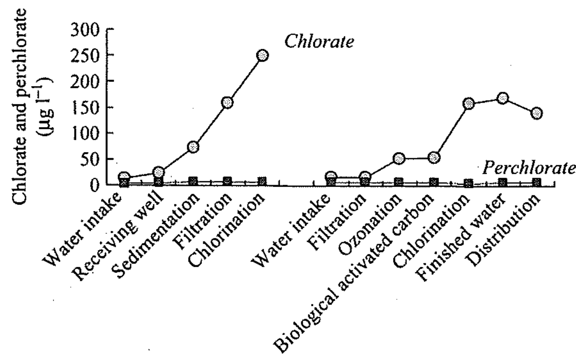


Figure 2 | Chlorate and perchlorate in water treatment plants using hypochlorite solutions (left-hand side, hypochlorite generated on-site; right-hand side, manufactured sodium hypochlorite solutions).

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 μg 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 μg 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.

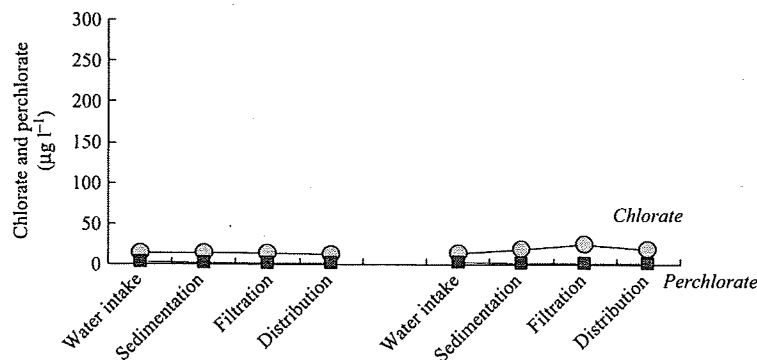


Figure 3 | Chlorate and perchlorate in water treatment plants using liquid chlorine.

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

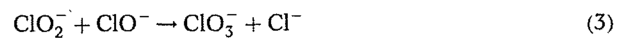
*The minimum is data detected above LO Q (limits of quantification).

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 $5{,}900\,mg\,l^{-1}$)

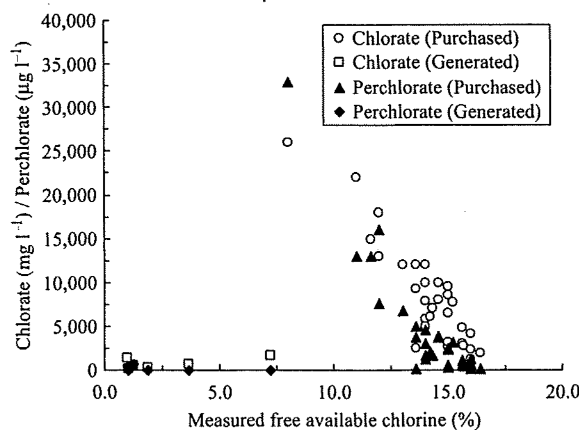


Figure 4 | Relationship between the measured FAC concentration and the concentrations of chlorate and perchlorate per measured FAC in the hypochlorite solution.

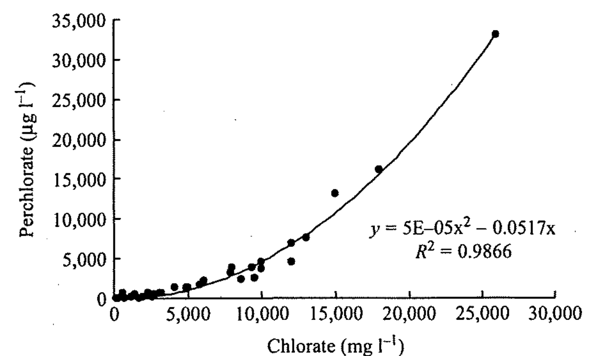


Figure 5 | Relationship between chlorate and perchlorate concentration in purchased hypochlorite solution.

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.

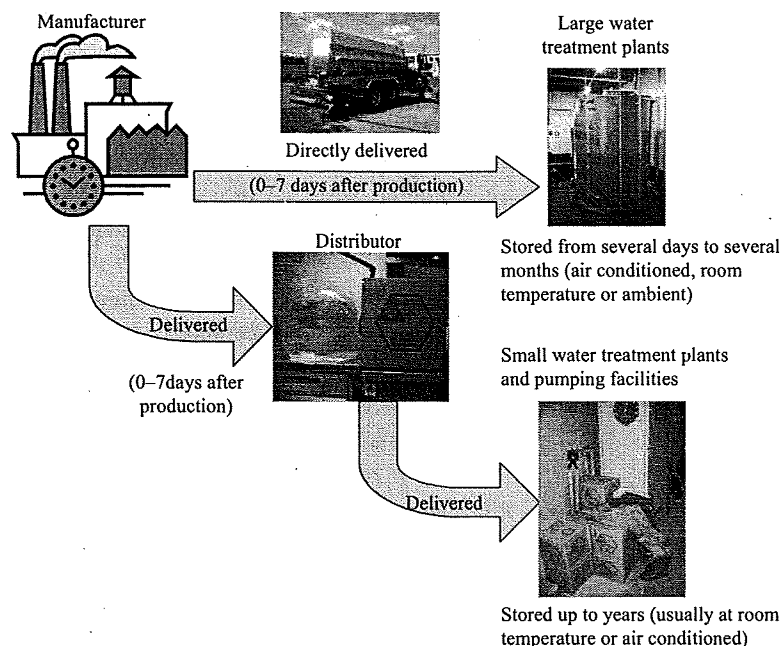


Figure 6 | Distribution channels for sodium hypochlorite.

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.

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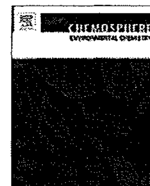

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Mutagenic activities of a chlorination by-product of butamifos, its structural isomer, and their related compounds

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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.

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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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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.

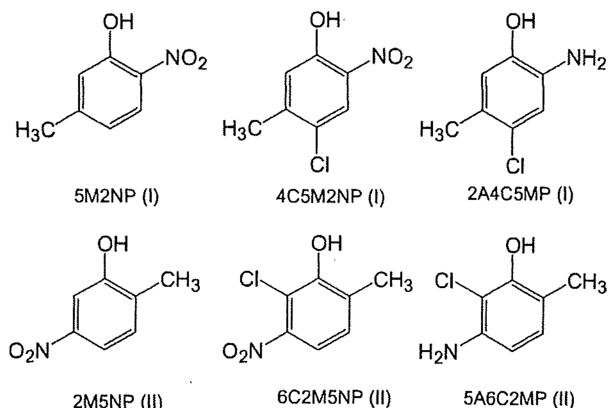

Fig. 1. Structures of MNPs and their related compounds. (I) group I; (II) group II.

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.

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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.

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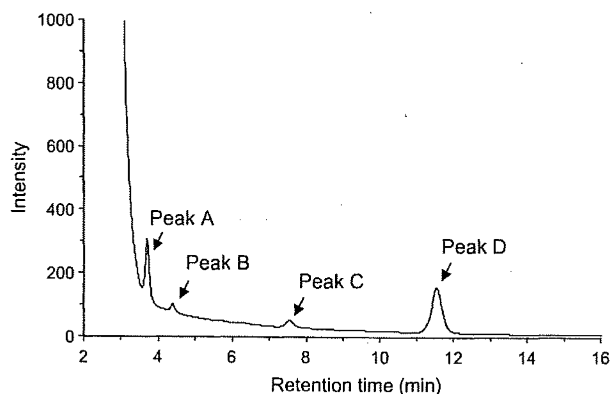

Fig. 2. DAD chromatogram of 4C5M2NP (I) in DMSO solution after incubation in the presence of S9 mix. (I) group I.

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.

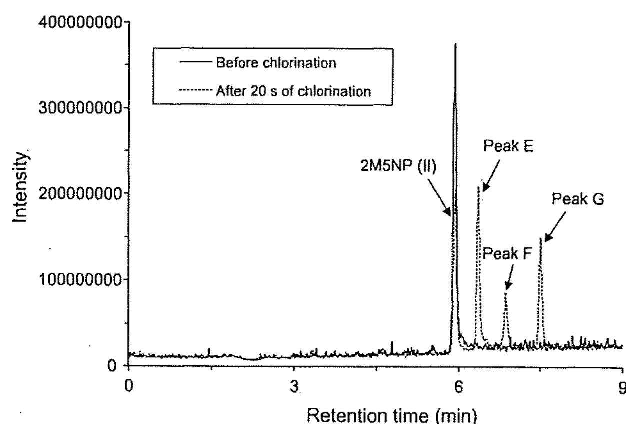

Fig. 3. Total ion monitoring chromatograms of 2M5NP (II) by UPLC/MS before chlorination and after 20 s of chlorination. Experimental conditions: 2M5NP (II) concentration, 25 mg L^{-1}; chlorine concentration, 120 mg L^{-1}; pH, 7.0 (5 mM phosphate buffer), temperature, 20 °C. (II) group II.

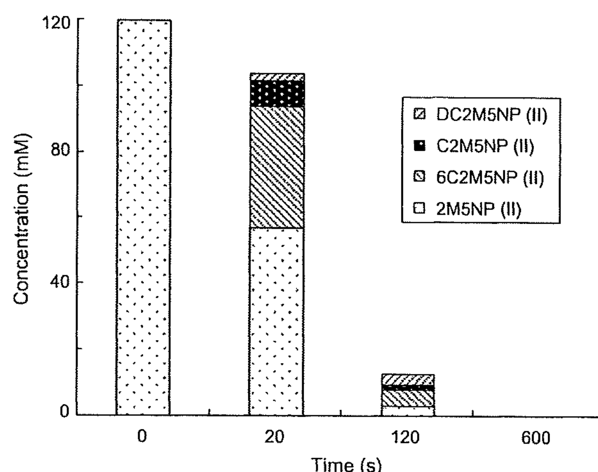

Fig. 4. Concentration profiles of 2M5NP (II) and its chlorination by-products in the extract in chlorination. Experimental conditions: 2M5NP (II) concentration, 25 mg L^{-1}; chlorine concentration, 120 mg L^{-1}; pH, 7.0 (5 mM phosphate buffer), temperature, 20 °C. (II) group II.

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

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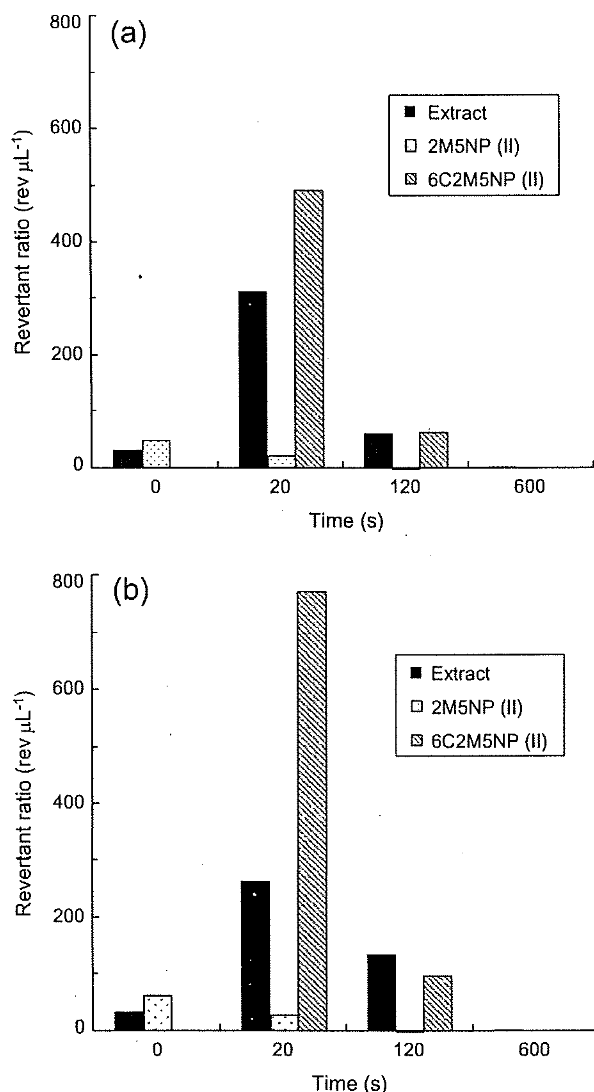

Fig. 5. Effects of chlorine on the mutagenic activities of the concentrated extracts of 2M5NP (II) in DMSO in YG1029 in the: (a) absence and (b) presence of S9 mix. Experimental conditions: 2M5NP (II) concentration, 25 mg L^{-1}; chlorine concentration, 120 mg L^{-1}; pH, 7.0 (5 mM phosphate buffer),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

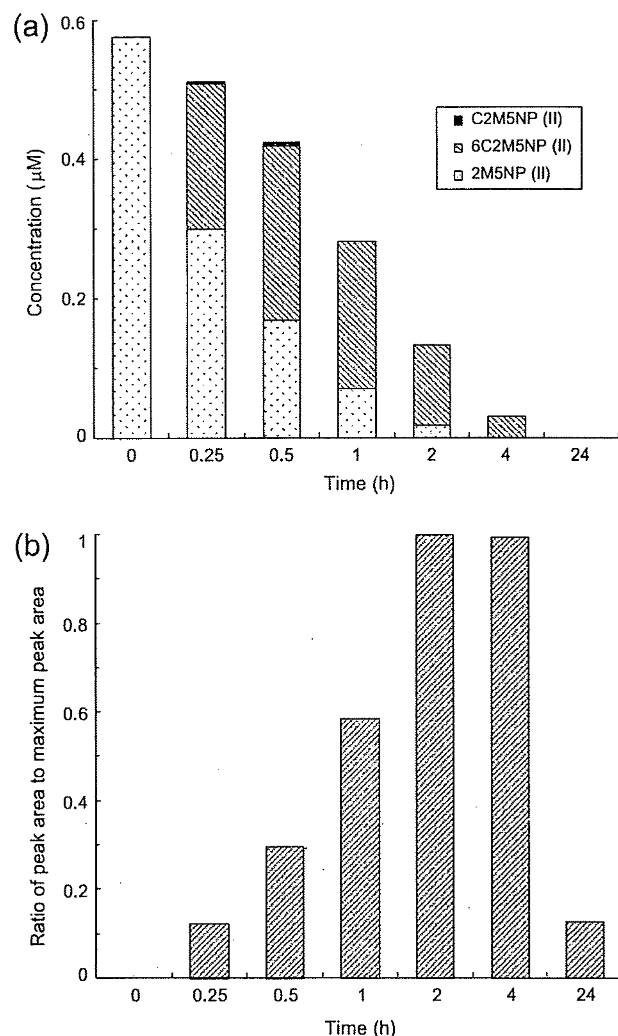

Fig. 6. Profiles of: (a) concentrations of 2M5NP (II), 6C2M5NP (II) and C2M5NP (II) and (b) ratio of peak area to maximum peak area of DC2M5NP (II) in chlorination. Experimental conditions: 2M5NP (II) concentration, 88 μg L^{-1} (0.58 μM); chlorine concentration, 0.95 mg L^{-1}; pH, 7.2 (5 mM phosphate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

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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.

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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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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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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

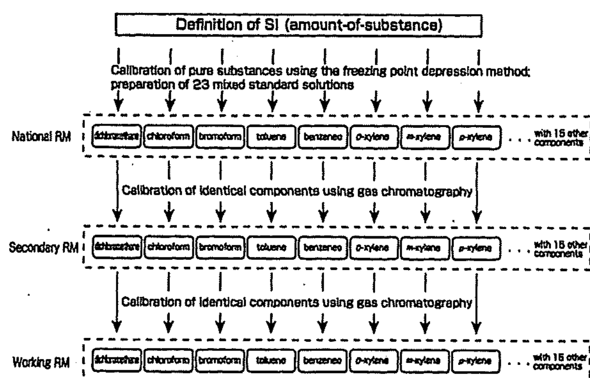

Fig. 1 Traceability system for RMs used in analysis of volatile organic compounds.

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).

A measurement method that can be applied to the calibration of a wide range of working RMs must satisfy the following conditions:
1) It must satisfy market demands regarding uncertainty, while also provide speed and simplicity of use.
2) It must be highly versatile and applicable to a wide variety of chemical compounds (general organic compounds for the purposes of this study).

Quantitative NMR is the most feasible candidate that can satisfy both conditions 1) and 2), although the answer is not yet fully established. Accordingly, in this study, we endeavored to establish quantitative NMR as a universal calibration technology for working RMs in organic compounds.

4.2 Principles of quantitative NMR

NMR is one of the main methods for determining the molecular structure of a chemical compound. It has an extensive track record in unraveling molecular structures, including the analysis of complex molecules such as proteins. Information obtained using NMR, such as chemical shift (the resonance peak position dependent on atomic bonding and the ambient environment) and spin-spin coupling (a split of the peak due to bonded nearby nuclei), provide hints about the chemical species and ambient environment of a molecule. In addition, the area ratio of various peaks, which resonates according to different chemical shifts, generally indicates the ratio of the number of atomic nuclei contributing to the peaks. As Figure 2 shows, the area ratio of ^{1}H NMR signals can easily be used to confirm the relative number of protons for the resonances, which is vital for the qualitative analysis of organic compounds.

Conventionally, this aspect of NMR was used exclusively to determine the chemical structure, solely by expressing the number of protons as a ratio in a molecule. However, the concept can be applied differently. If the molecular structure of an organic compound is already known and assignments of its ^{1}H NMR spectrum has been set, the number of protons contributing to each resonance peak is known, and this information can be applied to the quantitative analysis of chemical compounds. Thus, when the ^{1}H NMR measurement is performed by adding a reference chemical compound to a sample solution separately in an analyte (substance to be analyzed) solution, the spectra of the two chemical compounds overlay each other, as shown in Fig. 3. At this point, if the mass (weight), molecular weight, and purity of the added reference chemical compound (hereinafter, will be called the Primary Standard: PS) are known, the amount-of-substance (number of molecules) corresponding to peak I in Fig. 3 will also be known, and can be used as the criterion for finding the number of molecules in the analyte. To illustrate with a specific example, if the number of protons in PS (I) is the same as the number of protons in analyte (D) (the number is 6 for both), the ratio of the areas for peak I and peak D indicates the relative number of molecules. As such, the relationship can be expressed as follows:

(Peak area I)/(Number of molecules of PS) = (Peak area of D)/(Number of molecules in analyte)

Since the number of molecules in PS is already known, the

Table 1 Types of primary methods of measurement and their characteristics.

Analytical method	Primary direct method			Primary ratio method		
	Coulometry	Gravimetry	Freezing point depression method	Titrimetry	Isotope dilution mass spectrometry	Quantitative NMR
Outline of analytical method	Amount of electricity used in electrysis of specified substances is measured.	Settling quantity of specified substances in solution is measured.	Relationship between fraction melted and temperature around the melting point is measured.	The specified substance is measured using chemical reactions.	Mass spectrometry is performed using a stable isotope.	The ratio of areas of ^{1}H peaks with different chemical shifts is measured.
Main target substance	Metallic elements	Inorganic salts	High purity organic compounds	Acid, base, elements	Trace metals trace organic compounds	Organic compounds
Reference standard	Not required	Not required	Not required	Reference standards based on the principles of titration are required.	Required for each analyte	A reference standard for ^{1}H is required.
Uncertainty (less than 1 %)	○	○	○	○	○	△(Unknown value)
Rapid analysis	×	×	×	×	○	○
General applicability	×	×	×	×	×	○

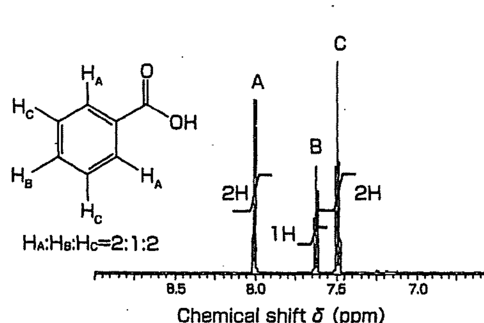

Fig. 2 Qualitative analysis of chemical compounds using ^{1}H NMR.

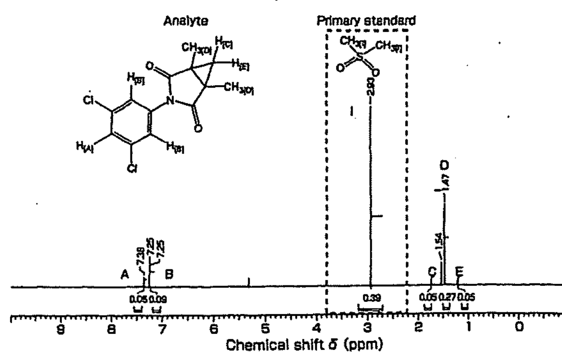

Fig. 3 Quantitative analysis of chemical compounds using ^{1}H NMR.

number of molecules in the analyte can be obtained. The mass (weight) and molecular weight of the target substance can then be used to determine the purity of the analyte[3]. Therefore, quantitative NMR is, in principle, a primary ratio method which can be used to obtain traceable measurement values for the number of protons — that is, amounts of substance in a sample.

In the example in Fig. 3, both the analyte and the PS are pure substances. After weighing the two substances individually, they are dissolved in a deuterated solvent, and quantitative NMR is used to measure the purity of the analyte using the mass ratio of the two substances. Working RMs, in contrast, are often supplied in the form of solution. If supplied at a certain concentration (about 0.1 %), quantitative NMR can be applied by dissolving the working RM in an appropriate deuterated solvent. The concentration of working RM can be found from the number of molecules obtained for the analyte, the mass of sample solution added, and the number of molecules in the analyte.

4.3 Feasibility of quantitative NMR

National metrology institutes in several countries (including AIST), which are members of the Consultative Committee for Amount of Substance (CCQM)[Term 12], have shown interest in the possibility of applying quantitative NMR as a primary ratio method, which was first suggested by Germany's Federal Institute for Material Research and Testing (BAM). In 2001, the Laboratory of the Government Chemist (LGC) in the United Kingdom and BAM served as pilot laboratories to conduct an international comparison[Term 13] for the quantitative analysis of ethanol in aqueous solution, with the participation by 10 institutes in key countries. On this occasion, measurements were conducted on the same sample using conventional analytical approaches such as gas chromatography (GC) as well as quantitative NMR[4]. The sample was precisely

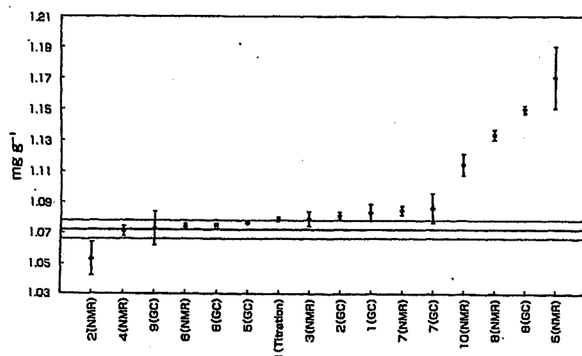

Fig. 4 Results of international comparison on quantitative analysis of ethanol in aqueous solution.
The solid line indicates the preparation value; the dotted line indicates uncertainty for the preparation value. No. 6 is the result for NMIJ/AIST. Participants: BAM (Germany), KRISS (Korea), LGC (UK), LNE (France), NIST (USA), NMi (Netherlands), NMIJ (Japan), NRC (Canada), NRCCRM (China), and VNIM(Russia).

produced by LGC, one of the pilot laboratories. The ethanol concentration was 1.072 mg/g ± 0.006 mg/g, but this value was not disclosed to the participants. Also, BAM separately supplied a deuterated water solution of PS (3-trimethylsilyl sodium propionate-d_4) of known concentration to the participating institutions that declared to conduct the quantitative NMR measurement.

The measurement results were reported individually to the pilot laboratory. Figure 4 is a summary of the results. Each data point represents a reported result. The adjacent error bar is the measurement uncertainty estimated by each participating institution (95 % confidence interval). The uncertainty of the quantitative NMR results from most institutions was in the range that could be described as percentage, and some of the results deviated significantly from the preparation values. In short, it was found that the quantitative NMR lacked accuracy compared to the conventional analytical methods such as GC. From the result of this international comparison, it was determined that the quantitative NMR did not offer sufficient technical accuracy. This view remains essentially unchallenged in the international scientific community today.

At the same time, Fig. 4 shows that the value reported by AIST closely matched the preparation value and its uncertainty was considerably smaller than the quantitative NMR findings of other participating institutions. This is why AIST takes a different stance on quantitative NMR. The uncertainty AIST reported to the pilot laboratory for quantitative NMR in the international comparison is illustrated in Fig. 5. Upon evaluating the relative standard uncertainties of each component, we found that the greatest factor was the uncertainty of the concentration of ^{1}H PS supplied by the pilot laboratory. Because the uncertainty of AIST's quantitative NMR measurement was much smaller, it became clear that a much smaller measurement uncertainty would have resulted if AIST had supplied its own more accurate PS.

It should be emphasized that the quantitative NMR offers a major advance in versatility. Whereas GC and other

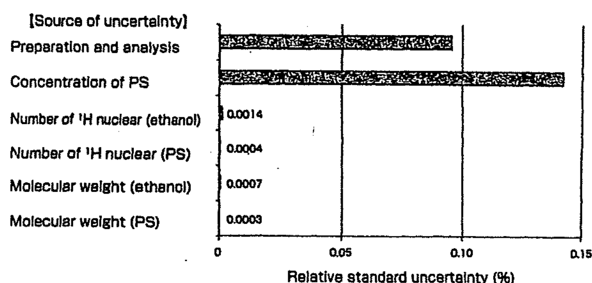

Fig. 5 Uncertainty for ^{1}H NMR in the international comparison on quantitative analysis of ethanol in aqueous solution.

conventional analytical calibration technologies applied in the international comparison can only be used to compare the concentrations of like chemical compounds (PS must be the same chemical compound as the measured substance), quantitative NMR can compare quantities of chemical compounds of different types (that is, PS does not have to be the same type of chemical compound as the measured substance). As such, although quantitative NMR requires at least one substance including ^{1}H, it can be used to measure any organic compound that includes proton, and a wide range of applications can be expected accordingly. The Authors believe that quantitative NMR can be applied in the calibration of working RMs by developing and integrating certain elemental technologies. These are discussed below.

5 Development and integration of elemental technologies to realize the quantitative NMR

5.1 Core elemental technologies

Figure 6 illustrates the elemental technologies developed by the authors, and the combination necessary to realize the potential of quantitative NMR as a universal calibration technology for working RMs. The features required of NMR differ greatly depending on whether the technology is optimized for qualitative analysis or for quantitative analysis, as in our case. With quantitative NMR, the highest priority is to observe the signal in accurate proportion to the number of atomic nuclei in the analysis, rather than improving measurement speed or improving the signal-to-noise ratio (S/N). We therefore revised the conditions for selecting the core elemental technologies.

The first elemental technology corrects a signal amplification issue. Generally speaking, NMR signals relax throughout its lifetime called the spin lattice relaxation time (T_1), which is the time taken for the atomic nuclei to settle from their excited state to their ground state. This period varies according to the environment of protons (such as bonding with other atoms). When NMR is performed for qualitative analysis, the sample is irradiated with microwave pulses with short cycle to increase the signal and to improve S/N. In such case, the delay time may be shorter than T_1, where excitation pulse is applied before all protons settled to their ground state. As result, differences in T_1 among the protons of analyte and PS make it impossible to obtain the peak area in correct proportion for the number of protons in each proton. We resolved this problem by measuring the relationship between repetition time and peak area. By taking delay time six times or greater than T_1 for the analyzed protons, it was demonstrated by experiment that 99.9 % or more of original signal intensity could be obtained, providing a stable peak-area ratio[5]. By ensuring that the delay time was sufficiently longer than the longest T_1 for all protons in the analyte, it was possible to obtain accurate peak-area ratio that was unaffected by the T_1 of the protons (though the measurement time increased several times longer than the conventional method).

The second elemental technology also concerns the S/N. Normally, S/N in the NMR signal is further improved by using an audio filter to narrow the bandwidth. However, this filter is not "flat" in sensitivity throughout the bandwidth, but exhibits severe loss of sensitivity at both ends of the filter bandwidth. Depending on the chemical shift, this loss of sensitivity can be in the range of several percents. Greater the chemical shift in the protons observed in the analyte and PS, more difficult it is to obtain an accurate peak-area ratio. To obtain flat sensitivity, we set the audio filter to cover 60 %~70 % of bandwidth and also widened the spectral width for data acquisition to 100 ppm, compared to less than 20 ppm in the conventional setting. This setting allowed the resulting spectrum to remain unaffected by sensitivity loss caused by filter for all chemical shifts. While such filter settings are not practical for ordinary NMR that involves handling of large volume data, we were able to solve several issues by taking an unconventional

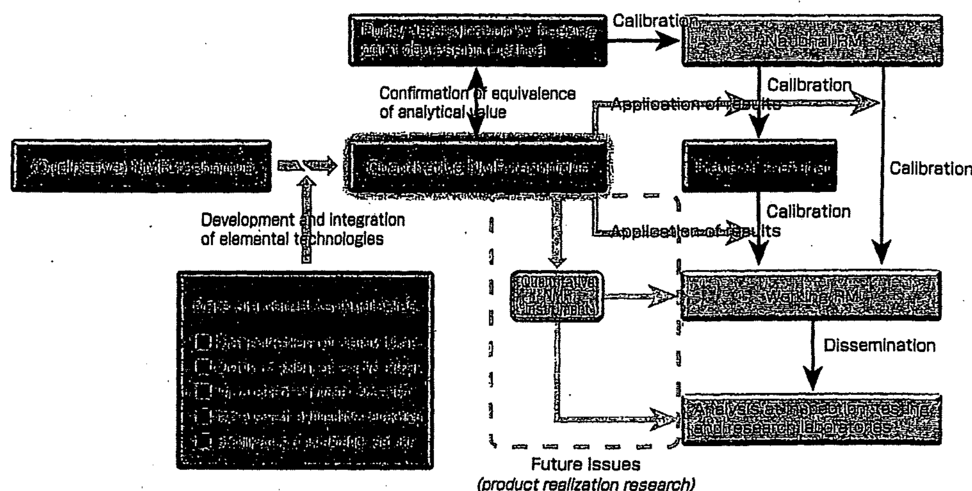

Fig. 6 Development of elemental technologies for the construction of universal calibration technology and the process of integration.

approach with priority on measurement accuracy[5].

In addition to the two elemental technologies described above, the Authors found that to improve the reproducibility of measurement results, phase correction, baseline correction, and peak area integration setting (range) were more important compared to other minor factors.

5.2 Use of transfer materials

Although quantitative NMR requires ^1H as the PS, the analyte does not have to be the same substance. The PS (limited to pure substances in this discussion) must satisfy the following conditions:

1) It must have as little impurities as possible, to keep the uncertainty for its purity value small.
2) It must dissolve easily in wide range of solvents, and must be stable in solution.
3) It must have low volatility (sublimability) and absorbency, so its mass (weight) can be measured easily.
4) Its chemical shift must not overlap with that of the target substance.

Although some national RMs satisfy these conditions for PS, many national RMs do not satisfy requirement 2), because a suitable solvent for dissolving both the PS and the analyte has not been found. Also, some national RMs do not satisfy 4), as the PS used depends on the analyte, and different PSs must be used with certain analytes.

The number of national RMs cannot be reduced if different PSs must be prepared according to various analytes. The Authors solved this problem using the calibration methods illustrated in Fig. 7, marshaling the advantages of quantitative NMR. We achieved this by selecting the transfer materials or chemical compounds whose chemical shifts do not overlap with either the PSs or the analytes. In Step 1, the PS (national RM) is used to calibrate the characteristic peak of the transfer material using quantitative NMR. In Step 2, the characteristic peak of the calibrated transfer material is adopted as the standard for calibration of the analyte. By adopting this two-step calibration method, the number of national RMs, which anchor the traceability system, can be minimized. Moreover, the transfer material does not need to be homogeneous or long-term stable like the RMs, so a wide range of materials is available for selection according to their match with a given analyte. The introduction of transfer materials to quantitative NMR was an important technological development in the process of synthesizing the elemental technologies.

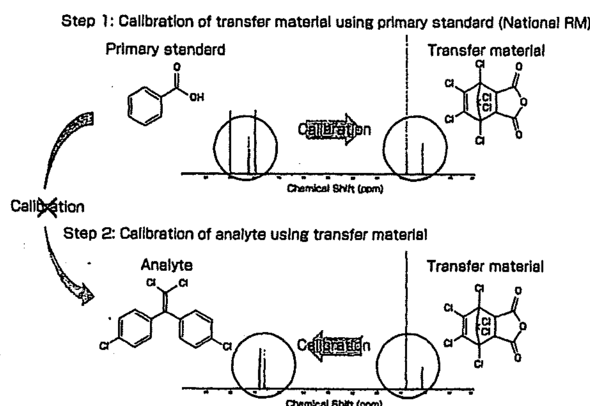

Fig. 7 Use of transfer material in quantitative NMR.

5.3 Evaluating the integrated technologies

In sections 5.1 and 5.2, we described how several elemental technologies were integrated to construct a calibration technology using quantitative NMR. Next, we demonstrated the reliability of the technologies by comparing them with long-established techniques. To do this, we first selected several target substances from commercially available, high-purity compounds. Their purity values were determined using the freezing point depression method, a well-established primary direct method that AIST has been using for the valuation of national RMs (see Table 1). Then we measured the same samples with the newly developed quantitative NMR to find the purity value, and checked whether the two values matched in the range of their respective uncertainties.

As the PS for measurements using quantitative NMR, we used benzoic acid (NIST SRM 350a, 99.9958 % ± 0.0027 %), a national RM distributed by the National Institute of Standards and Technology (NIST) of the United States. We performed the two-step calibration process described above using dimethyl sulfone or 1,4-bis-trimethylsilylbenzene-d_4 (1,4-BTMSB-d_4) as the transfer material, as the peak of the chemical shift for several substances overlaps the peak for benzoic acid. To dissolve the PS and the analyte, solvents were selected from a number of deuterated solvents, to minimize skewing of results from the protons of any impurities in the solvent. The solubility and other characteristics of the PS and analyte were also taken into consideration, and a solution with a concentration of about 1000 mg/L was prepared.

The analytical results are summarized in Table 2. Although in many cases the uncertainty was larger for the purity values by quantitative NMR compared to freezing point depression method, the values for the two methods matched within the uncertainty ranges, demonstrating that our calibration technology using quantitative NMR was sufficiently reliable[6]. The uncertainty for quantitative NMR was between 0.3 % and 1.2 % (k=2, 95 % confidence interval). Although this accuracy as a purity measurement technology is somewhat inferior to the freezing point depression method, quantitative NMR can be used to calibrate substances to which the freezing point depression method cannot be applied, including a wide range of organic compounds, and

it satisfies the market demand for the uncertainty levels in working RMs.

6 Issues for further study

We envision a transfer from the current one-to-one traceability system based on separate national RMs for each substance, to one-to-many traceability system in which several substances can be traced to just a few national RMs. So far, we made advancement for the development of universal calibration technology, a core technology applicable to numerous organic compounds. After establishing an ideal scenario for this project, we began by developing elemental technologies, using irradiation pulse delay time and optimization of audio filters. We then demonstrated that these calibration technologies could satisfy market requirements for uncertainty. We learned that the transfer materials could be used to minimize the number of national RMs required as standards for amount-of-substance. Finally, we plotted a roadmap toward an efficient traceability system, as illustrated in Fig. 8.

The system we outlined represents a quantum leap in the efficiency of traceability systems, since it removes the need to maintain one-to-one traceability chain from national RMs to working RMs for individual substances. It is an entirely new approach to RMs, unseen elsewhere in the world. The novelty of this technology, however, means that it is necessary to conduct numerous proving tests and to publish the results. The quantitative NMR technique must be standardized as an analytical method, and new international comparisons will be required at national metrology institutes

Table 2 Purity analysis results for organic compounds using quantitative NMR.

Terget substance	Freezing point depression method		Quantitative NMR				
	Reference value (%)	Uncertainty (%, k=2)	Analytical value (%)	Uncertainty (%, k=2)	Primary standard	Transfer material	Solvent
trans-Nonachlor	99.6	0.2	99.5	0.6	Benzoic acid	—	Acetone-d_6
cis-Nonachlor	99.8	0.2	99.9	0.5	Benzoic acid	—	Dichloromethane-d_2
Oxychlordane	99.9	0.1	99.3	0.5	Benzoic acid	—	Dichloromethane-d_2
Endrin	99.7	0.2	99.2	0.8	Benzoic acid	—	Dichloromethane-d_2
trans-Chlordane	99.8	0.3	99.5	0.6	Benzoic acid	—	Dichloromethane-d_2
cis-Chlordane	99.7	0.4	99.1	0.5	Benzoic acid	—	Dichloromethane-d_2
Trichlorfon (DEP)	99.7	0.3	99.6	0.5	Benzoic acid	—	Dichloromethane-d_2
Heptachlor	99.7	0.3	99.3	0.3	Benzoic acid	—	Dichloromethane-d_2
4,4'-DDT	99.6	0.3	99.9	1.2	Benzoic acid	Dimethyl sulfone	Acetonitrile-d_3
4,4'-DDE	99.7	0.3	99.8	0.7	Benzoic acid	Dimethyl sulfone	Acetonitrile-d_3
4,4'-DDD	99.8	0.2	99.9	0.6	Benzoic acid	Dimethyl sulfone	Acetonitrile-d_3
Procymidone	99.9	0.2	99.3	0.5	Benzoic acid	Dimethyl sulfone	Dichloromethane-d_2
Fenobucarb (BPMC)	99.8	0.2	99.8	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
Fenitrothion (MEP)	99.8	0.3	99.6	0.6	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
α-HCH	99.6	0.3	99.2	0.6	Benzoic acid	—	Dichloromethane-d_2
β-HCH	Inapplicable (thermal decomposition)		99.5	0.3	Benzoic acid	—	Dichloromethane-d_2
Atrazine	Inapplicable (thermal decomposition)		99.7	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
EPN	Inapplicable (Uncrystallized)		99.4	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
Diazinon	Inapplicable (Uncrystallized)		99.8	0.7	Benzoic acid	1,4-BTMSB-d_4	Dichloromethane-d_2
Malathion	Inapplicable (Uncrystallized)		99.5	0.7	Benzoic acid	—	Dichloromethane-d_2
Etofenprox	Inapplicable (Uncrystallized)		99.5	0.5	Benzoic acid	Dimethyl sulfone	Dichloromethane-d_2

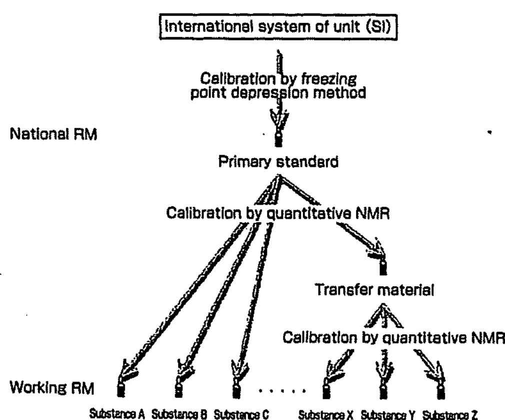

Fig. 8 Efficient traceability system with quantitative NMR.

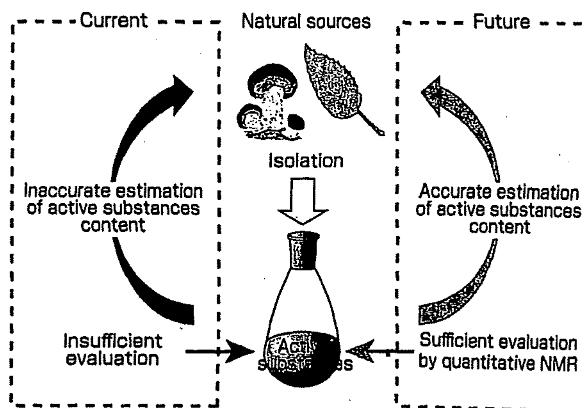

Fig. 9 Quantitative evaluation of active substances in natural sources using quantitative NMR.

around the world. More work must be done before one-to-many traceability can be firmly established.

At the same time, it is necessary to build the infrastructure that allows the industrial community to perform calibration of the wide range of working RMs that are in demand by the society. For this purpose, national RMs that are easy to use with quantitative NMR must be supplied along with sample applications. Automation tools are also necessary, covering all processes from measurement parameter sets using quantitative NMR to data analysis.

7 Future directions

Quantitative NMR has great potential marketability, as the necessary analytical equipment are commercialized (Fig. 6: Future issues). As reasonably priced, easy-to-use equipment, which are optimized for quantitative NMR, become available, and applications for nuclei other than ^1H are developed, they will find use not only in calibration technologies for working RMs, but also in quantitative analysis of several organic compounds occurring in numerous fields conducted at a wide variety of proving, testing, and research laboratories.

Many de facto commercial calibration standards are in use today, even though evaluation of their purity or concentration remains inadequate. For example, for active substances in natural sources, such as bioactive constituents and herbal medicines, quantitative analysis often depends on the samples of isolated constituents or the commercially available reagents. Quantitative NMR can offer highly reliable and effective quantitative analysis in such cases (see Fig. 9)[7], where the discovery of appropriate standard would normally be difficult.

Perhaps most exciting of all, an efficient traceability system based on this calibration technology for organic compounds may provide an effective scheme for responding flexibly to today's proliferating demand for RMs. Although core technologies other than quantitative NMR have not yet been demonstrated, universal calibration technologies that can be used similarly in the construction of a rational traceability system may be developed. The Authors hope that this paper will serve as a starting point for the development of such universal calibration technology.

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Finally, Dr. Shinichi Kinugasa (AIST) provided valuable assistance in writing this paper, for which we thank him sincerely.

Terminology

Term 1. Positive List System: Established in 2006 based on a revision of Japan's Food Sanitation Law, this system prohibits the sales of foods that contain agricultural chemical residues above a certain quantity. In cases where the safe (not harmful to human) quantity has been specified (called the residue level), the agricultural chemical must be below that quantity. In case where the safe quantity has not been specified, a uniform limit of 0.01 ppm is applied.

Term 2. Official Method of Analysis: A set of analytical procedures officially published and recognized in accordance with laws governing chemical compounds, to enable comparison of analytical results among different testing laboratories and samples. An official method of analysis must be robust and universally applicable. Examples used in Japan are Japanese Industrial Standard (JIS), Japanese Agricultural Standard (JAS), and Japanese Pharmacopoeia (JP).

Term 3. Certified reference material (CRM): In ISO Guide 35, which provides the international guidelines for RMs, this is defined as "reference material, characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability."

Term 4. Traceability: The characteristic of a measurement result, where the result can be linked to a known reference standard (usually a national standard) through an unbroken chain. In the 3rd version of

the *International Vocabulary of Metrology* (VIM), this term is amended to "metrological traceability" to distinguish from the term used to manage the shipping histories of foods and other goods.

Term 5. National metrology institute: A research institute that sets a country's official measurement standards. In Japan, it is the National Metrology Institute of Japan within the National Institute of Advanced Industrial Science and Technology.

Term 6. Primary method of measurement: The method used to define national RMs. It is defined as follows: "primary method of measurement is a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be expressed in terms of SI units."

Term 7. Coulometry: The method of measuring the amount-of-substance of an analyte from measurements of current and time when electrolysis is applied to a specific substance based on Faraday's Law. It is used in the analyses of inorganic ions of metallic elements as well as of trace amounts of moisture.

Term 8. Gravimetry: An analytical technique in which the quantity of an analyte in a sample is found by separating the analyte from the rest of sample using a reagent that reacts specifically to that component. The resulting mass is used to determine the quantity of the analyte. Generally, mass is found by precipitating the selected component out of the solution, but it can also be found by separating the selected component from the sample as gas, adsorbing the component using an adsorbent, and then calculating the mass from the amount adsorbed.

Term 9. Freezing point depression method: An analytical technique that finds the amount-of-substance fraction of impurities in a sample as a proportion of its amount-of-substance by measuring the temperature and enthalpy of impurities in a sample, as its freezing point decreases. It is generally used to determine the purity of high-purity organic compounds.

Term 10. Titrimetry: This is volumetric measurement in a limited sense. A solution that includes an RM that reacts with the sample is dropped into a sample solution, and the quantity of RM consumed before the equilibrium is reached is measured to find the quantity of the analyte in the solution. Depending on the chemical reaction used, the method includes neutralization (acid-base) titration, oxidation-reduction titration, complex formation titration, or precipitation titration.

Term 11. Isotope dilution mass spectrometry: A method of finding the quantity of an analyte in a sample using substance labeled with a stable isotope. The labeled substance is added to the sample, and the signal ratio of the mass spectrums of the analyte and the labeled substance are obtained. Because the chemical properties of the analyte and the labeled substance are roughly identical, the effect of the process of sample preparations with significant impurities can be cancelled (the signal ratio of the analyte and its labeled substance is maintained). In this technique, the concentration of the labeled substance for the RM must be known in advance.

Term 12. Consultative Committee for Amount of Substance (Comité Consultatif pour la Quantité de Matière: CCQM): One of the consultative committees formed under the aegis of the International Committee of Weights and Measures (Bureau International des Poids et Mésures: BIPM)) that consists of the Meter Convention member institutions. Established in 1993, this consultative committee discusses issues on metrology in chemistry.

Term 13. International comparison (CCQM inter-comparison): Comparison among calibration laboratories to confirm the degree of equivalence in the calibration and measurement capabilities and values assigned to RMs between various national metrology institutes. Normally, this process begins with an international comparison for research purposes, called a pilot study. After the technical groundwork has been established to a certain degree, an official international comparison, called a key comparison, is performed.

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