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Separation and analysis of dimethylaniline isomers by supercritical fluid chromatography—Electrospray ionization tandem mass spectrometry

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ABSTRACT

The assessment of human exposure to specific isomers of dimethylanilines (DMA's) is of interest for the evaluation of potential exposure-health outcome relationships. Improved analytical methods will help in identifying the environmental sources of such exposures. The separation of all six DMA isomers by supercritical fluid chromatography (SFC), without derivatization, is reported within. Further, the combination of SFC with electrospray ionization/tandem mass spectrometry provides selective detection in crude extracts of spiked (40 ppb of 3,5-dimethylaniline) raw materials. The raw materials chosen for analysis are commonly used in the manufacture of consumer hair-dye products.

1. Introduction

The significance of environmental exposure to low-levels of the chemical class of dimethylanilines is a topic of interest in the fields of epidemiology and toxicology. Most attention is focused on *specific* isomers such as 3,5-dimethylaniline. For example, Gan et al. measured the levels of various alkylaniline hemoglobin adducts, in samples collected from subjects (both cases and controls) in the Los Angeles Bladder Cancer Study [1]. They reported that the levels of 3,5-dimethylaniline (3,5 DMA) hemoglobin adducts were increased in women who regularly used permanent hair dyes. Other alkylanilines did not show an association between hemoglobin adducts and permanent hair-dye use. Cigarette smoking is an established risk factor for bladder cancer and it is considered to contribute to at least 50% of current cases in the US [2]. Cigarette smoke contains arylamines that are known bladder carcinogens (4-aminobiphenyl and 4-naphthylamine). All of the possible 2-substituted DMA's have also been detected in cigarette smoke [3].

Members of the DMA-class have been measured in various other contexts over the years. Some examples are 2,6 DMA as a metabolite of lidocaine [4,5]; 2,4 DMA from Amitraz, a mitocide in honey production [6]; DMA's released from their "sulfinamide-captured" form in human hemoglobin adducts [7,8] and DMA's as azo dye bacterial metabolism products [9]. The analytical methods employ mainly high-resolution-capillary GC (typically of DMA's as perfluoroalklyamide derivatives) or reverse-phase HPLC of some of the free amines. We are not aware of any published HPLC methods that separate all of the DMA isomers.

Detection methods typically employ varying degrees of selectivity and sensitivity, e.g., MS with electron ionization, MS with negative chemical ionization, or tandem mass spectrometry (MS/MS) with electrospray ionization. However, all mass spectral detection schemes produce similar signals for DMA isomers, so "separation" in another dimension (e.g., chromatography) is required when isomer-specific analysis is essential. Only capillary GC of pentafluoro-propionamide derivatized DMA's has been successfully used to separate all six DMA isomers [8]. However, concentration of derivatized samples, or attempts at removing the derivatizing reagents by evaporation, can lead to losses of the volatile analytes as well. Normal-phase HPLC conditions on polar-modified (diol and cyano classes) polymer skeletons (styrene-divinyl benzene) have been used to achieve baseline resolution of underivatized 2,6/2,4/and 3,5 DMA [10]. Supercritical fluid chromatography (SFC), often regarded as a normal-phase technique, offers higher separation efficiency than HPLC. We report within the normal-phase, SFC separation of all six DMA isomers *without derivatization*, and detection using electrospray ionization tandem mass spectrometry (MS/MS). This detection scheme has previously been reported for 2,6 DMA determinations in lidocaine metabolism [5]. The method has been initially applied to a short survey of hair-dye raw materials that are among those used at the highest volume worldwide by the international hair-dye industry.

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2. Experimental

2.1. Precautions

Alkylanilines are considered to be mutagenic and toxic. They may have carcinogenic activity as well. The compounds should be handled using appropriate personal protective equipment and ventilation in a properly rated fume hood.

Six dimethylaniline isomers were purchased from Sigma–Aldrich. The methyl group positional isomers are: 2,3 [CAS 87-59-2]; 2,4 [CAS 95-68-1]; 2,5 [CAS 95-78-3]; 2,6 [CAS 87-62-7]; 3,4 [CAS 95-64-7] and 3,5 [CAS 108-69-0] dimethylanilines. The stated purity was 98% or greater. Individual standards were made at 1.0 mg/mL in HPLC-grade methanol and were serially diluted by factors of 10× to a low-level of 1.0 μg/mL. For spiked raw materials, 4 μL of a solution of 1.0 ng/μL 3,5-dimethylaniline standard were added to 100 mg of solid, using a 10 μL syringe (40 part-per-billion standard). A swift movement of the plunger effectively "sprayed" the solution down on to the surface of the raw material, where it was allowed to dry for at least 15 min under ambient conditions.

Raw materials for testing were obtained from internal, manufacturing product supply (Procter and Gamble, Stamford, CT) or from well known, hair-colorant industry suppliers (J.H. Lowenstein, Brooklyn, N.Y., James-Robinson, Huddersfield, U.K. and Dupont, Wilmington, DE): 4-aminophenol [123-30-8]; 5-amino-2-methylphenol [2835-95-2]; 3-aminophenol [591-27-5]; 4-(methylamino)phenol sulfate [55-55-0]; 5-[(2-hydroxyethyl)amino]-2-methylphenol [55302-96-0]; 2-methylbenzene-1,4-diamine sulfate [615-50-9]; benzene-1,4-diamine [106-50-3]; N,N-bis(2-hydroxyethyl)-1,4 benzenediamine sulfate[54381-16-7]; 2-(4,5-diamino-1H-pyrazol-1H-yl)ethanol sulfate [155601-30-2] and 2,4-dihydro-5-methyl-2-phenyl-3H-pyrazole-3-one [89-25-8].

A raw-material sample (100 mg) was prepared in duplicate and spiked as described above. Two native samples (unspiked) of 100 mg were also weighed. The samples were transferred to individual glass vials (7.0 mL capacity and teflon-lined lid) containing 2.0 mL of 1.0N aqueous NaOH solution and the vial was shaken vigorously to disperse and dissolve the raw material. A 2.0 mL aliquot of HPLC-grade hexane was added next and the sample was gently shaken for one minute. The layers were allowed to separate and 1 mL of the upper, hexane layer was transferred to a conical glass vial, pre-marked at a volume of 100 μL. The vial was placed in a fume hood under a very gentle nitrogen stream and evaporated slowly (25 min.) to the volume mark. A ten microliter aliquot (5% of the original extract) was removed and injected for SFC–MS analysis. Recovery was evaluated by placing a 4 μL aliquot of 3,5-dimethylaniline solution in methanol (1.0 ng/μL) into a conical vial, allowing the methanol to evaporate in a fume hood at ambient conditions and adding 100 μL hexane to the sample.

Supercritical fluid chromatography (SFC) was carried out using a Berger Analytical SFC (Newark, DE) system. It was equipped with two columns in series (4.6 mm I.D. × 250 mm ethyl pyridine column, particle size 5 μm, pore size 60 angstrom, Princeton Chromatography, Cranbury, NJ). The flow rate was 2.0 mL/min or 3.0 mL/min, as specified within. The mobile phase composition was 5% by volume 10 mM ammonium acetate in methanol and 95% CO_2. The separation was done isocratically and isobarically (200 bar). Detection was performed using an HP 1050 diode array detector, while optimizing the separation of standards.

SFC/mass spectrometry was carried out with post-column addition of 10 mM ammonium acetate in methanol, via a "tee" to an ISCO model 250D syringe pump operating at a flow of 0.1 mL/min. For trace analysis, an Applied Biosystems ABI-4000 triple-quadrupole mass spectrometer was used. Typical electrospray ionization conditions were used (sprayer voltage 4–5.5 kV, turbo ion spray temperature 450 °C, orifice voltage 26 V). Resolution was set in the full-scan mode to at least a 20% valley between the protonated molecule and its [^{13}C]-isotope peak. The resolution of the first quadrupole was not altered for trace analysis in the multiple-reaction-monitoring (MRM) mode. The third quadrupole was run at the manufacturer's low-resolution setting. The collision-energy was set at 25 eV (laboratory frame of reference).

3. Results and discussions

The electrospray MS/MS (product-ion) spectra of the protonated molecule of DMA's are highly similar and cannot be used to establish the dimethyl substitution pattern of the standards, a priori. In contrast, the calculated [^{1}H] NMR spectra of dimethylaniline isomers have distinctly different line patterns and can unequivocally establish bond-to-bond linkages. Therefore, the [^{1}H] NMR spectrum of the purchased standard of 3,5-dimethylaniline was obtained, and the spectrum was found consistent with the 3,5-dimethyl substitution pattern (data not shown).

The normal-phase SFC separation of a mixture of six, underivatized dimethylaniline isomers is shown in Fig. 1, using MRM detection. The elution order was established by injection of the individual standards. In the mixture analysis, the 3,5-dimethylaniline isomer is well resolved from the other isomers. Initially a flow rate of 2.0 mL/min was used, and the retention time of 3,5-dimethylaniline was found to be 9.77 min with a standard deviation of 0.13 min (n = 16). In later analyses at the higher flow rate of 3.0 mL/min, the retention time was 5.22 min with a standard deviation of 0.17 min (n = 23). The separation factor was slightly higher at this flow rate. It is important to note that SFC allows DMA's to be readily analyzed without derivatization. Previous reports have alluded to the difficulty of consistently running these materials underivatized by capillary GC, where poor peak shape can arise as the analytical system becomes contaminated with sample matrix [11]. We observe no such problems here, injecting a high percentage (5%) of a simple, raw-material extract. This approach is simpler than using derivatization with capillary GC and does not lead to potential losses of volatile derivatives in sample concentration steps.

Detection by mass spectrometry—the interface of the SFC system to the mass spectrometer, with a post-column-tee for addition of ammonium acetate in methanol, prior to the ionization interface, has been previously described [12]. Electrospray ionization produces the protonated molecule (m/z 122) with little fragmentation. Collision-induced dissociation ("MS/MS" mode) causes formation of the radical cation of m/z 107 (loss of methyl radical) and the even-electron carbonium ion of m/z 105 (loss of ammonia), consistent with previous reports [5]. The sensitivity of the selected-reaction-monitoring method (detecting m/z 105$^+$ as a product ion of m/z

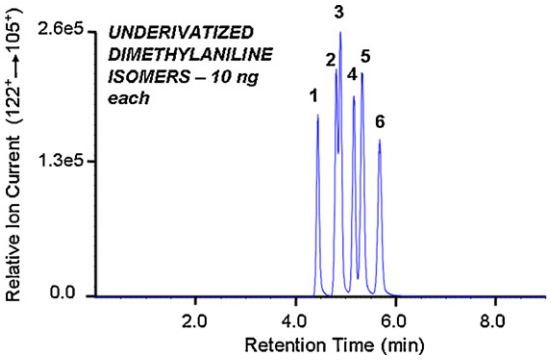

Fig. 1. SFC of a mixture of six, dimethylaniline isomers: (1) 2,6, (2) 2,5, (3) 2,4, (4) 2,3, (5) 3,5, (6) 3,4—10.0 ng each on-column, with detection by electrospray ionization tandem mass spectrometry (selected-reaction monitoring, m/z 122$^+$ to m/z 105$^+$).

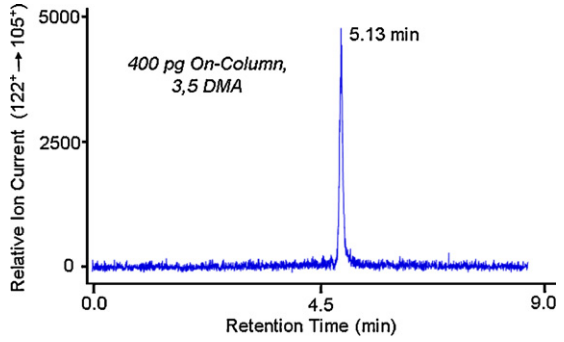

Fig. 2. SFC–electrospray ionization tandem mass spectrometry analysis (selected-reaction monitoring, m/z 122$^+$ to m/z 105$^+$) of 400 pg of 3,5-dimethylaniline (DMA).

122$^+$) is demonstrated in Fig. 2, where analysis of a standard is shown. The calculated mass delivered to the column is 400 pg or 3.3 pmols. The signal/noise (s/n) ratio is greater than 20:1 and the data have not been smoothed (which can improve s/n significantly). It is a reasonable hypothesis that the instrumental limit of detection is much lower than 400 pg injected.

Fig. 3 shows analysis of 3,5-dimethylaniline in the raw material benzene-1,4-diamine. The upper trace is for the spiked raw material (40 ppb) and the lower trace is for the unspiked raw material. The result supports the hypothesis that 3,5-dimethylaniline is not a contaminant at this level. This case is analytically difficult also, as the raw material is similar in physical–chemical behavior to the target analyte. It is likely that much "matrix" is present in the organic extract. In contrast, Fig. 4 shows the analysis of the phenolic raw material, 5-[(2-hydroxyethyl)-amino]-2-methylphenol, which will more favorably partition into aqueous base, leaving less matrix in the hexane extract. Since matrix will separate from the 3,5-dimethylaniline during sample preparation, the decrease in "chemical noise" during analysis of the organic extract of this phenolic compound (Fig. 4 versus Fig. 3) is not surprising. It is a reasonable hypothesis that the detection limit is well below 40 ppb in view of these data.

The only sample proving slightly problematic in analysis was 2-(4,5-diamino-1H-pyrazol-1-yl)ethanol sulfate. Chemical noise was significant in the SFC–MS/MS analysis. However, when spiked at 40-part-per-billion, the analyte signal was still visible at approximately twice the chemical noise on a descending baseline. The recovery was as described above. Thus, it is likely that the extract in this

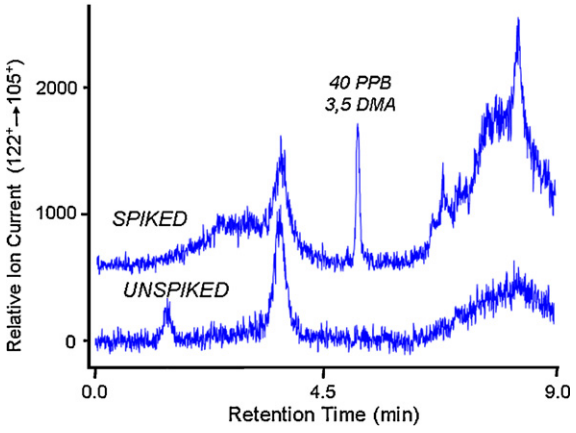

Fig. 3. SFC–electrospray ionization tandem mass spectrometry analysis (selected-reaction monitoring, m/z 122$^+$ to m/z 105$^+$) of 5% of a crude extract of spiked benzene-1,4-diamine raw material (upper, 40 ppb 3,5 DMA) versus the same raw material without spiking (lower).

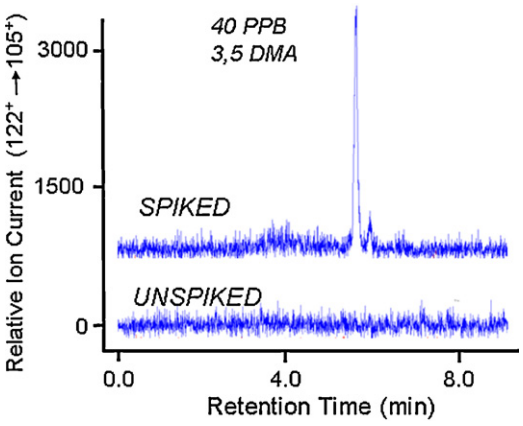

Fig. 4. SFC–electrospray ionization tandem mass spectrometry analysis (selected-reaction-monitoring, m/z 122$^+$ to m/z 105$^+$) of 5% of a crude extract of spiked 5-[(2-hydroxyethyl)-amino]-2-methylphenol raw material (upper, 40 ppb 3,5 DMA) versus the same raw material without spiking (lower).

case was highly matrix-loaded. The native sample did not show the signal for 3,5-dimethylaniline.

Furthermore, the compound 3,5-dimethylaniline was not detected in any of the native raw materials tested. Recoveries ranged from 50 to 85% with an average recovery across all raw materials of 60%. Here "recovery" is defined as relative peak area from a spiked sample compared to the neat standard at the same target concentration. Though the separation is robust, if native DMA is found, the sample should be spiked with the identified isomer and re-run to verify co-elution.

We did not adapt our method for analysis of hair-dye products under usage conditions, where mixing of a formulation containing the dye raw materials with basic hydrogen peroxide causes oxidative coupling to well known colored dye products. These formulations are more complicated than the raw materials and would quite likely require more extensive sample preparation than the simple extraction methods used here on organic, solid materials [13].

The data presented here show that DMA is detectable in typical raw materials, at 40 ppb. Further, the analytical methodology can very likely be extrapolated to the other isomers in the DMA class. Because of their physical–chemical similarity, it is reasonable to suggest they will have similar partitioning properties in the bulk extraction method described here. They also have unique chromatographic retention times. Finally, all of the isomers fragment similarly to 3,5-dimethylaniline in the MS/MS mode, with some variation in response factors (0.5–2× versus 3,5-dimethylaniline). Thus it is likely that any dimethylaniline isomer present at 40 ppb or higher would have been detected by this methodology.

Finally, the detection limit we found by performing just a crude extract (40 ppb in raw material) is reasonable for applying this method to further investigate human exposure issues [14]. It is based on a simple calculation that estimates a liberal upper limit of systemic exposure in humans. For example, the highest level of dye raw materials used is assumed to be 4% by weight in a hair-dye product. At a contaminant level of 40 ppb, and assuming 100% absorption of the DMA occurs, in a 60 kg person using 100 grams of product, the upper exposure would be 2.6×10^{-3} microgram per kg body weight.

4. Conclusions

The described analytical methodology has the sensitivity to specifically detect the 3,5-dimethylaniline isomer at a demonstrated level of 40 part-per-billion in simple, raw-material extracts.

The methodology quite likely can be extended to any dimethylaniline isomer as they have similar physical–chemical properties, are readily separated by SFC and are similarly detected by SRM.

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