PREPARATION OF ELECTROSPUN COATED THIN FILM EXTRACTION DEVICES FOR DETERMINATION OF CLINICALLY IMPORTANT ENDOGENOUS COMPOUNDS IN BIO-FLUIDS

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PREPARATION OF ELECTROSPUN COATED THIN FILM EXTRACTION DEVICES FOR DETERMINATION OF CLINICALLY IMPORTANT ENDOGENOUS COMPOUNDS IN BIO-FLUIDS

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ABSTRACT

PREPARATION OF ELECTROSPUN COATED THIN FILM EXTRACTION DEVICES FOR DETERMINATION OF CLINICALLY IMPORTANT ENDOGENOUS COMPOUNDS IN BIO-FLUIDS

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Changes in plasma concentrations of endogenous compounds such as cholesterol and steroid hormones may cause many vital problems threatening human life. Therefore, the development of novel rapid and reliable analytical methods for the quantitative determination of clinically important compounds in biofluids is of great importance. Coated blade spray (CBS), a technique which is based on solid-phase microextraction (SPME), consists of a sword like device which acts as an extractive tool for sampling and sample preparation, and when combined with mass spectrometry (MS), it functions as an electrospray ionization source. Therefore, it has gained attention in recent years as one of the most promising tools in the analytical toolbox. However, there are not many extractive phases suitable for certain studies. For that reason, this thesis was focused on preparation of new CBS probes with enhanced performance suitable for rapid analysis of spot sized plasma samples.

As a first step, poly(divinylbenzene) (PDVB) nanoparticles were synthesized by mini-emulsion polymerization method and then immobilized in polyacrylonitrile

(PAN) by electrospinning method. The new PDVB-PAN-coated blades were used to develop a CBS-MS method for determination of cholesterol, progesterone and testosterone in plasma samples. With this method, 10.0 ng/mL limits of quantification (LOQ) could be obtained for testosterone and progesterone with a sample volume of 200.0 µL while for cholesterol the LOQ was 200.0 ng/mL with the sample volume of 30.0 µL. The developed method requires only 7 minutes, including sample preparation and instrumental analysis, showing the applicability of the method for rapid clinical diagnosis.

Keywords: Solid Phase Microextraction, Mass Spectrometry, Coated Blade Spray, Electrospinning, Nanotechnology.

ÖZ

BİYO-SIVILARDAKİ KLİNİK ÖNEME SAHİP ENDOJEN BİLEŞİKLERİN TAYİNİ İÇİN ELEKTROSPUN KAPLI İNCE FİLM ÖZÜTLEME CİHAZLARININ HAZIRLANMASI

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Kolesterol ve steroid hormonları gibi endojen bileşiklerin plazma derişimlerindeki değişiklikler, insan hayatını tehdit eden birçok hayati soruna neden olabilir. Bu nedenle, biyolojik sıvılardaki klinik öneme sahip bileşiklerin nicel analizleri için yenilikçi, hızlı ve güvenilir analitik metotlar geliştirmek oldukça önemlidir. Kaplı bıçak spreyi (CBS), katı faz mikroekstraksiyon (SPME) temelli, örnekleme ve örnek hazırlama için ekstraktif araç olarak kullanılabilen kılıç benzeri bir probdan oluşan ve kütle spektrometre (MS) ile birleştirildiğinde elektrosprey iyonlaşma kaynağı olarak davranan bir tekniktir. Dolayısıyla, son yıllarda bu alanda en umut vadeden araçlardan biri olarak ilgi çekmektedir. Ancak, özel çalışmalara uygun çok fazla ekstraktif faz bulunmamaktadır. Bu sebeple, bu tezde spot boyutundaki plazma örneğinden hızlı analiz için uygun, performansı artırılmış yeni CBS problarının hazırlanmasına odaklanılmıştır.

İlk adım olarak mini emülsiyon polimerizasyon yöntemi ile poli(divinilbenzen) (PDVB) nanoparçacıkları sentezlenmiş ve daha sonra elektroeğirme yöntemi ile poliakrilonitril (PAN) fazına sabitlenmiştir. Yeni PDVB-PAN kaplı bıçaklar

testosteron, progesteron ve kolesterolün plazma örneklerinden tayini için bir CBS-MS yöntemi geliştirilmesinde kullanılmıştır. Bu yöntemle, testosteron ve progesteron için 200.0 µL numune hacminde 10.0 ng/mL tayin sınırı elde edilebilmişken, bu sınır kolesterol için 30.0 µL numune hacminde 200.0 ng/mL olarak bulunmuştur. Geliştirilmiş olan bu metodun numune hazırlama ve enstrümantal analiz için yalnızca 7 dakikaya ihtiyaç duyuyor olması metodun hızlı klinik tanı için uygulanabilirliğini göstermektedir.

Anahtar Kelimeler: Katı Faz Mikroekstraksiyon, Kütle Spektrometri, Kaplı Bıçak Spreyi, Elektroeğirme, Nanoteknoloji

I would like to dedicate this thesis to my grandmother, Sabriye Altunkıran, who died during my master's education.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

ACN	Acetonitrile
AIBN	Azobisisobutyronitrile
AIVN	2,2’-Azobis(2-methylbutyronitrile)
APCI	Atmospheric pressure chemical ionization
ASAP	Atmospheric solids analysis probe
BSA	Bovine serum albumin
CAR	Carboxen
CBS	Coated blade spray
CI	Chemical ionization
CW	Carbowax
DART	Direct analysis in real time
DBD	Dielectric barrier discharge
DBDI	Dielectric barrier discharge ionization
DESI	Desorption electrospray ionization
DI-SPME	Direct immersion solid phase micro extraction
DMF	Dimethyl formamide
DVB	Divinyl benzene
EI	Electron impact

ESI	Electrospray ionization
F.A	Formic acid
FAB	Fast atom bombardment
FAPA	Flowing atmospheric pressure afterglow
FTIR	Fourier-Transform Infrared Spectrometry
GC	Gas chromatography
HDL	High density lipoprotein
HLB	Hydrophilic lipophilic balance
HPLC	High-performance liquid chromatography
IPA	Isopropyl alcohol
IS	Internal standard
LC	Liquid chromatography
LDL	Low density lipoprotein
LI	Laser ionization
LLE	Liquid-liquid extraction
LPME	Liquid phase micro extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LTP	Low temperature plasma probe
MALDI	Matrix assisted laser desorption ionization
MDSPE	Magnetic dispersive solid phase extraction
MeOH	Methanol

MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
PA	Polyacrylate
PADI	Plasma assisted desorption ionization
PAN	Poly acrylonitrile
PBS	Phosphate-buffered saline
PDI	Plasma desorption ionization
PDMS	Polydimethylsiloxane
PDVB	Poly (divinyl benzene)
PNME	Packed needle micro extraction
PP	Protein precipitation
PVP	Polyvinylpyrrolidone
RDSE	Rotating disk sorptive extraction
RI	Resonance ionization
SBSE	Stir bar sorptive extraction
SEM	Scanning electron microscope
SI	Secondary ionization
SPDE	Solid phase dynamic extraction
SPE	Solid phase extraction
SPME	Solid phase micro extraction
TF-SPME	Thin film solid phase micro extraction

TFME	Thin film micro extraction
TGA	Thermal gravimetric analysis
TI	Thermal ionization
UHPLC	Ultra high-performance liquid chromatography
VLDL	Very low-density lipoprotein

LIST OF SYMBOLS

SYMBOLS

^{0}C	Degree Celsius
%	Percent
µL	Microliter
µm	Micrometer
cm	Centimeter
g	Gram
h	Hour
kHz	Kilohertz
kV	Kilovolt
L	Liter
M	Molar
m/z	Mass to charge ratio
min	Minute
mL	Milliliter
mm	Millimeter
ng	Nanogram
nm	Nanometer
sec	Second
V	Volt

v/v	Volume per volume
w/w	Weight per weight

CHAPTER 1

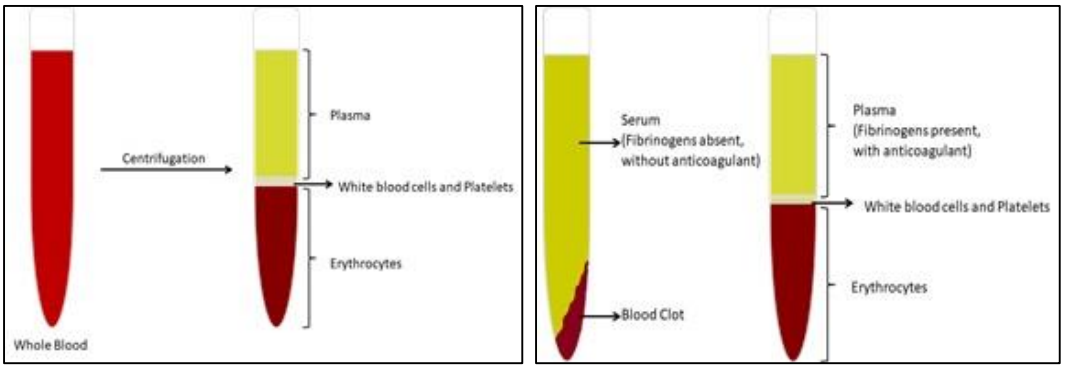
INTRODUCTION

The rapid determination of endogenous molecules in biofluids is of great significance when considering various psychological conditions such as cardiovascular diseases, hormonal syndromes, cancer, and psychological disorders that have increased in recent years. Therefore, new methods that can reduce the whole analysis time to only a few minutes, providing rapid clinical diagnosis are of paramount importance. In this context, coated blade spray (CBS), a technique based on solid-phase micro-extraction (SPME) can be used not only as the SPME extractive phase for sample preparation, but also as an electrospray ionization (ESI) source for mass spectrometry. It can thus combine sample preparation with mass spectrometric analysis, and this integration allows sensitive analysis even of droplet-sized samples while reducing the overall analysis time to a few minutes. However, existing SPME coatings have slow extraction and desorption kinetics which may affect the performance of the CBS for sensitive analysis. In this thesis, we proposed to synthesize home-made poly(divinylbenzene) nanoparticles and immobilize them into poly acrylonitrile (PAN) by electrospinning to produce novel CBS extractive phases with improved kinetics. The performance of the new CBS probes was evaluated by the use of the tool for determination of cholesterol and steroid hormones in spot sized biological samples.

1.1 Blood and Its Fractions

Biofluids are the liquids in the human body that are produced by the body itself such as serum, plasma, urine, blood, saliva, interstitial fluid, cytosol, cerebrospinal fluid (CSF), sweat, breath, seminal plasma, bile, earwax, amniotic fluid, or pus

which may develop from a blister or cyst. Biofluids include many compounds like drug molecules, proteins, or metabolites [1]. Thanks to the fact that biofluids contain such a variety of molecular information, they have been used to find out what is happening in the human body and to diagnose diseases. Therefore, this means that developing analysis methods to gather this information has also great importance. Of all the biofluids, whole blood is known as the richest informative and also the most complicated sample matrix. A blood sample ensures a temporary view of the body and discloses the general physiological state [2]. Blood is a dynamic fluid that constantly circulates in our veins and is the basis for life. About 45% of it consists of red blood cells (erythrocytes), less than 1% of it consists of white blood cells (leukocytes) and platelets, and about 55% of it consists of the fluid which is called plasma. Figure 1.1.a shows schematically how red blood cells, white blood cells, and plasma fractions are obtained when whole blood is separated according to their density by centrifugation. As a result of centrifugation of whole blood, erythrocytes are collected at the bottom. Above this, leukocytes together with thrombocytes are forming a whitish layer. At the top, the liquid called plasma is collected. Most of the plasma is made up of water, and this water contains nutrients, proteins, and other vital chemicals. The blood plasma's multi-chemical indicators-growth factors, chemokines, hormones, extracellular nucleic acids, cytokines, antibodies, etc., generate an ordered informational environment that inspects many different biological processes occurring at the same time in the human body [3]. In Figure 1.1.b, the difference between serum and plasma is shown schematically. Serum can be defined as the liquid portion formed after blood coagulation that does not contain coagulating proteins. The difference between serum and plasma is that there is no fibrinogen in the serum.



(a) **(b)**

Figure 1.1.a) Composition of human blood, b) difference between serum and plasma

1.2 Steroids and Steroid Hormones

Steroids are derivatives of cyclopentanoperhydrophenanthrene, formed as a result of the condensation of three cyclohexane rings in the phenanthrene order. Four fused rings of the steroid core also called gonane are indicated by letters A, B, C, and D, and carbon atoms in the molecule are numbered sequentially [4]. Figure 1.2 shows the cyclopentanoperhydrophenanthrene ring structure.

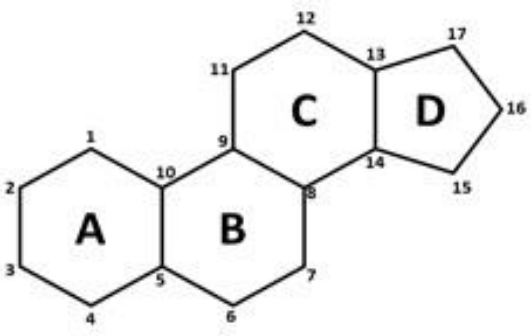


Figure 1.2. Cyclopentanoperhydrophenanthrene

The most important natural steroids are bile acids, sex and adrenal cortex hormones, and sterols. Steroid hormones are steroids that act as hormones. Steroid hormones can be categorized under five groups, namely, glucocorticoids,

mineralocorticoids, androgens, estrogens, and progestins. These hormones are synthesized in the adrenal cortex, ovaries, testicles, and corpus luteum. Their transportation process occurs in plasma by making complexes with proteins that provide ubiquitous distribution and raise their half-life because steroid hormones are hydrophobic molecules produced from cholesterol and therefore, insoluble in body fluids such as plasma [5]. Serum albumin acts as a nonspecific carrier. Specific steroid carrier plasma proteins bind steroid hormones more tightly than albumin. Figure 1.3 shows the transportation process of steroid hormones. In addition, their hydrophobicity allows them to freely pass from the blood to the cell membrane and from there to the cytoplasm of target cells.

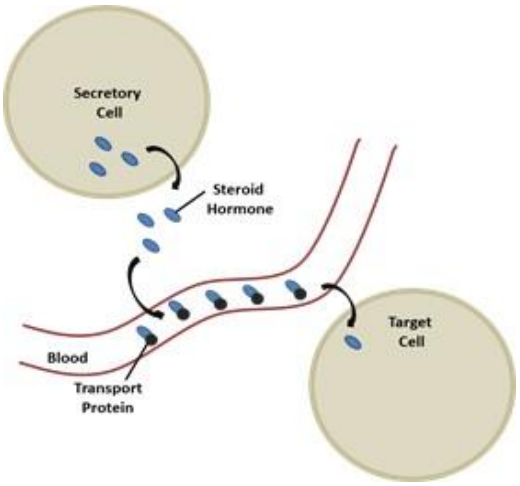


Figure 1.3. Transportation of steroid hormones

Steroid hormones play an essential role in regulating several vital processes such as secondary sex characteristics (secondary sex characteristics are changes in the body structure of girls and boys with puberty), gene expression, sodium homeostasis, and carbohydrate metabolism. For this reason, they are used as one of the basic indicators of disease and health [6]. For example, steroid hormones, especially cortisone, and sex hormones, which are androgens, estrogens, and progestogens, affect the immune system. Epidemiological evidence shows that steroid hormones affect the rate of immune system diseases such as systemic lupus erythematosus (SLE) and rheumatic diseases according to different ages and conditions [7].

Besides, there is a relationship between steroid hormones and the nervous system. Nerve cells in the brain secrete hormones, and even in some psychiatric diseases, there is a disorder in the hormones secreted in the brain [8]. Also, in recent studies has been shown that the nervous system is a target for sex steroid hormones which has broad effects on the differentiation, functioning, growth, and maturation of the brain cells [9]. Moreover, it is well known that steroid hormones have critical effects on determination of sexual characters. Both estrogens (hormone that enables the formation of female sex characteristics) and androgens (steroid hormone with masculinizing effects) affect sexual development and function. In men, androgens are responsible for expansion of secondary sexual characteristics, while in women, they play a less paramount role; however, adrenal androgens are responsible for most of the growth of pubic and axillary hair. The main task of estrogens in women is to stimulate the reproduction and maturation of individual cells in the human body, which are responsible for generation of many secondary sexual features. Also, progestins account for preparing the uterus for pregnancy and breasts for breastfeeding; in men, progestins and estrogens generally do not play an important role clinically in the generation of sexual characteristics, and men produce small amounts of estrogen, about 1/5 of a non – pregnant woman [10]. Some steroids, steroid drugs, and steroid hormones are listed in Table 1.1 with their molecular structures and brief definitions. In addition, the molecules specified in the table are given with their LogP values. LogP expresses the partition coefficient between octanol and water and thus is a property that gives information about the polarities of the molecules.

Table1.1. Some steroids and steroid hormones

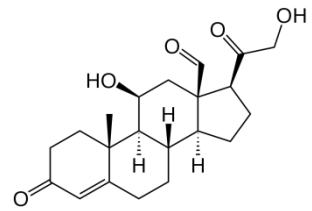
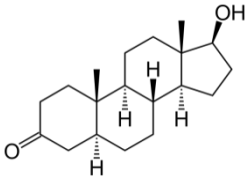
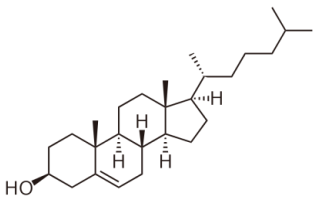
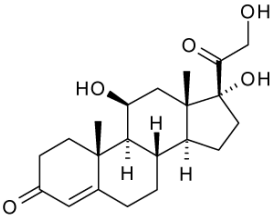
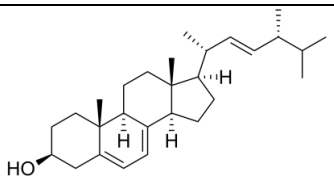
Molecule name and its physicochemical properties	**Molecular structure**	**Definition**
Aldosterone LogP = 1.08 MW = 360.4 g/mol		Steroid hormone produced in the adrenal glands. Plays a role in the regulation of sodium and potassium balance in the blood.
Androstanolone LogP = 3.55 MW = 290.4 g/mol		Anabolic steroid, a potent androgenic metabolite of testosterone that specifically mimics dihydrotestosterone (DHT), a naturally produced hormone in the male body.
Cholesterol LogP = 7.11 MW = 386.7 g/mol		Precursor compound of all steroid hormones. Produced naturally by all cells, especially in the liver. The human body needs a certain amount of cholesterol to form healthy cells and to form vitamin D.
Cortisol LogP = 1.28 MW = 362.5 g/mol		Corticosteroid hormone produced in the adrenal gland, associated with the body's response to stress.
Ergosterol LogP = 7.39 MW = 396.7 g/mol		A steroid alcohol found in yeast and bacteria, a precursor to vitamin D2. (Provitamin D2)

Table1.1. (Continued)

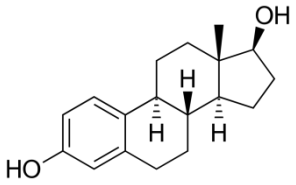
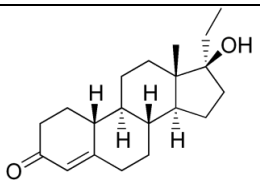
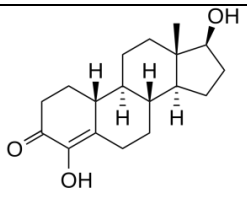
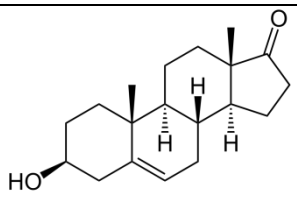
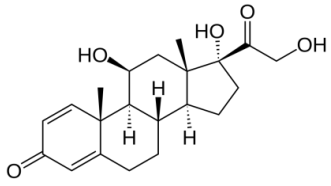
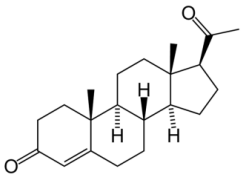
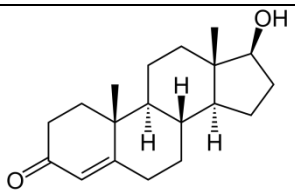
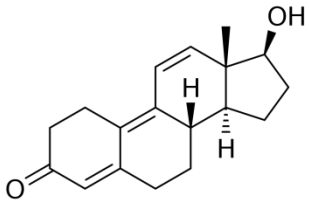
Molecule Name and its physicochemical properties	**Molecular Structure**	**Definition**
Estradiol LogP = 4.01 MW = 272.4 g/mol		The main estrogenic hormone found in human. Estrogens are steroid hormones that play an important role in the female menstrual cycle and are also found in all vertebrates and some insects.
Norethandrolone LogP = 3.87 MW = 302.5 g/mol		Androgen and anabolic steroid drug used to promote muscle growth and also to treat skin burns, physical trauma, etc.
Oxabolone LogP = 2.55 MW = 290.4 g/mol		A synthetic anabolic – androgenic steroid of the nandrolone group and is also a banned doping substance.
Prasterone LogP = 3.23 MW = 288.4 g/mol		Endogenous steroid hormone synthesized and secreted by the adrenal gland in mammals, serving as a precursor to male and female sex hormones.
Prednisolone LogP = 1.62 MW = 360.4 g/mol		A synthetic corticosteroid drug that suppresses the immune system and is 3 – 4 times stronger than cortisol, a natural hormone secreted from the adrenal gland.

Table1.1. (Continued)

Molecule Name and its physicochemical properties	**Molecular Structure**	**Definition**
Progesterone LogP = 3.87 MW = 314.5 g/mol		A steroid hormone that mainly prepares women for pregnancy.
Testosterone LogP = 3.32 MW = 288.4 g/mol		A steroid hormone from the androgen group, the male sex hormone.
Trenbolone LogP = 2.47 MW = 270.4 g/mol		A steroid primarily used to increase muscle growth and appetite in cattle.

1.2.1 Cholesterol

Cholesterol is a lipophilic, fat-like, waxy substance found in the membranes of animal cells [11,12]. It has various functions in human body. While cholesterol can be taken into the body by consuming foods of animal origin, it can also be synthesized in human body cells, especially in liver cells. It participates in the structure of cell membranes so that the human body can form healthy cells. It is used in the synthesis of steroid hormones, and is the precursor of vitamin D and bile acids/salts [13,14]. It has an essential role in the formation and stabilization of lipid microdomains, as well as in stabilizing and maintaining the permeability and fluidity of cell membranes [15].

Cholesterol is an amphiphilic sterol molecule that contains 27-carbon four fused rings (cyclopentanoperhydrophenanthrene), two methyls at the junction of the rings, and an eight-carbon chain attached to the fourth ring, with a double bond between the fifth and sixth carbons, and –OH at the third carbon [16,17]. Molecular structure of cholesterol can be seen in Table 1.1.

Lipoproteins are biochemical compounds consisting of both proteins and lipids. Lipoproteins in blood plasma are the primary source of circulating lipids because they transport low water-soluble lipid molecules throughout the body through the blood circulatory system [18,19]. Since cholesterol is a water-insoluble molecule, it cannot pass into the blood and cannot be distributed to the body by itself. Several lipoproteins are produced by the liver to help the transportation of cholesterol [20]. The cholesterol-carrying lipoproteins are classified traditionally as low-density lipoprotein (LDL), high-density lipoprotein (HDL), chylomicrons, and very-low-density lipoprotein (VLDL) [21]. Low-density lipoproteins transport cholesterol to tissues. LDL levels in the blood should be relatively low and are often referred as "bad cholesterol." If an individual's LDL cholesterol level is too high, it can build up on the walls of the arteries over time which is a circumstance called atherosclerosis. High-density lipoproteins export cholesterol from the tissues to the liver for removal from the body, thereby preventing the accumulation of cholesterol plaques in the arteries. HDL levels should be relatively high in the blood and are often referred as "good cholesterol." Chylomicrons transport fats from the intestines to the tissues, and very low-density lipoproteins (VLDL) transport triacylglycerols from the liver to other tissues [22]. Figure 1.4 illustrates the lipoprotein functions.

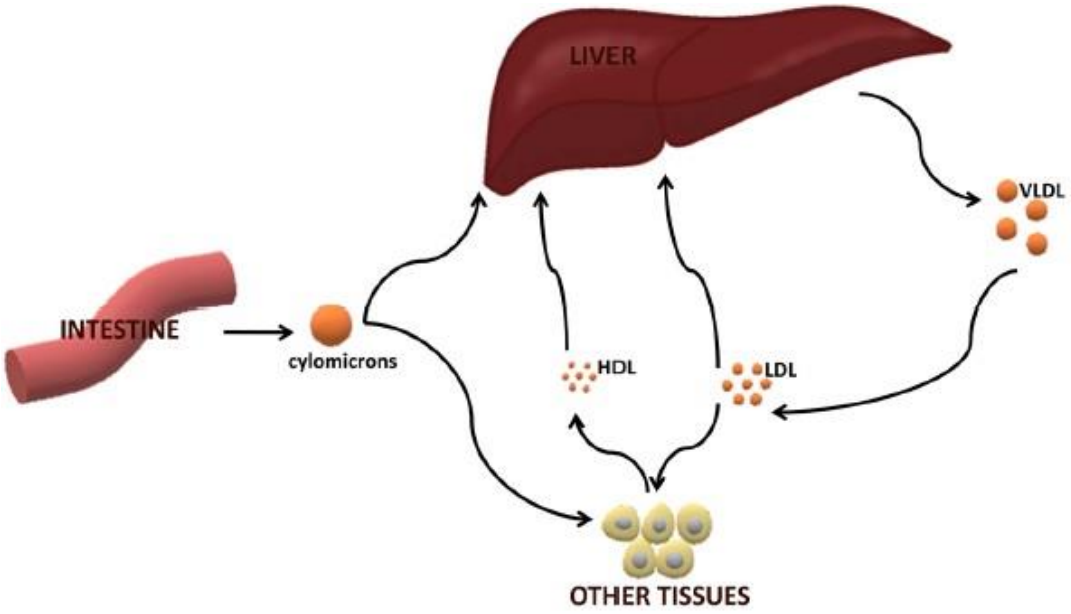


Figure 1.4. Working principles of lipoproteins (Redrawn from; Pratt & Cornely, 2013) [23]

The total cholesterol level in the blood is expected to be below 2000 µg/mL in a healthy human adult [24]. Generally, cholesterol levels of 2400 µg/mL and above are considered high cholesterol. According to the World Health Organization (WHO), it is estimated that high cholesterol causes 7.9 percent of the total deaths. Also, the cardiovascular diseases (CVD) are known as the main reason for death worldwide, with an estimated 17.9 million deaths every year, most of them causing strokes and heart attacks because of atherosclerosis [25–27]. In fact, cholesterol is an important contributor in the development of atherosclerosis, a disease that occurs due to the hardening of the arteries. Normally, arteries in the human body have a flexible structure that will adapt to all movements throughout human life and take shape according to the body, but in cases where cholesterol level is too high, the veins begin to thicken and lose their flexibility. As a result, as shown in Figure 1.5 the passage of blood through the arteries becomes difficult, and this restriction of blood flow causes the blood to not reach the tissues and organs timely and in sufficient amount. It is worth to mention that many fatal diseases such as cerebral hemorrhage and heart attack occur as a result of arteriosclerosis.

Moreover, some changes in HDL structure can be seen in patients with non-insulin-dependent diabetes mellitus (NIDDM), which leads to changes in the cholesterol return pathway, thus increasing the risk of atherosclerosis [28]. Mutations in genes encoding LDL receptors cause heterozygous familial hypercholesterolemia (HeFH), which is the most common autosomal inherited genetic disease of human metabolism [29]. Compared to the normal population, affected individuals have increased plasma LDL concentrations, posing a risk for early coronary artery disease (CAD) due to accelerated atherosclerosis [30].

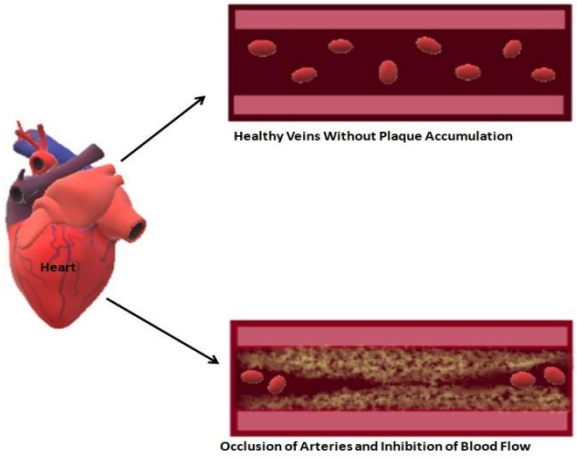


Figure 1.5. Schematic representation of atherosclerosis

It has also been shown that cholesterol can lead to cancer progression because it increases cell proliferation, invasion and migration; and also, cholesterol metabolism affects the immune system and thus antitumor immune responses [31,32].

Therefore, determination of cholesterol levels is critical in the early detection and prevention of atherosclerosis risk factors and vital diseases such as cancer.

1.2.2 Testosterone

Testosterone is an anabolic steroid that plays a significant role in the human body and is also known as the primary male sex hormone. Testosterone is an androstanoid with 17-beta-hydroxy and 3-oxo groups with an unsaturation between the fourth and fifth carbons, as can be seen in Table 1.1. The biosynthesis of the testosterone starts with cholesterol and takes place through enzymes.

For men, testosterone is produced by the Leydig cells of the testicles, and a small amount is also secreted by the adrenal glands [33]. In women, after being secreted by the ovaries and adrenal glands, a large part of it is converted to estradiol, which is known as the female sex hormone and is one of the three naturally produced estrogen hormones in the body.

Testosterone has many different and crucial functions in the body. First of all, testosterone has a variety of functions at different ages and periods such as in pregnant women, fetuses, children, and adolescents. Testosterone is known to show an increase along with estrogen in pregnancy for essential tasks such as supporting the myometrial function responsible for childbirth and cervical remodeling [34]. Also, the concentration of testosterone in the blood plasma is of great importance for the development of the reproductive organs of the fetus and the evaluation of genital abnormalities in newborn babies [35]. Testosterone begins to be secreted in higher amounts during puberty, assuring healthy growth and reproduction in men. Studies have shown that the changes in hormone levels, including testosterone during puberty, exceed most of the hormonal changes which take place in old age [36]. Secondly, testosterone has critical functions in metabolic activities. Studies have shown that various functions of testosterone in the human body, such as the distribution of stored fat in the body, the balance of muscle and fat in the body, regulation of bone density, and consequently reduced risk of osteoporosis, have great importance for human metabolism, especially for older men [37, 38]. Finally, the main task of the testosterone hormone is to produce sperm and regulate sexual functions. Studies have proven a linear relationship between nocturnal penile

bloating (NPT), criteria of sexual interest and behavior, number of daily erectile events, sperm motility, and testosterone level [39, 40].

The amount of testosterone in the blood varies in different gender and age groups. The testosterone hormone is secreted in small amounts in women compared to men, and it turns into estradiol over time. Standard testosterone levels should be between 0.3 μg/mL and 1.0 μg/mL in men and range from 0.015 μg/mL to 0.07 μg/mL in women, and thus it can be said that the testosterone level in women is about 5 percent of that in men. The amount does not always remain constant for men. While an increase in testosterone level is observed during adolescence, the amount of secretion begins to decrease with aging. When the amount of testosterone secreted in both women and men is too high or too low than normal values, it brings some health problems with it. Testosterone deficiency can lead to several health problems in men, such as decreased sexual desire and sperm count, increased breast size, impotence, reduced bone and muscle density, depression, and lack of attention. Sometimes, testosterone deficiency can develop due to chronic diseases such as type 2 diabetes mellitus (T2DM), while sometimes it can be caused by several genetic disorders such as Metabolic Syndrome (MetS), Klinefelter Syndrome, Myotonic Dystrophy, Prader-Willi Syndrome; and it can cause various diseases such as insulin resistance, Acne Fulminans, hypogonadism, and cardiovascular disease [41–45]. High testosterone levels in women cause also various health problems such as polycystic ovary syndrome, ovarian failure, and follicular cyst formation [46]. In addition, some masculine features such as an increase in body hair growth and muscle mass, a deepening tone of voice, and small breasts; and at the same time, it can cause many problems such as disruption in the menstrual cycle, hair loss, infertility, and stress. Furthermore, it has been reported that the testosterone hormone reduces HDL cholesterol levels [38]. This situation can cause various vital problems such as atherosclerosis, cardiovascular diseases (CVD), and increased LDL cholesterol.

In addition, various anabolic steroids, especially testosterone, are accepted as doping substances [47] as they are used for strength and endurance to the muscles

in sports branches such as body building, hammer throw, round shot, wrestling, boxing, and weightlifting.

Consequently, rapid analysis of testosterone is crucial both in sports competitions due to its banning and in clinical diagnosis due to the changes in testosterone levels in both genders and age groups are associated with various physical and psychological diseases.

1.2.3 Progesterone

Progesterone is an endogenous molecule, a derivative of cholesterol, and is also known as a female hormone. It has some fundamental functions in the human body, such as the menstrual cycle regulation and the continuation of pregnancy. Progesterone is a 21-carbon, cholesterol-derived steroid composed of four fused ring hydrocarbons. Oxo groups are located at positions 3 and 20, and there is an unsaturated bond between carbons 4 and 5. While progesterone contains oxygenated functional groups, methyl, and ketones in its structure, it also has a highly hydrophobic configuration due to the absence of polar functional groups, just as in the other steroid hormones. The molecular structure of the progesterone can be seen in Table 1.1.

Primarily, secretion and synthesis of the progesterone hormone in a woman of reproductive age occur in the ovaries. Progesterone is produced during ovulation, but if fertilization does not occur, progesterone secretion is interrupted, and the menstrual cycle takes place; if fertilization occurs, progesterone secretion continues for about eight weeks, and then the placenta takes over the function of secreting progesterone for the continuation of the pregnancy [48]. Although progesterone is known as a female hormone because it prepares women for pregnancy, progesterone is also produced in the adrenal glands of men. Therefore, the functions of this hormone cannot be limited to the reproductive system of women because the progesterone hormone is synthesized in both male and female bodies. Progesterone has vital roles in the remediation of various diseases such as osteoporosis, Alzheimer's disease, diabetic neuropathy, cerebral edema, and Carpal

Tunnel syndrome; and is also of great importance in hormone replacement therapy and raising HDL cholesterol [49].

Progesterone levels of women show variations in different periods. While the level of progesterone hormone before ovulation is below one ng/mL, this value increases up to 12.0 ng/mL during the ovulation period and to 20.0 – 24.0 ng/mL during the menstrual period. Progesterone levels during pregnancy are in between 11.0 and 90.0 ng/mL in the first trimester, in between 25.0 and 89.0 ng/mL in the second trimester, and in between 58.0 and 250.0 ng/mL in the third trimester. The expected level of progesterone hormone, which falls below one ng/mL in women after menopause, is below 0.20 ng/mL for men. Therefore, the effects of progesterone levels are also very critical in the diagnosis of diseases. Studies have shown that increased progesterone levels in the luteal phase of the menstrual cycle are associated with increased intensity in various psychological mood changes of women, such as anger, irritability, insomnia, fatigue, tearfulness, and sadness [50]. In addition, the decrease in progesterone level during pregnancy triggers premature birth [51]. Besides, imbalances in progesterone levels increase the risk of endometrial cancer [52]. Moreover, some studies showed that progesterone is a potential oncogenic hormone for prostate cancer in men, revealing the importance of progesterone levels [53].

In summary, rapid analysis of progesterone hormone is of great clinical importance due to its vital relationship both with the preparation of the body for pregnancy and various physical and psychological diseases.

1.3 Determination of Testosterone, Progesterone and Cholesterol

Various instrumental analysis and sample preparation methods have been used so far for the determination of the abovementioned analytes. Some examples of the methods used in the last five years are given in Table 1.2.

Table1.2. Some examples of methods developed for determination of testosterone, progesterone and cholesterol in the last five years

Analyte	Instrumental Method	Sample Preparation	Sample Volume	LOD/LOQ	Ref.	Year
Testosterone	LC–MS/MS	LLE	100 μL	0.02 ng/mL (LOQ)	[54]	2018
Testosterone & Progesterone (and some other steroid molecules)	UHPLC – MS/MS	LPME	20 mL	0.02 ng/mL (LOQ)	[55]	2021
Testosterone	LC–MS/MS	LPME	100 μL	0.01 ng/mL (LOQ)	[56]	2019
Testosterone (and some other steroid molecules)	LC – MS/MS	LLE	100 μL	0.074(LOD) 0.26 (LOQ)	[57]	2019
Testosterone	LC – MS/MS	LLE	100 μL	Not given	[58]	2019
Testosterone & Progesterone (and some other estrogen metabolites)	LC – MS/MS	SPE	6 mL	Not given	[59]	2020
Testosterone & Progesterone (and some other steroid hormones)	LC – MS/MS	TF – SPME	600 μL	0.006 ng/mL for T and 0.15 ng/mLforP (LOD) 0.02 ng/mL for T and 0.5 ng/mL for P (LOQ)	[60]	2020
Progesterone	DART – MS	SPE	8 mL	0.02 ng/mL (LOD) 0.07 ng/mL (LOQ)	[61]	2020
Progesterone	LC – MS/MS	LPME	500 μL	0.3 ng/mL (LOD) 1 ng/mL (LOQ)	[62]	2020

Table1.2. (Continued)

Analyte	Instrumental Method	Sample Preparation	Sample Volume	LOD/LOQ	Ref.	Year
Testosterone & Progesterone (and some other sex hormones)	HPLC – MS/MS	SPME	8 mL	0.06 ng/mL for T and 0.023 ng/mL for P (LOD) 0.2 ng/mL for T and 0.07 ng/mL for P (LOQ)	[63]	2020
Cholesterol (and some metabolites)	UHPLC–MS/MS	MDSPE	Not given	0.24 pg/mL (LOD) 1.6 pg/mL (LOQ)	[64]	2019
Cholesterol (and some related compounds)	GC – MS	LPME	200 – 600 μL	10 ng/mL (LOD) 30 ng/mL (LOQ)	[65]	2018
Cholesterol (and some other molecules)	GC – MS	DI – SPME	1.5 mL	0.34 μg/g (LOD)	[66]	2019

When the previous studies are examined, various instrumental analysis methods such as GC-MS, LC-MS, HPLC-MS, and ambient MS for the determination of steroids and steroid hormones have been used. Also, it has been observed that sample preparation methods such as solid phase extraction (SPE), liquid - liquid extraction (LLE), liquid phase micro extraction (LPME) and solid phase micro extraction (SPME) have been used.

1.3.1 Instrumental Analysis Techniques

As can be seen in Table 1.2, chromatographic methods such as gas chromatography (GC), liquid chromatography (LC) and high-performance liquid chromatography (HPLC) have been widely used in the determination of steroid hormones and cholesterol in recent years. The most commonly used detection system with all these chromatographic methods is mass spectrometry (MS), and in the literature,

there are also methods based on direct to MS without using any chromatographic separation. Among these methods ambient mass spectrometry has gained popularity in the recent years as allows performing rapid analysis. More details about ambient mass spectrometry can be found below.

1.3.1.1 Ambient Mass Spectrometry

Mass spectrometry is an analytical technique that allows us to identify the molecules or atoms based on mass/charge ratio. Mass spectrometry (MS) provides high sensitivity that allows both qualitative and quantitative analysis. Therefore, MS plays an essential role in various areas such as clinical applications, food analysis, environmental studies, pesticide analysis and forensic sciences.

Before MS analysis, analytes must first be ionized. There are various ionization methods used for this purpose, namely, electron impact (EI), chemical ionization (CI), thermal ionization (TI), atmospheric pressure chemical ionization (APCI), laser ionization (LI), fast atom bombardment (FAB), secondary ionization (SI), plasma – desorption ionization (PDI) matrix assisted laser desorption ionization (MALDI), resonance ionization (RI), and electrospray ionization (ESI). In addition to these techniques, a powerful and new MS – based ionization method, ambient ionization mass spectrometry, was introduced in the early 2000s [67].

Ambient ionization is an MS ionization technique in which ions are created under ambient conditions, allowing direct analysis of samples [68]. Since this method require minimal or no processes such as sample preparation or separation, it provides rapid analysis. In Figure 1.6, the ambient ionization process is illustrated.

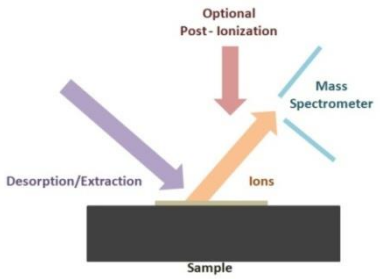


Figure 1.6. Representation of ambient ionization process

Ambient mass spectrometry allows direct probing of a sample under ambient conditions without any pretreatment. It also provides high-throughput, straightforward and rapid chemical analysis of samples. Different ambient ionization mass spectrometry approaches are used for various applications.

1.3.1.1.1. Different Ambient MS Techniques

Ambient ionization, of which various techniques are described below, was introduced in 2004 by presenting desorption electrospray ionization (DESI) technique [69].

Desorption electrospray ionization (DESI), which has various applications in biotechnology, pharmaceutical industry, food laboratories, forensic analysis, and environmental monitoring, is a similar method to electrospray ionization (ESI). It ionizes and desorbs analytes from a solid surface with the help of the sprayed aqueous solvent droplets and the created charged droplets can be suctioned by an atmospheric pressure capillary and enter the inlet of the MS [11,70,71].

Direct analysis in real-time (DART), is a technique in which the analytes can be directly desorbed from surfaces such as biofluids, polymers, glass, plants, clothes, banknotes, and tablets. DART differs from DESI by the feature of not requiring a liquid electrospray solvent; it utilizes an excited gas stream such as nitrogen or helium to ionize the analytes [72]. In addition, DART techniques thermally desorb the analytes from the sample surfaces using high temperatures [12].

The atmospheric solids analysis probe (ASAP), which provides ionization with high precision, is a practical and powerful device for direct analysis using atmospheric pressure ionization and heated nitrogen gas to vaporize the sample. In ASAP-MS, the analytes are volatilized at high temperatures and atmospheric pressure by using a gas such as nitrogen and ionized via a corona discharge [73].

The flowing technique, atmospheric-pressure afterglow (FAPA) technique, a type of atmospheric pressure chemical ionization method in which a helium discharge is used to produce reactive ions, is a very suitable technique for direct analysis and

portable mass spectrometry due to its high sensitivity and particularly uncomplicated design [74].

The plasma-assisted desorption ionization (PADI) method differs in some aspects from other direct ionization techniques, such as the discharge method used and the interaction of the plasma with the sample, a non-thermal atmospheric pressure plasma is produced and directed to the surface of the analyte [75].

Low-temperature plasma probe (LTP), which has a wide range of work areas, is a kind of ambient plasma-based ionization technique and has several advantages such as ease of operation, inexpensiveness, being suitable for on-site analysis, and being a relatively soft ionization method [13].

Dielectric barrier discharge ionization (DBDI) is a technique in which low-temperature plasma is generated by dielectric barrier discharges (DBD) obtained under ambient conditions. In DBDI a high voltage AC wave is applied between two electrodes to ionize the neutral gas-phase analytes [76].

Field desorption (FD) is an ion formation technique used to ionize the analyte molecules applied as a thin film to the surface of a specially prepared emitter which is placed in a high-intensity electric field [77-78]. FD, which is a kind of soft ionization method, is a very advantageous method with features such as a simple application method, high sensitivity, and low background production.

Paper spray ionization is a variation of electrospray ionization (ESI) and is a technique used in MS to generate ions from a sample that will be analyzed. In the early 2010s, it was found that the sample to be analyzed could be loaded directly on a paper and by applying a high voltage the analytes can be ionized and detected directly by MS [79]. It has several advantages, such as allowing analysis of a remarkably little amount of sample, low use of solvents, and paper being an inexpensive and available material. It has various application areas such as clinical studies, forensic science, and environmental analysis. In Figure 1.7, paper spray ionization is schematized.

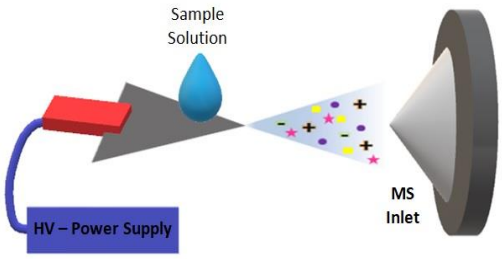


Figure 1.7. Paper spray ionization

All the ambient mass spectrometric techniques mentioned above may induce ionization enhancement or suppression as the matrix is transferred directly to the MS with the analyte ions. This circumstance may cause a loss of sensitivity in the signals obtained from the MS and require more maintenance for the instrument. In this case, the significance of sample preparation emerges. Coated blade spray (CBS), a technology that combines sample preparation and ambient ionization, was developed to overcome this drawback.

Coated blade spray (CBS) is a technology designed for extraction, desorption, and ionization from various complex matrices based on solid-phase micro-extraction (SPME), which can reduce the whole analysis time to several minutes due to its direct coupling to mass spectrometry [65]. In recent studies, the advantages of CBS technology, such as the simplicity and speed brought by direct coupling to mass spectrometry and the droplet approach, which provides rapid analysis from the small sample, were mentioned [66]. In this approach, a sample such as a microliter of plasma spot can be analyzed within a few minutes by direct coupling the CBS blade to the MS device. This method, which is very useful for in vitro studies, provides a significant benefit, especially for clinical studies that need a rapid diagnosis. It also provides rapid analysis with high sensitivity for the samples in microliters such as heel blood.

A typical CBS probe is shown in Figure 1.8. As can be seen from the figure, CBS is a small sword-shaped plate, made of stainless steel and coated with an extractive phase. Its sharp tip structure provides stable electro spray ionization and efficient way to transfer the produced ions to the MS inlet. The polymer is coated with a

biocompatible extractive phase suitable for extraction of small molecules. Since only small molecules are extracted from the matrix it minimizes ionization suppression and matrix effects compared to the other ambient ionization methods. The solid support is made of stainless steel which gives an extra stability during the ionization process.

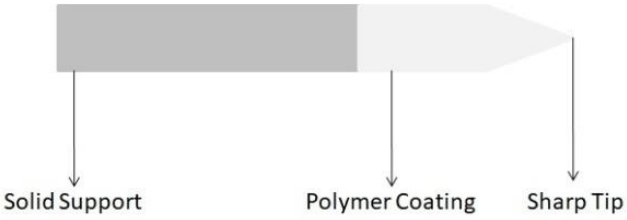


Figure 1.8. Coated blade spray

A typical droplet size application of CBS (extracted biofluid spot) for a small amount of biofluid is shown in Figure 1.9. As can be seen from the figure, the first step of a typical CBS process is introduction of sample on the blades surface. After waiting for the sorption of the analytes by the extractive phase, a quick washing is done. Next, the CBS probe is placed across the inlet of the MS instrument, and the extracted analytes are desorbed by dripping desorption solvent on it. Then, Taylor cone is formed by applying a high voltage to the blade. This process provides fast instrumental analysis as well as rapid sample preparation.

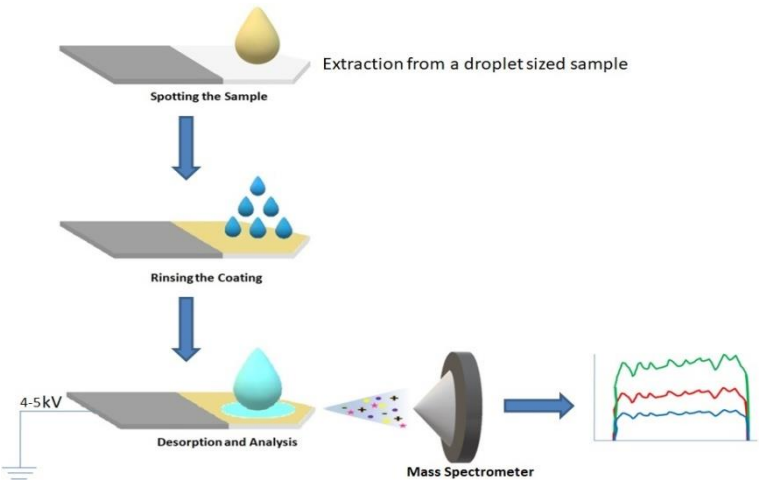


Figure 1.9. CBS with droplet approach

1.3.2 Sample Preparation Methods

Sample preparation is of great importance in chemical analysis, as it is the most time-consuming and error-prone stage of the entire analysis [80]. Also, to get rid of the matrix effect in instrumental analysis, effective sample preparation is required [81]. In addition to the commonly used traditional sample preparation methods, there are novel sample preparation methods based on microextraction reported in the literature.

1.3.2.1 Traditional Sample Preparation Methods

Traditional methods such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PP) are widely used for the determination of steroids and steroid hormones.

Solid-phase extraction (SPE) is an extraction technique in which compounds dissolved or suspended in a liquid mixture are separated from other compounds by adsorbing onto a solid stationary phase for purification, and pre-concentration of analytes before chromatographic separation. Although the SPE technique is well accepted it has various disadvantages such as being expensive, low selectivity, clogging problem of the solid stationary phase in complex matrices, and high impurity of the solid phase compared to the solvents in liquid phase extractions. Figure 1.10 shows the general steps of solid phase extraction. Firstly, the extractive phase is pre-conditioned with a solvent mixture such as MeOH:H_2O and is made ready for extraction. Then, the traces of organic solvents are removed with a washing step. After washing with water, the sample is loaded. While the analyte molecules (indicated by the green triangle in the Figure 1.10) are sorbed to the extractive phase, the other matrix components, which do not show affinity to the extractive phase, are removed. Finally, the target analytes are collected with the help of a suitable solvent. In this way, the separation of target analytes from the complex matrix is achieved.

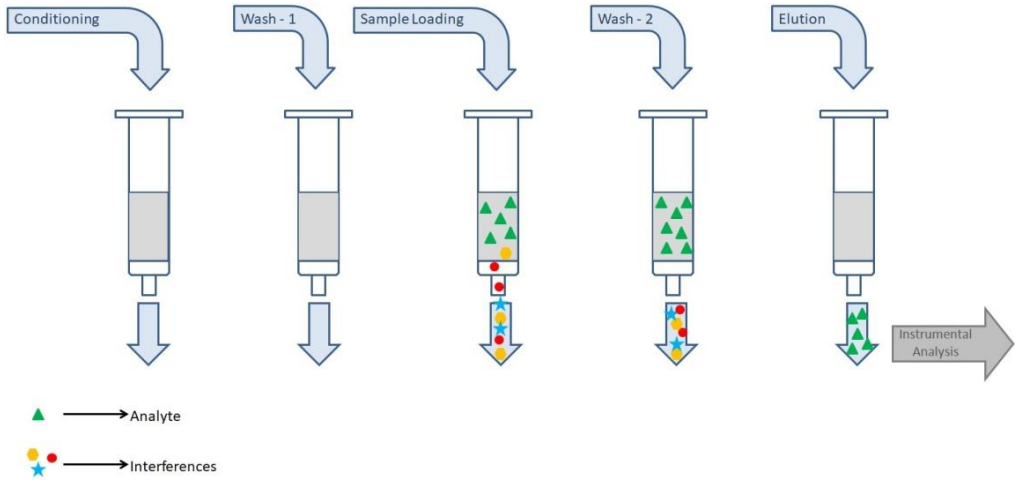


Figure 1.10. General steps of solid phase extraction

Liquid-liquid extraction (LLE), in other words solvent extraction, is a separation and purification method that separates a solute by transferring it from one solvent to another one in which two solvents are immiscible in each other. The general steps of liquid-liquid extraction are shown in Figure 1.11. First, the sample is mixed with two immiscible solvents and waited until the two solvent phases separate again. In this process, the target analytes distribute themselves between the two phases based on their affinity to the given phase. Then, the two phases are separated and collected in separate containers. Thus, target analytes are isolated from the sample matrix. While LLE has advantages such as high separation capacity and selectivity, it is a technique that is not environmentally friendly due to the use of large amounts of solvent, requires intensive labor because it is difficult to automate, and can easily cause human error problems.

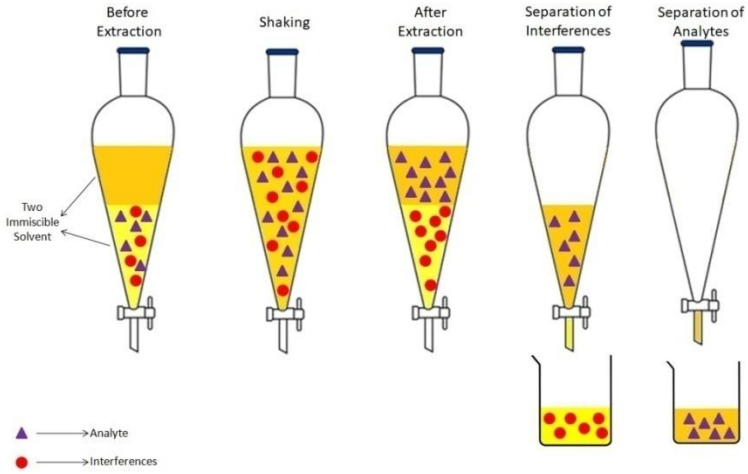


Figure 1.11. General steps of liquid – liquid extraction

Protein precipitation (PP) is a widely used sample preparation method that includes protein denaturation [82]. Figure 1.12 summarizes the steps of the PP method. First, a precipitating agent, such as a strong acid/base or organic solvent, is added to the biological sample and vortexed. It is then kept in an ice bath or freezer. After centrifugation, finally the supernatant containing the analyte is transferred to a vial for injection into the instrument.

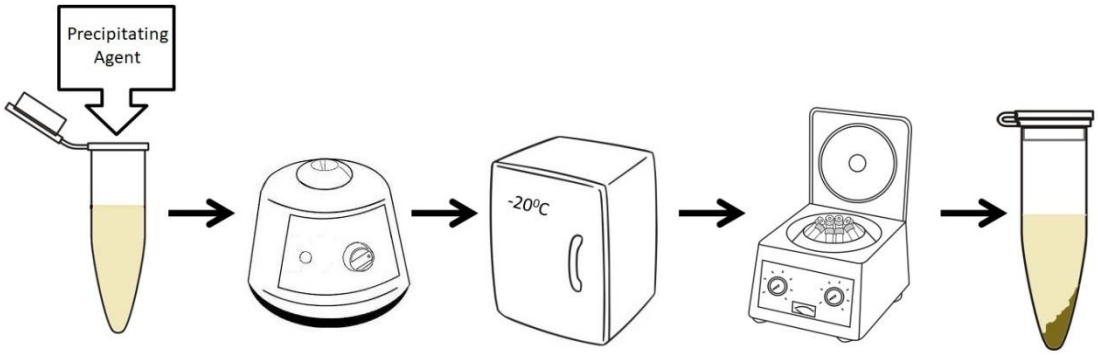


Figure 1.12. General steps of protein precipitation

1.3.2.2 Novel Sample Preparation Methods

Micro-extraction methods have attracted a lot of attention in recent years due to their various advantages such as minimizing the use of solvents, high pre-concentration rates, automation, and ease of direct analysis with chromatographic methods. The most common two micro extraction techniques are liquid phase micro extraction and solid phase microextraction.

In extraction with liquid-phase micro-extraction (LPME), which has all the advantages of micro-extraction methods, the analytes are usually in an aqueous matrix, and usually organic solvents are used as the extractive phase. LPME has drawbacks such as low sensitivity, low precision, low selectivity, and limited solvent preference. The other common technique is solid-phase micro-extraction (SPME) which allows performing the sampling and sampling preparation in single step. It has all the advantages of microextraction methods, such as a small sample requirement, non-exhaustive, and low solvent usage. More details about this technique is given in the next section.

1.3.2.2.1 Solid – Phase Micro Extraction

Solid-phase micro-extraction (SPME) was developed by Janusz Pawliszyn and his colleagues as a method suitable for rapid on-site and laboratory analysis to concentrate and isolate target analytes from complex matrices according to the principle of micro-extraction [83]. It is a convenient approach with various applications for different purposes and methods. In addition, its geometrical flexibility, compatibility with GC, LC, and direct to MS make SPME a very advantageous sample preparation method. One of the various advantages of SPME is that it can be made completely non-exhaustive technique, that is, only a little part of the target analyte is withdrawn from the matrix. Due to this feature, chemical changes in living systems can be monitored with minimal disturbance [84]. In addition, SPME can take any geometry; therefore, it has various application areas such as pharmaceutical research, clinical studies, food and environmental research. Shortening the sample preparation time and providing highly efficient pre-

concentration also makes the SPME an advantageous method. In addition, the availability of various commercial extractive phases (PDMS, PA, CW, CAR/PDMS, PDMS/DVB, DVB/CAR/PDMS, etc.) provides a significant advantage. Furthermore, the biocompatibility and sensitivity of the developed coatings make the SPME beneficial especially in clinical analyses.

Direct mode, membrane protected direct mode, and headspace mode of extraction are the three main extraction modes of SPME, and are schematically illustrated in Figure 1.13 [84]. In direct extraction, the coated fiber is immersed into the sample, and the analytes are transferred directly to the extractive phase; indirect extraction with membrane protection is carried out using a protective membrane in the matrix that may damage the extractive phase, while in headspace sampling, the extractive phase is placed at the headspace above the sample matrix to extract volatile analytes.

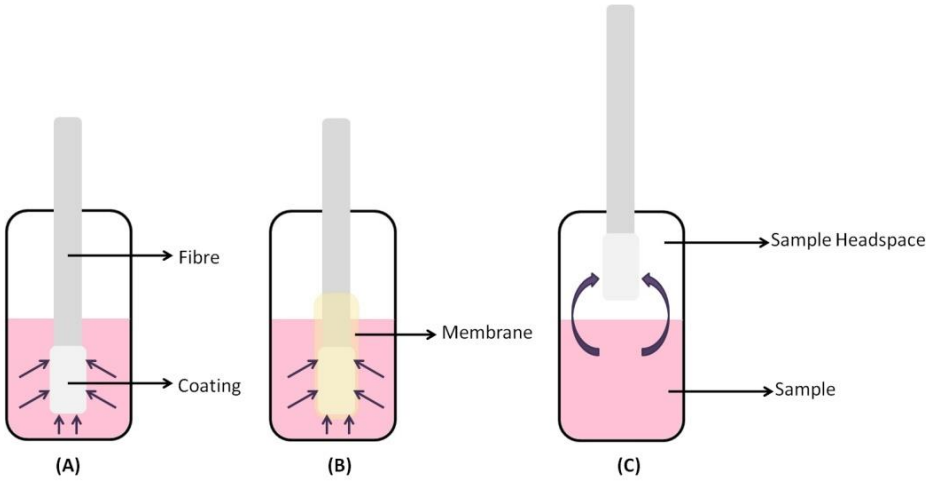


Figure 1.13. a) Direct extraction b) membrane protected extraction c) headspace extraction

To shorten the extraction time of the SPME technique, to perform more sensitive analyzes, and to increase its ease of use, configurations in different geometries have been developed, and these configurations can be classified into two main groups, namely, static and dynamic techniques [85]. Static SPME can be simply defined as the methods used for sample preparation from an immobile sample.

Static approaches include fiber SPME, shown in Figure 1.14, thin-film microextraction (TFME), stir-bar sorptive extraction (SBSE), rotating disk sorptive extraction (RDSE), and dispersive SPME. The static SPME techniques are schematically summarized in Figure 1.14. The fiber geometry is the most widely used conventional SPME configuration, is suitable for on-site applications, is adaptable to different matrices, and can be desorbed thermally directly to the injector port of gas chromatograph (GC), if it is thermally stable [84–86]. The other common SPME geometry is TFME. It uses a thin film layer with a large surface area to volume ratio and has higher extraction efficiency than fiber SPME [85,86] due to larger extractive volume used. SBSE is based on the same principle and has the same advantages as fiber SPME. In the case of SBSE a magnetic stir bar is coated with the extractive phase and thus has the advantage of being able to stir the sample and perform the extraction by itself [86]. Compared to SBSE, RDSE permits a larger surface area to be coated with extractive phase and has the benefit of being able to be stirred at high speed without damaging the extractive phase, resulting in better recovery and shorter extraction time than SBSE [87]. In dispersive SPME, dispersed sorbents are used to extract and pre-concentrate the analytes from the sample solution and require an additional drying process [88]. Dynamic SPME techniques include in-tube, in-needle, and in-tip SPME. In-tube SPME was developed by using an open tube capillary column to eliminate some drawbacks of traditional fiber SPME such as brittleness, and it has a higher sorption capacity than the fiber SPME, also another advantage is that it can be connected on-line by HPLC [89]. Solid-phase dynamic extraction (SPDE) is used by coating the inside of a needle syringe with the extractive phase, while in packed-needle micro-extraction (PNME) an extractive phase packed into a portable needle mounted inside the syringe is used, and all these in-needle SPME formats have advantages such as robustness and portability for on-site analysis and can be used to insert the needle directly into chromatographic instruments by solvent or thermal desorption routes [86]. In the in-tip SPME technique, the extractive phase is placed

on disposable tip. The schematic representations of dynamic SPME techniques are given in Figure 1.15.

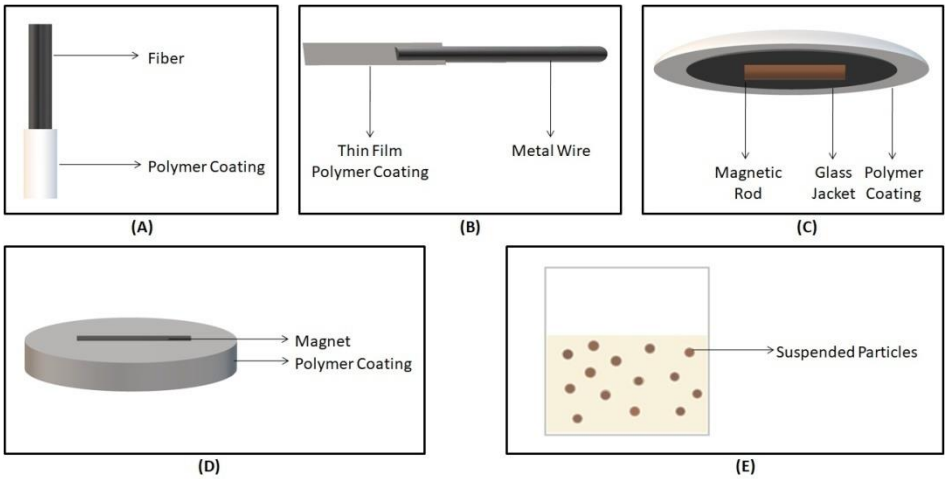


Figure 1.14. Static approaches of SPME a) fiber SPME b) TFME c) SBSE d) RDSE e) dispersive SPME

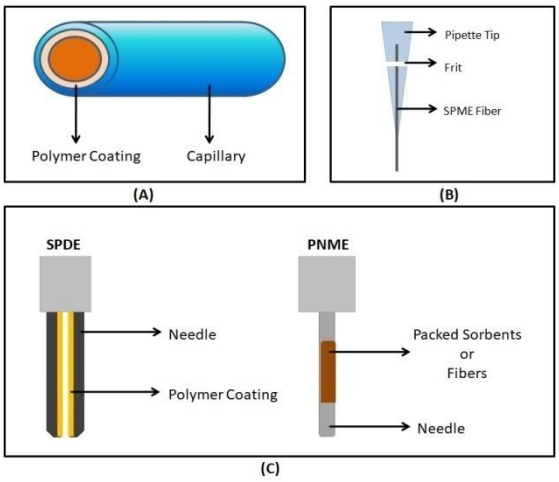


Figure 1.15. Dynamic approaches of SPME a) in – tube SPME b) in – tip SPME c) in – needle SPME

1.4. Extractive Phases Used for SPME

In SPME the analytes are sorbed to the extractive phase by two main mechanisms. Absorption is a case in which the analyte penetrates the entire volume of the extractive phase (liquid coatings), while adsorption is a phenomenon in which the

analyte is retained on the active surface of the extractive phase (solid coatings). The polarity of analytes gains importance in the selection of the extractive phase, for this reason, it is essential to choose the most suitable extractive phase according to the polarity of the analytes. A summary of the available commercial SPME coatings and their polarities and working principles is given in Table 1.3.

Table1.3. Commercially available SPME coatings

Type of Caoating	**Polarity**	**Extraction Mechanism**
Polydimethylsiloxane (PDMS)	Nonpolar	Absorbtion
Polyacrylate (PA)	Polar	Absorbtion
PEG (Polyethyleneglycol) (carbowax)	Polar	Absorbtion
Carbopack Z-PDMS	Bipolar	Adsorbtion
PDMS-DVB (Divinylbenzene)	Bipolar	Adsorbtion
DVB/Carboxen-PDMS	Bipolar	Adsorbtion
Carboxen-PDMS	Bipolar	Adsorbtion
C18	Nonpolar	Adsorbtion

As can be seen in Table 1.3, there are several commercially available SPME coatings. These coatings have the advantages of optimized chemical and physical properties, and being ready to use. However, their high costs can be considered as a disadvantage. Also, the variety of commercial SPME coatings, especially those suitable for LC, is quite limited. All these disadvantages have been a reason to develop new homemade SPME coatings.

Homemade SPME coatings have several advantages over commercial ones as they can be tuned for specific requirements. Some of these advantages are high selectivity, high sensitivity, high thermal and mechanical stability, and relatively cheap. The possibility of developing coatings in the desired thickness, volume, and geometry is also a highly critical advantage. The most common approach for preparation of new SPME phases is based on the use of commercial polymeric

materials or synthesis of homemade polymer particles with fine-tuned properties. Polymerization methods used for the synthesis are given in the next section.

1.5 Polymerization Techniques

Polymerization reactions are the reactions that lead to the formation of larger molecular chains by combining monomer units [90].

Polymerization reactions always require a monomer. A monomer is a molecule that can form a larger polymer chain by entering a polymerization reaction. It can be divided into two groups as natural and synthetic monomers. Natural monomers are organic molecules that exist in nature that combine to form larger biological molecules, for example amino acids and monosaccharides [91]. Synthetic monomers are molecules produced artificially by combining different atoms. Reaction initiators are molecules that enable the initiation of polymerization reactions. They can be divided into two groups as azo compounds and organic peroxides. Organic peroxides are organic compounds containing the peroxide functional group, and azo compounds are those expressed by the functional group whose general structure is RN=N+(O−)R. The molecules that provide the formation of the three-dimensional polymer network structure are called crosslinkers. Table 1.4 shows some examples of monomers, crosslinkers, and initiators.

Table1.4. Some examples of monomers, crosslinkers and initiators

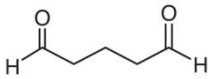
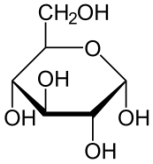
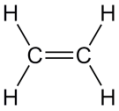
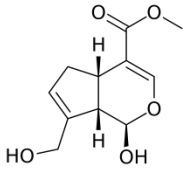
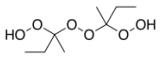
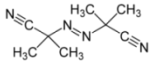
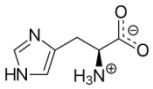
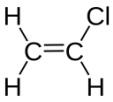
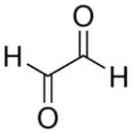
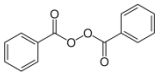
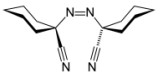
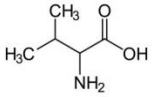
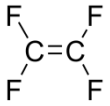
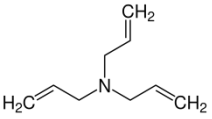
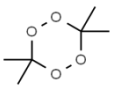
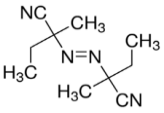
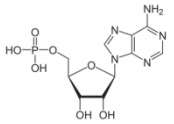
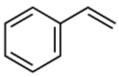
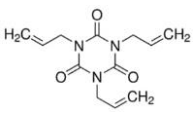
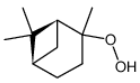
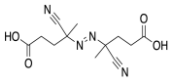
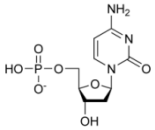
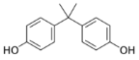
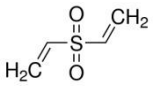
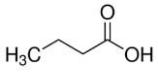
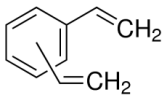
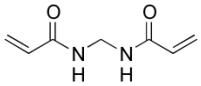
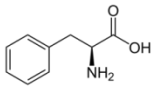
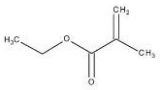
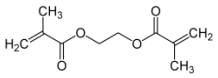
Monomers		**Cross – Linkers**	**Initiators**	
Natural	**Synthetic**	Gluteraldehyde 	**Organic Peroxides**	**Azo Compounds**
Glucose 	Ethylene 	Genipin 	Methyl ethyl ketone peroxide 	AIBN 
Histidine 	Vinyl chloride 	Glyoxal 	Benzoyl peroxide 	ABCN 
Valine 	Tetrafluoro ethylene 	Triallylamine 	Acetone peroxide 	AIVN 
Adenosine monophosphate 	Styrene 	Triallyl isocyanurate 	Pinane hydroperoxide 	ACVA 
Deoxtcytidine monophosphate 	Bisphenol A 	Divinyl sulfone 		

Table1.4. (Continued)

Monomers		**Cross – Linkers**	**Initiators**	
Natural	**Synthetic**		**Organic Peroxides**	**Azo Compounds**
Butyric acid 	Divinyl benzene 	N,N – methylenebisacrylamide 		
Phenylalanine 	Ethyl methacrylate 	Ethylene glycol dimethacrylate 		

There are several polymerization methods with different advantages and disadvantages. Some of those are bulk polymerization, solution polymerization, interface polymerization, suspension polymerization, and emulsion polymerization.

In bulk polymerization, the monomer is directly polymerized by adding a soluble radical initiator and then heating it under a specific temperature and pressure or exposing it to radiation. The most important feature of this process is that highly pure polymers can be produced. The most important disadvantages are that temperature control is challenging, and the viscosity increases rapidly due to the formation of high molecular weights [92].

Solution polymerization is an approach that eliminates the disadvantages of bulk polymerization since the low viscosity of the medium due to the solvent effect allows easy temperature control. In this method, the polymerization occurs in a solvent or diluent phase. However, the fact that the reaction takes place in the solvent both slows down the reaction rate and raises the cost as it requires the removal of the solvent from the product [93].

Interface polymerization is a kind of step polymerization, and polymerization takes place between two immiscible phases. At the end of the reaction, the polymer is formed between these two phases [94]. Although this method seems to be a relatively straightforward process, it also has difficulties such as controlling and optimizing numerous experimental variables. For example, polymerization time and temperature must be kept under control.

In suspension polymerization, the monomer is suspended in a suitable dispersion medium. Although suspension polymerization is a type of bulk polymerization, the reaction is faster than bulk polymerization. In addition to its benefits, such as ease of temperature control and low viscosity, it also has drawbacks such as requiring continuous stirring and requiring processes such as washing, filtering, and drying that make production expensive [95].

Emulsion polymerization is a process carried out in a suitable dispersion environment as in suspension polymerization [96]. The radical polymerization starts with an emulsion containing water, monomer, and surfactant. It has many advantages such as high polymerization rate, obtaining high molecular weight, easy control of the reaction steps, low viscosity of the medium, ease of production, being inexpensive, and the resulting product can generally be used directly. If a solid product is aimed, the need for processes such as drying, and purification increases the cost.

There are three types of emulsion polymerization methods. The first of these is the classical aqueous method, macro emulsion polymerization. The products formed as a consequence of this type of polymerization reaction are micron-sized particles that are not very stable both thermodynamically and kinetically [97]. The second is micro emulsion polymerization. Only micelles are formed, no droplets are created, and nm-sized, thermodynamically stable, and optically clear particles are obtained. Another emulsion polymerization method, which is the preferred method for polymer synthesis in this thesis, is mini emulsion polymerization. The particles obtained in this method have particle sizes in between micro and macro emulsion

polymerizations [98]. Even if it is not thermodynamically stable, kinetically stable particles are produced. The physical properties of the polymeric particles obtained with these three emulsion polymerization methods are summarized in Table 1.5.

Table1.5. Physical properties of the polymeric particles obtained with emulsion polymerization

Types of emulsion / Property	Macro emulsions	Mini emulsions	Micro emulsions
Particle size	1-100 μm	20-500 nm	10-100 nm
Shape	Spherical	Spherical	Spherical, Lamellar
Polydispersity	Often high (>40%)	Low (<10-20%)	Low (<10%)
Stability	Weakly kinetically	Kinetically	Thermo dynamically

1.6 Preparation of SPME Devices

In preparation of SPME devices, several different approaches are reported [99]. Most known methods include dip coating, spin coating, spray coating, and bar coating. These methods are schematically summarized in Figure 1.16.

In dip coating processes, the material to be coated is immersed in a liquid coating solution (or slurry of particles in glue) and then withdrawn from the solution at a certain speed. The greater the withdrawal rate, the thicker the coating will be, as more material will adhere to the support and will not have enough time to flow back into the solution. In addition to the withdrawal speed, the density, viscosity, and surface tension of the liquid coating solution are the factors affecting the coating thickness [100]. This method is generally used to obtain homogeneous and relatively easy-to-prepare coatings in micrometer size.

Spin coating is a procedure used to deposit thin films onto flat surfaces. Generally, a small amount of coating solution or slurry is applied in the middle of a flat support to be coated by rotating it for a certain period. The resulting coating

thickness depends on the concentration, viscosity, and surface tension of the coating solution, and is usually hundreds of micrometers thick. In addition, the rotation speed of the surface to be coated is one of the main factors affecting the coating thickness. The higher the angular velocity, the thinner the coating thickness obtained [101]. It is possible to acquire very homogeneous coatings with this coating method and usually is used for the preparation of flexible TFME probes.

The bar coating method is performed by placing a considerable amount of coating solution or slurry on the surface to be coated and spreading it with the help of a bar. This bar used is a long cylindrical rod with spiral wire around it [102]. The thickness of the coating obtained is related to the amount of gap between the surface to be coated and the bar. Just like in spin coating, coatings with hundreds of micrometer thicknesses can be obtained and usually is used for the preparation of flexible TFME probes.

In the spray coating method, the coating solution, or the slurry is sprayed directly onto the surface to be coated as fine droplets with the help of a pressurized inert gas. In this affordable and easy to apply method, coatings with micrometer or millimeter thickness and high mechanical stability can be obtained [99]. However, it is a disadvantage of this method that homogeneous coatings cannot be obtained.

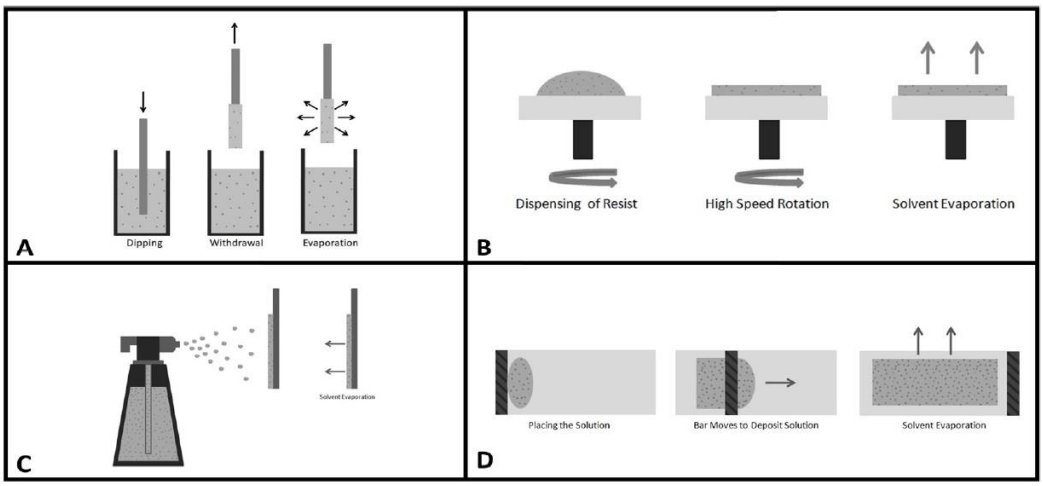


Figure 1.16. a) Dip coating, b) spin coating, c) spray coating, d) bar coating (Redrawn from; Olcer et al., 2019) [99]

More recently, electrospinning approach shown in Figure 1.17, has gained attention for preparation of the SPME coatings [103]. This approach is based on producing nanofibers from polymer solutions in a high electric field. It has crucial advantages such as the ability to acquire high surface area, nano to micro fibrous continuous phase in which polymeric particles (extractive phase) can be immobilized. The polymer solution taken into the syringe is fed to the needle tip at a certain speed with the help of an infusion pump. Meanwhile, with the high voltage applied to the needle tip, the polymer solution forms nanofiber jets with the help of a Taylor cone formation, and these are collected on a grounded metal collector plate. In this way, nanofiber structures are obtained.

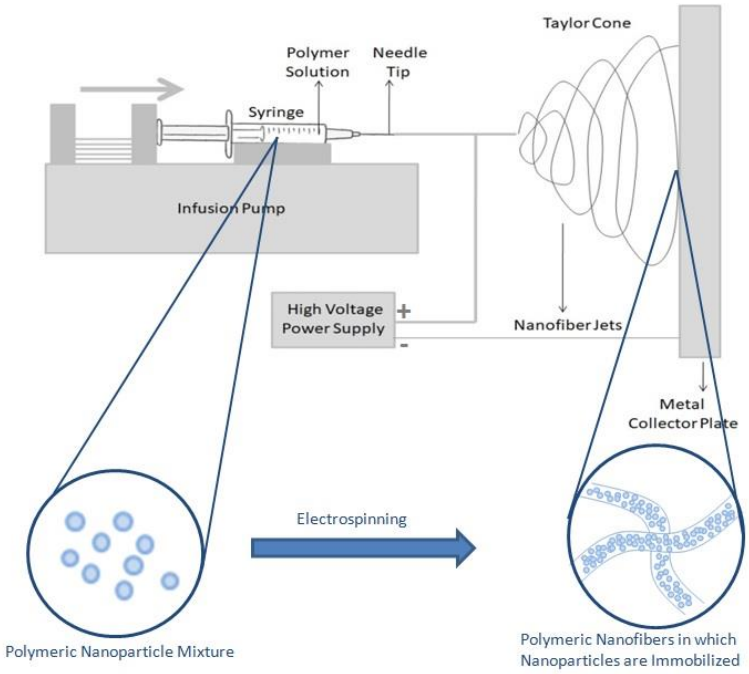


Figure 1.17. Electrospinning experimental set – up

1.7. Aim of the Study

As described in related parts of the Introduction Section, ambient mass spectrometry has great importance as a fast, simple, and selective technique. However, in this technique, the matrix and analyte are introduced simultaneously to mass spectrometer (MS) accompanying high background and ion suppression

during the analysis; thus, it prevents to take the full advantage of such system by decreasing the sensitivity. One of the simples and great approach to overcome abovementioned problem is to integrate a sample preparation method that is suitable for direct to MS analysis with ambient mass spectrometry. As shown in the related parts of the Introduction Section, coated blade spray form (CBS) of solid phase microextraction (SPME) can be used as a sampling/sample preparation probe and electrospray ionization source for direct to MS analysis. However, the current extractive phases are made with extractive particles embedded within a bulk polymer which results in slow analyte diffusion, limiting the sensitivity of the system.

In this thesis, new CBS probes prepared by immobilization of extractive nanoparticles in nanofibrous polymer structure was proposed as solution to abovementioned drawbacks of CBS. For this purpose, first of all, the extractive phase consisting of PDVB nanoparticles were synthesized by miniemulsion polymerization method. Then, synthesized particles were immobilized in PAN binder using the electrospinning technique. Finally, electrospun PAN-PDVB-CBS probes were hydrolyzed to give hydrophilic-lipophilic balance (HLB) property to the probe. The prepared hydrolyzed PAN-PDVB coated blades were then used to develop a new CBS-MS method for rapid determination of cholesterol, progesterone and testosterone in spot sized plasma samples. The new CBS probes showed improved extraction kinetics compared to classical CBS probes. Moreover, the method developed with these new CBS probes requires only 7 minutes to complete the sample preparation and instrumental analysis, proving the applicability of the method for rapid clinical diagnosis.

CHAPTER 2

EXPERIMENTAL

2.1 Reagents and materials

Reagents used for poly(divinylbenzene) (PDVB) nanoparticle synthesis: divinylbenzene (DVB) (Sigma-Aldrich), aluminum oxide (activated, basic) (Sigma-Aldrich), 2-2'-azobis(2-methyl-propionitrile) (Acros Organics), sodium dodecyl sulfate (Sigma-Aldrich), and hexadecane (Sigma-Aldrich).

Reagents used for polyacrylonitrile (PAN, MW: 150000) solution and hydrolysis of PAN: polyacrylonitrile (Sigma-Aldrich), dimethylformamide (Sigma-Aldrich), sodium hydroxide (anhydrous pellets) (Carlo Erba Reagents).

Reagents used for preliminary studies of immobilization of PDVB in a continuous phase: polyvinylpyrrolidone (Sigma-Aldrich), poly(vinyl alcohol) (Sigma-Aldrich), citric acid (Horasan Kimya).

Analytical Standards: testosterone (Sigma-Aldrich), progesterone (Sigma-Aldrich), cholesterol (Sigma-Aldrich).

Internal Standards: ergosterol (Sigma-Aldrich) was used as an internal standard for cholesterol, cortisone (Sigma-Aldrich) was used as an internal standard for testosterone and progesterone.

Formic acid, was added both in desorption solvent in direct to MS studies and in the mobile phase in LC-MS preliminary studies for protonation in positive ionization mode: formic acid 98-100% for HLPC (Merck).

Solvents used in desorption, LC-MS mobile phases, protein precipitation and stock solution preparation procedures: methanol (gradient grade for liquid chromatography) (Supelco), acetonitrile (gradient grade for liquid chromatography) (IsoLab), isopropyl alcohol (LC – MS grade) (IsoLab).

Chemicals needed for phosphate buffer saline preparation: sodium chloride (Sigma-Aldrich), potassium chloride (Sigma-Aldrich), potassium dihydrogen phosphate (IsoLab), sodium phosphate dibasic (Sigma-Aldrich).

Bovine serum albumin for preparation of synthetic plasma: bovine serum albumin, lyophilized powder (Sigma-Aldrich).

Plasma for real sample studies in method validation: human plasma pooled (BioWest).

2.2 Instruments and Apparatus

Vortex mixer (ISOLAB) was used for rapid mixing and homogenization of pre-polymerization mixture, analyte solutions, and various mixtures and solutions throughout the studies.

Ultrasonic baths (ELMA LC 30 and ELMA Elmasonic S 40 (H)) were used for various purposes such as degassing solutions/solvents, enabling faster dissolution of solids which cannot dissolve easily and homogenization.

Magnetic stirrer with hot plate (MSH-20A) was used for constant stirring and heating during polymer synthesis, mixing the polymer solutions and for other procedures that require prolonged mixing and/or heating at constant speed.

Ultrasonic processor (SONICS VibraCell VCX500/VCX750) was used for homogenization of pre-polymer mixtures in the polymer synthesis and during the preparation of the polymer mixture for electrospinning.

NüveNf 200 bench top centrifuge was used for centrifugation processes.

NE-300 Just Infusion™ syringe pump (New Era Pump Systems) and NE-1000 one channel programmable syringe pump (New Era Pump Systems) has been used as an infusion pump both in electrospinning and for various purposes in this thesis.

Scanning Electron Microscope (QUANTA 400F Field Emission SEM) was used for nanoparticle and nanofiber characterizations.

Simultaneous Thermal Analyzer (Perkin Elmer STA 6000) was used for thermal gravimetric analysis of the synthesized nanoparticles.

Fourier-Transform Infrared Spectrometry (Thermofisher Smart ITR ATR-FTIR) was used for characterization of different polymers, synthesized polymer nanoparticles, hydrolyzed polymers and cross-linked polymers.

Electrospinning device (nanoWEB Electro-Spin110), infusion pump (New Era Pump Systems) and DC power supply (Spellman SL30) was used for coating optimizations by electrospinning.

EUROSTAR IKA Werke digital mechanical stirrer was used as a revolver head for blade coating with electrospinning.

CAT AEK-SH10 heater-shaker was used for sample preparation steps (extractions and desorptions) in LC-MS studies.

Philip Harris 5kV D.C was used as external voltage source in CBS-MS analysis.

For CBS studies Agilent G61258 Single Quadrupole Mass Spectrometer was used.

For preliminary studies Agilent 1260 Infinity II Liquid Chromatography System was used.

The chromatographic column used for LC-MS studies was Agilent, Poroshell 120 EC-C18, 4.6x150 mm, 4μm.

2.3 Preparation of Novel Hydrophilic Lipophilic Balanced Coated Blade Spray (CBS) Probes

A new homemade extractive phase has been developed for coating CBS blades. For this purpose, PDVB nanoparticles were synthesized using the mini-emulsion polymerization technique and these nanoparticles were immobilized into the PAN polymer using the electrospinning method. Details of the experiments are given below.

2.3.1 Synthesis of PDVB Nanoparticles by Mini Emulsion Polymerization Method

Since the divinyl benzene monomer was stabilized with an inhibitor (p-tert-butylcatechol), it was first investigated whether the inhibitor removal is critical and affected nanoparticle synthesis. Also, to look at the effect of the reaction initiator on the synthesized nanoparticles, polymerization reactions with two different azo initiators (AIBN and AIVN) were investigated as described below.

2.3.1.1 PDVB Nanoparticle Synthesis by Removing the Inhibitor

In the first procedure applied, the inhibitor was removed from the divinylbenzene (DVB) monomer before poly(divinylbenzene) (PDVB) synthesis. For this purpose, activated basic alumina was filled into a clean, filter – tipped column, and 3.0 mL DVB monomer was passed through the column to remove the inhibitor. Inhibitor removed DVB monomer was stored in a light-blocked vial in the refrigerator until used.

For the synthesis of PDVB nanoparticles, firstly, 0.90 g of SDS (surfactant) was dissolved in 12.0 mL of distilled water in a 20 mL vial. In another 20 mL vial, 0.050 g of AIBN (reaction initiator), 0.162 mL of HD (co-stabilizer) and 3.0 mL of DVB (monomer) were mixed. This mixture was combined with the SDS solution and thoroughly mixed with the help of a vortex. It was further mixed in a magnetic stirrer at 990 rpm for 30 minutes. Following this step, ultrasonication (500 watt, 20

kHz) was carried out with the help of a sonotrode probe for 3 minutes to obtain a more homogeneous and well-dispersed polymer solution. This step was carried out in an ice bath to prevent premature polymerization due to heating. After this step, the mixture was polymerized for 18 h at 72 ^{o}C in an oil bath in which the stirring speed was adjusted to 990 rpm. The synthesis process is summarized in Figure 2.1.

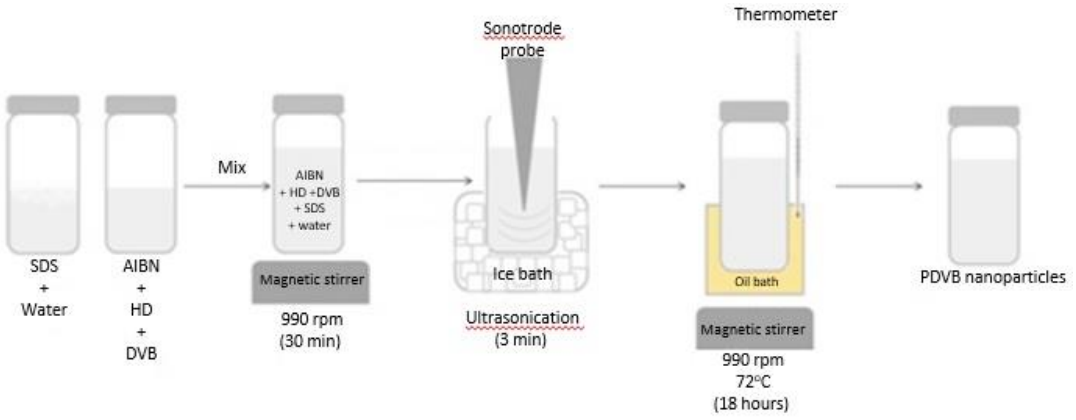


Figure 2.1. PDVB nanoparticle synthesis procedure

After the synthesis with the AIBN initiator (azobisisobutyronitrile) was completed, the same synthesis procedure was repeated using the AIVN initiator (2,2'-azobis(2-methylbutyronitrile)), which has a similar structure, instead of AIBN, to examine the effect of the initiator type on the polymer obtained in the reaction. The same procedure was applied by changing only the amount of AIVN (0.058 g) since the molecular weights of AIBN and AIVN molecules are different.

The separation of PDVB nanoparticles from the reaction media was also studied. At this stage, multi-step centrifugation, direct (without centrifugation/washing) drying, and centrifugation methods after adding methanol were tested. In centrifugation processes without adding methanol, the targeted precipitation was not achieved due to the stability of the polymer particles in solution. In direct drying processes without centrifugation and washing, products of the desired purity cannot be obtained and the drying process takes a long time. Therefore, among these methods, centrifugation by adding methanol found to be the most effective

method to separate the particles. The PDVB nanoparticle emulsion was mixed with methanol and centrifuged at 5000 rpm for 5 minutes the liquid that accumulated on the top of the collapsed polymer was removed. The remained polymer was centrifuged by the same process three more times. Finally, the obtained polymer particles were dried in an oven at 70 °C overnight and then slowly crushed in a mortar and powdered.

2.3.1.2 PDVB Nanoparticle Synthesis without Removing the Inhibitor

PDVB nanoparticle synthesis was performed without removing the inhibitor to prevent the loss of material that may occur during the removal of the inhibitor from the DVB monomer and to see whether the synthesis can be performed successfully without removing the inhibitor. For this experiment, the procedure schematized in Figure 2.1 was applied with DVB whose inhibitor was not removed. After the synthesis, the washing and drying steps described in the previous section were applied.

2.3.2 Immobilization of PDVB Nanoparticles into a Continuous Nanofibrous PAN Phase by Electrospinning

Different polymer mixtures described below were investigated to immobilize PDVB nanoparticles in a continuous nanofibrous phase and use them for coating the CBS blades.

2.3.2.1 Preliminary Studies for Electrospinning

Several mixtures were prepared and tested to find the best electrospinning polymeric composition in which PDVB particles can be immobilized. The composition of each mixture is given below.

Mixture 1: PDVB-PVP slurry

While preparing this mixture, 0.141 g of PDVB (synthesized by using AIVN initiator) which was obtained by drying directly in an oven at 70°C (SDS not

removed) was mixed with 4.0 mL of water in a 20 mL vial. Ultrasonication was carried out with the help of a sonotrode probe for 1 minute for thoroughly disperse the polymeric nanoparticles and obtain a homogeneous mixture. Then, 1.0 g of polyvinylpyrrolidone (PVP) was added to the mixture as the electrospinning polymer and mixed until the PVP was dissolved completely.

Mixture 2: PDVB-PVP slurry

This mixture was prepared with washed and centrifuged PDVB (SDS not removed). In this procedure, 0.0236 g of PDVB was mixed with 4.0 mL of water in a 20 mL vial and then ultrasonicated for 1 minute with the help of a sonotrode probe to obtain a homogeneous mixture. 1.0 g of PVP as the electrospinning polymer was added to the mixture and mixed until the PVP was dissolved completely.

Mixture 3: PDVB-PVP slurry

In this mixture, the milky PDVB nanoparticle mixture was directly used in liquid form without centrifugation and drying (SDS not removed). Briefly, 4.0 g of nanoparticle mixture was taken into a 20 mL vial, 1.0 g of PVP was added, and the PVP mixed until the PVP was dissolved completely.

Mixture 4: PDVB-PVP Ethanolic slurry

In this solution, ethanol was tried instead of water as a solvent. Firstly, 0.0364 g PDVB, which was centrifuged and dried after mixing with methanol, was added to the 1.0 g PVP mixture dissolved in 4.0 g ethanol and mixed thoroughly with the help of a vortex mixer.

The reason why PDVB particles were used in different amounts in the first four mixtures prepared is that different amounts of PDVB nanoparticles can be isolated in the synthesis at the initial stage and in the applied separation processes. These studies considered as preliminary studies for electrospinning. Based on information

from this preliminary study, two new mixtures containing controlled amounts of PDVB particles were prepared.

Mixture 5: PDVB-PVP Ethanolic Mixture

PDVB nanoparticles (0.20 g) were mixed with 4.0 g PVP solution (containing 20% by mass PVP in ethanol) using the same procedure applied in the preparation of Mixture 4.

Mixture 6: PDVB-PAN Mixture in DMF

PDVB nanoparticles (0.20 g) were mixed with 10.0 g PAN solution (containing 10% by mass PAN in DMF). The slurry was mixed first with the help of a vortex stirrer, and then ultrasonicated for one minute in an ice bath. The aim of using an ice bath in this process was to prevent solvent evaporation. Finally, the mixture was stirred with a magnetic stirrer until a homogeneous PDVB-PAN mixture was obtained.

2.3.2.2 Electrospinning of Polymer Mixtures

The electrospinning set-up used in the study is shown in Figure 2.2. First, the polymer mixture to be electrospinned was filled into a 13.0 mm diameter syringe and the syringe was placed in the infusion pump. Then the feed rate was adjusted to 2.4 mL/h. The alligator tip of the electrospinning device, which provides positive voltage, was connected to the needle tip of the syringe, and the grounding tip was connected to the collector plate. Then, 20.0 kV voltage was applied by taking security measures.

Various parameters, including the distance between the needle tip and collector plate, the feed rate of the polymer to the needle tip via the infusion pump, and the applied voltage, were evaluated to obtain an efficient electrospinning process and electrospun coating with a homogeneous fibrous structure. For this purpose,

voltages between 4.0-20.0 kV, distances between 5-20 cm, and feed rates between 0.1-2.4 mL/h were tried.

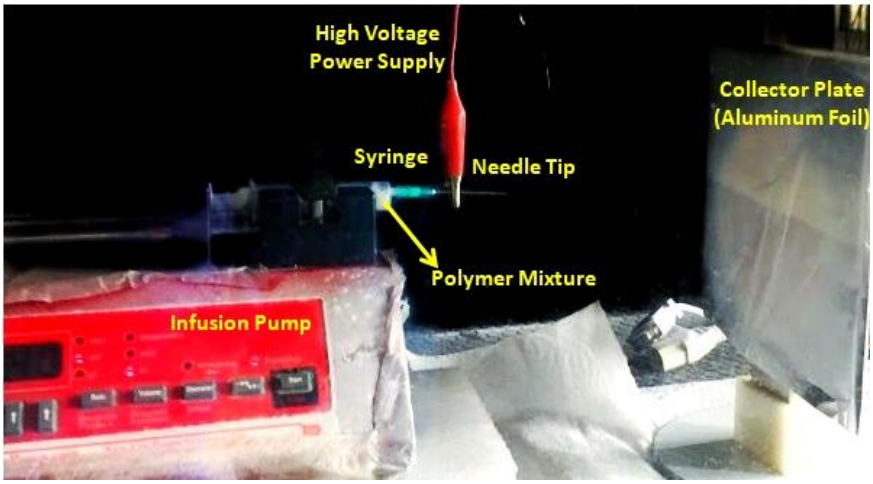


Figure 2.2. Electrospinning set up used in the study

2.3.3 Hydrolysis of PDVB-PAN Electrospun Nanofibers

Since PAN is not very suitable as an extractive phase on its own, PAN was hydrolyzed to introduce more polar functional groups suitable for extraction of polar analytes, and thus to give HLB properties to the produced PAN-PDVB coatings. For this purpose, firstly, 1.0 g of PDVB-PAN electrospun mat was added to 250.0 mL of 2.0 M NaOH solution, and a hydrolysis reaction was carried out in an oil bath at 70 ^{o}C for 1 hour (Figure 2.3). At the end of the reaction period, the polymer mat was neutralized by washing with water and left to dry for 24 hours at 70^{o} C in an oven. Characterization of the hydrolyzed fiber mat was done by SEM and FTIR.

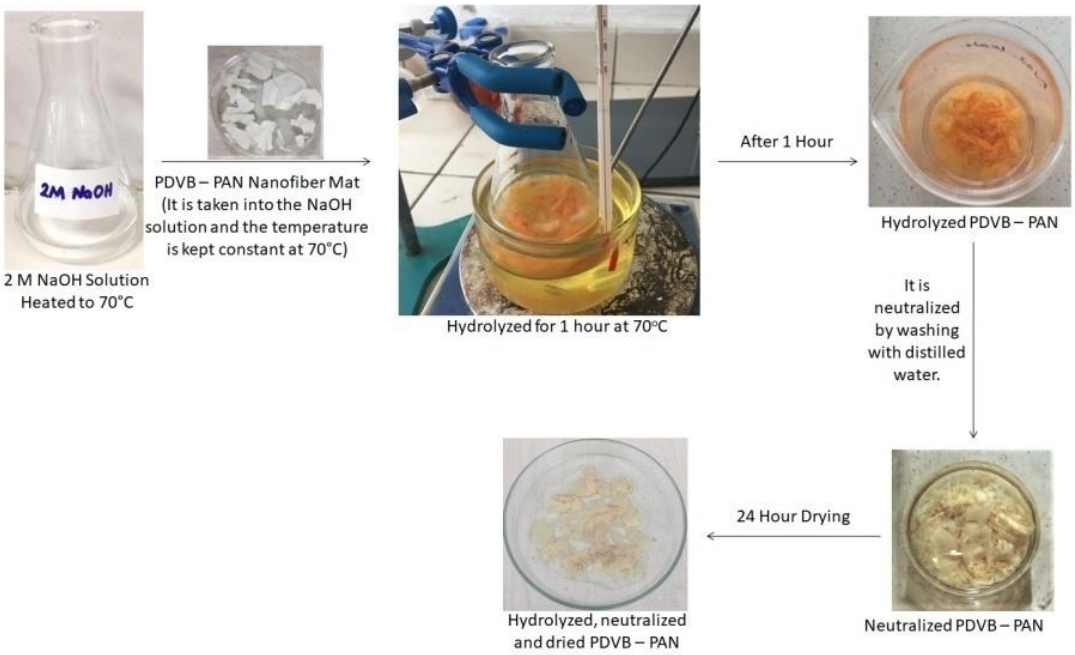


Figure 2.3. Hydrolysis procedure of PDVB-PAN mat

2.3.4 Characterization of Polymeric Nanoparticles and Nanofibers

Scanning electron microscope (SEM) images of the synthesized PDVB nanoparticles, electrospun nanofibers, and hydrolyzed nanofibers were taken. Apart from this, thermal gravimetric analysis (TGA) was performed to test the temperature resistance of the synthesized PDVB, and it was compared with the data in the literature. To see whether the synthesized PDVB nanoparticles and hydrolyzed electrospun PDVB-PAN were successful, the FTIR spectra were examined to determine their functional groups.

2.3.5 Preparation of Coated Blades by Electrospinning Method

Multifarious strategies and experimental setups were tried to find the most suitable coating method to coat the blades with the newly developed extractive phase. In the horizontal electrospinning setting, studies were carried out by fixing the blade on a flat surface, turning the blade upside down for certain periods, coating more than

one blade together, and using a rotating head. Afterward, same studies were carried out in a vertical electrospinning set up.

In this context, a vertical electrospinning setup with a rotating head was determined as the most convenient method, as schematized in Figure 2.4. For this system, the syringe filled with polymer solution was attached to the infusion pump and placed at a certain height, with its needle tip downwards, and the rotary head on which the blade is mounted was placed in a horizontal position across the syringe.

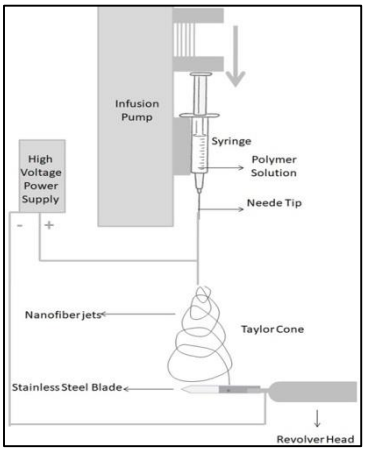


Figure 2.4. Vertical electrospinning set up for blade coatings

After the blades were coated, they were hydrolyzed under the previously mentioned hydrolysis conditions, and thus coatings with HLB properties that could be used in CBS studies were produced.

2.4 Analytical Methods for Instrumental Analysis of Target Analytes

An analytical method has been developed for direct to MS studies, and a suitable LC-MS method has also been developed for preliminary studies to be carried out before this method is generated.

2.4.1 LC-MS Method

LC ionization source conditions: Ionization mode was API-ESI, drying gas temperature was 350^0C, fragmentor voltage was 70 V, drying gas flow was 12 L/min, voltage was 3000 V and nebulizer pressure was 1811 Torr.

Chromatographic method: As a result of various studies, it has been concluded that the reverse phase method was the most suitable method for analyzing target analytes. In this method, Agilent, Poroshell 120 EC-C18, (4.6x150 mm, 4μm) column was used. Analyte separation was performed isocratically using MeOH:H_2O (80:20, v/v) containing 0.1% (v/v) formic acid with a mobile phase flow of 0.5 mL/min. In the analyses, the column temperature was 40.0 °C and the injection volume was 20.0 μL.

The observed adducts and the mass/charge ratios (m/z) used for the detection of the analytes are as follows: M+H adduct could be observed for testosterone and progesterone, and accordingly, the m/z of 289 and 315 were detected in SIM ion mode, respectively; and M+H-H_2O adduct was observed for cholesterol, and accordingly in SIM ion mode, 369 m/z ratio was used. A typical mass spectrum of selected ions is shown in Appendix A in Figure A.1.

2.4.2 CBS-Direct to MS Method

For the CBS-MS method the following parameters were used in positive ionization mode. Fragmentor voltage was 110 V, capillary voltage was 3000 V and external source voltage was 4.8 kV. The temperature of the drying gas used was 300 °C and the flow rate was 3.0 L/min. The nebulizer pressure was set to 725 Torr. Typical chronograms obtained for different analyte concentrations with CBS-MS are shown in Appendix A Figure A.2.

Internal standards added to the extraction solutions were selected as cortisone for testosterone and progesterone and ergosterol for cholesterol. The steroid structure of the molecules, the adducts they form, their m/z ratios, and similar behaviors

were effective in this selection. Another factor is that the molecules in question are not found in human blood plasma. Cortisone is an artificially produced form of the hormone cortisol, which is secreted in the adrenal glands. Ergosterol is a sterol molecule found in the cell membranes of fungi and serves many of the roles that cholesterol does in animal cells.

2.5 Evaluation of Extraction Capabilities of the Coated Blades

Various studies were carried out with coated blades in TFME configuration which were analyzed with LC-MS. The main purpose of evaluating the extractive phases in TFME format was to test the extraction capability and performance of the new extractive phase independently from CBS format which at the beginning was not optimized yet. In these investigations the novel extractive phase was compared to commercial coatings in two contexts, namely, extraction from different matrices, and extraction time profiles.

2.5.1 Comparison of Extraction Performance of the HLB-CBS and PAN-PDVB-CBS in Different Media

In this study, blades which were coated with HLB polymer using dip coating method and the HLB featured homemade PDVB-PAN electrospun coated blades were compared. Three different extraction media which were PBS, PBS-BSA, and PBS-BSA-ACN were used as media for the blades comparison. The main reason for testing different matrices is that in the real plasma sample, the analytes are bound to plasma proteins. To test whether we can perform sufficient extraction in protein – bound medium, extractions were carried out in phosphate-buffered saline (PBS), a medium that mimics biological samples free of binding matrix, in synthetic plasma (PBS-BSA) containing bovine serum albumin (3.5% w/w), which mimics protein-bound medium, and in synthetic plasma with the addition of acetonitrile (PBS-BSA-ACN) to mimic protein precipitation applied sample medium.

The following steps were applied sequentially during the experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and LC-MS analysis of desorption solution. Preconditioning was performed in 1.5 mL MeOH:H_2O (50:50, v:v) for 30 min without stirring. Following the pre-conditioning step, washing was applied using 1.5 mL ultra-pure water for 5 seconds without stirring to remove extra organic solvents from pre-conditioning. Following this step, extraction was performed with the following conditions: 1.5 mL sample volume, 500.0 ng/mL analyte concentrations for testosterone and progesterone, 1000.0 ng/mL analyte concentrations for cholesterol, 1000 rpm stirring rate and 60 min extraction time. After extraction, a second washing was applied using 1.5 mL ultra-pure water for 5 seconds without stirring to remove any loosely attached matrix components. Finally, desorption was performed in isopropyl alcohol with the following conditions: 1.5 mL desorption volume, 1000 rpm stirring speed and 30 min desorption time. Desorption solutions were analyzed with LC-MS method described in Section 2.4.1.

2.5.2 Comparison of Extraction Time Profiles of HLB-CBS and PAN-PDVB-CBS Coatings

In these experiments, the aim was to compare the extraction kinetics of HLB-coated blades prepared with the same procedure as commercial CBS and homemade PDVB-PAN coatings. The following steps were applied sequentially during the experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and LC-MS analysis of desorption solution. Preconditioning was carried out in 1.5 mL MeOH:H_2O (50:50, v:v) without stirring for 30 minutes as in all studies. Following the pre-conditioning step, washing was applied using 1.5 mL ultra-pure water without stirring for 5 seconds. Following this step, extraction was performed from PBS with the following conditions: 1.5 mL sample volume, 500.0 ng/mL analyte concentration for testosterone and progesterone, 1000.0 ng/mL analyte concentration for cholesterol, 1000 rpm stirring rate and extraction times were 5, 15, 30, 60, 120, and

180 minutes. After extraction, a second washing was applied using 1.5 mL ultra-pure water without stirring for 5 seconds. Finally, desorption was performed in isopropyl alcohol with the following conditions: 1.5 mL desorption volume, 1000 rpm stirring speed and 30 min desorption time. Desorption solutions were analyzed with LC-MS method described in Section 2.4.1.

2.6 Optimization of Signal from the Uncoated Blade

To test the device's performance, the analyses were performed to sketch a preliminary CBS-MS calibration. The following steps were applied consecutively during the experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and direct to MS analysis of desorption solution. Preconditioning was carried out in 1.5 mL MeOH:H_2O (50:50, v:v) without stirring for 30 min. Then, washing was applied using 1.5 mL ultra-pure water for 5 sec without stirring. After this step, extraction was performed from PBS-BSA solutions at 4 different concentrations of analyte mixtures (500.0, 1000.0, 2000.0, and 5000.0 ng/mL) with the following conditions: 1.5 mL sample volume, 1000 rpm stirring rate and 60 minutes extraction time. After extraction was completed, a second washing was applied using 1.5 mL ultra-pure water without stirring for 5 seconds. Finally, desorption was performed in isopropyl alcohol containing 0.1% F.A and 1000.0 ng/mL internal standard mixture (cortisone and ergosterol) with the following conditions: 1.5 mL desorption volume, 1000 rpm stirring speed and 30 min desorption time. Desorption solutions were analyzed with direct to MS method described in Section 2.4.2 by dripping onto the uncoated blade which was placed just in front of the MS inlet.

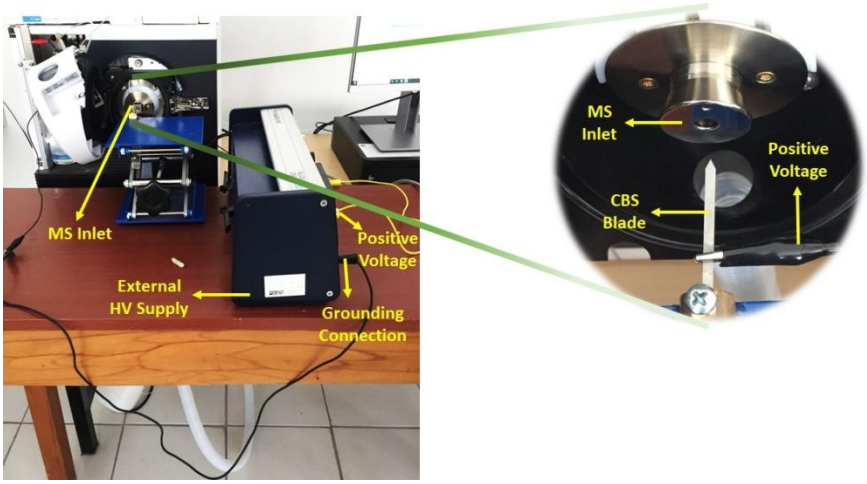


Figure 2.5. Experimental setup for using the CBS blade as an electrospray source with the help of an external voltage source

2.7 CBS Method Development for Direct-to-MS Studies

For CBS-MS studies, firstly, the optimization of sample and desorption solvent volumes was carried out. Then, the optimum desorption time was optimized to release the analytes extracted on the sorbent into the solvent. After that, the extraction times were studied. In this thesis, which aims to develop a rapid clinical diagnostic tool and method, it is of great importance to study the extraction kinetics. In addition, studies for breaking the binding between the analyte and protein were carried out as it may be needed in case of insufficient sensitivity in future studies. Afterwards, studies were carried out to improve the extractive phase and increase its extractive properties of the CBS.

2.7.1 Optimization of Sample and Desorption Solvent Volumes

One of the critical steps that will affect the sensitivity of the final method is the sample volume that can be placed on the CBS probe. Therefore, different amounts of PBS and synthetic plasma (PBS-BSA) were dripped onto the coated blade and kept for particular times. 10.0, 20.0, 30.0, 40.0, 50.0, 100.0 µL volumes of PBS and PBS-BSA were tried. Droplets with a volume greater than 30.0 µL overflowed

from the coating and even it could not stay on the blade and spilled to the ground. Volumes of 10.0, 20.0, and 30.0 μL were able to stand on the blade without overflowing, and the highest volume of 30.0 μL was preferred as the extraction volume.

For desorption studies, desorption solvent, which was isopropyl alcohol with 0.1% formic acid (F.A.), droplets in different amounts, which were 10.0, 15.0, 20.0, 25.0, 30.0 μL were dropped as in the same way with the extraction volume study. Here, on the other hand, solvent volumes above 15.0 μL were not preferred because they overflowed the coating and caused arcing in the device during the voltage application. Between 10.0 and 15.0 μL, 15.0 μL was preferred as desorption volume because it gave better results. After selecting the most suitable desorption volume, it was determined how long it took for the applied amount of solvent to evaporate spontaneously without applying any voltage. 15.0 μL of solvent was placed on the coated blades and waited for 5, 10, 15, 20, 60, 100, 120 seconds, and times longer than 60 seconds were not preferred as desorption time since the desorption solvent dried completely in ambient conditions for longer than 60 seconds. Times shorter than 15 seconds were not preferred in terms of application difficulty. After this preliminary study, desorption times of 15, 20 and 60 seconds were tested with the direct to MS method and the most suitable desorption time was determined.

2.7.2 Optimization of Desorption Time

The following steps were applied sequentially during the experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and direct to MS analysis of desorption solution. Preconditioning was performed in 1.5 mL MeOH:H_2O (50:50, v:v) for 30 min without stirring. Then, washing was applied using 1.5 mL ultra-pure water without stirring for 5 seconds. After this step, extraction was performed from PBS with the following conditions: 1.5 mL sample volume, 1.0 μg/mL analyte concentration for all analytes, 1000 rpm stirring rate and 30 minutes extraction time. After extraction,

washing was applied using 1.5 mL ultra-pure water without stirring for 5 seconds. After that, desorption was carried out with the following conditions: desorption volume was 15.0 μL, as determined in the previous study, and the desorption solvent was isopropyl alcohol with 0.1% formic acid. For desorption time, 15, 20, and 60 seconds were tried. After the desorption solvent was dropped onto the blade, waited for 15, 20 and 60 seconds for desorption to desorb the analytes, and then 4.8 kV voltage was applied to create an electrospray, and the extracted analytes were ionized and reached to the device. The desorption solutions were analyzed with direct to MS method described in Section 2.4.2. An important point here is that although the solvent did not completely dry in 15 seconds and 20 seconds, it was observed that the solvent was completely dry after 60 seconds of waiting, and for this reason, after 60 seconds of drying, another 15.0 μL of solvent was dropped, and then the voltage was applied.

2.7.3 Extraction Time Profiles

To understand the extraction kinetics of the newly developed coated blades, extraction time profile graphs were drawn first at long extraction times and then at shorter extraction times. At the same time, extraction kinetics for different PDVB-PAN ratios were studied and compared. In addition, to compare the coatings made with electrospinning with the coatings made by the more commonly used techniques, dip coating method, extraction kinetics were studied with both type of blades.

2.7.3.1 Extraction Time Profile Studies for Long Extraction Times by Static Extraction With Droplet Approach

The following steps were applied sequentially during the experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and direct to MS analysis of desorption solution. Preconditioning was performed in 1.5 mL MeOH:H_2O (50:50, v:v) for 30 min without stirring. Following the pre-conditioning step, washing was applied using

1.5 mL ultra-pure water for 5 seconds without stirring to remove extra organic solvents from pre-conditioning. After that, extraction was performed from PBS with the following conditions: 30.0 µL sample volume, 1.0 µg/mL analyte concentration for each analyte, static extraction and 1, 5, 15, 30, 60 minutes extraction times. After extraction, a second washing was applied using 1.5 mL ultra-pure water for 5 seconds without stirring. Finally, desorption was carried out with the following conditions: desorption volume was 15.0 µL, desorption solvent was isopropyl alcohol, and 60 sec desorption time (solvent dried and additional 15.0 µL solvent droplet was placed). Then, for the electrospray ionization, 4.8 kV external voltage was applied, and successful signals were obtained. The direct to MS analysis was performed by the method described in Section 2.4.2. The experimental steps are simply schematized in Figure 2.6. Same procedure was also applied by using synthetic plasma (PBS-BSA) samples.

Figure 2.6. Experimental setup of CBS – direct to MS with droplet approach

2.7.3.2 Extraction Kinetics Studies Using Short Extraction Times with Coatings Obtained with Different PDVB-PAN Ratios

Firstly, the blades were coated with polymer mixtures in which PDVB was added into PAN at 1%, 2%, and 3% by mass. Then, the following steps were applied consecutively during the extraction experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and direct to MS analysis of desorption solution. Preconditioning was performed in 1.5 mL MeOH:H_2O (50:50, v:v) for 30 min without stirring. After that, washing by using 1.5 mL ultra-pure water for 5 sec without any stirring. Then, extraction was performed with the following conditions from PBS containing 1.0 µg/mL internal

standard mixture (cortisone and ergosterol): extraction time concentration was 1.0 µg/mL analyte mixture, stirring rate was 1000 rpm, and extraction times were 30, 60, 120, and 300 sec. After extraction, a second washing was applied using 1.5 mL ultra-pure water for 5 sec without stirring. Finally, desorption was performed in isopropyl alcohol (0.1% F.A.) with the following conditions: 15.0 µL desorption volume direct to MS desorption, and 60 sec desorption time. The direct to MS analysis was done by using the method described in Section 2.4.2.

2.7.3.3 Extraction Kinetics Studies Using Short Extraction Times with Coatings Obtained by Electrospinning and Dip Coating

Since the PDVB-PAN polymer mixture containing 3% w/w PDVB gave the best results, this mixture was preferred in this study. The blades were coated with 3% PDVB-PAN using both electrospinning and dip coating methods. Then, the following steps were applied consecutively during the experiment: pre-conditioning of extractive phase, washing 1, extraction of analytes, washing 2, desorption of analytes, and direct to MS analysis of desorption solution. Preconditioning was performed in 1.5 mL MeOH:H_2O (50:50, v:v) for 30 min without stirring. Then, washing by using 1.5 mL ultra-pure water for 5 sec without any stirring. Next, extraction was performed with the following conditions from PBS containing 1.0 µg/mL internal standard mixture (cortisone and ergosterol): extraction time concentration was 1.0 µg/mL analyte mixture, stirring rate was 1000 rpm, and extraction times were 30, 60, 120, and 300 sec. After extraction, a second washing was applied using 1.5 mL ultra-pure water for 5 seconds without stirring. Finally, desorption was performed in isopropyl alcohol (0.1% F.A) with the following conditions: 15.0 µL desorption volume direct to MS desorption, and 60 sec desorption time. The direct to MS analysis was done by using the method described in Section 2.4.2.

2.7.4 Breaking of Protein Binding by Protein Precipitation Studies

By looking at the previous studies, it was concluded that in cases where sufficient sensitivity could not be achieved, it might be necessary to break protein binding in order to obtain results with better sensitivity. Acetonitrile is an organic solvent known as a protein denaturation agent [104]. Therefore, it was used to break the protein binding and extractions were conducted by adding different volumes of acetonitrile to the synthetic plasma containing the analytes (at 1.0 μg/mL concentration level). Synthetic plasma:ACN ratios (v:v) were tested as 5:1, 2:1, 1:2 and 1:1. After protein precipitation each sample was centrifuged and supernatant was used in the extraction step. The PDVB-PAN coated blades were preconditioned in 1.5 mL MeOH:H_2O (50:50, v:v) without stirring for 30 min. Following, quick washing to remove organic phase was performed using 1.5 mL ultra-pure water for 5 sec without stirring, and then, 30.0 μL protein precipitated synthetic plasma was transferred to the blade and extraction was performed under static conditions for 5 minutes. 1.0 μg/mL internal standard mixture (cortisone and ergosterol) was also added to the extraction solutions. Salts from the matrix were removed with the second wash using 1.5 mL ultra-pure water for 5 sec without stirring. Then the desorption was performed by using isopropyl alcohol (0.1% F.A.) with the following conditions: 15.0 μL solvent volume, direct to MS desorption, and 60 sec desorption time. The direct to MS analysis was done by using the method described in Section 2.4.2.

2.7.5 Further Improvement of the Extractive Phase

To increase the extraction capabilities of the extractive phase and to obtain results with higher sensitivity, firstly, it was tried to increase the amount of PDVB in the extractive phase, and then, in the light of the results obtained, the extraction kinetics of the new extractive phase were studied.

2.7.5.1 Characterization of Coatings Obtained with Increased PDVB Amounts by Scanning Electron Microscope

In the previous study, the extraction kinetics of PDVB-PAN mixtures with 1%, 2%, and 3% PDVB ratios by mass were compared and a significant increase in the obtained signal was observed as the PDVB ratio increased. For this purpose, PAN polymeric mixtures containing PDVB at ratios of 1%, 2%, 3%, 5%, 7%, and 10% by mass were prepared and coated on aluminum foil by electrospinning method. SEM images of the coatings were taken to see whether there was fiber formation in the obtained coatings and whether the PDVB particles were embedded in these fibers.

2.7.5.2 Extraction Kinetics Studies with Extractive Phases Obtained with Increased Amount of PDVB

For this experiment, firstly, CBS blades were coated by using both electrospinning and dip coating methods using PDVB-PAN mixture containing 10% PDVB. Care was taken to ensure that the coating thicknesses obtained in both techniques were equal. Both types of blades were preconditioned in 1.5 mL MeOH:H_2O (50:50, v:v) without stirring for 30 min. Then, the blades were washed quickly using 1.5 mL ultra-pure water for 5 sec without stirring. Following, extractions were performed from 1.5 mL PBS containing 1.0 µg/mL of analyte mixture (testosterone, progesterone and cholesterol) and 1.0 µg/mL internal standard mixture (cortisone and ergosterol) with continuous stirring at 1000 rpm. The tested extraction times were 30, 60, 120, and 300 seconds. After that, second washing was performed using 1.5 mL ultra-pure water for 5 sec without stirring, and then analytes were desorbed in 15.0 µL isopropyl alcohol (0.1% F.A.) for 60 sec with direct to MS technique. Finally, direct to MS analysis was performed by the method given in Section 2.4.2. According to the obtained signals, an extraction kinetics graph was drawn.

Then the same experiment was carried out in PBS-BSA. After this experiment, the same experiments were repeated in both PBS and PBS-BSA with a droplet approach using a 30.0 µL extraction volume with static extraction.

2.8 Validation Studies

For method validation studies, linearity, the lower limit of quantitation (LOQ), reproducibility, and accuracy studies were accomplished according to the bioanalytical method development and validation criteria appointed in the 'Bioanalytical Method Validation Guidance for Industry' [105] published by the FDA. Since the expected concentration of cholesterol in human blood plasma is above the linear range of the developed method, samples must be diluted before the extraction. But this is not the case for testosterone and progesterone. Therefore, cholesterol and the selected hormones were validated separately.

2.8.1 Cholesterol Method Validation

Linearity and lower limit of quantitation (LOQ):

As the first step of validation, the LOQ and linear range in CBS-MS were determined by matrix-matched CBS-MS. To determine the LOQ, firstly, the concentrations of the calibration points were back calculated by using the linear regression equation obtained in the calibration, and then the relative error in concentration was determined by comparing the found value with the nominal value. The lowest concentration with a maximum relative error of 20% (%RE) in back calculations was determined as the LOQ value for the method.

Carry-over test:

To test whether there was a carry-over from the previous analysis in the instrument or not, the blank analysis was executed by dropping 15.0 µL of isopropyl alcohol on the clean CBS blade before and after analyzing 5000.0 ng/mL analyte, which was the highest point of the calibration graph.

Determination of repeatability and accuracy:

Intra-day (N=3) and inter-day (N=3) reproducibility was evaluated following the determination of linear range and LOQ of the method. For this purpose, three quality control concentrations (300.0, 600.0, and 2000.0 ng/mL), which were in the linear range, were selected, spiked in the sample, and analyzed with the optimized method.

Comparison studies with reference method in real sample:

For these experiments, human blood plasma was purchased (pooled human plasma), and the developed method was tested in this sample. The results obtained were compared with LC-MS analysis after protein precipitation (PP), which is widely used in biological sample analysis. For PP study, protein precipitation was performed by adding ACN at a ratio of 3:1 to the plasma sample. After the addition of ACN, the plasma, which was kept in an ice bath for 30 minutes, was centrifuged, and the remaining liquid part was transferred to a separate vial and diluted 20000 times to ensure that the sample concentration is in the linear range of the CBS-MS method. Then, the amount of cholesterol in it was determined by LC-MS analysis. For developed CBS-MS method, due to the high concentration of cholesterol, the plasma sample was diluted 20000 times before analysis and the developed method was applied with 30.0 μL of diluted plasma. Since no cholesterol-free plasma could be found, the amount of cholesterol in the sample was determined by standard addition method.

2.8.2 Progesterone and testosterone

Linearity and lower limit of quantitation (LOQ):

As the first step of validation for testosterone and progesterone, the LOQ and linear range in CBS-MS were determined by matrix-matched CBS-MS. First of all, static extraction was performed on a CBS blade with 30.0 μL sample and SPME calibration was sketched after CBS-MS. In this calibration, the LOQ values for

testosterone and progesterone were found to be 25.0 ng/mL. Since even lower concentrations could be present in real samples, the final method was modified to have a larger volume of sample (200 µL with direct immersion of the probe and stirring). To determine the LOQ, firstly, the concentrations of the calibration points were back calculated by using the linear regression equation obtained in the calibration, and then the relative error in concentration was determined by comparing the found value with the nominal value. The lowest concentration with a maximum relative error of 20% (%RE) in back calculations was determined as the LOQ value for the method.

Carry-over test:

To test whether there was a carry-over from the previous analysis in the instrument or not, the blank analysis was executed by dropping 15.0 µL of isopropyl alcohol on the clean CBS blade before and after analyzing 500.0 ng/mL, which is the highest point of the calibration graphs.

Determination of repeatability and accuracy values:

Intra-day (N=3) and inter-day (N=3) reproducibility was evaluated following the determination of linear range and LOQ of the method. For this purpose, three quality control concentrations (15.0, 75.0 and 300.0 ng/mL), which were in the linear range, were selected, spiked in the sample, and analyzed with the optimized method.

Comparison studies with reference method in real sample:

The developed method was tested in the purchased pooled human plasma. The results obtained were compared with LC-MS analysis after protein precipitation (PP), which is widely used in biological sample analysis. The protein precipitation was performed by adding ACN (300.0 µL) at a 1:3 volume ratio to 100.0 µL plasma sample. After the addition of ACN, the sample was kept in an ice bath for 30 minutes, and then centrifuged. The remaining liquid part was transferred to a

separate vial and injected directly in the LC-MS method. The progesterone and testosterone concentrations were determined from the calibration graphs sketched against the calibration solutions prepared in the same solvent system. Due to the very low concentrations of progesterone and testosterone, the developed CBS-MS method was applied by using the plasma sample without any dilution (200.0 μL). In the CBS-MS method, progesterone and testosterone concentrations were determined by standard addition method.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Preparation of Novel Hydrophilic Lipophilic Balanced Coated Blade Spray (CBS) Probes

For the preparation of new HLB-featured coated blade spray (CBS) probes, firstly, PDVB nanoparticles were synthesized, and the synthesized particles were characterized by scanning electron microscopy (SEM), thermal gravimetric analysis (TGA) and Fourier transform infrared spectroscopy (FTIR). According to the results of these characterization studies, the most suitable synthesis conditions were found, and a successful synthesis was proven. Then, the synthesized PDVB nanoparticles were immobilized into polar polymers by electrospinning, and the most suitable polar continuous phase was determined. The results of these experiments are given below.

3.1.1 Characterization of Synthesized PDVB Nanoparticles

3.1.1.1 Scanning Electron Microscope (SEM)

As a result of the polymerizations, milky white nanoparticle mixtures were obtained in all three polymerization techniques. However, it was observed that the polymer mixture obtained with the AIVN initiator became viscous over time.

After they were turned into powder by drying and grinding, scanning electron microscope (SEM) images of polymeric nanoparticles obtained by each three methods were taken. SEM images of PDVB nanoparticle obtained with different conditions are given in Figures 3.1, 3.2 and 3.3. Based on the SEM results, all three syntheses were successful, and spherical nanoparticles were formed. In addition, it was found that the obtained particles have an average diameter of 40 – 50 nm as intended.

When the reaction initiators were compared, it was concluded that both AIVN and AIBN could be used and results in similar particle size and morphology. However, when the SEM images, shown in Figure 3.1, were examined in detail, it was seen that the nanoparticles obtained from the synthesis with AIVN mostly came together and formed a layer.

In addition, the nanoparticles synthesized without removing the inhibitor were also successfully formed, however, at some points, they were stuck to each other (Figure 3.3).

When all these results were taken into consideration, it was concluded that more homogeneous particles with more desired physical properties were obtained in polymer synthesis performed by using AIBN initiator and removing inhibitor (Figure 3.2). In this case, it was deemed more appropriate to perform polymer syntheses with these parameters throughout the thesis studies.

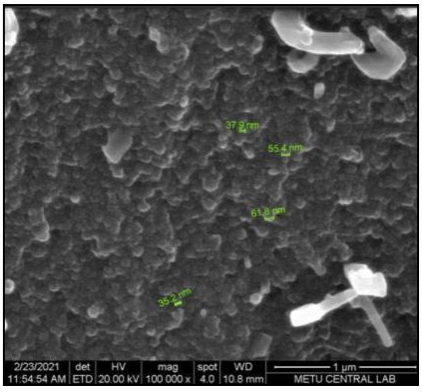


Figure 3.1. PDVB nanoparticles synthesized by removing the inhibitor. The reaction initiator used is AIVN.

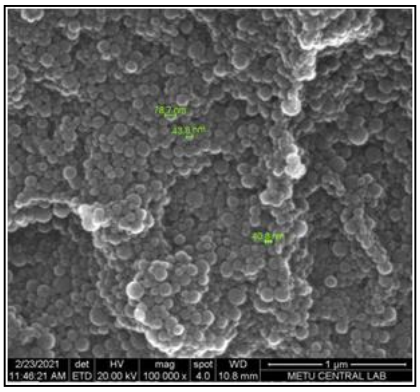


Figure 3.2. PDVB nanoparticles synthesized by removing the inhibitor. The reaction initiator used is AIBN.

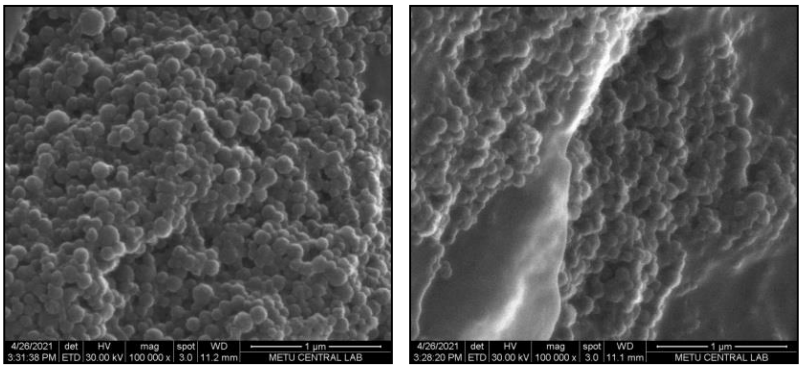


Figure 3.3. PDVB nanoparticles synthesized without removing the inhibitor. The reaction initiator used is AIBN.

3.1.1.2 Thermal Gravimetric Analysis (TGA)

The TGA result obtained for PDVB nanoparticles, synthesized using using AIBN and removing the inhibitor is shown in Figure 3.4. In this TGA characterization study, it was concluded that the approximately 2% mass loss observed at around 112 °C is due to tightly bound adsorbed water. It is known that PDVB undergoes thermal degradation at about 300 ˚C [106]. Similar to literature, the result of this study, indicates a significant amount of mass loss (more than 5%) that occurs after 295 ˚C, showing that the polymer begins to decompose after this temperature. It is

thought that approximately 4% mass loss between 120– 240 ˚C is caused by various reaction wastes (reactive, short-chain oligomer structures).

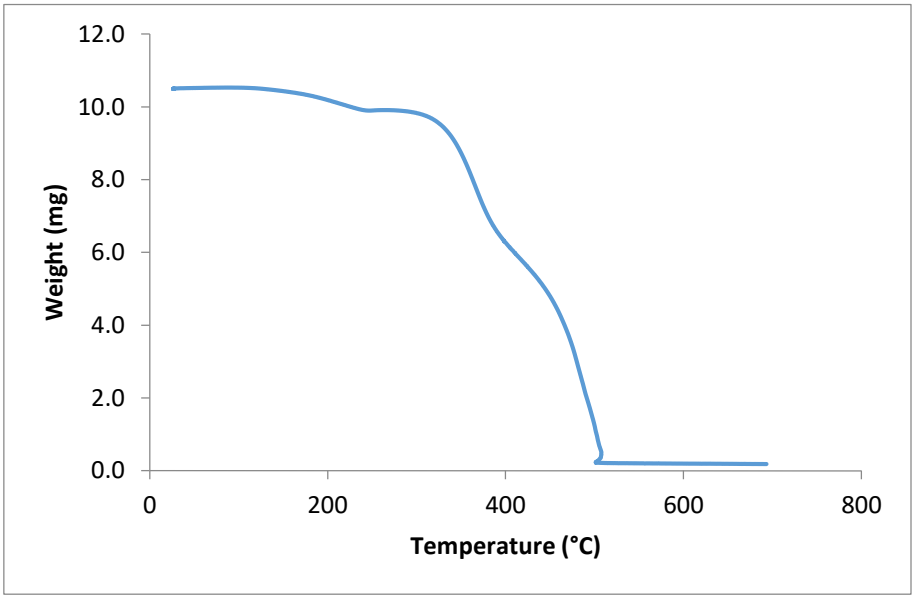


Figure 3.4. TGA curve of PDVB nanoparticles

3.1.1.3 Fourier Transform Infrared Spectrometer (FTIR)

The obtained FTIR spectrum of the synthesized PDVB nanoparticles is shown in Figure 3.5. It was reported by Sobiesiak et al. (2019) that the absorption bands in the range of 1400 – 1650 cm^{-1} are characteristic signals for the aromatic system [107]. These signals can be the signals of aromatic hydrocarbon regions between 1500-1400 cm^{-1} and 1600-1585 cm^{-1} due to C-C stretching vibrations. In addition, the peak at 2923 $cm^{-1}$is considered to be the C – H stretching peak. When the obtained FTIR spectrum was examined, it was concluded that the absorption bands were comparable with the literature, and PDVB nanoparticles were successfully synthesized.

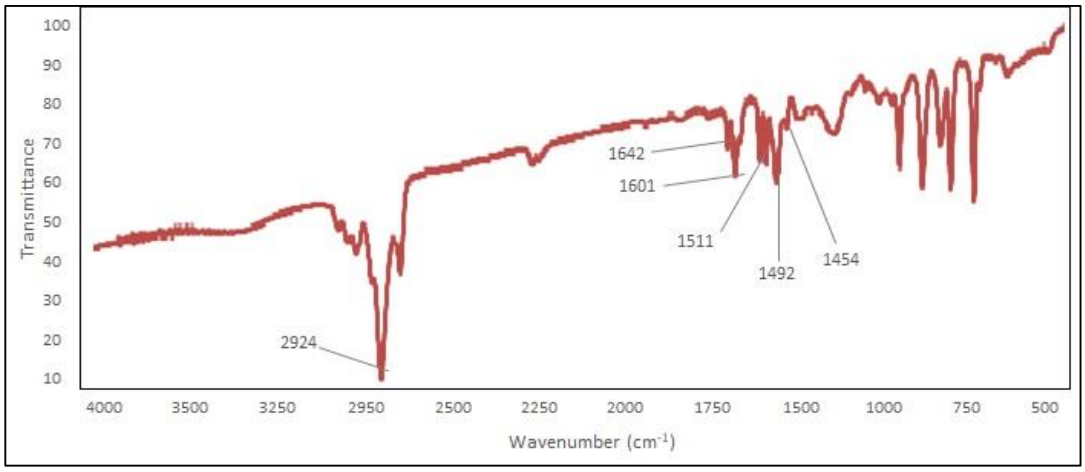


Figure 3.5. FTIR spectrum of PDVB nanoparticles

3.1.2 Immobilization of PDVB Nanoparticles into a Continuous Nanofibrous PAN Phase by Electrospinning

In studies of immobilizing PDVB nanoparticles into a continuous phase, polyvinyl pyrrolidone (PVP) and polyacrylonitrile (PAN) were tried as polar polymers. The compositions of these mixtures were given in Section 2.3.2.1. The results are given in the next sections.

3.1.2.1 SEM characterizations of prepared polymer mixtures

As shown in Figures 3.6, 3.7, and 3.8, although coating was obtained in Mixtures 1 and 3, no fiber structure was found. The SEM image of Mixture 2, which was prepared as PDVB-PVP slurry is unavailable, because although various parameters were tried, any coating on the collector could not be obtained. Even though Mixture 4 showed nodes, it formed a fiber structure. The most crucial difference between these four solutions is that Mixtures 1, 2, and 3 contain water, whereas Mixture 4 is prepared in ethanol. These results show that ethanol as a solvent would be more suitable for the immobilization of PDVB (lipophilic) particles in PVP (hydrophilic). In consideration of the information obtained from this preliminary study, two new mixtures containing controlled PDVB were prepared.

One of them was Mixture 5, which is shown in Figure 3.9, and the other one was Mixture 6, which is shown in Figure 3.10.

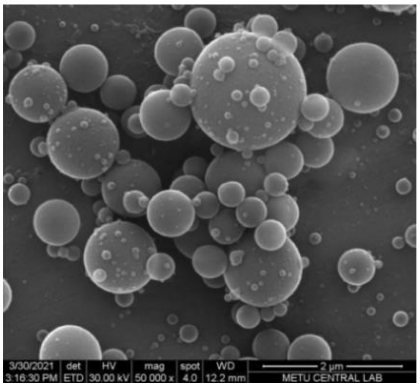


Figure 3.6. SEM image of PDVB-PVP slurry (Mixture 1) after electrospinning. The electrospinning conditions: 15.0 cm distance between needle tip and collector, 0.3 mL/h feed rate, 20.0 kV applied voltage.

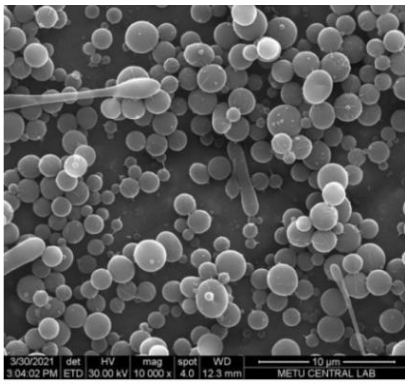


Figure 3.7. SEM image of PDVB-PVP slurry (Mixture 3) after electrospinning. The electrospinning conditions: 15.0 cm distance between needle tip and collector, 0.3 mL/h feed rate, 28.0 kV applied voltage.

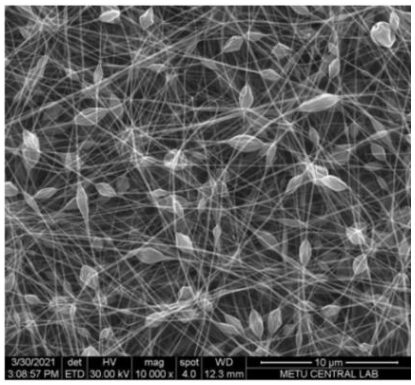


Figure 3.8. SEM image of PDVB-PVP ethanolic mixture (Mixture 4) after electrospinning. The electrospinning conditions: 15.0 cm distance between needle tip and collector, 0.3 mL/h feed rate, 15.0 kV applied voltage.

SEM images shown in Figure 3.9 confirm the formation of nanofibers and the immobilization of PDVB nanoparticles inside the fibers but also indicate the presence of large globules. While looking at the Mixture 6, a homogeneous coating could be obtained on aluminum foil. Looking at the SEM images, it was observed that the expected fiber structures could be obtained, and the PDVB nanoparticles were immobilized into these fibers.

When the images obtained by SEM are examined, it is clear that the mixture prepared with PAN (Mixture 6) provides the expected fiber structures better, and also retains the PDVB particles better in the fiber structure. Although PAN is not suitable for extraction of polar analytes directly, it can be made to have ion exchange groups by hydrolysis. Due to these properties, it stands out as a suitable polymer mixture.

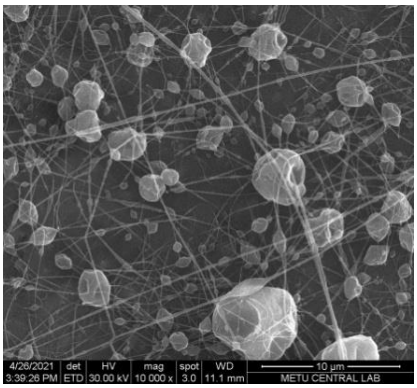


Figure 3.9. SEM image of PDVB-PVP ethanolic mixture (Mixture 5) after electrospinning. The electrospinning conditions: 15.0 cm distance between needle tip and collector, 2.4 mL/h feed rate, 20.0 kV applied voltage.

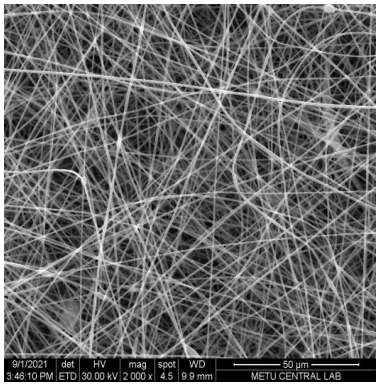


Figure 3.10. SEM image of PDVB-PAN mixture in DMF (Mixture 6) after electrospinning. The electrospinning conditions: 15.0 cm distance between needle tip and collector, 2.4 mL/h feed rate, 20.0 kV applied voltage.

3.1.2.2 Characterization of Hydrolyzed PDVB-PAN

3.1.2.2.1 Scanning Electron Microscope (SEM)

Electrospun PAN was hydrolyzed with 2.0 M NaOH at 70 ^{o}C as described in Section 2.3.3. When the SEM images for non – hydrolyzed PDVB-PAN (Figure 3.11) are examined, it was concluded that PAN presents the desired nanofiber structures and that the PDVB nanoparticles are immobilized into these continuous fiber structures. When the hydrolyzed PDVB-PAN SEM images (Figure 3.12) were

examined, it was concluded that although some changes were present in the fiber structures, the general morphology was preserved, and the PDVB nanoparticles were still embedded in the fibers. In this case, it can be said that the SEM images prove that the experiment was fruitful by the purpose.

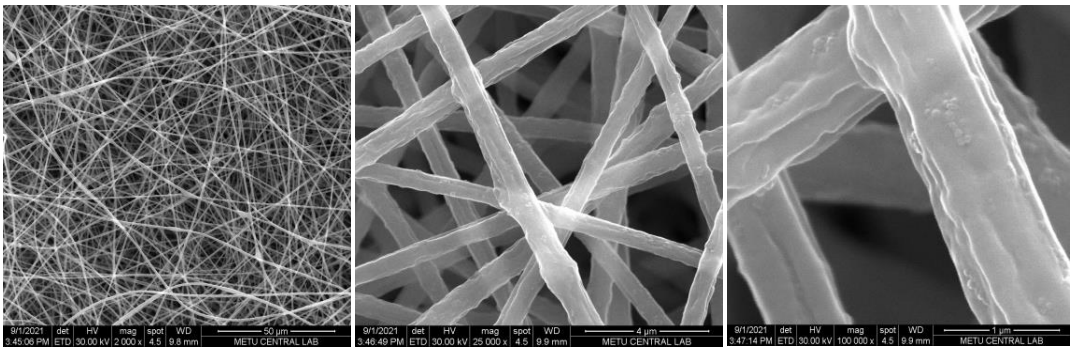


Figure 3.11. SEM images of non – hydrolyzed PDVB-PAN. Optimized electrospinning conditions: 15.0 cm distance between syringe needle tip and collector plate, 2.4 mL/h feed rate of the polymer solution to the needle tip, 20.0 kV applied voltage.

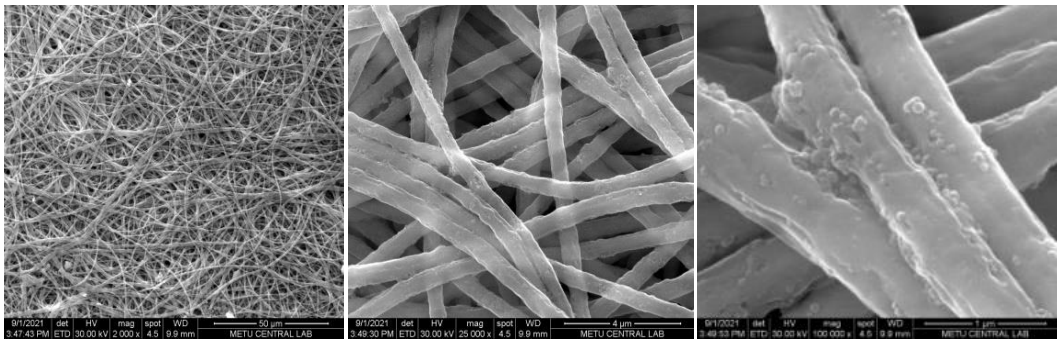


Figure 3.12. SEM images of hydrolyzed PDVB-PAN. Hydrolysis conditions: 2.0 M NaOH, 70.0 ^{0}C, 1 hour.

3.1.2.2.2 Fourier Transform Infrared Spectrophotometer (FTIR)

FTIR spectra of non – hydrolyzed PDVB-PAN, and hydrolyzed PDVB-PAN are given in Figure 3.13. When the obtained FTIR spectra are examined, the first of the two most prominent peaks for PDVB-PAN are the nitrile group (CN) stretching

peak at 2244 cm^{-1}, and the other is the C – H bending peak at 1454 cm^{-1} [108]. The characteristic bands for the carboxylate in the post – hydrolysis FTIR spectrum are in the range of 1510 – 1650 cm^{-1} for asymmetric vibration and 1280 – 1400 cm^{-1} for symmetric vibration. C=O stretching for amide groups and carboxylic acid groups peaks in the range of 1680 – 1720 cm^{-1}, and O – H bending for carboxylic acid groups peaks in the range of 1395 – 1440 cm^{-1}. In this case, the FTIR results show that some of the nitrile groups of PAN were converted to amide and some to carboxylic acid groups. According to the results, it can be accepted that the hydrolysis reaction was successful.

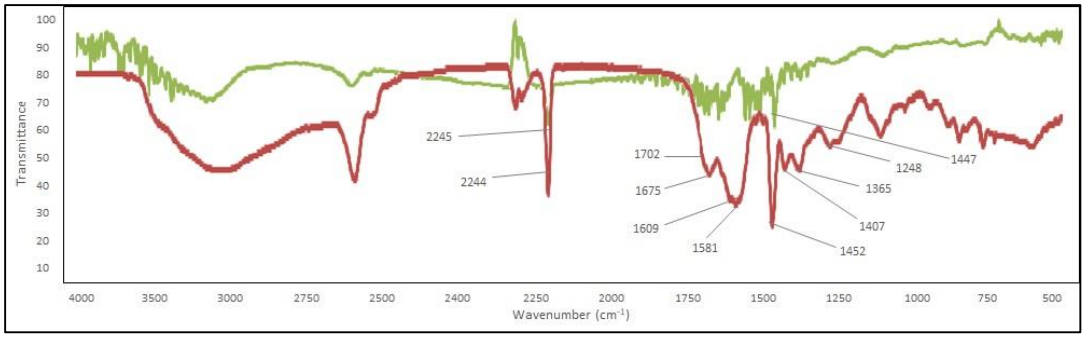


Figure 3.13. FTIR spectrum of PDVB-PAN mat before (Green) and after (Red) hydrolysis

3.1.2.3 Optimization of Blade Coatings by Electrospinning Method

As mentioned in Section 2.3.5, various experimental setups have been tried to coat the blades by electrospinning. Among the experimental setups tried, the most homogeneous, blade-focused, and successful coatings were obtained with the setup using a vertical setup and a rotating head. The vertical electrospinning system used in the study is shown in Figure 3.14.

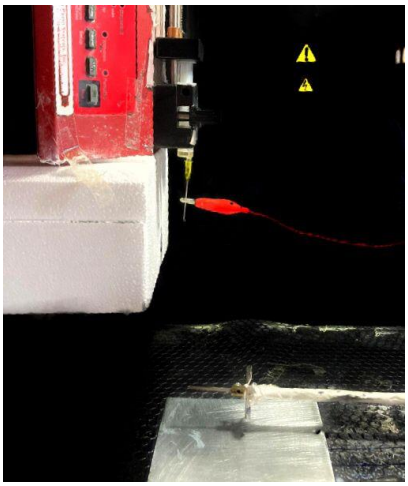


Figure 3.14. Vertical electrospinning set up for blade coatings

The main reason why more successful results in the vertical system could be achieved was that the blades were coated in the same direction as gravity. In coatings made in the horizontal arrangement, some amount of polymer mixture was coated on the blade by forming nanofiber jets, while some of the polymer mixtures with the effect of gravity hit the ground before they could reach the blade. In addition, the nanofiber jets caused the material loss by coating many areas around it instead of focusing only on the little blade at a certain distance away. When the vertical system was used, more homogeneous coatings could be obtained, only focused on the blade, at a much lower feed rate, in a shorter time. Thus, both time and material can be saved, as well as successful coatings could be done with the desired homogeneity. Another advantage of this system was that the blades were coated by rotating at a constant revolution speed. As a result, coatings made in the vertical system by rotating at a constant speed have been obtained in a very short time with equal and homogeneous coatings on both sides of the blades, and since the material loss is avoided during this process, it made this method more environmentally friendly and economical. It was also observed that the obtained coatings were not deformed under hydrolysis conditions, and their mechanical stability was quite high. An example of the produced coated blades is shown in Figure 3.15.

Figure 3.15. Example of prepared hydrolyzed PDVB-PAN coated blades

3.2 Evaluation of Extraction Capabilities of the Coated Blades

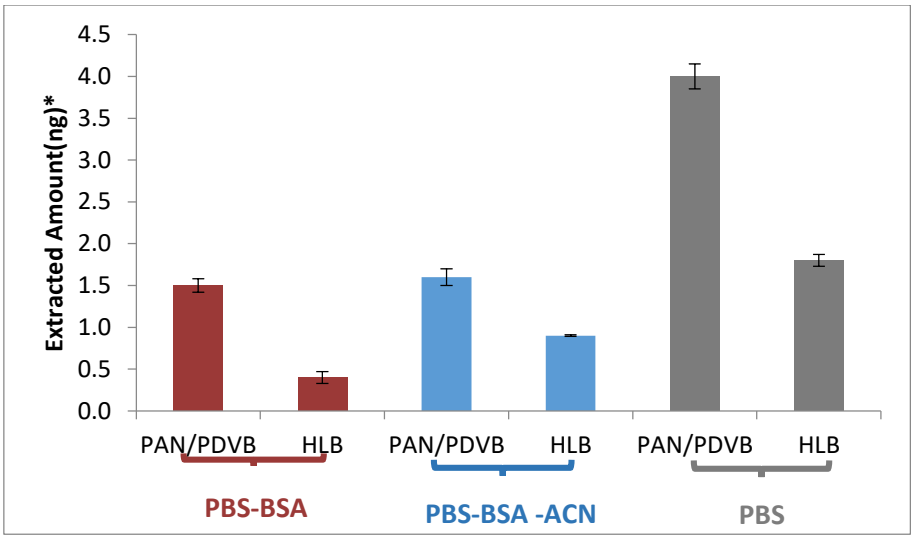
Before developing the CBS-direct to MS method, the extraction capabilities of the newly developed extractive phases were tested with the LC-MS method. The LC-MS method mentioned in Section 2.4.1 was used in these experiments.

3.2.1 Comparison of Extraction Performance of the HLB-CBS and PDVB-PAN-CBS in Different Media

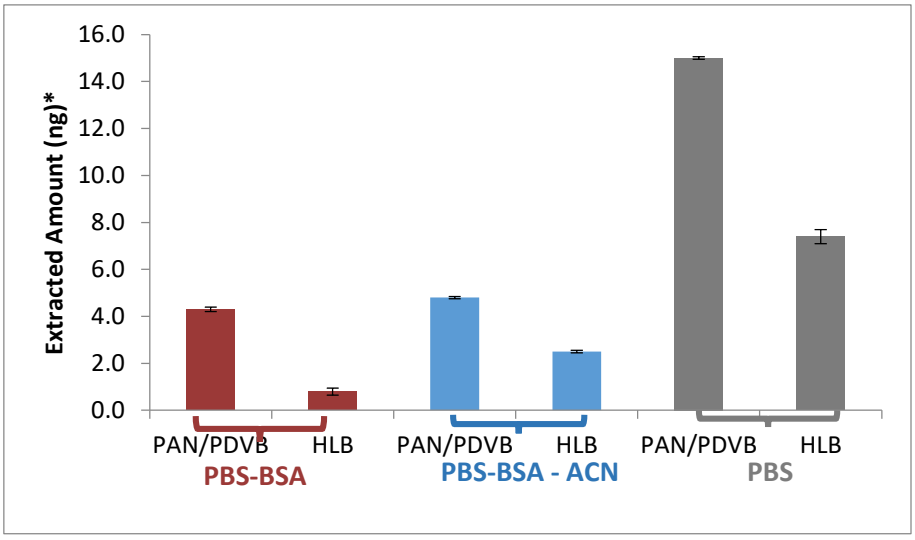
In this study, the newly developed HLB featured PDVB-PAN extractive phase was compared with the extractive phase obtained with commercial HLB. For this purpose, HLB-CBS was prepared using the dip coating method, as in commercial HLB-CBS. PDVB-PAN-CBS was prepared by electrospinning method as described in Section 2.3.2.2. The extractive properties of these two types of coatings were compared in different matrix environments. Experiments for comparison conducted by using PBS, PBS-BSA (synthetic plasma), and PBS-BSA-ACN as matrix, with the experimental conditions given in Section 2.5.1. The results obtained after the experiment are shown in Figure 3.16. It was observed that the newly developed HLB-featured PDVB-PAN coatings for all three analytes gave successful results compared to the coatings obtained with commercial HLB. It

should be noted here that the coating thicknesses obtained by dip coating and electrospinning differ. For this reason, the obtained extraction results were divided by the coating thicknesses and the coating thickness (hence the volume) was normalized and these results were compared. The results show the advantage of the newly developed extractive phase with HLB properties over commercially available HLB.

The data highlighted in grey in Figure 3.16 show the results of extractions performed in PBS. Although PBS is not the most suitable matrix for this study, it represents the binding free matrix and gives information about the sensitivity that can be obtained in the absence of the proteins. As expected, the highest extraction amounts were obtained in PBS among the tested matrices, since analytes were not bound to proteins in this matrix. The data shown in red are the results of extractions made in synthetic plasma (PBS–BSA), that is, the matrix where the analytes show protein binding. It should be noted here that SPME-based methods extract only the free forms of the analytes. The high protein binding of the analytes decreases the free analyte concentration and decreases the amount of analyte that can be extracted with the CBS probes. This explains why lower extraction was obtained in synthetic plasma compared to PBS. The results here support that breaking the protein-analyte bond for better sensitivity may also have benefits in real samples. For this reason, organic solvent that will reduce or break the protein-analyte interaction was added to the synthetic plasma sample and its extraction ability in this matrix was also investigated. The data shown in blue show the results of extraction from medium, to which acetonitrile has been added (10% of the sample volume). The fact that the amount of acetonitrile added gives very similar results to the PBS-BSA medium is due to either the insufficient amount of solvent added, or the added solvent modifies the matrix and increases the affinity of the analytes to this matrix, making extraction difficult. Therefore, it was concluded that the amount of organic solvent would need to be optimized in case of addition.



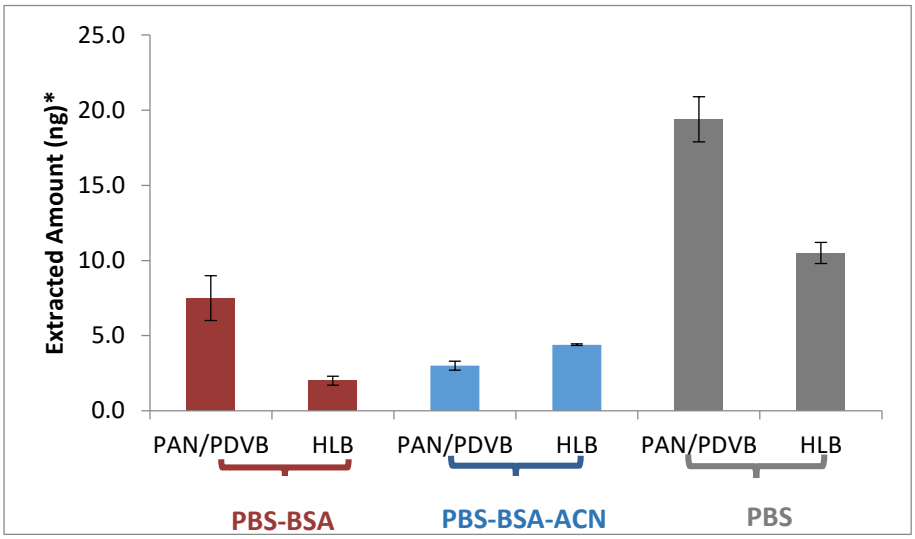
(a)



(b)

Figure 3.16. Extraction performances of dip-coated HLB and electrospun PDVB-PAN blades for a) progesterone, b) testosterone, c) cholesterol in different media (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, analyte concentrations are 500.0 ng/mL for testosterone and progesterone, 1000.0 ng/mL for cholesterol, extraction time is 60 min, desorption solvent is IPA,

desorption volume is 1.5 mL, desorption time is 30 min) *Values normalized to thickness.



(c)

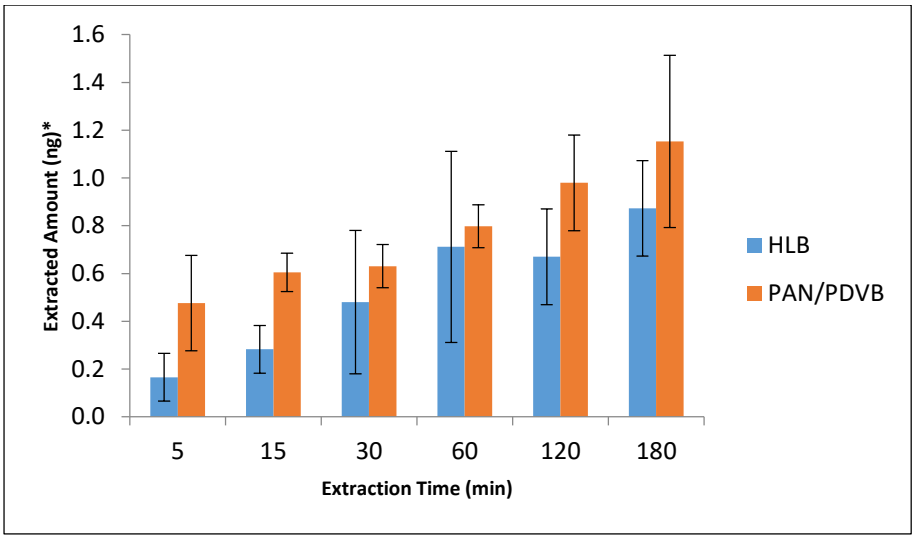
Figure 3.16. (Continued) Extraction performances of dip-coated HLB and electrospun PDVB-PAN blades for a) progesterone, b) testosterone, c) cholesterol in different media (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, analyte concentrations are 500.0 ng/mL for testosterone and progesterone, 1000.0 ng/mL for cholesterol, extraction time is 60 min, desorption solvent is IPA, desorption volume is 1.5 mL, desorption time is 30 min) *Values normalized to thickness.

3.2.2 Comparison of Extraction Time Profiles of HLB-CBS and PDVB-PAN-CBS Coatings

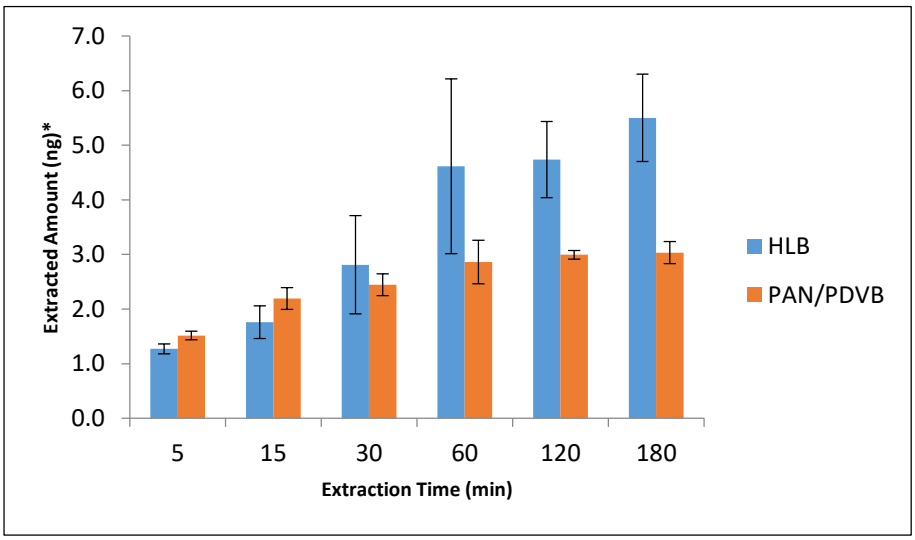
In this study, the most important aim was to prepare the CBS probes with extraction kinetics faster than commercial CBS blades. Therefore, in these pioneering studies, the extraction kinetics of dip-coated HLB (representative of commercial CBS) and newly developed PDVB-PAN were compared with extractions at 5, 15, 30, 60, 120 and 180 minutes. The conditions used in the

experiments carried out for this purpose are given in Section 2.5.2. The results of this experiment are shown in Figure 3.17. These results support that electrospun coated PDVB-PAN blades show faster extraction kinetics than HLB coated blades obtained by dip coating method for tested short extraction times.

It should be noted here that the size of commercially available HLB polymeric particles is 60 μm. These particles, which were immobilized into the PAN, both reduce the surface homogeneity and form thicker coatings. The most important disadvantages of thick coatings are that they have slow extraction and desorption kinetics. Considering that short extraction times are important for rapid diagnostic studies, the necessity of new, nano-sized, thin coatings with fast extraction/desorption kinetics has been demonstrated in this study. Due to the short molecular diffusion path in the nanostructures used, the sorption kinetics is fast, especially in the first stage of sorption. In addition, better extractions were obtained with the new coatings for progesterone and cholesterol given the longer extraction times (near equilibrium). Considering the logP value of testosterone (logP: 3.32), it can be concluded that this analyte is more polar than the others (progesterone and cholesterol). The fact that commercial HLB retains this relatively polar analyte better than the newly developed sorbent at near-equilibrium extraction times, perhaps indicates that commercial HLB has a more polar surface than PDVB-PAN. After these preliminary studies to examine the coating characteristics, optimizations were continued in direct to MS.

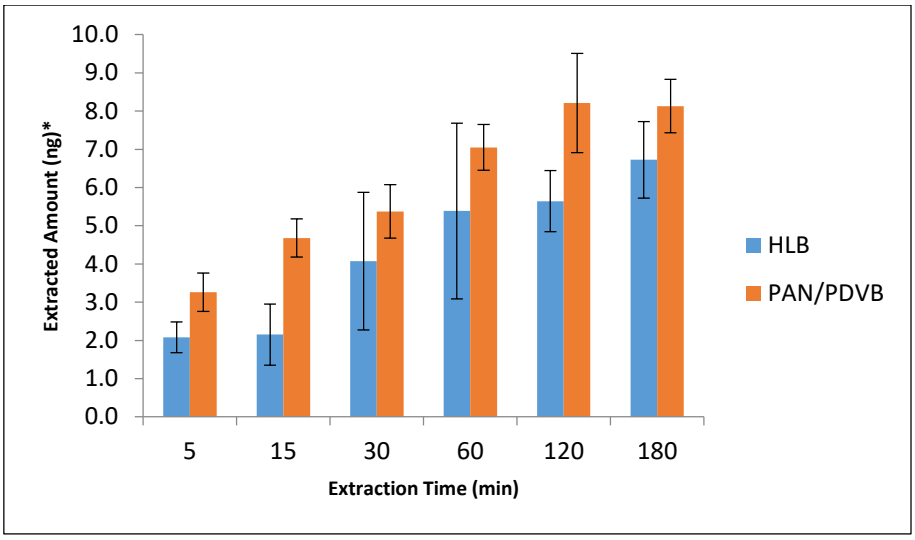


(a)



(b)

Figure 3.17. Extraction kinetics of dip-coated HLB and electrospun PDVB-PAN blades for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, analyte concentrations are 500.0 ng/mL for testosterone and progesterone, 1000.0 ng/mL for cholesterol desorption solvent is IPA, desorption volume is 1.5 mL, desorption time is 30 min) *Values normalized to thickness.

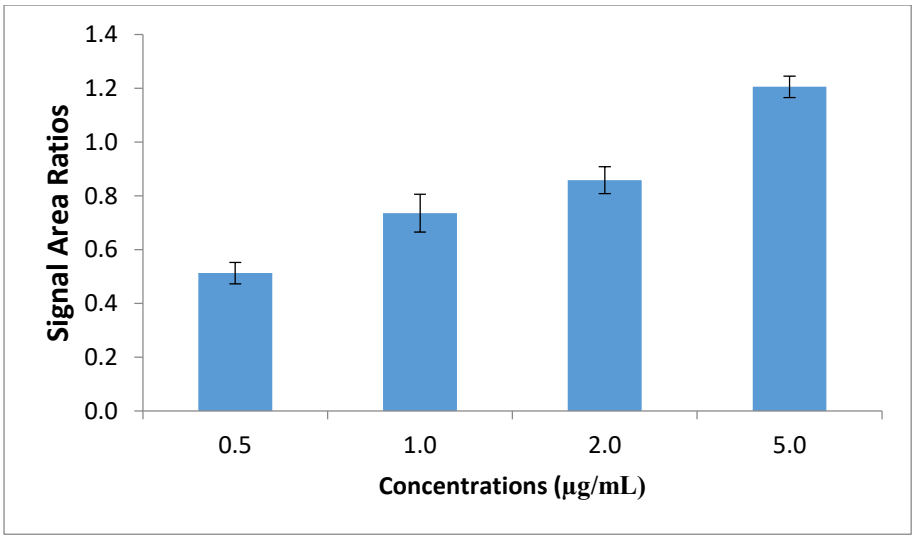


(c)

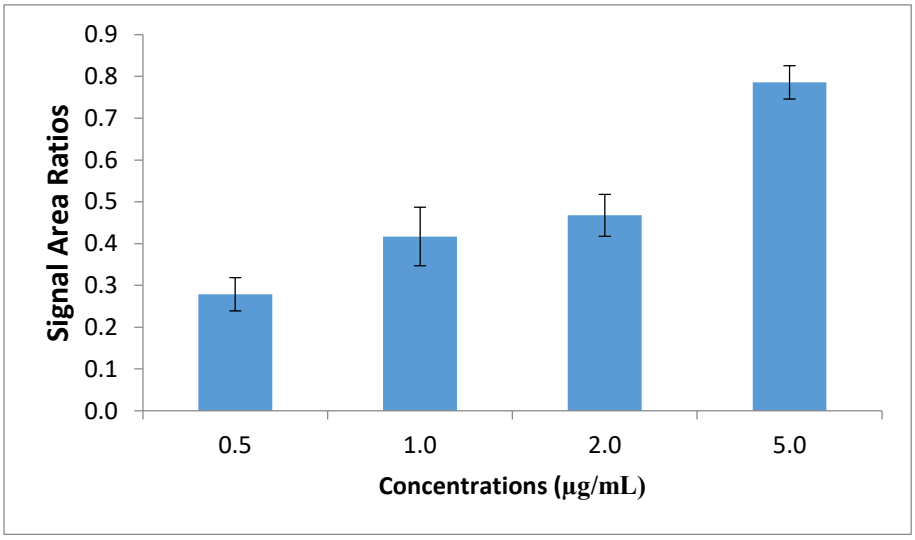
Figure 3.17. (continued) Extraction kinetics of dip-coated HLB and electrospun PDVB-PAN blades for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, analyte concentrations are 500.0 ng/mL for testosterone and progesterone, 1000.0 ng/mL for cholesterol desorption solvent is IPA, desorption volume is 1.5 mL, desorption time is 30 min) *Values normalized to thickness.

3.3 Optimization of Signal from the Uncoated Blades

This study was performed as preliminary investigation to test the CBS performance and see if we could get distinctive signals as the concentration increased. The CBS blades were used for extraction as described in Section 2.6 and then solvent desorbed analytes were placed on the bare blade for CBS-MS analysis. The results obtained in this experiment are given in Figure 3.18. As can be seen from the figure, the signal obtained from CBS-MS increases as analyte concentration increases. However, this experiment is only a preliminary study to test and show that the instrument can perform in CBS-MS mode.

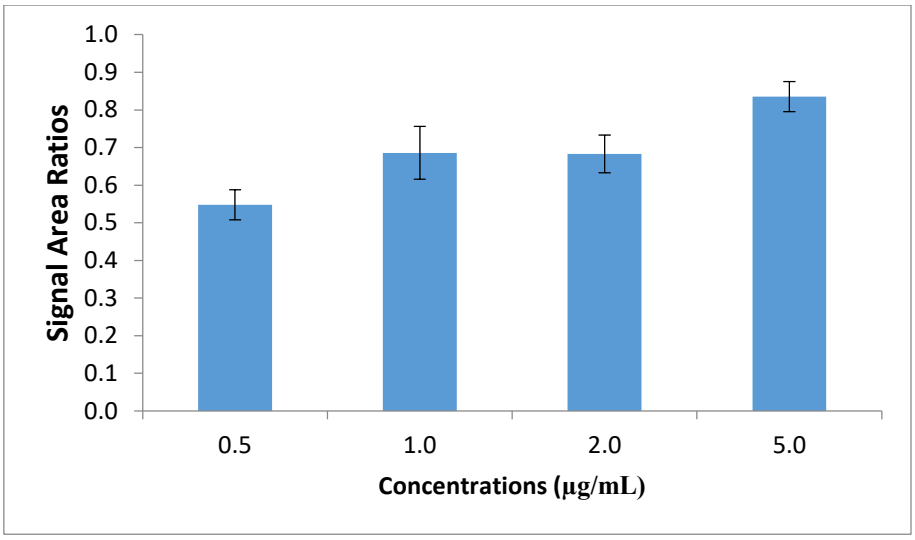


(a)



(b)

Figure 3.18. Extractions from PBS-BSA of a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, extraction time is 60 min, desorption solvent is IPA containing 0.1% F.A (v/v) and 1.0 µg/mL IS mixture, desorption volume is 1.5 mL, desorption time is 30 min)



(c)

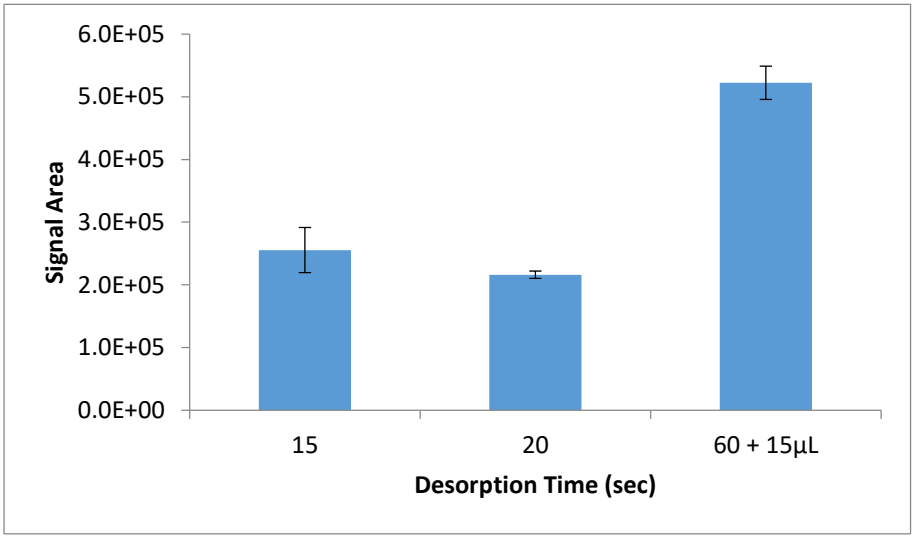
Figure 3.18. (continued) Extractions from PBS-BSA of a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 4 different concentrations of analyte mixtures (500.0, 1000.0, 2000.0, and 5000.0 ng/mL), extraction time is 60 min, desorption solvent is IPA containing 0.1% F.A (v/v) and 1.0 µg/mL IS mixture, desorption volume is 1.5 mL, desorption time is 30 min)

3.4 CBS Method Development for Direct to MS Studies

3.4.1 Optimization of Desorption Time

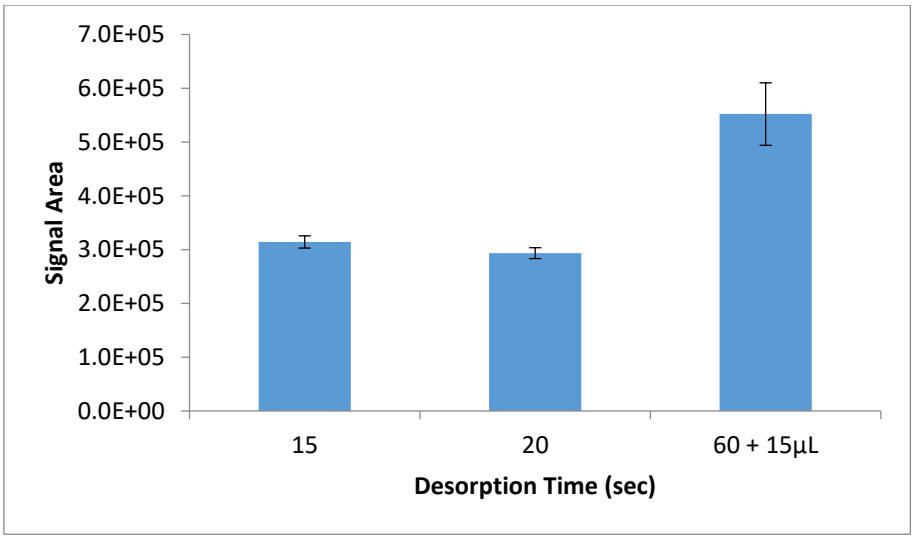
Optimization of desorption time is of great importance in order to obtain the most sensitive and reproducible result. For this purpose, three desorption times (15, 20 and 60 seconds) were tested using 15.0 µL of isopropyl alcohol (0.1% F.A.) as desorption solvent (with the conditions given in Section 2.7.2). After the desorption time was reached, the voltage was applied to the blade, an electrospray was produced and the analyte signal was collected. The most important reason for studying short desorption times at this stage is that the volume of the solvent is very small, and it completely evaporates from the surface in 60 seconds. For this reason, a second 15.0 µL of isopropyl alcohol was added to the blades that were

desorbed for 60 seconds, and the voltage was applied after this second 15.0 µL desorption solution added. Also, shorter times than 15 s were not studied because it was not possible to transfer the solvent to the blade surface at a reproducible speed. When the results shown in Figure 3.19 were examined, it was observed that there were no significant differences between 15 and 20 seconds of desorption time. As can be clearly seen in Figure 3.19, the most successful desorption time was determined as 60 seconds (completely evaporated) followed by a second 15.0 µL desorption solution. The higher signals obtained in this way shows that the analytes are desorbed and moved to the sorbent surface in the first solvent droplet and then easily transferred for analysis in the second droplet. Subsequent studies were continued under these conditions.

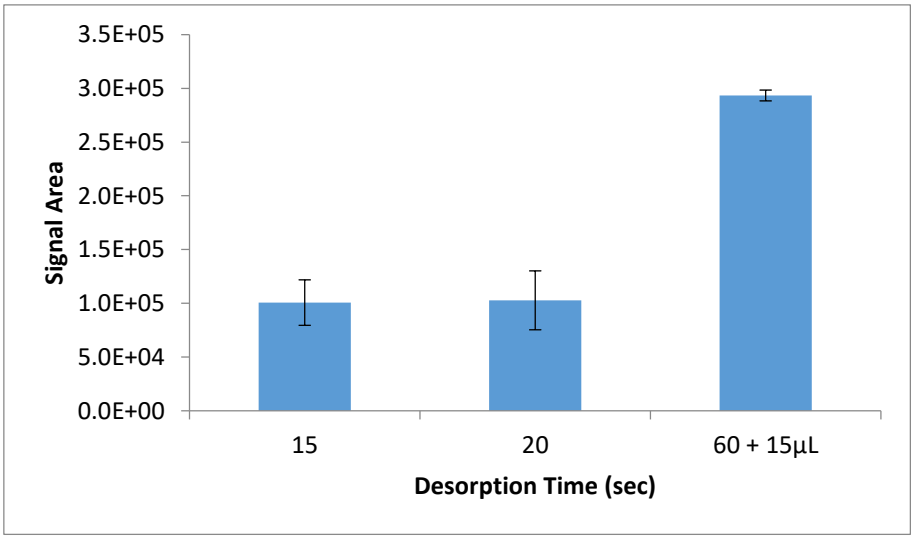


(a)

Figure 3.19. Determination of desorption time in CBS-MS studies for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for all analytes, extraction time is 30 min, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS analysis)



(b)



(c)

Figure 3.19. (continued) Determination of desorption time in CBS-MS studies for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for all analytes, extraction time is 30 min, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS analysis)

3.4.2 Extraction Time Profiles

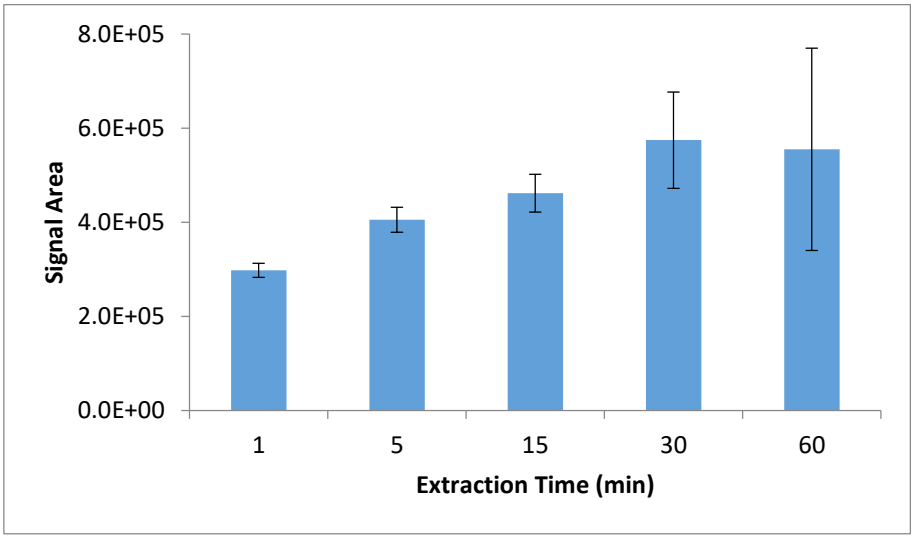
Extraction time is one of the most important parameters in SPME procedures, as it affects the sensitivity of the final method. In this context, the extraction time profiles of the analytes were determined by static extraction from both PBS and PBS-BSA medium from the droplet-sized sample (preliminary studies for plasma spot). After proving that the obtained extraction kinetics are fast and successful results can be obtained, studies have been carried out to make this extractive phase more effective. For this purpose, an extraction kinetics study was conducted with the PDVB-PAN coatings obtained with different PDVB ratios in order to investigate whether increasing the amount of PDVB in the extractive phase would give favorable results. After the most successful coating results were obtained for the coatings containing 3% PDVB (among tested 1, 2 and 3%), it was decided to coat the blades with 3% PDVB containing PDVB-PAN by both electrospinning and dip coating methods. Thus, the kinetics of the coatings obtained by using the same polymer mixture with two different approaches could be compared.

3.4.2.1 Extraction Time Profile Studies for Long Extraction Times by Static Extraction with Droplet Approach

In CBS-MS studies, if the sample volume is large enough, extraction can be performed by constant agitation of the blades or the sample. In such condition, the diffusion boundary layer is thin due to stirring and the sorption kinetics is relatively fast. In fact, static plasma spot sorption can also be performed at sample volumes below 50 µL, namely droplet size. However, in such conditions, the diffusion boundary layer is thick as the sorption will take place under static conditions. This requires the analytes to reach the coating by completely free diffusion. For rapid extraction in these conditions, nano-structured materials can solve the kinetic problem associated with static extraction conditions.

The experimental conditions used in these studies were given in Section 2.7.3.1 and the results were given in Figures 3.20 and 3.21 for PBS and synthetic plasma,

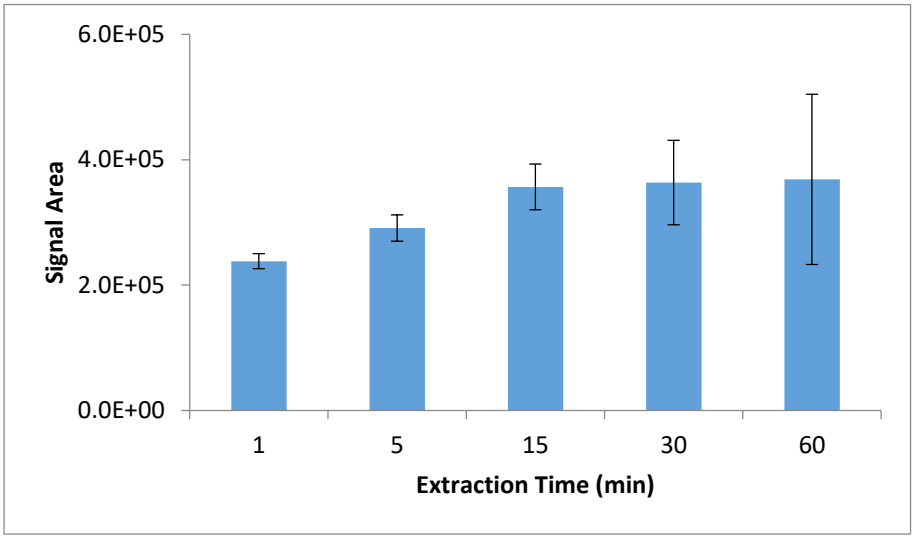
respectively. These results suggest that the extraction kinetics for all three analytes in both media is high. It has been observed that a signal close to equilibrium can be obtained even with short extraction times, for instance 5 to 15 minutes in PBS and 1 minute in synthetic plasma. The shortening of the extraction equilibrium time in the presence of binding matrix indicates that the free analyte concentration was low and was rapidly depleted by the extractive phase, and that the protein-analyte equilibrium did not produce free analyte of the sampling time, or the analyte release was slow under the static conditions used. It is also seen that the standard deviations are quite high in the experiments carried out in these droplet size static conditions. It has been observed that the standard deviation increases, especially when it comes to long extraction times. In general, it is thought that one of the main reasons for the high standard deviations is the use of very small sample volume (30.0 μL) and static extraction, and the other is that the desorption/ionization processes are carried out in a open system. Since the appropriate internal standard (analytes' isotopologues) could not be used in this study, it has become quite prone to error.



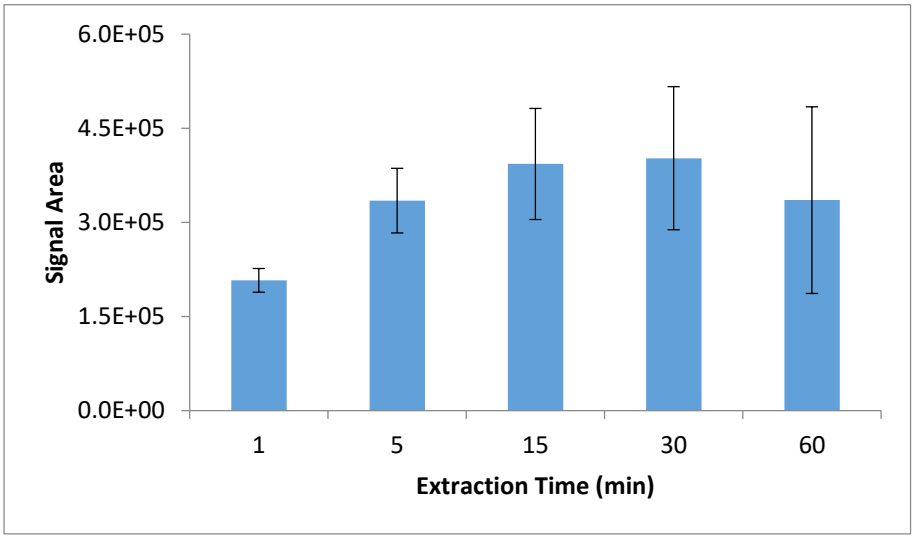
(a)

Figure 3.20. Static extraction time profiles in PBS with droplet approach for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction

volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec)



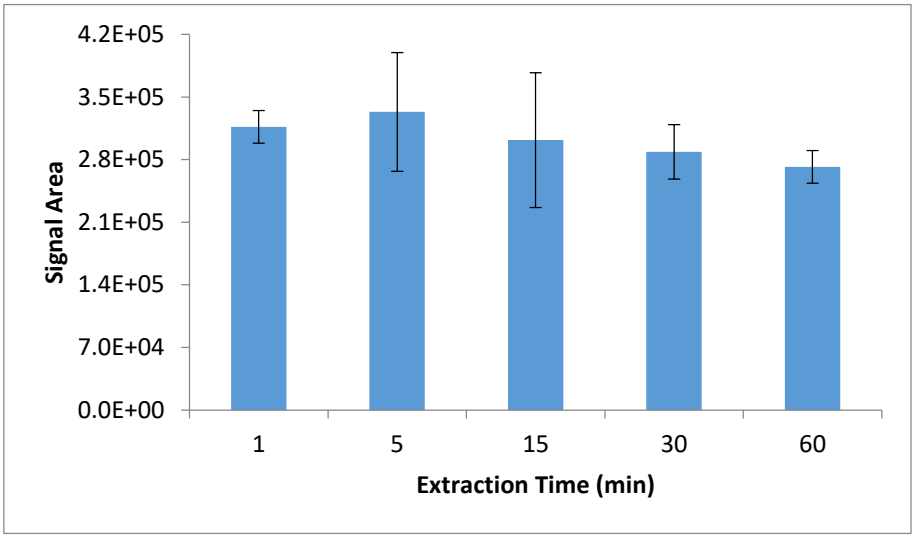
(b)



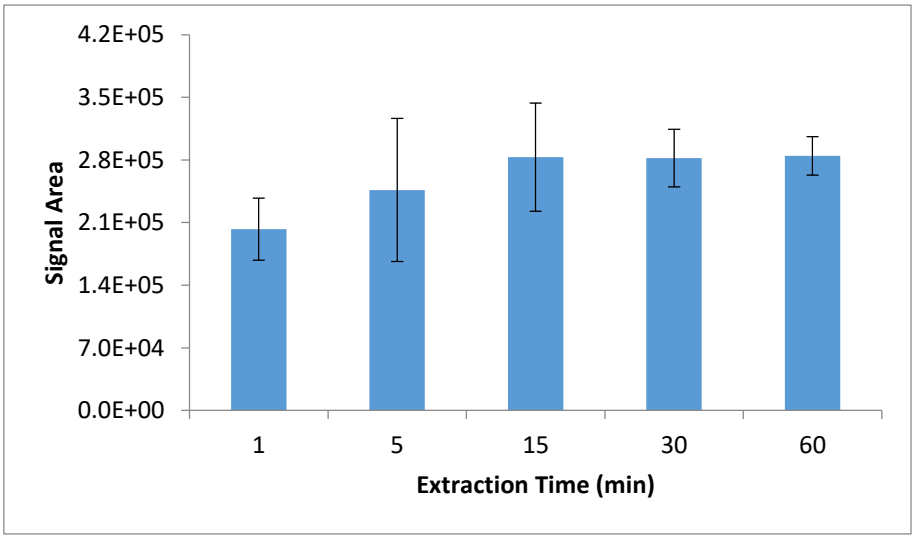
(c)

Figure 3.20. (continued) Static extraction time profiles in PBS with droplet approach for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte

concentration for each analyte, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec)



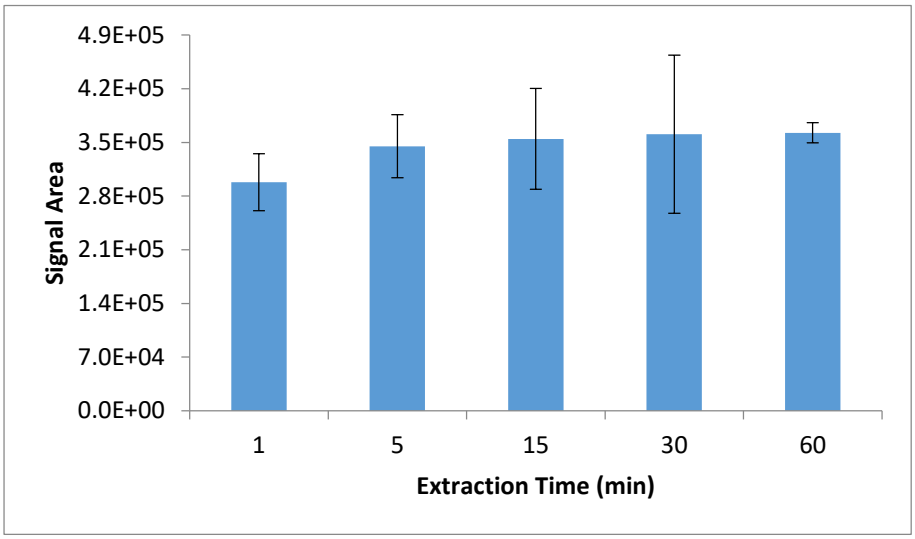
(a)



(b)

Figure 3.21. Static extraction time profiles in PBS-BSA by droplet approach for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each

analyte, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec)



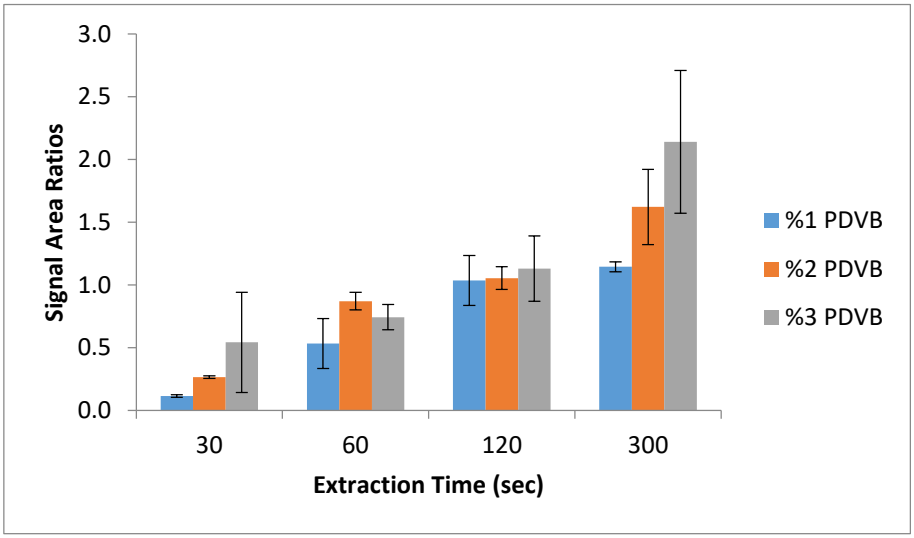
(c)

Figure 3.21. (continued) Static extraction time profiles in PBS-BSA by droplet approach for a) progesterone, b) testosterone, c) cholesterol. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec)

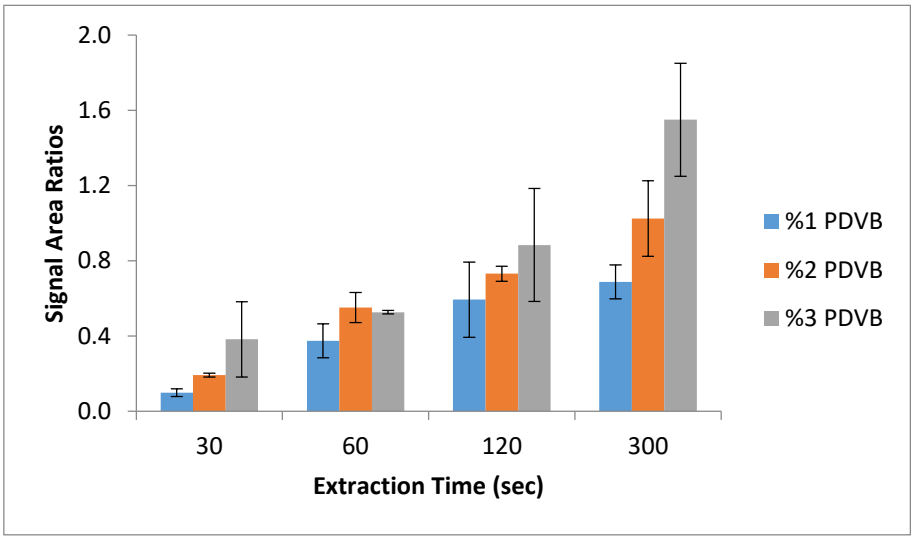
3.4.2.2 Extraction Kinetics Studies Using Short Extraction Times with Coatings Obtained with Different PDVB-PAN Ratios

The effect of the amount of PDVB in the PDVB-PAN on the sorption was tested with CBS probes prepared by increasing the ratio of PDVB (1%, 2% and 3%). Experiment details were given in Section 2.7.3.2 and the results obtained were shown in Figure 3.22. As can be seen from the figure, for all three analytes, the lowest signals were obtained at 1% PDVB, and the highest signals were obtained at

3% PDVB. These results led us to work on increasing the PDVB amount further and making the extractive phase more effective.



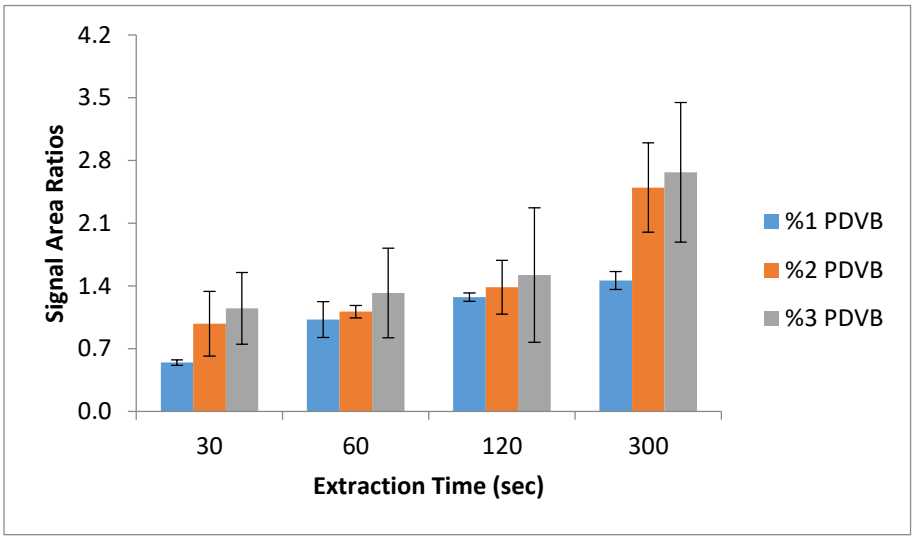
(a)



(b)

Figure 3.22. Extraction kinetics of a) progesterone, b) testosterone, and c) cholesterol for short extraction times with coatings obtained with different PDVB-PAN ratios (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is

1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, 60 sec desorption time)



(c)

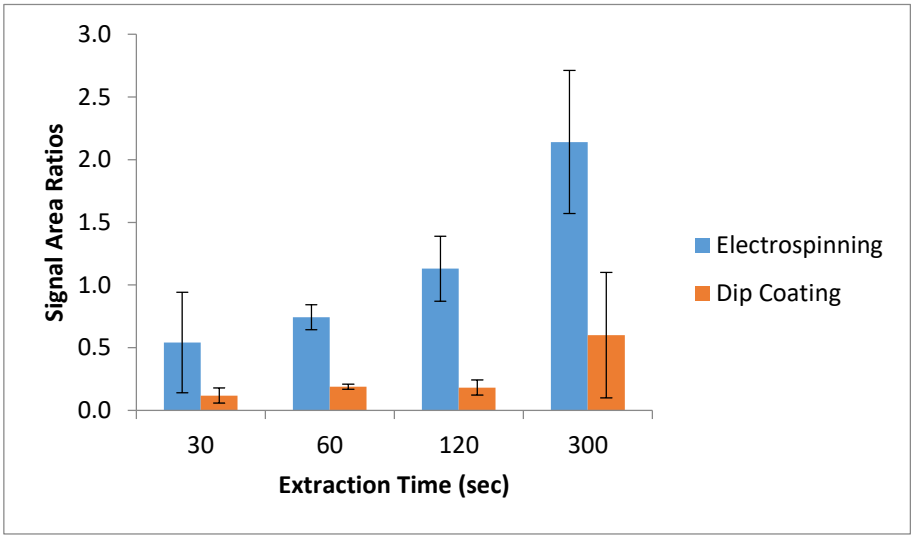
Figure 3.22. (continued) Extraction kinetics of a) progesterone, b) testosterone, and c) cholesterol for short extraction times with coatings obtained with different PDVB-PAN ratios (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, 60 sec desorption time)

3.4.2.3 Comparison of Extraction Kinetics of CBS Obtained by Electrospinning and Dip Coating

As PDVB-PAN containing 3% PDVB gave the highest sensitivity, the effect of two different coating methods on extraction kinetics was investigated by using the same PDVB-PAN mixture (PDVB-PAN containing 3% PDVB). Here, dip coating, which is the most widely used method in the production of CBS probes, and the electrospinning method were compared. The experimental conditions were given in Section 2.7.3.3, and the results of this experiment are given in Figure 3.23. By

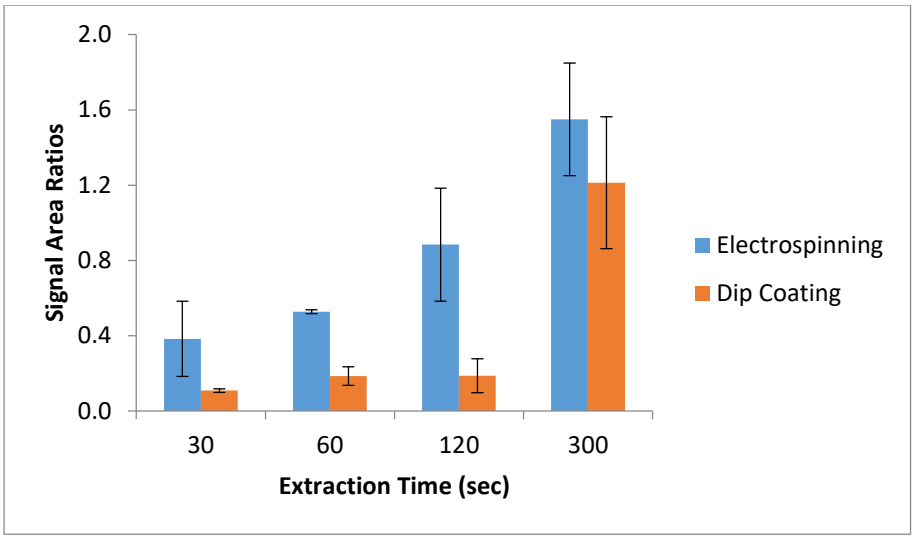
looking at the results, it was noticed that the sorption kinetics of the coatings obtained by electrospinning were faster than those obtained by dip coating. Thus, it has been proven that the chosen coating method (electrospinning) is more suitable for the purpose, more successful and more advantageous than the classical method.

These findings suggest that it may be necessary to investigate whether it is possible to immobilize PDVB at even higher amounts without impairing the electrospinning yield and whether it is possible to further increase the sensitivity.

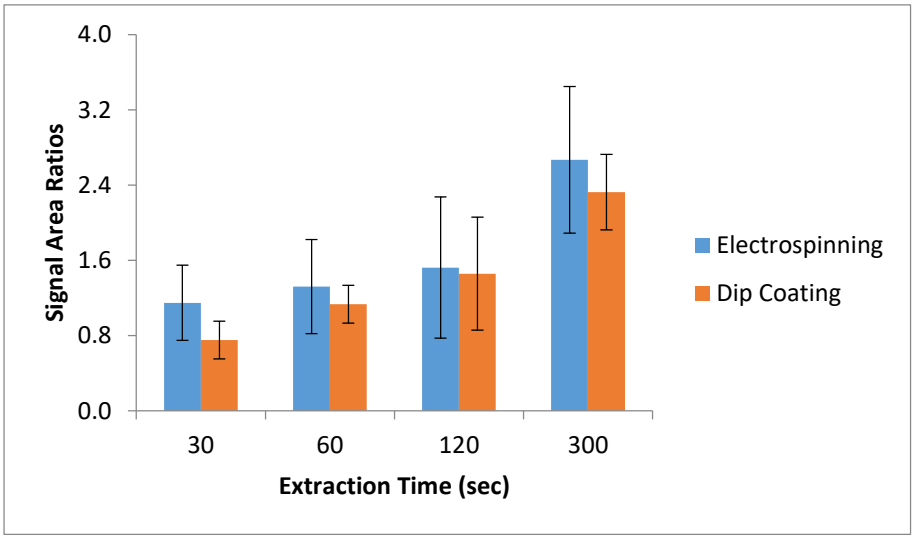


(a)

Figure 3.23. Extraction kinetics studies of a) progesterone, b) testosterone, c) cholesterol with the CBS blades obtained by electrospinning and dip coating (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec)



(b)



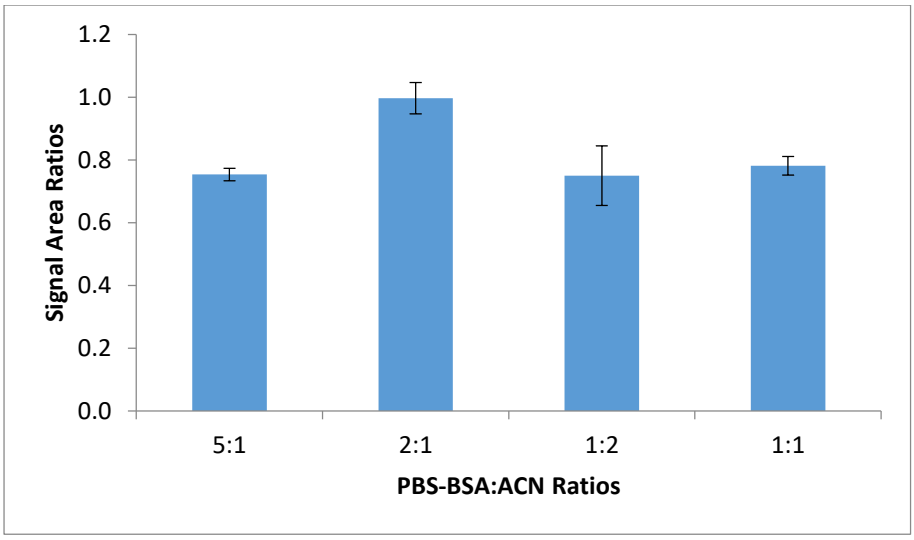
(c)

Figure 3.23. (continued) Extraction kinetics studies of a) progesterone, b) testosterone, c) cholesterol with the CBS blades obtained by electrospinning and dip coating (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec)

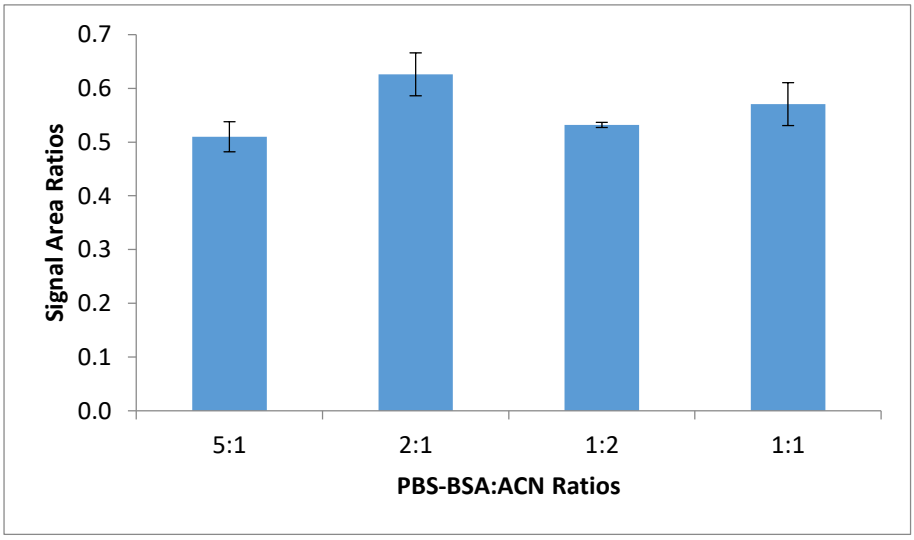
3.4.3 Breaking of Protein Binding by Protein Precipitation Studies

It may be necessary to increase the free concentration of analytes before sorption to obtain good sensitivity in plasma spot analysis. For this reason, CBS sorptions were performed by adding acetonitrile in various ratios that can change the analytes from protein bound state (≥98% bound) to the state without binding matrix component (all analytes are free), and the results were compared in CBS-MS mode.

The experimental procedure was given in Section 2.7.4 and the results obtained are given in Figure 3.24. As can be seen from the figure, the 2:1 PBS–BSA:ACN ratio resulted in higher signals for all three analytes. The lower signal for the 5:1 PBS–BSA:ACN ratio indicates that the protein precipitation was insufficient due to the low amount of organic solvent used in this experiment. The lower signal for 1:2 PBS–BSA:ACN ratio supports that the amount of organic solvent here both reduces the affinity of the analyte against the coating and causes a significant dilution in the sample. This showed that if sufficient sensitivity could not be achieved in method validation, the sample could be preprepared with 2:1 plasma:ACN.

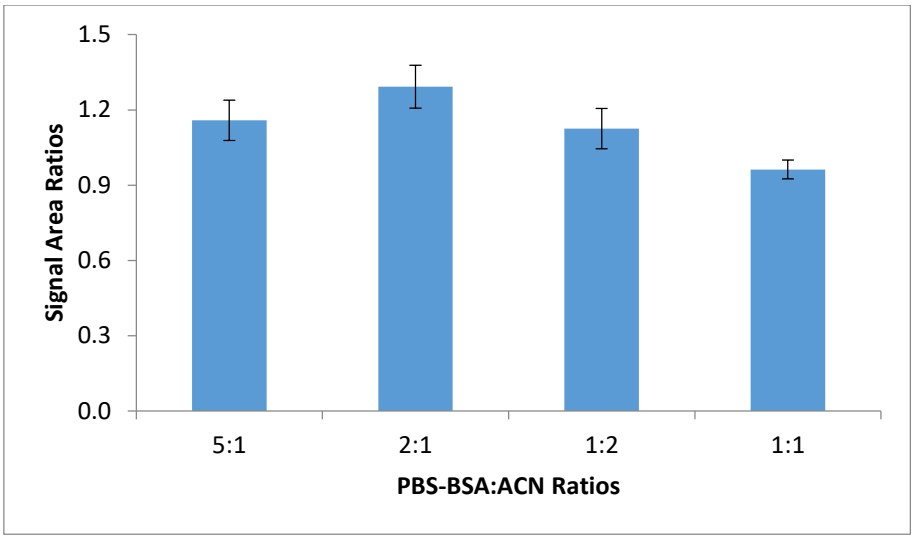


(a)



(b)

Figure 3.24. Breaking of protein binding study of a) progesterone, b) testosterone, c) cholesterol (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, extraction time is 5 min, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct to MS desorption, desorption time is 60 sec)



(c)

Figure 3.24. (continued) Breaking of protein binding study of a) progesterone, b) testosterone, c) cholesterol (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, extraction time is 5 min, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct to MS desorption, desorption time is 60 sec)

3.4.4 Further Improvement of the Extractive Phase

Due to the increase in the signals by increasing the amount of PDVB from 1% to 2% and 3%, it was concluded that further increasing the amount of PDVB would also increase the sensitivity, and it was decided to test it. First, whether the increase in the amount of PDVB has any negative effects on the formation of nanofibers was examined with SEM images. Then, extraction kinetics studies were carried out with the coatings obtained with the most suitable PDVB percentage.

3.4.4.1 Characterization of Coatings Obtained with Increased PDVB Amounts by Scanning Electron Microscope

In this characterization study, PDVB-PAN polymeric mixtures containing 1%, 2%, 3%, 5%, 7% and 10% by mass of PDVB were prepared and coated on aluminum foil by electrospinning method. SEM images of the coatings were taken to see whether there was nanofiber formation in the obtained coatings and whether PDVB particles were embedded in these fibers. When the SEM images given in Figure 25-30 were examined, it was seen that increasing PDVB ratios did not lead to negative results in the formation of a nanofibrous structure with the electrospinning method. It has been observed that PAN forms successful nanofiber structures in each of the electrospun polymer mixtures and PDVB nanoparticles were embedded in each of these nanofibers. In addition, the increase in the amount of PDVB embedded in nanofibers with increasing PDVB ratio was also clear. Considering these results, it was decided to continue to the method development studies with the extractive phase developed with 10% PDVB.

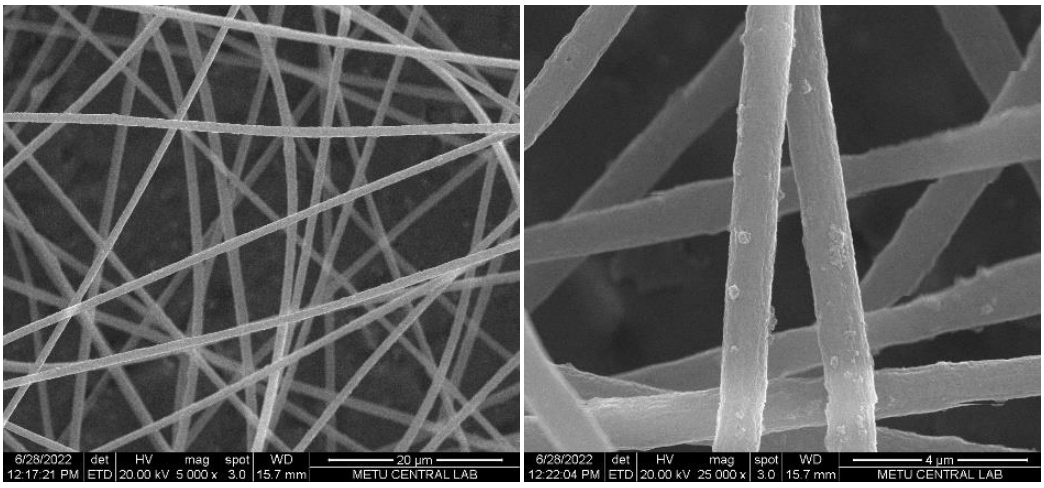


Figure 3.25. SEM images of electrospun PDVB-PAN polymer mixture containing 1% PDVB. (Electrospinning parameters: distance between needle tip and collector is 15.0 cm, feed rate is 2.4 mL/h, and applied voltage is 20.0 kV.)

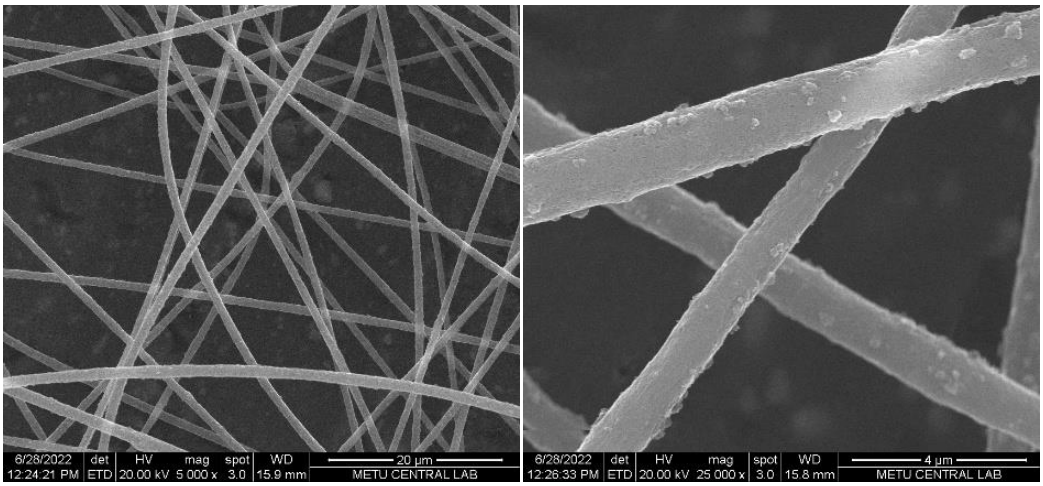


Figure 3.26. SEM images of electrospun PDVB-PAN polymer mixture containing 2% PDVB. (Electrospinning parameters: distance between needle tip and collector is 15.0 cm, feed rate is 2.4 mL/h, and applied voltage is 20.0 kV.)

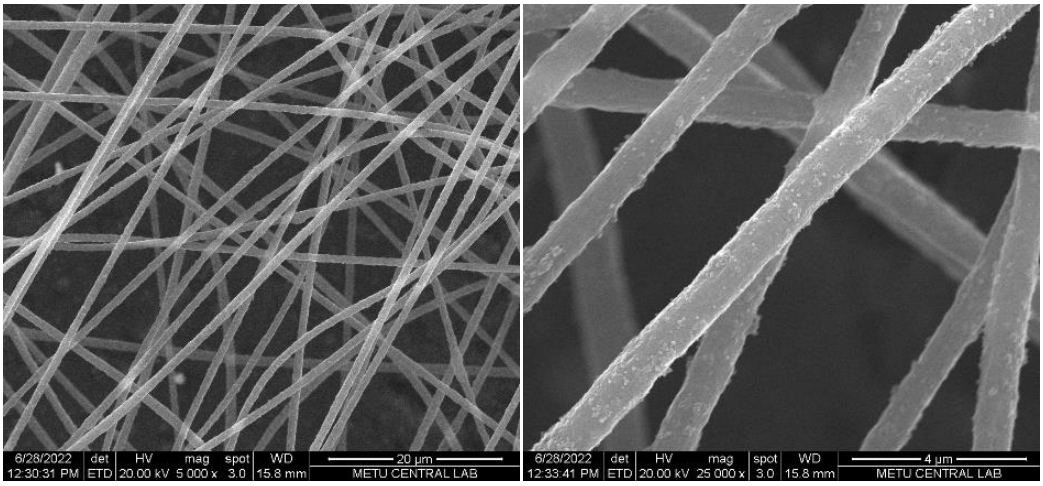


Figure 3.27. SEM images of electrospun PDVB-PAN polymer mixture containing 3% PDVB. (Electrospinning parameters: distance between needle tip and collector is 15.0 cm, feed rate is 2.4 mL/h, and applied voltage is 20.0 kV.)

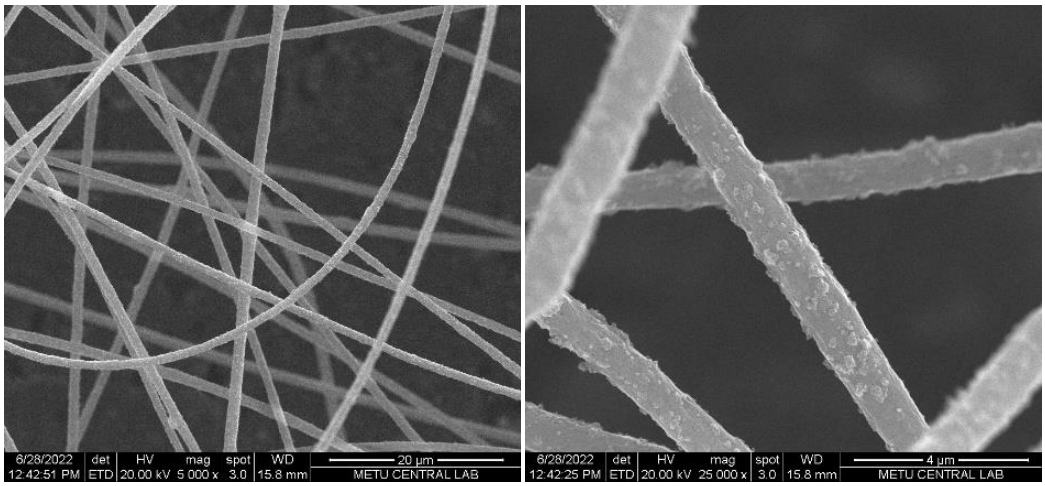


Figure 3.28. SEM images of electrospun PDVB-PAN polymer mixture containing 5% PDVB. (Electrospinning parameters: distance between needle tip and collector is 15.0 cm, feed rate is 2.4 mL/h, and applied voltage is 20.0 kV.)

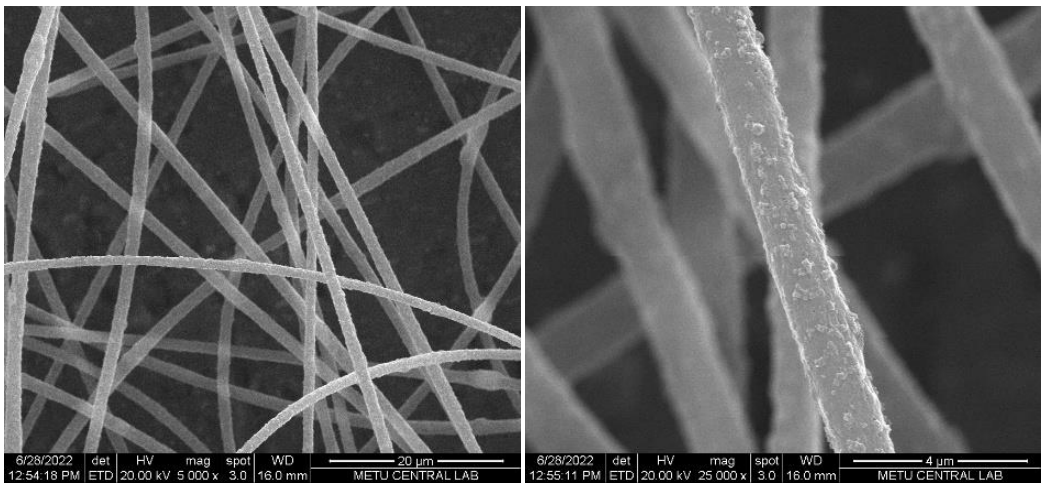


Figure 3.29. SEM images of electrospun PDVB-PAN polymer mixture containing 7% PDVB. (Electrospinning parameters: distance between needle tip and collector is 15.0 cm, feed rate is 2.4 mL/h, and applied voltage is 20.0 kV.)

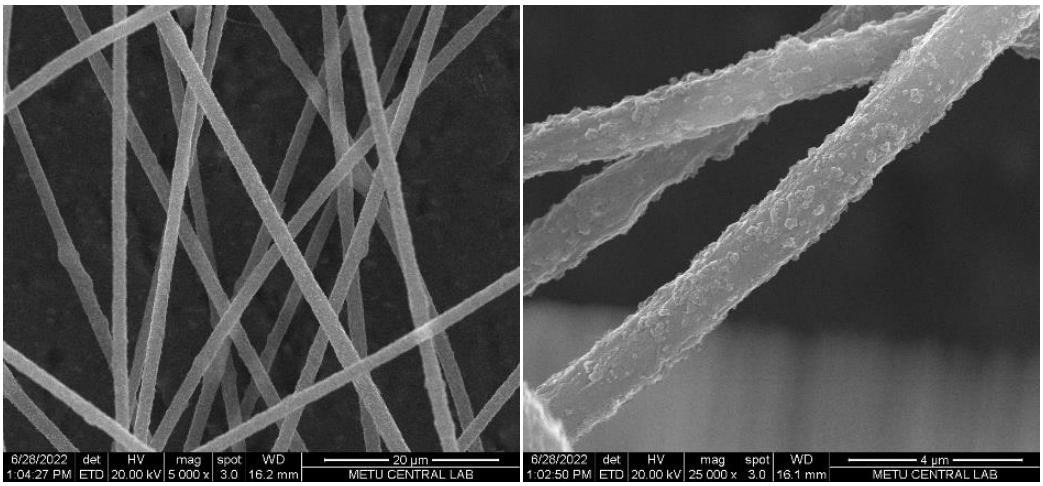


Figure 3.30. SEM images of electrospun PDVB-PAN polymer mixture containing 10% PDVB. (Electrospinning parameters: distance between needle tip and collector is 15.0 cm, feed rate is 2.4 mL/h, and applied voltage is 20.0 kV.)

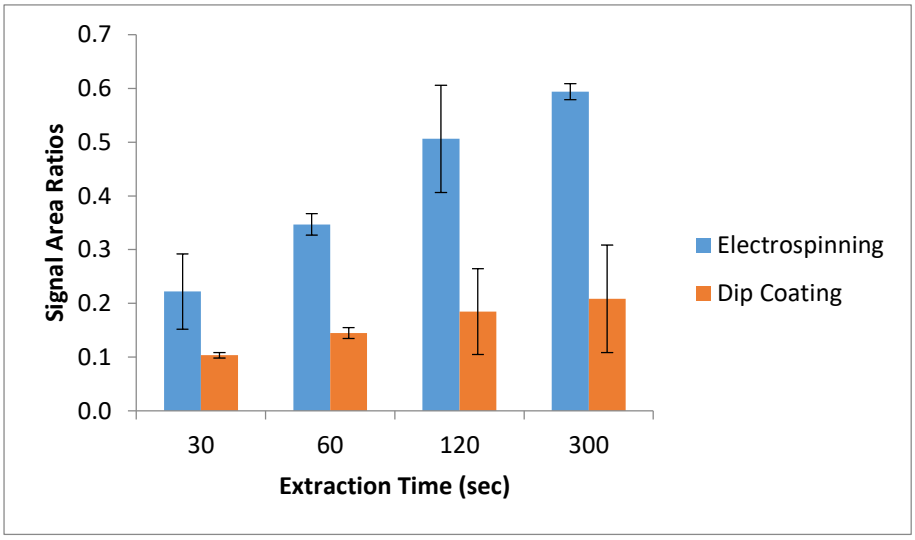
3.4.4.2 Comparison of Extraction Kinetics of CBS obtained by Electrospinning and Dip Coating using 10% PDVB

The previous extraction kinetics studies were repeated by dip coating and electrospinning methods using a PDVB-PAN polymer mixture containing 10% PDVB. In addition, the same studies were repeated for static extraction from the droplet size sample and extraction with continuous stirring in large volumes. Extractions from both PBS and PBS–BSA were attempted under both conditions and graphs showing extraction kinetics were plotted.

The sorption studies with constant stirring are given in Figures 3.31 and 3.32 for PBS and synthetic plasma, respectively. The obtained results support that the newly developed CBS-MS blades are more successful than the dip coating method. Another important finding here is that the difference between the two coatings is greater in PBS than in synthetic plasma. This shows that the free concentration of protein-bound analytes is released at a slower rate from the protein than the sorption rate of analyte by the coating when the amount of free analyte is depleted.

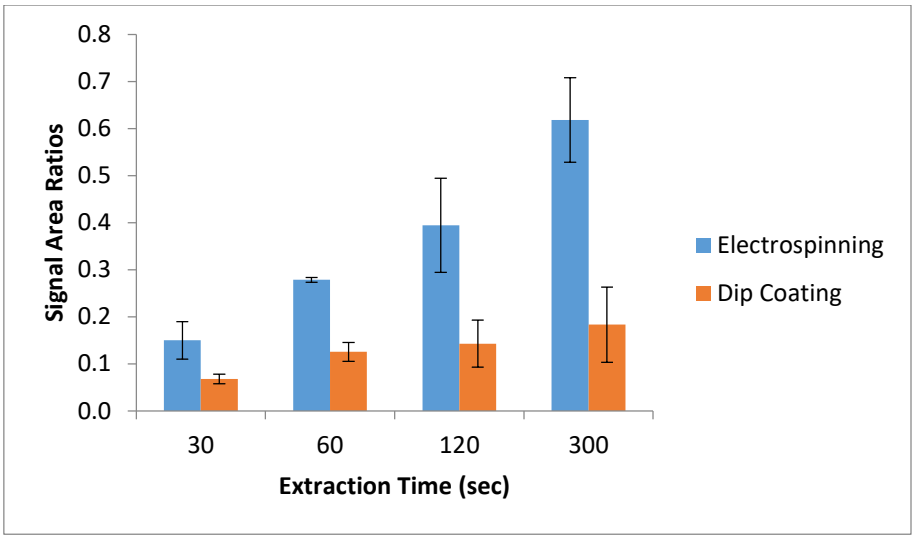
The results of the extraction time study performed with a droplet-sized sample under static conditions are given in Figures 3.33 and 3.34 for PBS and synthetic plasma, respectively. The results obtained here also highlight kinetic differences between the matrices. As expected, less analyte was extracted in this study (static extraction) than by extraction by stirring (due to smaller volume).

In conclusion, the graphs show that the extraction kinetics of coatings obtained by electrospinning are faster in both PBS and PBS - BSA media than those obtained by dip coating in both droplet size static and large volume constant mixing extractions.

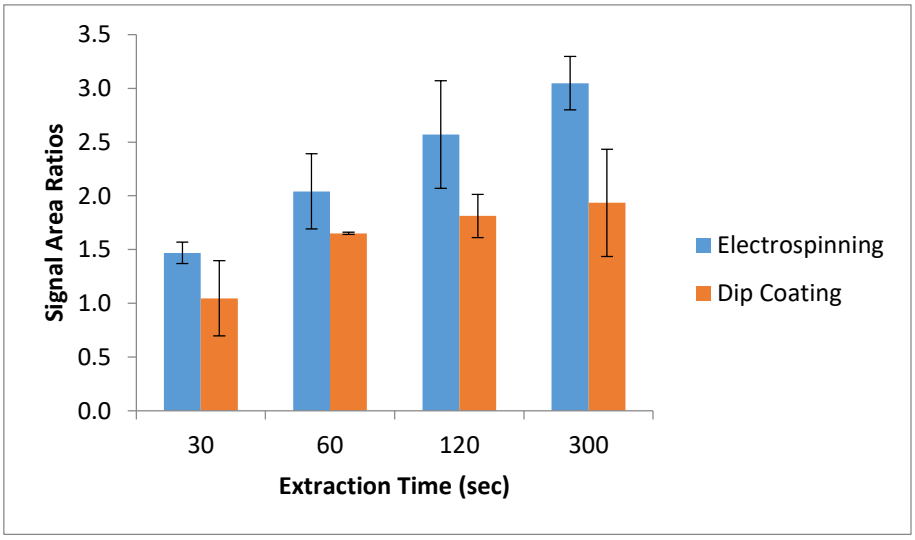


(a)

Figure 3.31. Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol with polymer solutions containing 10% PDVB from PBS for short extraction times using electrospinning and dip coating fabricated blades. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)



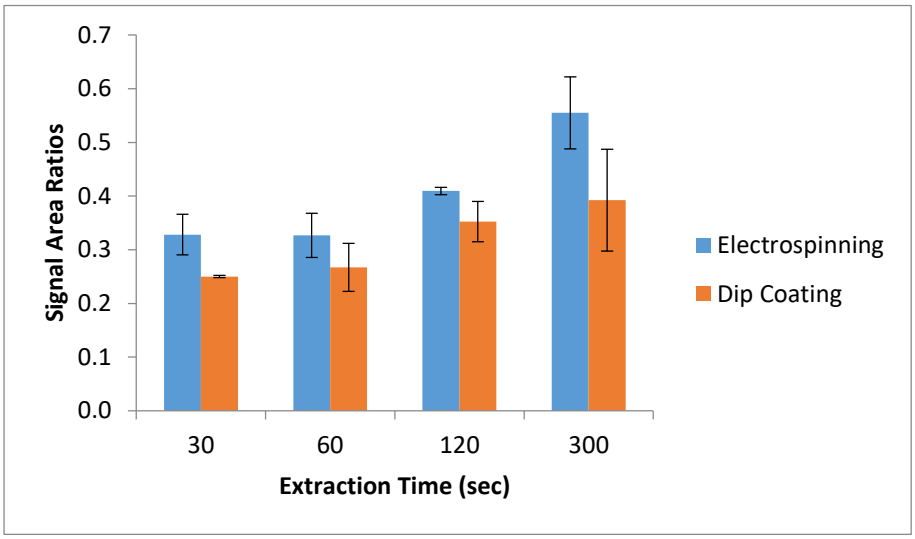
(b)



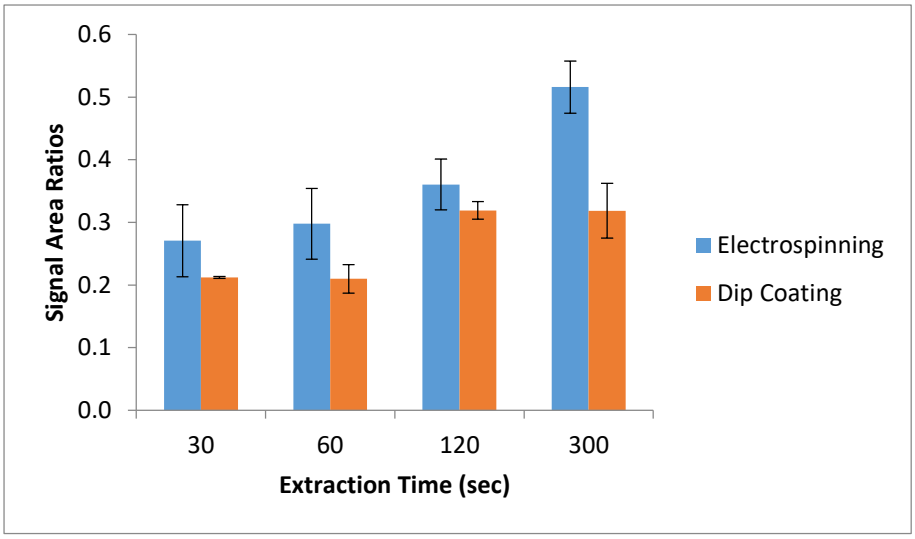
(c)

Figure 3.31. (continued) Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol with polymer solutions containing 10% PDVB from PBS for short extraction times using electrospinning and dip coating fabricated blades. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0

µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)



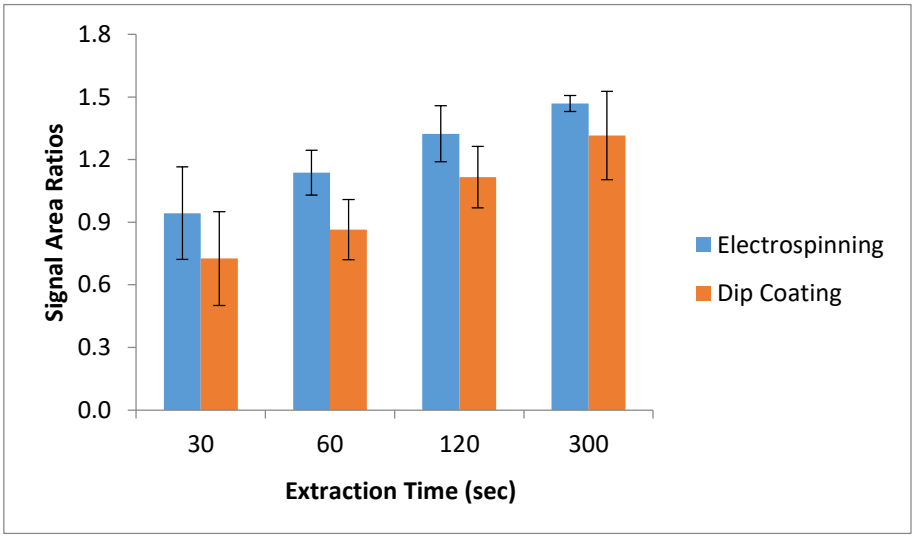
(a)



(b)

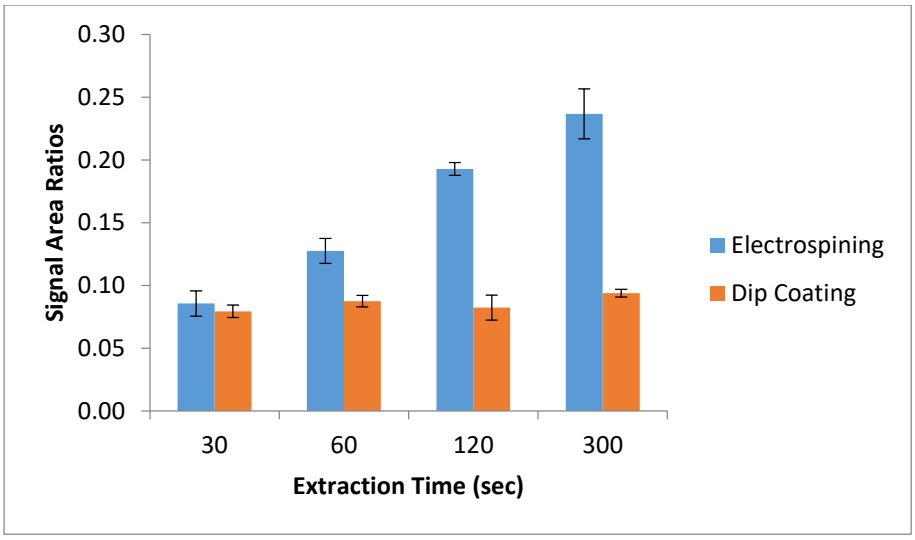
Figure 3.32. Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol with polymer solutions containing 10% PDVB from PBS-BSA for short extraction times using electrospinning and dip coating fabricated blades. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm,

1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)

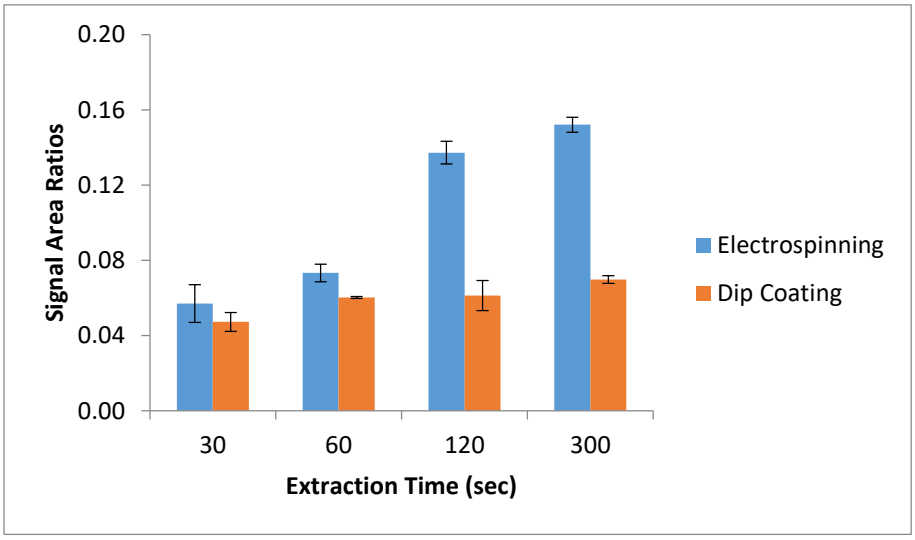


(c)

Figure 3.32. (continued) Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol with polymer solutions containing 10% PDVB from PBS-BSA for short extraction times using electrospinning and dip coating fabricated blades. (Experimental conditions: Extraction volume is 1.5 mL, stirring rate is 1000 rpm, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)



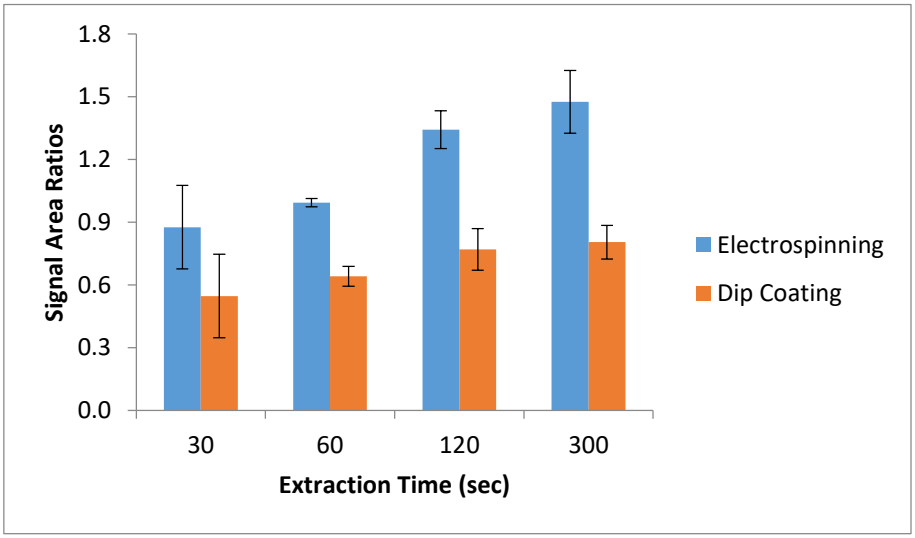
(a)



(b)

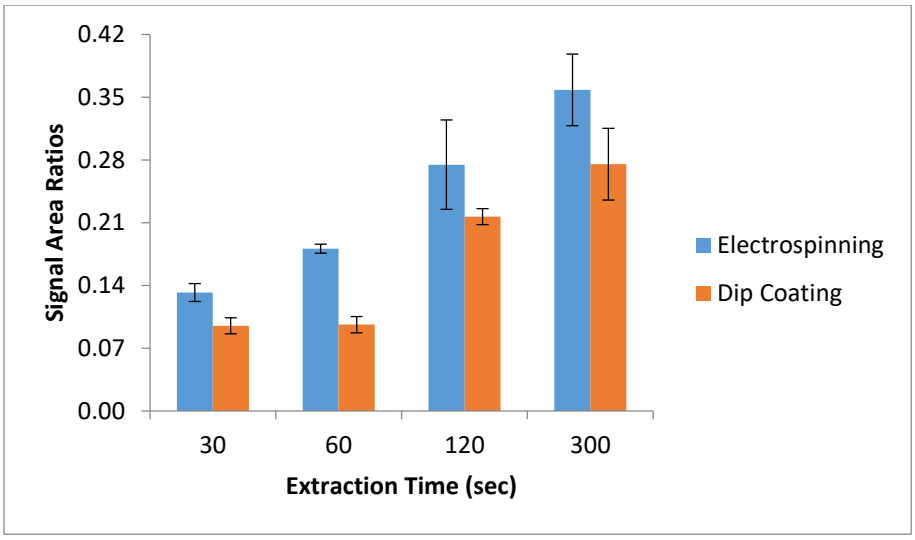
Figure 3.33. Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol from PBS with polymer solutions containing 10% PDVB for short extraction times using electrospinning and dip-coating blades. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is

IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)

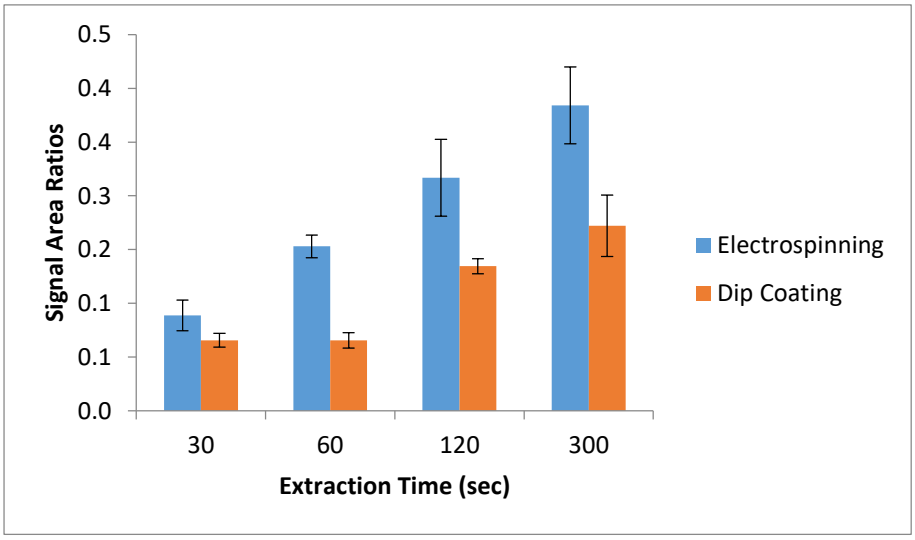


(c)

Figure 3.33. (continued) Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol from PBS with polymer solutions containing 10% PDVB for short extraction times using electrospinning and dip-coating blades. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)



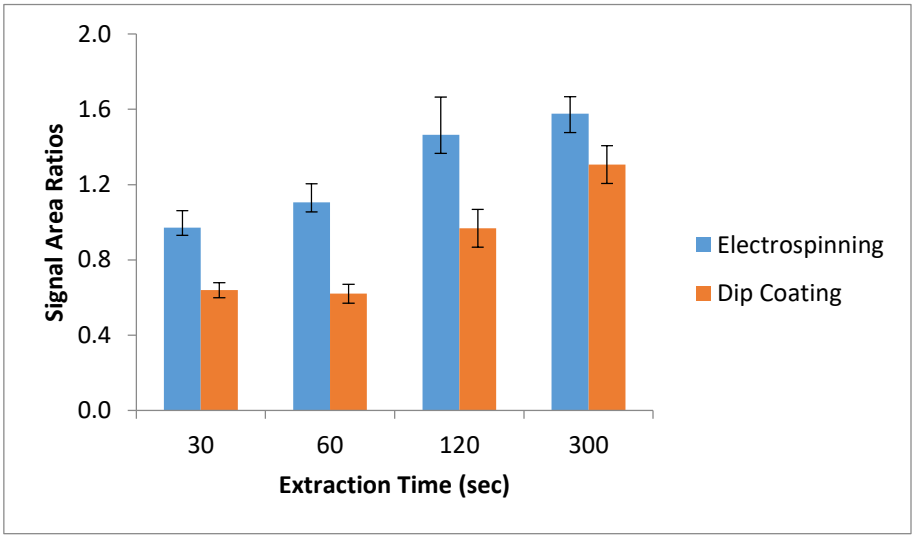
(a)



(b)

Figure 3.34. Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol from PBS-BSA with polymer solutions containing 10% PDVB for short extraction times using electrospinning and dip-coating blades. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is

IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)



(c)

Figure 3.34. (continued) Extraction kinetics study of a) progesterone, b) testosterone, c) cholesterol from PBS-BSA with polymer solutions containing 10% PDVB for short extraction times using electrospinning and dip-coating blades. (Experimental conditions: Extraction volume is 30.0 µL, static extraction, 1.0 µg/mL analyte concentration for each analyte, IS concentration is 1.0 µg/mL, desorption solvent is IPA containing 0.1% F.A (v/v), desorption volume is 15.0 µL, direct MS desorption, desorption time is 60 sec.)

3.5 Validation Studies

Method validation is important to ensure that the methods and procedures used in a measurement are suitable for the purpose of the study, data reliability, and accuracy. For this purpose, the expected concentrations of the selected analytes in human plasma were investigated. Cholesterol is found at levels higher than 1000 µg/mL, while testosterone and progesterone are found at ng/mL levels. Considering the linear operating range of the CBS-MS, it will not be possible to determine all

three analytes simultaneously in the same extraction. As the sample must be diluted for cholesterol analysis, and at such conditions the other two analytes will drop below the detection limits. Therefore, cholesterol has been validated separately from progesterone and testosterone.

3.5.1 Cholesterol Validation

Determination of linear range and limit of quantitation (LOQ):

As the first step of validation, the lower limit of quantitation (LOQ) and linear range in CBS-MS were determined by matrix-matched CBS-MS. The calibration graph obtained in a typical run is shown in Figure 3.35. The lowest concentration with a maximum relative error of 20% was determined as the LOQ value. The LOQ value determined for cholesterol in this study is 200.0 ng/mL.

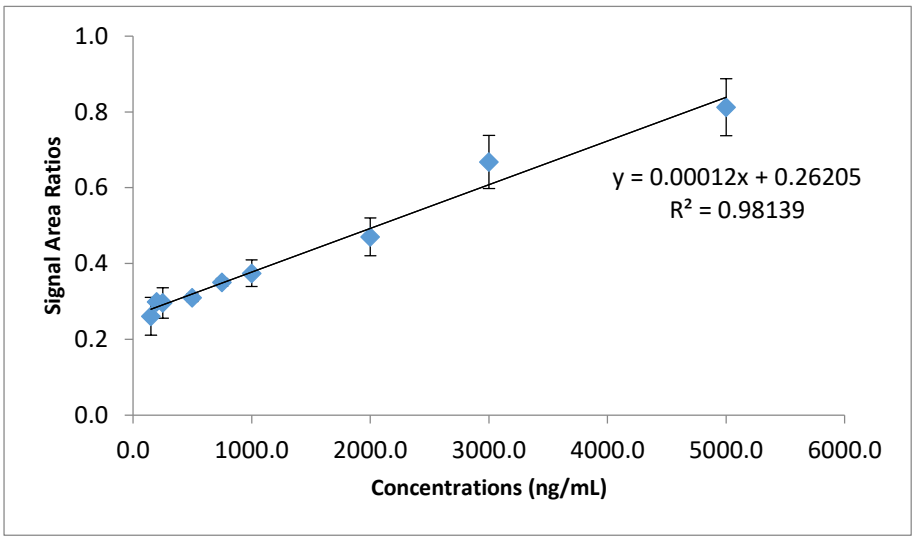


Figure 3.35. CBS-MS calibration graph obtained for cholesterol

Carry-over test:

After and before analyzing 5000.0 ng/mL, which is the highest point of the calibration graph, blank analysis was performed and it was tested whether there was a carry-over from the previous analysis in the MS. The results obtained are

shown in Figure 3.36. As can be seen, the signal received from the MS before and after 5000.0 ng/ml is similar. This shows that there is no accumulation of cholesterol in the system.

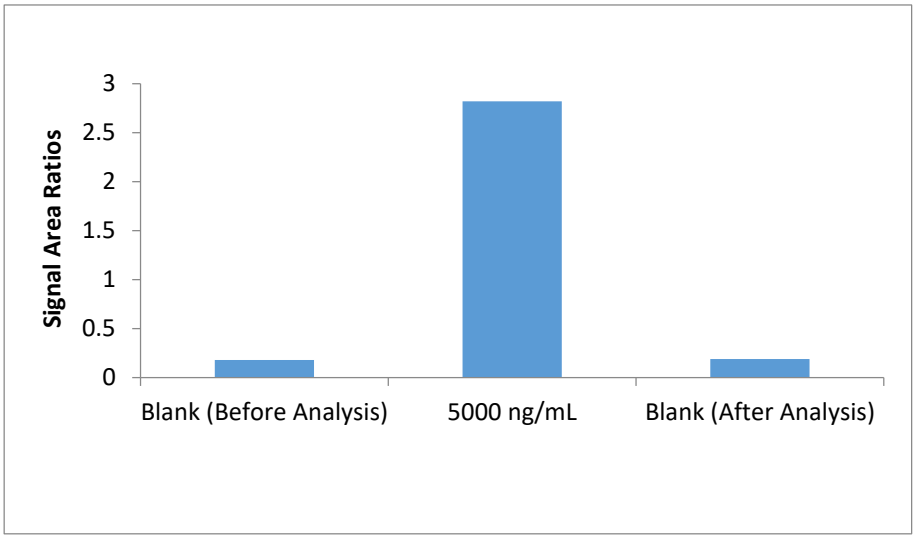


Figure 3.36. Carry-over test from previous analysis for cholesterol

Determination of repeatability and accuracy of the method:

For inter-day and intra-day repeatability, three quality control concentrations (300.0, 600.0 and 2000.0 ng/mL) were selected over the linear range of the calibration curve. The results obtained are summarized in Table 3.1. Here, the %RSD value over 15% (26.0%) at highest tested concentration was thought to be an adsorption problem since the error was negative and can be related to the molecule's lipophilic nature which means it is prone to have secondary adsorption to the surfaces.

Table3.1. Intra-day and inter-day reproducibility and accuracy of the developed method for cholesterol analysis by CBS-MS

QC	Repeatability (% Relative Standard Deviation)			Accuracy
ng/mL	Intra-day	Inter-day	Among Blades	Relative Error %
300.0	7.5	10.6	8.2	(+) 7.7
600.0	0.8	11.7	3.6	(+) 3.8
2000.0	4.4	**26.0**	4.2	(-) 12.6

Comparison studies with reference method in real sample:

The newly developed CBS-MS method was compared with the protein precipitation followed by LC-MS analysis using plasma sample. The experimental procedure is described in Section 2.8.1, the results of this study are given in Table 3.2. It was statistically examined whether the results of CBS-MS and PP-LC-MS differed significantly or not. First of all, it was checked whether there was a difference between the variances of the results obtained with the F-test and after it was proven that there was no difference, Student's t-test was applied, and the two results were compared at 95% confidence level. The t test results indicate that at 95% CL there is no difference between the results obtained with the two methods. These results show the reliability of the method.

Table3.2. Comparison of concentration of cholesterol in human plasma by CBS-MS and PP-LC-MS

	CBS-MS	PP-LC-MS
Concentration (μg/mL)	4560	4472
%RSD	2.9	3.4

After the validation studies for cholesterol were completed, validation studies were performed for testosterone and progesterone.

3.5.2 Testosterone and Progesterone Validation

Linearity and lower limit of quantitation (LOQ):

As the first step of validation for testosterone and progesterone, LOQs and linear ranges in CBS-MS were determined by matrix-matched CBS-MS.

In the validation studies for testosterone and progesterone, static extraction was performed on a CBS blade with 30.0 µL sample and SPME calibration was sketched after the CBS-MS. In this calibration, the LOQ values for testosterone and progesterone were found to be 25.0 ng/mL. Since lower concentrations can be found in real samples, in order to increase the sensitivity of the method, with increase in sample volume (200.0 µL) 10.0 ng/mL LOQ was reached for both analytes. Calibration graphs obtained in a typical run are shown in Figures 3.37 and 3.38 for testosterone and progesterone, respectively.

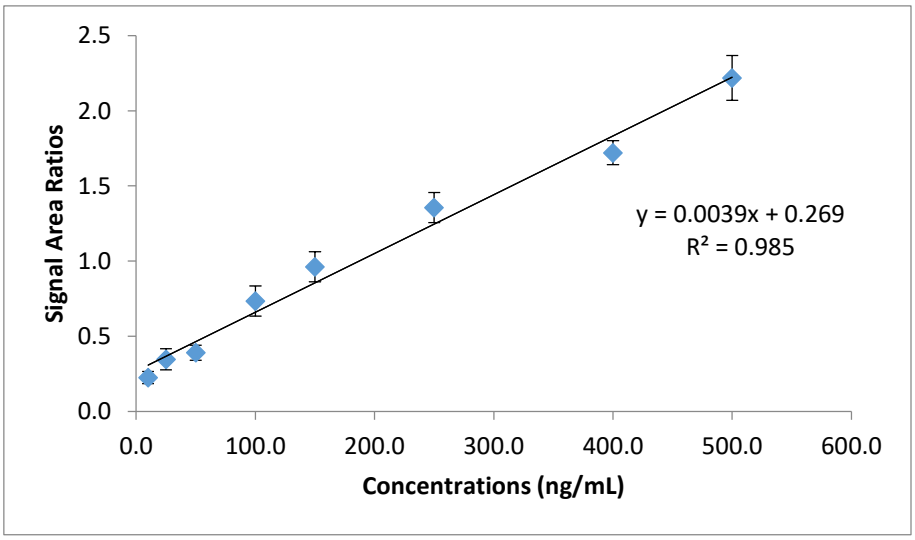


Figure 3.37. CBS-MS calibration graph for testosterone

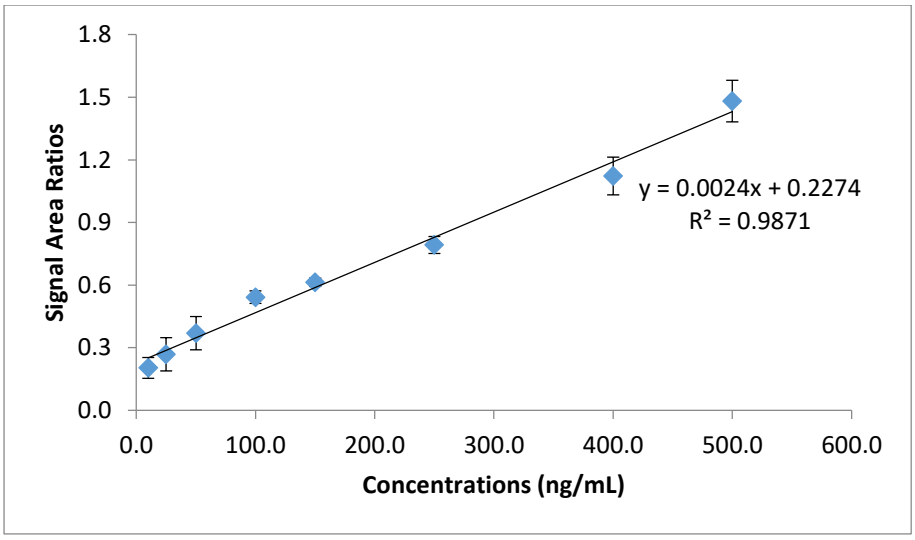


Figure 3.38. CBS-MS calibration graph for progesterone

Carry-over test:

After and before analyzing 500.0 ng/mL, which is the highest of the calibration graphs, blank analysis was performed and it was tested whether there was a carry-over from the previous analysis in the MS. The results obtained are shown in Figure 3.39. As can be seen, the signal received from the MS before and after 500.0 ng/ml is similar. This shows that there is no accumulation of testosterone and/or progesterone in the system.

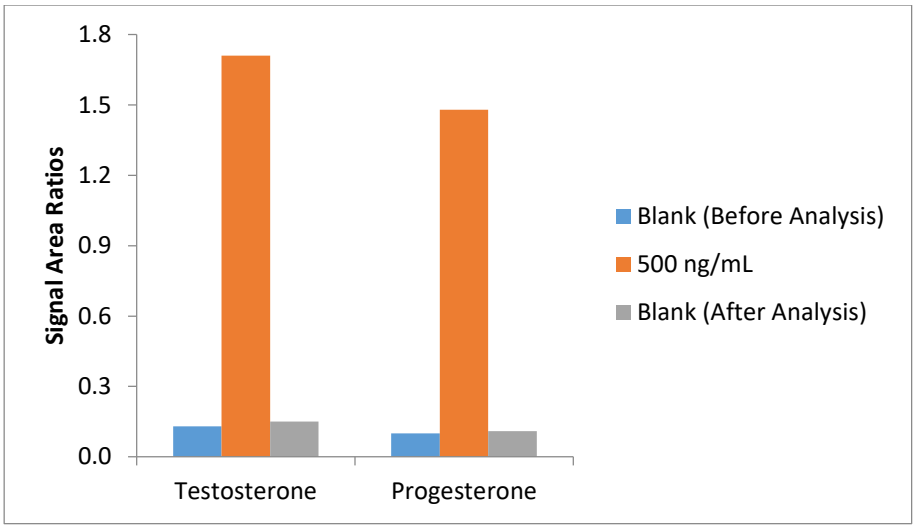


Figure 3.39. Carry-over test from previous analysis for testosterone and progesterone

Determination of repeatability and accuracy of the method:

For inter-day and intra-day repeatability, three quality control concentrations (15.0, 75.0 and 300.0 ng/mL) were selected over the linear range of CBS-MS. The experimental parameters used are summarized in Section 2.8.2. The results obtained in this study are summarized in Table 3.3 and 3.4 for testosterone and progesterone, respectively. The results show that, in general, both intra-day and inter-day reproducibly and accuracy are at acceptable levels. In case of testosterone, only at highest tested level, a high error (26.8%) was obtained. This observation could be related to slight modification of the matrix and change of the free concentration of analyte as a result of analyte spiking (by adding relatively large volume of organic solvent, especially at highest test level). In case of progesterone, high variations were obtained at low and mid-levels for inter-day reproducibility. Such variations, and errors can be further improved by the use of isotopologues of the analytes.

Table3.3. Intra-day and inter-day reproducibility of the developed method for testosterone analysis by CBS-MS

QC	Repeatability (% Relative Standard Deviation)			Accuracy
ng/mL	Intra-day	Inter-day	Among Blades	Relative Error %
15.0	6.3	10.0	8.6	(-) 5.2
75.0	1.7	2.4	1.6	(+) 13.6
300.0	0.7	1.3	3.0	**(+) 26.8**

Table3.4. Intra-day and inter-day reproducibility of the developed method for progesterone analysis by CBS-MS

QC	Repeatability (% Relative Standard Deviation)			Accuracy
ng/mL	Intra-day	Inter-day	Among Blades	Relative Error %
15.0	3.5	**22.4**	15.4	(+) 8.9
75.0	3.6	**17.2**	9.6	**(+) 16.0**
300.0	5.0	0.9	4.7	(+) 13.2

Comparison studies with reference method in real sample:

The newly developed CBS-MS method was compared with the protein precipitation followed by LC-MS analysis. The experimental procedure is described in Section 2.8.2, the results of this study are given in Table 3.5 and 3.6 for testosterone and progesterone, respectively. It was statistically examined whether the results differed significantly or not. First of all, it was checked whether there was a difference between the variances of the results obtained with the F-test and after it was proven that there was no difference, Student's t-test was applied, and the two results were compared at 95% confidence level. The t test results indicate that at 95% CL there is no difference between the results obtained with the two methods. These results show the reliability of the method.

Table3.5. Comparison of concentration of testosterone in human plasma by CBS-MS and PP-LC-MS

	CBS-MS	PP-LC-MS
Concentration (ng/mL)	132	147
%RSD	5.5	3.9

Table3.6. Comparison of concentration of progesterone in human plasma by CBS-MS and PP-LC-MS

	CBS-MS	PP-LC-MS
Concentration (ng/mL)	65	61
%RSD	8.8	14.7

CHAPTER 4

CONCLUSION

Within the scope of this thesis, a new extractive phase suitable for CBS-MS studies has been developed for rapid determination of the cholesterol, progesterone, and testosterone from the spot sized plasma samples.

For the preparation of the CBS probes, first of all, PDVB nanoparticles, as polymeric extractive phase with affinity for nonpolar analytes, were synthesized by the miniemulsion polymerization method. SEM images showed that the PDVB particles with 30-40 nm diameter were formed. The TGA of the PDVB particles, showed two stage weight lost. First lost, between 120– 240 ˚C, was caused by loss of various reaction wastes (reactive, short-chain oligomer structures) from the structure. Second lost, between 300-500 ºC was due to polymer decomposition which was in agreement with literature, proving that the polymer was synthesized successfully.

Next, the synthesized PDVB nanoparticles (30-40 nm) were immobilized into PAN nanofibrous phase by the electrospinning method. One of the major advantages of using electrospinning here is that it improves the diffusion kinetics of the analytes thanks to their nanofibrous structure, and the other is that it provides a large surface area for the analytes to interact with the extractive phase. During the optimization of electrospinning parameters, the effect of applied voltage, the feed rate and the distance between the collector and needle trip were studied. The smooth nanofibrous structure with efficient electrospinning was obtained with the following parameters: the distance between the needle tip and the collector was 15.0 cm, the feed rate was 2.4 mL/h, and the applied voltage was 20.0 kV. In addition, it has been found that the vertical set up of syringe was more efficient to obtain a homogeneous coating distribution on the blade. The SEM images also

showed that the PDVB nanoparticles were successfully immobilized within the PAN nanofibers.

To give HLB (hydrophilic-lipophilic balance) property to the PDVB-PAN extractive phase, electrospun PAN-PDVB coated blades were hydrolyzed with 2.0 M NaOH at 70.0 ^{o}C for 1 hour. The SEM images of HLB featured CBS coating showed that the PDVB nanoparticles were still embed within the PAN structure, and PAN nanofibers structure was preserved under the hydrolysis conditions. Besides, the hydrolysis of PAN was characterized by FTIR Spectroscopy. The characteristic bands for the carboxylate in the post – hydrolysis FTIR spectrum were observed in the range of 1510 – 1650 cm^{-1} for asymmetric vibration and 1280 – 1400 cm^{-1} for symmetric vibration. The broad peak at 3500 cm^{-1}, due to hydrogen bound OH also suggest the presence of carboxylic acid in the structure. Besides, O – H bending for carboxylic acid groups peaks were in the range of 1395 – 1440 cm^{-1}. In addition, C=O stretching for amide groups and carboxylic acid groups were observed in the range of 1680 – 1720 cm^{-1}. In overall, these results suggested that some of the nitrile groups of PAN were converted to amide and some to carboxylic acid groups showing that the weak ion exchange groups were introduced to the structure.

After the CBS probes were successfully prepared, the preliminary investigation of the coating's extraction performance was evaluated with LC-MS. For this purpose, a reverse phase method, using an Agilent, Poroshell 120 EC-C18, (4.6x150 mm, 4μm) column and MeOH:H_2O (80:20, v/v) containing 0.1% (v/v) formic acid, was developed under isocratic elution conditions. Using this developed LC-MS method, the extraction kinetics of newly developed PDVB-PAN coatings with HLB properties and CBS coated with commercial HLB particles using dip coating technique were compared. The extraction kinetics comparison showed that the new developed PDVB-PAN coatings have faster extraction kinetics.

Following, evaluation of the new CBS probes with LC-MS studies, a single quadrupole mass spectrometer and CBS blades were paired and optimized for rapid

analysis. In these studies, it has been observed that the voltage of the ESI source of the instrument was not sufficient to create electrospray from the blade. Therefore, ESI signal from the blades were obtained by the use of an external power source. During the optimization of electrospray parameters of CBS-MS, the effect of applied voltage and the distance between the blade and MS inlet were tested. These evaluations showed that the optimum distance between the MS input and the blade tip was 1 cm. Arc formation 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 and the best sensitivity was obtained when the voltage was set to 4.8 kV for the external source. Other instrumental parameters were set (or optimized in case of necessity) as follows. Fragmentor voltage was 110 V, capillary voltage was 3000 V. The temperature of the drying gas used was 300 °C and the flow rate was 3.0 L/min. The nebulizer pressure was set to 725 Torr.

After the CBS-MS coupling, the newly developed CBS coatings were optimized for direct to MS studies. For this purpose, the optimum desorption time and volume were studied, and the optimum analysis conditions were determined by examining the effect of parameter on the signal. In terms of solvent volume, it was found that solvent droplets larger than 30 µL were not stable on the blade and sometimes caused arcing during the voltage application. Very small volumes, less than 15 µL showed quick evaporation from the surface. Therefore, 15.0 µL was selected as optimum desorption volume. In these studies, also it was found that desorption time from the blade is critical, as short times may not be sufficient to desorb analytes and long times may result in complete solvent evaporation. The most successful desorption time was determined as 60 seconds, at which solvent evaporated completely, followed by addition of a fresh 15.0 µL desorption solution. The higher signals obtained in this way shows that the analytes were desorbed and moved to the sorbent surface in the first solvent droplet and then easily transferred for analysis in the second droplet.

In summary, the final developed method takes only 7 min in total, from extraction to analysis, with the following experimental parameters. Extraction conditions, sample volume: 30.0 μL, extraction time: 5 min static extraction. CBS-MS conditions, desorption solvent: isopropyl alcohol containing 0.1% FA (v/v), desorption solvent volume: 15.0 μL, desorption time: 60 s (to the dryness) followed by fresh 15.0 μL desorption solution and analysis, signal acquisition time: 60 s in positive ionization SIM mode, external voltage: 4.8 kV.

Method validation with these parameters shows that LOQ of 200.0 ng/mL can be reached for cholesterol, and 25.0 ng/mL for progesterone and testosterone, with a sample volume of 30.0 μL. However, considering that progesterone and testosterone can be found in lower concentrations in real samples, the method was modified for these hormones (200 μL sample volume and 5 min mixing extraction) and LOQ values of 10.0 ng/mL were achieved. The methods accuracy and precision results showed in general acceptable values for all analytes. In case of cholesterol, the %RSD value over 15% (26.0%) at highest tested concentration (2000.0 ng/mL) was most likely associated with secondary adsorption problem since lower value than the spiked amount was obtained and can be related to the molecule's lipophilic nature. In case of testosterone, only at highest tested level, a high relative error (26.8%) was obtained. This observation could be related to slight modification of the matrix and change of the free concentration of analyte because of analyte spiking (by adding relatively large volume of organic solvent, especially at highest test level). In case of progesterone, high variations were obtained at low and mid-levels for inter-day reproducibility. Such variations, and errors can be further improved using isotopologues of the analytes. Finally, the developed method (CBS-MS) was used for analysis of human plasma for determination of targeted analytes and compared to protein precipitation method followed by LC-MS analysis. The statistical evaluation of the results using t test for comparison of two experimental mean suggested that there were no significand differences between the results of the two methods at 95% CL, demonstrating the reliability of the method developed in this thesis.

In summary, the results obtained in this thesis show that the study reached its initial goals and was completed successfully. Although the analytical method parameters obtained in the study are generally prosperous and suitable for the targets, it will be possible to further improve the variations in cases where it is possible to obtain the isotopologues of all analytes. In addition, it is predicted that the obtained method sensitivities may reach lower levels if triple quadrupole is used instead of a single quadrupole mass spectrometer.

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APPENDICES

A. Representative Mass Spectra and Chronograms

Figure A.1 Mass spectrum of testosterone (m/z=289), progesterone (m/z=315), cortisone (m/z=361), cholesterol (m/z=369), and ergosterol (m/z=379)

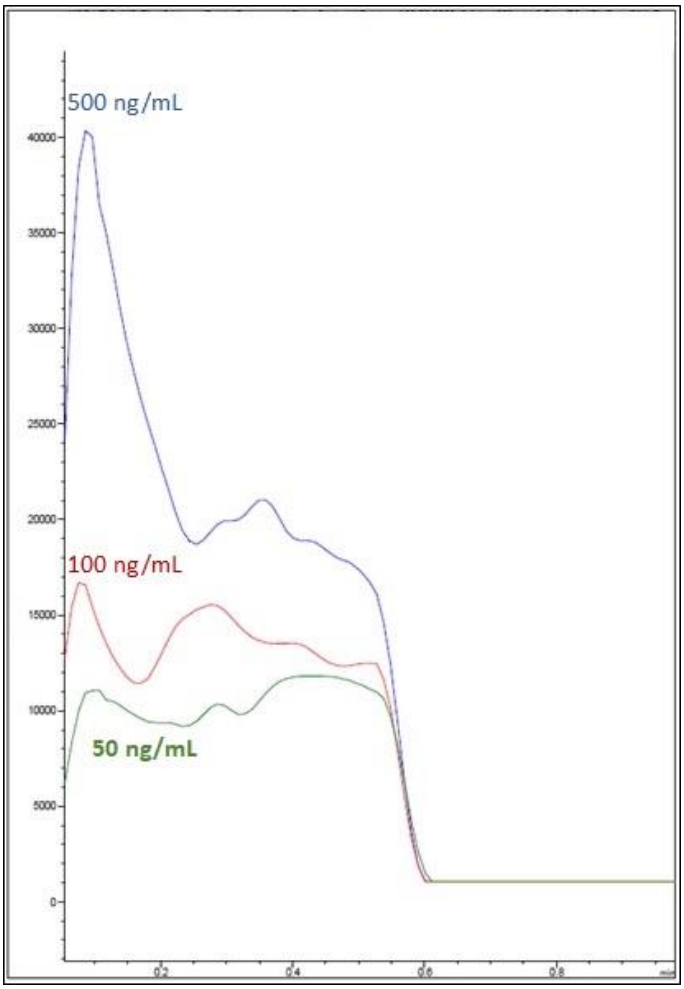

Figure A.2 Total ion chronograms obtained in a typical CBS-MS study