Identification of Aminobiphenyl Derivatives in Commercial Hair Dyes

Robert J. Turesky,*,† James P. Freeman,† Ricky D. Holland,† Daniel M. Nestorick,† Dwight W. Miller,† D. Luke Ratnasinghe,‡ and Fred F. Kadlubar‡

Division of Chemistry and Division of Molecular Epidemiology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, Arkansas 72079

Received June 4, 2003

A recent epidemiological study suggested that aromatic amines present in hair dyes may contribute to an increased risk of bladder cancer (Gago-Dominguez, et al. (2003) *Carcinogenesis* 24, 483−489). Moreover, a preliminary study linked frequent hair dye usage with elevated levels of DNA adducts of 4-aminobiphenyl (4-ABP) in human epithelial breast cells (Gorlewska, et al. *Proc. Am. Assoc. Cancer Res.* 43, 1018−1019). Therefore, we sought to determine if 4-ABP, a recognized human urinary bladder carcinogen, is present in commercial hair dyes. 4-ABP was isolated from dyes by solvent extraction with hexane, followed by silica gel chromatography, either with or without chemical treatment of the extract with Zinc/HCl, and a final purification with a mixed cation exchange reversed-phase resin. The identity of 4-ABP was confirmed by both HPLC with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) and gas chromatography with negative ion chemical ionization mass spectrometry (GC-NICI-MS) following chemical derivatization with pentafluoropropionic anhydride (PFPA). The levels of 4-ABP ranged from not detectable (<0.29 parts per billion (ppb)) up to 12.8 ppb. The noncarcinogenic isomer 2-aminobiphenyl (2-ABP) was also found at quantities up to 310 ppb. 4-ABP was detected in eight of the 11 hair dyes and found in black, red, and blonde hair dyes but not in brown hair dyes. 1,4-Phenylenediamine (PPD) is a key constituent for color development of many permanent hair dyes. Some batches of chemical research grade PPD were contaminated with 4-ABP (up to 500 ppb) and 2-ABP (up to 70 parts per million) and may be a source of ABP contamination in hair dyes. These analytical data demonstrate that 4-ABP is present in some hair dyes. Studies on dermal absorption and bioavailability of 4-ABP from hair dyes are required to determine if this aromatic amine contributes to the increased risk of bladder cancer reported in frequent users of hair dyes.

Introduction

The occurrence of urinary bladder tumors among workers in dyestuff factories was first reported by Rehn in 1895 (*1*). Since that time, numerous experimental and epidemiological studies have demonstrated that occupational exposure to arylamines such as 4-ABP,[1] 2-naphthylamine, and Bz is a cause for bladder cancer (*2*, *3*). 4-ABP was used as an antioxidant in the rubber industry prior to discovery of its carcinogenic activity (*4*). While the amounts of aromatic amines produced in dyestuff, chemical, and rubber factories have been greatly reduced in recent years, aromatic amines and nitroaromatics remain ubiquitous contaminants that may be found in some color additives (*5*, *6*), paints (*7*), food colors (*8*), leather and textile dyes (*9*, *10*), fumes from heated oils and fuels (*11*, *12*), and tobacco smoke (*13*). The United States Food and Drug Administration has set specifications for allowable limits of aromatic amines, including 4-ABP, in the food color additives FD&C Yellow Nos. 5 and 6, at not more than 5 and 15 ppb, respectively (Code of Federal Regulations: 21 CFR 74.705 and 21 CFR 74.706), resulting in a high user exposure of about 0.2 ng of 4-ABP per day (*14*).

The usage of hair dyes has increased dramatically over the past decade, and retail sales of home dyeing kits in the United States were reported to approach $1.6 billion in 2001 (*15*). Recent survey results have estimated that 42% of U. S. women and 25% of men use dyes (*15*). Similar hair dye usage estimates have been reported for Europe and Japan (*16*). Hair dyes are known to contain bacterial mutagens (*17*, *18*), and numerous epidemiological studies have been reported on hair dye usage and bladder cancer risk (*19*−*26*). A number of studies reported no significant association between the use of permanent hair dyes and death from bladder cancer (*19*−*23*); however, a recent investigation reported an increase in bladder cancer risk as a function of hair dye usage, with increased risk for duration of use (*16*, *24*, *25*).

* To whom correspondence should be addressed. Tel: 870-543-7301. Fax: 870-543-7686. E-mail: RTuresky@nctr.fda.gov.

† Division of Chemistry.

‡ Division of Molecular Epidemiology.

[1] Abbreviations: 2-ABP, 2-aminobiphenyl; 3-ABP, 3-aminobiphenyl; 4-ABP, 4-aminobiphenyl; Bz, benzidine; DEG, diethylene glycol; DPA, diphenylamine; GC-NICI-MS, gas chromatography-negative ion chemical ionization-mass spectrometry; HPLC-ESI-MS/MS, HPLC-electrospray ionization tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MCX, mixed mode, cation exchange reversed-phase resin; MS, mass spectrometry; NAT, *N*-acetyltransferase; 4-NO_2BP, 4-nitrobiphenyl; ppb, parts per billion; ppm, parts per million; PFPA, pentafluoropropionic anhydride; PPD, 1,4-phenylenediamine; SIM, selective ion monitoring; SRM, selective reaction monitoring; TMA, trimethylamine.

10.1021/tx030029r
Published on Web 08/28/2003

Women who used permanent hair dyes at least once a month experienced a 2.1-fold increased risk of bladder cancer relative to nonhair dye users, after adjustment for cigarette smoking (*16*). Moreover, when enzyme polymorphisms were considered as risk factors, individuals who were NAT2 slow acetylators had a 2.9-fold increased risk of bladder cancer while the corresponding relative risk in NAT2 rapid acetylators was 1.3-fold (*26*). Other studies also have reported an enhanced risk for bladder cancer development in individuals who were occupationally exposed to arylamines and displayed the NAT2 slow acetylator phenotype (*27*). In view of the fact that *N*-acetylation of arylamines by NAT2 is an important detoxification pathway in arylamine metabolism in experimental animals and humans (*28*−*30*), the recent epidemiological data on bladder cancer risk with hair dye usage and the association with NAT2 enzyme polymorphisms are suggestive of carcinogenic arylamine contamination in hair dye products. Consistent with this premise, a preliminary study has linked frequent hair dye usage with elevated levels of DNA adducts of 4-ABP in epithelial cells from human breast milk (*31*).

With the increased popularity of hair dye usage and the recent health concerns raised from epidemiological studies regarding permanent hair dyes and elevated bladder cancer risk, we sought to determine if 4-ABP may be present in some commonly used hair dye products. This paper describes an analytical method for the isolation, identification, and quantification of 4-ABP in commercial hair dyes by MS methods. We also report that PPD, a key ingredient in the development of color for many hair dyes (*32*), is a plausible source of ABP contamination.

Materials and Methods

Caution: *4-ABP and its derivatives are hazardous and should be handled with caution.*

Chemicals and Reagents. 4-ABP, 4-NO_2BP, DPA, Bz, PFPA (99% pure), TMA (40% solution in water), NH_3 (25% solution in water), and granular zinc were purchased from Aldrich (Milwaukee, WI). DEG was purchased from Fisher Scientific (Atlanta, GA). 2-ABP was purchased from K&K Laboratories, Inc. (Plainview, NY), and 3-ABP was obtained from K&K Laboratories, Division of ICN Biomedicals (Cleveland, OH). [$^2H_9$]-4-NO_2BP (99.3% isotopically pure) was purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). [$^2H_9$]-4-ABP was prepared by reduction of [$^2H_9$]-4-NO_2BP with Zn in C_2H_5OH/HCl at 60 °C for 2 h followed by purification with an Oasis MCX LP extraction cartridge as described below. The isotopic purity after reduction remained at 99.3% based upon HPLC-ESI-MS analyses vide infra. Oasis MCX LP extraction cartridges (0.5 g LP, 6 cm^3) and silica Sep-Pak cartridges (1 g, 6 cm^3) were purchased from Waters Corp. (Milford, MA). *n*-Hexane was purchased Burdick and Jackson, and methanol and ethyl acetate, HPLC grade, were obtained from Baker. Ethanol (95%) was obtained from AAPER Alcohol & Chemical Company (Shelbyville, KY). Permanent hair dyes and creme developer (30 strength) were purchased from local supermarkets or from a local hair salon in Little Rock, AR, and used according to the manufacturers' instructions with minor modifications. One part of hair dye was mixed with one part of creme developer for 15 min unless stated otherwise.

Dye Preparation and Extraction Method. An extensive purification procedure was required for the isolation of ABP isomers from hair dyes and is summarized in Figure 1. All hair dye samples were assayed in triplicate, and estimates of ABP content are reported as the mean ± SD. Hair dye (1 g) was placed in glass Pyrex tubes (12 mL) with Teflon-faced screw caps

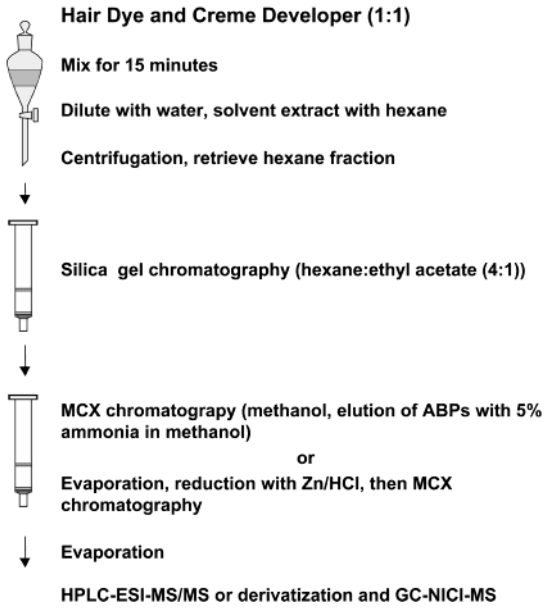

Figure 1. Scheme for the isolation of ABP isomers from hair dyes.

and mixed with commercial creme developer 30-strength (1 g) containing H_2O_2, followed by the addition of [$^2H_9$]-4-ABP (20 ng, 10 ppb). In some experiments, unlabeled 4-ABP (10 ppb) was also added. The mixture was gently shaken for 15 min at room temperature, and the color of the dye developed over time. The dye mixture was then diluted with distilled, deionized water (5 mL) followed by 2 N NaOH (0.3 mL) and extracted three times with *n*-hexane (4 mL). To disrupt emulsions, the samples were centrifuged at 3500 rpm for 10 min. The extraction with hexane was repeated twice. The pooled hexane fractions were applied to silica gel cartridges prewashed with hexane (5 mL). The cartridges were then washed with 20 mL of hexane:ethyl acetate (4:1), and both the hexane and the hexane:ethyl acetate eluents were collected into glass Pyrex tubes (50 mL).

The extracts were either treated with Zn/HCl as described below or further purified by solid phase extraction with a MCX cartridge, which had been prewashed with CH_3OH containing 0.01 N HCl. The samples were applied to the cartridges. The resins then were washed with CH_3OH (10 mL), and ABP isomers were eluted with 5% aqueous NH_3 (30% v/v) in CH_3OH (10 mL).

For samples that were treated with Zn/HCl prior to solid phase extraction with the MCX cartridges, the hexane:ethyl acetate eluents from the silica gel resin were transferred to a pear-shaped glass rotary evaporation flask and rotary evaporated to a volume of approximately 5 mL at 40 °C and 210 mbar pressure. DEG (20 μL) was added prior to evaporation to minimize loss of 4-ABP during evaporation. Using a glass syringe with a metal cannula, the concentrated extracts (∼5 mL) were quantitatively transferred to a graduated conical glass tube and the solvent was gently evaporated under a stream of nitrogen until only DEG remained.

The samples were dissolved in C_2H_5OH (1.5 mL), followed by addition of 1 N HCl (0.5 mL), and quantitatively transferred to glass Pyrex tubes (30 mL) with Teflon-faced screw caps. Zn (80 mg) was added, and the reduction was conducted at 60 °C for 2 h. Samples were then diluted with distilled, deionized water (8 mL) and applied to an MCX cartridge, which had been prewashed with CH_3OH (10 mL), followed by distilled, deionized water (10 mL). After sample application, the cartridges were washed with water (10 mL), followed by CH_3OH (10 mL). The ABP isomers were then eluted with 5% NH_4OH (30% v/v) in CH_3OH (10 mL).

Both the reduced and the nonreduced ammonical methanol dye extracts obtained from the MCX cartridges were transferred to a pear-shaped flask (25 mL) containing DEG (20 μL) and

carefully concentrated by rotary evaporation to a final volume of ~5 mL at 40 °C and 100 mbar pressure. The purified extracts were transferred to glass conical tubes and gently concentrated under a stream of nitrogen until only DEG remained. The samples were resuspended in CH_3OH (200 μL) and assayed by HPLC-ESI-MS/MS or derivatized with PFPA, as described below and assayed by GC-MS.

Solvent blanks containing distilled, deionized water (7 mL) and [$^2H_9$]-4-ABP (20 ng) were worked-up in the same manner and in parallel to the hair dye extracts to assess the possible cross contamination of hair dyes with ABP isomers that may be present in the organic solvents, glassware, or tubings used for extraction and concentration. The use of any plastics and rubber tubing that may contain ABP isomers was avoided whenever possible to minimize contamination.

Aminobiphenyl Analysis by HPLC-ESI-MS/MS. The chromatography was conducted with a capillary HPLC system from LC Packings/Dionex (Amsterdam, Netherlands) and comprised of an UltiMate quaternary pump, Switchos II column switching system, and a Famos autosampler. A Phenomenex Aqua C18 reversed-phase column (Torrance, CA) (3 μm particle size, 1 mm × 100 mm) was used for the chromatography. The purified hair dye extracts (1 μL) were loaded onto an LC-Packings Inertsil C18 trapping column (5 μm particle size, 1 mm × 15 mm) using the Famos autosampler and LC Packings Switchos pump system with a solvent containing 0.1% HCO_2H and 2 mM $NH_4CH_3CO_2$ at a flow rate of 50 μL/min. After they were loaded for 1 min, the samples were backflushed on to the Phenomenex column. The separation was done with a linear gradient starting from 0.1% HCO_2H (held for 1 min) to CH_3CN containing 0.1% HCO_2H over 10 min and then held for 5 min at this final solvent condition at a flow rate of 50 μL/min.

ABP detection and quantification were done by ESI-MS/MS with a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Manchester, U.K.). Quantitative analysis was done in positive ionization mode using the SRM transition $[M + H]^+ \rightarrow [M + H - 18]^+$ (*m*/*z* 170 → 152) for ABP and $[M + H]^+ \rightarrow [M + H - 19]^+$ (*m*/*z* 179 → 160) for [$^2H_9$]-4-ABP. The dwell time for each transition was set at 0.2 s. The capillary voltage was 3.2 kV, the cone voltage was set at 36 V, hexapoles 1 and 2 were set at 15 and 0 V, respectively, and the collision energy was 31 eV. The source and desolvation temperatures were 120 and 275 °C, respectively. The cone gas flow rate was 95 L/h, and the desolvation gas was set at 280 L/h. Argon was used as the collision gas and set a pressure of 2.2 mTorr. In some analyses, 5 μL of extract was assayed in order to obtain four transitions in SRM scan mode using 170 → 169 $[M + H - H^\bullet]^+$, 170 → 152 $[M + H - H^\bullet - NH_3]^+$, 170 → 127 $[M + H - NH_3 - C_2H_2]^+$, and 170 → 93 $[M + H - C_6H_5^\bullet]^+$ for corroboration of the identity of the ABP isomers (*33*, *34*). Product ion spectra of the dye extract analytes were obtained on the protonated molecules $[M + H]^+$ of the ABP isomers at *m*/*z* 170 scanning from *m*/*z* 50 to 200 at a scan speed of 150 amu/s using the same acquisition parameters as described above.

Aminobiphenyl Analysis by GC-MS. The extracts were derivatized with PFPA following purification with the MCX cartridges. A glass syringe containing a metal cannula was used to dispense the purified hair dye extract containing the internal standard (50 μL) into a septum-capped microvial (Chromacol, Trumbull, CT). The solvent was evaporated under a stream of argon until only DEG remained. Hexane (100 μL) was added, and the solution was vortexed. The solution was made alkaline by the addition of hexane:TMA (1.5:5) (5 μL). PFPA (2 μL) was added to the solution and mixed, and the derivatization was done at room temperature for 30 min (*35*). The volatile components were evaporated under argon, and then, hexane (100 μL) was added to the vial. The vial was vortexed, and the suspension was allowed to settle. A portion of the upper layer (hexane) was removed and placed into a new microvial for the GC-MS analysis.

The GC-MS analyses were performed on a ThermoFinnigan (San Jose, CA) TSQ 700 tandem quadrupole GC-MS, using a Varian (Palo Alto, CA) 3400 gas chromatograph and J&W Scientific (Folsom, CA) DB-5 ms capillary column (30 m × 0.25 mm × 0.25 μm). The carrier gas was helium at a pressure of 15 psi. The SPI injector, column oven, and transfer line were all temperature programmed. Injection volumes were 1 or 2 μL, using a CTC Analytics (Switzerland) model A200SE autosampler. Upon injection, the septum-equipped temperature programmable injector (SPI) was heated from 60 to 280 °C at 200 °C/min and held at 280 °C for 20 min. The column oven was held at 80 °C for 1 min, then heated to 280 °C at 10 °C/min, and held at 280 °C for 1 min. The GC-MS transfer line was held at 280 °C for 15 min before heating to 320 °C at 10 °C/min and then held at 320 °C for 3 min.

The TSQ700 MS was operated in the NICI mode with limited scan single quadrupole analysis. The first quadrupole (Q1) was scanned from *m*/*z* 200 to 310 with a 0.5 s cycle time. A commercial mixture of 10% NH_3 in N_2 was used as the reagent gas for production of thermalized electrons. The ion source was maintained at 150 °C (uncorrected) and 5 Torr pressure (uncorrected). For detection and quantification, the ions at *m*/*z* 295 (ABP isomers) and 304 ([$^2H_9$]-4-ABP), corresponding to $[M^\bullet - HF]^-$, were monitored. The retention times for the ABP isomers were as follows: 2-ABP−PFP (11.15 min), 3-ABP−PFP (13.58 min), [$^2H_9$]-4-ABP (13.89 min), and 4-ABP−PFP (13.93 min).

Additional analyses were performed on a ThermoFinnigan Voyager single quadrupole GC-MS using a Carlo Erba AS800 autosampler and GC 8000 TOP GC, which was equipped with a split/splitless injector operated in the splitless mode. The carrier gas was helium, controlled at 1 mL/min flow. The column was identical to the one for the other GC-MS analyses. The injector temperature was held at 275 °C, and the column was heated from 100 to 280 °C at 10 °C/min. The injection volume was 2 μL.

The Voyager MS was operated in the NICI mode with the combination ion source. Methane was the reagent gas, and the ion source pressure was controlled as a function of the backing pump vacuum (1.1×10^{-1} Torr). The ion source and transfer line temperatures were set at 200 and 250 °C, respectively. The quadrupole was operated in the selected ion monitoring (SIM) mode, collecting data for ABP and [$^2H_9$]-4-ABP ions at *m*/*z* 295 and 304, respectively.

Results

An extensive isolation procedure was required prior to the detection and quantification of ABP isomers in hair dyes (Figure 1). Both liquid−liquid partitioning and solid phase extraction methods were necessary to obtain extracts that were sufficiently clean to assay by HPLC-ESI-MS/MS and GC-NICI-MS. The dyes were assayed for ABP content either with or without treatment with Zn/HCl, which reduces any NO_2−BP, hydrazo-, azo-, and azoxy-ABP, or mix-dimer products that may be preformed in the hair dye portion or formed during the creme catalyzed oxidation of dye constituents back to the reduced ABP isomers (*36*).

The chemical composition of each dye was different, and the extent of emulsion produced during the initial solvent extraction with hexane varied as a function of the dye, which led to variable extraction efficiencies of 4-ABP. The employment of the MCX resin was critical for the success of this extraction method. The MCX resin effectively bound ABP while a number of lipophilic components that caused interferences in the MS assays were removed from the cartridge with CH_3OH. 4-ABP was then eluted with NH_3/CH_3OH (5% v/v). The overall recovery of [$^2H_9$]-4-ABP from the solvent blank workup was 89 ± 25% (mean ± SD, $N = 18$), and the recovery of [$^2H_9$]-4-ABP spiked at 10 ppb in the various hair dyes

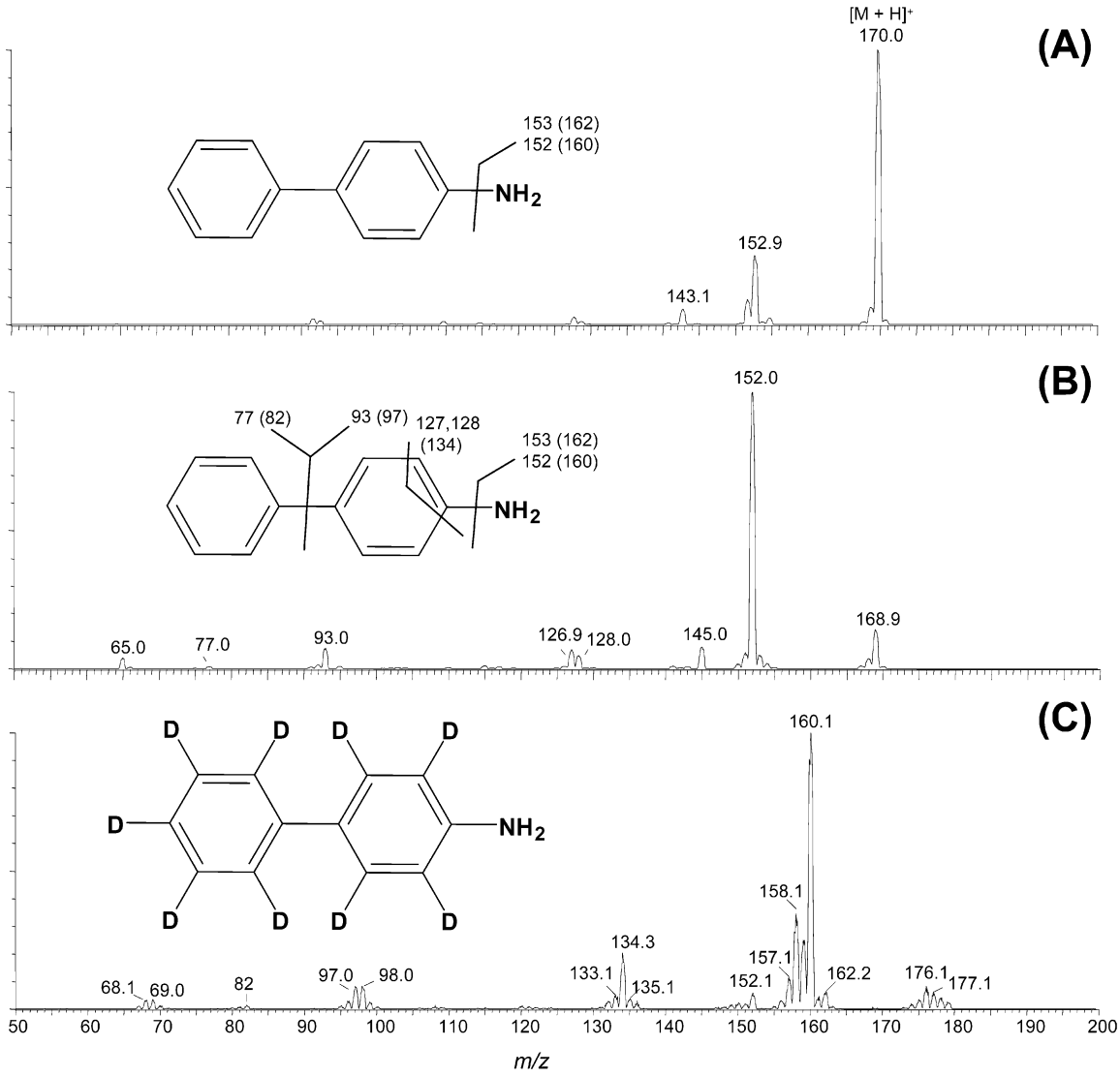

Figure 2. LC-ESI-MS/MS product ion mass spectra of 4-ABP and [$^2H_9$]-4-ABP and proposed mechanism of fragmentation. The mass assignments in parentheses represent the fragment ions of [$^2H_9$]-4-ABP. Fragmentation was done at either 16 and 31 eV for ABP (A and B, respectively) and at 31 eV for [$^2H_9$]-4-ABP (C).

was 53 ± 18% (mean ± SD, $N = 54$) based upon HPLC-ESI-MS/MS.

The LC-ESI-MS/MS product ion mass spectra of 4-ABP and [$^2H_9$]-4-ABP are presented in Figure 2. The collision energy conditions used to fragment ABP were optimized for maximum sensitivity for SRM analyses. Under low collision energy conditions (16 eV), the major fragment ion was observed at *m/z* 153 and attributed to loss of ammonia $[M + H - NH_3]^+$, but protonated 4-ABP $[M + H]^+$ at *m/z* 170 was still the principal ion in the spectrum. At the higher collision energy conditions used for ABP quantification (31 eV), there was elimination of a hydrogen atom as well, and the principal fragment ions for ABP were observed at *m/z* 169 $[M + H - H^\bullet]^+$, *m/z* 152 $[M + H - H^\bullet - NH_3]^+$, *m/z* 128 $[M + H - CH_2CNH_2^\bullet]^+$, *m/z* 127 $[M + H - NH_3 - C_2H_2]^+$, *m/z* 93 $[M + H - C_6H_5^\bullet]^+$, and *m/z* 77 $[C_6H_5^\bullet]^+$. In the case of [$^2H_9$]-4-ABP, there were important fragment ions seen in the product ion mass spectrum at *m/z* 176 $[M + H - HD]^+$, *m/z* 160 $[M + H - D^\bullet - NH_3]^+$, *m/z* 134 $[M + H - NH_3 - C_2D_2]^+$, *m/z* 97 $[M + H - C_6D_5^\bullet]^+$, and *m/z* 82 $[C_6D_5^\bullet]^+$. For quantitative measurements, the SRM transitions used for ABP isomers and [$^2H_9$]ABP were 170 → 152 and 179 → 160, respectively.

A calibration curve with at least five different concentrations of 4-ABP ranging from 0 to 1 ppb was used for quantification at low concentration levels. For higher concentration levels, a separate calibration curve was employed covering the 4-ABP concentrations from 0 to 10 ppb. Both calibration curves used a constant amount of [$^2H_9$]ABP, which was set at 10 ppb (Figure 3). Because of the intramolecular H−D scramble, the LC-ESI-MS/MS product ion mass spectrum of [$^2H_9$]-4-ABP contains more fragment ions than that observed for unlabeled ABP and the responses of the SRM transition $[M + H]^+ \rightarrow [M + H - 18]^+$ used for quantification are not equal. Therefore, the slope of the calibration curve is greater than unity when expressed as area response ratio vs amount ratio (ABP/[$^2H_9$]-4-ABP). The [$^2H_9$]ABP was also used as an internal standard to quantitate 2-ABP ($R^2 = 0.978$), which displayed a 4−5-fold greater response than 4-ABP in HPLC-ESI-MS/MS.

The HPLC-ESI-MS/MS analyses of a solvent blank workup, brown, and black hair dyes are presented in

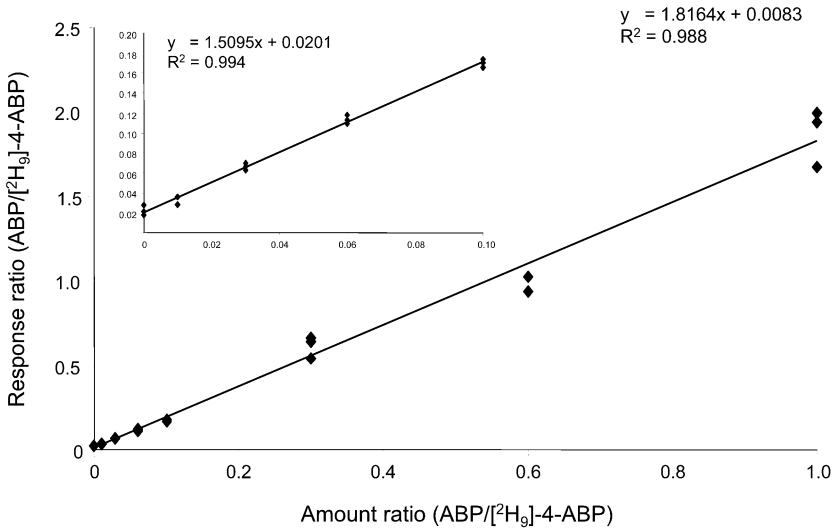

Figure 3. Calibration curve of 4-ABP with [$^2H_9$]-4-ABP set at 10 ppb. The full range is to 10 ppb. The insert shows the expanded range of 4-ABP up to 1 ppb.

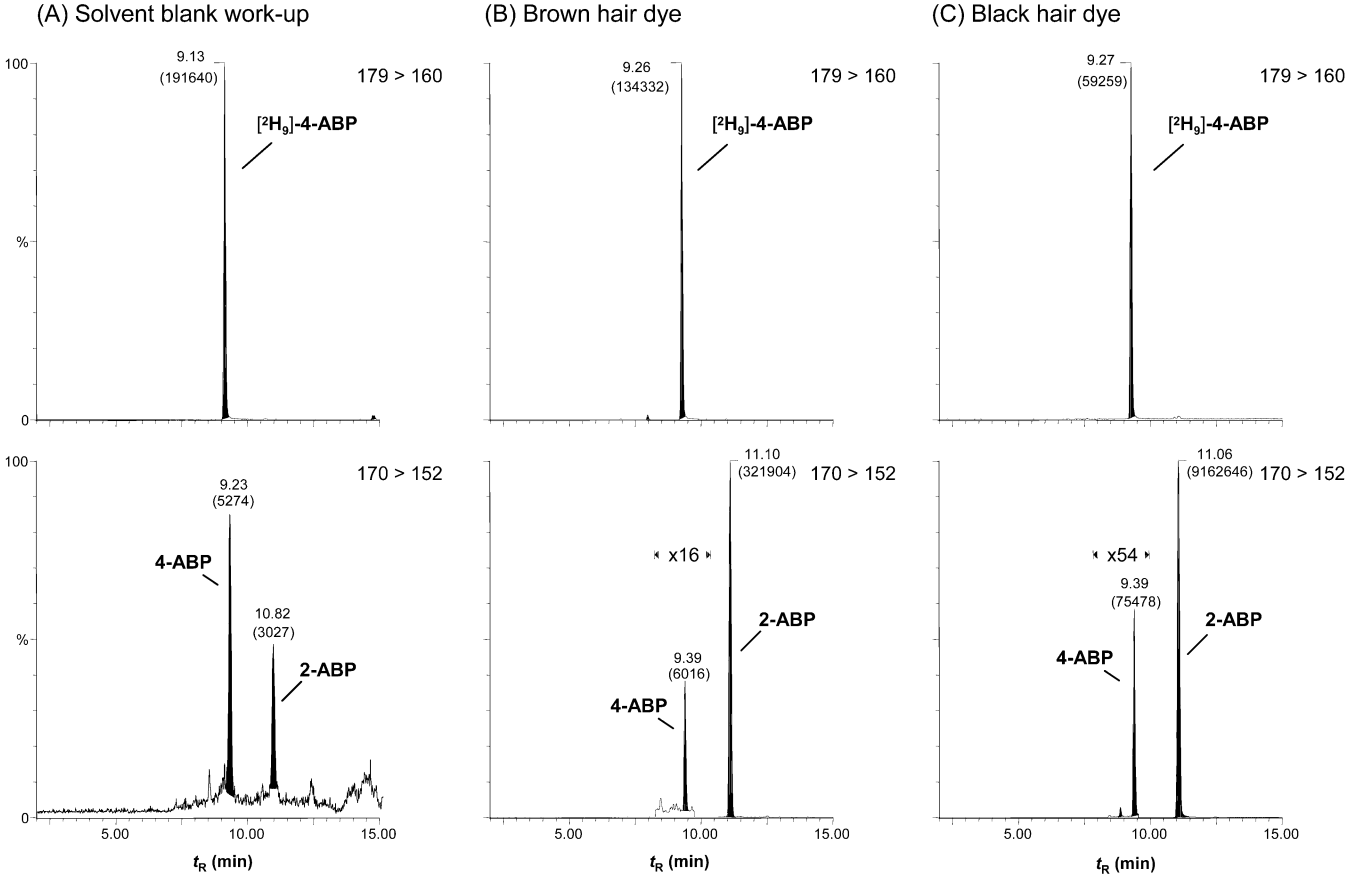

Figure 4. Analyses of ABP isomers in hair dyes by HPLC-ESI-MS/MS in SRM mode. (A) Blank solvent workup, (B) brown hair dye containing 4-ABP at levels <0.29 ppb (the LOD), and (C) a black hair dye containing high levels of ABP isomers. The chromatograms are scaled to the largest peaks, and the region of 4-ABP has been expanded in the hair dye chromatograms. Integrated areas are in parentheses.

Figure 4. The [$^2H_9$]-4-ABP elutes about 6 s earlier than 4-ABP because of the isotope effect. Very low levels of ABP isomers were detected in the solvent blanks that underwent the entire isolation procedure, indicating that there are trace amounts ABP isomers present in either the organic solvents, the solid phase extraction resins and holders, the tubings, or the various other labware. Trace levels of background 4-ABP have been previously reported in the laboratory surroundings during chemical analyses (*35*). The background levels of 2-ABP were estimated at 0.05 ± 0.05 ppb, and the background levels of 4-ABP were estimated at 0.08 ± 0.07 ppb (mean ± SD, $N = 18$). The LOD and LOQ were set at 3σ and 10σ SD units above the background level blanks based upon recommended guidelines for data acquisition and quality evaluation in environmental chemistry (*37*). The LOD and LOQ of 2-ABP are 0.20 and 0.55 ppb, respectively, and LOD and LOQ of 4-ABP are 0.29 and 0.78 ppb,

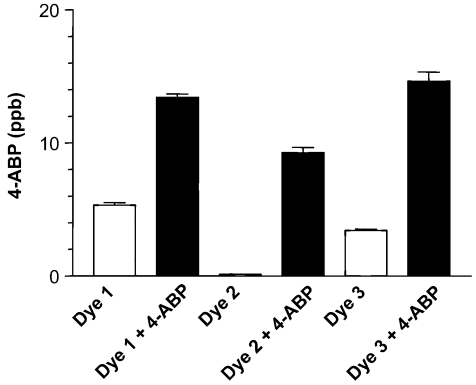

Figure 5. Estimates of 4-ABP added to three different hair dyes at 0 or 10 ppb and quantified by HPLC-ESI-MS/MS following chemical reduction with Zn/HCl. [$^2H_9$]-4-ABP was added to all dyes at 10 ppb.

respectively. The amount of 4-ABP present in this brown hair dye is below the LOD, while the amount present in the black hair dye is ~6 ppb. Significant amounts of 2-ABP (>LOQ) were detected in both dyes (Figure 4).

Three different hair dyes containing varying amounts of 4-ABP (<0.29−6 ppb) were mixed with creme developer and spiked with 4-ABP and [$^2H_9$]-4-ABP at a level of 10 ppb. After 15 min of color development with the creme mix containing H_2O_2, the hair dyes were partially purified and treated with Zn/HCl to reduce 4-ABP oxidation products to 4-ABP. The estimates of 4-ABP were within 5−23% of the expected target values, indicating that the analytical method with the Zn reduction step is quantitative and reproducible for 4-ABP measurements (Figure 5).

The identities of ABP isomers were corroborated by coelution of ABP standards with the hair dye extracts and by analysis of the dyes in the SRM scan mode using four characteristic transitions of ABP at 170 → 169, 170 → 152, 170 → 127, and 170 → 93, which were within ±15% relative abundance to reference compounds (data not shown). Moreover, product ion mass spectra were readily acquired on ABP analytes in many of these hair dye samples by injecting 5 μL of the extract and monitoring the protonated ABP molecule $[M + H]^+$ at *m*/*z* 170 and scanning from *m*/*z* 50 to 200. The mass spectra of the putative ABP derivatives in hair dyes were in excellent agreement with those of the reference compounds, and all major fragment ions of the ABP derivatives in hair dyes were present at similar relative abundance to those of the reference compounds (Figure 6). The product ion mass spectra of 2-ABP and 4-ABP are similar. The principal difference between the mass spectra of the two isomers is the fragment ion at *m*/*z* 128, which is more prominent in the spectrum of 2-ABP. Although 2-ABP and 4-ABP are readily resolved by HPLC, the 3-ABP and 4-ABP isomers are not separated. We evaluated several different solvent conditions and columns with different solid phase resins (C-8, C-12, C-18, phenyl, graphite, and a pyrenylethyl-modified resin) to separate 3-ABP from 4-ABP, as well as their acetylated derivatives, but none proved successful. The product ion spectrum of 3-ABP is also similar to those of 2-ABP and 4-ABP (data not shown). Thus, the peak identified as 4-ABP could also contain the 3-ABP isomer.

GC of ABP isomers following chemical derivatization with PFPA was utilized as a means to resolve 4-ABP and 3-ABP, which were separated by 20 s under these chromatographic conditions. GC-NICI-MS was also used as an independent means of quantification of 4-ABP employing the same calibration standards and levels as used for HPLC-ESI-MS/MS ($y = 1.32x + 0.048$; $R^2 = 0.994$). The LOD and LOQ for 4-ABP by GC-NICI-MS were comparable to the levels determined by HPLC-ESI-MS/MS and reflective of the trace contamination of 4-ABP found in the solvent blank workup. GC-NICI-MS analyses of a solvent blank workup, a weakly contaminated brown hair dye, and two contaminated black hair dyes are presented in Figure 7. Both 3-ABP and 4-ABP were present at trace levels in the brown hair dye and approached the LOD. The black hair dye extracts, which contained approximately 5 and 6 ppb of 3-ABP/4-ABP based upon HPLC-ESI-MS/MS estimates, were observed to contain significant amounts of 4-ABP while 3-ABP was found only at 9−13% of the peak area of 4-ABP. The signal response of equivalent amounts of 3-ABP and 4-ABP in GC-NICI-MS and HPLC-ESI-MS/MS was unity, demonstrating that 4-ABP was the principal isomer whereas 3-ABP was a minor contributor to the total ABP content in all hair dyes examined.

The hair dyes were assayed for ABP content either with or without treatment with Zn/HCl, which reduces any NO_2−BP, hydrazo-, azo-, and azoxy-ABP, or mixdimer products that may be preformed in the hair dye portion or formed during the creme-catalyzed oxidation of dye constituents back to the reduced ABP isomers (*36*). The ABP content quantified in various hair dyes by HPLC-ESI-MS/MS and GC-NICI-MS hair dyes is summarized in Table 1. 4-ABP and 2-ABP were above the LOQ in eight and 10, respectively, of the 11 hair dyes treated with Zn/HCl. The estimates of 4-ABP by HPLC-ESI-MS/MS are the sum of 4-ABP and any potential 3-ABP that coelutes. The estimates of 4-ABP by GC-NICI-MS were very similar to the combined 3-ABP + 4-ABP values determined by HPLC-ESI-MS/MS and further substantiated that 4-ABP as the principal carcinogenic isomer present in hair dyes. The amounts of ABP isomers estimated without reduction of the partially purified dye extracts with Zn/HCl were between 20 and >90% of the amounts observed with reduction. Because the recoveries of [$^2H_9$]ABP added to the hair dye and creme developer mixture without Zn/HCl reduction were not statistically different from those recoveries obtained with Zn/HCl treatment, some of the ABP may be present in the alkaline intermediate hair dye portions as N-oxidized or dimeric species.

The ABP isomers were below the LOD in the hair creme developer assayed without the hair dye portion, indicating that the ABP derivatives are present in the hair dye and not the creme. This observation was confirmed by assaying ABP content in a black hair dye with and without the creme developer (see dye 3). The 4-ABP content in the dye in the absence of the creme developer was found to be 2-fold greater than in the sample containing both hair dye and creme developer mixture, as expected, since addition of an equal volume of the creme developer, which has undetectable levels of 4-ABP, to the dye effectively dilutes the concentration of 4-ABP in the dye by a factor of 2. Effectively, the amount of ABP present in the hair dye portion is twice the value reported in the dye and creme mix in Table 1.

2-ABP and 4-ABP were detected in black, blonde, and red hair dyes. 2-ABP was also detected in brown hair

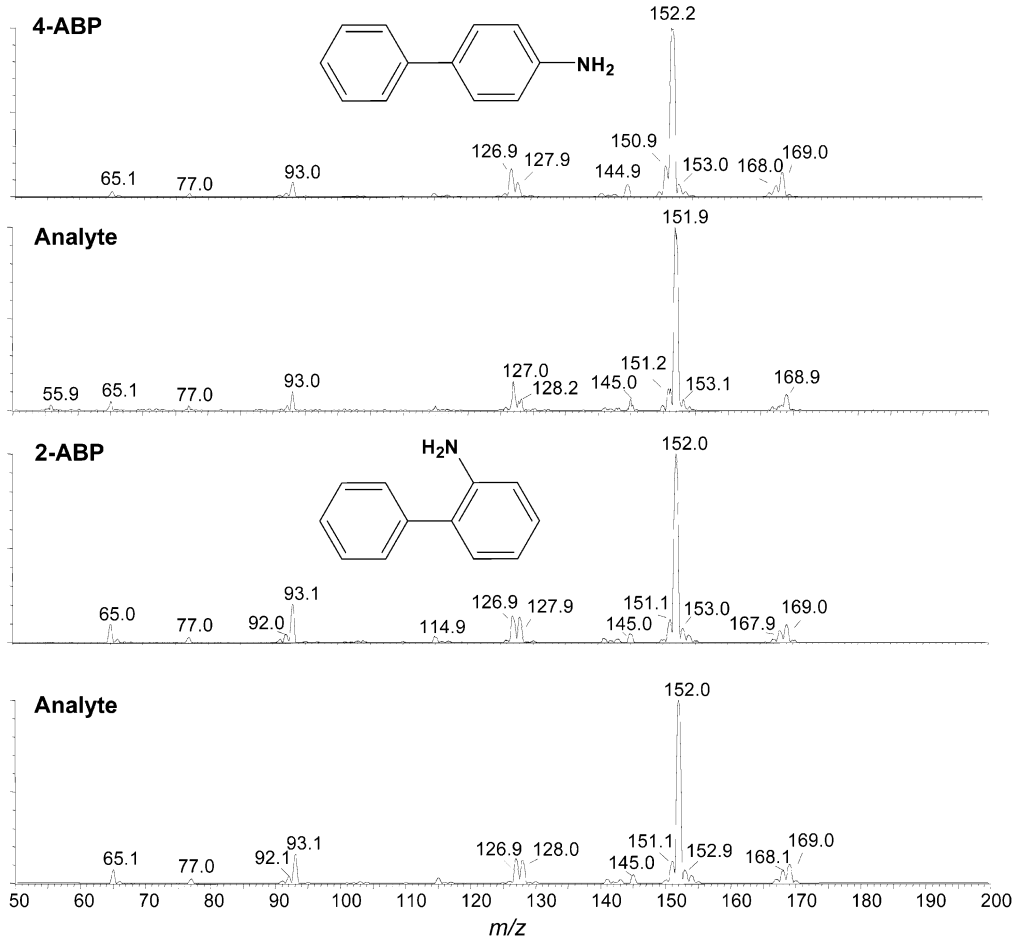

Figure 6. HPLC-ESI-MS/MS product ion mass spectra of synthetic 2-ABP and 4-ABP and analytes present in hair dye.

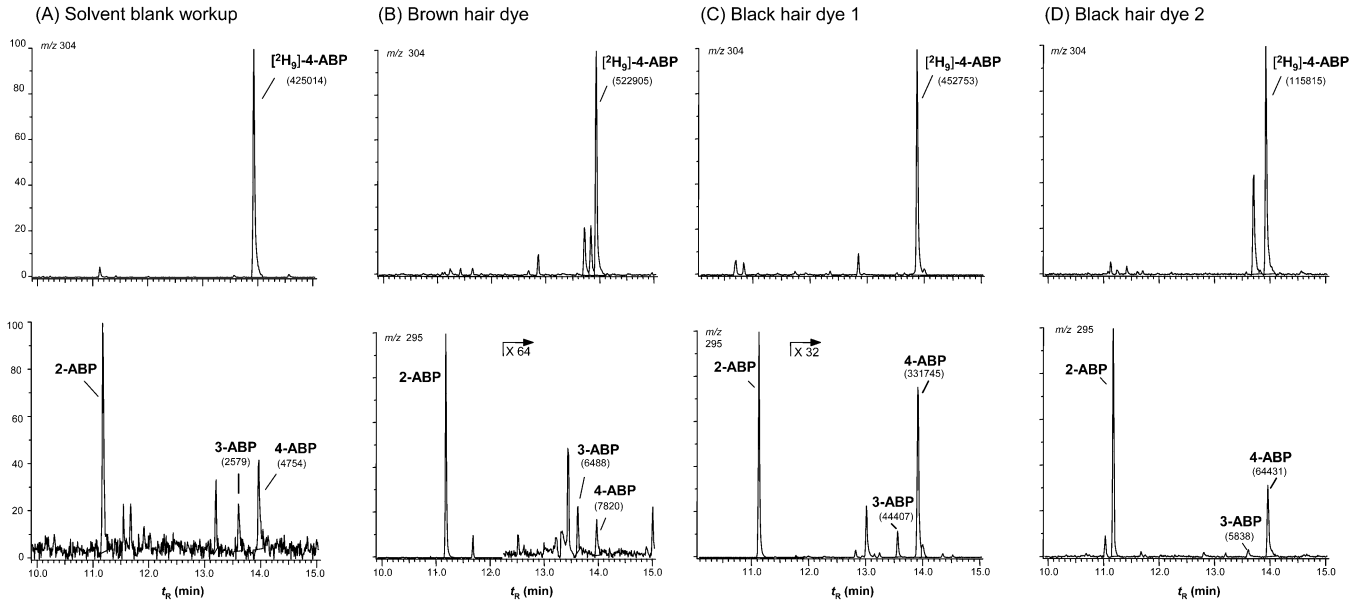

Figure 7. Analyses of ABP isomers in hair dyes by GC-NICI-MS. (A) Blank solvent workup, (B) brown hair dye containing 4-ABP at levels <0.29 ppb, (C, D) two different black hair dyes containing high levels of 4-ABP. Integrated areas are in parentheses.

dyes, while the amounts of 4-ABP were at or below the LOD in the brown dyes. The 4-ABP estimates in two black hair dyes that were assayed at least 3 weeks apart were within 10−25% of each other, while the 4-ABP content in the noncontaminated brown dye remained below the LOD (<0.29 ppb) on both occasions (Table 1). Two different lots of the same black (dye 3), brown (dye 7), and blonde hair dyes (dye 9) that were purchased 6 months apart were assayed for ABP. The 4-ABP content in the two lots of black and blonde hair dyes were within 1.8-fold of each other, while 4-ABP was near or below the LOD in the brown hair dyes. 2-ABP was below the LOD in the first lot of brown hair dye but present in significant quantities in the second lot (44 ppb), suggest-

Table 1. 4-ABP and 2-ABP Content in Hair Dyes and Creme Developer (1:1) Determined by HPLC-ESI-MS/MS and GC-NICI-MS[a]

		avg ± SD (ppb)			
		HPLC-ESI-MS/MS		GC-NICI-MS	
dye color	no.	4-ABP ± 3-ABP	2-ABP	4-ABP	comments
creme developer	0	<0.29	<0.20	not assayed	
black	1	3.96 ± 0.26	not assayed	not assayed	
black	1	5.33 ± 0.33	154 ± 32.2	5.30 ± 0.57	independent analysis; assayed more than 3 weeks apart
black	2	1.63 ± 0.25 (1.33 ± 0.84)	91.5 ± 5.5 (72.9 ± 20.8)	1.43 ± 0.38	
black	3	3.43 ± 0.17 (2.78 ± 0.06)	0.40 ± 0.06 (<0.20)*	3.78 ± 0.13	
black	3	3.81 ± 0.27	0.34 ± 0.02	not assayed	independent analysis; assayed more than 3 weeks apart
black	3	8.08 ± 0.66	1.9 ± 0.55	not assayed	no creme developer; 2 g of hair dye
black	3b	6.86 ± 0.45 (6.21 ± 0.56)	0.23 ± 0.03 (<0.20)	6.41 ± 0.40	same dye; different lot
black	4	1.27 ± 0.06 (0.82 ± 0.15)**	0.41 ± 0.05 (<0.20)*	1.08 ± 0.07	
black	5	0.37 ± 0.05	129 ± 33.3	<0.29	
brown	6	<0.29	2.97 ± 1.90	<0.29	
brown	6	<0.29	1.59 ± 0.30	not assayed	independent analysis; assayed more than 3 weeks apart
brown	7	0.33 ± 0.13	<0.20	<0.29	
brown	7b	<0.29 (<0.29)	44.2 ± 3.95 (26.4 ± 2.54)**	<0.29	same dye; different lot
wheat	8	<0.29	0.70 ± 0.06	<0.29	
blonde	9	2.75 ± 1.02 (0.54 ± 0.02)**	10.3 ± 1.91 (5.02 ± 0.53)**	1.80 ± 0.70	
blonde	9b	1.25 ± 0.08 (1.00 ± 0.37)	0.92 ± 0.13 (0.98 ± 0.37)	1.06 ± 0.06 (1.00 ± 0.39)	same dye; different lot
red	10	1.01 ± 0.02 (0.57 ± 0.06)**	4.41 ± 0.29 (2.44 ± 0.33)**	0.96 ± 0.05	
red	11	0.99 ± 0.09 (0.56 ± 0.03)**	14.1 ± 2.4 (7.78 ± 0.97)**	0.81 ± 0.26	

[a] Each dye was worked-up in triplicate. Values in parentheses are ABP content without Zn/HCl treatment. Student's *t*-test; *$p < 0.05$, **$p < 0.01$ for ABP content ± Zn/HCl treatment.

ing that there are batch to batch variations in ingredient purity.

The aromatic amine PPD, a key ingredient in the development of color for many hair dyes (*32*), was assayed as a possible source of contamination with ABP isomers. Three different preparations of PPD were purchased from vendors of chemical research and development products and screened for ABP isomers. The PPD was dissolved in 2% HCO_2H (10 mg/mL) and 3 μL (30 μg) assayed directly by HPLC-ESI-MS/MS or by GC-NICI-MS following solvent extraction with hexane and derivatization with PFPA using the same mass spectral acquisition parameters as for the hair dyes. ABP isomers were not detected in two of the PPD samples that were designated as 99% pure; however, 2-ABP and 3-ABP/4-ABP were detected in the third preparation, which was designated as 97% pure by the vendor (Figure 8). The identities of ABP derivatives were confirmed by HPLC-ESI-MS/MS product ion spectra and by GC-NICI-MS where the relative amounts of 3-ABP and 4-ABP (4:6 ratio) were determined (data not shown). The concentrations of 2-ABP, 3-ABP, and 4-ABP in PPD were estimated at 70 ppm, 310 ppb, and 500 ppb, respectively. A third peak was also detected by HPLC-ESI-MS/MS monitoring the product ions of the protonated ABP molecule $[M + H]^+$ at *m*/*z* 170 scanning from *m*/*z* 50 to 200. This peak was identified as DPA based upon coelution with the reference compound and the product ion mass spectrum, which was indistinguishable from that of the synthetic compound (Figure 8). The concentration of DPA exceeded 10 ppm based upon the PPD content. We also assayed for the carcinogen Bz as another possible contaminant present in PPD using the SRM transition $[M + H]^+ \rightarrow [M + H - 18]^+$ at *m*/*z* 185 → 167, but there was no evidence for the presence of this chemical (data not shown).

Discussion

An extraction scheme was developed to isolate ABP isomers from commercial hair dyes. The combined analyses by HPLC-ESI-MS/MS and GC-NICI-MS revealed that the urinary bladder carcinogen 4-ABP and the noncarcinogenic 2-ABP isomer (*38*) were present in some permanent hair dyes at quantifiable levels. The analysis of ABP isomers by HPLC-ESI-MS/MS was a more reliable method than by GC-NICI-MS because SRM scan modes with multiple transitions were used to quantify and corroborate the identity of the analytes (*34*). The identities of ABP analytes in some dyes were confirmed by HPLC-ESI-MS/MS with the acquisition of product ion mass spectra of the protonated molecules $[M + H]^+$, which were indistinguishable from those of the reference standard compounds, and thus fulfills the criteria of analyte confirmation in regulatory MS (*33*, *39*). The GC-NICI-MS method was less selective and did not meet the regulatory criteria for unequivocal identification of ABP

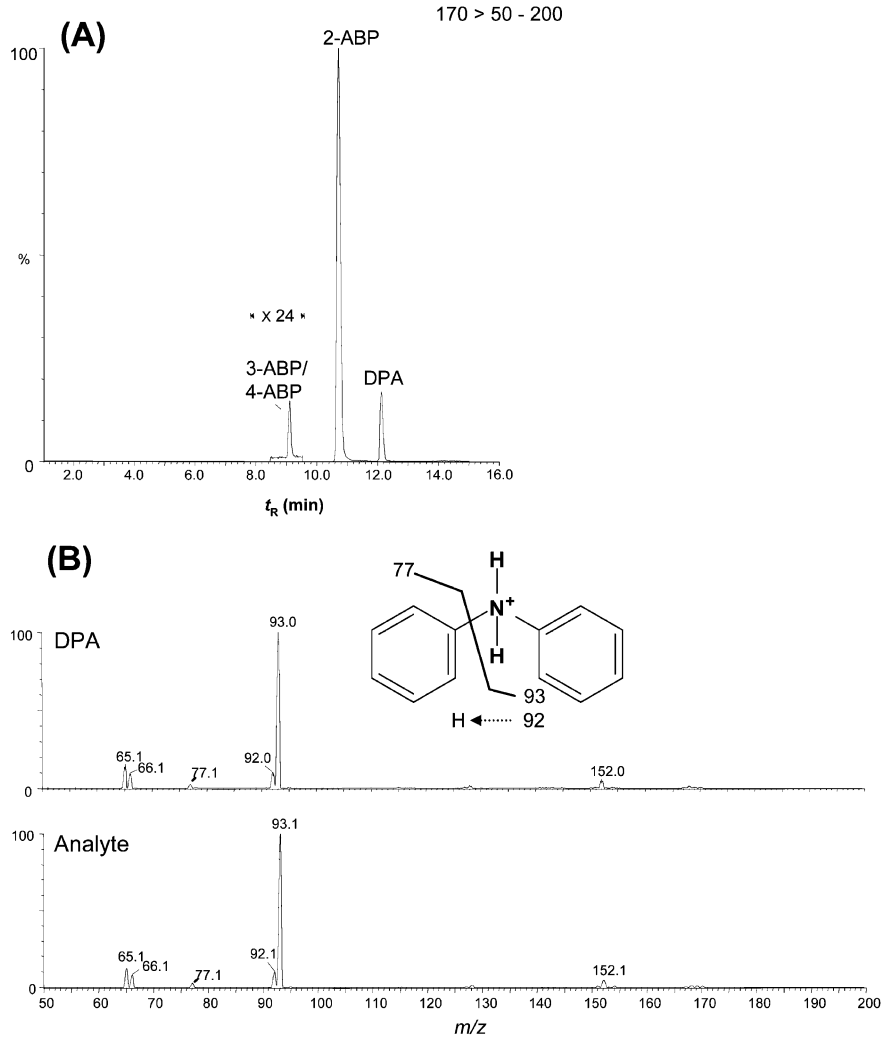

Figure 8. Analysis of PPD for contamination with ABP isomers by (A) HPLC-ESI-MS/MS in product ion scan mode of ABP $[M + H]^+$ at *m/z* 170 scanning from *m/z* 50 to 200, and (B) the product ion spectra of synthetic DPA and the analyte at 12.15 min are presented.

analytes since only one ion $[M^{\bullet} - HF]^-$ was monitored. We explored the analysis of ABP−PFP derivatives by GC-NICI-MS/MS, but the tandem MS did not provide significant and characteristic fragment ions attributable to the ABP moiety (Freeman, J. Unpublished data). Although 2-ABP and 4-ABP were well-resolved by HPLC, the separation of 3-ABP from 4-ABP proved to be a difficult task. Moreover, the product ion mass spectra of these ABP isomers were very similar. Consequently, the HPLC-ESI-MS/MS method provided strong evidence for the presence ABP derivatives in hair dyes but did not provide an unambiguous assignment of the isomer as either 3-ABP or 4-ABP (or both). However, the 3-ABP and 4-ABP isomers were readily separated by GC following derivatization with PFPA, and the GC-NICI-MS analyses were indispensable to establish that 4-ABP and not 3-ABP was the second principal ABP contaminant present in hair dyes. Ideally, the optimal method for ABP analyses is by HPLC-ESI-MS/MS where all three isomers are fully resolved. Methods of ABP isomer separation by HPLC are under continued investigation.

The amounts of 4-ABP found in hair dyes vary by more than 20-fold. In two of the most contaminated hair dyes samples, the amounts of 4-ABP equivalents following chemical reduction with Zn/HCl are 615 and 1130 ng per total application. The levels of 4-ABP present in hair dye samples without Zn/HCl treatment are within 20 to >90% of the values obtained following chemical reduction. In this regard, the oxidized products NO_2−BP, hydrazo-, azo-, or azoxy-, or mixed dimers that may form during the color development of hair dyes with creme containing H_2O_2 would be expected to be reduced to 4-ABP by bacteria in human skin, the intestinal flora, or by mammalian enzymes in vivo (*40*, *41*). In comparison to exposure of 4-ABP in hair dyes, variable amounts of 4-ABP have been reported in mainstream smoke of commercial cigarettes, a principal source of exposure to 4-ABP. The estimates of 4-ABP have been reported to range from 0.13 to 0.89 ng per cigarette (*42*), 2.4 ng per cigarette (*13*), and about 3.6 ng per cigarette (*43*). Thus, frequent treatments with hair dyes that are often done every 6 weeks may result in considerable exposure to 4-ABP over time.

Recent epidemiological data have revealed that frequent hair dye usage is a risk factor for urinary bladder cancer development (*16*, *24*, *25*), particularly in individuals who are NAT2 slow acetylators, where there is a 2.9-fold increase in risk. A concern has been raised that some carcinogenic arylamines may be present in hair dyes (*26*). 4-ABP produces bladder tumors in experimental animals and is recognized as a human bladder carcinogen (*2*, *4*). A major pathway of 4-ABP metabolism by humans is

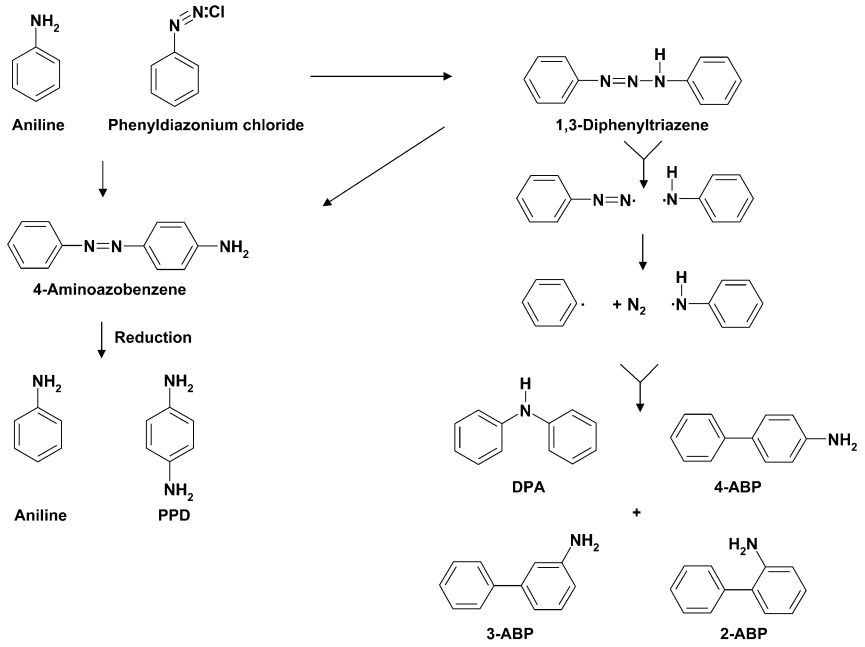

Figure 9. Synthesis of PPD through coupling of aniline with phenyldiazonium chloride salt. The formation of ABP isomers may occur through rearrangement of the 1,3-diphenyltriazene intermediate that is formed during the synthesis of PPD.

through cytochrome P450-mediated N-oxidation to produce the carcinogenic 4-hydroxyamino-biphenyl metabolite (*44*), which may bind to DNA (*28*) or proteins such as hemoglobin and serum albumin (*45*). N-Acetylation of 4-ABP by NAT2 is a competing pathway of metabolism and considered an important mechanism of detoxification of 4-ABP and other carcinogenic arylamines (*28*−*30*). As a consequence, individuals who are slow NAT2 acetylators may be at elevated health risk to this class of carcinogens, and these individuals who frequently use hair dyes that are contaminated with 4-ABP or other possible genotoxic aromatic amines may be at elevated health risk.

An accurate risk assessment of hair dye usage and the role of 4-ABP in elevated bladder cancer risk requires knowledge on the systemic absorption of ABP from the hair dyes. The extent of 4-ABP absorption through usage of hair dyes is not known, although several aromatic amines found in the environment and in hair dyes have been reported to be absorbed through the skin and skin of the scalp at appreciable levels (*17*, *46*, *47*). A preliminary study linked frequent hair dye usage with increased levels of DNA adducts of 4-ABP in epithelial cells from human breast milk (*31*), suggesting that a portion of the 4-ABP in hair dyes is absorbed and bioactivated. Moreover, environmental exposure to arylamines such as 4-ABP may account for a significant portion of nonsmoking-related bladder cancer in the general population (*48*).

The source of ABP contamination in hair dyes is not known. Some batches of PPD, a key ingredient for color formation in many permanent hair dyes (*32*), contain considerable amounts of ABP and DPA isomers and may be a source of ABP contamination in hair dyes. The origin of ABP contamination in chemical research grade PPD most likely occurs during the synthesis of PPD. There are several different methods of PPD synthesis including the reaction of NH_3 with 1,4-dichlorobenzene (*49*), nitration of acetanilide, or coupling of aniline with phenyldiazonium salt to form 4-aminoazobenzene followed by chemical reduction (*50*, *51*). The latter two synthetic methods may result in ABP isomer formation as minor side products. Trace amounts of biphenyl in either the benzene or the aniline used as precursors in the synthesis of PPD would undergo nitration to form principally 2-NO_2BP and 4-NO_2BP isomers (*52*) and be reduced to the respective ABP isomers during the reduction of 4-nitroaniline (or 4-nitroacetanilide) in the preparation of PPD. The coupling of aniline with the phenyldiazonium chloride salt to form 4-aminoazobenzene occurs through the formation of 1,3-diphenyltriazene as an intermediate product, which undergoes an intermolecular rearrangement to form 4-aminoazobenzene (Figure 9) (*53*). The formation of ABP and DPA isomers may occur at low levels during this rearrangement through the phenylamine radical and azophenyl radicals (*53*, *54*). The azophenyl radical eliminates N_2 to produce the phenyl radical, which may combine with the phenylamine radical to form the ABP isomers (Figure 9), although ABP formation through a phenyl cation pathway cannot be excluded (*53*). The PPD used in hair dye products may not be from the same suppliers as the research grade PPD. Furthermore, different methods of synthesis or purification of PPD could lead to vastly different amounts of contamination with ABP isomers.

The proportions of ABP isomers in hair dye products are variable (Table 1), and some differ from the relative amounts of ABP isomers present in research grade PPD. These findings suggest that either there are batch to batch variations of PPD contamination with ABP or that other aromatic amine constituents present in hair dye products may be sources of ABP contamination. The usage of hair dyes is growing in popularity worldwide. The purity of the PPD and also other aromatic amine constituents used in hair dyes may have an impact on the 4-ABP content and the safety of these products.

Acknowledgment. We are grateful for the helpful comments and discussions provided by Dr. F. Beland, Dr. D. Casciano, and Dr. D. Doerge of NCTR; Dr. P. Skipper of MIT; and Dr. P. Vouros of Northeastern University. The administrative aid of Ms. J. Dienstag, C. Teitel, and

R. York is also appreciated. Disclaimer: The views presented in this article do not necessarily reflect those of the United States Food and Drug Administration.

References

(1) Rehn, L. (1895) Blasengeschwulste bei fuchsinarbeitern. *Arch. Klin. Chir.* **50**, 588−600.

(2) Clayson, D. B. (1981) Specific aromatic amines as occupational bladder carcinogens. *Natl. Cancer Inst. Monogr.*, 15−19.

(3) Vineis, P., and Pirastu, R. (1997) Aromatic amines and cancer. *Cancer Causes Control* 8, 346−355.

(4) Hazardous substances databank (HSDB) (2003) http://toxnet.nlm.hih.gov.

(5) Stavric, B., Klassen, R., and Miles, W. (1979) Gas−liquid chromatographic-mass spectrometric determination of α- and β-naphthylamines in FD&C Red No. 2 (amaranth). *J. Assoc. Off. Anal. Chem.* **62**, 1020−1026.

(6) Davis, V. M., and Bailey, J. E., Jr. (1993) Chemical reduction of FD&C yellow No. 5 to determine combined benzidine. *J. Chromatogr.* **635**, 160−164.

(7) Garrigos, M. C., Reche, F., Pernias, K., Sanchez, A., and Jimenez, A. (1998) Determination of some aromatic amines in finger-paints for children's use by supercritical fluid extraction combined with gas chromatography. *J. Chromatogr. A* **819**, 259−266.

(8) Lancaster, F. E., and Lawrence, J. F. (1999) Determination of benzidine in the food colours tartrazine and sunset yellow FCF, by reduction and derivatization followed by high-performance liquid chromatography. *Food Addit. Contam.* **16**, 381−390.

(9) Cioni, F., Bartolucci, G., Pieraccini, G., Meloni, S., and Moneti, G. (1999) Development of a solid-phase microextraction method for detection of the use of banned azo dyes in coloured textiles and leather. *Rapid Commun. Mass Spectrom.* **13**, 1833−1837.

(10) Oh, S. W., Kang, M. N., Cho, C. W., and Lee, M. W. (1997) Detection of carcinogenic amines from dyestuffs or dyed substrates. *Dyes Pigm.* **33**, 119−135.

(11) Tokiwa, H., Nakagawa, R., and Horikawa, K. (1985) Mutagenic/carcinogenic agents in indoor pollutants; the dinitropyrenes generated by kerosene heaters and fuel gas and liquefied petroleum gas burners. *Mutat. Res.* **157**, 39−47.

(12) Chiang, T. A., Pei-Fen, W., Ying, L. S., Wang, L. F., and Ko, Y. C. (1999) Mutagenicity and aromatic amine content of fumes from heated cooking oils produced in Taiwan. *Food Chem. Toxicol.* **37**, 125−134.

(13) Patrianakos, C., and Hoffmann, D. (1979) Chemical studies on tobacco smoke LXIV. On the analysis of aromatic amines in cigarette smoke. *J. Assoc. Off. Anal. Chem.* **3**, 150−154.

(14) Department of Health and Human Services, Food and Drug Administration (1985) 21 CFR Parts 74, 81, and 82 (Docket No. 84N-0319) FD&C Yellow No. 5. *Fed. Regist.* **50**, 35774−35783.

(15) Reisch, M. S. (2003) Flush with color: Continuing progress in hair dye chemistry satisfies diverse desires of young and old. *Chem. Eng. News* **81**, 25−27.

(16) Gago-Dominguez, M., Castelao, J. E., Yuan, J. M., Yu, M. C., and Ross, R. K. (2001) Use of permanent hair dyes and bladder-cancer risk. *Int. J. Cancer* **91**, 575−579.

(17) Ames, B. N., Kammen, H. O., and Yamasaki, E. (1975) Hair dyes are mutagenic: identification of a variety of mutagenic ingredients. *Proc. Natl. Acad. Sci. U.S.A.* 72, 2423−2427.

(18) Rojanapo, W., Kupradinun, P., Tepsuwan, A., Chutimataewin, S., and Tanyakaset, M. (1986) Carcinogenicity of an oxidation product of *p*-phenylenediamine. *Carcinogenesis* **7**, 1997−2002.

(19) Hennekens, C. H., Speizer, F. E., Rosner, B., Bain, C. J., Belanger, C., and Peto, R. (1979) Use of permanent hair dyes and cancer among registered nurses. *Lancet* **1**, 1390−1393.

(20) Ohno, Y., Aoki, K., Obata, K., and Morrison, A. S. (2003) Case-control study of urinary bladder cancer in metropolitan Nagoya. *Natl. Cancer Inst. Monogr.* **69**, 229−234.

(21) Henley, S. J., and Thun, M. J. (2001) Use of permanent hair dyes and bladder-cancer risk. *Int. J. Cancer* **94**, 903−906.

(22) Hartge, P., Hoover, R., Altman, R., Austin, D. F., Cantor, K. P., Child, M. A., Key, C. R., Mason, T. J., Marrett, L. D., Myers, M. H., Narayana, A. S., Silverman, D. T., Sullivan, J. W., Swanson, G. M., Thomas, D. B., and West, D. W. (1982) Use of hair dyes and risk of bladder cancer. *Cancer Res.* **42**, 4784−4787.

(23) La Vecchia, C., and Tavani, A. (2001) Hair dyes and bladder cancer: an update. *Eur. J. Cancer Prev.* **10**, 205−208.

(24) Gago-Dominguez, M., Chan, K. K., Ross, R. K., and Yu, M. C. (2001) Permanent hair dyes and blader cancer risk. *Int. J. Cancer* **94**, 904−906.

(25) Yu, M. C., Skipper, P. L., Tannenbaum, S. R., Chan, K. K., and Ross, R. K. (2002) Arylamine exposures and bladder cancer risk. *Mutat. Res.* **506**−**507**, 21−28.

(26) Gago-Dominguez, M., Bell, D. A., Watson, M. A., Yuan, J. M., Castelao, J. E., Hein, D. W., Chan, K. K., Coetzee, G. A., Ross, R. K., and Yu, M. C. (2003) Permanent hair dyes and bladder cancer: risk modification by cytochrome P4501A2 and *N*-acetyltransferases 1 and 2. *Carcinogenesis* **24**, 483−489.

(27) Golka, K., Prior, V., Blaszkewicz, M., and Bolt, H. M. (2002) The enhanced bladder cancer susceptibility of NAT2 slow acetylators towards aromatic amines: a review considering ethnic differences. *Toxicol. Lett.* **128**, 229−241.

(28) Beland, F. A., and Kadlubar, F. (1990) Metabolic activation and DNA adducts of aromatic amines and nitroaromatic hydrocarbons. In *Chemical Carcinogenesis and Mutagenesis I* (Cooper, C. S., Ed.) Vol. 1, pp 267−325, Springer, Berlin.

(29) Hein, D. W., Doll, M. A., Fretland, A. J., Gray, K., Deitz, A. C., Feng, Y., Jiang, W., Rustan, T. D., Satran, S. L., and Wilkie, T. R., Sr. (1997) Rodent models of the human acetylation polymorphism: comparisons of recombinant acetyltransferases. *Mutat. Res.* **376**, 101−106.

(30) Hein, D. W., Doll, M. A., Fretland, A. J., Leff, M. A., Webb, S. J., Xiao, G. H., Devanaboyina, U. S., Nangju, N. A., and Feng, Y. (2000) Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. *Cancer Epidemiol. Biomarkers Prev.* **9**, 29−42.

(31) Gorlewska-Roberts, K., Green, B., Fares, M., Ambrosone, C. B., and Kadlubar, F. F. (2002) Carcinogen-DNA adducts in human breast epithelial cells. *Proc. Am. Assoc. Cancer Res.* **43**, 1018−1019.

(32) Corbett, J. F., and Menkart, J. (1973) Hair Coloring. *Cutis* **12**, 190−197.

(33) Brumley, W. C., and Sphon, J. A. (1981) Regulatory mass spectrometry. *Biomed. Mass Spectrom.* 8, 390−396.

(34) Bladwin, R., Bethem, R. A., Boyd, R. K., Budde, W. L., Cairns, T., Gibbons, R. D., Henion, J. D., Kaiser, M. A., Lewis, D. L., Matuski, J. E., Sphon, J. A., Stephany, R. W., and Trubey, R. K. (1997) 1996 ASMS Fall Workshop: Limits to Confirmation, Quantitation, and Detection. *J. Am. Soc. Mass Spectrom.* 8, 1180−1190.

(35) Bryant, M. S., Skipper, P. L., Tannenbaum, S. R., and Maclure, M. (1987) Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. *Cancer Res.* **47**, 602−608.

(36) March, J. (1992) *Advanced Organic Chemistry: Reactions, Mechanism and Structure*, p 1224, John Wiley & Sons, New York.

(37) MacDougall, D., Amore, F. J., Cox, G. V., Crosby, D. G., Estes, F. L., Freeman, D. H., Gibbs, W. E., Gordon, G. E., Keith, L. H., Lal, J., Langner, R. R., McClelland, N. I., Phillips, W. F., Pojasek, R. B., and Sievers, R. E. (1980) Guidelines for data acquistion and data quality evaluation in environmental chemistry. *Anal. Chem.* 52, 2242−2249.

(38) NTP (1982) Carcinogenesis bioassay of 2-biphenylamine hydrochloride in F344/N rats and B6C3F1 mice, NTP Report No. 233.

(39) Sphon, J. A. (1978) Use of mass spectrometry for confirmation of animal drug residues. *J. Assoc. Off. Anal. Chem.* **61**, 1247−1252.

(40) Platzek, T., Lang, C., Grohmann, G., Gi, U. S., and Baltes, W. (1999) Formation of a carcinogenic aromatic amine from an azo dye by human skin bacteria in vitro. *Hum. Exp. Toxicol.* **18**, 552−559.

(41) Chung, K. T., Stevens, S. E., Jr., and Cerniglia, C. E. (1992) The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18**, 175−190.

(42) Luceri, F., Pieraccini, G., Moneti, G., and Dolara, P. (1993) Primary aromatic amines from side-stream cigarette smoke are common contaminants of indoor air. *Toxicol. Ind. Health* **9**, 405−413.

(43) Grimmer, G., Schneider, D., Naujack, K. W., Dettbarn, G., and Jacob, J. (1996) Intercept-reactant method for the determination of aromatic amines in mainstream tobacco smoke. *Polycyclic Aromat. Compd.* **9**, 85−92.

(44) Butler, M. A., Iwasaki, M., Guengerich, F. P., and Kadlubar, F. F. (1989) Human cytochrome P-450$_{PA}$ (P450IA2), the phenacetin *O*-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7696−7700.

(45) Skipper, P. L., and Tannenbaum, S. R. (1990) Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis* **11**, 507−518.

(46) Wolfram, L. J., and Maibach, H. I. (1985) Percutaneous penetration of hair dyes. *Arch. Dermatol. Res.* 277, 235−241.

(47) Yourick, J. J., and Bronaugh, R. L. (2000) Percutaneous penetration and metabolism of 2-nitro-*p*-phenylenediamine in human and fuzzy rat skin. *Toxicol. Appl. Pharmacol.* **166**, 13−23.

(48) Skipper, P. L., Tannenbaum, S. R., Ross, R. K., and Yu, M. C. (2003) Nonsmoking related arylamine exposure and bladder cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **1**2, 503−507.

(49) German Patent (1907) Actien-Gesellschaft für anilin-fabrikation in Berlin. Verfahren zur Darstellung von *p*-Phenylendiamin. Patent no. 202170.

(50) Zollinger, H. (1961) Diazoamino Compounds. *Azo and Diazo Chemistry Aliphatic and Aromatic Compounds*, pp 177−189, Interscience Publishers, New York.

(51) Sidgwick, N. V. (1966) Aromatic Amines. In *The Organic Chemistry of Nitrogen* (Millar, I. T., and Springall, H. D., Eds.) pp 140−194, Oxford Press, Oxford.

(52) Billing, C. **J**., and Norman, R. O. C. (1961) The ortho: para-ratio in aromatic substitution. Part IV. The nitration of biphenyl. *J. Chem. Soc.* 3885−3888.

(53) Hegarty, A. A. (1978) Kinetics and mechanisms of reactions involving diazonium and diazo groups. In *The Chemistry of Diazonium and Diazo Groups* (Patai, S., Ed.) pp 511−592, Wiley, New York.

(54) Lippmaa, E., Saluvere, T., Pehk, T., and Olivson, A. (1973) Chemical polarization of ^{13}C and ^{15}N nuclei in the thermal decomposition of diazoaminobenzene (1,3-Diphenyltriazene). *Org. Magn. Reson.* 5, 429−436.

TX030029R