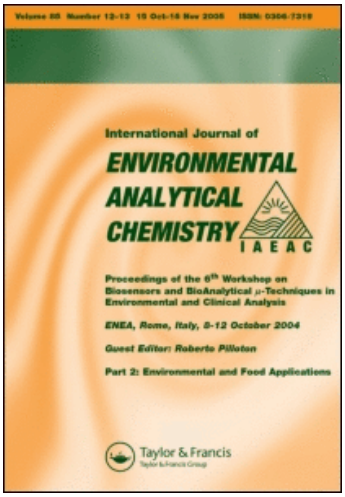

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Determination of selected non-steroidal anti-inflammatory drugs in wastewater by gas chromatography-mass spectrometry

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An analytical method for the simultaneous determination of the four most detected non-steroidal anti-inflammatory drugs (NSAIDs) in wastewater, namely ibuprofen (IBF), naproxen (NPX), diclofenac (DFC) and ketoprofen (KFN), was developed based on gas chromatography–mass spectrometry (GC-MS). Extraction from wastewater samples was performed by solid-phase extraction (SPE) with C18 cartridges, while meclofenamic acid (MFC) was used as surrogate. The optimisation of SPE procedure included the type of the extraction solvent used for the elution and the pH of the loading solution (acidic or neutral). The developed extraction procedure resulted in good repeatability and reproducibility with relative standard deviations (RSDs) less than 10% for all the compounds. Moreover, satisfactory recoveries (>98%) were obtained. Chromatographic analysis was achieved after derivatisation with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethyl chlorosilane (TMCS) in the presence of pyridine, a derivatisation mixture used for the first time for these compounds. The limits of detection (LODs) of the method varied from 0.37 (KFN) to 3.1 ng L^{-1} (NPX). The analytical method was successfully applied for the determination of these compounds in the raw and treated wastewater of two different wastewater treatment plants (WWTPs) of the city of Mytilene in Lesvos, Greece. The maximum concentrations were detected in raw wastewater originated from a hospital and they were 574 ng L^{-1} and 704 ng L^{-1} for IBF and KFN, respectively.

Keywords: wastewater; acidic pharmaceuticals; NSAIDs; SPE; derivatisation; GC-MS

1. Introduction

Nowadays, it becomes increasingly evident that pharmaceutical compounds constitute a group of novel organic emerging pollutants, which are continually introduced into the aqueous environment as a result of their extensive medical use and inefficient removal in wastewater treatment plants (WWTPs) [1]. Among these, ibuprofen (IBF), naproxen (NPX), diclofenac (DFC) and ketoprofen (KFN), classified as non-steroidal

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anti-inflammatory drugs (NSAIDs), share certain analgesic and antipyretic properties and, due to their high annual consumption, present significant research interest worldwide [1]. All of them show potential toxicological impact on aquatic life, with DFC being the most toxic [1,2]. According to literature, their occurrence in WWTPs, at concentrations ranging from $ng\,L^{-1}$ to $\mu g\,L^{-1}$ levels, is well documented [1–8] and their insufficient removal during sewage treatment results in discharge into different environmental compartments. The presence of these compounds has been verified in surface and drinking water [7–9] at concentrations ranging from $<0.1\,ng\,L^{-1}$ to few hundreds of $ng\,L^{-1}$.

Due to the trace levels usually found in the environment, various analytical methods have been developed for the determination of NSAIDs in the above-mentioned environmental matrices. First, a preconcentration step is required, which was usually performed by solid-phase extraction (SPE) after pH adjustment to 2–4 [3,5,9–13]. The majority of the existed extraction methods were based on the use of Oasis HLB cartridges using ethyl acetate [4,10,12,13] or methanol [12,14,15] as elution solvents. C18 cartridges have also been used for the extraction step using mostly methanol as the elution solvent [3,7,12,16,17], while two studies have investigated the extraction efficiency of the target compounds from mixed phase sorbents, like Oasis MCX [8] or Oasis MAX [11].

Regarding the use of C18 cartridges, contradictory results have so far been reported for the achieved absolute recovery of the analytical method, with recovery rates ranging from 67% (IBF) to 76% (DFC) [3], 87% (IBF) to 95% (NPX) [7] and 83% (DFC) to 103% (IBF) [17]. Moreover, contradictory results have been reported for the effect of the pH of the extracted sample. As was already mentioned, most of the studies reported extraction of NSAIDs after pH adjustment to 2–4 [12,13], without further investigation. As referred to in the literature, these substances are acidic compounds with pKa values of 4.15–4.91 and they should be protonated for their efficient adsorption onto reverse-phase sorbents by adjusting pH to 2–3 [13]. Debska *et al.* [17] showed that the extraction of NPX, DFC and IBF onto C18 cartridges were significantly improved after adjustment of the sample pH at 2, whereas the recoveries were <25% at pH 7. Gros *et al.* [14], using Oasis HLB for the SPE, showed that the recovery of IBF was better (100%) after adjusting pH at 2 than without pH adjustment (80%), whereas the behaviour of NPX was exactly the opposite. On the contrary, Thomas and Foster [4] and Rodil *et al.* [15] achieved quantitative recoveries for the studied NSAIDs using Oasis HLB cartridges after pH adjustment at 7. Recently, the first collaborative interlaboratory exercise for the determination of the four selected NSAIDs was performed and 6 out of the 14 participants extracted wastewater and river water samples at neutral pH, using Oasis HLB, with satisfactory results [18].

The chromatographic determination of the target compounds in the extracts is accomplished either by high performance liquid chromatography (LC) [7,12–19] or gas chromatography (GC) [3–6,8–13,20] coupled with mass spectrometry (MS). GC-MS quantitative analysis provides, in general, better sensitivity and lower limits of detection (LODs) when dealing with complex matrices such as wastewater [12]. It is less prone to matrix effects, avoiding signal suppression or enhancement effects that are commonly observed in electrospray ionisation (ESI) based LC-MS analysis [7,12–14,19]. However, there is a need for a derivatisation step prior to GC-MS determination of NSAIDs in order to improve their chromatographic behaviour (increased volatility, decreased polarity) and sensitivity [13]. Silylating agents are widely used nowadays, with the majority of derivatisations applied to NSAIDs to be based on *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and *N*-methyl-*N*-(*tert*-butyldimethysilyl)trifluoroacetamide (MTBSTFA) [12,13,18]. In general, *tert*-butyldimethylsilyl (TBDMS) derivatives were

considered more stable than their trimethylsilyl (TMS) analogues, also providing higher sensitivity [10,13,20,21]. On the contrary, MTBSTFA does not react unambiguously with the sterically hindered groups and the derivatisation reaction is usually not complete due to its lower reactivity [13,20,21].

Therefore, the objective of this study was to develop and validate a simple, precise and sensitive GC-MS method for the simultaneous determination of IBF, NPX, DFC, and KFN in wastewater, using C18 cartridges for SPE. The study was aimed at clarifying the elution efficiency of different organic solvents used for the extraction of NSAIDs and the effect of the pH of the loading solution on the extraction efficiency of the target compounds from wastewater samples using C18 cartridges. Moreover, the efficiency of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethyl chlorosilane (TMCS) and pyridine as derivatisation reagent was tested for the qualitative and quantitative determination of the proposed mixture (IBF, NPX, KFN, DFC and meclofenamic acid) in low levels ($ng\,L^{-1}$) in wastewater. The developed method was applied to wastewater samples collected from various wastewater treatment plants (WWTPs) in the island of Lesvos (Greece), so as to prove its applicability to real samples.

2. Experimental

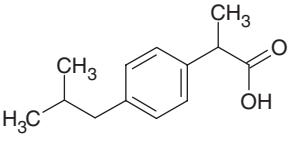
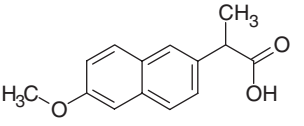
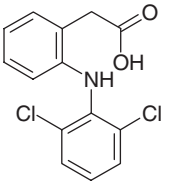
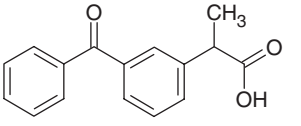
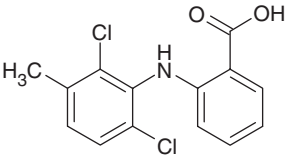
2.1 *Chemicals and standards*

Methanol (MeOH), dichloromethane (DCM) and ethyl acetate were of HPLC grade (Merck, Germany) and were used as received. BSTFA + 1% TMCS and pyridine, used for silylation, were purchased from Supelco (USA) and Carlo Erba-SDS (France), respectively. C18 (Sep-Pak, 6 mL, 500 mg) cartridges were supplied by Waters (Ireland). Analytical standards of IBF, NPX, KFN, DFC and meclofenamic acid (MFC), which was used as surrogate, were supplied by Dr Ehrenstorfer (Germany). All compounds were used without further purification (minimum purity >99%). CAS registry number, molecular weight and chemical structure of the target compounds are given in Table 1. Stock solutions of individual compounds were prepared in methanol at $1000\,mg\,L^{-1}$ and kept at $-18^{\circ}C$. HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (USA).

2.2 *Sampling*

Wastewater samples for the spiking procedure were collected in 1 L pre-cleaned amber glass bottles from the outlet of the WWTP of the campus of the University of the Aegean. An aliquot of the collected samples was analysed before spiking to determine possible background concentrations of these compounds. Following successful development, the method was applied for the determination of the target compounds in influents samples taken from two different WWTPs in the island of Lesvos (City of Mytilene and Hospital of Mytilene) and effluent samples originating only from the municipal WWTP of the City of Mytilene. Sampling was performed in November 2008. After sampling, the wastewater samples were immediately acidified at pH 2.5 and stored at $4^{\circ}C$ in the dark [10]. Extractions were performed within 48 h of their arrival.

Table 1. CAS registry numbers, molecular weights and chemical structures of the target non-steroidal anti-inflammatory drugs (NSAIDs).

Compound	Formula	MW ($g\,mol^{-1}$)	pKa	CAS number	Chemical structure
Ibuprofen (IBF)	$C_{13}H_{18}O_2$	206.28	4.91	15687-27-1	
Naproxen (NPX)	$C_{14}H_{14}O_3$	230.26	4.15	22204-53-1	
Diclofenac (DFC)	$C_{14}H_{11}Cl_2NO_2$	296.15	4.15	15307-86-5	
Ketoprofen (KFN)	$C_{16}H_{14}O_3$	254.28	4.45	22071-15-4	
Meclofenamic acid (MFC)	$C_{14}H_{11}Cl_2NO_2$	296.15	–	644-62-2	

2.3 *Extraction of wastewater samples*

Solid-phase extraction (SPE) was used as the isolation technique of the target compounds from the wastewater samples. The developed procedure is a modification of the previously described method of Gatidou *et al.* [22] used for the isolation of endocrine disrupting compounds from wastewater. Briefly, a volume of 100 mL of a blank wastewater sample was filtered through a pre-ashed (2 h, 60°C) glass fibre filter (GF/F, pore size 0.7 μm, Whatman, Brentford, Middlesex, UK) and spiked with 100 μL of $0.8\,mg\,L^{-1}$ standard solution of the target compounds (for optimisation and recovery studies) and 100 μL of $0.6\,mg\,L^{-1}$ of the surrogate MFC. The spiked samples were mixed in an ultrasonic bath for 10 min to ensure efficient distribution of the compounds in the solution and afterwards they were allowed to equilibrate for 10 min prior to extraction. Extraction was performed using C18 cartridges which were conditioned by 3×2 mL of ethyl acetate, 3×2 mL of

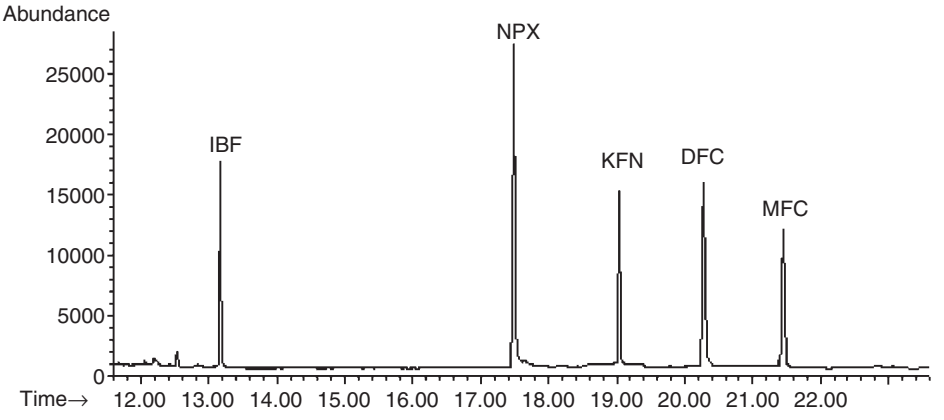


Figure 1. GC-MS-SIM chromatogram of a standard solution containing 800 μg L^{-1} of the target compounds and 600 μg L^{-1} of the surrogate (MFC).

methanol and 3 × 2 mL Milli-Q water at a flow rate of 0.5 mL min^{-1}. The samples were adjusted to pH 2.5 with HCl (2 M) and loaded onto the cartridge with a flow rate of 5 mL min^{-1}. After loading, cartridges were washed with 2 mL of Milli-Q water and then they were dried under vacuum for 60 min. The compounds were eluted with 3 × 2 mL of ethyl acetate at a flow rate 1 mL min^{-1}. The eluates were evaporated to dryness, under a gentle stream of nitrogen. Finally, the dried residues were subjected to derivatisation reaction.

2.4 *Derivatisation procedure*

The dried residues were derivatised by adding 50 μL of BSTFA + 1% TMCS along with 10 μL of pyridene. The vials were closed and vortex mixed for 1 min. The derivatisation reaction was performed at 70°C for 20 min. The derivatives were allowed to cool at room temperature for 10 min and then subjected to GC-MS analysis.

2.5 *GC–MS analysis*

For the analysis of the target compounds a Hewlett Packard Gas Chromatograph 5890 Series II connected to a Hewlett Packard Mass Spectrometer HP5971 MSD was used (Palo Alto, CA, USA). The separation of the compounds was achieved by using a DB5MS capillary column (60 m × 0.32 mM × 0.25 μm, Supelco, USA). The carrier gas was helium (0.9 mL min^{-1}). A sample volume of 1 μL was injected in splitless mode at an inlet temperature of 280°C. The column temperature was programmed as follows: at 80°C for 1 min, from 80 to 248°C at 15°C min^{-1}, 1 min at 248°C and from 248 to 280°C at 3°C min^{-1}. The MS transfer line temperature was maintained at 280°C, whereas the ion source temperature was 180°C. For the qualitative analysis, the full scan mode was used (mass range: 50–400). Quantitative analysis was carried out using selected ion monitoring (SIM) mode. The chromatographic separation of the target compounds in a standard solution is shown in Figure 1. Detailed information of the elution order, retention times and characteristic ions used for GC-MS analysis are shown in Table 2. Most of the selected

Table 2. Retention times and characteristic ions of the derivatised NSAIDs used for GC-MS (SIM) analysis. The underlined ions correspond to the derivatised molecules.

Analyte	Retention time (min)	Target ion (m/z)	Confirmation ions (m/z)
IBF	13.18	160	161, 263
NPX	17.58	185	243, 302, 141
KFN	18.94	282	283, 311
DFC	20.24	214	242, 216, 367
MFC	21.43	242	244, 367

ions of the target compounds are in agreement with those previously reported in the literature for the TMS derivatives of the compounds [8,9,20].

3. Results and discussion

3.1 *Optimisation of the extraction procedure*

The optimisation of the extraction procedure of the target compounds from wastewater samples included the type of the organic solvent for the elution and the pH of the loading solution. Ethyl acetate and a mixture of dichloromethane (DCM)-hexane were separately examined. DCM-hexane was tested because it was successfully used for the recovery of phenolic endocrine disrupting compounds from C18 cartridges in previous study of the group [22]. Methanol, regularly used for the elution of the target compounds from polymeric or C18 cartridges, was not tried due to incompatibility with the following derivatisation procedure and GC determination. The use of DCM-hexane mixture resulted in zero recoveries for the target compounds. In contrast, elution with ethyl acetate improved significantly the obtained absolute recoveries (higher than 78%). The results confirmed the appropriateness of ethyl acetate as the elution solvent. Recoveries were higher than those obtained by Koutsouba *et al.* [3] or Miao *et al.* [16] and similar to those reported by Hernando *et al.* [7] or Debska *et al.* [17], who used C18 cartridges and methanol as the elution solvent for the extraction of the target compounds from acidified (pH 2) wastewater samples.

Then, C18 cartridges were tested at different pH values of the loading solution (2.5, 5.4 and 7) for the extraction of the selected NSAIDs from wastewater samples. Six replicate experiments were performed at each pH value. The results are depicted in Figure 2 and denote that C18 cartridges were appropriate for the extraction of all the tested compounds at a wide pH range (2.5–7), since the absolute recoveries (based on peak area measurements) were higher than 71%. The results indicated strong interaction of the analytes with the C18 sorbent and similar behaviour at different levels of pH, revealing that the reverse-phase retention is dominant. Due to the fact that at pH 2.5, the obtained RSDs were sufficient (<6%) and with respect to the similar relative recoveries (98–107%) achieved for all analytes, this pH value was chosen for the isolation of the target compounds from wastewater samples. Moreover, the chromatograms of spiked wastewater samples at pH values of 2.5 and 7 showed similar matrix peaks, denoting that the matrix substances that co-extracted with the analytes are more or less the same at both pH values. The results of the present study are in agreement with the collaborative interlaboratory study recently published which clearly showed that extraction of NSAIDs at neutral pH resulted in accurate results [18].

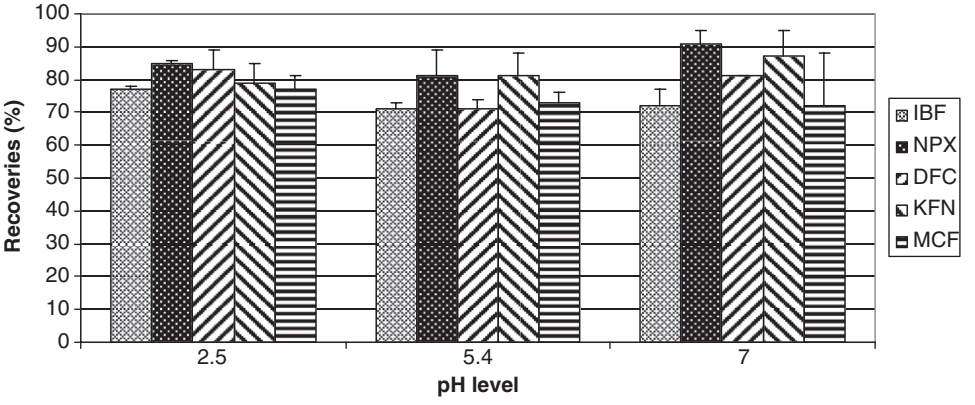


Figure 2. Recoveries of the target compounds from C18 cartridges at different pH values of the loading solution (2.5, 5.4 and 7).

Table 3. Calibration equations, coefficients of correlation (R^2), limits of detection and quantification of the target compounds in wastewater.

Compound	Calibration equation	R^2	Instrumental LOD (pg)	Method LOD (ng L^{-1})	Method LOQ (ng L^{-1})
IBF	$y = 0.0011x - 0.0046$	0.998	14	0.87	2.6
NPX	$y = 0.0021x - 0.2258$	0.994	10	3.1	10
DFC	$y = 0.0016x - 0.0919$	0.997	10	0.77	2.3
KFN	$y = 0.0012x - 0.1127$	0.997	16	0.37	1.1

3.2 *Validation of the method*

An instrument calibration was carried out with meclofenamic acid as internal standard (0.6 mg L^{-1}) for analyte concentrations ranging from 0.05 to 2 mg L^{-1} for each compound in methanolic standard solution, with three replicates per concentration. As shown in Table 3, the response of MS detector was linear for all the target compounds with coefficients of determination, $R^2 > 0.994$. Instrumental LOD was determined as three times the standard deviation of six replicate injections of the lowest concentration standard (50 μg L^{-1}). Instrumental LODs were approximately the same for all the compounds (10–16 μg L^{-1}).

The method LOD of each compound was determined as three times the standard deviation of five independent replicate extractions of a wastewater sample (100 mL) containing all the analytes at low concentrations (1.4 (DFC) to 65 (NPX) ng L^{-1}) and spiked with 0.6 μg L^{-1} of MFC. Limits of quantification (LOQs) were determined as 3.3 times of method LODs. According to the results (Table 3), the obtained LODs ranged from 0.37 ng L^{-1} (KFN) to 3.1 ng L^{-1} (NPX), whereas the LOQs varied from 1.1 ng L^{-1} (KFN) to 10 ng L^{-1} (NPX). The achieved LODs and LOQs are adequate for environmental monitoring of the target compounds and low enough, taking into account the complexity of the matrix and the relatively low volume of the extracted sample.

Table 4. Precision and recovery data of the analytical method developed for the determination of selected NSAIDs in wastewater samples.

	Wastewater extraction		
Compound	Intra-day precision RSD (%), ($n=5$)	Inter-day precision RSD (%), ($n=3$, $k=3$)	Recoveries (%) ($n=6$)
IBF	1.6	4.3	98.5 ± 3.6
NPX	1.6	8.0	107 ± 6
DFC	3.9	9.4	106 ± 3
KFN	4.3	7.9	99.8 ± 5.5

Additionally, the achieved LODs in the present work were lower than or similar to those previously obtained in studies with GC–MS [8,12,13]. The linearity of the method was also tested in the range of 0.01 $\mu g\,L^{-1}$ to 0.8 $\mu g\,L^{-1}$ in spiked wastewater samples at six different levels. The regression coefficients exceeded 0.99 for the calibration curves of all the compounds and consequently the method was found to be linear within the tested concentration ranges, at levels usually encountered in most of the wastewater samples.

For repeatability experiments, five replicates of a wastewater sample containing all the analytes, at concentrations ranged between 50 (DFC) to 704 (KFN) $ng\,L^{-1}$, were analysed during one day ($n=5$, intra-day precision). For reproducibility experiments three replicates ($n=3$) of a spiked sample (0.8 $\mu g\,L^{-1}$) were analysed at three different days ($k=3$) over a period of one week (inter-day precision). The results have shown satisfactory intra and inter-day precision of the analytical procedure with RSDs values less than 10% for all the compounds (Table 4).

In order to evaluate the trueness of the method, recovery experiments were performed. To accomplish this, a blank wastewater sample (100 mL) was spiked with 0.8 $\mu g\,L^{-1}$ for each compound with 0.6 $\mu g\,L^{-1}$ of MFC (IS). The recoveries ranged from 98% (IBF) to 107% (NPX) (Table 4), indicating the good accuracy of the developed extraction method and the advantage of the applied surrogate.

3.3 *Application of the analytical method to wastewater samples*

The analytical method developed in this study was applied to wastewater samples in duplicate, sampled from two different WWTPs in order to monitor the presence of the target compounds. The average sewage flows in the WWTPs included in this study varied from 120 for the hospital WWTP to 4500 $m^3\,d^{-1}$ for the WWTP serving the city of Mytilene, while both of them were equipped with secondary treatment (activated sludge process). A typical SIM chromatogram of an extract of a polluted influent wastewater sample from the hospital WWTP is shown in Figure 3(a), whereas the respective chromatogram of a spiked sample is presented in Figure 3(b). The range of concentrations detected in each sampling point is given in Table 5. Regarding the untreated sewage, NPX, IBF and KFN were detected in all samples, whereas DFC was not found in two samples. The maximum concentration of NSAIDs was determined for KFN (704 $ng\,L^{-1}$) and it was detected in the influent wastewater of the Hospital WWTP (Table 5). This observation

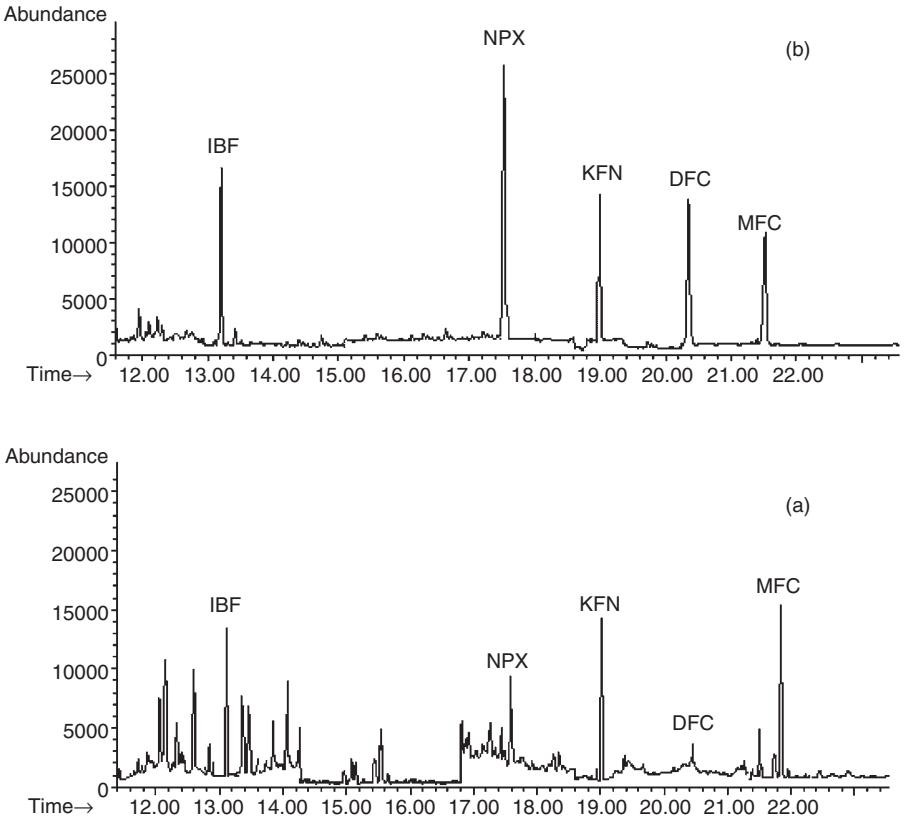


Figure 3. (a) Chromatogram in SIM mode of the extract of an influent wastewater sample from the hospital WWTP, containing 574 ng L^{-1} of IBF, 117 ng L^{-1} of NPX, 704 ng L^{-1} of KFN and 46 ng L^{-1} of DFC, plus 600 ng L^{-1} of MFC (surrogate); (b) Chromatogram in SIM mode of the extract of a spiked wastewater sample from the University WWTPs, spiked with 800 ng L^{-1} of the target compounds and 600 ng L^{-1} the surrogate.

Table 5. Levels of the target compounds in nine wastewater samples from different WWTPs of Mytilene, Lesvos, Greece.

	WWTP of the city of Mytilene		WWTP of the hospital of Mytilene
Compound	Influent (ng L^{-1})	Effluent (ng L^{-1})	Influent (ng L^{-1})
IBF	96–403	5–262	254–574
NPX	219–302	21–253	37–245
DFC	<LOQ–117	<LOQ–84	46–186
KFN	36–97	39–83	384–704

indicates the wide use of NSAIDs in medical practice. In most cases, concentrations of NSAIDs in effluent wastewater were lower than those detected in influent wastewater, indicating the ability of WWTP to partially remove these compounds. The concentrations of target compound detected in untreated and treated wastewater were similar or lower

than those previously reported by other authors worldwide [1]. The concentrations of all the compounds in municipal influents were lower than those found in Switzerland [5], Tokyo, Japan [6], Hungary [20], Galicia, Spain [23] and Mexico [24]. However, the concentrations of the target compounds in municipal effluents were higher than those found in Northern Virginia, USA [4] and in Marseilles, France [8] and only lower than those levels found in Hungary [20]. Similar levels of the selected NSAIDs were found in influent and effluent wastewater samples from five WWTPs of Croatia [14].

4. Conclusions

A simple, accurate and precise method was developed for the simultaneous determination of IBF, NPX, KFN and DFC in wastewater samples, using meclofenamic acid as surrogate. Derivatisation was successfully performed by using BSTFA + 1% TMCS together with pyridine. Sufficient isolation of all the compounds from the wastewater samples was obtained using C18 cartridges and ethyl acetate as the elution solvent. The investigation of the effect of sample pH on the extraction efficiency revealed a relatively strong retention of the analytes onto the C18 sorbent with similar behaviour at different pH levels (2.5–7). The method validation proved the satisfactory precision and recoveries for all the target compounds and adequate low method LODs for environmental monitoring, taking into account the complexity of the matrix and the low extracted volume of the sample. The analytical method was successfully applied to the analysis of the studied compounds in two different WWTPs in Lesvos Island (Greece). The highest detected concentrations were found in influent wastewater from the hospital WWTP. Further sampling campaigns are needed to investigate the removal efficiency of these compounds and study possible daily or/and seasonal variations in their concentration levels.

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