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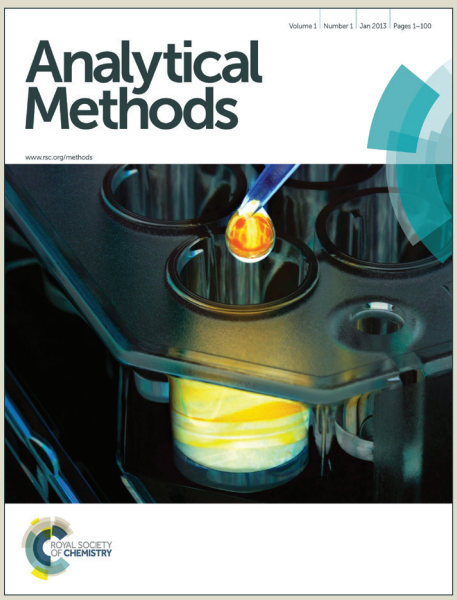

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ARTICLE TYPE

Cloud-point extraction followed by high pressure liquid chromatography with UV spectrophotometric detection for determination of permethrin in urine samples

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A new, effective cloud-point extraction (CPE) method for determination of the pesticide, permethrin, from the pyrethroid group, in human urine, by high-pressure liquid chromatography with UV spectrophotometric detection, was developed and validated. The key extraction conditions were as follows: surfactant 5% (w/v) Triton X-114, temperature and incubation time of 40°C and 30 min, respectively, and 100µL organic solvent (acetonitrile) for dissolving the micellar phase. The acetonitrile micellar phase with the isolated permethrin was analyzed by reverse-phase high-pressure liquid chromatography using the gradient flow of a mobile phase consisting of water and acetonitrile. The main analytical parameters of the developed method were: mean extraction recovery (89.9%), intra- (7.1, 9.1 and 13.6 %RSD for 0.25, 0.5 and 1.0 µg mL^{-} concentrations of permethrin, respectively) and interday (6.1, 10.3 and 14.1 %RSD for 0.25, 0.5 and 1.0 µg mL^{-1} concentrations of permethrin, respectively) repeatability, limit of detection (0.025 µg mL^{-1}) and limit of quantification (0.075 µg mL^{-1}), as well as the linear range (0.075 – 2.000 µg mL^{-1}, r^2 =0.9975). The evaluated parameters have enabled the proposed method to be hopefully useful for the monitoring of permethrin in urine samples taken from individuals exposed to this pesticide

Key words: permethrin, cloud-point extraction, HPLC-UV, urine

Introduction

Permethrin (3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate) (Table 1) belongs to synthetic pyrethroids. Pyrethroids constitute a class of insecticides that are employed for controlling a wide range of pests in both agricultural and urban environments, including residential applications.[1] Permethrin is especially commonly used in numerous formulations and other accessories (e.g. collars) to control insect pests on animals,[2] besides on humans (e.g. scabies, lice).[3]

The toxic action of pyrethroids is mainly caused by action on axons in the nervous system that interact with the sodium channels and affect the electric impulse transmission. These processes stimulate the nervous cells, inducing repetitive nerve activity with several electric shocks resulting in the total paralysis of the insect.[4] Generally, pyrethroids demonstrate effective toxicity on a large range of insect pests and have a much lower mammal to insect toxicity ratio than their counterparts from organophosphate or carbamate pesticide groups.[4] However, misuse of pyrethroids and human exposure to them may trigger such adverse effects as dizziness, headache, nausea, loss of appetite, or fatigue.[5]

Permethrin is excreted from the organism as a parent pesticide and metabolites with urine. Pharmacokinetic studies of permethrin are focused mainly on dermal adsorption from disinfected clothes.[6-8]

Pyrethroids, including permethrin, were determined in different biological samples, like plasma, serum, brain and urine, mostly using SPE as the sample preparation technique and various analytical methods. From the applied determination techniques, the following may be listed: HPLC-UV,[9-12] LC-MS/MS,[13] GC-MS[14] and GC-MS/MS.[15]

In this work we focused on the application of an unconventional extraction technique, cloud-point extraction (CPE), to isolate permethrin from urine samples and then determine it by the HPLC-UV method To the best of our knowledge this is the first generally available publication (except for a publication in Chinese[16]) where this extraction method was employed for analysis of human urine containing permethrin.

Experimental

Reagents and examined biological material

The surfactant Triton X-114 (analytical grade) was purchased from Sigma–Aldrich (Munich, Germany). The standard substances of permethrin and alprazolam also were also supplied by Sigma – Aldrich (Munich, Germany). The chemical structures

of permethrin and alprazolam (IS), as well as their basic physicochemical properties[17-19] have been placed in Table 1.

Table 1. Structures and physicochemical properties of permethrin and alprazolam (IS)

Methanol and acetonitrile (gradient grade) were purchased from Merck (Darmstadt, Germany). Acetic acid and sodium hydroxide were supplied by POCh (Gliwice, Poland). Blank (control) urine was obtained from an unexposed healthy volunteer, all experiments were performed in compliance with the relevant laws and institutional guidelines.

Standard solutions and preparation of samples

The stock methanolic solutions of permethrin (1mg mL^{-1}) and the internal standard - alprazolam (1 mg mL^{-1}) were stored in a refrigerator (at 4°C). The standard solutions were prepared by appropriate dilution of the stock solutions with acetonitrile and water (1:1, v/v). The urine samples were prepared by spiking with appropriate amounts of standard solutions of permethrin and alprazolam.

General CPE procedure

2 mL of the surfactant Triton X-114 at an appropriate concentration was added to 2 mL of control urine adjusted to a suitable pH value. The sample was incubated at 40°C for 30 min. The resultant micellar phase with the isolated permethrin was centrifuged at 13 000 rpm for 10 min and then was cooled in a refrigerator for 5 min. The upper water phase (with the surfactant below critical micellar concentration) was decanted off and the lower micellar phase was dried at 40°C for c.a. 1h. The dried micellar phase was dissolved with an appropriate volume of acetonitrile.

Apparatus and chromatographic conditions

The chromatographic system, Merck-Hitachi LaChrom, consisting of an L-7100 pump and an L-7455 UV spectrophotometric detector (Darmastadt, Germany) was equipped with the Eurospher II 100 C18 H column (250 mm x 4.6 mm x 5 µm) supplied by KNAUER (Germany), which was thermostatted to 25ºC. A mixture of water (A) and acetonitrile (B) was used as the mobile phase. The following optimal gradient conditions of the mobile phase flow were applied: 0min (20%A and 80%B), 5min (10%A and 90%B), 20min (10%A and 90%B) and 25min (20%A and 80%B). The flow rate was 1 mL min^{-1}. Permethrin and the internal standard were detected at $\lambda = 210$ nm.

Results and Discussion

Optimalization of CPE conditions

The key three factors influencing CPE efficacy: 1. sample pH, 2. surfactant concentration, and 3. acetonitrile volume (a solvent for dissolution of the micellar phase with the isolate analyte) were studied. Other experimental conditions, such as the temperature (40°C) and time (30 min) of incubation, speed (13 000 rpm) and time (10 min) of centrifugation, temperature (40°C) and time (c.a. 1h) of the micellar phase evaporation were selected on the basis of earlier examinations [20], and were kept constant during the sample preparation process.

In each experiment 2 mL aqueous Triton X-114 solution to a 2 mL urine sample was added. The extraction recovery of permethrin was considered as the optimization criterion. The study of optimal CPE conditions was conducted using one changeable variable.

The recovery values (RV, %) of permethrin were calculated using the following expression:

$RV\% = A_p/A_{st} \times k \times 100\ \%$ (1)

where: A_p - peak area of permethrin in a urine sample extract

A_{st} – peak area of permethrin in a standard solution (acetonitrile:water, 1:1, v/v)

k - correction coefficient = measured sample volume after dissolution of the final micellar phase with the solvent)/ volume of the solvent added)

For each surfactant concentration the diluted micellar phase volumes were measured and the experimentally determined values were considered in the correction coefficient (k).

Study of sample pH on permethrin recovery

For organic compounds, especially for highly ionizable species, the pH belongs to the critical factors regulating the partitioning of the analyte in the micellar phase. Maximum extraction efficiency is achieved at pH values where the uncharged form of the analyte prevails.

Taking into account the nonionizability of permethrin (Table 1), it was not possible to observe the essential sample pH effect on permethrin recovery. However, in order to confirm this prediction, an appropriate sample pH study was performed.

Control urine samples spiked with permethrin and IS were prepared at five pH levels: 4.5; 5.5; 6.5; 7.5 and 8.5 and treated according to a general CPE procedure and then subjected to chromatographic analysis. The acidic pH (4.5 and 6.5) or basic pH (7.5 and 8.5) were adjusted by the addition of appropriate amounts of 1M acetic acid or 0.5M sodium hydroxide, respectively. The pH 5.5 was achieved without any modification of the urine samples. The other optimized parameters: surfactant concentration (7.5% w/v) and acetonitrile volume (150 µL) were kept constant.

The results showed that the CPE efficacy was not essentially dependent on the sample pH in the examined pH range (Fig.1 left), and therefore an unchangeable sample pH was used for further study.

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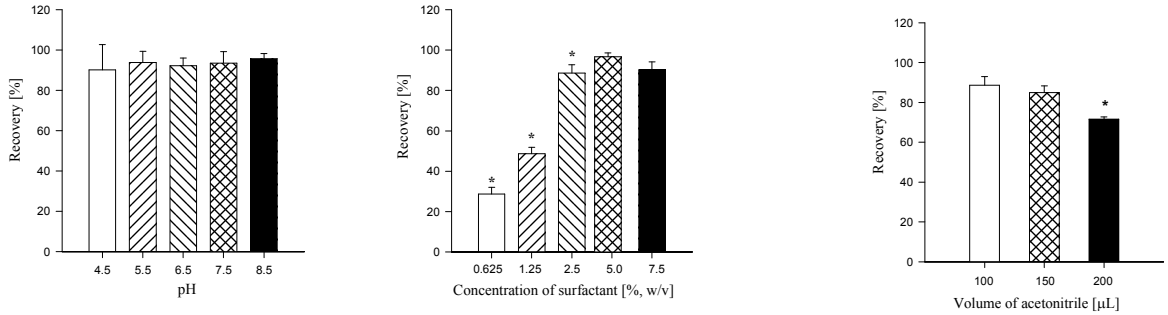

Figure 1. Effect of sample pH (left), surfactant concentration (middle) and volume of acetonitrile (right) on permethrin recovery from urine

* statistically significant from maximum recovery $p<0.05$

Study of surfactant concentration on permethrin recovery

Concentration of the used surfactant in the sample solution is another important factor influencing the efficacy of CPE. When selecting the surfactant concentration, one should consider the compromise between achievement of high preconcentration factors and the resultant micellar phase volume that should be sufficient for making reproducible extractions.

The surfactant Triton X-114 was added to urine samples at the five concentration levels: 0.625; 1.25; 2.5; 5.0 and 7.5 (w/v) and before the chromatographic measurements, the samples were prepared according to the CPE procedure using 150 µL acetonitrile for the dissolving of each obtained micellar phase. Taking into account the obtained results (Fig.1 middle), 5% Triton X-114 was selected for further study as the optimal surfactant concentration.

Study of the solvent volume on permethrin recovery

In order to achieve an appropriate high concentration coefficient of the analyte, the resultant micellar phase with the isolated analyte should be dissolved in a small amount of acetonitrile for analysis. However, some lost amounts of the analyte where there is an insufficient volume of the solvent should be also considered. The resultant micellar phases were dissolved in the following acetonitrile volumes: 100, 150 and 200 µL, and the obtained extraction results are given in the right of Fig.1 right (100 µL was the lowest volume which was able to be sampled by the used autosampler).

The use of 100 µL acetonitrile volume (providing a concentration coefficient of 9.7) turned out to be the best one (the lowest concentration coefficient with the completely dissolved micellar phase was obtained).

Chromatograms of: a blank urine, an urine extract containing permethrin and IS, and a standard substance mixture, are presented in Fig.2.

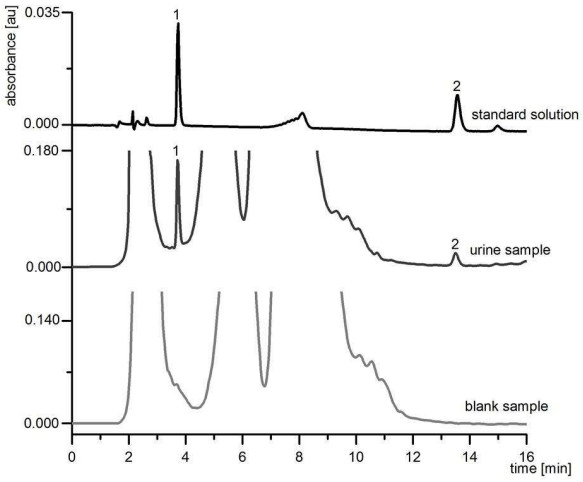

Figure 2. HPLC chromatograms of: blank urine, urine spiked with permethrin (peak 2- 1 µg mL^{-1}) and IS (peak 1 - 2.5 µg mL^{-1}), and standard mixture of permethrin (peak 2 -5 µg mL^{-1}) and IS (peak 1 - 5 µg mL^{-1}). The unnumbered peaks appearing in the range from 2 to c.a.12 min correspond to the surfactant.

Validation of the CPE-HPLC-UV method for the determination of permethrin in urine

Under the optimized conditions of CPE the following main analytical parameters of the method were determined: extraction recovery, intra- and interday repeatability, limits of detection and

quantification, and linear range.

Extraction recovery

The extraction recovery of CPE for permethrin was examined at its three concentration levels (0.25, 0.5 and 1.0 μg mL^{-1}) and calculated according to the equation (1). For each permethrin concentration, six urine samples after the CPE method were measured parallel with appropriate standard permethrin solutions by the HPLC-UV method. The mean recovery values for permethrin accounted were 75.7, 85.0 and 108.9% for 0.25, 0.5 and 1.0 μg mL^{-1} permethrin concentration levels, respectively (Table 2).

Intra- and interday repeatability

For the determination of measurement repeatability, two series (in two subsequent days) of four urine samples at the three concentration levels of permethrin (0.25, 0.5 and 1.0 μg mL^{-1}) and IS (1 μg mL^{-1}) were prepared using the CPE procedure. Repeatability of the quantitative parameter (ratio of permethrin and IS areas) was calculated as the relative standard deviation (RSD, %). Intraday repeatability accounted for 7.1, 9.1 and 13.6 %RSD for the low, middle and high permethrin concentration level, respectively. The repeatability of this parameter, between days, for the low, middle and high concentration levels of permethrin were 6.1, 10.3 and 14.1 %RSD, respectively (Table 2).

Limits of detection and quantification

In order to determine the LOD of permethrin, control urine was spiked with this pesticide at five concentrations: 0.125, 0.25, 0.5, 1.0 and 2.0 μg mL^{-1} and IS (1μg mL^{-1}) and then subjected to the CPE procedure. The LOD calculated from the expression: LOD = 3.3 x s/b (where: s – standard deviation for noise of the blank sample series and b – slope of calibration curve) was 0.025 μg mL^{-1}. The LOQ (three times the LOD value) was 0.075 μg mL^{-1} (Table 2) .

Linearity range

The linearity range of the developed method for the determination of permethrin in urine was examined in the range from 0.075 to 2.0 μg mL^{-1}, and the correlation coefficient was 0.9975 (Table 2).

Table 2. Retention times and validation parameters of the CPE-HPLC-UV method for determination of permethrin in urine

Based on the literature, permethrin has been determined in different biological materials (plasma, blood and urine) with the use of solid-phase extraction[9-12, 15] followed by such analytical techniques as HPLC-UV, LC-MS/MS and GC-MS or GC-MS/MS. In order to compare the obtained results for the determination of permethrin in urine with the proposed CPE-HPLC-UV method, the experimental conditions and analytical parameters of the above-mentioned methods are noted in Table 3. In three cases[9-11] the authors used the HPLC-UV method combined with solid-phase extraction for detection and quantification of permethrin in rat urine. The extraction recovery of permethrin from urine ranged from 77.9 to 83.9% and were a little lower or comparabed with the proposed CPE procedure (mean 89.9%) and the limits of detection (0.050 μg mL^{-1}) and quantification ranged from 0.100 to 0.150 μg mL^{-1} and were higher than those obtained in our method (LOD = 0.025 and LOQ = 0.075 μg mL^{-1}). However, the repeatability of the results in the current method (RSD%, mean 9.9 and 10.2 for intra- and interday measurements, respectively) is lower in comparison with the results repeatability obtained by the cited authors (RSD%, 4.2-4.3).[9-11]

Table 3. Methods for determination of permethrin in human urine

To our best knowledge, this was the first time when the unconventional extraction technique: cloud-point extraction (CPE) was employed for the isolation of permethrin from human urine. Permethrin as a highly hydrophobic compound (Table 1) is very susceptible to being extracted in the hydrophobic core of the micelles of the surfactant Triton X-114.

The obtained recoveries of permethrin from urine by the proposed CPE procedure are generally similar to those reported in the literature. Taking into account the limits of detection and quantification presented by the authors who involved the HPLC-UV technique for the determination of permethrin in urine, these parameters are comparable to our results with one exception which was when 10 mL-volume urine samples were used and LOD's values for trans- and cis-permethrin were lower by c.a. one order of magnitude.[12] The main advantages of the proposed CPE procedure over the reported conventional sample preparation methods are: simplicity, the requirement of simple and low cost laboratory instruments, and the ecological aspect (reduction of toxic organic solvent use) without any loss of the results' quality. Among the drawbacks of the proposed extraction procedure the requirement of the setting up of appropriate chromatographic separation conditions, usually using a gradient mobile phase flow (for avoiding interferences of the analyte with surfactant signals in the case of UV spectrophotometric detection use), as well as the necessity to rinse out the surfactant from the chromatographic column by acetonitrile (or methanol) between measurements should be noted.

Conclusions

A new HPLC-UV method preceded by a CPE procedure for the determination of permethrin in urine samples was developed and may be useful for the monitoring of permethrin in human urine taken from people dressed with uniforms disinfected with this pesticide and exposed to it. The proposed combination CPE technique with HPLC-DAD method could be a useful alternative for "classic" sample preparation techniques as: liquid-liquid extraction (high volume of toxic solvents) and solid phase extraction (relatively high cost of SPE columns). This extraction technique may be especially useful for isolation of appropriately high hydrophobic compounds like for example pesticides from pyrethroid group represented by permethrin.

The developed CPE-HPLC-UV method is simple, cheap and relatively quick, as well as being also characterized by good

analytical parameters (LOD, LOQ extraction recovery and repeatability), theat are comparable with those obtained with the use of conventional sample pretreatment techniques, or in some cases more advanced analytical methods. Additionally, the proposed extraction procedure requires small amounts (100 µL) of the organic solvent and the surfactant (which is relatively mild for environment), so we can classify its as a "green chemistry" method.

References

1 R. Budd, S. Bondarenko, D. Haver, J. Kabashima and J. Gan, *J. Environ. Qual*., 2007, **36**, 1006-1012.

2 A. Anadon , M.R. Martınez-Larranaga and M.A. Martinez, *Vet. J*., 2009, **182**, 7–20.

3 K. Gunning, K. Pippitt, B. Kiraly and M. Sayler, *Am. Fam. Physician*, 2012, **86**, 535-541.

4 V. Pérez-Fernández, M. A. García and M. L. Marina, *J. Chromatogr. A*, 2010**, 1217**, 968–989.

5 F. He, S. Wang, L. Liu, S. Chen, Z. Zhang and J. Sun, *Arch. Toxicol*., 1989, **63**, 54-58.

6 B. Rossbach, A. Niemietz, P. Kegel and S. Letzel, *Toxicol. Lett.*, 2014, **231**,147–153.

7 J. Cote, Y. Bonvalot, G. Carrier, C. Lapointe, U. Fuhr, D. Tomalik-Scharte, B. Wachall, M. Bouchard, LOS One. 2014, 9, e88517- e88517.

8 B. Rossbacha,, K. E. Appelb, K. G. Mrossc and S. Letzel, *Toxicol. Lett*., 2010, **192**, 50–55.

9 A. W. Abu-Quare and M. B. Abou-Donia, *Biomed. Chromatogr*. 2001, **15**, 464–470.

10 A. W. Abu-Qare and M. B. Abou-Donia, *J. Chromatogr. B*, 2000, **749**, 171–178.

11 A W. Abu-Qare and M. B. Abou-Donia, *J. Pharm. Biomed. Anal.,* 2001, **26**, 291–299.

12 M. E. Leon-Gonzalez, E. M. Plaza-Arroyo, L.V. Perez-Arribas and L.M. Polo-Dıez, *Anal. Bioanal. Chem*., 2005, **382**, 527–531.

13 J. M. Starr, S. E. Graham, D. G. Ross, et al., *Toxicology*, 2014, **320**, 15–24.

14 S. A. Cherstniakova, G. E. Garciaet al., *J. Anal. Toxicol*., 2006, **30**, 21-26.

15 R. Cazorla-Reyes, J. L. Fernández-Moreno, R. Romero-González, A. G. Frenich and J. .L. M. Vidal, *Talanta*, 2011, **85**, 183–196.

16 S. Zhang, X. Chen, Z. Yu, X.Shen, M. Gou and K. Bi, *Zhongguo Zhong Yao Za Zhi*, 2009, *34*, 2577-80.

17 D. A. Laskowski, *Rev. Environ. Contam. Toxicol*., 2002, **174**, 49-170.

18.National Pesticide Information Center, http://npic.orst.edu/factsheets/Permtech.html (available, Septemder 2014).

19 A. C. Moffat, M.D. Osselton and B. Widdop (Eds.*), Clarke's Analysis of Drugs and Poisons, 3rd edition*, Pharmaceutical Press, London, 2004.

20 K. Madej, K. Persona, M. Wandas and E. Gomółka, *J. Chromatogr. A*., 2013, **1312**, 42-48.

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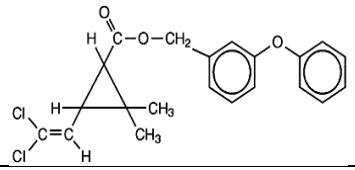
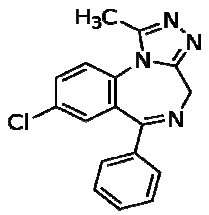
Table 1. Structures and physicochemical properties of permethrin and alprazolam (IS)

Compound	Structure	Physicochemical properties			
		Solubility in water (mg mL^{-1})	Log K_a	Log P	UV absorption spectrum [19, 20]
Permethrin		practically insoluble (5.5×10^{-3}) [18]	-[a] [18]	6.1 (20°C) [17] 4.19 (20°C) [18]	λ_{2max} = 207 nm λ_{1max} = 273 nm
Alprazolam (IS)		practically insoluble [20]	2.4 [19]	2.12 (25°C) [19]	λ_{1max} = 225 nm λ_{1max} = 260 nm

[a]) non ionizable compound

Table 2. Retention times and validation parameters of the CPE-HPLC-UV method for determination of permethrin in urine

Compound	Analytical parameter														
	RT [min]	**RRT[a]**	**Intra-day repeatability of identification parameter (RRT) [%, RSD], n=6**	**Inter-day repeatability of identification parameter (RRT) [%, RSD], n=12**	**Extraction recovery, ER [%], n=6**			**Intra-day precision of quantification parameter[b] [%, RSD], n=6**			**Inter-day precision of quantification parameter[b] [%, RSD], n=12**			**Limit of detection, LOD [µg mL^{-1}]**	**Limit of quantification, LOQ [µg mL^{-1}]**	**Linearity range [µg mL^{-1}] (r^2)**
					0.25	0.50	1.0	0.25	0.50	1.0	0.25	0.50	1.0			
					[µg mL^{-1}]			**[µg mL^{-1}]**			**[µg mL^{-1}]**					
Permethrin	13.63	3.62	0.01	0.03	75.7	85.0	108.9	7.1	9.1	13.6	6.1	10.3	14.1	0.025	0.075	0.075 -2.0 (0.9987)
Alprazolam (IS)	3.77	1.00	-	-	38.7[c]			3.9[c]			5.1[c]			-	-	-

[a]) relative retention time
[b]) ratio of permethrin and alprazolam areas
[c]) for 1 µg mL^{-1} of alprazolam

Table 3. Methods for determination of permethrin in urine samples

Extraction method	Basic extraction conditions			Analytical method	Analytical parameters					Ref.
	Column/eluent	Extraction solvent	Surfactant type and concentration/ extraction temperature/ extraction time		Recovery value (%)	Repeatability RSD (%)	Linearity (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	
SPE	Disposable C18 Sep-Pak Vac 3cc (500 mg) cartridges /methanol (1 mL) and then acetonitrile (1 mL)	-	-	HPLC-UV	77.9	9.1	0.100-1.000	0.050	0.100	[10]
SPE	Disposable C18 Sep-Pak Vac 3cc (500 mg) cartridges/ methanol (2 mL) and then acetonitrile (2 mL)	-	-	HPLC-UV	80.7	4.2	0.100-1.000	0.050	0.100	[9]
SPE	C18 Sep-Pak cartridges (500 mg) /dichloromethane (5 mL)	-	-	GC-IT-MS/MS	91.0	5.5	0.100-1.000	0.028	0.093	[15]
SPE	Disposable C18 Sep-Pak Vac 3cc (500 mg) cartridges/ acetonitrile (2 mL) and then metanol (2 mL)	-	-	HPLC-UV	83.9	4.3	0.100-1.000	0.050	0.150	[11]
SPE	RAM-C18 cartridges/0.5% methanol (5 mL, 1.5 mL min^{-1})	-	-	HPLC-UV	70 (trans-permethrin) 72 (cis-permethrin)	5.6[a] (trans-permethrin) 6.0[a] (cis-permethrin)	0.022-0.700 (trans-permrthrin) 0.050-0.100 (cis-premethrin	4.4[a] (trans-permethrin) 6.1[a] (cis-permethrin)	-	[12]

CPE	-	-	Triton X-114 5% (w/v)/ 40°C/ 25min	HPLC-UV	mean 89.9	mean 9.9 (intraday) mean 10.2 (interday)	0.075-2.000	0.025	0.075	(our method)

[a]) in units of μg L^{-1}