SPECIAL GUEST EDITOR SECTION

Simultaneous Determination of Four Nonsteroidal Anti-Inflammatory Drugs and Three Estrogen Steroid Hormones in Wastewater Samples by Dispersive Liquid–Liquid Microextraction Based on Solidification of Floating Organic Droplet and HPLC

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Background: **Nonsteroidal anti-inflammatory drugs (NSAIDs) and estrogen steroid hormones (estrogens) are pharmaceuticals intensively studied in environmental analysis due to their toxic effect on animal and human beings.** ***Objective:*** **Development of a simple, fast, and sensitive extraction method for the simultaneously analysis of four NSAIDs (ketoprofen, naproxen, ibuprofen, and diclofenac) and three estrogens (17ß-estradiol, 17α-ethynylestradiol, and estriol) from wastewater samples.** ***Method:*** **Dispersive liquid–liquid microextraction based on solidification of floating organic droplet followed by HPLC analysis with UV detection was developed. The influence of the main extraction parameters, e.g., the volume of extraction solvent and of disperser, the pH, and the ionic strength of sample were evaluated.** ***Results:*** **Good resolutions between the selected drugs were obtained using a reverse-phase column and a mobile phase of acetonitrile and water. This method provides good linearity (*r* > 0.999) in a concentration range of 1–100 µg/mL, good intra- and inter-day precision (RSD <7%) and low LOQs. The obtained enrichment factors were ranged between 162 and 180 for NSAIDs and between 118 and 185 for estrogens. The relative recoveries were situated >80% for all analysed drugs, except estriol (59%) both in synthetic and real wastewater samples.** ***Conclusions:*** **The developed method has been successfully applied for the analysis of the selected drugs in wastewater samples collected from the influent of a wastewater treatment plant.** ***Highlights:*** **Four NSAIDs and three estrogens from wastewater samples were simultaneously extracted and analysed using only 10 mL of sample and 50 µL of extraction solvent.**

Nonsteroidal anti-inflammatory drugs (NSAIDs) and estrogen steroid hormones (estrogens) are classes of pharmaceuticals intensively studied in environmental analysis as a result of their large use for the human and animal treatments. Having a toxic effect on many animal species and human beings, the occurrence and the fate of the residues of these drugs in the aquatic environment have attracted considerable attention in the recent years (1, 2).

Taking into consideration the negative effects of these drugs, the European Parliament amended Directives 2000/60/EC and 2008/105/EC (3) regarding the priority substances in the field of water policy and introduced diclofenac, 17α-ethynylestradiol, and 17ß-estradiol as emerging pollutants in the surface waters. Even if the occurrence of these drugs in the environmental compartments is in low quantities, they must be monitored and, therefore, the development of specific and sensitive methods of analysis is required.

Usually, the determination of these drugs in environmental samples involves a preconcentration step consisting of their extraction performed by different methods, e.g., solvent extraction (4–6), solid-phase extraction (7–13), assisted extraction (9, 14), or of late years, solid-phase (15–19) and liquid-phase (20–27) microextraction techniques, followed by their analysis by GC (4–7), LC (1, 7, 21–27), or micellar electrokinetic chromatography (20).

The liquid-phase microextraction (LPME) techniques, the dispersive liquid–liquid microextraction (DLLME) and DLLME based on solidification of floating organic droplet (DLLME-SFO) have become the most attractive alternative solvent-minimized sample preparation techniques which are applied for environment, food, and pharmaceutical samples. Their great advantage consists in using a few microliters of solvent to extract a wide variety of analytes from complex matrices. These techniques are much simpler and faster and have enrichment factors comparable with or better than the traditional LLE techniques (28).

According to our knowledge, even using the quick, easy, cheap, effective, rugged, and safe method (29), the NSAIDs and estrogens are analyzed separately, which means specific extraction and analysis methods for each of these drugs, and thus increasing working time and amount of waste (21–27).

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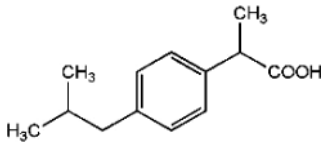
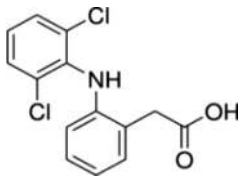
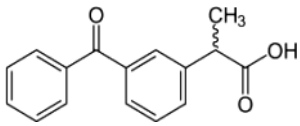
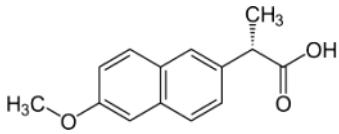
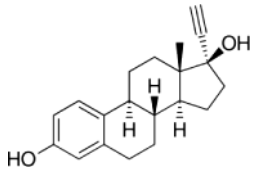
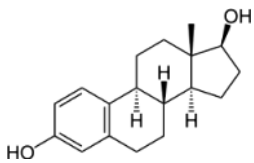
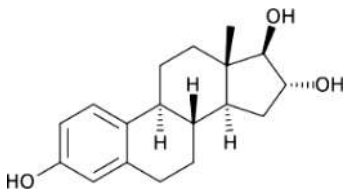
Table 1. Abbreviation and physicochemical properties of studied drugs

Name	Abbreviation	Structural formula	LogP	pKa
Ibuprofen	IBU		3.97	5.03
Diclofenac	DIC		4.51	4.15
Ketoprofen	KET		3.12	4.45
Naproxen	NAP		3.18	4.15
17α-Ethynylestradiol	EE2		3.67	10.21
17β-Estradiol	E2		4.01	10.08
Estriol	E3		2.45	10.54

The aim of this study was to develop a DLLME-SFO method followed by HPLC-UV for the simultaneous analysis of four NSAIDs and three estrogens in wastewater to reduce the working time and the amount of solvent used for analysis. The developed DLLME-SFO–HPLC-UV method was successfully applied to the analysis of these drugs in an influent of a wastewater treatment plant.

Materials and Methods

Reagents and Standards

For the proposed experiments, four NSAIDs (diclofenac, ibuprofen, ketoprofen, and naproxen) and three estrogens (17α-ethynylestradiol, 17β-estradiol, and estriol; Table 1) with >98% purity were purchased from Sigma-Aldrich (Paris, France).

A stock solution of 100 μg/mL of each drug was prepared in a mixture of acetonitrile and Milli-Q water (1+1, v/v) and then was stored at refrigerator at 4°C being stable for a long period of time. Acetonitrile of HPLC grade, 1-undecanol, NaCl and ortho-phosphoric acid were obtained from Merck (Darmstadt, Germany). Milli-Q water of 18.2· MΩ×cm resistivity was prepared using a Milli-Q-Plus ultrapure water system (Millipore, Milford, MA). Polyvinylidene difluoride (PVDF) syringe filters of 0.45 μm pore size were purchased from Phenomenex (Torrance, CA). The wastewater samples were collected at the entrance of the wastewater treatment plant (influent) from Cluj-Napoca, Romania, in a brown glass bottle and kept at 8°C before analysis. This plant treats municipal wastewater collected from households, hospitals, and industrial wastewater of Cluj-Napoca and its surroundings.

HPLC-UV Instrumentation and Method

For the analysis of the selected drugs, an HPLC system, Shimadzu model, equipped with a 10LC module pump, a 10LSD UV-Vis detector, and a manual injection valve containing a

sample loop of 5 µL was used. The separations were carried out on a reversed-phase column Nova-Pak C18 (3.9×300 mm, 4 µm particle size) purchased from Waters (Milford, MA). A mixture of acetonitrile and water (55+45, v/v) at a flow rate of 1 mL/min, in isocratic elution mode, was used as the mobile phase. The detection was carried out at a wavelength 220 nm. An Eppendorf centrifuge, model 5804 R (Eppendorf, Wien, Austria), was used for the centrifugation of samples and a pH-meter, model pH 211 (Hanna Instrument, Cluj-Napoca, Romania), for their pH measurement.

DLLME-SFO

For DLLME-SFO method, a volume of 10 mL water sample previously acidified to pH 3.0 with 0.3 mL 1% ortho-phosphoric acid solution was placed in a 15 mL screw-cap glass test tube with conical bottom. After adding 0.5 g NaCl, the sample was stirred for the salt dissolution. A volume of 200 µL extraction mixture containing 150 µL acetonitrile (disperser solvent) and 50 µL 1-undecanol (extraction solvent) was prepared and quickly injected into the water sample using a micropipet. The resulted aqueous sample was vigorously shaken for the dispersion of the fine droplets of 1-undecanol. After forming a cloudy solution, the test tube was centrifuged for 4 min at 4500 rpm in order to separate the two phases and then it was cooled in an ice-water bath for 5 min to solidify the 1-undecanol, which was then collected using a spatula and transferred into a conical vial. After melting the extract at room temperature, a volume of 5 µL was directly injected into HPLC for analysis.

For all experiments involved in the optimization of DLLME-SFO, a volume of 10 mL Milli-Q water sample spiked with 100 ng/mL each tested drug was used. Before extraction, the wastewater samples required a filtration step through 0.45 µm PVDF filters for removing the suspended particles.

Enrichment Factor (EF) and Extraction Recovery (ER)

To assess the DLLME-SFO efficiency, the EF and the relative ER (%) were considered (30). In LPME, EF (Equation 1) is defined as the ratio of the concentration of the analyte in the collected organic phase (C_{col}) and the initial concentration of the analyte into the sample (C_{aq}), whereas ER (Equation 2) is defined as the percentage of the total number of moles of analyte from the aqueous sample (n_{aq}) which is extracted into the collected organic phase (n_{col}).

$$EF = \frac{C_{col}}{C_{aq}} \quad (1)$$

$$ER\% = \frac{(n_{col})}{(n_{aq})} \times 100 = \frac{C_{col} \times V_{col}}{C_{aq} \times V_{aq}} \times 100 \quad (2)$$

where V_{col} = the volume of the collected organic phase; and V_{aq} = the volume of the aqueous sample.

For our aim, taking into consideration that the concentration of drugs in the samples is at parts per billion level, a value of ER between 80 and 120% was expected (31).

Results and Discussion

Optimization of DLLME-SFO Conditions

Based on literature data (21, 27), for the studied drugs in liquid matrices, we selected the most suitable solvents for extraction 1-undecanol and for dispersion acetonitrile.

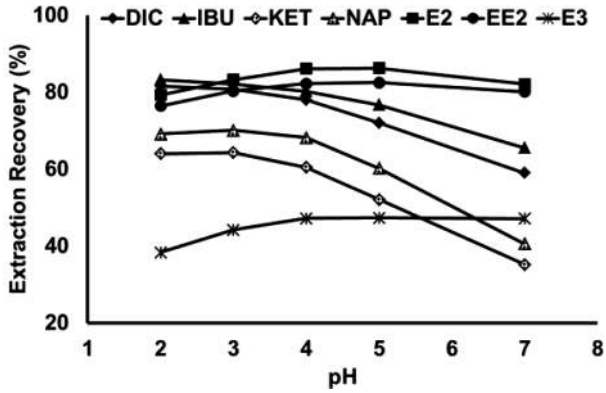


Figure 1. Effect of pH. Extraction conditions: 10 mL spiked Milli-Q water, acidified at different pH values with 1% ortho-phosphoric acid solution, 150 µL disperser solvent (acetonitrile), and 50 µL extraction solvent (1-undecanol).

Some parameters that can influence the extraction efficiency, namely the volume of the extraction and disperser solvent, respectively, as well as the pH and the ionic strength of sample, were studied.

The extraction efficiency was expressed by the relative ER of the studied drugs from 10 mL Milli-Q water sample spiked with 100 ng/mL of each drug. All experiments were done in triplicate.

Effect of pH

Considering the acidic character of the NSAIDs (pKa values between 4.15 and 5.03; Table 1), the influence of the sample pH was the first studied parameter. Four samples, each of them of 10 mL Milli-Q water spiked with drugs, were acidified at different (2, 3, 4, 5) pH values with different volumes of 1% ortho-phosphoric acid solution as follows: 0.3 mL for pH 2, 0.15 mL for pH 3, 0.10 mL for pH 4, and 0.05 mL for pH 5. A nonacidified sample of pH 7 was also considered. The resulted samples were extracted with 200 µL extraction mixture containing 150 µL acetonitrile (disperser solvent) and 50 µL 1-undecanol (extraction solvent), without NaCl addition. The results on pH effect have shown that the extraction efficiency of NSAIDs increases with decreasing pH values while the extraction efficiency of estrogens is not significantly affected by the pH variation (Figure 1).

These results can be explained by the fact that, at low pH value, the acidic compounds are in neutral form which facilitates their transfer to the extraction solvent (1-undecanol; 32). Because estrogens are very weak acids (pKa values between 10.08 and 10.54; Table 1), they are quite stable with the pH variation, thus they can be extracted with the same recovery percentage both at acidic and neutral pH. Therefore, the chosen pH value for the following experiments was 3.

Effect of Ionic Strength

For the aqueous sample solution, a way to enhance the extraction efficiency of the organic compounds is the salt addition (21–27) to can induce the salting-out effect which, in this study, was evaluated in the concentration range of 0–10% NaCl. For this purpose, in 10 mL Milli-Q water spiked with drugs and acidified to pH 3, different NaCl amounts were added.

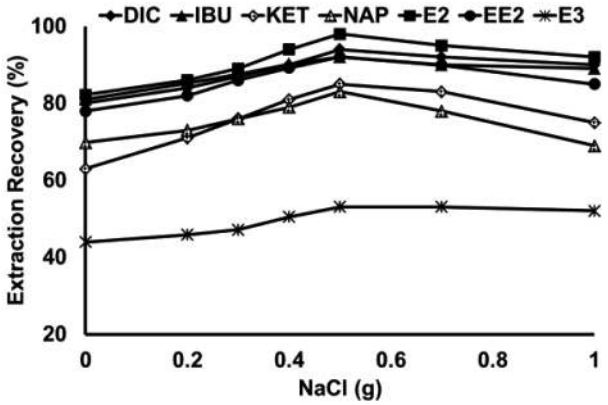


Figure 2. Effect of the ionic solution strength. Extraction conditions: 10 mL spiked Milli-Q water, pH 3, different NaCl amounts, 150 µL disperser solvent (acetonitrile), 50 µL extraction solvent (1-undecanol).

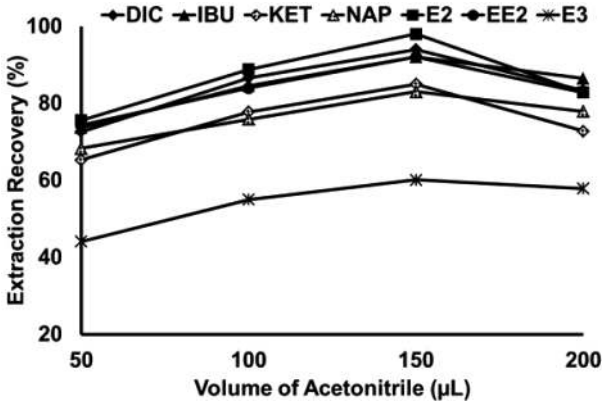


Figure 3. Effect of disperser solvent volume. Extraction conditions: 10 mL spiked Milli-Q water, pH 3, 0.5 g NaCl, different volumes of disperser solvent (acetonitrile), 50 µL extraction solvent (1-undecanol).

A water sample without NaCl addition was also considered. The obtained samples were extracted with 200 µL extraction mixture and analyzed by HPLC-UV. The results show that the ER of the drugs increases with increasing amount of NaCl from 0 to 5% and remains constant over 7% (Figure 2). Thus, an amount of 5% NaCl was selected for further experiments.

Effect of Disperser Solvent Volume

In DLLME-SFO, the disperser solvent has an important role because it is responsible for the dispersion of the extraction solvent in fine droplets into the water samples. This dispersion enhances the mass transfer of the target compounds to the extraction solvent and increases the ER. The disperser solvent should be miscible both with the extraction solvent and water samples, which is why we chose acetonitrile as disperser solvent. To study the influence of disperser solvent volume over the extraction efficiency, various experiments were performed by using different volumes of acetonitrile (50, 100, 150, and 200 µL) and 50 µL 1-undecanol as extraction solvent. The water sample consisted in 10 mL of Milli-Q water spiked with drugs, acidified at pH 3, and NaCl addition up to 0.5%.

The best ER was obtained for 150 µL acetonitrile, both for NSAIDs and estrogens. Moreover, the obtained diagrams have the same shape with a low ER at the lowest and at the highest disperser solvent volumes. This behavior can be explained by the fact that a low acetonitrile volume (50 µL) does not enhance the extraction efficiency because the cloudy state of solution was not well formed. A higher volume of acetonitrile (200 µL) decreased the extraction efficiency as a result of the solubility of the studied drugs in the disperser solvent (Figure 3; 32). Therefore, a volume of 150 µL acetonitrile was selected as disperser solvent for the further experiments.

Effect of Extraction Solvent Volume

For the optimization of the extraction solvent volume, samples of 10 mL Milli-Q water spiked with drugs, acidified at pH 3 and with 0.5 g NaCl addition for each of them, were extracted with different volumes of 1-undecanol (30, 40, 50, 60, and 100 µL). The volume of disperser solvent was maintained constant at 150 µL. The extraction efficiency was expressed both by ER and

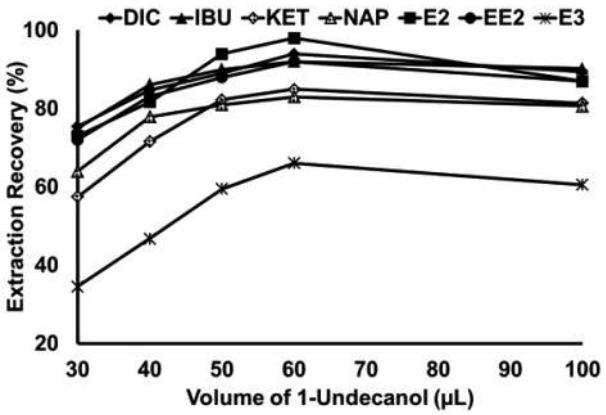


Figure 4. Effect of the extraction solvent volume. Extraction conditions: 10 mL spiked Milli-Q water, pH 3, 0.5 g NaCl, 150 µL disperser solvent (acetonitrile), different volumes of extraction solvent (1-undecanol).

EF. The results show that the extraction recoveries increased with increasing the extraction solvent volume (Figure 4), whereas the EFs decreased with increasing the extraction solvent volume as a result of the dilution of the drugs in the floated organic phase (Figure 5). Considering both parameters, ER and EF, the best compromise established for the optimal experimental conditions was to choose 50 µL extraction solvent for which ER was ranged between 59.3 and 92.5 and EF between 118 and 185 (Table 2).

Validation of DLLME-SFO

For the practical application, the optimum DLLME-SFO parameters experimentally validated were 10 mL Milli-Q water sample spiked with 100 ng/mL each drug and acidified at pH 3, 50 µL 1-undecanol as extraction solvent, 150 µL acetonitrile as disperser solvent, and 0.5 g NaCl. All samples were centrifuged for 4 min at 4500 rpm. The performance of the developed DLLME-SFO method was expressed in terms of accuracy (ER), intra- and interday precision, linearity, LOD, LOQ, and EF (Table 2).

The drug extraction recoveries from the spiked water sample were situated >80% for all studied drugs except estriol for which recovery was 59.3%.

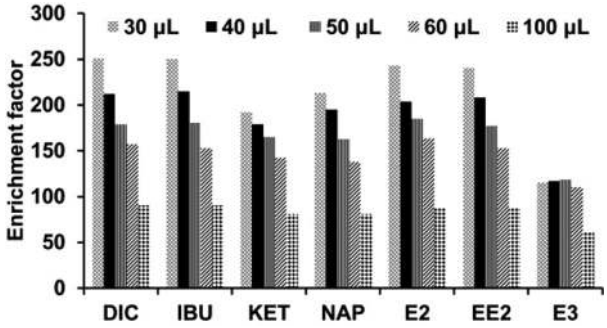


Figure 5. Enrichment factor. Extraction conditions: 10 mL spiked Milli-Q water, pH 3, 0.5 g NaCl, 150 µL volumes of disperser solvent (acetonitrile), different volumes of extraction solvent (1-undecanol).

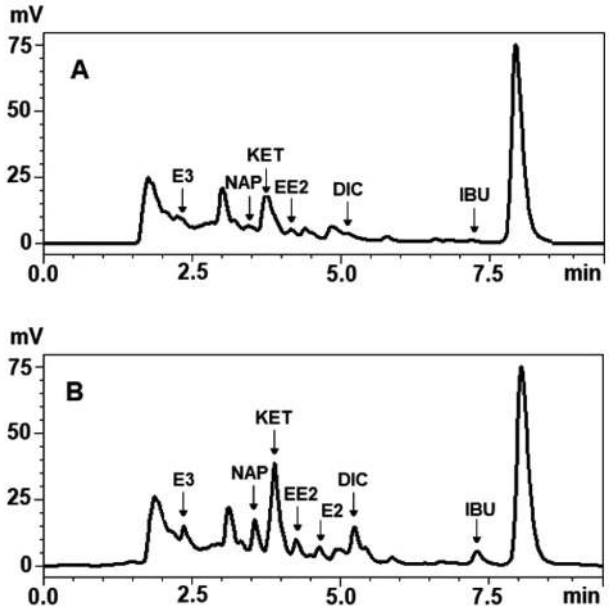


Figure 6. (A) Chromatograms of wastewater sample and (B) spiked wastewater sample with 200 ng each drugs. Extraction conditions: 10 mL water sample, pH 3, 0.5 g NaCl, 150 µL disperser solvent (acetonitrile), 50 µL extraction solvent (1-undecanol).

Intraday precision (repeatability) and interday precision (intermediary precision) were expressed by the RSD (%) by means of replicates of spiked Milli-Q water samples (Table 2). The obtained RSD values were situated under 7% for all studied drugs, the intraday RSD values ranged from 2.89 to 5.76%, whereas the interday RSD values ranged from 3.05 to 6.25%, which is in agreement with the requirements of the Validation Method Guide (31).

To study the linearity of the HPLC-UV method, different standard mixtures in concentration of 2, 5, 10, 50, and 100 µg/mL of each drug were prepared by successive dilution of the stock solution. The mixtures were analyzed by HPLC-UV at 220 nm and the calibration curves were plotted using the peak area versus the concentration of each drug. Good linearity for all studied drugs with correlation coefficients (r) >0.999 was obtained.

The LOD and the LOQ were found using standard deviation of the regression line (s) and the slope (S) of each calibration curve. Finally, the LOD and LOQ of the developed method were calculated taking into account the enrichment factors. Thus, for NSAIDs, the LOD was ranged from 0.075 to 0.19 µg/L and the LOQ from 0.22 to 0.59 µg/L, whereas for estrogens, the LOD ranged from 0.27 to 0.43 µg/L and the LOQ from 0.81 to 1.29 µg/L.

Analysis of Real Samples and Matrix Effect

The developed DLLME-SFO–HPLC-UV method was tested in the analysis of the studied drugs in wastewater samples collected from the influent of the wastewater treatment plant of Cluj-Napoca. Before extraction, the wastewater samples were filtered through a PVDF filter of 0.45 µm for removing the suspended particles and then acidified at pH 3 with 0.15 mL 1% ortho-phosphoric acid solution. The resulted samples were extracted and analyzed under the protocol described above. In these wastewater samples (Figure 6A), different amounts of the studied drugs were detected as follows: ibuprofen 1.85 µg/L, diclofenac 0.46 µg/L, ketoprofen 16.04 µg/L, naproxen 0.59 µg/L, 17α-ethynylestradiol 4.7 µg/L, and estriol 2.62 µg/L (Table 3).

For the evaluation of the matrix effect over the extraction efficiency of the developed DLLME-SFO method, a volume of 10 mL filtered wastewater sample was spiked with different amounts of studied drugs as follows: sample 1 with 200 ng each drug, sample 2 with 400 ng, and sample 3 with 600 ng. The resulted samples were acidified at pH 3 and then subjected to

Table 2. Figure of merits: linearity (study range of 2–100 µg/mL of each drug), correlation coefficient (r), LOD, LOQ, intraday precision, interday precision, ER, and EF

Drug	Calibration curve	r	SD	LOD, µg/L	LOQ, µg/L	Intraday[a] RSD, %	Interday[b] RSD, %	ER, %	EF
IBU	$y = 14170\,x + 559.7$	0.9997	140	0.18	0.55	3.45	4.35	90.1	180.2
DIC	$y = 24692\,x + 6686.5$	0.9988	186	0.14	0.42	4.80	5.74	89.3	178.6
KET	$y = 15617\,x + 397.95$	0.9997	154	0.19	0.59	3.98	4.52	82.4	164.8
NAP	$y = 41473\,x + 3252.6$	0.9996	151	0.075	0.22	4.02	4.87	81.3	162.6
EE2	$y = 8658\,x + 310.05$	0.9997	134	0.29	0.87	4.89	5.23	88.6	177.2
E2	$y = 8566.1\,x - 4731.8$	0.9999	129	0.27	0.81	2.89	3.05	92.5	185
E3	$y = 8138\,x + 187.85$	0.9996	125	0.43	1.29	5.76	6.25	59.3	118.6

[a] $n = 3$.

[b] $n = 9$.

Table 3. Analysis of the real samples and matrix effect

Drug	Concn in waste water sample, µg/L	Initial amt in waste water sample, ng	Found amt, ng					
			Sample 1[a]	ER,%	Sample 2[b]	ER,%	Sample 3[c]	ER, %
IBU	1.85	18.5	179.2	80.59	360.0	85.49	584.1	94.33
DIC	0.46	4.6	167.0	81.21	342.2	84.41	605.2	100.11
KET	16.04	160.4	341.0	90.3	514.4	88.49	735.2	95.79
NAP	0.59	5.96	172.7	83.39	334	82.01	506.1	83.36
EE2	4.7	47.0	211.3	82.13	388.2	85.3	606.4	93.22
E2	ND[d]	ND	160.5	80.25	345.8	86.45	496.2	82.70
E3	2.62	26.19	182.9	78.37	309.7	70.87	458.5	72.05

[a] Sample 1 = Wastewater spiked with 200 ng.

[b] Sample 2 = Wastewater spiked with 400 ng.

[c] Sample 3 = Wastewater spiked with 600 ng.

[d] ND = Not detected.

Table 4. Comparison of the proposed extraction method with other reports

Drugs	Method	Sample volume, mL	Extraction solvent, mL	Extraction time, min	LOQ	ER, %	Ref.
NSAIDs							
Clofibric acid, ibuprofen, naproxen, diclofenac, ketoprofen	LLE[a]	2.5	1.5	~25	0.18–4.1 µg/L	80–140	(6)
24 emerging pharmaceutical residues	SPE[b]	200	6	~40	10–1000 pg/L	72–138	(12)
Ketoprofen, naproxen	SBSE	20	150 µL	75	7.89–9.52 µg/L	91.9–114	(16)
Ketoprofen, flurbiprofen, diclofenac	SPME[c]	2	No solvent	17	0.05–0.25 µg/L	87.3–113	(17)
58 pharmaceuticals, Personal care products, Pesticides	DLLME	10	120 µL	10	0.012–1.25 µg/L	61–147	(23)
Diclofenac, ibuprofen, mefenamic acid	DLLME-SFO	10	60 µL	8	0.5–0.8 µg/L	69–78	(27)
Diclofenac, ibuprofen ketoprofen, naproxen,E2, EE2, E3	DLLME-SFO	10	50 µL	10	0.22–1.29 µg/L	59.3–92.5	This work
Hormones							
E1, E2, EE2, Genistein	LLE	1000	450	~60	0.5–5.0 ng/L	63.7–135	(4)
20 hormones	SPE	200	15	~30	1.7–15.0 ng/ L	70–114	(10)
7 hormones	SBSE[d]	50	3.5	30	0.34–1.37 µg/L	48.2–110	(18)
E1, E2, E3, EE2	SPME	10	0.9	~40	0.04–2.31 ng/L	86.9–98.7	(15)
E1, E2, E3, EE2, Dienestrol, zearalenone, 2-methoxy-estradiol,α-, β-Zearalanol,α-, β-Zearalenol	DLLME	7.5	185 µL	10	0.04–1.10 µg/L	43–91%.	(20)
E1, E2, E3, EE2	DLLME-SFO	5	10 µL	10	0.8 to 2.7 µg/L	87–116	(21)

[a] LLE = Liquid–liquid extraction.

[b] SPE = Solid-phase extraction.

[c] SPME = Solid-phase microextraction.

[d] SBSE = Stir-bar sorbtive extraction.

DLLME-SFO extraction and HPLC-UV analysis. The analysis of the spiked wastewater samples (Figure 6B) showed extraction recoveries exceeding 80% for all studied drugs, except estriol for which this value was between 72.05 and 78.37 (Table 3).

Comparing the extraction recoveries of the spiked wastewater samples (Table 3) with those obtained for spiked Milli-Q water samples (Table 2), it can be observed that no significant differences exist between samples, which means that no matrix effect occurs. As results, the developed DLLME-SFO–HPLC-UV method can be applied for the analysis of studied drugs in real wastewater samples.

Comparison with Other Literature Reports

To highlight the benefits of the developed method, its performance has been compared with other methods from literature (Table 4).

According to data from Table 4, it can be observed that the developed method reaches comparable or better performance with other methods used for the determination of NSAIDs and estrogens in water samples. Thus, the method has an LOQ in the range of micrograms per liter, a recovery >80%, needs a time of 10 min for sample processing and uses an extraction solvent volume in the order of tens µL compared with the order of tens mL in LLE or SPE. Moreover, both NSAIDs and estrogens are analyzed in a single run, whereas other reports give two runs for the analysis of these drugs.

Conclusions

A simple, fast, and inexpensive DLLME-SFO–HPLC-UV method for the simultaneous extraction and analysis of four NSAIDs and three estrogens in wastewater samples was developed.

The method is easy to operate, uses low volumes of sample and organic solvents of less toxicity, is fitted with the green analytical chemistry principles, and has performances comparable with those of the classical solvent extraction or solid-phase extraction. Our method has a sensitivity of micrograms per liter, a high EF, a high ER, and good repeatability.

Moreover, this method proved its applicability for the analysis of real wastewater samples and can open the way to a suitable and green alternative to the classical LLE and SPE techniques for the sample preparation in terms of performance and working time.

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