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**ANALYSIS OF PARACETAMOL, CAFFEINE AND FLUOXETINE IN STOKES  
BARDOLPH EFFLUENT USING SPE AND UPLC-QTOF-MS**

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## **Abstract**

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A method to achieve the chromatographic separation and mass identification of a group of pharmaceuticals was carried on using Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-QToF-MS). With a 1.7  $\mu\text{m}$  particle size column good chromatographic separation was achieved in 20 minutes. After a solid phase extraction of 700 mL of sample low limits of detection and quantification were obtained. This methodology was applied to real samples of Stoke Bardolph Sewage Treatment Works effluent, in Nottingham and fluoxetine was found at concentrations of 8 ppb which considering the flow of the effluent canal it is equal to 16500 doses of 60 mg. This project also includes a study of the efficiency comparing the number of plates between different conditions of analysis.

## **Objective**

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The aim of this work is to analyse and quantify a group of pharmaceuticals present in Stoke Bardolph Sewage Treatment Works effluent. For this reason, a method is developed starting from the information of other articles<sup>1</sup> and it includes how the mobile phase affects the chromatographic separation and a study of the efficiency depending on the chromatographic conditions. Furthermore, another goal of this article is to improve the skills with Liquid Chromatography as well as with Solid Phase Extraction.

# 1 Introduction

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In the recent years, pharmaceuticals and personal care products (PPCPs) have become a huge problem in our society due to their pollution in the rivers of the cities. The presence of these compounds and their metabolites have been detected in some rivers of the world leading to an increment of the rivers analysis<sup>2</sup>. The main problem is that these emerging contaminants reach the rivers by a lot of different sources and the goal of these articles is to assess the possible harmful effects that may have in the environment, the animal life as well as in the population even though some treatments to remove or reduce their concentration are done. Some of their potential risks in animals<sup>3</sup> are behavioural changes and chronic or reproductive damages among others.

A wide range of pharmaceuticals are usually studied<sup>4</sup> in river water analysis such as antiepileptics, antiphlogistics (agents to prevent or reduce the inflammation<sup>5</sup>) or antibiotics. However, the most analysed pharmaceuticals<sup>6</sup> are those that are more frequently used by the population, like Paracetamol, Ibuprofen or Caffeine which is a stimulant present in some pharmaceuticals. Furthermore, it is noticed that in some previous articles, pharmaceuticals like Naproxen and Fluoxetine were also present in high concentrations so it is important to know if they could be dangerous for the population.

The concentration of some pharmaceuticals in the aquatic environment varies between countries and also between regions of the same country. The level of pharmaceutical contaminants in the river of Nottingham has not been studied in as much detail as other rivers from which a wide bibliography can be found. The goal of this work is to analyse some of these main pharmaceuticals and the ones that can be in higher concentration in Stoke Bardolph Sewage Treatment Work (STW). This plant is located adjacent to the River Trent, and serves the majority of the Nottingham catchment<sup>7</sup>.

Because of these reasons, the compounds targeted for study in the Treatment Works effluent of Nottingham were Paracetamol, Ibuprofen, Caffeine, Naproxen and Fluoxetine.



This work is carried out in Nottingham Trent University and it is supervised by Dr. Quentin Hanley. In order to carry out this study knowledges in Liquid Chromatography as well as in Solid Phase Extraction were needed.

In the first part of the report one can find the characteristics of the pharmaceutical studied, its behaviour in aquatic environment and a description of the instrumentation used. Whereas in the second part, the optimization of the method developed and the results obtained from the analysis of the Stoke Bardolph STW are detailed.

## 2 Fundamentals

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### 2.1 Pharmaceuticals and Personal care compounds (PPCPs)

In this project, the five pharmaceuticals that are going to be studied are included in the group called Pharmaceuticals and Personal Care Products (PPCPs)<sup>8</sup>. This group includes a lot of emerging contaminants which are present at very low concentrations but have a risk due to their capacity of bioaccumulation in the environment and its toxicity<sup>9</sup>.

It is important to be aware that the main sources of PPCPs are the hospital and individual household discharges. Since many years, Environmental Protection Agencies developed a water quality criteria for the Priority Pollutants<sup>10</sup> and also Waste Water Treatment Plants (WWTPs) are present in most of the cities in order to reduce the contamination in the aquatic environment. However, in this kind of treatment plants not all PPCPs are completely removed and because of that, they are usually detected in the water surface at very low concentrations.

The principle characteristics of PPCPs are<sup>11</sup>:

- Persistence: most PPCPs are not easily removed by WWTPs and still are in the aquatic environment.
- Bioaccumulation: they and their metabolites are biologically active and can affect other aquatic organisms.

- Toxicity: the biggest problem of the presence of PPCPs in the aquatic environment is that some of them can interfere with the endocrine system and produce non wanted effects. However, the toxicity of PPCPs is only a problem when therapeutic levels are reached or exceeded.

### 2.1.1 Paracetamol

Paracetamol<sup>2</sup> which is the commonly name for acetaminophen or 4-acetaminophenol (Figure 1)<sup>12</sup>, is an analgesic mainly used to treat fever and headaches and it is one of the most common pharmaceuticals over the world.

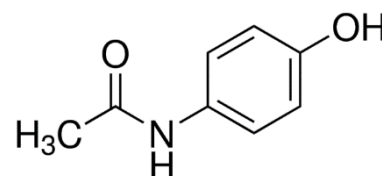


Figure 1. Structure of Paracetamol

Taken at therapeutic levels, it is known not to be danger for humans. The main problem is that paracetamol and its metabolites are usually thrown into the aquatic environment by the population. It is easily accumulated in environmental water because of its high solubility and hydrophilicity.

Although paracetamol is not very persistent in the environment, it can be found at high concentrations and it is a huge problem because of its transformation products. The hydrolytic product of paracetamol is 4-Aminophenol which has nephrotoxicity and teratogenic effects<sup>2</sup>.

Some studies says that the use of paracetamol is increasing and in 2014 it was found that 149.3 kilo tons of this pharmaceutical were sold<sup>13</sup>. Risk toxic doses of paracetamol have been fixed for adults at levels of 7.5-10 g per single ingestion<sup>14</sup>.

### 2.1.2 Caffeine

Caffeine<sup>15</sup> or 1,3,7-trimethylxanthine (Figure 2)<sup>12</sup> is one of the most detected drugs in the environment and is a stimulant that alters the central nervous system in humans. This stimulant is included in some pharmaceuticals as an analeptic and to intensify the effects of analgesic drugs.

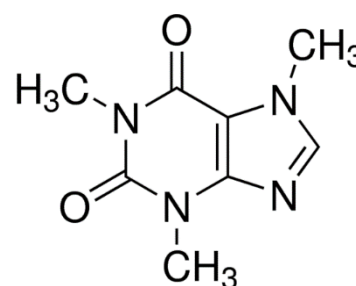


Figure 2. Structure of Caffeine

It is known that caffeine is a safe compound but at high concentrations it is toxic or even lethal.

It is thought that the greater number of caffeine present in the environment come from anthropogenic activities due to its presence in a lot of known beverages, foods and pharmaceuticals.

Although death is uncommon as a result of Caffeine poisoning<sup>14</sup>, oral doses of this pharmaceutical can be fatal in adults at 10 g.

### 2.1.3 Ibuprofen

(RS)-2-(4-(2-methylpropyl)phenyl)propanoic acid commonly known as Ibuprofen<sup>16</sup> (Figure 3)<sup>12</sup> is a nonsteroidal anti-inflammatory drug (NSAID) which is very used to treat pain and fever. Ibuprofen is one of the most detected pharmaceuticals in the aquatic environment

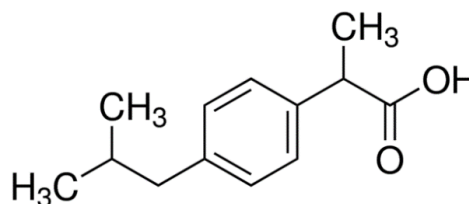


Figure 3. Structure of Ibuprofen

as it is one of the most used pharmaceuticals in the world. Due to its rapid biodegradation, it does not persist in the environment. On the other hand, it also has some transformation products that can present toxicology in the environment. It is known that Ibuprofen can affect the growth of some bacterial and fungal species and in combination with other pharmaceuticals it can block cell proliferation in humans' embryonic cells.

A long consumption of ibuprofen can result in renal papillary necrosis and other renal injury<sup>17</sup>. Moreover, doses higher than recommended may cause stomach bleeding. Thus, maximum levels of ibuprofen are at 800 mg per dose.

### 2.1.4 Naproxen

Naproxen<sup>18</sup> or (S)-(+)-2-(6-Methoxy-2-naphthyl) propionic acid (Figure 4)<sup>12</sup> is a non-steroidal anti-inflammatory drug (NSAID)<sup>19</sup> used to treat osteoarthritis, rheumatoid arthritis and ankylosing

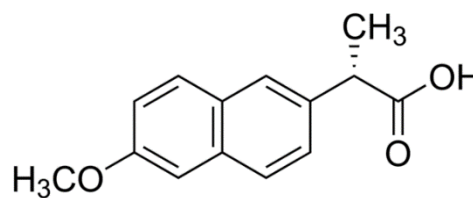


Figure 4. Structure of Naproxen

spondylitis. It is known that in the aquatic environment this pharmaceutical is quickly biodegraded and is not accumulated in aquatic organisms.

This drug is excreted almost in an unchanged form after its administration and the residuals of Naproxen can arrive to surface waters at concentrations that can impact on human health and ecosystem.

Massive Naproxen overdoses are set up at 70 g and may cause serious toxicity and can present altered mental status and renal seizures<sup>20</sup>. Furthermore, minor symptoms of nausea and stomach upset can be suffered with doses of 25 g.

### 2.1.5 Fluoxetine

Fluoxetine<sup>21</sup> (Figure 5)<sup>12</sup> also known as Prozac is an antidepressant chiefly used to treat depression or aids in the treatment of obsessive compulsive disorder among others. The main problem of the presence of this pharmaceutical in the aquatic environment is that it can be accumulated in the

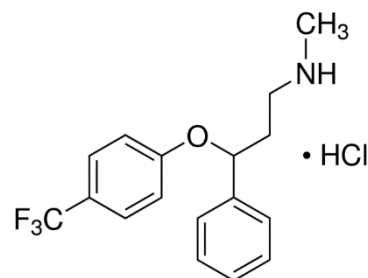


Figure 5. Structure of Fluoxetine hydrochloride

tissues of fish with the same concentration that it is in the environment. The activity of Fluoxetine has not been tested at low exposure levels. However, in other analysis that this compound was found, they did not show an influence of this. This behaviour could be because of Fluoxetine has not an important impact at low levels but may also be due to the nature of the species tested or its exposure.

Taking overdoses of Fluoxetine may result in a benign clinical course, with symptoms such as tachycardia, nausea, vomiting, tremor or drowsiness<sup>22</sup>. Hence, it is important to fix a maximum level which is at 80 mg per day<sup>23</sup>.

## 2.2 Ultra-Performance Liquid Chromatography - Mass spectrometry

The goal of coupling this two systems is to join their advantages and to obtain a final system that provides high sensitivity, resolution and faster analysis.

### 2.2.1 Ultra-Performance Liquid Chromatography (UPLC)

UPLC is a modern system used to improve the speed, resolution and sensitivity of liquid chromatography. <sup>24</sup>Although the principles of this technique are the same as High Performance Liquid Chromatography (HPLC), the main difference between UPLC and HPLC is the particle size of the column<sup>25</sup>, which is less than 2.0 µm for UPLC in comparison to 3.0 and 5.0 µm for HPLC.

Consequences of the smaller particle size:

- This instrument can work at very high backpressures compared to HPLC.
- The column length can be one third part of the column used in HPLC, thus a faster analysis is achieved and while preserving resolution.
- Less band spreading appears during migration through the column so that the sensitivity increases.

Using HPLC a loss of performance can occur due to the frictional heating of the mobile phase at high pressures. For this reason, the column diameter must be considered and minimizing the HPLC one, which is between 3.0 and 4.6 mm, the effects of frictional heating can be reduced. Consequently, smaller diameter columns are used in UPLC (1.0-2.1 mm)<sup>24</sup>.

### 2.2.2 Mass spectrometry (MS)

Using MS detector<sup>26</sup>, one can choose between different types of ion sources. However, electrospray ionisation (ESI) is widely used in this field and it works applying a strong electric field, under atmospheric pressure to a liquid passing through a capillary tube. With the use of this field, a charge accumulation at the liquid surface is caused, which will break to produce highly charged droplets. These droplets form a spray which is dispersed with a gas and pass through a heated inert gas to eliminate the solvent molecules. After this, the highly-charged droplets accumulate excess charge at the surface and the desorption of charged molecules happens from the surface. With the help of an electric field, these charged droplets reaches a point at which it is possible to eject the droplets to the gaseous phase<sup>27</sup>. Thus, a higher sensitivity is obtained for those compounds with a higher concentration in the surface.

Once the molecules are ionized, the analyser distinguishes between the different mass-to-charge ratios ( $m/z$ ) in order to classify and identify them (Figure 6).

The principle of separating ions is that the movement of the ions under a magnetic or electrical field is affected by their  $m/z$  ratio<sup>27</sup>. In a quadrupole, an assembly of four parallel metal rods in equal distance are connected electrically. This electrical field is responsible that ions travel in the direction of the rods.

The reason of using a mass spectrometry is because of its high sensitivity, detection limits, speed and wide range of applications.

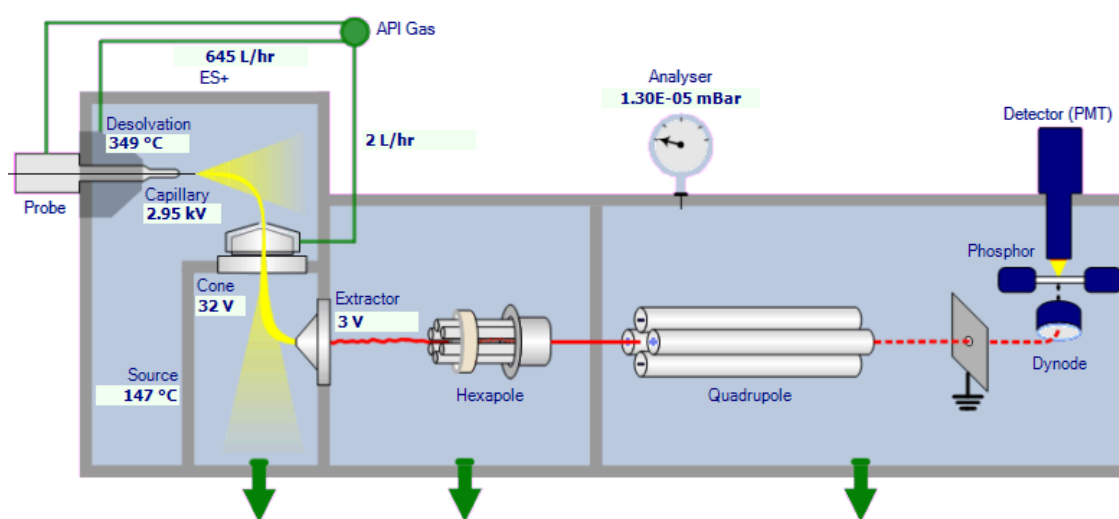


Figure 6. Scheme of the detector used

### 2.2.3 Quadrupole Time-of-Flight (QToF)

Once the UPLC and the MS are explained, a more sophisticated detector such as the QToF is studied<sup>28</sup>. First part of this detector has the same fundamentals as the one studied before (Section 2.2.2) whereas the second part has a different structure and fundamentals. This second part is the Time-of-Flight<sup>29</sup> (ToF), where the ions are guided with the use of an hexapole and introduced with a change on their paths of 90°. This orthogonal orientation allows a better separation between the ions by focusing optically the kinetic energy. Then, the ions are accelerated and directed to the reflectron, which is able to slow down the ions and focus them separately to the detector by distinguishing between the different kinetic energies (Figure 7)<sup>30</sup>.

With the use of QToF one can operate with single MS as well as with MS/MS mode, this last selecting a precursor ion in the first quadrupole and in the second producing a dissociation providing a fragment ion analysed in the ToF. With this detector, a better selectivity and sensitivity are obtained and a more accurate mass is achieved.

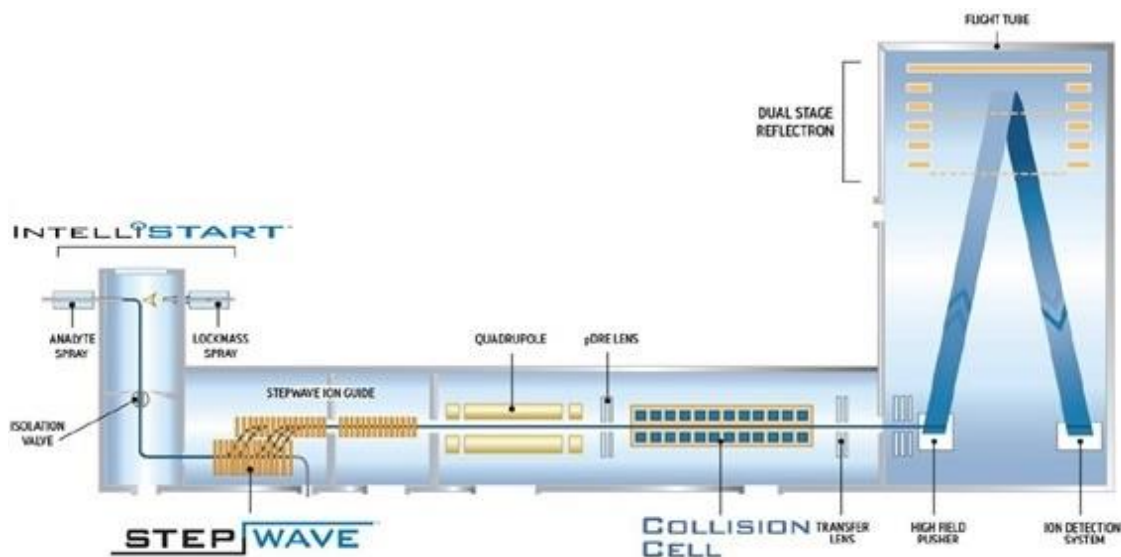


Figure 7. Scheme of the QTOF

### 3 Experimental Part

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In this section all the reagents, standards and the preparation that has been carried on in the laboratory are explained.

#### 3.1 Reagents and standards

In order to carry out this project some reagents have been needed and their characteristics have been studied so as to know their toxicology and how they had to be manipulated (Table 1 and Table 2).

Table 1. Toxicology of the solvents used<sup>12</sup>

<b>Compound</b>	<b>Chemical formula</b>	<b>Toxicology</b>	<b>Assay</b>
Methanol	CH <sub>3</sub> OH	Flammable and toxic	HPLC grade ≥99.9%
Acetonitrile <sup>31</sup>	CH <sub>3</sub> CN	Flammable, harmful and irritant	HPLC grade 99.8+%
Formic acid	HCOOH	Flammable, harmful and irritant	95-97%
Acetic acid glacial	CH <sub>3</sub> COOH	Flammable and irritant	≥99.85%
Water	H <sub>2</sub> O	-	For chromatography

Table 2. Toxicology of the pharmaceuticals used<sup>12</sup>

<b>Compound</b>	<b>Chemical formula</b>	<b>Toxicology</b>	<b>Assay</b>
Acetaminophen	CH <sub>3</sub> CONHC <sub>6</sub> H <sub>4</sub> OH	Harmful, irritant and toxic	Analytical standard
Ibuprofen	C <sub>13</sub> H <sub>18</sub> O <sub>2</sub>	Harmful and toxic	≥98%
Caffeine	C <sub>8</sub> H <sub>10</sub> N <sub>4</sub> O <sub>2</sub>	Harmful and toxic	99%
Naproxen Sodium	C <sub>14</sub> H <sub>13</sub> NaO <sub>3</sub>	Irritant and toxic	98.0-102.0%
Fluoxetine hydrochloride	C <sub>17</sub> H <sub>18</sub> F <sub>3</sub> NO·HCl	Biohazard, corrosive and toxic	Certified reference material

Methanol, formic acid, acetic acid glacial, water and the pharmaceuticals were provided by Sigma Aldrich whereas acetonitrile was provided by Alfa Aesar.



Things to be aware for handling the solvents:

- Use laboratory coat.
- Safety goggles.
- Nitrile gloves.
- Fume cupboard.

Things to be aware for handling the reagents:

- Use laboratory coat.
- Safety goggles.
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### **3.2 Instrumentation**

The instrument used for the analysis is a Water ACQUITY Single Quad Detector (SQD) Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS). The column used was an ACQUITY UPLC BEH C18 (2.1 x 100 mm) with a particle size of 1.7  $\mu\text{m}$ .

The temperature of the chromatograph was set at 40°C and for the mass spectra a positive Scan mode was done which started at 135 and ended at 350 m/z.

As the UPLC-MS had a pump failure, a Water UPLC-QToF-MS was used in order to continue the optimization. The column of this UPLC was an ACQUITY UPLC BEH C18 (2.1 x 100 mm) with a particle size of 1.7  $\mu\text{m}$ .

The conditions of the detector were:

- Ionization: ESI+
- Analyser mode: Sensitivity
- Collision Energy: 6 to 30 V
- Scan time: 0.1 seconds
- Capillary potential: 3000 kV
- Source temperature: 150°C
- Desolvation temperature: 250°C
- Desolvation gas flow: N<sub>2</sub> 600 L/h

### 3.3 Sample preparation

Water samples from the effluent canal leaving the Stoke Bardolph STW were collected with a bucket and with the help of a funnel the water was stored in a plastic bottle and kept in a fridge. Before doing the extraction, samples were filtered first with a microfibre glass filter using a Büchner funnel and vacuum. This was followed by solid phase extraction to obtain a cleaner more concentrated sample of the analytes of interest.

### 3.4 Solid Phase Extraction (SPE)

A solid phase extraction was done in order to concentrate the analytes of interest and to remove the possible interferences in the sample. The procedure found in the bibliography<sup>1</sup> was used as a beginning point taking advantage of the authors worked with a group of pharmaceuticals that included the ones that had been studied in this project.

The cartridges used were OASIS HLB and the procedure carried out for the SPE was divided in four steps<sup>1</sup>. First of all, the conditioning of the cartridge, using 5 mL of methanol followed by 5 mL of ultra-pure water. Secondly, the sample was run (the volume is discussed in Section 4.3). The sample was followed by 5 mL of 5% methanol in ultra-pure water in order to wash the cartridge. Finally, the elution was done with 5 mL of methanol/acetonitrile (50:50).

The 5 mL of eluted solution was evaporated to near dryness at 95°C under a stream of nitrogen.

After the evaporation a reconstitution was carried out in 1 mL of Methanol and the resultant solution was stirred using a vortex<sup>32</sup>.

### 3.5 Standards preparation

Individual standards of each pharmaceutical were prepared at a concentration of 1000 ppm (except Fluoxetine which was supplied as a 1000 ppm solution and it was diluted to 50 ppm which was used to prepare additional standards). Using these individual standards, a mixed standard was made using methanol as solvent. Working solutions were prepared from the mixed standard by dilution in

MeOH/H<sub>2</sub>O (15:85 v/v). The standard mix and the working solutions were prepared weekly and daily respectively in order to guarantee its stability.

## 4 Results and discussion

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In this section, all the optimizations and the results obtained are explained.

### 4.1 Mobile phase optimization

In order to achieve the chromatographic separation of the analytes of interest, an optimization of the mobile phase was needed with the help of different articles<sup>1</sup>.

The first step in the optimization was the recalculation of the flow rate due to the different column utilized. The column used in the article was a C18 (100 mm x 4.6 mm) with 2.7 μm of particle size whereas the column used experimentally was a C18 (100 mm x 2.1 mm) with 1.7 μm of particle size.

$$\text{Linear flow velocity} = \frac{\text{flow rate}}{\text{cross sectional area}} \quad \text{Equation 1}$$

Knowing that the flow rate applied in the article was 0.6 cm<sup>3</sup>/min, one can calculate its linear flow velocity with Equation 1.

$$\text{Linear flow velocity} = \frac{0.6 \text{ cm}^3/\text{min} \cdot \frac{1000 \text{ mm}^3}{1 \text{ cm}^3}}{\pi \cdot 4.6 \text{ mm}^2} = 9.03 \text{ mm/min}$$

Once the linear flow velocity was calculated, realizing that this had to be the same for the column used, the flow rate was determined.

$$9.03 \text{ mm/min} = \frac{\text{flow rate}}{\pi \cdot 2.1 \text{ mm}^2} ; \text{flow rate} = 125.04 \text{ mm}^3/\text{min} = 0.125 \text{ cm}^3/\text{min}$$

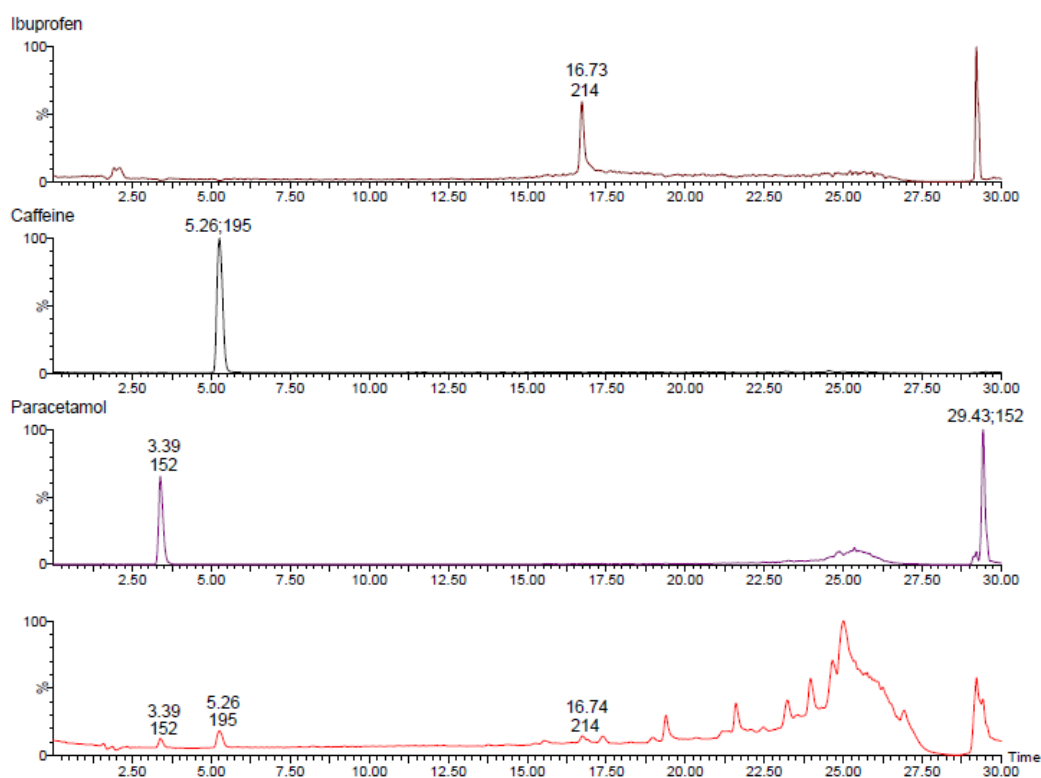
With the flow rate known and using ultra-pure water acidified with 0.1 % of formic acid as Solvent A and acetonitrile as Solvent B, a working solution of 10 ppm was analysed using the method in Table 3 (notice that only appears three compounds in the chromatogram, paracetamol, caffeine and ibuprofen because the other two were ordered) (Figure 8).

Knowing that paracetamol, caffeine and fluoxetine has to be analysed using ESI+ whereas naproxen and ibuprofen with ESI- due to its acidity, the MS method

applied first was ESI+ in order to identify all the compounds and optimize the ones that has to be studied with this type of ionisation.

Table 3. Method used

Time	Flow	% A	% B
Initial	0.125 mL/min	90.0	10.0
2.00		90.0	10.0
25.00		0.0	100.0
26.00		0.0	100.0
30.00		90.0	10.0

Figure 8. Chromatogram of the working solution of 10 ppm<sup>1</sup>.

With the help of the relation  $m/z$  found in the bibliography and with the analysis of individual standard solutions, the three pharmaceuticals were identified, paracetamol (152), caffeine (195) and ibuprofen (214). One problem found was

<sup>1</sup>In the chromatograms shown, at the bottom appears the entire one and then the single ion chromatograms of each compound.

that with paracetamol appeared two peaks and the analysis were not reproducible.

As paracetamol was known to appear between 3 and 4 minutes<sup>1</sup>, the peak at 29 minutes could appeared due to the fact that this peak had an average  $m/z$  of 152 corresponding to a different compound. Also with ibuprofen appeared another peak which had different  $m/z$  (305), so it could be thought that it was another compound.

After some analysis done using Solvent A mentioned before, it could be seen that some peaks appeared in the chromatogram and could affect the identification of them. With the help of the mass-to-charge ratio of these peaks it could be concluded that some of them appeared because of the use of formic acid in Solvent A<sup>33</sup>. This is thought because some peaks showed a  $m/z$  typical of a high concentration of formic acid.

Due to this problem, a modification of Solvent A was done and a change of formic acid for acetic acid was applied. At this point, mobile phase used was:

- Solvent A: Ultra-pure water acidified with 0.1 % of acetic acid.
- Solvent B: Acetonitrile.

Using this mobile phase with the same method as Table 3 and with a working solution of 10 ppm of all 5 compounds, an analysis was done (Figure 9).

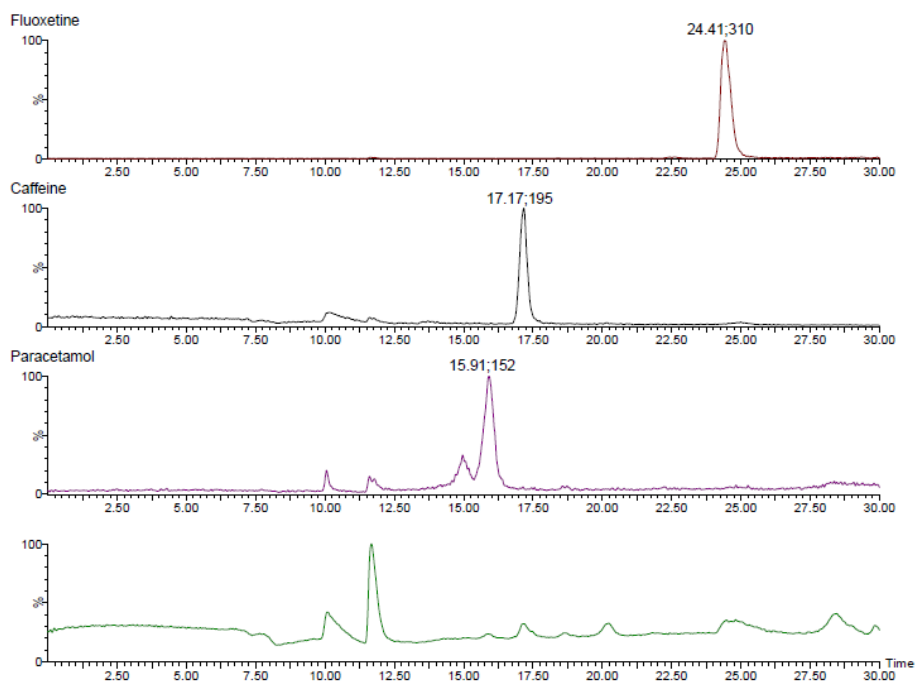


Figure 9. Chromatogram of the working solution of 10 ppm using new mobile phase

All five peaks of interest were not identified. However, using its mass-to-charge ratio paracetamol (152), caffeine (195) and fluoxetine (310) were seen. Hence, naproxen and ibuprofen were not successfully found.

Once Solvent A was changed, and an analysis of the five compounds was done using the same method as before, an increase of the flow rate was applied in order to see if all the peaks could be identified. Knowing that UPLC can work at high pressures, the flow rate employed was 0.600 ml/min (Figure 10).

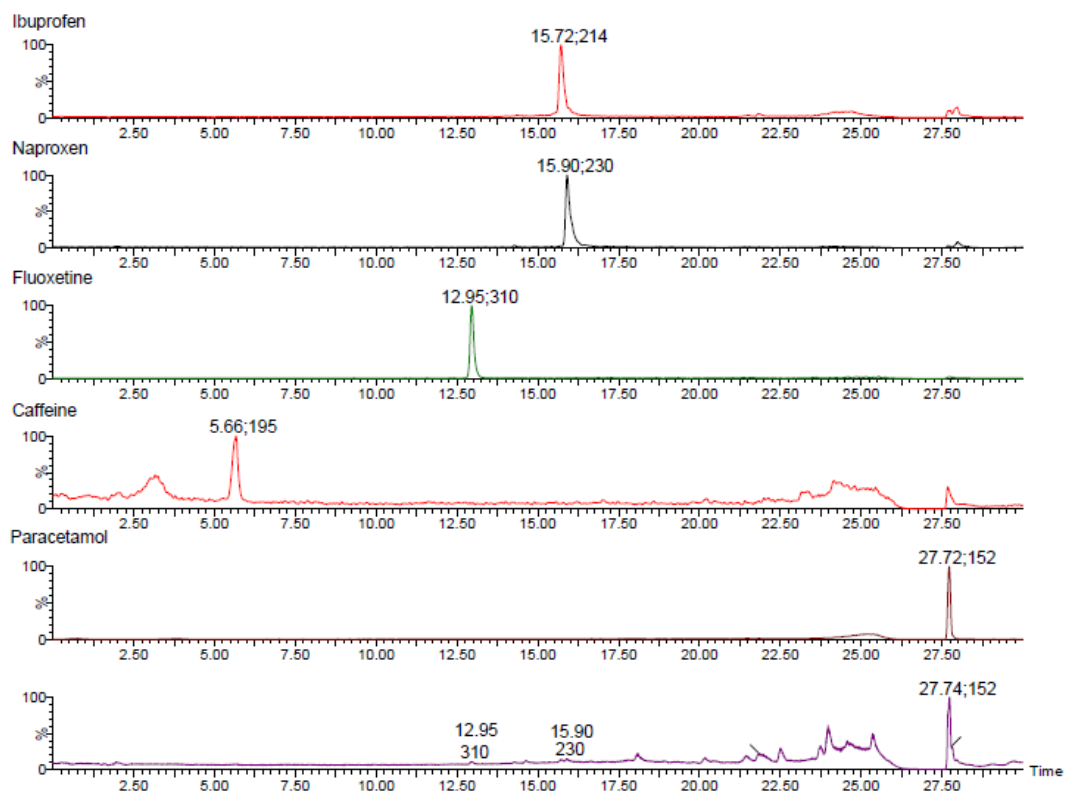


Figure 10. Chromatogram of the working solution of 10 ppm increasing the flow rate

With the change of the Solvent A and an increase of the flow rate, the identification of the five peaks of interest was achieved, paracetamol (152), caffeine (195), fluoxetine (310), naproxen (230) and ibuprofen (214).

In order to be sure of the retention times of the pharmaceuticals, a working solution of 50 ppm of each one was analysed (fluoxetine was 10 ppm due to there was not as much as the others) (Figure 11).

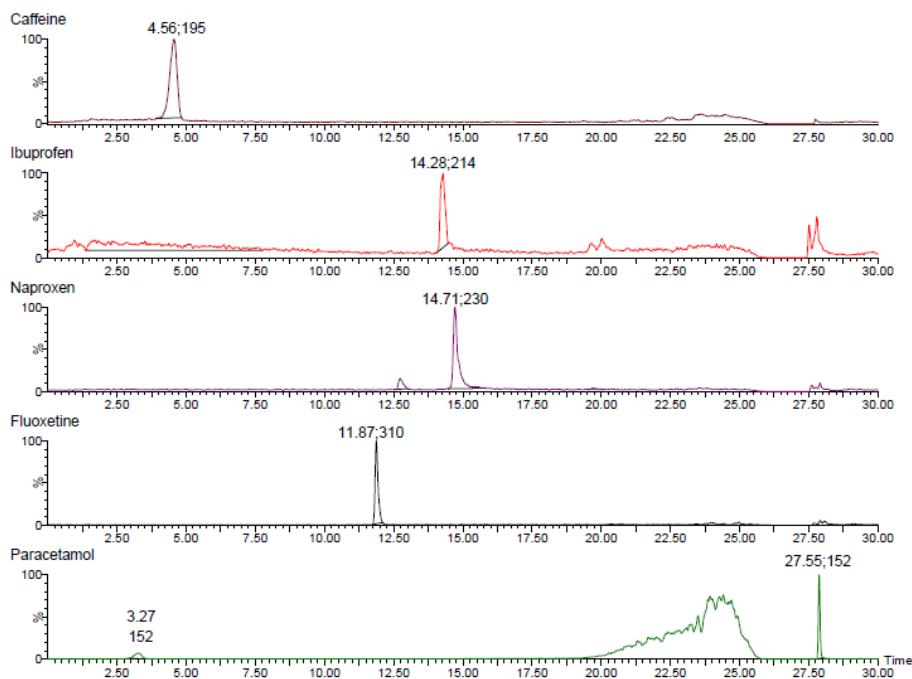
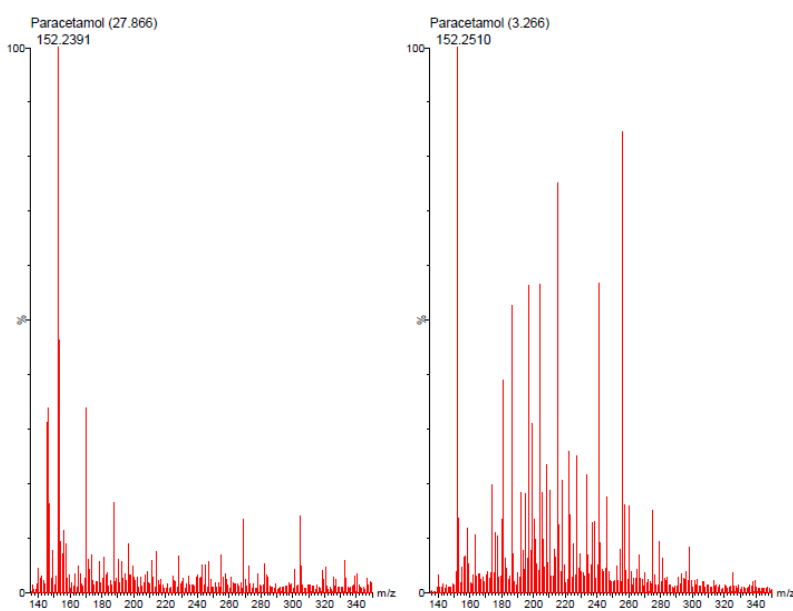


Figure 11. Chromatograms of individual standards

Although good results were obtained, the  $m/z$  of 152 which is the one that corresponds to paracetamol was found in two different times. Checking the mass spectra and knowing that paracetamol appears between 3 and 4 minutes<sup>1</sup>, one could think that the peak of paracetamol was the first one while the second one corresponded to another compound (Figure 12).

Figure 12. Mass spectra of the peaks of a  $m/z$  of 152



After this, more analysis in different days were done with the same method. In some of these, caffeine was not identified and it was found to be very unreproducible.

Because of that, different gradients were tried and with one of them caffeine (195), fluoxetine (310) and naproxen (230) could be identified in 10 minutes (Table 4) (Figure 13). Notice that working with ESI+, the only ones that could be quantified were caffeine and fluoxetine.

Table 4. New method used

Time	Flow	% A	% B
Initial	0.125 mL/min	80.0	20.0
2.00		80.0	20.0
8.00		30.0	70.0
10.00		80.0	20.0

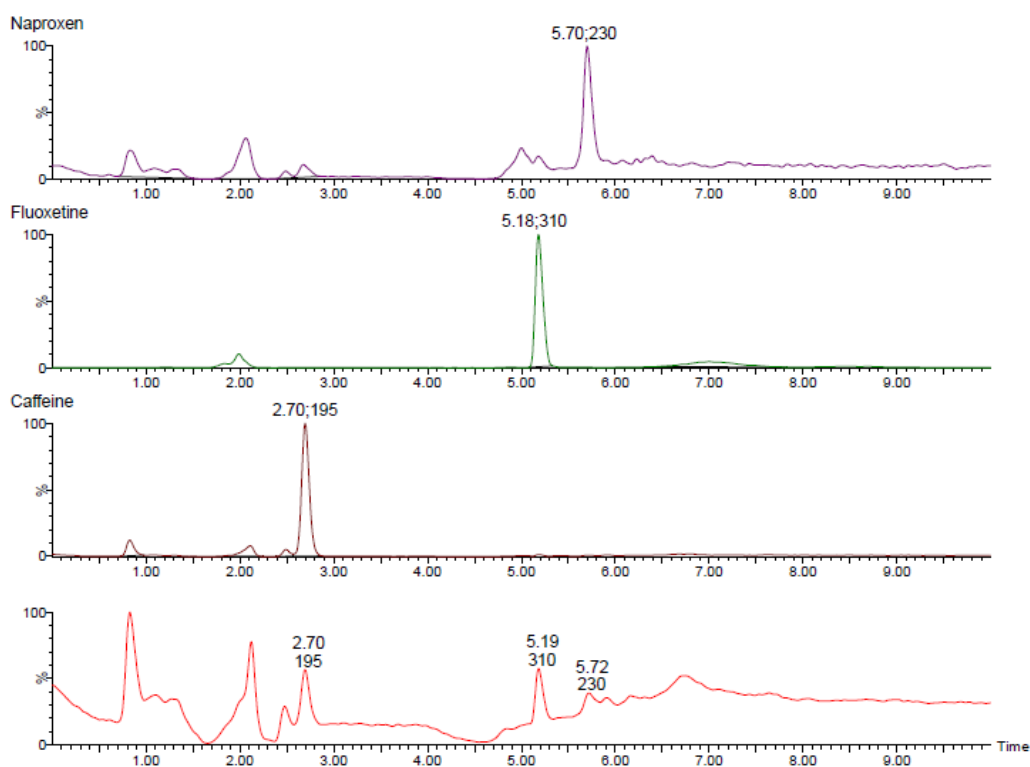


Figure 13. Chromatogram of the 10 minutes' analysis

However, it was found to be useless with concentrations less than 10 ppm and also not very reproducible.

After this new method and with the intention of reducing the time of the analysis to less than 30 minutes, more methods were searched and tried. Using one method found, the analysis of the five compounds could be achieved in 20 minutes<sup>34</sup>.

Table 5. Method finally used

<b>Time</b>	<b>% A</b>	<b>% B</b>
Initial	100.0	0.0
0.2	100.0	0.0
1.0	95.0	5.0
5.0	90.0	10.0
8.0	80.0	20.0
10.0	55.0	45.0
11.0	55.0	45.0
13.0	0.0	100.0
15.0	0.0	100.0
16.0	100.0	0.0
20.0	100.0	0.0

This method was first applied using the same solvents as before, Ultra-pure water acidified with 0.1 % of Acetic Acid as Solvent A and Acetonitrile as Solvent B. This method was applied only for ESI+, so only three compounds could be successfully analysed (the other two could be seen at different m/z compared with the bibliography<sup>34</sup>). However, in the article the authors used different solvents:

- Solvent A: 94.5% Ultra-pure Water, 5.0% Methanol and 0,5% Acetic Acid.
- Solvent B: 99.5% Methanol and 0.5% Acetic Acid.

It could be seen that with the solvents used before (ultra-pure water with 0.1% of acetic acid and acetonitrile) the method was not working as expected.

Because of that, as Solvent A was quite similar than the one that it had been used, a change of Solvent B was done and acetonitrile was changed for methanol.

With this change, the resolution of the peaks was much better and more compounds could be identified. As the authors used a flow rate of 0.07 mL/min an increment of that was done and 0.125 mL/min was tried. Using this flow rate and an injection volume of 1.0  $\mu$ L, caffeine, fluoxetine and ibuprofen were identified. In order to see if applying a different flow rate the other compounds could be seen, other flow rates were tried. This time, 0.300 mL/min was tried and with this, fluoxetine, ibuprofen and naproxen were identified.

Knowing that fluoxetine and ibuprofen were common between the two flow rates tried, an intermediate one was applied. With a flow rate of 0.200 mL/min fluoxetine, ibuprofen and naproxen were still the only peaks seen. For this reason, the flow rate was decreased to 0.150 mL/min and caffeine, fluoxetine, ibuprofen and naproxen were finally identified.

In order to recognize Paracetamol, an individual standard of it at 50 ppm was ran and it was seen at 1.64 minutes (Figure 14), which was the same retention time as another peak that appeared in the mix standard (Figure 15).

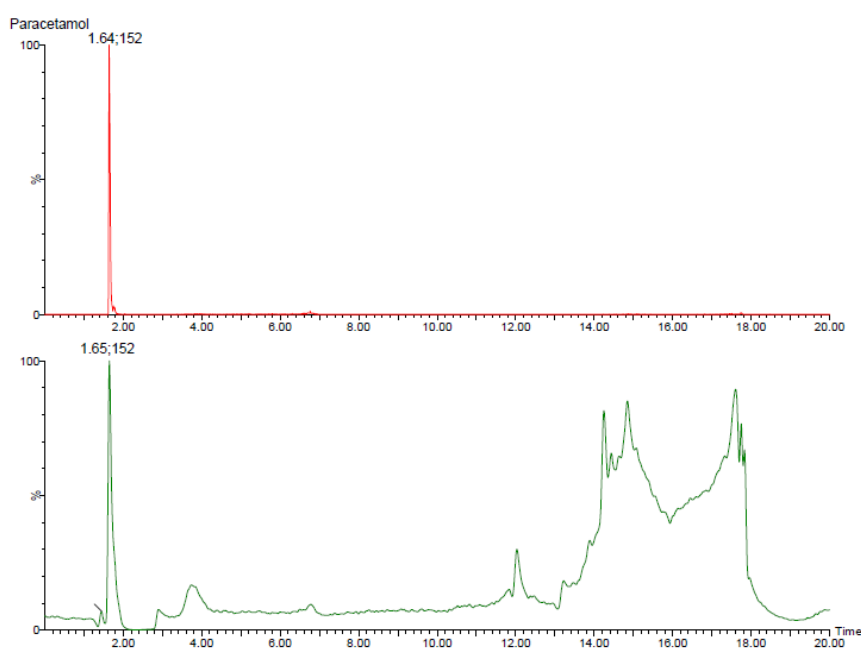


Figure 14. Analysis of the individual standard of paracetamol at 50 ppm

Finally, the flow rate to identify as much compound as possible was set to 0.150 mL/min, though paracetamol only at high concentrations (Figure 15).

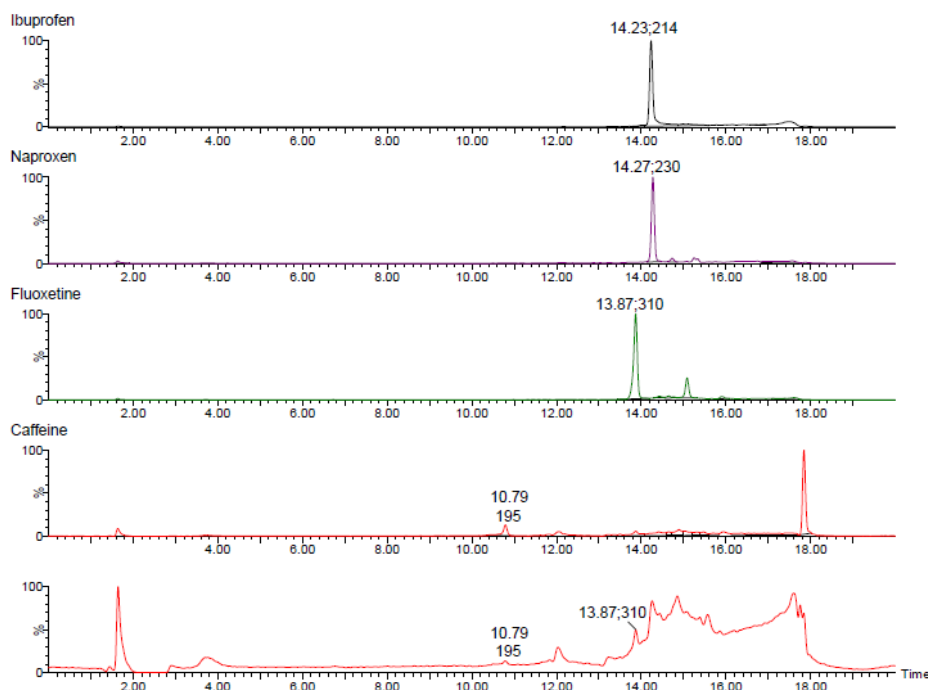


Figure 15. Chromatogram with the new method using 1  $\mu$ L as injection volume

## 4.2 Study of the number of theoretical plates

The number of theoretical plates is a mathematic concept related to the efficiency of the column<sup>35</sup>. This concept is a way to calculate the peak width for a peak at a definite retention time and it can be measured using Equation 3.

$$N=16 \times \left( \frac{t_r}{w_b} \right)^2 \quad \text{Equation 3}$$

Where:

- N: number of theoretical plates
- $t_r$ : retention time
- $w_b$ : peak width in the base line

A high value of N indicates that the column has a high number of plates which means that the column has a high efficiency. This means that the column with

more efficiency will have a narrower peak at a specific retention time than the other column.

As the column used in this study was the same in both instruments, the efficiency of the column depending on the chromatographic conditions is compared (Figure 16).

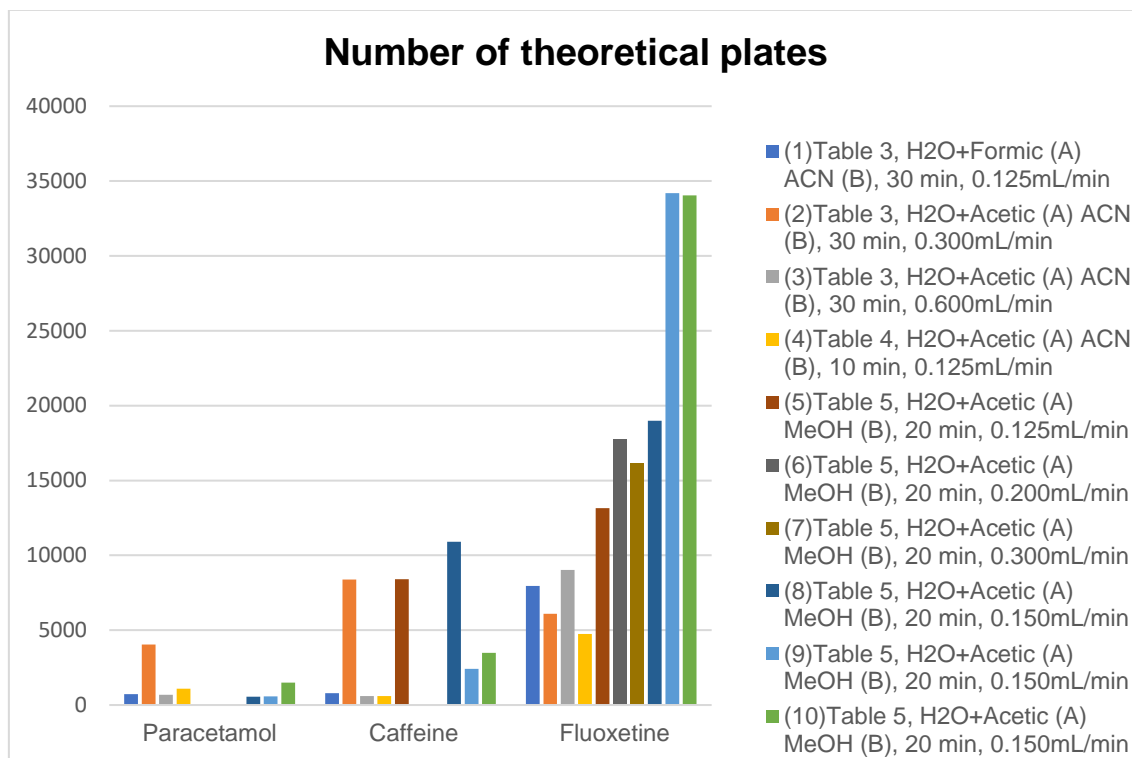


Figure 16. Comparison of the efficiency of the column in different conditions

Conditions (1) to (8) are the ones using UPLC-MS whereas conditions (9) and (10) are the ones using UPLC-QToF-MS. One thing to emphasize is that with conditions (5), (6) and (7) not all the pharmaceuticals are identified.

As it can be seen, conditions (8), (9) and (10) are the same and the ones that show the highest efficiency and with a big difference in the case of fluoxetine. It could be said that the use of methanol helped the separation of fluoxetine. Hence, the conditions final used (8), (9) and (10) are the ones with the highest number of theoretical plates and consequently with the highest efficiency of the column.

### 4.3 Injection volume optimization

In order to choose the optimal injection volume, first of all a bibliographic search was needed to know the range of volumes that it could be applied to the UPLC. Knowing that the injection volumes could go between 1 and 5  $\mu\text{L}$ <sup>36</sup> using Partial-loop mode different volumes were tried.

First of all, with 1  $\mu\text{L}$  of injection volume paracetamol was not identified and the peak of caffeine was very small as it can be seen in Figure 15.

After 1  $\mu\text{L}$ , 3  $\mu\text{L}$  was tried as injection volume and with this, a higher peak of caffeine was obtained and paracetamol (152) could be seen in some analysis (Figure 17). Although the peak of paracetamol was identified, when it was integrated the average m/z of the peak was different than 152.

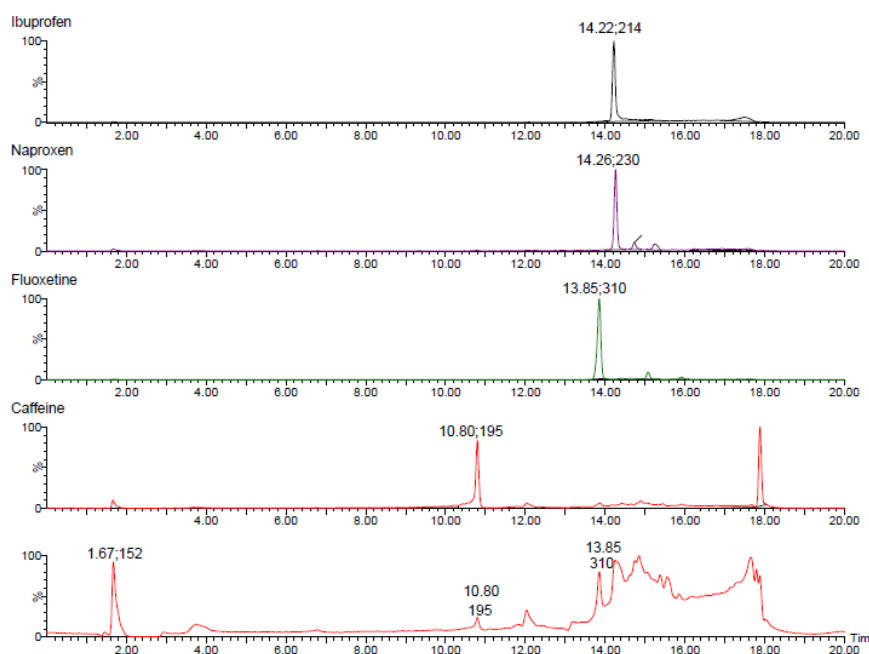


Figure 17. Chromatogram with the new method using 3  $\mu\text{L}$  as injection volume

The last injection volume tried was 5  $\mu\text{L}$ , with which the peaks were similar to 3  $\mu\text{L}$  and paracetamol was still showing the average m/z which was different from 152 (Figure 18).

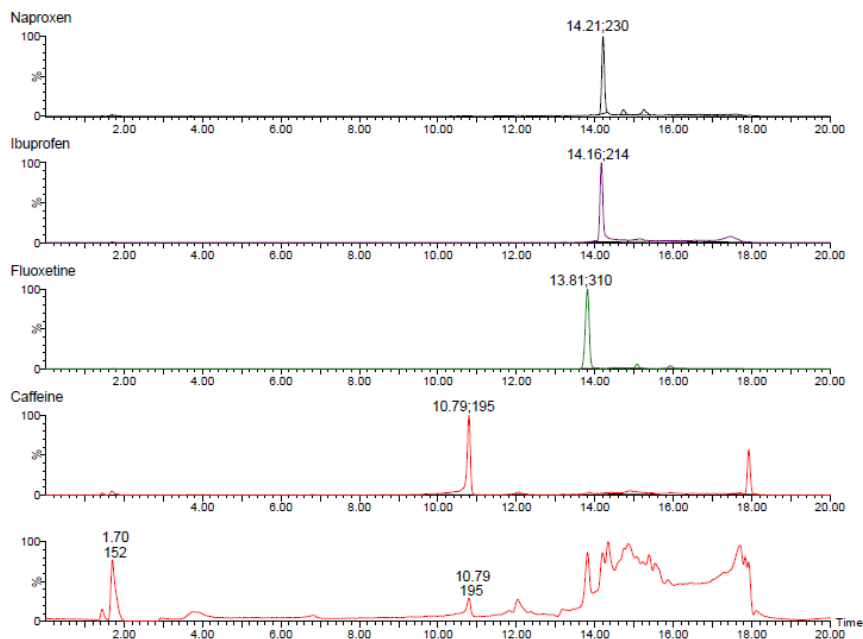


Figure 18. Chromatogram with the new method using 5  $\mu\text{L}$  as injection volume

In order to choose the optimal injection volume, retention times and areas of the peaks were compared in the same day and in different days. As the retention times were in the three injection volumes (1, 3 and 5  $\mu\text{L}$ ) approximately the same, the areas of caffeine and fluoxetine were compared (not paracetamol because it did not appear in all analysis) (Figure 19 and 20).

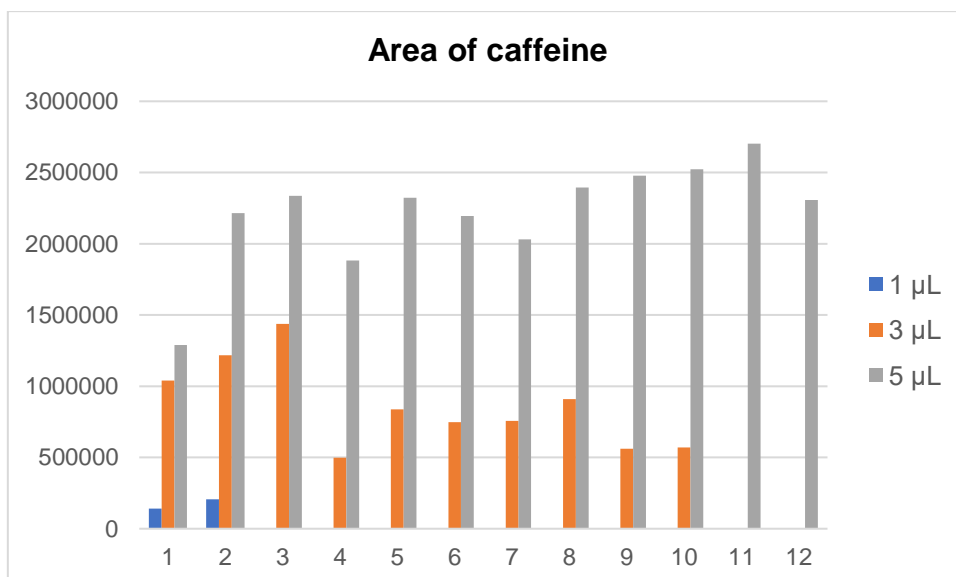


Figure 19. Comparison of areas of caffeine depending on the injection volume

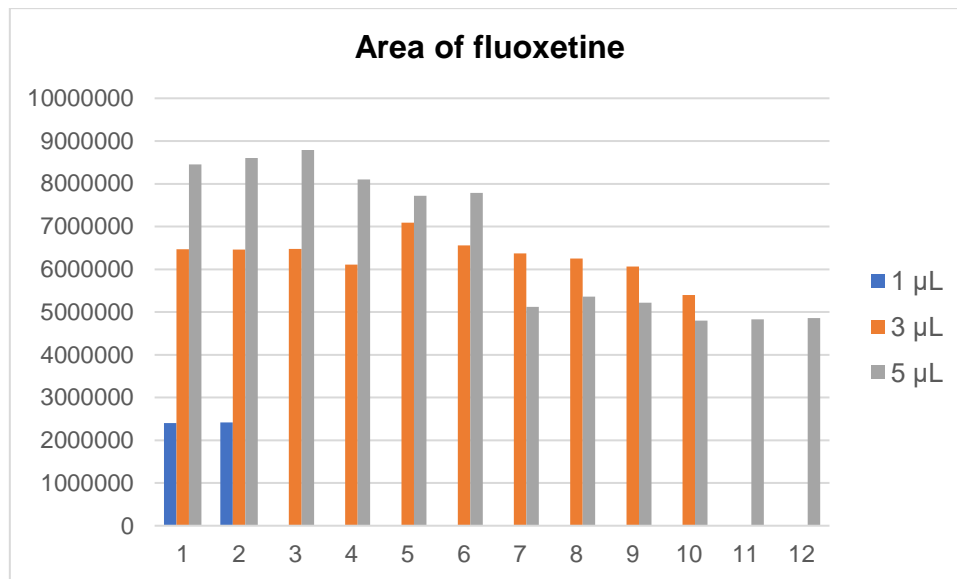


Figure 20. Comparison of areas of fluoxetine depending on the injection volume

As it can be seen, with 1  $\mu\text{L}$  low areas of caffeine and fluoxetine were obtained compared to the others.

Comparing 3 and 5  $\mu\text{L}$ , bigger areas were obtained with the second volume. One thing to emphasize is that using 3  $\mu\text{L}$  the area of caffeine was reproducible in the same day but it changed between different days whereas using 5  $\mu\text{L}$  the area was not changing as much as with 3  $\mu\text{L}$ .

For these reasons, the injection volume chosen was 5  $\mu\text{L}$ .

#### 4.4 Adaptation to UPLC-QToF-MS

Due to the UPLC-MS could not be used to finish the optimization because of a failure in the vacuum pump, a UPLC-QToF-MS was used. The column of this instrument was an ACQUITY UPLC BEH C18 (2,1 x 50 mm) with a particle size of 1.7  $\mu\text{m}$  and the method developed in the other UPLC was applied in this one.

Using ESI+, paracetamol, caffeine and fluoxetine were studied first. However, although the three compounds could be identified they did not present a good shape of the peaks (Figure 21).



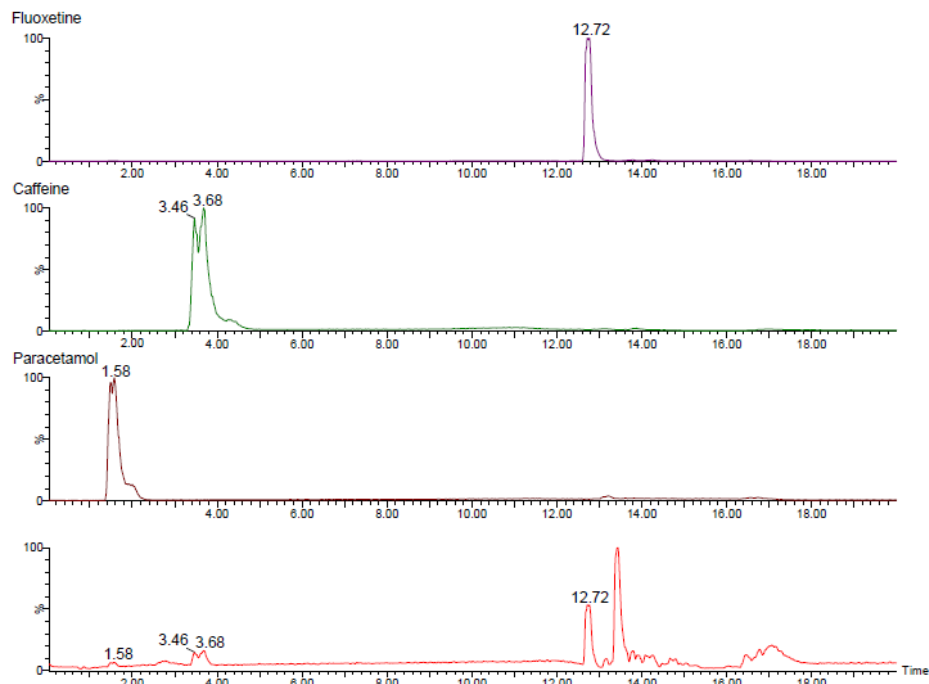


Figure 21. First analysis with UPLC-QToF-MS

If the mass spectra of the peaks were checked, it was observed that paracetamol (152), caffeine (195) and fluoxetine (310) were the main peaks. Thus, once the peaks were identified an optimization was needed.

In order to improve the efficiency of the peaks, less injection volume was tried (0.5  $\mu$ L). However, the shape of the peaks was the same, so in order to fix this problem a change of the column was tried and the one that it was used for the first UPLC was applied, also injecting 0.5  $\mu$ L.

With this column (2,1 x 100 mm) a good efficiency and resolution of the peaks were obtained (Figure 22). Also, it was seen to be very reproducible.

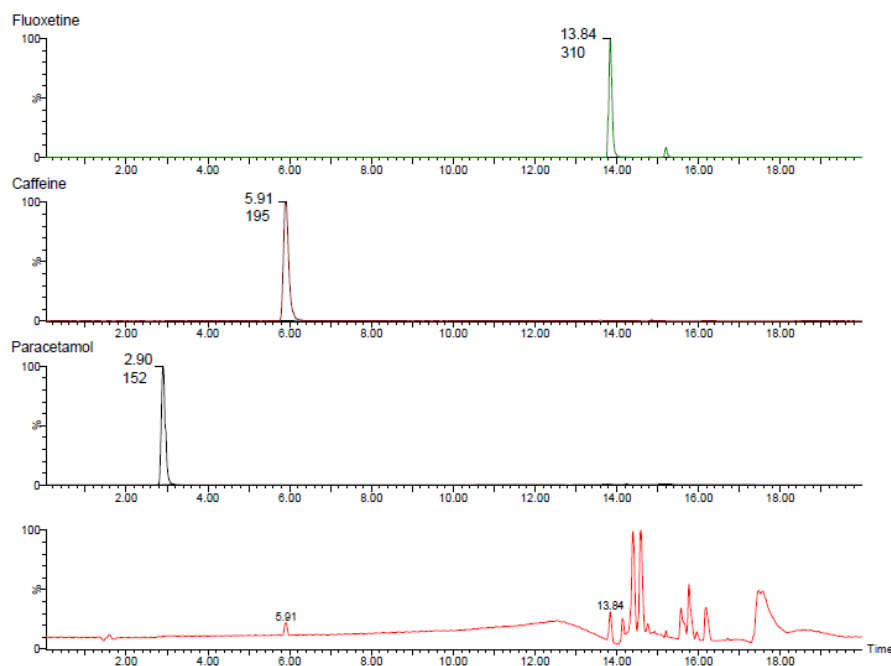


Figure 22. Analysis with the large column

To know if the peaks belonged to paracetamol, caffeine and fluoxetine, the mass spectrum of each compound was checked (Figure 23). As it can be seen, all the peaks showed the  $m/z$  that belongs to them.

Moreover, to confirm that the peaks were correctly identified, the MS-MS spectra were checked (Figure 24). For paracetamol, the fragment ion that has to appear is 134, which is the one in the spectrum. For caffeine, its fragment ion is 138, it also can be seen in its spectrum. Finally, the fragment ion for fluoxetine was supposed to be 148, but it cannot be observed in the spectrum probably due to the different voltage applied.

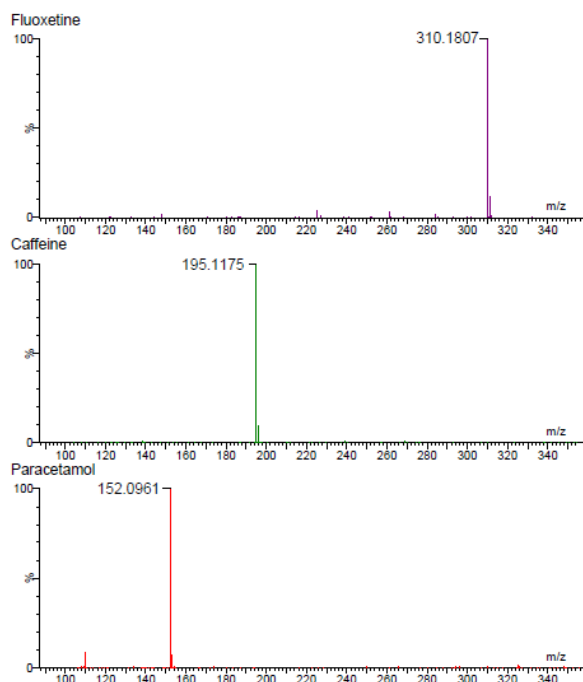


Figure 23. MS spectra

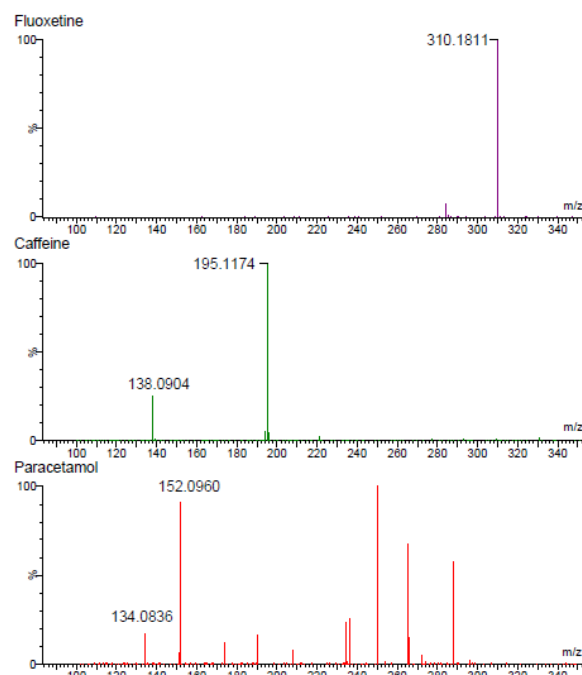


Figure 24. MS-MS spectra

## 4.5 Final procedure

Having the method defined and adapted to the new instrument, it was ready to be applied in real samples.

It has to be kept in mind that only paracetamol, caffeine and fluoxetine were finally analysed due to the short time that have had to adapt the method to the UPLC-QToF-MS and get some results about this method.

Thus, naproxen and ibuprofen were not analysed because they needed to be studied with ESI- due to its acidity.

At this point, the conditions of the UPLC were:

- Solvent A: Ultra-pure water acidified with 0.1 % of acetic acid.
- Solvent B: Methanol.
- Flow rate: 0.150 mL/min
- Gradient:

Table 5. Method finally used

<b>Time</b>	<b>% A</b>	<b>% B</b>
In	100.0	0.0
0.2	100.0	0.0
1.0	95.0	5.0
5.0	90.0	10.0
8.0	80.0	20.0
10.0	55.0	45.0
11.0	55.0	45.0
13.0	0.0	100.0
15.0	0.0	100.0
16.0	100.0	0.0
20.0	100.0	0.0

On the other hand, the conditions of the Q-ToF-MS were:

- Ionization: ESI+
- Analyser mode: Sensitivity
- Collision Energy: 6 to 30 V
- Scan time: 0.1 seconds
- Capillary potential: 3000 kV
- Source temperature: 150°C
- Desolvation temperature: 250°C
- Desolvation gas flow: N<sub>2</sub> 600 L/h

Retention times for each compound were:

- Paracetamol: 2.90 min
- Caffeine: 5.91 min
- Fluoxetine: 13.84 min

## 4.6 Calibration curves

With all the conditions optimized and the compounds identified, the calibration curves were carried on from concentrations of 0.05 ppm to 25 ppm analysing 16 different concentration per duplicate. All the data with its %RSD can be found in the Annex 7.1. Moreover, in order to help the interpretation of the calibration curves, a logarithmic plot is showed in the Annex 7.2.

For paracetamol, the linearity was between 0.5 ppm and 7 ppm because with 0.05, 0.1 and 0.3 ppm non-signal was obtained. Furthermore, if the other concentrations were plotted, the correlation coefficient ( $R^2$ ) was decreased to 0.9787 and it could be observed a change on the linearity (Figure 25).

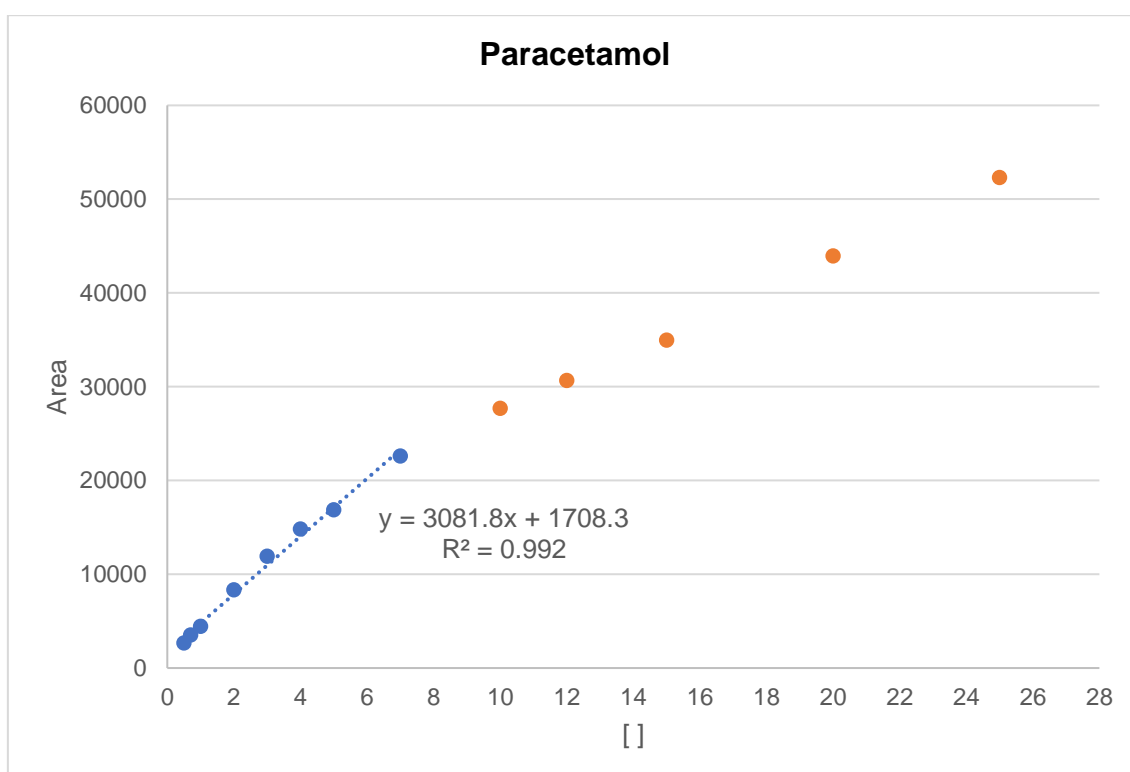


Figure 25. Linearity range of Paracetamol

In the case of caffeine, the linearity range was between 0.3 and 7 ppm due to with 0.05 and 0.1 ppm caffeine could not be identified. In addition, as it happened with paracetamol, the correlation coefficient decreased if all the concentration were plotted and it could be seen a changed on the linearity (Figure 26).

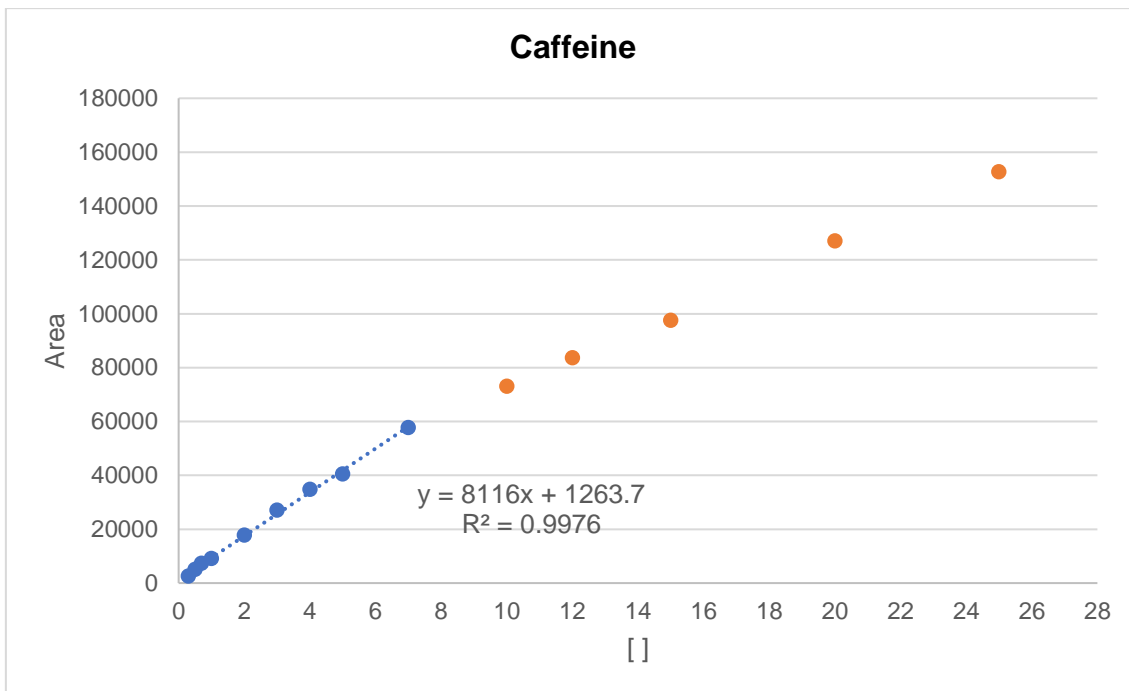


Figure 26. Linearity range of Caffeine

Finally, for Fluoxetine, the linearity range was the same as for Caffeine (Figure 27).

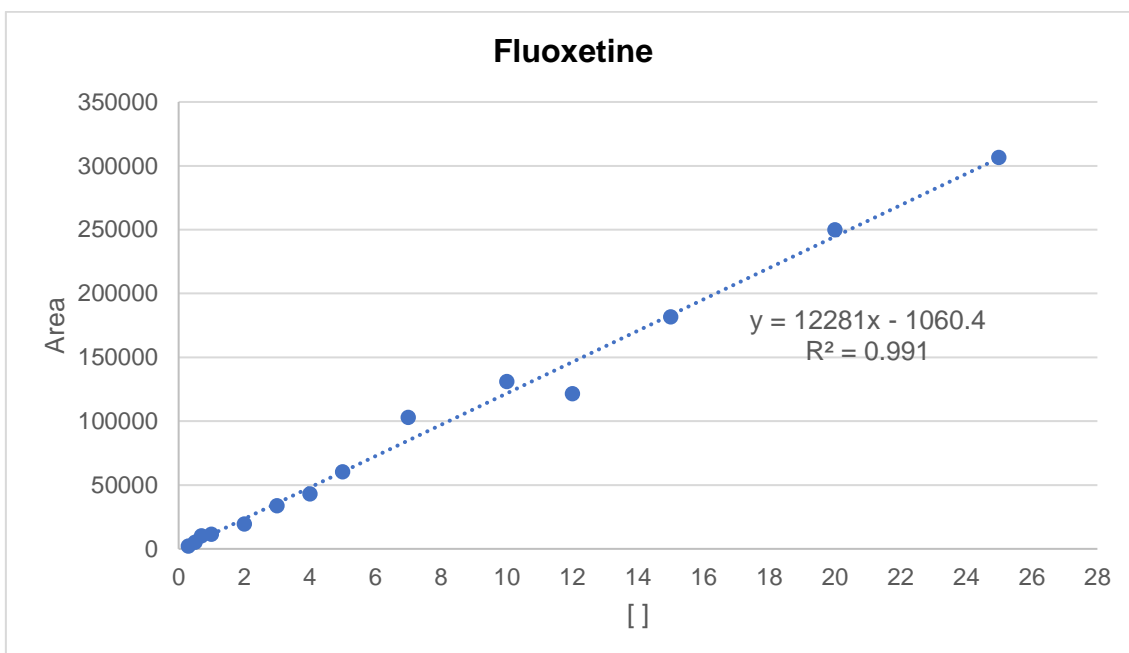


Figure 27. Linearity range of Fluoxetine

## 4.7 Limits of detection and quantification

Once the linearity range is studied, limits of detection (LOD) and quantification (LOQ) can be assigned. In this case, as the first concentration that can be detected and the first that can be integrated are the same, LOD and LOQ belong to the same concentration and to the first point in the linearity range. Thus, LOD and LOQ for paracetamol is 0.5 ppm whereas for caffeine and fluoxetine is 0.3 ppm.

## 4.8 Analysis of real samples

First step in the analysis of the Stoke Bardolph STW effluent water is to know the amount of water that had to be extracted in the SPE. Consequently, three different volumes were tried, 250, 500 and 700 mL.

Using a sample volume of 250 mL none of the compounds were found so the other two were proved. With 500 and 700 mL Fluoxetine could be identified and their areas were compared (Table 6).

Table 6. Areas of Fluoxetine obtained in Trent's River water

	<b>Area 1</b>	<b>Area 2</b>	<b>Average</b>
<b>500 mL</b>	4818	4175	4496.5
<b>700 mL</b>	5265	5194	5229.5

Comparing the two average areas, although more area per unit of mL was obtained with 500 mL, with 700 mL the concentration in the curve was not such in the end as with 500 mL. For this reason, 700 mL was the volume chosen to do the SPE.

The analysis of the sample showed three peaks: at 1.38, 8.49 and 13.86 minutes (Figure 28).

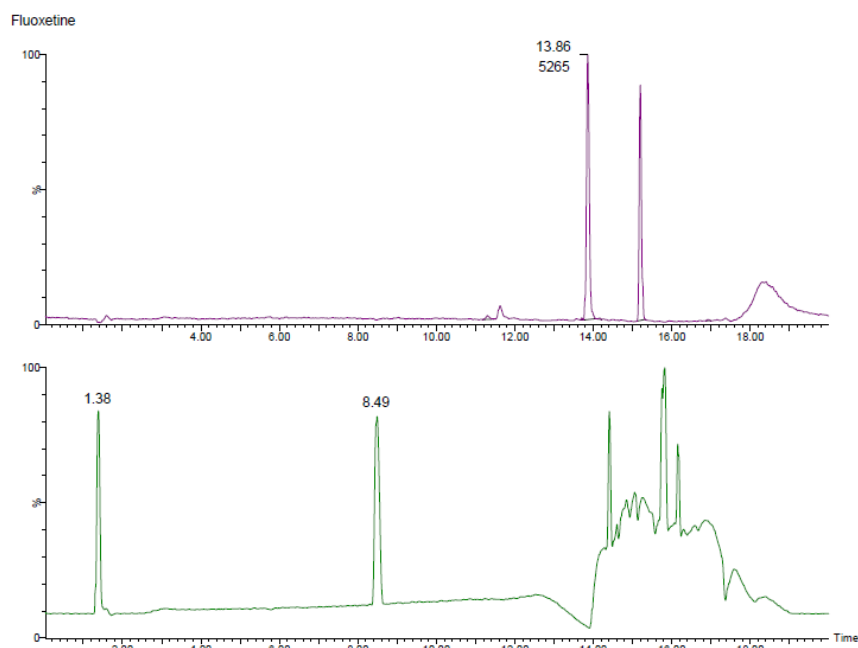


Figure 28. Chromatogram of the water from the effluent canal

With the help of the mass spectra it could be assign the first peak to a  $m/z$  of 203, the second one to 235 and the third one to 310, which corresponded to fluoxetine. The use of the elemental composition program was needed for the other two unknown peaks. The first one was assigned by a 59.16% to  $C_{14}H_7N_2$  whereas the second peak was assigned by 98.71% to  $C_{15}H_{27}N_2$ . Although a bibliographic research was done, it could not be assigned to any compound.

In order to know the concentration of fluoxetine in the effluent, the average area was replaced in the equation of the calibration curve of fluoxetine. Then, the dilution factor was applied and the concentration found was 683 ppt.

#### 4.9 Study of the recoveries

In order to study the recoveries of the whole method applied, 700 mL of water were measured and 0.5 mL of a 30 ppm mixed standard were added. Thus, the initial concentration was 20 ppb.

Beginning with this volume and concentration, the filtration and the SPE procedures were done. After the reconstitution, the sample was analysed (Table 7).



Table 7. Areas of the three pharmaceuticals to calculate the recoveries

	<b>Area 1</b>	<b>Area 2</b>	<b>Average area</b>
<b>Paracetamol</b>	7547	7577	7562
<b>Caffeine</b>	64401	66350	65375.5
<b>Fluoxetine</b>	12645	12786	12715.5

To calculate the recoveries, the average area was replaced in the equation of the calibration curve to obtain the concentration. Then, the factor of dilution was applied and the final concentration was obtained. As a way of example, paracetamol is done:

$$y = 2000.4x + 4945.4 \rightarrow x = 1.31 \text{ ppm}$$

$$1.31 \text{ ppm} \times \frac{1 \text{ mL}}{700 \text{ mL}} = 1.87 \times 10^{-3} \text{ ppm} = 1.87 \text{ ppb}$$

$$\text{Recovery} = \frac{1.87 \text{ ppb}}{20 \text{ ppb}} \times 100 = 9.35\%$$

Doing the same for the other compounds, the recoveries obtained were:

- Paracetamol: 9%
- Caffeine: 69%
- Fluoxetine: 8%

The SPE cartridges were made of a copolymer [poly(divinylbenzene-co-N-vinylpyrrolidone)]<sup>37</sup> that can retain a wide spectrum of both polar and nonpolar compounds. However, as it was observed in other articles, this cartridge may not retain some pharmaceuticals because of the matrix.

Once the recoveries are determined, the real concentration in the river can be calculated. Knowing that the concentration after the dilution factor was 0.68 ppb, if the recovery is applied the final concentration of fluoxetine found in the effluent was 8 ppb.

In order to calculate how much fluoxetine was in the river, the flow rate of the Stoke Bardolph was checked<sup>38</sup>. As it has a flow rate of 148000 m<sup>3</sup>/day, the total amount of fluoxetine can be calculated:

$$\frac{148000 \text{ m}^3}{\text{day}} \times \frac{1000 \text{ L}}{1 \text{ m}^3} \times \frac{8 \text{ } \mu\text{g}}{\text{L}} \times \frac{1 \text{ kg}}{10^9 \text{ } \mu\text{g}} = 1 \text{ kg/day}$$

Knowing that a normal dose for an adult person of Prozac (fluoxetine) is 60 mg<sup>23</sup>, the number of doses per day of this pharmaceutical are around 16500 doses.

## 5 Conclusions

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Once the project is finished, it can be said that a method to analyse 3 pharmaceuticals in 20 minutes with a good efficiency and resolution was successfully completed. Due to lack of time and the failure in the vacuum pump of the UPLC-MS, naproxen and ibuprofen could not be studied. Moreover, it has also been studied the effect of the mobile phase during the chromatographic separation developing, in this way, a suitable method for the compounds studied. It also has been studied the efficiency of the column with the different conditions that have been tried, resulting the optimal conditions as the highest efficiency of the column.

It has been observed that the recoveries using HLB cartridges were not suitable for the pharmaceuticals studied. If LOQs of the three compounds are calculated using the dilution factor and the recoveries, it could be observed that for caffeine (621 ng/L) it was a lower value than in some references<sup>1</sup> (1493 ng/L). However, for paracetamol and fluoxetine (7936 and 5357 ng/L) the LOQs were higher than compared to the same reference.

When the method has been applied to real samples, fluoxetine has been identified at a very low concentration. However, when the flow rate of the Stoke Bardolph effluent was applied, it could be observed that this belonged to 1 kg/day of this pharmaceutical.

Finally, the skills with Liquid Chromatography and Solid Phase Extraction have been successfully improved, obtaining a high ability in both techniques.

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## 7 Annex

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### 7.1 Data of the calibration curve

Table 8. Data of the calibration curve for paracetamol

	Paracetamol		
	Area	Average area	%RSD
0.05ppm	-	-	-
0.1ppm	-	-	-
0.3ppm	-	-	-
0.5ppm	2677 2669	2673	0.21
0.7ppm	3548 3477	3512.5	1.43
1ppm	4519 4379	4449	2.23
2ppm	8440 8212	8326	1.94
3ppm	12139 11711	11925	2.54
4ppm	14936 14698	14817	1.14
5ppm	17090 16662	16876	1.79
7ppm	22774 22398	22586	1.18
10ppm	28022 27343	27682.5	1.73
12ppm	31091 30223	30657	2.00
15ppm	35477 34468	34972.5	2.04
20ppm	44139 43738	43938.5	0.65
25ppm	52480 52145	52312.5	0.45

Table 9. Data of the calibration curve for caffeine

	<b>Caffeine</b>		
	<b>Area</b>	<b>Average area</b>	<b>%RSD</b>
0.05ppm	- -	-	-
0.1ppm	- -	-	-
0.3ppm	2496 2604	2550	2.99
0.5ppm	5159 5130	5144.5	0.40
0.7ppm	7434 7265	7349.5	1.63
1ppm	9075 9130	9102.5	0.43
2ppm	17981 17543	17762	1.74
3ppm	27437 26766	27101.5	1.75
4ppm	34992 34467	34729.5	1.07
5ppm	41328 39724	40526	2.80
7ppm	58217 57250	57733.5	1.18
10ppm	74054 72112	73083	1.88
12ppm	84426 82990	83708	1.21
15ppm	99315 95774	97544.5	2.57
20ppm	127944 126197	127070.5	0.97
25ppm	153668 151743	152705.5	0.89



Table 10. Data of the calibration curve for fluoxetine

	Fluoxetine		
	Area	Average area	%RSD
0.05ppm	- -	-	-
0.1ppm	- -	-	-
0.3ppm	2319 2305	2312	0.43
0.5ppm	5288 5271	5279.5	0.23
0.7ppm	10501 10011	10256	3.38
1ppm	11794 11352	11573	2.70
2ppm	19782 19135	19458.5	2.35
3ppm	33659 34219	33939	1.17
4ppm	44290 42073	43181.5	3.63
5ppm	62935 58132	60533.5	5.61
7ppm	104808 101155	102981.5	2.51
10ppm	130656 131799	131227.5	0.62
12ppm	123161 120325	121743	1.65
15ppm	186878 176511	181694.5	4.03
20ppm	253674 246288	249981	2.09
25ppm	298763 314528	306645.5	3.64

## 7.2 Calibration curves with logarithmic plot

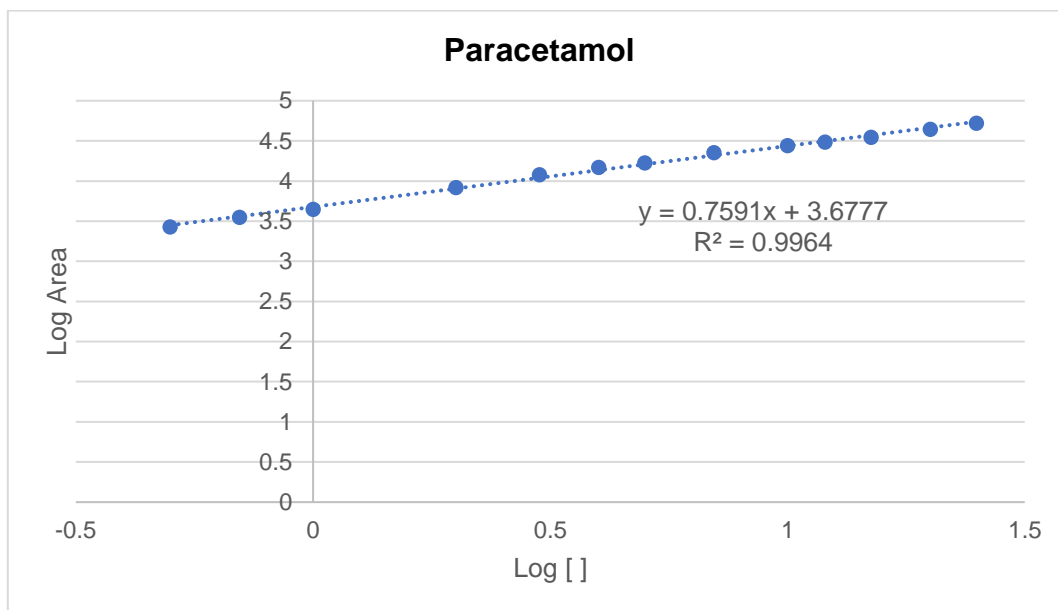


Figure 29. Logarithmic plot of the calibration curve of paracetamol

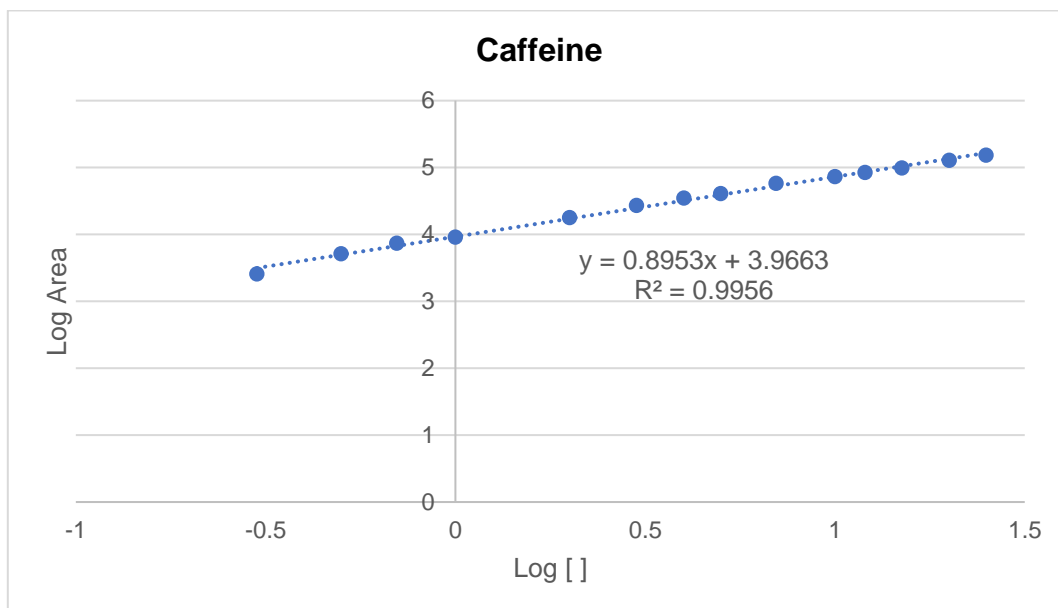


Figure 30. Logarithmic plot of the calibration curve of caffeine

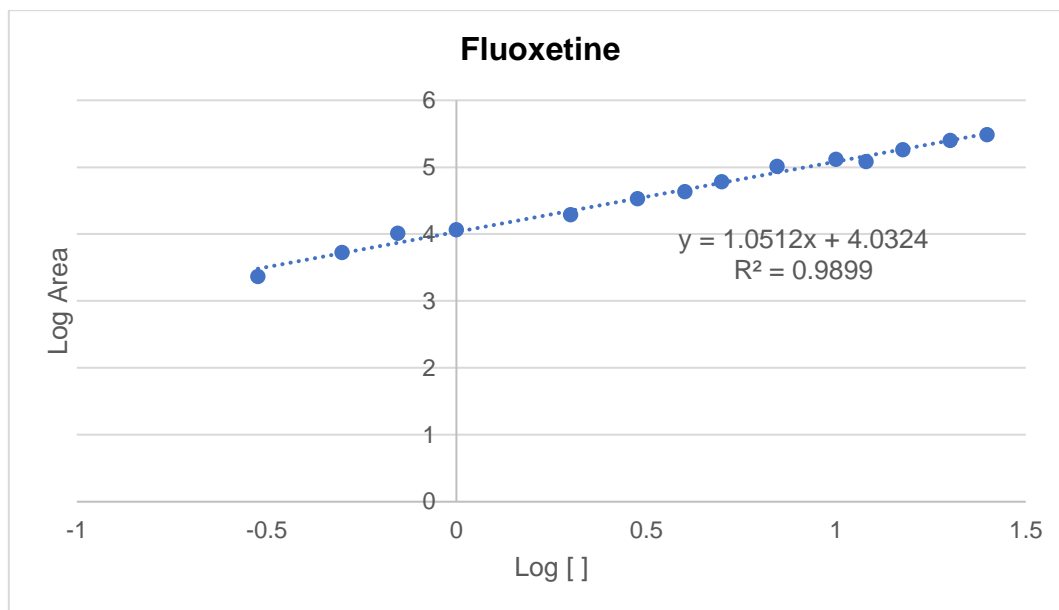


Figure 31. Logarithmic plot of the calibration curve of fluoxetine