Abbreviations list

EMEA ..... european medicines evaluation agency
ERA..... environmental risk assessment
FDA..... food and drug administration
GC-MS-SIM..... gas chromatography-mass spectrometry-selected-ion monitoring
HLB..... hydrophilic-lipophylic-balance
HPLC..... high-performance liquid chromatography
LC-ES-MS-MS ..... liquid chromarography-electrospray tandem mass spectromerty

MeOH..... methanol
MTBE..... methyl-terc-butyl ether
PEC ..... predicted environmental concentration
PNEC..... predicted no effect concentration
PPCPs..... pharmaceuticals and personal care products
STP..... sewage treatment plant
SPE..... solid phase extraction
UV ..... ultraviolet detection
WWTP..... wastewater treatment plant

1. INTRODUCTION

Pharmaceuticals have become recognised as relevant environmental contaminants in the course of the last decade. [1]

Residues of human and veterinary drugs were reported to be present in various environmental compartments including sewage[2], river water[3], groundwater[4] and even seawater.[5]

So far, a large variety of different compounds has been identified in the enviroment. Some of these are detected frequently and have a widespread distribution.[6]

At the beginning of the eighties the idea to make environmental risk assessment (ERA) for toxic substances was launched and it became compulsory for all new chemicals sold in EU from 1984. General principles and guidlines for ERA of new and existing chemicals have been introduced by European Medicines Evaluation Agency (EMEA) and the Food and Drug Administration (FDA), employing similar system. Both are based in the comparison between the predicted environmenatal concentrations (PEC) and the worst-case predicted no effect concentrations (PNEC) estimated from standart toxicity assays.[7,8]

At present, increasing attention is being paid to polar, often less persistent compounds, some of which, namely pharmaceuticals and personal care products, may act as if they were persistent because of their continuous input and permanent presence in aquatic systems.[9] These compounds and their bioactive metabolites have been continually introduced to the aquatic environment as complex mixtures via a number of routes, but primarily by both untreated and treated municipal wastewater.[10] The widespread presence in the aquatic environment can be explained by their extensive use in medical practices and incomplete removal in wastewater treatment plant (WWTP).

Their annual consumption ranges between a few kilograms and several hundred tonnes per individual compounds.2

Caffeine is one of the most widely consumed substances in the world and can be found in foods, beverages, condiments, tabacco, medication[11], and minor extent, as analeptic and in combination with analgesics to enhance their effect. Consequently, caffeine was detected in most non-target screening studies.[12]

Systematic research on the distribution of caffeine in the aquatic environment started recently and revealed its presence in surface and ground water in the ng/L to μg/L range.[13] These studies propose the use of caffeine as a tracer of the domestic sewage. The presence of caffeine in environmental waters is a distinct indicator of anthropogenic influences.

During the past few years, there has been significant progress in developing chromatographic methods for the analysis of caffeine, largely in consumables. However, due to very low concentration of caffeine in water samples, theses methods do not transfer to environmental sampling well. Chromatographic methods frequently employed include gas chromarography (GC) and high performance liquid chromatography (HPLC)[3,14,15]. Gas chromatography/mass spectrometry (GC/MS) was developed for determination of caffeine in tobacco[16], and in-vitro metabolites[17] and environmental samples.[18,19]

To improve the detection limit for environmental analysis, solid-phase extraction (SPE) was proposed for sample cleanup and pre-concentration of caffeine using various cartridges. Solid phase extraction (SPE) techniques require large samples (500-1000 mL water) and can be time-consuming and laborious.[20,21]

The aim of this study was to obtain a first overview about the contamination of waters from Mondego River and from source fountains near Coimbra city with caffeine. The samples were preconcentrated by Solid Phase Extraction (SPE) with Oasis polymeric column procedure and analysed

by Liquid Chromatography (LC) with UV detector. LC is commonly used for determination of caffeine in consumables, because of higher concentration of caffeine in beverages and food. GC/MS provide confirmation for detection of caffeine in complex solutions at below 1 μg/L, so this analytical method is more sensitive for environmental samples. We didn't have possibility in laboratory to use GC with MS detection, thereby we evaluate the applicability of the LC-UV system in the determination of caffeine residues in surface waters. The detection limit achieved with the proposed methodology for caffeine was 0.4 μg/L.

2. THEORETICAL PART

2.1. Sources

The consumption of pharmaceuticals is substantial. In the European Union (EU) about 3000 different substances are used in human medicine such as analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others. Also a large number of pharmaceuticals are used in veterinary medicine, among them antibiotics and anti-inflammatory. Sales figures are relatively high as reported for several countries.

Caffeine is one of the most widely consumed substances in the world and can be found in foods, beverages, condiments, tabacco, medication[22], and minor extent, as analeptic and in combination with analgesics to enhance their effect.

Consumption caffeine with combination other drugs:

-Panadol extra®
(caffeine 65 mg, paracetamol 500 mg)
-Saridon®
(Caffeine 50 mg, paracetamol 250 mg, Propylphenazonum 150 mg)
-Kinedry®l
(Caffeine 30 mg, Moxastini teoclas 25 mg)
-Ataralgin®
(caffeine 70 mg, Paracetamol 325 mg, Guaifenesinum 130 mg)

Consumption caffeine in beverages:

The global, average consumption is about 70 mg per person per day but varies in the different countries[23], for example, United Kingdom (UK) 440 (mainly tea), United States 210, and Switzerland 300 mg per persone per day (estimated from date on consumption of coffee, tea, and soft drinks).[24]

2.2 Fate and exposure routes

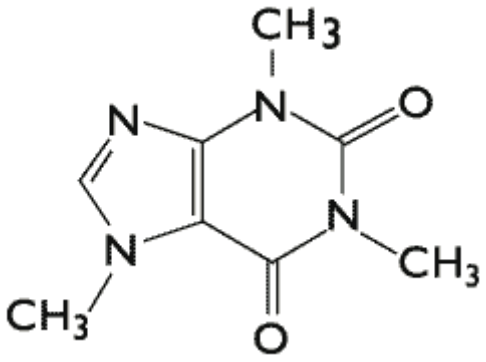
To date pharmaceuticals and personal care products (PPCPs) are used in high quantities throughout the world. Human use pharmaceuticals enter sewage effluents via urine and faeces and by improper disposal. These pharmaceuticals are discharged from private households and from hospitals and eventually reach municipal wastewater treatment plants (MWWTP). If PPCPs are only partially eliminated, residual amounts can reach surface waters or groundwater. However, direct inputs into natural waters are also possible through storm water overflow and leaks in the sewer system.[25]

The possible fate of drugs in the sewage treatment plant (STP) as for all other xenobiotics may be divided as follows:

a) The drugs or metabolites of the parent drug are mineralised by microorganisms to carbon dioxide and water, e.g. aspirin[26]
b) The drugs or metabolites of parent drug is more or less persistent in the STP, which implies that depending on the lipophylic properties or other binding possibilities e.g. ionic bindings, a part of the substance will be retained in the sludge. If the sludge is used as soil conditioner drug may be dispersed on agricultural fields. Again the fate of the drug depends on the lipophylic properties or other ability of binding to sludge or soil. Drugs that are mobile in the soil may be treating to the ground water or leach to a nearby stream. Depending on the ability of the drug to bind solids either organisms in the terrestrial ecosystem or aquatic ecosystem may be exposed.
c) The drug or metabolite of parent drug is persistent and at the same time very polar and nonbinding to solids. The substance will thus not be retained neither degraded in the STP and therefore easily reach the aquatic environment, and may affect the aquatic organisms.[1]

An unknown portion of drugs marketed for human treatment ends in the sewer system as surplus medical substances. After entering the STP the fate of these drugs will be almost identical with the excreted drugs. The only difference is that the waste water will not include the drug metabolites often produced by humans before excretion.[27]

2.3 Caffeine Generally



Caffeine is a xantine alkaloid compound that acts as a stimulant in humans. It is found in the leaves and beans of the coffee plant, in tea, yerba mate, and guarana berries, and in small quantities in cocoa, the kola nut. Overall, caffeine is found in the beans, leaves, and fruit of over 60 plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding upon them.

Caffeine is the worlds most widely consumed psychoactive substance. In North America, 90% of adults consume caffeine daily.[28]

Systematic name	1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione

Other names	1,3,7-trimethylxanthine, trimethylxanthine, theine, mateine, guaranine, methyltheobromine
Molecular formula	$C_8H_{10}N_4O_2$
SMILES	O=C1C2=C(N=CN2C)N(C(=O)N1C)C
Molar mass	194.19 $g \cdot mol^{-1}$
Appearance	Odorless, white needles or powder
CAS number	[58-08-2]
Properties	
Density and phase	1.2 $g \cdot cm^{-3}$, solid
Solubility in water	Slightly soluble
Other solvents	Soluble in ethyl acetate, chloroform, pyridine, pyrrole, tetrahydrofuran solution; moderately soluble in alcohol, acetone;

	slightly soluble in petroleum ether, ether, benzene.
Melting point	237 °C
Boiling point	178 °C (sublimes)
Acidity (pK_a)	10.4 (40 °C)

Caffeine is expected to persist in the water due mainly to its high solubility (13.5 g/L), low octanol-water partition coefficient (log K_{ow} = 0.01), and insignificant volatility, it fits the profile for a good, stable, dissolved marker directly related to human activity, with no potential biogenic sources.[14]

2.4. Pharmacology of caffeine

Caffeine is a central nervous system and metabolic stimulant,[29] and is used both recreationally and medically to reduce physical fatigue and restore mental alertness when unusual weakness or drowsiness occurs. Caffeine stimulates the central nervous system first at the higher levels, resulting in increased alertness and wakefulness, faster and clearer flow of thought, increased focus, and better general body coordination, and later at the spinal cord level at higher doses.[30]

2.4.1. Metabolism

Caffeine is completely absorbed by the stomach and small intestine within 45 minutes of ingestion. After ingestion it is distributed throughout all tissues of the body and is eliminated by first-order kinetics.[31]

The half-life of caffeine, the time required for the body to eliminate one-half of the total amount of caffeine consumed at a given time, varies widely among individuals according to such factors as age, liver function, pregnancy, some concurrent medications, and the level of enzymes in the liver needed for caffeine metabolism. In healthy adults, caffeine's half-life is approximately 3-4 hours.

Caffeine is metabolized in the liver by the cytochrome P450 (specifically, the 1A2 isoenzyme) into three metabolic dimethylxanthines[32], which each have their own effects on the body:

-Paraxanthine (84%) – has the effect of increasing lipolysis, leading to elevated glycerol and free fatty acid levels in the blood plasma.

-Theobromine (12%) – dilates blood vessels and increasing urine volume.

-Theophyllin (4%) - relaxes smooth muscles of the bronchi, and is used to treat asthma. The therapeutic dose of theophylline, however, is many times greater than the levels attained from caffeine metabolism.

Caffeine is extensively metabolized by humans with only approximately 3% excreted unchanged in the urine.[33]

The principal mode of action of caffeine is as an antagonist of adenosine receptors in the brain.[34] The caffeine molecule is structurally similar to adenosine, and binds to adenosine receptors on the surface of cells without activating them (an antagonist mechanism of action). Therefore, caffeine acts as a competitive inhibitor. The reduction in adenosine activity results in increased activity of the neurotransmitter dopamine, largely accounting for the stimulatory effects of caffeine. Caffeine can also increase levels of adrenalin, possibly via a different mechanism.[35]

Adrenaline, the natural endocrine response to a perceived threat, stimulates the sympathetic nervous system, leading to an increased heart rate, blood pressure and blood flow to muscles, a decreased blood flow to the skin and inner organs and a release of glucose by the liver.[36]

2.4.2. Tolerance and withdrawal

Because caffeine is primarily an antagonist of the central nervous system receptors for the neurotransmitter adenosine, the bodies of individuals who regularly consume caffeine adapt to the continual presence of the drug by substantially increasing the number of adenosine receptors in the central nervous system. This increase in the number of the adenosine receptors makes the body much more sensitive to adenosine, with two primary consequences. First the stimulatory effects of caffeine are substantially reduced, a phenomenon known as a tolerance adaptation. Second, because these adaptive responses to caffeine make individuals much more sensitive to adenosine, a reduction in caffeine intake will effectively increase the normal physiological effects of adenosine, resulting in unwelcome withdrawal symptoms in tolerant users.[37]

Adenosine, in part, serves to regulate blood pressure by causing vasodilatation, the increased effects of adenosine due to caffeine withdrawal cause the blood vessels of the head to dilate, leading to an excess of blood in the head and causing a headache and nausea. Reduced catecholamine activity may cause feelings of fatigue and drowsiness. A reduction in serotonin levels when caffeine use is stopped can cause anxiety, irritability, and inability to concentrate and diminished motivation to initiate or to complete daily tasks, in extreme cases it may cause mild depression.[38]

2.5. Occurrence of caffeine in environment

Caffeine concentrations in the effluents of wastewater treatment plants (WWTPs) were considerably lower (0.03–9.5 μg/L) than in corresponding influents, indicating an elimination of higher than 80%, primarily assigned to microbial degradation.[39] In the vast majority of WWTPs, caffeine removal was higher than 99.3%. A study realized in Swiss report that the caffeine concentrations in influents and effluents of WWTP ranged from 7-73 and 0.03-9.5 μg/L and indicated an efficient elimination of 81-99.9%.

Caffeine was detected in most non-target screening studies[12] (STP effluent), river water[40], seawater[5,41]. Systematic research on the distribution of caffeine in the aquatic environment started only recently and revealed its presence in surface and ground water in the ng/L to μg/L range.[42] Caffeine showed to be present throughout the North Sea in concentrations up to 16 ng/L.[43] Other report detected caffeine residues at concentrations as high as 320 μg/L in wastewater from La Pine, in Oregon (USA). Also, in domestic wastewater caffeine levels between 20 and 300 μg/L have been measured.[44]

The compound was also detected in surface water from then Mediterranean Sea off the Spanish coast (4–5 ng/L), but not in deeper water from the same location. Caffeine concentrations in lakes of the Swiss midland region varied from 6 ng/L to 164 ng/L and correlated with the population in the respective catchments areas, when normalized for the through flow of water (dilution) pointing out the suitability of caffeine as a quantitative anthropogenic marker.

Corresponding loads in untreated wastewater showed small variations when normalized for the population discharging to the WWTPs (15.8 ± 3.8 mg per person per day) reflecting a rather constant consumption. WWTP effluent loads were considerably lower (0.06 ± 0.03 mg per persone per day), apart from installations with low sludge age. Despite the efficient removal

in most WWTPs, caffeine was ubiquitously found in Swiss lakes and rivers (6-250 ng/L), except for remote mountain lakes (less than 2 ng/L).

Caffeine concentrations in lakes correlated with the anthropogenic burden by domestic wastewaters, demonstrating the suitability of caffeine as a marker.[45]

Caffeine was frequently detected in ground water also. The occurrence and the concentration of caffeine in ground water aquifers decreases as well depth increases. This implies that caffeine enters ground water mainly via leaching processes. Leaching of caffeine from domestic wastewater processing tanks[46] and sanitary landfills[47] has been reported in the literature and the presence of caffeine in ground water aquifers has been reported to be a positive indication of domestic wastewater contamination.

The loads of caffeine in untreated wastewater reflect not only consumption, metabolism, and excretion of the compound but also caffeine from beverages and foods that were poured out directly and potential degradation in the sewer system.[45]

2.6. Caffeine, anthropogenic marker

The importance of natural waters as ecosystems, drinking water and food resources, for agricultural irrigation, and for recreational activities requires a rigorous protection from contamination by xenobiotics and pathogenic microbes. Pollution may result from various domestic, industrial, or agricultural activities. Suitable markers are therefore necessary to detect and locate the source of water pollution.

An ideal marker for domestic wastewater should allow unambiguous elucidation of source of contamination allowing a distinction of these different sources of pollution and the quantification of the magnitude of pollution.[48]

Bacterial indicators such as faecal coliforms have traditionally been used to monitor the contamination of natural waters by municipal wastewaters, but their reliability has been questioned (e.g. because of their relatively short time of survival and their limited source specificity).[49] Alternatively, a series of chemical markers have been suggested to trace pollution caused by private, domestic activities.[50]

Possible candidates are human endogenous metabolites and constituents of pharmaceuticals, PPCPs, and food. Regular and constant consumption is a further prerequisite for a good marker, implying that consumer habits do not change or the compound is not phased out within the next years. Finally, the quantities discharged with wastewaters should be sufficient to permit analytical quantification after dilution in the environment.

Research has suggested that the presence of caffeine in the environment can serve as an indicator of the presence of human sewage. Caffeine is considered a good, stable, dissolved marker directly related to human activities

with no potential biogenic sources because of its high solubility (13.5 g/L), low octanol-water partition coefficient (log Kow=0.01) and negligiable volatility.[51]

As potential chemical marker for domestic wastewater contamination, caffeine[52] fulfils the basic requirements for a good chemical marker, *i.e.* source specificity and uniform, constant and high consumption to permit its analytical quantification after dilution/dissipation in the environment.

When consumed, caffeine is metabolized,[53] but small amount (0.5-10%) of ingested caffeine remains intact when excreted.[52,45] Most work in the past decade has focused on heavily polluted systems and efficiency of caffeine removal in sewage treatment plants.[46] However, with improvements in the analytical technique[54] and lowered detection limits, the scope of application has broadened to include stream, estuarine, and groundwater systems.[55] In many instances, there appears to be an association between elevated caffeine concentration and high population densities.[45]

2.7. Development of analytical methodology

Pure caffeine is a white powder, and can be extracted from a variety of natural sources. Caffeine extraction is an important industrial process and can be performed using a number of different solvents. Benzene, chloroform, trichloroethylene and dichloromethane have all been used over the years but for reasons of safety, environmental impact, cost and flavor, they have been superseded by the following main methods.

Analytical methodology for the determination of caffeine in environmental water samples typically involve an initial extraction of the analytes followed by clean-up and determination steps. Liquid-liquid extraction (LLE) by no hazardous organic solvents[56], Solid Phase Extraction (SPE) and supercritical carbon dioxide extraction are found described in the scientific literature. The clean-up is necessary for the removal of co-extractives and generally relies on SPE.

Analytical methods for the determination of caffeine in waters have been described in the literature for wastewater and natural waters. The analytical procedures include SPE, GC-MS-SIM or GC-MS-MS and the use of internal standard $^{13}C_3$-labeled caffeine.

LC-MS and GC-MS are the most widely used techniques. Buerge et al[45] used GC-MS/MS methodology to show that caffeine can be used as a chemical marker for surface water pollution by domestic wastewater.

A method of gas-chromatography-ion trap tandem mass spectrometry (IT-MS/MS) for analysis of acidic pharmaceuticals and caffeine in surface water and municipal wastewater was developed and a LOD of 20 ng/L was achieved for caffeine.[69]

Gardinali and Zhao[57] applied a method based on LLE coupled to LC-APCI-MS to determine trace amounts of caffeine in surface waters at 4.0 ng/L.

3. EXPERIMENTAL PART

3.1. Samples of caffeine

A total of twelve samples were collected in different site of Mondego River and from fountains near Coimbra city during three month's periods.

3.2. Reagents and materials

Standard of caffeine was purchased from Sigma-Aldrich (Steinheim, Germany). LC grade methanol was purchased from Carlo Erba (Milan, Italy). Acetic acid glacial 100% (Merck, Darmstadt, Germany). MTBE (Merck, Darmstadt, Germany)

Water was purified by distillation and passage through Milli Q system (Millipore, Bedford, MA)

Extraction cartridges Oasis HLB 6cc/ 200 mg (Waters Corp. Milford, MA)**.**HV filters (0.45 μm, Durapore, Ireland)

Preparation of standard caffeine solutions:

Stock standard solution (500 μg/mL) was prepared from 0.025 g standard of caffeine with LC water. Concentrations 10, 5, 2, 1, 0.8, 0.5, 0.4 μg/mL were prepared by a dilution of previous solutions with LC water.

Preparation of the mobile phase

The mobile phase used for the analysis was consisting of 190 ml acetic acid glacial 100% and 810 ml of LC water (adjusted to pH 3.0 with acetate of sodium). It was filtered through a 0.45 µm filter under vacuum and degassed by ultrasonication.

3.3. Apparatus and chromatographic conditions

The LC method described here was developed using a LC system, consisting of pump (model 305, Gilson Medical Electronics, France), a injector Model 7125 (Redone, Cotati, California, USA), a C_{18} Nucleosil column (Hichrom, UK), and UV/VIS 151 detector (Gilson, Medical Electronics, Villiers-le-Bel, France) operated at wavelength of 280 nm. The results were recorded on a SP 4270 integrator (Hewlett Packard, Philadelphia, USA). Caffeine was eluted isocratically using a mobile phase consisting of acetic acid glacial and LC water (190:810). The LC system was operated at room temperature and the flow rate was 1.3 mL/min.

3.4. Extraction and clean-up

The water samples were extracted by SPE by using Oasis HLB 6cc (200mg) cartridge. The cartridge was conditioned with 3 ml of MTBE, 3 ml of methanol and 3 ml of LC water. The water samples were filtered through a filter paper and then through membrane filter (polyamide 0.2 µm, NL 16 Schleicher and Schuell). Then 1 L of the sample was percolated through the cartridge. Washing was performed by 2 ml of 25% methanol.

Then the cartridge was eluted with 1 ml methanol followed by 6 ml of methanol/MTBE (1:9; v/v). This eluate was evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 1 ml of mobile phase for concentration 0.8-10 µg/L and in 0.250 ml of mobile phase for concentration 0.5 µg/L, then final filtration through membrane filters (0.45 µm). The volume of the eluate injected was 50 µl.

4. RESULT AND DISCUSSION

4.1 HPLC Conditions and optimization

The methodology reported here utilizes C_{18} Hichrom Nucleosil analytical column at room temperature with acetic acid glacial-LC water (190:810) (adjusted to pH 3.0 with acetate of sodium) as mobile phase. The isocratic analysis under the described conditions allows the elution of caffeine peak with good resolution (Fig. 1). We obtain peak shape at relatively low flow rate of 1.3 ml/min. The optimal mobile phase was selected by varying the proportion of the mobile phase constituents and pH.

At first we tried mobile phase consist of 190 ml acetic acid glacial 100% and 810 ml of LC water (adjusted to pH 3.0 with acetate sodium). Second mobile phase was prepared from the first one but with 5% of methanol. Subsequently we compare peaks of both mobile phases. We did´nt obtain good peak with methanol. It was not so high and narrow, retention time of caffeine was shorter than 7 minutes. Thereby we use mobile phase without methanol, where we obtained narrow, shape peak with good resolution.

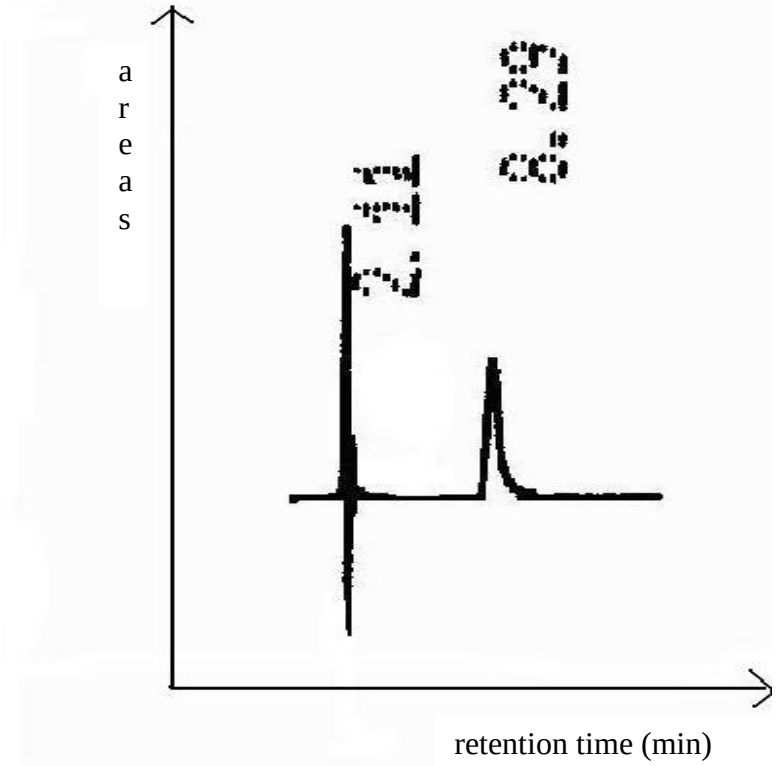
UV detection of caffeine is used in some scientific papers published on determination of caffeine in wastewater and surface water. In order to evaluated and optimize the sensitivity of detection we compare the results obtained at different wavelengths adopted by several authors, under the same chromatographic conditions: 273 nm[58], 276 nm[59] and 280 nm.[60] The higher peaks were obtained at 280 nm indicating a more sensitive detection.

The mean retention time for caffeine was 8.31 minutes. On the basis of six parallel determinations, during three days, the precision within-day and between-day (RSD) of caffeine retention times were 0.033 and 0.052 %, respectively, as we can observed in Table 1.

Table 1: Intra-day and between-day precision and RSD for caffeine retention times

DAY	MEAN	RSD within-day (%)	RSD Between-day (%)
1	8.35	0,029	0,053
2	8.37	0,034	0,065
3	8.22	0,036	0,039

Figure 1: chromatogram of standart of caffeine 0.5 µg/ı



4.2. Optimization of extraction and clean-up

The extraction method applied was based in Verenitch at al. method[61] with some modifications.

The variables optimised for the solid phase extraction were:

- type of the clean-up cartridge
- solvents used in the washing steps
- eluent solvents and the volumes for eluting caffeine from the cartridges

One liter of sample was filtered through 0.45 μm filters. In order to optimise the experimental conditions, two amounts of this sorbent, 60 mg and 200 mg, of the same particle size, were assayed. The accuracy results were higher for Oasis HLB 200 mg cartridges higher than 91% and lower for the 60 mg cartridge (76%). Therefore, Oasis 200 mg cartridges were chosen for this study. When the method was applied on the water samples, chromatograms not presented interfering peaks allowing the accurate determination of caffeine residues in this type of samples.

A mixture of 25% methanol/water to remove the polar co-extractives was more efficient in removing the interferences when compared with water. Therefore, it was used for washing the columns.

The elution of caffeine was performed using 1 mL of methanol followed by 6 mL of MeOH/MTBE (1:9, v/v). The volumes of methanol MeOH/MTBE (1:9, v/v) was optimized and a second elution with 1 mL of methanol didn't not improve the results. So, our results show that 1 ml is sufficient to elute all caffeine.

The extract was dried to dryness and then dissolved in 0.250 mL of mobile phase. For highly contaminated samples, such as the municipal wastewater effluent, the volume of mobile phase added was 1 mL instead.

Elution was performed by course of Verenitch method, but for dissolution we used different volume of mobile phase according to fortification levels.
For the fortification levels 1 and 10 µg/L 1 mL of mobile phase was added to dryness residue, but for fortification at 0.5 µg/L level 0.250 mL of mobile phase was added in order to achieve better sensitivity. Then we made final filtration through membrane filter, before injection in LC system.

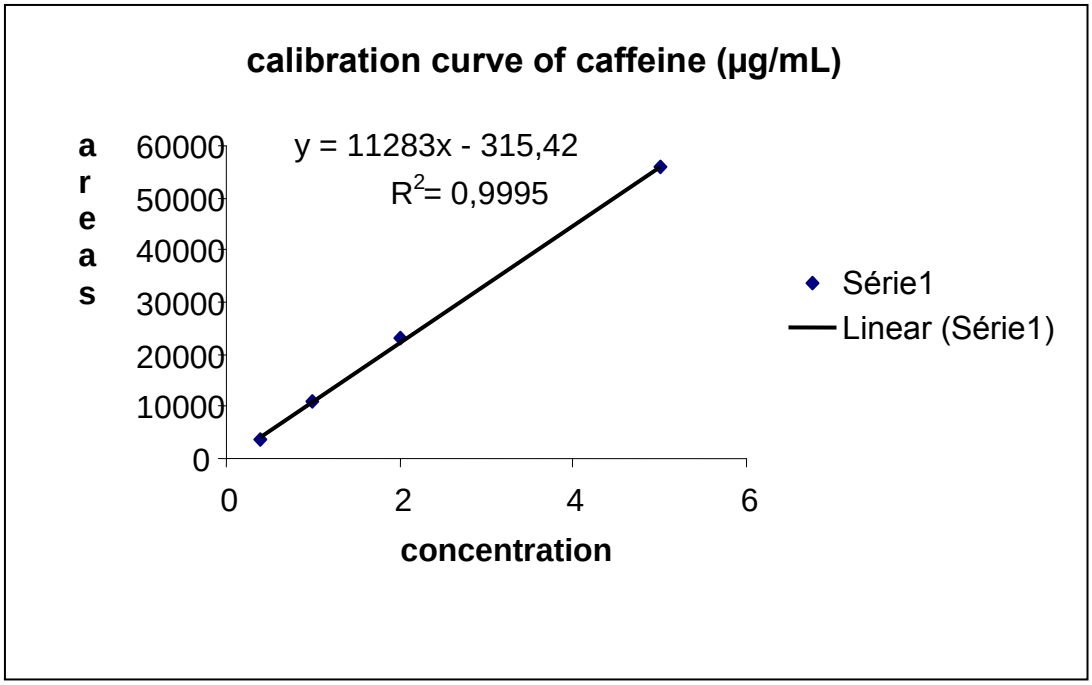
4.3. Validation

Method validation is one of the measures universally recognizes as a necessary part of a comprehensive system of quality assurance in analytical chemistry. Reliable analytical methods are required for compliance with national and international regulations in all areas of analysis providing date off the required quality. Method validation is therefore an essential component of the measures that a laboratory should implement to allow it to procedure reliable analytical data.

The main aim of validation of analytical method is to perform that the method is suitable for its intended purpose, such as implementation of legislation and for monitoring and risk assessment studies. Method validation makes use of a set of tests that both test any assumptions on which the analytical method is based and establish and document the performance characteristics of a method, thereby demonstrating whether the method is fit for a particular analytical purpose. Typical performance characteristics of analytical methods are: applicability, selectivity, calibration, trueness, precision, recovery, operating range, limit of quantification, limit of detection and sensitivity.

Linearity

The calibration curve was obtained using the linear least square regression procedure of the peak area versus the concentration. Within the concentration range described, 0.4-5 µg/L, linear plot was obtained for caffeine. The mean correlation coefficients (r^2) are above 0.9990.

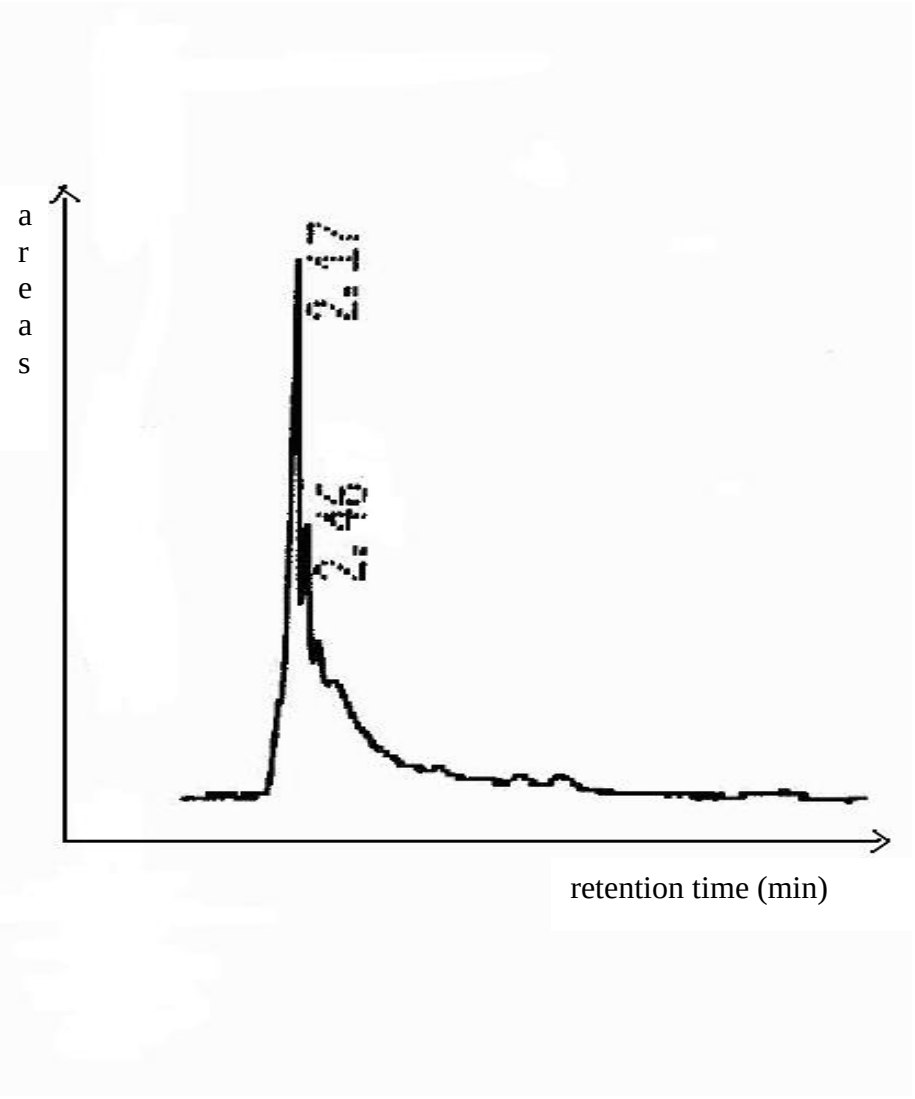


Specificity

In order to verify the absence of potential interfering substances around the retention time of caffeine, water blank samples (Fig. 2) were analyzed in order to assess the specifity of the method. In some water samples analysed no interference at retention time of caffeine was. In some samples from different points of collection of Mondego River and fountains near Coimbra city, there appeared interference before retention time of caffeine but didn't interfere with caffeine peak.

So, in order to obtain a simple, fast, robust and inexpensive method no further improvements were performed in the caffeine analysis.

Figure 2: Liquid chromatogram of a blank assay



Limit of quantification

The limit of quantification (LOQ) for caffeine was 0,4 µg/L, calculated according to the European Union Guidelines as the lower concentration that provides repeatabilities lower than 20%.

Accuracy and precision

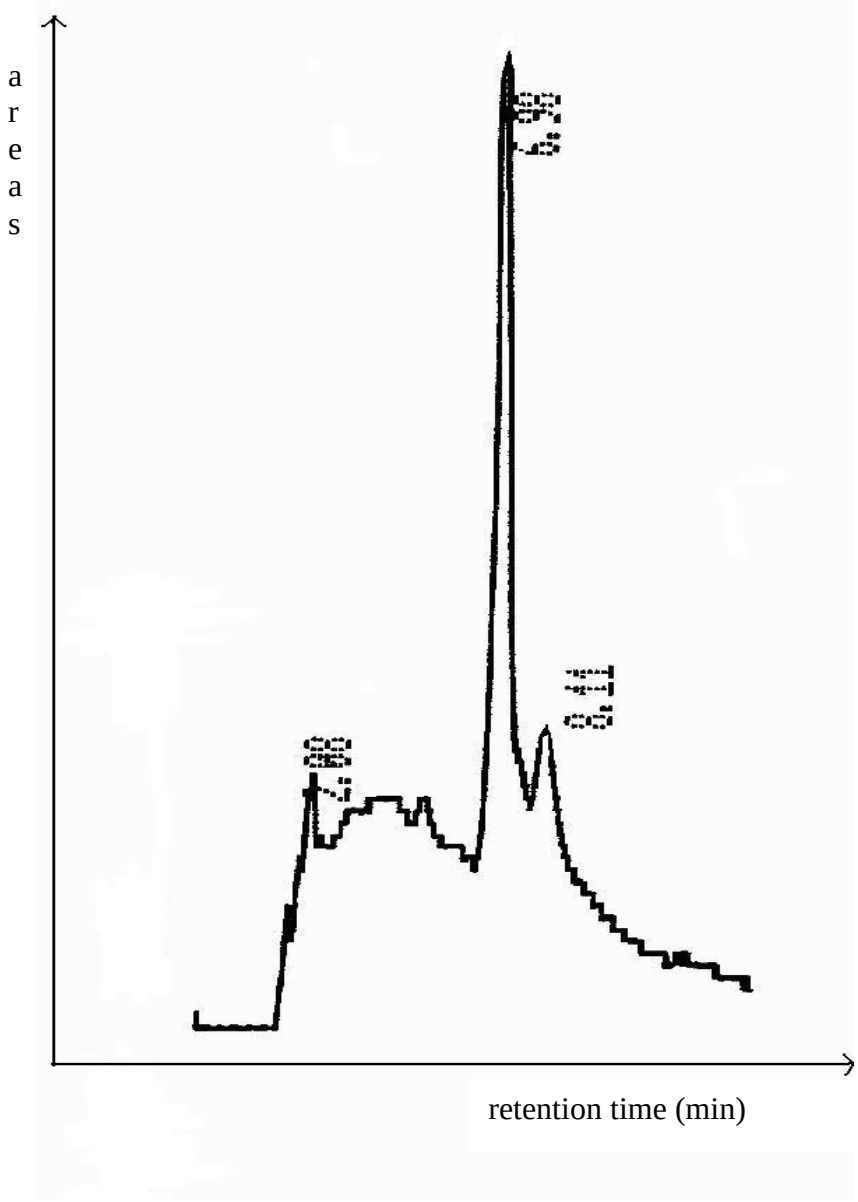
The accuracy of method was studied by spiking caffeine samples at three fortification levels (10.0, 1.0 and 0.5 µg/L). Fortification level 1µg/L is shown in Figure 3.

Within-day accuracy and the precision date were determined by analyzing, on the same day, three replicates of spiked samples at three levels. The between-day accuracy and precision were also determined by extracting batches of three fortification levels and analyzing them on three different days. Accuracy and intra-day and inter-day precision date are shown in Table 2. Recoveries were generally greater than 91.0 showing good accuracy of the method. For the three fortification levels, the relative standard deviation was less than 5.2 % demonstrating good method precision.

Table 2: Accuracy and inter- and intra assay validation results

FORTIFICATION LEVEL (µg/L)	RECOVERY MEAN (%)	RSD within-day (%)	RSD Between-day (%)
0.5	97.8	2,765	3,536
1	104.5	4,455	5,162
10	91.7	1,336	4,504

Figure 3: Chromatogram of spiked sample (1µg/L)



4.4. Evaluation of caffeine in samples

A total of twelve samples of caffeine were analysed under the conditions described and none of them showed to be contaminated with caffeine. These results could be explained by the poor sensitivity of the proposed method. Gardinali and Zhao[51] applied a method based on LLE coupled to LC-APCI-MS to determine trace amounts of caffeine in surface waters at 4.0 ng/L, and confirm its presence in 82% of the samples analyzed from Biscayne Bay and the Miami River.

Table 3: Monitoring of caffeine (sample, extraction method, elution solvent and mobile phase)

SAMPLE	EXTRACTION METHOD	ELUTION SOLVENT	MOBILE PHASE
WWTPs efflulents (USA, Maryland)	SPE (polysryrene-divinylbnezene sorbent)	Ethyl-acetate	
Surface water (Biscayne Bay, Florida)	Liquid-liquid extraction	Methylenchlord	Methanol:HPLC water (30:70)
Ground water of Northem Greece	SPE cartriges (Hyspere GP; 10mm*2mm)		A: 5 mmol/L phosphate buffer pH 3 B: Acetonitrile:HPLC water (90:10)
Streams in US	SPE (contain 0.5g of HLB)	Methnol	A: $NH_4H_2O_2/CH_2O_2$ pH 3.7 B: 100% C_2H_3N
Rivers and streams in Germany	SPE cartriges (with RP-C_{18} ec materiál)	Methanol	20 mmol/L amonia acetate in water-acetonitrile
Seawater (North Sea)	SPE(polysryrene-divinylbnezene sorbent, SDB 1, 2g)	Etylacetate+n-hexane/etylacetate (4:1, v/v)	
STP influents and effluents (Spain)	SPE oasis HLB catriges (200mg, 6cc)	Etylacetate	Acetonitrile

Table 4 : monitoring of caffeine (sample, column, analytical method, concentration range, LOD)

SAMPLE	COLUMN	ANALYTICAL METHOD	CONCENTRATION RANGE (µg/L)	LOD (µg/L)
WWTPs efflulents (USA, Maryland)	Factor four 5 ms (DB-5 type) capillary column (30m, 0.25 mm)	GC-MS	0.036	0.00769
Surface water (Biscayne Bay, Florida)	HPLC: C_{18} 5 µm (150 * 4.6 mm)	LC-APCI-MS	0.0769-0.119	0.004
Ground water of Northem Greece	HPLC: nucleosil 100-5C_{18}, reverse phase column (150mm*4.6mm)	HPLC-DAD 280 nm	0.28 (Aliakmon river) 2.7 (Loudias river) 1.5 (Axios river)	0.05
Streams in US	HPLC: reverse phase C_8 (150*2 mm)	HPLC/MS-ESI(+)	0.081-6.0	0.05
Rivers and streams in Germany	100 RP-C_{18} (125*3 mm) 5 µm	LC-ES-MS-MS	0.15-0.53 (Main) 0.35 (Rhein) 0.70 (Rodau)	0.005
Seawater (NorthSea)	DB 5-MS (30*0.25 mm)	GC-MS (70 eV)	0.002-0.0054 (central North Sea) 0.0097 (Danish coast) 0.008-0.009 (Germany)	0.00017
STP influents and effluents (Spain)	RP C_8 (150*4.6 mm)	LC-MS	79-118 (influents) 1.4-44 (effluents)	0.001

5. CONCLUSIONS

This project describes analytical method for the determination of caffeine in river water and fountains, based on solid phase extraction (SPE) and liquid chromatography with ultraviolet detection (LC-UV).

The proposed analytical methodology allows the simple, fast, low pollutant, accurate and precise determination of caffeine residues in surface waters. The SPE procedure through Oasis cartridges (200 mg) leads to a clean blank assays. The accuracy of our method was studied by spiking caffeine samples at three fortification levels (10.0, 1.0, 0.5 μg/L). Recoveries were generally greeted than 91.0 % showing good accuracy of the method. For the three fortification levels, the relative standard deviation was less than 5.2 demonstrating good method precision.

According our result we found out that LC with UV detection is not sensitive method enough for the quantification of the very low concentration of caffeine residues found in surface water.

Some published papers reported the determination by LC. Other scientific papers published on determination of caffeine in ground, surface and wastewater prefer LC-MS and GC-MS because of great sensitivity of those methods and analyzing environmental samples including low concentration level of caffeine. It is shown in table 3 and 4 (monitoring of caffeine). There compares various kind of samples, extraction method (they mostly used SPE), elution solvent, mobile phase, analytical method (in common use are GC with MS), different columns and also different sensitivity to environmental samples. From this table we can deduce that method using GC/MS are more sensitive for determination of caffeine in very low concentration.

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