DEVELOPMENT OF ELECTROSPUN FIBER COATED THIN FILM MICROEXTRACTION DEVICES FOR RAPID MASS SPECTROMETRIC DETERMINATION OF POLAR CANCER BIOMARKERS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

DEVELOPMENT OF ELECTROSPUN FIBER COATED THIN FILM MICROEXTRACTION DEVICES FOR RAPID MASS SPECTROMETRIC DETERMINATION OF POLAR CANCER BIOMARKERS

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One of the leading reasons of deaths around the world is cancer, and most deaths are associated with late diagnosis. In this regard, the development of rapid and reliable analytical methods that can be used in routine control and early diagnosis of the cancer is of great importance. Solid phase microextraction (SPME) is a sampling and sample preparation technique which offers a platform for development of tools suitable for clinical applications. Its thin film geometry when is made in sword like shape, so-called coated blade spray (CBS), can be also used as electrospray ionization source in direct to mass spectrometric analysis. However, the current CBS devices have low affinity for polar analytes.

In this thesis, new CBS devices providing high extraction kinetics and high affinity for polar analytes were prepared and an analytical method for determination of clinically important polar urinary metabolites was developed. For this purpose, various polymeric nanomaterials were prepared by the electrospinning method. Hydrolyzed polyacrylonitrile coated probe showed the best performance among all tested polymers and was selected to be used in the study. The extraction conditions were optimized for the determination of amino acids (phenylalanine and tryptophan),

nucleotides (8-Oxo-2'-deoxyguanosine (8-oxo-dG) and 8-Oxo-2'-deoxyadenosine (8-oxo-dA)) and neurotransmitters (serotonin and 5-hydroxyindoleacetic acid) which are essential biomarkers in cancer diagnosis, in urine.

Validation results showed that the limits of quantification of the final method for all the analytes were 50.0 ng/mL except 5-hydroxyindoleacetic acid which was 100.0 ng/mL, and the entire process including sample preparation and analysis takes only 6 minutes.

Keywords: Electrospinning, Coated Blade Spray, Mass Spectrometry, Solid Phase Micro Extraction

ÖZ

POLAR KANSER BİYO-İŞARETLEYİCİLERİNİN HIZLI KÜTLE SPEKTROMETRİK TAYİNİ İÇİN ELEKTROEĞRİLMİŞ FİBER KAPLI İNCE FİLM MİKRO EKSTRAKSİYON CİHAZLARININ GELİŞTİRİLMESİ

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Dünya çapında önde gelen ölüm nedenlerinden biri kanserdir ve ölümlerin çoğu geç tanı ile ilişkilendirilir. Bu bakımdan kanserin rutin kontrolünde ve erken tanısında kullanılabilecek hızlı ve güvenilir analitik yöntemlerin geliştirilmesi büyük önem taşımaktadır. Bir numune alma ve numune hazırlama tekniği olan katı faz mikro ekstraksiyon (SPME), klinik uygulamalara uygun araçların geliştirilmesi için bir platform sunmaktadır. Kılıç benzeri geometride yapıldığında, kaplı bıçak spreyi (CBS) olarak adlandırılan ince film geometrisi, doğrudan kütle spektrometrik analizde elektrosprey iyonizasyon kaynağı olarak da kullanılabilir. Bununla birlikte, mevcut CBS cihazlarının polar analitler için afinitesi düşüktür.

Bu tez çalışmasında, polar analitler için hazırlanan yüksek ekstraksiyon kinetiği ve yüksek afinite sağlayan yeni CBS cihazları hazırlanmış ve klinik olarak önemli polar üriner metabolitlerin tayini için analitik bir yöntem geliştirilmiştir. Bu amaçla elektroeğirme yöntemi ile çeşitli polimerik nanomalzemeler CBS ekstraktif kaplamaları olarak hazırlanmıştır. Hidrolize poliakrilonitril kaplı prob, test edilen tüm polimerler arasında en iyi performansı göstermiş ve çalışmada kullanılmak

üzere seçilmiştir. Ekstraksiyon koşulları, kanser teşhisinde temel biyo-işaretleyiciler olan amino asitler (fenilalanin ve triptofan), nükleotidler (8-okso-dG ve 8-okso-dA) ve nörotransmiterlerin (serotonin ve 5-hidroksi indol asetik asit) idrarda tayini için optimize edilmiştir.

Metot validasyon sonuçları geliştirilen CBS-MS metodu ile 5-hidroksi indol asetik asit için 100,0 ng/mL ve diğer analitler için 50,0 ng/mL tayin sınırına örnek hazırlama ve ölçüm dahil olmak üzere 6 dakika gibi kısa bir sürede ulaşılabileceğini göstermiştir.

Anahtar Kelimeler: Elektroeğirme, Kaplamalı Bıçak Spreyi, Kütle Spektrometre, Katı Faz Mikro Ekstraksiyon

To my family

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LIST OF ABBREVIATIONS

ABBREVIATIONS

ACN	Acetonitrile
AIMS	Ambient ionization mass spectrometry
CBS	Coated blade spray
DART	Direct analysis in real-time
DESI-MSI	Desorption electrospray ionization-mass spectrometry imaging
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophilic–lipophilic balance
HLB-SCX	HLB with strong cation exchange groups
IS	Internal standard
LC	Liquid chromatography
LLE	Liquid-Liquid Extraction
LOQ	Limit of quantification
MS	Mass spectrometry
nESI	Nano electrospray ionization
PA	Polyacrylate
PAN	Polyacrylonitrile
PVA	Poly(vinyl alcohol)
PDMS	Polydimethylsiloxane

PVP	Polyvinylpyrrolidone
SPE	Solid phase extraction
SEM	Scanning electron microscopy
SPME	Solid phase microextraction
TFME	Thin film microextraction
FTIR	Fourier-Transform Infrared Spectroscopy

LIST OF SYMBOLS

SYMBOLS

^{0}C	Degree Celsius
h	Hour
m/z	Mass to charge ratio
mL	Milliliter
µL	Microliter

CHAPTER 1

INTRODUCTION

Recently, analytical determination using liquid chromatographic (LC) or gas chromatographic separation (GC) coupled with mass spectrometric (MS) detection has been preferred in many clinical research areas. These methods allow to separate the analyte of interest from other components found in the matrix; therefore, they improve the selectivity of the method and reliability of the results. However, chromatographic separation is time consuming and could be the bottle neck of the analysis in clinical studies where rapid determination of various critical metabolites is required [1,2]. As an alternative technique, direct MS analyses in which the sample can be directly analyzed in its native state (without sample preparation and chromatographic separation), decreases the time that is spend for separation of analytes. However, the most crucial disadvantage of ambient mass spectrometry is its low analytical sensitivity due to introduction of the matrix components and analytes at the same time to the MS. Therefore, there is a need for an innovative sample preparation method which can eliminate problems of direct to mass spectrometry analysis, such as ion suppression and high background that diminish the analyte signal. In solid phase microextraction (SPME), which is sample preparation method that can combines sampling and sample preparation in a single step, fewer matrix effects are expected from biological fluids when directly coupled with a mass spectrometer as a result of minimal coextraction of matrix macromolecules [3,4]. Especially the thin-film geometry of SPME with a sword-like tip (coated blade spray, CBS) has recently become popular for clinical and bioanalytical applications as this sampler acts both as an extractive device and ionization source when is coupled with a mass spectrometer [5]. CBS has also high throughput capabilities in sample preparation which further decreases the time spend

for the analysis. Many studies have shown that this approach can be used for rapid and sensitive analyzes of various biological, environmental and food samples [6]. This thesis aims to prepare an SPME-based probe which can be coupled directly with MS for fast determination of various neurotransmitters, nucleotides and amino acids identified as cancer biomarkers in urine. For this purpose, new polar extractive phases with thin and homogeneous surface coatings were prepared for CBS-MS applications using the electrospinning approach as a coating method. The prepared polar extractive phase coated blades were optimized for the determination of various amino acids (phenylalanine and tryptophan), nucleotides (8-oxo-dG and 8-oxo-dA) and neurotransmitters (serotonin and 5-hydroxyindoleacetic acid) which are essential biomarkers in cancer research, in urine.

1.1 Electrospray Ionization

Generally, in the conventional LC-MS approaches, the first step involves a chromatographic separation, then separated analytes are ionized by electrospray ionization (ESI) and finally detected in the mass spectrometer [7]. Electrospray ionization works based on a spray of charged droplets containing analyte molecules dissolved in a solvent. A conductive capillary with a sharp tip facilitates to pump of the solvent-analyte solution, and voltage is used between the mass spectrometer's input and capillary. Electric field intensity is highest at the cone tip. The liquid coming out of the capillary is converted into a Taylor cone by the strong electric field [8]. Charged droplets, when released from the cone, are accelerated by the electric field and move faster in the direction of the mass spectrometer inlet. Nebulizing gas is used to promote spray generation and increase electrospray stability. The solvent in the droplet evaporates as it approaches the MS inlet. The charge density is higher in the smaller droplet. A Coulombic explosion occurs when the droplet reaches the Rayleigh limit, where the charges equal the surface tension [9]. After this explosion, a few small droplets are formed. The residual charged carriers (in positive ion mode) are bound to the analyte when the solvent has finally completely evaporated. These

charged species are easily detected by MS. The majority of MSs use a dry gas heated input system to assist the solvent evaporation.

1.2 Direct-to-MS technologies

Among many analytical techniques, mass spectrometry (MS) based methods have many advantages, such as high response speed, high specificity, and high sensitivity [10]. However, as mentioned before, prior to mass spectrometric (MS) analysis, sample pre-treatment and chromatographic separation are usually necessary for traditional MS-based analytical methods. Therefore, a fundamental issue limiting the efficiency of MS-based approaches is the practical sample preparation process. In 2004, the ambient mass spectrometric technique was introduced by Cooks and co-workers [11]. Ambient mass spectrometry (AMS) is completed in an open atmosphere on various samples in their natural environment or by using different surfaces or matrices. Ambient ionization mass spectrometry (AIMS) allows high-throughput, direct, rapid, in vitro, and in vivo analysis of gases, liquids, and solids. The most practical part of direct to mass spectrometric analysis is that the analyte is transferred directly to the mass spectrometer without the requirement for chromatographic separation [12–14]. Many other AIMS approaches have been developed over the last two decades, and several are now extensively used worldwide. The majority of the published scientific work is focused on direct analysis in real time (DART), desorption electrospray ionization (DESI) and paper spray ionization (PSI). As recently reviewed by Alberici et al., AIMS has a wide variety of applications in various scientific fields, including cancer pathology, forensic analysis, biomedicine, neurology microbiology, plant science, and pharmaceuticals [10]. However, a few particular AIMS approaches, such as the MasSpec Pen, SpiderMass and i-Knife, have been developed for real-time and in vivo analysis [15]. These approaches offer significant potential for the pharmacokinetic studies of human skin and intraoperative cancerous tissue identification. AIMS technology has the potential for in-field environmental analysis

or on-site clinical analysis because of its simplicity and ability to deliver quick analytical results, as less than 1 second is required from ion formation to signal acquisition for most MS [16]. In contrast, in most laboratories currently, chromatography based analytical procedures take several hours or days to complete the analysis of samples. The most practical part of direct to mass spectrometric analysis is that the analyte is transferred directly to the mass spectrometer without the requirement for chromatographic separation. The details of the techniques are given below.

1.2.1 DART

DART-MS could analyze a sample at atmospheric pressure, particularly in its natural environment [17]. The DART technique can be applied to compounds that are deposited or adsorbed on surfaces. DART-MS analysis depends on a gas-phase ionization process. The initial creation of ionized species in DART is accomplished with the help of a corona discharge, which produces excited noble gas atoms that trigger progressive gas-phase processes when released into the atmosphere [18]. The predominant mechanism of analyte ion production from a specific sample is particularly reliant on the analyte. The DART ion source is suitable for both polar and non-polar compounds. DART-MS is simple and convenient for safety and forensic applications such as illegal substances, warfare agents on baggage or screening for traces of explosives [19,20]. DART can also be applied for rapid determination and the identification of compounds from chemical synthesis or analyze liquid or solid bulk materials for quality control.

1.2.2 DESI

DESI is an ionization technique applied to condensed phase samples; it can be used for solid samples, liquids, frozen solutions, and adsorbed gases, including complex biological materials [21,22]. The technique is almost instantaneous in response time,

highly sensitive, and can be applied to proteins, small-molecule organic compounds and other biological molecules. The DESI approach combines desorption ionization and electrospray (ESI) ionization. Similar to ESI, it uses an electrospray source to ionize the analytes from the sample surface under ambient conditions. In DESI the electrospray source is directed to a surface of a sample which is placed in front of the inlet of the MS, enabling the analysis of a thin film or a surface top layer of the sample. A conventional DESI source contains two separate components to successfully transfer the ions: the surface on which the sample rests and a spray capillary. A solvent pump keeps the solvent flowing continuously through the capillary. Application of a high voltage to the spray capillary which is carrying the spray solvent is the first step in ionizing the analytes. As voltage is supplied to the spray solvent by a metal electrode, a charged primary microdroplet is emitted by pneumatic and electrophoretic forces [23]. The nebulizing gas stream accelerates the primary droplets to the surface, and these droplets extract the analytes. As a result, charged micro-droplets are created. Pneumatic nebulization and electric potential in the spray solution are applied to aid desolvation process which is the dissociation of solvent molecules [24]. As a result of the primary droplets' kinetic effect, secondary microdroplets comprising the dissolved analyte and a highly charged solvent are created. From the sample surface to the MS inlet, these secondary microdroplets are ionized [25]. Like the desolvation process in traditional ESI, droplet desolvation occurs as they pass through the air due to Coulomb fissions and solvent evaporation [26]. The nitrogen gas stream assists in transferring the droplets towards the heated inlet of the mass spectrometer.

1.2.3 Paper Spray

In this approach, a paper is cut into triangle shape, and then sample is placed on the paper with the wetting solution or simply preloaded onto the paper [27]. Applying high voltage to the paper substrate initiates the ionization process. The high voltage used on the paper can be positive or negative, generally ranging from 2.0 to 5.5 kV.

The stability of the signal and the strength of ion formation are affected by variables such as the distance between the mass spectrometer inlet and the paper spray tip, the volume of solvent, and the tip angle [28]. Numerous spray jets are produced from the edges and surface of the paper thanks to the paper's cellulosic fibers, and each one contributes to spread of a few microns of the solvent droplet. Paper spray is similar to nano electrospray ionization (nESI) with microliter spray solvent volumes and small droplet properties. However, nESI cannot analyze low and non-polar aromatic compounds with high sensitivity compared to paper spray chemical ionization [29]. The paper spray is used to analyze a variety of molecules, including small organic molecules, proteins, and peptides.

1.2.4 I knife

Takats group has developed a combination of MS and surgical techniques that applies the chemical analysis of tissue in situ during surgery [30], known as I knife. In this technique, ionization occurs during the use of electrocautery to stop bleeding or cut tissue at the surgical site [31]. A high-frequency current forms in the tissue, resulting in ionization through the tissue evaporation. The electrocautery handpiece is connected to the atmospheric interface of the mass spectrometer by a plastic tubing. Gaseous ions rapidly travel from the study site to the mass spectrometer. The results were similar in tissue specificity to DESI without any sample preparation. The atmospheric interface is adjusted to pass surgical smoke through the coil to improve signal-to-noise ratios before entering the MS. The ionized small and large molecular fragments are exposed to a collision surface so that they break up into many smaller fragments more appropriate for analysis. These molecules then transfer to the mass spectrometer. The main benefits of the I knife can be listed as integrates well with tools and instruments in use by surgeons, requires no sample preparation, and is rapid.

1.2.5 MasSpec Pen

The MasSpec Pen is a tool connected to an MS for cancer diagnosis and rapid tissue analysis that allows efficient extraction of biomolecules from a tissue sample [32]. The MasSpec Pen was developed as a disposable and straightforward device that can be used to interact with living tissues made of chemicals and biocompatible materials. The probe tip material is made of PTFE, and the conduit tubes are made of PDMS. These polymers are biocompatible materials commonly used in medical devices [33]. The MasSpec Pen has achieved highly selective mass spectra from biological samples characterized by metabolite ions, multiple charged protein ions and mono- and double-charged lipids like those produced by desorption electrospray ionization-mass spectrometry imaging (DESI-MSI) and ESI methods [34]. In fact, this approach follows the principles of a simple liquid-solid extraction and identifies molecules in tissue using a water droplet, without the need to use an electric field and gas when collecting the sample at the surface. Since only a discrete drop of water is deposited on a tissue sample for a certain period in this approach, the tissue integrity is preserved. The water droplet containing the analytes is transported to the MS and directly into a heated transfer tube, after which evaporation and ionization take place in the entrance region of the MS.

1.2.6 Coated blade spray

Coated blade spray (CBS) was developed for rapid, quantitative, and qualitative analysis of complex mixtures [35]. CBS is a thin film coated blade geometry of solid phase microextraction (SPME) [36–38]. The CBS device, coated with a polymer, is made of a stainless-steel sheet-like sword (Figure 1.1). It offers additional advantages such as a well-defined sharp tip, better electrical conductivity than paper spray, and convenient handling with large sample volumes without surface deformation.

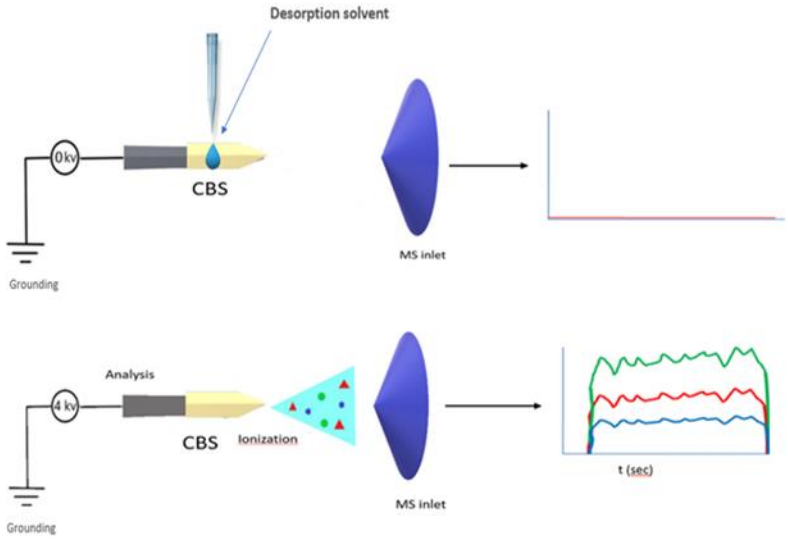


Figure 1.1. Experimental setup for CBS-MS

CBS has a dual function of both extracting analytes from complex samples and being used as an ESI source. An excellent compromise exists between direct coupling to mass spectrometry and sample preparation when CBS is employed in the analysis. Furthermore, the extractive properties of the device provide minimization in the matrix effects, and improvement of limits of quantification (LOQ), making it an ideal tool for rapid diagnosis [39]. In contrast to CBS, in the other ambient ionization approaches (PS, DESI and DART), there is no selective ionization. Hence, CBS is advantageous over other methods since it has an extractive phase and protects analytes against the presence of matrix components. Moreover, instead of a non-conductive, porous substrate of the paper spray, the substrate material of CBS made of stainless steel is assumed to produce a more stable electric field. In other words, more efficient and repeatable ions are generated during spraying because of the electric field between the MS inlet and the blade tip. In an identical sampling procedure, CBS can achieve better sensitivity than other SPME geometries due to its large sorbent volume dispersed in a highly big surface area [40]. In this way, even by simply increasing the interaction time between the sample and the coating from

15 s to 1 min, lower LODs can be obtained. Three factors affect the selection of appropriate desorption/ionization conditions for CBS process: 1. the desorption solvent affinity and analyte chemistry; 2. the analyte kinetics between the solvent drop and the extractive phase; and 3. spray ability of the solvent at the blade's tip. Moreover, CBS geometry allows for the automation of sample preparation and analysis [41]. CBS probes have been used successfully in biological fluids such as urine, blood, and plasma [42]. As one of the crucial problems with direct to mass spectrometric analysis is the matrix affects, the use of an extractive phase places a critical role in sensitive direct to MS analysis. In this regard, the coating of the blades, not only is important for the enrichment of small molecules from the sample but also minimizes the introduction of macromolecules and salts to a mass spectrometer, eliminating the matrix effect. Therefore, CBS offers substantial sensitivity compared to detection limits of SPME combined with LC-MS/MS or other direct to MS techniques.

The typical CBS procedure begins with quick preconditioning of the extractive phase, (see Figure 1.2) [43]. It then continues with the extraction part, which may take a very short time depending on the matrix and analyte concentration. The coated blade is immersed directly into the sample vial for extraction. After the extraction step, the blade is rinsed quickly with water to remove loosely attached matrix components. Then the blade is placed in front of an MS inlet in the shortest possible distance safe to avoid arc formation. Then the desorption solvent (5-20 μL) is added to the blade, waited for a short time, and a high voltage is applied. As the voltage is applied analytes are transferred to the MS inlet by forming a Taylor cone similar to ESI.

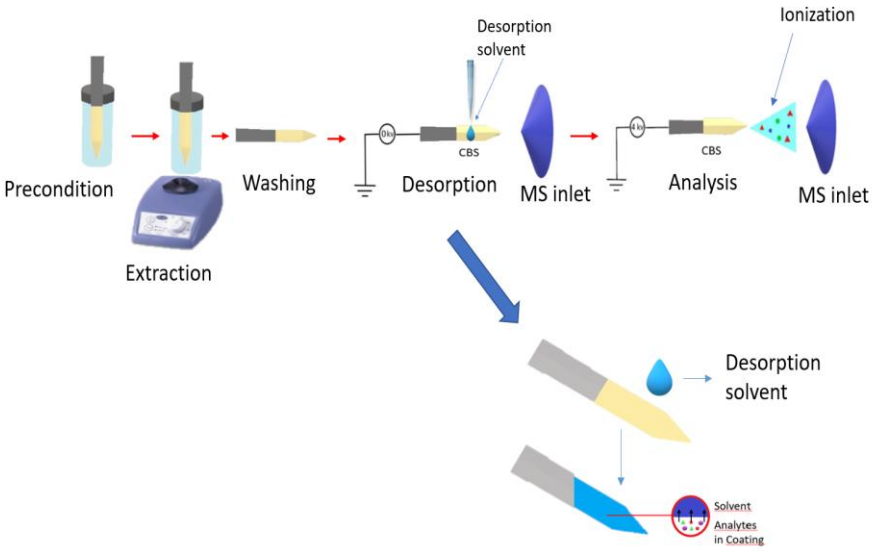


Figure 1.2. Typical protocol used for the CBS

1.3 Conventional sample preparation

As highlighted above, for sensitive analysis with direct to mass spectrometric approaches sample preparation plays an important role. There are many traditional sample preparation techniques used in conventional analytical analyses. One of the most straightforward techniques is “dilute and shot” and is usually employed for urine analysis [44,45]. It is the most rapid method, but the detection limit is high. Although the sample dilution followed by chromatographic separation reduces the overall matrix concentration, the analyte is diluted. This causes decreased sensitivity in analysis. Another simple sample preparation technique is protein precipitation (PPT) and usually is used in whole blood and plasma samples [46]. The PPT approach uses various salt solutions or agents such as heat, strong acids/bases, or a small amount of sample matrix miscible organic solvent to denature and precipitate the proteins and release the bound analytes to the solution. The sample is generally centrifuged to separate denatured proteins. PPT is useful as a rapid method for samples where the analytes are bound to the matrix and their interference is common.

Liquid-Liquid Extraction (LLE) is another method of sample preparation [47]. This method isolates the analytes from the matrix by mixing an aqueous sample with an immiscible organic solvent. When the analytes prefer the organic phase to the aqueous phase, analyte separation from the matrix occurs. The amount of solvent used, the laborious and time-consuming processes, and the low affinity for polar molecules are some drawbacks of LLE [48]. To address many of the limitations of LLE and PPT approaches, frequently solid phase extraction (SPE) is used for the separation of the target analytes from liquid samples. In this method, an aqueous sample is passed through a cartridge in which an extractive phase is immobilized [49]. Due to affinity of the analyte towards the extractive phase, the analytes are strongly hold on the sorbent while sample matrix is not retained. Following the sorption step, a desorption step with a strong eluent is performed to release the extracted analytes to a smaller volume suitable for instrumental analysis. However, extremely polar analytes have low breakthrough volumes due to restricted efficiencies brought on by low retention. Nevertheless, switching the sorbent type is one of the methods used in SPE to get around the breakthrough volume. SPE has several benefits over LLE, including, significantly less solvent consumption, a wider range of analyte selectivity, and better efficiency. The main issues with SPE based techniques in their applications to biological samples include repeatability problems, high cost per sample and low recoveries which requires extra care in quantitative analysis.

1.4 Solid phase microextraction (SPME)

SPME is a well-designed sampling and sample preparation technique developed by Pawliszyn and Belardi in the early '90s [50]. Compared to conventional SPE and LLE methods, SPME has great advantages such as versatility, minimal solvent use, in vivo and on-site applicability, and flexibility in design [51,52]. In SPME, the extraction phases are generally prepared by immobilization of extractive particles on thin support or bare fibers. Recoveries are low since SPME often uses small volumes

of extraction phases. Consequently, in most of the cases, SPME is a non-exhaustive extraction technique. Moreover, SPME can be performed in different modes, including headspace extraction, direct extraction and membrane-protected extraction providing flexibility and possibility for variety of applications. In a typical extraction, the analytes migrate between three phases: the fiber coating, a homogeneous matrix of air or water and the headspace or gas phase until equilibrium is achieved. The general equilibrium of the analyte in the system is associated with the mass of the analyte extracted on extractive coating. As the total amount of analyte is constant in the system the following mass balance exists for a typical SPME process:

$$C_o V_s = C_h^{\infty} V_h + C_f^{\infty} V_f + C_s^{\infty} V_s \qquad 1.1$$

Where, C_o is the analyte's initial concentration in the matrix; C_s^{∞}, C_h^{∞} and C_f^{∞} are concentrations of the matrix at equilibrium, headspace, and the analyte in the coating, respectively; V_s, V_h and V_f are volume of matrix, headspace, and coating, respectively. If the gas/sample matrix distribution constant is expressed in the equation as $K_{hs} = C_h^{\infty} / C_s^{\infty}$, and coating/gas distribution constant as $K_{fh} = C_f^{\infty} / C_h^{\infty}$, then $n = C_f^{\infty} / V_f$, the mass of the analyte absorbed by coating is defined as

$$n = \frac{K_{fh} K_{hs} V_f V_s C_o}{K_{fh} K_{hs} V_f + V_s + V_h K_{hs}} \qquad 1.2$$

$$K_{fs} = K_{fh} K_{hs} = K_{fg} K_{gs} \qquad 1.3$$

If the impact of moisture in the gaseous headspace is disregarded, Equation 1.2 can be represented as

$$n = \frac{K_{fs}V_fV_sC_o}{K_{fs}V_f+V_s+V_hK_{hs}} \quad 1.4$$

According to the equilibrium conditions, the equation expresses that the location of the fiber in the system does not affect the extraction amount of the analyte. It may be placed directly in the sample or in the headspace if the volumes of the sample, headspace and fiber coating are maintained constant. Assuming that the sample vial is filled, or the analyte is not present in the headspace of the sample, the term $K_{hs}V_h$, which relates to the capacity (C_rV_h) of the headspace in the denominator, is simplified, and result in the following formula:

$$n = \frac{K_{fs}V_fV_sC_o}{K_{fs}V_f+V_s} \quad 1.5$$

If the volume of the sample is much larger compared to the volume of the fiber ($V_f << V_s$); the equation is further simplified to Equation 1.6 which highlights the field sampling abilities of SPME based techniques as sample volume information becomes insignificant for quantitative purposes.

$$n = K_{fs}V_fC_o \quad 1.6$$

A typical SPME procedure is shown in Figure 1.3. The first step in this protocol is preconditioning step which prepares the coating for extraction. The coating is then washed to ensure that preconditioning solution is removed from the sorbent. In the extraction stage, the coating is immersed in the matrix, and the analyte extraction begins. Following the extraction, a second wash takes place to remove any loosely attached matrix components from the sorbent. Finally, the extracted analytes are transferred to the desorption solvent and resulting solution is analyzed with suitable instrument.

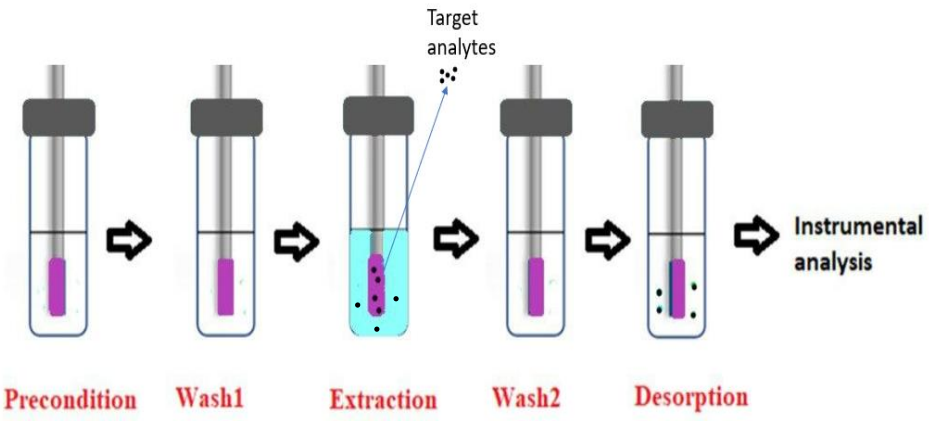


Figure 1.3. Typical SPME procedure

1.5 Thin film microextraction (TFME)

Thin film microextraction (TFME) is one of the popular geometries of SPME [53]. The relatively large volume of the extractive phase deposited as a thin film allows to extract higher amounts of analyte in a short time; therefore, improves the sensitivity of SPME [54]. This improvement can be realized in Equations 1.5 and 1.6 given in the previous section which show the intrinsic correlation between the amount of analyte extracted into the extractive phase at equilibrium (n or n_e^{eq}) and the effect of the volume of the extractive phase [55]. The time required to reach the equilibrium extraction (t_e) can be calculated by Equation 1.7; where, b, δ and D_s refer to thicknesses of extractive phase and boundary layer, and the diffusion coefficient of analyte within the sample, respectively. This equation shows that a thin layer should be used to achieve a faster extraction equilibrium.

$$t_e = 3\frac{\delta K_{es} b}{D_s} \qquad 1.7$$

Also, Equation 1.8 shows that the surface area of the extraction phase (A) is directly proportional to the initial extraction rate, expressed as the amount extracted (dn) per

a given time (dt). Thus, faster extraction kinetics is achieved by dispersing a relatively large volume of extraction phase in a large surface area with a thin layer.

$$\frac{dn}{dt} = \frac{D_S A}{\delta} C_O \qquad 1.8$$

1.6 Coating methods for SPME and TFME

Various methods have been used to coat supports such as metal wires or fused silica fibers with sorbents. Recently, the researchers have directed their efforts to more straightforward SPME preparation methods. These methods have resulted in higher functional surface area and better coating stability. The most used SPME coating methods are given below.

1.6.1 Spray coating

Spray coating is a process of forming a very fine droplets of coating slurry on the surface of the support [56]. Nebulization in the spray nozzle converts the coating liquid into fine droplets which is directed on the support surface to obtain physically stable coating. To obtain a uniform film on the support, various factors must be controlled. The solvent used the particle size, air pressure, the temperature, the flow rate, the distance between the spray nozzle and substrate, the speed of the moving nozzle during spraying and the concentration of the polymer solution, are all to be taken into consideration.

1.6.2 Dipping technique

The dipping method typically involves placing fibers or other suitable substrates for a short period of time in the slurry of the material to be deposited [57]. Gravitational force, inertia, surface tension, and viscous drag are the major forces used in the dip-

coating process. After the fiber is coated, the solvent is evaporated resulting in formation of a thin film of extractive phase.

1.6.3 Electrospinning

In recent years, nanostructured coating materials for SPME fibers have received increased attention [58] as nanostructured materials have a high surface-to-volume ratio, providing many active sites for adsorption and allowing efficient extraction and separation. One of the efficient ways to produce a nanostructured SPME phase is to use the electrospinning approach which is a well-establish technique for producing polymer fibers with various diameters in the nanometer, sub-micrometer, and micrometer range [59,60]. High voltage is used in the electrospinning process to initiate an electric field between a collector plate and the tip of the needle. (Figure 1.4). A Taylor cone is created when the electric field exceeds the surface tension of the droplets. The Taylor cone is produced, and then polymeric nanofibers travel toward the collector. SPME fibers with varying chemical compositions are produced using the electrospinning method by mixing numerous polymers and polymer blends. This method is versatile in varying the SPME fiber composition and facilitates the fabrication of high-surface-area fibers.

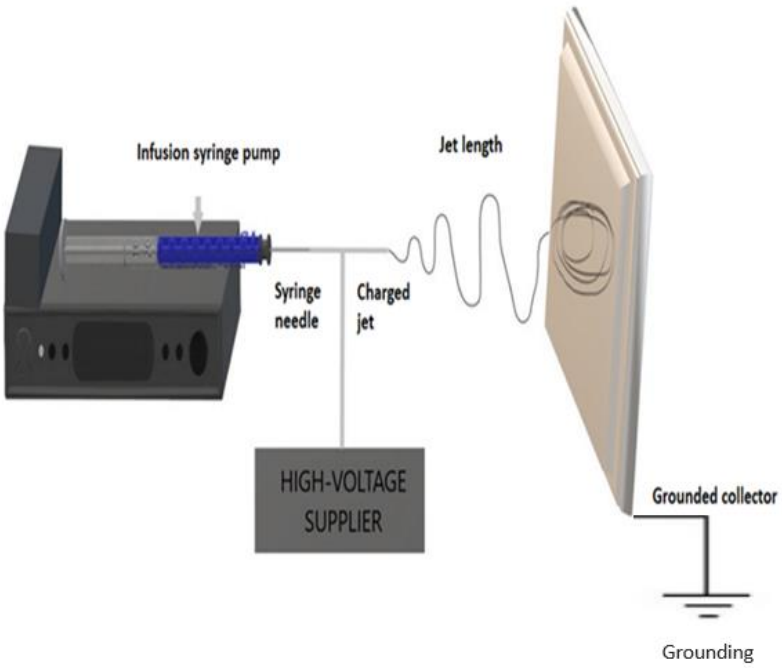


Figure 1.4. Schematic representation of electrospinning process

1.6.3.1 Factors affecting electrospinning process

In nanofiber production by electrospinning method, variables such as the distance between the tip of the syringe and the grounded collector plate, flow rate, voltage, ambient conditions, and solution properties affect the nanofiber structure [61]. By changing these parameters, nanofibers with adjustable diameters can be obtained. One of the parameters affecting the diameter of fibers in electrospinning process is voltage. At higher voltages, higher electrostatic interaction is seen, so the polymer solution is sprayed more. Since the polymer reaches the collector plate faster, the thickness of the fibers is less with this increase in jet velocity. For electrospinning to occur, the electric potential energy must be higher than the surface energy. A Taylor cone is formed when the surface energy and the electric potential energy are close to each other. The flow rate significantly affects the electrospun nanofibers' porosity, diameter, and form. Increases in flow are linked to larger pore sizes and diameters,

although extremely high flows increase the probability of beads formation [62]. The distance between the grounded plate and the needle tip is adjusted, and thus the shape and size of the fibers can be changed. Nevertheless, it has a lesser impact than other variables. Short distances can create defects on fiber, like roughness, undulation, waviness, folds, wrinkles, and misalignment [63]. Also, the fiber diameter can decrease with increasing distance between the grounded plate and the needle tip. Another critical parameter effecting the electrospinning process is the concentration of the polymer solution. In electrospinning, the concentration of polymer solution affects fiber shape. The diameter of nanofibers increases with increasing polymer concentration. However, the relationship between solution concentration and fiber diameter is frequently nonlinear. The nonlinear connection between polymer solution concentration and solution viscosity is related to this nonlinear relationship. The viscosity of a polymer solution grows gradually as the concentration rises until it reaches a certain point, at which point the viscosity increases dramatically. For electrospinning the molecular weight of the polymer solution is also important. It impacts molecular weight, viscosity, surface tension, surface charge density, and dielectric strength, among other things.

1.7 SPME extractive phases

Solid phase microextraction coating type is critical for extraction of reasonable amount of analytes [50]. The first coatings used in SPME were polyacrylate (PA) and polydimethylsiloxane (PDMS) which were coated on fused silica fiber. Later, different coatings were introduced to the market to extract the analytes with enhanced sensitivity. As polar coatings; polyacrylate, polydimethylsiloxane/divinylbenzene (PDMS/DVB), carbowax/templated resin (CW/TPR-100) and as non-polar coatings; polydimethylsiloxane (PDMS) were introduced as commercial products [64–66]. The most known Polydimethylsiloxane/Divinylbenzene Copolymer (PDMS/DVB) fiber consist of a mixture of liquid polydimethylsiloxane polymer and divinylbenzene polymer. The

pores in DVB provide strong retention of analytes and the porous structure makes this coating suitable for trace level analysis, especially for C6-C15 containing analytes. Although the PDMS-DVB extractive phase provides higher affinity for polar analytes compared to only PDMS, better selectivity can be achieved for the extraction of alcohols and amines when Carbowax/Divinylbenzene copolymer (CW/DVB) is used as extractive phase. The pore diameter of the extractive particles is also important and play critical role in the extraction as such the size of the analyte molecule to be extracted should be maximum in half the pore diameter size to ensure that the analyte can enter withing the pores. In fact, Carboxen®, one of the SPME coatings, has a wide variety of pore sizes; therefore, for analytes in the C2-C12 range, the PDMS/Carboxen® coating achieves good extraction sensitivity. However, larger molecules are difficult to desorb from the pores due to micro condensation process.

In addition to the commercial coatings, homemade coatings with tuned properties for specific group of analytes, or studies have been developed by different groups [67].

1.7.1 Extractive phases used in direct to MS

SPME is an effective sample preparation method that uses fiber coated with a polymeric phase [68]. The coating on the fiber mainly affects the selectivity of extraction. Table 1.1 shows a summary for extractive phases used in various studies in which SPME or TFME is coupled directly to MS [69–71].

Table 1.1 Extractive phases used in various studies in which SPME or TFME is coupled directly to MS

SPME coating	SPME geometry	SPME support	Ionization method
HLB particles-PAN	SPME	Nitinol wires	ESI
C18-PAN	SPME	Stainless steel sheet	CBS
C18-SCX	SPME	Nitinol wires	NanoESI
PDMS/DVB	SPME	Stainless steel	DESI
Graphite	SPME	Stainless-steel	DEP
TiO_2	SPME	Stainless steel needle	nanoESI
PDMS/DVB, CW/DVB	SPME	Titanium wires	DESI
PAN-over C18 − PAN	SPME	Stainless steel mesh	DART
RAM	SPME	Steel and platinum wire	nanoESI
CW/DVB	SPME	Titanium wires	DESI
SWNTs	IT-SPME	Glass capillary	DART
PAN-over C18 − PAN	TFME	Stainless steel mesh	DART
C18-PAN	TFME	Stainless steel mesh	DART
CW-DVB, PDMS, PDMS-DVB, PA	SPME	Titanium wires	DESI
Silica C18	SPME	Titanium wires	DESI
PDMS	SBSE	Stir bar	DART
MWNT	SPME	Glass capillary	PALDI
Mixed C18/SCX	TFME	Stainless steel rod	DESI
PDMS	TFME	PDMS substrate	DCBI
Graphite	SPME	Electrode	LTP
CNT	TFME	CNT film	DCBI

RAM: Restricted access material
MWNT: Multi-walled carbon nanotube
SWNT: Single-walled carbon nanotube
PA: Polyacrylate
CNT: Carbon nanotube
SCX: Strong cation exchange
CW: Carbowax
LTP: Low-temperature plasma
IT: In-tube

1.8 The role of SPME in diagnostic studies

1.8.1 Disease biomarker

A biomarker, in general, is anything that can be used as an indicator for a particular disease condition or other physiological states of an organism [72]. Biomarkers include metabolites, proteins, genes, and genetic variations from various sources such as tissues or body fluids. Proteins have received the most significant attention as prospective biomarkers because they are the biological endpoint that regulates many biological processes, are efficient in circulation, and can be evaluated with high analytical sensitivity.

1.8.2 Cancer biomarkers in urine

Cancer is one of the life-threatening diseases, with approximately 200 distinct forms having been found, resulting in over 1500 fatalities per day [73]. Despite today's technical breakthroughs, the survival rate of patients with this disease is extremely low due to the late detection of cancer. Cancer is caused by a combination of genetic accumulation, environmental factors, and inherited epigenetic abnormalities. Cancer's onset and course are linked to genetic and epigenetic alterations. These alterations cause tumorigenic changes and tumors by disrupting cellular signaling. The tumor mass, in cancer, expands due to uncontrolled cell proliferation. Because these procedures are dependent on the phenotypic features of the tumor, general methods such as ultrasonography, magnetic resonance imaging, and biopsy are insufficient for early-stage cancer identification. Urine has been investigated as a beneficial source of information for evaluating various cancer diseases [74].

Generally, urine is a rich source of intermediate and end products from multiple metabolic processes, and it is the carrier of blood wastes. The advantages of using urine in cancer diagnosis are that it is available in large quantities, can be collected without invasive treatments, and rich in metabolites. Thus, urine can provide

information from distant organs. However, many of these biomarkers are polar analytes. The main problem about the polar analyte is that their extraction from polar urine is more difficult compared to nonpolar analytes as polar analytes show high affinity towards the aqueous matrix. In fact, the extractive phases used for SPME show low affinity for polar analytes [75]. Therefore, the extraction and analysis of such analytes is challenging. Detecting alterations in concentrations of various amino acids, nucleotides and neurotransmitters in urine is considered important in cancer diagnosis. Table 1.2 shows urinary metabolites for which a link between cancer and alteration in their concentration has been found. For example, recent studies have shown that tryptophan is the most frequently altered metabolite in many types of cancer [76]. It has been observed that the amino acid levels of phenylalanine and tryptophan are decreased in esophageal cancer. Because of the obstructive nature of advanced esophageal cancer, up to 79% of patients developed some degree of malnutrition before being diagnosed as aromatic amino acid metabolism (especially phenylalanine and tryptophan) may become dysfunctional in gastroesophageal cancer [77]. Considering that tryptophan and phenylalanine are essential amino acids in the human diet, it is reasonable to see changes in the metabolism of tryptophan in this cancer. In a study comparing the metabolic profiles of normal, benign, and cancerous bladders, it was revealed that phenylalanine and tryptophan were increased in bladder tumors compared to benign bladder tissue. This study shows that tryptophan and phenylalanine are important in the diagnosis of bladder cancer.

In fact, 5-hydroxyindoleacetic acid (5-HIAA) which is urinary metabolite of serotonin is considered as the first-line test analyte in the diagnosis of bladder cancer [78]. Serotonin was also found at high levels in patients with carcinoid tumors. It has also been detected at high levels in various stages of the tumor in urothelial carcinoma [79]. Studies draw attention to nucleotides identified as another important biomarker group [80,81]. It has been found that the concentration of 8-Oxo-2'-deoxyguanosine (8-oxo-dG) is higher in the urine of cancer patients and older adults than in healthy people [82]. Elevated levels of 8-oxo-dG in cancer patients have been

particularly associated with DNA damage repair mechanisms [83]. These examples indicate how important it is to detect these polar metabolites in urine.

Table 1.2 Cancer biomarkers in urine

Biomarkers	Disease/Disorder	References
Phenylalanine, Tryptophan	Bladder Cancer	[84]
Phenylalanine, Tryptophan	Esophageal Cancer	[85]
Phenylalanine, L-Tryptophan	Lung Cancer	[86]
8-Hydroxy-2′-Deoxyguanosine	Breast Cancer	[87]
Serotonin	Colorectal Cancer	[88]
5-Hydroxyindoleacetic Acid	Bladder Cancer	[89]
Serotonin	Carcinoid Cancer	[90]
Adenosine	Lung Cancer	[91]
A-Methylacyl-Coa Racemase	Prostate Cancer	[92]
B-Alanine	Prostate Cancer	[93]
Tyrosine-Phosphorylated	Bladder Cancer	[94]
L-Ascorbic Stearate	Bladder Cancer	[95]
4-Hydroxyphenylacetate	Breast Cancer	[96]
Tetrahydrophenanthrene	Lung Cancer	[97]
Tryptophan	Colorectal Cancer	[98]
A-Methylacyl Coenzyme A Racemase	Prostate Cancer	[99]

1.9 Aim of the study

As described above, new analytical methods that can be used for reliable and rapid analyses is crucial in clinical diagnosis. CBS-MS with many advantages became one of the important technologies for such purposes. However, up to date only hydrophilic–lipophilic balance (HLB) type extractive phase made with dip coating approach were used for extraction of analytes with CBS. Although the dip coating is practical approach for making the CBS coating, in this approach the extractive particles are immobilized on the CBS surface in bulk PAN polymer, as such the analyte must diffuse through the entire coating to reach the maximum sensitivity in the system.

In this study, development of a novel CBS device with nanostructured extractive phases for rapid and sensitive analysis of polar analytes described as urinary biomarkers of cancer is proposed. In order to achieve the aim of the study, for the preparation of the novel extractive phase electrospinning approach which provides large surface area of interaction with analytes, thus fast extraction kinetics, was selected. The prepared electrospun coating also was functionalized with polar moieties (weak anion and cation exchange groups) by hydrolysis to provide stronger sorbent-analyte interaction; therefore, better sensitivity for polar analytes. The prepared polar extractive phase-coated blades were optimized for use in the determination of cancer biomarkers in urine.

CHAPTER 2

EXPERIMENTAL

2.1 Reagents and materials

Analytical standards, serotonin, 5-hydroxyindoleacetic acid (5-HIAA), tryptophan and phenylalanine were purchased from Sigma-Aldrich while 8-Oxo-2'-deoxyadenosine (8-oxo-dA) and 8-Oxo-2'-deoxyguanosine (8-oxo-dG) were purchased from Cayman and LGC, respectively. Polyvinylpyrrolidone (PVP, MW= 40000) and polyacrylonitrile (PAN, MW=150000) were purchased from Sigma-Aldrich and were used in the preparation of the electrospun based extractive phases. The NaOH used in the hydrolysis of PAN was obtained from Carlo Erba. Solvents such as N, N dimethylformamide (DMF, HPLC grade) and ethanol (HPLC grade) were purchased from Sigma Aldrich and used in the preparation of the PAN and PVP polymer solutions, respectively. Acetonitrile (ACN) (HPLC grade) and methanol (HPLC grade) were used as mobile phase in LC and various steps of sample preparation, and they were purchased from Sigma Aldrich. Commercial HLB cartridges were used for the SPE method (Supelco HLB, 500 mg sorbent). Chemicals used to prepare synthetic urine, sodium sulfite, urea, and disodium hydrogen phosphate were from Riedel-de Haën, and creatinine, potassium dihydrogen phosphate, and ammonium chloride were from Merck.

2.2 Instrumental

Agilent MSD single quadrupole mass analyzer was used in coated blade spray (CBS) studies. Philip Harris 5kv dc was used as an external voltage source for CBS. Electrospinning experiments for the coating of the blades were carried out using

nanoWEB Electro-Spin110 device equipped with Inc. NE 300 Spellman SL30 brand DC power supply and New Era Pump Systems. In method development studies, Agilent LC-MS with ESI ion source and Merck SeQuant ZIC-HILIC (particle size 3.5 µm, column dimensions 100 x 2.1 mm, and pore size 200 Å,) column was used for the separation of polar analytes. Elma Elmasonic D-78224 ultrasonic bath was used to facilitate the cleaning of the blades and to degas the mobile phases prior to use. For characterization studies of PAN and hydrolyzed PAN, FTIR Spectrometer (Bruker Alpha) was used. The morphology and diameter size of the resulting electrospun fibers were evaluated using scanning electron microscope (SEM) QUANTA 400F FE-SEM. The MSH-20A model magnetic stirrer was used in the preparation of polymeric solutions, crosslinking, and hydrolysis reactions. ISOLAB VORTEX mixer was used for homogenization of the samples.

2.3 Methods development for LC-MS

Several chromatographic columns and chromatographic conditions were tested to separate the analytes. As a result of these studies, it was found that the hydrophilic interaction liquid chromatography (HILIC) with SeQuant® ZIC-HILIC™ (3.5 µm, 200 Å 100 x 2.1 mm) column gave the best chromatographic method. Gradient separation of the analytes was achieved using the method given in Table 2.1 with a mobile phase flow of 0.35 mL/min. The mobile phase A used in the gradient consists of H_2O containing 0.1% formic acid and 1.0 mM ammonium formate, while the mobile phase B consists of ACN containing 0.1% formic acid and 1.0 mM ammonium formate. In the analyses, the column temperature was fixed at 40.0 °C and 20.0 µL of sample or standard was injected into the column. A quality control sample was prepared (QC, 100.0 ng/mL phenylalanine and atenolol) and the performance of the instrument was evaluated daily with this mixture. The selected analytes of the study can be seen in Table 2.2. The spectrum of the LC-MS is shown in Appendix A Figure A.1.

Table 2.1 The gradient used in the HILIC method

Time (min)	%A	%B
0	10	90
2	10	90
13	50	50
14	50	50
15	10	90
18	10	90

Mobile phase A: %0.1 formic acid 1 mM ammonium formate in H_2O

Mobile phase B: %0.1 formic acid 1 mM ammonium formate in acetonitrile

Table 2.2 Selected analytes

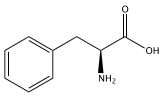
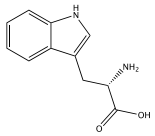
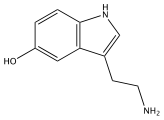
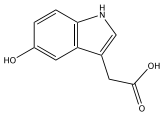
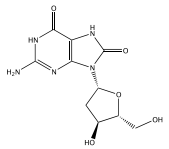
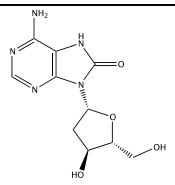
Analytes	Analyte Structure	LogP	MW	Adduct	m/z
Phenylalanine		-1.2	165.19	M+H	166.2
Tryptophan		-1.06	204.23	M+H	205.2
Serotonin		0.56	176.21	M+H	177.2
5-hydroxyindoleacetic acid		1.28	191.18	M+H	192.2

Table 2.2 (cont'd)

8-oxo-dG		−2.6	283.24	M+H	284.2
8-oxo-dA		−1.5	267.24	M+H	268.2

2.4 Coupling of the CBS to the MS

CBS was coupled to a single quadrupole MS and direct mass spectrometric analyses were performed. CBS blades were made of stainless-steel. The thickness of a blade was 0.5 mm, and the length of the probe was 50 mm while the width was 3 mm. An external high-power source was used as the voltage source for the blades. The extractive phase-coated blade spray (CBS) probe was connected to the MS as shown in Figure 2.1. The CBS blade was placed 1.0 cm far from the mass spectrometer inlet. A significant decrease in sensitivity was observed in shifts of more than 2 mm from inlet, and arc flashing was observed at distances closer than 1.0 cm. The external source voltage set directly form the instrument has been tested between 2 kV and 4.5 kV to obtain the highest signal possible. For the optimization of solvent amount, the CBS blade was placed at the fixed distance, MeOH/H_2O, (85:15; v/v) with 0.1% F.A. with 750.0 ng/mL tryptophan was introduced on the CBS blade. The amount of solvent was optimized and set at 10.0 µL. Arc lighting was also observed at 15.0 µL of solvent volume. The external voltage supplied to the blade was also optimized. At external source voltages lower than 4.0 kV, the analytes could not be transferred to the MS inlet. An external source voltage of 4.5 kV was applied from the high voltage source attached to the back of the CBS blade as provided better sensitivity compared to the same voltage settings directly on the instrument. At this stage, with the applied voltage Taylor cone was formed and the solvent and analyte ions were sprayed as vapor from the tip of the blade towards the mass spectrometer

inlet. Sufficient sensitivity was possible without the need for auxiliary (auxiliary and sheath) gases. This part has been optimized as summarized in Table 2.3. During the optimization studies, the signal obtained for 7.5 ng (750.0 ng/mL, 10.0 µL) tryptophan was studied with three replicates.

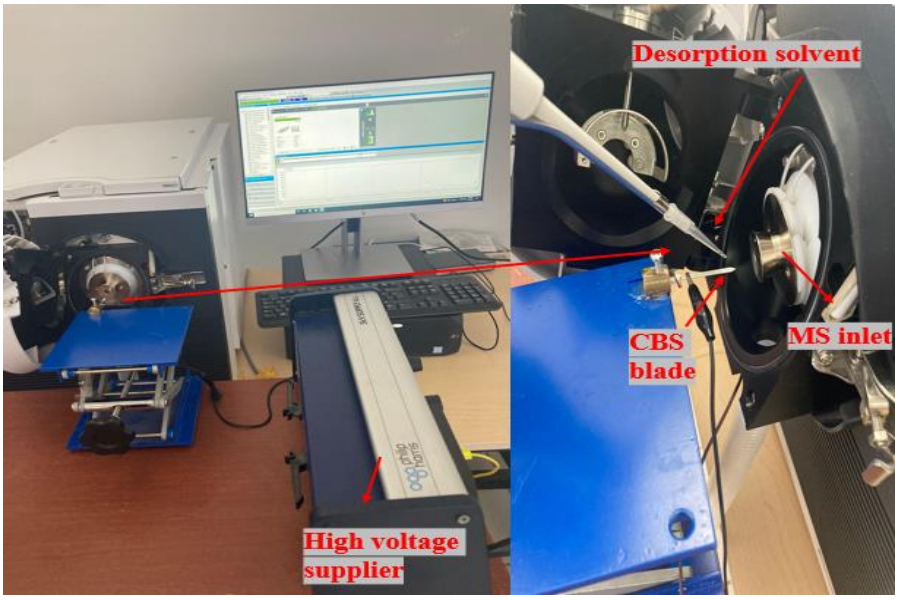


Figure 2.1. Experimental setup for the optimized CBS method

Table 2.3 Tested CBS-MS parameters

Trial number	Distance (cm)	External voltage (kV)	Drying gas (L/min)
1	1.0	4.5	3.0
2	1.0	4.0	3.0
3	1.0	3.0	3.0
4	1.0	4.5	1.0
5	1.0	4.5	3.0
6	1.0	4.5	5.0
7	1.0	4.5	6.0

2.5 Preparation of polar CBS blades

2.5.1 PAN as extractive phase for CBS

The extractive property of the polyacrylonitrile (PAN) polymer is quite low; therefore, the obtained polymer must be functionalized to be used as an extractive phase. The extractive phase preparation experiments proceeded in two steps, (1) electrospinning of PAN and (2) hydrolysis of electrospun PAN to a weak cation exchange polymer.

2.5.1.1 Electrospinning of PAN

For simplicity reason, initial electrospinning optimization studies were carried on an aluminum foil rather than on the blade. As a first step, 10% (w/w) PAN was dissolved in DMF by 24 hours stirring to obtain a homogeneous solution. Then, at various flow rates ranging from 0.4 mL/h to 2.4 mL/h, PAN solution was electrospun using a syringe pump and high voltage supply. The voltage applied to syringe needle and the distance between the needle and the grounded collector was varied to obtain the conditions providing the best nanofibrous morphology. Similar to electrospray ionization in ESI-MS, by application of the voltage to the needle tip a Taylor cone was formed and the electrospun fibers were collected in the grounded collector plate. The parameters optimized in this experiment performed for optimization of electrospinning of the PAN are given in Table 2.4.

Table 2.4 Experimental parameters evaluated for PAN electrospinning

Sample #	Polymer	Solvent	Distance (cm)	Flow rate (mL/h)	Voltage (kV)
1	10% PAN	DMF	15	2.4	14
2	10% PAN	DMF	15	2.4	8
3	10% PAN	DMF	21	2.4	14

Table 2.4 (cont'd)

4	10% PAN	DMF	21	2.4	20
5	10% PAN	DMF	21	0.4	20
6	10% PAN	DMF	21	1.0	20
7	10% PAN	DMF	8	2.4	20

2.5.1.2 Hydrolysis of electrospun PAN

The initial hydrolysis optimization was carried directly with electrospun mat. The hydrolysis of electrospun PAN was optimized by varying the NaOH concentration and the reaction time. For this purpose, 0.5 M, 2.0 M, and 5.0 M NaOH solutions and 1, 2, 2.5, 3, 5 and 8 hours of reaction times were tested. The hydrolysis reaction was performed in an oil bath (at 70 ^{0}C) by constant stirring at 700 rpm. During the hydrolysis process, the color of the PAN nanofibers changed from white to orange. After the reaction, the electrospun PAN was washed multiple times with distilled water to remove any traces of NaOH.

2.5.1.3 Coating the CBS blades with electrospun PAN nanofibers

During the adaptation of electrospinning process for CBS, horizontal and vertical electrospinning setups were tested. It has been found that the vertical set up is more efficient to prepare coatings with less material waste in shortest time. Figure 2.2 illustrated the electrospinning set up used in this study. In a typical experiment, the blade was mounted on the rotatable collector plate and electrospinning was performed for 3 minutes by applying a voltage to the syringe needle feeding the PAN solution. A fine tuning for electrospinning process was repeated with the conditions given in Table 2.4. After the blades were coated, they were hydrolyzed as described in the next section.

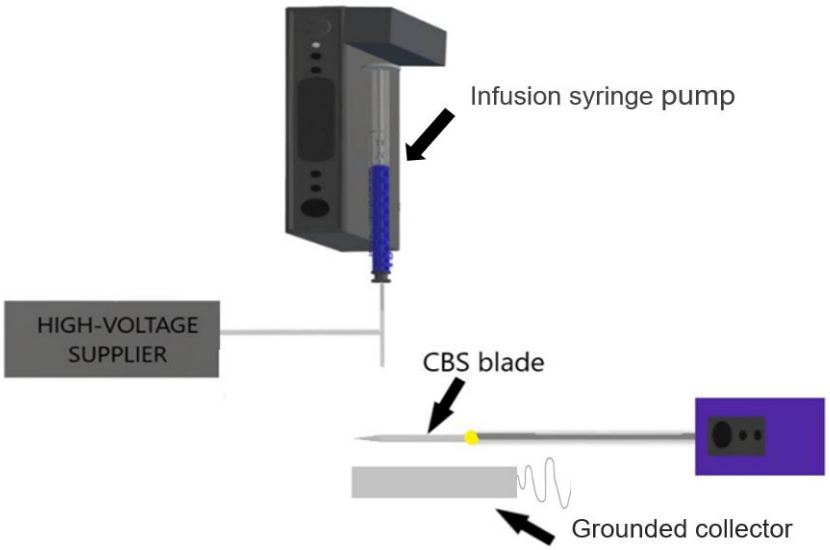


Figure 2.2. The schematic representation of the electrospinning process used in this study

2.5.1.4 Hydrolysis of electrospun PAN on CBS blades

After the CBS blades were coated with electrospun PAN, the hydrolysis process was applied directly to the blades. Three different hydrolysis procedures were applied to the blades. In the first procedure, the blades were hydrolyzed with 2.0 M NaOH for 30, 60, and 120 minutes at 70 °C. In the second procedure, the blades were hydrolyzed with 0.5 M NaOH for 60 min at 70 °C. In the third procedure, hydrolysis was performed with 5.0 M NaOH at 70 °C for 1, 2, 2.5, 3, 5 and 8 hours. After each hydrolysis reaction, the blades were washed with distilled water until neutral wash solutions were observed. As in the case of hydrolysis of electrospun PAN mat, the color of the coating on the blades changed from white to orange during the hydrolysis step. The steps involved in the preparation of functional electrospun-coated CBS blades are summarized in Figure 2.3. After 2.5 hours of hydrolysis with 5.0 M NaOH, coating loss was observed from the blades.

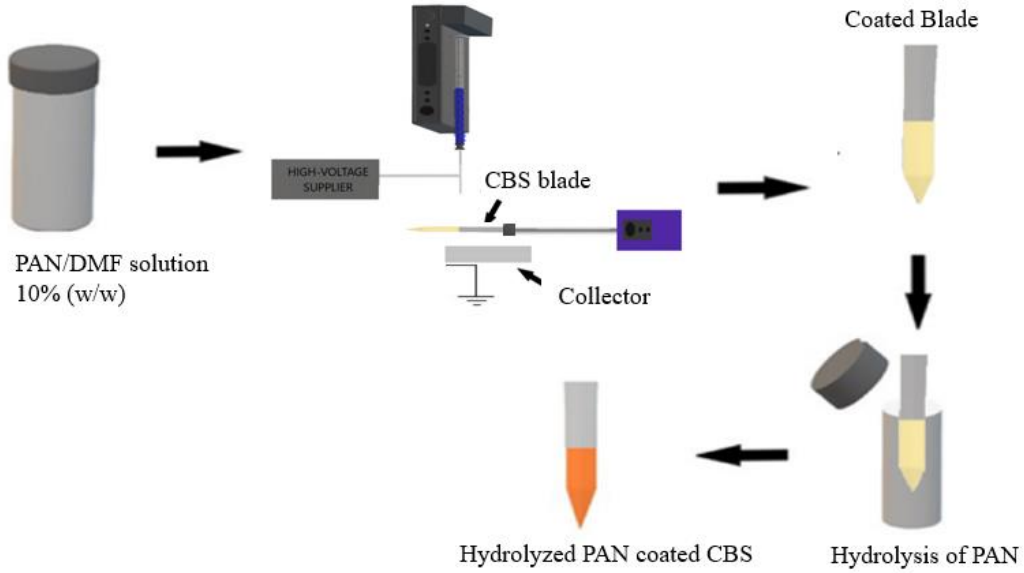


Figure 2.3. General representation of the preparation of CBS blades

2.5.2 PVP as extractive phase for CBS

PVP was selected as an alternative polar polymer to be used as an extractive phase. Differently from PAN, PVP is soluble in water; therefore, PVP experiments proceeded in two steps: (1) electrospinning of PVP and (2) crosslinking of electrospun PVP to avoid its solubility.

2.5.2.1 Electrospinning of PVP

Various concentrations of PVP (20%, 30%, 40%, 50% and 60%, w/w) were prepared in ethanol or water. The electrospinning of PVP was optimized as in case of PAN. The summary of tested electrospinning parameters is shown in Table 2.5.

Table 2.5 Tested parameters for optimization of electrospinning of PVP

Sample #	Polymer	Solvent	Distance (cm)	Flow rate (mL/h)	Voltage (kV)
1	30% PVP	Ethanol	8	0.4	8
2	30% PVP	Ethanol	8	0.4	14
3	30% PVP	Ethanol	8	0.4	20
4	30% PVP	Ethanol	15	0.4	14
5	30% PVP	Ethanol	15	0.4	20
6	30% PVP	Ethanol	15	2.4	20
7	30% PVP	Ethanol	15	0.4	20
8	30% PVP	Ethanol	15	0.4	8
9	20% PVP	Ethanol	15	0.4	8
10	20% PVP	Ethanol	15	0.4	14
11	20% PVP	Ethanol	8	0.4	14
12	20% PVP	Ethanol	8	0.4	8
13	20% PVP	Ethanol	8	0.4	20

2.5.2.2 Crosslinking of PVP electrospun fibers

PVP polymer has a highly polar structure therefore it is highly soluble in water. Consequently, the polymer must be crosslinked to eliminate its solubility in water and other polar solvents before used as an extractive phase. Hence, electrospun PVP was crosslinked using four different methods described below.

2.5.2.2.1 Crosslinking with initiator

20% (w/w) PVP was prepared in ethanol, and 2.5×10^{-4} mol of potassium peroxydisulfate ($K_2S_2O_8$) was added as an initiator. The resulting mixture was electrospun under pre-optimized conditions (flow rate 0.4 mL/h, voltage 20 kV, distance 15 cm). Following the electrospinning, the mats were kept in an oven at 80 °C for 1, 2, 3 and 4 hours to complete the crosslinking.

2.5.2.2.2 Crosslinking by heat treatment

PVP crosslinking was also investigated under heating the mat at 150 °C for 6, 12, 24 and 44 hours. These trials were performed with 30% PVP (dissolved in ethanol) with following electrospinning conditions: flow rate: 0.4 mL/h, voltage: 20 kV, distance: 15 cm.

2.5.2.2.3 Crosslinking with UV light

The next tested crosslinking method for PVP mat was curing with UV light at 254 nm wavelength for 15, 30, 60 and 120 minutes as described by Rosa et al. [100]. These trials were performed with 30% PVP (dissolved in ethanol) with following electrospinning conditions: flow rate: 0.4 mL/h, voltage: 20 kV, distance: 15 cm.

2.5.2.2.4 Heat followed by UV crosslinking

Finally, a combined method in which the PVP mat was first crosslinked at 150 °C at varying time intervals and sequentially subjected to UV light for 30 and 60 minutes was tested. Table 2.6 illustrates the tested parameters for heat and UV crosslinking of PVP mats. Following the crosslinking, each mat was tested to check whether they were soluble in water or not by keeping in water for 5 and 15 minutes.

Table 2.6 Optimized parameters for heat and UV crosslinking of fibers

Sample #	Crosslinking conditions:
1	150 °C/6 h
2	150 °C/6 h
3	30 min UV light 150 °C/12 h
4	150 °C/12 h 30 min UV light
5	150 °C/12 h 60 min UV light

2.5.3 PVA-PVP blend as extractive phase for CBS

For this study, 1.0 g of 10% polyvinyl alcohol (PVA) (dissolved in water) was mixed with 0.36 g of citric acid, and then 0.50 g of 30% PVP (dissolved in ethanol) solution was added following the protocol described by Truong et al. [101]. The resulting mixture was in the form of a gel. Prior to electrospinning, to dissolve the gel, the mixture was stirred at 50-60 °C for 15 minutes on a hot plate within a magnetic stirrer. The effect of the crosslinker amount was also tested by adding 0.10, 0.20 and 0.36 g of citric acid to the polymeric mixture. The electrospinning was performed with the following conditions: 20 kV voltage, the distance between the tip of the syringe and the grounded collector plate:12 cm and mixture flow rate: 1 mL/h. To complete the crosslinking of the electrospun material, the mat was kept in an oven at 130°C for 15, 30, 60 and 120 minutes.

2.6 Evaluation of coated blades with LC-MS

Before testing the novel CBS probes for direct to MS analyses, first, the extractive properties were characterized with classical method (LC-MS). The studies

performed are summarized below. Representative chromatograms of the analytes are shown in Appendix A Figure A.2.

2.6.1 Effect of hydrolysis time of electrospun PAN on analyte sorption

To find out the optimum hydrolysis conditions that leads to best sensitivity the extraction performances of blades produced with different hydrolysis conditions were tested. For this purpose, 3 replicates of unhydrolyzed PAN, hydrolyzed PAN for 1 hour with 5.0 M NaOH, 2 hours with 5.0 M NaOH, and 3 hours with 5.0 M NaOH were prepared. The extraction was performed using 1.5 mL of synthetic urine containing 250.0 ng/mL of each analyte (serotonin, phenylalanine, tryptophan, 8-oxo-dG, 8-oxo-dA and 5-hydroxyindoleacetic acid). The extractions were performed for 30 min using 1000 rpm as agitation speed. Desorption of analytes from the blades was carried out with 1.5 mL of acetonitrile/methanol/water (40/40/20, v/v/v) mixture containing 0.1% formic acid at 1000 rpm agitation for 30 min.

2.6.2 Optimization of extraction parameters

Increasing the overall repeatability and sensitivity of the method for coated blade sprays is important and for this reason the effect of pH, ionic strength, extraction time, desorption time and desorption solvents were investigated in three replicates. Since these parameters are independent of the CBS applications and only indicate the performance of the extractive phase these parameters were investigated in LC-MS.

2.6.2.1 Effect of pH on the extraction of analytes

Phosphate buffer solution with pH of 3.0, 5.0, 7.0 and 10.0 were prepared using suitable conjugate acid base pairs of phosphate and spiked with the analytes. Before extraction, firstly, the blades were preconditioned in 1.5 mL of methanol/water

(50/50, v/v) for 30 min. without mixing in such a way that the coating was completely submerged in the solution. Preconditioned blades were then quickly washed with distilled water (1.5 mL) and then submerged into 1.5 mL of buffer solution spiked with 500.0 ng/mL of all analytes (serotonin, phenylalanine, tryptophan, 8-oxo-dG, 8-oxo-dA and 5-hydroxyindoleacetic acid). The extractions were performed for 5 min using 1000 rpm as agitation speed. Desorption of analytes from the blades was carried out with 150.0 µL of acetonitrile/methanol/water (40/40/20, v/v/v) mixture containing 0.1% formic acid at 1000 rpm agitation for 15 min.

2.6.2.2 Effect of ionic strength on the extraction of analytes

Different amounts of NaCl were added to the solution to investigate the effect of salt concentration on extraction of the analytes. Prepared samples were no salt, 0.010 M, 0.10 M, and 1.0 M NaCl in ultra-pure water. Before extraction, firstly, the blades were preconditioned in 1.5 mL of methanol/water (50/50, v/v) for 30 minutes without mixing in such a way that the coating was completely submerged. Preconditioned blades were then quickly washed with distilled water (1.5 mL) and then submerged into 1.5 mL of solution spiked with 500.0 ng/mL of all analytes (serotonin, phenylalanine, tryptophan, 8-oxo-dG, 8-oxo-dA and 5-hydroxyindoleacetic acid). The extractions were performed for 5 min using 1000 rpm as agitation speed. Following that, the extracted analytes were desorbed from the coated blade spray with 150.0 µL of acetonitrile/methanol/water (40/40/20, v/v/v) mixture containing 0.1% formic acid at 1000 rpm agitation for 15 min.

2.6.2.3 Effect of extraction time on the extraction of analytes

The effect of extraction time on the extracted analytes was investigated with various extraction times. Investigated extraction times were 1 min, 2 min, 5 min, 15 min and 30 min. Before extraction, firstly, the blades were preconditioned in 1.50 mL of methanol/water (50/50, v/v) for 30 minutes without mixing in such a way that the coating was completely submerged. Preconditioned blades were then quickly

washed with distilled water (1.5 mL) and then submerged into 1.5 mL of synthetic urine solution (pH 6.4) spiked with 500.0 ng/mL of all analytes (serotonin, phenylalanine, tryptophan, 8-oxo-dG, 8-oxo-dA and 5-hydroxyindoleacetic acid). The extractions were performed for 1, 2, 5, 15 and 30 min using 1000 rpm as agitation speed. Following that, the extracted analytes were desorbed from the coated blade spray. Desorption of analytes from the blades was carried out with 150.0 µL of acetonitrile/methanol/water (40/40/20, v/v/v) mixture containing 0.1% formic acid at 1000 rpm agitation for 15 min.

2.6.2.4 Effect of desorption solvent on elution of the analytes

The effect of the desorption solution on elution of the analytes was investigated with 3 different solvents, namely, ACN:MeOH:H_2O (40:40:20, v/v/v) containing 0.1% formic acid, MeOH:H_2O (50:50, v/v) containing 0.1% formic acid, and MeOH:H_2O (85:15) containing 0.1% formic acid. Before extraction, firstly, the blades were preconditioned in 1.5 mL of methanol/water (50/50, v/v) for 30 minutes without mixing in such a way that the coating was completely submerged. Preconditioned blades were then quickly washed with distilled water (1.5 mL) and then submerged into 1.5 mL of synthetic urine solution (pH 6.4) spiked with 500.0 ng/mL of all analytes (serotonin, phenylalanine, tryptophan, 8-oxo-dG, 8-oxo-dA and 5-hydroxyindoleacetic acid). The extractions were performed for 5 min using 1000 rpm as agitation speed. Following that, the extracted analytes were desorbed from the coated blade spray with 150.0 µL of MeOH/H_2O (50:50; v/v) with 0.1% F.A, MeOH/H_2O (85:15; v/v) with 0.1% F.A and ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A at 1000 rpm agitation for 15 min.

2.7 CBS-MS

Following the preliminary investigation of the coatings with LC-MS, direct MS optimization studies were carried out to obtain the maximum sensitivity possible

from the CBS-MS system. For this purpose, the effect of desorption solvent, desorption volume and desorption time were optimized.

2.7.1 Effect of desorption volume

The effect of desorption volume was investigated in three replicates of four different desorption volumes: 5.0, 10.0, 15.0, and 20.0 µL of ACN:MeOH:H_2O (40:40:20; v/v/v) with 0.1% F.A. As a first step, extraction was performed. For this purpose, the blades were immersed in 1.5 mL of synthetic urine (pH 6.4) containing 500.0 ng/mL of mixed analyte and the extraction was performed for 5 min at 1000 rpm. Following the extraction, blades were washed in 1.5 mL of water for 2 seconds. The above-mentioned desorption volumes were tested in CBS-MS mode using 15 s as desorption time.

2.7.2 Effect of desorption solvent

The effect of the desorption solution on the signal obtained was investigated with three different solvents, namely, ACN/H_2O (85:15; v/v) with 0.1% F.A, MeOH/H_2O (85:15; v/v) with 0.1% F.A and ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. For this purpose first extractions were performed with the blades by immersing them in 1.5 mL of synthetic urine (pH 6.4) containing 500.0 ng/mL of mixed analyte for 5 min at 1000 rpm. Following the extraction, blades were washed in 1.5 mL of water for 2 seconds. 10.0 µL mixture of ACN/H_2O (85:15; v/v) with 0.1% F.A, MeOH/H_2O (85:15; v/v) with 0.1% F.A and ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A were investigated as desorption solvent in 3 replicates.

2.7.3 Effect of desorption time

The desorption time of the CBS process is very critical. For example, if the time is too long, the solvent may evaporate, making it impossible to produce an electrospray

after applying voltage. However, if it is too short, the analytes might not have enough time to be desorbed from the coating, which could impact the sensitivity. To evaluate the effect of desorption time, first extractions were performed with the blades. For this purpose, the blades were immersed in 1.5 mL of synthetic urine (pH 6.4) containing 500.0 ng/mL of mixed analyte and the extraction was performed for 5 min at 1000 rpm. Following the extraction, blades were washed in 1.5 mL of water for 2 seconds. The effect of desorption time was investigated in three replicates using three time points, namely, 15 s, 30 s, and 15 s with 10.0 µL fresh desorption solution added again after drying of the first 10.0 µL of desorption solution (ACN/MeOH/H_2O (40:40:20; v/v/v)) with 0.1% F.A.

2.7.4 Comparison of HLB-SCX and hydrolyzed PAN as CBS-MS coating

HLB-SCX modified with strong cation exchange groups was synthesized as described by Lendor, then 0.400 g of PAN was dissolved in 3.6 g of DMF and 0.21 g of HLB-SCX was added to this solution to prepare the electrospinning mixture [102]. After optimization the electrospinning conditions for this slurry, the CBS blades were coated. Coating parameters were as follows: applied voltage was 10 kV, needle-to-blade distance was 15 cm, and feed rate was 1.0 mL/h. In sorption studies, hydrolyzed PAN and HLB-SCX-coated CBS blades prepared at the same thickness were used and the extraction performances of these blades were compared. For this purpose, during the extraction process, the blades were immersed in 1.5 mL of synthetic urine (pH 6.4) containing 250.0 ng/mL mixed analyte and the extraction was performed for 5 minutes at 1000 rpm. Following the quick rinsing step CBS-MS analyses were performed with the conditions described for hydrolyzed PAN coated CBS-MS.

2.8 Validation studies

Linearity, limit of quantification (LOQ), repeatability (in terms of %RSD) and accuracy (in terms of %RE) studies for method validation were carried out according to the bioanalytical method development and validation standards given in the 'Bioanalytical Method Validation Guidance for Industry' (FDA, Bioanalytical Method Validation Guidance for Industry, 2018) guide published by the FDA. In a typical experiment, before extraction, the PAN-coated blades were preconditioned in 1.5 mL of methanol/water (50/50, v/v) for 30 minutes without mixing in such a way that the coating was completely submerged. Preconditioned blades were then quickly washed with distilled water (1.5 mL) and then immersed into 1.5 mL of synthetic urine solution spiked with of all analytes (serotonin, phenylalanine, tryptophan, 8-oxo-dG, 8-oxo-dA and 5-hydroxyindoleacetic acid). The extractions were performed for 5 min using 1000 rpm as agitation speed. Following that, the extracted analytes were detected using CBS-MS after fixing the CBS blades at a pre-optimized distance in front of the MS. For desorption 10.0 µL of desorption solution (ACN/MeOH/H_2O (40:40:20; v/v/v)) with 0.1% F.A. was pipetted on the surface of CBS and after 15 s 4.5 kV voltage was applied on the blade. The chronogram was collected for 45 s in SIM mode. Nicotine was used as internal standard for all analytes. 250.0 ng/mL of nicotine was spiked into the synthetic urine solution.

2.8.1 Linearity and limit of quantification (LOQ)

Linear operating range of the method for each analyte was determined by CBS-MS using matrix-similar synthetic urine standard solutions. Eight different concentrations of samples (50.0, 100.0, 150.0, 250.0, 500.0, 750.0, 1000.0, and 1500.0 ng/mL) were prepared in 3 replicates for the linearity studies. The FDA's "Bioanalytical Method Validation Guidance for Industry" (FDA, Bioanalytical Method Validation Guidance for Industry, 2018) handbook describes how to determine the limit of quantification (LOQ) for the bioanalytical method

development and validation standard. Based on this guideline, first, the nominal concentrations of the calibration points were back calculated using the acquired linear regression equation. Then, the LOQ values were determined as the lowest point providing maximum of 20% relative error in the back calculations.

2.8.2 Repeatability

The repeatability of the method was tested with 3 replicate analyzes for three QC levels using low, medium, and high concentrations that could be found in the linear operating range. Method repeatability was studied as intraday (three separate analyzes with three replicates in one day) and inter-day (analysis with three replicates on three different days).

2.8.3 Accuracy

Intraday accuracy was analyzed with low, medium, and high concentrations that can be found in the linear operating range and in 3 replicates for each level. Method accuracy was determined as percent relative error (%RE) by intraday (three analyzes in one day) and interday (three different days) studies.

2.8.4 Comparison of SPE-LC-MS and CBS-MS

The developed CBS-MS method was compared with the SPE, which was chosen as the reference method. Comparison of the method was performed using blind analysis of a sample. Concentration of the unknown sample was determined in CBS-MS mode by matrix-matched SPME calibration under conditions developed in method optimization for CBS-MS. As suggested by the producer's guide, cartridges were conditioned with 5.0 mL of methanol/water (50/50, v/v), then washed with 2.5 mL of water. As a next step, 5.0 mL (half-half diluted with water) sample was loaded into the cartridge. After sorption, washing was done with 2.5 mL of water and

finished with elution of analytes with 2.5 mL of ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. Concentration of the SPE eluate was determined after analysis of the eluent and standard calibration solutions in LC-MS using the HILIC method.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 CBS-MS optimizations

The coated blade spray was used as an extractive probe and electrospray source for analysis of polar analytes with mass spectrometry. Various parameters were investigated to obtain the best sensitivity from the CBS-MS system with repeatable results. The effect of various parameters on the signal was discussed in Chapter 2 (Section 2.7). Table 3.1 shows the optimized CBS-MS conditions. As an example, chronograms obtained during optimization of the voltage applied by the external voltage source is given in Appendix A in Figure A.3. As the external voltage increases, the signal increases. Under 3 kV, a sufficient signal could not be received. The best signal was obtained at 4.5 kV.

Table 3.1 CBS-MS conditions

Parameter	**Settings**
Ionization mode	CBS-MS
Polarity	Positive
Fragmentor voltage	110 V
Gas temperature	300 °C
Drying gas flow	1.0 L/min
Capillary Voltage	3000 V
Nebulizer pressure	700 Torr
External source voltage	4.5 kV

3.2 Preparation of polar CBS blades

Since each polymer has different moiety, it also has a different solubility in water. For example, PAN is insoluble in water, while PVP and PVA are highly soluble in water. However, any polymer to be considered as extractive phase should be insoluble in water. For this reason, PVP and PVA were crosslinked under various conditions described in Section 2.5, while PAN was modified with ion weak cation exchange (carboxylic acid) groups and weak anion exchange (amide) groups to have higher affinity for polar analytes. This section summarized the results obtained in preparation of electrospun materials, their hydrolysis and crosslinking optimizations and characterization results.

3.2.1 PAN as extractive phase for CBS

As mentioned before, the extractive property of the PAN polymer is quite low; therefore, the obtained polymers must be functionalized with amide groups and carboxylic acid groups in order to be used as an extractive phase. Studies with PAN progressed in two phases: (1) electrospinning of PAN and (2) hydrolysis of electrospun PAN to weak cation exchange polymer.

3.2.1.1 Electrospinning of PAN

Scanning electron microscope (SEM) images of electrospun PAN showed that nanofibers of different thicknesses were obtained at different applied voltages, distances, and flow rates. The representative images of electrospun PAN fibers under various conditions are given as inset in Table 3.2. According to the results shown as micrographs, the voltage has significant effect on the fiber diameter. For instance, the fiber diameter is *ca.* 200 nm at 14.0 kV and 800 nm at 20.0 kV. During this process the concentrated electric field at the needle tip generates multiple electrospinning jets on the grounded plate. However, the needle's input charges are shared among the multiple jets, reducing electrostatic forces stretching the fiber and resulting in a larger average fiber diameter. This also improves the distribution of fiber diameters in the collected fibers. Thus, the increase in voltage results in larger fiber diameters. Another parameter that affects the fiber thickness is the solution flow rate. Fiber diameter increased with increasing flow rate from 0.4 mL/h to 2.4 mL/h. Optimum conditions for PAN electrospinning were determined as applied voltage 20 kV, the distance between the needle tip and collector 21 cm, and flow rate 2.4 mL/h.

Table 3.2 Electrospun PAN fibers obtained under various electrospinning conditions

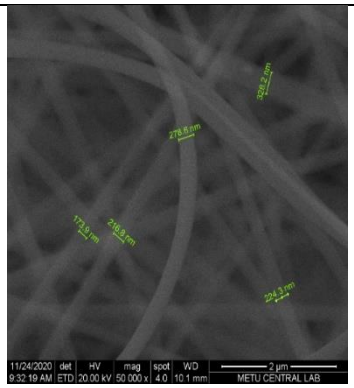
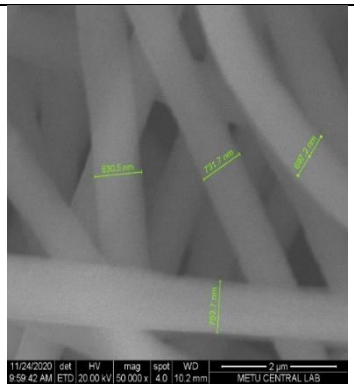
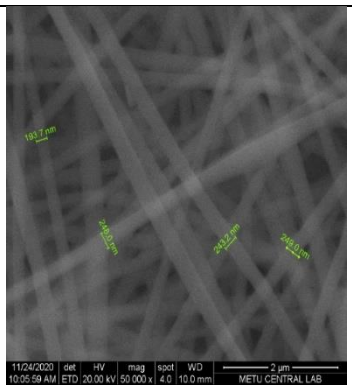
Sample	Parameters	SEM image	Results
1	Polymer: 10% PAN Solvent: DMF Voltage: 14 kV Distance: 15cm Flow rate: 2.4 mL/h		Fiber diameters were found in the range of 173-328 nm.
2	Polymer: 10% PAN Solvent: DMF Voltage: 20 kV Distance: 21 cm Flow rate: 2.4 mL/h		Fiber diameters were found in the range of 697-830 nm.

Table 3.2 (cont'd)

3	Polymer: 10% PAN Solvent: DMF Voltage: 20 kV Distance: 15cm Flow rate: 0.4 mL/h		Fiber diameters were found in the range of 193-247 nm.

3.2.1.2 Hydrolysis of electrospun PAN

Electrospun PAN was hydrolyzed with 0.5 M, 2.0 M and 5.0 M NaOH solutions as described in Section 2.5.1.2. The obtained SEM images (Table 3.3) for hydrolyzed electrospun PAN showed that the surface of PAN become rougher as the hydrolysis time increases. This observation was more obvious when electrospun PAN fibers hydrolyzed with higher concentration of NaOH at longer time. In overall, it was found that the fiber structures were preserved reasonably well under all experimental conditions suggesting that this coating is promising for the CBS blades. PAN hydrolyzed with 5.0 M NaOH solutions was preferred as the extractive phase because it had better sorption in CBS-MS studies.

Table 3.3 SEM images of hydrolyzed PAN

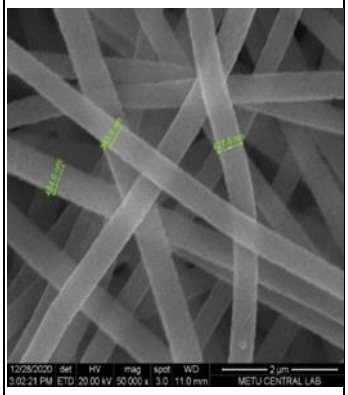
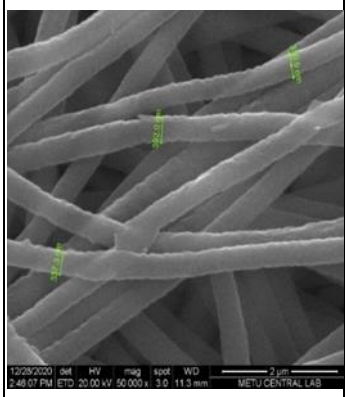
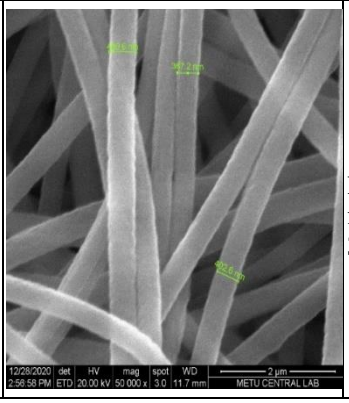
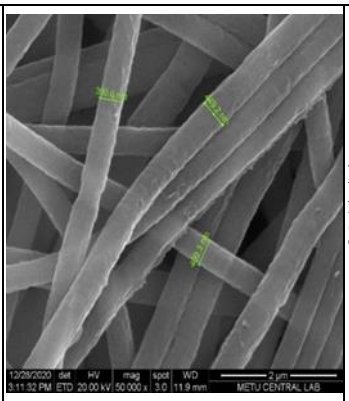
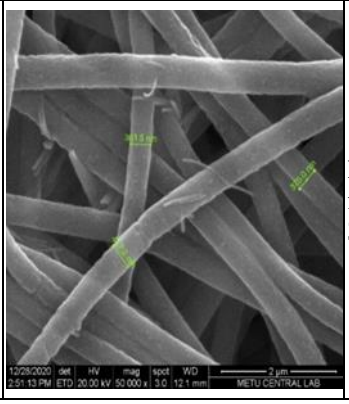
Sample	Parameters	SEM image	Results
1	Hydrolysis time: 1 h NaOH concentration: 0.5 M Hydrolysis temperature: 70 ºC Stirring: not applied.		Fiber diameters were found in the range of 363-454 nm.
2	Hydrolysis time: 1 h NaOH concentration: 2.0 M Hydrolysis temperature: 70 ºC Stirring: 700 rpm.		Fiber diameters were found in the range of 337-392 nm.
3	Hydrolysis time: 1 h NaOH concentration: 2.0 M Hydrolysis temperature: 70 ºC Stirring: not applied.		Fiber diameters were found in the range of 367-489 nm.

Table 3.3 (cont'd)

4	Hydrolysis time: 2 h NaOH concentration: 2.0 M Hydrolysis temperature: 70 ºC Stirring: not applied.		Fiber diameters were found in the range of 390-449 nm.
5	Hydrolysis time: 2.50 h NaOH concentration: 5.0 M Hydrolysis temperature: 70 ºC Stirring: not applied.		Fiber diameters were found in the range of 361-471 nm.

3.2.1.3 FTIR spectra of PAN based coatings

The FTIR spectra of unhydrolyzed electrospun PAN and PAN hydrolyzed in 5.0 M NaOH solution for 150 min were acquired after preparation of KBr pellets containing samples of polymers. Figure 3.1 shows the FTIR spectra of electrospun PAN and electrospun PAN hydrolyzed with 5.0 M NaOH for 2.50 h (spectral resolution of 4 cm^{-1}). The peaks attributed to C-N stretching at 1470 cm^{-1} and 2243 cm^{-1} were present in both unhydrolyzed and hydrolyzed PAN. The presence of these peaks in hydrolyzed PAN indicates that the nitrile groups in PAN were only partially hydrolyzed. In a study conducted by Jin et al. [103] for hydrolysis of PAN, it has been found that the alkaline hydrolysis process occurs in two steps; first step is formation of amide and second step is formation of carboxylic acid. In current study,

the strong peak at 1561 cm^{-1} which is only observed in hydrolyzed PAN shows the presence of amide ($–CONH_2$) in the hydrolyzed structure. Which suggest that in our case amide groups were also present in the final structure. As expected from the hydrolysis, carboxylic acid moieties were also formed in the polymer structure. This conversion is supported by the presence of peak of the carbonyl group in 1700 cm^{-1}. In addition, the broad OH stretching peaks at 3500-2900 cm^{-1} support the formation of carboxylic acid as a result of the hydrolysis. Figure 3.2 shows the FTIR spectra of electrospun PAN hydrolyzed with 5.0 M NaOH for 2.5 hours (spectral resolution of 2 cm^{-1}).

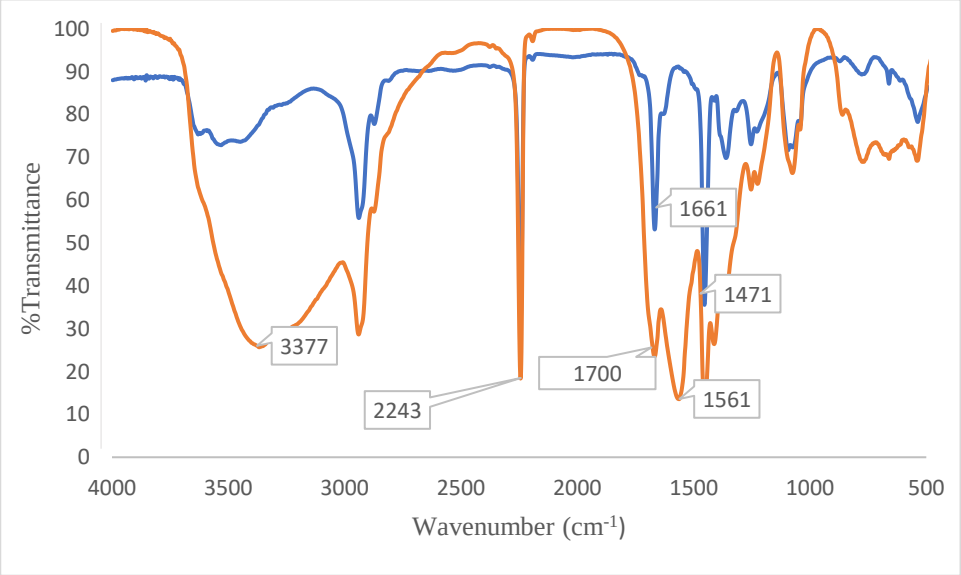


Figure 3.1. FTIR spectra of electrospun PAN (upper spectrum) and electrospun PAN hydrolyzed with 5.0 M NaOH for 2.5 hours (lower spectrum) (spectral resolution of 4 cm^{-1})

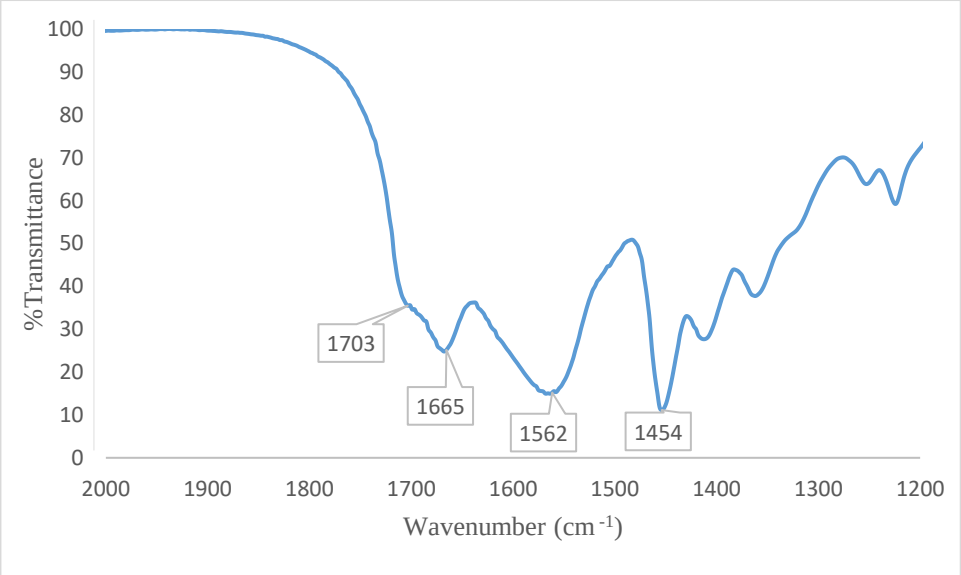


Figure 3.2. FTIR spectra of electrospun PAN hydrolyzed with 5.0 M NaOH for 2.5 hours (spectral resolution of 2 cm^{-1})

3.2.2 PVP

3.2.2.1 Electrospinning of PVP

In the electrospinning experiments with PVP, the effect of the parameters given in Section 2.5.2.1 was investigated. In these experiments it was not possible to obtain electrospun fibers with 50% and 60% PVP concentrations due to high viscosity of these mixtures. The densest fibers were obtained from 30% and 20% PVP solutions; therefore, various electrospinning parameters were investigated using these two polymeric solutions. The resulting morphologies were examined by scanning electron microscope (SEM). The findings are summarized in Table 3.4. As can be seen from the micrographs given in the table, fiber diameters considerably increased as the voltage was increased from 8.5 kV to 20 kV. The feed rate had also important effect on the fiber thickness. Especially with the increase of the flow rate from 0.4 mL/h to 2.4 mL/h, the fiber diameters became quite thick. Based on the results, the

distance between the needle tip and the collector did not affect the fiber morphology as a solvent that evaporates easily was used. 20% PVP solution in ethanol, with electrospinning conditions of 0.4 mL/h flow rate, 15 cm distance, 20 kV voltage, was easily electrospinned and fibers were obtained.

Table 3.4 Electrospun PVP fibers obtained under various electrospinning conditions

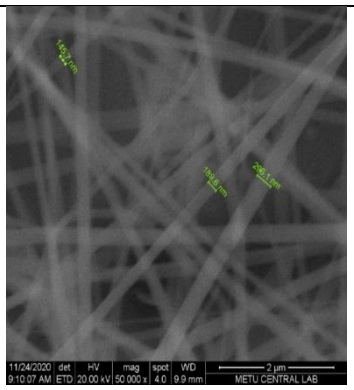
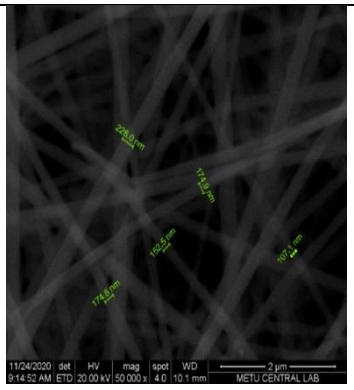
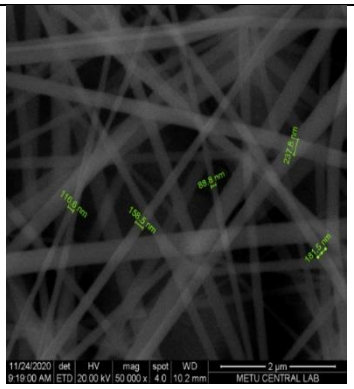
Sample	Experiment Conditions	SEM image	Comments
1	Polymer: 30% Solvent: Ethanol Voltage: 8.5 kV Flow rate: 0.4 mL/h Distance: 8 cm		Fiber diameters were found in the range of 145-296 nm. The fiber structures are smooth.
2	Polymer: 30% Solvent: Ethanol Voltage: 14 kV Flow rate: 0.4 mL/h Distance: 8 cm		Fiber diameters were found in the range of 107-226 nm.
3	Polymer: 30% Solvent: Ethanol Voltage: 14 kV Flow rate: 0.4 mL/h Distance: 15 cm		Fiber diameters were found in the range of 89-237 nm.

Table 3.4 (cont'd)

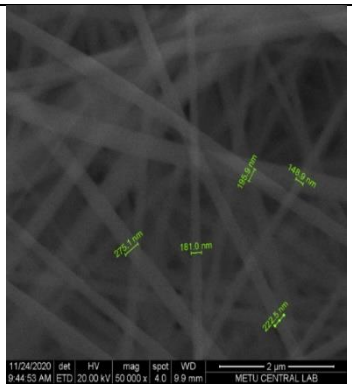
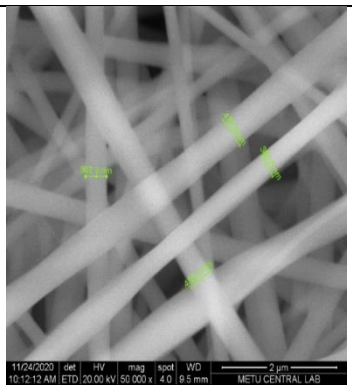
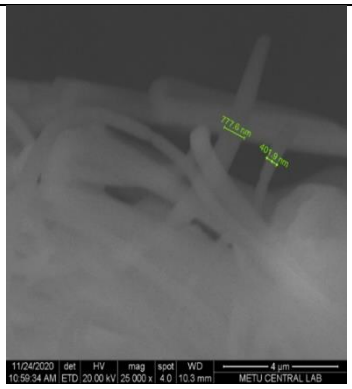
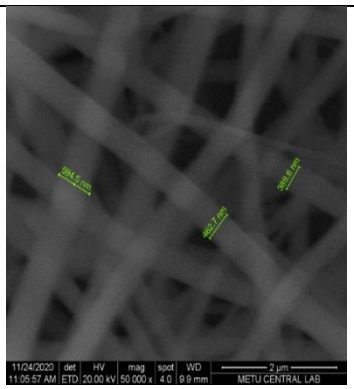
4	Polymer: 20% Solvent: Ethanol Voltage: 20 kV Flow rate: 0.4 mL/h Distance: 15 cm	No images available.	Electrospinning could be done similarly with 30% PVP.
5	Polymer: 30% Solvent: Ethanol Voltage: 20 kV Flow rate: 0.4 mL/h Distance: 15 cm		Fiber diameters were found in the range of 148 -275 nm.
6	Polymer: 30% Solvent: Ethanol Voltage: 20 kV Flow rate: 2.4 mL/h Distance: 15 cm		Fiber diameters in the range of 360-476 nm found.
7	Polymer: 30% Solvent: Ethanol Voltage: 20 kV Flow rate: 0.4 mL/h Distance: 15 cm		Fiber diameters were found in the range of 401-777 nm.

Table 3.4 (cont'd)

8	Polymer: 30% Solvent: Ethanol Voltage: 8.5 Flow rate: 0.4 mL/h Distance: 15 cm		Fiber diameters in the range of 390-594 nm found.

3.2.2.2 Crosslinking of electrospun PVP

3.2.2.2.1 Crosslinking with initiator

Tertiary amine and carbonyl groups in the structure of PVP have the property of making hydrogen bonds. Thus, a good interaction between the polymer and the selected polar analytes is expected. However, as mentioned before, the most important disadvantage of PVP is its solubility in water. This feature would cause a partial or full dissolution of the coating during the use. To avoid this problem, blocking the solubility of PVP is necessary, which is possible by crosslinking the polymer. The crosslinking reaction of PVP with potassium peroxydisulfate ($K_2S_2O_8$) is shown in Figure 3.3 [104]. Results of the studies conducted for crosslinking the polymer using initiator are summarized in Table 3.5. The results showed that, 20% PVP and 30% PVP solutions with different ratios of initiator are not capable to block the solubility of the polymer. Since the initiator has a low solubility in ethanol, the polymeric mixture was prepared in ethanol-water solution as well. Obtained solubility results from this experiment showed that this approach was not successful to break the solubility as well. When higher PVP concentrations were tested, similar and unsuccessful results were obtained. The effect of reaction time along with the concentration was also examined, but this also failed to block the solubility of PVP.

As can be seen from the results, water-resistant electrospun fibers with the desired stability could not be obtained with this approach.

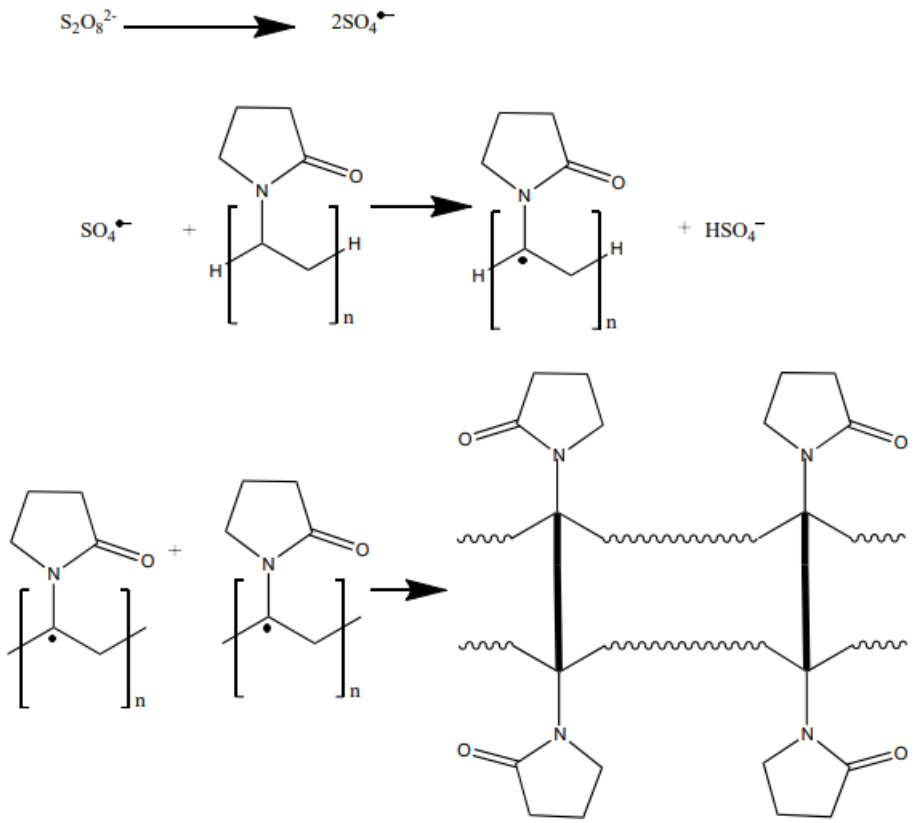


Figure 3.3. The expected crosslinking reaction of PVP with the initiator ($K_2S_2O_8$) (Beram et al., 2018)

Table 3.5 Crosslinking studies of PVP using initiator

Sample No.	Experiment conditions	Results
1	The prepared polymer was kept in an oven (80 °C) for 1 and 2 hours. Electrospinning conditions: Polymer: 20%, Solvent: Ethanol/Water (50/50, v/v) Initiator: in 1.1×10^{-4} mol/12 g polymer solution Voltage: 20 kV, Flow rate: 0.4 mL/h, Distance: 15 cm	The polymer was dissolved in a mixture of water and MeOH/H_2O (85:15, v/v) within 1 min.
2	The prepared polymer was kept in an oven (80 °C) for 1 and 2 hours. Electrospinning conditions: Polymer: 40%, Solvent: water, Initiator: 18×10^{-4} mol/ 5 g in polymer solution Voltage: 20 kV, Flow rate: 0.4 mL/h, Distance: 15 cm	
3	The prepared polymer was kept in an oven (80 °C) for 1 and 2 hours. Electrospinning conditions: Polymer: 30%, Solvent : Ethanol/Water (50/50, v/v) Initiator: 2.4×10^{-4} mol/ in 10 g polymer solution Voltage: 20 kV, Flow rate: 0.4 mL/h, Distance: 15 cm	
4	The prepared polymer was kept in an oven (80 °C) for 1 and 2 hours. Electrospinning conditions: Polymer: 30%, Solvent: Ethanol/Water (50/50, v/v) Initiator: in 24×10-4 mol/5.45 g polymer solution Voltage: 20 kV, Flow rate: 0.4 mL/h, Distance: 15 cm	
5	The prepared polymer was kept in an oven (80 °C) for 1 and 2 hours. Electrospinning conditions: Polymer: 30%, Solvent: Water Initiator: in 11×10^{-4} mol/10 g polymer solution Voltage: 20 kV, Flow rate: 0.4 mL/h, Distance: 15 cm	No fiber could be produced by electrospinning.

3.2.2.2.2 Crosslinking of PVP using heat

A second crosslinking approach was tested in which temperature-based crosslinking was used by exposing electrospun PVP fibers to 150 °C. In order to not exceed the glass transition temperature of the polymer higher temperatures were avoided. Table 3.6 summarizes the crosslinking studies by heat treatment. The results revealed that crosslinking with temperature was effective to stop the visible solubility of the fibers and resulted in hydrogel. When the effect of the heat treatment time on crosslinking was examined by immersing the resulting mat in water and MeOH/H_2O, the results showed that a period of 6 hours provided sufficient crosslinking to stop the solubility, but the resulting product was a hydrogel. Polymer coatings that are relatively more resistant to both water and methanol were obtained over the longer trials (24 hours). However, since gel formation has been observed in each case, these polymers are not suitable for use as an extractive phase.

Table 3.6 Crosslinking of PVP using heat (Electrospinning conditions: polymer: 30%, solvent: ethanol, voltage: 20 kV, flow rate: 0.4 mL/h, distance: 15 cm)

Sample	**Experiment conditions**	**Before immersion water**	**After immersion water**
1	Crosslinking conditions: 150 °C/24 hours		
2	Crosslinking conditions: 150 °C/44 hours		
3	Crosslinking conditions: 150 °C/6 hours		

3.2.2.2.3 Heat followed by UV crosslinking

PVP is a water-soluble and biocompatible polymer that can be crosslinked to form water insoluble PVP when exposed to UV light. Also, our results showed that it is possible to obtain PVP crosslinking at 150°C PVP, yet not perfect. The crosslinking mechanism of PVP formed by absorption of UV light was investigated by Rosa et al. and it was found that interpolymer chain bonds were formed by a radical reaction [105]. Based on this promising supporting information, the combined effect of heat and UV exposure was tested as described in Section 2.5.2.2.4 to make insoluble the PVP.

The obtained results for the tested parameters are summarized in Table 3.7. As can be seen from the SEM images given in the table; all of the coatings did survive the solubility test in water. However, the electrospun nanofibrous structure was affected. For instance, the nanofiber structure of the electrospun PVP, which was kept at 150 °C for 6 hours only, was destroyed. The fiber structure started to be protected, although not completely, in those samples crosslinked for 12 hours at 150 °C and for which UV exposure was used after the heating at 150 °C. The best results were obtained for the fibers that were kept first at 150 °C for 12 hours and then exposed to UV light for 30 minutes, according to the SEM images. Although promising results were obtained with this approach, the morphology of the fibers were not protected entirely. Therefore, PVP was not used in CBS blades for the extraction of the analytes.

Table 3.7 SEM images of PVP crosslinked with heat and UV light (Electrospinning conditions: polymer: 30%, solvent: ethanol, distance: 15 cm, voltage: 20 kV, flow rate: 0.4 mL/h)

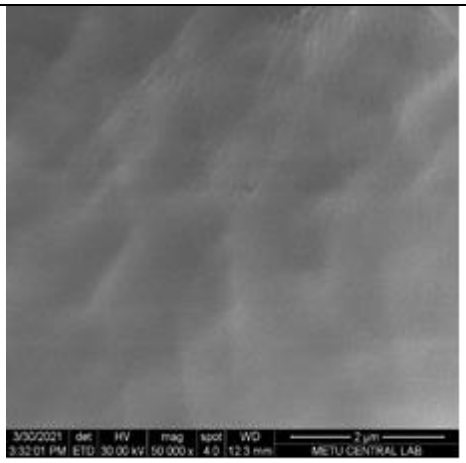
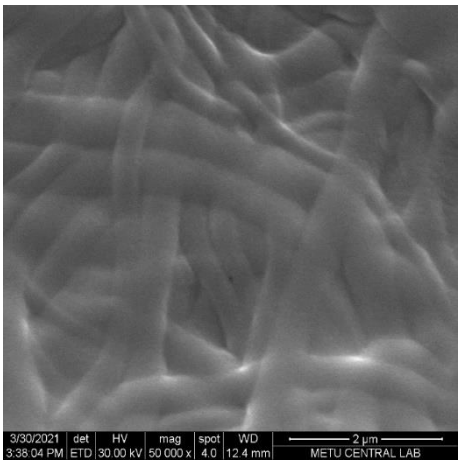
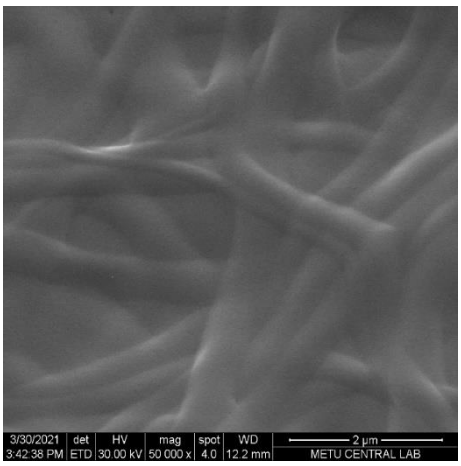
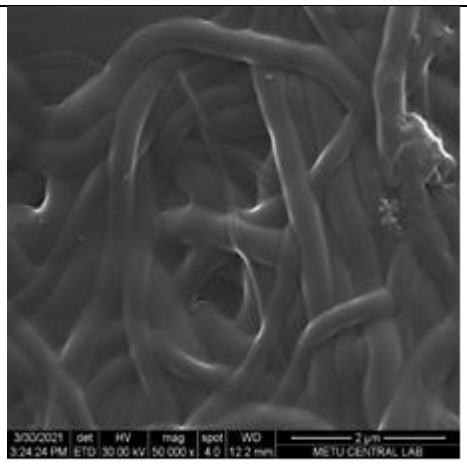
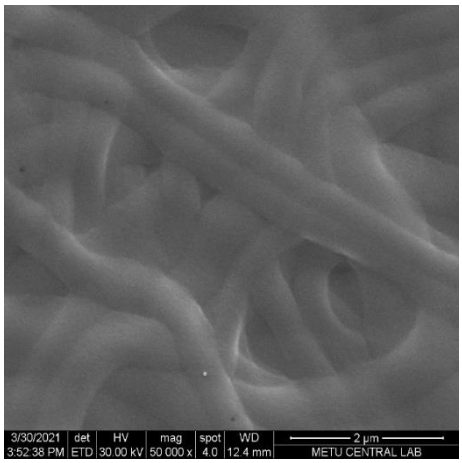
Sample No	Experiment conditions	SEM image
1	Crosslinking conditions: 150 °C/6 h	
2	Crosslinking conditions: 150 °C/6 h 30 min UV light	
3	Crosslinking conditions: 150 °C/12 h	

Table 3.7 (cont'd)

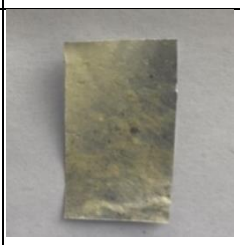
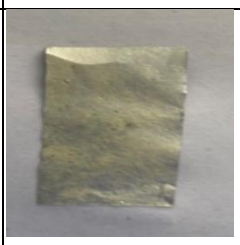
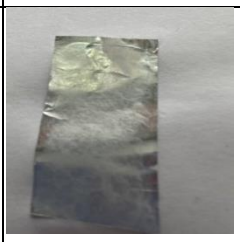
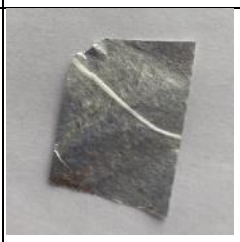
4	Crosslinking conditions: 150 °C/12 h 30 min UV light	
5	Crosslinking conditions: 150 °C/h 60 min UV light	

3.2.3 PVA-PVP blend as extractive phase for CBS

Citric acid shows an esterification reaction with alcohol groups in PVA [106]. This supports that it can be used as a crosslinker. Crosslinking with citric acid was tested by adding 0.10, 0.20 and 0.36 g of citric acid to the polymer solution. The water solubility test results for some parameters are summarized in Table 3.8. The findings show that the coating obtained with the citric acid crosslinker is water insoluble. The mixture containing 0.50 g (30% PVP)/1.0 g (10% PVA)/0.36 g (citric acid) was determined as the most suitable mixture because it is easily electrospun compared to the others. However, the nanofiber structure was not found in the SEM images obtained for these coatings. Moreover, very little coating could be obtained. For these reasons it was not considered further for CBS evaluations.

Based on the results summarized above for PAN, PVP and PVP-PVA, hydrolyzed PAN was selected as the most suitable extractive phase (which does not show any solubility and preserve its nanostructured morphology in aqueous environment) and used for CBS-MS studies.

Table 3.8 Crosslinking of electrospun PVA-PVP with citric acid

Sample no	**Experiment conditions**	**Before immersion water**	**After immersion water**	**Conclusion**
1	Electrospinning solution:1.0 g 10% PVA, 0.50 g 30% PVP and 0.36 g citric acid mixed Crosslinking conditions: 130 °C/30 min			The polymer was insoluble in water.
2	Electrospinning solution: 1.0 g 10% PVA, 0.50 g 30% PVP and 0.36 g citric acid mixed Crosslinking conditions: 130 °C/120 min			The polymer was insoluble in water.
3	Electrospinning solution: 1.0 g 10% PVA, 0.50 g 30% PVP and 0.10 g citric acid mixed Crosslinking conditions: 130 °C/30 min			The polymer was insoluble in water.
4	Electrospinning solution: 1.0 g 10% PVA, 0.50 g 30% PVP and 0.20 g citric acid mixed Crosslinking conditions: 130 °C/30 min			The polymer was insoluble in water.

3.3 CBS-LC-MS

3.3.1 Effect of electrospun PAN hydrolysis time on analyte sorption

The effect of the hydrolysis time of the electrospun PAN on the extraction performance of the blades was also investigated. For this purpose, PAN coated blades were hydrolyzed for 60, 120, 150, 180, 300 and 480 min. The coating on the blades of the hydrolysis times at 180, 300 and 480 minutes began to dissolve due to excessive hydrolysis and completely disappeared. Therefore, no extraction results are available for these coatings. Figure 3.4 shows the amount of analyte extracted during hydrolysis times of 60, 120 and 150 minutes. As the hydrolysis time increases, the amount of analyte extracted increases. The best extraction ability was obtained with PAN hydrolyzed for 150 minutes. A comparison between the extraction capacities of a hydrolyzed PAN coated blade and electrospun PAN showed that the extraction efficiencyof hydrolyzed PAN was higher than that of PAN.

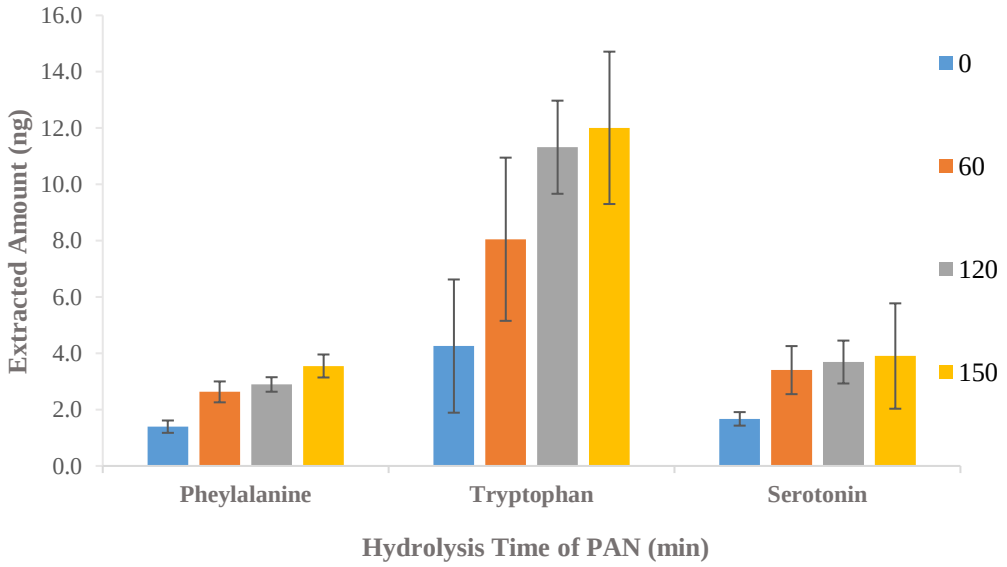


Figure 3.4. Effect of the hydrolysis time of PAN on extraction (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 min

3.3.2 Optimization of extraction parameters

3.3.2.1 Effect of pH on the extraction of analytes

In the case of polar analytes, the pH of the solution has an effect on the ionic form of the analyte. If the analyte has acidic or basic groups at certain pHs, it will be ionic and neutral at other pHs. The pKa values of the analytes in this study were as follows: phenylalanine= 2.20, tryptophan= 2.46, serotonin= 10.16 and 5-hydroxyindoleacetic acid = 4.22, 8-oxo-dG= 7.86. This indicates that the pH of the sample will affect the form of the analyte present in the sample. Since the hydrolyzed PAN sorbent has weak cation exchange groups and weak anion exchange groups, the solution pH will strongly affect the sorption process. Therefore, as sample pH, solutions buffered to pH 3.0, 5.0, 7.0 and 10.0 were used to investigate the effect of pH on sorption. The results obtained for the effect of pH on the sorption of analytes are shown in Figure 3.5. According to the results, tryptophan was extracted at highest amounts at pH 10.0. while for serotonin and 8-oxo-dG the highest extractions were achieved at pH 7.0. Phenylalanine and 8-oxo-dA were better extracted at pH 7 and 10. On the other hand, 5-hydroxyindoleacetic acid showed relatively better sorption at pH 3.0. Tryptophan and phenylalanine have an isoelectric point (pI) of 5.89 and 5.91, respectively. Tryptophan is negatively charged in the basic environment. The sorbent is positively charged due to the anion exchange group. Therefore, tryptophan has better sorption in basic media due to a positive charge and a negative charge interaction. The driving force for the phenylalanine extraction process is mainly the hydrogen bond interaction. 5-hydroxyindoleacetic acid having a carbonyl group is protonated in an acidic medium. Hydrogen bonds can exist between 5-hydroxyindoleacetic acid and sorbent. Serotonin pKa is 10.16 and 8-oxo-dG pKa is 7.86. It is better extracted in a neutral medium with π–π interactions and hydrogen bonding. However, optimum conditions vary from analyte to analyte. To obtain a reasonable sensitivity for all analytes in the sample, the pH of the sample should be

stabilized at a common pH, which can be chosen as pH 7.0 as it gives reasonable results for all analytes.

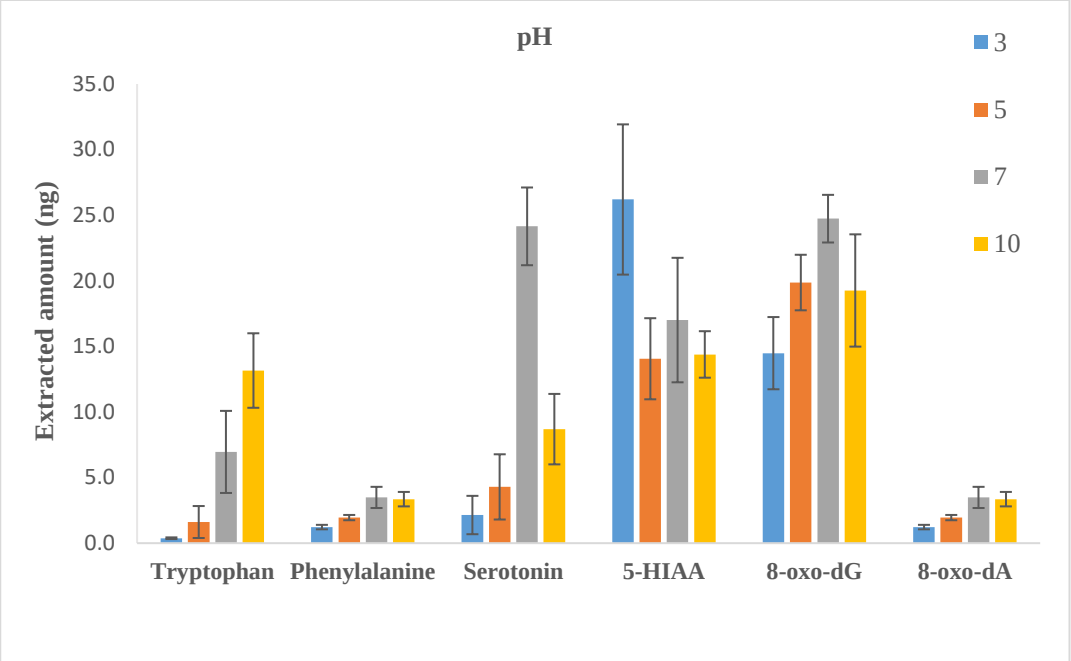


Figure 3.5. Effect of solution pH on sorption (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 min)

3.3.2.2 Effect of ionic strength on the extraction of analytes

In microextraction methods, one of the parameters that effect the sorption of analyte is the ionic strength. To alter the ionic strength of the sample, salts such as NaCl can be used. The added salt to the sample may have a significant effect especially for polar analytes as it decreases the analytes solubility in aqueous solution (salting out effect). Moreover, increasing the amount of salt increases the viscosity in the solution, thus causing a decrease in the mass transfer rate. In addition, the ionic strength of the urine samples may vary from person to person based on gender, hydration level, food intake; therefore, it may affect the analytical results if proper

control is not taken [107]. For these reasons, it is critical to investigate the effect of ionic strength on sorption of the selected analytes. The effect of ionic strength was investigated by adding NaCl to the sample to have 0, 0.010, 0.10 and 1.0 M NaCl in the final solutions. The amount of analyte extracted without salt and with different salt concentrations added is shown in Figure 3.6. As can be seen from the results given in the figure, the extraction ability of the coating decreased significantly by increase in the salt concentration of the sample, except for 5-hydroxyindoleacetic acid. The lower the salt content, the better the analytes were extracted. In the literature the salting out effect of analytes in presence of salt is reported as a method to improve the affinity of analytes towards the coating. However, the results of this study, suggested the opposite effect. This may be attributed to the nature of the sorbent as it has weak ion exchange moieties that can be saturated with salt ions, thus, decreasing the available sites for extraction of analytes.

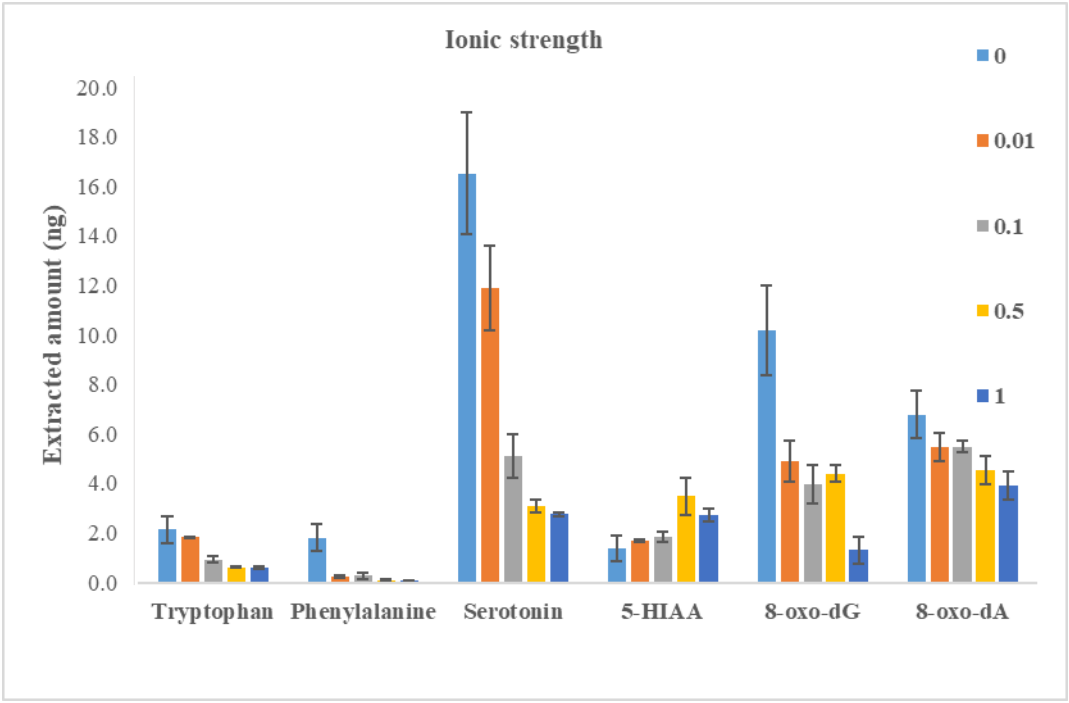


Figure 3.6. Effect of salt amount on sorption (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 min)

3.3.2.3 Effect of extraction time on the extraction of analytes

Five different extraction times (1, 2, 5, 15 and 30 minutes) were tested to determine the extraction time profiles of analytes. Figure 3.7 shows the extraction results. Since the analytes have different molecular structures, the amount of extracted analyte varies from analyte to analyte. As can be seen from the results, the maximum sorption was reached in about 5 minutes for most of the analytes. These results support that the sorption kinetic of the new coatings is fast. In addition, after reaching the maximum sorption for all analytes, a decrease in sorption amounts was observed at longer extraction times. This was an expected result for polar analytes as they show low affinity to the coating and in the presence of other ions displacement from the surface occurs. As a result, these experimental data show that nanofiber structures may indicate fast sorption equilibrium due to the higher surface area, but at the same time a low affinity for the analytes.

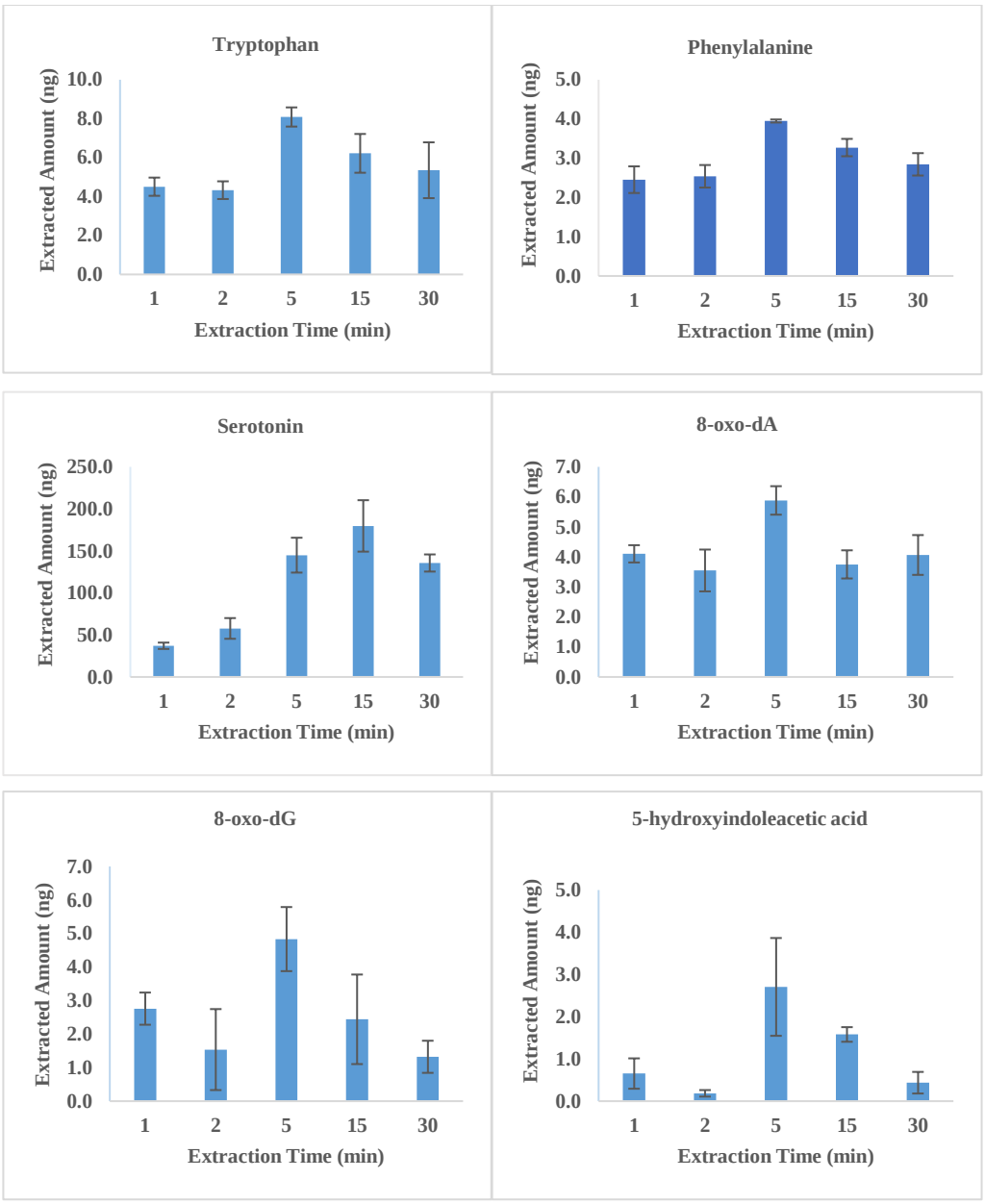


Figure 3.7. Extraction time profile of analytes (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption volume 150.0 µL, desorption time: 15 min)

3.3.2.4 Effect of desorption solvent on elution of the analytes

The desorption solvent was optimized from the extraction parameters. Solvents investigated were ACN:MeOH:H_2O (40:40:20, v/v/v) containing 0.1% formic acid, MeOH:H_2O (50:50, v/v) containing 0.1% formic acid, and MeOH:H_2O (85:15, v/v) containing 0.1% formic acid. ACN:MeOH:H_2O (40:40:20, v/v/v) containing 0.1% formic acid solvent performed better than other solvents. Figure 3.8 shows the extraction graph of the desorption solvent comparison. Based on the results, for phenylalanine and tryptophan ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. showed highest desorption amount. For serotonin, no significand difference was observing among desorbed amounts. However, ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. showed the lowest variation. Therefore, ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. was selected as the most suitable solvent among the tested.

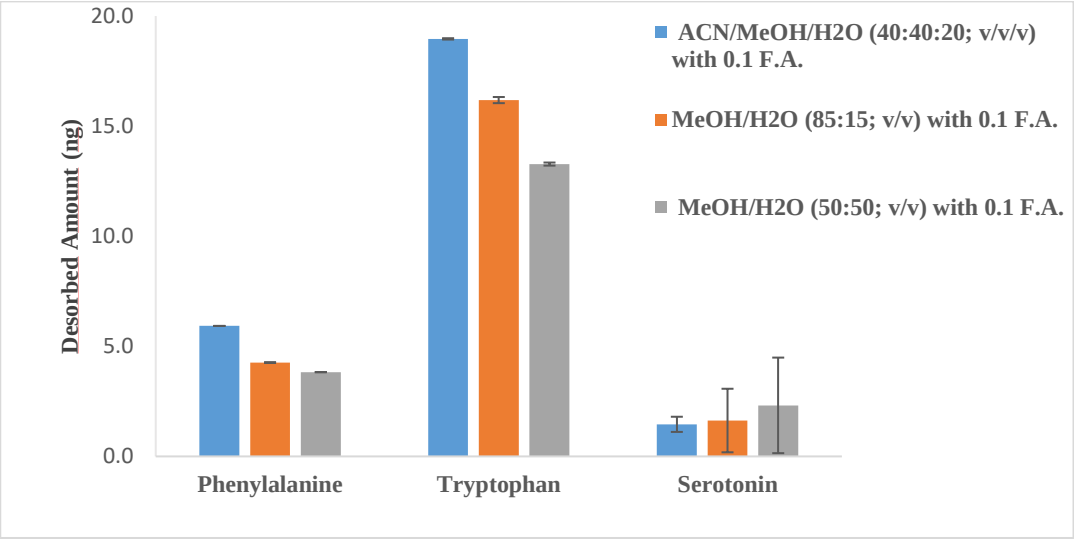


Figure 3.8. Desorption solvent optimizations (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption volume: 150.0 µL, desorption time: 15 min)

3.4 CBS-MS

3.4.1 Effect of desorption volume

The optimum desorption volume was investigated to obtain the best sensitivity from the CBS-MS. The desorption abilities of 5.0 µL, 10.0 µL, 15.0 µL and 20.0 µL solvents after 5 min extraction from synthetic urine were compared using CBS-MS (n=3). As mentioned before, the amount of solvent that the blade surface can carry is limited at the microliter level. Although the amount of analyte desorbed from the unit area will increase when the largest volume that can be used is selected, the analyte is diluted in the droplet. As can be seen in Figure 3.9, a volume of 10.0 µL of desorption solvent provided the best sensitivity while a 5.0 µL volume of desorption solvent was insufficient for the desorption of the analytes. Besides, a dilution effect was observed that reduced the concentration of solute in solution at 15.0 µL and 20.0 µL volumes.

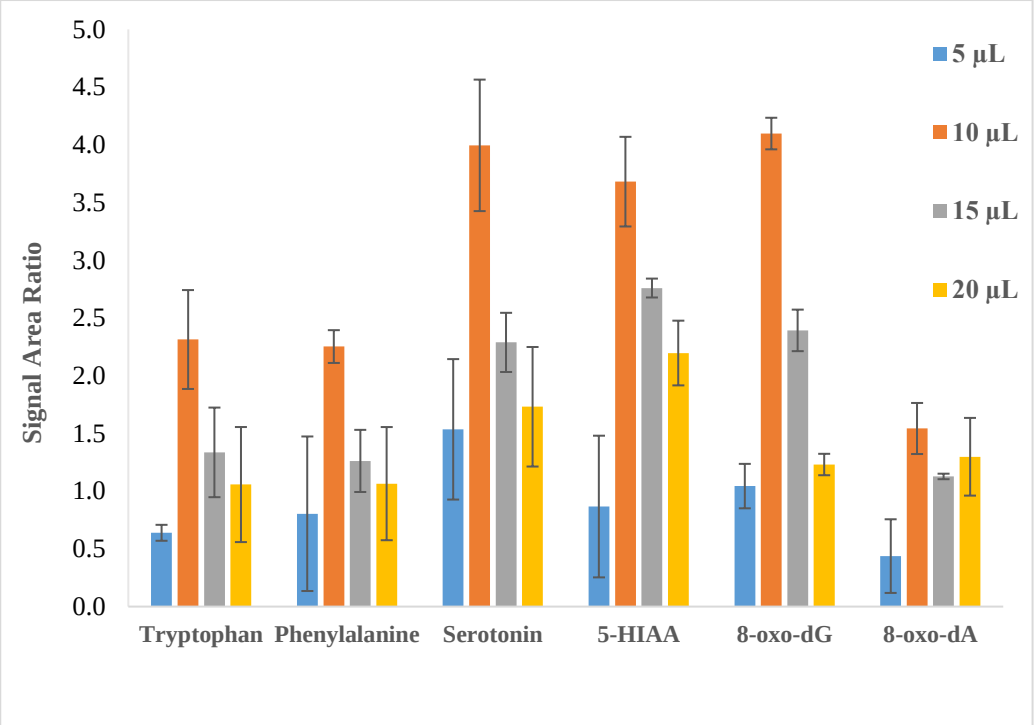


Figure 3.9. Effect of solvent volume on desorption and signal (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 sec, desorption volume: 5.0, 10.0, 15.0 and 20.0 µL)

3.4.2 Effect of desorption solvent

As can be seen from the above experimental results on desorption volume, very small volume of solvent is used for desorption of analytes in CBS-MS analyses. There are two main reasons for this. The first reason is that the amount of solvent that the blade surface can carry is approximately 30 µL. In case of using too much solvent, the solvent may drip from the blade surface to the ground during the electrospray formation with the application of voltage, affecting the repeatability. Another reason is the excess solvent that can cause dilution of the analytes in the droplet, thereby reducing the sensitivity. The most important problem when working with very small amounts of solvent is that the solvent evaporates before the voltage is applied during the desorption process and no signal can be obtained. Therefore, the desorption

solvent chosen for CBS studies should provide high desorption (high desorption kinetics) in the shortest time, low evaporation during desorption, and sufficient evaporation in the gas phase. In addition, the solvent's spray efficiency at the blade's tip and the analyte's chemistry and its affinity for the desorption solvent is essential for desorption/ionization conditions. The obtained results of this study are summarized in Figure 3.10. As can be seen from the figure, MeOH/H_2O (85:15; v/v/v) with 0.1% F.A. produced lower signal than ACN/H_2O (85:15; v/v/v) with 0.1% F.A. In this study, ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. showed the highest sensitivity as the best desorption solution, while MeOH/H_2O (85:15; v/v) with 0.1% F.A. gave the weakest signal. For further studies, ACN/MeOH/H_2O (40:40:20, v/v/v)) with 0.1% F.A. was chosen as the solvent.

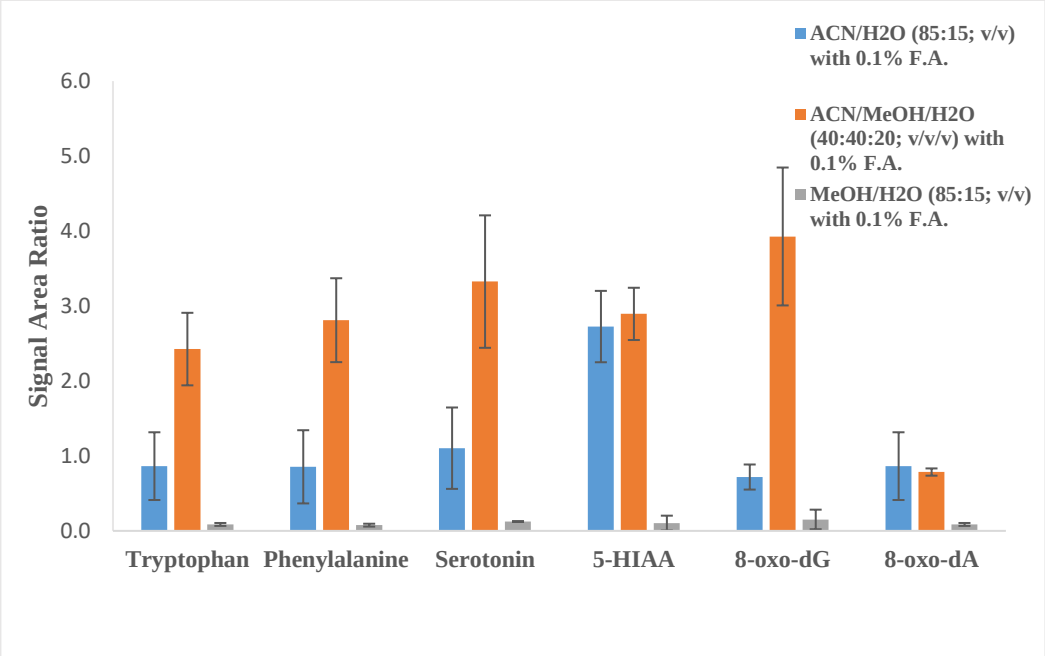


Figure 3.10. Desorption solvent optimizations (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 sec, desorption volume: 10.0 µL)

3.4.3 Effect of desorption time

As described at the beginning of this section, the desorption volumes available for CBS-MS are quite small (10.0 μL in this study) and therefore the solvent evaporates quickly from the surface of the CBS. This shows that sorbents with fast desorption kinetics should be used, and desorption should be completed as soon as possible. In this section, studies have been carried out to determine the time that provides maximum analyte desorption in the shortest possible time. Therefore, the desorption time has been optimized for the best sensitivity. After 5 minutes of extraction from synthetic urine, three methods of desorption were tested, and their results are given in Figure 3.11. The first one was 15 seconds of desorption with 10.0 μL of desorption solution. The second one was 30 seconds of desorption with 10.0 μL of desorption solution. And then the third one was 10.0 μL of desorption solution, let to be dried and then a fresh 10.0 μL of desorption solution was placed and kept for 15 seconds before the voltage application. The first method used provided the best signal intensity. The second and third methods provided lower signals for all the analytes.

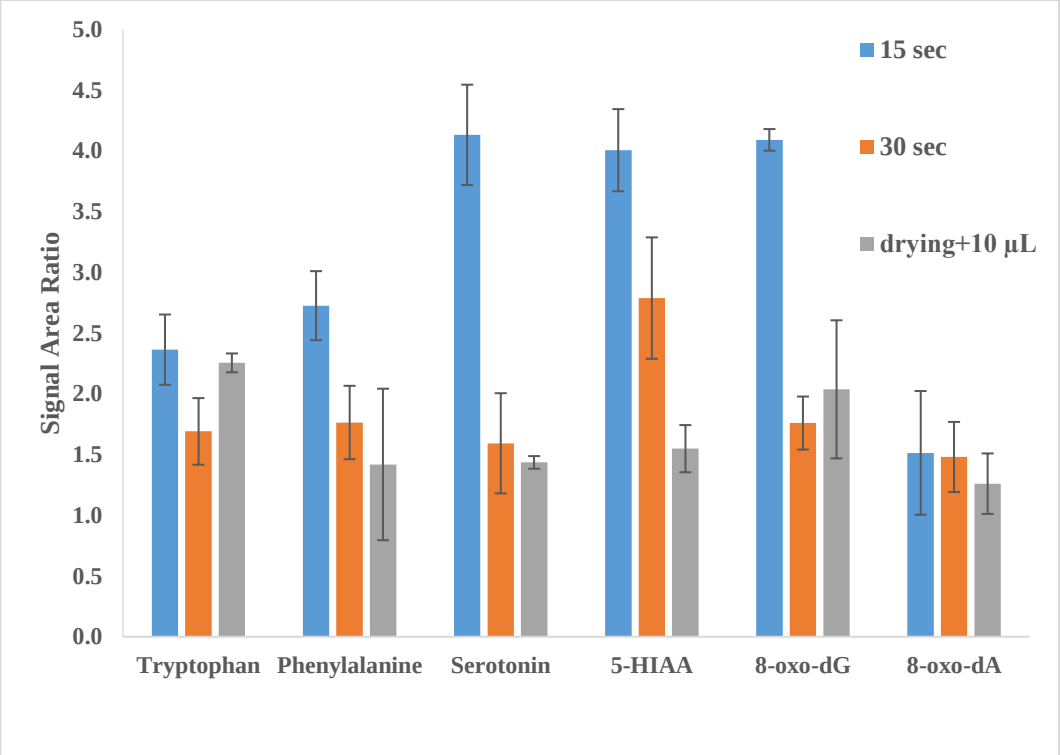


Figure 3.11. Optimization of desorption time (Extraction conditions; sample volume: 1.5 mL, analyte concentration: 500.0 ng/mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption volume 10.0 µL)

3.4.4 Comparison of HLB-SCX and hydrolyzed PAN as CBS-MS coating

Hydrophilic and lipophilic balanced HLB polymer with good sorption affinity for polar and nonpolar analytes was synthesized in the laboratory and functionalized with strong cation exchange groups (HLB-SCX). The electrospun HLB-SCX-PAN blades were prepared and compared with hydrolyzed PAN coated blades. The experimental conditions studied are summarized in Section 2.7.4. The results shown in Figure 3.12 show that hydrolyzed PAN containing weak ion exchange groups (weak cation exchange groups and weak anion exchange groups) has better extraction capacity than HLB-SCX-PAN for the selected analytes.

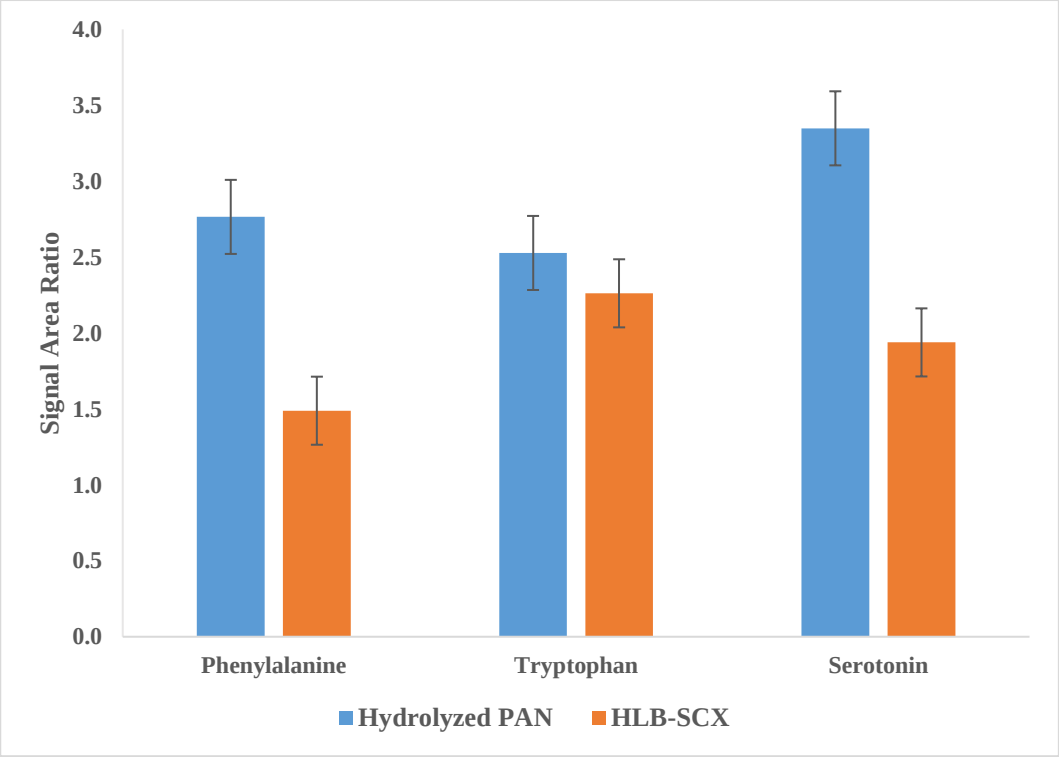


Figure 3.12. Comparison of hydrolyzed PAN and SCX-HLB-PAN coated CBS blades (Extraction conditions; sample volume: 1.5 mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 sec, desorption volume: 10.0 µL)

3.5 Validation studies

Linearity, limit of quantification (LOQ), repeatability and accuracy studies for method validation were carried out according to the bioanalytical method development and validation standard in the 'Bioanalytical Method Validation Guidance for Industry' (FDA, Bioanalytical Method Validation Guidance for Industry, 2018) guidance published by the FDA [108]. The experimental parameters used are given in Section 2.8 and obtained results are given below.

3.5.1 Linearity and limit of quantification (LOQ)

CBS-MS with matrix-matched synthetic urine standard solutions were used to determine the LOQ and linear range as the initial step of validation. For linearity studies, samples at 8 different concentrations were prepared in 3 replicates and the samples were used to calculate the linear range of the method. The expected coefficient of determination (R^2) values for the analyzed range are higher than 0.99. The LOQ for indole was calculated as 100.0 ng/mL, while the LOQ for tryptophan, serotonin, 8-oxo-dA, 8-oxo-dG and phenylalanine was determined as 50.0 ng/mL. The calibration graphs obtained are shown in Figure 3.13.

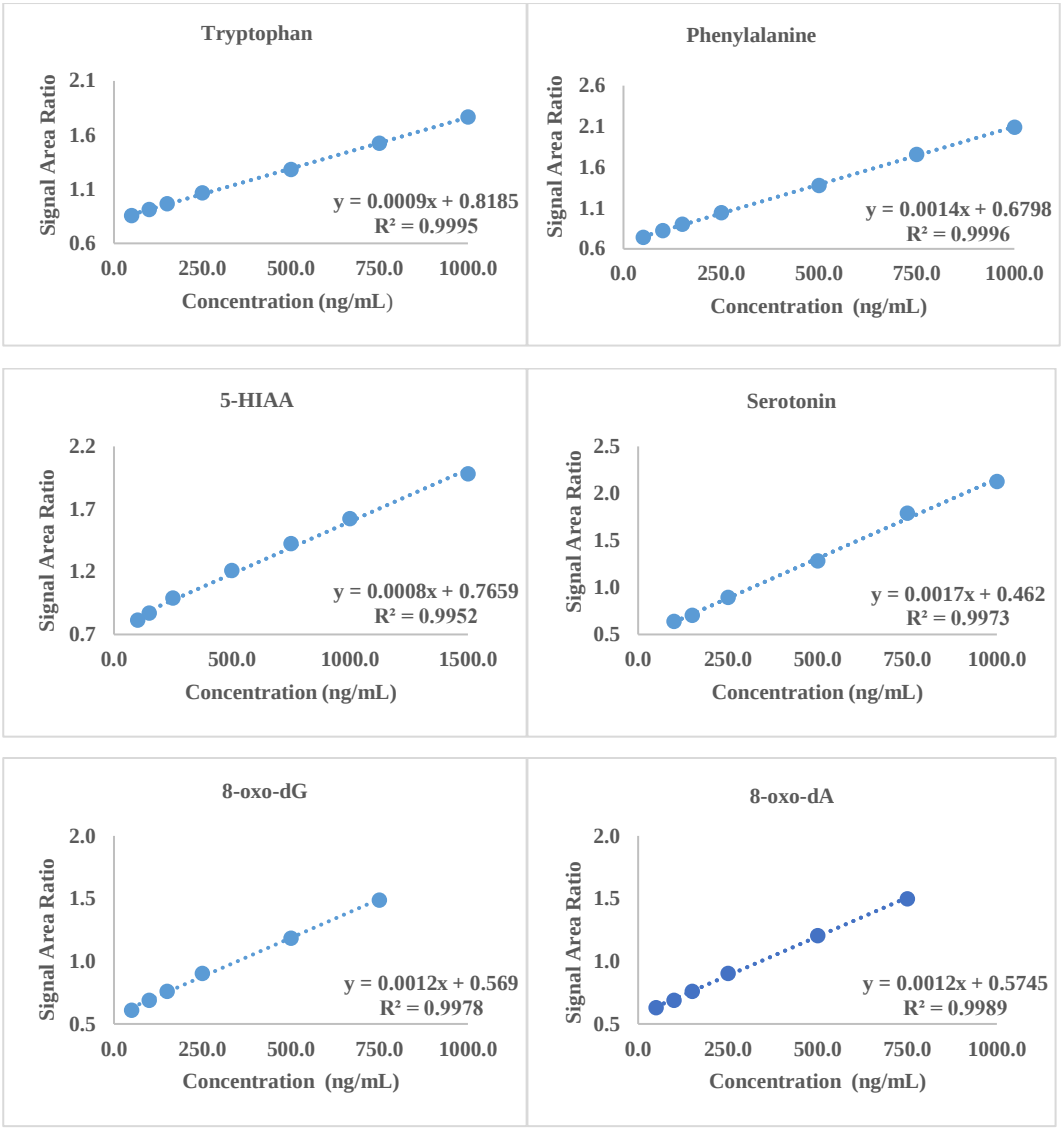


Figure 3.13. CBS-MS calibration graphs obtained for polar analytes (Extraction conditions; sample volume: 1.5 mL, desorption solvent: ACN:MeOH:H_2O (2:2:1, v/v/v) with 0.1% F.A., desorption time: 15 sec, desorption volume: 10.0 µL)

3.5.2 Repeatability

For the repeatability studies of the method, low, medium, and high concentrations that can be found in the linear operating range were determined and tested with 3 repetitive analyzes for each level. Method repeatability was studied as intraday (three

different analyzes with three replicates in one day) and inter-day (analysis with three replicates on three different days). The results obtained for intra-day and inter-day repeatability are shown in Table 3.9 and Table 3.10**,** respectively. As can be seen from these tables, the method showed a generally acceptable (<15%) repeatability for all analytes. In some cases, repeatability resulted in the 15-20% range. Nicotine was used as an internal standard in CBS-MS studies. For better repeatability, isotope-labelled internal standards of each analyte should be used.

Table 3.9 Intra-day repeatability values

	Percent relative standard deviation (%RSD)		
Analyte	**Low level 300.0 ng/ mL**	**Intermediate level 600.0 ng/ mL**	**High level 1200.0 ng/ mL**
Tryptophan	11.6	5.1	5.0
Phenylalanine	18.4	**17.7**	9.9
Serotonin	11.5	7.4	7.1
8-oxo-dA	9.7	**18.6**	10.4
8-oxo-dG	10.3	13.9	12.1
5-HIAA	13.6	10.1	8.4

Table 3.10 Inter-day repeatability values

	Percent relative standard deviation (%RSD)		
Analyte	**Low level 300.0 ng/ mL**	**Intermediate level 600.0 ng/ mL**	**High level 1200.0 ng/ mL**
Tryptophan	17.6	14.6	12.6
Phenylalanine	15.9	14.2	10.5
Serotonin	19.5	13.7	8.8
8-oxo-dA	11.3	12.1	14.5
8-oxo-dG	11.6	**17.6**	7.1
5-HIAA	6.19	11.8	12.5

3.5.3 Accuracy

Intraday accuracy was determined as low, medium, and high concentrations that could be found in the linear range and analyzed in 3 replicates for each level. Method accuracy was determined as relative error (%RE) by intra-day (three analyzes in one day) and inter-day (three separate days) studies. The results obtained for intra-day and inter-day accuracy are given in Table 3.11 and Table 3.12, respectively. As shown in the tables, all analytes show a generally acceptable (15%) relative error. In some cases, it gave a relative error of 15-25% in the results. In this study, nicotine was used as the internal standard for all analytes, and better results are possible with stable isotope-labelling internal standards.

Table 3.11 Inter-day accuracy (relative error)

	% Relative error (%RE)		
Analyte	**Low level 300.0 ng/mL**	**Intermediate level 600.0 ng/mL**	**High level 1200.0 ng/mL**
Tryptophan	0.6	1.3	3.2
Phenylalanine	**21.4**	6.4	3.1
Serotonin	9.9	11.2	12.1
8-oxo-dA	17.1	8.5	3.7
8-oxo-dG	5.1	2.8	6.5
5-HIAA	8.4	**26.8**	13.6

Table 3.12 intra-day accuracy (percent relative error)

	(%RE)		
Analyte	**Low level 300.0 ng/mL**	**Intermediate level 600.0 ng/mL**	**High level 1200.0 ng/mL**
Tryptophan	9.8	6.7	4.1
Phenylalanine	8.6	**23.3**	13.6
Serotonin	11.7	1.9	6.6
8-oxo-dA	14.6	7.2	8.4
8-oxo-dG	3.9	2.8	**17.1**
5-HIAA	12.8	8.9	9.4

3.5.4 Comparison of SPE-LC-MS and CBS-MS

The developed CBS-MS method was compared with the SPE as the reference method as described in Section 2.8 using blind analysis approach. In case of SPE, the desorption solvent obtained after extraction of the sample was analyzed in LC-MS and the analytes present in it quantified with instrumental calibration. For CBS-MS, the concentration of the analytes in the unknown sample was determined by matrix-matched SPME calibration under conditions developed in method optimization for CBS-MS. The results obtained from these experiments are given in Table 3.13. In case of CBS-MS, the obtained results were very close to the true value for all analytes. This supports the reliability of the developed method. Because SPE did not show exhaustive sorption under the working conditions applied here, it resulted in a very high error for all analytes when evaluated by direct instrument calibration. However, it should be kept in mind that, similar to CBS-MS, matrix matched calibration can be also prepared for SPE to obtain more accurate results. In fact, the SPE results obtained here, clearly shows the importance of the investigation of breakthrough volume of SPE prior to sampling, to ensure exhaustive extraction of the analytes which is the condition that do not require matrix matched calibration for reliable results.

Table 3.13 Comparison of SPE and CBS in the unknown sample (actual value 300.0 ng/ mL)

Analyte	**SPE-LC-MS**		**CBS-MS**	
	ng / mL	**% RE**	**ng / mL**	**% RE**
Tryptophan	115.4	61.5	286.7	4.4
Phenylalanine	21.1	92.9	267.9	10.7
Serotonin	6.5	97.8	265.6	11.4
8-oxo-dA	107.9	64.1	276.9	7.7
8-oxo-dG	106.7	64.4	271.9	9.3
5-HIAA	100.2	66.6	236.4	21.1

CHAPTER 4

CONCLUSION

In this thesis, new electrospun extractive phases were prepared for coated blade spray (CBS) and an analytical method was developed to determine clinically significant polar urinary metabolites.

The polymers tested to obtain polar CBS coatings were PAN, PVP and PVP/PVA. Electrospinning with all three polymers was successful and nanostructured materials were obtained. PVP and PVA are water-soluble polymers. Therefore, their solubility in water should be prevented before their use as a sorbent. For this reason, polymer crosslinking experiments were carried out and the following results were obtained.

For PVP, crosslinking experiments were made with UV, heat and $K_2S_2O_8$, and only those made with the heat were relatively successful. For the PVP/PVA mixture, crosslinking experiments were carried out with citric acid. Although insoluble coatings were obtained in crosslinking with citric acid, nanostructured morphologies were not observed. These polymers were not used in CBS-MS studies as they did not satisfy the criteria of being insoluble with nonofibrous polymer morphology.

The third polymer tested was PAN. Although this polymer does not have water solubility, its sorption property is also very low. Fortunately, nitrile groups in its structure can be easily hydrolyzed and converted into ion exchange groups. Therefore, to introduce ion exchange groups to PAN, hydrolysis of PAN in alkaline media was carried out. The hydrolysis time was optimized for this modification and the most successful values were obtained for PAN incubated in 5.0 M NaOH at 70 ^{o}C for 180 minutes. It was observed that the degree of hydrolysis increased for longer hydrolysis periods, but at the same time these conditions made PAN water soluble.

In this study, the hydrolyzed PAN coated CBS probes were coupled to a single quadrupole MS and optimized for rapid analysis. The studies conducted to optimize the CBS-MS coupling showed that an external voltage source is required to obtain stable electrospray. Optimizations for using the CBS probe as the ESI source showed that the best distance between the MS input and the blade tip was 1.0 cm. Arc flashing was observed at shorter distances, while the signal decreased or could not be received at longer distances. In addition, it was found that electrospray could not be obtained below 3.0 kV while 4.5 kV gave the highest signal.

Polar analytes, 8-oxo-dG, 8-oxo-dA, serotonin, 5-hydroxyindoleacetic acid, phenylalanine, and tryptophan, which were determined as biomarkers in various cancer types, were used as model analytes for optimization of extraction conditions and CBS-MS analysis conditions.

Extraction performances of CBS blades were determined in LC-MS by studying sample ion strength, pH, and extraction time. It was concluded that the selected analytes were significantly affected by the ionic strength and pH of the sample, and the effect could be an increase or decrease in sorption depending on the physicochemical properties of the analyte. This strongly suggest that in future when the method is used for real application, matrix normalization (for quantitation with matrix matched calibration) is necessary. Alternately, standard addition approach can be also used for reliable analyses. Moreover, in these optimizations, it was observed that the sorption kinetics were quite fast, as expected from the nanostructured sorbent, and equilibrium was reached in about 5 minutes of sorption time.

In CBS-MS studies, the instrumental response was optimized by studying the desorption time, desorption solvent type and volume, and optimum analysis conditions were determined. The sample volume in CBS-MS studies plays critical role as very small volumes may evaporate quickly from the surface and large volumes may results in excessive dilution of analytes. The method conditions obtained after optimizations with hydrolyzed PAN-CBS blades can be summarized

as follows. The best signal stability and intensity was obtained when 15 seconds was used as the desorption time, 10.0 μL was used as solvent volume, and ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. was used as desorption solution.

In conclusion, with the developed CBS-MS method the sample preparation and analysis can be completed in 6 minutes. This supports that the method is suitable for rapid diagnostic studies. The limits of quantification of the final method for 5-hydroxyindoleacetic acid was 100.0 ng/mL, while it was 50.0 ng/mL for the rest of the analytes with RSD% within 15-20% and ‘RE within a range of 15-25%, showing the reliability of the developed method.

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APPENDICES

A. CBS-MS and LC-MS Chronograms

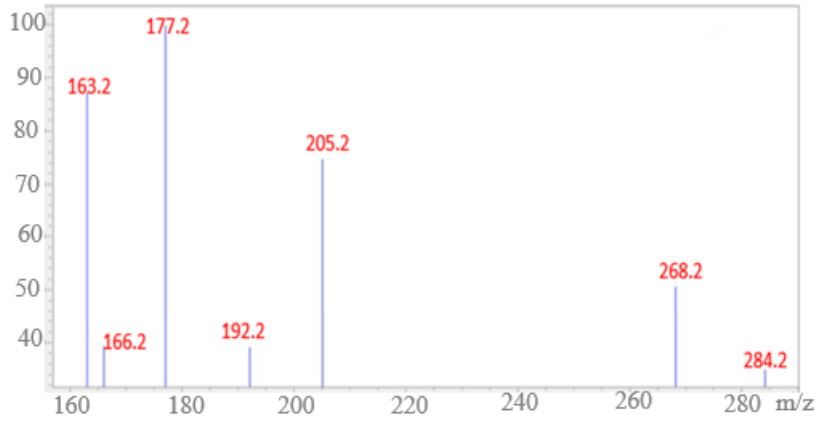

Figure A.1. Mass spectrum of nicotine (m/z=163.2), phenylalanine (m/z=166.2), serotonin (m/z=177.2), 5-hydroxyindoleacetic acid (m/z=192.2), tryptophan (m/z=205.2), 8-oxo-Da (m/z=268.2), and 8-oxo-dG (m/z=284.2) obtained in CBS-MS.

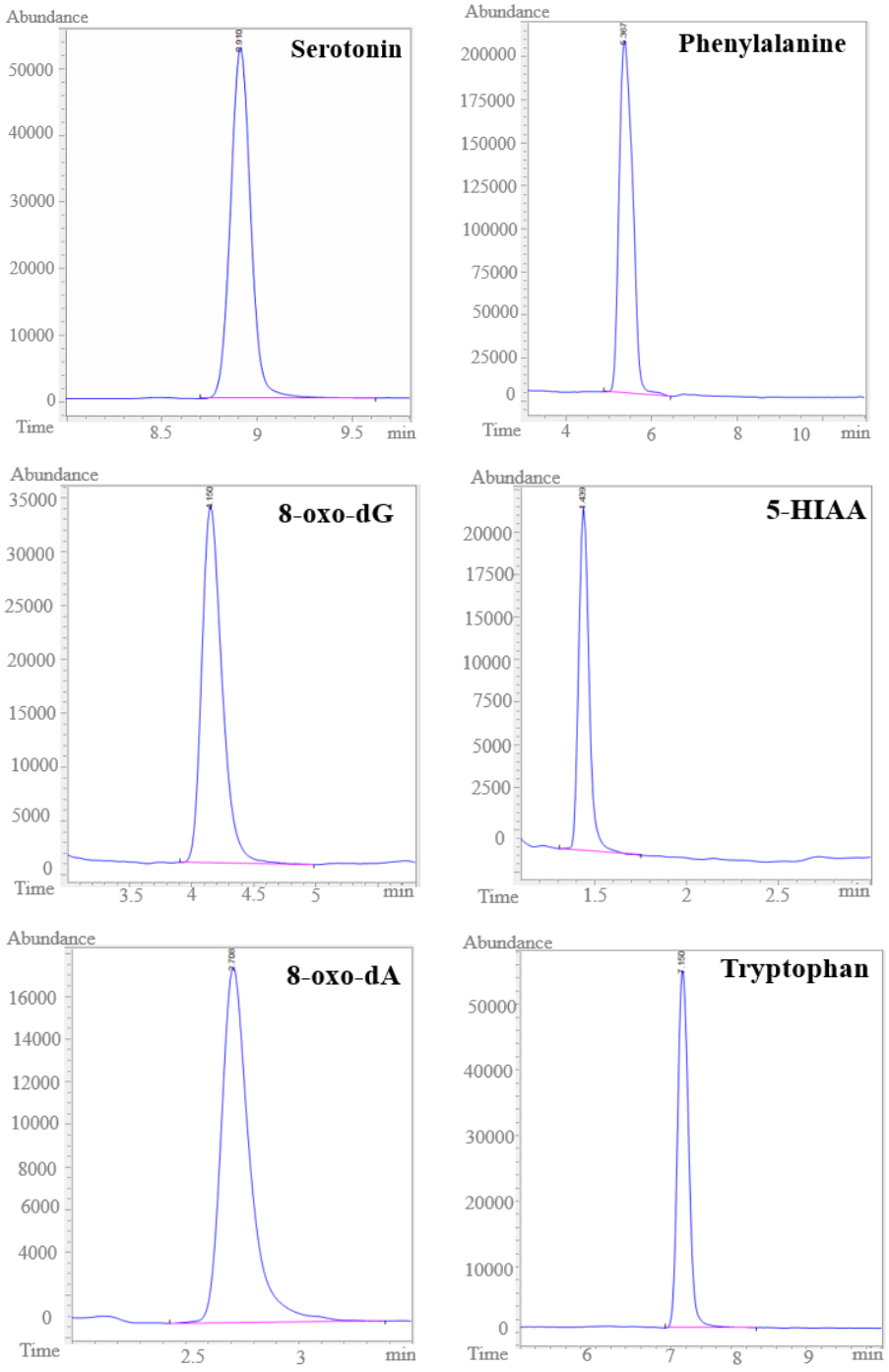

Figure A.2. LC-MS chromatogram of analytes obtained in SIM mode (concentration: 50.0 ng/mL)

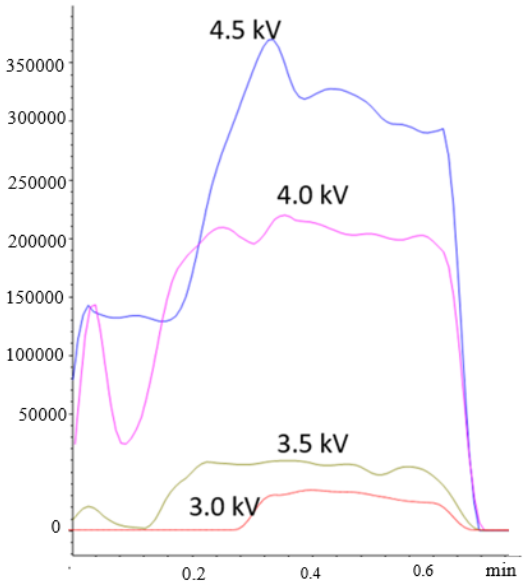

Figure A.3. Typical total ion chronograms obtained during the optimization of the voltage of the external voltage source in CBS-MS studies.