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Solid-phase microextraction–gas chromatography–mass spectrometry of biologically active substances in water samples

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Abstract

A solid-phase microextraction (SPME) method for determining trace amounts of polar, biologically active substances in water systems was developed and compared with solid-phase extraction followed by derivatization and GC–MS. SPME was examined with respect to the simultaneous determination of pharmaceuticals such as ibuprofen, paracetamol, phenazone, carbamazepine, and nonylphenols known to be xenoestrogens. The extraction performance of different SPME fibre coatings was studied. Coatings like polyacrylate and Carbowax–divinylbenzene proved to be the best suited. The optimum extraction time was found to be 30 min and the detection limits were between 0.2 and 50 μg/l. Low concentrations of accompanying organic matter did not impair these limits. One of the main pharmaceutical contaminants found in ground and river water around Leipzig (Germany) was ibuprofen, with a concentration in the ng/l range. The enantioselective metabolism of ibuprofen was investigated. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Water analysis; Environmental analysis; Extraction methods; Ibuprofen; Nonsteroidal anti-inflammatory drugs; Drugs

1. Introduction

Back in 1977, during water control studies [1], the penetration of the aquatic environment by pharmaceutical products and their metabolites became apparent. The pharmaceutical industry can be exonerated because of the stringent guidelines and waste control systems to which drug manufacture is subject. Nevertheless, the growing production of drugs for human healthcare and veterinary applications results in increasing amounts of highly biological active material being discharged into the environment. Nowadays it is known that the 'domestic' pathway, via municipal sewage treatment plants, is the main route of pharmaceutical substances into the aquatic environment [2–4]. Due to their polarity, persistence and water solubility, most drugs and metabolites are able to pass through the wastewater treatment plants. Low adsorption on sludge and soil may cause the contamination of surface and ground water. Consequently, the quality of drinking water produced from this ground water or river bank filtrate may be endangered [5]. The monitoring of wastewater effluents, surface and tap water has demonstrated the widespread distribution of pharmaceutical substances [2,6–11]. Although detailed knowledge about the ecotoxicological effects of these compounds is still lacking, these contaminants must be classified as environmentally relevant.

The widespread occurrence of endocrine disrupt-

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ing compounds and the consequences for the ecosystem are often discussed separately from pharmaceutical substances. Extensive ecotoxicological studies have revealed the variety of effects of hormone-disrupting chemicals on organisms [12–16]. A wide range of industrially produced and applied chemicals, such as pesticides (DDT, methoxychlor, toxaphene [17,18]), alkylphenols formed from surfactants [19,20] and plasticisers such as phthalates [21], display estrogenic effects.

A typical representative, and one of the first pharmaceuticals to be found in ground and drinking water, is clofibric acid, an active metabolite of clofibrate and related drugs used in medicine as blood-lipid regulators. Clofibric acid has been detected in the lower μg/l range of raw and treated water streams of sewage treatment plants [5,22]. Only improved analytical methods enabled the determination of lower concentrations (ng/l) in tap and drinking water [8,23,24]. One of the main problems when collecting data about the occurrence, distribution and behaviour of biologically active substances in the environment is that trace analysis is required for a great variety of compounds. The polar, water-soluble pharmaceuticals are present in trace amounts, and in the case of surface water or wastewater, they are accompanied by complex organic matter. Nowadays, modern analytical methods combine sample enrichment (solid-phase extraction, SPE), derivatization and GC–MS analysis using selected-ion monitoring for trace-level detection [25].

Investigations were often limited to the analysis of selected individual compounds, because the diverse range of analytes required highly specialised sample preparation and enrichment procedures. Ternes et al. [26] were the first to present the simultaneous determination of a group of different acidic drugs (antiphlogistics and lipid-regulating agents) by SPE–methylation–GC–MS. To avoid time consuming derivatization procedures, LC–MS–MS methods were developed [27].

The first investigations using solvent-free solid-phase microextraction (SPME)–GC–MS were developed for the determination of ibuprofen and the plasticiser *N*-butylbenzene sulfonamide [28].

The purpose of our study was to develop an SPME–GC–MS procedure as a fast, multicomponent method for the determination of a series of pharmaceuticals and their metabolites in wastewater effluents. The SPME technique presented is compared with the currently used method of SPE–derivatization–GC–MS regarding its capability and suitability in wastewater monitoring.

2. Experimental

2.1. Materials and methods

The pharmaceutical references used (Table 1) were supplied by Promochem (Wesel, Germany). The chlorinated phenols added were included in an environmental cocktail mixture (EPA 604, Supelco, Deisenhofen, Germany). The phenols were only used for comparative SPME studies recently described in detail in Ref. [29].

The wastewater samples were taken from the Elster and Pleisse rivers (Germany) at the point where the central municipal wastewater treatment plant of Leipzig and the surrounding area with a total of ca. 500 000 inhabitants discharges the treated water effluents. Further ground water samples were taken from the vicinity of a former dump south of Leipzig containing waste of both municipal and industrial origin.

The urinary samples used for the enantioselective study of the human ibuprofen metabolism were obtained from three healthy volunteers (two female, one male) 2 h after the oral administration of 200 mg ibuprofen (as the over-the-counter drug Dolormin). The urine samples were prepared in the same way for SPME analysis as the water samples (acidified, salted, see Section 2.1.1).

Degradation experiments were performed in a biofilm reactor. Complex riverine biofilms were grown in rotating annular reactors with Elbe river water as described elsewhere [30]. During the experiment, ibuprofen was added every second day. The samples were taken before and after addition. The 100-ml water samples were extracted with SPE Bakerbond C_{18}ec at pH 2. After drying, the analytes were extracted with 10 ml of acetone. After drying again, the extract was evaporated and diluted in 250 μl of methanol [31].

The deuterium-labelled [$^{2}H_{14}$]bisphenol A used as recovery standard for SPE experiments was prepared

Table 1
Pharmaceutical compounds investigated in this study

Analyte	Used as/effective as	SIM target ions	LOD (μg/l)	RSD (n=3) (%)
Ibuprofen	Analgetic, anti-inflammatory	161, 163, 206	0.2	10.0
Clofibric acid	Lipid regulator	128, 130, 214	10.0	13.0
Paracetamol	Analgetic, antirheumatic	109, 151	10.0	18.0
Caffeine	Drug additive	194, 109	0.5	10.0
Nonylphenols	Industrial detergent, xenoestrogen	107, 135, 220	0.2–1	13.0
Phenazone	Analgetic	188, 96	10.0	14.0
Gemfibrozil	Lipid regulator	122, 250	0.5	10.0
Naproxen	Anti-inflammatory	185, 230	5.0	17.0
$^2H_{14}$-Bisphenol A	Plasticizer, xenoestrogen	222, 240	2.0	12.0
Carbamazepine	Analgetic	193, 236	1.0	12.0
Indomethacine	Anti-inflammatory	139, 141, 357	0.5	18.0
Norethisteron	Androgene, steroid hormone	442, 194[a]	0.5[a]	10.0[a]
Propranolol	β-Adrenergetic blocking agent	73, 331, 316[a]	0.1[a]	3.0[a]
Metoprolol	β-Adrenergetic blocking agent	72, 339, 324[a]	0.1[a]	7.0[a]

[a] SPE–trimethylsilylation–GC–MS analysis.

from [$^2H_5$]phenol and [$^2H_6$]acetone followed by isotope exchange of the phenolic O^2H groups into OH by crystallisation from water. Its purity was checked by GC–MS, and the characteristic isotope cluster ions of m/z 222 (base peak) and m/z 240 (molecular ion) were chosen for quantification.

All solvents used and the derivatization reagent bis(trimethylsilyl)trifluoracetamide (BSTFA) were purchased from Merck (Darmstadt, Germany) and used without further purification.

2.1.1. Solid-phase microextraction (SPME)

The SPME fibre coatings were polyacrylate (PA of 85-μm film thickness), poly(dimethylsiloxane–divinylbenzene) (PDMS–DVB, 65-μm film thickness) and Carbowax–DVB (65-μm film thickness), all from Supelco. Before their first use, each fibre was conditioned using the procedure suggested by Supelco. Fibre blanks taken prior to analysis confirmed the quality of conditioning. A volume of 4 ml of each sample was extracted by completely immersing the fibre while the solution was stirred with a glass-covered magnetic stirrer at 1000 rpm. Prior to extraction, each sample was salted with 1 g NaCl and adjusted to pH 2 with 30% H_2SO_4 [32]. Furthermore, 2-bromo-2,3-dihydro-1H-indene-1-ol (2.5 μg/ml) was added as internal standard to check GC–MS operation.

The optimisation of the extraction yields of the analytes as a function of the extraction time was performed at analyte concentration levels between 6 and 10 ng/ml. According to the results of the extraction yield/extraction time investigations, an extraction time of 30 min was then applied for all experiments.

The stock mixtures were prepared in methanol at a concentration level of 10 μg/ml and were correspondingly diluted to a series of five standard solutions for calibration. All calibration points are the arithmetic mean of triplicate measurements.

2.1.2. Solid-phase extraction

A mixture of 100 mg of LiChrolut-EN and 250 mg of LiChrolut-RP-C_{18} (Merck, Darmstadt) was used for SPE. Prior to the enrichment of the analytes, the SPE target was conditioned with 6 ml of hexane, 6 ml of acetone and 12 ml of water of pH 2 (adjusted with 0.1 M H_2SO_4).

Standard experiments used 1 l of pure water spiked with the standard mixture to obtain an analyte concentration of 1 μg/l. Furthermore, the sample was adjusted to pH 2 and spiked with [$^2H_{14}$]bisphenol A as recovery standard. The samples passed the adsorbent at a flow-rate of 3 ml/min. The wastewater samples were treated analogously.

After drying the loaded adsorbent under a nitrogen stream for 1 h, the analytes were eluted by applying 3 ml of acetone and finally 1 ml of methanol. The

eluates were combined and carefully evaporated under a nitrogen stream to a volume of 500 μl. To improve the volatility of the polar analytes, 100 μl BSTFA was added to form trimethylsilyl derivatives. The reaction was completed by keeping the mixture for 1 h at 40°C and the reagent excess and solvent were removed under a nitrogen stream to a final volume of 250 μl. From this volume, 1 μl was injected into the GC–MS in splitless mode (1-min splitless time) for analysis.

For calibration purposes, standard mixtures were derivatised and analysed in the same manner at different levels.

2.1.3. Liquid–liquid extraction for GC–MS screening of river water

A volume of 500 ml of the aqueous samples was adjusted to pH 2 and 10 g of NaCl was added. Each sample was extracted three times using 50 ml *n*-hexane. The organic extracts were combined, dried over Na_2SO_4, and evaporated to a final volume of 200 μl. Subsequent GC–MS analysis of 1-μl splitless injected extract was performed in full scan as well as selected-ion monitoring (SIM) modes using the characteristic ions listed in Table 1.

2.2. Instrumental parameters

The GC–MS analyses were carried out with two GC–MS systems (5890 series II and 6890 gas chromatograph–mass-selective detector, Hewlett–Packard, San José, CA, USA) each equipped with an HP-5 MS column (30 m×0.25 mm I.D., 0.25-μm film thickness). A narrow bore insert of 2-mm internal diameter was used for SPME. The SPME fibres were desorbed at 280°C for 2 min in splitless injection mode. The GC oven temperature program started at 70°C. The initial temperature was held for 2 min and was then increased at a rate of 10°C/min to 250°C. After an isothermal period of 5 min, the temperature was increased to 280°C and was held for another 10 min. Helium was used as carrier gas at a pressure of 70 kPa (5890 GC–MS system) as well as 55 kPa (6890 GC–MS system).

The SPME(PA)–GC–MS enantioselective analysis of ibuprofen was carried out on the 5890 GC–MS system equipped with a 25 m×0.25 mm I.D. capillary column coated with a 0.25-μm film of the chiral stationary phase CP Chirasil-DEX CB (Varian/Chrompack, Middelburg, The Netherlands).

Because of the lower thermal stability of the chiral coating, the analyses were carried out isothermally at 180°C. At an injector temperature of 250°C, ibuprofen was desorbed for 2 min in splitless mode. The most abundant ions at m/z 161 and 163 as well as the molecular ion at m/z 206 were used as target ions for the detection of ibuprofen in the SIM mode.

A normal split/splitless liner was used to analyse the SPE eluates. The other instrumental and analytical conditions remained unchanged.

3. Results and discussion

The compounds most frequently detected in wastewater and related aquatic systems were chosen as target analytes during the development of this method [4]. They belong to different classes of pharmaceutical products and xenoestrogens. All target analytes are listed in Table 1. Chlorophenols were included in this study by way of comparison.

3.1. Determination of selected pharmaceutical compounds by SPE and GC–MS

SPE in combination with methylation of the H-acidic functional groups and subsequent GC–MS analysis [11] is the method currently used for the determination of pharmaceuticals and endocrine disrupting compounds. Often, better mass spectrometric selectivity can be obtained by using the trimethylsilyl (TMS) derivatization reaction. The derivatives formed can be easily identified by the typical mass spectrometric key fragments, such as m/z 73 $[(CH_3)_3Si]^+$], the corresponding molecular ions $(M+^{\cdot})$ and the most predominant ions $M-CH_3]^+$. Fig. 1 shows the resulting chromatogram of an SPE–TMS derivatization–GC–MS analysis of a standard mixture. Apart from the variable enrichment factors, the advantage of the SPE procedure is the enhanced detector sensitivity that results from the appropriate derivatives. Also, highly polar and thermally labile compounds such as propranolol and clofibric acid can be determined. Utilising the advantages of SPE necessitates the often time- and substance-consuming optimisation of all sub-steps. As shown in Fig. 1, the

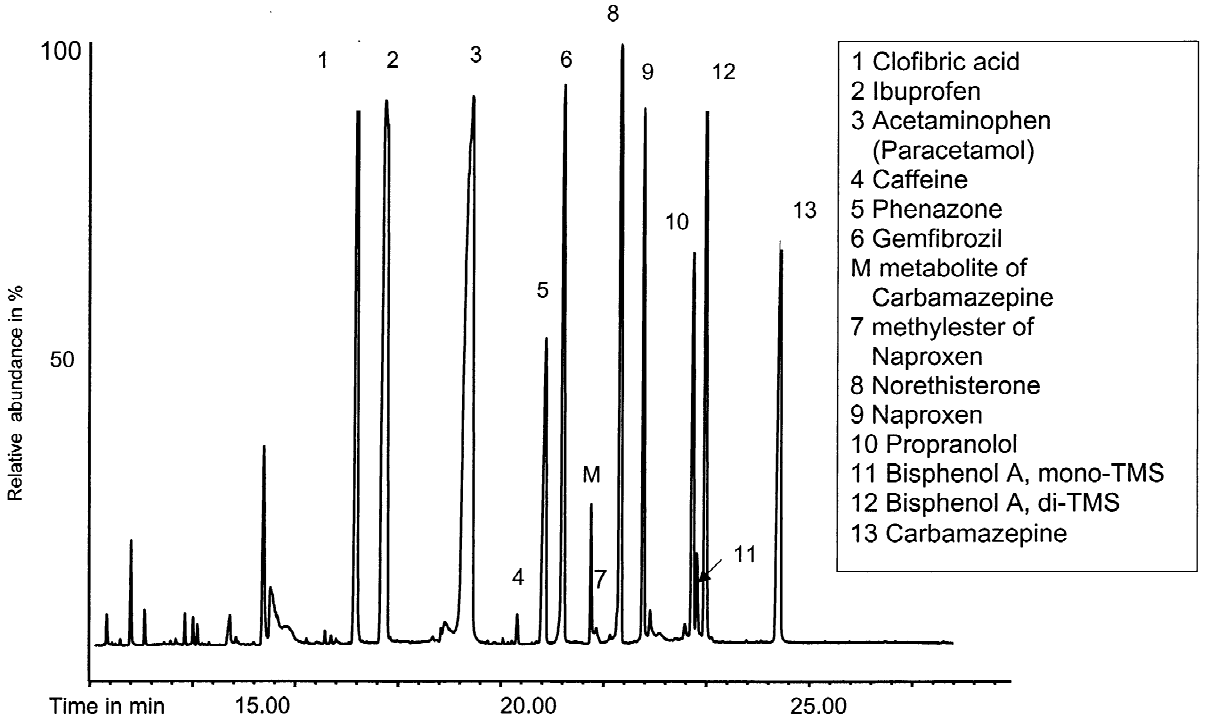

Fig. 1. Chromatogram of a SPE–derivatization–GC–MS procedure applied to a standard mix of pharmaceutical substances.

derivatization of the bisphenol A was incomplete under the conditions applied. The presence of both the mono- and di-TMS derivatives can cause problems in quantification. Because the detection of many of the analytes does not require any derivatization prior to GC–MS analysis, the prospects for the application of the solvent-free SPME method, which combines sample enrichment and analysis in a fast, simpler manner, are good.

3.2. Extraction time profile

Fig. 2 shows the extraction/time profile of selected pharmaceutical compounds using a polyacrylate-coated fibre. In agreement with the results presented in Ref. [28], the course of the ibuprofen curve indicates a continuously increasing slope over time. The partition equilibrium is probably not reached within 100 min. Optimum extraction yields are obtained for the other components in about 1 h. Because the extraction yields of most of the substances only increase by factors of about 1.1 between 20 and 60 min (except for ibuprofen: factor of 2.3), 30 min was chosen as the extraction time. This sampling time, co-ordinated with an appropriate GC–MS program, can be used for the automation of the complete analysis process.

Because of their similar physicochemical properties, the extraction behaviour of the pharmaceutical compounds is entirely comparable with that of chlorophenols. Therefore, simultaneous determination of such different substance classes is possible using the same SPME–GC–MS protocol.

3.3. Comparison of extraction efficiency using different fibre coatings

The results of the microextraction of a standard mixture containing chlorophenols and pharmaceuticals are shown in Fig. 3. The commercially available SPME fibres PDMS, PDMS–DVB, PA and Carbowax–DVB clearly differ in their ability to extract various substance classes. Generally speaking, although the more polar coatings PA and

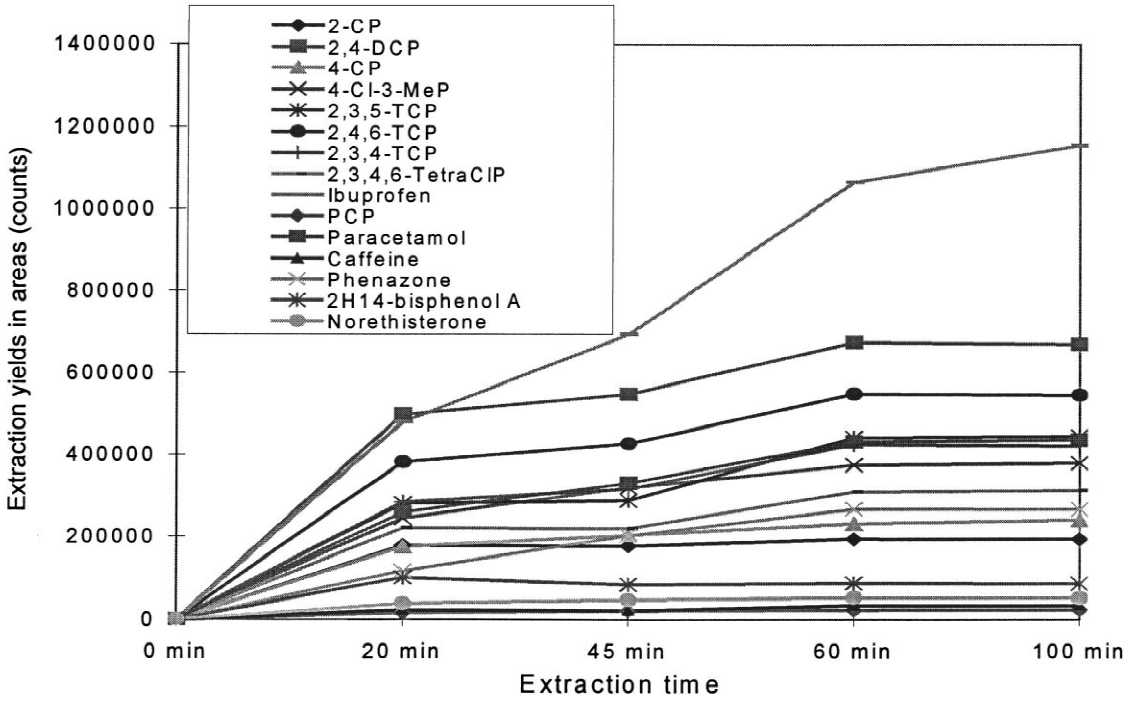

Fig. 2. SPME yield vs. time profile of selected pharmaceuticals and chlorophenols (CPs) at concentrations between 6 and 10 ng/ml in NaCl-saturated pure water samples (pH 2) using a polyacrylate fibre.

PDMS–DVB are best suited for the extraction of all substances investigated, the extraction yields obtained for pharmaceuticals are about 100 times smaller than those of chlorophenols. The only pharmaceutical that matches the extraction results of phenols is ibuprofen. In view of the comparable physicochemical properties of ibuprofen and some chlorophenols, their similar extraction behaviour is not unexpected. The water solubility and the octanol–water partition coefficient of ibuprofen (K_{ow}= 3.97) are quite similar to those of e.g. 2-chlorophenol (K_{ow}=2.15) and 2,3,4,6-tetrachlorophenol (K_{ow}= 4.42). Due to the much lower K_{ow} values of paracetamol (K_{ow}=0.46) or phenazone (K_{ow}=0.38) [29], in combination with their higher water solubility, their extraction yields are small. In particular, the results of the PA extraction support the preferential application of a more polar SPME fibre. The experiments using a Carbowax–DVB fibre prove the distinctive suitability of porous fibre coatings (Fig. 4). Furthermore, the more adsorptive extraction mechanism of the Carbowax–DVB coating enhances selectivity with regard to the extraction of *N*-heterocyclic molecules, such as carbamazepine or indomethacine. It should be mentioned that amino-substituted pharmaceuticals may best be extracted under neutral or weakly alkaline conditions (pH 6–8).

The limits of detection (LODs) ranging from 0.1 to 10 μg/l were obtained with a PA fibre under standard conditions. The LOD values listed in Table 1 partly depend on the lipophilic nature of the analyte, resulting in a high SPME yield. This behaviour is reflected by larger K_{ow} values, such as for nonylphenols. Moreover, the thermally labile character of the analyte determines its LOD in view of the high injection temperature for GC and the energy transferred during the mass spectrometric ionisation (electron impact using 70 eV).

At LOD levels, reproducibility varied between 10 and 18% RSD. An improved reproducibility of 6% RSD was obtained at the higher concentration levels.

The linearity of the calibration functions ranges from ng/l to higher μg/l. At this concentration level,

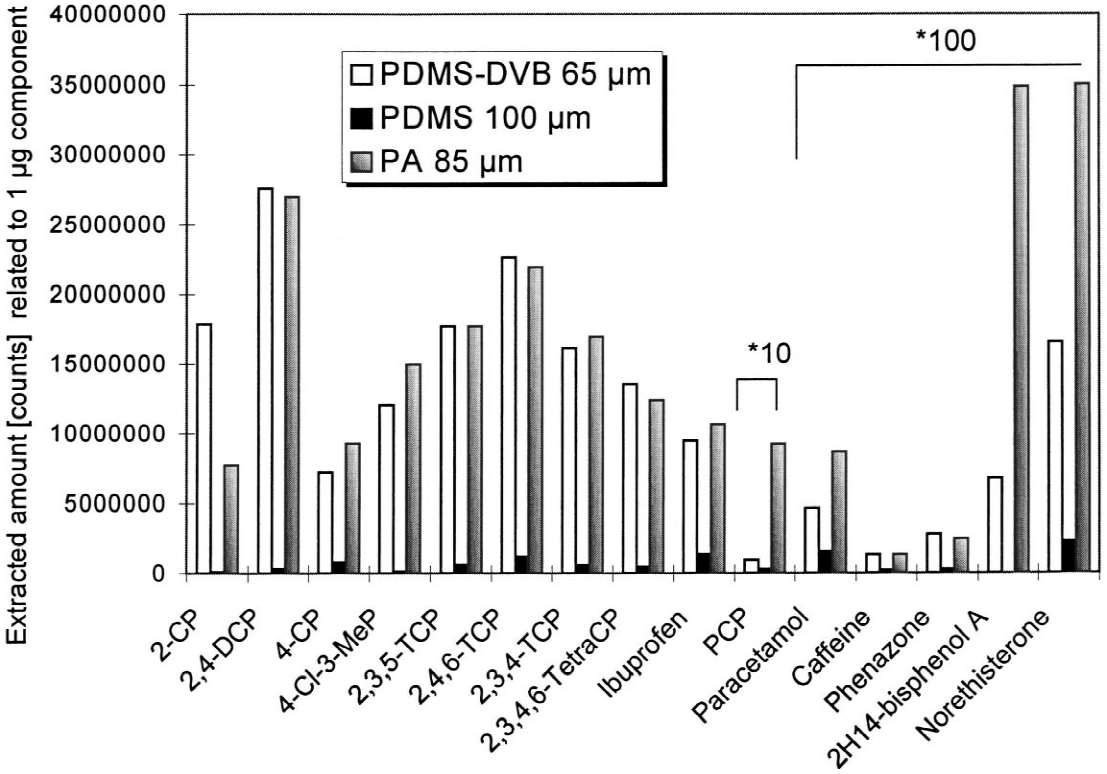

Fig. 3. Comparison of SPME efficiencies of pharmaceutical compounds and chlorophenols (CPs) related to an analyte concentration of 4 µg/ml.

carry over does not affect analysis. Care should be taken if higher concentrations of caffeine, ibuprofen and gemfibrozil (lower mg/l) are present — which is not very likely in real water samples.

Thermally labile compounds, such as the β-blocking agents metoprolol and propranolol or the lipid-regulating drug fenofibrate, are not detectable by GC–MS without derivatization.

Clofibric acid also causes problems. During desorption, the clofibric acid is decarboxylated in the hot GC injector and the resulting product is 4-chlorophenol. Interference from the 4-chlorophenol originally present is then possible. The difficulty in determining bisphenol A is probably caused by the manufacture of the SPME coating itself. Since bisphenol A undoubtedly appeared in considerable amounts in all blanks, it is assumed that the epoxy resins used to hold the silica rod and the metal holder together contain bisphenol A. Consequently, this compound requires an SPE GC–MS procedure for determination.

In agreement with the first in-tube SPME–LC–MS [33] analysis of propranolol, our initial experiments also demonstrate that electrospray (ESI)-MS appears to be the detection method of choice for such difficult substances. Detection limits obtained by flow injection analysis–ESI^+-MS are for propranolol and metoprolol at the ng/l level (10 ng/l and 100 ng/l respectively).

3.4. Matrix influence

In order to simulate wastewater conditions, a matrix-loaded water sample was prepared by spiking tap water with humic acids at a concentration of 0.1 mg/l. For comparison, tap water without any organic matter was used as a sample. The analyses of both solutions spiked with the same mix of pharma-

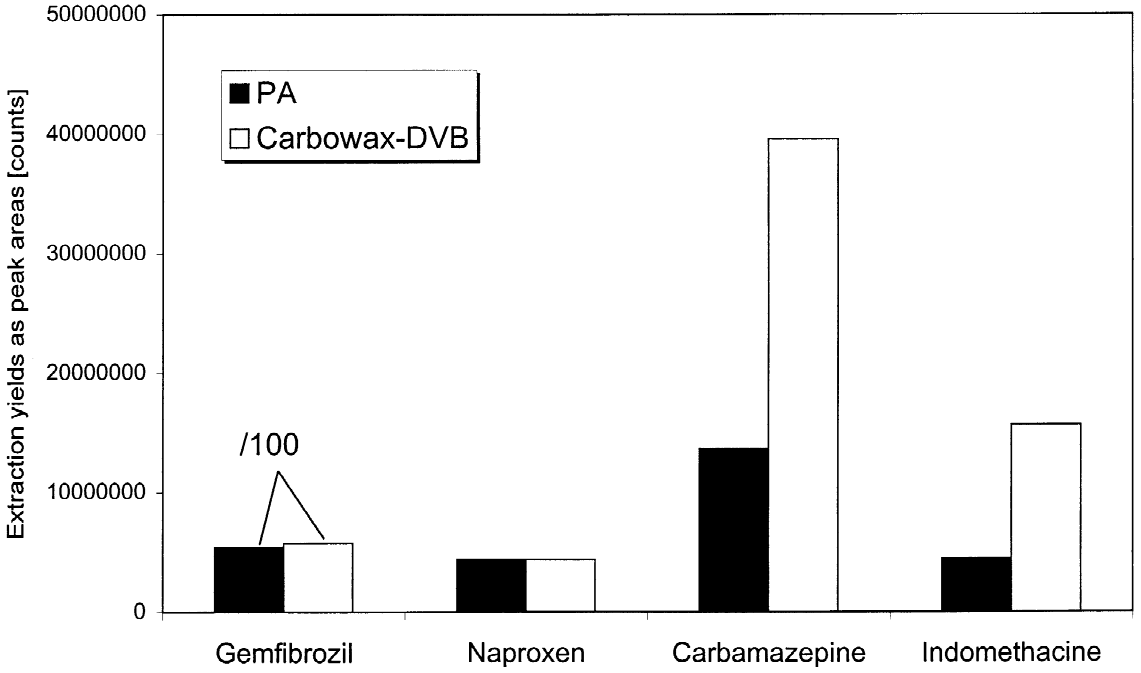

Fig. 4. Comparison of extraction yields of selected pharmaceuticals using polyacrylate and Carbowax–DVB.

ceuticals (1 μg/l of each) gave the extraction yields shown in Fig. 5. The loss of extraction efficiency in the presence of the organic matter is quite similar in all cases and does not exceed 30% of the extraction yields obtained in pure tap water. These deficits can be compensated for by extending the extraction time by about 15 min, because at this concentration, the organic matrix probably causes a delay in analyte diffusion [34,35]. Higher contents of organic matter can block the fibre surface and impede SPME analysis.

3.5. SPME–GC–MS analysis of real samples

Several ground water and river water samples were analysed by applying the SPME–GC–MS procedure developed. The samples came from water systems that are close to the municipal wastewater treatment plants in and around Leipzig. As the discharge of industrial and agricultural waste has drastically decreased during the last decade, only minor contamination is caused by commonly known hazards such as phenols, pesticides, halogenated and/or aromatic hydrocarbon fractions. GC–MS screening of river (Pleisse and Elster) and ground water showed, in addition to traces of alcohols and phenols, mainly saturated and aromatic hydrocarbons and both aliphatic and aromatic carboxylic acids as constituents. A mixture of isomeric nonylphenols stemming from degraded alkylphenolethoxylates widely used in Europe as industrial detergents was detected in ground water (south of Leipzig, waste dump influent) at a concentration level of 400 ng/l and in river water (Pleisse) about 40 ng/l. As a typical representative of pharmaceutical compounds, ibuprofen was detected in all water samples at concentrations between 200 and 400 ng/l. Even tap water contains traces of this substance (<1 ng/l).

Ibuprofen is used as a non-steroidal analgetic and anti-inflammatory agent. Since 1989, the drug has been available over the counter and is applied in relatively high amounts of 200–800 mg per tablet. Experiments indicate that just 2 h after the application of 200 mg (the normal dose recommended for acute headache) the drug is excreted in amounts of about 80 ng/ml urine. Pharmacological investigations followed the excretion of the drug and some metabolites over a period of 5 days [36]. Hence, the

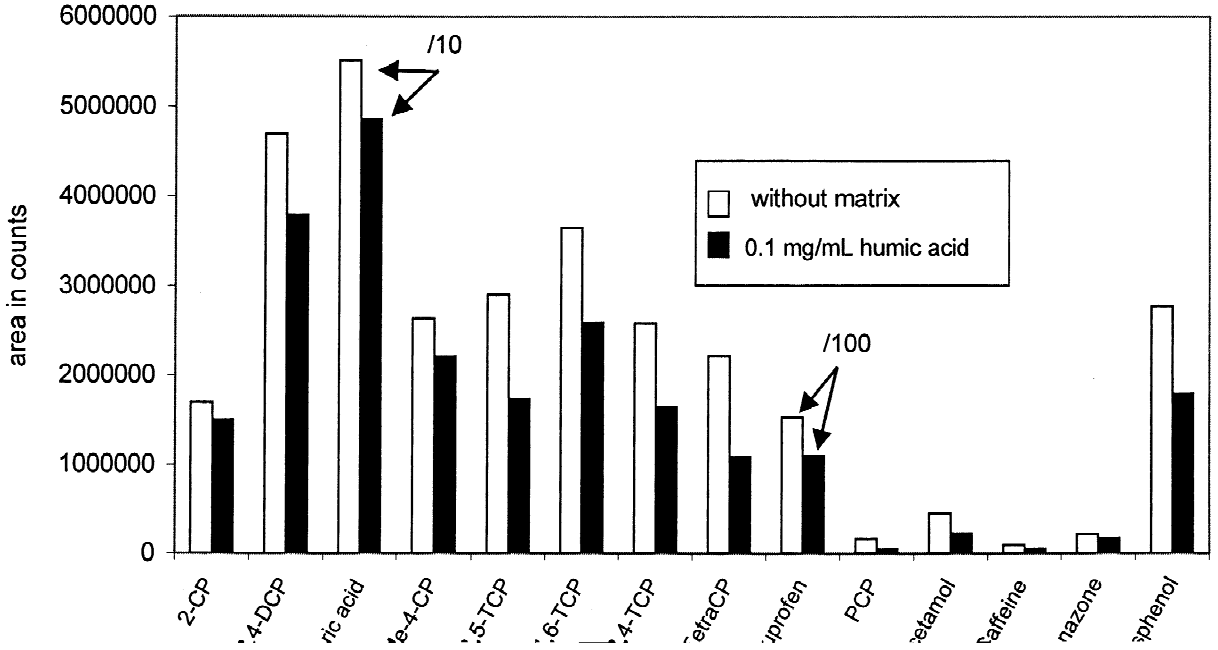

Fig. 5. Influence of redissolved organic matter on the microextraction of selected pharmaceutical compounds and chlorophenols (CPs) using a PA fibre.

wide distribution of ibuprofen in the aquatic environment is not surprising.

3.6. Enantioselective SPME–GC–MS analysis of ibuprofen

Because ibuprofen is applied as a racemic mixture, it was interesting to study the behaviour of individual enantiomers while passing through the human body as well as in microbial metabolism. Fig. 6 shows the partition of ibuprofen enantiomers analysed by SPME(PA)–GC–MS. Recent pharmacological studies provide information about the assignment of enantiomers as well as about their different biotransformation kinetics in the human organism [37]. Although both enantiomers have comparable pharmacological effects [38], the *S*-(+) enantiomer metabolises much faster then *R*-(−) form — which was also reflected in our experiments.

The initial enantiomeric composition of the applied drug Dolormin (Fig. 6, lower trace) changes during passage through the body. The three chromatogram traces above stem from urinary samples of different volunteers and indicate the enantioselective metabolism of ibuprofen by the clear decrease in the *S*-(+)-enantiomer. Thus, it was assumed that the preferentially released *R*-(−)-enantiomer contaminates the aquatic systems. However, enantioselective analysis of river water (Elster, Fig. 7) indicated the presence of *S*-(+)-ibuprofen. Consequently, completely different metabolism pathways lead to the degradation of the ibuprofen enantiomers.

The corresponding microbial degradation experiments proved the preferential metabolism of the *R*-(−)-enantiomer (Fig. 7), which reflects the naturally occurring reaction pathways in the aquatic environment. The ion traces shown in Fig. 7 indicate the different stages of microbial degradation that occurred in a biofilm reactor device. The results show clearly that the *R*-(−)-ibuprofen enantiomer is kinetically favoured in biotransformation in the experimental set-up applied. It is assumed that

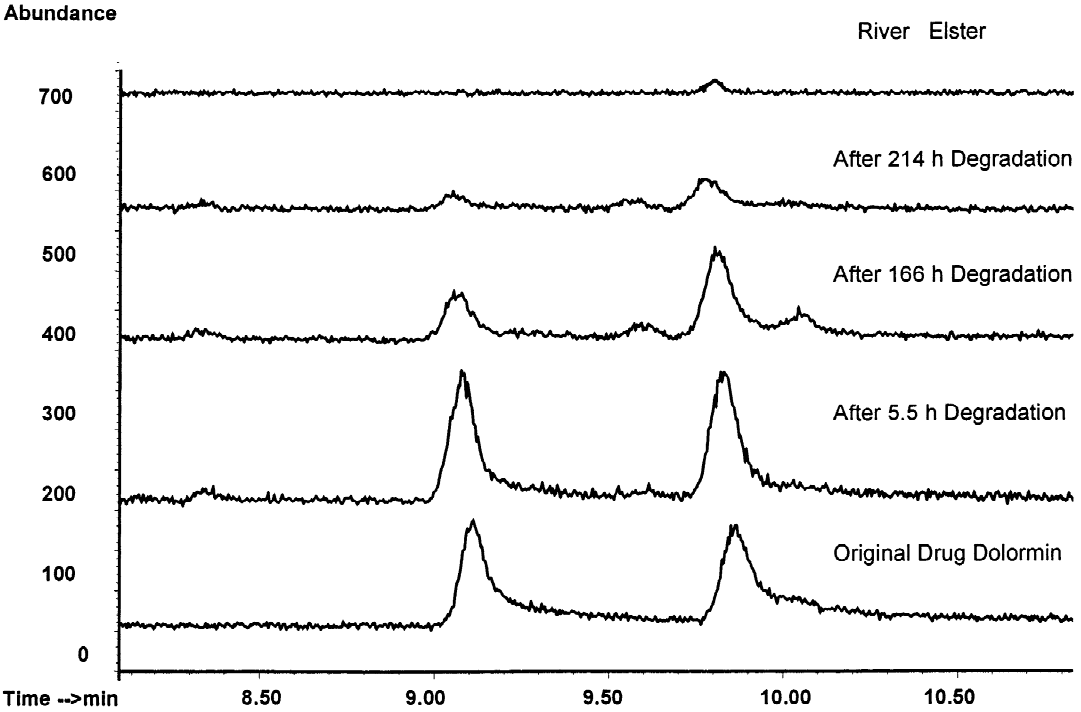

Fig. 7. Ion traces of the enantioselective SPME–GC–MS analysis of the original drug (lowest trace) and of time-dependent analysis of a microbial degradation of ibuprofen as well as the result of a river water (Elster) analysis (top trace).

in future, ESI-MS could be used as a very selective and sensitive detector for such polar compounds.

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