5

Structural elucidation of main ozonation products of the artificial sweeteners cyclamate and acesulfame

Purpose The two artificial sweeteners cyclamate (CYC) and acesulfame (ACE) have been detected in wastewater and drinking water treatment plants. As in both facilities ozonation might be applied, it is important to find out, if undesired oxidation products (OPs) are formed.

Methods For the separation and detection of the OPs, several analytical techniques, including nuclear magnetic resonance experiments, were applied. In order to distinguish between direct ozone reaction and a radical mechanism, experiments were carried out at different pH values with and without scavenging OH radicals. Kinetic experiments were used for confirmation that the OPs are formed during short ozone contact time applied in waterworks. Samples from a waterworks using bank filtrate as raw water were analyzed in order to prove that the identified OPs are formed in real and full-scale ozone applications.

Results In the case of CYC, oxidation mainly occurs at the carbon atom, where the sulfonamide moiety is bound to the cyclohexyl ring. Consequently, amidosulfonic acid and cyclohexanone are formed as main OPs of CYC. When ozone reacts at another carbon atom of the ring, a keto moiety is introduced into the CYC molecule. Acetic acid and the product ACE OP170, an anionic compound with *m/z* 170 and an aldehyde hydrate moiety, were identified as the main OPs for ACE. The observed reaction products suggest an ozone reaction according to the Criegee mechanism due to the presence of a C=C double bond. ACE OP170 was also detected after the ozonation unit of a full-scale drinking water treatment plant which uses surface water-influenced bank filtrate as raw water.

Conclusions Acesulfame can be expected to be found in anthropogenic-influenced raw water used for drinking water production. However, when ACE OP170 is formed during ozonation, it is not expected to cause any problem for drinking water suppliers, because the primary findings suggest its removal in subsequent treatment steps, such as activated carbon filters.

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5.1 Background, aim, and scope

In recent years, concerns arose about the environmental fate of so-called emerging contaminants released into the aquatic environment via wastewater treatment plants (WWTPs) due to incomplete removal during biological wastewater treatment. As a consequence, some of these organic trace pollutants were detected in the nanograms per litre to micrograms per liter range in WWTP effluents and were even found in raw waters used for drinking water production or in some cases also in finished potable water (Loraine and Pettigrove 2006; Benotti et al. 2009; Prasse et al. 2010). Acute toxic effects of low concentrations of these compounds are mostly unknown, but it should be aimed to minimize levels of such compounds in order to prevent from potential harmful and yet unknown risks.

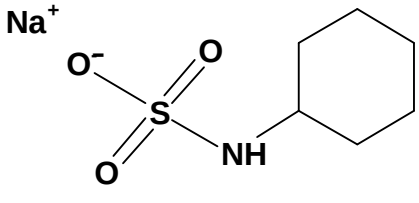
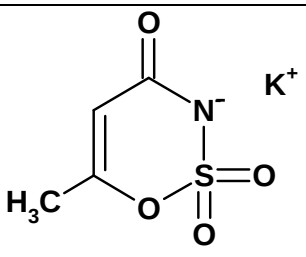
Different strategies to minimize the discharge of organic trace pollutants in the aquatic environment and for their removal during drinking water production are discussed in the literature. Upgrading WWTPs with powdered activated carbon or ozonation units as tertiary treatment seems to be a promising option. Several studies report not only about ozonation as an effective tool for the removal of pharmaceuticals, fragrances, X-ray contrast media, antibiotics, estrogens, plant-protecting agents, industrial chemicals, and food additives (Ternes et al. 2003; Huber et al. 2005; Dodd et al. 2006; Hollender et al. 2009; Rosal et al. 2010), but also about superior elimination of fecal and total coliforms (Ternes et al. 2003; Wert et al. 2007). Advanced wastewater treatment with ozone is considered to be cost effective and economically feasible (Joss et al. 2008; Schaefer et al. 2009). Ternes et al. (2003) assessed the costs for installation and ozone treatment with 10 g ozone/m³ wastewater to be ≤0.04 €/m³. An additional energy requirement of 12 % for upgrading a nutrient removal WWTP with a post-ozonation unit was reported by Hollender et al. (2009). The application of 0.6 g ozone per g dissolved organic carbon (DOC) was sufficient for the removal of the vast majority of 220 micropollutants in that case. Due to the benefits of micropollutant removal and disinfection, several full- or large-scale pilot plants, using ozonation as a tertiary wastewater treatment, are already established or planned for the near future.

The use of ozone in drinking water production has an even longer tradition and was already used for odor and color removal in the USA in the early 1900s (Rice 1999). Nowadays, ozonation is widely used for raw water treatment in drinking water treatment plants (DWTPs), in particular when indirectly surface water-influenced water (e.g. river bank filtrate) is used for the production of potable water.

The oxidation of organic trace pollutants during ozonation is often connected with a decrease of their original biological activity (Huber et al. 2004; Suarez et al. 2007; Dodd et al. 2009) and in most cases with the formation of more readily biodegradable compounds (Alvares et al. 2001). In aqueous solutions, there are two modes of attack to the trace pollutant: either by direct reaction of the O_3 molecule or after decomposition of O_3 by OH radicals. Direct reaction is favored by lower pH values and selectively occurs at sites with high electron density, such as double bonds or amines. The more nonselective reaction with OH radicals is the main oxidation pathway for some ozone-resistant micropollutants (Elovitz and von Gunten 1999). Although most of the hydroxyl radicals are likely to be scavenged in waters with a high matrix burden, they complicate the prediction of the nature of the oxidation products (OPs) even more. It was shown for wastewater and drinking water that ozonation can also lead to the formation of undesired by-products such as *N*-nitrosodimethylamine (NDMA) (Schmidt and Brauch 2008; Hollender et al. 2009). Thus, evidence is needed that harmless compounds are formed in ozonation units in WWTPs or DWTPs. The increasing use of ozone applications in water treatment resulted in an elevated number of publications dealing with the structural elucidation of potential OPs (e.g., Ramseier and von Gunten 2009; Benner and Ternes 2009a; Benner and Ternes 2009b; Dodd et al. 2010).

The aim of this work was to identify the main OPs of the artificial sweeteners cyclamate (CYC) and acesulfame (ACE) (Table 5-1), which were only recently detected in microgram-per-liter levels in WWTP effluents and which were also found in surface water as well as in raw water used for drinking water production (Buerge et al. 2009; Scheurer et al. 2010).

Table 5-1 Chemical structures of CYC and ACE with CAS registry number and mono-isotopic masses

	
Sodium cyclamate	**Potassium acesulfame**
CAS: 139-05-9	CAS: 55589-62-3
Monoisotopic mass: 201.04 (sodium salt), 178.05 (anion)	Monoisotopic mass: 200.95 (potassium salt), 161.99 (anion)

5.2 Materials and methods

5.2.1 Chemicals

Acesulfame potassium (>99 %), tertiary butyl alcohol (*t*-BuOH; 99.7 %), and cyclohexanone (99.5 %) were purchased from Fluka (Steinheim, Germany), sodium cyclamate from Supelco (Bellefonte, PA, USA) and para-chlorobenzoic acid (pCBA; 99 %) from Sigma-Aldrich (Steinheim, Germany). Acetic acid, formic acid, and oxalic acid dihydrate (analytical grade) were purchased from Merck (Darmstadt, Germany). The solvents used for this study were all of analytical grade.

5.2.2 Experimental setup

Ozonation experiments were performed as batch experiments in 1 L glass vessels filled with ultrapure water (200 mL) containing 25 mg/L CYC (=140 µmol/L) and ACE (=153 µmol/L), respectively. The batch setups were prepared at pH 3 and 7.5 with phosphate buffer (25 mM) with or without *t*-BuOH as a radical scavenger to be able to distinguish between reactions mainly driven by ozone or OH radicals. To assess if scavenging of OH radicals was sufficient, samples were spiked with 300 µg/L pCBA. The ozone stock solution was prepared by sparging ozone gas through a cooled reactor column (5 °C) filled with ultrapure water. The ozone concentration of the stock solution (ca. 17 mg/L) was measured by the indigo method (Bader and Hoigne 1981) before adding it to the batch vessel. Different compound to ozone ratios were achieved by adding defined amounts of the aqueous ozone solution and taking into account the dilution factor for subsequent calculations. For an even spread of the ozone, the test setup was slightly stirred on a magnetic stirrer for about two minutes. The samples were placed in the fume hood over night and analyzed the next day.

Similar tests were performed with Karlsruhe tap water (DOC = 0.9 mg/L, pH = 7.3) and with treated wastewater from the WWTP in Eggenstein-Leopoldshafen (DOC = 8.6 mg/L, pH = 7.6). The batch tests in these real matrices were repeated in 100 times lower compound concentrations (250 µg/L) and with realistic ozone concentrations applied in water treatment plants (0.2 mg/L to 2 mg/L) and WWTPs (0.25 mg/L to 1 mg/L per mg DOC) to investigate the influence of the present DOC on a more realistic basis.

Kinetic experiments for ACE (200 µg/L) with a waterworks relevant ozone dose of 1 mg/L and a contact time of 25 min were performed in ultrapure and potable water. Samples were directly injected every 4 min for analysis by rapid-resolution liquid chromatography-

mass spectrometry (LC-MS/MS) in order to track the degradation of the precursor and formation of identified OPs.

In order to perform nuclear magnetic resonance (NMR) experiments, higher concentrations (5 g/L) of both sweeteners were ozonated by passing the produced ozone gas directly through a gas-washing bottle head equipped with a filter disk into a separating funnel containing the test solution. Samples were taken after defined time intervals and stripped with nitrogen gas in order to remove remaining ozone.

5.2.3 Chromatographic separation and detection

Samples from the batch tests were analyzed with different LC and gas chromatography (GC) methods optimized for pCBA and the suspected OPs based on preliminary experiments. These separation techniques were coupled to different (also high resolution) mass spectrometers (MS) for detection and MS^2 experiments. MS^3 spectra were obtained by direct injection into the electrospray interface of the MS. Ion chromatography (IC) coupled with a conductivity detector was used to analyze carboxylic acids, which were suspected to be formed during ozonation. Furthermore, IC was also coupled with inductively coupled plasma mass spectrometry (ICP–MS) to rule out the formation of sulfate during ozonation of the sulfur containing sweeteners.

The organic carbon content of the batch samples was determined according to DIN EN 1484 (08/1997) with a total organic carbon (TOC) analyzer TOC-V_{CHP} equipped with an ASI-V auto sampler (both Shimadzu, Kyoto, Japan).

For structural elucidation of the proposed OPs, ^{1}H-NMR measurements were applied for the highly concentrated original aqueous solutions of OP mixtures. In a second approach, the results were confirmed by LC-HILIC-NMR/MS.

A detailed description of the applied methods is given in the supporting information (see chapter 5.5 and Table 5-2).

5.3 Results and discussion

The TOC balance for different CYC and ACE to ozone ratios for the batch test in ultrapure water without the addition of *t*-BuOH revealed that no significant loss of precursors or unknown organic oxidation products occurred till analysis. The results for the TOC measurements are displayed in Figure 5-7 in chapter 5.5.

For the batch tests where OH radicals were scavenged by the presence of *t*-BuOH, scavenging was controlled by pCBA measurements (method 3, Table 5-2). A constant pCBA concentration was observed for all tests where *t*-BuOH was added (data not shown).

5.3.1 Ozonation of cyclamate

For the CYC molecule, no preferred sites for a direct ozone attack could be expected. The cyclohexyl ring is saturated and the sulfonamide should be still protonated at pH 8 and therefore practically unreactive (Munoz and von Sonntag 2000). The ozonation of cyclohexane by OH radicals in gas-phase reactions or by ozonation has been reported before (Aschmann et al. 1997; Barletta et al. 1998). The second study describes the ozonation of cyclohexane as a pure compound and identified cyclohexanone and cyclohexanol as the main OPs.

In the case of CYC, we also identified cyclohexanone as one main ozonation product based on GC-MS/MS and ^{1}H-NMR measurements.

Figure 5-8 in chapter 5.5 shows the edited HSQC spectra of CYC and the main oxidation product after 80 min of ozonation. While CYC exhibits one single –CH proton resonating at 3.1 ppm, two pairs of equivalent $–CH_2$ groups and one $–CH_2$ according to the integral values between 1.1 and 2 ppm, the oxidation product shows only three NMR resonance signals corresponding to two pairs of equivalent $–CH_2$ groups and one additional $–CH_2$ between 1.7 ppm and 2.5 ppm. The loss of the downfield-shifted –CH, the significant downfield shift of the $–CH_2$ groups and the high degree of symmetry in the molecule is in agreement with the presence of cyclohexanone as being the major oxidation product of CYC. This has been corroborated by comparison with pure reference compound diluted in D_2O using NMR and GC-MS. Besides cyclohexanone, several minor NMR signals are present which are too low for further structural elucidation.

The formation of cyclohexanone was strongly dependent on the CYC to ozone ratio applied in the batch test (Figure 5-1). When ozone was present at the highest excess (1:10), 121 to 142 µmol/L cyclohexanone was formed in three out of four test series indicating an almost even mass balance on a molar scale. The non-scavenged batch test at pH 7.5 showed a lower conversion to cyclohexanone probably due to further oxidation by OH radicals. The formation of cyclohexanone represents a good example that several analytical techniques might be necessary for structural elucidation: from a highly polar precursor, predestinated for LC-MS, a typical GC compound is formed, which is not amenable for electrospray ionization anymore.

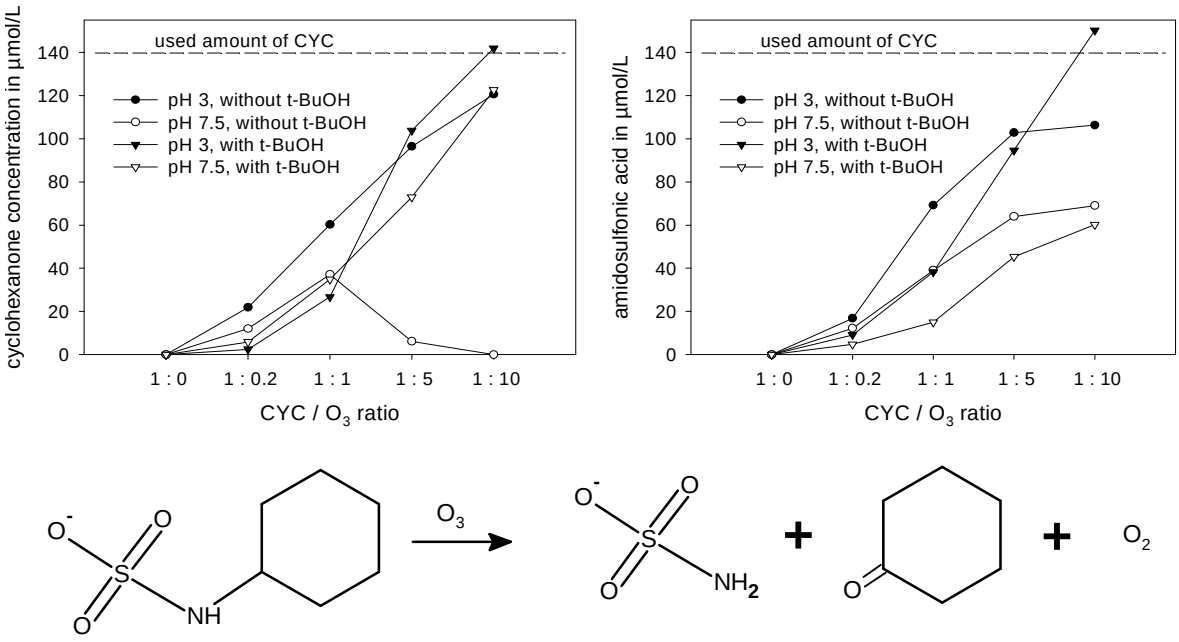


Figure 5-1 Formation of the OPs cyclohexanone (top left) and amidosulfonic acid (top right) in aqueous solution at different CYC to ozone ratios at pH 3 and 7.5 with and without the addition of *t*-BuOH as an OH radical scavenger (c_0 of CYC was 140 µmol/L and is indicated by the dashed line). Proposed main reaction pathway for the ozonation of CYC (bottom)

The fact that the ring of CYC yields cyclohexanone as one main OP, suggests that the cleaved side chain can lead to the formation of additional OPs. This led us to the identification of amidosulfonic acid as the second main OP of CYC (Figure 5-1). Quantification (method 2, Table 5-2) was possible with a reference standard. Amidosulfonic acid showed a higher yield at pH 3 compared with pH 7.5 for both the batch tests with and without the addition of *t*-BuOH. No further significant increase in concentration was observed between the CYC to ozone ratios of 1:5 and 1:10 in the non-scavenged batch, which corresponds to the complete reaction of CYC already at a ratio of 1:5. In batch tests with radical scavenging the yield of amidosulfonic acid increased with increasing CYC/ozone ratio due to residual precursor available for further conversion into amidosulfonic acid. The highest amidosulfonic acid concentration of about 140 µmol/L represents an almost even mass balance. Most likely an immediate oxidation of CYC to amidosulfonic acid occurs in the batch test without radical scavenging when adding ozone stock solution. Although the samples were measured the day after the ozone treatment, this time delay can be an explanation for lower concentrations due to a further hydrolysis of the OPs.

The OP amidosulfonic acid is also used as a precursor during production of both artificial sweeteners investigated in this study (Heravi et al. 2009; Yoshikubo and Suzuki 2000). In the case of CYC, cyclohexylamine and amidosulfonic acid are used for the synthesis (Alter and

Formann 1968). The US National Library of Medicine specifies the acute oral toxicity values (LD_{50}) to be 1,312 mg/kg for mouse and 3,160 mg/kg for rat (US NLM 2011).

IC-ICP/MS experiments with similar concentrations and a compound to ozone ratio of 1:2.5 proved that no or insignificant sulfate is formed during ozonation of cylamate neither with nor without scavenging of OH radicals. However, besides the main OPs of CYC, amidosulfonic acid and cyclohexanone, the formation of OPs with the same *m/z* = 192 (CYC OP192) was observed by high-resolution MS measurements (Figure 5-2 a, b and Figure 5-9). The molecular formula of $C_6H_{10}NO_4S$ indicated the introduction of a keto moiety most likely at the cyclohexyl ring of CYC forming three isomers of oxocylohexyl sulfamate. To confirm this assumption, we established a RP chromatographic separation (method 1,Table 5-2) for the three possible isomers. The elution order was assigned on the basis of the calculated octanol-water partition coefficients (logP) of the proposed structures (Chem Axon 2011). The three compounds revealed different fragmentation patterns also indicating the formation of three isomers. For CYC OP192, an α-cleavage next to the nitrogen heteroatom was observed for all three isomers. Amidosulfonate (*m/z* = 96) and the sulfite radical *(m/z* = 80) were generated as the main negatively charged fragments. The latter is known to be an important fragment in collision-induced dissociation of aliphatic sulfonates (Schultz et al. 2006; Frömel and Knepper 2008). Interestingly, the precursor CYC yields only the sulfite fragment and not amidosulfonate with the abovementioned instrumentation, which gives strong evidence that the introduced keto group influences the fragmentation pathway. In aliphatic compounds, an α-cleavage directly leads to the formation of fragment ions. However, in the case of alicyclic molecules a product ion with the same mass as the cyclic precursor ion can be formed by ring opening and formation of a C=C double bond, which might be the explanation for the high precursor signal in the spectrum of isomer 1.

In the case of isomer 2 with the keto group in β-position of the amidosulfonate group, an additional fragment ion with *m/z* = 122 was observed, indicating a ring cleavage at two positions. For isomer 3, the sulfite radical was the only MS fragment we observed under the applied fragmentation conditions. This might be explained by the position of the keto group, enabling the most stable possibility of a neutral aminocyclohexanone fragment of all isomers. Since the mass balance was almost even according to the quantitative results obtained from the two main OPs of CYC, cyclohexanone and amidosulfonic acid, the isolation of CYC OP192 was not feasible due to its low concentration. As a consequence, the quantification of the new OPs identified was not possible. However, we compared the peak area of a directly injected standard of the precursor CYC, which was commercially available

as a reference standard, with the highest peak area of the three isomers (pH 7.5, CYC to ozone ratio of 1:5) which could be directly observed in the reaction mixtures. Although the three isomers of CYC OP192 and CYC elute at different retention times and hence at different ionization conditions, a semi-quantitative estimation gave a maximum yield in the range of less than 10 % for CYC OP192 in the batch tests.

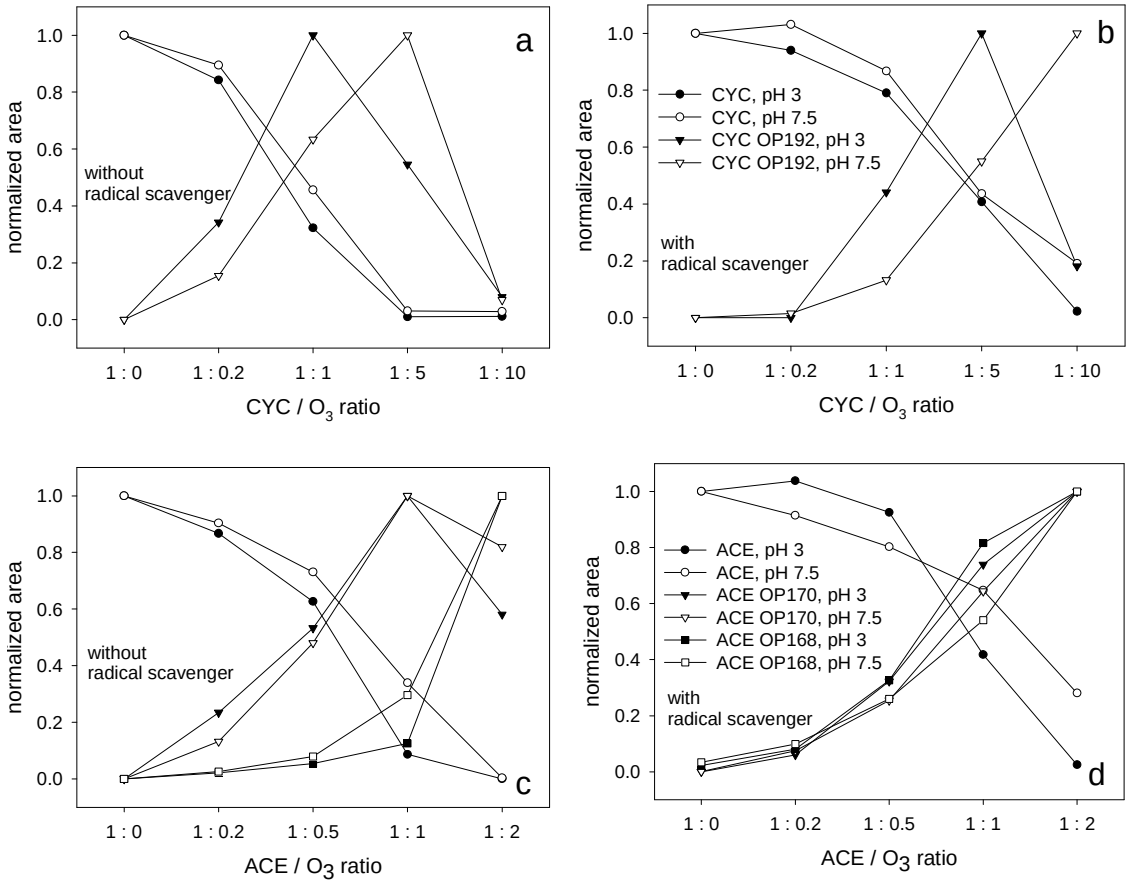


Figure 5-2 Normalized peak areas of CYC and CYC OP192 (a + b) and acesulfame and ACE OP170 and ACE OP168 (c + d) at two different pH values of the reaction mixture without (left) and with (right) scavenging of OH radicals. The normalized peak area of the suspected CYC OP192 refers to the most intensive isomer

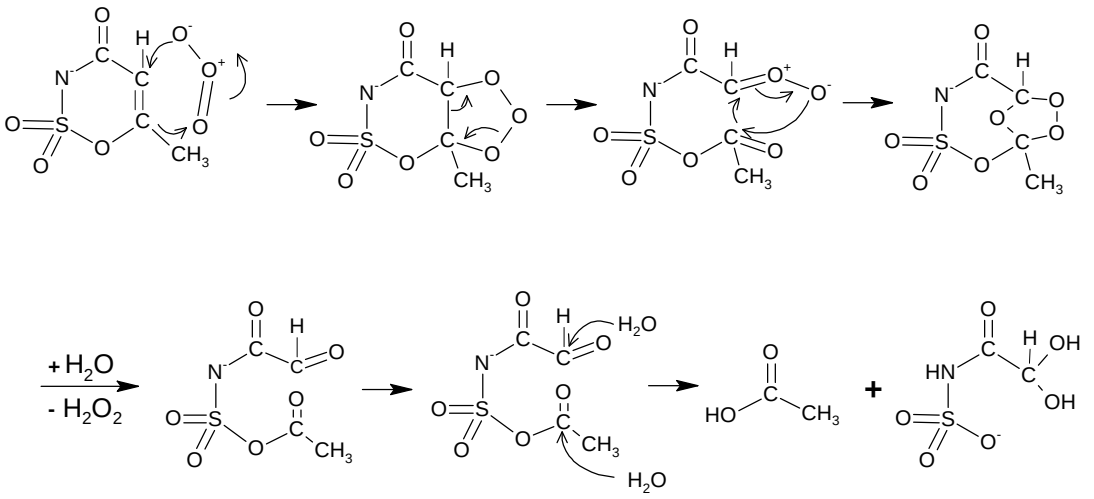
5.3.2 Ozonation of acesulfame

According to our previous work on artificial sweeteners (Scheurer et al. 2010), ACE was oxidized more readily by ozone compared with CYC. This can be understood due to the presence of a C=C double bond, which is known to be easily attacked by ozone according to the Criegee mechanism (Criegee 1975). Therefore, compound to ozone ratios were applied in a lower range for the batch tests in the buffered ultrapure water. As displayed in Figure 5-2 c, d, a complete transformation of ACE was observed in three of four test setups already

for a compound to ozone ration of 1:2 compared with a ratio of 1:10 necessary for comparable conversion of CYC. In the batch tests without *t*-BuOH, only slightly better oxidation yields were obtained, indicating that OH radicals play only a minor role in the ozonation of ACE.
According to the LC-ESI-MS results, the ozonation of ACE yields anionic OPs with molecular masses of 170 and 168 Da. Based on MS^2 high-resolution measurements and MS^3 experiments, the structures, oxidation, and fragmentation pathways displayed in Scheme 1 and Figure 5-3 are proposed.

Following the Criegee mechanism, a ring cleavage of the primary ozonide at the C=C double bond and a decomposition into a carbonyl and carbonyl oxide moiety can be suspected. At the subsequently formed aldehyde, the addition of water is assumed leading to the proposed aldehyde hydrate. Compounds with two hydroxyl groups attached to one carbon in general are impossible to isolate due to the elimination of water. However, chemicals are known (e.g., chloral hydrate and ninhydrin), where strong electron withdrawing groups in α-position support the formation of an aldehyde hydrate in water. Thus, due to the α-carbonyl function in the proposed structure of ACE OP170, in aqueous solution, the formation of a fairly stable aldehyde hydrate is straightforward.

ACE OP168 seems to be the direct oxidation product of ACE OP170. As it can be seen in Figure 5-2 C, after complete oxidation of ACE the maximum of ACE OP170 is reached and it decreases slightly in favor of the formation of ACE OP168. It has to be noted that in Figure 5-2 peak areas were normalized to the highest intensity of the corresponding transition. Therefore, a quantitative comparison between OP concentrations is not possible, but a graphical presentation of relative intensities is given in the supporting information (Figure 5-10). In all batch tests, we observed that ACE OP168 is only a minor product in the ozonation of ACE. In all cases the main product was ACE OP170, which remained also rather stable when remaining ozone was present. This is supported by the fact that no sulfate formation was observed by IC-ICP/MS even after complete ozonation of ACE when applying an ozone to ACE ratio of 2.5:1.



Scheme 1 Proposed reaction pathway for the oxidation of ACE resulting in ACE OP170 and acetic acid

If really an aldehyde hydrate moiety is formed during ozonation, in the case of ACE OP170 could not be clarified by these measurements. However, a coelution of a reaction product with a precursor mass of (-)151.9 Da together with ACE OP170 was observed for all measurements with different MS conditions (ion source temperature, fragmentor voltage, etc.). The loss of 18 Da indicates a loss of water supporting the expected little stability of ACE OP170 and that the water loss occurs partly as an in-source fragmentation. Both products, ACE OP170 and ACE OP168, have one and two ring double-bond equivalents, respectively, which supports the proposed structures in the way that a ring structure after ozonation cannot be expected.

For further confirmation of these two structures, deuterium exchange experiments were run with MS^2 high-resolution measurements. For this purpose, eluent A of LC method 2 (Table 5-2) was replaced by 20 mmol/L ammonium formate dissolved in D_2O. The combination of evidence about the exchangeable hydrogen atoms and MS^2 experiments can provide valuable information if the proposed fragmentation pathways and structures are valid. For ACE OP170, the protons of the aldehyde hydrate moiety and the proton bound to the nitrogen should be most easily exchangeable and therefore result in m/z (-)172.9. For ACE OP168 both remaining protons can be expected to be exchanged.

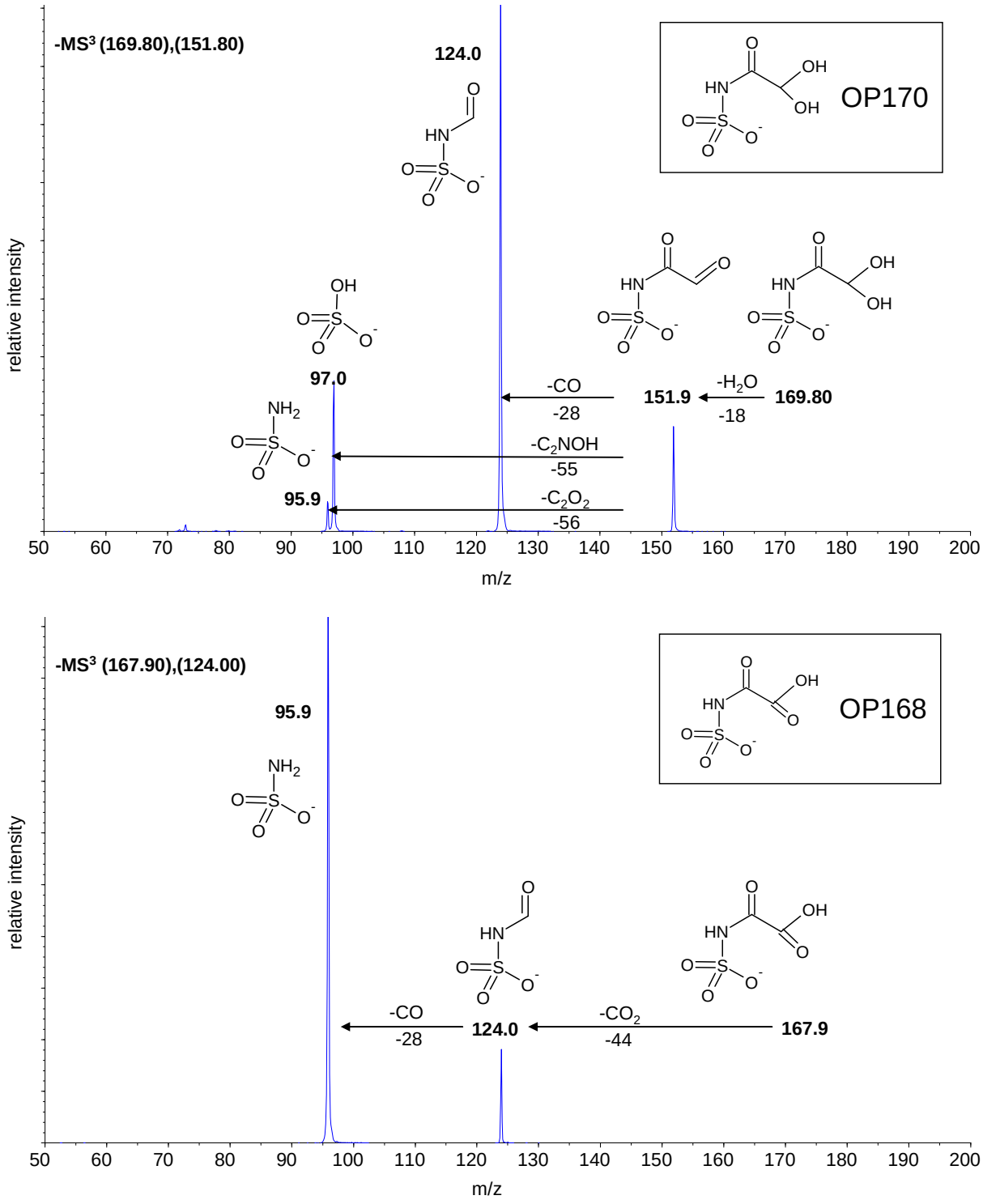


Figure 5-3 MS^3 fragmentation pathways for proposed ACE OP170 (top) and ACE OP168 (bottom) in negative ionization mode

As suspected for ACE OP170 an increase in m/z of three Da was observed with the modified eluent. The fragmentation led to a loss of 20 Da indicating a loss of D_2O, and consequently to the formation of a *m/z* fragment ion of (-)152.9 still carrying one nonexchangeable hydrogen from the precursor at the aldehyde moiety. The same was true for

the formylsulfamate fragment (m/z = 124.9), which also showed some exchange at the aldehyde hydrogen. As expected also the masses of the hydrogen sulfate fragment and the amidosulfonic acid fragment increased in 1 and 2 Da, respectively. In the D_2O exchange experiments, the formation of an additional fragment ion with a mass of 73 Da was observed. High-resolution measurement revealed the formation of a molecular ion with the formula C_2HDNO_2 as a consequence of the loss of SO_3.

For ACE OP168, all hydrogens were exchanged. Interestingly, the formation of the deuterated formylsulfamte fragment was strongly hindered in this case. It has to be mentioned that MS^3 (Figure 5-3) and MS^2 fragmentation experiments were carried out on different MS systems and are therefore not directly comparable. The complete suppression of the neutral loss of CO_2 in favor of the formation of amidosulfonic acid and the sulfite radical in contrast to experiments with D_2O is surprising.

The loss of two carbon atoms in the above-discussed OPs, left only minor possibilities for further oxidation products. However, as it has been shown that carboxylic acids are important oxidation by-products when applying ozone to tertiary wastewater (Wert et al. 2007), screening on some of these compounds was also performed. In fact, we observed high concentration of acetic acid in the batch test (Figure 5-12), which corresponds well with the proposed structures above. Formic and oxalic acid formation was also observed but with concentrations an order of magnitude lower (data not shown). Both can be expected when ACE OPs 170 and 168 are further oxidized, most likely by OH radicals, but it was also shown that ACE OP170 is rather persistent against further ozone attack which explains the low yield of these two carboxylic acids. The acetic acid formation in potable water was similar to the batch test, giving first evidence on the transferability of the oxidation mechanism to real matrices.

In order to obtain more information about the structure of the proposed OPs, we performed first NMR screening experiments with a high concentration of ACE (5 g/L). The results displayed in Figure 5-4 show that two main OPs (regions 4 and 6) are formed. The chemical shifts of the NMR signals (Figure 5-11) can be attributed to the formation of formic acid (8.25 ppm, region 1) and acetic acid (2.09 ppm, region 6). As signals referring to regions 1 and 3 (5.49 ppm) correlate, they seem to originate from the same minor compound. The shift in intensity at 75 min ozonation time might be explained by the disturbance of tautomerism possibly due to missing buffer in the sample for NMR measurements. The sample with an ozonation time of 180 min was analyzed two more times by ^{1}H-NMR two and

three weeks after the first measurement. The strong unknown signal at a shift of 5.25 ppm (region 4) was observed to be highly decomposed in favor of formic acid.

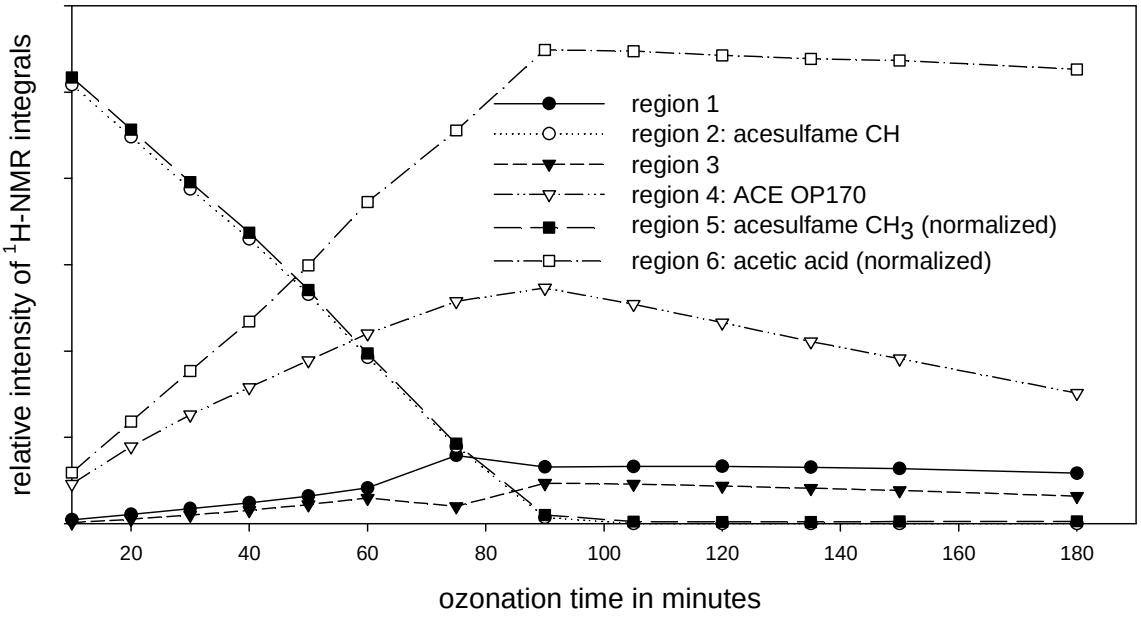


Figure 5-4 Integrated ^{1}H-NMR signals over ozonation time. Signals derived from more than one hydrogen are normalized. See also Figure 5-11 for corresponding ^{1}H-NMR spectra

In order to clarify if the remaining signal (region 4) can be assigned to ACE OP170, HPLC-NMR/MS-TOF were carried out using chromatographic conditions based on method 2 (replacement of water by D_2O for the aqueous buffer). Based on the optimized HILIC method and previous MS experiments, a certain retention time window could be used for storing the chromatographic fraction of interest into loops during the LC-NMR/MS run. The split of 2 % of the LC flow to the ion source of the mass spectrometer allowed for confirmation by the exact mass using the TOF-MS at the same time. After transfer of the loop corresponding to the peak with *m/z* = 170 into the NMR probe head and acquisition of an ^{1}H-NMR spectrum, a singlet with a chemical shift of 5.25 ppm could clearly be observed indicating a CH group with two hydroxyl moieties. This signal strongly corroborates the proposed dihydroxyacetyl sulfamate structure for ACE OP170 as shown in Figure 5-3. However, no resonance signals from ACE OP168 could be detected by ^{1}H-NMR. This is in agreement with the proposed structure of this compound (Figure 5-3) as the two remaining protons are fully exchangeable and hence cannot be detected in solutions with D_2O.

The formation of ACE OP170 and acetic acid is complete with the total oxidation of ACE (Figure 5-4). The further decrease of ACE OP170 can most likely be attributed to its further oxidation to oxalic acid and amidosulfonic acid (both detected by IC and LC screening).

Another possibility is the enhanced evaporation due to continuous sparging of ozone gas into the test solution which became strongly acidic (pH 2.2). Samples for NMR were also analyzed on carboxylic acids by IC and showed increasing oxalic acid and stable acetic and formic acid concentrations after complete ACE oxidation (Figure 5-13). The total sum of ^{1}H-NMR integrals decreased continuously over the test period to a final c/c_0 value of about 0.8 after 3 hours corroborating the assumption of evaporation.

For the reason of clarity, the identified OPs of both sweeteners and the tools used for identification are summarized in Table 5-3 in the supplementary material.

5.3.3 Ozonation of cyclamate and acesulfame in wastewater and tap water

In order to investigate ozonation of both artificial sweeteners in real matrices and at a more realistic environmental concentration, treated wastewater and tap water were spiked with 250 µg/L of the sweeteners and treated the same way as described above without the addition of *t*-BuOH. The wastewater samples were taken from a small WWTP described in detail in Scheurer et al. (2009) (STP 1). Influent and effluent samples were analyzed for both sweeteners. ACE concentrations in the STP influent and effluent were 39 and 34 µg/L, whereas for CYC, 145 and 0.58 µg/L were measured. The results confirm the persistence of ACE during conventional wastewater treatment and the good biodegradability for CYC reported before (Buerge et al. 2009; Scheurer et al. 2009). The applied ozone concentrations (0.2 mg/L to 5 mg/L ozone for drinking water and 0.25 mg/L to 2 mg/L ozone per mg DOC in wastewater) covered a range usually used in the corresponding treatment plants.

The results for ACE (Figure 5-14) in drinking water are in good agreement with our previous reported behavior of the compound, where 1 mg/L ozone was sufficient to remove 1 µg/L ACE from drinking water after a contact time of 40 min (Scheurer et al. 2010). As the DOC in the used drinking water is 0.9 mg/L, the results for drinking water and wastewater are comparable when contemplating the applied ozone dose per milligram DOC. A similar concentration was sufficient to remove ACE almost completely. However, 1 mg ozone per mg DOC in both drinking and wastewater treatment, represents a dosage rather in the upper range of the applied concentrations. Therefore, a considerable amount of ACE can be expected even after ozonation units.

In the case of CYC, the data for drinking water are in line with our previous findings, where 1 mg/L ozone led to a decrease of about 40 % CYC within one hour contact time (50 % after complete ozone depletion in this study), even though the compound concentration was lower. The formation of cyclohexanone was only observed for the drinking water samples and

not for the treated wastewater, which is likely due to matrix effects or further reaction with the wastewater matrix rather than due to a completely different oxidation pathway. The highest yield of cyclohexanone formation was observed when applying 0.5 mg/L and 1 mg/L ozone indicating a further oxidation of cyclohexanone. This is supported by the finding of the non-scavanged batch test at pH 7.5, where cyclohexanone was already assumed to be further oxidized. In drinking water also, an increase of amidosulfonic acid was measured with decreasing CYC concentrations, supporting the finding of the batch test performed in ultra pure water. In the treated wastewater, the background concentration of amidosulfonic acid was too high to track the formation of this compound with increasing ozone dose.

A kinetic experiment was chosen as an additional approach for ACE, as the compound was found in concentrations of several micrograms per liter in raw waters used for drinking water production. For ACE, it was therefore more important to prove that no other intermediates occur during ozonation in waterworks. This was necessary, because the ozone contact time in DWTPs is usually not more than one hour, but it was one day in the batch test. After adding ozone stock solution in defined amounts, the samples were immediately measured by LC-MS/MS (method 4) representing an overall ozone contact time of about 25 min (see materials and method section). The oxidation of the precursor ACE was much faster in ultrapure water due to missing DOC (Figure 5-5). Both, ACE OPs 170 and 168, were formed under these conditions in ultra pure and tap water. OP yields in tap water were lower and signal intensity fluctuation of the OPs was more pronounced. This might be explained due to the fact that OPs and ACE were separated but the OPs eluted with the matrix peak which might be more important for drinking water. This corresponds with observations during method development for artificial sweeteners, where especially for ACE strongly suppressed signals were observed when injecting samples without SPE clean-up. A complex formation of the OPs with present cations could also be an explanation for that phenomenon, but no adducts were observed during this study.

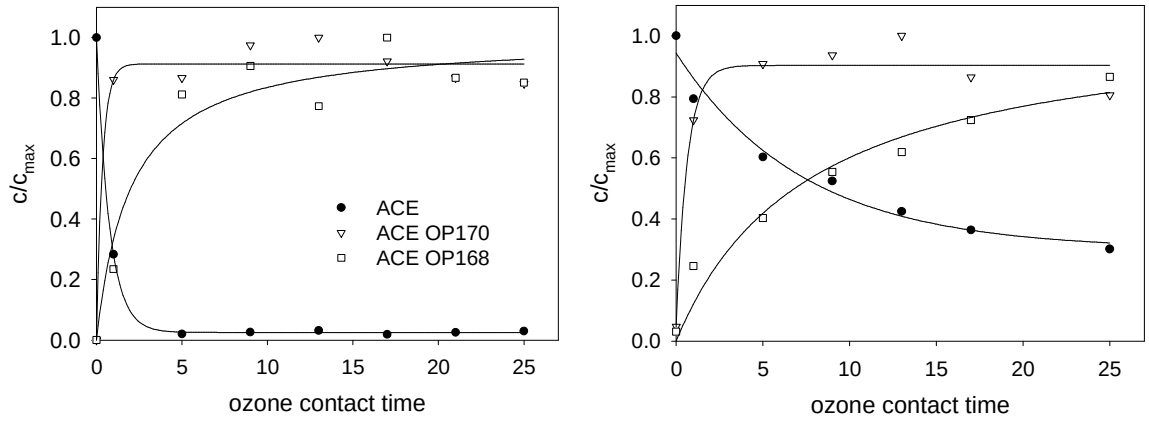


Figure 5-5 Degradation of ACE (200 µg/L) and formation of ACE OPs 170 and 168 in ultra_pure water (left) and tap water (right). Applied ozone concentration was 1 mg/L. Signals are normalized to the highest peak area observed for each compound

To confirm the relevance of ACE OP170 for real ozone applications, a DWTP located at the river Rhine was sampled. It uses a mixture of river bank filtrate and landside groundwater as raw water for drinking water production. Sampling points were the raw water, the water after the ozonation unit and after the following activated carbon filter. For detailed information about the treatment steps, see the description of waterworks D in Scheurer et al. 2010. The ozonated water was quenched with sodium sulfite in order to avoid further oxidation. In the raw water, no CYC and 0.51 µg/L ACE were measured. Due to insufficient ozone concentration and/or contact time, ACE was only partly removed and still present with 0.21 µg/L after the ozonation unit. Screening of ACE OP170 (after solid-phase extraction with a weak anion exchanger material and separation with LC method 2) confirmed the formation of the compound also in waterworks using ozonation, but also its removal by activated carbon (Figure 5-6). This is most likely due to biological activity in the upper part of the activated carbon filter and not due to the removal by adsorption. As the samples were not stabilized, a partial biological degradation of the OPs cannot be excluded, but the results are still adequate to prove the relevance for real water treatment processes.

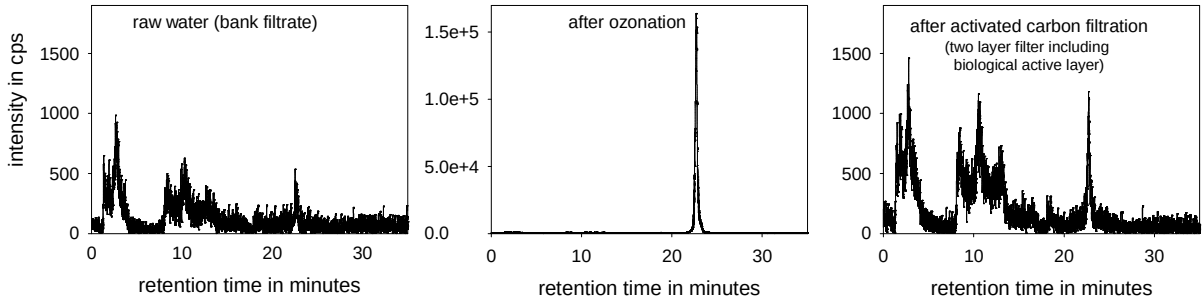


Figure 5-6 Chromatograms of MRM transition 169.8/151.8 (ACE OP170) of raw water (left), after ozonation (middle), and after subsequent activated carbon filtration (right) from a waterworks using bank filtrate of the Rhine river as raw water. Note the different ordinate scaling of the three graphs

5.4 Conclusions

For structural elucidation of unknown compounds, a wide range of analytical techniques for separation and detection is necessary. This work has shown that for polar analytes like artificial sweeteners, LC-MS is first choice, but it should be considered that also volatile compounds, more suitable for GC analysis, might be generated during ozonation. For the final structural elucidation, more advanced techniques like MS^n, high-resolution MS or even NMR measurements are necessary if no standards for the confirmation of the OPs are available.

In this study, the cleavage of the side chain of the CYC molecule during ozonation resulted in two main OPs, amidosulfonic acid and cyclohexanone. Based on the CYC concentrations found in WWTP effluents and the formation of these low to moderate toxic OPs during ozonation, the environmental relevance of ozone application in order to remove CYC seems little.

Based on previous studies, ACE has more environmental and drinking water relevance as CYC. As easily transformed during ozonation in DWTPs, it was important to elucidate that mainly acetic acid and ACE OP170, dihydroxyacetyl sulfamate, are formed in aqueous solution. This aldehyde hydrate might be a reactive compound, but primary findings showed its good removal in activated carbon filters downstream of an ozonation unit in a full-scale DWTP. Similar to the removal of other by-products formed during ozonation, such as NDMA, this combination of treatment steps can therefore be considered as an effective and safe tool to remove ACE from raw waters. For this purpose, ozone dose and contact time can be optimized, if necessary.

5.5 Supplementary material

Samples from the batch tests were analyzed with different liquid chromatography (LC), gas chromatography (GC), and ion chromatography (IC) methods coupled to different detection systems. The methods were optimized for pCBA and the suspected oxidation products (OPs) based on preliminary experiments.

Nuclear magnetic resonance (NMR), MS experiments and D_2O exchange experiments were used to confirm the structure of some OPs.

LC methods

For CYC and some OPs a Synergie Hydro RP column (250 x 3 mm, 4 µm) from Phenomenex (Aschaffenburg, Germany) was used for separation (method 1). More polar OPs were separated by a ZIC-HILIC column (150 x 2.1 mm, 3.5 µm) from dichrom (former SeQuant, Marl, Germany; (method 2)). Retention of pCBA (method 3) was achieved by using an Eclipse Plus C18 RRHD column (50 x 2.1 mm, 1.8 µm) from Agilent Technologies (Waldbronn, Germany). The same column was used for kinetic studies (method 4).

The optimized gradient programs and buffers used in the LC methods are summarized in Table 5-2. The analysis of ACE and CYC is described in detail in Scheurer et al. (2009).

The chromatographic methods were carried out on a series 1200 and an Infinity 1290 high performance (HP) LC system (both from Agilent Technologies, Waldbronn, Germany) equipped with a solvent cabinet, a micro vacuum degasser, a binary pump, a high-performance autosampler with two 54 vial plates, and a temperature controlled column compartment. The series 1200 HPLC system was connected to an API 4000 tandem mass spectrometer (Applied Biosystems / MDS Sciex Instruments, Concord, ON, Canada) with an electrospray interface and the series 1290 systems was coupled with a 6540 UHD Q-TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany) both operated in negative ionization mode. However, screenings for unknown OPs were also performed in positive ionization mode. The Infinity 1290 system was additionally connected to a diode-array detector, which was operated at 241 nm for the detection of pCBA.

GC method

GC analysis for the suspected oxidation products was carried out with an Auto System XL gas chromatograph connected to a Turbo Mass Gold mass spectrometer (both Perkin Elmer, Waltham, MA, USA). A ZB Multi Residue 1 column (30 m x 0.25 mm from Phenomenex (Aschaffenburg, Germany) was used for the separation of the analytes (flow rate 1.0 mL/min). The temperature program started at 40 °C and was held for 4 min, ramped 5 °C/min to 120 °C (held for 0 min), and ramped 45 °C/min to 300 °C and held for another 6 min.

IC methods

Detection of carboxylic acids:

Separation of carboxylic acids was achieved by using an Ion Pac AS IC column and an ICS3000 high performance IC system coupled with a conductivity detector (both Dionex, Sunnyvale, CA, USA). Eluents used for the gradient were ultra pure water (A), 200 mmol/L NaOH (B), and 10 mmol/L NaOH (C). The gradient (flow rate 1 mL/min) started with 8 mmol/L NaOH and held isocratic for 10 min and was then increased over 13 min to 126 mmol/L NaOH and held isocratic for another 12 min (total run time 35 min). Equilibration time with the starting condition was 10 min. Injection volume was 500 µL.

Detection of sulfate and other sulfur containing anionic species:

The coupling of ion chromatography with inductively coupled plasma mass-spectrometry (IC-ICP–MS) was used to obtain information about the number of anionic sulphur containing OPs and the mass balance of sulfur during ozonation. For this purpose the IC system described above, equipped with an electrochemical suppressor (ASRS 300, 4 mm, Dionex) was coupled to an ICP-MS 7500 ce system (Agilent) for the detection of sulfur oxide ion SO^+ (m/z = 48). Separation was done under isocratic conditions (25 mmol/L NaOH, flow rate: 0.5 mL/min) on an Ion Pac AS 20, 4 x 250 mm (Dionex, Sunnyvale, CA, USA) analytical column

NMR

For NMR measurements, the original aqueous solutions were diluted with 10 % (v/v) 1.5 mol/L KH_2PO_4 buffer in D_2O adjusted to a pH of 3 in 5 mm NMR tubes. NMR spectra were acquired on a 600 MHz AVANCE II NMR spectrometer equipped with a 5 mm TCI cryo probe. For the 1D NMR experiments, the noesygppr1d pulse program was used. For all experiments continual water presaturation RF of 25 Hz was applied during relaxation delay D1. Moreover, the noesygppr1d sequence was acquired using a total of 8 scans for acesulfame

and 32 scans for cyclamate reaction solutions. Data were acquired into 64 K complex data points, spectral width of 20 ppm, 10 ms of mixing time and relaxation delay of 10 s. With all types of experiments receiver gain was kept at constant value of 128. Free induction decays (FID)s were multiplied by an exponential function equivalent to that of a 0.3 Hz line-broadening factor and then Fourier transformed. Taking advantage from the baseopt digitalization mode, spectra were automatically phased, baseline corrected and referenced using Topspin. On representative samples we also acquired 2D-^{1}H-^{13}C-HSQC (Heteronuclear Single Quantum Coherence) and 2D-^{1}H-^{13}C-HMBC (Hetero Multiple Bond Correlation) experiments. 8 to 64 FIDs were acquired for each of the 400 increments. Sweep widths were adjusted according to the requirements obtained from the ^{1}H-NMR spectra.

For confirmation of the results HILIC-NMR/MS was used according to method 2 described above, but eluent A was replaced by D_2O (Deutero GmbH, Kastellaun) with 20 mmol/L NH_4COOH. The system consists of an Agilent 1200 HPLC system including a quaternary HPLC pump, auto sampler and diode array detector. The chromatography was done after injection of 30 µL of sample. 2 % of the post chromatographic flow was split to a MicroTOF time of flight mass spectrometer (Bruker, Rheinstetten, Germany) equipped with an electrospray ion source and operated in negative ionization mode. Calibration was done by infusion of 20 mmol/L lithium formate solution prior to the chromatographic separation. Peaks detected were stored in BPSU-loops (Bruker peak sampling unit, Bruker Biospin, Rheinstetten, Germany) prior to the transfer into a room temperature selective inverse NMR flow probe connected to an AVANCE III 500 MHz NMR spectrometer (Bruker Biospin, Rheinstetten Germany). NMR measurements were done using a WET solvent suppression pulse program (Smallcombe et al., 1995). Up to 1024 scans were collected into 32 K complex data points over a sweep width of 20 ppm and a relaxation delay of 3 s. Prior to Fourier transformation the free induction decay was multiplied to an exponential function leading to a peak broadening of 1 Hz.

Table 5-2 Compilation of liquid chromatographic methods used throughout this study

step	time				flow rate				eluent A			
	in minutes				in mL/min				in %			
Method No.	1[a]	2[b]	3[c]	4[a]	1[a]	2[b]	3[c]	4[a]	1[a]	2[b]	3[c]	4[a]
step 1	0	0	0	0	0.3	0.35	0.4	0.25	100	5	90	98
step 2	5	5	1	3	0.3	0.35	0.4	0.25	100	5	90	98
step 3	20	30	3		0.3	0.35	0.4		65	40	50	
step 4	23	32	6		0.3	0.35	0.4		100	40	50	
step 5		35	7			0.35	0.4			5	10	

[a] eluent A: water, eluent B: methanol; A and B with 20 mM ammonium acetate
[b] eluent A: water + 20 mM ammonium formate, eluent B: acetonitrile (for LC-NMR: water substituted by D_2O)
[c] eluent A: water, eluent B: methanol; A and B with 0.1% formic acid

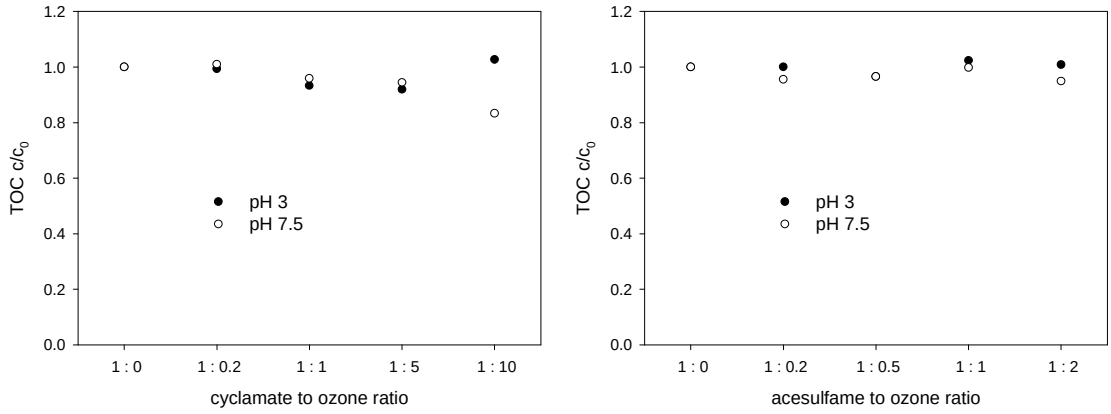


Figure 5-7 Normalized TOC concentrations after treatment with ozone for different cyclamate and acesulfame to ozone ratios and two different pH values in batch tests without t-BuOH radical scavenger

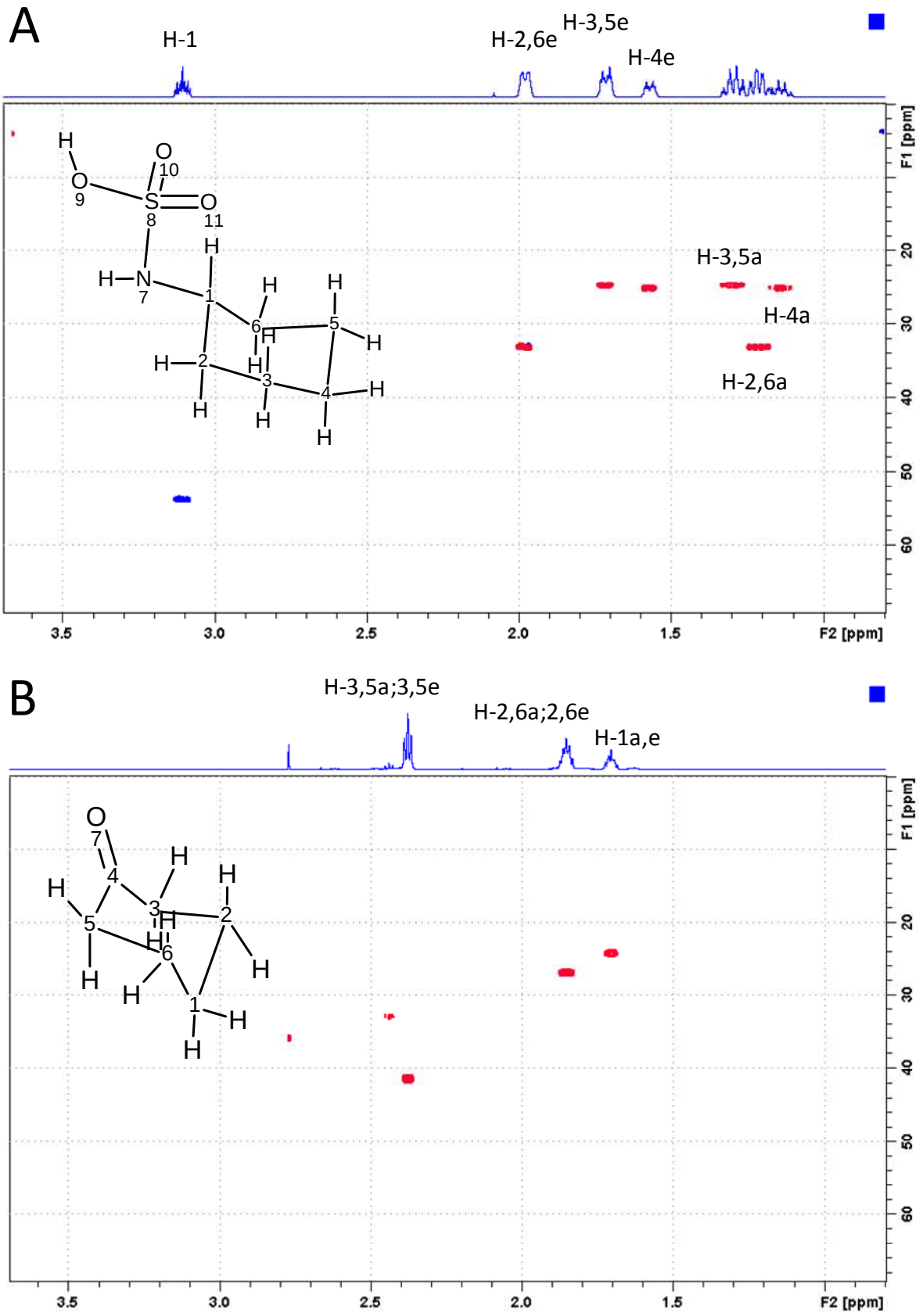


Figure 5-8 Edited HSQC spectrum of CYC before ozonation (A) and after 80 min ozonation (B). Blue signals indicate –CH groups, while red signals correspond to $–CH_2$ groups. The labeling for CYC distinguishes between axial (a) and equatorial (e) protons of the cyclohexane moiety

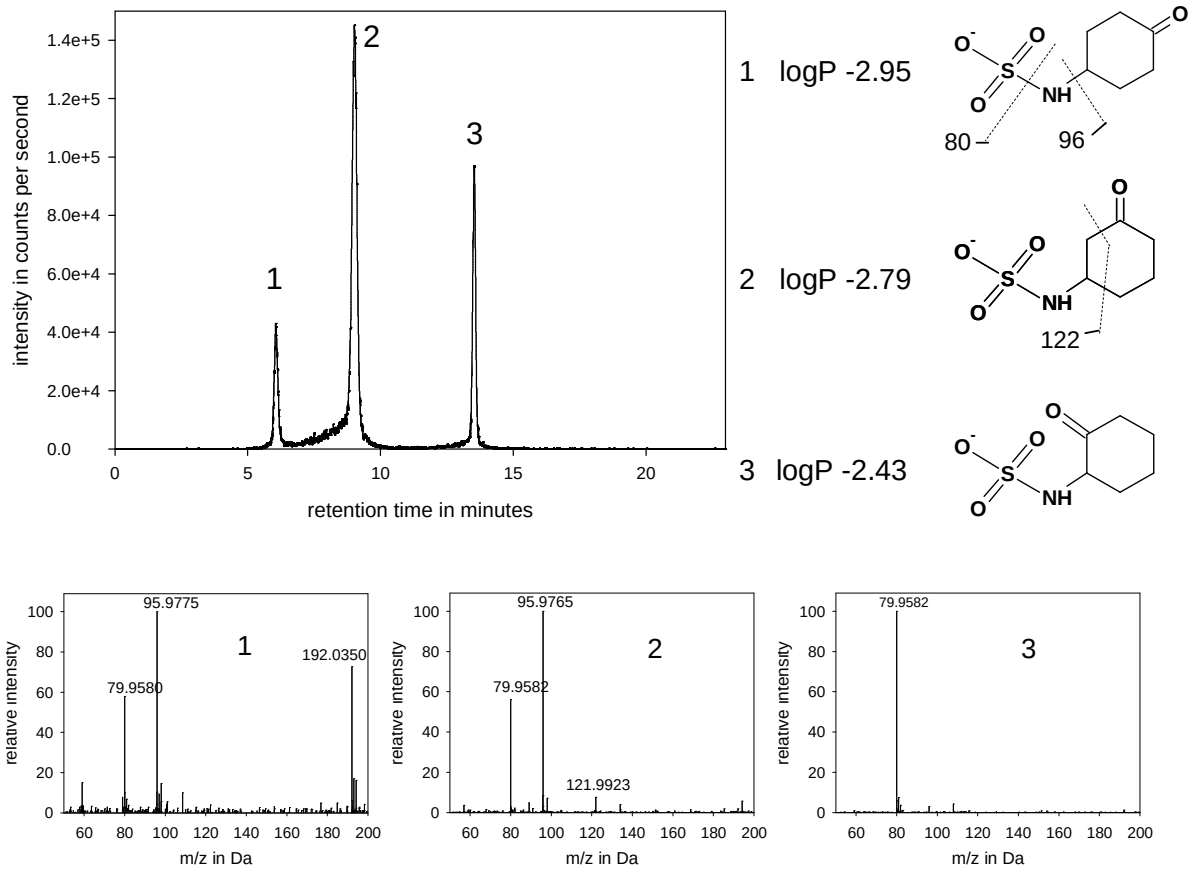


Figure 5-9 Extracted ion chromatogram of *m/z* = 192.03. Elution order of the expected isomers of the proposed structure of CYC OP192 was assigned on calculated logP values (ChemAxon, 2011) (top). MS/MS spectra from left to right correspond to the three peaks in the order of their elution (bottom)

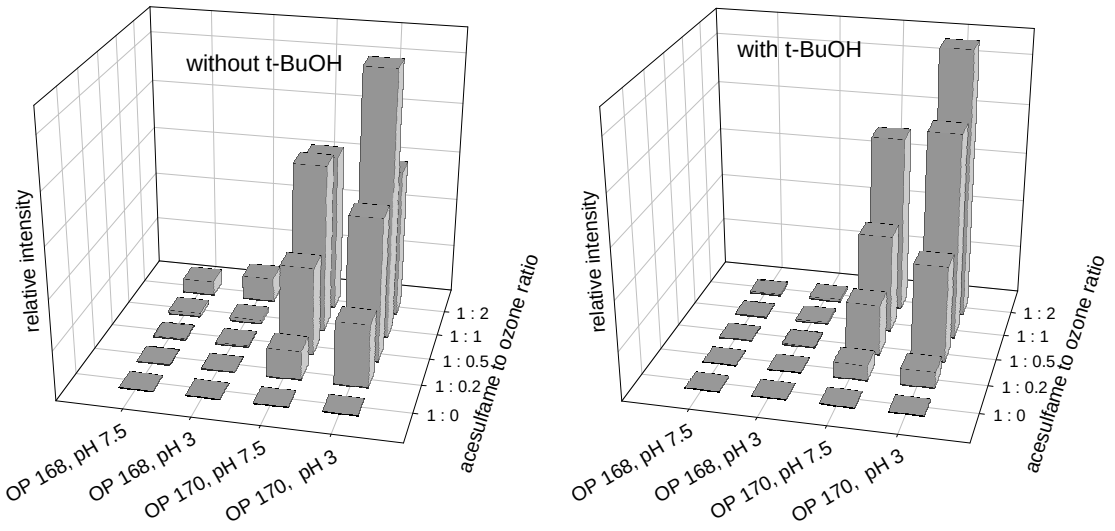


Figure 5-10 Relative intensities of ACE OPs 170 and 168 in batch tests with different pH values and with or without radical scavenging. Both figures are normalized to the same absolute peak area and are therefore directly comparable

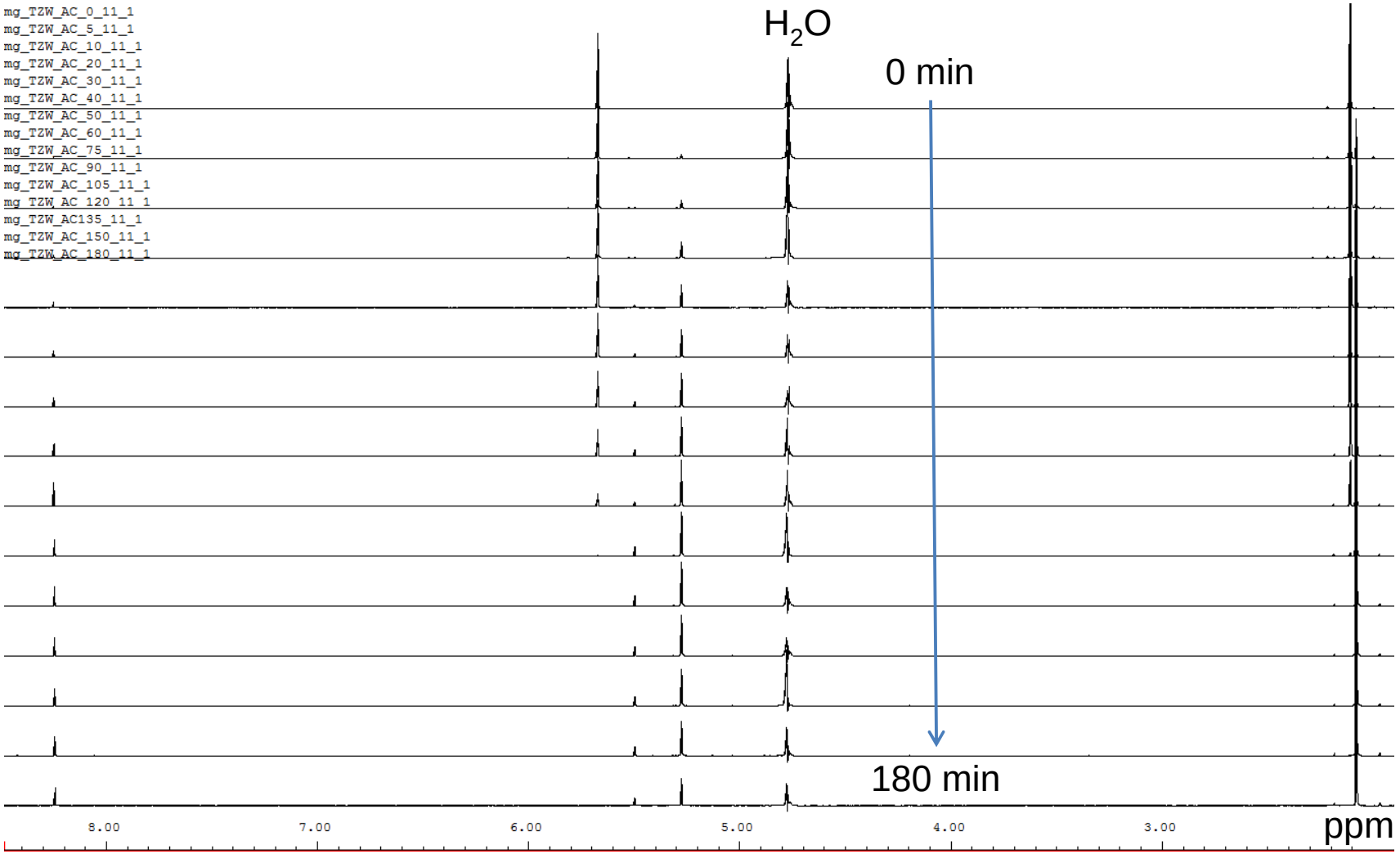


Figure 5-11 ^{1}H-NMR spectra for the ozonation of ACE after different ozonation times. Ozone gas was directly sparged into the aqueous test solution (C_0 ACE= 5g/L), maximum ozonation time was 180 min

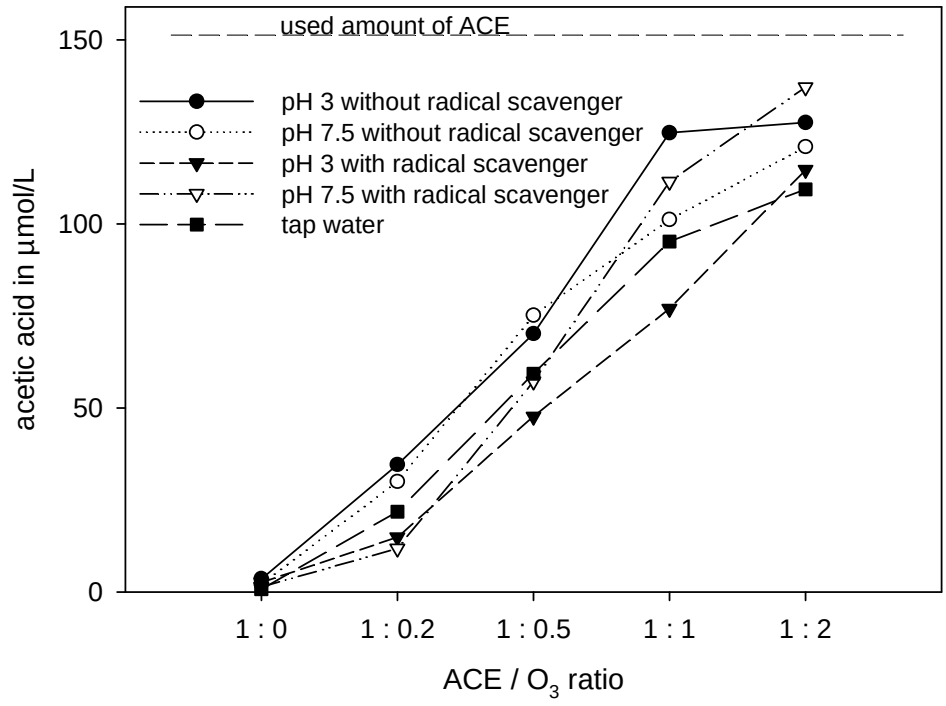


Figure 5-12 Formation of acetic acid in the batch test in ultra pure and tap water at different ACE to ozone ratios (c_0 of ACE was 153 µmol/L and is indicated by the dashed line)

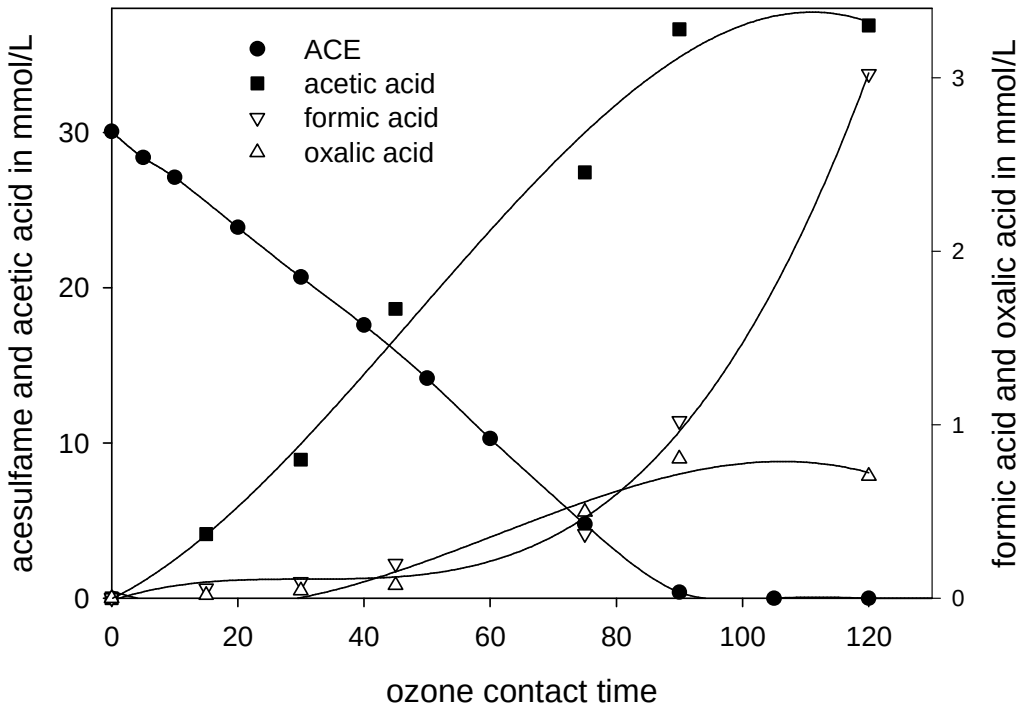
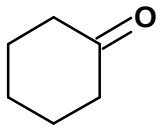
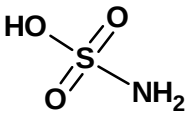
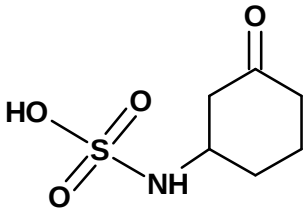
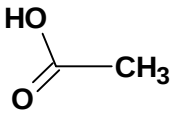
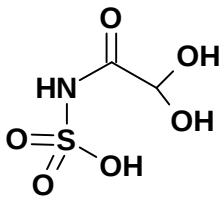
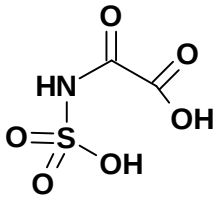
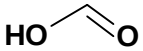
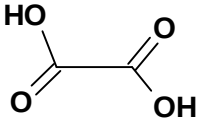


Figure 5-13 Formation of carboxylic acids during ozonation of ACE in ultra pure water, without radical scavenger. Ozone gas was directly sparged into the aqueous test solution

Table 5-3 Summary of the identified oxidation products of cyclamate and acesulfame and the used tools for their identification

name	chemical structure	tools used for identification
identified oxidation products of cyclamate		
cyclohexanone		- reference standard - ^{1}H-NMR - GC-MS and NIST database
amidosulfonic acid (sulfamic acid)		- reference standard - high resolution LC-Q-TOF
CYC OP192 (3-oxocyclohexyl)sulfamate*		- high resolution LC-Q-TOF - MS^2 experiments (high resolution L-Q-TOF)
identified oxidation products of acesulfame		
acetic acid		- reference standard - ^{1}H-NMR
ACE OP170 (dihydroxyacetyl) sulfamate		- ^{1}H-NMR - HILIC-LC ^{1}H-NMR - MS^2 experiments (high resolution LC-Q-TOF) - MS^3 experiments by direct injection - D_2O MS^2 experiments (high resolution LC-Q-TOF)
ACE OP168 (carboxycarbonyl) sulfamate		- MS^2 experiments (high resolution LC-Q-TOF) - MS^3 experiments by direct injection - D_2O MS^2 experiments with high resolution Q-TOF
formic acid		- reference standard - ^{1}H-NMR
oxalic acid		- reference standard - ^{13}C-NMR

* displayed is only one of three possible isomers, (2-oxocyclohexyl)sulfamate and (4-oxocyclohexyl)sulfamate are also identified oxidation products

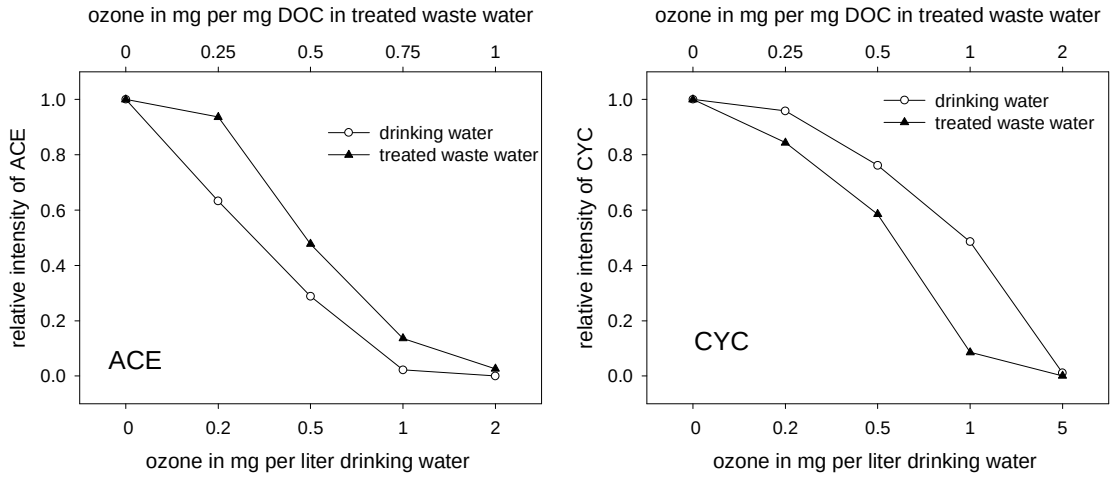


Figure 5-14 Decrease of ACE (left) and CYC (right) concentration in drinking water and treated waste water spiked with different ozone concentrations. Note the different ozone doses applied for the two sweeteners

5.6 References

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