Applied Occupational and Environmental Hygiene

ISSN: 1047-322X (Print) 1521-0898 (Online) Journal homepage: http://www.tandfonline.com/loi/uaoh20

# A Biological Monitoring Method for *o*-Toluidine and Aniline in Urine Using High Performance Liquid Chromatography with Electrochemical Detection

**Kenneth K. Brown , Alexander W. Teass , Steve Simon & Elizabeth M. Ward**

**To cite this article:** Kenneth K. Brown , Alexander W. Teass , Steve Simon & Elizabeth M. Ward (1995) A Biological Monitoring Method for *o*-Toluidine and Aniline in Urine Using High Performance Liquid Chromatography with Electrochemical Detection, Applied Occupational and Environmental Hygiene, 10:6, 557-565, DOI: 10.1080/1047322X.1995.10389074

**To link to this article:** https://doi.org/10.1080/1047322X.1995.10389074

# A Biological Monitoring Method for *o*-Toluidine and Aniline in Urine Using High Performance Liquid Chromatography with Electrochemical Detection

*Kenneth K. Brown, Alexander W. Teass, Steve Simon, and Elizabeth M. Ward**

Division of Biomedical and Behavioral Science and *Division of Surveillance, Hazard Evaluations, and Field Studies, National Institute for Occupational Safety and Health, 4676 Columbia Parkway, Cincinnati, Ohio 45226

A urinalysis method for *o*-toluidine and aniline was developed for biological monitoring. Urine specimens were made 4.7 M in sodium hydroxide and heated at 80°C for 2 hours to convert the metabolites acetanilide and *N*-acetyl-*o*-toluidine to the free amines. Extraction of the hydrolysate with butyl chloride and back extraction with 0.1 M aqueous hydrochloric acid gave an amine fraction, which was subjected to paired-ion reversed-phase liquid chromatography with coulometric electrochemical detection. For *o*-toluidine and aniline, respectively, the average recovery was 91 and 100 percent, the precision for field specimens was 13 and 16 percent relative standard deviation, and the limit of detection was 0.6 and 1.4 μg/L. This method was applied to 171 urine specimens from chemical plant workers. The median levels for *o*-toluidine were: exposed preshift, 11 μg/L; exposed postshift, 65 μg/L; nonexposed preshift, 0.7 μg/L; nonexposed postshift, 2.6 μg/L. For aniline the median levels were: exposed preshift, 11 μg/L; exposed postshift, 23 μg/L; nonexposed preshift, 2.0 μg/L; nonexposed postshift, 3.2 μg/L. The urinary levels of the two amines, especially *o*-toluidine, demonstrated significant uptake of the amines during the work shift and an accumulation of part of the dose with each passing work shift. BROWN, K.K.; TEASS, A.W.; SIMON, S.; WARD, E.M.: A BIOLOGICAL MONITORING METHOD FOR *o*-TOLUIDINE AND ANILINE IN URINE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION. APPL. OCCUP. ENVIRON. HYG. 10(6):557–565; 1995.

Investigating the incidence of cancer at a plant producing an antioxidant and an accelerator used in the manufacture of rubber, epidemiologists at the National Institute for Occupational Safety and Health (NIOSH) found an elevated risk of bladder cancer in workers employed there since 1957.[1] The chemicals used in this plant were reviewed as possible causes of these cancers. Among these chemicals, *o*-toluidine was classified as demonstrating "sufficient evidence," and aniline as demonstrating "limited evidence," for carcinogenicity in animals by the International Agency for Research on Cancer.[2] Consequently, NIOSH investigators planned a follow-up study of the plant to determine the extent of the exposure of the workers to *o*-toluidine and aniline. The work situation at the plant suggested the likelihood of both inhalation and dermal exposure of the workers to these two amines, both of which are adsorbed to a significant extent through the skin.[3,4] Thus, to obtain a complete picture of the extent of the workers' exposures, biological monitoring was necessary.

In previous studies, biological monitoring of workers for exposure to aniline has been accomplished by monitoring the end-of-shift urinary output of 4-aminophenol, a major metabolite of aniline. The American Conference of Governmental Industrial Hygienists has recommended a biological exposure index (BEI) for aniline of 50 mg of total 4-aminophenol per milligram of creatinine in urine obtained at the end of the work shift, "total" being free plus conjugated 4-aminophenol. The relationship of urinary 4-aminophenol to exposure to aniline is confounded by exposure of the workers to other chemicals, such as the common drug 4-acetylaminophenol (acetaminophen), which are metabolized or converted by the analytical procedure to 4-aminophenol. Additionally, application of this BEI requires that the samples be analyzed by a particular colorimetric method based on the indophenol reaction and having a relatively high detection limit of 10 mg/L.[4]

Other methods of biological monitoring for exposure to aniline have been reported. El-Bayoumy *et al.*[5] used urinalysis for aniline and *o*-toluidine to investigate smokers' and nonsmokers' exposures to these two amines. Lewalter and Korallus[6] determined the relative levels of aniline and metabolites in the urine of exposed workers and the relationship between those levels and the levels of aniline conjugated with blood protein. Stillwell *et al.*[7] studied the levels of hemoglobin adducts of aromatic amines, including *o*-toluidine and aniline, in the blood of smokers and nonsmokers.

In contrast to the documentation for aniline,[4–7] reports of biological monitoring for exposure to *o*-toluidine are limited to the two cited in the previous paragraph.[5,7] However, data on the metabolism of *o*-toluidine by rats are available. Cheever *et al.*[8] reported that, after receiving an oral dose of *o*-[*methyl*-$^{14}$C]toluidine, rats excreted 92 percent of the radioactivity in the 24-hour urine. While that 24-hour urine contained conjugates of 4-amino-2-methylphenol, roughly estimated to be 50 percent of the dose, also present was unchanged *o*-toluidine, representing 21 percent of the dose. Son *et al.*[9] found that rats injected with *o*-[*methyl*-$^{14}$C]toluidine excreted 53 to 74 percent of the radioactivity in the 24-hour urine, which contained 44 percent of the dose as ring-hydroxylated products, 5 percent as unchanged *o*-toluidine, and 0.2 percent as *N*-acetyl-*o*-

toluidine. These findings suggest that, like aniline, *o*-toluidine will be substantially metabolized by hydroxylation of the aromatic ring and rapidly excreted in the urine.

The investigation of the chemical plant required biological monitoring methods that would assess both the recent uptake and the accumulated doses of *o*-toluidine and aniline. To estimate recent exposures, we chose to measure the urinary levels of the parent amines and their *N*-acetyl metabolites, even though the ring-hydroxylated metabolites are the predominant species in the urine. This eliminated the ambiguity of aminophenol precursors from the interpretation of the biological monitoring results. The measurement of blood protein adducts is being investigated as a means of estimating the accumulated dose; this research will be reported elsewhere.

The urinalysis method had to meet three requirements. First, to quantify the differences between exposed and nonexposed workers, we needed to measure the background levels of the two amines, reported for nonsmokers to average 3 μg/24 hours (2 μg/L) for aniline and 4 μg/24 hours (2 μg/L) for *o*-toluidine.[5] Second, to remove the bias of acetylator status from the analytical results, the analytical method needed a hydrolysis step to convert the acetyl derivatives to the free amines. Humans of the metabolic phenotype to rapidly acetylate amines, fast acetylators, excrete 90 percent of the urinary aniline as acetanilide; slow acetylators excrete only 7 percent.[6] Third, to analyze the anticipated 200 specimens from this field investigation, we wanted maximum sample through-put.

In the method of El-Bayoumy *et al.*,[5] urine was treated with diethyl pyrocarbonate to stabilize the *o*-toluidine and aniline as the respective *N*-ethoxycarbonyl derivatives during sample storage, and reduced to a dry powder. The chloroform extract of the powder was hydrolyzed with 2 M sodium hydroxide at reflux for 2.5 hours. The amines in the chloroform extract of the hydrolysate were converted to the pentafluoropropionyl amides for quantification by gas chromatography with electron-capture detection. Although this method appeared to meet our needs for analytical range and hydrolysis of the acetyl derivatives, it seemed too labor intensive. Moreover, it seemed unwise to transport from the field to our laboratory sealed bottles of urine containing diethyl pyrocarbonate, since carbon dioxide is generated in its reactions with amines and water. Therefore, we sought another method. Among the other analytical methods for aromatic amines in urine, we had working knowledge of those for 4,4′-methylenedianiline[10,11] and 4,4′-methylenebis(2-chloroaniline). In the former, the urine was subjected to base hydrolysis, followed by solid-phase extraction of the hydrolysate. Base hydrolysis converted the *N*-acetyl derivatives to the free amine. The 4,4′-methylenedianiline was eluted from the solid-phase extraction bed and quantified by high performance liquid chromatography (HPLC) with electrochemical detection. Ullucci *et al.*[12] improved that method by fractionating the hydrolysate using liquid–liquid extraction with butyl chloride, instead of solid-phase extraction, and adding an ion-pairing reagent in the HPLC mobile phase. We successfully adapted that improved method to our analysis of urine for *o*-toluidine and aniline. This report describes the method and its evaluation and use with field samples.

## Methods

### *Reagents*

Water was purified by filtration and reverse osmosis using a Millipore Milli-RO4™ purifier, followed by activated charcoal treatment, ion-exchange treatment, and filtration using a Millipore Milli-Q™ purifier. The following were purchased: sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$), American Chemical Society (ACS) reagent grade (Fisher Scientific); methanol, HPLC grade (Baxter); sodium dodecyl sulfate, HPLC grade (Fisher Scientific); butyl chloride, HPLC grade (Fisher Scientific); anhydrous citric acid, ACS reagent grade (Fisher Scientific); aniline hydrochloride, 98 percent (Kodak); acetanilide, 99 percent (Aldrich); *o*-toluidine, 99+ percent (Aldrich); 85 percent phosphoric acid, ACS reagent grade (Mallinckrodt); sodium hydroxide, ACS reagent grade (J.T. Baker Co.); 0.1 M hydrochloric acid, certified standard (Mallinckrodt).

*N*-Acetyl-*o*-toluidine was synthesized from *o*-toluidine and acetic anhydride, and purified by recrystallization from water.[13] The resulting colorless needles melted at 106.5 to 107°C (uncorrected) (112°C in Reference 13). The mass spectrum (electron impact ionization) showed the molecular ion at m/z 149.0822, corresponding to $C_9H_{11}NO$ (calculated m/z 149.0841).

HPLC mobile phase was a 60:40 (v/v) mixture of 86 mM aqueous phosphate buffer and 150 mg/L sodium dodecyl sulfate in methanol. The final concentration of phosphate buffer was 52 mM and of sodium dodecyl sulfate was 60 mg/L. Before mixing, the pH of the buffer was adjusted with 8.5 percent phosphoric acid or 50 percent NaOH to 3.3 ± 0.05. Standard solutions were prepared in 0.1 M hydrochloric acid to contain both *o*-toluidine and aniline. Aliquots (1 to 7 ml) of these solutions were diluted with mobile phase to give 11 standard solutions of *o*-toluidine and aniline used for calibrating the HPLC. Standard solutions containing both *N*-acetyl-*o*-toluidine and acetanilide were prepared in methanol.

### *HPLC System*

The HPLC instrumentation consisted of the following components in sequence: Kontes 5-L solvent reservoir; Whatman in-line degasser and filter; Waters 510 HPLC pump set for a flow rate of 0.8 ml/min and a maximum pressure of 3400 psi; ESA pulse dampener; SSI in-line 0.2-μm filter; ESA guard cell at 1.0 V; Waters WISP™ 710 autoinjector making 50-μL injections; Waters Nova-Pak™ C18 guard column; Waters Nova-Pak C18 300 × 6-mm analytical column 4-μm, held at 30°C with a Waters column oven; SSI in-line 0.2-μm filter; and ESA 5011 detector cell with electrode 1 at 0.4 V and electrode 2 at 0.6 V. The ESA 5100 electrochemical controller for the ESA 5011 detector cell was set at a gain of 10 × 15 and a response time of 4 seconds and interfaced with a Hewlett-Packard 3396 recording integrator. The run time was 60 minutes. During idle periods, the HPLC mobile phase was 40:60 methanol/water. When this was replaced with the analytical mobile phase, 16 hours were required for the retention times to stabilize.

### *Samples*

Field urine specimens were collected in wide-mouth 500-ml high density polypropylene bottles. Aliquots (50 ml) for analysis were transferred to 2-oz polypropylene bottles containing 5 g of anhydrous citric acid as a preservative. The samples were immediately frozen on dry ice; at the laboratory they were stored at −68°C until analyzed.

Quality control samples were prepared as follows and were used for quality assurance and method development experiments. Anhydrous citric acid (150 g) was added to 1.5 L of urine from nonexposed, nonsmoking, unmedicated volunteers. This acidified urine was spiked with *o*-toluidine and aniline, or *N*-acetyl-*o*-toluidine and acetanilide, by adding the urine to 1 to 10 ml of the appropriate standard solution in a 250-ml volumetric flask. The spiked urine then was aliquotted into 10-ml high density polypropylene bottles and stored at −68°C. Samples of unspiked urine also were saved and subsequently analyzed. The nominal concentration of *o*-toluidine and aniline in a quality control sample was the sum of the amount added and the background level determined by analysis of the unspiked urine samples.

### *Analytical Procedure*

All glassware and caps were rinsed with methanol, 0.1 M hydrochloric acid, and purified water in that order before use. Samples were prepared for HPLC as follows:

1. Urine samples were thawed in a warm water bath and allowed to stand at room temperature for at least 10 minutes. A 4-ml aliquot of each was transferred to a 15-ml centrifuge tube, which contained 1 g ± 50 mg of sodium hydroxide pellets. Each tube was tightly capped and vortexed to aid dissolution of the sodium hydroxide.
2. The samples were heated for 2 hours at 80°C in a water bath.
3. After the samples cooled to room temperature, 8 ml of butyl chloride was dispensed into each tube. The tubes were recapped, tumbled for 10 minutes with a roto-torque at 50 rpm, and then centrifuged for 5 minutes at 3000 rpm.
4. From each sample, 5 ml of the butyl chloride (upper) layer was transferred to a fresh 15-ml centrifuge tube, to which was added 1 ml of 0.1 M hydrochloric acid. These tubes were capped, tumbled for 10 minutes with a roto-torque at 50 rpm, and then centrifuged for 5 minutes at 3000 rpm.
5. The lower layer (0.5 to 0.8 ml) was removed with a Pasteur-type disposable pipette and transferred to the barrel of a 3-ml plastic syringe fitted with a 10-mm (0.2-μm pore size) Anotop® syringe filter. The syringe plunger was inserted and the solution filtered into a 1-ml WISP vial for HPLC analysis.

The filtered extracts from step 5, hereafter called treated solutions, were analyzed in an alternating sequence of one standard solution after every pair of treated solutions. The standard solutions in mobile phase covered 11 levels which ranged from 2 to 140 μg/L *o*-toluidine and 1.4 to 101 μg/L aniline. Quantification was by calibration curve derived by quadratic regression of peak height versus mass of aniline and *o*-toluidine per 50-μL injection of the standard solutions.

Treated solutions with concentrations of analyte above 120 μg/L frequently saturated the detector. Such samples were reanalyzed using 5-μL injection volumes to extend the effective range to 1200 μg/L. Samples with concentrations above 1200 μg/L were reanalyzed using 1 ml of urine diluted with 3 ml of water and an injection volume of 5 μL.

## Results and Discussion

### *Hydrolysis*

The first step in the work-up of the urine specimens was the hydrolysis of *N*-acetyl-*o*-toluidine and acetanilide to the free amines, a step to make the analytical results independent of an individual's relative urinary output of free amine and acetyl derivative. The optimum conditions for the hydrolysis step were estimated by an experiment in which sodium hydroxide concentration and heating time were varied using a central composite design. A pool of spiked urine samples with citric acid was prepared with acetanilide and *N*-acetyl-*o*-toluidine at the level of 30 μg/L. These were analyzed as in the Methods section, except that sodium hydroxide concentration and hydrolysis time were varied over 20 specific combinations in the ranges of 1.0 to 9.5 M and 1.0 to 4.0 hours, respectively. Two samples were analyzed by each of the combinations. The water bath temperature was kept at a constant 80°C. The resulting recoveries of *o*-toluidine and aniline were examined as a function of sodium hydroxide concentration and hydrolysis time, the contour plots for which are shown in Figure 1.

The optima for the two hydrolyses, 4.8 M and 2.0 hours for *N*-acetyl-*o*-toluidine and 4.0 M and 3.0 hours for acetanilide, did not coincide, but each did lie in a region of high recovery for the other. As we were more interested in *o*-toluidine, we selected hydrolysis parameters close to the optimum for its recovery. Those parameters are still quite high on the broader recovery contour for aniline, so the method would still work well for aniline. Because the field samples were preserved with citric acid, enough sodium hydroxide (1 g) was added to field urine specimens to produce a final concentration of 4.7 M.

The adequacy of the hydrolysis conditions was best demonstrated during the analysis of the 171 field specimens. Among the quality control samples were 13 quality control samples spiked with 22 μg/L of *N*-acetyl-*o*-toluidine (16 μg/L *o*-toluidine) and 21 μg/L acetanilide (19 μg/L aniline, including the background level). The average recovery for these quality control samples was 83 percent for *o*-toluidine and 96 percent for aniline.

### *Extraction*

The work-up of the samples called for isolation of the basic fraction by extraction of the hydrolysate with butyl chloride, which in turn was to be extracted with 0.1 M hydrochloric acid. These extraction steps were tested with standard solutions of aniline and *o*-toluidine in 0.1 M hydrochloric acid, each at 300 μg/L, and the same made basic with solid sodium hydroxide. Equal volumes of the standard solution and butyl chloride were mixed by rotating at 50 revolutions per minute for specific periods of time from 5 to 30 minutes. The concentrations of the two amines in the aqueous phases were then determined by HPLC. Equilibrium was reached within 5 minutes. The partition coefficients were calculated to be: *o*-

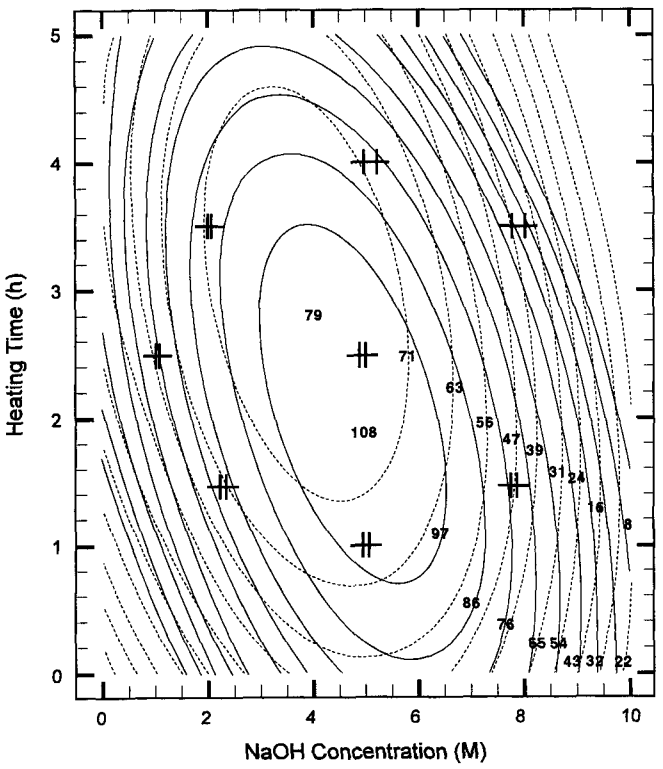


FIGURE 1. Contour plots of the recovery of *o*-toluidine and aniline from urine samples spiked with *N*-acetyl-*o*-toluidine and acetanilide. The procedure was as given in Methods, except that sodium hydroxide concentration and time at 80°C were varied. *o*-Toluidine recoveries are plotted as solid lines and aniline recoveries as dotted lines. The crosses mark the conditions of the experimental runs.

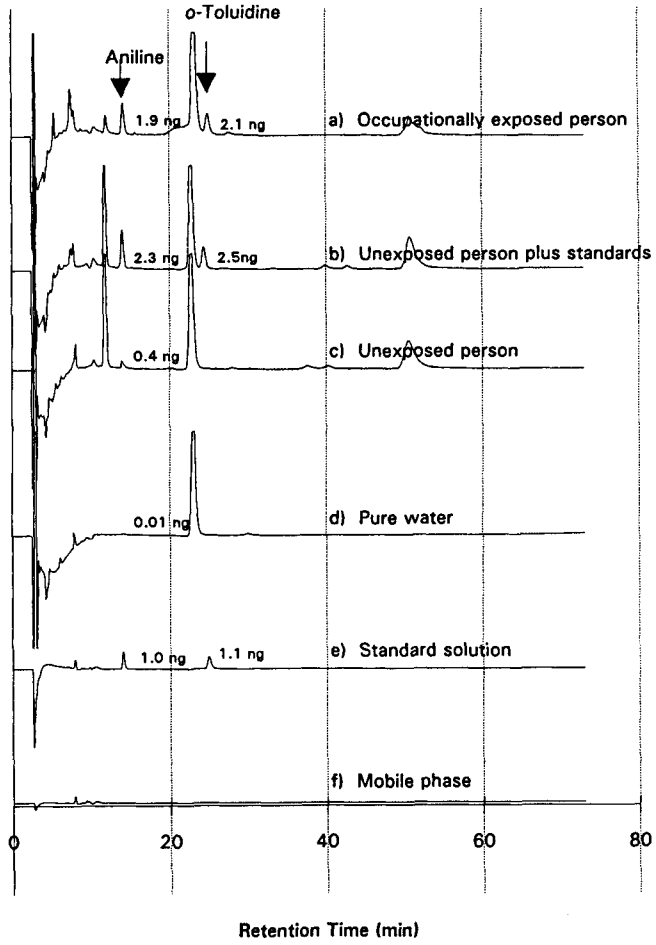


FIGURE 2. Liquid chromatograms from samples carried through the work-up procedure (a through d), standard solution of *o*-toluidine and aniline in 0.1 M hydrochloric acid (e), and mobile phase (f). HPLC conditions are given in Methods.

toluidine, 38; *o*-toluidinium chloride, 0.08; aniline, 10; anilinium chloride, 0.07.

To minimize the detection limit, we tried to maximize the volume of urine and minimize the butyl chloride-to-urine ratio, with the constraint that all be contained in a 15-ml centrifuge tube. If the butyl chloride-to-hydrolysate ratio was less than 2, then emulsions formed upon mixing of the phases. We settled on starting with 4 ml of urine and extracting the hydrolysate with 8 ml of butyl chloride. Using the partition coefficients above and these volumes, the potential recovery for the method was estimated to be 85 percent for *o*-toluidine and 90 percent for aniline. In practice, the average recoveries were higher.

During early use of the method, as much as possible of the 8 ml of butyl chloride was transferred for extraction with 0.1 M hydrochloric acid. Too frequently, trace quantities of the aqueous phase were included in the transfer, with the result that interfering peaks appeared in the liquid chromatograms. This problem was eliminated by centrifuging the mixtures and transferring only 5 ml of butyl chloride for back extraction.

### *Chromatography*

HPLC of standard solutions and treated solutions was performed on a 30-cm × 3.9-mm column of end-capped octadecylsilylated silica with a mobile phase of 52 mM dihydrogen phosphate and 60 mg/L sodium dodecyl sulfate in 40:60 methanol/water. Chromatograms thus obtained are shown in Figure 2. The treated solution giving trace a was from an exposed worker's urine, which contained 17 μg/L of *o*-toluidine and 15 μg/L of aniline. In contrast, the treated solution giving trace c was from urine from an unexposed person. Typical of the specimens we pooled for the quality control samples, this urine sample contained 3 μg/L aniline, but no detectable *o*-toluidine. When spiked to give nominal levels of 20 μg/L *o*-toluidine and 18 μg/L aniline, this urine yielded a treated solution giving the chromatogram in trace b.

The same HPLC column was used for much of the study, with about 500 injected samples passed through it. Column deterioration was monitored by tracking the efficiencies and retention times of the *o*-toluidine and aniline peaks from the standard solutions. Linear regression of these parameters against run number revealed that the column efficiency for *o*-toluidine was initially 7782 plates and dropped 13 percent over the period. For aniline the initial efficiency was 6068 plates and dropped 6.5 percent. The retention time of *o*-toluidine dropped from 18.3 to 16.8 minutes; that of aniline dropped from 11.4 to 10.8 minutes.

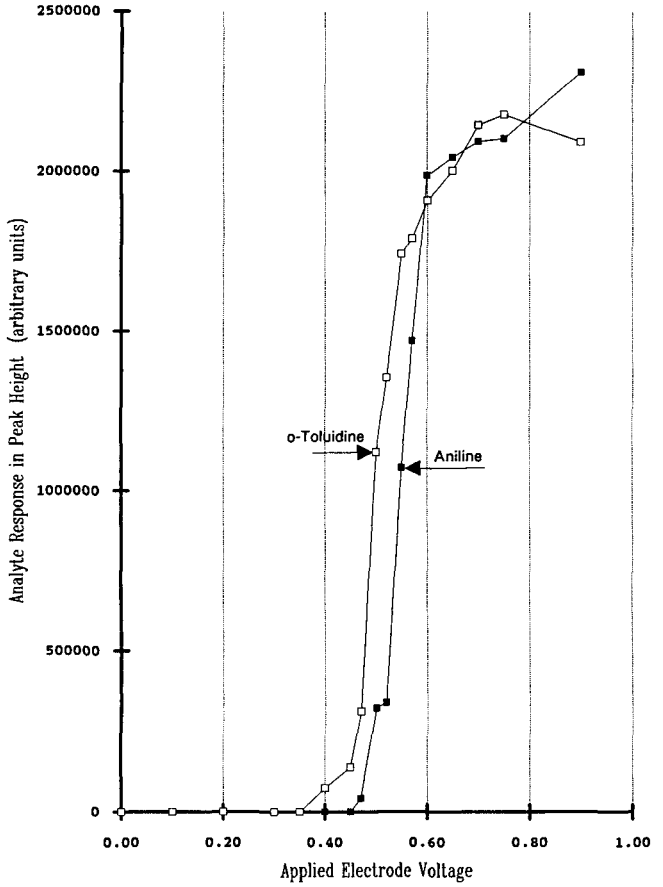


FIGURE 3. Hydrodynamic voltammograms for aniline (■) and *o*-toluidine (□) at 100 μg/L in 0.1 M hydrochloric acid using the HPLC conditions in Methods.

### *Detection*

Using standard solutions of *o*-toluidine and aniline at 100 μg/L each, the response of the electrochemical detector was measured as a function of applied potential, giving the hydrodynamic voltammograms shown in Figure 3. An optimal electrochemical window for the two detector electrodes was set at 400 mV for electrode 1 and 650 mV for electrode 2. Thus, electrode 1 removed easily oxidized compounds, and electrode 2 monitored the analytes at a potential on or close to the plateau for coulometric oxidation. This restricted the potential interferences with the method to basic organic compounds which survived or were produced by the base hydrolysis step and which were oxidized between 400 and 650 mV on glassy carbon.

The identification of aniline and *o*-toluidine in the field samples was done by retention time. However, there were situations when confirmation of identity was required, such as the presence of an unexpectedly large peak close to the target retention time. In these cases, identification was confirmed by reanalyzing the samples with electrode 2 set at 600 and 530 mV and comparing the 600 mV:530 mV response ratio with that of a standard solution. The average response ratio for *o*-toluidine was 1.4 with a standard deviation of 0.12 and for aniline was 3.7 with a standard deviation of 0.04. Unknown peaks matching these ratios within three standard deviations were considered to be *o*-toluidine or aniline.

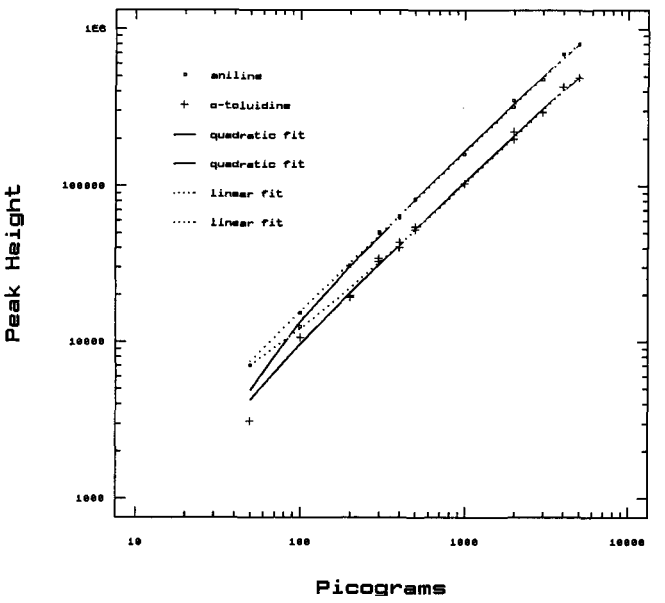


FIGURE 4. Calibration curves for aniline and *o*-toluidine generated using multiple regression analysis and linear regression data analysis.

The method was calibrated using external standards: standard solutions of *o*-toluidine and aniline in HPLC mobile phase. These covered 11 levels over the range 2 to 140 μg/L *o*-toluidine and 1.4 to 101 μg/L aniline and were introduced into the procedure at the HPLC step, such that a standard solution of randomly selected level was run before and after every two unknown test solutions. This sequence allowed precise tracking of retention time drifting and yielded calibration curves most representative of the detector response to the unknowns.

Calibration curves, constructed using peak heights, were found to deviate from linearity at the lowest decade of the curve for both *o*-toluidine and aniline. There the peak heights were lower than predicted by linear regression. As a quadratic equation gave the simplest curve that fit the data well, we used quadratic regression to construct the calibration curves. The multiple regression analysis of standard mass data and the standard mass data squared against the peak height provided coefficients for the quadratic curve as shown in Figure 4.

### *Limits of Detection*

The limits of detection were estimated from the analytical results from 18 samples of urine spiked with *o*-toluidine and aniline plus five blank samples, all from the same urine pool. The nominal concentrations were 0.12 to 15 μg/L *o*-toluidine and 3.9 to 15 μg/L aniline. Linear regression of the measured concentrations against the nominal concentrations gave lines, the slopes of which were estimates of the average recovery. The detection limits were defined as three times the standard error of estimate divided by the slope. For *o*-toluidine, the detection limit was 0.6 μg/L and the average recovery was 94 percent. The detection limit for aniline was 1.4 μg/L and the average recovery was 97 percent.

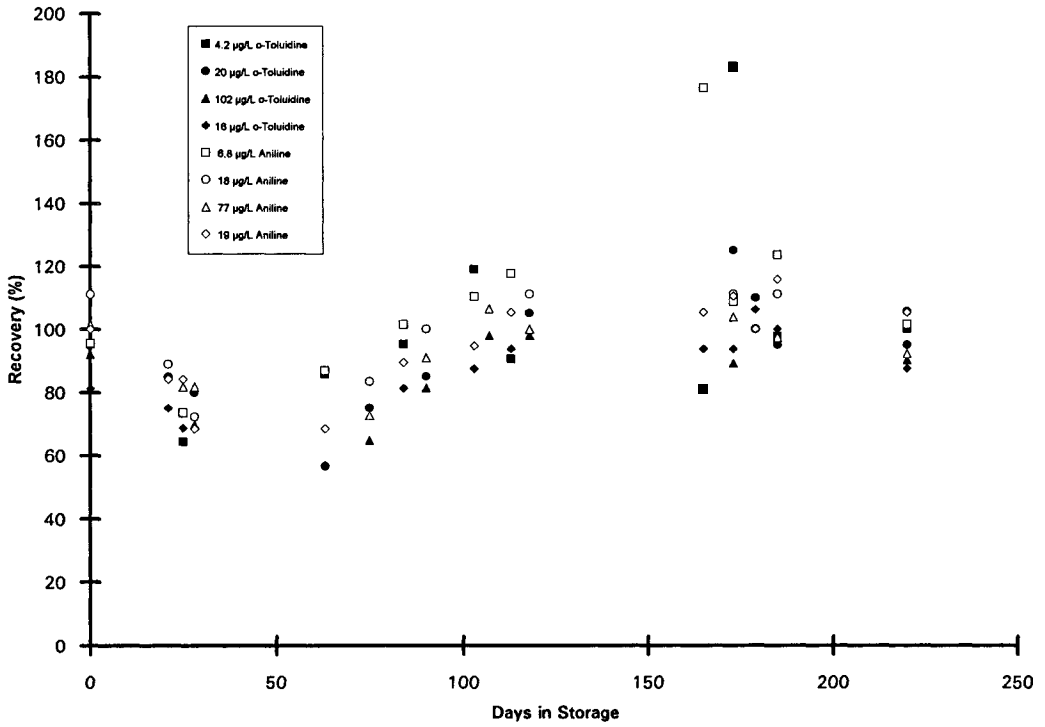


FIGURE 5. Recovery of *o*-toluidine and aniline from quality control samples as a function of age. The samples were stored at −68°C. The samples labeled 16 µg/L *o*-toluidine (◆) and 19 µg/L aniline (◇) were spiked with *N*-acetyl-*o*-toluidine and acetanilide; the remaining samples were spiked with *o*-toluidine and aniline.

### *Method Performance*

The 171 specimens obtained during the field investigation were analyzed with 39 quality control samples covering four levels between 4 and 102 µg/L for *o*-toluidine and 7 and 77 µg/L for aniline. The recoveries for these samples are plotted as a function of time in Figure 5. Overall, the recovery of aniline averaged 100 percent with a relative standard deviation of 17 percent, and the recovery of *o*-toluidine was 91 percent with a relative standard deviation of 20 percent. Over the 6-month period of analysis, there was no significant downward trend in recoveries, negating the possibility of analyte degradation during storage. Additionally, there existed no significant relationship between recovery and concentration.

As additional quality control, duplicate and, in 11 cases, triplicate aliquots of 56 specimens were analyzed on different days. The precision of these analyses is shown in Figure 6. The relative standard deviation appeared independent of concentrations, and averaged 13 percent for *o*-toluidine and 16 percent for aniline.

The laboratory background levels of aniline and *o*-toluidine were monitored by analyzing two water blanks with each lot of specimens. Aniline was detected in the water blanks in 14 of 16 lots and averaged 1.2 µg/L. *o*-Toluidine was found in only 2 of 16 lots and averaged 0.9 µg/L. The results for the field specimens and quality control samples were adjusted for these background levels before being reported.

### *Method Ruggedness*

After a change from the developing team to a trained analyst, the recoveries from the quality control samples became inexplicably low. To solve the problem, we tested the ruggedness of the method with a screening experiment in which we varied eight procedural steps in a 16-run fractional factorial design. We chose the eight steps we considered most likely to be subject to operator variation or error. Using urine spiked to the nominal concentrations of 40 µg/L *o*-toluidine and 42 µg/L aniline, 32 samples were analyzed by one of 16 modifications of the procedure dictated by the design, two by each modification. The eight steps, or factors, tested were:

1. quantity of sodium hydroxide added, 0.8 and 1.2 g, giving approximate concentrations of 3.4 and 5.9 M;
2. waiting time between the addition of sodium hydroxide to the urine and placing the samples in the heating bath, 0 and 120 minutes;
3. capping of the samples during hydrolysis, tightly capped and loosely capped centrifuge tubes;
4. light exposure during hydrolysis, samples covered and uncovered;
5. bath temperature, 75 and 85°C;
6. waiting time between hydrolysis and extraction with butyl chloride, 0 and 60 minutes;
7. lot of butyl chloride, old and new;
8. concentration of hydrochloric acid, 0.1 and 0.01 M.

The experimental data were subjected to statistical analysis of variance, the results of which are presented in Table 1. There the factors are listed in decreasing order by their contribution to the variance. The factors having the greatest effect were the quantity of sodium hydroxide and the hydrochloric acid concentration; using 1.2 g of sodium hydroxide or 0.01 M

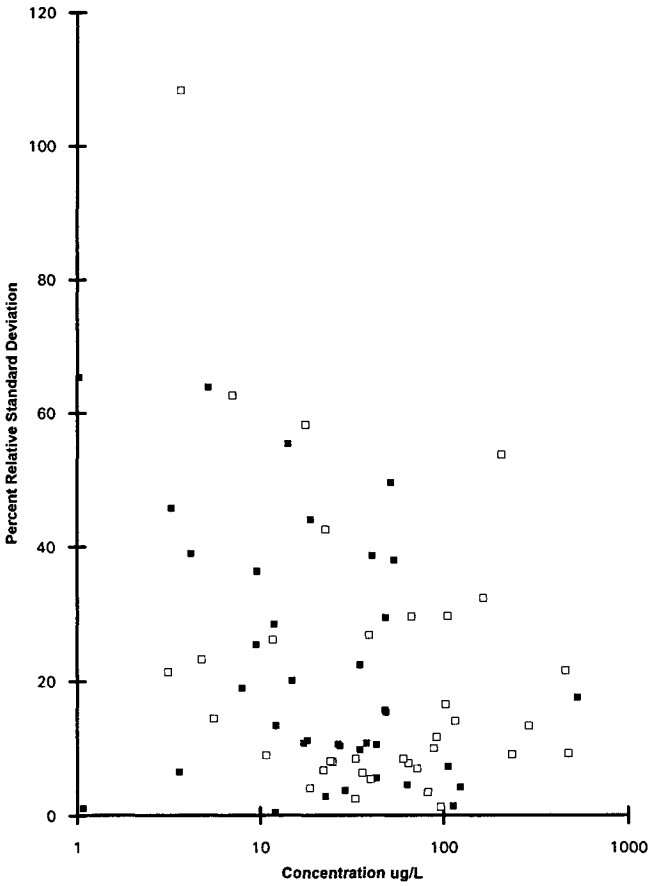


FIGURE 6. Precision estimates for the measurement of aniline and *o*-toluidine in split field specimens as percent relative standard deviation, plotted as a function of the average concentration of the splits.

hydrochloric acid caused substantially lower recoveries of both amines. Leaving the samples loosely capped during the hydrolysis step substantially reduced the recovery of *o*-toluidine, but not aniline. The significance of the other factors was marginal to none.

The revelations in Table 1 prompted us, in subsequent use of the method, to control more closely the quantity of sodium hydroxide to 1.0 g and the concentration of hydrochloric acid to 0.1 M and to tightly cap the centrifuge tubes containing the samples during the hydrolysis step. These procedural changes paid off, for recoveries from quality-control samples during subsequent analyses of field specimens were in control. As additional precautions, we continued to use the new lot of butyl chloride, to minimize the delay times on both sides of the hydrolysis step, and to cool the samples in an ice-water bath after hydrolysis.

### *Biological Monitoring*

In the industrial hygiene study of the plant, preshift and postshift urine specimens were obtained from 89 shift workers, 56 exposed to both *o*-toluidine and aniline and 33 not exposed to either amine. The workers (both controls and exposed) were pulled from all three shifts. Analysis of these 171 specimens gave the results presented in Figure 7. Of the results for *o*-toluidine, 16 were below the detection limit of 0.6 μg/L, with 13 from specimens yielding no chromatographic peak for *o*-toluidine. Twenty-two specimens had aniline levels below the detection limit of 1.4 μg/L, with 4 of those being smaller than the laboratory blank value for aniline. For completeness of data analysis, the results were not truncated at the detection limits until they were reported to the workers. In the case of the 17 having no chromatographic response or being below the laboratory blank, the values were approximated by the quotient of the detection limit ÷ $\sqrt{2}$.

The results in Figure 7 show that biological monitoring by measuring urinary *o*-toluidine and aniline was effective in revealing that those workers employed in the department where *o*-toluidine and aniline were used had been exposured to these compounds. In the more striking case of *o*-toluidine, the median postshift level for the exposed workers was twenty-sixfold higher than the median postshift level for the nonexposed workers. The preshift results suggest that *o*-toluidine and aniline are not completely eliminated from the bodies of many of the exposed workers between work shifts. One worker had exceptionally elevated levels of urinary aniline. As this worker was taking several undisclosed medications, the possibility of increased aniline, or an interfering substance, from metabolism of the medications must be considered. The correlation coefficient between aniline excretion and *o*-toluidine excretion was $r^2 = 0.87$ for postshift exposed samples after removing three outliers.

The relative extent of exposure during the work shift is shown in Figure 8, in which are plotted the preshift-to-postshift increases in urinary *o*-toluidine and aniline for the 77 workers for whom we had both specimens. Omitted are 15 small aniline preshift-to-postshift decreases, 9 for nonexposed workers and 6 for exposed workers. The data in Figure 8 suggest that, at that workplace during the period of the study, exposure to *o*-toluidine was greater than was exposure to aniline. The median increase in *o*-toluidine concentration for the exposed workers was 48 μg/L (range 10 to 526 μg/L), while for the nonexposed workers the median was 1 μg/L (range 0 to 5 μg/L). For aniline, the median increase for the exposed workers was 12 μg/L (range −23 to 1500 μg/L) and for the nonexposed workers was 2 μg/L (range −3 to 7 μg/L). There was an increase in aniline and *o*-toluidine excretion even for the unexposed, albeit the increase was only 1 and 2 μg/L, respectively. The unexposed workers were at the same plant facilities as the exposed workers. Their exposure to atmospheric background levels of aniline and *o*-toluidine or cigarette sidesteam smoke may explain their small increase.

Our method proved capable of estimating the background levels of urinary *o*-toluidine and aniline. Of the 64 specimens from nonexposed workers, only 15 (23%) were below the detection limit for *o*-toluidine (0.6 μg/L) and 21 (33%) below that for aniline (1.4 μg/L). Referring to Figure 7, the background levels in the nonexposed workers were comparable with those reported by El-Bayoumy *et al.*[5] for 19 healthy male adults: 0.3 to 13 μg/d (0.2 to 6 μg/L) for *o*-toluidine and 0.02 to 9 μg/d (0.01 to 4 μg/L) for aniline.

## Conclusions

We developed a method for the urinalysis for *o*-toluidine and aniline in which urine specimens were subjected to base hydrolysis, enrichment of the amine fraction, and then quantifi-

TABLE 1. Analysis of the Variance in the Eight-Factor Screening Experiment

| | *o*-Toluidine | | | Aniline | | |
|---|---|---|---|---|---|---|
| **Source*** | **Mean Square** | **F Value** | **P Value** | **Mean Square** | **F Value** | **P Value** |
| Concentration hydrochloric acid<br>*0.1 M* or 0.01 M | 4800 | 130 | 0.0001 | 5700 | 62 | 0.0001 |
| Added sodium hydroxide<br>*0.8 g* or 1.2 g | 740 | 19 | 0.0002 | 1200 | 13 | 0.0016 |
| Capping of vial<br>*Tight* or loose | 340 | 9 | 0.0063 | 3 | 0.04 | 0.85 |
| Butyl chloride lot<br>*New* or old | 99 | 3 | 0.12 | 600 | 7 | 0.018 |
| Postheating wait<br>*0 minutes* or 60 minutes | 110 | 3 | 0.10 | 530 | 6 | 0.026 |
| Preheating wait<br>*0 minutes* or 120 minutes | 150 | 4 | 0.055 | 210 | 2 | 0.15 |
| Light exposure<br>*Uncovered* or covered | 140 | 4 | 0.067 | 160 | 2 | 0.20 |
| Bath temperature<br>75°C or 85°C | 5 | 0.1 | 0.72 | 28 | 0.3 | 0.59 |
| Model | 800 | 21 | 0.0001 | 1100 | 11 | 0.0001 |
| Error | 38 | | | 93 | | |

*Factor setting giving higher recovery listed first in italics.

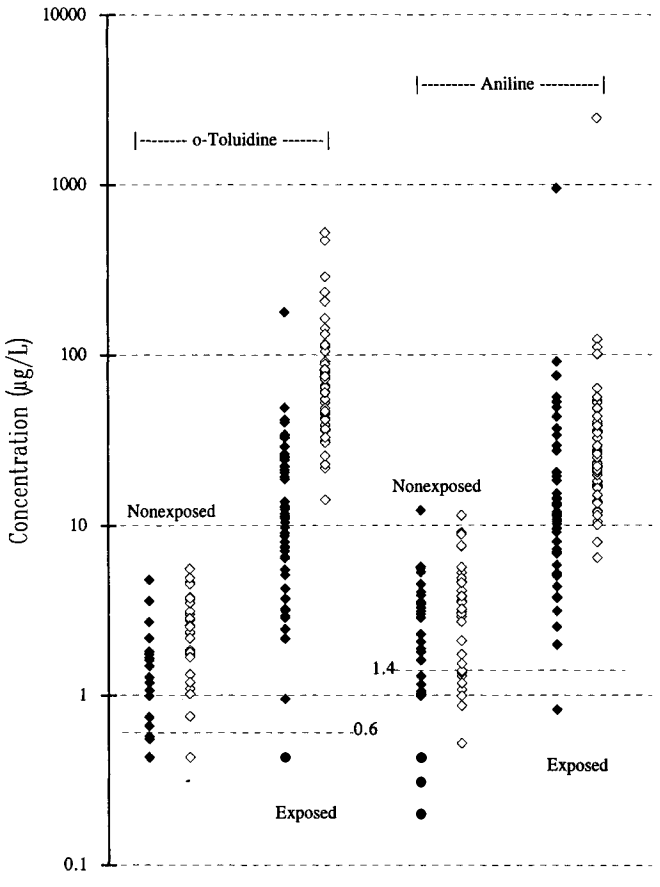


FIGURE 7. Workers' urinary *o*-toluidine and aniline concentrations in preshift (◆) and postshift (◇) specimens. The two dotted line segments represent the estimated detection limits for the method.

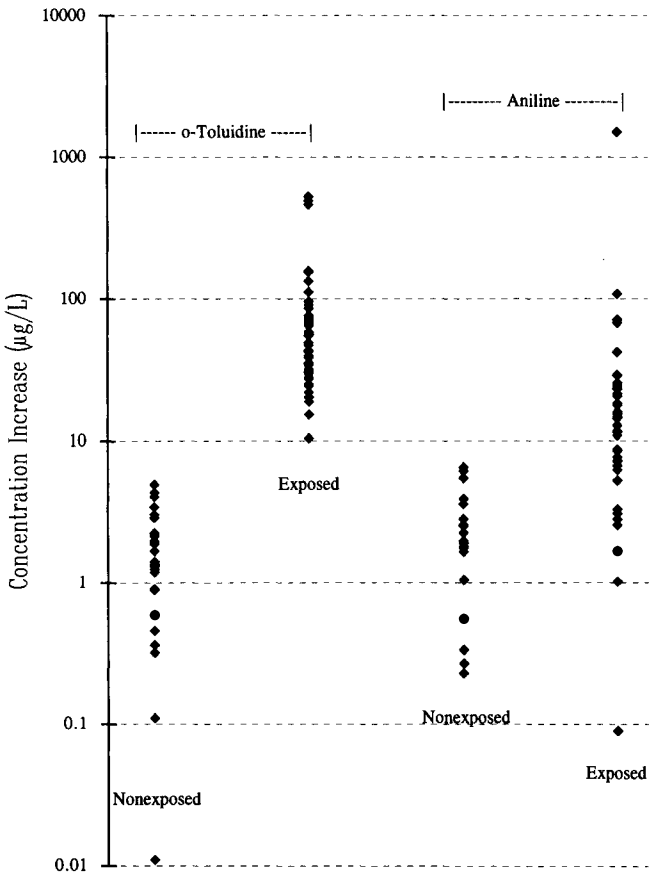


FIGURE 8. Preshift-to-postshift increases in urinary *o*-toluidine and aniline.

cation by HPLC. This method was used for biological monitoring of the exposure of workers to *o*-toluidine and aniline at a plant producing chemicals for tire manufacturing. The urinary levels of the two amines, especially *o*-toluidine, demonstrated significant uptake of the amines during the work shift and an accumulation of part of the dose with each passing work shift. As the levels of these amines in the work environment are reduced through implementation of improved controls and work practices, biological monitoring of these workers should demonstrate the resulting reduced exposures.

## Acknowledgments

The authors thank Dawn Ramsey, Terry Boyle, Barbara MacKenzie, and Shirley Robertson for their substantial assistance with the laboratory experiments; Lloyd Stettler, Delon Hull, and Marilyn Fingerhut for their encouragement and helpful discussions; Deanna Wild for statistical evaluation of some of the data; and Avima Ruder for substantial assistance in acquiring the urine specimens and notifying the workers of their results.

## References

1. Ward, E.; Carpenter, A.; Markowitz, S.; et al.: Excess Number of Bladder Cancers in Workers Exposed to Ortho-Toluidine and Aniline. J. Natl. Cancer Inst. 83:501–506 (1991).
2. International Agency for Research on Cancer: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42, Supplement 7, pp. 99–100, 362–363. IARC, Lyon (1987).
3. American Conference of Governmental Industrial Hygienists: *o*-Toluidine. In: Documentation of the Threshold Limit Values and Biological Exposure Indices, 5th ed., pp. 586–588. ACGIH, Cincinnati, OH (1986).
4. American Conference of Governmental Industrial Hygienists: Aniline. In: Documentation of the Threshold Limit Values and Biological Exposure Indices, 5th ed., pp. BEI51-BEI53. ACGIH, Cincinnati, OH (1986).
5. El-Bayoumy, K.; Donahue, J.M.; Hecht, S.S.; Hoffmann, D.: Identification and Quantitative Determination of Aniline and Toluidines in Human Urine. Cancer Res. 46:6064–6067 (1986).
6. Lewalter, J.; Korallus, U.: Blood Protein Conjugates and Acetylation of Aromatic Amines. New Findings on Biological Monitoring. Int. Arch. Occup. Environ. Health 56:179–196 (1985).
7. Stillwell, W.G.; Bryant, M.S.; Wishnok, J.S.: GC/MS Analysis of Biologically Important Aromatic Amines. Application to Human Dosimetry. Biomed. Environ. Mass Spectrom. 14:221–227 (1987).
8. Cheever, K.L.; Richards, D.E.; Plotnick, H.B.: Metabolism of *ortho*-, *meta*-, and *para*-Toluidine in the Adult Male Rat. Toxicol. Appl. Pharmacol. 56:361–369 (1980).
9. Son, O.S.; Everett, D.W.; Fiala, E.S.: Metabolism of *o*-[*methyl*-$^{14}$C]Toluidine in the F344 Rat. Xenobiotica 10:457–468 (1980).
10. Brown, K.; Boeniger, M.; Neumeister, C.: 4,4′-Methylenedianiline (MDA): Pilot Field Study of the Biological Monitoring of Aerospace Workers for Exposure Using HPLC-EC Analysis of Urine. Presented at the 199th American Chemical Society National Meeting, Boston (April 1990).
11. Neumeister, C.E.: Analysis of Urine to Monitor Exposures to Benzidine, *o*-Dianisidine, *o*-Toluidine, and 4,4′-Methylenedianiline. Appl. Occup. Environ. Hyg. 6:953–958 (1991).
12. Ullucci, P.A.; Griffin, R.M.; Matson, W.R.: Determination of 4,4′-Methylenedianiline (MDA) in Urine by Liquid Chromatography. Abstract 266. Presented at the American Industrial Hygiene Conference, Orlando, FL (May 1990).
13. Shriner, R.L.; Fuson, R.C.; Curtin, D.Y.: The Systematic Identification of Organic Compounds, 5th ed., pp. 259–260, 326. John Wiley & Sons, New York (1948).