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Determination of some phthalate acid esters in artificial saliva by gas chromatography–mass spectrometry after activated carbon enrichment

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ABSTRACT

The determination of six phthalate acid esters was achieved in artificial saliva using gas chromatography–mass spectrometry following activated carbon enrichment of samples. Central composite experimental design was applied to optimize method parameters, such as pH, adsorption time and amount of activated carbon. The best compromise of analytical conditions for the simultaneous determination of analytes from spiked artificial saliva were found to be: pH (3), adsorption time (30 min), activated carbon amount (1.8 g L^{-1}) and elution solvent (chloroform). These conditions were applied to study the migration of phthalate acid esters from different children's toys into saliva. A horizontal agitation method was applied to extract the analytes from plastic toys into saliva for 2 h at 37 °C. The detection limits of the method were in the range of 1.3–5.1 μg L^{-1}, while the relative standard deviation (%) values for the analysis of 100 μg L^{-1} of the analytes were below 3.0% (n = 5). Di-2-ethylhexyl phthalate was the main analyte found in these samples.

1. Introduction

Phthalate acid esters (PAEs), commonly known as phthalates, are low molecular weight, synthetic organic molecules included in polymer resins in order to improve properties such as elasticity, extensibility and processability [1]. PAEs are widely used in a variety of household and consumer products, such as; polyvinyl chloride (PVC), toys, personal care products, paints, industrial plastics, medical devices and pharmaceuticals. Due to both their low molecular weight and physically bonding in polymer, PAEs leach out from materials and pollute the surrounding environment [2]. The migration of PAEs from some products, especially toys and childcare products, has received considerable attention in recent years [3,4] because of their possible roles as carcinogens and endocrine disruptors and roles in peroxisome proliferation, mutagenic activity and infertility [5–9]. The exposure of children to PAEs could occur by ingestion, by dermal contact or both. For these reasons, the PAE contents in plastic toys and food packaging materials must not exceed the permitted concentration level, specific migration limits (SMLs) and tolerable daily intakes (TDIs) according to regulations [10–12]. Taking into account all of these considerations, the development of sensitive and reliable analytical methods is necessary to analyze not only the content of PAEs in these materials but also the efficiency of their migration from plastic toys.

The most common techniques for the analysis of PAEs are gas and liquid chromatography. In general, the amount of PAEs has been determined in a variety of matrices including water, food and other materials, by using different separation and pre-concentration techniques, such as liquid–liquid extraction (LLE) [13–18], solid-phase extraction (SPE) [19–22], solid-phase microextraction (SPME) [23–25], headspace [22,25] and thermal desorption [26,27]. However, only a few methods are available to study the migration of these compounds from toys into saliva. Importantly, there is also a possibility that the PAEs may be hydrolyzed by saliva and within the oral mucosa [28,29]. The Dutch Consensus group has developed a dynamic agitation method, commonly known as "Head over Heels", for the determination of diisononyl phthalate (DINP), which can be released from PVC toys [3]. This method was validated by Rijk and Ehlert [4] and by Simoneau et al. [30,31]. Bouma et al. also applied this agitation method to determine migration of di-2-ethylhexyl phthalate (DEHP) and DINP into saliva using high performance liquid chromatography (HPLC) [32]. Other approaches have been based on linear horizontal agitation (stringent and mild conditions) [33], ultrasonic extraction [34] and simulated chewing activity. The Instron Universal Testing Machine has also been used to determine migration of DINP from PVC products into artificial saliva [35]. All of these agitation methods are based on in vitro extraction procedures. Following agitation, liquid–liquid extraction of PAEs with

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organic solvents, such as n-hexane, dichloromethane, isooctane, etc., was performed; however, these compounds often cause some "blank" problems when samples are analyzed at low concentrations [36].

Exposure of the human foetus and infants to PAEs via maternal exposure is a matter of concern. The metabolic pathways of phthalate metabolites excreted in human urine are partly known for some PAEs, but our knowledge about metabolic distribution in the body and other biological fluids, including breast milk, is limited [37]. With the development of a multiple quantitative method using methylated with diazomethane and SPE followed by gas chromatography–mass spectrometry (GC–MS), it became possible to detect monoester PAEs in human urine in the low ng mL^{-1} range [38,39]. The method has since been modified several times. Currently several selective, sensitive and fast methods are advisable for quantitative analyses of a wide range of di-, monoester phthalates and secondary metabolites in several different fluids and tissues, such as of urine, serum, blood, milk and saliva [40–42].

The removal of toxic organic compounds from urban and industrial wastewater via adsorption onto activated carbon is a well-known and widely applied process. Due to the low solubility and highly hydrophobic character of PAEs, activated carbon would be an effective and reliable adsorbent for their removal from the aqueous phase. Activated carbon has been primarily used for the enrichment of elements from different matrices after the addition of complexing agents [43,44], but activated carbon has not been used for the removal of PAEs from saliva. Only a few studies have reported the use of an adsorption process for phthalate removal from water by activated carbon in the form of granules, powder or fiber [45–47].

The aim of our study was to develop an analytical procedure that is useful for the study of phthalate migration from plastic toys into saliva using GC–MS following enrichment of the analyte with activated carbon. Central composite experimental design was carried out to optimize the extraction parameters, including pH, adsorption time and the amount of adsorbent, and the optimized method was applied to study the migration of PAEs from different children's toys into saliva.

2. Materials and methods

2.1. Apparatus

Analysis was carried out using an Agilent 7890A GC gas chromatography-5975 C inert XL MSD model mass spectrometer (GC–MS) equipped with a 7683 B series auto sampler injection system (Agilent Technologies, Palo Alto, CA). An HP-5 ms fused-silica capillary column 30 m × 0.25 i.d., with 0.25 mm film thickness was employed. The GC oven temperature was held at 100 °C for 2 min, then increased to 280 °C at a heating rate of 7 °C min^{-1} and the temperature was held for 5 min. The detector temperature was 230 °C with a carrier gas (helium) at a flow rate of 1 mL min^{-1}. The injector temperature was set at 280 °C. Positive electron ionization (EI) mode at 70 eV was used with the scanning rate of 3.94 scans/s over the mass range of 40–400 amu. The MSD transfer line temperature was set at 280 °C. Selective ion monitoring acquisition mode was used for measurement collection. Retention times and the identification and quantification of ions selected for the target compounds are shown in Table 1. The total ion chromatogram of 0.1 mg L^{-1} standards is given in Fig. 1.

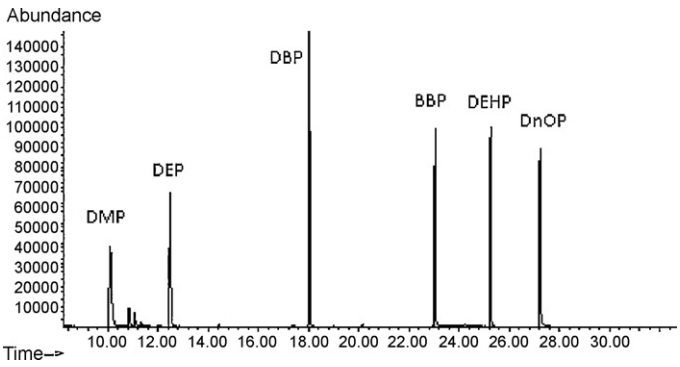

Fig. 1. GC–MS total ion chromatogram of 0.1 mg L^{-1} standards.

The residual total PAEs concentration in the saliva phase was analyzed spectrophotometrically using a Shımadzu 1700 UV–Vis spectrophotometer (Japan) at a wavelength of 230 nm.

A Mettler Toledo pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland) was used for pH adjustment of saliva samples. Stirring was performed using Variomag Poly multipoint 15 magnetic stirrers (Thermo Scientific, Langenselbold, Germany). In vitro saliva agitation was achieved by an orbital shaker equipped with an incubator system (Heidolph Instruments, GmbH &Co.KG, Schwabach, Germany). Ultrasonic extraction was performed using an Elma Ultrasonic LC 30 H model ultrasonic bath (Germany).

2.2. Reagents

All solvents used were of analytical reagent grade. The studied compounds were dimethyl-(DMP), diethyl-(DEP), dibutyl-(DBP), benzyl butyl-(BBP), di-2-ethylhexyl-(DEHP) and di-n-octyl (DnOP) phthalate esters. A multi-compound standard (Dr. Ehrenstorfer GmbH, phthalate ester mix 1, 2000 ng μL^{-1} in methanol, Ausburg, Germany) was used for GC–MS calibration. Artificial saliva was prepared by dissolving 0.17 g of $MgCl_2{\cdot}6H_2O$, 0.15 g of $CaCl_2{\cdot}6H_2O$, 0.76 g of $K_2HPO_4{\cdot}2H_2O$, 0.53 g of K_2CO_3, 0.33 g of NaCl and 0.75 g of KCl(analytical-reagent grade, Merck) in 1 L of water and the solution pH was adjusted to 6.8 ± 0.1 with 1% HCl (DIN V 53160-1). Activated carbon (Sigma C-5385, Sigma Chemical Co, St. Louis, USA) for adsorption experiments was in powder form. Millipore glass fiber prefilters (APFF02500) were used for the activated carbon filtration step (Millipore, County Cork, Ireland).

2.3. Determination of PAEs in toys

The toys were cut into pieces of that weighed about 0.01 g with scissors. A mass of 20 mg of each sample was weighed into a glass vial. A volume of 10 mL of organic solvent (chloroform) was then added to each sample. The vials were closed, and the samples were extracted in an ultrasonic bath for 30 min. Next the supernatant

Table 1
Retention times and selected ions for the analysis of the target compounds.

Name	Abbreviation	Retention time (t_R)	m/z
Dimethyl phthalate	DMP	10.081	77, 104, 135, 149, 163, 194
Diethyl phthalate	DEP	12.455	76, 105, 149, 177, 194, 222
Di-n-butyl phthalate	DBP	18.030	104, 149, 205, 223, 278
Benzyl butyl phthalate	BBP	23.040	91, 104, 132, 149, 206, 312
Di(2-ethyl-hexyl) phthalate	DEHP	25.251	113, 149, 167, 279
Di-*n*-octyl phthalate	DnOP	27.223	149, 261, 279, 390

Table 2
Experimental conditions obtained with the 2^3 central composite design.

	Factors	Levels			Star points ($\alpha = 1682$)
	Low (−1)	Central (0)	High (+1)	+α	−α
X_1 (pH)	2	4	6	0.7	7.4
X_2 (g L^{-1} AC)	0.5	1.0	1.5	0.015	1.85
X_3 (time/min)	30	60	90	10	110
Experiment	X_1	X_2	X_3		
1	−1	−1	−1		
2	1	−1	−1		
3	−1	1	−1		
4	1	1	−1		
5	−1	−1	1		
6	1	−1	1		
7	−1	1	1		
8	1	1	1		
9	0	0	0		
10	−1.682	0	0		
11	1.682	0	0		
12	0	−1.682	0		
13	0	1.682	0		
14	0	0	−1.682		
15	0	0	1.682		
16	0	0	0		
17	0	0	0		
18	0	0	0		
19	0	0	0		
20	0	0	0		

was diluted to obtain a calibration standard range, and 1 μL of the diluted supernatant was injected into the GC–MS system.

2.4. Adsorption of PAEs on activated carbon

The GC–MS experiments were carried out in batch mode. A volume of 50 μL of the phthalate ester mix standard was spiked into 50 mL of the saliva solution to obtain an initial concentration of 2 mg L^{-1} and the pH was adjusted by adding NaOH or HCl. The weighed portions (0.008–0.0925 g) of activated carbon were then immersed into 50 mL of the solutions. These samples were maintained under magnetic stirring between 30 and 110 min at 250 rpm and ambient temperature. The suspensions were filtered through a 25 mm diameter glass fiber prefilter using a vacuum pump, and the filters were dried in an oven at 70 °C for 15 min. Then the filters were sonicated with chloroform using an ultrasonic bath for 15 min, and the final solutions were injected into the GC–MS.

2.5. Migration studies of PAEs from toys into saliva

The toys containing DEHP as plasticizers were punched over a 3 cm diameter; the total surface area was approximately 14.2 cm^2. The samples were horizontally rotated at 200 rpm (rotation stroke length of 10 mm) in 100 mL glass Erlenmeyer flasks containing 25 mL of artificial saliva on a rotary shaker in an incubator at 37 °C for 60 min. After 60 min, the simulated saliva solution was replenished, and a second 25 mL portion of saliva was added; the two portions of saliva were combined. The adsorption procedure on activated carbon was then carried out under the optimum conditions.

3. Results and discussion

3.1. Activated carbon adsorption of PAEs

The effectiveness of PAE adsorption onto activated carbon from artificial saliva depended on the pH of a sample, the amount of adsorbent and the adsorption time. To determine the optimal conditions for this process, a central composite design (CCD) of five-level-three-factor was performed. The value of 1.682 was used to establish the rotatability condition. Twenty experiments were required in this design using five central points, and the experiments were performed randomly. The conditions set in each experiment are presented in Table 2. In all of the experiments, 50 μL of the standard PAE mix solution was spiked into 50 mL of artificial saliva, and the CCD was performed. Recovery values, which are expressed as *R*%, were used as the response for each PAE.

The model coefficients were calculated by backward multiple regression, and they were validated by the Analysis of Variance (ANOVA). The lack of fit (LOF) values were not significant ($p > 0.05$) for each PAE. The large adjusted R^2 values indicate a good relationship between the experimental data and the fitted model (Table 3).

The optimal experimental conditions obtained from the experimental data and the fitted model are shown in Table 4. PAE adsorption levels were found to be dependent on the saliva pH, and the uptake was observed to be greater in acidic media. The adsorption phenomena of the PAEs can be explained on the basis of hydrophobic and dispersion effects [45]. In acidic media, the protonation of surface functional groups could form hydrogen bonds with the oxygen atom of the ester groups, or adsorption can occur due to a strong specific interaction between the

Table 3
Semi-empirical expressions of recovery (*R* %) and model correlation coefficiencies (R^2).

Compounds	A semi-empirical equation	R^2	Adjusted R^2
DMP	$R\% = 68.5 + 2.79x_2 + 4.24x_1^2 + 8.26x_3^2$	0.879569	0.771182
DEP	$R\% = 67.8 + 3.50x_2 + 3.81x_1^2 + 8.23x_3^2 - 3.63x_2x_3$	0.883734	0.779095
DBP	$R\% = 65.9 + 3.33x_2 + 3.56x_1^2 + 2.17x_2^2 + 7.56x_3^2 - 3.01x_2x_3$	0.901795	0.81341
BBP	$R\% = 66.3 - 1.10x_2 + 3.42x_1^2 + 7.82x_3^2 - 3.76x_2x_3$	0.839298	0.694666
DEHP	$R\% = 64.9 + 4.19x_2 + 2.89x_1^2 + 1.95x_2^2 + 6.70x_3^2 - 3.68x_2x_3$	0.913919	0.836445
DnOP	$R\% = 65.5 + 3.67x_2 + 2.86x_1^2 + 6.73x_3^2 - 3.63x_2x_3$	0.907388	0.824038

Table 4
Optimum conditions for enrichment of each PAE onto activated carbon.

	DMP	DEP	DBP	BBP	DEHP	DnOP
pH	0.64	3.1	3.6	3.2	2.8	2.3
AC amount ($g\,L^{-1}$)	1.8	1.8	1.8	0.2	1.8	1.8
Time (min)	92	25	18	104	22	23
Log $K_{0/W}$ [48]	1.60	2.42	4.50	4.73	7.27	8.18

aromatic ring and the carbon surface. The decrease of adsorption observed at basic pH might be attributed to the increase in concentration of hydroxyl ions, leading to the formation of aqua-complexes and retarding adsorption [46]. Also in basic range (pH 9–11) saliva component precipitate as magnesium phosphate and it was observed that recovery values were decreased. After sorption process, the residual total PAEs concentration in the artificial saliva phase was also analyzed by UV–vis at 230 nm for different pHs. Recovery values are shown in Fig. 2. It was seen in Fig. 2, the optimum sorption pH was 3.0. Optimum adsorption time could also depend on competitive hydrophobic interactions, solubility, molecular weight and the substituent group of the PAE. Octanol/water partition coefficients ($K_{o/w}$) of PAEs which is the physicochemical parameter most commonly used to quantitatively characterize the hydrophobic nature of organic compounds are given in Table 4 [48]. Water solubility of PAEs decreases in the order: DMP ≥ DEP > DBP > BBP > DEHP ≥ DnOP; thus, the longer adsorption time for DMP compared with other compounds is probably due to the higher water solubility of DMP, and its adsorption time increased due to competitive hydrophobic interactions. The long chain hydrophobic PAEs have been suggested to strongly compete with the shorter chain of DMP for binding sites on the activated carbon [49]. Additionally, BBP adsorption time was longer than that of other PAEs. This observation can be explained by the presence of non-symmetric substituent groups (benzyl and allyl) in this compound, which give rise to some steric hindrance. The percentages of sorption of DMP, DEP, DBP, BBP, DEHP and DnOP were found 85%, 100%, 96%, 67%, 98% and 95% respectively in optimum conditions.

3.2. Selection of organic solvent for elution

The selection of an appropriate eluent is also of great importance for the efficiency of the activated carbon enrichment procedure. The candidate solvents should exhibit good sensitivity for chromatographic detection and have high affinity for the target compounds. Several elution solvents, including acetonitrile, acetone, methanol, chloroform, diethyl ether, hexane and a hexane/acetone mixture (1:1) were investigated. More highly polar solvents provided better recovery of all of the PAEs because these compounds have polar as well as nonpolar elution properties. As expected, nonpolar elu-

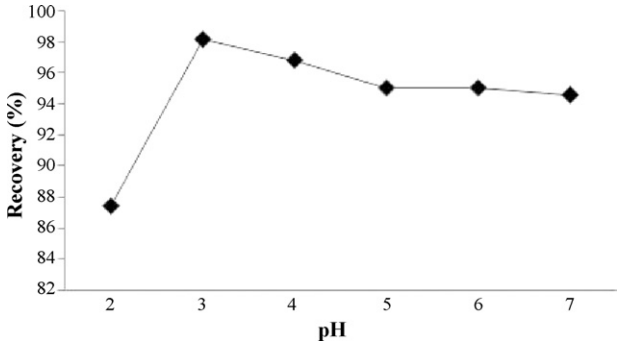

Fig. 2. The effect of pH on the recovery of total PAEs. The sorption recovery of total PAEs onto activated carbon at different pH. Conditions: saliva volume, 50.0 mL; amount of activated carbon, 1.8 $g\,L^{-1}$; spiked standard volume, 50.0 μL; stirring rate, 200 rpm; extraction time, 30 min.

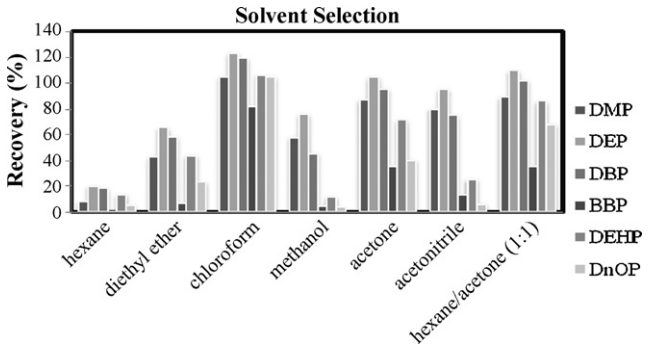

Fig. 3. The recovery of PAEs following enrichment onto activated carbon using different eluents. Conditions: saliva volume, 50.0 mL; pH, 3; amount of activated carbon, 1.8 $g\,L^{-1}$; spiked standard volume, 50.0 μL; stirring rate, 200 rpm; extraction time, 30 min; elution solvent volume, 5.0 mL.

ents, such as hexane and diethyl ether, did not show high affinity for the mentioned compounds. In general, elution of short-chain, hydrophobic PAEs (DMP, DEP, DBP) onto activated carbon was more efficient than elution of long-chain, hydrophobic PAEs (BBP, DEHP, DnOP). Among the tested solvents, chloroform demonstrated the best elution efficiency (Fig. 3) Elution solvent experiments showed that elution by chloroform can provide an adequate measure of irreversible adsorption.

3.3. Analytical figures of merit

Quality factors, including the limit of detection (LOD), linear range, linearity (R^2) and repeatability, were investigated to evaluate the analytical performance of the proposed method under the optimal conditions. The obtained values for the described quality factors are presented in Table 5. Linearities of the calibration curves were observed in the range of 0.1–1 $mg\,L^{-1}$ with correlation coefficients (R^2) ranging from 0.9982 to 0.9998. Inter-day precision tests ($n = 5$) were carried out by extracting a spiked saliva sample at 100 $\mu g\,L^{-1}$. The relative standard deviations (RSDs %) were below 3.0%, which indicated good method precision. The limits of detection (LODs) for each compound, based on a signal-to-noise ratio (S/N) ≥ 3, ranged from: 1.3 $\mu g\,L^{-1}$ for DMP, 2.1 $\mu g\,L^{-1}$ for DEP, 3.9 $\mu g\,L^{-1}$ for DBP, 5.1 $\mu g\,L^{-1}$ for BBP, 1.5 $\mu g\,L^{-1}$ for DEHP and 3.8 $\mu g\,L^{-1}$ for DnOP. This LOD value of DEHP is lower than the 25 $\mu g\,L^{-1}$ value obtained working with ultrasonic and solid phase extraction using Florisil cartridge and GC determination [21]. Kayali et al. [24] was found similar LOD value of DEHP to proposed method. A higher LOD and RSD% values were found by Kolon et al. [39] who was applied to the SPME followed by isotope dilution – fast GC–MS for the determination of PAEs in human serum. A similar study was shown by Freitas et al. [50] who was aimed to the development of a methodology based on activated carbon solid phase extraction (SPE) to characterize phthalates from landfill leachate samples and the LOD of DBP was determined as 30 $\mu g\,L^{-1}$.

Table 5
Analytical performance of the proposed method.

Name	Linear range ($mg\,L^{-1}$)	R^2	LOD[a] ($\mu g\,L^{-1}$)	RSD, %[b] ($n = 5$)
DMP	0.10–1.0	0.9998	1.3	2.3
DEP	0.10–1.0	0.9998	2.1	1.5
DBP	0.10–1.0	0.9995	3.9	0.9
BBP	0.10–1.0	0.9982	5.1	2.4
DEHP	0.10–1.0	0.9997	1.5	1.9
DnOP	0.10–1.0	0.9998	3.8	3.0

[a] Limit of detection for $S/N = 3$.
[b] Relative standard deviation at the concentration of 100 $\mu g\,L^{-1}$ of each PAE.

Table 6
Recovery of phthalates from plastic toys using different solvents.

	DMP	DEP	DBP	BBP	DEHP	DnOP
Methanol	87.7 ± 0.8	91.5 ± 1.3	85.7 ± 0.8	77.3 ± 2.0	86.7 ± 0.8	81.0 ± 1.8
Ethanol	84.7 ± 1.4	98.8 ± 5.7	87.8 ± 0.8	69.5 ± 5.6	86.5 ± 1.3	81.2 ± 2.0
Acetone	86.3 ± 1.0	94.5 ± 3.3	87.7 ± 1.8	80.8 ± 5.5	96.0 ± 2.2	85.7 ± 2.9
Acetonitrile	83.3 ± 0.6	90.5 ± 0.5	85.8 ± 0.8	83.7 ± 1.3	88.2 ± 0.8	87.2 ± 1.0
Diethyl ether	88.3 ± 0.6	96.7 ± 1.3	91.3 ± 0.3	83.5 ± 1.5	100.2 ± 0.3	87.7 ± 0.8
Chloroform	94.7 ± 1.5	103.2 ± 1.6	94. 8 ± 1.8	84.7 ± 6.7	98.3 ± 4.3	87.5 ± 4.3

Table 7
Migration of DEHP into artificial saliva and DEHP content of toys ($n = 3$).

Sample	Sample mass (g)	DEHP released (μg)	DEHP released rate (μg/10 cm^2/h)	[DEHP] in toys (% w w^{-1})
Toy 1	1.17 ± 0.06	5.02 ± 0.82	1.78 ± 0.29	27.8 ± 1.2
Toy 2	1.37 ± 0.25	5.17 ± 0.38	1.83 ± 0.13	33.9 ± 1.4
Toy 3	1.00 ± 0.13	n.d	n.d.	0.051 ± 0.004
Toy 4	1.47 ± 0.25	73.39 ± 5.30	25.97 ± 1.90	37.9 ± 2.0

Determination of PAEs in children's toys

The Standard Reference solution at the concentration of 2.0 mg L^{-1} of each PAE was added to the toy samples before solid–liquid extraction; thus, recovery studies were conducted for the selection of an appropriate extraction media. Methanol, ethanol, acetone and acetonitrile as well as diethyl ether and chloroform were investigated for this purpose. The obtained recoveries and standard deviations are shown in Table 6. Among the tested extraction solvents, chloroform demonstrated the best efficiency and was consequently selected. DEHP was found in three toy samples as the predominant plasticizer in the range of 27.8–37.9% (w/w). In just one sample, low amounts of DEHP (0.051%) were encountered.

3.4. Determination of PAE migration into saliva from toys

A horizontal agitation method was utilized to extract PAEs into the simulated saliva for 2 h at 37 °C. The optimized method was applied to the analysis of different kinds of toys, and DEHP was the sole analyte detected in these samples; the results are presented in Table 7. The migration and standard deviations were based on the migration results from three test specimens punched out of the toy sample. In the present study, it was observed that all of the toys had a DEHP release lower than the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) [11] guidance release value of 0.3 mg per 10 cm^2 of article mouthed over a 3 h time period. However, a trend is visible that the migration increases with increasing DEHP content. The DEHP release depends not only on the plasticizer content in the plastic but also on other factors such as surface roughness, coating type and thickness [32]. In addition, previous reports in the literature demonstrate that the concentration and rate of PAE release are dependent on conditions of migration using different agitation methods [33].

4. Conclusion

The activated carbon enrichment method has been shown to be a useful approach in the determination of the migration of regulated phthalates into saliva, which may then be tested to discern whether the concentration of phthalate exceeds the regulated limits. In comparison with solid and liquid microextraction methods [14,18,19,25] in water, activated carbon provided higher LOD. However, the enrichment factor of proposed method might be improved by choosing proper sample volumes. The low RSD values were probably because of the quantitative extraction. The described method can also applied to routine analyses for the determination of the target analytes in natural water and/or soft drink samples.

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