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Detection and identification of phenazone-type drugs and their microbial metabolites in ground and drinking water applying solid-phase extraction and gas chromatography with mass spectrometric detection

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Abstract

A new analytical method applying in situ derivatization was developed to enable the extraction of polar drug metabolites from water samples by solid-phase extraction (SPE). An additional derivatization by silylation was used to enhance the sensitivity of analyte detection by gas chromatography–mass spectrometry (GC–MS). Thus, the two metabolites 1,5-di-methyl-1,2-dehydro-3-pyrazolone (DP) and 4-(2-methylethyl)-1,5-dimethyl-1,2-dehydro-3-pyrazolone (PDP), postulated for the degradation of phenazone and propyphenazone, were identified and detected up to the μg/L level in raw and drinking water samples from public water supply.
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1. Introduction

The occurrence and fate of pharmaceutical residues in the aquatic environment has been recognized as one of the emerging issues in environmental chemistry [1–3]. One of the major sources for drug residues in the aquatic environment is their application in human medical care [4,5]. The presence of pharmaceutical residues from human medical care in surface- or ground water may, however, also be caused by other sources such as manufacturing residues. Especially in the industrialized countries, strong regulations and advanced manufacturing practices shall nowadays prevent such spills. But in the past, regulations were not as strong as they are today and in several cases the release of production residues was either tolerated or even accepted. Such spills could result in Superfund sites which may be responsible for today's detections of pharmaceuticals in surface water and especially in ground water samples.

Three pharmaceuticals namely phenazone, propyphenazone and dimethylaminophenazone, that have widely been used as analgesic and antipyretic drugs, have been detected in ground water samples from drinking water wells located in the northern districts of Berlin, Germany [6]. These residues were most likely caused by spills from a former production plant located in the city of Oranienburg upstream of Berlin. From there, production residues were released directly into the Upper Havel river and also into the subsoil underneath the production plant. In the past, large but unknown amounts of phenazone residues occurred in the surface water that is used downstream for ground water recharge in drinking water production. But even today, phenazone residues originating from the contaminated ground water underneath the former production plant (effluent conditions) are found in the Upper Havel river.

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Nine water works provide Berlin with drinking water only pumping and processing ground water mainly obtained by ground water recharge. The ground water is abstracted by means of vertical and horizontal filter wells with submersible pumps. It is aerated and processed through open and closed rapid filters without any addition of chemicals. Phenazone residues were found in raw water from all Berlin water works located near the Havel river. However, in the receiving drinking water treatment plants, a significant degradation of phenazone, propyphenazone and dimethylaminophenazone was observed [7]. Until now, the metabolic pathway responsible for the attenuation of these compounds has not exactly been enlightened. Lingens and coworkers [8] described the degradation pathway of phenazone by *Phenylabacterium immobile*. A number of aromatic or hererocyclic compounds structurally related to phenazone were also tested as sole sources of carbon and energy. Thus, propyphenazone and dimethylaminophenazone were identified as being also good growth substrates [9]. Due to the microbiological activity observed in the filters from the drinking water treatment plant, a degradation similar to that described above could take place. Thus, the metabolites 1,5-dimethyl-1,2-dehydro-3-pyrazolone (DP), 4-(2-methylethyl)-1,5-dimethyl-1,2-dehydro-3-pyrazolone (PDP) and 4-(*N*,*N*-dimethyl)-amino-1,5-dimethyl-1,2-dehydro-3-pyrazolone (DMADP) may occur as key metabolites in the metabolic pathway of the phenazone drugs.

Therefore, an analytical method had to be developed to examine the degradation pathway and to enable the sensitive detection of the postulated metabolites in water samples. In this paper, a method is reported for the determination of phenazone metabolites resulting from microbial degradation during drinking water purification. This method applies in situ derivatization, solid-phase extraction (SPE), additional derivatization to enhance the analytical sensitivity, and detection by gas chromatography–mass spectrometry (GC–MS). This paper also compiles first results of phenazone-type metabolites detected in the raw and purified drinking water of a drinking water treatment plant.

2. Experimental

2.1. Materials

Phenazone and dimethylaminophenazone were obtained from Sigma–Aldrich (Steinheim, Germany) and propyphenazone from Ferak (Berlin, Germany). 1,5-Dimethyl-1,2-dehydro-3-pyrazolone was synthesized according to a method described by Rojahn [10]. 4-(2-Methylethyl)-1,5-dimethyl-1,2-dehydro-3-pyrazolone and 4-(*N*,*N*-dimethyl)-amino-1,5-dimethyl-1,2-dehydro-3-pyrazolone were obtained after microbial degradation with phenylobacterium immobile [8]. *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) and acetic anhydride (AA) were purchased from Merck (Darmstadt, Germany). Extraction columns (6 mL) packed with 200 mg of poly(styrene–divinylbenzene) (PS–DVB) adsorbent, methanol, acetonitrile, and ethyl acetate were obtained from Baker (Deventer, The Netherlands). All solvents were of residue-free purity suitable for trace-level analysis. Sodium ethylate, 3-methyl-5-pyrazolone and methyl-*p*-toluenesulphonate for the synthesis of DP were purchased from Acros Organics (Geel, Belgium).

2.2. Synthesis of DP

5.43 g of sodium ethylate were dissolved in 60 mL of methanol. 9.84 g of 3-methyl-5-pyrazolone and 18.68 g of methyl-*p*-toluenesulphonate were added and the solution was stirred and heated under backflow. After 90 min of heating the resulting white residue was filtrated and washed thrice with dichloromethane. The organic phases were united and the solvent was removed by vacuum. After final removal of the solvent by nitrogen blow down, 2 g of DP were obtained as analytical standard.

2.3. Sample preparation/in situ derivatization

Water samples were collected in amber glass bottles and, if possible, analyzed immediately or stored at 4 °C for less than two days. Five hundred millilitres of the water sample were fortified with 200 ng of dichlorodiphenylamine used as surrogate standard. SPE of the polar metabolites DP, PDP, and DMADP was not satisfactory. Only low recoveries were achieved even after applying different SPE materials at varying pH conditions. The polarity of the analytes can, however, be reduced by replacing the active proton at the pyrazolone ring of the metabolites by a non-polar acetyl group (Fig. 4). This was achieved by directly (in situ) adding acetic anhydride to the water sample under basic pH conditions. Thus, 2 g of potassium carbonate were added to the sample and then 5 mL of acetic anhydride were rapidly dissolved by shaking the solution for 30 s. Sample extraction was performed by using PS–DVB cartridges and an automated extraction system (Autotrace SPE Workstation from Tekmar, Cincinnati, USA). After conditioning of the cartridges twice with 8 mL of methanol and water (pH $\sim$ 5.4), respectively, the samples were percolated through the cartridges at a flow rate of 10 mL/min. Then the cartridges were washed with 10 mL water (pH $\sim$ 5.4). After drying for 40 min by nitrogen flush, the cartridges were eluted with 10 mL of ethyl acetate. The eluate was reduced to a final volume of 500 μL by nitrogen blow down using a Zymark TurboVap II concentration workstation and used for the derivatization procedure as described in Section 2.4.

2.4. Additional derivatization (silylation) for GC–MS detection

The SPE eluate was dried under a gentle stream of nitrogen. Then the remaining residue was silylated to achieve

Table 1
Recoveries, LOD and LOQ values, ions for SIM and dwell time in the comparison of the two described methods

Compound	LOD[a,b] (ng/L)	LOQ[a,b] (ng/L)	Recovery (%)[a] ± RSD (%)[c]	*m/z* (SIM)[a]	*m/z* (SIM)[b]	Dwell time (ms)
DP	1/10	3/30	114 ± 10	169, 170, 155	111, 112, 154	80
DMADP	2/n.d.	6/n.d.	44 ± 5	269, 212, 197	140, 155, 197	80
PDP	1/5	3/20	95 ± 15	211, 212, 253	139, 154, 196	80

n.d.: not detected.
[a] Method 2: in situ derivatisation and silylation with MTBSTFA.
[b] Method 1: only in situ derivatisation with acetic anhydride.
[c] $n = 6$.

lower limits of detection (LODs) for the analysis by GC–MS. This was achieved by improving the gas chromatographic properties of the very polar DP, PDP and DMAAP replacing the acetyl moiety from the in situ derivatization by a *tert*-butyl dimethylsilyl moiety. The scheme in Fig. 4, shows the reaction mechanism for the generation of the less polar and more volatile compounds. Derivatization was performed at 55 °C for 30 min using 100 μL MTBSTFA (50% dissolved in acetonitrile). Finally, the derivatized sample was injected into the gas chromatograph.

2.5. Analysis by GC–MS

All GC–MS measurements were performed using an Hewlett-Packard HP6890 gas chromatograph combined with an HP5973 MS detector. Gas chromatographic separation was performed using an uncoated column as retention gap (1 m, deactivated 0.32 mm) and a 30 m HP-5MS column with 0.25 mm i.d. and 0.25 μm film thickness. Carrier gas was helium (99.999% purity) and the injection volume was 1 μL. The interface temperature was set to 280 °C. Mass spectrometric measurements were performed using electron impact ionization (EI) at 70 eV. Full scan mass spectra were obtained by scanning from 50 to 550 u. Using selected ion monitoring (SIM), three characteristic ions were selected for each compound and scanned in time windows corresponding to their expected retention times (Table 1).

2.6. GC temperature program for the analysis of the original and the acetylated analytes

The oven temperature was held at 70 °C for 1 min following injection, then programmed at 5 °C/min to 100 °C followed by 10 °C/min to 130 °C, then at 15 °C/min to 300 °C and finally held for 4 min.

2.7. GC temperature program for the analysis of the silylated analytes

The oven temperature was held at 70 °C for 1 min following injection, then programmed at 10 °C/min to 100 °C followed by 8 °C/min to 180 °C, then at 20 °C/min to 300 °C and finally held for 4 min.

2.8. Analysis of the phenazone-type drugs by high-performance liquid chromatography (HPLC)

The quantitation of phenazone, propyphenazone, and dimethylaminophenazone was carried out by HPLC with diode array detection (DAD). HPLC separation was achieved at 30 °C using a XTerra RP18 column (5 μm pore size, 3.9 mm diameter, and 150 mm length, Waters, Milford, MA USA). Eluent A was water (pH 8.5) and eluent B was acetonitrile. The following gradient was used for analyte separation: 30 min 95% A, 0.5 min 70% A and 0% A for 5 min. The solvent flow was set to 1.0 mL/min. The analytes were detected using a photodiode array detector from Waters (996 PDA). Full UV spectra were recorded by DAD, scanning from 200 to 550 nm. Quantitation limits of 0.05 μg/L were achieved for phenazone, propyphenazone, and dimethylaminophenazone, respectively.

3. Results and discussion

In Oranienburg, a small town located north of Berlin, a pharmaceutical production plant formerly produced several phenazone-type pharmaceuticals. It is supposed to that production residues were released into the soil and into the

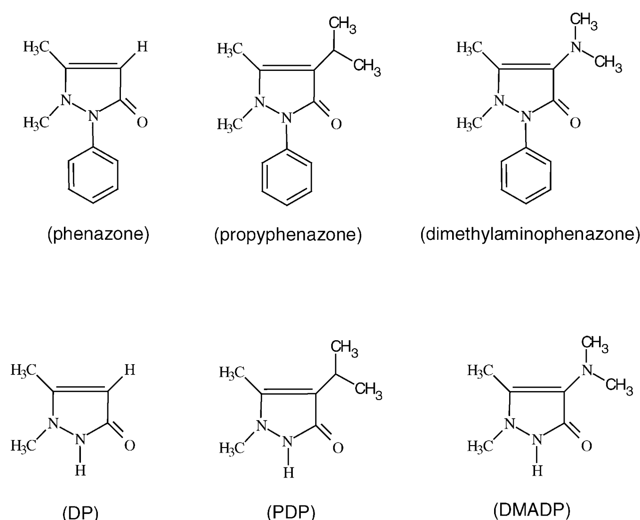


Fig. 1. Structures of the studied compounds.

neighboring Havel river. Although it is not clear when and how much of the pharmaceuticals were discharged into the environment, several pharmaceuticals such as phenazone, propyphenazone and dimethylaminophenazone have been detected in routine analysis of Berlin ground water samples [6]. A decrease of the concentrations of the phenazone-type pharmaceuticals was, however, observed during drinking water treatment. Thus, it was postulated that the phenazone-type compounds undergo a microbial degradation forming their corresponding dephenylated analogues. The structures of the pharmaceuticals and their postulated metabolites are shown in Fig. 1.

Due to the polarity of the phenazone-type metabolites, an in situ derivatization was developed to overcome difficulties in extracting the very polar compounds from the water matrix by SPE [11,12]. It also improves their gas chromatographic properties. The additional silylation using MTBSTFA additionally lowered the LODs remarkably [13,14].

3.1. Solid-phase extraction (SPE)

The original phenazone-type compounds phenazone, propyphenazone and dimethylaminophenazone can easily be extracted from water samples by SPE [6]. Under the same conditions, the extraction of their polar metabolites DP, PDP, and DMADP was not satisfactory. Thus, DP had a recovery of only 1% in recovery experiments with spiked water samples at various pH values between 2 and 11 using cartridges filled with both PS–DVB or reversed-phase octadecyl (RP-C_{18}) materials. PDP was extractable with recoveries of more than 50% but DMADP was not at all detectable in spiked water samples (10 μg/L). Because of the low recoveries, particularly those for DP and DMADP, an in situ derivatization method was used to lower the polarity of the compounds rendering them suitable for SPE.

3.2. In situ derivatization

The polarity of the analytes can be reduced by replacing the free, active proton at the pyrazolone ring of the metabolites by a non-polar acetyl group. This was achieved by directly adding acetic anhydride to the water sample under basic pH conditions. The best results were obtained by using 500 mL of the water sample and adding 2 g potassium carbonate. After basification, 5 mL of AA were added and the sample was stirred until all of the AA was rapidly dissolved. The conversion of the metabolites into their corresponding less-polar acetates was sufficient for the extraction of the analytes using PS–DVB cartridges. Additionally, the derivatization also enhanced the sensitivity of the detection by improving the chromatographic properties of the analytes. This resulted in much lower LODs for all three metabolites. Nevertheless, the LOD of DMADP was still too high for the trace-level determination of this compound in environmental samples. The mass spectra of the acetylated metabolites and a multiple ion detection (MID) chromatogram recorded by GC–MS applying SIM are shown in Figs. 2 and 3. The response of DMADP is still too small to be seen in the MID chromatogram.

3.3. In situ derivatization followed by silylation

To achieve lower LODs for all metabolites and to enable the analysis of DMADP at low concentration levels, a silylation method was used to convert the extracted analytes by replacing the acetyl moiety by a *tert*-butyl dimethylsilyl moiety. A scheme of the reaction mechanism is shown in Fig. 4.

In spiking experiments at a concentration level of 0.2 μg/L, recoveries of 100% were determined for DP and PDP, whereas DMADP was recovered with 45%. The lower recovery is most probably caused by the additional amino group in the molecule of DMADP. The recovery of this compound could, however, not be increased by varying the pH values. Using this combination of both derivatization methods, all three metabolites can be quantified down to a concentration of 6 ng/L, respectively. The electron impact mass spectra of the original and the silylated compounds are shown in Figs. 5 and 6. The quantitation of the analytes was carried out by external calibration which is linear between the LOQs and 5 μg/L. Recoveries, LODs and LOQs of the analytical method were determined with ground water samples spiked at 0.5 μg/L (recoveries) or less with each of the pyrazolone standard compounds (Table 1). Fig. 7 shows a MID chromatogram of a ground water sample spiked with the three metabolites at a concentration of 2 μg/L each.

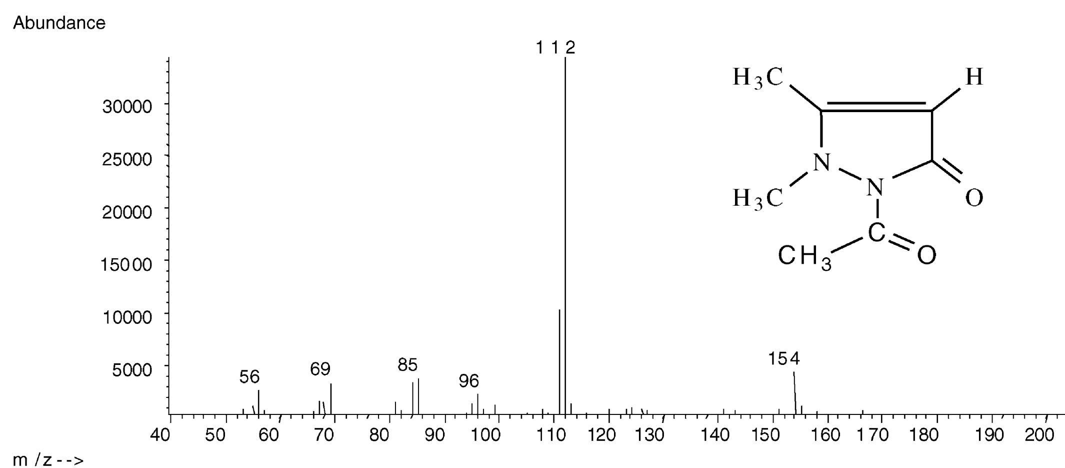
3.4. Screening of raw and drinking water

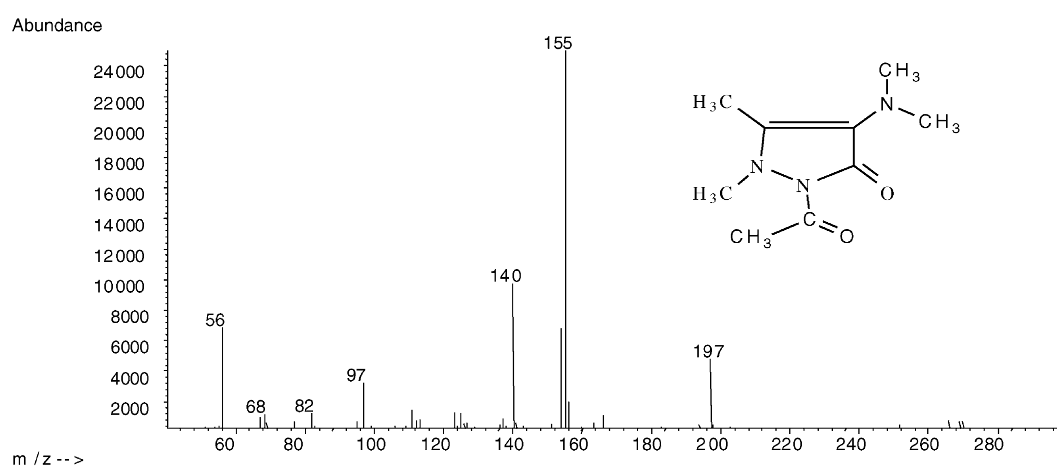
Analysis of two raw and two purified drinking water samples from two different waterworks revealed the suitability

Table 2
Concentration of DP, PDP and DMADP in two raw and drinking waters in the north west of Berlin

Sample type	Phenazone	DP	Propyphenazone	PDP	Dimethylaminophenazone	DMADP
Raw water[a]	2.50	1.15	0.88	0.32	0.24	<LOD
Drinking water[a]	0.25	1.10	0.08	0.24	<LOD	<LOD
Raw water[b]	1.10	0.98	0.39	0.25	<LOD	<LOD
Drinking water[b]	0.05	0.29	<LOD	0.10	<LOD	<LOD

All values in (μg/L).
[a] Sample collected in water works 1.
[b] Sample collected in water works 2.





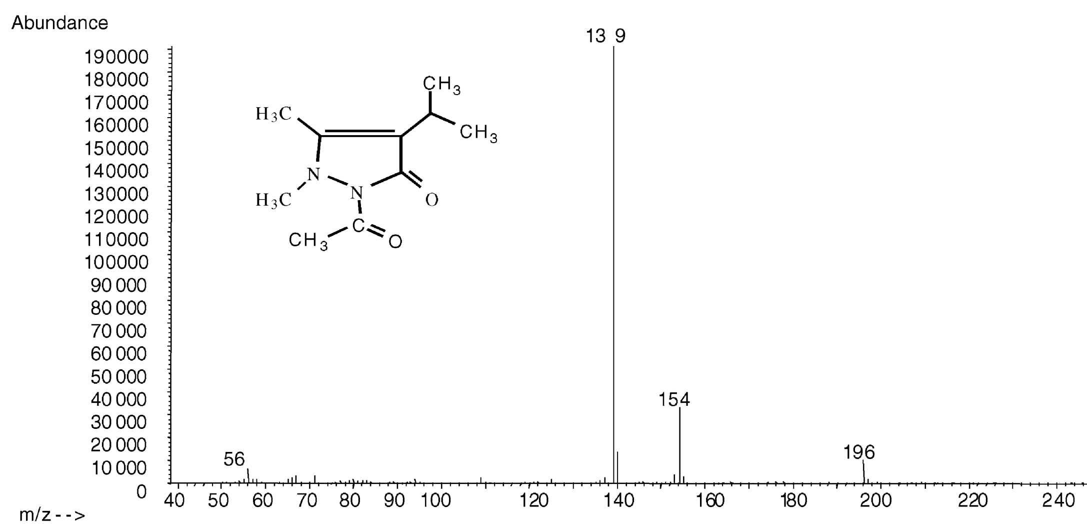


Fig. 2. Mass spectra of acetylated DP, PDP and DMADP (EI, 70 eV).

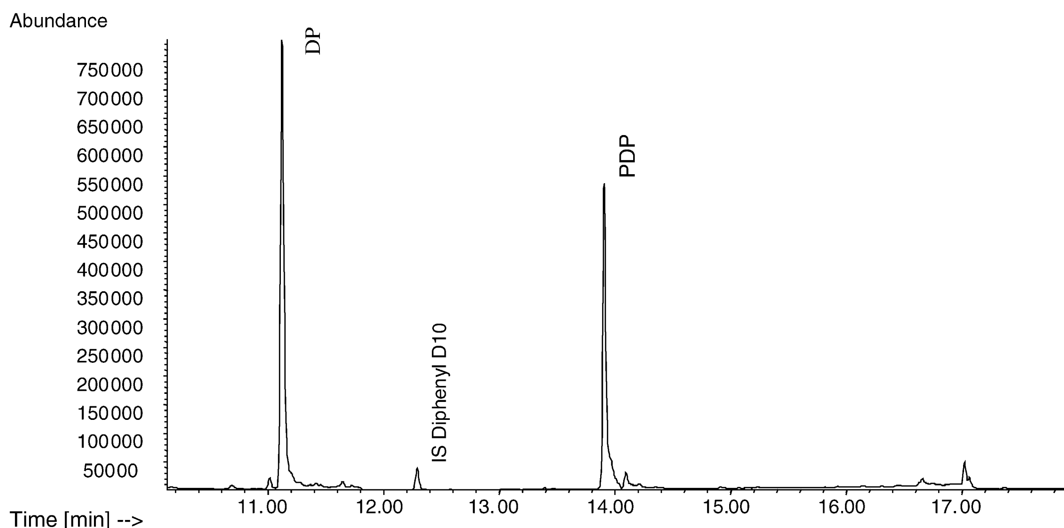


Fig. 3. Separation of a standard mixture of DP, PDP and DMADP (2 μg/L each) in ultra-pure water after in situ derivatization with AA; detection by GC–MS in MID mode.

of the above described methods. As compiled in Table 2, the three analgesic pharmaceuticals phenazone, propyphenazone and dimethylaminophenazone were detected in the raw water samples at concentrations up to 2.5, 0.88, and 0.24 μg/L, respectively. A significant reduction of the phenazone-type residues was observed during drinking water treatment. The final concentrations of phenazone, propyphenazone and dimethylaminophenazone detected in purified drinking water were more than ten times lower than those measured in the raw water samples. In the first water works 0.25 μg/L of phenazone were detected whereas in drinking water from the other water works only 0.05 μg/L were measured. Propyphenazone was determined at trace-level concentrations (0.08 μg/L) in the investigated drinking water, whereas dimethylaminophenazone was not detected in the drinking water from both water works. The results demonstrate that all three compounds are significantly degraded during conventional drinking water purification using aeration and sand filtration.

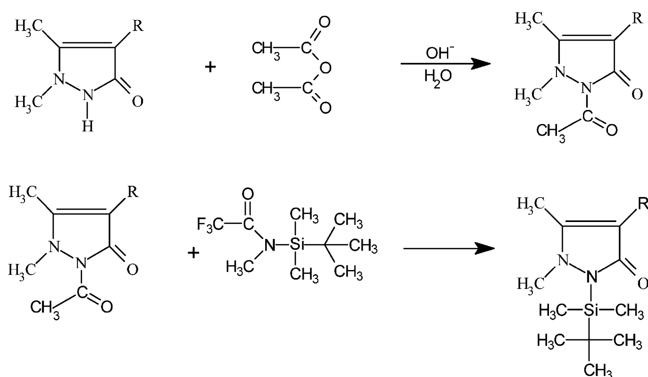


Fig. 4. Reaction pathway of the pyrazolones with acetic anhydride followed by silylation with MTBSTFA.

DP and PDP, the postulated metabolites of phenazone and propyphenazone, were identified in all raw and drinking water samples. Both metabolites were unambiguously identified in the water samples by their full scan mass spectra obtained for both the acetylated and the silylated derivatives. Quantitation of the analytes was performed by GC–MS with SIM as described in Section 2.5. DP was detected in both raw water samples at concentrations of 1.15 and 0.98 μg/L, respectively. Whereas a significant reduction of the DP concentrations was observed in one plant, only a slight decrease of its concentration was measured in the other water works. Similar results were also observed for PDP, the metabolite postulated for the degradation of propyphenazone. It was detected in the raw waters at concentrations of 0.32 and 0.25 μg/L, respectively. Again, a significant reduction of the concentrations of PDP in the drinking water samples was observed in one of the water works (from 0.32 to 0.10 μg/L), whereas in the other facility the concentrations decreased only slightly to 0.24 μg/L in the purified drinking water. The differences observed for both water works are not yet clear, but seem to depend on the filtration rate (2 m/h and 3.5 m/h, respectively). The results show, however, that both postulated metabolites have already been formed in the ground water. The results may also indicate that both metabolites are additionally formed from the parent compounds during drinking water treatment and will simultaneously be degraded to other metabolites which could not yet be identified. Additional investigations were also carried out to prove the toxicological relevance of DP. A recent study commissioned by the German Federal Environmental Protection Agency (UBA) [17] concluded that a lifetime consumption of 2 L of drinking water containing DP at a concentration of 3 μg/L will not cause any adverse human health effects and is tolerable for lifetime consumption. The maximum concentrations of DP in Berlin finished drinking water are clearly below this recommended concentration level.

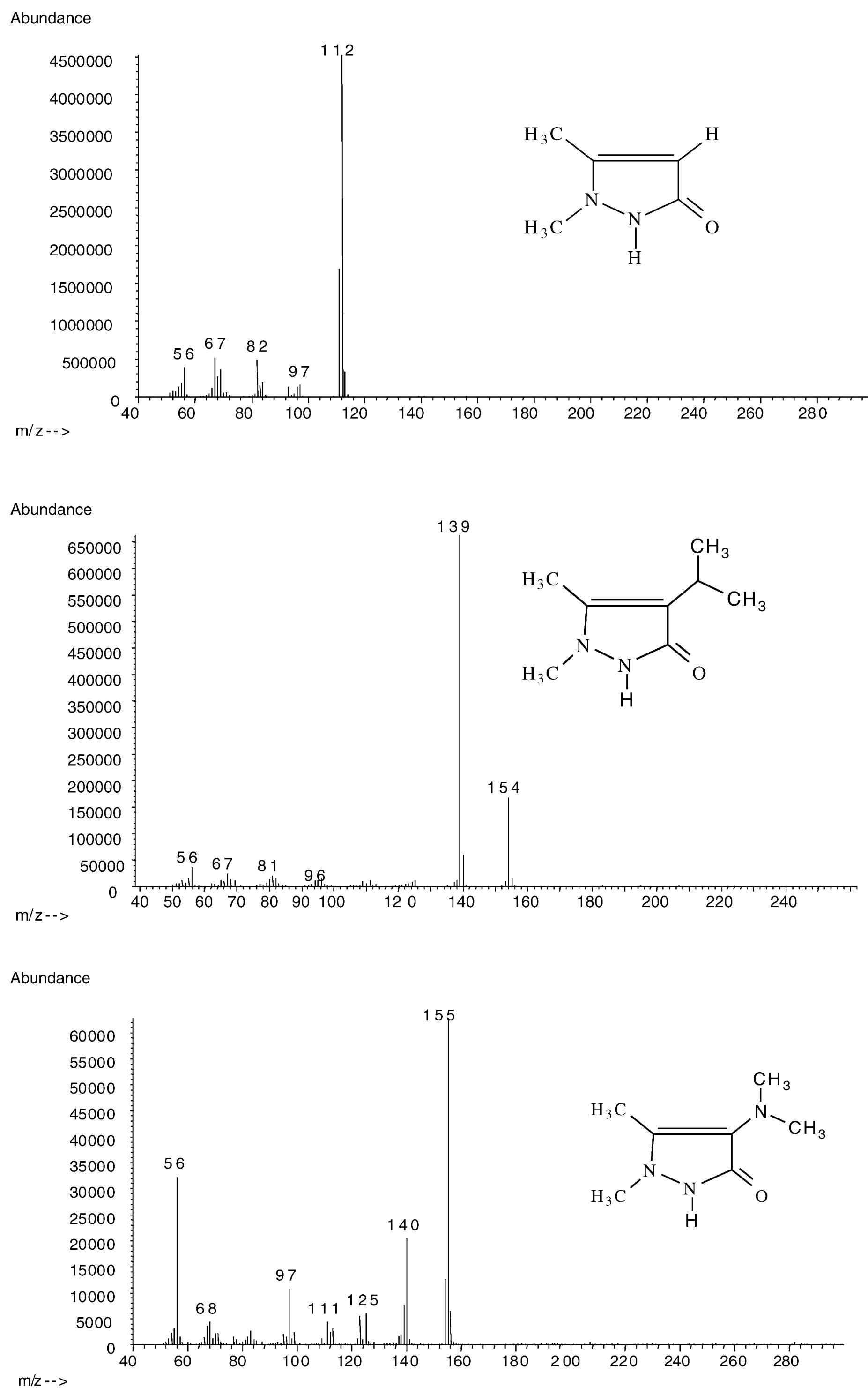
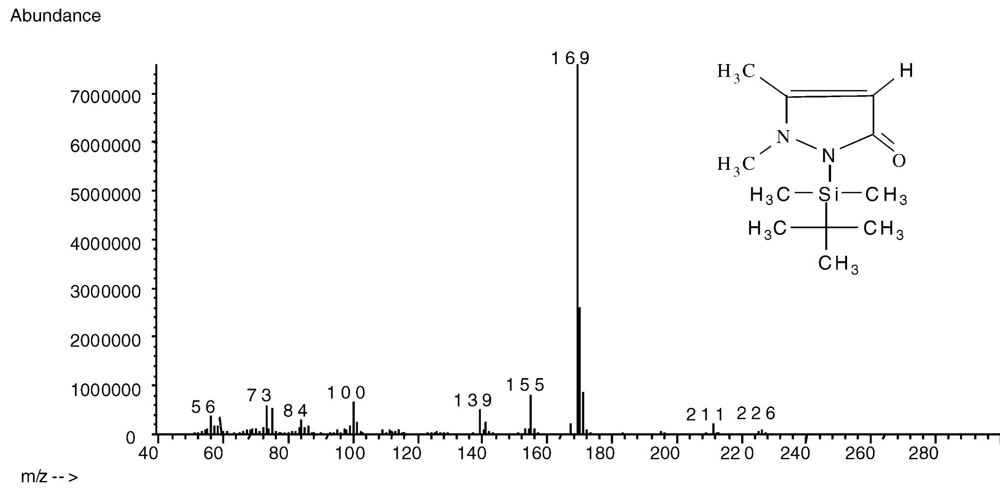
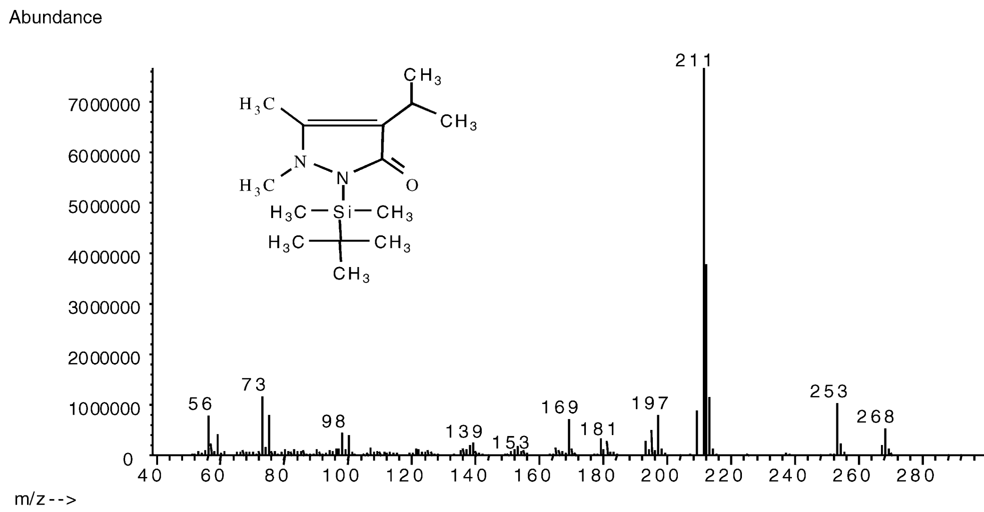


Fig. 5. Mass spectra of DP, PDP and DMADP (EI, 70 eV).

The third compound, DMADP, postulated as potential metabolite of dimethylaminophenazone could neither be detected in raw nor in purified drinking water. The observed degradation of dimethylaminophenazone during drinking water treatment seems to be subject to another mechanism. Thus, the oxidative and the photochemical transformation of dimethylaminophenazone to 1-acetyl-1-methyl-2-dimethyloxamoyl-2-phenylhydrazide (AMDOPH) and 1-acetyl-1-methyl-2-phenylhydrazide (AMPH) have been shown in two previous publications [15,16]. Recently, both compounds have also been detected by Reddersen et al. [6] in ground and drinking water in Berlin.





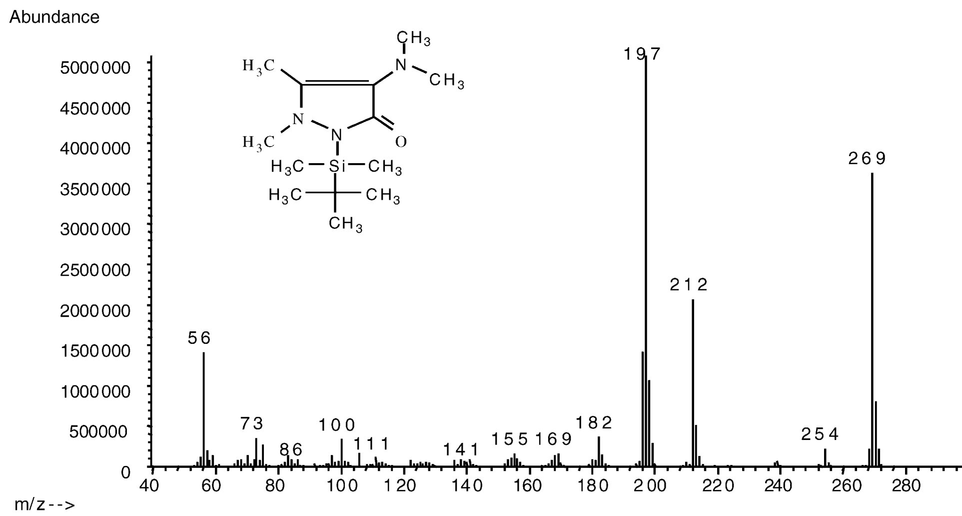


Fig. 6. Mass spectra of silylated DP, PDP and DMADP (EI, 70 eV).

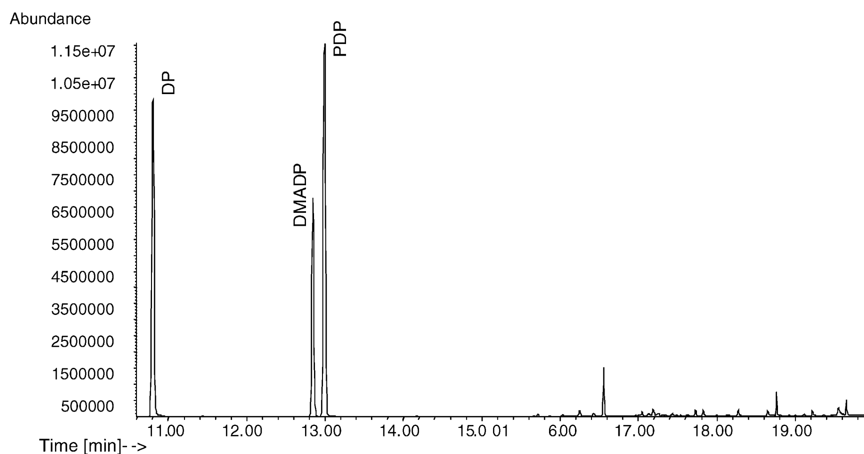


Fig. 7. MID chromatogram of a ground water sample spiked with DP, PDP and DMADP (2 μg/L each) after in situ derivatization with AA and silylation with MTBSTFA.

4. Conclusions

Residues of the analgesic drugs phenazone, propyphenazone and dimethylaminophenazone originating from spills of a former pharmaceutical production plant in the city of Oranienburg near Berlin were detected up to the μg/L level in raw water samples from two water works in Berlin, Germany. The results of our investigations show a significant decrease of the concentrations during conventional drinking water treatment. The drinking water concentrations for all three compounds are less than ten percent compared to those measured in the raw waters. Additionally, two metabolites (DP and PDP) postulated for the degradation of phenazone and propyphenazone were also detected up to the μg/L level in the raw and drinking water samples as well. Both compounds are already found in the ground water but may additionally be formed from the parent compounds during drinking water treatment and simultaneously be degraded to other metabolites which could not yet be identified. The maximum concentrations of DP in Berlin drinking water are clearly below the tolerable concentration level of 3 μg/L recommended by the German Federal Environmental Protection Agency.

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