Journal of Chromatography B, 908 (2012) 113–121

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Determination of five di-(2-ethylhexyl)phthalate metabolites in urine by UPLC–MS/MS, markers of blood transfusion misuse in sports

Núria Monfort[a,b], Rosa Ventura[a,b], Georgina Balcells[a], Jordi Segura[a,b,*]

[a] Grup de Recerca en Bioanàlisi i Serveis Analítics, Programa de Recerca en Neurociències, Institut Hospital del Mar d'Investigacions Mèdiques, Barcelona, Spain
[b] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, UPF, Barcelona, Spain

ARTICLE INFO

Article history:
Received 19 July 2012
Accepted 16 September 2012
Available online 21 September 2012

Key words:
Blood doping
Transfusion
Di-(2-ethylhexyl)phthalate (DEHP)
Plasticizers
Ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS)
Doping control

ABSTRACT

Di-(2-ethylhexyl)phthalate (DEHP) is the most commonly used plasticizer for polyvinyl chloride, which is found in a large variety of products, including most of the bags used for blood storage because of its protective role on erythrocytes survival. DEHP metabolites have been recently proposed as markers of the misuse of blood transfusion in athletes. In this study, a method to quantify the main five DEHP metabolites in urine has been developed: mono-(2-ethylhexyl)phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl)phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP), and mono-(2-carboxymethylhexyl)phthalate (2cx-MMHP). The method involved an enzymatic hydrolysis with β-glucuronidase from *Escherichia coli* followed by an acidic extraction with ethyl acetate. The hydrolysed extracts were analysed by ultraperformance liquid chromatography tandem mass spectrometry. Isotope labelled MEHP, MEOHP and 5cx-MEPP were used as internal standards. Analysis of all the metabolites was achieved in a total run time of 10 min, using a C_{18} column and a mobile phase containing deionized water and acetonitrile with formic acid, with gradient elution at a flow-rate of 0.6 mL min^{-1}. Detection of the compounds was performed by multiple reaction monitoring, using electrospray ionization in positive and negative ion modes. The method was validated for quantitative purposes. Extraction recoveries were greater than 90% and the limits of quantitation ranged from 1.2 to 2.6 ng mL^{-1}. Intra-day precisions were better than 8% for all metabolites while inter-assay precisions were better than 12%. Concentrations of DEHP metabolites were measured in a control group (n = 30, subjects reflecting the common environmental DEHP exposure), and in sportsmen (n = 464), to evaluate population distribution exposure to DEHP. Additionally, threshold concentrations indicating outliers of common exposure for DEHP metabolites are proposed.

1. Introduction

Di-(2-ethylhexyl)phthalate (DEHP) is one of the most widely used plasticizers in polyvinyl chloride products to increase flexibility. Thus, DEHP is used in numerous household products, floor tiles, furniture upholstery, and medical devices, among other products [1,2]. Especially relevant is its presence in bags for blood storage based on its properties to maintain erythrocytes stability [3,4]. Plasticizers are not chemically bound to the polymer and they leach, migrate or gas out into the atmosphere, into foodstuff or directly into the body fluids, exposing the environment and people. General population is ubiquitously exposed to DEHP [5–7]. Exposure to high concentrations of DEHP may produce a wide range of adverse effects. DEHP is a known reproductive and developmental toxicant and the possible effects of phthalates on the reproductive systems in children are well documented [8,9]. Furthermore, DEHP is also a suspected human endocrine disruptor/modulator [10]. However, direct links between the effects observed in animals and a decline in human reproductive health have not yet been established [11].

The metabolic pattern of DEHP is complex and several metabolites have been identified and characterized. Metabolism of DEHP involves very rapid hydrolysis to mono-(2-ethylhexyl)phthalate (MEHP) catalysed by unspecific lipases and formation of a glucuronide conjugate. Additionally, MEHP can undergo oxidations in the side chain to form several metabolites [12,13]. Nowadays, more than 15 metabolites of DEHP have been identified in human urine [14]. Oxidized metabolites mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl)phthalate (MEOHP) along with two other recently identified, mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP) and mono-(2-carboxymethylhexyl)phthalate (2cx-MMHP), are the major urinary DEHP metabolites in adult humans, excreted as conjugates with glucuronic acid [15,16]. A simplified metabolic pathway of DEHP is illustrated in Fig. 1.

* Corresponding author at: Grup de Recerca en Bioanàlisi i Serveis Analítics, Institut Hospital del Mar d'Investigacions Mèdiques, Dr. Aiguader, 88, 08003 Barcelona, Spain. Tel.: +34 93 3160470; fax: +34 93 3160499.
E-mail address: jsegura@imim.es (J. Segura).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.09.030

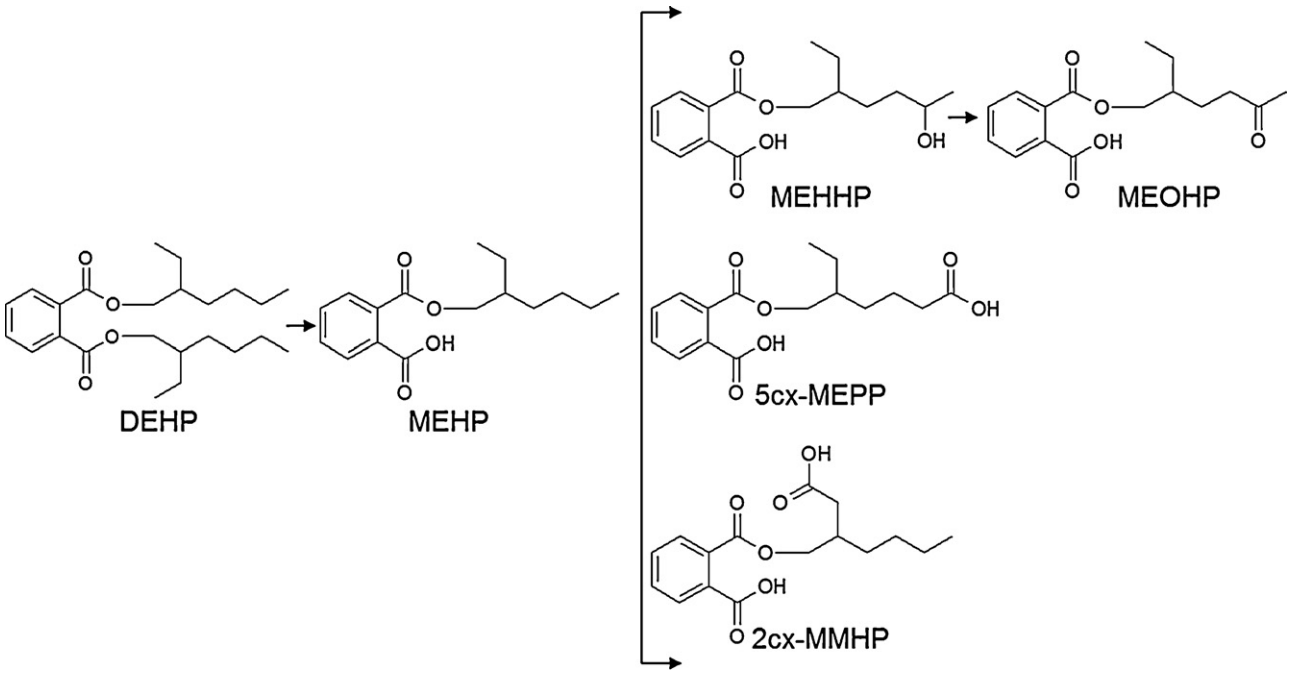

Fig. 1. Simplified metabolic pathway of DEHP: MEHP, mono(2-ethylhexyl)phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl)phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl)phthalate; 5cx-MEPP, mono-(2-ethyl-5-carboxypentyl)phthalate; 2cx-MMHP, mono-(2-carboxymethylhexyl)phthalate.

Secondary metabolites as well as the primary metabolite MEHP have been used as markers to predict exposure to DEHP [17–19]. It is known that adults and neonates who undergo a variety of serious medical procedures involving plastic materials experience much higher exposures to DEHP [20]. Workers from industries where materials containing DEHP are used have also high concentrations of DEHP metabolites in urine [21]. This has led to the development of analytical methods to detect DEHP metabolites in recent years addressed to the evaluation of exposure [22–26].

One of the processes leading to high and acute exposure to DEHP is blood transfusion. The use of blood transfusion is prohibited in sports by the World Anti-Doping Agency. Homologous blood transfusion (donor and receptor are different subjects) can be detected by the analysis of blood samples by flow cytometry through the identification of double populations of erythrocytes having different specific surface antigens [27,28]. However, autologous transfusion (donor and receptors are the same subject) invalidates the possibility of using the same analytical approach because all red blood cells bear the same surface antigens. Instead, DEHP is released from blood bags [29] and urinary concentrations of DEHP metabolites have demonstrated to be higher in patients subjected to homologous blood transfusion and in moderately trained volunteers who were subjected to a protocol of autologous blood transfusion [30–32]. So, the detection of high concentrations of DEHP metabolites in urine has been proposed as a marker of the misuse of both homologous and autologous blood transfusions in sports [33]. The ubiquitous exposure to DEHP of the general population makes the definition of commonly exposed population concentrations necessary. In recent works, common concentrations of some of the metabolites have been described [30,34–36].

In this study, a method to detect five DEHP metabolites in urine (MEHP, MEOHP, MEHHP, 5cx-MEPP and 2cx-MMHP) has been optimized and validated. Compared to previous studies [30–32,34–36], the methodology proposed allows the quantification of two additional DEHP metabolites (5cx-MEPP and 2cx-MMHP). The developed method has been used to investigate the concentrations of these five metabolites in general population and in samples from different sportive subjects in order to define basal population ranges of the metabolites.

2. Materials and methods

2.1. Chemicals and reagents

Standards of MEHP, MEHHP, MEOHP, 5cx-MEPP, 2cx-MMHP and their $^{13}C_4$ labelled (ring-1,2 $^{13}C_2$ and dicarboxyl $^{13}C_2$) analogues, MEHP $^{13}C_4$, MEOHP $^{13}C_4$ and 5cx-MEPP $^{13}C_4$,which were used as internal standards (ISTD), were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Ethyl acetate (HPLC grade), acetonitrile (LC gradient grade), formic acid (LC/MS grade) and other reagents (analytical grade) were purchased from Merck (Darmstadt, Germany). Milli-Q water was obtained from a Milli-Q purification system (Millipore Ibérica; Barcelona, Spain). β-Glucoronidase (*Escherichia coli*) was purchased from Roche Biomedical (Mannheim, Germany).

2.2. Standards and solutions

Stock standard solutions of DEHP metabolites and isotopically labelled ISTDs of 100 μg mL^{-1} were prepared in acetonitrile. Working standard solutions of 10 and 1 μg mL^{-1} were prepared by appropriate dilutions with acetonitrile of the stock solutions. All solutions were stored at −20 °C.

2.3. Sample preparation

Aliquots of urine samples (1 mL) were added with a concentration of 50 ng mL^{-1} of each ISTD (MEHP $^{13}C_4$, MEOHP $^{13}C_4$ and 5cx-MEPP $^{13}C_4$). The pH was adjusted to 6.5 with ammonium acetate buffer (250 μL) and, then, 30 μL of β-glucuronidase from *E. coli* were added. Samples were incubated at 55 °C for 60 min. After enzymatic hydrolysis, samples were acidified with phosphate buffer pH 2 (2 mL). The extraction was performed with 8 mL of ethyl acetate by shaking at 40 rpm for 20 min. After centrifugation

(3500 rpm, 5 min), organic layers were evaporated to dryness under nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 200 μL of a mixture of deionized water:acetonitrile (80:20, v/v, 0.01% formic acid) and aliquots of 5 μL were analysed by ultraperformance liquid chromatography tandem mass spectrometry (UPLC–MS/MS).

2.4. Instrumental analysis

Chromatographic separations were carried out on a Waters Acquity UPLC™ system using an Acquity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters Corporation, Milford, MA). The column temperature was set to 45 °C. The mobile phase consisted of deionized water with 0.01% formic acid (solvent A) and acetonitrile with 0.01% formic acid (solvent B). Separation was performed at a flow-rate of 0.6 mL min^{-1} with the following gradient elution: from 0 to 3 min, 20%B; linear increase to 50%B in 2 min; linear increase to 95%B in 1 min; 95%B during 2 min, decrease to 20%B in 0.2 min; and stabilization at initial conditions for 1.3 min. The total run time was 9.5 min. The sample volume injected was 5 μL.

The UPLC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corp.) with an electrospray (Z-spray) ionization source working in positive and negative ionization mode. Source conditions were set as follows: capillary voltage, 3 and 2.5 kV for positive and negative ionization mode, respectively; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas flow-rate, 50 L h^{-1} and desolvation gas flow-rate, 1200 L h^{-1}. High-purity nitrogen was used as desolvation gas and argon was used as collision gas. Electrospray ionization (ESI) working parameters (ionization mode, precursor and product ions, cone voltage and collision energies) were optimized for each compound using direct infusion of individual standard solutions of the compounds (10 μg mL^{-1}) at 10 μL min^{-1} with mobile phase (50:50, A:B) with a flow rate at 200 μL min^{-1}. Cone voltage was optimized to obtain the maximum signal of the precursor ion and the collision energy was adjusted to maximize the signal of the most abundant product ion for each compound. Detection and quantitation was performed in multiple reaction monitoring mode (MRM). MRM conditions are described in Table 1. Two specific transitions were monitored for each analyte. Data acquisition was performed in two acquisition groups with dwell times of 50 ms, interchannel delays of 5 ms and interscan times of 20 ms. All data were acquired and processed using MassLynx 4.1 software (Waters Corporation, Milford, MA).

2.5. Method validation

The following parameters were evaluated: linearity, limit of detection (LOD), limit of quantitation (LOQ), extraction recovery, matrix effect and intra- and inter-assay precisions.

Due to the ubiquitous presence of DEHP metabolites in urines from human population, the linearity of the method and the LOD and LOQ were evaluated using a calibration curve prepared in artificial urine. For the rest of the validation assays, calibration curves were prepared by using urines from healthy subjects with very low levels of DEHP metabolites diluted with water.

The artificial urine was prepared based on a protocol described elsewhere [37]: 0.1 g of lactic acid, 0.4 g of citric acid, 2.1 g of sodium bicarbonate, 10 g of urea, 0.07 g of uric acid, 0.8 g of creatinine, 0.37 g of calcium chloride·$2H_2O$, 5.2 g of sodium chloride, 0.0012 g of iron II sulphate·$7H_2O$, 0.49 g of magnesium sulphate·$7H_2O$, 3.2 g of sodium sulphate·$10H_2O$, 0.95 g of potassium dihydrogen phosphate, 1.2 g of di-potassium hydrogen phosphate and 1.3 g of ammonium chloride were dissolved in 1 L ultrapure water.

Six calibration levels were studied: 0, 5, 10, 50, 100 and 400 ng mL^{-1}. For the first validation assay, each calibration level was prepared in quadruplicate. The peak area ratios of the selected transitions of the analyte and the ISTD were plotted against concentrations. The ISTD used were: MEHP $^{13}C_4$ for the quantification of MEHP; MEOHP $^{13}C_4$ for the quantification of MEHHP and MEOHP; and 5cx-MEPP $^{13}C_4$ for the quantification of 5cx-MEPP and 2cx-MMHP. The Dixon test (α = 5%) was applied to detect outliers in the replicates of the area ratios at each concentration level. The behaviour of the variance over the calibration range (homo/heteroscedasticity) was evaluated by applying the Levene Test (α = 5%). To demonstrate the goodness for fit using the linear model, the *F* test (α = 5%) was applied to compare the variance attributable to lack of fit with that due to random error. Least-square regression analysis weighted by the concentration was applied to calculate the calibration curves and the coefficients of determination. The LOD and LOQ were defined as 3.3 and 10 times the value of the standard deviation of the noise, respectively. The standard deviation of the estimated concentration values of a sample spiked with 1 ng mL^{-1} for each analyte (n = 4) was used as a measure of the noise.

The extraction recovery was calculated by the analysis of three replicates of a blank urine spiked with 50 ng mL^{-1} of the compounds and three replicates of a blank sample spiked with the same concentration of analytes after the extraction procedure. The ratio of the peak areas between the analytes and the corresponding ISTD obtained from the extracted spiked samples was compared with ratios obtained for samples in which the analytes were added after the extraction procedure (representing 100% of extraction recovery).

Due to the impossibility to obtain true blank urine samples, the matrix effect was evaluated by standard additions. The concentrations levels of the analytes in 7 urine samples were calculated by both external calibration and standard addition calibration. For external calibration, a calibration curve was prepared in artificial urine. Five calibration levels were prepared in duplicate at the following levels: 0, 5, 10, 50, and 100 ng mL^{-1}. External calibration concentrations were calculated for each sample. Standard additions were carried out in every sample by addition of four concentration levels in duplicate (0, 5, 10, and 50 ng mL^{-1}). The matrix effect was calculated for each analyte as the ratio between the external calibration concentration and standard addition concentration.

The intra- and inter-assay precisions were estimated by using two quality control samples with low and high concentrations of DEHP metabolites. These quality control samples were actual samples obtained from subjects moderately exposed to DEHP (low control, concentrations around a range of 14–45 ng mL^{-1} for the different metabolites) and from patients subjected to blood transfusion highly exposed to DEHP (high control, concentrations range around 54–278 ng mL^{-1}). Intra-assay precision was calculated by the analysis of three replicates of the two control samples on the same day. Inter-assay precision was calculated after analysis of these two samples in eleven different days. Precisions were expressed as the relative standard deviation (RSD) of the concentrations measured.

2.6. Subjects

In order to evaluate basal concentrations of DEHP metabolites, urines from two different populations groups were quantified. The first group (control group) included 30 healthy subjects reflecting common environmental DEHP exposure. They were volunteers that did not receive any blood transfusion or any medical procedure involving plastic equipment. The volunteers were selected by the IMIM-Hospital de Mar Clinical Research Unit according to a clinical protocol approved by the ethical committee CEIC-IMAS

Table 1
Monitoring conditions in the UPLC–MS/MS system for DEHP metabolites. Transitions used for quantitation are underlined.

Compound	MM[a]	RT[b] (min)	ESI mode[c]	CV[d] (V)	CE[e] (eV)	Ion transitions (*m*/*z*)
MEHP	278.15	6.11	pos	15	15, 5	279 ≥ 149, 279 > 167
MEOHP	292.13	5.01	pos	10	10	293 ≥ 127
			neg	30	15	291 > 143
MEHHP	294.15	4.94	pos	12	7, 7	295 ≥ 167, 295 > 129
5cx-MEPP	308.13	4.83	pos	10	5, 5	309 ≥ 143, 309 > 161
2cx-MMHP	308.13	5.09	pos	10	5, 5	309 ≥ 143, 309 > 161
MEHP $^{13}C_4$	282.15	6.11	pos	15	10, 10	283 ≥ 153, 283 > 171
MEOHP $^{13}C_4$	296.13	5.01	pos	10	5, 10	297 ≥ 127, 297 > 109
5cx-MEPP $^{13}C_4$	312.13	4.83	pos	10	5, 5	313 ≥ 143, 313 > 161

[a] MM, monoisotopic mass.
[b] RT, retention time.
[c] ESI, electrospray ionization: pos, positive; neg, negative.
[d] CV, cone voltage.
[e] CE, collision energy.

(IMIM-Hospital del Mar, Barcelona, Spain, protocol 2006/2456/1). The average age was 23.67 ± 2.34 years (range: 21–31 years), and the gender distribution was 50% females and 50% of males. 24 h urine samples were collected and stored at −20 °C in polypropylene bottles until analysis.

In the second group (sportsmen group), a total of 464 official doping control urine samples covering different sports disciplines (mainly aquatics, athletics, cycling) were analysed. Spot urine samples were collected and stored at −20 °C in glass bottles until analysis.

Urine samples used as quality control samples were also obtained within the clinical protocol 2006/2456/1. The low quality control was a sample belonging to a volunteer of the "control group" described above. The high quality control was a urine sample collected from a hospitalized patient subjected to homologous blood transfusion [30].

2.7. Statistical analysis and calculations

All results of concentration were corrected by specific gravity, using the following formula:

$$\text{Adjusted concentration} = \text{concentration}\left[\frac{1.020 - 1}{\text{specific gravity of the sample} - 1}\right]$$

This mathematical correction by specific gravity is usually carried out in doping control analyses for some analytes [38]. Statistical analysis was performed on the logarithmic transformed concentrations with computer software SPSS 12.0 (SPSS, Inc., Chicago, IL). The program RefVal (RefVal 4.11, Oslo, Norway) was used for the determination of the upper reference limits (RL) and the detection of outliers. The 99.9% fractile of reference limits by parametric estimation was assessed [39]. Outlier detection occurred with Horn's algorithm. The outliers were removed from the statistics.

3. Results and discussion

3.1. Method development and validation

Different sample preparation protocols and different detection conditions were studied to analyse DEHP metabolites in urine, as per the data previously published [22–26]. A sample preparation based on a liquid–liquid extraction at pH 2 was finally selected to quantify DEHP metabolites in urine. A hydrolysis step to cleave the glucuronide conjugates was needed. β-Glucuronidase from *E. coli* was used; other preparations, such as β-glucuronidase from *Helix pomatia*, were not adequate due to its hydrolytic activity on phthalates to convert them to their monoesters [25]. The extraction was performed at pH 2 to obtain the protonated compounds. Thereafter, the samples were analysed by UPLC–MS/MS.

Regarding the LC–MS/MS optimization, mobile phases containing water, acetonitrile or methanol acidified with formic acid or acetic acid were studied. The mobile phases were evaluated taking into account chromatographic behaviour of the analytes and signal in ESI. Best results were obtained using a mobile phase with water and acetonitrile acidified with formic acid. The chromatographic separation was achieved with a total running time of 10 min, with all the compounds eluting between 4.8 and 6.1 min (Table 1).

ESI working parameters were optimized for each DEHP metabolite. Positive and negative ion modes were tested. In our conditions, positive ion mode was selected because higher signal was obtained. Protonated molecular ions $[M+H]^+$ were obtained for all the compounds formed by protonation of one of the carbonyl groups. No formation of adduct ions was observed. The cone voltage was optimized to maximize the signal of the protonated molecular ion and collision energy was adjusted to optimize the signal of the most abundant product ions. The optimal conditions for each metabolite are presented in Table 1. The product ion mass spectra of all metabolites and isotopically labelled internal standards at one of the optimal collision energies are presented in Fig. 2.

Pseudomolecular ions $[M+H]^+$ of DEHP metabolites showed a characteristic collision induced dissociation pattern. Ions resulting from the loss of water molecules were only observed at low collision energies (*m*/*z* 277 and 291, for MEHHP and 5cx MEPP, respectively). For all the compounds, except MEOHP and its isotopic labelled analogue, ions at *m*/*z* 167 (protonated phthalic acid) and at *m*/*z* 149 (protonated phthalic anhydride) were observed. The equivalent ions at *m*/*z* 171 and 153 were observed in the corresponding isotopic labelled analogues. Ions resulting from the protonated lateral chain were observed for all metabolites: *m*/*z* 113 for MEHP and MEHP $^{13}C_4$, *m*/*z* 127 for MEOHP and MEOHP $^{13}C_4$, *m*/*z* 129 for MEHHP, and at *m*/*z* 143 for 5cx MEPP, 2cx MMHP and 5cx MEPP $^{13}C_4$. A subsequent loss of a water molecule was observed for most of them (ions at *m*/*z* 109 for MEOHP and MEOHP $^{13}C_4$, *m*/*z* 111 for MEHHP, and *m*/*z* 125 for 5cx MEPP, 2cx MMHP and 5cx MEPP $^{13}C_4$). For compounds with an additional carboxylic acid function in the lateral chain (5cx MEPP, 2cx MMHP and 5cx MEPP $^{13}C_4$), additional ions resulting from the protonation of this group and including the lateral chain were also observed at *m*/*z* 161. The ions selected for quantitative purposes are indicated in Table 1.

Examples of chromatograms of urine samples from subjects with low and high exposure to DEHP (low and high quality control

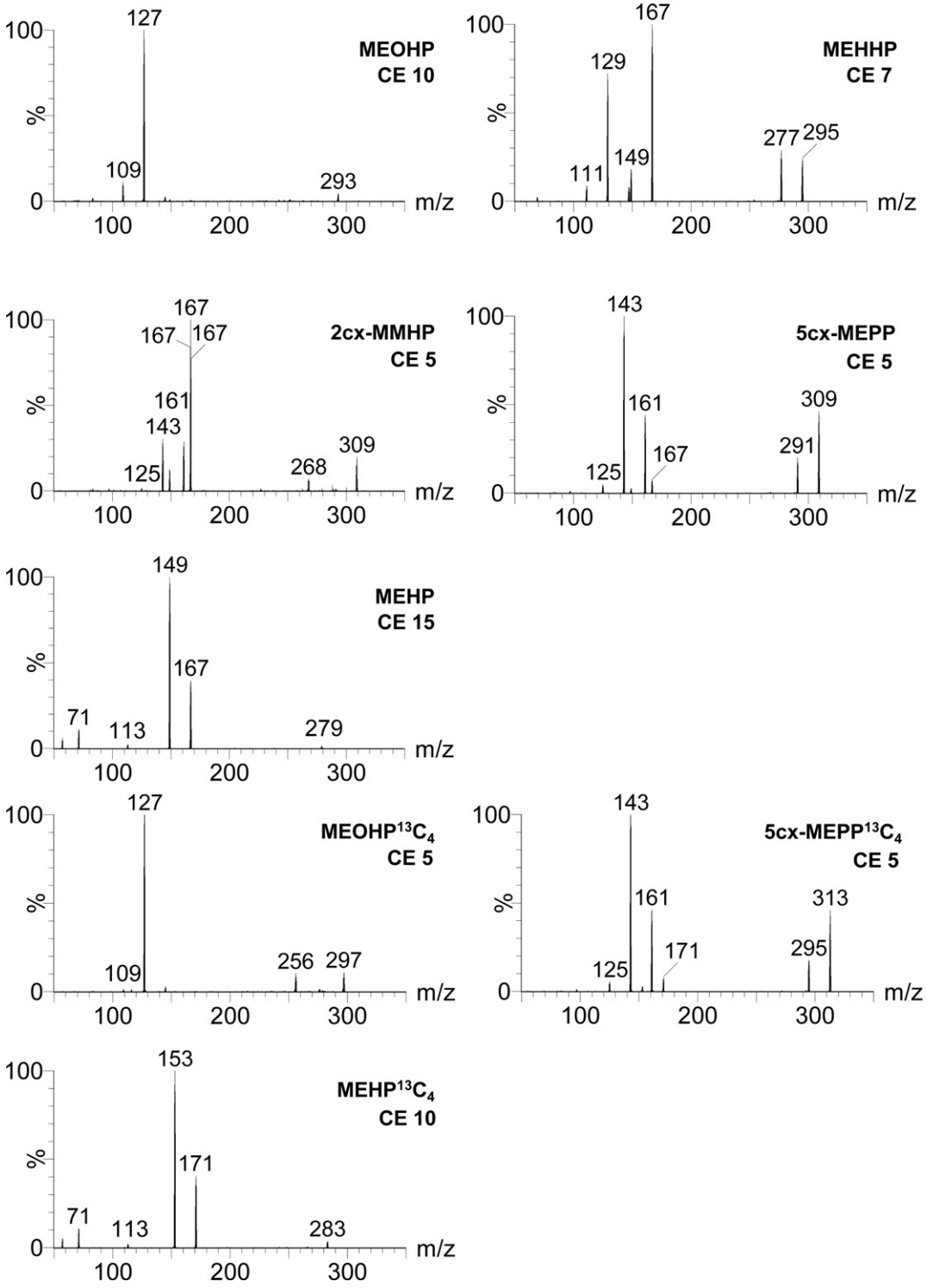

Fig. 2. Product ion mass spectra arising from the protonated molecular ions of MEHP (*m*/*z* 279), MEOHP (*m*/*z* 293), MEHHP (*m*/*z* 295), 5cx-MEPP (*m*/*z* 309), 2cx-MMHP (*m*/*z* 309), MEHP $^{13}C_4$ (*m*/*z* 283), MEOHP $^{13}C_4$ (*m*/*z* 297) and 5cx-MEPP $^{13}C_4$ (*m*/*z* 313) (CE, collision energy).

samples, respectively) are given in Fig. 3. As it can be observed, there is a great difference in intensities between both samples for all metabolites.

The method developed was linear in the range of concentrations studied. Coefficients of determination were always greater than 0.99. The F test for comparing variances was not significant, indicating adequate adjustment of the data to the proposed linear model over the calibration range. Extraction recoveries and LOQ are listed in Table 2. Extraction recoveries greater than 90% were obtained for all metabolites, and LOQ ranged from 1.2 to 2.6 ng mL^{-1}.

Regarding specificity of the method, no peaks at the retention time of the analytes and ISTDs were observed after analysis of artificial urine samples indicating no contamination due to reagents. In actual urine samples, low basal levels of the analytes were detectable in most of the samples due to ubiquitous exposition to DEHP.

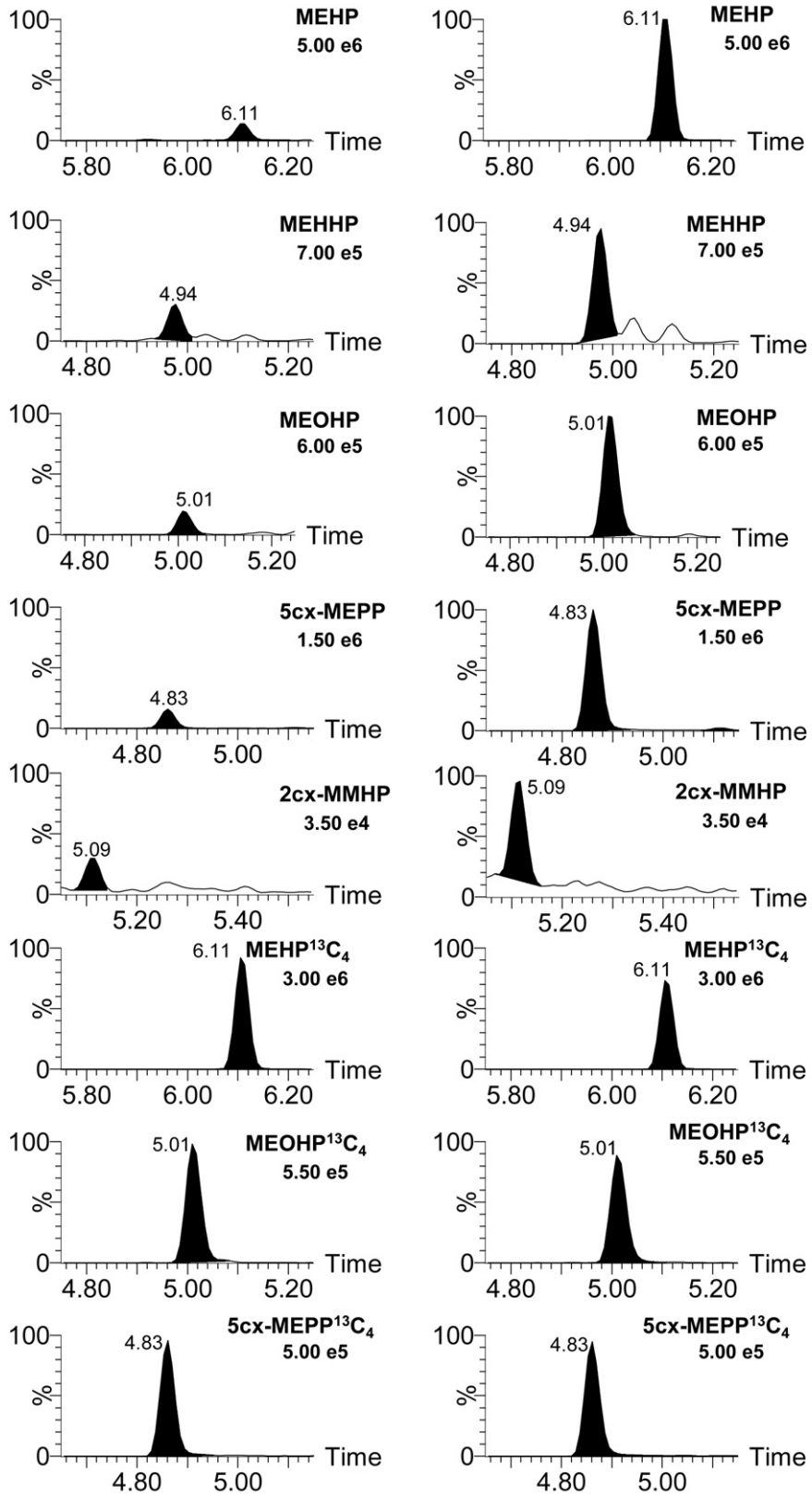

Fig. 3. Chromatograms of the selected transitions of DEHP metabolites and ISTDs. Left, low quality control sample; right, high quality control sample.

The intra- and inter-assay precisions obtained for the low and high concentration quality control samples are shown in Table 3. Intra-assay precision was always better than 8%, while inter-assay precision was better than 12%. The data indicates that the method provides adequate precision for the quantitation of the five DEHP metabolites in urine samples.

The matrix effect was found to be not significant for MEHP, 5cx-MEPP and 2cx-MMHP. For MEHHP and MEOHP, the ratios between

Table 2
Validation data: Extraction recoveries (ER) calculated at 50 ng mL^{-1} and limits of quantitation (LOQ) for the different DEHP metabolites.

Compound	ER (%), mean ± SD (ng mL^{-1})	LOQ (ng mL^{-1})
MEHP	97.1 ± 3.3	2.6
MEHHP	102.0 ± 1.1	1.9
MEOHP	94.8 ± 1.2	1.2
5cx-MEPP	93.8 ± 0.5	2.4
2cx-MMHP	90.2 ± 5.1	1.8

SD, standard deviation.

the concentrations calculated from the external calibration and the standard addition were 1.2 and 1.6, respectively, with a standard deviation for both of approximately 20% (n = 7 samples), showing a reproducible matrix enhancement effect especially for MEOHP. In order to correct the matrix effect isotopically labelled standards were used.

For MEHP, MEHHP and MEOHP, the developed methodology demonstrated sensitivity and reliability similar to previously described methods [34]. In addition, it offers the possibility of quantification of two additional DEHP metabolites (5cx-MEPP and 2cx-MMHP), which is important to have the largest number of potential markers.

3.2. Concentrations in urine samples from different population groups

The use of blood transfusions produces a huge increase in the concentration of all DEHP metabolites in urine [30–35]. However, as DEHP is ubiquitously present, all population is exposed to some extent. In order to have a criterion to suspect of an acute exposure to DEHP (e.g., blood transfusion), it is necessary to define the common range of metabolites concentrations. In this regards, the quantitation of the DEHP metabolites in urine from different population groups was performed.

Taking into account the possible differences of the urine samples regarding dilution status or variations due to sampling, specific gravity of the samples was measured. Concentrations were corrected by specific gravity, as it is usually performed in doping control laboratories to minimize the variations of the different dilution status of the samples [38]. The results of the specific gravity adjusted concentrations for all five DEHP metabolites in samples from the control group are shown in Table 4. Concentrations corresponding to percentiles 10th, 25th, 50th, 75th, and 90th are presented. The levels observed in the control group for the five DEHP metabolites are similar to first studies published [16]. However, they are slightly higher than the concentrations published in the literature in recent years probably due to the 24-h urine samples analysed in our work [7,17,40,41].

Distribution of the concentrations of the DEHP metabolites in the sports related samples is also described in Table 4. The levels observed in the sportsmen group for MEHP, MEHHP, MEOHP, and 5cx-MEPP are in accordance with published data [7,34,40–42]. Regarding 2cx-MMHP, the median concentration agrees with that described by Guo et al. [43], however it is greater the reported by other groups [7,40]. As it was previously described, all the concentrations of the athletes' samples were lower than those of the control group [30], and it could be due to the difference in the sample collection (spot urine samples vs. 24 h). Concentrations of phthalates in the first morning urine are known to be higher than those found in samples collected during the day [44]. For this reason, it may be expected than concentrations in spot urines collected during the day will be lower than those in 24 h urine samples.

Table 3
Validation data: results of intra- and inter-assay precision for the low and high quality control samples (LC and HC, respectively).

Compound	Conc.	Intra-assay precision (N = 3)		Inter-assay precision (N = 11)	
		Estimated Conc. (mean ± SD) (ng mL^{-1})	Precision (RSD%)	Estimated Conc. (mean ± SD) (ng mL^{-1})	Precision (RSD%)
MEHP	LC	14.9 ± 0.8	5.6	15.3 ± 1.1	6.9
	HC	131.2 ± 3.5	2.7	129.0 ± 2.7	2.1
MEHHP	LC	35.4 ± 1.6	4.6	36.0 ± 3.6	10.0
	HC	148.4 ± 1.3	0.9	137.8 ± 12.4	9.0
MEOHP	LC	29.2 ± 1.9	6.6	27.8 ± 1.3	4.5
	HC	159.0 ± 6.6	4.2	150.3 ± 4.4	2.9
5cx-MEPP	LC	44.9 ± 2.2	5.0	44.2 ± 1.8	4.0
	HC	277.5 ± 8.1	2.9	272.5 ± 6.4	2.4
2cx-MMHP	LC	18.4 ± 1.5	8.0	18.5 ± 2.2	12.0
	HC	57.0 ± 0.8	1.4	53.5 ± 5.3	9.8

Conc., concentration; SD, standard deviation; RSD, relative standard deviation.

Table 4
Distribution of concentrations of DEHP metabolites in the subjects of the control group and sportsmen, corrected for specific gravity.

Group	Compound	Concentration (ng mL^{-1})				
		Percentile				
		10th	25th	50th	75th	90th
Control (n = 30)	MEHP	5.1	8.1	16.0	22.7	26.4
	MEHHP	11.8	31.1	51.4	80.2	112.7
	MEOHP	9.1	20.2	38.2	65.1	111.7
	5cx-MEPP	17.4	33.2	55.0	109.2	143.1
	2cx-MMHP	10.7	22.5	34.0	60.5	169.0
Sportsmen (n = 464)	MEHP	2.5	3.5	5.5	9.4	15.3
	MEHHP	10.9	15.8	27.3	44.3	76.0
	MEOHP	5.1	8.5	13.6	22.0	39.8
	5cx-MEPP	12.3	18.8	28.4	46.7	81.8
	2cx-MMHP	8.4	12.8	21.1	33.8	66.2

Table 5
Threshold concentrations (99.9% reference limit) for the different DEHP metabolites in ng mL^{-1} corresponding to common exposure to DEHP among sportive subjects.

Metabolite	ng mL^{-1}
MEHHP	338.8
MEOHP	158.5
5cx-MEPP	331.1
2cx-MMHP	229.1

3.3. Threshold determination of phthalate metabolites

Taking into account the concentrations found in the sports related samples, threshold concentrations at 1:1000 risk for false positives (99.9% reference limit) were calculated for MEOHP, MEHHP, 5cx MEPP and 2cx MMHP (Table 5). The log-transformed values of the specific gravity adjusted concentrations of the DEHP metabolites obtained in samples from sportsmen ($n = 464$) were used to establish the reference limit values. For MEHP, the distribution of concentrations was not Gaussian, even after logarithmic transformation of the data, and the reference limits could not, therefore, be calculated [39]. The reference limits can be used as threshold concentrations to determine whether a high result may be suspicious for a transfusion process. The reference limits range from 158.5 to 338.8 ng mL^{-1} for a risk of 1:1000. The reference limit for MEOHP is in accordance with the value proposed by Solymos et al. [34]. However, for MEHHP the value proposed in the previous study (193.0 ng mL^{-1}) is lower than the value obtained with our data. This difference can be probably due to the different origin of the samples analysed in both studies.

The reference limits were evaluated using the concentrations detected in an experiment of autologous blood transfusion in 25 subjects performed by our group [31]. Concentrations of MEOHP the day of the reinfusion were higher than the reference limit in all subjects; for MEHHP and 5cx-MEPP, 92% and 88% of the samples, respectively, showed concentrations greater than their reference limit; and for 2cx-MMHP, only 32% of the subjects had concentrations greater than its reference limits. One day after blood reinfusion all metabolites were below the reference limit proposed, except 2cx-MMHP where 12% of the samples exceeded the reference limit. Therefore, MEHHP, MEOHP, and 5cx-MEPP can be used as markers of blood transfusion misuse during the first hours after the transfusion, while 2cx-MMHP could also be used to suspect beyond 24 h in some subjects. Substantial anecdotal information indicates that most athletes use transfusion for doping purposes just short time before competition and, in this situation, the detection window provided by DEHP metabolites measurement will be appropriate to suspect for blood doping.

The reference limits proposed for MEHHP and MEOHP were also compared with concentrations of these metabolites published in the literature obtained in samples from hospitalized patients subjected to blood transfusion [30,34]. The reference limits were appropriate to detect blood transfusion in most of the reported samples collected during the first 24 h after the transfusion [30,34] and also in some samples collected during 24–48 h after transfusion [30].

The possibility of reaching higher values of DEHP metabolites due to an uncontrolled exposure cannot be ruled out. For this reason, the threshold concentrations proposed were compared with concentrations of the metabolites obtained after longitudinal studies of individuals from a German cohort without special occupational exposure with a follow-up of several days [36]. The maximum concentrations detected for MEHHP and MEOHP along one week study were not higher than the reference limits proposed. However, maximum MEHHP concentrations described by Preau et al. in a study performed with an American cohort were higher than 338 ng mL^{-1} in some of the volunteers [45]. The difference may reflect a different lifestyle between the cohorts studied. For this reason, further investigations are still needed to clarify the origin of the increased DEHP exposures observed in some individuals not subjected to blood transfusion.

4. Conclusions

A methodology for the quantitation of five DEHP metabolites in urine has been developed and validated. The protocol developed is effective and it requires short time analysis due to its simple sample preparation and the use of UPLC as chromatographic separation technique which provides short total running times. The reliability of the method proposed makes it adequate as screening test to be applied in all urine samples in the antidoping control laboratories to suspect for blood transfusion practices.

Concentrations of DEHP metabolites in different population groups have been measured to obtain data that reflect the common environmental exposure. The concentrations found in normally exposed subjects (control group) and in sportsmen are usually low for all metabolites. Moreover, the basal concentrations obtained in the sportsmen group were used to establish threshold concentrations for four DEHP metabolites after common exposure.

Acknowledgements

This work was supported by grants from DIUE Generalitat de Catalunya (2009 SGR 492) and the World Antidoping Agency (Research Grant 06A6JS). Financial support from Consell Català de l'Esport (Generalitat de Catalunya) is also acknowledged. The background support of IMIM-Hospital del Mar is acknowledged. The authors thank the collaboration of Dr. M. Farré, E. Menoyo and M. Bosch for sample collection, Mr. S. Mojal for statistical analysis and to Pieter Van Renterghem for providing the RefVal program.

References

[1] European Commission Opinion on Medical Devices Containing DEHP plasticised PVC; Neonates and Other Groups Possibly at Risk from DEHP Toxicity, Scientific Committee on Medicinal Products and Medical Devices (SCMPMD), Brussels, 2002, Available at: http://ec.europa.eu/food/fs/sc/scmp/out43_en.pdf (accessed June 2012).
[2] FDA Public Health Notification, PVC Devices Containing Plasticizer DEHP, US Food and Drug Administration, Center of Devices and Radiological Health, Available at: http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/PublicHealthNotifications/ucm062182.htm (accessed June 2012).
[3] B. Horowitz, M.H. Stryker, A.A. Waldman, K.R. Woods, J.D. Gass, J. Drago. Vox Sanquinis. 48 (1985) 150–155.
[4] J. Simmchen, R. Ventura, J. Segura, Transfus. Med. Rev. 26 (2012) 27–37.
[5] H.M. Koch, A.M. Calafat, Philos. Trans. R. Soc. B 364 (2009) 2063–2078.
[6] H. Frederiksen, N. Skakkebæk, A. Andersson, Mol. Nutr. Food Res. 51 (2007) 899–911.
[7] H. Fromme, G. Bolte, H.M. Koch, J. Angerer, S. Boehmer, H. Drexler, R. Mayer, B. Liebl Int, J. Hyg. Environ. Health 210 (2007) 21–33.
[8] G. Lottrup, A.M. Anderson, H. Leffers, G.K. Mortensen, J. Toppari, N.E. Skakkebæk, K.M. Main, Int. J. Androl. 29 (2006) 172–180.
[9] C.R. Blystone, G.E. Kissling, J.B. Bishop, R.E. Chapin, G.W. Wolfe, P.M.D. Foster, Toxicol. Sci. 116 (2010) 640–646.
[10] G. Latini, A. Verrotti, C. De Felice, Curr. Drug Targets Immune Endocr. Metabol. Disord. 4 (1) (2004) 37–40.
[11] H.M. Koch, R. Preuss, J. Angerer, Int. J. Androl. 29 (2006) 155–165.
[12] P.W. Albro, S.R. Lavenhar, Drug Metab. Rev. 21 (1989) 13–34.
[13] M.J. Silva, D.B. Barr, J.A. Reidy, K. Kato, N.A. Malek, C.C. Hodge, D. Hurtz, A.M. Calafat, L.L. Needham, J.W. Brock, Arch. Toxicol. 77 (2003) 561–567.
[14] H.M. Koch, H.M. Bolt, R. Preuss, J. Angerer, Arch. Toxicol. 79 (2005) 367–376.
[15] M.J. Silva, E. Samandar, J.L. Preau, L.L. Needham, A.M. Calafat, Toxicology 219 (2006) 22–32.
[16] H.M. Koch, B. Rossbach, H. Drexler, J. Angerer, Environ. Res. 93 (2003) 177–185.
[17] D.B. Barr, M.J. Silva, K. Kato, J.A. Reidy, N.A. Malek, D. Hurtz, M. Sadowski, L.L. Needham, A.M. Calafat, Environ. Health Perspect. 111 (2003) 1148–1151.
[18] K. Kato, M.J. Silva, J.A. Reidy, D. Hurtz, N.A. Malek, L.L. Needham, H. Nakazawa, D.B. Barr, A.M. Calafat, Environ. Health Perspect. 112 (2004) 327–330.
[19] M.J. Silva, J.A. Reidy, J.L. Preau, E. Samandar, L.L. Needham, A.M. Calafat, Biomarkers 11 (2006) 1–13.

[20] A.M. Calafat, L.L. Needham, M.J. Silva, G. Lambert, Pediatrics 113 (2004) 429–434.
[21] G. Pan, T. Hanaoka, M. Yoshimura, S. Zhang, P. Wang, H. Tsukino, K. Inoue, H. Nakazawa, S. Tsugane, K. Takahashi, Environ. Health Perspect. 114 (2006) 1643–1648.
[22] R. Preuss, H. Koch, J. Angerer, J. Chromatogr. B 816 (2005) 269–280.
[23] K. Kato, M.J. Silva, L.L. Needham, A.M. Calafat, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 814 (2005) 355–360.
[24] M.J. Silva, E. Samandar, J.L. Preau, J.A. Reidy, L.L. Needham, A.M. Calafat, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 860 (2007) 106–112.
[25] B.C. Blount, K.E. Milgram, M.J. Silva, N.A. Malek, J.A. Reidy, L.L. Needham, J.W. Brock, Anal. Chem. 72 (2000) 4127–4134.
[26] H.M. Koch, L.M. Gonzalez-Reche, J. Angerer, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 784 (2003) 169–182.
[27] P.A. Arndt, B.M. Kumpel, Am. J. Hematol. 83 (2008) 657–667.
[28] S. Giraud, N. Robinson, P. Mangin, M. Saugy, Forensic Sci. Int. 179 (2008) 23–33.
[29] K. Inoue, M. Kawaguchi, R. Yamanaka, T. Highchi, R. Ito, K. Saito, H. Nakazawa, Clin. Chim. Acta 358 (2005) 159–166.
[30] N. Monfort, R. Ventura, A. Latorre, V. Belalcazar, M. López, J. Segura, Transfusion (Paris) 50 (2010) 145–149.
[31] N. Monfort, R. Ventura, P. Platen, T. Hinrichs, K. Brixius, W. Schänzer, M. Thevis, H. Geyer, J. Segura, Transfusion (Paris) 52 (2012) 647–657.
[32] N. Monfort, R. Ventura, P. Platen, T. Hinrichs, K. Brixius, W. Schänzer, M. Thevis, H. Geyer, J. Segura, Transfusion (Paris) 52 (3) (2012) 680–681.
[33] J. Segura, N. Monfort, R. Ventura, Drug Test. Anal. (2012), http://dx.doi.org/10.1002/dta.405.
[34] E. Solymos, S. Guddat, H. Geyer, U. Flenker, A. Thomas, J. Segura, R. Ventura, P. Platen, M. Schulte-Mattler, M. Thevis, W. Schänzer, Anal. Bioanal. Chem. 401 (2011) 517–528.
[35] E. Solymos, S. Guddat, H. Geyer, U. Flenker, A. Thomas, J. Segura, R. Ventura, P. Platen, M. Schulte-Mattler, M. Thevis, W. Schänzer, Anal. Bioanal. Chem. 401 (2) (2011) 579–580.
[36] E. Solymos, S. Guddat, H. Geyer, A. Thomas, M. Thevis, W. Schänzer, Drug Test. Anal. 3 (2011) 892–895.
[37] T. Brooks, C.W. Keevil, Lett. Appl. Microbiol. 24 (1997) 203–206.
[38] World Anti-doping Agency, Technical Document TD2004EAAS. 2004. Version 1.0. Available at: http://www.wada-ama.org/rtecontent/document/end_steroids_aug_04.pdf. (accessed September 2012).
[39] H.E. Solberg, Comput. Methods Programs Biomed. 48 (3) (1995) 247–256.
[40] M. Wittassek, G.A. Wiesmuller, H.M. Koch, R. Eckard, L. Dobler, J. Muller, J. Angerer, C. Schluter Int, J. Hyg. Environ. Health 210 (2007) 319–333.
[41] H. Frederiksen, N. Jorgensen, A.M. Andersson, J. Anal. Toxicol. 34 (2010) 400–410.
[42] J.A. Colacino, H. Robert, A. Schecter, Environ. Health Perspect. 118 (7) (2010) 998–1003.
[43] Y. Guo, Q. Wu, K. Kannan, Environ. Int. 37 (2011) 893–898.
[44] H. Frederiksen, L. Aksglaede, K. Sorensen, N.E. Skakkebaek, A. Juul, A.M. Andersson, Environ Res. 111 (2011) 656–663.
[45] J.L. Preau, L.Y. Wong, M.J. Silva, L.L. Needham, A.M. Calafat, Environ. Health Perspect. 118 (2010) 1748–1754.