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Studies on the Consumption of Pharmaceuticals among the Population using Wastewater Residues as Biomarkers

SUMMARY OF THE DOCTORAL THESIS

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INTRODUCTION

Numerous studies in the literature (Kummerer, 2009; Yi et al., 2020; Rastogi et al., 2021; Adeleye et al., 2022; Kock et al., 2023; Choi et al., 2024) draw attention to pharmaceutical residues present in aquatic environments of industrialized and developing countries, as they constitute a risk to the environment and public health. Residues largely arise from the consumption or production of pharmaceuticals. The consumption of pharmaceuticals reflects the socio-economic conditions of the country, as well as the health status of the population. The emergence of seasonal diseases (colds, viruses, flu, allergies) or a pandemic (SARS-CoV-2) lead to an increase in the consumption of some classes of pharmaceuticals (Samal et al., 2022).

The traditional collection of information on drug consumption among the population is carried out by centralizing medical prescriptions, data on drug sales held by pharmacies, companies, or through questionnaires, but all these methods have limitations (Castiglioni et al., 2014; Escola Casas et al., 2021).

A new method of monitoring substance use, called "wastewater based epidemiology" (WBE), has been developed in the last 20 years (Zuccato et al., 2005; Castiglioni et al., 2014; Gracia-Lor et al., 2017; Kasprzyk-Hordern et al., 2023). Since its first application in 2005 by Zuccato, the WBE method has experienced spectacular development. The method is based on measuring biomarkers (products of human metabolism) and/or parent compounds, excreted by the population, in the sewage system, and the information provided is qualitative, quantitative, almost in real-time and provides the anonymity of the person who consumed.

In Romania, data related to the consumption of pharmaceuticals among the population are difficult to access, and studies on this consumption based on wastewater-based epidemiology are very few and are carried out within European studies (Castiglioni et al., 2021; EUDA, 2024). These studies mainly focus on the consumption of illicit substances. As a result, this thesis presents the development of methods for the analysis and extraction of paracetamol together with four non-steroidal anti-inflammatory drugs (ketoprofen, naproxen, diclofenac and ibuprofen), five steroid hormones (estriol, 17-β-estradiol, estrone, 17-α-ethinylestradiol and hydrocortisone), as well as eight beta-blockers (atenolol, nadolol, pindolol, acebutolol, metoprolol, bisoprolol, propranolol and betaxolol) from wastewater samples and the application of the wastewater-based epidemiology method to estimate their consumption among the population.

The research conducted in this doctoral thesis concerns the aforementioned pharmaceuticals and focuses on the following aspects:

- ✓ Development of methods for the analysis of studied pharmaceuticals by high-performance liquid chromatography with photodiode array detection, as well as the quantification of these compounds in wastewater samples.
- ✓ Development of methods for the analysis of some studied pharmaceuticals (paracetamol, non-steroidal anti-inflammatory drugs, beta-blockers) by gas chromatography coupled with mass spectrometry with SIM mode, as well as their quantification in waste water samples.
- ✓ Development of methods for the analysis of some studied pharmaceuticals (paracetamol, nonsteroidal anti-inflammatory drugs, beta-blockers) by high-performance liquid chromatography coupled with tandem mass spectrometry, as well as their quantification in wastewater samples.
- ✓ Development of methods for the extraction of studied pharmaceuticals from wastewater, using liquid-liquid extraction (paracetamol and nonsteroidal anti-inflammatory drugs), solid-phase extraction (paracetamol, nonsteroidal anti-inflammatory drugs, steroid hormones, beta-blockers) and liquid-liquid dispersive microextraction (beta-blockers).
- ✓ Estimation of the consumption of studied pharmaceuticals by applying wastewater-based epidemiology, using the concentrations found in water samples collected from the influent of the Cluj-Napoca municipal wastewater treatment plant.

The novelty of this thesis lies in the fact that the wastewater-based epidemiology method used to estimate the consumption of the mentioned pharmaceuticals among the population in Romania is at its first attempt, and the simultaneous extraction of eight beta-blockers using liquid-liquid dispersive microextraction, to our knowledge, is not mentioned in the literature data.

This doctoral thesis is structured in two parts, a theoretical part that includes the literature study in the specifics of the thesis and an experimental part that presents the original contributions.

The theoretical part is structured in three chapters that include: *(i)* general data about the pharmaceuticals studied, *(ii)* information about their effects on the environment as polluting sources and the legislative norms in force, *(iii)* description of the wastewater-based epidemiology method, as well as the steps to follow, necessary for its application.

The experimental part is also structured in three chapters, in which the original contributions regarding the studied pharmaceuticals are presented: *(i)* analysis methods using different chromatographic techniques, *(ii)* extraction and microextraction methods, and *(iii)* estimation of pharmaceuticals consumption among the population under investigation.

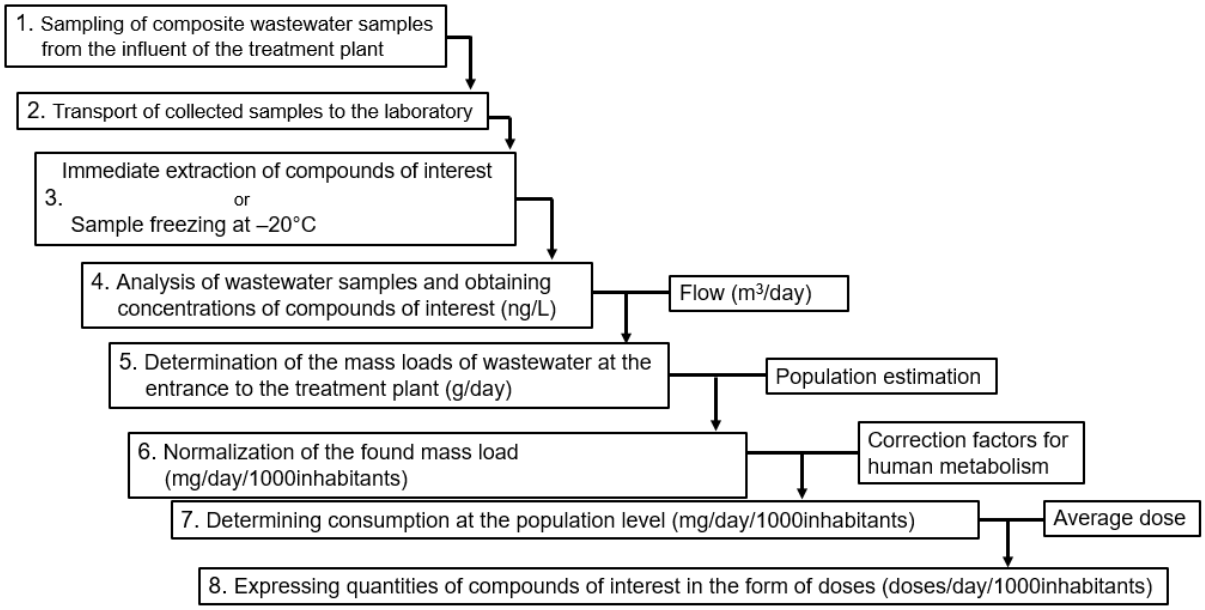
The results presented in this paper were achieved as part of the doctoral study and were disseminated at international scientific events, in the country and abroad, published or are in the process of being published in ISI-listed journals.

At the end of this doctoral thesis are attached:

- ✓ List of abbreviations frequently used in the doctoral thesis (Annex I),
- ✓ List of scientific publications and participation in scientific events (Annex II), and
- ✓ Note regarding the research internship accomplished abroad (Annex III).

Wastewater-based epidemiology (WBE) is a complementary method in studies of monitoring the consumption of compounds of interest among the population, as it provides additional information on short-term and long-term or occasional (sports or music events) consumption of these compounds, which allows monitoring and warning about the consumption of new psychoactive substances or ethnobotanical substances, and covers the entire system or almost the entire population connected to sewage system (Castiglioni et al., 2014; Lai et al., 2016). Furthermore, a detailed analysis of the data obtained with this method in conjunction with those from different data sets resulting from information campaigns, consumption reduction strategies, the presence of viruses, etc. can be particularly useful through the new information brought (Boogaerts et al., 2021).

However, the WBE method also presents several disadvantages generated by the lack of information regarding the purity, dosage or frequency of use of the compound of interest and, in some cases, the mode of administration, a single compound or concomitantly with other compounds. As a result, the method does not clearly reflect the number of consumers because the amount consumed is normalized (mg/day/1000 inhabitants or doses/day/1000 inhabitants), it does not distinguish between high consumption due to an increase in the number of consumers or an increase in the dose consumed, and it cannot estimate the price of the compound of interest (Castiglioni et al., 2014; Rice et al., 2020; Boogaerts et al., 2021).



Sequence of steps and data required to determine the consumption of compounds of interest through the wastewater-based epidemiology method.

Importance of the WBE study

Pharmaceuticals are therapeutic chemical compounds, specially created or isolated, to prevent, ameliorate, or treat various diseases. They contain active pharmaceutical substances, individually or in combinations, having the ability to cross cell membranes and produce the desired therapeutic effect (Calisto & Esteves, 2009). The increasing consumption of pharmaceuticals is caused by several factors: *(i)* increasing living standards and increasing demand for food products, especially animal protein (Kock et al., 2023), *(ii)* demographic aging of the population and vulnerability to chronic conditions, *(iii)* the increase in the number of drugs available for the treatment of various conditions and diseases, *(iv)* easy access to drugs, as well as *(v)* the influence of the pharmaceutical industry through intensively promoted advertisements (Escola Casas et al., 2021).

Pharmaceuticals have different chemical structures, but the usual classification is made by therapeutic classes or groups based on the mode of action and treatment of diseases, such as: antibiotics, analgesics and anti-inflammatories, hormones, antidepressants, antivirals, anticoagulants, sedatives, etc. (Samal et al., 2022).

Pharmaceuticals are generally polar compounds, with varied chemical structures, presenting one or more ionizable groups, lipophilic or hydrophilic properties (Patel et al.,

2019), acidic, basic or amphoteric character. Any change in the chemical structure of a drug can have consequences on its solubility and polarity, but also on some properties regarding their behavior in the environment, where drugs are found in different forms (neutral, cationic, anionic or zwitterionic), thus generating a complex behavior. Drugs retain their chemical structure for a long time after use, in order to be able to fulfill their role, therefore a fairly high percentage is released into the environmental factors, in the original form (Feng et al., 2013).

The progress and increase in the number of pharmceuticals available for the treatment of conditions and diseases can be observed through the increasing concentrations found in the environment, especially in the aquatic environment (Rastogi et al., 2021).

Pharmaceuticals and their metabolites end up in environmental factors, especially in water, following the discharge of treated water from sewage treatment plants, which is the point of transfer to environmental factors (Khumalo et al., 2023). Wastewater treatment plants receive domestic, hospital, industrial, and livestock wastewater. In most cases, wastewater treatment plants are designed to remove nutrients, pathogens, and particulate matter, but not pharmaceutical compounds (Adeleye et al., 2022; Kołecka et al., 2022). Pharmaceuticals can enter water and soil through the use of sewage sludge as fertilizer or waste from livestock farms, as well as from poorly managed sites where animal carcasses have been buried (Nguyen et al., 2023).

In domestic wastewater, the sources of pharmaceuticals, both in urban and in rural areas, are consumption, washing them off the body, or flushing expired, unwanted, or unused pharmaceuticals down the drain. In the European Union, expired medicines have a special collection system, as in Romania. After the expiry date, the pharmaceuticals must be taken to the special collection centers within the hospitals, to the points in charge of their management or to the pharmacies (Tit et al., 2016; Bungau et al., 2018).

Pharmaceuticals are specifically designed to provoke a biological response following use, but they can also produce a non-specific effect, due to their persistence and resistance to degradation (González-González et al., 2022). Their constant release into environmental factors produces mutagenic, genotoxic and ecotoxicological effects on plants, animals and humans (Samal et al., 2022; Huynh et al., 2023).

Monitoring the mass loads of pharmaceuticals in wastewater and their consumption is of particular importance, as it provides evidence and informs decision-makers regarding public and environmental health. To obtain quantitative data, mathematical equations are used and the biomarker or parent compound measured in the wastewater treatment plant influent is taken into account together with information related to human metabolism, namely drug excretion

rates, wastewater flow rate at the wastewater treatment plant entrance, as well as the number of inhabitants connected to the sewage system (Zuccato et al., 2005; van Nuijs et al., 2011; Lai et al., 2011; Han et al., 2022).

The metabolism and rate of excretion are specific to each drug and vary according to health status, lifestyle, age and gender (Holton et al., 2022). When estimating drug consumption, both parenteral and topical administration should be considered (Kannan et al., 2023).

Wastewater-based epidemiology offers the possibility of estimating drug consumption in order to assess their impact on the environment (Ceolotto et al., 2024).

Requirements for processing collected samples and analyzing studied pharmaceuticals necessary in wastewater-based epidemiology

Extraction methods

Despite technological advances in the field of analytical chemistry, most instruments are not capable of directly analyzing complex samples, especially samples with ultratrace level concentrations of compounds. The sample preparation stage for the actual analysis involves the isolation and/or preconcentration of the compounds of interest from different matrices, as well as the transposition of the compounds of interest into a form compatible with the analytical system, in order to achieve their separation and detection. It is the stage that requires around 80% of the time needed to complete an analysis and is of particular importance in the final result because it is the source of most errors, with effects on all subsequent stages (Robards et al., 2004; Chen et al., 2008; Xu & Lee, 2012).

When preparing samples for instrumental analysis, the following must be taken into account: (Chen et al., 2021):

(i) obtaining high efficiency in the extraction of compounds of interest,
(ii) compatibility between the resulting extract and the analytical system used,
(iii) the cost and time required to carry out the extraction process,
(iv) sustainability of the extraction method, according to green chemistry principles.

In recent years, miniaturized methods for sample preparation for analysis have been developed that respect the principles of green chemistry (reduced solvent volume (μL), replacement of toxic solvents, reduced energy consumption, waste reduction), have the

possibility of automation, present high recovery rates and preconcentration factors, and require a reduced sample volume (mL) (Seidi et al., 2019; Samadifar et al., 2023).

Miniaturized methods developed for both liquid-liquid extraction (Beldean-Galea, 2017) and ***solid-phase extraction*** (Samadifar et al., 2023) are based on the same classical extraction principles. *Liquid-liquid microextraction* (LLME) is performed in a single step, and the final extract does not require concentration, as the volume of solvent used is on the order of microliters. The extraction system is a tertiary one, consisting of the liquid sample, the extraction solvent and the dispersion solvent with a role in the dispersion of microdroplets of extraction solvent in the sample. *Solid-phase microextraction* (SPME) is performed by adsorption on a solid stationary phase of the compounds of interest from the sample to be analyzed, followed by their elution (Chen et al., 2021).

Microextraction of the studied drugs was performed by *Dispersive Liquid-Liquid MicroExtraction* (DLLME) (Rezaee et al., 2006); *Dispersive Liquid-Liquid Microextraction based on Solidification of Floating Organic Droplet* (DLLME-SFO(D) (Leong & Huang, 2008) and *solid-phase extraction* (SPE) on Strata X cartridges (Xu & Lee, 2012).

Chromatographic analysis techniques

Chromatographic analysis techniques are analytical separation techniques used for the separation, identification, and qualitative and quantitative determination of complex mixtures.

Currently, the increase in the performance of analytical instrumentation through high specificity and selectivity has made it possible to qualitatively and quantitatively evaluate drugs in various types of complex matrices, such as: biological fluids, food, soil, surface water, wastewater. Pharmaceuticals present a wide range of compounds with different physicochemical properties, which makes their determination a real analytical challenge due to the high number of compounds present in the environment, their nature (from non-polar to medium polar and very polar compounds, respectively from low to high volatility compounds), but also of the trace and ultratrace concentrations found in complex matrices.

Optimization of the parameters of analytical methods (liquid chromatography, gas chromatography, mass spectrometry or tandem mass spectrometry) is crucial in the development and successful application of the analytical method for the determination of compounds of interest in samples.

The remarkable increase in the number of publications on the presence of pharmaceuticals in waters around the world was recorded after the 2000s, as a result of the increasing consumption of pharmaceuticals in society, which leads to water pollution and, consequently, has negative effects on the health of the population, but also of animals (Moeder et al., 2000; Öllers et al., 2001; Jux et al., 2002; Hilton & Thomas, 2003; Cahill et al., 2004; Zuccato et al., 2005; Cui et al., 2006; MacLeod et al., 2007; Gros et al., 2008; Zhao et al., 2009; Parrilla Vázquez et al., 2010; van Nuijs et al., 2011; Herrero et al., 2012; Lai et al., 2013; Kankaanpää et al., 2014; Beldean-Galea et al., 2015; Lai et al., 2016; Baz-Lomba et al., 2017; Duan et al., 2018; Fang et al., 2019; Iancu et al., 2020; Yan et al., 2021; Ofrydopoulou et al., 2022; Santana-Viera et al., 2023; Li et al., 2024).

The frequently used analytical technique for the determination of pharmaceuticals in wastewater in WBE studies is high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (LC–QqQ-MS/MS) (Skees et al., 2018; Nguyen et al., 2018; Zhang et al., 2019; Lei et al., 2021; Galani et al., 2021; Tomsone et al., 2022; Han et al., 2022; Kannan et al., 2023; Gao et al., 2023; Li et al., 2024). Due to the polarity of the compounds, the separation is performed on C18 stationary phases (Nguyen et al., 2018; Zhang et al., 2019; Wang et al., 2020; Lei și colab., 2021; Yan et al., 2021; Han et al., 2022; Kannan et al., 2023; Gao et al., 2023). Mobile phases are water with acids (formic, acetic), salts (ammonium formate, ammonium acetate), acetonitrile or methanol (Skees et al., 2018; Nguyen et al., 2018; Zhang et al., 2019; Lei et al., 2021; Galani et al., 2021; Tomsone et al., 2022; Han et al., 2022; Kannan et al., 2023; Gao et al., 2023; Li et al., 2024). The ionization of compounds in the LC-MS/MS analysis method can be positive or negative (Galani et al., 2021).

THE SECOND PART – ORIGINAL CONTRIBUTIONS

Determination of the consumption of certain pharmaceuticals among the population of Cluj-Napoca using the wastewater-based epidemiology method

The wastewater samples were collected from the wastewater treatment plant of Cluj-Napoca municipality, Romania, which also includes four neighboring municipalities (Florești, Gilău, Baciu and Săvădisla). The wastewater treatment plant consists of three stages (mechanical, biological and chemical), each stage having a specific and well-defined role in the wastewater treatment process. In the WBE study, composite wastewater samples were collected from the wastewater treatment plant influent which consists of domestic water, industrial water, and stormwater from the sewer system serving a population of over 400,000.

Three case studies are presented on determining the consumption of certain pharmaceuticals among the population of Cluj-Napoca using the wastewater-based epidemiology method, namely:

(i) Paracetamol and non-steroidal antiinflammatory drugs,

(ii) Steroid hormones, and

(iii) beta-Blockers.

THE CASE STUDY No. 1. Paracetamol and antiinflamatory drugs

Paracetamol is used for symptoms associated with flu and colds, headaches, as well as for individuals allergic to aspirin (Boumya et al., 2021).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are generally safe and effective in the short-term management of moderate to severe pain (headache, toothache, musculoskeletal pain, post-operative pain, dysmenorrhea, and the common cold). They are non-narcotic analgesics, available over the counter or prescription, and are widely used alone or in combination with other pharmaceuticals (Garg, 2009). They are effective in relieving painful episodes in patients with chronic diseases (osteoarthritis), reduce the risk of developing cancer and cause tumor regression. The use of nonsteroidal anti-inflammatory drugs does not produce addiction like opioids (Bindu et al., 2020), but in case of abuse or prolonged use, gastrointestinal, cardiovascular, renal damage and even death may occur (Moore et al., 2015;

Bindu et al., 2020; Uzzaman et al., 2023). Nonsteroidal anti-inflammatory drugs are persistent, difficult to degrade, and accumulate in environmental factors (Rastogi et al., 2021).

Non-steroidal anti-inflammatories are derivatives of carboxylic acids (salicylic, acetic, anthranilic, enolic) which present themselves under a wide range of compounds with antipyretic, analgesic and anti-inflammatory properties (Feng et al., 2013; Harvey & Cuculici, 2013; Bindu et al., 2020; Huynh et al., 2023)

In the doctoral thesis, different approaches are presented regarding the possibilities of analysis and extraction from wastewater of paracetamol and four nonsteroidal anti-inflammatory drugs (paracetamol – PARA, ketoprofen – KET, naproxen – NAP, Diclofenac – DIC and ibuprofen – IBU), as well as how the consumption estimation was carried out in different periods of sampling (*September 2021* and *February 2022*).

Optimization of chromatographic analysis methods (HPLC-PDA, GC-MS-SIM, LC-MS/MS)

Analysis of paracetamol and studied nonsteroidal anti-inflammatory drugs by high performance liquid chromatography

Paracetamol and the studied four nonsteroidal anti-inflammatory drugs were analyzed by three high-performance chromatographic methods coupled with different detectors:

(i) High Performance Liquid Chromatography with Photodiode Array Detector, HPLC-PDA;
(ii) Gas Chromatography-Mass Spectrometry with Selected Ions Monitoring (GC-MS-SIM);
(iii) Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS).

Conclusions of the THE CASE STUDY No. 1.
Paracetamol and anti-inflammatory drugs

This study represents the first attempt in Romania to estimate the consumption of paracetamol and nonsteroidal anti-inflammatory drugs (ketoprofen, naproxen, ibuprofen and diclofenac) among the population using wastewater-based epidemiology.

For this purpose, the following analysis and extraction methods were developed and validated.

The ***HPLC-PDA*** method developed and validated for the studied pharmaceuticals shows acceptable linearity ($R^2 > 0.9969$) in the concentration range 0.25–10 mg/L, with good *intraday* (RSD ≤ 2.15%) and *interday* (RSD ≤ 3.19%) precision, low detection and quantification limits of the instrument (10–30 μg/L; 40–90 μg/L), as well as of the method (40–120 ng/L; 160–200 ng/L). However, the HPLC-PDA method was not sufficiently specific and selective for the analysis of paracetamol and nonsteroidal anti-inflammatory drugs in wastewater samples.

The developed and validated ***GC-MS-SIM*** method in the range of 0.667–10 mg/L has good linearity ($R^2 > 0.9872$), satisfactory *intraday* (RSD ≤ 12.23%) and *interday* (RSD ≤ 12.68%) precision, low limits of detection (30–150 μg/L) and quantification (90–440 μg/L) of both the instrument and the limits of detection (6–30 ng/L) and quantification (18–88 ng/L) of the method. This method has been successfully used in the quantification of paracetamol and the studied nonsteroidal anti-inflammatory drugs in water samples, due to the double identification based on the Selected Molecular Ion (*m/z*) and the retention time. The disadvantage of the method is the longer sample processing time, since the compounds require derivatization that was done with the BSTFA:TMCS (99:1, *v/v*) derivatization agent .

The developed and validated ***LC-MS/MS*** method shows satisfactory linearity ($R^2 > 0.9872$) in the concentration range 10–2000 ng/mL, with acceptable *intraday* (RSD ≤ 14.05%) and *interday* (RSD ≤ 7.58%) precision, having low detection (6–52 μg/L) and quantification (18.2–158 μg/L) limits of both the instrument and the detection (6–52 ng/L) and quantification (18.2–158 ng/L) limits of the method. This method allows the quantification of paracetamol and nonsteroidal anti-inflammatory drugs in wastewater samples, without the need for an additional step, as in the case of the GC–MS-SIM method.

After testing the two extraction methods (liquid-liquid extraction and solid-phase extraction), we observed that ***solid-phase extraction*** performed on the Strata-X cartridge, with a sample pH of 3, offers the best recovery rates for the studied compounds (paracetamol 41.57%; ketoprofen 91.89%; naproxen 91.62%; diclofenac 87.72%; and ibuprofen 93.75%). The other extraction cartridges considered were C18-U and C18-E, but the recovery rates were not satisfactory. In the case of liquid-liquid extraction, the presence of impurities in the final extract, following the use of ethyl acetate, disrupts the analysis, and when using the *n*-hexane:isopropranol mixture (3:2, *v/v*) no extraction of paracetamol occurs.

The ***GC-MS-SIM*** analysis method was applied to wastewater samples collected from septic tanks and from the influent of some treatment plants. The concentrations of the studied pharmaceuticals found range from undetectable to certain values, as follows: n.d.–224.11 ng/L for paracetamol; n.d.–2,705.10 ng/L for ketoprofen; n.d.–1,743.40 ng/L for naproxen; n.d.–16,967.80 ng/L for diclofenac; and from n.d.–2,436.70 ng/L for ibuprofen. Their determination, both in septic tanks and in different treatment plants, shows their common use for various conditions, as well as their persistence.

The ***LC-MS/MS*** method applied to wastewater samples collected from the influent of the Cluj-Napoca wastewater treatment plant, in the two periods, allowed the determination of the concentrations presented below: *September 2021 campaign* – concentrations varied in the ranges of 6.46–24.83 μg/L for paracetamol; 0.90–9.34 μg/L for ketoprofen; 1.28–1.92 μg/L for naproxen; 2.50–5.06 μg/L for ibuprofen; and 0.97–3.04 μg/L for diclofenac, while in the *February 2022 campaign* – concentrations varied in the ranges of 0.88–12.14 μg/L for paracetamol; 0.75–3.66 μg/L for ketoprofen; 1.44–2.62 μg/L for naproxen; 1.33–5.52 μg/L for ibuprofen; and 1.12–2.36 μg/L for diclofenac. These concentrations correspond to a consumption in *September 2021* between 44.60–185.57 g/day/1000inh paracetamol; 0.25–2.65 g/day/1000inh ketoprofen; 0.29–0.46 g/day/1000inh naproxen; 0.55–1.15 g/day/1000inh ibuprofen; and 0.23–0.73 g/day/1000inh diclofenac, and for *February 2022* between 6.65–93.46 g/day/1000inh paracetamol; 0.21–0.99 g/day/1000inh ketoprofen; 0.35–0.60 g/day/1000inh naproxen; 0.32–0.61 g/day/1000inh ibuprofen; and 0.27–0.57 g/day/1000inh diclofenac.

Ammoniacal nitrogen is the *hydrochemical parameter* taken into account for estimating the population connected to the sewage system (524,394 inhabitants for *September 2021*, respectively *498,718 inhabitants* for February 2022), being specific to urinary excretion, without being affected by industrial or domestic activities, but is affected by the rainy season.

The high values of estimated consumption of these drugs in the study may be associated with the large number of reported positive SARS-CoV-2 cases, the flu season, as well as rheumatic conditions.

Comparing the obtained values of drug consumption with other values from different studies, it is observed that the values of paracetamol are consistent with these values, while for ketoprofen and diclofenac the estimated values are higher, and for naproxen and ibuprofen they are lower.

THE CASE STUDY No. 2. Steroid Hormones

Hormones or chemical messengers are substances secreted by endocrine glands or other tissues that perform specific functions of stimulating and coordinating the activity of certain organs or the entire organism.

Endocrine glands, tissues that produce hormones, as well as hormones and hormone receptors, constitute the endocrine system. Depending on their molecular structure, hormones can be grouped into polypeptides, steroids, amines, and eicosanoids (Johnstone et al., 2014).

Steroid hormones (steroids) are present in plants, animals and humans. They can be classified into corticosteroids (adrenocorticoids) and gonadocorticoids (sex hormones: androgens, estrogens, progesterones) (Liu et al., 2021), forming a group of biologically active compounds, derived from cholesterol, having in its structure a cyclopentanoperhydrophenanthrene type nucleus formed by three cyclohexane rings and one cyclopentane ring (Cui et al., 2006). The process of forming steroid hormones is called steroidogenesis, and the main source is low-density lipoproteins (LDL cholesterol) (Samavat & Kurzer, 2015).

Depending on their origin, estrogen hormones can be classified into (Hamid & Eskicioglu, 2012; Domínguez-López et al., 2020): *(i)* natural estrogens (estrone, estriol, estradiol); *(ii)* synthetic estrogens (17-α-ethinylestradiol); *(iii)* plant estrogens or phytoestrogens (isoflavones, coumestans) and *(iv)* fungal estrogens or mycoestrogens (zearalenone).

Natural estrogens are secreted by the adrenal cortex, testicles, ovaries and placenta, being present in both the female and male body, but at lower concentrations (Cui et al., 2006; Ojoghoro et al., 2021). These hormones play a role in the growth and harmonious functioning of the body, the health of the reproductive, cardiovascular, bone, and gastrointestinal systems, as well as in cognitive behavior (Adeel et al., 2017; Ojoghoro et al., 2021; Liu et al., 2021). Hormone deficiency or hormonal imbalances can lead to cancer, obesity, diabetes, and heart disease (Almazrouei et al., 2023).

Veterinary medicine uses natural and synthetic estrogens to treat reproductive system dysfunctions in cows and mares, but also as growth promoters (Adeel et al., 2017; Ojoghoro et al., 2021; Almazrouei et al., 2023).

Hydrocortisone and 17-α-ethinylestradiol are part of the World Health Organization's list of essential medicines (WHO, 2021).

Endogenous excretion, therapeutic use or use in the zootechnical sector of steroid hormones constantly fuel their presence in environmental factors, especially in water (Adeel et al., 2017). Steroid hormones are endocrine disruptors and produce imbalances in hormone biosynthesis, metabolism, or activity (Almazrouei et al., 2023).

The case study no. 2 present the ***HPLC-PDA*** analysis method and the ***solid-phase extraction*** method of the steroid hormones studied from wastewater samples to determine the normalized mass loads with them.

The studied steroid hormones (estriol, 17-β-estradiol, estrone, 17-α-ethinylestradiol, hydrocortisone) are very weak acids (pKa values between 10.30 and 12.59), with a slightly lipophilic character (logP values between 1.61 and 3.94).

Conclusions of the CASE STUDY No. 2. Steroid Hormones

This study is the first attempt in Romania to investigate steroid hormone mass loads using wastewater-based epidemiology methodology.

For this purpose, an ***HPLC-PDA*** method was successfully optimized and validated. The method exhibits good linearity ($R^2 > 0.9977$) in a concentration range of 1.56–50 mg/L, high intraday precision (RSD ≤ 2.69%) and interday precision (RSD ≤ 3.11%), low detection and quantification limits of both the instrument (0.076–0.233 mg/L; 0.230–0.705 mg/L) and the method (0.114–0.163 μg/L; 0.345–1.057 μg/L).

Solid-phase extraction of steroid hormones from wastewater samples was performed using Strata-X cartridges, and the sample was acidified to pH 3. The recovery rates obtained were: estriol 93.22%; 17-β-estradiol 89.99%; estrone 80.01%; 17-α-ethinylestradiol 84.03% and hydrocortisone 86.75%.

Following the analysis of wastewater samples from *September 2021*, the hormone concentration was as follows: estriol 2.67–104.50 μg/L; 17-β-estradiol n.d.–2.47 μg/L; estrone n.d.–22.42 μg/L; 17-α-ethinylestradiol n.d.–0.65 μg/L and hydrocortisone n.d.–0.11 μg/L. For samples from *February 2022*, the hormone concentrations were: estriol 6.46–18.80 μg/L; 17-β-estradiol n.d.–0.03 μg/L; estrone n.d.–4.35 μg/L. The hormones 17-α-ethinylestradiol and hydrocortisone were not detected.

The corresponding mass load of these concentrations varies in the following ranges:

(i) ***September 2021***, the calculated values are: 0.60–23.81 g/day/1000inh for estriol; n.d.–0.56 for g/day/1000inh 17-β-estradiol; n.d.–5.11 g/day/1000inh for estrone; n.d.–0.15 g/day/1000inh for 17-α-ethinylestradiol; and n.d.–0.02 g/day/1000inh for hydrocortisone.

(ii) ***February 2022***, the calculated values are: 1.49–4.28 g/day/1000inh for estriol; n.d.–0.01 g/day/1000inh for 17-β-estradiol; n.d.–0.98 g/day/1000inh for estrone; values for 17-α-ethinylestradiol and hydrocortisone were not calculated because these hormones were not detected in the water samples.

The wastewater-based epidemiology applied in our research could be used as a tool to monitor trends in steroid hormone use in the community, but the following should be taken into account: *(i)* their stability (in sewage and in the sample), *(ii)* transformation due to microbial activity, *(iii)* specificity of hormones (excretion or consumption), *(iv)* human metabolism, *(v)* their affinity for solids due to their hydrophobicity, as well as *(vi)* accurate knowledge of the number of inhabitants connected to the sewage system.

THE CASE STUDY No. 3. beta-Blockers

beta-Blockers (β-blockers or β-adrenergic antagonists) are the first-line drugs for cardiovascular conditions and represent the basic therapy in the treatment of heart disease, especially heart failure, acute myocardial infarction, hypertension, angina pectoris, arrhythmias, but also anxiety, panic attacks, migraine, hyperthyroidism, as well as topical medication for open-angle glaucoma (Bekhradnia & Ebrahimzadeh, 2012; Dissanayake & Wahl, 2014; Parrilla Vázquez et al., 2014; Poirier & Tobe, 2014).

Due to their calming effects, beta-blockers can be abused to alleviate anxiety states, being present in the case of doping in athletes (Gonçalves et al., 2019; Chen et al., 2023), in the prevention of stress and death of animals on their way to slaughterhouses (Xu et al., 2019) or in the case of horse racing (Lee et al., 2007).

The adverse effects of beta-blocker use include bradycardia, asthma, dizziness, vomiting, depression, impotence, insomnia, coma, mutagenic potential, and in the case of accidental or intentional overdose, they lead to death, especially in patients with renal or hepatic failure (Barron et al., 2013; Dissanayake & Wahl, 2014; Chen et al., 2023). Nitro and N-nitroso derivatives of beta-blockers are genotoxic, carcinogenic, mutagenic and can cause liver damage. These derivatives are formed in the gastrointestinal tract, following reactions between the amine and amide functional groups of beta-blockers with gastric acid in the stomach (Sarvestani et al., 2021).

beta-Blockers are considered pseudo-persistent pollutants, with half-lives in water between 0.4–13.1 ± 0.9 days (Ramil et al., 2010; Xu et al., 2019). Maszkowska et al. (2014)

showed that beta-blockers affect testosterone levels in male organisms, similar to endocrine disruptors. Chronic or acute exposure of environmental organisms to pharmaceuticals causes negative effects associated with their growth, reproduction and even death (Xu et al., 2019).

In the doctoral thesis, different approaches are presented regarding the possibilities of analysis and extraction of beta-blocker drugs from wastewater. The determined beta-blocker concentrations will be used to estimate consumption among the population in two different periods (February 2024 and October 2024).

The studied beta-blockers are: atenolol, nadolol, pindolol, acebutolol, metoprolol, bisoprolol, propranolol, betaxolol. These are weakly alkaline compounds, being secondary amines, with pKa values between 9.21 and 9.67, having a hydrophilic character (logP 0.16–3.48) (Iancu et al., 2019).

Conclusions of the THE CASE STUDY No. 3. beta-Blockers

This study is the first attempt in Romania to investigate the consumption of beta-blockers (atenolol, nadolol, pindolol, acebutolol, metoprolol, propranolol, betaxolol) among the population using wastewater-based epidemiology.

For this purpose, the following analysis and extraction methods for beta-blockers have been developed and validated.

The ***HPLC-PDA*** analysis method for the studied beta-blockers presents well-defined retention times, good linearity ($R^2 > 0.9950$) in the concentration range of 1.56–50 μg/mL, high *intraday* (RSD ≤ 2.60%) and *interday* (RSD ≤ 1.68%) precision, low detection (0.07–0.15 μg/mL) and quantification (0.20–0.45 μg/mL) limits.

The ***GC–MS-SIM*** analysis method is distinguished by: good linearity ($R^2 > 0.9981$) in the concentration range of 1.56–100 μg/mL, satisfactory *intraday* (RSD ≤ 7.38%) and *interday* (RSD ≤ 14.91%) precisions, low limits of detection (0.13–0.69 μg/mL) and quantification (0.39–2.10 μg/mL).

The ***LC–MS/MS*** method exhibits well-defined retention times and specific molecular ions, good linearity ($R^2 > 0.99854$) in the concentration range of 1–200 ng/mL, and high accuracy (> 91.66%), low limits of detection (0.08–0.22 ng/mL) and quantification (0.15–0.44 ng/mL).

Solid phase extraction (SPE) using C18-U extraction cartridges allowed the isolation of the studied beta-blockers from wastewater samples. The recovery rate varied as follows:

atenolol 21.70%, nadolol 94.22%, pindolol 85.40%, acebutolol 89.30%, metoprolol 93.54%, bisoprolol 92.30%, propranolol 88.68%, betaxolol 91.94%, at pH 10.

The statistically optimized ***DLLME-SFO*** microextraction applied to wastewater samples shows good values of the enriched relative recovery degree ranging from 58.94 to 86.40%, with the exception of atenolol (1.58%) and nadolol (19.20%), and the enrichment factor with values between 60.14 and 86.40, with the exception of atenolol (1.61) and nadolol (19.60).

The statistically optimized ***DLLME*** microextraction applied to wastewater samples showed good values of the enriched relative recovery degree ranging from 63.34 to 82.13%. Atenolol was not extracted, while for nadolol the recovery was 3.95%. The enrichment factor had values between 190.03 and 246.38, except for nadolol (11.85), and for atenolol it was not calculated.

The optimized ***DLLME-SFO*** and ***DLLME*** microextractions allow the extraction of six beta-blockers out of the eight studied, with satisfactory recovery rates. By comparison with the other microextractions presented in other studies, only one study attempts to isolate a number of five drugs by DLLME microextraction.

LC-MS/MS analysis method was applied to wastewater samples collected from the influent of the treatment plant in order to quantify the beta-blockers studied. The concentrations found in the two campaigns have the following values: *February 2024* between 5.76–8.28 ng/L atenolol; n.d.–0.12 ng/L nadolol; n.d.–0.004 ng/L pindolol; n.d.–0.25 ng/L acebutolol; 0.09–0.13 ng/L propranolol; 0.15–0.25 ng/L betaxolol; and 25.51–124.59 ng/L metoprolol; in *October 2024* between 0.09–0.21 ng/L atenolol; n.d.–0.03 ng/L nadolol; and pindolol and acebutolol were not detected; 0.01–0.04 ng/L propranolol; n.d.–0.06 ng/L betaxolol; 35.01–57.84 ng/L metoprolol. These concentrations correspond to the following consumption values for *February 2024*: 2.36–3.07 mg/day/1000inh atenolol; n.d.-0.10 mg/day/1000inh nadolol; n.d.–0.01 mg/day/1000inh pindolol; n.d.–0.13 mg/day/1000inh acebutolol; 0.48–0.72 mg/day/1000inh propranolol; 0.23–0.38 mg/day/1000inh betaxolol; 54.85–276.45 mg/day/1000inh metoprolol; for *October 2024*: 0.03–0.08 mg/day/1000inh atenolol; n.d.–0.03 mg/day/1000inh nadolol; and for pindolol and acebutolol consumption was not calculated; 0.04–0.19 mg/day/1000inh propranolol; 0.05–0.07 mg/day/1000inh betaxolol; 68.93–111.65 mg/day/1000inh metoprolol.

In the application of wastewater-based epidemiology to determine pharmaceutical consumption among the population, its estimation was made based on ammonia nitrogen, this being the hydrochemical parameter resulting from urea hydrolysis, being specific to urine.

The study found that low ambient temperature increases the consumption of beta-blocker medications, due to the additional effort made by the cardiovascular system to supply oxygen and maintain a constant body temperature.

By comparing the estimated consumption from the study with other studies abroad, the amounts of metoprolol are consistent with those consumed abroad, while for atenolol, propranolol and betaxolol the estimated amounts are lower, and for nadolol, pindolol and acebutolol no consumption values were found in other studies.

FINAL CONCLUSIONS

The aim of this doctoral thesis was the development and validation of analytical methods for the quantification of paracetamol, non-steroidal anti-inflammatory drugs (ketoprofen, naproxen, diclofenac and ibuprofen), steroid hormones (estriol, 17-β-estradiol, estrone, 17-α-ethinylestradiol and hydrocortisone) and beta-blockers (atenolol, nadolol, pindolol, acebutolol, metoprolol, bisoprolol, propranolol and betaxolol) from wastewater samples, following their isolation by solid-phase extraction, in order to apply wastewater-based epidemiology to estimate the consumption of these pharmaceuticals among the population.

The results obtained in this doctoral thesis contribute through dispersive liquid-liquid microextraction applied to beta-blockers to the development of green, environmentally friendly extraction methods. Moreover, the method of wastewater-based epidemiology applied to the concentrations of pharmaceuticals found provides an overview of the pharmaceutical consumption among the population in different periods of time, constituting a rapid and non-invasive method of assessing the health status of the population.

This research conducted within the framework of this doctoral thesis can be summarized as follows:

1) A **literature study** was conducted regarding:

- ✓ Antipyretic and anti-inflammatory drugs used in case of SARS-CoV-2 infection, respectively, in case of seasonal infections;
- ✓ The use of steroid hormones for various imbalances of the reproductive system;
- ✓ The use of beta-blockers for the treatment of cardiac conditions, respectively the abuse due to their sedative effects;
- ✓ Chromatographic and extraction analysis techniques used in the determination of pharmaceuticals in wastewater samples;
- ✓ Wastewater-based epidemiology, respectively the constituent stages and the correct application of the method (•wastewater sampling from the influent of the wastewater treatment plant, •selection of the specific biomarker for the compound of interest, •determining the number of inhabitants served by the sewage treatment plant and •calculation of pharmaceutical consumption).

2) **Analysis** and **extraction methods** were developed and optimized for the three classes of pharmaceuticals studied and the wastewater-based epidemiology method was used to determine their consumption:

✓ *Paracetamol and nonsteroidal anti-inflammatory drugs*

•The developed and validated HPLC-PDA method used for the quantification of paracetamol and nonsteroidal anti-inflammatory drugs in wastewater samples was not sufficiently specific and selective.

•The developed and validated GC-MS method was successfully used to determine the concentrations of paracetamol and nonsteroidal anti-inflammatory drugs studied in wastewater samples, following the identification of the compounds based on the selected molecular ion (*m/z*) and retention time, but it required an additional work step, namely derivatization with BSTFA:TMCS (99:1, *v/v*).

•The developed and validated LC-MS/MS method allowed the quantification of paracetamol and the studied nonsteroidal anti-inflammatory drugs from wastewater samples, without the need for an additional step, with low detection (6–52 μg/L) and quantification (18.2–158 μg/L) limits.

•The optimal extraction method for the isolation of paracetamol and the studied nonsteroidal anti-inflammatory drugs was solid-phase extraction using the tested Strata-X cartridges at a sample pH of 3. The recovery rates obtained are: paracetamol 41.57%; ketoprofen 91.89%; naproxen 91.62%; diclofenac 87.72%; and ibuprofen 93.75%, respectively. Liquid-liquid extraction, as well as solid-phase extraction using C18-U and C18-E cartridges, did not provide satisfactory results.

•The concentrations of the studied pharmaceuticals found in water samples collected from the influent of the Cluj-Napoca municipal wastewater treatment plant, in the two sampling campaigns (September 2021, February 2022), had values ranging between: paracetamol 0.88–24.83 μg/L; ketoprofen 0.75–9.34 μg/L; naproxen 1.28–2.62 μg/L; ibuprofen 1.33–5.06 μg/L; and diclofenac 0.97–3.04 μg/L, respectively.

•Following the application of the wastewater-based epidemiology method to estimate the consumption of paracetamol and non-steroidal anti-inflammatory drugs, among the population it varied in the following ranges: 6.65–185.57 g/day/1000inh for paracetamol; 0.21–2.65 g/day/1000inh for ketoprofen; 0.29–0.60 g/day/1000inh for naproxen; 0.32–1.15 g/day/1000inh for ibuprofen; and 0.23–0.73 g/day/1000inh for diclofenac, respectively.

•The estimation of the number of inhabitants connected to the sewage system was made based on ammonia nitrogen, the hydrochemical parameter that best indicates the number of inhabitants.

•The high consumption of paracetamol and non-steroidal anti-inflammatory drugs obtained in the two investigated periods may be associated with the high number of cases of illness due to SARS-CoV-2 infection, respiratory viruses or osteoarticular pain.

✓ *Steroid hormones*

•The optimized and validated HPLC-PDA method for the analysis of the studied steroid hormones from water samples presents low detection (0.076–0.233 mg/L) and quantification (0.230–0.705 mg/L) limits, being efficient in their determination.

•The use of the solid phase extraction method using Strata-X extraction cartridges allowed the efficient isolation of steroid hormones, the recovery degrees obtained being the following: estriol 93.22%; 17-β-estradiol 89.99%; estrone 80.01%; 17-α-ethinylestradiol 84.03%; and, respectively, hydrocortisone 86.75%.

•The concentrations of steroid hormones present in the eighteen wastewater samples analyzed (two sampling campaigns: September 2021 and February 2022) were in the following value ranges: estriol 2.67–104.50 μg/L; 17-β-estradiol n.d.–2.47 μg/L; estrone n.d.–22.42 μg/L; 17-α-ethinylestradiol n.d.–0.65 μg/L; and hydrocortisone n.d.–0.11 μg/L, respectively.

•For steroid hormones, the wastewater-based epidemiology method was applied only to normalize the determined amounts of drugs in water samples. This normalization of amounts was chosen because the study could not distinguish between consumed and naturally excreted hormones, with the exceptions of 17-α-ethinylestradiol and hydrocortisone, but these hormones were detected in only one sample. The normalized values of steroid hormone mass loading are: 0.60–23.81 g/day/1000inh for estriol; n.d.–0.56 g/day/1000inh for 17-β-estradiol; n.d.–5.11 g/day/1000inh for estrone; n.d.–0.15 g/day/1000inh for 17-α-ethinylestradiol; and respectively n.d.–0.02 g/day/100inh for hydrocortisone.

•The estimation of the number of inhabitants connected to the sewage system was made based on ammonia nitrogen, the hydrochemical parameter that best indicates the number of inhabitants.

✓ *beta-Blockers*

•The developed and validated HPLC-PDA method was successfully applied in the quantification of beta-blocker drugs in water samples, with low detection (0.07–0.15 μg/mL) and quantification (0.20–0.45 μg/mL) limits.

•The developed and validated GC-MS method was effective in the determination of beta-blockers in water samples and is notable for low detection (0.13–0.69 μg/mL) and quantification (0.39–2.10 μg/mL) limits, but this method requires derivatization of the drugs with BSTFA:TMCS (99:1, v/v) prior to chromatographic analysis.

•The developed and validated LC-MS/MS method for the analysis of beta-blockers has low detection (0.08–0.22 ng/mL) and quantification (0.15–0.44 ng/mL) limits, being suitable for the determination of these drugs in wastewater samples.

•The solid-phase extraction method developed for the isolation of beta-blockers, using C18-U extraction cartridges, provided optimal recovery rates, as follows: nadolol 94.22%; pindolol 85.40%; acebutolol 89.30%; metoprolol 93.54%; bisoprolol 92.30%; propranolol 88.68%; betaxolol 91.94%; the exception being atenolol 21.70%.

•The need to apply the principles of green chemistry in the extraction step, as well as the limited number of studies available regarding these microextractions applied to beta-blockers, led to the attempt to isolate beta-blockers, using DLLME-SFO and DLLME microextractions. These microextractions provided satisfactory values for recovery rates and preconcentration factors. The recoveries for DLLME-SFO ranged from 59.99 to 87.55%, with the exceptions being atenolol 14.71% and nadolol 29.85%. The enrichment factors ranged from 61.22 to 89.34, with the exception of atenolol 15.01 and nadolol 30.46. The recovery rates using DLLME microextraction were between 53.04 and 92.10%, the exceptions being atenolol which was not detected and nadolol 3.14%. The enrichment factors ranged from 198.89 to 243.97, except for atenolol which was not detected and nadolol with a factor of 11.79.

•The concentrations of beta-blockers quantified in wastewater samples collected from the influent of the Cluj-Napoca municipal wastewater treatment plant, in the two investigated periods (February 2024 and October 2024) had values between: atenolol 0.09–8.28 ng/L; nadolol n.d.–0.12 ng/L; pindolol n.d.–0.004 ng/L; acebutolol n.d.–0.25 ng/L; propranolol 0.01–0.13 ng/L; betaxolol n.d.–0.25 ng/L; and metoprolol 25.51–124.59 ng/L, respectively.

•Estimation of the consumption of beta-blockers among the population using the wastewater-based epidemiology method showed values ranging from: 0.03–3.74 mg/day/1000inh for atenolol; n.d.–0.10 mg/day/1000inh for nadolol; n.d.–0.001 mg/day/1000inh for pindolol; n.d.–0.13 mg/day/1000inh for acebutolol; 0.04–0.72 mg/day/1000inh for propranolol; n.d.–0.38 mg/day/1000inh for betaxolol; and, respectively, 54.85–276.45 mg/day/1000inh for metoprolol.

•The estimation of the population connected to the sewage system for beta-blockers was made based on ammonia nitrogen, the hydrochemical parameter that best indicates the number of inhabitants.

•The estimated consumption of beta-blockers in February 2024 is higher compared to October 2024, due to the lower ambient temperature, which implies a greater need for beta-blockers to support the proper functioning of the cardiovascular system.

3) **The analysis methods and the extraction methods developed and validated** allowed the adequate quantification of the studied pharmaceuticals, but there were some exceptions that require additional efforts to improve their performance.

In conclusion, this study confirms the potential of the wastewater-based epidemiology method as an efficient and valuable method for estimating temporal trends in drug consumption among the population, but it requires high-performance or, in special situations, ultra-high-performance instrumental equipment for analysis.

Wastewater monitoring is a complementary method of public health surveillance, but also of the environment.

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Annex I.
List of abbreviations frequently used in the doctoral thesis

17-α-EE	17-α-Etinilestradiol
17-β-E	17-β-Estradiol
a	panta dreptei de regresie
ACE	Acebutolol
ACN	Acetonitril
AF	Acid formic
AINS	Antiinflamatoare nesteroidiene
ATE	Atenolol
BIS	Bisoprolol
BOD	*Biological Oxygen Demand* consumul biologic de oxigen
BSTFA	*N,O-bis(Trimethylsilyl)TrifluoroAcetamide* N,O-bis(Trimetilsilil)TrifluorAcetamidă
BTX	Betaxolol
COD	*Chemical Oxygen Demand* consumul chimic de oxigen
DAD	*Diode Array Detector* detector cu șir de diode
DIC	Diclofenac
DLLME	*Dispersive Liquid-Liquid MicroExtraction* microextracția dispersivă lichid-lichid
DLLME-SFO	*Dispersive Liquid-Liquid MicroExtraction based on Solidification of Floating Organic Droplet* microextracție dispersivă lichid-lichid cu solidificarea solventului de extracție
E1	Estriol
E2	Estronă
EF	*Enrichment (preconcentration) Factor* factorul de preconcentrare
ER	*Relative Enrichment Recovery* gradul de recuperare relativă îmbogățită
ESI	*ElectroSpray Ionization* ionizare prin electropulverizare
GC-MS	*Gas Chromatography–Mass Spectrometry* cromatografia în fază gazoasă cuplată cu spectrometria de masă

HC	*HydroCortisone* hidrocortizon
HLB	*Hydrophilic Lipophilic Balance* echilibru hidrofilic-lipofilic
HPLC	*High-Performance Liquid Chromatography* cromatografia în fază lichidă de înaltă performanță
HRMS	*High Resolution Mass Spectrometry* spectrometria de masă de înaltă rezoluție
HRT	*Hormone Replacement Therapy* terapie de substituție hormonală
IBU	Ibuprofen
ICH	*International Council for Harmonisation* Conferința Internațională pentru Armonizare
IT-MS	*Ion Trap Mass Spectrometer* spectrometru de masă cu trapă ionică
KET	Ketoprofen
K_{ow}	*n-Octanol-Water Partition Coefficient* coeficientul de partiție *n*-octanol-apă
LC	*Liquid Chromatography* cromatografia în fază lichidă
LD	*Limit of Detection* limita de detecție
LDM	*Limit of Detection of Method* limita de detecție a metodei
LLE	*Liquid-Liquid Extraction* extracția lichid-lichid
loc	locuitori
LogP	*Logarithm of n-Octanol-Water Partition Coefficient* coeficientul de partiție
LQ	*Limit of Quantification* limita de cuantificare
LQM	*Limit of Quantification of Method* limita de cuantificare a metodei
m/z	*Mass-to-Charge Ratio* raportul masă/sarcină
MRM	*Multiple Reaction Monitoring* monitorizarea reacțiilor multiple
MS/MS	*Tandem Mass Spectrometry* spectrometrie de masă în tandem

MSTFA	*N-Methyl-N-Trimethylsilyl)triFluoroAcetamide* N-metil-N-(trimetilsilil)trifluoracetamidă
MTP	Metoprolol
n	numărul de măsurători efectuate
n.d.	nedetectat
NAD	Nadolol
NAP	Naproxen
NH_4-N	Azot amoniacal
Orbitrap MS	*Orbitrap Mass Spectrometer* spectrometru de masă cu trapă orbitală
PARA	Paracetamol
PDA	*Photodiode Array Detector* detector cu fotodiode
PIN	Pindolol
pKa	constanta de aciditate
PRP	Propranolol
PTFE	*PolyTetraFluoroEthylene* politetrafluoroetilenă
PVDF	*PolyVinyliDen Fluoride* difluorură de poliviniliden
QqLIT-MS	*Quadrupole-Linear Ion Trap Mass Spectrometry* spectrometria de masă cuadrupol cu trapă de ioni
QqQ-MS, TQ-MS	*Triple-Quadrupole Mass Spectrometry* spectrometria de masă cu triplu cuadrupol
QTOF-MS	*Quadrupole Time-Of-Flight Mass Spectrometry* spectrometria de masă cu timp de zbor și cuadrupol
R^2	*Correlation Coefficient* coeficientul de corelare
RSD	*Relative Standard Deviation* deviația standard relativă
SARS-CoV-2	*Severe Acute Respiratory Syndrome Coronavirus 2* coronavirusul sindromului respirator acut sever 2
SD	*Standard Deviation* deviația standard
SIM	*Selected Ion Monitoring* monitorizarea ionului selectat
SPE	*Solid-Phase Extraction* extracția pe fază solidă

SUA	Statele Unite ale Americii
TMCS	*TriMethylChloroSilane* trimetilclorsilan
TN	azotul total
TP	fosforul total
t_R	*Retention Time* timpul de retenție
UHPLC	*Ultra-High-Performance Liquid Chromatography* cromatografia în fază lichidă de ultra-înaltă performanță
UPLC	*Ultra-Performance Liquid Chromatography* cromatografia în fază lichidă ultra performanță
UV/Vis	*UltraViolet-Visible* ultraviolet-vizibil
WBE	*Wastewater Based Epidemiology* epidemiologia bazată pe ape uzate

Annex II.
List of scientific publications and papers presented at scientific events based on research conducted during the doctoral study

Scientific publications:

M.-C. Herghelegiu, A. Ernault, M. S. Beldean-Galea, M.-V. Coman.

HPLC-PDA versus GC-MS in the analysis of paracetamol and non-steroidal anti-inflammatory drugs in wastewater.

Studia Universitatis Babes-Bolyai, Chemia 2023, 68(1), 19–35.

https://doi.org/10.24193/subbchem.2023.1.02 (Impact factor: 0.500)

Beldean-Galea M.S., **Herghelegiu M.-C.**, Pănescu V.-A., Vial J., Bruzzoniti M.C., Coman M.-V.

The effectiveness of liquid-phase microextraction of beta-blockers from aqueous matrices for their analysis by chromatographic techniques.

Molecules 2025, 30, 1016.

https://doi.org/10.3390/molecules30051016 (Impact factor: 4.2)

M.-C. Herghelegiu, M.-V. Coman, M.S. Beldean-Galea.

A pilot study for evaluation of steroid hormone consumption in Cluj-Napoca, Romania, through wastewater-based epidemiology –

Toxicological and Environmental Chemistry – accepted for publication (06.07.2025).

M.S. Beldean-Galea, **M.-C. Herghelegiu**, A. Combès, J. Vial, R. Tötös, M.C. Bruzzoniti, M.-V. Coman.

A surveillance of paracetamol and nonsteroidal anti-inflammatory drug consumption in Cluj-Napoca, Romania using wastewater-based epidemiology –

Metabolites – in final preparation (abstract accepted for publication).

Papers presented at scientific events:

***26th International Symposium on Separation Sciences**, 28/06–01/07/2022, Ljubljana, Slovenia.*

Poster: Analysis of non-steroidal anti-inflammatory drugs and paracetamol in wastewater by GC-MS and HPLC-PDA. A comparative study.

***M. C. Herghelegiu**, M.-V. Coman, M.S. Beldean-Galea

***51th International Symposium on High Performance Liquid Phase Separations and Related Techniques**, 18–22/06/2023, Duesseldorf, Germania.*

Poster: Estimating the consumption of nonsteroidal anti-inflammatory drugs and paracetamol in the SARS-CoV-2 pandemic in Romania through wastewater-based epidemiology.

***M.-C. Herghelegiu**, A. Combes, M.-V. Coman, M.S. Beldean-Galea

***27th International Symposium on Separation Sciences**, 24–27/09/2023, Cluj-Napoca, Romania.*

Poster: A pilot study for evaluation of steroid hormone consumption in Cluj-Napoca, Romania through wastewater based epidemiology

***M.-C. Herghelegiu**, M.-V. Coman, M.S. Beldean-Galea

Poster: Wastewater-Based Epidemiology as a surveillance tool to assess human consumption of beta-blockers

M.-C. Herghelegiu, E. Ea, V.-A. Pănescu, M.-V. Coman, M.S. Beldean-Galea

***Symposium Environment & Progress** 13/06/2024 – 15/06/2024, Cluj-Napoca, România.*

Oral presentation: Wastewater-based epidemiology as a monitoring tool for assessing human consumption of pharmaceuticals

***M.-C. Herghelegiu**, M.-V. Coman, M. S. Beldean-Galea

***28th International Symposium on Separation Sciences**, 22–25/09/2024 Messina, Italia.*

Plenary lecture: Wastewater-based epidemiology to assess pharmaceutical consumption in urban populations

M.-V. Coman, **M.-C. Herghelegiu**, M. S. Beldean-Galea

Oral lecture: The effectiveness of liquid phase microextraction of betablockers from aqueous matrices for their analysis by chromatographic techniques

M.-C. Herghelegiu, V. A. Pănescu, M.-V. Coman, M. S. Beldean-Galea

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*****Personally communicated pappers

Annex III

Research internships accomplished abroad within the doctoral program

Doctoral research internship accomplished at:

École Supérieure de Physique et de Chimie Industrielles de la Ville de Paris

(ESPCI Paris-PSL), Paris, France,

Department of Analytical Chemistry,

Laboratory of Analytical, Bioanalytical Sciences and Miniaturization (LSABM).

Period: 13 September 2022 – 11 November 2022

Experimental work performed using the laboratory's research infrastructure:

SPE extraction on Strata-X cartridges and LC-MS/MS analysis of paracetamol and four non-steroidal anti-inflammatory drugs (ketoprofen, naproxen, ibuprofen and diclofenac) from wastewater samples collected from the Cluj-Napoca wastewater treatment plant, in two campaigns (September 2021 and February 2022). Development and validation of the LC-MS/MS analysis method. Use of the method to determine the concentrations of the studied pharmaceuticals. Interpretation of the experimental results obtained.