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Preconcentration Strategy for Pharmaceuticals in Wastewater with Liquid Chromatography UV Detection

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Abstract

A preconcentration strategy has been developed for determining a selection of four pharmaceuticals: ketoprofen, naproxen, diclofenac, and ibuprofen, in wasterwater samples (influent and effluent). The method consisted of preconcentration by rotary evaporation and extraction of the pharmaceutical compounds by solid-phase extraction with a C18-sorbent at pH 4. The analytes were then separated and quantified by reversed liquid chromatography-diode array detection. Recoveries of the pharmaceuticals were between 96 and 105 % in an artificial sample, with the exception of ibuprofen (76 %). In a wastewater sample, lower recoveries were obtained (22 % for ibuprofen and 63 % for naproxen) due to interference with the matrix. The precision of the method, calculated as relative standard deviation, ranged between 8.49 to 26.3 % for ibuprofen and between 13.1 to 14.1 % for naproxen. The instrumental limit of detection was between 134 to 236 μg/L. The developed analytical method, combining rotary evaporation with solid-phase extraction, is a faster method to use than the more common approach to only use solid-phase extraction. However, the method needs further development to increase the recovery and precision.

Keywords: Non-steroidal anti-inflammatory drugs (NSAIDs), Wastewater, Rotary Evaporation, Solid-Phase Extraction, HPLC-DAD

1 Introduction

Today a wide range of different pharmaceutical products are used, and they play an important role in modern clinical treatments. Recently, several studies [1–5] have, however, found that residues of pharmaceuticals are also present in the aquatic environment, which has been deemed as reason for concern by for example the American Environmental Protection Agency (EPA) [6].

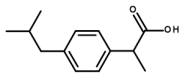
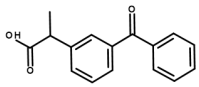
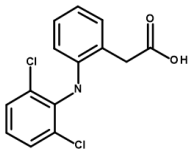
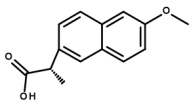
Pharmaceuticals reach the environment through several pathways, but the main one is believed to be through wastewater treatment plants where their removal is not possible by classical methods [7]. The concentrations that are found commonly lay in the ng/L range, meaning that the risks for humans are minimal [7]. Even though the concentrations are very low, there are reasons for concern since the substances are designed to disturb biological processes and can induce side-effects in non-target organisms [8, 9]. Cleuvers [10] could show that even though the biotoxicity of individual non-steroidal anti-inflammatory drugs (NSAIDs) is relatively low, mixtures of them a have significant higher biotoxicity for algaes. Further, Cleuvers [10] in agreement with other research [8] found that the risk for acute toxic effects are small, but that there are risks for chronic effects. With the current tools used in environmental assessment, these effects are often not considered since, the focus there lays on acute toxicity testing [8]. This together with the fact that it is harder to establish evidence for chronic toxic effects means that knowledge in the area still is lacking [7]. It should be noted that acute effects from pharmaceutical products have been reported, but primarily regarding the feminization of fish due to medicine that targets the endocrine system [11].

In order to clean wastewater from pharmaceuticals several different approaches are assessed, which include ultra-filtration membranes [12], ozonation [13] as well as different adsorption techniques [14, 15].

The most used approach for sample preparation for wastewater sample is to use solid-phase extraction (SPE) for both clean up and preconcentration [3, 5, 16–21].

However, due to the low concentrations of pharmaceuticals in wastewater, this technique requires large amounts of water to be loaded into the SPE to get a high enough elution concentration, which can be very time consuming. Thus, this paper investigates the possibility to use rotary evaporation as a first preconcentration step to quantify the amount of four different NSAIDs (ibuprofen, ketoprofen, diclofenac and naproxen) with liquid chromatography (LC) coupled to a diode array detector (DAD) in wastewater from Uppsala, Sweden. As of our knowledge, the combination of rotary evaporation and SPE has not been used before as sample preparation of pharmaceuticals in water. Further, this paper focuses on how the concentrations of the pharmaceuticals are impacted by the regular cleaning process at Uppsala Vatten. Table 1 shows the structures and pK_a-values for the investigated compounds.

Table 1: *Pharmaceutical compounds chemical structures and pK_a-values.*

Compound	Chemical Structure[a]	pK_a	Ref.
Ibuprofen		4.45	[22]
Ketoprofen		4.25	[22]
Diclofenac		4.06	[22]
Naproxen		4.38	[22]

[a] Structures drawn in BIOVIA Draw 2019

2 Experimental

2.1 Chemicals and stock solutions

Acetonitrile (ACN, chromatography grade), methanol (MeOH, hypergrade for LC-MS), ammonia (25% for analysis), and formic acid (80-100% pro analysis) were purchased from Merck Millipore (Darmstadt, Germany). Ammonium formate (AnalaR®) were obtained from BDH Laboratory Supplies (Poole, England). Pharmaceutical standards of ibuprofen (98%), ketoprofen, diclofenac (sodium salt), and naproxen were obtained from Sigma-Aldrich® (Missouri, USA).

Stock solutions of the compounds were prepared in MeOH with concentrations of 2000-2500 mg/L. Standard solutions were prepared from the stock solutions in 25:25:50 (v/v/v) MeOH : ACN : ammonium formate buffer (10 mM, pH 4) with the concentrations 23-31 mg/L and one mixture containing all four compounds. Test samples were prepared from the standard mixture with the concentration of 0.83-5.5 mg/L of the compounds in ammonium formate buffer. Artificial samples were prepared from the standard mixture solution in tap water with concentrations as expected in the wastewater samples (5 μg/L). All the solutions were stored in the dark.

2.2 Sample collection and pre-treatment

Samples were collected by spot checking at Uppsala Vatten Kungsängenverket 2022-10-05 between 10.00-11.00. All samples had a total volume of 2.5 L and were taken from both inlets to the wastewater treatment plant (one for the northern part, AB, and one for the southern part, C, of Uppsala) as well as the outlet. The samples were stored in the dark in plastic containers and acidified with formic acid to a pH of circa 2.5. After five days the samples were filtrated through cellulose filters (OA-filter, Stora Kopparberg - Grycksbo Pappersbruk, Sweden).

2.3 Sample preparation

The sample preparation was initialised by adjusting the pH of the sample to circa 4 (pH 3.95-4.05) with ammonia. 500 mL of the sample were put on rotary evaporation in a water bath at 80 °C (77.3 - 82.5 °C) during 35 minutes until approximately 10 mL of the initial volume were left. The pH of the solution was then checked and if needed adjusted to pH 4 (pH 3.95 - 4.05) with ammonia and formic acid. The sample was then transferred to glass centrifuge tubes and centrifuged during six minutes at 4500 rpm. In the next step of the preconcentration, SPE was used with only the supernatant from the centrifugation. The cartridge (Bond Elute LRC-C18 100mg/10mL, Agilent Technologies, California, USA) was conditioned with methanol and equilibrated with ammonium format buffer (10 mM, pH 4). 5 mL of sample were loaded and then washed with 1 mL MilliQ water and 1 mL of a solution with 75 % ammonium formate buffer and 25% organic phase consiting of 50% methanol and 50% acetonitrile. The rest of the sample was then loaded and the washing repeated. Elution was done with 3 mL 25 % ammonium formate buffer and 75 % organic phase consisting of 50 % methanol and 50 % acetonitrile. The flow rate was around 1 mL/min. The obtained eluate was homogenised by shaking before measurement.

2.4 HPLC-DAD analysis

A JASCO HPLC system with a Agilent 1100 diode array detector was used for the measurement. A 3 x 100 mm reversed phase 5 μm column (ACE® Equivalence 5 C18) was obtained from Avantor®. Isocratic eultion was used with the optimal mobile phase of 25:25:50 (v/v/v) of ACN, MeOH and ammonium formate buffer (10 mM, pH 4) and a flow rate of 0.8 mL/min. 20 μL was used as injection volume. Three wavelengths were found to be suitable for detection of the compounds; 258 nm (ketoprofen), 230 nm (naproxen), and 220 nm (diclofenac and ibuprofen).

2.5 Calibration

As calibration method, external calibration was used. Calibration solutions were prepared from the standard mixture in the range 5-7600 μg/L in ammonium formate buffer (10 mM, pH 4). A calibration curve was constructed for each compound from triplicate injection of the calibration solutions. The calibration curves were used to find the linear range, and instrumental limit of detection (LOD) and quantitation (LOQ). The LODs and LOQs were calculated as three and ten times the standard deviation of the residuals divided by the slope, respectively. In the beginning of each day, a calibration solution was measured to confirm the HPLC-DAD performance.

2.6 Method development

For method development, an optimisation of the isocratic separation and solid phase extraction was carried out. In the chromatographic separation, the retention factor was adjusted by modifying the composition of the organic solvent and water in the mobile phase. The tested concentrations were 60:40 and 50:50. The organic phases of these compositions were ACN and ACN:MeOH (50/50 v/v), respectively. This composition allowed a better separation of all analytes. A buffer solution of ammonium formate at pH 4 was used. For the SPE extraction, elution solutions from 10 % to 90 % organic phase were tested. From these experiments, 75 % organic phase solution was selected for elution. From the same experiments it was concluded that 25% of organic phase was suitable for washing.

2.7 Method validation

For method validation, SPE reproducibility, losses in rotary evaporation, recovery of the whole method, and instrumental and method precision were employed. The reproducibility of SPE was made by doing the SPE procedure (equal loading and elution volume) three times on a test sample containing all the four compounds. The relative standard deviation (RSD) was calculated for the compounds and compared. For losses in the rotary evaporation, a standard mixture of the compounds was used. Measurements before and after the rotary evaporation were made. Equation 1 was used to detect if losses of the compounds occur, where C_{before} and C_{after} is concentration before and after rotary evaporation, respectively.

$$Diff(\%) = \frac{C_{after} - C_{before}}{C_{before}} \times 100 \qquad (1)$$

For recovery of the whole method, an artificial sample as well as real spiked sample was used on the rotary evaporation and SPE. The artificial samples were also spiked with 4-7 μg/L of the compounds. Recovery was calculated with Equation 2, where C_{spiked} and $C_{unspiked}$ are the concentration of the artificial sample spiked and unspiked, and C_{spike} is the concentration of the added spike to the sample.

$$Recovery(\%) = \frac{C_{spiked} - C_{unspiked}}{C_{spike}} \times 100 \qquad (2)$$

The instrumental precision was assessed by injecting each standard for the calibration curve three times and calculating the RSD. To obtain the precision of the method, three sample preperation procedures were made for each sample.

3 Results and Discussion

3.1 Linearity, LOD, and LOQ

To find the linear range for the compounds, calibration solutions containing all four compounds, in the range 5-6400 μg/L were measured in a random order. From the calibration curve, instrumental LOD and LOQ were calculated. Table 2 shows the obtained linear equation, R^2, linear range, LOD, and LOQ for the four compounds. The R^2-values show a high linear correlation for all the compounds (>0.99) over a broad range (around 400-6000 μg/L). The LOD and LOQ varies between the compounds, where ketoprofen has both the lowest LOD and LOQ (134 μg/L, 447 μg/L) and diclofenac has the highest (236 μg/L, 788 μg/L). These LODs and LOQs are in the range as reported in another study of these compounds with HPLC-DAD [23]. LOD and LOQ are very sensitive to matrix and changes in the conditions, so further investigation of these factors can be done using these estimates as a starting point. It must be noted also that depending on the estimation approach it might be possible to have different limit of detection estimates [24].

3.2 Method validation

As an attempt to see if the rotary evaporation results in losses of the compounds, a standard mixture of the compounds with a concentrations of the analytes between 24.4 - 31.7 mg/L was used. Measurements of the mixture before and after rotary evaporation was employed for comparison. Figure 1 shows a plot of the percentage difference in concentration before and after the rotary evaporation (Equation 1) for the four compounds.

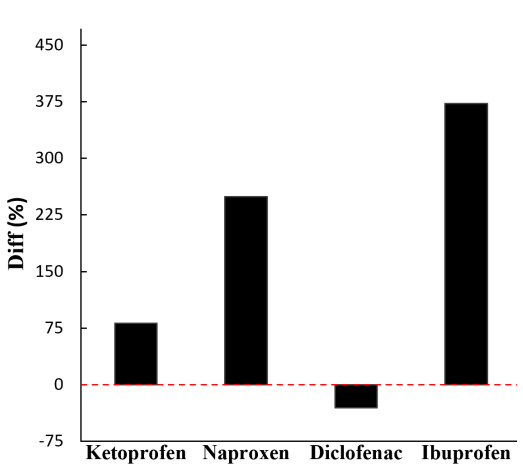


Figure 1: *Difference in percent between before and after rotary evaporation of standard sample mixture of ketoprofen, naproxen, dichlorfenac, and ibuprofen (26-40 mg/L of compounds).*

Table 2: Linear equation, R^2, linear range, LOD, and LOQ for ketoprofen, naproxen, diclofenac, and ibuprofen.

Compound	y=ax+b	R^2	Linear range (μg/L)	LOD (μg/L)	LOQ (μg/L)
Ketoprofen	y=0.108x+13.6	0.9996	384-6400	134	447
Naproxen	y=0.509x+65.2	0.9993	460-5720	167	557
Diclofenac	y=0.105x+50.3	0.9992	612-7610	236	788
Ibuprofen	y=0.0655+5.16	0.9988	402-5000	193	645

In Figure 1, it can be seen that all compounds increases in concentration after the rotary evaporation except diclofenac. It is expected to see an increase in concentration after rotary evaporation due to the decrease in solvent volume. The decease in concentration for diclofenac could be due to a much lower solubility in aqueous solvent (a magnitude lower compared to the other compounds) [25], which could be seen as precipitation in the remaining solution after the rotary evaporation.

The other compounds (ketoprofen, naproxen, and ibuprofen) all show higher concentrations after the rotary evaporation. The sample was concentrated about six times on the rotary evaporator, thus, it is expected that the concentration of the compounds should be six times higher than before the rotary evaporation. This was not the case for any of the compounds. Ketoprofen had a concentration closes to six times the original concentration, but all of them where lower than the expected concentration. This indicated that either losses occur during the process, however, no compounds where detected in the solvent receiving flask from the rotary evaporation, or that they precipitates (as diclofenac probably did) or degrades during the process. Degradation is also a possible reason for the lower diclofenac concentration. However, when using HPLC-DAD, it is hard to detect degradation products, and if degradation occurs, the concentration of them could be too low to be detected.

Ideally, the artificial sample should have been used to see if losses occur in the rotary evaporation since the same losses do not necessarily have to occur for samples with much lower concentrations. The artificial sample, which is constructed to have the same concentration expected in the wastewater sample (more than one-thousandth the concentration in the standard mixture), probability for precipitation of diclofenac should be much lower. However, due to the difficulty of measuring the artificial sample without concentrating it, it was not possible to use the artificial sample for looking at potential losses. Further studies should be carried out to find out about possible degradation and stability of the compounds and which compound(s) that is precipitating at the higher concentrations, and if this also occurs at lower concentrations.

The reproducibility test of the SPE was made by using a test sample containing all compounds and doing the SPE procedure but, with loading and eluting in the same volume. The RSDs were calculated for all compounds. All RSDs (%) for the four compounds were under 5 % (n=3) for all analytes except for diclofenac (RSD 47 %, n=3). This shows a good reproducibility of the SPE procedure for all compounds except diclofenac. The reason for the lower reproducibility of diclofenac could be due to its pK_a being very close to the ammonium formate buffers pH, thus, it could be partly charged and not be evenly retained by the C18-sorbent. The washes were measured from the SPE procedure and no compounds were detected, however, the eultion after the loading of the sample was not measured. Thus, it is not known if compounds gets eluted during the loading step which will affect the reproducibility.

The selectivity of the SPE is known to have a significant effect on the results, both in terms of preconcentration and isolation [26]. C18 is also known to be the least selective sorbent type [27]. An alternative option are hydrophobic–lipophilic balance (HLB) SPEs, which were used by for example De Santiago-Martín et al. [4] for a similar application. HLB SPE have been reported to be well suited for complex samples and, retain polar and non-polar acidic and basic analytes [28]. Further, they are less affected by over-drying the sorbent, resulting in a more robust method [29]. Even though the use of HLB-SPE cartridges has some advantages it is known that the recovery is dependant on the salt concentration in the sample [2]. The usage might therefore introduce other sources of error even though it might be better suited to obtain higher reproducibility for diclofenac as well.

Recovery was evaluated on a spiked artificial sample and calculated with Equation 2. Table 3 shows the obtained recoveries. As it can be seen, a good recovery was obtained for all compounds (96-105 %) except ibuprofen, which had a recovery of 76 %. The reason could be that the ibuprofen peak height is much lower than the other ones in the chromatography, thus, making it more difficult to integrate.

The precision of the instrument was determined by injecting the calibration solutions three times and calculate the RSD (%). The obtained RSD varies between 0.56 and 5.2 % for the calibration solutions and compounds. This indicates a good precision of the instrument.

Table 3: Recovery (%) of ketoprofen, naproxen, diclofenac, and ibuprofen from an artificial sample.

Compounds	Recovery (%)
Ketoprofen	105
Naproxen	105
Diclofenac	96
Ibuprofen	76

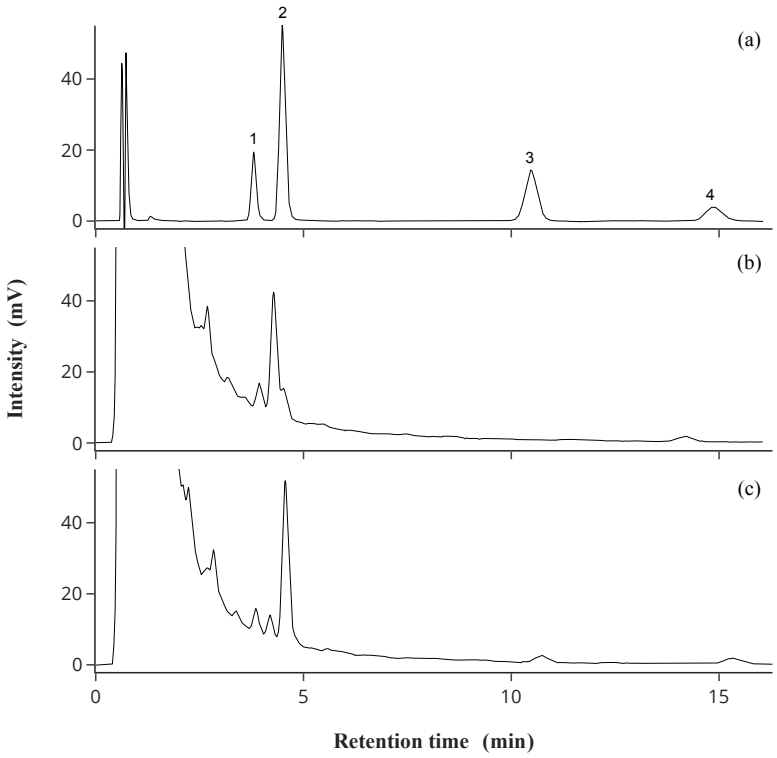


Figure 2: *Chromatograms for (a) calibration solution of (1) ketoprofen 646 μg/L, (2) naproxen 577 μg/L, (3) diclofenac 768 μg/L, and (4) ibuprofen 505 μg/L; (b) influent C sample; and (c) spiked influent C sample. Wavelenght: 220 nm.*

3.3 Wastewater samples

Figure 2 shows chromatograms of a calibration solution, influent C sample and spiked influent C sample at 220 nm. The compounds are eluting in the order: ketoprofen, naproxen, diclofenac, and ibuprofen, which is easiest seen for the calibration solution.

In Table 4 the concentration of the analytes in the sample can be found. The abbreviation ND (not detected) is used in the table for several compounds and samples. Not detected is meant as under the instruments detection limit, whereas <LOD is when a peak is observed under the methods detection limit. Below LOQ means that the concentration is above the LOD, but not high enough to be quantifiable.

It can be seen in the table, that in the case of naproxen and ibuprofen, a lower concentration of the analytes are found in the effluent compared to the influent, whereas the other analytes cannot be detected above the LOD in neither influent nor effluent. Ibuprofen and naproxen exhibit the highest concentrations in the influents, while ketoprofen and diclofenac might be present, but below the instruments detection limit. Ibuprofen is present above the LOD, but below LOQ, which means that no concentration can be reported. Both inlets were compared but no significant difference on the 95% confidence level of naproxen's concentration could be found (p-value 0.13, four degrees of freedom). The inlets, however, differ in additional peaks found in the chromatogram that are not one of the analytes of interest. In the AB inlet two additional peaks were found, whereas only one could be found in the C inlet. All extra peaks eluate at a similar retention time as naproxen, and absorb at 220 nm. These peaks could not be identified, but further experiments with mass spectrometry could provide possible candidates.

Table 4: *Detected amounts of ketoprofen, naproxen, diclofenac, and ibuprofen in wastewater samples (effluent, influent AB, and influent C) after correction for preconcentration. Values displayed as average of three sample preparations with the standard deviation.*

Sample	Effluent	Influent AB	Influent C
Ketoprofen	ND	ND	ND
Naproxen	<LOD	4.94 ± 0.70 μg/L	6.12 ± 0.80 μg/L
Diclofenac	<LOD	ND	ND
Ibuprofen	ND	<LOQ	<LOQ

ND = not detected, <LOD = peak observed under LOD

For several wastewater treatment plants throughout Sweden, the Swedish Environmental Research Institute (IVL) [30] have reported the average influent water concentrations for ketoprofen, naproxen, diclofenac, and ibuprofen of 3.0, 7.3, 0.37 and 7.5 μg/L, respectively. These concentrations might resemble in some extent the results found in the present study, where naproxen and ibuprofen are in greater concentrations. In the case of diclofenac, low selectivity of this method could have affected the detection due to low retention in the column and low solubility after the concentration step. Several factors could have affected the final concentrations in the wastewater sample including analyte degradation, time of sampling, and seasonal variation. It should be noted

that the samples taken here do not give a representative picture of the amount of the studied pharmaceuticals in the wastewater of Uppsala, since they are random spot samples and thereby only represent the content at a certain time and day. It is therefore, not possible to draw any conclusions about if the wastewater in Uppsala is more or less affected by pharmaceuticals in wastewater compared to other cities.

As mentioned above, the effluent exhibited lower concentrations of the studied NSAIDs. However, due to the fact that the samples were spot samples taken at only one point in time the influent and effluent can not be compared, since they are not done on the same water. Meaning, that from the type of sampling employed here it is not possible to draw any conclusions on the efficiency of the water treatment as any differences simply can be attributed to that the sample differed. To be able to judge the treatment as well as obtaining representative picture of the concentration of pharmaceuticals in the wastewater, samples that are pooled over a certain time period (at least 24 hours) are needed.

3.4 Recovery of wastewater samples

Recovery was evaluated in the influent C sample. As naproxen and ibruprofen are the only compounds found in the influent, recoveries in the sample can only be discussed for these compounds. The recoveries for the influent C sample are 22 % and 63 % for ibuprofen and naproxen, respectively. The recoveries reported here are low, and there are several possible explanations for this. Asimakopoulos et al. [1], who also used rotary evaporation as a preconcentration method for wastewater (but did not combine it with SPE), reported relatively low recoveries for some compounds. They obtain recoveries ranging from 44-127%, but since they look at different analytes than described here no direct comparison can be made. Asimakopoulos et al. [1] suggests that the low recoveries are due to heat degradation, which could be the case here as well. Like explained previously, degraded products are very difficult to detect with UV-detection. However, since the artificial sample did not give any indications of degrading as it gave high recoveries, it makes this explanation less likely.

An alternative explanation for the observed decrease in recovery is related to the water solubility of the analytes. All analytes are mostly non-polar and have a limited solubility in water. During the rotary evaporation, the sample volume is rapidly decreased resulting in an increase of the analyte concentration. This process can lead to problems regarding the solubility, especially since the volume decreases very rapidly at the end of the process. This might lead to precipitation of the analytes, which then would have been removed from further sample preparation by centrifugation. When performing the analysis with a artificial sample no such effects could be seen. The real samples however, have a much more complex matrix compared to the artificial samples, which could affect for example the solubility in the rotary evaporation. Another possible effect from the matrix that would decrease the recovery is if a reaction between an analyte and a matrix component occurs.

Another possible explanation for the low recoveries, is that the SPE cartridge is overloaded, meaning that the analytes eluate during the loading phase. Since the total amount of compounds is much higher compared to the artificial samples overloading could be a problem in the samples and can explain the large differences in recovery. Due to limited resources, no repetitions on recovery experiments could be done, which would have been beneficial to explore in more detail the possible causes.

However, since the artificial sample showed a good recovery it indicates that the method is, in principle, working, but that it needs to be developed further to be less affected by the matrix. Several other studies [3, 5, 16] use more complex filtering techniques that remove a larger fraction of other compounds. This could decrease matrix effects and also improve the results seen here. If matrix effects still are a problem, a mass spectrometer as a detector together with isotopically labeled standard for each analyte could provide a solution. The standards can be expected to behave similar e.g. have similar losses in the sample preparation, meaning that the losses can be accounted for. Even though this is an option, it should be noted that the losses still can be problematic due to the risk of obtaining concentrations below the LOD and LOQ. Furthermore, this approach does not provide a way of compensating for the losses with the investigated HPLC-DAD method.

The precision of the method was calculated as RSD (%) for the detected compounds in the influents. The precision ranged between 8.49 to 26.3 % for ibuprofen and between 13.1 to 14.1 % for naproxen. Thus, this is in the same range as reported by Asimakopoulos et al. [1]. But as stated above for recovery, the method needs to be developed further, which should result in a higher precision.

The sample preparation method presented here, combining rotary evaporation with SPE, as already mentioned, needs to be further optimised since the recovery and the precision of the compounds is quite low in the wastewater samples. Also, it is not known if the compounds degrades or not during the rotary evaporation. However, the method still has an advantage over only using SPE, that is the lower time consumption. The rotary evaporation was run for 35 minutes, to get a volume of about 10 mL. To do the SPE, it takes around 20 minutes, with loading 10 mL and eluting in 3 mL. It is common to use 100 mL or more to load to the SPE, to get a high enough concentration in the eluent [3, 18–20]. If a flow rate of around 1 mL/min is used, as in this study, it would take over 100 minutes to just load the sample. Thus, a much faster sample preparation time can be achieved by first using rotary evaporation to decrease the sample volume and then loading a lower volume to the SPE.

4 Conclusion

The developed method, based on rotary evaporation and solid-phase extraction followed by HPLC-UV analysis, was applied to wastewater of a treatment plant for the simultaneous quantitation of the pharmaceuticals: ketoprofen, naproxen, diclofenac, and ibuprofen. The studied chromatography separation and SPE conditions allowed recovery rates between 96 and 105 %, with the exception of ibuprofen (76%) in an artificial sample. However, in the wastewater sample, the recoveries were much lower (22 % for ibuprofen and 63 % for naproxen), which is due to matrix effects since the samples could not be cleaned enough by filtration before the rotary evaporation. The instrumental limit of detection LOD ranged from 134 to 236 μg/L. The presented preconcentration strategy is faster than the more common procedure with only SPE, but needs further optimisation to better account for matrix effects.

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