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DETERMINATION OF SULFONAMIDES IN HONEY USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) FROM BİNGÖL PROVINCE

MASTER THESIS

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Alaa Omar TAHIR

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LIST OF ABBREVIATIONS

HPLC	:	High performance liquid chromatography
UPLC	:	Ultra performance liquid chromatography
HPCE	:	High performance capillary electrophoresis
LC	:	Liquid chromatography
RP	:	Reversed phase
UV	:	Ultraviolet
FL	:	Fluorescence
ESI	:	Electrospray ionization
SPE	:	Solid phase extraction
LLE	:	Liquid-liquid extraction
HFRLM	:	Hollow fiber renewal liquid membrane
MS/MS	:	Tandem mass spectroscopy
SAs	:	Sulfonamides
UP	:	Ultrapure water
mL	:	Milliliter
g	:	Gram
kg	:	Kilogram
mg	:	Milligram
M	:	Molar
mM	:	Milli molar

L	:	Liter
µg	:	Microgram
nm	:	Nanometer
SA	:	Sulfonamide antibiotics
SNA	:	Sulfonamide non antibiotics
MRSA	:	Methicillin-resistant Staphylococcus aureus
AFB	:	American foulbrood
EFB	:	European foulbrood
LOQ	:	Limit of quantification
LOD	:	Limit of detection
Rs	:	Resolution
SD	:	Standard deviation
RSD	:	Relative standard deviation
N	:	Theoretical plate number
k’	:	Retention factor
A	:	Selectivity
T_R	:	Retention time
T_0	:	Void volume
R^2	:	Regression coefficient
W	:	Width at the base of analyte
MRL	:	Maximum residue level
ODS	:	Octadecylsilica
MSPD	:	Solid phase dispersion

SCX	:	Strong cation exchanger
SDZ	:	Sulfadiazine
STZ	:	Sulfathiazole
SFP	:	Sulfapyridine
SMZ	:	Sulfamerazine
ATS	:	α-Toluenesulfonamide
SMT	:	Sulfamethazine
PNS	:	p-Nitrobenzenesulfonamide
SDX	;	Sulfadoxine
SXZ	:	Sulfisoxazole
Ppb	:	Part per billion
CAN	:	Acetonitrile
TFA	:	Trifluoroacetic acid
HCl	:	Hydrochloric acid
KOH	:	Potassium hydroxide
STD	:	Standard

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BİNGÖL İLİNE AİT BALLARDA TERS FAZ-YÜKSEK PERFORMANSLI SIVI KROMATOGRAFİSİ (RP-HPLC) YÖNTEMİ İLE SÜLFONAMİDLERİN TAYİNİ

ÖZET

Bal, gıda ve ilaç olarak tüketilen en yaygın kullanılan doğal ürünlerdendir. Arıcılıkta, arıların bazı bakteriyel hastalık ve enfeksiyonlardan korunması için arıcılar tarafından birçok antibakteriyel grup kullanılabilir. Sülfonamidler uzun yıllardan beri kullanılan en etkili antibakteriyel ilaçlardan biridir. Gıda endüstrisinde ve veterinerlikte tedavi ve koruyucu amaçlı antimikrobiyal ajan olarak yaygın olarak kullanılırlar. Sülfonamidlerin kalıntıları balda farklı seviyelerde görülebilmektedir. Baldaki yüksek konsantrasyonda sülfonamidlerin varlığı insan vücudu için potansiyel bir risk oluşturabilir. Hızlı bir metod olarak, Ultraviyole Spektroskopik Algılamalı Ters Fazlı Yüksek Performanslı Sıvı Kromotografisi (RP-HPLC-UV) kullanılmıştır. Optimizasyon ve validasyondan sonra, Bingöl iline ait farklı ballarda 9 Sülfonamid'in (Sulfathiazole, Sulfadiazine, Sulfamethazine, Sulfamerazine, Sulfadoxine, Sulfisoxazole, Sulfapyridine, α-Toluenesulfonamide, p-Nitrobenzenesulfonamide) tespit analizleri gerçekleştirilmiştir. Metot trifloroasetik asit kullanılarak asit hidroliziyle başlayan örnek hazırlamadan oluşmuştur. Kromatografik ayrışmanın etkileri, örneğin, akış hızı, mobil faz organik modifiye edici, tampon pH tampon içeriği ve optimum analitik koşullar, kapsamlı bir şekilde çalışılmıştır. Sülfonamidlerin tespiti, izokratik mod, paslanmaz çelik kolon (150 mm, 4,6 mm I.D.) ve ultraviyole spektrometresi kullanılarak 220 nm dalga boyunda, 0,5 mL / dak. akış hızında yapılmıştır. Sülfonamidlerin Tespit limiti (LOD) 0,72 µg/kg olarak, kantifikasyon sınırı (LOQ) ise 2,37 µg/kg olarak alınmıştır. Bu metod, çeşitli ballarda Sülfonamidlerin tespitinde başarılı bir şekilde uygulanmıştır.

Anahtar Kelimeler: Sülfonamidler, Bal, Ters Faz, HPLC.

DETERMINATION OF SULFONAMIDES IN HONEY USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC) FROM BİNGÖL PROVINCE

ABSTRACT

Honey is one of the most widely natural product consumed as food and medicine. Many antibacterial groups can be used by beekeepers in apiculture to protect bees from some bacterial diseases and infections. Sulfonamides are one of the most effective antibacterial medicine that has been used for many years. They commonly used as antimicrobial agents in food industry and veterinary for therapeutic and protective purposes. Their residue can appear in honey at different levels. The presence of SAs in honey at the high level of concentration could have a potential risk for human body. Reversed-phase high-performance liquid chromatography with ultraviolet spectroscopic detection (RP-HPLC-UV) was used as a quick method. After optimization and validation, determination of 9 Sulfonamides (Sulfathiazole, Sulfadiazine, Sulfamethazine, Sulfamerazine, Sulfadoxine, Sulfisoxazole, Sulfapyridine, α-Toluenesulfonamide, p-Nitrobenzenesulfonamide) were carried out in different samples of honey from Bingol province. The method consisted of sample preparation that started with acid hydrolysis using trifluoroacetic acid. The influences of the chromatographic separation such as flow rate, mobile phase organic modifier, buffer pH, buffer content and optimum analytical condition were extensively studied. The detection of Sulfonamides carried out by the chromatographic separation using isocratic mode, stainless steel column (150 mm, 4.6 mm I.D.) and ultraviolet spectrometry detection with analytical wavelength at 220 nm, flow rate 0.5 mL/min. The LOD of Sulfonamides was 0.72 µg/kg (LOD of the method) with the limit of quantification (LOQ) 2.37 µg/kg. The method was applied successfully for Sulfonamides determination in various honey samples.

Keywords: Sulfonamides, Honey, Reversed Phase, HPLC.

1. INTRODUCTION

1.1. Honey

Honey is one of the most widely used natural, sweet and flavorful product which produced by Honeybees from plant nectars, secretions of plant and excretions of plant sucking insects. It is very important because of its medicinal properties and unique nutrition value which contain more group of useful substances for human health, in which is used as anti-inflammatory, antioxidant, antimicrobial and bacteriostatic properties, in addition to sunburn healing and wound effects (Buba et al. 2013 and Alimentarius 2001). Usually honey is a more concentrated solution which contain 16% of water, large amount of carbohydrates which around 80-85%, 0.1-0.4% protein, antibiotics, vitamins, antioxidants, enzymes, amino acids and around 0.2% of ash content (Ajani 2009) and (National Honey Board 2003). According to the origin, honey can be classified in different categories as follows: (1) Blossom Honey, which obtained mainly from the nectar of flowers, (2) Honeydew Honey, produced by bees after they collect 'honeydew' (3) Monofloral Honey, in which the bees feed mainly on one type of plant and it is named according to the plant, and (4) Multifloral or Polyfloral Honey that has several botanical sources, none of which is predominant (Alvarez-Suarez et al. 2014). The constituents depend on the honey types such as; Clover Honey is produced by both New Zealand and Canada, it is lighter than amber color with a sweet taste and you may feel a little sour aftertaste. Manuka Honey is produced from New Zealand's Manuka tree and it can be used as medicine to cure wounds, and it has an antibacterial property which can heal infection such as MRSA, Manuka taste is a bit like medicinal if compared with other types of honey. Sourwood Honey comes from sourwood trees in the Appalachian Mountains run from Northern Georgia up to Southern Pennsylvania, it could cure cancer and has a light color with a taste like a caramel taste. Buckwheat Honey is produced from New York and has a very dark color and a lingering aftertaste, this thick honey is rich in iron, and has more antioxidants than its lighter counterparts. Hence, can be noticed that the physical and chemical properties of honey are

Differed from one country from the other countries due to different types of weather and environmental effects also different types of flowers as a source of nectar which absorbed by honeybee to produce honey (Ramírez and Montenegro 2004).

Water content is a major component in honey and quantitatively depends on some environmental factors during production such as weather, amount of water inside of hive as a humidity and the way of treating bees during of storage time and extraction (Molan 2002). Honey with 18% or less than that amount of water content, can be stored safely for a long time without going through a fermentation process with the same taste and smell, but honey in case of above 18% of water content and storing for a long time, the fermentation will take place when the yeasts grow in both number and size by using honey sugar as a source of energy and they make a spoil of the honey taste and smell (Bogdanov et al. 2008).

For a thousand years ago honey was a most important natural source available of carbohydrates and sweetener (Manyi-Loh et al. 2011), essentially the amount of carbohydrates in honey contained 95-99% of sugar which has been studied for more years ago, the major sugars in honey are fructose and glucose, their structures are shown in Figure 1.1.

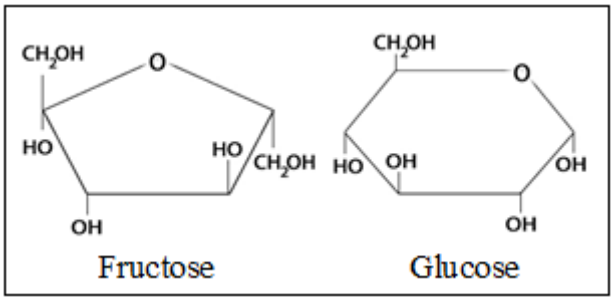


Figure 1.1. Structure of sugars in honey

They are the simple sugars and building block of more complexes sugars in honey which contain about 80% of solid materials content (Cantarelli et al. 2008 and Ebenezer et al. 2010).

Protein investigation is difficult due to a high amount of sugar present and low amount of protein around 0.1-0.4%, therefore, there is only a limited study of protein available (White Jr, 1957). Vitamin content present in honey but in trace level such as niacin, pantothenic acid, ascorbic acid and riboflavin along with minerals like; iron, copper, phosphorus, zinc, magnesium, manganese and potassium (Ajibola et al. 2007 and Ajibola et al. 2012). Also, there are several enzymes available in natural honey which is useful for food digestion process especially starch and carbohydrates (Willson and Crane 1975). The amino acids are important required by the honeybee for making of tissues, enzymes, etc. This content contains two amino acids groups such as essential amino acids in which the bee does not have the ability to produce and need to take them from an external source, and non-essential amino acids in which the bee can transform metabolically from one to another, the essential amino acids (e.g. tyrosine from phenylalanine, cysteine from methionine, etc.) (Hrassnigg and Crailsheim 2005). Honey is acceptable and suitable to use in the medical profession as a natural source for antibacterial and can use for treating some infectious and diseases caused burns and wounds, and their effectiveness as an antibiotic is widely reported (Molan 1992 and Al-Jabri 2005).

1.1.1. Honey in Bingöl-Turkey

Turkey is one of the biggest honey and bee wax producers in the world. In recent years, it has ranked as the fourth largest honey producer only after China, USA, and Argentina. In fact, Turkey produces 92% of the world's pine honey, definitely in its South Aegean regions and West Mediterranean. Nearly in every region of Turkey beekeeping is became traditional agricultural activity. In Turkey 20% of the world's 25 bee sub-species could be found. Due to this diversity, bee farmers are advised to raise native bee species of their region alternatively of commercial bees. Turkey has strong views in beekeeping in all regions of Turkey and 75% of beekeepers are migratory. The honey of different regions within Turkey has different local behaviors. Around 70% of plants in Turkey are floristic and it is became an extensive place of a variety of honey types, nectar and honeydew, both unifloral and multifloral.

According to (Bingöl Investment Opportunities November 2011) nearly 1000 different diversities of wild flowers, 109 of them are prevalent to Turkey, and grow in the Bingöl

Mountains that cut through Bingöl province. Honeys in Bingöl are generally obtained from flowers that naturally grow on the high plains of the province. The honey has abide taste and translucent appearance, and the aroma of unique flowers. Bingöl is greatly suitable for apiculture because of its geographic features and Bingöl honey has a great possible to be a trademark. According to data of (2009), 784.499 tons of honey and 82.384 bee colonies were produced in Bingöl. The province has four honey packaging abilities. The establishment of a queen bee production creativity will increase the amount of honey production in the province. Bingöl can get the competitive advantage with the production of organic honey in the sector. When the countries that import honey in the world are taken into consideration, it is so important to develop the product which is already demanded in high amounts (Saner et al. 2004) and (The Turkish Beekeeping and Honey Sector 2015).

1.2. Antibiotics

The term "antibiotic" is widely defined as a compound that produced by a micro-organism that selectively inhibits the growth and increase of some other micro-organisms (Directive 2002). Antibiotics are substances that can inhibit fungi and bacteria from increasing and growing their number by killing them outright. Antibacterial antibiotics play a major role to heal infections, regrettably; the effectiveness of antibacterial decrease with increasing of use over time because of a bacteria can make a resistance to those antibacterial due to excessive use. To found and make a new antibiotic medicine is challenging also need the high budget cost to make a test experimentation and result and additionally, a carefulness is necessary to use the antibacterial due to some side effects such as hypersensitivity. As healers in past years reported that increased infections of strains resistant to some antibiotic substance mainly due to their excessive and abusive use of these antibacterial compounds. Therefore scientists searched for a suitable and new antibacterial and among all possible alternatives, the use of natural compound has been recommended. Among these, the honeybee products such as honey which is historically known as a non-toxic very effective antimicrobial (Cooper et al. 2002).

The infection by bacteria is very scary when bacteria can produce some enzyme with the ability to digest protein, which can destroy the body cells (Church 1954). Also, bacteria can damage the growth of protein factor when released by the body to arrange healing

process for regenerating damaged tissues (Postmes and Vandeputte 1999). In addition, there are several toxically microbes which can release a toxin that destroy the body cells (Davis and Arnold 1974). In other hands, bacteria can be a very effective factor to damage tissue by holding antigens that fluctuate response of prolonged inflammatory immune which makes an excess preparing of active radicals. The wounds caused by bacteria can use oxygen, therefore the oxygen level in wounds tissue reached to that point which tissues growth is damaged (Christopher 2003). The bacterial infection consequences will be; uncured sore cells and the size will increase, failure of skin grafts, pain, and swelling. The infection process can be prevented by using natural honey, which can help the immune system of body by its anti-inflammatory effect, antioxidant activity and cell growth stimulation (Abuharfeil et al. 1999).

1.2.1. Types of Antibiotics

Many classification ways of antibiotics available but the most used and common ways of classification depend on their mode of action, chemical structure and activity spectrum (Calderón and Sabundayo 2007), others include route of administration (injectable, oral and topical). Some antibiotics can kill bacteria and some are only able to inhibit them from growing and increasing size. Antibiotics having similar molecular structure will always show a similar action and affectivity, toxicity, and similar side effects. Some classes of antibiotics according to their molecular arrangement structures included of Macrolides, Beta-lactams, Glycopeptides, Sulfonamides, Oxazolidinones, Quinolones, Aminoglycosides and Tetracyclines (Van Hoek et al. 2011), (Frank and Tacconelli 2012) and (Adzitey 2015). From table 1.1. the classification of some groups of antibacterial can be seen based on chemical or molecular structure.

Table 1.1. Classification of antibiotics (according to British national formulary 2012) based on molecular structure

Antibiotics Classes	**Drug Names**
β-lactams	Cephalosporacin, Penicillin
Quinolones	Ciprofoxacin, Nalidixic Acid
Tertracyclines	Doxycyline, Tertracycline
Sulfonamides	**Sulfadiazine, Sulfamerazine**
Aminoglycosides	Gentamycin
Macrolides	Erythromycin, Roxithromycin, Azithromycin

1.3. Sulfonamides (SAs)

Sulfonamide also called sulfa drug is an organic sulfur compounds consists of p-aminobenzoyl ring group with an amino group at the N4 position and the different functional group at the N1 position (Tilles 2001). In chemistry, the functional group of **s**ulfonamide (also spelled sulphonamide) is -S (=O)-NH, a sulfonyl group connected to an amine group. Relatively this group is unreactive. The center of amine is no longer basic. Difficulty the S-N bond is cleaved. Because of the rigidity of the functional group, sulfonamides are normally crystalline. Because of this reason, the formation of a sulfonamide is a classic method to change an amine into a crystalline derivative which can be recognized by its melting point. Sulfonamides can be used to preparing many other important drugs (Actor et al. 2005).

$RSONH_2$ is the general formula of a sulfonamide compound, in which R maybe alkyl, aryl or hetero aryl etc. R1 and R2 maybe hydrogen, alkyl, aryl or hetero aryl groups. As an example; (methanesulfonamide $CH_3SO_2NH_2$). Many of sulfonamides can be prepared from the reaction of a sulfonic acid by using amine group instead of the hydroxyl group (Ainsa 2002), Representation of general structure of sulfonamides can be shown in Figure 1.2.

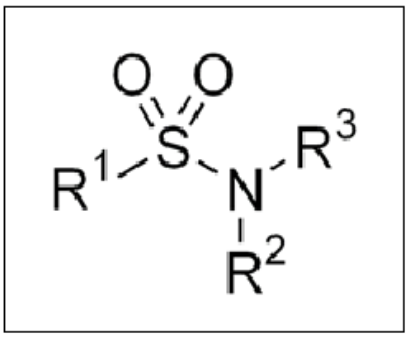


Figure 1.2. General structure of sulfonamides

Chemically sulfa drugs are amphoteric. They act as the weak organic acid with pKa (4.79-8.56). However, they are weakly soluble in water, at alkaline pH their solubility is increased. SAs are polar compounds its solubility ranged (0.1 to 8 g/L) relying on the compound within the group (Sarmah 2006). Their occurrence is in the neutral form in which between pH (2.5 – 6), and negatively charged in alkaline conditions. Sulfonamides are described as having a low chelating ability, and low binding constants (Sukul and Spiteller 2006). They also have a low sorption coefficient and they are expressed to be the majority mobile antibiotics (Tolls 2001 and Boxall 2002).

Most of the sulfonamide derivatives are prepared from exchanging of (H) atom on the N atom of sulfonamides group at the position of N1, and others obtained from the exchanging of the (H) atom on the nitrogen of aromatic group at the position N4. By substitution at N^1 and N^4 positions, about 5000 compounds are produced. Among them, 30 are of clinical importance. Sulfanilamide and its derivatives are prevalently known as sulfonamide or sulfa drug. The introduction of numerous substituents ensued in the products with various pharmacodynamics, pharmacokinetic and physicochemical properties. In Figure 1.3. the structure of some common sulfonamide drugs are shown (Anand 1975).

1.3.1. Structure Activity Relationship

The activity of sulfonamides is extremely structure dependence, it can be detailed as follows; (i) Free para-amino group is important for antibacterial activity. (ii) More potent sulfa drugs were produced by the substitution of heterocyclic aromatic components at the N^4 position. (iii) In any substitution of benzene ring causes loss of activity. (iv)

SO_2 NH_2 group is not important as such sulfur atom is straight linked with the benzene ring. (v) The further negative SO_2 group at N^1 exhibits larger antibacterial activity. (vi) Substitutions prepared in the amide NH_2 (N^1) which have variable antibacterial activity. (vii) The para NH_2 group (N^4) can be exchanged or substituted by such chemical groups that can be changed into the free NH_2 group in the body (Tačić et al. 2017).

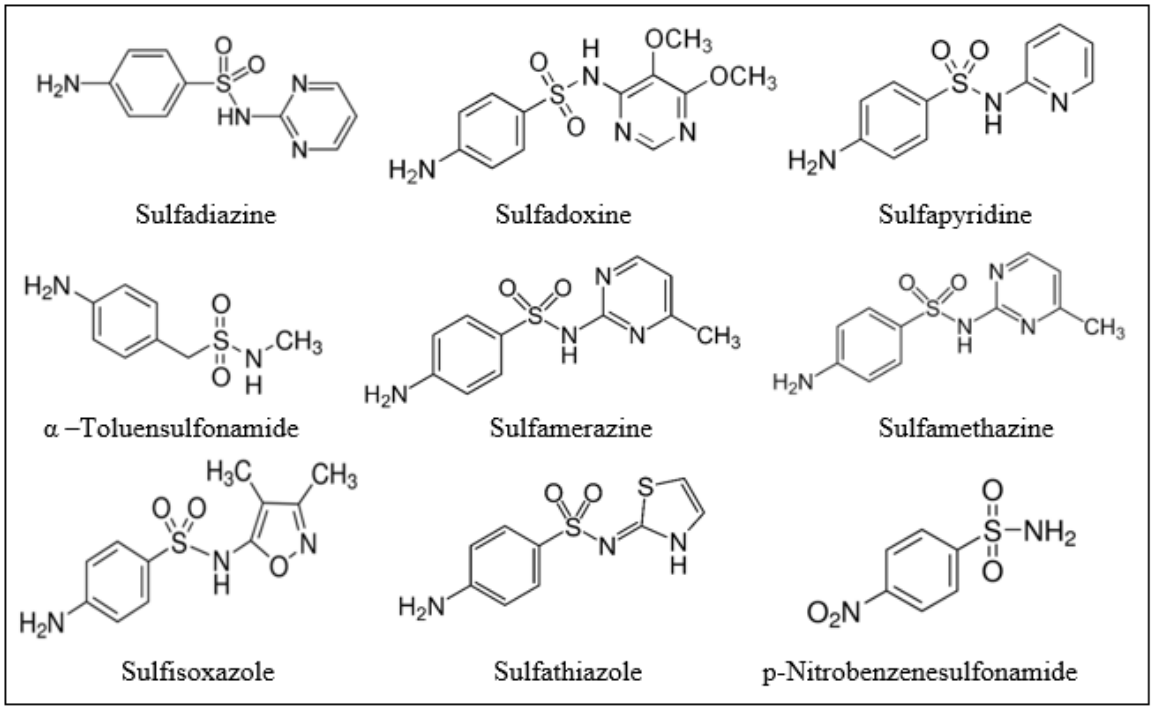
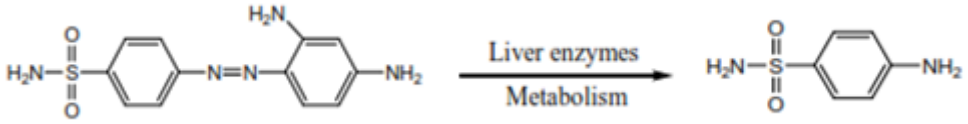


Figure 1.3. Structure of some common SAs

1.3.2. History of Sulfonamides

Sulfonamide is one of a group of antibiotics, commonly referred to as “Sulfa drugs” which were discovered in the 1930's. Exactly in 1935, by Gerhard Domagk, he was made a breakthrough discovery by finding that a red dye, 4'-sulfamyl-2, 4- diaminoazo-benzene, which was later named Prontosil. It was inactive in vitro, but it was active to inhibit streptococcal infection in vivo (Bendjeddou et al. 2016). The lack of correlation between in vitro and in vivo antibacterial tests prompted Domagk to resort to in vivo testing, a very fortunate decision, since otherwise the fate of sulfonamides might have been different. Gerhard Domagk had explained that the Prontosil dye behaved as an antimicrobial when injected into an infected mouse but not when tested against bacteria

growing in a test tube. After that, the reason for that strange action was understood (Bhattacharjee 2016). Domagk's discovery led to the other serious discovery of antibiotics, and he gained the noble price award at 1939, one year after Domagk's discovery of the anti-streptococcus activity of the dye Prontosil, in 1936 from the Pasteur Institute in Paris, Ernest Fourneau revealed that Prontosil breaks down in the human body to produce sulfanilamide as shown from the equation 1.1., which is the active agent that kills streptococcus bacteria. By Fourneau's discovery, a flurry of research on structural derivatives of sulfanilamide generated which led to in the development of a group of greatly successful antibiotics that have protected millions of lives (Nicolaou et al. 2017).



(1.1)

Prontosil Sulfanilamide

Domagk's discovery rapidly resulted in the development of a multiplicity of sulfonamides, all of which were principally substituted from sulfanilamides. In the meantime, there have been many correspondents of sulfanilamide developed as pharmacological agents that show an extensive range of biological activities. For instance, Amprenavir is used in HIV therapy, Glibenclamide was used as a hypoglycaemic agent, Sulfathiazole for an antibacterial agent. Acetazolamide was used as a carbonic anhydrase inhibitor, E7070 for an anticancer agent, and Furosemide as a diuretic. Also in 1938, the effective antibiotic Sulfapyridine was shown against pneumonia. While in 1941 Sulfacetamide was found greatly which successfully used in fighting urinary tract infections starting. And the Succinoyl-sulfathiazole since 1942 has been used against gastrointestinal tract infections. During World War II Sulfathiazole was used very efficiently to cure the infection in fighters with weapon wounds. Sulfa drug, is an effective antibiotic which never gained widespread use due to its greater human toxicity against its various derivatives (Supuran et al. 2004).

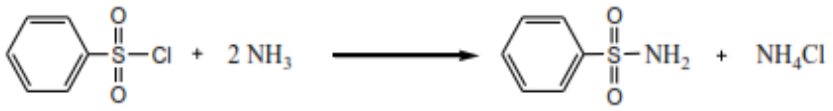
1.3.3. Sulfonamides in Environment

The usage of antibiotic has benefited the animal industry and facilitated providing reasonable animal proteins to the developing human population. Though, the wide use of antibiotic outcomes in the inhibition of sensitive organisms whereas selecting for the resistant ones, agricultural use is contributing significantly to the spread and appearance of antibiotic resistance in the environment. Up to now, predominantly the scientists are focused on the spread and appearance of bacteria resistant and genes into the environment as an effect of veterinary treatment, particularly through manure, direct animal contact, and food products. However, environmental pollution with the residues of antibiotic might also be a significant factor in the variety and distribution of antibiotic resistant bacteria. The persistence of antibiotics in the environment depends on climate, soil type, and also on physical and chemical characteristics of the diverse types of antibiotics. Observing studies were showed that considerable concentrations of the residues of antibiotic can occur in water and soil, and in locations close to animal farming.

Because of using huge amounts of sulfonamides in animal husbandry mainly as veterinary medicine cause to the harmful effects on popular health and environment, and also they are commonly known as most contaminant antibiotic in honey. However, they are generally used in the treatment of humans and animals but also they have a risk as environmental impurities. They clearly have the potential to enter waters, sediments, soils, animal waste, surface waters, and groundwater, and have been perceived in environmental samples, for instance, sulfamethazine's detectable concentration was found in soil agricultural seven months after the manure application. In another study, the concentration of sulfachloropyridazine above to 590 µg/L was detected in drainage waters seven days after application of manure on clay loam soil. SAs are expected to occur in the environment in a bioavailable form fixed on their relative stability and physiochemical properties, therefore they may carriage a risk with detail to antimicrobial resistance expansion (Pikkemaat et al. 2016).

1.3.4. Synthesis of Sulfonamides

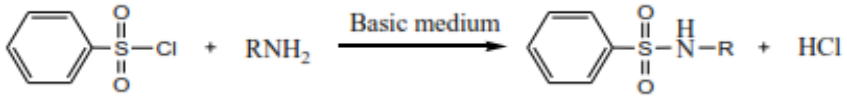
Because of the widely used of sulfonamides, it is required to find the effective and general way for their preparation and synthesis. Therefore the interest in the synthesis of these compounds is continuing. Up to now, many synthetic methods have been developed. The most common method for sulfonamides preparation is by the reaction of proper sulfonyl halide, either aromatic or aliphatic, with amines or ammonia. Thus, similarly, heterocyclic sulfonamides were prepared through the reaction of heterocyclic sulfonyl halide with amines or ammonia.



(1.2)

Sulfonyl chloride Ammonia Sulfonamide Ammonium chloride

The equation 1.2. represented the synthesis of sulfonamides using an aromatic sulfonyl halide with ammonia as a reactant to produce aromatic sulfonamides, and the equation 1.3. represents the synthesis of sulfonamides from the reaction of aromatic sulfonyl halide with amines.



(1.3)

Sulfonyl chloride Amine Sulfonamide Hydrogen chloride

Typically the reaction between amines and sulfonyl halide is catalyzed by a basic catalyst such as potassium carbonate, sodium carbonate, triethylamine or pyridine.

The method of sulfonyl chloride denotes the most direct and simple route for the preparation of sulfonamides. This is because of the ease with which the necessary sulfonyl chlorides in high yields are obtained (Kołaczek et al. 2014).

1.3.5. Application of Sulfonamides

SAs derivatives can be used in biological applications, they are widely used as antimicrobial, anti-cancer, anti-inflammatory anti-diabetic, anti-epileptic, antifungal (Asker et al. 2017), antiviral, antineoplastic, antithyroid agents as well as HIV protease inhibitors. Also, some derivatives are well recognized as an antimetabolite considering this type of compounds a "privileged structure" in medicinal chemistry (Camargo-Ordoñez et al. 2011). And in another study new sulfonamides have shown good cytotoxic effects against breast cancer cells. Sulfonamides are the first effective chemotherapeutic agent that used cure the bacterial infection in human beings (Saeedi et al. 2014).

SAs are commonly used as a microbial agent to treat the infections by bacteria and inhibit the growth of gram negative and gram positive bacteria, some fungi, and protozoa. They are also used in the clinic to treat several infections such as; gastrointestinal infections and urinary tract infections, eye infections and as a prophylaxis of rheumatic fever. Sulfonamides that are aromatic or hetero-aromatic can inhibit the growth of cancer. They are also used as an anti-tumor by preventing the carbonic anhydrase. SAs antibiotics are used as veterinary medicines to treat infections in livestock herds (Afroz et al. 2012). In other hand, sulfonamides can be used to inhibit many enzymes such as Serine protease, cyclooxygenase, matrix metalloproteinase and carbonic anhydrase (Rehman et al. 2017). Inhibition of carbonic anhydrases has been done by the drugs containing sulfonamide functional group and these carbonic anhydrase inhibitors are also reported as potential anticancer, antiglaucoma (as substituted heterocyclic and aromatic sulfonamides), diuretics, and anti-obesity agents. Therapeutically sulfonamides are being widely used in human (especially where other antibiotics are non-tolerable to patients) and veterinary practice (Abdul Qadir et al. 2015).

For agricultural purposes, many derivatives of sulfonamide have been reported due to their antifungal and herbicidal properties. The new developed sulfa drugs with different mechanism of action have become an emerging demand to overcome existing drugs resistant pathogens. As the pathogenic organisms (bacteria, fungi, and mold) are exposed or treated medically with routine antibiotic drug molecules, they become considerably resistant with the emergence of new species as permutation, conjugation, transduction,

or transformation (Abdul Qadir et al.2015). SAs are classified into two groups; sulfonylartylamine, non sulfonylartylamine and sulfonamide moiety-containing drugs and the other is based on cross reactivity theory i.e., Sulfonamides Antibiotics (SA) and Sulfonamides Non-Antibiotics (SNA) (Shakoor 2013).

1.3.6. Classification of Sulfonamides

Classifications of sulfonamides are based on chemical structure, duration of action, the spectrum of activity and therapeutic applications. Common classification of sulfonamides is based on their therapeutic applications. There are three groups of sulfonamides according to their duration of action:

1.3.6.1. Short Acting Sulfonamides

These are preferred for systemic infections as they are rapidly absorbed and rapidly excreted. For example, sulfadiazine, sulfadimidine or sulfamethazine, and sulfamethoxazole have been used for the treatment of the infection of the urinary tract (Locuson et al. 2007).

1.3.6.2. Intermediate or Moderate Acting Sulfonamides

These are used for infections requiring prolonged treatment. For example, sulfacetamide, sulfadoxine. The sulfadoxine is an ultralong-lasting sulfonamide can be used to treat malaria when combined with pyrimethamine. It is also used, usually when it is combined with other drugs can be used to treat or prevent several livestock infections (Boison et al. 1996).

1.3.6.3. Long Acting Sulfonamides

These are rapidly absorbed and slowly excreted. For example, sulfametopyrazine, sulfasalazine, which is marketed as azulfidine in the U.S. and salazopyrin & sulazine in Europe and Hong Kong, was developed over 70 years ago specifically to treat rheumatoid arthritis. Sulfapyridine can be reacted with salicylate with an azo bond to produce mesalazine and its derivatives such as sulfasalazine. It may be abbreviated SSZ. In addition

to these, there are different types of sulfonamides which have been used in various types of infections. For example, sulfabenzamide used in mucous membrane, sulfacetamide sodium used for superficial ocular, sulfadiazine used in urinary tract infection and sulfamethizole used in bacterial infections (O'Shea and Moser 2008).

1.3.7. Side Effects of Sulfonamides

Sulfonamides are potential hazard compounds if they used in high dosage, they may cause a hypersensitivity reaction, urinary tract disorder, and intense allergic reaction. Around 3% of the population when treated with sulfonamides as an antibacterial agent, they have adverse reactions. It observed that 60% of HIV patients have more prevalence (Tilles 2001). Reactions of hypersensitivity are rarely happening with nonantibiotic sulfonamides, and it has been noted that those patients with hypersensitivity reaction by antibiotic sulfonamides, do not have increased hypersensitivity with nonantibiotic agent sulfonamides (Slatore CG and Tilles 2004). The arylamine group at the position of N4 is responsible to make allergic side effect of sulfonamide antibiotics, it present in sulfasalazine, sulfadiazine, antiretroviral, sulfamethoxazole, fosamprenavir and amprenavir. Thus, it recommended to those patients with the allergic reaction of arylamine sulfonamides to use those antibiotics with lack of arylamine group or non-aryl sulfonamide (Knowles et al. 2001).

Chemical structure of two regions of the sulfonamide antibiotic is involved in the hypersensitivity reactions related with the N1 heterocyclic ring, which causes a type I hypersensitivity reaction, and with the N4 amino nitrogen which forms reactive metabolites that cause either direct cytotoxicity or immunologic response in a stereospecific process.

1.4. Sulfonamides in Honey

Honey is the most important healthy and consumable natural product in whole of the worlds, however many antibiotics used by beekeepers in apiculture, including sulfonamides to prevent some bacterial disease known as American and European foulbrood (AFB and EFB) they reported that a strong and destructive disease that can affect

honeybees, caused by *Paentibacillus* and *Melissoccuus pluton* (*Bacillus larvae*) the outcome of treating bee with makes honey contamination by trace amount of sulfonamides and it can be detected and measured quantitatively, sulfonamides commonly used as an antibacterial agent in the food industry and veterinary for therapeutic and prophylactic purposes, therefore sulfonamides residue can be found and detect in more animal and harvest food products such as egg, meat, milk, fish and honey. And also used as food additives (Gunes et al. 2009).

Therefore, determination of sulfonamides in food products is the main concern for preventing those side effects that mentioned before caused by high dosage of sulfonamides and monitoring of sulfonamides content, therefore honeybee treating with sulfa drugs forbids by European Union. Regulatory agencies are responsible for declaring that harmful residues of these drugs are not present in honey. The amount of sulfonamides in honey is different from one country to another country and generally, the LOQ range between 20-50 ng/g. Currently, the actual detection of sulfonamides refers that in Europe no maximum residue level (MRL) for sulfonamides in honey exist. Based on the initially concerned compounds (sulfa drugs and metabolites) and the residue level of these compounds recommended being below the (limit of quantitation LOQ) using most precise and accurate analytical method, Switzerland concerned of LOQ approximately 50 µg/kg for honey. (Maudens et al. 2004). Many countries are prohibited use of sulfa drugs for treating bees, but due to importing honey from other countries by some EU countries, therefore the action limits were established by many EU countries such as Switzerland, UK, and Belgium, which amounts sulfonamides residue allowed between 0.01 to 0.05 mg/kg, and (EU Commission Regulation) (EU) No.37/2010 established 100 µg/kg, the Brazilian honey sulfonamides content arranged to be below of 100 g/kg (da Presidência et al. 2007). France was at 50 and 15 g/kg respectively. In some country such as turkey, using sulfa drugs or antibiotics has been prohibited and illegal by the authorized ministry and according to Turkish Food Codex Honey Communique harmonized with European Commission Honey Directive, therefore, there is no MRL established for total sulfonamides residue in honey (Gunes et al. 2009). Sulfathiazole was first recommended to control AFB, but now, for many months Sulfathiazole residue in honey was founded and remained, therefore, it is banned to use anymore, and this fact caused a major concern due to the possible appearance of resistance phenomena in consumer's health. However, it is well-known that in various

honey samples these compounds can be found according to the origin of the sample (Kujawski et al. 2008). In addition; the aromatic group of SAs can react with reducing sugar to produce different sugar bonded compound with different chromatographic behavior. Due to many problems coming from the excess level of SAs, therefore the sulfa drugs residue analysis and the quantification of them in honey must be strongly concerned, their MRLs values should be regulated and the use of them must be monitored under the control limit in the food products mainly honey to detect the presence and amount of SAs and class of level in order to prevent the side effects and for ensuring of safely used (Sheth and Sporns 1991) and (Tashakkori et al. 2014).

1.5. Analyzing Techniques for Sulfonamides in Honey

Many techniques and methods are available for detecting and determination of sulfonamides in honey such as Colorimetric technique (Schwartz and Sherma 1986), Thin layer chromatography (Zotou and Vasiliadou 2006), Enzymes immunoassay (Thomson and Sporns 1995), LC, LC-MS and GC, GC- MS, Radioreceptor assay (Schwaiger et al. 2000), Biosensor immunoassay (BIA), High performance capillary electrophoresis (HPCE) (Shao et al. 2005), Capillary electrophoresis (Bateman et al. 1997), and RP-HPLC. Among those techniques, HPLC is one of the best ways of analyzing sulfonamides in biological and water matrices. Because it is more precise and accurate for both quantitative and qualitative analysis with a capability of separate components so; by using this technique all the components can obtain separately with high purity as from Figure 1.4. can be seen. During the past 5 years, multiple analytical methods based on RP-HPLC for the determination of sulfonamides in honey have been published. Some of them use UV-VIS detection, others use pre-column derivatization with fluorescence detection (Martel and Zeggane 2003).

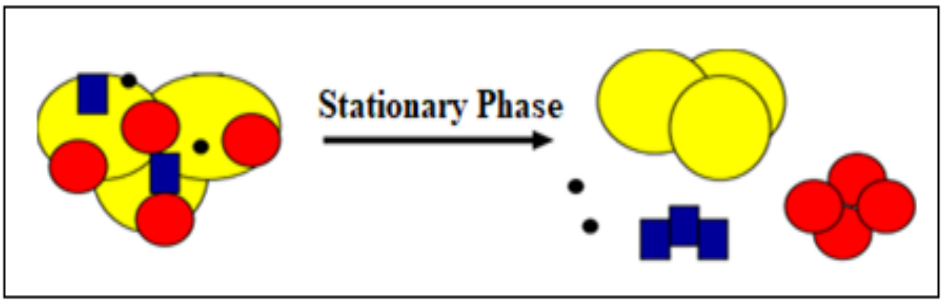


Figure 1.4. Separation of the mixture into its components

The liquid chromatographic technique is one of the best analytical technique that based on the separation of components due to differences in their molecular structure and/or composition. Generally, the chromatographic technique involves sample passing through a column that contains stationary phase and the sample moved and held by a liquid mobile phase, the separation based on the difference of molecular affinity to the stationary phase finally all components will separate with different elution or retention time. Consequently, a molecule with high affinity to the stationary phase displays a slow moving through a column with a high retention time and a component that has a weak interaction with stationary phase elutes quickly and faster with law retention time as shown from the Figure 1.5., therefore, a separation by LC depends on the variety of moving speed through a column (Sundaram et al. 2009).

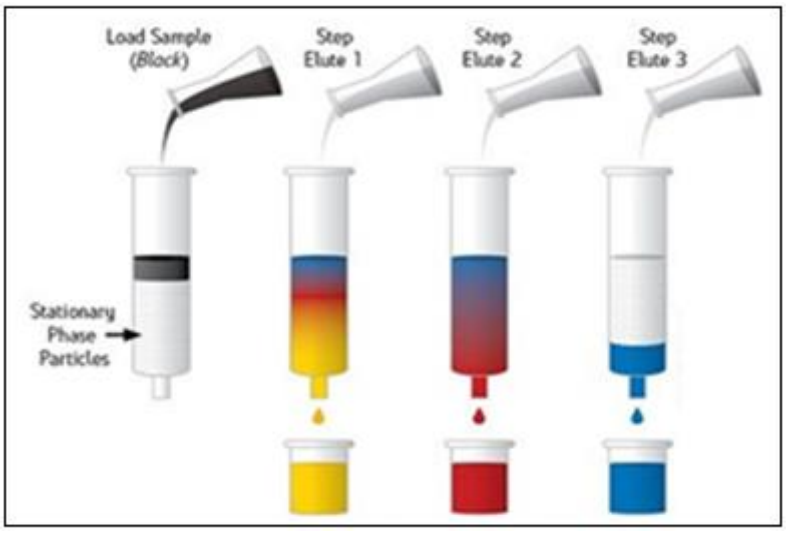


Figure 1.5. Separation of mixture components by LC

1.6. High Performance Liquid Chromatography (HPLC)

HPLC expression was developed by Horváth et al. in 1967 during his experimentation at his lab with a material that contains a special porous and was needed a pressure over 1000 psi so; Horvath said, "this is not LC anymore, this is high-pressure LC (HPLC)" (Horvath et al. 1967) and then another Hungarian, Halasz thirty five years ago changed the fundamental for the fine particles that packed inside column as a stationary phase with that new condition he separated 15 compounds (Halász et al. 1975).

HPLC comes from a high improvement of column liquid chromatography based on a pump that flows down of mobile phase through a column instead of by gravity, for this reason, it necessary to use a pump to generate a pressure up to 42000 kpa that makes analyzing by HPLC is much faster than column liquid chromatography, as a reminder that all chromatographic separation techniques even HPLC are based on the same principle; the basis of sample components separation depends on the difference of analyte constituents affinities for the mobile phase and the stationary phase inside of the column used in the separation (Snyder et al. 2011). Since the development date of HPLC up today there are continuous improvements especially in the stationary phase, here some variations of HPLC, according to physical properties of (stationary phase) of the process in the system.

1.6.1. HPLC Historical Background

The Russian botanist Mikhail Tswett in 1906 that described the separation phenomena when the mixture of natural colors (plant pigment) was passed through a tube of $CaCO_3$ or alumina and using petroleum ether, just after Tswett discovery, a modern Liquid Chromatography (LC) was used a liquid materials as a mobile phase for holding the analyte constituents through a column packed that contained a solid material called the stationary phase (phase of separation), there are two main modern chromatographic techniques that are very widely used in chemical analysis as a separation technique which are: Gas Chromatography (GC) and High-Performance Liquid Chromatography (HPLC).

HPLC is the most widely used analytical technique for separation and analyzing purpose in drug synthesis manufacture for controlling the quality and purity and researches. HPLC

can be used for qualitative and quantitative analysis of the unknown sample and determine the types and amount of constituents and separation of mixtures for later analysis preparative HPLC. To analyze a mixture to by chromatography technique a trace amount of sample mixture is injected into the system by manual injection or autosampler then passed through a column that contain a large amount of micro size particle as stationary phase using a liquid material or solvent and different molecules exist the column at different times this is named separation. The separation of mixture components based on the different interaction force between the analyte constituents with a stationary phase. A stationary phase included in a stainless steel tube (column) the most common stationary phase that used in this technique is silica gel (surface-modified silica particles) and a mobile phase (solvent) is moved through the system and carrying the analytes through a column that contains a stationary phase. In HPLC, the stationary phase contains a very small with a standard particle size in the unit of micro for column chromatography is 60 microns and for HPLC is generally 5 microns , this is to maximize the interaction area between analyte constituents with stationary phase which makes the excellent separation, the solvent, and mobile phase flow and passing through such a dense and small particle phase is difficult and needs for pumping to generate pressurized flow by several thousand pounds per square inch (psi) which makes flow of ml/min. Therefore, HPLC during analyzing called high-performance liquid chromatography and according to base on working called high-pressure liquid chromatography (Fekete et al. 2009).

1.6.2. Instrumentation

Every HPLC instrument have these main components: solvent reservoir, pump, injector, column, detector, integrator, and display system.

1.6.2.1. Solvent Reservoir

The solvent reservoir is a glass that contains a mobile phase, in HPLC the mobile phase maybe polar or nonpolar liquid depending on the type of analyte that presents in a sample.

1.6.2.2. Pump

HPLC pump section duty is to receive the mobile phase from a solvent reservoir and forced it down into a column then to the detector, this section can generate a pressure over 42000 kpa and the operation pressure depends on the column dimension, particle size flow rate and mobile phase content.

1.6.2.3. Sample Injector

The injection section of HPLC can be automatized in an autosampler, it should give the infusion of the sample fluid inside the scope of 0.1 mL to 100 mL of volume using microsyringe, and it should have a great repeatability and work at the high pressure greater than 4000 psi.

1.6.2.4. Column

Columns are typically made of cleaned metal commonly stainless steel with 50 - 300 mm length, the diameter ranged between 2 and 5 mm. A column internally contains with a particle size around 3–10 µm, always the oven temperature and mobile phase temperature should be constant during a period of analyzing because the viscosity of mobile phase can get decreased and the result of separation will fluctuate. The column is always used to separate sample constituents according to the difference between their molecular structures.

1.6.2.5. Detector

The HPLC detector designed to a place and installed just after the column distinguishes the analytes as they elute from the chromatographic column. The most common detectors are UV-spectroscopy, mass-spectrometric, fluorescence and electrochemical detectors.

1.6.2.6. Data Collection Device or Integrator

After the detection of each separated components, the detector can send a signal and gathered on the chart recorders or electronic integrators that vary in complexity and in their

ability to process, save and calculation reprocess chromatographic data as shown from the Figure 1.6.

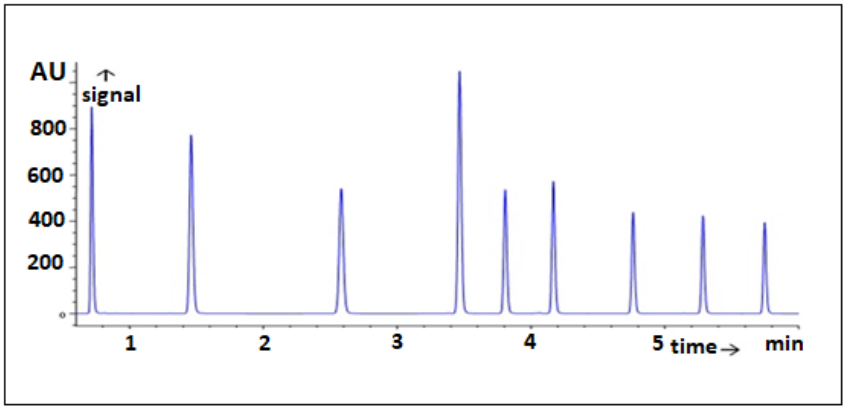


Figure 1.6. Detector signals are shown by chromatograph

The chromatograph is easy to read, that shows the recorded output of detector as a series of peaks, each peak representing an individual component in the mixture passing through the detector. The amount of each analyte can be seen by calculating the area of each peak and using in the automatized mathematical calculation with computerized calculation software the amount of each analyte can be determined (Thammana 2016). All of HPLC sections are connected together by stainless steel tubing in a series way by a stainless steel tube as shown in the Figure 1.7.

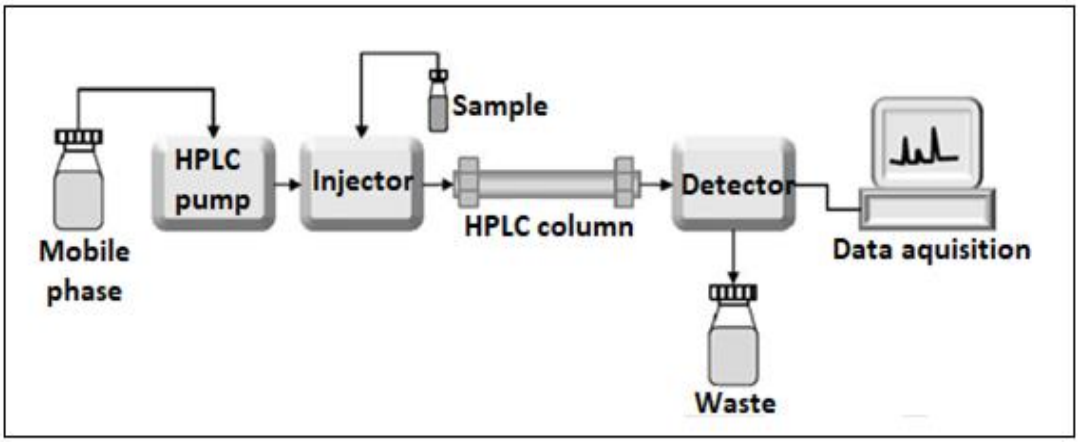


Figure 1.7. HPLC instrumentation

1.6.3. Types of HPLC

1.6.3.1. Normal Phase HPLC

Separation by this type of HPLC is based on the polarity. Normal phase uses nonpolar mobile phase such as methylene chloride, chloroform, hexane and diethyl ether, with a column that contains polar stationery phase commonly used silica, and this method can be used to separate a mixture of polar analytes when more polar constituent remains more times at the surface of stationary phase than less polar parts (Majors and Hopper 1974).

1.6.3.2. Size-Exclusion HPLC

The separation of sample constituents based on the size of molecules when a column is filled with controlled pore size substance. The bigger molecule size elutes first while smaller molecules retain later (Lues et al. 1998).

1.6.3.3. Ion Exchange-HPLC

This technique can be used to analyzing a sample that contains an ionizable constituent and the surface of stationary phase has an opposite ionically charged against the sample ions using the aqueous buffer as a mobile phase for elution, the separation by this technique depends on both pH and ionic strength. Therefore, the stronger charged analyte remain more time than weaker to the elution process (Bhattacharyya and Rohrer 2012).

1.6.3.4. Reverse Phase HPLC

This type of HPLC contains nonpolar stationary phase (hydrophobic) with polar mobile phase (hydrophilic) , the solution of water with methanol or acetonitrile or a mix of both used as a common mobile phase. The separation by this technique depends on the hydrophobicity interaction between the solute molecule in the mobile phase and the hydrophobic stationary phase, thus, more polar analyte remains for shorter time inside of the column and elutes firstly (Aguilar and Hearn 1996) and (Dorsey and Cooper 1994). The mixture of analytes is initially applied to the sorbent (separation phase) with aqueous

buffer solution. Then, the solute elutes by using the mixture of mobile phase with the organic modifier. The elution process can be done by either isocratic pump mode or gradient, if isocratic used, the flow of organic solvent will be constant if gradient mode used, the flow of organic solvent changes and increases during a period of analysis (Aguilar and Hearn 1996). The organic modifier used for increase molecular hydrophobicity (Aguilar 2004). RP-HPLC is a most powerful technique for the analysis and separation because of the following factors:

- More conditions can be used to achieve excellent resolution.
- Easy to use and selectivity can be optimized through changes in mobile phase characteristics and composition.
- Generally has high recoveries.
- Good reproducibility separation over a period of time.

Because of these factors, RP-HPLC is one of the best ways of analyzing and quantification of sulfonamides in honey. Figure 1.8. represents the schematic configuration of RP_HPLC.

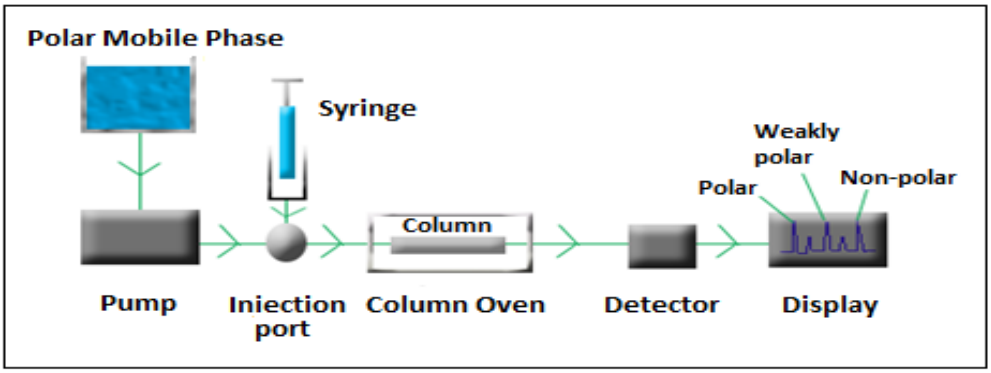


Figure 1.8. RP-HPLC schematic configuration

There are many detectors can be used with HPLC to see the presence of the analyte which has different components from the mobile phase composition then can convert the signal and information into the electrical signal (Thammana 2016). Here some common detector used with HPLC such as

- Diode Array
- UV-Visible
- Refractive Index

- Fluorescence
- Light Scattering
- Conductivity
- Mass Spectrometer

High performance liquid chromatography coupled with UV detector is one of the best ways that can be used to study of sulfonamides in honey.

1.7. Applications of Reversed Phase HPLC

RP-HPLC is typically used applicable for when the analytes molecule weight is very small (M.Wt. < 2000 Da). The method with this technique can be developed using C18 or Octadecylsilane (ODS) column initially. RP can also is applicable when extended to analyses and separation of weak electrolytes such as weak acid and weak bases with extreme control of mobile phase composition especially the organic modifier and buffer to control the analytes ionization process during the elution time, another application of RP is to analyze of amino acids, peptides and protein using the column of short chain alkyl stationary phases. Other compounds with M.Wt above 2000 can be also analyzed with RP technique but needs to use of additives, pH control and may need special column treatment (Aguilar 2004)

1.8. Goals and Objectives

1.8.1. Goal

The main goal of this master thesis is to develop an optimized method for measuring of sulfonamides residue in different honey samples by RP-HPLC-UV with isocratic elution mode.

1.8.2. Objectives

1. The explanation of the theoretical background and types of Honey and its importance.

2. Discuss basic theory behind antibiotics, types of antibiotics, and sulfonamides with advantage and side effects.
3. Discuss the basic theory of chromatography.
4. In order to understand HPLC and to utilize its practical applications effectively, some basic concepts of instrumentation and components of high performance liquid chromatography are mentioned.
5. Checking suitable chromatographic condition for analyzing.
6. Preparation of mobile phases with some different pH to make a good peak diagnosis in order to reach a good peak resolution condition.
7. Preparation of sulfonamides standards and making HPLC calibration then Validation.
8. Appling a procedure for sample preparation before injecting and analyzing.
9. Injecting prepared samples and calculation.
10. Determination of the amount of sulfonamides in honey samples.

2. LITERATURE REVIEW

Due to the importance of sulfonamides and health care of the human body, there are many scientific articles and research published about analyzing of sulfonamides and determination quantitatively in a different matrix of food products by HPLC as mentioned from the researches below.

Bernal et al. showed an analytical method for detecting a trace amount of sulfonamides residue in honey by HPLC, the sample was prepared and treated with methanol to prevent emulsion, turbidity and to breakdown sulfonamide-sugar bond as mentioned before that honey contains around 80% of sugar, the limit of quantification founded between the range of 4-15 ng/g and the percentage of recoveries ranged between 56%-96% (Bernal et al. 2009). In 2008 some researchers developed another method for sulfonamides in honey using an internal standard technique with sulfapyridine, this experiment managed and validated by Granja et al. also there are some changes with proposed method for the determination and analysis of sulfonamides using Brazilian honey sample. The limit of detection was calculated as 3 ppb for sulfathiazole, 5 ppb for sulfadimethoxine and 4 ppb for sulfamethazine, also the average recovery percentage was 61% for sulfathiazole, 86% for sulfadimethoxine and 94.5% for sulfamethazine at a level of 100 µg/kg (Granja et al. 2008).

The fast method Salting-out liquid-liquid extraction (LLE) used water-miscible organic solvents as the extractants was improved by Liu et al. for the determination of sulfa drugs residue in different matrix and honey in 2010, this method mentioned liquid-liquid extraction during sample preparation step and 1mL microsyringe as a separation phase tool, acetonitrile used as an extraction solvent in very low volume. The calculation showed that the average recovery percentage valued between the range of 31.97% to 66.54% and this developed method was more accurate with law RSD, therefore, it was accepted for a real application (Liu et al. 2010).

Tsai with his collaborators in 2010, are another researchers that they used a simple sugaring-out assisted liquid-liquid extraction method combined with high-performance liquid chromatography with fluorescence detection (HPLC-FL) for the extraction and determination of sulfonamides in honey., acid hydrolysis was used to breakdown sulfonamides-sugar bond then sugar separated and released, the sulfonamides residue derivatized with fluorescamine, they were partitioned into the organic layer below the honey/mobile phase system. The recoveries were 80.9-99.6 % for honey at three levels (5, 20 and 100 ng/g) and the linearity was obtained from (2 to 200 ng/ml) and Limits of detection for the sulfonamides studied were found to range from 0.6 to 0.9 ng/g. (Tsai et al. 2010).

Bedendo and his collaborators were described a precise and sensitive analysis using hollow fiber renewal liquid membrane (HFRLM) extraction followed by high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) for determination of five SAs in honey samples. In this method, the organic solvent presented directly into the sample matrix extracts the SAs and carries them over the polypropylene porous membrane. The most important parameters affecting the extraction efficiency were optimized by multivariable designs (sample mass and pH, a buffer for stripping phase and pH, time and temperature of extraction, volume and type of extractor solvent and use of salt to saturate the sample). Limit of detection in the range of 5.1–27.4 μg kg^{-1} and linearity coefficient of correlation higher than 0.987 were obtained for the target analytes. The results obtained for the proposed method show that HFRLM–LC–MS/MS can be carried out for determination of the five SAs studied in honey samples with excellent precision, accuracy, practicality and short analysis time (Bedendo et al. 2010).

A fast and selective method was developed by Chen et al. for the determination of sulfonamides (SAs) in the honey based on the magnetic molecularly imprinted polymer. The sample was blending and stirring for the extraction solvent and polymers. After completing the extraction, the polymers, along with the captured analytes, were definitely separated by an adscititious magnet from the sample matrix. The analytes eluted from the polymers were determined by liquid chromatography-tandem mass spectrometry. Under the optimal conditions, the detection limits of SAs are in the range of 1.5−4.3 ng g−1. The relative standard deviations of intra- and interday ranging from 3.7% to 7.9% and from

4.3% to 9.9% are obtained, respectively. The proposed method was successfully applied to determine sulfonamides including sulfadiazine, sulfamerazine, sulfamethoxydiazine, sulfamonomethoxine, sulfadimethoxine, sulfamethoxazole and sulfaquinoxaline in different honey samples. The recoveries of SAs in these samples from 67.1% to 93.6% were obtained (Chen et al. 2009).

In 2002 liquid chromatography (LC) method in combination with tandem mass spectrometry (MS–MS) has been developed by Verzegnassi et al, and applied to the separation and detection of 10 different sulfonamides in honey. In the method, a simple hydrolysis of the honey sample was used to release sugar-bound sulfonamides followed by liquid–liquid extraction of the 10 analytes, filtration, and analysis by LC–MS–MS. Conditions for RP-LC and electrospray ionization (ESI) MS–MS in the positive ion mode were optimized for the 10 compounds, monitoring two characteristic mass transitions simultaneously for each analyte. The procedure is a qualitative confirmatory method for 10 sulfonamides at the low μg/kg level in honey. The recoveries of the analytes in honey ranged from 44 to 73% at a fortification level of 50 μg/kg (Verzegnassi et al. 2002).

A solid phase extraction (SPE) for sample preparation step using acetate buffer (pH 5.0) was settled in 2002 by Posyniak et al. liquid chromatography analysis for SAs residues in honey was used, The liquid chromatography (LC) separation was carried out on the reversed phase C18 column, the mobile phase contains acetic acid and acetonitrile, with gradient pump mode and fluorescence detection, after pre-column derivatization. Recoveries were above 80%, and LOQ was 0.1 mg/kg for sulfacetamide and 0.2 mg/g of sulfamethazine and sulfathiazole (Posyniak et al. 2002).

Separation and quantification of six sulfonamides (sulfamethoxazole, sulfacetamide, sulfamethazine, sulfadiazine, sulfathiazole, and sulfanilamide) using HPLC-fluorescence detection method was developed by Bonta and his co-workers in 2009, the analytes were extracted from honey with extraction mixture (acetonitrile and dichloromethane) after hydrolysis by acid then cleaned by a C18 SPE and pre-column derivatization with fluorescamine. RP-HPLC with gradient pump mode performed, 30 min time of analysis. LOD for sulfadiazine and sulfamethazine was at 2μg/kg, and 3μg/kg for sulfanilamide, sulfacetamide and sulfathiazole and 5μg/kg for sulfamethoxazole. The sample preparation

step was efficient and the recoveries of the analytes in spiked honey ranged between 47 to 79% (Bonta et al. 2009).

In 2013 a new RP liquid chromatography method (HPLC-FLD and LC-MS/MS) developed by Tolgyesi and his co-workers. Their method used for determining the amount of sulfa drugs residue in incurred and spiked honey sample. The optimization of sample preparation carried out using Qasis (hydrophilic-lipophilic balance) and SPE cartridge used for sample preparation step procedure. They used three different medium for sulfonamides elution (Acidic, Basic and neutral). the average recovery founded that is ranged between 66.8 -90 %, which is higher than the other conditions by 10% .the strata-XL used to test for sample clean-up. Mobile phase contains ternary (methanol-acetonitrile and phosphate buffer solution pH 5) and Varian C18 column. This condition of analysis showed a good selectivity for determination of sulfonamides, a computer simulation used to control ternary gradient pump mode, using Dry-lab software LC-MS/MS analysis and separation of analytes was carried out on a kinetex-XB core-shell type. LOQ (0.01-0.05 µg/kg) and the time of analysis faster and shorter (6 min), this developed method validated based on European Union Commission Decision 2002/657/EC and it was successful for quantification of more than four hundred of honey samples. The result of sulfonamides concentration was 0.03 up to 686 µg/kg (Tölgyesi et al. 2013).

Another validated procedure of sulfonamides residue in honey was developed by Szczesna et.al in 2009. they used RP-HPLC for separation and fluorescence detector to detection, identification, and quantification of sulfonamides after pre-column derivatization by fluorescamine, after calculation of quantification limit with LOD of each analyte the developed method was validated, the hydrolysis parameters (Temperature and mixing condition) sulfonamides-sugar bond was carried out and determined to control the repeatability and recovery of sulfonamides. To achieve a good separation of 4-aminobenzoic acid which occurred in some types of honey from sulfamethazine and analysis of other sulfonamides such as sulfacetamide, sulfadimethoxine, sulfamerazine, sulfachlor pyridazine, sulfathiazole, sulfamethoxy pyridazine, sulfamethoxazole, sulfanilamide, sulfamethazine. The calculation of LOQ and LOD of each sulfonamide showed that LOQ=0.3 and LOD=0.2 µg/kg. In the normal analysis, the recovery percentage

was 37% for sulfacetamide and 93% for sulfanilamide. The repeatability coefficient ranged between 1.8 for sulfadimethoxine and 1.4 for sulfadimethoxine (Szczêsna et al. 2009).
In 2016, Kivrak et al. were developed a more accurate method for detection of antibiotics in honey including of sulfonamides using UPLC with tandem mass spectroscopy detection (UPLC-MS/MS), and the method validation successfully applied for 23 analytes of antibiotics. The developed method consists of sonication for ultrasonic extraction and filtration followed the analysis by UPLC–ESI–MS/MS, the method was involved of the study of the optimum condition of analysis for the weight sample and temperature of extraction in term of recovery percentage. Determination of antibiotics included sulfonamides was fulfilled by UPLC separation with a column specification of BEH C18 (100 mm x 2.1 mm, 1.7 µm) and gradient pump mode for elution of mobile phase the mass spectrophotometry detection. This method was applied to detecting the antibiotic residue in the honey sample which found in some turkey markets at Muğla city (Kivrak et al. 2016).

In 2014, Tashakkori et al. were developed a cheap, fast, simple and selective analytical method by HPLC using cetyltrimethylammonium bromide-silica gel for preconcentration for sulfamerazine, sulfadiazine, sulphamethoxazole, sulfamethazine and antibiotics detection and quantification by RP-HPLC. The investigation parameters such as pH value, class and amount of surfactant, mobile phase flow rate and the eluting volume agent was studied. The limit of detection of sulfonamides was 3-13µg L-1 (LOD of the method) with the linearity of 0.010-2.0 µg/mL. The method was applied successfully for sulfonamides determination in various honey samples (Tashakkori et al. 2014).

The first automated flow injection system combined with re-generable antigen microarrays was developed in 2011 by Wutz and his collaborators for determination of antibiotics residue in honey samples. The scheme depends on the indirect competitive immunoassay format by monoclonal antibodies attached to the surface of the microarray. The slide was coated by epoxy-activated poly (ethylene glycol) at the surface. This method has a fast analysis result for 4 analytes without extraction and purification technique. The quality control parameters of this method were studied, the recoveries of sulfadiazine (89%±20%), enrofloxacin (92%±6%), sulfamethazine (130%±21%), and streptomycin (93%±4%) (Wutz et al. 2011).

In 2005 Krivohlavek again was talked about that the sulfonamides are stable chemotherapeutics and can be used as an antibacterial drug to avoid bacterial disease affecting bees, therefore the sulfonamides residue could be available in honey after bees treatment with antibacterial agents, and the concentration must be monitored in the range of desired value. For this reason, they developed a reliable, simple and fast analyzing method for quantification of 11 sulfonamides after the method was optimized. The sample was prepared by solid phase extraction after homogenization using a Chroma bond C18 end-capped cartridge followed by LC/MS analyses. The specification parameters of the method were studied, the limit of detection was 25 μg/kg was achieved for all sulfonamides. Other important parameters also investigated and approved such as buffer pH of mobile phase and temperature (acetic acid buffer) (Krivohlavek et al. 2005).

Sheridan et al. in 2008 were developed a new method for determination of 14 sulfa drugs and chloramphenicol in the honey sample. These antibacterial agents have been avoided to use in production by animals. However the residue of them have been found in many samples, therefore the multiresidues analysis technique and method is needed in honey. The sample was prepared by following acid hydrolysis to cleavage sugar-sulfonamides bong, solid phase extraction carried out to remove and release other interferences. The chromatographic separation used with LC-electrospray ionization coupled with tandem mass spectroscopic detection in negative mode for all antibiotics residue with includes 15 analytes. This method was designed to confirm the food and drug quality control in the U.S. it is can be used to provide 4-EU identification points. In 25 different countries, 116 samples have been taken, 38% of samples contain at least one analyte of antimicrobial, and 13 samples contains 5 different target compounds (Sheridan et al. 2008).

In the year 2017, Korkmaz and his collaborators have developed a new method for determination of sulfonamides residue and tetracycline in pine honey from Aegean Region of Turkey, which Turkey is one of the world's largest pine honey producer around 95% export share. Due to the importance of antibiotics residue in honey around the worldwide, therefore a good quality control is required. For this reason, 59 samples of natural pine honey were collected and quantified the number of sulfonamides and antibiotic residue in the carried out through a competitive enzyme-linked immunoassay method, 35 samples consisting tetracycline group of antibiotics with the range of concentration 6-42 ppb,

whereas in 24 samples of honey the ratio of antibiotics residue was bellowed the value of the detection limit 4 ppb. the sulfonamides were found and observed in 31 honey samples with the range of the concentration of 3-31 ppb and 28 sample of them contains the sulfonamides with the concentration below the detection limit value 2ppb in conclusion; there no maximum residue limit of sulfonamides in honey samples (Korkmaz et al. 2017).

Thompson and Noot in 2005, developed a new simple and fast analytical method for quantification of 7 sulfonamides in the honey sample, the sample was prepared for acid hydrolysis to break-down sugar-sulfonamides bond, then filtered the sample, and the acidified honey sample was injected directly into HPLC-MS/MS system. Using gradient pump mode for sample analyte elution, the sulfonamides extraction carried out by an automated system. The mobile phases flow arranged using the six-port valve. The bulk honey sulfonamides matrix has been removed. The contamination of MS chamber can be observed in a minimum quantity with injection over 600 honey samples. The internal standard technique has been used, with excellent accuracy and good precision was obtained. The LOD for sulfonamides were calculated and ranged between 0.5-2 g/kg (Thompson and Noot 2005).

A selective and sensitive method was described by Maudens and Lambert for the simultaneous quantitative analysis and determination of 14 sulfonamides in honey. After acidic hydrolysis of the honey sample to release and break-down the sugar-sulfonamides bonds with adjustment of the pH, the sulfonamides are extracted into acetonitrile. This fraction is dried and evaporated. The residue is reconstituted in an acetic acid solution and loaded onto a strong cation exchanger (SCX). The eluate is evaporated, re-dissolved into an acetic acid solution and an aliquot is injected. Separation is achieved on RP C18 column, running a gradient program with an acetate buffer and acetonitrile. A post-column derivatization with fluorescamine allows fluorescence detection at the analytical wavelength of 420 nm and the emission wavelength of 485 nm. The method has a limit of quantification between 2 and 5 μg/kg for all compounds. From the limit of quantification up to 100 μg/kg, the method showed good linearity for every compound ($r^2 > 0.995$) (Maudens and Lambert 2004).

3. METHODOLOGY

Honey contents with sulfonamides resulted from honey bee treatment with antibiotics to protect their life from some bee diseases, but because of the serious side effects of antibiotics if present in high level, a good quality control method for analyzing and calculating sulfonamides in honey is necessary, in this study, for honey qualification, a new method developed according to the method development which explained from the Figure 3.1.

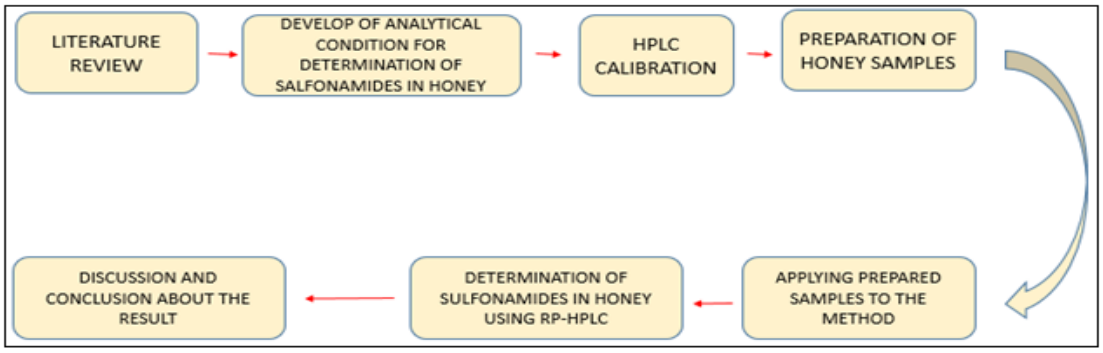


Figure 3.1. Stages of research: Sulfonamides in honey by RP-HPLC

This section will be explained how objectives will be achieved, in relation way, first of all, beginning with document review.

3.1. Document Review

The aim of document review is to conduct a review of the topic under study concerning technical information such as selection of information based on the level of relevance, content analysis of selected bibliography, using scientific articles and reference books about types of sulfonamides and determination the quantity of them and finally methods for evaluating of determination of sulfonamides in honey using HPLC.

3.2. Analytical Method Development and Procedures for the Determination of Sulfonamides in Honey Using RP-HPLC-UV

3.2.1. Chemicals

Chemicals were used in this method are shown in the table below:

Table 3.1. List of chemicals that used in this study

Reagent	CAS Number	Company
Thioeurea	62-56-6	Sigma-Aldrich
Sulfadiazine	68-35-9	Sigma
Sulfamerazine	127-79-7	Sigma-Aldrich
Sulfapyridine	144-83-2	Sigma-Aldrich
Alpha-toluene sulfonamide	4563-33-1	Alfa-Aesar
Sulfamethazine	57-68-1	Sigma
p-Nitrobenzenesulfonamide	121-52-8	Sigma-Aldrich
Sulfadoxine	144-83-2	Fluka
Sulfisoxazole	119K1308V	Sigma
Sulfathiazole	72-14-0	Fluka
Acetonitrile	SZBA175S	Sigma-Aldrich
Methanol	SZBB12CV	Sigma-Aldrich
Trifluoroacetic acid	1695585	Fisher Scientific Uk
Hydrochloric acid	7647-01-0	Sigma-Aldrich
Sodium phosphate monobasic	13472-35-0	Sigma-Aldrich
Sodium phosphate dibasic	K41462580035	Merck

The purity of chemicals and reagents grade should be used in all tests. All reagents certification should be confirmed according to ACS with enough purity and sufficient to be used in a method without losing accuracy.

3.2.2. Apparatus

Also, many instruments and equipment were used in this study as shown in the table below:

Table 3.2. List of instruments and apparatus that used in this study

Instrument and Apparatus	Serial No.	Company
Hplc	L20224606065	Shimadzu
Micropipette 10-100µl	----------	Ecopipette
Micropipette 100-1000 µl	----------	Ecopipette
Ultrapure water system	100936102	Human Power I
pH meter	4120600	J.P.SELECTA
Balance	6A7700259	AND(A&D)
Microfilter	367-256-451	AFG Scientific
Classic filter paper	207011	Macherey-Nagel
Oven	253.3.6/12/936	Memmert
Ultrasonic bath	NHM1120509-519C-T.E.LEF	Elma(Elmasonic)
Megnatic stirrer	0025002970	IKA C-MAG

3.2.3. Preparation of the Standard Solutions

The standard solution of these sulfonamides (Sulfadiazine, Sulfamerazine, Sulfadoxine, Sulfathiazole, Sulfamethazine, Sulfapyridine, Sulfisoxazole, α-Toluenesulfonamide, and p-Nitrobenzenesulfonamide) with Thiourea in different concentration have been prepared according to the rule 3.1.

$$ppb_{solute} = \frac{mass\ of\ solute}{mass\ of\ solution} * 10^9 \quad (3.1)$$

The scientific bases used to prepare the standards to determine sulfonamides in honey by the following points: (1) The greatest possible weight of reagent with more than 99% assay to avoid systematics analytical errors. (2) The use of a mother solution maintaining the same proportion of a natural mixture of the sulfonamides in the original honey. (3) Use no more than two dilutions of mother solution, to prevent the increasing of error when measuring the volumes of the standards.

In this sense, 0.0200 grams of each reagent (9 in total) were taken, which were weighed successively in the same flask, then diluted in 100 mL methanol. This mother solution (A) is equivalent to 200 ppb (0.2 ppm) of a mixture of 9 different sulfonamides.

Firstly, 1 ml of this stock solution (A) was taken and completed with mobile phase in order to the volume become 10 mL to obtain 20 ppb. (Mother solution B). Secondly, 5 mL of mother solution B was taken and completed to a 10 mL volumetric flask with mobile phase to achieve 10 ppb. Finally, the standard solution of 1 ppb and 0.1 ppb were prepared from the stock solution B (20 ppb) by dilution way when 0.5 taken from stock solution B for preparing 1 ppb, and 0.05 taken for preparing 0.1 ppb.

Using this procedure it is guaranteed that no more than two dilutions exist and no more than one weighing to avoid major errors in the preparation of the calibration standards.

3.2.4. Preparation of Buffer Solution and Mobile Phase

5Mm of the phosphate buffer solution was prepared from the mixed solutes of sodium phosphate dibasic $Na_2HPO_4.2H_2O$ and sodium phosphate monobasic$NaHPO_4.H_2O$, the mixed solutes diluted in ultra-pure water (UP), the pH of the solution adjusted by adding of a diluted solution of (hydrochloric acid HCl) to decreasing the pH value and diluted (potassium hydroxide KOH) for raising, the pH adjusted at 5.
Because of the buffer prepared with ultrapure water, and water is highly polar solvent, therefore, the organic modifier must be used to reduce the polarity of the mobile phase and arranging the analytes elution. A mixture of acetonitrile with methanol 1:1 used as an organic modifier. So the mobile phase in RP-HPLC is polar mobile phase contains 80% buffer solution with 20% of organic modifier (acetonitrile: methanol 1:1).

3.2.5. pH and Flow Rate

The pH value always used to arrange and detection the acidity or alkalinity of the mobile phase in HPLC, it can be explained and calculated as the negative logarithm of hydrogen ions concentration in aqueous solution, the mobile phase pH must be kept constant and controlled carefully because it has a strong effect on the peak separation and resolution especially when the analyte is polar as in reversed phase HPLC.

The buffer solution is used to control pH stability in the mobile phase. In this study, the peaks separation diagnosed when different mobile phase prepared with different pH using phosphate buffer solution.

At the first time a mobile phase prepared with 5 mM buffer solution at pH: 8, the standard solution of thiourea and each sulfonamide injected individually, then the mixture of them (thiourea, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, α-toluenesulfonamide, sulfamethazine, p-nitrobenzene sulfonamide, sulfadoxine, sulfisoxazole) were injected at a flow rate: 0.5 ml/min, from the Figure 3.2, the chromatographic separation can be seen:

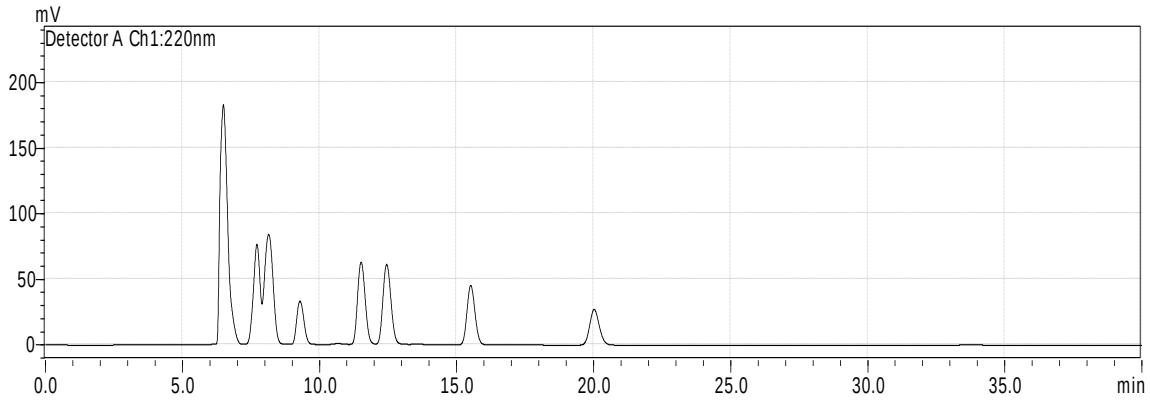


Figure 3.2. Chromatographic separation of SAs at pH: 8, flow rate: 0.5 mL/min

From the Figure 3.2., a poor peak separation chromatograph can be seen due to the absence of two peaks of (sulfamerazine and sulfadoxine), and overlap of another two peaks (sulfadiazine with sulfisoxazole) at minute 8. In this condition, the missing peak was Sulfamerazine peak due to ionization effect when the pKa value of sulfamerazine is equal to 8 which is fairly close to the pH of mobile phase and also another reason due to the column stationary phase is also affected by pH, it means that at high pH (equal or greater than 8) the column silica itself will be damaged by dissolution when even after the bonding and end-capping process is completed on high purity silica, there are still free (unbounded) silanols (Si-O-H) present on the silica surface. These silanols has a weakly acidic property which can react with basic materials such as at pH: 8 and then can has effect on the chromatographic resolution, therefore, the separation capacity will decrease, for this reason, preparing for another buffer pH was suggested, buffer solution with pH: 7 prepared and the mixture of standards injected at a flow rate 0.5 ml/min, the chromatographic separation can be seen from the Figure 3.3.

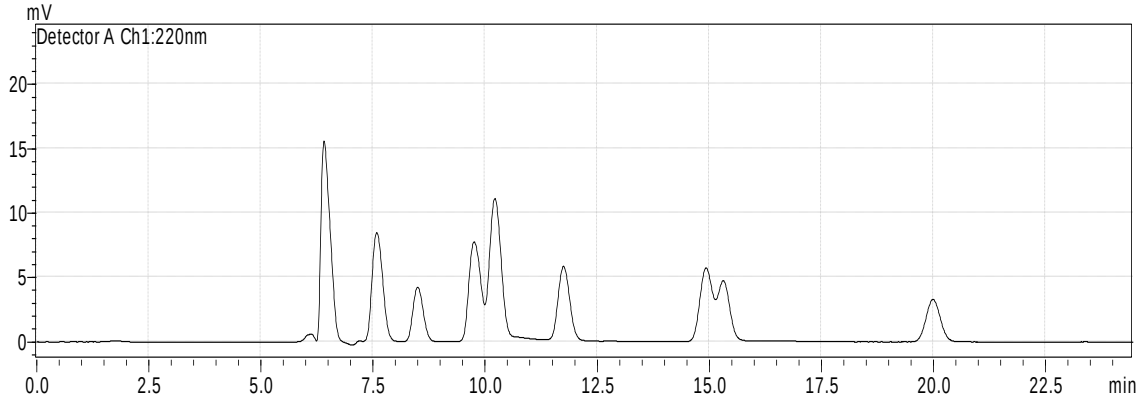


Figure 3.3. Chromatographic separation of SAs at pH: 7, flow rate: 0.5 mL/min

From the Figure 3.3. can be noticed that a very poor peak separation, at min: 10 the peaks of (sulfadoxine and sulfathiazole) are mixed by a big overlap and at min: 15 peaks of a-toluene sulfonamide and sulfamethazine separated with insufficient peak resolution and much more overlap. And also in this condition a poor chromatographic separation can be seen with law peak resolution, sulfamerazine peak has disappeared due to the same effect as in condition at pH 8. When the ionization state of ionizable functional groups on sulfamerazine molecule will depend primarily on the pH of the mobile phase and will result in a shift in retention, selectivity and will be difficult to decipher from the baseline for sulfamerazine peak. It means that still at this pH the column separation is insufficient., then another mobile phase prepared with pH: 6 By lowering buffer pH, the separation capacity increased when pH: 6 prepared and the overlap problem was solved as from the Figure 3.4.

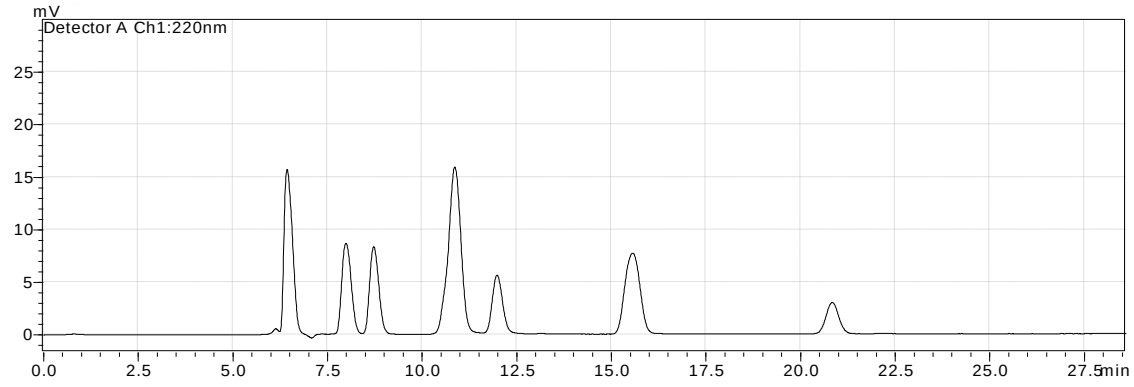


Figure 3.4. Chromatographic separation of SAs at pH: 6, flow rate: 0.5 mL/min

The Figure 3.4 showed that by lowering pH in reversed phase HPLC, the peak resolution gets increased and the overlap effect at the minute 10, 15 was solved, but still is not sufficient peak separation, At this condition pH: 6 the separation resolution of three analyte peaks were disappeared, the missing peaks were (sulfadoxine, sulfamerazine, and α-toluenesulfonamide), due to ionization effect when and the pH of mobile phase was fairly close to the pKa of those sulfonamides which the pKa value of those three sulfonamides ranged between 6.3-8. Subsequently, the peaks were broad and difficult to decipher from the baseline. Decreasing the mobile phase pH to 5 was very sufficient to neutralize (sulfadoxine, sulfamerazine, and alpha-toluenesulfonamide) and visualize sharp, corresponding peaks. By following that consequence (lowering pH) a buffer of pH 5 was prepared and the mixture of standards injected again at the flow rate of 0.8, in this condition, the peak separation can be seen from the Figure 3.5.

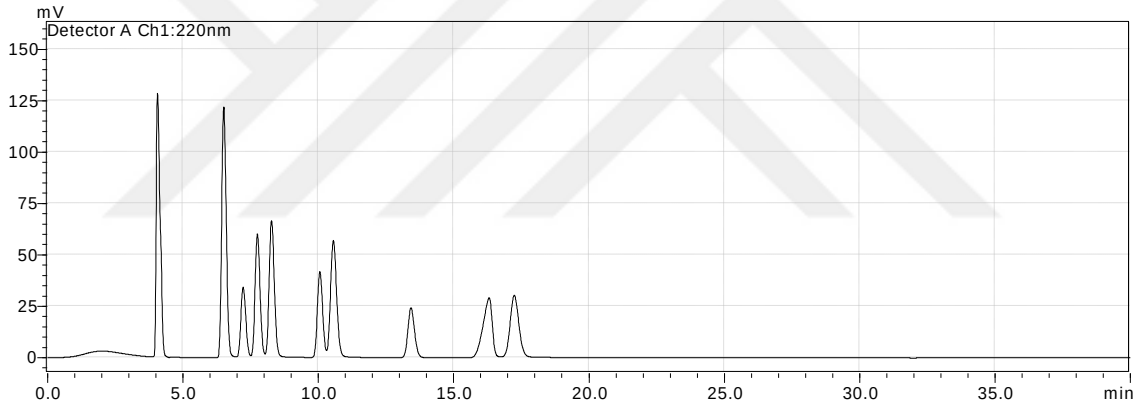


Figure 3.5. Chromatographic separation of SAs at pH: 5, flow rate: 0.8 mL/min

This is the best condition according to others (pH: 8, 7 and 6) and all peaks separated individually, but the resolution needs a little improvement at minutes 9 and 11, therefore, the standards injected again at flow rate 0.5 as shown in the Figure 3.6.

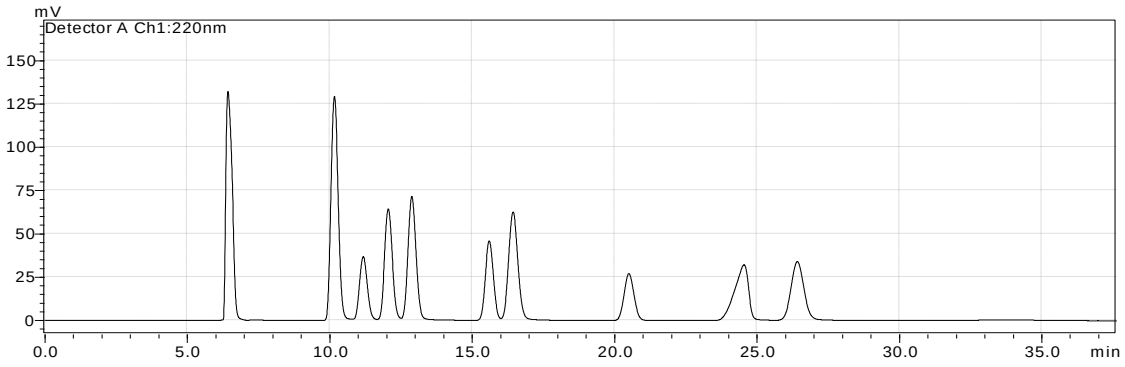


Figure 3.6. Chromatographic separation of SAs at pH: 5, flow rate: 0.5 mL/min

Now all peaks separated individually with no overlap of any peak, and the column separation efficiency is in better condition with sufficient peak resolution. It is clear to understand that the peak resolution is extremely pH dependable and however time of analysis gets shorter with increase of mobile phase flow rate but the peak resolution is decreased means there is a reverse relationship between them according to increasing and decreasing, when the sample injected at flow rate of 0.5 the peaks separated with more distance between them and maximum time of analysis is 26 min , but when the flow rate increased to 0.8, the distance between them decreased and the last peak separated at min:17 due to increasing rate of analyte elution from the column. According to literature the optimum condition of analysis is required so; a new buffer with pH: 4 prepared for a new mobile phase and the standard injected again as shown in the Figure 3.7.

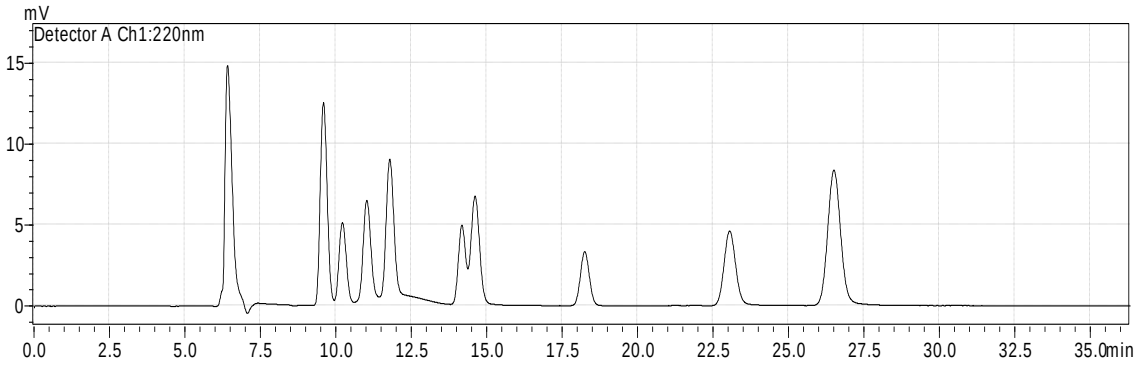


Figure 3.7. Chromatographic separation of SAs at pH: 4, flow rate 0.5 mL/min

In this condition again the separation fluctuated and insufficient at min: 11 and 15, with longer time 27 min for analyzing.

The separation and resolution of all standards obtained at pH: 5 and flow rate: 0.5 as the best condition, therefore, in this study, this condition selected as an analytical condition and the concatenation of each peak of SAs standards with their retention times were mentioned in the table below:

Table 3.3. The sequence of SAs standards at pH: 5 flow rate 0.5 mL/min

ID#	Names of SAs	Ret. Time
1	Thiourea	6.418
2	Sulfadiazine	10.154
3	Sulfathiazole	11.168
4	Sulfapyridine	12.046
5	Sulfamerazine	12.873
6	Alpha-toluenesulfonamide	15.592
7	Sulfamethazine	16.428
8	P-nitrobenzenesulfonamide	20.495
9	Sulfadoxine	24.540
10	Sulfisoxazole	26.410

3.2.6. Analytical Condition for HPLC Method

The optimum condition of sulfonamides analysis to obtain all analyte peaks individually with a sufficient peak separation and resolution by RP-HPLC with isocratic elution mode obtained when the phosphate buffer prepared at pH 5, this condition reaches after a long investigation of peak separation and resolution by many trails of injection and various buffer pH, to protecting GC precision and repeatability of analysis, the condition of all sections must be maintained constant as shown from the next tables:

Autosampler: SIL 20A (Shimadzu Brand)

Table 3.4. Autosampler section condition

Parameter	**Value Optimized**
Rack capacity	200 vials
Vial capacity	1,5 Ml
Rinsing volume	200 mL
Purge time	15 min
Rising mode	Before and after each injection.
Injection volume	5µL

Pump LC-20AT

Table 3.5. Pump section condition

Parameter	**Value Optimized**
Working mode	Isocratic Flow
Controlled by:	Constant flow thru column.
Analytical flow	0.5 mL/min
Pressure	63 bar

Oven CTG-10ASvp.

Table 3.6. Oven section condition

Parameter	**Value Optimized**
Working mode	Isothermal
type:	Isolated/Lichrosphere columns
Temperature	35° C

Detector SPD-20A

Table 3.7. Detector section condition

Parameter	**Value Optimized**
Lamp	Deuterium D2
Cell temp	40°C
Wavelength setup	220 nm and 265 nm
Ratio change	10
Runtime	40 min

HPLC -Column.

Table 3.8. HPLC column specification

Parameter	**Value Optimized**
Column brand	Kromasil
Specification	150 mm × 4.6 mm I.D.
Mobile Phase	Phosphate buffer + Acetonitrile+ methanol 1:1

3.2.7. Calibration

The system calibrated with four points of analyte concentrations applied to this study was a linear calibration listed in the table below:

Table 3.9. List of analytes and standards for calibration

Calibration Point #	Analytes	Prepared Concentration (ppb)
1	SDZ, STZ, SFP, SMZ, ATS, SMT, PNS, SDX, SXZ	0.1
2		1
3		10
4		20

Protocol of calibration:

1. Measure each standard
2. Execute regression analysis.
3. Validate results of calibration based on criteria of:
    a) Positive response factor magnitude: > 10^3
    b) Regression coefficient (R^2): minimum: 0.9500
    c) External calibration.

4. Determine conformity, recovery factor, precision and accuracy, lower detection limit (LOD) and lower quantification limit (LOQ).

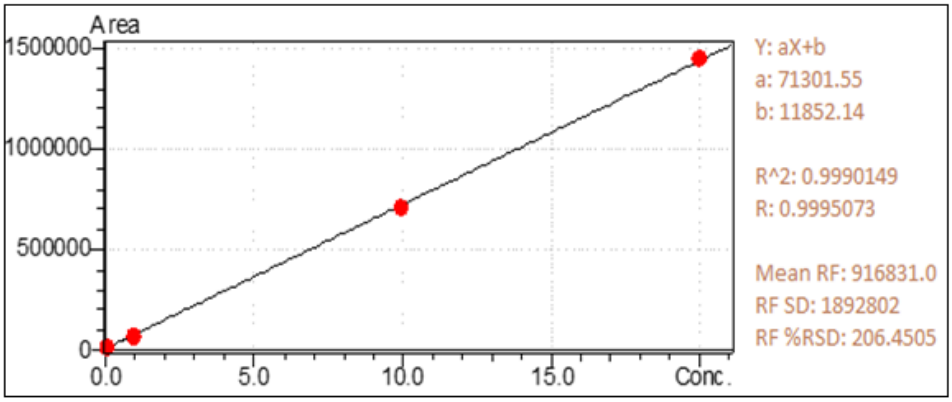


Figure 3.8. Calibration curve

3.2.8. Sample Preparation

Seven honey samples were obtained in different places in and around Bingol province as shown in the table below:

Table 3.10. List of sample names

Sample Names	Sample name abbreviations
Kıractepe köyü	S1
Karliova I	S2
Hazarshah köyü solhan	S3
Karliova II	S4
Togs yaylası genç	S5
Tanrıverdı köyü genç	S6
Bingol market	S7

The samples were stored in the refrigerator at 4 ^{o}C (to preventing photodegradation of sulfonamides). In this investigation the matrix of sulfonamides was very viscose, the sample preparation is necessary to reducing matrix effect.

An aliquot of honey sample (1 g) was weighed into a small volumetric flask 10 mL capacity using the high accuracy electronic analytical balance. The sample dissolved with 20 ml of 1% trifluoroacetic (TFA) acid mixed with acetonitrile 1:1.

The sample solution was shaken for 3 min continuously and after that was sonicated for 30 min in ultrasonic bath system ULTRASONIC BATH (37 kHz, 1000 W, Elmasonic S120H), with raising temperature up to 50 ºC. After the sample completely dissolved in TFA and ACN, and homogenized with sonication to break down sugar-analyte bond completely, then filtered with a classical technique of filtration (MACHEREY-NAGEL,) slow filter paper to remove the bulk sulfonamide matrix (honey residue except of sulfonamides) as a solid and precipitate phase. After removing the solid residue, the liquid was passed through a highly small porous membrane (0.20 µm) (AFG Scientific) into the autosampler vials and injected into the system (RP-HPLC-UV SHIMADZU BRAND).

3.3. Peak Study

In this study, thiourea used as a void marker to calculate T_o, T_o is a more important parameter in RP-HPLC when the mobile phase consists of a methanol-water-acetonitrile mixture, the use of it is important to calculate parameters that required for optimization processes such as the capacity factor or retention factor which is necessary to measure other chromatographic relationships like selectivity (α) as shown from the Figure 3.9.

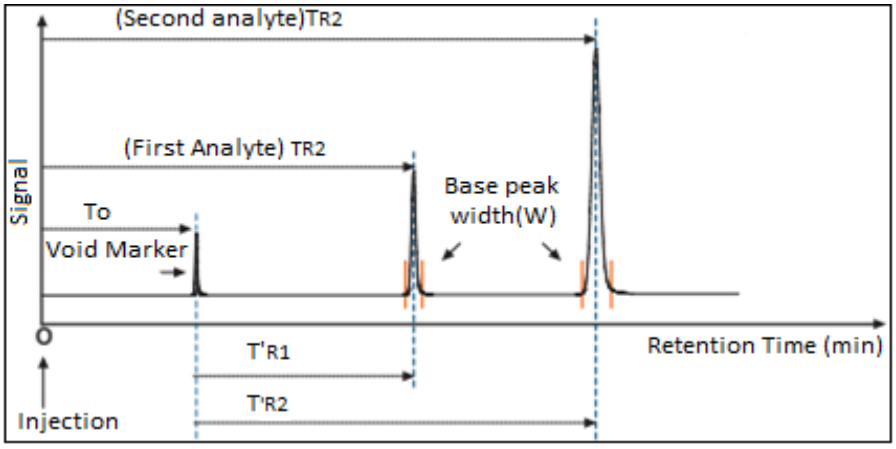


Figure 3.9. Void marker

Where:

T_o: Void volume marker retention (min).

T_{R1}: Retention time of the first analyte (min).

T_{R2}: Retention time of the second analyte (min).

$$T'_{R_1} = T_{R_1} - T_o$$

$$T'_{R_2} = T_{R_2} - T_o$$

The retention factor or capacity factor represented by k' is used to explain and calculation of analytes movement through the column as defined by the equation below:

$$k' = \frac{T_R - T_o}{T_o} \quad (3.2)$$

When the retention factor of some analytes is smaller than one, means that very fast elution and will be very difficult to calculate retention time accurately. High retention factor value (Greater than 20) means very difficult elution, the ideal range of retention factor is (1 to 5). The division of the retention factor between two peaks called selectivity factor which expressed by (α).

$$\alpha = \frac{k'_2}{k'_1} \quad (3.3)$$

Where:

α: Selectivity.

k'_1: Retention factor of analyte 1.

k'_2: Retention factor of analyte 2.

The selectivity indicates the separation power

Where:

$\alpha \geq 2$ Easy Separation.

α = 1.5-2 Possible separation.

α= 1.2 -1.5 Difficult separation.

α ≤ 2 Very difficult separation.

The efficiency of the column is usually expressed by theoretical plate number (N), the theoretical plate is a measurement of column effectiveness and efficiency by mathematical calculation, as shown in the next figure and equations.

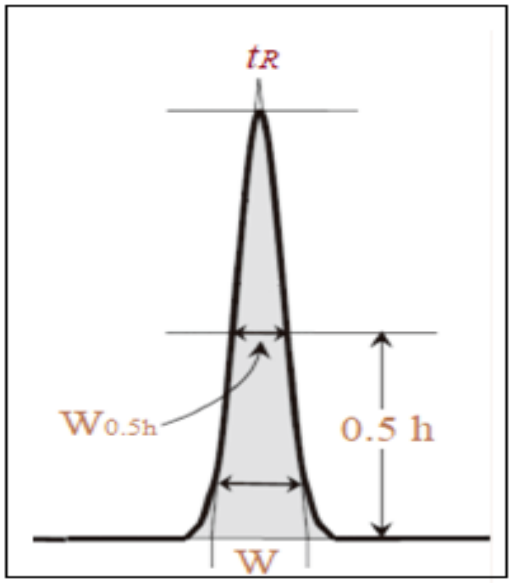


Figure 3.10. Theoretical plate parameter

According to the United States Pharmacopeia

$$N = 16 * \left[\frac{t_R}{W}\right]^2 \quad (3.4)$$

And according to the German Pharmacopeia

$$N = 5.54 * \left[\frac{t_R}{W_{0.5}}\right]^2 \quad (3.5)$$

Where:

N: Theoretical plats number.

t_R: Retention time of the analyte.

W: width at the base of the analyte.

$W_{0.5}$: width at half-height of the analyte.

The N value has a directly proportional relationship with a column efficiency when the column theoretical plate is high means it has high efficient separation with a sharper peak shape than a column with a lower theoretical plate, the value of N ranged between 2000-1200, and it depends on the mobile phase flow rate, viscosity, molecular weight of the analyte and temperature.

The relationship between those three important parameters (N, k' and α) can be explained as peak resolutions shown from the equation 3.6.

$$R_S = \frac{1}{4}\sqrt{N}\left[\frac{(\alpha-1)}{\alpha}\right]\left[\frac{K}{K+1}\right] \quad (3.6)$$

3.4. Peak Resolution and Diagnosis

The compounds with ionizable functional groups such as carboxylic, amino or pyridinyl group, are the most common ionizable groups in pharmaceutical compounds. as mentioned before; in reverse phase HPLC the polar mobile phase is used therefore controlling of mobile phase pH and composition is one of the most critical and main parameters that can be used to control and adjust HPLC chromatographic separation, retention and peak resolution, another important criterion that can affect resolution in RP-HPLC is selectivity and efficiency of the column.

For HPLC it is most important to reach the value of peak resolution of 1.5 in minimum time, it means that the optimum peak resolution. If the resolution value between two peaks is equal or greater than 1.5 will ensure that the sample constituents are separated very good and the concentration of each analyte can be calculated accurately based on peak area. The peak resolution calculation explained in the Figure 3.11.

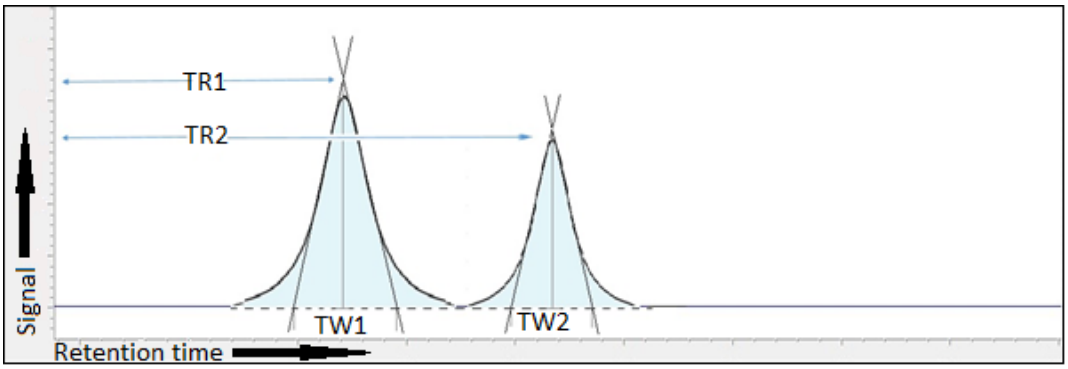


Figure 3.11. Determination of resolution between two peaks

Figure 3.11. represents the separation of two peaks, the resolution between them explained below:

$$Rs = \frac{t_{R_2} - t_{R_1}}{(t_{w_1} + t_{w_2}) * 0.5} \quad (3.7)$$

According to the above equation, resolution in HPLC expressed as the distance between two peaks represents by retention time divided by their width.
Where: Rs: Resolution

t_{R1} and t_{R2}: the retention times of peaks, t_{W1} and t_{W2}: the widths of the peaks taken at their bases.

As an example, the peak resolution calculated between sulfadiazine with sulfathiazole where: sulfathiazole peak exhibits a retention time of 11.168 min and a width of 0.75 min and the Sulfadiazine peak exhibited a retention time of 10.154 min with a width of 0.75 min:

$$Rs = \frac{11.168 - 10.154}{(0.75 + 0.75) * 0.5}$$

$$R_s \frac{1.736}{0.75} \quad \rightarrow \quad R_s = 1.352 \quad \approx R_S = 1.3$$

According to that value, both peaks separated sufficiently, Rs = 1.3 means more than 98% purity has been achieved at 98% of peak recovery, to achieve 100% recovery, the value of Rs should be 1.5 Figure 3.12. represents the separation results with different resolutions.

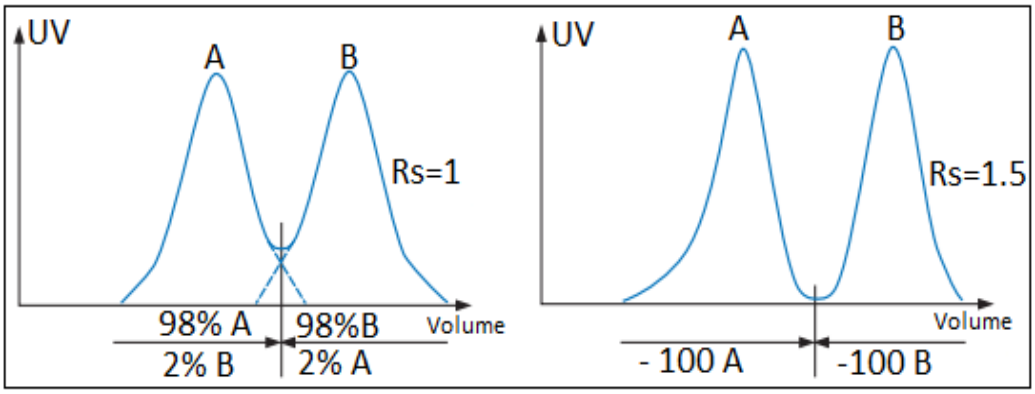


Figure 3.12. Separation results with different resolutions

4. RESULTS AND DISCUSIONS

Honey is contained of several classes of compounds that include carbohydrates, carboxylic acids, amino acids, proteins, minerals and antibiotics including sulfonamides. Sulfa drugs carry aromatic amino groups, which can react with the sugars forming bases. Therefore acidic hydrolysis conditions are needed to break-down such interactions, and in the case of this research, the optimum pH was found at 5. The main problem faced in this application was to reduce as much it can the matrix interferences, some researches recommend to use cation exchange for eliminating such matrix effects but this resource was not available at the moment of developing this study.

The way of controlling that matrix effect comes from the strong ionic bond which formed between sulfonamides and sulfonic acid moieties on this sorbent, therefore the strong wash by an organic solvent is necessary to wash out organic impurities and eluting sulfa drugs separately.

In order to present results, the most convenient way is to show validations results firstly and, then results related to the determination of sulfa drugs in honey done by this research.

4.1. Validation Results

The method was developed and applied to analysis of different honey samples, therefore the study of some important parameters for that technique is quite important such as the validations which can establish the documentary evidence showing that procedure or method carried out in testing with the desired level of compliance at all stages. In the pharmaceutical industry, it is very important parameters that make assured that the process will consistently produce the expected results. Validation results are related to recovery, precision, and accuracy.

To improve the application of sulfonamides analysis response at desired wavelengths, all standard solutions of sulfa drugs were injected into the system individually, the standards were prepared at 4 different concentrations 0.1-20 ppb, all standard solution was prepared in a methanol HPLC grade, and also methanol used as an organic modifier for preparation of mobile phase, the results of all injections have been seen in a good shape of peaks and steady detector baseline response. The quantification limit (LOQ) and detection limit (LOD) was calculated according to:

$$LOD = 3 * \frac{S.D.}{Slope} \quad (4.1)$$

$$LOQ = 10 * \frac{S.D.}{Slope} \quad (4.2)$$

Table 4.1. showed the validation results of this specific application in details, the calibration curve can be used for determination of nine different types of sulfa drugs with a scope from 0.1 ppb up to 20 ppb.

Table 4.1. Validation parameters of the method

Analytes	**STD 0.1ppb**	**STD 1ppb**	**STD 10 ppb**	**STD 20 ppb**	**Validation parameter**	**Validation result**	**Criteria of acceptance**	**Evaluation**
SDZ	0.135	0.987	10.188	22.631	Standard deviation (□)	1.51	≤ 5%	Conform
STZ	0.109	0.821	11.603	20.921	Variation coefficient (CV)	0.13	≤ 5%	Conform
SFP	0.395	1.098	12.388	20.45	Precision	98.49	≥95%	Conform
SMZ	0.152	2.297	13.448	18.755	Accuracy	99.87	≥95%	Conform
ATS	0.195	1.301	12.407	22.13	Recovery	84.80	≥80%	Conform
SMT	0.103	1.065	13.621	20.322	LOD	0.72 ppb		
PNS	0.302	3.003	10.090	19.857	LOQ	2.37 ppb		
SDX	0.089	0.958	9.917	22.986				
SXZ	0.206	1.936	10.020	21.369				
$\overline{X}$	**0.19**	**1.5**	**11.52**	**21.05**				**100%**

Additionally, taking about validation, indicated that precision and accuracy are totally in control, for example, the acceptance criteria for both are less than 5% but results are bellow of this specification, therefore, application have enough accuracy and precision to report detailed sulfa drugs.

The prepared honey samples applied to the method, and the result of peak separation can be seen from the following figures:

Sample one

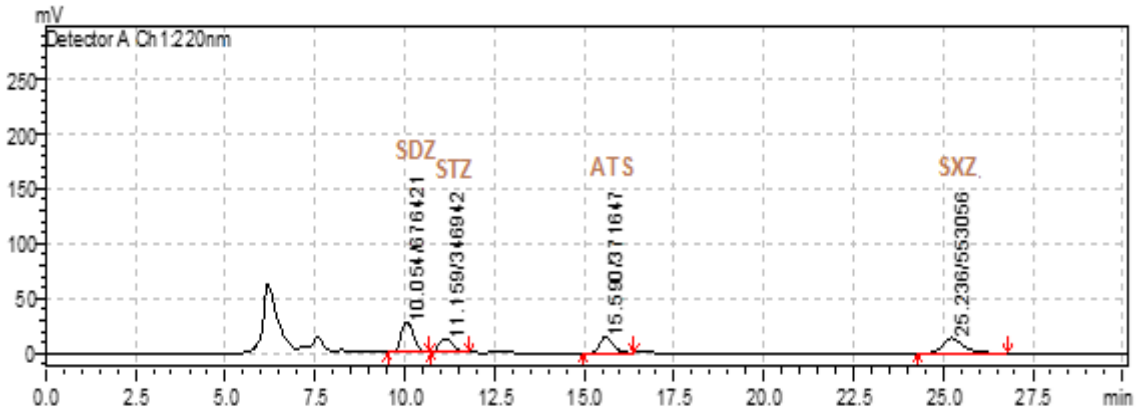


Figure 4.1. HPLC chromatogram of the sample number 1

In Figure 4.1. was able to calculate the concentration of four different SAs (SDZ, STZ, ATS, and SXZ) at obtained optimum condition in which polar mobile phase with pH 5, flow rate 0.5 mL/min at pressure of 63 bar, with 35^0C oven temperature and 220 nm analytical wavelength, the first peak couldn't recognize due to the lack of standards, thus, the first peak was unknown.

Sample two

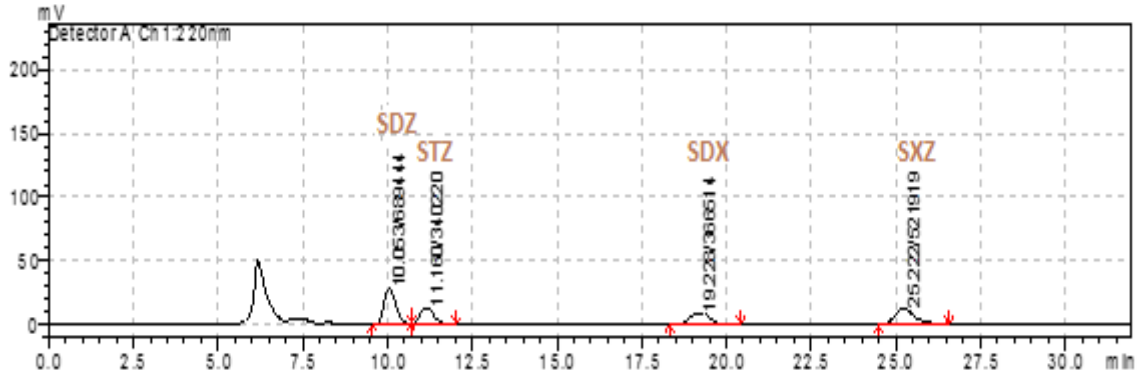


Figure 4.2. HPLC chromatogram of the sample number 2

Sample two contained four SAs (SDZ, STZ, SDX, and SXZ) at the same optimized system condition. Their separation peaks can be seen from the Figure 4.2.

Sample three

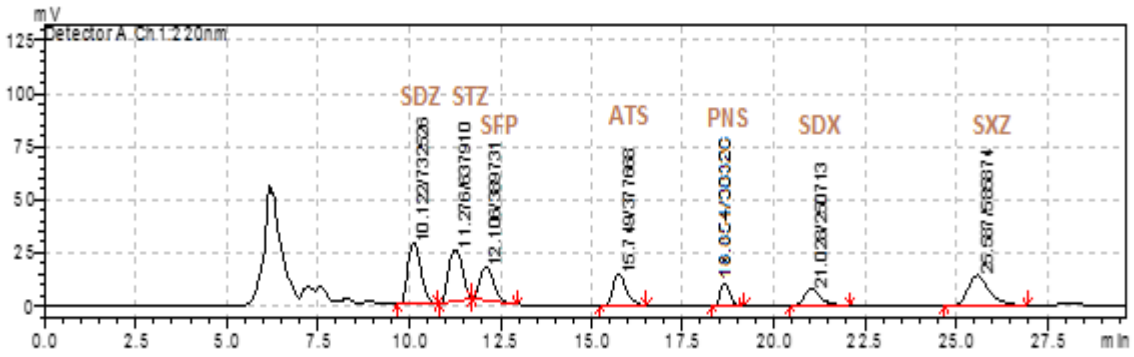


Figure 4.3. HPLC chromatogram of the sample number 3

Figure 4.3 represents the separation of seven sulfonamides in sample three (SDZ, STZ, SFP, ATS, PNS, SDX, and SXZ) also in the same system condition. The analysis chromatographic separation of sample number 4,5,6,7 can be seen from the next figures:

Sample four

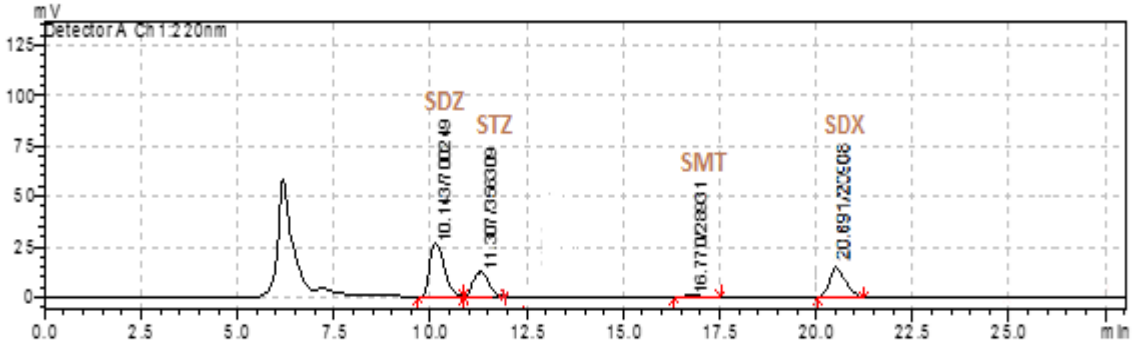


Figure 4.4. HPLC chromatogram of the sample number 4

In S4 four SAs peaks separated which contain (SDZ, STZ, SMT, and SDX) at same optimized condition of the HPLC system.

Sample five

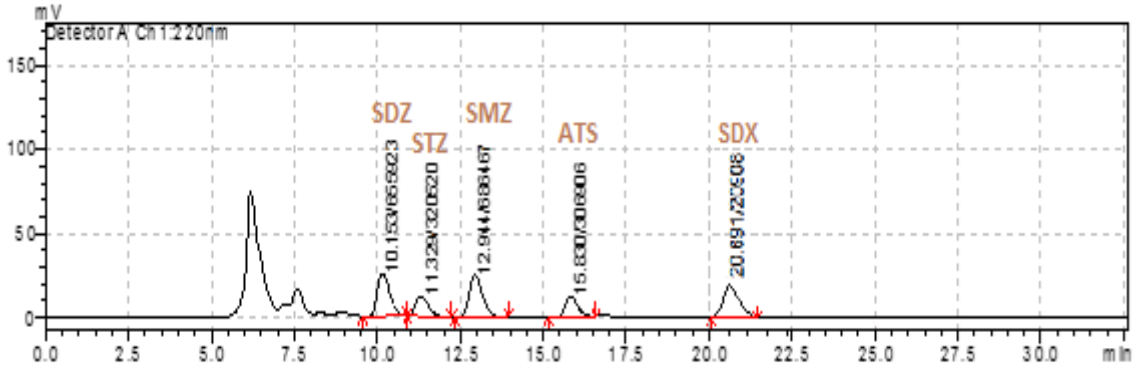


Figure 4.5. HPLC chromatogram of the sample number 5

When S5 at the same analytical condition was analyzed, five peaks of SAs recognized such as (SDZ, STZ, SMZ, ATS, and SDX).

Sample six

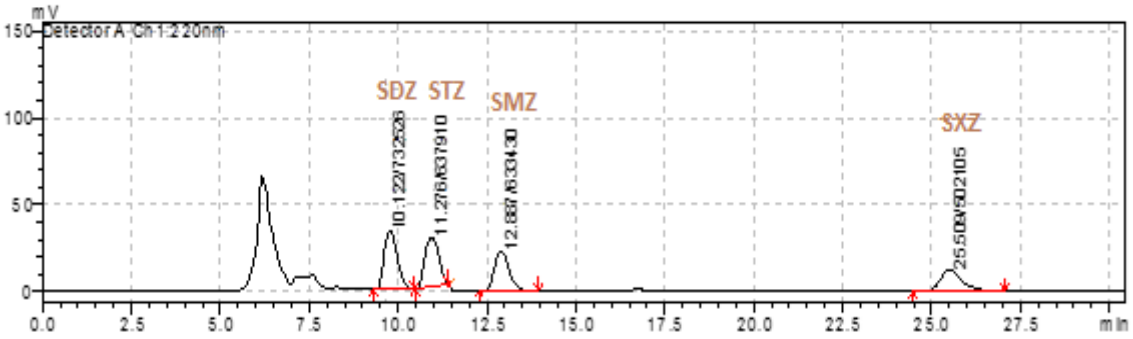


Figure 4.6. HPLC chromatogram of the sample number 6

S6 contained four SAs (SDZ, STZ, SMZ, and SXZ) but in S7 contained two SAs (STZ and SMT) and their separation can be seen from the Figure 4.7.

Sample seven

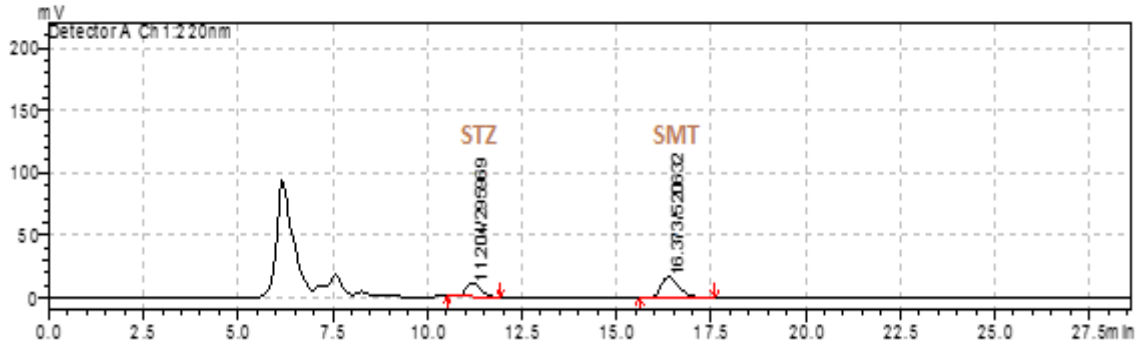


Figure 4.7. HPLC chromatogram of the sample number 7

The recovery factor even had excellent result perhaps may be improved using techniques that can mitigate side matrix effects, like cation exchanges or extraction cartridges. Recovery factor in this way can be increased from 84.8% up to 95%, this is convenient when pure sulfa drugs are intended to collect.

Related to LOD and LOQ can be said this method is adequate to determine sulfa drugs at routine levels, when concentrations of analytes are over 2.4 ppb can be reported with 95% of confidence and also this method will be useful for identify contamination of honey at lower concentrations like 0.72 ppb, lower concentration cannot be detected, will be only for presumptive tests. The result of all analyzed samples with their level of SAs can be seen from the tables below:

Table 4.2. Statistical analysis of SAs determination in sample number 1

First Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	0.547	0.33	2.0	7.3	90.9
STZ	0.475	0.2	2.3	6.8	85.35
SFP	…….	2.7	2.9	5.8	66.3
SMZ	…….	0.40	2.0	6.2	109
ATS	1.241	0.37	2.1	7.5	88.84
SMT	……	0.37	2.1	9.9	95.2
PNS	……	1.15	1.3	7.5	99.06
SDX	…..	1.20	1.21	4.5	99.17
SXZ	0.607	0.2	2.24	7.3	100.02

Table 4.3. Statistical analysis of SAs determination in sample number 2

Second Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	0.400	0.33	2.0	7.3	90.9
STZ	0.132	0.2	2.3	6.8	85.35
SFP	---	2.7	2.9	5.8	66.3
SMZ	---	0.40	2.0	6.2	109
ATS	---	0.37	2.1	7.5	88.84
SMT	---	0.37	2.1	9.9	95.2
PNS	---	1.15	1.3	7.5	99.06
SDX	1.244	1.20	1.21	4.5	99.17
SXZ	0.033	0.2	2.24	7.3	100.02

Table 4.4. Statistical analysis of SAs determination in sample number 3

Third Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	1.344	0.33	2.0	7.3	90.9
STZ	1.536	0.2	2.3	6.8	85.35
SFP	2.812	2.7	2.9	5.8	66.3
SMZ	---	0.40	2.0	6.2	109
ATS	0.442	0.37	2.1	7.5	88.84
SMT	---	0.37	2.1	9.9	95.2
PNS	1.222	1.15	1.3	7.5	99.06
SDX	1.490	1.20	1.21	4.5	99.17
SXZ	0.131	0.2	2.24	7.3	100.02

Table 4.5. Statistical analysis of SAs determination in sample number 4

Fourth Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	1.035	0.33	2.0	7.3	90.9
STZ	0.529	0.2	2.3	6.8	85.35
SFP	---	2.7	2.9	5.8	66.3
SMZ	---	0.40	2.0	6.2	109
ATS	---	0.37	2.1	7.5	88.84
SMT	0.456	0.37	2.1	9.9	95.2
PNS	---	1.15	1.3	7.5	99.06
SDX	2.753	1.20	1.21	4.5	99.17
SXZ	---	0.2	2.24	7.3	100.02

Table 4.6. Statistical analysis of SAs determination in sample number 5

Fifth Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	0.865	0.33	2.0	7.3	90.9
STZ	0.105	0.2	2.3	6.8	85.35
SFP	---	2.7	2.9	5.8	66.3
SMZ	1.064	0.40	2.0	6.2	109
ATS	1.239	0.37	2.1	7.5	88.84
SMT	---	0.37	2.1	9.9	95.2
PNS	---	1.15	1.3	7.5	99.06
SDX	1.999	1.20	1.21	4.5	99.17
SXZ	---	0.2	2.24	7.3	100.02

Table 4.7. Statistical analysis of SAs determination in sample number 6

Sixth Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	0.656	0.33	2.0	7.3	90.9
STZ	0.982	0.2	2.3	6.8	85.35
SFP	---	2.7	2.9	5.8	66.3
SMZ	0.448	0.40	2.0	6.2	109
ATS		0.37	2.1	7.5	88.84
SMT	---	0.37	2.1	9.9	95.2
PNS	---	1.15	1.3	7.5	99.06
SDX	---	1.20	1.21	4.5	99.17
SXZ	0.841	0.2	2.24	7.3	100.02

Table 4.8. Statistical analysis of SAs determination in sample number 7

Seventh Sample	Result µg/kg	LOD µg/kg	LOQ µg/kg	RSD%	RC%
SDZ	---	0.33	2.0	7.3	90.9
STZ	0.094	0.2	2.3	6.8	85.35
SFP	---	2.7	2.9	5.8	66.3
SMZ	---	0.40	2.0	6.2	109
ATS	---	0.37	2.1	7.5	88.84
SMT	1.119	0.37	2.1	9.9	95.2
PNS	---	1.15	1.3	7.5	99.06
SDX	---	1.20	1.21	4.5	99.17
SXZ	---	0.2	2.24	7.3	100.02

The concentration of sulfonamides calculated in each honey samples in unit of µg/kg, and according to those standards which established by many EU countries, therefore our samples are sufficient to use, in which the total sulfonamides residue in sample 1 (Kıractepe köyü) was equal to 2.8 µg/kg, in sample 2 (Karliova I) can be determined that the total sulfonamides residue was 1.8 µg/kg. The third sample (Hazarshah köyü solhan) after pretreatment, the calculation showed that the total sulfonamides were equal to 9 µg/kg. While total SAs residue in sample 4 (Karliova II) was 4.8 µg/kg, sample 5 (Togs yaylası genç) contained 5.3 µg/kg of SAs residue, the sixth sample (Tanrıverdı köyü genç) contained 3 µg/ kg of sulfonamides residue, the last sample which obtained from Bingol market was lighter and less viscose than others and contained 1.2 µg/kg residue of sulfonamides.

5. CONCLUSION

Determination of sulfa drugs using RP-HPLC by the configuration of this study is capable to determine 9 individual sulfa drugs (SDZ, STZ, SFP, SMZ, ATS, SMT, PNS, SDX and SXZ) from 0.1 ppb up to 20 ppb. Accuracy and precision of method using analytical and instrumentation setup were, precision: 98.5% and accuracy 99.87%, that's means RP-HPLC is a suitable technique to quantify this antibiotics family as well as the top of art. The separation technique optimized after a long investigation of buffer pH and flow rate of the mobile phase that prepared from the mixture of phosphate buffer solution with methanol: acetonitrile as an organic modifier. The peak resolution increased with increasing mobile phase buffer pH until pH: 5 which detected as an optimum pH to have the highest peak resolution at flow rate 0.5 mL/min. It is recommended to improve recovery of this method including a preparation sample step that can be able to isolate matrix effects like cation exchange cartridges. Due to the lack of sulfonamide standards, some peaks couldn't identified as appears at the minute of 5-7. Isocratic method is suitable for separation using reverse phase and longer column for improving separation and selectivity at high concentrations levels. Seven different honey samples were analyzed, which obtained from Bingol province. After pretreatment and acid hydrolysis to break-down sugar-sulfonamides bond and to reducing matrix effect. Results indicated that honey is in quality control specification, and according to the EU COMMISSION REGULATION (EU) No 37/2010 established 100 µg/kg as MRLs of SAs in honey, means that the samples are good and total sulfonamides residue are under the control when Sample 1 contains 2.8 µg/kg, Sample 2: 1.8 µg/kg, Sample 3: 9 µg/kg, Sample 4: 4.8µg/kg, Sample 5: 5.3 µg/kg, Sample 6: 3 µg/kg and Sample 7: 1.2 µg/kg.

REFERENCES

Abdul Qadir M, Ahmed M, & Iqbal M (2015) Synthesis, characterization, and antibacterial activities of novel sulfonamides derived through condensation of amino group containing drugs, amino acids, and their analogs. Biomed research international

Abuharfeil N, Al-Oran R, & Abo-Shehada M (1999) The effect of bee honey on the proliferative activity of human B-and T-lymphocytes and the activity of phagocytes. Food and Agricultural Immunology 11(2): 169-177

Actor P, Chow AW, Dutko F J, & McKinley MA (2005) Chemotherapeutics. Ullman's Encyclopedia of Industrial Chemistry

Adzitey F (2015) Antibiotic classes and antibiotic susceptibility of bacterial isolates from selected poultry; a mini review

Afroz S, Abbasi MA, Tanveer W, Khan KM, Ashraf M, Ahmad I & Ambreen N (2012) Synthesis, characterization and biological screening of sulfonamides derived form 2-phenylethylamine. Pakistan journal of pharmaceutical sciences 25(4)

Aguilar MI (2004) Reversed-phase high-performance liquid chromatography. In HPLC of Peptides and Proteins (pp. 9-22). Springer, Totowa, NJ

Aguilar MI, & Hearn MT (1996) [1] High-resolution reversed-phase high-performance liquid chromatography of peptides and proteins. In Methods in enzymology (Vol. 270, pp. 3-26). Academic Press

Ainsa J (2002) Stuart B. Levy: The antibiotic paradox. How the misuse of antibiotics destroys their curative powers, 2nd edn. International Microbiology 5(3): 155-156

Ajani OO (2009) Physical characterization of some honey samples from North-Central Nigeria. International Journal of Physical Sciences 4(9): 464-470

Ajibola A, Chamunorwa JP, & Erlwanger KH (2012) Nutraceutical values of natural honey and its contribution to human health and wealth. Nutrition & metabolism 9(1)

Ajibola A, Idowu GO, Amballi AA, Oyefuga OH, & Iquot IS (2007) Improvement of some haematological parameters in albino rats with pure natural honey. J Biol Sci Res 2(1): 67-69

Alimentarius C (2001) Revised codex standard for honey. Codex stan 12: 1982

Al-Jabri AA (2005) Honey, milk and antibiotics. African Journal of Biotechnology 4(13)

Alvarez-Suarez JM, Gasparrini M, Forbes-Hernández TY, Mazzoni L, & Giampieri F (2014) The composition and biological activity of honey: a focus on Manuka honey. Foods 3(3): 420-432

Anand N (1975) Sulfonamides and sulfones. In Mechanism of Action of Antimicrobial and Antitumor Agents (pp. 668-698). Springer, Berlin, Heidelberg

Asker FW, Mahamad ZZ, Eliwei AG, & Nief OA (2017) Synthesis and characterization of some sulfonamide dervatives. International Journal of Applied Chemistry 13(2): 169-177

Bateman KP, Locke SJ, & Volmer DA (1997) Characterization of isomeric sulfonamides using capillary zone electrophoresis coupled with nano-electrospray quasi-MS/MS/MS. Journal of mass spectrometry 32(3): 297-304

Bedendo GC, Jardim IC,SF, & Carasek E (2010) A simple hollow fiber renewal liquid membrane extraction method for analysis of sulfonamides in honey samples with determination by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1217(42): 6449-6454

Bernal J, Nozal MJ, Jiménez, JJ, Martín MT, & 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(43): 7275-7280

Bhattacharjee MK (2016) Chemistry of Antibiotics and Related Drugs. Springer

Bhattacharyya L, & Rohrer JS (Eds.) (2012) Applications of ion chromatography in the analysis of pharmaceutical and biological products. John Wiley & Sons

Bogdanov S, Jurendic T, Sieber R, & Gallmann P (2008) Honey for nutrition and health: a review. Journal of the American College of Nutrition 27(6): 677-689

Boison JO, Nachilobe P, Cassidy R, Keng L, Thacker PA, Peacock A & Bulmer WS (1996) Determination of trimethoprim and sulphadoxine residues in porcine tissues and plasma. Canadian journal of veterinary research 60(4): 281

Bonta V, MARGHITAS LA, Dezmirean D, & BOBIS O (2009) Determination of six sulfonamide residues in honey by HPLC with fluorescence detection. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies 66(1-2)

Boxall AB, Blackwell P, Cavallo R, Kay P, & Tolls J (2002) The sorption and transport of a sulphonamide antibiotic in soil systems. Toxicology letters 131(1-2): 19-28

Buba F, Gidado A, & Shugaba A (2013) Analysis of biochemical composition of honey samples from North-East Nigeria. Biochem Anal Biochem 2(3): 139

Calderón CB, & Sabundayo BP (2007) 2 Antimicrobial Classifications. Antimicrobial Susceptibility Testing Protocols, 7

Camargo-Ordoñez A, Moreno-Reyes C, Olazarán-Santibáñez F, Martínez-Hernández S, Bocanegra-García V, & Rivera G (2011) Efficient synthesis of sulfonamide derivatives on solid supports catalyzed using solvent-free and microwave-assisted methods. Química Nova 34(5): 787-791

Cantarelli MA, Pellerano RG, Marchevsky EJ, & Camiña JM (2008) Quality of honey from Argentina: Study of chemical compositttion and trace elements. The Journal of Argentine Chemical Society 96(1-2): 33-41

Chen L, Zhang X, Sun L, Xu Y, Zeng Q, Wang H, & Ding L (2009) Fast and selective extraction of sulfonamides from honey based on magnetic molecularly imprinted polymer. Journal of agricultural and food chemistry 57(21): 10073-10080

Christopher W (2003) Antibiotics Actions, Origins, Resistance.1st Edition, ASM Press, Washington, USA. pp. 3-18

Church J (1954 March) Honey as a source of the anti-stiffness factor. In Fed Proc Am Physiol Soc (Vol. 13, No. 1, p. 26)

Cooper RA, Halas E, & Molan PC (2002) The efficacy of honey in inhibiting strains of Pseudomonas aeruginosa from infected burns. The Journal of burn care & rehabilitation 23(6): 366-370

Da Presidência GP, & de Administração de Pessoal SR, AP (2007) Ato n. 134/SRAP. SERH. GDGCA. GP, de 30 de março de 2007. Ato n. 134/SRAP. SERH. GDGCA. GP, de 30 de março de 2007: (134)

Davis C, Arnold K (1974) Role of meningococcal purpura. Journal of Experimental Med.140: 159-171

Directive EC (2002) 657/EC of 12 August, 1990 on implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Communities, L221

Dorsey JG, & Cooper WT (1994) Retention mechanisms of bonded-phase liquid chromatography. Analytical chemistry 66(17): 857A-867A

Ebenezer IO, & Olugbenga MT (2010) Pollen characterisation of honey samples from north central Nigeria. Journal of Biological Sciences 10(1): 43-47

Fekete S, Fekete J, Molnár I, & Ganzler K (2009) Rapid high performance liquid chromatography method development with high prediction accuracy, using 5 cm long narrow bore columns packed with sub-2 μm particles and Design Space computer modeling. Journal of Chromatography A 1216(45): 7816-7823

Frank U, & Tacconelli E (2012) The Daschner Guide to In-Hospital Antibiotic Therapy: European Standards. Springer Science & Business Media

Granja RH, Niño AMM, Rabone F, & Salerno AG (2008) A reliable high-performance liquid chromatography with ultraviolet detection for the determination of sulfonamides in honey. Analytica chimica acta 613(1): 116-11

Gunes ME, Gunes N, & Cibik R (2009) Oxytetracycline and sulphonamide residues analysis of honey samples from Southern Marmara Region in Turkey. Bulgarian Journal of Agricultural Science 15(2): 163-167

Halász I, Endele R, & Asshauer J (1975) Ultimate limits in high-pressure liquid chromatography. Journal of Chromatography A 112: 37-60

Horvath CG, Preiss BA, & Lipsky SR (1967) Fast liquid chromatography. Investigation of operating parameters and the separation of nucleotides on pellicular ion exchangers. Analytical chemistry 39(12): 1422-1428

Hrassnigg N, & Crailsheim K (2005) Differences in drone and worker physiology in honeybees (Apis mellifera). Apidologie 36(2): 255-277

Kivrak I, KIVRAK Ş, & Harmandar M (2016) Development of a rapid method for the determination of antibiotic residues in honey using UPLC-ESI-MS/MS. Food Science and Technology (Campinas) 36(1): 90-96

Knowles S, Shapiro L, Shear NH (2001) "Should Celecoxib Be Contraindicated in Patients Who Are Allergic to Sulfonamides?".Drug Safety. 24 (4): 239–247

Korkmaz SD, Kuplulu O, Cil GI, & Akyuz E (2017) Detection of sulfonamide and tetracycline antibiotic residues in Turkish pine honey. International Journal of Food Properties 20(sup1): S50-S55

Krivohlavek A, Šmit Z, Baštinac M, Žuntar I, & Plavšc-Plavšic F (2005) The determination of sulfonamides in honey by high performance liquid chromatography–mass spectrometry method (LC/MS). Journal of separation science 28(13): 1434-1439

Kujawski MW, & Namieśnik J (2008) Challenges in preparing honey samples for chromatographic determination of contaminants and trace residues. TrAC Trends in Analytical Chemistry 27(9): 785-793

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(1-2): 74-80

Locuson CW, Gannett PM, Ayscue R, & Tracy TS (2007) Use of simple docking methods to screen a virtual library for heteroactivators of cytochrome P450 2C9. Journal of medicinal chemistry 50(6): 1158-1165

Lues JFR, Botha WC, & Smit EJ (1998) Ion-exchange HPLC analysis of a broad spectrum of organic acids from matured Cheddar cheese and assessment of extraction methods. Food research international 31(6-7): 441-447

Majors RE, & Hopper MJ (1974) Studies of siloxane phases bonded to silica gel for use in high performance liquid chromatography. Journal of Chromatographic Science 12(12): 767-778

Manyi-Loh CE, Clarke AM, & Ndip N (2011) An overview of honey: therapeutic properties and contribution in nutrition and human health. African Journal of Microbiology Research 5(8): 844-852

Martel AC, & Zeggane S (2003) HPLC determination of sulfathiazole in French honeys. Journal of liquid chromatography & related Technologies 26(6): 953-961

Maudens KE, & Lambert WE (2004) Quantitative analysis of 14 sulfonamides in honey after acidic hydrolysis by HPLC with post-column derivatisation and fluorescence detection. J. Chromatography A 1047: 85

Molan PC (1992). The antibacterial activity of honey: 1. the nature of the antibacterial activity. Bee world 73(1): 5-28

Molan PC (2002) Re-introducing honey in the management of wounds and ulcers-theory and practice

National Honey Board (2003) Honey Health and Therapeutic Qualities. National Honey Board, Longman 28

Nicolaou KC, & Rigol S (2017) A brief history of antibiotics and select advances in their synthesis. The Journal of Antibiotics

O'Shea R, & Moser HE (2008) Physicochemical properties of antibacterial compounds: implications for drug discovery. Journal of medicinal chemistry 51(10): 2871-2878

Postmes T, & Vandeputte J (1999) Recombinant growth factors or honey? Burns: journal of the International Society for Burn Injuries 25(7): 676

Pikkemaat MG, Yassin H, Fels-Klerkx HJ, & Berendsen BJA (2016) Antibiotic residues and resistance in the environment (No. 2016.009). RIKILT Wageningen UR

Posyniak ANDRZEJ, Sniegocki T, & Zmudzki J (2002) Solid phase extraction and liquid chromatography analysis of sulfonamide residues in honey. Bulletin-Veterinary Institute in Pulawy 46(1): 111-118

Posyniak A, Zmudzki J, Niedzielska J, Sniegocki T, & Grzebalska A (2003) Sulfonamide residues in honey. Control and development of analytical procedure. Apiacta 38: 249-256

Ramírez R, & Montenegro G (2004) Certification of the botanical origin of honey and corbicular pollen from VI Region of Chile (Litueche). Ciencia e Investigación Agraria 31(3): 197-211

Rehman H, Abdul Qadir M, Shad HA, Khan ZI (2017) One Pot Synthesis of Some Novel Sulfonamide Derivatives Containing -NH2 Group: Spectral Characterization and Biological Evaluation. Med Chem (Los Angeles) 7: 252-256.doi: 10.4172/2161-0444.1000465

Saeedi M, Goli F, Mahdavi M, Dehghan G, Faramarzi MA, Foroumadi A, & Shafiee A (2014) Synthesis and biological investigation of some novel sulfonamide and amide derivatives containing coumarin moieties. Iranian journal of pharmaceutical research: IJPR 13(3): 881

Saner G, Engindeniz S, Tolon B, & Cukur F (2004) The economic analysis of beekeeping enterprise in sustainable development: A case study of Turkey. Apiacta 38(4): 342-351

Sarmah AK, Meyer MT, & Boxall AB (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65(5): 725-759

Schwaiger I, & Schuch R (2000) Bound sulfathiazole residues in honey-need of a hydrolysis step for the analytical determination of total sulfathiazole content in honey. Deutsche Lebensmittel-Rundschau 96(3): 93-98

Schwartz DP, & Sherma J (1986) Practical screening procedure for sulfathiazole in honey. Journal-Association of Official Analytical Chemists 69(1): 72-74

Shakoor MD (2013) Sulfa allergy: Cross-reactivity versus multiple concurrent allergies

Shao B, Dong D, Wu Y, Hu J, Meng J, Tu X., & Xu S (2005) Simultaneous determination of 17 sulfonamide residues in porcine meat, kidney and liver by solid-phase extraction and liquid chromatography–tandem mass spectrometry. Analytica Chimica Acta 546(2): 174-181.

Sheridan R, Policastro B, Thomas S, & Rice D (2008) Analysis and occurrence of 14 sulfonamide antibacterials and chloramphenicol in honey by solid-phase extraction followed by LC/MS/MS analysis. Journal of agricultural and food chemistry 56(10): 3509-3516.181

Slatore CG, Tilles SA (2004) "Sulfonamide hypersensitivity". Immunology and Allergy Clinics of North America 24(3): 477–49

Snyder LR, Kirkland JJ, & Dolan JW (2011) Introduction to modern liquid chromatography. John Wiley & Sons

Sukul P, & Spiteller M (2006) Sulfonamides in the environment as veterinary drugs. In Reviews of environmental contamination and toxicology (pp. 67-101). Springer, New York, NY

Sundaram H, Vijayalakshmi N, & Srilatha K (2009) High Performance Liquid Chromatography and its role in identification of Mycobacteriae: An Overview. NTI Bulletin 45: 1-4

Supuran CT, Innocenti A, Mastrolorenzo A, & Scozzafava A (2004) Antiviral sulfonamide derivatives. Mini reviews in medicinal chemistry 4(2): 189-200

Szczêsna T, Rybak-Chmielewska H, Waœ E, & Pohorecka K (2009) Study on sulphonamide residues in honey. Journal of Apicultural Science 53(2)

Tačić A, Nikolić V, Nikolić L, & Savić I (2017) Antimicrobial sulfonamide drugs. Advanced Technologies 6(1): 58-71

Tashakkori P, Bozkurt SS, & Merdivan M (2014) Extraction and preconcentration of sulfonamides in honey samples by cetyltrimethylammonium bromide coated silica gel and their determination by HPLC 42(2): 175-182

Thammana M (2016) A Review on High Performance Liquid Chromatography (HPLC). Department of Pharmacy, Vignan Institute of Pharmaceutical Technology, Duvvada, India: 25

Thompson TS, & Noot DK (2005) Determination of sulfonamides in honey by liquid chromatography–tandem mass spectrometry. Analytica Chimica Acta 551(1-2): 168-176

Tilles SA (2001) Practical issues in the management of hypersensitivity reactions: sulfonamides. Southern medical journal 94(8): 817-817

Tölgyesi Á, Berky R, Békési K, Fekete S, Fekete J, & Sharma VK (2013) Analysis of sulfonamide residues in real honey samples using liquid chromatography with fluorescence and tandem mass spectrometry detection. Journal of Liquid Chromatography & Related Technologies 36(8): 1105-1125

Tolls J (2001) Sorption of veterinary pharmaceuticals in soils: a review. Environmental science & technology 35(17): 3397-3406

Tsai WH, Chuang HY, Chen HH, Wu YW, Cheng SH, & Huang TC (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(49): 7812-7815

Van Hoek AH, Mevius D, Guerra B, Mullany P, Roberts AP, & Aarts HJ (2011) Acquired antibiotic resistance genes: an overview. Frontiers in microbiology 2: 203

Verzegnassi L, Savoy-Perroud MC, & Stadler RH (2002) Application of liquid chromatography–electrospray ionization tandem mass spectrometry to the detection of 10 sulfonamides in honey. Journal of Chromatography A 977(1): 77-87

White Jr JW (1957) The composition of honey. Bee World 38(3): 57-66

Willson RB, & Crane E (1975) Uses and products of honey. Honey: A Comprehensive Survey. E. Crane, ed

Wutz K, Niessner R, & Seidel M (2011) Simultaneous determination of four different antibiotic residues in honey by chemiluminescence multianalyte chip immunoassays. Microchimica Acta 173(1-2): 1-9

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

PERSONAL BACKGROUND

My name is Alaa Omar Tahir and I was born on the 19th of May, 1990 in Erbil city, Kurdistan Region-Iraq, I have finished primary, secondary, and high school from Erbil. I have graduated from SALAHADDIN University/college of science/Environmental sciences department successfully in 2012. In addition, after four years, I hold the bachelor certification in that field. I started to work at high food Pack Company (2013) as an employee sales, and from (2014-2016) as a science teacher at Balla typical secondary school, In 12th of October of 2016, I started to study of my master degree in biochemistry at Bingöl University in Turkey, then I got master degree in 2018.

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