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Pandemic impact on the consumption patterns of pharmaceuticals and other emerging contaminants

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Abstract

The initial COVID-19 outbreak occurred in the end of 2019 and it rapidly spread across the world. Governments implemented measures worldwide against the spread of the COVID-19 pandemic. The Portuguese government adopted such procedures as lockdowns and the obligation of remote work whenever possible, which resulted in changes of lifestyle and personal behaviours, possibly altering the consumption of some products, such as pharmaceuticals and illicit drugs, which may have impacted their environmental occurrence patterns. After consumption, pharmaceuticals and their excreted metabolites may reach wastewater treatment plants (WWTPs), where conventional biological treatment is inadequate to completely eliminate them. The treated effluents from these plants may, therefore, constitute a risk for aquatic organisms and human health. Most of these compounds are considered contaminants of emerging concern (CECs) and their occurrence over the course of the pandemic may give insights about their consumption trends. This project aims to apply wastewater-based epidemiology (WBE) to the occurrence patterns of pharmaceuticals in Portugal over the pandemic.

The present work developed a protocol to analyse CECs in wastewater comprising liquid-liquid extraction (LLE) and solid-phase extraction (SPE) as alternatives, followed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) for the identification and quantification of the target compounds. An optimized SPE procedure was developed using Oasis® HLB cartridges to extract 25 mL of samples with a pH adjusted to 5 by 5.5 mL of ethanol (EtOH) as elution solvent. The SPE-UHPLC-MS/MS method was capable of quantifying 17 different CECs in wastewater and was validated according to international guidelines concerning linearity and range of application, recovery, accuracy, precision, and instrument and method limits of detection and quantification. The matrix effect and process efficiency of each CEC were also evaluated. Wastewater samples collected from the influent of a WWTP in Portugal from April to November 2020, were analysed for CECs concentrations to establish occurrence patterns through three phases of the pandemic, including the first wave (FW), from March 18th to July 1st, the reopening phase (RO), until October 14th, and the second wave (SW), until the end of 2020.

It was found that the frequencies of detection (FOD) of fluoxetine and remdesivir decreased from the FW to the RO, while the FODs of cocaine and metoprolol slowly increased over time. Additionally, the measured concentrations of atenolol and tramadol increased significantly between the FW and the RO. Further studies following this work can be performed, or in other contexts, using the developed SPE-UHPLC-MS/MS method.

Keywords: wastewater; COVID-19 pandemic; contaminants of emerging concern; solid-phase extraction; ultra-high performance liquid chromatography tandem mass spectrometry.

Sumário

O surto inicial de COVID-19 ocorreu no fim de 2019 e alastrou-se rapidamente pelo mundo. Governos a nível mundial implementaram medidas contra o alastramento da pandemia de COVID-19. O governo português adotou procedimentos como confinamentos e a obrigação de trabalho remoto quando possível, os quais levaram a mudanças de estilo de vida e comportamentos pessoais, possivelmente alterando o consumo de alguns produtos, como fármacos e drogas ilícitas, o que poderá terá afetado os seus padrões de ocorrência no meio ambiente. Após consumo, os fármacos e os seus metabolitos excretados podem ser descarregados em estações de tratamento de águas residuais (WWTPs), nas quais o tratamento biológico convencional é inadequado para os eliminar completamente. Os efluentes tratados destas estações podem, portanto, constituir um risco para organismos aquáticos e para a vida humana. A maioria destes compostos são considerados contaminantes de preocupação emergente (CECs) e a sua ocorrência ao longo da pandemia pode revelar informação acerca das suas tendências de consumo. Este projeto procura aplicar epidemiologia baseada em águas residuais (WBE) aos padrões de ocorrência de fármacos em Portugal ao longo da pandemia.

O presente trabalho desenvolveu uma metodologia de processamento de amostras de águas residuais, incluindo como alternativas a extração líquido-líquido (LLE) e extração em fase sólida (SPE), seguidas por cromatografia líquida de ultra-alta eficiência associada a espetrometria de massa em tandem (UHPLC-MS/MS) para identificação e quantificação dos compostos alvo. Um procedimento SPE otimizado foi desenvolvido usando cartuchos Oasis® HLB para extrair 25 mL de amostras com o pH ajustado a 5 por 5.5 mL de etanol (EtOH) como solvente. O método SPE-UHPLC-MS/MS foi capaz de quantificar 17 CECs diferentes em água residual, e foi validado de acordo com diretrizes internacionais relacionadas com linearidade e gama de aplicação, recuperação, exatidão, precisão, e limites de deteção e quantificação do instrumento e do método. O efeito matriz e eficiência do processo também foram avaliados para cada CEC. Amostras de água residual recolhidas no influente de uma WWTP localizada em Portugal, entre Abril e Novembro de 2020, foram analisadas em relação a concentrações de CECs para estabelecer padrões de ocorrência ao longo de três fases da pandemia, incluindo a primeira onda (FW), entre 18 de Março e 1 de Julho, a fase de reabertura (RO), até 14 de Outubro, e a segunda vaga (SW), até ao final de 2020.

Foi determinado que as frequências de deteção (FOD) de fluoxetina e remdesivir decresceram entre a FW e a RO, enquanto as FODs de cocaína e metoprolol aumentaram lentamente com o tempo. Adicionalmente, as concentrações determinadas de atenolol e tramadol aumentaram significativamente entre a FW e a RO. Novos estudos em seguimento deste trabalho podem ser efetuados, ou noutros contextos, aplicando o método SPE-UHPLC-MS/MS desenvolvido.

Palavras-chave: água residual; pandemia COVID-19; contaminantes de preocupação emergente; extração em fase sólida; cromatografia líquida de ultra-alta eficiência associada a espectrometria de massa em tandem.

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List of Acronyms and Abbreviations

- ACE2 – angiotensin-converting enzyme 2;
- ACN – acetonitrile;
- AOP(s) – advanced oxidation process(es);
- BBD – Box-Behnken design;
- CEC(s) – contaminant(s) of emerging concern;
- CF – concentration factor of the optimized SPE method;
- DoE – design of experiments;
- EtOH – ethanol;
- FA – formic acid;
- FOD – frequency of detection;
- FW – first wave;
- IDL – instrument detection limit;
- IQL – instrument quantification limit;
- IS(s) – internal standard(s);
- LC – liquid chromatography;
- LC-MS/MS – liquid chromatography tandem mass spectrometry;
- LLE – liquid-liquid extraction;
- MDL – method detection limit;
- MeOH – methanol;
- MQL – method quantification limit;
- MS – mass spectrometer (spectrometry);
- NSAIDs – non-steroidal anti-inflammatories;
- PSB –post-spiked blank;
- PTFE – polytetrafluoroethylene;
- QC – quality control;
- RO – reopening phase;
- RSD – relative standard deviation;
- SARS-COV-2 – severe acute respiratory syndrome coronavirus 2;
- SPE – solid-phase extraction;
- SRM – selected reaction monitoring;
- SW – second wave;
- UHPLC-MS/MS – ultra-high performance liquid chromatography with tandem mass spectrometry;
- UP – ultrapure;
- UV – ultraviolet;
- WBE – wastewater-based epidemiology;
- WHO – World Health Organization;
- WWTP(s) – wastewater treatment plant(s).

List of Symbols

- A/A_{IS} – ratio of chromatographic peak areas of a given target CEC and its assigned IS;
- A_b – chromatographic peak area of a target CEC in blank wastewater;
- A_s – chromatographic peak area of a target CEC in spiked wastewater or spiked UP water;
- A_t – chromatographic peak area of a target CEC in the 200 μg/L stock solution;
- b – y-intercept of method-adjusted calibration curve;
- C_b – target CEC concentration in blank wastewater;
- C_{PSB} – target CEC concentration in PSB wastewater;
- C_s – target CEC concentration in spiked wastewater;
- C_t – measured target CEC concentration in 200 μg/L stock solution;
- C_{WW} – target CEC concentration in a wastewater sample;
- m – slope of method-adjusted calibration curve;
- m/z – mass/charge ratio;
- ME – matrix effect for a given target CEC;
- R – target CEC recovery in spiked wastewater or spiked UP water extracts;
- R_{opt} – target CEC recovery of the optimized SPE method for a given target CEC;
- r^2 – correlation coefficient of method-adjusted calibration curve;
- PE – process efficiency for a given target CEC;
- S/N – signal-to-noise ratio.

1. Frame of the study

Coronavirus disease 2019, also known as COVID-19, surged in Wuhan, China in late 2019 and rapidly spread worldwide, being classified as a pandemic by the World Health Organization (WHO) on March 11th, 2020. In order to curb the spread of the virus responsible for COVID-19, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), several governments across the world instituted a policy of restricted citizen movement, through measures such as lockdowns and closing of non-essential commerce.

The COVID-19 pandemic and the restrictions implemented resulted in a change of lifestyle, which could have affected the consumption of pharmaceutical products such as antibiotics, analgesics, antidepressants, anti-inflammatories, chronic medication, as well as that of illicit drugs. After consumption, parent drugs and their metabolites are excreted into the sewage system and eventually reach wastewater treatment plants (WWTPs). Conventional WWTPs are usually not able to completely remove pharmaceutical residues due to their low biodegradation and specifically those whose occurrence frequency and levels may have increased because of the COVID-19 pandemic. The effluents from these plants are discharged into surrounding water bodies like rivers, lakes, and streams, which may lead to bioaccumulation and biomagnification of these pharmaceutical residues in aquatic organisms, which can pose later risks to human health. As such, pharmaceuticals are considered contaminants of emerging concern (CECs) and their consumption needs to be regularly monitored over the course of the COVID-19 pandemic.

The aim of this study was to monitor the environmental occurrence of several pharmaceutical compounds and some illicit drugs in a region of Portugal over the COVID-19 pandemic, via the application of wastewater-based epidemiology (WBE), for which wastewater samples have been collected over the course of the pandemic (since April 2020). For that, an analytical method for processing and analysing the wastewater samples was developed and validated to allow the establishment of occurrence patterns over the different phases of the pandemic in Portugal. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were selected as sample preparation techniques, whereas the identification and quantification technique was ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

2. Introduction

2.1. COVID-19 pandemic and Portuguese government response

SARS-CoV-2 causes COVID-19, which is a respiratory illness that is highly transmissible and capable of causing severe breathing difficulties and pneumonia-like symptoms. SARS-CoV-2 strains generally have higher transmissibility than most other coronaviruses, possessing a higher basic reproduction number **[1,2]**.

SARS-CoV-2 is primarily directly transmitted through respiratory droplets ejected during exhalation, talking, coughing, and sneezing, forming aerosols. In addition to the respiratory passageways, SARS-CoV-2 can also infect the digestive tract through the angiotensin-converting enzyme 2 (ACE2) receptor. Indirect modes of transmission include other bodily secretions, such as saliva and urine, contaminated surfaces in the immediate environment of an infected host, or fomites (*e.g.*, medical instruments used on the infected person). As such, the risk of contracting COVID-19 increases with proximity to an infected host **[3,4]**. Some of the most common modes of SARS-CoV-2 transmission are illustrated in **Figure 1**:

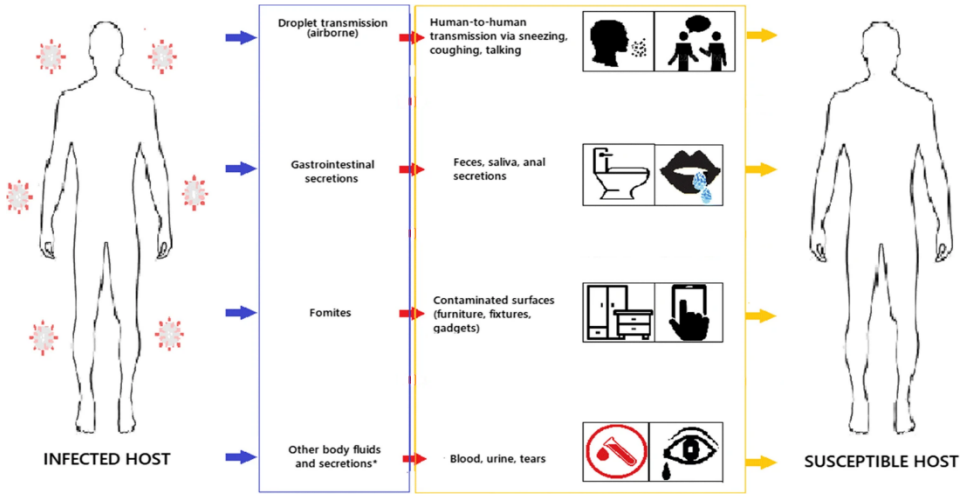

Figure 1. Most common modes of transmission of SARS-CoV-2. Reprinted (adapted) from **[4]** with the permission of Springer Nature

Considering SARS-CoV-2 ease of transmission in enclosed spaces, it is expected that public transportation vehicles would have favourable conditions for its spread. Since the Chinese Spring Festival holiday, which attracts 3 billion public transport passengers per year, occurred during the early stage of the COVID-19 pandemic in China, SARS-CoV-2 infections began increasing exponentially. Although effective measures were later taken to curb the virus' spread in public transportation, like mask mandates and frequent disinfection, before the first lockdown of Wuhan on January 23rd, 5 million residents had already left the city, and as such COVID-19 had already disseminated into the general Chinese population **[5,6].**

COVID-19 then quickly disseminated from China across the world, with the first major outbreaks occurring in Iran, South Korea, and Japan. In Europe, Italy suffered the second biggest outbreak, after

the original one, with an exponential increase in infections, which was correlated with an increase in test and tracing, which led the Italian government to issue a lockdown of the country on March 8th, 2020. An early infection rate peak in Italy occurred on March 21st of the same year, and from then COVID-19 quickly spread to the rest of Europe **[7,8]**.

The rapid spread of COVID-19 across the world caused problems related to healthcare capacity in many countries, straining the healthcare systems and putting a significant number of hospitals at risk of being overloaded due to exponentially increased hospitalization rates. In some of the most densely populated cities of the world, such as Tokyo, the medical system was close to a full collapse **[9]**.

Limitations in hospital capacity, staff and supplies forced some spaces to implement crisis standards of patient care, which consist of extreme operating conditions, in which the core of medical decisions changes from reaching the best outcome for individual patients to assuring the immediate medical needs of larger patient groups. Abandoning conventional standards of hospital care leads to many preventive and elective procedures being suspended, which can cause the progression of serious diseases within patients that would have benefitted from earlier diagnosis and intervention **[10]**.

The rapid spread, high infection rate and hospital overload and its consequences led governments worldwide to implement strict measures to promote hygiene and to reduce the movement of citizens, such as quarantining close contacts of infected ones, restricting public gathering and movement, widespread testing for SARS-CoV-2, obligating mask wearing, implementing social-distance measures, promoting recommendations for hand washing and disinfection of public surfaces, preventing the spread of false information related to the pandemic, and promoting a widespread test availability and compulsory testing of some groups during outbreaks **[11]**.

Due to the spread of COVID-19 within Europe and its classification as a pandemic by the WHO on March 11th, 2020, the Portuguese government approved and implemented measures in the following day to control COVID-19 spread, with a state of emergency being declared shortly after, on the 18th. These measures were similar to those adopted by other countries and included the closing of restaurants and non-essential commerce, the cancellation of events, the obligation of remote work whenever possible, remote home school activities, some local and district-wide quarantines, and the establishment of curfews **[12,13]**. For most of 2020, there were no approved COVID-19 vaccines available in Portugal, and the Directorate-General of Health followed WHO directives. In this sense, there were no approved drugs for the treatment of COVID-19, except for hydroxychloroquine, remdesivir, and lopinavir/ritonavir for severe cases of pneumonia **[14]**. Over the course of the pandemic, these measures were adjusted, introduced, and removed depending on the infection and death rate, vaccine availability, available hospital capacity and expected citizen movement. The severity of the restrictions imposed by the Portuguese government can be estimated via the Oxford COVID-19 Government Response Tracker project Stringency Index. The Stringency Index is calculated from the policy of indicators of a specified government, which can take values from 1 to

100, reflecting the severity of them measures of that government. During the main active phase of COVID-19 in Portugal, the Stringency Index varied between 11, that was achieved in April 2022, and 88, which was reached between February and March 2021 **[15]**. The variation of the Portuguese Stringency Index and daily deaths over the course of the pandemic is presented in **Figure A1** of **Annex A**.

On April 21st, 2022, in lieu of an overwhelming majority of vaccinated citizens and stable death and infection rates, the Portuguese government repealed the general mask mandate with exceptions for healthcare facilities, elderly care facilities and public transportation; terminated mandatory COVID-19 testing under certain circumstances; stopped demanding the EU COVID Digital Certificate or a negative test or a third vaccination certificate for access to certain establishments **[16]**. As such, the main active phase of the COVID-19 pandemic in Portugal can be considered to have lasted from March 2020 to April 2022 regarding government measures. However, these measures led to an expected increase of infections not accompanied by healthcare overflows nor extremely high incidence of intensive care needs or death, due to the positive impact of the broad vaccination of the Portuguese population.

2.2. Lifestyle and consumption changes due to the COVID-19 pandemic

The reduction of exposure to outbreaks has been associated with significant changes in lifestyle, caused by measures like social-distancing, quarantining, and event cancellation, as these ultimately result in restricted freedom of movement of the population, with people spending much more time at home. These changes include dietary changes, reduced physical activity and increased screen time and can, in turn, lead to psychological effects among individuals **[17]**.

Due to the modified lifestyle imposed by the COVID-19 pandemic and the restrictions implemented, the consumption of some products was inevitably altered, through changes in their demand, supply, and prescription. The consumption of many pharmaceuticals like antibiotics, analgesics, antidepressants, anti-inflammatories, chronic medication, as well as illicit drugs, was very likely affected. For instance, during the first waves of COVID-19, infected patients were commonly prescribed with antibiotics despite rare bacterial co-infection. Several factors, including younger age, dry cough, flu symptoms and fever have been associated with incorrect prescriptions. Additionally, people might have intentionally sought out and consumed antibiotics and antivirals despite an unknown potential of protection, due to panic and lack of understanding. Widespread antibiotic abuse has severe consequences, such as adverse effects and development of microbial resistance **[18,19]**.

COVID-19 infection symptoms include acute pain, headaches and widespread myalgia, and as such, it is likely that the consumption of analgesics and antiepileptics like carbamazepine increased during the pandemic **[20,21]**. A study from Innsbruck found that codeine and acetaminophen, both analgesics, had higher concentrations in wastewater than the usual values just before lockdown,

decreasing during lockdown to levels below those usual, possibly indicating respectively higher demand and then difficulty in acquiring them during quarantine **[22]**. Another study, from central Italy, found a significant increase in wastewater concentrations of carbamazepine during a lockdown period **[23]**.

Non-steroidal anti-inflammatories (NSAIDs), particularly ibuprofen but also diclofenac and naproxen, were initially thought and reported by the media to exacerbate COVID-19 symptoms and increase the risk of infection, because these pharmaceuticals were proposed to cause upregulation of ACE2 receptors, which are used by SARS-CoV-2 to enter the cells. Even though more recent studies suggest that NSAIDs do not potentiate harsher COVID-19 infections, it is likely that their consumption was negatively impacted during the pandemic. Non-opioid analgesics, particularly acetaminophen, were recommended as an alternative to NSAIDs, and therefore their consumption may have increased **[24,25]**. A study performed in Athens found that the consumption of diclofenac and ibuprofen was reduced by over 50% in 2020 in comparison to previous years **[26]**. The concentration of diclofenac in the rivers of KwaZulu-Natal in South Africa decreased significantly during the pandemic **[27]**.

Regarding opioid analgesics, such as tramadol, some European countries attempted to ensure continued provision for patients undergoing opioid substitution treatment by facilitating their access to the medication. Consequently, an increased availability of illegally diverted prescription opioids was reported **[28]**. A study on suspected abusers of illicit drugs in Turkey showed that blood and urine samples revealed that the use of tramadol in combination with other drugs increased from 13% in a group of suspected drug users in 2019 to 82% during a complete lockdown period in 2020 **[29]**.

The glucocorticoid dexamethasone is a steroidal anti-inflammatory, which was reported in June 2020 as one of the first compounds to successfully delay respiratory failure and death in critical COVID-19 patients, which combined with its wide availability and low cost, hassled to a considerably increased usage worldwide during the pandemic **[30]**. This has been verified in the United States, as dexamethasone was previously not detected in 2014, while its concentration in January 2021 was predicted to reach a maximum peak of 55.6 ng/L **[31]**.

The COVID-19 pandemic had extreme effects on the social, physical and mental state of people, with a significant rise in mental pathologies being reported worldwide, mostly attributed to loneliness resulting from lockdowns. As such, the pandemic had a significant effect on people who already had depression or who were at risk of developing it. For example, the prevalence of depression more than tripled in the United States and doubled in the United Kingdom. For antidepressants, rises in prescriptions during the COVID-19 pandemic were reported, potentially derived from the impact of the pandemic on mental health **[32]**. Additionally, fluoxetine and fluvoxamine were reported to trigger mechanisms that might be useful in combating a COVID-19 infection, which led to these drugs being administered for certain COVID-19 patients beyond their antidepressant effects **[33]**.

Another study discovered a significant association between the use of the antidepressants fluoxetine, citalopram, venlafaxine, paroxetine or mirtazapine and a reduced risk of intubation or death for COVID-19 patients. This is thought to result from the effect of these compounds on reducing the plasma levels of some pro-inflammatory mediators, which have been associated with severe COVID-19 **[34]**. As expected, increased concentrations of citalopram and fluoxetine in wastewater effluents and rivers were reported across the world during the COVID-19 pandemic, while a trifold increase of the concentration of venlafaxine in untreated wastewater of Athens in comparison to pre-COVID years, was also reported **[35,36]**.

Considering the restrictions implemented to combat the pandemic, it is possible that the availability of illegal drugs was reduced, due to a decrease in trafficking of these drugs across the world. This could have led to higher drug prices and decreased drug purity, leading to a lower overall consumption of traditional illicit drugs and even a possible shift to novel psychotropic drugs as substitutes. In addition, lockdown measures difficulted the meetings with drug dealers, possibly collapsing local drug street markets **[37]**. Another important consequence of the restrictions is that the usual recreational events and establishments in which drugs tend to be used were compromised. However, the restrictive measures might have intensified drug users' mental health problems by limiting access to detoxification centres, leading to an increase in drug abuse **[38]**. A study performed in Innsbruck found that cocaine sales during lockdown decreased, which was attributed to the shutdown of the hospitality industry and event cancellation, leading to a lower demand for drugs in recreational settings. The same study found that the concentration of morphine, an opioid, in Innsbruck wastewater was significantly higher in 2020 than in 2019. However, the authors reported that the population normalized mass loads of morphine showed no trend during quarantine and suggested that the increase was not directly related with the lockdown **[22]**. Another study, from India, found that the average consumption of morphine per patient during the 2020 lockdowns was almost double that of 2019, resulting from a much higher number of prescriptions. A possible justification for this is that during the lockdowns, there were significant delays in cancer-related therapies and surgeries, which aimed to reduce hospital overload, increasing opioid needs for the affected patients. Another reason suggested by the authors was that the few patients admitted were those that were in severe pain and therefore had a true requirement of morphine, increasing the likelihood of it being prescribed **[39]**.

In a recent study on the consumption habits of alcohol, stimulant drinks, illegal substances, and pharmaceuticals (antidepressants, sleep inducers, tranquillizers, and/or anxiolytics) in the Portuguese population, performed via a questionnaire, it was concluded that the COVID-19 pandemic led to an increase in the consumption of all these classes of substances **[40]**.

Certain pharmaceuticals, namely the two antimalarials chloroquine and hydroxychloroquine, were initially reported to have some beneficial effects on COVID-19 patients, such as reducing their viral

load, with this effect being allegedly reinforced by the antibiotic azithromycin **[41]**. The effect of hydroxychloroquine effect on SARS-CoV-2 viral load was suggested to occur due to an increase in intracellular pH, reducing the activity of ACE2 receptors' glycosylation pathway, and thus resulting in a lower probability of cells being infected **[42]**. Chloroquine has an equivalent mechanism of action to that of hydroxychloroquine, however it has lower cytotoxic concentration threshold and as such its use was not recommended whenever hydroxychloroquine was available **[43]**. Azithromycin can inactivate the endocytic activity of coronaviruses, preventing them from entering and infecting new cells **[44]**. Because of the dissemination of these early findings, a significant increase in the prescriptions of these compounds was reported in the early stages of the pandemic in 2020, indicating a widespread increase in their consumption **[45]**. However, since then other studies have increasingly reported no significant relationship between hydroxychloroquine or chloroquine usage and the reduction of COVID-19 symptoms, leading some governments to revoke their previous emergency use authorization in certain hospitalized patients **[42]**. One study performed in the Lombardy region of Italy found that sales of azithromycin in March 2020 were 160% higher than those registered in March 2019 and that the wastewater concentrations of hydroxychloroquine and azithromycin were positively correlated with the occurrence of COVID-19 infections and deaths, suggesting that people sought and consumed these pharmaceuticals because of anxiety towards the pandemic **[46]**. Another study performed during the initial surge of COVID-19 in Athens, found an increase by 387% of hydroxychloroquine concentrations in wastewater and a rising of 36% of azithromycin concentrations in comparison to the same months of 2019 **[26]**.

Access to chronic medications, such as β-blockers (*e.g.*, atenolol, propranolol, and metoprolol), might have also been affected by the disrupted supply chains, quarantines, social-distance measures and difficult access to healthcare facilities and refilling medication, whereas a decline in newly diagnosed patients that needed to start therapy may have led to lower overall consumption. Additionally, financial pressures and personal stress may have caused the restocking of chronic medication to become a lower priority. Clement et al. suggested that some patients may have discontinued some necessary medication **[47]**. For example, a decrease in medical consultations and lowered pharmacy attendance was observed in Austria during quarantine **[48]**. In Athens, it was found that a significant reduction in the consumption of cardiovascular drugs such as β-blockers and other antiarrhythmics had occurred during the first lockdown period **[26]**.

2.3. Wastewater treatment and environmental impact of COVID-19 pandemic-motivated consumption changes

The above-mentioned reported variations on consumption patterns and wastewater concentrations are expected to have implications on the occurrence patterns of the referred substances in the receiving environment, as well. After consumption, both the parent pharmaceuticals and their

metabolites are excreted and transferred through the sewage system until reaching WWTPs. Some compounds have a relatively low metabolic turnover, such as amoxicillin, which is 80% excreted in its parent form, whereas others are metabolised into metabolites that may be more toxic for ecosystems or metabolites that are converted back to the parent form in the WWTPs **[49]**. Additionally, drugs incorrectly disposed can also end up in the sewage system. In WWTPs, the conventional biological treatment is generally inadequate to remove traces of most of these small organic molecules, due to their slow metabolism and the inhibitory effect of some drugs on nitrifying microorganisms **[49,50]**. Thus, the treated effluents from these plants contain pharmaceutical and metabolite residues that may constitute a risk for aquatic organisms and human health when discharged by WWTPs in surface/ground waters, as they increase the pharmaceutical load of the receiving water bodies. Additionally, they may present a bioaccumulation potential and consequently may undergo bioaccumulation followed by biomagnification in aquatic life. Kumar et al. highlighted that these phenomena could cause human health risk if the contaminated water or aquatic organisms are consumed **[51]**. Therefore, CECs should be monitored, specially over the course of the pandemic, when the inputs of many of them may be higher. This is especially true for drugs which have been used to treat COVID-19 symptoms, such as hydroxychloroquine, chloroquine, and azithromycin **[52]**.

Azithromycin is mostly excreted in parent form, since it has a low metabolic assimilation rate despite a long residence time in the body. Consequently, large amounts of parent azithromycin can reach WWTPs, where it is not fully removed. Collado et al. found that conventional primary and secondary treatments do not significantly reduce wastewater azithromycin concentration, while tertiary ultraviolet (UV) treatment only results in a slight decrease. This might contribute to the development of antimicrobial resistance after effluent discharge, since trace amounts of azithromycin can be found in receiving waters **[53,54]**.

Both hydroxychloroquine and chloroquine have a metabolic assimilation rate of around 30%, originate the same metabolites and are excreted as 70% parent form. These compounds can cause organ failure in aquatic organisms, stunt or prevent the development of plants and bioaccumulate due to their resistance to acids, hydrolysis, chelation, heat, and UV light treatment. They are also highly soluble and have poor biodegradability, persistently contaminating groundwater and potentially harming non-target organisms **[52,55]**. Similarly to azithromycin, they are both poorly removed by conventional WWTP. Kuroda et al. estimated the average removals of hydroxychloroquine and chloroquine by biodegradation as 6 and 63%, respectively, with half-lives ranging from weeks to months **[56]**.

A study performed at WWTPs near Rome found that despite the increased pharmaceutical concentrations in the influent of a large WWTP during the COVID-19 pandemic, their average removal efficiencies were not significantly different than those recorded before. On the other hand, a smaller WWTP presented reduced removal efficiencies towards higher influent concentrations,

suggesting that plants with lower capacity are less resistant to influent concentration fluctuations. In both cases, the plants were not efficient in reducing the increased pharmaceutical concentrations, which were inevitably discharged through the effluent into receiving water bodies, from which the pharmaceuticals can infiltrate sediments and wetlands **[23,57]**.

Since secondary treatment processes in WWTPs like activated sludge tanks have varying but generally low removal efficiencies of pharmaceuticals that were overused in treating COVID-19, new processes should be investigated and implemented to prevent further damage to the environment **[57]**.

Alternative processes with higher pharmaceutical removal efficiencies that have been demonstrated as promising alternatives as tertiary treatment include advanced oxidation processes (AOPs), which may be efficient to remove toxic compounds like pharmaceuticals, dyes, and pesticides. AOPs generally have high reaction rates and a broad organic compound spectrum of action **[57]**. However, AOPs have not been widely applied at full-scale WWTPs and many aspects are currently being investigated, specifically regarding reactor design, chemical mechanisms, and the influence of operational variables **[49]**.

Examples of AOPs include combined hydrogen peroxide/UV light treatment, photocatalysis, ultrasonic processes, Fenton and photo-Fenton processes, and ozonation in the presence of catalysts, UV light, or hydrogen peroxide, among others. Some advantages of the application of AOPs for removing organic pollutants include reduced sludge production in comparison with biological treatment, the capability of removing some trace heavy metals, and the possibility of additionally removing pathogens when using UV light, while their main disadvantages are the necessity of removing residual hydrogen peroxide and high installation and maintenance costs **[58]**.

Other processes that are efficient in removing pharmaceuticals from aqueous matrices are several different forms of photodegradation, adsorption and conventional oxidation. However, adsorption uses costly materials and AOPs high amounts of energy, hampering the scale-up of these technologies to full-scale WWTP. As such, more research needs to be conducted on these topics, particularly towards the development of new, economical, and sustainable adsorption materials, energy optimization in the case of AOPs, or the development of hybrid biological/chemical technologies capable of treating effluents in a shorter operation time **[31]**.

2.4. WBE

WBE is a non-intrusive approach that consists in monitoring pollutants and biomarkers in wastewater samples, which if collected at the influent of WWTPs, allow to infer information related to drug use and exposure to pollutants by the population served by the respective WWTP. It finds a niche in estimating the real-time consumption of licit and illicit drugs through the detection and quantification of parent drugs and their metabolites in wastewater. In this way, WBE does not require personal contact and is more reliable and unbiased than survey-based investigations, making it an

ideal approach for the current pandemic context, where research is hampered by restrictions that restrict social interaction **[59,60]**.

WBE can be used to study the consumption of illegal drugs and to determine the fraction of prescribed pharmaceuticals that are actually consumed by the patients. In this sense, disparities between estimated consumption and prescriptions can indicate either poor compliance with the medication regimen or diversion of drugs. Over their course through the sewage system towards the WWTP inlet, parent pharmaceuticals and their metabolites may undergo partial biodegradation but do not commonly react with other compounds, being therefore overall stable and preserved. However, a major drawback associated with WBE methods is the various uncertainties related with measuring an analyte concentration in wastewater as well as estimating the *per capita* consumption of a drug. The WBE methodology is also incapable of estimating the consumption of individuals within the WWTP catchment area **[59,61,62]**.

In order to determine the consumption rate of a given drug by individuals served by a WWTP, apart from determining the drug concentration in wastewater, other parameters must be known such as the wastewater flow rate, the estimative of the population served, and specific correction factors for each analyte. The population served can be either lifted from a recent census, or otherwise estimated via the quantification of known human biomarkers in the samples. The advantage of normalizing the drug mass loads to the served population via biomarkers is that differences in drug loads are not incorrectly attributed to changes in population. Correction factors are needed to account for differences in the metabolic degradation rate and travel time of different pharmaceuticals, owing to variations in wastewater matrices, as well as differences in the sewage system. Correction factors for some analytes may also be correlated with known stable metabolites that can serve as internal controls, such as hydroxycotinine in the case of nicotine **[36,62,63]**.

Knowing the concentrations of the target compounds in the wastewater influents allows the establishment of spatial and temporal consumer patterns of the population served by a particular WWTP, providing information about the health and lifestyle of that population. Therefore, WBE can support decision makers in understanding the pandemic impact on different communities and is an efficient tool to recommend measures to curb the health and environmental risks imposed by these possible emerging contaminants. It can also judge the effects of the measures taken regarding improper pharmaceutical consumption **[59,61]**.

The workflow for a typical WBE study is summarized in **Figure 2**: wastewater samples are collected at a WWTP inlet and stored to be prepared and then analysed with the goal of determining the target pharmaceuticals concentrations in the wastewater. Then, the individual consumption of the pharmaceuticals is estimated and analysed to determine the causes of possible variations, which in turn can allow decision makers to implement measures to curb those variations **[59]**.

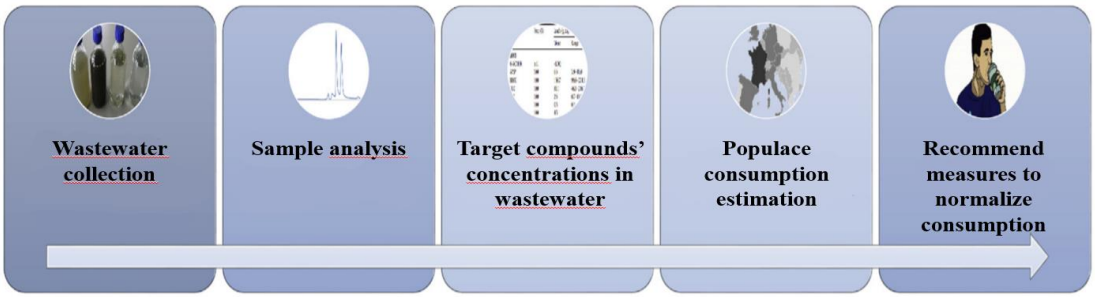

Figure 2. Typical WBE study workflow. Reprinted (adapted) from **[59]** with the permission of Elsevier

Other than WWTP inlets, wastewater samples collected for the WBE framework can also be recovered at the neighbourhood level through manholes, which allows researchers to refine the resolution of monitoring data as a more accurate information can be obtained for a smaller population. Data recovered from downstream WWTP inlets and upstream manholes can complement each other to better study the habits of a given population. **Figure 3** illustrates these sample collection points **[62]**.

Figure 3. Possible WBE sample collection points and relative position on the sewage system. Reprinted from **[62]** with the permission of Springer Nature (open access article distributed under the terms of the Creative Commons CC BY license)

Since wastewater samples are complex matrices that contain compounds capable of interfering with target analyte identification and quantification, samples are first processed by filtration or centrifugation in order to separate suspended solids from the liquid phase, and then preconcentrated and cleaned up by an extraction technique. Usually, SPE is selected to extract polar compounds from complex matrices such as wastewater. After processing, samples are typically analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS), since this technique is capable of selectively analysing multiple compounds in a single run and at concentrations as low as in the µg/L range, meaning that such levels in the extracts correspond to ng/L levels in the samples. Depending on the target analytes and the matrix itself, sample preparation and analytical methods need to be developed and optimized for an accurate wastewater analysis **[59,62]**.

2.4.1. Wastewater pre-treatment and processing

Cleanup of wastewater matrices is necessary to prevent analytical interferences, while preconcentration increases the sensitivity of the analytical method for detection and quantification of the target analytes. SPE is capable of both cleaning up and preconcentrating, making it one of the most used techniques for aqueous sample processing **[64]**.

SPE is based on the use of disposable cartridges containing silica-based adsorbents, which are loaded with liquid samples and subjected to vacuum suction. Cartridges are essentially small hollow columns, resembling open syringes, where the adsorbent material is packed between porous plastic holders, as illustrated in **Figure 4**. Typically, multiple cartridges are mounted on an SPE manifold to allow for multiple and simultaneous sample processing. To counteract clogging of the adsorbent bed, SPE manifolds possess a vacuum port, connected to purge liquid recipients and a pump which establishes a vacuum on the side of the cartridges opposite the liquid insertion site, and speeds up the SPE process by suctioning the liquids inserted onto the cartridges **[65,66]**.

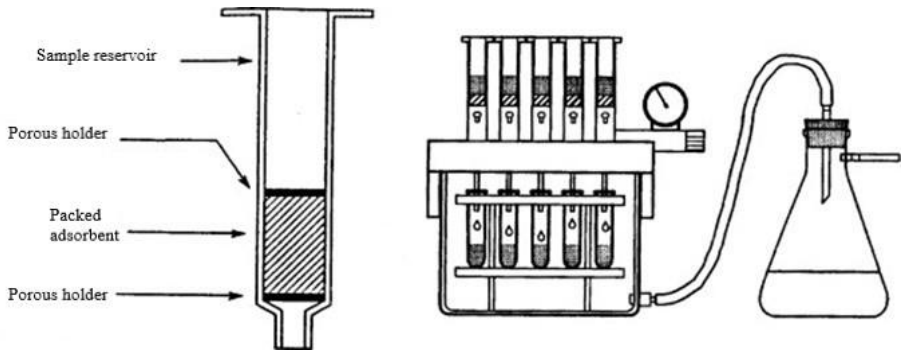

Figure 4. SPE cartridge and vacuum extraction manifold system diagram. Reprinted from **[65]** with the permission of Elsevier (License number: 5338851350117)

The first typical step of the SPE procedure is the conditioning of the packed adsorbent by the elution solvent (*e.g.*, methanol (MeOH), acetonitrile (ACN) or ethanol (EtOH)), followed by the equilibration of the adsorbent bed with an aqueous buffer, such as ultrapure (UP) water. Afterwards, the liquid sample is loaded onto the cartridges, during which the analytes ideally adsorb to the active sites of the bed and interfering compounds remain on the liquid phase, which is purged out of the manifold system. Separation of the analytes and the interferents occurs by differences in adsorbent affinity and liquid sample solubility. A slower sample flow rate in SPE is generally preferable to better promote mass equilibrium through the full length of the adsorbent bed. The subsequent step is the washing of the bed by loading an aqueous solution (*e.g.*, UP water, acidified water, alkalinized water) which will elute matrix interferents, with a small amount of the target analytes being lost in this step. This is followed by drying the adsorbent bed with air or nitrogen to remove residual water. Finally, collection recipients, such as tubes, are placed under the cartridges before loading the elution solvent, which will remove the analytes from the adsorbent because of higher affinity to the solvent. The analytes can be concentrated during this step by employing a lower volume of elution solvent

than the original sample volume **[64,67,68]**. **Figure 5** illustrates the different steps of an SPE experiment.

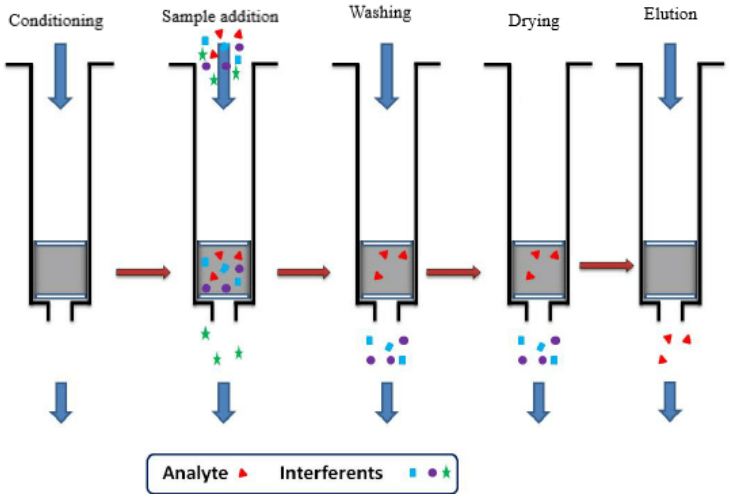

Figure 5. Schematic representation of SPE steps. Reprinted (adapted) from **[69]** with the permission of IntechOpen (open access book)

After collecting the eluted extracts, they are evaporated, and the analytes can be further concentrated by reconstitution in a smaller and exactly known volume of the mobile phase used in the case of reverse phase liquid chromatography (LC) **[68]**.

The main advantages of SPE in comparison with other sample preparation techniques, like LLE, are multiple and simultaneous sample processing, generally higher recoveries, no risk of forming an emulsion, lower environmental impact due to the low volume of solvents required, which also translates in reduced costs, and ability to concentrate large sample volumes into a much smaller extract volume. The disadvantages of SPE are related to the complexity of method development, high cost of cartridges, and potential clogging of the adsorbent bed **[66,68,70]**.

The efficiency of an SPE method is directly related to mass equilibrium and depends on the choice of the adsorbent and the elution solvent, as well as other factors like sample pH, sample flow rate, sample loading volume, and elution solvent loading volume. Each of these factors affect the establishment of favourable interactions between the adsorbent, the analytes and the interferents **[71]**.

The most used SPE cartridges for wastewater processing are Oasis® HLB cartridges, which consists of a reverse phase adsorbent made up of two monomers, hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. This adsorbent can capture acidic, basic, and neutral compounds and functions at a pH range of 1-14. As this adsorbent is water wettable, it has a high retention capacity even if the adsorbent bed is dried **[64,72]**. In conventional SPE, polar analytes are retained on a polar adsorbent and nonpolar interferents are purged, while in reverse phase SPE nonpolar analytes are retained on a nonpolar adsorbent while polar interferents are purged. As such, reverse phase SPE is applied to aqueous samples **[73]**.

LLE may also be applied to wastewater samples even with the previously mentioned disadvantages (such as lower recoveries than SPE), for purposes as preliminarily testing the affinity of different

solvent candidates for developing a SPE method with the target analytes, or comparing the recoveries of optimized LLE and SPE methods **[74]**. The principle of LLE is the transfer of a solute between two different and immiscible liquid phases, driven by differences in affinity, including polarity and solubility. Ideally, the target analytes and the interferents are obtained in different phases, and the analytes are concentrated by transferring into a smaller volume of liquid **[75]**. The LLE procedure consists of combining an aqueous sample with an organic hydrophobic solvent in a container that is then agitated to ensure adequate mixing. Then, the two liquid phases are separated, and the result is the concentration of hydrophilic compounds in the aqueous sample and hydrophobic compounds in the organic solvent. Other inherent disadvantages of conventional LLE methods include large solvent requirements, long, time-consuming processes, and multiple extractions may even be required to separate the majority of the target analytes from the aqueous sample **[76,77]**.

2.4.2. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

The preferred technique to analyse liquid samples with a high variety of polar/semi-polar analytes is LC-MS/MS, which combines compound separation by LC with their detection by a mass spectrometer (MS). The better separation achieved in the chromatography step, the lower the ion suppression effect in the MS. LC separates individual compounds in a mixture by a mass transfer process that occurs when the liquid sample is injected onto a mobile phase that passes through a stationary phase of opposite polarity. In reverse phase LC, the mobile phase is hydrophilic while the stationary phase is lipophilic, and the opposite applies in normal phase LC. The stationary phase can consist of different materials, depending on the separation mechanism, and it is generally a porous solid packed into a column where the liquid phase moves through. The compounds are separated based on the relative affinity of each one to the two phases, as well as solubility in the liquid phase. Chemicals with higher affinity for the stationary phase have a longer retention time and are eluted out of the column later than chemicals with lower affinity **[78,79]**.

The mobile phase is usually a mixture of an aqueous and an organic solvent such as MeOH or ACN, and mobile phase gradients typically perform better than isocratic mobile phases because a better separation of the analytes can be achieved and signal/noise ratio is increased. UHPLC utilizes high pressures to pump the liquid phase through the column and allows the use of column particles with a diameter below 2 μm as packing material, since a pump will compensate for the mobile phase pressure drop as it travels the column, and this results in a higher separation resolution **[80]**.

MS functions by ionizing the target analytes and identifying and quantifying the ions, as well as the fragments formed from the ions, based on their mass/charge ratio (m/z). There are different kinds of ion sources and mass analysers. An electrospray ionization source is apt for moderately polar molecules, being well suited for metabolites and xenobiotics. In this kind of ion source, a liquid sample, which can be previously subjected to coupled LC, is nebulised in an electrified metal capillary to form a spray of charged droplets which are instantly evaporated by heat or dry nitrogen. This results

in the simultaneous ionization and separation from the liquid phase of the target analytes. The ionization source can be operated to detect positive or negative ions. The most common type of mass analyser is the quadrupole analyser, a set of four metal rods that can detect a wide range of m/z values. Quadrupoles can also act as collision cells, where nitrogen or argon gas collides with ions coming from the ionization source and causing their fragmentation. A very common MS setup is the triple quadrupole MS, where the collision cell is placed between two quadrupole mass analysers, with the advantage of higher analytical specificity, as the first mass analyser can be set to detect a certain parent target ion, and the second mass analyser to detect specific product ions resulting from the parent ions fragmentation in the collision cell. In complex samples, multiple compounds could possibly form different fragments with the same m/z, but the triple quadrupole MS avoids any mix-ups. Multiple fragment ions from the same parent ion can be detected to confirm the presence of a target analyte more convincingly **[81]**.

A considerable disadvantage of a triple quadrupole MS in comparison to single quadrupole is the decrease in the detected analyte signal due to losses in the collision cell, where the signal is spread across multiple ion fragments, from which only one or two are detected. If a target analyte produces a low concentration of ionic fragments, triple quadrupole MS can produce a lower signal/noise ratio than single quadrupole **[81]**.

In complex sample matrices, quantitative MS analysis limitations are due to issues such as ion suppression and other matrix effects which are amplified by the imperfections of the MS system like contaminated ionization source or unsteady sample flow rate, which can all result in significant inter-day response deviations. To counteract this, internal standards (ISs) are used, which are stable isotopes of target analytes with identical chemical properties but separately identified and quantified in MS. Stable isotope dilution is performed when ISs are spiked into the samples to be analysed and therefore also consider analyte losses in sample preparation and processing, yielding highly accurate and precise results **[81]**.

2.5. Objectives

This project aims to contribute to the application of WBE to determine whether the consumption of various licit and illicit drugs in Portugal changed during the pandemic, establishing their variation patterns, and the possible human health and environmental risks that those changes may pose. The compounds to be studied include pharmaceuticals such as those used to treat COVID-19 (approved and not approved), and representatives of the following classes: antidepressants, anti-inflammatories, analgesics, antibiotics, antivirals, chronic medication, and illicit drugs. In this way, the data can be associated with changes in the behaviour and consumer patterns of the population **[22,59]**. Depending on the results, measures to curb the health and environmental risks imposed by these possible emerging contaminants may be recommended. Moreover, conclusions can be drawn about the need

for information about the consumption of non-prescribed drugs. To the best of our knowledge, this is the first project applying WBE in Portugal for assessing pharmaceutical consumption variations caused by the COVID-19 pandemic. To this end, wastewater samples have been collected over the course of the pandemic.

As part of this project, the present work focused on the development and application of a sample preparation methodology – LLE and SPE – able to clean-up wastewater influent samples and preconcentrate the target analytes, thus allowing their determination at residual levels. Initially, LLE experiments with UP water and wastewater were conducted and an optimized LLE method was developed for comparison with an unoptimized SPE method. Then, the SPE parameters were studied regarding the recovery of the target CECs, and an optimized SPE method was chosen to process wastewater samples. UHPLC-MS/MS was used to perform all determinations. The SPE-UHPLC-MS/MS method was validated, and quality assurance/control were accounted for. Wastewater samples collected from the influent of a mixed urban and industrial zone WWTP in Portugal over 2020 at different phases of the COVID-19 pandemic in Portugal were analysed for the target CECs concentrations to establish occurrence patterns across different phases of the pandemic.

3. State of the art

The increasing environmental occurrence of CECs is a problem that urges immediate action. Under the context of the COVID-19 pandemic, some researchers reported an increased consumption of licit and illicit drugs. These small organic molecules can contaminate soil and surface waters due to their excretion rates into wastewater **[31]**. As such, there is a need to develop analytical methods for detecting and quantifying residues of organic pollutants in contaminated aqueous matrices (*e.g.*, wastewater) that are usually found in the ng L^{-1} to µg L^{-1} range **[31]**. Due to laborious, expensive and time-consuming aqueous sample preparation and processing techniques, there has been a research trend on the development of methods capable of analysing low concentrations of multiple target compounds and with different chemical properties, including polar, nonpolar, neutral and ionic compounds. For sample processing, SPE has been increasingly used over LLE due to generally higher recoveries and lower organic solvent requirements and the capacity to clean up some interferents, lowering matrix effects **[82]**. **Table 1** lists some analytical methods developed for the preparation of wastewater matrices for the analysis of multiple small organic CECs, such as licit and illicit drugs:

Table 1. Analytical methods developed for wastewater sample processing and quantification of multiple small organic CECs

Target CECs	Sample pre-treatment	Sample processing	Sample analysis	Method performance	Reference
19 pharmaceuticals 4 illicit drugs	Centrifugation 17000g, 5 min.	SPE Cartridges: Strata X-C. Conditioning: 2 mL MeOH + 2 mL water. Sample loading: 20 mL sample/MeOH 50% (*v/v*). Washing: 4 mL water/MeOH 50% (*v/v*). Drying: vacuum, 10 min. Elution: 750 µL of 2% FA in isopropanol/ACN (2:8, *v/v*). Evaporation: nitrogen, 60 ºC. Reconstitution: water/ACN 50% (*v/v*).	LC-MS/MS System: Agilent 1100 series HPLC. MS: QTrap 4000. Column: Phenomenex Kinetex C8 100 Å 2.6 µm, 100 X 2.1 mm i.d. Mobile phase: linear gradient of an aqueous 0.5% acetic acid solution and MeOH. Flow rate: 200 µL/min.	Caffeine, nicotine, cocaine, amphetamine, MDMA, methamphetamine, lidocaine, venlafaxine, carbamazepine, metoprolol, morphine, tramadol, acetaminophen, codeine and trimethoprim quantified. MDLs and analyte detection range not reported.	**[22]**
8 antiretroviral drugs 2 metabolites	Filtration 2.7 µm followed by 0.45 µm Whatman GF/D glass microfiber filters.	SPE Cartridges: Strata SDB-L. Conditioning: 4 mL ACN + 4 mL MeOH + 4 mL deionised water. Sample loading: 50 mL. Drying: nitrogen. Elution: 4 mL MeOH/dichloromethane /FA (49:49:2, *v/v/v*). Evaporation: nitrogen. Reconstitution: MeOH/water (3:7, *v/v*).	LC-MS/MS System: Waters Acquity UHPLC. MS: Xevo TQ-S triple quadrupole. Column: Waters HSS T3 1.8 µm, 150 X 2.1 mm i.d. Mobile phase: gradient of an aqueous 0.1% FA solution and ACN. Flow rate: 300 µL/min.	Efavirenz, emtricitabine, lamivudine, nevirapine, ritonavir, 8,14-dihydroxy-Efavirenz and 12-hydroxy-Nevirapine quantified. MQLs in the range of 0.43-0.92 ng/mL. Analytes detected in the range of 0.128-625 ng/mL.	**[83]**

1 antiviral 9 macrolides 10 fluoroquinolones 12 sulfonamides 40 glucocorticoids	Chemical treatment Na_2EDTA (0.25 g) and ascorbic acid (150 mg) added to 1 L water samples. Filtration 0.45 µm glass fibre filters.	SPE Cartridges: Oasis® HLB. Conditioning: 10 mL MeOH + 10 mL HPLC grade water. Sample loading: 1 L. Washing: 10 mL HPLC grade water. Drying: vacuum. Elution: 10 mL MeOH/ammonia (95:5, *v/v*). Evaporation: nitrogen Reconstitution: MeOH/water (20:80, *v/v*). Filtration of extracts Unspecified 0.22 µm membrane.	LC-MS/MS System: Waters Acquity UPLC. MS: Xevo triple quadrupole. Column: CEH-C18 1.7 µm, 100 X 2.1 mm i.d. Mobile phase: gradient of an aqueous 0.1% FA solution and MeOH with 0.1% FA. Flow rate: 250 µL/min.	Quantification of 27 target analytes, including 1 antiviral, 9 sulfonamides, 3 fluoroquinolones, 4 macrolides and 10 glucocorticoids. MQLs in the range of 0.05-2.76 ng/mL. Analytes detected in the range of 2.61-1122 ng/L.	**[84]**
112 pharmaceuticals including NSAIDs, antihypertensives, diuretics, antiepileptics, antilipidemics, antibiotics, analgesics, antivirals and others	Filtration Whatman GF/F 0.7 µm glass fibre filters.	SPE Cartridges: multilayer with Oasis® HLB, Isolute ENV+, Strata-X-AW and Strata-X-CV. Conditioning: 3 mL MeOH + 3 mL water. Sample loading: 100 mL, pH adjusted to 6.5 (FA 0.1 M). Drying: vacuum, 1 h. Elution: 4 mL MeOH/ethyl acetate 50% (*v/v*) with 2% ammonia. Evaporation: nitrogen. Reconstitution: MeOH/water 50% (*v/v*). Filtration of extracts Phenomenex Regenerated Cellulose syringe filters (0.2 µm).	LC-MS/MS System: Thermo Accela UHPLC. MS: TSQ Quantum Access triple-200 quadrupole. Column: Waters Atlantis T3 C18 3 µm, 100 X 2.1 mm i. d. Mobile phase: gradient of an aqueous 0.01% FA solution and MeOH. Flow rate: 100 µL/min.	Quantification of 95 of the target analytes. MQLs not reported but MDLs in the range of 0.1-104.7 ng/L. Analytes detected in the range of 5-58239 ng/L.	**[26]**
46 pharmaceuticals 6 personal care products 19 illicit drugs 7 generic chemicals	Centrifugation 3000 rpm, 15 min (centrifuge model or distance to centre of rotation not reported).	LLE 525 µL of sample mixed with 525 µL of ACN, vortexed and centrifuged at 14000 rpm for 20 min at 4ºC. Filtration of extracts Laboratory Sciences polyethylene external filter tips (10-90 µm).	LC-MS/MS System: Thermo Ultimate 3000 HPLC. MS: Q-Exactive (positive electrospray ionization only). Column: Agilent SB-C18 RRHD 1.8 µm, 150 X 2.1mm i. d. Mobile phase: gradient of an aqueous 0.1% FA solution and ACN with 0.1% FA. Flow rate: 200 µL/min.	Detection of 11 pharmaceuticals, 1 personal care product, 7 illicit drugs and 1 generic chemical. MDLs and quantification range not reported (quantification not performed).	**[85]**

Acetaminophen Diclofenac Salbutamol Ceftriaxone	Chemical treatment Addition of sodium thiosulfate for dichlorination (80 mg/L).	SPE Cartridges: unspecified C18 adsorbent packed into disks. Conditioning: 10 mL MeOH + 20 mL UP water. Sample loading: 200 mL. Washing: 10 mL MeOH/water 15% (*v/v*). Drying: vacuum, 15 min. Elution: 15 mL MeOH. Evaporation: nitrogen, 45 ºC. Reconstitution: MeOH/water 50% (*v/v*).	LC-MS/MS System: Shimadzu AHT2010 HPLC-MS/MS. MS: integrated photodiode array detector. Column: Phenomenex C18 column 250 X 4.6 mm i.d. Mobile phase: gradient of an aqueous 0.02% ammonium hydroxide solution and MeOH with 0.02% ammonium hydroxide. Flow rate: 200 µL/min.	Quantification of all target analytes, with MDLs not reported. Acetaminophen detected at 4.60 µg/mL, diclofenac up to 4.2 µg/mL, salbutamol up to 18.7 µg/mL and ceftriaxone up to 29.15 µg/mL.	**[86]**
9 antiviral drugs	Chemical treatment Wastewater samples mixed with MeOH to 5% (*v/v*), in order to inhibit microbial growth. Filtration Whatman 0.7 µm glass fibre filters.	SPE Cartridges: Oasis® HLB. Conditioning: 10 mL MeOH + 10 mL distilled water Sample loading: 500 mL. Washing: 100 mL MeOH/water 5% (*v/v*). Drying: vacuum, 2 h. Separate elutions: 4 mL MeOH, 3 mL dichloromethane and 3 mL ethyl acetate. Evaporation: nitrogen. Reconstitution: MeOH. Filtration of extracts Unspecified 0.22 µm nylon membrane filter.	LC-MS/MS System: Agilent 1290 series UHPLC. MS: Agilent 6470 series triple quadrupole (positive electrospray ionization only). Column: Agilent ZORBAX eclipse plus C18 1.8 µm, 50 X 2.1 mm i.d. Mobile phase: linear gradient of distilled water with 5 mM ammonium acetate and MeOH with 0.2% FA. Flow rate: 300 µL/min.	Abacavir, zidovudine, efavirenz, nevirapine, ritonavir, lopinavir, lamivudine, and telbivudine quantified. MQLs in the range of 0.026-3.846 ng/L. Analytes detected in the range of 0.97-3043 ng/L.	**[87]**
Acetaminophen Diclofenac Ibuprofen Naproxen Sulfadiazine Sulfamethoxazole	Sequential filtrations Unspecified quantitative paper filter, 1 µm glass microfilter and 0.45 µm PTFE membrane.	SPE Cartridges: Oasis® HLB Conditioning: 400 µL aqueous 5 mg/L EDTA solution. Sample loading: 400 mL. Washing: 3 mL water. Drying: nitrogen, 15 min. Elution: 8 mL MeOH. Filtration of extracts Unspecified 0.2 µm cellulose nitrate membrane. Evaporation: nitrogen, 5 psi and 35ºC. Reconstitution: UP water.	LC-MS/MS System: Dionex Ultimate 3000. MS: Thermo TSQ Endura triple quadrupole. Column: Phenomenex Kinetex EVO C18 2.6 µm, 50 X 2.1 mm i.d. Mobile phase: linear gradient of an aqueous 0.5% FA solution with 0.01 mM ammonium acetate and MeOH. Flow rate: 300 µL/min.	Acetaminophen, diclofenac, ibuprofen, naproxen, sulfadiazine and sulfamethoxazole quantified. MQLs in the range of 2.24-40 ng/mL. Analytes detected in the range of 1.5-19 ug/L.	**[88]**

35 pharmaceuticals	Sequential filtrations Whatman 1 µm glass fibre filters and 0.45 µm nylon membrane filters.	SPE Cartridges: Oasis® HLB. Conditioning: 6 mL MeOH + 6 mL HPLC grade water. Sample loading: 100 mL. Washing: 5 mL HPLC grade water. Drying: vacuum, 20 min. Elution: 6 mL MeOH. Evaporation: nitrogen. Reconstitution: MeOH/water 10% (*v/v*).	LC-MS/MS System: Spark Holland Symbiosis Pico HPL MS: 4000 QTRAP. Column: Merck Purospher Star RP-18 5 µm, 125 X 2 mm i.d. Mobile phase: linear gradient of an aqueous 0.1% FA solution and ACN with 0.1% FA. Flow rate: 300 µL/min.	Diclofenac, indomethacin, naproxen, salicylic acid, gemfibrozil, bezabirate, sulfamethoxazole, trimethoprim, atenolol and carbamazepine quantified. MQLs in the range of 7.5-262 ng/L. Analytes detected in the range of 4-14900 ng/L.	**[89]**
35 pharmaceuticals and personal care products	Filtration Whatman GF/F 47 mm glass fibre filters. Chemical treatment Unspecified amount of Na_2EDTA and *L*-ascorbic acid added to samples to prevent target compound degradation. Sample pH adjusted to 4 with HCl 1 M.	SPE Cartridges: Oasis® HLB. Conditioning: 12 mL MeOH + 12 mL deionized water + 6 mL acidified (pH = 4) deionized water. Sample loading: 100 mL. Washing: 10 mL UP water. Drying: pressurized air, 10 bar. Elution: 8 mL MeOH/water 50% (*v/v*). Evaporation: nitrogen. Reconstitution: MeOH/water 50% (*v/v*) with 0.025% FA. Filtration of extracts 0.22 µm Whatman 13 mm PFTE microfiltration membrane.	LC-MS/MS System: Dionex Ultimate 3000. MS: AB SCIEX API 3200 triple quadrupole. Column: Agilent XDB C18 3.5 µm, 150 X 3 mm i.d. Mobile phase: gradient of an aqueous 0.01% FA solution and MeOH. Flow rate: 300 µL/min.	33 pharmaceuticals and personal care products detected. MDLs and detected concentration range not reported.	**[90]**

FA – formic acid; MDL – method detection limit; MQL – method quantification limit; PTFE - Polytetrafluoroethylene

From the studies summarized in **Table 1**, it is clear that wastewater samples that are aimed to be analysed by LC-MS/MS typically undergo at least one pre-treatment step, either chemical treatments with the objective of preserving the target analytes, or a solid/liquid separation process (*e.g.*, centrifugation or filtration). For sample processing, the most common process is SPE and the most common cartridges are Oasis® HLB, whereas the most common elution solvent is MeOH, often mixed with another solvent (*e.g.*, water or ethyl acetate). After elution, the solvent is evaporated with nitrogen, and reconstituted, usually in MeOH or a MeOH/water mixture. Regarding the analysis of the wastewater samples, C18 chromatography columns are the most used, while the typical mobile phase is a gradient of water and an organic solvent, often with a small fraction of formic acid (FA), with flow rate in the 100-300 µL/min range. The detected concentrations of the target CECs in these studies are in the ng/L and low µg/L range.

4. Materials & Methods

4.1. Wastewater sampling

Wastewater samples were collected from the influent of a WWTP served by a mixed urban and industrial zone, located in Portugal. Samples were attempted to be collected bi-weekly since April 2020, on Mondays and Thursdays, but pandemic lockdown measures led to gaps in some days. Samples were collected using a glass bottle with the aid of an extensible bottle holder and were immediately frozen at -20 ºC until pre-treatment, processing, and analysis.

The WWTP serves a population-equivalent of under 200 thousand, handling an average flow rate of roughly 40 thousand cubic metres of liquid influent every day. It employs conventional treatment processes, starting with a pre-treatment of the influent through screening of bigger solid particles, followed by the primary treatment, consisting of decantation and surface scraping to remove remaining solids. The liquid sludge from the primary treatment undergoes secondary treatment in aerated bioreactors to remove small organic compounds. The WWTP is also equipped with a secondary decantation process for the effluent from the aerated tanks, to remove any suspended solids dragged from the bioreactors.

4.2. Chemicals and materials

In this study, 17 CECs were selected as target analytes: 2 analgesics, 1 antibiotic, 3 antidepressants, 5 anti-inflammatories, 1 antiviral, 3 β-blockers, 2 antimalarials, and 2 illicit drugs. **Table 2** lists the main class, molar mass and p*K*a of the different compounds, while **Table B1** of **Annex B** lists the chemical structures. Azithromycin, chloroquine, and hydroxychloroquine were also specifically used to treat COVID-19 symptoms. Powder form analytical standards of the licit pharmaceuticals with a minimum purity of 98% were acquired from different corporations: remdesivir was acquired from Ambeed (Arlington Heights, USA); chloroquine was supplied by the European Pharmacopoeia (Strasbourg, France); hydroxychloroquine, naproxen, carbamazepine, tramadol, venlafaxine hydrochloride, fluoxetine hydrochloride, citalopram hydrobromide, azithromycin, atenolol, propranolol hydrochloride, metoprolol tartrate and dexamethasone were acquired from Sigma-Aldrich (Steinhein, Germany); acetaminophen, ibuprofen and diclofenac sodium were supplied by the University of Minho (Braga, Portugal). Both illicit drugs (cocaine and morphine) were supplied by Lipomed (Arlesheim, Switzerland) as methanolic stock solutions.

Table 2. Target CECs and their main class, molar mass and p*K*a **[91,92]**

Class	CEC	Used to treat COVID-19	Molar mass (g/mol)	p*K*a
Analgesics	Tramadol	X	263.38	9.41
Antiepileptics	Carbamazepine	X	236.27	13.9
Antibiotics	Azithromycin	✓	748.98	12.43
Antidepressants	Citalopram	X	324.4	9.78
	Fluoxetine	X	309.33	10.05
	Venlafaxine	X	277.4	10.09
Anti-inflammatories	Dexamethasone	X	392.46	12.42
	Diclofenac	X	296.15	4.15
	Naproxen	X	230.26	4.19
Antivirals	Remdesivir	X	602.6	10.23
β-blockers	Atenolol	X	266.34	14.08
	Metoprolol	X	267.37	14.09
	Propranolol	X	259.34	14.09
Antimalarials	Chloroquine	✓	319.9	10.1
	Hydroxychloroquine	✓	335.87	15.59
Illicit drugs	Cocaine	X	303.35	8.61
	Morphine	X	285.34	10.26

Stock solutions of isotopically labelled ISs of some of the pharmaceuticals (azithromycine-D3, fluoxetine-D3 and diclofenac-D4) were acquired from Sigma-Aldrich (Steinhein, Germany), while ISs of the illicit drugs (cocaine-D3 and morphine-D3) were supplied by Lipomed. From these, a 2500 µg/L ISs stock solution was prepared in ACN.

Stock solutions of each pharmaceutical analytical standard at 1000 mg/L were prepared in MeOH by dissolving known amounts of each in this solvent. A 400 µg/L working solution containing the 19 target CECs was prepared by dilution of a given volume of each stock solution in ACN, and a 200 µg/L stock solution of ACN/water 50% (v/v) was prepared by dilution of this working solution in UP water. Similarly, calibration curve solutions with concentrations of 10, 25, 50, 100, 150 and 200 µg/L of all target CECs in ACN/water 50% (v/v) were also prepared by diluting the 400 µg/L working solution in UP water.

EtOH (HPLC grade), ACN and MeOH (MS grade) were acquired from VWR International (Fontenay-sous-Bois) and used to prepare the stock solutions, for the extraction assays or as mobile phase for chromatography. FA for UHPLC-MS/MS (>98%) was supplied by TCI Chemicals (Belgium). UP water was produced by a Milli-Q water system from Merck (Darmstadt, Germany).

1 mol/L HCl and 0.02 mol/L NaOH aqueous solutions were used for wastewater acidification and alkalinization respectively. A pHenomenal pH 1100L pH meter (VWR, Germany) was used for pH measurements. Oasis® HLB (Hydrophilic-Lipophilic-Balanced) cartridges (150 mg, 6 mL) obtained from Waters (Milford, MA, USA) were used for SPE. 0.22 µm (13 mm Ø) PTFE syringe filters were acquired from VWR (Germany).

4.3. Sample pre-treatment and processing

Pre-treatment of wastewater samples consisted of a preliminary centrifugation with a Hettich Zentrifugen Rotofix 32 A at 4000 rpm for 10 min, followed by a subsequent decantation of the liquid phase to be extracted by SPE. The sample processing method was optimized for the recovery of the target CECs through several sets of extraction tests involving LLE and SPE under varying conditions. The recovery of the analytes in each assay was calculated with the objective of identifying the extraction parameters yielding the highest recoveries.

4.3.1. Overview of the extraction optimization tests

Each set of recovery tests was performed after the previous one and based on the results of the previous one. In total, five sets were performed, labelled A, B, C, D and E. The techniques, sample matrices tested and objective of each set of recovery tests are presented in **Table 3**:

Table 3. Test sets performed for optimizing the recovery of the target CECs and corresponding technique, sample matrices and objective

Test set	Technique	Sample matrix	Objective
A	LLE	UP water and wastewater	Verify the applicability of LLE to the wastewater samples.
B	LLE and SPE	UP water and wastewater	Compare the performance of LLE and SPE.
C	SPE	UP water and wastewater	Perform SPE with the same conditions as test set B, but with a second elution.
D	SPE	Wastewater	Select the SPE solvent that yields the highest overall recovery.
E	SPE	Wastewater	Optimize the remaining SPE parameters (sample pH, loaded sample volume and solvent elution volume).

Across all sets of recovery tests, different sample types were extracted, including spiked UP water, blank wastewater, spiked wastewater. Additionally, blank extracts were spiked after extraction, herein called post-spiked blank (PSB) wastewater. For spiking UP water and wastewater samples, 125 µL of the working solution of 400 µg/L were added to each sample.

In all assays, the LLE or SPE extracts were collected in glass tubes and evaporated to dryness for 2 hours at 45ºC, using a LABCONCO Centrivap Concentrator device (Kansas City, USA). Afterwards, the dried extracts were reconstituted in 250 µL of ACN/water 50% (v/v) and filtered through a 0.22 µm PTFE syringe filter, being the theoretical concentration of a fully recovered extract of 200 µg/L. Then, the reconstituted extracts of spiked UP water, blank wastewater and spiked wastewater were injected into the UHPLC-MS/MS system to calculate recoveries. Reconstituted extracts of PSB wastewater were firstly mixed with an equal volume of the working solution of 400 µg/L to achieve a theoretical spiked concentration of 200 µg/L, before being injected into the UHPLC-MS/MS system.

4.3.2. LLE optimization tests procedures and calculations

LLE assays were performed with varying parameters to extract the CECs from wastewater samples (test set A) and the recoveries of the CECs in each assay were calculated for identifying the extraction conditions yielding the highest recoveries.

Briefly, three different sample types (spiked UP water, blank and spiked wastewater) were subjected to LLE with ACN in test set A, by varying solvent/sample volume ratios (30, 40, 50%), with assays being performed in duplicate. The pH of the wastewater and UP water samples was unaltered. The extraction conditions for this set of experiments are listed in **Table 4**:

Table 4. LLE conditions used in test set A to extract spiked UP water, blank and spiked wastewater, with varying ACN/sample volume ratios (30, 40, 50%)

Solvent fraction (%)	V sample (mL)	V solvent (mL)
30	31.5	13.5
40	27	18
50	22.5	22.5

The LLE procedure was executed as follows: a certain volume of either wastewater or UP water was poured into a 50 mL Falcon tube and a corresponding volume of solvent was added to each sample. Then, all Falcon tubes were vortexed for 10 seconds and refrigerated at -20º C for a minimum of 24 hours. After freezing the aqueous phase, the unfrozen supernatant extraction solvent was decanted into glass tubes to be evaporated and reconstituted in 250 µL of mobile phase, before injection into the UHPLC-MS/MS system for analysis.

The LLE was repeated by using the best conditions for the overall CECs (test set A, solvent fraction 50%) to be compared with SPE (test set B).

The LLE recoveries were calculated based on the comparison of the areas of chromatographic peaks of each CEC in the extracts and those areas obtained for the 200 µg/L stock solution (diluted in the same solvent as reconstitution solvent), which concentration corresponded to the theoretical concentration of a fully recovered extract of a spiked sample. Recovery (R) of each CEC in the spiked wastewater extracts was calculated via **Equation 1**:

$$R(\%) = \frac{(A_s - A_b)}{A_t} * 100\% \quad (\mathbf{1})$$

A_s – chromatographic peak area of a target CEC in spiked wastewater; A_b – chromatographic peak area of a target CEC in blank wastewater; A_t – chromatographic peak area of a target CEC in the 200 µg/L stock solution

The recovery (R) of each CEC in the spiked UP water extracts was calculated via **Equation 2**:

$$R(\%) = \frac{A_s}{A_t} * 100\% \quad (\mathbf{2})$$

A_s – chromatographic peak area of a target CEC in spiked UP water; A_t – chromatographic peak area of a target CEC in the 200 µg/L stock solution

4.3.3. SPE optimization tests procedures and calculations

Four sets of SPE assays (test sets B, C (both preliminary), D and E) were performed to optimize the extraction conditions to achieve the best overall recovery for the target CECs. The same experimental setup was used for all SPE assays, consisting of a WAT200607 extraction manifold coupled with a sealed vacuum glass container, both acquired from Waters (Milford, MA, USA), connected to two liquid trap containers in sequence, and a pump to establish a vacuum inside the glass container. The pump exhaust pipe was directed towards an active exhaust chamber. The experimental setup was as shown in **Figure 6**:

Figure 6. Experimental setup for SPE assays. Left – full setup; Middle – closeup of extraction manifold (with cartridges and tubes in place); Right – closeup of liquid trap containers and pump

Oasis® HLB cartridges were selected, and the parameters studied included the conditioning and elution solvent, sample pH, sample loading volume, and solvent elution volume. Generally, the SPE procedure was executed as follows: cartridges were conditioned sequentially with 4 mL of solvent and 4 mL of UP water under atmospheric pressure. Next, the sample was loaded at a regular flow rate of *ca.* 2 mL/min, supported by vacuum pump to counteract adsorbent blocking by residual solids.

Then, a washing step was performed with 4 mL of UP water, which was followed by a drying step for 45 min. The elution was performed drop by drop into glass tubes, which was then evaporated, and the dried extracts were reconstituted before injection into the UHPLC-MS/MS system for analysis.

Three sample types (spiked UP water, blank and spiked wastewater) were subjected to duplicate SPE using MeOH as solvent (test set B). This procedure was repeated with a second elution being performed, also with MeOH, that was collected into a separate glass tube (test set C). Then, two sample types (blank and spiked wastewater) were subjected to SPE with MeOH, ACN or EtOH (test set D), each blank being performed in duplicate and spiked wastewater in triplicate. The SPE procedure for these three sets of experiments was similar, with unaltered sample pH, a sample loading volume of 45 mL and a solvent elution volume of 4 mL.

For preliminary sets B and C, the SPE recoveries obtained in the spiked wastewater and spiked UP water extracts were calculated by comparing the areas of chromatographic peaks, respectively by **Equations 1** and **2**.

For test set D onwards (test set E and validation), the concentrations of each CEC in the extracts were estimated by interception of the calibration curve built with standard solutions ranging from 10 to 200 µg/L in ACN/water 50% (v/v)). The recoveries (R) of each CEC in the spiked wastewater extracts were calculated by **Equation 3**:

$$R(\%) = \frac{(C_s - C_b)}{200} * 100\% \qquad (\mathbf{3})$$

C_s – target CEC concentration in spiked wastewater; C_b – target CEC concentration in blank wastewater

4.3.4. Design of experiments (DoE)

Based on the results of test set D, the last test set (E) used the best performing solvent (EtOH), and a design of experiments (DoE) was employed for optimizing the remaining SPE parameters (sample pH, sample loading volume and solvent elution volume), through Minitab 21 (Minitab Statistical Sofware, PA, USA). Blank and spiked wastewater samples were extracted, with spiked wastewater assays performed in triplicate. One blank extract of each assay was also post-spiked (PSB) with a concentration equivalent to a fully recovered extract, which at this stage allowed for more accurate estimation of recoveries, since the matrix effect is equivalent in the spiked wastewater and the PSB wastewater samples. The SPE parameters of test set E were generated by Minitab 21, by applying three-factor Box-Behnken Design (BBD), which required the input of the limiting extremes of each parameter: (i) pH 5 – 10, (ii) solvent elution volume 4 – 7 mL, and (iii) sample loading volume input as 25 – 50 mL to avoid the cartridges fouling whereas providing a preconcentration factor able to allow the determination of the target CECs. The designated coded values for the variables, -1, 0 and 1, were used to represent low, middle, and high levels, respectively, and the coded and actual values of the variables are listed in **Table C1**. 15 unique sets of SPE parameters were generated, each corresponding to a test, including a triplicate on the centre point, occupying tests 13, 14 and 15, all

of which were merged into test 13. The general procedure of these assays was the same as the previous SPE tests, with only a single elution being performed, and the sole solvent being EtOH. Sample pH, sample loading volume and solvent elution volume varied for each test, as listed in **Table 5**:

Table 5. SPE conditions used for test set E

Test	Sample pH	Volume of sample loaded (mL)	Volume of elution solvent (mL)
1	5	37.5	4
2	10	37.5	4
3	5	37.5	7
4	10	37.5	7
5	5	25	5.5
6	10	25	5.5
7	5	50	5.5
8	10	50	5.5
9	7 (natural pH)	25	4
10	7 (natural pH)	25	7
11	7 (natural pH)	50	4
12	7 (natural pH)	50	7
13	7 (natural pH)	37.5	5.5

As with test set D, in test set E the concentration of each analysed sample was estimated through the calibration curve solutions. The recoveries (R) of each CEC (test set E) were calculated by **Equation 4**:

$$R(\%) = \frac{(C_s - C_b)}{(C_{PSB} - C_b)} * 100\% \quad (4)$$

C_s – target CEC concentration in spiked wastewater; C_b – target CEC concentration in blank wastewater; C_{PSB} – target CEC concentration in PSB wastewater

4.3.5. Method validation and samples processing

Before pre-treatment, dated wastewater samples were spiked with 25 µL of the 2500 µg/L ACN ISs stock solution, for a theoretical ISs concentration in the reconstituted extracts of 250 µg/L, in

order to apply the ISs calibration method. Each CEC was assigned an IS for concentration calculations, and this information is presented in **Tables B2** and **B3** of **Annex B**.

The optimized SPE conditions corresponded to test 5 of test set E (**Table 5**), which gave the highest overall recoveries of the target CECs: blank and spiked wastewater samples were subjected to SPE as previously described, with an acidified sample pH of 5, a sample loading volume of 25 mL, and a solvent elution volume of 5.5 mL. As such, the concentration factor of the optimized SPE method (CF) was 100 and the IS concentration in each sample was 2500 ng/L.

The blank wastewater samples were prepared in duplicate and the spiked blank wastewater samples were prepared in triplicate. Eight triplicates of blank wastewater samples were spiked with different volumes of the 400 µg/L working solution (concentrations in the spiked blank wastewater were 50, 100, 250, 500, 1000, 2500, 5000 and 10000 ng/L) and with a fixed volume of ISs working solution, as a mean to establish method-adjusted calibration curves that accounted for both the incomplete recovery of the target CECs and the matrix effect. For each CEC, the measured chromatographic peak area in each sample was divided by the chromatographic peak area of the corresponding IS, obtaining the area ratio for that CEC in that sample (A/A_{IS}). In the case of the method-adjusted calibration curves, the area ratio in the blank wastewater samples was subtracted from the area ratio in each spiked wastewater sample. The slope (m), y-intercept (b) and determination coefficient (r^2) of the method-adjusted calibration curve of each CEC are listed in **Table C2 of Annex C.** The CEC concentrations of each wastewater sample collected in the year of 2020 (C_{WW}) were obtained by interception of the area ratio (A/A_{IS}) into the corresponding method-adjusted calibration curves (**Equation 5**):

$$\frac{A}{A_{IS}} = m * \frac{C_{WW}}{2500} + b \qquad (\mathbf{5})$$

A/A_{IS} – ratio of chromatographic peak areas of a given target CEC and its assigned IS; C_{WW} – target CEC concentration in a wastewater sample; m – slope of method-adjusted calibration curve; b – y-intercept of method-adjusted calibration curve

4.4. UHPLC-MS/MS analysis

All identification and quantification analysis were performed via UHPLC-MS/MS with a Shimadzu Nexera X2 UHPLC system (consisting of two LC-30AD pumps, a SIL-30AC autosampler, a DGU-20A degassing unit, a CTO-20AC column oven, a CBM-20A system controller with a Shimadzu LC Solution Version 5.41SP1 software) (Kyoto, Japan) and an Ultra Fast Mass Spectrometry series LCMS-8040 triple quadrupole MS.

Different mobile phases were tested, composing of a binary mixture of UP water and ACN, and using a Cortecs UPLC C18+ 1.6 µm, 100 X 2.1 mm i. d. column acquired from Waters (Milford, USA), to select the one that yielded chromatographic peaks with the highest resolution. For analysing diclofenac, ibuprofen and naproxen, elution was performed in a gradient, with ACN starting at 30%

gradually increasing to 70%, with a total run time of 5 minutes. For analysing the remaining target analytes, UP water was acidified with 0.1 % FA, and elution was performed in a gradient, with ACN starting at 20% gradually increasing to 80% within 1 minute, with a total run time of 5 minutes. For both gradients, the total flow rate was of 200 µL/min and the injection volume was of 5 µL.

An electrospray ionization source was operated in positive and negative modes. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the MS were 4.5 kV, 12.5 dm^3 min^{-1}, 2.5 dm^3 min^{-1}, 250 °C and 400 °C, respectively. Selected reaction monitoring (SRM) was applied to identify and quantify the target analytes. To select the precursor ion of each compound, as well as the two most abundant fragments of each chosen precursor ion, each 1000 mg/L stock solution of individual CECs was diluted to 10 mg/L, and each IS stock solution was also diluted in MeOH to 10 mg/L, all being injected into the UHPLC-MS/MS system by flow injection analysis. SRM1 was used for quantification and the ratio between SRM1 and SRM2 as well as the retention time for confirming the identification of the analytes.

4.5. Quality assurance/quality control of the SPE-UHPLC-MS/MS method

The SPE-UHPLC-MS/MS method was validated according to the International Conference on Harmonization Guidelines **[93]**, considering the following parameters: linearity and range of application, recovery, accuracy, precision, and instrument and method limits of detection and quantification.

Linearity was evaluated by analysing the correlation coefficient (r^2) of the method-adjusted calibration curves obtained by spiking wastewater samples with different volumes of working solution and processing them through the optimized SPE method before analysis, each one prepared in triplicate. Eight different spiked concentrations of the standard samples were prepared: 50, 100, 250, 500, 1000, 2500, 5000 and 10000 ng/L, all spiked with 2500 ng/L of ISs. Information on the method-adjusted calibration curves is presented in **Table C2 of Annex C**. In order to estimate accuracy and precision, three quality control (QC) samples were prepared at three different concentrations from those used to define the method-adjusted CEC calibration curves (350, 3500 and 7000 ng/L), all in triplicate, and were also initially spiked with 2500 ng/L of ISs before processing by the optimized SPE method. The accuracy of the method was evaluated as the percentage of agreement between the method results for the QC samples, calculated through the method-adjusted calibration curves (**Equation 5**) and the nominal concentration of each target CEC. Intra-batch precision was expressed as the relative standard deviation (RSD) of the replicate measurements of the triplicate QC samples at three different concentration levels in the same day. The instrument detection (IDL) and quantification (IQL) limits of each CEC were determined by the LC Solution software, by injection of the ACN/water 50% (*v/v*) calibration curve solutions and based on the signal-to-noise (S/N) ratio, being IDL the minimum detectable concentration that yields a S/N value of 3.3

and IQL the minimum detectable concentration that yields a S/N value of 10. Method detection (MDL) and quantification (MQL) limits of each CEC were calculated via **Equations 6** and **7**, respectively:

$$MDL = \frac{IDL}{CF * PE} \quad (\mathbf{6})$$

IDL – instrument detection limit of a given target CEC; CF – concentration factor of the optimized SPE method (100); PE – process efficiency for a given target CEC

$$MQL = \frac{IQL}{CF * PE} \quad (\mathbf{7})$$

IQL – instrument quantification limit of a given target CEC; CF – concentration factor of the optimized SPE method (100); PE – process efficiency for a given target CEC

The process efficiency (PE) of the SPE-UHPLC-MS/MS method for a given CEC was calculated through **Equation 8**. It evaluates the overall performance of the method, by accounting for both the recovery of the optimized SPE method (R_{opt}) and the matrix effect of the UHPLC-MS/MS analysis.

$$PE(\%) = \frac{R_{opt}(\%) * (100 + ME(\%))}{100} \quad (\mathbf{8})$$

R_{opt} – recovery of the optimized SPE method for a given target CEC; ME – matrix effect for a given target CEC

For assessing the recovery of the optimized SPE method for each CEC, **Equation 4** was applied as previously described, with all samples processed through the optimized SPE method.

For assessing the matrix effect (ME) of the wastewater over the ionization of each CEC during analysis, measured concentrations determined through the ACN/water 50% (v/v) calibration curve of a blank wastewater sample, a PSB wastewater sample and the stock solution at 200 µg/L in ACN/water 50% (v/v) were compared through **Equation 9**. In case of no significant matrix effects, matrix effect is close to 0%, while for ionization enhancement and suppression it yields a value of >0% and <0% respectively.

$$ME(\%) = (\frac{(C_{PSB} - C_b)}{C_t} - 1) * 100\% \quad (\mathbf{9})$$

C_b – target CEC concentration in blank wastewater; C_{PSB} – target CEC concentration in PSB wastewater; C_t – measured target CEC concentration in 200 µg/L stock solution

4.6. Data analysis

With the aim of checking whether CEC concentrations differed significantly between three phases of the 2020 COVID-19 pandemic in Portugal, t-Tests were performed, with the Excel Data Analysis ToolPak, assuming: (i) unequal variances; (ii) significance level of 95%; (iii) two-tail p-value. For the data sets with >30% of points not quantified, the data analysis was not performed.

5. Results and Discussion

5.1. UHPLC-MS/MS optimization

The simultaneous determination of the 17 target compounds at trace levels was provided by their separation by UHPLC and their identification and quantification by tandem MS detection, applying a triple quadrupole mass analyser.

The precursor ion of each analyte and IS was firstly identified by flow injection analysis into the MS detector through the direct injection of individual stock solutions of each compound, which were scanned from a m/z of 100 to a m/z of 1000, under both positive and negative modes. From all the CECs analysed, 15 had a protonated main precursor ion, displaying a superior response to the positive ionization mode, and 2 had a deprotonated main precursor ion, displaying a better response to the negative ionization mode. After choosing the main precursor ion, the transitions of each CEC were confirmed by flow injection analysis of the individual stock solutions into the MS detector and the most abundant fragments were determined. Most of the studied CECs displayed at least two transitions. Only 2 pharmaceutical compounds (*i.e.*, fluoxetine and tramadol) demonstrated a poor fragmentation and only one SRM could be monitored, a drawback overcome by the ISs calibration method, using the respective surrogate standards during the wastewater sample analyses. The SRM instrument parameters, including ionization mode, main precursor ion, two most abundant transition fragments (SRM1 and SRM2), declustering potential, collision energy and collision cell exit potential of all CECs and ISs are listed respectively in **Tables B2** and **B3** of **Annex B**. The ratios of the transition fragments (SRM1/SRM2) of the CECs, used to confirm their identity, are also presented in **Table B2**, whereas the retention times, an additional identity confirmation parameter, are listed in **Table D1 of Annex D**.

Regarding chromatographic separation, a sub-2 μm particles column was used, which permits short and high-resolution chromatographic runs. Since various CECs were analysed, with varying physicochemical properties, whatever optimized mobile phases are chosen will inevitably result in different sensitivities for different compounds **[94]**. A mobile phase consisting of a binary mixture with a gradient of acidified water and an organic solvent, in this case ACN, was selected to analyse most of the CECs, except for naproxen and diclofenac which were analysed using a mobile phase consisting of water and ACN under isocratic mode.

5.2. Wastewater processing optimization

The wastewater sample processing procedure was optimized with the objective of maximizing the overall recoveries of the different target CECs. For this purpose, several sets of extraction tests consisting of LLE and/or SPE, were performed, each studying the effect of different LLE or SPE parameters on the recoveries. Basic information on the techniques, samples matrices and objectives of each set of tests are presented in **Section 4.3.1** (**Table 3)**.

5.2.1. LLE optimization (test set A)

Different parameters can affect recovery in LLE, namely the organic solvent, the ratio between solvent and sample volumes, the pH of the aqueous sample, the ionic strength of the aqueous sample, the use of chelating and dechlorination agents, the temperature of the extraction medium, the contact time between the two phases, and the mixing efficiency **[95,96]**. In a previous study using 2 model compounds (venlafaxine and diclofenac), it was found that ACN was the best performing extraction solvent **[97]**. Therefore, in this work, ACN was tested for LLE and the solvent fraction in the extraction mixture was optimized, by testing fractions of 30, 40 and 50%. Wastewater and UP water samples were studied as matrices, without pH adjustment. The recovery results are presented in **Figure 7**.

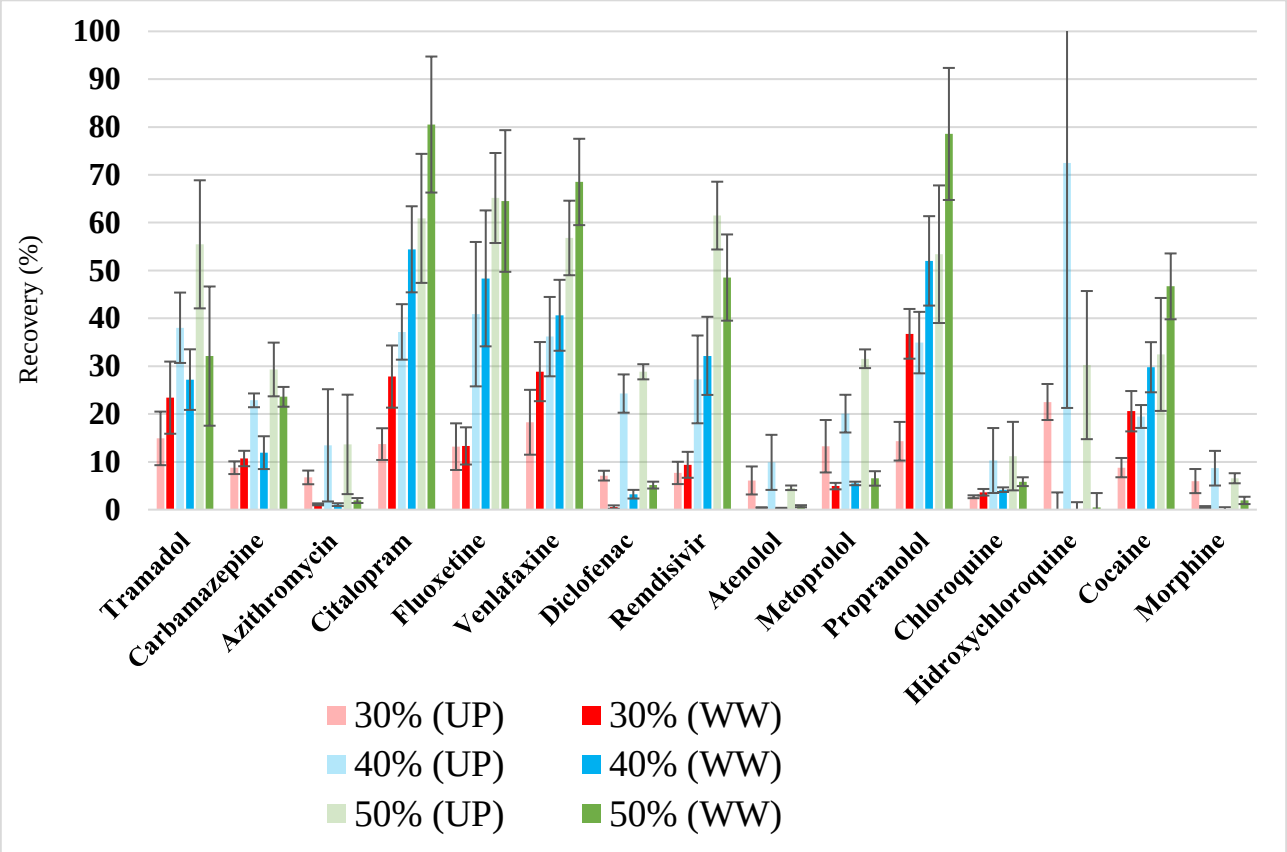

Figure 7. Recoveries obtained by LLE of the analytes from either UP water (UP) or wastewater (WW), using a fraction of ACN as extracting solvent with a ratio of solvent/sample volume of 30% (red), 40% (blue), or 50% (green). Each colour represents one condition tested, with wastewater being fully coloured and UP samples being watermark coloured

Dexamethasone and naproxen were not recovered in any sample. The recoveries of the other target CECs varied depending on the solvent fraction and the type of matrix (*i.e.*, WW and UP), as expected. Generally, recoveries increased with the rising of solvent fraction (50% of ACN), which agrees with previous studies, with higher LLE recoveries having been reported up to a 7:1 ratio of solvent to aqueous sample **[98]**. High standard deviations of recoveries were observed, that are represented by the error bars (**Figure 7**), indicating a lack of reproducibility in this LLE methodology. The pharmaceutical compound hydroxychloroquine was only detected in the extracts recovered from

spiked UP water samples, which can be ascribed to the matrix effect. In general, it was possible to confirm that the wastewater matrix could have caused ionization suppression or enhancement for different compounds since the overall recoveries were different between UP water and wastewater. It is noteworthy that, for a solvent fraction of 30%, lower recoveries were generally obtained in the extracts of the wastewater matrix, while an opposite trend was registered for solvent fractions of 40 and 50%, where lower recoveries were observed in the extracts of UP water. This effect is recognized widely due to the heterogeneous composition of wastewater samples, which components coelute with the target analytes and may generate a suppression of their signals or less commonly an enhancement **[99]**.

5.2.2. LLE and SPE performance comparison (test set B)

In the next assays (set B), the LLE procedure using 50% of ACN was selected for comparison with the reference SPE protocol proposed by Oasis®, which uses MeOH as solvent for conditioning and elution. For this set of experiments, the sample pH was not adjusted, and 45 mL of sample were loaded, with an elution solvent volume of 4 mL. The recovery results obtained for wastewater and UP water samples in test set B are presented in **Figure 8**.

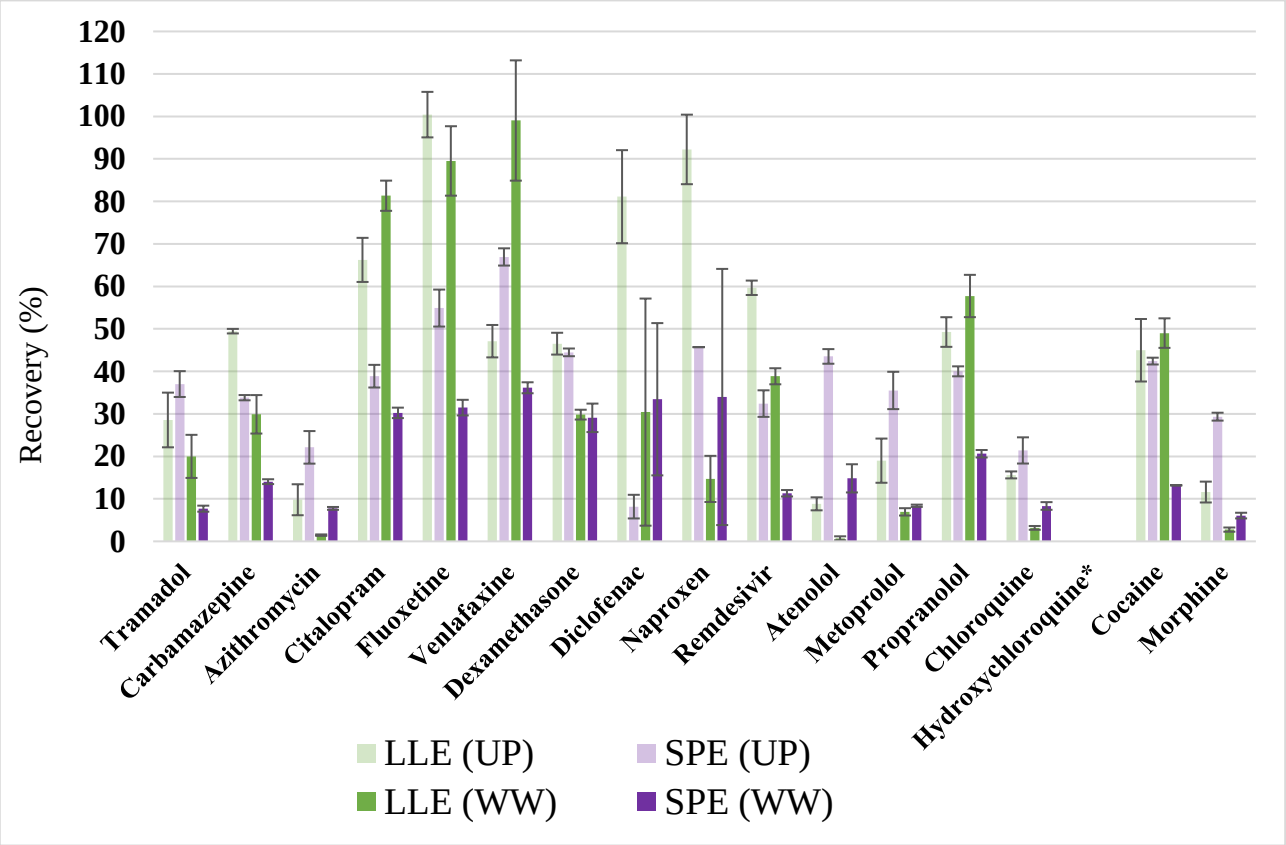

Figure 8. Recoveries obtained by LLE and SPE of either UP water (UP) or wastewater (WW). LLE (green) was performed using a fraction of ACN as extracting solvent with a ratio of solvent/sample volume of 50% and SPE (purple) was performed using MeOH as extracting solvent, with unadjusted sample pH, 45 mL of sample loaded and 4 mL of solvent used for elution. Each colour represents one condition tested, with wastewater being full coloured and UP samples being watermark coloured

UP water samples generally led to higher recoveries than wastewater samples, for both LLE and SPE (**Figure 8**), possibly due to the matrix effects of wastewater extracts in the ionization source, as observed in the first set of experiments (**Figure 7**). However, SPE gave a lower reduction of recoveries for wastewater samples than LLE, likely due to the better clean-up effect of SPE on interferents present in the wastewater matrix **[82,100]**. Hydroxychloroquine recoveries could not be determined. The optimized LLE method presented generally higher recoveries for both UP water and wastewater than the reference SPE method, but the SPE results were more reproducible, with generally lower standard deviations, except for naproxen in wastewater. As such, it was worthwhile to further optimize the SPE procedure aiming for better and more reproducible recoveries.

5.2.3. Second SPE elution (test set C)

In order to verify the possibility of recovering a higher amount of the target CECs that could be retained on the sorbent of the Oasis® HLB cartridge after the elution with 4 mL of MeOH, the SPE procedure described in section 5.2.3 was repeated, only differing in the elution step, that consisted of a 2-step elution, *i.e.*, 2 x 4 mL of MeOH (**Figure 9**).

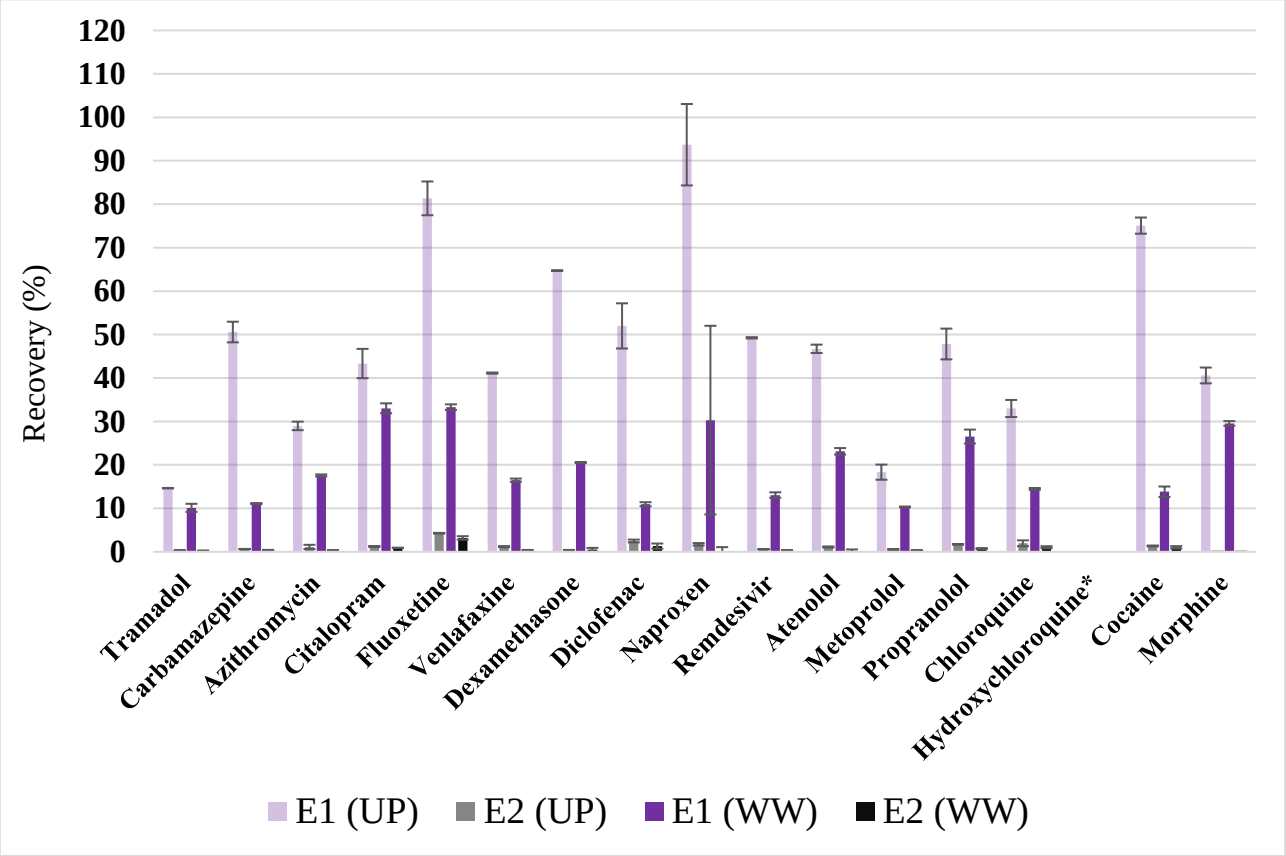

Figure 9. Recoveries obtained by SPE of either UP water (UP) or wastewater (WW). SPE was performed using MeOH as extracting solvent, with unadjusted sample pH, 45 mL of sample loaded and 4 mL of solvent used for elution. Two elutions were performed, and the first (E1, purple) and the second (E2, grey) were collected and analysed separately. Each colour represents one condition tested, with wastewater being full coloured and UP samples being watermark coloured

The recoveries of all CECs in the second step elution for both UP water and wastewater were negligible (**Figure 9**). Hydroxychloroquine recoveries could not be determined. In fact, the recommendation of the manufacturer of the cartridges is to perform an optional second elution only for very nonpolar analytes **[101]**, which is not the case. Since these recoveries were very low, the next steps of this study only considered a 1-step elution.

5.2.4. SPE solvent choice (test set D)

In the following set of experiments (D), three solvents were investigated for both conditioning and elution: MeOH, EtOH, and ACN. The remaining SPE conditions were the same used in the previous experiments (*i.e.*, sample pH not adjusted, sample loading volume of 25 mL, and solvent elution volume of 4 mL) and the recoveries are summarized in **Figure 10**.

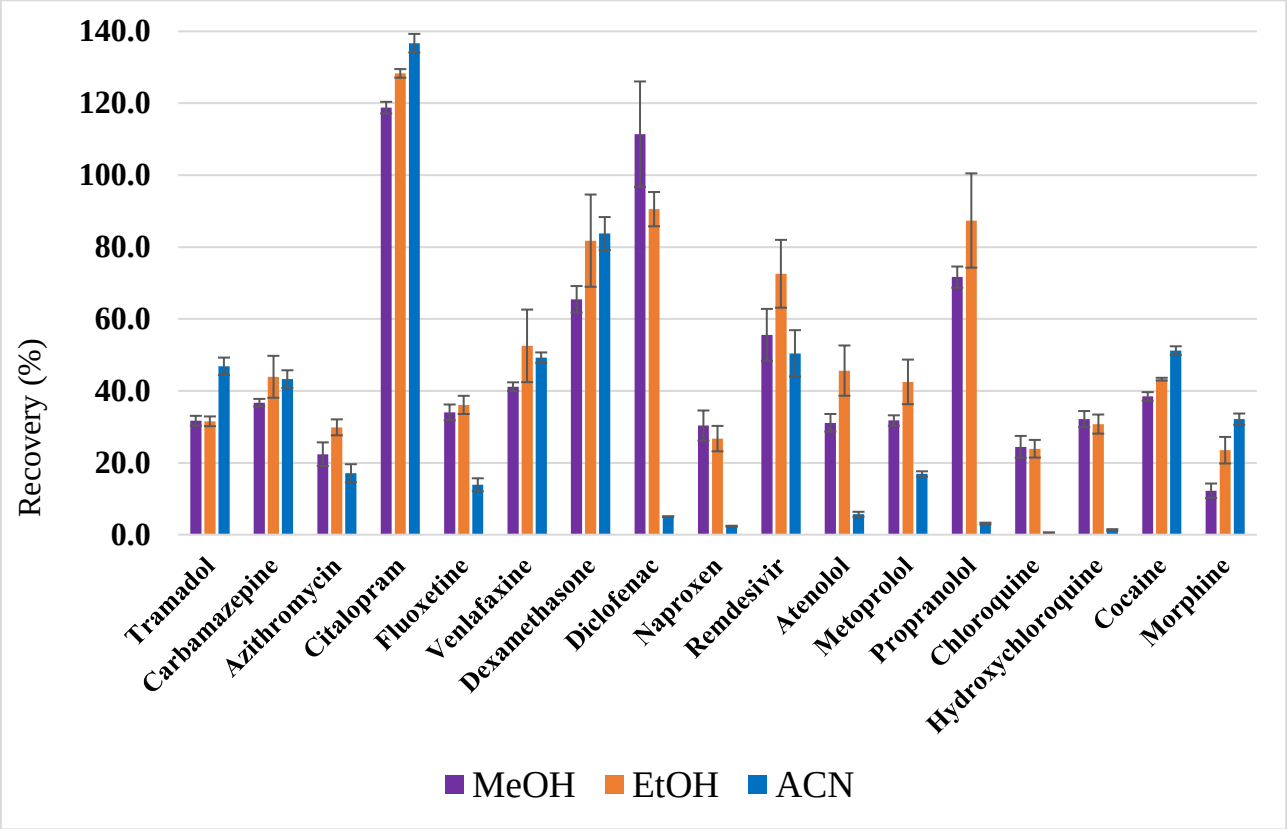

Figure 10. Recoveries obtained by SPE of wastewater. SPE was performed using MeOH (purple), EtOH (orange) or ACN (blue) as extracting solvent, with unadjusted sample pH, 45 mL of sample loaded, and 4 mL of solvent used for elution. Each colour represents one condition tested

It was possible to conclude that ACN performed worse than either MeOH or EtOH (**Figure 10**). Although ACN gave higher recoveries for some CECs, such as cocaine and morphine, in other cases (diclofenac, naproxen, atenolol, propranolol, chloroquine and hydroxychloroquine) the recovery approached zero, which was decisive to disregard ACN. A previous study performed by Babić and Periša (**[102]**) reported similar results when applying ACN for recovering pharmaceutical compounds from wastewater through SPE using Oasis® HLB cartridges, with much inferior recoveries in comparison to MeOH and EtOH.

Comparing the recovery results obtained when using MeOH or EtOH, they were similar. In the case of EtOH, higher recoveries were obtained for most CECs, with the exceptions of diclofenac, naproxen, chloroquine, and hydroxychloroquine, but within the error margin. Similar results were found by Babić and Periša (**[102]**). As EtOH is considered a solvent that follows the guidelines of green analytical chemistry, due to ease of recovery, low toxicity, renewability, and biodegradability **[103]**, it was chosen as SPE solvent for the rest of this study.

5.2.5. SPE parameters optimization (test set E)

After selection of the best performing solvent (EtOH), DoE was employed (test set E) via three-factor BBD, aiming to optimize the following SPE parameters to extract the CECs from wastewater and preconcentrate them: (i) sample pH (5, 7 (natural sample pH), 10); (ii) elution volume (4, 5.5, 7); and (iii) loading volume (25, 37.5, 50). The design of the tests generated through BBD in coded form and the recoveries for each CEC in each test are presented in **Tables C3**, **C4**, **C5** and **C6** of **Annex C**, and the recoveries are also illustrated in **Annex C**, **Figures C1** to **C17**. **Figure 11** shows the mean recovery of the 17 CECs for each condition tested (**Table 5**).

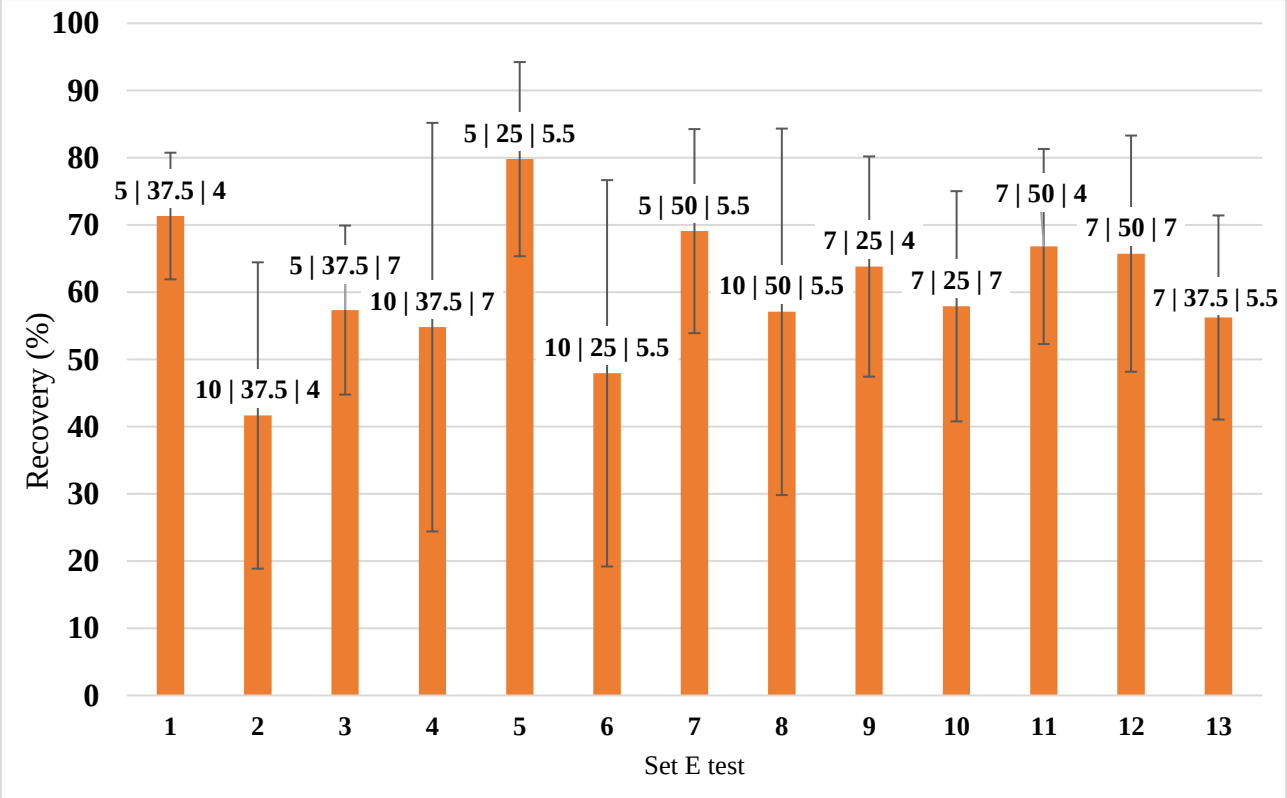

Figure 11. Mean results of each test of test set E. SPE parameters varied within each test, and they are shown on top of the corresponding bar according to the pattern: sample pH | sample loading volume (mL) | elution volume (mL)

Considering that various parameters can affect the recovery of a SPE method (sample loading volume, sample pH, solvent, and solvent elution volume, among others), a multivariable framework is more adequate for optimizing SPE **[104]**. The test conditions that gave the highest recoveries were 1, 5 and 7 (performed with acidified wastewater), while the test conditions giving the lowest

recoveries were 2, 4 and 6 (performed with alkalinized wastewater), showing the importance of sample pH for the extraction of these multi-class CECs from wastewater (**Figure 11**). As presented in **Table 2**, most of the target CECs have a high p*K*a, meaning that they are weak acids and tend to ionize under an alkaline medium. As such, in alkalinized wastewater, a significant portion of the CECs would be ionized, therefore having high affinity for the UP water used in the washing step, resulting in losses and subsequently lower overall recoveries. The opposite would occur in acidified wastewater, as the CECs would be in their non-ionized form, therefore having a greater affinity for the sorbent and for a solvent less polar than water, as EtOH **[77]**. Similar results were reported by Babić and Periša, who showed that recoveries obtained for pharmaceutical compounds (praziquantel, febantel, albendazole, ciprofloxacin, and sulfamethazine) were significantly lower for a pH of 8 in comparison with a pH of 4, using the same solvent (EtOH) and SPE cartridges (Oasis® HLB) **[102]**.

Regarding the sample loading volume, the breakthrough volume, which is the sample loading volume that yields maximum extraction efficiency and starting from which efficiency begins to decline due to analyte washout **[94]**, was similar to that of test 5 (sampling loading volume of 25 mL). In terms of solvent elution volume, the lower amount used for test 1 (4 mL) was not enough for a complete elution of the retained CECs **[105]**, resulting in slightly lower recoveries than test 5 (5.5 mL).

The main goal of the optimization of the sample preparation method was the development of a single SPE procedure, enabling the extraction of a wide range of compounds. Test 5 had the highest mean recovery, of 79.9 ± 14.4 %. As a result, and according to the higher recovery values obtained for most of the target CECs, the selected conditions for analysing the wastewater samples were those of test 5: EtOH as elution solvent, sample pH of 5, 25 mL of sample loading volume and 5.5 mL of solvent elution volume.

5.3. Quality assurance/quality control of the SPE-UHPLC-MS/MS method

A SPE-UHPLC-MS/MS method was developed and optimized in this study for detecting and quantifying 17 CECs in wastewater, including pharmaceuticals and illicit drugs. This method was then validated according to the International Conference on Harmonization Guidelines **[96],** concerning linearity and range of application, recovery, accuracy, precision, and instrument and method limits of detection and quantification. For each target CEC, the linearity and range of application of the method-adjusted calibration curves are listed in **Table C2** of **Annex C**, the retention time, instrument and method detection and quantification limits are listed in **Table D1** of **Annex D**, and the recovery, accuracy, intra-batch precision, process efficiency and matrix effect are listed in **Table D2**, also in **Annex D**.

The recovery of each CEC by the optimized SPE method was determined by analysing spiked wastewater, blank wastewater and PSB wastewater samples. CEC concentrations in blank wastewater

were deducted from the spiked and PSB wastewater samples. CEC concentrations of spiked and PSB wastewater samples were compared, allowing for accurate recovery calculations, as the matrix effect is the same for both sample types. The recoveries of the optimized SPE method are illustrated in **Annex C**, **Figures C1** to **C17**, test 5 of each figure. The recovery values obtained were reproducible and in the range of 38.6% – 110%, with only fluoxetine and chloroquine having a recovery lower than 70% (38.6% and 59.8%, respectively). Babić and Periša (**[102]**) reported similarly high recoveries for pharmaceuticals in an acidic wastewater sample extracted through SPE using Oasis® HLB cartridges and EtOH, in the range of 75.2% – 95.1%, while Gracia-Lor et al. (**[106]**) reported recoveries in the range of 52% – 103%, in a similar context.

Method-adjusted calibration curves were prepared by processing eight aliquots of wastewater samples (in triplicate) that were spiked with different concentrations of the target CECs and a fixed volume of an ISs solution, after which the optimized SPE method was employed for further analysis of the extracts by the ISs calibration method. Five ISs were used for five sets of target CECs. The method-adjusted calibration curves ranged from 50 ng/L (or the MQL when this value was superior) to 10000 ng/L. Linearity was evaluated through the correlation coefficient (r^2) which was always superior to 0.98. IDL and IQL were in the range of 0.12 – 1.14 µg/L and 0.35 – 3.45 µg/L, respectively, while MDL and MQL were in the range of 1.44 – 21.6 ng/L and 4.36 – 65.5 ng/L, respectively.

Accuracy and intra-batch precision were evaluated by processing three QC wastewater samples (in triplicate), spiked with different concentrations (low, medium. And high range) from the method-adjusted calibration curves. Accuracy was in the range of 80.6% – 113%, while intra-batch precision, evaluated by the RSD of the QC triplicates, was less than 19.9%, both in accordance with the International Conference on Harmonization Guidelines **[93]**.

The matrix effect of the wastewater over the ionization of each CEC in the ionization source of the MS was determined by utilizing PSB wastewater samples and an ACN/water 50% (*v/v*) stock solution, both with the same CECs concentrations. A negligible matrix effect is close to 0%, while ionization enhancement and suppression yield a value of >0% and <0% respectively. Matrix effect values were in the range of -59.4% – 72.6%, with most CECs presenting ionization suppression. Chloroquine (-0.2%), remdesivir (5.9%) and fluoxetine (-6.0%) presented negligible matrix effect. Morphine (-40.7%), carbamazepine (-47.9%), citalopram (-47.4%), atenolol (-41.3%) and naproxen (-59.4%) had the most significant ionization suppression, while azithromycin (72.6%), diclofenac (23.6%) and hydroxychloroquine (21.2%) had the highest ionization enhancement.

The process efficiency of the extraction and analysis of the various CECs was in the range of 29.2% – 145%, with azithromycin (145%) and diclofenac (102%) having a value over 100%, possibly due to high matrix effect. Morphine (48.2%), atenolol (48.3%), naproxen (29.2%) and fluoxetine (36.3%) were the only CECs with a process efficiency under 50%, which in the case of fluoxetine

can be explained by its low recovery and by the matrix effects (suppression of signal) in the other cases. Gracia-Lor et al. (**[106]**) reported similarly high process efficiency values for pharmaceutical concentrations in the ng/L and low µg/L range in wastewater that was extracted through SPE with Oasis® HLB cartridges.

5.4. Occurrence of pharmaceuticals and illicit drugs in WWTP influent over 2020

The determined concentrations of each CEC in the wastewater influents are listed in **Tables E1** and **E2** of **Annex E**. All target CECs were quantified in at least one sample, with the exceptions of remdesivir and cocaine, which were only detected.

The wastewater samples were grouped according to their collection date (April – November 2020). These groups corresponded to different phases of the pandemic, characterized by varying degrees of restrictions in Portugal. After the initial declaration of a state of emergency on March 18th, restrictions slowly began being lifted on May 4th, and the state of emergency was lifted on July 1st, beginning a relatively relaxed period of restrictions **[13,107,108]**. This lasted until the 14th of October, when a state of calamity replaced a state of contingency and heavier restrictions were slowly implemented again as the pandemic situation worsened **[109]**. Summarizing, samples collected during the first period belong to the group defined as the first wave (FW), samples collected during the second period belong to the group defined as the reopening phase (RO), and samples collected during the third period belong to the group defined as the second wave (SW). **Table E3** summarizes the minimum, maximum and average concentrations of each CEC, as well as the standard deviations, calculated for each group of samples belonging to three different phases of the pandemic in terms of the severity of restrictions. **Table 6** presents the frequency of detection (FOD) in the wastewater samples of each CEC, for each phase of the pandemic:

Table 6. FOD of CECs in wastewater samples collected over each phase of the pandemic

CEC	FW (n = 18)	RO (n = 27)	SW (n = 11)
Tramadol	100	100	100
Carbamazepine	100	100	100
Azithromycin	100	100	100
Citalopram	100	100	100
Fluoxetine	72	26	18
Venlafaxine	100	100	100
Dexamethasone	100	100	100
Diclofenac	100	100	100
Naproxen	100	100	100
Remdesivir	78	33	36
Atenolol	100	100	100
Metoprolol	22	30	82
Propranolol	100	100	100
Chloroquine	100	100	100
Hydroxychloroquine	100	100	100
Cocaine	0	4	27
Morphine	100	100	100

The FOD of carbamazepine, azithromycin, citalopram, venlafaxine, dexamethasone, diclofenac, naproxen, tramadol, atenolol, propranolol, chloroquine, hydroxychloroquine, and morphine was 100% for all the three periods in study. On the other hand, the detection frequencies of fluoxetine decreased over time, contrary to metoprolol and cocaine which increased. In turn, the FOD of remdesivir decreased between the FW and the RO, being kept approximately constant between the RO and the SW.

Fluoxetine was released on the market in 1987 and is one of the most prescribed antidepressants throughout the world **[35]**. In March 2020, corresponding to the first stage of the pandemic but before the start of the monitoring campaign of this study, 2.2 million packages of antidepressants were sold in Portugal, 28% higher than the same month in 2019 **[110]**, an increase that can be attributed to the impact of the pandemic on mental health **[32]**. The subsequent decrease of detection frequency in the RO could be due to the ease of the restriction measures, which has been reported to have a positive effect on mental health **[111]**. Despite the need of tapering the dosage instead of abruptly stopping this type of medication, this reduction of detection frequency of fluoxetine may indicate a simultaneous incorrect and doctor-supervised withdrawal of the antidepressant. The decrease of FOD from the RO to the SW can be related to the general population being accustomed to the routine of increasing and decreasing restrictions, and even that some people welcomed the return of the restrictive measures, due to the possibility of working from home and having more rest and relaxation time **[112,113]**. However, it should be noted that the FODs of citalopram and venlafaxine, other antidepressants, remained constant.

Metoprolol is a β-blocker, a chronic pharmaceutical used to treat high blood pressure **[114]**. For the initial lockdowns, patients had difficulty accessing chronic medication, a higher likelihood of discontinuing treatment, and fewer new patients began taking these chronic drugs **[47]**. It is possible that the relaxation of pandemic measures over the RO had an opposite effect and facilitated access to chronic medication, slightly increasing the FOD of metoprolol. For the SW, the FOD of this β-blocker increased considerably, which could be due to measures taken by the Portuguese government to ensure the delivery of chronic medication to patient homes. Although this program was approved in March 2020, the responsibility of distribution and delivery of chronic medicine was decentralized and assigned to local pharmacies, pharmacy suppliers, hospitals, city halls and even churches **[115]**. It is possible that the speed of implementation of this program varied by location in accordance with the available resources, perhaps being slower to establish in the region the WWTP serves, from whence the wastewater samples were collected, and as such the highest FOD occurred in the SW.

Cocaine, an illicit drug, was undetected during the FW, which could be due to several reasons, such as a decrease in drug trafficking worldwide, difficulty in contacting drug dealers during lockdowns, and the shutdown of the tourism industry, recreational events and establishments, leading to lower demand **[37,38]**. The fact that the detection frequency increased only to 4% in the RO

suggests a slow recovery and reestablishment of the illicit drug market following the initial impact of pandemic measures. The jump to 27% in the SW would then be the continuation of this recovery, largely unaffected by the return of restrictions. The United Nations Office on Drugs and Crime reported that the coca leaf market started to recover only when restrictions in Colombia were lifted in July 2020, and only reached pre-COVID levels at the end of the same year. It also reported that the quantities of drugs seized after the second quarter of 2020 were the same or even higher than pre-COVID amounts **[116]**.

Remdesivir is a broad-spectrum antiviral. In the FW of the pandemic, people suffering panic attacks might have self-medicated with various antibiotics and antivirals despite an unknown potential of protection **[18]**, which could explain the high FOD for the FW. Over 2020, several studies were unable to prove that remdesivir could treat COVID-19 **[117]**, and the WHO advised against its use for COVID-19 patients **[118]**, factors that can explain its drop in FOD in the later phases of 2020. It should be noted that subsequent studies proved that remdesivir was in fact effective in reducing the hospitalization and death risk of COVID-19 **[119]**.

Data analysis was performed to verify whether CEC concentrations differed significantly between the three phases of the pandemic in Portugal during 2020, except for CEC concentration data sets (organized by pandemic phase) with >30% of wastewater samples not quantified. The results obtained are presented in **Table 7**:

Table 7. Significance of difference between CEC concentration averages across the different phases of the pandemic. p-values with statistical significance (< 0.05) in bold

	p-values (95% confidence)		
CEC	FW v. RO	RO v. SW	FW v. SW
Tramadol	**0.00002**	0.17437	**0.03717**
Carbamazepine	0.19023	0.27170	0.12523
Azithromycin	0.73891	0.27593	0.10571
Citalopram	0.67733	0.11384	-
Fluoxetine	-	-	0.25595
Venlafaxine	0.78619	0.28043	0.18715
Dexamethasone	0.76464	0.09883	0.59238
Diclofenac	0.28027	0.12433	0.99586
Naproxen	0.84570	0.89044	0.03717
Remdesivir	-	-	-
Atenolol	**0.00590**	0.30678	0.13025
Metoprolol	-	-	-
Propranolol	-	0.09488	-
Chloroquine	0.08856	-	-
Hydroxychloroquine	0.12521	0.54986	0.09218
Cocaine	-	-	-
Morphine	0.81656	0.28391	0.34104

Significant concentration differences between pandemic phases were found for tramadol, which increased between the FW and the RO and between the FW and the SW. Regarding atenolol, it is

possible to observe an augment between the FW and the RO. No significant differences were found between the RO and the SW for any CEC.

Tramadol is an opioid analgesic which was reported to have been diverted after being prescribed and sold to the black-market during 2020 lockdowns in European countries, as an increased availability of diverted prescription opioids and benzodiazepines was registered, including other compounds like buprenorphine and methadone **[120]**. For example, in Lisbon, an increase in illicit benzodiazepines and opioids (including tramadol) consumption was associated with difficulties in obtaining stronger drugs like heroin and cocaine during the lockdown of the FW **[28]**. In Portugal, tramadol is approved as an opioid analgesic for treating chronic pain, and access to prescription opioids was facilitated during lockdown for patients going through opioid substitution treatment, enhancing the risk of diversion to the black-market **[28,121]**. This could help to explain the increase in tramadol concentration from the FW to the RO, as drug abusers were still unable to acquire cocaine despite relaxing of restrictions, as the cocaine market would only recover later **[116]**, and sought illegally diverted opioids like tramadol as substitutes.

Atenolol is, like metoprolol, a β-blocker to treat high blood pressure **[122]**. As such, it is possible that its difference in wastewater concentration between the FW and the RO is caused by similar reasons: in the FW, chronic medication access was harder due to lockdowns and other restrictive measures **[47]**, and afterwards the RO lifted the more severe restrictions, acting to counter the tendency to discontinue chronic medication. It is also possible that atenolol had a higher availability in Portugal than metoprolol, and therefore its delivery to chronic patient homes was more effective, increasing its consumption earlier than the SW, as was the case for metoprolol.

6. Conclusions

In this study, an analytical method to analyse 17 different CECs in wastewater influent samples was developed through LLE and SPE recovery tests and using UHPLC-MS/MS for identification and quantification. The performance of LLE was studied by extracting the analytes with ACN from UP water and WW samples in assays which differed by solvent/sample ratio, between 30 and 50%. A solvent fraction of 50% was found to yield the highest overall recoveries. SPE assays were then performed by applying a reference protocol proposed by the manufacturer of the HLB cartridges employed in all SPE assays, with MeOH as solvent, unadjusted sample pH, 45 mL of sample, and 4 mL of solvent used for elution. Recoveries for the SPE method were lower than those of LLE, but more reproducible; the SPE procedure was then optimized. First, a second consecutive elution was performed in the same conditions, and the extract analysed separately, with the recoveries of all analytes being negligible. Overall, UP water and WW recoveries were different, confirming the presence of significant interference of the matrix components on the extraction. The reference protocol was repeated with three different conditioning and elution solvents, including MeOH, EtOH and ACN. ACN performed worse than MeOH and EtOH. EtOH had slightly higher recoveries than MeOH for most CECs and as such was selected as SPE solvent for subsequent tests. Finally, the remaining SPE parameters, including sample pH, sample loading volume, and solvent elution volume, were optimized through the employment of DoE. The SPE procedure chosen used EtOH as solvent, sample pH of 5, sample volume of 25 mL, and solvent elution volume of 5.5 mL. The SPE-UHPLC-MS/MS method was validated according to international guidelines concerning linearity and range of application, recovery, accuracy, precision, and instrument and method limits of detection and quantification. The matrix effect and process efficiency of each CEC were also evaluated.

WWTP influent CEC concentrations were determined by analysing wastewater samples collected from April to November 2020, a period spanning three phases of the pandemic in Portugal: the FW, from March 18th to July 1st; the RO, until October 14th; the SW, for the rest of 2020. The FOD of fluoxetine decreased over time, possibly due to ease of restriction measures and some settling of the population towards the pandemic. The FOD of cocaine slowly increased from the FW onwards, as the market for that illicit drug may have collapsed in the first lockdowns and then slowly recovered. The FOD of remdesivir also decreased from the FW, as studies that failed to prove its efficiency for treating COVID-19 were published and people became more informed. The FOD of metoprolol increased over time, and the concentrations of atenolol increased significantly after the FW, likely due to difficulty in accessing chronic medication in the first lockdowns, which was then mitigated by the establishment and development of government programs to reinstate the access to prescription drugs. The concentrations of tramadol increased significantly between the FW and the RO, suggesting its diversion into the black-market as a substitute for harder-to-obtain drugs like cocaine.

7. Recommendations for future work

The SPE-UHPLC-MS/MS method developed and validated in this work was used to analyse 17 CECs in wastewater samples from the influent of a mixed urban and industrial zone WWTP in Portugal. The samples studied in this work were collected over the course of 2020, but the same processing and analysis method can be applied again for analysing the remaining wastewater samples, collected during 2021 and 2022, in future studies. Additional testing for further optimizing the method may also be performed by studying new conditions for the SPE procedure. For example, the addition of NaCl to raise the ionic strength of the wastewater and promote extraction by the organic solvent could be tested, as well as the addition of a chelating agent to bind soluble metals present in the wastewater matrix, preventing them from binding to the target analytes and decreasing their extraction recoveries.

The occurrence patterns of the CECs over different phases of the pandemic in Portugal during 2020 were evaluated in this work, and future works can carry out equivalent research for 2021 and 2022. Furthermore, other metrics related to the occurrence patterns determined in this study can also be assessed. For example, population-normalised consumption patterns can be established from the CECs concentrations determined in this study and from the daily wastewater influent flow rate, the estimated population served by the WWTP, and stability and metabolic excretion-related correction factors for each analyte.

Further studies following this work could apply the same SPE-UHPLC-MS/MS method, but focus on the analysis of wastewater samples collected upstream from the WWTP influent, at manholes from different neighbourhoods, to research the consumption of CECs at a smaller level of population aggregation. This data could complement the results of this study, giving more insight into the behaviour of the population served by the WWTP. Also, upstream wastewater sampling could allow for the measurement of more unstable metabolites than the CECs studied in this work, due to shorter travel time to the sampling location.

Other than differing in the location of sampling, future research can also vary regarding the day of wastewater collection. The samples analysed in this work were collected on Mondays and Thursdays, but sampling on Saturdays or Sundays as well would allow the comparison of consumption of pharmaceuticals and illicit drugs between weekdays and the weekend.

Another possible application of the SPE-UHPLC-MS/MS method developed in this work is the evaluation of the overall capacity of a WWTP to remove different CECs, by sampling both the influent and effluent and comparing the concentrations. It is also possible to sample other points within the WWTP, such as before and after a certain unit process, to verify the effectiveness of a specific treatment in removing organic pollutants.

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Annexes

Annex A – COVID-19 pandemic and Portuguese government response

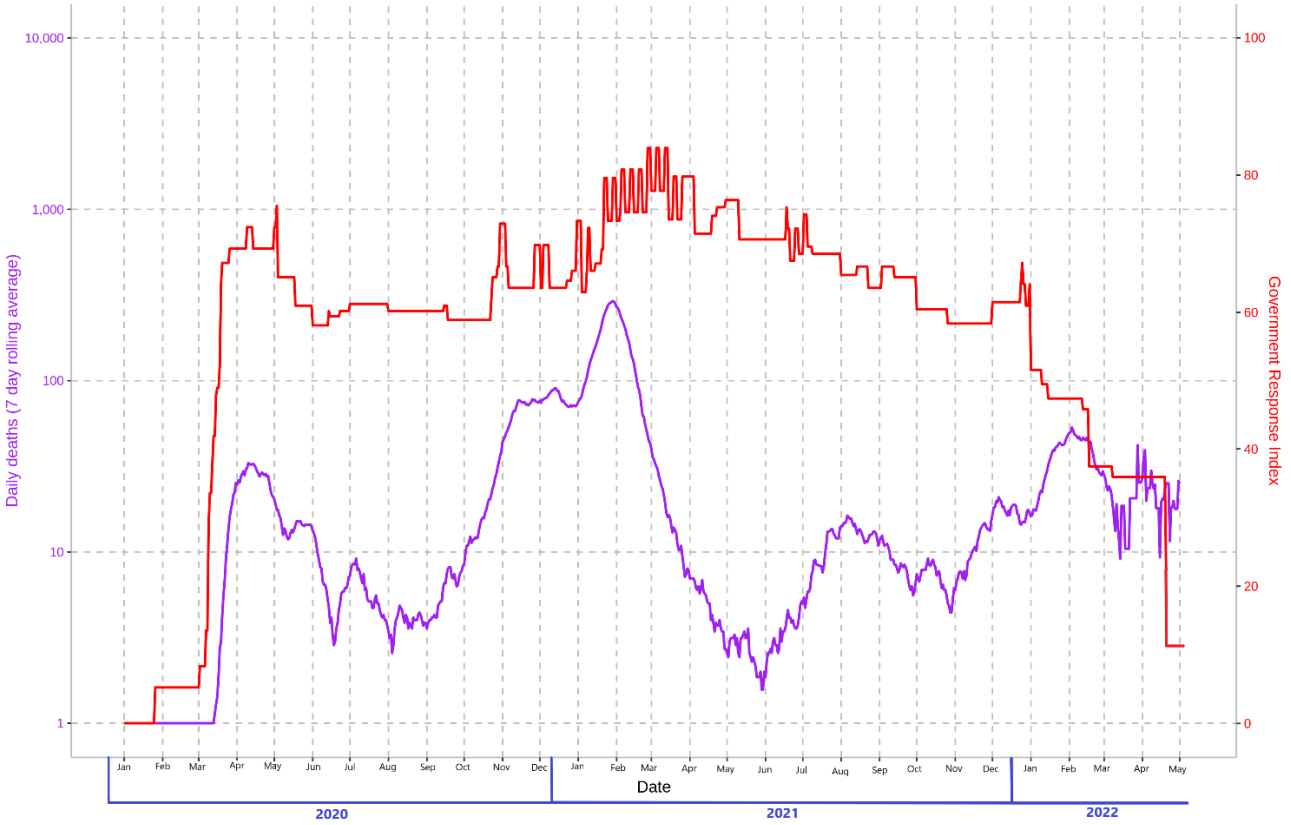
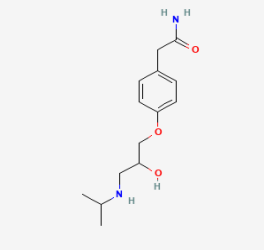
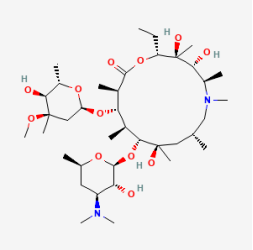
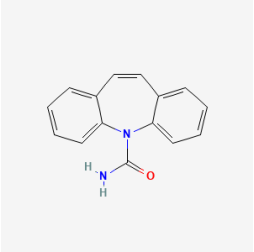
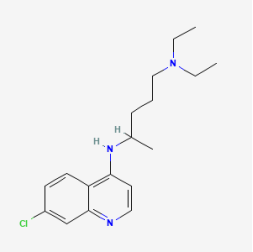
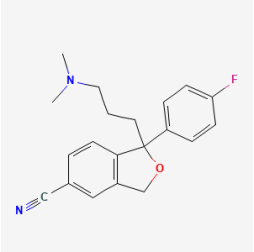
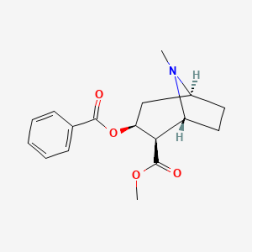
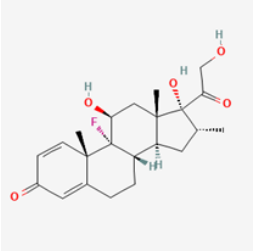
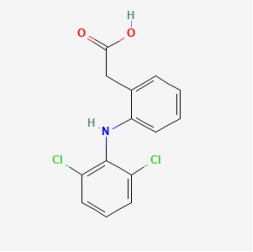
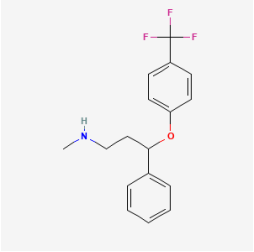
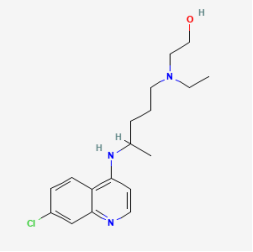
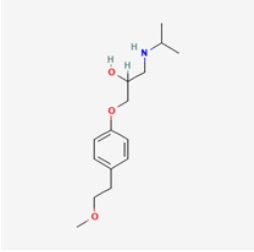
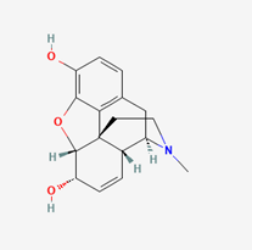


Figure A1. Portuguese Stringency Index and daily deaths over the main phase of the COVID-19 pandemic. Reprinted (adapted) from **[15]** with the permission of the Blavatnik School of Government, University of Oxford (Creative Commons CC BY 4.0 license)

Annex B – Additional information on the target CECs and their MS parameters

Table B1. Chemical structure of the screened CECs **[91]**

CEC	Chemical structure	CEC	Chemical structure
Atenolol		Azithromycin	
Carbamazepine		Chloroquine	
Citalopram		Cocaine	
Dexamethasone		Diclofenac	
Fluoxetine		Hydroxychloroquine	
Metoprolol		Morphine	

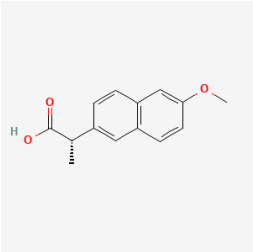
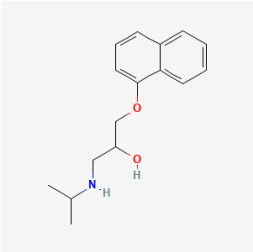
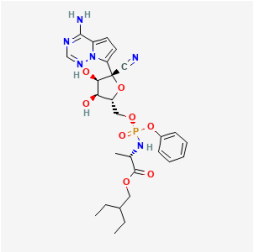
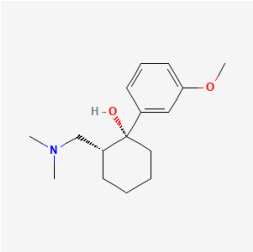
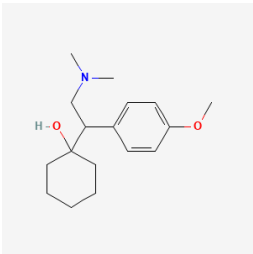
Naproxen		Propranolol	
Remdesivir		Tramadol	
Venlafaxine			

Table B2. SRM instrument parameters and IS sets of the target CECs

CEC	IS set	ESI mode	Precursor ion (m/z)	Quantification (SRM1)				Confirmation (SRM2)				Ion ratio
				Product ion	DP (V)	CE (V)	CXP (V)	Product ion	DP (V)	CE (V)	CXP (V)	
Tramadol	2	+	263.80	58.05	-27	-20	-21	-	-	-	-	n.a.
Carbamazepine	2	+	236.90	194.05	-19	-19	-19	192.00	-19	-22	-18	4.76
Azithromycin	5	+	749.50	158.10	-36	-42	-15	591.20	-36	-31	-40	1.05
Citalopram	2	+	324.80	109.00	-30	-28	-20	262.10	-30	-19	-27	2.70
Fluoxetine	2	+	310.10	44.05	-15	-13	-16	-	-	-	-	n.a.
Venlafaxine	2	+	277.80	58.05	-28	-21	-23	260.25	-28	-12	-11	2.78
Dexamethasone	5	+	393.20	373.10	-11	-10	-23	355.20	-11	-13	-16	1.64
Diclofenac	1	-	294.00	250.05	10	11	17	214.05	10	19	22	25.00
Naproxen	1	-	229.00	170.05	16	15	18	169.10	16	30	30	1.01
Remdesivir	5	+	603.20	402.15	-28	-16	-26	200.15	-28	-40	-20	1.67
Atenolol	2	+	266.80	145.00	-26	-26	-24	190.05	-26	-18	-11	1.67
Metoprolol	2	+	267.80	116.10	-25	-18	-21	72.10	-25	-22	-26	1.12
Propranolol	2	+	259.80	116.15	-25	-17	-20	183.10	-25	-17	-11	1.51
Chloroquine	5	+	320.20	247.10	-15	-20	-25	142.15	-15	-22	-27	2.33
Hydroxychloroquine	5	+	336.20	247.00	-16	-23	-25	178.90	-16	-36	-17	3.45
Cocaine	4	+	303.70	182.05	-29	-20	-30	82.05	-29	-32	-13	3.70
Morphine	3	+	286.10	165.05	-28	-45	-26	152.00	-28	-55	-29	1.10

Table B3. SRM instrument parameters and assigned sets of the ISs

IS	IS set	ESI mode	Precursor ion (m/z)	Quantification (SRM1)				Confirmation (SRM2)			
				Product ion	DP (V)	CE (V)	CXP (V)	Product ion	DP (V)	CE (V)	CXP (V)
Diclofenac-d4	1	-	298.00	254.10	21	11	17	217.05	21	20	24
Fluoxetine-d5	2	+	315.10	44.00	-16	-14	-15	-	-	-	-
Morphine-d3	3	+	289.10	165.00	-14	-46	-30	151.95	-14	-53	-27
Cocaine-d3	4	+	306.90	185.15	-29	-21	-30	85.10	-29	-32	-14
Azithromycin-d3	5	+	752.50	594.25	-36	-31	-40	158.00	-36	-44	-27

Annex C – Wastewater processing optimization

Table C1. Selected variables and respective levels investigated in the BBD

Variables	Selected levels		
	-1	0	+1
(i) sample pH	5	7 (Unchanged)	10
(ii) solvent elution volume (mL)	4.0	5.5	7.0
(iii) sample loading volume (mL)	25.0	37.5	50.0

Table C2. Range, slope (m), y-intercept (b) and linearity (r^2) of the method-adjusted calibration curves of each CEC

CEC	Range (ng/L)	Slope (m)	Y-intercept (b)	r^2
Tramadol	50-5000	2.2325	-4.1939E-02	1.000
Carbamazepine	50-5000	1.1638	-2.2442E-02	0.993
Azithromycin	50-5000	1.2518	-1.0336E-02	0.999
Citalopram	50-5000	1.6150	-1.5152E-02	0.999
Fluoxetine	53-5000	1.5836	1.0412E-02	0.998
Venlafaxine	50-10000	1.4781	3.8317E-02	0.995
Dexamethasone	50-5000	0.7865	-3.8206E-02	0.984
Diclofenac	50-5000	0.6449	1.0748E-02	0.999
Naproxen	66-5000	0.2568	-3.8184E-03	0.992
Remdesivir	50-5000	3.8583	2.6305E-02	0.986
Atenolol	50-2500	0.1013	1.4085E-03	0.986
Metoprolol	50-10000	0.2389	2.1401E-02	0.991
Propranolol	50-5000	0.5189	1.6536E-03	0.999
Chloroquine	50-5000	4.5128	-4.3393E-02	0.999
Hydroxychloroquine	50-5000	0.3556	-8.4195E-03	0.995
Cocaine	50-10000	0.6202	6.2990E-03	0.997
Morphine	50-10000	0.5194	1.1600E-02	0.993

Table C3. Design of set E tests (in coded form) by BBD and corresponding recovery values for tramadol, carbamazepine, azithromycin, citalopram and fluoxetine. Parameters: (i) sample pH; (ii) loading volume (mL); (iii) elution volume (mL).

Test	Parameters			Recovery (%)				
	i	ii	iii	Tramadol	Carbamazepine	Azithromycin	Citalopram	Fluoxetine
1	-	0	-	56	67	75	95	72
2	+	0	-	33	58	32	61	15
3	-	0	+	50	51	61	85	61
4	+	0	+	50	68	39	69	12
5	-	-	0	82	80	84	104	39
6	+	-	0	34	72	21	76	6
7	-	+	0	74	71	93	95	70
8	+	+	0	47	72	50	82	16
9	0	-	-	62	71	45	87	24
10	0	-	+	54	61	46	81	33
11	0	+	-	49	78	49	87	47
12	0	+	+	56	72	49	84	39
13	0	0	0	48	66	33	75	44

Table C4. Design of set E tests (in coded form) by BBD and corresponding recovery values for venlafaxine, dexamethasone, diclofenac, naproxen and remdesivir. Parameters: (i) sample pH; (ii) loading volume (mL); (iii) elution volume (mL).

Test	Parameters			Recovery (%)				
	i	ii	iii	Venlafaxine	Dexamethasone	Diclofenac	Naproxen	Remdesivir
1	-	0	-	74	73	83	68	69
2	+	0	-	39	66	3	4	58
3	-	0	+	42	68	57	40	52
4	+	0	+	47	72	4	13	64
5	-	-	0	88	83	83	72	83
6	+	-	0	58	74	5	5	58
7	-	+	0	71	77	47	49	72
8	+	+	0	69	86	0	13	64
9	0	-	-	72	75	67	58	75
10	0	-	+	61	71	58	58	63
11	0	+	-	60	81	77	73	74
12	0	+	+	57	79	77	68	73
13	0	0	0	43	79	67	76	70

Table C5. Design of set E tests (in coded form) by BBD and corresponding recovery values for atenolol, metoprolol, propranolol and chloroquine. Parameters: (i) sample pH; (ii) loading volume (mL); (iii) elution volume (mL).

Test	Parameters			Recovery (%)			
	i	ii	iii	Atenolol	Metoprolol	Propranolol	Chloroquine
1	-	0	-	70	60	75	51
2	+	0	-	54	47	72	46
3	-	0	+	68	50	78	45
4	+	0	+	96	73	79	48
5	-	-	0	82	79	110	60
6	+	-	0	74	64	73	31
7	-	+	0	62	75	70	65
8	+	+	0	71	61	101	49
9	0	-	-	83	76	71	33
10	0	-	+	75	65	75	16
11	0	+	-	81	60	88	38
12	0	+	+	84	61	106	34
13	0	0	0	69	53	67	33

Table C6. Design of set E tests (in coded form) by BBD and corresponding recovery values for hydroxychloroquine, cocaine and morphine. Parameters: (i) sample pH; (ii) loading volume (mL); (iii) elution volume (mL).

Test	Parameters			Recovery (%)		
	i	ii	iii	Hydroxychloroquine	Cocaine	Morphine
1	-	0	-	69	73	75
2	+	0	-	279	24	17
3	-	0	+	49	42	64
4	+	0	+	111	28	59
5	-	-	0	73	75	81
6	+	-	0	176	23	41
7	-	+	0	83	67	75
8	+	+	0	93	19	22
9	0	-	-	54	70	60
10	0	-	+	31	62	50
11	0	+	-	62	61	69
12	0	+	+	51	55	73
13	0	0	0	53	39	41

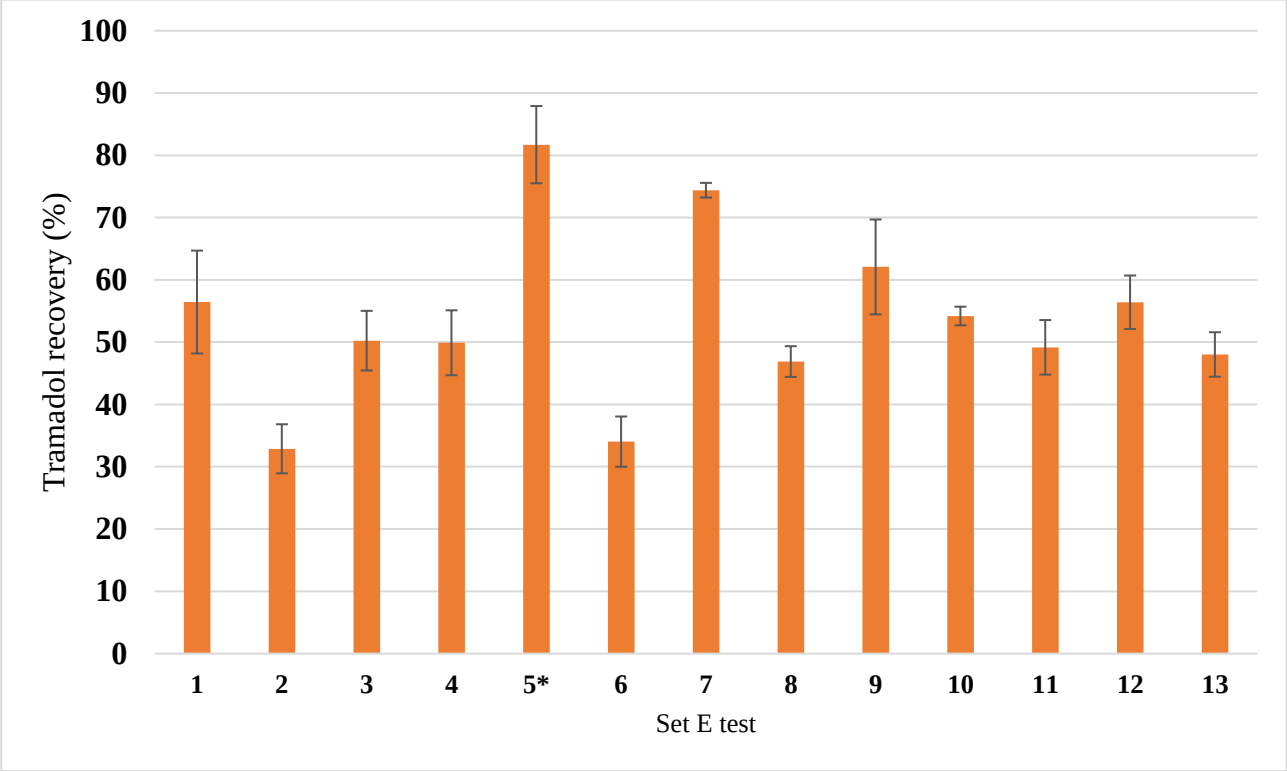


Figure C1. Recovery results of tramadol from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

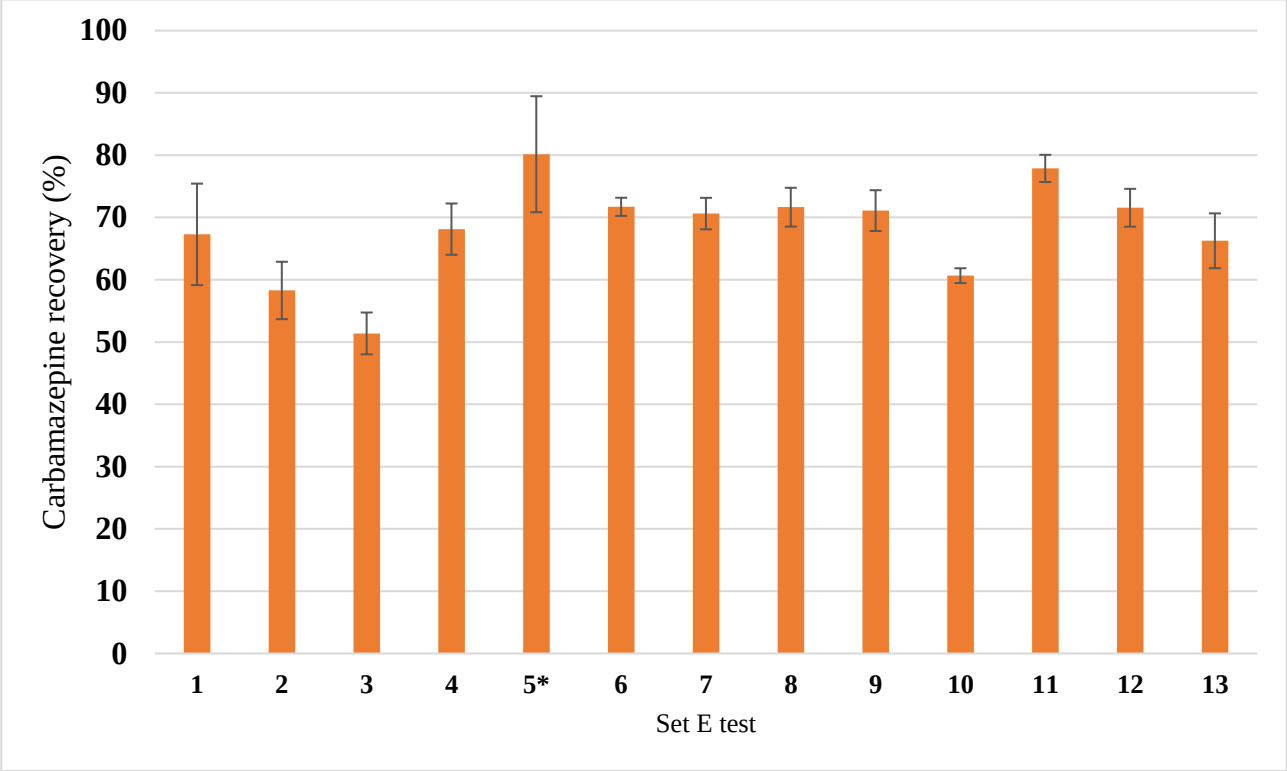


Figure C2. Recovery results of carbamazepine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

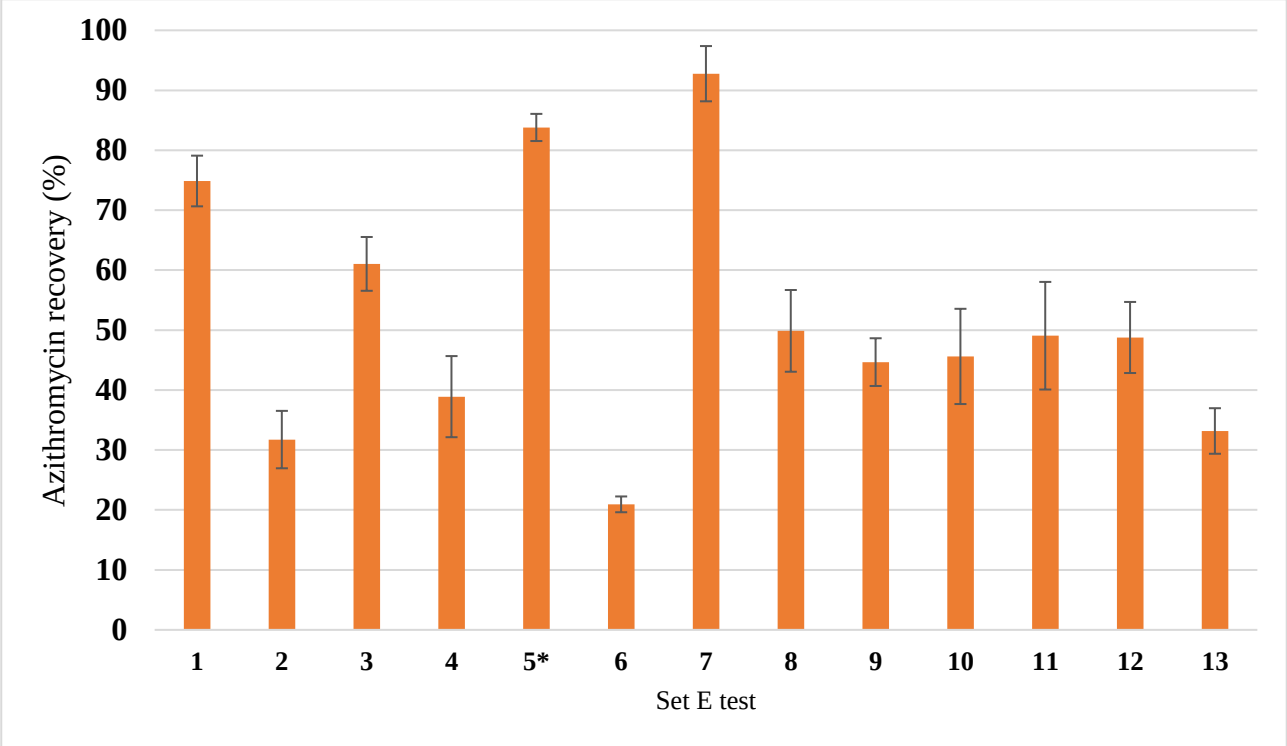


Figure C3. Recovery results of azithromycin from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

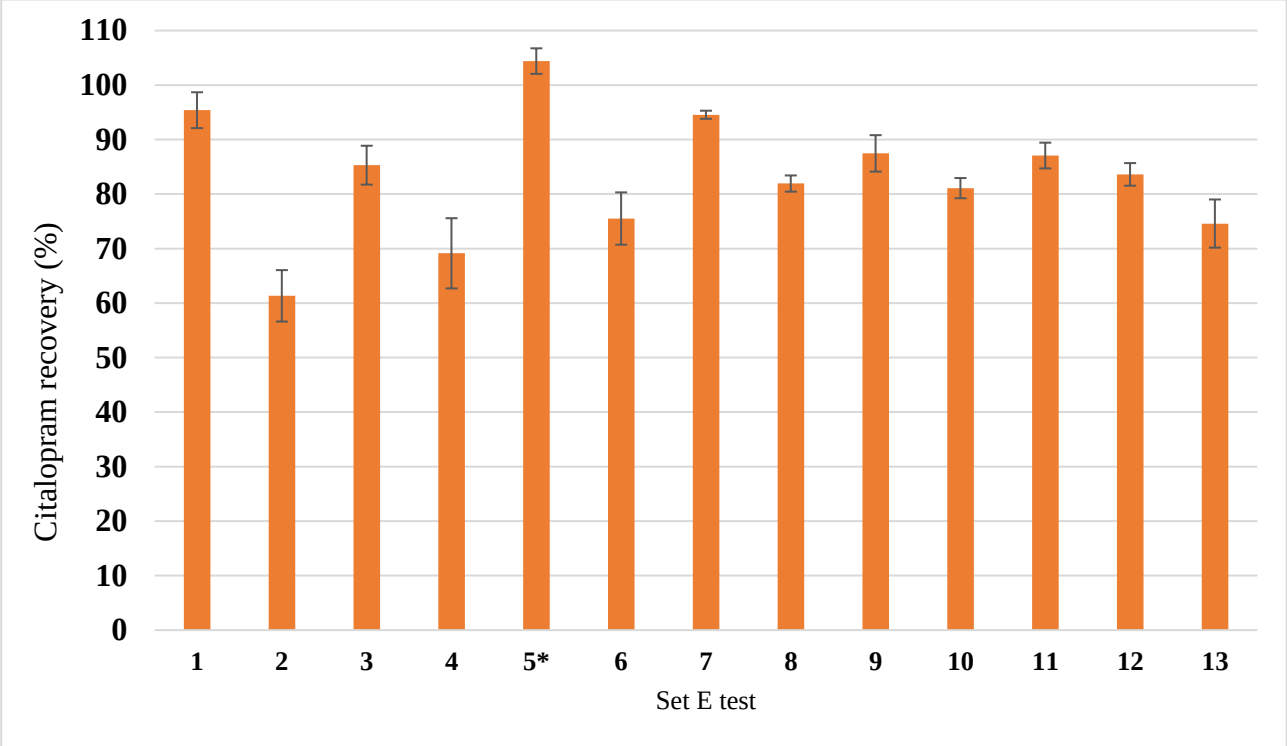


Figure C4. Recovery results of citalopram from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

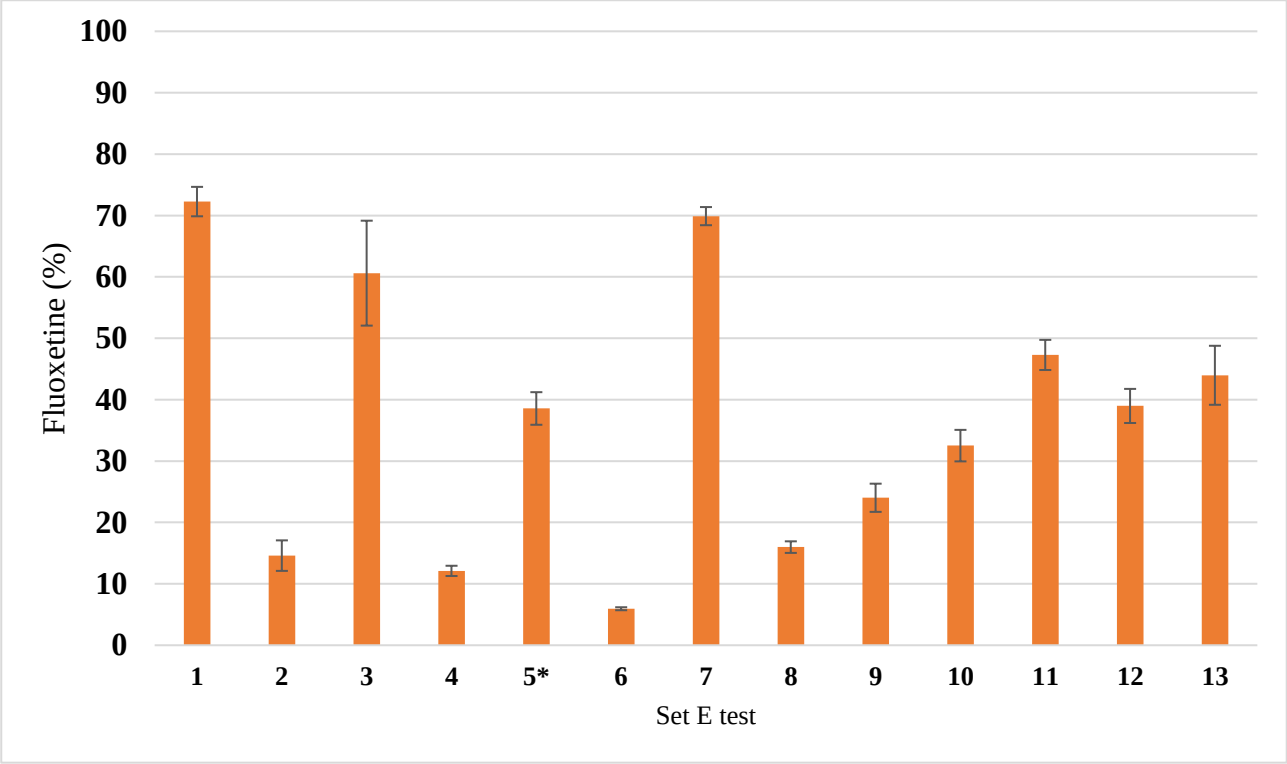


Figure C5. Recovery results of fluoxetine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

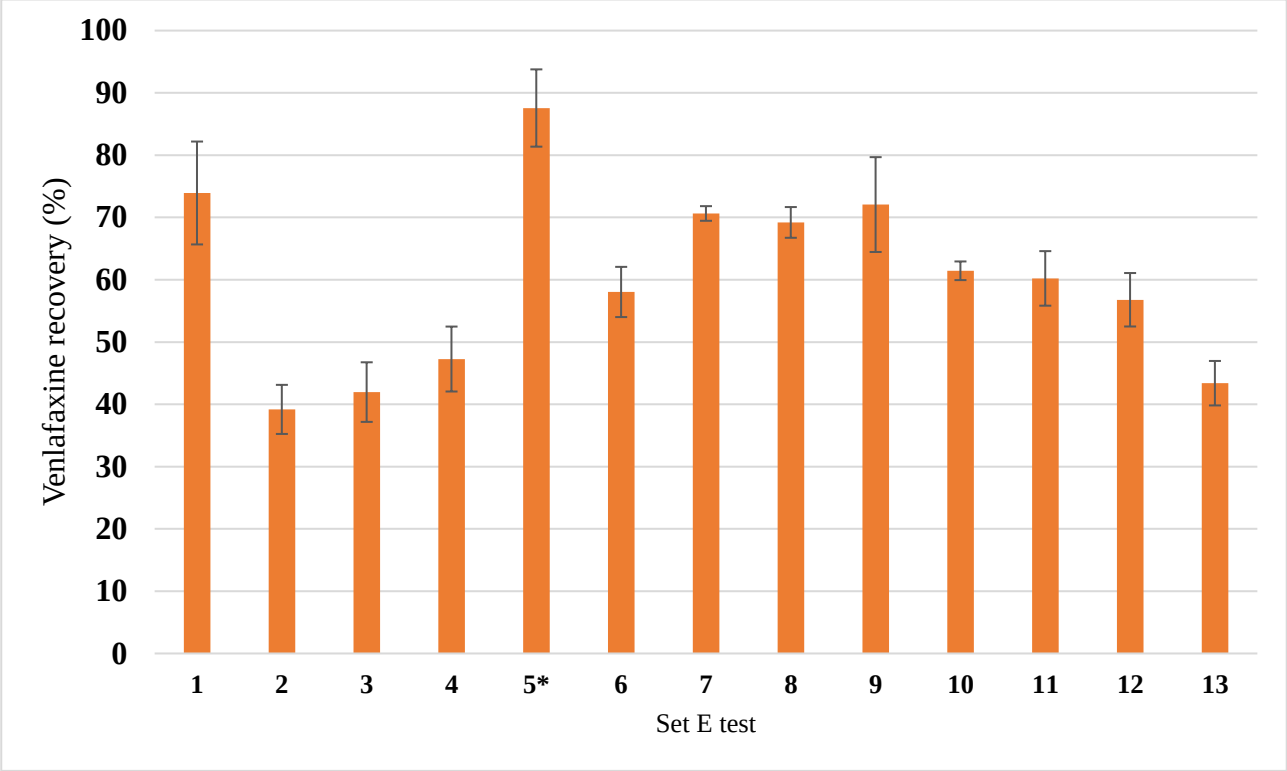


Figure C6. Recovery results of venlafaxine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

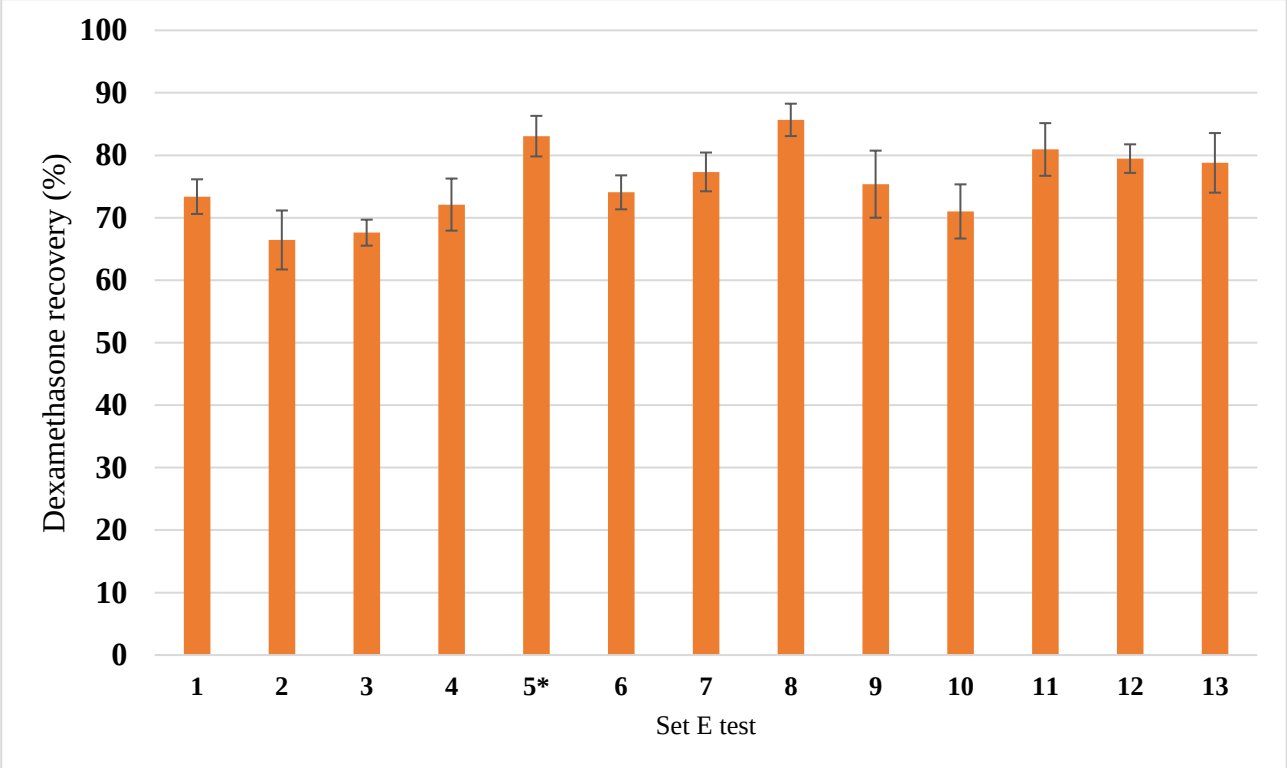


Figure C7. Recovery results of dexamethasone from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

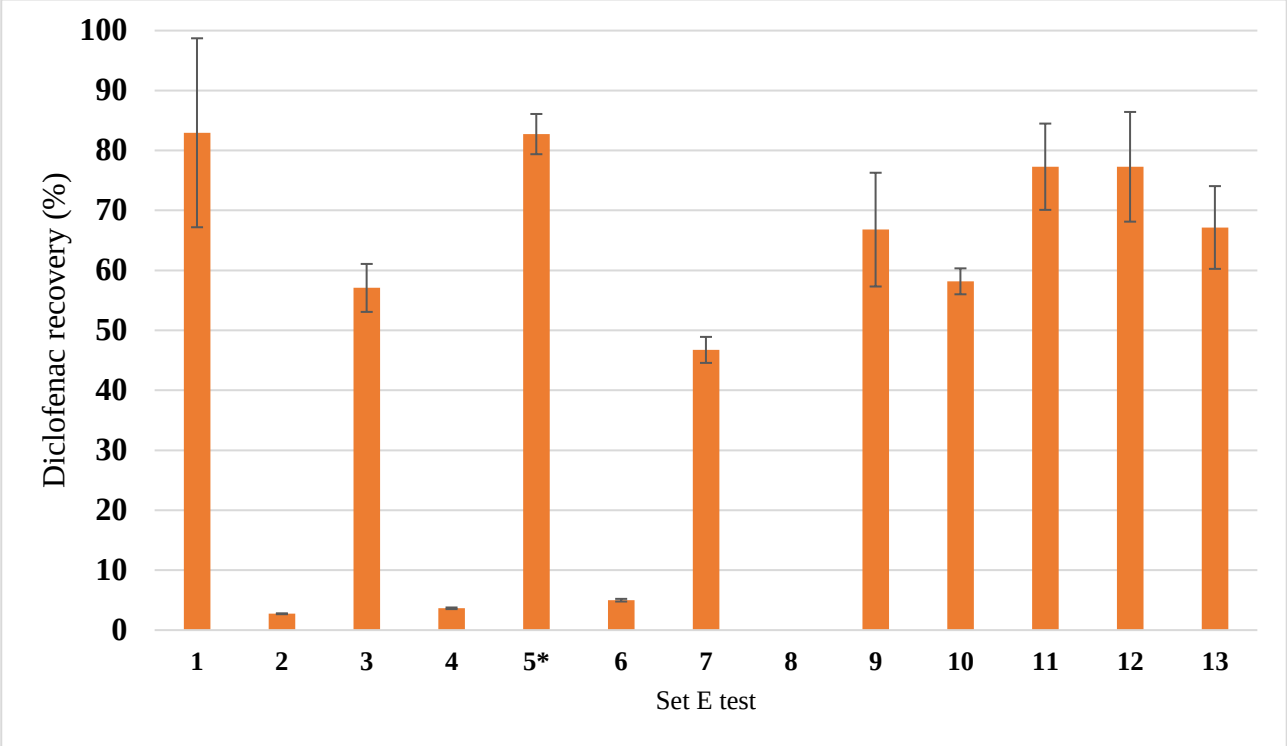


Figure C8. Recovery results of diclofenac from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

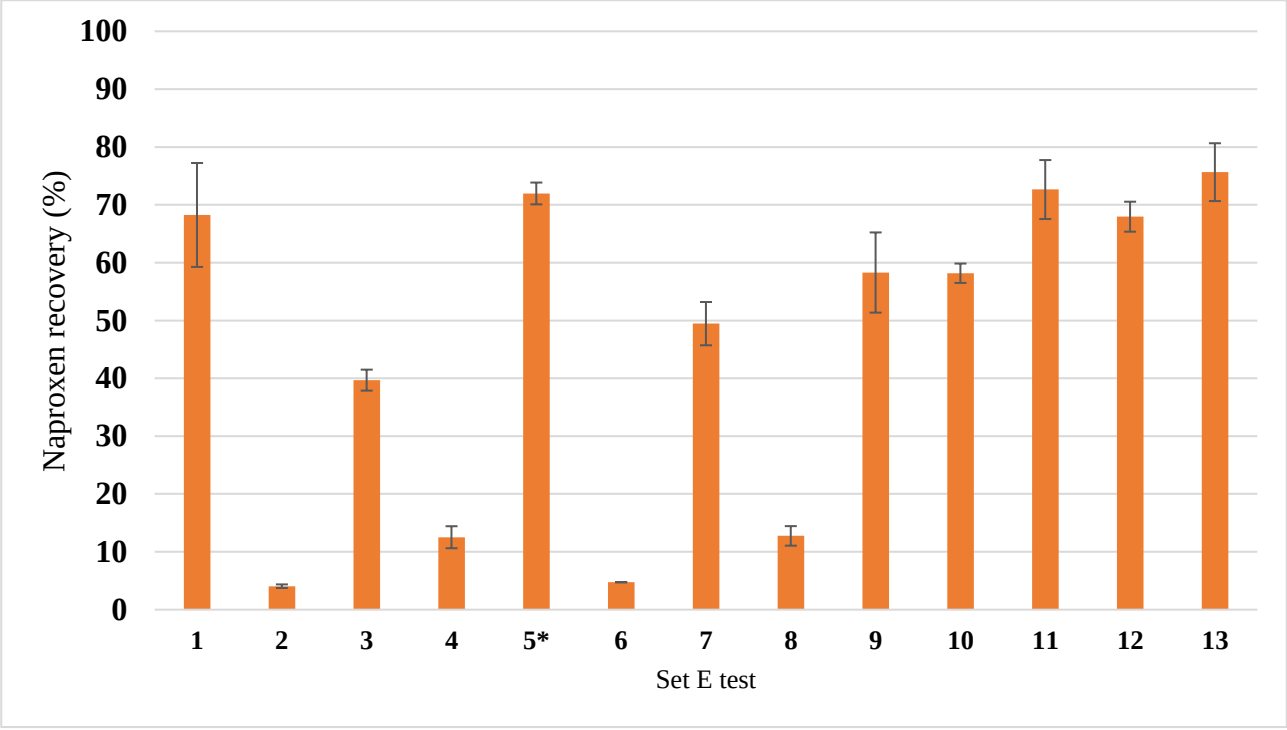


Figure C9. Recovery results of naproxen from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

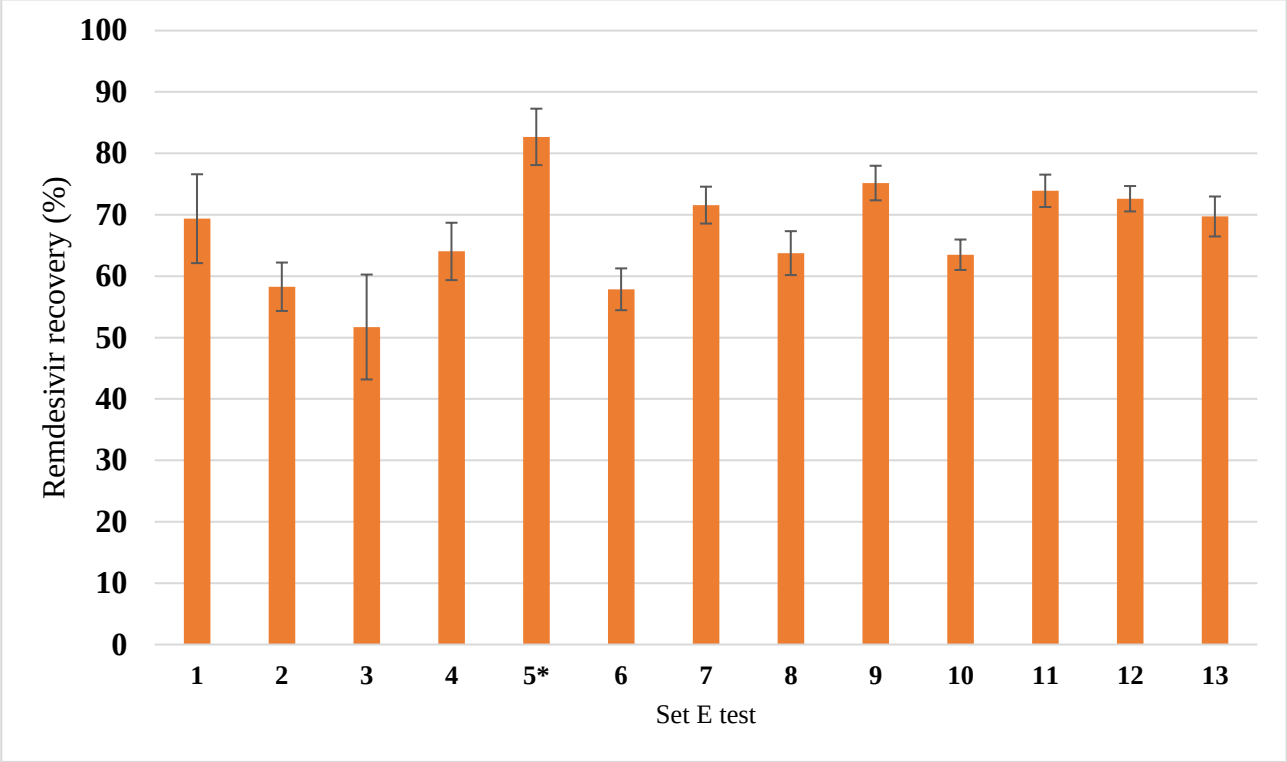


Figure C10. Recovery results of remdesivir from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

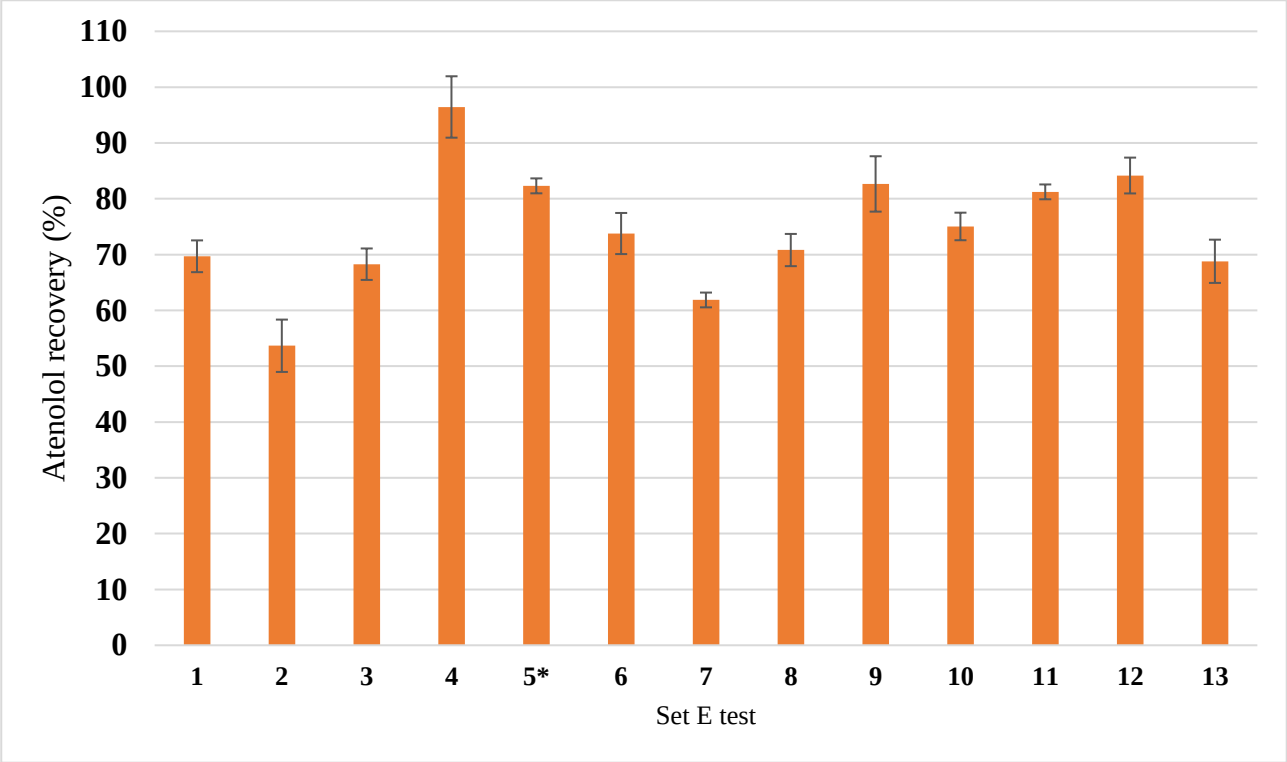


Figure C11. Recovery results of atenolol from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

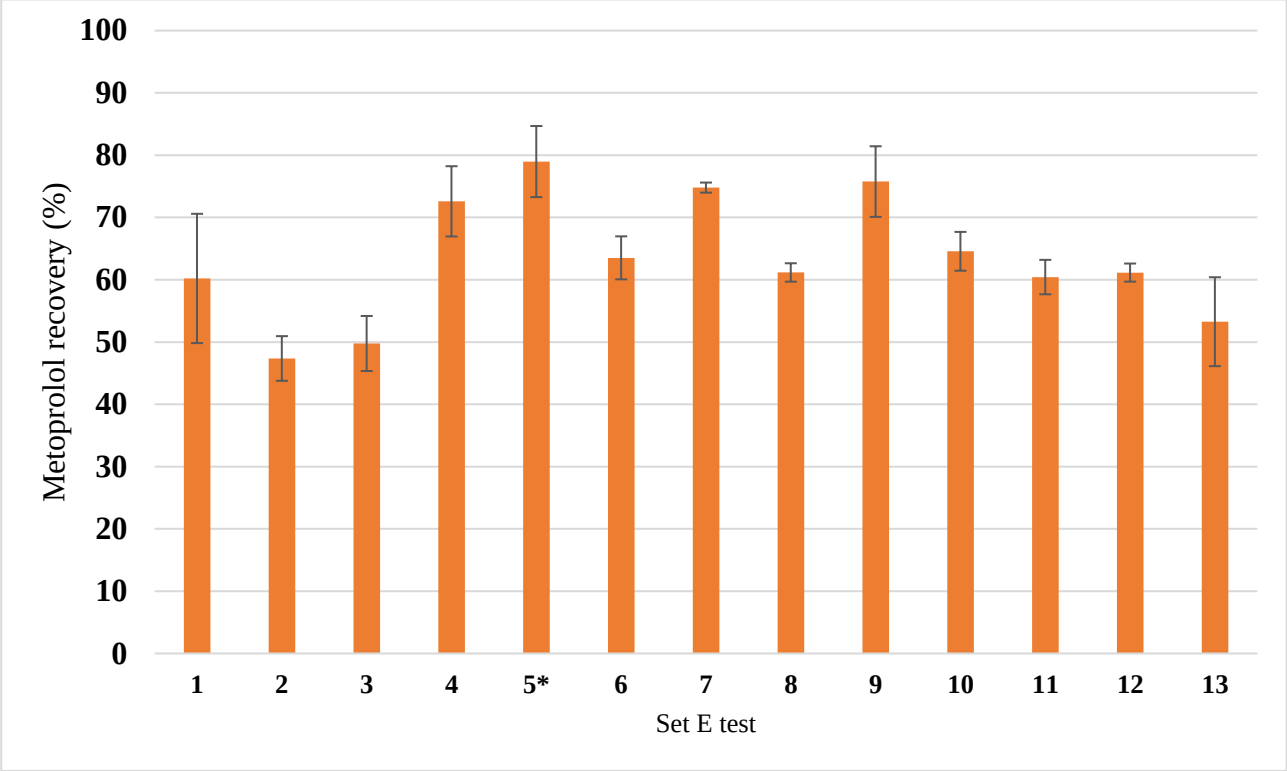


Figure C12. Recovery results of metoprolol from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

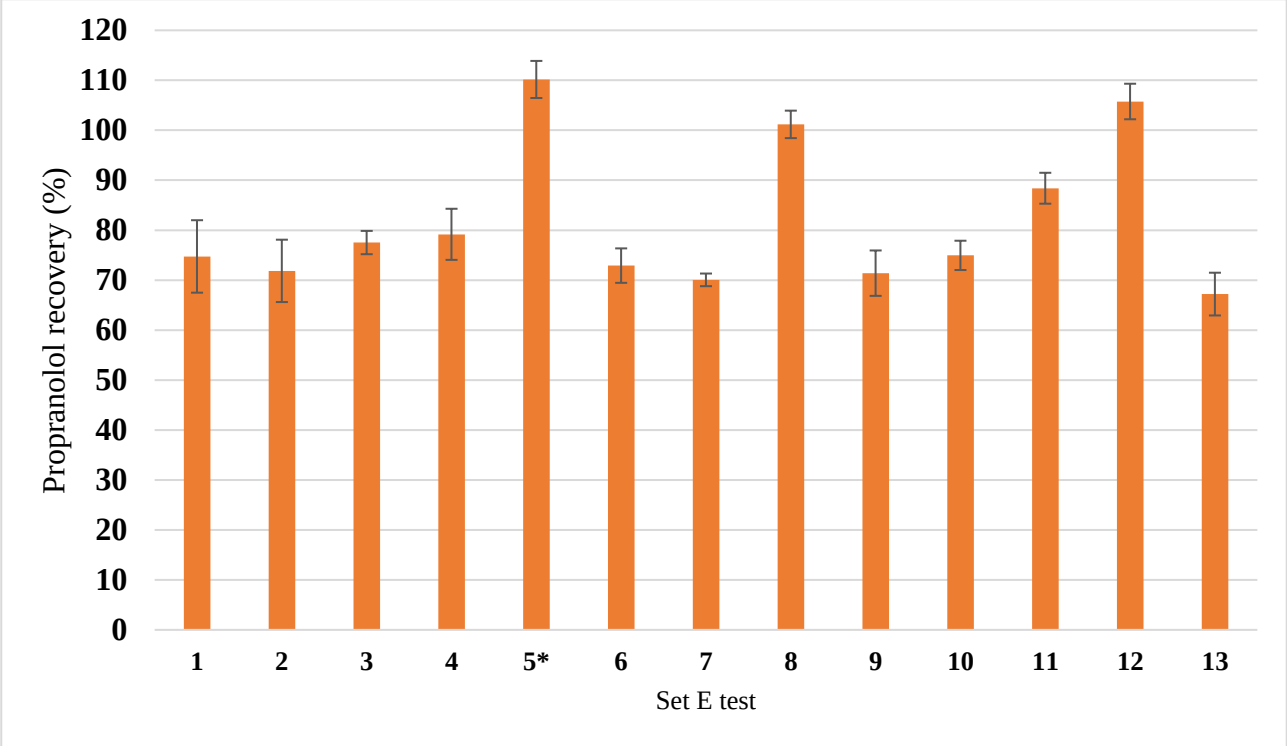


Figure C13. Recovery results of propranolol from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

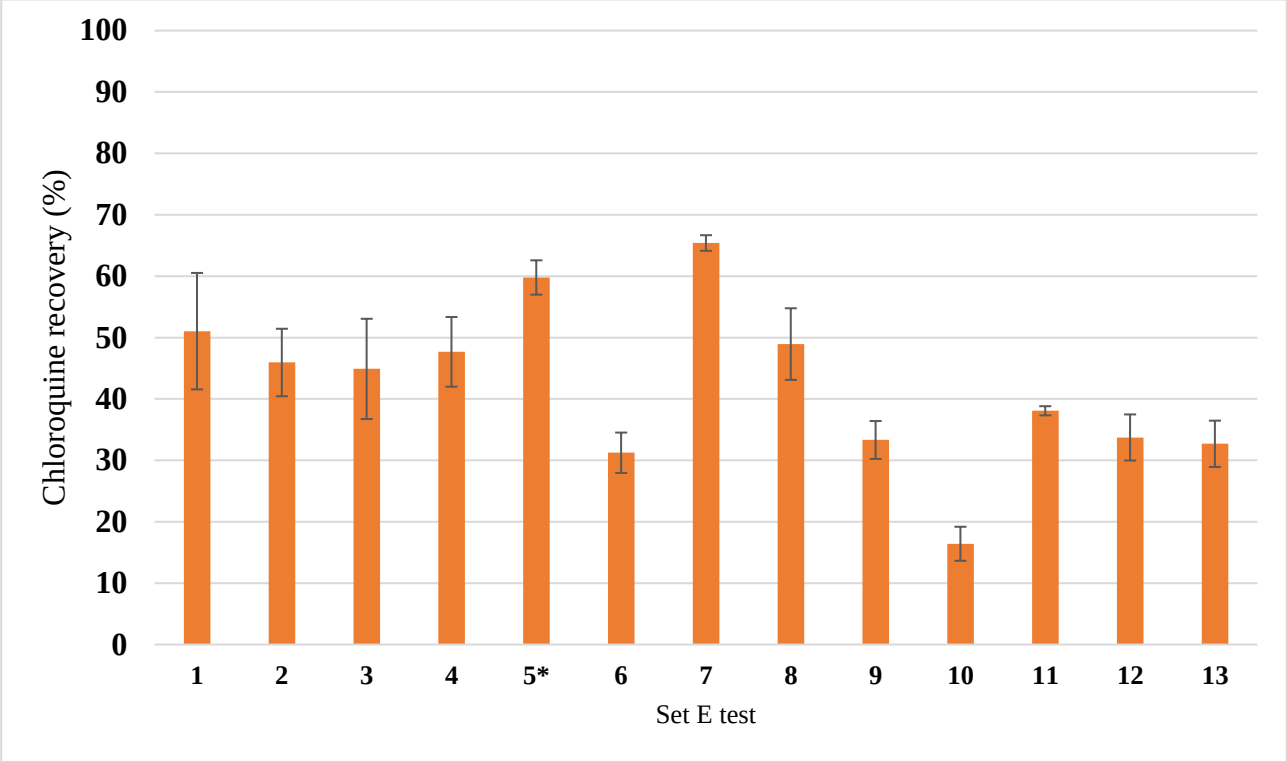


Figure C14. Recovery results of chloroquine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

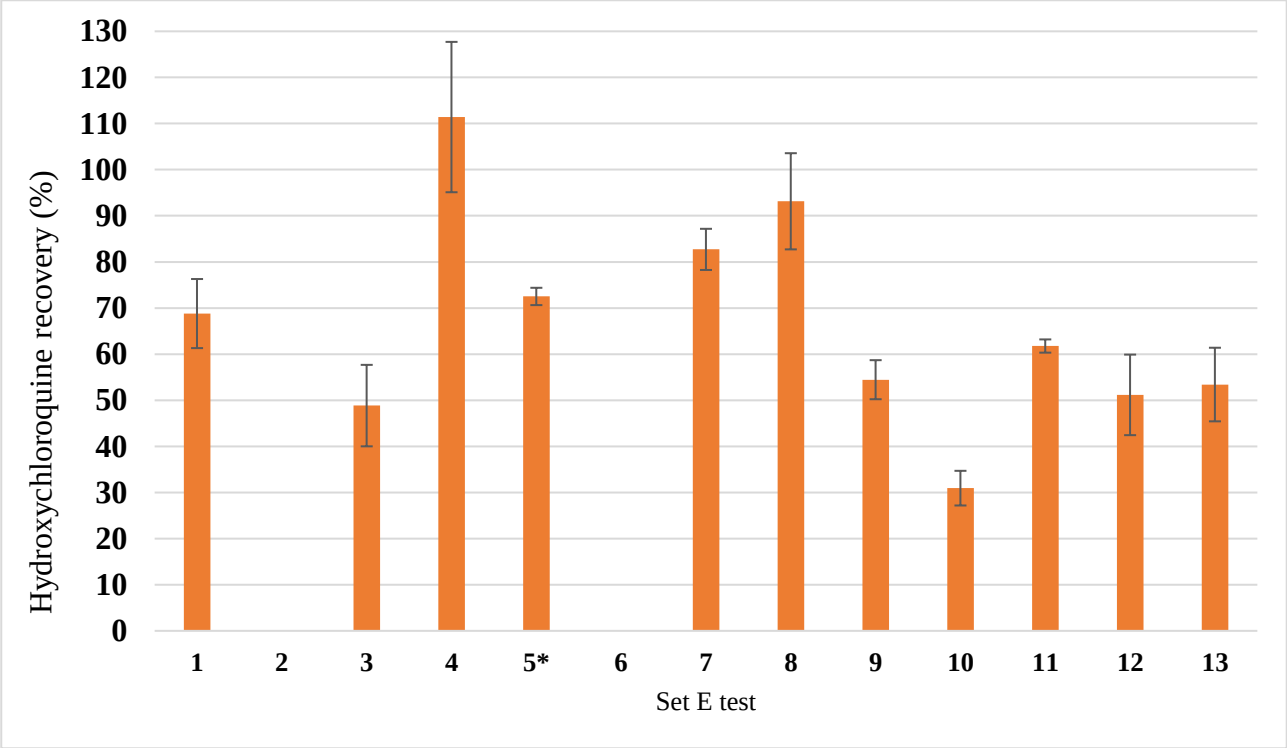


Figure C15. Recovery results of hydroxychloroquine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL). Results of tests 2 (279%) and 6 (176%) are not represented and were not accounted for in calculating average recovery.

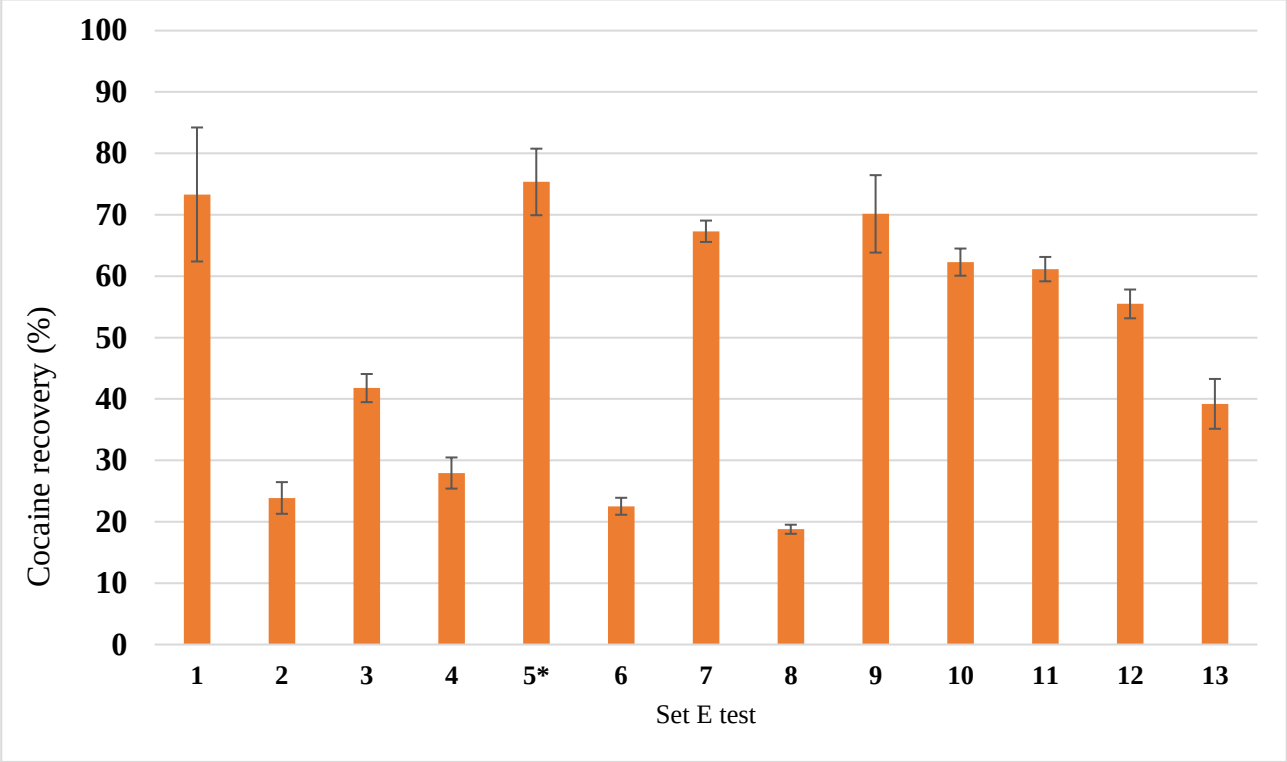


Figure C16. Recovery results of cocaine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

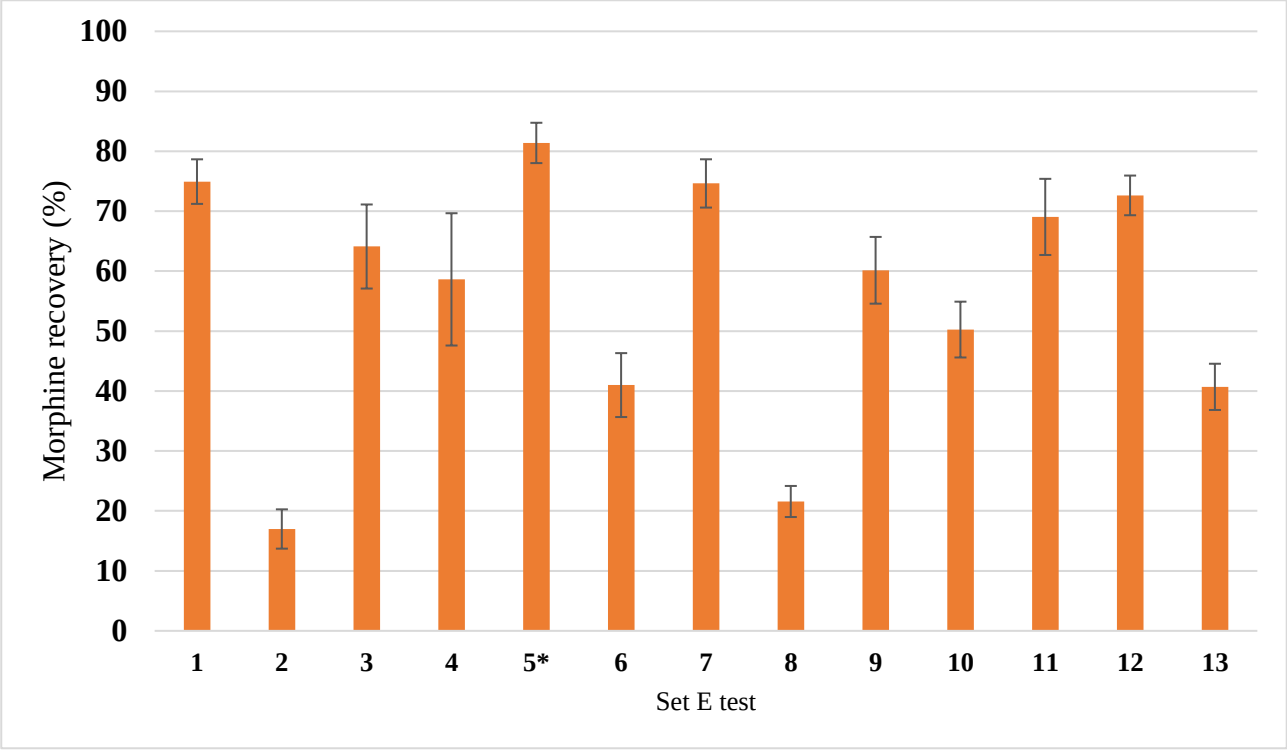


Figure C17. Recovery results of morphine from test set E. Test 5 was selected for wastewater processing (EtOH, WW pH acidified to 5, sample loading volume of 25 mL, solvent elution volume of 5.5 mL).

Annex D – Quality assurance/quality control of the SPE-UHPLC-MS/MS method

Table D1. Retention time, instrument and method detection and quantification limits of the target CECs

CEC	Retention time (min)	IDL (ug/L)	IQL (ug/L)	MDL (ng/L)	MQL (ng/L)
Tramadol	1.003	0.12	0.35	1.61	4.81
Carbamazepine	3.133	0.25	0.77	6.07	18.43
Azithromycin	0.863	0.87	2.65	6.04	18.31
Citalopram	1.679	0.62	1.87	11.24	34.07
Fluoxetine	2.937	0.63	1.91	17.42	52.71
Venlafaxine	1.073	0.12	0.35	1.61	4.81
Dexamethasone	3.167	0.36	1.10	6.26	18.96
Diclofenac	2.118	0.96	2.91	9.39	28.42
Naproxen	1.550	0.63	1.91	21.61	65.54
Remdesivir	3.160	0.13	0.38	1.44	4.36
Atenolol	0.838	0.42	1.28	8.72	26.40
Metoprolol	0.996	0.47	1.42	9.21	27.86
Propranolol	1.210	0.68	2.05	9.61	29.12
Chloroquine	0.860	0.70	2.12	11.70	35.48
Hydroxychloroquine	0.857	1.14	3.45	12.93	39.20
Cocaine	1.045	0.47	1.43	8.79	26.67
Morphine	0.908	0.60	1.82	12.46	37.78

Table D2. Recovery of the optimized SPE method, accuracy, intra-batch precision, process efficiency and matrix effect of the target CECs

CEC	Recovery (%)	Accuracy (%)	Intra-batch precision RSD (%)	Process efficiency (%)	Matrix effect (%)
Tramadol	81.7	80.7 ± 3.7	1.9 – 13.6	64.1	-21.5
Carbamazepine	80.4	89.8 ± 2.7	2.41 – 3.81	41.9	-47.9
Azithromycin	83.8	90.8 ± 3.1	2.77 – 4.01	144.7	72.6
Citalopram	104.4	91.8 ± 3.2	1.73 – 6.35	54.9	-47.4
Fluoxetine	38.6	96.4 ± 3.7	1.57 – 7.23	36.3	-6.0
Venlafaxine	87.6	113 ± 6	1.5 – 11.5	72.1	-17.7
Dexamethasone	83.1	103 ± 5	2.86 – 6.13	58.2	-29.9
Diclofenac	82.7	92.7 ± 1.1	0.74 – 4.41	102.3	23.6
Naproxen	72.0	91.6 ± 8.2	2.1 – 15.2	29.2	-59.4
Remdesivir	82.7	89.6 ± 3.9	2.00 – 6.52	87.6	5.9
Atenolol	82.3	80.6 ± 1.6	3.50 – 15.7	48.3	-41.3
Metoprolol	79.0	95.8 ± 5.1	3.6 – 19.9	51.1	-35.3
Propranolol	110.2	87.3 ± 2.6	1.88 – 4.04	70.6	-36.0
Chloroquine	59.8	113 ± 6	4.8 – 12.9	59.6	-0.2
Hydroxychloroquine	72.5	111 ± 8	5.8 – 11.3	87.9	21.2
Cocaine	75.5	102 ± 2	0.51 – 3.80	53.6	-29.0
Morphine	81.4	109 ± 12	10.0 – 19.4	48.2	-40.7

Annex E – Occurrence of pharmaceuticals and illicit drugs in WWTP influent over 2020

Table E1. Concentrations of tramadol, carbamazepine, azithromycin, citalopram, fluoxetine, venlafaxine, dexamethasone, diclofenac and naproxen, in wastewater samples collected in a WWTP influent during 2020. ND – not determined

Date \ CEC	Tramadol	Carbamazepine	Azithromycin	Citalopram	Fluoxetine	Venlafaxine	Dexamethasone	Diclofenac	Naproxen
07/apr	259.7	144.0	324.4	77.78	153.7	87.09	328.4	600.5	683.4
20/apr	277.5	143.0	203.1	70.63	118.5	75.30	234.3	657.3	1035
04/may	372.0	205.0	260.4	92.01	220.2	136.8	240.0	1101	768.8
07/may	411.9	208.2	140.5	80.90	<MQL	159.6	615.6	958.8	958.2
09/may	419.1	174.6	128.2	232.4	107.2	362.4	216.9	1050	686.6
14/may	324.1	202.8	145.0	77.28	ND	139.9	430.6	899.7	1046
18/may	285.3	114.7	127.6	122.9	127.8	157.8	148.8	642.4	683.6
21/may	518.9	453.3	371.8	156.5	<MQL	400.9	823.2	2332	2103
24/may	468.7	200.7	208.4	102.0	137.5	198.2	371.4	1336	1069
27/may	320.6	168.7	188.1	101.6	135.9	164.5	345.7	849.6	763.0
01/jun	500.6	237.3	199.8	86.86	109.8	215.2	247.1	1188	793.4
04/jun	313.4	208.6	113.6	78.97	ND	131.5	722.0	900.9	1097
07/jun	523.8	207.1	201.9	83.10	121.1	172.9	279.0	958.9	1456
12/jun	458.4	270.3	182.5	100.4	<MQL	187.2	310.2	978.6	980.7
17/jun	384.3	205.6	187.5	69.72	ND	176.3	524.1	853.7	1207
22/jun	350.7	167.8	137.9	78.24	88.19	81.27	262.0	705.7	471.2
25/jun	356.7	218.4	113.5	74.84	ND	150.9	655.8	785.2	1403
29/jun	521.7	240.9	212.8	84.03	ND	188.3	361.8	1222	745.1
02/jul	320.9	251.9	139.0	85.94	ND	168.0	376.2	1019	1119
06/jul	345.0	155.6	116.0	80.59	83.78	88.83	199.5	760.7	671.2
09/jul	358.5	249.7	121.3	94.59	ND	210.5	537.6	1423	1027
13/jul	530.6	195.8	146.2	77.93	ND	162.0	319.4	1093	884.0
16/jul	434.4	284.0	181.3	102.3	ND	235.8	438.4	1400	1265
20/jul	766.3	194.8	163.0	83.92	<MQL	104.2	272.4	771.7	1076

23/jul	409.6	204.3	74.67	81.58	ND	134.0	428.7	695.3	1012
27/jul	636.6	229.0	209.9	93.42	<MQL	156.4	527.1	1168	1025
30/jul	480.5	315.7	326.0	102.8	ND	310.8	814.8	1434	1837
04/aug	537.9	225.3	198.4	107.1	ND	183.2	352.2	996.9	779.8
06/aug	579.0	280.4	140.6	145.4	ND	178.6	691.7	997.5	1034
10/aug	921.6	248.2	238.5	114.0	56.59	148.6	335.7	978.5	974.9
14/aug	624.8	213.8	147.0	105.4	<MQL	161.7	255.0	1083	693.4
17/aug	455.2	176.8	153.3	85.77	<MQL	65.63	156.7	730.3	858.7
24/aug	339.2	139.5	141.9	70.76	ND	53.66	339.3	828.3	428.7
27/aug	730.3	250.4	160.2	125.7	ND	215.4	427.2	1042	1081
31/aug	605.7	203.0	162.9	99.61	ND	130.8	258.8	1008	741.6
03/sep	894.9	230.5	52.83	117.7	ND	309.8	543.6	1212	1124
07/sep	765.2	245.2	282.7	87.56	ND	276.5	300.0	1321	1244
14/sep	772.8	270.1	213.0	108.0	ND	224.3	233.8	1223	1225
17/sep	784.1	255.5	291.2	123.1	75.05	317.4	1223	1371	1179
22/sep	653.7	230.6	196.3	105.8	ND	150.7	278.0	1155	698.8
24/sep	545.0	209.1	181.6	92.92	ND	176.7	513.2	1001	803.9
28/sep	580.7	240.0	253.6	113.6	ND	162.0	298.3	1450	852.4
05/oct	530.4	255.7	197.3	115.3	ND	122.4	285.0	1124	1124
08/oct	1061	398.0	317.1	140.7	ND	397.9	559.8	1870	2262
12/oct	501.6	210.5	174.1	107.4	ND	120.4	220.8	1004	510.6
15/oct	884.0	320.9	378.9	133.2	ND	273.4	593.8	1282	2057
19/oct	652.0	195.2	254.5	126.5	ND	211.9	312.0	1236	917.4
22/oct	370.9	164.2	127.3	84.65	ND	104.0	194.0	445.6	546.6
27/oct	297.8	140.6	101.5	80.60	ND	<50	203.7	488.0	611.9
29/oct	477.6	152.1	193.0	93.06	<MQL	144.8	274.6	727.2	732.1
02/nov	542.4	208.6	198.5	80.92	ND	119.6	238.1	1128	943.0
09/nov	482.0	239.3	169.9	91.12	ND	<50	166.9	794.9	535.4
12/nov	>5000	592.3	430.6	851.3	<MQL	1152	421.1	1508	1179
16/nov	1934	503.8	203.4	305.3	ND	317.3	202.6	1044	1274

19/nov	1321	238.3	256.0	192.8	ND	132.7	519.9	977.0	1375
25/nov	2173	444.2	134.6	464.4	ND	349.5	292.1	539.9	807.5

Table E2. Concentrations of remdesivir, atenolol, metoprolol, propranolol, chloroquine, hydroxychloroquine, cocaine and morphine in wastewater samples collected in a WWTP influent during 2020. ND – not determined

CEC / Date	Remdesivir	Atenolol	Metoprolol	Propranolol	Chloroquine	Hydroxychloroquine	Cocaine	Morphine
07/apr	<50	51.20	82.44	<MQL	116.2	812.7	ND	388.5
20/apr	<50	87.04	ND	<MQL	112.6	707.4	ND	324.5
04/may	<50	171.2	259.7	<50	127.9	1772	ND	388.9
07/may	ND	200.7	ND	<MQL	94.05	1558	ND	179.2
09/may	<50	141.9	85.27	<50	127.3	1397	ND	179.8
14/may	<50	161.2	ND	<50	67.49	711.7	ND	341.1
18/may	<50	63.68	ND	<MQL	94.91	515.4	ND	102.9
21/may	<50	298.9	ND	103.0	129.1	3637	ND	1215
24/may	<50	173.6	ND	<50	106.0	978.3	ND	597.3
27/may	<50	88.78	ND	<50	115.7	1128	ND	582.7
01/jun	<MQL	225.1	ND	<50	69.05	1087	ND	393.3
04/jun	<50	85.94	ND	<50	69.80	947.8	ND	229.2
07/jun	<50	161.6	ND	52.89	122.9	1523	ND	358.0
12/jun	ND	144.1	438.0	65.67	58.41	1519	ND	903.4
17/jun	ND	100.7	ND	<50	70.70	1477	ND	635.7
22/jun	<50	113.5	ND	<50	104.8	583.6	ND	252.1
25/jun	<50	124.9	ND	<50	140.1	921.7	ND	350.3
29/jun	ND	200.9	ND	<50	90.72	947.8	ND	193.0
02/jul	ND	100.8	ND	<50	62.39	337.5	ND	122.6
06/jul	ND	99.65	131.0	<MQL	65.20	759.9	ND	300.4
09/jul	<50	172.9	ND	<50	87.99	570.5	ND	781.1
13/jul	ND	146.9	ND	<50	124.1	1277	ND	258.0

16/jul	<50	221.5	ND	84.00	73.90	558.5	ND	675.4
20/jul	ND	160.5	338.5	<50	59.64	755.5	ND	364.3
23/jul	<50	121.8	ND	<50	104.7	659.6	ND	440.2
27/jul	ND	228.5	ND	<50	185.8	1264	ND	161.9
30/jul	<MQL	285.0	ND	69.50	111.5	749.8	ND	808.3
04/aug	ND	117.8	177.8	70.53	60.86	1136	ND	893.8
06/aug	ND	254.0	ND	106.2	64.47	798.5	ND	257.4
10/aug	ND	219.9	527.6	76.02	96.03	852.8	ND	272.5
14/aug	ND	204.5	56.38	78.09	52.73	1339	ND	257.1
17/aug	ND	99.02	228.9	59.19	54.21	370.1	ND	260.5
24/aug	ND	134.6	ND	59.25	55.51	547.4	ND	289.8
27/aug	<50	268.0	ND	79.96	62.08	1052	ND	219.4
31/aug	ND	129.7	391.1	74.71	<MQL	1419	ND	364.1
03/sep	<50	278.4	ND	58.31	57.99	1161	ND	560.4
07/sep	ND	310.2	ND	58.71	<50	939.7	ND	431.7
14/sep	ND	261.3	ND	72.05	<50	1036	ND	452.6
17/sep	<50	226.5	ND	95.51	145.3	1685	ND	419.1
22/sep	ND	206.3	<MQL	73.12	56.17	543.2	ND	304.0
24/sep	ND	240.2	ND	68.98	139.8	841.7	ND	453.9
28/sep	<50	282.5	ND	<50	94.96	1209	ND	290.4
05/oct	ND	201.2	ND	78.32	<50	1074	ND	359.3
08/oct	<50	508.5	ND	68.82	66.28	1903	<MQL	1143
12/oct	ND	166.4	ND	56.11	56.01	692.8	ND	108.9
15/oct	<50	223.0	ND	70.33	68.79	734.5	<MQL	922.9
19/oct	ND	126.7	613.6	104.4	<50	2185	ND	534.2
22/oct	ND	93.32	300.1	<50	<50	640.5	ND	325.4
27/oct	ND	113.2	ND	<50	<50	409.5	ND	115.5
29/oct	<50	216.5	96.43	87.92	89.18	893.1	<MQL	942.0
02/nov	ND	116.5	478.2	69.41	<50	693.1	ND	406.5
09/nov	ND	114.7	245.3	72.97	<50	365.0	ND	250.1

12/nov	<MQL	>2500	678.0	567.3	63.78	714.9	<MQL	1195
16/nov	ND	697.1	1861	329.9	<50	1203	ND	470.2
19/nov	<50	438.9	<MQL	109.9	95.73	706.7	ND	511.6
25/nov	ND	1170	159.3	206.5	63.15	731.5	ND	283.4

Table E3. Minimum (Min), maximum (Max), average (Avg) and standard deviation (SD) of the concentrations of target CECs for each phase of the pandemic. NQ – not quantified

	FW				RO				SW			
CEC	Min (ng/L)	Max (ng/L	Avg (ng/L)	SD (ng/L)	Min (ng/L)	Max (ng/L	Avg (ng/L)	SD (ng/L)	Min (ng/L)	Max (ng/L	Avg (ng/L)	SD (ng/L)
Tramadol	260	524	393	87	321	1061	599	186	298	2173	914	637
Carbamazepine	115	453	210	70	140	398	236	50	141	592	291	148
Azithromycin	114	372	192	68	53	326	184	66	102	431	223	98
Citalopram	70	232	98	39	71	145	103	18	81	851	228	228
Fluoxetine	88	220	132	34	57	84	72	11	NQ	NQ	NQ	NQ
Venlafaxine	75	401	177	82	54	398	184	80	104	1152	312	309
Dexamethasone	149	823	395	188	157	1223	414	220	167	594	311	135
Diclofenac	601	2332	1001	379	695	1870	1117	261	446	1509	925	337
Naproxen	471	2103	997	369	429	2262	1020	366	535	2058	998	433
Remdesivir	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Atenolol	51	299	144	61	99	509	209	86	93	1171	331	334
Metoprolol	82	438	216	147	56	528	264	151	96	1861	554	531
Propranolol	53	103	74	21	56	107	73	13	69	567	180	159
Chloroquine	58	140	101	25	53	186	84	35	63	96	76	14
Hydroxychloroquine	515	3638	1235	685	338	1904	946	378	365	2186	844	474
Cocaine	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Morphine	103	1215	423	272	109	1143	417	244	116	1195	542	322