Analytical method for the simultaneous determination of a broad range of opioids in influent wastewater : optimization, validation and applicability to monitor consumption patterns

Analytical method for the simultaneous determination of a broad range of opioids in influent wastewater: optimization, validation and applicability to monitor consumption patterns

Tim Boogaerts[a,*], *Maarten Quireyns*[a], *Adrian Covaci*[a], *Hans De Loof*[b] & *Alexander L.N. van Nuijs*[a,*]

[a]*Toxicological Centre, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium*

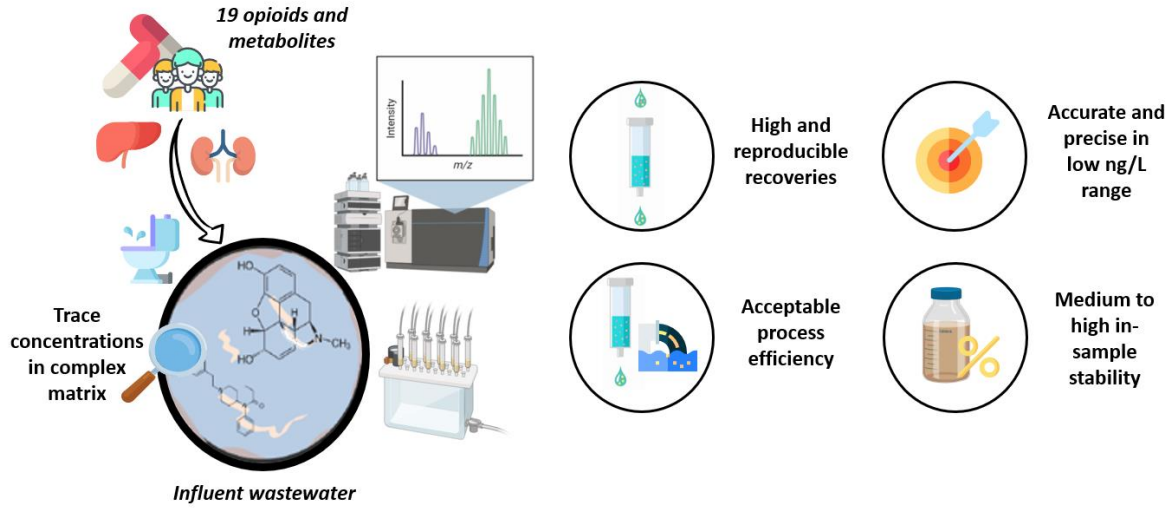
[b]*Laboratory of Physiopharmacology, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium*

** Corresponding author; tim.boogaerts@uantwerpen.be*

Abstract

Wastewater-based epidemiology (WBE) employs the analysis of human metabolic biomarkers in influent wastewater (IWW) to estimate community-wide exposure to xenobiotics (e.g. prescription opioids). The low ng/L range of concentrations of these biomarkers and the complex matrix composition pose bioanalytical challenges related to sample preparation and detection/quantification. Therefore, a sensitive analytical method for the detection and analysis of 19 opioid biomarkers was optimized and validated according to the European Medicines Agency guidelines. Oasis HLB cartridges were used for sample concentration and the Atlantis T3 column with gradient elution resulted in sufficient separation of the analytes. Absolute recoveries (RE) were highly reproducible and ranged between 50-93% with the exception of 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). The lower limit of quantification (LLOQ) ranged between 1 and 100 ng/L and was based on the analyte's concentrations found in IWW. Process efficiency was acceptable for all biomarkers for which an isotope-labelled deuterated analogue was available. All biomarkers showed high benchtop stability with the exception of buprenorphine, EDDP, fentanyl and normorphine. Apart from buprenorphine and hydrocodone, all analytes under investigation were detected at least once above LLOQ levels in five locations in Belgium, including Antwerp, Boom, Brussels, Ostend and Koksijde. The highest population-normalized mass loads were found for tramadol, O-desmethyltramadol and codeine. The proposed methodology was able to evaluate spatial differences in opioid use.

Graphical abstract



Credit author statement

Tim Boogaerts – Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, roles/writing – original draft, writing -review and editing

Maarten Quireyns – Data curation, formal analysis, investigation, methodology, validation, visualization, roles/writing – original draft, writing -review and editing

Adrian Covaci – Conceptualization, writing – review and editing, funding acquisition, supervision

Hans De Loof – Writing – review and editing, supervision
Alexander L.N. van Nuijs – Conceptualization, methodology, writing – original draft, writing – review and editing, funding acquisition, supervision

Keywords:

- *Wastewater-based epidemiology*
- *Opioids*
- *Mass-spectrometric analysis*
- *Validation*
- *Stability*
- *Process efficiency*

Highlights

- Validation of an analytical method for quantification of 19 opioids in wastewater
- Process efficiency acceptable for all analytes when deuterated analogue was available
- High in-sample stability for 15 opioid biomarkers
- Spatial differences in opioid use between five Belgian catchment areas

Introduction

Chronic pain affects about 20% of the adult population and has substantial financial costs [1]. Although opioids (e.g. tramadol, oxycodone, fentanyl, etc) are widely considered as the most effective pharmaceuticals for the treatment of acute pain, the risk of addiction exists to the current day in spite of a continued and still promising search for safer opioids [2]. In the United States, the drastically increased use of prescription opioids has led to a public health crisis of epidemic proportions in the last decades, as reflected by the four-fold increase in admissions, morbidity and mortality for substance use disorder treatment [3]. In Belgium, the use of prescription opioids has increased with 35% with the highest increase reported for oxycodone [4]. For policy makers to monitor the burden of (prescription) opioids and react swiftly and decisively, objective and timely data on the use and misuse of opioids in high spatio-temporal resolution is necessary [5–8]. New patterns can become established in a short time, and could develop into major problems before they have even been identified. This suggests that alternative and complementary approaches are needed in order to rapidly provide an objective and clear picture on opioid consumption within different communities.

Wastewater-based epidemiology (WBE) is an analytical approach based on the chemical analysis of specific human metabolic excretion products (biomarkers) in influent wastewater. These biomarkers are collected, transported and pooled by the sewage system. Therefore, wastewater contains a wealth of information about the catchment population connected to a specific wastewater treatment plant (WWTP), as illustrated in Figure 1 [9]. For biomarkers stable in wastewater and efficiently conveyed to a wastewater treatment plant (WWTP), it is reasonable to assume that the collective amount excreted by the target population within a given period (day, week, month, season, year, etc) is reflected by the mass load reaching the WWTP in the corresponding interval. Concentrations of biomarkers in influent wastewater need to be analyzed using sensitive and selective analytical techniques (e.g. chromatography coupled with mass spectrometry) and these concentrations can then be multiplied with daily flow rates to obtain the mass loads of biomarkers (in mg/day). The mass loads can be divided by the size of the population present in the catchment area of a WWTP, resulting in population-normalized loads (in mg/day/1000 individuals). This allows results to be compared between different WWTP and/or different time points. The WBE approach can be applied to estimate different health-related aspects of populations. It has been used successfully in measuring use of alcohol, tobacco, illicit drugs and other pharmaceutical classes, dietary habits, and to investigate population exposure to pollutants such as pesticides, flame retardants, plasticizers, etc [10–17]. WBE could especially be useful to monitor quickly developing changes in the consumption patterns of pharmaceuticals in near real time and to measure the extent of illegal consumption [9,18,19].

In contrast to the monitoring of amphetamine-type drugs using WBE, opioids have been only recently received more attention [17,19–22]. Additionally, most WBE applications only include a minor selection of opioid biomarkers (mostly morphine, codeine, heroine and methadone). However, only a few of these

publications quantitatively measure a broad range of biomarkers simultaneously [17,19–23] and others only provide semi-quantitative measurements [24,25].

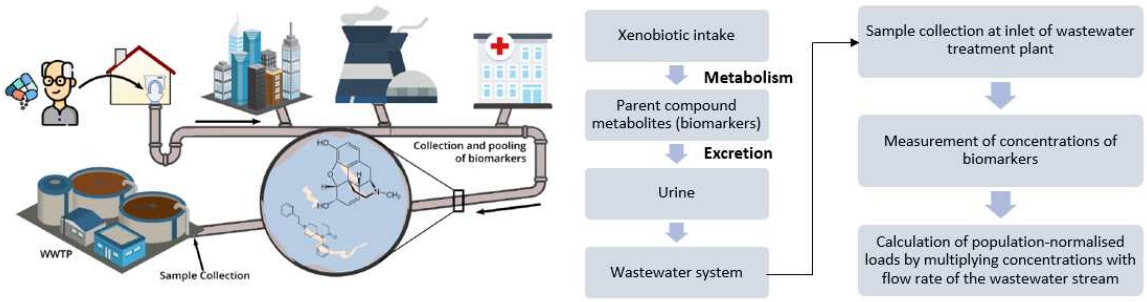


Figure 1 Schematic overview of the WBE approach

The aim of this study was to develop, validate and apply a analytical method capable of measuring a broad range of opioids and metabolites simultaneously at low levels (ng/L) in influent wastewater (IWW). The validated method will be applied to different catchment areas in Belgium to evaluate opioid consumption at a population level.

Materials and methods

Reagents and materials

Reference standards and deuterated internal standards (IS) for investigated compounds were purchased from Cerilliant Corporation (Texas, US), Chiron AS (Trondheim, NO), Grünenthal Gmbh (Aachen, DE), LGC Limited (Teddington, UK), National Measurement Institute (Canberra, AU) and Toronto Research Chemicals (Ontario, CA). Reference and deuterated standards were of analytical grade and purchased as neat powders or as solutions at concentrations of 1 mg/mL or 100 µg/mL in acetonitrile (AcN) and methanol (MeOH). AcN and MeOH were purchased from Fischer Scientific (Loughborough, UK). Formic acid (analytical grade) was obtained from Merck (Darmstadt, DE). Ultrapure water was prepared using an Elga LabWater Purelab Flex system (Veolia Water Solutions & Technologies, Tienen, BE). Oasis® MCX and HLB solid-phase extraction (SPE) cartridges (60 mg, 3 mL) were purchased from Waters (Massachusetts, USA). SPE was performed on a Supelco 12- or 24-port VISIPREP™ vacuum manifold with a Welch 2023 self-cleaning dry vacuum system. Samples were dried in a REACTI-TERM III #TS-18824 evaporator from Thermo Fischer Scientific (Massachusetts, United States). Centrifugal filters (modified nylon 0.2 µm, 500 µL) were acquired from Avantor (Pennsylvania, United States). An overview of all investigated analytes and the corresponding IS is given in Table 1.

Samples and sample treatment

Influent wastewater (IWW) samples were collected from five different Belgian cities covering approximately 12% of the Belgian population (Table 2). IWW samples (500 mL) were collected over a 24h period during a normal week (i.e. with no special events occurring), in a time- or volume-proportional manner, in order to be representative for an entire day. During sample collection, the sample collection bottle was cooled (<4 °C) and after transport samples were stored at -20 °C until analysis. Daily flow rates (m^3/day) were obtained from the Flanders Environment Agency [26].

Method development

Liquid chromatography tandem mass spectrometry

The liquid chromatography (LC) system consisted of an Agilent® 1290 Infinity II Ultra High Performance LC (UHPLC), with a degasser, a column thermostat, a binary pump, and an autosampler. In the final protocol, chromatographic separation was carried out with an Atlantis® T3 column (150 mm x 2.1 mm, 3 µm) maintained at 30 °C. The mobile phase consisted of (A) ultrapure water with 0.1% v/v formic acid, and (B) methanol with 0.1% v/v formic acid. A gradient was optimised as follows: 0-0.5 min: 5% B; 0.5-5 min: increase to 25% B; 5-16 min: increase to 30% B; 16-21 min: increase to 95% B; 21-21.1 min decrease to 5% B and equilibration at 5% B up to 25 min. The flow rate was 0.275 mL/min and the injection volume was 4 µL.

The mass spectrometry (MS) system consisted of an Agilent® 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI), operated in positive ionization mode. The source parameters were optimised as follows: gas temperature 300 °C; gas flow 5 L/min; nebulizer pressure 32 psi; sheath gas temperature 350 °C; sheath gas flow 11 L/min; and capillary voltage 3500 V. Dynamic multiple reaction monitoring (dMRM) was used and at least two MRM transitions were used for each analyte: one transition, usually the most abundant, was used as quantifier (Q) and the other was used as qualifier (q). Identification of the analytes of interest in the extracted samples was based on the quantifier/qualifier (Q/q) ratio and the relative retention time (RRt) (i.e. the ratio of the Rt of the compound to that of the IS). A tolerance level for the Q/q ratio of 30% relative standard deviation (RSD) was set for the identification of the compounds of interest within each batch. In addition, the RRT must be within ±2.5% RSD of that of the calibration standard [27,28].

Sample preparation

An aliquot of 100 mL IWW was spiked with 100 µL of internal standard mixture resulting in a concentration of 100 ng/L (except for TRA-D_6 with a final concentration of 500 ng/L). The aliquot was centrifuged for 30 min at 2465*g* prior to SPE to remove solid particles.

In order to obtain the highest and most reproducible absolute recoveries (RE), different SPE cartridges (Oasis HLB (60 mg, 3 mL) and Oasis MCX (60 mg, 3 mL)),washing, and elution conditions were tested. The RE was determined for each analyte by comparing the peak areas of the analyte spiked pre- and post-extraction at 200 ng/L in tap water, following this equation [29]:

$$RE\ (\%) = \frac{peak\ areas\ of\ the\ standards\ spiked\ pre\ extraction}{peak\ areas\ of\ the\ standards\ spiked\ post\ extraction}$$

Equation 1 Absolute recovery (RE) calculations used to evaluate the different SPE conditions

In the finalized protocol, Oasis HLB cartridges were gravitationally conditioned with 4 mL MeOH and subsequently 4 mL ultrapure water at pH 7. The supernatant from the centrifugal tubes was loaded under vacuum on the SPE cartridges. After loading of the samples, washing of the cartridges was done with 3 mL 20% (v/v) methanol in ultrapure water, under vacuum. The SPE cartridges were afterwards vacuum-dried for at least 30 minutes. Elution of the analytes from the SPE cartridges was performed with 8 mL of 2% v/v formic acid in methanol. Subsequently, the eluent was evaporated at 37 °C under a gentle nitrogen stream and reconstituted in 150 µL of 5% methanol/95% ultrapure water + 0.1% v/v formic acid, which was the same as the starting mobile phase conditions. The reconstituted extract was vortexed for 2.5 minutes, centrifuged on a centrifugal filter (0.2 µm) at 10 000*g,* transferred to an autosampler vial with glass insert and finally analyzed with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Method validation

The analytical method was validated according to the guidelines for bioanalytical method validation by the European Medicines Agency (EMA) with minor adjustments [30]. Tap water was used as an alternative matrix for method validation because it is not possible to obtain blank influent wastewater matrix. Yet, tap water is not the most ideal alternative because of lower matrix effects compared to real influent wastewater. For this reason, standard addition experiments were applied to investigate whether the IS robustly compensates for matrix interferences (see below).. Among performance features, linearity, precision, accuracy, lower limit of quantification (LLOQ), calibration range and specificity/selectivity were assessed during method validation using tap water.

Selectivity was evaluated by analyzing three zero blank samples (i.e containing no analytes or IS) for interferences. The response of the quantifier transition in the zero blank should be <20% of the response of the LLOQ for each biomarker and <5% for the response of the IS. The confirmation criteria based on RRT and Q/q ratios (described earlier) were applied to distinguish matrix interferences from the analytes of interest in IWW. Carry-over was assessed for each biomarker by injecting a zero blank sample after the highest calibration level and comparing the response of the quantifier transition in this zero blank to the response of the quantifier transition of the LLOQ sample. Limits were set at <20% for the analyte and <5% for the IS.

For each biomarker, a seven-level calibration curve was prepared in tap water; linearity and curve fitting were evaluated by testing weighting factors 1/x or $1/x^2$. Back-calculated concentrations of the seven calibration standard levels should be within 15% of the nominal value, or within 20% at the LLOQ.

Within- and between-run accuracy and precision were determined using four quality control (QC) levels. The LLOQ was equal to the lowest calibration level, the QC low (QCL) within three times the LLOQ, the QC medium (QCM) between 30-50% of the calibration curve range, and the QC high (QCH) was between 75-100% of the calibration curve range. For within-run and between-run accuracy and precision four and twelve replicates were considered respectively for each QC level. For accuracy, mean concentration levels should be 15% of the nominal values for the QC level; except for the LLOQ which should be within 20%. For precision, the coefficient of variation (CV) should not exceed 15%; except for the LLOQ where the CV should not exceed 20%.

The recommendations from Matuszewski et al. concerning process efficiency (PE) were modified and applied [12,29]. PE is the product of matrix effects (ME) and RE during sample preparation, as defined by Matuszewski et al [29]:

$$PE = \frac{ME\ x\ RE}{100} = \frac{peak\ areas\ for\ standards\ spiked\ before\ extraction\ in\ blank\ matrix}{peak\ areas\ obtained\ in\ neat\ solution\ standards}\ x\ 100\%$$

Equation 2 Calculation of the process efficiency according to Matuszewski et al [29].

Standard addition was used to evaluate PE given the impossibility of obtaining blank influent wastewater matrix:

$$PE = \frac{(concentration\ in\ spiked\ sample\ before\ extraction\ (C2) - concentration\ in\ a\ corresponding\ native\ sample\ (C1))}{concentration\ obtained\ in\ neat\ solution\ standard\ (A)}\ x\ 100\%$$

Equation 3 Calculation of the process efficiency through standard addition experiments.

Thus, the measured concentration in the C2 sample equals the sum of spiked and native concentration (Figure S1). The proposed set-up was employed to test whether the method is sensitive enough to distinguish the compounds under investigation from the matrix interferences, and to assess if the IS corrects for potential ME and/or losses during sample preparation.

Six samples of pooled IWW originating from different sources were spiked to evaluate PE (Figure S1). Multiple sources of IWW were used to mimic different IWW matrices. Each IWW pool (N = 6) was further subdivided in 100 mL aliquots of non-spiked 'control' samples (C1 in Equation 3), and spiked samples (C2 in Equation 3), as illustrated by Figure S1. The C2 samples were spiked with 100 µL of a spiking mixture with biomarker concentrations ranging from 250 to 1000 ng/L to ensure that spiked concentrations were substantially higher (up to 10 times) compared to native concentrations. Native concentrations measured in the control sample were subtracted from the concentrations found in the spiked IWW samples (if >LLOQ), as illustrated in Eq 2. It was accepted that the IS compensates for possible loss during sample preparation and matrix effects when PE was within 80-120% bias [12,29].

Stability experiments

The experimental set-up to assess in-sample stability was done accordingly to McCall et al [31]. In short, a large wastewater pool was made by subsampling different IWW sources. This IWW pool was subsequently divided in 3 aliquots of 1000 mL, including a non-spiked 'control' IWW sample and two spiked IWW samples (Figure S2). Metabolites (M) and parent (P) compounds were spiked separately in different pools to insure there was no interference from overlapping metabolization pathways. The M and P pools were spiked with high concentrations of compounds (250-1000 ng/L) to ensure that spiked biomarkers were present in substantially higher amounts compared to control samples. During the experiment, the aliquots were placed at room temperature, and on a magnetic stirrer (300 rpm) to simulate sewer currents. The time point the aliquots were spiked was considered the as time point 0 h. At specific time intervals (2 h, 6 h, 10 h, and 24 h) two aliquots of 100 mL were taken from each sample and prepared according to the sample protocol outlined earlier, including the SPE drying step. The cartridges were stored at -20 °C until the moment of elution, which was the next day. The aliquots taken at time interval 24 h were eluted after the drying step, along with the other stored SPE cartridges. The time interval was set to 24 h since this encompasses the residence times in most sewer systems in Belgium.

Native biomarker concentrations measured in the control non-spiked samples, when above LLOQ, were subtracted from the concentrations in the spiked samples. Mean concentrations at time intervals 2 h, 6 h, 10 h and 24 h were normalized against time point 0 h to evaluate the stability.

Each biomarker was categorized as either high (<20% transformation), medium (20-60%), low (60-100%) or variable (i.e. varying results found in different studies) stability over a 24-h period [31]. Biomarkers with medium to low (and variable) stability are unreliable to perform further back-calculations and should, if transformation is not corrected for, be excluded in WBE studies.

Results and discussion

Method development & optimization

Liquid chromatography-tandem mass spectrometry

For LC optimization, a standard mixture with all analytes of interest was injected on different reversed-phase LC (RPLC) columns: a Kinetex® EVO C18 (100 mm x 2.1 mm, 2.6 µm) column, a Kinetex® Biphenyl (100 mm x 2.1 mm, 2.6 µm) column and an Atlantis T3 (150 mm x 2.1 mm, 3 µm) LC column. This was done in combination with the use of different organic phases (MeOH or AcN) and buffer conditions (0.1% v/v HCOOH or 0.04% v/v HCOOH). Most of the analytical methods found in literature applied RPLC with the use of modified C_{18} LC columns [17,20,21]. The Atlantis® T3 column resulted in the best retention for the most polar compounds, such as MOR and norMOR, and was therefore chosen for chromatographic separation (Figure 2). norMOR eluted within the first 2 minutes when using the Kinetex® EVO C18 and Kinetex®

Biphenyl LC column (Figure S3). Initially, mobile phase A and B consisted respectively of ultrapure water and acetonitrile both with 0.1% v/v formic acid using a standard gradient of 0-0.5 min: 5% B; 0.5-15 min: increase to 95% B; 15-17 min: 95% B; 17-17.1 min: decrease to 5% B. Lowering the percentage of aqueous mobile phase at the beginning of the run did not result in sufficient retention of norMOR on these columns. For the organic mobile phase, MeOH with 0.1% v/v formic acid was chosen because this resulted in better retention and sensitivity of the analytes of interest. All compounds eluted within 21 minutes as illustrated by Figure 2.

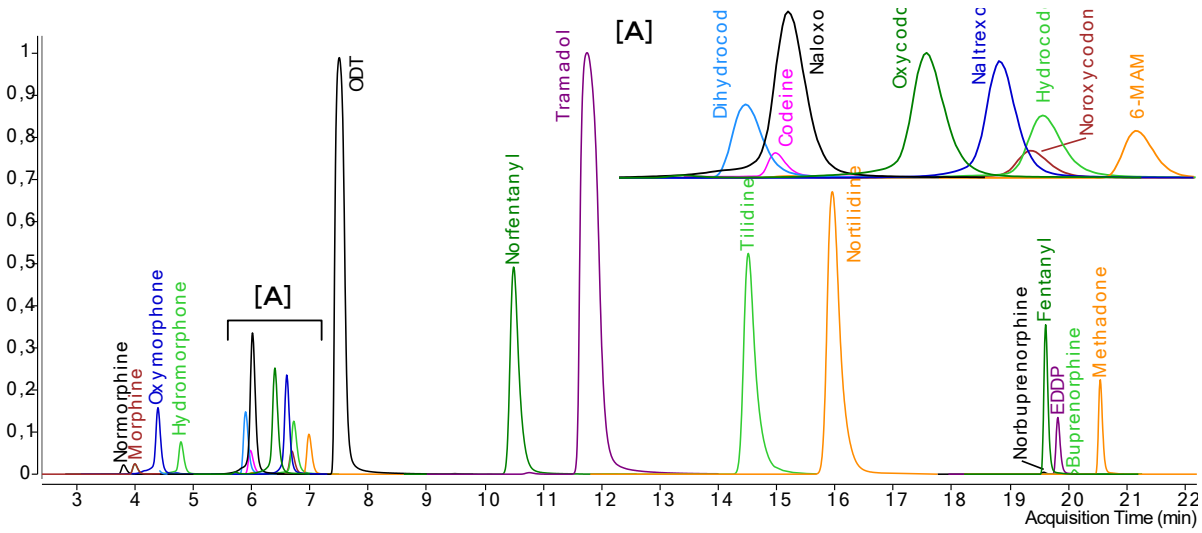


Figure 2 Chromatographic overview of all MRM quantifier transitions in tap water spiked at the highest calibration level. The y-axis scale was represented as the abundance relative the most abundant peak (in this case tramadol)

Compound-dependent MS parameters, including fragmentor voltage, collision energy and ionization mode, were determined by injecting 1 ppm standard solutions without LC column. For each analyte, the transition with the highest signal-to-noise ratio (S/N) and absolute abundance was used as quantifier transition and the two remaining abundant transitions were used as qualifier transition (Table S1). Biomarkers which share the same transitions were separated sufficiently with the Atlantis T3 column. The effect of co-eluting matrix interferences on peak intensity was also considered when choosing quantifier and qualifier transitions since matrix-induced suppression or enhancement could result in poor or improved peak intensities.

TRA shared its transitions with a coeluting interferent (Figure S4). A reference standard was used to confirm the identity of the interference as O-desmethylvenlafaxine (ODV), a metabolite of the antidepressant venlafaxine and previously quantified in wastewater [12,32]. As a result, chromatographic separation was further optimized to separate these compounds. Baseline separation was achieved by increasing the aqueous gradient time at the moment of elution.

norHCD, norCOD and 6-MAM did not go through method validation due to sensitivity issues. This is less of a concern for norHCD as the parent drug hydrocodone is, since 2008, no longer available on the Belgian

market [33]. This is more important for norCOD since it is a distinct metabolite of COD, especially because of the overlap in metabolic pathways between heroin (HER), MOR and COD (Figure S5).

Furthermore, PIR was excluded from the final method due to poor reproducibility of the reference standard peaks in tap water. In some European countries, PIR is considered the first-choice opioid analgesic for pre- and post-operative pain [34]. It should be noted that the global use of PIR is rather limited [35]. Consumption in Belgium is also low [4], making this exclusion of less concern. In 2017, piritramide was only dispensed in Austria, Belgium, Curaçao, Czechia, Germany, Italy, Luxembourg, the Netherlands, Slovakia, and Slovenia [35].

Sample preparation

Most of the multi-analyte sample preparation methods used for the extraction of opioids in IWW applied SPE with Oasis MCX (or Strata-X-C) and Oasis HLB cartridges with loading volumes between 50-500 mL [20–25,36] and used (acidified) ultrapure water as a washing solvent. Methanol (with or without pH modifier) and dichloromethane/isopropanol mixtures were used most frequently as elution solvents. RE was assessed to select the most optimal SPE conditions, including elution solvents, washing solvents, pH modifiers and solvent volumes. Initially, extraction with both Oasis HLB and MCX cartridges resulted in high RE (Table S2), which was in line with the findings of others [17,21]. However, the MCX procedure was discarded in an early stage because of the high variability in RE among the different SPE experiments compared to the HLB procedure. It should be noted that the RE of EDDP was low, yet highly reproducible with the HLB extraction. However, the combination of a highly sensitive detection and considerable concentrations of EDDP in influent wastewater allow the use of the HLB protocol. Table S2 indicates that an elution volume of 8 mL resulted in the highest RE with the HLB procedure. Higher elution volumes did not yield in further improvements. Overall, the use of MeOH and MeOH with acidic modifier (i.e. 2% v/v FA) as an elution volume was similar. However, MeOH with 2% v/v FA was selected as elution solvent for sample preparation since it resulted in slight improvements for a minor selection of biomarkers including norOXY and OMP.

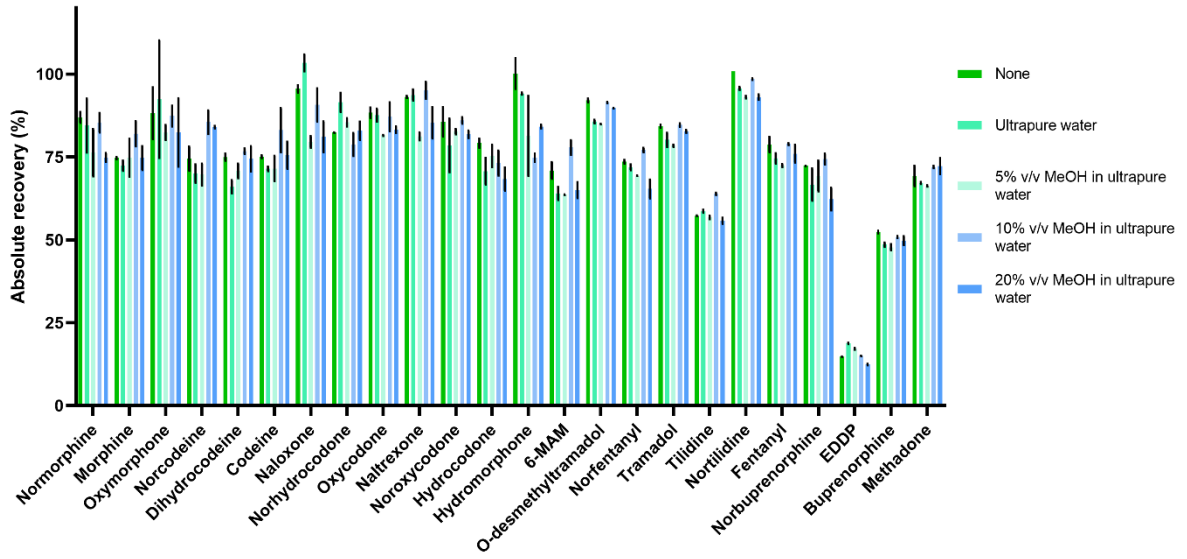


Figure 3 Absolute recoveries (RE) (in % ± SD, n = 2) in tap water for all compounds per protocol. Colours represent the different washing solvents tested.

In the analytical method proposed by Krizman-Matasic et al., co-eluting matrix interferences were reported with the use of Oasis HLB [21], emphasizing the need for further optimization of the washing solvent. Figure 3 illustrates the RE (in % ± SD) using the HLB procedure with 8 mL of 2% v/v FA in MeOH as elution solvent and varying washing solvents. Washing of the HLB cartridges with 3 mL of 20% v/v MeOH proved to have a positive effect on the peak shape and signal intensity of the investigated compounds, potentially by washing away matrix interferences. No substantial loss in absolute recoveries (<20%) was observed with this washing solvent. RE were highly reproducible and ranged between 50-93% with the exception of EDDP.

Method validation

Performance criteria

For 19 compounds, the performance criteria met the requirements for method validation provided by the EMA guidelines, as illustrated in Table S3.

The method proved to be selective as analysis of three zero blank samples did not result in any interference. No significant carry-over occurred for most biomarkers (< 3%), however, carry-over was higher for methadone (17%) and fentanyl (10%) but still less than 20% of the LLOQ as recommended by EMA. Additionally, injection of IS did not interfere with the analytes and vice versa.

A linear calibration curve ranging from the low ng/L to low µg/L range was obtained for 21 compounds in tap water. The LLOQ of the analytes of interest was between 1 ng/L and 100 ng/L, based on their concentrations found in IWW. A weighting of $1/x^2$ was considered more appropriate for biomarkers with measured concentrations at the lower end of the calibration curve, whereas 1/x was used for higher concentrations. Most biomarkers favored a curve weight of $1/x^2$ based on their low concentrations in IWW, as illustrated in Table S3.

The within-run and between-run accuracy and precision measured at the QC levels met the acceptance criteria. Within-run and between-run accuracy and precision results (Table S3) at four different spiking levels (i.e. LLOQ, QC_{low}, QC_{mid}, QC_{high}) were respectively within the range of <15% bias and <15%CV.

The LLOQs in the present study range between 1-15 ng/L for all biomarkers of investigation with the exception of tramadol which has a LLOQ of 100 ng/L. From an analytical perspective, it would be probably possible to achieve lower LLOQs, however, the relevance of an LLOQ needs to be considered from a WBE perspective. From a WBE point-of-view, the analytical approach needs to sensitive enough to pick up trends in consumption patterns of prescription drugs. Even though it is theoretically possible to obtain lower detection levels, this might be less of interest because these concentrations correspond with very low levels of consumption. For this reason, the proposed LLOQs of this analytical methodology are appropriate to capture relevant consumption patterns in the use of prescription opioids.

Process efficiency

The PE experiment was designed to investigate whether the IS was able to correct for ME and/or loss of analyte during extraction (i.e. RE). Deviations in PE originate from differences between the analyte and the corresponding IS in wastewater due to different signal suppression or enhancement. This is especially important for biomarkers for which no deuterated analogue was available as IS (Table 1). Figure 4 summarizes the results of the PE standard addition experiment. PE were within ±20% bias for all compounds for which an isotope-labelled analogue was available. For DHC, HCD, norMOR and norOXY, the following IS COD-D_6, HMP-D_3, MOR-D_3 and OXY-D_6 respectively were able to compensate for potential losses due to matrix interferences or during SPE. For NLX and NTX, no isotope-labelled deuterated analogues were available during method development. No alternative IS was found for NLX and NTX and therefore these compounds were excluded from the final method. The high reproducibility of the PE of NLX would potentially allow the use of a correction factor (CF) to accurately measure concentrations of NLX in wastewater, but more research is needed to establish such a CF.

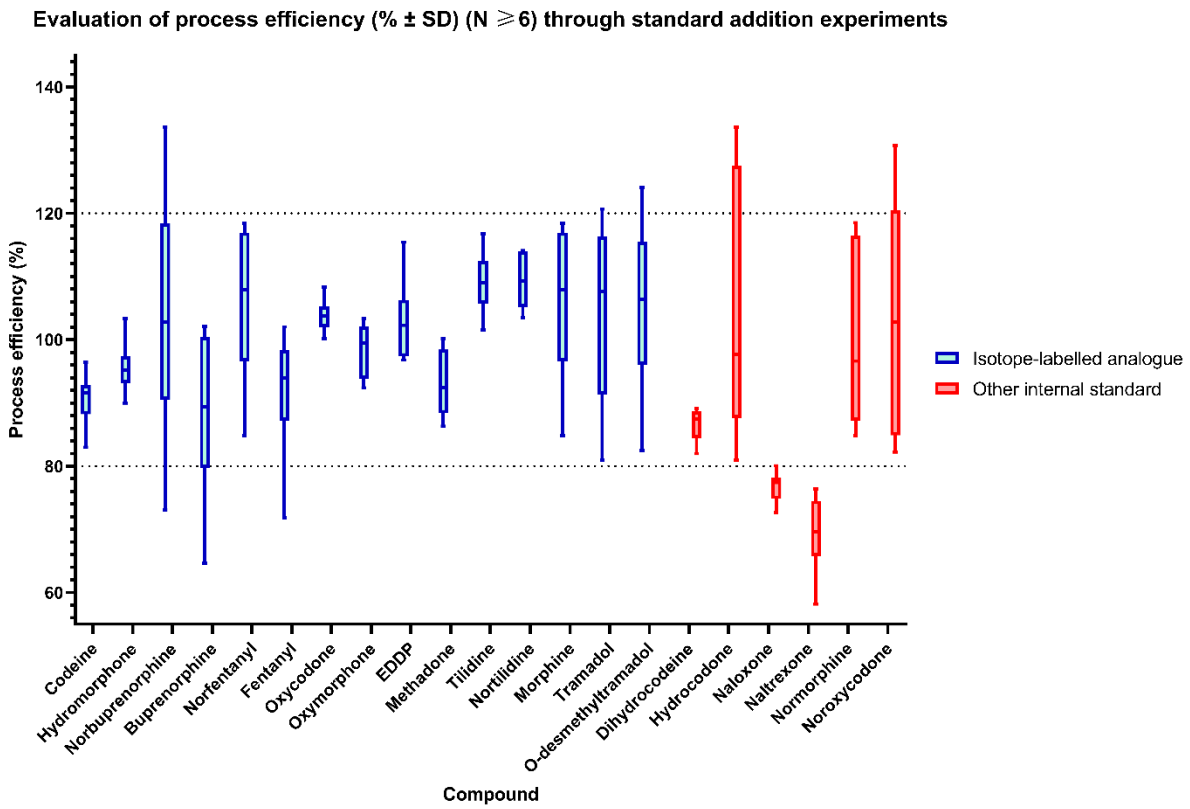


Figure 4 Estimation of process efficiency through standard addition experiments in influent wastewater.

Stability

Benchtop stability of the analytes under investigation was investigated as in-sample biomarker transformation could result in over- or underestimation of population-normalized mass loads. This study did not evaluate in-sewer degradation in presence of a biofilm under gravity or rising main sewer conditions or in a pilot sewer study. The in-sewer transformation of the targeted biomarkers could lead to additional uncertainty. Additionally, this study did not investigate in-sample stability at -20 °C. However, McCall et al. indicate that most opioid compounds (e.g. morphine, oxycodone, fentanyl,…) are sufficiently stable during sample storage at -20 °C, with the exception of some compounds such as 6-MAM and heroin [31]. Furthermore, we did not assess potential loss due to sorption to solid particulate matter, which would potentially lead to uncertainty in back-estimating biomarker population-normalised mass loads. Baker et al. found that the average proportion of solid particulate matter was >10% with regard to MTD, EDDP and FEN, but was acceptable for most opioid biomarkers (e.g. norCOD, DCD, TRA,…) [37]. It should also be noted that PE also evaluates if any potential loss of analyte due to sorption to particulates is corrected.

Figure 5 shows the residual percentages of the analytes of interest at five different time points (0 h, 2 h, 6 h, 10 h and 24 h). 16 compounds showed high stability (<20% transformation) over 24 h. Stability of BUP, norMOR, FEN and EDDP was only medium, with more than 20% but less than 40% in-sample transformation. However, the use of BUP and FEN could be easily monitored through the use of their metabolites norBUP and norFEN. Similarly, the parent compound MTD could be used in the case of EDDP, which proves to be

stable in IWW (with 12% of the initial MTD load transformed). As illustrated by Figure S5, there is overlap between the metabolic pathways of MOR, COD and HER and norMOR, therefore it is more appropriate to measure MOR use. However, the benchtop stability of norMOR should be taken into account to accurately measure its concentrations in influent wastewater. To our knowledge, it was the first time in-sample stability was determined for HMP, norTIL, ODT and TIL. Even though the residual percentage of BUP and FEN decreased in the IWW pools spiked with parent compound (P1 and P2, see Figure S2), we did not observe a parallel increase in norBUP and norFEN in these pooled IWW samples. This means that BUP and FEN are not degraded in norBUP and norFEN, further proving the suitability of these metabolite as alternative biomarkers.

The results found in this study were comparable with the results found by Baker et al for most compounds under investigation (Table S4) [38]. However, for BUP, MOR, OMP and norMOR, the data did not match as Baker et al did report in-sample formation of MOR and OMP and no more than 20% transformation was observed for BUP and norMOR in this study. According to McCall et al, these biomarkers should strictly be excluded from the analytical method due to variable stability. Therefore, OXY use should be monitored through its metabolite norOXY and for MOR a more specific and stable metabolite should be further explored.

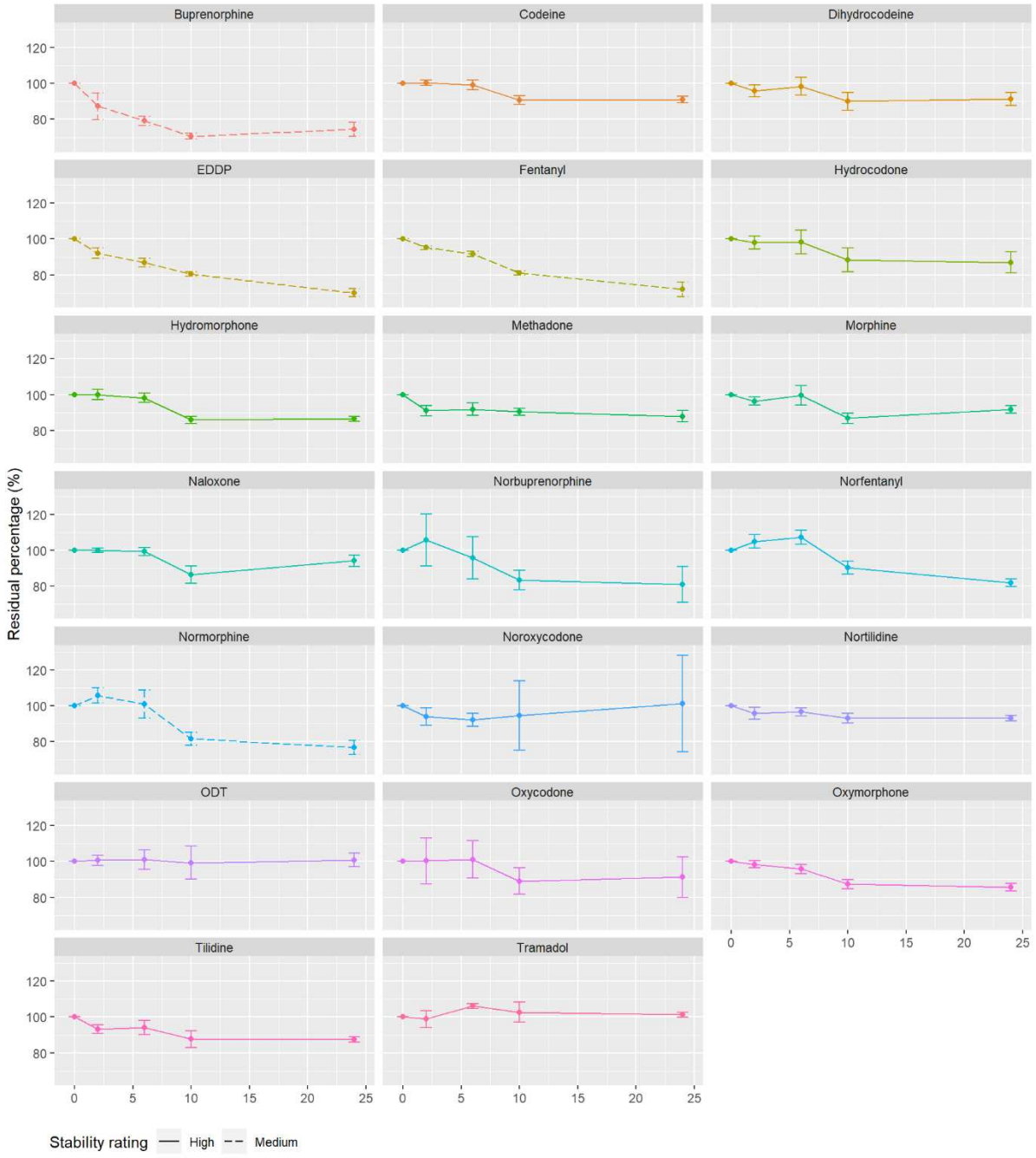


Figure 5 Benchtop stability in wastewater of opioids, transformation of biomarker at each time point. Mean residual percentages of four spiked samples, normalised against time of spiking are reported for time points 2 h, 6 h, 10 h and 24 h. The error bars represent the relative standard deviation (%RSD) between the concentrations measured in the replicate wastewater pools.

Method application

Spatial comparison of prescription opioid use

The applicability of the validated method was evaluated on 35 different IWW samples from 5 different WWTPs (Table 2). Apart from BUP and HCD, every validated biomarker was detected (>LLOQ) at least once. Calculated population-normalised mass loads of the investigated analytes are shown in Table S5. Highest population-normalised mass loads were reported for TRA, ODT and COD which is in line with sales and

dispensing data [39,40]. Although fentanyl is prescribed in a relatively high number of patients in Belgium [4], population-normalised mass loads of norFEN and FEN are close to the LLOQ level because of its low dose. The magnitude of the population-normalized mass loads found in this study were also comparable with the results of other WBE applications on prescription opioids [17,20,22,23,41].

Figure 6 summarizes the total opioid population-normalized mass loads in all locations under investigations. Note that only one biomarker (preferably the metabolite as it represents actual consumption) was included for each opioid prescribed in Belgium. A limitation of this study is that only one week of sampling within the same time period (i.e. Sep 2019) was included for the spatial comparison, and consumption rates might be different in the sampling period compared to the rest of the year. Spatial differences were found for the majority of prescription opioids among the different locations.

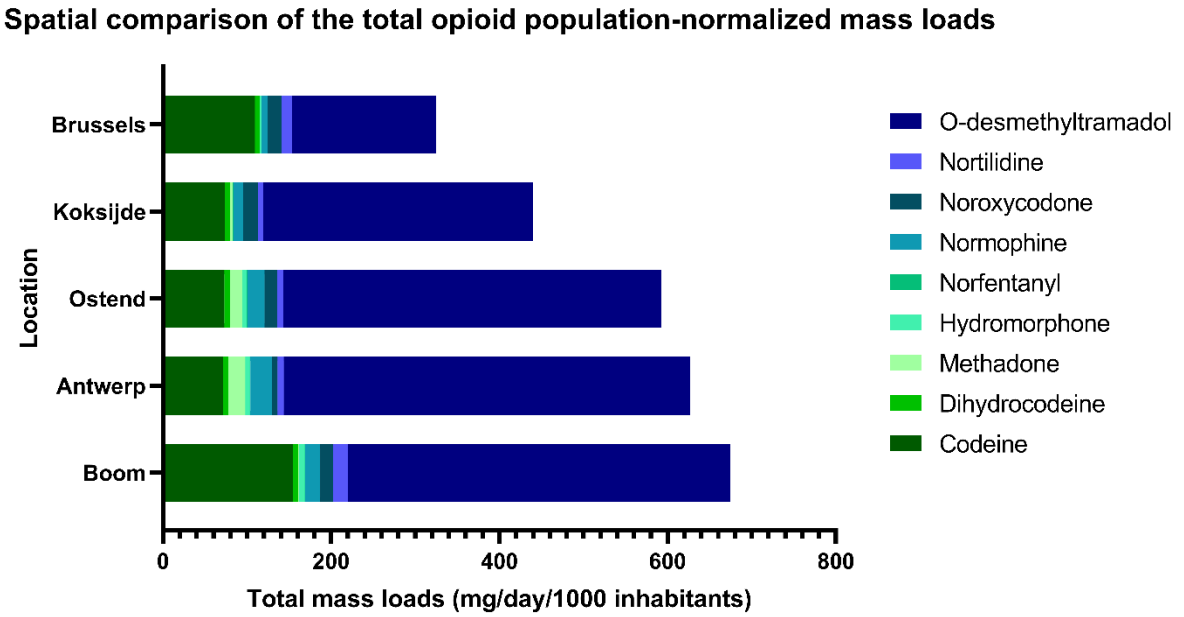


Figure 6 Spatial analysis of total opioid consumption among different catchment areas.

At this moment HER use can only be measured based on morphine concentrations, after the correction for morphine originating from consumed morphine and codeine. In the future, a specific biomarker for HER needs to be found in order to investigate consumption patterns of this compound with less uncertainty. The in-sewer transformation of 6-monoacetylmorphine (6-MAM) is substantial and paralleled with an increase of morphine, as reported by Senta et al. [42]. This implies that at this stage it is difficult to estimate HER consumption from 6-MAM.

Investigation of metabolite/parent compound ratios

For 6 compounds, the parent compound and metabolite were included in the analytical method. However, BUP was never detected and FEN was only detected occasionally. Table 3 illustrates the metabolite to parent compound ratios (M/PC). M/PC are expected to be relatively constant for each location. It should

be noted that (accidental) discharge of the parent drug can as also result in a decrease of the metabolite/parent ratio.

The mean M/PC of EDDP/MTD is 1.33 ± 0.19, which was comparable with the average EDDP/MTD ratio of 1.97 found in 44 other wastewater studies [43]. In all locations the population-normalised mass loads of EDDP were higher than MTD. The norMOR/MOR ratio was relatively constant within each location. Based on the mean influent concentrations of only 7 IWW samples, Boleda et al. reported a M/PC ratio of 7.9%, which was lower compared to this study [44]. However, deviations in the norMOR/MOR ratio could be the result of overlapping metabolic pathways for MOR. MOR could also be metabolized from COD and HER and therefore COD and HER use could potentially influence the observed norMOR/MOR ratios. Baker et al found a OMP/OXY ratio of 1.66 which is in line with the results found in this study [17]. No relevant literature could be found for ratios norOXY/OXY and norTIL/TIL. As illustrated in Table 3, the metabolite was always measured in higher concentrations than the parent drug. Variations in these ratios appear to be relatively high, which could be related to their lower concentrations found in IWW compared to MTD, MOR and their metabolites. It should be noted that this variability is not related to in-sample stability since OXY, TIL and their metabolites showed <20% transformation for 24h.

Note that if the population (or rather the number of people using a drug) attached to a WWTP is low, the variance associated with ratios will increase. This is because interindividual metabolization variability (poor metabolizer, high metabolizer, …) will be more predominant.

Conclusions

A sensitive analytical method based on SPE and LC-MS/MS was developed and validated for the simultaneous measurement of 19 prescription opioids and their metabolites at trace concentrations in influent wastewater. The PE was acceptable for all compounds for which an isotope-labelled deuterated analogue was available. However, for NLX and NTX, no IS was found able to compensate for ME and potential losses during extraction and therefore these compounds were excluded from the final method. All compounds with the exception of BUP, EDDP, FEN and norMOR showed high benchtop stability.

Apart from BUP and HCD, all biomarkers were detected at least once in Belgian IWW. Wherever possible metabolite/parent compound ratios were determined but, apart from EDDP/methadone, literature data for comparison is scarce. The M/PC ratios were in line with the results found in other WBE studies on opioids. Overall, metabolites were found in higher levels compared to parent compounds among all location.

Acknowledgements

This study was funded by the European Union's Justice Programme—Drugs Policy Initiatives, EuSeME (project number 861602) and the Research Foundation Flanders (project number G060920N).

Figures

Figure 1 Schematic overview of the WBE approach

Figure 2 Chromatographic overview of all MRM quantifier transitions in tap water spiked at the highest calibration level. The y-axis scale was represented as the abundance relative the most abundant peak (in this case tramadol)

Figure 3 Absolute recoveries (RE) (in % ± SD, n = 2) in tap water for all compounds per protocol. Colours represent the different washing solvents tested.

Figure 4 Estimation of process efficiency through standard addition experiments in influent wastewater.

Figure 5 Benchtop stability in wastewater of opioids, transformation of biomarker at each time point. Mean residual percentages of four spiked samples, normalised against time of spiking are reported for time points 2 h, 6 h, 10 h and 24 h. The error bars represent the relative standard deviation (%RSD) between the concentrations measured in the replicate wastewater pools.

Figure 6 Spatial analysis of total opioid consumption among different catchment areas.

Tables

Table 1. Target biomarkers and corresponding internal standard for quantification purposes. (*) = compounds for which no isotope-labelled analogue was used; (**) = not transferred to method validation

Pharmaceutical	Biomarker monitored in wastewater	Abbreviation	Internal standard
Buprenorphine	Buprenorphine	BUP	Buprenorphine-D_4
	Norbuprenorphine	norBUP	Norbuprenorphine-D_3
Codeine	Codeine	COD	Codeine-D_6
	Norcodeine*,**	norCOD	Codeine-D_6
	Morphine and metabolites		
Dihydrocodeine	Dihydrocodeine*	DCD	Codeine-D_6
Fentanyl	Fentanyl	FEN	Fentanyl-D_5
	Norfentanyl	norFEN	Norfentanyl-D_5
Heroine	6-Monoacetylmorphine**	6-MAM	6-MAM-D_3
	Morphine	MOR	Morphine-D_3
Hydrocodone	Hydrocodone*	HCD	Hydromorphone-D_3
	Norhydrocodone*,**	norHCD	
	Dihydrocodeine		
Hydromorphone	Hydromorphone	HMP	Hydromorphone-D_3
Methadone	Methadone	MTD	Methadone-D_9
	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP	EDDP-D_3
Morphine	Morphine		Morphine-D_3
	Normorphine*	norMOR	Morphine-D_3
Naloxone	Naloxone*	NLX	Codeine-D_6
Naltrexone	Naltrexone*	NTX	Norfentanyl-D_5
Oxycodone	Oxycodone	OXY	Oxycodone-D_6
	Noroxycodone*	norOXY	Oxycodone-D_6
	Oxymorphone		
Oxymorphone	Oxymorphone	OMP	Oxymorphone-D_3
Piritramide	Piritramide*,**	PIR	*
Tilidine	Tilidine	TIL	Tilidine-D_6
	Nortilidine	norTIL	Nortilidine-D_3
Tramadol	Tramadol	TRA	Tramadol-D_6
	O-desmethyltramadol	ODT	O-desmethyltramadol-D_6

Table 2 Overview of the sampling locations and periods

WWTP (City)	Period	Population serviced by WWTP	Sampling mode
Antwerp-South (Antwerp)	23/09/2019-29/09/2019	130,218	Time-proportional
Boom (Boom)	23/09/2019-29/09/2019	30,600	Time-proportional
Brussels-North (Brussels)	23/09/2019-29/09/2019	953,987	Volume-proportional
Ostend (Ostend)	23/09/2019-29/09/2019	159,000	Time-proportional
Wulpen (Koksijde)	23/09/2019-29/09/2019	78,441	Time-proportional

Table 3. Percentual mass load ratios of metabolite/parent drug per location. N.d.: The M/PC ratio could not be determined because either the parent or metabolite could not be detected in IWW.

	EDDP/MTD (± SD)	**OMP/OXY (±SD)**	**NorMOR/MOR (± SD)**	**norOXY/OXY (±SD)**	**norTIL/TIL (± SD)**
AZ	1.11 ± 0.12	n.d.	0.26 ± 0.02	n.d.	1.30 ± 0.28
BOO	n.d.	1.06 ± 0.78	0.38 ± 0.03	1.61 ± 0.85	1.76 ± 0.63
BRU	n.d	1.57 ± 0.66	0.12 ± 0.01	4.80 ± 1.29	1.71 ± 0.64
OOS	1.41 ± 0.14	2.26 ± 0.70	0.29 ± 0.01	2.73 ± 0.37	1.87 ± 0.55
WUL	1.46 ± 0.12	2.57 ± 0.40	0.28 ± 0.03	3.25 ± 0.45	n.d
Mean	**1.33 ± 0.19**	**1.87 ± 0.68**	**0.27 ± 0.09**	**3.17 ± 1.34**	**1.66 ± 0.25**

Reference list

1. Phillips CJ. The Cost and Burden of Chronic Pain. *Rev pain*. 2009;3(1):2-5. doi:10.1177/204946370900300102

2. Siuda ER, Carr R 3rd, Rominger DH, Violin JD. Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. *Curr Opin Pharmacol*. 2017;32:77-84. doi:10.1016/j.coph.2016.11.007

3. Hagemeier NE. Introduction to the opioid epidemic: the economic burden on the healthcare system and impact on quality of life. *Am J Manag Care*. 2018;24(10 Suppl):S200-S206.

4. National Institute for Health and Disability Insurance. Statistics on pharmaceuticals dispensed to public pharmacies (Farmanet). Published 2017. https://www.riziv.fgov.be/nl/statistieken/geneesmiddel/Paginas/Statistieken-geneesmiddelen-apotheken-farmanet.aspx

5. Singer E. Introduction: Nonresponse Bias in Household Surveys. *Public Opin Q*. 2006;70(5):637-645. http://www.jstor.org/stable/4124219

6. Fehr A, Tijhuis MJ, Hense S, Urbanski D, Achterberg P, Ziese T. European Core Health Indicators - status and perspectives. *Arch Public Health*. 2018;76:52. doi:10.1186/s13690-018-0298-9

7. Wittwer R, Hubrich S. Nonresponse in Household Surveys: A Survey of Nonrespondents from the Repeated Cross-sectional Study "Mobility in Cities – SrV" in Germany. *Transp Res Procedia*. 2015;11:66-84. doi:https://doi.org/10.1016/j.trpro.2015.12.007

8. European Monitoring Centre for Drugs and Drug Addiction. *Comorbidity of Substance Use and Mental Disorders in Europe*.; 2015.

9. Choi PM, Tscharke BJ, Donner E, et al. Wastewater-based epidemiology biomarkers: Past, present and future. *TrAC Trends Anal Chem*. 2018;105:453-469. doi:https://doi.org/10.1016/j.trac.2018.06.004

10. Rousis NI, Zuccato E, Castiglioni S. Wastewater-based epidemiology to assess human exposure to pyrethroid pesticides. *Environ Int*. 2017;99:213-220. doi:10.1016/j.envint.2016.11.020

11. Boogaerts T, Covaci A, Kinyua J, Neels H, van Nuijs ALN. Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach. *Drug Alcohol Depend*. 2016;160:170-176. doi:10.1016/j.drugalcdep.2016.01.002

12. Boogaerts T, Degreef M, Covaci A, van Nuijs ALN. Development and validation of an analytical procedure to detect spatio-temporal differences in antidepressant use through a wastewater-based approach. *Talanta*. 2019;200:340-349. doi:https://doi.org/10.1016/j.talanta.2019.03.052

13. Been F, Bastiaensen M, Lai FY, van Nuijs ALN, Covaci A. Liquid Chromatography-Tandem Mass Spectrometry Analysis of Biomarkers of Exposure to Phosphorus Flame Retardants in Wastewater to Monitor Community-Wide Exposure. *Anal Chem*. 2017;89(18):10045-10053. doi:10.1021/acs.analchem.7b02705

14. Choi PM, Tscharke B, Samanipour S, et al. Social, demographic, and economic correlates of food and chemical consumption measured by wastewater-based epidemiology. *Proc Natl Acad Sci U S A*. 2019;116(43):21864-21873. doi:10.1073/pnas.1910242116

15. González-Mariño I, Baz-Lomba JA, Alygizakis NA, et al. Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. *Addiction*. 2020;115(1):109-120. doi:10.1111/add.14767

16. Lai FY, Been F, Covaci A, van Nuijs ALN. Novel Wastewater-Based Epidemiology Approach Based on Liquid Chromatography-Tandem Mass Spectrometry for Assessing Population Exposure to Tobacco-Specific Toxicants and Carcinogens. *Anal Chem*. 2017;89(17):9268-9278. doi:10.1021/acs.analchem.7b02052

17. Baker DR, Barron L, Kasprzyk-Hordern B. Illicit and pharmaceutical drug consumption estimated via

wastewater analysis. Part A: chemical analysis and drug use estimates. *Sci Total Environ*. 2014;487:629-641. doi:10.1016/j.scitotenv.2013.11.107

18. Causanilles A, Rojas Cantillano D, Emke E, et al. Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater. *Environ Int*. 2018;115:279-284. doi:10.1016/j.envint.2018.03.039

19. Tscharke BJ, Chen C, Gerber JP, White JM. Temporal trends in drug use in Adelaide, South Australia by wastewater analysis. *Sci Total Environ*. 2016;565:384-391. doi:10.1016/j.scitotenv.2016.04.183

20. Centazzo N, Frederick B-M, Jacox A, Cheng S-Y, Concheiro-Guisan M. Wastewater analysis for nicotine, cocaine, amphetamines, opioids and cannabis in New York City. *Forensic Sci Res*. 2019;4(2):152-167. doi:10.1080/20961790.2019.1609388

21. Krizman-Matasic I, Kostanjevecki P, Ahel M, Terzic S. Simultaneous analysis of opioid analgesics and their metabolites in municipal wastewaters and river water by liquid chromatography–tandem mass spectrometry. *J Chromatogr A*. 2018;1533:102-111. doi:https://doi.org/10.1016/j.chroma.2017.12.025

22. Jacox A, Wetzel J, Cheng S-Y, Concheiro M. Quantitative analysis of opioids and cannabinoids in wastewater samples. *Forensic Sci Res*. 2017;2(1):18-25. doi:10.1080/20961790.2016.1270812

23. Rice J, Kannan AM, Castrignanò E, Jagadeesan K, Kasprzyk-Hordern B. Wastewater-based epidemiology combined with local prescription analysis as a tool for temporalmonitoring of drugs trends - A UK perspective. *Sci Total Environ*. 2020;735:139433. doi:https://doi.org/10.1016/j.scitotenv.2020.139433

24. Duvallet C, Hayes BD, Erickson TB, Chai PR, Matus M. Mapping Community Opioid Exposure Through Wastewater-Based Epidemiology as a Means to Engage Pharmacies in Harm Reduction Efforts. *Prev Chronic Dis*. 2020;17:E91. doi:10.5888/pcd17.200053

25. Endo N, Ghaeli N, Duvallet C, et al. Rapid Assessment of Opioid Exposure and Treatment in Cities Through Robotic Collection and Chemical Analysis of Wastewater. *J Med Toxicol Off J Am Coll Med Toxicol*. 2020;16(2):195-203. doi:10.1007/s13181-019-00756-5

26. Flamish Environment Agency. Geoloket Geoviews. Published 2020. http://geoloket.vmm.be/Geoviews/index.php?res=low

27. Munich P, Bonn M, Heidelberg A. *Guideline for Quality Control in Forensic-Toxicological Analyses*.; 2018.

28. European Commission. *COMMISSION DECISION of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods Andthe Interpretation of Results*.; 2002.

29. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem*. 2003;75(13):3019-3030.

30. Committee for Medicinal Products for Human Use (CHMP). *Guideline on Bioanalytical Method Validation*.; 2011.

31. McCall A-K, Bade R, Kinyua J, et al. Critical review on the stability of illicit drugs in sewers and wastewater samples. *Water Res*. 2016;88:933-947. doi:10.1016/j.watres.2015.10.040

32. Ma L-D, Li J, Li J-J, et al. Occurrence and source analysis of selected antidepressants and their metabolites in municipal wastewater and receiving surface water. *Environ Sci Process Impacts*. 2018;20(7):1020-1029. doi:10.1039/c8em00077h

33. BELGISCH CENTRUM VOOR FARMACOTHERAPEUTISCHE INFORMATIE. *Gecommentarieerd Geneesmiddelenrepertorium 2017*.; 2017. https://www.bcfi.be/ggr_pdfs/GGR_NL_2017.pdf

34. Hinrichs M, Weyland A, Bantel C. [Piritramide : A critical review]. *Schmerz*. 2017;31(4):345-352. doi:10.1007/s00482-017-0197-y

35. International Narcotics Control Board. *Narcotic Drugs - Estimated World Requirements for 2019, Statistics for 2017*.; 2019. https://www.incb.org/documents/Narcotic-Drugs/Technical-Publications/2018/INCB-Narcotics_Drugs_Technical_Publication_2018.pdf

564 36. Gushgari AJ, Driver EM, Steele JC, Halden RU. Tracking narcotics consumption at a Southwestern U.S.
565 university campus by wastewater-based epidemiology. *J Hazard Mater*. 2018;359:437-444.
566 doi:10.1016/j.jhazmat.2018.07.073

567 37. Baker DR, Kasprzyk-Hordern B. Multi-residue determination of the sorption of illicit drugs and
568 pharmaceuticals to wastewater suspended particulate matter using pressurised liquid extraction, solid
569 phase extraction and liquid chromatography coupled with tandem mass spectrometry. *J Chromatogr A*.
570 2011;1218(44):7901-7913. doi:10.1016/j.chroma.2011.08.092

571 38. Baker DR, Kasprzyk-Hordern B. Critical evaluation of methodology commonly used in sample collection,
572 storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and
573 wastewater by solid phase extraction and liquid chromatography-mass spectrometry. *J Chromatogr A*.
574 2011;1218(44):8036-8059. doi:10.1016/j.chroma.2011.09.012

575 39. IPhEB asbl - IFEB vzw. IPhEB Monthly barometer. Published 2020. https://www.ipheb.be/index_nl.html

576 40. IQVIA. *IQVIA Sales Statistics on Opioids.*; 2020. https://www.iqvia.com/locations/united-
577 states/solutions/commercial-operations/essential-information/sales-information

578 41. Tscharke BJ, Chen C, Gerber JP, White JM. Trends in stimulant use in Australia: A comparison of
579 wastewater analysis and population surveys. *Sci Total Environ*. 2015;536:331-337.
580 doi:10.1016/j.scitotenv.2015.07.078

581 42. Senta I, Krizman I, Ahel M, Terzic S. Assessment of stability of drug biomarkers in municipal wastewater
582 as a factor influencing the estimation of drug consumption using sewage epidemiology. *Sci Total*
583 *Environ*. 2014;487:659-665. doi:https://doi.org/10.1016/j.scitotenv.2013.12.054

584 43. Thai PK, Lai FY, Bruno R, et al. Refining the excretion factors of methadone and codeine for wastewater
585 analysis - Combining data from pharmacokinetic and wastewater studies. *Environ Int*. 2016;94:307-314.
586 doi:10.1016/j.envint.2016.05.033

587 44. Boleda MAR, Galceran MAT, Ventura F. Monitoring of opiates, cannabinoids and their metabolites in
588 wastewater, surface water and finished water in Catalonia, Spain. *Water Res*. 2009;43(4):1126-1136.
589 doi:10.1016/j.watres.2008.11.056

590