Microchemical Journal 180 (2022) 107567

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Multiple headspace solid-phase microextraction (MHS-SPME) methodology applied to the determination of volatile metabolites of plasticizers in human urine

M.L. Alonso [a,*], I. San Román [a], L. Bartolomé [b], N. Monfort [c], R.M. Alonso [a], R. Ventura [c,d]

[a] FARMARTEM Research Group, Analytical Chemistry Department, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), P.O. Box 644, 48080 Bilbao, Spain
[b] Central Analysis Service (SGIker), Faculty of Science and Technology, University of the Basque Country (UPV/EHU), P.O. Box 644, 48080 Bilbao, Spain
[c] Doping Control Research Group, IMIM (Institut Hospital del Mar d'Investigacions Mèdiques), 08003 Barcelona, Spain
[d] Department of Experimental and Health Sciences, Pompeu Fabra University, 08003 Barcelona, Spain

ARTICLE INFO

Keywords:
DEHP metabolite
2-Ethylhexanol
4-Heptanone
Multiple headspace solid-phase micro-extraction (MHS-SPME)
Human urine

ABSTRACT

In the present work, an optimization and validation of an analytical method for the determination of two plasticizer metabolites, 2-ethylhexanol and 4-heptanone in urine, were carried out by multiple headspace solid-phase microextraction (MHS-SPME) coupled to GC–MS. The validation study was successfully performed in terms of stability, method selectivity, linearity, accuracy, recovery, intermediate precision, repeatability, limit of detection (LOD) and limit of quantification (LOQ) in urine samples. Finally, two population group studies were developed in urine samples of volunteers with plastic exposure. First group represents the common plastic exposure of general population and the second one was carried out with healthy moderately trained individuals who have received blood transfusion. This study demonstrates that significantly increased levels of 2-ethylhexanol were found ($p < 0.05$) in urine samples of volunteers in the early hours after receiving blood transfusion.

1. Introduction

Di(2-ethylhexyl) phthalate (DEHP), the most common member of phthalates, is used as plasticizer in polyvinyl chloride (PVC) plastics and may constitute up to the 40% of the finished product. Some examples of its use are films for wrapping food, toys or medical devices such as dialysis tubes, infusion sets and storage bags for blood or parenteral nutrition and continuous ambulatory peritoneal dialysis fluids [1]. DEHP is not chemically bound to a polymeric matrix, so can migrate from the product, reaching the human body [2,3,4]. Some studies indicate that DEHP produces a wide range of toxic effects in animals and in multiple organs including the heart, lungs, kidneys and reproductive tract. It has been identified as a liver carcinogen in rodents, however DEHP has been shown to have no toxic effect in primates [5]. In 2002, the European Commission issued a report specifying that DEHP does not possess any risk to adult humans, although in the case of children it could have toxic effects on reproductive development. Its Established Tolerable Daily Intake (TDI) is 48 μg/kg per day for adults and 20 μg/kg per day for neonates and children [1,6]. Despite the toxic effects, DEHP is the only plasticizer listed in the European Pharmacopoeia that can be part of medical and pharmaceutical products [7,8].

DEHP is rapidly hydrolyzed in the body to mono-(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH), Fig. 1 [7,9,10]. MEHP is mostly metabolized to form oxydized metabolites, mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl)phthalate (MEOHP), mono(2ethyl-5-carboxypentyl)phthalate (MECPP) and mono[2-(carboxymethylhexyl] phthalate (MCMHP). On the other hand, 2-EH is oxidized to 2-ethylhexanoic acid and this by β oxidation to 2-ethyl-3-hydroxyhexanoic acid, 2-ethyl-3-oxohexanoic acid and 4-heptanone.

The three major metabolites (MEHP, MEHHP and MEOHP) have low half-life in the urine. 2-EH and 4-heptanone represent the first and last products of the phase-I metabolism of DEHP. Moreover, unlike the metabolites derived from MEHP, these metabolites do not undergo phase-II metabolism, so that samples do not require a previous enzymatic hydrolysis to the analysis [11,12]. Therefore, in this work a reliable method is presented for the quantitation of 2-EH and 4-heptanone in urine samples. These metabolites can come from different sources.

* Corresponding author.
E-mail address: marialuz.alonso@ehu.eus (M.L. Alonso).

https://doi.org/10.1016/j.microc.2022.107567
Received 6 January 2022; Received in revised form 26 April 2022; Accepted 4 May 2022
Available online 8 May 2022

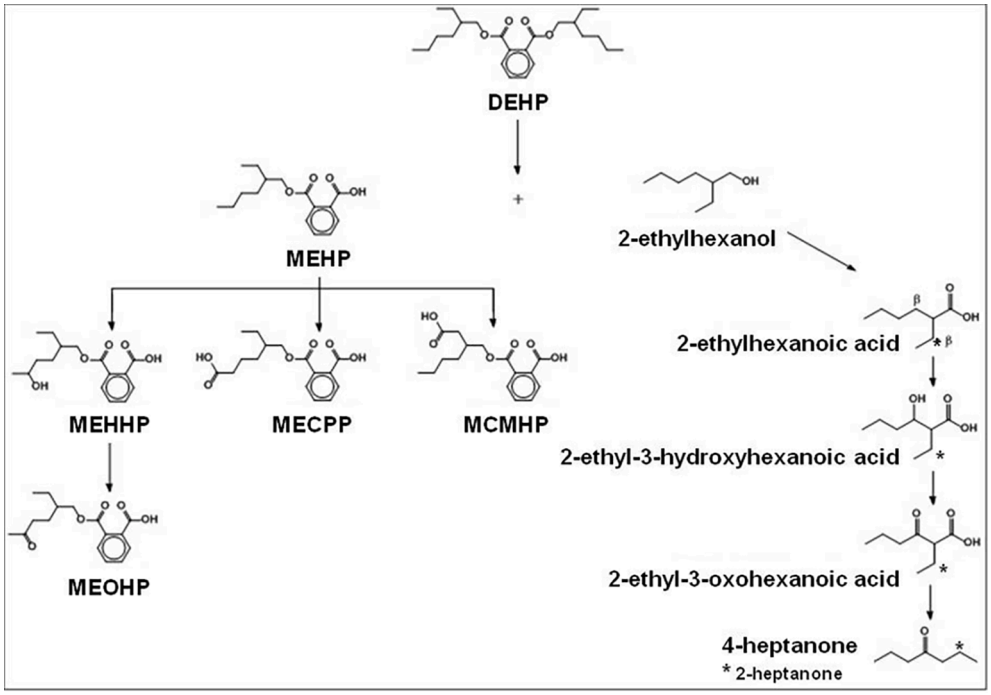

Fig. 1. Oxidation pathway of DEHP metabolites [7,9,10].

Both compounds are large scale industrial chemicals used in several products as solvents, preservatives, colorants, formulating agents or flame retardants, among others. The main uses for 2-EH is in the production of plasticizers, coatings, adhesives and other speciality chemicals. The largest market for 2-EH has been the plasticiser DOP which is used in the manufacture polyvinyl chloride (PVC) products [13].

Taking into account there is research that suggest that DEHP metabolites in urine can be used as plastic exposure markers [14–17], two groups of population will be object of study. One of them consisted of volunteers with a common plastic exposure and the other one which involved healthy moderately trained individuals who have received transfusion of blood using PVC infusion tests and blood storage bags.

The volatility of these target analytes makes the use of headspace solidphase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC/MS) an adequate method for their analytical determination. For 4-heptanone, it is reported in the literature that Wahl et al. used HS-GC/MS method for the analysis of this metabolite in human plasma and urine obtained from healthy volunteers and patients with different diseases [18,19]. 4-heptanone is also considered as endogenous volatile organic metabolites (EVOMs) and potential biomarker of cancer from human urine; and has been determined using octadecyl functionalized core–shell magnetic silica nanoparticle as sorbent and further GC/MS analysis [20]. It has been also included in the analysis of carbonyl volatile compounds as biomarkers in human urine inside a pilot study to discriminate individuals with smoking habits [21] and in a study of differential expression of urinary volatile organic compounds (VOCs) by sex, male reproductive and pairing status [22]. This compound has been also determined in a source separation sanitation system, where it was the most dominant odorants emitted [23]. It was identified in urine collected from children with celiac disease [24] and in domestic cat urine by HS-SPME-GC–MS olfatimetry [25]. Walker et al. analyzed the metabolites of DEHP in urine from individuals with normal and increased plasticizer exposure by HS-SPME-GC/MS [26] and hypothesized that 4-heptanone arises from in vivo β-oxidation of 2-ethylhexanoic acid from plasticizers. For 2-EH a GC/MS method previous derivatization has been reported [12]. This compound has been also determined as possible volatile biomarker of lung cancer by headspace-programmed temperature vaporization-mass spectrometry [27].

Nowadays, the multiple headspace solid-phase microextraction (MHS-SPME) methodology occurs in which there is no external calibration [28–30]. MHS-SPME employs the peak areas of a few HS-SPME consecutive extractions of a same sample to calculate the amount of analyte of a complete extraction. The sum of the areas obtained in each extraction results in a total area value that can be represented by the following equation:

$$A_T = \Sigma\, A_i = A_1 / (1 - \beta) \quad (1)$$

being A_T the theoretical total area of analyte, A_i the peak area of the i^{th} step, A_1 the area of the first extraction and β a constant with values between 0 and 1. The value of β can be obtained from linear regression analysis of the following equation, where (i-1) represent the extractions performed:

$$\ln A_i = \ln A_1 + (i - 1) \cdot \ln \beta \quad (2)$$

Then, A_T is estimated by means of Eq. (1) from the response obtained from A_1 and from the β parameter obtained experimentally. Once obtained the A_T value, the real concentration of the target analyte in the original matrix can be obtained from a simultaneous external calibration graph, constructed apart with standard compounds [30].

Therefore, known the advantages of MHS-SPME methodology, the aim of this work was to optimize and validate a MHS-SPME method for quantitative purposes of 2-EH and 4-heptanone, the volatile metabolites of DEHP in urine samples, in terms of sample stability, selectivity, linearity, recovery, accuracy, precision (repeatability and intermediate precision), limit of detection and limit of quantification. Then, concentrations of metabolites of DEHP were obtained for the two population groups studied: first group represents the common plastic exposure of general population and the second one healthy moderately trained volunteers, who have also received blood transfusion. Variations in urine dilution supported normalization by specific gravity.

2. Materials and methods

2.1. Reagents, standard solutions and materials

4-heptanone (98%), 2-EH (99.6%), DEHP (99%) and synthetic urine (Cerilliant, surine negative urine control) were purchased from Sigma Aldrich (St. Louis, MO, USA), while sodium chloride (NaCl, ≥99,5%), sodium hydroxide (NaOH, 99%), hydrochloric acid (HCl, 37%), monopotassium dihydrogen (KH_2PO_4, 99,5%) and dipotassium monohydrogen phosphate (K_2HPO_4, 99,5%), used for sample conditions optimization, were obtained from Merck (Darmstadt, Germany). Methanol (HPLC gradient grade, 99.8%) was acquired from Teknokroma (Steinheim, Germany).

Methanolic stock solution containing the target compounds was prepared at 25 mg/L and stored at 4 °C in sealed glass vials completely filled to eliminate headspace. Working solutions were prepared at the concentration level of 0.2 mg/L in water. Standard aqueous solutions were prepared using ultrapure reagent grade water purified by a Milli-Q gradient system (Millipore, Bedford, MA, USA). KH_2PO_4/K_2HPO_4 buffer (1 M, pH 6.4) and NaOH (6 M) solutions were prepared using Milli-Q water.

Pooled urine sample spiked with 2-EH at a concentration of 0.02 mg/L, due to the low basal level of this target compound, was used for extraction method optimization. Spiked urine pool was stored at −20 °C.

For HS-SPME, SPME fibers coated with 100 μm polydimethylsiloxane (PDMS), 85 μm polyacrylate (PA), 75 μm carboxen-polydimethylsiloxane (CAR/PDMS) and 65 μm polydimethylsiloxane-divinylbenzene (PDMS/DVB) obtained from Supelco (Bellefonte PA, USA) were used. All SPME fibers were thermally conditioned in accordance with the manufacturer's recommendations.

2.2. Urine samples

Urine samples were collected in order to carry out the two population group studies. In both cases the samples were of healthy volunteers and were approved by the Ethics Committee of the Ruhr-University Bochum (Reg. No. 3200–08). Before the beginning of the study, subjects gave their written informed consent for their enrollment.The first study consisted of a group of people with common exposure to plastics. Samples (n = 30, average age = 25 ± 5 years, range 19–37 years, 15 men and 15 women) were collected from the first morning void and stored at −20 °C. In the second group, six healthy moderately trained volunteers received autologous blood transfusion. Urine samples were collected before and from 1 to 3 days after blood transfusion and were supplied by Hospital del Mar Medical Research Institute (IMIM) [14,15]. Urines stored at −20 °C, corresponding to day −1 (one day before reinfusion), day 0 (reinfusion) and 1, 2 and 3 (3 consecutive days after blood reinfusion) were taken.

After being thawed at room temperature, specific gravity of centrifuged urine samples was measured by means of a refractometer. Concentrations were then corrected for specific gravity. This mathematical correction process, usually carried out in doping control for some analytes, was calculated by applying the following formula [15,31]:

$$\text{Concentration adjusted} = \text{Concentration} \times [(1.020 - 1) / (\text{specific gravity of the sample} - 1)] \quad (3)$$

2.3. Optimization of the MHS-SPME method

MHS-SPME parameters can affect the extraction process, and in order to get the highest recovery values of the analytes the optimization of parameters such as fiber type, fiber desorption time, sample conditions (pH, salt addition), sample agitation, extraction temperature and time was performed.

For the fiber selection, four different fiber coatings were tested: 100 μm PDMS, 85 μm PA, 75 μm CAR/PDMS and 65 μm PDMS/DVB. Once the fiber was selected, the influence of desorption time (1, 2 and 6 min) was studied.

In order to select the sample conditions (pH and addition of salt) which may enhance the release of analytes to the headspace, the influence of the addition of HCl, phosphate buffer, NaOH and NaCl was studied. After that, since the agitation of the sample during the sample preheating time could also increase the release of the volatile compounds, different agitation speeds were tested (0, 500, and 750 rpm).

Finally, different extraction times (1, 5, 70 15, 30, 45 min) at different temperatures (50 and 60 °C) were studied in order to reach distribution equilibrium in the system. For this purpose, each sample was extracted seven times under previously optimized conditions and at the different times and temperatures to establish when the linearity of neperian logarithm of A_i ($\ln A_i$) versus number of extractions (i-1) plot was achieved (Equation 1–2). 10 mL headspace vials with soft silicone rubber seals and aluminum caps were used for every analysis.

2.4. GC/MS method

An Agilent 6890 series gas chromatograph equipped with a PAL COMBI-xt sample injector and a 5973-N mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA) was used. The GC separation column was a 30 m × 0.25 mm × 0.25 μm DB-1MS capillary column. The injector and detector temperatures were set at 260 °C and 300 °C, respectively. Oven temperature was programmed with an initial temperature of 50 °C for 2 min, followed by an increase at a rate of 20 °C/min up to 130 °C, and finally the oven temperature was increased at a rate of 40 °C/min up to 250 °C. The carrier gas was helium (99.999%) at a flow rate of 1.3 mL/min. Data were acquired in selected ion monitoring (SIM) mode using specific ions to monitor each compound (m/z 43, 71, 114 for 4-heptanone and m/z 57, 43, 98 for 2-EH). The injections were performed in splitless mode.

2.5. MHS-SPME-GC/MS method validation

Sample stability, method selectivity, calibration model (linearity), recovery, accuracy, precision (repeatability and intermediate precision), LOD and LOQ were evaluated in method validation [32,33].

The stability of the DEHP metabolites in the prepared samples was tested by using synthetic urine spiked at a concentration level of 20 μg/mL and stored at − 20, 4 °C and room temperature, respectively, up to 3 months. Since samples were frozen and thawed several times through the study, freeze/thaw stability of analytes was evaluated over nine freeze/thaw cycles.

The approach used to establish method selectivity was to verify that there were no interfering peaks at the retention time of the analytes. Several blank samples belonging to different sources (empty vials, SPME fiber blanks, water blank samples and synthetic urine samples) were run to verify the absence of interfering peaks.

Calibration curves of the method were obtained by representing the total area against the standard concentration used before for the linearity study, where the slope obtained in the multiple extractions was used to calculate the total area present in the vial using Eq. (1).

Intra-day and inter-day accuracy and precision (repeatability and intermediate precision) were evaluated analyzing three concentration levels and three replicates of the spiked urine pool over three consecutive days (n = 3 + 3 + 3). Accuracy was expressed as relative error, RE% = (Absolute error / "True" value) * 100%. Precision was expressed as RSD% of the calculated concentrations.

Recovery percentage (REC%) was obtained from the ratio (in percentage) between the analyte concentration obtained and the analyte concentration with which urine pool was spiked. Recovery was determined from the solutions used for accuracy and precision studies.

LOD and LOQ were calculated from the peak area value for the first

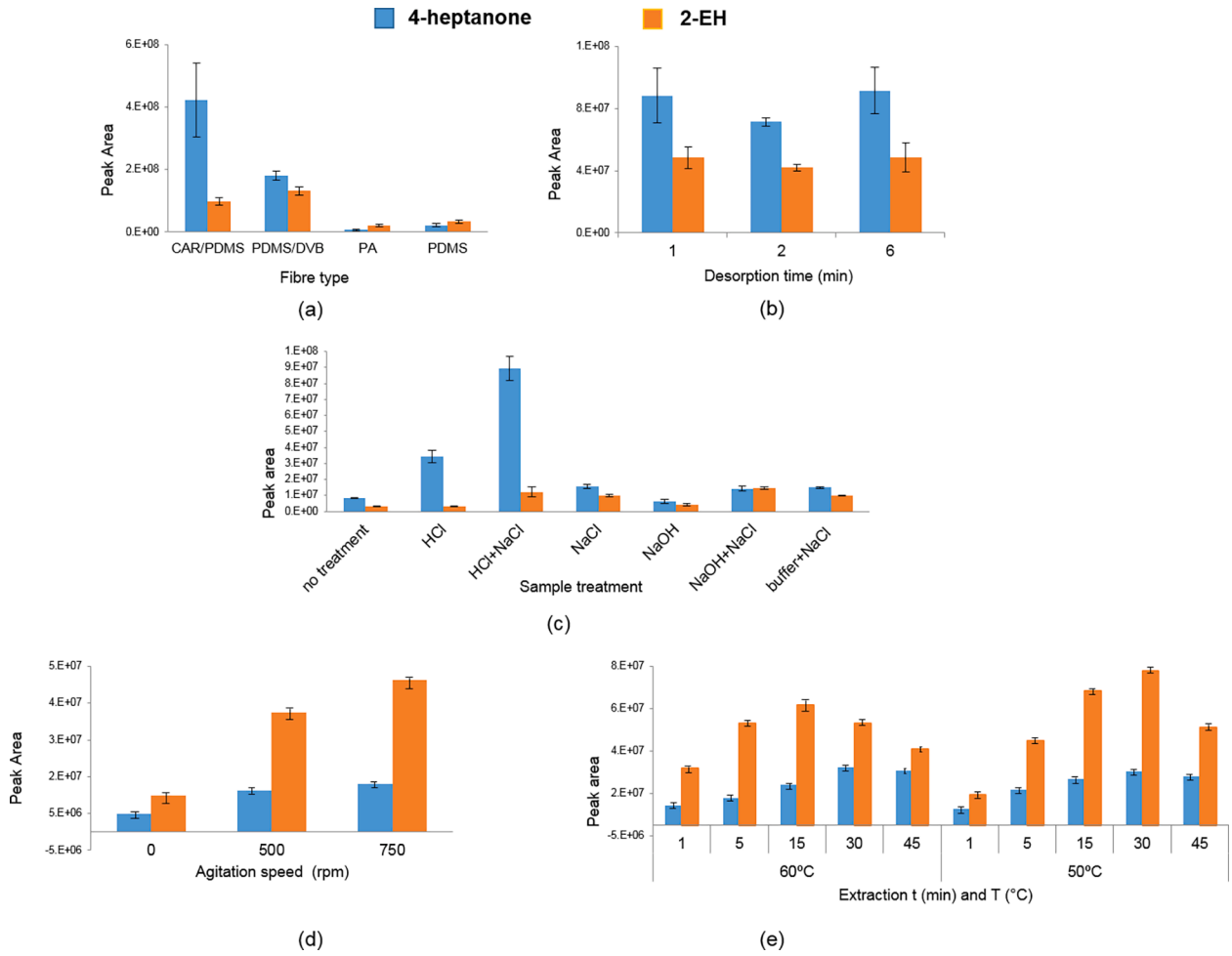

Fig. 2. Peak areas of 4-heptanone and 2-EH extracted with different (a) SPME fibres: 85 μm PA, 65 μm PDMS/DVB, 75 μm CAR/PDMS and 100 μm PDMS; (b) desorption times with PDMS/DVB fiber: 1, 2 and 6 min; (c) conditions of pH and NaCl (no treatment, HCl addition (pH 1–2), HCl and NaCl addition, NaCl addition, NaOH addition (pH 11–12), NaOH and NaCl addition, phosphate buffer (1 M, pH 7.0) and NaCl addition; (d) agitation speed of the vial in the extraction. Conditions of extraction: 1 mL of urine, 0.5 g NaCl and 0.5 mL KH_2PO_4/K_2HPO_4 (1 M); concentration of analytes, 200 μg/L; type of fiber, PDMS/DVB; time of desorption, 2 min; temperature of desorption, 260 °C; (e) extraction times (1, 5, 15, 30 and 45 min) and temperatures (50 and 60 °C).

extraction of a blank plus three and ten times, respectively, the standard deviation of five blank replicates. This parameter was calculated in this way due to the impossibility to obtain an experimental decay from a blank sample. Finally, the chromatographic signal of the blanks was interpolated in the linear calibration to determine the concentrations of both parameters [30].

3. Results and discussion

3.1. Optimization of the MHS-SPME method

Seven factors were selected to optimize MHS-SPME: fiber type, desorption time, agitation speed, extraction temperature, extraction time and other parameters that can affect the extraction process such as salt addition and sample pH control (Fig. 2). Fiber type and desorption time were optimized using a standard solution of target analytes. The rest of the parameters were evaluated in urine matrix since the diffusion of the analytes from the matrix is often very different in comparison with that for the standard.

In MHS-SPME it is essential to extract a significant amount of analyte in order to observe an exponential decay of peak areas versus the number of extractions [28]. Although the fiber that provided the highest chromatographic signal was CAR/PDMS, the obtained chromatograms shown peak tails and lack of resolution. Since high chromatographic signals with good resolution and good repeatability results were obtained using the 65 μm PDMS/DVB fiber, it was used in all remaining experiments (Fig. 2a). Once the most suitable fiber was selected, desorption time of 1, 2 and 6 min were studied [26,34]. Similar chromatographic signals were obtained for the studied desorption times (Fig. 2b). Nevertheless, the repeatability of the extraction was favored at 2 min desorption time.

With regard to the sample conditions, salt concentration and sample pH control can be used to enhance extraction [2,7,26,34]. In some works the use of HCl (pH = 1 – 2) is recommended in order to promote the analyte release, as well as the addition of NaCl [34], while other works reported that the addition of NaOH (pH = 11 – 12) favors the release of ketones into the HS [26]. Also, the use of a buffer is very common when urine samples are analyzed [35,36]. According to this previous data, the influence of the addition of HCl, phosphate buffer, NaOH and NaCl was studied as summarized in Fig. 2c. Extraction temperature and time were set at 60 °C and 10 min [2,7,19]. The results showed a great increase in the release of 4-heptanone after the addition of HCl and the biggest increase for both analytes was seen when NaCl was also added. NaOH and/or NaCl addition also enhanced the analyte extraction, although the signal corresponding to 4-heptanone did not increase at the same level as with the acid. Nevertheless, as reported Walker and co-workers [26], HCl not only favors the release of the analytes, but also could produce the decarboxylation of 2-ethyl-3-oxohexanoic acid to 4-heptanone as

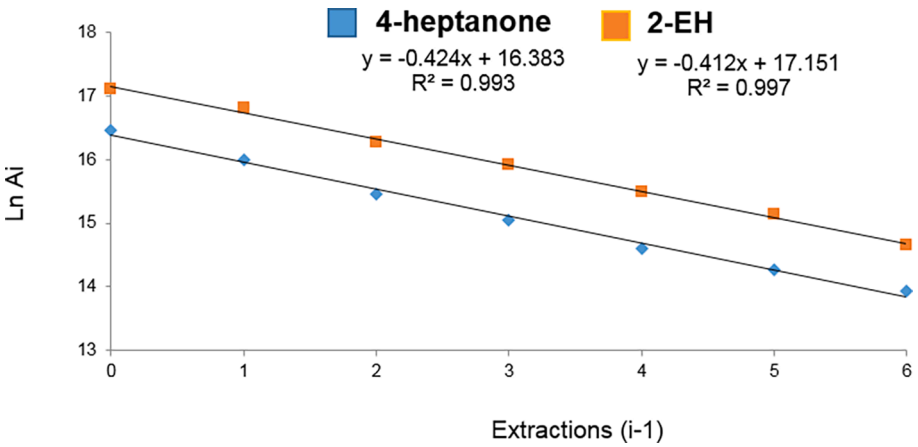

Fig. 3. Extraction plot of 7 consecutive extractions of 4-heptanone and 2-EH. 1 g NaCl and 33 μL NaOH (6 M) were added to 1 mL urine sample and extracted 1 min at 50 °C.

can be seen in Fig. 1. This fact would not allow carrying out a study on 4-heptanone monitoring over time if necessary. Moreover, the addition of HCl introduces higher variability of the results than the rest of sample treatments, which leads to a worse repeatability. The sensitivity obtained without the addition of the acid was acceptable, so for all the reasons mentioned above, the addition of HCl was discarded.

All urine collection and storage containers should be sterile, clean and free of particles or interfering substances. Nevertheless, sometimes PVC bottles are used for urine sample collection where the migration of DEHP to the sample may occur. This way, additional experiments were performed in the event that DEHP could hydrolyze in the presence of an acid or a base when PVC bottles are used. In order to confirm or exclude this possibility, the analysis of water samples spiked with DEHP and after adding HCl or NaOH was carried out. The results suggested the appearance of a peak corresponding to 2-EH when NaOH was added. Since the hydrolysis of DEHP in an alkaline medium was concluded, the addition of NaOH was also discarded.

The control of sample pH is important to obtain reproducible results and, therefore, 0.5 mL of phosphate buffer (1 M, pH 7.0) as well as 1 g of NaCl were added to urine aliquots. The resulting pH was 5.5 ± 0.2. In addition, the sample volume was reduced to 1 mL because of the substantial increment in sensitivity after NaCl addition.

Once sample conditions were set, the effect of sample agitation was evaluated, which could also help improving analyte extraction. Different stirring speeds (0, 500 and 750 rpm) were tested [30]. Stirring at 750 rpm resulted in an increase of the chromatographic signal of 4-heptanone and 2-EH by more than 100% (Fig. 2d).

Finally, the extraction time (1, 5, 15, 30 and 45 min) and temperature (50 and 60 °C) were investigated by means of a four-step MHS-SPME procedure [37], Fig. 2e. The experiments carried out at 50 °C and 1 and 5 min extraction time and also at 60 °C and 1 min provided linear $\ln A_i$ vs. (i-1) plots as well as good chromatographic signals. However, the conditions that showed the best correlation coefficients (0.999 and 0.978 for each analyte) were selected, 50 °C and 5 min.

When MHS-SPME is used for quantitative purposes the examination of the $\ln A_i$ versus (i-1) representation is recommended. This representation should be linear through successive extractions. Additionally, the correlation coefficient as well as the β value should also be examined to ensure the absence of possible undesirable matrix effect or any adsorption effect [37]. In this sense, seven successive extractions were carried out under previously optimized conditions. The results showed lack of linearity for both compounds from the fourth extraction on. One of the causes could be adsorption effects of the various solid compounds present in the sample such solid particulate [29]. Although different strategies were considered (the use of new headspace vials, centrifugation of the samples to avoid suspended solids or avoid NaCl addition), the adsorption effect was not eliminated. Target analytes appeared to be linked in a certain way to sample matrix. Therefore, the addition of NaOH was reconsidered with the aim of eliminating this possible link and therefore the lack of linearity.

Although NaOH hydrolyzes the DEHP that migrates from the container to the sample when PVC bottles are used, the amount of 2-EH produced from this hydrolysis should be comparable for all samples, if they have stored following the same conditions, and thus, the effect would be eliminated.

Since the addition of NaOH can have an influence on the sample equilibration time, the extraction time was readjusted. It was seen that the lack of linearity disappeared after the addition of NaOH and the best results were obtained at 1 min extraction time with correlation values greater than 0.99 (see Fig. 3).

The same experiment was repeated over three different days with three replicates per day in order to confirm the reliability of the results. Once the seventh extraction profile was checked, it was noticed that not significant changes were observed if only four successive extractions were considered for calculation of neither β nor A_T values. Therefore, it was decided to reduce the number of extractions to four, reducing this way analysis time.

Similarly, the optimum extraction time of the standard solutions was established by extracting, in this case, 2 μL of 5 mg/L standard solution at different times (1, 5, 15, 30 y 45 min) at 50 °C. The best results were also obtained at 1 min extraction time.

In summary, 10 mL headspace vials were filled with 1 mL aliquots of urine and after alkalization with NaOH (33 μL, 6 M), 0.5 g NaCl were added. The extraction was performed at 50 °C for 1 min under agitation (750 rpm) using a 65 μm PDMS/DVB SPME fiber and the extracted analytes were desorbed for 2 min in the injector of the GC/MS at 260 °C. The current 4 extraction MHS-SPME-GC/MS method was used to analyze and quantitate the volatile metabolites of DEHP in urine samples.

3.2. *MHS-SPME-GC/MS method validation*

The MHS–SPME method developed was validated in terms of sample stability, method selectivity, calibration model (linearity), recovery, accuracy, precision (repeatability and intermediate precision), LOD and LOQ.

Within this study, the stability of the DEHP metabolites in the prepared samples was tested. No significant decrease of the volatile metabolites concentrations was observed in the samples stored at − 20 and 4 °C. Nevertheless, sample storage at room temperature appeared to affect its stability (data not shown). On the other hand, as observed, the analytes were stable after several freeze/thaw cycles indicating sample

Table 1
Intra-day and inter-day values of repeatability (RSD%) and accuracy (RE%) at each concentration level; and LOD and LOQ values.

	4-heptanone			2-EH		
	Concentration (mg/L)	RSD %	RE %	Concentration (mg/L)	RSD %	RE %
Intra-day	0.0080	7	7.5	0.002	5	4.3
	0.027	6	5.4	0.004	16	11.0
	0.23	12	9.0	0.013	18	13.0
Inter-day	0.018	14	7.2	0.052	22	10.6
LOD (mg/L)	0.00001			0.0005		
LOQ (mg/L)	0.00002			0.001		

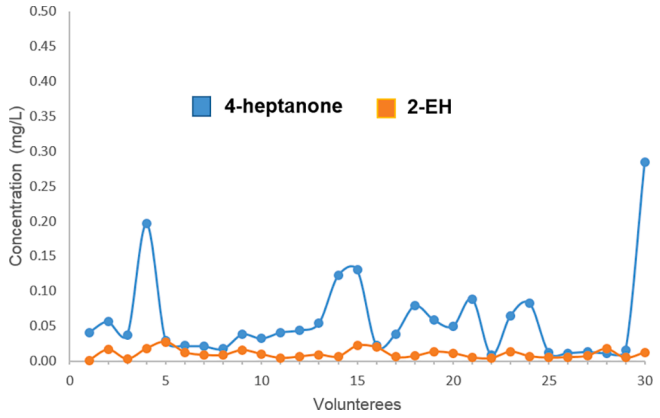

Fig. 4. Concentrations of 4-heptanone and 2-EH in urine obtained from 30 volunteers with daily exposure to plastics.

stability and ensuring accurate quantification.

With regard to selectivity, no significant interfering peaks were detected at the target compounds retention times. As expected, low basal levels of the analytes were always detectable with stable relative ion ratios identical to the reference compound.

The experiments showed linear correlation between A_T and analyte concentration for the concentration range of 0.00002 – 0.23 mg/L and 0.001 – 0.052 mg/L for 4-heptanone and 2-EH, respectively with r^2 values $\geq$ 0985.

Precision and accuracy were evaluated at three concentration levels in terms of RSD% and RE%, respectively. The method presented slightly better RSD% values for 4-heptanone and at low and medium concentration levels. Table 1 shows the intra-day and inter-day repeatability and accuracy from the analysis of a spiked urine pool; and LODs and LOQs obtained for each target compound. REC% values were between 90 and 110% for both compounds.

3.3. Urine sample analysis

Urine samples corresponding to two population groups of healthy volunteers were analyzed by the developed and validated method.

3.3.1. Population group 1

Metabolite concentrations were determined from 30 urine samples of individuals with common exposure to plastics. In Fig. 4 results obtained for 2-EH and 4-pentanone are shown. The results showed mean concentration levels of 0.06 ± 0.01 mg/L for 4-heptanone and 0.01 ± 0.01 mg/L for 2-EH.

3.3.2. Population group 2

Concentrations of 4-heptanone and 2-EH were determined in urine from healthy moderately trained individuals, who have been exposed to a blood transfusion. In Fig. 5, the results of the reinfused volunteers urine can be seen. A statistically significant increase (test t, $p < 0.05$) in the concentration of 2-EH in all urine samples collected the day of the reinfusion (day 0) compared to the samples collected the day before ($p = 0,03$) was observed. Nevertheless, the concentration returned to baseline the following day.

It can be observed that there is no statistically significant increase in the concentration of 4-heptanone in any of the samples with respect to the levels obtained for the samples collected the day before reinfusion (p value range: 0.06–0.49).

These results, as the ones obtained by Monfort et al. [14,15], indicated that the first metabolite of the metabolic process of the DEHP (in this case 2-EH) is which is affected after blood transfusion.

The quantitative results were corrected to the physiological amount of the respective analytes in the used blank matrix and adjusted to a standard urine density of 1.020 g/mL according to WADA guidelines [31].

4. Conclusions

The developed MHS-SPME method coupled to GC/MS is simple, selective, sensitive, precise and allows satisfactory identification and quantification of 4-heptanone and 2-EH metabolites in urine real samples at trace levels. Besides, it is capable of detecting the target metabolites by using small sample volume (1 mL urine). The quantified

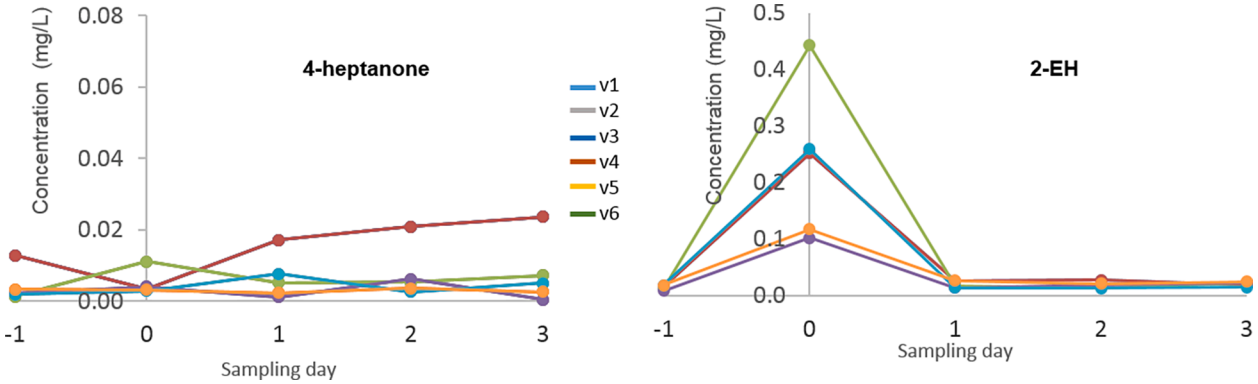

Fig. 5. Concentrations of 4-heptanone and 2-EH in urine obtained from reinfused volunteers (v1-v6). Urines corresponding to day −1 (one day before reinfusion), day 0 (reinfusion) and 1, 2 and 3 (3 consecutive days after blood reinfusion) were taken. Sample conditions: to 1 mL of urine, 0.5 g NaCl and 33 μL NaOH (6 M) were added. Extraction conditions: 1 min extraction time, 50 °C extraction temperature, PDMS/DVB fiber, 750 rpm agitation speed, 2 min time of desorption at 260 °C.

volatile compounds offer the advantage that, unlike the metabolites derived from MEHP, do not undergo phase-II metabolism, so that a previous enzymatic hydrolysis is not required. This fact makes the analytical procedure easier and less time consuming.

Due to MHS-SPME technique could make the analysis independent from the matrix analyzed, as future perspective, the method developed would manage to be applied to the analysis of these compounds in other matrixes as plasma. Moreover, it would be possible to widen the method to the determination of other endogenous volatile organic metabolites (EVOMs).

CRediT authorship contribution statement

M.L. Alonso: Writing – original draft, Investigation, Methodology, Validation. **I. San Román:** Investigation, Methodology, Validation. **L. Bartolomé:** Supervision, Writing – review & editing. **N. Monfort:** Conceptualization, Writing – review & editing. **R.M. Alonso:** Funding acquisition, Project administration, Visualization, Visualization, Resources, Supervision, Writing – review & editing. **R. Ventura:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors thank the SGIker's Analytical Research Service (SCAB), financed by the National Program for the Promotion of Human Resources within the National Plan of Scientific Research, Development and Innovation, "Ministerio de Ciencia, Innovación y competitividad" (MINECO), "Fondo Social Europeo (FSE)" and "Gobierno Vasco/Eusko Jaurlaritza, Dirección de Política Científica", the Bizkaia Council. I. San Román thanks the University of the Basque Country for her postdoctoral grant.

Funding

This work was funded by University of the Basque Country (UPV/EHU) (project GIU19/068). I. San Román was funded by the University of Basque Country (UPV/EHU).

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

[1] ATSDR, agencia para sustancias toxicas y registro de enfermedades). Reseña Toxicológica de Di(2-etilhexil) ftalato (DEHP). Atlanta, GA: US Department of Health and Human Services Public Health Service (2002). www.atsdr.cdc.gov/es.

[2] X. Cao, Determination of phthalates and adipate in bottled water by headspace solid-phase microextraction and gas chromatography/mass spectrometry, J. Chromatogr. A. 1178 (2008) 231–238, https://doi.org/10.1016/j.chroma.2007.11.095.

[3] H.G. Wahl, Q. Hong, D. Stube, M.E. Maier, H.U. Haring, H.M. Liebich, Simultaneous analysis of the di(2-ethylhexyl)phthalate metabolites 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine by gas chromatography-mass spectrometry, J. Chromatogr. B. 758 (2001) 213–219, https://doi.org/10.1016/s0378-4347(01)00183-9.

[4] M.S. dos Santos, D. Budziak, E. Martendal, E. Carasek, Determination of phthalates and adipate in physiological saline solutions by solid-phase microextraction and gas chromatography, Anal. Sci. 25 (2009) 865–868, https://doi.org/10.2116/analsci.25.865.

[5] C.C. Peck, P.W. Albro, Toxic potential of the plasticizer Di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man, Environ. Health Perspect. 45 (1982) 11–17, https://doi.org/10.1289/ehp.824511.

[6] Y. Wu, Z. Song, J.C. Little, M. Zhong, H. Li, Y. Xu, An integrated exposure and pharmacokinetic modeling framework for assessing population-scale risks of phthalates and their substitutes, Environ. Int. 156 (2021), 106748, https://doi.org/10.1016/j.envint.2021.106748.

[7] H.G. Wahl, Q. Hong, D. Stube, M.E. Maier, H.U. Haring, H.M. Liebich, Simultaneous analysis of the di(2-ethylhexyl)phthalate metabolites 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine by gas chromatography-mass spectrometry, J. Chromatogr. B. 758 (2) (2001) 213–219, https://doi.org/10.1016/s0378-4347(01)00183-9.

[8] 2007/47/CE. https://www.boe.es/doue/2007/247/L00021-00055.pdf (accessed 31 December 2021).

[9] H. Frederiksen, N. Skakkebæk, A.M. Andersson, Metabolism of phthalates in humans, Mol. Nutr. Food Res. 51 (2007) 899–911, https://doi.org/10.1002/mnfr.200600243.

[10] H.M. Koch, A.M. Calafat, Human body burdens of chemicals used in plastic manufacture, Philos. Trans. R. Soc. B. 364 (2009) 2063–2078, https://doi.org/10.1098/rstb.2008.0208.

[11] Y.J. Zhang, J.L. Guo, J.C. Xue, C.L. Bai, Y. Guo, Phthalate metabolites: Characterization, toxicities, global distribution, and exposure assessment, Environ. Pollut. 291 (2021), 118106, https://doi.org/10.1016/j.envpol.2021.118106.

[12] H.M. Koch, H.M. Bolt, J. Angerer, Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP, Arch. Toxicol. 78 (2004) 123–130, https://doi.org/10.1007/s00204-003-0522-3.

[13] S. Nalli, O.J. Horn, A.R. Grochowalski, D.G. Cooper, J.A. Nicell, Origin of 2-ethylhexanol as a VOC, Environ. Pollut. 140 (1) (2006) 181–185.

[14] N. Monfort, R. Ventura, A. Latorre, V. Belalcazar, M. López, J. Segura, Urinary di-(2-ethylhexyl)phthalate metabolites in athletes as screening measure for illicit blood doping: a comparison study with patients receiving blood transfusion, Transfusion 50 (2010) 145–149, https://doi.org/10.1111/j.1537-2995.2009.02352.x.

[15] N. Monfort, R. Ventura, P. Platen, T. Hinrichs, K. Brixius, W. Schänzer, M. Thevis, H. Geyer, J. Segura, Plasticizers excreted in urine: indication of autologous blood transfusion in sports, Transfusion 52 (2012) 647–657, https://doi.org/10.1111/j.1537-2995.2011.03331.x.

[16] J.-P. Fong, F.-J. Lee, I.-S. Lu, S.-N. Uang, C.-C. Lee, Estimating the contribution of inhalation exposure to di-2-ethylhexyl phthalate (DEHP) for PVC production workers, using personal air sampling and urinary metabolite monitoring, Int. J. Hyg. Environ. Health 217 (1) (2014) 102–109.

[17] J. Dong, Y. Ma, K. Leng, L. Wei, Y. Wang, C. Su, M. Liu, J. Chen, Associations of urinary di-(2-ethylhexyl) phthalate metabolites with the residential characteristics of pregnant women, Sci. Total Environ. 707 (2020), https://doi.org/10.1016/j.scitotenv.2019.135671.

[18] H.G. Wahl, A. Hoffmann, D. Luft, H.M. Liebich, Analysis of volatile organic compounds in human urine by headspace gas chromatography–mass spectrometry with a multipurpose sampler, J. Chromatogr. A 847 (1999) 117–125, https://doi.org/10.1016/s0021-9673(99)00017-5.

[19] H.G. Wahl, Q. Hong, S. Hildenbrand, T. Risler, D. Luft, H. Liebich, 4-Heptanone is a metabolite of the plasticizer di(2-ethylhexyl) phthalate (DEHP) in haemodialysis patients, Nephrol. Dial. Transplant. 19 (2004) 2576–2583, https://doi.org/10.1093/ndt/gfh425.

[20] Z. Qiao, R. Perestrelo, E.M. Reyes-Gallardo, R. Lucena, S. Cárdenas, J. Rodrigues, J. S. Câmara, Octadecyl functionalized core–shell magnetic silica nanoparticle as a powerful nanocomposite sorbent to extract urinary volatile organic metabolites, J. Chromatogr. A 1393 (2015) 18–25.

[21] I. Calejo, N. Moreira, A.M. Araújo, M. Carvalho, M.L. Bastos, P.G. de Pinho, Optimisation and validation of a HS-SPME–GC–IT/MS method for analysis of carbonyl volatile compounds as biomarkers in human urine: Application in a pilot study to discriminate individuals with smoking habits, Talanta 148 (2016) 486–493, https://doi.org/10.1016/j.talanta.2015.09.070.

[22] M.K. Jones, T.B. Huff, E.W. Freeman, N. Songsasen, B. Senthilkumaran, Differential expression of urinary volatile organic compounds by sex, male reproductive status, and pairing status in the maned wolf (Chrysocyon brachyurus), PLoS ONE 16 (8) (2021) e0256388.

[23] B. Liu, A. Giannis, A. Chen, J. Zhang, V.W.C. Chang, J.-Y. Wang, Determination of urine-derived odorous compounds in a source separation sanitation system, J. Environ. Sci. 52 (2017) 240–249, https://doi.org/10.1016/j.jes.2016.04.013.

[24] N. Drabińska, M. Starowicz, U. Krupa-Koza, Headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry for the determination of volatile organic compounds in urine, J. Anal. Chem. 75 (2020) 792–801, https://doi.org/10.1134/S106193482006008.

[25] C. Banik, J.A. Koziel, J.Z. Li, Simultaneous chemical and sensory analysis of domestic cat urine and feces with headspace solid-phase microextraction and GC-MS-olfactometry, Separations. 8 (2021) 15, https://doi.org/10.3390/separations8020015.

[26] V. Walker, G.A. Mills, Urine 4-heptanone: a β-oxidation product of 2-ethylhexanoic acid from plasticisers, Clin. Chim. Acta 306 (2001) 51–61, https://doi.org/10.1016/s0009-8981(01)00390-4.

[27] A. Pérez, A. García, M. del Nogal, J.L. Pérez, B. Moreno, A.P. Crisolino, Headspace-programmed temperature vaporization-mass spectrometry for the rapid determination of possible volatile biomarkers of lung cancer in urine, Anal.

Bioanal. Chem. 408 (2016) 5239–5246, https://doi.org/10.1007/s00216-016-9618-5.
[28] O. Ezquerro, B. Pons, M.T. Tena, Multiple headspace solid-phase microextraction for the quantitative determination of volatile organic compounds in multilayer packagings, J. Chromatogr. A 999 (2003) 155–164, https://doi.org/10.1016/s0021-9673(02)01524-8.
[29] B. Kolb, L.S. Ettre, Static Headspace-Gas Chromatography: Theory and Practice, second ed., Wiley-VCH, New York, 2006.
[30] I. San Román, M.L. Alonso, L. Bartolomé, R.M. Alonso, R. Fañanás, Analytical strategies based on multiple headspace extraction for the quantitative analysis of aroma components in mushrooms, Talanta 123 (2014) 207–217, https://doi.org/10.1016/j.talanta.2014.01.021.
[31] World Anti-doping Agency (WADA) Montreal: international standards for laboratories. http://www.wada-ama.org/, 2009. (accessed 31 December 2021).
[32] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224, https://doi.org/10.1016/j.forsciint.2006.05.021.
[33] O. González, M.E. Blanco, G. Iriarte, L. Bartolomé, M.I. Maguregui, R.M. Alonso, Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect, J. Chromatogr. A 1353 (2014) 10–27.
[34] C.L. Silva, M. Passos, J.S. Cámara, Solid-phase microextraction, mass spectrometry and metabolomic approaches for detection of potential urinary cancer biomarkers-a powerful strategy for breast cancer diagnosis, Talanta 89 (2012) 360–368, https://doi.org/10.1016/j.talanta.2011.12.041.
[35] Y.u. Ait Bamai, A. Araki, T. Kawai, T. Tsuboi, E. Yoshioka, A. Kanazawa, S. Cong, R. Kishi, Comparisons of urinary phthalate metabolites and daily phthalate intakes among Japanese families, Int. J. Hyg. Environ. Health 218 (5) (2015) 461–470.
[36] E.H.M. Koster, I.S. Niemeijer, G.J. de Jong, Multiple solid-phase microextraction of drugs from human urine, Chromatographia 55 (2002) 69–73, https://doi.org/10.1007/BF02492317.
[37] I. San Roman, L. Bartolome, M.L. Alonso, R.M. Alonso, M. Ezcurra, DATINK pilot study: An effective methodology for ballpoint pen ink dating in questioned documents, Anal. Chim. Acta 892 (2015) 105–114, https://doi.org/10.1016/j.aca.2015.08.038.