Table 1 Perchlorate concentrations in raw and finished water samples from water-purification plants

Plant	Sample type[a]	Perchlorate concentration/μg L^{-1}	
		IC/ESI-MS/MS[b]	LC/ESI-MS[c]
A	Raw/finished water	0.09/0.12	< 0.1/0.1
B	Raw water	39.8	36.1
C	Raw/finished water	10.8/10.3	10.5/10.1
D	Raw/finished water	2.34/1.37	2.3/1.4
E	Raw/finished water	0.48/0.67	0.5/0.6
F	Raw/finished water	4.60/6.03	4.1/6.4
G	Raw/finished water	7.96/7.91	7.5/8.0

a. Raw water at plant B was raw ground water and the other raw waters were raw surface waters.
b. Perchlorate concentrations were determined by IC/ESI-MS/MS (MRL: 0.05 μg/L).
c. Perchlorate concentrations were determined by LC/ESI-MS, the proposed method.

Experimental

Standards and reagents

A 1000 mg L^{-1} standard solution of perchlorate was obtained from GFS Chemicals (Powell, OH). ^{18}O-enriched sodium perchlorate ($NaClO_4$) obtained from Cambridge Isotope Laboratories (Andover, MA) was used for an internal standard (IS) of perchlorate. One thousand milligrams per liter standard solutions of fluoride, chloride, nitrite, nitrate, sulfate and chlorate were obtained from Kanto Chemical (Tokyo, Japan) or Wako Pure Chemical (Osaka, Japan). Ammonium carbonate ($(NH_4)_2CO_3$) and ammonium chloride (NH_4Cl) were obtained from Sigma-Aldrich (St. Louis, MO). Twenty-five percent (v/v) ammonium hydroxide (NH_4OH) aqueous solutions, sodium ascorbate, and acetonitrile (high-performance liquid chromatography grade) were obtained from Wako Pure Chemical (Japan). For preparing the standard and stock solutions and eluents as well as diluting the samples, ultrapure water prepared by a Gradient A10 water purification system (Millipore, Bedford, MA) was used.

Sample collection and preparation

For a recovery study of perchlorate, river and tap-water samples were collected in October 2006. For investigating the presence of perchlorate in water samples from water-purification plants, raw and finished water samples from seven water-purification plants (Plants A - G) were collected in September 2006 (Table 1). The river water of the Tone River Basin, the largest basin in Japan, is widely contaminated by perchlorate, owing to the discharge of industrial effluents containing perchlorate in the upper Tone River Basin.[9] All of the water-purification plants investigated are located in the Tone River Basin, and, except for Plant A, the water intake points of the plants are downstream of the discharging points of industrial effluents containing perchlorate. All of the sample solutions collected were refrigerated at 4°C. River and raw-water samples were filtered with 0.2-μm polytetrafluoroethylene (PTFE) disposable filters (Advantec Toyo, Tokyo, Japan). Residual free chlorine in finished and tap-water samples was quenched using NH_4Cl or sodium ascorbate. ^{18}O-enriched $NaClO_4$ was added to the sample solutions and mixed before analysis (its concentration in sample solution: 1.0 μg L^{-1}).

Sample analysis

The perchlorate concentrations in the sample solutions were analyzed by LC/ESI-MS. The separation was performed using an Agilent 1100 series binary pump (Agilent Technologies, Palo Alto, CA) with anion exchange columns (*i.e.*, IonPac AG21 (2 × 50 mm) as a guard column and IonPac AS21 (2 × 250 mm) as a separation column (Dionex, Sunnyvale, CA)). The IonPac AS21 is suitable for separating highly retainable ions, such as perchlorate. The eluent, which was a mixture of 73 mmol L^{-1} $(NH_4)_2CO_3$ and 20 mmol L^{-1} NH_4OH aqueous solutions and acetonitrile (55/45), was isocratically eluted at 0.2 mL min^{-1}. These ammonium species (*i.e.*, $(NH_4)_2CO_3$ and NH_4OH) are the types of the eluent typically used in LC/MS; the eluent used in this study seemed to be nontoxic and much easier to handle. The injection volume was 100 μL. The detection was performed using an Agilent 1100 VL mass spectrometer (Agilent Technologies) operated in the negative-ion ESI mode. The optimized conditions were as follows: dry gas temperature (nitrogen), 350°C; dry gas flow, 10 L min^{-1}; nebulizer pressure, 20 psi; capillary voltage, 1000 V; fragmentor voltage, 110 V. Perchlorate analysis was performed by selective ion monitoring (SIM) and the monitored ions were *m/z* 99 (quantification), *m/z* 101 (identification) for perchlorate, and *m/z* 107 for ^{18}O-enriched perchlorate. For investigating the presence of perchlorate in water samples from water-purification plants, perchlorate concentrations were also determined by IC/ESI-MS/MS. The analytical conditions for IC/ESI-MS/MS are described elsewhere.[22] In some cases, the retention times of coexisting anions were investigated by LC/ESI-MS/MS or non-suppressed IC/CD under the same LC conditions as those of the LC/ESI-MS system.

Results and Discussion

Limit of quantification of perchlorate

Figure 1 shows SIM chromatograms of 0.1 μg L^{-1} perchlorate and 1 μg L^{-1} ^{18}O-enriched perchlorate in an ultrapure water sample. The separation column used in this study was a type of hydroxide-selective anion column.[23] However, the pH of the eluent in this method was much lower than those of the other methods (EPA method 331.0 and IC/ESI-MS), although the same separation column was used for perchlorate analysis.[20,23] That is, the pH of the mixture of the $(NH_4)_2CO_3$ and NH_4OH aqueous solutions was 9.2, and that of the 200 mmol L^{-1} methylamine aqueous solution, the eluent used in the EPA 331.0, was 12. This was because, in this study, carbonate salt was used as the eluent, and an organic solvent (*i.e.*, acetonitrile) was mixed in it. Also, $(NH_4)_2CO_3$ and NH_4OH aqueous solutions are generally used as the eluents in LC/MS. In the case of EPA 331.0, if the components of the LC system are normally not tolerant to high-pH solutions, the materials have to be replaced with suitable ones.[20] Therefore, it was considered that the effect of the alkaline pH of the eluent used in this study on the components in the LC system was much smaller than that of other alkaline solutions used in the LC system, such as a methylamine aqueous solution.

The LOQ of perchlorate obtained by LC/ESI-MS was determined from the signal-to-noise ratio (*S/N*) and the standard deviation (SD) by repetition analysis. The *S/N*s of the SIM chromatograms of 0.1 μg L^{-1} perchlorate were 10 for *m/z* 99 (quantification) and 4.4 for *m/z* 101 (identification), and that of 1 μg L^{-1} ^{18}O-enriched perchlorate was 125 for *m/z* 107 (Fig. 1). Also, the *S/N* of the peak of 0.05 μg L^{-1} perchlorate was 5.0 for *m/z* 99. Thus, when the LOQ was defined as 10*S/N*s, the LOQ

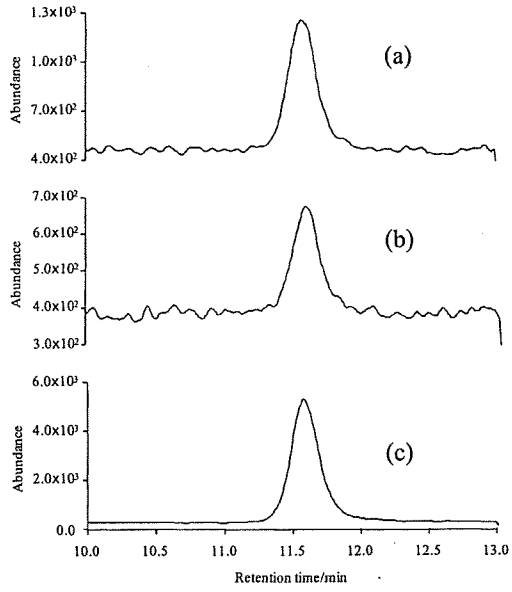

Fig. 1 SIM chromatograms of 0.1 μg L^{-1} perchlorate and 1.0 μg L^{-1} ^{18}O-enriched perchlorate in ultrapure water sample. (a) *m/z* 99, perchlorate (quantification), (b) *m/z* 101, perchlorate (identification), (c) *m/z* 107, ^{18}O-enriched perchlorate.

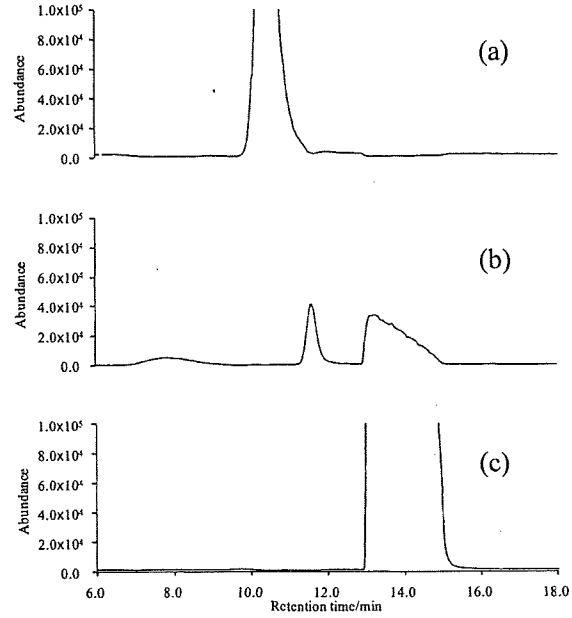

Fig. 2 SIM chromatograms of 1.0 μg L^{-1} perchlorate in an ultrapure water sample containing 100 mg L^{-1} chloride, 10 mg L^{-1} nitrate and 100 mg L^{-1} sulfate. (a) *m/z* 62, nitrate, (b) *m/z* 99, perchlorate, (c) *m/z* 97, sulfate.

of perchlorate was 0.1 μg L^{-1}. Li and George[18] reported that the *S/N*s of the peaks of multiple reaction monitoring (MRM) chromatograms of 0.05 μg L^{-1} perchlorate in deionized water sample determined by LC/ESI-MS/MS are 11 for *m/z* 99 - 83 (quantification) and 1.3 for *m/z* 101 - 85 (identification). Asami *et al.*[22] reported that the *S/N* of the peaks of 0.05 μg L^{-1} perchlorate in an ultrapure water sample by IC/ESI-MS/MS is 280 for *m/z* 99 - 83 (average of 5 replications). Thus, it was considered that the detection sensitivity for perchlorate of the proposed method was lower than that of IC/ESI-MS/MS, but not very different from that of LC/ESI-MS/MS. For an LOQ determination from the SD value by repetition analysis, 0.1 μg L^{-1} perchlorate was selected because this concentration was the LOQ determined from the *S/N*s. When the LOQ was defined as 10SD (n = 5), the LOQ was calculated to be 0.03 μg L^{-1}. In the case of the calibration curve, when the range of the perchlorate concentration was from 0.1 to 10 μg L^{-1} in the ultrapure water sample, its linearity was observed (R^2 = 0.9997). Although the values of LOD, LOQ or MRL are dependent on the studies, the LOQ and MRL used in this study were not very different from those in other studies. Thus, it was considered that the proposed method is sufficient for determining the perchlorate concentration in water samples.

Separation of perchlorate from common anions

Figure 2 shows SIM chromatograms of 1.0 μg L^{-1} perchlorate in an ultrapure water sample containing coexisting anions (100 mg L^{-1} chloride, 10 mg L^{-1} nitrate, and 100 mg L^{-1} sulfate). Because the mass scan range in Fig. 2 was *m/z* 50 - 350, the *m/z* 35 of chloride was beyond the scan range, and the chloride peak was not observed. However, when these anions were analyzed by LC/ESI-MS/MS, the chloride peak was observed at a much earlier retention time than the nitrate peak (data not shown). In the case of nitrate, the tail of the nitrate peak slightly overlapped with the perchlorate peak, but most of the nitrate and perchlorate peaks were separated. The presence of nitrate in the sample seemed to affect the ionization suppression of perchlorate, but the degree was not very large (see next section). Also, the shape of the perchlorate peak was not affected by the presence of nitrate. The sulfate peak was observed later than the perchlorate peak. Sulfate, the most important anion, should be separated from perchlorate, because the minor sulfate isotope (^{34}S) has an *m/z* 99 signal as $H^{34}SO_4^-$, and the *m/z* is the same as that of the quantification for perchlorate. Furthermore, the retention times of nitrite, chlorate and fluoride were also investigated. The LC/ESI-MS/MS system was used for nitrite and chlorate, and a non-suppressed IC/CD system was used for fluoride. The retention time of fluoride was earlier than that of chloride, and those of nitrite and chlorate were earlier than that of nitrate; therefore, all of those peaks did not affect the perchlorate analysis. From these results, it was shown that the combination of the column and the eluent resulted in a rapid and successful isocratic separation of perchlorate from coexisting anions.

In general, IC/ESI-MS(/MS) is superior to separate these anions from perchlorate, because the IC system is designed to separate ions. However, sodium and potassium-based aqueous solutions are usually used as eluents in IC/ESI-MS(/MS); therefore, a suppressor is required to remove these nonvolatile ions. Moreover, an organic solvent, such as acetonitrile, must be mixed as a postcolumn solvent with an additional LC pump to improve the sensitivity.[19,21,22] Therefore, the analytical system using IC/ESI-MS(/MS) is inevitably too complex to control. On the other hand, the LC/ESI-MS(/MS) method is considered to be simpler and less expensive than the IC/ESI-MS(/MS) method. Also, in many cases, MS(/MS) systems are currently connected to the LC systems for the analysis of organic micropollutants. For such laboratories, routine switching from the LC/MS(/MS) system to the IC/MS(/MS) system and *vice versa* can be avoided even if they have only one MS(/MS) system. Considering these aspects, it is considered that the LC/ESI-MS(/MS) method is more applicable if the separation of perchlorate from coexisting anions is achieved like in the case of this study.

Recovery studies

Recovery studies were performed by spiking the matrix samples with 1.0 μg L^{-1} perchlorate. The matrix samples were river and tap water, and ultrapure water containing 100 mg L^{-1} chloride, 20 mg L^{-1} nitrate, and 100 mg L^{-1} sulfate (synthesized water). Residual free chlorine in a tap-water sample was quenched by NH_4Cl or sodium ascorbate. The mean percentage recoveries of perchlorate were 102% for a raw-water sample, 101% for a tap-water sample with NH_4Cl, 98% for a tap-water sample with sodium ascorbate, and 99% for a synthesized water sample. The relative standard deviation (RSD, n = 5) was less than 3% for each case. The direct recoveries of the ^{18}O-enriched perchlorate (*i.e.*, the percentages of perchlorate peak areas in the samples to that in ultrapure water) ranged from 85 to 89% for each sample. Ionization suppressions seemed to occur to some degree, although the determination of perchlorate by the IS method was not affected.

Determination of perchlorate in water samples from water-purification plants by LC/ESI-MS and a comparison of the measurement results with those determined by IC/ESI-MS/MS

The perchlorate concentrations in raw and finished water samples from seven water-purification plants were determined by LC/ESI-MS and IC/ESI-MS/MS (Table 1). The lowest calibration standard, 0.1 μg L^{-1}, was set to be the MRL of perchlorate by LC/ESI-MS. Perchlorate was detected in 12 out of 13 samples by LC/ESI-MS, and the perchlorate concentrations detected were from 0.1 to 36.1 μg L^{-1}. The remaining sample was raw water from Plant A. Compared to the interim HAL for perchlorate (15 μg L^{-1}), the perchlorate concentration in a raw-water sample from Plant B was higher than the interim HAL. Also, the perchlorate concentrations in raw and finished water samples from Plants C and G were higher than 50% of the interim HAL, and those from Plants D and F were higher than 10% of the interim HAL. From these results, it was shown that drinking water from the Tone River Basin is widely contaminated by perchlorate, as reported in a previous study.[9] Thus, it was considered that perchlorate is a type of contaminant that must be paid attention, although Japanese people generally take sufficient iodine from marine foods.[9] When the perchlorate concentrations of the 12 samples determined by LC/ESI-MS and IC/ESI-MS/MS were statistically compared using a paired t-test (level of significance: 0.05), the difference for each sample was not statistically significant.

Conclusions

In this study, an analytical method for perchlorate in a water sample by LC/ESI-MS using an anion exchange column and a volatile and weakly alkaline eluent in an isocratic mode was proposed. Perchlorate was chromatographically separated from coexisting anions, particularly chloride and sulfate, using the proposed analytical method. The perchlorate concentrations in raw and finished water samples from water-purification plants determined by LC/ESI-MS were not statistically different from those determined by IC/ESI-MS/MS, the previously developed analytical method.

Acknowledgements

The authors thank Dr. Sekiguchi (Dionex) for advice concerning the chromatographic conditions, and Ms. Yoshida (National Institute of Public Health) for help with the experiment. This study was financially supported by a research grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (19560557).

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Bromate, chlorate, chlorite and perchlorate in sodium hypochlorite solution used in water supply

Mari Asami, Koji Kosaka and Shoichi Kunikane

ABSTRACT

A survey was conducted to reveal the concentrations of bromate, chlorite, chlorate and perchlorate as impurities in sodium hypochlorite solutions and those of chlorate and perchlorate in raw and processed waters including a metropolitan area. High concentrations of bromate (max. 414 mg l^{-1}) and chlorate (max. 260,000 mg l^{-1}) were found in purchased sodium hypochlorite solutions for drinking water disinfection that had been stored for a long time, more than two years at a maximum. In the survey of chlorate and perchlorate in raw and processed waters in the Tone River Basin, the highest concentration of chlorate in raw water was 78 μg l^{-1} and that of perchlorate was 40 μg l^{-1}. Chlorate and perchlorate concentrations in 32 purchased sodium hypochlorite solutions and six on-site-generated hypochlorite solutions were also analysed. In the purchased sodium hypochlorite solutions, perchlorate concentrations ranged from 0.170 to 33.0 mg l^{-1}. In hypochlorite solutions whose measured FAC (free available chlorine) concentration was lower than the manufacturer-specified FAC concentrations, the chlorate and perchlorate concentrations were higher than those in relatively fresh sodium hypochlorite solutions. In on-site-generated hypochlorite solutions, the maximum concentrations of chlorate and perchlorate were 1,700 mg l^{-1} (140 mg g^{-1} of measured FAC) and 0.660 mg l^{-1} (0.053 mg g^{-1} of measured FAC), respectively.

Key words | bromate, chlorate, chlorite, perchlorate, sodium hypochlorite solution

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INTRODUCTION

Sodium hypochlorite solution is frequently used as a disinfectant and as an oxidizing agent in waterworks. Since the concentration of residual chlorine should be maintained at more than 0.1 mg l^{-1} in distribution systems, sodium hypochlorite solution is commonly used as a residual disinfectant in over 80% of treatment facilities in Japan. It is usually added at the final stage of the treatment process, and sometimes added at the first and/or the middle stage as an oxidizing agent. A 12% (or 6%) stock solution has been widely used in waterworks for the chlorination of water; more recently, on-site generation of hypochlorite has been introduced into 10% of treatment facilities (JWWA 2006).

doi: 10.2166/aqua.2009.014

The duration of storage of stock solutions is sometimes longer, over several months, for example, in small water treatment facilities. In addition, attention should be paid to impurities in hypochlorite solutions, especially in raw waters containing ammonia, which is a chlorine-consuming compound, since the injection ratios of hypochlorite solutions are relatively high in ammonia-contaminated raw water.

For example, in April 2004, bromate concentration in chlorinated drinking water in Hokkaido was found to be 0.168 mg l^{-1}, 16.8 times higher than the concentration limit stipulated by Japanese standards (*Hokkaido News* 2004). Later analysis showed bromate at a concentration of

668 mg l^{-1} in the sodium hypochlorite solution used in the water treatment process, illustrating the fact that sodium hypochlorite solution can be a major source of bromate in chlorinated drinking water.

Bromate, known as a carcinogenic ozonation by-product, was introduced in the Japanese drinking water quality standard in 2004 and has been regulated to be less than 0.01 mg l^{-1}. Chlorate and chlorite, known by-products of chlorine dioxide disinfection, have also been listed as chemicals to be monitored. The criterion has been set at 0.6 mg l^{-1} for both chlorate and chlorite based on their oxidative property for red blood cells in humans (MHLW 2003). In a national survey of monitored items (MHLW 2005), chlorate concentrations exceeded the criterion in 14 of the 598 monitored finished waters. The principal criterion for including a monitored compound in the list of drinking water standards is the detection of the compound in finished water at a concentration near or above one-tenth of its threshold standard. Accordingly, chlorate was introduced into the drinking water standards in April 2008. Perchlorate has only been recently addressed as a contaminant of concern in drinking water, though it is naturally occurring and was identified in Chilean salt caliche in the early 1900s (Dafert 1908). More recently it has been used as a chemical propellant in rocket fuels and an oxidizing agent in many products. Perchlorate is known to interfere with the iodine uptake of the thyroid gland (Greer *et al.* 2002; National Research Council 2005). In 2005, the United States Environmental Protection Agency (US EPA) established an official reference dose (RfD) of 0.7 μg kg^{-1} day^{-1} of perchlorate and specified its drinking water equivalent level (DWEL) to be 24.5 μg l^{-1} (US EPA 2005), based on a report by the National Academy of Sciences (NAS) (National Research Council 2005).

Chlorate and perchlorate have been detected in Japanese aquatic environments, especially in the Tone River Basin, which is one of the largest water sources for drinking water supply in the Tokyo metropolitan area (Asami *et al.* 2007; Kosaka *et al.* 2007). The maximum concentration of chlorate and perchlorate in river water affected by industrial effluents was measured at 9,000 μg l^{-1} and 15,000 μg l^{-1}, respectively. One of the highest concentrations of perchlorate was attributable to unintentional production of perchlorate in an electrolysis process.

All oxyhalides listed here (i.e. bromate, chlorate, chlorite and perchlorate) are industrial chemicals and are also known to exist in chlorinated drinking waters as impurities from sodium hypochlorite solutions. The concentrations of bromate, chlorate, chlorite and perchlorate in hypochlorite solution have been shown to increase during storage (Gordon *et al.* 1995 for chlorate; Weinberg *et al.* 2003 for bromate). However, the quality of the hypochlorite solution used in water treatment plants and the parameters which may contribute to increased rates of production of undesirable oxyhalide species are not well known. In this study, we investigated the concentrations of bromate, chlorate, chlorite and perchlorate in raw, processed and finished waters and hypochlorite solutions collected from various water treatment plants.

MATERIALS AND METHODS

Bromate, chlorate and chlorite in stored sodium hypochlorite solutions

The primary study was conducted to detect bromate, chlorate and chlorite concentrations in hypochlorite solutions. Thirty-seven samples were collected from hypochlorite solutions used in water supply facilities from 11 prefectures including Kanto (east), Kansai (west), Hokkaido (north) and Okinawa (south) regions in Japan. The water supply facilities include 28 treatment plants and 9 distribution facilities. Out of 37 facilities, 14 stored the sodium hypochlorite solutions in an air-conditioned environment. Samples were collected and stored in cool and dark conditions and analysed within 2 days. Sodium hypochlorite solutions were diluted 10,000 times by pure water (MilliQ Gradient A10 water purification system, Millipore, Bedford, Massachusetts) and the concentration of free available chlorine (FAC) was analysed by the DPD method. Chlorate was analysed using ion chromatography (IC, DX-500, Dionex, Sunnyvale, California), electric conductivity with an Ion Pac AG19/AS19 (4 mm) column and KOH generator. Bromate and chlorite were analysed by the IC-post-column colouring method using the same eluent reacted with 1.2 mM l^{-1} $NaNO_2$ and 1.5 M KBr 1.0 M l^{-1} H_2SO_4 solution, according to the official Japanese notification method (MHLW 2004).

Chlorate and perchlorate in raw, processed and finished waters and hypochlorite solutions

An intensive survey of chlorate and perchlorate concentrations in source and finished waters was conducted in conjunction with the Ministry of Health, Labour and Welfare, Japan. Raw, processed and finished water and hypochlorite solutions were collected from water treatment plants, especially in the Tone River Basin, to quantify the effect of industrial effluents. The Tone River is the largest water source in the Tokyo Metropolitan area and has been previously found to be contaminated by chlorate and perchlorate (Kosaka *et al.* 2007). More than ten other large cities and water supply bodies previously reporting high concentration of disinfection by-products (DBPs) were selected (MHLW 2006). In addition, 32 purchased and six on-site-generated hypochlorite solutions were collected and analysed. Chlorate and perchlorate concentrations were analysed with IC-tandem mass spectrometry (MS/MS) (Dionex ICS-2000 and API 3200QTrap, Applied Biosystems) as described elsewhere in detail (Kosaka *et al.* 2007). ^{18}O-enriched $NaClO_4$ (Cambridge Isotope Laboratories) was used as an internal standard for perchlorate. The minimum reporting limits (MRLs) for perchlorate and chlorate were set to be 0.05 and 0.05 $mg\,l^{-1}$, respectively, except the MRL for chlorate of the sample waters in several water treatment plants was 0.1 $mg\,l^{-1}$.

RESULTS AND DISCUSSION

Bromate, chlorate and chlorite in hypochlorite solutions

In the 37 sodium hypochlorite solutions collected, the concentration of measured free available chlorine (FAC) in the solution ranged from 0.04 to 15%, and the average concentrations of bromate in the solution were 96 $mg\,l^{-1}$ (maximum 414 $mg\,l^{-1}$). When the concentrations were converted into their finished water, bromate concentration was below 0.001 $mg\,l^{-1}$; chlorate and chlorite concentrations were below 0.20 and 0.003 $mg\,l^{-1}$, respectively, assuming the dose of the hypochlorite solution to be 1 $mg\,l^{-1}$. However, in some cases, chlorate concentration in the hypochlorite solution was extremely high when the measured FAC in the solution was much lower than its manufacturer-specified FAC at the time of purchase. The concentration of FAC is a critical factor for controlling residual chlorine, chlorate and bromate, because, if the sodium hypochlorite solutions which contain lower FAC than manufacturer-specified are used for disinfection, bromate and chlorate concentration may increase subsequently because of the increased amount of hypochlorite solution used in order to accomplish residual chlorine concentration.

Bromate concentration varied as shown in Figure 1. One factor is that bromate concentration varied among manufacturers. Though the number of samples was limited in this study, the bromate concentrations in sodium hypochlorite solutions of one manufacturer ranged from 5.4 to 49.5 $mg\,l^{-1}$ ($n = 7$), while those of another manufacturer ranged from 24.5 to 96.5 $mg\,l^{-1}$ ($n = 7$).

The other factor seems to be the timing of the purchase of hypochlorite solutions. It was recently reported that the concentration of bromate is largely dependent upon the salts used to produce hypochlorite solutions and can be controlled by changing the salt to those salts whose concentration of bromide is lower or by refining the salts. It is also reported that the manufacturers have changed the salts to refined types in accordance with the revision of the standard (JWWA 2006). Therefore, the high bromate concentration in older samples may be attributable to the bromide present in salts that were used as a basic ingredient in the production process.

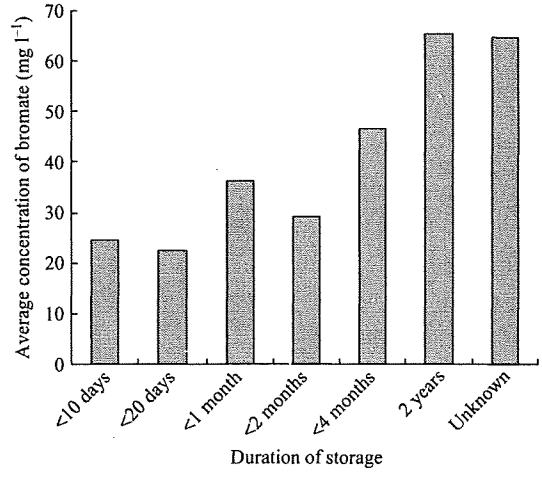

Figure 1 | Concentration of bromate in the hypochlorite solutions in relation to the duration of storage after purchase of each hypochlorite solution.

Weinberg *et al.* (2003) indicated the mechanism of bromate formation in hypochlorite solution as described in Equation (1) below. Since no residual bromide was found in any of the samples, and the reaction has been reported in the literature to occur very rapidly, the majority of bromide in sodium hypochlorite solution was expected to react to produce bromate.

$$Br^- + 3ClO^- \rightarrow BrO_3^- + 3Cl^- \quad (1)$$

Thus the authors consider that the high concentration of bromate might depend mainly on the bromide concentration in salts, while bromate concentration was higher in those sodium hypochlorite solutions that had been stored for more than two years or for an unknown period after the purchase of each hypochlorite solution.

Chlorite concentration was rather low in the hypochlorite samples, with an average of 145 $mg\,l^{-1}$ and a maximum of 397 $mg\,l^{-1}$. Chlorite concentrations in the treated water were below 0.003 $mg\,l^{-1}$ and 0.03 $mg\,l^{-1}$ assuming respective hypochlorite solution doses of 1 $mg\,l^{-1}$ and 10 $mg\,l^{-1}$. So chlorite concentrations were not expected to be very high in hypochlorite solutions and finished water.

The average concentration of chlorate in the hypochlorite solutions was 15,300 $mg\,l^{-1}$ (maximum 260,000 $mg\,l^{-1}$), and was largely different between samples and FAC levels. Chlorate concentrations in finished water were estimated to be 0.20 $mg\,l^{-1}$ and 2.0 $mg\,l^{-1}$ when the dose of the sodium hypochlorite solution was assumed to be 1 $mg\,l^{-1}$ and 10 $mg\,l^{-1}$, respectively. Thus, further study was conducted as described in the next section.

Chlorate and perchlorate in raw, processed and finished water

Chlorate and perchlorate concentrations in raw, processed and finished water in water treatment plants are shown in Table 1. Out of the 368 samples, chlorate was detected in 93.2% of the raw water samples and 100% of the processed and finished water samples. Perchlorate was detected in 98.8% of the raw water samples and 94.9% of the processed and finished water samples. The highest concentration of chlorate in raw water (78 $\mu g\,l^{-1}$) was found in groundwater apparently affected by the chlorate and perchlorate contamination in the Tone River. The concentrations of chlorate and perchlorate, and the ratio of their concentrations, were higher in the samples taken from the Tone River Basin. Perchlorate concentrations in raw, processed and finished waters at the same treatment plant were almost unchanged during the process. Chlorate concentrations were much higher in processed and finished waters, especially in the smaller facilities located in remote areas. The maximum concentration of chlorate in this study was 2.9 $mg\,l^{-1}$ (2,900 $\mu g\,l^{-1}$) due to chlorate in sodium hypochlorite solution used for disinfection.

Figures 2 and 3 show some examples of chlorate and perchlorate concentration through different stages of treatment in water treatment plants using different types of chlorine disinfectant. In Figure 2, the water treatment plant shown on the left-hand side used hypochlorite generated on-site while the plant on the right used manufactured sodium hypochlorite solutions. Chlorate concentrations increased during the treatment process. Both plants showed a large increase in chlorate concentrations. Figure 3 shows

Table 1 | Chlorate and perchlorate concentrations in raw, processed and finished water in water treatment plants

	Chlorate ($\mu g\,l^{-1}$)			Perchlorate ($\mu g\,l^{-1}$)		
	Detection rate	Min*	Max	Detection rate	Min*	Max
Raw water of water treatment plants						
Tone River Basin	116/116	0.06	78	114/116	0.09	40
Other than the Tone River Basin	62/75	0.08	53	55/55	0.06	2 .5
Processed water and finished water from water treatment plants						
Tone River Basin	178/178	0.17	2,900	168/178	0.05	24

*The minimum is data detected above LO Q (limits of quantification).

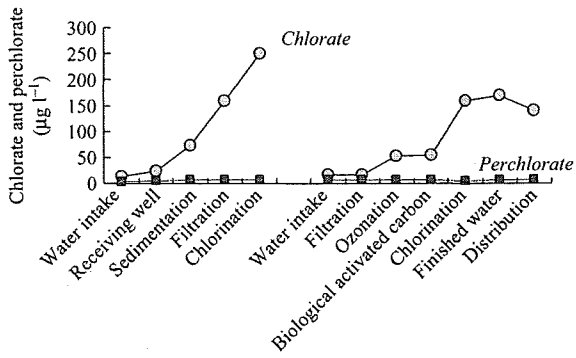

Figure 2 | Chlorate and perchlorate in water treatment plants using hypochlorite solutions (left-hand side, hypochlorite generated on-site; right-hand side, manufactured sodium hypochlorite solutions).

an example of two treatment plants where liquid chlorine was used in the disinfection processes. As such, concentrations of chlorate and perchlorate through the stages of treatment were not noticeably increased when liquid chlorine was used.

Chlorate and perchlorate in hypochlorite solutions

For the analysis of chlorate and perchlorate concentrations, hypochlorite solutions were collected mainly from the water treatment plants investigated in the previous section. Of these, 32 sodium hypochlorite solutions were purchased and 6 hypochlorite solutions were generated on site. Measured FAC concentrations in the purchased sodium hypochlorite solutions ranged from 8.0 to 16.4%, while the FAC in on-site-generated hypochlorite solutions ranged from 1.0 to 7.2%. Chlorate concentration in the purchased sodium hypochlorite ranged from 1,200 $mg\,l^{-1}$ to 26,000 $mg\,l^{-1}$. The chlorate concentration varied and was above 10,000 $mg\,l^{-1}$ in 10 samples. Perchlorate concentrations in the purchased hypochlorite solutions ranged from 170 to 33,000 $\mu g\,l^{-1}$, as shown in Table 2.

FAC and concentration of chlorate and perchlorate

Figure 4 shows the relationship between the measured FAC concentration and the concentrations of chlorate and perchlorate per measured FAC in the hypochlorite solutions. The concentrations of chlorate and perchlorate in purchased hypochlorite solutions were higher in instances when the measured FAC was low. The maximum chlorate concentration was 26,000 $mg\,l^{-1}$ (320 $mg\,g^{-1}$ of measured FAC). The maximum concentration of perchlorate was 33,000 $\mu g\,l^{-1}$ (0.420 $mg\,g^{-1}$ of measured FAC) with the hypochlorite solution whose FAC was 8.0% while the manufacturer-specified FAC concentrations was '12.0% or above'. The manufacturer-specified FAC concentrations in the hypochlorite solutions were similar in range, from 12 to 13.2%, except for four samples for which information could not be obtained. However, the FAC concentrations ranged from 8.0 to 16.4% for purchased hypochlorite solutions. The chlorate and perchlorate concentrations were higher in hypochlorite solutions in which the FAC was lower than the manufacturer-specified FAC concentrations, while no difference was observed among nine different manufacturers. Thus, it was considered that the concentrations of chlorate and perchlorate increased with the decay of FAC in the hypochlorite solutions.

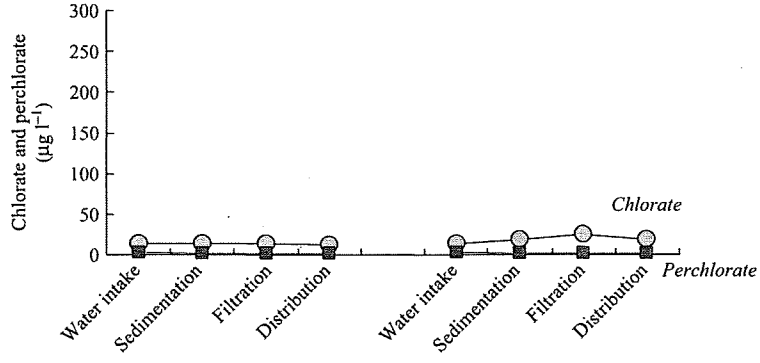

Figure 3 | Chlorate and perchlorate in water treatment plants using liquid chlorine.

Table 2 | Chlorate and perchlorate concentrations in hypochlorite solutions

	Chlorate ($mg\,l^{-1}$)			Perchlorate ($\mu g\,l^{-1}$)		
	Detection rate	Min*	Max	Detection rate	Min*	Max
Sodium hypochlorite (Purchased)	32/32	1,200	26,000	32/32	170	33,000
Sodium hypochlorite (On-site generation)	6/6	160	1,700	6/6	13	660

*The minimum is data detected above LO Q (limits of quantification).

Figure 5 shows the relationship between chlorate and perchlorate concentration in purchased hypochlorite solution. Across the concentration range tested, the concentration of perchlorate was approximately three orders of magnitude lower than chlorate, though the ratio increased with the chlorate concentration, especially when the chlorate concentration was higher than $10{,}000\,mg\,l^{-1}$. Therefore, their relationship was expressed as an approximate equation of the second order.

Hypothesized mechanism of generation of chlorate and perchlorate in hypochlorite solutions

It is reported that, in hypochlorite solution, chlorate is generated from a disproportionation reaction of hypochlorous acid (HOCl) expressed by Equations (2) and (3), or a decomposition reaction of hypochlorite ion (ClO^-) through chlorite ion (ClO_2^-). It is of note that the reaction rate in Equation (2) is much faster than that in Equation (3) (Gordon *et al.* 1995).

$$ClO^- + ClO^- \rightarrow ClO_2^- + Cl^- \quad (2)$$

$$ClO_2^- + ClO^- \rightarrow ClO_3^- + Cl^- \quad (3)$$

As shown in Figure 5, chlorate concentrations in the hypochlorite solutions were almost 1,000 times higher than perchlorate concentrations. The ratio of perchlorate to chlorate concentration was elevated under conditions where there was a corresponding increase in chlorate concentration from the decay of FAC in the hypochlorite solution. In addition, perchlorate was not found in stored chlorate standard solutions (data not shown). Considering these results, a hypothetical explanation for the presence of perchlorate in hypochlorite solution is from the reaction between chlorate and hypochlorite.

The manufacturer-specified FAC concentrations in the purchased sodium hypochlorite solutions normally ranged from 12 to 13.2%. Therefore, from the higher FAC samples (i.e. above 14%), chlorate and perchlorate concentrations in relatively fresh purchased hypochlorite solutions could be obtained. Chlorate concentrations in the solutions ranged from 1,200 to $12{,}000\,mg\,l^{-1}$ (average $5{,}900\,mg\,l^{-1}$)

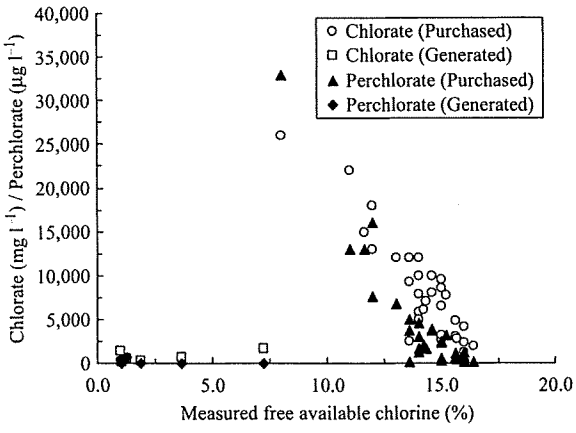

Figure 4 | Relationship between the measured FAC concentration and the concentrations of chlorate and perchlorate per measured FAC in the hypochlorite solution.

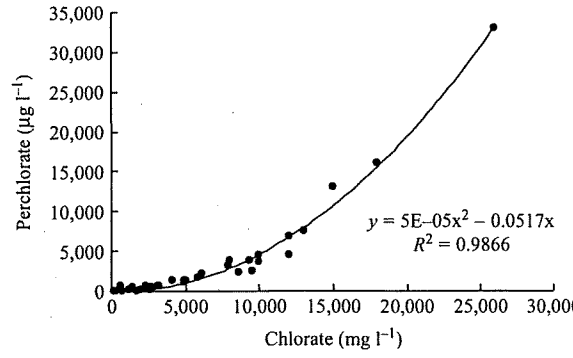

Figure 5 | Relationship between chlorate and perchlorate concentration in purchased hypochlorite solution.

and perchlorate concentrations ranged from 170 to 4,400 $\mu g l^{-1}$ (average 1,800 $\mu g l^{-1}$). The ratios of perchlorate to chlorate concentration ranged from 7.9×10^{-5} to 5.0×10^{-4} (average 2.7×10^{-4}), rather lower compared with the average of all solutions.

From these results, perchlorate concentrations in new hypochlorite solutions are normally low, but increase during storage. This might be controlled by controlling chlorate formation during storage.

Chlorate and perchlorate concentrations in the on-site-generated hypochlorite solutions

The on-site-generated hypochlorite solutions had concentrations of chlorate ranging from 160 to 1,700 $mg l^{-1}$ and perchlorate from 0.013 to 0.660 $mg l^{-1}$. Their concentrations per measured FAC are shown in Figure 4. Maximum ratios of chlorate and perchlorate were 140 and 0.053 $mg g^{-1}$ of measured FAC, respectively. The apparatus used to generate hypochlorite solutions was different in each of the companies, although the electrodes used in the apparatus were the same (titanium oxide). Thus, the reason for the difference of the chlorate and perchlorate concentrations between the on-site-generated hypochlorite solutions does not seem to be related to the material of the electrode. In addition, although the storage conditions of each on-site-generated hypochlorite solution were unknown, these conditions may have affected chlorate and perchlorate concentrations as in the case of the purchased hypochlorite solutions.

There were no reports of investigations on perchlorate generation in on-site-generated hypochlorite solutions. However, Tock *et al.* (2004) reported generation of perchlorate in water storage tanks that employed the electron voltage technique to prevent corrosion. In that study, perchlorate was generated in proportion to the contact time. It was thought that the same phenomenon might occur in on-site generation tanks.

Estimation of chlorate and perchlorate concentration due to hypochlorite solutions

Using the manufacturer-specified FAC, chlorate and perchlorate concentrations in hypochlorite solution, maximum chlorate and perchlorate load concentrations in hypochlorite solution were calculated. Chlorine dosage was assumed to be 10 $mg l^{-1}$. As in the case shown in Figure 5, the manufacturer-specified FAC concentration was taken as 12.5% for purchased and 1.0% for on-site-generated hypochlorite solutions when no data were available. For the purchased hypochlorite solutions, calculated load concentrations of chlorate ranged from 92 to 2,100 $\mu g l^{-1}$; most of the measured values ranged from 100 to 1,000 $\mu g l^{-1}$. The chlorate concentrations in four hypochlorite solutions exceeded 1,000 $\mu g l^{-1}$. For on-site-generated hypochlorite solutions, the load concentrations of chlorate ranged from 160 to 1,400 $\mu g l^{-1}$. It was shown that the load concentrations of chlorate in on-site-generated hypochlorite solutions were usually lower, but not necessarily lower than those in the purchased hypochlorite solutions.

For perchlorate, the calculated load concentrations in purchased hypochlorite solutions ranged from <0.05 to 2.8 $\mu g l^{-1}$, with most values falling below 0.5 $\mu g l^{-1}$. The load concentration of perchlorate in four hypochlorite solutions exceeded 1.0 $\mu g l^{-1}$. The load concentrations in on-site-generated hypochlorite solutions ranged from <0.05 to 0.67 $\mu g l^{-1}$. These values were much lower than those for purchased hypochlorite solutions.

Distribution channel of sodium hypochlorite solutions

Interviews were conducted with the manufacturers, distributors and users (waterworks) involved with the distribution channel of sodium hypochlorite solutions to identify the duration of time in each distribution process. One route is direct delivery of the products from manufacturer to large facilities by trucks. The other is indirect delivery of solutions distributed by trucks to packaging facilities followed by distribution in small packages to rather small facilities. The two main routes of distribution are shown in Figure 6. Normal distribution from manufacturer to the large treatment plants and distributors is accomplished 0–7 days after production. On the other hand, indirect delivery of small packages takes longer than direct distribution. It takes 0–7 days to deliver from manufacturer to distributors and more days to deliver to the smaller water treatment facilities, though the number of interviews was limited.

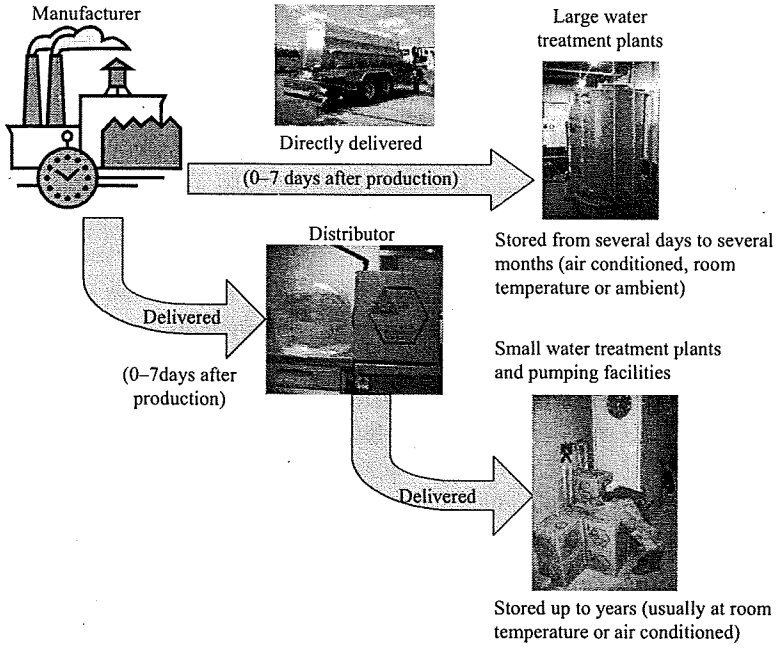

Figure 6 | Distribution channels for sodium hypochlorite.

In this study, initial concentrations of chlorate and perchlorate in hypochlorite solutions at the manufacturers were not studied; however, the concentrations of chlorate and perchlorate are not noticeably high in relatively fresh solutions. Control of the distribution process and storage is the key factor to control chlorate and perchlorate as far as this study is concerned. Since the temperature of storage and the impurities in the sodium hypochlorite solutions have been pointed out to affect the decay of hypochlorite (JWWA 2006), further study is needed to prove the factors affecting degradation of hypochlorite in sodium hypochlorite solutions. In addition, further research is required on the relationship between concentrations of chlorate and perchlorate and their generation conditions in on-site generation facilities for hypochlorite.

CONCLUSIONS

1. Higher concentrations of bromate and chlorate were found in hypochlorite solutions stored for longer periods of time, more than two years at a maximum; their maximum concentrations were 414 and 260,000 $mg\,l^{-1}$, respectively.
2. A survey was conducted to reveal the concentrations of chlorate and perchlorate in raw, processed and finished water and hypochlorite solutions. The highest concentration of chlorate in raw water was 78 $\mu g\,l^{-1}$ and that of perchlorate was 40 $\mu g\,l^{-1}$.
3. In purchased hypochlorite solutions tested, perchlorate concentrations ranged from 0.170 to 33.0 $mg\,l^{-1}$.
4. In hypochlorite solutions whose measured FAC concentration was lower than the manufacturer-specified FAC concentration, the chlorate and perchlorate concentrations were higher than those in the relatively fresh hypochlorite solutions. The ratio of perchlorate to chlorate concentration in the relatively fresh hypochlorite solutions was around 10^{-3}.
5. In on-site-generated hypochlorite solutions, the maximum concentrations of chlorate and perchlorate were 1,700 $mg\,l^{-1}$ (140 $mg\,g^{-1}$ of measured FAC) and 0.660 $mg\,l^{-1}$ (0.053 $mg\,g^{-1}$ of measured FAC), respectively.
6. Perchlorate in hypochlorite solutions was considered to be produced by degradation of hypochlorite and reaction with chlorate.

ACKNOWLEDGEMENTS

The authors express special thanks to the water supply bodies for providing samples and information for this research. This study was financially supported by the grant-in-aid for Basic Science Research (C)-19560557. The authors also thank Dr Shane Snyder and Dr Benjamin Stanford, Southern Nevada Water Authority, for their advice in preparing this manuscript.

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First received 7 February 2008; accepted in revised form 28 July 2008. Available online January 2009

Chemosphere 78 (2010) 482–487

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Mutagenic activities of a chlorination by-product of butamifos, its structural isomer, and their related compounds

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ARTICLE INFO

Article history:
Received 14 July 2009
Received in revised form 30 September 2009
Accepted 1 October 2009
Available online 27 October 2009

Keywords:
Methylnitrophenol
Chloromethylnitrophenol
Butamifos
Chlorination
Ames assay
YG1029 strain

ABSTRACT

The mutagenic activities of 5-methyl-2-nitrophenol (5M2NP), a chlorination by-product of butamifos, its structural isomer 2-methyl-5-nitrophenol (2M5NP), and related compounds were evaluated by the Ames assay. The mutagenic activities of 5M2NP and 2M5NP were negative or not particularly high. However, those of their chlorinated derivatives were increased in *Salmonella typhimurium* strain TA100 and the overproducer strains YG1026, and YG1029 in the absence and/or presence of a rat liver metabolic activation system (S9 mix), particularly for YG1029. The mutagenic activities of 6-chloro-2-methyl-5-nitrophenol (6C2M5NP) in YG1029 in the absence and presence of S9 mix were 70 000 and 110 000 revertants mg^{-1}, respectively. When nitro functions of 6C2M5NP and 4-chloro-5-methyl-2-nitrophenol (4C5M2NP) were reduced to amino functions, their mutagenic activities were markedly decreased. The mutagenic activities of 5M2NP and 4C5M2NP were lower than those of 2M5NP and 6C2M5NP, respectively. Thus, it was shown that substituent position is a key factor for the mutagenic activities of methylnitrophenols (MNPs) and related compounds. The mutagenic activities of the extracts of 2M5NP in chlorination increased early during the reaction time and then decreased. The main chlorination by-product contributing to the mutagenic activities of the extracts of 2M5NP in chlorination was 6C2M5NP. The results of chlorination of 2M5NP suggested that MNPs were present as their dichlorinated derivatives or further chlorination by-products in drinking water.

1. Introduction

Butamifos is a type of organophosphorus pesticide (OP) and is used as a herbicide. In Japan, pesticides are categorized as management items in the drinking water regulation; the list consists of 102 pesticides, including butamifos (Ministry of Health, Labour, and Welfare).

It has been reported that OPs with a P=S bond are rapidly transformed into compounds with a P=O bond (i.e., oxons) in chlorination, a disinfection process used in drinking water treatment (Magara et al., 1994; Onodera et al., 1995a; Qi and Simo, 1999; Stephen and Timothy, 2006). OPs with a P=S bond are structurally classified into subgroups. The initial chlorination by-products of OPs for phosphorothionate and phosphorodithionate subgroups were reported to be their oxons (Duirk et al., 2009). It was also reported that the transformation yields of OPs with P=S bonds to their oxons in chlorination were high (from around 70% to around 100%) for phosphorothionate, phosphorodithionate, and phosphonothionate subgroups (Kamoshita et al., 2007). On the other hand, those for OPs in a phosphoramidothionate subgroup (i.e., butamifos and isofenphos) were around 50%. The remaining 50% of their chlorination by-products consisted of phenolic compounds produced by cleavage of the P–O–aryl bond. In the case of butamifos, 5-methyl-2-nitrophenol (5M2NP) was produced as a phenolic compound. As phenolic compounds are reactive with chlorine (Deborde and von Gunten, 2008), the phenolic compounds produced from butamifos and isofenphos in chlorination were further transformed into their chlorinated derivatives (Kamoshita et al., 2007).

It has also been reported that the P–O–aryl bonds of OPs were cleaved by ultraviolet (UV) irradiation in water, with production of phenolic compounds as decomposition products (Katagi, 1993; Hirahara et al., 2004). 5M2NP and 3-methyl-4-nitrophenol (3M4NP) were produced from butamifos and fenitrothion, respectively. Moreover, it was reported that 4-chloro-2-methyl-6-nitrophenol (4C2M6NP) was detected in environmental water at concentrations up to 360 ng L^{-1} (Chiron et al., 2009). In their study, 4C2M6NP was produced by nitration of 4C2MP, which was mainly derived from the decomposition of (4-chloro-2-methylphenoxy)-acetic acid (MCPA), a herbicide. Based on these previous studies,

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doi:10.1016/j.chemosphere.2009.10.002

methylnitrophenols (MNPs) and their chlorinated derivatives were considered to be common compounds originating from pesticides in environmental and drinking water.

With regard to the toxicity, OPs are known to inhibit acetylcholinesterase (AChE) (Fulton and Chambers, 1985; Rompas et al., 1989; Tahara et al., 2005). In the case of OPs with a P=S bond, the levels of AChE inhibition of oxons are much higher than those of the parent OPs (Tahara et al., 2005). The mutagenic activities of OPs and their oxons were also investigated to evaluate those of OPs in drinking water (Onodera et al., 1995b). For butamifos, those of other chlorination by-products (e.g., 5M2NP and its chlorinated derivatives) were not reported. Thus, evaluation of the mutagenic activities of 5M2NP and its chlorinated derivatives, including their isomers, was considered important because the toxicities of nitro aromatic compounds are of concern (Watanabe et al., 1989, 1990; Heng et al., 1996).

In this study, we investigated the mutagenic activities of 5M2NP, a chlorination by-product of butamifos, its structural isomer, and their related compounds. 2-Methyl-5-nitrophenol was used as a structural isomer of 5M2NP. This is because as long as our knowledge, both MNPs and their chlorinated derivatives that are commercially available are only 2M5NP and its chlorinated derivative (i.e., 6-chloro-2-methyl-5-nitrophenol (6C2M5NP)) among the structural isomers of 5M2NP. The effects of chlorination on the mutagenic activities of MNPs were also investigated.

2. Methods

2.1. Reagents and solutions

The ultrapure water used was obtained with a Gradient A10 water purification system (Millipore, Bedford, MA). 5M2NP (98+%) was purchased from Wako Pure Chemical (Osaka, Japan). 4-Chloro-5-methyl-2-nitrophenol (4C5M2NP; 99%) was purchased from Acros Organics (Geel, Belgium). 2-Methyl-5-nitrophenol (2M5NP; >98.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). 6C2M5NP was purchased from Apin Chemicals (97.9%; Oxon, UK). 5-Amino-6-chloro-2-methylphenol (5A6C2MP; 99%) was purchased from AK Scientific (Mountain View, CA). 2-Amino-4-chloro-5-methylphenol (2A4C5MP; 99.3%) was custom ordered from Tokyo Chemical Industry. Except for 6C2M5NP, the purity data of reagents were obtained from the suppliers (i.e., catalogs and labels of the reagent bottle). In case of 6C2M5NP, its purity data was obtained from the analytical results by Tokyo Chemical Industry because we could not obtain the purity data from the supplier. These chemicals were used for the experiments without further purification. The structures of the compounds investigated are shown in Fig. 1. To understand the relationships between the compounds, 5M2NP and its related compounds were designated as group I, and 2M5NP and its related compounds were designated as group II. Here, symbols (I) and (II) are attached to the compound names (or abbreviations) in groups I and II, respectively. Chlorine solution was prepared by bubbling chlorine gas produced from commercially available sodium hypochlorite solution (Wako Pure Chemical) into sodium hydroxide solution.

2.2. Ames assay

Mutagenic activities of the target compounds and the extracts of 2M5NP (II) in chlorination were evaluated by the Ames assay. The Ames assay was performed in the presence or absence of rat liver metabolic activation system (S9 mix). The assay was performed using the *Salmonella typhimurium* strains TA98 and TA100 and the overproducer strains YG1026 and YG1029. TA98 and TA100 strains were kindly provided by Ames (Department of Biochemistry, University of California, Berkeley, CA). YG 1026 and YG1029 strains were kindly provided by Nohmi (National Institute of Health Sciences, Tokyo, Japan). Both YG1026 and YG1029 were developed from TA100 and are sensitive to the mutagenic activities of typical nitroarenes, and nitroarenes and aromatic amines, respectively (Watanabe et al., 1989, 1990). That is, the overproducer strains from TA100 strain were only used. This is because positive results were obtained for 4C5M2NP in the presence of S9 mix using TA100 strain when the Ames assay was performed for 5M2NP and 4C5M2NP using TA98 and TA100 strains, as preliminary experiments. In the presence of S9 mix, preincubation was conducted at 37 °C for 20 min. Mutagenic activity was calculated from the slope of the linear potion of the dose–response curve using the statistical model of least squares linear regression (Bernstein et al., 1982; Endo et al., 2003). The slope was taken as the revertant ratio.

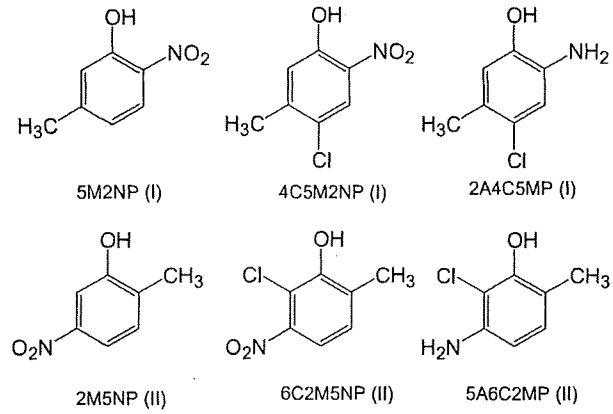

Fig. 1. Structures of MNPs and their related compounds. (I) group I; (II) group II.

2.3. Metabolite identification

To identify the metabolites of 4C5M2NP (I) by S9 mix, 4C5M2NP (I) in dimethylsulfoxide (DMSO) solution was incubated in the presence of S9 mix at 37 °C for 20 min. After incubation, the mixture was centrifuged (14 000g, 990 min) for ultrafiltration with Microcon® centrifugal filter devices (Ultracel YM-3 (3000 D); Millipore). The components in the permeate were identified as the metabolites of 4C5M2NP using high-performance liquid chromatography (HPLC) with a diode array detector (DAD) and liquid chromatography coupled with mass spectrometry (LC/MS).

2.4. Chlorination

Several brown glass bottles (1 L) were prepared containing 25 mg L^{-1} 2M5NP (II) at pH 7.0 (5 mM phosphate buffer). Chlorination (120 mg L^{-1}) was performed at 20 °C by adding a small amount of chlorine solution with stirring. At selected time intervals, the reaction was terminated by adding sodium thiosulfate ($Na_2S_2O_3$; Wako Pure Chemical) solution to each of the bottles. After chlorination, 150 g sodium chloride (Wako Pure Chemical) was added to 0.9 L from the 1-L sample and liquid–liquid extraction was conducted using 20 mL of *n*-hexane (Wako Pure Chemical)/diethyl ether (Wako Pure Chemical) solution. The extraction was conducted five times. The extracts were combined and evaporated using a rotary evaporator. The residue was dissolved into 1 mL of methanol (Wako Pure Chemical). Aliquots of 100 μL of the 1 mL of methanol solution were used for determination of 2M5NP (II) and its chlorination by-products. The remaining 900 μL of methanol solution was dried and redissolved in DMSO (Wako Pure Chemical), which was used for the Ames assay.

2M5NP (II) was also chlorinated with a lower concentration of chlorine at pH 7.2 (5 mM phosphate buffer) at 20 °C to determine the fates of MNPs and their chlorination by-products in the actual chlorine disinfection process. The sample volume used in the experiment was 300 mL. 2M5NP (II) concentration was 88 μg L^{-1} (0.58 μM) and chlorine concentration was 0.95 mg L^{-1}. At selected time intervals (up to 24 h), sampling was performed in vials containing quenching agent, $Na_2S_2O_3$ solution. Chlorine concentration at 24 h was 0.59 mg L^{-1}.

2.5. Analysis

MNPs and related compounds were identified or determined by LC/MS, ultra-performance liquid chromatography coupled with mass spectrometry (UPLC/MS), or HPLC with the DAD. In case of LC/MS and HPLC with the DAD, separation was performed using a HP1100 (Agilent Technologies, Palo Alto, CA) with a ZORBAX Eclipse XDB-C18 (2.1 × 150 mm, 3.5 μm, double endcapped; Agilent Technologies) column. Mixture of 0.1% acetic acid aqueous solution and acetonitrile (ACN) was used for mobile phase. For LC/MS, detection was performed using a HP1100 MSD (Agilent Technologies) operated in atmospheric pressure chemical ionization (APCI) negative-ion mode. The analytical conditions are shown in Table S1. In case of UPLC/MS, separation was performed using an ACQUITY UPLC system (Waters, Milford, MA) with a BEH C18 (2.1 × 150 mm, 1.7 μm, endcapped; Waters) column. Mixture of 0.1% formic acid aqueous solution and ACN was used for mobile phase. Detection was performed using an ACQUITY TQD (Waters) operated in electrospray/chemical ionization (ESCI) negative-ion mode. The analytical conditions are shown in Table S2. Standards of chloro-2-methyl-5-nitrophenol (C2M5NP) (II), a structural isomer of 6C2M5NP (II), and dichloro-2-methyl-5-nitrophenol (DC2M5NP) (II) were not obtained, so they were determined using 6C2M5NP (II) as a standard. That is, C2M5NP (II) was determined by LC/MS or UPLC/MS, and DC2M5NP (II) was determined by HPLC with the DAD. Thus, it was considered that the concentrations of these two compounds were estimated values. Chlorine was determined spectrophotometrically using *N,N*-diethyl-*p*-phenylenediamine (Wako Pure Chemical) (Japan Water Works Association, 2001).

3. Results and discussion

3.1. Mutagenic activities of target compounds

Table 1 shows the results of the mutagenic activities of MNPs and their related compounds by the Ames assay. Their mutagenic activities at each dose are also shown in Tables S3–S8. Dose–response curves of the assays are shown in Figs. S1 and S2. The mutagenic activities of 5M2NP (I) were negative for all four strains in the absence and presence of S9 mix. However, 4C5M2NP (I), one of the chlorination by-products of 5M2NP (I) (Kamoshita et al., 2007), was positive in TA100, YG1026, and YG1029 strains in the absence and/or presence of S9 mix. In addition, the highest revertant ratio of 4C5M2NP (I) (5100 revertants mg^{-1}) was observed by YG1029 strain in the presence of S9 mix although that by this strain in the absence of S9 mix was negative. It was reported that the mutagenic activities of butamifos and its oxon were negative on evaluation using strains TA98 and TA100 in the absence and presence of S9 mix (Onodera et al., 1995a,b). They were also negative for mutagenic activities in strains YG1026 and YG1029 in the absence and presence of S9 mix in the present study (data not shown). Therefore, it was considered that the reaction pathway through 5M2NP (I) would produce mutagenic compounds in chlorination of butamifos. We previously reported that from the mass balance, the initial chlorination by-products of butamifos were butamifos oxon and 5M2NP (I) (Kamoshita et al., 2007). However, it was supposed that other minor chlorination by-products of butamifos might be produced, and that the chlorination by-products might also induce the mutagenicity.

Next, identification of the metabolites of 4C5M2NP (I) by S9 mix was performed because some of them were considered to be mutagenic, particularly in YG1029. Fig. 2 shows the DAD chromatogram of 4C5M2NP (I) in DMSO solution after incubation in the presence of S9 mix. For comparison, Fig. S3 shows the DAD chromatogram of DMSO solution. Several peaks (i.e., peaks A – D) were observed after incubation of 4C5M2NP (I). These peaks were fractionated and the fractions were analyzed by LC/MS (scan mode) (Fig. S4). By comparison of retention times and mass spectra of LC/MS between the peaks and standard reagents, peaks A and D were identified as 2A4C5MP (I) and 4C5M2NP (I), respectively. From the mass spectrum of LC/MS, m/z 202 and 204 were observed for peak C. The molecular weight (MW) of 4-chlorohydroxy-5-methyl-2-nitrophenol (4CH5M2NP) (I) is 203.5. Thus, peak C may be 4CH5M2NP (I). However, in the case of the fraction of peak B, the mass spectrum of LC/MS was not obtained.

The proposed metabolic pathway of 4C5M2NP (I) is shown in Fig. S5. After incubation, 4C5M2NP (I) was decomposed by 10% and the molar conversion yield of 2A4C5MP (I) to was 10%. From the results, it was considered that 2A4C5MP (I) was the main metabolite of 4C5M2NP (I) by S9 mix, i.e., the main metabolic pathway was reduction of the nitro function to an amino function. Weak mutagenic activities of 2A4C5MP (I) were observed with strains TA100, YG1026, and YG1029 in the presence and/or absence of S9 mix (Table 1). YG1029 was sensitive to the mutagenic activity of aromatic amines, but the activity of 2A4C5MP (I) was low. The mutagenic activities of 4C5M2NP (I) in the presence of S9 mix were higher than those of 2A4C5MP (I) in the absence of S9 mix, particularly for YG1029. Therefore, 2A4C5MP (I), a major metabolite, was considered to contribute less to the mutagenic activity of 4C5M2NP (I) in the presence of S9 mix and some other minor metabolites were considered to have high mutagenic activities.

Table 1
Mutagenic activities of MNPs and their related compounds.

Target compound	Mutagenic activity (revertants mg^{-1})							
	TA98		TA100		YG1026		YG1029	
	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
5M2NP (I)	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
4C5M2NP (I)	neg.	neg.	neg.	700	1300	1200	neg.	5100
2A4C5MP (I)	neg.	neg.	340	320	530	390	280	270
2M5NP (II)	neg.	neg.	350	320	4000	4200	2600	3400
6C2M5NP (II)	(470)	(490)	7900	9400	31 000	49 000	70 000	110 000
5A6C2MP (II)	neg.	neg.	neg.	neg.	neg.	neg.	neg.	700

(I) group I; (II) group II; neg., negative; values in parentheses are tentatively positive.

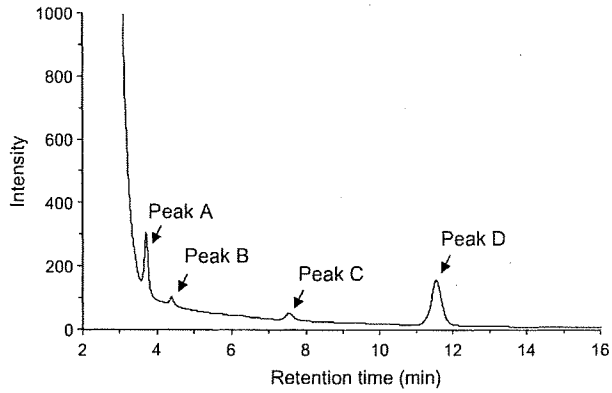

Fig. 2. DAD chromatogram of 4C5M2NP (I) in DMSO solution after incubation in the presence of S9 mix. (I) group I.

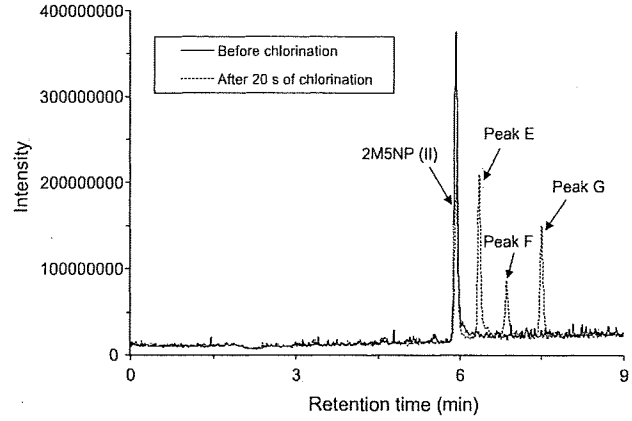

Fig. 3. Total ion monitoring chromatograms of 2M5NP (II) by UPLC/MS before chlorination and after 20 s of chlorination. Experimental conditions: 2M5NP (II) concentration, 25 mg L^{-1}; chlorine concentration, 120 mg L^{-1}; pH, 7.0 (5 mM phosphate buffer), temperature, 20 °C. (II) group II.

In the case of 2M5NP (II) and its related compounds, several points of their mutagenic activities were similar to those of 5M2NP (I) and its related compounds. That is, the mutagenic activities of 2M5NP (II) and its chlorinated derivative 6C2M5NP (II) were negative in TA98 and positive in TA100, YG1026, and YG1029, and YG1026 and YG1029 were more sensitive although 6C2M5NP (II) was only tentatively positive for mutagenic activity in TA98. In addition, the mutagenic activities of 6C2M5NP (II) were much higher than those of 2M5NP (II). Moreover, when the nitro function of 6C2M5NP (II) was changed to an amino function in 5A6C2MP (II), the mutagenic activities were significantly decreased. These results suggested that the mutagenic activities of MNPs and their chlorinated derivatives involved base pair substitutions and their nitro functions were associated with these mutagenic activities. On the other hand, the intensities of the mutagenic activities of 2M5NP (II) and its related compounds were higher than those of 5M2NP (II) and its related compounds. Particularly, those of 6C2M5NP (II) in YG1029 in the absence and presence of S9 mix were very high (70 000 and 110 000 revertants mg^{-1}, respectively). Thus, it was shown that substituent position is a key factor in the mutagenic activities of MNPs and their related compounds.

3.2. Chlorination of 2M5NP (II)

The effects of chlorination on the mutagenic activity of MNP were evaluated. As the MNP, 2M5NP (II) with higher mutagenic activity was used. YG1029 was used as the test strain because it showed the highest sensitivity to the mutagenic effects of 2M5NP (II) and its chlorinated derivative 6C2M5NP (II). Fig. 3 shows the total ion monitoring chromatograms of UPLC/MS of the extracts of 2M5NP (II) before chlorination and after 20 s of chlorination at pH 7.0 (5 mM phosphate buffer) at 20 °C. The 2M5NP (II) concentration was 25 mg L^{-1}, and the chlorine concentration was 120 mg L^{-1}. The recovery of 2M5NP (II) in the sample before chlorination was 84%.

Several peaks (2M5NP, peaks E–G) were observed with 20 s of chlorination. Fig. S6 shows mass spectra of peaks E–G by LC/MS (scan mode). By comparison of retention times and mass spectra between peaks and a standard reagent, peak E was identified as 6C2M5NP (II). From the mass spectra, peaks G and H were considered to be C2M5NP (II), a structural isomer of 6C2M5NP (II), and DC2M5NP (II), respectively.

Fig. 4 shows the concentration profiles of 2M5NP (II) and its chlorination by-products in the extract. As described above (Section 2), the concentrations of C2M5NP (II) and DC2M5NP (II) were determined using 6C2M5NP (II) as a standard. Thus, the concentrations of C2M5NP (II) and DC2M5NP (II) were estimated values. From the mass balance, it was shown that 6C2M5NP (II) and C2M5NP (II) were major and minor chlorination by-products of 2M5NP (II), respectively, and they were further transformed into DC2M5NP (II). DC2M5NP (II) was not detected at 600 s on HPLC with a DAD. However, the peak of DC2M5NP (II) was observed by UPLC/MS. The peak areas of DC2M5NP (II) by HPLC with the DAD and UPLC/MS at 600 s were much lower than those at 120 s, indicating that DC2M5NP (II) was transformed into unidentified compounds. Fig. S7 shows the proposed reaction pathway of 2M5NP (II) in chlorination.

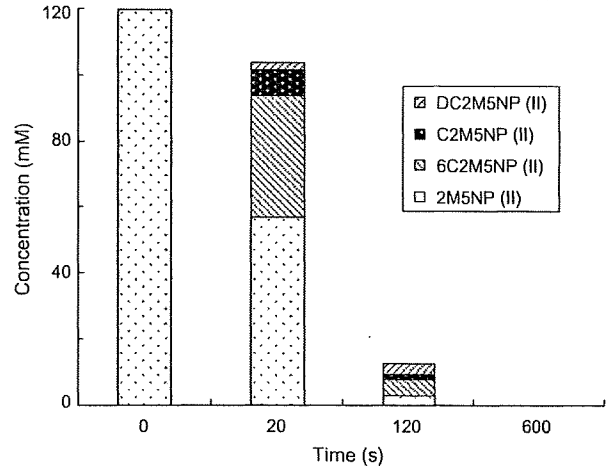

Fig. 4. Concentration profiles of 2M5NP (II) and its chlorination by-products in the extract in chlorination. Experimental conditions: 2M5NP (II) concentration, 25 mg L^{-1}; chlorine concentration, 120 mg L^{-1}; pH, 7.0 (5 mM phosphate buffer), temperature, 20 °C. (II) group II.

Fig. 5a and b show the effects of chlorine on the mutagenic activities of the concentrated extracts of 2M5NP (II) in DMSO in YG1029 in the absence and presence of S9 mix, respectively. The unit of horizontal axis in the figure is revertants μL^{-1} of the concentrated extracts of the sample in DMSO. The dose–response curves of the extracts of 2M5NP (II) in chlorination are also shown in Fig. S8. From the concentrations of 2M5NP (II) and 6C2M5NP (II) in the extract and their revertant ratios (Table 1), the contributions of 2M5NP (II) and 6C2M5NP (II) to the mutagenic activities in these

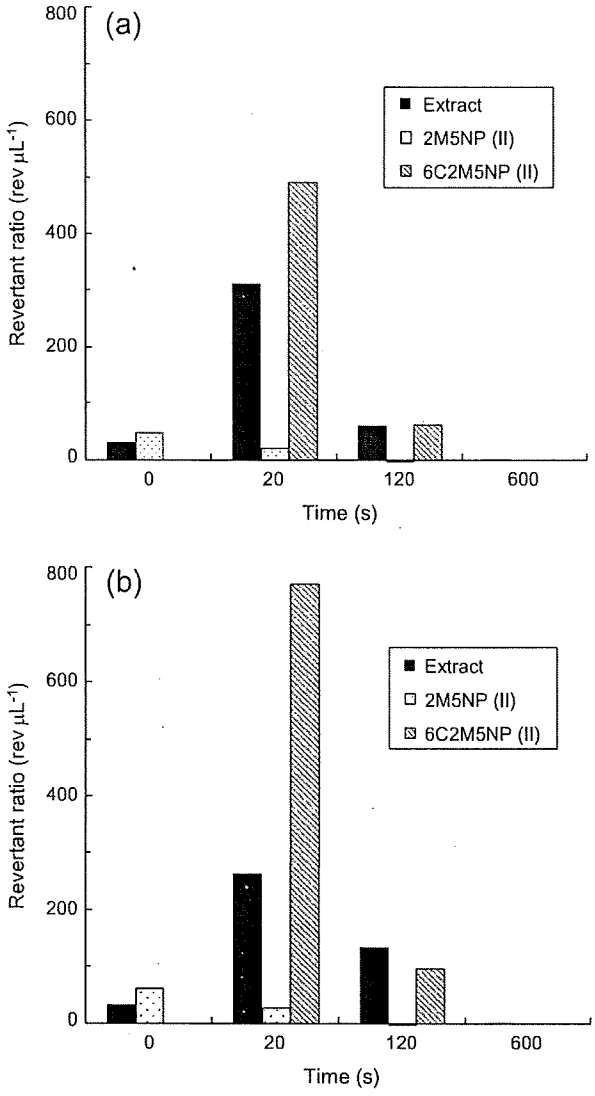

Fig. 5. Effects of chlorine on the mutagenic activities of the concentrated extracts of 2M5NP (II) in DMSO in YG1029 in the: (a) absence and (b) presence of S9 mix. Experimental conditions: 2M5NP (II) concentration, 25 mg L^{-1}; chlorine concentration, 120 mg L^{-1}; pH, 7.0 (5 mM phosphate buffer), temperature, 20 °C. (II) group II.

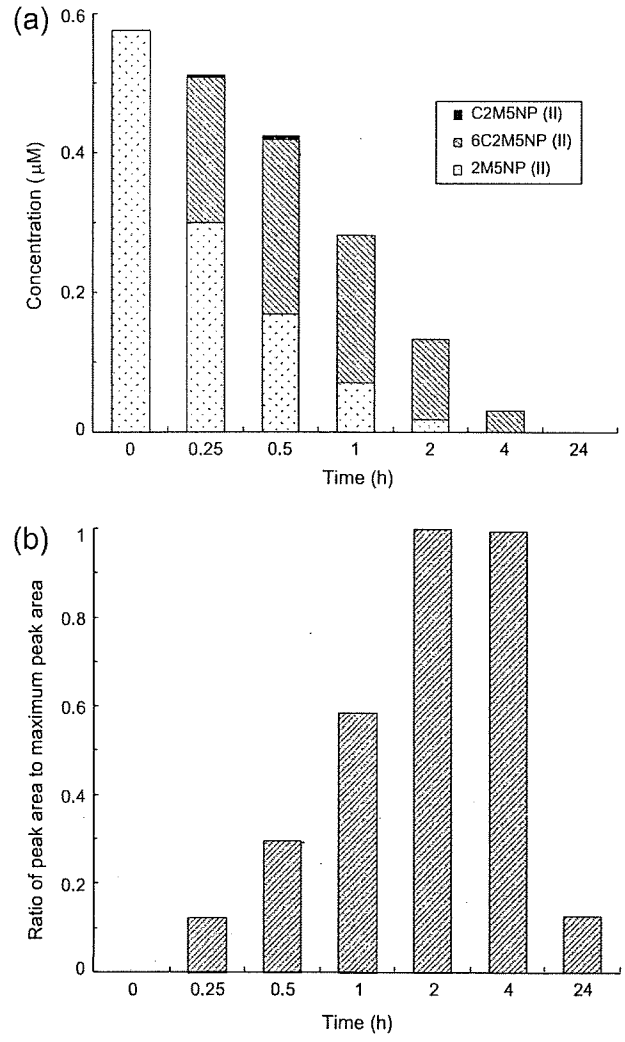

Fig. 6. Profiles of: (a) concentrations of 2M5NP (II), 6C2M5NP (II) and C2M5NP (II) and (b) ratio of peak area to maximum peak area of DC2M5NP (II) in chlorination. Experimental conditions: 2M5NP (II) concentration, 88 μg L^{-1} (0.58 μM); chlorine concentration, 0.95 mg L^{-1}; pH, 7.2 (5 mM phosphate buffer), temperature, 20 °C. (II) group II.

extracts were also calculated. Both in the absence and presence of S9 mix, the mutagenic activities of the extract were the highest at 20 s. Subsequently, they decreased and became lower than those before chlorination at 600 s. The profile of the calculated contributions of 6C2M5NP (II) to the mutagenic activities in the absence and presence of S9 mix indicated that 6C2M5NP (II) was the main chlorination by-product contributing to the mutagenic activities of 2M5NP (II) in chlorination, and the mutagenic activity of 6C2M5NP (II) was the highest among the chlorination by-products. The calculated contribution of the mutagenic activity of 6C2M5NP (II) in the presence of S9 mix at 20 s was much higher than the mutagenic activity of the extract. The reason for this was not clear in this study. It was also found that after decomposition of DC2M5NP (II), the mutagenic activity of the extract became lower than that of 2M5NP (II) before chlorination.

To understand the fates of MNPs and their chlorination by-products in the actual chlorine disinfection process, 2M5NP (II) was chlorinated at a lower chlorine concentration. Fig. 6 shows the profiles of 2M5NP (II) and its chlorination by-products at pH 7.2 (5 mM phosphate buffer) at 20 °C. Like the case of Fig. 4, C2M5NP (II) concentration was estimated value. The concentrations of 2M5NP (II) and chlorine were 88 μg L^{-1} (0.58 μM) and 0.95 mg L^{-1}, respectively. 2M5NP (II) was decomposed rapidly and disappeared within 4 h. The level of 6C2M5NP (II) produced from 2M5NP (II) was highest at 0.5 h (47 μg L^{-1}; 0.25 μM), and disappeared by 24 h. C2M5NP (II) was detected as the minor chlorination by-product, as in the case in Fig. 4. In addition, the presence of DC2M5NP (II) was confirmed from the peaks of m/z 220 and 222. The presence of DC2M5NP (II) was also observed after 24 h although its concentration was maximum at 2 h. When butamifos, a precursor of 5M2NP (I) in chlorination, was chlorinated at pH 7.2 at 22 °C (butamifos concentration, 1 mg L^{-1}; chlorine concentration 2.5 mg L^{-1}), similar results were obtained (Kamoshita et al., 2007). That is, at 24 h, 4C5MNP (I) and its structural isomer were not detected, and dichloro-2-methyl-5-nitrophenol (DC5M2NP) (I) was detected. These results suggested that MNPs were generally present in the form of dichloromethylnitrophenols and their further chlorination by-products in drinking water.

4. Conclusions

(1) The mutagenic activities of 5M2NP (I) and 2M5NP (II) were negative or not particularly high. However, those of their chlorinated derivatives were increased in TA100, YG1026, and YG1029, particularly for YG1029. The highest levels of mutagenic activity were observed for 6C2M5NP (II), a chlorinated derivative of 2M5NP (I), in YG1029, i.e., 70 000 and 110 000 revertants mg^{-1} in the absence and presence of S9 mix, respectively.
(2) When the nitro functions of 6C2M5NP (II) and 4C5M2NP (I) were reduced to amino functions, their mutagenic activities were markedly decreased.
(3) The mutagenic activities of the extract of 2M5NP (II) in chlorination were increased early in the reaction time and then decreased. 6C2M5NP (II) was the main chlorination by-product contributing to the mutagenic activities of the extracts of 2M5NP (II) in chlorination.
(4) When 2M5NP (II) was chlorinated at 0.95 mg L^{-1} at pH 7.2, 6C2M2P (II) produced was further transferred within 24 h. At 24 h, DC2M5NP (II) was still present.

Acknowledgment

This study was financially supported in part by a research grant from the Ministry of Health, Labour, and Welfare, Japan.

Appendix A. Supplementary material

Analytical methods of MNPs and their related compounds including Tables S1 and S2; mutagenic activities of MNPs and their related compounds (Tables S3–S8); dose–response curve of the Ames assay of MNPs and their related compounds (Figs. S1 and S2); DAD chromatogram of DMSO solution (Fig. S3); mass spectra of peaks A, C, and D by LC/MS (Fig. S4); proposed metabolic pathway of 4C5M2NP (I) (Fig. S5); mass spectra of peaks E–G by UPLC/MS (Fig. S6); proposed reaction pathway of 2M5NP (II) in chlorination (Fig. S7); dose–response curve of the extracts of 2M5NP (II) before and after chlorination (Fig. S8). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2009.10.002.

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Expansion of organic reference materials for the analysis of hazardous substances in food and the environment

—Realization of an efficient metrological traceability using the quantitative NMR method —

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[Translation from *Synthesiology*, Vol.2, No.1, p.12-22 (2009)]

Reference materials are indispensable for accurate analysis of hazardous substances in food and the environment. For organic substances, however, the dissemination of reference materials is hopelessly unable to catch up with today's rapidly proliferating analytical needs. To solve this problem, analytical techniques were improved to develop a method in which a single primary reference material could provide accurate quantitative measurements for a wide variety of organic compounds. In pursuit of this goal, we turned our attention to the ^{1}H NMR method. We improved upon the method to allow precise comparisons of signal quantities from protons with different chemical shifts, enabling calibration at an acceptable level of uncertainty for a variety of organic reference materials using a primary reference material for protons. This result opens the prospect of highly efficient metrological traceability, reducing the required number of national reference materials to a minimal level.

Keywords : Chemical metrology, metrological traceability, reference material, nuclear magnetic resonance spectroscopy, primary method of measurement

1 Introduction

Our modern lives are surrounded by chemical compounds, and a wide range of laws and regulations controls these chemical compounds, to ensure safety and to prevent adverse impact on the environment and human health. In recent years, public concern for safety has increased in Japan, prompting an increase in the number of chemical compounds subject to regulation, limitations, and other regulatory controls. For example, in May 2006, the Food Sanitation Law was revised to introduce the "Positive List System[Term 1]" for agricultural chemical residue in foods. With the enforcement of stringent regulations, the number of control subjects expanded from approximately 250 to about 800 kinds of agricultural and other chemical compounds traded domestically and internationally. At the same time, several new Official Methods of Analysis[Term 2] were established to measure the regulated chemical compounds, and as result, the use of advanced analytical equipment capable of conducting multiple simultaneous measurements, such as gas chromatograph/mass spectrometer (GC/MS) and liquid chromatograph/mass spectrometer (LC/MS), increased in food and environmental analyses. In this situation, many laboratories that inspect and test chemical compounds are increasingly employing GC/MS and LC/MS to conduct analyses.

While these analytical equipment are capable of simultaneously measuring multiple components, it is necessary to calibrate the sensitivity of the analytical instrument for each analyte in the samples to ensure the reliability of analytical results. To perform this calibration, reference materials (RMs) that serve as "yardstick" are required for individual analytes. In this type of analysis, the accuracy of inspection and testing results are crucial, and the reliability of the "yardstick" is of paramount importance. The use of certified reference materials (CRMs)[Term 3, 1] or equivalent RMs is highly recommended in such cases, and therefore various testing and inspection laboratories are working swiftly to acquire the RMs necessary to handle the ever-increasing list of regulated materials.

2 Current problems with RMs

The characterization of RMs by metrologically appropriate procedures is achieved by using measurement methods that offer traceability[Term 4] to SI definitions (in this case, amount-of-substance). Normally, this is a task performed by the national metrology institute[Term 5] of a country, and the RMs produced are known as the national reference materials (primary RMs). Generally, national RMs offer the highest standards of accuracy, and are scrupulously prepared with labor, time, and expense. Normally, they are not transferred directly to the inspection and testing laboratories that perform the actual analysis, because this is not practical due to the quantities and costs involved. Instead, secondary RMs are calibrated based on the national RMs, and working RMs are in turn calibrated using the secondary RMs. In this way, a pyramid structure is constructed, with few higher-order RMs at the top and a larger population of lower-order RMs reproduced below. Order in this proliferation of RMs is enforced through traceability to the original set of accurate "yardstick" or the national RMs. In essence, this concept is similar to the traceability systems where scales are calibrated using a series of weights, and the current RM traceability

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Received original manuscript October 6,2008, Revisions received December 26,2008, Accepted December 26,2008

system is not unique to reference materials. However, it is different from the weight system in a number of ways.'

Figure 1 is a schematic diagram of the traceability system for the RMs used in the analysis of volatile organic compounds that are used to test the quality of river and tap water in Japan. The national RM is in the form of a single solution incorporating 23 volatile compounds. Traceability to SI is obtained using pure substances for each component that is valuated using the freezing point depression method. The secondary and working RMs also consist of a single solution incorporating 23 volatile organic compounds, but in this case, calibration from upper-order to lower-order standards is conducted separately for each component. Because the lower-order traceability system requires a one-to-one correspondence, the pyramid structure breaks down for these RMs. In other words, the national RM for a given component must be used to calibrate the secondary RM for the same component, and calibration of working RMs for the same component is performed using this secondary RM. Because this is one-to-one calibration of the same chemical compounds, commercially available analytical technologies such as gas chromatography can be used for calibration down to the working RM level while maintaining excellent reliability. This practical system of traceability is used throughout the world.

The drawback of this traceability system is that it requires a wide array of national RMs to match each chemical compound subject to be analyzed. Development of these national RMs is a major bottleneck in the traceability system because it requires enormous time, labor, and expense. The construction of a more efficient traceability system based on an entirely new concept is needed to address the rapidly proliferating demand for RMs prompted by increasingly tight regulation of chemical compounds through the positive list system discussed above.

3 Research target: Construction of an efficient traceability system

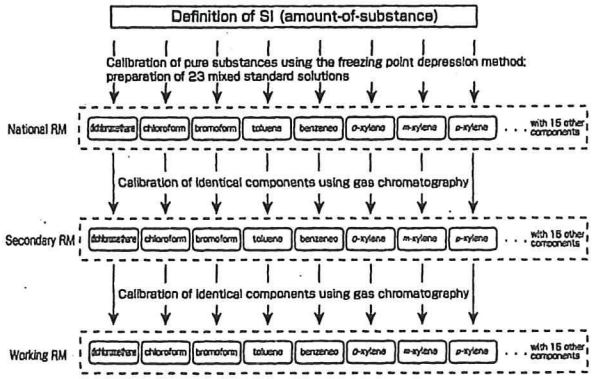

Fig. 1 Traceability system for RMs used in analysis of volatile organic compounds.

To recapitulate the previous chapter, the problem with present traceability system for RMs lies in the dependence on national RMs. Because the system consists of the series of "calibration chains" for the same chemical compound, the system cannot respond promptly to the need for RMs to analyze the growing number of chemical compounds. Although this problem can be solved if minimum types of higher-order RMs could be used to calibrate a wide range of working RMs, this is not possible with current calibration technology, which is designed to calibrate like chemical compound with like chemical compound. An entirely new calibration technology must be developed and introduced: a universal calibration technology capable of analyzing chemical compounds independently of their molecular structure.

The aim of this study is to develop a new calibration technology to realize a system that efficiently secures traceability to the wide range of working RMs without creating national RMs for each chemical compound. Given that the vast majority of chemical compounds subject to strengthened regulation in recent years are organic compounds, we developed a universal calibration technology targeting organic compounds.

4 An analytical method that achieves our objectives: Quantitative NMR

4.1 The required calibration technology

Absolute values for amount-of-substance can be obtained using SI-traceable measurements. This type of measurement is known as the primary method of measurement[Term 6, [2]]. Table 1 shows a list of analytical methods certified as primary methods of measurement, divided into primary direct methods and primary ratio methods. The primary direct methods, also called the absolute measurement methods, are defined as "the methods for measuring the value of an unknown without reference to a standard of the same quantity." Examples of primary direct methods are coulometry[Term 7], gravimetry[Term 8], and the freezing point depression method[Term 9]. Because these analytical methods yield absolute values for amount-of-substance, they are appropriate for valuing the national RMs. However, in general, they tend to be slow and their application are limited to short list of substances, and they are not suitable candidates as universal calibration technology that is the objective of this study. Primary ratio methods, on the other hand, are already in practical use. They are defined as "methods for measuring the value of a ratio of an unknown to a standard of the same quantity; its operation must be completely described by a measurement equation." They include titrimetry[Term 10] and isotope dilution mass spectrometry[Term 11]. Another analytical approach that qualifies as a primary ratio method, though not well established as an analytical technology, is the quantitative nuclear magnetic resonance (quantitative NMR).