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Development and validation of method for analysis of some ototoxic solvents in saliva matrix by headspace gas chromatography/mass spectrometry

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ABSTRACT

The aim of this study was to develop an analytical method to monitor the saliva matrix for ototoxic solvents absorption: the method is based on headspace gas chromatography/mass spectrometry and represents an alternative biological monitoring for investigating low exposure to hazardous ototoxic solvents. Simultaneous determination of toluene, ethylbenzene, xylenes and styrene has been carried out and the method has been optimized for both instrumental parameters and samples treatment. Chromatographic conditions have been set in order to obtain a good separation of xylene isomers due to the interest in *p*-xylene as ototoxic one. Method validation has been performed on standards spiked in blank saliva by using two internal standards (2-fluorotoluene and deuterated styrene-d_8). This method showed the possibility to detect the target compounds with a linear dynamic range of at least a 2 orders of magnitude characterized by a linear determination coefficient (r^2) greater than 0.999. The limit of detection (LOD) ranged between 0.19 ng/mL (styrene) and 0.54 ng/mL (*m*-xylene) and the lower limit of quantification (LLOQ) ranged between 0.64 ng/mL (styrene) and 1.8 ng/mL (*m*-xylene). The method achieved good accuracy (from 99 to 105%) and precision for both intra- and inter-assay (relative standard deviation ranging from 1.7 to 13.8%) for all six compounds concerned. The repeatability was improved by adding sodium sulphate to the matrix. Saliva samples resulted stable for at least 7 days after collection, if stored in headspace vials, at the temperature of 4 °C. An evaluation of the main sources of uncertainty of the method is also included: expanded uncertainties ranges between 10 and 16% for all of the target compounds. In summary, the headspace gas chromatography/mass spectrometry method is a highly sensitive, versatile and flexible technique for the biological monitoring of exposure to ototoxic solvents by saliva analysis.

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1. Introduction

Saliva seems to be a suitable matrix for biological monitoring of exposure to hazardous solvents. The major advantage of saliva with respect to other matrices, such as blood and urine, is that it is non-invasive and less confidential. Salivary analysis is generally acceptable by studied subjects and can be applied to a wide variety of compounds, including hazardous solvents [1–3]. Ethylbenzene, toluene, *p*-xylene and styrene have shown ototoxicity characterized by an irreversible hearing loss, measured by behavioural or electrophysiological methods, associated with damage to outer hair cells in the cochlea of the exposed animals [4,5]. Moreover, recent studies have demonstrated a synergic action of ototoxic solvents and noise in producing acoustic damage, even if the dose–response relationship at very low levels of exposure has not been still addressed [6–9]. Some ototoxic compounds are widely used as solvents: for example, toluene or xylenes are used as solvents in oil and rosin extraction, printing inks, paints or varnishes or as additive to aviation and automotive fuels. Ethylbenzene and *p*-xylene are used in chemical synthesis. Styrene and its homologues are used in the plastics industry, particularly in the production of glass-reinforced unsaturated polyester resins [10]. In addition, a simultaneous occurrence of such ototoxic solvents can take place in workplaces resulting in a multiple exposure for workers [11].

So far, not many studies were published on the saliva matrix to monitor exposure to chemicals and few authors reported the use of saliva as a tool for biological monitor of ototoxic solvents. For example, Wang and Lu [12] have applied solid-phase microextraction and gas chromatography–mass spectrometry for measuring chemicals in the saliva of synthetic leather workers, Ernstgård et al. have investigated biological samples of exhaled air, blood, saliva, and urine in an inhalation toxicokinetic study on isopropyl alcohol and *m*-xylene exposure [13]. The headspace of biological matrices was monitored for the presence of isopropyl alcohol, its metabolite

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Table 1
Names and CAS number of target compounds, their retention times and monitored ions (*m*/*z*).

Peak no.	Name	CAS-number	*tR* (min)	Qualifier ion (*m*/*z*)
1	Toluene	108-88-3	14.94	91, 92
2	2-Fluorotoluene	95-52-3	16.32	109
3	Ethylbenzene	100-41-4	18.88	91, 106[a]
4	*m*-Xylene	108-38-3	19.38	91, 106[a]
5	*p*-Xylene	106-42-3	19.71	91, 106[a]
6	*o*-Xylene	95-47-6	22.00	91, 106[a]
7	Styrene-d_8	19361-62-7	25.65	112
8	Styrene	100-42-5	25.71	104[a], 78

[a] Used for quantitation.

acetone, and *m*-xylene and the authors proposed that the compounds measured in saliva might be a useful indication of internal exposure. Rose et al. studied concentrations of acetone in both blood and saliva during isopropyl alcohol exposure and concluded that a high correlation was found between these two biological matrices for either individual subjects or the entire study group [14]. The aim of the present study was to develop an analytical method by static headspace (SHS) gas chromatography–mass spectrometry (GC–MS) for measurement of multi-component mixtures of ototoxic solvents in saliva samples. Saliva samples are ideal candidates for headspace since they can be placed directly in a vial with little or no preparation [15,16].

This method will be useful in establishing an alternative exposure monitoring approach for workers occupationally exposed to airborne toluene, ethylbenzene, xylene isomers and styrene levels well below the threshold limit value–time weighted average (TLV–TWA) [17], (at the time-weighted average concentration for a normal 8 h workday and 40 h workweek to which nearly all workers may be repeatedly exposed, day after day, without adverse effect).

2. Experimental

2.1. Chemicals and supplies

All reagents were analytical grade. The analytical reference standards of ethylbenzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene and the internal standard 2-fluorotoluene were purchased from Chem-Service (Steinheim, Germany); styrene standard was purchased from Riedel-de Haën (Buchs, Switzerland); deuterium labelled styrene-d_8 was purchased from Isotec, Inc. (Miamisburg, OH, USA). Methyl alcohol was purchased from Sigma–Aldrich (Steinheim, Germany) and sodium sulphate from E. Merck (Darmstadt, Germany). Purified water was obtained from a Milli-Q Plus system (Millipore, Milford, MA, USA) and screw top 20 mL clear vials (75.5 mm × 22.5 mm) with ultraclean 18 mm screw cap W/Septa were supplied by Agilent. The analytes were separated on a DB-WAXetr column having a length of 30.0 m, an id of 320 μm and a film thickness of 1.00 μm (J&W 123-7334) (California, USA). Helium with 99.999% purity for GC–MS was used as carrier gas (Air Liquid, Milan, Italy). Control human saliva samples for calibration, blank samples and quality control samples were obtained from healthy, non-smoking volunteers.

2.2. Instrumentation

The analysis of toluene, ethylbenzene, *m*-xylene, *p*-xylene, *o*-xylene and styrene in saliva was performed by a static headspace sampling device (G1888A, Agilent Technologies), with a 70 location autosampler, coupled with a gas chromatograph (6890N Agilent Technologies) equipped with a single quadrupole mass spectrometric detector (5973 MSD System, Agilent Technologies). The headspace device was a "*pressurised loop system*": it initially regulates the vial at a certain temperature and pressure and then the valve is turned and the loop filled with the sample headspace. The sample is then flushed into the transfer line leading to the analytical column. The flushing into the transfer line has been realised by a split injector. Helium flow rate was 1.5 mL/min split. Injector temperature has been set at 250 °C; ion source and quadrupole temperatures have been set at 230 °C and 150 °C, respectively. The column oven temperature has been initially set at 50 °C, then raised to 120 °C with 2.5 °C/min increments. Electron impact at 70 eV was selected as the ionization mode. Detection was performed in the positive single-ion monitoring (SIM) mode; in some cases more than one ion was monitored, but only one was used for quantitation. The ions selected for each compound are shown in Table 1.

2.3. Blank samples, standard solutions and quality control samples preparation

Blank saliva samples from healthy volunteers, who were not been exposed to the target chemicals and gave their informed consent, were collected in a polycarbonate sterile tube with screw cap and conical bottom. This approach is convenient, simple, and makes possible to collect larger volumes of saliva than the sampling methods using sterile cotton swabs, within few minutes. After collection, 1 mL of saliva was sealed in 20 mL headspace vial and saturated with an excess of sodium sulphate. The vials were then closed with an open-centre aluminium screw cap with PTFE/silicone septum and stored at the temperature of 4 °C until use. A standard mixture stock solution, about 430 ng/L, was prepared by diluting the target compounds in methyl alcohol and kept at −20 °C until use. From this standard stock solution, working standard solutions for calibration were prepared in saliva matrix at five levels for each compound (ranging from 0.52 ng/mL to 36 ng/mL).

Blank saliva samples were also used in preparing zero samples, blank samples + internal standards, and quality control samples at two levels (about 3.4 and 17 ng/mL) for method validation. The quantitative determination of the target compounds was performed with the internal standard method: for this purpose 2-fluorotoluene and deuterium labelled styrene-d_8, were added to each sample just before GC analysis reaching the final concentration of 4.0 and 3.9 ng/mL, respectively.

2.4. Static headspace parameters optimization

SHS parameters, which affect the partitioning between aqueous phase and headspace for all the ototoxic compounds analysed, were studied in order to optimize the extraction yield of the target compounds from the matrix. This optimization was carried out by using 20 mL volume vials and by fixing the phase ratio β to 19/1.

Equilibration time: the time required in reaching the static thermodynamic equilibrium between liquid and vapour phase influences the method sensitivity [18]. Several tests were performed in order to adjust the time value for the most advantageous equilibrium: time intervals ranging from 5 to 20 min, incremented

by five, were used in verifying the increase in peak area of quality control samples.

Equilibration temperature: the concentration of volatile organic compounds in the headspace phase, from which method sensitivity depends, can be also increased by lowering the partition coefficient value (K); this can be obtained raising the equilibration temperature. Temperatures from 70 °C to 100 °C were tested.

Salt's concentration: the analyte's concentration in the headspace depends, finally, on the ionic strength of sample. Experiments aimed at verifying the increase in sensitivity due to salt saturation were performed [19].

Stirring mode: efficiency of analyte's transfer from liquid phase to vapour phase was analysed by switching selectively different stirring modes.

Optimization of vial pressurization: once the equilibrium between liquid and vapour is reached, the vial has to be pressurized by carrier gas in order to achieve the best reproducibility.

Split ratio: experiments aimed at selecting the optimum ratio for splitting were carried out too.

2.5. Method validation

All samples were analysed in triplicate and the average peak area for each analyte was used. The ChemStation Software (Agilent Technologies) integrated the peak areas generated by analysis of standard working solutions. The calibration curve was built with five level concentrations in the range 0.52–34.6 ng/mL of the target compounds plus the zero sample reporting peak areas normalized to that of internal standard (A_x/A_i) versus the ratio of nominal concentration to that of internal standard (C_x/C_i) for each target analyte. The calibration curves were fitted by a linear regression analysis, following the equation $(A_x/A_i) = B_1(C_x/C_i) + B_0$, where "$B_1$" is the slope of the regression line and "B_0" is the intercept. Internal standard 2-fluorotoluene was used for determination of ethylbenzene, toluene and xylenes. The same data processing was adopted in the case of styrene, by using deuterated styrene as internal standard. The concentration of each analyte was expressed as ng/mL of saliva.

The sensitivity of the method was expressed in terms of limit of detection (LOD) and the lower limit of quantification (LLOQ). The limit of detection (LOD) and the lower limit of quantification (LLOQ) are defined as three times and ten times the standard deviations of the appropriate blank baseline value. Because no peaks were generated in blank saliva samples from donors not exposed to the target compounds, both parameters were determined empirically by analysing decreasing concentrations of spiked saliva samples.

Accuracy was expressed as recovery percentage. For this purpose, quality control samples were used (7 replicates for each concentration level). The concentrations of the analyte in quality control samples were calculated on a calibration curve analysed in the same day.

Accuracy was expressed by its mean at 95% confidence interval, using the equation:

$$\%R = 100 \times \frac{\mathrm{OV} - \mathrm{BV}}{\mathrm{KV}}$$

where $\%R$ is the recovery percentage, OV is the observed value of quality control samples (ng/mL), BV is the background value of the zero sample (ng/mL) and KV (known value) is the theoretical value (ng/mL).

The precision was tested for both intra- and inter-day repeatability. Three independent sets of quality control samples were analysed at two levels on 1 day for intra-assay precision, whereas three independent sets of quality control samples were analysed on 7 different days, three of them not consecutive, for inter-assay precision. Precision is expressed as relative standard deviation (RSD).

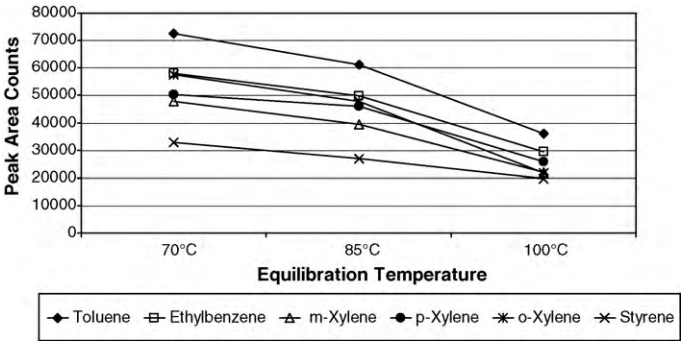

Fig. 1. Effect of equilibration temperature on peak area counts of analysed compounds.

2.6. Samples stability

In order to evaluate the short-term bench top stability, quality control samples (6 replicates for each level) were prepared and stored in both polycarbonate microcentrifuge tubes (1.5 mL) and headspace screw top vials (20 mL). Three samples for each level were analysed immediately (control samples) and the remaining ones after 3 h on the bench at room temperature (stability samples).

To verify the long-term (more than 7 days) stability of saliva samples during storage, we tested two different temperatures ($T_1 = -20$ °C and $T_2 = 4$ °C) at two different storage conditions, in polycarbonate microcentrifuge tubes (1.5 mL) and in the headspace screw top vials (20 mL). Each level was analysed in triplicate after 1, 2, 3, 7 and 10 days. The frozen samples were thawed and kept at ambient temperature for 1 h before analysis.

3. Results and discussion

3.1. Static headspace parameters optimization

Equilibration time: since not significant differences in peak area counts were observed during the tests, the shortest equilibration time, 5 min, was chosen.

Equilibration temperature: during the tests, a considerable decrease in peak area counts was observed when temperature rises to 100 °C due to water evaporation that interferes with mass spectrometry detection (see Fig. 1). The best signal to noise ratio, at equilibration time of 5 min, was obtained at 70 °C.

Salt's concentration: in the present study salt did not improve the analytical sensitivity. However, an improvement in the chromatographic signal repeatability was observed as consequence of salt addition. Saliva samples ionic strength was normalized by adding sodium sulphate (≅0.7 g).

Stirring mode: no significant difference in efficiency of analyte's transfer from liquid to vapour phase was observed by varying the sample-stirring mode: nevertheless, a high-stirring mode was used.

Pressurization: a carrier gas pressure of 10.3 kPa, lasting for 0.15 min, resulted as optimal for 1 mL sample analysis.

Table 2 summarizes all SHS and GC parameters selected.

3.2. Assay performance (linearity, sensitivity, accuracy, precision)

Gas chromatography with mass spectrometric detection is a very selective instrumental technique because it allows a good chromatographic separation and analytes identification through their characteristic ion fragmentation. A typical chromatogram of standard working solution at 34.6 ng/mL is shown in Fig. 2. All the target analytes were well separated. The calibration line was linear in the range from 0 to 34.6 ng/mL and the determination

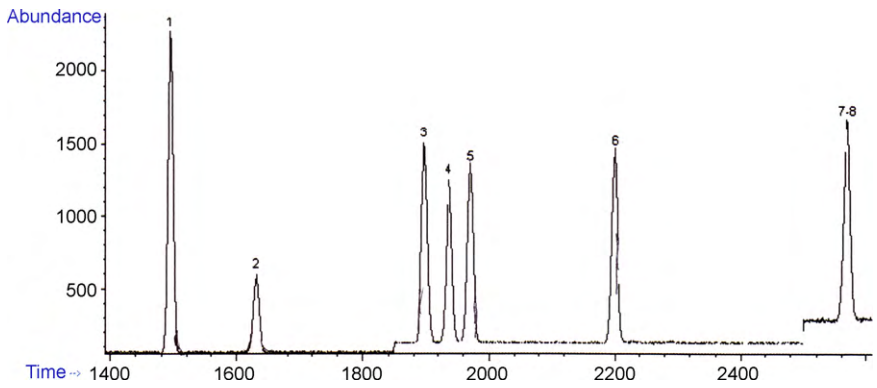

Fig. 2. SHS-GC–MS chromatogram of a standard solution of analysed solvents (concentration of analytes 34.6 ng/mL). Peaks: 1, toluene; 2, 2-fluorotoluene; 3, ethylbenzene; 4, *m*-xylene; 5, *p*-xylene; 6, *o*-xylene; 7, styrene d_8; 8, styrene.

Table 2
Optimum static headspace and gas chromatographic parameters for analysis of ototoxic solvents in saliva samples.

SHS	
Equilibration parameters	5 min, T = 70 °C, high stirring mode
Vial pressurization	10.3 kPa, lasting for 0.15 min
Loop filling	1 mL, at 120 °C lasting for 0.5 min, 0.1 min equilibration
Transfer line	T = 120 °C, injection time = 0.5 min
GC	
Inlet	Split ratio 1:5 at T = 250 °C, headspace liner: P/N 5183-4709
Carrier gas	Constant flow (1.5 mL/min)
Oven temperature programme	50–120 °C by 2.5 °C/min
Run time	28 min

coefficients always higher than 0.999. The method enabled the detection of the ototoxic compounds with a LOD ranging from 0.19 ng/mL (styrene) to 0.54 ng/mL (*m*-xylene) and a LLOQ ranging from 0.64 ng/mL (styrene) to 1.8 ng/mL (*m*-xylene).

The accuracy of the method, expressed as mean recovery percentage, has always resulted greater than 99% for all the target compounds, ranging from 107 to 121% at the lowest level and from 99 to 105% at the highest level experimented. The recovery data, at the 95% confidence interval, are summarized in Table 3.

The precision of the method at intra-assay level was well below 10% for both levels experimented. The inter-assay precision ranged from 10 to 14% at the low level and from 6.9 to 11% at the high level. Table 4 summarizes validation results for all target compounds.

3.3. Uncertainty evaluation

The uncertainty was evaluated following the recommendations in the EURACHEM/CITAC Guide to Quantifying the Uncertainty in Analytical measurements [20]. The estimation of uncertainty was carried out on the basis of "*top down*" empirical model using the data derived from the method validation experiments. The main components of uncertainty were calculated through the calibration data, the recovery and the repeatability assays, respectively. Therefore, the calibration uncertainty $u(c_0)$, the recovery uncertainty $u(R)$ and the repeatability uncertainty $u(rep)$ were used to obtain the combined uncertainty u_c, by the following formula based on the theory of variance propagation:

$$u_c = \sqrt{u(c_0)^2 + u(R)^2 + u(rep)^2}$$

The calibration uncertainty $u(c_0)$ is given by

$$u(c_0) = \frac{S}{B_1}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{S_{xx}}}$$

with the residual standard deviation S given by

$$S = \sqrt{\frac{\sum_{j=1}^{n}[A_j - (B_0 + B_1 * c_j)]^2}{n-2}}$$

and

$$S_{xx} = \sum_{j=1}^{n}(c_j - \bar{c})^2$$

with p, number of measurements to determine c_0; n, number of measurements for the calibration; c_0, determined chromatographic concentration of each standard solution; c, mean value of the different calibration standards (n number of measurements); i, index for the number of calibration standards; j, index for the number of measurements to obtain the calibration curve; A_j, detector response (A_x/A_i) of the ith calibration standard; c_i, concentration of the ith calibration standard (C_x/C_i); B_1, the slope and B_0 the intercept values.

The calibration uncertainty $u(c_0)$ was evaluated at the concentrations of the quality control samples (high level = 16.8 ng/mL and low level = 3.5 ng/mL). The results are shown in Table 5.

Table 3
Absolute recoveries (mean % and 95% CI) for all the ototoxic analytes at low and high concentration levels.

Compound	Nominal concentration (ng/mL)		Absolute recovery (mean %; [95% CI[a], %]) (n = 7)	
	Low		High	
Toluene	3.5	17	115 [104–127]	103 [94–111]
Ethylbenzene	3.5	17	116 [108–125]	105 [95–115]
m-Xylene	3.4	17	121 [112–131]	104 [93–115]
p-Xylene	3.4	17	119 [112–126]	104 [94–114]
o-Xylene	3.5	18	107 [100–114]	100 [92–108]
Styrene	3.6	18	115 [108–122]	99 [90–108]

[a] 95% CI = mean ± 1.96*RSD/$(n)^{1/2}$.

Table 4
Analysis of solvents in saliva samples: limit of detection (LOD), lower limit of quantification (LLOQ) and intra- and inter-assay precision (RSD).

Name	LOD (ng/mL)	LLOQ (ng/mL)	Intra-day assay, RSD (%)		Inter-day assay, RSD (%)	
			Low	High	Low	High
Toluene	0.22	0.73	6.2	1.7	14	8.0
Ethylbenzene	0.39	1.3	6.8	7.2	11	10
m-Xylene	0.54	1.8	3.2	4.1	12	11
p-Xylene	0.33	1.1	9.1	4.3	12	9.7
o-Xylene	0.38	1.3	3.7	2.2	10	8.5
Styrene	0.19	0.64	6.2	1.7	10	6.9

In order to evaluate whether the recoveries R of each analyte at the two spiked quality control levels were statistically significant different from one, a t-test was performed using the formula:

$$\left|1 - R\right| \leq t_{\alpha/2,\nu} u(R),$$

where $t_{\alpha/2,\nu}$ is the two sided t tabulated value for the degrees of freedom associated with $u(R)$.

The experimental values obtained at the low spiked level were found statistically different from one, for all the target analytes, excepted for styrene; therefore, the recovery uncertainty was included in the expanded uncertainty. The contribution to the combined uncertainty measure u_{rel} arising from the repeatability, $u(rep)$, was calculated at both concentration levels and are shown in Table 5.

The expanded uncertainty U was calculated from the following expression:

$$U = u_c k$$

with $k = 2$, assuming a normal distribution at a confidence level of 95%.

The uncertainty details at the quality control levels are shown in Table 5.

The extended uncertainty resulted ranging from 11 to 16% and from 10 to 14% at low and high level, respectively. Fig. 3 shows the uncertainty contributions and the extended relative uncertainty for toluene.

3.4. Evaluation of the samples stability during storage

The short-term stability study of quality control samples stored at 4 °C demonstrated no vapour loss, both at the low level at and the high level of concentration.

Good sample stability until 7 days was assured in headspace vials. Sample loss resulted well below 10% for both storage temperatures (4 °C and −20 °C) and it decreased at less then 5% when samples storage lasted for 2 days. With storage time greater than 7 days, sample loss can reach values greater than 35%.

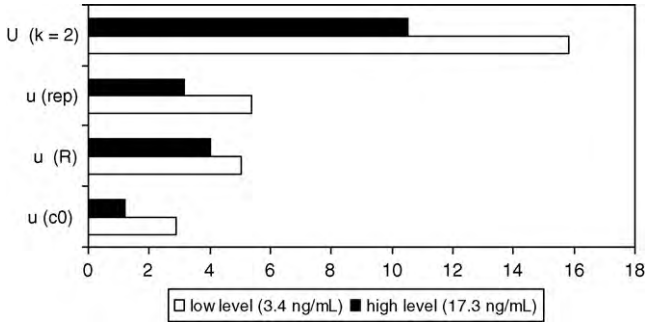

Fig. 3. Calibration uncertainty $u(c_0)$ (%), recovery uncertainty $u(R)$ (%), repeatability uncertainty $u(rep)$ (%) and extended uncertainty U (%) at low and high level of toluene determination.

Unlike the storage in the headspace devices, storing samples in polycarbonate tubes does not assure vapour preservation. Therefore, after saliva collection from donors in polycarbonate tubes, samples must be immediately transferred to headspace vials. In addition, the analysis of samples stored in such devises has to be performed as soon as possible and not later than 7 days after sampling.

Fig. 4 shows the ototoxic vapour loss during the 10 days storing time for the earliest and latest eluted compound (toluene and styrene, respectively) in different storage devices for the high level quality control samples.

4. Real samples

As a preliminary feasibility study, the method presented was applied to the analysis of saliva samples collected from workers exposed to styrene in a fiberglass industry and to xylenes in a research laboratory. Concerning the fiberglass industry, the good relation between the potential exposure associated to the tasks performed and the salivary levels of styrene [21] indicates that saliva sampling is a promising technique for the biological mon-

Table 5
Detail of uncertainty contributions in saliva analysis.

Compound	C (ng/mL)	$u(c_0)$ (%)	$u(R)$ (%)	$u(rep)$ (%)	U (%) ($k = 2$)
Toluene	3.5	2.9	5.0	5.4	16
	17	1.2	4.0	3.2	10
Ethilbenzene	35	3.5	3.6	4.1	13
	17	1.4	4.9	4.1	13
m-Xylene	35	3.4	4.0	4.4	14
	17	1.4	5.4	4.4	14
p-Xylene	35	3.2	3.1	4.6	13
	17	1.3	4.8	3.9	13
o-Xylene	35	2.4	3.3	3.8	11
	18	1.0	4.0	3.3	11
Styrene	36	4.4	3.1	3.9	13
	18	1.8	4.8	2.7	11

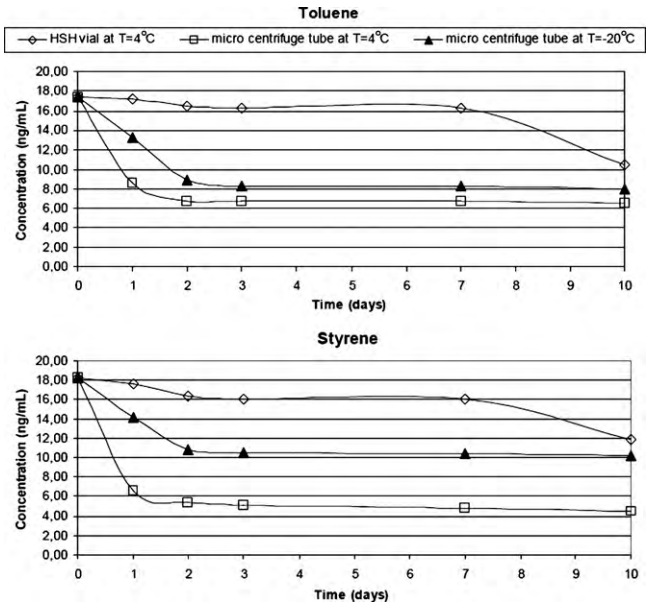

Fig. 4. Toluene and styrene stability at different storage conditions of saliva samples.

itoring of occupational exposure. Further data analysis and studies aimed at verifying the correlation between the salivary styrene and urinary phenylglyoxylic (PGA) and mandelic (MA) acids are in progress. Regarding the research laboratories, few saliva samples were above detection limit, even if airborne xylenes were not detected. The significance of this finding should be further investigated.

5. Conclusions

A new method was designed to develop a highly sensitive indicator of ototoxic solvents absorption. The method allows the determination in saliva of toluene, ethylbenzene, *para*-xylene (independently from *ortho*- and *meta*-xylene, which can also been measured) and styrene by means of headspace analysis and GC–MS. The method allows an easy sample preparation, a reproducible separation and precise and accurate detection of the solvents concerned. Since the ototoxic solvents content in saliva matrix resulted stable in the storage condition proposed, the method can be used in biological monitoring, classically performed on urine samples. As well, the high sensitivity of this method results useful in measuring multiple exposures to low levels of ototoxic solvents, allowing the possibility to improve the performance of the studies aimed at correlating the ototoxic dose to the response in hearing damage.

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