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Determination of antibiotic compounds in water by solid-phase extraction–high-performance liquid chromatography–(electrospray) mass spectrometry

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Abstract

We developed a sensitive method based on solid-phase extraction and high-performance liquid chromatography coupled to mass spectrometry using an electrospray interface for the determination of four tetracyclines and two quinolones in water. The method was applied to river, well and sewage-treatment-plant waters. For the solid-phase extraction of 1000 ml river water samples, recoveries were between 88 and 112% and limits of detection were as low as 4 and 6 ng l^{-1}. Recoveries were higher than 64% for 1000 ml well water samples for the majority of the compounds. For the influent and effluent of the sewage-treatment-plant sample volumes of 100 and 250 ml were extracted, respectively. The method developed allowed ciprofloxacin to be determined in samples from the influent and effluent of the sewage-treatment-plant at 0.58 and 0.60 $\mu g\ l^{-1}$, respectively.
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1. Introduction

In recent years concern about the presence of pharmaceutical compounds (e.g. antiinflamatory, lipid regulators, antiepileptic drugs, antibiotics, etc.) in the environment, their possible adverse effect on humans and ecological systems, and new strains of resistant bacteria has considerably increased [1–4].

After they have been administered, a considerable amount of some of these pharmaceuticals is excreted unmetabolized and they can persist in the environment. Because they cannot be totally removed from sewage-treatment-plants (STPs) they can be found in their effluents [5–7] and can therefore reach surface waters [5,8–10]. Some of them are also widely used in veterinary medicine to treat bacterial infections and promote growth so they may be present in wells near farms which use natural manure as a fertiliser [11]. Their widespread use and potential adverse effects because of their possible persistence in the environment have increased interest in their determination, although none of them are on the list of priority and hazardous substances of the Water Framework Directive of the European Union [12].

Tetracyclines (TCs) and quinolones (Qs), two antibiotic families that are extensively used in human

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and veterinary medicine to treat and prevent bacterial infections, and promote animal growth in husbandry, are the target compounds of this paper. Such techniques as gas chromatography (GC) [3,11], capillary electrophoresis (CE) [13–15] and high-performance liquid chromatography (HPLC) [6,16–19] have been used to determine pharmaceutical compounds that include some antibiotics of these two families in several matrixes, mainly in biological samples. For instance, Hurtand-Pessel et al. [20] and Turnipseed et al. [21] determined Qs in muscle by HPLC coupled to mass spectrometry (MS) using different interfaces while Hernández et al. [14,15] used CE to analyse several Qs in plasma. Some authors have also studied the presence of Qs [7,16,22] and TCs [19,23–25] in waters and HPLC coupled to MS or tandem MS have been the techniques of choice, although UV–Vis [26] and diode array detectors [27] have also been used.

As pharmaceuticals appear at low concentrations in the environment, enrichment steps are needed. Solid-phase extraction (SPE) [6–8,17,23] has been the preferred technique, although liquid–liquid extraction (LLE) [28] and solid-phase microextraction (SPME) [3] have been used in some cases. For TCs, SPE has been the most commonly used preconcentration technique [19,23–25]. Snow et al. [24] reported a method for analysing three TCs in different waters by SPE–HPLC–MS–MS with limits of detection (LODs) in the low μg l^{-1} range. Lindsey et al. [25] reported a SPE–HPLC–MS method for determining TCs in ground and surface waters with good recoveries and limits of quantification (LOQs) of 1 μg l^{-1}.

The aim of this study is to develop a sensitive method based on HPLC–MS with an electrospray ionization (ESI) interface and preconcentration by SPE for the simultaneous determination of six antibiotics (four TCs and two Qs) in waters.

2. Experimental section

2.1. Reagents and standards

Enrofloxacin (ENR) and ciprofloxacin (CPR) were purchased from Cenavisa (Reus, Spain), tetracycline (TC) and oxytetracycline (OTC) from Acros Organics (NJ, USA), doxycycline (DC) from Sigma (St. Louis, MO, USA) and chlortetracycline (CTC) from Merck (Damstadt, Germany). Individual standard solutions of 500 mg l^{-1} were prepared in HCl 0.01 *M*. These solutions were stored at 4 °C for a maximum of 3 months. A mixed standard solution of 0.25 mg l^{-1} was prepared daily in HCl 0.01 *M*. Working standard solutions were prepared by dilution with HCl 0.01 M.

Acetonitrile (ACN), used as the organic component of the mobile phase and methanol (MeOH), used for the extraction procedure were HPLC-grade and they were supplied by SDS (Peypin, France). Chlorhydric acid (HCl), potassium hydrogenphosphate (K_2HPO_4) and acetic acid (HAc) from Prolabo (Bois, France) were used to adjust the pH of the sample and the mobile phase, respectively. Ultrapure water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nitrogen of 99.995% purity from Carburos Metálicos (Barcelona, Spain) was used in the extraction procedure.

2.2. Instrumentation

The chromatographic system was an HP1100 series LC–mass selective detector (Agilent Technologies, Barcelona, Spain) with an ESI interface and equipped with an automatic injector, a degasser, a quaternary pump, a column oven and a photodiode array detection (DAD) system. The chromatographic column was a Kromasil 100 C_{18} (25.0×0.46 cm) with a 5-μm particle size (Teknokroma, Barcelona, Spain).

Subsequently, 500 mg OASIS HLB cartridges (Waters, Milford, MA, USA) and 200 mg Isolute ENV+ cartridges (International Sorbent Technology, Mid. Glamorgan, UK), connected to a manifold (Teknokroma, Barcelona, Spain) and a pump as a vacuum source were tested for the SPE procedure.

2.3. Chromatographic conditions

A binary mobile phase with a gradient elution was used. Solvent A was Milli-Q water with 1% acetic acid (pH 2.8) and solvent B was acetonitrile. The gradient was 10% B for the first 10 min, which increased to 20% in 10 min, to 40% in 5 min and

then to 45% in 4 min. Finally, B was increased to 100% in 2 min, kept at 100% for 5 min and returned to the initial composition in 4 min. The mobile phase flow-rate was 1 ml min^{-1}, and the column temperature was kept at 35 °C. The injection volume was 50 μl and all the compounds eluted within 27 min.

The mass spectrometer simultaneously acquired in full-scan and under selected-ion monitoring (SIM) acquisition modes. Each compound was analysed separately by flow injection analysis (FIA), operating in the positive mode, to find the optimum parameters of the ESI interface. Optimized variables were averaged for the range of analytes of interest as follows: the drying gas was operated at a flow-rate of 13 l min^{-1} and 350 °C, the nebulizer pressure was set at 40 p.s.i. (1 p.s.i.=6894.76 Pa), and the capillary voltage at 2000 V. The fragmentor voltage was operated at 75 V for all the compounds except for ENR, which was set at 100 V.

Two ions (Table 1) were acquired for each analyte and they were used for quantification in the SIM mode.

The diode array detector was used to optimize the separation and the SPE procedure and it was set at 274 nm.

2.4. Solid-phase extraction

Antibiotics were extracted using 500 mg Oasis HLB cartridges from Waters. The cartridges were preconditioned with 5 ml of MeOH and 2 ml of MilliQ water. Sample volumes of 100 and 250 ml were extracted for the influent and effluent of the STP, respectively and 1000 ml was used for Ebre River and well samples. The pH of the sample was adjusted to 2.8 (HCl) and then the sample was passed through the cartridge at a flow-rate of 15 ml min^{-1}. The analytes retained were eluted with 5 ml of MeOH.

Table 1
Ions selected for the quantification in SIM mode. Their relative abundance is shown in parentheses

Compound	*m/z*	*m/z*
OTC	461 (100)	443 (10)
CPR	332 (100)	158 (25)
TC	445 (100)	427 (11)
ENR	360 (100)	302 (6)
CTC	479 (100)	445 (13)
DC	445 (100)	428 (13)

The extract was concentrated to dryness under a stream of N_2 and was then redissolved with 1 ml of a mixture of MeOH–water (50:50, v/v). After filtration with a 0.22-μm nylon syringe filter (Teknokroma, Barcelona, Spain), 50 μl of this solution was injected into the HPLC–(ESI)MS system.

After the cartridge was rinsed with 10 ml of acetonitrile it could be reused. About 10 real samples or 20 standards could be extracted with the same cartridge.

2.5. Sample preparation

Water samples from the Ebre River and wells were collected in pre-cleaned 2.5 l amber glass bottles.

STP water samples were collected at the influent and effluent streams of the STP of Tarragona in pre-cleaned amber glass bottles.

STP samples were filtered with Rundfilter MN 615 filter paper (Teknokroma, Barcelona, Spain) to eliminate the suspended matter and then they were filtered with the 0.45-μm membrane filter (Whatman, Maidstone, UK), while Ebre River and well water samples were filtered with the second filter only. Then, the samples were acidified to pH 2.8 with HCl and stored at 4 °C until analysis.

3. Results and discussion

3.1. Chromatographic separation

The compounds studied were separated with a C_{18} column. Other authors [24,25,27] have reported that there could be strong bindings between TCs and the silanol groups of the column and as a consequence tailing peaks could be observed. To avoid tailing peaks, we used a fully endcapped column and added 1% acetic acid to the aqueous solvent of the mobile phase because it is an additive that does not affect the absorbance or clog the interface [23].

First, MeOH was used as the organic solvent of the mobile phase but the analytes were not completely separated. A gradient elution using ACN allowed the optimum separation of the pharmaceuticals in 27 min.

3.2. HPLC–(ESI)MS

The parameters affecting the ESI interface were optimized by FIA for all the compounds individually in the positive and negative ion modes. The positive ion mode, in agreement with the literature [18,19,24], provided a better response to all the compounds so optimised variables were averaged for the range of analytes of interest in this mode. The interval tested for each parameter and the optimum value were the following: drying gas (3–13 l min^{-1}) 13 l min^{-1}, the capillary voltage (1000–6000 V) was set at 2000 V and the nebulizer pressure (20–60 p.s.i.) and the fragmentation voltage (50–150 V) were operated at 40 p.s.i. and 75 V, respectively (1 p.s.i.=6894.76 Pa). The mass spectrometer was managed in the full-scan and the SIM modes simultaneously but, as we want the limits of detection to be very low and there was no response at these levels in the scan mode, the SIM mode was used for the quantification. The two most intense ions in the spectrum were selected to be recorded in the SIM mode (Table 1) and they were used for quantification in this mode of acquisition. The mass peak corresponds to the molecular mass of the compounds and in the case of TCs the second ion of major intensity corresponds to losses of small molecules like H_2O and NH_3.

All the compounds showed good linearity between 1 and 200 μg l^{-1} in Milli-Q water (pH 2.8, HCl) with r^2>0.9992 by direct injection. Limits of detection calculated as signal-to-noise ratio of 3 were 0.2 μg l^{-1} for OTC, TC, ENR and CPR and 0.5 μg l^{-1} for CTC and DC in the SIM mode.

3.3. SPE procedure

TCs and Qs have different pK_a values, which should be considered in the pH adjustment of the sample. Both acid and basic mediums were tested to prepare samples using HCl (pH 2.8) or K_2HPO_4 (pH 9.0). The recoveries for Qs were better in basic than in acid conditions. On the other hand, recoveries for TCs were greater when the acid medium was used. Since recoveries increased more for TCs than for Qs and were acceptable for Qs acidifying the sample, pH 2.8 (HCl) was the sample conditioning chosen.

Standard solutions were used to test two different polymeric sorbents: 200 mg Isolute ENV+ and 500 mg Oasis HLB. Both highly-crosslinked polymeric sorbents can be used to retain hydrophilic and hydrophobic compounds with great capacity and neither of them has silanol groups which can interact with TCs. The Isolute ENV+ is a styrene–divinylbenzene polymer while OASIS HLB cartridge is a divinylbenzene–vinylpirrolidone which has an hidrophylic group on its structure.

Three replicates of different sample volumes (100, 250, 500 and 1000 ml) were tested to find the breakthrough volumes. The recoveries obtained at different sample volumes are shown in Table 2. While the recoveries between 250 and 500 ml for the Isolute ENV+ cartridge decreased significantly, recoveries were good for the preconcentration of 1000 ml samples using the Oasis HLB sorbent, which may be due to the hydrophilic group. We decided to use this sorbent with this sample volume and this is also in agreement with the literature for some of the compounds [15,24,25].

Table 2
Comparison of recoveries obtained with three replicates of different sample volumes using Isolute ENV+ and Oasis HLB cartridges with RSDs lower than 15%

	100 ml		500 ml		1000 ml
	Oasis HLB	Isolute	Oasis HLB	Isolute	Oasis HLB
OTC	96	84	93	18	86
CPR	106	78	87	38	79
TC	95	100	86	28	85
ENR	108	80	88	79	88
CTC	93	80	88	52	87
DC	99	82	90	71	90

We used 5 ml of MeOH as the eluting solvent because the analytes retained were eluted quantitatively. To increase the preconcentration factor, an evaporation to dryness was carried out in a N_2 stream. Then 1 ml of MeOH–water (50:50, v/v) was used for redissolving the residue to avoid peak distorsion observed when 1 ml of MeOH was used.

3.4. Method validation

Ebre River water was used in the validation of the method. Linearity was tested following the procedure developed in the range 10–500 ng l^{-1} and in the SIM mode. All the compounds showed r^2>0.9987 when this type of water was used as the matrix. This sample was used as a blank as no analyte peaks were found. Recoveries were between 88 and 112% when 1000 ml samples spiked at 50 ng l^{-1} were analyzed with the method. Repetitivity and reproducibility, expressed as relative standard deviation (RSD), were obtained by analysing three replicates of 1000 ml spiked samples at 50 ng l^{-1}. RSDs were lower than 8 and 10% respectively. Limits of detection, calculated as signal-to-noise ratio of 3, were 4 ng l^{-1} for OTC, TC, ENR and CPR and 6 ng l^{-1} for CTC and DC in the SIM mode. These results are shown in Table 3.

Water collected from wells not contaminated with the compounds studied was also used to determine the applicability of the method. As there was no suspended matter and a high organic content was not expected in the sample, 1000 ml was preconcentrated. Recoveries were between 41 and 87% for samples spiked at 50 ng l^{-1} levels when this water was used as the matrix. Some values were slightly lower than for river water. Fig. 1 shows a chromatogram of a blank and a spiked sample for this type of water.

Influent and effluent waters from the Tarragona STP were also analyzed. As recovery was low when 1000 ml were preconcentrated, because of the complexity of the matrix, the sample volume was reduced to 100 and 250 ml for the influent and effluent samples, respectively. The recoveries for OTC, TC, CPR and ENR were between 68 and 89% when effluent samples spiked at 320 ng l^{-1} were analyzed and between 73 and 103% for influent samples spiked at 2 μg l^{-1} (Table 4). Recoveries were lower only for CTC and DC probably due to the extraction procedure or ion suppression caused by the high contamination of the sample.

Some authors reported that a strong metal chelator added to the sample may improve TC recoveries [19,24,25], as TCs have a strong tendency to form chelates with free metals in the sample. EDTA 0.25 *M* was added to the different samples analyzed to check whether chelation between TCs and free metals in the sample occurred but there was no significant improvement in recovery so we decided to work without it.

3.5. Application to real samples

Samples from five different wells were analysed. These waters had high nitrate contents because of contamination from animal excrements of nearby farms or fertilisers. As the animals are treated with antibiotics and a considerable amount of them is excreted unmetabolized, these antibiotics can also

Table 3
Recoveries, repetitivity and reproducibility obtained with three replicates of 1000 ml Ebre River water samples spiked at 50 ng l^{-1}

	Recoveries (%)	Repetitivity, RSD (%, n=3)	Reproducibility, RSD (%, n=3)	LOD (ng l^{-1})
OTC	106	1	5	4
CPR	103	7	8	4
TC	99	5	10	4
ENR	108	5	5	4
CTC	88	8	9	6
DC	112	2	3	6

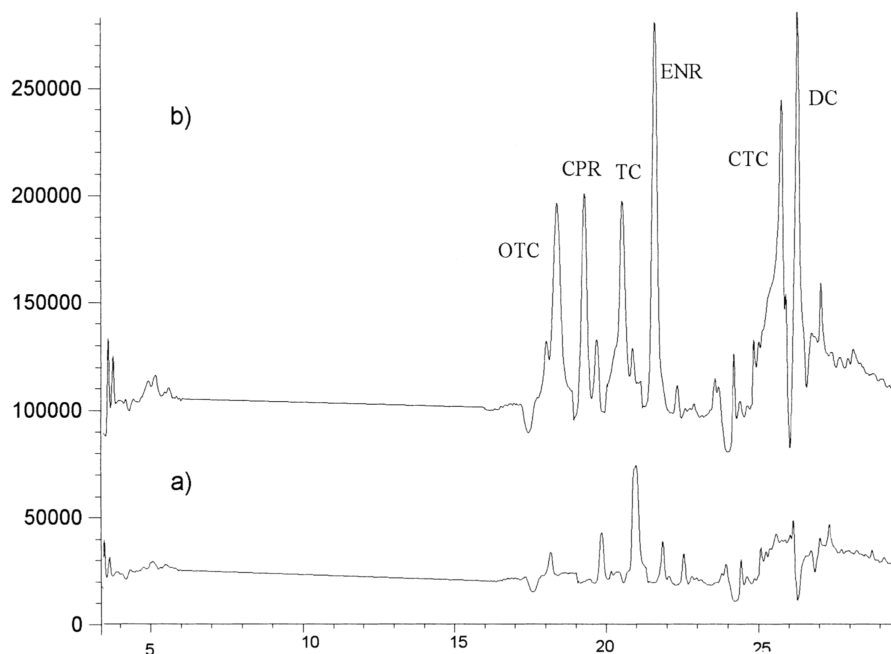

Fig. 1. Chromatogram obtained with: (a) 1000 ml well water sample; (b) 1000 ml well water sample spiked at 50 ng l^{-1}. Time scale in min.

contaminate the wells. Fortunately, none of the compounds were found in these samples.

Several water samples from the influent and effluent of the Tarragona STP were tested. The factors used to confirm the presence of the compounds in these samples were: retention time, and the relative abundance of the two ions selected. Peaks at the same retention time than ENR, TC and CPR and TC and CPR appeared in the chromatograms of the influent and effluent samples, respectively. In both cases only the presence of CPR was confirmed by the factors defined above because the deviation between the ion abundances of the other compounds was larger than 15%, the acceptable value we had fixed.

Fig. 2 shows a spectrum and a chromatogram obtained in the SIM mode for a STP sample in which CPR had been shown to be present. Concentrations in the influent and effluent samples of the STP were 0.58 and 0.60 μg l^{-1}, respectively.

Table 4
Recoveries obtained with three replicates of waters from different origins

	Well water[a]	STP water	
		Influent[b]	Effluent[c]
OTC	64	88	89
CPR	56	87	89
TC	41	73	68
ENR	71	103	80
CTC	87	34	27
DC	64	50	23

[a] 1000 ml spiked at 50 ng l^{-1}.
[b] 100 ml spiked at 2 μg l^{-1}.
[c] 250 ml spiked at 320 ng l^{-1}.

4. Conclusions

HPLC coupled to MS with an ESI interface, operating in the positive ion mode, was a powerful tool for determining the antibiotics studied at low concentration levels in waters of different origins.

SPE with an Oasis HLB cartridge makes it possible to preconcentrate 1000-ml samples of Ebre River and well water with good recoveries, which means that detection limits can be lower. When influent and effluent waters from the STPs were

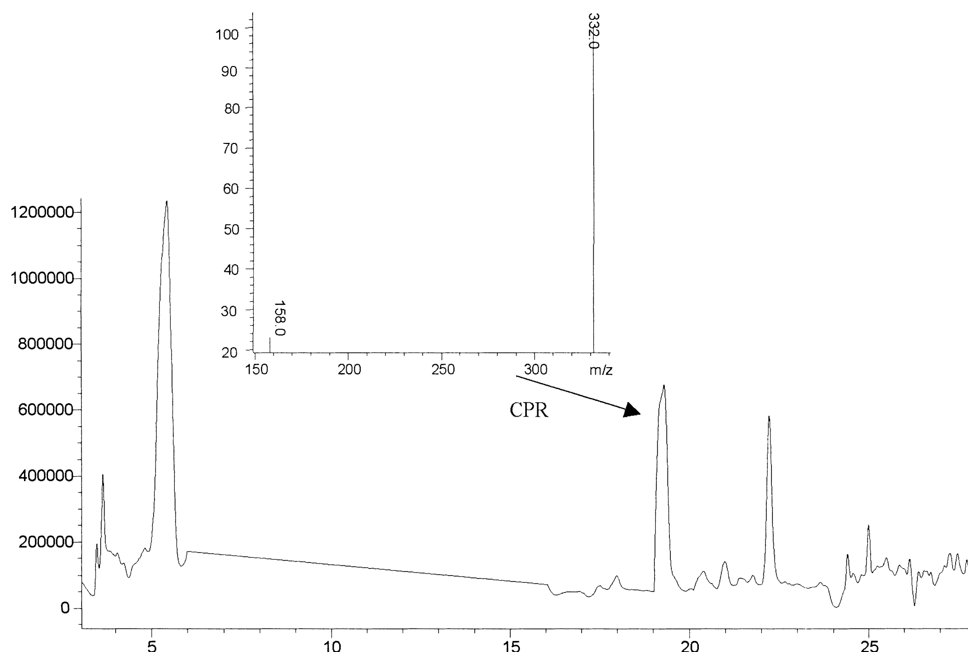

Fig. 2. Chromatogram obtained by preconcentration of a 250 ml sample from the effluent of the STP in Tarragona where CPR was found at 0.60 μg l^{-1} and the corresponding spectrum. Time scale in min.

analyzed volume samples of 100 and 250 ml were extracted.

The method developed enabled CPR to be determined in influent and effluent samples of the STP at 0.58 and 0.60 μg l^{-1}, respectively.

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References

[1] T.A. Ternes, M. Stumpf, B. Schuppert, K. Haberer, Wasser 90 (1998) 295.
[2] T.A. Ternes, R. Hirsch, J. Mueller, K. Haberer, Fresenius J. Anal. Chem. 362 (1998) 329.
[3] M. Moeder, S. Schrader, M. Winkler, P. Popp, J. Chromatogr. A 873 (2000) 95.
[4] T. Haberer, Toxicol. Lett. 131 (2002) 139.
[5] M. Stumpf, T.A. Ternes, R.D. Wilken, S.V. Rodrigues, W. Baumann, Sci. Total Environ. 225 (1999) 135.
[6] T. Ternes, M. Bonerz, T. Schmidt, J. Chromatogr. A 938 (2001) 175.
[7] E.M. Golet, A.C. Alder, A. Hartmann, T.A. Ternes, W. Giger, Anal. Chem. 73 (2001) 3632.
[8] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Environ. Sci. Technol. 36 (2002) 1202.
[9] R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, F. Ballwarz, K.L. Kratz, J. Chromatogr. A 815 (1998) 213.
[10] M. Winkler, J.R. Lawrence, T.R. Neu, Water Res. 35 (2001) 3197.
[11] R. L Seiler, S.D. Zaugg, J.M. Thomas, D.L. Howcroft, Ground Water 37 (1999) 405.
[12] Water Framework Directive (2000/60/EC), European Union, Brussels, 2000.
[13] S. Pedersen-Bjergaard, T. Grounhaug Halvorsen, Chromatographia 52 (2000) 593.
[14] M. Hernández, F. Borrull, M. Calull, J. Chromatogr. B 742 (2000) 255.
[15] M. Hernández, C. Aguilar, F. Borrull, M. Calull, J. Chromatogr. B 772 (2000) 163.
[16] A. Alder, Chimia 54 (2000) 14.
[17] C.D. Metcalfe, B.G. Koening, X.S. Miao, J. Chromatogr. A 952 (2002) 139.
[18] D.A. Volmer, B. Mansoori, S.J. Locke, Anal. Chem. 69 (1997) 4143.

[19] G. Hamscher, S. Sczesny, H. Höper, H. Nau, Anal. Chem. 74 (2002) 1509.
[20] D. Hurtaud-Pessel, P. Sanders, B. Delepine, Analyst 123 (1998) 2743.
[21] S.B. Turnipseed, C.C. Walker, J.E. Roybal, A.P. Pfenning, J.A. Hurlbut, J. AOAC Int. 81 (1998) 554.
[22] K. Kümmerer, Chemosphere 45 (2001) 957.
[23] A.M. Kamel, P.R. Brown, B. Munson, Anal. Chem. 71 (1999) 968.
[24] D.D. Snow, J. Zhu, D.A. Cassada, S.J. Monson, R.F. Spalding, J. Chromatogr. A 928 (2001) 177.
[25] M.E. Lindsey, M. Meyer, E.M. Thurman, Anal. Chem. 73 (2001) 4640.
[26] M.C. Carson, G. Bullock, J. Bebak-Williams, J. AOAC Int. 85 (2002) 341.
[27] S. Mou, X. Ding, J. Chromatogr. A 897 (2000) 205.
[28] S.R. Müller, M.Y. Haller, C.S. McArdel, A.C. Alder, M.J.-F. Suter, J. Chromatogr. A 952 (2002) 111.