of the pregnant body containing fetuses. Further, altered physical condition during the gestation and lactation periods could make their toxic susceptibility higher. However, considering that no other dose-related changes were found in detailed clinical and functional observations, the toxicological significance of this forelimb grip weakness is unclear. Effects on grip strength were examined in 90-day repeated oral dose toxicity studies of PFBA, perfluorohexanoic acid (PFHxA) and perfluorobutane sulfonic acid (PFBS) in rats, but no dose-related changes were reported (Chengelis *et al.,* 2009; Lieder *et al.,* 2009a; van Otterdijk, 2007b).

Body weight gain of male and female rats was markedly inhibited at 1,000 mg/kg/day in the present study. Although decreases in body weight coincided with the reduction of food consumption during PFOdA administration, they were hardly recovered during the 14-day recovery period when food consumption was not different between the control and 1,000 mg/kg/day group. The effects on body weight are typically observed in rodents given PFAAs at relatively high doses (ATSDR, 2009). Interestingly, these effects are not associated with reduced food intake in many cases. For example, 7-day or 10-day dietary administration of PFOA at 0.02% caused more than 10% reduction of body weight, compared with the control (Xie *et al.,* 2003; Yang *et al.,* 2000). In these studies of PFOA, food intake was not reduced significantly. Yang *et al.* (2002) reported that body weight gain was not inhibited in PPARα-null mice given PFOA with the same dose regimen, suggesting that PPARα activation is involved in the body weight effects of PFAAs. In the present study, however, no clear effects indicating the alteration of lipid metabolism were found as mentioned above. The other mechanisms may contribute to the effects of PFOdA on body weights.

PFOdA affected various hematological parameters at 200 mg/kg/day and above. Anemic effects observed in males (i.e. reduced red blood cell count, hemoglobin level and hematocrit) were previously reported in repeated oral dose studies of PFBA, PFHxA, PFOA, PFBS and PFOS in rats (Chengelis *et al.,* 2009; Goldenthal *et al.,* 1978; Lieder *et al.,* 2009a; Sibinski, 1987 ; van Otterdijk, 2007b); however, the mechanism has yet to be revealed. In the present study, increased serum total bilirubin and hepatic hemosiderin deposition indicated a hemolytic effect of PFOdA. A decrease in reticulocyte ratio also suggests a decline in hematopoietic function, but histopathological examination of bone marrow did not reveal any abnormalities. At the end of the recovery period, the reticulocyte ratio was considerably increased, but reduced red blood cells, hemoglobin and hematocrit did not indicate recovery. In females, these anemic changes were not found, but the number of basophils was increased at 1,000 mg/kg/day. Its etiology and toxicological significance is unknown. Blood clotting parameters were also affected in females. Prolongation of APTT suggests the inhibition of an intrinsic and/or common pathway in blood coagulation. Shortening of PT was considered to be toxicologically insignificant, but showed that PFOdA does not inhibit the extrinsic pathway of coagulation. At the end of the recovery period, this effect on APTT was also observed in males. Elevation of basophils and prolongation of APTT were not reported in previous studies of other PFAAs (ATSDR, 2009). In order to gain a better understanding of the etiology of the hematological effects occurring in male and female rats given PFOdA, additional investigations are needed.

In the 1,000 mg/kg/day group, other dose-related changes were found in blood biochemical variables, organ weights and histopathological findings. In males, serum total protein was reduced, and this change seemed to result primarily from a decrease in the globulin α_1 fraction, which was also found in females given 200 and 1,000 mg/kg/day. Since most serum proteins, with the exception of γ-globulin, are synthesized, and also degraded in the liver (Ove *et al.,* 1972), observed effects on serum protein may be attributable to hepatic effects of PFOdA, although other etiologies could be responsible. An elevated serum level of BUN, observed in both sexes, increases the likelihood that the hepatic protein catabolism was increased because urinalysis parameters and the gross and microscopic appearance of the kidneys were not affected by PFOdA treatment. In the pancreas, zymogen granules were decreased in both sexes. This change might result from increased pancreatic secretion of digestive juice. Lipids are known to stimulate pancreatic secretion, and long chain fatty acids stimulate this more intensively than medium chain fatty acids (Ioannidis *et al.,* 2008), which might explain why such effects on the pancreas were not reported for the other PFAAs (ATSDR, 2009).

As for the reproductive/developmental effects, all pups were stillborn in one pregnant female given 1,000 mg/kg/day. Although the incidence was low, the possibility that this change was treatment-related could not be ruled out because of the very low incidence of such effect (all pups stillborn) in the laboratory's historical control data (approximately 0.5~1.0%). Another pregnant female given 1,000 mg/kg/day was found moribund during late pregnancy as described above, suggesting that PFOdA might have a certain effect on the maintenance of pregnancy. In the 1,000 mg/kg/day group, the number of corpora lutea was slightly decreased. Such effects on the

number of corpora lutea has not been reported so far for the other PFAAs (ATSDR, 2009; Luebker *et al.,* 2005a). Ovarian follicle development is known to be regulated by the hypothalamic-pituitary-ovarian axis; however, the present result that no effects were found on estrous cyclicity, on the copulation, fertility or delivery index, or on the weight and histopathology of male and female reproductive and endocrine organs denies the possibility that PFOdA affected the axis. Since the recent studies indicate that many other intra-ovarian signaling cascades affect follicular development (Richards and Pangas, 2010), PFOdA might act on such cascades.

PFOdA administration slightly decreased the delivery, live birth and viability index at 1,000 mg/kg/day, and the values were outside the normal range for this strain of rat in the laboratory that performed this study (historical control range for the last twelve years: 84.5-97.0%, 97.7-100.0% and 96.2-100%, respectively). Further, the birth weight of pups was decreased and postnatal body weight gain was inhibited at 1,000 mg/kg/day. Such effects on prenatal and postnatal development could be attributed to secondary effects due to maternal toxicity such as inhibition of body weight gain, but the lipophilic property of PFOdA also indicates the possibility that it was transferred via placenta and/or breast milk and affected the fetuses/pups directly. Previous studies demonstrated developmental effects of the other PFAAs, which were observed even at doses which produced no maternal toxicity (ATSDR, 2009; Butenhoff *et al.,* 2004; Case *et al.,* 2001; Das *et al.,* 2008; Harris and Birnbaum, 1989; Lau *et al.,* 2006, 2003; Luebker *et al.,* 2005a, 2005b; Thibodeaux *et al.,* 2003). Abbott *et al.* (2007) reported an increase in the incidence of full litter loss, reduction of neonatal survival and body weight gain and delayed eye opening in mice given PFOA during days 1-17 of gestation at 0.6 mg/kg/day and above. Interestingly, such developmental effects were not detected in PPARα knockout mice given the same dose. Investigating the PPARα agonistic activity of PFOdA might provide useful information to understand the mechanism of the developmental effects as well as hepatotoxicity.

In summary, oral gavage administration of PFOdA primarily affected the liver, causing centrilobular hepatocyte hypertrophy and necrosis. Other effects included inhibition of body weight gain, anemia, prolongation of APTT and decreased pancreatic zymogen granules. These toxic effects observed at the end of the 42- to 56-day administration period were also detected after the 14-day recovery period. PFOdA also showed reproductive/developmental toxicity: the number of corpora lutea and implantation, total number of pups born, the number of live pups and birth weight of pups were decreased, and the postnatal body weight gain was inhibited. Based on these findings, the NOAEL of PFOdA was considered to be 40 mg/kg/day for repeated dose toxicity and 200 mg/kg/day for reproductive/developmental toxicity.

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Chlorine Demands of Amino Acids and Amino Sugars in Water

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ABSTRACT

Nitrogenous compounds are ubiquitous in drinking water sources and they increase the chlorine demand of water, shift the breaking point, and also cause strong odors. The present study was performed to investigate the chlorine demands of selected amino acids and amino sugars in water. The results indicated that the chlorine demands of the precursors were in the order: aromatic amino acids (except histidine) > S-amino acids > non-S-amino acids and amino sugars. Aromatic amino acids were expected to have the highest chlorine demand due to chlorine substitution in both the aliphatic and aromatic parts of these molecules. However, the chlorine demand of histidine was threefold lower than those reported previously, which may be attributed to the experimental conditions or shorter contact time used in this study. The chlorine demands of most compounds did not show marked differences at contact times of 15 - 96 h, although some of the nitrogenous organic compounds showed an increasing trend in chlorine demand with time when our 24 h study was compared with previous studies conducted at 72 and 96 h. Chlorine demand in 24 h showed a good correlation with predicted data. Kinetic studies are required to understand how fast the precursors can react with chlorine in typical water treatment contact times and chlorine doses. The information presented here will be useful in controlling disinfection byproducts.

Keywords: amino acids, amino sugars, chlorine demand, QSPR model

INTRODUCTION

Microbial contamination of water is not simply a problem of the past, and the morbidity and mortality rates associated with waterborne diseases are highly related to poverty or the lack of access to sanitary and safe potable water supply systems (Galal-Gorchev, 1996; Hermer, 1999; Ballester and Sunyer, 2000). Epidemiological data have shown that cases, outbreaks, and mortality rates of cholera are higher in developing countries than in developed countries (Swerdlow *et al.*, 1997; WHO, 1998; Andersson and Bohan, 2001; Lee *et al.*, 2002; Hutin *et al.*, 2003; Griffith *et al.*, 2006). Thus, potable water must be routinely disinfected using chlorine (chlorine gas and hypochlorites) or alternative disinfectants to eliminate pathogens (USEPA, 1999). While developed countries can afford both chlorine and alternative disinfectants, such as ozone or chlorine dioxide, chlorine is the only reliable, affordable, and convenient disinfectant in many countries (Galal-Gorchev, 1996; Chaidou *et al.*, 1999; WQHC, 2002). The advantages of chlorine over alternative disinfectants are that it has a wide biocidal spectrum, does not require high levels of technical skill to use, and has low investment and operation costs (Galal-Gorchev, 1996; PNL, 1998; USEPA, 1999; ACC 2008).

While chlorine has played a major role in reducing waterborne diseases worldwide (Galal-Gorchev, 1996; Lee *et al.*, 2002), it has posed a new challenge to potable water

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supply authorities because chlorine has high oxidizing power (Larson and Weber, 1994). It reacts with traces of different classes of naturally occurring and anthropogenic organic compounds, which are collectively called dissolved organic matter, to form chlorinated organic compounds (Crittenden *et al.*, 2005). This is because water treatment processes do not completely remove components of dissolved organic matter (Ribas *et al.*, 1991; Volk *et al.*, 2002; Volk *et al.*, 2005), and dissolved organic matter is a complex mixture of various natural and anthropogenic organic molecules of unknown structures (Clesceri *et al.*, 1998; Peuravuori and Pihlaja, 2007).

Nitrogenous organic compounds are one of the classes of dissolved organic matter present in water, and the most abundant forms are amino acids, amino sugars, nucleic acids, and proteins derived from natural and anthropogenic sources (NRC, 1987). Although water treatment can remove most nitrogenous organic compounds, it may still leave trace amounts of organic compounds at the point of chlorination (Pietsch *et al.*, 2001; Dotson and Westerhoff, 2009). For example, the reaction of chlorine with amino acids produces trihalomethanes and haloacetic acids (Hureiki *et al.*, 1994; Bull *et al.*, 2006; Hong *et al.*, 2009) as well as odorous chloroaldimines (Brosillon *et al.*, 2009). Although all nitrogenous organic compounds have reduced nitrogen (amine, amide, imine) with a lone pair of electrons in their structure, they are not equally reactive toward chlorine. The reactivity of organic compounds with chlorine at a given pH may be influenced by a number of factors, such as the number and oxidation states of amine groups, number and relative positions of OH and NH_2 in aromatic rings, and number of aliphatic sulfur atoms (Hureiki *et al.*, 1994; Bull *et al.*, 2006; Arnold *et al.*, 2008; Hong *et al.*, 2009; Luilo and Cabaniss, 2010). Some studies have indicated that activated aromatic amino acids had higher chlorine demands than sulfur-containing amino acids (hereafter denoted as S-amino acids), followed by non-sulfur-containing amino acids (hereafter denoted as non-S-amino acids) after an incubation period of 72 - 96 h at pH 7 - 8 (Hureiki *et al.*, 1994; Hong *et al.*, 2009). However, the typical potable water treatment contact time is much less than 72 h. Thus, the chlorine demands reported over such a long contact time scale represent those that occur both at the treatment plant and in the distribution system due to the reaction of residual chlorine with traces of amino acids. Another study investigated the chlorine demands of five amino acids with a contact time of 15 h at pH 7, and their results were comparable to those determined in other studies performed over longer time scales (de Laat *et al.*, 1982; Hureiki *et al.*, 1994; Hong *et al.*, 2009). These observations suggest that these amino acids are mostly depleted between 15 and 72 h. There are gaps in chlorine demand data for all amino acids and amino sugars at contact times of 15, 24 and 48 h. There is also a lack of kinetic data important for estimating chlorine dose sufficient to sustain chlorine residuals in distribution systems. As it is still difficult to deduce these figures from the literature due to the lack of consistency between studies, our experiments obtained data under consistent conditions to allow comparison, especially with regard to contact time.

This study was performed to determine the chlorine consumptions of amino acids and amino sugars as representative nitrogenous organic compounds in water with a contact time of 24 h. The results were compared to those obtained using other contact times to determine the impact of time on chlorine demands, to predict which amino acids are most likely to be depleted at water treatment plants and which will be depleted in storage tanks or distribution systems.

MATERIALS AND METHODS

Model compounds and reagents

Pure reagents, nitrogenous organic compounds, and ammonium chloride were used in this study for the bench-scale chlorination experiments. All reagents used were of analytical grade unless otherwise noted. The 20 amino acids were purchased from Wako Pure Chemicals (Osaka, Japan); three amino sugars and ammonium chloride were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water purified with Gradient A10 (Millipore, Bedford, MA, USA) was used for all experiments. Phosphate buffer was prepared with potassium dihydrogen phosphate and disodium hydrogen phosphate obtained from Wako Pure Chemicals (Osaka, Japan). Hypochlorite (10% - 15%) used as the chlorine source was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Chlorination and chlorine demand

The solutions of model nitrogenous organic compounds (5 μM) and ammonia (5 μM) in glass flasks were buffered at pH 7 using phosphate buffer solution (1 mM), and one buffer solution was used as a control. Each buffered solution was dosed with a small amount of hypochlorite solution while stirring using a magnetic stirrer. The control was subjected to the same reaction conditions except no nitrogenous organic compound or ammonia was present. The sample solutions were added to the flasks to minimize the headspace. After mixing for about 1 min, all flasks were placed in the dark at 20°C for 24 h without mixing. Chlorine residual was set at 1.0 ± 0.2 mg Cl_2/L after 24 h. To obtain the samples at 1.0 ± 0.2 mg Cl_2/L after 24 h, several flasks dosed with different amounts of chlorine were prepared for each compound. That is, chlorine doses were different among the nitrogenous organic compounds and ammonia solutions (i.e., 1.1 - 5.3 mg Cl_2/L). The chlorine residuals for control, nitrogenous organic compounds, and ammonia solutions at 24 h were determined using the standard *N*, *N*-diethyl-*p*-phenylenediamine (DPD) titration method (Eaton *et al.*, 2005).

Prediction of chlorine demand

Models are valuable tools for validating experimental data. Although there are many empirical models for predicting chlorine demand and disinfection byproduct formation in drinking water, there has been only one previous report of a Quantitative Structure-Property Relationship (QSPR) model (Luilo and Cabaniss, 2010). In this study, the QSPR was used as a model because the structure of a compound is the determining factor in chlorination reaction at optimal contact time, pH, and temperature. Here, the QSPR included eight constitutional descriptors mentioned below.

The QSPR model is summarized by Equation 1. This QSPR was calibrated using 159 compounds and was validated using 42 external data. The QSPR had coefficient of determination of calibration R_c^2 equal to 0.86 and a residual standard deviation (SDE) of 1.24 mol-Cl_2/mol-Cp (Luilo and Cabaniss 2010). The brief description of each descriptor in the model is described below.

$$Cl_2\ demand = 7.61 \times RAI + 1.16 \times ArOH + 3.00 \times ACN + 1.23 \times CI + 2.37 \times AS + 1.01 \times O:C + 0.49 \times ArORact - 0.72 \times ArORnact \quad \text{.... (Eq. 1)}$$

The ring activation index (RAI) is the ratio of the sum of strong aromatic ring activators

(NH_2 and OH) to the number of rings. Carbonyl index (CI) is motivated by the observation that carbonyl compounds undergo chlorine substitution reaction via keto-enol tautomerization and that β-dicarbonyl compounds (e.g., 3-oxopentanedioic acid), consume more chlorine than simple ketones (e.g., 2-propanone). The symbol ArOH is the number of phenolic groups in aromatic ring, AS is the number aliphatic sulphur, ACN is the number of aliphatic carbon bonded to amines, O : C is the ratio of atomic oxygen to carbon, ArORact is the number of ring-activating alkoxy groups in aromatic ring, i.e., alkoxy groups (weak ring activators) attached to aromatic rings that have no strong ring activators (NH_2 and OH) on them; and ArORnact is the number of non-activating groups in aromatic ring, i.e., alkoxy groups attached to aromatic rings that have strong ring activators (NH_2 and OH) on them. A value of zero was given to a molecule that did not have any one of the descriptors. The predictive power of the QSPR model for chlorine demand was evaluated using external validation data (any sets of data that were not used in calibration of the model). The q^2 (coefficient of determination of validation) can be calculated using Equation 3 below (Golbraikh and Tropsha, 2002; Tropsha *et al.*, 2003) and is calculated differently from R_c^2, which can easily be calculated manually using Equation 2. However, most statistical software can generate R_c^2 and residual SDE (how far each data point is from the best fitted line or model) when performing simple or multiple linear regression between response and one variable or a list variables.

$$R_c^{\,2} = 1 - \frac{\sum (y_i - \hat{y}_i)^2}{\sum (y_i - \bar{y})^2} \quad \text{..... (Eq. 2)}$$

where, y_i and $\hat{y}_i$ are experimental and predicted values respectively and $\bar{y}$ is the mean of experimental data in calibration dataset. On the other hand the q^2 for external validation, q^2_{ext}, was calculated using Equation 3 (Golbraikh and Tropsha, 2002; Tropsha *et al.*, 2003).

$$q^2_{ext} = 1 - \frac{\sum_{i=1}^{test} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{test} (y_i - \bar{y}_{tr})^2} \quad \text{..... (Eq. 3)}$$

where y_i and $\hat{y}_i$ are experimental and predicted Cl_2 demands (from external validation data) respectively; $\bar{y}_{tr}$ is the average of the experimental Cl_2 demands in the entire calibration data (Golbraikh and Tropsha, 2002; Tropsha *et al.*, 2003). Since the q^2 may be influenced by the outliers in training and external data sets (Eq. 2), Golbraikh and Tropsha (2002) recommended using slope, k and R^2 of regression line obtainable from the plot of predicted values against the experimental values (normal regression) or vice versa (reverse regression) in addition to q^2. The symbols, k_i and R_i^2 are slope and R^2, respectively, and are obtained from the regression plot of predicted Cl_2 demand and experimental Cl_2 demand with y-intercept. Whereas k_o and R_o^2 are slope and R^2, respectively, when the y-intercept in the regression in the same plot is set to zero (regression through origin). The essence of this test is that if the QSAR/QSPR model is close to perfection, k_i and k_o, and R_i^2 and R_o^2 should not be significantly different from

each other and both should be closer to 1. Thus, the QSAR/QSPR model is regarded to have high predictive power if it meets the following criteria: $R_c^2 > 0.6$, $q^2 > 0.5$; $(R_i^2 - R_o^2)/R_i^2 < 0.1$; $0.85 \leq k \leq 1.15$ (Golbraikh and Tropsha, 2002; Tropsha *et al.*, 2003).

The root mean square error (RMSE) and model bias deviation (MBD) are also useful in checking the predictive power of the model and were computed using Equations 4 and 5,

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N}} \quad \text{..... (Eq. 4)}$$

$$MBD = \frac{\sum_{i=1}^{N}(\hat{y}_i - y_i)}{\sum_{i=1}^{N} y_i} \times 100\% \quad \text{..... (Eq. 5)}$$

where y_i and $\hat{y}_i$ are experimental and predicted Cl_2 demand and N is the total number of observations. The RMSE of external validation is expected to be closer to the residual (SDE) of model calibration for a model with high predictive power. The MBD provides a qualitative diagnosis of the predictive power of the model. A MBD of zero indicates that the model has no prediction bias. However, a negative MBD indicates that the model predicts lower than the experimental value and positive MBD indicates that the model predicts higher than the experimental value. However, the magnitude of MBD does not necessarily indicate how many data were biased higher or lower than expected because one data point (compound) that is either over-predicted or under-predicted may drive total residuals (numerator in the Equation 5) up or down, respectively.

Therefore, in this work the eight descriptors represented in Equation 1 were calculated from the structure of each amino acid and amino sugar in accordance with the procedure described in the literature (Luilo and Cabaniss, 2010). A value of zero was given to any of the eight descriptors if a molecule lacked functional groups needed to calculate it. The descriptors for each molecule were substituted in the QSPR (Equation 1) to estimate the chlorine demand while the predictive power of the QSPR on the model compounds were determined in accordance with the methods described in literature (Golbraikh and Tropsha, 2002; Tropsha *et al.*, 2003).

RESULTS AND DISCUSSION

Chlorine demand of nitrogenous compounds

The chlorine demands (mg Cl_2/L) for 20 amino acids, 3 amino sugars, ammonia and ultrapure water (blank) were determined from the difference between chlorine dose and chlorine residual. The chlorine demand of each model compound was corrected by subtracting the chlorine demand of the blank (0.1 mg Cl_2/L). The net chlorine demand of each compound was further transformed into a unit of mole of chlorine per mole of compound (mol-Cl_2/mol-Cp) (Table 1). The results showed that tyrosine and tryptophan had chlorine demands of 10.99 mol-Cl_2/mol-Cp and 12.54 mol-Cl_2/mol-Cp, respectively, whereas that of histidine was 4.54 mol-Cl_2/mol-Cp.

Table 1 - Chlorine demands of amino acids and amino sugars.

Compounds	Cl_2 demand (mg/L)	Compound (μmol/L)	MW (g/mol)	Cl_2 demand (μmol/L)	Cl_2 demand (mol/mol)
Amino acid					
Glycine	1.65	5	75.07	23.24	4.65
Alanine	0.90	5	89.09	12.68	2.54
Valine	0.85	5	117.15	11.97	2.39
Isoleucine	0.90	5	131.17	12.68	2.54
Leucine	0.90	5	131.17	12.68	2.54
Serine	1.20	5	105.09	16.90	3.38
Threonine	1.40	5	76.12	19.72	3.94
Methionine	2.35	5	149.21	33.10	6.62
Cysteine	2.65	5	121.16	37.32	7.46
Aspartic acid	1.40	5	133.10	19.72	3.94
Glutamic acid	0.95	5	147.13	13.38	2.68
Lysine	1.60	5	146.19	22.54	4.51
Arginine	2.00	5	174.20	28.17	5.63
Histidine	1.50	5	155.15	21.13	4.23
Asparagine	2.05	5	132.12	28.87	5.77
Glutamine	1.30	5	146.14	18.31	3.66
Proline	1.20	5	115.13	16.90	3.38
Tryptophan	4.45	5	204.23	62.68	12.54
Phenylalanine	1.00	5	165.19	14.08	2.82
Tyrosine	3.90	5	181.19	54.93	10.99
Amino sugar					
Glucosamine	1.60	5	179.19	22.54	4.51
Galactosamine	1.89	5	179.19	26.62	5.32
Mannosamine	1.50	5	179.19	21.13	4.23
Ammonia	0.85	5	17.03	11.97	2.39

*Amino acids are listed from simple to complex.

The low chlorine demand for histidine determined in the present study relative to previous 72 h and 96 h studies may be attributable to the shorter contact time (Hureiki *et al.*, 1994; Hong *et al.*, 2009). However, the chlorine demand for tyrosine in the present study, 10.99 mol-Cl_2/mol-Cp, was similar to 11.40 mol-Cl_2/mol-Cp reported in a previous 15-h study (de Laat *et al.*, 1982). As these two data for tyrosine were not significantly different from each other despite the 9 h difference, it is likely that most of the tyrosine reacts in less than 15 h. However, contact time longer than 24 h may increase the chlorine demand of tyrosine. Thus, the chlorine demand of tyrosine in the present study was lower than 13.40 and 13.20 mol-Cl_2/mol-Cp reported previously (Hureiki *et al.*, 1994; Hong *et al.*, 2009). There were no significant differences in chlorine demands between 72 and 96 h, indicating that tyrosine is depleted in less than 72 h. Figure 1 shows a comparison of the chlorine demands in the present study with those reported in the literature.

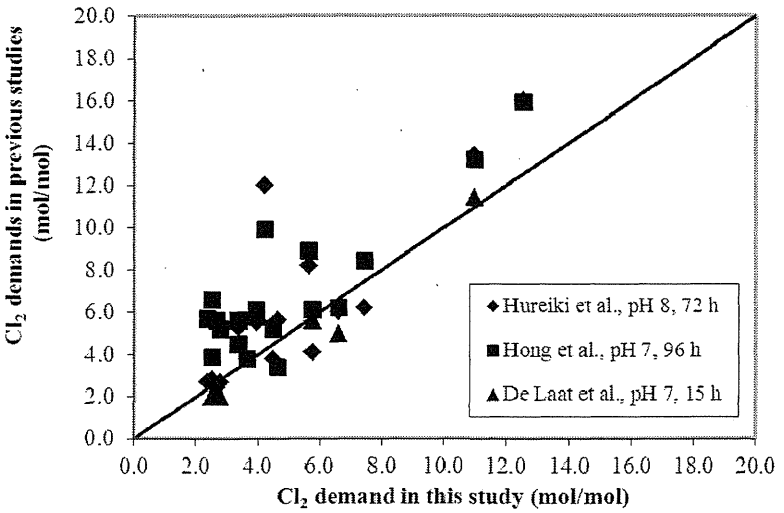

Fig. 1 - Chlorine demands in the present study and those reported in the literature.

The high chorine demand of aromatic amino acids is generally related to the structure because each has an aromatic ring with an alanyl substituent. The contribution of the alanyl substituent is expected to be around 3 mol-Cl_2/mol-Cp and the rest should be contributed by the aromatic ring through electrophilic substitution reaction. Tyrosine has a phenol group that activates the ring favoring electrophilic substitution reaction. The chlorine demand of the 4-hydroxyphenyl ring is expected to contribute about 9 mol-Cl_2/mol-Cp based on the results of previous studies for phenol or 4-hydroxytoluene (de Laat *et al.*, 1982; Gallard and von Gunten, 2002). Similarly, tryptophan is expected to undergo electrophilic substitution reaction in the aromatic heterocyclic indole moiety, and its contribution is expected to be around 10 - 13 mol-Cl_2/mol-Cp depending on the contact time (de Laat *et al.*, 1982; Hureiki *et al.*, 1994; Hong *et al.*, 2009).

The chlorine demand of histidine, which has imidazole and alanyl moieties, was 4.23 mol-Cl_2/mol-Cp. Our study also showed that alanine had a chlorine demand of 2.5 mol-Cl_2/mol-Cp. If the alanyl substituent in the histidine is taken into account, the imidazole moiety contributed only 1.3 mol-Cl_2/mol-Cp. This contribution is lower than expected from the electrophilic substitution in histidine, which has two endocyclic amines. Other studies indicated that the chlorine demands of histidine at 72 and 96 h were 12.0 and 9.9 mol-Cl_2/mol-Cp, respectively (de Laat *et al.*, 1982; Hureiki *et al.*, 1994; Hong *et al.*, 2009). However, it is difficult to clarify the reason why the 96-h study had lower chlorine demand than the 72 h study. If the chlorine demands of alanine at 72 and 96 h were taken into account in each study, the imidazole moiety is expected to contribute about 6 and 9 mol-Cl_2/mol-Cp, respectively. This suggests that there is either an error in our data or that histidine undergoes electrophilic substitution very slowly in the first 24 h as studies with longer contact times indicated higher chlorine demands than observed in our experiments (Hureiki *et al.*, 1994; Hong *et al.*, 2009).

S-amino acids had intermediate chlorine demands, with values of 7.46 and 6.62 mol-Cl_2/mol-Cp for cysteine and methionine, respectively. These results are not markedly different from those reported in previous studies performed with contact times

of 72 and 96 h (Hureiki *et al.*, 1994; Hong *et al.*, 2009). S-amino acids have two basic groups (sulfur and amine) at which chlorine substitution takes place. Aside from these two common amino acids, water may be contaminated with traces of cystine, thiamine, and biotin, all of which have sulfur in their structures. These contaminants and any other sulfur-containing organic molecules may contribute to higher chlorine demand in water treatment plants. Another group of amino acids with relatively high chlorine demand closer to those of the S-amino acids are arginine and asparagine. These amino acids possess amide and imine groups that can undergo chlorine substitution, and they have chlorine demands of 5.63 and 5.77 mol-Cl_2/mol-Cp, respectively. However, this study showed that chlorine demand for arginine was about 3 units lower than those reported in previous studies with longer reaction times (Hureiki *et al.*, 1994; Hong *et al.*, 2009). It was found from the structure that there are 3 amines and 1 imine groups in arginine. However, 2 amines and 1 imine that are connected to the carbon-6 may not be as basic as the α-amine group due to resonance stabilization in the former. Thus, the reactions of these groups in arginine (or its byproducts) with chlorine is slow and it may most likely be incomplete in the 24 h contact time used in this study. On the other hand, the chlorine demand of asparagine was comparable to or higher than those described in previous studies (Hureiki *et al.*, 1994; Hong *et al.*, 2009). Similarly, the chlorine demand of glutamine was similar to those in previous studies. Thus, the reactions of these compounds or their byproducts with chlorine were completed in less than 24 h.

The chlorine demand of asparagine was larger than that of glutamine. Although glutamine has an amide group, similar to asparagine, the two are structurally different since asparagine has one methylene bridging amine and amide groups, while glutamine has an ethylene group bridging the amine and amides. The difference in their chlorine demands was considered to be due to the difference in the methylene and ethylene groups. It should be noted that the pKa of the methylene group is lower than that of the ethylene group, but the difference is small.

Six amino acids have chlorine demands ranging between 3 and 4, i.e., proline, serine, threonine, lysine, glycine, and aspartic acid (Table 1). These values were generally lower than those reported in previous studies conducted with longer contact times (Hureiki *et al.*, 1994; Hong *et al.*, 2009). This suggests that these amino acids may continue reacting even in distribution systems, because it is expected that treated water may still be in the distribution system after 24 h. While glutamic acid showed a chlorine demand of 2.68 mol-Cl_2/mol-Cp, both leucine and isoleucine, which isomers, showed the same chlorine demand of 2.54 mol-Cl_2/mol-Cp (Table 1). These results for the latter are similar to those of the 72 h study (Hureiki *et al.*, 1994) but lower by about 3 units compared to the 96-h study (Hong *et al.*, 2009).

Phenylalanine and alanine consumed 2.82 and 2.54 mol-Cl_2/mol-Cp, respectively, and the results are not significantly different from the previous 15 h and 72 h studies (de Laat *et al.*, 1982; Hureiki *et al.*, 1994). However, Hong *et al.* (2009) reported chlorine demands of 3.90 and 5.2 mol-Cl_2/mol-Cp for alanine and phenylalanine, respectively, which are higher than those in our experiments and in the other two studies mentioned above. The chlorine demands were increased by 1 and 2 units from those in the 15 h and 72 h studies, respectively. These observations indicated that alanine and phenylalanine would have reacted with chlorine if the contact time was increased to 96 h. Although

phenylalanine is an aromatic amino acid, it is not as reactive as tyrosine. The difference is that phenylalanine lacks strong ring-activating substituents, such as OH and NH_2. Therefore, electrophilic substitution may occur very slowly in the phenyl ring, which is why the chlorine demand of phenylalanine is expected to be slightly higher than that of alanine and less than that of tyrosine (Hong *et al.*, 2009).

Amino sugars are nitrogenous organic compounds that have amine groups, and can react with chlorine similar to amino acids. In this study, glucosamine, galactosamine, and mannosamine were tested for chlorine consumption under the same conditions as those used for the amino acids. The results indicated that the chlorine demands were very close to each other and were also similar to those of some amino acids despite the differences in their structures (Table 1). These results also indicated that high levels of amino sugars in water sources may contribute significantly to the total chlorine demand of water. Finally, all nitrogenous compounds may contain amine, amide, or imine groups in their structures. In the present study, ammonia was used as the simplest nitrogenous compound in water. Chloramines are formed after the reaction of ammonia and chlorine. In the case of the nitrogenous organic compounds used in the present study, *N*-chloramines were considered to be formed by chlorination. This was supported by the frontier electron density (FED) calculation, which showed that the nitrogen in the amine group had the highest electron density of all atoms in alanine (Chu *et al.*, 2009). Thus, it was considered that the amine group was an important site for chlorine substitution in nitrogenous compounds, as discussed previously (Bull *et al.*, 2006). However, chlorination contact time at water treatment plants and distribution systems may not be sufficient to allow complete reaction with some compounds in water. Although there have been few reports of amino acids in tap water, an unpleasant smell in tap water indicates the formation of odorous compounds (e.g., chloroaldimines). Thus, the presence of such odorous compounds was considered to indicate the importance of determining the chlorine demand under the conditions used for chlorination in actual distribution systems.

Prediction of chlorine demand

Further analysis of the data reported in this work has shown that there is a weak linear relationship (R^2 = 0.265, F = 7.93, P = 0.01, N = 24) between the molecular weight (MW) and chlorine demand at 95% confidence level (Fig. 2). The weak relationship may be attributed to the fact that only certain sites of the molecule (functional groups) are involved in chemical reaction with chlorine and not the whole molecule.

Thus, the number of functional groups and relative position in the molecule are highly important in chemical reaction. That is why two molecules with different molecular weights may have the same chlorine demands as shown by the following pairs of molecules: lysine vs. glucosamine, alanine vs. leucine or serine vs. proline (Table 1). Although the MW of tyrosine differs from that of amino sugars by 2 units, its chlorine demand was 2 times higher than that of amino sugars (Table 1). Thus, models based on structure of molecules may be more useful for predicting chlorine demands than those models that were derived using bulk water parameters (e.g., pH, turbidity, UV-absorption, temperature) or molecular properties (e.g., MW, molecular volume, molecular surface area).

We used the QSPR model for predicting the chlorine demand in drinking water (Luilo and Cabaniss, 2010) to estimate the chlorine demand of the amino acids and amino sugars studied. The results showed that all compounds, with the exception of arginine and glutamine were predicted within ± 2.48 standard deviations of prediction, for 2% error margins for both sides (Fig. 3).

The standard deviation of prediction is ± 2SDE, and in this case the SDE for QSPR calibration at 95% confidence interval was 1.24 mol-Cl_2/mol-Cp. Any predicted data

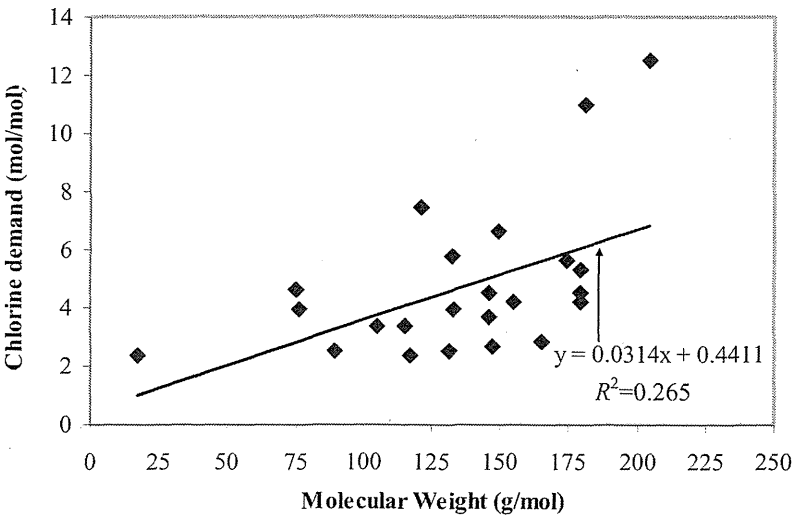

Fig. 2 - Weak correlation between chlorine demands and molecular weights of the 23 model compounds.

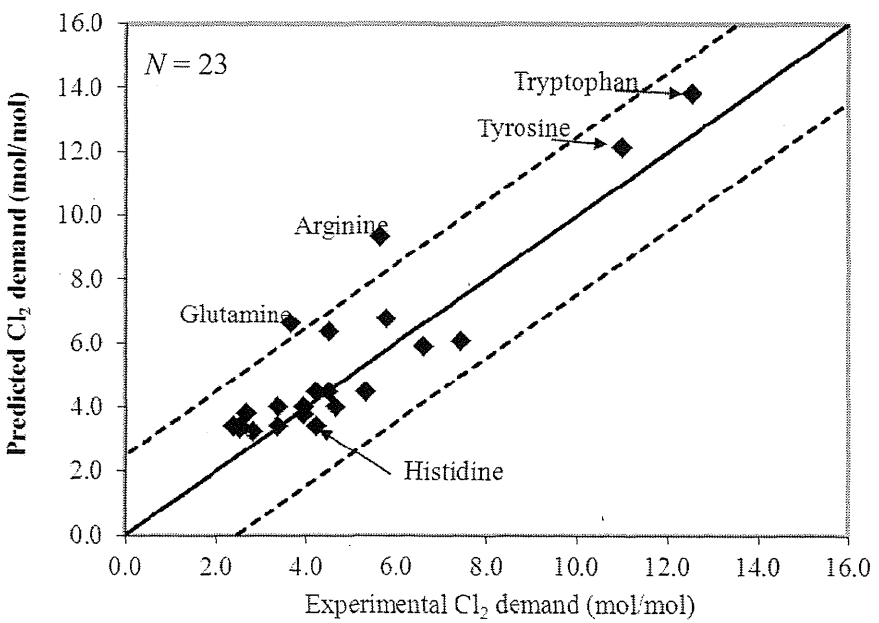

Fig. 3 - Relationship between predicted and experimental chlorine demands for the 23 amino acids. The solid line is for the ideal model and dotted lines are standard deviation of prediction margins (± 2.48 mol-Cl_2/mol-Cp).

point outside ± 2.48 margins implies that its prediction by the QSPR has some uncertainty and therefore, the predicted value may not be reliable.

To determine the reliability of the prediction, we used the coefficient of determination for cross-validation, q^2, as described previously (Luilo and Cabaniss, 2010). The value of q^2 was 0.74 which is much higher than the cut off value of 0.5 (Golbraikh and Tropsha, 2002) and RMSE equal to 1.30 mol-Cl_2/mol-Cp is closer to the model's standard error of 1.24 mol-Cl_2/mol-Cp. However, the MBD of 11.9% indicates that the model predicted 15 out of 24 compounds, which is slightly higher than expected. Although chlorine demand prediction data for tryptophan and histidine were close to the observed data, the chlorine demand prediction for histidine may not be as reliable as that for tryptophan. This is because the QSPR calibration data did not include any molecules with endocylic heteroatoms such as nitrogen (e.g., imidazole, indole, pyrrole). Thus, one limitation of QSPR is that it cannot give reliable prediction of chlorine demands of aromatic molecules without exocylic ring activators such as histidine because RAI descriptor is zero. However, RAI may be calculated in tryptophan because the amine group, which is exocyclic to the benzene ring (pyrrolyl moeity) may induce electrophilic susbstitution reaction in the benzene ring through resonance. However, the model cannot estimate the contribution of chlorine demand from the chlorine substitution at the pyrrolyl ring which has the endocyclic amine group.

The predictive power of the QSPR on model compounds is tested by generating regression line in a plot of predicted chlorine demand against the observed chlorine demand with and without intercept (Fig. 4). A good prediction should not give significantly different slopes that are closer to 1 ($0.85 \leq k \leq 1.15$); y-intercept, b, should

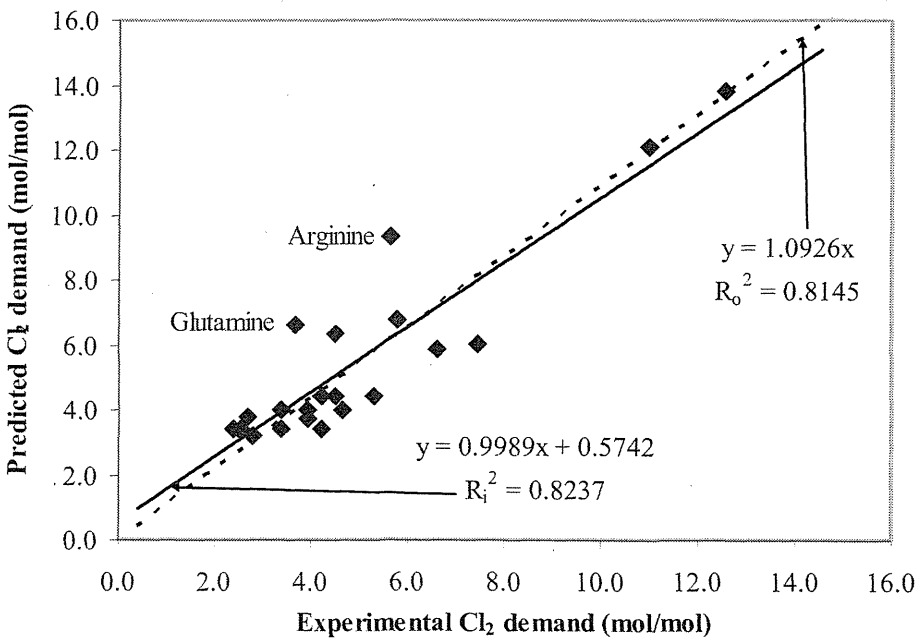

Fig. 4 - Normal regression of predicted chlorine demand against the experimental chlorine demand with y-intercept (solid line) and without y-intercept (dotted line) for 23 model compounds.

be closer to zero (b ≈ 0); and the ratio $(R_i^2 - R_o^2)/R_i^2 < 0.1$ (Golbraikh and Tropsha, 2002; Tropsha *et al.*, 2003) where R_i^2 is obtained using regression line with intercept and R_o^2 is obtained from regression line through origin. In Fig. 4, k_i is 0.998; k_o is 1.092; R_i^2 is 0.82; R_o^2 is 0.81 and the ratio $(R_i^2 - R_o^2) / R_i^2$ is 0.01, which imply that the predictive strength of the model is high.

It is interesting that arginine has three amines and one imine. Despite having the greatest number of reduced nitrogens (amine and imine) in the molecule, its chorine demand (5.63 mol-Cl_2/mol-Cp) was comparable to those of asparagine (2 amine groups) and amino sugars (1 amine group). It is possible that if the contact time used in the present study was extended to 72 h or more, arginine would have consumed more chlorine. This is because chlorination of arginine for 72 h and 96 h indicated chlorine demands of 8.20 and 8.90 mol-Cl_2/mol-Cp, respectively (Hureiki *et al.*, 1994; Hong *et al.*, 2009), which are close to the predicted value of 9.34 mol-Cl_2/mol-Cp. These observations imply that arginine reacts slowly with chlorine and therefore, requires a longer contact time for depletion. Thus, arginine is likely to continue reacting with residual chlorine in the distribution system.

CONCLUSIONS AND RECOMMENDATIONS

Amino acids and amino sugars are included among the dissolved organic matter that must be removed from raw water. However, conventional water treatment processes cannot remove all fractions of nitrogenous compounds, and are therefore likely to contribute to the chlorine demand of water. This study showed that the aromatic amino acids consumed 10 - 13 mol-Cl_2/mol-Cp, whereas the chlorine consumption of S-amino acids was between 6 and 8 mol-Cl_2/mol-Cp. The remaining amino acids and the three amino sugars showed chlorine consumption ranging between 2 and 6 mol-Cl_2/mol-Cp. The findings of our 24-h study were compared to those of the previous studies with contact times of 72 h and 96 h, and the results indicated an increasing trend in chlorine demands with time for some of the nitrogenous compounds. The QSPR predictions showed that arginine was overpredicted, while the rest were within the model prediction error.

The results of the present study indicated that chlorine demand in 24 h showed a good correlation with prediction data. Furthermore, kinetic studies are required to determine how fast the precursors can react with chlorine at typical water treatment contact times and chlorine doses. The data will provide insight into the amounts of chlorine demand and production of disinfection byproducts, especially nitrogenous compounds related to odor, while the results of longer contact times, such as 72 h and 96 h, might be useful to drive the reaction to completion.

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「報　文」

水道水質試験の標準液調製における不確かさと定量精度に影響を及ぼす要因

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要旨：水道水質試験の定量分析では、試料調製から機器分析の一連の試験操作に起因して、得られる値にばらつきが生じるが、この不確かさの評価法は明確にされていない。本研究では、農薬ブタミホスの定量分析をモデル実験として、標準液調製時の不確かさを検証した。その結果、天秤、調製器具、実験者、測定機器に不確かさを生じ、0.05 mg/L の測定結果に付随する合成標準不確かさは1.63%となった。さらに、qNMR によりブタミホス市販標準品３社３製品の絶対純度を測定した結果、90.3、94.7、94.8%であり、このメーカー間差が大きな不確かさを与え、分析精度に影響を及ぼしていることが明らかとなった。

キーワード：不確かさ※、信頼性、精度管理、純度※、定量分析※
分類項目：水質管理一般（120101）、試験方法一般（120201）、試験機器・器具・試薬（120203）、精度管理（120204）、機器分析（120205）

1. はじめに

定量分析では、様々な誤差要因により、得られる値にある程度のばらつきが生じる。この測定量のばらつきを数値として具体的に表した「不確かさ」は、計測の分野で急速に広まってきた考えであり、「測定の結果に付記される、合理的に測定量に結び付けられ得る値のばらつきを特徴づけるパラメータ」と国際的に定義されている[1,2]。分析法の開発過程では、一般に併行精度を立証するバリデーションが行われるが、得られた分析値の真度と確度を評価するものではないため、一連の操作および測定の不確かさの評価が別途重要視され始めている。実際に、近年、分析結果の信頼性を示す指標の一つとして不確かさを表記することが要求されつつある。しかし、水道水質試験の場合は、定量分析値を得るまでの過程に不確かさの要因が多いため、試料および標準液の調製、機器分析における不確かさの具体的な評価法は明確にされていない。このような背景から、水道水質試験では、得られた分析結果の信頼性を確保するため、標準手順書の作成、内部精度管理や外部精度管理等の実施を要求している。また、前処理から機器分析、データ解析までの各作業段階における誤差要因の低減のために、標準品（標準物質）の入手先、標準液調製、試料採取、機器の管理等のトレーサビリティの証明を要求している。

一方、岩村らは[3]、試薬メーカー３社の市販農薬混合標準液について、ガスクロマトグラフ／質量分析計（GC/MS）により、標準液中に含まれる68種の農薬の濃度を定量し、５％の危険率で一

元配置分散分析および多重比較したところ、68物質中30物質に有意差が認められたと報告している。このように市販標準品の純度や市販標準液の濃度を評価した報告[3~6]は少ないが、市販標準品の純度および市販標準液中の物質の濃度が試薬メーカー間で異なる可能性は否定できない。すなわち、異なる試薬メーカーの市販標準品を用いて精度管理を行った場合、室内および室間精度は現実には検証できず、結果として各機関において得られた定量値の信頼性が厳密な意味で確保できないこともあり得ると考えられる。

そこで本研究では、水道水質試験における標準液調製時に生じる不確かさを評価し、定量精度の信頼性を確保するための要因を明らかとする目的で、水環境中で検出される恐れのある農薬として水質管理目標設定項目にも挙げられているブタミホスを測定対象としたモデル実験を行った。

2. 装置および調査方法等

2.1 標準品および試薬

ブタミホス市販標準品として、市販残留農薬試験用標準品3社3製品A（林純薬工業株式会社、Cat. No. 990-52021、Lot J091016037、純度99.9％（GC/FID））、B（和光純薬工業株式会社、Cat. No. 020-10931、Lot ALK8029、純度99.8％（GC/FID））、C（関東化学株式会社、Cat. No. 04346-96、Lot 708X7110、純度99.2％（GC/FID））を用いた。フタル酸ジエチル（diethyl phthalate: DEP）は独立行政法人産業技術総合研究所の認証標準物質（NMIJ CRM 4022-b、純度99.98±0.01 w/w％（99.74±0.09 mol/mol％））を用いた。高純度ヘキサメチルジシラン（hexadimethyldisilane: HMD）は和光純薬工業株式会社特注品、重メタノールはIsotec（99.8 atom％ D）を用いた。アセトンおよびアセトニトリルは高速液体クロマトグラフ用（和光純薬工業株式会社）、ギ酸は試薬特級（和光純薬工業株式会社）を用いた。精製水はElix純水装置システム（日本ミリポア株式会社）より得たものを用いた。

2.2 試料調製に用いた器具および機器

20 mLメスフラスコ（IWAKI PYREX、許容誤差0.04 mL（20℃））、100 μLマイクロシリンジ（HAMILTON SYRINGE 80665、Lot 279510、許容誤差1％以内）、2.0 mLメスピペット（PYREX pipet 7077-2N、disposable glass、serological、individual wrap、sterile、7740 glass、許容誤差1％以内）を試料調製に用いた。

天秤は、ウルトラミクロ天秤XP2U（メトラー・トレド株式会社、最小計量値0.6 mg）およびセミミクロ天秤R200D（ザルトリウス・メカトロニクス・ジャパン株式会社、最小計量値4.8 mg）を用いた。なお、各天秤の最小計量値は、USP-NF Weights and Balances[7]に準じ、実測値より計算した。

液体クロマトグラフ／フォトダイオードアレイ検出器（LC/PDA）は、Acquity UPLC/PDA（日本ウォーターズ株式会社）を用いた。

核磁気共鳴装置（NMR）は、オートサンプラー付きJNM-ECA600（日本電子株式会社、600 MHz）を用いた。

2.3 試料調製および不確かさの評価

ブタミホス標準原液は、市販標準品製品A 20 mgを精密に量り取り、メスフラスコで20 mLに定容して1,000 mg/Lのアセトン溶液とし（図-1 段階1）、－20℃で保存したものを用いた。0.05、0.5、5 mg/Lの標準液は、ブタミホス標準原液をアセトンで希釈し、用時調製した。すなわち、5 mg/L標準液は1,000 mg/L標準原液をマイクロシリンジで100 μL とり、メスフラスコで20 mLに定容した（段階2）。また、0.5 mg/L標準液は5 mg/L標準液からメスピペットで2.0 mL とり20 mLに定容し（段階3）、同様に0.5 mg/L標準液からメスピペットで2.0 mL とり20 mLに定容し、0.05 mg/L標準液とした（段階4）。

実験者の調製の不確かさは、アナログ計器の読み取りの偏りとして求めた。希釈に用いたメスフラスコ、メスピペットおよびマイクロシリンジについて、精製水を正確に量り取り、その重量を秤量し、25回試行において得られた実験標準偏差（SD）により相対標準偏差（RSD）を算出した。また、調製した標準液を液体クロマトグラフ／フォトダイオードアレイ検出器（LC/PDA）に付し、クロマトグラム上に観察されたブタミホスのピーク面積値について、25回測定におけるRSDを算出し、繰り返し測定における不確かさとした。な