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Simultaneous analysis of opioid analgesics and their metabolites in municipal wastewaters and river water by liquid chromatography-tandem mass spectrometry

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Highlights

- LC-MS/MS method for the determination of 27 opioid analgesics was developed
- Keeping SPE catridges at -20 ^{o}C was the best way to ensure stability of opioids
- Opioid analgesics are common constituents of municipal wastewater and river water
- Metabolites of opioids contributed significantly to the overall mass balance
- Conjugated opioids may represent a significant percentage of the total concentration

Abstract

Although published literature provides a clear demonstration of widespread occurrence of opioid analgesics (OAs) in the aquatic environment, analytical methods suitable for a systematic study of this pharmaceutical class, which would include a broad spectrum of opioid analgesics and their metabolites, are still missing. In this work, a comprehensive multiresidue method for quantitative analysis of 27 opioid analgesics and their metabolites, including 2 morphine glucuronide conjugates, was developed and validated for three matrices: raw wastewater (RW), secondary effluent (SE) and river water. The method comprised different classes of opioid analgesics, including natural opiates (morphine and codeine), their semi-synthetic derivatives (hydrocodone, hydromorphone,

oxycodone, oxymorphone and buprenorphine) as well as fully synthetic opioids such as methadone, fentanyl, sufentanil, propoxyphene and tramadol. The optimized enrichment procedure involved mixed-mode, strong cation-exchange sorbent in combination with a sequential elution procedure. The extracts were analyzed by reversed-phase liquid chromatography using a Synergy Polar column coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS). Accurate quantification of target OAs was achieved using 19 deuterated analogues as surrogate standards. Method accuracies for RW, SE and river water varied in the range from 91-126%, 74-120% and 75-116%, respectively. Careful optimization of the procedure allowed reliable determination of OAs with method quantification limits in the low ng/L range (RW: 0.3-2.6 ng/L; SE: 0.2-1.9 ng/L; river water: 0.1-0.8 ng/L). The developed method was applied for analysis of RW, SE and river water samples from Croatia. The concentrations of individual OAs in municipal wastewater varied in a wide range (from <MQL to 808 ng/L) and the most prevalent representatives were tramadol, codeine, morphine and methadone and their derivatives. Elevated concentrations of morphine glucuronides (up to 459 ng/L) found in raw municipal wastewater indicated their importance in the overall morphine mass balance.

Keywords: pharmaceuticals, opioid analgesics, liquid chromatography-tandem mass spectrometry, wastewater, surface water

1. Introduction

Opioid analgesics (OAs) are a type of prescription drugs, which are used to treat moderate to severe pain, particularly of visceral origin. Their mode of action is based on binding to opioid receptors, which are found principally in the central and peripheral nervous system and gastrointestinal tract. They also find a significant application in heroin addiction treatment. On the other hand, all opioid analgesics themselves are well known for their addictive properties and therefore should be considered as potential drugs of abuse.

OAs comprise a number of structurally diverse chemical compounds, such as natural (e.g. morphine and codeine), semisynthetic morphine-like opioids (e.g. oxycodone, buprenorphine, ethylmorphin) and fully synthetic opioids (e.g. methadone, fentanyl, pentazocine, propoxyphene etc.). Tramadol, which was also included in this research, is structurally not an opioid, but does act as one by showing agonist activity at the μ-opioid receptor [1].

The major sources of opioid compounds to the aquatic environment are human excretion and dumping of unused medications to the sewer. According to the available pharmacokinetic data [2] most of the OAs are extensively metabolized in the human body. Consequently, they are only partly excreted as unchanged drugs, while a significant percentage is excreted as metabolites [2]. For example, morphine is significantly metabolized after administration. Up to 87% of the dose is eliminated in urine with 75% present as morphine-3-glucuronide and only about 10% as unchanged morphine. In addition, several minor metabolites such as normorphine, morphine-6-glucuronide, diglucuronide, and sulphate conjugate are also formed. Codeine is biotransformed in man via *O*-demethylation to morphine and *N*-demethylation to norcodeine and excreted mainly in conjugated form. The metabolism of buprenorphine also leads to the number of products (primarily by *N*-dealkylation), which are excreted mainly as conjugated buprenorphine and norbuprenorphine. The metabolism of methadone is even more complex. The most important transformation is mono- and di-*N*-demethylation followed by spontaneous cyclisation to 2-ethylidene-1,5-dimethyl-3,3,-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3,-diphenylpyrrolidine (EMDP). The major urinary excretion products are methadone (5-50%) and EDDP (3-25%), while conjugated forms seem to be less important. The metabolic pattern of tramadol shows several major products. Approximately 29% of an oral dose is excreted as unchanged drug, 20% as free and conjugated *O*-desmethyltramadol, 17% as nortramadol and 20% as free and conjugated *O*-desmethylnortramadol. Since the main source of OAs in the environment are municipal wastewater effluents, the human pharmacokinetics data clearly show that their metabolic products should be taken into account when assessing their behavior and fate in the aquatic environment.

There have been numerous reports in the literature which addressed the issue of environmental occurrence of opioid analgesics. The review by Verlicchi et al. [3] on the occurrence of various pharmaceuticals in urban wastewater showed that opioid analgesics, notably codeine and tramadol, were among the most abundant compounds. Moreover, codeine was highly ranked regarding potential environmental risk. The risk posed by OAs can be significantly enhanced in the watersheds influenced by discharges from pharmaceutical formulation facilities [4, 5]. Nevertheless, most of the literature published so far dealt only with a limited number of selected representatives of OAs, mainly as a part of broader scope studies, aimed to cover several therapeutic classes simultaneously [6-8]. Furthermore, OAs have been often included in the studies dealing with sewage epidemiology of illicit drugs [9-13] because they themselves have a significant record of abuse. Such studies focus primarily on influent wastewater and include only heroin-related metabolites (morphine, 6-monoacetyl morphine and morphine glucuronide), codeine and methadone along with its main metabolite EDDP [9, 11].

Most of the published analytical methods for the determination of OAs comprise a rather limited number of parent compounds and/or their human metabolites [14-19]. The most comprehensive list of OAs was covered by a multiresidue LC-MS/MS method developed by Baker and Kasprzyk-Hordern [20], who employed solid-phase extraction in combination with ultra-performace liquid chromatography-tandem mass spectrometry for wide scope multiclass assessment of 65 pharmaceuticals and illicit drugs, including simultaneous determination of twenty OAs and their metabolites. However, this method does not include some of the most polar representatives of OA-derived compounds such as morphine derivatives dihydromorphine and hydromorphone and especially the two morphine glucuronides. Given the prominence of glucuronide conjugates in the human excreta [2], their determination in wastewater might be very important for the correct assessment of the total concentration of OAs and thus for the comprehensive assessment of their behavior and fate in the aquatic environment. In order to achieve this goal, further improvement of analytical methodology is required, especially in terms of improved HPLC separation of polar OAs, which include some structurally related isobaric compounds.

The aim of this study was therefore to develop and validate a dedicated multiresidue LC-MS/MS method for simultaneous determination of 27 OAs, which should comprise both parent compounds and their major metabolites, including glucuronide conjugates, in environmental aqueous samples. A special emphasis was on improvement of chromatographic separation and sample cleanup for a more reliable determination of OAs in heavily loaded matrices. In order to improve the overall analytical reliability, stability of target compounds during sample preparation and storage was also systematically investigated. Finally, the method was applied for determination of opioid analgesics in

wastewater and river water samples from Croatia in order to assess their occurrence in the aquatic environment, in particular to demonstrate relative importance of so far neglected glucuronide conjugates.

2. Experimental

2.1 Chemicals and materials

The developed method included 27 analytes, involving both parent compounds and their human metabolites, and 19 deuterated analogues used as surrogate standards for quantitation (Table 1). Additional information about structures, nomenclature and properties can be found in Table S1 in Supplementary material. Standard solutions of all target analytes and their deuterated analogues were purchased from Lipomed (Arlesheim, Switzerland) at concentration of 1 mg/mL and 0.1 mg/mL, respectively. Mixed standard solutions were prepared in MeOH at concentration of 10 µg/mL for analytes and 2 µg/mL for their deuterated analogues, and kept in the dark at -20 °C. LC-MS grade methanol and acetonitrile were delivered by BDH Prolabo (UK). Acetic and formic acid, also LC-MS grade, and phosphoric acid were purchased from Sigma-Aldrich (Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA * 2H_2O$) was also purchased from Sigma-Aldrich. 25% ammonia solution in water was supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained using an Elix-Milli-Q-system (Millipore, Bedford, USA). Solid-phase extraction cartridges, Oasis HLB (200 mg / 6 mL) and Oasis MCX (150 mg / 6 mL) were purchased from Waters (Milford, MA, USA), while Strata NH_2 cartridges (200 mg / 3 mL) were purchased from Phenomenex (Torrance, California, USA). Whatman glass fiber filters (GF/D, 47 mm; pore size, 2.7 µm) were delivered by GE Healthcare (Maidstone, UK).

2.2 Sample collection and preparation

All wastewater samples for method optimization and validation, including raw wastewater (RW) and secondary effluent (SE) were collected at the central WWTP of the city of Zagreb, while wastewater samples for method evaluation were collected in Zagreb and Split. River water samples were collected along the Sava River in the city of Zagreb. All wastewater samples were 24 h-composite samples, while river samples were grab samples. Sample volumes for RW, SE and river water analyses were 125 mL, 250 mL and 500 mL, respectively. After the filtration through glass-fiber filters (GF/D), the mixture of surrogate standards (15 ng of each) was added to the samples. The enrichment of opioid analgesics from the filtered samples was performed using solid phase extraction (SPE). Two

types of SPE cartridges were tested during the method development: Oasis HLB and Oasis MCX. In the final procedure, samples were extracted by using Oasis MCX cartridges at pH 2. The pH was adjusted by addition of 85% phosphoric acid. The cartridges were previously preconditioned with 5 mL of MeOH, 5 mL of ultrapure H_2O and 5 mL of 25 mM H_3PO_4 at the flow rate of approximately 5 mL/min. Before the elution, cartridges were washed with 6 mL of ultrapure water and subsequently dried under N_2 (30 min). If the samples could not be analyzed on the same day, the cartridges containing adsorbed analytes were wrapped up in aluminum foil and stored at −20 °C until analysis. The elution of the target opioids from the cartridge was performed using sequential elution. The first fraction was eluted with 6 mL of pure MeOH and this fraction, containing only interferences from wastewater matrix, was discarded. The second fraction, containing all target OAs, was eluted with 6 mL of 0.5% NH_3 in MeOH. This purified extract was evaporated to dryness under N_2 using a TurboVap evaporator (Caliper Life Science, Hopkinton, Massachusetts, USA). The residue was redissolved in 500 µL of H_2O/MeOH (8:2, *v*/*v*) containing 50 mM ammonium acetate.

2.3 LC-MS/MS analyses

LC-MS/MS analyses were performed on a instrument (Thermo Electron, USA) consisting of an HPLC system equipped with a Surveyor autosampler and a quarternary MS pump interfaced to a triple quadrupole mass spectrometer (Quantum AM) equipped with an electrospray ionization source. During the method development, four different HPLC columns as well as several different eluents and elution gradients were tested. The tested HPLC columns used in this study included Synergi Polar (4 µm, 150 mm x 3 mm) and Gemini C_{18} (5 µm, 150 mm x 3 mm) columns supplied by Phenomenex (Torrance, California, USA), YMCPro C_{18} (3 µm, 150 mm x 2.1 mm) supplied by YMC Europe, Schernbeck, Germany and ACE C18-PFP (3 µm, 150 mm x 3 mm) supplied by Advanced Chromatography Technologies Ltd (Aberdeen, UK). In the optimized procedure, Synergi Polar column was used with 0.1% acetic acid in water (*v*/*v*) as eluent A and 0.1% acetic acid in MeOH (*v*/*v*) as eluent B at the flow rate of 400 µL/min. Gradient elution was performed as follows: 0–20 min, from 10 to 50% B; 20–21 min, from 50 to 65% B; 21–26 min, from 65 to 85% B; 26–31 min, from 85 to 88% B; 31–32 min, from 88 to 95% B; 32–33 min, 95% B (isocratic hold); 33–34 min, from 95 to 10% B; 34–44 min, 10% B (reconditioning to initial conditions). The injection volume was 15 µL. The target analytes were analyzed in positive ionization mode. The capillary voltage was 3500 V and the capillary temperature was 300 °C. The desolvation gas (N_2) and auxiliary gas (N_2) were 40 and 10 arbitrary units, respectively. Identification and quantification of target compounds was performed in multiple reaction monitoring (MRM) mode, using argon as collision gas. Two most abundant

precursor/product ion transitions were selected for each analyte and deuterated surrogate. First transition was used for quantification and second for additional confirmation. The collision energy and tube lens offset were optimized for each analyte and surrogate separately. The MRM parameters for all compounds are presented in Table 1. Quantification of all analytes was performed using the corresponding deuterated internal standards.

2.4 Method validation

The method validation included determination of instrument detection limit (IDL), extraction recovery, matrix effect, accuracy, repeatability and method quantification limit (MQL) for each matrix (RW, SE, river water) in experiments performed in quadruplicate.

Linearity range was determined from 12-point internal standard calibration curves obtained by injecting standard solutions containing analytes in the concentration range from 0.5 to 1500 ng/mL and internal standards at the fixed concentration of 15 ng/mL. Linearity was verified by F-test, at 95% confidence level, according to IUPAC recommendations [21]. Sigma Plot was used for the statistical analysis. IDLs were determined by repetitive injection of low concentrations standard solution until signal to noise ratio was equal to 3. MQLs were determined as the minimum concentration of target analytes in standard solutions that can be measured and fulfilling the following criteria: bias from the calibration curve less than 25-30%, peak shapes acceptable, signal to noise ratio at least 8 and RSD of four replicates below 19%, taking into account method accuracy and sample volume for each sample matrix.

Extraction recovery was determined from the model experiment in which real water samples were spiked with target analytes either before (A_{be}) or after extraction (A_{ae}). Samples were spiked at 1 µg/L, 500 ng/L and 50 ng/L for RW, SE and river water, respectively. Analytes already present in the original sample were also taken into account (A_{orig}). The extraction recovery was calculated by comparing the average responses of analyte spiked to samples after extraction to the samples spiked before extraction according to the following equation:

$$\text{Extraction recovery (\%)} = \frac{Abe - Aorig}{Aae - Aorig} \times 100$$

The method accuracy was assessed from model experiment in which samples were spiked both with analytes and internal standards. Samples were spiked with target compounds at 1 µg/L, 500 ng/L and 50 ng/L for RW, SE and river water, respectively, while the internal standards were added at 15 ng in

all samples. Non-spiked sample was also analyzed to correct for analytes already presented in original samples. Accuracy was calculated from the following equation:

$$\text{Accuracy (\%)} = \frac{c2-c1}{c0} \times 100$$

where c_0, c_1 and c_2 represent nominal spiked concentration, average concentration measured in original sample and average concentration in spiked sample, respectively.
Repeatability (method precision) was determined in the same experiment and calculated as the relative standard deviation (RSD) of the analysis of spiked samples.
Matrix effect (signal suppression or enhancement) was determined by comparing the average response of target analyte spiked in to the final water extracts (A_{fe}), after extraction and evaporation, with the average response of analyte in matrix-free standard solutions (A_{std}) according to the following equation:

$$\text{Matrix effect (\%)} = \frac{Afe-Aorig-Astd}{Astd} \times 100$$

In that experiment RW, SE and river water extracts were spiked at the concentration levels of 1 µg/L, 500 ng/L and 50 ng/L, respectively. Contributions of target analytes from the original sample (A_{orig}) were also taken into account.

2.5 Stability experiments

Stability of target compounds during storage was studied for three scenarios: a) storage of water samples, b) storage of Oasis MCX cartridges after sample enrichment and c) storage of the final extracts.
Stability of opioid analgesics during water sample storage was tested as follows. Raw wastewater sample was filtered, spiked at concentration of 1 µg/L and then divided into five identical subsamples. One of these subsamples was processed immediately, including SPE, evaporation and LC-MS/MS analysis, according to the procedure described in Section 2.2-2.3 (above). Analyses in this experiment were conducted in triplicate. The remaining four subsamples were frozen and stored at -20 °C. These subsamples were processed and analyzed after being stored for 7, 30, 70 and 100 days.

Stability of target compounds on Oasis MCX sorbents (cartridges) and in SPE extracts after extraction was evaluated as follows. The target analytes were spiked into 800 mL of ultrapure water at the concentration of 1 µg/L. The spiked sample was homogenized and divided into 15 subsamples of 50 mL. In each subsample, deuterated surrogates (15 ng) were also added. All subsamples were acidified to pH 2 and extracted on SPE cartridges according to the procedure described in Section 2.2. Three of them were eluted, evaporated and analyzed on the same day. The remaining subsamples were divided into two sets. The first set of MCX cartridges were eluted immediately after the extraction and the extracts were frozen at −20 °C until the analysis. The second set of cartridges was kept frozen at −20 °C until the day of analysis, when the cartridges were eluted and analyzed. In this experiment, the stored samples were analyzed after 7 and 30 days. At each point, the analyses in this experiment were conducted in triplicate.

The results of the stability experiments were analyzed using SigmaPlot software.

3. Results and discussion

3.1 LC-MS/MS optimization

In order to achieve the best chromatographic separation of the selected opioid analgesics, four different reverse-phase HPLC columns were tested: Synergy POLAR-RP, YMCPro C_{18}, Gemini C_{18} and ACE C_{18}-PFP. YMCPro C_{18} and Gemini C_{18} are reverse phase columns, whose selectivity is mostly based on hydrophobic interactions, while the column ACE C_{18}-PFP, which is also strongly hydrophobic, contains a specially developed ligand combining a C_{18} chain with integrated pentafluorophenyl functionality. This functionality provides additional π-π interactions as a basis for retention and separation of the target compounds. In contrast, Synergi Polar is a polar endcapped, ether-linked phenyl phase, which provides both polar and aromatic reversed-phase selectivity.

Different types of eluents were tested, including water, methanol and acetonitrile as solvents and addition of acetic or formic acid as modifiers. Acidic modifier was added to promote protonation of basic compounds and increase the MS signal. Formic acid addition (0.1%) to both eluents was selected in the optimized procedure. Regarding strong solvent, methanol provided slightly better performance than acetonitrile. Preliminary experiments showed that Gemini C_{18} column was suitable only for separation of the most lipophilic OAs, while the hydrophilic OAs (e.g. MOR, glucuronides, norMOR) were very poorly retained with very bad peak shapes (Figure S1a, Supplementary info), which is probably due to the relatively low carbon load of this column (14%). The alternative C_{18}

column YMCPro with carbon load of 16% provided a much better separation of OAs (Figure S1b), however some of the isobaric compounds such as morphine and hydromorphone coeluted on this column, which precluded their reliable determination.

On the other hand, ACE C_{18}-PFP column proved to be much more efficient and allowed satisfactory separation and good peak shapes for most of the target compounds (Figure S1c), except for the most polar analyte M3G. The best separation with satisfactory retention and peak shapes of polar OAs was achieved with Synergi Polar column (Figure 1) and therefore this column was selected for further method development. This separation is clearly superior to that shown by Baker and Kaszprzyk [20] using a generic method based on BEH C_{18} column. Of 27 target analytes, there were only three co-eluting analyte pairs (DHCOD and OTRA, FNT and BUP and EDDP and PP). However, selectivity of the determination for the co-eluting compounds was fully assured by highly specific MRM detection, using the two most abundant precursor/product ion transitions for each analyte (see Table 1). The first transitions were used for quantification of analytes, while the second transitions were used as additional criteria for confirmation. M3G and M6G, which are structural isomers with identical product ions, were fully separated on the Synergi Polar column. MRM detection provided highly sensitive determination for most of the target OAs, however for some analytes, such as NOC, norBUP and PP, the sensitivity was relatively low (IDLs 5-15 pg). In order to improve sensitivity, for BUP and norBUP, several product ions were tested, which were previously used in literature [12, 17] and the transitions *m*/*z* 468 to *m*/*z* 396 and *m*/*z* 414 to *m*/*z* 187 were found to be the most sensitive for BUP and norBUP, respectively.

3.2 Optimisation of the extraction procedure

For the optimization of the extraction procedure, two types of SPE cartridges were compared, Oasis HLB and Oasis MCX, which have been widely used for the extraction of similar types of basic analytes [11, 12, 15, 20, 22]. Besides at the original sample pH (approximately 7.5), Oasis HLB cartridges were also tested at basic conditions (pH 10). Basic conditions were chosen to suppress dissociation of the target compounds, all of which are weak bases, and in that way to enhance their hydrophobic interaction with the sorbent and, consequently, the extraction recoveries. Unlike HLB sorbent, which achieves adsorption through hydrophilic and lipophilic interactions, Oasis MCX has a more complex mode of retention, involving hydrophobic, hydrophilic and cation-exchange groups. This provides a basis for enhanced retention and improves selectivity when extracting basic compounds. The MCX

cartridges were tested at pH 2 to promote the interaction of protonated analytes with the cation exchange moiety.

Preliminary experiments, comparing three enrichment procedures described above, were performed using spring water spiked with target opioid analgesics and results are presented in Figure S2 (Supplementary material). Acceptable recoveries (57-115%) were achieved for all target analytes using each of the three procedures. The experiment showed that for some analytes, such as codeine and tramadol derivatives, better recoveries were obtained using HLB cartridges, especially at pH 10 (57-115%). In contrast, SFNT showed better recovery on MCX cartridge. Furthermore, this experiment demonstrated that most of the target compounds couldn't be completely desorbed from the HLB column using pure methanol and therefore an additional elution using 0.5% ammonia solution in methanol was required (not shown). The two eluates were analyzed separately and then added up to obtain the total concentrations. Based on these preliminary results, HLB enrichment at pH 10 seemed to be the most promising procedure.

However, this could not be verified in a more complex matrix such as wastewater. Firstly, the adjustment of pH to 10 in raw wastewater with ammonia solution resulted in the formation of a very fine calcium carbonate precipitate, which prevented percolation of the sample due to the cartridge clogging. To solve this problem, it was necessary to add high concentration of Na_2EDTA (1 g/L) to wastewater samples before solid-phase extraction. The comparison of the three SPE protocols for the recovery of target compounds from raw wastewater is shown in Figure S3. The recoveries using HLB cartridges were similar at pH 10 and pH 7.5, except for EDDP and TRA, whose recovery appeared to be much higher at natural pH (7.5). The recoveries of the lipophilic OAs using MCX cartridges were only slightly better from those obtained by HLB-based procedure, however for the polar OAs, such as morphine glucuronides, OM and HM, MCX showed a clearly superior performance. Morphine glucuronides were completely lost during the enrichment using HLB cartridges, indicating problems with their retention in complex real matrices.

Additional problem when using HLB cartridges was co-extraction of some matrix components, which caused a significant peak-shape deterioration (Figure 2A). This problem could be rectified only after an additional clean up step using aminosilica cartridges as described earlier [23] (Figure 2B). This additional step could be skipped when using MCX cartridges for the enrichment (Figure 2C), since this procedure applied sequential elution as described earlier [23, 24]. The predominant part of interfering compounds was eluted in the methanol fraction, before the final elution of the target analytes with 0.5% ammonia solution in methanol.

3.3 Method validation

Method validation parameters, including linearity, instrument detection limits, extraction recovery, matrix effect, repeatability and method quantification limits, were determined for three different matrices: raw wastewater, secondary effluent and river water, and the results are presented in Table 2. For most of the analytes, linearity was attained in the range from 0.5 ng/mL to 500 ng/mL with few exceptions. For morphine-like opioids, such as norMOR, MOR and DMOR, linearity range was narrower (up to 200 ng/mL), while for TRAM and its metabolites linearity range extended up to 1500 ng/mL. The IDLs varied in wide range from 0.02 pg up to 15 pg (injected on column). MQL were generally in the low ng/L range (RW: 0.3-2.6 ng/L; SE: 0.2-1.9 ng/L; river water: 0.1-0.8 ng/L).
Very good extraction recoveries of OAs were achieved from all three water matrices for most of the target OAs (from 79% to 97% for RW, from 73% to 116% for SE and from 73% to 102% for river water). Somewhat lower recoveries in raw wastewater were obtained only for EDDP (44%) and TRA (63%). For EDDP, some losses were observed during the evaporation step in river water matrix.
Regarding matrix effects, the experiments showed relatively low effects in all examined matrices. For almost all analytes, matrix effects were below 20% with only few exceptions. A slightly more pronounced ion suppression (up to 38%) was observed in raw wastewater and river water for early eluting polar OAs (M3G, M6G and norMOR) but also for BUP and norBUP. On the other hand, a signal enhancement up to 27% was observed for some late eluting OAs (MTHD and SFNT) in the secondary effluent extracts. Low matrix effects were probably a result of sequential elution from MCX cartridges [23, 24], employing elution with pure methanol (which removed a large percentage of matrix components) before the actual elution of the target OAs with basified methanol (0.5% NH_3).
The losses during the sample preparation and matrix effects were successfully compensated using 19 surrogate standards. For those analytes for which isotopically labelled standards were not available (norMOR, M6G, DMOR, norCOD, OTRA, NOC, EMOR and NTRA) the surrogate standard was chosen based on the maximum similarity of the chemical structures and retention times. Another criterium was also the similarity of the extraction recoveries between the analyte and surrogate, determined in preliminary experiments. The morphine derivatives, norMOR and DMOR, were determined based on MOR-d3 as surrogate, however, this surrogate was not appropriate for the determination of EMOR. Instead, a more closely eluting NFNT-d_5 was found more suitable internal standard for EMOR. Similarly, the two tramadol derivatives (OTRA and NTRA) were quantified using DCOD-d_3 and NFNT-d_5, respectively.
Careful selection of appropriate surrogate standards allowed determination of all target OAs with very good accuracies in all examined matrices (from 80% to 120%). Exceptions were somewhat lower accuracies for the determination of norBUP in secondary effluent (74%) and norCOD in river water

(75%) as well as for OTRA (126%). It should be stressed that for these compounds deuterated analogs were not available in this work.

The analytical repeatability of the target analytes was better than 11% for all matrices. The only exception was repetability of EDDP determination in raw wastewater (15%), which was probably related to lower recoveries of this compound (44%).

Overall, our analytical quality assurance parameters compare very well with the best literature achievements [20], while providing analysis of an extended range of OAs.

3.4 Stability of opioid analgesics in samples

Stability of opioid analgesics in wastewater samples during sample storage and analysis is an essential part of accurate assessment of these compounds in real systems. Losses of target compounds during storage can lead to a significant underestimation of the true concentrations, while formation from co-occurring precursors would lead to an overestimation of the target compounds. Among selected opioid compounds, both options are very likely [25-27]. When analyzing large number of samples collected in a sampling campaign, which cannot be processed on the same day, there are two strategies to minimize possible pitfalls due to the sample deterioration: immediate storage of the samples by freezing at low temperature (typically -20 °C) or extraction of the samples on the same day with options to store either adsorption cartridges or the final extract until instrumental analysis. In this work, stability of the target opioid analgesics was examined for raw wastewater, which is the most complex matrix regarding possible biotic and abiotic processes. Determination of stability of OAs during wastewater sample storage at -20 °C showed that some analytes were stable even during prolonged storage time of 100 days with only slight changes in concentrations, which didn't exceed 20% (Table 3). On the other hand, for some analytes such as SFNT, PP, FNT, norBUP, NFNT, NTRA, EMOR, NOC, HM and NHC, a statistically significant decrease of concentrations (>25%) was observed already after 7 days. A gradual decrease for EMOR, NFNT and BUP continued during the prolonged storage of 100 days. The most unstable opioid compounds were morphine glucuronides and 6-AM, which is in accordance with our earlier observations [26]. The loss of glucuronides exceeded 50% after 30 days and the concentration continued to decrease till the end of experiment. It should also be noted that M3G was significantly less stable than M6G. Similarly, the concentration of 6-AM decreased by 86% on the day 100. It is interesting that decrease of M3G, M6G and 6-AM was accompanied by a significant increase in the concentration of MOR (up to 195% after 100 days). This shows that transformation between these analytes can occur during prolonged storage. 6-AM is an exclusive biomarker of heroin, so its stability in wastewater samples is essential

for a reliable estimation of heroin consumption using wastewater-based epidemiology approach. Our results show that estimation of heroin consumption based on 6-AM might be underestimated, if the samples were kept frozen before extraction. In addition, norBUP, also increased during the experiment, while its precursor BUP decreased.

Comparison of OAs stability on the MCX cartridges and in the SPE extracts stored at -20 °C after extraction of target compounds is presented in Figure 3. Most of the opioid analgesics were found to be stable on MCX cartridges over a period of 30 days (loss <20%). A moderate, statistically significant decrease of the concentrations (up to 31%) was observed for norMOR, DMOR, MOR and HC. Only for M6G and EDDP a more pronounced concentration loss (up to 43%) was noticed, however the difference between the days 7 and 30 was not significant. A significant decrease in OA concentration in SPE extracts was observed for 6-AM (43%), EMOR (42%), DMOR (49%) and 6-morphine glucuronide (56%). Generally, opioid analgesics were more stable if the MCX cartridges were stored at -20 °C after SPE rather than SPE extracts. The stability of opioids during storage of SPE cartridges at -20 °C was also demonstrated by Baker and Kasprzyk-Hordern [25], who reported that OAs, adsorbed onto MCX cartridges, were stable over a period of 6 weeks, and Gonzalez-Marino et al. [28], who showed that OAs adsorbed onto HLB cartridges were stable for three months. Consequently, storage of MCX cartridges was selected as the best way to preserve the samples after extraction and to avoid sample alterations.

3.5 Method application

The developed method was applied for the analysis of target opioid analgesics in real samples, including raw water, secondary effluent and river water, collected in the area of the cities of Zagreb and Split (Croatia). The results are presented in Table 4. The measured concentrations of individual opioids varied in wide ranges, from <MQL (low ng/L) to almost 1 µg/L. The most abundant opioid compounds were TRA and its metabolites NTRA and OTRA with the total concentration in WW exceeding 1 µg/L. It is interesting to note that TRA metabolites contributed significantly to the total concentration. This is in agreement with published literature [6, 7, 15]. Other relatively abundant opioid compounds found in untreated municipal wastewater included COD (237-625 ng/L), MOR (142-445 ng/L), M3G (4-370 ng/L), M6G (5-89 ng/L), MTHD (71-94 ng/L) and its metabolite EDDP (115-175 ng/L). Such results are not surprising since tramadol and codeine belong to the widely prescribed opioid analgesics for the treatment of moderate-severe pain in Europe [29]. On the other hand, MOR and its glucuronide conjugates originate mainly from the heroin abuse, while only a smaller part (10-15%) is associated with the therapeutic use [7, 30]. Although literature data often suggest that morphine glucuronides are quickly deconjugated by wastewater bacteria and biofilms

[31], our data from Split show that contribution of conjugated MOR can reach up to 50% of the total MOR concentration calculated on the molar basis. This indicated that the contribution of conjugated morphine must be taken into account when assessing the total morphine in municipal wastewater as a basis for the estimation of heroin consumption. Alternatively, heroin consumption can be estimated using 6-AM as exclusive heroin biomarker, and therefore, it is important to emphasize that the developed method allows its reliable measurement in low ng/L concentrations.

Moreover, in addition to its use as analgesic, the presence of MTHD and its metabolite EDDP can be related to extensive use of MTHD for the treatment of heroin addiction in Croatia. Other opioids, found in measurable concentrations, were norMOR, 6-AM and BUP, while most of the other semi-synthetic OAs were not detected. It is interesting to note that the concentration of BUP (3-11 ng/L) was much lower than that of MTHD and EDDP, indicating that its use for the treatment of heroin addiction is less popular in Croatia.

It is interesting to note that the concentrations of OAs in secondary effluents were in the same range as those in raw wastewater, indicating that conventional biological wastewater treatment was rather inefficient in removing these compounds from wastewater. Lack of any removal was observed for TRA and its derivatives and methadone, while the removal efficiencies of COD and MOR were 21-28% and 65-67%, respectively. The elimination of minor opioids 6-AM and BUP were 50-67% and 20-43%, respectively. These findings are in a good agreement with our previously published data [11] and the recent literature [7, 20]. The only opioid which was efficiently eliminated in conventional WWTP was norMOR (>90%).

As could be expected, considering a relatively large dilution factor (typically >50), the concentrations of OAs in the Sava River were rather low (<MQL-72 ng/L) with prevalence of those compounds which were the most abundant opioid constituents in municipal wastewater (TRA, NTRA, OTRA, COD and EDDP). This could be primarily result of dilution since the results on removal efficiency in WWTP indicated that these compounds are rather refractory to biodegradation. The measured concentrations are lower than those reported by Baker and Kasprzyk-Hordern [20], probably as a combined result of lower usage prevalence and a high dilution factor in the Sava River.

4.0 Conclusions

The developed analytical method allows reliable determination of 27 opioid analgesics and their metabolites at trace concentrations in different aqueous matrices thus providing a basis for their comprehensive study in the aquatic environment. Application of the method to real wastewater and

river water samples showed widespread occurrence of OAs in the aquatic environment. Inclusion of the number of OA metabolites along with their parent compounds indicated that the relative contribution of metabolites to the overall mass balance could be significant, which has often been neglected in literature. In particular, we showed that conjugated OAs may play an important role. Results showed that opioid analgesics and their metabolites can reach significant levels in municipal wastewaters, raising concerns about their possible ecotoxic effects. The method is expected to be an important tool for systematic monitoring of OAs and assessment of their behavior and fate in the aquatic environment as well as for wastewater-based epidemiology to study their potential abuse.

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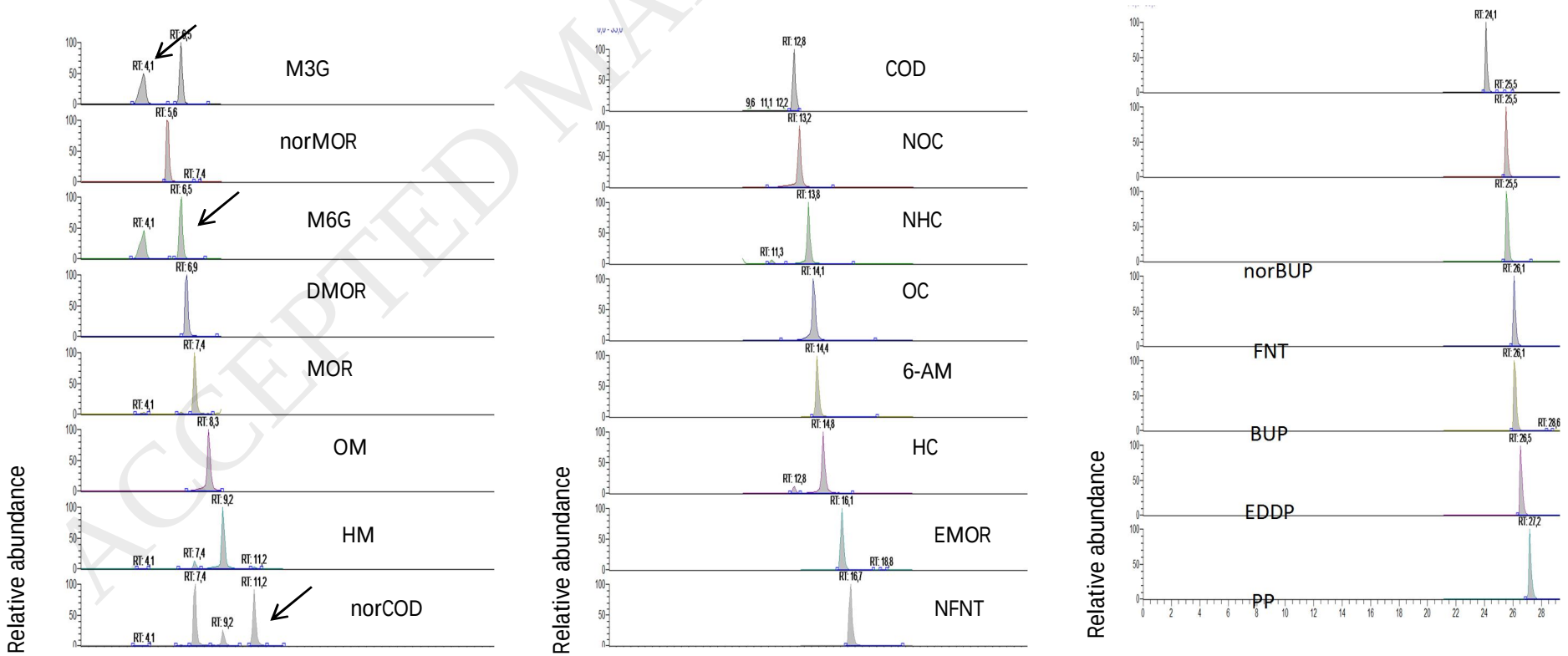
FIGURE CAPTIONS

Figure 1. MRM chromatogram of standard mixture of opioid analgesics (100 pg/uL). HPLC column: Synergi Polar (4 µm, 150 mm x 3 mm). For abbreviations of analyzed substances see Table 1.



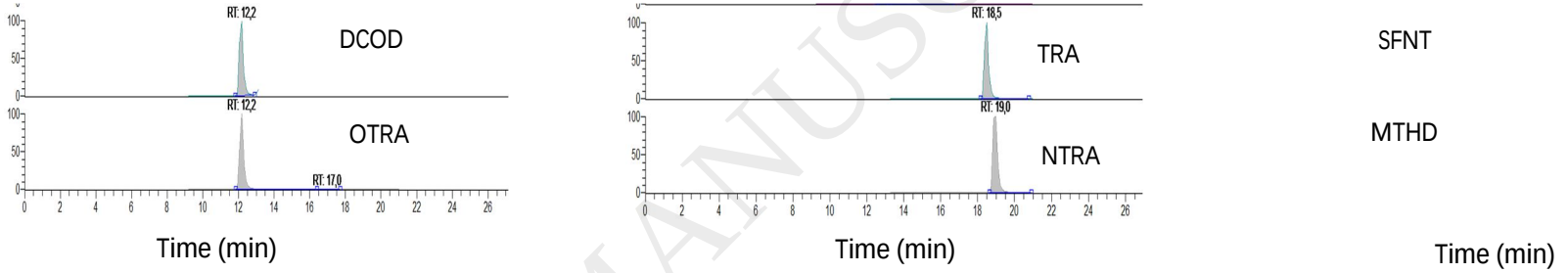


Figure 1. MRM chromatogram of standard mixture of opioid analgesics (100 pg/µL). HPLC column: Synergi Polar (4 µm, 150 mm x 3 mm). For abbreviations see Table 1.

Figure 2. MRM chromatogram of raw wastewater extract spiked with target opioid analgesics (1 µg/L) obtained on: A) Oasis HLB cartridges without extract clean up; B) Oasis HLB cartridges with extract clean up on Strata NH_2 and C) Oasis MCX cartridges by fractionated elution. For abbreviations see Table 1.

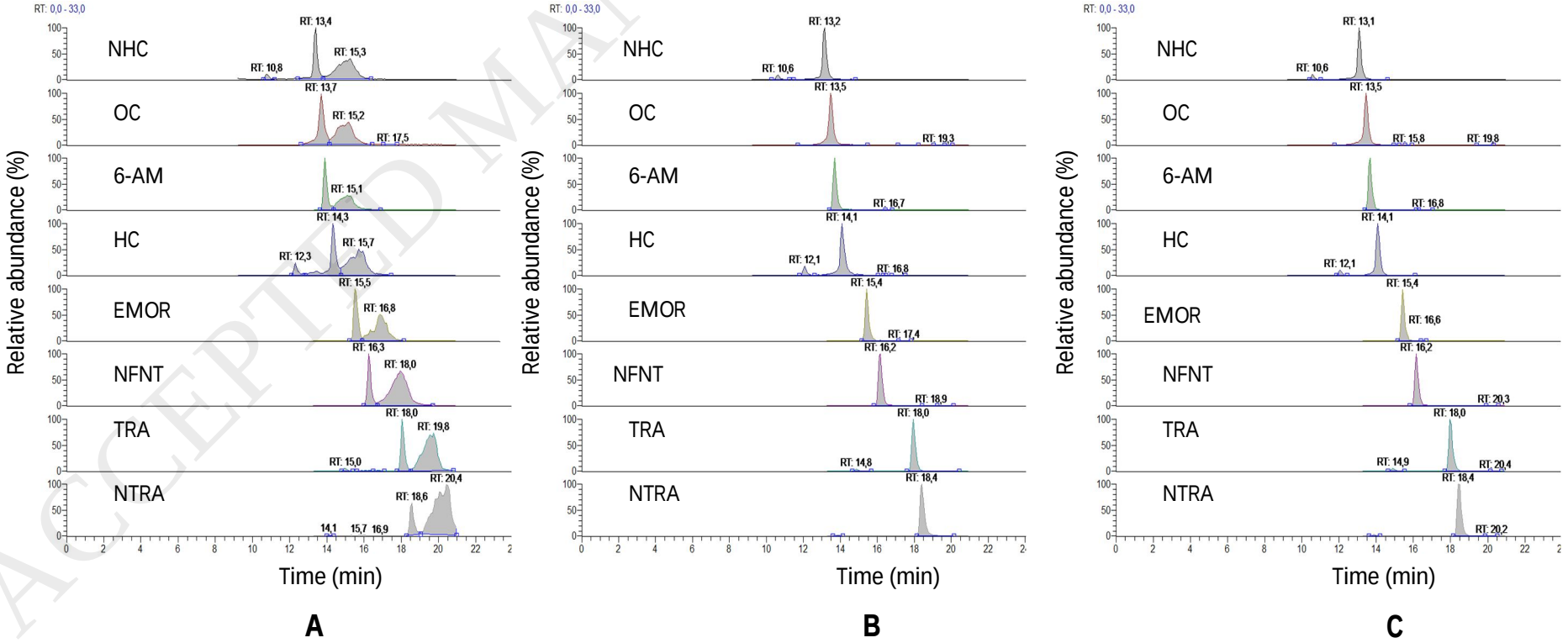


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Figure 3. Stability of opioid analgesics (spiking level: 1 µg/L, n=3) stored on Oasis MCX cartridges and in SPE extracts at -20 °C for 7 and 30 days. For abbreviations of analyzed substances see Table 1.

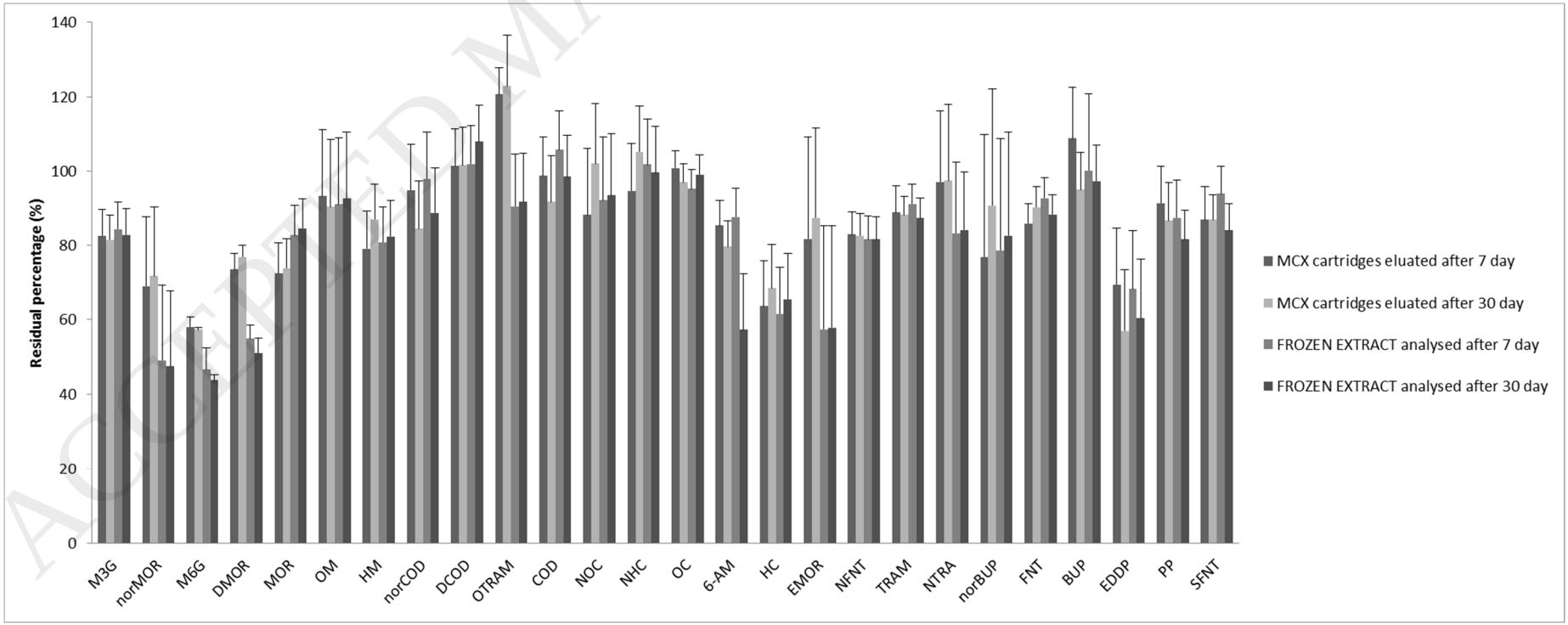

Figure 3. Stability of opioid analgesics (spiking level: 1 µg/L, n=3) during storage after their extraction on to Oasis MCX cartridges and in SPE extracts during 7 and 30 days (T= -20 °C). For abbreviations see Table 1.

Table 1. List of opioid analgesics analysed in this study and the applied LC-MS/MS parameters.

Common name	Abbreviation	Internal standard	RT (min)	Precursor ion (*m/z*)	Product ion 1 (*m/z*)	CE (1) / V	Product ion 2 (*m/z*)	CE (2) / V
Morphine-3-β-D-glucuronide	M3G	M3G-d_3	4.1	462	286	27	201	41
Normorphine	norMOR	MOR-d_3	5.6	272	165	44	201	25
Morphine-6-β-D-glucuronide	M6G	MOR-d_3	6.5	462	286	35	201	36
Dihydromorphine	DMOR	MOR-d_3	6.9	288	185	35	187	31
Morphine	MOR	MOR-d_3	7.4	286	165	42	201	27
Oxymorphone	OM	OM-d_3	8.3	302	284	20	227	29
Hydromorphone	HM	HM-d_3	9.2	286	185	29	157	40
Norcodeine	norCOD	COD-d_3	11.2	286	165	37	225	17
Dihydrocodeine	DCOD	DCOD-d_3	12.2	302	199	27	201	25
O-desmethyl-cis-tramadol	OTRA	DCOD-d_3	12.2	250	58	20	232	12
Codeine	COD	COD -d_3	12.8	300	165	37	215	20
Noroxycodone	NOC	NOC-d_3	13.2	302	284	15	187	23
Norhydrocodone	NHC	NHC-d_3	13.8	286	199	26	183	38
Oxycodone	OC	OC-d_6	14.1	316	298	17	187	27
6-acetylmorphine	6-AM	6-AM-d_3	14.4	328	165	34	211	23
Hydrocodone	HC	HC-d_3	14.8	300	199	28	183	27
Ethylmorphine	EMOR	NFNT-d_5	16.1	314	229	24	183	27
Norfentanyl	NFNT	NFNT-d_5	16.7	233	84	17	177	13
Tramadol	TRA	TRA-O-CD_3	18.5	264	58	14	246	10
N-desmethyl-cis-tramadol	NTRA	NFNT-d_5	19.0	250	44	15	232	9
Norbuprenorphine	norBUP	norBUP-d_6	24.1	414	187	39	211	45
Fentanyl	FNT	FNT-d_5	25.5	337	188	24	105	32
Buprenorphine	BUP	BUP-d_6	25.5	468	396	45	187	45
EDDP[a]	EDDP	EDDP-d_3	26.1	278	234	31	249	23
Propoxyphene	PP	PP-d_5	26.1	340	266	8	58	13
Sufentanil	SFNT	SFNT-d_5	26.5	387	238	19	110	35
Metadone	MTHD	MTHD-d_3	27.2	310	265	14	105	26
Morphine-3-β-D-glucuronide -d3	M3G-d_3	-	4.1	465	289	29	204	38
Morphine-d3	MOR- d_3	-	7.3	289	165	37	201	24
Oxymorphone -d3	OM- d_3	-	8.2	305	287	19	230	27
Hydromorphone -d3	HM- d_3	-	9.2	289	185	32	157	43
Dihydrocodeine -d3	DCOD- d_3	-	12.1	305	201	31	199	32
Codeine -d3	COD- d_3	-	12.7	303	165	37	215	25
Norhydrocodone -d3	NHC- d_3	-	13.7	289	202	29	183	30
Oxycodone -d6	OC- d_3	-	14.0	322	304	20	190	29
6-acetylmorphine -d3	6-AM- d_3	-	14.3	331	165	38	210	24
Hydrocodone -d3	HC- d_3	-	14.7	303	199	32	183	28
Norfentanyl -d5	NFNT- d_5	-	16.6	238	84	25	56	27
Tramadol –O-CD_3	TRA-O-CD_3	-	18.3	267	58	16	249	9
Norbuprenorphine-d6	norBUP- d_6	-	24.1	417	211	43	188	23
Fentanyl -d5	FNT- d_5	-	25.5	342	188	23	105	34
Buprenorphine -d6	BUP- d_6	-	25.6	472	400	45	59	45
EDDP-d3	EDDP- d_3	-	26.0	281	237	28	249	20

Propoxyphene -d5	PP- d_5	-	26.1	345	271	7	58	7
Sufentanil -d5	SFNT- d_5	-	26.6	392	238	19	111	35
MTHD-d3	MTHD- d_3	-	27.1	313	268	12	105	30

CE – collision energy, RT –retention time

[a]2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

Table 2. Method validation parameters for the determination of opioid analgesics in raw wastewater, secondary effluent and river water (spiking level: 1 µg/L for RW, 500 ng/L for SE, 50 ng/L for river water; n=4).

				Raw wastewater (RW)					Secondary effluent (SE)					River water				
	Linearity range	r^2	IDL (pg)	R (%)	Extraction recovery (%)	Matrix effect (%)	Accuracy (%)	MQL (ng/L)	R (%)	Extraction recovery (%)	Matrix effect (%)	Accuracy (%)	MQL (ng/L)	R (%)	Extraction recovery (%)	Matrix effect (%)	Accuracy (%)	MQL (ng/L)
M3G	0.5-500	0.999	0.4	4	90	-25	111	0.3	2	78	-18	109	0.2	3	82	-38	94	0.1
norMOR	0.5-200	1.000	0.2	3	89	-27	102	0.9	4	82	-11	106	0.5	5	81	-25	80	0.2
M6G	0.5-500	0.999	1	1	89	-17	119	0.7	11	73	-9	96	0.4	4	79	-24	82	0.2
DMOR	0.5-200	0.999	0.07	4	92	-15	124	1.0	1	84	-5	119	0.6	4	86	-9	107	0.3
MOR	0.5-200	0.997	0.1	3	95	-21	125	0.9	4	79	-7	109	1.1	5	73	-13	100	0.3
OM	0.5-500	0.999	1	2	91	-16	117	0.3	4	81	-8	114	0.2	3	87	-15	98	0.1
HM	0.5-500	0.999	0.05	4	88	-28	110	2.6	3	81	-8	100	1.7	7	90	-16	98	0.8
norCOD	0.5-500	0.999	0.3	2	92	-12	106	1.8	4	84	5	113	1.1	4	85	-15	75	0.4
DCOD	0.5-500	0.999	0.3	2	93	-1	120	2.8	1	85	4	112	1.5	3	87	2	95	0.7
OTRA	0.5-1500	0.999	0.02	3	97	1	126	0.4	5	79	-6	93	0.2	3	87	3	98	0.1
COD	0.5-500	1.000	0.1	2	91	-7	116	0.4	2	81	-15	102	0.2	3	91	0	83	0.1
NOC	0.5-500	0.998	5	2	89	-9	105	2.5	5	81	8	100	1.5	3	85	-11	91	0.6
NHC	0.5-500	1.000	0.07	2	88	-6	104	1.1	3	84	15	107	0.7	3	92	-16	86	0.3
OC	0.5-500	0.999	1	1	90	4	115	0.4	6	88	10	120	0.2	3	89	-2	98	0.1
6-AM	0.5-500	0.998	0.2	3	93	-11	119	0.4	5	93	8	118	0.2	4	102	-8	94	0.1
HC	0.5-500	0.994	0.08	1	88	-5	107	1.1	3	86	4	101	0.6	4	96	-8	100	0.3
EMOR	0.5-500	0.999	0.02	2	91	-4	115	3.5	1	88	6	120	1.9	11	87	-5	116	0.8
NFNT	0.5-500	0.999	0.05	4	91	2	120	0.8	4	84	20	111	0.5	6	86	-3	94	0.2
TRA	0.5-1500	1.000	0.3	8	63	-4	106	1.2	5	83	-16	79	0.5	4	92	7	101	0.3
NTRA	0.5-1500	1.000	0.05	3	92	0.2	116	1.2	6	87	5	103	0.6	3	87	1	93	0.3
norBUP	0.5-500	0.998	5	4	91	-26	113	0.6	4	86	5	74	0.4	3	87	-13	89	0.2
FNT	0.5-500	0.999	0.04	3	90	-12	114	0.4	6	89	18	120	0.2	7	87	0	97	0.1
BUP	0.5-500	0.995	0.4	1	86	-31	115	0.3	8	87	2	114	0.2	2	84	-10	98	0.1
EDDP	0.5-500	0.999	0.08	15	44	-9	104	0.7	4	116	7	109	0.4	0	109	1	103	0.2
PP	0.5-500	0.999	15	2	79	-19	108	0.6	5	89	14	109	0.5	4	90	-1	99	0.2
SFNT	0.5-500	0.997	0.06	3	89	-4	108	0.8	5	91	27	117	0.5	4	93	-3	98	0.2

MTHD	0.5-500	0.997	0.1	6	79	-8	91	2.6	4	90	24	110	1.7	2	89	3	102	0.7

For abbreviations see Table 1.

IDL - instrumental detection limit. MQL - method quantification limit; R – repeatability

Table 3. Stability of opioid analgesics in raw wastewater sample (spiking level 1 µg/L; n = 3) during sample storage at -20 °C.

	Residual percentage (%)			
	7 days	**30 days**	**70 days**	**100 days**
M3G	40*	38*	1*	0*
norMOR	84*	92*	114*	112
M6G	55*	28*	45*	32*
DMOR	85*	74*	76*	83*
MOR	118*	139*	183*	195*
OM	79*	90*	100	88*
HM	76*	90*	80*	71*
norCOD	83*	103	111	119
DCOD	79*	106	95	85*
OTRA	79	120	130*	96
COD	83	95	105	118
NOC	72*	86	112*	78*
NHC	66*	96	112	97
OC	79*	89*	93	89*
6-AM	70*	59*	29*	14*
HC	78*	79*	83*	60*
EMOR	85*	65	60*	69*
NFNT	75*	50*	60*	53*
TRA	91*	88*	91	59
NTRA	78*	98	96*	76
norBUP	73*	70*	97	78
FNT	72*	84*	87*	70*
BUP	67*	59*	57*	57*
EDDP	82*	103	89*	80
PP	74*	82*	92*	79*
SFNT	76*	79*	87*	73*
MTHD	80*	87*	99	78

For abbreviations see Table 1.

*Significant change (t-test; $p < 0.05$).

Table 4. Occurrence of opioid analgesics in raw wastewater (RW), secondary effluent (SE) and river water in Croatia.

	c (ng/L)							
OA	RW-ST-1[a] (24/08/2016)	RW-ST-2[a] (09/11/2016)	RW-ZG-1[b] (21/03/2017)	RW-ZG-2[b] (24/03/2017)	SE-ZG-1[b] (21/03/2017)	SE2[b] (24/03/2017)	River water 1[c] (23/03/2017)	River water 2[c] (23/05/2017)
M3G	296	370	5.0	4.0	<MQL	<MQL	<MQL	<MQL
norMOR	26	16	21	23	<MQL	<MQL	1.8	<MQL
M6G	65	89	5.0	5.0	<MQL	<MQL	<MQL	<MQL
DMOR	6.2	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
MOR	445	287	148	142	52	47	<MQL	0.3
OM	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
HM	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
norCOD	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	1.6	<MQL
DCOD	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
OTRA	671	298	624	671	859	890	10	7.6
COD	237	625	400	478	289	379	4.0	1.1
NOC	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	2.0	<MQL
NHC	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	2.8	<MQL
OC	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
6-AM	24	30	3.1	1.9	1.0	0.9	<MQL	<MQL
HC	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
EMOR	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
NFNT	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
TRA	717	586	748	752	808	721	23	72
NTRA	189	135	208	214	246	249	5.3	4.2
norBUP	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	1.8	<MQL
FNT	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
BUP	11	3	4.9	7.1	4.1	3.9	<MQL	<MQL
EDDP	175	115	159	148	176	182	1.7	0.9
PP	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL

SFNT	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
MTHD	94	73	71	76	65	64	<MQL	<MQL

For abbreviations of analyzed opioid analgesics (OA) see Table 1.

MQL - method quantification limit; [a]city of Split; [b] Wastewater treatment plant of the city of Zagreb; [c] Sava river