Measurement Methods for Aromatic Amines in Urine, Shampoo-water Mixture, and River Water using Solid Phase Extraction Liquid Chromatography/tandem Mass Spectrometry

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Summary

Methods for measuring hair dye ingredients in urine, shampoo-water mixture, and river water were developed using solid phase extraction (SPE) and liquid chromatography/tandem mass spectrometry (LC/MS/MS). Coloring of hair results in the deposition of aromatic amine constituents on the hair and scalp, a portion of which passes through the skin, accumulating in the human body or being excreted. Ingredients in hair dye have been implicated in many kinds of adverse effects in humans, for example, dermatitis, allergies, and cancer. For purposes of this study, we checked for aromatic amines routinely present in hair dyes, and selected three target chemicals that are the most frequently used, namely, 2-methyl-5-aminophenol, 2,5-diaminotoluene, and 4-aminophenol. These amines are extremely water soluble and highly susceptible to oxidation. In the case of river water analysis, fifty milliliters of the sample with added ascorbic acid, as the antioxidant, was passed through an ion exchange cartridge. In the analysis of shampoo-water mixture, ten milliliters of the sample diluted nineteen fold with 1% ascorbic acid was passed through the ion exchange cartridge. In the case of urine, the sample containing ascorbic acid was diluted with a mixture of methanol and 2-propanol followed by centrifugation, and the supernatant was filtered.

Before concentrating the eluted samples under a stream of nitrogen, acetonitrile was added as a keeper solvent to the solution eluted from the SPE cartridge, and the temperature of the solution was maintained below 37 °C to prevent the evaporation and oxidation of the target chemicals. The recoveries of the target chemicals were 96.5 ~ 123.8% (RSD 3.8 ~ 12.8%) for river water, 75.8 ~ 129.0% (RSD 3.3 ~ 8.3%) for shampoo-water mixture, and 75.8 ~ 127% (RSD 7.7 ~ 14.8%) for urine.

Key words: hair dye, aromatic amines, liquid chromatography/tandem mass spectrometry, solid phase extraction

Introduction

Hair dying 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, the production of hair dye increased for the fifth consecutive year, reaching 30,777 tons. 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 and journals, and hair dye ingredients have been implicated in many kinds of adverse effects in humans, for example, dermatitis, allergies, and cancer.

The aromatic amines 5-aminophenol (5A2MP), 2,5-diaminotoluene (2,5DAT), 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. No chronic toxicity has been reported for amino cresols, except for the case of 4-aminophenol, which was shown to have a strong cytotoxic effect in a gene mutation study. 2,4-diaminotoluene has been shown to induce genotoxic effects in the human hepatoma
cell line HePG2, such as the inhibition of the repair mechanism of damaged DNA and micronucleus formation.\textsuperscript{25} 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.\textsuperscript{26}

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 this is immediately excreted in urine and feces, while the rest is transferred to other organs by secondary deposition and gradually metabolized. However, little is known about the exposure of humans to aromatic amines through hair dyeing. This is, in part, due to the difficulties in developing accurate measurement methods for the aromatic amines and their metabolites in water, 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 urine, which are predominantly composed of water. In addition, the recoveries of these aromatic amines are reduced owing to rapid oxidation during cleanup time. Consequently, very few methods are available for measuring hair dye ingredients.

The levels of oxidation dyes and their intermediates in hair dye ingredients with the methods above. The analysis of the intact molecules of aromatic amines and their conjugates is then of great significance in order to assess human exposure to them.

Therefore, as a step toward the accurate analysis of human exposure to aromatic amines in hair dye, we describe a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method combined with solid phase extraction (SPE) and solvent dilution to determine the concentration of free intact aromatic amine molecules in river water, shampoo-water mixture, and urine.

**Experimental**

**Reagents**

Ascorbic acid (99.6%), formic acid (98%), and distilled water for HPLC were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Pure water (10.00 µS/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\textsubscript{3}OH) was obtained from Yoneyama Yukinori 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), respectively. 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. The working solutions (1 mg/L in ACN) of the three were prepared by diluting the respective standard solutions. The calibration curves of the aromatic amines were good in the range of 1–300 µg/L.

**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). In addition, we used a US-2 ultrasonic cleaner (As One, Osaka, Japan), a CF15R centrifuge (HTATCHI High-tech, Tokyo, Japan), and an in-house fabricated concentrator using nitrogen.

**LC/MS/MS conditions**

The operation conditions used in the LC and MS studies are summarized in Table 1. An Agilent 1100 Series LC 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 LC/MS/MS system with an ESI source was used for determining the aromatic amines by selected reaction monitoring (SRM).

**Sample preparation for 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 1mL/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\textsubscript{3}OH/MeOH (5/95, v/v) into a 10 mL test tube. Then 150 µL of ACN was added as a keeper solvent prior to concentrating the eluted solution to reduce 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 process of concentration.

However, the concentrated solution still contained some water from the 28 w/v% NH\textsubscript{3}OH. 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\textsubscript{3}OH/0.75 mL of MeOH) in the concentrated solution. In contrast, the NH\textsubscript{3} of the 28 w/v% NH\textsubscript{3}OH was mostly removed during the concentration process because the boiling point of NH\textsubscript{3} is -33.3 °C. Then, 1 mL of ACN was added to the concentrated solution and further concentrated to completely remove the 0.75 mL of MeOH 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 LC/MS/MS analysis. We subsequently found that the solvent exchange from MeOH to ACN in the concentration process was not necessary for the LC/MS/MS analysis.

**Sample preparation for shampoo-water mixture**

Aromatic amines are expected to be present in 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. Shampoo-water mixture containing 4.7 g/L of shampoo liquid of...
which concentration was equivalent to the average concentration in shampoo waters from 5 shampooed people, was used in this experiment. A 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 was 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 Inertsep C18 and Oasis MCX Plus Short cartridges, which were conditioned with 5 mL of MeOH followed by pure water prior to use. The C18 cartridge was used upstream for removing hydrophobic species in the shampoo-water mixture, and the MCX cartridge was used downstream for collecting the aromatic amines passing through ACN in the eluted solution. The recovery experiments were carried out by adding 0.1 µg of the aromatic amines to 10 mL of shampoo-water mixture, followed by the method described above.

**Sample preparation for urine**

The flow chart for sample preparation and analysis is shown in Fig. 2-C. Twelve milligrams of ascorbic acid was added to 1 mL of human urine, obtained from a person who had not dyed his hair for at least 6 months. The 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 LC/MS/MS analysis. The recovery experiments were performed by adding 1 µg of each aromatic amine to urine, followed by the method described above.

**Result and Discussion**

**Method for river water analysis**

At first, we examined the effects of keeper solvents and the sample solution temperature on the recoveries of the aromatic amines in order to minimize evaporation losses during the concentration process. The effects of the keeper solvents were examined using a recovery standard containing 0.1 µg of each of the three target chemicals in 5 mL of 28 w/v% NH₂OH/MeOH (5/95, v/v).

Four solvents (150 µL), which exhibit π-π interactions or are capable of hydrogen bonding with aromatic amines, were examined as the keeper solvent, namely, ACN, PEG, 4E3MP, and Eugenol. The recovery standards, with four different keeper solvents added, were compared for the recovery efficiencies in the concentration process under nitrogen and 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 2,5DAT and 4AP were below 50% and 40% on using PEG and 4E3MP, respectively. The recoveries of all the target chemicals were below 40% when using Eugenol. On using ACN as the keeper solvent, the recoveries of 5A2MP and 2,5DAT 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). Given that the boiling points of 5A2MP, 2,5DAT, and 4AP, are 241 °C, 273 °C, and 284 °C, respectively, we suspected the role of another type of interaction or process, for example, oxidation, to account for the reduced recovery of 4AP. Then, we examined the effect of the sample solution temperatures on the recovery of aromatic amine by reducing the temperature of the solution from 55 °C to 37 °C; in the latter case, the temperature of the heater block was 125 °C. At 37 °C, the recoveries of the aromatic amines were 113.0% for 5A2MP, 128.8% for 2,5DAT, and 92.9% for 4AP. These results suggest that the loss of 4AP during the concentration process would mostly be due to oxidation at the higher temperature employed.

Next, both breakthrough and elution profiles of the aromatic amines from the SPE cartridge were obtained by SRM with the apparatus shown in Fig. 4. The MCX cartridge is packed using a divinylbenzene-N-vinylpyrrolidone copolymer with sulfo groups, which 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 can be eluted...
Sample water spiked with the aromatic amines was loaded on to the MCX cartridge in the above setup (Fig. 4-A), and the aromatic amine could be monitored for their breakthrough profiles from the MCX cartridge. Formic acid and ascorbic acid were respectively examined for subjecting sample water to acidic and acidic-reductive. After adding 0.1 μg of the standard reagents to the acidic-reductive formic acid and to the acidic aqueous solution, 50 mL of 1% formic acid was passed through the MCX cartridge using an LC pump. Then, we replaced the MCX cartridge with a new MCX cartridge before 1% ascorbic acid was passed through it. We did not observe any peak corresponding to the aromatic amines as they passed through the MCX cartridges, suggesting that the aromatic amines were being retained or decomposed in the cartridges. To confirm this, we put three syringes upstream of the MCX cartridge; all three syringes were filled with 28 w/v% NH₄OH/MeOH (5/95, v/v). To elute the aromatic amines from the MCX cartridge, we passed distilled water from the LC pump to the three syringes (Fig. 4-B). Doing so, helped to avoid flowing highly concentrated NH₄OH through the HPLC pump. At the same time, it avoided over diluting the solution with distilled water; therefore, we restricted the number of syringes to three. In this way, we obtained the elution chromatograms of the aromatic amines from both the cartridges (Fig. 5). The aromatic amines were retained in both the cartridges. However, a significant difference was observed between the two elution chromatograms shown in Fig. 5. The elution chromatogram from the cartridge through which 1% formic acid solution was passed 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 for the cartridge through which 1% ascorbic acid solution was passed exhibited peaks of higher intensity and larger area. Therefore, in the case of river water analysis, we decided to add 1% ascorbic acid solution to the sample. The recoveries from river water were 124% for 5A2MP, 98% for 2,5DAT, and 97% for 4AP (Table 2). Fig. 6 shows SRM chromatograms of the aromatic amines in river water. Strong 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.

**Method for shampoo-water mixture analysis**

First, we examined the effect of ascorbic acid concentration (1, 5, or 20 w/v%) on the recovery of aromatic amines from shampoo-water mixture. The most reliable results were obtained with the sample containing 1% ascorbic acid, where the mean recoveries of 5A2MP, 2,5DAT, and 4AP were 69% with a 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 for the 1% ascorbic acid sample. For samples containing 20% ascorbic acid, the recovery of 2,5DAT was approximately 4 times higher than the spiked amount (in other words, the recovery of 2,5DAT 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 2,5DAT, the amine with no hydroxyl group. This could possibly be due to the increase in the ionization efficiency, which was 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, we conclude that the phenolic hydroxyl group might suppress the increase in the ionization efficiency. The recoveries from shampoo-water mixture were 84% for 5A2MP, 80% for 2,5DAT, and 112% for 4AP (Table 2).

Method for urine analysis

Because large amounts of organic impurities were contained in the urine sample, it was difficult to collect aromatic amines by SPE, even after diluting the urine sample with large amounts of water. The urine sample was diluted with organic solvents to remove large organic-solvent-insoluble molecules. Many water-soluble organic solvents were examined for their suitability in diluting the urine sample, but no single solvent showed good recoveries for the aromatic amines. Next, we examined the suitability of 4 mL of MeOH/IPA at varying volume ratios (1:3, 1:1, and 3:1) for diluting 1 mL of urine. The results are shown in Table 4. Good recoveries were obtained for the three aromatic amines with an equimolar ratio of MeOH/IPA, while the recovery of 5A2MP was low at a MeOH/IPA ratio of 3:1. Considering that the LogPow 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, which were removed from the urine sample. The recoveries from urine were 129% for 5A2MP, 107% for 2,5DAT, and 76% for 4AP (Table 2).

To summarize, our results show that aromatic amine concentrations of around 1 ng/mL in river water, around 10 ng/mL in shampoo water, and around 160 ng/mL in urine could be measured with good recoveries. The present method improved not only the selectivity but also the sensitivities of the aromatic amines compared to very
few previously reported methods. For example, the limit of detection (LOD) for 2,5DAT was 161 ng/mL, which is 1/160 of that obtained using HPLC/UV, and the LOD for 4AP was 149 ng/mL, which was 1/33 of that obtained using thin layer chromatography.

### Conclusion

Because the aromatic amines in hair dye are extremely water soluble and highly susceptible to oxidation, it has been difficult to determine the concentration of the dye and their metabolites in river water, shampoo-water mixture, and urine. As a step toward solving this problem, we developed an LC/MS/MS method for measuring 5A2MP, 2,5DAT, and 4AP levels in river water, shampoo-water mixture, and urine. Especially, 2,5DAT and 4AP were more susceptible to oxidation than 5A2MP, making it necessary to prepare the sample at low room temperature and also shorten the clean-up time.

In the analysis of river water, the aromatic amines in the river water was added with ascorbic acid could be efficiently collected with an MCX cartridge. In the analysis of shampoo-water mixture, the aromatic amines could be determined by diluting the sample with nineteen fold with 1% ascorbic acid followed by SPE and LC/MS/MS. In the analysis of urine, the urine sample diluted with 4 times larger volume of MeOH/IPA (1/1, v/v) was filtrated and subjected to LC/MS/MS analysis. It was essential for the river water analysis to add ACN to the eluted solution and also essential to keep the solution temperature below 37 ℃ in the concentration process. In the case of urine, it was also essential for measuring the aromatic amines to remove large water-soluble molecules from the urine sample.

The methods presented as a step for analyzing human exposure and environmental impacts by dyeing hair. With those methods, three of free aromatic amines could be quantified in environmental water, shampoo-water mixture, and urine. 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. Further study is essential to develop measurement method for aromatic amine metabolites in environmental and biological media.

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