ADDIS ABABA UNIVERSTIY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF CHEMISTRY

ANALYTICAL CHEMISTRY STREAM

OPTIMIZED QUECHERS METHOD FOR THE DETERMINATION OF CIPROFLOXACIN IN WASTEWATER BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-DIODE ARRAY DETECTOR (HPLC-DAD)

By: Teshome Gezahegn Ijo

Advisors: Dr. Feleke Zewge

Prof. B. S. Chandravanshi

October 2018

Addis Ababa, Ethiopia

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A THESIS SUBMITTED TO THE OFFICE OF GRADUATE PROGRAMS OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

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Teshome Gezahegn Ijo

Approved By:	Signature	Date
Dr. Feleke Zewge Advisor	_____	_____
Prof. B.S. Chandravanshi Advisor	_____	_____
Dr. Merid Tessema Examiner	_____	_____
Dr. Negussie Negash Examiner	_____	_____

October, 2018

DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other University and that all the sources of materials used for this thesis has been duly acknowledged.

Name: Teshome Gezahegn Ijo Signature_____ Date_____

This thesis has been submitted for examination with our approval as university advisors.

Advisors	Signature	Date
1. Dr. Feleke Zewge	_____	_____
2. Prof. B.S. Chandravanshi	_____	_____

DEDICATION

This work is dedicated to my mom Boga Mideksa who is in heaven and my dad Gezahegn Ijo which is not married for 20 year until now and also to my beloved brothers Adugna, Ashenafi, Belete and my sister Hawi Gezahegn.

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LIST OF ABBREVIATIONS

APIs	Active pharmaceutical ingredients
AOAC	Association of the Official Analytical Chemists
ASTP	Addis Ababa Sewage Treatment Plant
BOD	Biological oxygen demand
BW	Bottled water
COD	Chemical oxygen demand
Cipro	Ciprofloxacin
CQ	Conventional QuEChERS
DAD	Diode array detector
dSPE	dispersive Solid-phase extraction
FA	Formic acid
FQs	Fluoroquinolones
GC/MS	Gas chromatography/Mass spectrometry
HPLC	High performance liquid chromatography
HWW	Hospital waste water
K_{ow}	Octanol-water partition coefficients
LOD	Limit of detection
MWWTP	Municipal Wastewater Treatment Plant
MS/MS	Tandem mass spectrometry
PPs	Pharmaceutical products
PPCP	Pharmaceutical and personal care products
PWW1	Pharmaceutical waste water 1
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RP	Reverse phase
rpm	revolution per minute
SPE	Solid-phase extraction
TW	Tap water
tR	Retention time
WWTP	Wastewater treatment plant
UHPLC	Ultra high performance liquid chromatography

USDA	United States Drug Administration

Abstract

An optimized QuEChERS-HPLC/DAD method was developed for the detection and determination of a pharmaceutically emerging pollutant ciprofloxacin (1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl quinolin-3-carboxylic acid) from river water and waste water sample from pharmaceutical, hospital and sewage treatment plant. The ciprofloxacin level was determined with high performance liquid chromatography diode array detector (HPLC-DAD). A sample of ciprofloxacin was used to develop an analytical method in the optimization experiment, which involved the selection of the most suitable salt, pH and different parameters for the extraction of ciprofloxacin from liquid sample. The usefulness of the method was verified based on the recovery. Different salt types were tested. The obtained results showed that the best recovery of 95% was obtained using $MgSO_4$ with the addition of acetonitrile in the sample extraction steps. The optimized method was applied for the determination of ciprofloxacin in five waste water samples. The method was validated using real sample: distilled water, tap water, bottled water and waste water samples spiked with ciprofloxacin at different concentration levels. The percentage recovery ranged from 86 to 120% with relative standard deviation (RSD) less than 6% for spiked level 0.02, 0.1, 1.25, 2.5 and 5 μg/mL. The calibration curve was linear in the range 0.005 to 5.0 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were 0.45 μg/L and 1.5 μg/L, respectively. The optimized method was adequate for the detection and determination of ciprofloxacin residue in waste water sample. The analytical parameters of the newly optimized method were compared with the literature methods and showed the optimized method is comparable with most of the literature methods.

1. Introduction

The use of pharmaceuticals, like many other anthropogenic compounds, are growing and their input to the aquatic environment is increasing making them of concern to the environmental, even if the use of pharmaceutical is increasing the occurrence of different anthropogenic toxic substances in water has been a subject of major scientific interest during the last decades. Particular emphasis has been paid to substances such as Heavy metals, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAH). However in recent years, there has been growing interest in the determination of emerging contaminants which is not currently covered by existing regulations on water quality or have not been previously studied like many other anthropogenic compounds of heavy metal and organic pollutant. These compounds may be candidates for future regulation, depending on research on their toxicity and potential effects in the environment and on human health. Their determination in water matrices has been widely performed in the framework of monitoring programs, and a number of policies and directives have been enforced in order to protect the environment from exposure to harmful chemicals. Emerging pollutants present as a new global water quality challenge with potentially serious threats to human health and ecosystems, their concentrations have been regulated in order to safeguard water quality (Vieno *et al.*, 2017).

The occurrences of emerging pollutant in the aquatic and terrestrial environment are of increasing concern. The fact that these substances are continually being introduced in to the environment through different pathways, Such as from discharge of domestic sewer system, disposal (flushing) of unused or expired drugs, medical waste, discharge from waste water treatment plant (WWTP), and leakage from septic system. The pharmaceutical industries enter the environment during various stages of the product lifecycle from their production and consumption to disposal of both untreated waste and final effluent of waste water treatment plant (WWTP). Simultaneously agricultural waste-storage facilities and via application of human and agricultural waste to land and also pharmaceuticals used in aquaculture are directly added to receiving waters, formulated as feed additives. Nowadays increasing concern is raised from a large number of "emerging pollutants", as shown in the Figure 1. The term emerging pollutants describes pollutants currently being released into the environment, without having been regulated

yet. The reasons that prevent their regulation are mostly the lack of information regarding their occurrence and toxicity, the lack of appropriate analytical methods for their determination, or both (Batt *et al*., 2006; Galan *et al*., 2008; and Nikolaou, 2013).

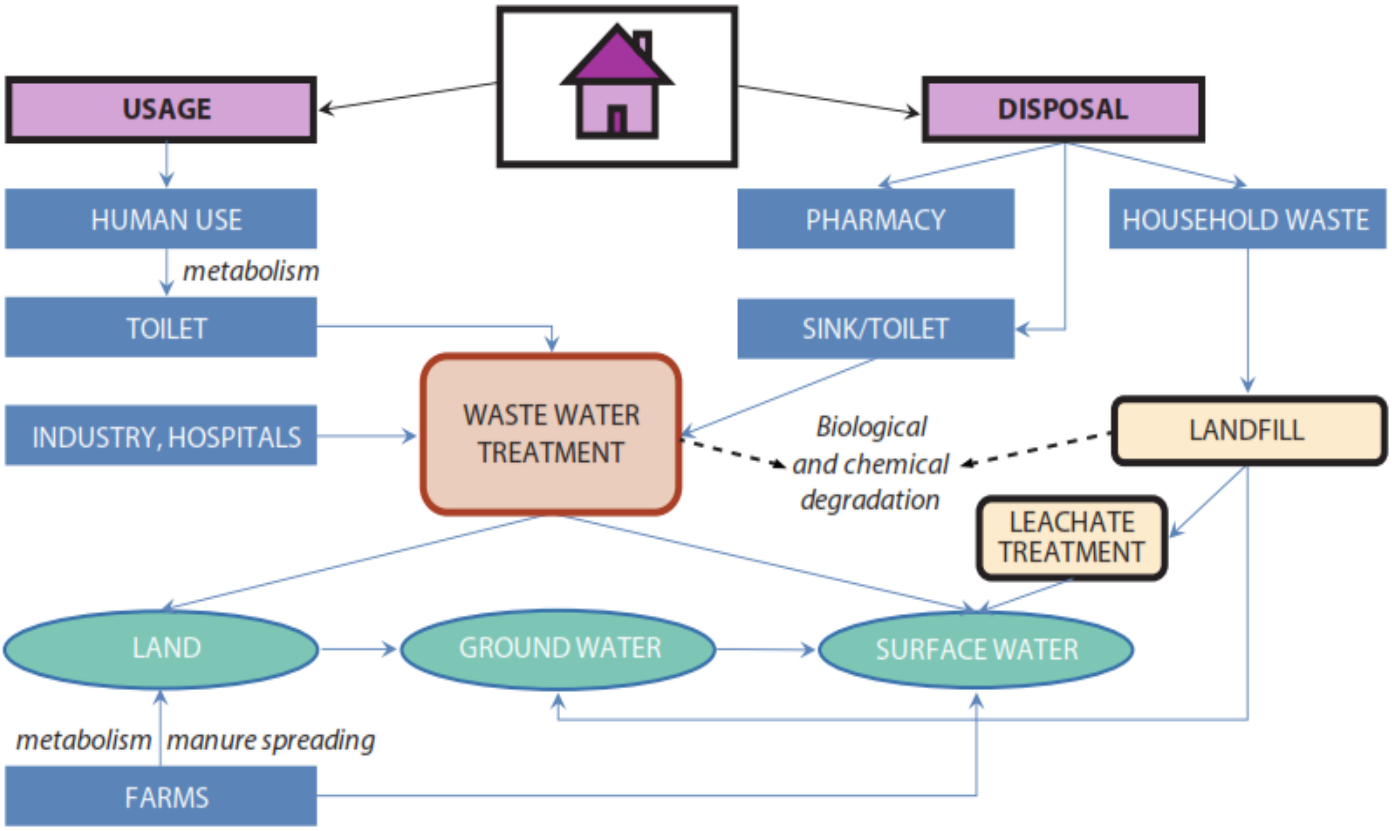


Figure 1. Sources of emerging pollutants in water (Nikolaou *et al.*, 2007)

Emerging pollutants have traditionally received little attention as potential environmental pollutants as stated above. However, in recent years there has been an increase in the number of publications reporting the level of contamination from this source of pollutants in the environment. Although their toxicity to aquatic and terrestrial organisms is relatively unknown and a number of reported investigations have shown that emerging pollutants pose a real threat to the environment. There has also been growing interest in the determination of emerging contaminants that are not currently covered by existing regulations on water quality or have not been previously studied. They may be candidates for future regulation, depending on research on their toxicity and potential effects in the environment and on human health. Several groups of compounds have been considered as emerging pollutants (Hernandez *et al.*, 2007). Among the

major categories of emerging pollutants are pharmaceuticals and illicit drugs, steroid estrogens (hormones and contraceptives) and personal care products. Pharmaceuticals can be classified into numerous therapeutic classes including antibiotics, anti-inflammatories, stimulants, etc. The list of individual compounds belonging to these categories keeps growing, an increasing number of such compounds and their oxidation/degradation products are being detected in trace levels in the environment, as the related techniques for their determination and quantification are being continuously improved (Nikolaou, 2013; McEneff, 2014).

One of the major emerging pollutants which are given a large attention by the scientists is pharmaceuticals and related compounds. This is due to both the number of pharmaceutical compounds licensed for human use and their annual consumption have increased dramatically in the world because they have an important role in the treatment and prevention of disease in both humans and animals. Because of their nature they can also have unintended effects on animals and micro-organisms in the environment. Although the effects of pharmaceuticals are investigated in safety and toxicology studies, the potential environmental impacts of their production and use are less understood. They have recently become a topic of research interest despite this there is no monitoring of the level of contamination caused by such pollutants in most waters to date (Lacey *et al.,* 2008 and Babic *et al*., 2010).

Meanwhile there are many gaps in the fields of analytical method development and optimization, sample preparation, ecotoxicological data and risk assessment, data on occurrence and fate of pharmaceutical compounds in the environment and in biota. Research has shown that these compounds are not effectively removed during conventional wastewater treatment; therefore they are released into the surface waters, as mixtures of parent compounds, their metabolites and transformation by-products. Some pharmaceuticals can persist in the environment and, either via the food chain or via drinking water, can make their way back to humans, while the properties and fate of metabolites and transformation products are still largely unknown (Fatta *et al*., 2007). Recent research has shown that some of these compounds are associated with adverse developmental effects in aquatic organisms at environmentally relevant concentrations and the effects of human exposure to low-dose mixtures of pharmaceuticals, as well as those of the exposure to low-dose pharmaceuticals mixed with other low-dose synthetic pollutants have not

yet been determined. Concern is raised about practices such as wastewater reuse for irrigation, discharge into the sea and other aquatic environments, groundwater replenishment, etc. and emphasize that such practices can raise concerns about the potential effects on non-target organisms, including plants, animals, and humans (Khetan and Collins, 2007).

There have been numerous reports in order to find a solution to the concerns described above. Researchers are working on the development and optimization of analytical methods, and their application for the determination and detection of pharmaceuticals in water and wastewater. During the last decade, a large number of related papers have been published, increasing the knowledge of the levels and fate of individual compounds in the aquatic and terrestrial environment (Farre *et al*., 2001; Renew and Huang, 2004; Hordern *et al*., 2007; Gracia-Lor *et al*., 2011; Khattab *et al*., 2014; Kihampa, 2014).

Nevertheless, there are many researches done in developed world which shown the existence of pharmaceuticals and related compounds in water and wastewater by further improving of the sample preparation, instrumentation and analytical techniques (Santos *et al*., 2005; Gros *et al*., 2013). But the work on such area in developing country is limited. The consumption and production of pharmaceuticals is increased dramatically, simultaneously the disposing of such compounds is also increasing without monitoring by regulatory bodies but the research side is not given as such emphasis in developing world. Therefore the aim of these research work is to develop an optimized sample preparation and analytical techniques which is a new optimized QuEChERS method for the detection and determination of pharmaceutical specifically ciprofloxacin from waste water by using high performance liquid chromatography-diode array detector.

1.1 Statement of the problem

Data about pharmaceutical production were collected from different sources, according to the information obtained, Ethiopia has licensed for production of pharmaceuticals product for at least 11 companies (i.e. industries) out of which at least 7 of the manufacturing industries are in Addis Ababa and 4 of them are around Oromia region. The contribution of manufacturing

factories to emission of pharmaceutical product to the environment is not properly monitored by EPA of Ethiopia, considering this product as emerging pollutants. Most of the pharmaceuticals industries do not have WWTP but some of them collect the waste water to reservoir and check some physico-chemical parameter (i.e. BOD, COD, temperature, etc.) before discharging to the environment. There is no comprehensive information about the potential disposal of pharmaceutical effluent in Ethiopia. Consequently pharmaceuticals are introduced in to the environment through different path way. Even though pharmaceuticals are important to cure the life of many peoples and animals, the disposal mechanism of many of this pharmaceuticals effluent result threat to aquatic biota and human. Hence in recent years they are emerged as environmental pollutants, so it is important to determine the amount of pharmaceutical emerging pollutants.

1.2 Objective

1.2.1 General objective

The main objective of this research is to determine emerging pollutant antibiotics (i.e. ciprofloxacin) residue from wastewater by using HPLC-DAD.

1.2.2 Specific objectives

- ✓ To develop optimum working procedure for sample preparation and extraction of selected pharmaceutical pollutant by using HPLC.
- ✓ To develop optimized method for determination of selected pharmaceutical pollutant in waste water.
- ✓ To estimate the amount of pharmaceutical ciprofloxacin in waste water sample.

1.3 Purpose of the study

The occurrence of pharmaceuticals substances in the environment is of global concern and also in Ethiopia; the extent of their impact on human health and biota is largely unknown. It is not much studied in developing countries therefore the output of this research work will help the country to maintain an evidence for the presence of such pollutant in our environment and also it will be used as a reference for those who are studying in such areas.

2. LITERATURE REVIEW

2.1 Emerging pharmaceutical pollutants in the environment

Pharmaceuticals were first introduced as environmental contaminants by Richardson and Bowron (1985), but their negative environmental effect was only later acknowledged in the late nineties when they were described as 'agents of subtle change'. As previously mentioned, pharmaceutical concern in the environment results from the rapid technological advances in recent years, which have enabled the improvement of analytical performance, in terms of resolution and sensitivity and the detection of these now-termed CECs (chemicals of emerging concern) (McEneff, 2014).

There are over 4000 pharmaceuticals are currently in use for medical and veterinary purposes, and in agriculture as part of growth promotion of livestock (Boxall *et al*., 2012). As forecasted, by 2020 the global use of medicines is estimated to reach 4.5 trillion doses worth a total of 1.4 trillion $ (IMS, 2015). The relatively recent awareness of pharmaceutical products (PPs) impact on environment is reflected in literature since the 1990s through the exponentially increasing number of studies concerning this emergent class of water pollutants. This rising interest is not only connected with the widespread and growing use of human and veterinary PPs consumption, but also with the analytical techniques improvement allowing detecting traces of substances (ng/L or less) in any type of water (Mompelat *et al*., 2008).

The great majority of studies on pharmaceutical products in water concerns their analysis, occurrence and fate in wastewater (WW) and wastewater treatment plant (WWTP), with an emphasis on processes efficiency with respect to their removal. As the majority of organic micro pollutants, the contamination origin is above all anthropogenic and continuously released in wastewater or directly in the environment (via human and animal excretion). Considering that they are metabolized to some extent after administration, unmetabolized active compounds are excreted in urine (generally 55–80%) and feces as a mixture of metabolites or conjugated compounds. Accordingly, they can enter water bodies through effluent of urban wastewater

treatment plants (UWWTP), hospital and pharmaceutical treatment plants due to their removal inefficiently by conventional systems (Mirzaei *et al*., 2017).

As some pesticides and other priority substances, PPs are ubiquity substances more often persistent and bioaccumulable in the environment mainly in surface water (SW) and sometimes in groundwater (GW). For drinking water (DW), studies are less numerous but the risk is higher (Mompelat *et al*., 2008).

Pharmaceuticals enter the environment during various stages of the product lifecycle from their production and consumption to disposal. Freshwater and marine pollution is of concern because pharmaceutical compounds and their bioactive metabolites are continually introduced to the aquatic environment via various pathways. Main pathways, in general, are discharges of untreated and treated wastewater. Emissions from manufacturing facilities are generally assumed to be very low compared to inputs occurring during the consumption phase.

However, there might be exceptions to this rule, and in other regions of the world emissions from production may be very high. The main pathway of human consumed pharmaceuticals to the marine environment is via direct discharges of effluents from municipal wastewater treatment plants (MWWTPs) in coastal areas as well as via rivers carrying effluents from inland MWWTPs. Other sources include land application of sewage and sludge whereby pharmaceuticals may leach into surface and ground waters. Pharmaceuticals also enter the environment via agriculture, aquaculture and veterinary practices (Vieno *et al.*, 2017).

Pharmaceutical compounds are widespread contaminants of the aquatic environment. Since traditionally they have not been viewed as environmental contaminants, the study of their presence in the environment is in some ways a new area of research which has taken in recent years. Our current knowledge indicates that residues of pharmaceuticals at trace quantities are widely spread in aquatic systems. Antibiotics constitute a large group of pharmaceuticals, which are widely administered in human and veterinary medicine. The extensive use of these antibiotics may result in their presence in the environment. Antibiotics are believed to be of greatest concern among all pharmaceuticals due to the potential risk of antibiotic resistance. Studies in the United

States of America and Europe have detected antibiotic resistant bacteria in drinking water supplies. According to previous studies and publications, one of the most prevalent groups of antibiotics found in the environment, and particularly in surface waters, is that of the widely used, highly potent fluoroquinolones (FQs).

Administered FQs are largely excreted as unchanged compounds in urine, and consequently discharged into hospital sewage or municipal wastewater. Despite lots of studies with positive detection of antibiotics and other pharmaceuticals in soils and environmental waters and despite of their negative effects on human health, there is no defined limit value for the occurrence of these pollutants in soils or natural waters. Therefore, more monitoring and surveillance studies are needed at local level to determine exactly how the antibiotics make their way into public waterways and to obtain a better understanding of the transport and environmental fate of antibiotics.

Insufficiently treated wastewaters from pharmaceutical production in one of the world's largest centers for bulk drug production located in India resulted in very high concentrations of ciprofloxacin (up to 6.5 μg/mL), cetirizine (up to 1.2 μg/mL), norfloxacin (up to 0.52 μg/mL), and in freshwater lakes (Fick *et al*., 2009). Three antibiotics from the group of fluoroquinolones, enrofloxacin, norfloxacin and ciprofloxacin, were determined in muscle tissues of rainbow trout from fish farms at maximum concentrations of 22, 60 and18.5 ng/g, respectively (Wagil *et al*., 2014).

Ciprofloxacin is fluoroquinolone derivative with broad spectrum antibacterial activity which is active against a wide range of bacteria. It is widely used in the treatment of urinary tract infections, lower respiratory tract infections, bacterial diarrhea, skin and soft tissue infections, bone and joint infections, gonorrhea, and in surgical prophylaxis. In most of the cases, it would appear that for treatment of above said infections. Physicians prescribe ciprofloxacin as a first choice of drug (Oishi *et al*., 2011).

Ciprofloxacin have an amino group (piperazinyl) in the heterocyclic ring and have two dissociation constants. The reported pK_a values of ciprofloxacin were 5.76 (strong acidic) and 8.68 (strong basic). They are in their zwitterion form in neutral condition and in cationic form in acidic condition. The chemical structures are shown in Figure 2 (Turiel *et al*., 2003; Gros *et al*., 2013; Khattab, 2014).

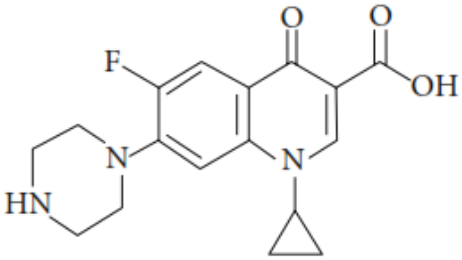


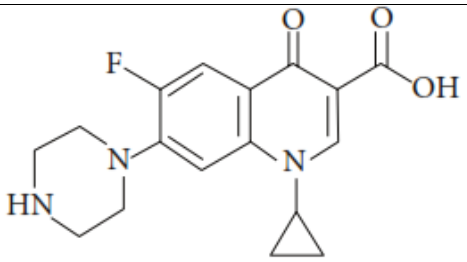
Figure 2. The chemical structure of Ciprofloxacin (MF: $C_{17}H_{18}FN_3O_3$)

It is very important to have information on the physical and chemical properties of an analyte (e.g., log K_{ow}, pK_a) because that may help determine whether a compound is likely to concentrate in some specific conditions. Log K_{ow} is an indicator of the lipophilicity of the compound, high log K_{ow} is typical for hydrophobic compounds, whereas a low K_{ow} signifies a compound soluble in water. Most pharmaceuticals have acidic and/or basic functionalities; their ionization rate depends on acidic dissociation constants (i.e. pK_a values) and is controlled by solution pH (e.g., pK_{a1} and pK_{a2} values for certain flouroquinoles (i.e. ciprofloxacin) are in the ranges 5.7 to 6.3 for carboxylic group and 7.6 to 8.3 for protonated amino group, respectively) (Pavlovic *et al*., 2007).

These different chemical species (cationic, neutral, or anionic) often have vastly different properties. Unfortunately, the pK_a values of many relevant pharmaceuticals are either not known accurately or not available at all. But, with this knowledge, one can choose the best option for analyzing pharmaceuticals (pK_a value enables adjustment of the pH value of sample solution; log K_{ow} shows affinity of pharmaceuticals towards water (polar/non-polar compounds)).

Sample preparation must also be tailored to the final analysis, considering the instrumentation to be used and the degree of accuracy required, whether quantitative or qualitative.

Table 1. Physicochemical properties of ciprofloxacin.

Compound	Structure	Log K_{ow}	Water solubility (g/L)	pK_a
Ciprofloxacine		0.28	30 (20 ^{o}C)	pK_{a1} = 5.90 pK_{a2} = 8.89

Different methods are available for the determination of the selected FQs, CIP in environmental aqueous samples. Water samples are analyzed after solid-phase extraction, by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Turiel *et al*., 2003, Kihampa, 2014), fluorescence detection (Nakata *et al.*, 2005), mass spectrometry detection (Jay and Ching-Hua, 2004), tandem mass spectrometric detection (Gomez *et al.*, 2006), ultra high-performance liquid chromatography (UHPLC) with fluorescence and mass spectrometry detection (Marcela *et al*., 2010). The aim of this work was to develop simple, accurate, and sensitive HPLC methods for the simultaneous determination of the selected FQs, CIP, in wastewater (Khattab, 2014).

2.2 Analytical methods for determination of pharmaceutical pollutants

Different analytical methods have been developed and applied, for the analysis of different groups of pharmaceuticals, according to the properties of the compounds, therefore chemical analysis of pharmaceutical residues in the environment has been closely tied to the analytical capabilities, especially in the mass spectrometry field. The development of more sensitive and versatile instruments has enabled their detection and quantitation at low environmental levels and has also provided the required tools to ensure a precise identification (Gros *et al*., 2006; Botitsi *et al*., 2007; Da silva *et al*., 2011).

2.3 Sample preparation for extraction of pharmaceutical pollutants

Sample preparation is the crucial step in environmental analysis. It is highly influenced by the physical and chemical properties of analytes studied and by matrices. However the sample extraction for the analysis of all the target analyte from the sample is done by using different appropriate sample extraction methods. The most commonly employed technique for liquid samples is liquid-liquid extraction (LLE) and SPE (Pavlovic *et al*., 2007).

In the most instances, the preconcentration and clean-up has been performed by SPE. SPE has been the mostly preferred technique, which replaced classical liquid-liquid extraction (LLE) and become the most common sample preparation technique in the environmental area. SPE offers some advantages over LLE such as improved selectivity, specificity and reproducibility, lower organic solvent consumption, shorter sample preparation time, and easier operation and the possibility of automation (Seifrtova *et al*., 2009).

However, the SPE method is expensive because of the cost of the cartridge and the cleaning of the homogenizer probe creates a risk of pollution and cross-reactivity, and therefore the SPE method is somewhat tedious and cannot be performed in many centers (Kostopoulou and Nikolaou, 2008), but the QuEChERS method is a simple, cheap, fast and reliable method (Cerqueira *et al*., 2014).

This method is widely used to determine pesticides and was first introduced in by Anastassiades and Lehotay in 2003 (Anastassiades *et al*., 2003). In most published paper the sample preparation for emerging pollutant were done by using solid phase extraction (SPE), a combination of two SPE materials can also be performed, either in series or classifying the analytes into two or more groups, according to their physico-chemical properties (Benito-Pena *et al*., 2006; Khattab *et al*., 2014), the commonly used cartilage for the solid phase extraction are Oasis HLB, Oasis MCX or C18 and also Lichrolut ENV+, and StrataX is also used (Diaz-Cruz *et al*., 2008; Seifrtova *et al*., 2009; Madikizela *et al*., 2014; Olaitan *et al*., 2014; Mirzaei *et al*., 2017).

2.3.1 Solid-phase extraction (SPE)

Solid phase extraction (SPE) is a powerful method for sample preparation and is used by most chromatographers today and it is a form of (step-wise) chromatography designed to extract, partition, and /or adsorb one or more components from a liquid phase (sample) onto a stationary phase (adsorbent or resin). An adsorbed substance can be removed from the adsorbent by step-wise increase of elution strength of the eluent (step gradient technique). SPE extends a chromatographic system's lifetime, improves qualitative and quantitative analysis, and the demand placed on an analytical instrument is considerably lessened (Petrovic *et al*., 2006; Olaitan *et al*., 2014).

After extraction, elution of the analytes from the cartridges is performed with pure organic solvents, mostly methanol or acetonitrile. Analyte losses can occur in some cases, for example in the case of antibiotics, in particular tetracycline, sulphonamides and polypeptide antibiotics, Na_2EDTA is added as chelating agent before extraction, to avoid formation of complexes of the antibiotics with metal ions. Particular attention is required also for the container material, with PTFE being preferable than glass, because the compounds tend to bind to the glass, resulting in significant losses (Diaz-Cruz and Barcelo, 2006; Pavlovi *et al*., 2013).

In SPE a purification step is usually included in the analytical methods, in order to minimize the matrix effects, especially in complex environmental samples. For the purification (cleanup) of the extract, the extracts are usually diluted with an appropriate volume of MilliQ water, until the organic solvent content is below 10%. SPE is applied, using mostly the same cartridges and conditions as the analysis of pharmaceuticals in water samples (Jacobsen *et al*., 2004; Baranowska and Kowalski, 2011).

2.3.2 Liquid-liquid extraction (LLE)

The liquid-liquid extraction technique has been used for a long time. It is based on the principle of organic compounds partitioning between the aqueous sample and an immiscible organic solvent. Its use in environmental analysis is limited due to the use of large volumes of solvents and analyte loss due to multistage operation (JJemba, 2008; Kihampa, 2014). It is prone to contamination, poses disposal challenge for toxic solvents, tedious and this method is relatively

laborious and time-consuming (Payan *et al.*, 2010). The QuEChERS approach is similar to LLE in terms of simplicity and is also similar to SPE with high selective performance (Anastassiades *et al*., 2003; Payá *et al*., 2007).

2.3.3 QuEChERS method

QuEChERS is the Multiresidue method of Choice. QuEChERS (pronounced "catchers"), an acronym for Quick, Easy, Cheap, Effective, Rugged and Safe, it covers a variety of sample preparation and clean-up techniques for the analysis of multiple pesticide residues. Originally designed for the analysis of fruits, vegetables, and a wide range of agricultural products, since its development and publication by scientists at the USDA in 2003 (Anastassiades *et al*., 2003).

QuEChERS has gained significant popularity as the method of choice. It combines several sample preparation steps and extends the range of analytes recovered over older, tedious extraction methods. A driving force in the growth of QuEChERS is the emerging need to determine trace amounts of analytes in a high throughput environment matrix include: (1) animal products meat, fish, kidney, chicken, milk, honey, (2) cereals and grain products, (3) food products like wines, juices, fruit and vegetables (Wong *et al*., 2010), etc. The expansion of the QuEChERS methodology indicates not only its power for sample extraction and clean-up but also addresses the concern about detecting a vast array of pesticides, herbicides, fungicides, antibiotics, and other compounds throughout the entire food supply. QuEChERS in its basic form involves three steps: (i) liquid micro-extraction, (ii) solid-phase clean-up and (iii) LC-UV, DAD, FL or LC-MS or MS/MS or GC/MS analysis.

QuEChERS continues to undergo modifications for improved sample preparation in a broad array of analytes in a vast array of matrices. Due to the large number of QuEChERS methods now published, QuEChERS is considered an "approach" rather than a "method." QuEChERS has now become a generic technique with many modifications; each variation is designed to accomplish one thing quick sample extraction and clean-up (Tsartsali and Samanidou, 2015). Modifications to the original method have been introduced to increase sample throughput while reducing costs, minimize degradation of susceptible compounds (e.g. base and acid labile analyte) and expand the range of matrices amenable by this approach.

2.3.4 The three primary QuEChERS methods

1) *Original QuEChERS method* (Anastassiades and Lehotay, 2003). Sodium chloride is used to reduce polar interferences. It provides the cleanest extraction because it uses fewer reagents. It does not use acetic acid which may be problematic in GC/MS analysis and it uses dispersive clean-up procedures.

2) *AOAC 2007.01* employs 1% acetic acid in acetonitrile and sodium acetate buffer to protect base sensitive analytes from degradation. A USDA study has demonstrated that this method provides superior recovery for pH sensitive compounds when compared to the other two QuEChERS methods (kachhawaha *et al*., 2017). The approach uses acetic acid in the extraction step. The acetic acid can overload the PSA sorbent used in the clean-up step making it ineffective and possibly causing GC resolution issues.

3) *EN 15662* the European method includes sodium chloride to limit polar interferences and several buffering reagents to preserve base sensitive analytes. Sodium hydroxide is used in the citrus step should be avoided as it can add impurities to the extract as well as damage the sorbent used in the clean-up step.

The QuEChERS method has several advantages over most traditional extraction methods: high recoveries (>85%) are achieved; very accurate results are obtained; high sample throughput is possible; solvent usage and waste generation is very low; no chlorinated solvents are used; a single person can perform the method without much training or technical skill; very little bench space is needed, thus the method can be carried out in a small mobile laboratory if needed; ACN is added by a dispenser to an unbreakable vessel that is immediately sealed, thus solvent exposure to the worker is minimal; inexpensive reagents are used; and few equipment are needed (Anastassiades *et al*., 2003; Lehotay, 2011).

2.3.5 QuEChERS extraction reagents and their function

QuEChERS significantly improves laboratory efficiency and throughput. A batch of 20 extracts can be prepared in less than 60 min by a single analyst. This procedure requires only a few milliliters of solvent and is capable of generating recoveries of 90-110% with RSD < 5% for a wide range of GC and LC amenable compounds (Zhang and Cheng, 2017). Solvent: Solvent extraction techniques are designed to remove as much analyte from matrix as possible and it is

necessary to select appropriate solvent to minimize co-extracting compounds. Magnesium sulfate, anhydrous: Facilitates solvent partitioning and improves recovery of polar analytes. Acetic acid: is used to adjust pH. Acetonitrile: organic solvent providing the best characteristics for extracting the broadest range of analyte with the least number of co-extractable. Amenable for both LC and GC analysis. Buffers: prevents degradation of pH sensitive analytes by maintaining optimal pH. Sodium Chloride: reduces the amount of polar interferences. The schematic flow chart for main steps of originally developed QuEChERS is shown in Figure 3.

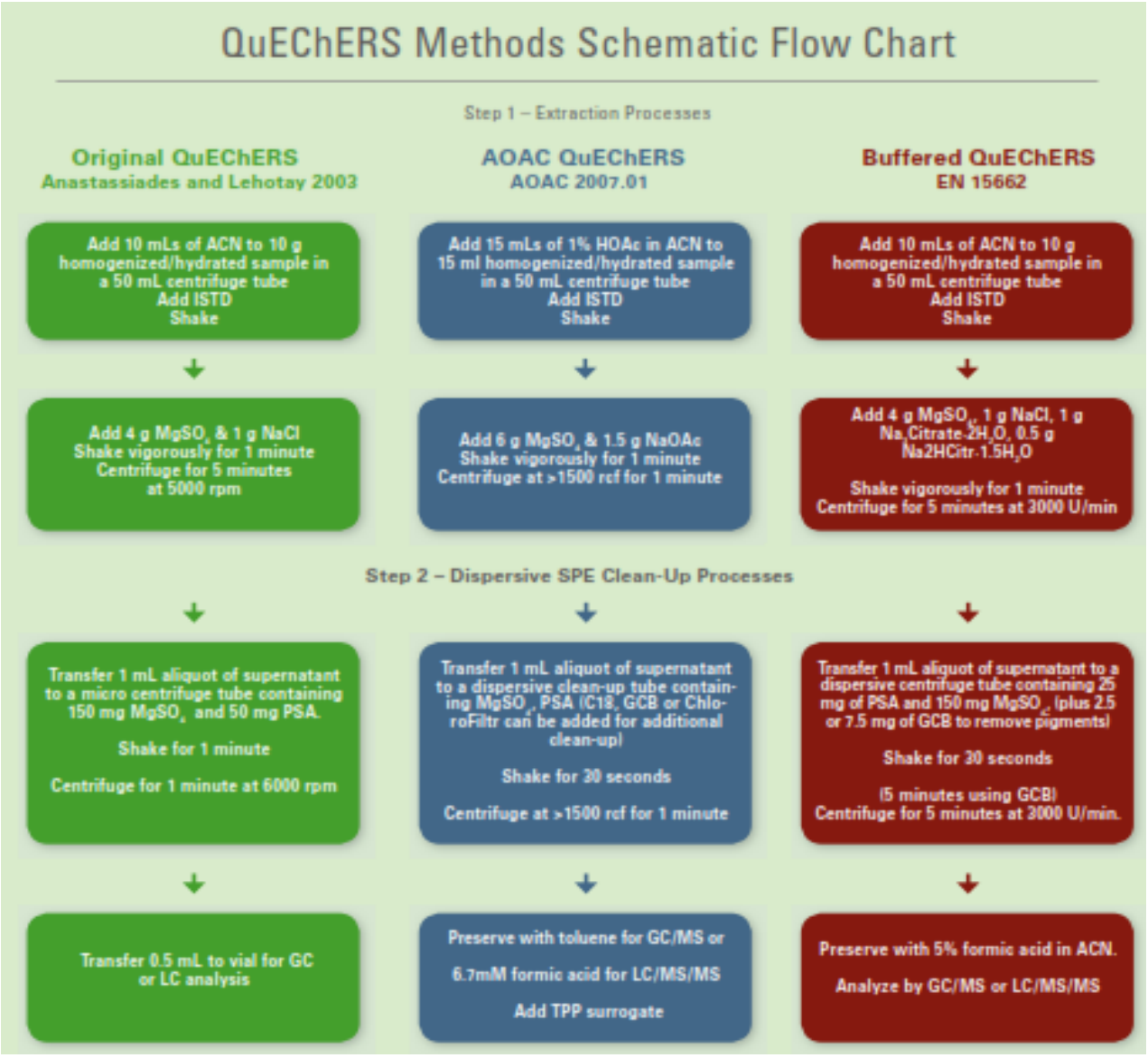


Figure 3. QuEChERS methods schematic flow chart

2.3 Analytical method for separation of pharmaceuticals

There are several analytical methods dealing with the analysis of pharmaceuticals at different concentration ranges mostly chromatography is used. Chromatography is the most commonly used separation technique in environmental sample analysis and most current analytical methods for the separation and detection of pharmaceuticals are based on gas chromatography-mass spectrometry (MS) or to a larger extent on coupling liquid (LC) chromatography with ultraviolet (UV), diode array detector (DAD), fluorescence (FL) detectors (Turiel *et al*., 2003; Alawi and Alahmad, 2012; Kihampa, 2014) or mass-spectrometry (MS) and tandem mass spectrometry (MS/MS) (Daniele *et al*., 2016).

The analytical methodology used for the analysis of pharmaceuticals in water samples is usually based on liquid chromatography (LC) with mass spectrometry (MS) (LC-MS/MS) (Carmona *et al*., 2014). LC-MS/MS has become the preferable analysis method, due to its versatility, specificity and selectivity, gradually replacing GC-MS and LC-MS (Mirzaei *et al*., 2017).

However GC-MS can still be successfully applied in some cases, especially for non-polar and volatile pharmaceutical compounds. The disadvantage of GC-MS application is that it requires a time-consuming derivatization step, during which there are risks of analyte losses (Kolpin *et al*., 2002; Weigel *et al*., 2004).

Another technique used for separation is reversed-phase LC, with UV, fluorescence and DAD detectors. The mobile phases mostly used are acetonitrile, methanol, or mixtures. The sensitivity of the method can be improved with use of mobile phase modifiers, buffers and acids, usually ammonium acetate, tri-n-butyl amine (TrBA), formic acid and acetic acid (Gros *et al*., 2006b; Pavlovi *et al*., 2013).

3 EXPERIMENTAL

3.1 Materials and methods

3.1.1 Reagents and chemicals

Pharmaceuticals standards of ciprofloxacin were high purity grade (>99%) purchased from Sigma-Aldrich, acetonitrile (Sigma-Aldrich, ECHROMASOLV for HPLC, UV and GC >99%), methanol (Sigma-Aldrich, HPLC grade, >99.9%), acetic acid (Fisher chemical UK, 99%), ammonium solution (Fisher chemical UK, 35%), ethyl acetate (Fine Chem industries, Mumbai >99%), formic acid (Sigma-Aldrich, 85%) and ethanol (Fisher scientific, UK 99.9%) were purchased. The different salt used, were magnesium sulfate (Fine Chem industries, Mumbai, 70%), sodium chloride (Sigma-Aldrich, 99.5%), ammonium acetate (BDH chemical Ltd, England 96%), and sodium acetate anhydrous (BDH chemical Ltd, England 96%) distilled and deionized water was used throughout the study.

3.1.2 Standard and working solutions of ciprofloxacin

Stock standard solution of ciprofloxacin (20 mg/L) was prepared by dissolving 5 mg of ciprofloxacin in 250 mL distilled water using volumetric flask. The standard can be stored at -20 °C (1 months) if it is not required to use. For calibration working solutions in the range of 0.005-5.0 μg/mL were prepared. Working solutions were prepared from 10 μg/mL of intermediate stock solution by taking 12.5 mL of aliquot from 20 mg/L in 25 mL. A series of solutions for calibration were prepared of concentrations 5, 2.5, 1.25, 0.6, 0.02, 0.01, and 0.005 μg/mL by serial dilutions of the intermediate standard solutions. Working series of solutions were prepared every day during analysis.

3.1.3 Apparatus and instruments

The apparatus and instrument used were beakers (50 mL, 250 mL), measuring cylinders (10 mL, 25 mL, 100 mL and 500 mL), micro pipettes (10-100 μL, 1-5 mL, Huawei), volumetric flasks (25 mL, 50 mL, 100 mL and 250 mL), spatula, micro filter, Whatman filter paper (11 cm diameter, China) and vortex, centrifuge (Model 800-1, Japan), oven (Griffin), mortar and pestle and analytical balance (Adam equipment), sonicator, pH meter were used after calibration with

buffer solutions of pH 4, 7 and 12. Analysis was performed using high performance liquid chromatograph (HPLC) (Agilent 1260 Infinity, Germany) coupled to a diode array detector (DAD).

3.2 Extraction method: Conventional and optimized QuEChERS

In order to extract aqueous sample for PPs (pharmaceutical products) different types of extraction methods where monitored. Because these compounds are usually present at very low concentrations in the environment, a sample preconcentration step is necessary before the analysis. Typically, SPE is the preferred technique for the extraction of PPCPs from water (Moreno-Bondi *et al*., 2009; Pavlovi *et al*., 2013).

Although SPE is widely recognized as an established and robust sample preparation technique for aqueous matrixes, it has certain limitations. The SPE method is expensive because of the cost of the cartridge (i.e. three times the international cost locally) and the cleaning of the homogenizer probe creates a risk of pollution and cross-reactivity, and therefore the SPE method is somewhat tedious and requires time-consuming procedure, and there is a risk of degradation of compounds due to long analysis times. These limitations of the commonly used SPE-based methods motivated us to look for another sample preparation technique with a shorter analysis time and lower cost. The QuEChERS method was initially developed for residue analysis of pesticides in fruits and vegetables. It was first introduced in 2003(Anastassiades *et al*., 2003) but, over the years, it has been applied with modifications to extract various organic compounds from varied matrixes. There are studies that have used the QuEChERS method for the analysis of PPCPs in sediments (Carmona *et al*., 2014), drinking water treatment plant sludge (Cerqueira *et al*., 2014), and limited report in aqueous sample in swage and surface water (Kachhawaha *et al*., 2017).

The goal of the present study was to develop an efficient analytical method for the extraction of antibiotics specifically ciprofloxacin in different aqueous matrixes using QuEChERS as an approach for sample preparation, followed by chromatographic separation and detection by HPLC-DAD. The target compound is ciprofloxacin but the method can serve practically for at

least 19 PPCP by changing some parameter Kachhawaha *et al*., (2017). The compounds were selected on the basis of significance, prescription rate and effectiveness and also ciprofloxacin exhibit different physical and chemical properties such as varied pK_a and K_{ow} values, etc.

A conventional AOAC 2007.01 QuEChERS approach for sample preparation was initially used to determine selected PPs in different aqueous matrixes and was assessed for its extraction efficiency. In the CQ method, the recommended proportion of magnesium sulfate and sodium acetate was used for extraction (Anastassiades *et al*., 2003). But our standpoint is a modified QuEChERS method. However, in the modified QuEChERS method magnesium sulfate (4 g) and 0.2 g ammonium acetate salt and 10 mL of 1% acetic acid (HoAC) in acetonitrile (ACN) as extracting solvent for 10 mL of waste water sample were used (Kachhawaha *et al*., 2017).

In the optimization of QuEChERS for determination of ciprofloxacin from waste water different parameters were tested respectively. These are extraction solvent (i.e. ACN, ACN in 1% HOAc, ethyl acetate (EA) and Diethyl ether (DE)), amount of solvent used (i.e. 5 mL and 10 mL), QuEChERS-salt combination and amount (i.e. 2.0 g, 3.0 g, 4.0 g and 5.0 g) used for extraction (i.e. $MgSO_4$ with NaCl; original QuEChERS by Anastassiades and Lehotay 2003 and EN 15662 , $MgSO_4$ with NaOAc; AOAC 2007, $MgSO_4$ with NH_4OAc; (Kachhawaha *et al*., 2017)). Further optimization were carried out by changing pH of the sample which are pH values of 3, 4.5, 5.5, 6.5 and 8.0 which are obtained by adding NH_4OH and HOAc for pH adjustments and finally extraction time (i.e. 2, 4, 6 and 8 min) and rotation speed (i.e. 2000, 2500, 3000, 3500 and 4000 rpm) for extraction were experimented and optimized. Consequently after this optimization were done 3 to 5 mL of aliquot of the extract from the original layer were pipetted to sample container or holder for concentration and it was evaporated to low volume under a gentle stream of nitrogen to near dryness and reconstituted in a known volume of distilled water prior to HPLC-DAD analysis.

3.2.1 Newly optimized QuEChERS method

According to the optimized method the water and waste water sample (10 mL) were extracted by using 5 mL of ACN in the presence of $MgSO_4$ salt but prior to addition of 5 mL of ACN to 50 mL falcon tube, the pH of the sample were adjusted after calibrating the pH meter by three point

(i.e. 4,7 and 12) before adjusting the pH of the sample were around 5.5 to 8.0, so by adding NH_4OH drop by drop the optimum pH of the sample were obtained at pH 3 then 5 mL of ACN and 4 g of magnesium sulfate were added simultaneously then the mixture was instantly and rigorously vortex mixed or shacked manually by using hand for 6 min, followed by centrifugation at 4000 rpm for 5 min. After extraction 3 mL of aliquot were extracted out to sample container or vial and it was evaporated to low volume under a gentle stream of nitrogen to near dryness and reconstitute in 300 μL of distilled water prior to HPLC-DAD analysis. Newly optimized QuEChERS method is summarized in Figure 4.

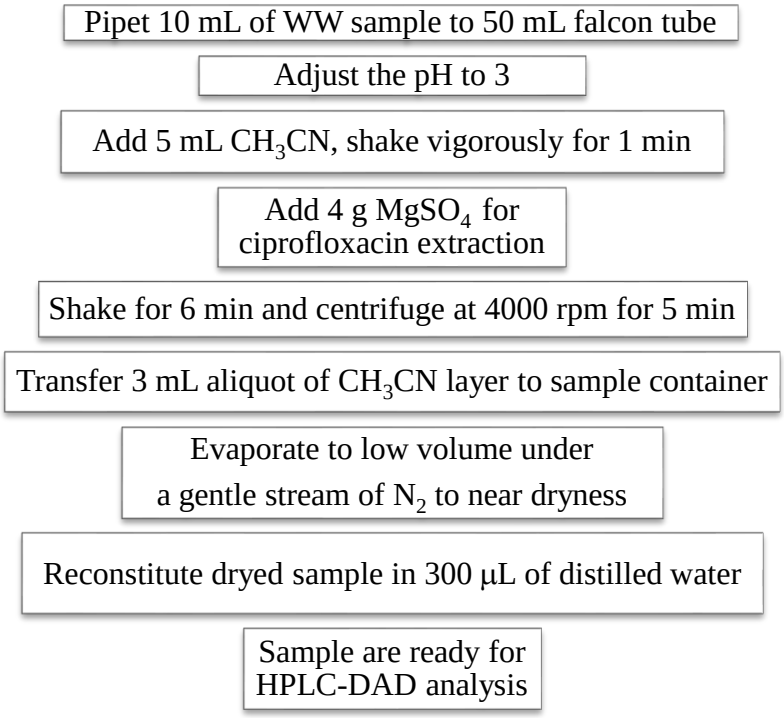


Figure 4. Optimized QuEChERS method

3.3 Analysis by HPLC-DAD

For analysis different chromatographic conditions were tested to obtain optimal peak shape and resolution. These are mobile phase selection by referring to different published paper that is between ACN with 2% HOAc and ACN with 0.1% formic acid. Composition of the injection solvent or mobile phase was ACN: 0.1% formic acid 14:86, 20:80, 30:70, 40:60, 50:50%, flow rate (i.e. 0.8, 0.9, 1.0, 1.1 and 1.2 mL/min), optimization of column temperature; 25, 30 and 40

^{o}C and injection volume; (i.e. 5, 10 and 20 μL) and the DAD wavelength were set at 277 nm for ciprofloxacin determination. All the analysis were done in triplicate from three separate extracts, and each of the triplicate samples were also analyzed by HPLC.

3.4 Method validation

Validation studies were carried out in order to demonstrate the performance of the optimized method. The calibration curves and linear ranges of the detector response were evaluated by analyzing standard solutions of ciprofloxacin and matrix-matched standards within the range of 0.005–5 μg/mL. The standard criteria was used for the determination of LOD (S/N of 3) and LOQ (S/N of 10). The precision was determined based on repeatability in recoveries (reported as RSD) and evaluated by carrying out the extraction and analysis of the fortified samples at different spiking concentration (0.02, 0.1, 1.25, 2.5 and 5 μg/mL) using replicates each. The accuracy of the analytical method was evaluated through recovery studies for four kinds of water samples (distilled, tap water, and potable water and real sample). Indeed the presence of target analyte was established by its retention times.

3.5 Application to environmental sample

The applicability of the optimized method was evaluated by analyzing a real sample that was collected from two pharmaceutical effluent (PWW1 and 2), one from outlet of ditch and the other from WWTP, ASTP, hospital and river simultaneously potable water, tap water and distilled water were also examined. After sampling all the samples were transported through ice box were immediately transported to the laboratory and filtered promptly and extracted. During analysis all sample were analyzed along with blank and matrix-spike control sample. All samples were analyzed in triplicate from three separate extracts, and each of the triplicate samples was also analyzed by HPLC-DAD.

4. RESULTS AND DISCUSSION

4.1 HPLC-DAD optimization

The best chromatography separation was achieved on a RP-C18 Column at 40 ^{o}C column temperature (25 cm, 4.6 mm, 5 μm) which was purchased from Agilent Technology and the system was operated isocratically at flow rate of 0.9 mL/min. For routine analysis a mobile phase consisting of a mixture of 30:70 v/v ACN:0.1%FA was used and the sample was injected through a fixed sample loop having a volume of the 20 μL. The UV-DAD detector was set at 277 nm. All samples were analyzed in triplicate from three separate extracts, and each of the triplicate samples was analyzed by HPLC-DAD for detection and determination of ciprofloxacin.

4.2 Optimized QuEChERS method

The method chosen for the extraction of ciprofloxacin from waste water was based on the approach of QuEChERS extraction. It involves a salting out liquid-liquid extraction step of the QuEChERS extraction which has been optimized in order to obtain the best responses by HPLC-DAD for the target analyte, ciprofloxacin. By preparing known amount of the target compound (ciprofloxacin) the method were optimized. The signals corresponding to each experiment done in triplicate were normalized for ciprofloxacin with respect to the maximum response and compared in order to choose the best response among the different tested conditions.

4.2.1 Type and volume of solvents

In order to choose appropriate solvent several organic solvents (i.e. ACN, 1% HOAc in ACN, ethyl acetate, diethyl ether) were tried for extraction of ciprofloxacin from aqueous sample. The results obtained showed that ACN gives good extraction efficiency than the other solvent (Figure 5). Therefore, ACN was chosen as it is generally used for the extraction of pharmaceuticals from aqueous samples. Another advantage of acetonitrile is its compatibility with the chromatographic applications, acetonitrile is recommended for QuEChERS, because upon the addition of salts, it is separated more easily from water than other organic solvent. The polarity of acetonitrile is

higher than that of acetone and ethyl acetate. High polarity solvents used in salting out systems have been investigated for extraction or concentration of analytes.

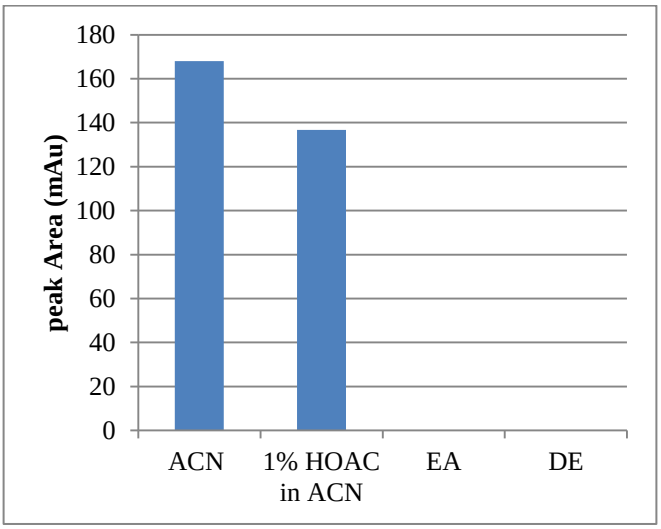


Figure 5. Responses of ciprofloxacin (replicate measurements, n = 3) obtained for the extraction of 10 mL of water using different solvent. (i.e. EA - ethyl acetate, DE - diethyl ether)

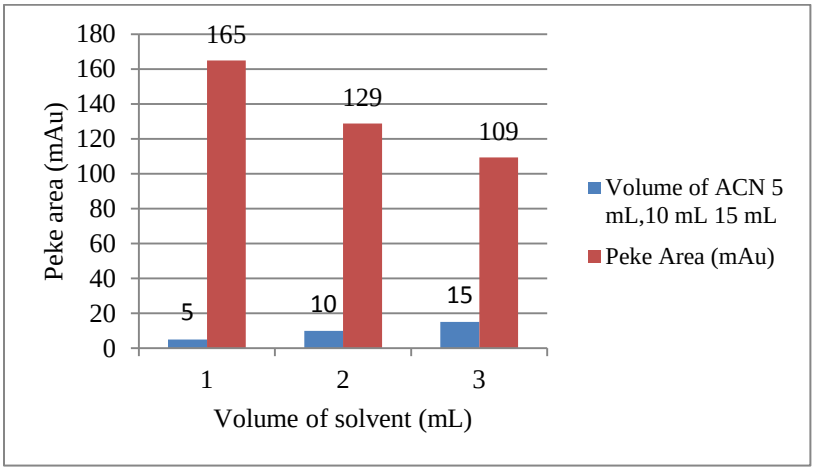


Figure 6. Normalized responses of ciprofloxacin (replicate measurements, n = 3) obtained for the extraction of aqueous sample using different ratios of acetonitrile/water (QuEChERS, no dSPE clean-up).

In order to obtain good extraction the best ratio of solvent and water should be chosen. Thus three different volume ratios were tested that is 5 mL/10 mL and 10 mL/10 mL and 15 mL/10 mL ACN/sample. The results indicated that best results were obtained with 5 mL of ACN solvent in 10 mL of aqueous sample (Figure 6). ACN has shown to offer excellent performance for the extraction of the broadest range of compounds showing the least interference (Correia-Sá *et al.*, 2012).

4.2.2 Choice of salt combination

In order to enhance the partition of the analyte different salt composition and buffer were recommended by QuEChERS method, such as $MgSO_4$ and NaCl by original QuEChERS (Anastassiades *et al*., 2003), $MgSO_4$ and NaOAc by AOAC QuEChERS 2007.01, and $MgSO_4$ and NH_4OAc modified QuEChERS (Kachhawaha *et al*., 2017), and $MgSO_4$ alone as new trial, so the extraction were performed in all case with 10 mL of aqueous sample and 5 mL ACN using 4 g for $MgSO_4$ and 0.2 g for the rest of salt. The result is presented in Figure 7.

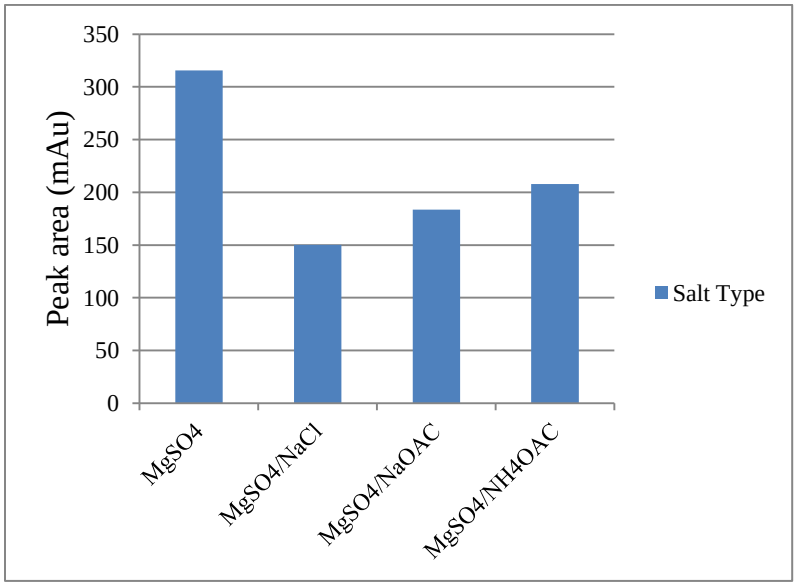


Figure 7. Responses of ciprofloxacin obtained using different salt combination of $MgSO_4$, $MgSO_4$/NaCl, $MgSO_4$/NaOAc and $MgSO_4$/NH_4OAc (replicate measurements, n = 3) for the extraction of 10 mL of aqueous sample spiked at 2.5 µg/mL (QuEChERS, 10 mL water, 5 mL ACN).

Among the tested salts anhydrous magnesium sulfate allows optimum salting-out of ACN and yielding maximum overall recoveries especially of polar analytes. However, $MgSO_4$ contributes to remaining parts of water in the acetonitrile layer and to co-extracting some undesirable polar compounds from sample matrix like sugars. To bind a vast majority of the water fraction, the amount of magnesium sulfate added has to exceed the saturation concentration (Rejczak and Tuzimski, 2015). In this case, an addition of $MgSO_4$ improved antibiotic extraction efficiency, particularly for ciprofloxacin, so the signal intensities were mostly higher with the $MgSO_4$ salt. Therefore this salt was used for all subsequent extraction.

4.2.3 Amount of salt

After examining the salt combination, in order to enhance partition of aqueous sample, $MgSO_4$ were selected to increase the efficiency of extraction. Optimum salt amount were also assessed that is 2.0, 3.0, 4.0, and 5.0 g of $MgSO_4$ from this four trials, 4 g was obtained as the optimum amount for extraction of ciprofloxacin from aqueous sample as shown in Figure 8. Thus 4 g of $MgSO_4$ was used for all subsequent extractions.

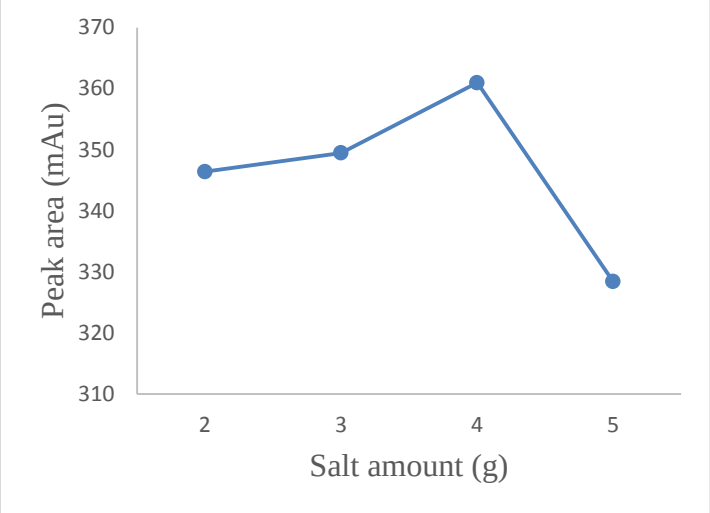


Figure 8. Responses of ciprofloxacin obtained using different amount of $MgSO_4$ (replicate measurements, n = 3) for the extraction of 10 mL of aqueous sample spiked at 2.5 µg/mL (QuEChERS, 10 mL water, 5 mL ACN).

4.2.4 Optimization of pH

After all the parameters were optimized the pH of the aqueous sample were optimized by using different pH 3.0, 4.5, 5.5, 6.5 and 8.0, without optimization the pH of the sample was around 5.5 to 8.0. The results obtained (Figure 9) indicated that the response of ciprofloxacin decreased when the pH of the water solution was increasing, so pH 3 was obtained as the optimum pH. When a water sample is acidified to a pH that is less than the pK_a value of target compounds the acids are non-ionized which leads to their adsorption through the reversed-phase interactions (Lee *et al*., 2005). Therefore, in subsequent experiments, the pH in all the sample solutions was adjusted to 3.

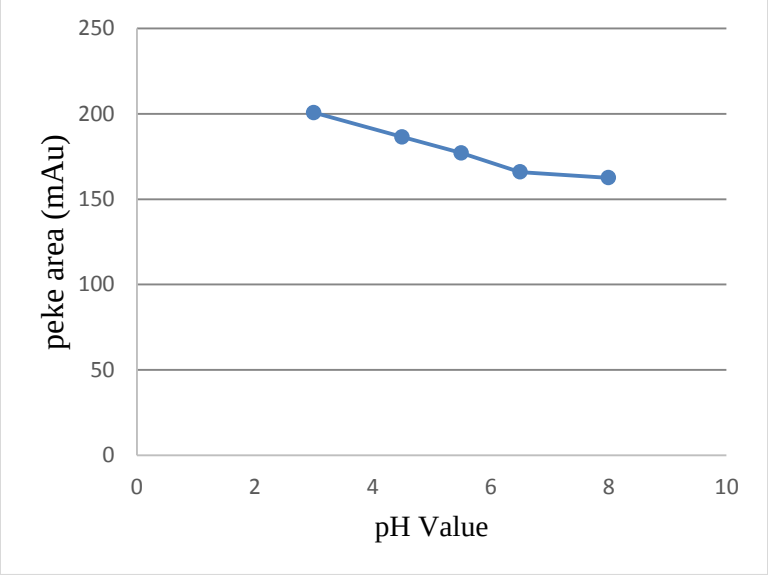


Figure 9. Responses of ciprofloxacin (replicate measurements, n = 3) obtained for the extraction of aqueous sample using different pH values

Depending on the target analytes, pH adjustment could be necessary to enhance extraction retention. However, it is necessary to take into account that the use of polar solvents and acidic conditions also helps to extract the organic substances, leading to an increase in the matrix effects (Vazquez-Roig *et al*., 2013). The pH of water sample solution significantly influences the chemical form of analytes in samples (Figure 10). Antibiotics from group of FQs, ciprofloxacin have acidic and/or basic functional groups and therefore their ionization is controlled by solution pH.

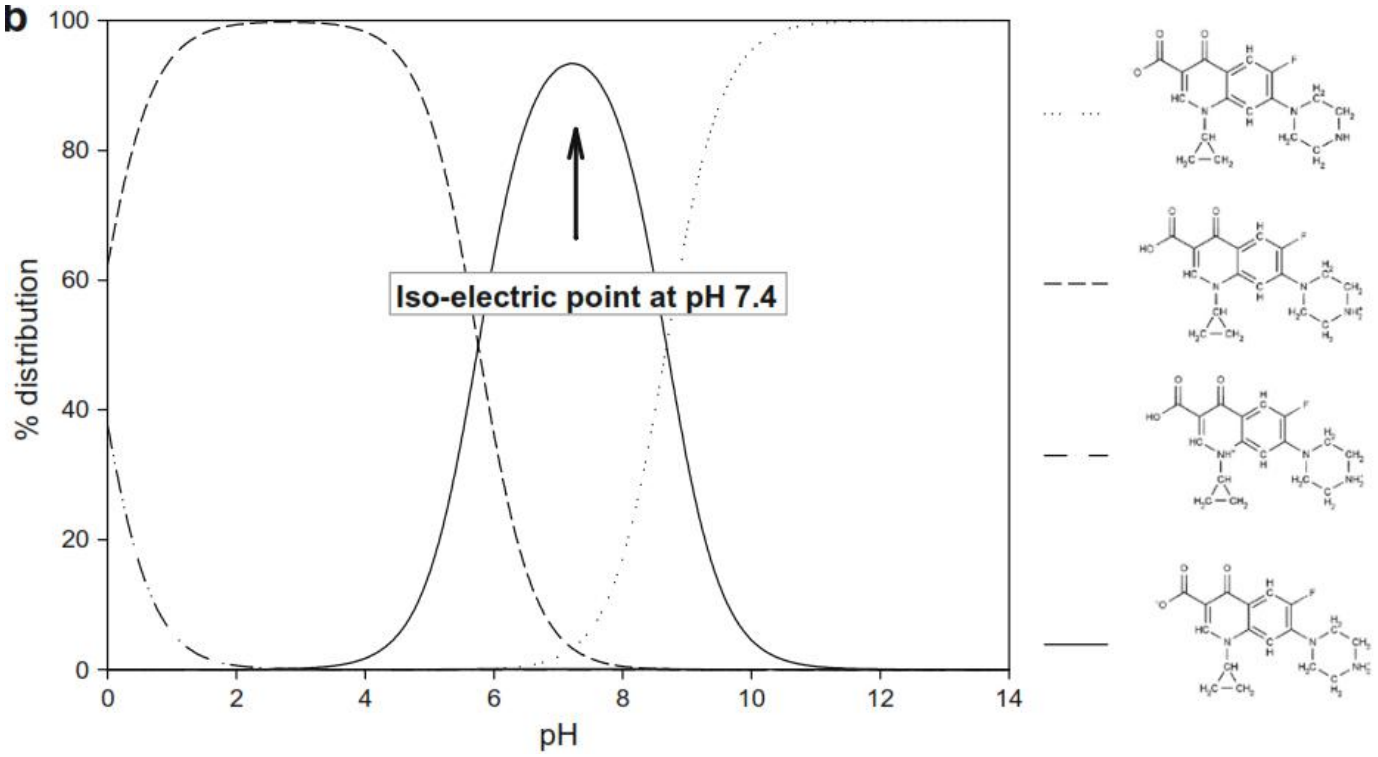


Figure 10. Chemical structure of ciprofloxacin; at different pH ciprofloxacin carries different electrical charges and chemical species (Kummerer, 2009).

At acidic conditions they are in cationic form, which is important for their retention during the extraction. At basic conditions, the anionic species of both acidic and piperazinylic quinolones are less retained in comparison to cationic, zwitterionic and neutral species (Seifrtova *et al*., 2009).

4.2.5 Extraction time and centrifugation speed

The effect of centrifugation was examined by choosing five different speeds: 2000, 2500, 3000, 3500, and 4000 rpm. The phase separation of the solution was obtained after addition of salt and vortex mixing but, a very clear solution was appeared when the solution was centrifuged. The results show (Figure 11) as the centrifugation speed increases the extraction efficiency of analyte is also increased promptly. Therefore, the speed 4000 rpm was chosen as optimum centrifugation speed for the extraction of the analyte.

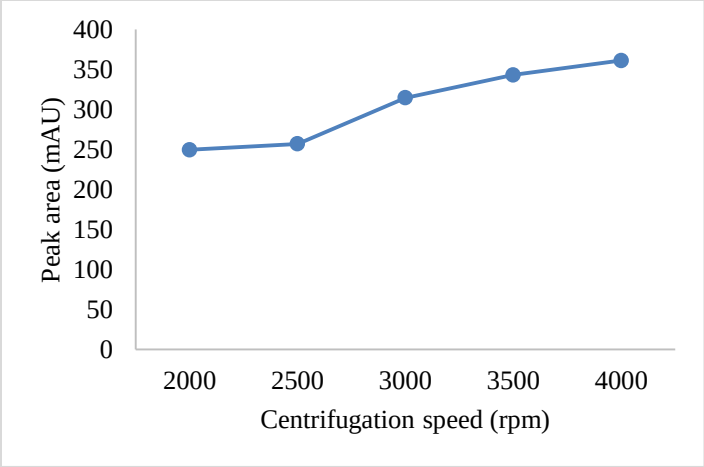


Figure 11. Selection of optimum centrifugation speed

The influence of extraction time was evaluated in water spiked with ciprofloxacin experimenting the following point 2, 4, 6, 8 and 10 min, using a vortex mixer or mechanically with hand as homogenization device. Recoveries improved when the extraction time was increased from 1 to 6 min (Figure 12). Therefore 6 min was used for all subsequent extractions as appropriate extraction time.

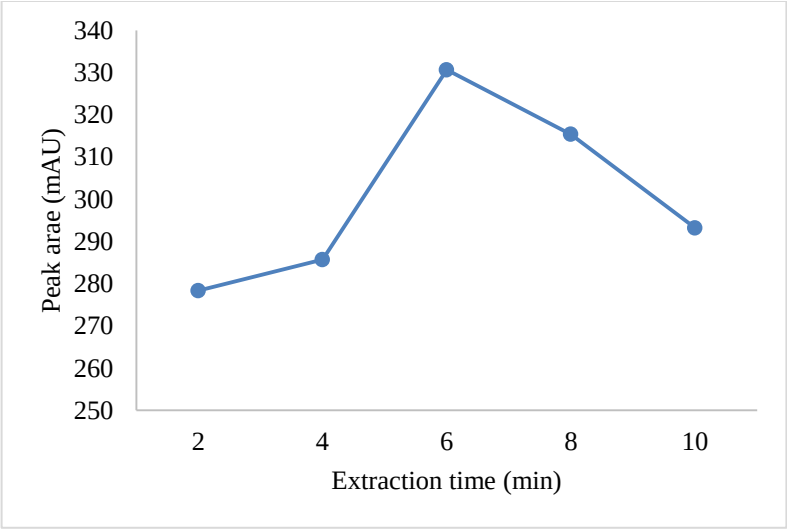


Figure 12. Responses of ciprofloxacin obtained using different extraction time (replicate measurements, n = 3) for the extraction of 10 mL of aqueous sample spiked at 2.5 µg/mL (QuEChERS, 10 mL water, 5 mL ACN, 4 g $MgSO_4$).

4.3 Method validation

Calibration curves were plotted using seven different concentrations of ciprofloxacin and matrix-matched standards within the range of 0.005–5 μg/mL. Linearity was verified by calibration curves using standard concentrations of 0.005, 0.01, 0.02, 0.6, 1.25, 2.5, and 5μg/mL (Figure 13). The detector response of ciprofloxacin was linear within the given range, with correlation coefficients (R^2) = 0.998. Recoveries of the analytes in tap water, bottled water and real samples (waste water samples) spiked at the different concentration were in the ranges of 86 to 120%. The precision (RSDs) were <6% at all the spiked levels. Slightly higher RSDs were obtained for the tap water; however, the overall performance of the method was in the acceptable range in terms of recovery (80–120%) and precision (%). The LODs and LOQs were 0.45 μg/L and 1.5 μg/L, respectively (Table 2), which are mostly comparable to most of the literature, reported methods. The validation results are summarized in Table 3.

Table 2. Results of LOD and LOQ

Blank	1	2	3	4	5	6	7	SD	LOD (μg/L)	LOQ (μg/L)
	5.8	5.9	5.5	5.6	5.7	5.6	5.5	0.15	0.45	1.5

Table 3. Validation results in different water matrices

Types of sample	Spiked with CIP (μg/mL) (n = 3)	X_i	X_{ii}	Mean Peak area (mAu)	SD	RSD	Experimentally found concentration (μg/mL)	Recovery (%)
TW	0.02	35	37	36	1.0	2.78	0.02	100
TW	0.1	89.3	99.7	95	5.2	5.50	0.12	120
TW	2.5	303	307	305	2.0	0.66	2.16	86.4
BW	0.1	87.5	85.3	86	1.1	1.27	0.11	108
BW	2.5	328	316	322	5.9	1.82	2.28	91.2
DW	2.5	256	260	258	2.1	0.79	2.52	101
DW	5	509	535	522	12.9	2.47	5.13	103
HWW	1.25	200	180	190	9.95	5.24	1.46	117
HWW	-	-	-	-	-	-	BDL	

Where: TW = Tap water, DW = Distilled water, BW = Bottled water, HWW = Hospital waste water BDL = Below detection limit

5. REAL SAMPLE ANALYSIS

5.1 Detection and determination of ciprofloxacin in waste water by using HPLC- DAD

The method was applied to waste water samples from two pharmaceutical industries (PWW1 and PWW2), hospital (HWW), ASTP, river (RW) and tap water, bottled water and distilled water samples collected. The extracts of water samples were analyzed to determine the concentration of ciprofloxacin. Peak identification was performed by comparing the retention times of analytes to those of pure standard of ciprofloxacin. Quantitative determination of ciprofloxacin was done after constructing calibration of standard working solutions of ciprofloxacin (Figure 13). The chromatograms are shown in Figures 14-20.

5.1.1 Calibration curve of ciprofloxacin for HPLC method

Calibration curve was obtained using seven different concentration of ciprofloxacin in the range of 0.005 to 5 µg/mL (Table 4) for the determination of ciprofloxacin. The calibration curve equation was obtained from peak area versus concentration.

Table 4. Concentration versus peak area of ciprofloxacin standard using HPLC-DAD.

Concentration (µg/mL)	0.005	0.01	0.02	0.6	1.25	2.5	5.0
Peak area (mAU)	15.0	29.2	34.1	78.5	128.3	255.9	509

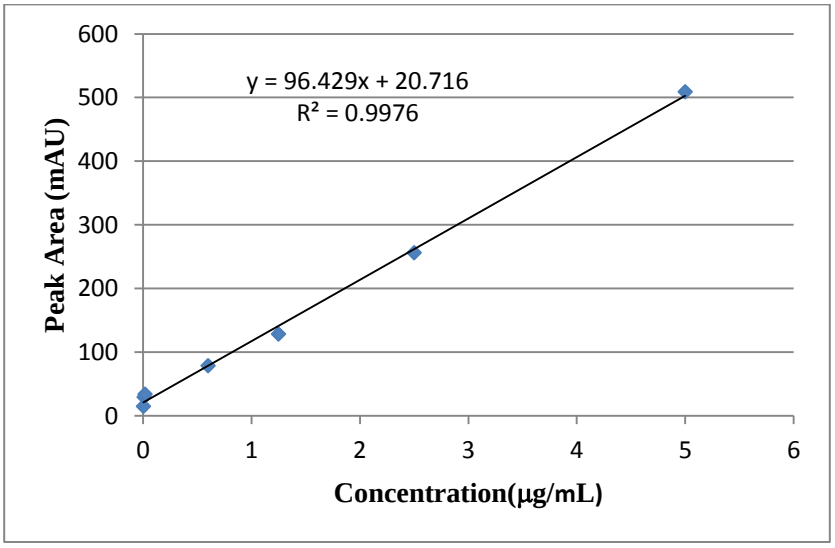


Figure 13. Calibration curve of ciprofloxacin standard by using HPLC-DAD

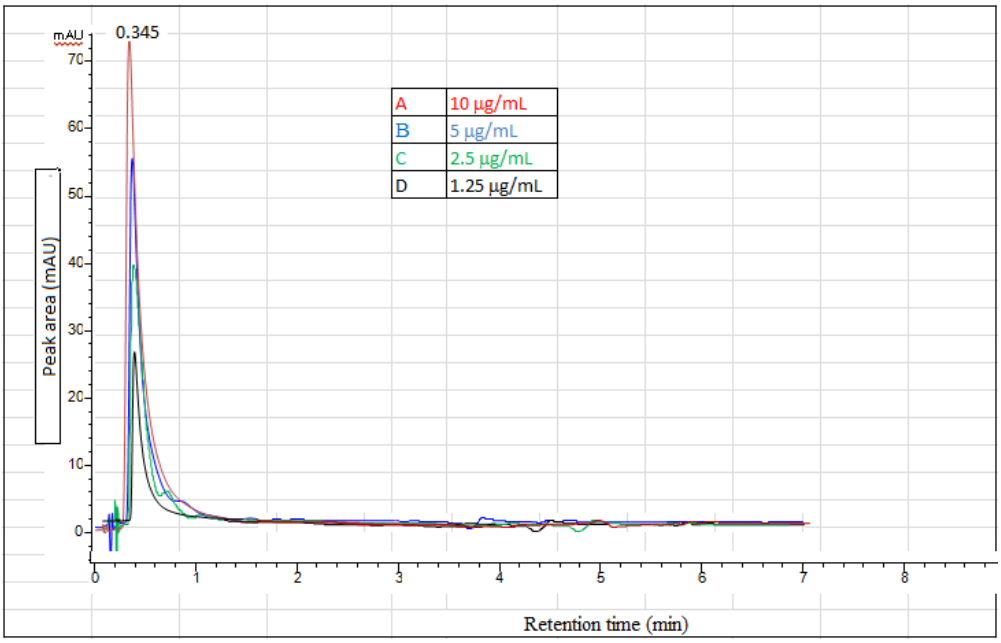


Figure 14. HPLC chromatogram of different concentration of ciprofloxacin standard solution

The ciprofloxacin level of the sample were calculated from the regression equation of Figure 13, y = 96.43x + 20.7, where y is the peak area of ciprofloxacin at 277 nm, x is the slope of the equation which is the concentration of ciprofloxacin and R^2 = 0.998 is the linear regression coefficient. The LOD and LOQ were calculated as 3σ and 10σ of the blank solutions (n = 7). The results are given in Table 3. The low values of LOD and LOQ indicate that the newly 0ptimized method is sensitive and can be used for the quantification of traces of ciprofloxacin in the waste water samples.

The method was successfully applied to waste water sample. The results obtained for the concentrations of ciprofloxacin (µg/mL) in the sample were detected only in ASTP sample and concentration was 0.82 ± 0.5 µg/mL. The RSDs of the results in waste water sample were RSD<1% indicating that the analytical procedure provided precise results.

Table 5. Results of sample analysed

Types of sample	X_i	X_{ii}	Mean Peak area (mAu)	SD	RSD	Experimentally found concentration (μg/mL)
HWW	-	-	-	-	-	BDL
PWW1	-	-	-	-	-	BDL
PWW2	-	-	-	-	-	BDL
ASTP	117	118	118	0.5	0.43	0.828 ±0.5
RW	-	-	-	-	-	BDL
TW	-	-	-	-	-	BDL
BW	-	-	-	-	-	BDL
DW	-	-	-	-	-	BDL

Where: TW = Tap water, DW = Distilled water, BW = Bottled water, HWW = Hospital waste water, ASTP = Addis Ababa Sewage Treatment, RW = River water PWW1 and 2 Pharmaceutical waste water 1 and 2. BDL = Below detection limit.

Table 5 indicates ciprofloxacin was not detected in most of the sample except in one sample from ASTP. The presence of ciprofloxacin in ASTP indicate that the potential of APIs in waste water.

From different literature we can understand that the occurrence of APIs in aquatic environments can adversely affect living organisms on different organization level and lead to alteration in the ecological function of river and lakes. Further contamination of drinking water and food sources with these compounds may lead to unintended human exposures and potential effects on health. So far over 130 different pharmaceuticals have been detected in waste water generated from pharmacological factories and different water sources (Fick *et al*., 2009, Cardoso *et al*., 2014).

Inorder to know the potential toxicity and environmental impact it is necessary to analyze many samples and study in the future. Since pharmaceutical pollutant (ciprofloxacin) is present in one

of the waste water sample from Addis Ababa Sewage Treatment, other pharmaceutical pollutants might be present in other waste waters. Therefore further studies are required to assess the potential risk of pharmaceutical to the aquatic environment.

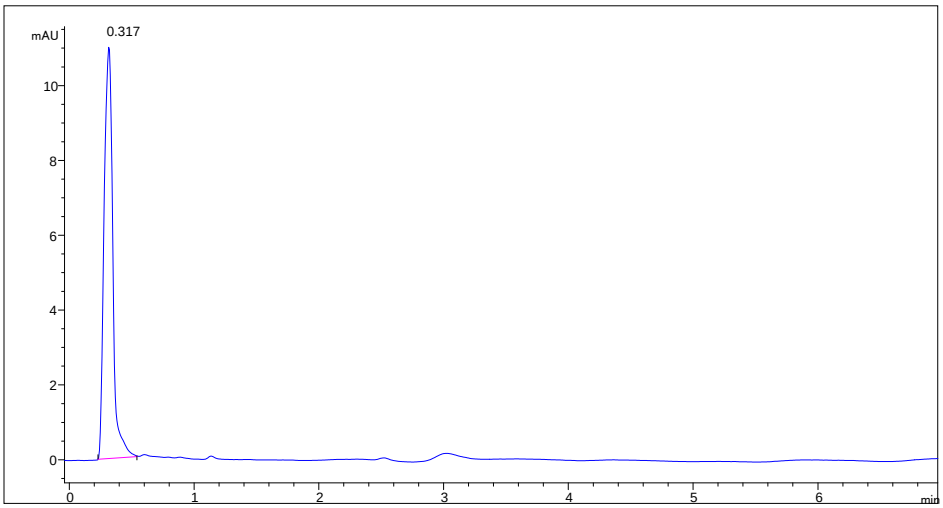


Figure 15. HPLC chromatogram of TW spiked with 0.02 μg/mL ciprofloxacin.

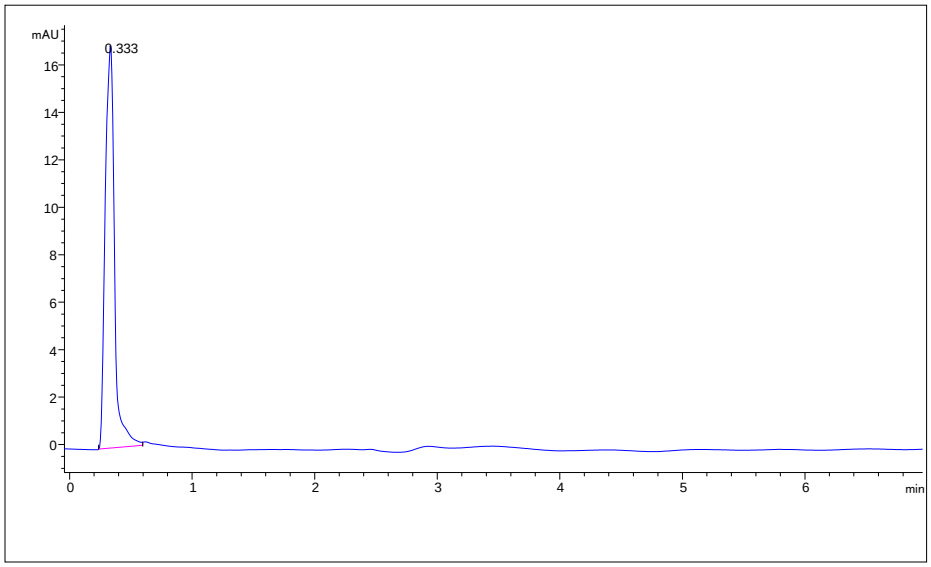


Figure 16. HPLC chromatogram of TW sample spiked with 0.1 μg/mL ciprofloxacin

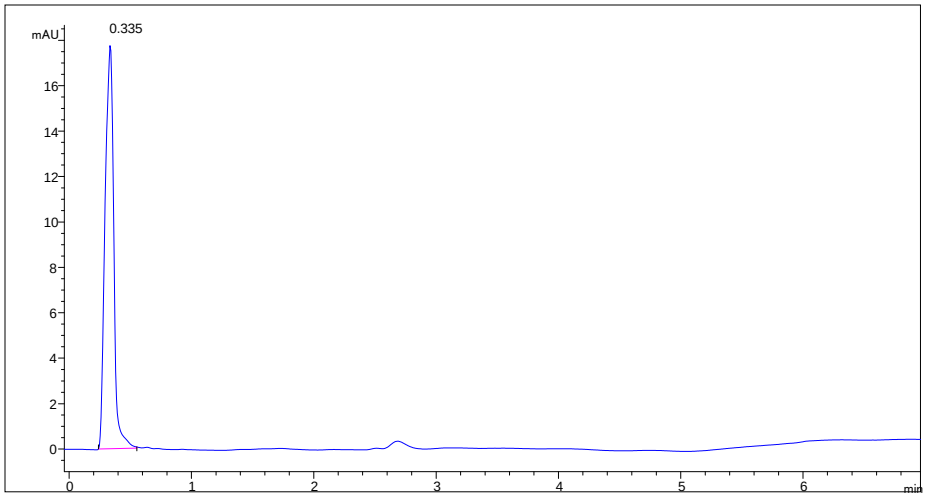


Figure 17. HPLC chromatogram of BW sample spiked with 0.1 µg/mL ciprofloxacin

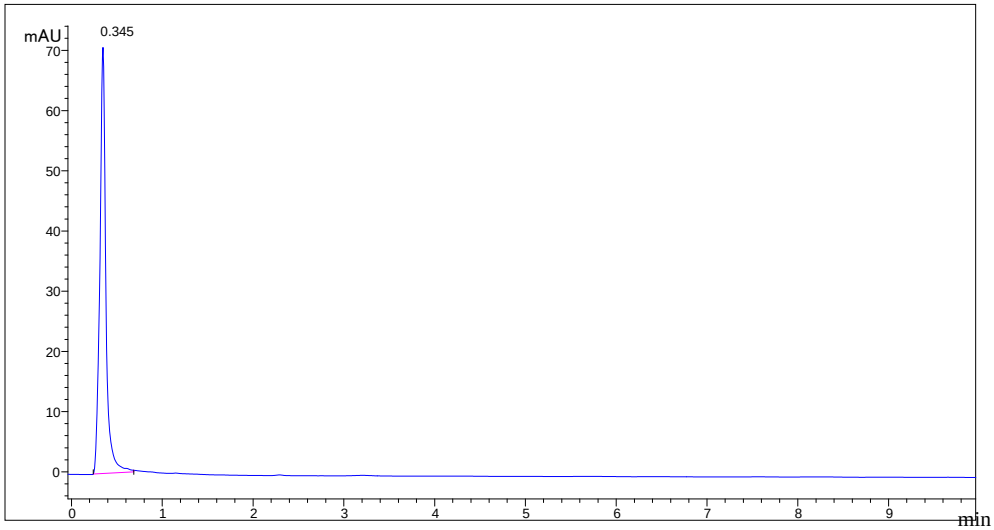


Figure 18. HPLC chromatogram of BW spiked with 2.5 µg/mL ciprofloxacin

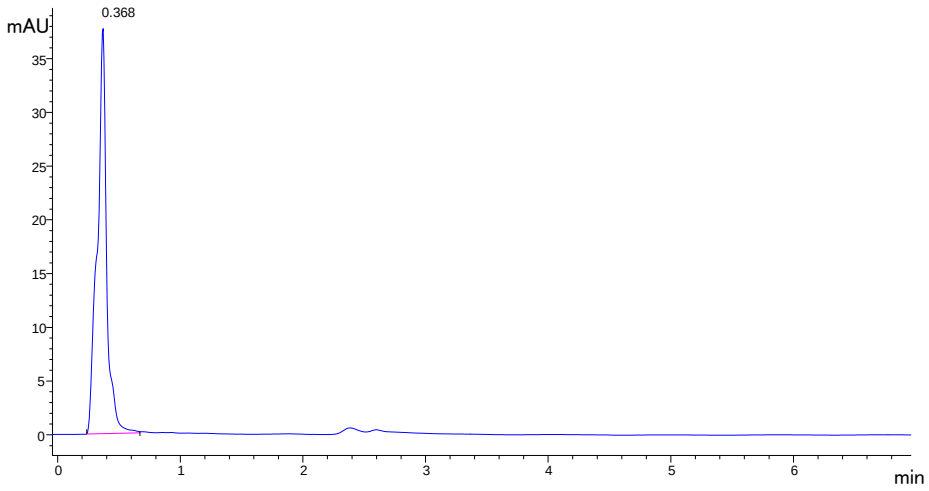


Figure 19. HPLC chromatogram of HWW spiked with 1.25 µg/mL ciprofloxacin**.**

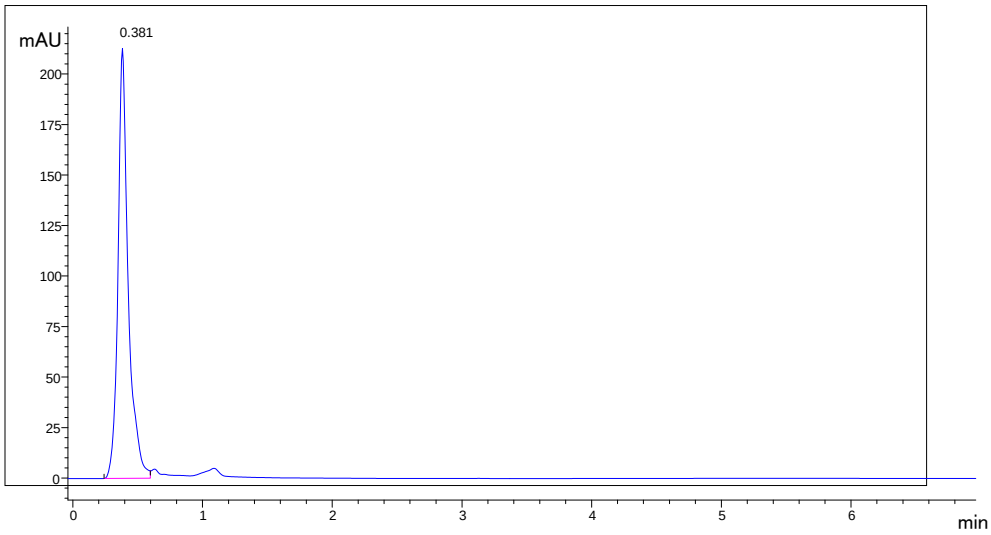


Figure 20. HPLC chromatogram of ciprofloxacin of ASTP sample (concentration factor = 10)

5.1.2 Comparison of optimized method with the literature methods

The analytical parameters of the newly developed method were compared with the literature methods (Table 6). The newly developed method is comparable with most of the literature methods.

Table 6. Comparison of optimized method with the literature methods

Sample preparation	Analytical technique	Linear range	LOD (μg/L)	LOQ (μg/L)	References
Optimized QuEChERS	HPLC-DAD	0.005-5 μg/mL	0.45	1.5	This work
Modified QuEChERS	LC-MS/MS	0.01-10 μg/L	0.001-0.167	0.002-0.25	Kachhawaha *et al*., 2017
SPE	HPLC-MS	0.01-0.5 μg/mL	0.004	0.013	Reverte *et al*., 2003
SPE	UHPLC-FD	0.1-5 μg/mL	25	100	Seifrtova *et al*., 2010
SPE	UHPLC-MS/MS	0.001-0.5 μg/mL	0.5	1.0	Seifrtova *et al*., 2010
SPE	HPLC-DAD	0.5-20 μg/L	0.25	0.5	Asperger *et al*., 2014
SPE	LC-MS-MS	0.01-10 μg/L	1.0 ng/L	10.0 ng/L	Babic *et al*., 2010
SPE	LC-MS/MS	0.5 – 600 μg/L	0.001	0.0034	Afonso-Olivares *et al*., 2013

6. CONCLUSION

An optimized QuEChERS-HPLC/DAD method has been developed for the determination of ciprofloxacin. The newly optimized method was successfully applied for the determination of ciprofloxacin in water samples. This work is an addition to very few reports available on the presence of pharmaceutical products in aquatic environment. It is an initiative towards catalyzing research on pharmaceutical products in Ethiopian aquatic environment. Since pharmaceutical pollutant (ciprofloxacin) is present in one of the waste water sample from Addis Ababa Sewage Treatment, other pharmaceutical pollutants might be present in other waste waters. Therefore further studies are required to assess the potential risk of pharmaceutical to the aquatic environment.

Based on this work the QuEChERS-HPLC/DAD concept has been used here in principal to extract and determine pharmaceutical products (ciprofloxacin) in aqueous matrices. The optimized method can be efficiently used for qualitative and quantitative analysis of pharmaceutical products in water and waste water. It is also advantageous method than expensive method SPE and other conventional methods.

The optimized method is easy, cheap, effective, rugged and safe and the applicability of this method serve as a useful tool to determine the amount of pharmaceutical discharge to the aquatic environment, to evaluate the efficiency of WWTP in eliminating pharmaceutical compound and also used for routine monitoring and regulation purpose.

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