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SPE–GC–MS for the sampling and determination of unmetabolized styrene in urine☆

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Abstract

The urinary excretion of unmetabolized styrene can be a very good indicator for biomonitoring styrene in occupationally exposed people. The use of a new urine sampling system, involving a solid-phase extraction cartridge, offers several advantages for determining styrene. The advantages are especially related to the pre-analytical phase of styrene determination, which may be influenced by many variables. The effect on styrene recovery of sorbent type, eluting solvent, elution volume, elution flow-rate, and the addition of methanol to the washing solvent, was evaluated by experimental design methodology. As a result, Oasis HLB cartridges were selected for urine sampling, as well as 1.5 mL of ethyl acetate at 0.5 mL/min for eluting the retained styrene. These conditions were then applied to the validation of the solid-phase extraction combined with GC–MS method for the sampling and analysis of unmetabolized styrene in urine. The overall uncertainty was in the 12–22% range and the limit of detection was 2.2 μg/L for a 4 mL urine sample. The stability of styrene has been studied both in cartridges and in vials under different storage periods. After 1 month period the styrene stored on cartridges at room temperature remained stable, whereas this is not the case for styrene recovery from vials. The results obtained indicate that on-site solid-phase extraction of urine can provide a simple, accurate and reproducible sampling and analytical method for the biomonitoring of styrene in urine.

Keywords: Styrene; Urine; SPE; GC–MS; Biomonitoring

1. Introduction

Styrene in urine has been proposed as a specific and quantitative biomarker of occupational exposure. Good correlations have been found between environmental styrene concentrations and styrene concentrations in the urine of workers in fibreglass-reinforced plastic industries [1–4].

The first technique used for the determination of volatile compounds from urine was solvent extraction [5,6]; this technique posed several problems, the most important of which was evaporation of the solvent accompanied by the loss of low boiling point compounds. Alternatively, manual [3,7,8] and automated [2,9] headspace determinations have been described. A purge and trap method, based on the concentration of styrene in a solid sorbent and subsequent analysis by thermal desorption–gas chromatography, has also been applied [10]. More recently, reliable and accurate methods using solid-phase microextraction have been developed to determine volatile organic concentrations in urine [11–13]. However, all the above mentioned methods entail the handling, transporting and storage of liquid urine samples with the requirement that the urine sample must be analysed as soon as possible in order to avoid the loss of volatile compounds. It is necessary to take into account that the pre-analytical phase, including sample collection, storage and transport, plays an important role for the quality of the final results [14].

In field studies, urine is generally sampled in glass or plastic containers, refrigerated, and delivered to the laboratory, where the sample is transferred to vials for analysis. Transport from the sampling site with storage at low temperature is difficult for liquid samples in bottles. Additionally, several processes may affect the stability of samples during storage and, especially, during transport, such as precipitation of the urinary components, the instability of the organic chemicals of interest and, in

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the case of volatile organic compounds, loss of the analytes of interest [15].

Solid-phase extraction (SPE) for liquid samples has become a widely used laboratory technique, although its use has mainly focused on assessing water contamination [16–19] and, to a lesser extent, on the determination of drugs in toxicological studies [20–22]. The use of SPE cartridges has shown potential for the on-site sampling of water from remote areas, prior to further analysis in the laboratory [23].

Recently, a new sampling system that combines the sampling, transportation and preservation of biological fluids has been developed at the Institut National de Recherche et de Sécurité (Vandoeuvre, France) with the objective to overcome the disadvantages related to handling liquids in urine sampling. The system, which consists on a special syringe joined to an SPE cartridge by means of an adaptor, is being currently tested for different metabolites from organic compounds that are commonly present in occupational environments [24–26]. As a part of this study, the aim of this work was firstly to develop a sampling and analysis method for the determination of styrene in urine, using the aforementioned sampling system, which was used to collect and concentrate the styrene. The study estimated the influence of different variables that could affect the SPE extraction efficiency—including type of sorbent, elution solvent, elution volume, elution flow-rate and the use of a methanol fraction in the washing solvent—and evaluated the simultaneous effect of the more significant variables on styrene extraction using experimental design methodology. The experimental conditions selected were then applied to study the analytical performance of the method for the determination of styrene in urine to monitor occupational exposure to styrene. Additionally, the stability of styrene at three different storage periods in SPE cartridges was tested and the obtained results compared with those of styrene stability in a urine matrix.

To our knowledge, this is the first study that considers the use of an on-site sampling system, involving a SPE cartridge, for biological monitoring of styrene in urine.

2. Experimental

2.1. Chemicals and urine sampling device

Styrene and anhydrous sodium sulfate was supplied by Fluka (Buchs, Switzerland); methanol and dichloromethane were purchased from Merck (Darmstadt, Germany); ethyl acetate from Riedel-de Haën (Seelze, Germany). The high purity water was from a Milli-Q water system (Millipore, Bedford, MA, USA). Certified styrene standard in methanol (199.9 μg/mL) was from Supelco (Bellefonte, PA), and was stored at −18 °C.

The device used for urine sampling, transport and storage relies on a special syringe (S-Monovette®, 4.5 mL, Sarstedt, Germany) with an adaptor (Multi-adapter, Sarstedt, Germany) providing a Luer connection between the syringe and the SPE cartridge. A pipette cone tip is placed on the other side of the cartridge.

Two solid-phase extraction (SPE) cartridges were used: Sep-Pak® Plus C18 cartridges (500 mg sorbent), and Oasis® HLB Plus cartridges (225 mg), both of them purchased from Waters (Milford, MA, USA).

2.2. Preparation of styrene standards

A stock standard solution of styrene was prepared in methanol. The work standard solutions of styrene used for calibration were prepared daily by diluting different amounts of the stock standard solution in dichloromethane or ethyl acetate. These were injected in triplicate in the same run as the samples.

Work solutions of styrene used for solid phase extraction were also prepared from the stock standard solution of styrene by diluting with urine from a non-exposed subject.

An independent styrene solution was prepared from a commercially standard solution of styrene in methanol in order to perform accuracy and repeatability measurements.

2.3. Collection of urine samples and solid-phase extraction procedure

The SPE cartridges were conditioned before use by passing 3 mL of the elution solvent through them, followed by 3 mL of methanol and 3 mL of Milli-Q water. After that, the cartridge was joined through the adapter to the 4.5-mL syringe and a cone tip was also attached to the other extreme. Fig. 1 shows a scheme of the sampling device used. The cone tip was plunged into the urine and an aliquot withdrawn through the SPE cartridge by pulling the syringe piston to the locking point. When sampling was finished, the cartridge was disconnected from the sampling device.

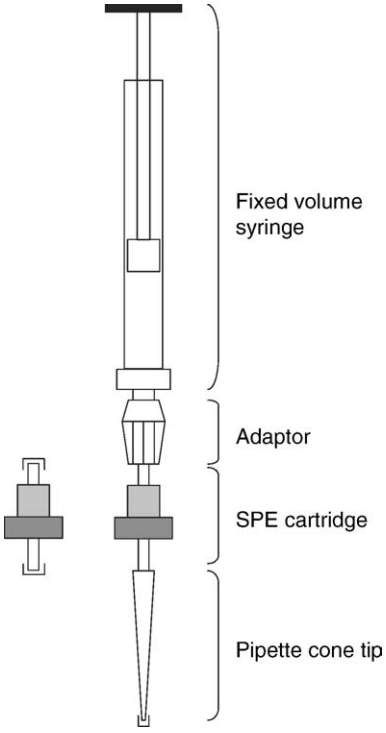

Fig. 1. Scheme of the sampling device used.

Finally, the cartridge was rinsed with 2 mL of Milli-Q water containing, or not, 5% (v/v) methanol. The styrene retained on the cartridge was desorbed with volumes between 1 and 4 mL of the eluting solvents at 0.5 or 4 mL/min flow-rates, dispensed by an HPLC pump. Anhydrous sodium sulfate was used to remove any residual water. A 1-μL aliquot of the organic extract was injected into the GC column.

The influence of the SPE variables on styrene extraction was studied with solutions of 170 μg/L of styrene in urine. An experimental design was applied to determine the significant variables affecting styrene recovery. The experimental matrix designs were developed and evaluated using the Statgraphics Plus 5.1 software package (Manugistics, 2000)

2.4. Gas chromatography and mass spectrometry analysis

Analyses were made on a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a Hewlett-Packard 7683 autosampler and a split-splitless injector operating in splitless mode. The operating temperature of the injector was 250 °C. The column used was an HP1-MS (crosslinked methyl silicone) capillary column (50 m length, 0.2 mm i.d.) with 1 μm phase thickness (Hewlett-Packard, Palo Alto, CA, USA). The column temperature was held at 120 °C, and helium was used as the carrier gas at a constant flow of 1.2 mL/min. The injection volume was 1 μL.

A Hewlett-Packard MSD 5973 mass-spectrometer, in the selected ion monitoring mode, focused at *m/z* 104 molecular ion, with the source at 230 °C, was used. Mass spectra were obtained at an energy level of 70 eV. Standard autotunes with perfluorotributylamine were made on a daily basis. The styrene retention time was 6.5 min.

2.5. Study of the styrene stability

The study has been performed on a pool of urine specimens from unexposed volunteer people. The urines were spiked with different amounts of a stock standard solution of styrene in methanol in order to have three concentration levels: 5, 25 and 100 μg/L of styrene.

For each one of the concentration levels, three series of eight samples were taken using the sampling device, described in Fig. 1, with the Oasis HLB cartridge previously conditioned. Once the spiked urine was sampled, luer male and female plugs were used to seal the cartridges, which were stored in the dark at room temperature.

Each set of cartridges was analyzed at three storage times (1, 7 and 30 days). Previous to the analysis, the cartridge was rinsed with two millilitres of Milli-Q water. The elution step was performed with 1.5 mL of ethyl acetate at 0.5 mL/min. Anhydrous sodium sulfate was used to remove any residual water. Analysis was carried out following the conditions described in the previous section.

Samples of the spiked urine of each concentration level, prepared for the described stability study, were stored in 10-mL vials at −18 °C. A series of eight vials of the stored urine was extracted and analysed for styrene in parallel to the analysis of every set of cartridges. In this way, a total of 144 samples were analysed.

The stability was evaluated as the ratio *R* of the mean values from eight measurements at each of the three storage times to the amount of spiked styrene.

3. Results and discussion

3.1. Recovery experiments

The effectiveness of the SPE method depends on several parameters, such as the cartridge stationary adsorbent, pH, sample pretreatment, the solvents used for washing and eluting, elution volume, and the flow rate during the different steps [27].

In our case, since the sampling device was designed to be used on-site for the direct sampling of urine, no pre-treatment (pH or ionic strength modification, or addition of an organic component) of the sample is possible. So, the tested variables that may affect the extraction efficiency are: type of sorbent, eluting solvent, elution volume, elution flow-rate and the addition of methanol to the washing solvent.

The first step in developing a method using SPE is to select the most suitable sorbent. C18 packing is the most popular and widely used reversed phase to retain organic compounds from aqueous and biological samples. Oasis® HLB is a co-polymer consisting of two monomer components, divinylbenzene and *n*-vinylpyrrolidone, and has the same applicability as the C18 sorbent has. However, Oasis could be advantageous in field applications because it can be used without conditioning, and dryness does not alter the cartridge behaviour [28,29].

As regards the possible eluent solvents, both dichloromethane and ethyl acetate are suitable when gas chromatography is to be used [30,31].

It has been reported that washing with water/methanol is successful in removing urine matrix interferences [20]. A 5% methanol mixture in water has been found suitable to eliminate matrix compounds without affecting analyte recoveries [32,33]. Therefore, in this study, the presence, or not, of 5% of methanol in the washing solvent was tested.

To ascertain the individual effects of the mentioned variables, a half-fractional factorial design for five variables at two levels was applied. Table 1 shows the list of factors and the low and high levels studied for the experiment. A total of 16 runs were performed and a replicate was carried out on different days and using different solutions. The design matrix used and the results obtained are listed in Table 2. The ANOVA analysis identified

Table 1
List of factors and their levels for the half-fractional factorial experiment

Factors	Low	High
Sorbent (A)	C18	OASIS
Eluting solvent (B)	EA	DCM
Elution volume (mL) (C)	1.0	4.0
Elution flow rate (mL/min) (D)	0.5	4.0
Methanol fraction (%) (E)	0	5

EA: ethyl acetate; DCM: dichloromethane.

Table 2
Design matrix and response results (% of recovery) in the 2^{5-1} half fractional factorial design for styrene determination

Sorbent	Eluting solvent	Elution volume	Elution flow-rate	Methanol	Recovery (%)	
OASIS	DCM	1	0.5	5	54.3	58.2
C18	DCM	1	0.5	0	59.5	50.5
OASIS	DCM	1	4.0	0	33.3	45.6
OASIS	EA	4	0.5	5	90.0	97.8
C18	EA	4	0.5	0	93.2	83.3
C18	EA	1	0.5	5	82.8	83.6
OASIS	EA	4	4.0	0	74.8	77.7
OASIS	DCM	4	0.5	0	54.8	64.3
C18	EA	1	4.0	0	43.2	46.3
C18	DCM	4	4.0	0	40.9	43.8
OASIS	EA	1	0.5	0	89.8	83.3
C18	DCM	1	4.0	5	41.9	31.8
OASIS	EA	1	4.0	5	67.2	68.4
C18	DCM	4	0.5	5	44.7	57.4
C18	EA	4	4.0	5	63.2	52.3
OASIS	DCM	4	4.0	5	38.4	41.4

EA: ethyl acetate; DCM: dichloromethane.

seven factors as being statistically significant for the recovery data. The Pareto chart (Fig. 2) shows each one of the estimated effects in decreasing order of magnitude. The length of each bar is proportional to the standardized effect, which is the estimated effect divided by its standard error. The vertical line can be used to judge which effects are statistically significant. Any bars that extend beyond the line correspond to effects, which are statistically significant at the 95% confidence level. In our case, the most significant effects contributing to the recovery were eluting solvent and elution flow-rate and, to a lesser extent, the sorbent used and the elution volume. The use of ethyl acetate and the Oasis HLB sorbent improved the efficiency of styrene extraction. The presence of methanol in the washing solvent had no influence on the extraction recovery. Additionally, the data showed that the recovery was higher at the low level of elution flow-rate. Although higher elution volumes had a minor positive effect, low elution volumes allow enrichment of the styrene concentration in the eluate. Thus, an elution volume of 1.5 mL should allow easy manipulation of samples, while maintaining a reasonable enrichment of analyte.

Selecting the OASIS cartridge for urine sampling and 1.5 mL of ethyl acetate at 0.5 mL/min for eluting the retained styrene, the model predicted a recovery of 88.5% (80.8–96.6%, 95%

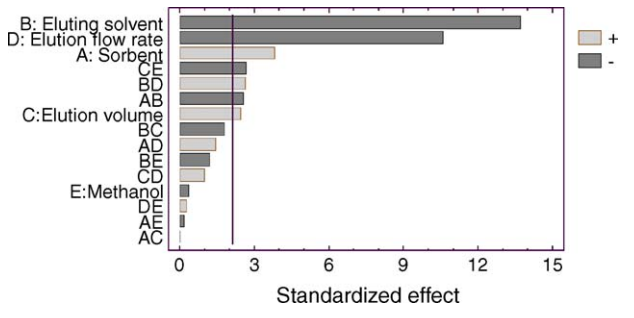

Fig. 2. Pareto chart of the effect obtained from a 2^{5-1} half fractional factorial design.

Table 3
Styrene recovery for spiked urine samples

	Extracted urinary styrene (μg/L)	Recovery (%)
	151.1	86.5
	145.2	83.1
	154.7	88.5
	145.6	83.4
	173.9	99.5
	170.0	97.3
Mean ± S.D.	156.8 ± 12.4	89.7 ± 6.4

confidence limits). A confirmatory experiment was carried out to verify the results from the analysis. Table 3 shows the results obtained by performing six replicate extractions from a urinary solution of styrene of 174.7 μg/L. As can be seen the results obtained agree fairly well with predictions and the obtained recovery is nearly 90%. Therefore, the conditions proposed were used for further validation studies.

3.2. Analytical performance

The repeatability and accuracy of the method was determined by conducting six replicate extractions of independent styrene standards at two levels of concentration, 4.4 and 100 μg/L that were prepared from a commercial standard. The results in Table 4 shows that the relative standard deviation (R.S.D.) was 5.3% for the lower level of concentration and 9.0% for the higher level of the urine standard. These R.S.D. values were in agreement with the values reported in the literature [11–13], and somewhat higher than those reported for a purge-and-trap method [10].

The accuracy of the method was also checked; the mean value data of the six replicate analyses are shown in Table 4. Good correspondence was obtained between the standard concentration values and the SPE recovery results. The results of the overall uncertainty of the measuring procedure are also shown in Table 4. Overall uncertainty is expressed, on a relative basis, by a combination of bias and precision [34]. The results for uncertainty of the proposed method were satisfactory and similar to

Table 4
Precision and accuracy of the method in standard styrene samples

	Styrene concentration in spiked urine samples	
	100 μg/L	4.4 μg/L
	82.2	4.4
	105.3	4.5
	93.5	4.6
	98.4	4.4
	102.8	4.2
	89.8	4.5
Mean	95.3	4.5
R.S.D. (%)	9.03	5.31
Overall uncertainty[a]	21.90	12.19

[a] $((|\bar{x} - x_{true}| + 2s)/(x_{true})) \times 100$ where: $\bar{x}$ is the mean value of the repeated measurements; x_{true} the true concentration value; s the standard deviation of measurements.

those obtained in methods for unmetabolized contaminants in biological fluids [13,35].

Calibration curves were obtained in a concentration range between 3.5 and 300 μg/L. From the linear regression analysis performed on calibration data, an instrumental limit of detection of 6.6 μg/L was obtained [36]. This value corresponds to a detection limit of 2.2 μg/L of styrene in urine for a 4-mL sample, a value that is 20 times lower than the proposed biological exposure index [2–4,8]. The limit of detection is bigger than that reported for other methods. However, sampling a higher volume of urine can increase sensitivity. With the sampling device described here, the volume of urine that was passed through the cartridge corresponds to the volume of the syringe attached to the cartridge. However, if low concentrations of analyte are expected a higher volume of urine can be sampled, increasing the sensitivity in this way. Fig. 3 shows a representative mass chromatogram of the SPE extract corresponding to the tested levels of concentration in Table 4. A chromatogram of the SPE extract corresponding to the low level of styrene concentration in urine sampled by using two syringes consecutively (i.e. corresponding to 8 mL of urine) has also been included.

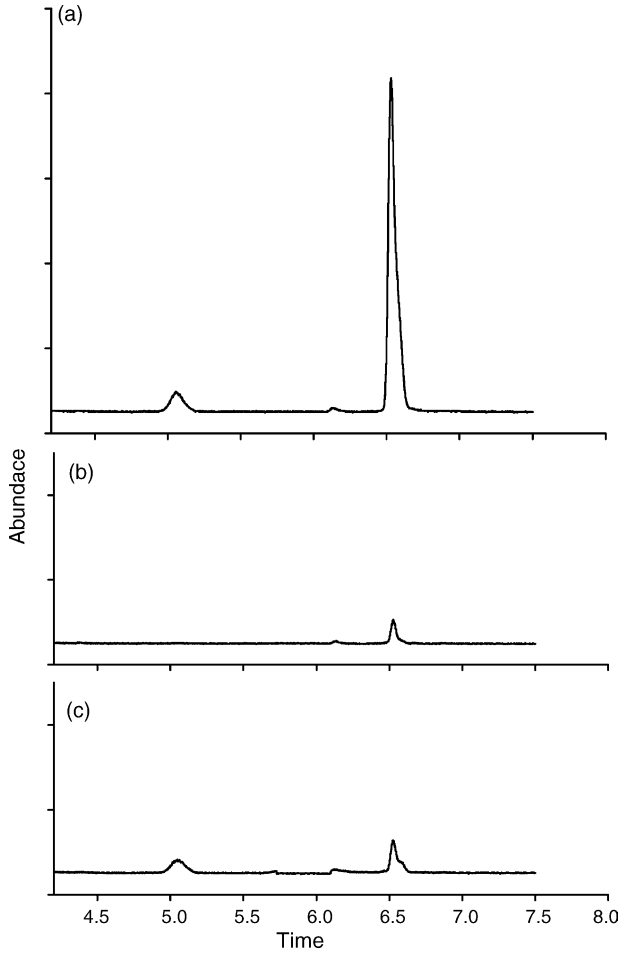

Fig. 3. Mass chromatograms of *m*/*z* 104 obtained from standard urine samples: (a) 4 mL of 100 μg/mL styrene solution, (b) 4 mL of 4.4 μg/mL styrene solution, and (c) 8 mL of 4.4 μg/mL styrene solution.

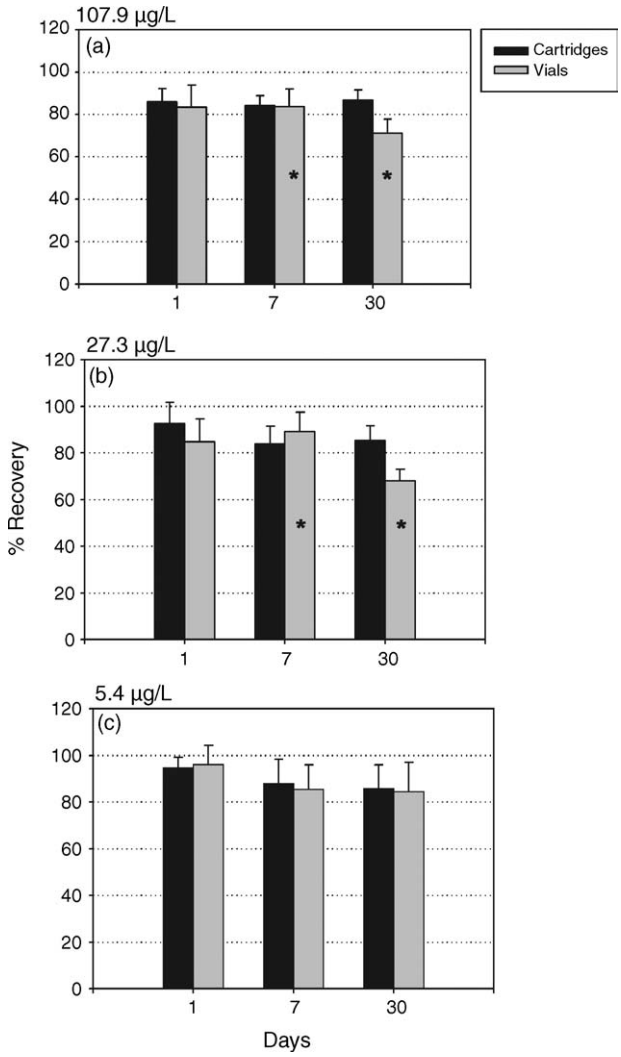

Fig. 4. Study of the stability of styrene both in cartridge and urine samples after 1, 7 and 30 days of storage in the dark at room temperature. Mean values of recovery and standard deviation ($n = 8$, $*n = 6$). (a) 107.9 μg/mL; (b) 27.3 μg/mL; (c) 5.4 μg/mL.

3.3. Study of the styrene stability

Experiments were conducted to study the stability of styrene on cartridges stored after urine extraction using the validated method. The results were compared with those obtained extracting urine after sample conservation. The study was carried out at three levels of urinary styrene concentration, that correspond approximately to 0.1, 0.5 and 2 times the published biological limit value for the urinary excretion of styrene [2–4,8]. Eight samples were analyzed at each one of the concentration level and each one of the storage periods specified in the Section 2.5. Fig. 4 shows the results obtained for the mean recovery in each case.

Urine was stored in vials having, on one side, the minimum headspace to avoid evaporative loss of the analyte, and, on the other side, enough headspace to avoid breakdown of the glass vials. However, in some cases (marked with an asterisk in Fig. 4) two vials of the set were broken. This is one of the problems that can arise from sample conservation in vials.

Table 5
Analysis of variance of storage data

Source	Sum of squares	Df	Mean square	*F*-ratio	*P*-value
(a) Storage in cartridges					
Styrene concentration: 107.9 μg/L					
Between groups	27.35	2	13.673	0.48	0.6227
Within groups	592.50	21	28.214		
Total (Corr.)	619.84	23			
Styrene concentration: 23.7 μg/L					
Between groups	344.36	2	172.182	2.78	0.0851
Within groups	1301.86	21	61.993		
Total (Corr.)	1646.22	23			
Styrene concentration: 5.4 μg/L					
Between groups	342.93	2	171.464	2.33	0.1219
Within groups	1545.26	21	73.584		
Total (Corr.)	1888.18	23			
(b) Storage in vials					
Styrene concentration: 107.9 μg/L					
Between groups	626.88	2	313.441	3.96	0.0400
Within groups	1265.97	16	79.123		
Total (Corr.)	1892.86	18			
Styrene concentration: 23.7 μg/L					
Between groups	1444.34	2	722.172	10.71	0.0011
Within groups	1078.43	16	67.402		
Total (Corr.)	2522.77	18			
Styrene concentration: 5.4 μg/L					
Between groups	656.09	2	328.046	2.99	0.0721
Within groups	2305.88	21	109.804		
Total (Corr.)	2961.97	23			

The recoveries for styrene stored on cartridges ranged from 83.7 to 94.4%, whereas on vials ranged from 68.0 to 96.1%.

ANOVA test has been carried out on recovery results for each level of concentration in order to compare results for each storage period. The ANOVA table decomposes the variance of recovery into two components: a between-group component, and within-group component. The *F*-ratio is a ratio of the between-group estimate to the within-group estimate. When the *p*-value of the *F*-test is greater or equal to 0.05, there is not a statistically significant difference between the recoveries from one period of storage time to another at the 95% confidence level.

Table 5a shows the results of ANOVA for cartridge storage and Table 5b, for vials conservation. The results on Table 5a shows that there is no statistically significant differences between the recoveries obtained for styrene from OASIS HLB cartridges at the three storage periods in the range of styrene concentrations studied. However, from the results on Table 5b, it can be seen that the styrene recoveries, for the 107.9 and 23.7 μg/L concentration levels, are statistically different. The differences arise from the recoveries obtained after 30 days of storage, which are lower. Changes in styrene concentration, with respect 1-day recovery value, were between 14 and 20%.

The potential of SPE cartridges to store analytes from water matrix has been studied. Studies dealing with the stability of pesticides have shown that, in general, it is better when they are stored in SPE at −20 °C than in water matrix [37,38]. Other compounds, such as antibiotics were degraded faster when stored in SPE cartridges [39]. Results of the present study have shown that SPE cartridges are also a good alternative to storage of the urine matrix.

4. Conclusions

The method described offers the possibility of extracting styrene from urine using an on-site sampling device. The SPE sorbent cartridge used may then be easily transported to the laboratory for further analysis. With the described procedure, typical concentrations of styrene in the urine of occupationally exposed workers can be determined for the assessment of occupational exposure to this contaminant. Studies on analyte stability have shown that no significant loss of the compound was observed after 30 days of storage at room temperature. The sampling device seems suitable for routine use in the measurement of urinary excretion of styrene. It also has the potential for the measurement of other volatile organic compounds in urine.

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