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Identification of disinfection by-products of selected triazines in drinking water by LC-Q-ToF-MS/MS and evaluation of their toxicity

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During the development of an on-line solid phase extraction-liquid chromatography-ultraviolet detection (SPE-LC-UV) analytical method for determination of eight selected triazines; ametryn, atrazine, cyanazine, metrybuzine, prometryn, propazin, simazine, and terbutryn, in drinking water, it was observed that the retention times of three of them (ametryn, prometryn, and terbutryn) in Milli-Q water were different from those in chlorinated Milli-Q water, indicating the formation of new products. The cause of this change was found in the oxidation of the molecules as a result of chlorination with sodium hypochlorite. Experiments performed at varying concentrations of triazines and hypochlorite showed that the extent of the reaction depended on their relative concentrations. At the maximum admissible level of 100 ng/l for individual pesticides in drinking water, no apparent transformation was observed in the absence or at low concentrations (0.05 mg/l) of hypochlorite; however, on increasing the concentration of hypochlorite to the level typically present in drinking water (0.9 mg/l) the transformation was complete. The reaction is quite fast; within 1 h the parent compound is completely degraded and after 22 h the concentrations of the by-products are constant. Investigation of the by-products by ultra performance liquid chromatography-quadrupole-time of flight- tandem mass spectrometry (UPLC-Q-ToF-MS/MS) has shown that all three triazines follow a similar transformation pathway, forming four new molecules whose structure have been elucidated. The acute toxicity of the new products was investigated using a standard method based on the bioluminescence inhibition of *Vibrio fischeri*, and the by-products showed a higher toxicity than that of the parent compounds.

Keywords: triazines; disinfection by-products; UPLC-QToF; *Vibrio fischeri*; chlorination; identification

Introduction

Triazines are, because of their high use and physical-chemical characteristics which allow for mobility and moderate persistence, among the most frequently reported pesticides in ground water.[1]

Even though newer pesticides (including triazines) are relatively labile and as such do not persist long in the environment, there is such a widespread use that it is expected that there will always be some level of exposure.[2] The lability of the pesticides also makes it very important to monitor their transformation products as these can at times be present in concentrations higher than that of the parent compound.[3] This fact has been recognized by the European Union (EU) and the World Health Organization (WHO), who have both included specific transformation/degradation products in their guidelines for drinking water quality.[4–6]

Owing to their labile nature, there is also a risk of transformation during the disinfection process of drinking water. Sodium hypochlorite (NaClO) is one of the most used disinfectants worldwide,[7] underlining the importance of identifying possible degradation products resulting from this procedure. In the disinfection process, hypochlorous acid (HClO) is the main responsible reagent for pathogen destruction, but both HClO and ClO^- react with organic compounds giving addition, substitution, or oxidation products.[8] A further interesting fact is that while chlorinated, s-triazines do not react with chlorine, the non-chlorinated s-triazines react very quickly with this compound.[9]

One of the major concerns regarding the disinfection by-products (DBPs) from chlorination is that NaClO is known to produce genotoxic DBPs[10] and can thus increase the acute toxicity of these practically nontoxic s-triazines. Also, the major chlorinated degradation products of Atrazine, Simazine, and Propazine are considered to be endocrine-disrupting chemicals by the U.S. Environmental Agency, and thus, monitoring these compounds is compulsory.[11] This raises the question of the

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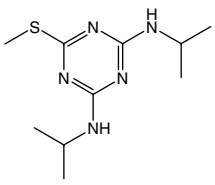
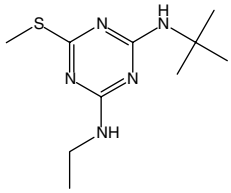
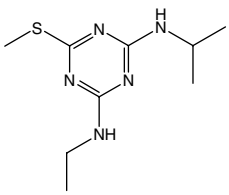
Name	Prometryn	Terbutryn	Ametryn
Structure			
Empirical formula	$C_{10}H_{19}SN_5$	$C_{10}H_{19}SN_5$	$C_9H_{17}SN_5$
Molecular weight	241.1361	241.1361	227.1205
CAS no.	7287-19-6	886-50-0	834-12-8

Figure 1. Structures of the studied compounds.

possible endocrine-disrupting properties of DBPs of the remaining triazines, thus making their identification even more vital.

The current paper deals with the transformation of three triazines (prometryn, terbutryn, and ametryn) as a result of the chlorination of drinking water. Out of the eight tested compounds (ametryn, atrazine, cyanazine, metrybuzine, prometryn, propazin, simazine, and terbutryn), only these three were degraded in the presence of hypochlorite. Figure 1 shows their chemical structure, empirical formula, molecular weight, and chemical abstract number (CAS) number. It has been investigated at which level of chlorination, the degradation occurs and the degradation products have been identified by ultra performance liquid chromatography-quadrupole-time of flight- tandem mass spectrometry (UPLC-Q-ToF-MS/MS). Furthermore, the acute toxicity of the degradation products has been determined by the bioluminescence inhibition of the marine bacteria *Vibrio fischeri*.

Experimental

Reagents and solutions

Acetonitrile (ACN) and extra pure water was purchased from Scharlau Chemie SA (Spain), and were filtered through 0.45 μm filters before being used. Methanol high performance liquid chromatography (HPLC) gradient grade 240 nm far UV, NaClO solution 15% w/v extra pure and sodium sulfite, extra pure, Ph Eur BP E221 were all purchased from Scharlau Chemie SA (Spain).

For the liquid chromatography-quadrupole-time of flight- tandem mass spectrometry(LC-Q-ToF-MS/MS) studies, water (CHROMASOLV Plus for HPLC) was from Sigma–Aldrich (Madrid, Spain) and acetonitrile (CHROMASOLV LC-MS grade) was from Riedel–de–Häen (Seelze, Germany).

High purity standards of ametryne, prometryn, and terbutryn were purchased from Riedel–de–Haën (Seelze, Germany) and SIGMA–ALDRICH Laborchemikalien GmbH (Seelze, Germany).

Stock standard solutions (1000 mg/l) for each triazine were prepared in methanol and stored at 4 °C in the dark. Working solutions of the individual standards at 1 mg/l and mixtures of all of them were prepared in the range 50–2000 ng/l by appropriate dilution of the stock standard solutions.

Solutions for the identification study of the transformation products were made by mixing 0.8 ml triazine in water (10 mg/l) with 0.2 ml hypochlorite solution (60 mg/l); obtaining final concentrations of 8 mg/l triazine and 12 mg/l hypochlorite. Samples were left at room temperature for the duration of each experiment.

On-line SPE-LC-UV analyses

Fully automated on-line solid phase extraction (SPE) was performed with a Symbiosis Environ system from Spark Holland (Emmen, The Netherlands) consisting of a Symbiosis Environ (P-2) unit equipped with an autosampler Endurance, a high pressure dispenser (HPD) using a mix mode option and an Automatic Cartridge Exchange (ACE). Aqueous standards (50 ml) were preconcentrated (at 5 ml/min) on polymeric cartridges PLRP-s (12.5 mg crosslinked styrene-divinylbenzene polymer, 15–25 μm particle size, from Spark Holland), previously conditioned with 2 ml acetonitrile (ACN) and 2 ml water (flow rate 5 ml/min). After washing with 1 ml water (5 ml/min), elution to the chromatographic system was carried out with the liquid chromatography (LC) mobile phase. The chromatographic system consisted of an Agilent 1100 LC pump (Palo Alto, CA, USA) connected in series with an Agilent 1100 photodiode array (UV-DAD) detector (Milford, MA).

Separation of the triazines was achieved on a Mediterranean Sea C_{18} column (150 × 4.6 mm, 5 μm) from Teknokroma (Sant Cugat del Vallés, Spain) using acetonitrile/water at 1 ml/min as the mobile phase. The gradient started with 35% ACN, which was held isocratic for 18 min, increased to 80% in 22 min, was held under these conditions for 5 min, returned back to the starting conditions over 5 min, and followed by 5 min equilibration. The last triazine is eluted at 36.6 min and corresponds to Terbutryn. Total analysis time was 55 min and the detection was performed at 220 nm.

UPLC-Q-ToF-MS/MS analysis

UPLC was performed on a Waters Acquity UPLC system (Waters Corp., Mildford, MA) equipped with a binary solvent delivery system, an autosampler and a UV detector. Chromatographic separation was performed on a 50 × 2.1 mm Waters Acquity C18 1.7 μm column. The injection volume was 10 μl and the flow rate was 0.35 ml/min. Triazines were analyzed using positive ionization. The elution was performed by an ACN/water gradient. The gradient was linearly increased from 10 to 90% ACN in 10 min, then increased to 95% over 2 min. Total run time, including re-equilibration of the column to the initial conditions, was 16 min. Mass spectrometry was performed on a Q-ToF-Micro (Waters Corp.). The nebulization gas (nitrogen) was set to 600 l/h at a temperature of 350 °C; the cone gas (nitrogen) was set to 50 l/h, and the source temperature to 120 °C. The capillary and cone voltages were set to 3000 and 30 V, respectively. For MS experiments, the Q-ToF-Micro instrument was operated in a wide pass quadrupole

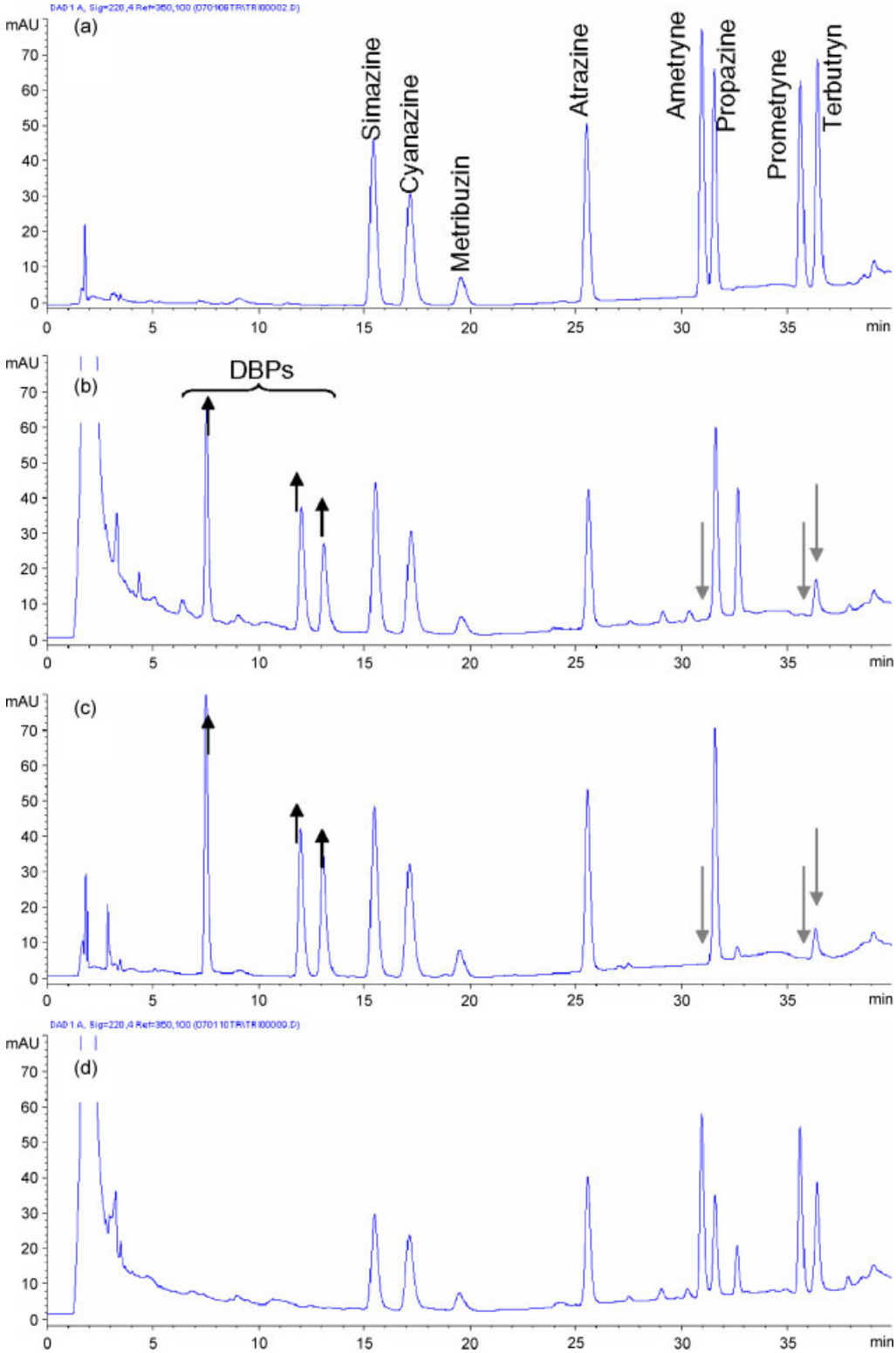


Figure 2. On-line SPE-LC-UV analysis of aqueous standard mixtures of the eight triazines (2 µg/l) prepared in (a) Milli-Q water, (b) tap water, (c) Milli-Q water spiked with 1 mg/l hypochlorite, and (d) tap water previously spiked with sodium sulphite.

mode with the time of flight (TOF) data being collected between *m/z* 50 and 1000 (PI) and low collision energy of 10 eV. Data were collected in the centroid mode, with a scan accumulation time of 1 s. The MS/MS experiments were performed at variable collision energies (10–40 eV). All analyses were performed using an independent reference spray (lockSpray) to ensure accuracy and reproducibility. Val-Tyr-Val was used as the lock mass (*m/z* 380.2029) at a flow rate of 10 µl/min. The lockSpray frequency was set at 11 s, meaning that every 11 s the flow from the lockspray was introduced into the mass spectrometer for 1 s, thus giving the

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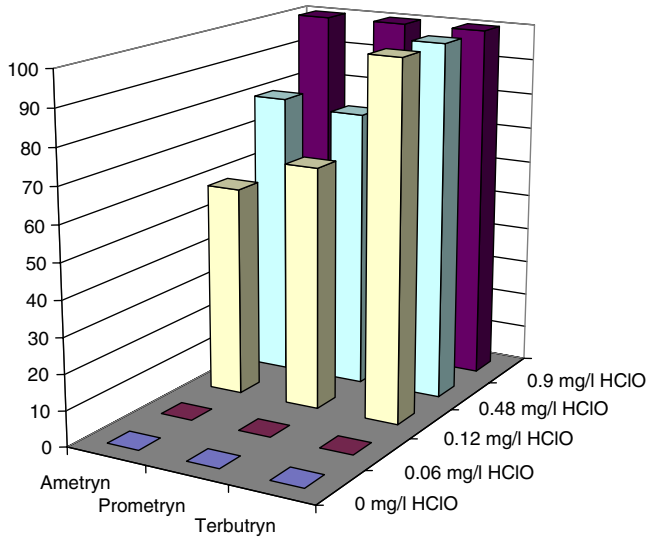

Figure 3. Formation of disinfection by-products of ametryn, prometryn, and terbutryn as a function of the concentration of hypochlorite.

software the possibility to perform ongoing correction of the exact mass of the analyte. Data were averaged over 10 spectra/min. The Masslynx software (Waters Corp.) has a feature that calculates all possible elemental compositions from the accurate mass. When using previous knowledge and basic organic chemical rules to eliminate nonfeasible formulae, this is a strong tool for forming hypotheses about the identity of an unknown compound. The final identification can then be performed based on the accurate mass measurements of the parent ions and fragments obtained in MS/MS experiments.

Acute toxicity

For the acute toxicity evaluation, a method based on the bioluminescence inhibition of the marine bacteria *V. fischeri,* NRR-11177 was applied. This marine bacterium naturally emits light, thanks to the action of the enzyme bacterial-luciferase. The bioluminescence is directly proportional to the metabolic status of the cell. A toxic substance will cause changes in the cellular state, which may be on different levels – cell wall, cell membrane, the electron transport chain, enzymes, cytoplasmatic constituent, but in all these cases changes are rapidly reflected in a decrease of the light production that can be easily measured by a photomultiplier in a luminometer. This work was carried out using the ToxAlert100 (Merck).

The percentage of inhibition (%*I*) is determined by comparing the response given by a saline control solution to that corresponding to the sample.

$$\%I = [1 - (\text{dilution light/control light})] \times 100$$

Measuring the inhibition values in a wide number of sample concentrations it is possible, by fitting an inhibition curve, to calculate the concentration where 50% of the specie is affected by this substance (EC_{50}).

Main advantages of this method are rapid, robust, cost effective, and standard responses, and it has been applied in different works for the characterization of organic pollutants,[12,13] degradation products, and mixtures.[14]

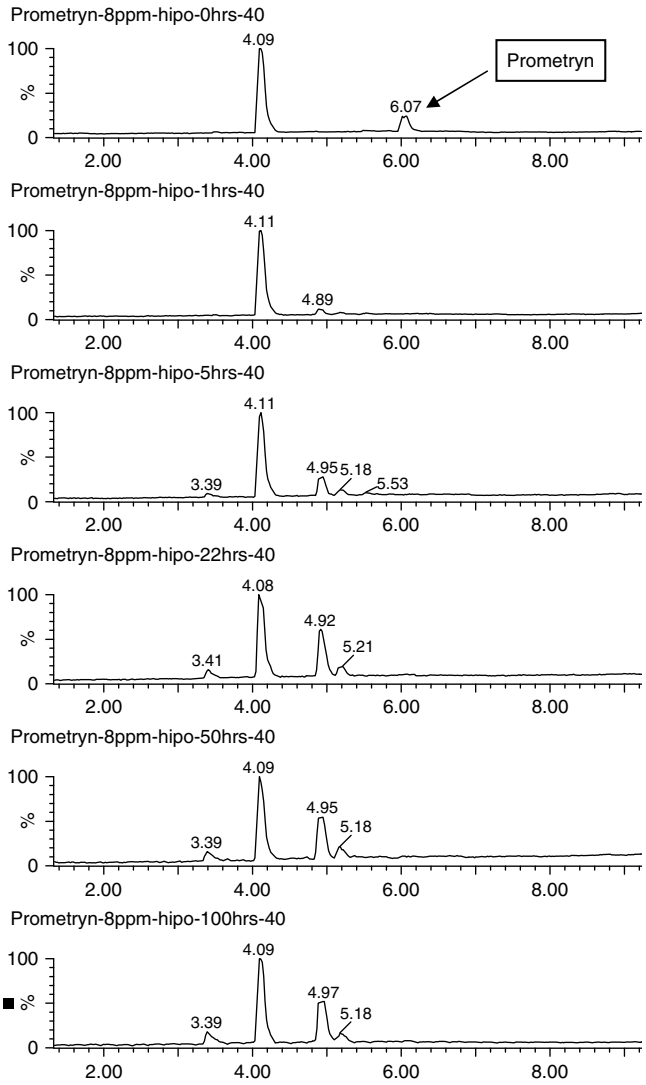

Figure 4. Kinetics of formation of prometryn by-products in the presence of hypochlorite.

Results and Discussion

Transformation of triazines in the presence of chlorine

Figure 2 shows the on-line solid phase extraction-liquid chromatography-ultraviolet detection (SPE-LC-UV) analysis of aqueous standard mixtures of the eight triazines (2 µg/l) prepared in Milli-Q water, tap water, Milli-Q water spiked with 1 mg/l hypochlorite, and tap water previously spiked with sodium sulfite (12 mg/l, in order to block the hypochlorite). As it can be seen, the peaks corresponding to three of the eight target triazines, namely, ametryn (RT 30.95 min), prometryn (35.62 min), and terbutryn (36.44 min), which are visible in Milli-Q water, disappear in the chlorinated solutions (tap water and Milli-Q water added with HClO), but remain the same in the solution prepared in tap water previously treated with sodium sulfite (to block any residual hypochlorite). The disappearance of these three peaks goes hand-in-hand with the appearance of three new peaks at shorter retention times (7.49, 11.97, and 13.04 min) which correspond to the formation of three main transformation products.

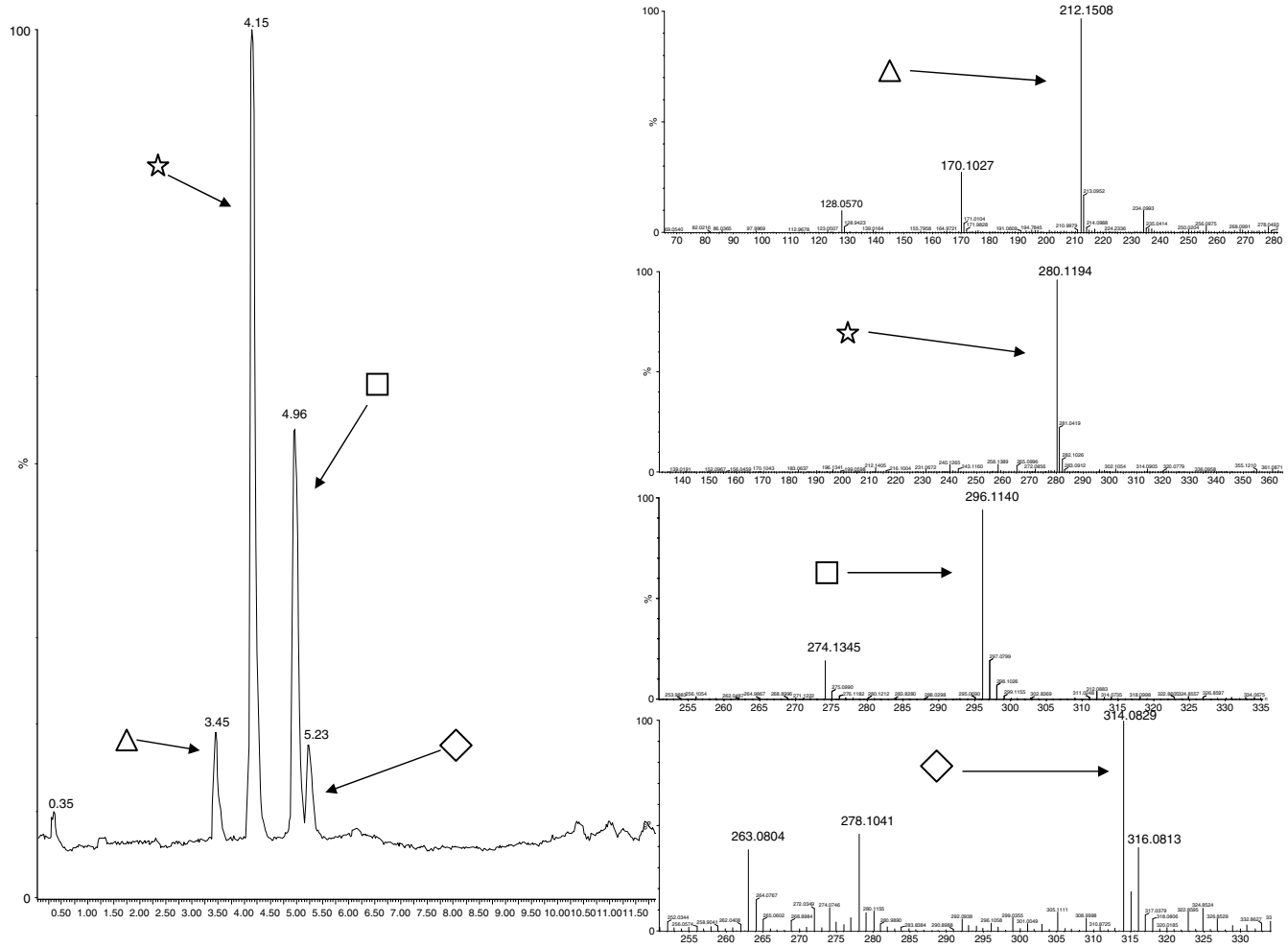

Figure 5. Chromatogram and MS spectra of degradation products of prometryn.

The peak appearing at 32.7 min in the chromatograms in Fig. 2 (b), (c) and (d) is due to a previous contamination of the HPLC equipment and even occurred in chromatograms of standard solutions prepared in Milli-Q water.

Determination of the effect of the concentration of hypochlorite

For a further investigation of the effect of chlorination, standard mixtures of the triazines (10 mg/l) were prepared with 0.9 and 12 mg/l hypochlorite (usual concentrations in drinking water are between 0.5 and 1 mg/l). Results showed that ametryn, prometryn, and terbutryn (10 ppm) were only degraded in the samples with 12-mg/l hypochlorite. It was suspected that the reason for the apparent lack of degradation with 0.9-mg/l hypochlorite was because of the comparatively high concentration of triazines.

To test this hypothesis, solutions containing 100 ng/l of each triazine were prepared in different hypochlorite solutions with concentrations ranging from 0 to 0.9 mg/l. The results (Fig. 3) confirmed that without the presence of hypochlorite, no degradation takes place and that degradation increases with the hypochlorite concentration and at 0.9 mg/l of hypochlorite, the triazines are fully degraded.

Time development of the formation of disinfection by-products

To study the time development of the formation of DBPs and simultaneously identify their structure, 12 mg/l hypochlorite was added to the samples with 8 mg/l triazine in water and these samples were injected into the UPLC-Q-ToF-MS/MS system at different time intervals during 1 week. Figure 4 shows the development of the by-products of prometryn.

From Fig. 4 it can be seen that prometryn is fully degraded within 1 h leading to the formation of one by-product. It can also be noted that after 5 h, the presence of a second new product can be detected. Furthermore, after 22 h a third and a fourth product can be observed, but with much lower responses. There is no significant change after 22 h. An identical pattern was observed for terbutryn and ametryn with the only difference being that the second, third and fourth by-products developed to a lesser extent for ametryn.

The relatively fast reaction times imply that the reaction products are likely to be present at the time that the drinking water arrives to the consumer as the average residence time of the drinking water in the distribution net has been reported to be 36–48 h.[15]

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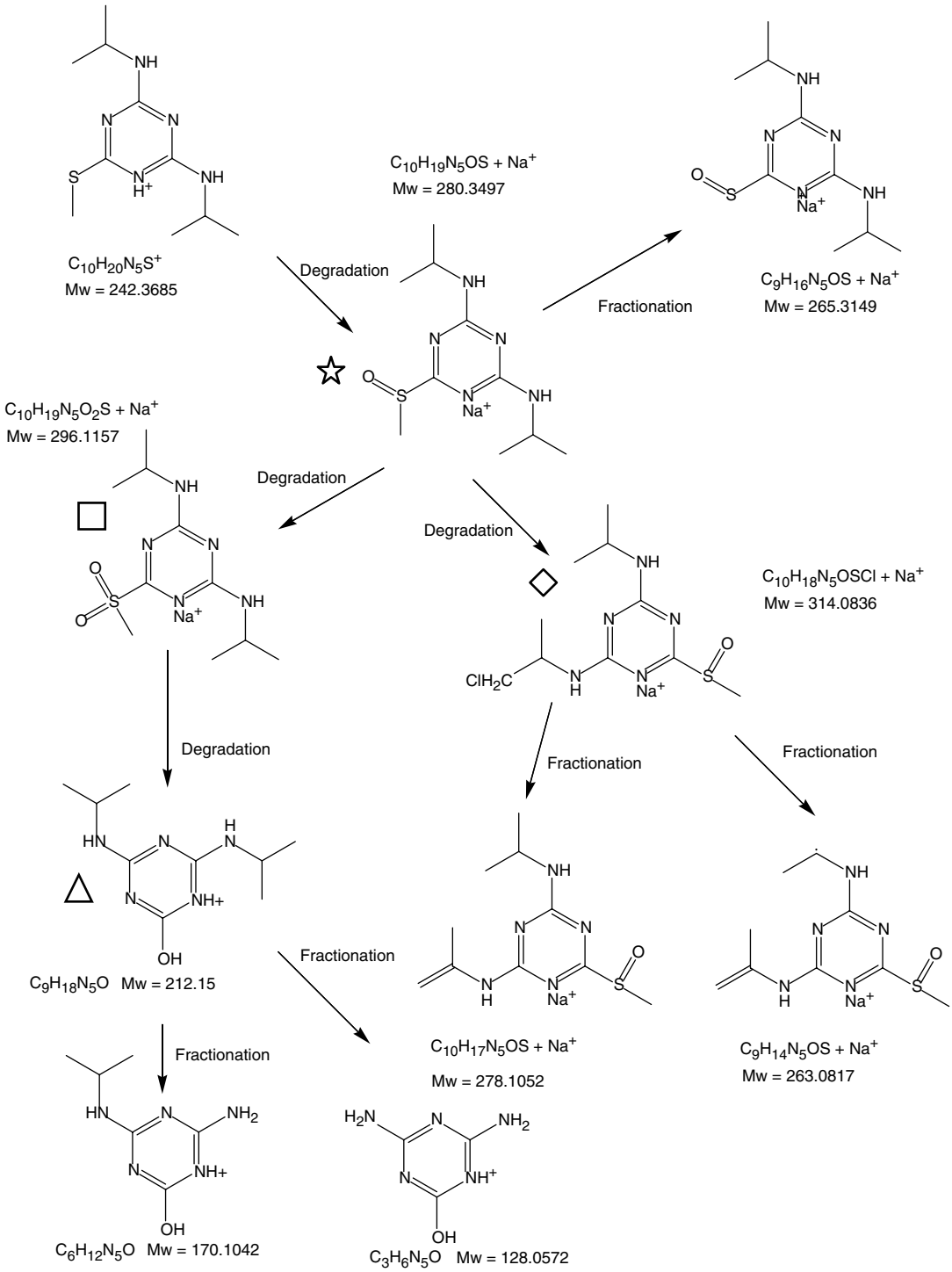

Figure 6. Reaction pathway of prometryn.

Identification of disinfection by-products

In Fig. 5, an enlarged chromatogram of the degradation products of prometryn is shown along with the MS spectra of the four peaks. It can be seen that the two major products formed have masses of 280.1194 Da and 296.1140 Da, respectively and the two minor products have masses of 212.1508 Da and 314.0829 Da.

The mass 280.1194 Da could correspond to an oxygenation of the sulphur, resulting in the following ion: $[C_{10}H_{19}N_5OS + Na]^+$, marked with a star in Fig. 6. The added sodium is a typical result of positive ionization using an electrospray.

The specification of the Q-ToF-Micro states a maximum mass error of 5 ppm for the range of masses discussed in this paper. If we calculate the number of possible elemental compositions valid for the mass 280.1194 with a window of ±5 ppm, without any restrictions on the type or number of atoms, we get more than 130 possible elemental compositions. However, if the type and number of atoms is restricted by the information from the

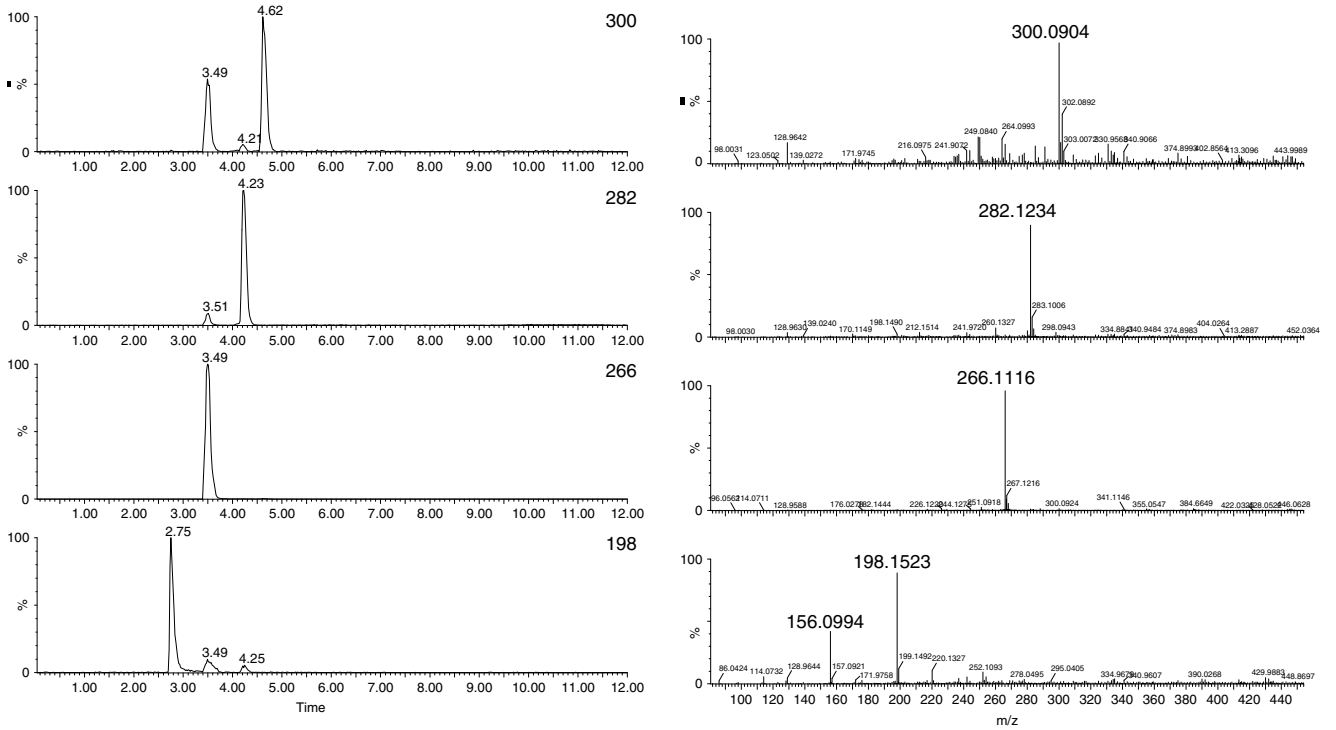

Figure 7. Ion extracted chromatograms and MS spectra of degradation products of ametryn in the presense of hypochlorite.

spectra (e.g. no halogens) and the possible degradation scenarios under the experimental conditions (i.e. maximum three oxygen atoms, ten carbon atoms, five nitrogen atoms and no phosphor or fluorine), the calculation leads to only one possible elemental composition. This fact alone can be used as a confirmation of the suggested elemental composition, but in the current work it has been sought to always have a second fact for final confirmation.

In order to verify the hypothesis that the mass 280.1194 Da corresponds to the ion: $[C_{10}H_{19}N_5OS + Na]^+$, an MS/MS run was performed. In this run, the quadrupole was filtering ions, permitting only the ion with mass 280 Da to pass to the collision cell where 20 eV was applied. In this run, the molecule fractured with a neutral loss of 15 Da. This could correspond to a loss of a methyl unit. Literature confirms that this fractionation pattern strongly indicates an oxygenation of the sulphur.[16]

The mass 296.1140 Da could correspond to a further oxygenation of the sulphur, resulting in the following ion: $[C_{10}H_{19}N_5O_2S + Na]^+$ (marked with a square in Fig. 6). This hypothesis could not be confirmed by MS/MS as it was not possible to fractionate the molecule. But the hypothesis is supported by the fact that the mass 274.1345 Da was found at a low abundance. This mass corresponds to the following ion: $[C_{10}H_{20}N_5O_2S]^+$ which is the same molecule as above, but ionized with hydrogen instead of sodium, thus confirming the hypothesis.

The mass 212.1508 Da is likely a further degradation of the sulfone above (296 Da) to an alcohol, $C_9H_{17}N_5O$ (marked with a triangle in Fig. 6). In this case, there is no need to perform MS/MS to confirm the structure since this molecule is already fragmented by the cone voltage, as can be seen in the mass spectra shown in Fig. 5. The fragmentation corresponds to the loss of first one C_3H_6 group resulting in the ion $[C_6H_{12}N_5O]^+$, mass 170.1027 Da and later two C_3H_6 groups leading to the ion $[C_3H_6N_5O]^+$, mass 128.0570 (see Fig. 6).

The fourth transformation product has the mass 314.0829 Da. Again it can be seen from the mass spectra (Fig. 5, bottom right) that the molecule has been fragmented already in the cone. It should also be noted that the characteristic pattern for the presence of chloro is present for the parent compound, but not for the fragments. The parent compound of this spectra is most likely another degradation product of the 280 Da sulfoxide. The degradation lead to a substitution of a hydrogen atom for a chloro atom; resulting in the molecule $C_{10}H_{18}N_5OSCl$ (marked with a diamond in Fig. 6). This thesis is supported by the presence of fragments with masses 278.1041 Da (loss of HCl) and 263.0804 Da (loss of HCl and CH_3).

For terbutryn, the same transformation products are seen as for prometryn, with the same masses, as terbutryn has the same mass as prometryn. Ametryn is undergoing the same transformations, but to a much lower extent (see Fig. 7).

The formation of the first three degradation products can be confirmed by the literature,[9,17,18] however, the fourth degradation product has previously not been mentioned in the literature.

Toxicology

The *V. fischeri* toxicity studies were carried out with 30 min exposure time. It was found that EC_{50} values were very high (18–26 mg/l - see table 1), indicating very low acute toxicity which is in correspondence with a previous literature.[19,20]

After treatment with hypochlorite, the acute toxicity increased considerably (e.g. prometryn becomes five times more toxic), but EC_{50}s still remained rather high (5.2–15 mg/l) and were still not considered toxic. However, these results indicate that in future works, the synergistic effects with other compounds present in the same samples should be studied.

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Table 1. Cell toxicity of the three triazines before and after treatment with hypochlorite

Compound	EC_{50} in Milli-Q + 2% NaCl	EC_{50} in H_2O hypochlorite + 2% NaCl
Ametryn	18 ± 1 mg/l	9.2 ± 3 mg/l
Prometryn	26 ± 1 mg/l	5.2 ± 2 mg/l
t-Butryn	24 ± 1 mg/l	15 ± 3 mg/l

Conclusions

Three triazines, i.e. ametryne, prometryne, and terbutryn, out of eight tested, have been shown to react with hypochlorite in water and form four main DBPs, which in the *V. fischeri* toxicity studies are more toxic than the parent compounds. At typical hypochlorite and pesticides concentrations (0.5–1 mg/l and 100 ng/l, respectively) in drinking water the transformation of the parent compounds into the corresponding by-products is complete. In addition, the reaction is quite fast; within 1 h the parent compound is completely degraded and after 22 h the concentrations of by-products are constant, which means that the reaction products are likely to be present at the time that the drinking water arrives to the consumer. The four main degradation products have been identified with UPLC-Q-ToF-MS/MS of which only three have previously been described in the literature. Because some s-triazines are suspected endocrine-disrupting compounds and, further, that DBPs formed during water chlorination are often genotoxic, the identified by-products should be subjected to a more profound investigation in terms of toxicity to evaluate potential risks of exposure through chlorinated drinking water.

Acknowledgements

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