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Analytical Method Development for Measuring
Chemical Substances from Wastewater Treatment Plants
by High Performance Liquid Chromatography/Mass
Spectrometry

Thesis for the Degree of Philosophiae Doctor (Applied Biology)

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Preface

This thesis is submitted to the Chubu University for partial fulfillment of the requirements for the degree of philosophiae doctor.

This doctoral work has been performed at the Environmental Analysis Laboratory for Toxic Chemicals, Graduate School of Bioscience and Biotechnology, Chubu University, Kasugai, with Professor Suzuki Shigeru as main supervisor and with Professor Yamamoto Atsushi and Professor Tsutsumiuchi Kaname as co-supervisors.

Abbreviations and Acronyms

2,5-DAT	2,5-diaminotoluene
28 w/v% NH ₄ OH	ammonia water (28 w/v%)
4AP	4-aminophenol
4I3MP	4-isopropyl-3-methylphenol
5A2MP	5-amino-2-methylphenol
ACN	acetonitrile
BHT	2,6-di-tert-butyl-p-cresol
BPA	bisphenol A
Br _y BPA	brominated bisphenol A congener
Cl _x BPA	chlorinated bisphenol A congener
Eugenol	4-allyl-2-methoxyphenol
GC	gas chromatography
HILIC	hydrophilic interaction chromatography
HOMOs	highest occupied molecular orbitals
HPLC	high performance liquid chromatography
IPA	2-propanol
LOD	limit of detection
MDL	method detection limit
MeOH	methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo- <i>p</i> -dioxin
PEG	polyethylene glycol 200
POPs	persistent organic pollutants
PPCPs	pharmaceutical and personal care products
Q-ToFMS	quadrupole-time of flight mass spectrometer
RSD	relative standard deviation
SPE	solid phase extraction
SRM	selected reaction monitoring
TCS	triclosan (5-chloro-2-(2, 4-dichlorophenoxy) phenol)
TLC	thin-layer chromatography
WWTP	wastewater treatment plant

Summary

This thesis reports on the development of several high performance liquid chromatography/mass spectrometry (HPLC/MS) methods for the analysis and source identification of hazardous water pollutants, and discusses the results of environmental measurements using these methods. Analytical chemistry sets the basis for understanding risks posed by chemical substances to humans and ecosystems.

This thesis consists of 5 chapters. The outlines of each chapter are as follows:

The first chapter presents the current situation and problems surrounding the environmental analysis and environmental chemistry of hazardous chemical substances. Persistent organic pollutants (POPs) have been studied through environmental monitoring and quantitative analyses by many researchers. Chemical substances in pharmaceutical and personal care products (PPCPs), such as drugs, antibiotics, antimicrobials, and cosmetics, are becoming of interest globally as new environmental pollutants. Recently, compounds such as polar substances, non/semi-volatile substances, and heat-labile substances have polluted the environment and adversely affected humans, living organisms, and ecosystems. In order to understand the environmental concentrations and environmental dynamics of chemical substances, it is of great importance for environmental researchers to develop quantitative and qualitative analysis methods such as mass spectrometry coupled with gas- and liquid-chromatography, and conduct environmental science studies related to hazardous pollutants.

The second chapter discusses solid phase extraction high performance liquid chromatography/tandem mass spectrometry (SPE-HPLC/MS/MS) analysis methods for aromatic amines, and their application to shampoo-water mixture, urine after hair dyeing, river water, and other environmental media. Aromatic amines used for hair dyeing are extremely water soluble, highly susceptible to oxidation and volatile, evaporating during the sample concentration process. Therefore, the analysis methods include concentration processes under low temperature with added acetonitrile as the evaporation inhibitor. Recoveries of aromatic amines were 76 to 124%, with relative standard deviation (RSD) of 3.3 to 13%. 0.7 to 43% of the aromatic amines in the applied hair dye were detected in the shampoo-water mixture. A maximum of 0.1% of the 2,5-diaminotoluene (2,5-DAT) contained in the hair dye was excreted in urine. A maximum of 2% of the applied 4-aminophenol (4AP), which is more hydrophilic than 2,5-DAT, was excreted in urine. In sewage water effluent, 2,5-DAT concentrations were 0.25 µg/L or less, 100 times lower

than a previously reported concentration (25 µg/L). No toxicity data were found for the concentration levels reported here.

The third chapter discusses a measurement method for halogenated bisphenol A in wastewater by HPLC/MS/MS. Chlorinated bisphenol A congeners (Cl_xBPAs), which are often found in the environment, are probably unintentional products of the sterilization of bisphenol A (BPA) or brominated bisphenol A (Br_yBPA) at wastewater treatment plants (WWTPs). Therefore, the measurement method includes chromatographic separation of all eleven halogenated BPAs, including structural isomers possibly produced in WWTPs. The measurement results show that the concentrations of BPA, Cl₁₋₃BPAs, and Br₄BPA decreased after sterilization, but that of Cl₄BPA increased. This suggests that BPA, Cl₁₋₃BPAs, and Br₄BPA could be precursors of Cl₄BPA in the sterilization process in WWTPs. However, the amount of Cl₄BPA produced was much lower than the decrease in the precursor species.

The fourth chapter presents accurate mass measurements with high performance liquid chromatography/quadrupole – time of flight mass spectrometry (HPLC/Q-ToFMS) carried out for qualitative analysis of wastewater samples before and after sterilization. Sixteen halogenated organic compounds were found in treated sewage effluent water after the sterilization process. Three of them, namely monochlorophenol, dichloro-dihydroxybenzene, and Cl₄BPA, possibly originated from halogenated BPAs. The other thirteen compounds were not identified, but some of them were probably a kind of clinical medicines and a kind of industrial reagents.

The fifth chapter summarizes the studies presented in chapters 2 to 4. In those studies, several measurement methods were developed to determine the behaviors of toxic chemicals in water and during the sewage treatment process. However, the number of compounds that need to be determined in water is increasing at an accelerated pace. Therefore, it is necessary to develop quantitative methods for simultaneous determination in multiple environmental media with higher sensitivity, precision, and accuracy, using newly-developed technologies. In terms of qualitative analysis, further research is needed for target screening analysis and non-target environmental analysis.

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First Chapter

Introduction

It has been reported by the American Chemical Society that over 100 million kinds of chemicals exist in the world in 2018,¹⁾ benefiting people and making their lives more comfortable and more affluent. On the other hand, there are many hazardous chemical substances that influence human health and ecosystem functions. The first chapter presents the current situation and problems associated with hazardous chemical substances, and their environmental chemistry and environmental analysis.

1 Background in Environmental Chemistry

In Japan, Four Big Pollution Diseases were caused one after another in the latter half of the 1950s. With these serious problems, enhanced public interest in environmental pollution has emerged, and environmental laws including the Basic Pollution Control Act (1967) were enacted.

Polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs), which are persistent organic pollutants (POPs), have been studied since the 1960s and 1970s respectively, when those chemical substances were reported to be accumulative in the environment and to deeply influence human health and ecosystems.

In recent years, diversified production and use of chemical substances has caused a variety of chemical pollution sources and pathways to the environment. There has been global concern regarding the unintentional presence of pharmaceutical and personal care products (PPCPs; diverse organic groups, such as antibiotics, hormones, antimicrobial agents, synthetic musks, etc.) in various compartments of the aquatic environment (i.e., sewage, river, ocean) and in the human body. The chemical substances in PPCPs are mostly polar and can be absorbed in the human body. On the other hand, some of them are hardly decomposed in the human body and in sewage treatment plants, and are discharged into the environment.²⁾

2 Background in Environmental Analysis

2-1 Quantitative Analysis

Development of analytical methods for environmental chemical substances is indispensable for evaluating environmental pollution and human exposure.

Environmental pollution with polar, semi-volatile, and heat-labile compounds, which adversely affect humans, living things, and ecosystems, is one area of concern. The polar, semi-volatile, and heat-labile compounds are usually difficult to analyze by gas chromatography/mass spectrometry, which is

a mature method in environmental analysis. The compounds can be analyzed mostly by high performance liquid chromatography/mass spectrometry (HPLC/MS), which has been rapidly progressing since the beginning of the 2000s. Improvements in instrument performance have contributed to the progress in HPLC/MS. On the other hand, the environment receives all kinds of substances, which means that interference with co-existing matrices must be prevented when analyzing the compounds of interest in the environmental media. In order to accurately determine the target compound, it is necessary to understand the chemistry of the instrumental analysis itself in addition to understanding the characteristics of the individual compounds and developing appropriate pretreatment methods for each environmental media.

In addition, as mentioned above, there are more than 100 million kinds of chemical substances in the world, therefore there is a need to develop efficient analysis methods, such as the simultaneous analysis of target compounds and related compounds. In order to obtain high precision and accuracy in simultaneous analysis, significant experimental knowledge, computing estimation, and informatics knowledge of the detailed physico-chemical properties and behaviors of chemical substances are needed.

2-2 Qualitative Analysis

In environmental analysis, qualitative methods to identify environmental pollutants quickly and comprehensively are also of great importance. To comprehensively identify unintentionally produced chemical substances in wastewater treatment plants (WWTP), a screening analysis and a non-target analysis assisted by accurate mass analysis were studied.

As the main identification method, accurate mass spectral databases were used. The databases (Mass Bank,²⁾ etc.) contain mainly submissions of mass spectra for compounds related to metabolomics. Thus, the compounds identified using these databases were very few. There are two ways to perform the qualitative analysis of environmental chemicals: one is to use accurate mass databases of environmental chemicals, which must be further developed; the other is to develop mass fragment databases based on chemical structure analysis.^{4, 5)}

3 Study Subject and Outline

In this study, measurement methods for (A) aromatic amines from PPCPs, and (B) chlorinated bisphenol A congeners (Cl_xBPAs) and their analogous compounds showing POPs-like properties were developed.

3-1 The Second Chapter

Aromatic amines contained in PPCPs have become of interest as new environmental pollutants in recent years. Quantitative methods for aromatic amines in shampoo-water, human urine, and sewage water effluent were

developed using high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) coupled with solid phase extraction or liquid-liquid extraction. Human urine, shampoo-water, and sewage water effluent are an important primary releasing factor for chemicals from PPCPs, an exposure and excretion indicator, and a primary releasing factor contributing to the environmental burden, respectively. Aromatic amines contained in hair dyes are highly polar and easily oxidized. Since all the samples originating from hair dyeing are water samples, it is not easy to extract the highly polar aromatic amines. The author developed these analytical methods in order to reveal the concentration of aromatic amines in each sample.

3-2 The Third Chapter

An HPLC/MS/MS analysis method coupled with liquid-liquid extraction was developed to determine halogenated bisphenol A congeners in sewage treatment water. The basic skeleton of the halogenated bisphenol A congeners is bisphenol A (BPA), and the congeners show POPs-like properties. Because the congeners are also structurally similar to estrogen, they were designated as Class I chemical substances in 2001. On the other hand, Cl_xBPAs species have reportedly higher binding activity to estrogen receptors,⁵⁻⁷⁾ yet remain unregulated as of 2017. Using the analysis method, the author aimed to clarify the concentration of these chemicals in wastewater before and after the sterilization process. During the wastewater treatment process, chlorine sterilization is a possible source of Cl_xBPAs. It has been suggested that Cl_xBPAs are frequently detected in influent water, are unintentionally produced from BPA and four kinds of brominated BPAs (Br_yBPAs) during the sterilization process.

3-3 The Fourth Chapter

In addition, halogenated organic compounds produced by the sterilization process in WWTPs were identified by accurate mass analysis. It has been suggested that some halogenated organic compounds other than Cl_xBPAs are unintentionally produced by the sterilization process in WWTPs. However, it is difficult to identify those compounds using accurate mass databases, in which few mass spectra of environmental compounds are registered, and impossible to reveal whether the compounds are persistent in the environment. The author tried to estimate the structures of the compounds by combining available identification methods.

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Second Chapter

A High Performance Liquid Chromatography/Tandem Mass Spectrometry Method Development and Application to Study Anthropogenic Emission Sources of Hazardous Aromatic Amines in Water

1 Introduction

Hair dyeing has become a fairly common practice in recent years, with many people frequently using some type of cosmetic dye to change or enhance the color of their hair. In 2012, hair dye production increased for the fifth consecutive year, reaching 30,777 tons.¹⁾ The beauty industry produces a wide range of hair colors by combining diverse aromatic amines. The toxicity of aromatic amines has been discussed in the reports of scientific committees^{2,3,6)} and in scientific journals.^{7,8)} Hair dye ingredients have been implicated in many kinds of adverse effects in humans,²⁻⁸⁾ for example: dermatitis,^{2,3)} allergies,⁴⁾ and cancer.⁵⁾

The aromatic amines 5-amino-2-methylphenol (5A2MP), 2,5-diaminotoluene (2,5-DAT), and 4-aminophenol (4AP) are major ingredients in hair dye (Fig. 1). Nephrotoxicity in rats and chromosomal aberrations in human lymphocytes have been shown to be induced by 4AP.⁷⁾ No chronic toxicity has been reported for amino cresols, except for 4-amino-*m*-cresol, which was shown to have a strong cytotoxic effect in a gene mutation study.⁶⁾ 2,4-diaminotoluene has been shown to induce genotoxic effects in the human hepatoma cell line HePG2, such as inhibition of the damaged DNA repair mechanism and micronucleus formation.⁸⁾ An investigation of the chronic toxicity of 2,5-DAT showed that it induced an increase in the number of cells with structural chromosomal aberrations, indicating genotoxic (clastogenic) activity.²⁾

Considerable amounts of aromatic amines from hair dye are washed into domestic wastewater when shampooing dyed hair. Moreover, a portion of the aromatic amines deposited on the scalp can pass through the skin; some of which is immediately excreted in human urine and feces, while the rest is transferred to other organs by secondary deposition, and gradually metabolized. However, little is known about human exposure to aromatic amines through hair dyeing. This is, in part, due to the difficulties in developing accurate measurement methods for aromatic amines and their metabolites in water, human urine, and other biological fluids. These difficulties arise from the fact that aromatic amines and

their metabolites are highly hydrophilic and susceptible to oxidation. The high hydrophilicity of these amines makes it difficult to recover them from samples such as river water, shampoo-water mixture, and human urine, which are predominantly composed of water. In addition, the recoveries of these aromatic amines are reduced owing to rapid oxidation during cleanup. Consequently, very few methods are available for measuring hair dye ingredients.

The levels of aromatic amines and their intermediates in hair dye products were measured by thin layer chromatography.⁹⁾ High performance liquid chromatography (HPLC) measurements for hair dye have been reported for seven aromatic amines,¹⁴⁾ including the three aminophenol isomers¹⁵⁾ and phenolic compounds.¹⁶⁾ For human urine measurements, only a gas chromatography (GC) report for *p*-phenylenediamine derivatives¹⁷⁾ and a thin-layer chromatography (TLC) report for *p*-aminophenol¹⁸⁾ were found.

However, it is difficult to measure aromatic amines and their intact conjugate molecules in hair dye ingredients with the methods above. The analysis of intact molecules of aromatic amines and their conjugates is then of great significance in order to assess human exposure. Here we describe three high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) methods combined with solid phase extraction (SPE) and solvent dilution to determine the concentration of free intact aromatic amine molecules in environmental water, shampoo-water mixture, and human urine respectively, because shampoo-water and urine are the main sources of aromatic amines from hair dye to environmental water. The author also applied the methods to discussing the behaviors of aromatic amines in shampoo-water from colored hair, urine excreted after hair-dyeing, and wastewater treatment plant (WWTP) effluent.

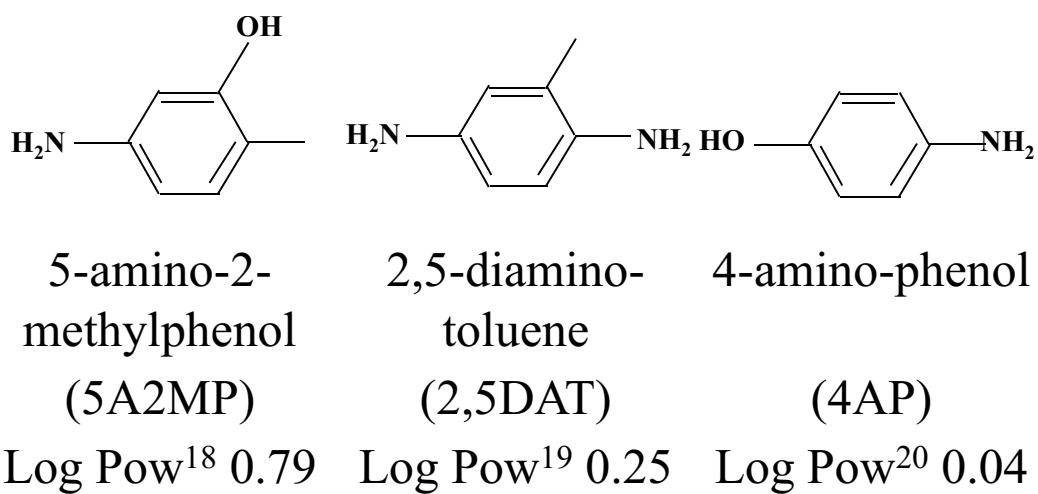


Figure 1 Chemical Structures, Mass Transition Ion Pairs for Selected Reaction Monitoring, and Log P_{ow} Values of Three Aromatic Amines: 5A2MP: 5-Amino-2-methylphenol, 2,5-DAT: 2,5-Diaminotoluene, and 4AP: 4-Aminophenol

2 Experimental

2-1 Target Chemicals and Reagents

Ascorbic acid (99.6%), formic acid (98%), and distilled water for HPLC were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Pure water ($\leq 1.00 \mu\text{S}/\text{cm}$) was obtained from Kobayashi-Shokai (Aichi, Japan). Acetonitrile (ACN) for HPLC was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) for HPLC was purchased from Merck (Whitehouse Station, NJ, USA). Ammonia water (28 w/v%, abbreviated as “28 w/v% NH_4OH ”) was obtained from Yoneyama Yakuhin Kogyo Co., Ltd. (Osaka, Japan). 2-propanol (IPA) and polyethylene glycol 200 (PEG) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-isopropyl-3-methylphenol (4I3MP) and 4-allyl-2-methoxyphenol (Eugenol) were obtained from Tokyo-Kasei (Tokyo, Japan). 5A2MP (97%) and 2,5-DAT (98%) were purchased from Tokyo-Kasei (Tokyo, Japan), while 4AP (98%) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Standard solutions (1000 mg/L) of 5A2MP and 4AP were made in ACN, while that of 2,5-DAT was made in a 1:1 (v/v) mixture of ACN and water. Working solutions (1 mg/L in ACN) of the three compounds were prepared by diluting their respective standard solutions. The calibration curves for the aromatic amines were good in the range of 1-300 $\mu\text{g}/\text{L}$.

2-2 Materials

An Oasis MCX Plus Short Cartridge was purchased from Waters (Milford, MA, USA), and a DISMIC filter (13CP045AN, 0.45 μm polytetrafluoroethylene filter) was obtained from Advantec (Tokyo, Japan). A C18 SPE cartridge (500 mg, 6 cc) and an SPE Manifold were obtained from GL Sciences (Tokyo, Japan). An ultrasonic cleaner US-3 (As One, Osaka, Japan), a centrifuge separator CF15R (HITACHI High-tech, Tokyo, Japan), and an in-house fabricated nitrogen-based concentrator were also used.

2-3 Instrument Conditions

The operation conditions used in the HPLC and MS studies are summarized in Table 1. An Agilent 1100 Series HPLC system was used; the amide column TSK Gel Amide 80 (Tosoh, Tokyo, Japan) was used in the hydrophilic interaction chromatography (HILIC) mode, which required a long equilibration time (15 min). An API3000 HPLC/MS/MS system with an ESI source was used for determining the aromatic amines by selected reaction monitoring (SRM).

Table 1 Operating Conditions for High Performance Liquid Chromatography/Tandem Mass Spectrometry

Agilent 1100/API 3000					
Column	Tosoh TSK Gel Amide 80 (2.2 mm×150 mm, 3 μm), 40°C				
Guard column	GL Sciences Inertsil Amide (1.5 mm×10 mm, 3 μm), 40°C				
Gradient profile	Time (min)	0	3	3.1	8
Mobile phase	A: 0.1 % formic acid	10	10	80	80
	B: Acetonitrile	90	90	20	20
Flow rate	0.2 mL/min				
Post time	15 min (Acetonitrile 90 %)				
Injection volume	10 μL				
Curtain gas/ Nebulizer gas	14 psi/ 14 psi				
Ion source temperature	400°C				
Entrance potential	5500 V				
Declustering potential (volts)	5-amino-2-methylphenol: 66/37 2,5-diaminotoluene: 91/39				
Collision energy (volts)	4-aminophenol: 16/29				
Monitor ion sets in Selected Reaction Monitoring	5-amino-2-methylphenol:124/71 (+) 2,5-diaminotoluene:123/108 (+) 4-aminophenol:110/92 (+)				

2-4 Sample Preparations

2-4-1 River Water

The flow chart for sample preparation and analysis is shown in Fig. 2-A. Ascorbic acid (0.5 g), used as an antioxidant, was added to 50 mL of river water (Uchitsu river in Kasugai Aichi). Then, the sample was loaded at a flow rate of 1 mL/min into a cation exchange cartridge (Oasis MCX Plus Short Cartridge), which was conditioned with 5 mL of MeOH followed by 5 mL of pure water prior to use. After washing the cartridge with 5 mL of MeOH, the aromatic amines were eluted with 5 mL of 28 w/v% NH_4OH /MeOH (5/95, v/v) into a 10 mL test tube. Then 150 μL of ACN were added as keeper solvent prior to concentrating the eluted solution to reduce the evaporation losses of the aromatic amines. Subsequently, the solution in the test tube was concentrated to 1 mL or less on a heater block. The temperature of the heater block was set such that the temperature of the solution was below 37°C. The head space was purged with nitrogen gas during the concentration process.

However, the concentrated solution still contained some water from the 28 w/v% NH_4OH . Therefore, despite the eluted solution being concentrated to 1 mL, the ratio of water/MeOH was 1/4 (0.25 mL of water from 28 w/v% NH_4OH /0.75 mL of MeOH) in the concentrated solution. In contrast, the NH_3 from the 28 w/v% NH_4OH was mostly removed during the concentration process because the boiling point of NH_3 is -33.3°C. Then, 1 mL of ACN was added to the concentrated solution and further concentrated to completely remove the dissolved MeOH (approx. 0.75 mL) from the concentrated solution, to adjust its composition and make it similar to that of the mobile phase. Finally, the above solution was concentrated to 1 mL or less, diluted up to 1 mL with ACN, and subjected to HPLC/MS/MS analysis. It was subsequently found that the change of solvent from MeOH to ACN during the concentration process was not necessary for the HPLC/MS/MS analysis.

2-4-2 Shampoo-Water Mixture

Aromatic amines are expected to be present at a higher concentration in shampoo-water mixture than in river water. Thus, it was not necessary to concentrate the solution eluted from the MCX cartridge. The flow chart for sample preparation and analysis is shown in Fig. 2-B. This experiment used a shampoo-water mixture containing 4.7 g/L of shampoo liquid, equivalent to the average concentration in shampoo water from five shampooed people. Shampoo typically comprises many surfactants, whose main component is an amphiphilic molecule containing a hydrophilic head group and a long hydrophobic chain.

Ten milliliters of the shampoo-water mixture were diluted with 190 mL of 1% ascorbic acid to prepare the sample solution, which was loaded at a flow rate of 1 mL/min into a series of Inertsil C18 and Oasis MCX Plus Short cartridges

conditioned with 5 mL of MeOH followed by pure water prior to use. The C18 cartridge was used upstream for removing hydrophobic species from the shampoo-water mixture, and the MCX cartridge was used downstream for collecting the aromatic amines passing through the C18 cartridge. After washing the MCX cartridge with 5 mL of MeOH, the aromatic amines in the MCX cartridge were eluted with 3 mL of 28 w/v% NH_4OH /MeOH (5/95, v/v). The eluted solution was diluted to 5 mL with MeOH and subjected to HPLC/MS/MS analysis. In the above preparation, it was not necessary to change the solvent from MeOH to ACN in the eluted solution. The recovery experiments were carried out by adding 0.1 μg of aromatic amines to 10 mL of shampoo-water mixture, then applying the method described above.

2-4-3 Human Urine

The flow chart for sample preparation and analysis is shown in Fig. 2-C. Twelve milligrams of ascorbic acid were added to 1 mL of human urine, obtained from a person who had not dyed his hair for at least 6 months. The human urine was then diluted with 4 mL of MeOH/IPA (1/1, v/v). The diluted solution was sonicated for 10 min and centrifuged at 3000 rpm for 10 min. Then, the supernatant was filtered through the DISMIC filter and subjected to HPLC/MS/MS analysis. The recovery experiments were performed by adding 1 μg of each aromatic amine to human urine, then applying the method described above.

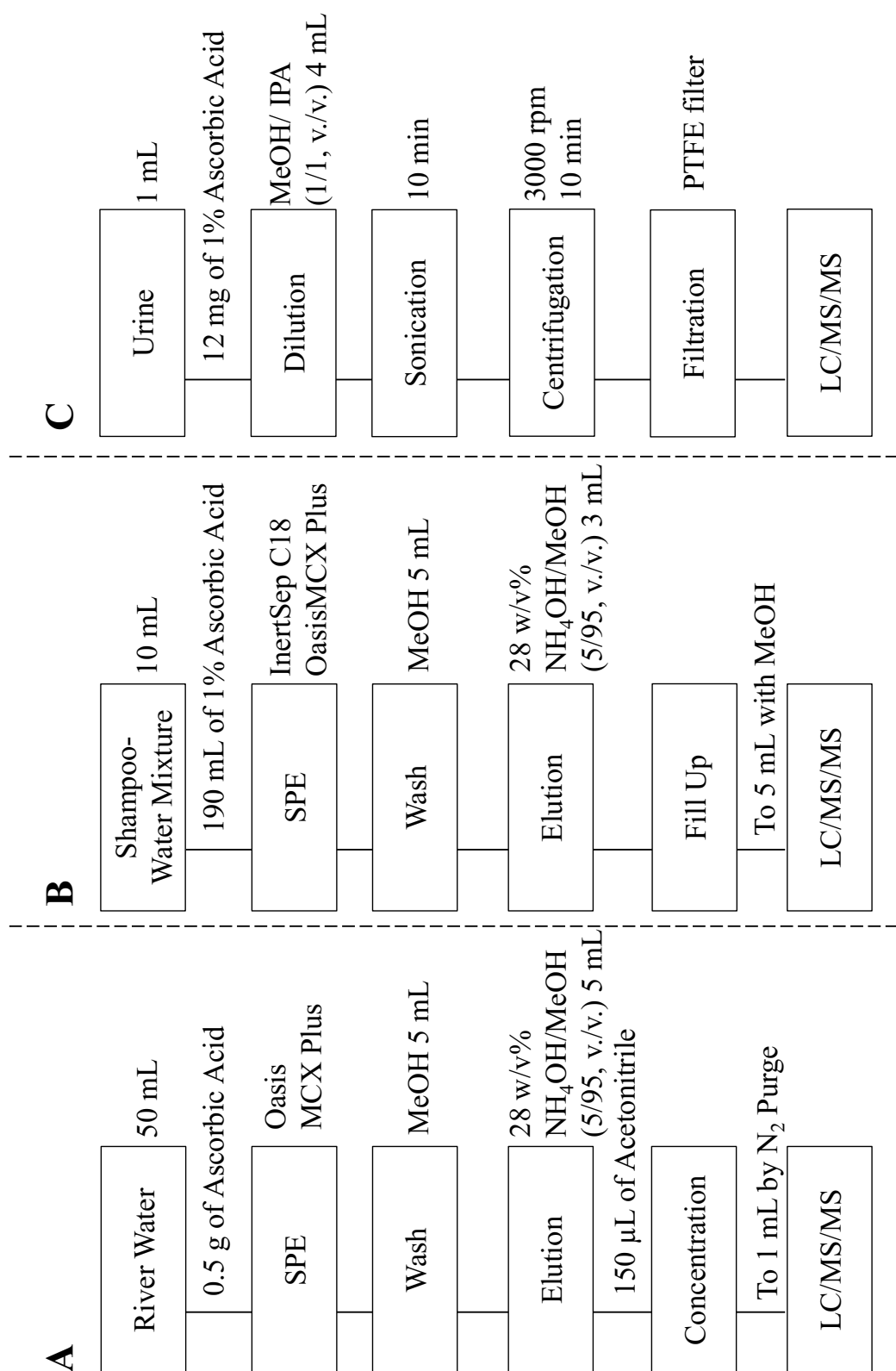


Figure 2 Analytical Scheme for the Determination of Aromatic Amines in River Water (A), Shampoo-Water Mixture (B), and Human Urine (C).

3 Results and Discussion

3-1 Recoveries from River Water, Shampoo-Water Mixture, and Human Urine

Table 2 shows recoveries from river water, shampoo-water mixture, and human urine.

Table 2 Recoveries and Limits of Detection (LOD) of Aromatic Amines in Pure Water, River Water, Shampoo-Water Mixture, and Human Urine

		n [*]	5A2MP	2,5-DAT	4AP
Pure Water (2 ng/mL) **	Average (%)		100	102	93
	RSD *** (%)	7	9.5	0.9	15
	LOD (ng/mL)		0.4	0.03	0.6
River Water (2 ng/mL) **	Average (%)		124	98	97
	RSD *** (%)	7	13	4.9	3.8
	LOD (ng/mL)		0.5	0.2	0.2
Shampoo-water Mixture (10 ng/mL) **	Average (%)		84	80	112
	RSD *** (%)	7	5.9	4.7	3.7
	LOD (ng/mL)		11	9.1	7.2
Human Urine (1000 ng/mL) **	Average (%)		129	107	76
	RSD *** (%)	7	3.3	8.3	7.7
	LOD (ng/mL)		64	161	149

* Number of Experiments

** Prepared Concentration

*** Relative Standard Deviation

5A2MP: 5-amino-2-methylphenol, 2,5-DAT:2,5-diaminotoluene

4AP: 4-aminophenol

3-2 Method Qualification for River Water Analysis

3-2-1 Decrease in Recovery in the Concentration Process

The factors affecting the recoveries of aromatic amines include losses due to oxidation and evaporation when concentrating the dichloromethane solution.

(A) Additives in Concentrating Solution from Solid Phase Cartridge

In the keeper solvent experiment, 5 mL of a 28 w/v% $\text{NH}_4\text{OH}/\text{MeOH}$ (5/95, v/v) solution containing 0.1 μg of each of the three target chemicals and a keeper solvent were concentrated to 1 mL with a nitrogen gas concentrator. Four solvents (150 μL) that exhibit π - π interactions or are capable of hydrogen bonding with aromatic amines were examined as keeper solvent candidates, namely ACN, PEG, 4I3MP, and Eugenol. The recovery efficiencies of the standards in the concentration process were compared at a solution temperature of 55°C (the temperature of the heater block was 200°C). The results are shown in Fig. 3.

The recoveries of all target chemicals were below 40% when using Eugenol. When using PEG and 4I3MP, the recoveries of 2,5-DAT and 4AP were below 50 % and 40%, respectively. When using ACN as the keeper solvent, the recoveries of 5A2MP and 2,5-DAT were 92.7% and 103.8%, respectively, while the recovery of 4AP was low (35.7%). This is owing to the vapor pressures of the aromatic amines being low enough for the retention of the amines in the recovery standard containing added ACN (owing to π - π interactions between ACN and the amines).

(B) Temperature of Concentrating Solution from Solid Phase Cartridge

In the experiment on the temperature during the concentration process, 5 mL of a 28 w/v% $\text{NH}_4\text{OH}/\text{MeOH}$ (5/95, v/v) solution containing 0.1 μg of each of the three target chemicals and 150 μL of ACN as a keeper solvent were concentrated to 1 mL at 37°C (in experiment A, the temperature was 55°C). The recoveries of 5A2MP, 2,5-DAT, and 4AP were improved to 113.0%, 128.8%, and 92.9%, respectively. These results suggest that the loss of 4AP during the concentration process occurs mostly at higher temperatures.

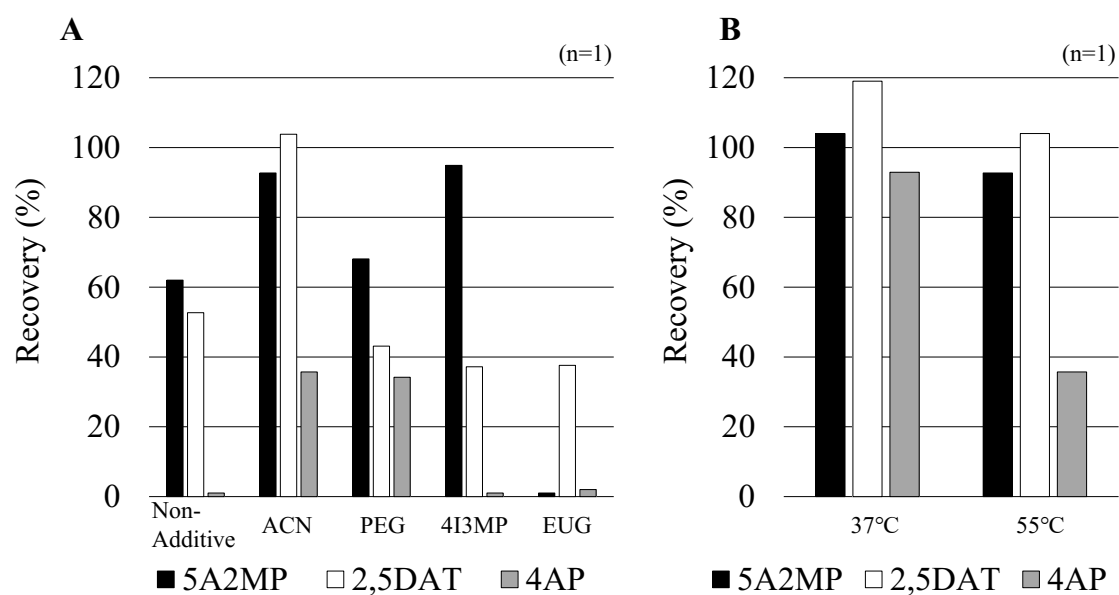


Figure 3

(A) Effect of the Keeper Solvent Added during the Concentration Process on the Recoveries of Aromatic Amines

ACN: acetonitrile, PEG: polyethyleneglycol, 4I3MP: 4-isopropyl-3-methylphenol, EUG: 4-allyl-2-methoxyphenol.

(B) Effect of Temperature during the Concentration Process on the Recoveries of Aromatic Amines.

3-2-2 Determination of Breakthrough Volumes and Simulation of Elution Profiles for Concentrating the Aromatic Amines by Solid Phase Extraction

Both the breakthrough volume and the elution profiles of aromatic amines on the SPE cartridge were obtained by SRM with the apparatus shown in Fig. 4. The MCX cartridge, packed with divinylbenzene-N-vinylpyrrolidone copolymer with sulfo groups, binds weak basic compounds such as the target chemicals by electrostatic interaction. Therefore, if aromatic amines are loaded onto the MCX cartridge under acidic conditions, they will be eluted with short retention times. Sample water spiked with the aromatic amines was loaded onto the MCX cartridge in the setup shown in Fig. 4-A, where the breakthrough profiles of the aromatic amines eluted from the MCX cartridge could be monitored. Formic acid and ascorbic acid were examined for subjecting the sample water to acidic and acidic-reductive conditions, respectively. After adding 0.1 μg of the standard reagents to the MCX cartridge, 50 mL of 1% formic acid were passed through the MCX cartridge using an HPLC pump. Then, the MCX cartridge was replaced with a new MCX cartridge before 1% ascorbic acid was passed through it. No peak corresponding to aromatic amines was observed as they passed through the MCX cartridges, suggesting that the aromatic amines were being retained or decomposed in the cartridges. To confirm this, three syringes were set upstream of the MCX cartridge; all three syringes were filled with 28 w/v% $\text{NH}_4\text{OH}/\text{MeOH}$ (5/95, v/v). To elute the aromatic amines from the MCX cartridge, distilled water was passed from the HPLC pump to the three syringes (Fig. 4-B). With the setup above, it was possible to avoid passing highly concentrated NH_4OH solution through the HPLC pump system. Also, it was possible to avoid over diluting the solution with distilled water.

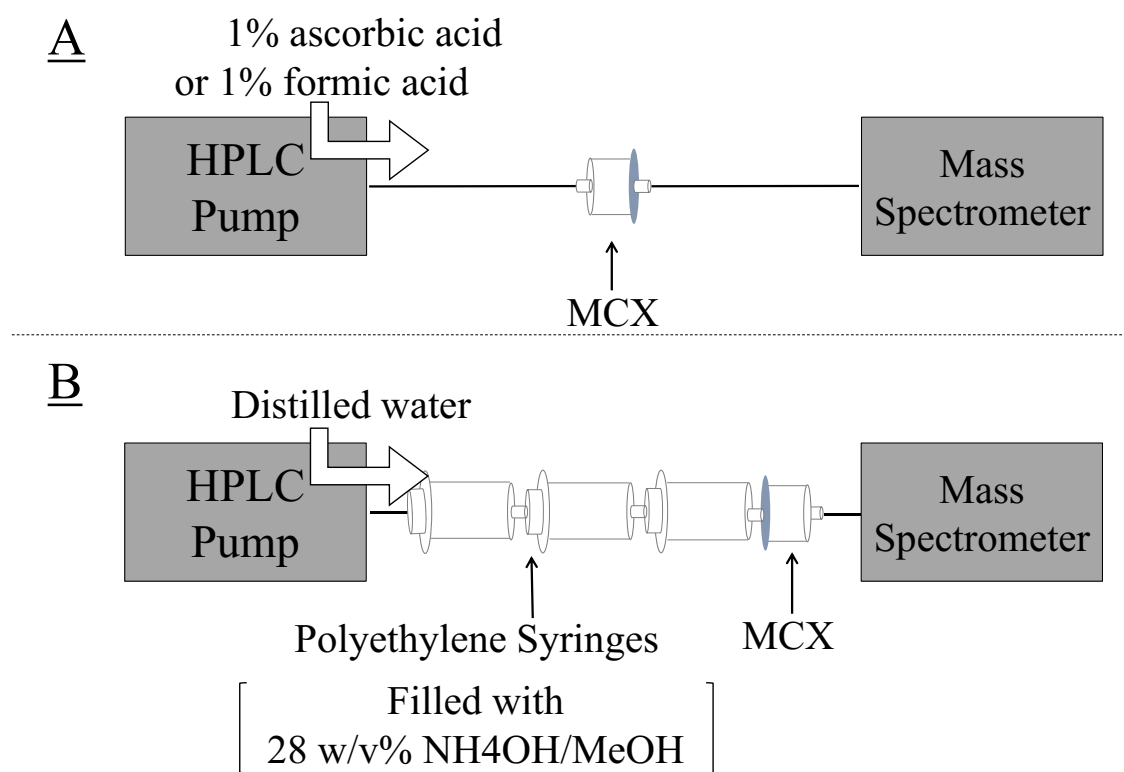


Figure 4 Instrumental Scheme for Monitoring the Breakthrough Volume and Elution Profiles of Aromatic Amines from the Solid Phase Extraction Cartridge.

The aromatic amine elution chromatograms from the MCX cartridges with mobile phases of 1% formic acid solution and 1% ascorbic acid solution are shown in Fig. 5. The aromatic amines were retained in the MCX cartridges with both mobile phases. However, a significant difference was observed in the chromatograms between the two mobile phases (Fig. 5).

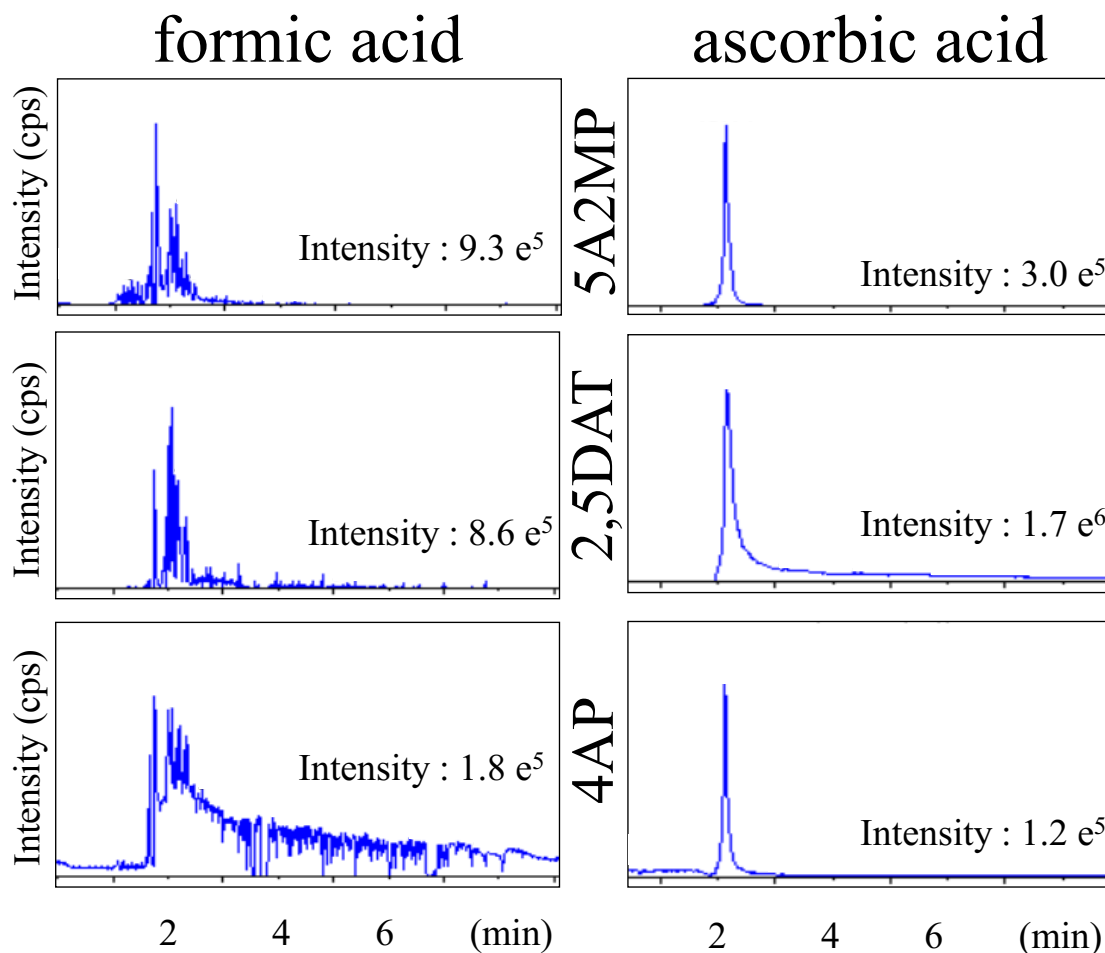


Figure 5 Elution Profiles of Aromatic Amines from the Cation-Exchange Cartridge after Adding the Aromatic Amines to Solid Phase Extraction Using 1% Formic Acid Aq. (Left) or 1% Ascorbic Acid Aq. (Right).

The elution chromatogram with 1% formic acid solution exhibited peaks of lower intensity and smaller area. This might be due to irreversible adsorption on the cartridge or partial decomposition. On the other hand, the elution chromatogram with 1% ascorbic acid solution exhibited peaks of higher intensity and larger area. Therefore, the river water sample was added using 1% ascorbic acid solution. The recoveries from river water were 124% for 5A2MP, 98% for 2,5-DAT, and 97% for 4AP (Table 2). Fig. 6 shows the SRM chromatograms of the aromatic amines in river water. High and sharp peaks corresponding to the aromatic amines were observed in the chromatograms of the spiked river water sample, while no aromatic amine was found in those of the original river water sample.

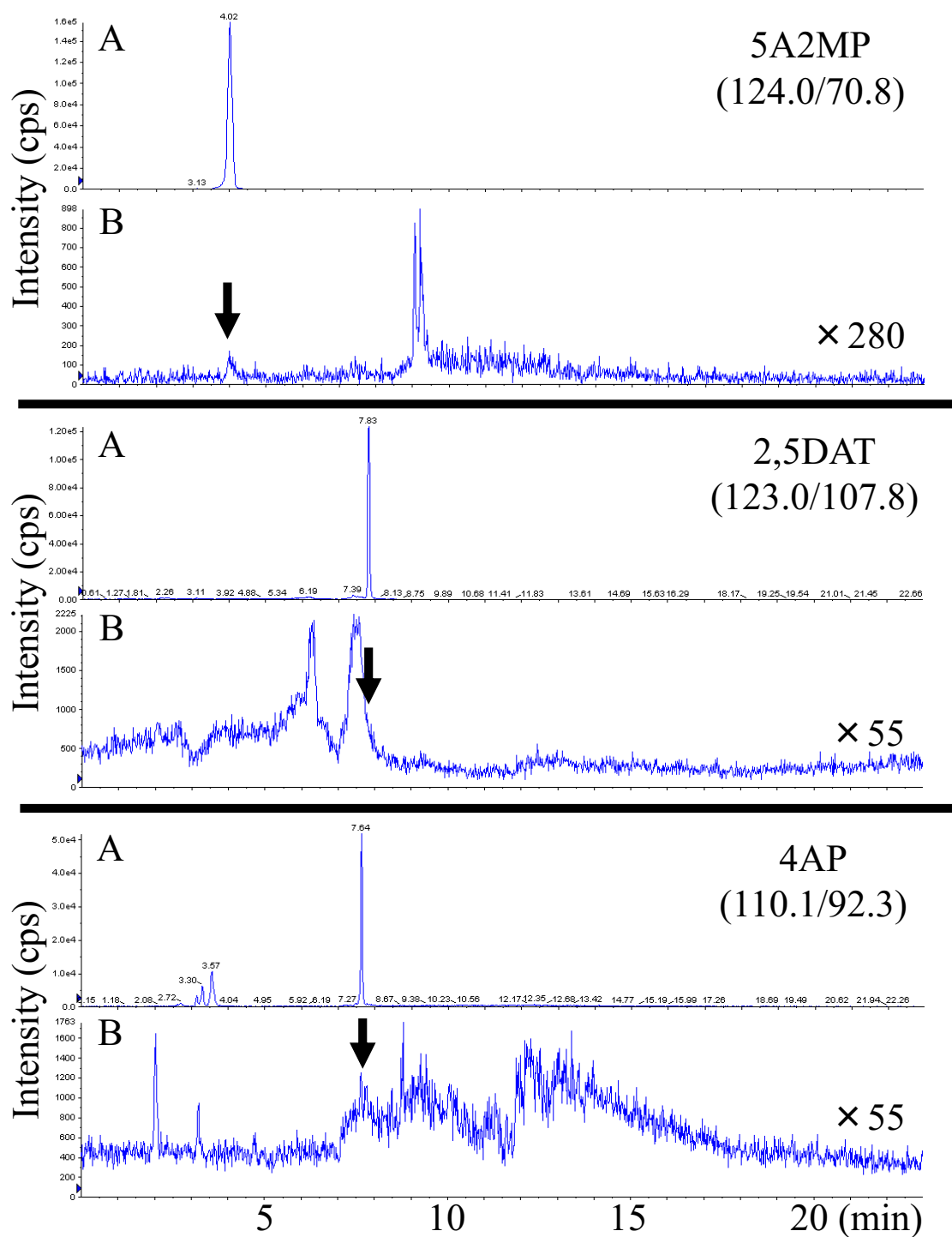


Figure 6 Selected Reaction Monitoring Chromatograms of Aromatic Amines in River Water Spiked with the Three Aromatic Amines (0.1 $\mu\text{g/mL}$ each) (A), and in a Non-Spiked Sample (B).

3-3 Method Qualification for Shampoo-Water Mixture Analysis

The effect of the ascorbic acid concentration (1, 5, or 20 w/v%) on the recovery of aromatic amines from shampoo-water mixture was examined. The most reliable results were obtained with the sample containing 1% ascorbic acid, where the mean recoveries of 5A2MP, 2,5-DAT, and 4AP were 69% with a relative standard deviation (RSD) of 4.5%, 103% with a RSD of 10%, and 98% with a RSD of 6.5%, respectively (Table 3).

For samples containing 5% ascorbic acid, the recoveries of the three aromatic amines were between 70 and 80% of those obtained with 1% ascorbic acid. For samples containing 20% ascorbic acid, the recovery of 2,5-DAT was approximately four times higher than the spiked amount (in other words, the recovery of 2,5-DAT was approximately 400%), while no aromatic amine was found in the non-spiked shampoo-water mixture containing the same concentration of ascorbic acid. This phenomenon was observed only for 2,5-DAT, the amine with no hydroxyl group. This could possibly be due to increased ionization efficiency induced by residual ascorbic acid in the shampoo-water mixture, or by impurities in the solution eluted from the MCX cartridge, compared to the non-spiked shampoo-water mixture containing the same concentration of ascorbic acid. Considering that no increase in the ionization efficiency was observed for 5A2MP and 4AP, it was concluded that the phenolic hydroxyl group might suppress the increase in ionization efficiency. The recoveries from shampoo-water mixture were 84% for 5A2MP, 80% for 2,5-DAT, and 112% for 4AP (Table 2).

Table 3 Effect of Ascorbic Acid Concentration on the Recovery of Aromatic Amines from Shampoo-Water Mixture

Ascorbic acid		n *	Recovery (%)		
			5A2MP	2,5-DAT	4AP
1%	Average (%)	3	69	103	98
	RSD ** (%)		4.5	10	6.5
5%	Average (%)	3	55	73	76
	RSD ** (%)		3.8	11	9.3
20%	Average (%)	3	72	408	121
	RSD ** (%)		13	450	36

* Number of Experiments

** Relative Standard Deviation

5A2MP: 5-amino-2-methylphenol, 2,5-DAT: 2,5-diaminotoluene

4AP: 4-aminophenol

3-4 Method Qualification for Human Urine Analysis

Because large amounts of organic impurities were contained in the human urine sample, it was difficult to collect aromatic amines by SPE, even after diluting the human urine sample with large amounts of water. The human urine sample was diluted with organic solvents to remove large organic solvent-insoluble molecules. Many water-soluble organic solvents were examined, but no single solvent showed good recoveries for aromatic amines from human urine samples. Then, MeOH/IPA solvent mixtures in ratios of 1/3, 1/1, and 3/1 were examined as dilution solvents to remove large organic solvent-insoluble molecules from human urine. The results are shown in Table 4. Good recoveries were obtained for the three aromatic amines in 1/1 MeOH/IPA, while the recovery of 5A2MP was low in the 3/1 composition. Considering that the $\text{Log}P_{\text{ow}}$ values of 5A2MP were higher than those of the other aromatic amines (Fig. 1), the lower recovery of 5A2MP could be due to the loss of hydrophobic interactions with the organic impurities removed from the human urine sample. The recoveries from human urine were 129% for 5A2MP, 107% for 2,5-DAT, and 76% for 4AP (Table 2).

Table 4 Effect of the Dilution Solvent Composition on the Recoveries of Aromatic Amines from Human Urine Samples

MeOH/IPA (v/v)		n *	Recovery (%)		
			5A2MP	2,5-DAT	4AP
1/3	Average (%)	3	140	45	31
	RSD ** (%)		15	8.3	9.3
1/1	Average (%)	3	126	99	83
	RSD ** (%)		2.1	3.3	4.3
3/1	Average (%)	3	75	51	45
	RSD ** (%)		44	15	17

* Number of Experiments

** Relative Standard Deviation

MeOH: Methanol, IPA: 2-propanol, 5A2MP: 5-amino-2-methylphenol, 2,5-DAT: 2,5-diaminotoluene

4AP: 4-aminophenol

4 Study of Anthropogenic Emission Sources of Hazardous Aromatic Amines in Water

4-1 Overview

Shampoo-water mixtures and urine were collected as representing environmental release sources of hazardous aromatic amines. Wastewater treatment plant effluent was collected as representing a major source of hazardous aromatic amines release to the environment.

Five volunteers colored their hair with hair dyes, then washed their scalps and hair to discharge shampoo-water. All portions of the shampoo-water were collected and prepared according to the HPLC/MS/MS method discussed here. Three volunteers provided urine excreted within 24 hours after dyeing their hair. Additionally, a total of nine WWTPs effluent samples were collected in the Aichi prefecture.

4-2 Study Results

4-2-1 Concentration Levels of Hazardous Aromatic Amines in Shampoo-Water Mixture Eluted from Colored Hair

Aromatic amines in shampoo-water mixtures collected from five volunteers after using hair dyes and washing their hair were quantitated. Table 5 shows the hair dye product information, the concentration of aromatic amines in shampoo-water mixtures, and the elution percentages from hair dyes into shampoo-water mixtures. The elution percentages from shampoo-water mixture ranged from 0.7 to 43%. In hair dyes A and D, the elution percentages of the aromatic amines were very low. The results above might be due to the fact that some hair dyes contain aromatic amines mixed in the application to produce dyeing agents hard to dissolve in shampoo-water by diazo-coupling. The aromatic amines were likely to convert to diazo-compounds that are retained in hair, scalp, and other human body parts, or evaporate into the air.

No other measurement results for aromatic amine concentrations in shampoo-water mixture collected after dyeing hair exist for comparison.

Table 5 Aromatic Amines in Hair Dyes and Shampoo-Water Mixtures Collected after Hair Dying

	Color	Hair Dye (g)	Aromatic Amines in Hair Dye (mg)		Aromatic Amines in Shampoo Water Mixture (mg)	Elution Percentage (%)
A	Black	70	4AP	66	0.7	1.0
B	Brown	70	5A2MP	25	7.0	28
C	Brown	100	2,5-DAT	0.3	0.5×10^{-1}	19
			4AP	0.3	0.4×10^{-1}	13
D	Blond	210	2,5-DAT	2.0×10^3	14	0.7
			4AP	2.6×10^2	14	5.3
E	Brown	60	2,5-DAT	5.6	2.4	43
			4AP	3.4×10^2	63	17

4AP: 4-aminophenol 5A2MP: 5-amino-2-methylphenol 2,5-DAT: 2,5-diaminotoluene

4-2-2 Hazardous Aromatic Amines in Human Urine Excreted after Hair Dyeing

Three volunteers colored their hair with hair dyes containing 2,5-DAT and 4AP. Urine samples were collected just before and within 24 hours after dyeing. 51 to 370 μg of 2,5-DAT and 47 to 292 μg of 4AP were excreted into urine in 24 hours (Fig. 7). The amount of aromatic amines in urine corresponded to <0.01 to 1.9% of the amount in the applied hair dye.

2,5-DAT and 4AP behave almost similarly in human urine (Fig.8), which suggests that 2,5-DAT and 4AP have similar chemical properties and therefore behave similarly during metabolism in the human body. These results show evidence of human exposure to aromatic amines when using hair dyes. The aromatic amines were excreted in human urine, with large individual differences due, for example, to differences in metabolism or in the amount of diazo-compounds produced from the aromatic amines, etc.

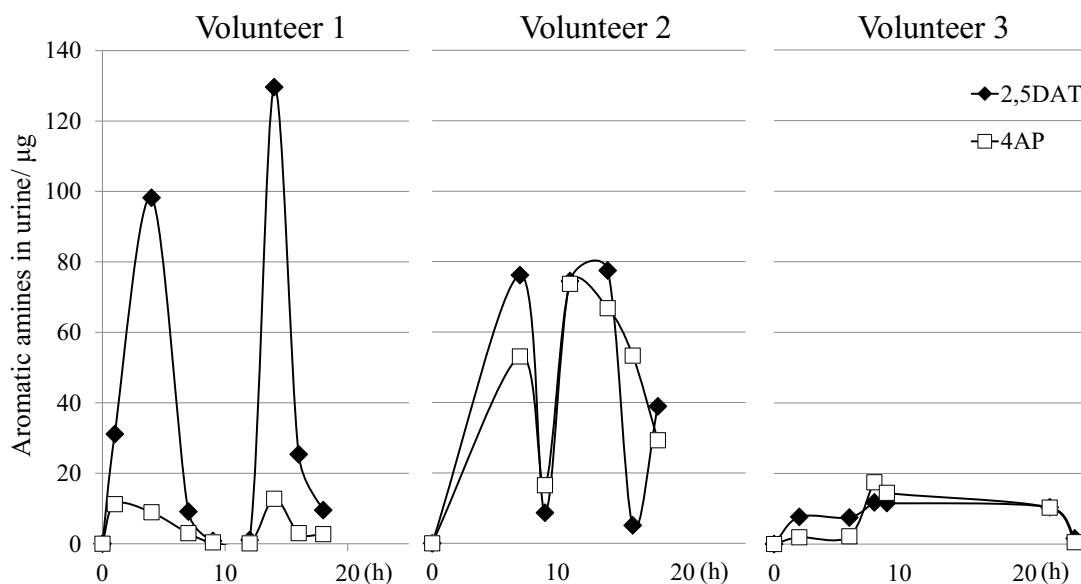


Figure 7 Analysis Results of Aromatic Amines Excreted into Human Urine after Using Hair Dyes

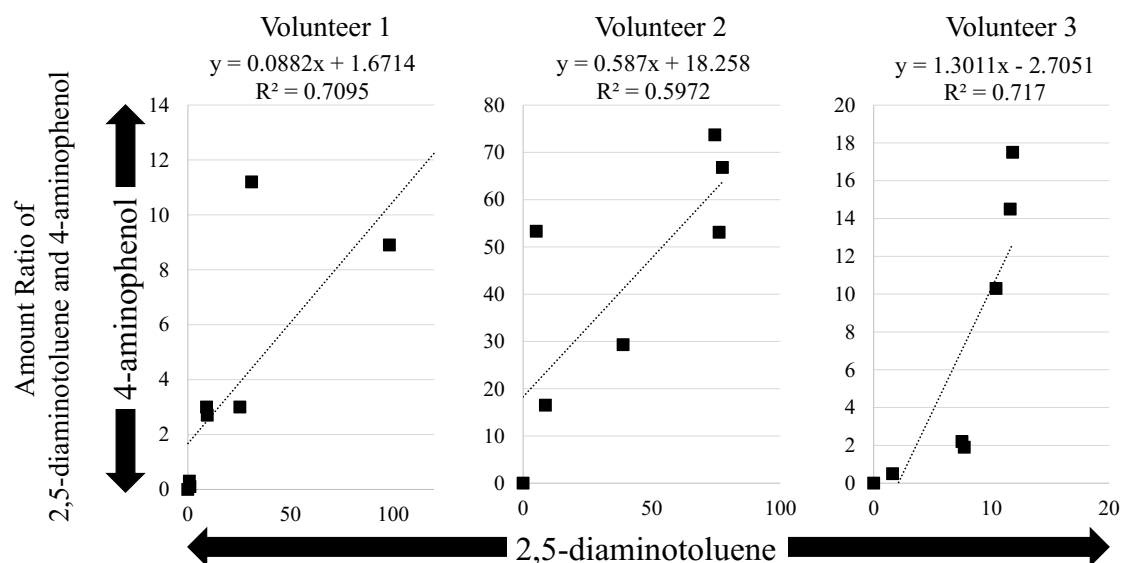


Figure 8 Ratio of 2,5-Diaminotoluene and 4-Aminophenol Excreted into Human Urine

In a previous report, 2,5-DAT was found to rapidly absorb dermally, with 90% of the adsorbed amount excreted within 24 hours after hair dying.²²⁾ In the present study, a maximum of 0.1% of the 2,5-DAT applied while hair dyeing was excreted into human urine. A maximum of 2% 4AP, which is more hydrophilic than 2,5-DAT, was excreted into urine. Both of these results are much lower than previous result.²²⁾ On the other hand, it has been discussed that free aromatic amines, especially 4AP, would be metabolized to N-acetylated species and glucuronic acid conjugates.²³⁾ In a previous study,²³⁾ aromatic amines were found to be likely converted to aromatic amine metabolites in the human body.

4-2-3 Hazardous Aromatic Amines in Effluent from Wastewater Treatment Plants

Nine effluent samples were collected at nine WWTPs in the Aichi prefecture. Fig. 9 shows that aromatic amines were mostly below the LODs at all plants. Although most data were below the LODs, we tried to approximate the concentrations levels. 4AP, which was found in higher concentrations than 2,5-DAT and 5A2MP, is widely used as a pharmaceutical intermediate, a dye intermediate, an anti-oxidant inhibitor, etc. The higher concentration level of 4AP compared to the other two aromatic amines might also be due to its stronger hydrophilicity, which makes it less likely to be removed in WWTPs.

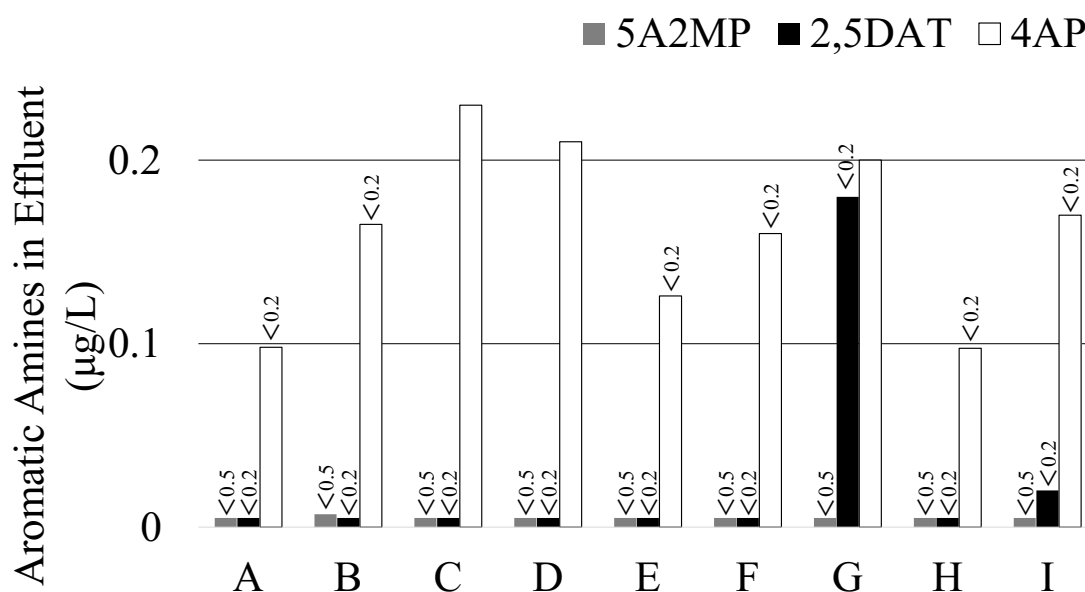


Figure 9 Analysis Results of Aromatic Amines in Effluent from Nine Wastewater Treatment Plants

Eight water samples were collected for every three hours at the same place from a river downstream of a WWTP, which received more domestic than industrial wastewater. Fig. 10 shows the time-dependent concentration profile of aromatic amines in that river. It suggests that aromatic amines are likely to be released throughout the day and to be discharged not only from domestic sources but also from industrial ones.

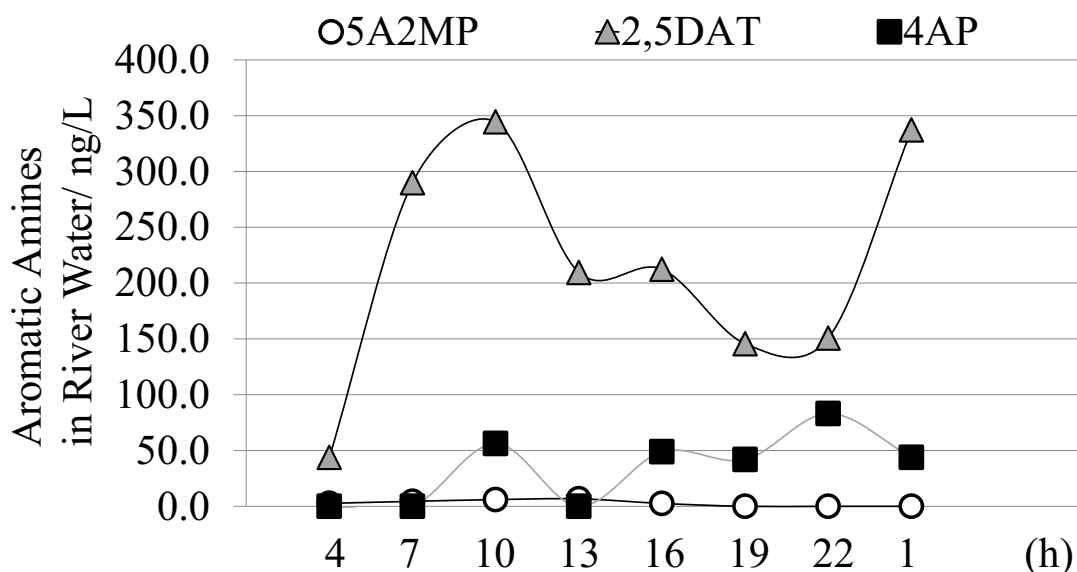


Figure 10 Time-Dependent Concentration Profiles of Aromatic Amines in a River Downstream of a Wastewater Treatment Plant.

An investigation of the chronic toxicity of 2,5-DAT showed that it induced an increase in the number of cells with structural chromosomal aberrations, indicating genotoxic (clastogenic) activity at or above $25\mu\text{g/L}$.²⁾ In the present study, 2,5-DAT was found at levels below $0.25\mu\text{g/L}$ in river waters, too low to cause the chronic toxicity issues previously reported. No toxicity data were found for the low concentrations reported here.

5 Conclusions

Because aromatic amines are extremely water soluble and highly susceptible to oxidation, it has been difficult to determine their concentration in hair dye and their metabolites in river water, shampoo-water mixture, and human urine. As a step toward solving this problem, we developed HPLC/MS/MS methods for measuring 5A2MP, 2,5-DAT, and 4AP in river water (LOD: approx. 1 ng/mL), shampoo-water mixture (LOD: approx. 10 ng/mL) and human urine (LOD: approx. 160 ng/mL). Because 2,5-DAT and 4AP are more susceptible to oxidation than 5A2MP, it is necessary to prepare the samples at low room temperature and with shorter cleanup time. These new methods for aromatic amines have not only improved selectivity but also sensitivity compared to the very few previously reported methods. For example, the LOD for 2,5-DAT was previously 161 ng/mL in human urine, which is 1/160 of that obtained using HPLC/UV;⁹⁾ the new method LOD for 4AP is 149 ng/mL in human urine, which is 1/33 of that previously obtained using thin layer chromatography.²¹⁾

In the analysis of river water, the aromatic amines added with ascorbic acid were efficiently collected with an MCX cartridge. In the analysis of shampoo-water mixture, the aromatic amines could be determined by diluting the sample nineteen-fold with 1% ascorbic acid aqueous solution, followed by SPE and HPLC/MS/MS. In the analysis of human urine, the sample was diluted fourfold with MeOH/IPA (1/1, v/v), filtrated, and subjected to HPLC/MS/MS analysis. For the river water analysis, it was essential to add ACN to the eluted solution and to keep the solution temperature below 37°C during the concentration process. For human urine, it was also essential to remove large water-soluble molecules. The methods presented here are a first step for analyzing human exposure and environmental impacts from dyeing hair. With these methods, three aromatic amines could be quantified in environmental water, shampoo-water mixture, and human urine.

Aromatic amines in shampoo-water mixture, urine after dyeing hair, and river water were determined with the HPLC/MS/MS methods developed in the present study.

The percentages of aromatic amines transferred from hair dye into the shampoo-water mixture were low (0.7 to 43%). This suggests that aromatic amines are likely to convert to diazo-compounds, remain as residues on hair, scalp, and other parts of the human body, or evaporate. There are no previous measurement data for aromatic amines from hair dying in shampoo-water mixture to compare our results with.

A maximum of 0.1% 2,5-DAT from hair dyeing was excreted in urine. For 4AP, which is more hydrophilic than 2,5-DAT, a maximum of 2% of the amount applied when hair dying was excreted into urine. Both of these values

are extremely low compared to previous results that found 2,5-DAT rapidly absorbed dermally, with 90% excreted within 24 hours after hair dying²²⁾. On the other hand, it has been discussed that free aromatic amines, especially 4AP, could be metabolized to N-acetylated species and glucuronic acid conjugates.²³⁾ As discussed in a previous report, aromatic amines were likely converted to aromatic amine metabolites.

In river water downstream of a WWTP, 2,5-DAT were found below 0.25 µg/L. An investigation of the chronic toxicity of 2,5-DAT showed that it induced an increase in the number of cells with structural chromosomal aberrations, indicating genotoxic (clastogenic) activity at levels greater than or equal to 25µg/L.²⁾ In our study, 2,5-DAT was detected below 0.25 µg/L in effluent, extremely low compared to the concentration reported in a previous study. No toxicity data exist for such low concentrations.

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Third chapter

High Performance Liquid Chromatography - Tandem Mass Spectrometry Method Development and Application to Studying Unintentionally Generated Chlorinated Bisphenol A Congeners in Wastewater

1 Introduction

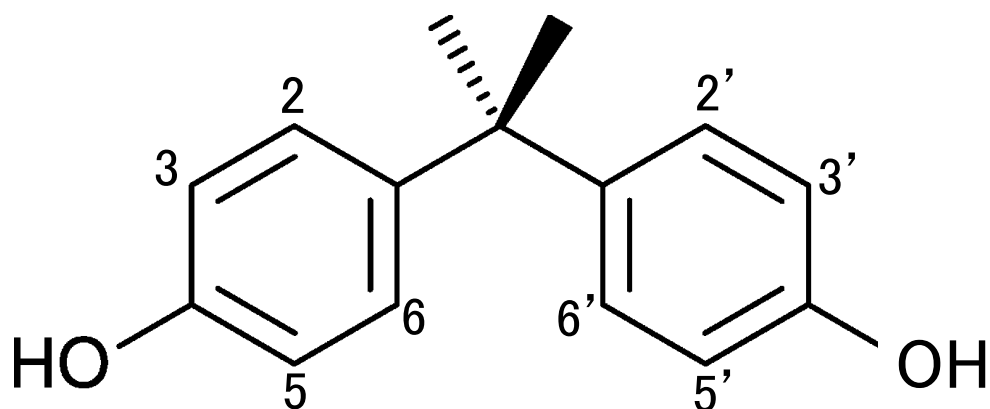
In recent years, halogenated organic compounds that are unintentionally produced and released into the environment have become a topic of increasing environmental concern. Previous reports have indicated an increase in mutagenic activity in industrial effluent, leachates, and treated wastewater, similar to that caused by chlorinated organic compounds.¹⁾

Five chlorinated bisphenol A (BPA) congeners (Cl_xBPAs , shown in Fig. 1) and five brominated BPA congeners (Br_yBPAs) can be unintentionally produced from BPA by chlorination with sodium hypochlorite in wastewater treatment plants (WWTPs). The photoproducts of Cl_xBPAs and Br_yBPAs also contain chlorophenols, which are precursors of halogenated dioxins.^{2, 3)} Cl_xBPAs bind to estrogen receptors more strongly than BPA, and Cl_xBPAs and Br_yBPAs differ in their levels of toxicity. For example, compared to BPA, Cl_1BPA and 3,3'- Cl_2BPA have 1.4- and 9.0-fold higher affinity to estrogen receptor α binding assay, respectively.⁴⁾⁻⁶⁾ According to the scientific literature⁴⁾⁻¹⁹⁾, BPA congeners have high environmental persistence^{16), 17)}, are endocrine disruptors toxic^{4), 5), 7), 10)-12), 15), 17), 18)} to rats^{5), 9)} and humans¹⁰⁾⁻¹⁵⁾, and adversely affect soil and sludge quality^{7), 8)}. In Japan, BPA is considered a Class 1 Designated Chemical Substance according to the Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture.²⁰⁾ However, there are no strict regulations on the discharge of Cl_xBPAs , despite their greater toxicity compared to BPA.

BPA, triclosan (TCS; 5-chloro-2-(2, 4-dichlorophenoxy) phenol), and Br_4BPA have been detected in human serum by gas chromatography/mass spectrometry (GC/MS).¹⁴⁾ TCS in wastewater also reacts with free chlorine to generate chlorinated TCS derivatives, which are known as potential sources of polychlorodibenzo-p-dioxins.²¹⁾ BPA congeners such as Cl_xBPAs , Br_yBPAs , and other derivatives might be more persistent than the original compound. BPA and Cl_xBPAs have been detected in drinking water by high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS).⁶⁾ Gas chromatography (GC) has been used for measuring BPA in soil.^{7), 8)} In GC

analysis, the BPA congeners are derivatized to less polar species by silylation, acylation, alkylation, and esterification. However, these steps might change the resulting profiles due to different reaction efficiencies among the congeners. Given the aforementioned different toxicities of BPA congeners, their separation as intact molecules and subsequent quantification are important. In addition, the simultaneous determination of BPA congeners is of great significance when evaluating their individual behaviors in many environmental fields. However, to the best of our knowledge, there is no reported method for separating individual BPA congeners and simultaneously quantifying the intact molecules.

The author described an HPLC/MS/MS method for the quantification of halogenated BPA congeners with full chromatographic separation. The method was applied to simultaneously determine these chemicals in wastewater before and after sterilization with sodium hypochlorite, to capture species unintentionally produced during this process.



Name	Monitor ion	Log Pow
bisphenol A	227 / 211	4.04
3-monochlorobisphenol A	216 / 209	4.65
3-monobromobisphenol A	305 / 79	4.81
3,3'-dichlorobisphenol A	295 / 215	5.25
3,5-dichlorobisphenol A	295 / 215	5.25
3,3'-dibromobisphenol A	385 / 79	5.58
3,5-dibromobisphenol A	385 / 79	5.58
3,3',5-trichlorobisphenol A	329 / 278	5.86
3,3',5-tribromobisphenol A	463 / 79	6.35
3,3',5,5'-tetrachlorobisphenol A	365 / 313	6.46
3,3',5,5'-tetrabromobisphenol A	543 / 79	7.12

Figure 1 Chemical Structures of the Bisphenol A Congeners and the Mass Transition Ion Pairs for Selected Reaction Monitoring.

2 Experimental

2-1 Reagents

Dichloromethane 300, HPLC-grade distilled water, polychlorinated biphenyl (PCB) analysis grade anhydrous sodium sulfate, and guaranteed reagent grade acetic acid were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Pure water ($\leq 0.1 \mu\text{S/m}$) was obtained from Meinan-Service Co., Inc. (Aichi, Japan). HPLC reagent grade methanol (MeOH) and acetonitrile (ACN) were purchased from Merck KGaA (Darmstadt, Germany). Guaranteed grade ammonia solution (28%) and p-aminophenol ($>98\%$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Resorcinol and 2,6-di-tert-butyl-p-cresol ($>99.0\%$) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Environmental analysis grade BPA ($>99.0\%$) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Br₄BPA and Cl₄BPA were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). ¹³C₃ BPA ($>95.0\%$) was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Ring-¹³C₁₂ Br₄BPA ($>99\%$) was purchased from Cambridge Isotope Laboratories Inc. (Massachusetts, USA). Mono-, di-, and tri-chloro/bromo-substituted BPAs were separately synthesized in our laboratory. The Cl_xBPAs were synthesized from BPA and sulfuryl chloride, and the Br_yBPAs were synthesized by dissolving BPA in bromine water. The synthesized Cl_xBPA and Br_yBPAs were isolated by silica gel column chromatography, and their purities were confirmed by GC/MS, ¹H-NMR, and ¹³C-NMR.

Standard solutions of BPA congeners were prepared at a final concentration of 1 mg/L in ACN. The working solutions of BPA congeners were prepared at concentrations of 0.01 or 0.1 mg/L in ACN/H₂O (1/1, v/v) by diluting the corresponding standard solutions. The calibration curves were linear in the range of 0.03 to 10 $\mu\text{g/L}$.

2-2 Apparatuses

The HPLC/MS/MS operating conditions are summarized in Table 1. For quantitative analysis, a Prominence series LC-20AD HPLC system (Shimadzu Co., Kyoto, Japan) was used with an HPLC column (InertSustain C18, GL Sciences Inc.) in reverse-phase mode. An API4000 (Sciex Pte., Ltd., Framingham, MA, USA) MS/MS system with an ESI source was used for measuring the BPA congeners by selected reaction monitoring (SRM). Concentration was performed with a rotary evaporator (N-1000 with water bath SB-1000, TOKYO RIKAKIKAI CO, LTD) and a nitrogen gas concentrator (fabricated in-house) under nitrogen flow.

Table 1 Operating Conditions for High Performance Liquid Chromatography/Tandem Mass Spectrometry

Prominence (Shimadzu Co.)/API 4000 (Sciex Pte., Ltd.)				
Analysis Column	InertSustain C18 (2.1 mm × 150 mm, 3 μm), 20°C			
Gradient Profile		Time (min)	0	25
Mobile Phase (%)	A: 0.01 v/v % CH ₃ COOH in H ₂ O		75	25
	B: 0.01 v/v % CH ₃ COOH in Acetonitrile		0	100
Others	Flow Rate: 0.2 mL/min	Injection Vol.: 40 μL		
Curtain Gas/Nebulizer Gas	30 psi/40 psi			
Ion Source Temperature	200°C			
Ion Spray Voltage	-4500 V			
	Monitor Ion (<i>m/z</i>)	Declustering Potential (V)	Collision Energy (V)	
BPA (bisphenol A)	227/211	-75	-30	
3-chloroBPA	261/209	-75	-32	
3,3'-dichloroBPA	295/215	-95	-42	
3,5-dichloroBPA	295/215	-80	-42	
3,3',5-trichloroBPA	329/278	-110	-36	
3,3',5,5'-tetrachloroBPA	365/313	-80	-34	
3-bromoBPA	305/79	-70	-46	
3,3'-dibromoBPA	385/80	-90	-60	
3,5-dibromoBPA	385/80	-85	-68	
3,3',5-tribromoBPA	463/79	-95	-72	
3,3',5,5'-tetrabromoBPA	543/80	-75	-70	
¹³ C ₃ BPA	230/214	-75	-26	
¹³ C ₁₂ 3,3',5,5'-tetrabromoBPA	555/79	-115	-94	

2-3 Sample Preparation

A schematic diagram of the sample preparation is shown in Fig. 2. BPA congeners in 500 mL of water were extracted twice with 100 mL dichloromethane. The organic phases were combined, resulting in a total volume of 200 mL, and then dehydrated with anhydrous sodium sulfate. After adding 2 mL of ACN as an evaporation inhibitor, the dichloromethane solution was concentrated to 10 mL or less with an evaporator at 40°C and atmospheric pressure. After adding 50 µL of resorcinol/MeOH solution (0.1 w/v %) as a polymerization inhibitor, the concentrated solution was further evaporated to dryness in a glass test tube. The precipitates in the test tube were dissolved in 1 mL of 28 w/v % ammonium in ACN at 0.1 v/v %, and concentrated to near dryness. The residue in the test tube was dissolved in ACN /distilled water (1/1, v/v) and filled up to 0.2 mL before HPLC/MS/MS analysis. All sample preparation steps were performed under light shielding to prevent photodegradation of the chemicals, particularly of the halogenated BPAs.²²⁾

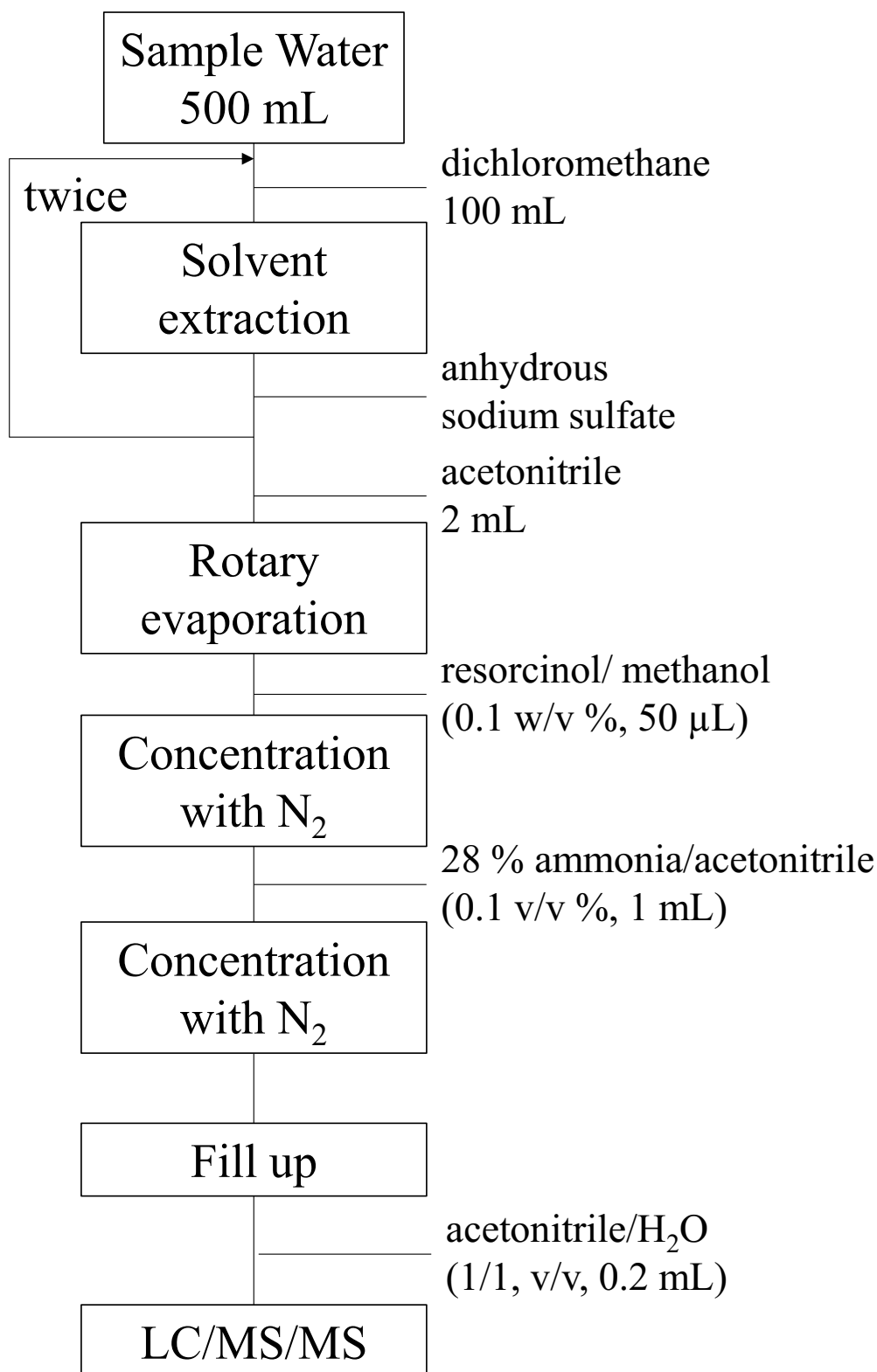


Figure 2 Sample Preparation Scheme for the Determination of Bisphenol A Congeners in Wastewater

3 Results and Discussion

3-1 Recoveries from Wastewater

The recoveries from wastewater are shown with their respective method detection limit (MDL) in Table 2.

Table 2 Recoveries and Method Detection Limit of the bisphenol A Congeners in Wastewater (n = 5)

		Recovery (%)	RSD* (%)	MDL** (ng/L)
	BPA (bisphenol A)	50.6	4.3	0.32
1-halogen	3-chloroBPA	61.3	14	0.04
	3-bromoBPA	69.8	10	0.45
2-halogen	3,3'-dichloroBPA	68.8	14	0.04
	3,5-dichloroBPA	66.6	6.8	0.04
	3,3'-dibromoBPA	77.4	14	0.50
	3,5-dibromoBPA	69.9	14	0.45
3-halogen	3,3',5-trichloroBPA	77.9	14	0.05
	3,3',5-tribromoBPA	69.8	9.9	0.45
4-halogen	3,3',5,5'-tetrachloroBPA	75.9	16	0.05
	3,3',5,5'-tetrabromoBPA	73.2	12	0.47

* Relative Standard Deviation

** Method Detection Limit (MDL) = $t(n-1, 0.05) \times \sigma_{n-1} \times 2$

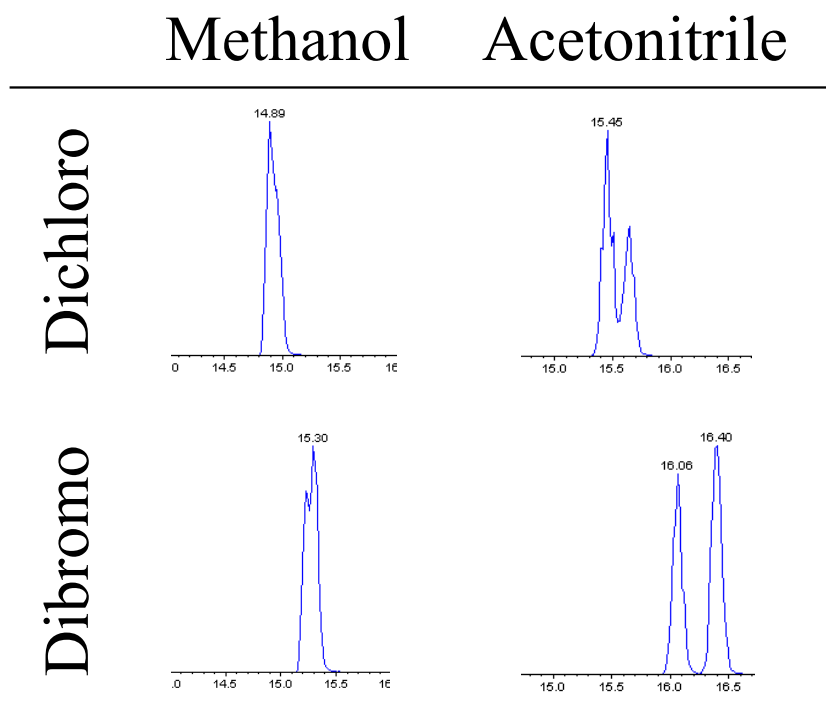
3-2 Method Qualification

3-2-1 Solvent Optimization for the Separation of Halogenated Bisphenol Congeners by High Performance Liquid Chromatography

To evaluate precisely the individual behaviors of the halogenated BPAs, it is necessary to separate them, without derivatization, by HPLC systems. The peak shapes of halogenated BPAs were improved by the addition of 0.01 v/v % acetic acid to the mobile phase. Baseline separation in the mobile phase with 0.01 v/v % ACN/water was achieved for the structural isomers of 3,3'-Br₂BPA and 3,5-Br₂BPA (Fig. 3A).

At a column temperature of 20°C, baseline separation was achieved for BPA and all halogenated BPAs, including the Cl₂BPA and Br₂BPA congeners (Fig. 3B). The slower diffusion rates of the analytes in the mobile phase led to better separation at lower temperature.

(A) Mobile Phase



(B) Column Oven

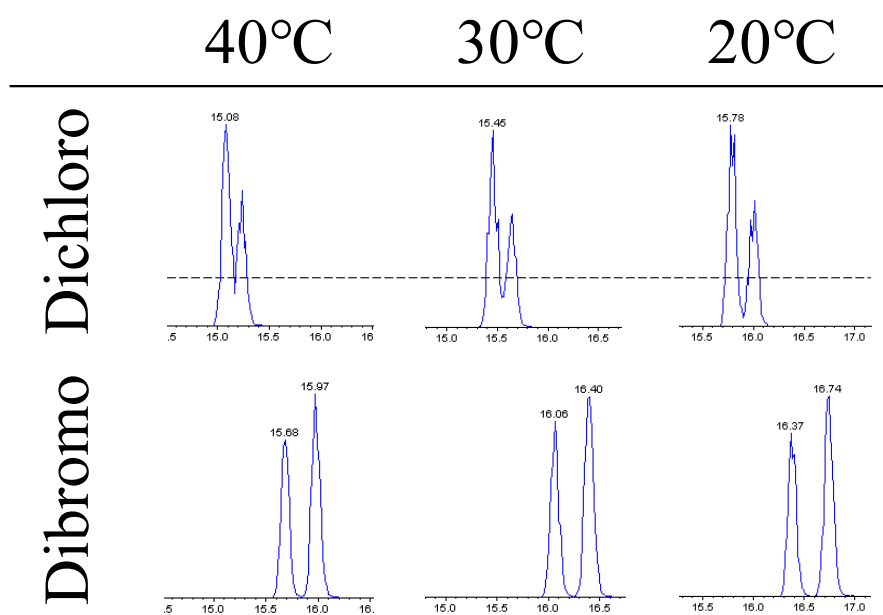


Figure 3 Effect of the Mobile Phase Composition and Operating Temperatures on the Separation of Halogenated Bisphenol A Isomers.

3-2-2 Factors Affecting the Recovery of Halogenated Bisphenol A Congeners from Wastewater Samples

The factors affecting the recoveries of halogenated BPAs include losses due to adsorption onto the glassware, evaporation, polymerization, and oxidization when concentrating the dichloromethane solution.

(A) Additives for Concentrating the Dichloromethane Solution

Three kinds of reagents, namely 2,6-Di-tert-butyl-p-cresol (BHT), 4-aminophenol (4AP), and resorcinol, were evaluated as additives to the solution before concentrating, to improve the recoveries of the halogenated BPA congeners. 0.1 ng of each halogenated BPA were prepared in 5 mL of dichloromethane with 50 µg of the additives, concentrated to near dryness, and diluted to 0.2 mL with an ACN/water (1:1, v/v) mixture (Fig. 2). The resulting recoveries are shown in Table 3.

There were no significant differences between the recoveries with or without the addition of 4AP, except for Cl₃BPA and Br₁BPA. After the addition of resorcinol, the recoveries of the non-, mono-, and di-halogenated BPA congeners were 80 to 100%, while those of the tri- and tetra-halogenated BPA congeners were 20 to 50%. The highest recoveries were obtained with the additive solvent of resorcinol.

(B) Rinsing Solution in Combination with the Additive Resorcinol

The use of rinsing solutions in combination with the additive resorcinol was also evaluated, because the recoveries of the BPAs substituted with multiple chlorine and bromine atoms tended to be low when using the additive solvents (17 to 55% for BPAs substituted with three and four chlorine and bromine atoms). As the lower recoveries were suspected to be due to adsorption, the following acid and basic organic solution mixtures were evaluated for their ability to extract the BPA congeners adsorbed onto glass test tubes: acetic acid/MeOH (0.1 v/v %) and ammonia/MeOH (0.028 w/v %) solutions.

With ammonia/MeOH (0.028 w/v %), the recoveries of all BPA congeners were 84 to 115% (Table 3). With acetic acid/MeOH (0.1 v/v %) washing, the recoveries of the halogenated BPAs, except for BPA, mono halogenated BPAs, and 3,3'-Br₂BPA, ranged from below the MDLs (defined in Table 3) to 66%. Those low recoveries suggest that halogenated BPA congeners adsorb onto the siloxane sites of the glass test tube under acidic conditions. In contrast, the recoveries of BPA, Cl₁BPA, and 3,3'-Br₂BPA were higher than the added amounts (150, 138, and 139%, respectively). The non-, mono-, and di-halogenated BPAs had higher recoveries than the tri- and tetra-halogenated BPAs, and the recoveries of the 3,3'-di-halogenated isomers were higher than those of the 3,5-di-halogenated isomers. The Highest Occupied Molecular Orbitals (HOMOs) of the 3,3'-dihalogenated BPAs, when drawn by the Extended Hückel

Method (ChemBio 3D Ultra 15.1, PerkinElmer Informatics Inc., Waltham, MA, USA), are delocalized on the π -systems of the two benzene rings and bromine atoms, while the HOMOs of the 3,5-isomers are localized on the benzene ring with two bromine substituents. Therefore, the 3,3'-halogenated BPAs are more stable against electrophilic attack, for example by acidic protons, than the 3,5-dihalogenated ones. The higher recoveries of the 3,3'-isomers, compared to the 3,5-isomers, may be due to their increased stability to electrophilic attack. On the other hand, the recovery of 3,3',5,5'-Br₄BPA was nearly 0% ("N.D." means below the MDL of 0.47 ng/L, as shown in Table 2. If all 0.1 ng of 3,3',5,5'-Br₄BPA added to the 5 mL of dichloromethane were recovered, the concentration of 3,3',5,5'-Br₄BPA should be 20 ng/L. Thus, "N.D." represents a recovery rate of <2.4%). Thus, in the recovery experiment with a solution of acetic acid/MeOH (0.1 v/v %) as the rinsing solution, 3,3',5,5'-tetrahalogenated and 3,3',5-trihalogenated BPAs would be partially or fully de-halogenated to produce fewer halogenated species, among which the 3,3'-dihalogenated, 3-halogenated, and non-halogenated BPAs would be more stable against electrophilic attack under acidic conditions. All these factors would cause the high recoveries, exceeding 100%, of the three BPA congeners.

Therefore, 50 μ g of resorcinol was added to the solution extracted from wastewater as an anti-oxidative compound before the sample was concentrated, and ammonia/MeOH (0.028 w/v %) was used to wash the glass test tubes used for concentration.

Table 3 Effect of the Additive Solvents and the Test Tube Rinsing Solutions on the Recoveries of the Halogenated Bisphenol A Congeners in the Concentration Process (n = 3)

			Additive Solvents			Rinsing Solvents with Res. (Additive)	
		Additive -Free (%)	4AP (%)	BHT (%)	Res (%)	Acidic Sol. (%)	Basic Sol. (%)
	BPA (bisphenol A)	N.D.	N.D.	68.6	82.5	150	83.6
1-halogen	3-chloroBPA	118	120	88.6	101	138	89.1
	3-bromoBPA	37.4	69.0	113	98.4	90.9	94.7
2-halogen	3,3'-dichloroBPA	13.9	28.2	106	91.7	57.2	89.9
	3,5-dichloroBPA	12.8	24.1	58.2	48.8	14.3	85.8
	3,3'-dibromoBPA	N.D.	N.D.	67.9	82.9	139	84.4
	3,5-dibromoBPA	85.9	88.9	93.7	66.7	49.0	97.8
3-halogen	3,3',5-trichloroBPA	N.D.	29.0	49.9	30.2	66.3	105
	3,3',5-tribromoBPA	96.1	114	48.4	55.2	23.1	92.9
4-halogen	3,3',5,5'-tetrachloroBPA	114	122	14.7	27.9	57.5	99.2
	3,3',5,5'-tetrabromoBPA	N.D.	N.D.	N.D.	17.1	N.D.	115

4AP: 4-aminophenol BHT: 2,6-di-tert-butyl-*p*-cresol Res: resorcinol

Acidic: acetic acid/methanol (0.1 v/v %) Basic: 28 w/v % ammonia/methanol (0.1 v/v %)

4 Summary of the Method Development for Chlorinated Bisphenol A Congeners

An HPLC/MS/MS method was developed for simultaneously measuring BPA, Cl_xBPAs, and Br_yBPAs unintentionally produced in WWTPs. The goal of the first study was to develop a non-derivatizing measurement method based on HPLC/MS/MS to analyze BPA, mono-, di-, tri-, and tetra-substituted BPAs with chlorine or bromine groups in the aromatic rings (eleven chemicals in total) via full separation and selective quantitation. The goal of the second study was to develop a method of preparing a wastewater sample for analyzing the halogenated BPAs. During the concentration process, some compounds were polymerized and adsorbed onto the glass test tubes. After the addition of resorcinol as a polymerization inhibitor, the recovery rates of the non-, mono-, and di-halogenated BPA congeners were 80 to 100%, while those of the tri- and tetra-halogenated BPA congeners were still 20 to 50%. Since the tri- and tetra-halogenated BPAs are more strongly adsorbed onto glass test tubes than the non-, mono-, and di-halogenated BPA congeners, the glass test tubes were washed with an ammonia/MeOH (0.028 w/v %) solution to improve the recovery rates for all the halogenated BPAs. The limit of detection for these chemicals was found to be within 0.01 to 0.16 µg/L, with recoveries of 50.6 to 77.9 % and relative standard deviation of 4.3 to 16 %.

5 Study of Unintentionally Generated Chlorinated Bisphenol A Congeners in Wastewater Treatment Plants

5-1 Overview

Wastewater samples were collected before and after the sterilization process at three municipal WWTPs (labeled “A,” “B,” and “C”) in Kasugai, Aichi Prefecture, using brown glass bottles to prevent oxygen dissolution in the water. Sample preparation was started around 30 min after collection, as this corresponds to the time when the treated WWTP effluent is likely to be discharged into rivers.

5-2 Results and Discussion

Fig. 4 shows the concentrations of BPA and halogenated BPAs in wastewater before and after sterilization. Plants A and C receive much less industrial wastewater and more domestic wastewater than plant B. Some of the BPAs were observed at concentrations below the MDL, but we would like to refer to their measured concentrations as semi-quantitative. Before the sterilization process, BPA and Br₄BPA, which are often contained in industrial products, were higher in concentration than the other halogenated BPAs. At plants A and C, there were almost no significant differences in the concentrations of BPA and Br₄BPA after the sterilization process. Therefore, we believe that no new compounds derived from BPA and Br₄BPA were formed during the

sterilization processes at Plants A and C.

At Plant B, the concentrations of almost all halogenated BPAs decreased after sterilization. In particular, the concentrations of BPA and Br₄BPA were significantly lower after sterilization, while that of Cl₄BPA tended to increase. These observations suggest that Cl₄BPA and other halogenated BPAs, especially Br₄BPA, were produced from BPA by the sterilization procedure. Similar results have been reported in laboratory experiments.^{4), 21)}

Cl_xBPAs bind to estrogen receptors more strongly than BPA, however there are no studies of toxic effects in fish or other organisms. For example, compared to BPA, Cl₁BPA and 3,3'-Cl₂BPA have 1.4- and 9.0-fold higher affinity, respectively, for estrogen receptor α in a binding assay.⁴⁾⁻⁶⁾ Some of the halogenated BPAs were observed at concentrations below the MDL. Cl_xBPAs, which are intentionally produced in WWTPs, were detected at 0.08 to 0.27 ng/L in this study.

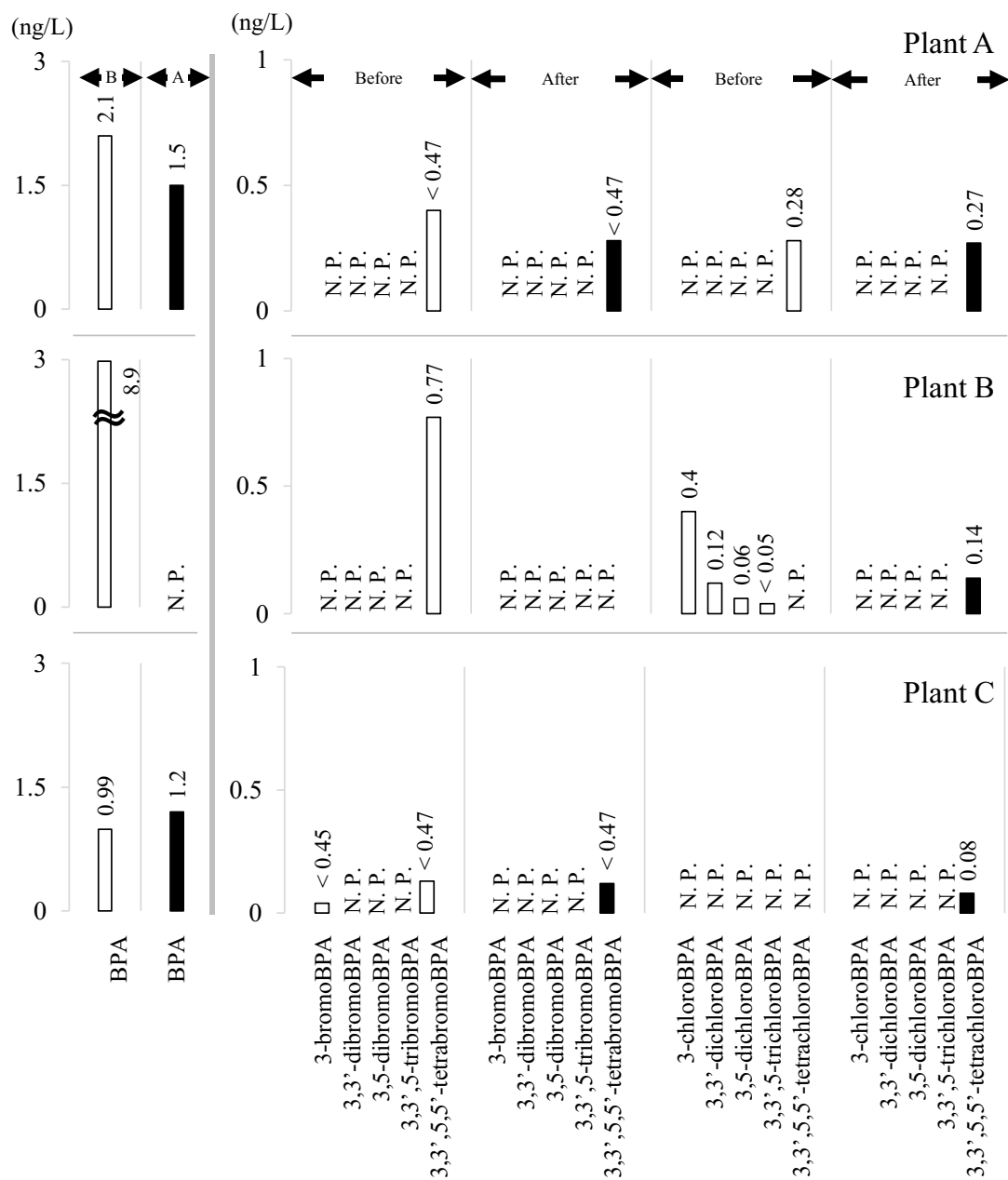


Figure 4 Quantitative Results for Bisphenol A and Halogenated Bisphenol A Congeners in Wastewater Before/After Sterilization at Three Wastewater Treatment Plants

6 Summary of the Study of Unintentionally Generated Chlorinated Bisphenol A Congeners in Wastewater Treatment Plants

The wastewater samples were collected before and after the sterilization process at three municipal WWTPs (labeled “A,” “B,” and “C”) in Kasugai, Aichi Prefecture.

At plants A and C, which receive much less industrial wastewater, there were almost no significant differences in the concentrations of BPA and Br₄BPA after the sterilization process. Therefore, we believe that no new compounds derived from BPA and Br₄BPA were formed during the sterilization process. In the case of Plant B, the concentrations of almost all halogenated BPAs decreased after sterilization, while that of Cl₄BPA tended to increase. These observations suggest that Cl₄BPA and other halogenated BPAs, especially Br₄BPA, were produced from BPA by the sterilization procedure.

7 Conclusions

An HPLC/MS/MS method was developed for simultaneously measuring BPA, Cl_xBPAs, and Br_yBPAs unintentionally produced in WWTPs. The first study aimed to develop a non-derivatizing measurement method based on HPLC/MS/MS to analyze BPA, mono-, di-, tri-, and tetra-substituted BPAs with the chlorine or bromine groups (eleven chemicals in total) via full separation and selective quantitation. The second study aimed to develop a method of preparing a wastewater sample for measuring halogenated BPAs, considering loss of halogenated BPA congeners in the concentration process during sample preparation. The limit of detection for the chemicals was found to be within 0.01 to 0.16 µg/L, with recoveries of 50.6 to 77.9 % and relative standard deviation of 4.3 to 16 %.

Using the above method, we measured wastewater samples collected before and after the sterilization process at WWTPs. The concentrations of BPA, Cl_xBPAs, and Br_yBPAs in sterilized wastewater were determined in order to understand the behavior of these chemicals at WWTPs. The concentrations of BPA, Cl₁₋₃BPAs, and Br₄BPA decreased after sterilization, but that of Cl₄BPA increased. Therefore, BPA, Cl₁₋₃BPAs, and Br₄BPA could be precursors of Cl₄BPA in the sterilization process at WWTPs. However, the amount of Cl₄BPA produced was much lower than the reduction in the precursor species.

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Forth Chapter

A High Performance Liquid Chromatography/High Resolution Mass Spectrometry Study Identifying Unintentionally Generated Halogenated Organic Compounds at Wastewater Treatment Plants

1 Introduction

As mentioned in the third chapter, in recent years, halogenated organic compounds that are unintentionally produced and released into the environment have become a topic of increasing environmental concern. Previous reports have indicated an increase in mutagenic activity in industrial effluent, leachates, and treated wastewater, similar to that caused by chlorinated organic compounds.¹⁾ We reported that tetrachlorinated bisphenol A (BPA) congeners were unintentionally produced at wastewater treatment plants (WWTPs) by the sterilization process.²⁾ As a next step, we also identified a few other halogenated compounds, produced in the sterilization process, by accurate mass measurement with high performance liquid chromatography/quadrupole- time of flight mass spectrometry (HPLC/Q-ToFMS).

2 Experimental

2-1 Reagents

Dichloromethane 300, distilled high performance liquid chromatography (HPLC)-grade water, polychlorinated biphenyl (PCB) analysis grade anhydrous sodium sulfate, and guaranteed reagent grade acetic acid were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Pure water ($\leq 0.1 \mu\text{S/m}$) was obtained from Meinan-Service Co., Inc. (Aichi, Japan). HPLC reagent grade methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Guaranteed grade ammonia solution (28%) and p-aminophenol (>98%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Resorcinol and 2,6-di-tert-butyl-p-cresol (>99.0 %) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2-2 Apparatuses

The HPLC/Q-ToFMS operating conditions are summarized in Table 1. A 1290 Infinity II HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) was used with an HPLC column (InertSustain C18, GL Sciences Inc.) in reverse-phase mode. An Agilent 6545 Q-ToF LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA) with ESI in positive and negative

modes was operated in the range of m/z 30–1000 at the rate of 5 spectra/s. The five most intense precursor ions in the spectrum were chosen for analysis.

Table 1 Operating Conditions for High Performance Liquid
Chromatography/Quadrupole –Time of Flight Mass Spectrometry

1290 Infinity II (Agilent Technologies Inc.)/Agilent 6545 Q-ToF LC/MS				
Analysis Column	InertSustain C18 (2.1 mm × 150 mm, 3 μm), 20°C			
Gradient Profile		Time (min)	0	25
Mobile Phase (%)	A: 0.01 v/v % CH ₃ COOH in H ₂ O		75	25
	B: 0.01 v/v % CH ₃ COOH in		0	100
	Acetonitrile			
Others	Flow Rate: 0.2 mL/min	Injection Vol.: 40 μL		
Ionization	AJS (Positive/Negative)			
Drying Gas/Sheath Gas	10 L/min (350°C)/12 L/min (400°C)			
Nebulizer Gas	345 kPa			
Nozzle Vol/Fragmentor	0/120 (V)			
Collision Energy	10 and 30 eV			
Mass Range	<i>m/z</i> 30-1000 (Rate: 5 spectra/sec)			

2-3 Sample Preparation

A schematic diagram of the sample preparation is shown in Fig. 1. Halogenated organic compounds in 500 mL of water were extracted twice with 100 mL dichloromethane. The organic phases were combined, resulting in a total volume of 200 mL, and then dehydrated with anhydrous sodium sulfate. After adding 2 mL of acetonitrile as an evaporation inhibitor, the dichloromethane solution was concentrated to 10 mL or less with an evaporator at 40°C and atmospheric pressure. After adding 50 µL of resorcinol/methanol solution (0.1 w/v %) as a polymerization inhibitor, the concentrated solution was further evaporated to dryness in a glass test tube. The precipitates in the test tube were dissolved in 1 mL of 28 w/v % ammonium in acetonitrile at 0.1 v/v %, and concentrated to near dryness. The residue in the test tube was dissolved in acetonitrile/distilled water (1/1, v/v) and filled up to 0.2 mL before HPLC/Q-ToFMS analysis. All sample preparation steps were performed under light shielding to prevent photodegradation of the chemicals, particularly of the halogenated (and especially brominated) organic compounds.³⁾

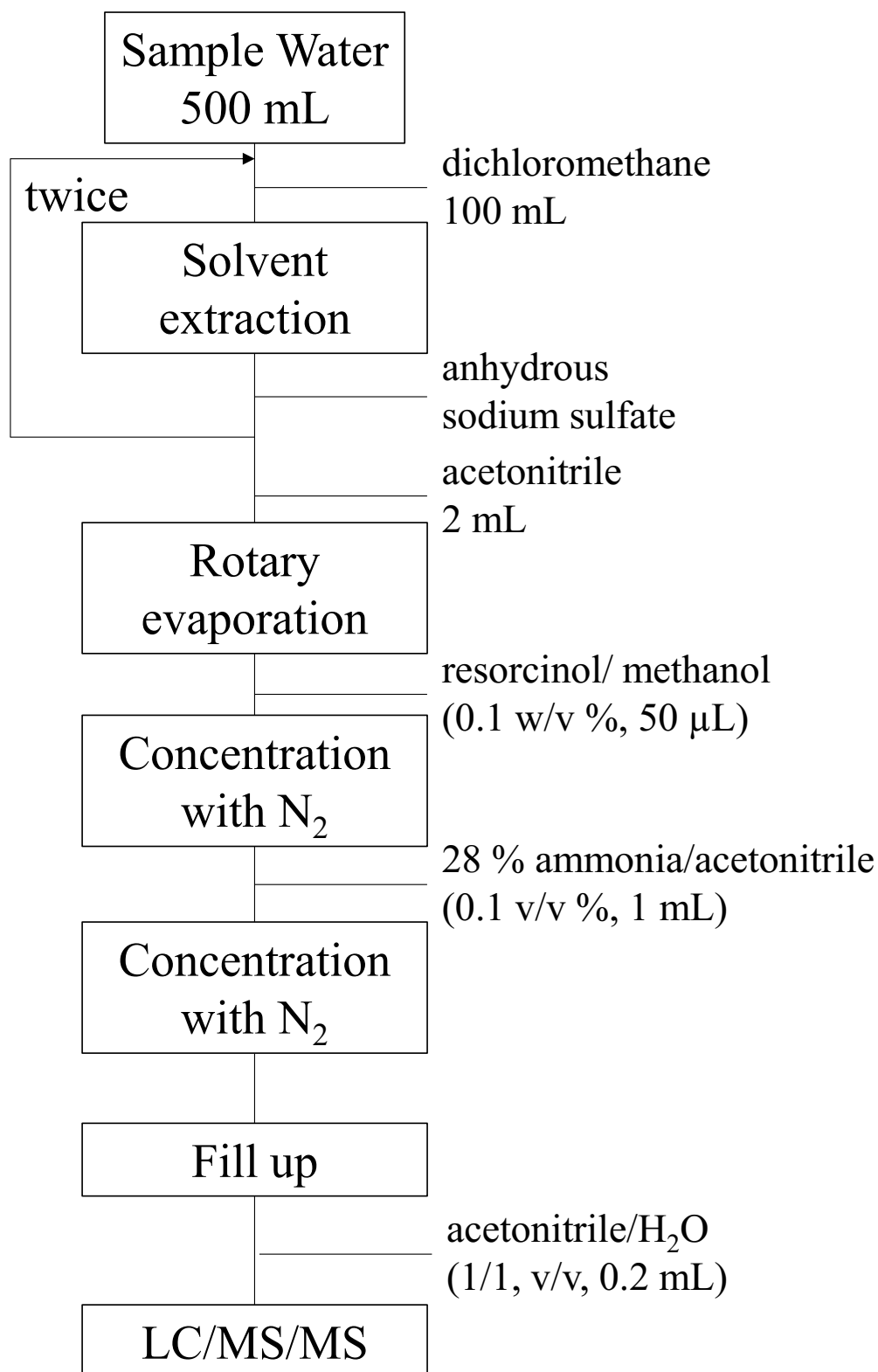


Figure 1 Sample Preparation Scheme for the Determination of Bisphenol A Congeners in Wastewater

3 Identification of Unintentionally Generated Halogenated Organic Compounds at Wastewater Treatment Plants by High Performance Liquid Chromatography/High Resolution Mass Spectrometry

3-1 Overview

Wastewater samples were collected before and after the sterilization process at a municipal WWTP in Kasugai, Aichi Prefecture, using brown glass bottles to prevent oxygen dissolution in the water. Sample preparation was started around 30 min after collection, as this corresponds to the time when the treated effluent is likely to be discharged into rivers.

3-2 Results and Discussion

Accurate mass measurements of wastewater samples collected before (Bf) and after (Af) sterilization were carried out via HPLC/Q-ToFMS, using the conditions given in the bottom rows of Table 1. In order to determine the unintentionally produced species, the total ion chromatogram of Bf was subtracted from that of Af using Mass Hunter Qualitative Analysis B 07.00 software (Agilent Technologies Inc., Santa Clara, CA, USA). The resulting total ion chromatogram contained sixteen halogenated organic compounds as negative ions, but no positive ions.

As a target analysis, we searched for chlorinated compounds that might be produced from halogenated BPAs by sterilization, using their elemental compositions and isotopic mass ratios. Three such compounds,^{4), 5)} namely monochlorophenol, dichloro-dihydroxybenzene, and Cl₄BPA, were retrieved from the WWTP effluent after the sterilization processes.

A non-target analysis of halogenated organic compounds was also performed using Molecular Structure Correlator (Agilent Technologies Inc., Santa Clara, CA, USA). A total of thirteen compounds were confirmed from a number of candidate chemicals in the chemical structure database ChemSpider that fit the molecular formulae, by comparing the isotope ratio of the precursor ion and the corresponding product ion. The chemical formulae of the species labeled 5 and 11 in Table 2 are identical to those of certain clinical medicines. Chemical 7 might be a chemical reagent, but its origin is unknown.

Table 2 Formulae of the Halogenated Organic Compounds Produced by the Sterilization Process at Wastewater Treatment Plants

ID	CE* (eV)	RT** (min)	Formula
1	10	7.7	C ₁₀ H ₅ ClN ₃ O ₂
2	10	8.8	C ₂₃ H ₃₀ ClNO ₆ S
3	10	9.8	C ₉ H ₁₂ BrN ₂ O ₂
4	10	11.9	C ₂₂ H ₁₉ ClN ₂ OS
5	10	12.6	C ₂₂ H ₂₂ ClN ₆ O
6	10	14.5	C ₇ H ₅ Cl ₂ O ₂
7	10	18.5	C ₁₀ H ₉ Cl ₂ O ₃
8	10	18.8	C ₁₈ H ₁₇ ClNO ₄
9	30	7.6	C ₁₀ H ₆ ClN ₃ O ₂
10	30	12.5	C ₂₂ H ₂₃ ClN ₆ O
11	30	16.6	C ₁₇ H ₁₅ ClO ₄
12	30	18.4	C ₁₀ H ₁₀ Cl ₂ O ₃
13	30	20.0	C ₁₂ H ₈ Cl ₂ O ₂

*CE: Collision Energy

**RT: Retention Time

The mass spectrum of compound 13 ($C_{12}H_8Cl_2O_2$) is shown in Fig. 2. Its likely molecular structure identified by mass fragment and neutral loss analysis⁶⁾ is also shown in the figure. The molecule was cleaved at the points divided by the dotted lines (1) through (4). Therefore, compound 13 was theorized to be a decomposition product of triclosan. The structures of the other compounds in Table 2 have not yet been determined. Nevertheless, despite the large uncertainty in their chemical structures, the unintentionally generated products can often be identified by combining multiple tools and methods as described above.

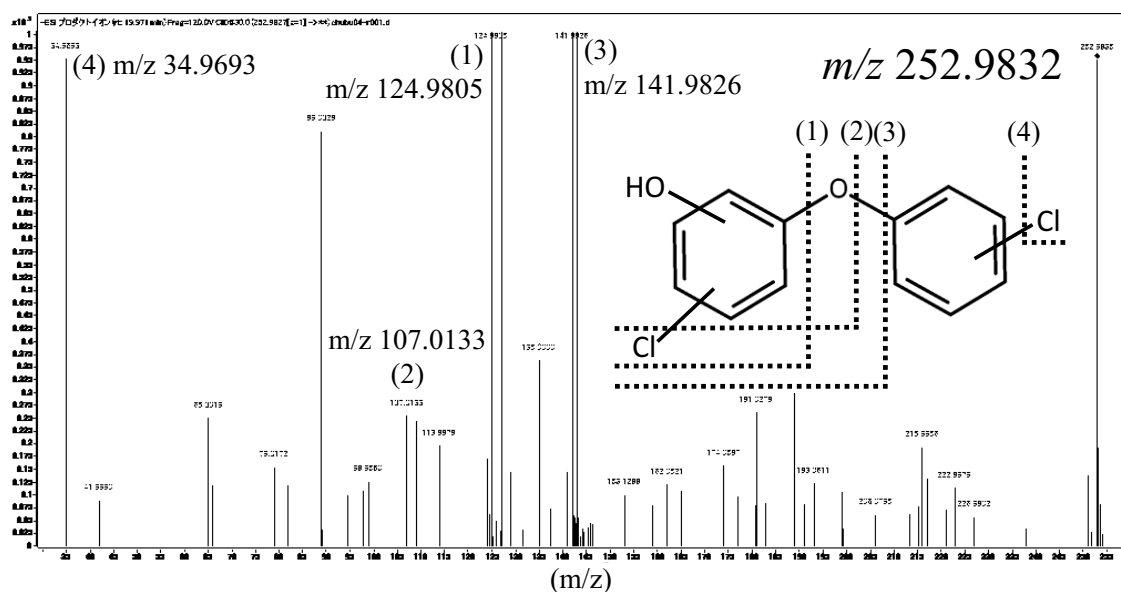


Figure 2 Fragment Analysis of the Mass Spectrum of One Organic Halogenated Compound Suspected to be Unintentionally Produced at Wastewater Treatment Plants (Table 2; #13)

4 Conclusions

Halogenated organic compounds possibly produced by reaction with hypochlorite were also found in wastewater after the sterilization process. Chlorophenol (a dioxin precursor), pharmaceuticals, and a possible decomposition product of triclosan were detected in the WWTP effluent. A few other halogenated compounds were identified, although their exact structures and sources were not immediately evident. Further studies combining multiple qualitative analytical tools and methods are essential for the identification of such unknown compounds in wastewater.

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Fifth Chapter

Conclusion

The second chapter presents newly developed high-sensitivity quantitative methods for aromatic amines. Using newly developed extraction methods that accounted for evaporation and oxidation during sample preparation, hydrophilic aromatic amines were extracted from aqueous samples such as shampoo-water mixture, human urine, and effluent. The methods presented here are a key step for analyzing the potential human exposure and environmental impacts of hair dyeing. The author also used the above method to measure aromatic amines in shampoo-water mixture, urine after hair dying, and river water. The levels of aromatic amines in shampoo-water mixture were low, accounting for 0.7 to 43 % of the aromatic amines in hair dye. Those results suggest that aromatic amines likely convert to diazo-compounds, or remain as residue on human hair, scalp, or other body parts, or evaporate into the air. A maximum of 0.1% of 2,5-DAT in hair dye was excreted in urine, as well as a 2% maximum of 4AP, which is more hydrophilic than 2,5-DAT. Both of these results are extremely low compared to the findings of a previous study. In effluent, 2,5-DAT are detected at levels below 0.25 $\mu\text{g/L}$, which is 1/100 of the concentration reported in a previous study (25 $\mu\text{g/L}$). There are no toxicity data available for this low concentration.

In the third chapter, quantitative high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) methods were developed and used to measure bisphenol A (BPA), chlorinated BPA congeners (Cl_xBPAs), and brominated BPA congeners (Br_yBPAs) unintentionally produced at WWTPs. The first study aimed to develop a measurement method based on HPLC/MS/MS to analyze BPA, mono-, di-, tri-, and tetra-substituted BPAs with the chlorine or bromine groups (eleven chemicals in total) via full separation. The second study aimed to develop a method of preparing wastewater samples for halogenated BPAs analysis considering the loss of halogenated BPAs in the concentration process during sample preparation. The limit of detection for the chemicals was found to be 0.01 to 0.16 $\mu\text{g/L}$, with recoveries of 50.6 to 77.9% and relative standard deviation of 4.3 to 16%. Using the above method, we measured wastewater samples collected before and after the sterilization process at a WWTP. The concentrations of BPA, $\text{Cl}_{1-3}\text{BPAs}$, and Br_4BPA decreased after sterilization, but that of Cl_4BPA increased. Therefore, BPA, $\text{Cl}_{1-3}\text{BPAs}$, and Br_4BPA could be precursors of Cl_4BPA in the sterilization process at WWTPs.

The fourth chapter reports that halogenated organic compounds, possibly produced by sterilization with hypochlorite, were also found in

wastewater after the sterilization process. Chlorophenol (a dioxin precursor), pharmaceuticals, and a possible decomposition product of triclosan were detected in WWTP effluent. Further studies combining multiple qualitative analytical tools and methods are essential for the identification of such unknown compounds in wastewater.

The studies presented here have led to the development of several measurement methods for water pollutants. However, the number of target compounds in water analysis has been continuously increasing. Therefore, in quantitative analysis, there is a need for developing better techniques for multiple simultaneous analyses, at lower concentration levels, with higher precision and efficiency. In qualitative analysis, there is a need for techniques for target screening and non-target analysis.

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Publications

- 1) M. TAKAZAWA and S. SUZUKI, Measurement methods for aromatic amines in urine, shampoo-water mixture, and river water using solid phase extraction liquid chromatography/tandem mass spectrometry, *J. Environ. Chem.*, vol. 25(2), pp.87-94 (2015)
- 2) M. TAKAZAWA, S. SUZUKI, T. NAKANO, S. TSUNOI, and M. SHINOMIYA, Quantitative and Qualitative Analysis of Organic Halogenated Compounds Unintentionally Generated in Wastewater Treatment Plants using Liquid Chromatography/ Mass Spectrometry and High Resolution Mass Spectrometry, *J. Environ. Chem.*, vol. 27(4), pp.137-144 (2017)