Automatic capillary liquid chromatography tandem mass spectrometry method for pharmaceutical products in environmental water samples

Thesis for the Master's degree in chemistry

Deniz Demir

60 study points

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Abstract

The use of active pharmaceutical ingredients (APIs) is increasing continuously and thereby constitutes a potential source of surface water contamination. Environmental concentrations of APIs are usually at trace levels, generally in low ng/L concentrations, making them challenging to detect. However, these low concentrations can be sufficient to induce toxic effects on the aquatic environment.

This project focuses on selected APIs, which are probably the most common in the aquatic environment. Several of the compounds selected are on The Norwegian Institute of Public Health's (NIPH's) list over the most prescribed APIs in Norway throughout 2014.

A rapid, sensitive and selective method was developed and validated for screening of seven APIs (acetaminophen, atenolol, fluoxetine, sulfapyridine, sulfamethoxazole, trimethoprim and xylazine) in surface water. The method include an on-line automatic filter and filter back flush (AFFL) solid phase extraction (SPE) in combination with capillary liquid chromatography (capLC) coupled to a triple quadrupole mass analyser (MS). By combining large volume injection (100 µL) and the AFFL-SPE-system, a rugged and high throughput switching system was obtained.

The analytes were loaded under non-eluting condition and further trapped on a reversed phase (RP) Kromasil (C_{18}, 5 mm x 1 mm ID, 5 µm) SPE-column with a flow rate of 150 µL/min. A 10 minutes gradient was applied using an ACE C_{18} column (150 mm x 0.3 mm ID, 3 µm) as analytical column with a flow rate of 4 µL/min. The mobile phases (MP) for loading and MP A consisted of type 1 water with 0.1 % formic acid (FA) and the organic MP (MP B) consisted of 100 % methanol with 0.1 % FA (pH 2.7). The total analysis time was 15 minutes including 6 minutes reconditioning.

This study demonstrates that the AFFL-SPE-capLC system combined with a triple quadrupole MS enables the detection of selected classes of APIs in surface water in a concentration range of 10-100 ng/L. The calculated concentration limit of detection (cLOD) for the selected APIs was in the 2– 18 ng/L range and calculated concentration limit of quantification (cLOQ) in the 5 – 54 ng/L range. Acceptable linearity (R^2 = 0.9777-0.9998) and generally high apparent recovery values (from 69-288 %) with generally relative standard deviation (RSD %) lower than 20 % were found.

Preface

This study was carried out at the University of Oslo, Faculty of Mathematics and Natural Science at the Department of Chemistry within the research group of bioanalytical chemistry from August 2015 to June 2017. Professor Elsa Lundanes, Assoc. prof. Dr. Steven Ray Wilson, Dr. Silvija Abele and Dr. Hanne Røberg-Larsen served as my supervisors.

This study was a part of a sub-project within a cooperation project between the University of Oslo and the University of Latvia.

My time as master student has been a true journey. I would like to thank my supervisors for giving me the opportunity to take a master's degree in bioanalytical chemistry and providing me with a challenging and interesting task. I would especially like to thank Elsa for her guidance and support whenever I was facing a problem.

I am also extremely grateful to Inge Mikaelsen and Hanne R. L. for helping me during trouble shooting when the AFFL-SPE-LC instrument failed. I would like to thank Silvija and Sunniva Furre Amundsen for their work on this project.

Furthermore, I would like to thank all of my fellow students and office-partners. Thank you, Kamilla Bjørseth, Esma Benn Hassine and Beatrix Rácz for all the interesting discussions and for spreading joy and happiness.

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Oslo, Norway, May 2017

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1 Abbreviations and symbols

1.1 Abbreviations

Abbreviation	Meaning
ACN	Acetonitrile
AFFL	On-line automated filtration and filter back flush
AGC	Automatic gain control
API	Active pharmaceutical ingredient
a.u.	Arbitrary unit
capLC	Capillary liquid chromatography
cLOD	Concentration limit of detection
cLOQ	Concentration limit of quantification
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FA	Formic acid
HCD	Higher energy collisional dissociation
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
GC	Gas chromatography
ID	Inner diameter
IS	Internal standard
LC	Liquid chromatography
MeOH	Methanol
MP	Mobile phase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio

nanoLC	Nano liquid chromatography
PP	Pharmaceutical product
ppm	Parts per million
NCE	Normalized collision energy
PRM	Parallel reaction monitoring
Q	Quadrupole
RF	Radio frequency
RP	Revered phase
SD	Absolute standard deviation
SP	Stationary phase
SIM	Selected ion monitoring
SPE	Solid phase extraction
SPE-LC	On-line solid phase extraction liquid chromatography
SRM	Selected reaction monitoring
SSS	Standard stock solution
TICC	Total ion current chromatogram
UHPLC	Ultra high performance liquid chromatography
UPLC	Ultra pressure/performance liquid chromatography (product of water)
UV	Ultraviolet

1.2 Symbols

Symbol	Meaning
A	Peak area
A_{is}	Peak area of internal standard
C	Concentration
C_{is}	Concentration of internal standard
H	Plate height
k	Retention factor
L	Length of column
n	Number of replicate(s)
N	Plate number
n_m	Number of molecules in the mobile phase
n_s	Number of molecules in the stationary phase
$\boldsymbol{R'}$A	Apparent recovery
R_s	Resolution
R^2	Correlation coefficient
t_M	Hold-up time of an unretained compound
t_R	Retention time
w	Peak width
w_{av}	Average peak width (10 % or 50 % of the peak height)
$\boldsymbol{\sigma}$	Standard deviation

2 Introduction

2.1 Background

2.1.1 Active pharmaceutical ingredients (APIs)

The continuously increasing contaminations of APIs in surface water is an issue receiving growing attention worldwide [1]. Recent studies reports detection of APIs belonging to different pharmaceutical groups, in different aquatic environments [2] e.g. rivers [3, 4], marine water [2, 5] and drinking water [6]. APIs access these locations from various sources like sewage effluent, the improper disposal of drugs, and residues during production of pharmaceuticals [3].

Figure 1 shows an example of an API cycle, starting with the manufacturing process causing contamination of surface water by the residues of APIs. APIs are used in hospitals and in households by humans as medical treatment, but APIs may not be completely absorbed by the human body. The surface water is exposed to the pharmaceutical residues from human consumption, but also from the numerous sources as above mentioned. The residues reach the wastewater treatment plants which work as a filter [7].

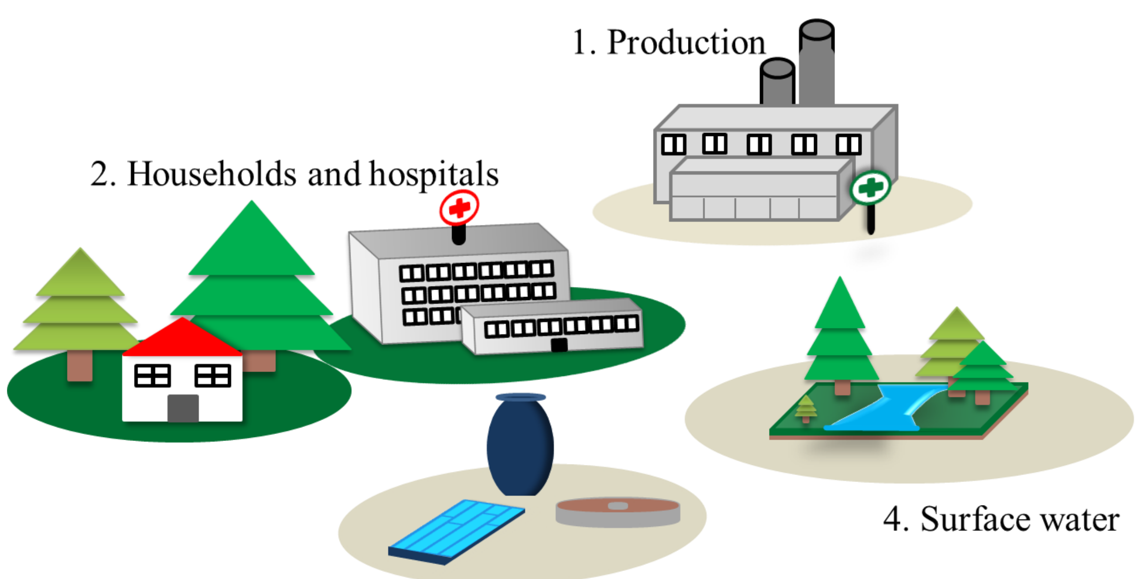


Figure 1 Cycle of an API starts with manufacturing and will further be subjected to human consumption. Residues passing the wastewater treatment plants will reach the surface water.

The filter may not completely eliminate all these substances, which differ in properties and concentrations. Several types of filters and methods [8] are necessary to treat the waste water to eliminate API residues, but some is not eliminated and will pass through the treatment plants and reach surface waters [7, 9].

Concentrations of APIs found in environmental water are usually at trace levels [6, 10], generally in the low ng/L, making them challenging to detect even by a sensitive mass spectrometer [11]. However, even these low concentrations of APIs can be sufficient to induce toxic effects on aquatic species. APIs are disturbing the aquatic environment by leading to unwanted biological, undesirable ecological and detrimental effects on aquatic species [10].

An example may be residues of antibiotics reaching the environmental water. An overuse of antibiotics results in bacterial resistance [12], meaning antibiotics are no longer effective at killing or limiting the growth of bacteria in organisms. Residues from antibiotics reaching the surface water may potentially adversely affect aquatic organisms and humans through drinking water [9]. Antibiotics at trace levels can have an impact on cell functions by changing the genetic expression or may cause transfer of antibiotic resistance [9].

This project, which is conducted in cooperation with the University of Latvia (Riga, Latvia) focuses on selected APIs which are probably the most common ones in surface water. They are representative for different pharmaceutical groups, namely anti-inflammatory, anti-hypertensive, antibiotics, lipid regulators, psychiatric drugs, stimulants, sedation medication and statins. Several of them are on The Norwegian Institute of Public Health's (NIPH's) list over the most prescribed APIs in Norway in 2014 [13].

Table 1 shows the structure of the compounds. The initial list of the APIs also included some hormones, but these were never considered to be a part of this method and were removed from the list (**Table 1**) which presents the target analytes in this study. Sulfapyridine and the internal standards: atenolol-d7, fluoxetine-d5 and sulfamethoxazole-(phenyl-$^{13}C_6$) were later added to the study.

Table 1 Selected APIs and the internal standards with their trivial name and structure.

Name of API	Structure
Acetaminophen	
Atenolol	
Atenolol-d_7	
Atorvastatin	
Azithromycin	
Caffeine	

Carbamazepine	
Ciprofloxacin	
Clarithromycin	
Diclofenac sodium salt	
Erythromycin a dehydrate	
Fluoxetine hydrochloride (Prozac)	
Fluoxetin-d_5 hydrochloride	

Gemfibrosil	
Ibuprofen	
Ketoprofen	
Losartan potassium	
Metoprolol tartrate salt	
Naproxen	
S-Propranolol hydrochloride	
Pravastatin sodium salt hydrate	

Salicyclic acid	
Simvastatin	
Sulfapyridine	
Sulfamethoxazole	
Sulfamethoxazole-(phenyl-$^{13}C_6$)	
Trimethoprim	
Valsartan	
Xylazine hydrochloride	

Physiochemical properties of the analytes

The physiochemical property of an analyte is of interest regarding the development of both chromatographic and mass spectrometric methods as well as the principle of the analytical techniques. The APIs differ in structure, polarity (**Appendix Figure A-1**) and acidity (**Appendix Figure A-2**).

2.1.2 Active pharmaceutical ingredients and statistical information

APIs are used by humans mostly as protection to prevent or cure diseases [7]. The sale of pharmaceuticals in Norway has in 2013-2014 increased with 8.9 % according to the report "Pharmaceutical Consumption in Norway 2010-2014» from the NIPH [14]. NIPH indicates that the total sales of pharmaceuticals in 2015 were 22 billion NOK [15]. Increasing amounts of APIs are consumed as a result of improved medical care. APIs are in many cases not completely absorbed by the human body, but partially excreted or metabolized in the body [7], resulting in trace level contamination of surface water. A summary and an overview of the classes, the specific compounds within these classes and the general usage of the groups are described in **Table 2.**

Table 2 The APIs included in this project, classified by pharmaceutical group. The main usage of the group is stated and the compounds are identified using their trivial names. The * indicates that the compounds was one of the most prescribed APIs in Norway in 2014.

Group	Usage	Compound
Analgesic/nonsteroidal anti-inflammatories	For controlling pain, inflammation and fever [16].	Acetaminophen* Ibuprofen* Ketoprofen Naproxen Salicylic acid* Diclofenac*
Antibiotics	For prevention or treatment of a bacterial infection [17].	Azithromycin Ciproflaxin Clarithromycin Erythromycin Sulfamethoxazole Trimethoprim Sulfapyridine
Anti-hypertensives	To treat high blood pressure and heart failure [18].	Losartan Valsartan
Beta-blockers	Management of irregularities in heartbeat including in treatment of hypertension, altering myocardial processes and decreasing the incidence of heart failure [19].	Atenolol Metoprolol* Propanolol
Lipid regulators	For reducing cholesterol [20, 21].	Gemfibrosil
Psychiatric drugs	For treatment of mental illness such as depression, panic attacks [22] and may decreasing nerve impulses that cause pain [23].	Carbamazepine Fluoxetine

Stimulant	For increasing alertness or energy As a stimulant consumed in different amounts differ in effects, and may cause increased in energy availability and enhanced short-term memory [24].	Caffeine (not necessarily an API)
Sedations	For reducing anxiety, and as a muscle relaxant [25].	Xylazine
Statins	For treatment of lipid disorders to reduce cardiovascular risk, to lower inflammation and to lower cholesterol [26].	Atorvastatin* Simvastatin* Pravastatin

2.2 Chromatography

2.2.1 Chromatographic theory

Chromatography is a technique used for separation of compounds within a mixture [27]. Separation is achieved when different sample compounds interact differently with a stationary phase (SP). The SP is located inside a column and a sample is transported through the column using a mobile phase (MP). The MP can either be a liquid, as used in liquid chromatography (LC), or a gas used in gas chromatography (GC). Each compound within a sample will be distributed between the two phases in the column [28]. This is described by the retention factor, k and shown in **Equation 1,** where n_s is the number of molecules in the stationary phase, and n_m is the number of molecules in the mobile phase.

$$k = \frac{n_s}{n_m} \qquad \textbf{(1)}$$

The interaction between different sample compounds and the SP must be different to obtain separation. The speed of the compounds will differ, because a compound is more retained on the column if it has high affinity to the SP, compared to a compound which has low affinity to the SP. The outlet of the column is connected to a detector to measure the intensity of the band of eluting sample component as a function of time, called retention time (t_R). The relation between k and t_R is given by **Equation 2,** where t_M is the time a component would be eluted elute at if it has no interaction with the SP [28].

$$k = \frac{t_{R-}t_R}{t_M} \qquad \textbf{(2)}$$

The efficiency of a column depends on physical processes, both external and within the column. The solute ideally elute in (close to) Gaussian curves with standard deviation σ. The column efficiency, given by number of plates *N* **(Equation 3)** [28]

$$N = \left(\frac{t_R}{\sigma}\right)^2 \qquad \textbf{(3)}$$

A high N value is an indication of an efficient column and N depends on the column length (L). Plate height, H is a measure of band broadening, and N is inversely proportional to the H. The correlation between plate height and number of plates is given in **Equation 4**.

$$N = \frac{L}{H} \qquad \textbf{(4)}$$

Resolution, R_s **(Equation 5)** illustrates how well two bands close to each other can be distinguished from one another. Δt_R is the difference in elution time for two components and w_{av} is the average of band width for the two components [27].

$$R_s = \frac{\Delta t_R}{w_{av}} \quad \textbf{(5)}$$

2.2.2 Liquid chromatography

In high performance LC (HPLC), high pressure is used to force solvent (MP) through a column containing small particles with SP to give high-resolution [27]. The small particles, which most often are porous throughout, have diameters of 3-5 µm. The efficiency of a packed column increases as the size of the SP particles decreases. Chromatography with 1.5-2 µm diameter particles is commonly called ultra high performance LC (UHPLC) [27]. An advantage of UHPLC is the ability to substantially decrease run times. This is due to the use of short columns (i.e., 50 mm length) packed with sub-2 µm particles at higher linear velocities without compromising the separation. The most common columns in LC are packed, however both monolithic and open tubular columns can be used [9].

2.2.3 Reversed phase chromatography

Reversed phase (RP) chromatography is the most common separation mechanism in LC where the separation is based on the difference in hydrophobicity of analytes. The most common RP SP is a nonpolar hydrocarbon chain, chemically bonded to silica-based particles. Among the commercially available RP materials, C_4, C_8, and C_{30} chains, C_{18} (octadecyl) bonded silica is the most frequently used (**Figure 2**), which generally supports a pH range limited to pH 2-8 [29].

The MP is often a polar organic solvent mixed with an aqueous buffer, making it more polar than the SP. A nonpolar compound is more strongly retained on the hydrophobic SP than a polar compound. The traditional silica-based C_{18} stationary phases are generally used for 80-90% of all LC separations. Currently there are numerous selectivity options for RP HPLC and UHPLC columns, which facilitate widespread adoption of LC for all types of separations [30].

RP gives the opportunity to use gradient elution, meaning continuously changing composition of the MP and by increasing the percentage of organic solvent the eluting strength will increase. RP makes it possible to analyze complex samples where target analytes have different hydrophobicity. APIs selected for method development in this study differ in hydrophobicity**,** which makes the choice of C_{18} SP favorable [31].

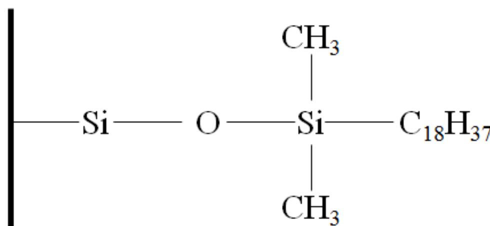


Figure 2 The structure of C_{18} bonded to silica material [Adapted from [32]].

2.2.4 Column

In addition to being characterized by type, e.g. packed or monolithic, the LC columns are characterized based on their inner diameter (ID) (**Table 3**). Conventional LC columns with an ID in a range of 3-5 mm are still dominating the analytical laboratories due to higher loading capacity and being more robust than a column with smaller ID. A limited amount of a sample with low abundant analytes of interest needs a sensitive method, which can detect the analytes within the complex matrix background. Narrow ID columns such as capillary LC (capLC) and nanoLC are used with smaller volumetric flow rates. A strongly reduced radial dilution (**Figure 3**) of chromatographic bands gives increased sensitivity with concentration-sensitive detection [33]. The drawback of narrow ID columns is that typically smaller injection volumes can be used without extensive band broadening. However large injection volumes can be performed if the sample is dissolved in a solvent with lower elution strength than the MP. By using a column with 0.3 mm ID instead of 4.6 mm ID, a signal 250 times more sensitive should be expected [34]. A benefit of using low flow rates is also formation of smaller droplets into the electrospray ionization mass spectrometry (ESI-MS) causing higher signal and increased sensitivity.

Larger impact from dead volumes and easily clogged LC column due to small dimensions of valves and tubings are the disadvantages of narrow ID columns [27]. The same column length and SP for conventional columns and narrow ID columns are possible [27].

Table 3 Typical IDs of columns used in LC [Adapted from [35]].

Column type	ID (mm)
Conventional	3-5
Narrow bore	2
Micro	0.5-1
Capillary	0.1-0.5
Nano	0.01-0.10
Open tubular	0.005-0.05

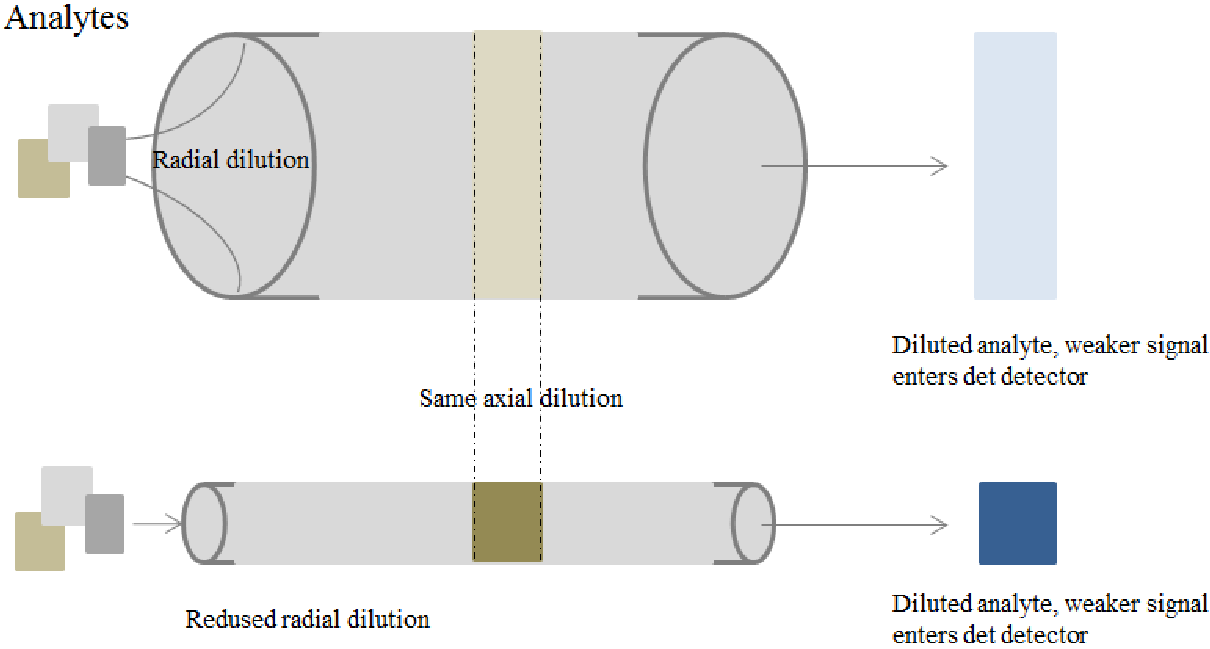


Figure 3 Illustration of radial dilution. Radial dilution is a function of the square of the radius of the column [Adapted from [34]].

2.2.5 Solid phase extraction

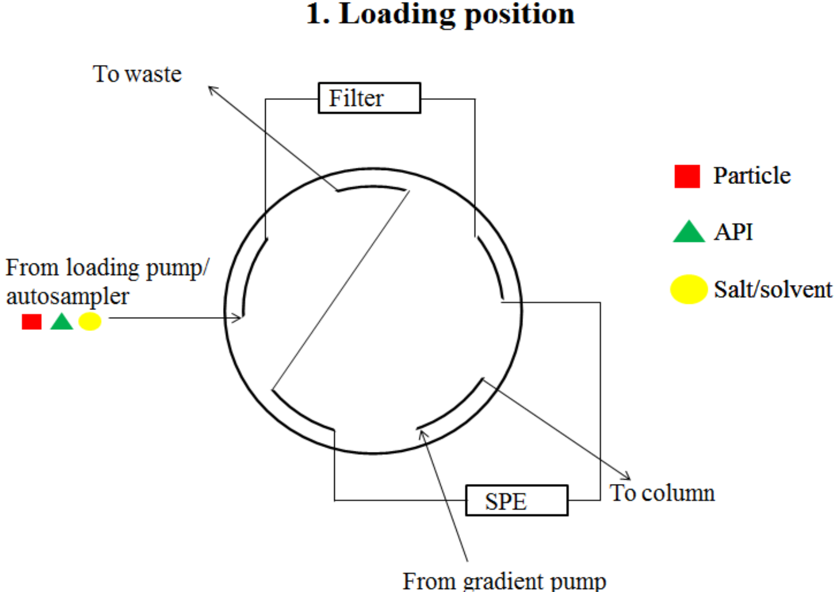
Solid phase extraction (SPE) is a sample preparation process which uses a small chromatography column [36]. A frit is placed both under and above a layer of SP particles. The SPE is used to isolate and concentrate components of interest in a sample, including pharmaceuticals from environmental water samples. Traditionally SPE has been carried out off-line prior to the LC separation. However, it is also possible to carry out on-line SPE-LC, utilizing large volume injection [37].

2.2.6 Large volume injection by on-line solid phase extraction

In order to detect trace levels of APIs in aquatic environment, high sensitivity is required and this may be achieved by using large volume injection. Large volume injection on an SPE-column, also called pre-column or trap-column, followed by a separation column (analytical column) may increase the sensitivity. A switching valve is used to switch between the two columns, and the system is called a column switching system [38]. The on-line SPE-column is used to trap the analytes in the sample using a non-eluting MP, providing fast and effective analyte enrichment and clean-up. The purpose of such a column switching systems is to obtain low detection limits using an SPE-column to be able to load a large sample volume without large band boarding. The easily clogging of SPE-columns represent the disadvantage of using a switching system [39]. The switching valve may have six, eight or ten ports dependent on the set-up wanted. The SP of the SPE-column can be of the same type as that of the analytical column. If efficient phase focusing is needed on the analytical column to avoid band broadening, phase focusing can be accomplished by using an SPE-column giving a lower retention factor than the analytical column. That means the band eluting from the SPE-column is refocused to a narrow band at the inlet of the analytical column [39].

Figure 4 shows a valve switching system (10 port) called an on-line automatic filter and filter back flush system (AFFL-system) [40]. The AFFL-system is used to avoid blockage and pressure build-up, which can be a problem arising with an on-line SPE. In order to act as a safeguard for the SPE- and analytical column a filter of stainless steel is incorporated into the system. In load position, the sample is on-line filtrated and analytes retained/focused on an SPE-column. In the inject position, the analytes retained by the SPE-column are transferred to the analytical column for separation by the eluting MP from the gradient pump.

At the same time the MP from the loading pump runs through the filter in back flush mode, transporting the trapped particles to waste [40].



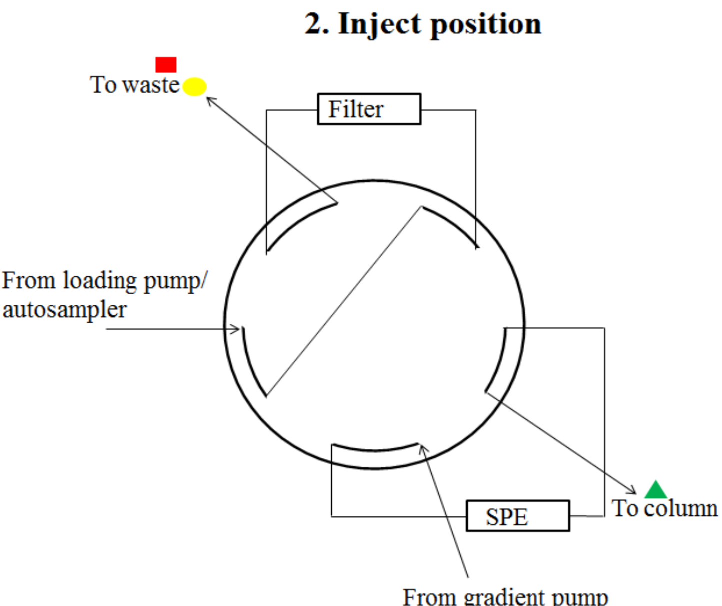


Figure 4 The AFFL-SPE-LC set-up for robust column switching. In load position, the sample is on-line filtrated through an incorporated filter and analytes retained/focused on an SPE-column. In the inject position, the analytes retained by the SPE-column are transferred to the analytical column for separation by the eluting mobile phase (MP) from the gradient pump. At the same time the MP from the loading pump runs through the filter in back flush mode, transporting the trapped particles to waste

2.2.7 Internal standard

An internal standard (IS) is a compound which is known and added of a known and constant concentration to the sample and calibration solutions to enable quantitation of the target analyte. The IS corrects for loss of analyte, compensates for sample to sample recovery differences and corrects for variable instrumental conditions, such as injection volume, retention time, and MS response [41]. The IS used must be separated (in time or mass) from the analyte and other compounds in the sample, but not be present in the sample. Considering sample preparation, extraction e.g. the IS has to behave like the analyte and be added in a concentration that give equal peak height/ peak area as the target analyte [28]. Stable isotope labelled (SIL) ISs e.g. deuterated ISs are often the choice when using MS as detector. They are available of high purity, have a retention time close to the target analyte and behave like the target analyte [42]. The calibration curve is constructed by using **Equation 6** when an IS is used.

$$\frac{\mathrm{A}}{A\ is} = \frac{\mathrm{C}}{C\ is} \qquad \textbf{(6)}$$

A is the peak area of the analyte of interest and A_{is} the peak are to the IS to the corresponding analyte. C is the concentration of the analyte of interest and C_{is} is the concentration of the corresponding IS. However, concentration of IS used was the same throughout the study, a simpler version of **Equation 6** was used, and given in **Equation 7**.

$$\mathrm{C} = \frac{\mathrm{A}}{A\ is} \qquad \textbf{(7)}$$

2.2.8 Calibration curve

A calibration curve illustrates the response of an analytical method to known quantities of analyte [27]. The curve is established by plotting the ratio of the analyte peak area and the peak area of the IS as a function of analyte concentration (**Equation 7**). The mathematical equation (regression equation) relates the instrument response to the analyte concentration [43].

2.2.9 Limit of detection and limit of quantification

The concentration detection limit (cLOD) is the lowest concentration of analyte detectable by the method at a specific level of confidence [44].

The lowest level that the performance is acceptable for a typical value is defined as the concentration limit of quantification (cLOQ) [44].

2.3 Mass spectrometry

2.3.1 Electrospray ionization

Liquid chromatography-mass spectrometry (LC-MS) is a combined technique where electrospray ionization (ESI) is the most common interface (**Figure 5**) [37]. A challenge is that LC is performed with liquids as MP, while a MS measures ions in gas phase at high vacuum. The interface should be able to convert the analytes from the liquid phase into ions in gas phase. The eluent from the analytical column enter a stainless steel capillary and ESI is performed by applying an electrical potential between the conductive capillary and a counter electrode, which is the source block of the mass detector. The potential is usually in the range 1-5kV [39] resulting highly charged droplets. The magnitude of this potential is depended on the source design, the inner diameter of the capillary and the nature of the solution to be sprayed. The capillary is held a constant positive potential in the positive ESI mode and the positive ions in the solution are repelled from the capillary [39]. At the end of the capillary tube, a capillary tip is formed, a characteristic Taylor cone, which emits a fine jet of small droplets when the electric field strength is high enough. By using high voltage and nitrogen gas, an aerosol of charged analyte ions is formed. The analytes are either ionized in the MP or easily ionized in the ESI process. After the Taylor cone, the formed larger droplets will gradually decrease in size by evaporation of the volatile solvent due to heated air around (higher thermal energy), moving toward the entrance of the MS. The charge of the ions increases relative to the radius with the evaporation. Inside the droplets there are repulsive forces, and at a curtain point, it overcomes the surface tension and leads to splitting of the droplets. The droplets will reach a size where gas-phase ions can be produced and detected with an MS analyser [39].

Two mechanisms have been proposed for the formation of gas-phase ions: one by Dole and one by Iribarne and Thomson (**Figure 5**). Dole proposed a theory called charge residue theory in the case of high mass ions (*m/z,* mass to charge ratio). The theory involves continuous evaporation of the solvent which leads to splitting of the droplet into small and smaller

droplets. The expectation is formation of single ion at the end of the process [45]. Solvent evaporation from such a droplet will lead to gas-phase ion.

Iribarne and Thomson proposed a new theory believed to favor ions with relatively low mass values called ion evaporation theory. The coulomb effect will break the highly charged droplet into smaller droplets and the ions are believed to be evaporated directly from the surface.

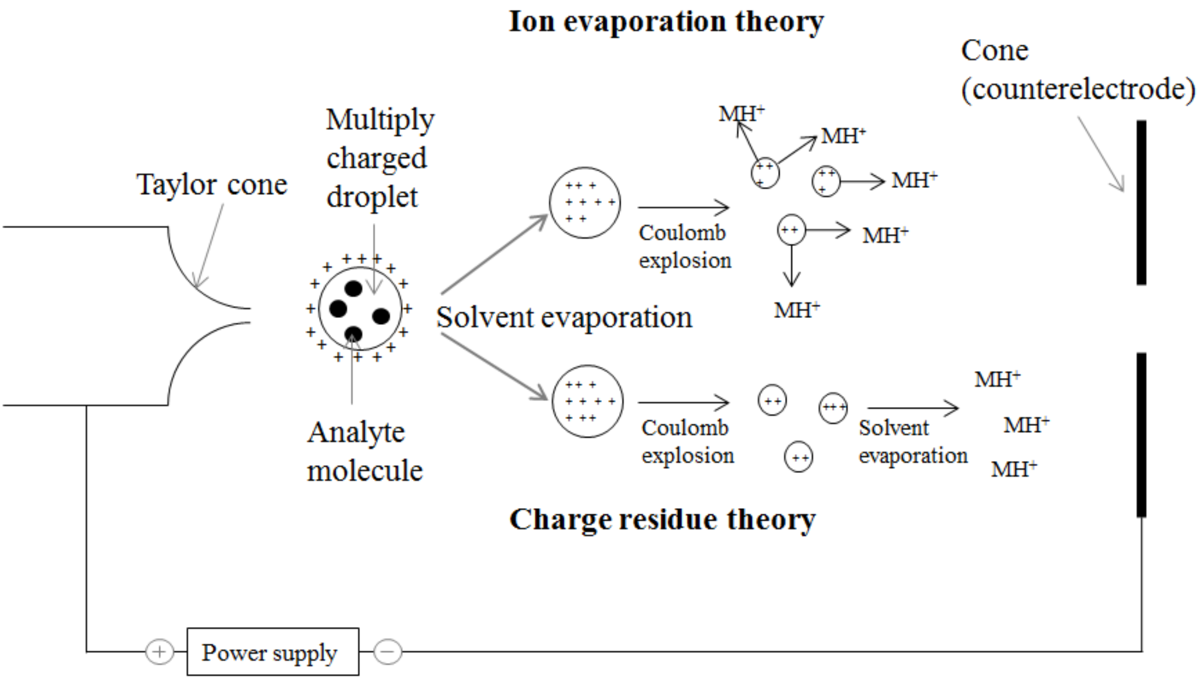


Figure 5 The principle of electrospray ionization both with charged residue theory and ion evaporation theory [Adapted from [45]].

2.3.2 Mass spectrometers

A mass analyser is used for identification of a compound or its fragments. The requirement is that the compound/fragments must be of gas-phase ions and charged to be separated according to their *m/*z. An MS is generally equipped with an ion source used to generated ions which are introduced into the mass analyser and sorted according to their *m/z* value and detected by the detector. The detector generates a signal in response to the ions hitting the detector at their *m/z* value [46]. The mass analysers, also known as a mass filters, are used to separate the ions, before their *m/z* value and intensity are registered.

Fragment ions are used for identification and distinction of co-eluting compounds which is a common scenario in complex samples. The mass analysers such as time of flight (TOF), ion trap, quadrupole (Q) and orbitrap are used for *m/z* analysis. Only the two used in this thesis will be described below.

A compound can be identified by observing the *m/z* to the precursor ion in an MS spectrum. MS is generally used to produce two types of spectra, MS spectra and MS/MS spectra. LC-MS data can be produced by using different MS modes. The common modes are full scan resulting in the total ion current chromatogram (TICC) plot, selected ion monitoring (SIM), and selected reaction monitoring (SRM) or also called multiple reaction monitoring (MRM). SRM is the most common targeted quantification method [47]. Parallel reaction monitoring (PRM)-based MS is comparable in performance to SRM and PRM [48].

2.3.3 Hybrid quadrupole Orbitrap mass analyser

The orbitrap mass analyser of the hybrid quadrupole Exactive Orbitrap (Q Exactive) mass spectrometer is a high-resolution mass analyser ([49]) with a high resolving power ($R_{s=}$150 000) and mass accuracy (2-5 ppm) [50]. An illustration of the Q Exactive Orbitrap mass spectrometer is shown in **Figure 6**, and as observed it consists of two mass analysers (a fragmentation cell); a Q (serving as a mass filter) and an orbitrap mass analyser (serving as a both *m/*z separator and detector).

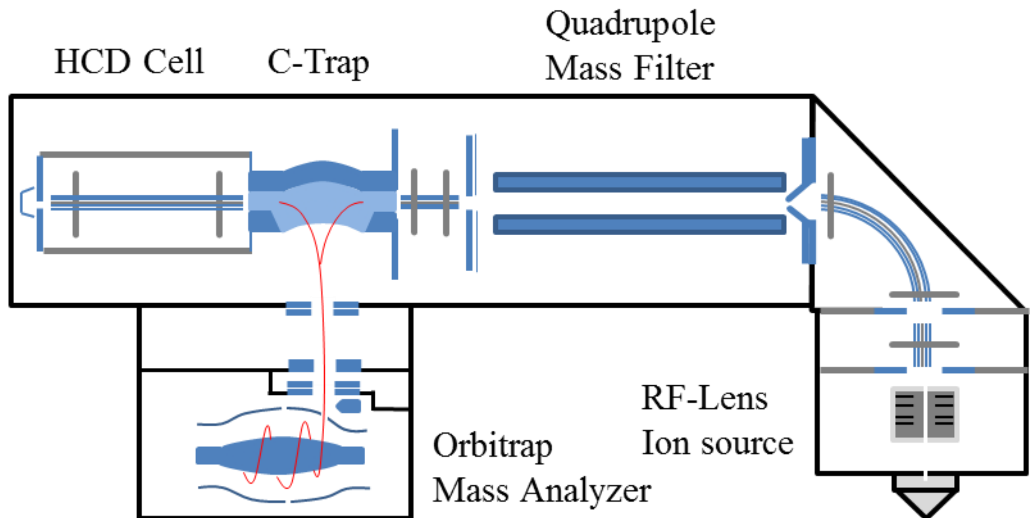


Figure 6. Schematic illustration of the process in Q Exactive Orbitrap mass analyser [Adapted from [51]].

In a Q Exactive MS the ions are transported from the ion source for separation from neutral molecules by a beam guide. The Q mass filter (see also **Chapter 2.3.4**) which is positioned between the optics and the detector is used for selection of the precursor ion. The higher energy collisional dissociation (HCD) cell is positioned next to the C-trap for fragmentation of the precursor ion. The Q mass filter allows transfer of ions with a specified mass range into the C-trap for relaxation [51]. In the Q Orbitrap MS the selected ions are transferred to the C-trap where the ions are relaxed and stored. In MS mode the ion are pulsed into the Orbitap mass analyser from the C-trap with the same injection energy. While in MS/MS mode the ions are transferred through the C-trap to the HCD cell. In the HCD cell the ions are fragmented by a collision gas (nitrogen) and subsequently transferred back to the C-trap for relaxation before they are pulsed into the Orbitrap mass analyser. Orbitrap mass analyser is composed of an outer barrel-like electrode and a spindle-like central electrode which is separated by an insulator. The outer electrodes have the shape of cups facing each other. The ions are injected between the central and outer electrodes. A voltage is applied between the outer electrode and the central electrode causing circulation of ions around the central electrode and oscillation along the horizontal axis at the same time. Outer electrodes are used as receiver plates for image current detection of these axial oscillations. The spin radius of the ions is dependent of their the *m/z* value which result in different frequencies, which will be detected and transformed to *m/z*-values by Fourier transformation [52].

2.3.4 Triple quadrupole mass analyser

A triple quadrupole (QqQ) MS is also called a tandem mass analyser (MS/MS) [53], and commonly used for quantitative analyses. The resolution of a triple quadrupole is 0.2 Da [54] and the accuracy is > 100 ppm [28]. The instrument consists of two quadrupole (Q1 and Q3) mass analysers and a collision cell (q2) positioned between the mass analysers [55]. A quadruple consists of four cylindrically formed identical rods. The ions enter an oscillating electrical field which is created between these rods. The both opposite rods are connected electrically to each other. The oscillating electrical field is created by applying a certain direct current (DC) and a radio frequency (RF) on one of the pair and the opposite DC and RF on the other pair. By varying the potential applied to the rods, ions of different masses are selected to reach the detector. Only ions with a specific *m/z* are stabilized through the rods to enter the detector when a certain DC and RF are applied. The ions with higher or lower *m/z* than the *m/*z range will be unstable and be ejected from the Q. The ions will be separated in

time by continuously increasing the potential. By selecting a range of potentials or single potentials only some ions will pass. A mass spectrum in a single Q appears when these ions hit a detector which makes signals with intensities corresponding to number of ions hitting. [28, 55]. In a QqQ, the quadrupoles are separated by the Einzel lenses in the gaps supporting the transition of ions through the instrument [56].

In MS/MS mode (**Figure 7**), the Q1 operates as a mass filter used to select the precursor ion and allows transfer into the q2. In q2 the collision gas (nitrogen, N2 or argon, Ar) is provided at a pressure of 10^{-8} to 10^{-6} bar to break the precursor ion into fragments called product ions. When the ions finally enter the Q3, the fragments will be analysed and be detected [27].

As described above, a QqQ allows MS/MS generating an increase in selectivity and sensitivity. The instrument may both do SRM/MRM and both of these are performed in this study. The principle of SRM is that one product ion from the precursor ion is monitored and of MRM that several product ions from the precursor ion are monitored [48]. The chromatograms using these modes are obtained by plotting the intensity of ions as a function of time. In an extracted ion chromatogram one or a set of chosen *m/z* is plotted. In a TICC the signal of a complete mass spectrum is plotted, meaning the sum of the different ion currents from the ions with different *m/z* values are plotted as a function of time.

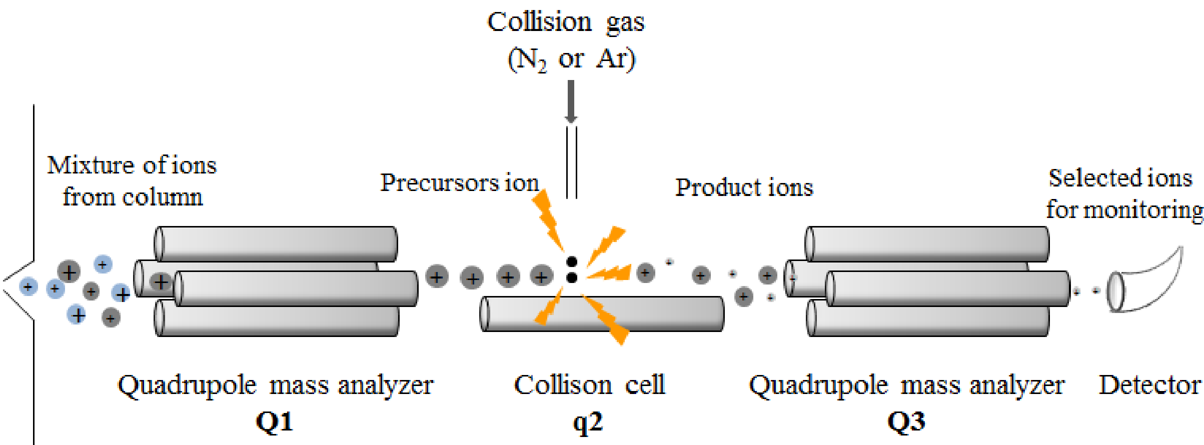


Figure 7 The principle of selected reaction monitoring [Adapted from [27]].

2.4 Methods used for detection of the APIs in water samples

One of the challenges in keeping water resources clean is that the mixture of the APIs used by the society is continuously changing. In this context, several publications [2, 57, 58] share experiences with techniques and methods suited for the detection of various the APIs in environmental water samples.

MS is often a preferred choice for detection of APIs in water samples, where either triple quadrupole tandem MS [59] or Orbitrap high resolution MS [3] is operated in either ESI mode or atmospheric pressure chemical ionization (APCI) mode. As reported by R. D. Briciu et al. [60] and M. Stas et al. [61], APCI and ESI are the currently used LC-MS interfaces. However, R. D. Briciu et al. report that low fragmentation repeatability was observed in the APCI interface for some APIs compared to the ESI interface. Generally, the ESI interface was suitable for the APIs investigated in their study and resulted in higher sensitivity compared to the APCI interface. ESI tandem quadrupole TOF-MS has also been used for screening of 13 APIs in water at low concentrations (1-100 ng/L) as described by Stolker at al. [62].

Since most of the APIs are polar and non-volatile and thermally labile, GC is not suitable as chromatographic method of the APIs without derivatization. Derivatization of the hydroxyl- and carboxyl-groups is required for GC detection. However, GC has been used by C. Hao et al. ([63]) for determination of selected APIs in water samples.

Although LC-MS (and GC-MS) is highly sensitive, sample preparation is necessary to remove possible interferences and enrich the analytes of interest to achieve the LODs required. A classical approach for environmental water sample preparation is SPE, which is generally used to isolate and enrich the analytes in environmental water samples. The sample preparation can either be performed off-line [3, 58] or on-line. In this context, off-line means that at least some steps in the sample preparation has to be done manually and that there is no physical connection between the SPE and the LC. As described ([2, 3, 58]) in the off-line approach, the operator has to collect the final eluent and place it in the injector/autosampler by hand. Filtration is usually the first step of the sample preparation if the subsequent extraction of the sample is based on the SPE. This is due to the suspended solids which could easily clog the adsorbent bed [2, 3, 60].

In the sample preparation using SPE, the column requires preconditioning prior to applying the aqueous sample to the column, the application is followed by elution. In addition in off-line SPE evaporation to dryness and enrichment of analyte by dissolving it in a low volume of an appropriate solvent is common. An ideal sample preparation step should be fast, accurate, and precise, and consume little organic solvent. Therefore automatization, ideally of the whole procedure is attractive. This eliminates the manual steps where loss of analytes is possible and external filtration which is time consuming. Compared to SPE, pressurized liquid extraction (PLE) [57] and liquid-liquid extraction (LLE) [64] are also suitable approaches for sample preparation of water samples. SPE is considered to be the most appropriate technique for sample preparation of water samples and it is preferred over conventional techniques such as LLE [64].

For the LC separation of APIs, a mixture of water-ACN or water-MeOH are frequently used with gradient elution by increasing the amount of organic solvent from 10 to 50 to 100%. ([3, 10, 65]) MP additives such as acetic acid or FA are used to improve MS detection of the APIs with ESI [64].

C_{18} as SP has been the most widely used SPE material for extraction of APIs, with efficient extraction from wastewater and surface water samples [64]. Analytical columns of different ID, length and particle size can be used for separation of APIs as seen in several publications ([3, 10, 58]). The analytical column used by B. J. A. Berendsen et al. was an UPLC analytical column (2.1 ID x 100 mm) with a particle size of 1.7 µm [65]. The same particle size, but a shorter column was preferred by R. Loos et al. [5]. However, a larger particle size has been used by S. Esteban et al. (C_{18}, 2 mm ID x 125 mm, 5 µm) [66] and by T. Benijs (C_{18}, 2 mm ID x 100mm, 3µm) [58]. The ID of analytical columns was generally of 2 mm, however 0.3 mm ID was used in the present study for improvement of sensitivity and reduction of radial dilution of APIs in the column by the MP.

2.5 The aim of the study

The aim of this study was to develop and validate a method for screening of selected APIs in surface water samples by using capillary high performance liquid chromatography mass spectrometry (capLC-MS). The method should provide high sensitivity and minimal sample preparation by using an AFFL-SPE-capLC-MS/MS platform.

This thesis work is a sub-project within a cooperation project between the University of Oslo and the University of Latvia.

3 Experimental

3.1 Chemicals and materials

3.1.1 Chemicals

Type 1 water was obtained from a Milli-Q Water Purification System delivered from Merck Millipore *(Billerica, MA, USA)* and Optima LC-MS water was purchased from Fisher Scientific AS *(Waltham, MA, USA).* Hipersolv Chromanorm HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from VWR International *(Radnor, Pennsylvania, USA).* Toluene was purchased from Rathburn Chemicals *(Walkerburn UK)*. Formic acid (FA, 98 % purity) was purchased from Sigma-Aldrich *(St. Louis, Missouri, USA).* The analytical standards of caffeine, ibuprofen, ketoprofen, naproxen, salicylic acid, diclofenac, acetaminophen, xylazine hydrochloride (HCl), carbamazepine, fluoxetine HCl, diazepam, clarithromycin, erythromycin (dihydrate), trimethoprim, azithromycin, sulfamethoxazole, ciprofloxacin, valsartan, losartan, propranolol HCl, atenolol, pravastatin (sodium salt hydrate), gemfibrozil, atorvastatin calcium, simvastatin, metoprolol tartrate salt, sulfamethoxazole and sulfapyridine were purchased from Sigma-Aldrich. All the analytical standards were of high purity grade (≥ 95 %).

3.1.2 Sample preparation equipment and consumables

For direct infusion MS experiments was an SGE Analytical Science syringe (500 µL) form Trajan Scientific and Medical *(Ringwood, Australia)* used in combination with a Fusion 101 Pump from Chemyx Precision Syringe *(Stafford, Texas, USA).* Automate pipettes were delivered from Thermo Fisher Scientific *(Waltham, MA, USA)*. A 5510 Ultrasonic bath was purchased from Branson *(St. Louis, MO, USA).* All solutions were prepared in Eppendorf tubes delivered from Eppendorf AS *(Hamburg, Germany).*

3.2 Standard stock solutions and sample

3.2.1 Standard stock solutions

The solvent used for making analytical standard solutions of the APIs are shown in **Table 4**. This information was obtained from the University of Latvia.

The standard stock solutions (SSSs) with the concentration of 1 mg/mL were prepared by weighing an appropriate amount of the solid standard which was dissolved in 10 mL of the chosen solvent (**Table 4**). All the solutions were sonicated for 25 minutes and acidified with a few drops of FA. The SSSs of 1 mg/mL were aliquated into 1.5 mL Eppendorf tubes (1 mL in each tube) and stored at -20 ˚C until the day of analysis. The preparations of SSSs were done by a visiting master student, Diana Dzabijeva from the University of Latvia. Sulfapyridine was prepared by PhD Hanne Røberg-Larsen in the same way as the other analytical standards.

Table 4 The APIs and their corresponding stock solvents used for preparation of the stock solutions (1 mg/mL).

Name of compound	Stock solvent
Acetaminophen	ACN/MeOH (80/20)
Atenolol	ACN/MeOH (80/20)
Atorvastatin calcium salt	ACN
Azithromycin	ACN
Caffeine	ACN
Carbamazepine	ACN
Ciprofloxacin	Toluene/MeOH (50/50)
Clarithromycin	ACN/MeOH (60/40)
Diclofenac sodium salt	ACN/MeOH (80/20)
Erythromycin (dihydrate)	ACN
Fluoxetine	ACN/MeOH (80/20)
Gemfibrozil	ACN
Ibuprofen	ACN
Ketoprofen	ACN
Losartan (potassium)	ACN/MeOH (80/20)
Metoprolol	ACN/MeOH (80/20)
Naproxen	ACN/MeOH (80/20)
S-Propranolol (hydrochloride)	ACN/MeOH/H_2O (50/25/25)
Pravastatin (sodium salt hydrate)	ACN
Simvastatin	ACN
Sulfapyridine	ACN
Sulfamethoxazole	ACN
Trimethoprim	ACN
Valsartan	ACN
Xylazine (hydrochloride)	ACN

3.2.2 Working solutions

SSSs of 1 mg/mL were diluted using MeOH/type 1 water (50/50, v/v) with 0.1% FA, to obtain appropriate working solutions: A_1 (100 µg/mL), A_2 (10 µg/mL) and A_3 (1 µg/mL) (**Table 5**) and stored in Eppendorf tubes at -20 ˚C. All subsequent dilutions were done with solvent B* consisting of 0.1 % FA in type 1 water (see **Table 5**) and were stored at 4 ˚C. **Figure 8** illustrates how the standard solutions (working solutions) were prepared from the standard stock solution.

Table 5 Working solutions with the name given of the series, the solvent and their concentration. A*: dissolved in MeOH/type 1 water (50/50, v/v) with 0.1 % FA and B*: dissolved in type 1 water with 0.1 % FA.

Series name	Solvent	Concentration	Unit
SSS	SSS solvent	1	mg/mL
A_1	A*	100	µg/mL
A_2	A*	10	µg/mL
A_3	A*	1	µg/mL
A_4	B*	100	ng/mL
A_5	B*	10	ng/mL
A_6	B*	1	ng/mL
A_7	B*	100	ng/L
A_8	B*	10	ng/L

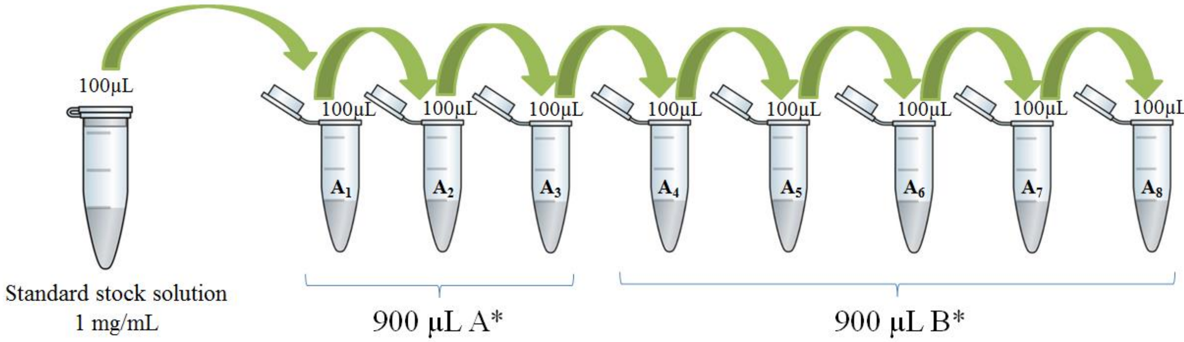


Figure 8 A general illustration of how the working solutions were prepared and µL of solvent used for dilution for all the APIs. A*: dissolved in MeOH/type 1 water (50/50, v/v) with 0.1% FA and B*: dissolved in type 1 water with 0.1 % FA.

3.2.3 Internal standard solutions

The internal standards sulfamethoxazole-(phenyl-$^{13}C_6$) (99.5 %), atenolol-d7 (≥97 % isotopic purity) and fluoxetine-d5 hydrochloride (98 atom % D) were purchased from Sigma-Aldrich and were pre-weighed by the supplier (see **Table 6**). The standard of atenolol-d7 was dissolved in 5 mL of the ACN/MeOH (80/20, v/v) and fluoxetine-d5 hydrochloride was dissolved in 10 mL of ACN/MeOH (80/20, v/v). The Sulfamethoxazole-(phenyl-$^{13}C_6$) solution was prepared by PhD Hanne Røberg-Larsen, by dissolving the solid standard in 10 mL of ACN. Preparation of IS_1, IS_2 and IS_3 were done by further dilution from stock solutions using solvent A*. All subsequent dilutions were done with solvent B*. Stock solutions and IS_1, IS_2 and IS_3 were stored in Eppendorf tubes at -20 ˚C. The working solutions IS_4-IS_6 were stored at 4 ˚C. Concentration of stock solutions and working solutions are shown in **Table 7**. **Table 8** shows the IS used for the API.

Table 6 Pre-weighed analytical standards from supplier and the stock solvent used for dilution of each IS.

Name of internal standard	Stock solvent	Amount of purchased IS (mg)
Atenolol-d7	ACN/MeOH (80/20)	2
Fluoxetine-d5 hydrochloride	ACN/MeOH (80/20)	10
Sulfamethoxazole-(phenyl-$^{13}C_6$)	ACN	10

Table 7 Concentration of stock solutions and working solutions prepared. The A* indicates that the dilution was done by using MeOH/type 1 water (50/50, v/v) and 0.1 % FA, while B* indicates that the dilution was done by using in type 1 water with 0.1 % FA.

	Atenolol-d7	Fluoxetine-d5 hydrochloride	Sulfamethoxazole-(phenyl-$^{13}C_6$)	Unit
Stock solution	0.4	1	1	mg/mL
IS_1 (A*)	100	100	100	µg/mL
IS_2 (A*)	10	10	10	µg/mL
IS_3 (A*)	1	1	1	µg/mL
IS_4 (B*)	100	100	100	ng/mL
IS_5 (B*)	100	100	10	ng/mL
IS_6 (B*)	1	1	1	ng/mL
IS_6 (B*)	100	100	100	ng/L

Table 8 The internal standard used for each API in method development and validation.

Internal standard	**Atenolol-d7**	**Fluoxetine-d5 hydrochloride**	**Sulfamethoxazole-(phenyl-$^{13}C_6$)**
Analyte (s)	Atenolol	Fluoxetine	Acetaminophen Sulfapyridine Sulfamethoxazole Trimethoprim Xylazine

3.2.4 Water sample collection and preparation

The water sample was collected 27th Mars 2017 in the Sognsvann creek located in Oslo (**Figure 9**). The coordinates of the sampling point are *59.948842, 10.712272*. The sample was transported to the laboratory and one part was filtrated through a non-pyrogenic 0.2 µm cellulose acetate membrane filter (7 bar max, FP 30/0.2 CA-S) from Schleicher and Schuell *(Dassel, Germany).* The filtrated sample was acidified with 0.1 % FA and stored at 4 ˚C before analyses. One part of the non-filtrated water sample was stored at -20 ˚C, while it was acidified with 0.1 % FA and stored at 4 ˚C before analysis.

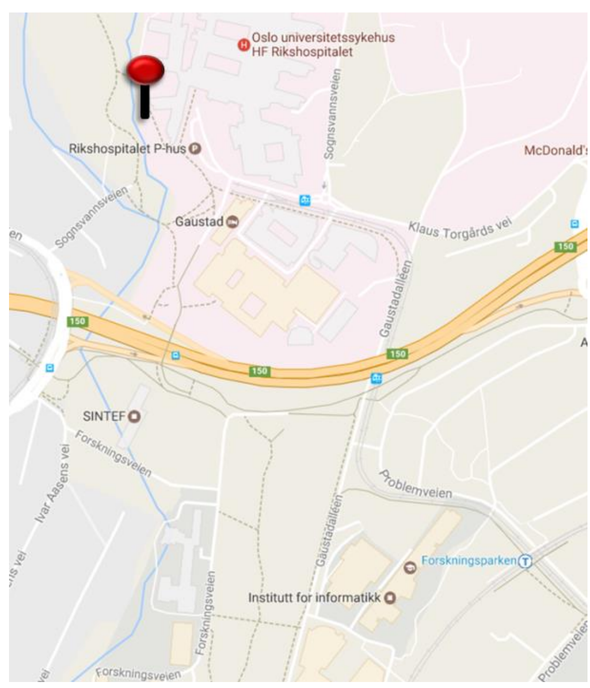


Figure 9 Map of the location of the Sognsvann creek (Rikshospitalet) where the water sample was collected.

The filtrated and acidified water sample from the Sognsvann creek was added the internal standards of different concentrations and analysed using the Quantiva QQQ MS set-up. **Figure 10** illustrates the preparation of the water sample from the Sognsvann creek by adding the internal standards.

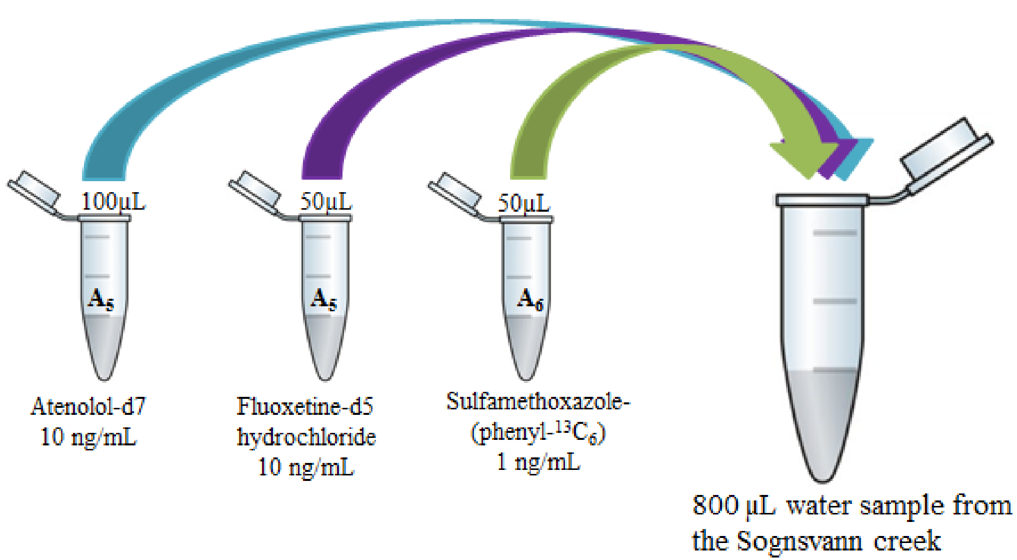


Figure 10 An 800 µL water sample from the Sognsvann creek was filtrated through a non-pyrogenic 0.2 µm cellulose acetate membrane filter and acidified (0.1 % FA). The filtrated water sample was added different concentrations of the internal standards: 1000 ng/L of atenolol-d7, 500 ng/L of fluoxetine-d5 hydrochloride and 50 ng/L of sulfamethoxazole-(phenyl-$^{13}C_6$).

3.2.5 Validation standard solutions

Validation solutions were prepared at concentration levels 10-100 ng/L (**Table 9**). The validation solutions were thoroughly mixed by vortex and aliquoted into vials and analysed by the final LC-MS method. The validation solutions were made in three matrices, type 1 water with 0.1 % FA (MP A) and acidified filtrated and no-filtrated water sample from the Sognsvann creek. For the IS used for each analyte, see **Table 8.** The same concentrations of both the APIs and the internal standards were used to make the calibration curve and linearity curves.

Table 9 Validation solutions (VAL 1-5) with the spiked concentrations of the APIs and the internal standards: atenolol-d7 and fluoxetine-d5 hydrochloride were of ≥ 95 % HPLC purity and sulfamethoxazole-(phenyl-$^{13}C_6$) of 98 % HPLC purity. Solvent of HPLC grade was used for the dilutions.

	Concentration of APIs	Concentration of atenolol-d7	Concentration of fluoxetine-d5 hydrochloride	Concentration of sulfamethoxazole-(phenyl-$^{13}C_6$)
VAL 1	10 ng/L	1000 ng/L	500 ng/L	50 ng/L
VAL 2	25 ng/L	1000 ng/L	500 ng/L	50 ng/L
VAL 3	50 ng/L	1000 ng/L	500 ng/L	50 ng/L
VAL 4	75 ng/L	1000 ng/L	500 ng/L	50 ng/L
VAL 5	100 ng/L	1000 ng/L	500 ng/L	50 ng/L

3.2.6 MS tune solutions

MS tune solutions for the Quantiva QQQ MS and the Q Exactive MS were prepared with a concentration of 10 µg/mL (A_2) for each of the APIs. These solutions were used for tuning of the MS parameters by direct infusion. The MS tune solution was used for tuning of the MRM transitions on the Quantiva QQQ MS, and PRM transitions the Q Exactive MS. The *m/z* of the precursor ion was observed in full scan, before the optimization of the conditions was started. When using the Quantiva QQQ MS, parameters such as collision energy and RF-lenses were optimized and the product ions were identified. Low mass exclusion was used and set to *m/z* 90 for the both mass analysers. Optimization of the Q Exactive MS was done manually by changing the fragmentation energy (NCE) and observing the intensity of the fragments.

3.3 Instrumentation

3.3.1 The AFFL-system

The system consisted of two pumps: an Agilent 1100 series G1378A CapPump from Agilent Technologies *(Palo Alto, CA, USA)* as the gradient pump, and an Agilent 1100 series G1310A QuatPump as the loading pump. Column switching was performed with a CapLC® selector 10-port two-position switching valve from Waters *(Milford, Massachusetts, US).* Injections were done by a G1313A ALS standard autosampler *(Agilent Technologies)* either with a 100 µL loop or with an extended loop (1500 µL). By using an external loop, multiple injections of 100 µL could be combined into a larger volume.

The SPE-column HotSep (AQ Kromasil C_{18}, 5 µm particles, 1 mm ID x 5 mm) and the analytical columns HotSep (C_{18}, 2 µm particles, 0.3 mm ID x 100 mm or 150 mm) were from G&T Septech AS *(Ski, Norway)* and were used in preliminary experiments. An ACE column (C_{18}, 3 µm particles, 0.3 mm ID x 150 mm) from Advanced Chromatography Technologies *(Aberdeen, Scotland)* was used as the analytical column for method validation. The SPE-column used for method validation was a HotSep (Kromasil C_{18}, 5 µm particles, 1 mm ID x 5 mm) from G&T Septech AS.

In preliminary experiments the waste tubing from the valve was connected to a UV detector (**Figure 11**), to observe possible breakthrough from the SPE-column.

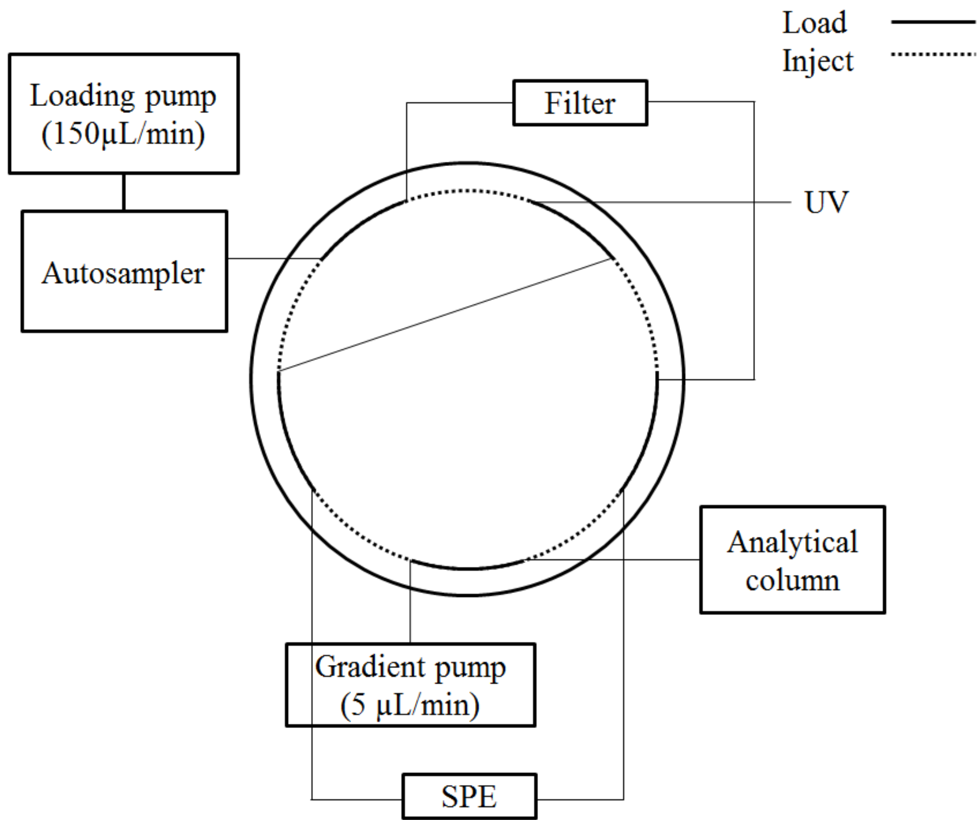


Figure 11 The LC-UV set-up for measurement of breakthrough.

The AFFL-system had a 2 µm stainless steel filter screen placed inside a union (1/16″, 0.25 mm bore) both purchased from Vici (Valco Instrumens Co. Inc, *Houston, Texas, USA*).

The tubings used in the system were Polymicro Technologies™ fused silica capillary tubings from Molex *(Wellington court, IL, US),* with IDs of 50 µm and 100 µm. **Figure 12** shows the AFFL set-up with tubing ID.

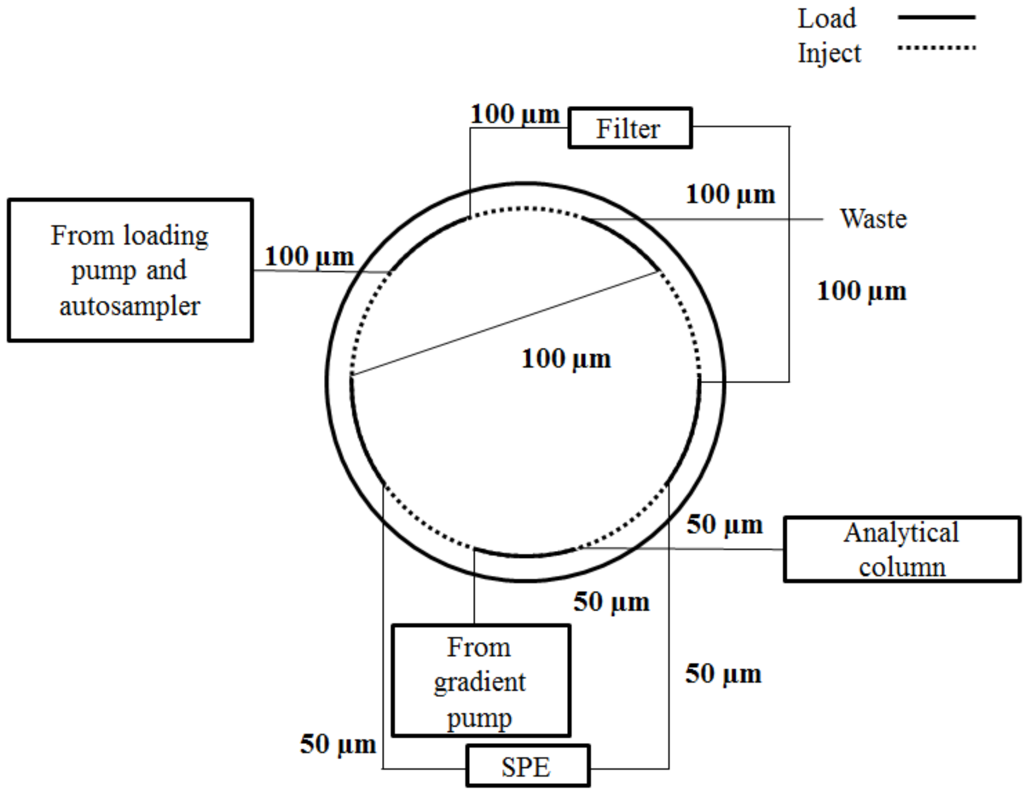


Figure 12 The AFFL-SPE 10-port-valve set-up in back flush mode, with ID of the tubings used.

3.3.2 The gradient programs

The flow rate of the gradient pump was set to 3.5 µL/min and maximum pressure of 400 bar. In preliminary experiments, the analytes were separated using a 15 minutes gradient program (1), see **Table 10**. The total analysis time was 20 minutes. Analytes were loaded in 1.6 minutes in loading position, by using MP A (non-eluting conditions, type 1 water with 0.1 % FA). After loading, the valve was switched to inject position. The gradient program started from 35 % MP B (MeOH with 0.1 % FA) to 95 % B in 7 minutes and was held at 95 % for 7 minutes, before returned to starting conditions which was held for 6 minutes.

Table 10 Gradient program (1) used for separation of APIs. Mobile phase (MP) A was type 1 water with 0.1 % FA and MP B was MeOH with 0.1 % FA.

Time (min)	MP B (%)
0	35
7	95
13	95
14	35

The gradient program was later optimized. The analytes were separated in 10 minutes (**Table 11**). Analytes were loaded in 1.6 minute in loading position, and after loading the valve was switched to inject position. The gradient program started from 47 % MP B and increased to 99 % MP B in 4 minutes and was held at 99 % for 4 minutes. The total analysis time was 15 minutes including 6 minutes reconditioning.

Table 11 Gradient program (2) used for separation of APIs selected for the current method. Mobile phase (MP) A was type 1 water with 0.1 % FA and MP B was MeOH with 0.1 % FA.

Time (min)	MP B (%)
0	47
4	99
8	99
9	47

3.3.3 The detectors and the mass analysers

The detectors used were either a Dionex Ultimate 3000 RS variable wavelength UV detector (now Thermo Scientific, Waltham, MA, US) operated at 254 nm, a TSQ Quantiva (Quantiva QQQ) MS or a high-resolution mass spectrometer (Q Exactive Orbitrap) both from Thermo Scientific. The Q Exactive MS was used in preliminary experiments, while the TSQ Quantiva was used for method validation. The optimal conditions for both mass analysers were obtained by tuning of the analytes by direct infusion. The Quantiva QQQ MS was operated in MRM mode, while the Q Exactive MS was operated in target MS^2 mode (PRM).

The MRM transitions by using Quantiva QQQ MS and the PRM transitions by using Q Exavtive MS were obtained by tuning of the analytes by direct infusion. The default values of sheath gas and auxiliary gas were used in arbitrary units (a.u.). Both mass analysers were controlled by the software Xcalibur, version 3.0. The ESI-MS parameters of each mass analyser are given in (**Table 12**). The precursor ion and the product ions of each analyte were identified during tuning by direct infusion, see **Appendix, Chapter 7.2, Table A-1** and **Table A-2**.

Table 12 The parameters used for the Quantiva QQQ MS and the Q Exactive MS. (– indicates that the instrument does not have a value) a.u. is arbitrary units.

	Parameters	Q Exactive MS	Quantiva QQQ MS
Ion source	Ion source type	H-ESI-source	H-ESI-source
	Polarity	Positive	Positive
	Spray voltage	3500	3500
	Sheat gas (a.u.)	5	5
	Auxillary gas (a.u.)	1	1
	Ion transfer tube temperature (°C)	350	325
	Vapoization temperature (°C)	50	50
Mass analyser	CID gas (mTorr)	-	0.5
	Type of fragmentation gas	HCD cell: N_2	q2: Ar
	Collision energy (V)	PRM dependent	MRM dependent
	Resolution	35 000	Q1: 0.2 Da
		-	Q3: 0.4 Da
	AGC target in HCD cell	$2e^5$	-
	Maximum injection time (ms)	100	-
	Cycle time (s)	-	1
	Scan range (*m/z*)	100-1000	100-1000

3.4 Quantification

The calibration curves used to quantify the APIs in filtrated water samples was constructed using linear regression by using Microsoft Excel (2010 version). The ratio of analyte peak area and the IS peak area were plotted as a function of the concentration of the analyte (**Equation 7**), see **Chapter 3.2.5** for concentrations used. The quantifier and qualifier characterizing the target analyte for identification were established based on the highest intensity of the target analyte ions. The precursor ion, product ion and their collision energy and RF-lens values are given in **Table 13**. The extracted ion chromatograms (EIC) were smoothened (Gaussian smoothing by 9 points) before integration and integration of the peak areas were performed manually.

Table 13 The precursor ions, product ions, collision energies and the RF-lens values of the APIs and internal standards (IS). * indicates that the *m/z* was used as a quantifier and ** indicates that the *m/z* was used as the qualifier. The numbers in italic were removed from the method to minimize background noise and interferences.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	RF-lens (V)
Acetaminophen	152.075	110.076*	28	66
		93.112**	20	66
		109.383	28	66
Atenolol	267.205	145.130*	34	72
		190.064**	23	72
		133.087	39	72
Atenolol-d7 (IS)	274.340	145.0990*	38	70
		190.124**	26	70
		133.133	42	70
Fluoxetine	310.205	148.065*	8	53
		117.087**	59	53
		183.073	56	53
Fluoxetine-d5 HCl (IS)	315.265	153.089*	12	50
		122.245**	59	50
		314.177	12	50
Sulfapyridine	250,045	184.050*	24	67
		155.955**	25	67
		92.060	37	67
Sulfamethoxazole	254.010	155.980*	23	67
		108.040**	33	67
		92.065	37	67
Sulfamethoxazole-(phenyl-$^{13}C_6$) (IS)	260.000	161.960*	23	64
		114.030**	34	64
		98.12	38	64
Trimethoprim	291.150	230.140*	31	85
		260.999**	34	85
		123.165	35	85
Xylazine	221.095	120.141*	45	77
		164.064**	33	77
		105.121	45	77

3.5 Calculations

Mixtures of the APIs with increasing concentrations (10, 25, 50, 75 and 100 ng/L) were prepared in MP A, filtrated and non-filtrated water sample from the Sognvann creek. The same APIs were spiked in the same matrix on three consecutive days and analysed. The number of replicates (n) for each concentration spiked in MP A, filtrated and non-filtrated water sample are given in **Table 14**. The ratios between the peak area of the analyte and that of the IS of the corresponding analyte were calculated by Microsoft Excel (version 2010). An example of calculated ratios is shown in **Table 15**.

Table 14 Number of replicates of each concentration of the APIs in MP A, filtrated and non-filtrated water sample from the Sognsvann creek.

Concentration (ng/L)	MP A Number of replicates:	Filtrated/Non-filtrated water sample Number of replicates:
10	10	6
25	3	3
50	10	6
75	3	3
100	10	6

Table 15 Example of calculated ratios between the peak area of the analyte (X) and the peak area of the corresponding internal standard (IS). The replicates in consecutive days (day 1, day 2 and day 3) of the analyte with same concentration in the same matrix.

Compound X		Peak area of analyte X/ Peak area of corresponding IS		
Concentration of analyte (ng/L)	**REPLICATE**	**DAY 1**	**DAY 2**	**DAY 3**
	1	0.20	0.30	0.24
	2	0.22	0.24	0.21
	3	0.24	0.33	0.24
	4	0.21	0.27	0.24
	5	0.20	0.29	0.24
	6	0.21	0.26	0.23
	7	0.24	0.26	0.23
	8	0.20	0.29	0.22
	9	0.20	0.29	0.25
	10	0.20	0.28	0.22

3.5.1 Repeatability

Single Factor Anova

A typical summary output of a single factor Anova test done by Microsoft Excel for an analyte with a specific concentration and matrix is given in **Figure 13**. The standard deviation (SD) for within-day (red) and between-day (blue) replicates was calculated for all concentrations in all matrices. The total SD (green) was used as error bar for that concentration of the analyte in the figures.

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	2.11341275	0.21134127	0.000289671		
DAY 2	10	2.81742540	0.28174254	0.000575967		
DAY 3	10	2.29362470	0.22936247	0.000140311		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.02674925	2	0.013374623	39.88655276	8.69312E-09	3.35413
Within Groups	0.00905355	27	0.000335317			
Total	0.03580279	29				

Figure 13 The *count* is the number of replicates analysed for the same concentration each *group* (day). The sum, average and variance of the replicates each day are calculated. The *between group* variance (blue) and *within group* variance (red) are summed as *total* (green), which is used as standard deviation (SD) for the API in a specific matrix and of a specific concentration.

3.5.1 The linearity curve and regression analysis

Linearity curves for each API were established for day 1, day 2 and day 3 in same matrix. The calculated average for each day by the single factor Anova was used to calculate the average of the days (the mean). In addition, the mean for day 1, day 2, and day 3 was plotted for each matrix, see **Table 16**. By plotting the mean as a function of concentration (10-100 ng/L) and using the calculated SD as error bar (**Table 16**) the figure was plotted (representing three consecutive days for each matrix).

Table 16 Example of the calculated mean (the average) of the average of each day and the standard deviation (SD) used as error bar (calculated by single factor Anova, see Figure 13) for increasing concentration of API in matrix.

Concentration (ng/L)	Mean (A A SM /A SM-IS)	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.24	0.04	15	3	10
25	0.60	0.03	5		
50	1.09	0.16	14		
75	1.56	0.09	6		
100	2.08	0.42	20		

By doing regression analysis using Microsoft Excel with a 95 % confidence level, the linear correlation (R^2) of the curve, for an API in the matrix was established. An example of a summary output of a regression statistics is shown in **Figure 14**. The number bolded in green is the R^2 of the curve. The precision, for all APIs, was expressed as relative standard deviation (RSD %) which was calculated by using the **Equation 7**.

$$RSD\ \% = \frac{SD}{Mean} \times 100 \qquad \textbf{(7)}$$

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,99958421
R Square	**0,99916859**
Adjusted R Square	0,99889145
Standard Error	0,02448111
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2,160756329	2,160756329	3605,319026	1,0177E-05
Residual	3	0,001797974	0,000599325		
Total	4	2,162554303			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0,06649405	**0,020589136**	3,229569889	0,048231614	0,000970234	0,132017872	0,000970234	0,132017872
X Variable 1	**0,02013443**	0,000335326	60,04430886	1,0177E-05	0,01906727	0,021201585	0,01906727	0,021201585

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0,26783833	-0,027022903	-1,274589455
2	0,56985475	0,025862125	1,219839039
3	1,07321544	0,015800836	0,745278169
4	1,57657614	-0,011905598	-0,561551417
5	2,07993683	-0,002734461	-0,128976336

Figure 14 A typical summary output of a regression analysis using Microsoft Excel. The number bolded in green is the linearity of the curve, while the numbers bolded in red are used for calculations of concentration limit of detection (cLOD) and concentration limit of quantification (cLOQ).

3.5.1 Limit of detection and limit of quantification

The numbers bolded in red (**Chapter 3.5.1 Figure 14**) were used for calculations of cLOD and cLOQ as shown in the guideline from ICH Harmonized Tripartit [67]. The calculated *Standard Error* represents the SD, while the *X Variable 1* represents the slope of the curve. These values were used to calculate the cLOD and cLOQ by using **Equation 8** and **Equation 9**, respectively [43, 67].

$$cLOD = 3 \times \frac{SD}{slope} \qquad \textbf{(8)}$$

$$cLOQ = 10 \times \frac{SD}{slope} \qquad \textbf{(9)}$$

3.5.2 Apparent recovery

Apparent recovery (The $R'A$ %) was investigated for analytes spiked in filtrated and non-filtrated water sample. The calculations were performed by using filtrated and non-filtrated water sample as the quantity observed value, *slope a*, while the *slope b* represented the slope of MP A as the reference value [68]. Thus, by multiplied with 100 the apparent recovery was calculated in percentage.

$$R'A\,\% = \frac{slope\ a}{slope\ b} \times 100 \qquad \textbf{(10)}$$

4 Results and discussion

4.1 Method development

The initial aim of this study was to develop a sensitive method for screening of selected APIs in environmental water samples. To achieve low enough detection limits (low ng/L concentration) capLC-MS with large volume injection was chosen. For enrichment of analytes and removal of unwanted salts and particles an in-house built AFFL-SPE system was chosen for the method. By combining large volume injections (100 µL) with the AFFL-system and narrow column (0.3 mm ID), the wanted low cLOD (ng/L range) could be reached with a sensitive MS instrumentation.

The initial goal was to develop a method for the APIs presented in **Table 1.** However**,** during method development several of the APIs were removed from the method for various reasons.

The flow sheet in **Figure 15** shows the main steps in the method development and validation, and where some APIs were removed from the method.

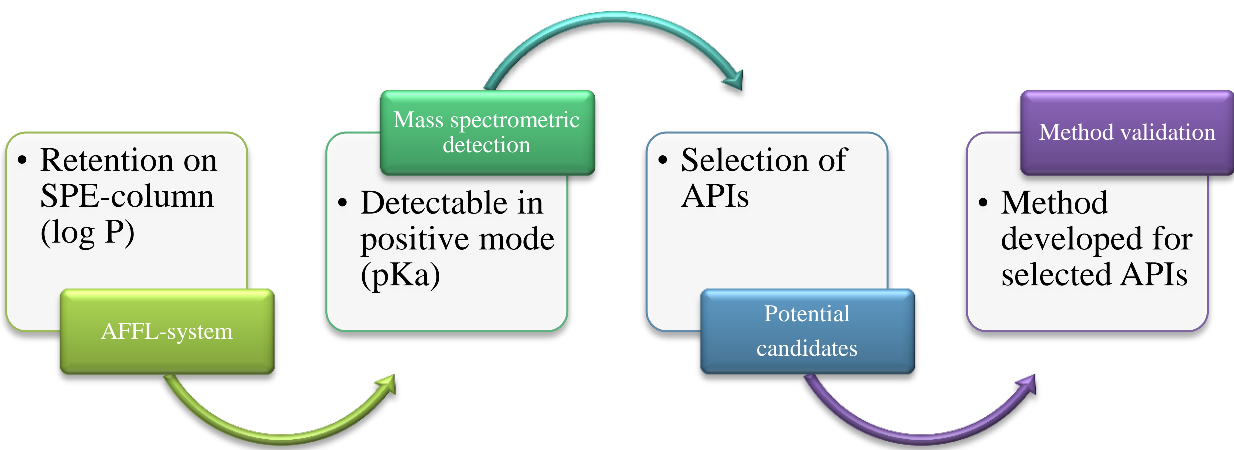


Figure 15 Flow sheet if the steps in the method development. The APIs need to have retention on the SPE-column incorporated into the AFFL-system to be included in the method. APIs without retention on the SPE-column will be eliminated from the method. The APIs with retention on the SPE-column was further monitored with the mass analyser. In mass spectrometric detection, the APIs needed to be detectable in positive mode to be potential candidates for the method. A further selection of APIs was done due to time limitation in the project.

4.1.1 Optimlaization of the AFFL-SPE-LC system

Injection volume

In order to obtain as low as possible cLOD, large injection volumes are preferred. To investigate if the analytes had sufficient retention on the SPE-column, the loading capacity was investigated using the AFFL-system and UV detector operated at 254 nm (**Figure 11**). An external loop was installed into the injector to increase the possible injection volume up to 1500 µL. A solution of caffeine was used as a test solution to investigate possible overloading and breakthrough on the SPE-column. Among the APIs, caffeine was the analyte with the theoretically lowest hydrophobicity (log P=-0.13) and acidity (pK_a (strongest basic)=0.92), and was expected to have the lowest retention on the column. The prediction was that the other APIs should be better retained. By injection of increasing volumes **(Appendix Chapter 7.3, Table A-3)** of the caffeine standard solution onto the SPE-column under non-eluting conditions, the volume loading capacity was estimated. Breakthrough (eluting of analyte during loading) of the SPE-column was found by an increase in UV absorbance during loading of the sample. The loading flow rate was 150 µL/min and the total volume of the sample loop within the injector was 1500 µL (meaning that 10 minutes loading time was needed for empting the loop). An injection volume of 800 µL was found to be retained on the SPE-column, without breakthrough. Thus, in some preliminary experiments 800 µL were used as injection volume.

However, in later experiments using LC-MS unstable signal intensities were observed. This issue was addressed to injection, and new experiments showed decreased sensitivity with 800 µL injection volume compared to 100 µL (**Figure 16**). The used SPE-column was unable to retain the injected amount and some of the injected amount was sent to waste during loading.

Hence, the external loop was removed and 100 µL was further used as injection volume.

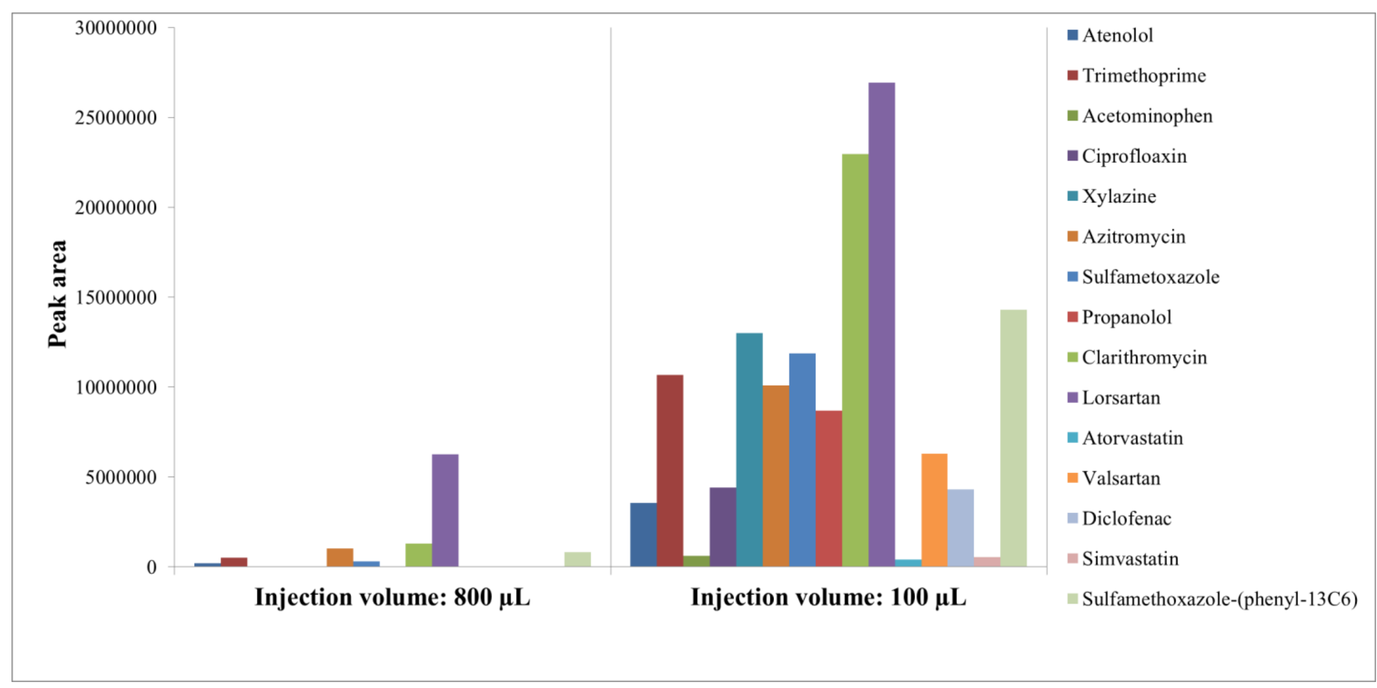


Figure 16 The peak area of selected APIs injecting 800 µL with a concentration of 25 ng/L (corresponding 0.02 ng on the column) in minutes 10 minute versus 100 µL with a concentration of 100 ng/L (corresponding to 0.01 ng) on column in 1 minute on the AFFL-SPE-LC set-up. The loading mobile phase was type 1 water with 0.1 % FA with a loading flow rate of 200 µL/min in both cases.

Gradient elution

The gradient program 1 which was used in initial experiments was later optimized for the selected APIs for the current method (the gradient program 2). In order to get faster analysis the gradient was started at 47 % organic (MP B) instead of 35 % (gradient program 1), meaning shorter retention time of APIs. In order to elute the APIs earlier, the final percentage of MP B was increased to 99 % instead of 95 %.

Hence, gradient program 2 was used for the selected APIs in the current method.

Loading flow rate and gradient flow rate

In order to obtain faster analysis the loading flow rate (pump 2) was increased from 150 µL/min to 200 µL/min. However, this increase resulted in reduced peak intensity and distorted peak shape of some compounds in combination with gradient 2. Therefore the loading flow rate was kept at 150 µL/min and the sample was loaded within 1.6 minutes.

The gradient flow rate (pump 1) in preliminary experiments was 3.5 µL/min. In order to obtain shorter gradient and faster analysis, the gradient flow rate was increased to 4 µL/min. This did not affect the peak intensity (**Figure 17**).

By changing the gradient flow rate and gradient program the APIs were separated within 10 minutes and the total analysis time was decreased from 20 minutes to 15 minutes including 6 minutes for reconditioning.

Hence, 4 µL/min was further used as the gradient flow rate.

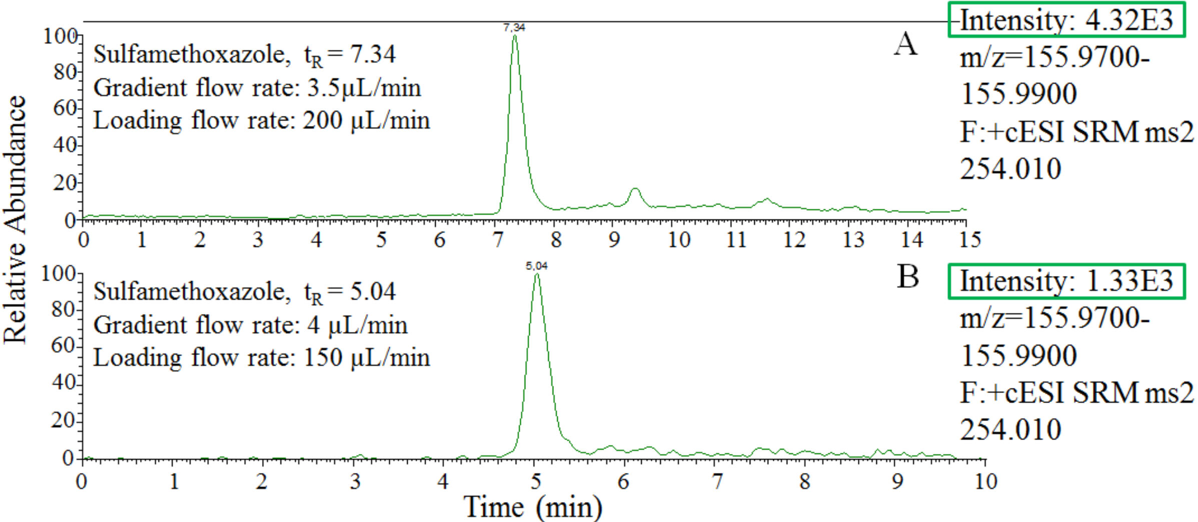


Figure 17 EIC of sulfamethoxazole (10 ng/L) with different gradients (A: gradient 1, B: gradient 2). The chromatographic system in A and B consisted of an AFFL-SPE-LC-MS set-up with a Hotsep Kromasil 1 mm x 5 mm C_{18} (5 µm) SPE-column. A HotSep 0.3 mm x 150 mm C_{18} (2µm) was used in A as the analytical column, while an ACE 0.3 mm ID x 150 mm C_{18} (3µm) was used in B. The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA. The detection was carried out by the Quantiva QQQ MS.

Organic solvents

ACN (LC-MS-grade) was used as the organic solvent in the experiments with UV detection and initial experiments with MS detection. Sensitive MS detection of the APIs (i.e. ionization) can be affected by the organic solvent used [69], thus ACN and MeOH (LC-MS-grade) as organic modifier was compared by PhD Hanne Røberg-Larsen. MeOH improved ionization of most of the APIs **(Appendix Figure A-3 and A-4).**

Hence, MeOH was chosen as the organic solvent in the method.

Retention of the APIs on SPE-column

Retention of the APIs on the chosen SPE-column was investigated by individual injections of 800 µL (100 ng/mL) on the AFFL-SPE-LC-UV set-up. It was expected that acidic compounds having low hydrophobicity, were poorly retained on the SPE-column, and lost to waste. By connecting the waste tube to the UV detector (**Figure 11**), the analytes not retained on the SPE-column could be detected, see **Table 17**.

Table 17 Retention (YES or NO) of APIs on the SPE-column (Hotsep Kromasil C_{18} 1 mm ID x 5 mm, 5 µm) was investigated using the AFFL-LC-UV set-up (Figure 11). Only the loading pump with type 1 water with 0.1 % FA at a flow rate of 150 µL/min was utilized, with the injector set in loading position throughout the experiment. The UV detector operated at 254 nm.

Name of compound	Retention on SPE-column (YES or NO)
Acetaminophen	YES
Atenolol	YES
Atorvastatin	YES
Azithromycin	YES
Caffeine	YES
Carbamazepine	YES
Ciprofloxacin	YES
Clarithromycin	YES
Diclofenac sodium salt	YES
Erythromycin a dihydrate	YES
Fluoxetine hydrochloride	YES
Gemfibrosil	YES
Ibuprofen	YES
Ketoprofen	YES
Losartan potassium	YES
Metoprolol tartrate salt	YES
Naproxen	YES
S-Propranolol hydrochloride	YES
Pravastatin sodium salt hydrate	YES
Salicyclic acid	NO
Simvastatin	YES
Sulfamethoxazole	YES
Trimethoprim	YES
Valsartan	YES
Xylazine hydrochloride	YES

Salicylic acid was removed from the method because of no retention on the SPE-column.

Technical challenges with the AFFL-SPE-LC system

Sample clean-up and enrichment were achieved using the rugged AFFL-system with the SPE-column without pressure build-up. In some cases pressure build-up was however observed and this constitutes the drawback of such a system as well as the narrow tubings and dead volumes. Pressure build-up indicates clogged tubings, filter in AFFL, filter in the MP reservoirs or clogged columns. Particles from the rotor and/or stator in the valve may cause pressure build-up. Particles not removed by the incorporated filter may be transferred to the SPE-column and cause pressure build-up, which is easily observed by the gradient pump. If the filter is clogged by particles this is observed by increased pressure on the loading pump. If filter and SPE-column are changed routineously, the use of such a system with incorporated clean-up and analyte enrichment is rugged.

Maintenance of AFFL-SPE-LC-system is needed to ascertain correct delivery of the MPs, injection volume (auto sampler) and flow rate both of the gradient pump and loading pump. Deviation may either be observed by the pressure or visible errors (e.g. leakage) in the LC set-up. Correct delivery of the MPs and injected volume by the autosampler is not visible, and need to experimentally be measured. In order to investigate clogged narrow tubings, the tubing was disconnected, and observed if the pressure went down. The tubings were not changed at the first time, but the ends which are usually where the tubings may be clogged were removed.

4.1.2 Optimization of the mass spectrometric parameters

The MS parameters need to be optimized to obtain low cLOD. Both a triple Q MS and a Q Orbitrap MS allows identification of a compound by a precursor ion and its product ions. In positive mode, the precursor ion will have a mass of $[M+nH]^{n+}$, where n=1 with an ESI interface for these APIs. The MRM transitions, the precursor and the product ions were optimized using both a Quantiva QQQ MS **(Appendix Chapter 7.2, Table A-1)** and a Q Exactive MS **(Appendix Chapter 7.2, Table A-2)** in preliminary experiments. The Quantiva QQQ MS was used for final method development and validation due to practical issues (available instrument time). For the quantifier, the qualifier, the collision energy and the RF-lens values for selected APIs, see **Table 13**. Direct infusions of the APIs were done on the Quantiva QQQ and the Q Exactive MS using a concentration of 10 µg/mL of standard solutions (A_2) both in preliminary and final experiments.

In preliminary experiments the analytes were monitored by MRM on the Quantiva QQQ MS. The total ion current chromatogram (TICC) (**Figure 18**) indicated that the compounds mostly were detected within 9-13 minutes and the most hydrophobic API was eluted after 29 minutes.

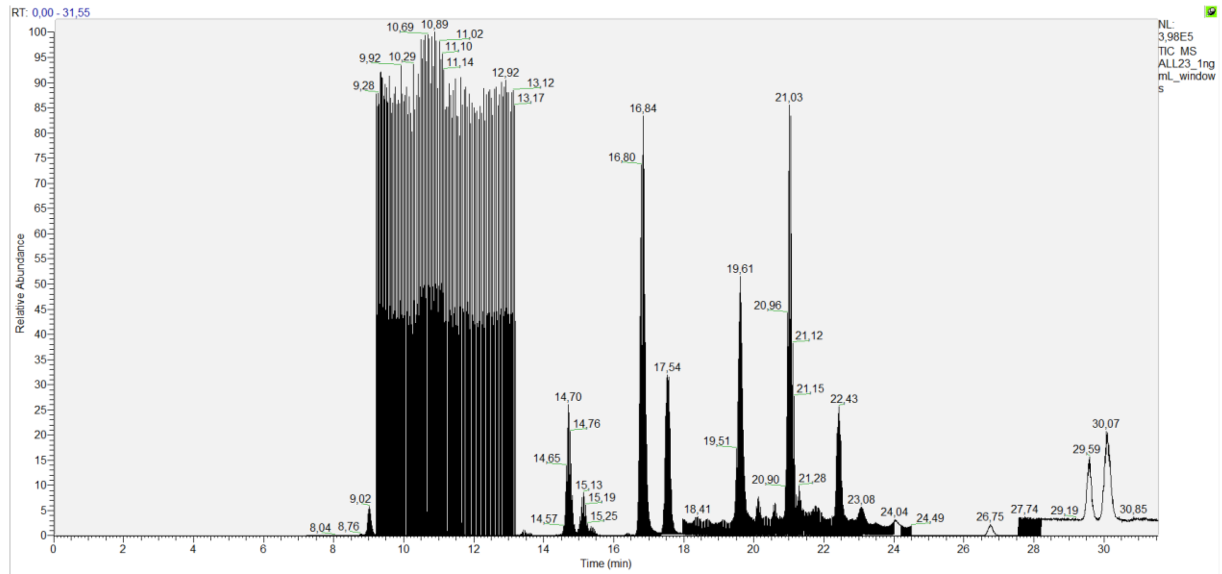


Figure 18 TICC for all 23 APIs monitored by MRM. An injection of 800 µL of a mixture with concentration of 1 ng/mL each was performed with the AFFL set-up using a Hotsep Kromasil 1 mm x 5 mm C_{18} (3 µm) SPE-column and a HotSep 0.3 mm x 150 mm C_{18} (2µm) analytical column. The loading MP and MP A was type 1 water with 0.1 % FA and the organic mobile phase (MP B) was ACN/type 1 water (95/5, v/v) with 0.1 % FA. Detection was carried out by the Quantiva QQQ MS.

In ESI, the eluent from the analytical column contains ionized or ionizable compounds which enter the stainless steel capillary. The ionization process is performed in the MP by pH adjustment and the mode of ionization (positive or negative) is dependent of the charge of the ions. Detection is either performed in the negative mode, for detection of deprotonated ions or in the positive mode detecting the protonated ions. The pKa value of a compound is useful to verify if it is ionisable in acidic or basic pH. Highly basic compounds (high pKa value) allow ionization in low pH (acidic MP). The basic compound would be protonated and thus form positively charged ions. The protonated ions would be most responsive to analysis with positive mode due to their tendency to form positive ions as the molecular ions, $[M+nH]^{n+}$. An acidic compound under these conditions (low pH) would be deprotonated and form negatively charged ions $[M-nH]^{n-}$, and thus responsive with negative mode [40].

The APIs are expected to be suited for ionization in different modes: negative mode, positive mode or in both modes. The APIs were examined in the mode they were expected to give best response. Unfortunately, it was established from the direct injections that the instrument used (Quantiva QQQ MS) did not perform well enough in negative mode or polarity switching between negative and positive mode, due to technical challenges with detector setting. Hence it was decided to continue with analytes that were ionisable in positive mode, excluding e.g. acids. Analytes excluded from the method at this point is shown in **Table 18**.

Table 18 The APIs with the mode they are expected to be detected: negative, positive or both modes and if they were included or not (YES/NO) in the method.

Name of compound	Predicted ionization mode	Kept (YES/NO)
Acetaminophen	Positive	YES
Atenolol	Positive	YES
Atorvastatin	Positive/negative	NO
Azithromycin	Positive	YES
Caffeine	Positive	YES
Carbamazepine	Positive	YES
Ciprofloxacin	Positive	YES
Clarithromycin	Positive	YES
Diclofenac sodium salt	Positive/negative	NO
Erythromycin (dihydrate)	Positive	YES
Fluoxetine hydrochloride	Positive	YES
Gemfibrosil	Positive/negative	NO
Ibuprofen	Negative	NO
Ketoprofen	Positive/negative	NO
Losartan potassium	Positive	YES
Metoprolol tartrate salt	Positive	YES
Naproxen	Positive/negative	NO
S-Propranolol hydrochloride	Positive	YES
Pravastatin sodium salt hydrate	Negative	NO
Simvastatin	Positive	YES
Sulfamethoxazole	Positive	YES
Trimethoprim	Positive	YES
Valsartan	Positive	YES
Xylazine hydrochloride	Positive	YES

The APIs ionizable in negative mode which were removed from the method at this point were: atorvastatin, diclofenac, gemfibrozil, ibuprofen, ketoprofen, naproxen and pravastatin. Thus, at this point, the remaining APIs to include in the method were: acetaminophen, atenolol, azithromycin, caffeine, carbamazepine, ciprofloxacin, clarithromycin, erythromycin, fluoxetine, losartan, metoprolol, propranolol, simvastatin, sulfamethoxazole, trimethoprim, valsartan and xylazine.

From this list (**Table 18**), a selection of APIs was done based on their usage and their MS response during method development (the reasons for elimination of the various APIs can also be found in **Appendix Chapter 7.5, Table A-4**. Development of a method representing as many as possible pharmaceutical groups and as many as possible APIs from each group was wanted. Also considering time available the number of APIs had to be limited, but at least one API was selected from each group.

Hence, the method was developed for the seven APIs shown in Figure 19 and five of the eight API groups were represented in the method. An IS is however, expensive and therefore only three ISs were used in the present study.

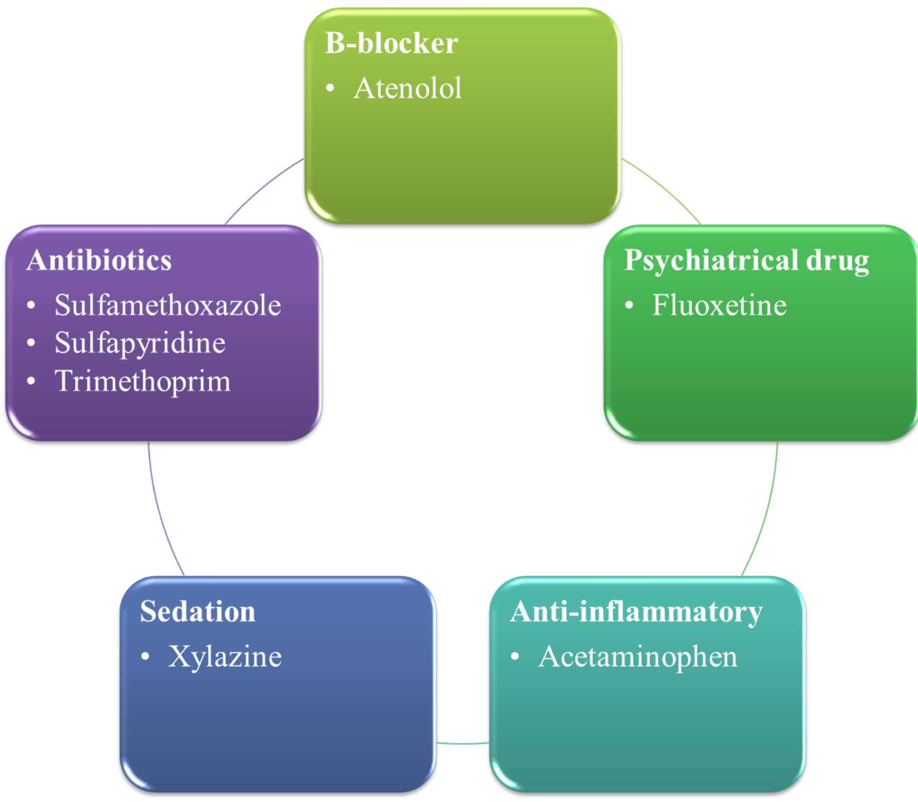


Figure 19 Pharmaceutical groups and the APIs included in the method from each group.

Further method development for the selected APIs (**Figure 19**) was done by using the Quantiva QQQ MS. Direct infusion was done to confirm the MRM-transitions (**Table 13**). The precursor ion of the analyte was selected in MS1 and the product ions were monitored in MS2. For each product ion the cone voltage and collision energy were optimized by the software. The *m/z* transition of the highest intensity was chosen as the quantification *m/z*, and a qualification *m/z* was chosen to ensure the identification. The TICC of the APIs is shown in **Figure 20**.

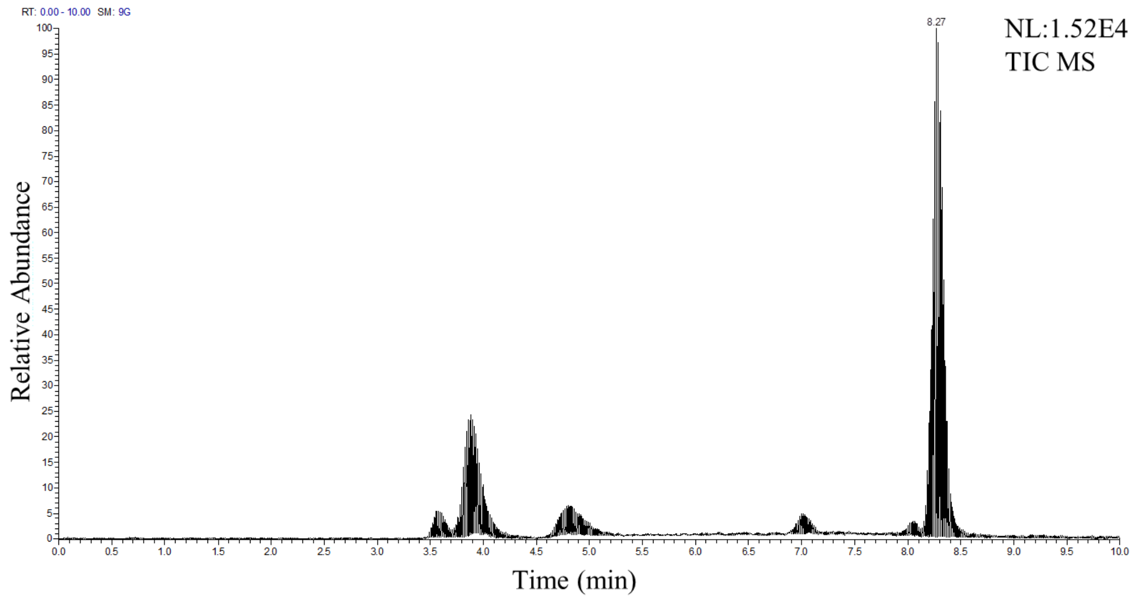


Figure 20 TICC for acetaminophen, atenolol, fluoxetine, sulfapyridine, sulfamethoxazole, trimethoprim, xylazine and the internal standards monitored by MRM. An injection of 100 µL of a mixture with concentration of 100 ng/L each API and the internal standard; atenolol-d7 (1000 ng/L), fluoxetine-d5 hydrochloride (500 ng/L) and sulfamethoxazole-(phenyl-$^{13}C_6$) (50 ng/L) was performed. The chromatographic system consisted of an AFFL-SPE-LC-MS set-up with a 1 mm ID x 5 mm C_{18} (5 µm) SPE-column (Kromasil) and a 0.3 mm ID x 150 mm C_{18} (3µm) analytical column (ACE). The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA. Gradient program 2 was used and the detection was carried out by the Quantiva QQQ MS.

4.1.3 Chromatographic separation of the selected APIs

The APIs were separated using a gradient program (2). The retention times of the APIs (100 ng/L) and the internal standards are shown in the chromatograms in **Figure 21**. Chromatograms of selected APIs were obtained by using the quantifier and the qualifier as the *m/z* transitions (**Table 13**). The peak areas of the APIs were integrated manually using the software, Xcalibur. Integration of the peak area of acetaminophen was difficult compared to the other APIs, due to background noise.

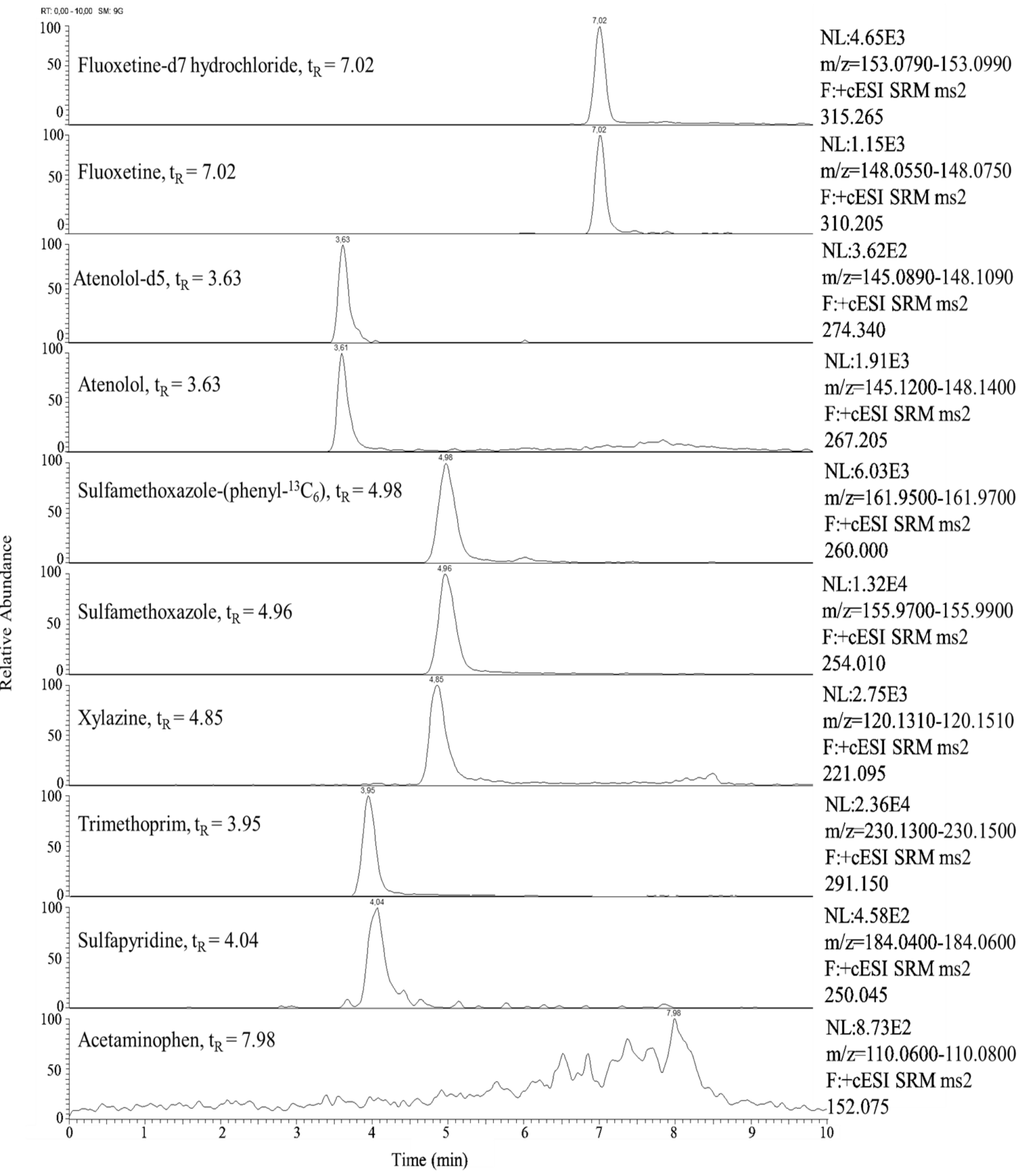


Figure 21 The EIC chromatograms showing the APIs (100 ng/L) and the internal standards, atenolol-d7 (1000 ng/L), fluoxetine-d5 hydrochloride (500 ng/L) and sulfamethoxazole-(phenyl-$^{13}C_6$) (50 ng/L) spiked in MP A. The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed using gradient program 2 on an ACE C_{18} column (0.3 mm ID x 150 mm, 3 µm) with a flow rate of 4 µL/min. A Kromasil C_{18} (1 mm ID x 5 mm, 5 µm) SPE-column was used. The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA.

4.2 Method validation

4.2.1 AFFL-system

The AFFL set-up used was equipped with an on-line sample filtration by a stainless steel filter and subsequent back flushing of the filter making it clean and ready for the next injection. The incorporated filter eliminated the need of single use filters and manual handling of these which is time consuming. Without an on-line filter, a common consequence of using on-line SPE is that the particles and precipitants form samples can cause easily clogged SPE-columns. By back flushing the analytes from the SPE-column, these particles may be transported to the analytical column, resulting in a clogged analytical column. In order to avoid these problems, a procedure including off-line filtration ([70, 71]) or SPE is often required as sample preparation step.

The automatized system has been successfully used for analysis of biological samples such as blood, cells etc. ([72, 73]). The aim of the current study was to investigate if such a system could be used for analysis of water samples, without any external filtration as a pre-treatment. The on-line SPE column was employed to automatically enrich the target analyte prior to capLC separation and MS detection. The AFFL-system should provide automation to avoid human mistakes during sample preparation. It was found that the AFFL-system could be used to analyse more than 100 creek water samples without pressure build-up in the system, showing a rugged system (**Figure 22**).

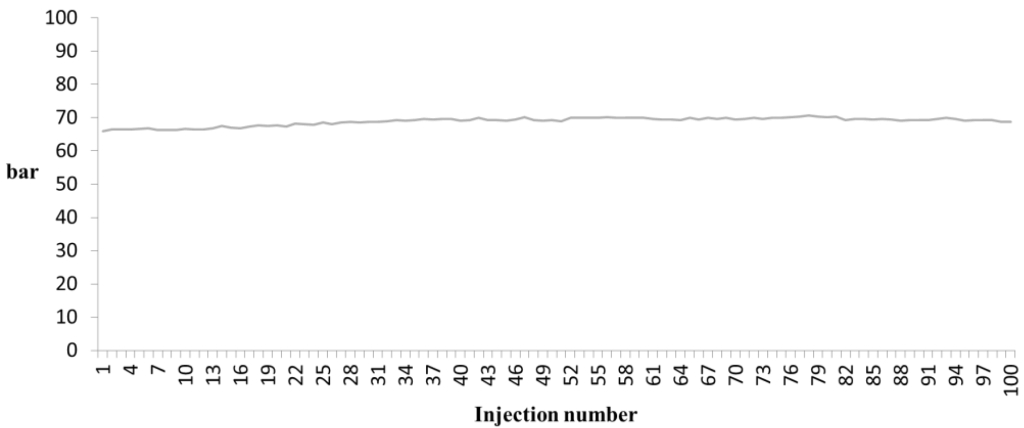


Figure 22 Pressure on pump 1 (loading pump) by injection number of 100 µL non-filtrated spiked creek water [From Hanne Røberg-Larsen].

4.2.2 Validation

Validation of the method was done according to the Eurachem Guide, *The Fitness for Purpose of Analytical Methods,* second edition 2014, which is an integral part of the international conformity assessment standards and guides [44]. The method was validated with regard to cLOD, cLOQ, precision (within-day and between-day repeatability), apparent recovery, and linearity including standard solutions of the APIs and the internal standards spiked in MP A, filtrated and non-filtrated water samples. All validation data are shown in **Table 19, 21, 22** and **23,** and will be discussed below (**Chapter 4.2.3-4.2.8** and **Chapter 4.3-4.4**).

4.2.3 Limit of detection

For validation purposes it is sufficient to prove an approximate value for LOD due to the level at which detection of analyte becomes problematic [44]. The cLOD was calculated by **Equation 8** based on the linearity curve as described in **Chapter 3.5.1**. According to the Eurochem Guide, the cLOD is normally calculated by multiplying with a suitable factor (f=3) [44]. The raw data are shown in **Appendix Chapter 7.7.1-7.7.7.**

By the criteria, 10 replicates were used for examination of the cLOD at the lowest concentration (10 ng/L) [44]. Replicate analyses were done by AFFL-SPE-LC-MS/MS of reagent blanks (MP A) spiked with low concentrations of analyte as described [44]. The cLOD of the analyte spiked in filtrated and non-filtrated water sample was calculated using 6 replicate analysis, instead of 10 (as done for MP A) due to time limitations. The calculated cLODs in the different matrices are shown in **Table 19** in **Chapter 4.2.5**.

The calculated cLOD for the selected APIs was in the 2– 18 ng/L range.

4.2.4 Limit of quantification

cLOQ was estimated similar to cLOD, and in this case another multiplier value (10) was used [44], see **Equation 9.** For the calculated cLOQs in the different matrices, see **Table 19** in **Chapter 4.2.5**.

The calculated cLOQ for the selected APIs was in the 5– 54 ng/L range.

The APIs corrected with their labelled IS such as sulfamethoxazole, showed lower cLOD and cLOQ. Of the analytes investigated, acetaminophen showed high calculated values of cLOD and cLOQ in all matrices. The reason could be that the IS used did not behave as acetaminophen, as well as the high background observed on the chromatograms which did integrations difficult.

Generally, the calculated cLOD and the cLOQ values are reflected in the chromatograms, where the APIs were spiked in MP A, filtrated and non-filtrated water samples, see Appendix Chapter 7.6, Figure A-5, A-6 and A-7.

4.2.5 Apparent recovery

Apparent recovery ($R'A\,\%$) is defined as "the quantity observed value/reference value, obtained using an analytical procedure that involves a calibration graph" [68]. The 100 % recovery does not require 100 % yield for any separation or enrichment stage. The requirement is that the yield for the test and the calibration must be the same [68]. $R'A$ was calculated based on **Equation 10** (**Chapter 3.5.2**) from IUPAC recommendations 2002 [68] as described in **Chapter 3.5.2**. The $R'A$ was investigated for analytes spiked in filtrated and non-filtrated water sample, see **Table 19**.

Generally, high apparent recovery values were found of the APIs spiked in both filtrated (from 106-241 %) and non-filtrated (from 69-288 %) water samples from the Sognsvann creek.

Matrix effects

The apparent recovery examined for all compounds showed generally high values. The potential source of these variations could be ion suppression or ion enhancement which can theoretically be avoided by using suited IS. Ideally and theoretically an suited IS should correct for loss of analyte, compensate for sample to sample recovery differences and correct for variable instrumental conditions, such as injection volume, retention time, and MS response. Since, the APIs with their suited IS also showed high apparent recovery values, the signal may be affected by interferences in the complex matrices. Water samples are complex matrices containing compounds from the aquatic environment which are not possible to control.

The quantifiers used as MRM-transition for the APIs could also be common *m/z* values for other compounds causing ion enhancement. A compatible IS may improve the ability to compensate for ion suppression and ion enhancement.

Table 19 Validation data for measurement of the selected APIs spiked in different matrices: MP A, filtrated and non-filtrated water samples from the Sognsvann creek. The linearity (R^2), cLOD and cLOQ calculated for three days are shown. See supplementary details (Chapter 3.2.5) about the concentrations levels of the validation solutions and internal standards used.

Name of API and matrix	R^2 (STD)	cLOD (ng/L)	cLOQ (ng/L)	Apparent recovery (%)
Acetaminophen				
MP A	0.9777	18	54	
Filtrated	0.9882	13	39	215
Non-filtrated	0.9853	14	43	69
Atenolol				
MP A	0.9919	11	32	
Filtrated	0.9970	5	19	139
Non-filtrated	0.9980	5	16	150
Fluoxetine				
MP A	0.9985	5	14	
Filtrated	0.9974	6	18	241
Non-filtrated	0.9954	8	24	288
Sulfapyridine				
MP A	0.9957	8	23	
Filtrated	0.9941	9	27	106
Non-filtrated	0.9970	6	19	81
Sulfamethoxazole				
MP A	0.9992	3	10	
Filtrated	0.9981	5	15	135
Non-filtrated	0.9998	2	5	151
Xylazine				
MP A	0.9997	2	6	
Filtrated	0.9916	11	33	133
Non-filtrated	0.9965	7	21	125
Trimethoprim				
MP A	0.9928	9	26	
Filtrated	0.9878	13	39	259
Non-filtrated	0.9936	9	28	127

Acceptable linearity (R^2 = 0.9777-0.9998) was obtained even if some APIs were quantified using a non-ideal IS.

4.2.6 Linear range in standard solutions

The method was checked for linearity in the range from 10 ng/L to 100 ng/L for the selected APIs. The analytes were spiked in MP A, filtrated and no-filtrated water sample from Sognsvann creek and analysed at 10 ng/L, 25 ng/L, 50ng/L, 75 ng/L and 100 ng/L concentration levels, see **Table 14** for number of replicates for each concentration.

Linearity equations (y=ax+b) for the APIs are shown in **Table 20** and the linearity curves for all the APIs in the different matrices are shown in **Figure 20**. Linearity curves for atenolol, fluoxetine and sulfamethoxazole were established by using their ideal IS.

Table 20 The linearity equation of each API spiked in different matrices (MP A, filtrated and non-filtrated water sample from the Sognsvann creek). Note: without the equations for trimethoprim.

Name of API	MP A	Filtrated	Non-filtrated
Atenolol	y = 0.0438x + 0.2599	y = 0.0610x + 0.1962	y = 0.0656x - 0.1887
Acetaminophen	y = 0.0013x + 0.0567	y = 0.0028x + 0.0444	y = 0.0009x + 0.0430
Fluoxetine	y = 0.0017x + 0.0156	y = 0.0041x + 0.0261	y = 0.0049x - 0.0163
Sulfapyridine	y = 0.0016x - 0.0007	y = 0.0017x + 0.0442	y = 0.0013x + 0.0059
Sulfamethoxazole	y = 0.0201x + 0.0665	y = 0.0271x + 0.0859	y = 0.0304x + 0.0146
Xylazine	y = 0.0053x + 0.0044	y = 0.0068x + 0.0660	y = 0.0069x + 0.0074

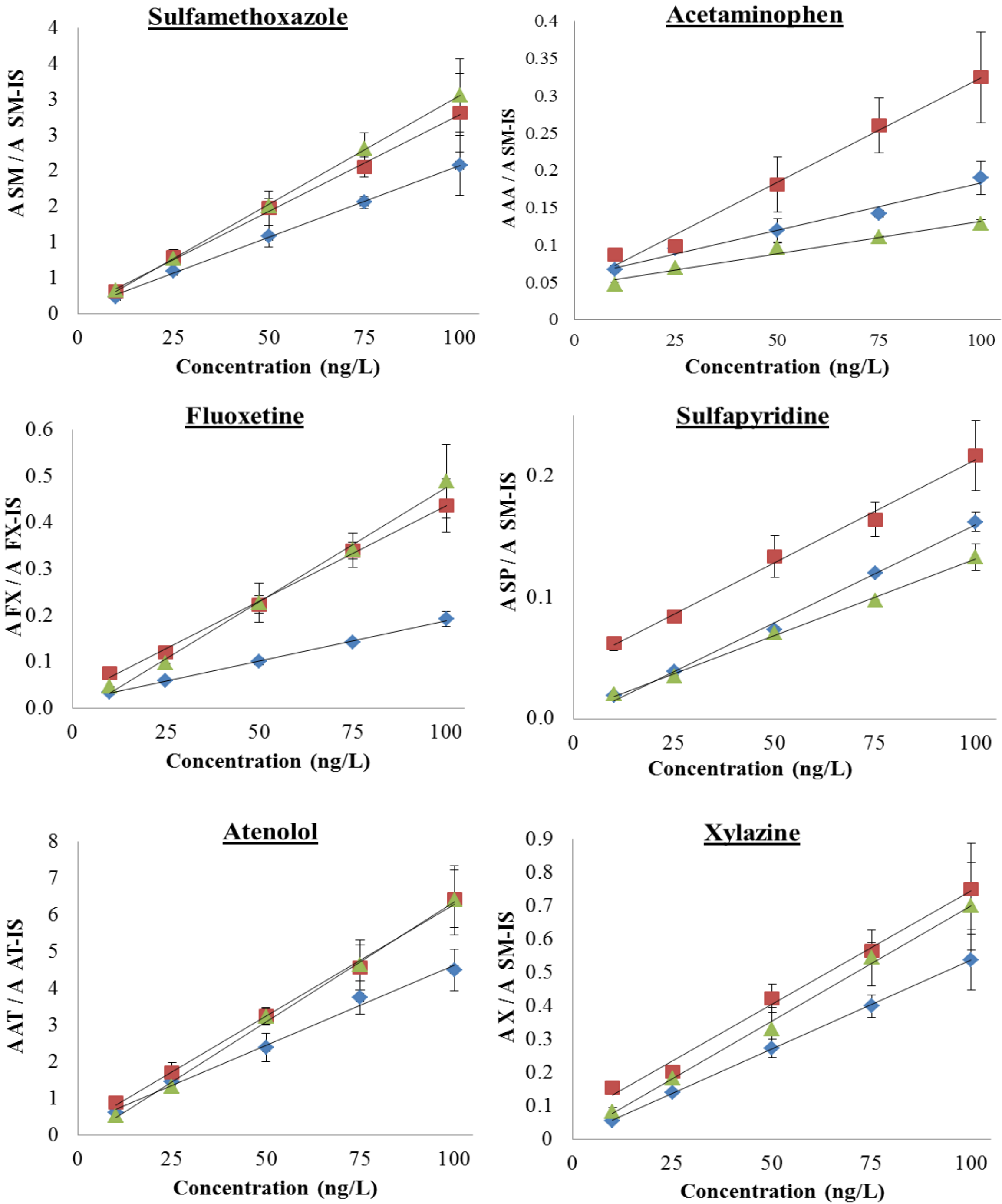


Figure 23 Linearity curves form 10 ng/L to 100 ng/L for atenolol (AT) acetaminophen (AA), fluoxetine (FX), sulfamethoxazole (SM), sulfapyridine (SP) and xylazine (X) spiked in MP A (blue), filtrated (red) and non-filtrated (green) water sample from the Sognsvann creek. Deuterated internal standards, atenolol-d7 and fluoxetine-d5 were used for AT and FX, respectively. The internal standard, sulfamethoxazole-(phenyl-$^{13}C_6$) was used for AA, SP, SM, and X. The curves were established by 10 replicates for low (10 ng/L), medium (50 ng/L) and high (100 ng/L) concentrations spiked in MP A, and 6 replicates for the same concentrations spiked in filtrated and non-filtrated water samples. 3 replicates were used for concentrations of 25 ng/L and 75 ng/L independent of spiking in MP A, filtrated and non-filtrated water samples. Standard deviations (SD) were calculated as shown in Appendix Chapter 7.7.

The linearity curves for the APIs with suited internal standards shows similar trend with respect to closeness of the linear curve for the filtrated, non-filtrated and MP A compared with the curves for the APIs without their own IS. The samples were filtrated by using the AFFL-system. External filtration of the water sample as a sample preparation did not affect the slope of the curves.

The linearity equation (**Table 20**) and linearity curve (**Figure 23**) for trimethoprim are not presented due to high values of SD of between-day repeatability, see the values in **Table 21-23**.

4.2.7 Repeatability

The within-day (n = 10, number of replicates) and between-day (n = 3, number of days) repeatability were calculated by analysis of variance (Anova, single factor) using Excel, see **Appendix Chapter 7.7.1-7.7.7.** The repeatability was analysed at low (10 ng/L), medium (50 ng/L) and high (100 ng/L) concentration levels, the calculated RSD for each concentration in the different matrices are given in **Table 21-Table 23**.

Table 21 The within-day repeatability and the between-day repeatability of analytes spiked in MP A were established by calculation of (mean (A/ A is)), standards deviation (SD) and % relative standard deviation (RSD %). The calculation of within-day repeatability was based on 10 replicates (n = 10) of low (10 ng/L), medium (50 ng/L) and high (100 ng/L) concentrations and the calculation of between-day repeatability was based on 3 consecutive days.

MP A	Within-day repeatability (n=10)			Between-day repeatability (n=3)		
	Mean A / A $_{is}$	**SD**	**RSD %**	**Mean A / A $_{is}$**	**SD**	**RSD %**
Atenolol						
L	0.61	0.05	9	0.6	0.11	18
M	2.24	0.14	6	2.4	0.39	16
H	4.59	0.13	3	4.5	0.56	13
Acetaminophen						
L	0.12	0.01	11	0.081	0.003	4
M	0.20	0.02	9	0.136	0.003	3
H	0.27	0.02	7	0.196	0.004	2
Fluoxetine						
L	0.041	0.006	15	0.034	0.003	15
M	0.099	0.010	10	0.101	0.008	7
H	0.174	0.013	7	0.193	0.016	8
Sulfapyridine						
L	0.019	0.002	12	0.019	0.0007	3
M	0.074	0.004	6	0.073	0.003	4
H	0.170	0.016	9	0.162	0.008	5
Sulfamethoxazole						
L	0.23	0.01	5	0.24	0.04	15
M	1.12	0.02	2	1.1	0.16	14
H	2.13	0.05	2	2	0.4	20
Trimethoprim						
L	0.56	0.03	5	0.4	0.33	86
M	2.31	0.08	3	2	2.4	124
H	4.28	0.45	10	4	6.0	156
Xylazine						
L	0.06	0.01	13	0.055	0.001	3
M	0.31	0.02	5	0.3	0.027	10
H	0.57	0.04	7	0.5	0.09	17

Table 22 The within-day repeatability and the between-day repeatability of analytes in filtrated water sample from the Sognsvann creek were established by calculation of (mean (A/ A is)), standards deviation (SD) and % relative standard deviation (RSD %). The calculation of within-day repeatability was based on 6 replicates (n = 6) of low (10 ng/L), medium (50 ng/L) and high (100 ng/L) concentrations and the calculation of between-day repeatability was based on 3 consecutive days.

Filtrated	Within-day repeatability (n=6)			Between-day repeatability (n=3)		
	Mean A / A $_{is}$	**SD**	**RSD %**	**Mean A / A $_{is}$**	**SD**	**RSD %**
Atenolol						
L	0.87	0.06	7	0.88	0.05	5
M	3.22	0.17	5	3.26	0.23	7
H	6.35	0.19	3	6.4	0.78	12
Acetaminophen						
L	0.09	0.01	12	0.086	0.007	8
M	0.17	0.03	18	0.181	0.037	20
H	0.31	0.04	14	0.325	0.061	19
Fluoxetine						
L	0.053	0.008	16	0.076	0.008	10
M	0.239	0.027	11	0.224	0.019	8
H	0.477	0.036	7	0.437	0.057	13
Sulfapyridine						
L	0.057	0.010	18	0.062	0.006	10
M	0.108	0.012	11	0.134	0.017	13
H	0.192	0.030	15	0.217	0.029	13
Sulfamethoxazole						
L	0.30	0.05	17	0.32	0.06	18
M	1.52	0.10	7	1.48	0.24	16
H	2.76	0.21	8	2.81	0.55	19
Trimethoprim						
L	1.51	0.17	11	1.6	1.3	82
M	5.42	0.25	5	6	14	243
H	9.57	0.38	4	10	13	137
Xylazine						
L	0.15	0.03	19	0.16	0.02	11
M	0.39	0.06	16	0.42	0.04	10
H	0.65	0.03	5	0.8	0.14	18

Table 23 The within-day repeatability and the between-day repeatability of analytes in non-filtrated water sample from the Sognsvann creek were established by calculation of (mean (A/ A is)), standards deviation (SD) and % relative standard deviation (RSD %). The calculation of within-day repeatability was based on 6 replicates (n = 6) of low (10 ng/L), medium (50 ng/L) and high (100 ng/L) concentrations and the calculation of between-day repeatability was based on 3 consecutive days.

Non-filtrated	Within-day repeatability (n=6)			Between-day repeatability (n=3)		
Name of API	**Mean A / A $_{is}$**	**SD**	**RSD %**	**Mean A / A $_{is}$**	**SD**	**RSD %**
Atenolol						
L	0.49	0.03	5	0.52	0.02	4
M	3.23	0.15	5	3.23	0.23	7
H	6.36	0.15	2	6.4	0.94	15
Acetaminophen						
L	0.036	0.003	7	0.047	0.003	6
M	0.084	0.004	5	0.095	0.007	7
H	0.117	0.012	11	0.128	0.005	4
Fluoxetine						
L	0.048	0.005	11	0.047	0.001	3
M	0.282	0.039	14	0.23	0.042	19
H	0.556	0.052	9	0.49	0.078	16
Sulfapyridine						
L	0.015	0.003	17	0.0250	0.0005	2
M	0.065	0.011	17	0.070	0.0026	4
H	0.113	0.013	11	0.133	0.011	8
Sulfamethoxazole						
L	0.29	0.03	9	0.33	0.03	9
M	1.44	0.05	3	1.51	0.11	7
H	2.83	0.20	7	3.06	0.52	17
Trimethoprim						
L	0.53	0.06	10	0.7	0.3	46
M	2.12	0.14	6	2.5	3	105
H	4.22	0.29	7	4.7	4.4	93
Xylazine						
L	0.10	0.02	19	0.08	0.01	15
M	0.39	0.05	13	0.33	0.06	19
H	0.75	0.03	5	0.70	0.13	19

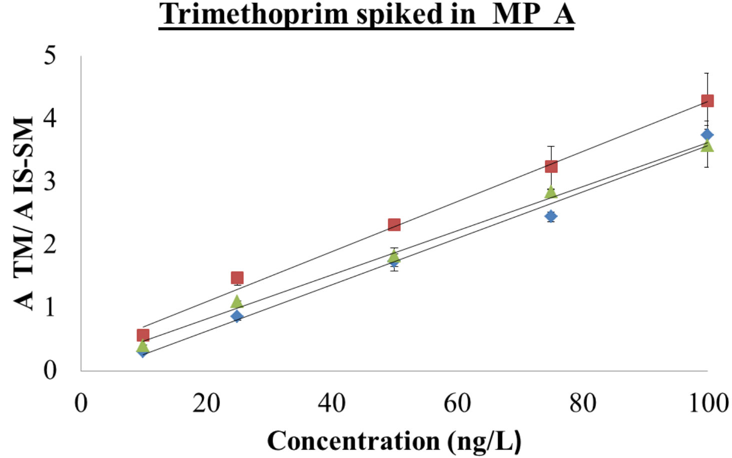
Although the precisions generally were satisfactory (RSD % < 20) for the APIs, it would be better if suited internal standards for each API, which are commercially available, were used instead of sulfamethoxazole (sulfamethoxazole-(phenyl-$^{13}C_6$).

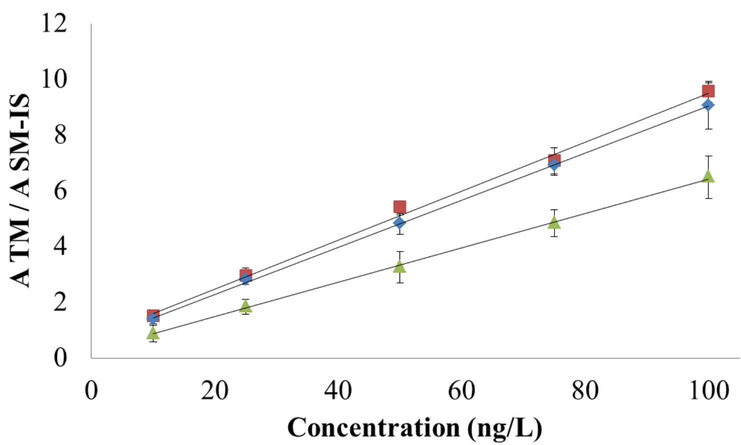
Trimethoprim showed higher RSD values of between-day repeatability in all matrices compared to within-day repeatability and those of the other APIs. However, trimethoprim showed high intensity and low baseline MS/MS noise, see **Appendix Chapter 7.6** for the chromatograms of trimethoprim. The electron multiplier detector of the MS was changed during the validation of the method. This change caused the need for mass calibration of the instrument several times during the analyses. The change of detector with the accompanying need for often re-calibration could be the reason for high RSD values for the between-day repeatability of trimethoprim in addition to the non-ideal IS.

The statistical calculations show the variation in between-day and within-days repeatability, where a higher value was observed for between-day variations, see **Appendix Chapter 7.7.7**. In order to compare the within-day repeatabilities, it is shown with the linearity equations (**Table 24**) and linearity figures, see **Figure 24.**

Table 24 The linearity equation of trimethoprim spiked in different matrixes (MP A, filtrated and non-filtrated water sample from the Sognsvann creek). The equations represent the within-day repeatability for each matrix.

DAY	MP A	Filtrated	Non-filtrated
DAY 1	y=0.0369x+0.1051	y=0.0878x+0.7402	y=0.0414x+0.1190
DAY 2	y=0.0398x+0.3017	y=0.0844x+0.6138	y=0.0513x+0.3657
DAY 3	y=0.0351x+0.1193	y=0.0619x+0.2625	y=0.0461x+0.9890





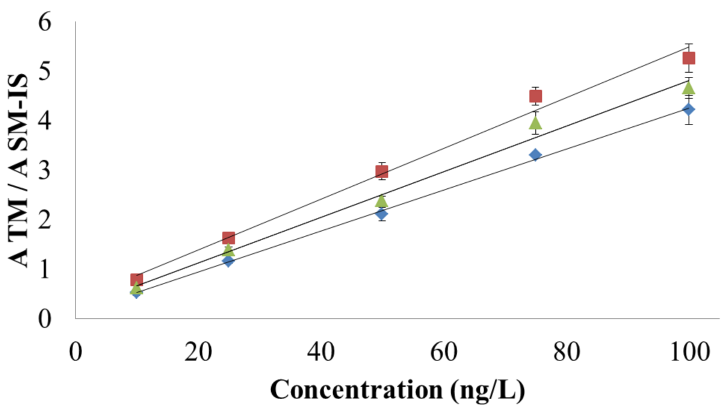


Figure 24 The linearity graphs for trimethoprim in the range 10-100 ng/L where the analyte was spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek. The blue, red and green slope represents day 1, 2 and 3, respectively. Sulfamethoxazole-(phenyl-$^{13}C_6$) was used as the IS. The curves were established for 10 replicates for low (10 ng/L), medium (50 ng/L) and high (100 ng/L) concentrations spiked in MP A, and 6 replicates for same concentrations spiked in filtrated and non-filtrated water samples, 3 replicates were used for concentrations of 25 ng/L and 75 ng/L independent of the matrices. The standard deviations (SD) is shown as error bars, for calculations see Appendix Chapter 7.7.

4.2.8 Need for pre-filtration

The idea behind spiking of the APIs in the filtrated and non-filtrated water sample was to compare the effect of external filtration. If filtrated and non-filtrated water samples gave the same results minimal sample clean-up and preparation are required. The linearity graphs showed similar slope of the curves for the analytes with their ideal IS used, in both filtrated and non-filtrated water samples, indicating that pre-filtration of the sample was not necessary.

4.3 Application of the method

A filtrated water sample from the Sognsvann creek was spiked with the internal standards and analysed by the AFFL-SPE-LC-MS. The chromatograms in **Figure 25** were obtained by using the quantifier as the *m/z* transitions for each API (**Table 13**). The used concentration of atenolol-d7 and fluoxetine-d5 was high compared to the concentration of sulfamethoxazole-(phenyl-$^{13}C_6$) and the method detection limits for the analytes. Atenolol-d7 and fluoxetine-d5 were included later than sulfamethoxazole-(phenyl-$^{13}C_6$) and the MS-conditions were not optimal during the analysis.

As expected no detectable APIs were found in the water sample from the Sognsvann creek with the method developed for selected APIs.

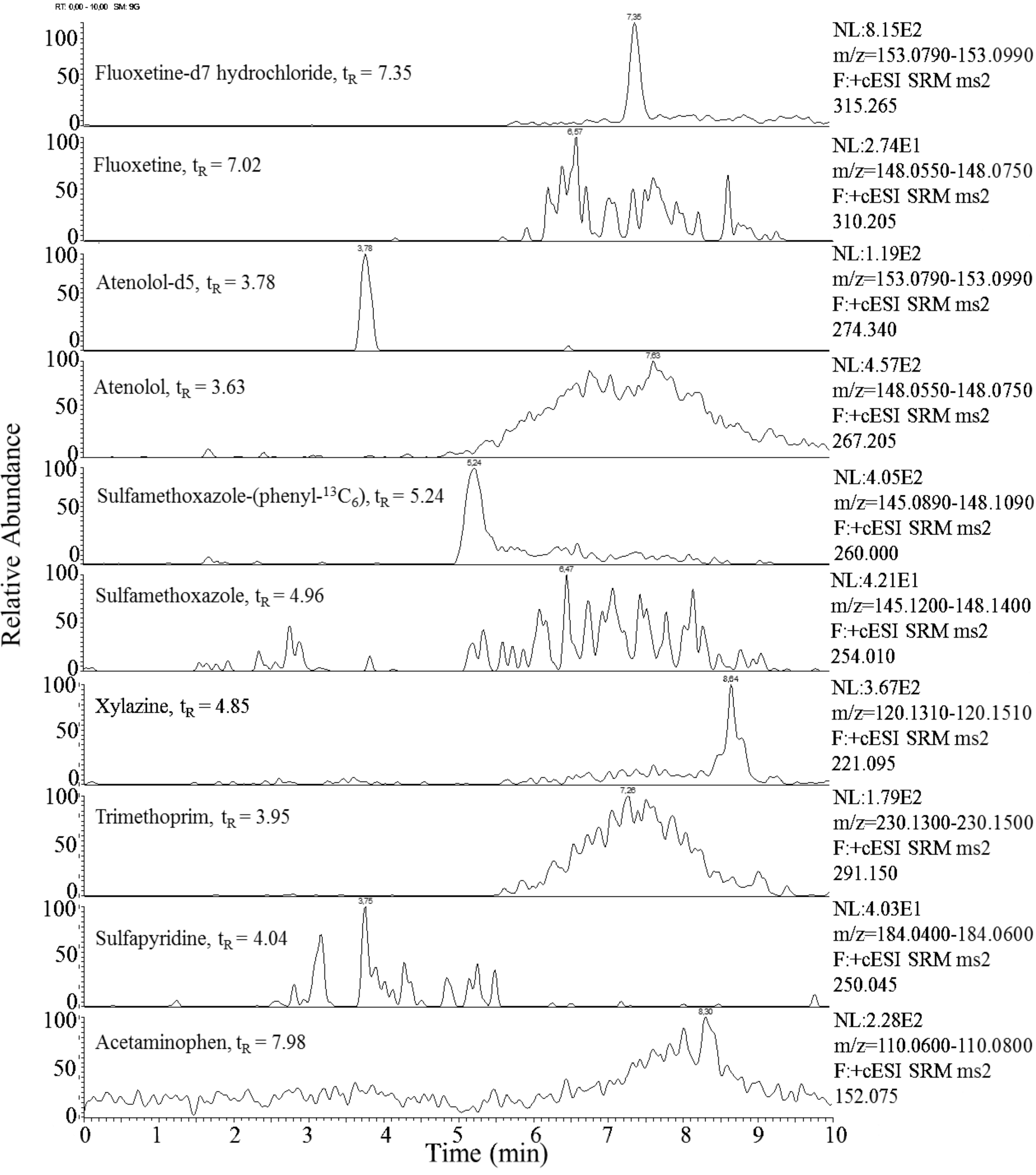


Figure 25 EIC of filtrated water sample from Sognsvann creek added the internal standards, atenolol-d7 (1000 ng/L), fluoxetine-d5 (500 ng/L) and sulfamethoxazole-(phenyl-$^{13}C_6$) (50 ng/L). The APIs were monitored by MRM using the Quantiva QQQ MS. The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed using gradient program 2 on an ACE C_{18} column (0.3 mm ID x 150 mm, 3 µm) with a flow rate of 4 µL/min. A Kromasil C_{18} (1 mm ID x 5 mm, 5 µm) SPE-column was used The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA.

4.4 Comparison with other studies

Several methods have been developed and validated for determination of selected APIs in water samples ([2, 3, 57, 58]). The quantification limits found in the present study are in-line with what has been obtained by others ([1, 10, 66]).

The method developed and validated by C. L. Chitescu et al. for selected APIs (43 APIs) in surface water using Exactive Orbitrap MS allowed quantification of the APIs in the concentration range of 10-100 ng/L. By using a hybrid quadrupole Orbitrap MS better sensitivity and lower cLOD values were obtained. However, the same cLOD level was achieved using a triple quadrupole MS with MRM monitoring in our study.

Generally, high apparent recovery values (from 81-288 %) were found with relative standard deviation (RSD %) lower than 20 % for all APIs except trimethoprim (between-day) in the present study. High recoveries (80-125 %) were also reported by A. L. Batt et al [10], who developed a method for APIs in water samples. They report that use of non-ideal internal standards could be the reason for ion suppression or ion enhancement which is the same argument as used in our study.

Maria Angelis, K. et al. who quantified APIs and personal care product residues in surface water and drinking water by SPE and LC-ESI-MS/MS, validated a method for higher linear range (200 ng/L – 2500 ng/L) comparted to this study (10 ng/L – 100 ng/L) and higher cLOD (µg/L) and cLOQ (0.05 µg/L – 1 µg/L) were calculated compared to this study (cLOQ: 2 ng/L – 18 ng/L, cLOQ: 5 ng/L – 54 ng/L), however same mass analyser was used. Their study showed generally high recovery values (from 65-120 %) with relative standard deviation (RSD %) lower than 20 %. As reported the limitation in LC-MS is the susceptibility of API interfaces to co-extracted matrix components, typically results in either signal suppression or enhancement [74].

The method by A. L. Batt et al. had a total analysis time of 48 minutes using SPE-UPLC-MS/MS. The lengthy sample preparation in their method has been avoided using the AFFL-SPE-LC switching platform in the present study. We found that use of MeOH instead of ACN as the organic MP improved the ionization of selected API. The same has been reported by R. Loos et al. in 2013 [5]. They optimized the chromatographic separation by testing the same mobile phases and concluded that better chromatographic peaks could be obtained by using a mixture of water-MeOH. They used automatized SPE (off-line) of one-liter water samples

followed by LC-MS. They washed the SPE cartridges with water to remove the salts from the extracts in order to reduce LC-MS matrix effects causing ion suppression. In our study the incorporated SPE-column was washed with organic solvent (47 %) subsequently during loading of analytes however, ion suppression has been found for some APIs in the present study.

A report by S. Esteban et al. in 2013 [66] describes a method for selected APIs in water samples using an on-line column system for enrichment of APIs and subsequent separation on the analytical column followed by ESI triple quadrupole MS SRM detection. The APIs were detected in ng/L range and R^2 were higher than 0.99. However, they have not addressed possible matrix effects.

Larger sample loading volumes (100-500 µL) were tested by X, Yi. et al. using a SPE-LC-ESI-MS/MS method. They observed that the increased sample loading volume from 100 to 500 µL only caused a slight loss of the compounds [75], similar to the present study. Compared to the report from I, Tlili. et al. the loss of analyte during loading was not considered however, 1 mL was used as injection volume with the same instrumental set-up as X, Yi. et al [76].

5 Conclusion

A sensitive method for screening of seven APIs in surface water has been developed. The method was validated for these APIs belonging to five groups using three labelled internal standards. The method is selective and suitable for determination of the selected APIs in the concentration 10-100 ng/L by using the AFFL-SPE-capLC-MS/MS platform. Total analysis time was 15 minutes. The method is suitable for analysis of water samples without the need for sample preparation. By using the rugged AFFL-system with incorporated 0.2 µm filter and SPE-column (1mm ID x 5mm, 5µm) for sample clean-up and enrichment, a number of injections are possible without pressure build-up. The drawback by such a system is the technical challenges by using narrow tubings and need for maintenance of the LC-system. High sensitivity (10 ng/L) was reached using a capLC column.

Further work could be to expand the method for more APIs. Lower detection limits may be reached (1 ng/L) by using a nanoLC column and/or a high sensitivity and resolution mass analyser. Considering matrix effects, inclusion of a labelled IS for each API is necessary to compensate for interferences causing, ion suppression and ion enhancement.

6 References

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7 Appendix

7.1 Physiochemical properties

The theoretical log P value of each compound is shown in **Figure A-1** and theoretical pKa value in **Figure A-2**. The acidity of a compound is expressed with a pK_a value, which is the negative log of the acid dissociation constant (K_a) [77]. The log P is the logarithm of the octanol-water partition coefficient, which is used as a measure of polarity [77] of a compound. The hydrophobicity of a compound increases with increased log P values.

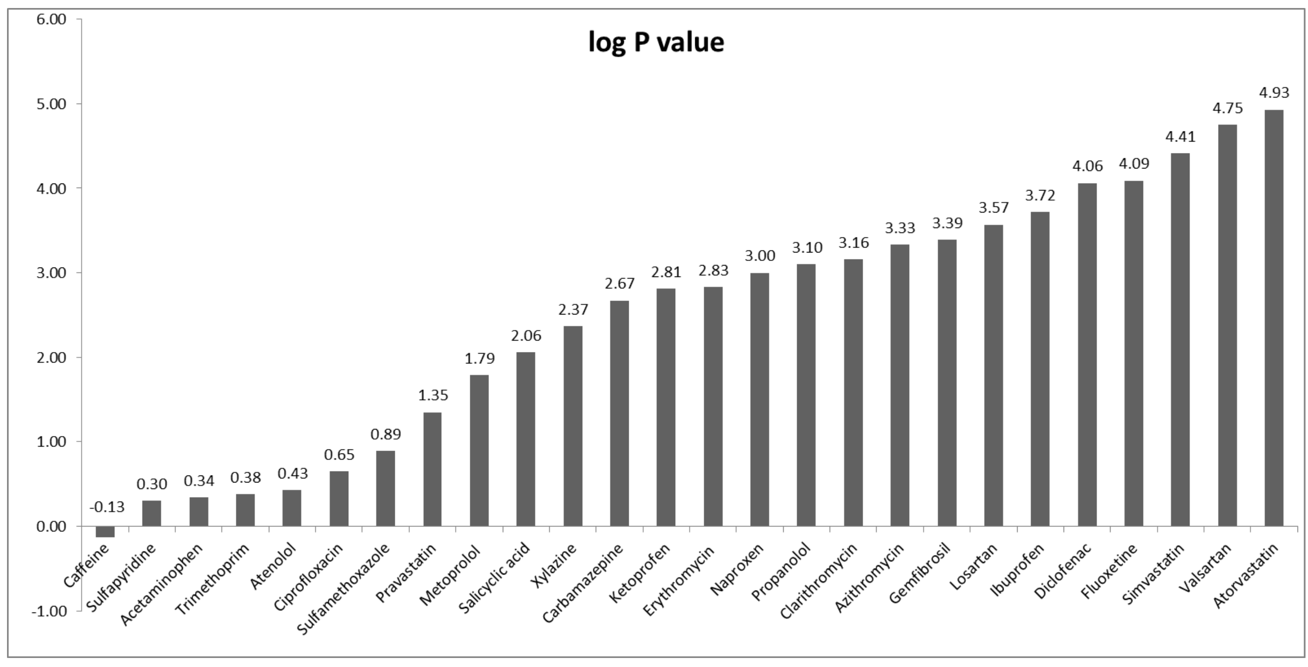

Figure A- 1 Log P values of the compounds obtained from ChemSpider [78].

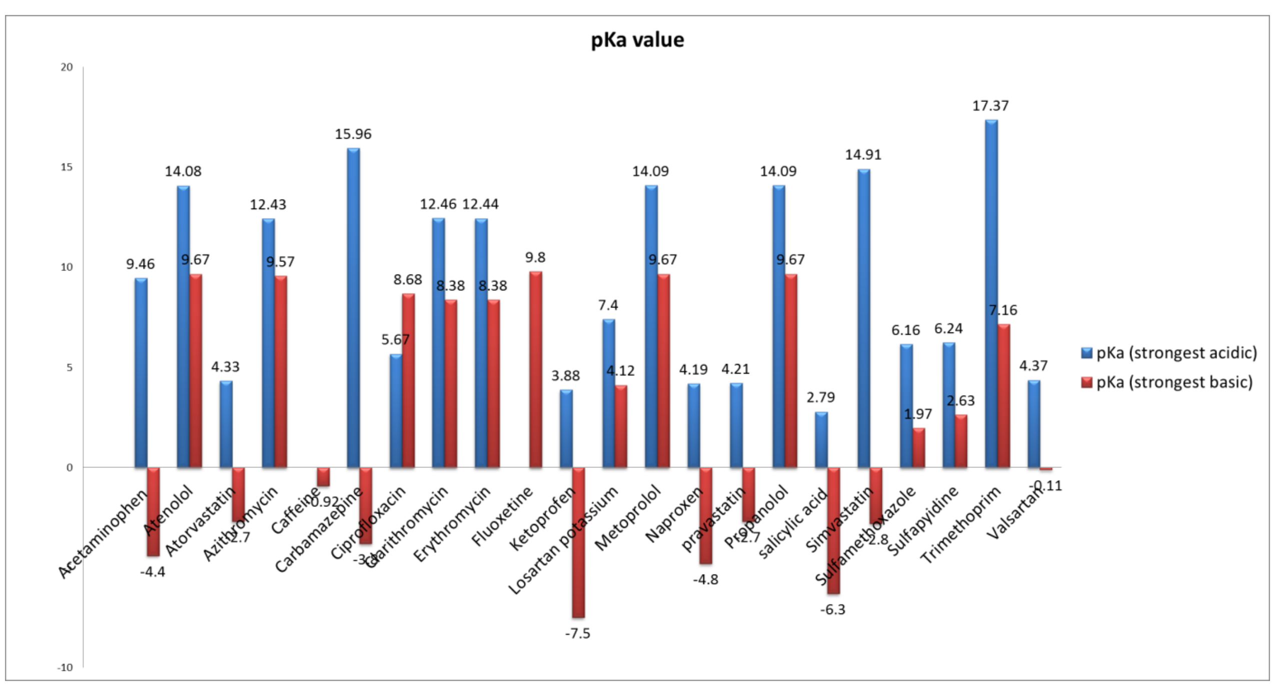

Figure A- 2 The pKa values of the compounds, both the acidic and basic, obtained from Drugbank [79].

7.2 Mass spectrometry of the APIs

The precursor ion and its product ions for each API monitored by MRM using the Quantiva QQQ and the Q Exactive are given in **Table A-1** and **Table A-2**, respectively.

Table A- 1 The APIs were examined by direct injection (10 µg/mL) in positive mode MRM monitoring by Quantiva QQQ MS. Molar mass, precursor ion, and product ions for selected APIs are shown. Fragments in italics were later removed from the analytical method, as they could be a source for background noise. Note: Pravastatin is not included as the compound was not compatible with positive mode detection using the Quantiva QQQ MS.

Name of API	Molar mass (g/mol)	Precursor ion (*m/z*)	Product ions (*m/z*)
Acetaminophen	151	152	93, *109,* 110
Atenolol	266	267	133, 145, 195
Atorvastatin	558	559	250, 276, 440
Azithromycin	749	750	*83,* 158, 591
Caffeine	194	195	110, 123, 138
Carbamazepine	236	273	179, 192, 194
Ciprofloxacin	331	332	231, 245, 314
Clarithromycin	747	748	158, 558, 590
Diclofenac	295	296	*214*, 215, 250
Erythromycin	733	734	116, 158, 576
Fluoxetine	309	310	117, 148, *183*
Gemfibrosil	250	251	*77,* 152, 215
Ibuprofen	206	207	*77,* 91, 105
Ketoprofen	254	255	*77*, 105, 209
Losartan	422	423	180, 205, 207
Metoprolol	267	268	*77*, *91*, 103
Naproxen	230	231	153, 170, 185
Propranolol	259	260	127, 129, 155
Simvastatin	418	419	143, 199, 225
Sulfapyridine	249	250	*92,* 155, 184
Sulfamethoxazole	253	254	*92*, 108, 155
Trimethoprim	290	291	123, 230, 261
Valsartan	435	436	180, 190, 207
Xylazine	220	221	*105*, 120, 164

Table A- 2 APIs were examined by direct injection (10 µg/mL) in positive mode PRM monitoring by Q Exactive MS. Molar mass, precursor ion, product ions and fragmentation energy (NCE) for selected APIs are shown. Fragments in italics were later removed from the analytical method, as they could be a source for background noise. Note: Pravastatin is not included as the compound was not compatible with positive mode detection using the Q Exactive QQQ MS. Note: caffeine, carbamazepine, ibuprofen, ketoprofen and metoprolol were not included because of difficulties during method optimization on negative mode of ionization.

Compound	Molar mass (g/mol)	Precursor ion (*m/z*)	Product ions (*m/z*)	NCE
Acetaminophen	151	152.0710	110.0606	25
Atenolol	266	267.1709	145.0651, 190.0866, 208.0973, 225.1235	35
Atorvastatin	558	559	380.1660, 422.2123	20
Azithromycin	749	750	296.2148, 591.4222	15
Ciprofloxacin	331	332.1412	231.0569, 314.1303	50
Clarithromycin	747	748.4860	158.1179, 558.3644, 590.3908	15
Diclofenac	295	296.0245	215.0500, 250.0189, 278.0138	10
Erythromycin A	733	734.4708	158.1179, 522.3433, 540.3538, 558.3646, 576.3749	15
Fluoxetine	309	310.1419	148.1224, 117.0705	25
Gemfibrozil	250	251. 1646	205.1591, 233.1539	20
Losartan	422	423.1704	207.0920, 377.1531, 405.1595	15
Naproxen	230	231.1021	170.0730, 185.0966	40
Propranolol	259	260.1650	116.1075, 157.0652, 183.0810, 218.1182	30
Simvastatin	418	419.2798	199.1486, 243.1743, 255.1641, 267.1747, 285.1853, 303.1959	15
Sulfapyridin	249	250.0630	156.0108	30
Sulfamethoxazole	253	254.0599	108.0450, 147.0795, 156.0116, 188.0822	25
Trimethoprim	290	291.1456	123.0670, 245.1037, 230.1166, 261.0986, 275.1143	45
Valsartan	435	436.2352	235.0980, 291.1496, 306.1717, 352.1772, 362.2229, 408.2286, 418.2230	15
Xylazine	220	221.1113	90.0380, 147.0920, 164.0531	50
Sulfamethoxazole-(phenyl-$^{13}C_6$)	259	260.0790	194.1017, 166.1068, 162.0313, 153.0990, 114.0647	30

7.3 Loading capacity and loading pump flow

The loading capacity was measured by observing the breakthrough, see **Table A-3**.

Table A- 3 The estimation of breakthrough for various injection volumes using AFFL-SPE-UV setup.

Injection volume (µL)	Time 1 (min)	Time 2 (min)	Avarage time (min)
200	14.5	Not done	14.5
400	13.5	Not done	13.5
600	14.4	14.5	14.5
800	10.9	10.3	10.6

7.4 Organic solvents

Organic solvents such as ACN and MeOH were compared as organic MP. The analytes were prepared at a concentration of 1000 ng/L for MRM monitoring. The comparison was done by using some representative compounds, which are also included in the method. **Figure A-3** shows the chromatograms of the analytes using ACN with 0.025 % FA as MP B and **Figure A-4** shows the chromatograms of analytes using the same concentration while the MP B was MeOH with 0.1 % FA.

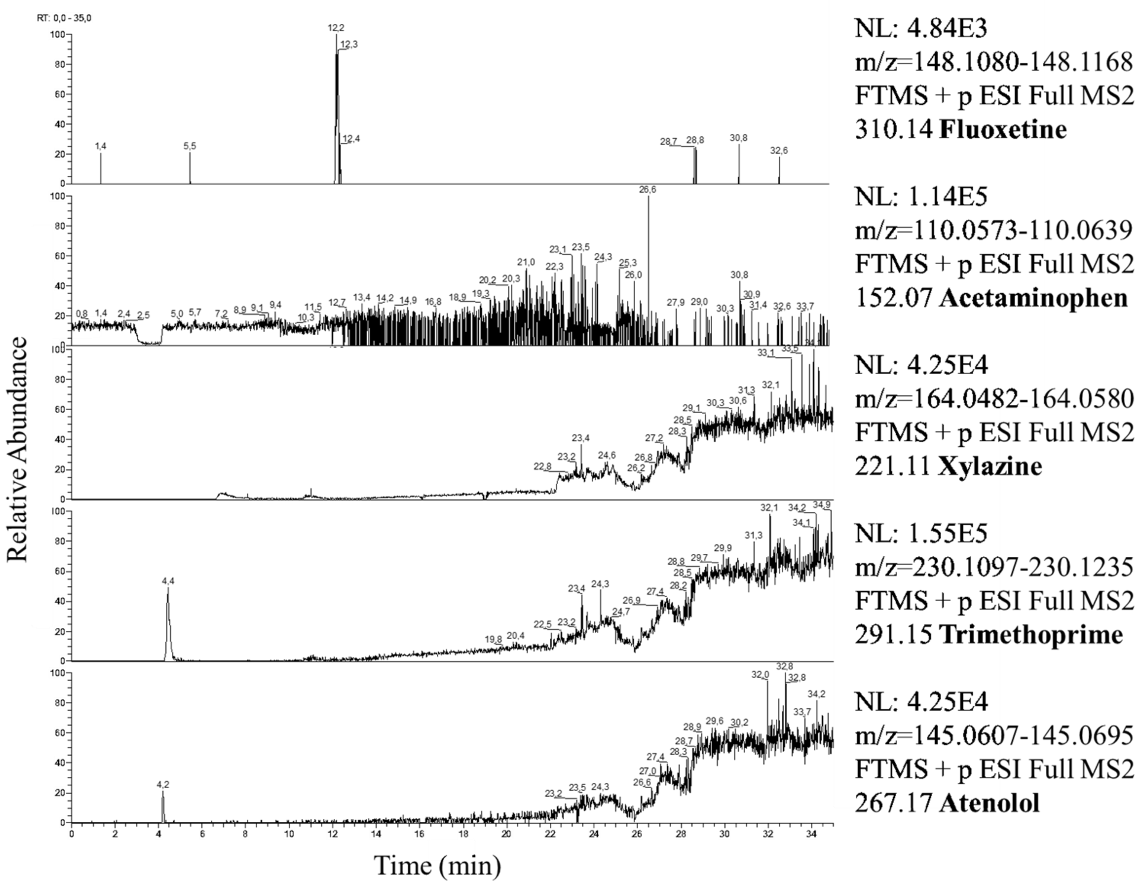

Figure A- 3 EIC of the selected APIs (1 µg/L) where ACN with 0.025 % FA was used as organic solvent (MP B). The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed on a HotSep C_{18} column (100 mm x 0.3 mm ID, 2 µm) with a flow rate of 4 µL/min. The loading flow rate was 200 µL/min and loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA.

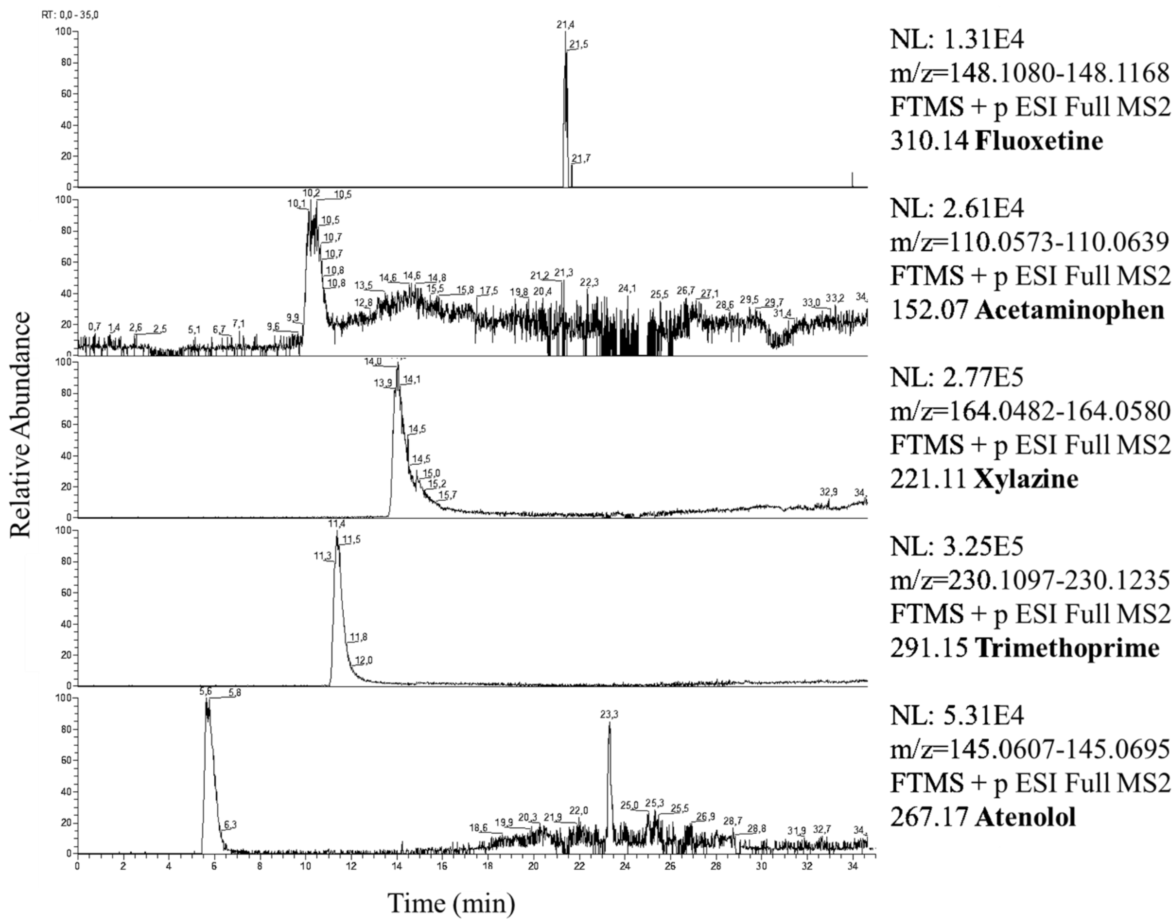

Figure A- 4 EIC of the selected APIs (1 µg/L) where MeOH with 0.1 % FA was used as organic solvent (MP B). The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed on a HotSep C_{18} column (100 mm x 0.3 mm ID, 2 µm) with a flow rate of 4 µL/min. The loading flow rate was 200 µL/min and loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA.

7.5 Elimination of compounds

The reason for removal of the APIs from the method is given in **Table A-4**.

Table A- 4 The compounds eliminated from the method and the reason for elimination.

Name of compound	Reason eliminated
Atorvastatin	Visible in MS with loss of H_2O ($[M+H-H_2O]^+$) in direct infusion using 0.1 % MeOH as dissolving solvent. The peak was disappeared after some days at same concentration (100 ng/L) it has been visible before.
Azithromycin	Carry over approximately 4 %.
Caffeine	Very high baseline, with no distinctive peak in MS, despite giving a clear peak in UV.
Carbamazepine	Eliminated due to time available.
Ciprofloxacin	MS-chromatograms indicate poor solubility and tailing. Stock solution was dissolved with toluene and then dissolved in MP B. Peak shape was improved, but it causes approximately 4 % carry over.
Clarithromycin	Carry over approximately 4 %.
Diclofenac sodium salt	Incompatible with positive mode ESI.
Erythromycin a dihydrate	Eliminated due to time available.
Gemfibrosil	Same *m/z* values as polyethylene glycol (PEG), which is often present in some of the equipment used, no basic groups, so the peaks are most likely due to interferences.
Ibuprofen	Incompatible with positive mode ESI. MS-chromatograms indicate impurities of the standard utilized, no basic groups, so the peaks are most likely due to interferences.

Ketoprofen	Two peaks always present in blank samples; possible an interference, no basic groups, so the peaks are most likely due to interferences.
Losartan potassium	Signals in blank are observed. The signals from the most intense peaks are not from Losartan, but are present in water in same concentration. They are enriched on SPE-column, when loaning for a long time. The fragment ion with *m/z* 401.1613 could be used as quantification for Losartan.
Metoprolol tartrate salt	Eliminated due to time available.
Naproxen	Incompatible with positive mode ESI.
S-Propranolol hydrochloride	Carry over approximately 2-4 %.
Pravastatin sodium salt hydrate	Found to be incompatible with positive mode ESI.
Salicyclic acid	Not retained on SPE-column, incompatible with positive mode ESI.
Simvastatin	Eliminated due to time available.
Valsartan	Eliminated due to time available.

7.6 Chromatograms of the selected APIs and the internal standards

The chromatograms of the selected APIs at a concentration of 10 ng/L spiked in MP A, filtrated and non-filtrated water samples from the Sognsvann creek are shown in **Figure A-5, A-6** and **A-7**, respectively. The quantifier (**Table 13**) of each API was used for quantification, see **Table 12** for the optimized MS conditions of the Quantiva QQQ MS.

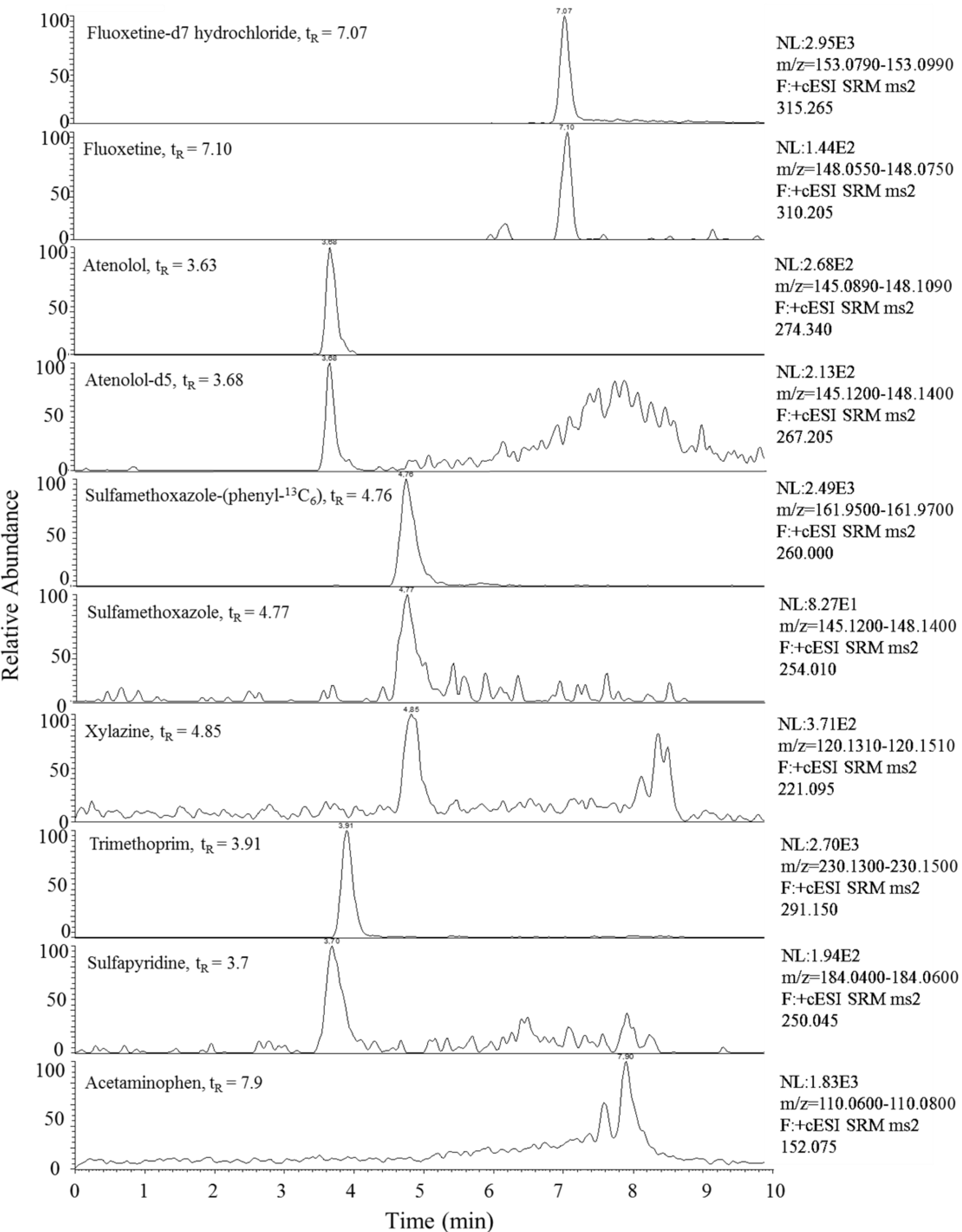

Figure A- 5 EIC of the selected APIs and the internal standards spiked in MP A monitored by MRM using the Quantiva QQQ MS. The concentration of the APIs was 10 ng/L, and concentration of atenolol-d7, fluoxetine-d5 hydrochloride and sulfamethoxazole-(phenyl-$^{13}C_6$) were, 1000 ng/L, 500 ng/L and 50 ng/L, respectively. The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed using gradient program 2 on an ACE C_{18} column (150 mm x 0.3 mm ID, 3 µm) with a flow rate of 4 µL/min. The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA.

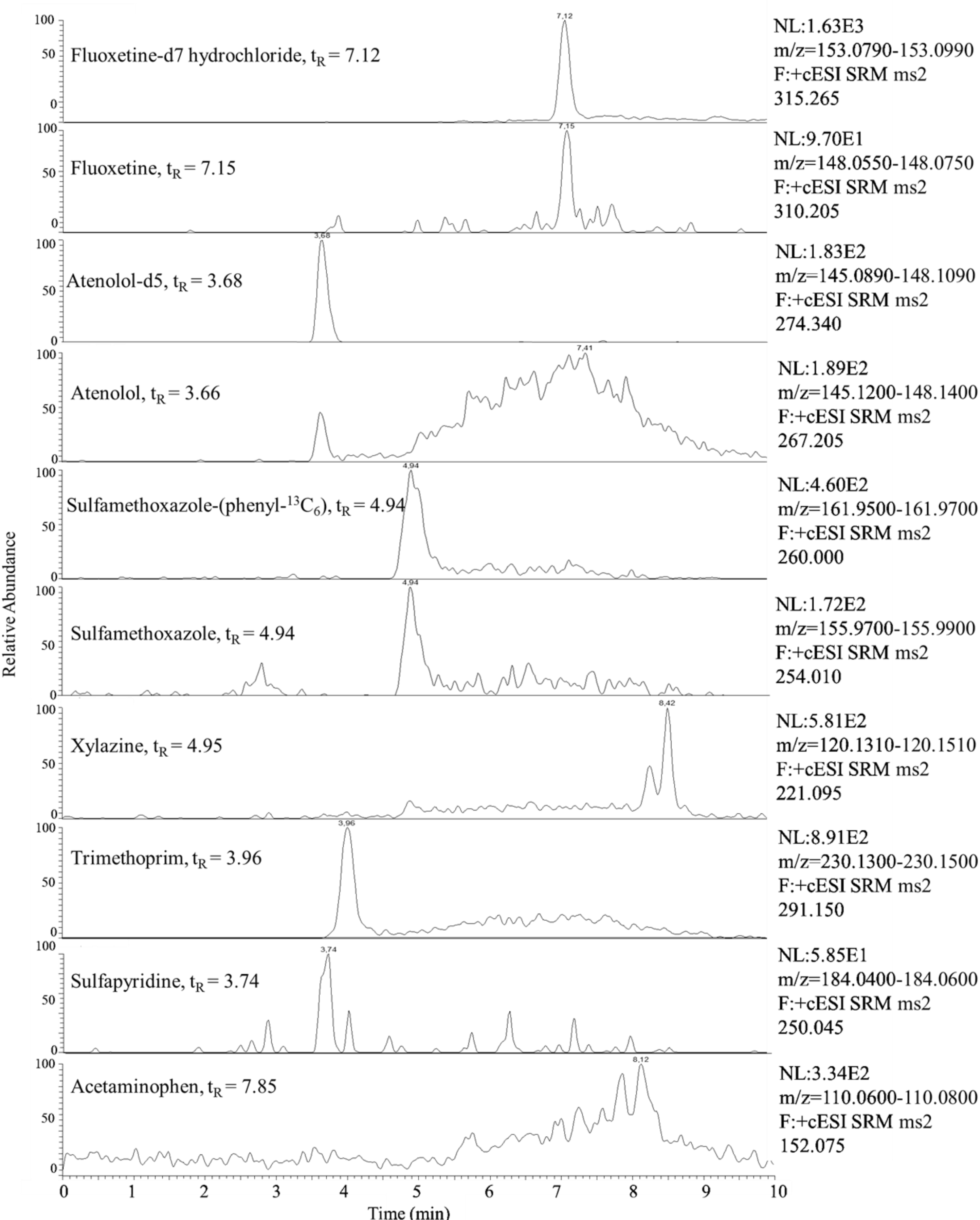

Figure A- 6 EIC of the selected APIs and the internal standards spiked in filtrated water sample from Sognsvann creek monitored by MRM using the Quantiva QQQ MS. The concentration of the APIs was 10 ng/L, and concentration of atenolol-d7, fluoxetine-d5 hydrochloride and sulfamethoxazole-(phenyl-$^{13}C_6$) were, 1000 ng/L, 500 ng/L and 50 ng/L, respectively. The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed using gradient program 2 on an ACE C_{18} column (150 mm x 0.3 mm ID, 3 µm) with a flow rate of 4 µL/min. The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA.

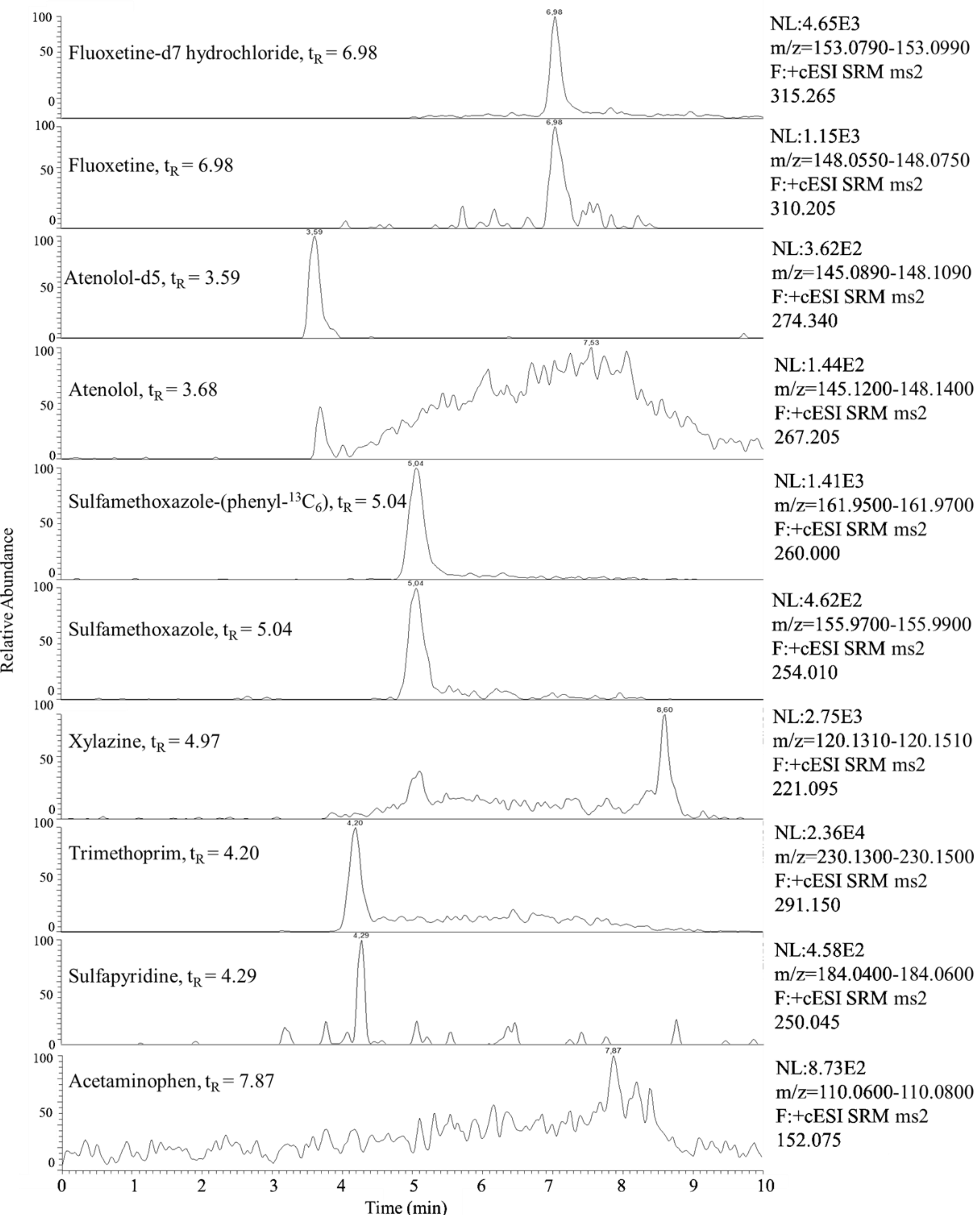

Figure A- 7 EIC of the selected APIs and the internal standards spiked in non-filtrated water sample from Sognsvann creek monitored by MRM using the Quantiva QQQ MS. The concentration of the APIs was 10 ng/L, and concentration of atenolol-d7, fluoxetine-d5 hydrochloride and sulfamethoxazole-(phenyl-$^{13}C_6$) were, 1000 ng/L, 500 ng/L and 50 ng/L, respectively. The chromatographic system consisted of an AFFL-SPE-LC-MS set-up where gradient elution was performed using gradient program 2 on an ACE C_{18} column (150 mm x 0.3 mm ID, 3 µm) with a flow rate of 4 µL/min. The loading mobile phase (MP) and MP A was type 1 water with 0.1 % FA and the MP B was 100 % MeOH with 0.1 % FA.

7.7 Raw data for method validation

7.7.1 Acetaminophen (AA)

The calculated mean, SD and RSD for acetaminophen spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table A-5.** The regression analyses of acetaminophen in the matrices are shown in **Table A-6.** The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-7, A-8, and A-9**, respectively.

Table A- 5 The concentrations of acetaminophen (AA) spiked in MP A (i), filtrated (ii) and no-filtrated (iii) water sample from the Sognsvann creek. The ratios between the peak area of AA and peak area of the IS (SM-IS) (sulfamethoxazole-(phenyl-$^{13}C_6$) was calculated for all replicates in a single day. The average (the mean) of the average calculated for each day was calculated. The corresponding SD and RSD of the mean were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A AA/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.067	0.005	8	18	54
25	0.095	0.002	2		
50	0.119	0.015	13		
75	0.141	0.004	3		
100	0.189	0.022	12		

i)

Concentration (ng/L)	Mean A AA/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.086	0.007	8	13	39
25	0.098	0.007	7		
50	0.181	0.037	20		
75	0.260	0.037	14		
100	0.325	0.061	19		

ii)

Concentration (ng/L)	Mean A AA/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.047	0.003	6	14	43
25	0.069	0.001	1		
50	0.091	0.002	2		
75	0.110	0.002	2		
100	0.128	0.005	4		

iii)

Table A- 6 The regression analysis for acetaminophen spiked in MP A (A), filtrated (B) and no-filtrated (C). The *standard error* and the *coefficient of X variable 1* (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the confidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.988799842
R Square	**0.977725127**
Adjusted R Square	0.97030017
Standard Error	0.008029203
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.008489218	0.008489218	131.6809055	0.001420493
Residual	3	0.000193404	6.44681E-05		
Total	4	0.008682622			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.056725411	**0.006752732**	8.400364909	0.003538772	0.035235205	0.078215618	0.035235205	0.078215618
X Variable 1	**0.001262032**	0.000109979	11.47523009	0.001420493	0.00091203	0.001612033	0.00091203	0.001612033

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.069345727	-0.002185811	-0.314347149
2	0.088276201	0.006359784	0.914617129
3	0.119826991	-0.000482057	-0.069325835
4	0.151377781	-0.01024632	-1.473549981
5	0.182928571	0.006554404	0.942605836

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.994091753
R Square	**0.988218413**
Adjusted R Square	0.984291217
Standard Error	0.012879599
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.041742174	0.041742174	251.634622	0.000544674
Residual	3	0.000497652	0.000165884		
Total	4	0.042239826			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.044444474	**0.010832019**	4.103064726	0.026198815	0.009972156	0.078916793	0.009972156	0.078916793
X Variable 1	**0.002798491**	0.000176416	15.86299537	0.000544674	0.002237056	0.003359927	0.002237056	0.003359927

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.072429385	0.013972362	1.252670503
2	0.114406752	-0.016058914	-1.439737176
3	0.184369029	-0.003797872	-0.340492373
4	0.254331306	0.005471986	0.490582461
5	0.324293583	0.000412439	0.036976585

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.992644779
R Square	**0.985343657**
Adjusted R Square	0.980458209
Standard Error	0.004533851
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.004145891	0.004145891	201.6895332	0.000756393
Residual	3	6.16674E-05	2.05558E-05		
Total	4	0.004207559			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.042961031	**0.003813066**	11.2667938	0.0014993	0.030826152	0.055095909	0.030826152	0.055095909
X Variable 1	**0.000881953**	6.21017E-05	14.20174402	0.000756393	0.000684317	0.001079588	0.000684317	0.001079588

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.051780559	-0.005252695	-1.337778613
2	0.065009851	0.003704198	0.943400882
3	0.087058671	0.003565532	0.908084922
4	0.109107492	0.000666042	0.169630515
5	0.131156312	-0.002683078	-0.683337706

Table A- 7 The single factor Anova test of acetaminophen spiked at different concentrations (10, 25, 50, 75 100 ng/L) in MP A. The test was used to establish absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor					**10 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	0.663247786	0.066324779	0.000100021		
DAY 2	10	0.804894865	0.080489487	5.98474E-05		
DAY 3	10	0.54665484	0.054665484	4.38537E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.003344857	2	0.001672429	24.62806338	8.17903E-07	3.354130829
Within Groups	0.001833501	27	6.79074E-05			
Total	0.005178358	29				

Anova: Single Factor					**25 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.252901	0.084300	0.000017		
DAY 2	3	0.338879	0.112960	0.000063		
DAY 3	3	0.259944	0.086648	0.000298		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001519168	2	0.000759584	6.018939431	0.036804198	5.14325285
Within Groups	0.000757194	6	0.000126199			
Total	0.002276362	8				

Anova: Single Factor					**50 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	1.003689025	0.100368903	0.000110086		
DAY 2	10	1.47086749	0.147086749	5.63779E-05		
DAY 3	10	1.105791516	0.110579152	0.00021133		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.01206537	2	0.006032685	47.90456282	1.31461E-09	3.354130829
Within Groups	0.003400146	27	0.000125931			
Total	0.015465516	29				

Anova: Single Factor					**75 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.3769026	0.1256342	0.0000166		
DAY 2	3	0.5106465	0.1702155	0.0000930		
DAY 3	3	0.3826340	0.1275447	0.0001886		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00381	2	0.001905973	19.17061347	0.002477597	5.14325285
Within Groups	0.00060	6	9.94216E-05			
Total	0.00441	8				

Anova: Single Factor					**100 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	1.664300	0.166430	0.000119		
DAY 2	10	2.225315	0.222531	0.000164		
DAY 3	10	1.794875	0.179487	0.000248		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.017	2	0.0086	48.72023907	1.10009E-09	3.354130829
Within Groups	0.005	27	0.0002			
Total	0.022	29				

Table A- 8 The single factor Anova test of acetaminophen spiked at different concentrations (10, 25, 50, 75 100 ng/L) in filtrated water sample from the Sognsvann creek. The test was used to establish absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				10 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.40	0.07	0.000142786		
DAY 2	6	0.57	0.09	0.000152129		
DAY 3	6	0.59	0.10	0.000402806		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.004	2	0.001808457	7.775851578	0.004818576	3.682320344
Within Groups	0.003	15	0.000232574			
Total	0.007	17				

Anova: Single Factor				25 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.286164261	0.095388087	7.64625E-05		
DAY 2	3	0.41729977	0.139099923	1.28231E-05		
DAY 3	3	0.476710013	0.158903338	0.0002349		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006337087	2	0.003168543	29.32152549	0.000799628	5.14325285
Within Groups	0.000648372	6	0.000108062			
Total	0.006985459	8				

Anova: Single Factor				50 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.820763388	0.136793898	0.000259879		
DAY 2	6	1.02259527	0.170432545	0.00095803		
DAY 3	6	1.406922158	0.234487026	0.000245756		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.029556965	2	0.014778482	30.29068371	5.40224E-06	3.682320344
Within Groups	0.007318331	15	0.000487889			
Total	0.036875296	17				

Anova: Single Factor				75 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.58070212	0.193567373	0.000467325		
DAY 2	3	0.728116826	0.242705609	0.000287019		
DAY 3	3	1.029410681	0.343136894	0.000306467		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.034872051	2	0.017436025	49.30951168	0.000188635	5.14325285
Within Groups	0.002121622	6	0.000353604			
Total	0.036993673	8				

Anova: Single Factor				100 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	1.660536903	0.27675615	0.000884521		
DAY 2	6	1.831821714	0.305303619	0.001705782		
DAY 3	6	2.352349782	0.392058297	0.000928823		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.043271834	2	0.021635917	18.44427977	9.07084E-05	3.682320344
Within Groups	0.017595632	15	0.001173042			
Total	0.060867466	17				

Table A- 9 The single factor Anova test of acetaminophen spiked at different concentrations (10, 25, 50, 75 100 ng/L) in non-filtrated water sample from the Sognsvann creek. The test was used to calculate absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				10 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.216633699	0.036105617	8.23754E-06		
DAY 2	6	0.360224747	0.060037458	0.000162706		
DAY 3	6	0.260643109	0.043440518	6.22105E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001803984	2	0.000901992	11.60594833	0.000899902	3.682320344
Within Groups	0.001165771	15	7.77181E-05			
Total	0.002969756	17				

Anova: Single Factor				25 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.18089158	0.060297193	1.03701E-05		
DAY 2	3	0.238874896	0.079624965	2.31306E-05		
DAY 3	3	0.198659966	0.066219989	5.54236E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000588336	2	0.000294168	9.924213282	0.01250695	5.14325285
Within Groups	0.000177849	6	2.96414E-05			
Total	0.000766184	8				

Anova: Single Factor				10 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.504513817	0.084085636	1.53198E-05		
DAY 2	6	0.605448059	0.10090801	0.000144126		
DAY 3	6	0.521273792	0.086878965	9.68769E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000975218	2	0.000487609	5.7069809	0.014352675	3.682320344
Within Groups	0.001281612	15	8.54408E-05			
Total	0.00225683	17				

Anova: Single Factor				75 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.294995819	0.09833194	1.72138E-05		
DAY 2	3	0.374268828	0.124756276	0.000324698		
DAY 3	3	0.318697161	0.106232387	8.4051E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001103797	2	0.000551899	3.886946991	0.082657743	5.14325285
Within Groups	0.000851926	6	0.000141988			
Total	0.001955723	8				

Anova: Single Factor				100 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.700245646	0.116707608	0.000155763		
DAY 2	6	0.830348109	0.138391352	0.000465855		
DAY 3	6	0.781924462	0.130320744	0.000123772		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001441274	2	0.000720637	2.900375134	0.086117605	3.682320344
Within Groups	0.00372695	15	0.000248463			
Total	0.005168224	17				

7.7.2 Atenolol (AT)

The calculated mean, SD and RSD for atenolol spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table 10**. The regression analyses of atenolol in the matrices are shown in **Table 11**. The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-12, A-13, and A-14**, respectively.

Table A- 10 The concentrations of atenolol (AT) spiked in MP A (i), filtrated (ii) and no-filtrated (iii). The ratios between the peak area (A) of AT and peak area of the IS (AT-IS) (atenolol-d7) was calculated for all replicates in a single day. The average (the mean) of the average calculated for each day was calculated. The corresponding SD and RSD of the mean were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A AT/ A AT-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.60	0.11	18	11	32
25	1.45	0.24	17		
50	2.38	0.39	16		
75	3.75	0.46	12		
100	4.50	0.56	13		

i)

Concentration (ng/L)	Mean A AT/ A AT-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.88	0.05	5	6	19
25	1.69	0.28	17		
50	3.26	0.23	7		
75	4.57	0.63	14		
100	6.44	0.78	12		

ii)

Concentration (ng/L)	Mean A AT/ A AT-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.52	0.02	4	5	16
25	1.32	0.19	14		
50	3.23	0.23	7		
75	4.63	0.69	15		
100	6.40	0.94	15		

iii)

Table A- 11 The regression analysis for atenolol spiked in MP A (A), filtrated (B) and no-filtrated (C) water sample from the Sognsvann creek. The *standard error* and the *coefficient of X variable 1* (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the confidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.995944811
R Square	**0.991906067**
Adjusted R Square	0.989208089
Standard Error	0.166656204
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	10.21116188	10.21116188	367.6479842	0.000309803
Residual	3	0.083322871	0.02777429		
Total	4	10.29448475			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.259872045	**0.140161442**	1.854090836	0.160770079	-0.186184218	0.705928308	-0.186184218	0.705928308
X Variable 1	**0.043769741**	0.002282748	19.17414885	0.000309803	0.036505019	0.051034462	0.036505019	0.051034462

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals
1	0.697569454	-0.098975297	-0.685764019
2	1.354115568	0.097932066	0.678535854
3	2.448359092	-0.070607414	-0.489213223
4	3.542602615	0.203729697	1.411569352
5	4.636846139	-0.132079052	-0.915127964

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998514623
R Square	**0.997031453**
Adjusted R Square	0.996041937
Standard Error	0.140578731
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	19.91248349	19.91248349	1007.595435	6.87056E-05
Residual	3	0.059287139	0.01976238		
Total	4	19.97177062			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.195572514	**0.118229728**	1.654173767	0.196665178	-0.180687246	0.571832274	-0.180687246	0.571832274
X Variable 1	**0.061122219**	0.001925555	31.74264379	6.87056E-05	0.054994242	0.067250195	0.054994242	0.067250195

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals
1	0.8067947	0.075089924	0.61678161
2	1.723627979	-0.032363396	-0.265829903
3	3.251683444	0.005628437	0.046231454
4	4.779738909	-0.184490414	-1.515386996
5	6.307794374	0.136135449	1.118203834

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999021595
R Square	**0.998044148**
Adjusted R Square	0.997392197
Standard Error	0.122220716
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	22.86781396	22.86781396	1530.858324	3.67323E-05
Residual	3	0.044813711	0.014937904		
Total	4	22.91262767			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	-0.180718818	**0.102790244**	-1.758132007	0.176968462	-0.507843249	0.146405614	-0.507843249	0.146405614
X Variable 1	**0.065501119**	0.001674099	39.12618463	3.67323E-05	0.060173388	0.07082885	0.060173388	0.07082885

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals
1	0.474292374	0.050394613	0.476111487
2	1.456809162	-0.11642005	-1.09989778
3	3.094337142	0.133584428	1.262061096
4	4.731865122	-0.099329314	-0.938430205
5	6.369393102	0.031770322	0.300155402

Table A- 12 The single factor Anova test of atenolol spiked at different concentrations (10, 25, 50, 75, 100 ng/L) in MP A. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor					**10 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	6.136197392	0.613619739	0.002854244		
DAY 2	10	6.366538566	0.636653857	0.002144377		
DAY 3	10	5.455088771	0.545508877	0.002045366		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.044923558	2	0.022461779	9.56636302	0.000723249	3.354130829
Within Groups	0.063395883	27	0.002347996			
Total	0.108319441	29				

Anova: Single Factor					**25 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	4.706101186	1.568700395	0.037828708		
DAY 2	3	4.317405369	1.439135123	0.009878319		
DAY 3	3	4.044922159	1.348307386	0.03753996		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.073609916	2	0.036804958	1.295234915	0.340724216	5.14325285
Within Groups	0.170493974	6	0.028415662			
Total	0.24410389	8				

Anova: Single Factor					**50 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	24.15922624	2.415922624	0.020947969		
DAY 2	10	23.69287022	2.369287022	0.009228527		
DAY 3	10	23.48045389	2.348045389	0.01045201		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.024111351	2	0.012055676	0.890188447	0.422287647	3.354130829
Within Groups	0.365656559	27	0.013542836			
Total	0.389767911	29				

Anova: Single Factor					**100 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	45.9062564	4.59062564	0.017202933		
DAY 2	10	44.44197885	4.444197885	0.010156259		
DAY 3	10	44.79477734	4.479477734	0.02238975		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.116798708	2	0.058399354	3.521643964	0.043750759	3.354130829
Within Groups	0.447740479	27	0.016582981			
Total	0.564539187	29				

Anova: Single Factor					**100 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	45.9062564	4.59062564	0.017202933		
DAY 2	10	44.44197885	4.444197885	0.010156259		
DAY 3	10	44.79477734	4.479477734	0.02238975		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.116798708	2	0.058399354	3.521643964	0.043750759	3.354130829
Within Groups	0.447740479	27	0.016582981			
Total	0.564539187	29				

Table A- 13 The single factor Anova test of atenolol spiked at different concentrations (10, 25, 50, 75, 100 ng/L) in filtrated water sample form the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				**10 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	5.197241059	0.866206843	0.003453004		
DAY 2	6	5.32472765	0.887454608	0.00321373		
DAY 3	6	5.351954529	0.891992421	0.002245968		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002273911	2	0.001136955	0.382697186	0.688490813	3.682320344
Within Groups	0.04456351	15	0.002970901			
Total	0.04683742	17				

Anova: Single Factor				**25 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	4.790319539	1.59677318	0.061033707		
DAY 2	3	4.839396744	1.613132248	0.006784468		
DAY 3	3	5.591664967	1.863888322	0.006804562		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.134496727	2	0.067248364	2.703533791	0.145523023	5.14325285
Within Groups	0.149245474	6	0.024874246			
Total	0.283742201	8				

Anova: Single Factor				**50 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	19.33428582	3.22238097	0.028801388		
DAY 2	6	19.60770598	3.267950996	0.011639442		
DAY 3	6	19.68962206	3.281603677	0.002369717		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011540704	2	0.005770352	0.404364269	0.674461833	3.682320344
Within Groups	0.214052737	15	0.014270182			
Total	0.225593441	17				

Anova: Single Factor				**75 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	13.01353135	4.337843783	0.074373957		
DAY 2	3	13.97667287	4.658890955	0.040533133		
DAY 3	3	14.12875316	4.709584386	0.07568249		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.243832186	2	0.121916093	1.919036073	0.226842143	5.14325285
Within Groups	0.38117916	6	0.06352986			
Total	0.625011346	8				

Anova: Single Factor				**100 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	38.10733013	6.351221688	0.03750416		
DAY 2	6	38.8108667	6.468477783	0.030155765		
DAY 3	6	39.07253999	6.512089999	0.071749451		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.083059262	2	0.041529631	0.893690912	0.429845297	3.682320344
Within Groups	0.697046881	15	0.046469792			
Total	0.780106143	17				

Table A- 14 The single factor Anova test of atenolol spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in non-filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				**10 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	2.94053782	0.490089637	0.000637664		
DAY 2	6	3.299398279	0.549899713	0.000913864		
DAY 3	6	3.204429679	0.534071613	0.00031152		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011524377	2	0.005762188	9.27864363	0.002383873	3.682320344
Within Groups	0.009315243	15	0.000621016			
Total	0.02083962	17				

Anova: Single Factor				**25 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	4.007676087	1.335892029	0.014726348		
DAY 2	3	4.353301026	1.451100342	0.009619842		
DAY 3	3	3.547003901	1.182334634	0.014515725		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.109087835	2	0.054543917	4.210594214	0.072019582	5.14325285
Within Groups	0.077723829	6	0.012953972			
Total	0.186811664	8				

Anova: Single Factor				**50 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	19.35670848	3.22611808	0.023286371		
DAY 2	6	19.35075084	3.225125141	0.013452341		
DAY 3	6	19.39512895	3.232521492	0.009942548		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000193391	2	9.66956E-05	0.006214204	0.993807621	3.682320344
Within Groups	0.233406297	15	0.01556042			
Total	0.233599688	17				

Anova: Single Factor				**75 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	13.77202696	4.590675653	0.03795758		
DAY 2	3	13.33572189	4.445240631	0.146312466		
DAY 3	3	14.58507342	4.861691141	0.025574196		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.268031767	2	0.134015884	1.915933688	0.227271886	5.14325285
Within Groups	0.419688482	6	0.06994808			
Total	0.687720249	8				

Anova: Single Factor				**100 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	38.16834686	6.361391143	0.023766124		
DAY 2	6	38.02744576	6.337907626	0.025680259		
DAY 3	6	39.02514902	6.504191503	0.118813626		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.097187492	2	0.048593746	0.866404557	0.440471707	3.682320344
Within Groups	0.841300045	15	0.05608667			
Total	0.938487537	17				

7.7.3 Fluoxetine (FX)

The calculated mean, SD and RSD for fluoxetine spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table A-15**. The regression analyses of fluoxetine in the matrices are shown in **Table A-16**. The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-17, A-18, and A-19**, respectively.

Table A- 15 The concentrations (10-100 ng/L) of fluoxetine (FX) spiked in MP A (i), filtrated (ii) and no-filtrated (iii) water sample from the Sognsvann creek. The ratios between the peak area (A) of FX and peak area of the IS (FX-IS) (fluoxetine-d5 hydrochloride) was calculated for all replicates in a single day. The average (the mean) of the average calculated for each day was calculated. The corresponding SD and RSD of the mean were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A FX/ A FX-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.034	0.005	15	5	14
25	0.060	0.001	1		
50	0.101	0.008	7		
75	0.143	0.001	0		
100	0.193	0.016	8		

i)

Concentration (ng/L)	Mean A FX/ A FX-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.076	0.008	10	6	18
25	0.121	0.008	6		
50	0.224	0.019	8		
75	0.340	0.018	5		
100	0.437	0.057	13		

ii)

Concentration (ng/L)	Mean A FX/ A FX-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.047	0.001	3	8	24
25	0.097	0.001	1		
50	0.227	0.042	19		
75	0.341	0.037	11		
100	0.489	0.078	16		

iii)

Table A- 16 The regression analysis for fluoxetine spiked in MP A (A), filtrated (B) and no-filtrated (C). The *standard error* and the *coefficient of X variable 1* (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the confidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999228309
R Square	**0.998457213**
Adjusted R Square	0.997942951
Standard Error	0.002885277
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regression	1	0.01616292	0.01616292	1941.533467	2.57E-05
Residual	3	2.49745E-05	8.32482E-06		
Total	4	0.016187894			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.015578536	**0.00242658**	6.419956723	0.007659217	0.007856	0.023300995	0.007856077	0.023300995
X Variable 1	**0.001741391**	3.95206E-05	44.06283544	2.57305E-05	0.001616	0.001867163	0.001615619	0.001867163

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.032992446	0.001309875	0.524217947
2	0.059113312	0.00056598	0.226507744
3	0.102648088	-0.001511452	-0.604889923
4	0.146182864	-0.003390587	-1.356927995
5	0.18971764	0.003026184	1.211092227

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998697588
R Square	**0.997396872**
Adjusted R Square	0.996529162
Standard Error	0.008842957
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.089885292	0.089885292	1149.459441	5.6412E-05
Residual	3	0.000234594	7.81979E-05		
Total	4	0.090119885			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.026109301	**0.007437116**	3.510675498	0.039178823	0.002441079	0.049777524	0.002441079	0.049777524
X Variable 1	**0.004106584**	0.000121125	33.90367887	5.6412E-05	0.00372111	0.004492058	0.00372111	0.004492058

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.067175141	0.008975718	1.172036329
2	0.128773901	-0.007986128	-1.04281705
3	0.231438502	-0.007247565	-0.946376594
4	0.334103102	0.006140928	0.801873552
5	0.436767702	0.000117046	0.015283762

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.997710126
R Square	**0.995425495**
Adjusted R Square	0.993900661
Standard Error	0.014097001
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.129729707	0.129729707	652.8087274	0.000131493
Residual	3	0.000596176	0.000198725		
Total	4	0.130325883			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-0.016264053	**0.011855881**	-1.371813176	0.263709151	-0.053994756	0.02146665	-0.053994756	0.02146665
X Variable 1	**0.004933511**	0.000193091	25.55012187	0.000131493	0.004319008	0.005548014	0.004319008	0.005548014

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.033071058	0.013494971	1.10538756
2	0.107073726	-0.009998466	-0.818985105
3	0.230411504	-0.002992635	-0.245129981
4	0.353749283	-0.012601226	-1.032179939
5	0.477087062	0.012097356	0.990907465

Table A- 17 The single factor Anova test of fluoxetine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) spiked in MP A. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor						**10 ng/L spiked in MP A**
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	0.444964902	0.04449649	7.06234E-05		
DAY 2	10	0.407011327	0.040701133	3.97786E-05		
DAY 3	10	0.177093426	0.017709343	7.8183E-06		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.004201928	2	0.002100964	53.31481431	4.2E-10	3.354130829
Within Groups	0.001063982	27	3.94068E-05			
Total	0.005265911	29				

Anova: Single Factor						**25 ng/L spiked in MP A**
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.214316177	0.071438726	1.40642E-05		
DAY 2	3	0.168585242	0.056195081	2.33925E-05		
DAY 3	3	0.154212211	0.05140407	3.94706E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00065671	2	0.000328355	12.80514236	0.006839	5.14325285
Within Groups	0.000153855	6	2.56424E-05			
Total	0.000810564	8				

Anova: Single Factor						**50 ng/L spiked in MP A**
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	1.118436531	0.111843653	0.000499083		
DAY 2	10	0.986425249	0.098642525	0.000106496		
DAY 3	10	0.929237295	0.09292373	2.50303E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001883126	2	0.000941563	4.479300715	0.020896	3.354130829
Within Groups	0.005675486	27	0.000210203			
Total	0.007558612	29				

Anova: Single Factor						**75 ng/L spiked in MP A**
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.450436084	0.150145361	4.22105E-05		
DAY 2	3	0.423370868	0.141123623	0.00015402		
DAY 3	3	0.411323543	0.137107848	5.72224E-06		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000267495	2	0.000133747	1.986812632	0.217718	5.14325285
Within Groups	0.000403906	6	6.73176E-05			
Total	0.000671401	8				

Anova: Single Factor						**100 ng/L spiked in MP A**
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	1.986709093	0.198670909	0.000434758		
DAY 2	10	1.744886055	0.174488605	0.000165726		
DAY 3	10	2.050719581	0.205071958	0.000580948		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.005203662	2	0.002601831	6.606809771	0.004617	3.354130829
Within Groups	0.010632884	27	0.000393811			
Total	0.015836546	29				

Table A- 18 The single factor Anova test of fluoxetine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				10 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
0.06440281	5	0.251275488	0.050255098	4.85546E-05		
0.103935698	5	0.519978938	0.103995788	4.96763E-05		
0.071741512	5	0.371008463	0.074201693	5.89009E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.007248649	2	0.003624324	69.19653535	2.5806E-07	3.885293835
Within Groups	0.000628527	12	5.23773E-05			
Total	0.007877176	14				

Anova: Single Factor				25 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.299191326	0.099730442	0.000228783		
DAY 2	3	0.472143698	0.157381233	0.000304738		
DAY 3	3	0.315754941	0.105251647	0.000185785		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006071591	2	0.003035796	12.66134536	0.007028725	5.14325285
Within Groups	0.001438613	6	0.000239769			
Total	0.007510204	8				

Anova: Single Factor				50 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	1.433019522	0.238836587	0.00074068		
DAY 2	6	1.431489813	0.238581635	0.000695674		
DAY 3	6	1.170927528	0.195154588	0.000788538		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.007588181	2	0.00379409	5.115873847	0.020233893	3.682320344
Within Groups	0.011124465	15	0.000741631			
Total	0.018712645	17				

Anova: Single Factor				75 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	1.126686514	0.375562171	0.002385496		
DAY 2	3	1.007343585	0.335781195	1.25328E-05		
DAY 3	3	0.928166172	0.309388724	0.003446962		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.006658014	2	0.003329007	1.708646163	0.258628033	5.14325285
Within Groups	0.011689981	6	0.00194833			
Total	0.018347995	8				

Anova: Single Factor				100 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	2.859231888	0.476538648	0.001273653		
DAY 2	6	2.673711319	0.445618553	0.002383664		
DAY 3	6	2.330982269	0.388497045	0.002960291		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.023940486	2	0.011970243	5.426542498	0.016859381	3.682320344
Within Groups	0.033088038	15	0.002205869			
Total	0.057028524	17				

Table A- 19 The single factor Anova test of fluoxetine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in non-filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				**10 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.28768452	0.04794742	2.90077E-05		
DAY 2	6	0.329735408	0.054955901	1.80768E-06		
DAY 3	6	0.220768598	0.036794766	5.0764E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001006655	2	0.000503327	18.50936031	8.90199E-05	3.682320344
Within Groups	0.000407897	15	2.71931E-05			
Total	0.001414551	17				
Anova: Single Factor				**25 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.308644663	0.102881554	5.83713E-05		
DAY 2	3	0.290588933	0.096862978	0.000363359		
DAY 3	3	0.274443741	0.091481247	1.05303E-07		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000195153	2	9.75767E-05	0.693943756	0.535664578	5.14325285
Within Groups	0.000843671	6	0.000140612			
Total	0.001038824	8				
Anova: Single Factor				**50 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	1.694714987	0.282452498	0.001508071		
DAY 2	6	1.227522753	0.204587125	0.000301908		
DAY 3	6	1.171301905	0.195216984	0.001143172		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.027521701	2	0.013760851	13.97915544	0.000373965	3.682320344
Within Groups	0.014765753	15	0.000984384			
Total	0.042287454	17				
Anova: Single Factor				**75 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	2	0.887072691	0.443536346	0.000878922		
DAY 2	2	0.506893527	0.253446763	8.12875E-05		
DAY 3	2	0.68229223	0.341146115	2.63278E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.03620599	2	0.018102995	55.05012045	0.004320028	9.552094496
Within Groups	0.000986537	3	0.000328846			
Total	0.037192527	5				
Anova: Single Factor				**100 ng/L spiked in non-filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	3.33895155	0.556491925	0.002755031		
DAY 2	6	2.531031382	0.421838564	0.000416812		
DAY 3	6	2.935336601	0.489222767	0.00160456		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.054394596	2	0.027197298	17.0822899	0.000135923	3.682320344
Within Groups	0.023882013	15	0.001592134			
Total	0.07827661	17				

7.7.4 Sulfamethoxazole (SM)

The calculated mean, SD and RSD for sulfamethoxazole spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table A-20**. The regression analyses of sulfamethoxazole in the matrices are shown in **Table A-21**. The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-22, A-23, and A-24**, respectively.

Table A- 20 The concentrations of sulfamethoxazole (SM) spiked in MP A (i), filtrated (ii) and no-filtrated (iii) water sample from the Sognsvann creek. The ratio between the peak area (A) of SM and peak area of its IS (IS-SM) (sulfamethoxazole-(phenyl-$^{13}C_6$) was calculated for all replicates in a single day. The average (the mean) of the average calculated for each day was calculated manually. The corresponding SD and RSD of the mean were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A A SM A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.24	0.04	15	3	10
25	0.60	0.03	5		
50	1.09	0.16	14		
75	1.56	0.09	6		
100	2.08	0.42	20		

i)

Concentration (ng/L)	Mean A A SM A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.32	0.06	18	5	15
25	0.80	0.11	14		
50	1.48	0.24	16		
75	2.06	0.14	7		
100	2.81	0.55	19		

ii)

Concentration (ng/L)	Mean A A SM A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.33	0.03	9	2	5
25	0.78	0.06	8		
50	1.51	0.11	7		
75	2.31	0.22	10		
100	3.06	0.52	17		

iii)

Table A- 21 The regression analysis for sulfamethoxazole spiked in MP A (A), filtrated (B) and no-filtrated (C). The *standard error* and the *coefficient of X variable* 1 (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the confidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999584208
R Square	**0.999168588**
Adjusted R Square	0.998891451
Standard Error	0.024481107
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2.160756329	2.160756329	3605.319026	1.0177E-05
Residual	3	0.001797974	0.000599325		
Total	4	2.162554303			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.066494053	**0.020589136**	3.229569889	0.048231614	0.000970234	0.132017872	0.000970234	0.132017872
X Variable 1	**0.020134428**	0.000335326	60.04430886	1.0177E-05	0.01906727	0.021201585	0.01906727	0.021201585

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.267838331	-0.027022903	-1.274589455
2	0.569854748	0.025862125	1.219839039
3	1.073215444	0.015800836	0.745278169
4	1.576576139	-0.011905598	-0.561551417
5	2.079936835	-0.002734461	-0.128976336

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999046888
R Square	**0.998094684**
Adjusted R Square	0.997459579
Standard Error	0.049838824
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3.90356683	3.90356683	1571.542208	3.53173E-05
Residual	3	0.007451725	0.002483908		
Total	4	3.911018555			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.085928958	**0.04191552**	2.050051109	0.13275752	-0.047464933	0.219322848	-0.047464933	0.219322848
X Variable 1	**0.027062456**	0.00068266	39.64268165	3.53173E-05	0.024889928	0.029234983	0.024889928	0.029234983

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.356553516	-0.034125168	-0.790635631
2	0.762490354	0.032857955	0.761275961
3	1.439051749	0.040627627	0.941289131
4	2.115613145	-0.056978515	-1.320117874
5	2.792174541	0.017618101	0.408188413

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999884261
R Square	**0.999768536**
Adjusted R Square	0.999691381
Standard Error	0.019503707
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	4.929146349	4.929146349	12957.98295	1.49467E-06
Residual	3	0.001141184	0.000380395		
Total	4	4.930287533			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.014564534	**0.016403036**	0.887917006	0.439989527	-0.037637246	0.066766314	-0.037637246	0.066766314
X Variable 1	**0.030410408**	0.000267149	113.8331364	1.49467E-06	0.029560221	0.031260596	0.029560221	0.031260596

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.318668618	0.009805228	0.580510295
2	0.774824743	0.003032409	0.179531237
3	1.535084952	-0.029030078	-1.718701344
4	2.29534516	0.013664106	0.808971913
5	3.055605369	0.002528334	0.149687898

Table A- 22 The single factor Anova test of sulfamethoxazole spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in MP A. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor — **10 ng/L spiked in MP A**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	10	2.11341275	0.21134127	0.000289671
DAY 2	10	2.81742540	0.28174254	0.000575967
DAY 3	10	2.29362470	0.22936247	0.000140311

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.02674925	2	0.013374623	39.88655276	8.69312E-09	3.354130829
Within Groups	0.00905355	27	0.000335317			
Total	0.03580279	29				

Anova: Single Factor — **25 ng/L spiked in MP A**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	3	1.59233562	0.53077854	8.216E-05
DAY 2	3	2.006919298	0.668973099	0.000451053
DAY 3	3	1.762196942	0.587398981	0.000680449

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.028957947	2	0.014478974	35.78994576	0.000462601	5.14325285
Within Groups	0.002427325	6	0.000404554			
Total	0.031385273	8				

Anova: Single Factor — **50 ng/L spiked in MP A**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	10	10.25830416	1.025830416	0.000601998
DAY 2	10	11.22504871	1.122504871	0.009549054
DAY 3	10	11.18713555	1.118713555	0.000539877

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.059958673	2	0.029979336	8.412553114	0.001445941	3.354130829
Within Groups	0.096218362	27	0.003563643			
Total	0.156177035	29				

Anova: Single Factor — **75 ng/L spiked in MP A**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	3	4.37830161	1.45943387	0.003852655
DAY 2	3	4.491856329	1.497285443	0.000825734
DAY 3	3	4.959628524	1.653209508	0.007205316

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.063294053	2	0.031647027	7.989182145	0.020345466	5.14325285
Within Groups	0.023767409	6	0.003961235			
Total	0.087061463	8				

Anova: Single Factor — **100 ng/L spiked in MP A**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	10	19.92531751	1.992531751	0.004808308
DAY 2	10	21.13525776	2.113525776	0.027501359
DAY 3	10	21.25549595	2.125549595	0.002233297

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.108259578	2	0.054129789	4.701083728	0.017708959	3.354130829
Within Groups	0.310886679	27	0.011514321			
Total	0.419146257	29				

Table A- 23 The single factor Anova test of sulfamethoxazole spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

10 ng/L spiked in filtrated water sample

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DAY 1	6	1.781266903	0.296877817	0.003100554
DAY 2	6	1.909667706	0.318277951	0.003540809
DAY 3	6	2.112775655	0.352129276	0.003077452

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009313203	2	0.004656602	1.437397856	0.268457963	3.682320344
Within Groups	0.048594079	15	0.003239605			
Total	0.057907282	17				

25 ng/L spiked in filtrated water sample

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DAY 1	3	2.087729205	0.695909735	0.012429142
DAY 2	3	2.445511463	0.815170488	0.00323019
DAY 3	3	2.62489411	0.874964703	0.015432395

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.049859157	2	0.024929579	2.405422339	0.170952268	5.14325285
Within Groups	0.062183455	6	0.010363909			
Total	0.112042612	8				

50 ng/L spiked in filtrated water sample

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DAY 1	6	9.130761339	1.521793556	0.010553967
DAY 2	6	8.129209284	1.354868214	0.005466539
DAY 3	6	9.37425816	1.56237636	0.003468272

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.145141329	2	0.072570665	11.17114677	0.001069491	3.682320344
Within Groups	0.097443888	15	0.006496259			
Total	0.242585217	17				

75 ng/L spiked in filtrated water sample

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DAY 1	3	5.863113183	1.954371061	0.019734546
DAY 2	3	6.169620148	2.056540049	0.005124044
DAY 3	3	6.494978341	2.16499278	0.01255008

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.066562006	2	0.033281003	2.668980497	0.148200229	5.14325285
Within Groups	0.074817339	6	0.012469556			
Total	0.141379345	8				

100 ng/L spiked in filtrated water sample

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
DAY 1	6	16.55080623	2.758467706	0.042865045
DAY 2	6	16.69310295	2.782183824	0.039347197
DAY 3	6	17.33235838	2.888726396	0.015534629

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.057762201	2	0.0288811	0.886404855	0.43265407	3.682320344
Within Groups	0.488734355	15	0.03258229			
Total	0.546496555	17				

Table A- 24 The single factor Anova test of sulfamethoxazole spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in non-filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor — **10 ng/L spiked in non-filtrated water sample**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	6	1.745535808	0.290922635	0.00081414
DAY 2	6	2.197580558	0.366263426	0.000413122
DAY 3	6	1.969412862	0.328235477	0.000971464

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.017029216	2	0.008514608	11.61755453	0.000895812	3.682320344
Within Groups	0.010993632	15	0.000732909			
Total	0.028022848	17				

Anova: Single Factor — **25 ng/L spiked in non-filtrated water sample**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	3	2.108622429	0.702874143	0.001133943
DAY 2	3	2.669482395	0.889827465	0.000143797
DAY 3	3	2.222609545	0.740869848	0.000431149

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.05858359	2	0.029291795	51.42254586	0.000167505	5.14325285
Within Groups	0.003417777	6	0.000569629			
Total	0.062001366	8				

Anova: Single Factor — **50 ng/L spiked in non-filtrated water sample**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	6	8.618965792	1.436494299	0.00221442
DAY 2	6	9.363373971	1.560562329	0.00566006
DAY 3	6	9.126647964	1.521107994	0.004988607

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.048217996	2	0.024108998	5.622833281	0.015057483	3.682320344
Within Groups	0.064315435	15	0.004287696			
Total	0.112533431	17				

Anova: Single Factor — **75 ng/L spiked in non-filtrated water sample**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	3	6.405338683	2.135112894	0.00438811
DAY 2	3	7.384464162	2.461488054	0.006208937
DAY 3	3	6.991280555	2.330426852	0.018957241

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.161845326	2	0.080922663	8.214306953	0.019144607	5.14325285
Within Groups	0.059108575	6	0.009851429			
Total	0.2209539	8				

Anova: Single Factor — **100 ng/L spiked in non-filtrated water sample**

SUMMARY

Groups	Count	Sum	Average	Variance
DAY 1	6	16.98193247	2.830322078	0.038133232
DAY 2	6	18.30425988	3.05070998	0.012445374
DAY 3	6	18.41290946	3.068818243	0.010567851

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.211558314	2	0.105779157	5.189793301	0.019366465	3.682320344
Within Groups	0.305732284	15	0.020382152			
Total	0.517290598	17				

7.7.5 Sulfapyridine (SP)

The calculated mean, SD and RSD for sulfapyridine spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table A-25**. The regression analyses of sulfapyridine in the matrices are shown in **Table A-26**. The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-27, A-28, and A-29**, respectively.

Table A- 25 The concentrations of sulfapyridine (SP) spiked in MP A (i), filtrated (ii) and no-filtrated (iii) water sample from the Sognsvann creek. The peak area to SP/peak area to the IS (SM-IS) (sulfamethoxazole-(phenyl-$^{13}C_6$) was calculated for a single day. The mean of three days and the corresponding SD and RSD were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A SP/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.019	0.0007	3	8	23
25	0.039	0.0003	1		
50	0.073	0.0027	4		
75	0.120	0.0003	0.3		
100	0.162	0.0082	5		

i)

Concentration (ng/L)	Mean A SP/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.062	0.006	10	9	27
25	0.084	0.001	2		
50	0.134	0.017	13		
75	0.164	0.014	9		
100	0.217	0.029	13		

ii)

Concentration (ng/L)	Mean A SP/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.0205	0.0005	2	6	19
25	0.0346	0.0008	2		
50	0.0704	0.0026	4		
75	0.0973	0.0006	1		
100	0.1331	0.0110	8		

iii)

Table A- 26 The regression analysis for sulfapyridine spiked in MP A (A), filtrated (B) and no-filtrated (C). The *standard error* and the *coefficient of X variable 1* (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the cofidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.997847554
R Square	0.995699742
Adjusted R Square	0.994266322
Standard Error	0.004440521
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	0.013696921	0.013696921	694.6325206	0.000119837
Residual	3	5.91547E-05	1.97182E-05		
Total	4	0.013756076			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	-0.000715713	0.003734573	-0.191645106	0.860257985	-0.012600792	0.011169366	-0.012600792	0.011169366
X Variable 1	0.001603053	6.08233E-05	26.35588209	0.000119837	0.001409486	0.00179662	0.001409486	0.00179662

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals
1	0.015314817	0.00374504	0.973849685
2	0.039360612	-0.000481263	-0.125146355
3	0.079436936	-0.006164463	-1.602989664
4	0.11951326	0.000290572	0.075559529
5	0.159589584	0.002610114	0.678726806

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.997048127
R Square	0.994104967
Adjusted R Square	0.992139955
Standard Error	0.005497899
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	0.015291874	0.015291874	505.9029688	0.000192437
Residual	3	9.06807E-05	3.02269E-05		
Total	4	0.015382555			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.044227975	0.004623851	9.565182092	0.00242416	0.029512818	0.058943132	0.029512818	0.058943132
X Variable 1	0.001693818	7.53066E-05	22.49228687	0.000192437	0.001454159	0.001933477	0.001454159	0.001933477

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals
1	0.061166156	0.001024669	0.215206972
2	0.086573426	-0.002274252	-0.477651545
3	0.128918877	0.005059219	1.062566356
4	0.171264328	-0.006984491	-1.466923175
5	0.21360978	0.003174855	0.666801393

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998492047
R Square	0.996986368
Adjusted R Square	0.995981824
Standard Error	0.002908748
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	0.00839716	0.00839716	992.4766061	7.02777E-05
Residual	3	2.53824E-05	8.46081E-06		
Total	4	0.008422543			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.005912632	0.002446319	2.41695008	0.094426607	-0.001872648	0.013697912	-0.001872648	0.013697912
X Variable 1	0.00125517	3.98421E-05	31.50359672	7.02777E-05	0.001128375	0.001381966	0.001128375	0.001381966

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals
1	0.018464334	0.002078341	0.825049796
2	0.037291886	-0.002712215	-1.076681929
3	0.068671141	0.001725695	0.68505804
4	0.100050396	-0.002796774	-1.110249559
5	0.13142965	0.001704952	0.676823652

Table A- 27 The single factor Anova test of sulfapyridine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in MP A. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor					**10 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	0.142411107	0.014241111	3.55005E-06		
DAY 2	10	0.189685358	0.018968536	4.94164E-06		
DAY 3	10	0.23969925	0.023969925	1.2516E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000473374	2	0.000236687	33.80006659	4.45525E-08	3.354130829
Within Groups	0.000189069	27	7.00256E-06			
Total	0.000662443	29				

Anova: Single Factor					**25 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.097138493	0.032379498	1.30139E-05		
DAY 2	3	0.13293771	0.04431257	3.9183E-06		
DAY 3	3	0.11983793	0.039945977	2.17299E-07		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000218717	2	0.000109358	19.1302985	0.002491162	5.14325285
Within Groups	3.4299E-05	6	5.71651E-06			
Total	0.000253016	8				

Anova: Single Factor					**50ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	0.697569293	0.069756929	0.000190356		
DAY 2	10	0.737971607	0.073797161	1.86983E-05		
DAY 3	10	0.762633273	0.076263327	6.71509E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000215796	2	0.000107898	1.17193093	0.325033166	3.354130829
Within Groups	0.002485846	27	9.20684E-05			
Total	0.002701642	29				

Anova: Single Factor					**75 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.361624781	0.120541594	1.70651E-07		
DAY 2	3	0.363692407	0.121230802	0.000111328		
DAY 3	3	0.352917301	0.1176391	4.70798E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.17998E-05	2	1.08999E-05	0.206205105	0.819199843	5.14325285
Within Groups	0.000317157	6	5.28595E-05			
Total	0.000338957	8				

Anova: Single Factor					**100 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	1.662313819	0.166231382	0.000242045		
DAY 2	10	1.704115991	0.170411599	0.000259951		
DAY 3	10	1.499561153	0.149956115	0.000145154		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002335951	2	0.001167976	5.414396656	0.01053919	3.354130829
Within Groups	0.00582435	27	0.000215717			
Total	0.008160301	29				

Table A- 28 The single factor Anova test of sulfapyridine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				10 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.339464408	0.056577401	0.000123889		
DAY 2	6	0.287026461	0.047837743	7.63956E-05		
DAY 3	6	0.492943981	0.08215733	0.000221623		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.003817097	2	0.001908548	13.57085262	0.000431861	3.682320344
Within Groups	0.002109538	15	0.000140636			
Total	0.005926635	17				

Anova: Single Factor				25 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.225490239	0.075163413	0.000155124		
DAY 2	3	0.233576714	0.077858905	1.06689E-05		
DAY 3	3	0.299625617	0.099875206	1.37944E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001102656	2	0.000551328	9.209905784	0.014832926	5.14325285
Within Groups	0.000359175	6	5.98625E-05			
Total	0.001461831	8				

Anova: Single Factor				50 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.649513239	0.108252207	0.000143104		
DAY 2	6	0.714244546	0.119040758	3.65586E-05		
DAY 3	6	1.047847941	0.174641324	0.000224007		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.015230661	2	0.007615331	56.59573625	1.0274E-07	3.682320344
Within Groups	0.002018349	15	0.000134557			
Total	0.01724901	17				

Anova: Single Factor				75 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.398426331	0.132808777	9.39823E-06		
DAY 2	3	0.438275412	0.146091804	0.000396224		
DAY 3	3	0.641816797	0.213938932	0.00101106		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.011361774	2	0.005680887	12.02998157	0.007952221	5.14325285
Within Groups	0.002833364	6	0.000472227			
Total	0.014195138	8				

Anova: Single Factor				100 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	1.14922393	0.191537322	0.000879618		
DAY 2	6	1.254753071	0.209125512	0.001017207		
DAY 3	6	1.498146418	0.24969107	0.001745445		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.010673535	2	0.005336767	4.395694546	0.031443867	3.682320344
Within Groups	0.018211345	15	0.00121409			
Total	0.02888488	17				

Table A- 29 The single factor Anova test of sulfapyridine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in non-filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				10 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.092632795	0.015438799	8.73419E-06		
DAY 2	6	0.149155452	0.024859242	1.69996E-05		
DAY 3	6	0.127979902	0.021329984	1.60267E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000271813	2	0.000135906	9.763291551	0.001925451	3.682320344
Within Groups	0.000208802	15	1.39201E-05			
Total	0.000480615	17				

Anova: Single Factor				25 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.097784073	0.032594691	4.24081E-05		
DAY 2	3	0.075423845	0.025141282	1.18202E-05		
DAY 3	3	0.138009123	0.046003041	2.06794E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00067055	2	0.000335275	13.42753902	0.006090406	5.14325285
Within Groups	0.000149815	6	2.49692E-05			
Total	0.000820365	8				

Anova: Single Factor				50 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.392821746	0.065470291	0.000117039		
DAY 2	6	0.474195957	0.079032659	0.000128722		
DAY 3	6	0.400125348	0.066687558	0.00014359		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000675642	2	0.000337821	2.60295683	0.107052237	3.682320344
Within Groups	0.001946754	15	0.000129784			
Total	0.002622396	17				

Anova: Single Factor				75 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.277918308	0.092639436	5.9279E-06		
DAY 2	3	0.305553723	0.101851241	1.61946E-05		
DAY 3	3	0.291810568	0.097270189	0.00022578		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000127287	2	6.36436E-05	0.770185499	0.503820237	5.14325285
Within Groups	0.000495805	6	8.26342E-05			
Total	0.000623092	8				

Anova: Single Factor				100 ng/L spiked in non-filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.676995991	0.112832665	0.000163546		
DAY 2	6	0.948611237	0.158101873	0.000288598		
DAY 3	6	0.693782888	0.115630481	0.000205063		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.007721896	2	0.003860948	17.62437258	0.00011541	3.682320344
Within Groups	0.00328603	15	0.000219069			
Total	0.011007926	17				

7.7.6 Xylazine (X)

The calculated mean, SD and RSD for xylazine spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table 30**. The regression analyses of xylazine in the matrices are shown in **Table 31**. The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-32, A-33, and A-34**, respectively.

Table A- 30 The concentrations of xylazine (X) spiked in MP A (i), filtrated (ii) and no-filtrated (iii) water sample from the Sognsvann creek. The ratios between the peak area (A) to X and peak area to the IS (SM-IS) (sulfamethoxazole-(phenyl-$^{13}C_6$) was calculated for a single day. The mean of three days and the corresponding SD and RSD were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A X/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.055	0.001	3	2	6
25	0.141	0.004	3		
50	0.273	0.027	10		
75	0.400	0.033	8		
100	0.538	0.091	17		

i)

Concentration (ng/L)	Mean A X/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.16	0.02	11	10	33
25	0.20	0.01	4		
50	0.42	0.04	10		
75	0.56	0.03	5		
100	0.75	0.14	18		

ii)

Concentration (ng/L)	Mean A X/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.08	0.01	15	6	21
25	0.18	0.02	9		
50	0.33	0.06	19		
75	0.54	0.08	15		
100	0.70	0.13	19		

iii)

Table A- 31 The regression analysis for xylazine spiked in MP A (A), filtrated (B) and no-filtrated (C). The *standard error* and the *coefficient of X variable 1* (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the confidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999855811
R Square	0.999711643
Adjusted R Square	0.999615523
Standard Error	0.003811687
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.151112132	0.151112132	10400.7549	2.07837E-06
Residual	3	4.35869E-05	1.4529E-05		
Total	4	0.151155719			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.004387118	0.003205711	1.368532011	0.264621684	-0.005814884	0.014589119	-0.005814884	0.014589119
X Variable 1	0.005324589	5.221E-05	101.9840914	2.07837E-06	0.005158433	0.005490744	0.005158433	0.005490744

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.057633004	-0.002776777	-0.841188053
2	0.137501834	0.003406048	1.031817578
3	0.27061655	0.001996511	0.604816707
4	0.403731267	-0.00421477	-1.276809159
5	0.536845983	0.001588988	0.481362927

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.995783881
R Square	0.991585538
Adjusted R Square	0.988780717
Standard Error	0.026370484
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.245844936	0.245844936	353.5290168	0.000328419
Residual	3	0.002086207	0.000695402		
Total	4	0.247931143			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.066007844	0.022178143	2.976256622	0.058772824	-0.004572904	0.136588592	-0.004572904	0.136588592
X Variable 1	0.006791521	0.000361206	18.80236732	0.000328419	0.005642003	0.007941038	0.005642003	0.007941038

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.133923051	0.021895448	0.958749409
2	0.235795861	-0.034073946	-1.49201677
3	0.405583879	0.017123479	0.749796257
4	0.575371896	-0.010848732	-0.475040074
5	0.745159914	0.005903751	0.258511179

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.998236051
R Square	0.996475214
Adjusted R Square	0.995300285
Standard Error	0.017391925
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.256537152	0.256537152	848.1153941	8.89095E-05
Residual	3	0.000907437	0.000302479		
Total	4	0.257444589			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.007447905	0.014626982	0.509189484	0.645708351	-0.03910168	0.053997491	-0.03910168	0.053997491
X Variable 1	0.006937636	0.000238223	29.12242081	8.89095E-05	0.006179504	0.007695769	0.006179504	0.007695769

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.076824269	0.006826463	0.453228749
2	0.180888813	0.001819356	0.120792344
3	0.354329721	-0.023608119	-1.567411748
4	0.527770629	0.017182903	1.140822965
5	0.701211537	-0.002220603	-0.147432311

Table A- 32 The single factor Anova test of xylazine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in MP A. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor					**10 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	0.523771817	0.052377182	1.43405E-05		
DAY 2	10	0.600478363	0.060047836	5.96901E-05		
DAY 3	10	0.521436639	0.052143664	4.0972E-05		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000404565	2	0.000202282	5.276815171	0.011630806	3.354130829
Within Groups	0.001035023	27	3.83342E-05			
Total	0.001439587	29				
Anova: Single Factor					**25 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.410070522	0.136690174	9.25355E-05		
DAY 2	3	0.39720109	0.132400363	0.000663928		
DAY 3	3	0.460899326	0.153633109	0.000676346		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000756295	2	0.000378147	0.791761092	0.495268682	5.14325285
Within Groups	0.002865618	6	0.000477603			
Total	0.003621913	8				
Anova: Single Factor					**50 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	2.609229485	0.260922949	0.000324171		
DAY 2	10	3.08003451	0.308003451	0.000280269		
DAY 3	10	2.489127831	0.248912783	0.000257496		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.019508416	2	0.009754208	33.94988488	4.26905E-08	3.354130829
Within Groups	0.007757423	27	0.000287312			
Total	0.027265839	29				
Anova: Single Factor					**75 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	1.022651772	0.340883924	0.000112294		
DAY 2	3	1.434135147	0.478045049	0.001252035		
DAY 3	3	1.138861551	0.379620517	0.000108237		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.030001086	2	0.015000543	30.5600244	0.000714327	5.14325285
Within Groups	0.00294513	6	0.000490855			
Total	0.032946217	8				
Anova: Single Factor					**100 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	5.632648265	0.563264827	0.002014064		
DAY 2	10	5.745876658	0.574587666	0.001501759		
DAY 3	10	4.77452419	0.477452419	0.000365402		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.056424107	2	0.028212054	21.80655527	2.30918E-06	3.354130829
Within Groups	0.03493103	27	0.001293742			
Total	0.091355138	29				

Table A- 33 The single factor Anova test of xylazine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				10 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.873850621	0.14564177	0.0009231		
DAY 2	6	0.85219067	0.142031778	0.000342992		
DAY 3	6	1.078691691	0.179781948	0.001139783		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.005207318	2	0.002603659	3.246627619	0.067362465	3.682320344
Within Groups	0.012029371	15	0.000801958			
Total	0.017236689	17				

Anova: Single Factor				25 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.724551007	0.241517002	0.001058118		
DAY 2	3	0.828140889	0.276046963	0.001347116		
DAY 3	3	0.867971081	0.289323694	0.000178345		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00365407	2	0.001827035	2.121516098	0.200987079	5.14325285
Within Groups	0.005167158	6	0.000861193			
Total	0.008821227	8				

Anova: Single Factor				50 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	2.363750969	0.393958495	0.004023503		
DAY 2	6	2.455193773	0.409198962	0.000441835		
DAY 3	6	2.789787695	0.464964616	0.000741584		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.016767898	2	0.008383949	4.830464135	0.024022256	3.682320344
Within Groups	0.026034607	15	0.00173564			
Total	0.042802506	17				

Anova: Single Factor				75 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	1.537917259	0.512639086	0.004637908		
DAY 2	3	1.711506621	0.570502207	0.001272415		
DAY 3	3	1.8312846	0.6104282	0.000156149		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.014504936	2	0.007252468	3.586500678	0.094492979	5.14325285
Within Groups	0.012132943	6	0.002022157			
Total	0.02663788	8				

Anova: Single Factor				100 ng/L spiked in filtrated water sample		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	3.909643282	0.651607214	0.000965018		
DAY 2	6	4.674800562	0.779133427	0.005571077		
DAY 3	6	4.93470213	0.822450355	0.001814532		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.094653341	2	0.04732667	17.00231685	0.000139285	3.682320344
Within Groups	0.041753136	15	0.002783542			
Total	0.136406477	17				

Table A- 34 The single factor Anova test of xylazine spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in non-filtrated water sample from the Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor			**10 ng/L spiked in non-filtrated water sample**			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	0.314276227	0.052379371	7.54986E-06		
DAY 2	6	0.62987547	0.104979245	0.00046737		
DAY 3	6	0.561561475	0.093593579	0.00019076		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.009189982	2	0.004594991	20.70810181	4.84346E-05	3.682320344
Within Groups	0.003328401	15	0.000221893			
Total	0.012518383	17				

Anova: Single Factor			**25 ng/L spiked in non-filtrated water sample**			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	0.388984092	0.129661364	0.000438867		
DAY 2	3	0.671945667	0.223981889	0.000135171		
DAY 3	3	0.583443767	0.194481256	0.000550174		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.013968267	2	0.006984134	18.63741397	0.00266531	5.14325285
Within Groups	0.002248424	6	0.000374737			
Total	0.016216691	8				

Anova: Single Factor			**50 ng/L spiked in non-filtrated water sample**			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	1.580515785	0.263419298	0.000463664		
DAY 2	6	2.317467716	0.386244619	0.00244129		
DAY 3	6	2.055005338	0.34250089	0.000688519		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.046506944	2	0.023253472	19.41308742	6.89022E-05	3.682320344
Within Groups	0.017967368	15	0.001197825			
Total	0.064474311	17				

Anova: Single Factor			**75 ng/L spiked in non-filtrated water sample**			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	1.240629674	0.413543225	0.00026927		
DAY 2	3	1.832191846	0.610730615	0.001701938		
DAY 3	3	1.831760275	0.610586758	0.00112692		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.077709042	2	0.038854521	37.62386555	0.000402736	5.14325285
Within Groups	0.006196257	6	0.001032709			
Total	0.083905298	8				

Anova: Single Factor			**100 ng/L spiked in non-filtrated water sample**			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	3.535666214	0.589277702	0.002726721		
DAY 2	6	4.524047149	0.754007858	0.001155427		
DAY 3	6	4.522123452	0.753687242	0.00069696		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.108333248	2	0.054166624	35.48722971	2.05548E-06	3.682320344
Within Groups	0.022895542	15	0.001526369			
Total	0.13122879	17				

7.7.7 Trimethoprim (TM)

The calculated mean, SD and RSD for trimethoprim spiked in MP A, filtrated and non-filtrated water sample from the Sognsvann creek are shown in **Table A-35**. The regression analyses of trimethoprim in the matrices are shown in **Table A-36.** The single factor Anova tests of the API in MP A, filtrated and non-filtrated water sample are shown in **Table A-37, A-38, and A-39**, respectively.

Table A- 35 The concentrations of trimethoprim (TM) spiked in MP A (i), filtrated (ii) and no-filtrated (iii) water sample from the Sognsvann creek. The ratios between the peak area (A) to TM and peak area to the IS (SM-IS) (sulfamethoxazole-(phenyl-$^{13}C_6$) was calculated for a single day. The mean of three days and the corresponding SD and RSD were calculated. The cLOD and cLOQ were calculated for the lowest concentration.

Concentration (ng/L)	Mean A TM/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.42	0.358	86	9	26
25	1.19	1.045	88		
50	1.95	2.428	124		
75	2.84	2.019	71		
100	3.87	6.031	156		

i)

Concentration (ng/L)	Mean A TM/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	1.56	1.28	82	13	39
25	2.29	0.86	38		
50	5.66	13.77	243		
75	7.65	8.88	116		
100	9.71	13.31	137		

ii)

Concentration (ng/L)	Mean A TM/ A SM-IS	SD	RSD %	cLOD (ng/L)	cLOQ (ng/L)
10	0.65	0.30	46	9	28
25	1.40	0.34	0		
50	2.49	2.62	105		
75	3.92	0.09	2		
100	4.72	4.37	93		

iii)

Table A- 36 The regression analysis for trimethoprim spiked in MP A (A), filtrated (B) and no-filtrated (C) water sample from the Sognsvann creek. The *standard error* and the *coefficient of X variable* 1 (slope of the curve) were used in calculations of cLOD and cLOQ. The units in all cases are ng/L and the confidence level was 95 %.

A)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.997323569
R Square	0.994654301
Adjusted R Square	0.992872401
Standard Error	0.112632016
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	7.081293524	7.081293524	558.1987794	0.000166147
Residual	3	0.038057913	0.012685971		
Total	4	7.119351437			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.141264756	0.094725941	1.491299579	0.232682679	-0.160195465	0.442724976	-0.160195465	0.442724976
X Variable 1	0.036449594	0.00154276	23.62623075	0.000166147	0.031539844	0.041359343	0.031539844	0.041359343

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.505760695	-0.088160017	-0.903814229
2	1.052504603	0.132754062	1.360991239
3	1.96374445	0.010464653	0.107283356
4	2.874984297	-0.101815433	-1.043809206
5	3.786224143	0.046756734	0.479348839

B)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.99681505
R Square	0.993640243
Adjusted R Square	0.991520325
Standard Error	0.155984529
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	11.40441448	11.40441448	468.7161731	0.000215665
Residual	3	0.07299352	0.024331173		
Total	4	11.477408			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.231156436	0.131186334	1.762046612	0.176271967	-0.186337029	0.648649902	-0.186337029	0.648649902
X Variable 1	0.046256513	0.002136574	21.64985388	0.000215665	0.039456981	0.053056045	0.039456981	0.053056045

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	0.693721567	-0.044837967	-0.331920258
2	1.387569264	0.013355412	0.09886558
3	2.543982092	-0.049798772	-0.368643409
4	3.70039492	0.220947992	1.635603006
5	4.856807748	-0.139666664	-1.033904919

C)

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.993893465
R Square	0.987824219
Adjusted R Square	0.983765625
Standard Error	0.441796156
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	47.50594544	47.50594544	243.3907676	0.000572306
Residual	3	0.58555153	0.195183843		
Total	4	48.09149697			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.463750653	0.371560042	1.248117667	0.300528335	-0.71871923	1.646220535	-0.71871923	1.646220535
X Variable 1	0.094408344	0.006051434	15.60098611	0.000572306	0.075149979	0.113666709	0.075149979	0.113666709

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	1.407834089	0.153936307	0.402335635
2	2.823959245	-0.535866996	-1.400568792
3	5.184167836	0.473243112	1.236891875
4	7.544376428	0.106944058	0.27951434
5	9.90458502	-0.198256481	-0.518173058

Table A- 37 The single factor Anova test of trimethoprim spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in MP A. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor					**10 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	2.990178405	0.299017841	0.000630243		
DAY 2	10	5.567039731	0.556703973	0.000904735		
DAY 3	10	3.957702598	0.39577026	0.000612076		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.338876112	2	0.169438056	236.7494954	7.61145E-18	3.354130829
Within Groups	0.019323494	27	0.000715685			
Total	0.358199606	29				

Anova: Single Factor					**25 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	2.560308657	0.853436219	0.00164999		
DAY 2	3	4.819655875	1.606551958	0.077261226		
DAY 3	3	3.28736345	1.095787817	0.000325295		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.886797622	2	0.443398811	16.78767045	0.003484815	5.14325285
Within Groups	0.158473022	6	0.02641217			
Total	1.045270644	8				

Anova: Single Factor					**50 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	17.27359174	1.727359174	0.019346688		
DAY 2	10	23.14059709	2.314059709	0.005676576		
DAY 3	10	18.1422268	1.81422268	0.021952141		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.005332809	2	1.002666404	64.03349305	5.64157E-11	3.354130829
Within Groups	0.422778637	27	0.015658468			
Total	2.428111446	29				

Anova: Single Factor					**75 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	7.49765235	2.49921745	0.005997308		
DAY 2	3	10.71982609	3.573275363	0.115212195		
DAY 3	3	8.676810293	2.892270098	0.00249582		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.771858956	2	0.885929478	21.48483484	0.001839383	5.14325285
Within Groups	0.247410646	6	0.041235108			
Total	2.019269602	8				

Anova: Single Factor					**100 ng/L spiked in MP A**	
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	10	37.36522781	3.736522781	0.054299546		
DAY 2	10	42.84509449	4.284509449	0.198452054		
DAY 3	10	35.74222235	3.574222235	0.109526543		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.770462608	2	1.385231304	11.47100368	0.000247811	3.354130829
Within Groups	3.260503289	27	0.120759381			
Total	6.030965897	29				

Table A- 38 The single factor Anova test of trimethoprim spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in filtrated water sample from Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor				**10 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	9.089608245	1.514934708	0.035245464		
DAY 2	6	8.349775803	1.391629301	0.02344436		
DAY 3	6	10.67248309	1.778747182	0.103108237		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.469322998	2	0.234661499	4.351007007	0.032344099	3.682320344
Within Groups	0.808990306	15	0.053932687			
Total	1.278313304	17				

Anova: Single Factor				**25 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	8.855259444	2.951753148	0.088824018		
DAY 2	3	8.433227626	2.811075875	0.001515685		
DAY 3	3	10.16861992	3.389539972	0.067747752		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.54606811	2	0.273034055	5.181322983	0.049305248	5.14325285
Within Groups	0.316174911	6	0.052695818			
Total	0.862243021	8				

Anova: Single Factor				**50 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	32.50500778	5.417501297	0.063731592		
DAY 2	6	29.01323983	4.835539972	0.139647667		
DAY 3	6	40.31514945	6.719191574	0.318147168		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.16243984	2	5.581219921	32.10510319	3.80037E-06	3.682320344
Within Groups	2.607632136	15	0.173842142			
Total	13.77007198	17				

Anova: Single Factor				**75 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	21.24853784	7.082845946	0.218899362		
DAY 2	3	20.72682729	6.908942431	0.106409007		
DAY 3	3	26.88651924	8.962173082	0.226633146		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.777869022	2	3.888934511	21.1377532	0.001919876	5.14325285
Within Groups	1.103883031	6	0.183980505			
Total	8.881752053	8				

Anova: Single Factor				**100 ng/L spiked in filtrated water sample**		
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	57.42705895	9.571176492	0.14331798		
DAY 2	6	54.35336569	9.058894281	0.677886373		
DAY 3	6	62.93348907	10.48891485	0.580040222		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.299271123	2	3.149635562	6.74322445	0.008145113	3.682320344
Within Groups	7.006222879	15	0.467081525			
Total	13.305494	17				

Table A- 39 The single factor Anova test of trimethoprim spiked at different concentrations (10, 25, 50, 75, and 100 ng/L) in non-filtrated water sample from Sognsvann creek. The test was used to establish the absolute standard deviation (*total*), between-day and within-day variance of each concentration.

Anova: Single Factor			10 ng/L spiked in non-filtrated water sample			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	3.198204641	0.533034107	0.003668152		
DAY 2	6	4.704152376	0.784025396	0.015301862		
DAY 3	6	3.777547782	0.629591297	0.00250952		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.192339618	2	0.096169809	13.43182859	0.000453843	3.682320344
Within Groups	0.107397673	15	0.007159845			
Total	0.299737291	17				

Anova: Single Factor			25 ng/L spiked in non-filtrated water sample			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	3.508788488	1.169596163	0.002471907		
DAY 2	3	4.897860405	1.632620135	0.002565534		
DAY 3	3	4.201673189	1.40055773	0.003286417		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.321587404	2	0.160793702	57.95162411	0.000119236	5.14325285
Within Groups	0.016647717	6	0.002774619			
Total	0.338235121	8				

Anova: Single Factor			50 ng/L spiked in non-filtrated water sample			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	12.71685576	2.119475959	0.018762284		
DAY 2	6	17.88425208	2.98070868	0.029134273		
DAY 3	6	14.29419193	2.382365322	0.009456998		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.337694781	2	1.168847391	61.13905586	6.14716E-08	3.682320344
Within Groups	0.286767772	15	0.019117851			
Total	2.624462554	17				

Anova: Single Factor			75 ng/L spiked in non-filtrated water sample			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	3	9.939408708	3.313136236	0.002669319		
DAY 2	3	13.4979369	4.4993123	0.032809784		
DAY 3	3	11.8547406	3.9515802	0.050575636		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.114634805	2	1.057317402	36.8597041	0.000426345	5.14325285
Within Groups	0.172109478	6	0.028684913			
Total	2.286744282	8				

Anova: Single Factor			100 ng/L spiked in non-filtrated water sample			
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
DAY 1	6	25.32524471	4.220874118	0.086748332		
DAY 2	6	31.61146873	5.268578122	0.08015441		
DAY 3	6	27.97182607	4.661971012	0.042327488		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.320444673	2	1.660222336	23.80471991	2.21761E-05	3.682320344
Within Groups	1.046151147	15	0.06974341			
Total	4.36659582	17				