DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CHROMATOGRAPHIC DETERMINATION OF SULFONAMIDES IN MILK AND HONEY

by
Sezer ÖZGENÇ

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İZMİR

CHROMATOGRAPHIC DETERMINATION OF SULFONAMIDES IN MILK AND HONEY

A Thesis Submitted to the
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M. Sc. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"CHROMATOGRAPHIC DETERMINATION OF SULFONAMIDES IN MILK AND HONEY"** completed by **SEZER ÖZGENÇ** under supervision of **PROF. DR. MELEK MERDİVAN** 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.

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CHROMATOGRAPHIC DETERMINATION OF SULFONAMIDES IN MILK AND HONEY

ABSTRACT

The sulfonamides are collected in animal tissues and organs and then they accumulate in human kidney and hepatic by following the chain of consumption. For these reasons, determination of sulfonamides becomes essential because of inhibiting folic acid synthesis and causing permanent damage.

In this study, preconcentration process was performed for food products such as milk and honey with a surfactant Triton X-114 and high pressure liquid chromatography diode array detector (HPLC-DAD) was used to determine sulfadiazine (SDA), sulphamethoxazole (SMZ). Cloud point extraction (CPE) which is simple, fast, new and economic and uses surfactants fundamentally as an alternative instead of organic solvents, concerns green chemistry concept was performed for the extraction of sulfonamides. Quantitative analysis of extracts was performed in methanol/water (22:78, v/v) isocratic elution at 1.0 mL/min flow rate with 35 degrees Celsius at 270 nm after pH, salt & surfactant concentration, incubation time and temperature optimizations were completed.

Detection and quantitation limits of the method were found as 5.68 and 20.09 ppb and 6.06 and 21.86 ppb for SDA and SMZ, respectively, the linear range of quantitation for analytes was approximately 0.025 - 2.000 ppm, the percentages of average recovery as 65.2 and 99.4 and the relative standard deviation percentage as 0.51 and 2.17 were found for SDA and SMZ, respectively.

The results of the amount of sulfonamides in two kinds of milk and ten kinds of honey were determined as in the range of 0.062 - 0.104 ppm and 0.017 - 0.643 ppm (SDA), 0.018 - 0.038 ppm and 0.006 - 0.162 ppm (SMZ), respectively.

Keywords: sulfonamide, sulphadiazine (SDA), sulphamethoxazole (SMZ), antibiotic analysis, residue, cloud point extraction, honey, milk, HPLC

SÜT VE BALDA SÜLFONAMİDLERİN KROMATOGRAFİK TAYİNİ

ÖZ

Sülfonamidler, hayvanın doku ve organlarında toplanıp, tüketim zincirini takiben insan vücudunda böbrek ve karaciğerde birikirler. Bu sebeplerden ötürü, sülfonamidlerin analizi, folik asit sentezini engellediği ve birçok kalıcı hastalığa sebep olduğu için önem kazanmıştır.

Bu çalışmada, süt ve bal gibi gıda ürünlerine Triton X-114 yüzey aktif maddesi kullanılarak önderiştirme işlemi uygulandı ve DAD dedektörlü yüksek performanslı sıvı kromatografisi (HPLC) ile sülfadiazin (SDA) ve sülfametoksazol (SMZ) tayini gerçekleştirildi. Sülfonamidlerin ekstraksiyonu, organik çözücülere alternatif olarak yüzey aktif madde kullanımını esas alan, çevreyle dost, basit, hızlı, yeni ve ekonomik bir yöntem olan bulutlanma noktası ekstraksiyonu kullanılarak yapıldı. pH, tuz ve yüzey aktif madde derişimi, inkübasyon süresi ve sıcaklık optimizasyonları tamamlandıktan sonra, ekstraktların metanol:su (22:78, v/v) izokratik elüsyonu, 1,0 mL/dk akış hızı, 270 nm ve 35 Celsius derecede kantitatif analizleri gerçekleştirildi.

Doğruluk için gözlenebilme ve tayin sınırları SDA ve SMZ için sırasıyla 5,68 - 20,09 ppb ve 6,06 - 21,86 ppb değerleri arasında, derişim aralığı yaklaşık olarak 0,025 - 2,000 ppm, geri kazanım yüzdesi SDA ve SMZ için sırasıyla 65,2 ve 99,4 ve standart sapma yüzdesi SDA ve SMZ için sırasıyla 0,51 ve 2,17 değerleri bulunmuştur.

İki tür süt ve on tür baldaki sülfonamidlerin miktarı yaklaşık olarak sırasıyla 0,062 - 0,104 ppm ve 0,017 - 0,643 ppm (SDA), 0,018 - 0,038 ppm ve 0,006 - 0,162 ppm (SMZ) arasında değişen değerlerde hesaplanmıştır.

Anahtar sözcükler: sülfonamid, sülfadiazin (SDA), sülfametoksazol (SMZ), antibiyotik analizi, kalıntı, bulutlanma noktası ekstraksiyonu, bal, süt, HPLC

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CHAPTER ONE
INTRODUCTION

1.1 Antibiotics

Antibiotics are pharmacologically and biologically active chemical agents especially designed for the treatment and prevention of animal diseases. At present, veterinary drugs are extensively used in animal production. This is related to the gigantic growth and intensification of animal production (Botsoglou & Fletouris, 2001). Antibiotics and other chemotherapeuticals are administered in therapeutical quantities especially in the therapy and prevention of specific animal diseases. The most important and most frequently used group of veterinary drugs is that of antimicrobial agents (Fischer et al, 2003). 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 these compounds can have a harmful effect on human health, such as allergic reactions in some hypersensitive individuals as well as generation of drug resistant bacterial strains in humans (Vincent, Chedin, Yasar & van Holst, 2008; Koesukwiwat, Jayanta & Leepipatpiboon, 2007; Huebra, Vincent & van Holst, 2007), and for these reasons their use in animal husbandry must be subjected to strict control (Wang, Yang, Zhang, Mo & Lu, 2008). Consequently, 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). At present, however, no method exists which can detect all of these agents at the levels of the established MRL values. In order to provide for the high technological quality of raw milk and, at the same time, the safety of the milk and milk products for the consumer, the IDF

has developed a so called integrated system of checking veterinary drugs in milk and milk products. The system recommends the use of various methods for the antibiotic detection and specifies the responsibility for the health safety of milk and milk products of particular subjects in the whole of the technological process of producing and processing milk (Honkanen, Buzalski & Reybroeck, 1997; Honkanen, Buzalski & Suhren, 1999).

In the following list are grouped characteristics of each family of antibiotics, indications, major adverse events, cons-indications.

Figure 1.1 Family of antibiotics

1.1.1 Kinds of Antibiotics

The antibiotics are classified according to three criteria and although that each category contains several drugs but each one of them is unique in some features and effects.

- According to spectrum,
- According to type of the action of antibiotic and

- According to route of the administration of the drug (Classification of antibiotics, 2011, http://www.healthy-market.org/classification.php).

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

Sulfonamides (SAs) belong to a class of antimicrobial drugs that are widely used for food producing animals as growth promoters as well as for therapeutic and prophylactic purposes. Residues of SAs in the food chain are of an increasing concern due to their carcinogenic potency and their contribution to an increase of antibiotic resistance (Crosby & Horwood, 1991).

The chemical class of sulfonamides shares a common p-aminobenzoyl ring moiety with an aromatic amino group at the N_4-position, differing in the substitution at the N_1-position.

Sulfonamides, a series of synthetic antimicrobial agents containing a sulphanilamide group and a distinct five- or six-membered heterocyclic ring (Lai & Hou, 2008), are widely applied to prevent and treat bacterial infective disease (Sun et al., 2009) because of their broad spectrum of activity and low costs (Lara, García-Campan, Neusüss, & Alés-Barrero, 2009).

Most of the papers reviewed refer to the analysis of animal tissues such as muscle, liver, kidney, skin and fat. Other matrices analyzed are salmon muscle, skin and liver tissues (Zheng, Liu, Hall, Kitts & McArlene, 1994; Kitts, Zheng, Burnsflett & McErlane, 1995; Gehring, Rushing, Churchwell, Doerge, McErlane & Thomson, 1996). With respect to whole analytes, most studies deal with sulphameth (SMZ), sulphadiazine (SDA), sulphamerazine (SMR) and sulphathiazole (STZ) using other examples with lower frequency.

1.2.1 Sulfadiazine

Sulfadiazine eliminates bacteria that cause infections by stopping the production of folic acid inside the bacterial cell and is commonly used to treat urinary tract infections. In combination, sulfadiazine and pyrimethamine can be used to treat toxoplasmosis, a disease caused by toxoplasma gondii (Sulfadiazine, 2011, http://en.wikipedia.org/wiki/Sulfadiazine).

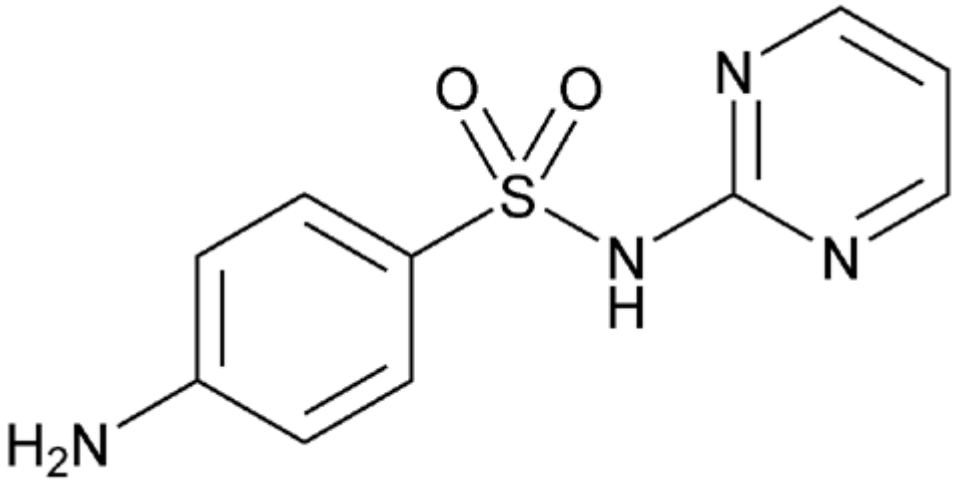


Figure 1.2 Structure of sulfadiazine

1.2.2 Sulphamethoxazole

Sulfamethoxazole is a sulfonamide bacteriostatic antibiotic. 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 (SMZ-TMP) 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 (Sulphamethoxazole, 2011, http://en.wikipedia.org/wiki/Sulfamethoxazole).

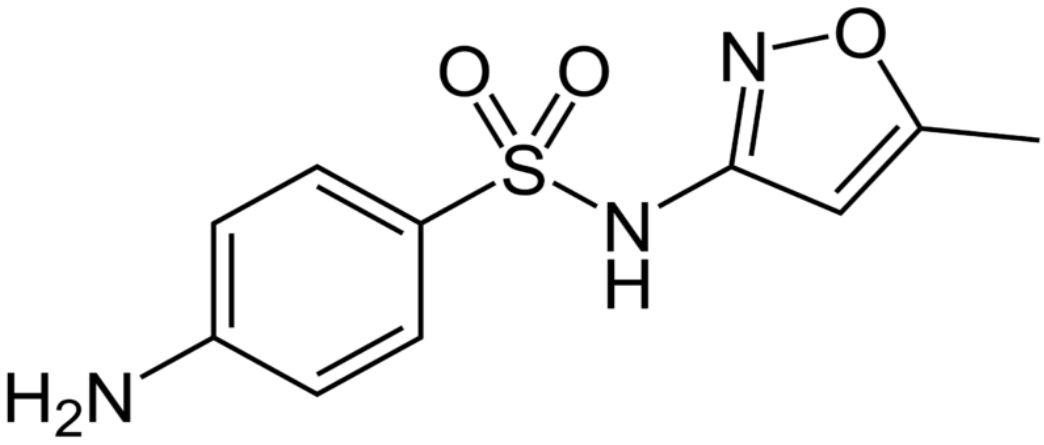


Figure 1.3 Structure of sulphamethoxazole

1.3 Contaminated Food Products

If the proper withdrawal periods are not observed before slaughtering or milking of the medicated animals, meat, honey and milk from these animals may be contaminated with residual sulfonamides (Saschenbrecker & Fish, 1980; Franco, Webb & Taylor, 1990; McEvoy, Mayne, Higgins & Kennedy, 1999).

1.4 Effects of Sulfonamides

At present, sulfonamides and other drugs (chlortetracycline, penicillin and several ionophores) 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. In addition, some sulfonamides have been found to be potentially carcinogenic and this fact has become a cause for

considerable debate in food safety. It has been estimated that approximately 5% of human patients medicated with sulfonamides received unwanted effects from the drugs (Bevill, 1984; Montanaro, 1998).

1.5 Maximum Residue Limits

The presence of sulfonamide residues in food was considered harmful to consumers. In order to protect consumers from risks related to drug residues, maximum residue limits (MRL) have been established by law in many countries.

Table 1.2 Maximum residue limit (MRL) values for sulfonamides in food listed by The Japan Food Chemical Research Foundation (38).

Food	MRL (mg/L)
SULFADIAZINE Pig, muscle, fat, liver, kidney, chicken	0.10
Milk	0.07
Egg	0.02
SULPHAMETHOXAZOLE Pig, muscle, fat, liver, kidney, chicken	0.02
Chicken	0.05
SULFADOXINE Cattle, muscle, fat, liver, kidney, chicken	0.10
Pig, edible offal	0.02
Milk	0.06

In contrast to other countries, in Turkey only one limit for whole sulfonamides and whole animal organs and tissues has been determined by Tarım ve Köyişleri Bakanlığı. Table 1.3 shows the maximum tolerable limits of sulfonamides in foods.

Table 1.3 Maximum residue limit (MRL) value for sulfonamides in Turkey

Food	MRL
SULFONAMIDES Any organ and tissue	0.10 µg/kg

Gıda, Tarım ve Hayvancılık Bakanlığı, 2011

Long-term use of SAs could lead to unwanted residues in animal-derived food products and pose potential dangers to human health, such as toxicity, the generation of resistant bacterial strains and allergic hypersensitivity reactions (Li, Cai, Shi, Mou, & Jiang, 2007). To ensure the safety of food to consumers, the European Union (EU) has established maximum residue limits (MRLs) of 100 µg/kg for SAs in foods of animal origin, including milk (Prada, Reviejo, & Pingarron, 2006).

1.6 Analysis of Sulfonamides

Sulfonamides in food may be determined by a number of different analytical methods, based, for example, on enzyme immunoassay (Thomson & Sporns, 1995), thin-layer chromatography (Sherma, Bretschneider, Dittamo, & Dibiase, 1989), gas chromatography (Tarbin, Clarke & Shearer, 1999), and reversed-phase high-performance liquid chromatography (HPLC) (Posyniak, Zmudzki & Mitrowska, 2005; Zotou & Vasiliadou, 2006).

1.6.1 Related Studies for Determination of SAs in Food Products by HPLC

There are some articles about analyzing of sulfonamides in food products in literature until now. Literatures concerning the determination of sulfonamides in food products were given below.

In 2007, an analytical method to determine six commonly used sulfonamides (sulfadiazine, sulfapyridine, sulfamerazine, sulfisoxazole, sulfamethoxazole and sulfadimethoxine) in pork and beef meat was developed utilizing no halogenated solvents for extraction and two SPE cartridges for sample clean-up and pre-concentration of analytical components prior to liquid chromatography analysis. The HPLC determination was performed using a RP C18 column and sulfonamides were detected at 266 nm. The newly developed sample pretreatment procedure effectively removed the potential matrix interferences from endogenous compounds of meat. Average recoveries of analytes from spiked meat ranging from 71 to 78% of six sulfonamides were determined (Bele, Matea, Dulf & Mirela, 2007).

In one of the latest studies, an efficient and environmentally friendly analytical methodology is proposed for extracting and preconcentrating seven sulfonamides from milk prior to high performance liquid chromatography with ultraviolet detection (HPLC–UV). It is based on the induction of micellar organized media by using Triton X- 100 as an extracting solvent. Optimization of the factors affecting de-emulsification and phase separation was performed. The limits of detection (LOD) of the sulfonamides ranged from 2.23 to 9.79 μg/L, the linear range of quantitation for all analytes was approximately 0.05–2.00 mg/L and the correlation coefficients of the calibration curves were P0.9999. The average recoveries and relative standard deviations were in the range of 67.0–105.7% and 0.93–8.31%, respectively, for fortified samples at 0.05, 0.10 and 0.20 mg/L of each sulfonamide (Zhang, Duan & Wang, 2010).

Thompson and Noot, developed a simple and rapid analytical method for the determination of residues of seven sulfonamide antibiotics in honey. Sample preparation consisted of acid hydrolysis to release sugar-bound sulfonamides. After filtration, acidified honey solutions were injected directly into a liquid chromatograph–tandem mass spectrometer (LC–MS/MS) system. Using gradient elution programming, analyte extraction and sample cleanup were automated. A six-port valve system was utilized to divert eluent from the extraction column into the MS/MS after the bulk of the honey matrix had been selectively removed. Minimal

contamination of the MS source chamber was observed even after the injection of over 600 honey samples. Using internal standard quantitation, excellent accuracy and good precision were obtained. The method detection limits for the sulfonamides studied were found to range from 0.5 to 2.0 μg/kg (Thompson & Noot, 2005).

Huang and his friends presented a simplified and rapid determining/identifying method for residual sulfonamides (SAs) in milk by using Ether-type stationary phase. The target analytes were extracted by mixing with ethanol–acetic acid (97:3, v/v) followed by centrifugation. The procedure used a Ether-type C8 column, isocratic elution with acetonitrile–water (5:95, v/v), and a photo-diode array detector. The linear range of determination was 50–10,000 μg/L for sulfanilamide and 100–10,000 μg/L for sulfadiazine, sulfamerazine, sulfamethazine. Average recoveries of four SAs (spiked 0.5, 1.0 and 1.5μg/mL) ranged from 80.1% to 87.6%, with relative standard deviations between 3.4% and 5.8%. The total time and solvent required for the analysis of one sample were <15 min and <1.0mL of ethanol and 0.6mL of acetonitrile, respectively. The developed procedure was nearly harmless to the human and environment (X. Huang, Yuan & B. Huang, 2007).

In Brasil, a method for the determination of residual sulfonamides in honey, using sulfapyridine as an internal standard has been developed, optimized and validated. Some changes were implemented on current available methodologies for the analysis of sulfonamides in honey in order to adopt such procedures to Brazilian honey samples. Sulfonamides were extracted from honey with dichloromethane after dissolution with 30% sodium chloride, and cleaned up with solid phase extraction on Florisil columns. The eluate was analyzed by high-performance liquid chromatography with ultraviolet detection. The limit of detection was determined at 3 μg/kg, 4 μg/kg and 5 μg/kg for sulfathiazole, sulfamethazine and sulfadimethoxine, respectively with average recoveries of 61.0% for sulfathiazole; 94.5% for sulfamethazine and 86.0% for sulfadimethoxine at the 100 μg/kg level. As the final step of validation procedure, the analysts were submitted to a blind spiked sample prepared by the quality assurance officer which results were successfully obtained regarding recovery and deviations (Granja, Nino, Rabeno & Salerno, 2008).

1.7 Chromatographic Determination

1.7.1 High Pressure Liquid Chromatography (HPLC)

HPLC is the most widely used of all of the analytical separation techniques (Skoog & James, 1992). The reasons behind this popularity are its sensitivity, its ready adaptability to accurate quantitative determinations, its suitability for separating nonvolatile species or thermally fragile ones, and above all, its widespread applicability to substances that are of prime interest to industry, to many fields of science, and to the public. In analytical HPLC, the focus is to obtain information about the sample compounds. The information that can be obtained includes identification, quantification and resolution of a compound. Bonded-phase packings are classified as reversed-phase when the bonded coating is nonpolar in character and as normal-phase when the coating contains polar functional groups. Most commonly, the R group of the siloxane in these coatings of the reversed-phase is a C8 chain or a C18 chain. 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 upon 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	$Rs = 2\ [(t_R)_B - (t_R)_A] / [W_A + W_B]$

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.

1.7.2 Screening Methods and Clean-up Procedure

The first test for establishing antimicrobial agent residues in milk (microbial inhibitor test) was developed as early as 1952. It was then known, too, that the presence of these agents could cause the inhibition of the starter cultures used in dairy industry, and for this reason the development of such methods was initiated so as to establish the inhibitor agent levels in milk. It was important that the methods be relatively cheap, simple to carry out, and capable of detecting a wide variety of antimicrobial agents. Of the methods used, microbial inhibitor methods suited best these requirements. A drawback which limits their use is a long incubation period (Mitchell et al. 1998).

HPLC methods tend to prevail due to their good qualitative and quantitative analysis and simple procedure. Fluorescence detectors (Bernal, Nozal, Jiménezb, Martínb, & Sanz, 2009) are sometimes used for their inherent sensitivity, but pre- or post-column derivatization with fluorescence reagents is required. Recently, mass spectrometric detectors (Lu, Chen, & Lee, 2007) have attracted attention and become a preferred choice, although they are very expensive. For HPLC with a UV detector, several matrix influences may occur when sulfonamides are monitored (Lu et al., 2007), but these can be eliminated by using an appropriate extraction and preconcentration procedure. At present, preconcentration and clean-up techniques for sulfonamides mainly focus on solid phase extraction (Zayas-Blanco, Garcia-Falcon & Simal-Gandara, 2004), matrix solid phase dispersion (Kishida & Furusawa, 2001) and liquid–liquid extraction (Pang, Cao, Zhang, Jia, Fan & Li, 2005). These methods are time-consuming and present a threat to the environment and human health due to the use of organic solvents, although they have their respective advantages. Other novel methods, such as solid phase microextraction, reduce the demand for organic solvents (Lu et al., 2007).

1.8 Cloud Point Extraction Procedure

Separation and preconcentration procedures are considered of great importance in analytical and environmental chemistry. This enables elimination or minimization of matrix effects and concomitants, lowering the detection limit of many analytes with different techniques and enhancing the detectability for many analytes.

The cloud point procedure (CPE) procedure is based on the following phenomenon: an aqueous solution of some surfactant becomes turbid and separates into two isotropic phases if some condition such as temperature or pressure is changed or if an appropriate substance is added to the solution. The surfactant solution becomes turbid because it attains the cloud point. At this point, the original surfactant solution separates into a surfactant phase of small volume, which is rich in the surfactant and containing the analyte or metal (organic or inorganic species)

trapped by micellar structures and a bulk diluted aqueous phase (Hinze & Pramauro, 1993).

The use of micellar systems such as CPE has attracted considerable attention in the last few years mainly because it is in agreement with the “green chemistry” principles. Green chemistry can be defined as those procedures for decreasing or eliminating the use or generation of toxic substances for human health and for the environment (Anastas, 1999). CPE is a green method for the following reasons:

- It uses as an extractor media diluted solutions of the surfactants that are inexpensive, resulting in the economy of reagents and generation of few laboratory residues,

- Surfactants are not toxic, not volatile, and not easily flammable, unlike organic solvents used in liquid–liquid extraction (Bezerra, Marcos de Almeida, Arruda, Zezzi-Ferraira & Costa, 2005).

The small volume of the surfactant-rich phase obtained with this approach permits the design of extraction strategies that are simple, inexpensive, and highly efficient when compared to those extractions that use organic solvents. The main limitation of CPE is the relatively low partition coefficients of several metal species with determinate chelates. However, it can be circumvented with the use of highly hydrophobic ligands (Quina & Hinze, 1999; Pramauro & Prevot, 1995). Another advantage of CPE to traditional procedures such as solid-phase extraction (SPE), liquid–liquid extraction (LLE) and coprecipitation is the high preconcentration factor that can be obtained starting from the small initial volumes of the sample. Those techniques often demand an additional stage of metal reextraction of the collector phase for a solution that can be submitted to an analytical technique. Reextraction of the analyte generates a larger final solution volume and to obtain a high preconcentration factor uses a larger volume of the sample. Thus, CPE presents a high capacity to concentrate a wide variety of metal with quantitative recoveries and high preconcentration factors, since the metal can be collected in small volumes (0.2–0.5 mL) of the surfactant phase. This allows preconcentration factors identical

to those of other techniques without an additional reextraction step (Bezerra et al, 2005).

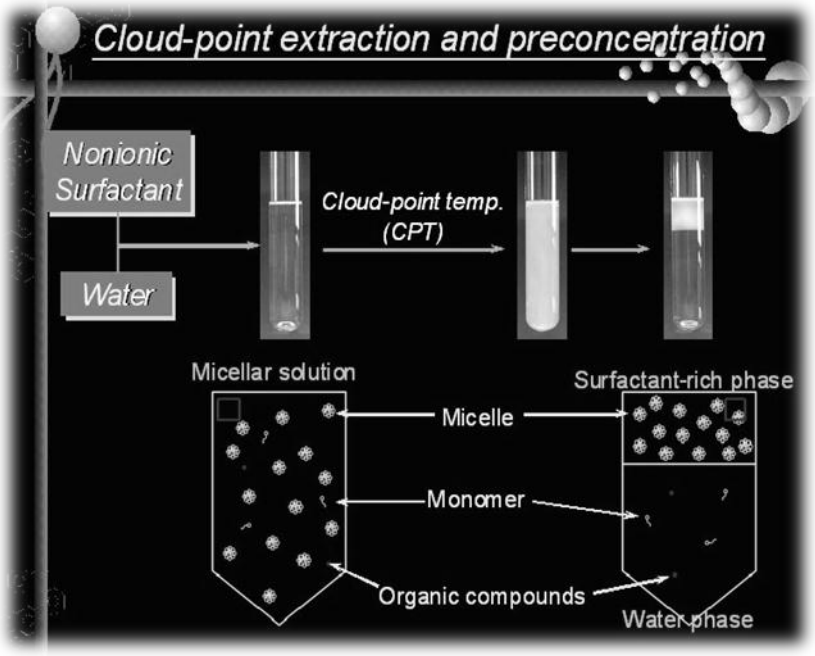


Figure 1.4 Basic principle of cloud point extraction

Cloud point extraction can be utilized for the preconcentration and separation of organic substances and metal ions. The metal can be in ionic form or in hydrophobic chelates that are produced after reaction under appropriate conditions (Stalikas, 2002). Then, a thorough optimization of the chemical and operational parameters is required to ensure reliable quantitative separation and high preconcentration efficiency for the subsequent metal ion determination. Thus, the characteristics of each spectroanalytical technique must be considered for developing a method.

1.9 Surfactants

Surfactants are amphiphilic organic substances. Their molecules present a long hydrophobic hydrocarbon chain and a small charged group or polar hydrophilic. 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). The most usual chemical classification of surfactant is based on

the hydrophilic group nature. The four general groups of surfactants are defined as non-ionic, cationic, anionic, and amphoteric (or zwitterionic) (Myers, 1991). Table 1.5 presents some characteristics and examples of each of the four groups.

Table 1.5 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 ($ROSO_3^-$)	$CH_3(CH_2)_{11}SO_4^-Na^+$ Sodium dodecil sulfate (SDS)
Cationic	The hydrophilic group carrying a positive charge as, for example, the quaternary ammonium halides ($R_4N^+Cl^-$)	$CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$ 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	$CH_3(CH_2)_{11}(OCH_2CH_2)_{23}OH$ Polyoxyethylene (23) dodecanol (Brij 35)
Zwitterionic	Its molecules present both the anionic and cationic groups and, depending of pH, its prevalence the anionic, cationic, or neutral species	$CH_3(CH_2)_{11}N^+(CH_3)_2(CH_3)COO^-$ 4-(Dodecyldimetyl ammonium) butyrate (DAB)

The most intensely studied and discussed type of microscopically ordered molecular aggregates is also the simplest in terms of structure, the micelles. Micelles are supramolecular structures of colloidal dimensions formed by surfactants

molecules that aggregate in a spontaneous way in aqueous solution when critical micelle concentration (CMC) is attained. 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 predominantly in a nonassociate monomer form. However, when the CMC is attained, the formation process is favored (Figure 1.5). Micelles are not static structures. An important micelle characteristic is its dynamic equilibrium with the dissolved surfactant monomers, which remain at an approximately constant concentration after reaching the CMC. 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).

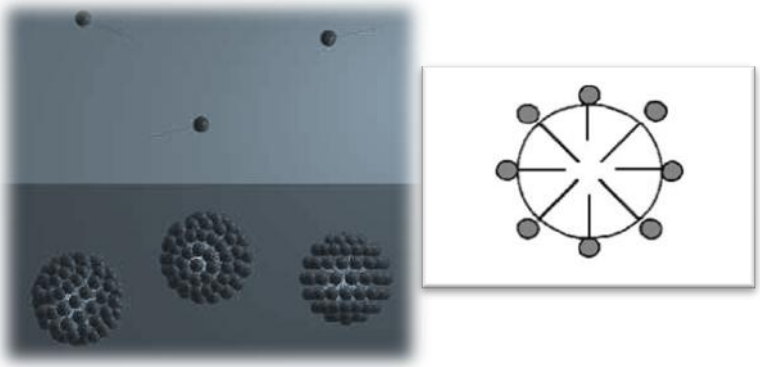


Figure 1.5 Before CRM and after CRM

As we can see, cloud point is also a measure of the hydrophil/lipophil balance of a surface-active agent. When a surfactant can have its cloud point run in an aqueous solution, it is therefore a water soluble surface active agent; whereas, when a surfactant must have its cloud point run in an aqueous/solvent mixture, it is either water dispersible or oil soluble in character. Nonionic surface-active agents are less soluble at elevated temperatures in aqueous solutions and, therefore, exhibit a cloud point which varies with the hydrophilic/lipophilic balance of the nonionic surface-active agent.

1.10 Purpose of the Study

Determination of sulfonamides becomes essential because of inhibiting folic acid synthesis and causing permanent damage in human nature by following the chain of consumption.

Sulfonamides are used widely in many countries also in Turkey. And only there are a few articles in literature of Turkey. For these purposes, the development of analytical methodologies for determination of sulfonamides in food products is getting more and more important.

The aim of this study was to determine the amount of sulphadiazine and sulfamethoxazole in food samples such as milk and honey by using a CPE method.

To achieve this goal we aimed at:

(1) Controlling of working wavelengths for SDA and SMZ,

(2) Optimization for cloud point extraction procedure,

(i) Effect of pH,

(ii) Effect of surfactant concentration (v/v, %),

(iii) Effect of salt type & concentration (w/v, %),

(iiii) Effect of incubation time (min),

(iiiii) Effect of equilibration temperature (^{O}C).

(3) Optimization for HPLC method,

(i) Determination of oven temperature (^{O}C),

(ii) Selection of mobile phase ratio (v),

(iii) Control of flow rate (mL/min),

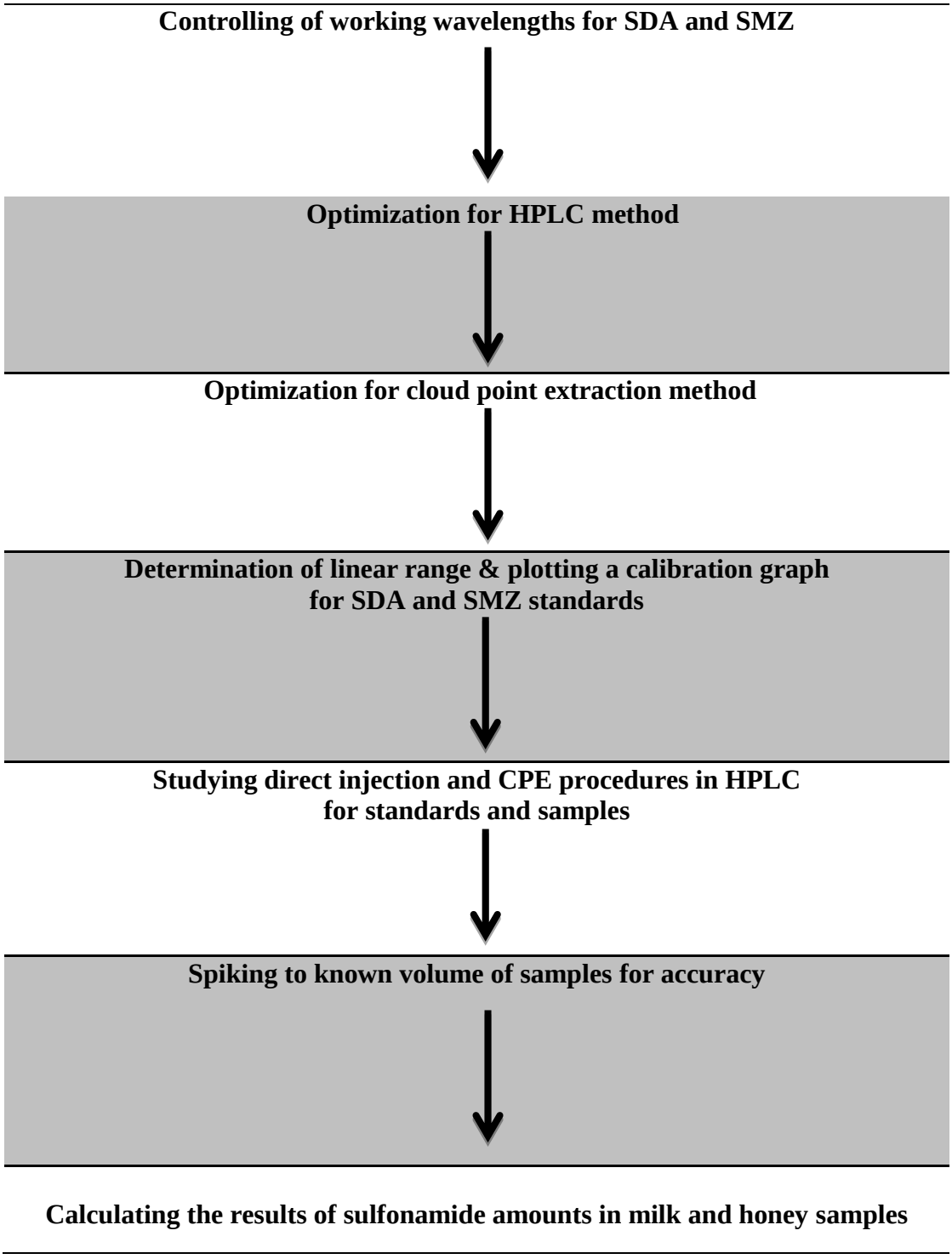
(4) Determination a linear range and plotting a calibration graph for standards of SDA and SMZ,

(5) Studying direct injection and CPE procedures in HPLC for standards and samples,

(6) Spiking to known volume of samples for accuracy,

(7) Calculating the results of sulfonamide amounts in milk and honey samples.

Table 1.6 Flowchart of the study



CHAPTER TWO
EXPERIMENTAL METHODS AND MATERIALS

2.1 Preparation of Reagent Solutions

A stock solution of sulfonamide (approximately 200.0 mg/L) was prepared by dissolving 20.0 mg of sulfonamide (Sigma–Aldrich) in 100.0 mL methanol (Panreac) and stored at -20 ^{o}C.

A series of working sulfonamide standard solutions from 25.0 µg/L to 10.0 mg/L were prepared of the stock solutions, followed by perform CPE procedure and calibrate the HPLC detector response.

Surfactant solution was prepared by dissolving 10.0 mL of Triton X-114 (Sigma-Aldrich) in the final volume of 100.0 mL ultra pure water.

Salt solutions were prepared as 10% (w/v) by dissolving sodium sulphate (Sigma-Aldrich) in 100.0 mL of ultra pure water and same procedure were performed for other salt solutions which include sodium chloride and sodium carbonate from (Sigma-Aldrich).

3.0 mL of milk were added to 6.0 mL of organic solvent (5:1, Acetone : Acetonitrile) in order to make milk denatured and centrifuged in 5000 rpm for 20 minutes. Then upper phase was evaporated in a nitrogen atmosphere to eliminate organic solvent and residue was preconcentrated by CPE in order to be analysed.

In addition to preparing unspiked milk solutions, spiked milk solutions which include 0.4 mg/L of SDA and SMZ standard solutions were prepared. Then 3.0 mL of spiked milk solution were taken for CPE procedure in the final volume of 10.0 mL as 0.12 mg/L which was preconcentrated to 1.2 mg/L in order to calculate the percentage recovery.

Honey solutions were prepared by dissolving 2 grams of honey in 10.0 mL of ultra pure water and 3.0 mL of this solutions were used for CPE procedure.

In addition to preparing unspiked honey solutions, spiked honey solutions which include 0.4 mg/L of SDA and SMZ standard solutions were prepared. Then 3.0 mL of spiked honey solution were taken for CPE procedure in the final volume of 10.0 mL as 0.12 mg/L which was preconcentrated to 1.2 mg/L in order to calculate the percentage recovery.

2.2 Apparatus

Schimadzu UV-1201 spectrophotometer in figure 2.1 was used to determine the wavelengths for working solutions of SDA and SMZ.

Figure 2.1 Schimadzu 1201 UV spectrophotometer

The instrument used was an Agilent Technologies 1100 series liquid chromatographic system equipped with a DAD detector at 270 nm controlled by Chemstation 3D software (Figure 2.2). This system includes Agilent 1100 series quaternary pump (model G1311A). ACE C18 reverse phase (4x250 mm, 5µm)

HPLC analytical column was used to separate SDA and SMZ from the matrix. Injections were performed by 100 μL syringe which was from Hamilton to HPLC apparatus comprising the following injection system with 20 μL injection loop. The separations were achieved by isocratic elution.

Figure 2.2 The Agilent Technologies 1100 series HPLC system

Millipore Milli-Q Advantage A10 Ultrapure Water Purification System was used for needs in almost every step.

2.3 Procedure for Optimization of HPLC for Separation

The optimized parameters for the proposed procedure in HPLC were of oven temperature, mobile phase ratio and flow rate.

2.3.1 Optimization of Oven Temperature

A suitable mobile phase methanol/water (22:78, v/v) was preferred depending on the literatures. 5.0 mg/L concentration of sulfonamide standard solution was injected to HPLC at 1.0 mL/min flow rate and the effect of oven temperature was studied from 25 to 50 °C.

2.3.2 Optimization of Mobile Phase Ratio

Sulfonamide working solutions as 5.0 mg/L was injected into the HPLC system at a flow rate of 1.0 mL/min and oven temperature at 35 °C. A mobile phase as methanol/water at different ratios (18:82, 22:78, 25:75) was tested to get the best retention time.

2.3.3 Optimization of Flow Rate

After optimization of mobile phase ratio, the retention time at different sulfonamide concentration was controlled in methanol/water (22:78, v/v) mobile phase at 35 °C oven temperature while the flow rate of mobile phase was increased from 1.0 to 2.0 mL/min.

2.4 Procedure for Optimization of CPE for Separation

According to the effective conditions such as pH, salt type and concentration, surfactant concentration, incubation time and equilibration temperature, experiments were tested for stable phase separation. Different chosen parameters as can be seen in Table 2.1, 2.2, 2.3, 2.4 and 2.5 were used for better CPE result in all optimization studies.

2.4.1 pH Effect

All pH parameters were performed in settled experimental conditions such as sulfonamide standard solution (5.0 mg/L), salt solution (Na_2SO_4, 1%), surfactant solution (0.3%), incubation time (20 min.) and equilibration temperature (50 ^oC).

Table 2.1 Chosen parameters of pH optimization

pH
2.8
3.9
5.0
5.9
7.0
8.0

2.4.2 Surfactant Concentration

Surfactant optimization parameters were performed in settled experimental conditions such as sulfonamide standard solution (5.0 mg/L), salt solution (Na_2SO_4, 1%), pH (3.9), incubation time (20 min.) and equilibration temperature (50 ^oC).

Table 2.2 Chosen parameters of surfactant optimization

Triton Conc. (v/v %)
0.10
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
2.50

2.4.3 Salt Type and Concentration

All salt types (Na_2SO_4, Na_2CO_3, NaCl) and their concentration parameters were performed in settled experimental conditions such as sulfonamide standard working solution (5.0 mg/L), surfactant solution (2%), pH (3.9), incubation time (20 min.) and equilibration temperature (50 ^oC).

Table 2.3 Chosen parameters of salt type & concentration optimization

Na_2SO_4 (w/v %)	**Na_2CO_3 (w/v %)**	**NaCl (w/v %)**
0.25	0.25	0.25
0.50	0.50	0.50
0.75	0.75	0.75
1.00	1.00	1.00

2.4.4 Incubation Time

All incubation time parameters were performed in settled experimental conditions such as sulfonamide standard solution (5.0 mg/L), Triton X-114 solution (2%), pH (3.9), Na_2SO_4 concentration (0.75%) and equilibration temperature (50 ^oC).

Table 2.4 Chosen parameters of incubation time optimization

Incubation Time (min)
10
20
30
40
50
60

2.4.5 Equilibration Temperature

All equilibration temperature parameters were performed in settled experimental conditions such as sulfonamide standard solution (5.0 mg/L), surfactant solution (2%), pH (3.9), Na_2SO_4 concentration (0.75%) and incubation time (20 min).

Table 2.5 Chosen parameters of equilibration temperature optimization

Equilibration Temperature (^oC)
20
30
40
50
60
70

2.5 Sampling and Storage

Ten honey samples (Balkovan, Balparmak for Kids, Balparmak, Bergama Çam, Bergama Çiçek, Bitlis Karakovan, Iranian Darraye, Iranian Ghasemlu, Konya Geven and Village) and two milk samples (Pınar UHT, Sek Daily) were purchased from the market. All honey samples were stored in their original bottles, darkness at +4 °C because of the storage conditions and milk was used freshly.

2.6 Sample Preparation

All samples were filtered through from 0.45 μm filter paper (Millipore Millex-HV, Hydrophilic PVDF) and added to vials.

3.0 mL of milk were added to 6.0 mL of organic solvent (5:1, Acetone:Acetonitrile) in order to make milk denatured and centrifuged in 5000 rpm for 20 minutes. Then upper phase was evaporated in a nitrogen atmosphere to eliminate organic solvent and residue was preconcentrated by CPE in order to be analysed.

Besides the direct usage of milk and honey samples, spiked versions of samples were prepared for checking the accuracy of the results.

Spiked milk solutions which include 0.4 mg/L of SDA and SMZ standard solutions were prepared. Then 3.0 mL of spiked milk solution were taken for CPE procedure in the final volume of 10.0 mL as 0.12 mg/L which was preconcentrated to 1.2 mg/L in order to calculate the percentage recovery.

Honey solutions were prepared by dissolving 2.0 grams of honey in 10.0 mL of ultra pure water and 3.0 mL of this solutions were used for CPE procedure.

In addition to preparing unspiked honey solutions, spiked honey solutions which include 0.4 mg/L of SDA and SMZ standard solutions were prepared. Then 3.0 mL of spiked honey solution were taken for CPE procedure in the final volume of 10.0 mL as 0.12 mg/L which was preconcentrated to 1.2 mg/L in order to calculate the percentage recovery.

2.6.1 CPE Procedure

All milk samples were added to tubes with an organic solvent (acetone/acetonitrile, 5:1) and evaporated to dryness under N_2 stream. Residues were preconcentrated by CPE then dissolved in HPLC mobile phase and analyzed. Performed CPE procedure for milk can be seen below, respectively.

All samples were analyzed with and without spiked SDA and SMZ solutions.

- ✓3.0 mL milk sample + 6.0 mL solvent (5:1, Acetone:ACN)
- ✓Centrifuged at 5000 rpm for 20 minutes
- ✓Supernatant phase was taken (upper phase)
- ✓Evaporated in a nitrogen atmosphere to eliminate organic solvent
- ✓Residue was taken
- ✓Added 1.0 mL pH: 3.9 phosphate buffer

- ✓Added 0.75 mL 10% Na_2SO_4 (for optimum 0.75% value)
- ✓Added 2.0 mL 10% Triton X-114 (for optimum 2% value)
- ✓Final volume was made up to 10.0 mL with ultra-pure water
- ✓Mixture was equilibrated at 50 ^oC for 20 minutes
- ✓Centrifuged at 5000 rpm for 10 minutes
- ✓Solution was kept in an ice bath for 10 minutes
- ✓Rich phase was taken
- ✓Final volume was made up to 1000 µL with mobile phase (78:22, Pure Water/Methanol)
- ✓Injection to HPLC

A different procedure was performed for honey samples as can be seen below.

- ✓3.0 mL honey solution
- ✓Added 1.0 mL pH: 3.9 phosphate buffer
- ✓Added 0.75 mL 10% Na_2SO_4 (for optimum 0.75% value)
- ✓Added 2.0 mL 10% Triton X-114 (for optimum 2% value)
- ✓Final volume was made up to 10.0 mL with pure water
- ✓Mixture was equilibrated at 50 ^oC for 20 minutes
- ✓Centrifuged at 5000 rpm for 10 minutes
- ✓Solution was kept in an ice bath for 10 minutes
- ✓Rich phase was taken
- ✓Final volume was made up to 1000 µL with mobile phase (78:22, Pure Water/Methanol)
- ✓Injection to HPLC

2.7 Statistical Analyze for Validation of Method

The samples employed in this study are of great chemical complexity and are difficult to reproduce accurately. Thus, the method of standard additions was used in the validation of this method.

The limit of detection (LOD) (3 s) was calculated using the following relation.

$S_m = S_{bl} + k\ s_{bl}$

$S = S_{bl} + mc$

$LOD = c_m = (S_m - S_{bl}) / m$

Where:

S_m: Minimum distinguishable analytical signal,

S_{bl}: The average signal of blank solution,

s_{bl}: The standard deviation of blank solution,

k: The constant which is 3,

m: The slope of the calibration curve.

The limit of quantitation (LOQ) (10 s) was calculated as the lowest concentration for which acceptable data of recovery and precision were obtained.

Once the procedures were optimized, the study of the repeatability was undertaken. Detection and quantitation limits of the method were found as 5.68 and 20.09 ppb and 6.06 and 21.86 ppb for SDA and SMZ, respectively, the linear range of quantitation for analytes was approximately 0.025 - 2.000 ppm, the percentages of average recovery as 65.2% and 99.4% and the relative standard deviation percentage as 0.51% and 2.17% were found for SDA and SMZ, respectively.

CHAPTER THREE
RESULTS AND DISCUSSIONS

3.1 Optimization Procedure for SDA and SMZ in HPLC

A successful use of HPLC for determining the amount of SDA and SMZ in milk and honey samples depended on the choice of the right combination of operating conditions: flow rate of the mobile phase, the mobile phase composition and temperature of the column. This choice in turn required a basic understanding of the various factors that control HPLC separations. So oven temperature, mobile phase composition and flow rate were optimized before this analyze in this study.

After HPLC and CPE optimizations, 5.0 mg/L of sulfonamide solution which include both of SDA and SMZ were given to HPLC in order to get the optimum chromatogram which is shown in figure 3.1 and chromatography was performed at 3.9 pH, in 0.75% Na_2SO_4 solution, 2% Triton X-114 solution, 20 min incubation time and at 50 ^oC equilibration temperature.

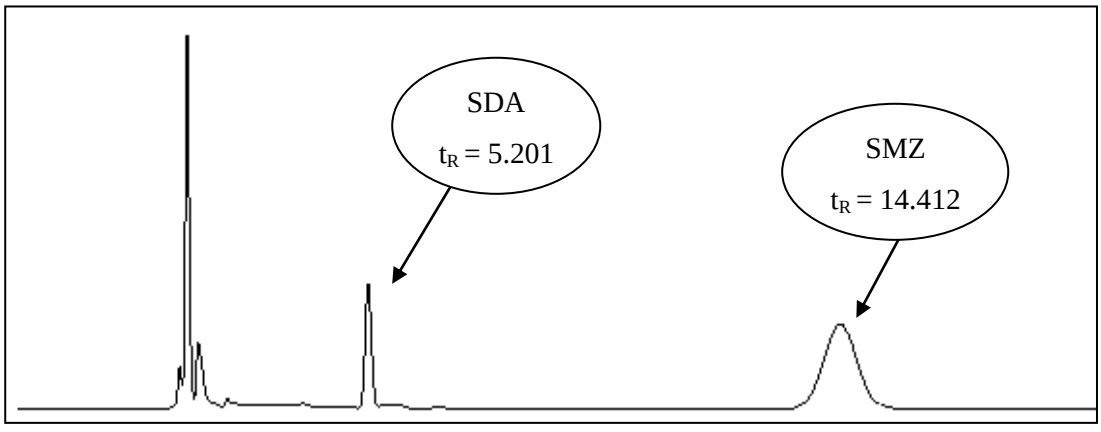


Figure 3.1 Chromatogram of mixed SDA and SMZ solution in optimum parameters

3.1.1 Optimization of Oven Temperature

Temperature effects in HPLC are not as significant as in gas chromatography. Volatile solvents are not allowed to rise to higher temperatures too much, and the stability of the attached bonded ligands on the adsorbent surface may be influenced

by the high temperature. So, the main temperature range is from ambient temperature to 60 or 70 °C (Kazakevich & McNair, 1996-2002).

Stabilization of the column under the elevated temperature usually leads to the stabilization of the retention times. Origin of this effect is not well understood yet. Possible explanation is that the solvent viscosity decreased and more uniform stabilized temperature with absence of local temperature fluctuations due to the solvent friction lead to the more uniform adsorption-desorption process (Kazakevich & McNair, 1996-2002). Another effect is the increase of the column efficiency. At the elevated temperature viscosity of liquids decrease and the diffusion coefficient increase. From the Van Deemter equation the second term will increase which will lead to the decrease of the efficiency at the very low flow rates (which is not important). The last term will decrease which will lead to the increasing of the efficiency at the common flow rates. It also widens the flow rate range with optimum efficiency (Kazakevich et al, 1996-2002).

In order to get best oven temperature value, 5.0 mg/L of mixed standard solution including SDA and SMZ was injected to HPLC and 35 °C was chosen as the best parameter as can be seen in table 3.1.

Table 3.1 Optimization of Oven Temperature

Oven Temperature (°C)	**Retention Time (min) (SDA)**	**Retention Time (min) (SMZ)**
25	4.84	15.32
30	4.77	15.17
35	4.65	14.68
40	4.69	14.95
45	4.82	15.65
50	5.01	15.72

3.1.2 Optimization of Mobile Phase Ratio

In order to get best mobile phase ratio, 5.0 mg/L of mixed standard solution including SDA and SMZ was injected to HPLC and 22:78 (methanol:water) was chosen as the best parameter as can be seen in table 3.2.

Table 3.2 Optimization of mobile phase ratio optimization

Mobile Phase Ratio (22:78, v/v %)	**Retention Time (min) (SDA)**	**Retention Time (min) (SMZ)**
18:82	4.84	15.00
22:78	4.80	14.98
25:75	4.81	15.22

3.1.3 Optimization of Flow Rate

In order to get best flow rate, 5.0 mg/L of mixed standard solution including SDA and SMZ was injected to HPLC and 1.0 mL/min was chosen as the best parameter as can be seen in table 3.3.

Table 3.3 Optimization of flow rate optimization

Flow Rate (mL/min)	**Retention Time (min) (SDA)**	**Retention Time (min) (SMZ)**
1.0	4.91	14.91
1.5	4.80	14.76
2.0	4.51	14.11

3.2 Calibration Curves of SDA and SMZ

In order to verify the linearity of the response of SDA and SMZ solutions at the specified wavelengths for the working concentration, standard solutions of sulfonamides were prepared and injected to HPLC. Different standards of

sulfonamides as mixture solutions were prepared in HPLC mobile phase as 22:78, methanol:water.

The calibration graph was constructed by plotting the SDA and SMZ peak areas against different levels of sulfonamides' concentration which ranged from 0.10 to 1.0 mg/L. Regression equations of the calibration curves had of y = 95.954x - 0.437 and y = 86.229x - 1.3418 where y was the peak area, x was the concentration of SDA and SMZ in the standard solutions, respectively. The curves also were linear within the given range, with the regression coefficient of 0.9993 and 0.9998 (Figure 3.2 and 3.3), respectively.

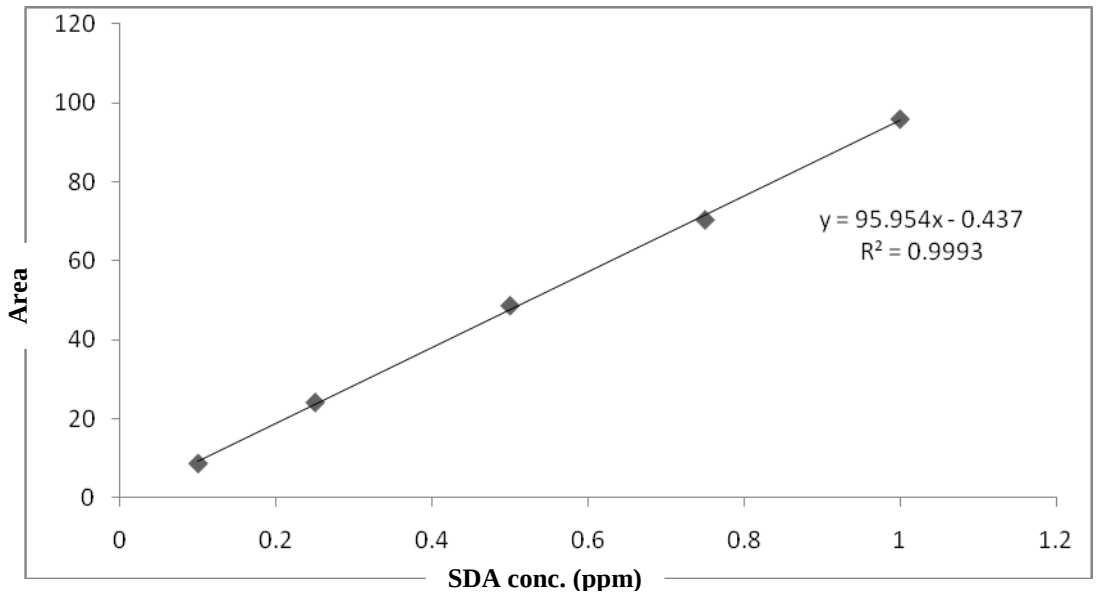


Figure 3.2 Calibration graph of SDA solution

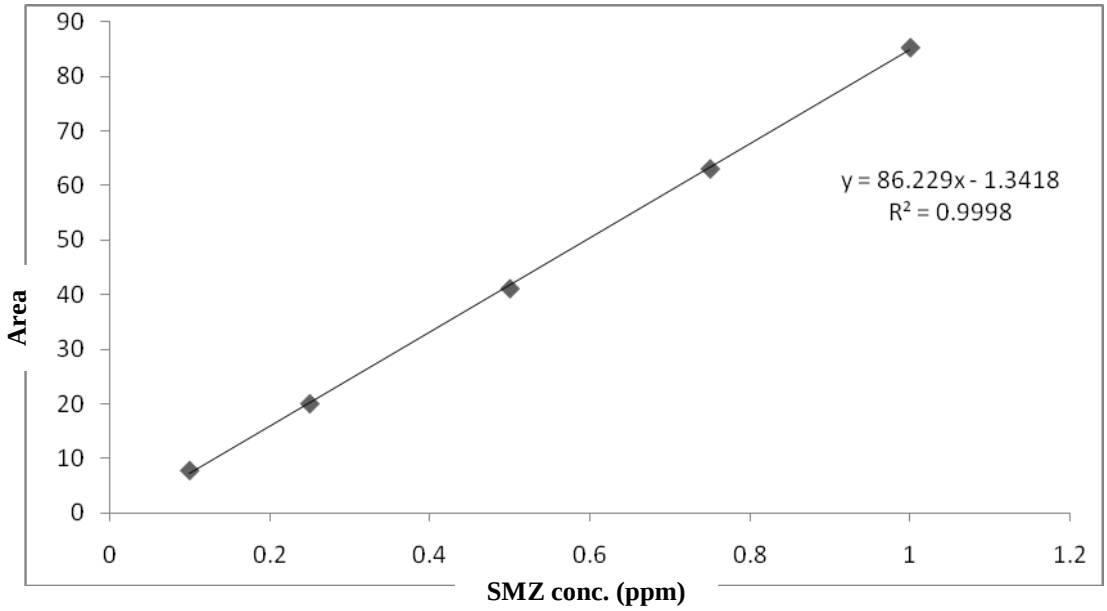


Figure 3.3 Calibration graph of SMZ solution

3.3 Determination of Sulfonamides in Food Products

A range of highly sensitive and sophisticated analytical methods has been developed for the determination of sulfonamides in several food samples and matrices. However, the determination step is usually preceded by a number of operations such as sampling, sample preparation, extraction and clean-up.

3.4 Determination of CPE Conditions

In this study, CPE procedure was performed to all milk and honey samples. During the optimization studies, only SMZ (test solution) was used as a sulfonamide.

3.4.1 pH Effect

5.0 mg/L of SMZ standard solution was injected to HPLC for pH effect controlling.

pH 3.9 was preferred as the best optimization value according to HPLC results as can be seen in Figure 3.4.

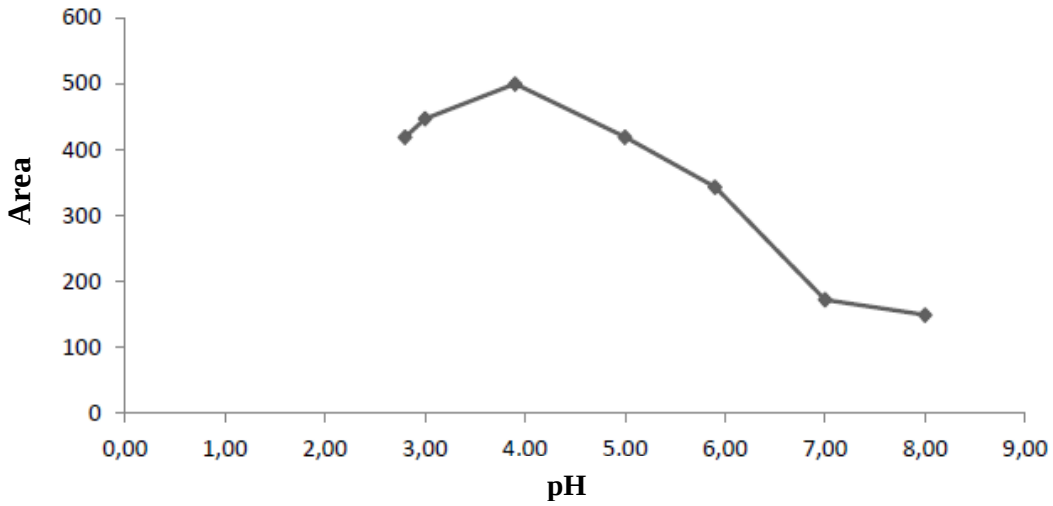


Figure 3.4 Optimization graphic of pH effect for 5.0 mg/L SMZ

3.4.2 Surfactant Concentration

5.0 mg/L of SMZ standard solution was injected to HPLC for surfactant concentration controlling.

2% surfactant concentration was preferred as the best optimization value according to HPLC results as can be seen in Figure 3.5.

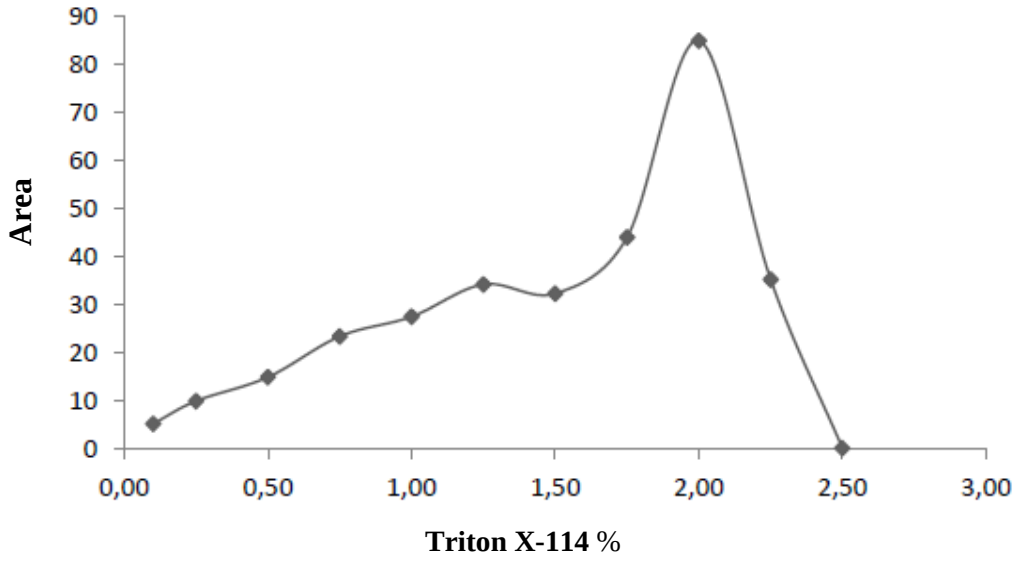


Figure 3.5 Optimization graphic of Triton X-114 concentration for 1 mg/L SMZ

3.4.3 Salt Type and Concentration

5.0 mg/L of SMZ standard solution was injected to HPLC for salt type and concentration controlling.

Na_2SO_4 (0.75%) salt concentration was preferred as the best optimization value according to HPLC results. In the presence of Na_2CO_3, phase separation didn't occur because of alkaline salt specialties. Peak areas of NaCl salt were not as high as the areas of Na_2SO_4 salt. (Figure 3.6 and 3.7)

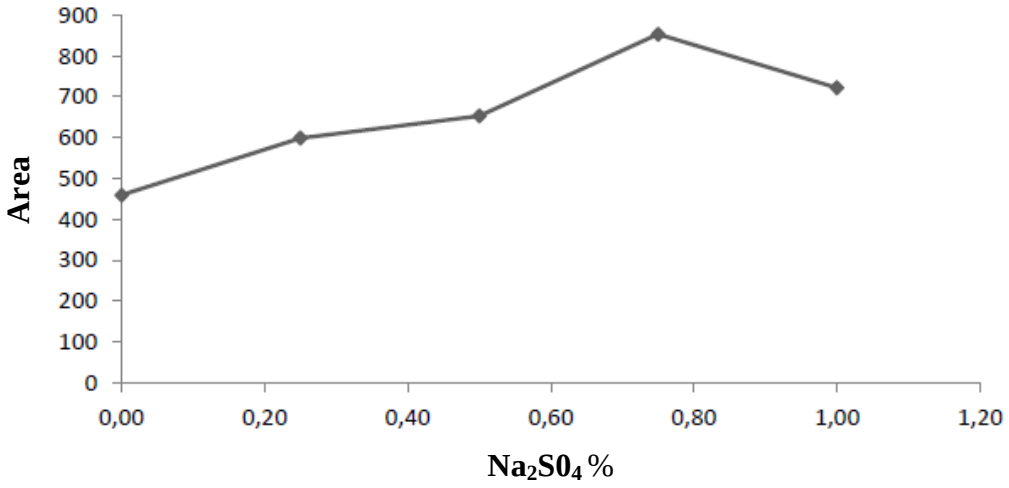


Figure 3.6 Optimization graphic of Na_2SO_4 concentration for 5.0 mg/L SMZ

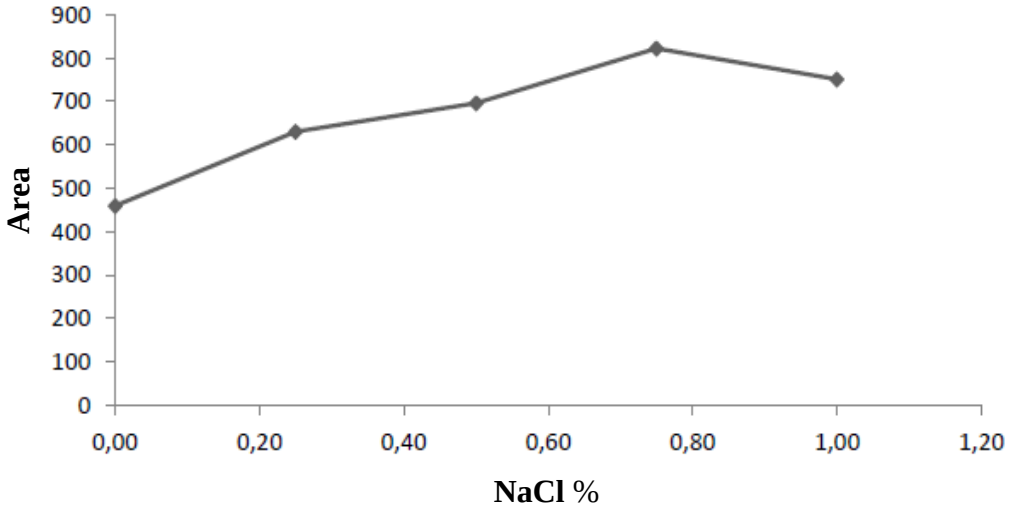


Figure 3.7 Optimization graphic of NaCl concentration for 5.0 mg/L SMZ

3.4.4 Incubation Time

5.0 mg/L of SMZ standard solution was injected to HPLC for incubation time controlling.

20 minute was preferred as the best incubation time value according to HPLC results as can be seen in Figure 3.8.

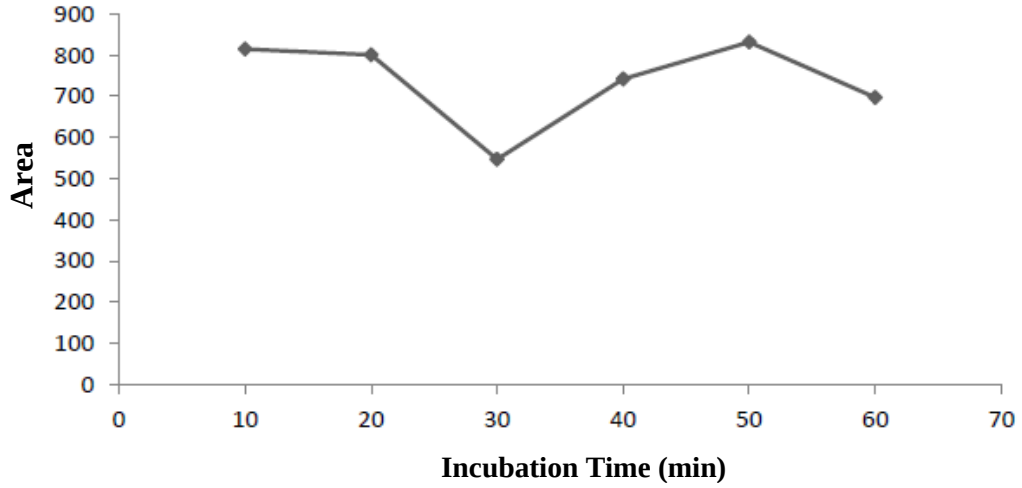


Figure 3.8 Optimization graphic of incubation time for 5.0 mg/L SMZ

3.4.5 Equilibration Temperature

5.0 mg/L of SMZ standard solution was injected to HPLC for equilibration temperature controlling.

50 ^{o}C was preferred as the best equilibration temperature value according to HPLC results as can be seen in Figure 3.9.

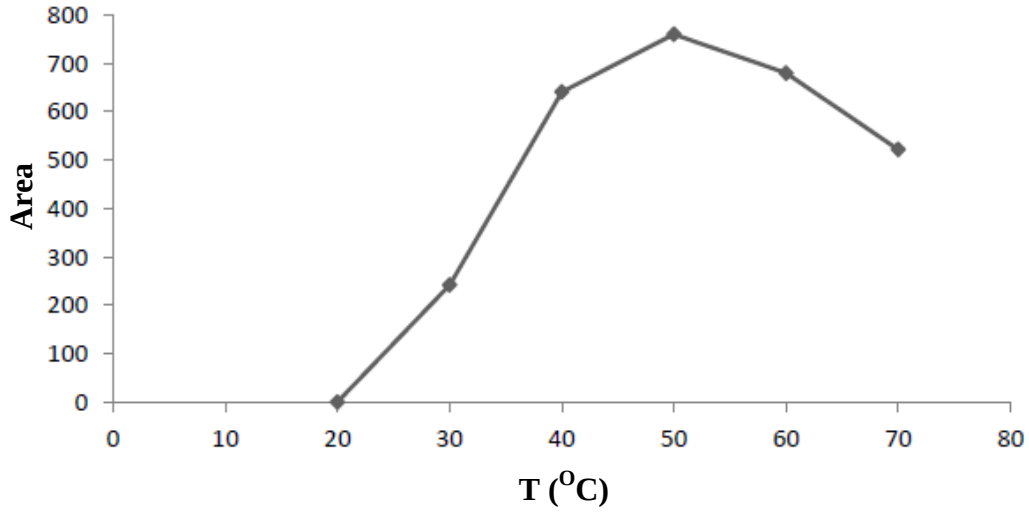


Figure 3.9 Optimization graphic of equilibration temperature for 5.0 mg/L SMZ

It should be noted that sample preparation is the time determining step in the whole analytical procedure with 2/3 of the total analysis time. It is the primary source of errors and differences in the results obtained by different laboratories.

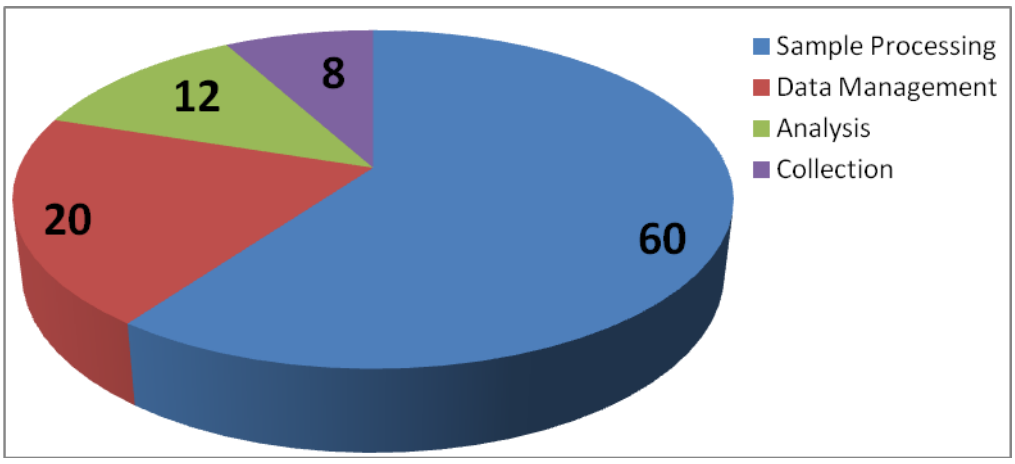


Figure 3.10 The distribution of time that spend on sample analysis

3.5 Enrichment Factor and Recovery

After proposed CPE procedure, volumes of rich phases were determined as 600±50 µL and this means that our total volume in tube which includes 10.0 mL of solution was preconcentrated into 600 µL. Thus, enrichment factor is calculated as 10.0 mL / 600 µL = 16.7 (approximately).

0.1 mg/L of mixed sulfonamide solution was studied for preconcentration and the average recoveries of SDA and SMZ were in the range of 65.2 - 99.4, respectively.

Table 3.4 Calculation of recovery for SDA

Average Area of preconcentrated SDA (1.0 mg/L)	37.8395
Rich Phase Vol. (µL)	600
Preconcentration factor	16.7
Preconcentration factor after dilution	10
Area of 1.0 mg/L SDA standard solution from calibration curve	58.0047
Recovery %	37.8395/58.0047 = **65.2%**

Table 3.5 Calculation of recovery for SMZ

Average Area of preconcentrated SMZ (1.0 mg/L)	55.0918
Rich Phase Vol. (µL)	600
Preconcentration factor	16.7
Preconcentration factor after dilution	10
Area of 1.0 mg/L SMZ standard solution from from calibration curve	55.3944
Recovery %	55.0918/55.3944 = **99.4%**

3.6 Analytical Figures of Merit

Under the optimum conditions, calibration range, detection limit, the limit of quantitation and repeatability for the chromatographic method for sulfonamides were analyzed in milk and honey samples.

The calibration study is one of the most important steps in HPLC studies for all kinds of matrix to determine the linear working range. The calibration curve of the proposed method was plotted the concentration of SDA and SMZ against the peak area and the linearity was obtained over the 0.025 - 2.000 mg/L range with the regression coefficient of 0.9993 and 0.9998, respectively. The statistical calculations were based on the triplicate injections and readings for each standard solution and milk & honey samples such as 5.68 - 20.09 µg/L (based on 3s) and 6.06 - 21.86 µg/L (based on 10s) for detection and quantitation limit for SDA and SMZ, respectively. The linear range of quantitation for analytes was approximately 0.025 - 2.000 ppm, the percentages of average recovery as 65.2% and 99.4% and the relative standard deviation percentage as 0.51% and 2.17% were found for SDA and SMZ, respectively. Table 3.6 summarized the statistical data in this study.

Table 3.6 Summary of analytical statistical data

	SDA	**SMZ**
Linear Working Equation	y = 95.954x - 0.437	y = 86.229x - 1.3418
LOD (µg/L)	5.68	6.06
LOQ (µg/L)	20.09	21.86
Linear Working Range (mg/L)	0.025 - 2.000	0.025 - 2.000
Regression Coefficient (R^2)	0.9993	0.9998

The accuracy and precision of the proposed method was evaluated using recovery index. Standard mixture of the studied sulfonamides (0.12 mg/L) was fortified into milk and honey samples prior to CPE procedure. The recoveries were calculated and summarized in the table below. It shows high recoveries which are in the acceptable recoveries for trace analysis established by the Association of Official Agricultural Chemists (AOAC) and European commission (≥ 70% and ≤ 110%).

Therefore, this method has been proven to be suitable for the determination of sulfonamides in milk and honey samples. It is expected that, this method will be effective for multi residues analysis in other matrices as well.

3.7 Results of SDA and SMZ in Milk and Honey Samples

After accuracy was ensured, quantitative results were calculated according to 65.2% SDA and 99.4% SMZ recovery and also preconcentration factor after dilution (given in table 3.4 and 3.5) was considered in order to calculate the amount of sulfonamide residues in milk and honey.

3.0 mL of milk samples and 2.0 grams of honey samples (except honey 3 sample which was prepared for 5.0 grams) were considered in order to determine the amount of sulfonamides in milk and honey for each mL and gram, respectively.

Results of recoveries of with and without spiked milk and honey samples for accuracy and quantitative results of sulfonamides in milk samples (for mL) and honey samples (for gram) were given in table 3.7.

Table 3.7 Results of recoveries of with and without spiked milk and honey samples for accuracy and quantitative results of sulfonamides in milk samples (for mL) and honey samples (for gram)

Samples	Added (mg/L)	Found (mg/L)[a] or (mg/kg)[b]		Recovery %	
		SDA	SMZ	SDA	SMZ
Milk 1	-	0.104 [a]	0.038 [a]	-	-
	0.120	0.221	0.164	98.61 ± 2.09	103.94 ± 3.17
Milk 2	-	0.062	0.018		
	0.120	0.178	0.134	97.62 ± 2.45	97.10 ± 4.84
Honey 1	-	0.021 [b]	0.006 [b]		
	0.120	0.144	0.138	102.46 ± 0.65	109.10 ± 6.99
Honey 2	-	0.017	ND*		
	0.120	0.140	0.118	102.85 ± 0.50	98.42 ± 4.45
Honey 3	-	0.019	0.006		
	0.120	0.143	0.127	103.02 ± 5.47	100.96 ± 3.91
Honey 4	-	0.030	0.010		
	0.120	0.154	0.137	102.49 ± 3.77	105.31 ± 6.22
Honey 5	-	ND*	0.048		
	0.120	0.121	0.184	101.11 ± 2.27	109.61 ± 7.51
Honey 6	-	0.027	0.015		
	0.120	0.149	0.135	101.61 ± 0.81	99.65 ± 4.12
Honey 7	-	0.643	0.162		
	0.120	0.837	0.295	109.67 ± 6.93	104.51 ± 1.19
Honey 8	-	0.110	0.052		
	0.120	0.243	0.157	105.48 ± 4.58	91.37 ± 3.92
Honey 9	-	ND*	0.015		
	0.120	0.128	0.135	106.25 ± 4.66	99.81 ± 1.69
Honey 10	-	ND*	0.017		
	0.120	0.135	0.149	112.16 ± 5.43	108.67 ± 7.02

*ND=Non dedected

3.8 Chromatograms of Milk and Honey Samples

After all optimum conditions were performed, milk and honey solutions were injected to HPLC. Chromatograms which are the mirrors of results can be seen in the figures below.

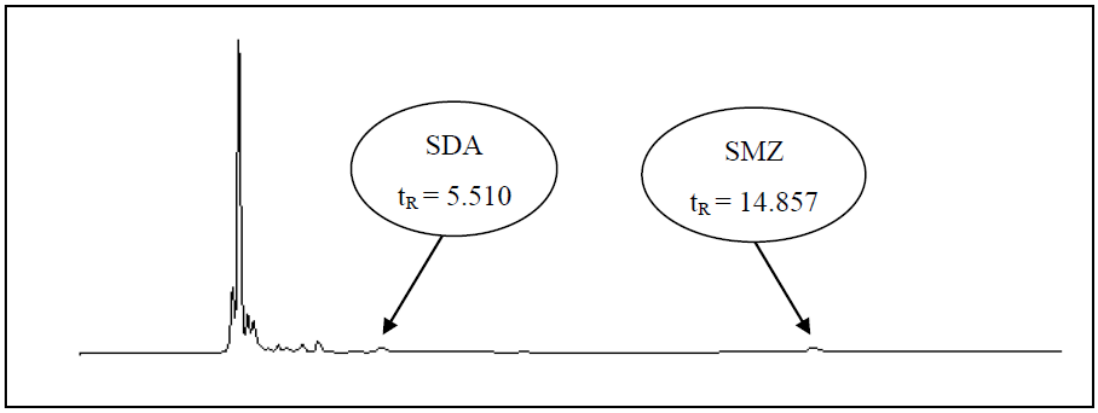


Figure 3.11 Chromatogram of unspiked milk sample 1 after CPE procedure

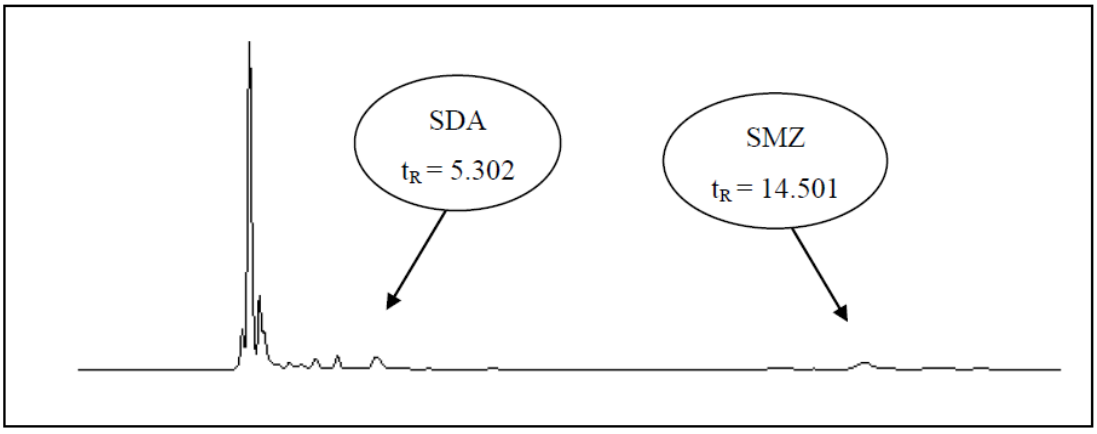


Figure 3.12 Chromatogram of spiked milk sample 1 after CPE procedure

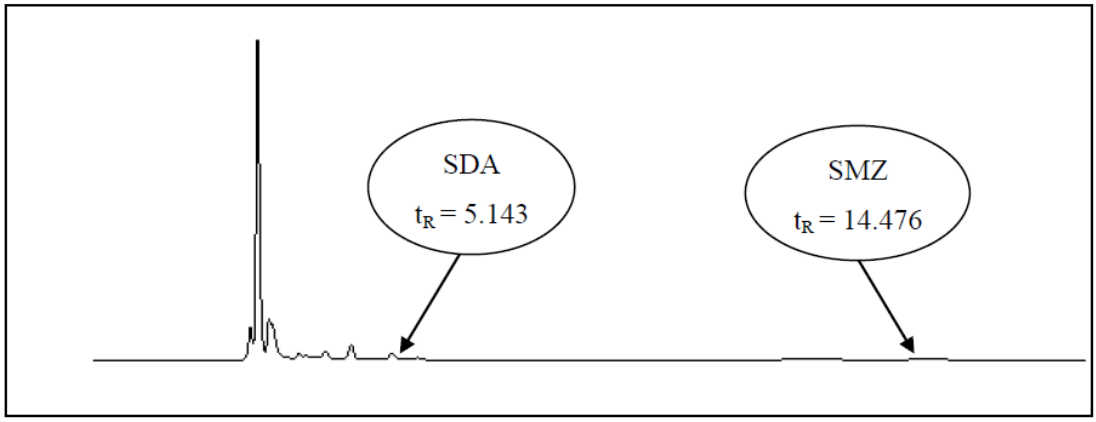


Figure 3.13 Chromatogram of unspiked milk sample 2 after CPE procedure

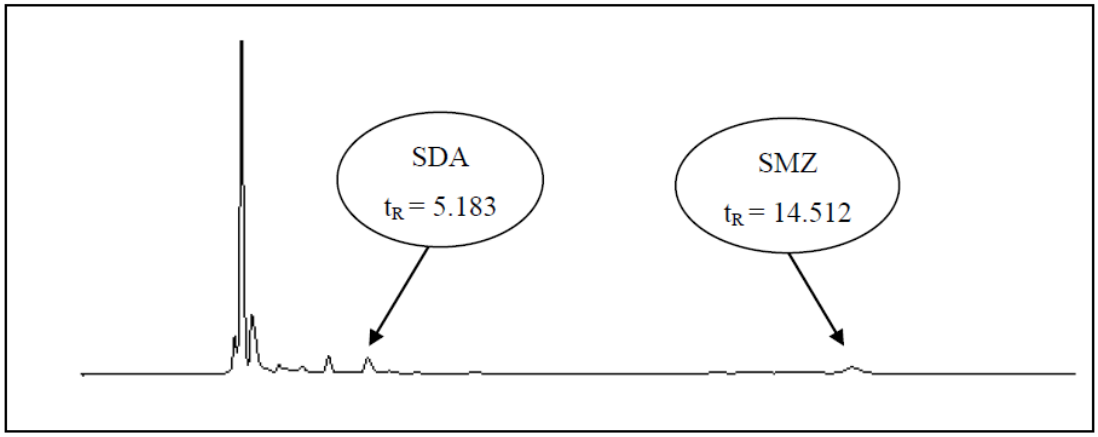


Figure 3.14 Chromatogram of spiked milk sample 2 after CPE procedure

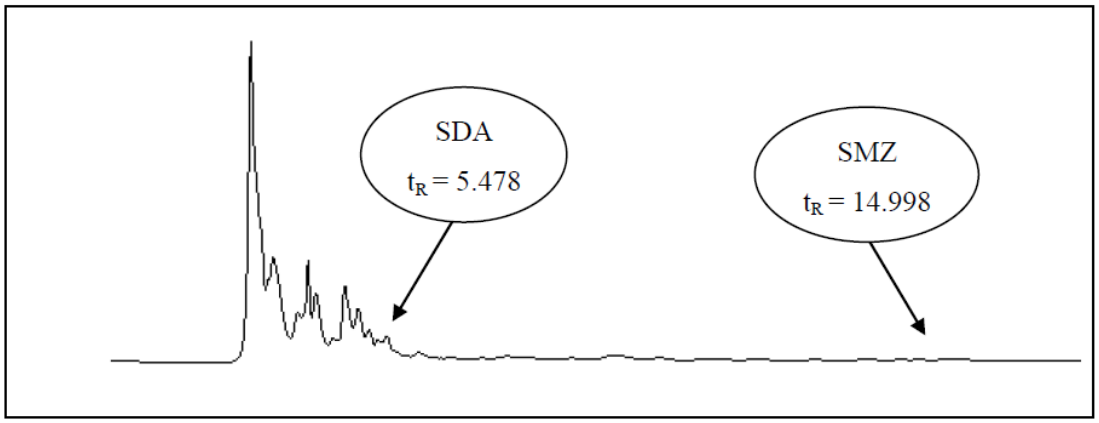


Figure 3.15 Chromatogram of unspiked honey sample 1 after CPE procedure

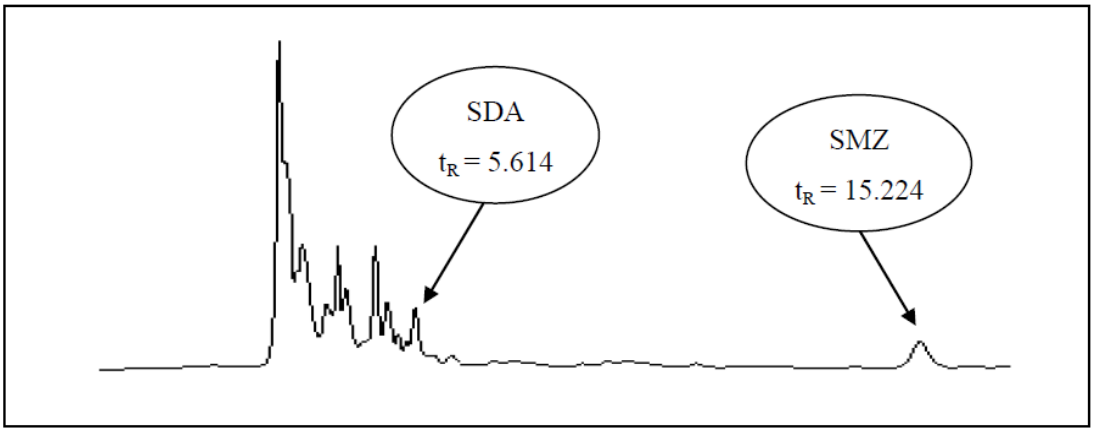


Figure 3.16 Chromatogram of spiked honey sample 1 after CPE procedure

Figure 3.17 Chromatogram of unspiked honey sample 2 after CPE procedure

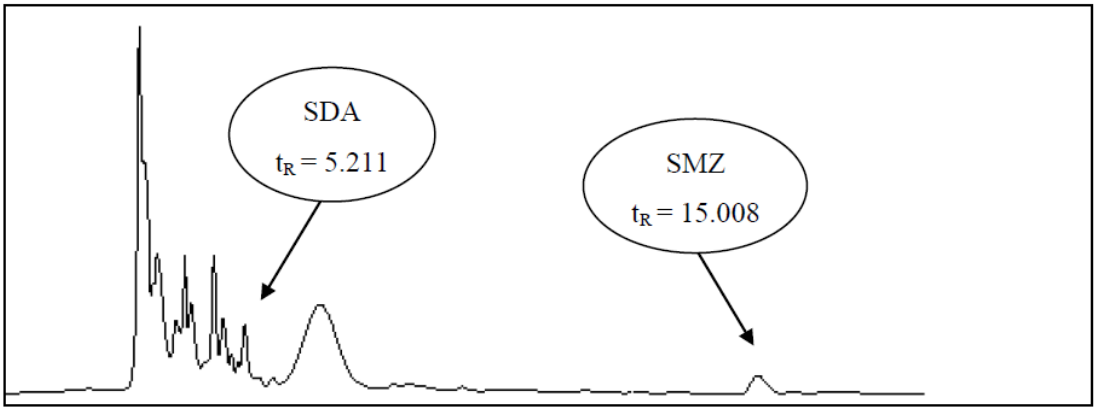


Figure 3.18 Chromatogram of spiked honey sample 2 after CPE procedure

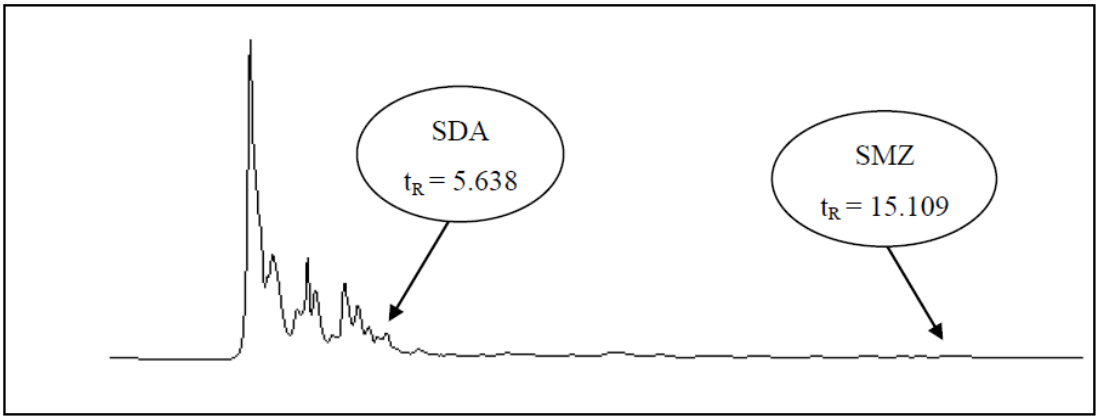


Figure 3.19 Chromatogram of unspiked honey sample 3 after CPE procedure

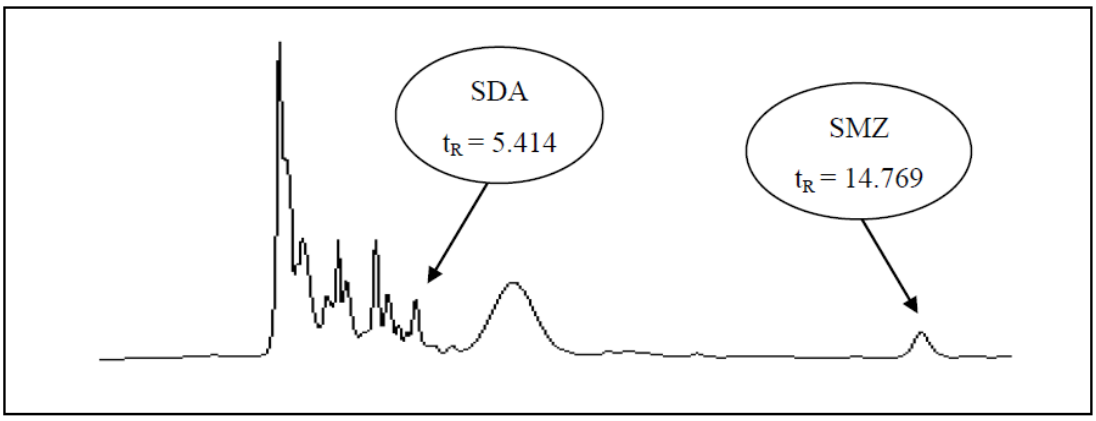


Figure 3.20 Chromatogram of spiked honey sample 3 after CPE procedure

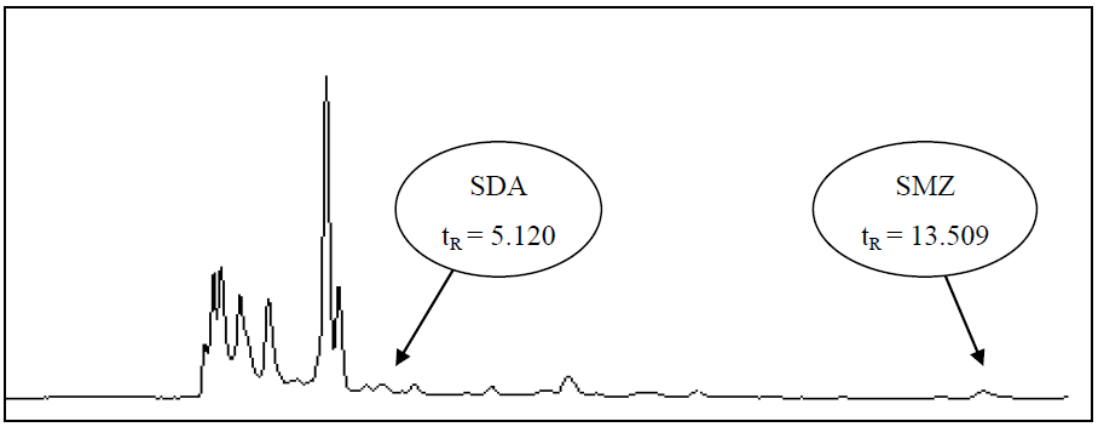


Figure 3.21 Chromatogram of unspiked honey sample 4 after CPE procedure

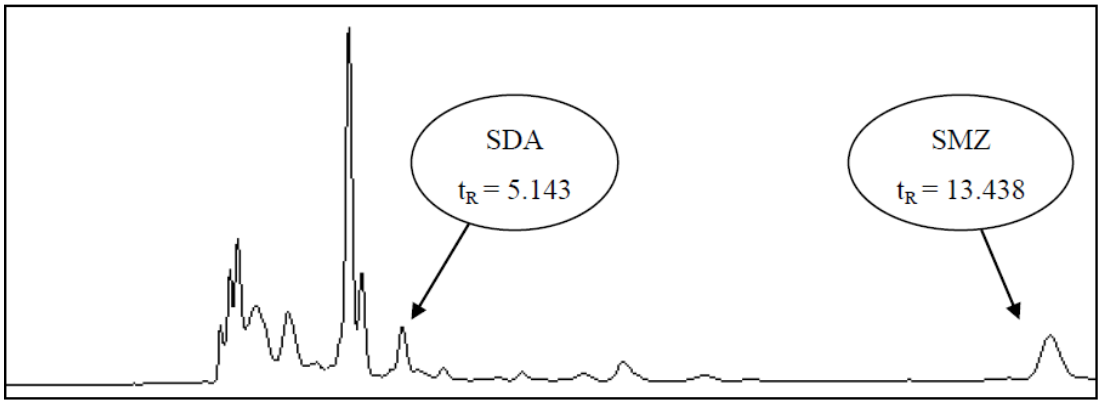


Figure 3.22 Chromatogram of spiked honey sample 4 after CPE procedure

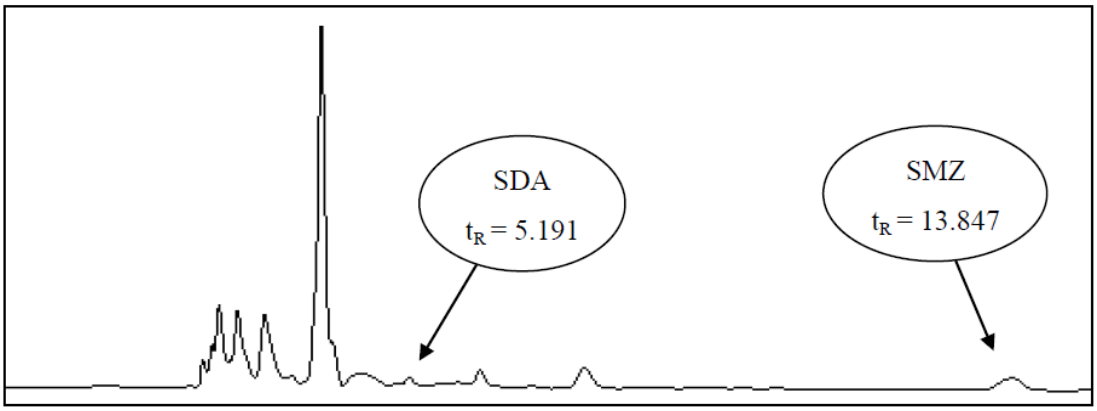


Figure 3.23 Chromatogram of unspiked honey sample 5 after CPE procedure

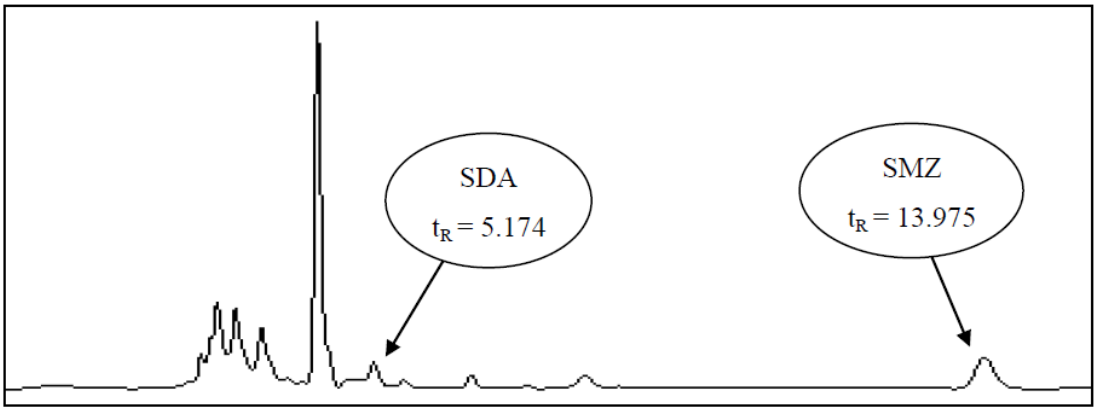


Figure 3.24 Chromatogram of spiked honey sample 5 after CPE procedure

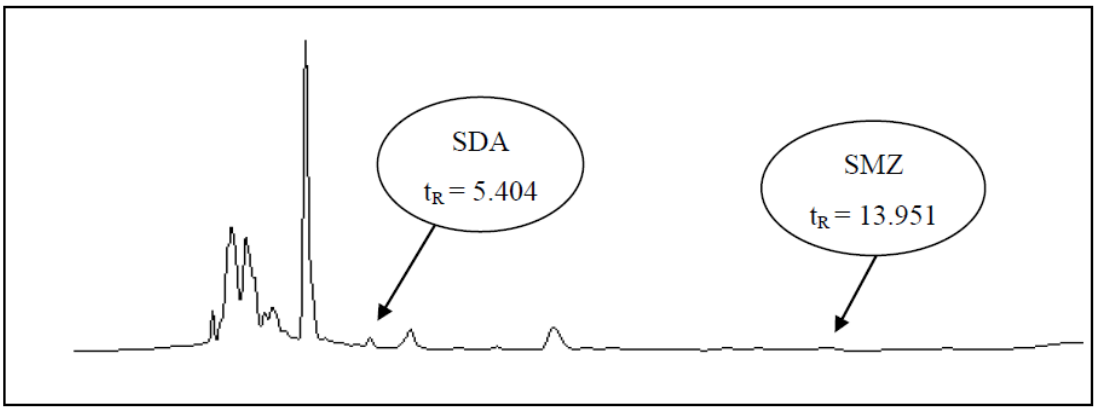


Figure 3.25 Chromatogram of unspiked honey sample 6 after CPE procedure

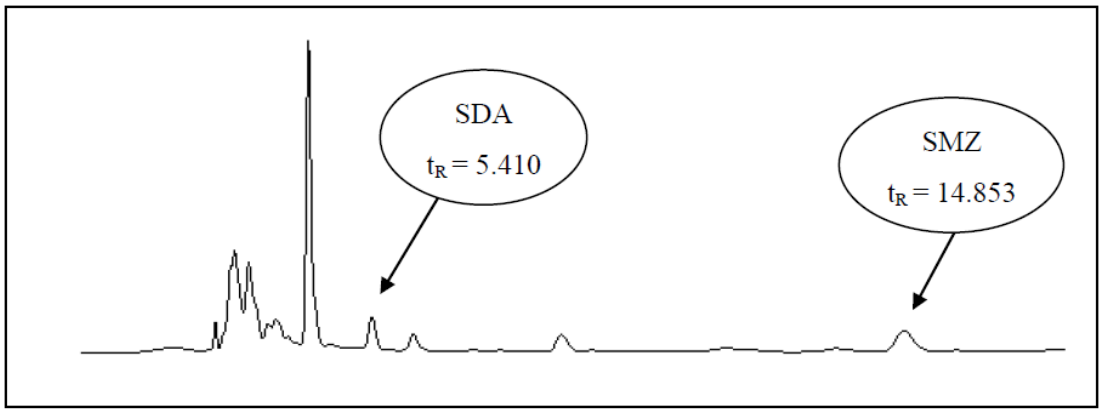


Figure 3.26 Chromatogram of spiked honey sample 6 after CPE procedure

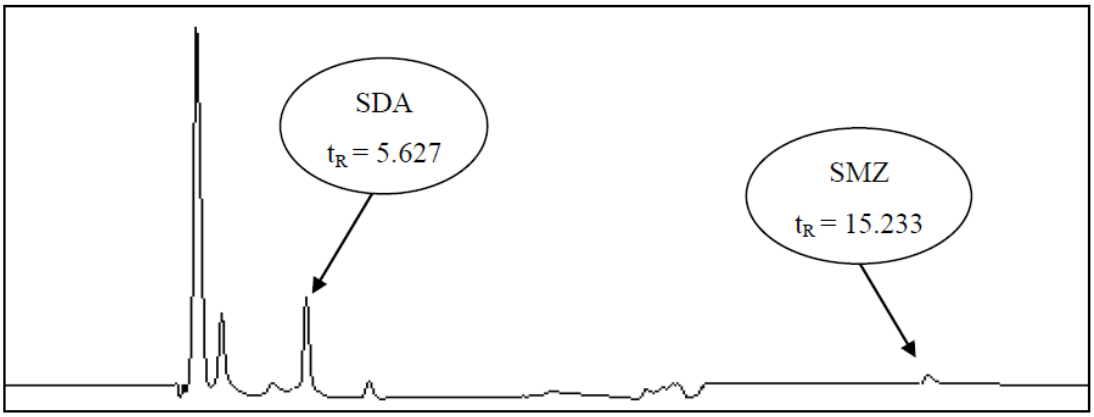


Figure 3.27 Chromatogram of unspiked honey sample 7 after CPE procedure

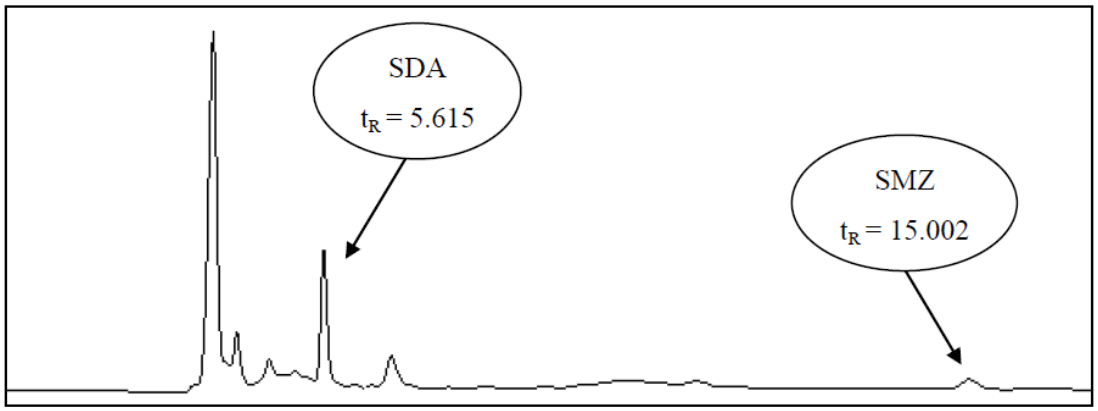


Figure 3.28 Chromatogram of spiked honey sample 7 after CPE procedure

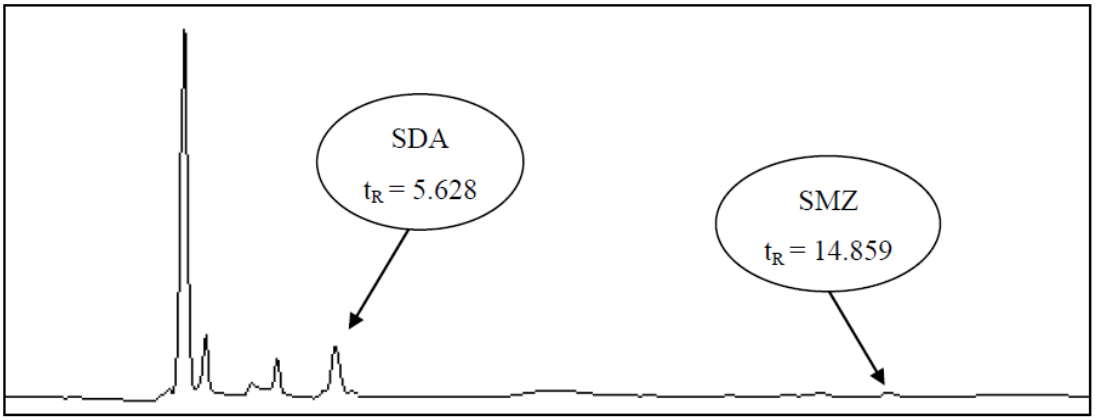


Figure 3.29 Chromatogram of unspiked honey sample 8 after CPE procedure

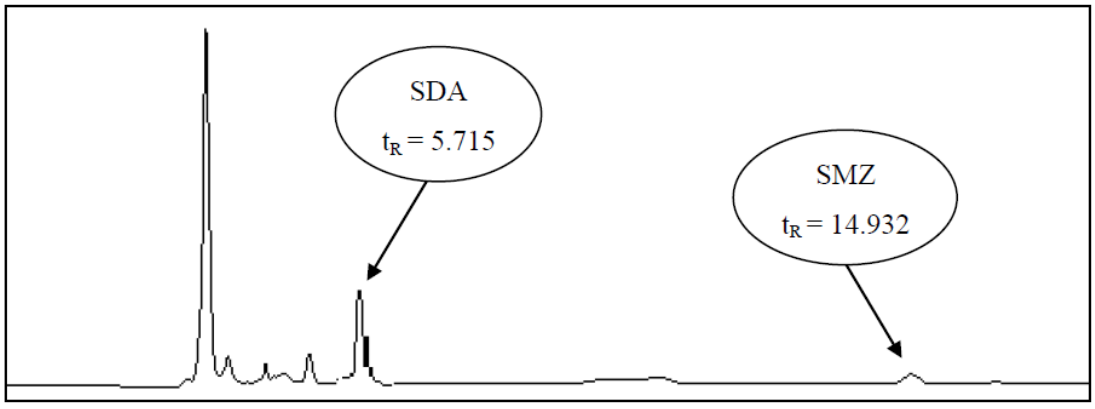


Figure 3.30 Chromatogram of spiked honey sample 8 after CPE procedure

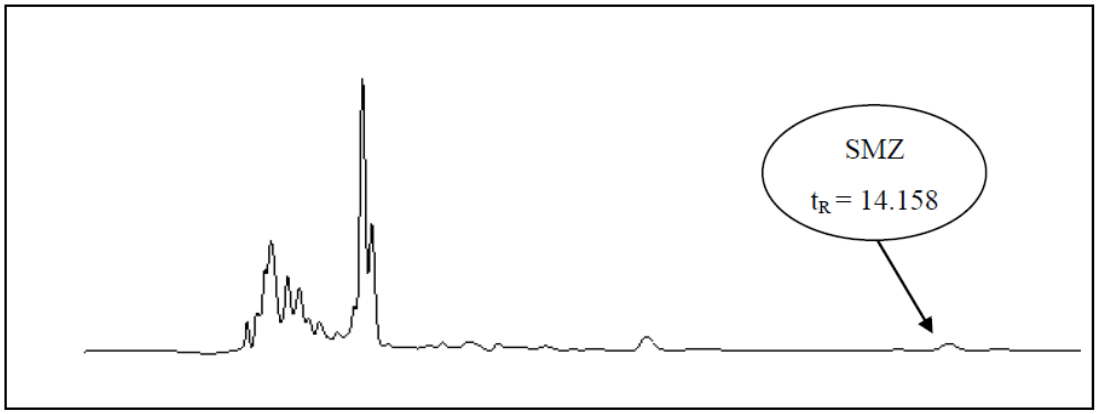


Figure 3.31 Chromatogram of unspiked honey sample 9 after CPE procedure

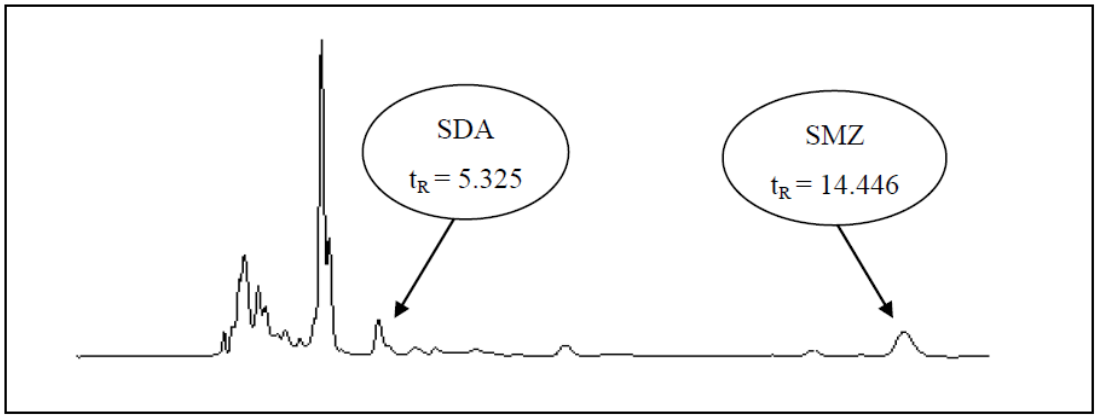


Figure 3.32 Chromatogram of spiked honey sample 9 after CPE procedure

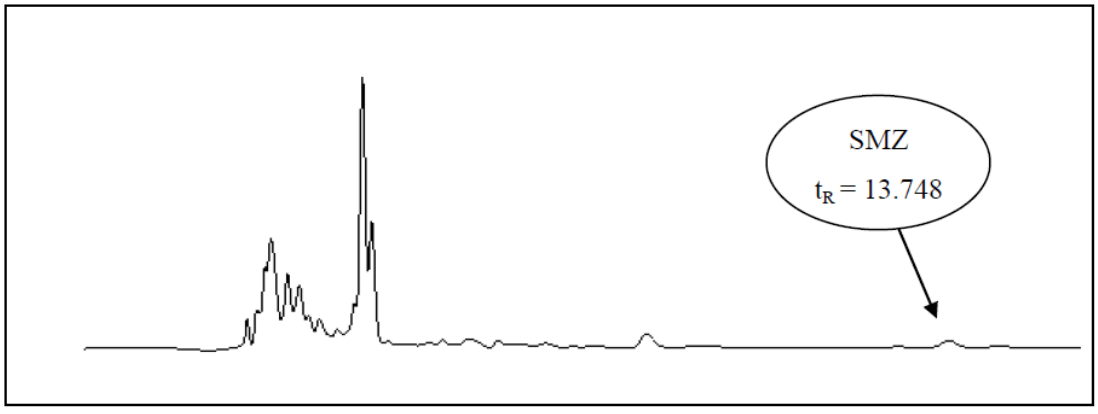


Figure 3.33 Chromatogram of unspiked honey sample 10 after CPE procedure

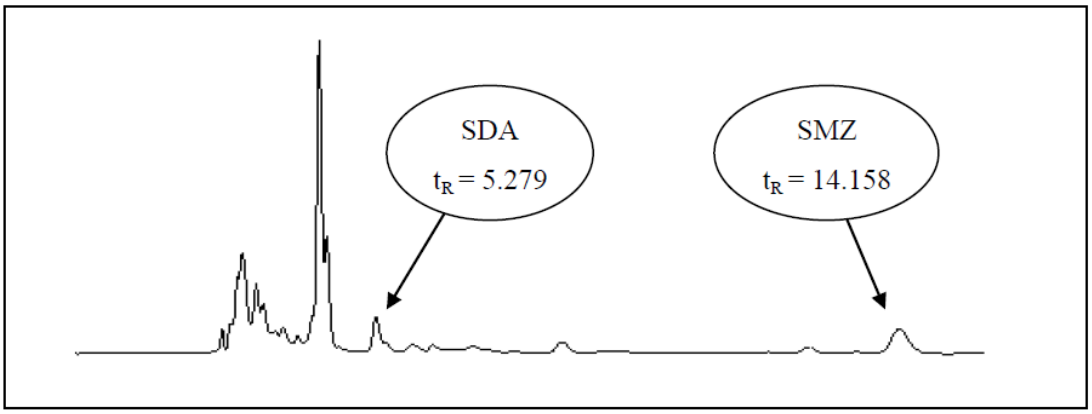


Figure 3.34 Chromatogram of spiked honey sample 10 after CPE procedure

CHAPTER FOUR
CONCLUSION

Sulfonamides have been found to be potentially carcinogenic and this fact has become a cause for considerable debate in food safety. Human nature always faces with these food products that contain antibiotic residues which effects human life seriously.

Contaminated foods with sulfonamides decrease the quality of foods or feed and cause several economic losses in the world. For these reasons, determination of sulfonamides becomes essential.

Sulfonamides are used widely in many countries also in Turkey and only there are a few articles in literature of Turkey. For these purposes, the development of analytical methodologies for determination of sulfonamides in food products is getting more and more important.

In this study, RP-HPLC-DAD determination of sulfonamides in food products was purposed after selection of wavelengths for SDA and SMZ solutions. In this case, some parameters were optimized in CPE procedure before HPLC analyses such as pH 3.9, 0.75% (w/v) Na_2SO_4 concentration, 2% (v/v) Triton X-114 concentration, 50 ^{O}C equilibration temperature and 20 min incubation time.

For accuracy, spiked SDA and SMZ standard solutions were compared to direct readings and the results of quantitative analyses in milk and honey samples were presented in good agreement.

In this work, milk samples were prepared by dissolving in the organic solvent to separate fat and casein phase, then evaporation was performed to dry under N_2 stream. The evaporation step was not required in honey samples. Detection and quantitation limits of the CPE method were found as 5.68 - 20.09 μg/L and 6.06 - 21.86 μg/L, respectively, the linear range of quantitation for SDA and SMZ was

approximately 0.025 - 2.000 mg/L, the average recoveries and the relative standard deviations were in the range of 65.2 - 99.4% and 0.51 - 2.17%, respectively.

Table 4.1 Summary of analytical statistical data

	SDA	**SMZ**
Linear Working Equation	y = 95.954x - 0.437	y = 86.229x - 1.3418
LOD (µg/L)	5.68	6.06
LOQ (µg/L)	20.09	21.86
Linear Working Range (mg/L)	0.025 - 2.000	0.025 - 2.000
Regression Coefficient (R^2)	0.9993	0.9998

In this study, the amount of SDA and SMZ in milk was found between the range of 0.062 - 0.104 mg/L and 0.018 - 0.038 mg/L. On the other side, the amount of SDA and SMZ in honey was found between the range of 0.017 - 0.643 mg/L and 0.006 - 0.162 mg/L.

When the amounts of sulfonamides' levels in honey from Turkey were compared to the levels of Iranian honey, sulfonamide residues in Iranian honey are higher than Turkish products. These data of food products are in good agreement with the hypothesis that milk and honey samples might contain significant concentration sulfonamide in milk and honey.

Therefore, this method has been proven to be suitable for the determination of sulfonamides in milk and honey samples. It is expected that, this method will be effective for multi residues analysis in other matrices as well. But according to the recovery results, it might be said that, SMZ gives better results than SDA in this proposed method.

In the future, amount of the other kinds of sulfonamides such as sulfafurazole, sulfamethoxine, sulfamethoxypryidazine (etc.) will be analyzed in meat, fat, chicken and also in fish as a following study. For this purpose, HPLC parameters will be optimized for the new analysis of sulfonamides. Direct injection and extraction procedures could be also studied such as CPE method for different samples.

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