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Monitoring of anti-inflammatory drugs in hydric media from Bragança district

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"The knowledge that you have emerged wiser and stronger from setbacks means that you are, ever after, secure in your ability to survive."

J. K. Rowling

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ABSTRACT

Emerging micropollutants are chemical substances present in different matrices at very low concentrations, ranging from nanograms to micrograms per liter. Non-Steroidal Anti-Inflammatory Drugs (NSAID) are some of the most prescribed drugs worldwide and several studies report the presence of these substances in various hydric media including drinking water, surface water, and sewage water, among others. Since they are present in very low concentrations, their identification, quantification and removal are not easy tasks.

The main objective of this work is to gather some knowledge about the most recent analytical methodologies in the field, in order to start the evaluation of this environmental and health problem in the Bragança region. Therefore, this work is based on the development of an analytical methodology to extract, detect and quantify non-steroidal anti-inflammatory drugs and on the validation of this methodology using real samples from different hydric media of the Bragança region.

From an initial set of ten pharmaceutical drugs belonging to different pharmacological classes, a group of five NSAID, acetyl salicylic acid, diclofenac, ibuprofen, ketoprofen and naproxen, was selected as the prototype acidic molecules due to their relatively low pKa (3.49 to 4.91). After an extensive literature review, the solid phase extraction (SPE) was selected as extraction technique using an adsorbent (Chromabond® HLB) applicable for a wide range of analyte polarities.

In order to improve the SPE recoveries, several experimental measurements were performed using different flow-rates for the adsorbent conditioning, sample loading, washing and elution steps. Extractions were performed with individual and mixture standards prepared in ultrapure water with the aim of evaluating the analytes recovery.

The high performance liquid chromatography with photo diode array detector (HPLC-DAD) was the selected instrumental method of analysis with a Nucleosil C18 column under reversed phase mode conditions. Several studies were carried out to optimize the mobile phase composition, mobile phase pH, elution flow-rate and wavelengths for detector monitorization.

After the optimization of some SPE/HPLC-DAD operating conditions, the developed methodology was validated analyzing six real samples collected from different aqueous sources in Bragança region: city tap water, well water, swimming pool, “river A” at two different points and “river B”. The river A, location 1, was contaminated with naproxen and ketoprofen and the well water sample indicated the presence of ketoprofen.

For these samples, taking into account the limits of quantification (LOQ) of the developed analytical method, only river A contamination could be evaluated with a measured concentration of 11.230 ± 0.767 ppb for ketoprofen. The concentrations of naproxen in river A and ketoprofen in well water sample were below the LOQ but above the limit of detection (LOD), proving the presence of these compounds.

Responses were also observed for acetyl salicylic acid in river A, location 1, and in pool water samples, but their values were below the LOD, therefore, it is not possible to confirm the presence of this NSAID in those samples.

Keywords: Emerging Pollutants; Non-Steroidal Anti-Inflammatory Drugs; Hydric Media; Solid Phase Extraction, High Performance Liquid Chromatography - Diode Array Detection.

RESUMO

Micropoluentes emergentes são substâncias químicas presentes em diferentes matrizes em concentrações muito baixas, variando de nanogramas a microgramas por litro. Os Anti-Inflamatórios Não-Esteróides são alguns dos fármacos mais prescritos em todo o mundo e diversos estudos reportam a presença destas substâncias em várias matrizes aquosas incluindo água potável, água superficial, águas residuais, entre outros. Como estão presentes em concentrações muito baixas, a sua identificação, quantificação e remoção não são tarefas fáceis.

O principal objetivo deste trabalho é adquirir algum conhecimento sobre as mais recentes metodologias analíticas neste campo, a fim de iniciar a avaliação deste problema ambiental e de saúde na região de Bragança. Portanto, este trabalho é baseado no desenvolvimento de uma metodologia analítica para extrair, detetar e quantificar anti-inflamatórios não-esteróides (AINEs) e na validação desta metodologia utilizando amostras reais de diferentes matrizes aquosas da região de Bragança.

De um conjunto inicial de dez compostos pertencentes a diferentes classes farmacológicas, um grupo de cinco AINEs, ácido acetil salicílico, diclofenaco, ibuprofeno, cetoprofeno e naproxeno, foi selecionado, sendo constituído por moléculas protótipo de caráter ácido devido a seus valores relativamente baixos de pKa (3.49 a 4.91). Após uma extensa revisão bibliográfica, a Extração em Fase Sólida (EFS) foi a técnica selecionada utilizando um adsorvente (Chromabond® HLB) aplicável para uma vasta gama de polaridade dos analitos.

Com o objetivo de aperfeiçoar as recuperações da EFS, várias medições experimentais foram realizadas usando diferentes caudais para as etapas de condicionamento do adsorvente, carregamento da amostra, limpeza e eluição. Foram feitas extrações dos compostos em padrões individuais e em misturas preparadas em água ultrapura a fim de avaliar a recuperação dos analitos.

A cromatografia líquida de alta eficiência acoplada ao detetor de arranjo de díodos (CLAE-DAD) foi o método instrumental de análise selecionado, com uma coluna Nucleosil C18 sob condições de fase reversa. Diversos estudos foram feitos para otimizar

a composição da fase móvel, o pH da fase móvel, o caudal de eluição e os comprimentos de onda monitorizados no detetor.

Depois da otimização de algumas das condições de operação de EFS/CLAE-DAD, a metodologia desenvolvida foi validada com a análise de seis amostras reais recolhidas em diferentes matrizes aquosas da região de Bragança: água de torneira da distribuição municipal, água de poço, água de piscina, “Rio A” em dois pontos distintos e “Rio B”. O Rio A, ponto 1, estava contaminado com naproxeno e cetoprofeno e a água de poço indicou a presença de cetoprofeno.

Para estas amostras, considerando os limites de quantificação do método desenvolvido, apenas a contaminaçao do rio A pode ser avaliada com uma concentração medida de 11.230 ± 0.767 ppb para o cetoprofeno. As concentrações de naproxeno no rio A e de cetoprofeno na água de poço estavam abaixo do limite de quantificação, mas acima dos limites de deteção, provando a presença destes compostos.

Houve também resposta para o ácido acetil salicílico no Rio A, ponto 1, e na água de piscina, mas seus valores estavam abaixo do LD, portanto, não se pode confirmar a presença destes AINEs nestas amostras.

Palavras-chave: Poluentes emergentes; Anti-Inflamatórios Não-Esteróides; Matrizes Aquosas; Extração em Fase Sólida; Cromatografia Líquida de Alta Eficiência acoplada a Detector de Arranjo de Diodos.

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LIST OF ABBREVIATIONS

CAS	Chemical Abstracts Service
CV	Coefficient of Variation
DAD	Diode Array Detector
EDC	Endocrine Disrupting Chemicals
ESI	Electrospray ionization
ETP	Effluent Treatment Plant
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IT	Ion Trap
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit Of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
NSAID	Non-Steroidal Anti-Inflammatory Drugs
POP	Persistent Organic Pollutants
PPCP	Pharmaceuticals and Personal Care Products
PPB	Parts per billion
PPM	Parts per million
RP	Reverse Phase
SAID	Steroidal Anti-Inflammatory Drugs

SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
TOF	Time of Flight
UV	Ultraviolet

1. Motivation and objectives

1.1 Introduction

Due to the increasing anthropic action and industrial activities, the quality of water has been impaired and the contact and ingestion of contaminated water can cause injurious effects to living beings [1, 2].

Recently, some compounds that did not used to be found in the environment and were not considered as pollutants have been identified mainly in aqueous matrices, the so-called emerging pollutants. These are contaminants that can affect living beings and the environment but are not usually present in national or international environmental legislations. Such substances may be regulated in the future, if toxicity, health effects and bioaccumulation studies will stress this need [3]. Emerging micropollutants are some of these compounds that are found in very low concentrations ranging from nanograms to micrograms per liter. These substances, even in small quantities, may cause adverse reactions to living organisms with which they come into contact [4]. Different types of products can be sources of emerging micropollutants into the environment, including pharmaceuticals, cosmetics, synthetic hormones, surfactants, pesticides, cleaning products and others [5]. Medical drugs, in turn, can also be subdivided into different classes, such as analgesics and anti-inflammatories, antibiotics, antiepileptic, contrasting, synthetic steroids and others [6].

Pharmaceuticals are emerging micropollutants of significant relevance, since they normally have high pharmacological potency, which means that they can cause significant effects even at low concentrations. Furthermore, they are also designed to be persistent, keeping their chemical properties for long periods of time [7, 8]. As a result of being compounds found in low concentrations, the analysis and the study of drugs become challenging tasks. More sophisticated analysis methods are necessary to detect the substances in micro and nanograms amounts [4]. Thereby, it is recognized the demand to develop an effective method for quantifying, and consequently, monitoring drugs as emerging micropollutants in aqueous matrices.

1.2 Objectives

1.2.1 Main objective

The main objective of this work is to contribute to the development and validation of an experimental methodology that could be applied to the monitoring of emerging micropollutants in aqueous matrices of Bragança region.

1.2.2 Specific objectives

The analytical methodology development includes both solid phase extraction (SPE) technique and high performance liquid chromatography coupled with diode array detector (HPLC-DAD) main operation conditions optimization.

For SPE, different flow-rates will be studied for all the main steps (conditioning, loading, washing and elution) in order to raise recoveries.

The optimization of HPLC-DAD method will be performed by means of the selection of the most promising operating conditions to improve the compounds separation. Among these, the selection of the solvent composition, the solvent pH value, the mode of operation (isocratic or gradient) and the wavelength(s) for each analyte.

The experimental methodology must be validated with the determination of the most relevant statistical parameters, such as, the calibration curves using a confidence level of 95%, the intermediate precision, the repeatability and the limits of quantification and detection.

The developed methodology will be implemented by collecting and analyzing different types of aqueous samples and different locations from the Bragança region.

1.3 Report organization

This master thesis report is organized in five main chapters. The first one presents a brief introduction to the relevance of the proposed work, the main objectives to be fulfilled and the organization of this report.

In the second chapter it is presented an extensive state of the art, which includes some important definitions. The review of some recent published work, in the field of extraction techniques for the analysis of micropollutants and instrumental methods of analysis is also done. Besides, this introductory chapter will present the description of the most relevant equations and statistic parameters used for the experimental methodology validation.

The third chapter is dedicated to list all the equipment, reagents and experimental procedures used in this work. In this chapter, it is presented the experimental work which is split in two main parts. The first one is dedicated to the development of the analytical methodology to extract and to quantify the selected anti-inflammatory drugs. The second one is directed to the implementation of the developed methodology using real aqueous samples from the Bragança region.

In the fourth chapter, the main experimental results are presented and discussed.

The fifth and final chapter gathers the main conclusions obtained in this work and presents some considerations and suggestions for future works.

2. Literature review

2.1 Water and environment

Water is an indispensable substance for human life, having applications ranging from its need for metabolic functions to its use for energy production [1, 2]. Nevertheless, in 2014 it was estimated that more than 700 million people had no access to potable water and, in the coming decades, the supply of this resource would be reduced to 75% of the currently available amount. It is estimated that, in each year, between 12500 to 15000 km^3 of water are used, and 4000 km^3 are exclusively destined to industry, irrigation and domestic use. Furthermore, it is known that only 0.5% of the planet's water is freshwater available for consumption [9]. This information is even more alarming when considering the quality of water available for human consumption. It is known that with the increasing anthropic action and the industrial activities, the quality of this resource has been negatively affected [1].

2.1.1 Pollution in hydric media

Population and urban growth are strongly associated with environmental degradation and increasing need for basic sanitation, waste treatment and access to drinking water. The response to this type of needs is not always achieved with the desired speed and efficacy, causing a high risk to the health of the population of regions without access to the types of treatments mentioned [10]. Contact and ingestion of contaminated water can cause harmful effects to living beings and are associated with most part of the diseases that affect the world population, especially parasitic diseases, such as schistosomiasis [9].

In addition to the water pollution caused by microbiological contamination, there are other factors, such as contamination by toxic metals, greatly emphasized between 1970 and 1980. In the last decades, it was raised the interest about the presence of substances with less perceptible effects, and generally found in low concentrations, the recently called emerging pollutants [1].

2.2 Emerging pollutants

With the recent advances in analytical techniques, new compounds can be identified in aqueous matrices [4]. The emerging pollutants are substances that have been detected in different environmental matrices and, until some time ago, were not considered as contaminants [5, 6]. These pollutants are present in the environment

worldwide and usually are sourced from domestic, commercial and industrial wastewater [11].

These contaminants may cause harm, but since their detection and quantification have only received some attention from the competent authorities recently, some are not legislated yet. Such substances are candidates for future regulation in case the toxicity, effects on public health and bioaccumulation studies indicate this requirement [3].

The expression “emerging micropollutants” is widely used to refer to these compounds, since they are usually found at low concentrations in the order of micro (μg/L) or nanogram per liter (ng/L) [4, 6]. These substances, even in small quantities, may cause adverse reactions to the organisms which they come into contact with [4].

2.2.1 Legislation

Due to the low concentrations in which emerging micropollutants are found, their presence was only perceived with the development of new analytical methods and equipment [4]. In this way, most of the ruling legislations cover just the compounds that were already identified several years ago, such as organochlorine pesticides and aromatic hydrocarbons [12]. The methods of analysis and quantification of emerging micropollutants are still under development and study, as well as their effects on different organisms [6, 8–11]. Thus, the maximum amounts in which these substances could be found in nature have not been established yet in order to regulate these values [1, 12, 13].

Although legislation, usually, does not present limits for the concentration of emerging micropollutants in different environmental matrices, there are guidelines and monitoring activities for some candidate compounds for a future regulation [1, 12, 13]. In the European Union, for example, Directive 2008/105/EC presents list of 33 priority substances and their maximum allowable concentrations in aqueous matrices, which includes pollutants already characterized and traditionally legislated, such as benzene, naphthalene and hexachlorobenzene. In addition, this Directive indicates 11 substances for possible classification as priority in the future [5, 14–16].

Directive 2013/39/EU already presents 45 compounds in the list of priority substances and establishes a surveillance list with compounds that indicate risk to the aquatic environment and whose monitoring data are still insufficient. Among the substances on the watch list are diclofenac, 17-beta-estradiol and 17-alpha-eylestradiol,

being them, respectively, an anti-inflammatory, a natural hormone and a synthetic hormone utilized in contraceptives [15, 17].

2.2.2 *Emerging micropollutant classes*

Emerging micropollutants belong to very different groups of substances, such as pharmaceuticals, cosmetics, surfactants, pesticides, cleaning products, illicit drugs, flame retardants, nanomaterials and others [5, 18]. Due to the lack of a specific legislation for emerging micropollutants, there is no standard classification for them. However, the most common classification found in the literature is separated in Persistent Organic Pollutants, Pharmaceuticals and Personal Care Products and Endocrine Disrupting Compounds [6, 7, 19].

Persistent organic pollutants, also called POP, are organic compounds of difficult degradation, either by chemical, physical or photolytic agents. Due to this characteristic, these substances can accumulate in human or animal tissues and can also be inserted into the food chain. In this group, there are also some pesticides, such as hexachlorobenzene and DDT (dichlorodiphenyltrichloroethane), brominated flame retardants and organometallic compounds [11]. As a result of bioaccumulation and biomagnification properties, these compounds may be carcinogenic or mutagenic [1].

Pharmaceuticals and Personal Care Products (PPCP) form a very diverse class. It includes medicines, fragrances, disinfectants, sunscreen agents, preservers agents, X-ray contrasting substances, illicit drugs, veterinary drugs, cosmetic additives and others [6, 20–22]. Many of these compounds are water soluble, so their presence is quite common in environmental hydric systems due to wastewater, leachate of sanitary landfills or septic tanks [11].

Endocrine Disrupting Compounds (EDC) are substances capable of inhibiting the function of natural hormones or assuming their function, altering their production. By modifying the endocrine functions of a living being, they can cause behavioral, reproductive or development changes of the organism. Some compounds belonging to this class are natural or synthetic hormones, such as 17-beta-estradiol, 17-alpha-estradiol, and some pesticides, such as carbofuran [11].

Table 1 presents some studies that aimed to measure some emerging micropollutants found in aqueous matrices.

Table 1. Some studies on emerging micropollutants found in the literature.

Class	Pollutant	Matrix	Method of analysis	Average concentration (ng/L)	Year	Reference
PPCP	Acetylsalicylic acid	Sewer	SPME/GC-MS	27000	2018	[24]
	Azithromycin	Surface water	SPE/UHPLC–MS/MS	30.00	2018	[25]
	Bezafibrate	Sewer	SPE/LC-MS	95	2011	[11]
	Caffeine	Surface water	SPE/HPLC-DAD	4.46	2011	[26]
		Treated water	SPE/HPLC-MS/MS	8.93	2013	[14]
	Diclofenac	Sewer water	SPE/LC-MS	100	2011	[11]
		Sewer	SPME/GC-MS	300	2018	[24]
	Ibuprofen	Sewer	SPME/GC-MS	10100	2018	[24]
	Naproxen	Sewer	SPME/GC-MS	11000	2018	[24]
	Miconazole	Sewer water	SPE/LC-MS	13.9	2011	[11]
	Sulfamethoxazole	Sewer water	SPE/LC-MS	13	2011	[11]
	Triclosan	Surface water	SPE/HPLC-DAD	28.43	2011	[26]
	Trimethoprim	Sewer	SPE/LC-MS	61	2011	[11]
EDC	Bisphenol A	Sewer	SPE/LC-MS	165	2011	[11]
		Surface water	SPE/HPLC-DAD	6.21	2011	[26]
		Treated water	SPE/HPLC-MS/MS	5	2013	[14]
	Estriol	Surface water	SPE/HPLC-DAD	5.39	2011	[26]
	Estrone	Surface water	SPE/HPLC-DAD	4.9	2011	[26]
	Testosterone	Surface water	SPE/HPLC-DAD	1.34	2011	[26]
POP	Atrazine	Surface water	SPE/HPLC-DAD	3.59	2011	[26]
		Treated water	SPE/HPLC-MS-MS	2.65	2013	[14]
		Surface water	SPE/UHPLC–MS/MS	556.66	2018	[25]

This information shows that compounds from all the mentioned classes were found in the recent years in different aqueous matrices and with a wide range of concentrations.

2.2.2.1 Pharmaceuticals and personal care products

Pharmaceutical and Personal Care Products (PPCP) are known as pseudo persistent pollutants because although they are not really resistant to degradation like

POP, they are continuously reintroduced into the environment. Due to their massive consume, substantial amounts reach hydric media again, maintaining significant amounts in water systems [20, 21, 24]. Drugs are defined as therapeutic substances used to prevent or treat diseases in humans or animals. The personal care products are used to improve the quality of human life [22].

Pharmaceutical compounds are generally organic, moderately soluble in water and lipophilic. They are substances with medicinal properties that can be administered topically (local application on skin or mucosa), internally (usually orally) or parenterally (such as injections), under different forms [28]. These substances are produced with high pharmacologic activity, that means they perform their functions even at low concentrations [6]. The pharmaceutical industry is one of those with largest expansion in the global economy. It is estimated that in 2010, the sale of drugs produced profits of more than 780 billion euros. The European Federation of Pharmaceutical Industries and Associations (EFPIA) data show that the investments in research and development for pharmaceutical industries are rising year after year, with a perspective of 140 billion euros until 2020. In Europe, pharmaceutical industry employed more than in 747 thousand people in 2016 [29]. Worldwide, the antibiotics are the most prescribed medications and each year more than 12000 tons of these compounds are prescribed [30].

The presence of these chemical compounds in the environment does not automatically means an environmental problem. However, from the first evidence that such products may cause damage or other consequences to aquatic life or human beings, more attention should be given to this problem [22]. Despite their importance in society, preventing and healing diseases, drugs can cause harm to health when consumed through an incorrect method or in wrong quantities. It was believed that the main cause of inadequate ingestion of these compounds was self-medication, which could lead to consumption in higher quantities than the recommended ones. However, the development of more sensitive analytical techniques in recent decades has evidenced the contamination of water by drugs, that combined with their high pharmacological activity, can also cause reactions in the organisms with which they come into contact [9, 27].

The spreading of medications and water contamination can occur by different routes: launching of residues during the production of these compounds in industries, excretion of substances by the organism after ingestion, disposal of expired products and

use of animal manure as fertilizers [6]. Figure 1 presents a flowchart with the main routes of environment contamination by drugs [10, 25].

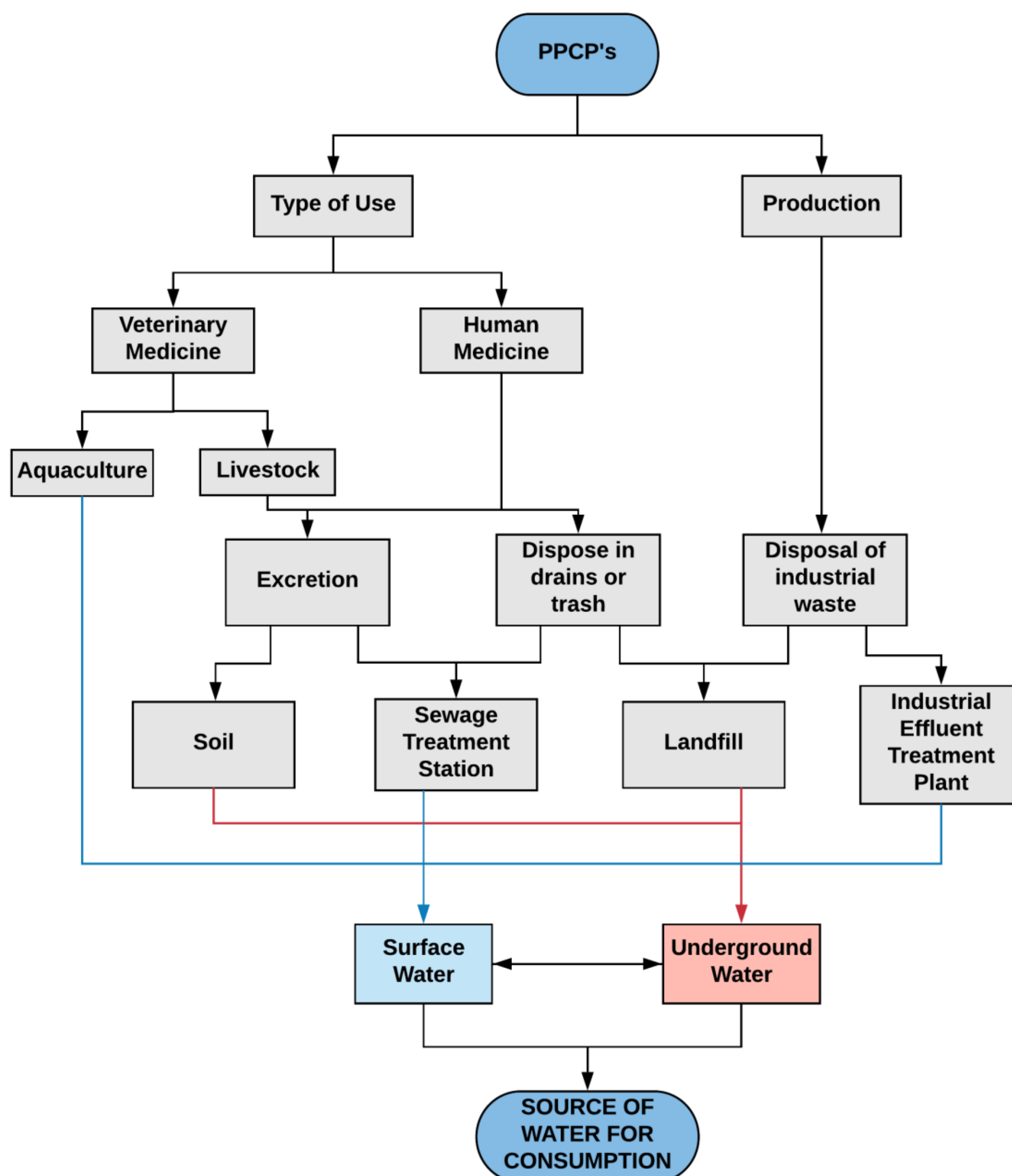

Figure 1. Sources and routes of contamination of water for consumption.

Among the water contamination pathways exposed above, such as aquaculture, soil, sewage treatment plants, landfills, industrial wastewater treatment plants and direct discards of residues from the pharmaceutical industries, the last two are the most controlled ones, as there are rules legislating and regulating the quality of the effluents thrown by the plants [6]. However, it is difficult to measure the elimination of these compounds by the organisms. It is known that the quantities ingested from a medicine are

not fully metabolized, and a part of them are discarded, mainly by urine. This happens both in humans and in animals and in different quantities according to each organism. However, animal manure is usually deposited directly into the soil without going through any sort of prior treatment [9, 13, 25, 27].

Although not considered persistent pollutants and found in low concentrations, the drugs can reach aquatic life and cause unexpected reactions. The most noticeable effects caused by these substances are the natural selection of microorganisms that can be resistant to some antibiotics and the harmful influence on the reproductive system of aquatic beings [8].

Different studies have found PPCP in fish, algae and mosquitoes [22]. Since these compounds were not developed to act in these living beings, the active principles and their derivatives can cause alterations or accumulate in their organism. In addition, certain drugs may promote adverse reactions when interacting with each other, and may impair human and animal health [32]. Another aggravating factor is the fact that these compounds are developed to present a high pharmacological potency, which means that they can cause the desired effects in a substantial way even at low concentrations and retain their physicochemical properties long enough to serve to the therapeutic purpose developed [4, 10, 11, 21].

The physicochemical characteristics of these compounds hinder their removal by conventional processes of water and wastewater treatment. There are several studies showing that although different treatment techniques are used, significant amounts of PPCPs still reach the water systems [2, 4, 21, 22, 29, 30]. Usually, the processes used to remove PPCP are physical, biological or chemical. The most common physical method is adsorption, which consists of transporting the pollutants from the effluent to the surface of the adsorbent material, which may be activated charcoal, graphene, carbon nanotubes, among others [21]. The methods of biological degradation are those that have lower cost and less complex operation, because they use microorganisms that incorporate pollutants as compounds for their metabolic functions [21]. In the literature, the most mentioned chemical process for degradation of these compounds is oxidation. Some of the procedures described apply ozone treatment, ultraviolet radiation treatment and ionizing radiation [21].

The drugs can be subdivided in different classes according to the application they are intended for. Among them there are analgesics and anti-inflammatories, antibiotics, antiepileptic, contrasting, synthetic steroids and others can be cited [9, 20].

Analgesics and anti-inflammatories are drugs used to reduce pain and inflammation and do not require medical prescription. The most common are paracetamol (acetaminophen), aspirin (acetylsalicylic acid), diclofenac and ibuprofen [9, 20].

Antibiotics have the function of assisting the treatment of microbiological infections eliminating bacteria in the organism, both of animals and humans. In this group, it is possible to mention sulfamethoxazole, azithromycin, amoxicillin and penicillin [6, 9, 31].

Lipid regulators are substances that help to control cholesterol and triglycerides in the blood. Some of these compounds found in studies in aqueous matrices were clofibrate and bezafibrate, the latter being one of the most used drugs in the world [6, 20, 21].

Beta-blockers can inhibit the release of adrenaline and noradrenaline, promoting the control of cardiovascular diseases and arterial hypertension. The most consumed are atenolol, propranolol and metoprolol [12, 20, 22, 31].

Table 2 shows some studies in which the authors quantified the concentrations of some drugs in the environment. These studies evidences that a wide range of pharmaceuticals can be found in different water matrices and in different concentrations.

Table 2. Classes of pharmaceutical drugs quantified in selected literature studies.

Class	Pollutant	Matrix	Method of analysis	Average concentration (ng/L)	Year	Reference
Anti-inflammatory	Diclofenac	Sewer	SPE/LC-MS	100	2011	[11]
		Groundwater	SPE/LC-ESI-MS/MS	0.41	2013	[36]
	Ibuprofen	Groundwater	SPE/LC-ESI-MS/MS	87.05	2013	[36]
		Effluent	LLE/GC-MS	15530	2014	[37]
Antibiotic	Azithromycin	Surface water	SPE/RP-LC-MS-MS	0.45	2014	[38]
		Groundwater	SPE/LC-ESI-MS/MS	15.09	2013	[36]
	Sulfadiazine	Surface water	SPE/RP-LC-MS-MS	21.45	2014	[38]
	Sulfamethoxazole	Sewer	SPE/LC-MS	13	2011	[11]
		Surface water	SPE/RP-LC-MS-MS	9.65	2014	[38]
		Surface water	SPE/LC and SPE/GC	113	2014	[39]
	Sulfathiazole	Surface water	SPE/RP-LC-MS-MS	2.1	2014	[38]
	Trimethoprim	Sewer	SPE/LC-MS	61	2011	[11]
		Surface water	SPE/RP-LC-MS-MS	13.1	2014	[38]
		Surface water	SPE/LC and SPE/GC	0.7	2014	[39]
Antiepileptic	Carbamazepine	Surface water	SPE/LC and SPE/GC	72	2014	[39]
Antifungal	Miconazole	Sewer	SPE/LC-MS	13.9	2011	[11]
Beta-blocker	Atenolol	Surface water	SPE/LC and SPE/GC	0.8	2014	[39]

Table 2. Classes of pharmaceutical drugs quantified in selected literature studies (continuation).

Class	Pollutant	Matrix	Method of analysis	Average concentration (ng/L)	Year	Reference
Stimulating	Caffeine	Surface water	SPE/HPLC-DAD	4.46	2011	[26]
		Effluent	LLE/GC-MS	360	2014	[37]
		Treated water	SPE/HPLC-MS/MS	8.93	2013	[14]
Lipid regulators	Gemfibrozil	Surface water	SPE/LC and SPE/GC	1.2	2014	[39]
		Groundwater	SPE/LC-ESI-MS/MS	0.41	2013	[36]
	Bezafibrate	Groundwater	SPE/LC-ESI-MS/MS	3.36	2013	[36]
		Sewer	SPE/LC-MS	95	2011	[11]

2.3 Compounds of interest

In an effort to select the compounds to be analyzed in this study, it was necessary to investigate which of the multiple types of drugs would be more frequently found in aqueous matrices of Bragança.

After examining studies with a similar theme conducted in northern Portugal or in regions with socio-economic characteristics similar to Bragança, the following compounds were selected: ibuprofen, diclofenac, naproxen, ketoprofen, acetylsalicylic acid, azithromycin, sulfamethoxazole, carbamazepine, acetaminophen and caffeine [8, 36–39].

2.3.1 Anti-inflammatory

Ibuprofen, diclofenac, naproxen, ketoprofen and acetylsalicylic acid are non-steroidal anti-inflammatory drugs (NSAID), one of the most widely used drug classes worldwide [44]. As all the anti-inflammatories, their function is to combat inflammations and to reduce pain and fever, however the difference from steroidal anti-inflammatory drugs (SAID) is the performance at different moments of inflammatory action, each one of these two classes being indicated for different forms of inflammation [45]. NSAID are usually recommended for musculoskeletal, urinary and intestinal inflammations, while allergic diseases are often treated with SAID [40, 41]. NSAID, however, are more used because the cases that require them are more frequent than those that require SAID [45].

2.3.1.1 Ibuprofen

The propanoic 2-(4-isobutylphenyl) acid, whose chemical structure is presented in Figure 2, is the third most consumed drug in the world, with a production that reaches kilotons [43]. It is a compound that, under ambient conditions, presents itself in the form of a white powder of weak odor and that can be absorbed by ingestion or contact with skin or mucosa [42, 43].

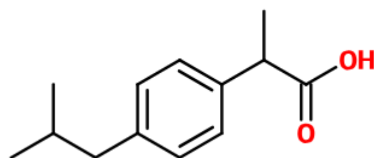

Figure 2. Ibuprofen chemical structure.

2.3.1.2 Diclofenac

Diclofenac is a NSAID derived from phenylacetic acid, usually used as sodium or potassium salt, commonly consumed orally or topically. It is a drug indicated for intervention in mild to moderate pain and clinical and post-operative inflammations [48]. Its chemical structure is presented in Figure 3.

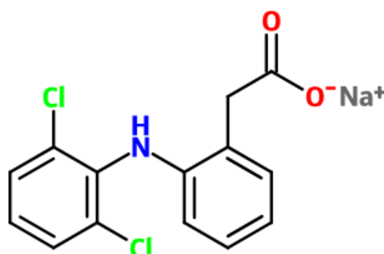

Figure 3. Sodium diclofenac chemical structure.

2.3.1.3 Naproxen

Naproxen is the active ingredient of medicinal products such as *Naprosyn*®, *Flamaprox*® and *Flanax*®, used for the relief of acute pain with inflammatory component, musculoskeletal circumstances such as torticollis, or post-traumatic conditions [49]. A manner to consume the compound is in the form of sodic salt, whose chemical structure is shown in Figure 4.

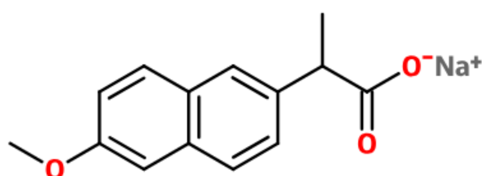

Figure 4. Sodium naproxen chemical structure.

2.3.1.4 Ketoprofen

Ketoprofen is the main compound present in Profenid®, Flamador®, Artrosil®, Menarini®. It is an anti-inflammatory drug mostly used to treat rheumatism consequences, arthritis and general trauma [47, 48]. Ketoprofen chemical structure is presented in Figure 5.

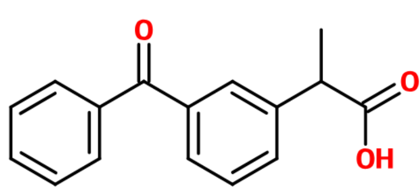

Figure 5. Ketoprofen chemical structure.

2.3.1.5 Acetylsalicylic acid

The acetylsalicylic acid is an anti-inflammatory drug that also avoids the grouping of platelets, preventing clots as well. It is an odorless white crystal usually consumed in pills and the main compound of Aspirin® [49, 50] . The chemical structure of acetylsalicylic acid is showed in Figure 6.

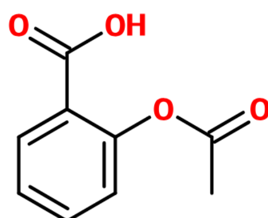

Figure 6. Acetylsalicylic acid chemical structure.

2.3.2 Antibiotics

Antibiotics are natural or synthetic substances used to treat or prevent bacterial infections, killing bacteria or avoiding their proliferation [46–48]. The year 1928 is considered the milestone for the treatment of bacterial diseases due to the discovery of penicillin by Alexander Fleming, although this was not the first identified antibiotic and was adopted as a therapeutic compound only in 1940 [55]. Currently there are several classes of these drugs, divided by the mechanism of antibiotic action, since each one acts in one of the many enzymes necessary for the metabolism of bacteria [55].

2.3.2.1 Azithromycin

Azithromycin is an antibiotic of the macrolide group, which acts interfering in the synthesis of bacterial proteins. It is generally indicated to combat respiratory infections such as pneumonia, chronic bronchitis, among other diseases [55]. Its chemical structure is displayed in Figure 7.

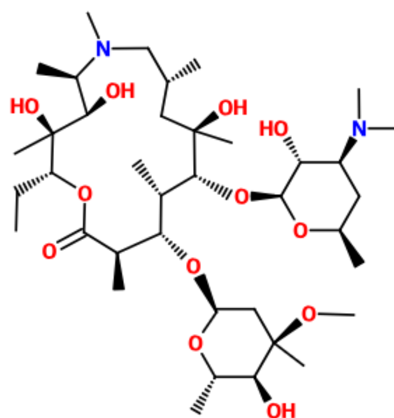

Figure 7. Azithromycin chemical structure.

2.3.2.2 Sulfamethoxazole

Sulfamethoxazole, presented in Figure 8, is an antibiotic belonging to the sulfonamides group, which blocks the necessary enzymes for the synthesis of nucleic acids from bacteria. It is widely used to cure urinary infections and HIV-infected patients affected by some opportunistic infections such as pneumocystosis [55].

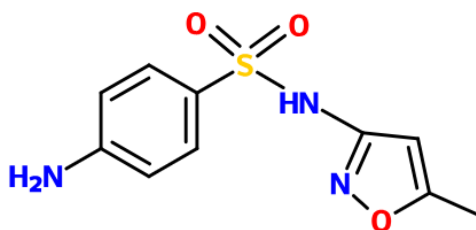

Figure 8. Sulfamethoxazole chemical structure.

2.3.3 Other commonly used drugs

2.3.3.1 Carbamazepine

Synthesized for the first time in 1953, carbamazepine is an antiepileptic, but it is also used to control hyperactivity, bipolarity and schizophrenia. As epileptic crises occur when there is uncontrolled electrical activity in the brain, these episodes happen often, but unpredictable. Antiepileptic agents act in such a way to inhibit neuronal activity or reduce their excitatory activity [57]. The action of the carbamazepine as an antiepileptic happens in order to prevent the flow of sodium ions in the neuronal membranes, stabilizing them and restricting the synapses of excitatory impulses [57]. The Figure 9 presents its chemical structure.

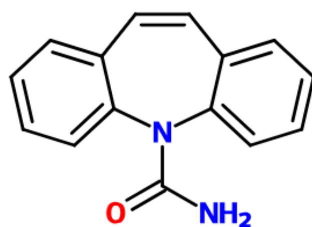

Figure 9. Carbamazepine chemical structure.

2.3.3.2 Acetaminophen

Acetaminophen or paracetamol (Figure 10) is an analgesic and antipyretic drug. It is the active principle of commercial products as Tylenol®, Cimegripe®, Panadol® and Vick Pyrena®. This compound is typically used to treat toothache, adverse reactions of vaccines, headache and flu symptoms, such as fever [55, 56].

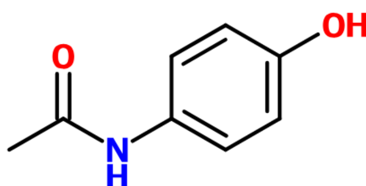

Figure 10. Acetaminophen chemical structure.

2.3.3.3 Caffeine

The caffeine (Figure 11) is a compound present in over sixty species of plants. It was isolated in 1820, but its effects were recognized only in 1981. It is present in several foods and beverages consumed worldwide besides in medical drugs. Different analgesics contain caffeine combined with acetaminophen or NSAID [57, 58] . Due to these two possible sources of caffeine to the environment, this molecule may be an indicator, showing that the matrix can be contaminated with other compounds. That means that despite not being a proven harmful substance, the detection of caffeine shows a high probability of presence of other PPCP [62].

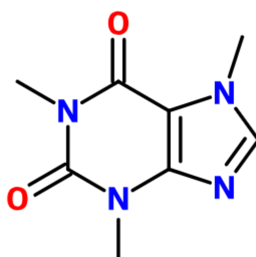

Figure 11. Caffeine chemical structure.

Table 3 summarizes some physicochemical characteristics of the compounds of interest discussed in this section.

Table 3. Some physicochemical data of the compounds of interest.

Compound Name	Molecular Formula	CAS	Molar mass (g/mol)	Solubility (mg/L)	pKa	Reference
Acetaminophen	$C_8H_9NO_2$	103-90-2	151.17	14000	9.38	[63]
Acetylsalicylic acid	$C_9H_8O_4$	50-78-2	180.16	4600	3.49	[53]
Azithromycin	$C_{38}H_{72}N_2O_{12}$	117772-70-0	748.99	2.37	8.74	[64]
Caffeine	$C_8H_{10}N_4O_2$	58-08-2	194.194	11000	10.4	[65]
Carbamazepine	$C_{15}H_{12}N_2O$	298-46-4	236.27	18	13.9	[66]
Diclofenac sodium	$C_{14}H_{10}Cl_2NO_2Na$	15307-79-6	318.10	2.4	4.15	[67]
Ibuprofen	$C_{13}H_{18}O_2$	15687-27-1	206.30	21	4.91	[47]
Ketoprofen	$C_{16}H_{14}O_3$	22071-15-4	254.29	51	4.45	[68]
Naproxen Sodium	$C_{14}H_{13}NaO_3$	26159-34-2	252.00	15.9	4.2	[69]
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	723-46-6	235.30	610	5.7	[16]

2.4 Review of experimental methodologies for drugs analysis

It was necessary to research similar studies in the literature in order to understand the approach generally used to analyze the selected compounds. Thus, it was possible to verify which methods of extraction, identification and quantification can be used in a more effective way. Table 4 presented below, gathers the information obtained.

Table 4. Main analytical methods found in literature for some pharmaceutical drugs.

Compound	Matrix	Extraction method	Quantification method	LOQ* (ppb)	Average concentration (ppb)	Reference
Ibuprofen	Affluent ETP**	SPE (Bond Elut® 500 mg/5 mL)	HPLC-DAD	170	<LOQ	[70]
	Affluent ETP	SPE (Oasis MAX 6cc 150 mg)	HPLC-DAD	0.1	62	[71]
	Effluent ETP	SPE (Oasis MAX 6cc 150 mg)	HPLC-DAD	0.1	3.15	[71]
	Hospital effluent	LLE (Chloroform)	HPLC-UV	20	77.1	[72]
Diclofenac	Affluent ETP	SPE Bond Elut® 500 mg/5 mL	HPLC-DAD	170	0.77	[70]
	Effluent ETP	SPE Bond Elut® 500 mg/5 mL	HPLC-DAD	170	0.93	[70]
	Surface water	SPE (Sampliq - OPT Agilent Technologies® 60mg/3mL)	HPLC-DAD	1.1	<LOQ	[73]
	Affluent ETP	SPE (Oasis® MAX 6cc 150 mg)	HPLC-DAD	0.4	11.2	[71]
	Effluent ETP	SPE (Oasis® MAX 6cc 150 mg)	HPLC-DAD	0.4	1.7	[71]
	Hospital effluent	LLE (Chloroform)	HPLC-UV	12	93.3	[72]
	Sewer	SPE (Strata SAX® 500mg/6 mL e Strata X® 500mg/6mL)	HPLC-MS-IT-TOF	0.0165 – 0.0293	0.1	[11]
Azithromycin	Wastewater	SPE (Oasis® HLB 200 mg/6 mL)	LC-MS-MS	0.013	2.686	[74]
Carbamazepine	Blood	SPE (Oasis® HLB 3cc)	UPLC-MS/MS	162.79	-	[57]
	Pool water	EPS (ENVI-18 Tube)	GM-MS	0.00071	-	[75]

Table 4. Main analytical methods found in literature for some pharmaceutical drugs (continuation).

Compound	Matrix	Extraction method	Quantification method	LOQ (ppb)	Average concentration (ppb)	Reference
Naproxen	Affluent ETP	SPE (Oasis® MAX 6cc 150 mg)	HPLC-DAD	0.4	17.5	[71]
	Effluent ETP	SPE (Oasis® MAX 6cc 150 mg)	HPLC-DAD	0.4	0.85	[71]
	Hospital effluent	LLE (Chloroform)	HPLC-UV	8	119	[72]
Sulfamethoxazole	Sewer	SPE (Loss SAX® 500mg/6 mL And Loss X® 500mg/6mL)	HPLC-MS-IT-TOF	0.0051 – 0.0074	0.013	[11]
	Surface water	SPE (C18 Waters Sep-Then ®)	HPLC-UV	1.1	<LOD	[76]
Ketoprofen	Surface water	SPE (Oasis HLB 6cc 200 mg)	HPLC-DAD	0.26	1.55	[77]
	Wastewater	SPE (Oasis HLB 6cc 200 mg)	HPLC-DAD	0.26	4.3	[77]
Acetylsalicylic acid	Wastewater	SPE (Oasis MCX 60 mg)	UPLC–ESI/MS/MS	0.0005	0.966	[78]
	Surface water	SPE (Oasis HLB 6mL 500mg)	HPLC-DAD	0.548	0.469	[79]
Acetaminophen	Surface water	SPE (Oasis HLB 6mL 500mg)	HPLC-DAD	0.020	0.047	[79]
	Surface water	SPE (Oasis MCX 60 mg)	UPLC–ESI/MS/MS	0.012	0.212	[78]
Caffeine	Wastewater	SPE (Oasis HLB 6cc 200 mg)	GC-MS	-	0.016	[80]

* LOQ: Limit of quantification

** ETP: Effluent treatment plant

From the information collected in Table 4, it is concluded that the most used extraction method is solid phase extraction (SPE). The SPE technique allows the study of samples with small concentrations, as is the case of micropollutant analyses, besides not needing large volumes of toxic solvents, as in the liquid phase extraction [11]. The selection of the cartridges for SPE is related to the affinity between the analyte and the adsorbent present in the cartridge. Both studies in Table 4 and studies by other authors do not present a conclusion of the most appropriate cartridge [70].

Regarding the quantification techniques, the most widely used method among the studies mentioned above was high performance liquid chromatography (HPLC) coupled to a diode array detector (DAD). However, many studies also use gas chromatography (GC) and other coupled techniques to quantification, such as mass spectrometry (MS), tandem mass spectrometry (MS/MS), spectrophotometric detector in the ultraviolet region (UV) and flame ionization detector (FID) [10, 13, 59].

2.4.1 Solid phase extraction

The solid phase extraction (SPE) technique was created during the 80s and has the purpose to extract and pre-concentrate the analytes from complex media. Unlike Liquid-Liquid Extraction, SPE does not use high amounts of solvents, because it uses solid sorbent. The sample passes through the sorbent that retains the desired compounds due to specific polar interactions [1, 11, 68, 79].

The SPE cartridge is a syringe shape tube that contains the solid sorbent packed between two filters called frits. Presently, there is a massive variety of sorbents in the market, being each one of them employed for a specific type of interaction between the analyte and the matrix. For this reason, several solids were developed, to attend to different specifics purposes [11, 68, 79].

There are basically four steps during the SPE procedure as illustrated in Figure 12 that must be optimized in order to provide maximum recovery.

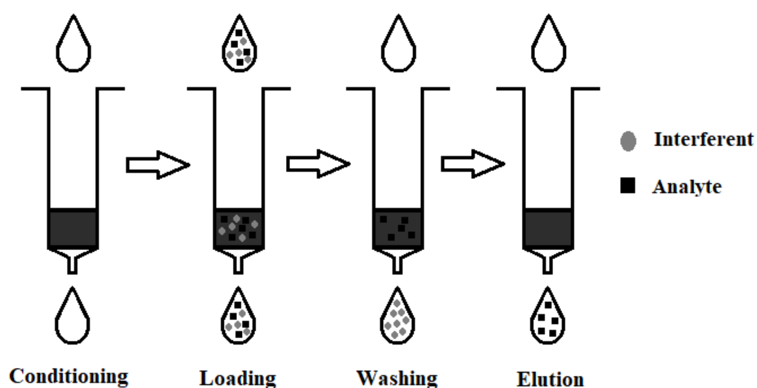

Figure 12. The four steps used in the SPE basic procedure.

The first stage is the solid conditioning by adding a solvent that is able to activate the sorbent and to clean impurities that can be inside the cartridge. The second step is to percolate the sample through the solid, in order to retain the compound(s) of interest. This retention happens due to the polar interactions and due to the adsorption process. In this moment, some amount of impurities can also be retained in the solid. For this reason, there is the third step, the washing, which consists of adding a weak solvent to remove the impurities without removing the compound(s) of interest from the sorbent. Finally, the last stage is to elute the analyte(s) from the solid adding small volumes of a stronger solvent [11, 68, 79, 80].

Figure 13. SPE equipment used in this work.

2.4.2 High performance liquid chromatography

Chromatography is a technique that allows the separation of substances to promote their identification, quantification or purification. The sample passes through a stationary phase carried by a mobile phase. The separation is based on the different polar interactions between the compounds and the mobile and stationary phases. The analytes that interact more with the stationary phase take longer to cross it, while the other compounds leave the system faster. This is the main separation principle of chromatography [4, 11, 68, 71].

The main difference between gas chromatography (GC) and liquid chromatography (LC) is that for the first one, the mobile phase is a gas and for the second one it is a liquid. The stationary phase in GC is normally a capillary column and in LC is normally a packed column. Concerning chromatographic analysis of PPCP, the most used technique is high performance liquid chromatography (HPLC), because these compounds tend to be less volatile and to suffer degradation with high temperatures [11, 68]. After the chromatographic separation, the substances need to be identified by a detector [4, 11, 71]. Figure 14 shows a basic HPLC equipment layout.

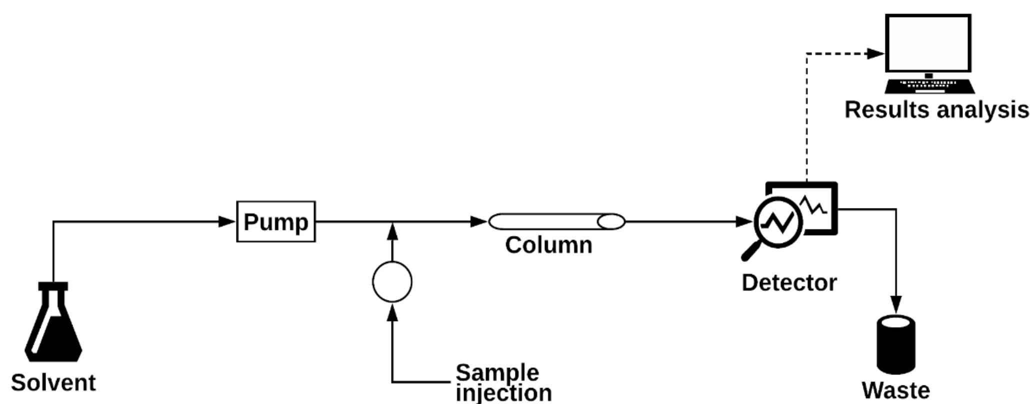

Figure 14. Basic HPLC equipment layout.

As each analyte has different affinities with the phases, they left the column in different moments and are detected separately. Usually, the most used parameter to identify the compounds is their retention time, that represents the time required for an analyte to be detected after the sample has been injected [81].

For pharmaceuticals chromatographic separations, the most indicated solvent mode of HPLC is the reversed phase (RP-HPLC). This mode of chromatography employs a column

that is less polar than the mobile phase, which is normally a mixture of acetonitrile with water. The main stationary phases are, generally, silica supports modified with C_8 or C_{18} carbon chains [81, 82].

This type of chromatography is indicated to polar compounds due to the speed of analysis comparing to normal phase chromatography. It happens because, for a same set of experimental conditions, the retention of a compound in RP-HPLC depends on its polarity. Less polar molecules tend to interact more with the stationary phase, increasing its retention time, while more polar substances interact more with the mobile phase than with the stationary one, presenting a lower retention time [81, 82]

2.4.2.1 Diode Array Detector (DAD)

Different HPLC detectors respond to different physicochemical properties, such as UV absorbance, mass charge ratio, fluorescence, refractive index, among others. Each detector measures one of these properties of the mobile phase as it flows and the detector response changes as the mobile phase contents changes [85].

Most HPLC methods use UV-visible detectors, due to their low cost, acceptable limits of detection and quantification, ease of use and because most of compounds has reasonably UV-visible absorbance. There are three main types of UV-Vis detectors: single wavelength, multiple wavelength or photodiode array (DAD) [81, 83, 84].

The single wavelength detectors can only use one wavelength to the analysis, being the most common the value 254 nm, obtained from a low pressure mercury lamp. This is the reason why many studies with UV detector use this wavelength [86]. The multiple wavelength detector can be programmed to operate with a specific wavelength. This selection is made with a diffraction grating that rotates, directing a single wavelength, through a slit, to the flow cell [81, 84]. The photodiode array detector has a diffraction grating also, but it is located after the flow cell, so that several wavelengths pass through the sample and then reach the array of photodiodes. This way, it is possible to collect chromatograms at an entire light spectrum with a single run and a chromatogram at any of these wavelengths can be then displayed. Another information that can be displayed after the analysis is the UV spectrum of each analyte, that allows the selection of an optimum

wavelength for each analyte in the final HPLC method [79, 81, 84]. Figure 15 illustrates the operation of a photodiode array detector.

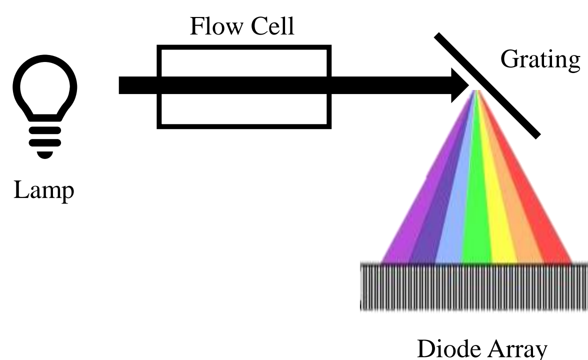

Figure 15. Photodiode array detector scheme.

The light from the emission source is collimated by a set of lenses and passes through the sample. After, the beam impinges the diffraction grating, separating the wavelengths and allowing that each one of them reaches a diode. The diodes convert the light intensity to a measurable output voltage sign, then an internal calibration system transforms this sign into an absorbance value for each wavelength, resulting in the absorbance spectrum of the sample [79, 81, 84].

Figure 16. HPLC-DAD system used in this work.

2.5 Analytical methodology validation

The analytical method development includes the validation process to measure its efficiency. The validation consists in guarantee, through objective criteria, that the method attends to its purpose, leading to reliable results [11, 68, 80, 85].

The validation parameters applied in this study are presented in the next sub-sections.

2.5.1 Calibration curve and linearity

During the method development it is necessary to build a calibration curve for each analyte, that is the graph between the standard solutions concentration, at X-axis, and their detected signal (in this case an area under the chromatographic peak), at the Y-axis [68, 71, 80, 85].

The method of least squares was used in order to do the line of regression of the obtained data. Since the results should fit into a straight line, the calibration plots take the algebraic form of equation (1), where a is the graph interception on y-axis, b is its slope, y is the area of the peak and x is the analyte concentration [88].

$$y = a + bx \tag{1}$$

The method of least squares is based on two main equations. Equation (2) gives the slope of least squares line, the b coefficient. The intercept of least squares lines, a coefficient, is calculated through equation (*3*), where $\bar{x}$ and $\bar{y}$ are the mean values of concentration (x_i) and area (y_i) results, respectively [88].

$$b = \frac{\sum_i[(x_i - \bar{x}) \times (y_i - \bar{y})]}{\sum_i(x_i - \bar{x})^2} \tag{2}$$

$$a = \bar{y} - b\bar{x} \tag{3}$$

Another important parameter is the product–moment correlation coefficient, r, calculated through equation (4). This method is used to estimate how well the behavior of the experimental data fits to a straight line. The closer the correlation coefficient is to the unit, the better the experimental data can be described by a linear model [88].

$$r = \frac{\sum_i[(x_i - \bar{x}) \times (y_i - \bar{y})]}{\{[\sum_i(x_i - \bar{x})^2] \times [\sum_i(y_i - \bar{y})^2]\}^{1/2}} \tag{4}$$

It is also possible to calculate the error $S_{y/x}$ associated to the slope and to the intercept of the regression line. Equation (5) shows its calculation, where n is the amount of adjusted points and $\hat{y}_i$ are the values estimated with the regression line equation [88].

$$S_{y/x} = \sqrt{\frac{\sum_i(y_i - \hat{y}_i)^2}{n-2}} \tag{5}$$

The standard deviations of slope and intercept can be calculated using equations (6) and (7), respectively [88].

$$S_b = \frac{S_{y/x}}{\sqrt{\sum_i(x_i - \bar{x})^2}} \tag{6}$$

$$S_a = S_{y/x} \times \sqrt{\frac{\sum_i x_i^2}{n \times \sum_i(x_i - \bar{x})^2}} \tag{7}$$

The $S_{y/x}$ parameter can be used to identify and reject outliers, abnormal results that inevitably occur in calibration procedures. A criterion usually applied is when the y-residual $(y_i - \hat{y}_i)$ is more than twice the value of the $S_{y/x}$ value. When this happens, the point must be removed from the fitting procedure [88].

The t-Student statistic distribution is used to provide the confidence limits (CL) for the slope and intercept as shown in equations (8) and (9) [88].

$$CL(b) = b \pm t \times S_b \tag{8}$$

$$CL(a) = a \pm t \times S_a \tag{9}$$

This statistic methodology can be used to find a concentration corresponding to an experimental measured signal, and the error associated to this concentration can be estimated, using equation (10) [88].

$$S_{x_0} = \frac{S_{y/x}}{b} \times \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \times \sum_i(x_i - \bar{x})^2}} \tag{10}$$

2.5.2 Limit of detection

The limit of detection (LOD) is the smaller amount of an analyte which can be detected, not necessarily quantified, within the experimental conditions stablished for the developed method. It can be valued trough three different ways by visual observation, with the signal/noise relationship and by means of the analytical curve parameters methods [11, 68, 79].

The most usual way is the third one, that uses analytical curve parameters applying them to equation (11) [70].

$$LOD = 3 \times \frac{S_a}{b} \tag{11}$$

2.5.3 Limit of quantification

The limit of quantification (LOQ), is the smaller amount of an analyte which can be measured within the experimental conditions stablished for the developed method [68, 79]. It can be calculated by the following equation (12) [70].

$$LOQ = 10 \times \frac{S_a}{b} \tag{12}$$

2.5.4 Precision

Precision is the parameter that evaluates the results from different analysis for the same sample in the same conditions. It can be determined in two different cases: repeatability and intermediate precision [68, 74, 79, 80].

The first one expresses the precision among data obtained in a short interval of time, with the same materials, by the same operator in the same day. Usually, it can be evaluated with three different standard concentrations in triplicate or with six repetitions for a single standard concentration [70], [76], [81], [82].

The intermediate precision refers to the closeness among data achieved in different days, by different operators, with different materials. This approach is known as the most representative for the variability of results [70], [76], [81], [82].

In both cases, the precision can be expressed as coefficient of variation (CV) or relative standard deviation (RSD), calculated using equation (13), where SD is the standard deviation and $\bar{X}$ is the average result [73].

$$CV(\%) = RSD(\%) = \frac{SD}{\bar{X}} \times 100 \tag{13}$$

2.5.5 *Accuracy*

The method accuracy is the closeness between the experimental data and the true values. The exact value only can be defined by an absolutely perfect measurement, in doing so, this value cannot be defined [11, 68, 74, 79, 80, 85].

Accuracy is expressed as percentage systemic error and it is intrinsic to the method. A systemic error occurs due to the loss of the substance due to the small extraction recovery, inexact volumetric measurements or interfering substances in the sample [76].

The most usual process to evaluate the accuracy is through recovery measurements with the real matrix. Recovery represents the amount of a certain compound recovered in the process in relation to the real amount present in the sample, that is the reason why it can be employed to estimate accuracy [11, 68, 79].

For recovery measurements, the samples are fortified (spiked) with known quantities of analyte and the results obtained after extraction are compared with the initial ones before extraction for the same samples. Usually a real "clean" sample is used to avoid other interferers beyond those that already exists in the analyzed matrix [68, 79, 80].

The recovery percentage is calculated through the equation (14), where C_1 is the measured concentration in the eluted sample, C_2 is the measured concentration in not-fortified sample and C_3 is the concentration that was added to the "clean" sample [11].

$$R = \frac{C_1 - C_2}{C_3} \times 100 \tag{14}$$

2.5.6 *Selectivity*

Selectivity represents the ability of the method to measure precisely the compound of interest without suffering interference from other substances or other analytes. If the

developed method is able to respond to different analytes distinguishing the responses among them, it is a selective method.[68, 74, 78].

This parameter is not measured, but it can be evaluated in different ways for chromatographic methods, such as comparing the analysis results of the matrix with and without the analytes and verifying if any other compound presents the same retention time that the compounds of interest. Another method is to employ detectors like photodiode array detector, which allows the analysis for several wavelengths and is also able to compare the peaks from the standards and the real samples to verify the compound purity [76].

3. Materials and methods

3.1 Materials

3.1.1 Solvents

- Acetonitrile HPLC grade, Carlo Erba, +99.9%
- Ultrapure water (resistivity value below 18.2 MΩ.cm - Type I)
- Commercial ethanol, Aga, 96%
- Methanol HPLC grade, Carlo Erba, +99.9%
- Trifluoracetic acid, Sigma Aldrich, +99%

3.1.2 Standards and reagents

- Ketoprofen, Sigma Aldrich, +98%
- Naproxen, Alfa Aesar, +95%
- Ibuprofen, Alfa Aesar, +99%
- Diclofenac, Alfa Aesar, +95%
- Acetylsalicylic acid, Alfa Aesar, +99%
- Sulfamethoxazole, Sigma-Aldrich, +98%
- Azithromycin, Sigma-Aldrich, +98%
- Carbamazepine, Sigma-Aldrich, +97%
- Acetaminophen, Alfa Aesar, +98%
- Caffeine, Alfa Aesar, +99%

3.1.3 Equipment

- RHPLC Jasco system, Extreme model, equipped with a PU-4180 Pump, PDA MD-4010 detector, an Interface box LC-Net II/ADC and a manual Rheodyne injection valve
- Chromatographic column EC Nucleosil 100-5, C18, 150mm L x 4.6mm ID, with a particle size diameter of 5 µm, Macherey-Nagel
- Analytical balance ADA 210/C, ±0.0002 g, Adam Equipment
- pH meter HI 2020-02, Hanna
- SPE cartridges, Chromabond HLB, 60 µm;6 mL/500 mg, Macherey-Nagel
- Visiprep™ SPE Vacuum Manifold, Supelco

- Millipore Direct Q3 UV Water Purification System, Merck

3.2 Experimental methodology

The study is divided in two main experimental steps. The first experimental task is to develop and optimize an experimental methodology to detect and quantify pharmaceutical drugs that belong to the class of non-steroidal anti-inflammatory drugs in different hydric media of the Bragança region. The proposed methodology is based on the solid phase extraction of analytes followed by detection and quantification using high performance liquid chromatography with a photo diode array detector. In this step, the main statistic parameters needed for method validation should be determined, such as the calibration curves, limits of detection and quantification, repeatability, accuracy and recoveries. The second experimental task will be the implementation of the developed methodology using some real aqueous samples collected from different points of the Bragança region.

3.2.1 Analytical method development

During the experimental work, all glassware was cleaned with detergent and abundantly rinsed with tap water, then rinsed with distillated water and finally with ethanol to remove any impurities.

The stock solutions were prepared by measuring 100 mg of each standard into a 100 mL volumetric flask and completing the remaining volume with the solvent used in each study, reaching a final concentration of 1000 mg/L. All prepared standard solutions were transferred to chromatography vials, sealed with film and stored at -18ºC until analysis.

3.2.1.1 Effect of mobile phase composition on chromatographic separation

The first variable to be optimized was the HPLC mobile phase composition. Several literature studies considering liquid chromatography of pharmaceutical drugs [11, 68, 77, 85] mentioned the use of a solvent mixture between acetonitrile (ACN) and water (W) in different compositions, under either isocratic or gradient modes of operation.

Initially, three mobile phases with different solvent proportions were tested in isocratic mode: 60ACN:40W, 50ACN:50W and 40ACN:60W. Due to the different polarities of acetonitrile and water, the interactions between the analytes and the mobile phase changes

for different compositions. Thus, the retention times and the dispersion of the chromatographic peaks are, generally, different for each solvent composition.

For preliminary studies, and due to the available standards quantity, ketoprofen was selected as the prototype NSAID for preliminary measurements. Three ketoprofen standard solutions with a concentration of 100 ppm were prepared using the three different solvent compositions. These solutions were analyzed using the HPLC system with a flow-rate of 1 mL/min and a 20 μL sample loop and the detector was set with a 254 nm wavelength, since some studies showed this value an appropriate wavelength for ketoprofen analysis [79].

3.2.1.2 Effect of mobile phase pH on chromatographic separation

Since reverse-phase HPLC was the selected analytical method, it was important to control the pH value in the mobile phase, because the compounds of interest present a wide range of pKa values. At lower pH values, the concentration of H^+ is higher, what inhibits the ionization of acid compounds (smaller pKa). When the compound is less ionized, it is less polar and has more interactions with the stationary phase (C_{18} column), enhancing the separation of acid compounds [79, 80, 87].

In order to study the pH effects on the retention times and dispersion, three 60acetonitrile:40water mobile phases were evaluated, each one with a differed content of trifluoracetic acid (TFA): 0%, 0.01% and 0.1%. Three ketoprofen standards samples at 100 ppm were prepared in 60ACN:40W solvent with the respective amounts of TFA for each analysis. The system was set up with a flow-rate of 1 mL/min and a wavelength of 254 nm, for the three analysis.

Besides that, since the employed chromatographic column works in a pH range from 2 to 8, it was necessary to define a safe proportion of TFA in the mobile phase. For these experiments, the pH values were measured in 60ACN:40W mixtures prepared with different contents of TFA that ranged from 0% to 0.1%.

3.2.1.3 Study of the elution order and selection of the individual wavelengths

Individual stock solutions at 1000 ppm for the ten standards were prepared with 60ACN:40W:0.01TFA. The working solutions at 100 ppm were prepared by diluting the stock solutions with the same solvent. The equipment operated with the 60ACN:40W:0.01TFA mobile phase, with a flow-rate of 1 mL/min and a wavelength range

from 190 to 650 nm. The aim of these measurements was to find out the elution order of the compounds and the wavelength that could allow a maximum absorbance value.

The HPLC-DAD equipment can work up to four chromatograms in real time during each analysis, each one for one predefined wavelength. However, after each run, the software allows to create several virtual digital channels with different functions. One of the virtual channels that can be generated is a total chromatogram for a given wavelength that gives a maximum absorbance value. Doing this for each individual standard analysis, it was possible to find out the exact wavelength corresponding to the maximum absorbance for each compound.

3.2.1.4 Standard mixture analysis

From the stock solutions, a 60ACN:40W:0.01TFA mixture with each one of the ten compounds at 1 ppm was prepared and analyzed under different mobile phase compositions to verify if the compounds could be completely baseline resolved. Initially, the studied %acetonitrile:%water based compositions were 20:80, 30:70, 40:60, 50:50, 60:40, 70:30 and 80:20, all solutions containing 0.01% of trifluoracetic acid. For all these analyses, the flow-rate was kept at 1 mL/min. The monitored wavelengths were 202, 228, 254 and 280 nm.

After, a 60ACN:40W:0.01TFA standard mixture, with NSAID only, was prepared with a concentration of 100 ppm for each anti-inflammatory to perform several gradients in order to study if a solvent gradient mode of operation could improve the baseline resolution for acetylsalicylic acid, diclofenac, ibuprofen, ketoprofen and naproxen. The wavelengths were set at 219, 224, 254 and 275 nm. The studied solvent gradients are presented in Table 5.

Table 5. Solvent gradient compositions studied for NSAIDs analysis.

Gradient	Time (min)	ACN (%)	W (%)	Flow rate (mL/min)
1	0	15	85	1.0
	4	15	85	
	30	40	60	
	80	70	30	
2	0	50	50	1.0
	4	50	50	
	20	80	20	
3	0	60	40	1.0
	4	60	40	
	20	80	20	
4	0	23	77	0.8
	60	23	77	
5	0	60	40	0.8
	60	60	40	
6	0	60	40	1.2
	60	60	40	

3.2.1.5 Linearity parameters of the HPLC analysis

The calibration curves were built for each analyte, by preparing a stock mixture solution with all the five NSAID (acetylsalicylic acid, diclofenac, ibuprofen, ketoprofen and naproxen) with individual concentration of 1000 ppm in methanol. Using the stock solution, 18 standard solutions were prepared for all the five standards with successive dilutions in methanol for the final concentrations from 100 ppm to 1 ppb.

The analyses were carried out with 60ACN:40W:0.01% TFA using a flow-rate of 1.2 mL/min. The monitored wavelengths were 219 nm for ibuprofen, 224 nm for acetylsalicylic acid and naproxen, 254 nm for ketoprofen and 275 nm for diclofenac. Each standard solution was injected three times.

In order to evaluate the repeatability and intermediate precision, as explained in section 2.5.4, three concentrations were analyzed in triplicate for each analyte, considering their linear range. In Table 6 are presented the concentration values used for the precision studies for each substance.

For the repeatability evaluation, the standards with the selected concentrations were analyzed in three times in a row, at the same day, with the same solvents. For the intermediate precision, the standards were analyzed in different days, with prepared mobile phases prepared at the day of the analyses. The parameters were obtained using the equation (13) to calculate de coefficient of variation for each concentration.

Table 6. Concentrations applied in precision studies for each analyte.

Analyte	Concentration 1 (ppb)	Concentration 2 (ppb)	Concentration 3 (ppb)
Acetylsalicylic acid	40	250	500
Diclofenac	250	500	750
Ibuprofen	50	250	500
Ketoprofen	30	40	50
Naproxen	30	40	50

3.2.1.6 Solid phase extraction operating conditions

The cartridge was chosen based on published references for pharmaceuticals extraction, as those referred in Table 4, comparing their results and searching for the composition of commercially available solids. It was concluded that an adsorbent based on poly (N-vinyl pyrrolidone-divinylbenzene), such as the Chromabond HLB 60 μm/6 mL/500 mg was the most suitable for this application.

The adopted SPE methodology was based on the experimental procedure proposed by the manufacturer [90]. The solvents and the volumes for each stage are presented in Table 7. The flow-rates were not referred by the manufacturer, therefore, in order to optimize them, for each step in each extraction, the dripping was adjusted to be as constant as possible and the cartridge emptying time was registered in each stage.

Table 7. Volumes for SPE steps [90].

Step	Solvent	Volume (mL)
Conditioning A	Methanol	5
Conditioning B	Water	5
Loading	Aqueous sample	10
Washing	Water	5
Elution	Methanol	8

The first extractions were carried out employing individual standard solutions prepared in ultrapure water. The concentration for each compound was different due to their solubility in water. The values are listed in Table 8.

Table 8. Standard solution concentration in the individual SPE extractions.

Compound	Concentration (ppm)
Acetylsalicylic acid	420
Diclofenac	6
Ibuprofen	19
Ketoprofen	35
Naproxen	9

After all the individual extractions measurements, a mixture was prepared measuring 20 mL of each individual standard solution for a total mixture volume of 100 mL. The concentrations in the final standard mixture solution for each compound are presented in Table 9.

Table 9. Standard mixture solution concentrations for the SPE extraction.

Compound	Concentration (ppm)
Acetylsalicylic acid	84
Diclofenac	1.2
Ibuprofen	3.8
Ketoprofen	7
Naproxen	1.8

One of the most important parameters to evaluate the SPE experimental methodology is the recovery. The measurements are usually carried out with spiked samples, prepared by adding known amounts of the analyte into real samples that do not contain any trace of the studied compound. In this study, the recovery was calculated through the analysis of samples

prepared in ultrapure water, therefore, the term C_2 of equation (14) is equal to zero. So, in the modified equation (15) C_1 is the concentration of analyte in the eluted solution and C_3 is the concentration of analyte in the standard solution. Considering that the loading is done with 10 mL of the sample and the elution uses 8 mL of methanol, the elute is more concentrated than the original sample, for this reason, the concentration basis recovery may present values above 100%.

$$R\ (c/c) = \frac{C_1}{C_3} \times 100 \tag{15}$$

Another way to evaluate the extraction is the mass basis recovery given by the equation (16), where m_2 represents the analyte mass added to the cartridge and m_1 is the recovered mass measured after the elution.

$$R\ (m/m) = \frac{m_1}{m_2} \times 100 \tag{16}$$

Another way to evaluate the extraction is to calculate the recovery in a mass basis, using equation (15). However, the mass basis recovery, equation (16) is a useful parameter for the daily evaluation, since it ranges from 0 to 100%, being easier to understand its meaning.

After each extraction procedure, the loading and washing wastes were analyzed to check if there was some loss of analyte in the wastes, as well as the eluate and the sample were also analyzed to calculate the extraction recovery. In this recovery studies, all the following HPLC analysis were performed with a 60acetonitrile:40water:0.01TFA solvent composition with a flow-rate of 1.2 mL/min and the DAD detector was set at 219, 224, 254 and 275 nm wavelengths.

3.2.2 Implementation of the developed methodology

The experimental implementation of the developed methodology was done by collecting six samples in different locations and hydric sources in the Bragança region. The samples were collected in sterile amber flasks, stored in ice and transported to the laboratory. Then where 15 mL of each sample were measured and filtered with a 0.45 µm polytetrafluoroethylene filter and store in freeze (-18ºC) until the moment of analyses. These samples were named from S1 to S6, as described below.

- S1 – Tap water from municipal water distribution system;
- S2 – Tap water from groundwater that supplies the Polytechnic Institute of Bragança;
- S3 – Surface water from River A, location 1, collected at Polis;
- S4 – Surface water from River A, location 2, collected inside IPB campus;
- S5 – Surface water from River B;
- S6 – Pool water from municipal sports facilities.

The SPE extraction procedure was carried for each sample, at room temperature according to the developed methodology. In Table 10 is presented the SPE procedure applied for all samples (S1 to S6).

Table 10. Final optimized flow-rates used in the SPE procedure for all samples.

Step	Solvent	Volume (mL)	Flow-rate (mL/min)
Conditioning 1	Methanol	5	0.22
Conditioning 2	Water	5	0.51
Loading	Sample	10	0.53
Washing	Water	5	0.87
Elution	Methanol	8	0.18

The eluted samples were stored in the freezer until the moment of their analysis. Each eluted solution was injected three times. In Table 11 is shown the chromatographic operating conditions for the optimized HPLC method.

Table 11. Operating conditions and parameters for HPLC analysis.

HPLC-DAD	
Mobile phase composition	60acetonitrile:40water:0.01TFA
Flow-rate	1.2 mL/min
pH	2.5
Column	Nucleosil C18 (150mm x 3 mm)
Sample injection volume	20 μL
Monitored wavelengths	219, 224, 254 and 275 nm
Time of analysis	5 min

4. Results and discussion

4.1 Analytical methodology development

This section comprises the main results that lead to the final analytical methodology developed in this work.

4.1.1 Effect of mobile phase composition in chromatographic separation

The obtained chromatograms for the different studied mobile phases are overlaid in Figure 17. These results show that the increase of acetonitrile content in the mobile phase decrease significantly the retention time of ketoprofen and also improves the shape of the chromatographic peak (it is thinner and taller), decreasing the dispersion. This fact could be important in order to obtain higher resolution and improve the analytes identification.

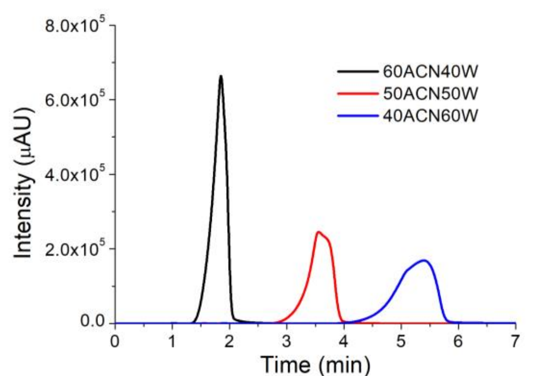

Figure 17. Study of ketoprofen (100 ppm) separation using different acetonitrile:water mobile phase volumetric compositions without pH modifier (0%TFA).

This behavior was already expected, since different compositions result in different interactions between the analytes, the solvent and the stationary phase. In this case, due to the relative polarity of ketoprofen, the rise of acetonitrile content will increase the solvent apolarity, impairing the interaction with the stationary phase and promoting the interaction with the solvent.

4.1.2 Effect of mobile phase pH on chromatographic separation

In Figure 18 it is shown the experimental chromatograms obtained with 0, 0.01 and 0.1% of TFA in the mobile phase. All the three mobile phase solutions were prepared with 60ACN:40W with each one of the above referred proportions of acid. It must be emphasized that all the standard solutions were prepared in the same solvent compositions as the studied mobile phase.

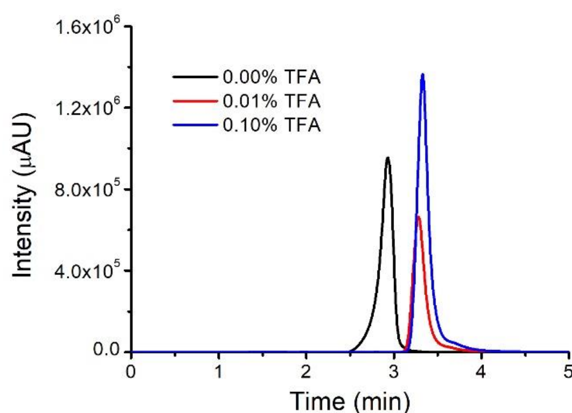

Figure 18. Study of ketoprofen (100 ppm) separation using 60% acetonitrile and 40% water as mobile phase composition with different acidic modifier contents.

As expected, the more acid is the mobile phase, less ionized and less polar are the analyte molecules, increasing the retention times. The peak shape was also improved with the increase of TFA. These results show that 0.01 and 0.1%TFA led to very similar retention times, however, the 0.1%TFA content presents higher intensity value, what can result in better detection limits. Knowing that the pH value of the mobile phase influences the separation and that the used chromatographic column (C18) has a safety working pH range from 2 to 8, the pH values for several 60ACN:40W mixtures were evaluated as a function of the amount of TFA. These results are presented in Table 12.

Table 12. Effect of TFA content on the pH value using 60ACN:40W mobile phase composition.

% TFA	**0**	**0.001**	**0.005**	**0.01**	**0.02**	**0.03**	**0.04**	**0.05**	**0.10**
pH	8.19	3.46	2.57	**2.50**	2.32	2.19	2.09	2.02	**1.66**

As referred above, the 0.1%TFA content presents better results if compared with the 0.01%TFA. Nevertheless, the column lifetime will be affected considering that 0.1%TFA represents a pH value of 1.66 that is below the minimum value recommended by the manufacturer.

4.1.3 Study of the elution order and selection of the individual wavelengths

After each individual NSAID analysis, one virtual channel was used to generate the chromatogram at the wavelength with the highest absorbance. One wavelength was obtained for each one of the individual compounds and was selected for monitoring the detector signal for that compound. In Appendix A are presented the absorption spectrum for each analyte.

The retention time of the compounds using the 60ACN:40W:0.01%TFA mobile phase with a flow-rate of 1 mL/min and the optimum selected wavelengths, are described in Table 13.

Table 13. Maximum absorbance wavelength and retention time for each compound of interest.

Micropollutant	UV-DAD Wavelength (nm)	Retention time (min)
Azithromycin	198	1.243
Acetaminophen	245	1.783
Caffeine	272	1.967
Acetylsalicylic acid	224	2.210
Sulfamethoxazole	268	2.210
Carbamazepine	284	2.820
Ketoprofen	254	3.310
Naproxen	224	3.403
Diclofenac	275	4.913
Ibuprofen	219	5.237

Usually, the criteria employed to recognize one compound in a chromatogram is its retention time. However, this parameter depends on the HPLC operating conditions and other compounds can present the same retention time, resulting in wrong identification and quantification. Using a DAD detector, it is possible to analyze the sample in the whole spectrum, allowing the introduction of the wavelength as a new parameter for the identification and/or quantification of the compounds [81]. This way, it is possible to study a compound at the wavelength where it responds the most. For this reason, knowing the maximum absorbance wavelength is very important to identify the substances in a mixture.

4.1.4 Standard mixture analysis

In this step, a mixture containing all the ten compounds was used with individual concentrations of 1 ppm. This mixture was analyzed, and the obtained chromatograms are presented in Figure 19.

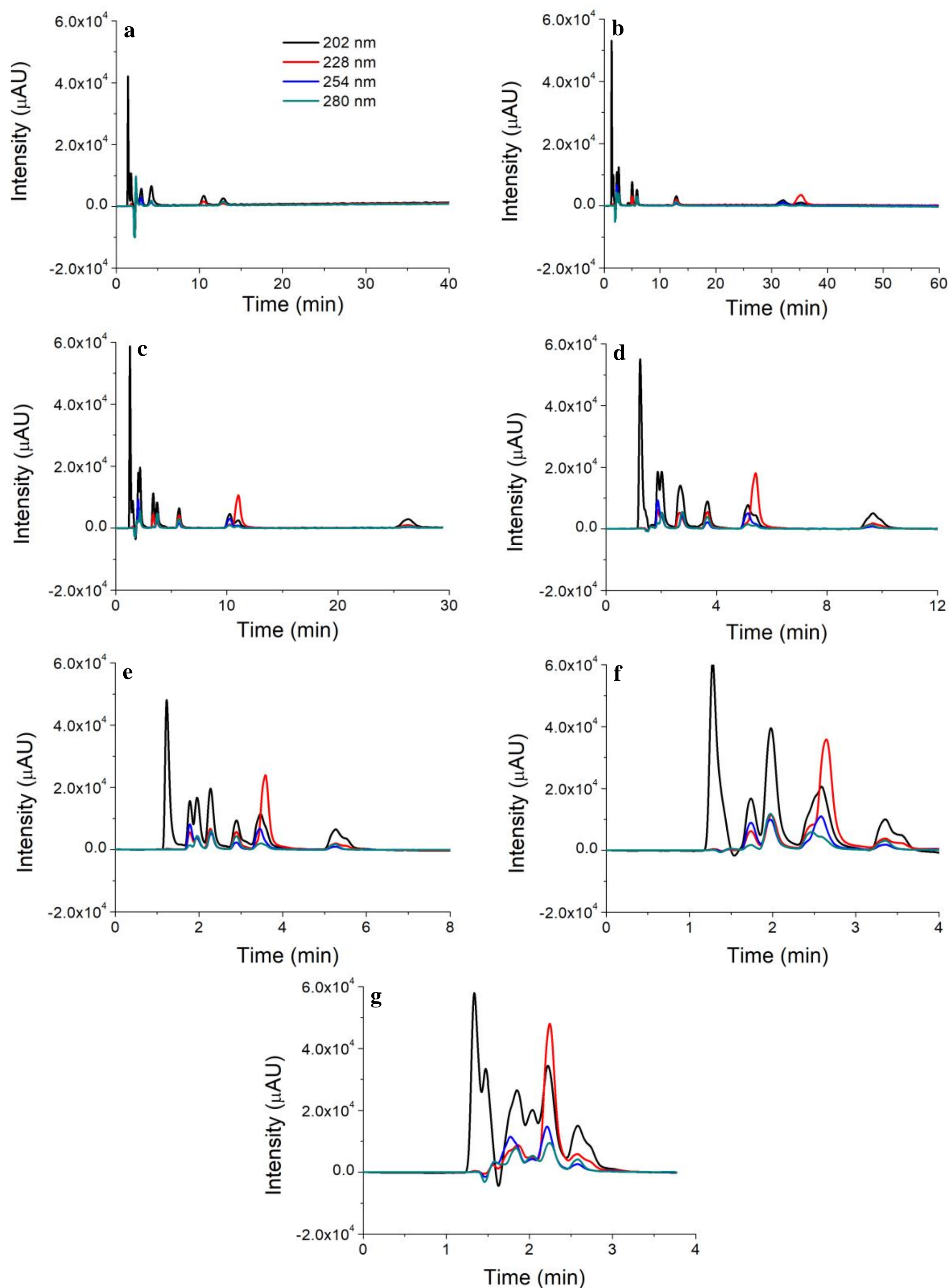

Figure 19. HPLC-DAD chromatograms for different acetonitrile:water volume proportions. **19a:** 20ACN80W; **19b:** 30ACN70W; **19c:** 40ACN60W; **19d:** 50ACN50W; **19e:** 60ACN40W; **19f:** 70ACN30W and **19g:** 80ACN20W.

The results showed that increasing the water content in the composition, increases the retention time and the dispersion of the peaks. That means that, in order to separate all the peaks, it would be necessary to use a mobile phase with a higher percentage of water, nevertheless this would result in lower peaks, prejudicing the limit of detection of the method. Based on these results and on the considerations about the pH and pKa explained on topics 3.2.1.2 and 4.1.2, it was decided that it would be better to develop two different methods. The first one, that will be presented in this work, will be developed for compounds with low pKa values, between 3.5 to 4.9, (acidic compounds, such as the anti-inflammatory drugs) and the second one for compounds presenting higher pKa values (basic compounds).

For this reason, some mobile phase gradients were set up and tested in order to try to optimize the baseline separation, improving the quality of the method for the acid compounds, the NSAID. The Figure 20 presents the results of this study.

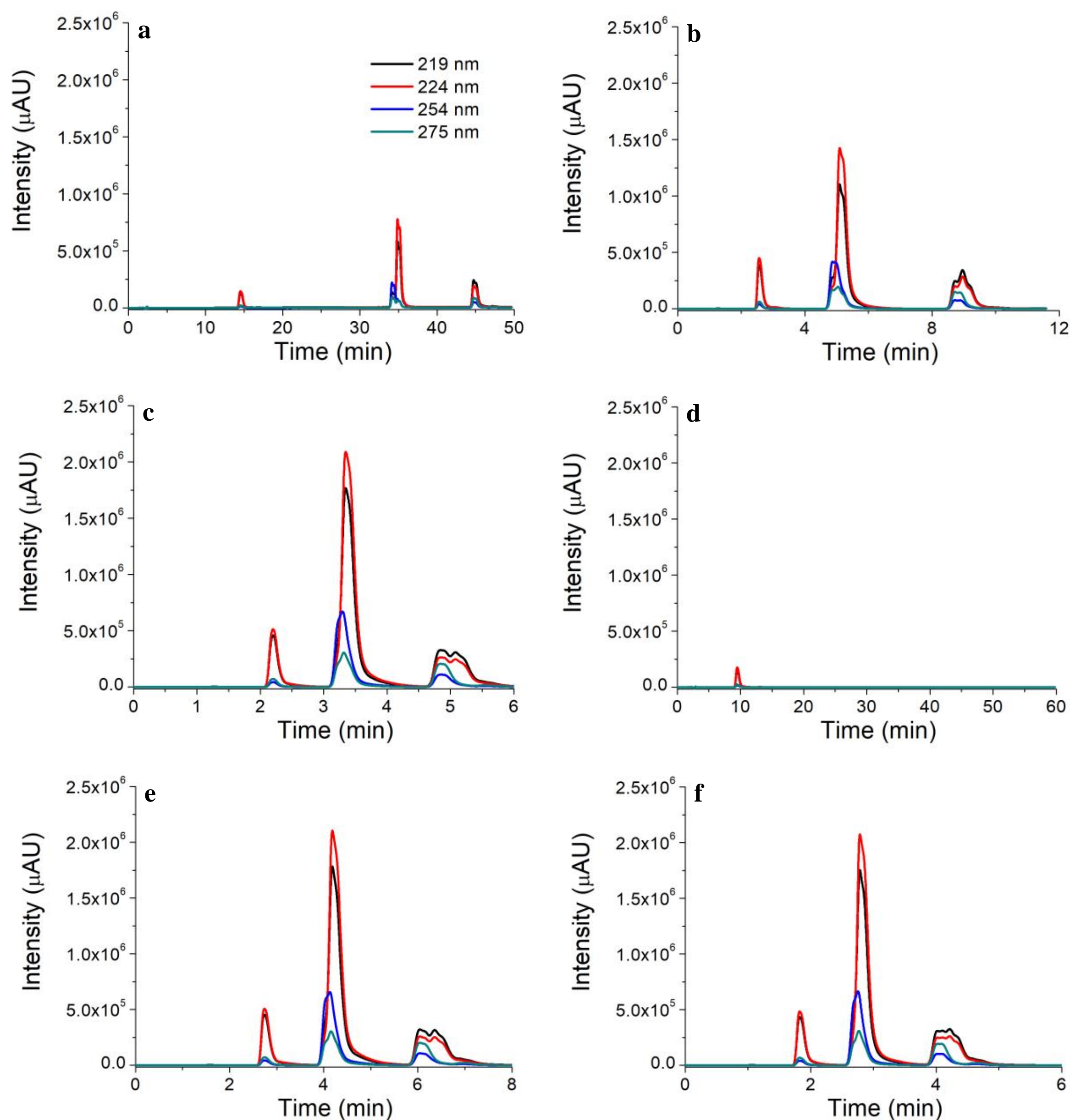

Figure 20. HPLC-DAD chromatograms for NSAIDs analysis with the 6 gradients previously referred in Table 5, page 39.
20a: Composition 1; **20b:** Composition 2; **20c:** Composition 3; **20d:** Composition 4; **20e:** Composition 5 and **20f:** Composition 6.

Composition 6 was chosen to be used in the final method despite of not presenting a baseline resolution for all the 5 selected compounds. However, each compound responds in a different wavelength from the others, making possible an individual identification and quantification. The individual retention times obtained for each NSAID with 60ACN:40W: 0.01TFA mobile phase at 1.2 mL/min are presented in Table 14.

Table 14. Retention times for each analyte with composition 6.

NSAID	Retention time (min)
Acetylsalicylic acid	2.1
Ketoprofen	2.7
Naproxen	2.8
Diclofenac	4.0
Ibuprofen	4.0

4.1.5 Linearity parameters of the HPLC analysis

In order to calculate the linearity parameters and the linear equation for each one of the five NSAID, only the lowest 6 to 8 concentrations levels were considered, since these compounds are considered to be found in very low quantities. The linearity results are presented in Table 15 and all the experimental and calculated data for this linearity study is presented in Appendix B.

Table 15. Calibration curve and linearity parameters obtained for the selected compounds.

Compound	Linear range (ppb)	n	Slope	Intercept	R^2	LOD (ppb)	LOQ (ppb)
Acetylsalicylic acid	40 - 5000	8	21.71 ± 0.29	359.80 ± 536	0.9989	74.00	246.67
Diclofenac	100 - 5000	6	34.80 ± 0.33	-2324.30 ± 696.09	0.9996	60.01	200.04
Ibuprofen	30 - 500	6	79.09 ± 0.48	283.85 ±112	0.9999	4.24	14.12
Ketoprofen	5 - 50	6	70.45 ± 1.73	-39.15 ± 52.57	0.9976	2.24	7.46
Naproxen	5 - 50	6	197.912 ± 11.07	-241.762 ± 336.05	0.9876	5.09	16.98

Comparing these results with other already published studies, referred in Table 4, the achieved limits of detection and quantification were a little higher than those from the compared studies. Considering only similar methodologies (SPE/HPLC-DAD) it can be observed that for acetylsalicylic acid, the obtained LOQ value (246.67 ppb) is higher than the presented in reference [79] (0.548 ppb). The obtained LOQ value for diclofenac (200.04 ppb) is similar to the referred in reference 67 (170 ppb), but considerably higher than that the referred in reference [71] (0.4 ppb). For ibuprofen the obtained LOQ value (14.12 ppb) is between the value referred in reference [70] (170 ppb) and the value referred in reference

[71] (0.1 ppb). For ketoprofen the obtained LOQ value (7.46 ppb) is higher than the value referred in reference [77] (0.26 ppb). For naproxen the obtained LOQ value (16.98 ppb) is higher than the values referred in reference [71] (0.4 ppb).

Different reasons can explain these differences in the LOQ and LOD values obtained in this work from others already published. The most important hypothesis can be related to the fact that after a carefully reading of other works, it is possible to find different methods for the LOD and LOQ calculation. Another one could be related to the pH value of the mobile phase. Results presented in Figure 18 show that the pH values and the size of peak are related. The chromatogram for the analysis with lower pH showed higher peaks, what could consequently improve the limits of detection and quantification. Nevertheless, the employed chromatographic column was not designed to work in pH values under 2, so the 0.01% TFA proportion was the best one for the available column.

Concerning to the precision parameters, Table 16 shows the results for coefficients of variation (CV) for the analyses of the concentrations selected (Table 6) to evaluate the repeatability and the intermediate precision.

Table 16. Coefficients of variation for the selected concentrations for each compound for repeatability and intermediate precision.

Compound	Repeatability (%)			Intermediate precision (%)		
	C1	C2	C3	C1	C2	C3
Acetylsalicylic acid	1.96	2.29	2.32	4.77	9.27	2.37
Diclofenac	2.02	0.94	1.84	1.91	1.54	0.43
Ibuprofen	1.28	1.64	0.25	0.97	2.34	4.91
Ketoprofen	2.07	3.26	2.28	4.92	3.93	2.39
Naproxen	3.45	4.76	1.55	6.58	7.65	2.69

The repeatability and intermediate precision results for the developed SPE/HPLC-DAD methodology ranged from 0.25 to 4.76% and 0.43 to 9.27%, respectively. As expected, the intermediate precision present higher mean values than the repeatability since some of the operating conditions may change, such as small variations in the chromatographic column pressure, mobile phase proportions, sample temperature and others.

However, the obtained results were acceptable given that other published works use to consider that precise analytical methodologies present 20% as a maximum CV value in repeatability and intermediate precision studies [67, 70, 78].

It is possible to compare these results with the precision studies performed for other similar methodologies (SPE/HPLC-DAD). For acetylsalicylic acid, the calculated results for repeatability were better than the reported values, 22.3 and 3.8%, and the intermediate precision is between the 20.3 and 3.8% values [24, 88]. Regarding to diclofenac, the achieved precision parameters were slightly better than those from other works, 2.9, 2.6 and 1.9% for repeatability and 3.5, 4.4 and 1.25% for intermediate precision [24, 67, 88]. Comparing ibuprofen results, the obtained values were better than the ones from literature, 3.5, 16.7 and 3.9% for repeatability and 9.7, 8.8 and 4.4% for intermediate precision [24, 67, 88]. The ketoprofen results for repeatability were similar to the 2.3% referred in reference [91] and much better than the 11.3% for intermediate precision from the same work. Both repeatability and intermediate precision results for naproxen in reference [91] were equal to 14.3%, a higher value than the calculated ones in this work for the same analyte.

4.1.6 Solid phase extraction operating conditions

Firstly, the individual standard solutions were extracted, and then, the mixture with the five NSAIDs was also extracted. This practice was implemented in order to compare the recovery values for the compounds individually and in the mixture, consequently, it would be possible to verify if the adsorbent would present more selectivity for one individual compound when in the presence of the others. Nevertheless, the recovery value parameter was calculated using the mixture with the five compounds, since the real samples are mixtures as well.

4.1.6.1 Individual extractions

During the individual extractions, it was noticed that the loading and washing wastes did not carry the analyte. This means that the analyte was kept in the adsorbent and the most important steps are the elution and loading. For this reason, the flow-rates for these two stages should be lower. Table 17 shows the flow-rates for each step in the individual extractions and their recovery values. These experimental recovery values were calculated using equation (15) and (16).

Table 17. Experimental recovery and flow-rate values for the individual NSAIDs extractions.

		Ketoprofen	Naproxen	Diclofenac	Ibuprofen	Acetylsalicylic acid
Flow-rate (mL/min)	Conditioning 1	0.29	0.40	0.20	0.34	0.27
	Conditioning 2	0.41	0.32	0.51	0.54	0.47
	Loading	0.52	0.50	0.53	0.37	0.46
	Washing	1.00	1.23	1.46	0.79	1.02
	Elution	0.30	0.25	0.27	0.16	0.15
Recovery (% c/c)		111.38	113.44	111.78	106.46	128.63
Recovery (% m/m)		89.10	90.75	89.42	85.17	102.90

After these studies, it was decided that the flow-rates for the loading and elution stages should be around 0.5 and 0.2 mL/min, respectively, to ensure higher recovery results with a reasonable flow-rate that could be manually controlled.

4.1.6.2 SPE extractions of NSAID mixture

The extraction procedure was performed in triplicate with the conditions that were selected based on the individual extraction studies. Table 18 presents the experimental flow-rates and recoveries, as well as the standard deviation and coefficient of variation.

Table 18. SPE extraction recoveries (based in concentration and mass) and flow-rate values for NSAIDs mixture extractions.

		SPE 1	**SPE 2**	**SPE 3**	**Average**	**SD**	**CV (%)**
Flow-rate (mL/min)	Conditioning 1	0.25	0.20	0.20	0.22	0.02	10.88
	Conditioning 2	0.54	0.55	0.45	0.51	0.04	8.76
	Loading	0.50	0.54	0.56	0.53	0.02	4.68
	Washing	0.84	0.79	0.97	0.87	0.08	8.75
	Elution	0.17	0.19	0.18	0.18	0.01	4.54
Recovery (% c/c)	Ketoprofen	122.47	109.31	123.08	118.29	6.35	5.37
	Naproxen	122.95	110.37	129.43	120.92	7.91	6.54
	Diclofenac	110.23	114.59	122.32	115.71	5.00	4.32
	Ibuprofen	117.47	122.06	132.83	124.12	6.44	5.19
	Acetylsalicylic acid	118.43	102.55	121.94	114.31	8.44	7.38
Recovery (% m/m)	Ketoprofen	97.98	87.45	98.46	94.63	5.08	5.37
	Naproxen	98.36	88.30	103.55	96.74	6.33	6.54
	Diclofenac	88.18	91.70	97.85	92.58	4.00	4.32
	Ibuprofen	93.98	97.64	106.26	99.29	5.15	5.18
	Acetylsalicylic acid	94.74	82.04	97.55	91.44	6.75	7.38

Since the available SPE equipment does not allow the direct control of the flow-rate, it was not simple to obtain the exact value stipulated for the method. The flow-rate control was done by monitoring of emptying time of the cartridge. This is the reason why the average, the standard deviation and the variation coefficient of the measured flow-rates were also calculated. Despite this, the flow-rates for the most important steps, loading and elution, were satisfactory, as it can be observed by their coefficients of variations below 5%.

In addition, it was concluded that the selected adsorbent, Chromabond HLB, was appropriated for these compounds, because it was not shown a major interaction for one NSAID in relation with the others. It can be also observed that the mass basis recoveries range between 91.44% for acetylsalicylic acid and 99.29% for ibuprofen. These values are in agreement with the higher recovery values referred in literature. Some published studies that analyzed the same class of pharmaceutical drugs, using the Chromabond HLB adsorbent or similar products of other brands but with the same solid adsorbent, such as Oasis HLB or Strata X, refer most of the recovery values above 80% [75, 77, 88, 89].

4.2 Implementation of the developed methodology

SPE/HPLC-DAD analysis of a blank sample

During the HPLC studies for mobile phase selection, it was noticed that between 1 and 2 minutes, there is a normal change in the baseline of the chromatogram when the injected samples were prepared in methanol. This variation in the signal is related to the different compositions between solvent used to eluate the samples and the solvents, acetonitrile and water, used in mobile phase. This variation in the baseline is almost imperceptible when the samples have high concentrations, however it can hamper the analysis for very small concentrations. For this reason, it was decided to do a blank analysis with only methanol and analyze it in the HPLC system for all the wavelengths selected for the NSAID quantification. In Figure 21 are presented the chromatograms obtained for methanol with the four wavelengths.

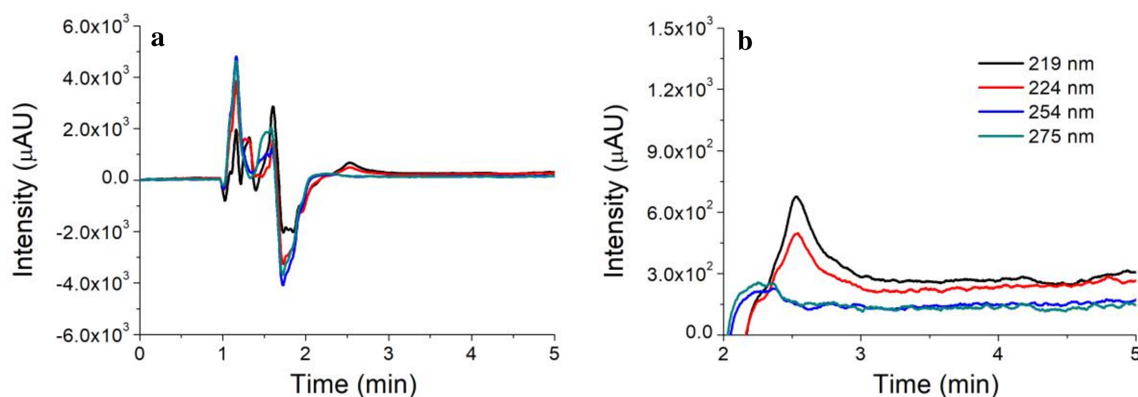

Figure 21. 21a: HPLC-DAD chromatograms obtained for blank analysis (pure methanol) using the four wavelengths (219, 224, 254 and 275 nm) and **21b:** zoom of the most important zone.

The presented chromatogram shows some peaks between 1 and 1.5 minutes related with some impurities present in acetonitrile, water and trifluoracetic acid. Between 1.5 and 2 minutes, approximately, it can be observed a negative peak, for all the four wavelengths, due to methanol, and a peak at 2.5 minutes for both 219 nm and 224 nm wavelengths. Acetylsalicylic acid is the only compound that could be affected (2.1 min and 224 nm), however, due the very small intensity of this signal this peak can be neglected.

SPE/HPLC-DAD analysis of the six samples

As referred before, six samples were collected from different sources and locations in the Bragança region. The obtained chromatograms using the developed methodology (HPLC-DAD after SPE) are presented in Figures 20 to 26.

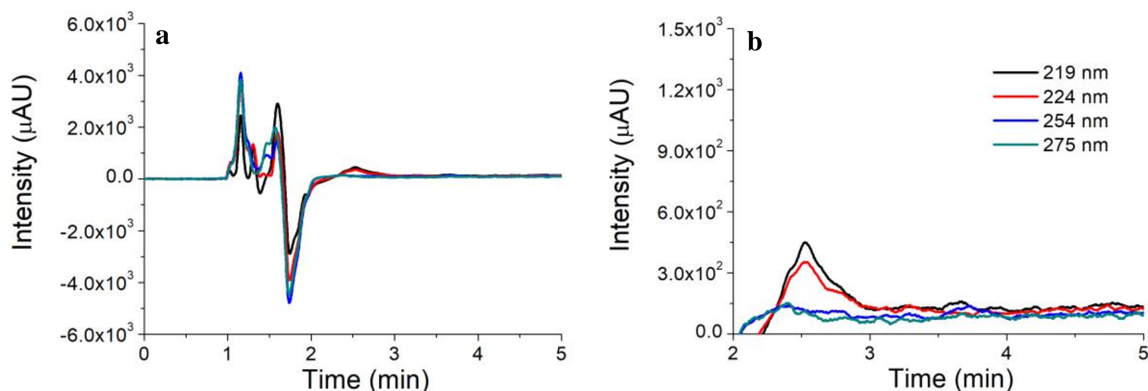

Figure 22. 22a: HPLC-DAD chromatograms obtained for S1 using the four wavelengths (219, 224, 254 and 275 nm) and **22b:** zoom for the most important zone.

S1 is a sample of tap water collected from the municipal water distribution system. The obtained HPLC-DAD chromatogram, presented in Figure 22, shows at 2.526 min the methanol peak and at 3.668 min a very small peak, although, this retention time is not related with none of the five studied NSAID.

S2 is a sample of tap water collected from the groundwater that supplies the Polytechnic Institute of Bragança (IPB). The obtained HPLC-DAD chromatograms are presented in Figure 23.

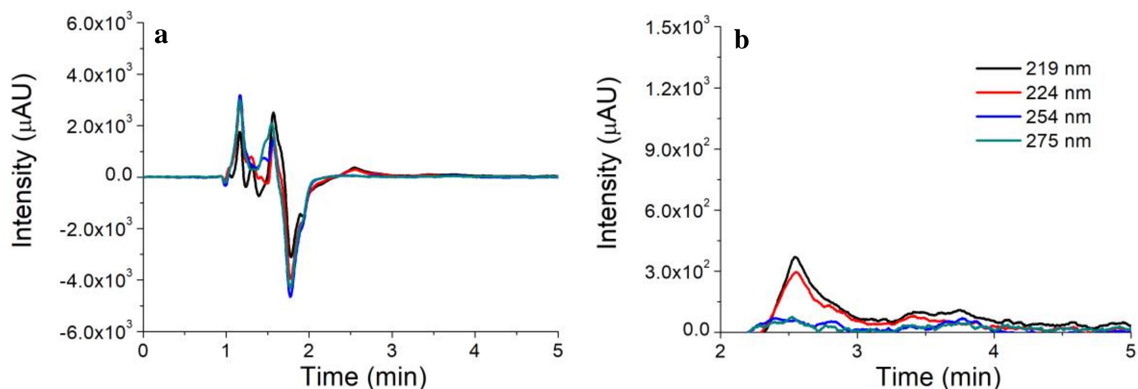

Figure 23. 23a: HPLC-DAD chromatograms obtained for S2 using the four wavelengths (219, 224, 254 and 275 nm) and **23b:** zoom for the most important zone.

The four chromatograms for S2, shows for the 254 nm a small signal, exactly in the same retention time of ketoprofen.

S3 is a sample of surface water collected from River A, location 1, in the section that crosses part of IPB campus. The obtained HPLC-DAD chromatograms are presented in Figure 24.

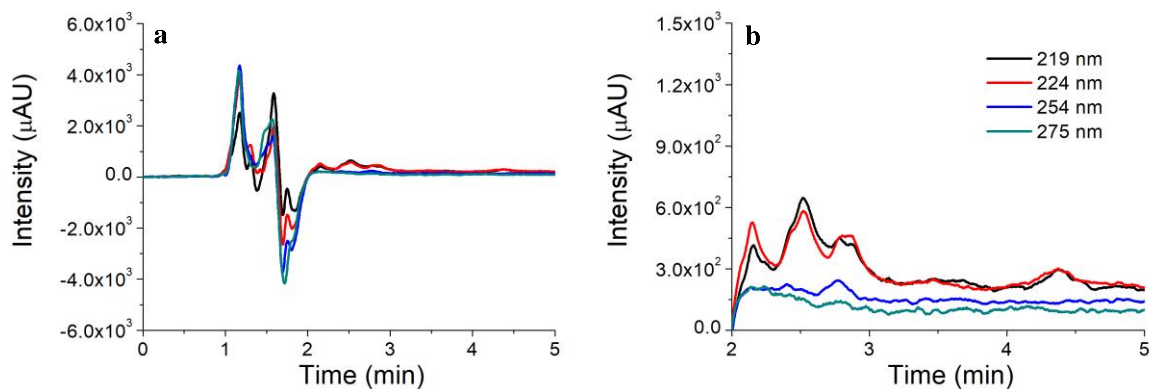

Figure 24. 24a: HPLC-DAD chromatograms obtained for S3 using the four wavelengths (219, 224, 254 and 275 nm) and **24b:** zoom for the most important zone.

The analyzes of S3 presented in the four obtained chromatograms shows one peak at 2.519 min due to the methanol, and also presents two new signals at 2.140 and 2.830 min for 224 nm wavelength, corresponding to acetylsalicylic acid and naproxen, respectively. Besides, in 254 nm curve, there is a ketoprofen signal at 2.741 min. Another compound is clearly noticeable at 4.385 min using the two lowest wavelengths of 219 and 224 nm but is not related with any of the five studied NSAIDs.

S4 is a sample of surface water collected from River A, location 2, at the “Polis” passage. The obtained HPLC-DAD chromatograms are presented in Figure 25.

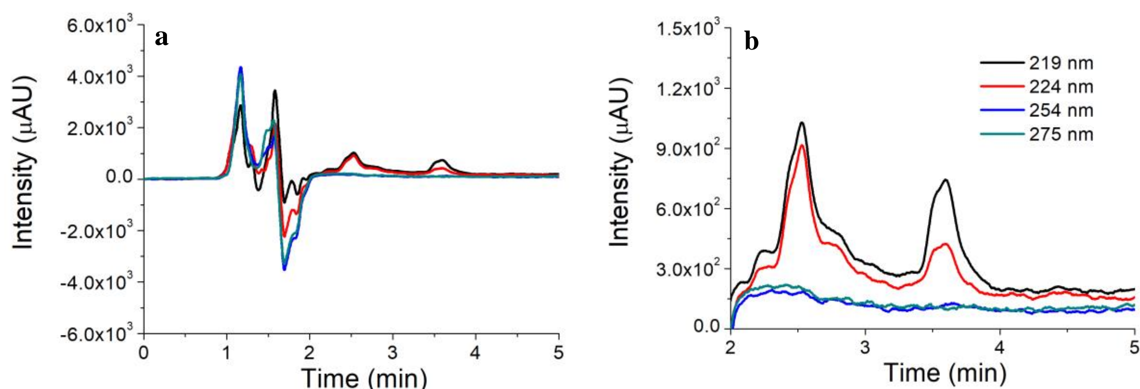

Figure 25. 25a: HPLC-DAD chromatograms obtained for S4 using the four wavelengths (219, 224, 254 and 275 nm) and **25b:** zoom for the most important zone.

The S4 obtained chromatograms reveal a new compound, responding using 219 and 224 nm with a retention time of 3.589 min, so it cannot be identified as one of the five NSAID compounds of this study.

S5 is a samples of surface water collected from River B. The obtained HPLC-DAD chromatograms are presented in Figure 26.

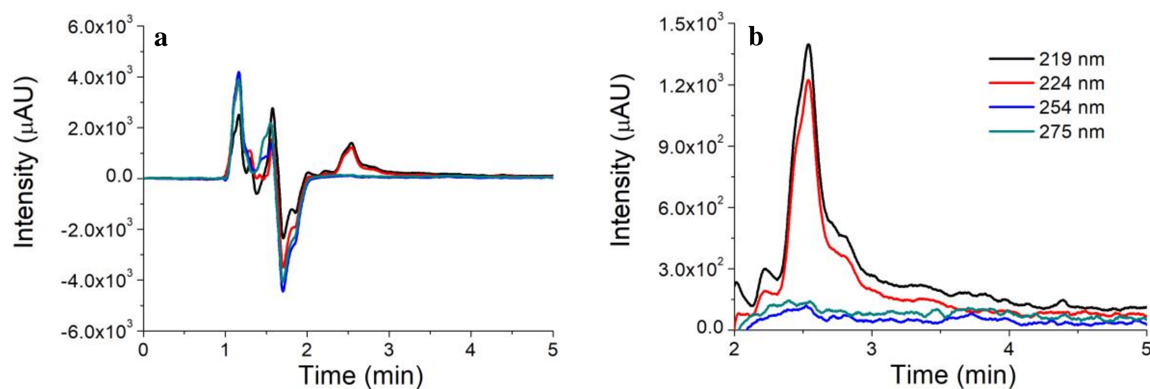

Figure 26. 26a: HPLC-DAD chromatograms obtained for S5 using the four wavelengths (219, 224, 254 and 275 nm) and **26b:** zoom for the most important zone.

The analysis of the results obtained for S5 shows at 2.010 and 2.219 min two small compounds that cannot be identified as the studied five NSAID.

The last collected sample S6 is a pool water sample from Bragança municipal sports facilities, and the obtained HPLC-DAD chromatogram can be seen in Figure 27.

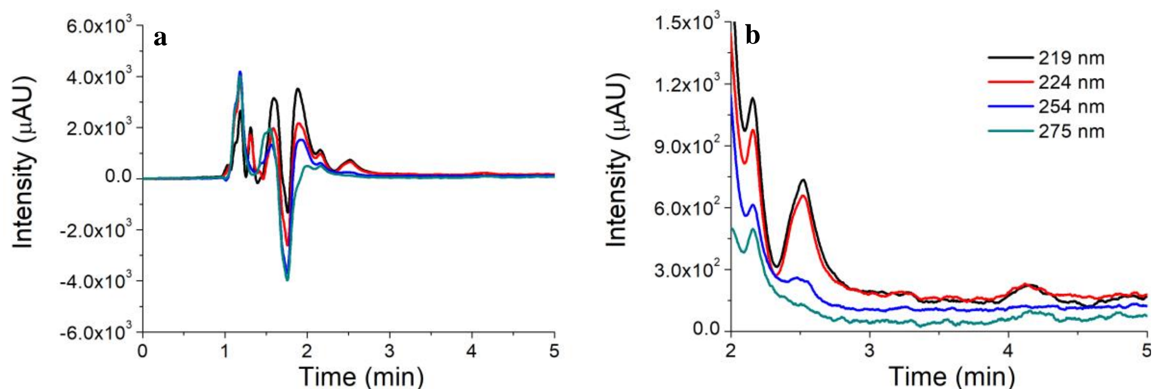

Figure 27. 27a: HPLC-DAD chromatograms obtained for S6 using the four wavelengths (219, 224, 254 and 275 nm) and **27b:** zoom for the most important zone.

The pool water S6 sample analysis, shown in Figure 27, presents more peaks than the other samples. But most of them are in the beginning of the graph and they do not interfere in the analytes' retention times. It can be observed a small peak, at 2.155 minutes for 224 nm, which identifies acetylsalicylic acid.

A summary of the obtained average concentration, resulting from the triplicate analysis of the contaminated samples is presented in Table 19. The presented concentrations were obtained with the HPLC-DAD linear regression parameters and SPE recovery obtained for each compound.

Table 19. Calculated concentration based on linear regression and recovery values for the five NSAID and for the contaminated samples.

Sample	Compound	Concentration (ppb)
S2	Ketoprofen	<LOQ
S3	Acetylsalicylic acid	<LOD
	Ketoprofen	11.230 ± 0.767
	Naproxen	<LOQ
S6	Acetylsalicylic acid	<LOD

From the five peaks that were detected and integrated in the samples, only one can be quantified since it is the only concentration above the limit of quantification. Ketoprofen found in S3 corresponds to a concentration of 11.230 ppb. Despite not being possible to quantify the other detected NSAID compounds, it is allowed to say that S2 and S3 were contaminated with ketoprofen and naproxen, respectively, since the concentrations for these NSAID were above the limit of detection and below the limit of quantification.

Other published works reported the water contamination by ketoprofen. It is known that the average concentration for a same analyte may variate in different matrices and regions due to the amount of the consumption of this drug and the contamination sources. The works that analyzed and found ketoprofen in surface water samples presented results ranging from 0.04 to 2.00 ppb, with an average concentration of 0.55 ppb, which is lower than the calculated value from this work [74, 76, 89, 90]. The authors who quantified ketoprofen in wastewater treatment plants divided the samples into influent and effluent samples. The obtained concentration in influent samples ranged from 1.7 to 260 ppb and presented an average value of 57.38 ppb. Regarding to the wastewater treatment plant's effluent samples, the values ranged from 1.2 to 160 ppb, with an average concentration of 37.74 ppm. Both of these values were higher than the found for ketoprofen in surface water in this work.

Besides that, it is noticeable that S1 and S4 showed the presence of compounds at 3.668 and 3.589 min (for 219 nm) and S3 at 4.385 min (for 219 nm and 224 nm), which were not identified as the compounds studied in this work.

5. Conclusions

The aims of this work were to develop and to validate an experimental methodology to identify and quantify non-steroidal anti-inflammatory drugs. Five compounds belonging to this class of pharmaceutical drugs (acetylsalicylic acid, diclofenac, ibuprofen, ketoprofen and naproxen) were selected to develop and validate the experimental methodology in real samples from different hydric media of the Bragança region.

From a careful review of the state of the art in this field, several extraction and quantitative analysis methods were found adequate. Taking into account the instrumental equipment available inside the group and the costs associated with the use of other more sophisticated technologies, it was decided to develop the analytical methodology using solid phase extraction followed by high performance liquid chromatography with photo diode array detector. The development of the analytical methodology involved the selection of an appropriate adsorbent for the solid phase extraction and the optimization of the flow-rate for each one of the four steps needed for SPE. Besides that, it was necessary to optimize some of the operating conditions of HPLC-DAD analysis, such as the mobile phase composition, pH, and flow-rate, as well as the DAD wavelength to be set for each analyte detection and quantification.

The initial set of compounds comprised ten pharmaceutical drugs from different classes. After several preliminary experimental measurements, it was decided that would be better to develop two different methods: one for the low pKa compounds, which cover the non-steroidal anti-inflammatory drugs, and another method for the compounds with higher pKa. This work focused on the acid compounds, while the other method is being developed by other member of this research group.

The developed SPE/HPLC-DAD experimental methodology is based in a first SPE extraction using the Chromabond® HLB adsorbent with different solvents and flow-rates from 0.2 to 0.9 mL/min for each one of the four SPE steps. The SPE extraction applied for the mixture of the five studied NSAID led to mass recovery values above 91%, with coefficients of variation from 4% to 7%, values that are similar or even higher than some others found in literature.

The second stage of the methodology is based in HPLC-DAD analysis of the eluted samples, collected in the last step of SPE. The HPLC method was developed under reversed

phase mode, using a non-polar C18 stationary phase and a polar acetonitrile:water:trifluoracetic acid (60:40:0.01 (%v:v:v)) mobile phase composition.

Taking advantage of diode array detector capabilities, four wavelengths were set in real time analysis in order to promote the selectivity of the five NSAID compounds since each one of them presents better detector response in different wavelengths. The selected wavelengths were 219 nm for ibuprofen, 224 nm for acetylsalicylic acid and naproxen, 254 nm for ketoprofen and 275 nm for diclofenac.

The statistical parameters for the calibration curves were obtained for a confidence level of 95%. The linearity of the five calibration curves was confirmed since the obtained determination coefficients values are in the range between 0.9876 for naproxen and 0.9999 for ibuprofen. The obtained limits of quantification are in the range between 7.5 ppb for ketoprofen and 246.7 ppb for acetylsalicylic acid. The preliminary HPLC measurements with ketoprofen standard solutions using different pH values showed that lower pH values increase chromatographic signals. This fact can suggest that the use of a chromatographic column that allows to work with lower pH values could improve the results obtained for the limits of detection and quantification.

After the development of SPE/HPLC-DAD methodology, the method was implemented analysing six real aqueous samples of the Bragança region. The samples were collected in different locations, including surface water, tap water, groundwater and pool water. From the six samples analysed, three of them were contaminated with one or more NSAID from the set of 5 compounds studied. Nevertheless, only ketoprofen was identified and quantified in S3, collected from the surface water of river A, inside IPB campus. The measured concentration of ketoprofen was 11.230 ± 0.767 ppb, a high value when compared with values referred in other published works.

S3 was also contaminated with naproxen, but it was not possible to quantify this compound. S2, collected from the groundwater that supplies the IPB campus, was also contaminated with ketoprofen.

As suggestions for future works, it would be interesting to improve this experimental methodology using a different HPLC adsorbent that can tolerate more extreme pH values, in order to verify if the LOD and LOQ values would be enhanced. Another option is to use a mobile phase with pure methanol, since this solvent is employed for the SPE elution step

and therefore the baseline resolution could be improved for the first 3 minutes of the HPLC analysis.

Besides that, other extraction procedures can be evaluated, changing the SPE adsorbent, trying different solvents or using solid phase micro extraction (SPME). It also could be studied the use of more sophisticated analytical methods such as the HPLC coupled with mass spectrometry (LC-MS) or with tandem mass spectrometry (LC-MS/MS). It can be also considered other instrumental methods such UV-Vis, Gas chromatography coupled with mass spectrometry, such as GC-MS or GS-MS/MS.

The scope of the analysed compounds could be enlarged to other classes of pharmaceutical drugs or even other types of emerging micropollutants.

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APPENDICES

Appendix A: Absorption spectra

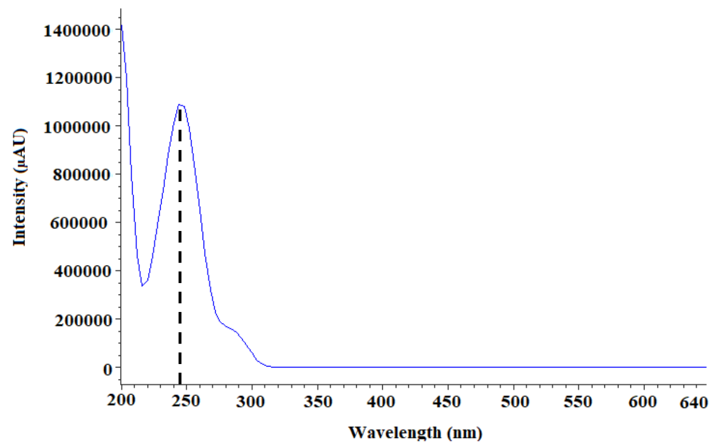

Figure A1. Acetaminophen absorbance spectrum.

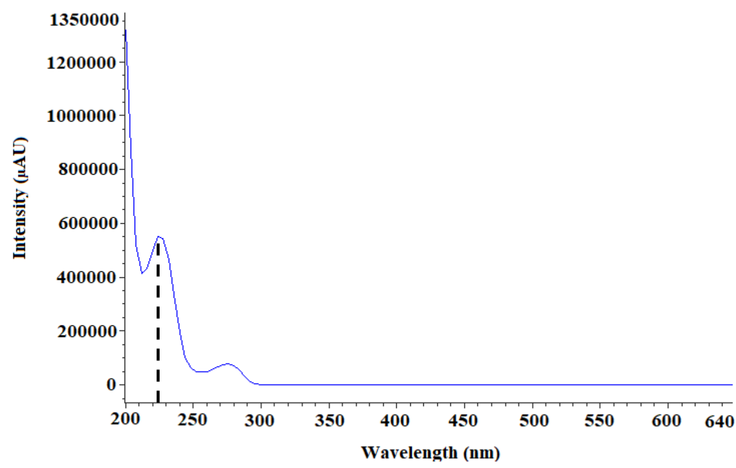

Figure A2. Acetylsalicylic acid absorbance spectrum.

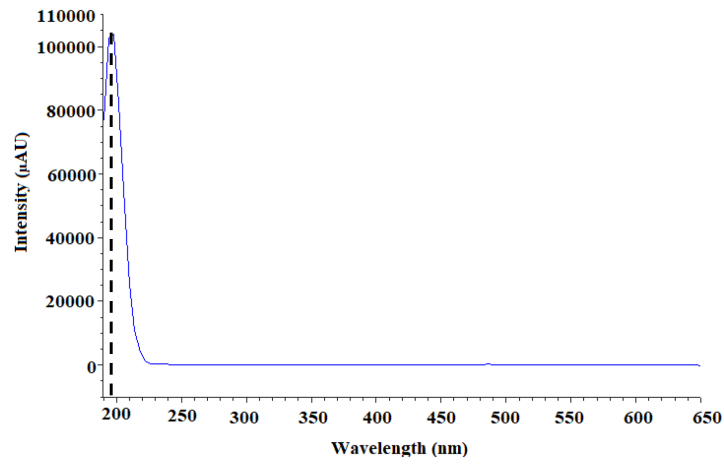

Figure A3. Azithromycin absorbance spectrum.

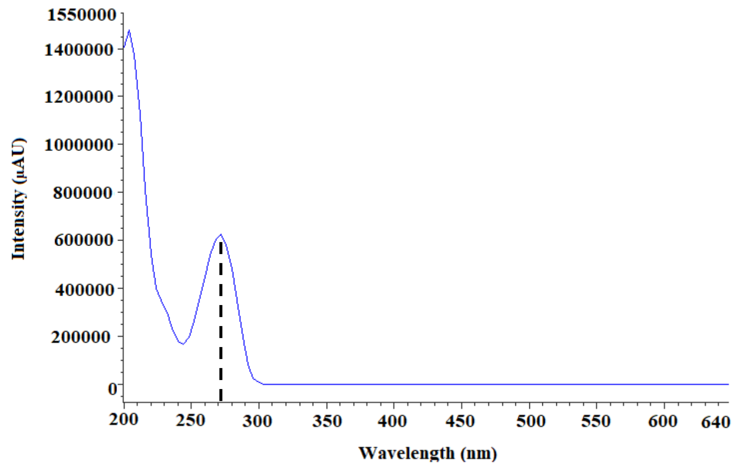

Figure A4. Caffeine absorbance spectrum.

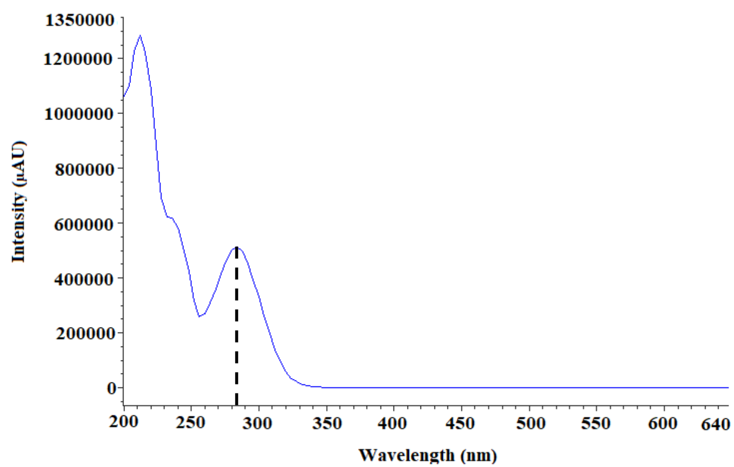

Figure A5. Carbamazepine absorbance spectrum.

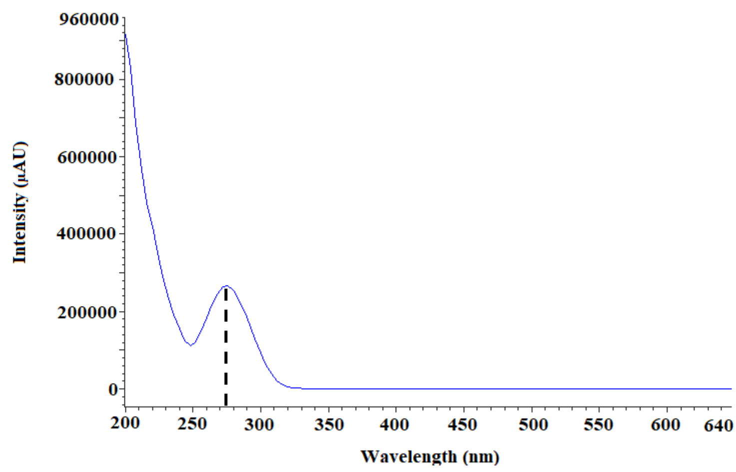

Figure A6. Diclofenac absorbance spectrum.

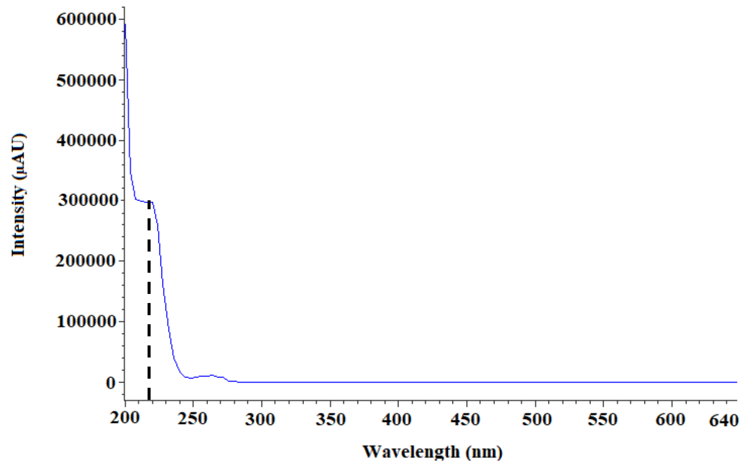

Figure A7. Ibuprofen absorbance spectrum.

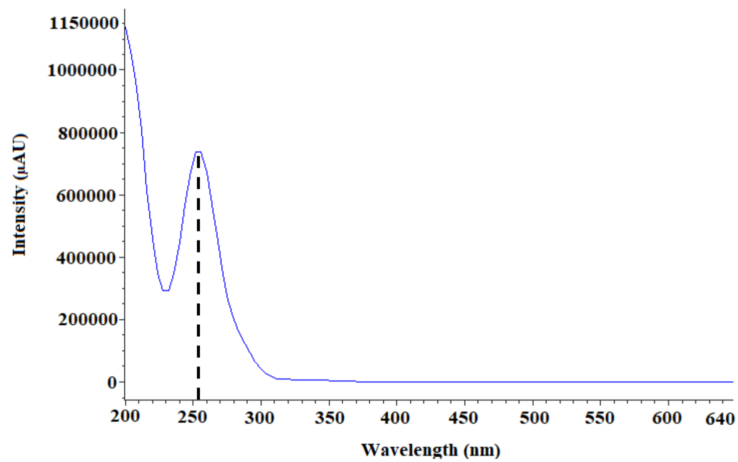

Figure A8. Ketoprofen absorbance spectrum.

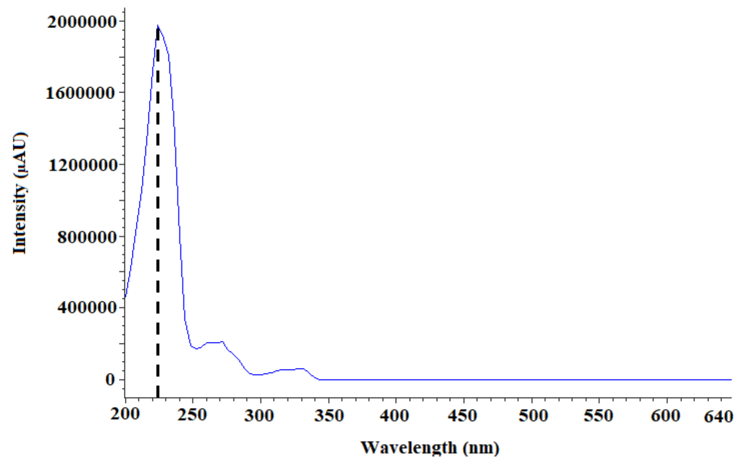

Figure A9. Naproxen absorbance spectrum.

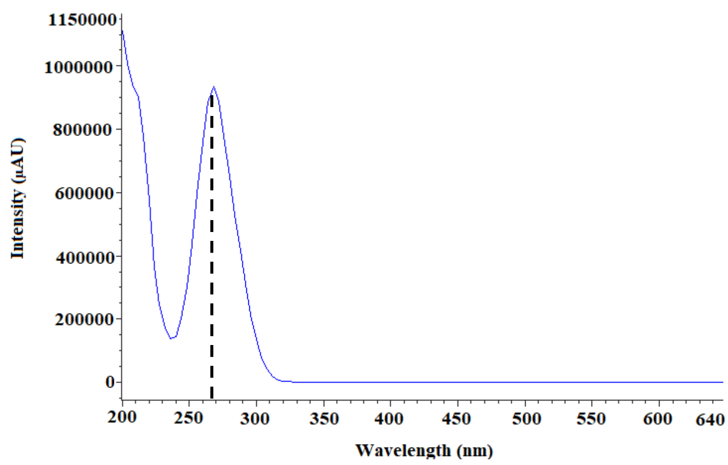

Figure A10. Sulfamethoxazole absorbance spectrum.

Appendix B: Calibration curves data

Ibuprofen data

Table A 1. Ibuprofen data for the calibration curve.

C (ppb)	A1	A2	A3	$\bar{A}$	CV%
100000	9408289	9417965	9703830	9510028	1.4
75000	6760827	6541913	6578263	6627001	1.4
50000	4171459	4259441	4372266	4267722	1.9
25000	2268008	2270698	2270063	2269590	0.1
10000	873997	879961	881060	878339	0.4
5000	448892	446874	450199	448655	0.3
1000	84068	83043	84431	83847	0.7
750	58781	61054	59532	59789	1.6
500	39890	39653	39722	39755	0.3
250	20625	19824	20356	20268	1.6
100	8096	7632	8256	7995	3.3
50	4250	4146	4269	4222	1.3
40	3309	3939	3742	3663	7.2
30	2599	2417	2535	2517	3.0

Table A 2. Calculations for the ibuprofen parameters.

	C (ppb)	$\bar{A}$
Experimental data	30.00	2517
	40.00	3663
	50.00	4222
	100.00	7995
	250.00	20268
	500.00	39755
Average	161.67	13070

	x_i	y_i	x_i-x_{med}	$(x_i$-$x_{med})^2$	y_i-y_{med}	$(y_i$-$y_{med})^2$	$(x_i$-$x_{med})(y_i$-$y_{med})$
	30	2517	-131.67	17336.1111	-10553	1.11E+08	1.39E+06
	40	3663	-121.67	14802.7778	-9407	8.85E+07	1.14E+06
	50	4222	-111.67	12469.4444	-8848	7.83E+07	9.88E+05
	100	7995	-61.67	3802.7778	-5075	2.58E+07	3.13E+05
	250	20268	88.33	7802.7778	7198	5.18E+07	6.36E+05
	500	39755	338.33	114469.4444	26685	7.12E+08	9.03E+06
Total	970	78420	0.00	170683.3333	0	1.07E+09	1.35E+07

	x_i	y_i	x^2	y_i*	y_i-y_i*	$(y_i$-$y_i*)^2$	**Outlier**
	30.00	2517	900.0	2657	140	1.95E+04	0.71
	40.00	3663	1600.0	3447	216	4.66E+04	1.09
	50.00	4222	2500.0	4238	17	2.78E+02	0.08
	100.00	7995	10000.0	8193	198	3.93E+04	1.00
	250.00	20268	62500.0	20056	212	4.50E+04	1.07
	500.00	39755	250000.0	39829	74	5.42E+03	0.37
Total	970	78420	327500	78420	856	1.56E+05	-

Sy/x	198
Sb	0.48
Sa	112

IC	**a**	310.09
	b	1.33

a	284	±	310
b	79	±	1

Acetylsalicylic acid data

Table A 3. Ibuprofen data for the calibration curve.

C (ppb)	A1	A2	A3	$\bar{A}$	CV%
100000	2828605	2910756	2809693	2849685	1.5
75000	1992982	1986558	2001609	1993716	0.3
50000	1222264	1284938	1297224	1268142	2.6
25000	658732	652427	649510	653556	0.6
10000	262511	288325	310654	287163	6.9
5000	114416	109042	104635	109364	3.7
1000	21691	20981	21949	21540	1.9
750	13796	14988	13964	14249	3.7
500	10921	10345	10482	10583	2.3
250	7106	6901	7299	7102	2.3
100	3144	3411	2876	3144	6.9
50	2631	2516	2556	2568	1.9
40	1289	1336	1277	1301	2.0

Table A 4. Calculations for the acetylsalicylic acid parameters.

	C (ppb)	$\bar{A}$
Experimental data	40	1301
	50	2568
	100	3144
	250	7102
	500	10583
	750	14249
	1000	21540
	5000	109364
Average	961.25	21231.375

	x_i	y_i	x_i-x_{med}	$(x_i-x_{med})^2$	y_i-y_{med}	$(y_i-y_{med})^2$	$(x_i-x_{med})(y_i-y_{med})$
	40.00	1301	-921.25	8.49E+05	-19930	3.97E+08	1.84E+07
	50.00	2568	-911.25	8.30E+05	-18663	3.48E+08	1.70E+07
	100.00	3144	-861.25	7.42E+05	-18087	3.27E+08	1.56E+07
	250.00	7102	-711.25	5.06E+05	-14129	2.00E+08	1.00E+07
	500.00	10583	-461.25	2.13E+05	-10648	1.13E+08	4.91E+06
	750.00	14249	-211.25	4.46E+04	-6982	4.88E+07	1.48E+06
	1000.00	21540	38.75	1.50E+03	309	9.52E+04	1.20E+04
	5000.00	109364	4038.75	1.63E+07	88133	7.77E+09	3.56E+08
Total	7690	169851	0	1.95E+07	0	9.20E+09	4.23E+08

	x_i	y_i	x^2	y_i*	y_i-y_i*	$(y_i-y_i^*)^2$	**Outlier**
	40.00	1301	1.60E+03	1228	73	5.28E+03	0.06
	50.00	2568	2.50E+03	1445	1123	1.26E+06	0.87
	100.00	3144	1.00E+04	2531	613	3.76E+05	0.48
	250.00	7102	6.25E+04	5788	1314	1.73E+06	1.02
	500.00	10583	2.50E+05	11216	633	4.01E+05	0.49
	750.00	14249	5.63E+05	16645	2396	5.74E+06	1.86
	1000.00	21540	1.00E+06	22073	533	2.84E+05	0.41
	5000.00	109364	2.50E+07	108925	439	1.93E+05	0.34
Total	7690	169851	2.69E+07	169851	7123	9.98E+06	-

Sy/x	1290
Sb	0.29
Sa	536

IC	**a**	1310.5
	b	0.71

a	360	±	1311
b	22	±	1

Naproxen data

Table A 5. Naproxen data for the calibration curve.

C (ppb)	A1	A2	A3	$\bar{A}$	CV%
100000	26692759	26974104	27476592	27047818	1.2
75000	19787347	19420711	19495736	19567931	0.8
50000	12614911	12839374	13149833	12868039	1.7
25000	6994468	6962072	6977961	6978167	0.2
10000	2720206	2725576	2743295	2729692	0.4
5000	1397153	1402288	1400894	1400112	0.2
1000	258156	261249	261839	260415	0.6
750	174623	193000	180513	182712	4.2
500	124409	123236	124586	124077	0.5
250	61280	59730	59133	60048	1.5
100	19994	19132	20414	19847	2.7
50	9895	10277	10109	10094	1.5
40	6842	6974	7621	7146	4.8
30	5341	5354	5748	5481	3.4
20	4002	4076	4142	4073	1.4
10	1319	1523	1628	1490	8.6
5	977	933	919	943	2.6

Table A 6. Calculations for the naproxen parameters.

	C (ppb)	$\bar{A}$
Experimental data	5.000	943
	10.00	1490
	20.00	4073
	30.00	5481
	40.00	7146
	50.00	10094
Average	25.83	4871.11

	x_i	y_i	x_i-x_{med}	$(x_i-x_{med})^2$	y_i-y_{med}	$(y_i-y_{med})^2$	$(x_i-x_{med})(y_i-y_{med})$
	5.00	943	-20.83	4.34E+02	-3928	1.54E+07	8.18E+04
	10.00	1490	-15.83	2.51E+02	-3381	1.14E+07	5.35E+04
	20.00	4073	-5.83	3.40E+01	-798	6.36E+05	4.65E+03
	30.00	5481	4.17	1.74E+01	610	3.72E+05	2.54E+03
	40.00	7146	14.17	2.01E+02	2275	5.17E+06	3.22E+04
	50.00	10094	24.17	5.84E+02	5223	2.73E+07	1.26E+05
Total	155	29226.66667	0	1.52E+03	0	6.03E+07	3.01E+05

	x_i	y_i	x^2	y_i*	y_i-y_i*	$(y_i-y_i^*)^2$	Outlier
	5.00	943	2.50E+01	748	195	3.81E+04	0.45
	10.00	1490	1.00E+02	1737	247	6.12E+04	0.57
	20.00	4073	4.00E+02	3717	357	1.27E+05	0.83
	30.00	5481	9.00E+02	5696	215	4.61E+04	0.50
	40.00	7146	1.60E+03	7675	529	2.80E+05	1.23
	50.00	10094	2.50E+03	9654	440	1.93E+05	1.02
Total	155	29227	5.53E+03	29227	1983	7.46E+05	-

Sy/x	432
Sb	11.07
Sa	336.05

IC	**a**	933.01
	b	30.75

a	-242	±	933
b	198	±	31

Ketoprofen data

Table A 7. Ketoprofen data for the calibration curve.

C (ppb)	A1	A2	A3	$\bar{A}$	CV%
100000	8475609	8547683	8754666	8592653	1.4
75000	6051785	5946034	5963888	5987236	0.8
50000	3784701	3825845	3916112	3842219	1.4
25000	2063372	2047769	2044084	2051742	0.4
10000	790090	769753	799352	786398	1.6
5000	407470	408140	406871	407494	0.1
1000	75106	74771	75314	75064	0.3
750	52724	53223	51879	52609	1.1
500	34692	34430	34392	34505	0.4
250	18038	17517	17780	17778	1.2
100	6375	6598	6600	6524	1.6
50	3412	3608	3517	3512	2.3
40	2709	2566	2776	2684	3.3
30	2096	2077	2178	2117	2.1
20	1459	1321	1548	1443	6.5
10	696	624	594	638	6.7
5	265	308	299	291	6.4

Table A 8. Calculations for the ketoprofen parameters.

	C (ppb)	$\bar{A}$
Experimental data	5.000	291
	10.00	638
	20.00	1443
	30.00	2117
	40.00	2684
	50.00	3512
Average	25.83	1780.78

	x_i	y_i	x_i-x_{med}	$(x_i$-$x_{med})^2$	y_i-y_{med}	$(y_i$-$y_{med})^2$	$(x_i$-$x_{med})(y_i$-$y_{med})$
	5.00	291	-20.83	4.34E+02	-1490	2.22E+06	3.10E+04
	10.00	638	-15.83	2.51E+02	-1143	1.31E+06	1.81E+04
	20.00	1443	-5.83	3.40E+01	-338	1.14E+05	1.97E+03
	30.00	2117	4.17	1.74E+01	336	1.13E+05	1.40E+03
	40.00	2684	14.17	2.01E+02	903	8.15E+05	1.28E+04
	50.00	3512	24.17	5.84E+02	1732	3.00E+06	4.18E+04
Total	155.00	10685	0	1.52E+03	0	7.57E+06	1.07E+05

	x_i	y_i	x^2	y_i*	y_i-y_i*	$(y_i$-$y_i*)^2$	**Outlier**
	5.00	291	2.50E+01	313	22	4.88E+02	0.33
	10.00	638	1.00E+02	665	27	7.47E+02	0.40
	20.00	1443	4.00E+02	1370	73	5.31E+03	1.08
	30.00	2117	9.00E+02	2074	43	1.82E+03	0.63
	40.00	2684	1.60E+03	2779	95	9.05E+03	1.41
	50.00	3512	2.50E+03	3483	29	8.43E+02	0.43
Total	155	10685	5.53E+03	10685	289	1.83E+04	-

Sy/x	68
Sb	1.73
Sa	52.57

IC	**a**	145.96
	b	4.81

a	-39	±	146
b	70	±	5

Diclofenac data

Table A 9. Diclofenac data for the calibration curve.

C (ppb)	A1	A2	A3	$\bar{A}$	CV%
100000	3617815	3651070	3714509	3661131	1.1
75000	2581630	2517210	2538916	2545919	1.1
50000	1609478	1621581	1673184	1634748	1.7
25000	873182	870295	873512	872330	0.2
10000	337150	337989	337699	337613	0.1
5000	172127	172677	171385	172063	0.3
1000	30794	31795	31722	31437	1.4
750	21750	22749	22333	22277	1.8
500	14431	14390	14698	14506	0.9
250	7847	7757	7481	7695	2.0
100	2483	2585	2563	2544	1.7

Table A 10. Calculations for the diclofenac parameters.

	C (ppb)	$\bar{A}$
Experimental data	100.00	2544
	250.00	7695
	500.00	14506
	750.00	22277
	1000.00	31437
	5000.00	172063
Average	1266.67	41753.72

	x_i	y_i	x_i-x_{med}	$(x_i-x_{med})^2$	y_i-y_{med}	$(y_i-y_{med})^2$	$(x_i-x_{med})(y_i-y_{med})$
	100.00	2544	-1166.67	1.36E+06	-39210	1.54E+09	4.57E+07
	250.00	7695	-1016.67	1.03E+06	-34059	1.16E+09	3.46E+07
	500.00	14506	-766.67	5.88E+05	-27247	7.42E+08	2.09E+07
	750.00	22277	-516.67	2.67E+05	-19476	3.79E+08	1.01E+07
	1000.00	31437	-266.67	7.11E+04	-10317	1.06E+08	2.75E+06
	5000.00	172063	3733.33	1.39E+07	130309	1.70E+10	4.86E+08
Total	7600	250522	0	1.73E+07	0	2.09E+10	6.01E+8

x_i	y_i	x^2	y_i*	y_i-y_i*	$(y_i-y_i*)^2$	Outlier

	100.00	2544	1.00E+04	1156	1388	1.93E+06	1.02
	250.00	7695	6.25E+04	6375	1320	1.74E+06	0.97
	500.00	14506	2.50E+05	15075	569	3.23E+05	0.42
	750.00	22277	5.63E+05	23775	1497	2.24E+06	1.10
	1000.00	31437	1.00E+06	32474	1037	1.08E+06	0.76
	5000.00	172063	2.50E+07	171668	395	1.56E+05	0.29
Total	7600	250522	2.69E+07	250522	6206	7.47E+06	-

Sy/x	1366
Sb	0.33
Sa	696.09

IC	**a**	1932.67
	b	0.91

a	-2324	±	1933
b	35	±	1