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Solid phase microextraction—Liquid chromatography (SPME-LC) determination of chloramphenicol in urine and environmental water samples

A. Aresta, D. Bianchi, C.D. Calvano, C.G. Zambonin *

Università degli Studi di Bari, Dipartimento di Chimica, Via Orabona, 4, 70126 Bari, Italy

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ABSTRACT

A solid phase microextraction—liquid chromatography with ultraviolet detection (SPME-LC-UV) method for the determination of the antimicrobial agent chloramphenicol was developed. The performances of three commercially available fibers were compared; the Carbowax/TPR-100 was found to provide the most efficient extraction. All the aspects influencing the fiber adsorption (extraction time, temperature, pH, salt addition) and desorption (desorption and injection time, desorption solvent mixture composition) of the analyte were investigated. The method was eventually applied to the determination of the drug in both biological (urine) and environmental (tap and sea water) samples. The optimized procedure required a simple sample pretreatment, isocratic elution, and provided enough sensitivity for the analyte determination in the considered samples. The investigated linear ranges were 37–1000 ng/ml (urine), 0.1–10 ng/ml (tap water), 0.3–30 ng/ml (sea water). Within-day and between-days RSD% ranged between 5.5–6.2 and 8.7–9.0 (urine), 5.1–6.0 and 8.4–8.8 (tap water), 5.4–5.7 and 8.6–8.9 (sea water). Estimated LOD and LOQ were 37 and 95 ng/ml (urine), 0.1 and 0.3 ng/ml (tap water), 0.3 and 0.7 ng/ml (sea water).

1. Introduction

A growing interest has been observed in the past few years in the determination of a wide range of emerging contaminants whose presence in aqueous matrices has still not been regulated, such as algal and cyanobacterial toxins, hormones and other endocrine-disrupting compounds, surfactants, perfluorinated compounds, pharmaceuticals or personal-care products. Among pharmaceuticals, antibiotics, a term that comprises a wide spectrum of substances, are of particular concern; the large amounts of antibiotics used in both human and veterinary medicine have led to their occurrence in the environment. After their application and excretion, residual human antimicrobials frequently end up entering into municipal sewage-treatment plants. Antibiotics can induce bacterial resistance, even at low concentrations, through continuous exposure. In fact, the increasing use of these compounds has resulted in genetic selection of more harmful bacteria in recent years [1].

In view of the above consideration, sensitive and selective methods for their determination both in biological fluids and the aquatic environment are highly advisable. LC has become the technique of choice for antibiotics determination, since they are polar compounds, insufficiently volatile or too thermally unstable to allow their direct GC determination without a previous derivatization step. The state-of-the-art of the environmental analysis of antibiotics has been recently thoroughly reviewed, focusing on sample preparation, analyte stability and matrix effects [1] and on analytical methods based on LC–MS^2 for antibiotics determination in surface ground and waste waters [2,3], respectively.

Chloramphenicol (CAP) is an antimicrobial agent used since 1950, active against both Gram-positive and Gram-negative bacteria [4]. It inhibits bacterial protein synthesis by blocking the transfer of soluble ribonucleic acid to ribosome. It is widely used in the treatment of serious infections including typhoid fever and other forms of salmonellosis and also in animal production, even if recent research revealed that CAP has serious side effects on the haemopoietic system, i.e. mile anemia with reticulocytopenia, sometimes accompanied by leucopenia and thrombocytopenia and aplastic anemia [5]. As a consequence, it has been banned for use in foodstuffs of animal origin in the European Union and United States [6,7]. However, it is sometimes used topically for eye infections; nevertheless, the global problem of advancing bacterial resistance to newer drugs has led to renewed interest in its use [8]. Furthermore, in low-income countries, chloramphenicol is still widely used because it is exceedingly inexpensive and readily available.

In humans, CAP is eliminated primarily following biotransformation: much as 90% of administered chloramphenicol is eliminated in urine as the chloramphenicol glucuronide con-

* Corresponding author. Tel.: +39 080 5442506; fax: +39 080 5442026.
E-mail address: zambonin@chimica.uniba.it (C.G. Zambonin).

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jugate. Nevertheless, in the environment the main metabolite chloramphenicol glucuronide is deconjugated by bacterial to chloramphenicol, thus reactivating the parent drug [9].

In view of the above considerations, sensitive and accurate analytical methods for CAP determination are highly desirable. In fact, several papers have been published to date in order to monitor CAP levels in various matrices, namely animal tissues [10–15], honey [10,16–19,22], seafood [12,18,19,20], urine [21,22], milk [10,11,22] and many others, especially by means of gas-chromatography (GC) [13], liquid chromatography (LC) [16,21], GC–mass spectrometry (MS) [12,15,22], LC–MS [21], and LC–MS/MS [14,17,18,21]. On the contrary, the literature dealing with its determination in environmental water samples is more limited [23–25]; multiresidue (including CAP) methods were recently developed by LC-UV-DAD [23,24] and SPE-LC/MS/MS [25].

Most of the existing methods for CAP determination are time consuming require complex isolation procedures to separate the analyte from the original matrix and employ toxic solvents. These drawbacks can be avoided adopting solid phase microextraction (SPME), a technique that can be applied in combination with GC [26] or can be easily coupled to LC [27] by means of a dedicated interface mounted instead of the injector loop. Compared to traditional extraction techniques, SPME is cheap (one fiber can be generally used for hundreds of extractions), rapid and simple; furthermore, no harmful solvents are needed.

In this work, a solid phase microextraction (SPME)-LC-UV method for the determination of chloramphenicol was developed for the first time using a Carbowax/Templated Resin (CW/TPR-100) coated fiber and successfully applied to its determination in human urine, tap and sea water samples.

2. Experimental

2.1. Chemicals

Chloramphenicol was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Stock solutions (1 mg/ml) of chloramphenicol were prepared in methanol and stored at 4 °C in the dark. Dilute solutions were prepared just before use. Organic solvents (Aldrich Chemical Co.), were HPLC grade. Mobile phase was filtered through a 0.45 μm membrane material (Whatman Limited, Maidstone, UK) before use. β-Glucuronidase from bovine liver was obtained from Sigma.

2.2. Apparatus

The SPME interface (Supelco, Bellefonte, PA, USA), consisted of a standard six-port Rheodyne valve equipped with a fiber desorption chamber (total volume: 60 μl), installed in place of the sample loop.

The TurboVap II Concentration Evaporator Workstation, a microprocessor-controlled concentrator used for sample preparation that provides fast automated sample evaporation, was purchased by Caliper LifeSciences (Hopkinton, MA, USA). The system uses a gas vortex shearing action and optical sensors to provide fast and efficient simultaneous (6 samples) evaporation of organic solvents. Units may be configured to accommodate up to 200 ml glassware.

The LC system used in this study includes a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA, USA) and a Luna C18 (150 × 4.6 mm i.d, particle size 5 μm) chromatographic column (Phenomenex, Torrance, CA, USA). Mobile phase was degassed by an SCM 1000 vacuum membrane degasser (Thermo Separation Products). The detector was a photodiode-array (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer.

2.3. Chromatographic and detection conditions

The mobile phase used was an acetonitrile/ammonium acetate buffer (10 mM, pH 4.6) mixture (70:30, v/v). The flow rate was 1.0 ml min^{-1} and temperature was ambient. The detection wavelength was 278 nm (10 Hz frequency, 5 nm bandwidth). Spectra were acquired in the 220–380 nm range (2 Hz frequency, 5 nm bandwidth).

2.4. Solid phase microextraction

Fibers coated respectively with a 50 μm thick Carbowax/Templated Resin (CW/TPR-100) film, a 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB) film and a 85 μm thick polyacrylate (PA) film (Supelco) were employed for comparative studies. A manual SPME device (Supelco) was used to hold the fiber. Working standard solutions were prepared by spiking 5 ml of an ammonium acetate buffer (10 mM, pH 5.0) solution with different amounts of CAP (10–1000 ng/ml) into 7 ml clear vials (Supelco). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The optimized extraction was carried out under magnetic stirring at 50 °C for 30 min in the presence of 200 mg/ml of sodium chloride. Chloramphenicol optimized desorption was performed in static desorption mode by soaking the fiber in an acetonitrile/ammonium acetate buffer (10 mM, pH 4.6) mixture (70:30, v/v) into the desorption chamber of the interface for 5 min. Then, the valve was changed to the inject position and the fiber was exposed for 10 s to the mobile phase stream.

In order to evaluate percentages of desorption and carryover, the fiber was left in the chamber after each experiment and a second chromatographic run was performed leaving the interface valve in the inject position (dynamic desorption); this operation mode ensured a total desorption of the analyte remained on the fiber.

2.5. Urine samples

Samples were collected from healthy donors in the early morning. Then, 5 ml of all samples were subjected to deconjugation (pH 5.0, 37 °C) by β-glucuronidase (5000 U) for 90 min. Finally, 1.5 g of sodium sulphate were added in order to disrupt bindings between CAP and urine proteins, filtered through a 0.45 μm Millex-HV Hydrophilic polyvinylidene fluoride filter (Millipore, Billerica, MA, USA) and subjected to the SPME procedure.

2.6. Water samples

All samples were stored at −20 °C. All samples (100 ml sea water, 300 ml tap water, respectively) were filtered through a 0.45 μm nylon membrane (Whatman Limited, Maidstone, UK) and evaporated (at 90 °C using a nitrogen pressure of 1.3 bar; evaporation speed was about 1.5 ml/min) in the TurboVap II Concentration Evaporator Workstation to a final volume of 5 ml (30% NaCl was also added to tap water). Finally, acetic acid was added to obtain a pH value of 5.0 and samples were subjected to SPME.

2.7. Quantitation

Calibration curves were constructed spiking drug free samples with variable amounts of chloramphenicol, in order to cover the following concentration ranges: 37–1000 ng/ml (urine), 0.1–10 ng/ml (tap water) and 0.3–30 ng/ml (sea water). Samples were spiked by adding small aliquots of CAP standard solutions and left to equilibrate overnight. Three replicates for each concentration were performed. The within-day ($n = 3$) and between-days ($n = 3$ over 10 days) coefficient of variation for chloramphenicol were calculated

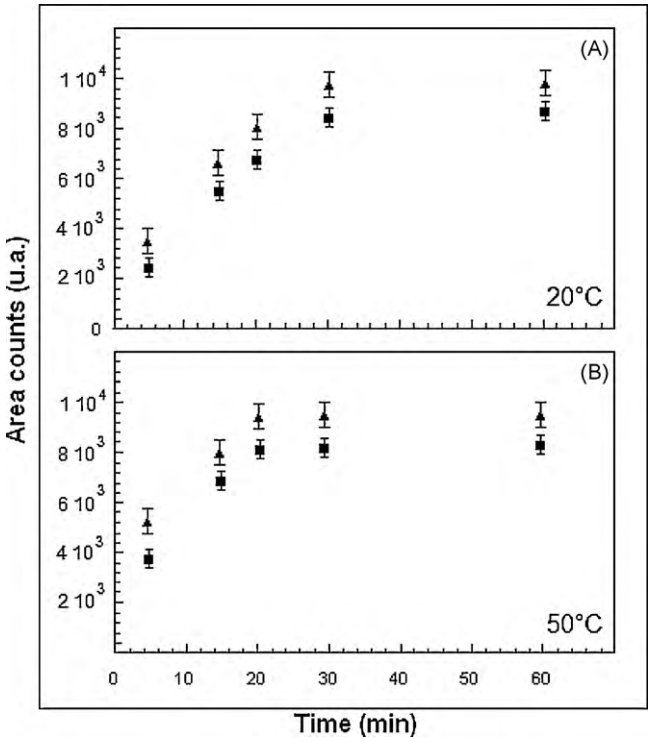

Fig. 1. Extraction time profiles obtained with the Carbowax (▲) and PDMS/DVB (■) coated fibers at (A) 20 °C and (B) 50 °C. The CAP concentration is 100 ng/ml.

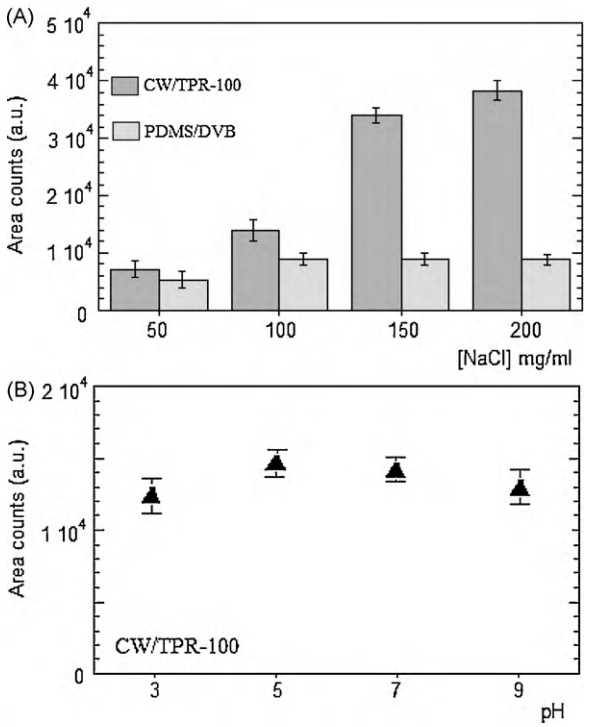

Fig. 2. Effect of (A) salt addition (CW/TPR-100 and PDMS/DVB) and (B) pH (CW/TPR-100) on CAP extraction.

on drug free urine and water samples spiked with variable amounts of chloramphenicol (see Tables 2–4). LOD and LOQ were always calculated as 3- and 10-fold the standard deviation of the intercept of the calibration curves [28]

3. Results and discussion

3.1. SPME optimization

Choice of fiber coating material. Preliminary experiments were performed in order to compare the extraction efficiency obtained using the CW/TPR-100, PA and PDMS/DVB coated fibers, respectively. The CW/TPR-100 and PDMS/DVB were able to extract the analyte to similar extent and were then chosen for the prosecution of the work; the PA fiber did not show satisfactory results and was discarded.

Extraction time and temperature. Adsorption times ranging from 5 to 60 min were investigated, both at room temperatures and at 50 °C, in order to establish the equilibration time for analyte partition between the aqueous and the polymer phase. The extraction time profiles for the CW/TPR-100 and PDMS/DVB fibers were then obtained by plotting the area counts vs. the extraction time, as reported in Fig. 1A and B. As apparent, similar extraction efficiencies (slightly higher in the case of the CW/TPR-100) were observed for the two fibers and no significant differences were obtained increasing the extraction temperature; however, equilibrium conditions were reached faster (after about 20 min) at 50 °C. Thus, the selected extraction conditions were 20 min at 50 °C. Under equilibrium conditions, the fiber-solution distribution coefficients, K_{f-s}, of chloramphenicol could be calculated as the ratio between the concentration of the analyte in the fiber coating and in the solution; $\log K_{f-w}$ values of 1.15 and 1.06 were estimated for the CW/TPR100 and PDMS/DVB fibers, respectively.

Effect of ionic strength and pH. Salt addition often improves the recovery, especially in the case of polar (hydrophilic) compounds that are difficult to extract. Thus, experiments were performed by increasing progressively the ionic strength of the extraction solutions. The relevant results are shown in Fig. 2A. A 6-fold CAP signal enhancement (i.e. improved extraction efficiency) was obtained using the CW/TPR100 fiber by the addition of 200 mg/ml of sodium chloride, that was selected as optimal concentration, since higher salt levels did not produce a further signal improvement. Positive results were observed also in the case of the PDMS/DVB fiber, even if the amount of the increase was definitely lower; thus, this fiber was no more considered in the present study. As far as the pH influence was concerned, it did not produce significative response variations on CAP extraction (see Fig. 2B).

Desorption conditions and "carry-over". Sample transfer from the fiber to the column is not a crucial step in gas-chromatography (GC) since problems arising from slow desorption kinetics can be simply counteracted by increasing the injector temperature and/or the desorption time and refocusing the injection band on the GC column head. In the case of SPME interfaced to LC, analyte transfer represents more than a problem. As it can be seen from Table 1, dynamic desorption mode (which ensures quantitative recoveries) causes a significant increase of both peak width and peak asymmetry compared to conventional loop injection (20 μl), deteriorating chromatographic efficiency and resolution. Thus, a static desorption technique was evaluated as possible alternative. The fiber was soaked in the static mobile phase contained in the desorption chamber (60 μl volume) for a variable period of time before injection into the LC column. The best conditions were reached after

Table 1
Peak width at 10% peak height (min) and peak symmetry factors for SPME injection (static or dynamic mode) and conventional loop injection (20 μl). For instrumental conditions see Section 2. The symmetry factors were calculated at 10% peak height.

Injection mode	Peak width	Peak symmetry
Conventional loop	0.43	1.05
SPME "dynamic desorption"	0.54	1.50
SPME "static desorption"	0.45	1.09

Table 2
Within-day ($n = 3$) and between-days ($n = 3$, for 10 days) precision obtained on drug free urine samples spiked with variable amounts of chloramphenicol.

Chloramphenicol (ng/ml)	Precision RSD%	
	Within-day	Between-days
50	6.2	9.0
100	5.8	8.9
500	5.5	8.7

5 min of static desorption in mobile phase and 10 s of exposition to the mobile phase stream. As shown in Table 1, the peak shape observed for conventional loop injection is now quite well preserved even if, under these conditions, complete sample transfer could not be necessarily achieved (recovery of 96.0 ± 2.4%).

3.2. Linear range, detection limits and precision

The response of the developed SPME-LC procedure was linear in the range 13–1000 ng/ml. The unweighted regression line peak area counts (arbitrary unit) vs. [chloramphenicol] (μg/ml) was described by the following equation: $y = (-1.5 \pm 0.7) + (266.5 \pm 1.5)\, x$; $R^2 = 0.9996$. The estimated LOD and LOQ obtained on standard solutions were 13 and 31 ng/ml, respectively. The within-day precision of the method was investigated on standard solutions in the concentration range 50 and 500 ng/ml by performing daily three replicates. The same solutions were analyzed three times each day for a period of 10 days for the between-days precision evaluation. The within-day RSD% ($n = 3$) and between-days ($n = 3$ over 10 days) RSD% were 4.2 and 5.1, respectively, and were find to be not concentration dependent for standard solutions.

3.3. Urine samples analysis

Once the study on extraction and desorption conditions was completed, the procedure was applied to urine samples previously subjected to deconjugation by β-glucuronidase, since CAP is mainly eliminated in human urine as chloramphenicol glucuronide conjugate. A slight loss of sensitivity was observed during the analysis of urine samples due to matrix effect. Thus, a calibration curve in urine was constructed; it resulted linear in the range 37–1000 ng/ml. The unweighted regression line peak area counts (arbitrary unit) vs. [chloramphenicol] (μg/ml) was described by the following equation: $y = (-2.0 \pm 1.3) + (157.1 \pm 1.7)\, x$; $R^2 = 0.9994$. The estimated LOD and LOQ were 37 and 95 ng/ml, respectively, calculated according to IUPAC (see above). The method resulted to possess enough sensitivity for the analyte detection at its usual urinary concentration [29]. Table 2 reports the obtained within-day and between-days coefficients of variation for chloramphenicol in urine samples.

Fig. 3 reports the SPME-LC-UV chromatograms obtained from a blank urine sample (lower trace) and a urine sample spiked with a known amount of chloramphenicol. As apparent, the target analyte was well resolved from matrix components. Furthermore, the separation was performed under simple isocratic elution conditions in less than 10 min.

3.4. Water samples analysis

The developed procedure was then applied to drinking and sea water samples. In this case, samples were not incubated with β-glucuronidase, since CAP glucuronide is deconjugated in the environment by bacterial, thus reactivating the parent drug [9]. The calibration curve in drinking water samples resulted linear in the range 0.1–10 ng/ml. The unweighted

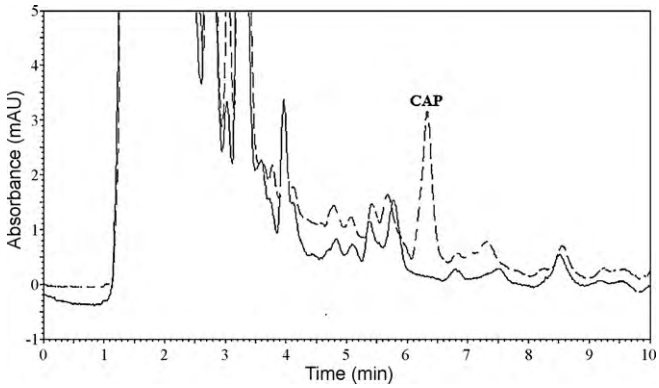

Fig. 3. SPME-LC-UV chromatograms relevant to urine samples, blank (down, full line) and spiked with chloramphenicol at 0.4 μg/ml (up, dotted line).

Table 3
Within-day ($n = 3$) and between-days ($n = 3$, for ten days) precision obtained on drug free tap water samples spiked with variable amounts of chloramphenicol.

Chloramphenicol (ng/ml)	Precision RSD%	
	Within-day	Between-days
0.5	6.0	8.8
5	5.9	8.6
10	5.1	8.4

regression line peak area counts (arbitrary unit) vs. [chloramphenicol] (μg/ml) was described by the following equation: $y = (-3.8 \pm 1.7) + (75386.4 \pm 23.4)\, x$; $R^2 = 0.9994$. The estimated LOD and LOQ were 0.1 and 0.3 ng/ml, respectively. Table 3 reports the obtained within-day and between-days coefficients of variation for chloramphenicol in drinking water.

Fig. 4 reports the SPME-LC-UV chromatograms obtained from a drug free drinking water (lower trace) and a spiked drinking water (upper trace) samples. As apparent, the analyte was clearly detected and well resolved from matrix components.

As far as sea water samples are concerned, calibration curve resulted linear in the range 0.3–30 ng/ml. The unweighted regression line peak area counts (arbitrary unit) vs. [chloramphenicol] (μg/ml) was described by the following equation: $y = (-8.1 \pm 2.8) + (53662.0 \pm 7.9)\, x$; $R^2 = 0.9992$. The estimated LOD and LOQ were 0.3 and 0.7 ng/ml, respectively. Table 4 reports the obtained within-day and between-days coefficients of variation for chloramphenicol in sea water.

Fig. 5 reports the SPME-LC-UV chromatograms obtained from a drug free sea water (lower trace) and a spiked sea water (upper trace) samples; also in the present case, no significant interferences

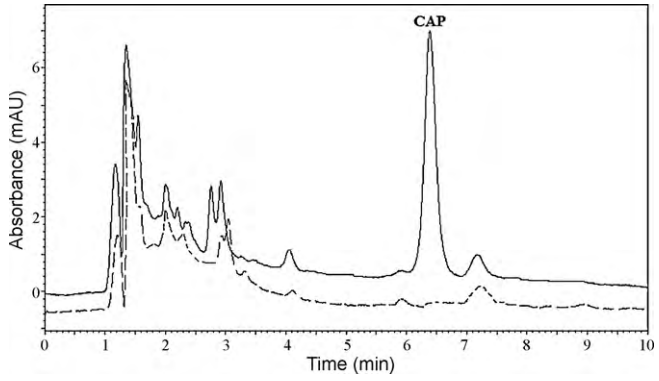

Fig. 4. SPME-LC-UV chromatograms relevant to drinking water samples, blank (down, dotted line) and spiked with chloramphenicol at 1 ng/ml (up, full line).

Table 4
Within-day ($n = 3$) and between-days ($n = 3$, for 10 days) precision obtained on drug free sea water samples spiked with variable amounts of chloramphenicol.

Chloramphenicol (ng/ml)	Precision RSD%	
	Within-day	Between-days
1	5.7	8.9
10	5.5	8.7
30	5.4	8.6

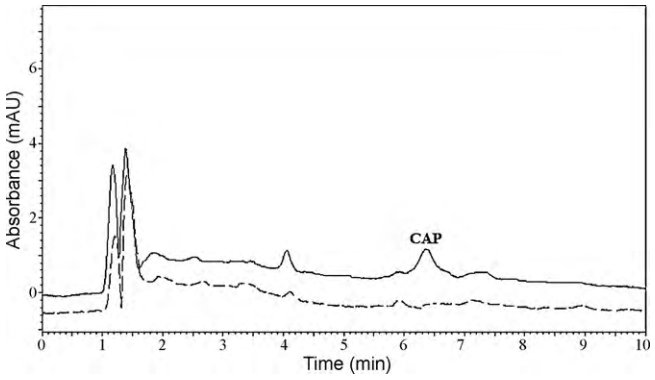

Fig. 5. SPME-LC-UV chromatograms relevant to sea water samples, blank (down, dotted line) and spiked with chloramphenicol at 1 ng/ml (up, full line).

from matrix components were observed and the analyte signal was clearly detectable.

It is worth noting that a significant increase in terms of sensitivity could be easily achieved with this method replacing the UV detector with ESI-MS, since the mobile phase used appears compatible with the ESI source.

4. Conclusions

An SPME (Carbowax fiber)-LC-UV method for the determination of chloramphenicol was developed for the first time and applied to the determination of the drug in urine, drinking and sea water samples. The proposed sample pretreatment is simple, cheap and totally solventless. The chromatographic step required a short (less than 10 min) simple isocratic elution, even in the case of a very complex matrix such as urine. Detection limits achieved in urine are sufficient to permit CAP detection at its usual concentration levels [29]; the results obtained in tap and sea water are comparable to those [23,24] obtained with analogue analytical instrumentation in similar matrices employing traditional extraction techniques.

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