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Analysis of Diazepam Residue from Water Samples by Triple Phase-Suspended Droplet Microextraction Coupled to High Performance Liquid Chromatography and Diode Array Detection

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New pre-concentration technique, triple phase suspended droplet microextraction (SD-LPME) and liquid chromatography-photodiode array detection were applied to determine diazepam in water samples. The analyte was extracted from 4.5 mL sample volumes directly into 10 μL of extraction solvent. The microextraction process is consisted of a free suspended micro droplet of an aqueous solvent which is delivered to the surface of an immiscible organic solvent. After the optimized extraction time, the suspended micro-droplet is withdrawn by a HPLC microsyringe, injected to and analyzed by HPLC-DAD. The effective parameters such as organic solvent, pH, extraction time, micro droplet volume and agitation speed were investigated. Method was evaluated and enrichment factor 839.8, linearity range from 25 to 5000 ng mL^{-1} with an average of relative standard deviation (n = 5) 5.62 % for diazepam using a photodiode array detector were determined. All experiments were carried out at room temperature.

Key Words: Suspended droplet liquid phase microextraction, Benzodiazepine, Diazepam, Water analysis, HPLC-DAD.

INTRODUCTION

There are numerous observations of pharmaceuticals (or their metabolites) as contaminants in wastewater, surface water and groundwater. This implies a potential for indirect human exposure to pharmaceuticals *via* drinking water supplies. The group of benzodiazepines is one of the most used classes of drugs in the world, with diazepam being the most well known compound[1]. Diazepam (7-chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepine-2-[1*H*]-one) marketed under brand names valium, stesolid, diazemuls, seduxen, bosaurin, diapam, antenex, ducene, apozepam and pax (South Africa)[2] is a drug which is a benzodiazepine derivative. It possesses anxiolytic, sedative, skeletal muscle relaxant. It is listed as one of the top 20 most used pharmaceuticals[3-5].

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Consequently, benzodiazepines are frequently encountered in clinical, forensic toxicological and water sample analysis. Because of low concentration of diazepam in environmental sample pre-treatment and a pre-concentration step is generally required for determination of trace amounts of diazepam as the pollutants[6].

Solid-phase microextraction (SPME) is a solvent free sample preparation technique suitable for small sample volumes. The method was originally developed for analysis of aqueous samples in environmental analysis In spite of its advantage of being a solvent free technique SPME has not been widely implemented in drug analysis. The time to reach equilibrium applied in bioanalysis of drugs is quite long using SPME, in addition the recovery is low[7,8].

Recently in the field of liquid phase microextraction, Yangcheng and coworkers developed a new sampling method termed directly suspended droplet micro extraction (DSDME)[9]. In this work, we used of a new application of this method, based on a three phase extraction system which is compatible with CE and HPLC. In this mode of DSDME, the acceptor solution is another aqueous phase providing a three-phase system, where the analyte is extracted from an aqueous sample, through the thin layer of organic solvent and into an aqueous acceptor solution. Parameters including type of organic solvent, pH, extraction and back extraction times and stirring rate were investigated. This study suggests a simple, economical, fast and safe method for the determination of diazepam in aqueous samples[10,11].

EXPERIMENTAL

Diazepam was supplied from Loghman Pharmacy Co., Tehran, Iran. Analytical reagents grade methanol, acetonitrile, 1-octanol, *n*-hexane, benzyl alcohol, toluene, dichloromethane were purchased from Merck (Darmstadt, Germany). All of these compounds were HPLC grade.

De-ionized water and plain water were purified in a Milli-Q filtering system (Millipore). Stock solutions of the analytes (2.0 μg mL^{-1}) were prepared separately in methanol and they were stored at 4 ºC. Daily working solutions were prepared by combining aliquots of each stock solution and diluting, to appropriate concentrations with NaOH 0.1 M (pH = 9) and stored in a refrigerator.

Instrumentation: Separation, identification and quantification were carried out on a KNAVER HPLC system, KNAVER Jahre35 (Germany). This system was equipped with a Wellchrome K-2800 diod-array detector and was fitted with Smart line autosampler 3800 injector and a 50 μL-sample loop. Analyte was separated on PerfectSil Targetat C_{18} MZ-Analytical column (250 mm × 4.6 mm, 5μm); ODS-3 5 μm. Flow rate was 1 mL min^{-1} under room temperature. A RP-18 guard column was fitted upstream of the analytical column. The mobile phase was delivered by KNAVER K-1001 HPLC pump. The other equipments were; solvent organizer KNAVER K-1500, degasser KNAVER K-500, Sonobath (LIARRE-Italy), pH meter 744 (Metrohm-Switzerland), HPLC syringe 10, 25μL (Hamilton- Switzerland). The system was equipped by Eurochrom HPLC Software, Version 3.05.

Chromatographic conditions: The isocratic mobile phase consisted of three parts: aqueous solution (75 %) (adjusted to pH = 5 with HCl), methanol (20 %) and acetonitrile (5 %).The flow-rate was maintained at 1.0 mL min^{-1}. The mobile phase was degassed by sonication. A diode array detector operated at 200 nm was used to quantify diazepam. After finishing a sequence of samples the HPLC column was washed with methanol and water for 1h at flow rate of 1.0 mL min^{-1}.

Extraction process: In this research, liquid phase microextraction (LPME) was performed in a three-phase system where analyte in its neutral form was extracted from aqueous sample, through a thin layer of an organic solvent on the top of the sample and into an aqueous micro droplet suspended at the top of the organic phase[12,13]. In the latter, pH was selected to ionize the analytes to prevent back-extraction into the organic phase again. In this three-phase system providing an aqueous micro droplet extract, high performance liquid chromatography (HPLC) was typically used in the final chromatographic analysis. In the three phase mode when both extraction and back-extraction are included, excellent clean-up has been observed.

For extraction of acidic compounds, pH in the sample has to be adjusted into the acidic region to suppress analyte solubility, whereas pH in the acceptor solution should be high to promote analyte solubility. In this manner, the acidic compounds may easily be extracted into the organic phase and further into the acceptor phase without back-extraction to the organic phase again[14].

As a result, the suspended droplet microextraction was carried out as follow. The sample solution was placed in a 5 mL cylindrical glass vial. A 7 mm × 3 mm magnetic stir bar was placed into the 4.5 mL aqueous sample solution to ensure efficient stirring during the extraction. Afterward 400 μL organic solvent was delivered into the vial. Turn on the magnetic stirrer and adjust it to the 1000 rpm for 30 s. Consequently make a steady and gentle vortex. An aluminium foil was used to cover the vial during extraction to prevent the evaporation of the organic phase. A 10 μL suspended droplet of aqueous acceptor phase, adjusted at pH 9 (with NaOH) was placed into the surface of organic solvent. So, the analyte is back-extracted into the aqueous microdrop (acceptor phase). The solution was agitated with a stirring rate of 300 rpm during the extraction process. A 50 μL flat-cut HPLC microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland), was used for delivery and removal of the acceptor phase.

Before each extraction, the syringe was rinsed with acetone and then with de-ionized water for 10 times to avoid the analyte carry-over and air bubble formation. Eventually, aqueous droplet was withdrawn into the microsyringe and injected to HPLC for further analysis. The experimental set-up is illustrated in Fig. 1.

RESULTS AND DISCUSSION

Method optimization: Various parameters were investigated to determine the optimal sample extraction procedure. All the optimization experiments were accomplished on the analyte in concentration of 2 μg mL^{-1}.

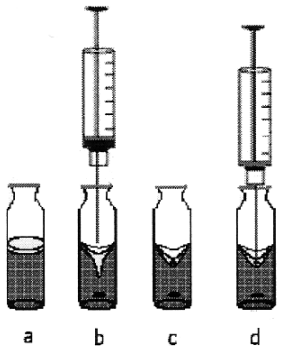

Fig. 1. Microextraction device; different steps in the developed method: (a) Organic solvent is added to the aqueous donor phase, extraction step (T_1); (b) Magnetic stirrer should be turned on, vortex is formed and aqueous droplet is added into the vortex; (c) back extraction occurs (T_2); (d) After the back extraction, droplet is withdrawn with the microsyringe

Organic solvent: Analyte was extracted from the aqueous sample solution (donor phase) into the organic phase and further into the aqueous acceptor phase. Since the analyte of interest is an acidic substance, the aqueous sample makes strongly acidic to ensure that the analyte is in their neutral form and the analyte could be easily extracted into the organic phase. Moreover, the basic solution (acceptor phase) provides a higher solubility of the analyte than the organic phase and prevents from re-entering the organic phase; thus, choosing the most suitable extraction organic solvent is very important for achieving high enrichment factors and good selectivity for the target analyte. The organic phase must therefore be immiscible with both the acceptor and donor phases. The solubility of the analyte should be higher in the organic phase than in the donor phase to promote the extraction of the analytes. In this manner, the solubility of the analyte should be lower in the organic phase compared to the acceptor phase in order to achieve a high degree of recovery of analyte in the acceptor phase.

Different organic solvents were investigated: *n*-Hexane, 1-octanol, benzylalcohol-octanol (30:70), toluene, hexane-dichloromethane (30:70), 1-octanol was chosen as the most suitable organic solvent. Because aqueous droplet just stable in 1-octanol[15,16] (Table-1)[17].

TABLE-1
CHARACTERISTICS OF ORGANIC SOLVENTS [Ref. 17]

Solvent	Density (g/cm^3)	Solubility in water (g/L)	Surface tension (dyne/cm)	Viscosity C.P. (25 °C)	log $P_{o/w}$
1-Octanol	0.830	0.0003	27.5	10.64	3.00
Benzyl alcohol	1.040	35.0000	39.0	4.43	1.10
Toluene	0.870	0.5300	28.5	0.59	2.69
n-Hexane	0.659	0.0130	18.4	0.31	3.90
Dichloromethane	1.325	13.0000	26.5	0.43	NA

NA = Not available.

Selection of pH conditions: Using the Henderson-Hasselbalch equation[18] the degree of protonation at varying pHs was calculated using pK values. When pH of the solution is equal to the pK of the analyte, equal amounts exist in ionized and unionized forms. Decreasing the pH of the solution, in case of acidic analytes, decreased the proportion which was associated at pH 1-7. A considerable portion of the analyte was present in the uncharged form and was amenable for extraction by LPME. The pH of the solution is an important variable when extracting the analyte by microextraction. In accordance with the Henderson-Hasselbalch equation, the extractable amount depends to a large extent on the pH of the solution and the pK value of the analyte. The lower the pH, greater the proportion which is capable of partitioning as a neutral molecule.

Adjustment of pH within the sample and acceptor phase are of high importance in three phase LPME in order to maximize distribution constants from the sample into the organic phase and so minimize the K_D from the organic phase to the acceptor phase.

The extraction involves pH adjustment of the sample solution to a pH where the analyte is uncharged, because the analyte must be extracted through the organic solvent and uncharged molecules have a better tendency for going into organic phase. The acceptor solution has a pH, which the analyte is charged and preventing it from back diffusion into the organic solvent. For practical applications, pH of the donor and acceptor phases should differ from the pKa values of the analytes by at least 2.5 units[19-21]. The effects of sample pH in the range of 1-7 were investigated. Thus, as shown in Fig. 2 in this study we used pH 2 for donor and pH 9 for acceptor phases, respectively.

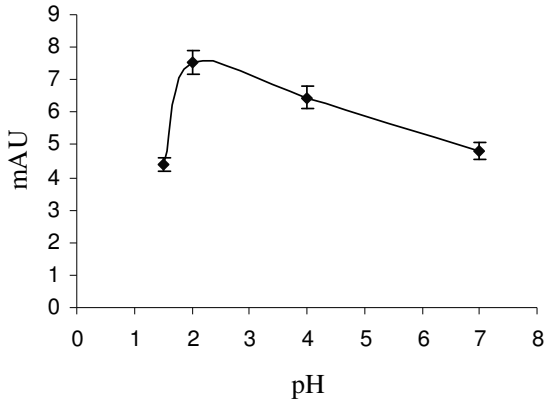

Fig. 2. Sample pH effect on the extraction process

Agitation rate: A more efficient means of agitating solutions in the three phase liquid phase microextraction, was required to minimize the static layer of solvent next to the droplet so that mass transport of the analyte into the acceptor phase was only limited by diffusion[22]. The static layer of organic phase near to the droplet was

found to be minimized by agitating the solution (*e.g.* by magnetically stirring each sample) minimizing diffusion problems and hastening the extraction process.

The effect of stirring of the sample speeds, the partition process thereby decreasing the amount of time to reach equilibrium. Stirring also aids in achieving homogeneity of the solution therefore a more representative analysis can be performed. The amount of analyte extracted is limited by the rate of diffusion through the sample matrix.

As a result, agitation of the sample is routinely applied to accelerate the extraction kinetics and enhance mass transfer in LLLME[23]. However, the aqueous droplet (acceptor phase) may be lost in case of more agitation. Therefore, as shown in Fig. 3, a stirring speed of 300 rpm was selected for this work.

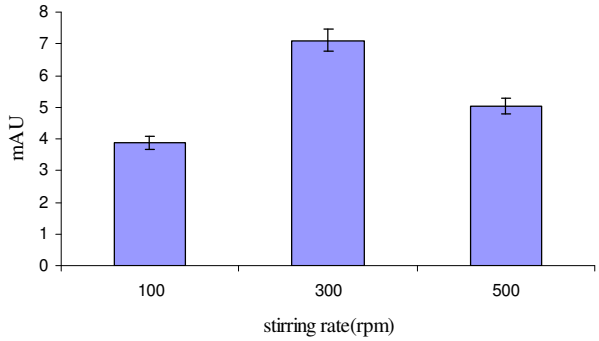

Fig. 3. Effect of stirring speed on the extraction process

Extraction time: Equilibration was determined in the order of 10 s to 5.0 min for the analyte in this study. The rate of partitioning was greatest at the periods ranging from 30 to 60 s, after it there was no significant improvement in the extraction results. Consequently, at *ca.* 30 s, equilibrium was attained between the analyte in solution and the acceptor aqueous solution.

Back extraction time: Extraction efficiency depends on the period of extraction and back extraction times. Hence, the function of extraction time was studied with respect to extraction efficiency. As shown in Fig. 4, the enrichment factors increased with increasing back extraction time. It took only 1 min for the back extraction to attain equilibrium and the EF did not increase significantly after too long[24].

Phase volume: It was found that LPME was much more sensitive to the magnitude of partition coefficients than LLE, because LPME is carried out with a very high volume ratio between sample and acceptor solution. Thus, whereas LLE may be accomplished with relatively large volumes of solvent to compensate for poor partition coefficients, LPME suffered from low recoveries either if the partition coefficient from the sample to the organic phase, or the partition coefficient from the organic phase to the acceptor phase, was low. The application area of three-phase LPME is therefore inferior compared to LLE, but for good three-phase LPME candidates,

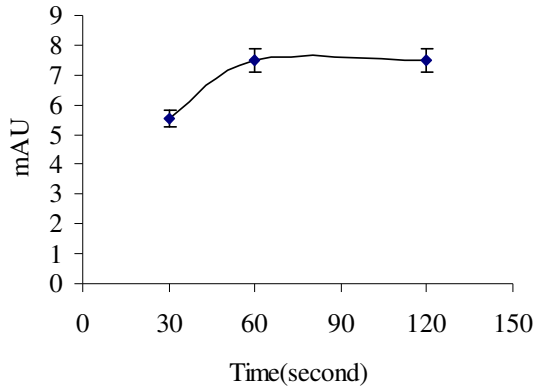

Fig. 4. Effect of extraction time on the extraction efficiency when using DSDME technique with 1-octanol as solvent. Other extraction conditions: analyte concentration 2 μg mL^{-1}, stirring speed 300 rpm, 4.5 mL donor sample volume, microdroplet volume 10 μL

preconcentration values are much higher in LPME than LLE. In addition, because three-phase is more selective in nature, it also provides higher sample clean up that LLE. Even in comparison with LLE with back extraction, which itself is known to be a very efficient clean up from biological samples.

The SDME theory reveals that the amount of analyte extracted by the drop is related to the volume of the drop and the use of a large drop results in an enhancement of the analytical sensitivity. In order to suspend a large liquid drop, efforts have been made to prepare a stable droplet. In these experiments, donor volume was kept constant and for change the phase volume ratio different volumes of drop were evaluated. The efficiency and enrichment factor can be improved by the increasing the volume ratio of donor and aceptor phases[25-27]. For studying aqueous droplet volumes of 5, 10, 15 and 20 μL were investigated. For 20 μL an aqueous droplet was not stable and it (acceptor phase) may be lost. Thus, as shown in Fig. 5 the volumes of the donor and acceptor phases were adjusted at 4.5 mL and 10 μL in this procedure, respectively.

Effect of ionic strength: It is common, in some microextraction applications, to add salt to an aqueous solution in order to reduce the solution's solvating power[23,28]. Hence, moderately water soluble compounds may be 'salted out' and go into the organic phase. In this case, addition of salt to the solution has been tried and discarded. The addition of salt had no effect with respect to the partitioning of analyte into the organic solvent and no salting out effect was observed. This was tested at various molar concentrations (0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 M) of NaCl for the diazepam. The salting-out effect appears to be more important for the less polar compounds. Salting-out only works for uncharged species of molecules which exhibit low water solubilities.

Linearity: The linearity of the method was studied in triplicate. The calibration curve (ranged from 25 to 5000 ng/mL) was constructed *versus* diazepam concentration (ng mL^{-1}).

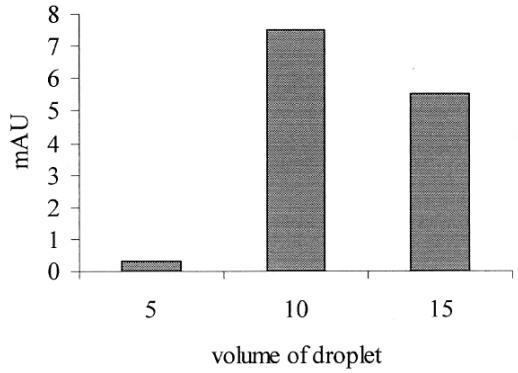

Fig. 5. Effect of microdroplet volume on the extraction efficiency. Extraction conditions: analyte concentration 2 μg mL^{-1}; 400 μL, 1-octanol as organic solvent; back extraction time 60 s; stirring speed 300 rpm; 4.5 mL donor sample volume

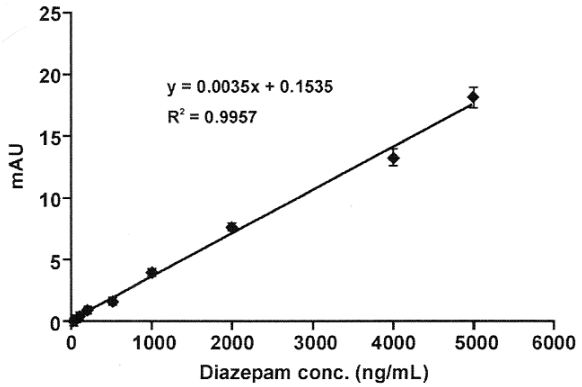

Fig. 6. Calibration curve (ranged from 25 to 5000 ng/mL)

TABLE-2
PERFORMANCE OF THE DSDME METHOD

Analyte	Enrichment factor	RSD % (n=3)	Linear range (ng/mL)	Correlation Coefficient (r^2)	LOD (ng/mL) (n=5)	LOQ (ng/mL) (n=5)
Diazepam	839.8	5.62	25-5000	0.9957	20	25

The equation describing the linearity showed an intercept of 0.1535 and a slope of 0.0035 having a correlation coefficient of 0.9957.

Sensitivity: The limit of quantitation (LOQ) of the method as signal/noise of 10 was equal to 25 ng/mL. Considering a signal/noise of 3, a LOD of 20 ng/mL was determined.

DSDME conditions: 4.5 mL aqueous sample with pH = 2 as the donor phase; 400 µL organic phase, 1-octanol; 10 µL aqueous drop with pH = 9 as the acceptor phase; T_1 = 30 s, T_2 = 60 s, stirring speed 300 rpm.

Extraction from natural waters: The standard solution of diazepam was spiked into real water samples to demonstrate the potential of this method as a viable extraction technique for environmental water samples.

The spiked real water data was compared to the HPLC water standards. A 1:1 correlation represents that the measured amount of analyte in each matrix is the same. Extraction recovery of diazepam from real water samples, clinical waste water, tap water and industrial waste water were assessed by calculating the ratio of the peak height of analyte in real water samples (spiked with 50 ng/mL of diazepam) extract and aqueous solution. An average recovery of 89.5 ± 6.4 % (mean ± SD) was obtained for tap water in concentrations (100, 1000 and 2000 ng/mL) studied (n = 5). In case of industrial waste water 78.9 ± 8.5 % and clinical waste water 104 ± 7.4 % were obtained (Table-3).

TABLE-3
RELATIVE RECOVERY AND RSD OF DSDME TECHNIQUE

Diazepam	Clinical waste water	Tap water	Industrial water
Relative recovery (%)	104	89.5	78.9
RSD (%)	7.4	6.4	8.5

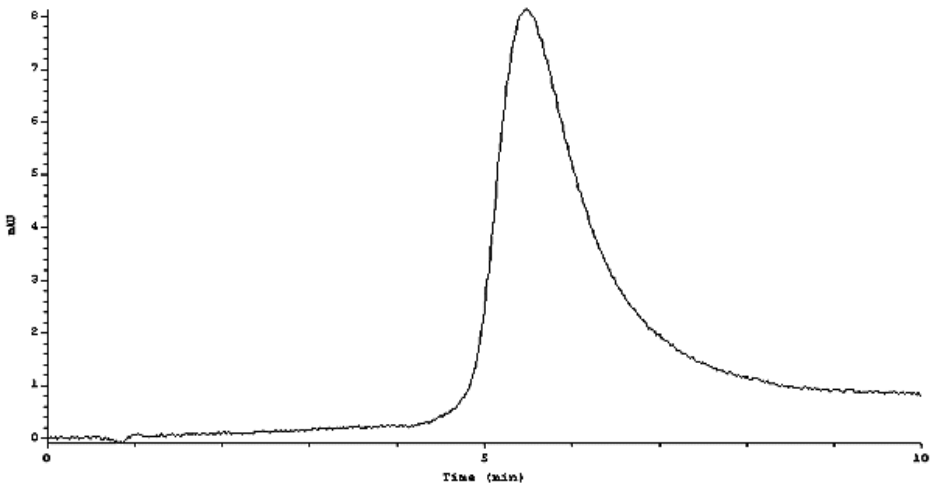

Fig. 7. Chromatograms of drinking water from the Mashhad water-supply network: the samples were spiked with 2 µg mL^{-1} of diazepam under optimum conditions

Comparison of DSDME with other sample preparation techniques: SD-LPME has a short extraction time, higher enrichment factor, quantitative recovery and lower solvent consumption.The mainly competing method (traditional liquid-liquid extraction) has lower enrichment factor and higher solvent consumption. It

uses about 10 mL or more solvent and EF for analytes is about 10 or less in most cases, whereas SD-LPME uses extraction solvent in the micro liter range with higher EF (about 839.8 in this study). Also with solid phase extraction (SPE) it is possible to obtain higher EF such as the presented technique, but it is very time-consuming in comparison with SD-LPME. The review of some methods which were used for determination of diazepam in the environmental and biological samples was shown in Table-4.

TABLE-4
COMPARISON OF SOME METHODS WHICH WERE USED FOR DETERMINATION OF DIAZEPAM

Year	Matrix	Extraction method	Detection	LOD	LOQ
1998	Water	SPME	GC-MS	0.02 g/mL	–
2001	Ground water	SPE	GC-MS	–	20 ng/L
2001	Plasma	LLE	HPLC	5 ng/mL	–
2002	Serum	SPME	HPLC	24 ng/mL	81 ng/mL
2003	Waste water	–	HPLC	18 ng/mL	61 ng/mL
2003	Water	–	LC-MS	–	20 ng/L
2004	Whole blood	DI-SPME	LC-MS	20-35 ng/mL	–
2004	Waste water	–	LC-MS	0.1 ng/mL	0.4µg/L
2005	Sludge	SPE	LC-MS	–	20 ng/g
2006	Tissue	SPE	GC-MS	2 µg/kg	–
2006	Water	SPME	IMS [2]	10 ng/mL	–
2007	Urine	LLE	GC-MS	–	0.15 ng/mL
2007	Urine	LLE	LC-MS	–	10 ng/mL
2007	Waste water	SPE	LC-MS	0.0002 µg/L	0.001 µg/L
	DLR	r^2	RSD%	Recovery %	References
1998	0.1-2µg/mL	–	12.5	–	**8**
2001	20-50 ng/L	–	–	102	**29**
2001	10-200 ng	0.9900	6.4	87	**30**
2002	50-50000 ng/mL	0.9990	4.6	–	**31**
2003	–	0.9972	5.2	–	**32**
2003	–	–	–	–	**33**
2004	50-1000 ng/mL	0.9960	10	–	**34**
2004	0.1-100 µg/L	0.9900	5.66	71.53	**5**
2005	–	–	11	59	**35**
2006	10-500 ng/mL	0.9999	3.9	74.7	**36**
2006	50-1000 ng/mL	0.9980	–	–	**37**
2007	0.1-1 ng/mL	0.9970	5.7	74.5	**38**
2007	0.05-40 ng/mL	0.9992	9.9	85.3	**39**
2007	–	–	4	101	**40**

[1]Dynamic linearity range. [2]Ion molecular spectroscopy.

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