compound containing two different isotopes. As shown here, the isotopic composition of a sample is altered intentionally by spiking with an isotopically enriched material. At the beginning, the most abundant isotope in the sample is isotope 1 while the spike is enriched with isotope 2. After spiking of sample, the isotopic ratio of final mixture is expected to be intermediate with the sample and spike. This relation can be expressed by isotope dilution equations [170].

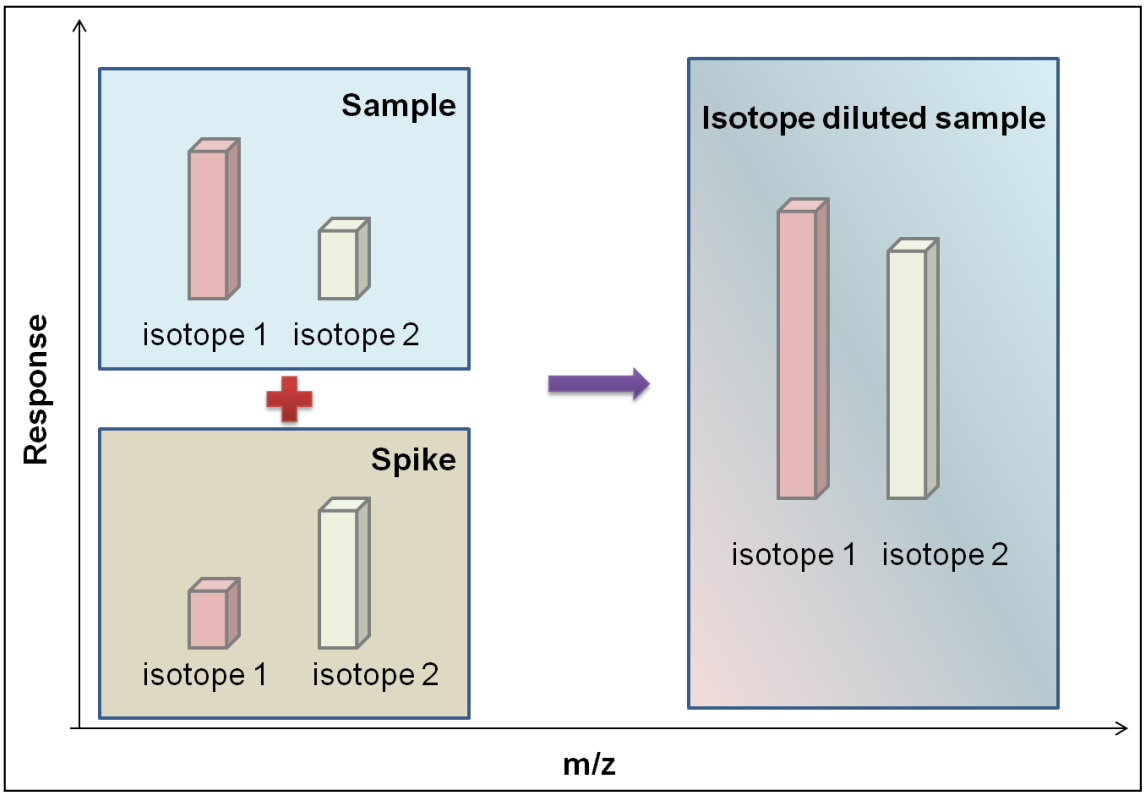



**Figure 2.7** A schematic illustration of IDMS principle

#### 2.4.3.3 Isotope dilution equation

The different equation forms can be used to express the concept of isotope dilution. In this study, the formulations reported by Pagliano et al. are used [171]. If the amount of analyte in the sample is represented with $n_A$ and $n_B$ for the same analyte in the spike, the amount of analyte in the final mixture $n_{AB}$ can be expressed as:

$$n_{AB} = n_A + n_B \tag{2.7}$$

The mass balances for natural (x) and isotopic form (y) of analyte can be also obtained as:

$$n_{AB}{}^{x} = n_A{}^{x} + n_B{}^{x} \tag{2.8}$$

$$n_{AB}{}^{y} = n_A{}^{y} + n_B{}^{y} \tag{2.9}$$

The isotopic ratio of the final mixture ($R_{AB}$) can be obtained via dividing equation (2.8) by equation (2.9):

$$R_{AB} = \frac{n_{AB}{}^{x}}{n_{AB}{}^{y}} = \frac{n_A{}^{x} + n_B{}^{x}}{n_A{}^{y} + n_B{}^{y}} = \frac{n_A i_A{}^{x} + n_B i_B{}^{x}}{n_A i_A{}^{y} + n_B i_B{}^{y}} \tag{2.10}$$

where $i^x$ and $i^y$ represent the isotope abundances of natural (x) and isotopic forms (y) of the analyte. After rearranging of equation (2.10), $n_A$ is obtained as:

$$n_A = n_B \left( \frac{i_B{}^{x} - R_{AB} i_B{}^{y}}{R_{AB} i_A{}^{y} - i_A{}^{x}} \right) \tag{2.11}$$

Equation 2.11 represents the basic form of isotope dilution but further arrangements are required to simplify the equation by eliminating $i^x$ and $i^y$:

$$n_A = n_B \left( \frac{R_B - R_{AB}}{R_{AB} - R_A} \right) \frac{\sum R_A}{\sum R_B} \tag{2.12}$$

The equation (2.12) can be also written in the form of mass fraction:

$$\omega_A = \omega_B \frac{m_{B(AB)}}{m_{A(AB)}} \left( \frac{R_B - R_{AB}}{R_{AB} - R_A} \right) \frac{\sum R_A}{\sum R_B} \frac{M_A}{M_B} \tag{2.13}$$

where $\omega_A$ and $\omega_B$ represent the mass fractions of the analyte in the sample and spike, respectively while $m_{A(AB)}$ and $m_{B(AB)}$ represent the masses of aqueous solution of sample and spike required to prepare the blend, respectively.

In the most of mass spectrometry systems, the measured isotope ratio (r) is recorded as proportional to theoretical isotope ratio (R) and so, the measured ratio can be linked to the theoretical ratio using mass bias calibration factor (K) with the following equation:

$$R = K \times r \tag{2.14}$$

However, it should be noted that this equation may not be valid for all measurement devices. There are numerous practical challenges for further improvement of the isotope dilution equation that obviates some of the variables in the equation. The improved variations of isotope dilution include single ($ID^1MS$), double ($ID^2MS$), triple ($ID^3MS$), and quadruple ($ID^4MS$) dilutions [171].

#### 2.4.3.4 Single dilution

Single dilution ($ID^1MS$) is the simplest variation of isotope dilution strategy which requires the exact knowledge of the isotopic composition of the spike beforehand. In this variation, the minimum number of variables ($r_{AB}$) is measured while the majority of variables are known ($r_{AB}$, $\omega_B$, K, $M_A$, $M_B$, $\Sigma R_A$ and $\Sigma R_B$) in advance:

$$\omega_A = \omega_B \frac{m_{B(AB),aq}}{m_{A(AB),aq}} . \frac{R_B - K.r_{AB}}{K.r_{AB} - R_A} . \frac{\sum R_A}{\sum R_B} . \frac{M_A}{M_B} \tag{2.15}$$

Herein, the equation (2.14) is integrated with equation (2.15) and for the identical measurement ratios, the mass bias factor cancels out giving the following equation:

$$\omega_A = \omega_B \frac{r_B - r_{AB}}{r_{AB} - r_A} . \frac{m_{B(AB),aq}}{m_{A(AB),aq}} . \frac{\sum R_A}{\sum R_B} . \frac{M_A}{M_B} \tag{2.16}$$

The final equation (2.16) represents the origin of all isotope dilution methods. This equation requires the exact knowledge of the mass fraction of spike ($\omega_B$), the isotopic composition of the spike ($r_B$) and the isotopic composition of the sample ($r_A$). It also includes many other unknown variables such as $M_A$, $M_B$, $\Sigma R_A$ and $\Sigma R_B$. As can be deduced from the final equation, the quantification of an analyte in the

sample applying single dilution strategy is carried out using isotopic material as the primary calibrator. Since it is often difficult to obtain information about the chemical purity of isotopic standard with high accuracy, further improvements on the single dilution method are introduced [14, 171].

#### 2.4.3.5 Double dilution

The double dilution method ($ID^2MS$) is also known as "reverse dilution" which refers to the use of a second blend to eliminate the mass fraction of spike ($\omega_B$) from the isotope dilution equation. The potential problems in the traditional isotope dilution method arising from the availability of enriched materials in very small quantities and the unequal chemical purity of enriched and natural materials can be effectively overcome by the double dilution design. This can be achieved by combining two isotope dilution equation using the same spike solution ($\omega_B$) in two dilutions. Herein, natural primary standard (A*) can be used to obtain $\omega_B$ with the following equation:

$$\omega_B = \omega_{A^*} \cdot \frac{R_{A^*} - R_{A^*B}}{R_{A^*B} - R_B} \cdot \frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}} \cdot \frac{\sum R_B}{\sum R_{A^*}} \cdot \frac{M_B}{M_{A^*}} \qquad (2.17)$$

By embedding the equation (2.17) to the traditional isotope dilution equation (2.16), the double dilution formula is obtained as:

$$\omega_A = \omega_{A^*} \cdot \frac{R_{A^*} - R_{A^*B}}{R_{A^*B} - R_B} \cdot \frac{R_B - R_{AB}}{R_{AB} - R_A} \cdot \frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}} \cdot \frac{m_{B(AB),aq}}{m_{A(AB),aq}} \frac{\sum R_A}{\sum R_{A^*}} \cdot \frac{M_A}{M_{A^*}} \qquad (2.18)$$

As a result, two variables, $\omega_B$ and $\Sigma R_B$ cancel out and this reveals the first significant advantage of double spiking strategy. A second advantage can be attained by assuming the isotopic compositions of analyte in the sample and standard as identical ($R_A = R_{A^*}$ and $M_A = M_{A^*}$, which is always the case) and that provides the simplification of equation (2.18) as:

$$\omega_A = \omega_{A^*} \cdot \frac{R_A - R_{A^*B}}{R_{A^*B} - R_B} \cdot \frac{R_B - R_{AB}}{R_{AB} - R_A} \cdot \frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}} \cdot \frac{m_{B(AB),aq}}{m_{A(AB),aq}} \qquad (2.19)$$

In practice, the identical mass bias factor can be applied to the equation (2.19) by measuring all isotope amount ratios in the same sequence and the formula becomes:

$$\omega_A = \omega_{A^*}.\frac{r_A - r_{A^*B}}{r_{A^*B} - r_B}.\frac{r_B - r_{AB}}{r_{AB} - r_A}.\frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}}.\frac{m_{B(AB),aq}}{m_{A(AB),aq}} \quad (2.20)$$

Additionally, the exact matching can be applied to double dilution to redesign the formula by taking $r_A = r_{A^*}$, i.e.;

$$\frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}}.\frac{m_{B(AB),aq}}{m_{A(AB),aq}} = \frac{m_{A^*(A^*B),aq}}{m_{A(A^*B),aq}}.\frac{m_{B(AB),aq}}{m_{B(AB),aq}} \approx 1 \quad (2.21)$$

$$\omega_A \approx \omega_{A^*} \quad (2.22)$$

The exact matching design is based on the addition of natural standard and spike at the equal amounts of analyte in the sample. This design makes all isotope ratio measurements irrelevant to the final value ($\omega_A$). Moreover, the value of $r_B$ is not required to be known with high precision [171].

#### 2.4.3.6 Triple dilution

Triple dilution ($ID^3MS$) is an alternative approach to the exact matching double dilution strategy for the indirect measurement of $r_B$ value. This can be achieved by augmenting double dilution with an additional blend. Thereby, two isotope dilution equations get involved in the formula design:

$$\omega_A = \omega_{A^*-1}.\frac{r_A - r_{A^*B-1}}{r_{A^*B-1} - r_B}.\frac{r_B - r_{AB}}{r_{AB} - r_A}.\frac{m_{A^*(A^*B-1),aq}}{m_{B(A^*B-1),aq}}.\frac{m_{B(AB),aq}}{m_{A(AB),aq}} \quad (2.23)$$

$$\omega_A = \omega_{A^*-2}.\frac{r_A - r_{A^*B-2}}{r_{A^*B-2} - r_B}.\frac{r_B - r_{AB}}{r_{AB} - r_A}.\frac{m_{A^*(A^*B-2),aq}}{m_{B(A^*B-2),aq}}.\frac{m_{B(AB),aq}}{m_{A(AB),aq}} \quad (2.24)$$

where A*B-1 and A*B-2 represent the additional blends of spike and natural standard. By combining these equations, $r_B$ can be removed from the final formula:

$$\omega_A = \frac{m_{A^*-2} r_1 + m_{A^*-1} r_2}{r_3}.\frac{m_{B(AB),aq}}{m_{A(AB),aq}} \quad (2.25)$$

where

$$m_{A^*-i} = \omega_{A^*-i}\frac{m_{A^*(A^*B-i),aq}}{m_{B(A^*B-i),aq}} \quad (2.26)$$

$$r_1 = (r_{AB} - r_{A^*B-1})(r_A - r_{A^*B-2}) \quad (2.27)$$

$$r_2 = (r_{AB} - r_{A^*B-2})(r_{A^*B-1} - r_A) \quad (2.28)$$

$$r_3 = (r_{AB} - r_A)(r_{A^*B-1} - r_{A^*B-2}) \quad (2.29)$$

This model equation of triple dilution is designed according to the use of two blends of standard and spike (A*B-1 and A*B-2), and the single blend of sample and spike (AB). In the meantime, the model can be also designed based on the use of single blend of A*B and two blends of AB. In that case, the following formulations are obtained:

$$\omega_A = \frac{r_{3^*}}{m_{A-2}r^*_1 + m_{A^*-1}r^*_2} \cdot \frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}} \quad (2.30)$$

where

$$m_{A-i} = \frac{1}{\omega_{A^*}} \frac{m_{A(AB-i),aq}}{m_{B(AB-i),aq}} \quad (2.31)$$

$$r_{1^*} = (r_{A^*B} - r_{AB-1})(r_A - r_{AB-2}) \quad (2.32)$$

$$r_{2^*} = (r_{A^*B} - r_{AB-2})(r_{AB-1} - r_A) \quad (2.33)$$

$$r_{3^*} = (r_{A^*B} - r_A)(r_{AB-1} - r_{AB-2}) \quad (2.34)$$

The exact matching design can be also applied to triple dilution strategy and this is achieved with a similar approach (equation 2.21 and 2.22) as in the exact matching double dilution. Despite the similar underlying principle in exact matching double dilution and exact matching triple dilution, the conceptual difference between these two strategies cannot be ignored. In double dilution, $r_B$ is excluded numerically from the formula with an effective experimental approach. On the other hand, in triple dilution, $r_B$ is not present as a variable in the design because it is removed analytically by expanding the complexity of measurement [171, 172].

#### 2.4.3.7 Quadruple dilution

Quadruple dilution (ID$^4$MS) provides an additional elimination of the isotopic composition of primary standard ($r_A$). In molecular spectrometry, the direct measurement of $r_A$ is not always possible, especially for the minor isotopes ($r_A$>50).

In such case, the elimination of mass bias correction in equations of isotope dilution designs is needed and this can be achieved by only measuring all isotopic amount ratio values in the equation (2.16). Therefore, quadruple dilution is applied with the combination of equations in three double dilutions. There are three different ways for designing quadruple dilution: (i) utilizing three blends of AB and one blend of A*B, ii) utilizing two blends of AB and two blends of A*B and iii) utilizing one blend of AB and three blends of A*B. By assuming $R_A$=$R_{A^*}$, the following equation is obtained to represent the quadruple dilution model:

$$\omega_A = -\frac{m_{A^*-2}m_{A^*-3}r_4 + m_{A^*-1}m_{A^*-3}r_5 + m_{A^*-1}m_{A^*-2}r_6}{m_{A^*-1}r_4 + m_{A^*-2}r_5 + m_{A^*-3}r_6} \cdot \frac{m_{B(AB),aq}}{m_{A(AB),aq}} \quad (2.35)$$

where

$$m_{A^*-i} = \omega_{A^*-i}\,\frac{m_{A^*(A^*B-i),aq}}{m_{B(A^*B-i),aq}} \quad (2.36)$$

$$r_4 = (r_{AB} - r_{A*B-1}) \cdot (r_{A*B-2} - r_{A*B-3}) \quad (2.37)$$

$$r_5 = (r_{AB} - r_{A*B-2}) \cdot (r_{A*B-3} - r_{A*B-1}) \quad (2.38)$$

$$r_6 = (r_{AB} - r_{A*B-3}) \cdot (r_{A*B-1} - r_{A*B-2}) \quad (2.39)$$

The exact matching can be also applied in quadruple dilution using the same approach with other variations ($r_{AB}$=$r_{A*B-2}$). In this design, all variables are obtained indirectly by measuring the sample and standard blends [18, 171].

#### 2.4.3.8 The advantages of IDMS

IDMS is regarded as the most accurate and highly qualified strategy of modern quantitative analysis. In IDMS, superior accuracy is achieved when the sample is homogeneous and the complete equilibrium between the analyte and its isotopic analogue is reached. The main advantages include [4]:

- Once isotopic equilibrium is reached, the variations in the sensitivity of the instrument including signal drift and matrix effect do not influence on the quantitative result.

- After complete equilibration, any possible losses of analyte during sample preparation have also no influence on the result.
- There is no requirement for the detailed evaluation of total recovery, dilution factor or preconcentration factor of analyte in every step of the analytical procedure because the amount of analyte is determined by measuring the ratio between its natural and isotopic form.
- The uncertainty is definable and only depends on the measurement of isotopic ratio values. The accuracy of the method is directly related to these measurements which are performed by mass spectrometers with high precision.
- Considering the complex matrices, the operational procedure of IDMS is less time consuming and provides greater accuracy when compared to other classical calibration strategies such as external calibration and standard addition. Unlike classical strategies, there is no need to conduct any external calibration or standard additions to the sample matrix.

#### 2.4.3.9 The disadvantages of IDMS

Despite all the advantages, there are some disadvantages of IDMS methodology which include:

- It requires mass spectrometry systems that have a higher cost when compared to spectrophotometry systems.
- Specifically trained personnel are essential to perform the whole procedure.
- The availability of isotopic standards is still limited and most of them have high costs.
- The isotopic equilibration has to be guaranteed. Otherwise, the measured isotopic ratio will not represent the accurate value.

# 3
# ERECTILE DYSFUNCTION DRUGS

## 3.1 General Information

Erectile dysfunction (ED) is defined as the inability to attain or maintain an erection sufficient for satisfactory sexual activity [173]. ED primarily affects men older than 40 years old with a prevalence of 52% [174] and has been predicted to affect 322 million men by the year 2025 [175]. The impotence of ED reported to be 75% physical but neurogenic, psychogenic, or endocrinologic basis are still present in many cases [176]. Both experimental and clinical studies have led to the emergence of effective medical therapies concerning the deeper understanding of the pathophysiology of ED. In 1998, the discovery of phosphodiesterase-5 (PDE-5) inhibitors introduced oral therapy as a treatment option for ED [177]. PDE-5 is an enzyme that selectively cleaves cyclic guanosine monophosphate (cGMP) to 5'-guanosine monophosphate (5'-GMP) and is located in the smooth muscle of the corpus cavernosum. Herein, PDE-5 inhibitors are structurally similar to cGMP and competitively bind to PDE-5 to prevent cGMP hydrolysis which result in the maintenance of erection [178].

Sildenafil citrate (Viagra®) was the first approved PDE-5 inhibitor by The United States Food and Drug Administration (FDA) in 1998 [179] followed by tadalafil (Cialis®) [180] and vardenafil hydrochloride (Levitra®) [181] approval in 2003. In addition to these drugs, avanafil (Stendra®) has recently been developed and approved by the FDA in 2012 [182]. The chemical structures of approved PDE-5 inhibitors are presented in Figure 3.1. Besides that, a number of PDE-5 inhibitors have been developed and marketed in many countries [183]. All of these drugs have been developed based on the nitric oxide (NO) role in smooth muscle relaxation of penile cavernous tissues. Herein, NO-cGMP pathway plays a fundamental role in the physiology of erection and smooth muscle relaxation in corpora cavernosa (CC)

[184]. Therefore, ED drugs aim to preserve and strengthen the activity of the NO-cGMP pathway by preventing the breakdown of cGMP [185].

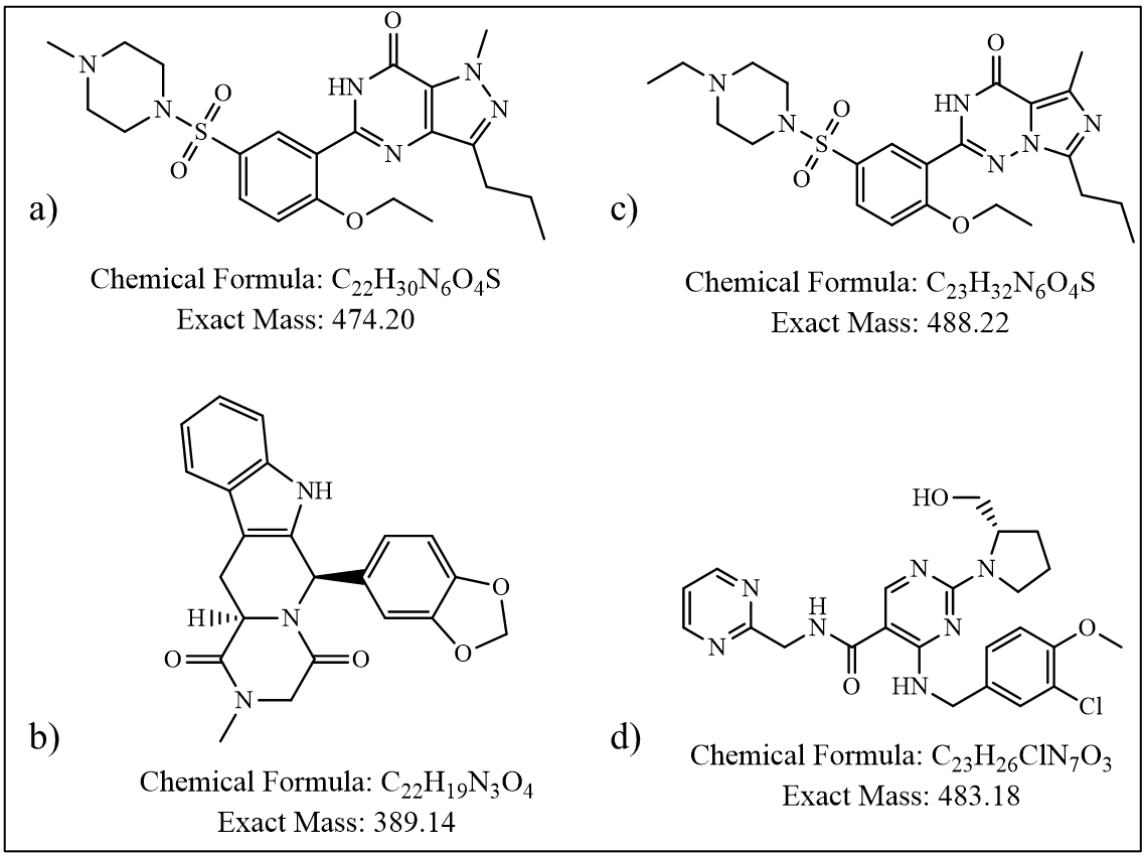



**Figure 3.1** Chemical structures of a) sildenafil, b) tadalafil, c) vardenafil and d) avanafil

### 3.1.1 Mechanism of action

Penile erection normally results from the relaxation of the corpus cavernosum smooth muscles which is mediated by spinal reflex [186]. The dynamic balance between the contraction of smooth muscle and relaxation of vascular components determines the contractile tone of the penis [187]. In the healthy male, sexual stimulation starts with the activation of muscarinic receptors in the endothelial cells by acetylcholine and that leads to the release of NO. NO diffuses into the cytoplasm of smooth muscle cells and activates guanylyl cyclase [188]. Hereby, the increase of cGMP result in muscle relaxation and vasodilation of the penis [188]. PDE-5 inhibitors modulate the duration and intensity of intracellular response in the corpus cavernosum of the penis by catalyzing the hydrolysis of cGMP to its inactive form [189]. This action leads to the increasing concentration of cGMP result in the

vasodilation and antiproliferative effect on the smooth muscle cells [188]. On the other hand, PDE-5 is also found in the pulmonary tissue, heart, renal tubules and platelets and its inhibition also leads to positive cardiovascular and pulmonary effects for hypertension, pulmonary arterial hypertension and coronary artery disease [178].

### 3.1.2 Risk factors

Sildenafil, tadalafil, vardenafil and avanafil are efficacious PDE-5 inhibitors for the treatment of erectile dysfunction disorder. They are considered as first-line therapy and have a similar mechanism of action [188]. They differ from each other with their pharmacokinetics and are selected based on clinical effects and patient requirements [190]. The daily dosages of sildenafil, tadalafil, vardenafil and avanafil are 25-100 mg/day, 5-20 mg/day, 5-20 mg/day and 50-200 mg/day, respectively [178]. ED drugs have common side effects including headache, flushing, dyspepsia, visual abnormalities, back pain and nasal congestion [190]. Furthermore, they are not recommended for people who have low blood pressure unstable angina, severe cardiac failure and severe liver impairment or dialysis patient. Additionally, men who have a stroke or myocardial infarction within the last 6-8 weeks should not take one of these drugs [191].

### 3.1.3 Counterfeit PDE-5 inhibitors

Counterfeiting of pharmaceutical drugs poses permanently growing problem since the first cases were reported in about 1990 [192]. The estimated global trade for counterfeit medicines ranges between 75-200 billion dollars [193]. The identity and ingredients of these drugs are deliberately and fraudulently mislabeled with fake packaging and usually do not contain the correct active ingredients or the correct dosage of the ingredients [194]. They may lead to serious even cause death about their poor product quality (e.g. toxic elements, impurities and contaminants) and user characteristics (e.g. contra-indications) [195].

Among counterfeit drugs, counterfeit PDE-5 inhibitors are a common target for manufacturers due to the high cost of prescription drugs and the embarrassment by the condition [190]. Accordingly, 35.8 million counterfeit sildenafil tablets were

intercepted between 2004 and 2008, and the amount of illicit sildenafil exposure is comparable with the legal use of sildenafil in Europe [190]. The sildenafil concentrations of these illicit samples were reported as only 10% of the samples contained active ingredients within 10% of advertised ingredients on samples [193]. Therefore, it is quite difficult to estimate the actual exposure of illicit PDE-5 inhibitors.

Undoubtedly, the most significant risk factor of counterfeit PDE-5 inhibitors is their unregulated content that may change their side-effect profile, effectiveness and severity of drug interactions [193]. Furthermore, the users of counterfeit PDE-5 inhibitors bypass the legitimate healthcare system that results to fail in the diagnosis of other medical comorbidities associated with erectile dysfunction including cardiovascular disease, diabetes, hypertension, hyperlipidemia and metabolic syndrome [193]. Given these risk factors, a strict regulation and control of the counterfeit drugs must be considered and applied for the detection of illicit ingredients.

### 3.1.4 Analytical methods for the determination of PDE-5 inhibitors

Until now, several analytical methods have been developed for the determination of PDE-5 inhibitors and sometimes their analogues in several different matrices including biological, environmental and food matrices. Analytical methods to determine PDE-5 inhibitors described in the literature are summarized in Table 3.1. The most used instrumental techniques are HPLC with various types of detectors such as UV-Vis, electrochemical detector (ECD), diode array detector (DAD) and charged aerosol detector (CAD), GC-MS, GC-MS/MS, LC-MS and LC-MS/MS.

In a study conducted by Weinmann and coworkers, post-mortem detection of sildenafil and its metabolites in urine was proposed using LC-MS and MS/MS systems. In this study, sildenafil and its three metabolites were detected in a urine sample taken from a volunteer who had ingested 25 mg sildenafil, 8.0 h ago before the collection [196]. In another study conducted by Zou and coworkers, synthetic PDE-5 inhibitors and their analogues including sildenafil, tadalafil, homosildenafil, acetildenafil, vardenafil and hyroxyhomosildenafil were screened in dietary

supplements and, LC-ESI-MS/MS and HPLC-DAD systems were used to the determination of these compounds. The detection limits were ranged between 0.9-2.0 and 0.02-0.21 μg $mL^{-1}$ for UV and MS detectors, respectively [197]. Later on, the detection of synthetic PDE-5 inhibitors in dietary supplements, herbal medicines, counterfeit drugs and illicit products was studied by several researchers [198-202]. In these studies, the detection limits of compounds were obtained at ng $mL^{-1}$ levels for LC-MS/MS methods while at μg $mL^{-1}$ for GC-MS/MS and HPLC methods. These studies concluded that sildenafil and tadalafil were predominantly and illegally involved in unapproved medicines, dietary supplements and other illicit products.

The synthetic PDE-5 inhibitors were also determined in biological samples including plasma, hair and breast milk samples. Al-Ghazawi and coworkers reported the determination of sildenafil and N-desmethyl sildenafil in human plasma using the HPLC-ECD method [203]. Four years later from this study, Bartosova and coworkers also studied the determination of sildenafil and and N-desmethyl sildenafil along with vardenafil and N-desethyl vardenafil in plasma with HPLC-ECD [204]. The detection limits for the studied compounds were comparable within two studies and ranged between 2.0-4.0 ng $mL^{-1}$.

Lee and coworkers studied on the determination of five erectile drugs including sildenafil, vardenafil, tadalafil, udenafil, mirodenafil and their selected metabolites in human and rat hairs. In this study, the samples were prepared by solid phase extraction using C18 mixed-mode cartridges and analyzed using LC-MS/MS system. LODs of target compounds in this study were varied between 0.05-1.0 ng/10 mg hair [205]. In another study, Wollein and coworkers investigated the transfer of sildenafil into breast milk of a lactating woman. Sildenafil and its metabolite (N-desmethyl sildenafil) was reported in breast milk as the first time with this study [206].

The determination of PDE-5 inhibitors were also studied in environmental such as waste water and sewage sludge samples. Nieto and coworkers proposed the determination of three PDE-5 inhibitors including sildenafil, tadalafil and vardenafil in wastewater and sewage sludge samples. For this purpose, solid phase extraction and pressurized liquid extraction methods were used for the preparation of real samples and prepared samples were analyzed with LC-MS/MS system. The

proposed method provided the detection of analytes with LOD values between 1.0-3.0 ng $g^{-1}$. This was the first study that reported the presence of sildenafil in environmental samples at low ng $g^{-1}$ levels [207].

Causanilles and coworkers also studied the determination sildenafil, tadalafil and vardenafil compounds in wastewater samples. They applied the direct injection method and used LC-MS/MS for the analysis. LOD values of the developed method were reported between 1.8-7.2 ng $L^{-1}$ [208].

**Table 3.1** Analytical methods described in the literature to determine PDE-5 inhibitors

| Ref., Year | PDE-5 inhibitors and analogues | LOD/LOQ | Preconcentration procedure | Instrumental technique | Sample |
|---|---|---|---|---|---|
| **[196], 2001** | sildenafil | 14/25 ng $mL^{-1}$ | solid phase extraction | LC-MS/MS | urine |
| **[197], 2006** | sildenafil | 1.3/4.5 μg $mL^{-1}$<br>0.03/0.1 μg $mL^{-1}$ | - | HPLC-UV<br>LC-MS/MS | dietary supplements |
| | tadalafil | 0.9/3.2 μg $mL^{-1}$<br>0.21/0.7 μg $mL^{-1}$ | | | |
| | vardenafil | 2.0/7.0 μg $mL^{-1}$<br>0.02/0.07 μg $mL^{-1}$ | | | |
| **[203], 2007** | sildenafil | 3.448/7.858 ng $mL^{-1}$ | - | HPLC-ECD | human plasma |
| **[209], 2009** | sildenafil | 0.5 ng $mL^{-1}$ | - | LC-MS | plasma |
| **[207], 2010** | sildenafil | 1.0/3.0 ng $g^{-1}$ | solid phase extraction | LC-MS/MS | waste water, sewage sludge |
| | tadalafil | 3.0/5.0 ng $g^{-1}$ | | | |
| | vardenafil | 2.0/5.0 ng $g^{-1}$ | | | |

**Table 3.1** Analytical methods described in the literature to determine PDE-5 inhibitors (continues)

| Ref., Year | PDE-5 inhibitors and analogues | LOD/LOQ | Preconcentration procedure | Instrumental technique | Sample |
|---|---|---|---|---|---|
| **[204], 2011** | sildenafil | 3.0/7.7 ng $mL^{-1}$ | - | HPLC-ECD | plasma |
| | vardenafil | 3.0/9.3 ng $mL^{-1}$ | | | |
| **[198], 2013** | sildenafil | 0.15/0.5 ng $mL^{-1}$ | - | LC-MS/MS | illicit erectile dysfunction products |
| | tadalafil | 0.06/0.23 ng $mL^{-1}$ | | | |
| | vardenafil | 0.014/0.046 ng $mL^{-1}$ | | | |
| **[199], 2014** | vardenafil, sildenafil, tadalafil | 1.0-5.0/5.0-100 ng $g^{-1}$ | - | LC-MS/MS | herbal medicines and dietary supplements |
| **[200], 2014** | tadalafil | 0.1/0.3 μg $mL^{-1}$ | - | LC-MS | supplements for women sexual desire enhancement |
| **[205], 2015** | sildenafil | 0.5/1.0 ng (10 mg hair)$^{-1}$ | solid phase extraction | LC-MS/MS | hair |
| | tadalafil | 1.0/1.0 ng (10 mg hair)$^{-1}$ | | | |
| | vardenafil | 0.1/2.5 ng (10 mg hair)$^{-1}$ | | | |

**Table 3.1** Analytical methods described in the literature to determine PDE-5 inhibitors (continues)

| Ref., Year | PDE-5 inhibitors and analogues | LOD/LOQ | Preconcentration procedure | Instrumental technique | Sample |
|---|---|---|---|---|---|
| **[201], 2016** | sildenafil | 0.1/0.03 μg $g^{-1}$ | - | GC-MS/MS | dietary supplements |
| **[202], 2016** | sildenafil | 28/92 ng $mL^{-1}$ | - | HPLC-UV | illicit/counterfeit drugs, and wastewater samples |
| **[208], 2016** | sildenafil | 1.8/6.0 ng $L^{-1}$ | - | LC-MS/MS | waste water |
| | tadalafil | 2.3/7.5 ng $L^{-1}$ | | | |
| | vardenafil | 7.2/24 ng $L^{-1}$ | | | |
| **[206], 2016** | sildenafil | 1.64/4.49 ng $mL^{-1}$ | solid phase extraction | LC-MS/MS | breast milk of a lactating woman |

## 3.2 Experimental Study

### 3.2.1 Chemicals and reagents

Chemicals and reagents used in this study were of high-purity grade. The preparations and dilutions of standards were made with ultrapure water obtained from a Milli-Q instrument. Acetonitrile, methanol, ammonium formate and formic acid were purchased from Merck. Citric acid, ammonia, ammonium iron (II) sulfate hexahydrate [$(NH_4)_2Fe(SO_4)_2.6H_2O$] and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were purchased from Sigma-Aldrich. Sildenafil, tadalafil and avanafil analytical standards were obtained from Toronto Research Chemicals while vardenafil was obtained from Santa Cruz Biotechnology. Zopiclone was supplied from Eczacıbaşı, Turkey and used as the internal standard. The stock solutions of individual analytes were prepared using their analytical standards at the concentration of 1,000 μg $g^{-1}$ and they were stored at +4.0 °C until use.

Isotopically labeled standards of tadalafil-d3 and sildenafil-d8 were supplied from Toronto Research Chemicals. Isotopically labeled vardenafil-d5 and avanafil-13C5-15N-d2 were obtained from Santa Cruz Biotechnology and AptoChem, respectively.

### 3.2.2 Instrumentation

An Agilent 1260 HPLC system consisted of a binary pump, a column oven, an autosampler and a degasser coupled to an Agilent 6530 QTOF-MS detector with an ESI source was utilized for method development and quantification studies. The accurate mass data obtained for precursor ions of molecules were processed using Agilent MassHunter 7.0 software. A photograph of the LC-MS system with a Q-TOF mass analyzer is represented in Figure 3.2.

An Agilent Poroshell 120 column (EC-C18, 3.0 × 150 mm, 2.7 μm) was used for chromatographic separation of four PDE-5 inhibitors and I.S. The parameters utilized for the detection of compounds in mass spectrometer is represented in Table 3.2.

Citric acid-coated magnetic nanoparticles were characterized using X-Ray Diffraction (XRD), Fourier-Transform Infrared Spectrophotometer (FTIR), Scanning

Electron Microscope (SEM) and Transmission Electron Microscope (TEM) images. Attenuated total reflection (ATR) - FTIR spectrum was taken in the wavelength range of 650-4000 $cm^{-1}$ using the Perkin Elmer Spectrum 100 instrument. XRD pattern of magnetic nanoparticles was performed using a PANalytical X'Pert Pro analyzer. The scanning angle was ranged between 10-90° with a rate of 3 $min^{-1}$ at 45 kV and 40 mA using a Cu-Kα radiation source.

A Zeiss EVO LS10 model SEM and a Jeol 2100 High-resolution TEM were used to take the images of magnetic nanoparticles and investigate their morphologies.

**Figure 3.2** A photograph of LC-MS system with a Q-TOF mass analyzer

**Table 3.2** Operating parameters of the LC-QTOF-MS method [210]

| | Parameter | Value |
|---|---|---|
| **Q-TOF MS** | Drying gas temperature | 300 °C |
| | Drying gas flow | 10 L $min^{-1}$ |
| | Nebulizer gas flow | 35 psig |
| | Capillary voltage | 4.0 kV |
| | Fragmentor voltage | 200 V |
| **LC** | Injection volume | 5.0 µL |
| | Flow rate | 0.55 mL $min^{-1}$ |
| | Column type | Agilent EC-C18, 3.0 × 150 mm, 2.7 µm |
| | Column temperature | 40 °C |
| | Mobile phase | A: 10 mM ammonium formate buffer at pH 4.6<br>B: 0.10% formic acid in acetonitrile |

### 3.2.3 Synthesis of Magnetic Nanoparticles

The synthesis of citric acid functionalized $Fe_3O_4$-MNPs was carried out by the modification of a previously reported procedure [211] that includes the co-precipitation route of $Fe^{2+}$ and $Fe^{3+}$ ions in alkaline conditions. The synthesis procedure comprised the dissolved amounts of 5.883 g of $(NH_4)_2Fe(SO_4)_2.6H_2O$ and 8.109 g of $FeCl_3.6H_2O$ in ultrapure water following heating to 80 °C. Then, ammonia solution (25%, 30 mL) and citric acid solution (3.0 mL of 1.0 g $mL^{-1}$ citric acid in ultrapure water) were added drop wise into the mixture while stirring. The final solution was kept at 80 °C for 2 hours under the nitrogen atmosphere. Upon the completion of the reaction, citric acid coated-MNPs (CA-MNPs) were settled at the bottom of the flask using a neodymium magnet and washed several times with ultrapure water to remove any remaining impurities. The synthesized MNPs were finally dried in an oven at 50 °C for 24 hours to be used in the preconcentration process [212].

### 3.2.4 Sample Preparation

Biological fluids including human plasma and urine samples were taken from healthy volunteers in our laboratory and, diluted in 1:10 ratio followed by filtration with a 0.45 μm membrane filter before use.

The magnetic solid phase microextraction process can be briefly explained as: 0.50 mL of buffer at pH 6.0 was added into the 8.0 mL of an aqueous solution containing selected analytes. After that, 100.0 mg of CA-MNP was added to the resulting solution and vortexed for 100 s to adsorb analytes onto its surface. Then, magnetic nanoparticles were collected by an external magnetic field and separated to another tube for the desorption of adsorbed molecules. The desorption step was carried out using 100 μL of acetonitrile as eluent phase. The magnetic separation was applied again to separate the eluent phase to a glass vial. The eluent containing target molecules were spiked with the internal standard at a final concentration of 1.00 μg $g^{-1}$ and analyzed. This process was repeated at least three times for each samples [212].

### 3.2.5 Optimization strategy for MSPE procedure

The influential parameters of the MSPE procedure including buffer pH, type of eluent and mixing type were optimized using one variable-at-a-time strategy to obtain maximum extraction efficiency. Other variables including amounts of eluent, buffer and sorbent material were optimized with a response surface methodology called as Box-Behnken design. The evaluation of experimental data resulted from the averages of triplicate analysis was processed using the trial version of Design Expert 7.0 software [212].

### 3.2.6 Preparation of blends for ID$^4$MS application

The calibration and standard blends were prepared separately for the application of the ID$^4$MS strategy before and after the extraction process. The calibration was conducted according to the exact matching strategy (Equation 2.35) reported by Pagliano and coworkers [171]. Accordingly, three calibration blends having 0.5, 1.0 and 2.0 isotopic ratios (A*B-1, A*B-2 and A*B-3, respectively) and the sample blend having 1.0 isotopic ratio (AB-1) were utilized for the quantitative determination of

the sample blend. All preparations were adjusted carefully to keep the final volume of blends equal to each other. The purpose of this adjustment was to simplify the final calculation by ensuring the same preconcentration factor for all blends [213].

## 3.3 Results and Discussion

### 3.3.1 Characterization Study

FTIR spectra of citric acid and CA-MNPs are represented in Figure 3.3. The stretching vibrations of hydroxyl groups were presented at 3368 $cm^{-1}$ with a broad peak and the stretching vibrations of C=CH-H were presented with the peaks at 3185 and 3055 $cm^{-1}$. The peaks 1587 and 1408 $cm^{-1}$ originating from symmetric and asymmetric C-O bonds proved the presence of citric acid on the surface of iron oxide [212].

The confirmation of the crystalline structure of magnetic iron oxide was performed by XRD pattern. As illustrated in Figure 3.4, the reflections of inverse cubic spinel structure of iron oxide at (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0) planes were displayed at 2θ values of 30.51°, 35.59°, 43.17°, 52.81°, .43° and 63.57°. The average size of nanoparticles was calculated as 35.5 ± 2.9 nm using Debye-Scherrer equation. SEM and TEM images are presented in Figure 3.5 also confirmed the nanosized and spherical structures of nanoparticles [212].

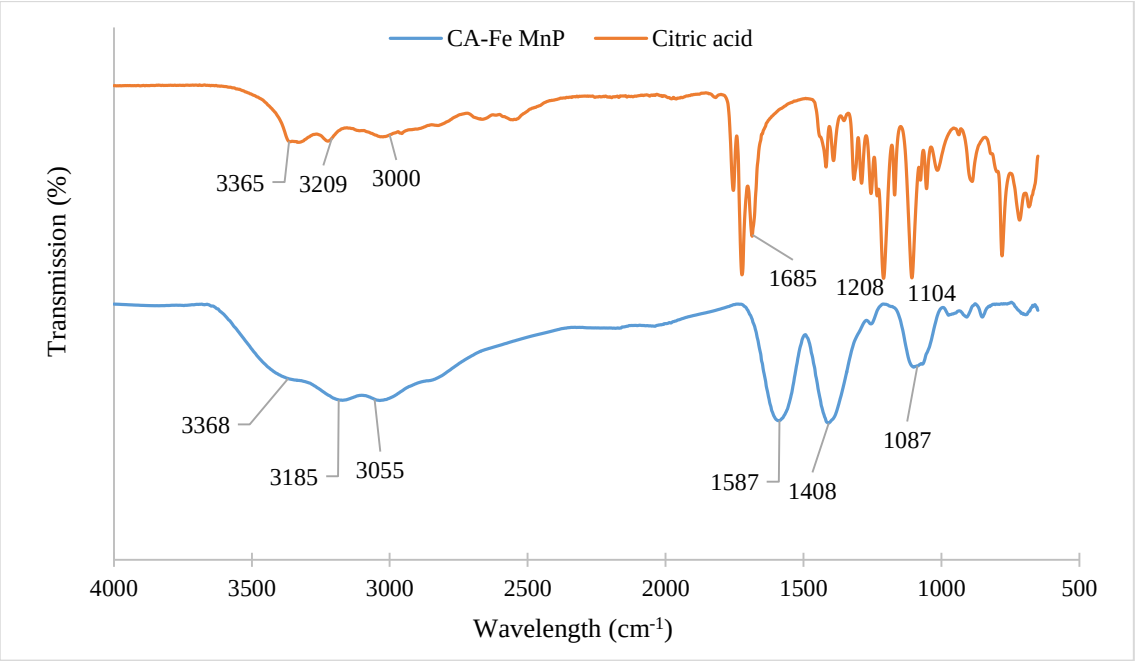



**Figure 3.3** FTIR spectra of citric acid and CA-MNPs [212]

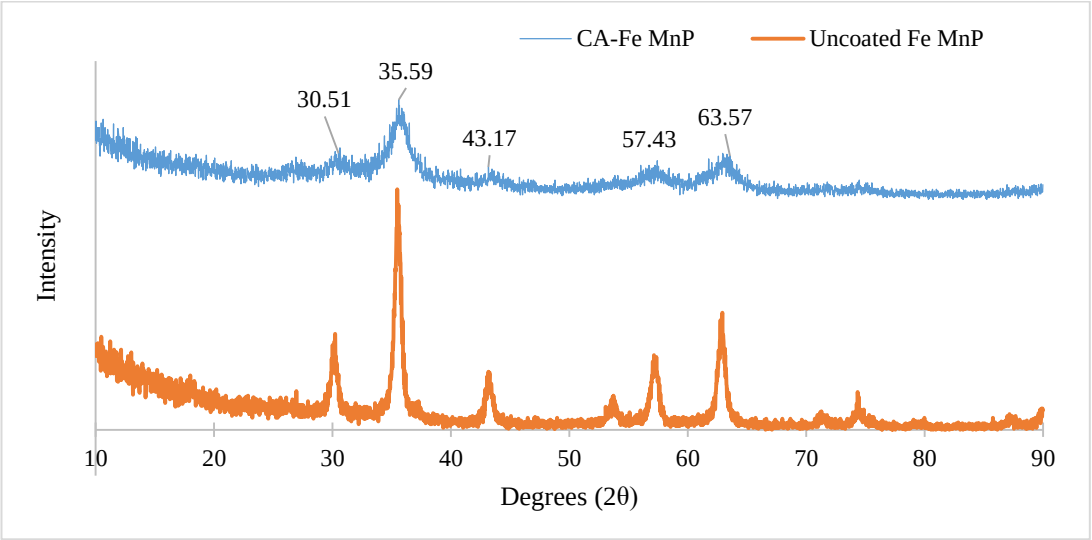



**Figure 3.4** XRD patterns of bare and citric acid-coated iron oxide MNPs [212]

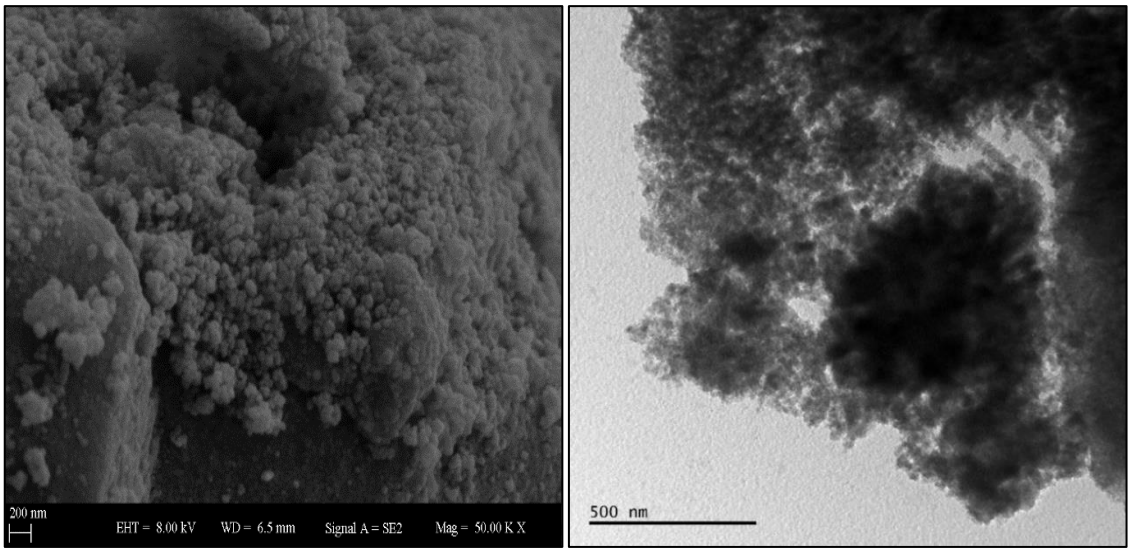



**Figure 3.5** SEM (left) and TEM (right) images of CA-MNPs [212]

### 3.3.2 Chromatographic Separation Study

Chromatographic separation of four different ED drugs including vardenafil, avanafil, sildenafil and tadalafil was achieved by using operating parameters of LC-QTOF-MS method which is presented in Table 3.2. The gradient elution of the method is also reported in Table 3.3. Four analytes were chromatographically separated from each other in only 5.5 min with a total analysis period of 8.0 min. The retention times (RTs) of I.S., vardenafil, sildenafil, avanafil and tadalafil are 1.561, 2.427, 2.900, 3.399 and 5.287 minutes, respectively and they were reproducible for sequential runs. Identical RTs were also observed for isotopic analogues. The extracted ion chromatograms of analytes are represented in Figure 3.6 indicated good selectivity with completely separated sharp peaks [210].

**Table 3.3** Gradient elution used in the developed method [210]

| Time, min | A % | B% |
|---|---|---|
| 0.00 | 65.0 | 35.0 |
| 3.30 | 65.0 | 35.0 |
| 3.31 | 20.0 | 80.0 |
| 4.50 | 20.0 | 80.0 |
| 4.51 | 65.0 | 35.0 |

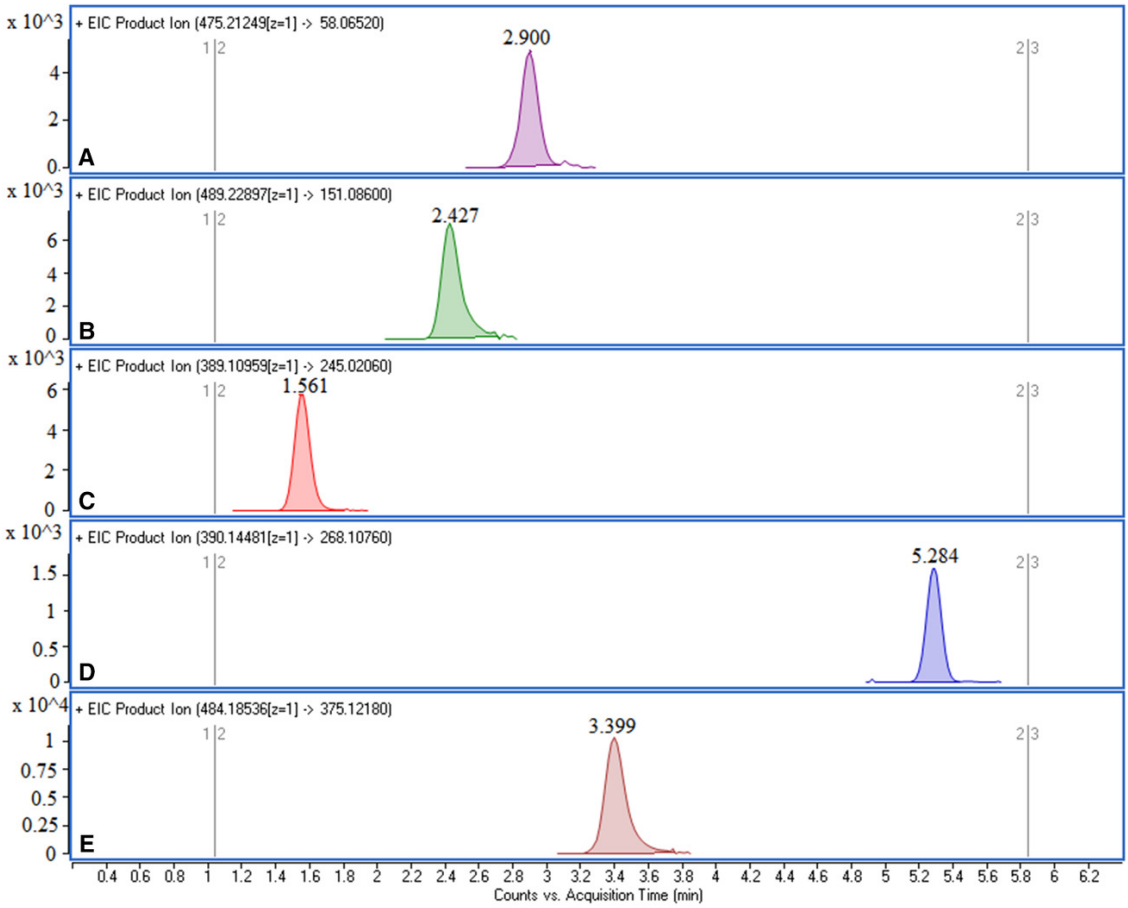


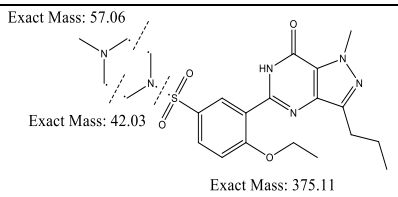
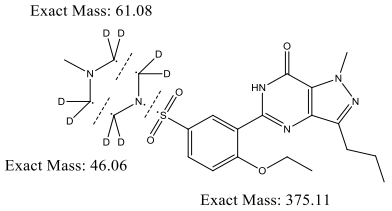
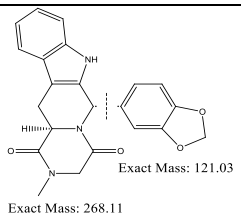
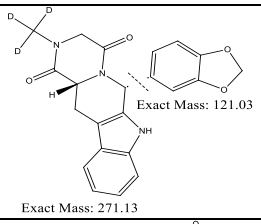
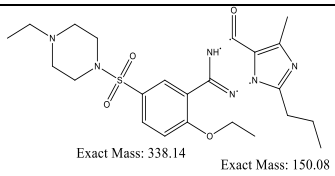
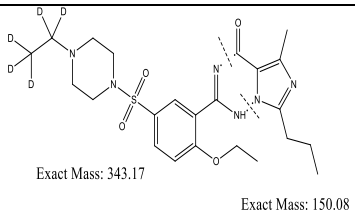
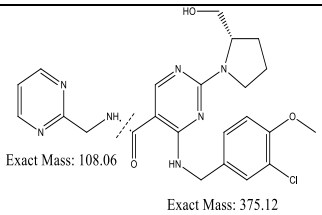
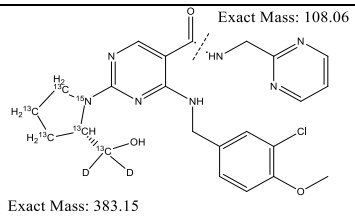

**Figure 3.6** Extracted ion chromatograms of A) sildenafil, B) vardenafil, C) I.S., D) tadalafil and E) avanafil (1,000 ng g-1 standard solutions) [210]

### 3.3.3 Mass Spectrometry

The spectrometric acquisition was studied by targeted MS/MS mode which enables to identify and quantify analytes with both precursor and product ions. Compounds were easily confirmed with their protonated molecular ion and fragmentations in high resolution (<5.0 ppm mass accuracy). The precursor and product ions used for quantitative determination of vardenafil, sildenafil, avanafil and tadalafil were 489.22897 > 151.08595, 475.21249 > 58.06524, 484.18536 > 375.12175 and 390.14481 > 268.10764, respectively. The collision energies (CEs) were selected according to the highest response resulted from fragmentations. The CE values were 50, 40, 30 and 5 V for vardenafil, sildenafil, avanafil and tadalafil, respectively [210].

The mass spectra of each compounds are presented in Figure 3.7 and the proposed fragmentation patterns of compounds are represented in Table 3.4. The fragmentation of vardenafil resulted in the formation of the product ion with m/z 151.0859 with the cleavage of the amide bond of tetrazabicyclonona and weak N-N bond [214]. The observed fragments m/z 283.1177 and 58.0652 for sildenafil were attributed to the loss of sulfonyl-methylpiperazine ring and secondary fragmentation of methylpiperazine. The fragmentation of tadalafil occurred with the loss of benzodioxole ring structure (m/z 135.0437) which leads to a change in pyrazinopyridoindole-1,4-dione moiety (m/z 268.1076). The fragmentation pathway of avanafil exhibited the elimination of aminoethylpiperazine and 3-chloro-4-methoxy-methylbenzene resulting in the peaks at m/z 375.1217 and m/z 155.0254. Isotopic analogues of selected compounds also showed identical fragmentation characteristics. The proposed fragmentation pathways were confirmed with the mass spectra results to prevent any interference that may result from other substances in matrices [210].

**Table 3.4** The precursor and product ions for natural and isotopic analytes [213]

| Name | Fragmentation | Precursor ion (m/z) | Product ion (m/z) |
|---|---|---|---|
| Sildenafil |  | 475.2125 | 58.0652 |
| Sildenafil-d8 |  | 483.2620 | 62.0900 |
| Tadalafil |  | 390.1448 | 268.1076 |
| Tadalafil-d3 |  | 393.1612 | 271.1263 |
| Vardenafil |  | 489.2290 | 151.0860 |
| Vardenafil-d5 |  | 494.2581 | 151.0860 |
| Avanafil |  | 484.1854 | 375.1218 |
| Avanafil-13C5-15N-d2 |  | 492.2109 | 383.1484 |

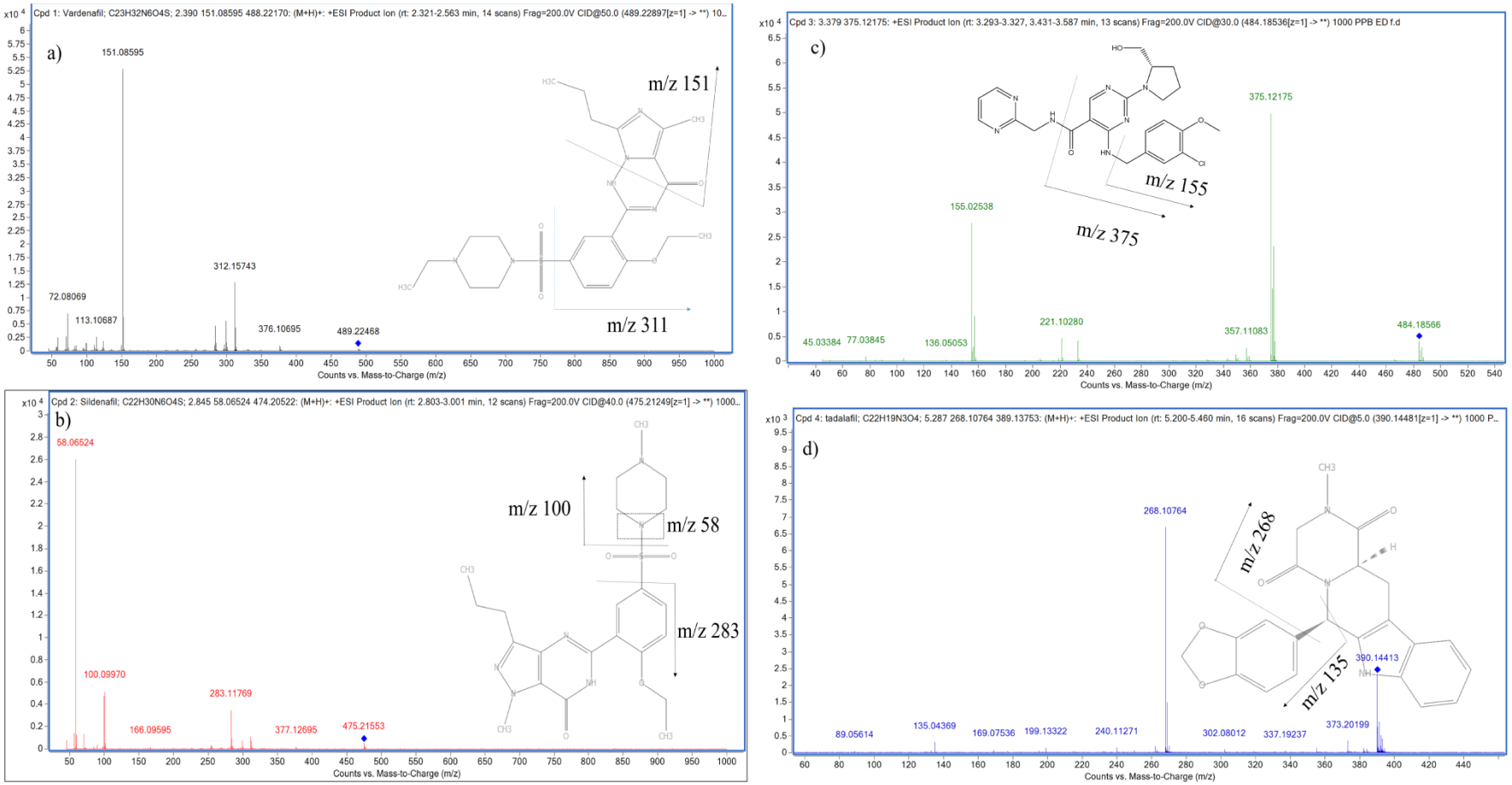



**Figure 3.7** Full scan product ion mass spectra and proposed fragmentations of a) vardenafil, b) sildenafil, c) avanafil and d) tadalafil [210]

### 3.3.4 Optimization Study

#### 3.3.4.1 One-variable-at-a-time optimization

The experimental variables including pH of the buffer, mixing type and eluent type were optimized by one-variable-at-a-time methodology. An aqueous standard solution containing of 100 ng $g^{-1}$ of each analytes was used for all steps of optimization. The extraction efficiency was examined by the ratio value of the analyte peak area to the internal standard peak area.

pH optimization was performed in a wide range between pH 2.0 – 13. As presented in Figure 3.8, the maximum extraction efficiency was obtained for pH 6.0 buffer and thus, pH 6.0 was selected for further analysis [212].

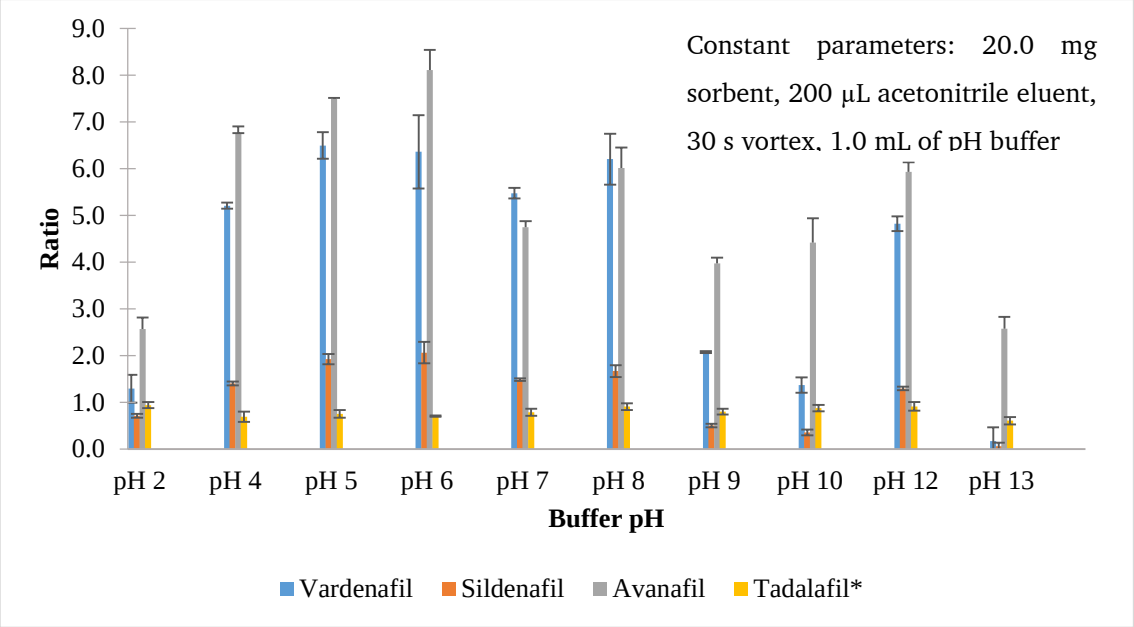



**Figure 3.8** The influence of pH on the extraction efficiency of vardenafil, sildenafil, avanafil and tadalafil (*multiplied by 10 for normalization) [212]

The optimum eluent type was determined using different eluents including methanol, acetonitrile and 2-propanol. As presented in Figure 3.9, the maximum ratio value was obtained for acetonitrile eluent indicating acetonitrile had the maximum desorption capacity among other examined solvents. Therefore, acetonitrile was selected for subsequent analysis [212].

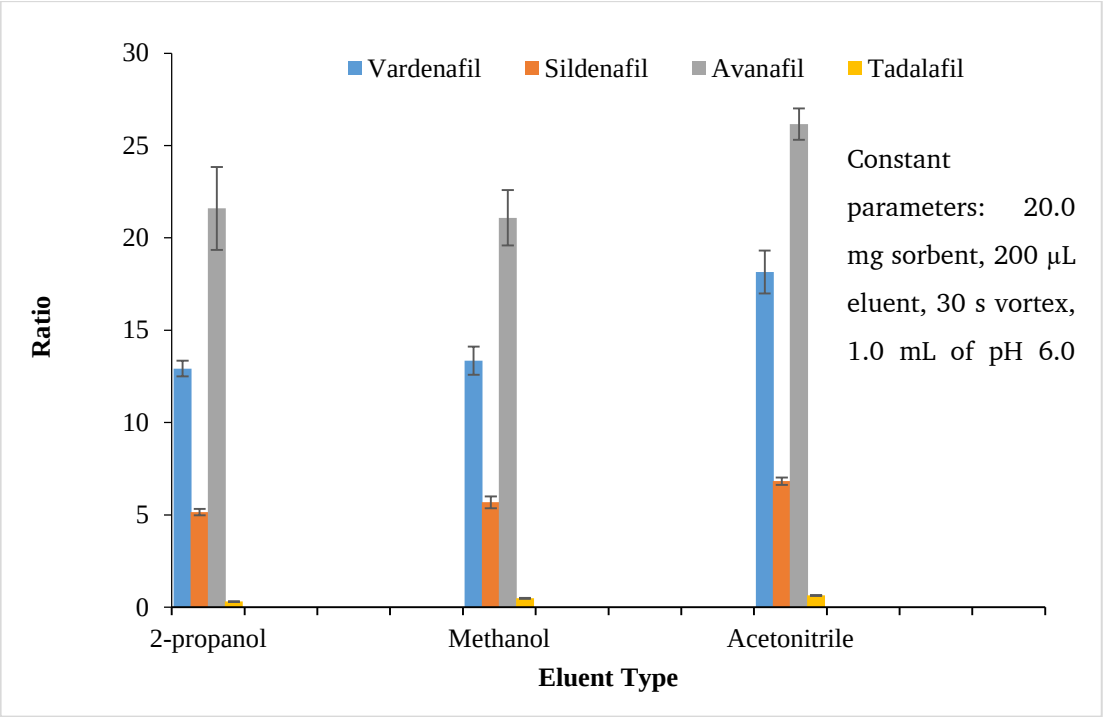



**Figure 3.9** The influence of eluent type on the extraction efficiency of vardenafil, sildenafil, avanafil and tadalafil

The mixing type was another important parameter to obtain maximum interaction between sorbent material and target molecules. Vortex, ultrasonication and manual shaking were examined as different mixing types. As presented in Figure 3.10, vortex mixing was selected due to relatively higher ratio values and lower %RSDs [212].

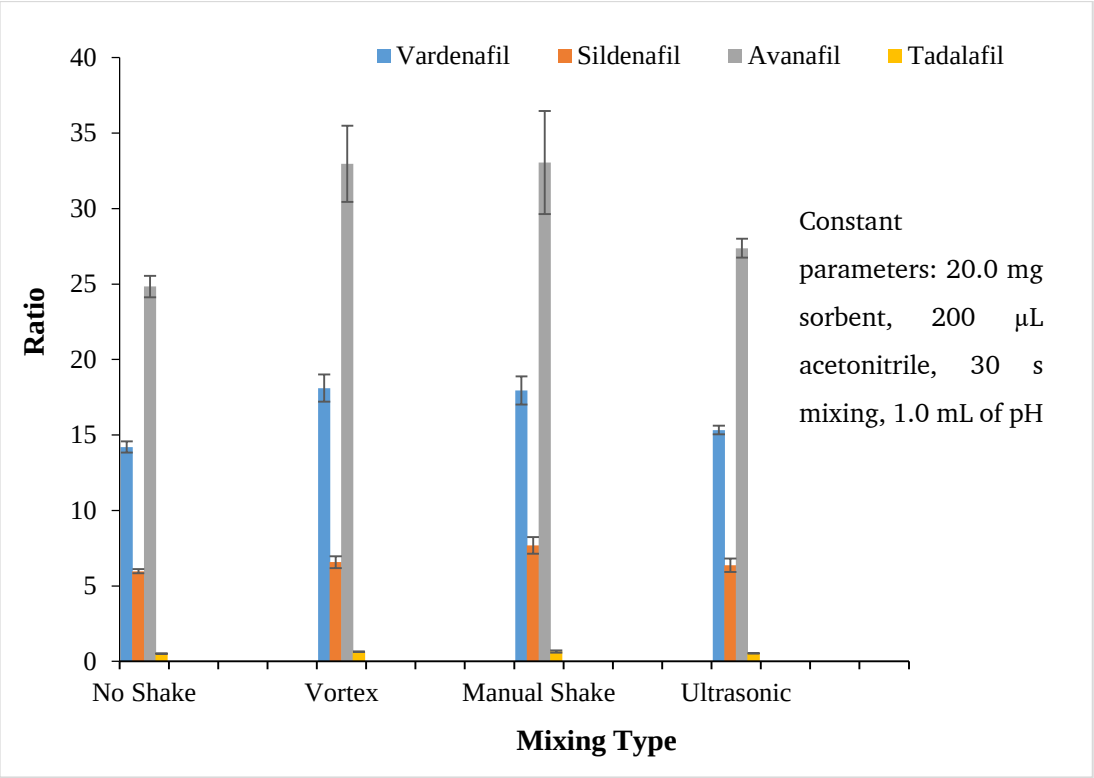



**Figure 3.10** The influence of mixing type on the extraction efficiency of vardenafil, sildenafil, avanafil and tadalafil

### 3.3.4.2 Experimental design

The optimization of four experimental variables such as sorbent amount, eluent volume, buffer volume and mixing period were optimized using Box-Behnken experimental design (BBD). The design was constructed at three levels with 29 trials including 5 center points. The average results of three replicate experiments were presented as the responses for each run. The design matrix is given in Table 3.5. The explanation for the behavior of the system can be given as (3.1) [215]:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} {x_{ii}}^2 + \sum \beta_{ij} x_{ij} + \varepsilon \qquad (3.1)$$

where $\beta_0$ is the constant term, $\beta_i$ is the linear effect, $\beta_{ii}$ is the quadratic effect, $\beta_{ii}$ is the coefficient of the interaction parameters, Y represents the response (dependent variable) and ε is the residual related to the experiments or random error. $x_1, x_2, \ldots x_k$ are the coded independent variables which affect the response.

A multiple function (multiple response, $R_m$) was utilized to examine the effects of main factors and their interactions that was calculated with the following equation (3.2), taking the first run as an example:

$$R_m = \frac{13.7}{34.0} + \frac{4.4}{11.3} + \frac{0.53}{1.18} + \frac{21.7}{49.0} = 1.68 \quad (3.2)$$

The multiple response model which were generated from a multiple regression analysis was explained by the equation below:

$$R_m = 3.0267 + 0.045A - 0.011B - 0.0003C - 1.169D - 0.0000651AB + 0.00124BD - 0.00297CD - 0.0000687A^2 + 0.0000122B^2 + 0.0000694C^2 + 0.234D^2 \quad (3.3)$$

The accuracy of the model was evaluated by analysis of variance (ANOVA) study which is presented in Table 3.6. The model yielded high accuracy with a determination coefficient ($R^2$) of 0.979. The closeness of $R^2$ and adjusted- $R^2$ proved the good agreement between the observed data and predicted values. The low probability (p-value) of <0.0001 showed that the model is highly significant. The non-significant lack of fit value proved the applicability of the model for the experiments [212].

**Table 3.5** The matrix for Box-Behnken design [212]

| Run | Factor 1 (A) CA MnP amount (mg) | Factor 2 (B) Volume of eluent (µL) | Factor 3 (C) Mixing Period (s) | Factor 4 (D) Volume of pH 6 (mL) | Ratio | | | | Rm |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Vardenafil | Sildenafil | Tadalafil | Avanafil | |
| **1** | 100 | 300 | 10 | 1.75 | 13.7 | 4.4 | 0.53 | 21.7 | 1.68 |
| **2** | 100 | 300 | 120 | 1.75 | 17.2 | 5.73 | 0.53 | 25.8 | 1.99 |
| **3** | 55 | 100 | 10 | 1.75 | 21.7 | 7.4 | 0.75 | 28.8 | 2.51 |
| **4** | 55 | 300 | 65 | 1.75 | 10.0 | 3.05 | 0.26 | 11.9 | 1.03 |
| **5** | 10 | 300 | 10 | 1.75 | 3.77 | 1.09 | 0.13 | 3.67 | 0.39 |
| **6** | 55 | 500 | 65 | 0.50 | 5.83 | 1.87 | 0.22 | 8.50 | 0.70 |
| **7** | 55 | 500 | 120 | 1.75 | 7.77 | 2.37 | 0.24 | 10.2 | 0.85 |
| **8** | 55 | 300 | 120 | 0.50 | 17.9 | 5.67 | 0.46 | 25.1 | 1.93 |
| **9** | 10 | 300 | 65 | 0.50 | 3.53 | 1.15 | 0.16 | 5.30 | 0.45 |
| **10** | 55 | 100 | 65 | 0.50 | 34.0 | 11.3 | 0.92 | 47.0 | 3.74 |
| **11** | 55 | 300 | 65 | 1.75 | 9.40 | 4.13 | 0.33 | 11.7 | 1.18 |
| **12** | 10 | 500 | 65 | 1.75 | 1.87 | 0.56 | 0.083 | 2.16 | 0.22 |
| **13** | 55 | 300 | 10 | 3.0 | 17.8 | 5.50 | 0.50 | 18.5 | 1.81 |
| **14** | 10 | 300 | 65 | 3.0 | 2.99 | 0.74 | 0.11 | 2.79 | 0.30 |
| **15** | 100 | 300 | 65 | 0.50 | 21.0 | 7.40 | 0.75 | 32.3 | 2.56 |
| **16** | 100 | 100 | 65 | 1.75 | 29.9 | 10.9 | 1.18 | 49.0 | 3.84 |
| **17** | 100 | 300 | 65 | 3.0 | 17.6 | 5.60 | 0.50 | 20.7 | 1.86 |
| **18** | 55 | 300 | 65 | 1.75 | 9.10 | 2.71 | 0.27 | 9.73 | 0.94 |
| **19** | 55 | 100 | 120 | 1.75 | 26.8 | 8.93 | 0.72 | 33.2 | 2.86 |
| **20** | 55 | 500 | 65 | 3.0 | 6.33 | 1.94 | 0.19 | 7.53 | 0.67 |

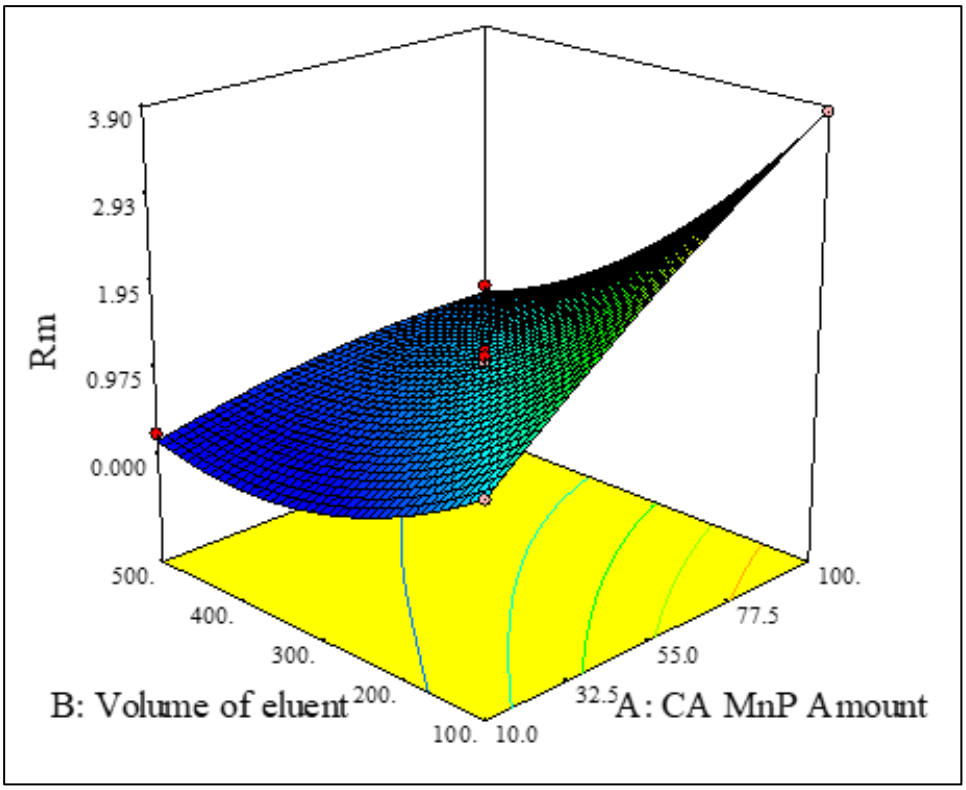
**Table 3.5** The matrix for Box-Behnken design [212] (continues)

| Run | Factor 1 (A) CA MnP amount (mg) | Factor 2 (B) Volume of eluent (µL) | Factor 3 (C) Mixing Period (s) | Factor 4 (D) Volume of pH 6 (mL) | Ratio | | | | Rm |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Vardenafil | Sildenafil | Tadalafil | Avanafil | |
| **21** | 55 | 300 | 10 | 0.50 | 13.7 | 4.53 | 0.48 | 19.9 | 1.62 |
| **22** | 55 | 300 | 65 | 1.75 | 10.6 | 3.37 | 0.30 | 12.4 | 1.12 |
| **23** | 55 | 300 | 120 | 3.0 | 13.2 | 4.13 | 0.31 | 14.1 | 1.31 |
| **24** | 55 | 500 | 10 | 1.75 | 7.50 | 2.40 | 0.26 | 9.30 | 0.84 |
| **25** | 55 | 300 | 65 | 1.75 | 9.43 | 3.50 | 0.33 | 10.1 | 1.07 |
| **26** | 10 | 300 | 120 | 1.75 | 3.83 | 1.11 | 0.12 | 3.77 | 0.39 |
| **27** | 55 | 100 | 65 | 3.0 | 23.8 | 7.63 | 0.68 | 25.3 | 2.47 |
| **28** | 10 | 100 | 65 | 1.75 | 10.4 | 3.27 | 0.27 | 10.6 | 1.04 |
| **29** | 100 | 500 | 65 | 1.75 | 5.97 | 1.87 | 0.23 | 7.0 | 0.67 |

**Table 3.6** The ANOVA analysis for multiple response function [212]

| Source | Sum of Squares | df | Mean Square | F Value | p-value (Prob > F) |
|---|---|---|---|---|---|
| **Model** | 26.2 | 11 | 2.38 | 71.3 | < 0.0001 |
| **A-CA MnP Amount** | 8.03 | 1 | 8.03 | 240 | < 0.0001 |
| **B-Eluent Amount** | 13 | 1 | 13 | 390 | < 0.0001 |
| **C-Mixing Period** | 0.0183 | 1 | 0.0183 | 0.547 | 0.47 |
| **D-Buffer Amount** | 0.552 | 1 | 0.552 | 16.5 | 0.000812 |
| **AB** | 1.37 | 1 | 1.37 | 41.1 | < 0.0001 |
| **BD** | 0.386 | 1 | 0.386 | 11.5 | 0.00342 |
| **CD** | 0.167 | 1 | 0.167 | 5.01 | 0.0389 |
| **$A^2$** | 0.125 | 1 | 0.125 | 3.75 | 0.0695 |
| **$B^2$** | 1.55 | 1 | 1.55 | 46.4 | < 0.0001 |
| **$C^2$** | 0.286 | 1 | 0.286 | 8.57 | 0.00941 |
| **$D^2$** | 0.866 | 1 | 0.866 | 25.9 | < 0.0001 |
| **Residual** | 0.568 | 17 | 0.0334 | | |
| **Lack of Fit** | 0.535 | 13 | 0.0411 | 4.86 | 0.0694 |
| **Pure Error** | 0.0339 | 4 | 0.00847 | | |
| **Cor Total** | 26.8 | 28 | | | |
| **$R^2$** | 0.979 | | | | |
| **Adj $R^2$** | 0.965 | | | | |

The main and interactive effects of four factors were evaluated by 3-D response surface plots as presented in Figure 3.11. The increase in sorbent amount lead to the increase in response value and the eluent volume resulted in a negative effect on the response value. The buffer volume was another significant parameter that affected the response value positively while the mixing period affected response value negatively. As a result of the quadratic model, the optimum values were selected as: 100 mg of CA-MNP sorbent, 100 s of vortex mixing, 0.50 mL of pH 6.0 and 100 μL of acetonitrile as eluent [212].



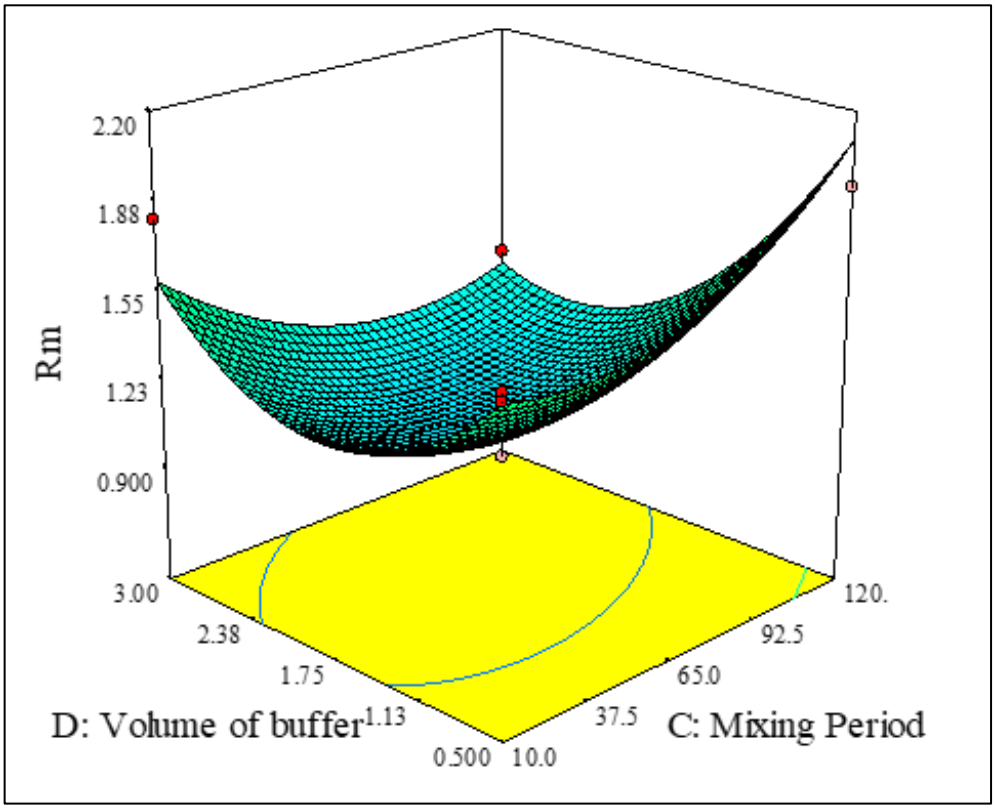




**Figure 3.11** Response surface plots obtained from the experimental design [212]

### 3.3.5 Analytical figures of merit

The method performance was evaluated for linearity, LOD and LOQ, and the correlation coefficient. Calibration standards were prepared at final concentrations ranging from 5.0 - 2,000 ng $g^{-1}$ and 0.25 - 50 ng $g^{-1}$ for direct analysis [210] and preconcentration process [212], respectively. The peak areas of each analyte were used for quantification. LOD and LOQ values were calculated from ten replicate measurements of the lowest concentrations with the signal to noise ratios of 3 and 10, respectively. Equation 3.4 was used for the calculations of LOD and LOQ.

$$LOD = \frac{3xSD}{m}; LOQ = \frac{10xSD}{m} \quad (3.4)$$

where SD is the standard deviations of ten measurements and m is the slope of the calibration plot. Under the optimum conditions, the method performance was determined and the summary of the results are presented in Table 3.7. The linear ranges for the preconcentration process were obtained between 0.25-25.0 ng $g^{-1}$ for vardenafil and avanafil; 0.50-25.0 ng $g^{-1}$ for sildenafil and 5.0-25.0 ng $g^{-1}$ for tadalafil. The enrichment factors were calculated by comparing LOD values before and after the extraction procedure. High correlation coefficients ($R^2$) were obtained between 0.9991-0.9997 indicating good linearity. The repeatability was satisfactory and varied between 6.57-9.57%. The LOD values were between 0.06 and 0.94 ng $g^{-1}$ for target analytes and the enrichment factors were obtained between 10.5 and 28.0 [212].

**Table 3.7** Analytical figures of merit of the proposed method [210, 212]

| | **Sildenafil** | **Tadalafil** | **Vardenafil** | **Avanafil** |
|---|---|---|---|---|
| **LOD/LOQ, (ng g$^{-1}$)** | 0.14/0.47 (2.19/7.28)* | 0.94/3.13 (9.81/32.71)* | 0.13/0.43 (2.28/7.56)* | 0.06/0.19 (1.63/5.43)* |
| **%RSD** | 8.48% (6.71%)* | 6.57% (7.46%)* | 9.57% (7.72%)* | 6.82% (7.60%)* |
| **Linear Range, (ng g$^{-1}$)** | 0.5 - 25.0 (5.0 - 500)* | 5.0 - 25.0 (25.0 - 1000)* | 0.25 - 25.0 (5.0 - 1000)* | 0.25 - 25.0 (5.0 - 1000)* |
| **Correlation Coefficient ($R^2$)** | 0.9995 (0.9995)* | 0.9997 (0.9987)* | 0.9991 (0.9998)* | 0.9991 (0.9995)* |
| **Enrichment Factor** | 15.6 | 10.5 | 17.5 | 28.0 |

*(Analytical figures of merit for direct analysis were presented in parenthesis).

### 3.3.6 Recovery

A recovery study was performed to examine the effectiveness of the proposed method for practical applications. Therefore, urine and plasma samples were spiked with different concentrations of analyte mixtures. No interfering or analyte peaks were detected in blank samples. To obtain high percent recoveries, the matrix-matching study was applied to samples. The recovery results are summarized in Table 3.8 and varied from 83.0% to 109.5 % for urine samples and from 93.4% to 116.8 % for plasma samples. These results proved that the proposed method can be used for accurate determination of target analytes in biological samples.

**Table 3.8** Percent recoveries of sildenafil, tadalafil, vardenafil and avanafil in spiked human urine and plasma samples [212]

| Analyte | Human urine (%Recovery) | | |
|---|---|---|---|
| | **8.0 ng g$^{-1}$** | **12.0 ng g$^{-1}$** | **20.0 ng g$^{-1}$** |
| **Sildenafil** | 83.4 ± 7.5 | 83.2 ± 1.9 | 104.1 ± 2.6 |
| **Tadalafil** | 88.0 ± 7.8 | 93.9 ± 7.2 | 83.0 ± 9.2 |
| **Vardenafil** | 96.6 ± 4.2 | 98.2 ± 5.1 | 84.9 ± 3.1 |
| **Avanafil** | 93.7 ± 6.1 | 109.5 ± 2.9 | 93.6 ± 6.7 |
| | **Human plasma (%Recovery)** | | |
| | **50.0 ng g$^{-1}$** | **100.0 ng g$^{-1}$** | **150.0 ng g$^{-1}$** |
| **Sildenafil** | 104.9 ± 5.5 | 104.1 ± 2.8 | 95.0 ± 5.7 |
| **Tadalafil** | 116.8 ± 8.7 | 105.2 ± 5.8 | 100.0 ± 4.6 |
| **Vardenafil** | 106.6 ± 5.5 | 105.6 ± 5.9 | 93.4 ± 2.1 |
| **Avanafil** | 104.9 ± 6.0 | 100.5 ± 3.6 | 101.1 ± 2.3 |

### 3.3.7 ID$^4$MS

#### 3.3.7.1 Equilibration period

Despite the impressive advantages of IDMS, there is still a possible risk for obtaining incorrect results, if the adequate effort is not spent for the isotopic equilibration. In the lack of isotopic equilibration, every effect that is present on the analyte and the isotopic analogue during instrumental analysis and sample preparation will differ from each other and these variations cannot be corrected anymore. Therefore, it is an essential task to establish a complete equilibrium between the analyte and isotopic analogue at the early stage of the analytical procedure.

To determine the equilibration period between analyte and its isotopic analogue, different periods between 0.25 and 3.0 h have been tested by the analysis of blends compositions. The change in the isotopic ratio of blend compositions with increased periods is graphically presented in Figure 3.12. As illustrated in that figure, isotopic

equilibrium has been established in 1.0 hour for all analytes, thus, 1.0 h was determined as the equilibration period [213].

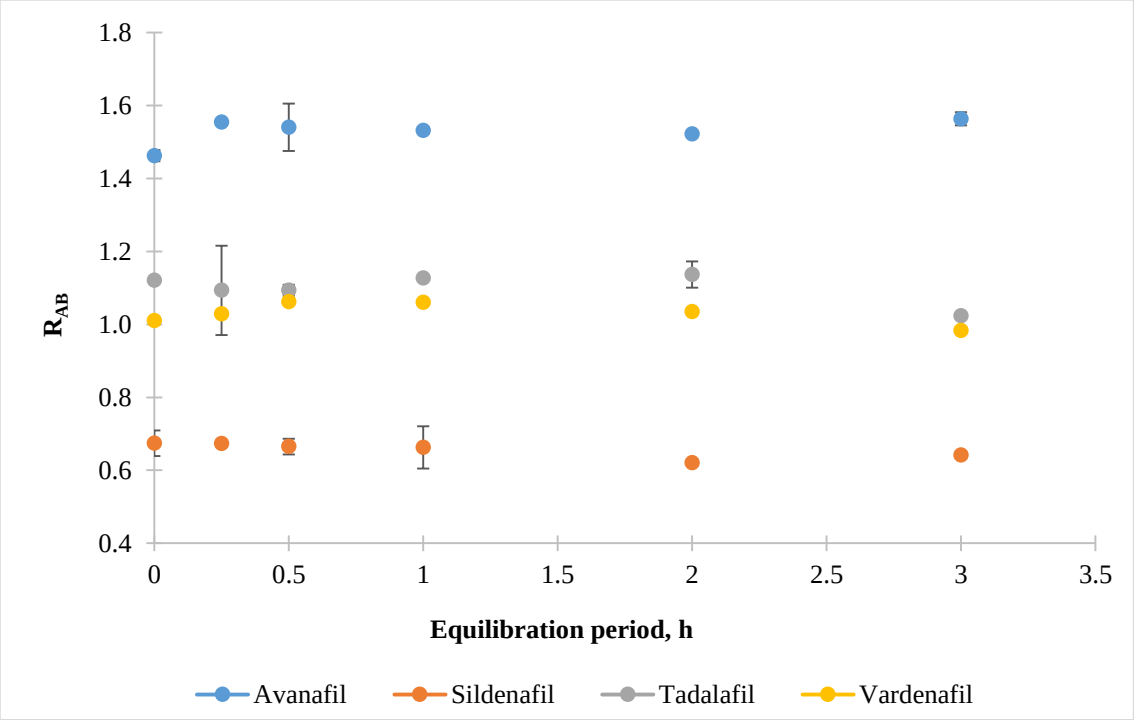



**Figure 3.12** The investigation of isotopic equilibration period for each analytes [213]

### 3.3.7.2 Direct ID⁴MS

At first, the ID⁴MS strategy was applied to samples without preconcentration. The study was carried out by establishing calibration and sample blends which were consisted of isotopically labeled standard and calibration/sample solutions at the concentration of 250 µg $kg^{-1}$. Calibration blends and sample blends were prepared and analyzed to measure the actual isotopic ratios following the isotopic equilibration (kept 1.0 h prior to analysis).

Three replicate measurements of calibration blends and five replicate measurements of sample blends were used to calculate the spiked amounts of analytes in plasma and urine samples. The isotopic ratios of each blend were determined by dividing the peak areas of the analyte and its isotopic analogue. The measured isotopic ratios and weighed amounts of sample, calibration and

isotopically labeled standard solutions were utilized to obtain the experimental mass fractions of target analytes using Equation 2.35. The comparison of experimental and theoretically spiked mass fractions of analytes provided the percent recovery values which are summarized in Table 3.9. The obtained percent recovery values varied between 99.90% –100.78% and 100.70% – 101.06% providing very low %RSD values of 0.80% – 1.33% and 0.67% – 1.12% for plasma and urine samples, respectively [213].

#### 3.3.7.3 ID$^4$MS combined with preconcentration

ID$^4$MS strategy was also combined with the MSPE process with the aim of obtaining quantitative accuracy and precision even at a low concentration of analytes. Therefore, all blends were prepared at 50.0 μg kg$^{-1}$ concentration with a total mass of 8.0 g. After the preparation of blends with isotopic ratios as in direct ID$^4$MS method, optimum conditions of MSPE process were utilized to blends after 1.0 h equilibration period and the final eluent phases were sent to the instrument for analysis. The results for the obtained percent recovery values are presented in Table 3.9.

The percent recoveries for ID$^4$MS combined MSPE process were ranged between 98.26% –100.92% and 97.50% – 99.57% with %RSD values below than 1.95% and 1.96% for plasma and urine samples, respectively. The results indicated that isotope dilution provided a great improvement in accuracy and precision when compared with classical matrix matching strategy (93.4% – 116.8% with %RSDs ≤ 5.9% for plasma; 83.0% – 109.5% with %RSDs ≤ 7.8% for human urine). The obtained results also showed that all shortcomings caused by the sample preparation steps or chromatographic and spectrometric acquisitions can be completely compensated even in complex matrices by the application of isotope dilution strategy. The proposed study can be regarded as a reference method for highly accurate and precise determination of PDE-5 inhibitors even at very low levels in complex matrices [213].

**Table 3.9** Percent recoveries of erectile compounds in human urine and plasma samples for direct ID$^4$MS and ID$^4$MS after preconcentration with MSPE [213]

| | **Direct ID$^4$MS (250 µg kg$^{-1}$)** | | **ID$^4$MS after preconcentration (50 µg kg$^{-1}$)** | |
|---|---|---|---|---|
| | **Human urine** | **Human plasma** | **Human urine** | **Human plasma** |
| **Vardenafil** | 100.70 ± 1.12 (%) | 100.78 ± 1.33 (%) | 97.50 ± 1.96 (%) | 98.26 ± 1.95 (%) |
| **Sildenafil** | 100.92 ± 0.79 (%) | 100.14 ± 1.16 (%) | 99.30 ± 1.19 (%) | 98.74 ± 1.79 (%) |
| **Avanafil** | 100.81 ± 0.79 (%) | 100.51 ± 0.80 (%) | 99.57 ± 1.85 (%) | 100.35 ± 0.49 (%) |
| **Tadalafil** | 101.06 ± 0.67 (%) | 99.90 ± 0.87 (%) | 98.31 ± 1.77 (%) | 100.92 ± 1.59 (%) |

# 4
# AMINO ACIDS

## 4.1 General Information

Amino acids are the most important substances containing amino and acid groups. They are building blocks of proteins, some of the hormones, vitamins and antibiotics. They play several significant roles in lipid transport, neurotransmission and metabolic processes [216]. Amino acids can be found either in free forms (about 500 amino acids) or as protein constituents (also known as proteogenic). Only 20 of amino acids are known as proteogenics that link covalently through peptide bonds with different combinations and sequences [217]. These combinations give form to different products including enzymes, antibodies, antibiotics, hormones, transporters and several different structures with the diversity of biological activities [218]. The chemical and physical properties of these structures are determined by their amino acid constituents. Therefore, the functions of amino acids become increasingly important with respect to the recent developments in biotechnology.

### 4.1.1 History of amino acids

Amino acids were discovered after the first isolation of asparagine from *Asparagus satiuus* in 1806 by Vauquelin and Robiquet [219]. The physiological importance of amino acid properties was firstly demonstrated by Piria in 1848 through the asparagine role during the life cycle of plants [220, 221]. Despite the early discovery of amino acids, their analysis was only described in 1954 by Moore and Stein [222]. They developed a photometric method with consistent and reproducible results for the identification and quantification of amino acids through chromatography. Thereafter, they were awarded in 1972 by Nobel Prize in Chemistry for work associated with the 124-amino acid sequence in ribonuclease A [223]. To date, numerous methodologies have been reported for amino acid determination based

on chromatographic studies with spectrophotometric or spectrofluorometric detections.

### 4.1.2 Structure and stereochemistry of amino acids

The most abundant amino acids are protein amino acids known as α-amino acids. However, a large number of amino acids are present naturally in living systems that are not α-amino acids and protein constituents [224]. They are found rarely and do not play important roles in cellular metabolism [224]. Other standard 20 amino acids are involved in several biochemical processes and they have both carboxyl group and the amino group bonded to α-carbon atom which is also linked to a side chain group (except for glycine) and a hydrogen [218]. The general formula of α-amino acids is shown in Figure 4.1.

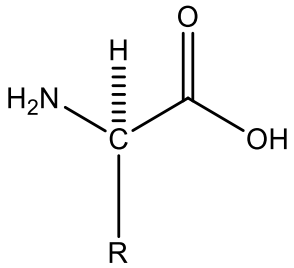



**Figure 4.1** The general formula of α-amino acids

Except for glycine, all α-amino acids have asymmetric α-carbon atom which constitutes the chirality center of the molecule. The chiral center refers to four different substituents to carbon atom and means that biomolecule cannot be superimposed on its mirror image. All molecules with chiral center exhibit optical activity and are classified by D and L nomenclatures according to the direction of optical rotation. The clockwise rotation of polarized light refers to D- amino acids while counter-clockwise rotation refers to L- amino acids [218]. In any case, D- and L- stereoisomers have the same chemical and physical properties but they possess different biological activities. D- amino acids are rarely found in nature; generally in plants, bacterial cells or in several antibiotics. The protein amino acids are generally composed of L- amino acids and present in higher animals [225].

### 4.1.3 The protein amino acids

Despite the great functional diversity of protein structures, they all consist of a linear arrangement of amino acids in a polypeptide chain. As aforementioned, proteins are generally composed of 20 standard amino acids and all amino acids contain carbon, hydrogen, oxygen and nitrogen except two of them also contain sulfur [226]. Amino acids are joined together via the formation of peptide bonds by the nucleophilic addition-elimination reaction between the carboxylic group of one amino acid and the amino group of another amino acid. The amino acid sequence of a protein is encoded within the genetic information. Proteins differ from each other according to their amino acid sequence, amino acid residues and their overall amino acyl composition [227, 228]. All standard amino acids have L- configurations and they are grouped in Table 1 according to the chemical properties of side chains [228, 229]. The common abbreviations of amino acids are also presented in Table 4.1.

Each amino acids have specific characteristics based on the side chains and they can be classified according to their interaction with water in four subsections: nonpolar, polar, acidic and basic. Nonpolar amino acids have poor interaction with water that enable the stabilization of three-dimensional protein structures. On the other hand, polar amino acids easily interact with water and cover the surface of molecule with the ability of participation to hydrogen bonding. Acidic amino acids are negatively charged in physiological pH whereas basic amino acids are positively charged in physiological pH and they can easily form ionic bonds [228].

**Table 4.1** The standard amino acids

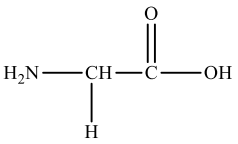
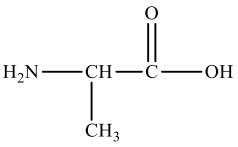
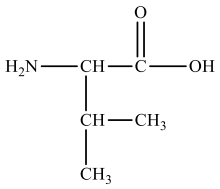
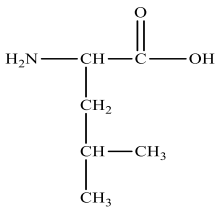
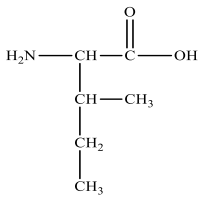
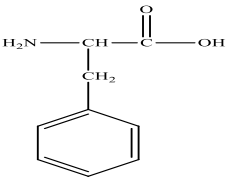
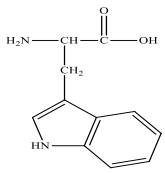
| Name | Symbol | Abbreviation | Structure |
|---|---|---|---|
| **Nonpolar Amino Acids** | | | |
| Glycine | G | Gly |  |
| Alanine | A | Ala |  |
| Valine | V | Val |  |
| Leucine | L | Leu |  |
| Isoleucine | I | Ile |  |
| Phenylalanine | F | Phe |  |
| Tryptophan | W | Trp |  |

**Table 4.1** The standard amino acids (continues)

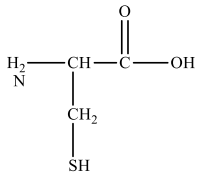
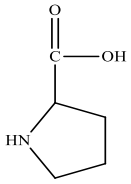
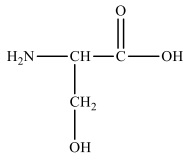
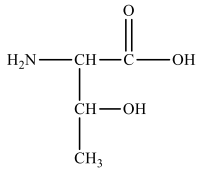
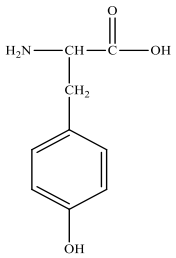
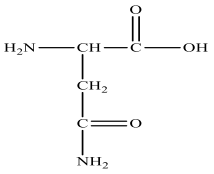
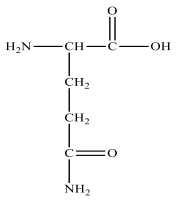
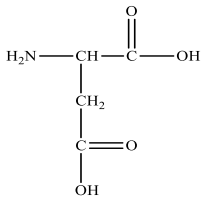
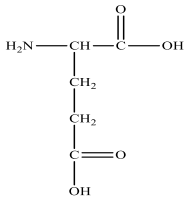
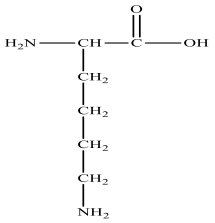
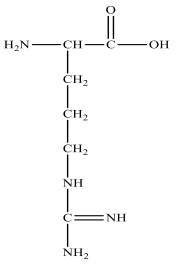
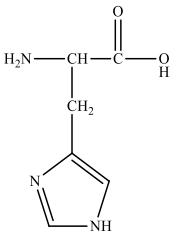
| Name | Symbol | Abbreviation | Structure |
|---|---|---|---|
| Cysteine | C | Cys |  |
| Proline | P | Pro |  |
| **Polar Amino Acids** | | | |
| Serine | S | Ser |  |
| Threonine | T | Thr |  |
| Tyrosine | Y | Tyr |  |
| Asparagine | N | Asn |  |
| Glutamine | Q | Gln |  |

**Table 4.1** The standard amino acids (continues)

| Name | Symbol | Abbreviation | Structure |
|---|---|---|---|
| **Acidic Amino Acids** | | | |
| Aspartic acid | D | Asp |  |
| Glutamic acid | E | Glu |  |
| **Basic Amino Acids** | | | |
| Lysine | K | Lys |  |
| Arginine | R | Arg |  |
| Histidine | H | His |  |

### 4.1.4 Amino acids and human health

Amino acids are key players for modulating human nutrition and health maintenance. They have significant functions on growth, development, reproduction and survival of organisms with their special features resulting from their functionality and chirality [230]. Therefore, human needs ingestion of amino acids, peptides and proteins from a variety of food sources to maintain their health. Amino acids are traditionally classified according to growth or nitrogen balance of mammals as nutritionally essential or non-essential amino acids [231]. In this context, essential amino acids are defined as amino acids whose carbon skeletons are not produced by animal cells and need to be supplied in the diet. Despite that, non-essential amino acids can be produced by human organisms in sufficient amounts and human does not further require them in nutrition [232]. Histidine, isoleucine, leucine, methionine, lysine, phenylalanine, tryptophan, threonine and valine are known as essential amino acids whereas arginine, alanine, asparagine, aspartate, cysteine, glutamate, glycine, glutamine, serine, proline and tyrosine are non-essential for humans and other animals [233].

It had been long assumed that animals or humans synthesized non-essential amino acids adequately to maintain growth and nitrogen balance. However, evidences from recent studies reported that young and gestating mammals cannot produce adequate amounts of some of the non-essential amino acids (e.g. proline, glutamine, glutamate, and arginine) to benefit embryonic survival, intestinal growth and development [234, 235]. Furthermore, these non-essential amino acids have also significance on the regulation of cell signaling pathways, thereby on energy and nutrient metabolism and regulation of gene expression [233]. Herewith, the nutritional and dietary amino acids requirements need to be evaluated by considering cell and tissue functions of amino acids for impact upon both old-age quality of life and longevity of humans and animals. The main physiological functions of amino acids are presented in Figure 4.2.

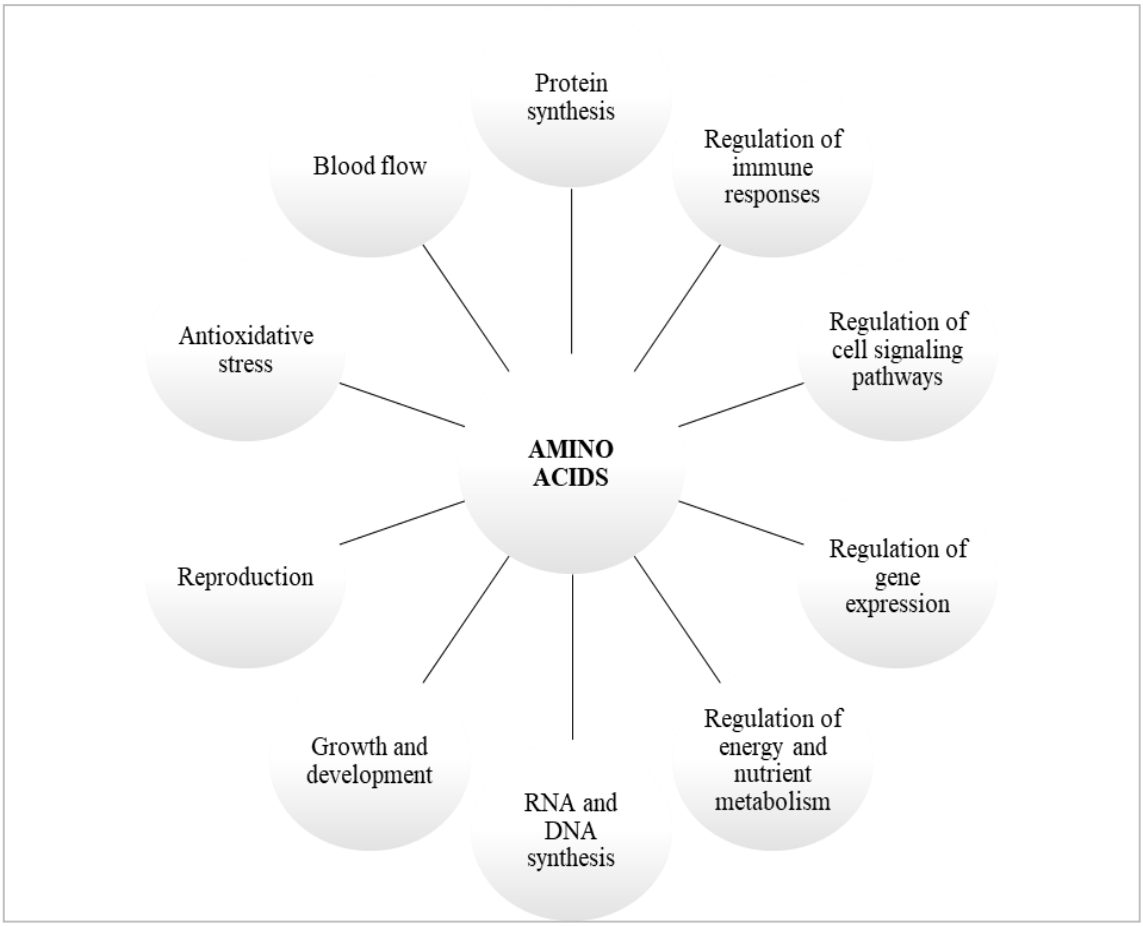



**Figure 4.2** Major functions of amino acids in nutrition and metabolism

### 4.1.5 Amino Acid-Related Diseases

In view of the enormous functions of amino acids, variations (decrease or increase) of their physiological concentrations can cause various clinical symptoms which are not directed to only a single disorder [236]. The biochemical abnormality in the metabolic pathway of amino acids is known as aminoacidopathies which are autosomal recessive and inherited in countries where the consanguinity rates are high [237].

Aminoacidopathies are caused by the inability of the body to breakdown or metabolize amino acids related to the deficiency of an enzyme or transporter, or by the defect in detoxification of amino acid by-products in the urine cycle [237]. The more common aminoacidopathies are phenylketonuria, tyrosinemia type 1, urea cycle defects, maple syrup urine disease, isovaleric aciduria, propionic aciduria, methylmalonic aciduria, homocystinuria, glutaric aciduria type 1 and nonketotic

hyperglycinemia [238]. Major amino acid metabolism disorders, their prevalence and diagnosis are listed in Table 4.2.

**Table 4.2** Amino acid metabolism disorders

| **Disorder** | **Deficiency,** [238] | **Prevalence** | **Diagnosis,** [239] |
|---|---|---|---|
| **Phenylketonuria** | Phenylalanine hydroxylase | 1:10,000 [239] | Phenylalanine levels, phenylalanine/tyrosine ratio=3 |
| **Tyrosinemia type 1** | Fumarylacetoacetase deficiency, hepatorenal tyrosinemia | 1:100,000 [240] | Tyrosine levels |
| **Maple syrup urine disease** | Branched-chain alpha-keto acid dehydrogenase deficiency | 1:185,000 [241] | Leucine > 250 μmol/L<br>Valine > 250 μmol/L<br>Leu/Phe > 4.0<br>Val/Phe > 3.5 |
| **Argininemia** | Arginase deficiency | 1:350,000 [242] | Arginine > 100 μmol/L |
| **Classical homocystinuria (HCU)** | Cystationine synthase | 1:200,000 [239] | Methionine, homocysteine, total homocysteine levels |
| **Glutaric aciduria type 1** | Glutaryl-CoA dehydrogenase | 1:100,000 [243] | Glutaric acid and glutarylcarnitine levels |
| **Nonketotic hyperglycinemia (NKH)** | Deficiency in the mitochondrial glycine cleavage system | 1:55,000 [244] | Glycine levels |

Disorders of amino acid metabolism are caused by the deficiency of an enzymatic step which results in the accumulation of metabolites and endogenous product deficiencies [237]. These accumulations mostly affect the brain and liver resulting in varied clinical symptoms after many months or within-day following birth [245, 246]. In the case of late diagnosis and treatment, serious medical consequences including mental retardation, developmental delays and even death may occur with irreversible damages [247]. Therefore, the abnormal physiological levels and dynamic changes of amino acids need to be screened for the diagnosis and management of one or a group of disorders.

### 4.1.6 Analytical methods for amino acid determination

It is well known that physiological levels of a specific or a group of amino acids are directly related to different aminoacidopathies [237]. Besides that, there is also a growing recognition besides the physiological roles of amino acids that may become biomarkers of specific physiological and pathological states. This idea has been enlightened researchers about the evaluation of amino acids for improved non-invasive diagnosis, characterization and prognostic biomarkers [248-250]. This can be achieved by simultaneous detection and quantification of free amino acids in biological fluids including urine, plasma, amniotic fluid and cerebrospinal fluid breast milk. To date, several analytical techniques have been developed for the determination of free amino acids with or without derivatization using GC-MS, HPLC, LC-MS and LC-MS/MS systems. These studies are summarized in Table 4.3.

In the previous reports, the determination of underivatized amino acids with LC systems have carried out using RP separation with the addition of ion-pairing agents into the mobile phases [251], HILIC separation [252-254] or mixed-mode chromatography which comprises hydrophobic chromatography together with anion/cation-exchange mechanisms [255, 256]. The applicability of these separation modes for the determination of amino acids has been successfully tested in biological and environmental samples. The quantification of four amino acids in hydrolyzed protein/peptide structures was reported with the detection

limits ranged between 0.003 - 0.04 nmol $mL^{-1}$ and the developed HILIC method was compared with commercially available derivatization method that included aminoquinolylhydroxysuccinimidyl carbamate as derivatization agent. This comparison revealed that more accurate and precise data were obtained for HILIC separation [252].

The quantification studies of underivatized amino acids were also carried out in human urine and serum as biological samples. Joyce and co-workers reported an analytical method for the determination of 18 free amino acids in urine samples providing detection limits of 0.001 - 0.30 nmol $mL^{-1}$ [254] while Choi and co-workers found the detection limits for 23 free amino acids between 0.010 - 0.50 nmol $mL^{-1}$ using LC-MS/MS in human serum [256]. On the other hand, the determination of amino acids in environmental samples was reported for atmospheric aerosol [255] and soil samples [253] followed by solid phase extraction process. These methods showed satisfactory performance in terms of accuracy and precision.

In recent years, derivatization techniques have been commonly utilized for the determination of amino acids using many types of derivatization reagents including alkyl chloroformates such as ethylchloroformate, methyl chloroformate and propyl chloroformate: ninhydrin; iTRAQ (isobaric tag for relative and absolute quantitation), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and o-phthaldialdehyde for RPLC and GC-MS systems [257].

A variety of methods have been reported for the determination of amino acids in biological, environmental and food samples. GC-MS has been widely used for amino acid profiling in biological fluids such as urine, serum, plasma, whole blood [258-263]; tobacco [264], soybean seed [263] and plant samples [265]. The described methods differed from each other in terms of sample preparation, analysis period, the number of target amino acids, detection limits and accuracy.

Mudiam and coworkers reported very low detection limits in the range of 0.36 – 3.68 ng $mL^{-1}$ applying in situ derivatization and DLLME prior to analysis. This method provided low consumption of organic solvent, fast and simple analysis

procedure, cost-effectiveness and good repeatability. The method was applied to hair, urine and soybean seed samples resulting in acceptable percent recovery values [263]. Li and coworkers also applied the DLLME method based on the solidification of floating organic droplet for the preconcentration of isopropyl chloroformate derivatives of amino acids in tobacco samples [264]. They found detection limits ranged between 0.12 – 2.82 $\mu$g $mL^{-1}$ and indicated that method performance parameters showed satisfactory reproducibility.

RPLC systems with different detectors such as UV, FLD and MS have been also successfully applied for the determination of derivatized amino acids in plasma, urine, serum samples [257, 260, 266-268], animal tissue hydrolysates [269], soil [270], beer [271] and tea samples [272]. Chen and coworkers reported very low detection limits between 0.0005 - 015 nmol $mL^{-1}$ for 20 amino acids using N-phosphorylation labeling prior to LC-MS/MS analysis [268]. They indicated that derivatization greatly improved the chromatographic resolution and the signal intensity for target amino acids.

Krumpochova and coworkers compared different approaches for amino acid determination including GC-MS, RPLC-MS/MS after derivatization with propyl chloroformate and HILIC-MS without derivatization. They also investigated the real-life applicability of each approach for complex biological samples. As a result, it was concluded in this study that HILIC-MS resulted in the least accurate and longest period for analysis. On the other hand, the GC-MS method presented more precise results for some amino acids and the RPLC method was the only method enabled the analysis of all amino acids [269].

The choice of analytical system for the determination of amino acids mostly depends on the critical issues such as the overall set of target amino acids, sample matrices, the expense of systems and the intended use of the analytical method. The derivatization methods usually face many analytical problems such as instability of derivatives, long periods for sample preparation, inability for the derivatization of secondary amino groups and interferences caused by derivatization reagents [255].

Besides that, all analytical procedures still have some drawbacks including low throughput and contamination of the analytical system because of complex matrices. Therefore, it is still a challenging task to develop an analytical method providing simple, fast and robust procedures with accurate and precise results, and high sample throughput.

**Table 4.3** Analytical methods described in the literature for the determination of amino acids

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **without derivatization** | | | | | | | | |
| **[252], 2009** | Ile, Phe, Pro, Val | 0.003 - 0.04 nmol $mL^{-1}$ / 0.01 - 0.10 nmol $mL^{-1}$ | 89 min | - | - | HILIC-ESI-MS | hydrolyzed protein/peptide | 94.6 - 100.8 |
| **[255], 2013** | Phe, Pro, Ile, Leu, Met, Val, Trp, Tyr, Ala, Thr, Gln, Gly, Ser, Asn | 9.5 - 26 ng $mL^{-1}$ | 5.0 min | - | SPE | LC-ESI-MS/MS | atmospheric aerosol | 70 - 103 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[253], 2016** | Ala, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, Sar | 13 -384 ng $g^{-1}$ / 43 - 1267 ng $g^{-1}$ | 12 min | - | SPE | HILIC-ESI-MS/MS | soil | 74 - 117 |
| **[254], 2016** | Phe, Trp, Leu, Ile, Met, Val, Pro, Tyr, Ala, Thr, Gly, Glu, Gln, Ser, Asn, His, Arg, Lys | 0.001 - 0.3 nmol $mL^{-1}$ / 0.01-0.5 nmol $mL^{-1}$ | 15 min | - | - | HILIC-ESI-MS/MS | human urine | - |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[256], 2017** | Ala, Asn, Asp, Cystine, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, Orn | 0.010 - 0.50 nmol $mL^{-1}$ / 1.0 - 5.0 nmol $mL^{-1}$ | 22 min | - | - | LC-ESI-MS/MS | human serum | 56.8 - 118.8 |
| **with derivatization** | | | | | | | | |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[259], 2005** | Ala, Gly, Val, Leu, Ile, Thr, Met, Asp, Gln, Phe, Try, Tyr | 0.16 - 2.44 nmol $mL^{-1}$ | 22 min | N,O-bis(trimethylsilyl) trifluoroacetamide | - | GC-MS | neonatal blood samples | 91 - 103 |
| **[258], 2008** | Ala, Gly, Val, Leu, Ile, Thr, Ser, Pro, Asp, Met, Glu, Phe, Lys, His, Tyr, Cystine | 0.03 - 12 nmol $mL^{-1}$ / 0.3 - 30 nmol $mL^{-1}$ | 30 min | propyl chloroformate | - | GC-MS | biological fluids | 94.3 -123.7 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[271], 2009** | Asn, His, Asp, Glu, Ser, Thr, Gly, Ala, Pro, Met, Val, Trp, Phe, Leu, Ile, Cys, GABA, Tyr, Lys | 2.40 - 6.50 nmol $mL^{-1}$ / 9.60 - 26.0 nmol $mL^{-1}$ | 45 min | 4-chloro-3,5-dinitrobenzotrifluorid | - | HPLC-UV | beer samples | 97.0 - 103.9 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[260], 2009** | Ala, Arg, Asn, Asp, Cys-Cys, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Tau, Thr, Trp, Tyr, Val | 0.3 - 30 nmol $mL^{-1}$ | 20 min | propyl chloroformate | - | GC-MS | urine | 98 - 111 |
| | | 0.5 - 10 nmol $mL^{-1}$ | 25 min | iTRAQ agent | | LC- MS/MS | | 91 - 106 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[266], 2009** | Tau, Asn, Ser, Gln, Gly, Thr, Ala, Vali Tyr, His, Ile, Phe, Orn, Arg | ≤ 5.0 nmol $mL^{-1}$ / 10 nmol $mL^{-1}$ | 30 min | ninhydrin | - | LC- MS/MS | plasma and urine samples | |
| **[270], 2009** | His, Ser, Arg, Gly, Asp, Glu, Thr, Ala, Pro, Cys-cys, Lys, Tyr, Met, Val, Ile, Leu, Phe | 0.20 - 0.60 nmol $mL^{-1}$ | 50 min | 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate | | LC- ESI-MS | soil | 82.8 - 111.0 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[261], 2010** | Val, Leu, Ile, Met, Phe | 1.5 - 24.2 nmol $mL^{-1}$ | 18 min | propyl chloroformate | - | GC-MS | whole blood | > 69.8 |
| **[273], 2010** | Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val | 0.05 - 5.0 nmol $mL^{-1}$/ 0.5 - 15 nmol $mL^{-1}$ | 15 min | methyl chloroformate | - | GC-MS | plant samples | 90.2 - 107.4 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[262], 2010** | D-Ala, L-Ala, Gly, D-Val, D-Leu, L-Ile, D-Pro, D-Thr, L-Thr, D- Asp, D-Ser, L-Ser, D-Met, L-Met, D-Asn, L-Asn, D+L-Phe | 0.029-16.41 nmol $mL^{-1}$/ 0.061-31.25 nmol $mL^{-1}$ | 30 min | methyl chloroformate | - | GC-MS | human serum and urine | 77.2 - 176.1 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[257], 2010** | Glu, Asp, Ser, Gly, Glu, His, Citrulline, Thr, Ala, Arg, Pro, Tyr, Val, Met, Ornithine, Lys, Ile, Leu, Phe, Try | 0.12 - 1.86 nmol $mL^{-1}$ / 0.39 - 6.2 nmol $mL^{-1}$ | 12 min | ninhydrin | - | LC-ESI-MS | human plasma | 96 - 102 |
| **[264], 2013** | Ala, Gly, Val, Leu, Ile, Pro, Asn, Met, Phe, Cys, Tyr | 0.12 – 2.82 µg $mL^{-1}$ / 0.4 - 9.39 µg $mL^{-1}$ | 26 min | isobutyl chloroformate | DLLME | GC-MS | tobacco | 84.6 - 108.5 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[272], 2013** | Asp, Glu, Asn, Ser, Gln, His, Gly, Thr, Cys, Arg, Ala, Theanine, Lys, Tyr, GABA, Met, Val, Trp, Phe, Ile, Leu | 0.2 - 19.2 nmol $mL^{-1}$ / 0.8 - 63.9 nmol $mL^{-1}$ | 30 min | o-Phthaldialdehyde | - | HPLC-FLD | tea samples | - |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[263], 2013** | Ala, Gly, GABA, Val, Leu, Ile, Thr, Ser, Glu, Pro, Asp, Met, Phe, Cys, Lys, His, Tyr, Try, ornithine | 0.36 – 3.68 ng $mL^{-1}$ / 1.26 – 12.01 ng $mL^{-1}$ | 10 min | ethyl chloroformate | DLLME | GC-MS | hair, urine and soybean seed samples | 89 - 100 for urine, 90 - 97 for hair, 91 - 96 for soybean samples |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[267], 2013** | Asp, Asn, Ser, Glu, Gln, Gly, His,, Arg, Thr, Ala, Pro, Tyr, Val, Met, Lys, Ile, Leu, Phe, Try | 0.17 - 8.06 nmol $mL^{-1}$ / 0.52 - 24.42 nmol $mL^{-1}$ | 113.0 min | 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate | - | HPLC-UV and FLD | plasma | 87.6 - 109.3 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[268], 2014** | Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val | 0.0005 - 015 nmol $mL^{-1}$/ 0.0020 - 0.50 nmol $mL^{-1}$ | 20 min | N-phosphorylation labeling | - | RPLC-MS/MS | human serum | 85.5 - 117.4 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[269], 2015** | Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val | 0.05 nmol $mL^{-1}$ | 7.0 min | propyl chloroformate | - | GC-MS | animal tissue hydrolysate | 96.6 - 108.2 |
| | | 0.1-0.5 nmol $mL^{-1}$ | 20 min | propyl chloroformate | | RPLC-MS/MS | | 93.4 - 111.2 |
| | | 0.001-1.0 nmol $mL^{-1}$ / 0.03-100 nmol $mL^{-1}$ | 45 min | - | | HILIC-MS/MS | | 98.6 - 105.3 |

**Table 4.3** Analytical methods described in the literature for the determination of amino acids (continues)

| Ref., Year | Amino acids | LOD/LOQ | Analysis period | Derivatization agent | Preconcentration method | Instrumental technique | Sample | Recovery (%) |
|---|---|---|---|---|---|---|---|---|
| **[265], 2016** | Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val | 0.32 - 8.97 nmol $mL^{-1}$ / 0.64 - 17.97 nmol $mL^{-1}$ | 13 min | methyl chloroformate | SPE | GC-MS | plant samples | 95.0 - 100 |

## 4.2 Determination of Amino Acids without Derivatization

### 4.2.1 Experimental Study

#### 4.2.1.1 Chemicals and reagents

Amino acid mixture was purchased from Sigma-Aldrich containing 2.50 μmol $mL^{-1}$ of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine and 1.25 μmol $mL^{-1}$ of cysteine in 0.1 N HCl. Stable isotope labeled cell free amino acid standard mixture (98 atom % $^{15}N$) consisted of aspartic acid (60 mmol $L^{-1}$, $^{15}N$), threonine (35 mmol $L^{-1}$, $^{15}N$), serine (35 mmol $L^{-1}$, $^{15}N$), glutamic acid (40 mmol $L^{-1}$, $^{15}N$), proline (20 mmol $L^{-1}$, $^{15}N$), glycine (100 mmol $L^{-1}$, $^{15}N$), alanine (100 mmol $L^{-1}$, $^{15}N$), valine (40 mmol $L^{-1}$, $^{15}N$), methionine (10 mmol $L^{-1}$, $^{15}N$), isoleucine (30 mmol $L^{-1}$, $^{15}N$), leucine (45 mmol $L^{-1}$, $^{15}N$), tyrosine (10 mmol $L^{-1}$, $^{15}N$), phenylalanine (16 mmol $L^{-1}$, $^{15}N$), histidine (5.0 mmol $L^{-1}$, $^{15}N$), lysine (15 mmol $L^{-1}$, $^{15}N$), arginine (15 mmol $L^{-1}$, $^{15}N$), glutamine (20 mmol $L^{-1}$, $^{15}N$), asparagine (20 mmol $L^{-1}$, $^{15}N$), tryptophan (20 mmol $L^{-1}$, $^{15}N$) and cysteine (20 mmol $L^{-1}$, $^{15}N$) were also obtained from Sigma-Aldrich.

#### 4.2.1.2 Instrumentation

The positive ion mode of the ESI system was utilized using targeted MS/MS experiments. The detector parameters were as follows: 200 °C source temperature, 10 L $min^{-1}$ drying gas flow rate, 3.0 kV capillary voltage and 150 V fragmentor voltage.

Various analytical columns were performed for chromatographic separation of underivatized amino acids including ACE 3 SIL (100 x 4.6 mm, 3.0 μm); Intrada Imtakt (150 x 3.0 mm, 3.0 μm); and ACE 3 SIL (150 x 3.0 mm, 3.0 μm). In order to achieve maximum chromatographic resolution, different mobile phase systems with various gradient conditions were performed. Herein, some of the studied chromatographic system conditions and resulted chromatograms were presented by numbering each conditions as listed in Table 4.4.

**Table 4.4** Some examples of chromatographic systems tested for the separation of amino acids

| | Mobile phases | Gradient elution | Column | Flow Rate, mL $min^{-1}$ |
|---|---|---|---|---|
| **Condition-1** | 10 mM ammonium formate in ultrapure water at pH 4.50 – A; Acetonitrile - B | 0-1.0 min, 100% B; 1.0-5.0 min, 100-60% B; 5.01-7.0, 25% B; 7.01-8.0, 5.0% B; 8.01-10.5, 100% B | ACE 3 SIL (100 x 4.6 mm, 3.0 µm) | 0.60 |
| **Condition-2** | 100 mM ammonium formate in ultrapure water at pH 3.0 : acetonitrile (9:1) – A Acetonitrile : 100 mM ammonium formate in ultrapure water at pH 3.0 (9:1) - B | 0-3.0 min, 90% B; 3.00-6.30 min, 90-65% B; 6.31-7.0, 65-15% B; 6.31-20.0, 15.0% B; 20.01-24.0, 90% B | ACE 3 SIL (100 x 4.6 mm, 3.0 µm) | 0.60 |
| **Condition-3** | 100 mM ammonium formate in ultrapure water at pH 3.0 – A; Acetonitrile - B | 0-0.50 min, 85% B; 0.50-4.50 min, 85-65% B; 4.51-6.00, 20% B; 6.01-16.0, 0.0% B; 16.01-20.0, 85% B | Intrada Imtakt (150 x 3.0 mm, 3.0 µm) | 0.60 |
| **Condition-4** | 100 mM ammonium formate in ultrapure water at pH 3.0 : acetonitrile (9:1) – A Acetonitrile : 100 mM ammonium formate in ultrapure water at pH 3.0 - B | 0-1.0 min, 90% B; 1.0-4.50 min, 90-60% B; 4.51-15.00, 0% B; 6.01-16.0, 0.0% B; 15.01-20.0, 90% B | Intrada Imtakt (150 x 3.0 mm, 3.0 µm) | 0.60 |
| **Condition-5** | 20 mM ammonium formate in ultrapure water at pH 3.0 : acetonitrile (9:1) – A Acetonitrile : 20 mM ammonium formate in ultrapure water at pH 3.0 (9:1) - B | 0-0.50 min, 90% B; 0.50-3.30 min, 90-75% B; 3.31-6.0, 35% B; 6.01-8.0, 90 % B | ACE 3 SIL (150 x 3.0 mm, 3.0 µm) | 0.60 |

#### 4.2.1.3 Preparation of Real Samples

Human urine and plasma were collected from healthy volunteers in our research laboratory, pooled and filtered using a 0.22 μm PTFE syringe filter. All samples were freshly prepared by spiking different concentrations of amino acid standard solutions. The spiked urine samples were directly injected into the instrument while plasma samples were injected after protein precipitation. Protein precipitation was carried out by adding 400 μL of acetonitrile to 100 μL of plasma. The mixture was vortexed for 15 s and centrifuged for 15 min at 10000 rpm. The supernatant was sent to the instrument after additional filtration using a 0.22 μm PTFE syringe filter. All samples were 20-fold diluted with acetonitrile prior to analysis for matrix matching strategy.

On the other hand, ID$^4$MS was applied to undiluted samples. Three calibration blends at different isotopic ratios ($R_{A*B}$ proportional to 0.50, 1.0 and 2.0) and one sample blend ($R_{AB}$ proportional to 1.0) were prepared using primary, spiked sample and enriched solutions. The required amounts of solutions for the preparation of blends are represented in Table 4.5. All sample and calibration blends were prepared gravimetrically to reduce the measurement uncertainty that comes from the sample preparation. The accuracy was presented as the percent recoveries calculated by comparing theoretical and spiked concentrations of target amino acids, except cystine because cystine was not present in enriched material. Since the concentrations of amino acids in the isotopic mixture and real samples, the ID$^4$MS strategy was carried out at three levels to evaluate the applicability of the method for variable amounts of multiple analytes. A three-point calibration was applied for all levels. These levels were determined as low (25 μmol $kg^{-1}$), mid (80 μmol $kg^{-1}$), and high (140 μmol $kg^{-1}$), according to mean concentrations of amino acids in human urine and plasma.

**Table 4.5** Theoretical masses calculated for the preparation of blends

| | Human plasma | | Human urine | | |
|---|---|---|---|---|---|
| **Blend** | **A or A*, g** | **B, g** | **A or A*, g** | **B, g** | **Proportional Target Ratio** |
| **AB** | 0.1934 | 0.200 | 0.3000 | 0.3000 | 1.0000 |
| **A*B-1** | 0.0946 | 0.200 | 0.1439 | 0.3000 | 0.5000 |
| **A*B-2** | 0.1934 | 0.200 | 0.3000 | 0.3000 | 1.0000 |
| **A*B-3** | 0.3922 | 0.200 | 0.6135 | 0.3000 | 2.0000 |

### 4.2.2 Results and Discussion

#### 4.2.2.1 Chromatographic Separation

Since it is not mandatory to separate all identical peaks of analytes for LC-MS/MS studies, good chromatographic resolution generally results in higher analytical selectivity and sensitivity. Considering the amino acid mixture including acidic, basic and neutral amino acids together, the complete separation was not expected. Chromatographic separation in this study aimed to achieve maximum possible separation with sharp peak shapes. Therefore, different mobile phase systems with various gradient elutions were performed to achieve satisfactory chromatographic separation with the best resolution.

In the first trial, Condition 1 was tested and the resulted chromatograms are represented in Figure 4.3. As seen in this figure, peak tailing and broadening occurred for valine, serine, aspartic acid and glutamic acid when the pH of mobile phase A was 4.50. At the second stage, the mobile phase pH value was adjusted to 3.0 and Condition 2 was utilized.

As seen in Figure 4.4, acidic mobile phase conditions resulted in sharper chromatographic peaks except for basic amino acids including lysine, arginine and

histidine. Therefore, acidic pH was used for the next studies and another analytical column was tried to obtain better chromatographic peak shapes for basic amino acids. The resulted chromatograms for Intrada column utilizing Condition 3 is presented in Figure 4.5. Herein, the peak shapes of some amino acids were broad and tailing. Additionally, two different peaks were observed for lysine. Therefore, mobile phase composition and gradient elution were changed to obtain better chromatographic peaks. The extracted ion chromatogram obtained for Condition 4 is presented in Figure 4.6. Most of the peaks were sharp and also separated adequately from each other. However, the analysis period was long (20.0 min) and the chromatographic peak of arginine was broad. Therefore, the different analytical column was performed utilizing Condition 5.

Finally, well-resolved peaks with sharp peak shapes were obtained with sufficient separation. In addition to that, the chromatographic run was only 8.0 min. Sharp quantifiable peaks were obtained for all analytes between retention times of 2.91-5.60 minutes. Therefore, the proposed chromatographic system was used for further validation of the analytical method. The chromatographic parameters including injection volume, flow rate and column temperature have been studied to obtain optimum values. The optimum injection volume, flow rate and column temperature were obtained as 5.0 μL, 0.60 mL/min and 35.0 °C, respectively. The resulted chromatograms are represented in Figure 4.7.

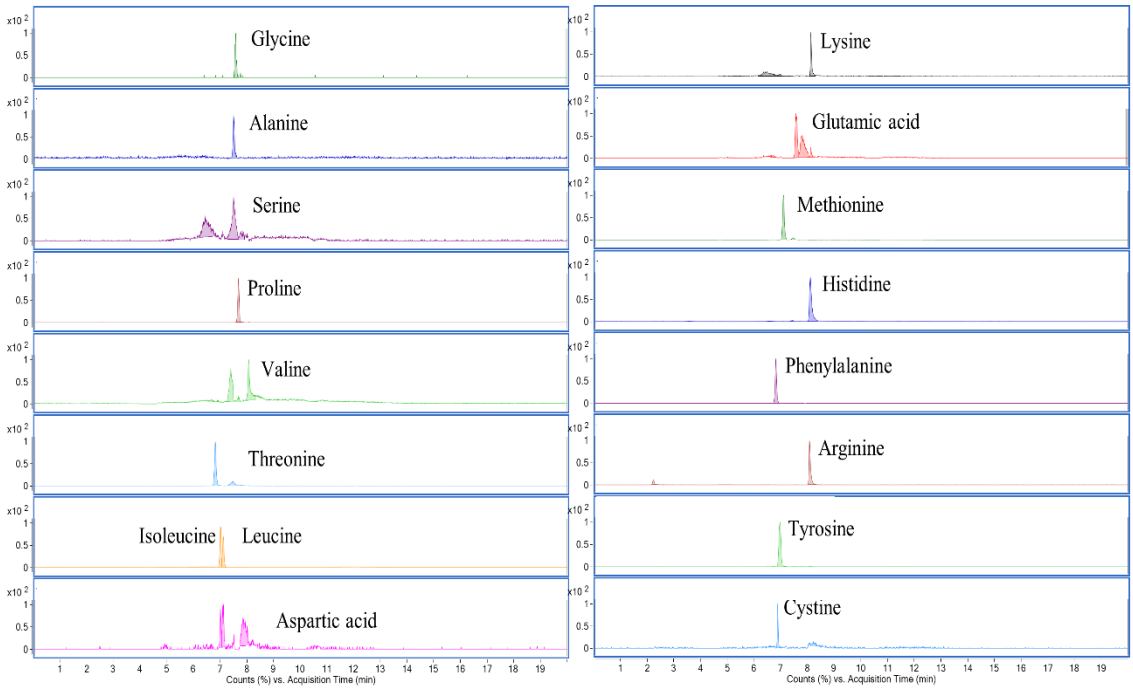



**Figure 4.3** Extracted ion chromatograms of amino acids for Condition 1

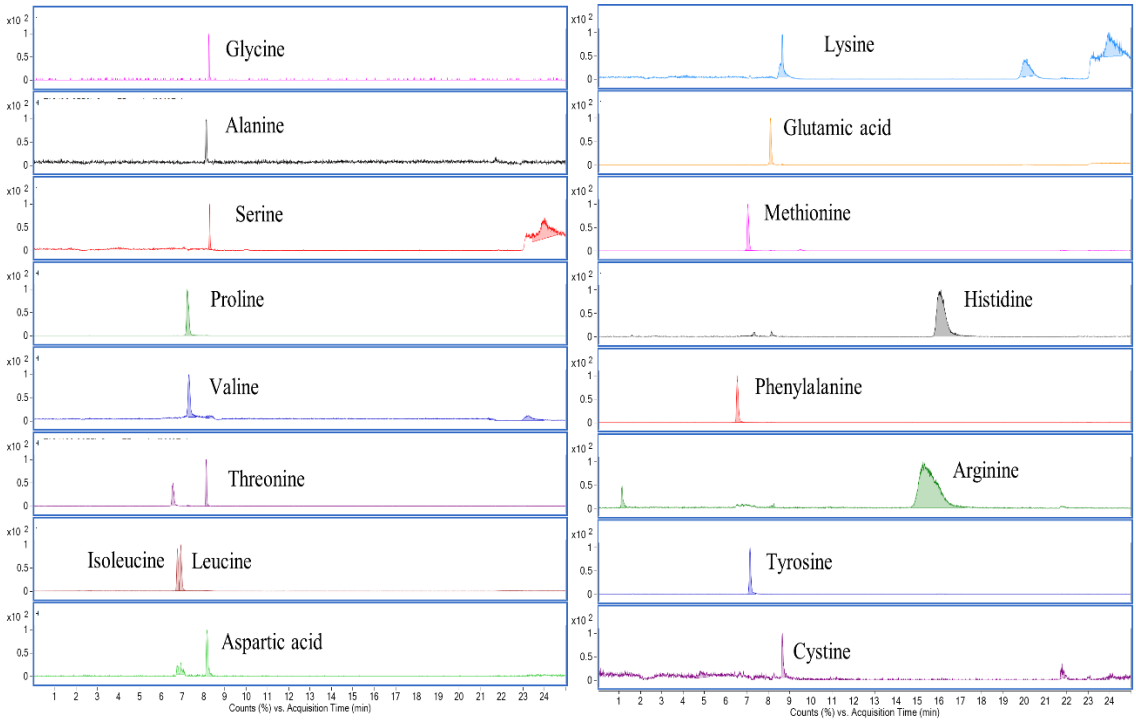



**Figure 4.4** Extracted ion chromatograms of amino acids for Condition 2

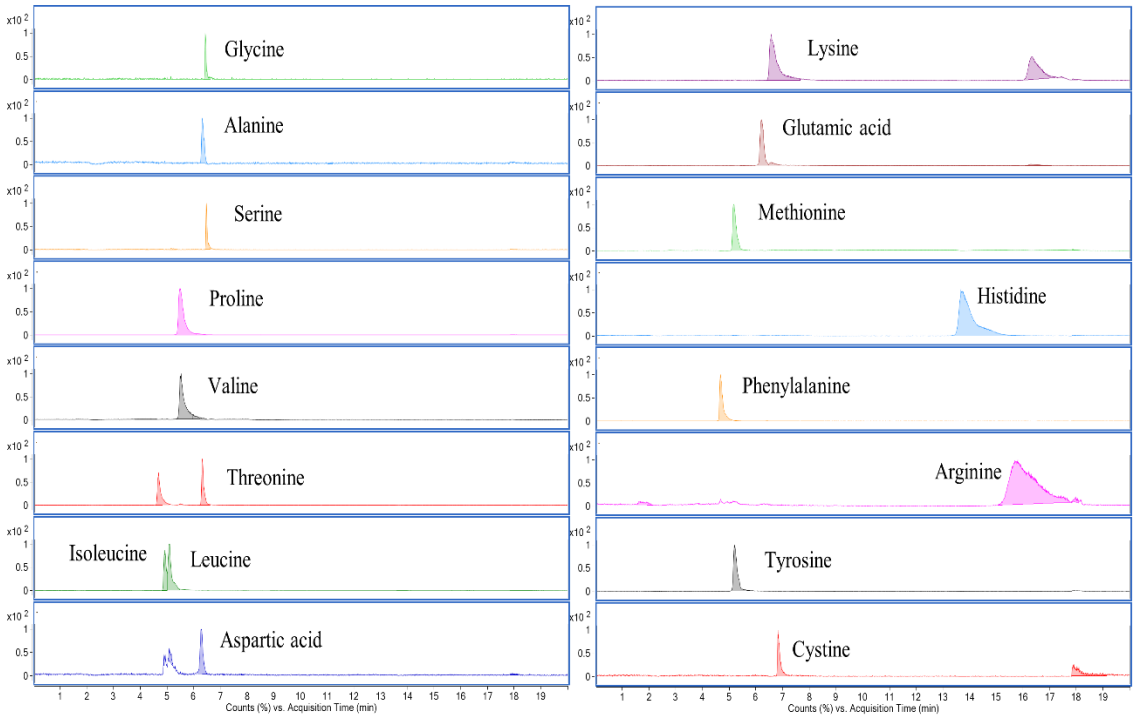



**Figure 4.5** Extracted ion chromatograms of amino acids for Condition 3

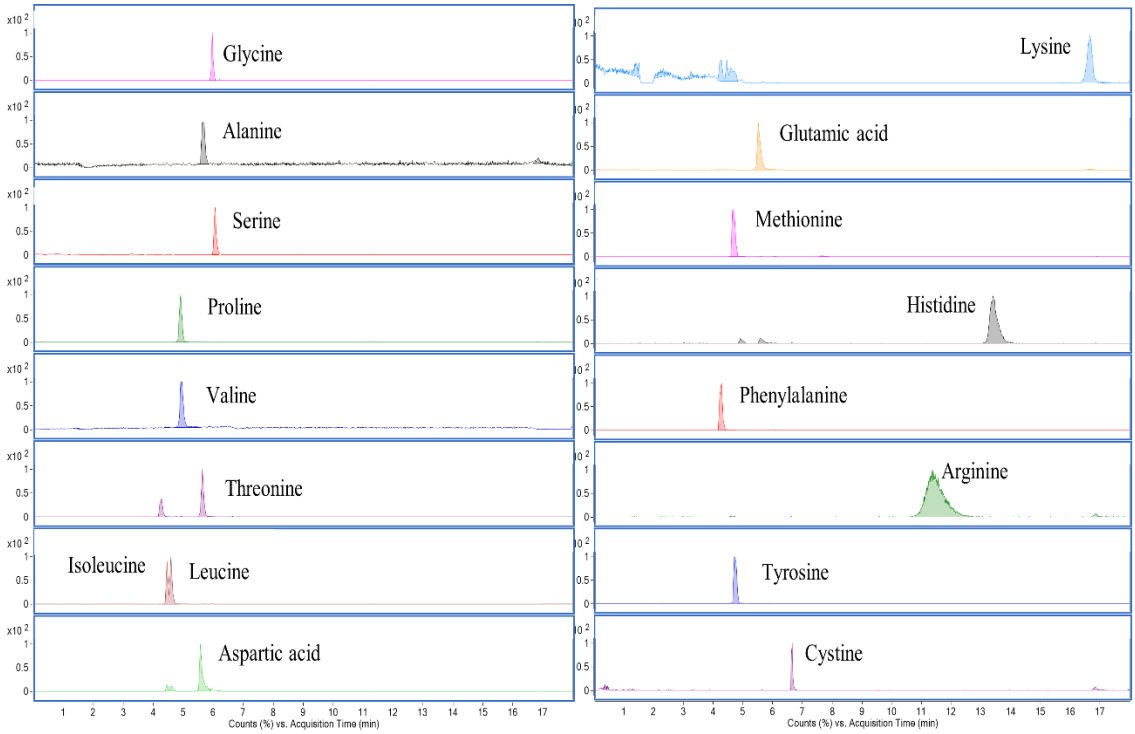



**Figure 4.6** Extracted ion chromatograms of amino acids for Condition 4

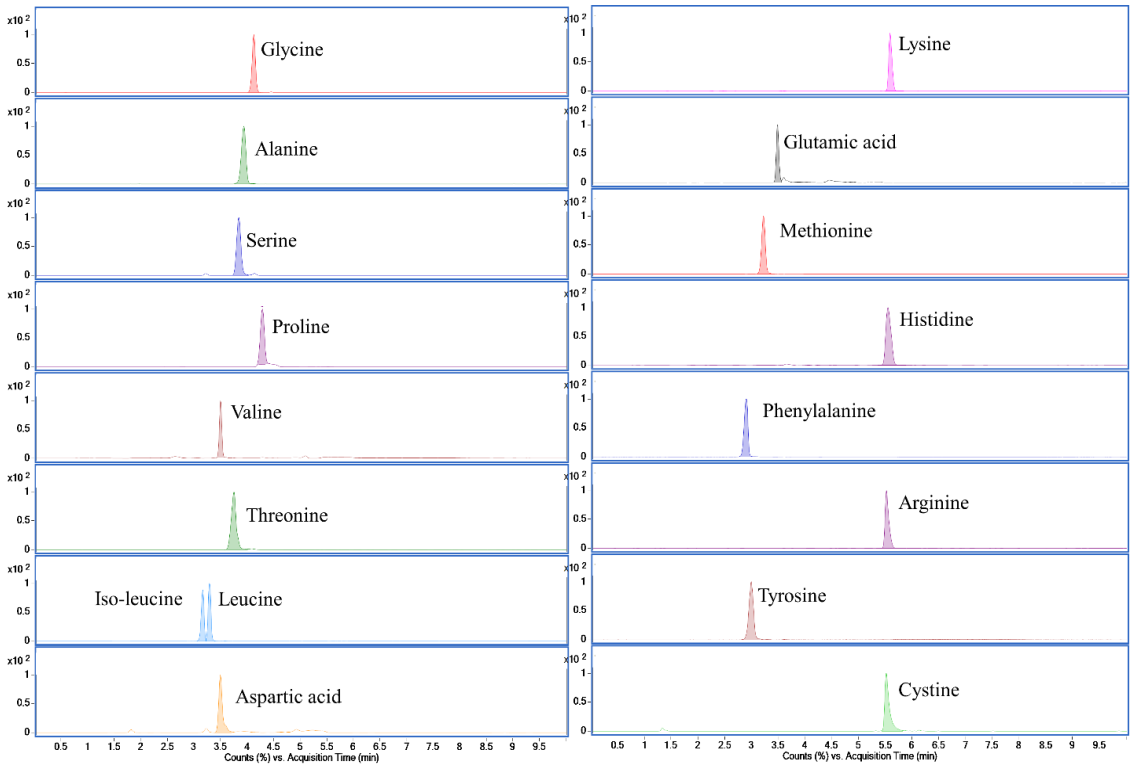



**Figure 4.7** Extracted ion chromatograms of amino acids for Condition 5

#### 4.2.2.2 Tandem Mass Spectrometry

In this study, the tandem mass spectrometry (MS/MS) method was used for the qualitative and quantitative determination of amino acids. Therefore, the targeted mode was applied to analytes after selecting the mass-to-charge-ratios (m/z) of analytes and their retention times. Fragmentation of analytes was applied by collision-induced dissociation (CID) and, the most intensive fragment ion was selected as quantifier while the second was chosen as qualifier. The precursor, quantifier and qualifier ions of each amino acids were listed with their retention times and applied collision energies in Table 4.6.

**Table 4.6** The summary of precursor, quantifier and qualifier ions, retention times and collision energies used for amino acids

| Analyte | Precursor ion (M+H)+ | Quantifier (m/z) | Qualifier (m/z) | CE (V) | RT (min) |
|---|---|---|---|---|---|
| **Alanine** | 90.055 | 44.0448 | 55.0526 | 15 | 4.003 |
| **Arginine** | 175.1198 | 70.0651 | 60.0553 | 25 | 5.566 |
| **Aspartic acid** | 134.0448 | 74.021 | 88.037 | 15 | 3.500 |
| **Cystine** | 241.0311 | 120.0092 | 74.0218 | 15 | 5.524 |
| **Glycine** | 76.0378 | 30.0328 | 43.0178 | 5 | 4.147 |
| **Glutamic acid** | 148.0604 | 84.0444 | 130.0494 | 15 | 3.509 |
| **Histidine** | 156.0768 | 110.0713 | 83.0599 | 15 | 5.608 |
| **Iso-leucine** | 132.1019 | 86.0954 | 69.0691 | 10 | 3.281 |
| **Leucine** | 132.1019 | 86.0954 | 44.0495 | 10 | 3.146 |
| **Lysine** | 147.1128 | 84.0808 | 130.086 | 15 | 5.600 |
| **Methionine** | 150.0583 | 56.0490 | 104.0529 | 10 | 3.230 |
| **Phenylalanine** | 166.0863 | 120.0797 | 131.0484 | 10 | 2.917 |
| **Proline** | 116.0706 | 70.0651 | 43.0538 | 10 | 4.290 |
| **Serine** | 106.0499 | 60.0472 | 42.0319 | 10 | 3.859 |
| **Threonine** | 120.0655 | 74.059 | 56.0495 | 15 | 3.757 |
| **Tyrosine** | 182.0812 | 91.0532 | 136.075 | 30 | 3.019 |
| **Valine** | 118.0863 | 72.0807 | 55.0541 | 10 | 3.551 |

#### 4.2.2.3 Method Validation

The calibration plot for each amino acid was constructed by analyzing amino acid calibration standard solutions at the concentration range of 0.25 – 100 μmol $kg^{-1}$ by LC-QTOF-MS/MS system. All experiments were carried out at least triplicates and the average values of triplicate measurements were used to construct calibration curve. The working range of each analyte was determined according to the linear region of the calibration curve. Peak areas of each analytes were plotted vs. actual concentrations, and linearity were evaluated according to correlation coefficient ($R^2$ > 0.99). The analytical figures of merit for the developed method is summarized in Table 4.7. LOD and LOQ values of amino acids were ranged between 0.03 – 2.26 μmol $kg^{-1}$ and 0.09 – 4.96 μmol $kg^{-1}$, respectively. The developed method showed good linearity for each analyte with $R^2$ values of higher than 0.9978. The repeatability of the method at the lowest concentration was varied between 2.3% and 12.3%.

**Table 4.7** Analytical figures of merit of amino acids for the developed method

| Analyte | LOD (µmol kg$^{-1}$) | LOQ (µmol kg$^{-1}$) | Linear Range (µmol kg$^{-1}$) | $R^2$ | %RSD | Repeatability and Reproducibility, %RSD | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 0.80 µmol kg$^{-1}$ | | | 8.0 µmol kg$^{-1}$ | | | 20.0 µmol kg$^{-1}$ | | |
| | | | | | | Intra-day | Inter-days | Inter-analysts | Intra-day | Inter-days | Inter-analysts | Intra-day | Inter-days | Inter-analysts |
| **Alanine** | 0.13 | 0.45 | 0.50-25 | 0.9993 | 8.4 | 5.1 | 7.9 | 8.2 | 3.1 | 9.6 | 6.2 | 1.5 | 4.3 | 2.3 |
| **Arginine** | 0.14 | 0.46 | 0.50-25 | 0.9991 | 6.5 | 3.4 | 11.5 | 7.6 | 1.5 | 7.2 | 7.2 | 1.3 | 6.4 | 4.2 |
| **Aspartic acid** | 1.27 | 4.25 | 5.0-100 | 0.9995 | 2.8 | - | - | - | 3.3 | 7.4 | 9.1 | 6.7 | 9.2 | 8.2 |
| **Cystine*** | 1.11 | 3.71 | 3.75-50 | 0.9990 | 3.5 | - | - | - | 3.3 | 4.6 | 3.4 | 3.5 | 4.8 | 4.8 |
| **Glutamic acid** | 0.98 | 3.27 | 5.0-75 | 0.9998 | 12.0 | - | - | - | 1.2 | 3.3 | 3.7 | 0.5 | 1.4 | 1.0 |
| **Glycine** | 1.49 | 4.96 | 5.0-100 | 0.9997 | 9.3 | - | - | - | 2.8 | 8.7 | 9.2 | 0.5 | 1.9 | 1.9 |
| **Histidine** | 0.17 | 0.57 | 0.50-75 | 0.9990 | 12.3 | 8.4 | 8.7 | 8.7 | 4.4 | 7.4 | 4.1 | 2.2 | 5.9 | 4.6 |
| **Iso-leucine** | 0.03 | 0.09 | 0.10-25 | 0.9994 | 3.1 | 6.6 | 5.7 | 6.6 | 4.4 | 8.0 | 6.8 | 2.3 | 3.2 | 3.5 |
| **Leucine** | 0.08 | 0.25 | 0.25-75 | 0.9998 | 2.6 | 7.4 | 8.0 | 8.9 | 0.9 | 9.4 | 9.2 | 2.1 | 6.5 | 6.5 |
| **Lysine** | 0.29 | 0.96 | 1.0-100 | 0.9992 | 11.0 | - | - | - | 4.8 | 8.6 | 9.4 | 5.0 | 8.5 | 6.8 |
| **Methionine** | 0.07 | 0.24 | 0.25-25 | 0.9998 | 3.0 | 7.8 | 11.3 | 8.3 | 5.9 | 4.5 | 4.4 | 1.2 | 5.2 | 6.5 |
| **Phenylalanine** | 0.05 | 0.16 | 0.25-7.5 | 0.9989 | 3.2 | 5.5 | 7.8 | 6.4 | - | - | - | - | - | - |
| **Proline** | 0.03 | 0.09 | 0.10-1.0 | 0.9978 | 4.5 | 0.9 | 4.9 | 1.9 | - | - | - | - | - | - |
| **Serine** | 0.20 | 0.66 | 0.75-25 | 0.9995 | 2.9 | - | - | - | 2.4 | 7.2 | 5.2 | 4.3 | 4.5 | 5.4 |
| **Threonine** | 2.26 | 7.54 | 7.50-100 | 0.9999 | 2.7 | - | - | - | 7.6 | 11.0 | 9.4 | 5.2 | 5.4 | 5.2 |
| **Tyrosine** | 0.07 | 0.23 | 0.25-10 | 0.9983 | 2.3 | 3.7 | 7.6 | 5.9 | 0.7 | 6.3 | 3.9 | - | - | - |
| **Valine** | 0.16 | 0.52 | 0.50-7.50 | 1.0000 | 4.9 | 9.0 | 6.6 | 7.1 | - | - | - | - | - | - |

*The concentrations of cystine for precision assay were 0.40, 4.0 and 10 µmol kg$^{-1}$.

The precision of the developed method was evaluated in terms of intra-day, inter-days and inter-analysts using control standards at the concentrations of 0.80, 8.0 and 20.0 μmol $kg^{-1}$. The standards were analyzed repeatedly five times within a single day for intra-day, for three consecutive days for inter-days and also they were prepared by three different analysts to determine precision. The precision was evaluated as %RSD and presented in Table 4.7. The results of %RSD were varied between 0.5% - 9.0% for intra-day, 1.4% - 11.5% for inter-day and 1.0% - 9.4% for inter-analyst studies which confirmed the acceptable limits for precision.

#### 4.2.2.4 Extraction Rates and Matrix Effect

Plasma samples were prepared for analysis using the protein precipitation method to avoid possible clogging of the system, unstable background and high interference. The extraction rates were assessed to determine analyte losses during the precipitation procedure. The spiking experiments before and after precipitation protocol were carried out and the signals were compared for the evaluation of extraction rates. The resulting data for extraction rates are represented in Table 4.8. The extraction rates were varied between 90.0% - 99.5% with %RSD values of 0.7% - 7.8% for 4 amino acids. Lower extraction rates were obtained as 82.8%, 88.8% and 77.9% with %RSD below than 5.4% for alanine, arginine and aspartic acid. The results showed that the quantitative determination of amino acids can be carried out with proper adjustments regarding extraction rates.

The matrix effect is a major problem for electrospray ionization mainly caused by competition between complex matrix components and target molecules. The matrix effect may result in ion enhancement as well as ion suppression. The control and prediction of the matrix effect can be very difficult because of the variability of sample components. In this study, the effect of matrix components on electrospray ionization efficiency was evaluated by the percentage obtained from analyte peak areas in real samples ($A_{sample}$) divided by standard solutions ($A_{standard}$). Equation 4.1 was utilized for the calculation of the percent matrix effect (%ME). The %ME results for human urine and plasma samples are represented in Figure 4.8 and 4.9, respectively.

$$\%ME = \left(\frac{A_{sample}}{A_{standard}} - 1\right) \times 100 \quad (4.1)$$

As seen in Figure 4.8 and 4.9, ion suppression at high levels was observed for the majority of target analytes in both samples. The percentage of matrix effect for human urine and plasma samples was found to be between 3.6-95.7% and -8.25-94.8%, respectively.

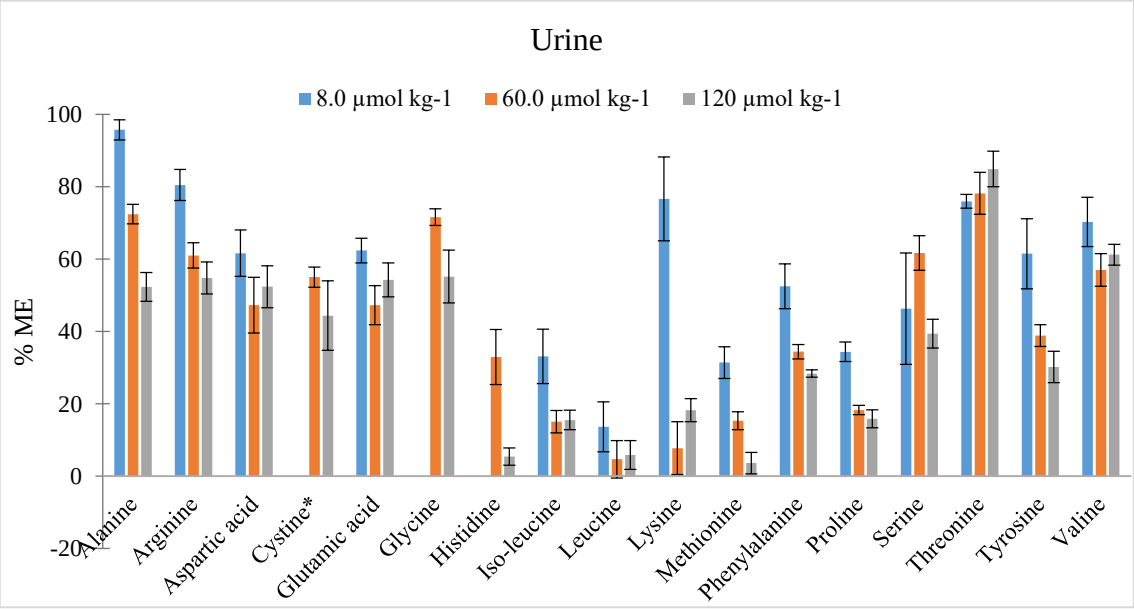



**Figure 4.8** %ME of amino acids in human urine

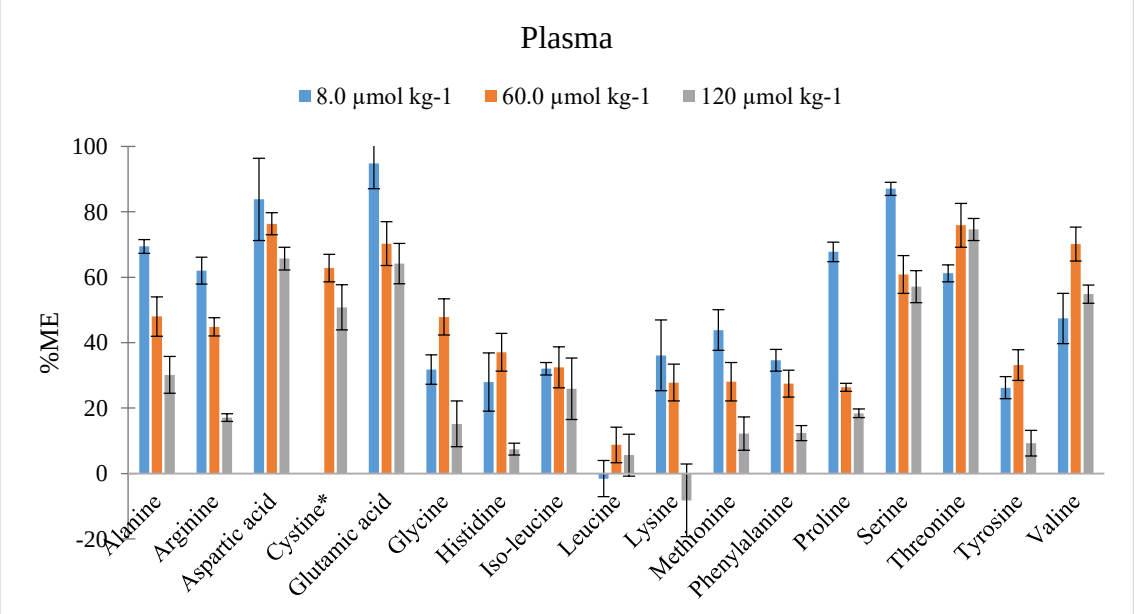



**Figure 4.9** %ME of amino acids in human plasma

#### 4.2.2.5 Recovery

To compensate for the matrix effect, the matrix matching method was applied to urine and plasma samples. Different concentrations of amino acids were spiked to real samples to construct the calibration plots and the percent recovery values were calculated using the linear equations generated from the calibration plots followed by a blank correction. The obtained percent recovery values for human plasma and urine are presented in Table 4.8 and 4.9, respectively.

As represented in Table 4.8, the percent recovery values for plasma samples are varied between 77.9 and 127.4% with %RSDs ranged from 1.3 to 13.5%. As represented in Table 4.9, the percent recovery values for urine samples were varied between 80.1 and 127.2% with %RSDs ranged from 1.9 to 12.1%. The acceptable percent recovery values confirmed the application of the proposed method to urine and plasma samples with satisfactory accuracy and precision. To increase the accuracy and precision of the proposed method, isotopic dilution strategy was applied for further studies.

**Table 4.8** The percent recovery values and extraction rates for different spiked concentrations of amino acids in human plasma

| Analyte | Recovery (%) | | | | | Extraction Rates (%) |
|---|---|---|---|---|---|---|
| | 4.0 µmol $kg^{-1}$ | 8.0 µmol $kg^{-1}$ | 20 µmol $kg^{-1}$ | 40 µmol $kg^{-1}$ | 80 µmol $kg^{-1}$ | |
| **Alanine** | - | 109.9 ± 2.1 | 98.7 ± 3.8 | 99.8 ± 4.0 | 94.9 ± 2.5 | 82.8 ± 5.4 |
| **Arginine** | 90.9 ± 1.3 | 98.0 ± 4.1 | - | 77.9 ± 4.8 | 107.1 ± 4.3 | 88.8 ± 3.3 |
| **Aspartic acid** | - | - | - | - | 88.0 ± 2.5 | 77.9 ± 4.8 |
| **Cystine** | - | - | - | 110.1 ± 7.7 | - | 93.0 ± 2.6 |
| **Glutamic acid** | - | - | - | 99.1 ± 3.2 | 98.0 ± 2.4 | 99.5 ± 1.7 |
| **Glycine** | - | - | - | - | 102.4 ± 7.1 | 90.1 ± 3.0 |
| **Histidine** | - | 99.3 ± 8.9 | 118.0 ± 5.3 | 94.8 ± 6.2 | 94.1 ± 8.8 | 97.6 ± 3.6 |
| **Iso-leucine** | 104.4 ± 6.0 | 91.3 ± 1.9 | 108.6 ± 4.3 | 93.4 ± 8.6 | 106.2 ± 1.7 | 90.6 ± 3.5 |
| **Leucine** | 91.5 ± 4.2 | 105.2 ± 5.5 | 96.6 ± 4.3 | 84.0 ± 6.0 | 99.2 ± 5.9 | 93.2 ± 1.8 |
| **Lysine** | - | - | - | - | 95.7 ± 11.3 | 96.2 ± 4.1 |
| **Methionine** | 80.5 ± 8.7 | 89.4 ± 6.2 | 116.2 ± 3.6 | 86.2 ± 5.6 | 105.4 ± 6.0 | 96.3 ± 1.2 |
| **Phenylalanine** | 80.3 ± 6.1 | 109.2 ± 3.3 | 105.5 ± 3.8 | 89.7 ± 4.1 | 103.3 ± 3.6 | 99.0 ± 7.8 |
| **Proline** | - | 123.3 ± 3.0 | 103.3 ± 3.9 | 88.0 ± 3.0 | 97.9 ± 3.1 | 96.5 ± 2.2 |
| **Serine** | - | - | 107.4 ± 5.9 | 86.7 ± 4.2 | 96.2 ± 9.3 | 92.5 ± 2.8 |
| **Threonine** | - | 114.5 ± 2.6 | 111.6 ± 13.5 | - | 98.3 ± 2.8 | 92.3 ± 0.7 |
| **Tyrosine** | - | - | 117.4 ± 3.3 | 88.3 ± 7.0 | 127.4 ± 3.9 | 97.4 ± 2.6 |
| **Valine** | - | - | - | - | 95.1 ± 6.7 | 97.5 ± 3.0 |

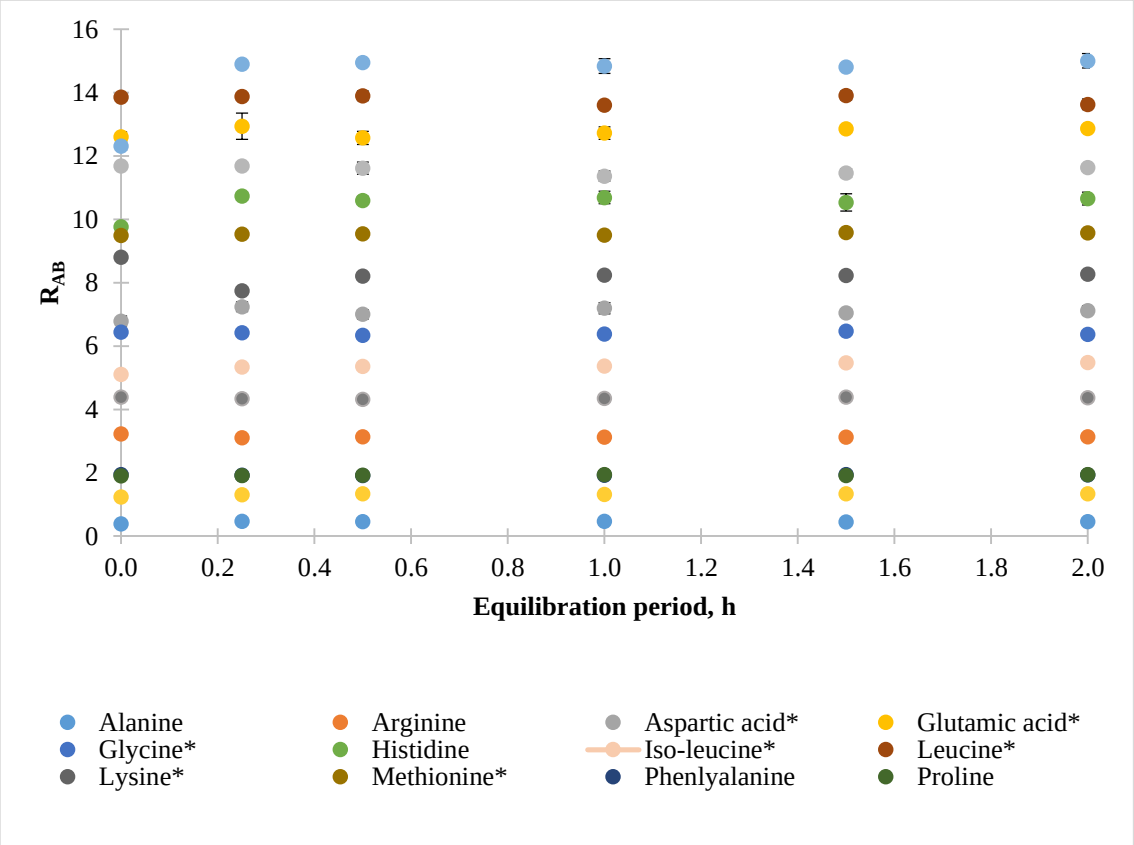
**Table 4.9** The percent recovery values for different spiked concentrations of amino acids in human urine

| Analyte | Recovery (%) | | | | |
|---|---|---|---|---|---|
| | **4.0 µmol kg$^{-1}$** | **8.0 µmol kg$^{-1}$** | **20 µmol kg$^{-1}$** | **40 µmol kg$^{-1}$** | **80 µmol kg$^{-1}$** |
| **Alanine** | - | - | - | - | 98.3 ± 2.6 |
| **Arginine** | - | 92.8 ± 4.3 | 96.4 ± 6.4 | 101.6 ± 4.4 | 103.0 ± 2.5 |
| **Aspartic acid** | - | - | 127.2 ± 12.1 | 80.1 ± 5.3 | 103.3 ± 6.0 |
| **Cystine** | - | - | - | 114.5 ± 6.3 | - |
| **Glutamic acid** | - | - | - | 95.7 ± 2.0 | 106.2 ± 5.4 |
| **Glycine** | - | - | - | - | 103.5 ± 5.9 |
| **Histidine** | - | - | - | 101.5 ± 1.9 | 102.2 ± 2.9 |
| **Iso-leucine** | 108.0 ± 5.6 | 88.9 ± 7.5 | 103.5 ± 9.3 | 93.9 ± 3.5 | 106.9 ± 6.7 |
| **Leucine** | 97.5 ± 7.1 | 97.6 ± 6.9 | 104.3 ± 3.3 | 92.8 ± 4.3 | 100.9 ± 7.2 |
| **Lysine** | - | - | - | 106.7 ± 9.0 | 91.2 ± 11.8 |
| **Methionine** | 88.6 ± 5.4 | 101.8 ± 4.4 | 91.3 ± 10.9 | 107.6 ± 1.9 | 105.5 ± 8.4 |
| **Phenylalanine** | 84.6 ± 5.9 | 94.3 ± 6.2 | 98.2 ± 2.9 | 103.0 ± 5.5 | 99.1 ± 1.9 |
| **Proline** | 94.6 ± 4.3 | 95.5 ± 2.7 | 98.7 ± 3.2 | 103.0 ± 3.1 | 105.1 ± 2.4 |
| **Serine** | - | - | - | 93.5 ± 9.1 | 113.9 ± 12.0 |
| **Threonine** | - | 89.4 ± 1.9 | 118.1 ± 6.1 | 103.4 ± 3.2 | 95.6 ± 2.9 |
| **Tyrosine** | 89.9 ± 3.0 | 119.3 ± 9.7 | 96.6 ± 3.1 | - | 98.8 ± 3.0 |
| **Valine** | - | - | 86.4 ± 5.4 | 101.0 ± 11.4 | 95.7 ± 8.9 |

#### 4.2.2.6 ID⁴MS

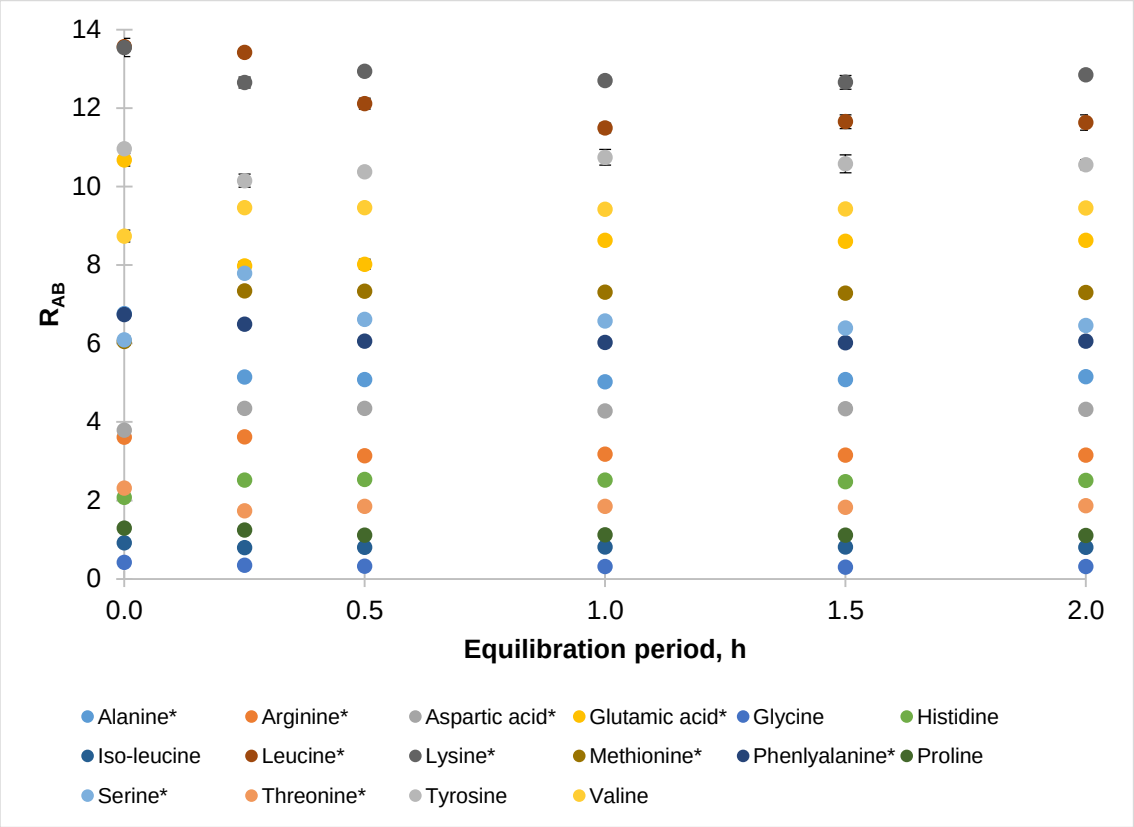
- ***Isotopic equilibration***

In the present work, the isotopic equilibration has been studied for 16 amino acids in two different biological matrices, human urine and plasma, separately. For this purpose, natural and spike blends were prepared triplicate for each period and different periods including 0.25, 0.50, 1.00, 1.50 and 2.00 h were tested to investigate the adequate period for isotopic equilibration. The results for human urine and plasma are presented in Figure 4.10 and 4.11, respectively.




* represents the normalized values for better visibility

**Figure 4.10** The investigation of the period for isotopic equilibration in human urine




* represents the normalized values for better visibility

**Figure 4.11** The investigation of the period for isotopic equilibration in human plasma

As seen in Figure 4.10 and 4.11, adequate periods for equilibrium were different for each analyte. To ensure isotopic equilibration for all analytes, the equilibration period was determined according to the analyte which had the longest equilibration period. Therefore, 1.0 h was chosen as an optimum equilibration period for both urine and plasma samples.

- ***Direct ID⁴MS***

ID⁴MS strategy was applied for the determination of 16 amino acids in human urine and plasma samples. The actual target blend ratios were different for each amino acids due to the concentration differences in the enriched material and the actual weighed amounts of samples and standards. The analysis of sample, calibration and blank blends were performed after waiting for 1.0 to reach isotopic equilibration.

The actual weighed amounts were used for the calculations and calculations were done separately for 16 different amino acids.

A representative example calculations are given for valine in Table 4.10 and 4.11 for human urine, for phenylalanine in Table 4.12 and 4.13 for human plasma. A representative illustration of the relationship between the measured and theoretical isotopic ratios for blends of spike and the primary standard are also presented Figure 4.12 and 4.13 for valine in human urine and plasma, respectively. A similar procedure was performed for amino acids in different matrices and the obtained percent recovery values are represented in Table 4.14 and 4.15 for human urine and plasma, respectively.

**Table 4.10** Actual weighed amounts for the preparation of blends and measured ratio values (An example for valine in human urine, mid level)

| **Human urine** | | **Valine, µmol kg$^{-1}$** | |
|---|---|---|---|
| $\omega_A$ | | 80.000 | |
| $\omega_B$ | | 80.000 | |
| $\omega_{A^*}$ | | 159.988 | |
| **Blend** | **$m_{A(AB)}$/g**<br>**A, Blank or A*, g** | **$m_{B(AB)}$/g**<br>**B, g** | **$r_{A^*B}$ (measured)** |
| **AB-Blank** | 0.2990 | 0.3014 | 0.0824 |
| | 0.3000 | 0.2999 | 0.0845 |
| | 0.3004 | 0.2999 | 0.0842 |
| **AB** | 0.3005 | 0.2997 | 0.7501 |
| | 0.3003 | 0.3003 | 0.7515 |
| | 0.3003 | 0.3003 | 0.7539 |
| **A*B-1** | 0.1442 | 0.3001 | 0.3465 |
| | 0.1438 | 0.3009 | 0.3417 |
| | 0.1437 | 0.3000 | 0.3419 |
| **A*B-2** | 0.3002 | 0.3004 | 0.7509 |
| | 0.3001 | 0.2997 | 0.7515 |
| | 0.3003 | 0.2994 | 0.7524 |
| **A*B-3** | 0.6296 | 0.3000 | 15.476 |
| | 0.6297 | 0.3016 | 15.450 |
| | 0.6298 | 0.3001 | 15.456 |

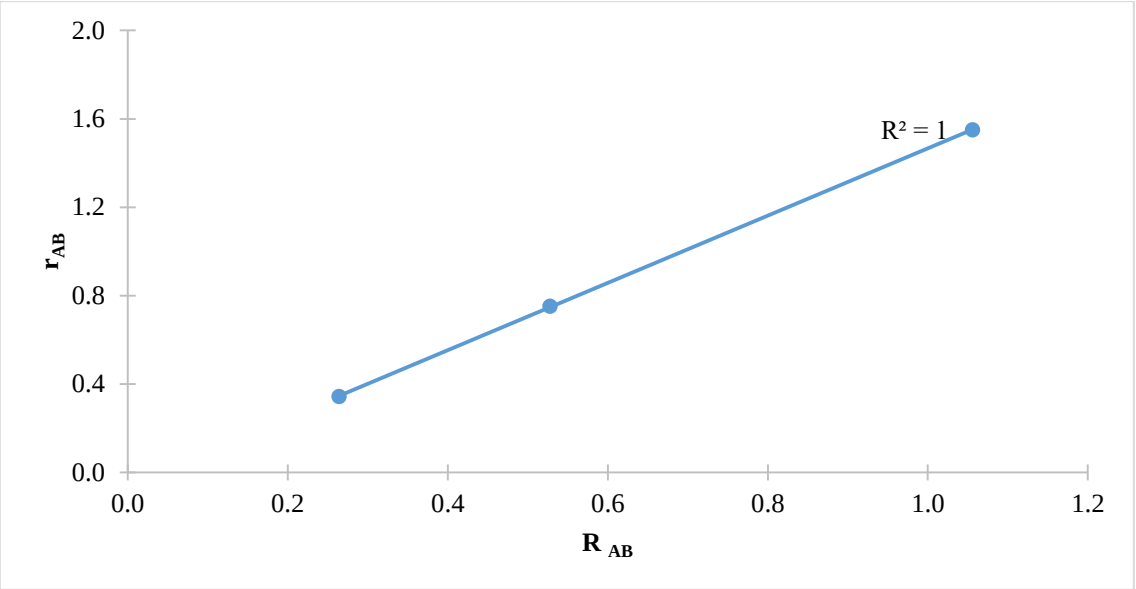



**Figure 4.12** Theoretical blend ratio vs. measured blend ratio (An example for valine in human urine, mid level)

**Table 4.11** The results obtained from the ID⁴MS model (An example for valine in human urine, mid level)

| Sample blends | $r_{AB}$ | $w_A$, µmol kg$^{-1}$ | Experimental amount, Average, µmol kg$^{-1}$ | %RSD | Theoretical spiked amount, µmol kg$^{-1}$ | Recovery, % |
|---|---|---|---|---|---|---|
| **AB-1** | 0.7501 | 79.6526 | | | | |
| **AB-2** | 0.7515 | 80.1605 | 80.0251 | **0.41%** | 80.0000 | **100.03%** |
| **AB-3** | 0.7539 | 80.2621 | | | | |

**Table 4.12** Actual weighed amounts for the preparation of blends and measured ratio values (An example for phenylalanine in human plasma, low level)

| **Human plasma** | | **Phenylalanine, µmol kg$^{-1}$** | |
|---|---|---|---|
| $\omega_A$ | | 25.2342 | |
| $\omega_B$ | | 21.1386 | |
| $\omega_{A^*}$ | | 25.1625 | |
| **Blend** | **$m_{A(AB)}$/g**<br>**A, Blank or A*, g** | **$m_{B(AB)}$/g**<br>**B, g** | **$r_{A^*B}$ (measured)** |
| **AB-Blank** | 0.1942 | 0.2003 | 0.6027 |
| | 0.1938 | 0.1994 | 0.6140 |
| | 0.1940 | 0.1998 | 0.6029 |
| **AB** | 0.1936 | 0.1999 | 0.9040 |
| | 0.1937 | 0.2006 | 0.8816 |
| | 0.1936 | 0.2006 | 0.9067 |
| **A*B-1** | 0.0951 | 0.2006 | 0.4630 |
| | 0.0948 | 0.2001 | 0.4882 |
| | 0.0946 | 0.2011 | 0.4497 |
| **A*B-2** | 0.1929 | 0.2000 | 0.9283 |
| | 0.1934 | 0.2003 | 0.8982 |
| | 0.1934 | 0.1998 | 0.8972 |
| **A*B-3** | 0.3921 | 0.2000 | 1.8127 |
| | 0.3923 | 0.2001 | 1.7389 |
| | 0.3922 | 0.2031 | 1.7612 |

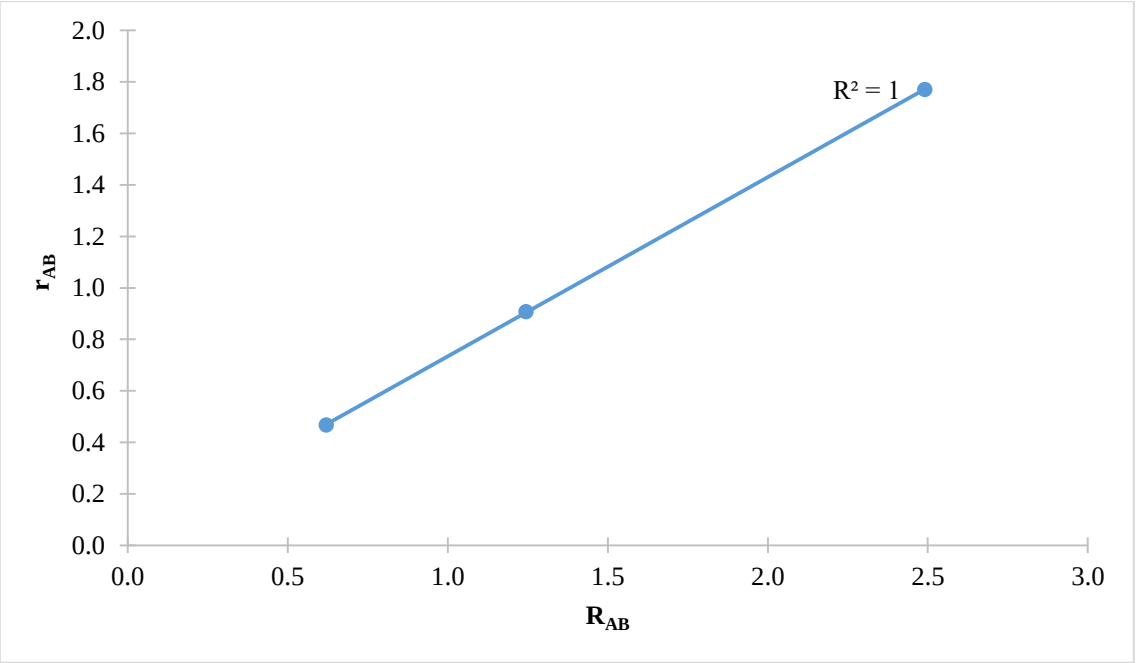



**Figure 4.13** Theoretical blend ratio vs. measured blend ratio (An example for phenylalanine in human plasma, low level)

**Table 4.13** The results obtained from the ID⁴MS model (An example for phenylalanine in human plasma, low level)

| Sample blends | $r_{AB}$ | $w_A$, µmol kg$^{-1}$ | Experimental amount, Average, µmol kg$^{-1}$ | %RSD | Theoretical spiked Amount, µmol kg$^{-1}$ | Recovery, % |
|---|---|---|---|---|---|---|
| **AB-1** | 0.9040 | 24.7453 | | | | |
| **AB-2** | 0.8816 | 24.9912 | 25.1523 | **1.41%** | 25.2342 | **99.31%** |
| **AB-3** | 0.9067 | 25.4434 | | | | |

**Table 4.14** The accuracy results obtained from the ID$^4$MS model for 16 amino acids in human urine

| | Low level | | Mid level | | High level | |
|---|---|---|---|---|---|---|
| **Analyte** | **Recovery** | **%RSD** | **Recovery** | **%RSD** | **Recovery** | **%RSD** |
| **Alanine** | - | - | 100.42 | 0.78 | 99.22 | 0.80 |
| **Arginine** | 101.78 | 0.76 | 100.69 | 1.26 | 99.23 | 0.65 |
| **Aspartic acid** | - | - | - | - | 99.42 | 0.78 |
| **Glutamic acid** | - | - | - | - | 100.37 | 0.67 |
| **Glycine** | - | - | - | - | 99.75 | 0.66 |
| **Histidine** | 100.92 | 2.44 | 100.62 | 1.15 | 99.50 | 0.21 |
| **Isoleucine** | - | - | 99.93 | 0.65 | 99.35 | 1.03 |
| **Leucine** | - | - | 100.77 | 0.80 | 100.74 | 1.21 |
| **Lysine** | - | - | 100.13 | 0.62 | 99.14 | 0.96 |
| **Methionine** | 100.98 | 0.70 | 100.37 | 0.93 | 99.26 | 1.05 |
| **Phenylalanine** | 101.78 | 2.28 | 100.55 | 0.47 | 99.70 | 0.63 |
| **Proline** | 101.50 | 0.80 | 100.29 | 0.84 | 99.41 | 0.68 |
| **Serine** | - | - | - | - | 99.98 | 0.45 |
| **Threonine** | - | - | - | - | 99.49 | 0.24 |
| **Tyrosine** | - | - | 100.89 | 0.85 | 100.16 | 2.21 |
| **Valine** | - | - | 100.03 | 0.41 | 99.13 | 0.93 |

**Table 4.15** The accuracy results obtained from ID$^4$MS model for 16 amino acids in human plasma

| | Low level | | Mid level | | High level | |
|---|---|---|---|---|---|---|
| **Analyte** | **Recovery** | **%RSD** | **Recovery** | **%RSD** | **Recovery** | **%RSD** |
| **Alanine** | 99.63 | 0.85 | 99.65 | 0.83 | 99.76 | 0.25 |
| **Arginine** | 99.59 | 0.88 | 100.42 | 0.67 | 101.14 | 0.72 |
| **Aspartic acid** | - | - | - | - | 100.37 | 0.93 |
| **Glutamic acid** | - | - | 100.17 | 0.15 | 99.95 | 0.86 |
| **Glycine** | - | - | - | - | 100.82 | 0.79 |
| **Histidine** | - | - | 99.93 | 0.87 | 99.69 | 0.37 |
| **Isoleucine** | 100.14 | 1.52 | 99.82 | 0.57 | 100.59 | 1.02 |
| **Leucine** | 99.29 | 0.70 | 100.41 | 0.82 | 101.29 | 0.79 |
| **Lysine** | - | - | - | - | 100.33 | 1.00 |
| **Methionine** | 100.05 | 0.69 | 101.04 | 1.28 | 98.79 | 1.56 |
| **Phenylalanine** | 99.68 | 1.36 | 100.72 | 0.79 | 99.39 | 1.11 |
| **Proline** | 101.68 | 1.40 | 100.59 | 0.45 | 100.22 | 0.80 |
| **Serine** | - | - | - | - | 99.75 | 0.96 |
| **Threonine** | - | - | - | - | 100.45 | 0.12 |
| **Tyrosine** | - | - | 100.15 | 0.64 | 99.28 | 1.62 |
| **Valine** | 98.70 | 0.56 | 100.00 | 0.80 | 100.48 | 0.89 |

As seen in Figure 4.12 and 4.13, good agreement was achieved for measured and theoretical values with high correlation coefficients. As summarized in Table 4.14, the percent recovery for urine samples was ranged between 99.13 - 101.78% providing high precision values between 0.21 – 2.44%. As represented in Table 4.15, the percent recovery values for human plasma were varied between 98.70% - 101.68% with precision values in the range of 0.12 - 1.62%. As a consequence, the ID$^4$MS strategy was applied to human urine and plasma sample successfully and this strategy provided superior accuracy and precision in complex biological samples. The results demonstrated the high metrological quality of ID$^4$MS strategy over the traditional methods.

## 4.3 Determination of Amino Acids with Derivatization

### 4.3.1 Experimental Study

#### 4.3.1.1 Chemicals and reagents

2-Naphthoyl chloride (98%) was obtained from Sigma-Aldrich and used as a derivatization agent. Sodium carbonate ($Na_2CO_3$) was also supplied from Sigma Aldrich and used to adjust the pH of solutions.

#### 4.3.1.2 Instrumentation

The detection of amino acid derivatives was carried out in positive ion - targeted MS/MS mode of the system with the following parameters: 300 °C source temperature, 10 L min$^{-1}$ drying gas flow rate, 4.0 kV capillary voltage and 200 V fragmentor voltage.

An Agilent Poroshell 120 column (EC-C18, 3.0 × 150 mm, 2.7 µm) was used for the chromatographic separation of amino acid derivatives. Mobile phase combinations consisted of 20 mM ammonium formate in ultrapure water at pH 3.0 : acetonitrile (9:1) as mobile phase A and acetonitrile : 20 mM ammonium formate in ultrapure water at pH 3.0 (9:1) as mobile phase B was utilized for chromatographic separation. The following gradient elution was applied at a flow rate of 0.40 mL min$^{-1}$: 0-0.60 min, 15% B; 0.60-2.0 min, 15-45% B; 2.0-4.0 min, 45%B; 4.0-7.0 min, 45-

80% B; 7.0-8.0 min, 80% B and 8.01-12.0 min, 15% B. The volume of injection was 20.0 µL and the temperature of the column oven was adjusted to 30.0 °C.

#### 4.3.1.3 Preparation of Samples/Standards

Serum and urine samples were taken from healthy volunteers and pooled before use. All sample and standard solutions were prepared using the derivatization procedure. Briefly, 0.50 g of sample/standard solution was taken into a microcentrifuge tube. This was followed by the addition of 50 uL of 150 mM $Na_2CO_3$ in ultrapure water and 50 uL of 2-naphthoyl chloride in acetone (at a concentration of 10,000 mg $kg^{-1}$). The resulting mixtures were sonicated for 70.0 min to complete the derivatization reaction. Serum and urine samples were 10-fold diluted using ultrapure water before derivatization. Protein precipitation was performed for serum samples by the addition of acetone with the final concentration of 11.0% (w/w). The resulting samples were vortexed, centrifuged and the supernatant was transferred to a vial for analysis.

To apply the ID$^4$MS strategy, isotopically enriched material was also derivatized before spiking into samples/standards. The theoretically required amounts of sample, enriched and primary solutions are presented in Table 4.16. Accuracy and precision of the ID$^4$MS strategy were evaluated at three different concentration levels: 5.0 (low), 20.0 (mid) and 40.0 (high) µmol $kg^{-1}$ for all amino acids.

**Table 4.16** Theoretical masses calculated for the preparation of blends

| Blend | A or A*, g | B, g | Proportional Target Ratio |
|---|---|---|---|
| **AB** | 0.1675 | 0.1675 | 1.0000 |
| **A*B-1** | 0.0835 | 0.1675 | 0.5000 |
| **A*B-2** | 0.1675 | 0.1675 | 1.0000 |
| **A*B-3** | 0.3325 | 0.1675 | 2.0000 |

### 4.3.2 Results and Discussions

#### 4.3.2.1 Derivatization and chromatographic separation

The derivatization of amino acids for chromatographic separation and determination generally provides better detection and simplifies the chromatographic system. Furthermore, the high polarity of amino acids makes their separation difficult on reversed phase systems. In this study, acyl chloride labeling of amino acids was studied and confirmed by the identification of formed derivatives using Q-TOF MS/MS.

For amino acids containing more than one amino groups, the derivatization could occur with multiple acyl groups while the others derivatized with one acyl group. For example, lysine has two amino groups and could be labeled from either one or two amino groups, as illustrated in Figure 4.14. Extracted ion chromatograms presented in Figure 4.15 showed that both derivatives of lysine were observed, while a single labelled molecule resulted to double peaks, a double labelled molecule had a sharp single chromatographic peak. Therefore, double labelled molecule of lysine selected for quantification.

For histidine, arginine and cystine, only single labelled forms were observed in the total ion chromatogram. All amino acid derivatives were fragmented in the collision cell and confirmed with product ions. A representative fragmentation of the lysine derivative is illustrated in Figure 4.16. As seen in that figure, the fragments of the derivatization agent were observed as m/z 155.0478 and 127.0532 by the cleavage of C-N bond. The characteristics peaks of lysine were also observed with ions at m/z 238.1213 with the subsequent loss of $H_2O$ and at m/z 84.0805 upon the loss of $NH_3$ and the elimination of $CO_2$+$H_2O$ from lysine structure. The precursor and product ions, collision energies and retention times of amino acid derivatives are listed in Table 4.17.

The extracted ion chromatograms 17 amino acids are presented in Figure 4.17. Each amino acids showed well-resolved single peaks with satisfactory peak shapes. The retention times of amino acids were varied between 3.91 and 8.24 minutes. The total chromatographic run was 12.0 min.

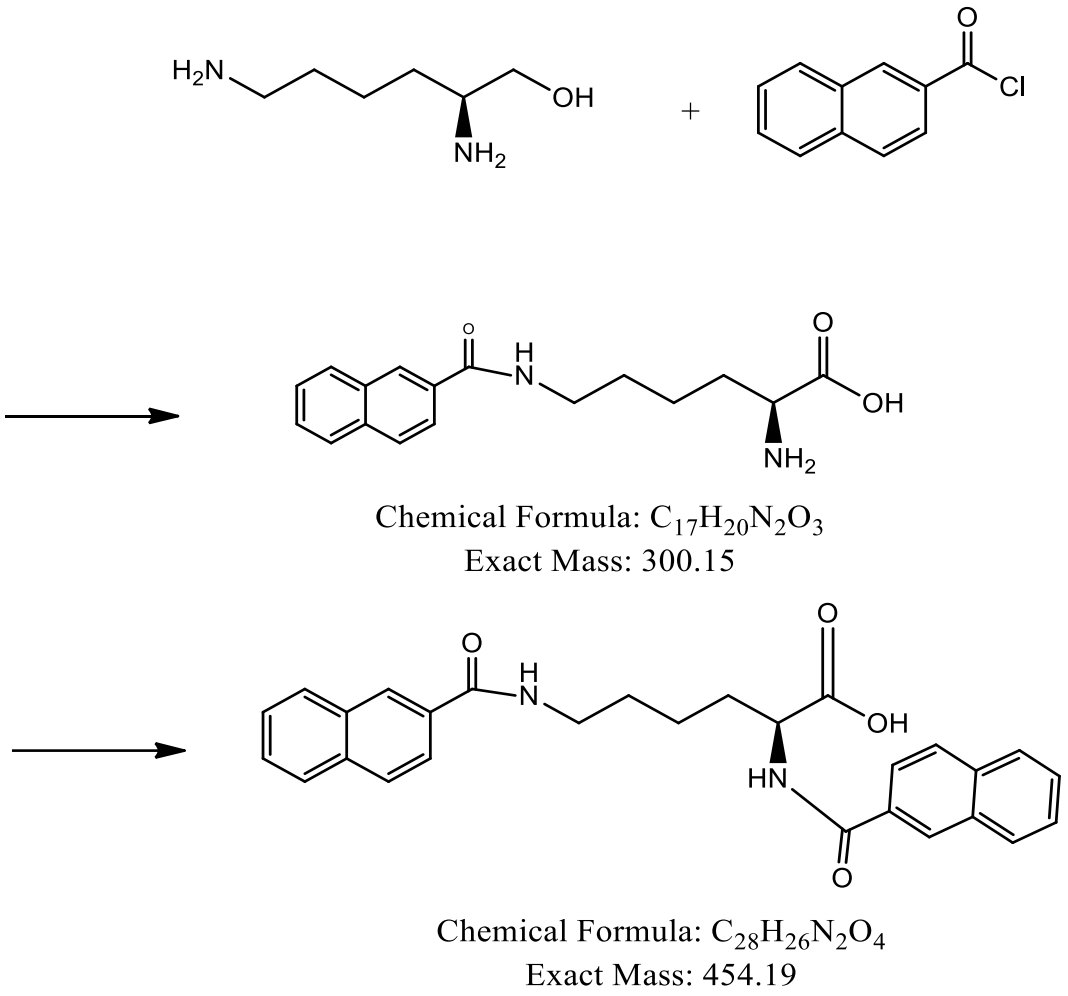



**Figure 4.14** The reaction scheme of 2-Naphtoyl chloride with amine groups on lysine

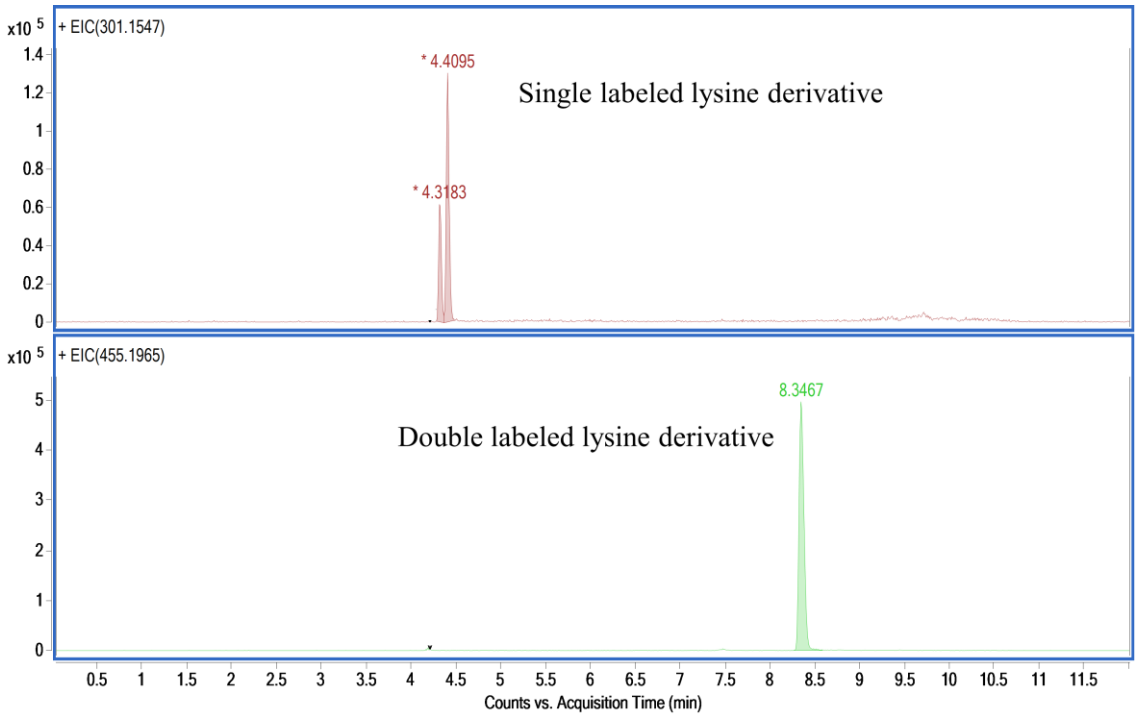



**Figure 4.15** Extracted ion chromatograms of single and double labelled lysine derivatives

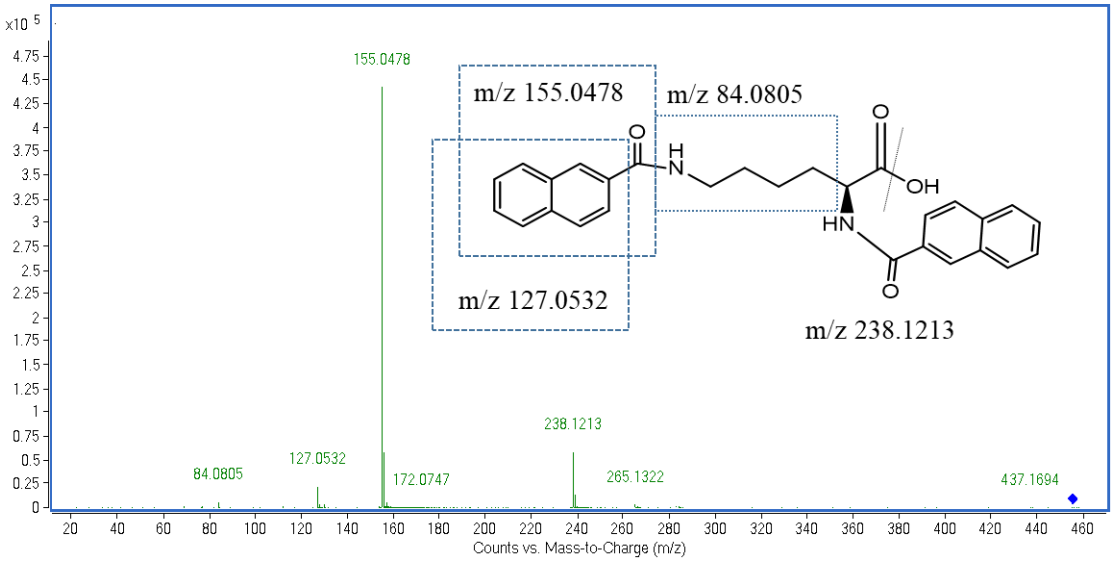



**Figure 4.16** Fragmentation and tandem mass spectrum of lysine derivative

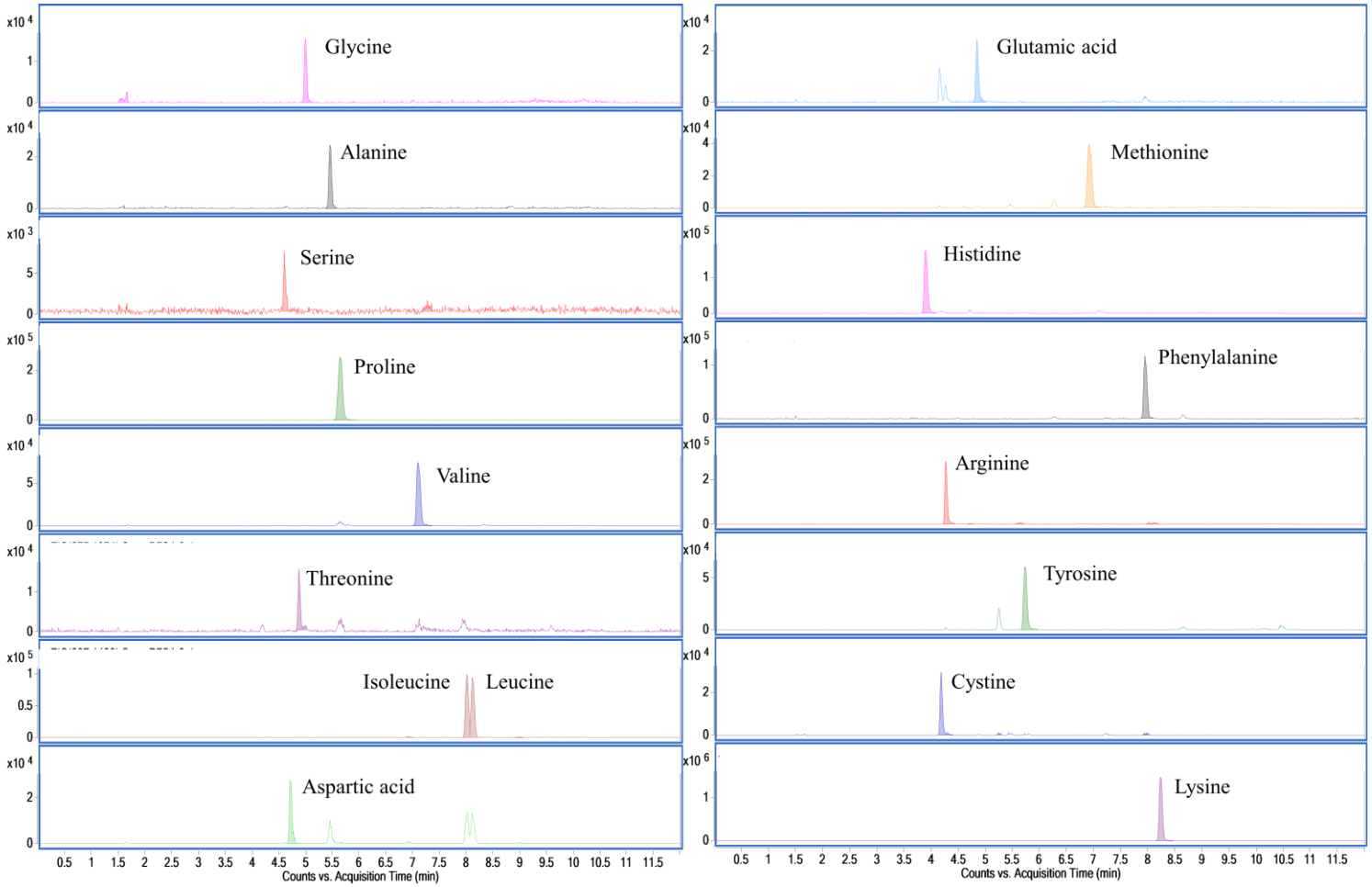



**Figure 4.17** Extracted ion chromatograms of amino acid derivatives

**Table 4.17** Retention times, collision energies, precursor and product ions of amino acid derivatives used in this study

| Amino acid derivatives | Precursor ion (M+H)+ | Quantifier (m/z) | RT (min) | CE (V) |
|---|---|---|---|---|
| **Alanine** | 244.0968 | 44.0431 | 5.4604 | 30 |
| **Arginine** | 329.1608 | 70.0653 | 4.2835 | 30 |
| **Aspartic acid** | 288.0866 | 155.0475 | 4.7146 | 25 |
| **Cystine** | 395.0730 | 274.0554 | 4.1842 | 15 |
| **Glycine** | 230.0812 | 77.0491 | 5.0046 | 10 |
| **Glutamic acid** | 302.1023 | 130.0499 | 4.8555 | 10 |
| **Histidine** | 310.1186 | 110.0695 | 3.9125 | 15 |
| **Iso-leucine** | 286.1438 | 86.1089 | 8.0107 | 15 |
| **Leucine** | 286.1438 | 86.1089 | 8.1185 | 15 |
| **Lysine** | 238.1219 | 238.1219 | 8.237 | 15 |
| **Methionine** | 304.1002 | 104.0672 | 6.919 | 10 |
| **Phenylalanine** | 320.1281 | 120.0978 | 7.9549 | 10 |
| **Proline** | 270.1125 | 70.0652 | 5.6345 | 10 |
| **Serine** | 260.0918 | 127.0568 | 4.6151 | 30 |
| **Threonine** | 274.1074 | 127.0568 | 4.8803 | 35 |
| **Tyrosine** | 336.1231 | 136.0768 | 5.7339 | 10 |
| **Valine** | 272.1281 | 120.0978 | 7.1013 | 10 |

#### 4.3.2.2 Optimization of Derivatization

The experimental variables of derivatization reaction including the buffer concentration, reagent concentration and reaction period were optimized using BBD. The design was established with 17 trials consisting of 5 center points. The design matrix is presented in Table 4.18. The multiple function representing the responses of 17 amino acids was used to obtain the model equation. The model equation was generated as follows:

$$R_m = -17.40 + 0.314A + 0.00115B + 0.0863C + 0.000013AB - 0.00136A^2 - 0.00000016B^2 - 0.00060900C^2 \quad (4.2)$$

The ANOVA results of the model are summarized in Table 4.19. The model presented good agreement between observed and predicted data with high $R^2$ (0.988) and adjusted $R^2$ (0.979). Low probability and non-significant lack of fit showed that the model was satisfactory with the 95% confidence level.

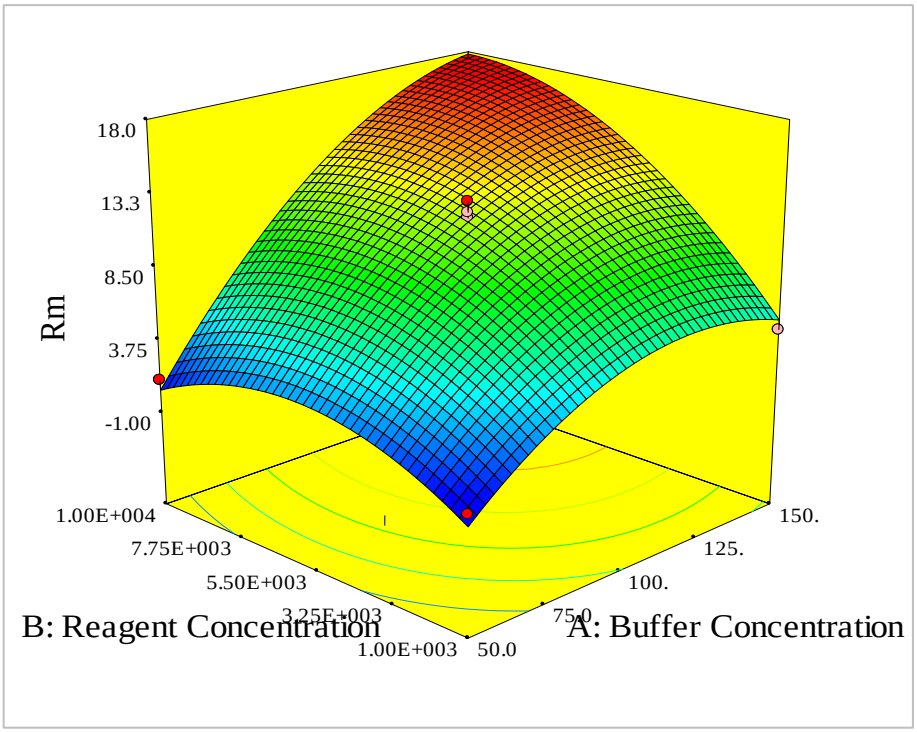
The main and interactive effects of parameters are presented by 3-D response surface and one factor plots in Figure 4.18. It was observed that the concentrations of buffer and derivatization reagent were the most influential parameters on the response, unlike ultrasonication period, which was not significant. Besides that, a significant interaction between buffer and reagent concentration was also observed. Therefore, maximum concentrations of buffer and reagent (150 mM buffer and 10,000 mg $kg^{-1}$ reagent) were selected as optimum values. Addition to that, 70.0 min ultrasonication period was selected with respect to quadratic effect of this parameter.

**Table 4.18** BBD design matrix for derivatization reaction

| Run | Factor 1 | Factor 2 | Factor 3 | Response |
|---|---|---|---|---|
| | A:Buffer Concentration | B:Reagent Concentration | C:Ultrasonication Period | Rm |
| | mM | mg $kg^{-1}$ | min | |
| **1** | 150 | 5500 | 10 | 12.80 |
| **2** | 100 | 1000 | 10 | 3.81 |
| **3** | 100 | 10000 | 10 | 10.00 |
| **4** | 100 | 1000 | 120 | 3.98 |
| **5** | 50 | 5500 | 10 | 0.58 |
| **6** | 150 | 5500 | 120 | 14.20 |
| **7** | 150 | 10000 | 65 | 17.10 |
| **8** | 100 | 5500 | 65 | 11.80 |
| **9** | 100 | 5500 | 65 | 11.80 |
| **10** | 100 | 5500 | 65 | 12.20 |
| **11** | 50 | 1000 | 65 | 0.31 |
| **12** | 50 | 10000 | 65 | 1.16 |
| **13** | 100 | 5500 | 65 | 12.90 |
| **14** | 100 | 5500 | 65 | 12.90 |
| **15** | 100 | 10000 | 120 | 11.50 |
| **16** | 50 | 5500 | 120 | 0.66 |
| **17** | 150 | 1000 | 65 | 4.52 |

**Table 4.19** ANOVA results for the model of derivatization reaction

| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F |
|---|---|---|---|---|---|
| **Model** | 507 | 7 | 72.4 | 109 | < 0.0001 |
| **A-Buffer Concentration** | 264 | 1 | 264 | 399 | < 0.0001 |
| **B-Reagent Concentration** | 92.2 | 1 | 92.2 | 139 | < 0.0001 |
| **C-Ultrasonication Period** | 44562 | 1 | 44562 | 31048 | 0.207 |
| **AB** | 34.4 | 1 | 34.4 | 52.1 | < 0.0001 |
| **$A^2$** | 48.4 | 1 | 48.4 | 73.2 | < 0.0001 |
| **$B^2$** | 41.2 | 1 | 41.2 | 62.4 | < 0.0001 |
| **$C^2$** | 43904 | 1 | 43904 | 44003 | 0.00120 |
| **Residual** | 34820 | 9 | 0.661 | | |
| **Lack of Fit** | 29312 | 5 | 0.961 | 12844 | 0.132 |
| **Pure Error** | 42005 | 4 | 0.287 | | |
| **Cor Total** | 513 | 16 | | | |
| **$R^2$** | 0.988 | | | | |
| **Adj $R^2$** | 0.979 | | | | |



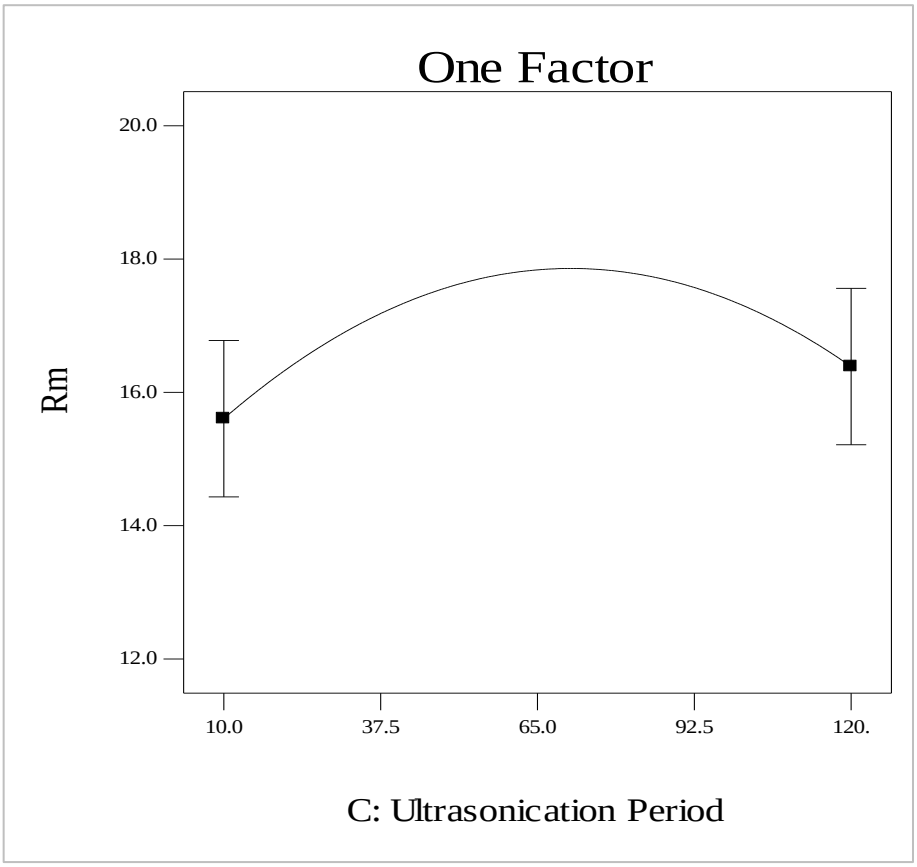




**Figure 4.18** The main and interactive effects of parameters on the response

#### 4.3.2.3 Method Validation

Standard solutions were prepared at different concentrations of amino acids in the range of 0.06 – 100 μmol $kg^{-1}$ and derivatization procedure was applied to standard solutions under the optimum conditions. The analytical figures of merit for the developed method is represented in Table 4.20. All amino acids showed good linearity with $R^2$ values above 0.9992. LOD and LOQ values were varied between 0.015 – 0.266 μmol $kg^{-1}$ and 0.051 – 0.888 μmol $kg^{-1}$, respectively. The precision of the method was evaluated at three different levels with intra-day, inter-days and inter-analysts assays. The precision was found to be between 2.7 – 14.1% for low level (0.60 μmol $kg^{-1}$), 3.7 – 12.2% for mid-level (2.50 μmol $kg^{-1}$) and 4.3 - 11.9% for high level (10.0 μmol $kg^{-1}$).

**Table 4.20** Analytical figures of merit of amino acids for the derivatization method

| Analyte | LOD (µmol kg$^{-1}$) | LOQ (µmol kg$^{-1}$) | Linear Range (µmol kg$^{-1}$) | $R^2$ | %RSD | Repeatability and Reproducibility, %RSD | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 0.60 µmol kg$^{-1}$ | | | 2.50 µmol kg$^{-1}$ | | | 10.0 µmol kg$^{-1}$ | | |
| | | | | | | Intra-day | Inter-days | Inter-analysts | Intra-day | Inter-days | Inter-analysts | Intra-day | Inter-days | Inter-analysts |
| **Alanine** | 0.016 | 0.054 | 0.06 - 49.60 | 0.9994 | 5.5 | 4.3 | 5.5 | 6.9 | 5.1 | 7.6 | 12.0 | 5.1 | 5.3 | 11.4 |
| **Arginine** | 0.016 | 0.054 | 0.06 - 3.38 | 0.9998 | 4.1 | 6.2 | 9.5 | 4.9 | 5.3 | 7.1 | 6.3 | - | - | - |
| **Aspartic acid** | 0.067 | 0.223 | 0.24 - 12.20 | 0.9995 | 5.0 | 8.8 | 14.1 | 10.0 | 5.5 | 6.2 | 10.0 | 7.9 | 7.8 | 5.7 |
| **Cystine*** | 0.034 | 0.112 | 0.12 - 13.01 | 0.9999 | 4.3 | 8.9 | 11.3 | 9.4 | 6.3 | 6.1 | 6.2 | 8.4 | 9.1 | 10.6 |
| **Glutamic acid** | 0.070 | 0.234 | 0.24 - 12.20 | 0.9992 | 4.7 | 9.0 | 9.4 | 8.0 | 6.7 | 6.7 | 7.2 | 7.1 | 8.4 | 11.9 |
| **Glycine** | 0.026 | 0.088 | 0.10 - 12.20 | 0.9994 | 4.0 | 6.1 | 7.9 | 6.9 | 4.8 | 4.6 | 6.2 | 7.9 | 9.1 | 7.8 |
| **Histidine** | 0.018 | 0.061 | 0.06 - 26.02 | 0.9994 | 5.3 | 6.4 | 8.4 | 7.7 | 7.2 | 8.8 | 6.7 | 5.2 | 5.1 | 7.4 |
| **Iso-leucine** | 0.028 | 0.093 | 0.10 - 12.20 | 0.9998 | 5.3 | 5.3 | 8.2 | 6.5 | 4.9 | 7.2 | 11.9 | 5.1 | 7.4 | 4.3 |
| **Leucine** | 0.018 | 0.061 | 0.06 - 12.20 | 0.9995 | 4.3 | 3.7 | 4.5 | 6.0 | 7.8 | 7.6 | 10.3 | 2.8 | 5.4 | 6.0 |
| **Lysine** | 0.015 | 0.051 | 0.06 - 26.02 | 0.9992 | 5.3 | 2.7 | 7.6 | 9.9 | 6.1 | 6.8 | 8.0 | 4.9 | 4.8 | 10.1 |
| **Methionine** | 0.028 | 0.093 | 0.10 - 12.20 | 0.9999 | 3.7 | 6.5 | 10.0 | 9.7 | 3.1 | 7.9 | 6.5 | 4.9 | 5.1 | 9.7 |
| **Phenylalanine** | 0.029 | 0.095 | 0.10 - 12.20 | 0.9995 | 4.4 | 4.3 | 7.4 | 8.6 | 4.6 | 5.7 | 12.2 | 5.8 | 5.8 | 6.5 |
| **Proline** | 0.026 | 0.088 | 0.10 - 12.20 | 0.9996 | 4.5 | 6.4 | 9.2 | 7.0 | 4.2 | 6.8 | 4.2 | 6.4 | 7.5 | 8.1 |
| **Serine** | 0.066 | 0.221 | 0.24 - 12.20 | 0.9997 | 3.7 | 6.5 | 8.4 | 10.7 | 4.7 | 5.6 | 8.9 | 7.4 | 10.0 | 9.8 |
| **Threonine** | 0.017 | 0.057 | 0.06- 12.20 | 0.9998 | 3.5 | 7.2 | 9.7 | 8.9 | 3.7 | 6.5 | 7.3 | 7.1 | 8.7 | 7.8 |
| **Tyrosine** | 0.266 | 0.888 | 0.91-75.09 | 0.9998 | 10.1 | - | - | - | 5.9 | 6.0 | 6.8 | 5.2 | 9.2 | 7.6 |
| **Valine** | 0.017 | 0.057 | 0.06 - 6.10 | 0.9997 | 4.2 | 3.1 | 8.1 | 10.3 | 5.6 | 6.1 | 8.7 | - | - | - |

*The concentrations of cystine for precision assay were 0.30, 1.25 and 5.0 µmol kg$^{-1}$.

#### 4.3.2.4 Recovery Study and Matrix Effect

The effects of serum and urine matrices on the ionization of amino acid derivatives were studied by comparing analyte signals at the concentration of 10.0 μmol $kg^{-1}$ in standard solution and the matrix medium. %ME results for human serum and urine are represented in Figure 4.19 and 4.20, respectively. As seen in Figure 4.19, while ion enhancement with %ME of between -4.5% and -94.3% was observed for alanine, aspartic acid, glutamic acid, glycine, histidine, lysine, serine and valine derivatives, others including arginine, cysteine, isoleucine, leucine, phenylalanine and tyrosine suffered from ion suppression effect in human serum. On the other hand, the matrix effects for proline, serine, threonine and valine were not significant (≤10%). As seen in Figure 4.20, there was a significant matrix effect for amino acids except for isoleucine, lysine and methionine. The relative standard deviations were below 10%.

The recovery was evaluated by matrix matching using spiked samples with different concentrations of amino acids. The extraction rates of amino acids from human serum were evaluated with spiking human serum before and after precipitation. The resulting data are represented in Table 4.21 and 4.22 for human serum and urine, respectively. Data showed that high extraction rates of 16 amino acids were obtained within a range of 95.5 - 102.9% with %RSD values below 8.5%. On the other hand, a low extraction rate (59.4%) was obtained for cystine. Nevertheless, satisfactory variability of cystine (8.3%, as %RSD) also comprised the accurate determination of cystine in human serum using a proper correction factor or matrix matching strategy. The average percent recovery values of amino acids were evaluated for all spiked levels and obtained in the range of 80.6 – 121.8% and 78.1 - 118.8% for human serum and urine, respectively. In addition to that, %RSDs were less than 10.3 and 14.3% for human serum and urine, respectively.

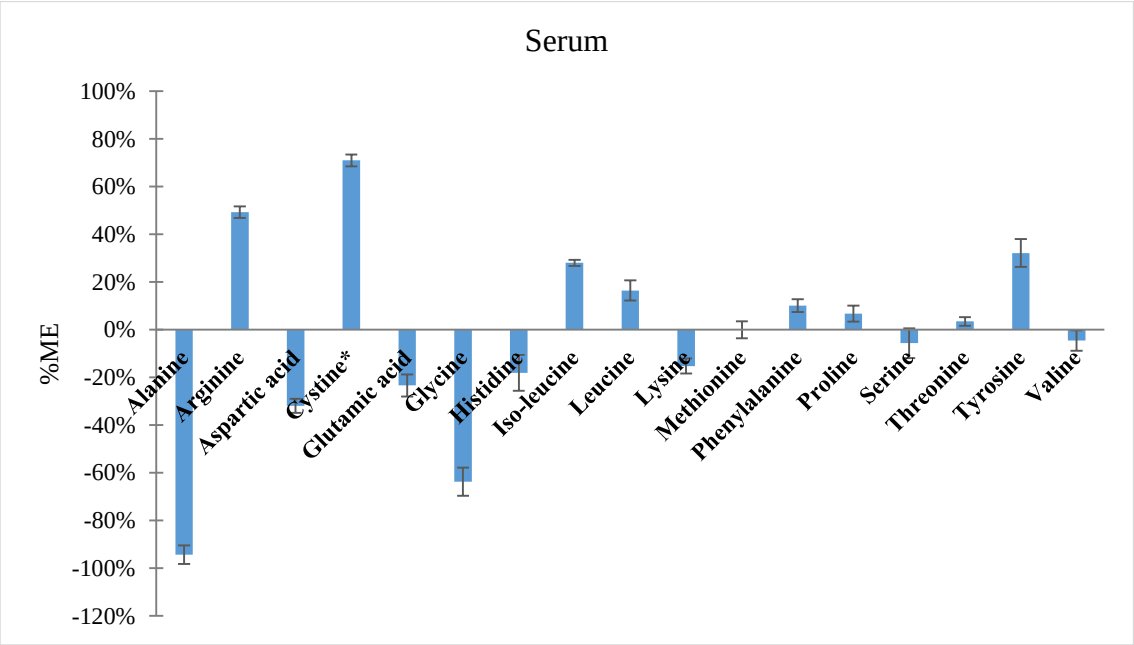



**Figure 4.19** %ME of amino acid derivatives in human serum

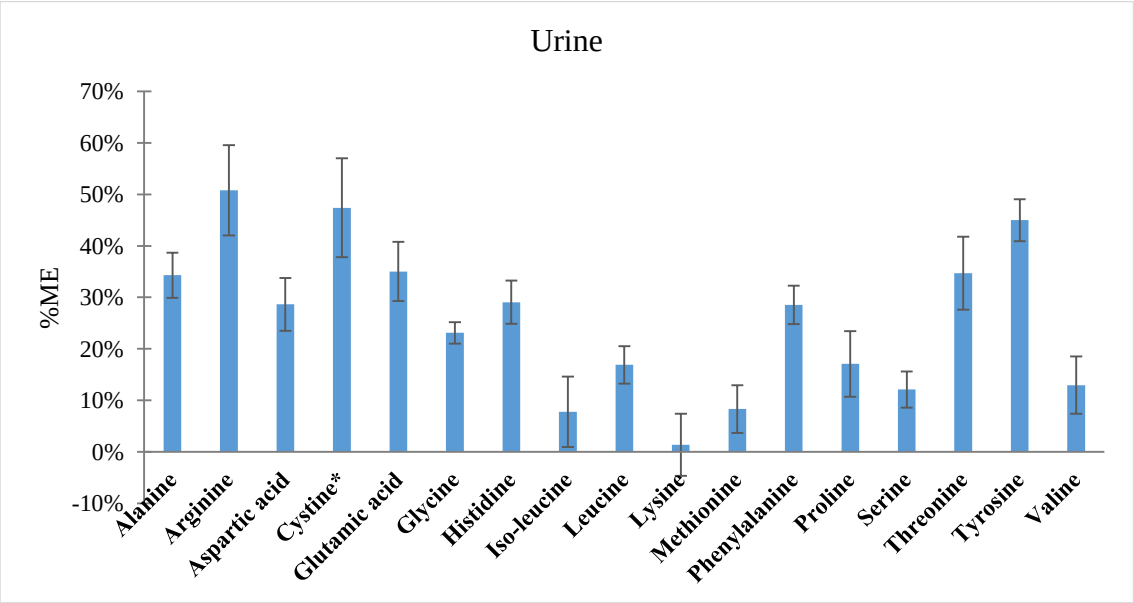



**Figure 4.20** %ME of amino acid derivatives in human urine

**Table 4.21** The percent recovery values and extraction rates for different spiked concentrations of amino acid derivatives in human serum

| Analyte | Recovery (%) | | | | | Extraction Rates (%) |
|---|---|---|---|---|---|---|
| | 2.9 μmol $kg^{-1}$ | 5.2 μmol $kg^{-1}$ | 10.4 μmol $kg^{-1}$ | 19.9 μmol $kg^{-1}$ | 37.9 μmol $kg^{-1}$ | |
| **Alanine** | - | 96.1 ± 2.3 | 108.7 ± 3.9 | 108.0 ± 4.0 | 99.8 ± 2.2 | 99.4 ± 4.2 |
| **Arginine** | 90.4 ± 1.4 | 103.4 ± 1.5 | 106.9 ± 2.4 | 97.3 ± 7.2 | 98.0 ± 4.8 | 99.7 ± 6.9 |
| **Aspartic acid** | 90.9 ± 5.2 | 106.2 ± 2.6 | 106.0 ± 3.0 | 99.3 ± 5.6 | - | 99.3 ± 5.8 |
| **Cystine** | 108.1 ± 9.0 | 102.4 ± 8.3 | 94.5 ± 2.5 | 94.6 ± 2.8 | - | 59.4 ± 8.3 |
| **Glutamic acid** | - | - | 102.3 ± 4.6 | 101.4 ± 9.5 | 101.5 ± 7.4 | 100.7 ± 4.3 |
| **Glycine** | 80.6 ± 4.1 | 102.3 ± 6.9 | 108.2 ± 5.0 | 105.1 ± 5.0 | 100.5 ± 5.8 | 102.9 ± 4.8 |
| **Histidine** | 114.8 ± 6.0 | 106.6 ± 2.4 | 103.3 ± 7.5 | 98.3 ± 4.4 | 102.8 ± 3.6 | 98.7 ± 5.5 |
| **Iso-leucine** | 82.8 ± 2.5 | 95.8 ± 5.6 | 103.9 ± 1.2 | 105.9 ± 5.8 | 100.3 ± 5.1 | 101.9 ± 4.2 |
| **Leucine** | 91.6 ± 2.0 | 100.5 ± 3.0 | 101.8 ± 4.2 | 103.0 ± 3.3 | 102.2 ± 2.2 | 101.7 ± 2.5 |
| **Lysine** | 95.9 ± 2.3 | 109.6 ± 4.0 | 107.4 ± 3.2 | 102.6 ± 3.4 | - | 95.5 ± 3.3 |
| **Methionine** | 86.2 ± 7.7 | 90.7 ± 6.7 | 104.4 ± 3.5 | 106.6 ± 6.9 | 102.9 ± 4.1 | 99.5 ± 6.4 |
| **Phenylalanine** | 109.2 ± 1.9 | 102.3 ± 3.7 | 109.2 ± 2.7 | 104.0 ± 4.7 | - | 101.1 ± 3.5 |
| **Proline** | 86.9 ± 4.4 | 93.5 ± 3.0 | 107.6 ± 3.4 | 108.9 ± 1.4 | - | 101.5 ± 2.4 |
| **Serine** | 86.8 ± 6.3 | 114.9 ± 2.7 | 107.4 ± 6.2 | 104.1 ± 5.0 | - | 98.1 ± 8.5 |
| **Threonine** | 101.7 ± 6.5 | 112.2 ± 10.3 | 109.0 ± 1.9 | 92.9 ± 4.5 | - | 96.7 ± 8.5 |
| **Tyrosine** | 121.8 ± 3.6 | 103.5 ± 0.9 | 90.8 ± 5.8 | 92.1 ± 5.7 | 99.2 ± 12.1 | 99.5 ± 6.7 |
| **Valine** | - | 95.1 ± 4.2 | 100.6 ± 4.3 | 103.6 ± 6.2 | 96.6 ± 5.3 | 100.4 ± 3.0 |

**Table 4.22** The percent recovery values for different spiked concentrations of amino acid derivatives in human urine

| Analyte | Recovery (%) | | | | |
|---|---|---|---|---|---|
| | **2.8 µmol kg$^{-1}$** | **5.3 µmol kg$^{-1}$** | **10.5 µmol kg$^{-1}$** | **22.3 µmol kg$^{-1}$** | **35.5 µmol kg$^{-1}$** |
| **Alanine** | - | - | - | 103.6 ± 4.6 | 103.2 ± 2.2 |
| **Arginine** | - | 105.5 ± 4.7 | 104.2 ± 8.8 | 99.4 ± 6.0 | - |
| **Aspartic acid** | 104.9 ± 6.9 | 106.2 ± 5.1 | 100.2 ± 3.6 | - | - |
| **Cystine** | 117.5 ± 9.8 | 100.0 ± 9.6 | 97.6 ± 7.4 | 102.5 ± 2.3 | - |
| **Glutamic acid** | - | 102.4 ± 10.1 | 101.8 ± 5.8 | 102.5 ± 7.5 | - |
| **Glycine** | - | - | - | 108.1 ± 2.0 | 102.4 ± 5.2 |
| **Histidine** | - | 103.9 ± 7.0 | 104.9 ± 4.2 | 101.4 ± 3.3 | 101.8 ± 2.4 |
| **Iso-leucine** | - | 97.4 ± 10.9 | 106.5 ± 6.8 | 102.0 ± 5.9 | - |
| **Leucine** | - | 94.7 ± 7.5 | 102.7 ± 3.6 | 101.6 ± 6.0 | - |
| **Lysine** | - | 96.4 ± 8.7 | 100.7 ± 6.0 | 104.1 ± 4.5 | - |
| **Methionine** | - | 96.5 ± 5.7 | 101.8 ± 4.6 | 103.1 ± 3.2 | - |
| **Phenylalanine** | - | 87.6 ± 6.5 | 106.7 ± 3.7 | 105.0 ± 2.6 | - |
| **Proline** | - | 78.1 ± 4.7 | 104.0 ± 6.4 | 107.7 ± 3.0 | 102.3 ± 2.7 |
| **Serine** | - | 102.5 ± 0.3 | 112.7 ± 3.5 | 105.1 ± 4.7 | - |
| **Threonine** | - | 103.9 ± 6.3 | 96.8 ± 7.1 | 99.3 ± 7.2 | - |
| **Tyrosine** | - | 118.8 ± 13.4 | 91.1 ± 4.1 | 105.1 ± 7.9 | - |
| **Valine** | - | 94.8 ± 8.5 | 100.0 ± 5.6 | 101.3 ± 6.9 | - |

#### 4.3.2.5 ID$^4$MS Strategy

To increase the accuracy of the developed method, the ID$^4$MS strategy was also applied to derivatized amino acids. For this purpose, calibration, blank and sample blends were prepared and after that, the derivatization procedure was applied to all blends to compensate for analytical errors coming from sample preparation. The blank correction was applied before calculating the experimental spiked amount of amino acids. The percent recovery was evaluated by comparing experimental and theoretical spiked concentrations. Representative examples for the preparation of blends and obtained isotope ratios are represented in Table 4.23 and 4.24 for histidine at low level for serum and in Table 4.25 and 4.26 for arginine at high level for urine samples, respectively. The average isotope ratios of calibration blends were measured as 0.3747, 0.7590 and 1.5183 for histidine; 0.7552, 1.4796 and 2.7784 for arginine. The isotope ratios of calibration blends were varied for each amino acid to be proportional to 0.5, 1.0 and 2.0. Good agreement between measured and theoretical isotope ratios was obtained for all amino acids ($R^2 \geq 0.999$). Figure 4.21 and 4.22 are represented as examples for histidine and arginine, respectively.

**Table 4.23** Actual weighed amounts for the preparation of blends and measured ratio values (An example for histidine in human serum, low level)

| **Human serum** | | **Histidine, µmol kg$^{-1}$** | |
|---|---|---|---|
| $\omega_A$ | | 4.7541 | |
| $\omega_B$ | | 1.4350 | |
| $\omega_{A^*}$ | | 5.4060 | |
| **Blend** | **$m_{A(AB)}$/g**<br>**A, Blank or A*, g** | **$m_{B(AB)}$/g**<br>**B, g** | **$r_{A^*B}$ (measured)** |
| **AB-Blank** | 0.1658 | 0.1665 | 0.9239 |
| | 0.1668 | 0.1687 | 0.9553 |
| **AB** | 0.1671 | 0.1651 | 0.7764 |
| | 0.1669 | 0.1680 | 0.7585 |
| | 0.1680 | 0.1655 | 0.7736 |
| **A*B-1** | 0.0867 | 0.1702 | 0.3619 |
| | 0.0849 | 0.1673 | 0.3794 |
| | 0.0861 | 0.1725 | 0.3827 |
| **A*B-2** | 0.1695 | 0.1721 | 0.7701 |
| | 0.1682 | 0.1707 | 0.7558 |
| | 0.1682 | 0.1702 | 0.7510 |
| **A*B-3** | 0.3337 | 0.1680 | 1.5397 |
| | 0.3327 | 0.1714 | 1.5071 |
| | 0.3322 | 0.1648 | 1.5080 |

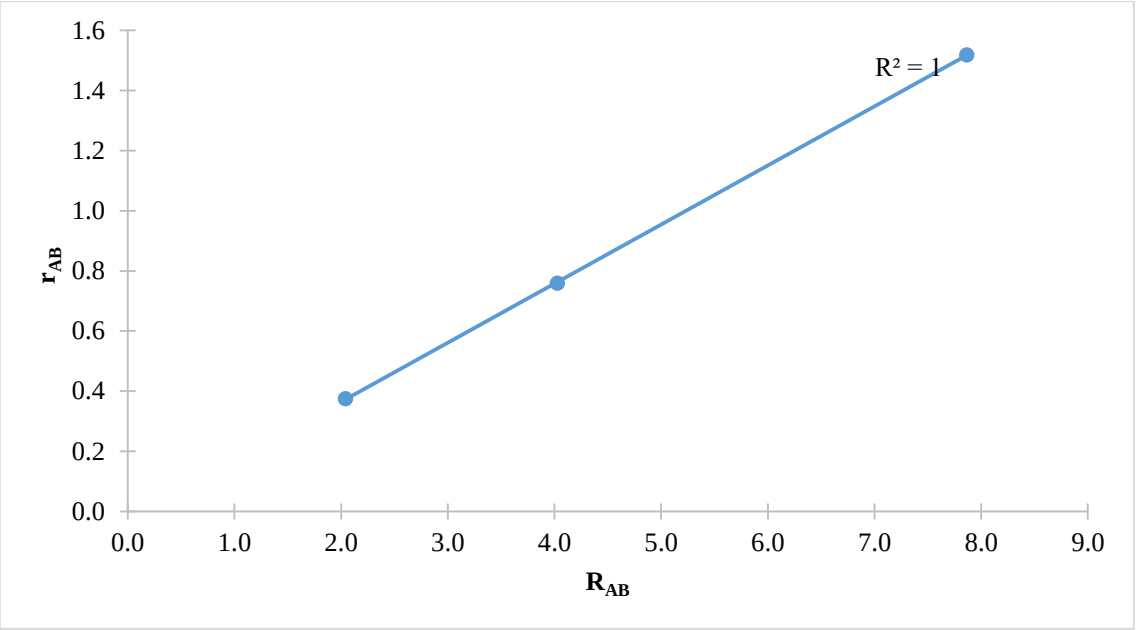



**Figure 4.21** Theoretical blend ratio vs. measured blend ratio (An example for histidine in human serum, low level)

**Table 4.24** Recovery results obtained from the ID⁴MS model for histidine at low level in serum

| Sample blends | $r_{AB}$ | $w_A$, µmol kg$^{-1}$ | Experimental amount, Average, µmol kg$^{-1}$ | %RSD | Theoretical spiked amount, µmol kg$^{-1}$ | Recovery, % |
|---|---|---|---|---|---|---|
| **AB-1** | 0.7764 | 5.0186 | | | | |
| **AB-2** | 0.7585 | 5.3716 | 5.2603 | **3.98%** | 5.1014 | **103.11%** |
| **AB-3** | 0.7736 | 5.3907 | | | | |

**Table 4.25** Actual weighed amounts for the preparation of blends and measured ratio values (An example for arginine in human urine, high level)

| **Human urine** | | **Arginine, µmol kg$^{-1}$** | |
|---|---|---|---|
| $\omega_A$ | | 38.1432 | |
| $\omega_B$ | | 21.5953 | |
| $\omega_{A^*}$ | | 40.5053 | |
| **Blend** | **$m_{A(AB)}$/g**<br>**A, Blank or A*, g** | **$m_{B(AB)}$/g**<br>**B, g** | **$r_{A^*B}$ (measured)** |
| **AB-Blank** | 0.1678 | 0.1664 | 0.1538 |
| | 0.1647 | 0.1692 | 0.1518 |
| **AB** | 0.1662 | 0.1703 | 1.5287 |
| | 0.1680 | 0.1694 | 1.3766 |
| | 0.1672 | 0.1639 | 1.3800 |
| **A*B-1** | 0.0852 | 0.1672 | 0.7443 |
| | 0.0864 | 0.1663 | 0.7494 |
| | 0.0848 | 0.1681 | 0.7720 |
| **A*B-2** | 0.1646 | 0.1720 | 1.5200 |
| | 0.1686 | 0.1638 | 1.4717 |
| | 0.1686 | 0.1671 | 1.4471 |
| **A*B-3** | 0.3340 | 0.1712 | 2.8212 |
| | 0.3312 | 0.1729 | 2.7344 |
| | 0.3352 | 0.1710 | 2.7797 |

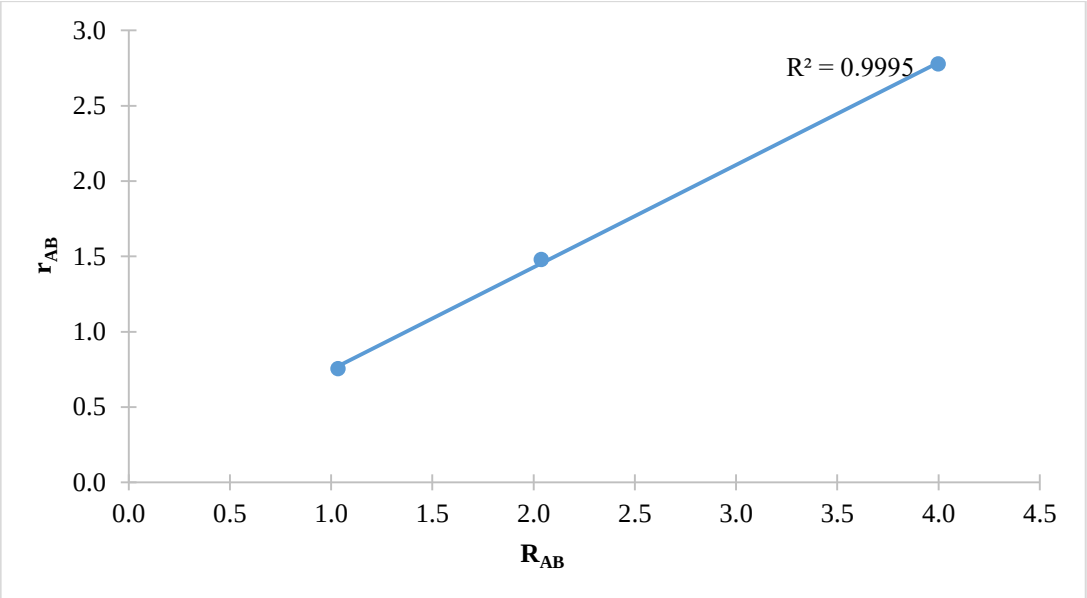



**Figure 4.22** Theoretical blend ratio vs. measured blend ratio (An example for arginine in human urine, high level)

**Table 4.26** Recovery results obtained from the ID⁴MS model for arginine at high level in urine

| Sample Blends | $r_{AB}$ | $w_A$, µmol kg$^{-1}$ | Experimental amount, Average, µmol kg$^{-1}$ | %RSD | Theoretical spiked amount, µmol kg$^{-1}$ | Recovery, % |
|---|---|---|---|---|---|---|
| **AB-1** | 1.5287 | 39.9499 | | | | |
| **AB-2** | 1.3766 | 39.3032 | 39.1171 | **2.40%** | 38.1432 | **102.55%** |
| **AB-3** | 1.3800 | 38.0983 | | | | |

The results obtained from the ID⁴MS equation for each amino acids are summarized in Table 4.27 and 4.28 for serum and urine, respectively. The accuracy of ID⁴MS method for serum samples were varied between 98.00% - 103.11%, 97.18% - 103.11% and 98.00% - 103.64% for low, mid- and high levels, respectively. For urine samples, high accuracy was also obtained with the percent recovery values

within a range of 100.71% - 103.70%, 98.47% – 104.22% and 98.15% - 103.95% for low, mid- and high levels, respectively. The precision of the method was expressed as %RSD and obtained between 0.86% - 4.23% for serum and 0.42% - 3.84% for urine matrices.

**Table 4.27** The accuracy results obtained from the ID$^4$MS model for 16 amino acids in human serum

| Analyte | Low level | | Mid level | | High level | |
|---|---|---|---|---|---|---|
| | Recovery | %RSD | Recovery | %RSD | Recovery | %RSD |
| **Alanine** | 98.65 | 1.13 | 100.17 | 1.47 | 99.09 | 0.86 |
| **Arginine** | 99.64 | 2.13 | 103.11 | 1.50 | 99.76 | 2.58 |
| **Aspartic acid** | - | - | 97.31 | 3.12 | 98.76 | 2.89 |
| **Glutamic acid** | - | - | 101.98 | 3.84 | 101.56 | 3.46 |
| **Glycine** | 102.99 | 4.23 | 102.66 | 2.22 | 98.00 | 2.97 |
| **Histidine** | 103.11 | 3.98 | 98.67 | 3.64 | 97.81 | 1.13 |
| **Isoleucine** | 99.35 | 2.52 | 97.18 | 2.43 | 99.09 | 1.47 |
| **Leucine** | 98.27 | 2.04 | 99.88 | 2.47 | 103.64 | 1.69 |
| **Lysine** | 100.03 | 1.37 | 100.77 | 2.94 | 101.74 | 2.12 |
| **Methionine** | 101.26 | 2.19 | 102.02 | 3.67 | 98.52 | 1.57 |
| **Phenylalanine** | 100.58 | 2.35 | 100.21 | 3.23 | 98.95 | 2.11 |
| **Proline** | 98.20 | 1.13 | 102.32 | 2.58 | 98.36 | 1.46 |
| **Serine** | - | - | 98.00 | 3.39 | 101.72 | 2.96 |
| **Threonine** | - | - | 98.28 | 3.83 | 100.47 | 1.59 |
| **Tyrosine** | - | - | 101.59 | 3.74 | 100.62 | 1.24 |
| **Valine** | 98.00 | 1.46 | 100.31 | 2.75 | 101.24 | 3.49 |

**Table 4.28** The accuracy results obtained from the ID$^4$MS model for 16 amino acids in human urine

| Analyte | Low level | | Mid level | | High level | |
|---|---|---|---|---|---|---|
| | Recovery | %RSD | Recovery | %RSD | Recovery | %RSD |
| **Alanine** | 101.55 | 2.78 | 102.14 | 2.75 | 102.27 | 2.11 |
| **Arginine** | 101.57 | 2.29 | 102.13 | 0.42 | 102.55 | 2.40 |
| **Aspartic acid** | - | - | 103.50 | 3.31 | 103.44 | 2.59 |
| **Glutamic acid** | - | - | 103.78 | 2.58 | 103.70 | 1.46 |
| **Glycine** | - | - | 103.28 | 2.51 | 101.44 | 2.67 |
| **Histidine** | - | - | 103.63 | 1.54 | 103.95 | 1.47 |
| **Isoleucine** | 102.29 | 1.56 | 99.50 | 0.73 | 102.63 | 1.69 |
| **Leucine** | 100.71 | 2.49 | 98.47 | 0.61 | 98.15 | 2.01 |
| **Lysine** | 102.73 | 2.20 | 99.60 | 2.94 | 103.43 | 2.14 |
| **Methionine** | 102.82 | 1.00 | 101.23 | 2.49 | 103.24 | 3.32 |
| **Phenylalanine** | 102.87 | 0.83 | 103.00 | 2.63 | 103.03 | 1.94 |
| **Proline** | 103.35 | 2.39 | 104.22 | 2.75 | 102.21 | 3.08 |
| **Serine** | - | - | 102.07 | 3.30 | 103.26 | 1.70 |
| **Threonine** | - | - | 101.40 | 3.84 | 103.93 | 1.71 |
| **Tyrosine** | - | - | 103.41 | 1.12 | 103.31 | 1.73 |
| **Valine** | 103.70 | 1.98 | 100.41 | 3.44 | 103.64 | 1.38 |

# 5
# RESULTS AND DISCUSSION

As the most outstanding quantification method, isotope dilution paved the way for accurate results in analyzing complex biological samples. This method is going to be more popular in the future regarding its capability to overcome analytical errors encountered in sample preparation and instrumental processes. The potential of isotopic dilution strategy paved the way toward the development of highly accurate and reliable analytical methods even for complex biological samples. The challenge to combine this strategy with preconcentration and/or derivatization methods is very important for definite quantitative results even at trace levels.

Herein, ID$^4$MS was successfully applied for the determination of erectile drugs and amino acids in biological samples. In the first part of the study, ID$^4$MS was combined with the MSPE method for the simultaneous determination of four different erectile drugs including sildenafil, tadalafil, vardenafil and avanafil in human urine and plasma samples. In this regard, citric acid coated MNPs were synthesized, characterized and used as solid sorbent in the MSPE process successfully. The experimental variables in the MSPE procedure for erectile drugs were optimized using experimental design.

In the second part, 16 free amino acids were simultaneously determined with two different approaches; with and without derivatization. Underivatized amino acids were quantified using HILIC method in only 8.0 min. Derivatization was achieved for all amino acids in a one-pot reaction using 2-naphtoyl chloride as derivatization reagent. The optimization of experimental variables in the derivatization procedure was also studied by experimental design. Under the optimum conditions, amino acid derivatives were simultaneously determined using the RPLC method in 12 minutes.

In the preset study, for each experimental case; low detection limits, wide linear ranges and acceptable repeatability were obtained. Furthermore, satisfactory percent recovery values were achieved for applied matrices using matrix matching strategy.

- The accuracy of the developed method for erectile drugs was greatly improved with the application of ID$^4$MS; from 83.0 - 109.5% to 97.50 – 99.57% for urine samples and from 93.4 to 116.8% to 98.26 - 100.92% for plasma samples.
- Superior precision was also achieved for ID$^4$MS with %RSD ≤ 1.95% and 1.96% for plasma and urine samples, respectively.
- For free amino acids, the accuracy of HILIC- ID$^4$MS method was obtained in the range of 99.13 - 101.78% with %RSDs ≤ 2.44% for urine; 98.70 - 101.68% with %RSDs ≤ 1.62% for plasma samples which were ranged between 80.1 - 127.2% for urine and 77.9 - 127.4% for plasma with %RSDs ≤ 13.5% when matrix matching strategy was used.
- For amino acid derivatives, the accuracy of RPLC - ID$^4$MS was also greatly improved from 78.1 - 118.8% to 98.15 - 104.22% for urine and from 80.6 – 121.8% to 97.18 - 103.64% for serum matrices.

  The precision of method was below than 3.84% and 4.23% for urine and serum samples, respectively.

These results confirmed that the application of ID$^4$MS provided full compensation of analyte losses during the whole analytical procedure with great extraction recoveries. ID$^4$MS could be used for accurate and precise quantification of trace organic analytes when matrix interference is high and matrix matching is not possible. Although isotopically enriched materials have high costs today, upon the increase in the availability of these products, isotope dilution is expected to be used as a definitive reference method for toxicological, clinical and environmental laboratories in the coming decades.

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# Publications from the thesis

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## Papers

1. E. Ö. Er, E. Akkaya, B. Özbek, and S. Bakırdere**,** "A powerful combination of quadruple isotope dilution strategy with dispersive magnetic solid phase extraction for the accurate and precise multi-analyte determination of tadalafil, sildenafil, avanafil and vardenafil in human plasma and urine samples using LC-ESI-Tandem MS", *Microchemical Journal*, vol. 152, p. 104302, 2020.
2. E. Ö. Er, E. Akkaya, B. Özbek, and S. Bakırdere**,** "Development of an analytical method based on citric acid coated magnetite nanoparticles assisted dispersive magnetic solid-phase extraction for the enrichment and extraction of sildenafil, tadalafil, vardenafil and avanafil in human plasma and urine prior to determination by LC-MS/MS", *Microchemical Journal*, vol. 147, pp. 269-276, 2019.
3. E. Öztürk Er, B. Özbek, and S. Bakırdere**,** "Accurate and sensitive determination of sildenafil, tadalafil, vardenafil, and avanafil in illicit erectile dysfunction medications and human urine by LC with quadrupole-TOF-MS/MS and their behaviors in simulated gastric conditions", *Journal of Separation Science*, vol. 42, no. 2, pp. 475-483, 2019.
4. E. Ö. Er, B. Özbek, and S. Bakırdere**,** "A sensitive and selective analytical method for the simultaneous determination of sildenafil and tadalafil in water, energy drinks and sewage sludge matrices by LC-QTOF-MS/MS", *Measurement*, vol. 124, pp. 64-71, 2018.

## Conference Papers

1. Er, Elif Öztürk, Erhan Akkaya, Belma Özbek and Sezgin Bakırdere, "Determination of Selected PDE-5 Inhibitors in Human Urine by LC-QTOFMS/MS after Citric Acid Coated Magnetic Nanoparticle Assisted Phase Microextraction", *11th Aegean Analytical Chemistry Days (AACD 2018)*, Crete, Greece, 25 September 2018, pp. 48.
2. Er, Elif Öztürk, Sezgin Bakırdere and Belma Özbek, "A Sensitive and Selective Method for Simultaneous Determination of Sildenafil and Tadalafil in Tap Water and Sewage Sludge Samples by LC-QTOF-MS/MS" *7th International IUPAC Conference on Green Chemistry*, Moscow, Russia, 02 October 2017, pp. 49.

## Projects

1. Researcher in the project titled with "Determination of Analytes in Biological Fluids By Q-TOF LC/MS Using Isotope Dilution Method", Coordinator: Prof. Dr. Belma ÖZBEK, FDK-2017-3186, 13.11.2017-13.05.2021.