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# Exhaled breath condensate as matrix for toluene detection: A preliminary study

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**Abstract**

The study was designed to investigate whether exhaled breath condensate, obtained by cooling exhaled air in spontaneous breathing, could be a suitable matrix for toluene quantitative analyses. Nine healthy subjects were exposed for a short period (20 min) to a known concentration of toluene. Exhaled breath condensate samples were collected before and at the end of the exposure, while the environmental concentration of toluene was continuously monitored. Toluene was analysed by head-space gas-chromatography mass spectrometry, and assay repeatability was also estimated *in vitro*. Baseline and post-exposure measurement of hippuric acid, the urinary toluene metabolite, was performed to assess current toluene exposure. Before the exposure toluene concentrations in the exhaled breath condensate were lower than the detectable limit in all subjects, while after the exposure toluene was detectable with a median value 0.35 $\mu g\ l^{-1}$ (range 0.15–0.55 $\mu g\ l^{-1}$) in all the exhaled breath condensate samples. As compared with the standard calibration in distilled water, the curves obtained by exhaled breath condensate were linear and comparable with the range examined *in vivo* for toluene. A significant correlation was found between the environmental toluene levels and toluene in the exhaled breath condensate at the end of exposure. Furthermore, a significant relationship between increased exhaled breath condensate toluene levels and urinary hippuric acid after the exposure was found. In conclusion, exhaled breath condensate is a promising matrix for toluene assessment, although its application in humans requires further investigations.

**Keywords:** *Toluene, solvents, exhaled breath condensate, head-space*

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## Introduction

Toluene is the most utilized volatile organic solvent (Inoue et al. 1994), and is present in about 80% of paints, 62% of inks, 56% of thinners and 51% of adhesives (Inoue et al. 1983, Kumai et al. 1983). Persistent exposure to toluene is responsible for chronic systemic intoxication, producing mucous membrane irritation, decrements in central nervous system function, and endocrine disruption (Angerer and Kramer 1997) in thousands of industrial plants workers. Therefore, monitoring toluene and its metabolites, as biomarkers of toluene exposure, is crucial. Several biological indicators

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of exposure, such as relative toxicant levels in blood and urine, metabolite levels in urine, or measurable biochemical changes related to exposure have been generally used (American Conference of Governmental Industrial Hygienists 2003). Compared with other routine biomarkers, the direct measurement of toluene in blood, urine and exhaled air has been shown to be a reliable marker (Pierce et al. 1998). In particular, the measurement of toluene in exhaled breath condensate (EBC) provides an accurate reflection of concentrations at a target tissue site in and relevant to the potential appearance of toxicity. However, the method for toluene sampling in the exhaled air is quite difficult.

EBC obtained by cooling exhaled air under condition of spontaneous breathing is a new method to collect volatile and non-volatile substances from the lower airways (Scheideler et al. 1993, Mutlu et al. 2001). Although, consisting predominantly of water, EBC has dissolved within it non-volatile compounds. In addition, EBC traps potentially volatile water-soluble compounds and other volatile organic compounds (Effros et al. 2002). Recently, EBC has been used as new matrix to assess inhaled mixtures of transition elements and hard metals such as cobalt and tungsten in individuals occupationally exposed (Goldoni et al. 2004). Whether EBC can be also used as matrix for monitoring organic solvents is not known yet.

The aim of the present pilot study was to investigate whether EBC can be used as a suitable matrix for toluene quantitative analyses in subjects exposed to this solvent.

## Materials and methods

### *Subjects*

Nine healthy Caucasian volunteers (six males, mean age 32 years (range 25–50 years) with a mean body weight of 64 kg (range 51–90 kg) were enrolled in the study. All subjects were non-smokers, with no occupational or other organic solvents exposure, and were recruited among the personnel of the hospital. All volunteers abstained from drinking alcoholic beverages for at least 18 h.

The experiment was conducted with the informed consent of the participants and after approval of our local ethical committee.

### *Experimental design*

*Experiment 1.* All the subjects were asked to simulate an occupational activity by cleaning shoes with toluene for 20 min while wearing protective gloves. The exposure was carried out in a 40 $m^3$ room. The temperature and relative humidity were continuously measured. The toluene concentration in the air was continuously monitored by a Sapphire CONTEC Engineering (Foxboro, USA) infrared spectrophotometer, positioned close to the head of subjects. Measurements were carried out at 13.77 μm (with 3.66 μm as the reference wavelength) and the toluene concentration was recorded every 3 min. EBC was collected once immediately before exposure and at the end of exposure. Lung function was also assessed in each subject in a different day by a computerized spirometer (FL 2000 SensorMedics, USA).

*Experiment 2.* Five subjects were re-exposed on a different day as in experiment 1. EBC was collected once immediately before exposure and at the end of exposure after mouth and nasal washing (with 0.9% wt/vol. NaCl solution). Furthermore, urine

samples were collected at 0 and 4 h from the onset of exposure for the assay of toluene metabolite hippuric acid.

*Experiment 3.* Three subjects were re-exposed in a different day as in experiment 1. EBC was collected once immediately before exposure and at 10 min, 1 and 5 h after the end of exposure.

### *Exhaled breath condensate (EBC) sampling*

EBC was collected through a condenser, which allowed the non-invasive collection of non-gaseous components in the expiratory air (EcoScreen, Jaeger, Wyrzburg, Germany). Subjects breathed through a mouthpiece and a two-ways non-rebreathing valve, which also acted as a saliva trap. Patients were asked to breathe normally, wearing a nose-clip, for a period of 15 min. The condensate (at least 2 ml) was immediately transferred in glass vials (10 ml volume) for analysis. Soon after introducing the samples, the vials were closed with 20-mm butyl rubber lined with PTFE septa and crimped with perforated aluminium seals. The analysis was performed within 24 h.

### *Toluene analysis*

Toluene was analysed by using head-space gas-chromatography mass spectrometry (Shimadzu GC-17° interfaced with the GCMS-QP 5000 mass detector). The chromatograph conditions were: an SPB 20 (0.25 mm i.d. × 60 m in length and 0.25 μm in film thickness) capillary column; helium as the carrier gas; column flow = 1.3 ml $min^{-1}$. For analysis, the column was maintained at 50°C for 2 min, heated at 10°C $min^{-1}$ and then kept at 100°C. A split ratio of 1:10 was taken. The masses detected were *m/z* 91 and 92.The EBC samples were heated at 50°C for 5 min. Then, 250 μl of head-space gas were taken by a microsyringe whose needle perforated the septa of the closed vials and injected into the gas chromatograph. The approximate retention time for toluene was 7.20 min.

### *Calibration and estimation of the repeatability of the assay*

Toluene (laboratory grade purity) was purchased from Carlo Erba (Milan, Italy). Standard curves were prepared daily by adding variable amounts of toluene (0.1–0.6 μg $l^{-1}$) to 2 ml of distilled water. Furthermore, for calibration and estimation of the repeatability of the assay, four calibration samples from EBC of unexposed subject spiked with 0, 0.1, 0.20, 0.30, 0.40, 0.60 μg $l^{-1}$ of toluene were used. To estimate the possibility of storing the samples, the analysis was performed both immediately after the preparation of samples and 24 h later.

### *Urine hippuric acid assay*

The urine samples were analysed by high-performance liquid chromatography (HPLC) for creatinine and hippuric acid. An Agilent 1100 liquid chromatograph (Agilent, USA) equipped with a Jasco UV 975 diode array detector was used. A reversed-phase 5 μm (4.6–150 mm) Nucleosil C18 column was performed for separation. The mobile phase was a mixture of acetonitrile and 1% phosphoric acid in

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water. The flow rate was 0.5 ml $min^{-1}$ and the sample volume was 10 μl. The detector wavelength was at 240 nm.

### *Statistics*

Least-squares linear regression analysis was established to estimate the slopes ($b$) and intercepts ($a$) of the calibration curves:

$$y = bx + a,$$

where $y$ is the chromatographic area of the analyte and $x$ is the sample concentration of the analyte (μg $l^{-1}$). The limit of detection (LOD) of the assay was calculated according to the expression:

$$\text{LOD} = (3 \;\text{Sy}\; a)/b,$$

where Sy is the standard error of the estimate, $a$ is the intercept and $b$ is the slope.

The repeatability of the assay (as coefficient of variation, CV%) was estimated by repeated analysis of EBC (five per concentration) spiked with toluene at the concentration of 0.1 and 0.6 Ìg $l^{-1}$. Correlation was performed using Spearman's test. A $p < 0.05$ was considered as statistically significant.

## Results

The calibration curves obtained in the EBC were similar as compared with the standard calibration curves and linear in the range assessed for toluene ($r^2 = 0.983$, $p = 0.001$) (Figure 1).

The method error, expressed as the relative standard deviation, was estimated from six identically prepared samples to 1.9% at high toluene concentration of 0.6 μg $l^{-1}$ to 2.5% at low toluene concentration of 0.1 μg $l^{-1}$. The LOD was 0.05 μg $l^{-1}$. During the exposure, the environmental concentration of toluene was always lower than 100 parts per million (mean value of 70 parts per million).

In the EBC of all the subjects, toluene was lower than the detectable limit before the exposure. Toluene was detectable in all the samples of EBC after exposure (Figure 2).

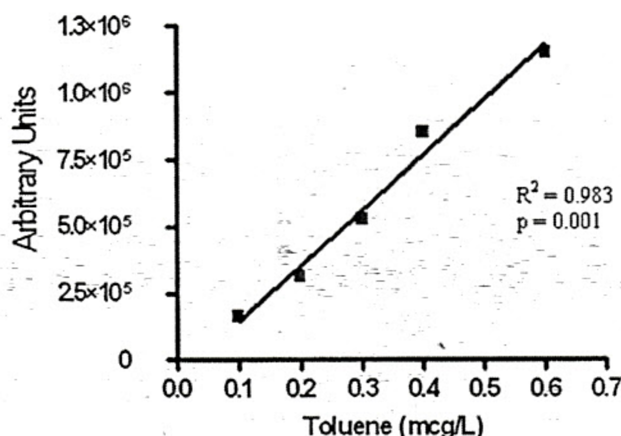


Figure 1. Calibration curve for toluene in exhaled breath condensate. The calibration curve was prepared by adding variable amounts of toluene (0.10–0.60 μg $l^{-1}$) to 2 ml of the exhaled breath condensate of an unexposed subject.

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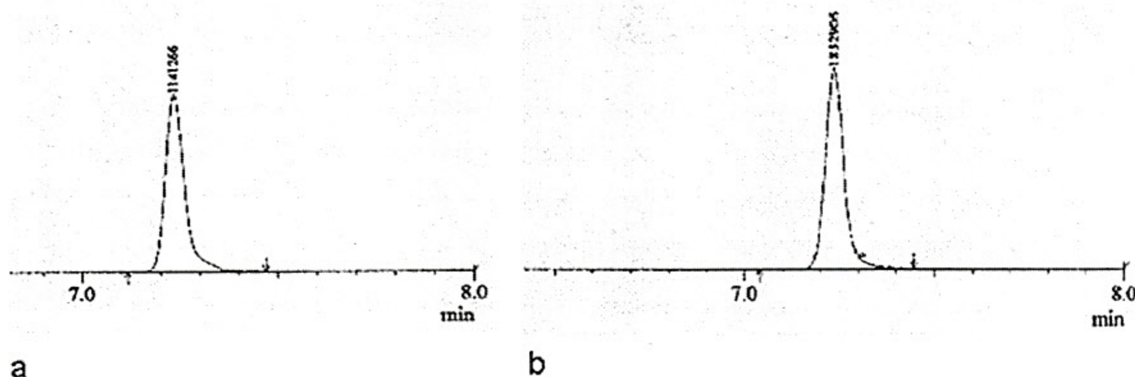


Figure 2. (a) Representative standard toluene gas-chromatographic trace (0.60 μg $l^{-1}$) and (b) representative toluene gas-chromatographic trace (0.97 μg $l^{-1}$) in the exhaled breath condensate of a subject after exposure.

The mean value was 0.33 μg $l^{-1}$ (range 0.15–0.55 μg $l^{-1}$). A significant correlation between the environmental toluene levels and the toluene in the EBC at the end of exposure was found ($r=0.75$, $p=0.02$) (Figure 3).

Lung function tests were within normal range in all volunteers ($FEV_1$ (Forced Expiratory Volume at 1s) = 110.2% ± 6.2%, FVC (Forced Vital Capacity) 108.0% ± 4.1%). No correlation between post-exposure toluene in the EBC and lung volumes was found.

Toluene was detectable in the samples of EBC in the five subjects after the second exposure also after the mouth and nasal washing. The mean value was 0.36 μg $l^{-1}$ (range 0.06–1.2 μg $l^{-1}$). An increase in hippuric acid was found after the exposure in all subjects (from 335.6 ± 118.9 to 533.6 ± 258.0 mg $l^{-1}$). A significant correlation between post-exposure toluene in the EBC and both total levels of post-exposure hippuric acid and the difference between pre- and post-exposure levels of hippuric acid was achieved ($r=0.88$, $p=0.04$; and $r=0.90$, $p=0.03$, respectively).

In three subjects, the toluene was detectable at 10 min, 1 and 5 h after the end of the exposure (Figure 4), with an evident decrease in the toluene levels.

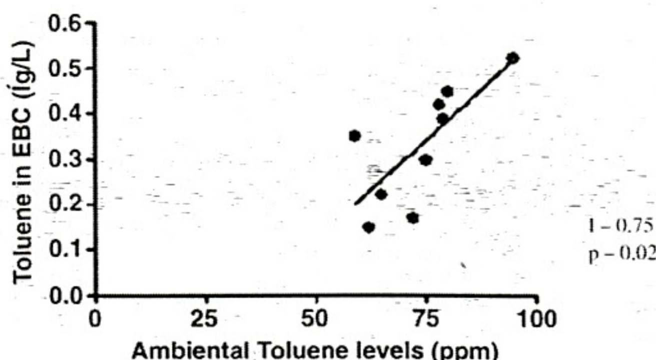


Figure 3. Correlation between the toluene levels in the environmental setting and toluene levels in the exhaled breath condensate of healthy non-smokers. Each point represents one individual at the end of toluene exposure.

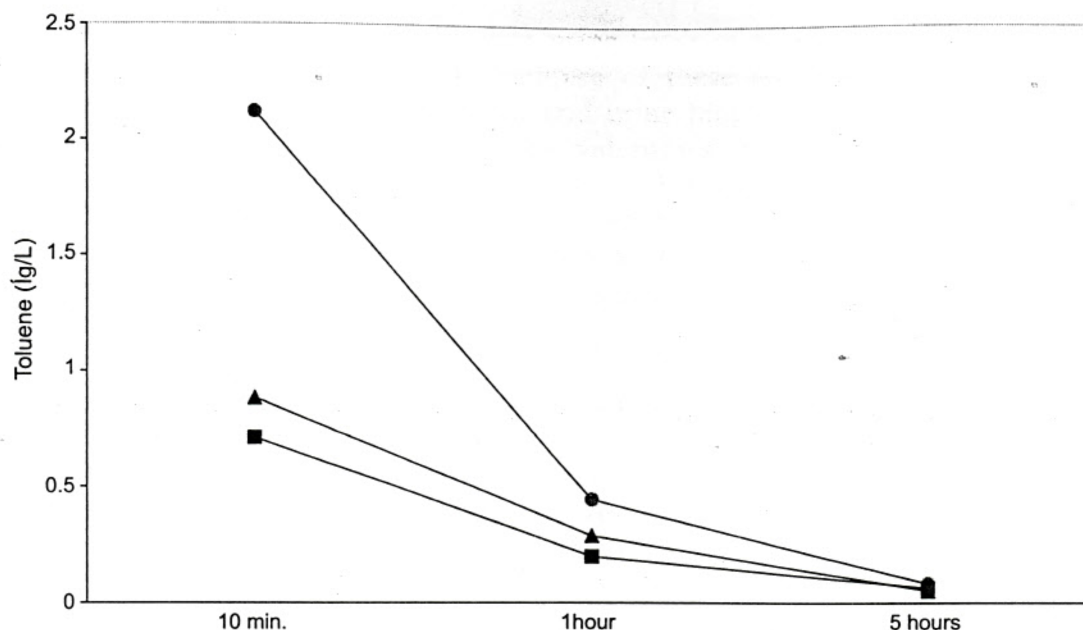


Figure 4. Toluene levels in the exhaled breath condensate of three subjects at 10 min, 1 and 5 h after the end of exposure. The mean environmental toluene exposure for subject 1 (circle) was 96 ppm, while for patients 2 and 3 (triangle and square) it was 80 ppm.

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## Discussion

It has been shown that the volatile organic compound toluene could be assayed on EBC after a brief inhalation exposure in healthy volunteers. The subjects were exposed for 20 min by cleaning shoes with toluene limiting its dermal absorption by protective gloves. At that time, a steady level of exhaled breath toluene concentration, reflecting its initial fast elimination from the blood could be supposed (Heinrich-Ramm et al. 2000). Since all the volunteers included in the study were non-smokers and were asked to abstain from ethanol intake the day before that may interfere with toluene metabolism (Wallen et al. 1984), it is unlikely that changes in toluene metabolism at the time of the study could have influenced the results.

Although sparingly soluble in water, the present authors believe that exhaled toluene could be collected in EBC where volatile substances with a high water partition coefficient may be trapped (Hunt 2002). Indeed, the concentrations of toluene measured were not from only EBC, but also from exhaled breath. However, although it is uncertain whether distal airways and alveoli contribute to either the water volume or the non-volatile solute content of EBC during quiet breathing, they might significantly contribute to levels of volatile compounds (Scheideler et al. 1993). Furthermore, although total ventilation was not measured during EBC collection, we did not find any correlation between toluene EBC levels and baseline lung absolute volumes making unlikely an effect of retarded expiration of inhaled toluene on EBC post-exposure levels.

Toluene was undetectable before the exposure, while it was detectable in all collected EBC samples after the exposure. Furthermore, there was a significant relationship between toluene EBC levels and toluene environmental concentration. One could exclude a possible contamination from salivary toluene as in the second experiment in five subjects toluene detectability on EBC was reproduced after nasal

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and mouth washing. Moreover, baseline urine levels of toluene metabolite hippuric acid increased in all post-exposure samples of these subjects, with a significant association between EBC toluene levels and urine hippuric acid. This may suggest that EBC toluene levels in the healthy volunteers might reflect actual toluene exposure. Compared with data obtained from other authors using breath sampling methods, the toluene levels found in EBC resulted in higher levels than found in breath sampling from non-occupationally exposed subjects (Rahman and Kelly 2003), suggesting that EBC analysis may be very sensitive to exposure conditions despite the fact that the levels of toluene in the environmental air were always lower than permissible limits (American Conference of Governmental Industrial Hygienists 2003). Moreover, the curves obtained in the EBC were similar as compared with the standard calibration curves and were linear in the range investigated for toluene. The curves of toluene in the EBC measured immediately after the preparation and 24 h after the preparation yielded comparable results. Taken together, these findings indicate that EBC is a suitable and stable matrix for toluene detection and analysis with specificity being assumed as the unchanged volatile organic compound is measured.

The authors believe that toluene detection in EBC is a potential tool that should be compared in the field with recommended measurements of toluene concentration either in blood or alveolar air (Ghittori et al. 2004). Furthermore, they are aware that owing to the very fast kinetics of toluene, its volatility, and the relatively prolonged time required to collect a sufficient amount of EBC, further standardization of sampling time and duration are required. However, the aim of this preliminary study was to assess whether the EBC was suitable as a matrix for quantitative detection of toluene after a controlled exposure. In this regard, EBC may be a simpler biological matrix than blood and urine for analysis representing a clean matrix, consisting mostly of water. Moreover, in contrast to another matrix such as blood sampling, EBC is a non-invasive assay and requires no specialized training. Unlike urine sampling, EBC can be collected at any time and in virtually any environment. Furthermore, the method of breath analysis by the collection of exhaled air is non-invasive, but also it bears some limitations related to practical and analytical deficiencies with sampling, shipping and storage of alveolar air.

The present study demonstrates that toluene can be measured in the EBC of subjects after a short period of exposure. However, the potential use of this matrix into monitoring solvent exposure should require further studies.

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