EXTRACTION OF SULFONAMIDES FROM HONEY USING ADMICELLE SOLID PHASE EXTRACTION SORBENT PRIOR TO LIQUID CHROMATOGRAPHIC DETERMINATION

A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of
Dokuz Eylül University In Partial Fulfillment of the
Requirements for the Degree of Master of
Science in Chemistry

by

Paniz TASHAKKORI

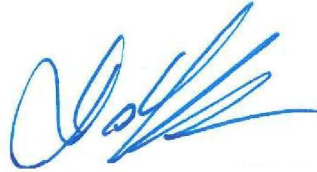
July, 2013
İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"EXTRACTION OF SULFONAMIDES FROM HONEY USING ADMICELLE SOLID PHASE EXTRACTION SORBENT PRIOR TO LIQUID CHROMATOGRAPHIC DETERMINATION"** completed by **PANIZ TASHAKKORI** under supervision of **ASSOC. PROF. DR. SERAP SEYHAN BOZKURT** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

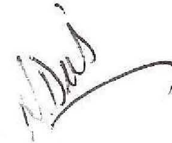
Assoc. Prof. Dr. Serap Seyhan Bozkurt

Supervisor



Prof. Dr. Delya Sponza

(Jury Member)



Assoc. Prof. Dr. M. Nalan Tüzmen

(Jury Member)

Prof.Dr. Ayşe OKUR
Director
Graduate School of Natural and Applied Sciences

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Serap Seyhan Bozkurt for her intelligent supervision, constructive guidance, inspiration, encouragement and friendship throughout the completion of this work. Thanks a lot because without your support, I could not have done this work.

I would like to thank Prof. Dr. Melek Merdivan for the experience she shared with me through meeting and discussion. It was a great honor for me to make use of her experience.

I want to thank Dr. Mine Antep for helping me during laboratory studies, discussion and for her valuable contribution.

I would like to express my appreciation to Dokuz Eylul University for providing the financial support through AFP project numbered as 2011.KB.FEN.036. Also, I would like to thank Prof. Dr. N.Bizsel for providing HPLC which was financially supported by project TSTRI, COST 110Y193.

I would also like to express my deep gratitude for my family, and my fiance for their continuous and strong moral support, encouragement and patience throughout my endeavour for realization of this study.

Finally, I would prefer to say to all my friends.

Paniz TASHAKKORI

EXTRACTION OF SULFONAMIDES FROM HONEY USING ADMICELLE SOLID PHASE EXTRACTION SORBENT PRIOR TO LIQUID CHROMATOGRAPHIC DETERMINATION

ABSTRACT

A new solid phase extraction method for four sulfonamides as sulfadiazine, sulfamethazine, sulfamerazine and sulfamethoxazole in honey was developed. Fortified honey samples were studied in order to check the accuracy of this method.

The sorbent used in the solid phase extraction method was prepared by modifying silica at pH 9 with surfactant such as cetlytrimethyl ammonium bromide. The FTIR and SEM analysis of the prepared sorbent were performed.

The proposed method was optimized in terms of some parameters; pH of sample solution, amount of surfactant, eluent type and volume, flow rate using test solutions containing sulfonamides at a concentration of 0.5 mg per L. The optimized values are; pH 6, 50 mg of surfactant, 4 mL of methanol: water with ratio of 1:1 as desorption solvent mixture and 1.5 mL per min for flow rate of loading of sample.

The analysis of sulfonamides were carried out on HPLC-DAD using mobile phase as methanol: acetonitrile: 0.05 M formic acid with ratio of 10:15:75. Chromatographic separation was obtained by isocratic elution. The sulfonamides were detected at 272 nm. The detection limit of the HPLC method was in the range of 3-13 ng per mL for all analytes.

The linear ranges of sulfonamides were from 0.010 to 2.000 mg per L. The precision of this method as relative standard deviation percentage, RSD percentage, at 0.1 mg per L was found less than 5.91 percentage for all analytes for 5 replications.

The amounts of sulfonamides in honey samples by this method were found in the range of 0 to 1.30 ppm. The recovery as percentage of this method using fortified honey samples were above mostly 70 percentage for all studied sulfonamides.

Keywords: Sulfonamides, honey, solid phase extraction, HPLC.

BALDA SÜLFONAMİDLERIN SIVI KROMATOGRAFİSİ İLE TAYİNİ ÖNCESİ ADMİSEL KATI FAZ EKSTRAKSİYONU SORBENTİ KULLANILARAK AYRILMASI

ÖZ

Baldan sülfadiazin, sülfametazin, sülfamerazin, sülfametoksazol gibi sülfonamidlerin ekstraksiyonu için yeni katı faz ekstraksiyon yöntemi geliştirildi. Yöntemin doğruluğunu kontrol etmek için bal örneklerine sülfonamid eklendi.

Katı faz ekstraksiyon yönteminde kullanılan sorbenti hazırlamak için pH 9 da setiltrimetil amonyum bromür yüzey aktif maddesi silika ile modifiye edildi. Hazırlanan sorbentin FTIR ve SEM analizleri yapıldı.

Önerilen yöntem örnek çözeltisinin pHsı, yüzey aktif madde miktarı, elüent türü ve hacmi, akış hızı açısından optimize edildi. Bu parametreler için yapılan çalışmalarda 0,5 ppm sülfonamidleri içeren sentetik çözeltiler kullanıldı. Optimizasyon sonucu elde edilen sonuçlar; pH 6, 50 mg yüzey aktif madde, elüent çözücüsü olarak 1:1 oranında 4 mL metanol:su ve örneğin yükleme akış hızı dakikada 1,5 mL olarak belirlendi.

Sülfonamidlerin analizi, mobil faz olarak 10:15:75 oranında methanol: asetonitril: 0,05 M formik asit kullanılarak HPLC-DAD ile gerçekleştirdi. Kromatografik ayırmada izokratik elüsyon kullanıldı. Sülfonamidler 272 nm'de tayin edildi. HPLC yönteminin gözlenebilme sınırı tüm analitler için mililitrede 3-13 ng olarak bulundu.

Sülfonamidlerin 0,01 ile 2,000 ppm derişim aralığında doğrusal olduğu gözlendi. Yöntemin tekrarlanabilirliği yüzde bağıl standart sapma, BSS, olarak tüm analitler için 5 paralel çalışma sonucunda litrede 0,1 mg için 5,91 den az bulundu. Bu yöntem ile bal örneklerinde bulunan sülfonamid miktarının litrede 0 ile 1,30 mg aralığında olduğu

gözlendi. Yöntemin yüzde geri kazanımı sülfonamid eklenmiş bal örneklerinde çoğunlukla yüzde 70 üzerinde bulundu.

Anahtar sözcükler: Sülfonamid, bal, katı faz ekstraksiyonu, HPLC.

CONTENTS

Page

MSc THESIS EXAMINATION RESULT FORM ii

ACKNOWLEDGEMENT iii

ABSTRACT iv

ÖZ vi

LIST OF FIGURES xi

LIST OF TABLES xiii

CHAPTER ONE – INTRODUCTION 1

1.1 Antibiotics 1

1.1.1 Kinds of Antibiotics 2

1.2 Sulfonamides (SAs) 3

1.2.1 Sulfadiazine 4

1.2.2 Sulfamethazine 4

1.2.3 Sulfamerazine 5

1.2.4 Sulfamethoxazole 5

1.3 Contamination of Food Products by Antibiotics 6

1.3.1 Honey 6

1.3.2 Effects of Sulfonamides 7

1.3.3 Maximum Residue Limits of Sulfonamides 7

1.4 Analysis of Sulfonamides in Food Products (honey) 8

1.4.1 Screening Methods 8

1.4.2 Clean-up Procedures 9

1.5 Solid Phase Extraction Method 9

1.5.1 Silica 10

1.5.2 Surfactants 11

1.5.3 Hemimicelle/Admicelle 13

1.6 High Performance Liquid Chromatography 14
1.7 Related Studies for Determination of SAs in Honey by HPLC 16
1.8 Purpose of the Study 18

CHAPTERTWO-EXPERIMENTAL METHODS AND MATERIAL 19

2.1 Reagents 19
2.2 Preparation of Reagent Solutions 19
2.3 Apparatus 20
2.3.1 Fourier Transform Infrared (FTIR) Spectrometer 20
2.3.2 Scanning Electron Microscope (SEM). 20
2.3.3 HPLC 20
2.4 Liquid Chromatographic Conditions for Sulfonamides 21
2.5 Calibration Curves of Sulfonamides and Determination of Limit of Detection and Limit of Quantification in HPLC 22
2.6 Honey Samples 23
2.7 Preparation of Honey Sample Solutions 23
2.8 Preparation of Silica-CTAB Sorbent 23
2.9 Solid Phase Extraction Procedure for Determination of Sulfonamides in Honey Samples 24

CHAPTER THREE – RESULT and DISCUSSION 25

3.1 Type of Surfactants 25
3.2 FTIR Analysis 25
3.3SEM Analysis 26
3.4 Optimization Parameters of Solid Phase Extraction Method Used for Determination of Sulfonamides in Honey Samples 28
3.4.1 Effect of pH 28
3.4.2 Effect of Surfactant Amount 29

3.4.3 Effect of Eluent Type and Volume 29
3.4.4 Effect of Flow Rate 30
3.5Preconcentrationfactor and Recovery 31
3.6 Analytical Performance 32
3.7 Determination of Sulfonamides in Honey Samples 35

CHAPTER FOUR – CONCLUSION 38

REFERENCES 39

LIST OF FIGURES

Page

Figure 1.1Structure of sulfonamide.. 3

Figure 1.2Structure of sulfadiazine... 4

Figure 1.3Structure of sulfamethazine.. 5

Figure 1.4Structure of sulfamerazine.. 5

Figure 1.5Structure of sulphamethoxazole 6

Figure 1.6A typical SPE sequence 10

Figure 1.7Surfactant.. 13

Figure 1.8Hemimicelle and Admicelle 14

Figure 2.1The SHIMADZU 20A series HPLC system.. 21

Figure 2.2a) The HPLC chromatogram of four sulphonamides and b) four sulphonamides with CTAB.. 22

Figure 3.1Extractability of surfactants for studied sulfonamides 25

Figure 3.2The FTIR spectra of a) silica, b) silica-CTAB, and c) silica-CTAB/sulfonamide 26

Figure 3.3Scanning electron micrograph of silica at 100 and 2500 times of magnification... 27

Figure 3.4Scanning electron micrograph of silica-CTAB at 100 and 2500 times of magnification... 27

Figure 3.5Scanning electron micrograph of silica-CTAB/sulfonamide at 100 and 2500 times of magnification. 27

Figure 3.6pH effect on sorption of sulfonamides (N = 3)...................................... 28

Figure 3.7Effect ofAmount of surfactant on sorption of sulfonamides (N = 3). Exp. conditions: 250 mg sorbent, c_{int}(SAs) = 0.50 µg mL^{-1}, pH = 6, sample volume: 10 mL 29

Figure 3.8Type of elution solvent/mixture on sorption of sulfonamides (N = 3). Exp. conditions: 250 mg sorbent, c_{int}(SAs) = 0.50 µg/mL, pH = 6, sample volume: 10 mL, V_{eluent}: 2 mL.. 30

Figure 3.9Effect of flow rate on sorption of sulfonamides (N = 3) 31
Figure 3.10Effect of sample volume on sorption of sulfonamides 32
Figure 3.11Instrumental calibration curves of SDA 33
Figure 3.12Instrumental calibration curves of SMR.. 33
Figure 3.13Instrumental calibration curves of SMT 34
Figure 3.14Instrumental calibration curves of SMX.. 34

LIST OF TABLES

Page

Table 1.1Kinds of antibiotics 2

Table 1.2Maximum residue limit (MRL) value for sulfonamides.............................. 7

Table 1.3Classification and characteristics of surfactants 12

Table 1.4Important relationships in HPLC 15

Table 3.1Analytical performance of the proposed method....................................... 34

Table 3.2Levels of sulfonamides in honey samples (N=3)....................................... 36

CHAPTER ONE
INTRODUCTION

1.1 Antibiotics

Antibiotics, by far, are the most important compounds made by microbiological synthesis. Since the discovery of early antibiotics more than 5500 natural microbial compounds which display antibiotic activity have been described. Only a relatively small number of these products has found practical use, indeed about 150 antibiotic compounds are currently being product exclusively by fermentation processes and find application in medical, veterinary and agricultural practice as products with antibacterial, antifungal, antiviral, antitumor, piscicidal or insecticidal activity.

Antibiotics have indeed found extensive application in the treatment of infectious diseases of man, animals and to a smaller extent, plants. Several compounds are used in animal nutrition, and a few in food conservation. Nowadays, animal source food take place in human diet in developing countries in conjuction with increasing incomes, changing life-style in city life (Delgado, Rosegrant, Steinfeld, Ehui &Courbois, 1999). The widespread usage of antibiotics in veterinary practices can lead to the presence of residues in foodstuffs of animal origin (Samanidou, Tolika & Papadoyannis, 2008). Residues of antibiotics can have a harmful effect on human health, such as allergic reactions in some hypersensitive individuals and may compromise the human immune system. The presence of low levels of residues in foodstuffs for long period has led to the problem of resistance in pathogens in human body (Vincent, Chedin, Yasar & van Holst, 2008; Koesukwiwat, Jayanta & Leepipatpiboon, 2007). Therefore their use in animal husbandry must be subjected to strict control (Wang, Yang, Zhang, Mo & Lu, 2008).

In many countries, governmental authorities have established monitoring programmes to determine antibiotic levels in foods, as well as the highest allowable residue levels (Ramrrez & et al. 2003). In addition, authorities around the world have laid down a large number of regulations to ensure food safety and reduce human

exposure. In the European Union, maximum residue limits (MRLs) have been established for antibacterials in animal-derived foods, and in the case of feeds no antibiotics other than coccidiostats and histomonostats can be marketed and used as feed additives. Moreover, medicated feeds, which contain active principles at therapeutic levels, must be prepared from authorized medical premixes and used under veterinary prescription (European Union, 1990). Currently, no MRLs have restricted for use with bee products. Antibiotics have had MRLs fixed for their utility in large animals but are illegal for use with bees. Honey is considered to be natural healthy food containing primarily sugar and water (Bernal, Nozal, Jimenez, Mratín & Sanz, 2009). On the other hand, all antibiotics are not detected by one method at their MRL levels. For example produce a good technological quality of raw milk and also, healty and reliable milk and milk products for consumer, veterinary drug in milk and milk products must be checked. For this purpose, the antibiotic detection has been done by the use of various methods.

1.1.1 Kinds of Antibiotics

Antibiotics are classified with respect to bacterial spectrum or type of activity or mostly chemical structure (Table 1.1). Antibiotics having similar structure show generally similar effectiveness, toxicity and allergic potential.

Table 1.1 Kinds of antibiotics

Aminoglycosides	Lincosamides
Ansamycins	Lipopeptide
Carbacephem	Macrolides
Carbapenems	Monobactams
Cephalosporins (1st generation)	Nitrofurans
Cephalosporins (2nd generation)	Penicillins
Cephalosporins (3rd generation)	Polypeptides
Cephalosporins (4th generation)	Quinolones
Cephalosporins (5th generation)	**"Sulfonamides"**
Glycopeptides	Tetracyclines

1.2 Sulfonamides (SAs)

Sulfonamide is an organic sulfur compounds shares a common p-aminobenzoyl ring moiety with an aromatic amino group at the N4-position, differing in the substitution at the N1-position (Figure 1.1).

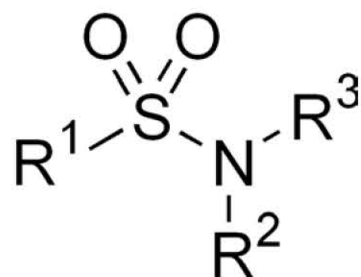


Figure 1.1 Structure of sulfonamide.

Historically, sulfonamides have been used as synthetic antimicrobial agents (Lai & Hou, 2008), for prevent and treat bacterial infective diseases (Sun & et al, 2009) because of their broad spectrum of activity and low costs (Lara, García- Campan, Neusüss, & Alés-Barrero, 2009). Using the large quantities of sulfonamides in animal husbandry in particular as veterinery medicines, we can obtain a reliable estimation of the effects and consequences of prolonged use of anti-infectives on people's health and environment (Baran, Adamek, Ziemianska & Sobczak, 2011). Therefore residues of sulfonamides in the food chain are of an increasing concern due to their carcinogenic potency and their contribution to an increase of antibiotic resistance.

Most of the papers reviewed refer to the analysis of sulfonamides in different matrices such as muscle, liver, kidney, skin and fat. Other matrices analyzed are egg, milk, fish, honey (Tsai, Huang, Chen, Wu & Huang, 2010; Fernandez-Torres & et al. 2011; Bernal, Nozal, Jimenez, Mratín & Sanz, 2009). With respect to whole analytes, most studies deal with sulfamethazine (SMT), sulfadiazine (SDA), sulfamerazine (SMR) and sulfathiazole (STZ) using other examples with lower frequency. However sulfathiazole was initially recommended to control, at

this moment, its use banned because there were found residues many months after being applied.

1.2.1 Sulfadiazine (SDA)

In low concentrations, sulfadiazine (pK_a 6.5) acts as a bacteriostat and as a bactericide in high concentrations (Figure 1.2). Mechanism depends on its competitive antagonism with para-aminobenzoic acid. In most animals, other than ruminants cattle, sulfadiazine is rapidly absorbed and distributed throughout the entire body parts. This is readily crosses the blood-brain barrier depending on the type of animal. Sulfadiazine can be acetylated or hydroxylated in the liver. Acetyl sulfadiazine is not active bacteriologically, but has toxic properties in all the body excretions. The rate of excretion is dependent on the pH of the tubular fluid and on the renal flow.

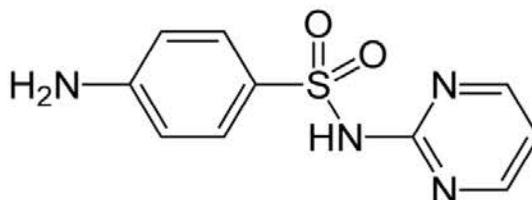


Figure 1.2 Structure of sulfadiazine.

1.2.2 Sulfamethazine (SMT)

Sulfamethazine (pK_a 7.45) is primarily used as a veterinary antibacterial drug in food animals and has been detected in airborne dust collected from a pig-fattening farm (Figure 1.3). Sulfamethazine has been used as a tool for phenotyping people as slow acetylators or fast acetylators for NAT2 substrates. Sulfamethazine-induced thyroid cancer is thought to involve a cascade of effects starting with the inhibition of the enzyme thyroid peroxidase, leading to decreased thyroxine synthesis, with a consequent increase in thyroid stimulating hormone secretion from the pituitary (Choquet-Kastlevsky, Vial, & Descotes, 2002).

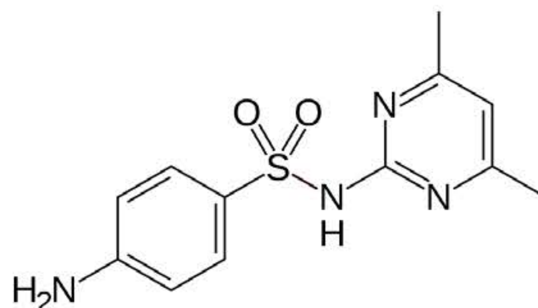


Figure 1.3 Structure of sulfamethazine.

1.2.3 Sulfamerazine (SMR)

Sulfamerazine (pK_a 6.98) is most often used as part of a synergictic combination with trimethoprim (Figure 1.4). Its primary activity is against susceptible forms of Streptococcus, Staphylococcus aureus, Escherichia coli, Haemophilus influenzae and oral anaerobes. It is commonly used to treat furunculosis, columnaris and bacterial kidney disease infection of fish (Brander, Pugh & Bywater, 1982, Kaya, Pirincci & Bilgili, 1997).

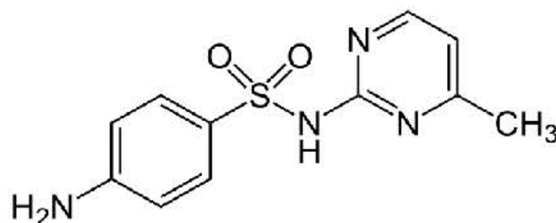


Figure 1.4 Structure of sulfamerazine.

1.2.4 Sulfamethoxazole (SMX)

Sulfamethoxazole (pK_a 5.81) is a sulfonamide bacteriostatic antibiotic (Figure 1.5). It is commonly used to treat urinary tract infections. In addition it can be used as an alternative to amoxicillin-based antibiotics to treat sinusitis. It can also be used to treat toxoplasmosis and it is the drug of choice for Pneumocystis pneumonia, which affects primarily patients with HIV. The most common side effect of sulfamethoxazole/trimethoprim is gastrointestinal upset. Allergies to sulfa-based

medications typically cause skin rashes, hives, or trouble breathing or swallowing and nausea, severe stomach, or abdomen pains (Ma, Cheng, Xu, Qu & Fang, 2007).

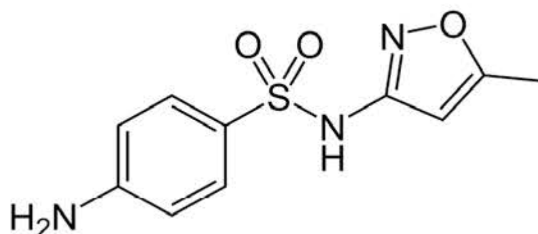


Figure 1.5 Structure of sulphamethoxazole.

1.3 Contamination of Food Products by Antibiotics

Antibiotics for the treatment and prevention of bacterial infections generally used as antimicrobial agents in veterinary and human medicine. The ingestion of antibiotics for a long time can promote the growth effect because it is also used as an additive in animal feed. Appropriate withdrawal period are not observed before slaughter or milking medicated animals, food ingredients derived from animals may be contaminated with antibiotics. These residues may cause adverse effects in some humans (Seifrtova, Novakova, Lino, Pena & Solich, 2009).

1.3.1 Honey

Prevention and treatment of bacterial bee diseases with sulfonamides can cause the remaining of these compounds in honey. Residues of these antibacterial drugs in honey are of major concern because of their contribution in development of antibiotic-resistant pathogenic bacteria. The free aromatic amino group of sulfonamides could react with reducing sugars to form a variety of different sugar-bound compounds, which had a different chromatographic behavior. Sulfonamides were not destroyed in these reactions (Maudens, Zhang, Lambert, 2004).

1.3.2 Effects of Sulfonamides

Sulfonamides are the most common contaminating antimicrobials in animal feed, generating potentially serious problems in human health, such as allergic or toxic reactions. Furthermore, the main risk from the excessive use of antimicrobials in animals is that bacteria may develop resistance. Some sulfonamides are also potentially carcinogenic, causing considerable debate about food safety. Approximately 5% of human patients medicated with sulfonamides may have adverse response to these drugs (Bevill, 1984). In addition, after use, large amounts of sulfonamides have been discharged into the environment in the form of unused waste. Differentiation has seen in the retainability of sulfonamides in the environment, their spreading rate and their accumulation in the biosphere. However, it could be mentioned that the antibiotics cause significant changes in the biosphere due to their high biological activity. (Baran, Adamek, Ziemianska & Sobczak, 2011).

1.3.3 Maximum Residue Limits of Sulfonamides

The Australian Pesticides and Veterinary Medicines Authority regulates the maximum residue limits for agricultural and veterinary chemicals in agricultural production. The presence of antibiotics above the maximum residue limits in food could be dangerous for consumers. Consumers can be proptected by designating the maximum residue limits in many countries by official authority. The European Union regulations for sulfonamides in foods are given In Table 1.2 (EC,1990).

Table 1.2 Maximum residue limit (MRL) value for sulfonamides

	FOOD	MRL (µg/kg)
All sulfonamide groups	muscle, fat, liver, kidney,	100
	chicken	100
	milk	100
	egg	100
	honey	100

1.4 Analysis of Sulfonamides in Food Products Such as Honey

Various methods are available to measure sulfonamide residues in honey and other matrixes. Those methods included mainly: colorometric procedures (Schwartz, & Sherma, 1986), enzyme immunoassay (Thomson & Sporns, 1995), thin-layer chromatography (Sherma & et al. 1989), gas chromatography (Tarbin, Clarke & Shearer, 1999), and reversed-phase high performance liquid chromatography (HPLC) (Posyniak, Zmudzki & Mitrowska, 2005; Zotou & Vasiliadou, 2006).

1.4.1 Screening Methods

Screening methods are used to detect the presence of a substance or class of substances at the level of interest and are specifically designed to avoid false compliant results (Rodríguez, Ortiz, Sarabia & Herrero, 2010).

The first test, microbial inhibitor test, for establishing antimicrobial agent residues in milk was developed as early as 1952. Then, it was understood that the presence of these agents could cause the inhibition of the starter cultures used in dairy industry, and the methods have been developed to check and restrict the level of these agents in milk. The microbial inhibitor methods are preferred because of their advantages as cheapness, simplicity and detecting wide range of antimicrobial agents. However, their disadvantage is long incubation time (Mitchell, Griffiths, McEwen, McNab, & Yee, 1998).

Nowadays, high performance liquid chromatography is used because of attaining good qualitative and quantitative evaluation using different detection systems. Fluorescence detector is a good alternative, mainly due to its intristic sensitivity, but sometimes the analytes need to be derivatized with a fluorescence reagents before or after column. Mostly, analyte is derivatized after column with fluorescamine for the HPLC determination of sulphonamides (Bernal, Nozal, Jimenez, Mratín & Sanz, 2009).

At the present time, mass spectrometric detectors have been prefered in spite of their cost (Lu, Chen, & Lee, 2007). Some matrix influences may seen during monitoring of sulfonamides by HPLC with a UV dedector, but these can be eliminated by using an appropriate extraction and preconcentration procedures.

1.4.2 Clean-up Procedures

Mostly, direct analysis of extract containing analyte is not possible because of possible presence of matrix interferences. Clean-up procedure is generally required in order to prevent interference effects coming from matrix and/or reduce the detection limits of methods. Extensive clean-up of extracts may result in the partial loss of some compounds as well as increased labour and cost demands. However, inadequate clean-up can lead to adverse effects related to the quality of the produced data. The generally used sample clean-up techniques are liquid–liquid extraction, solid-phase extraction, solid-phase microextraction, and matrix solid-phase dispersion. Solid-phase extraction is being substantially used in food analysis, especially for sample clean-up. Normal or reversed solid-phase extraction cartridges provide the possible simple purification of analyte from matrix, reduction of the volume of solvent consumed, automation and high sample throughput (Hamide, & Glibert, 2010).

1.5 Solid Phase Extraction (SPE) Method

SPE is currently being used as a preconcentration or separation technique whenever complex matrices or low concentration analytes have to be analyzed. It is now the most common sampling technique in many areas of chemistry, including environmental, pharmaceutical, clinical food and industrial chemistry. The basic principle of SPE is the transfer of analytes from the aqueous phase to the active sites of the adjacent solid phase. This is termed as solid-phase extraction. The transferring of solute from aqueous solution to solid sorbent is encouraged by optimizing experimental conditions between aqueous phase, analyte and sorbent. After sorption

onto solid sorbent, the analyte is eluted with a suitable solvent and further analyzed (Martinez, Cugat, Borrull & Callul, 2000; Bruzzoniti, Sarzanini & Mentásti, 2000).

In a typical SPE system, the column is firstly conditioned, and activated with a solvent, water or blank solution. Then, the aqueous sample solution is passed through the column and the analyte with or without interfering compunds is trapped. Preferably, the interferences are mostly removed by rinsing the column after loaded with analyte. Finally, the analyte is eluted with an organic solvent or solvent mixture and further preconcentration takes place by evaporation under N_2 (Figure 1.6).

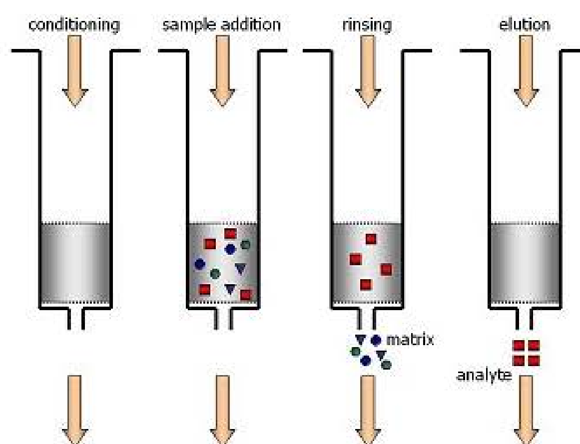


Figure 1.6 A typical SPE sequence.

Solid phase extraction is prefered to liquid-liquid extraction because it has some advantages as flexibility, low cost, less reagent consumptiont, absence of emulsion, speed and simplicity, sampling in the field, safety and ease of automation (Pyrzynska & Trojanowicz, 1999).

1.5.1 Silica

Generally, silica gel is defined as absorbing moisture when it is stored in packs. However, it removes moisture by adsorbing on surface of its numerous pores as material.

Silica gel is used as a stationary phase in several types of chromatography. In thin layer chromatography, aluminum, glass or plastic sheets coated with silica gel are used as stationary phase. In column chromatography, silica gel having different particle sizes is used as stationary to get intended separation of solutes. Non polar solutes are primarily eluted from the column because of the polarity of silica gel. This type of chromatography is known as normal-phase chromatography. But, when silica gel surface is modified by attaching C2, C8, C18 groups, the polar components elute first. This method is named as reverse phase chromatography. Besides hydrophobic groups, the hydroxyl groups on silica gel surface can be functionalized by such as amine or cyano groups to provide special silica gels that show unique stationary phase parameters. They are called as functionalized silica gels which are also used in organic synthesis and purification as insoluble reagents.

Silica gel has been modified chemically by covalent bound chelating groups. They can given off metal ions from aqueos media. The mechanical strength of silica gel surface has been progressed by grafting polyamines which bound covalently to chelating groups. Silica gel can act as reducing agent by combining with alkali metals (Dye & et al. 2005).

1.5.2 Surfactants

Surfactants (Figure 1.7) are amphiphilic organic compounds. Their molecules contain both long hydrophobic hydrocarbon groups (their tails) and hydrophilic groups (their heads). A typical surfactant has a R-X structure, where R is a hydrocarbon chain, which can have between 8 and 18 atoms of carbon, and X is the polar or ionic head group (Maniasso, 2001). Its most usual chemical classification is based on the hydrophilic group nature. Surfactant can be catinoic, anionic, non-ionic, and zwitterionic. In Table 1.5, the classification, the characteristics and the examples of surfactants are given.

Table 1.3 Classification and characteristics of surfactants

Classification	Characteristic	Example
Anionic	The hydrophilic group carrying a negative charge such as carboxyl (RCOO), sulfonate (RSO_3), or sulfate ($ROSO_3^-$)	$CH_3(CH2)_{11}SO_4$ $-Na^+$ Sodium dodecil sulfate (SDS)
Cationic	The hydrophilic group carrying a positive charge as, for example, the quaternary ammonium halides (R_4N^+Cl)	$CH_3(CH2)_{15}N^{+(}CH3)_3Br^-$ Cetyl trimetyl ammonium bromide (CTAB)
Nonionic	The hydrophilic group has no charge but derives its water solubility from highly polar groups such as polyoxyethylene or polyol groups	$CH_3(CH2)_{11}(OCH_2CH_2)_{23}OH$ Polyoxyethylene (23) dodecanol (Brij 35)
Zwitterionic	Its molecules present both th anionic and cationic groups and, depending of pH, its prevalence the anionic, cationic, or neutral species	$CH_3(CH2)_{11}N^{+(}CH_3)_2$ $(CH_3)COO^-$ 4-(Dodecyldimetyl

Besides the composition, the critical micelle concentration (CMC) is acquired. Micelles are molecular clusters possessing both hydrophilic and hydrophobic phases that are generated by surfactants that associate spontaneously in aqueous solutions starting at a certain concentration, termed the CMC (Dantas & et al. 2003, 2002). The CMC of a surfactant depends on several factors, such as its molecular structure, and experimental conditions such as ionic strength, counterions, temperature, etc. Below the CMC, the surfactant is primarily in a nonassociate monomer form. However, when the CMC is reached, the formation process is prefered. Micelles are thermodynamically stable and easily reproducible, but they can be destroyed by water dilution when the surfactant concentration is below its CMC (Maniasso, 2001; Myers, 1991; Sanz-Medel, Campa, Gonzalez & Fernandez-Sanchez, 1999; Pelizzett & Pramauro, 1985).

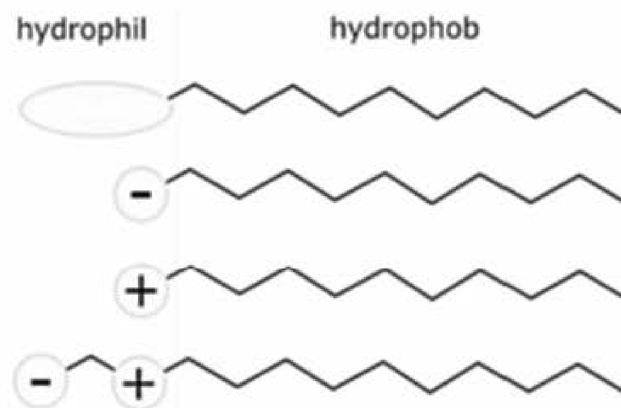


Figure1.7 Surfactant.

1.5.3 Hemimicelle/Admicelle

Recently, hemimicelles and admicelles have been come up as excellent sorbent materials for solid phase extraction of analytes (Merino, Rubio, & Perez-Bendito, 2004). These sorbent materials are prepared by the adsorption of ionic surfactants on the surface of metal oxides, such as alumina, silica and ferric oxyhydroxides. The adsorption of ionic surfactants on metal oxides is ensured at four steps (Nagashima, & Blum, 1999). Initially, the surfactant is adsorbed on the metal oxide by the coulombic attraction between the charged oxide surface and the oppositely charged surfactant head group. The charge of solid particle is not affected from this one-to-one charged exchange of surfactant and the adsorbed counter ions. Then, the primary aggregate formation which is named as hemimicelles are formed by the strong interaction between adsorbed monomers and this promotes the adsorption capability of the solid particle. The adsorption is enforced by hydrophobic and electrostatic attraction. This causes the formation of hemimicelles and admicelles. Finally, surfactant forms micelles in solution, the surface charge of sorbent is completely neutralized and adsorption at this point is solely because of hydrophobic ineractions with the surfactant tails. The process of solubilization of organic compounds in admicelle is named as adsolubilization (Li, Cai, Shi, Mou, & Jiang, 2007). Micelles form when the surfactant concentration is above the critical micelle concentration (CMC), but admicelle aggregates form slightly below the CMC.

Formation of hemimicelles, admicelles or mixed situations depends on the concentration of surfactant in solution, pH of solution, concentration of electrolyte, and type of surface material. There are several papers in the literature concerning the hemicelle and admicelle formation on sorbent preparations.

There is much attention in the application of surface adsorbed surfactants that include admicellar catalysis and surface modification.

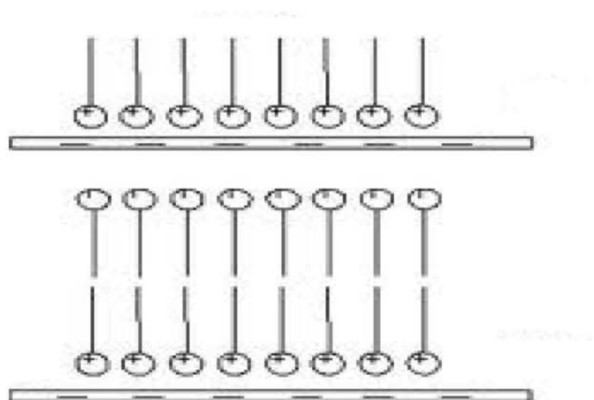


Figure 1.8 Hemimicelle and Admicelle.

1.6 High Performance Liquid Chromatography

High performance liquid chromatgoraphy is the most common used technique of all of the analytical separation techniques (Skoog & James, 1992). In addition HPLC technique can provide a valuable tool for generating highly pure preparations for characterizing the antimicrobial activities (Joshi, 2002). Its advantages are its sensitivity, its adaptability to accurate quantitative determinations, its suitability for separating nonvolatile species or thermally decomposing compounds. HPLC is used analytically to separate a mixture of compounds with the purpose of identifying, quantifying and resolution the individual components of the sample mixture. Normal-phase HPLC, or adsorption chromatography, separates analytes based on their affinity for a polar stationary surface such as silica, so it is based on analyte ability to engage in polar interaction with the sorbent surface. Reversed phase HPLC has a non-polar stationary phase. One common stationary phase is silica which has been surface-modified with RMe_2SiCl, where R is a straight chain such as C8 or

C18. The mechanisms by which these surfaces retain solute molecules are at present not entirely clear (Alshana, 2004).

Improving the resolution of a chromatographic column is based on varying one of the three parameters (N, k', and α) (Table 1.4).

Table 1.4 Important relationships in HPLC

Name	Equation
Number of theoretical plates	$N = 16 (t_R/W)^2$
Retention factor	$k' = (t_R - t_M) / t_M$
Selectivity factor	$\alpha = [(t_R)B - t_M] / [(t_R)_A - t_M]$
Resolution	$R_s = 2 [(t_R)_B - (t_R)_A] / [W_A + W_B]$

Where,

t_R : retention time, time between injection of a sample and appearance of a solute peak at the detector.

t_M : dead time, time required for an unretained species to pass through a column.

$(t_R)_A$: retention time of species A.

$(t_R)_B$: retention time of species B.

W_A : peak width at its base (in units of time) for species A.

W_B : peak width at its base (in units of time) for species B.

"The retention factor (k') is experimentally the most easily manipulated of the three because of the strong dependence of this constant upon the composition of the mobile phase. For optimal performance, k' should be in the ideal range" of 1 to 10; for complex mixtures, however, this range must often be expanded to perhaps 20 in order to provide time for peaks of all of the components to appear. Sometimes, adjustment of k' alone does not suffice to produce individual peaks with no overlap. If resolution is very poor (below 0.5) variation in selectivity factor (α) must be resorted to keep k' within a reasonable range. This can be achieved by choosing a different stationary phase or by changing the mobile phase identity.

1.7 Related Studies for Determination of SAs in Honey by HPLC

There are numerous articles about analysis of sulfonamides in honey and other food production in literature until this time. Several papers about the determination of sulfonamides by HPLC is given below:

In 2008, a method for the determination of residual sulfonamides in honey,using sulfapyridine as an internal standard was developed, optimized and validated by Granja et.al. Some changes were carried out using proposed method for the analysis of sulfonamides in Brazilian honey samples. The limit of detection was found as 3 μg/kg for sulfathiazole, 4 μg/kg for sulfamethazine and 5 μg/kg for sulfadimethoxine with average recoveries of 61.0% for sulfathiazole; 94.5% for sulfamethazine and 86.0% for sulfadimethoxine at 100 μg/kg level (Granja, Nino, Rabone & Salerno, 2008).

Bernal and co-workers presented a novel method for the simultaneous analysis at trace level of sulfonamides in honey by using methanol in the sample treatment step to avoid the emulsion formation and to break the N-glycosidic bond between sugars and sulfonamides. Recovery percentages of the analytes on fortified honey samples were ranged from 56% to 96% with relative standard deviations below 10%. The quantitation limits were determined between 4 and 15 ng/g (Bernal & et al.2009).

In 2010, a convenient, fast and eco-cost method for determination of residual sulfonamides in honey and other food matrices was developed. In this study, a novel liquid–liquid extraction was proposed. Using a micro syringe as the phase separation tool, the very less volume of the extraction solvent (acetonitrile) was required. The absolute recoveries ranged from 31.97% to 66.54%. Although the recoveries were not high, the method was accurate with low RSD values. Therefore, it was acceptable for real applications (Liu, Jiang, Li, Xu, & Xie, 2010).

Also in 2010, Tsai with co-workers presented a simple liquid–liquid extraction method combined with high-performance liquid-chromatography with fluorescence

detection for the extraction and determination of sulfonamides in honey. During sample preparation sugar was released from sulfonamides using acid hydrolysis. After derivatization of sulfonamides with fluorescamine, they were partitioned into the organic layer under the honey/water/acetonitrile system. Linearity was obtained from 2 to 200 ng/mL with the coefficient of determination higher than 0.998. Under the optimal conditions, recoveries were determined as 80.9–99.6% for honey fortified samples at three levels (5, 20, and 100 ng/g) (Tsai & et al. 2010).

A simple, rapid, and sensitive method for the quantitation of five sulfonamide residues in milk was developed by stir bar sorptive extraction coupling to high performance liquid chromatography with diode array detection. The analytes were enriched by SBSE method using poly (vinylimidazole–divinylbenzene) monolithic coater. In this study, the limit of detection and the limit of quantification were calculated at a concentration at which signal-to-noise ratios were equal to 3 and 10, and those were in the range of 1.30–7.90 ng/mL and 4.29 26.3 ng/mL. The recoveries varied from 54.8% to126% and the relative standard deviations for reproducibility were less than 13% for the target analytes in the milk samples (Huang, Qui, Yuan, 2009).

Yu and Hu were developed a simple, rapid, sensitive, cheap and less sample consuming method of stir bar sorptive extraction–high performance liquid chromatography tandem mass spectrometry for the determination of six sulfonamides in milk and milk powder samples. The preparation of stir bar was simple with good mechanic strength and it could be reused for more than 20 times. The detection limits of the proposed method for six sulfonamides were in the range of 0.9–10.5 μg/L for milk and 2.7–31.5 μg/kg for milk powder. Additionally, the recoveries of the studied sulfonamides except SMX were ranged from 87% to 99% for the spiked milk sample and from 71% to 115% for the spiked milk powder sample. However, the recovery of 68% and 120% were obtained for SMX in the spiked milk and milk powder samples due to the matrix interference (Yu, & Hu, 2012).

In 2011, an efficient method is provided to detect simultaneously sulfonamides by using matrix solid-phase dispersion (MSPD). With MSPD, clean up steps or the addition of chemical agents to further separate the drugs from interfering substances before HPLC analysis of extract are not necessary. In the MSPD method, the results indicated that the average recoveries ranged from 81% to 103% and relative standard deviation of the peak areas changed from 0.3% to 6.1%. Also the limit of detection was in the range 2–10 mg/kg and the limit of quantification (LOQ) was7–34 mg/kg (Yu, Mu, & Mei, 2012).

1.8 Purpose of the Study

Because of the development of analytical methodologies for determination of sulfonamides in food products is getting more and more important, we aimed the preconcentration of sulfonamides by using hemimicelle/admicelle sorbents and determination of them by HPLC in this study.

Ways to get this goal, we aimed at:

(1) Checking of chromatographic conditions such as mobile phase and working wavelengths by DAD dedector to get stable retention time of SDA, SMT, SMR and SMX in HPLC,

(2) Quantitative analysis procedure for sulfonamides by HPLC,

(3) Preparation of Hemimicelle/Admicelle sorbents using siilca gel and cetyltrimethyl ammonium bromide

(4) Optimization of conditions for a solid-phase extraction method,

(i) Effect of pH

(ii) Effect of surfactant concentration

(iii) Effect of type, concentration and volume of eluent

(iiii) Effect of loading flow rate

(5) Analysis of anaytical performance of proposed method,

(6) Determination the amounts of sulfonamides in honey samples,

(7) Spiking known concentration sulfonamide standards to honey samples for checking accuracy.

CHAPTER TWO
EXPERIMENTAL METHODS AND MATERIALS

2.1 Reagents

Four sulfonamides [sulfadiazine (SDA), sulfamethazine (SMT), sulfamerazine (SMR), sulfamethoxazole (SMX)], were obtained from Sigma-Aldrich. HPLC-grade acetonitrile and methanol were purchased from LAB SCAN. All other solvents were of analytical grade. Formic acid was from Merck. Cetyltrimethyl ammonium bromide (CTAB), didecyldimethyl ammonium bromide (DDAB) and tridodecylmethyl ammonium chloride (TDMAC) was obtained from Fluka. Silica (70- 230 mesh, 60 Å and surface area >500 m^2/g) was supplied by Merck. Ultrapure water (Milli-Q plus system, Millipore Bedford, MA, USA) was used throughout the work.

2.2 Preparation of Reagent Solutions

A stock solution (approximately 200 mg/L) of each sulfonamide was prepared by solving 20 mg of related sulfonamide in 100 mL methanol and stored at 4^0 C in refrigerator. They were stable at least one month.

A series of sulfonamide mixture working standard solutions from 0.10 µg/mL to 10 µg/mL were daily prepared from stock solutions of each sulfonamide, followed by dissolution in HPLC mobile phase and were stored at 4^0 C until use. They were used to calibrate the HPLC detector response.

The surfactant solutions were prepared by dissolving 50 mg of CTAB, DDAB, and TDMAC in 25 mL ultrapure water.

A mixture of 0.0258 g of potassium hydrogen phosphate and 0.3198 g potassium dihydrogen phosphate in 25 mL was used as a buffer pH 6.

0.01 mol/L tris-HCl for pH 9 was prepared by dissolving 0.01 g tris [hydroxymethyl] aminomethane hydrochloride in 25 mL ultrapure water. A buffer of pH 4 was prepared using citric acid/ sodium hydroxide/ hydrogen chloride.

2.3 Apparatus

A Watson Marlow 323i (Falmouth, England) peristaltic pump was used to pass the test and honey sample solutions through the columns in solid phase extraction experiments. A Selacta pH 2001 model pH meter was used for all pH adjustments. A Selecta diaphragm pump for filtering of sorbent was used. For solid phase extraction experiments, Varian cartridges (plastic container, 10 mm x 100 mm) equipped with 20 μm polypropylene frits were used.

2.3.1 Fourier Transform Infrared (FTIR) Spectrometer

A Perkin Elmer Spectrum 100 model FTIR spectrometer was used to record the FTIR spectra of KBr discs in the range of 4000-700 cm^{-1}, 20 co-added interferograms were scanned at 2 cm^{-1} resolution.

2.3.2 Scanning Electron Microscope (SEM)

The experiments were carried out with a Philips SEM XL-300 FEG. The operated voltage is 7 kV. The samples were mounted on aluminum stubs.

2.3.3 HPLC

HPLC (Figure 2.1) analyses were carried out on a SHIMADZU model 20A liquid chromatographic system equipped with a diode array detector (DAD), (SPD-M20A) controlled by Chemstation 3D software. This system includes SHIMADZU quaternary pump (LC-20AD/T). Injections were performed by 250 μL syringe which was from Hamilton to HPLC apparatus comprising the following injection system with 20 μL injection loop.

Figure 2.1 The SHIMADZU 20A series HPLC system.

2.4 Liquid Chromatographic Conditions for Sulfonamides

The separation of four sulfonamides was performed on a Hypersyl Gold C_{18} column (5µm, particle size, 250 mm x 4 mm) and the oven temperature was set to 35°C. The mobile phase consists of methanol: acetonitrile: 0.05 M formic acid (10:15:75, v:v:v) (Ito & et al. 2000). The diode array detector was set at 272 nm, the mobile phase at isocratic condition was pumped at a flow rate of 1.0 mL/min and the injection volume was 20 µL. The chromatograms of four sulfonamides (a) and four sulfonamides with CTAB (b) obtained were given Figure 2.2.

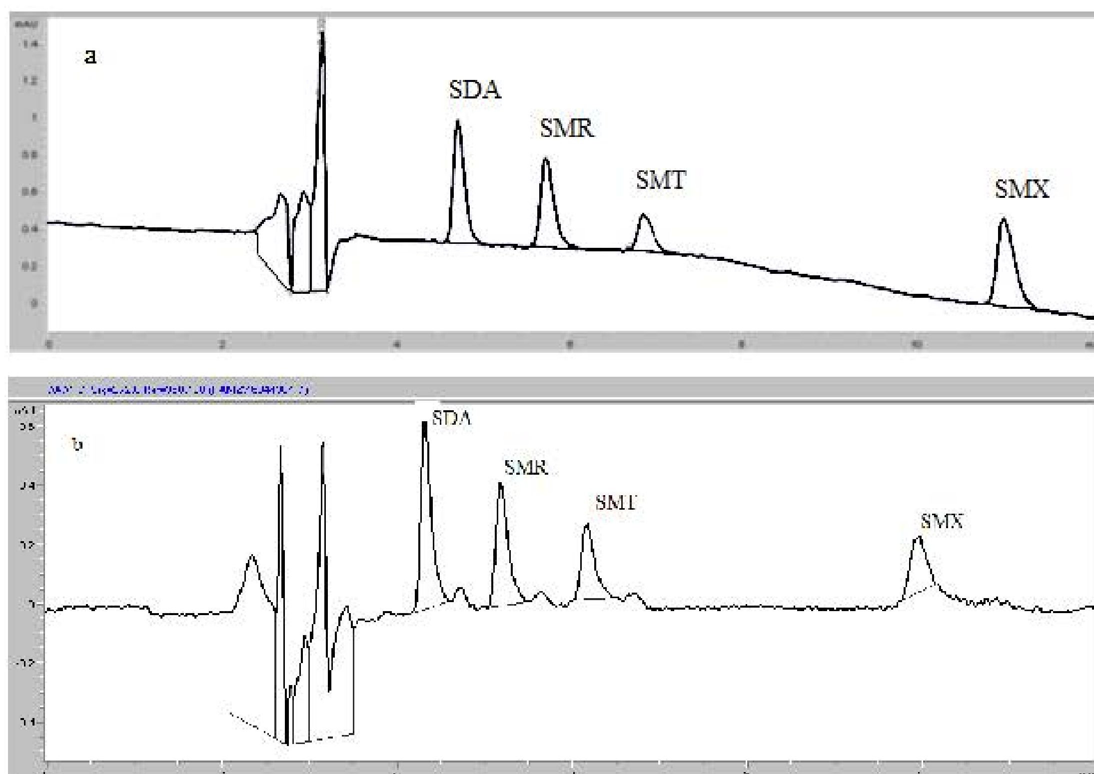


Figure 2.2 a) The HPLC chromatogram of four sulphonamides and b). four sulphonamides with CTAB at concentration of $5x10^{-3}$ mol/L.

2.5 Calibration Curves of Sulfonamides and Determination of Limit of Detection and Limit of Quantification in HPLC

To confirm the linearity of the response of sulfonamides at the specified wavelengths, each standard solution of sulfonamides was injected to HPLC. The working standard solutions of sulfonamides at concentrations from 10 to 2000 ng/mL were prepared in methanol and also in HPLC mobile phase. In both solvent conditions, the sample peak was seen normally and no fluctuations were also seen in baseline. Then, methanol was preferred as dilution solvent for standard sulfonamide solutions, and the honey samples. All samples were injected as triplicate. The limit of quantitation (LOQ) has been calculated as the lowest concentration for which acceptable data of recovery and precision were obtained. For determining the limit of detection (LOD) and LOQ, 0.01 µg/mL of each sulfonamide was injected 10 times and the standard deviation was calculated. LOD and LOQ were calculated based on the given equations below:

LOD = 3 x S.D./slope

LOQ = 10 x S.D./slope

2.6 Honey Samples

Four honey samples (pine, flower, lavander) were purchased from the local market in Izmir. All studied samples were stored in their original bottles, in darkness at +4° C.

2.7 Preparation of Honey Sample Solutions

For extraction honey samples, 2 grams of honey sample was weighted (accurate 0.01 g) and added to 50 mL of polyethylene tube containing 5 mL of 2 mol/L HCl solution. Later, it was stirred using magnetic stirrer for 30 min at room temperature. Then, 20 mL of 30% (w/v) NaCl aqueous solution was added to this solution and the final solution was transferred to 100 mL of separating funnel. The mixture after adding 25 mL of dichloromethane was shaken for 5 min manually. After waiting for phase separation in the funnel, the 25 mL of lower aqueous phase was separated to test tube and 5 mL portion of this aqueous honey sample solution was used for solid-phase extraction procedure (Granja, Niño, Rabone & Salerno, 2008). For accuracy of extraction experiments, 0.1-0.25 mg/L of mixed sulfonamide standard solution was spiked to honey samples.

2.8 Preparation of Silica-CTAB Sorbent

50 mg CTAB was mixed with 25 mL of 0.01 mol/L tris-HCl (pH 9) solution, then, this mixture was added to 2 g of silica and stirred using magnetic stirrer for 1 h. The last mixture was filtered using glass sintered funnel by diaphragm pump. After filtration, the solid prepared sorbent was dried at 110 ^{0}C for 2 h.

2.9 Solid Phase Extraction Procedure for Determination of Sulfonamides in Honey Samples

To prepare admicelle column, a 250 mg of silica-CTAB sorbent was put on a polyethylene SPE cartridge. It was conditioned by passing distilled water and then phosphate buffer (pH 6). A 5 mL of aqueous honey sample solution or test solution having sulfonamides was firstly mixed with 5 mL of pH 6 phosphate buffer solution and then passed through the column at a flow rate 1.5 mL/min using peristaltic pump and sulfonamides were eluted with 4 mL of methanol-water (1:1) mixture. A 20 μL aliquot of eluate was injected into the HPLC system. All values were the average values of three replicates.

CHAPTER THREE
RESULTS AND DISCUSSIONS

3.1 Type of Surfactants

In this study, three types of surfactants were tested for highest sorption capacity of sulfonamides. These surfactans are cetyltrimethyl ammonium bromide, didecyldimethyl ammonium bromide and tridodecylmethyl ammonium chloride. Within the studied surfactants, the highest sorption was yielded by CTAB which has the longest alkyl group (Figure 3.1). The other surfactants have more than one but shorter alkyl groups. Therefore, it could be concluded that CTAB showed the greatest extractability by forming admicelles.

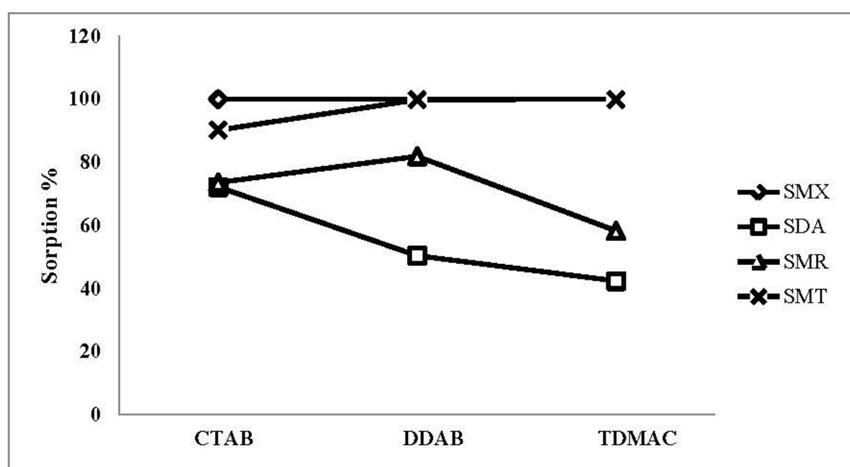


Figure 3.1 Extractability of surfactants for studied sulfonamides.

3.2 FTIR Analysis

The characterization of silica, silica-CTAB and silica-CTAB/sulfonamide were investigated by FTIR analysis. The FTIR spectra of silica, silica-CTAB and silica-CTAB/sulfonamide were given in Figure 3.2. The presence of 2820 cm^{-1} peak showed that the interaction between silica and surfactant. This peak was the aliphatic

$-CH_2$ vibration in the CTAB and this peak was shifted to 2910 cm^{-1} by sorption of sulfonamide on silica-CTAB sorbent. The peak of 3095 cm^{-1} was the aromatic =CH vibration in the sulfonamide. This clarified that sulfonamide was adsorbed to surface of admicelle sorbent.

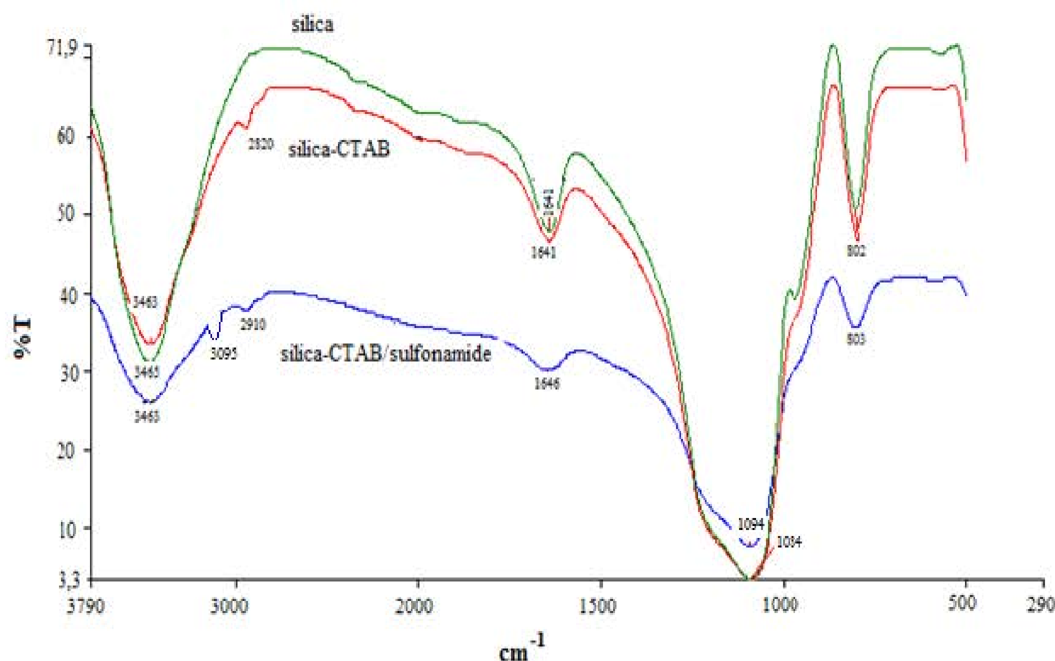


Figure 3.2 The FTIR spectra of a) silica, b) silica-CTAB, and c) silica-CTAB/sulfonamide.

3.3 SEM Analysis

Surface morphologies of silica, silica-CTAB and silica-CTAB/sulfonamide were investigated through scanning electron microscope analysis with .Philips model SEM at IYTE University. The applied voltage was low because the sample was not covered with gold layer. The morphology free silica was seen as small naked particles at 100 (in regular sequence) and 2500 magnification (Figure 3.3). The small particles on silica was seen when the silica was interacted with surfactant and the order of particles was disrupted (Figure 3.4). In sorption of sulfonamides to sorbent surface, smaller particles on final sorbent were clearly seen. So, the interaction between them is clearly observed (Figure 3.5).

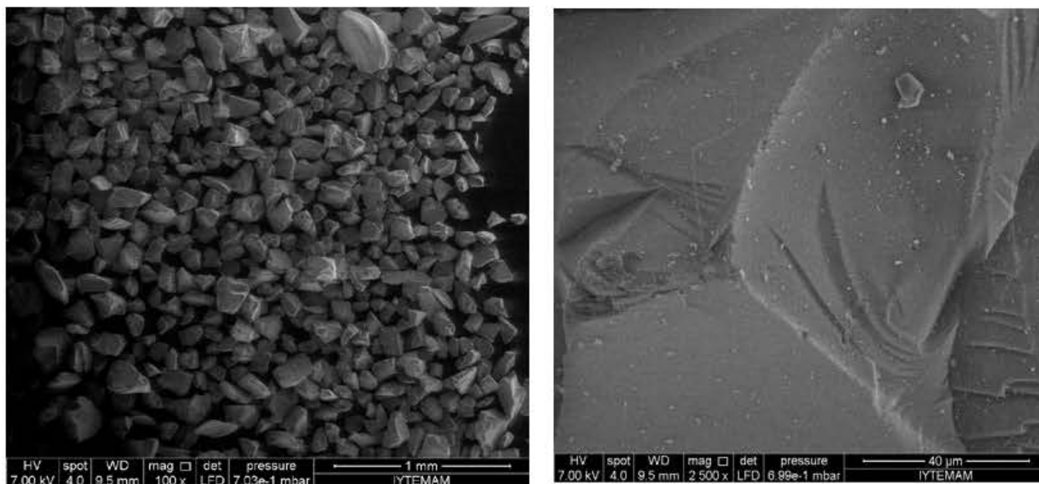


Figure 3.3 Scanning electron micrograph of silica at 100 and 2500 times of magnification.

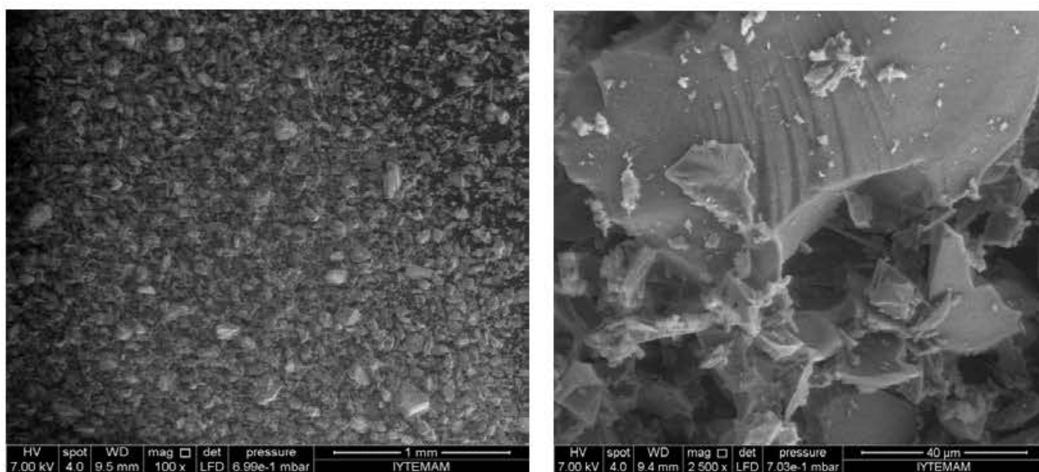


Figure 3.4 Scanning electron micrograph of silica-CTAB at 100 and 2500 times of magnification.

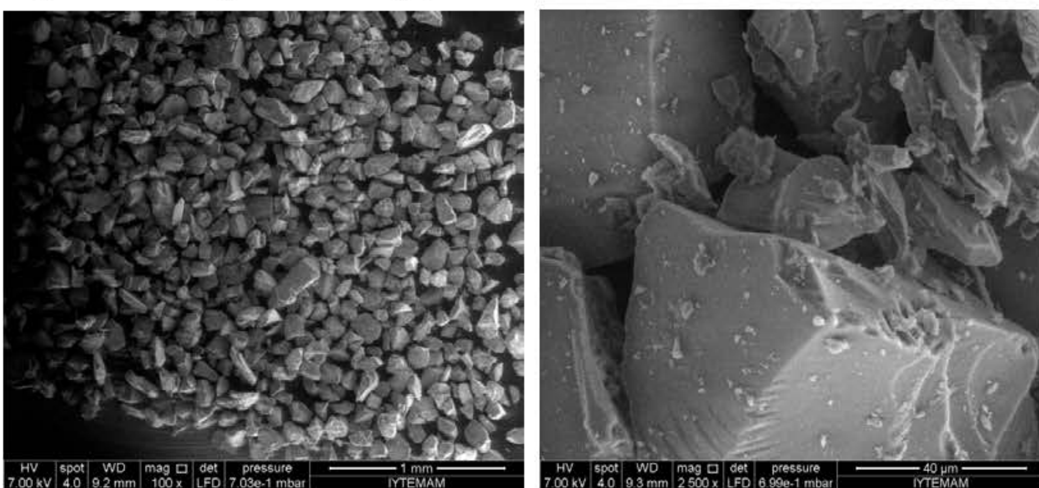


Figure 3.5 Scanning electron micrograph of silica-CTAB/sulfonamide at 100 and 2500 times of magnification.

3.4 Optimization Parameters of Solid Phase Extraction Method Used for Determination of Sulfonamides in Honey Samples

In this study, pH, surfactant concentration, type of eluent/mixture, ratio of solvents in eluent mixture, volume of eluent mixture and loading flow rate as optimization parameters using test solutions containing sulfonamides at a concentration of 0.50 mg/L were studied in the proposed solid-phase extraction procedure.

3.4.1 Effect of pH

In order to evaluate the effect of pH on the extraction efficiency, the pH of the 10 mL test solutions containing 0.50 mg/L of sulfonamides was adjusted from 4 to 8. For pH adjustments, phosphate buffer solutions were used. In these experiments, SPE cartridge was filled with 250 mg of silica gel-CTAB. The analyte ions, SMX, SDA, SMT and SMR, were retained by silica-CTAB between pH 5 to pH 8, quantitatively as shown in Figure 3.6. But, sorption was slightly higher at pH 6 for all analytes. So, for further experiments, pH 6 was chosen as the best sorption pH value.

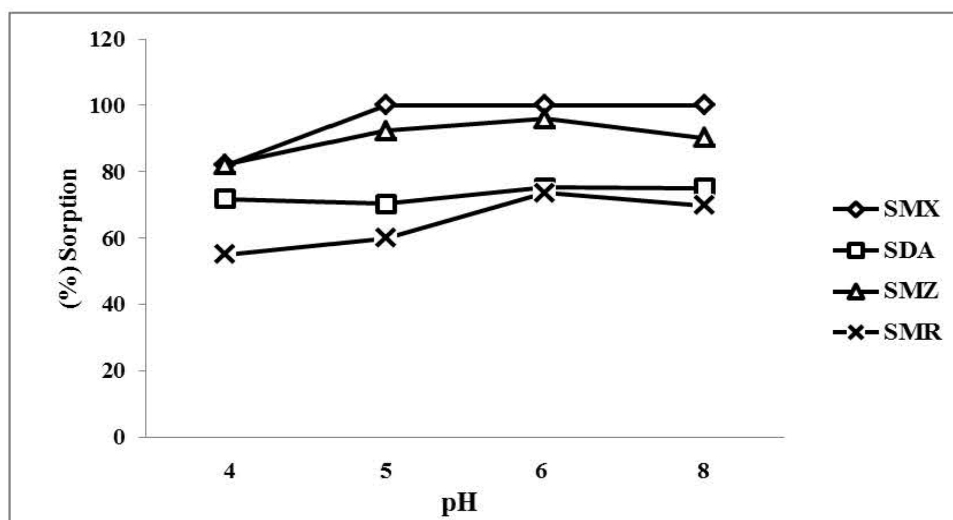


Figure 3.6 pH effect on sorption of sulfonamides (N = 3).

3.4.2 Effect of Surfactant Amount

The effect of CTAB amount on the sorption of sulfonamides was investigated. In these experiments, silica-CTAB sorbents including CTAB amount from 0 to 60 mg at pH 9 were prepared. When CTAB was not used, no sulfonamides were sorbed by silica. The sorption percentage of analytes after 50 mg of CTAB begun to decrease slightly (Figure 3.7). This could be reported as hemimicelle and admicelle formation was occurred with silica. Above this amount, micelle formation started to begin. Therefore, 50 mg of CTAB was used in further experiments.

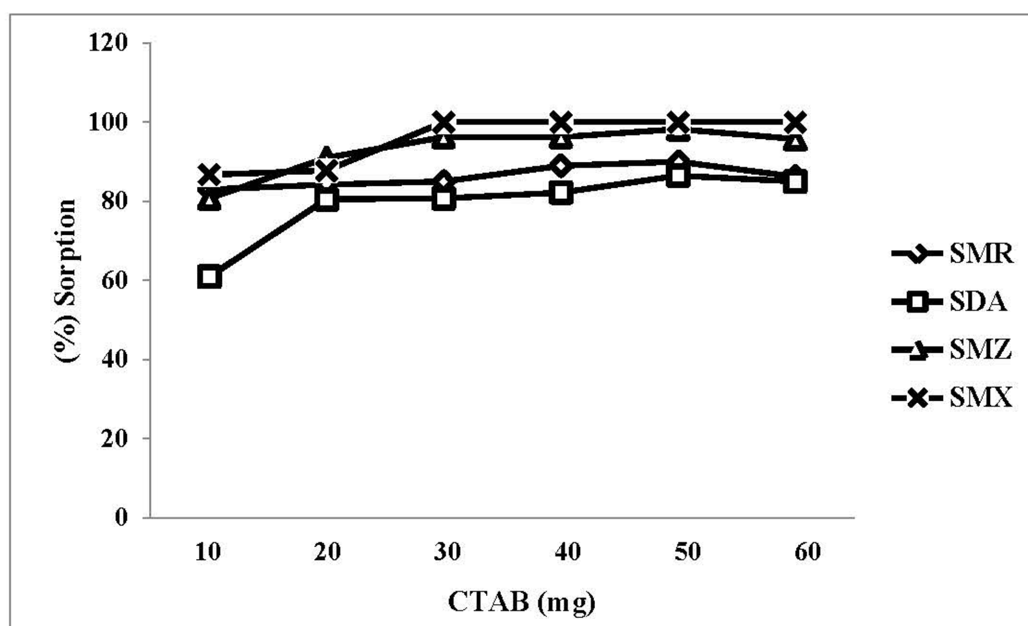


Figure 3.7 Amount of surfactant on sorption of sulfonamides (N = 3). Exp. conditions: 250 mg sorbent, c_{int} (SAs) = 0.50 µg mL^{-1}, pH = 6, sample volume: 10 mL.

3.4.3 Effect of Eluent Type and Volume

Several eluent solvent or solvent mixtures were tested to get satisfactory recovery of analytes from solid phase. By the methanol-ammonia mixture, the worst results for all of them were obtained except SDA. All analytes were desorbed above 60% using 2 mL of methanol-water (1:1) mixture (Figure 3.9). After determining the type of eluent mixture, the volume of eluent mixture from 1 to 10 mL was controlled for

complete recovery of analytes. The highest elution (≥95) recovery for 0.1 and 0.5 μg/mL SAs was achieved by eluting with 4 mL of methanol-water (1:1) mixture.

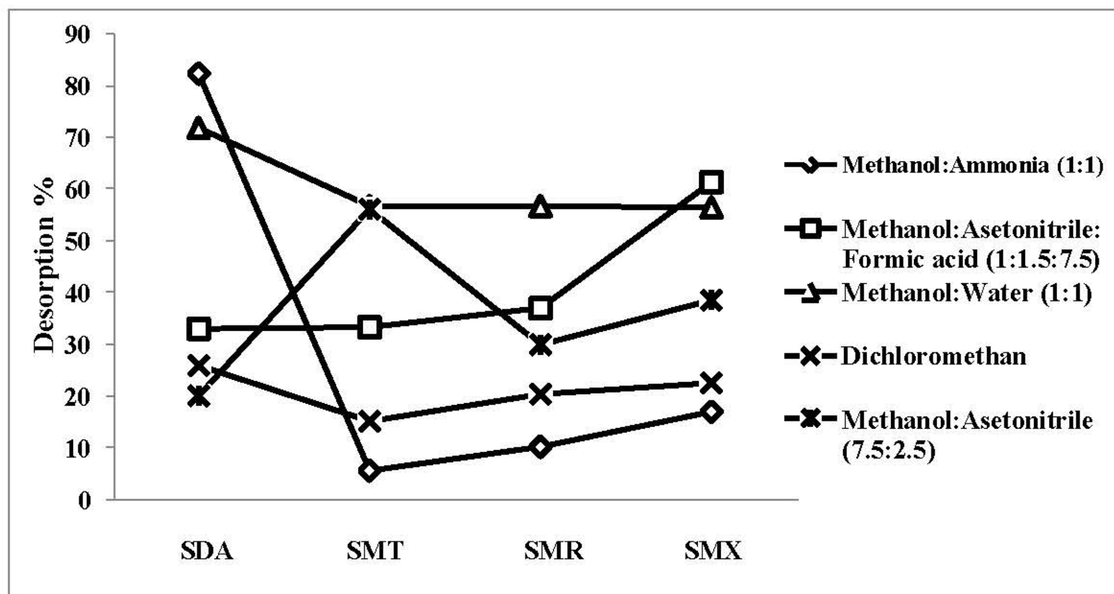


Figure 3.8 Type of elution solvent/mixture on sorption of sulfonamides (N = 3). Exp. conditions: 250 mg sorbent, c_{int} (SAs) = 0.50 μg/mL, pH = 6, sample volume: 10 mL, V_{eluent}: 2 mL.

3.4.4 Effect of Flow Rate

The flow rate of the sample solution is a measurement of the contact time between the sample solution and the sorbent. For reaching optimum loading flow rate, the tested flow rates were 1, 1.5, 2 and 2.5 mL/min (Figure 3.10). No change was seen for SMR and SMX at all tested flow rates. But, SDA and SMT, at 1.5 mL/min, maximum percentage of sorption was observed. So, 1.5 mL/min as loading flow rate was recommended for further studies.

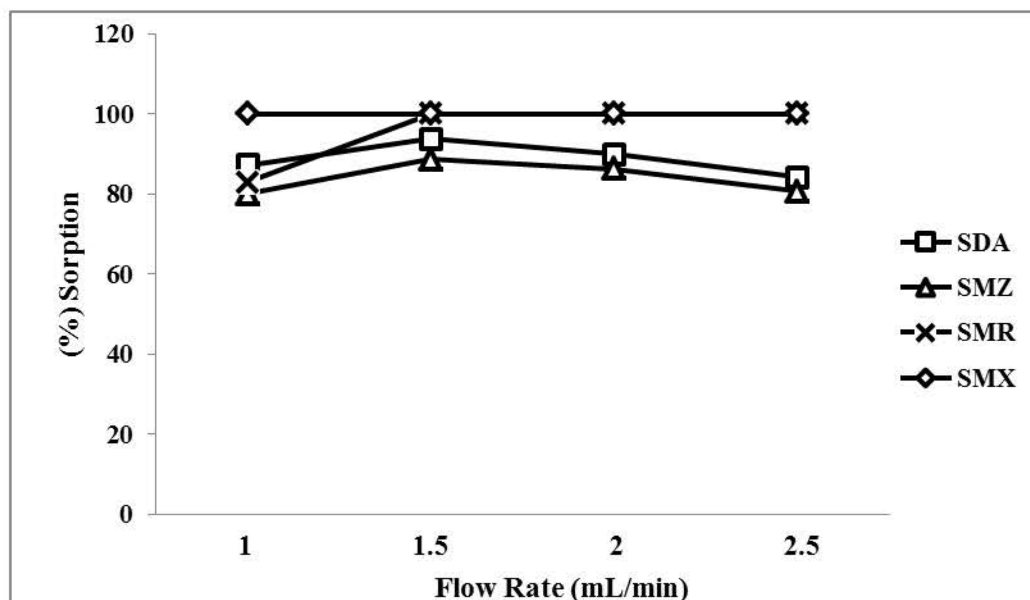


Figure 3.9 Effect of flow rate on sorption of sulfonamides (N = 3).

3.5 Preconcentration Factor and Recovery

To calculate the preconcentration factors of sulfonamides, 10-250 mL solutions containing 1 μg of sulfonamides were passed through the column and eluted with 4 mL of methanol-water (1:1) mixture. The sorptions and recoveries of the sulfonamides were calculated. The average of sorptions of sulfonamides was 70-100% until 100 mL. But after 100 mL the sorptions and recoveries of sulfonamides were decreased significantly. The results were given in Figure 3.10. Thus the preconcentration factors of sulfonamides were determined as 100 mL / 4 mL = 25.

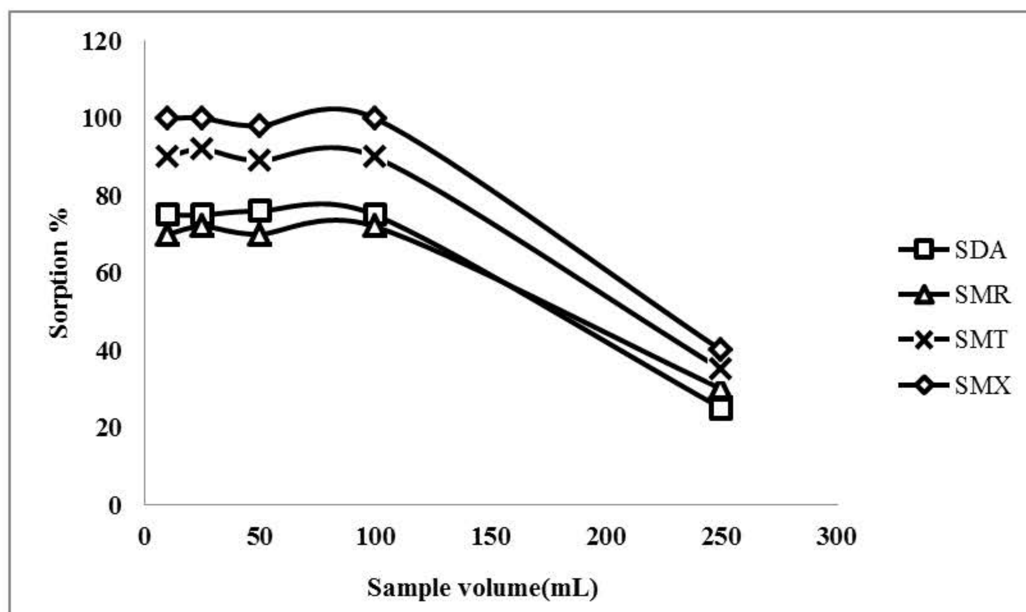


Figure 3.10 Effect of sample volume on sorption of sulfonamides.

3.6 Analytical Performance

Under the optimum conditions, calibration range, detection limit, the limit of quantitation and repeatability for the analytical method for sulfonamides were analyzed.

The instrumental calibration curves of SDA-SMT-SMR-SMX standard solutions by triplicate injections were plotted (Figure 3.11-3.14). The curves of SAs were linear in the range of 0.010-2.000 μg/mL with the regression coefficients of 0.9982-0.9996. The proposed extraction method calibration curve of each sulfonamide was established by applying SPE method to test standard solution containing different amount of analytes in order to check the effect of extraction method. The detection and quantification limits of the proposed method were determined by analyzing blank honey samples following the procedure given in section 2.9. The RSD% studies were carried out at 0.10 μg/mL of each analyte by carrying out five replicated determination using proposed procedure. The dynamic range, precision as RSD%, LOD and LOQ of the proposed method for each analyte were given in Table 3.1.

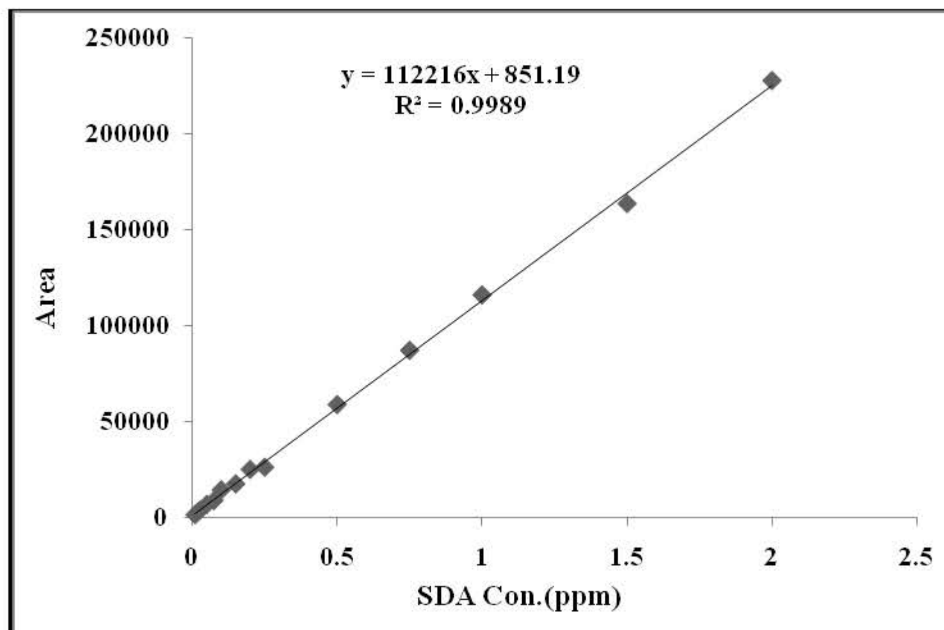


Figure 3.11 Instrumental calibration curves of SDA.

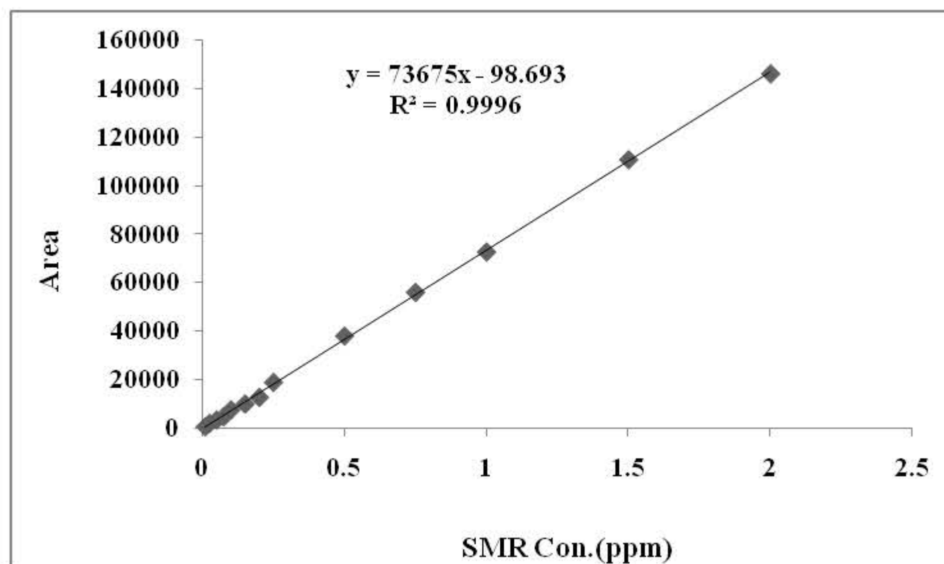


Figure 3.12 Instrumental calibration curves of SMR.

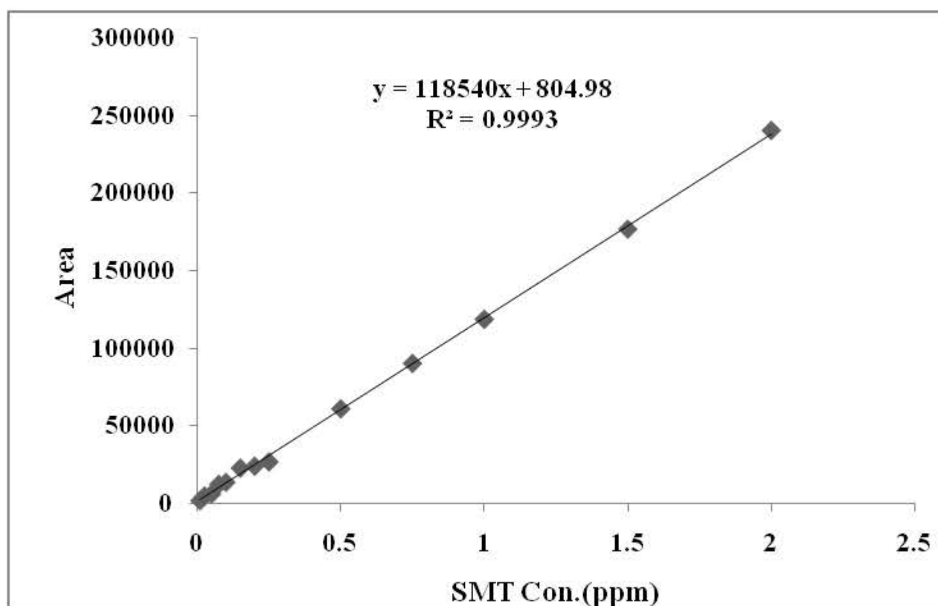


Figure 3.13 Instrumental calibration curves of SMT.

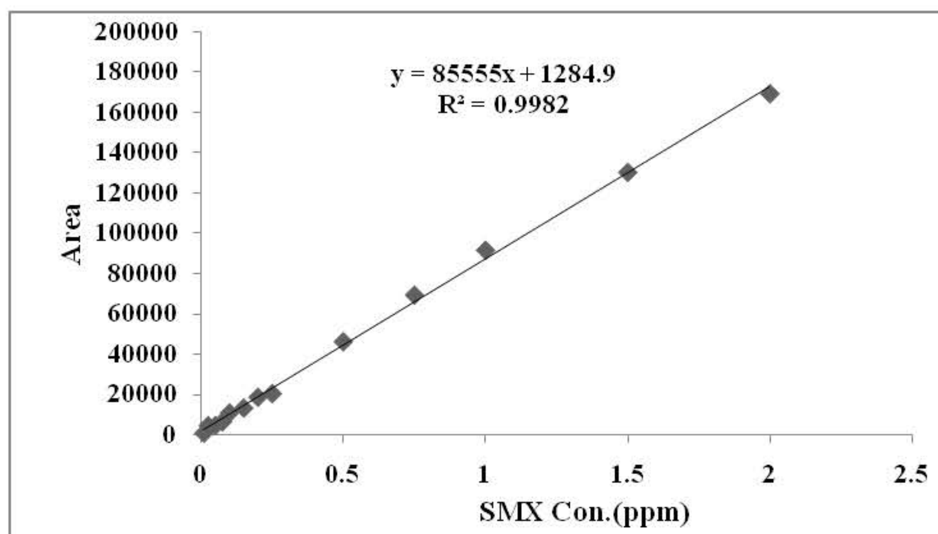


Figure 3.14 Instrumental calibration curves of SMX.

Table 3.1 Analytical performance of the proposed method

Parameters	SDA	SMR	SMT	SMX
Linear range (μg/mL)	0.01-2.00	0.01-2.00	0.01-2.00	0.01-2.00
LOD (μg/L)	5	13	3	4
LOQ (μg/L)	11	23	11	8
RSD (%)	5.91	3.33	2.84	1.98

3.7 Determination of Sulfonamides in Honey Samples

The proposed method was applied to honey samples. The results were given in Table 3.2. Also, to check the reliability of this method, the mixture of the studied sulfonamides (0.1 and 0.25 mg/L) was fortified into honey samples prior to solid-phase extraction procedure. The results given in Table 3.2 demonstrated that the recovery of spiked samples were satisfactorily provided and indicated the capability of this method for the determination of these analytes. A good agreement was obtained between the added and the measured amounts of analytes. It also shows high recoveries which are in the acceptable recoveries limit (between ≥ 70% and ≤ 107%) for trace analysis established by the Association of Official Agricultural Chemists (AOAC) and European Commission. The amount of sulfonamides in all studied honey samples were mostly above the MRL of European Union (EC, 1990).

Table 3.2 Levels of sulfonamides in honey samples (N=3)

Samples	**Added (µg/mL)**	**Found (µg/mL)**	**RSD(%)**	**Recovery(%)**
Honey1 (pine)				
SDA	-	0.78±0.02	1.35±0.02	
	0.10	0.85±0.02	1.02±0.03	97±1
	0.25	1.02±0.03	6.22±0.09	99±1
SMR	-	0.29±0.01	1.05±0.03	
	0.10	0.41±0.01	1.10±0.03	105±1
	0.25	0.53±0.02	6.59±0.09	98±1
SMT	-	0.26±0.01	0.85±0.02	
	0.10	0.33±0.01	0.90±0.02	92±1
	0.25	0.41±0.01	4.25±0.04	80±2
SMX	-	0.15±0.01	0.95±0.02	
	0.10	0.22±0.01	0.94±0.02	88±1
	0.25	0.29±0.01	1.55±0.03	73±2
Honey 2 (flower)				
SDA	-	0.31±0.01	0.11±0.01	
	0.10	0.39±0.01	0.78±0.02	95±1
	0.25	0.56±0.02	2.90±0.04	100±1
SMR	-	0.04±0.01	0.98±0.03	
	0.10	0.10±0.01	0.95±0.03	71±3
	0.25	0.28±0.01	2.85±0.04	97±1
SMT	-	0.06±0.01	1.14±0.02	
	0.10	0.13±0.01	1.02±0.03	81±2
	0.25	0.26±0.01	1.49±0.03	84±1
SMX	-	0.23±0.01	1.28±0.02	
	0.10	0.30±0.01	1.08±0.02	91±1
	0.25	0.45±0.02	2.46±0.04	94±1

Table 3.2 Levels of sulfonamides in honey samples (N=3) (continued)

Samples	**Added (µg/mL)**	**Found (µg/mL)**	**RSD(%)**	**Recovery(%)**
Honey3 (lavender)				
SDA	-	1.30±0.03	3.46±0.04	
	0.10	1.38±0.03	1.10±0.02	99±1
	0.25	1.60±0.04	1.22±0.03	103±1
SMR	-	0.80±0.02	3.53±0.04	
	0.10	0.75±0.02	1.14±0.02	83±2
	0.25	1.04±0.03	2.09±0.03	99±1
SMT	-	-	-	
	0.10	0.07±0.01	1.05±0.02	70±3
	0.25	0.20±0.01	6.05±0.09	80±2
SMX	-	0.18±0.01	4.46±0.04	
	0.10	0.27±0.02	1.07±0.02	96±1
	0.25	0.45±0.02	1.28±0.02	105±1
Honey 4 (flower)				
SDA	-	0.12±0.01	5.88±0.04	
	0.10	0.20±0.01	1.05±0.02	91±1
	0.25	0.38±0.01	2.12±0.02	103±1
SMR	-	0.11±0.01	3.33±0.03	
	0.10	0.18±0.01	0.65±0.02	82±2
	0.25	0.37±0.01	0.96±0.02	103±1
SMT	-	-	-	
	0.10	0.09±0.01	0.72±0.01	90±1
	0.25	0.26±0.01	0.98±0.02	104±1
SMX	-	0.04±0.01	3.28±0.03	
	0.10	0.14±0.01	0.85±0.01	100±1
	0.25	0.31±0.02	0.96±0.02	107±1

CHAPTER FOUR
CONCLUSION

In this study, the preparation of silica containing surfactants was firstly achieved. The preparation of this sorbent was easy. It was low-cost sorbent and utilized conveniently.

The IR analysis of silica, silica-surfactant (CTAB) and sulfonamide sorbed silica-CTAB were done using FTIR spectrometer. The results showed that the interaction between silica and surfactant was provided. Also, the loading of sulfonamides on the prepared sorbent was accomplished. SEM analysis of the prepared sorbent was carried out. Besides this, loading of sulfonamides on sorbent was showed by scanning electron micrographs.

Secondly, the optimization parameters of the proposed solid phase extraction methods were studied. These parameters were pH of sample solution, the amount of surfactant in the prepared sorbent, the type and the volume of desorption solvent mixture, the flow rate of loading sample.

The chromatographic separation of sulfonamides; sulfadiazine, sulfamethazine, sulfamerazine and sulfamethoxazole, were achieved using mobile phase as the mixture of methanol, acetonitrile and formic acid by diode array detector. The instrumental calibration curves of the studied sulfonamides were linear upto 2 µg/mL with regression coefficients, > 0.99.

The proposed solid phase extraction method was successfully applied to honey samples. The high recoveries of analytes were achieved. The proposed environmentally friendship solid phase extraction technique has good analytical performance in terms of dynamic range, limit of detection, limit of quantification and precision.

REFERENCES

Alshana, U. (2004). Separation and quantitation of some platinum group metals by RP-HPLC. *4th AACD Congress,* Middle East Technical University. Graduate School of Natural and Applied Sciences, Ankara, Turkey.

Baran, W., Adamek, E., Ziemianska, J., & Sobczak, A. (2011). Effects of the presence of sulfonamides in the environment and their influence on human health. *Journal of Hazardous Materials, 196,* 1– 15.

Bernal, J., Nozal, M. J., Jimenezb, J. J., Martínb, M. T., & Sanz, E. (2009). A new and simple method to determine trace levels of sulfonamides in honey by high performance liquid chromatography with fluorescence detection. *Journal of Chromatography A, 1216*, 7275–7280.

Bevill, R. F. (1984). Sulphonamides. In T. H. Jukes, H. L. Dupont, L. M. Crawford, (Ed.), *CRC Handbook series inzoonoses, section D, antibiotics, sulphonamides, and public health* (355–365). Boca Raton, FL: CRC Press.

Brander, G. C., Pugh, D. M., & Bywater, R. J. (1982). *Vetarinary applied pharmacology and therapeutics. anthelmintics* (403-422). London: Bailliere Tindall.

Bruzzoniti, M. C., Sarzanini, C., & Mentasti, E. (2000). Preconcentration of contaminants in water analysis. *Journal of Chromatography A, 902*(1), 289-309.

Choquet-Kastylevsky G., Vial, T., & Descotes, J. (2002). Allergic adverse reactions to sulfonamides. *Current Allergy & Asthma Reports, 2*(10), 16-25.

Commission of the Europan Communities, (1990). *Council Regulation EEC 2377/90. Official Journal L.224* (1).

Council Directive 90/167/EEC (1990). Laying down the conditions governing the preparation, placing on the market and use of medicated feding stuffs in the community. *Official Journal of European Union, 92*, 42-48.

Dantas, T. N. C., Neto, A. A. D., Moura, M. C. P. A., Neto, E. L. B., Forte, K. R., & Leite, R. H. L. (2003). Heavy metals extraction by microemulsions. *Water Research, 37*(11), 2709-2717.

Dantas, T. N. D., Neto, M. H. D., & Neto, A. A. D. (2002). Gallium extraction by microemulsions. *Talanta, 56,* 1089-1097.

Delgado, C., Rosegrant, M., Steinfeld, H., Ehui, S., & Courbois, C. (1999). *Livestock to 2020: The next food revolution. Food, Agriculture, and the Environment Discussion Paper 28.* International Food Policy Research Institute, Food and Agriculture Organization of the United Nations, International Livestock Research Institute, May 1999, Retrieved May 13, 2004 from http://www.fao.org/ag/aga/agal/lvst2020/20201/pdf.

Dye, J. L., Cram, K. D., Urbin, S. A., Redko, M. Y., Jackson, J. E., & Lefenfeld, M. (2005). Alkali metals plus silica gel: powerful reducing agents and convenient hydrogen sources. *Journal of American Chemical Society, 127* (26), 9338 -9339.

Fernandez-Torres, R., Bello Lopez, M.A., Olias Consentino, M., Callejon Mochon,M., Ramos Payan, M. (2011). Enzymatic-microwave assisted extraction and high-performance liquid chromatography–mass spectrometry for the determination of selected veterinary antibiotics in fish and mussel samples. *Journal of Pharmaceutical and Biomedical Analysis, 54,* 1146-1156.

Granja, R. H. M. M, Nino, A. M., Rabone, F., & Salerno, A. G. (2008). A reliable high-performance liquid chromatography with ultraviolet detection for the determination of sulfonamides in honey. *Analytica Chimica Acta, 613*(1),116–119.

Hamide, Z. S., & Glibert, J. (2010). Immunoaffinity column clean-up techniques in food analysis. *Review, 878*(2), 115-32.

Huang, X., Qiu, N., & Yuan, D. (2009). Simple and sensitive monitoring of sulfonamide veterinary residues in milk by stir bar sorptive extraction based on monolithic material and high performance liquid chromatography analysis. *Journal of Chromatography A, 1216,* 8240–8245.

Joshi, S. (2002). HPLC separation of antibiotics present in formulated and unformulated samples. *Journal of Pharmaceutical and Biomedical Analysis, 28,* 795–809.

Kaya, S., Pirincci, I., & Bilgili, A. (1997). *Veteriner uygulamalı farmakoloji 2.* Ankara: Medisan Press.

Koesukwiwat, U., Jayanta, S., & Leepipatpiboon, N. (2007). Solid-phase extraction for multiresidue determination of sulfonamides, tetracyclines, and pyrimethamine in Bovine's milk. *Journal of Chromatography A, 1149* (1), 102–111.

Lai, H. T., & Hou, J. H. (2008). Light and microbial effects on the transformation of four sulfonamides in eel pond water and sediment. *Aquaculture, 283*, 50–55.

Lara, F. J., García-Campan, A. M., Neusüss, C., & Alés Barrero, F. (2009). Determination of sulfonamide residues in water samples by in-line solidphase extraction capillary electrophoresis. *Journal of Chromatography A, 1216*, 3372–3379.

Li, J. D., Cai, Y. Q., Shi, Y. L., Mou, S. F., & Jiang, G. B. (2007). Determination of sulfonamide compounds in sewage and river by mixed hemimicelles solid-phase extraction prior to liquid chromatography–spectrophotometry. *Journal of Chromatography A, 1139* (2), 178–184.

Liu, J., Jiang, M., Li, G., Xu, L., & Xie, M. (2010). Miniaturized salting-out liquid–liquid extraction of sulfonamides from different Matrices. *Analytica Chimica Acta, 679,* 74–80.

Lu, K. H., Chen, C. Y., & Lee, M. R. (2007). Trace determination of sulfonamides residues in meat with a combination of solid-phase microextraction and liquid chromatography–mass spectrometry. *Talanta, 72,* 1082–1087.

Ma, M. L., Cheng, Y. Y., Xu, Z. H., Xu, P., Qu, H., Fang, Y. Y. (2007). Evaluation of polyamidoamine dendrimers as drug carriers of anti-bacterial drugs using sulfamethoxazole as a model drug. *European Journal of Medicinal Chemistry, 42* (1), 93–98.

Maniasso, N. (2001). Micellar media in analytical chemistry. *Quimica Nova, 24* (1), 87–93.

Martinez, D., Cugat, M. J., Borrull, F., & Callul, M. (2000). Solid-phase extraction coupling to capillary electrophoresis with emphasis on environmental analysis. *Journal of Chromatography A, 902* (1), 65-89.

Maudens, K.E., Zhang, G.F., Lambert, W.E. (2004). Quantitative analysis of twelve sulfonamides in honey after acidic hydrolysis by high-performance liquid chromatography with post-column derivatization and fluorescence detection. *Journal of Chromatography A, 1047,* 85-92.

Merino, F., Rubio, S., & Perez-Bendito, D. (2004). Evaluation and optimization of an on-line admicelle-based extraction-liquid chromatography approach for the analysis of ionic organic compounds. *Analytical Chemistry, 76* (14), 3873-3886.

Mitchell, J. M., Griffiths, M. W., McEwen, S. A., McNab, W. B., & Yee, A. J. (1998). Antimicrobial drug residues in milk and meat: cause, concerns,

prevalence, Regulations, tests, and test performance. *Journal of Food Protection, 61*, 742–756.

Myers, D. (1991). *Surfaces, interfaces, and colloids.* Principles and Applications New York: VCH Publishers.

Nagashima, K., & Blum, F. D. (1999). Adsorption of Sodium n-Decylbenzenesulfonate onto Alumina: Relationships between Macro- and Microscopic behavior. *Journal of Colloid & Interface Science, 214* (1), 8-15.

Pelizzetti, E., & Pramauro, E. (1985). Analytical applications of organized molecular assemblies. *Analytica Chimica Acta, 169* (1), 1–29.

Posyniak, A., Zmudzki J., & Mitrowska, K. (2005). Dispersive solid phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography. *Journal of Chromatography A, 1087* (1-2), 259-264.

Pyrzynska, K., & Trojanowicz, M. (1999). Functionalized cellulose sorbents for preconcentration of trace metals in environmental analysis. *Critical Reviews in Analytical Chemistry, 29* (4), 313-321.

Ramrrez, A., Gutierrez, R., Draz, G., Gonzalez, C., Perez, N., & Vega, S., (2003). High-performance thin-layer chromatography- bioautography for multiple antibiotic residues in cow's milk. *Journal of Chromatography B, 784,* 315–322.

Rodríguez, N., Ortiz, M. C., Sarabia, L. A., & Herrero, A. (2010). A multivariate multianalyte screening method for sulfonamides in milk based on front-face fluorescence spectroscopy. *Analytica Chimica Acta, 657,* 136-146.

Samanidou, V. F., Tolika, E. P., & Papadoyannis, I. N. (2008). Chromatographic residue analysis of sulfonamides in food stuffs of animal origin. *Separation Purification Review, 37* (4), 325–371.

Sanz-Medel, A., Campa, M. R. F., Gonzalez, E. B., & Fernandez-Sanchez, M. L. (1999). Organised surfactant assemblies in analytical atomic spectrometry. *Spectrochimica Acta B, 54* (2), 251–287.

Schwartz, D. P., & Sherma, J. (1986). Practical screening procedure for sulfathiazole in honey. *Journal Of The Association Of Official Analytical Chemists, 69* (1), 72-74.

Seifrtova, M., Novakova, L., Lino, C., Pena, A., Solich, P. (2009). An overview of analytical methodologies for the determination of antibiotics in environmental waters. *Analytica Chimica Acta, 649*, 158-179.

Sherma, J., Bretschneider, W., Dittamo, M., Dibiase, N., Huh, D., & Schwartz, D. P. (1989). Spectrometric and thin-layer chromatographic quantification of sulfathiazole residues in honey. *Journal of Chromatography*, *463*(1), 229-233.

Skoog, D. A., & James, J. L. (1992). *Principles of instrumental analysis* (4th ed.). Fort Worth: Saunders College Publishing.

Sun, L., Chen, L. G., Sun, X., Du, X. B., Yue, Y. S., & He, D. Q. (2009). Analysis of sulfonamides in environmental water samples based on magnetic mixed hemi micelles solid-phase extraction coupled with HPLC–UV detection. *Chemosphere, 77*, 1306–1312.

Tarbin, J. A., Clarke, P., & Shearer, G. (1999). Screening of sulphonamides in egg using gas chromatography-mass-selective detection and liquid chromatography-mass spectrometry. *Journal of Chromatography B*, *729*, 127-128.

Thomson, C. A., & Sporns, R. (1995). Direct ELISAs for sulfathiazole in milk and honey with special emphasis on enzyme conjugate preparation. *Journal of Food Science*, *60* (2) , 409-415.

Tsai, W. H., Chuang, H., Chen, H. H., Wu, Y. W., & Cheng, S. H. (2010). Application of sugaring-out extraction for the determination of sulfonamides in honey by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A, 1217,* 7812–7815.

Tsai, W.H., Huang, T.C., Chen, H.H., Wu, Y.W., Huang, J.J., Chuang, H.Y. (2010). Determination of sulfonamides in swine muscle after salting-out assisted liquid extraction with acetonitrile coupled with back-extraction by a water/acetonitrile/dichloromethane ternary component system prior to high-performance liquid chromatography. *Journal of Chromatography A, 1217,* 250-255.

Vincent, U., Chedin, M., Yasar, S., & von Holst, C. (2008). Determination of ionophore coccidiostats in feedingstuffs by liquid chromatography tandem mass spectrometry: Part I. Application to targeted feed. *Journal of Pharmaceutical* & *Biomedical Analysis, 47* (4), 750–757.

Wang, L., Yang, H., Zhang, C., Mo, Y., & Lu, X. (2008). Determination of oxytetracycline, tetracycline and chloramphenicol antibiotics in animal feeds using subcritical water extraction and high performance liquid chromatography.*Analytica Chimica Acta, 619* (1), 54–58.

Yu, C., Hu, B. (2012). C18-coated stir bar sorptive extraction combined with high performance liquid chromatography–electrospray tandem mass spectrometry for the analysis of sulfonamides in milk and milk powder. *Talanta, 90,* 77– 84.

Yu, H., Mu, H., & Mei, Y. (2012). Determination of fluoroquinolones, sulfonamides, and tetracyclines multiresidues simultaneously in porcine tissue by MSPD and HPLC–DAD. *Journal of Pharmaceutical Analysis, 2* (1), 76–81.

Zotou, A., & Vasilliadou, C. (2006). Selective determination of sulfonamide residues in honey by SPE-RP-LC with UV detection. *Chromatographia, 64* (5-6), 307-311.