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Original research paper

Analysis of diclofenac in water samples using *in situ* derivatization-vortex-assisted liquid-liquid microextraction with gas chromatography-mass spectrometry

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A novel micro-extraction technique for a rapid and sensitive analysis of diclofenac (DCF) in water samples has been developed. DCF was derivatized and extracted simultaneously using vortex-assisted liquid-liquid micro-extraction (VALLME) prior to gas chromatography with mass spectrometry detection. The effects of extraction solvent volume, extraction and derivatization time and ionic strength of the sample were studied using 2^3 factorial experimental design. The optimum extraction conditions were as follows: 200 μL of chloroform, 25 μL of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) derivatization reagent, vortex extraction and derivatization time 5 min at 3000 rpm. The extraction recovery for different fortification levels was 98 %. Also, the proposed micro-extraction method exhibited results comparable with the solid phase extraction of real water samples. The proposed one-step VALLME and derivatization method is simpler and faster than the conventional extraction and derivatization methods used for the determination of DCF in real water samples.

Keywords: diclofenac, GC-MS, *in situ* derivatization, vortex-assisted liquid-liquid micro-extraction, water

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Pharmaceutical compounds (PhCs) in water have become a major concern for human health and the environment because PhCs are often resistant to biodegradation in aquatic and terrestrial ecosystems. In recent years, determination of pharmaceuticals in different environmental media has become a relevant topic. The most important source of PhCs in an aquatic environment is urban wastewater. After being used, these compounds are excreted into the sewerage system. Also, unused or expired drugs are at the disposal to the sewerage system. Conventional wastewater treatment plants (WWTP) do not provide for the removal of PhCs (1, 2). Diclofenac (DCF, $C_{14}H_{11}Cl_2NO_2$, M_r 296.16, pK_a 4.15, log K_{ow} 4.51)

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is a nonsteroidal anti-inflammatory drug (NSAID). It is one of the mostly detected PhCs in the environment because it is not effectively removed by the current conventional WWT processes. In the literature, different removal rates for DCF have been determined, such as 0 % (3), 17 % (4), 69 % (5), 100 % (6) in wastewater treatment plants. Recent studies have indicated that DCF has been detected in groundwater, some drinking water, surface water, seawater, wastewater, WWTP effluents and in sludge in many countries at concentrations generally in the ng L^{-1} to μg/L range. The maximum concentration of DCF of 3.5 μg L^{-1} has been detected in effluent wastewaters at five different sewage treatment plants in Spain (7). Up to 717 ng L^{-1} DCF was determined in surface water in China (8). In Mexico City, 1 and 20-32 ng L^{-1} DCF, resp., has been reported in groundwater and in surface water (9). In river and pond waters in Germany DCF has been measured in 10 out of 27 water samples in concentrations of up to 15 μg L^{-1} (10). DCF has also been detected up to a concentration of 380 ng L^{-1} in the groundwater of Barcelona (11). Extensive and continuous release of DCF from domestic and hospital wastewaters is a potential risk to non-target organisms, even at concentrations of ≤ 1 μg L^{-1} (12). According to the results of toxicity tests, DCF exhibited the highest acute toxicity within the class of NSAIDs. DCF is listed among priority substances in the European Union Water Framework Directive (2000/60/EC). Recently, DCF was included in the watch list in Directive 2013/39/EU and is monitored by the EU member states in water media.

DCF in aqueous samples can be determined by chromatographic techniques, *i.e.*, high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), tandem mass spectrometry (MS-MS), fluorescence (FL) or ultraviolet detection (UV), or gas chromatography (GC) coupled with MS and MS-MS after sample pre-concentration steps such as solid phase extraction (SPE). DCF should be derivatized prior to GC analysis. SPE was generally used for determination of DCF in water samples (13). However, there are some disadvantages of SPE methods; analytes may be adsorbed and complex matrices can cause settling in cartridges. The SPE method requires a disposable cartridge with a manifold system and a large volume of organic solvents (14). In recent years, for extraction and pre-concentration of DCF residues in water, several micro-extraction based methods have been developed, including solid-phase micro-extraction (SPME) (15), stir bar sorptive extraction (SBSE) (16), fiber liquid-phase micro-extraction (HF-LPME) (17), ultrasound-assisted emulsification micro-extraction (USAEME) (18), sonication-assisted emulsification micro-extraction combined with vortex assisted porous membrane protected micro-solid-phase extraction (SAEME-VA-μ-SPE) (19), dispersive liquid-liquid micro-extraction and single-drop micro-extraction (20). However, these techniques have some drawbacks. For instance, the SPME method has a high price and fiber fragility, as well as carryover problems. LPME, SDME and DLLME methods are complex and difficult for automation. These methods also suffer from droplet instability and relatively low precision. The ultrasound energy used in the USAEME method may degrade the analytes and can lead to low recovery. There is recently a growing interest in simultaneous micro-extraction and derivatization methods.

In this study, a novel analytical method for determining the residues of DCF in waters was developed by VALLME with *in situ* derivatization followed by GC-MS. Chang *et al*. (21) employed this method successfully for determination of aliphatic amines. Thus far, VALLME with *in situ* derivatization has not been used for the residue analysis of DCF in water samples.

EXPERIMENTAL

Chemicals

DCF sodium was purchased from Fluka (Switzerland). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Merck (Germany). Sodium chloride and all organic solvents were also purchased from Merck. Deionized water was prepared with the aid of a Millipore Milli-Q Plus water purification system (Millipore, USA). Stock solutions of DCF were prepared in methanol. Working solutions were prepared by diluting the standard stock solution with deionized water and storing it in the dark at 4 °C until use.

Real water samples

Tap water was obtained from the laboratory. Bottled water was taken from the market in Konya, Turkey. The surface water sample was collected from the Altınapa dam in Konya. Wastewater influent and effluent samples were taken from domestic WWTP from Konya. One-L amber glass bottles were used for sample collection. All samples were collected on the day before being analyzed. Sample bottles were rinsed with the sample prior to sample collection. Dam and wastewater samples were filtered using membrane filters (Sartorius, Germany) before the extraction.

Instrumentation

Quantification of DCF was carried out using a gas chromatograph equipped with a quadrupole mass selective detector with electron ionization and a programmed temperature vaporizing (PTV) injector (Agilent Technologies, USA). DB-5 MS capillary column (30 m length, 0.25 mm i.d. and 0.25 μm film thickness) was used for separation. The oven program was as follows: initial temperature 80 °C for 1 min, 15 °C min^{-1} to 300 °C, hold at 300 °C for 2 min (run time: 17.67 min). Helium was used as carrier gas at a constant flow-rate of 1.9 mL min^{-1}. PTV was operated in splitless mode. One μL injection was applied with an Agilent 7683 B Series automatic injector. The temperature of the ion source was 150 °C while MS transfer line was at 270 °C. MS detector operated in the selected ion monitoring (SIM) mode. Ions at *m*/*z* 214, 242 and 277 were monitored. Quantitation was based on the ion at *m*/*z* 214 monitoring.

A vortex agitator (Wiggen Hauser, Germany) was used for the extraction and derivatization process.

A 100-μL Hamilton syringe (Hamilton Bonaduz AG, Switzerland) was used to introduce the organic solvent into the aqueous sample.

VALLME with in situ *derivatization*

To determine VALLME efficiency with *in situ* derivatization, spiking experiments were carried out. Firstly, some factors affecting the extraction process such as solvent type (dichloromethane, chloroform, 1,2-dichlorobenzene, bromoform, *n*-hexane, cyclohexane, petroleum ether, 2-propanol, isooctane, *n*-pentane, toluene, diethylether, methanol, and

ethyl acetate), derivatization reagent volume (25–200 μL) and the vortex agitation speed (0–3000 rpm) were tested. Then, 2^3 factorial experimental design was applied for simultaneous optimization of the extraction solvent volume, extraction and derivatization time, and ionic strength of the sample. To determine extraction efficiency of different solvents, 5 mL of an aqueous standard solution containing 5 μg L^{-1} DCF was placed in a 10-mL Falcon tube. A hundred μL of derivatization reagent (MSTFA) and 100 μL of extraction solvent were injected into the tube. Then, the tube was strongly vortex shaken for 2 min at 1200 rpm. As a result, DCF derivatized with MSTFA was extracted from the aqueous bulk to the organic phase. After that, the mixture was centrifuged for 5 min at 4000 rpm and the lower organic phase was removed using a 100-μL syringe and put into the glass microvial for analysis. Extraction solvent volume, extraction and derivatization time, and ionic strength of the sample (addition of NaCl into the sample), were termed factors 1, 2 and 3, resp. The experiments were randomly carried out (in duplicate) to avoid any systematic error. Low and high levels for factors 1, 2 and 3 were selected as 100 and 200 μL, 2 and 5 min, 0 and 0.05 g mL^{-1}, resp. The experimental matrix design is given in Table I.

Table I. Factorial matrix

Experiment No.	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
1–9	–	–	–	100	2	0
2–10	+	–	–	200	2	0
3–11	–	+	–	100	5	0
4–12	+	+	–	200	5	0
5–13	–	–	+	100	2	0.05
6–14	+	–	+	200	2	0.05
7–15	–	+	+	100	5	0.05
8–16	+	+	+	200	5	0.05

Factor 1: solvent volume (μL); factor 2: extraction and derivatization time (min); factor 3: ionic strength of the sample (g L^{-1}). –/+: denoting lower/upper factor level.

Solid phase extraction (SPE)

An oasis HLB cartridge (Waters, USA) was used for SPE extraction of DCF from water samples. Traditional SPE procedure was performed according to the US EPA method for DCF extraction (22). Five mL of de-ionized water following 5 mL of methanol were used to condition the cartridge. The water sample (200 mL) was passed and the cartridge was dried for 10 min under vacuum. After extraction, elution of the DCF from the cartridge was performed with 10 mL of methanol. Derivatization with MSTFA was performed prior to GC analysis. After 200 μL of MSTFA and 5 μL of pyridine were added into the 1-mL extract, it was incubated for 30 min at 80 °C and then cooled to room temperature.

RESULTS AND DISCUSSION

Optimization of extraction parameters: solvent type, derivatization reagent volume and agitation speed

Selection of an appropriate extraction solvent is very important for efficient extraction in the VALLME procedure. It should meet some criteria. For example, it must be immiscible with water, with excellent gas chromatographic behavior and high affinity to analytes (23).

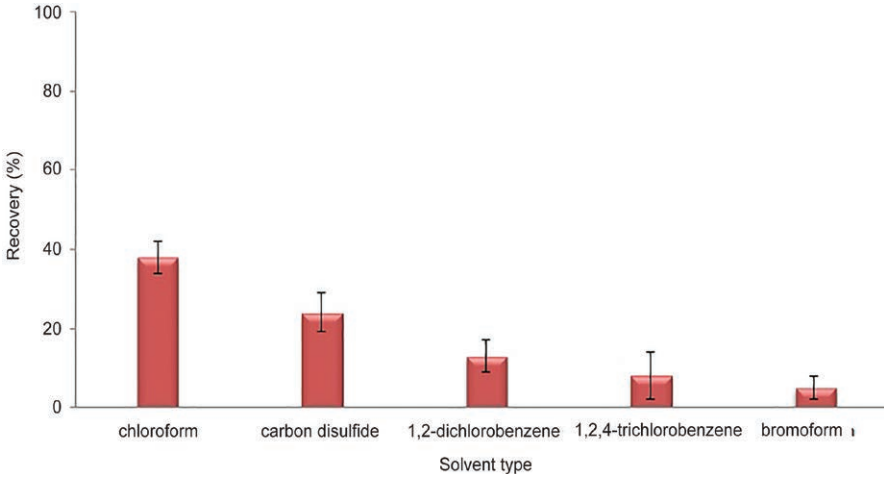

Fig. 1. Recovery of DCF in different organic solvents (mean ± RSD, n = 4).

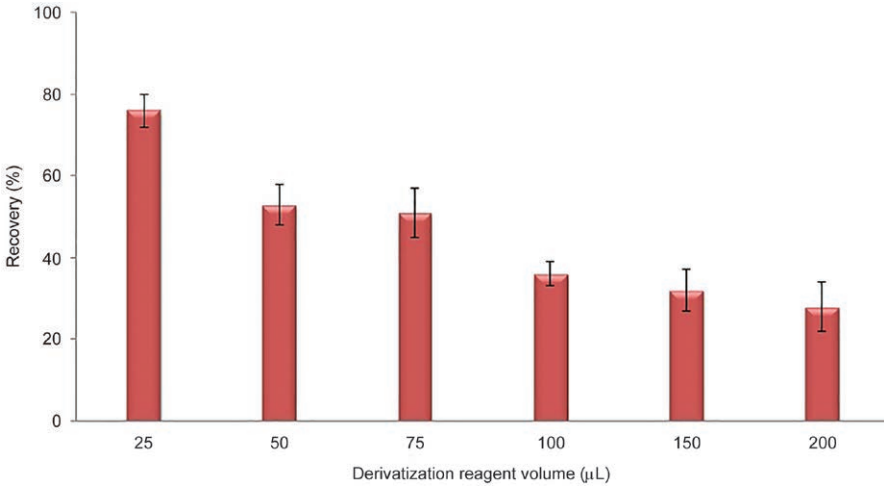

Fig. 2. Effect of the derivatization reagent (MSTFA) volume on DCF recovery (mean ± RSD, n = 4).

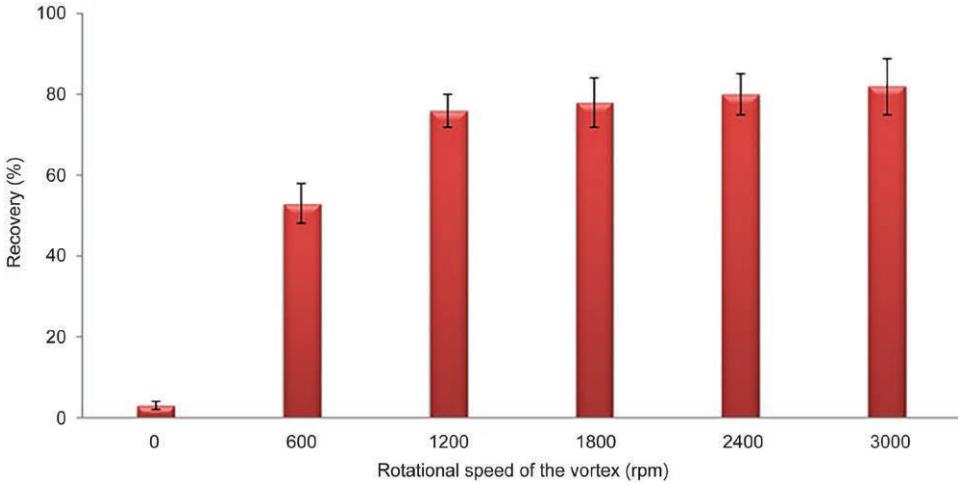

Fig. 3. Effect of vortex agitation speed on DCF recovery (mean ± RSD, $n = 4$).

In this study, extraction solvents with lower (petroleum ether, *n*-hexane, isooctane, diethyl-ether, cyclohexane, 2-propanol, *n*-pentane, toluene, ethyl acetate) and higher density than water (carbon disulfide (CS_2), 1,2-dichlorobenzene, dichloromethane, 1,2,4-trichlorobenzene, chloroform, bromoform) were studied. After centrifugation, chloroform, CS_2, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene and bromoform appeared at the bottom of the tube while the other extraction solvents were not collected because of their high solubility in water. As seen in Fig. 1, the best recoveries were obtained with chloroform. In comparison with the other solvents used, chloroform had a lower dipole moment. The lower dipole moment allowed better interaction between solvent molecules and DCF. Fig. 2 shows the effect of derivatization reagent volume, ranging from 25 to 200 μL, on extraction with MSTFA. As the MSTFA volume increased, the recovery values of DCF decreased. Therefore, 25 μL MSTFA was selected as optimal. Extraction and derivatization were performed with the support of vortex agitation. It affected the extraction equilibrium between DCF and chloroform and the mass transfer process of the DCF. When the agitation speed was increased up to 3000 rpm, extraction efficiency of DCF increased from 3 to 82 % (see Fig. 3).

Factorial experimental design

The extraction solvent volume, extraction and derivatization time and water ionic strength were simultaneously optimized using a 2^3 factorial design (24). The effect of each factor was evaluated using the analysis of variance (*p*-values at 5 % significance level). All factors were determined as significant factors. Factors 1 (solvent volume) and 2 (extraction and derivatization time) exhibited positive effects while factor 3 (ionic strength of the sample) had a negative effect. When the extraction solvent volume was increased from 100 to 200 μL, DCF recovery increased from 82 to 93 % (see Fig. 4). The number of submicron droplets increased with increasing the volume of chloroform. Thus, the solvent and water

contact surface increased and higher extraction efficiency was obtained. When the extraction time and derivatization time were increased from 2 to 5 min, the recovery of DCF increased from 93 to 98 %. It was observed that higher mass transfer from the aqueous phase to solvent phase was achieved with the increase in extraction time. When NaCl concentration in the water sample increased from 0 to 0.05 g mL^{-1}, the extraction of DCF decreased from 82 to 51 %. Because of the increasing ionic strength of the sample, the mass-transfer process and extraction efficiency decreased. The interaction between factors 1 and 2 was determined as significant with a positive effect. Interactions between factors 1 and 3 and factors 2 and 3 were found to be significant factors with a negative effect. The optimum conditions for VALLME and derivatization of DCF from the water sample were found as: 5 mL aqueous sample, 200 μL chloroform, 25 μL MSTFA, extraction and derivatization time 5 min at 3000 rpm, centrifugation 5 min at 4000 rpm with no addition of NaCl.

Analytical performances of the optimized method

Analytical performances of the method were evaluated through limits of detection (*LOD*) and quantification (*LOQ*), correlation coefficient (R^2), repeatability and extraction recovery. Method validation was carried out according to the official document (25). *LOD* and *LOQ* were calculated at a signal-to-noise ratio (*S*/*N*) of 3 and 10, resp. (26). *LOD* and *LOQ* were determined as 0.002 and 0.007 μg L^{-1}. R^2 value for the calibration line drawn at seven concentrations points in the concentration range of 0.001–10 μg L^{-1} of DCF was 0.9998. RSD below 1.20 % was obtained from six injections of 0.1 μg L^{-1} of DCF. The optimized VALLME *in situ* derivatization was applied to distilled water spiked with DCF. Recoveries obtained for fortifications of 0.1, 1.0 and 5.0 μg L^{-1} DCF were 99.3, 101.1 and 98.5

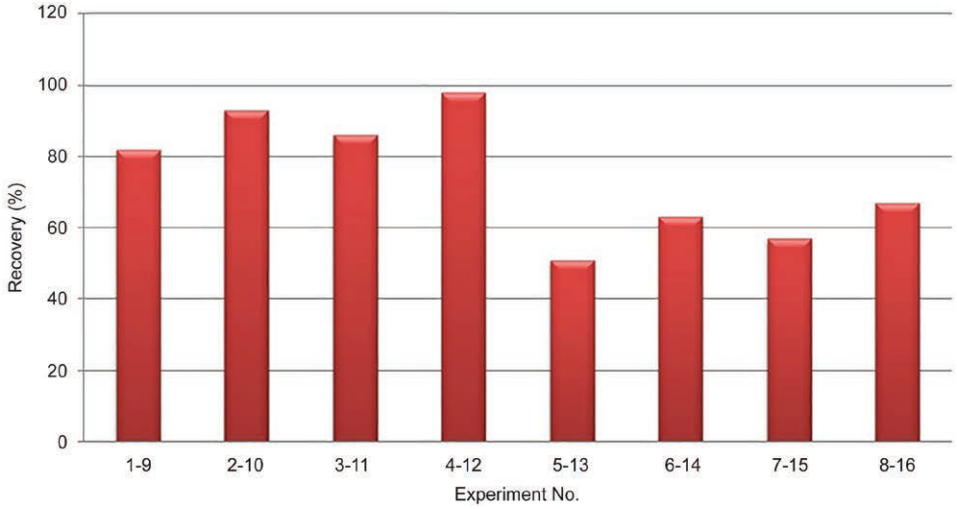

Fig. 4. Effects of the factorial experimental design factors used in VALLME with the *in situ* derivatization procedure (see Table I) on DCF recovery (mean, $n = 2$).

Table II. Comparison of the proposed method with the SPE method

DCF	Recovery (%)	
	VALLME *in situ* derivatization method	SPE method
Tap water	99.3 ± 5	88.4 ± 4
Bottled water	101.2 ± 6	87.5 ± 5
Surface water	98.4 ± 5	85.5 ± 6
Influent water	96.1 ± 6	85.1 ± 6
Effluent water	99.3 ± 4	80.2 ± 7

[a] DCF fortification: 1 μg L^{-1}.
[b] Mean ± RSD, $n = 4$.

%, resp. When statistical evaluations were carried out between spike levels 0.1–1.0, 0.1–5.0 and 1.0–5.0 μg L^{-1}, no significant differences ($p < 0.05$) were observed.

Real water samples including tap, surface and bottled water, domestic wastewater and treated domestic wastewater were fortified with 1 μg L^{-1} of DCF and the optimized VALLME *in situ* derivatization and traditional SPE method were performed. Blank analysis was also carried out with distilled water to be sure that no interfering compounds were present. Tap water, bottled water, surface water and effluent water samples were free of DCF contamination. However, DCF was present in influent water samples at a concentration of 35.8 ng L^{-1}. The obtained results are given in Table II. As seen in Table II, sample matrix did not adversely affect the efficiency of the VALLME *in situ* derivatization procedure. The results also showed that the efficiency of the optimized method was higher than that of the SPE method.

Comparison of the proposed method with other methods

Performance of the proposed method was compared with the other, previously reported extraction methods for DCF determination in water samples (18, 27–33). The proposed method brings several advantages. The RSD values and linearity range of the proposed method are comparable with different extraction techniques given in the literature (see Table III). *LOD* obtained with VALLME with *in situ* derivatization (GC-MS) is generally better than those obtained with MNPs-based dispersive-micro-SPE (HPLC-UV), USAEME with *in situ* derivatization (GC-MS), graphene oxide-based DSPE with *in situ* derivatization (GC-MS), and SALLE with *in situ* derivatization (GC-MS). The extraction and derivatization time, sample and solvent volume of the optimized procedure are much lower than in the SPE derivatization (GC-MS) method. The proposed method has shown to be comparable with USAEME with *in situ* derivatization (GC-MS) in regard to extraction efficiency. The extraction recovery of the method is generally higher than in other methods given in Table III. The new method also reduces solvent waste and does not require any additional equipment for derivatization.

Table III. Comparison of VALLME with in situ derivatization-GC-MS with other extraction methods for diclofenac

Method	*LOD/LOQ* (μg L^{-1})	Linearity range (μg L^{-1})	RSD (%)	Extraction and derivatization time (min)	Sample volume (mL)	Solvent volume (mL)	Extraction recovery (%)	Reference
SPE-derivatization (GC-MS)[a]	0.0012–0.00237	0.005–0.2	3.9–6.2	210–310	1000–2000	12	65.9–76.2	27
MNPs based dispersive-micro-SPE (HPLC-UV)[b]	1.5	5–500	5.8	6	5	0.05	76.7	28
USAEME with *in situ* derivatization (GC-MS)[c]	0.003	0.05–10	6.9	5	5	0.04	99	29
Graphene oxide-based DSPE with *in situ* derivatization (GC-MS)[d]	0.016	0.05–50	6	1.16	5	1.15	60–67	30
USAEME with *in situ* derivatization (GC-MS)[c]	0.01	0.02–5	4.4–8.3	3	10	0.1	85–104	18
DLLME (LC-MS)[e]	0.0001	0.25–500	5–6	1	6	0.08	77–88	31
SA ion-pair LLE with injection port derivatization (GC-MS)[f]	0.00275	0.1–1000	4.9	5	1	0.2	78.2	32
SALLE with *in situ* derivatization (GC-MS)[g]	0.08	0.08–1000	3.8–4.1	17	2.5	1.5	80–180	33
VALLME with *in situ* derivatization (GC-MS)[h]	0.002/0.007	0.001–10	4–6	5	5	0.2	96.1–101.2	Present study

[a] SPE-derivatization (GC-MS): solid phase extraction-derivatization (gas chromatography-mass spectrometry).

[b] MNPs based dispersive-micro-SPE (HPLC-UV): magnetic nanoparticles based dispersive-micro-solid phase extraction (high performance liquid chromatography--ultraviolet detection).

[c] USAEME with *in situ* derivatization (GC-MS): ultrasound assisted emulsification microextraction with *in situ* derivatization (gas chromatography-mass spectrometry).

[d] Graphene oxide-based DSPE with *in situ* derivatization (GC-MS): graphene oxide-based dispersive solid-phase extraction with *in situ* derivatization (gas chromatography-mass spectrometry).

[e] DLLME (LC-MS): dispersive liquid-liquid microextraction (liquid chromatography- mass spectrometry).

[f] SA ion-pair LLE with injection port derivatization (GC-MS): sonicated assisted ion-pair liquid-liquid extraction with injection port derivatization (gas chromatography-mass spectrometry).

[g] SALLE with *in situ* derivatization (GC-MS): salting out assisted liquid-liquid extraction with *in situ* derivatization (gas chromatography-mass spectrometry).

[h] VALLME with *in situ* derivatization (GC-MS): vortex assisted liquid-liquid microextraction with *in situ* derivatization (gas chromatography-mass spectrometry).

CONCLUSIONS

The paper presents a novel analytical method based on simultaneous VALLME and MSTFA derivatization combined with GC-MS for the determination of DCF in water and wastewater. Experimental parameters (extraction solvent type, the amount of derivatization reagent, and vortex agitation speed) influencing the extraction process were initially optimized. Other critical variables involving the volume of extraction solvent, extraction and derivatization time, and ionic strength of the sample were optimized using a 2^3 factorial experimental design. VALLME with the *in situ* derivatization-GC-MS method can be considered an emerging alternative to SPE. Lower organic solvent consumption decreases environmental pollution and waste treatment costs.

The method was intended for determination of DCF residues in real water samples such as tap, bottled and surface water and also influent and effluent samples taken from Konya domestic WWTP.

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