Talanta ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Talanta

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

Development of an analytical method for the targeted screening and multi-residue quantification of environmental contaminants in urine by liquid chromatography coupled to high resolution mass spectrometry for evaluation of human exposures

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ARTICLE INFO

Article history:
Received 20 March 2015
Received in revised form
9 June 2015
Accepted 15 June 2015

Keywords:
Exposome
LC–HRMS
Urine
ToF
Organic contaminants

ABSTRACT

The aim of this study was to develop an analytical method and contribute to the assessment of the Exposome. Thus, a targeted analysis of a wide range of contaminants in contact with humans on daily routines in urine was developed. The method focused on a list of 38 contaminants, including 12 pesticides, one metabolite of pesticide, seven veterinary drugs, five parabens, one UV filter, one plastic additive, two surfactants and nine substances found in different products present in the everyday human environment. These contaminants were analyzed by high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC–HRMS) with a quadrupole-time-of-flight (QqToF) instrument from a raw urinary matrix. A validation according to the FDA guidelines was employed to evaluate the specificity, linear or quadratic curve fitting, inter- and intra-day precision, accuracy and limits of detection and quantification (LOQ). The developed analysis allows for the quantification of 23 contaminants in the urine samples, with the LOQs ranging between 4.3 ng.mL^{-1} and 113.2 ng.mL^{-1}. This method was applied to 17 urine samples. Among the targeted contaminants, four compounds were detected in samples. One of the contaminants (tributyl phosphate) was detected below the LOQ. The three others (4-hydroxybenzoic acid, sodium dodecylbenzenesulfonate and O,O-diethyl thiophosphate potassium) were detected but did not fulfill the validation criteria for quantification. Among these four compounds, two of them were found in all samples: tributyl phosphate and the surfactant sodium dodecylbenzenesulfonate.

1. Introduction

The impact of the environment on human health has been conclusively demonstrated. In recent decades, scientists have shown that many chronic diseases are related to our environment. For example, a team from INSERM (the French National Institute of Health and Medical Research) has demonstrated that farmers using pesticides were twice as likely to develop Parkinson's disease [1]. According to ANSES (the French agency for food, environmental and occupational health and safety), the environment is responsible for 90% of childhood cancers and 50% for several cancers in adults [2]. The work of Rappaport explains that environmental factors could be responsible for 90% of chronic diseases risks [3]. Several studies also demonstrate the contamination of environmental matrices (water [4], soil and sediments [5], bees [6], gastropods [7]) and the effects of the pollutants on the health [8].

In this context, a new term was born in 2005: the Exposome. It corresponds to all types of exposures humans are subjected throughout their lives via lifestyle, diet, and social environment, as well as the body responses to these exposures [9,10]. Concerning environmental exposures, the Exposome is equivalent to the human genome [9]. To characterize the Exposome, there are different strategies. One of them is the so called top-down strategy [3] which corresponds to an identification and quantification of the chemicals in biological samples. The use of a biological matrix provides a comprehensive view of the Exposome together with the internal chemical environment of each individual. The development of tools and methods to assess the exposome would achieve the human biomonitoring of environmental contaminants, determine the effects of the environment on human health and thus help protect people from risk exposures by triggering new preventive measures and regulations.

The exposures the man is facing are numerous and all environmental contaminants present in everyday life are part of the Exposome. So the concept of the Exposome highlights the need to develop measurement methods to evaluate human exposures.

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http://dx.doi.org/10.1016/j.talanta.2015.06.038
0039-9140/

Furthermore, the Exposome is an unstable concept that evolves over time. Therefore, it is necessary to rapidly establish sensitive routine measurement methods. Several multi-residue methods were developed to analyze environmental contaminants, such as pesticides [11], veterinary drugs [12] and parabens [13] in foodstuffs inter alia. This type of method was also developed in biological matrices. Various contaminants, such as pesticides or UV filters, has been measured in plasma [14] or blood serum [15] and in less commonly used biological matrices, such as placental tissues [16], meconium [17] or human milk [18]. The urine is one of the biological matrices in which the diversity of the endogenous and exogenous compounds sought is very large. For example, urine samples were analyzed to find parabens [19], pesticides [20], veterinary drugs [21] and also UV filters [22]. Multiple residue analysis has been frequently performed by liquid chromatography coupled to mass spectrometry tandem (LC–MS/MS) by operating in multiple reaction monitoring (MRM) mode. This method is the most sensitive for the quantification of environmental contaminants in various matrices. Indeed, the ionization, transfer and fragmentation parameters are optimized for each targeted substance which leads to a sensitive, specific and selective method. However, it has some limitations, such as measuring only the targeted compounds. In contrast, liquid chromatography coupled to high resolution mass spectrometry (LC–HRMS) allows for the screening of both targeted and non-targeted contaminants. Thus, the use of LC–HRMS enables the detection of metabolites, degradation products or other non-targeted compounds by measuring the exact monoisotopic mass in full-scan mode. More and more multi-residue methods are performed on HRMS instruments, such as time-of-flight (ToF) [23,24], quadrupole-time-of-flight (Qq-ToF) [25], or Orbitrap [26]. Several scientists have used LC-ToF to measure compounds in biological samples, especially in urine [27,28].

Urine appears to be the preferential biological matrix for studying the Exposome: it is easier to obtain (non-invasive, readily available) and includes a large number of endogenous and exogenous metabolites. The choice of a liquid (95% of water) homogenous biological matrix limits the sample preparation step and enables direct injection into the instrument. Different researchers showed that the use of diluted urine was preferable to more selective techniques, such as solid phase extraction [29,30]. Furthermore, this generic strategy was used in several studies [28,31] since it is time-saving and allows for the analysis of large cohorts.

In this study, the analysis of a wide range of contaminants in contact with man during daily routines was developed. The main objective of this study was to develop a method to determine if the targeted environmental contaminants were present in the urine of an individual and quantify them. These measures could represent an indicator of exposure. A total of 38 compounds have been chosen, classified in seven families: 12 pesticides, those to which the population is the most exposed via fruit and vegetable consumption [32,33], (carbendazim, imazalil, cyprodinil, ethylene thiourea, 2-phenylphenol, diuron, linuron, methamidophos, methomyl, acephate, dimethoate and omethoate); one metabolite of pesticide (O,O-diethyl thiophosphate potassium, metabolite of parathion, an insecticide found in fruit and vegetable); seven veterinary drugs ingested by humans via foods and chosen according to their frequent administration of cattle, sheep, poultry, goats and pigs [34,35], (marbofloxacin, difloxacin, danofloxacin, enrofloxacin, clorsulon, dicyclanil and levamisole); five parabens (propylparaben, butylparaben, methylparaben, ethylparaben and isopropylparaben); one UV filter (cyasorb UV9); one plastic additive (bisphenol A); two surfactants (perfluorooctanoic acid and sodium dodecylbenzenesulfonate); and nine substances present in different products use in daily routines (tributyl phosphate, 4'-hydroxyacetophenone, dibutylphosphate, bis(2-ethylhexyl) phosphate, perfluoropentanoic acid, undecafluorohexanoic acid, perfluorononanoic acid, 4-hydroxybenzoic acid and heptafluorobutyric acid). For example, 4'-hydroxyacetophenone is used as perfume in soaps, detergents, cosmetics, food, beverages and tobacco.

The developed analytical strategy consists in the detection and also the quantification of the analytes for which the standard validation requirements were met. Urine did not undergo sample preparation in order to retain all the desired contaminants that have different physico-chemical properties and to reduce the duration of the analytical process. After development and validation, the method was applied to the first morning urine of several healthy subjects.

2. Materials and methods

2.1. Standards and reagents

Diuron, carbendazim, marbofloxacin, difloxacin, tributyl phosphate, omethoate, methamidophos, levamisole, cyprodinil, bisphenol A, dibutyl phosphate, bis(2-ethylhexyl) phosphate, perfluoropentanoic acid, undecafluorohexanoic acid, O,O-diethyl thiophosphate potassium, perfluorononanoic acid, clorsulon, acephate, butylparaben, propylparaben, methylparaben, ethylparaben, dimethoate, imazalil, perfluorooctanoic acid, cyasorb UV 9, linuron, danofloxacin, enrofloxacin, dicyclanil, heptafluorobutyric acid, 2-phenylphenol, 4-hydroxybenzoic acid, sodium dodecylbenzenesulfonate, ethylene thiourea, 4'-hydroxyacetophenone and methomyl were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Isopropylparaben was obtained from Tokyo Chemical Industry (Tokyo, Japan).

Individual stock solutions were prepared at concentrations of 1 $mg.mL^{-1}$ in methanol (MeOH), acetonitrile (MeCN), N,N-dimethylformamide (DMF) or water, depending on the specific solubility properties of the compound. These solutions were stored for six months at −18 °C, except for those prepared in water which were stored for one month at 4 °C. During these periods, the compounds were stable. Mixtures of solutions of compounds were prepared from the individual stock solutions and diluted to obtain the appropriate concentrations.

MeOH, MeCN, formic acid, acetic acid, ammonium formate, ammonium acetate (all LC–MS grade), isopropanol (HPLC grade), and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). DMF was purchased from BiosolveChimie (Dieuze, France). Pure water was obtained from a MilliQ device from Millipore (Saint-Quentin-en-Yvelines, France).

2.2. Sampling procedure

For the analytical development and validation, the human urine samples were collected from eight healthy volunteers (4 females and 4 males) at the same moment after lunch, for convenience. The samples were provided by subjects aged from 22 to 61. After collection, each urine sample was aliquoted and kept frozen at −20 °C until analysis, as recommended for stability reasons [36]. All individual urine samples were collected, aliquoted and frozen at the same moment so that the state of degradation of the compounds in urine was the same in each sample. In effect, the urine is a rich source of nutrients for microorganisms, including bacteria, yeast and fungi. Furthermore, urine contains enzymes that modify the contaminants and molecules contained in the urine [37].

After development, the method was applied to the first morning urine of 17 colleagues, 10 males and seven females. For its application to real samples, the developed method was applied to

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first morning urine because the biological matrix is generally more concentrated at this time of day [38]. All urine samples were collected without preservatives and aliquoted in glass bottles.

2.3. Sample preparation

Urine did not undergo sample preparation to avoid the loss of information and analyze all the desired contaminants that have different physico-chemical properties. Moreover, the elimination of the sample preparation step limits the duration of the analytical process. Unlike the more selective techniques, the lack of sample preparation makes the analysis simple and fast [29,30] and this is of particular importance in the context of the Exposome which often requires high throughput methods. However, after a cycle of freezing and thawing, urine becomes cloudier. Indeed, urine samples can contain high concentrations of materials in suspension, including calculi, cellular components or precipitated protein fragments [36]. To inject the clearest solution possible and not obstruct the injector or column, urine was allowed to stand for a few minutes as the proteins and suspended solids settled to the bottom of the tube. Only the supernatant was used for analysis.

The quantification of the analytes was performed using a matrix-matched calibration in pooled urine. The analytical development and validation were conducted on a mixture of the eight individual urine samples. For the development, pooled urine was spiked at 1 $\mu g.mL^{-1}$ by the addition of a mixture of targeted compounds. Each collected urine sample is different from one individual to another due to factors, such as age, gender, diet, urine collection time and volume [39]. As a result, the concentrations of contaminants and metabolites in urine are highly variable [40]. Accordingly, for the development and quantification of analytes, urine was pooled to average the differences of each urine sample. For the calibration, the aliquots of pooled urine were spiked with a mixture of standard solutions of contaminants at suitable concentrations. This spike causes a dilution of each range point with a dilution factor equal to 0.12 for the validation and calibration. All real urine samples were diluted by adding MilliQ water to have the same dilution factor that each range point. Moreover, the dilution factor is low which avoid loss of information and observe a maximum of contaminants in the sample. This step of dilution was not optimized.

2.4. HPLC–HRMS analysis

The liquid chromatography was performed on an UHPLC Ultimate 3000 (Thermo Scientific®, MA, USA) series LC system consisting of a ternary pump, autosampler and column compartment. The compounds ionized in the positive mode were separated by HPLC with an XSelect CSH reversed phase (2.1 × 100 mm; 3.5 μm) column from Waters® (Mississauga, Canada). The mobile phases were (A) 0.1% formic acid and 10 mM ammonium formate in MilliQ water and (B) 0.1% formic acid and 10 mM ammonium formate in MeCN with the following gradient: 2% (B) for 3 min, from 2% to 100% (B) in 7 min, and 100% (B) for 10 min. The compounds ionized in the negative mode were separated by HPLC with a Kinetex reversed phase (2.1 × 100 mm; 2.6 μm) column from Phenomenex®. The mobile phases were (A) 0.05% acetic acid in MilliQ water and (B) 0.05% acetic acid in MeCN with the following gradient: 5% (B) for 3 min, from 5% to 32% (B) in 12 min, from 32% to 50% (B) in 1 min, from 50% to 100% (B) in 14 min and 100% (B) for 5 min. For both ionization modes, the flow rate was 0.5 $mL.min^{-1}$, the injection volume was 5 μL in full loop, and the oven temperature was 50 °C.

A micrOTOF Q II (Bruker Daltonics® GmbH, Bremen, Germany) mass spectrometer, quadrupole and orthogonal acceleration time-of-flight tandem equipped with an electrospray ionization (ESI) source were coupled to the chromatographic system. In the positive electrospray ionization mode, the m/z range was from 50 to 600, and the capillary voltage was +2500 V. In the negative electrospray ionization mode, the m/z range was from 50 to 1000, and the capillary voltage was −3000 V. For both ionization modes, the source temperature was 250 °C, the nebulizer pressure (nitrogen) flow was 3 bars, and the dry gas (nitrogen) flow was 12 $L.min^{-1}$. Data were collected during 20 min from 50 to 600 m/z and during 35 min from 50 to 1000 m/z in the positive and negative ionization modes, respectively, using micrOToF Control software (Bruker Daltonics® GmbH, Bremen, Germany). Each individual standard solution at 1 $\mu g.mL^{-1}$ was infused into the mass spectrometer at 5 $\mu L.min^{-1}$. An external calibration of the mass spectrometer was performed before each sequence with a sodium formate solution, which was constituted of 200 mg of NaOH, 250 mL of isopropanol and 1 mL of formic acid. Moreover, close external calibration was achieved on each sample at the beginning of the acquisition by means of a valve system and syringe pump. All samples were acquired with a segment in the method corresponding to the calibration with sodium formate clusters. The segment was inserted in the method from 0.1 min to 0.5 min. The mean standard deviation for each sample was less than 1 ppm. The mass resolution was set at 7000 FWHM (m/z 200).

2.5. Data handling

The data analysis was performed using DataAnalysis software version 1.3 (Bruker Daltonics® GmbH), with the application QuantAnalysis. The samples were analyzed in scan mode. After that, an extract ion chromatogram at the exact mass corresponding to the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecule, with a 5 mDa mass window, was performed. If a chromatographic peak was present at the same retention time of the standard, the experimental accurate mass was measured and compared with the theoretical exact calculated from the molecular formula using the IsotopePattern program (Bruker Daltonics® GmbH). In addition to the use of accurate mass, the deviation of the isotopic pattern between the experimental spectrum and theoretical one was used. However, this criterion was not used as identification criteria because at low concentration, at a ratio signal to noise equal to ten, the experimental isotopic pattern was not visible with our apparatus. To confirm identification, we could use an additional identification ion or specific fragments of targeted compounds. Badoud et al. [31] have used these additional criteria when it was possible. However, these additional ion and fragments were not visible at low concentration. Thus, with a HRMS instrument, usually only two identification criteria are used. In our study, analyte identification was based on: (i) the comparison of the retention time of the analyte and a standard compound ($\pm$ 0.1 min); and (ii) the difference of the theoretical exact monoisotopic mass of the ion in the mass spectrum and the measured accurate monoisotopic masses of the analyte ($\leq$ 5 ppm).

The quantification of the analytes was performed using a matrix-matched calibration in pooled urine with an 18-points range from 2.5 $ng.mL^{-1}$ (C1) to 250.0 $ng.mL^{-1}$ (C18). These 18-points range covers all concentration levels of calibration range of each compound. All the injections were carried out in duplicate.

2.6. Validation of the method

The validation procedure of the entire method was conducted using a within-laboratory protocol employing spiked urine from volunteers. The analytical methods were validated based on the bioanalytical validation method guidelines adopted by the Food and Drug Administration (FDA) [41]. The validation procedure

includes: the calibration curve, inter- and intra-day precision, accuracy and limits of detection and quantification. The stability of targeted analytes in urine was not assessed because our method was developed for the analysis of fresh urine sample or with only one freeze and thaw cycle.

The mathematical model best fitting the calibration curve was determined by assessing the signal response of each compound from spiked pooled urine over a concentration range from 2.5 to 250.0 ng.mL^{-1}. For each compound, the model was evaluated in duplicate on three days.

Intra-day precision was determined according to three concentrations (low, medium and high) in five replicates for each concentration. The high concentration corresponded closest to the highest concentration of the standard curve, which was approximately 225.0 ng.mL^{-1} for all molecules. The medium concentration corresponded to the concentration in the middle of the range, which was approximately 150.0 ng.mL^{-1}. The tested low concentrations were 112.5 ng.mL^{-1}, 62.5 ng.mL^{-1} and 30.0 ng .mL^{-1} in the positive electrospray ionization mode, and 62.5 ng.mL^{-1} 20.0 ng.mL^{-1} and 5.0 ng.mL^{-1} in the negative electrospray ionization mode. Because each compound did not have the same calibration range and LOQ, the low concentration was different for each molecule. However, groups of molecules have been made to have the least different concentrations for the low concentration. Inter-day precision was determined for three concentrations, the same as for intra-day precision, in five replicates for each concentration and on three days. The intra-day repeatability and inter-day reproducibility were expressed as relative standard deviations (RSD, %) for each concentration.

Accuracy was determined according to the same three concentrations (low, medium and high) for inter- and intra-day precision in five replicates for each concentration.

The limit of detection (LOD) is defined as the lowest concentration of the compound that can be detected [42], and the limit of quantification (LOQ) is associated with the smallest concentration that can be quantified with accuracy comprised between 80% and 120% and precision less than 20%. In the field of chemical analysis and in practice, several approaches exist to assess these two values [43]. The LOD and LOQ were determined as the analyte concentration that produced a peak signal of three and 10 times the background noise from the extracted ion current chromatograms, respectively. Nonetheless, some compounds were already present in the urinary matrix. The determination of these limits was therefore assessed on the standard deviation of a blank. For these substances, the detection and quantification limits were obtained as follows: Mean Blank Noise+3 standard deviation of blank and Mean Blank Noise+10 standard deviation of blank, respectively [44]. In the first step, it was necessary to inject a series of samples of the blank matrix (without spiking) to determine the standard deviation of the blank. In the second step, the samples that were spiked, ranging from 1.0 ng.mL^{-1} to 5000 ng.mL^{-1} were injected. After this first estimation of LOD and LOQ, the range was narrowed from 2.5 ng.mL^{-1} (C1) to 250.0 ng.mL^{-1}. These experiments led to the values of LOD and LOQ.

The FDA guidelines recommend evaluating matrix effects but do not provide guidance on how to determine them. Therefore, this criterion was assessed according to the European Medicines Agency (EMA) guidance for bioanalytical method validation, which details the procedure [45]. This guideline is widely used for biological matrix [46,47]. Thus, the matrix effects were determined using six lots of blank matrix from individual donors and six blanks of MilliQ water spiked at two concentrations (low and high), the same high and low concentrations chosen for the determination of precisions and accuracy. According to the EMA guideline, the matrix effects should be evaluated for each analyte and internal standard (IS). However, there are many different strategies of quantification. We have chosen to make a matrix-matched quantification but not by internal standard. Therefore, the matrix effects were calculated without IS as the following:

Matrix effect=[(peak area of urine spiked/peak area of water spiked)−1]*100

It is important to determine the matrix effects with the use of the LC–ESI–MS system. Indeed, electrospray source ionization can reduce or enhance the analyte response. Moreover, the absence of sample preparation steps can result in significant matrix effects despite the time savings. Indeed, some endogens compounds to the matrix can cause ionization competition. The EMA guideline recommends not using pooled matrix. Therefore, the matrix effects were determined on six urine samples from six different healthy donors (three males and three females). The responses were different from one sample to another because the urine samples varied considerably in composition. Therefore, area values for urine were averaged before the matrix effect computation.

3. Results and discussion

3.1. Selection of the targeted substances

In urine samples, generally metabolites are estimated for majority of organic molecules. However, the majority of the targeted compounds are parent molecules because the standards of metabolites are not always available. These contaminants have been selected according to (i) their frequency of interaction with humans and (ii) potential hazard. Selected contaminants can interact with human through diet, personal care products or by everything which surrounds humans during daily routines. Some of these substances have a proven negative effect on humans, and others do not. For example, bisphenol A, which was recognized as an endocrine disruptor, was used in baby bottles until 2010 in France [48]. However, this contaminant is still used in receipts and cans, among other products. Other substances are controversial and suspected to be toxic, in particular parabens [49,50]. People are also exposed to many other contaminants which ANSES judge as priority substances, especially pesticides and veterinary drugs [32,35]. Some of selected pesticides are banned in the EU but these compounds are still found in fruit and vegetable. These contaminants have been also selected according to (iii) different physico-chemical properties, and (iv) analytical feasibility using LC–MS. Indeed, the range of molecular weight extends from 102.16 g.mol^{-1} to 464.08 g.mol^{-1} for ethylene thiourea and perfluorononanoic acid, respectively. The polarity range is wide with log $K_{o/w}$ between −0.9 and 6.8, for acephate and sodium dodecylbenzenesulfonate, respectively. The range of pK_a extends from 0.4 to 13.5, for clorsulon and diuron, respectively. These compounds were analyzed in the raw urinary matrix by high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC–HRMS) with a ToF mass spectrometer.

3.2. LC–HRMS optimization

During the optimization of the experimental parameters, some compromises have been made to obtain a suitable analysis for all the contaminants since the number of contaminants to be analyzed is high and they have very different physico-chemical properties

3.2.1. IC separation

For the analysis in positive mode, an XSelect CSH reversed phase (2.1 × 100 mm; 3.5 μm) column was chosen because it improved the peak shape for basic compounds and provided the best separation. The addition of formic acid and ammonium formate in

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the mobile phase was tested during the development of the method. Indeed, formic acid and ammonium formate were identified as the preferred organic modifiers for optimal detection (3.1.2.). To fully optimize the separation, various gradients composed of MeCN/water with formic acid and ammonium formate were used. Different concentrations of formic acid and ammonium formate were tested. For the majority of the molecules, the optimum range is obtained with 0.1% formic acid and 10 mM of ammonium formate. The conditions were also selected to obtain the best signal-to-noise ratio and area. Moreover, a compromise has been made to obtain an optimal separation and detection. The separation of the twenty targeted analytes was performed in 20 min.

For the negative mode analysis, a Kinetex reversed phase (2.1×100 mm; 2.6 μm) column was chosen because a core shell column yields narrow peaks and increases resolution. An organic modifier was added to improve retention. The addition of acetic acid in the mobile phase was tested. Indeed, the best results with organic modifier were obtained with acetic acid for the ionization (3.1.2.). The impact of concentration of acetic acid in the mobile phase on the contaminants response is demonstrated in Fig. 1. Only the most significant responses are illustrated. Three trends were observed. The normalized area of bisphenol A, butylparaben and propylparaben decreased gradually with the percentage of acetic acid. We observed the opposite phenomenon to sodium dodecylbenzenesulfonate, dibutyl phosphate and bis(2-ethylhexyl) phosphate. Their normalized area increased gradually with the percentage of acetic acid. In case of the ESI–MS analysis, it is well known that pK_a is an important parameter for the ionization mode prediction [51]. Regarding these three compounds (dibutyl phosphate $pK_a = 2.32$, sodium dodecylbenzenesulfonate $pK_a = 2.55$ and bis(2-ethylhexyl) phosphate $pK_a = 1.47$), it can be concluded that the previously quoted molecules exhibits a preferential ionization in the negative mode. According to the literature, the improvement in the response of these compounds may be explained by the fact that this weak acid (i) enhances the formation of negatively charged droplets by providing cations (H^+) that can be easily reduced by electrochemical reaction and (ii) provides high gas-phase proton affinity anions ($H_3C–COO^-$) that efficiently deprotonate the analytes [52]. The last observed trend was for 4-hydroxybenzoic acid, the normalized area did not vary significantly for different concentrations of acetic acid. Nevertheless, the normalized area of the majority of compounds decreased gradually with the percentage of acetic acid as for bisphenol A, butylparaben and propylparaben. Considering the different responses of the targeted compounds as a function of the percentage of acetic acid in the negative mode, a compromise was needed. The chosen acetic acid concentration was 0.05%. With the optimized conditions, the compounds were separated in 35 min. The most important goal was to separate both isomers propylparaben and isopropylparaben. It was possible with these conditions because the retention times (RT) of propylparaben and isopropylparaben were 14.6 and 14.3 min, respectively.

Urine is a complex biological fluid, which was not subjected to sample preparation. So the obtained chromatograms are rich in information. Even with an extract ion current chromatogram and an accuracy of 50 Da, components contained in urine were detected with similar monoisotopic masses of targeted contaminants, explaining why a large number of peaks are visible on the chromatogram, as illustrated in Fig. 2.

3.2.2. Mass parameters

Detection and quantification were realized in scan mode by monitoring the most intense ions of each contaminant e.g. $[M+H]^+$ or $[M-H]^-$ to obtain the best sensitivity. Indeed, the use of QqToF mode caused a loss of sensitivity.

The optimization of the detection in HRMS was realized in several steps. First, each individual standard solution was infused into the mass spectrometer to characterize each contaminant and create our internal database which will be used for the targeted screening. The ionization mode (positive and negative) for each targeted molecule and its majority adduct ion were then determined. Table 1 summarizes the exact monoisotopic masses of adduct ions and ionization mode used for each compound. After this step, the ionization conditions in terms of solvent composition were optimized by infusing the mixture of molecules in the positive and negative modes. Various organic modifiers were added to compare the intensity of each molecule. For the optimization of the detection in mass spectrometry, the best response was obtained without organic modifiers in the negative mode and with ammonium formate and formic acid in the positive mode. However, these conditions were not optimal for the separation in the negative mode. Therefore, compromises had to be made. Indeed, the best separation and detection were obtained with acetic acid in the negative mode. During this step, the transfer settings were also optimized. The MS parameters permit the analyte ions to be separated from the drying gas and solvent and optimally transferred to the detector, with minimal losses.

After the chromatographic development, the optimization of electrospray ionization source settings was realized to obtain a good signal and high sensitivity for all desired compounds. These parameters include the incoming pressure of the nebulizer gas, flow rate of the drying gas, drying gas temperature and voltage applied to the metal cap of the glass capillary at the entrance of the vacuum system.

The parameter that exhibits the greatest impact on the ionization of the compounds in ESI interface is the drying gas temperature [53]. Four temperatures were tested for the ESI interface (for both positive and negative modes), ranging from 200 to 350 °C with steps of 50 °C. The impact of the drying gas temperature on

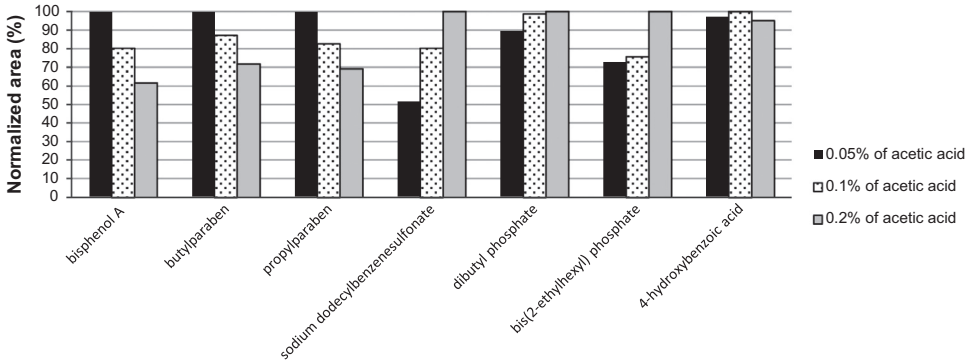

Fig. 1. Normalized area of seven targeted compounds with the use of percentage of acetic acid in the mobile phase, in the negative electrospray ionization mode.

Please cite this article as: A. Cortéjade, et al., Talanta (2015), http://dx.doi.org/10.1016/j.talanta.2015.06.038

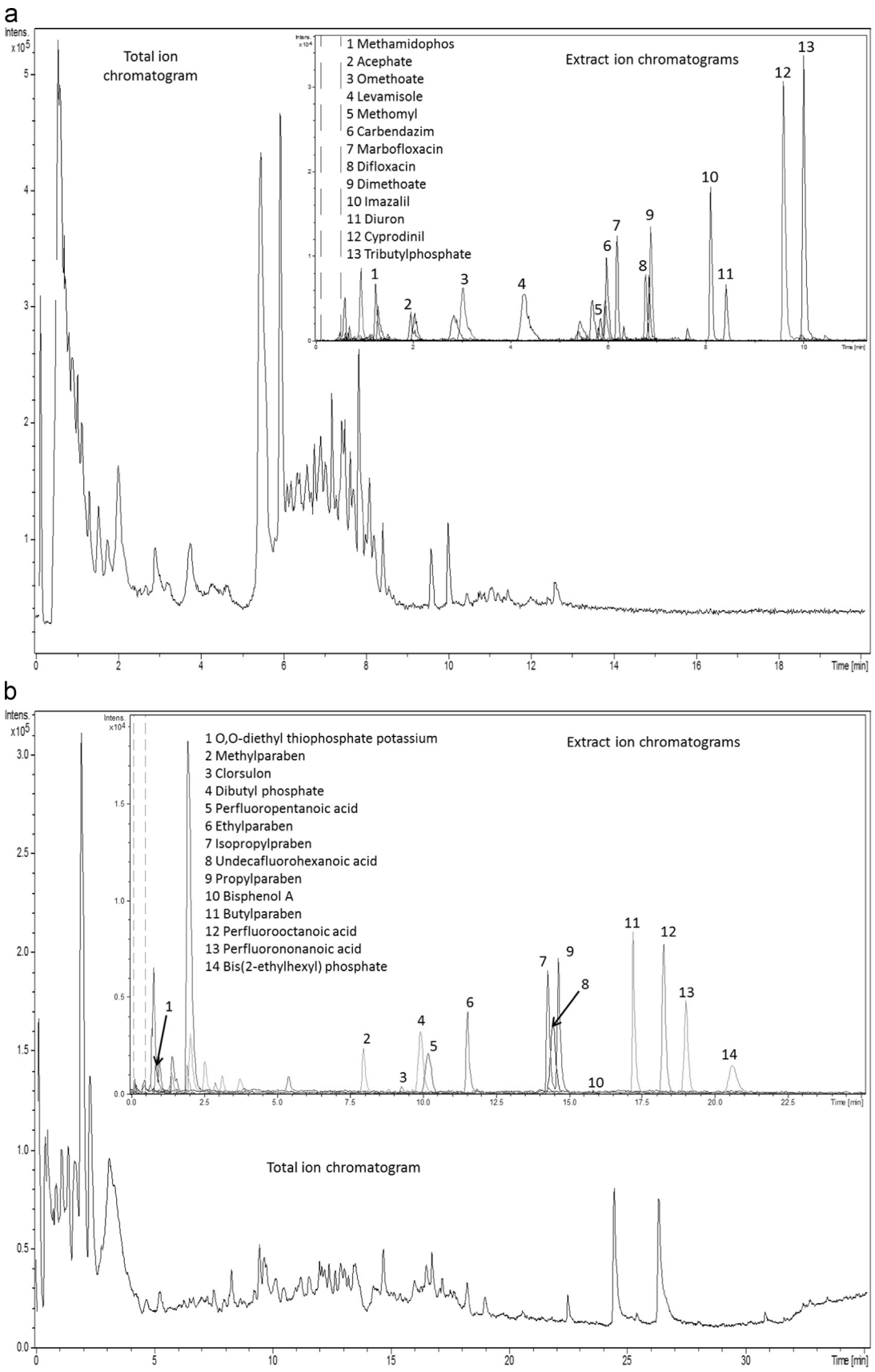

Fig. 2. Chromatograms of the 27 targeted contaminants in the urine spiked by 250.0 ng · g^{-1} in the negative (a) and the positive (b) electrospray ionization mode.

Table 1
Selected compounds and their molecular formulae, CAS number, retention time (RT) and exact mass of their adduct ion covered by the proposed methods.

Compound	Formula	CAS RN	RT (min)	Exact mass of adduct ion (Da)
Positive mode				$[M+H]^+$
Diuron	$C_9H_{10}Cl_2N_2O$	330-54-1	8.43	233.0248
Carbendazim	$C_9H_9N_3O_2$	10605-21-7	5.94	192.0773
Dimethoate	$C_5H_{12}NO_3PS_2$	60-51-5	6.89	230.0075
Imazalil	$C_{14}H_{14}Cl_2N_2O$	35554-44-0	8.10	297.0561
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	115550-35-1	6.18	363.1469
Difloxacin	$C_{21}H_{19}F_2N_3O_3$	98106-17-3	6.77	400.1473
Tributyl phosphate	$C_{12}H_{27}O_4P$	126-73-8	10.05	267.1725
Omethoate	$C_5H_{12}NO_4PS$	1113-02-6	3.11	214.0303
Methamidophos	$C_2H_8NO_2PS$	10265-92-6	1.27	142.0092
Levamisole	$C_{11}H_{12}N_2S$	14769-73-4	4.25	205.0799
Cyprodinil	$C_{14}H_{15}N_3$	121552-61-2	9.62	226.1344
Methomyl	$C_5H_{10}N_2O_2S$	16752-77-5	5.88	163.0541
Acephate	$C_4H_{10}NO_3PS$	30560-19-1	2.01	184.0197
Cyasorb UV 9	$C_{14}H_{12}O_3$	131-57-7	9.62	229.0865
Linuron	$C_9H_{10}Cl_2N_2O_2$	330-55-2	9.03	249.0198
Ethylene thiourea	$C_3H_6N_2S$	96-45-7	0.73	103.0330
Danofloxacin	$C_{19}H_{20}FN_3O_3$	112398-08-0	6.45	358.1567
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	93106-60-6	6.53	360.1723
Dicyclanil	$C_8H_{10}N_6$	112636-83-6	2.87	191.1045
4'-hydroxyacetophenone	$C_8H_8O_2$	99-93-4	6.43	137.0603
Negative mode				$[M-H]^-$
Dibutyl phosphate	$C_8H_{19}O_4P$	107-66-4	10.01	209.0943
Perfluoropentanoic acid	$C_5HF_9O_2$	2706-90-3	10.37	262.9755
Undecafluorohexanoic acid	$C_6HF_{11}O_2$	307-24-4	14.60	312.9723
Methylparaben	$C_8H_8O_3$	99-76-3	8.03	151.0395
Ethylparaben	$C_9H_{10}O_3$	120-47-8	11.57	165.0552
Isopropylparaben	$C_{10}H_{12}O_3$	4191-73-5	14.31	179.0708
Propylparaben	$C_{10}H_{12}O_3$	94-13-3	14.67	179.0708
Butylparaben	$C_{11}H_{14}O_3$	94-26-8	17.20	193.0865
Clorsulon	$C_8H_8Cl_3N_3O_4S_2$	60200-06-8	9.35	377.8944
Perfluorononanoic acid	$C_9HF_{17}O_2$	375-95-1	19.18	462.9627
Perfluorooctanoic acid	$C_8HF_{15}0_2$	335-67-1	18.35	412.9659
Bis(2-ethylhexyl) phosphate	$C_{16}H_{35}O_4P$	298-07-7	20.80	321.2195
O,O-diethyl thiophosphate potassium	$C_4H_{10}KO_3PS$	5871-17-0	1.01	169.0088
2-phenylphenol	$C_{12}H_{10}O$	90-43-7	16.85	169.0653
Heptafluorobutyric acid	$C_4HF_7O_2$	375-22-4	2.96	212.9787
4-hydroxybenzoic acid	$C_7H_6O_3$	99-96-7	1.81	137.0239
Sodium dodecylbenzenesulfonate	$C_{18}H_{29}NaO_3S$	25155-30-0	21.46	325.1837
Bisphenol A	$C_{15}H_{16}O_2$	80-05-7	15.80	227.1072

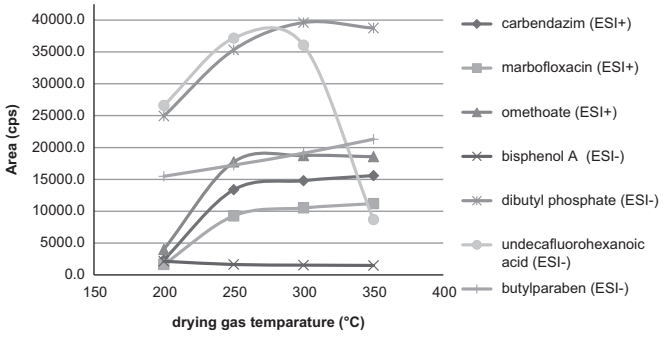

Fig. 3. Influence of the drying gas temperature on the response of seven targeted compounds.

the ionization of seven target compounds is demonstrated in Fig. 3. Only the most significant responses are illustrated. In the positive mode, the area of the majority of compounds increased gradually with temperature. For example, the response of marbofloxacin and carbendazim increased by a factor of six. Nevertheless, this phenomenon was observed for all contaminants ionized in the positive mode, except for six compounds (linuron, methomyl, dicyclanil, acephate, methamidophos and omethoate). These contaminants may have undergone degradation with the increase of the drying gas temperature. The area of five compounds gradually increased with temperature from 200 to 250 °C and decreased from 250 to 350 °C. For omethoate, the area increased from 200 to 250 °C, and then seemed to stabilize up to 350 °C. Considering the different responses of the target compounds as a function of the drying gas temperature in the positive mode, a compromise was needed and the final temperature was 250 °C. In the negative mode, different phenomena were observed. For example, the responses of parabens gradually increased with temperature as for butylparaben, while the response of bisphenol A gradually decreased with temperature. On the other hand, the response of dibutyl phosphate gradually increased with temperature from 200 to 300 °C and decreased from 300 to 350 °C. In addition, the response of undecafluorohexanoic acid gradually increased with temperature from 200 to 250 °C and decreased from 250 to 350 °C. A compromise had to be found between the maximum drying gas temperature and the minimum decomposition temperature of each compound because this parameter is compound-dependent. Finally, the drying gas temperature was set to 250 °C in both positive and negative modes.

Three incoming pressures of the nebulizer gas were tested in the positive and negative ionization modes. In the positive mode, the response of the majority of compounds increased gradually with the pressure of the nebulizer gas. However, for six

Table 2
Performances and validation of the LC–HRMS method for each compound (concentration range, model of regression (MOR), limit of detection (LOD), limit of quantification (LOQ), matrix effects, correlation coefficient (R^2) for three days (D1, D2 and D3), inter- and intra-day precision and accuracy obtained on three levels of dynamic range (low (C1), middle (C2), and high levels (C3)).

Compound	Range (ng mL^{-1})	MOR	LOD (ng.mL^{-1})	LOQ (ng.mL^{-1})	Matrix effect (%)		R^2			Inter-day precision (%RSD)			Intra-day precision (%RSD)			Accuracy (%)		
					C1	C3	D1	D2	D3	C1	C2	C3	C1	C2	C3	C1	C2	C3
Positive mode																		
Diuron	27.3–202.1	Quadratic	4.6	27.3	−35	−25	0.995	0.996	0.995	6	4	2	5	5	9	100	56	95
Carbendazim	30.0–222.4	Linear	16.0	30.0	−41	−68	0.994	0.994	0.995	10	6	6	2	3	8	120	101	89
Dimethoate	14.9–240.6	Quadratic	4.1	14.9	−37	−40	0.996	0.994	0.996	10	3	5	6	3	8	95	101	94
Imazalil	18.7–208.0	Linear	8.1	18.7	55	103	0.997	0.995	0.997	6	1	1	2	4	6	103	99	100
Marbofloxacin	13.1–213.6	Linear	3.6	13.1	175	−12	0.998	0.990	0.996	6	4	4	3	2	9	111	96	98
Difloxacin	15.9–172.0	Quadratic	3.0	15.9	150	9	0.988	0.994	0.996	13	5	5	9	1	9	100	98	104
Tributyl phosphate	21.1–291.0	Quadratic	4.9	21.1	39	77	0.997	0.995	0.999	4	2	2	9	2	5	92	105	99
Omethoate	34.7–258.2	Quadratic	10.1	34.7	−10	−23	0.997	0.991	0.997	9	5	2	1	3	5	118	105	98
Methamidophos	30.1–221.2	Quadratic	8.5	30.1	−54	−55	0.994	0.988	0.992	21	7	3	7	6	7	120	108	93
Levamisole	38.1–178.4	Quadratic	16.1	38.1	17	2	0.995	0.993	0.997	5	2	2	3	3	5	100	103	102
Cyprodinil	13.7–222.6	Quadratic	3.7	13.7	18	30	0.998	0.998	0.999	4	2	2	2	1	6	101	102	98
Methomyl	113.2–216.6	Quadratic	41.8	113.2	−20	−26	0.895	0.956	0.967	7	9	4	15	2	5	101	102	83
Acephate	63.9–228.3	Linear	46.0	63.9	35	−44	0.968	0.948	0.958	3	7	7	5	10	28	109	84	93
Negative mode																		
Dibutyl phosphate	12.2–199.8	Linear	7.8	12.2	−5	−23	0.997	0.999	0.998	5	2	2	11	9	3	92	92	85
Perfluoropentanoic acid	6.5–166.2	Quadratic	3.7	6.5	−9	−2	0.998	0.998	0.999	22	14	13	6	4	2	116	100	102
Undecafluorohexanoic acid	4.3–193.7	Quadratic	2.2	4.3	−8	−4	0.999	0.999	0.999	19	3	2	5	3	1	89	102	101
Methylparaben	17.7–198.7	Quadratic	14.4	17.7	−55	−65	0.993	0.998	0.997	19	3	5	7	15	5	90	83	95
Ethylparaben	11.9–191.6	Linear	7.6	11.9	−56	−66	0.994	0.993	0.990	12	4	6	7	10	2	101	112	100
Isopropylparaben	14.0–228.9	Quadratic	2.6	14.0	−51	−58	0.998	0.999	0.997	7	2	2	12	11	3	101	109	102
Propylparaben	13.8–225.9	Quadratic	8.8	13.8	−54	−59	0.998	0.999	0.999	4	2	2	9	13	8	99	112	109
Butylparaben	7.3–188.1	Quadratic	4.2	7.3	−43	−59	0.998	0.999	0.998	6	3	1	9	9	3	100	107	99
Clorsulon	19.6–270.4	Quadratic	10.5	19.6	−64	−75	0.997	0.989	0.993	11	7	2	16	33	4	106	125	98
Perfluorononanoic acid	4.8–213.8	Quadratic	2.4	4.8	−69	−51	0.989	0.986	0.992	9	5	6	4	8	1	34	89	100

contaminants, the best response was obtained with a pressure of 3 bars. There was no significant difference between 3 and 4 bars for the majority of molecules, except for two carbamates (carbendazim and methomyl). In the negative mode, the best response was obtained with 3 bars for the majority of molecules. However, for two contaminants (bisphenol A and 2-phenylphenol), the best significant response was obtained with 1 bar. Considering the different responses of the target compounds as a function of the pressure of the nebulizer gas in the positive and negative modes, the value was set to 3 bars for both modes.

To maximize the ionization in the source, four flow rates of the drying gas were compared: 3, 5, 8 and 12 L.min^{-1}. In the positive mode, the best response from the majority of compounds was obtained with 12 L.min^{-1}. Nevertheless, the response of bisphenol A increased by a factor of two from 12 to 8 L.min^{-1}. With the value of 12 L.min^{-1}, most of the target compounds showed the best response in the negative mode. Therefore, to achieve the best sensitivity given the flow rate and the composition of mobile phase and matrix, a flow rate of 12 L.min^{-1} was selected for the drying gas in both positive and negative ionization mode.

The cap capillary voltage role is crucial for efficient ion transfer to obtain the best sensitivity. Different cap capillary voltage values were tested, ranging from −3500 V to −5500 V and from +2500 V to +4500 V with steps of 1000 V in the positive and negative ionization modes, respectively. In the positive mode, the best areas were obtained mainly with −5500 V. However, in this condition, repeatability was not satisfactory (RSD > 20%). The second best areas were obtained with −3500 V with a higher signal-to-noise ratio and satisfactory repeatability. In the negative mode, the area of the majority of compounds decreased gradually with the increasing cap capillary voltage. Nevertheless, this model was not applied to five contaminants. The area of two compounds (bisphenol A and 2-phenylphenol) gradually increased with cap capillary voltage. Three analytes (sodium dodecylbenzenesulfonate, 4-hydroxybenzoic acid and O,O-diethyl thiophosphate potassium) were not sensitive to the cap capillary voltage and their normalized responses were roughly equal over the range of values. The best compromise to detect the maximum number of substances was to use +2500 V. Finally, the cap capillary voltage was set to −3500 V and +2500 V in the positive and negative modes, respectively.

3.3. Validation of the method

The validation study was conducted to demonstrate the performance of the methods. The developed analytical methods were validated by characterizing the best mathematical fit to the calibration curve for each day of validation D1, D2 and D3, inter- and intra-day precision, accuracy, and limits of detection and quantification. The results are summarized in Table 2. As the matrix was urine, a biological matrix, the bioanalytical validation method from the Food and Drug Administration (FDA) guidelines was used to validate the method because this method is the one most commonly used in cases of biological samples [54, 55], particularly for urine [56]. However, it is more restrictive in terms of number of repetition as others guidelines, such as the recommendations provided by the International Conference Harmonization ICH/2005 directives [57]. For example, the FDA guidelines recommend five repetitions, while ICH directives recommend three. Because there was no sample preparation step, the mixture of compounds was prepared every day of validation. Since the samples were provided by healthy subjects, we decided to focus on the compounds for which small amounts could be measured i.e. having rather low LOQs. Thus, only the compounds with an estimated LOQ less than 200 ng.mL^{-1} and a concentration range corresponding to a suitable mathematical model underwent the method validation processes. Thus, only 27 compounds have undergone the method validation process. Indeed, 11 compounds (sodium dodecylbenzenesulfonate, cyasorb UV 9, ethylene thiourea, heptafluorobutyric acid, 4-hydroxybenzoic acid, dicyclanil, 4'-hydroxyacetophenone, danofloxacin, enrofloxacin, 2-phenylphenol and linuron) have not undergone the validation process. The ranges of concentration corresponding to suitable mathematical models are varied, according to the compounds. Therefore, the validation protocol has been adapted for our study. The plan of validation is represented in Table 3.

The FDA guidelines recommend a minimum of six standard points for the calibration curve. For six contaminants (carbendazim, imazalil, marbofloxacin, acephate, dibutyl phosphate, and ethylparaben), a linear model provides the best fit (R^2, accuracy and precision) to the calibration curves. In contrast, for all others substances, a quadratic model provides the best fit. The adequacy of the chosen model was evaluated using the correlation coefficient (R^2). The correlation coefficients were higher than 0.99 over the three days for all compounds, except for six contaminants. For methomyl and acephate, their correlation coefficients were greater than 0.89. For perfluorononanoic acid, clorsulon, difloxacin and methamidophos, the correlation coefficients were greater than 0.98 or higher than 0.99 throughout the validation process. It was not possible to validate the analytical protocol for four contaminants (bisphenol A, bis(2-ethylhexyl) phosphate, O,O-diethyl

Table 3
Validation plan on three days for each compounds (concentration range, number of point per calibration range and concentration for the three spiked levels of dynamic range (low (C1), middle (C2), and high levels (C3)) at day 1 (D1), day 2 (D2) and day 3 (D3)).

Compound	Range (ng.mL^{-1})	Number of point per range	Concentration for precision, accuracy and matrix effects (ng.mL^{-1})		
			C1 D3	C2 D2	C3 D1
Positive mode					
Diuron	25.0–250.0	12	30.0	150.0	225.0
Carbendazim	25.0–250.0	11	30.0	150.0	225.0
Dimethoate	25.0–250.0	14	30.0	150.0	225.0
Imazalil	25.0–250.0	12	30.0	150.0	225.0
Marbofloxacin	25.0–250.0	14	30.0	150.0	225.0
Difloxacin	25.0–250.0	12	30.0	150.0	225.0
Tributyl phosphate	5.0–250.0	14	30.0	150.0	225.0
Omethoate	75.0–250.0	11	30.0	150.0	225.0
Methamidophos	10.0–250.0	11	30.0	150.0	225.0
Cyprodinil	10.0–250.0	14	30.0	150.0	225.0
Acephate	10.0–250.0	8	62.5	150.0	225.0
Levamisole	25.0–250.0	8	62.5	150.0	225.0
Methomyl	75.0–250.0	6	112.5	150.0	225.0
Negative mode					
Perfluorononanoic acid	25.0–250.0	14	5.0	150.0	225.0
Bis(2-ethylhexyl) phosphate	5.0–250.0	9	5.0	150.0	225.0
Undecafluorohexanoic acid	5.0–250.0	16	5.0	150.0	225.0
Dibutyl phosphate	5.0–250.0	14	20.0	150.0	225.0
Perfluoropentanoic acid	5.0–250.0	16	20.0	150.0	225.0
Methylparaben	10.0–250.0	14	20.0	150.0	225.0
Ethylparaben	25.0–250.0	14	20.0	150.0	225.0
Isopropylparaben	5.0–250.0	16	20.0	150.0	225.0
Propylparaben	5.0–250.0	16	20.0	150.0	225.0
Butylparaben	5.0–250.0	16	20.0	150.0	225.0
Clorsulon	75.0–250.0	14	20.0	150.0	225.0
Perfluorooctanoic acid	10.0–250.0	16	20.0	150.0	225.0
O,O-diethyl thiophosphate potassium	25.0–250.0	9	62.5	150.0	225.0
Bisphenol A	75.0–250.0	9	62.5	150.0	225.0

thiophosphate potassium and perfluorooctanoic acid) in the desired concentration range because all points did not produce a defined model. Bisphenol A range was not wide enough for the validation. O,O-diethyl thiophosphate potassium was coeluted with an endogenous substance present in urine. The analytical protocol for perfluorooctanoic acid was not validated because a potential endogenous interfering present in urine had an excellent response with this method. Thus, the results of validation process were presented only for 23 compounds.

For the determination of the LOD and LOQ several strategies are described in the literature [43]. Among them, the one relying on the signal-to-noise ratio is faster and therefore preferred. However, a urine sample free of all targeted analytes could not be provided. Indeed, three compounds were already present in pooled urine: perfluorooctanoic acid, sodium dodecylbenzenesulfonate and 4-hydroxybenzoic acid. For each compound, their presence was confirmed by only two criteria: retention time and exact mass. But from a certain concentration, above the LOQ, isotopic profiles are visible and its correspond to a third identification criteria. Thus, for all compounds, we have confirmed identification of targeted substances with three criteria for the top of the calibration range. In this case, a determination of LOD and LOQ was established from urine and urine spiked by the analyte. The LODs and LOQs were determined each day of validation and the final chosen value is the highest value obtained on three days. The limits of detection and quantification are listed in Table 2. To our knowledge, the concentrations of the targeted contaminants in the urine are not reported in the literature. Indeed, the targeted compounds are not systematically researched in urine. For example, only the most hazardous pesticides are researched in environmental or biological matrices. The values of LOD that we obtained for the targeted pesticides were similar to the concentrations of pesticides found in urine (7.62 $ng.mL^{-1}$ for 2,4-dichlorophenol, 10.56 $ng.mL^{-1}$ for 2,5-dichlorophenol) [58]. However, the response of compound and their LOD are different according to the molecule inter alia. The LODs of compounds varied between 2.4 and 46.0 $ng.mL^{-1}$. The method offered a LOD below 17 $ng.mL^{-1}$ for all contaminants, except for methomyl (41.8 $ng.mL^{-1}$) and acephate (46.0 $ng.mL^{-1}$). The LOQs of compounds ranged from 4.3 to 113.2 $ng.mL^{-1}$. The method offered a LOQ below 40 $ng.mL^{-1}$ for all contaminants, except for methomyl (113.2 $ng.mL^{-1}$) and acephate (63.9 $ng.mL^{-1}$). Various studies have quantified these contaminants by LC-ToF. In our knowledge, the targeted pesticides have not yet been quantified by a HRMS instrument, so we will compare the results obtained in other matrices. The multi-residue analysis of P. Sivaperumal et al. [24] yielded a LOQ of 10.1 $\mu g.kg^{-1}$ for dimethoate in fruit and vegetable, which is comparable to the value we obtained (14 $ng.mL^{-1}$). For acephate and carbendazim, the LOQs were better for P. Sivaperumal et al. than with our method. However, our results were obtained without sample preparation, only by diluting urine by a factor of 0.12, while P. Sivaperumal et al. extracted and concentrated their compounds. In the veterinary drugs family, several teams (M.P. Hermo et al. and D. Ortelli et al.) have obtained LOD values similar to ours for marbofloxacin and difloxacin. As expected for a generic method, the LOQs for some analytes are higher than those reported in the literature. For example, for perfluoropentanoic acid we obtained a limit of quantification of 4.8 $ng.mL^{-1}$ which could be improved by purification and sample concentration as shown in the literature (13.3 $pg.mL^{-1}$) [59]. This is also the case of levamisole (38.1 $ng.mL^{-1}$) for which the determined LOQ is higher than the values determined by GC–MS (0.15 $ng.mL^{-1}$) [60]. In our knowledge, parabens had not yet been quantified by LC-ToF. Few studies analyze these contaminants in urine. One such study [61] related to the analysis of diuron in human urine was based on the injection of urine to an HPLC-UV–vis detector without sample preparation. The obtained LOD and LOQ for diuron were 14.0 $ng.mL^{-1}$ and 40.0 $ng.mL^{-1}$, respectively. Mass spectrometry is more sensitive than a UV–vis absorbance detector; our results were better with 4.6 $ng.mL^{-1}$ and 27.3 $ng.mL^{-1}$ for the LOD and LOQ, respectively. Similarly, studies by R.K. Moos et al. [19] and A.G. Asimakopoulos et al. [22] related to the determination of parabens in urine samples by LC–MS/MS in MRM mode obtained better LOQs than our results because these teams used sample preparation. Moreover, tandem mass spectrometry in MRM mode is more sensitive than the high resolution mass spectrometer we used. Dibutyl phosphate was quantified by N. Van den Eede et al. [62] by LC–MS/MS in MRM mode with a LOQ amounting to 3.4 $ng.mL^{-1}$. Despite the fact that we did not use solid phase extraction technique, we had a higher LOQ (12.2 $ng.mL^{-1}$).

Inter- and intra-day precision were determined according to three concentrations levels. The obtained intra-day RSD values indicated satisfactory repeatability, with RSD < 20% for the low concentration and RSD < 15% for the medium and high concentrations for the majority of compounds. Acephate and clorsulon exhibited values of RSD for intra-day precision of 28% and 33% for the medium and high concentrations, respectively. As recommended in the FDA guidelines, all of the RSDs for inter-day reproducibility were less than 15% for the medium and high concentrations. For the low concentration, the RSD values of only two substances for inter-day precision were slightly higher than 20%. The RSD values amounted to 21% and 22% for methamidophos and perfluoropentanoic acid, respectively.

Accuracy, the degree of closeness of the determined value to the true value, was assessed based on the same three concentrations as for the inter- and intra-day precision. The accuracy was calculated as the following:

Accuracy = 100 − [(average calculated concentration/exact concentration) − exact concentration]*100

The calculated concentration was determined by using the equation of the calibration curve.

For the medium and high concentrations, most accuracy values were between 85% and 115%, except for diuron, which exhibited an accuracy of 56% at the medium concentration, and clorsulon with 125% at the medium concentration. For three other compounds (methomyl, acephate and methylparaben), the accuracy values were slightly outside the expected range (83%, 84% and 83%, respectively). All accuracy values ranged from 80% to 120% for the low concentration, except for perfluoropentanoic acid at 34%.

The impact of urine matrix on the method was evaluated by spiking water and urine with known amounts of contaminants at two different concentrations. The results are summarized in Table 2. A matrix effect was observed for all substances. Given that the urine sample was analyzed without sample preparation and diluted weakly, we expected to achieve significant matrix effects. For all contaminants ionized in the negative ionization mode, the matrix effects were negative, indicating a signal inhibition. In the positive ionization mode, there were more significant suppression signals than enhancements. An enhanced response was observed for five compounds (imazalil, difloxacin, tributyl phosphate, levamisole and cyprodinil) and a reduced response for six contaminants (diuron, carbendazim, dimethoate, omethoate, methamidophos and methomyl) at both concentration levels. For marbofloxacin and acephate, a phenomenon was highlighted: the signals were enhanced at low concentrations with matrix effects of 175% and 35%, respectively, and the signals were reduced at high concentrations with matrix effects of −12% and −44%, respectively. C. Jansson et al. [63] showed that the influence of the matrix could be significantly variable with concentration, particularly for pesticides. Indeed, the composition of urine varies from donors. In addition, urine contains over 3000 components [64] that can

interact and cause biological reactions according to the conditions, resulting in different matrix effects depending on the injection. Despite the fact that we had no sample preparation step, we obtained matrix effects for four parabens equal or better than those observed by other authors [22].

3.4. Application to various urinary samples

The multi-residue analytical method was applied to 17 first morning urines samples from colleagues of the laboratory. The objective was to demonstrate the applicability of the developed analytical method and thus to establish a wide screening of the presence of the target compounds in urine. To compensate for the matrix effects, the quantification method was based on a matrix-matched calibration method. Therefore, the urine pool used in validation and analytical development spiked with the 38 studied compounds was employed.

Of the 38 targeted environmental contaminants, four compounds were detected (tributyl phosphate, sodium dodecylbenzenesulfonate, 4-hydroxybenzoic acid and O,O-diethyl thiophosphate potassium), which represented 11% of all targeted contaminants. Tributyl phosphate was validated and detected in the samples. However, its concentration was lower than the LOQ. Total ion current chromatogram and extract ion current chromatogram of the majority adduct ion of tributyl phosphate in the first morning urine sample were illustrated in Fig. 4. Three others substances were detected. Nonetheless, sodium dodecylbenzenesulfonate and 4-hydroxybenzoic acid did not undergo the validation method process; thereby, they were unable to be quantified. O,O-diethyl thiophosphate potassium was unable to be quantified because this compound was not validated. Moreover, it was coeluted with an endogenous substance present in urine. The presence of all targeted compound was confirmed by only two criteria: retention time and exact mass. At low concentrations, isotopic profiles are not visible. This is why only two criteria are used in this case. To confirm identification, we could use more specific adduct or specific fragments of these targeted compounds. However, at low concentration, we have not visualized other specific adducts. Moreover, fragmentation does not allow visualizing fragments at this concentration.

Tributyl phosphate was already detected in human in adipose tissue [65]. According to some studies, O,O-diethyl thiophosphate potassium was found in different human samples, such as fecal samples [66], amniotic fluid [67] and urine [68]. Of the samples analyzed, this metabolite of pesticide was detected in 73% of the urine donors. To our knowledge, sodium dodecylbenzenesulfonate and 4-hydroxybenzoic acid have not been found in human samples. The results of our analyses indicated that two contaminants (tributyl phosphate and sodium dodecylbenzenesulfonate) were found in all samples. Indeed, these compounds are used in textiles and paper, which largely come in contact with donors of these samples in their daily lives. However, the analyses were performed on only 17 urine samples from donors with similar lifestyles. This application to various urinary samples demonstrated the feasibility of the method. It would be interesting to analyze samples from different cohorts and thus compare the results by statistical tools according to defined criteria; for example, the frequency of consumption of fruit and vegetable, with or without their skin, for pesticide research.

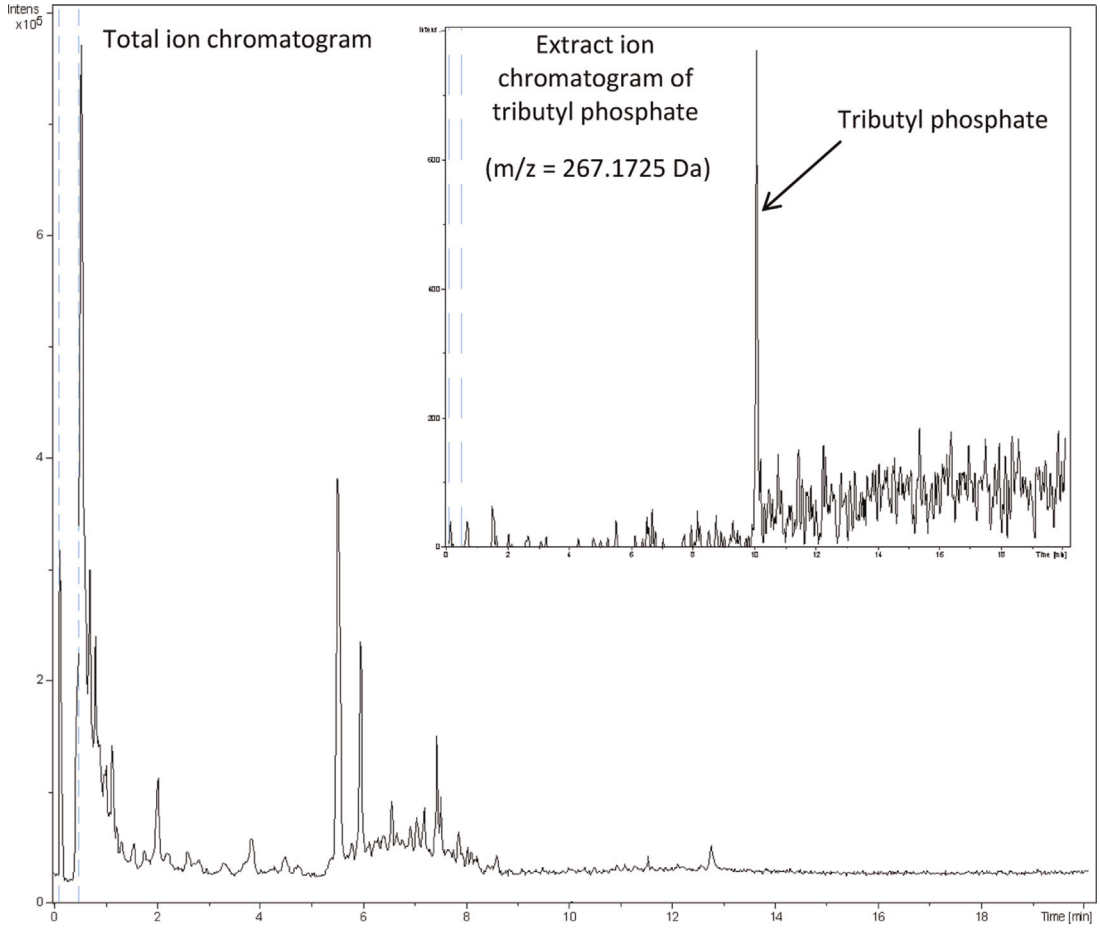

Fig. 4. Chromatograms from the analysis of one healthy donor urine containing tributyl phosphate in the positive electrospray ionization mode.

Please cite this article as: A. Cortéjade, et al., Talanta (2015), http://dx.doi.org/10.1016/j.talanta.2015.06.038

4. Conclusions

The developed method enables the detection of 38 contaminants of different chemical families, including the quantification of 23 compounds at low concentration levels ($ng.mL^{-1}$) in human urine. The applicability of the methodology is demonstrated by analyzing 17 first morning urine samples. Four contaminants were found in these samples. However, the samples were obtained from donors with similar lifestyles. It would be interesting to test this method on other cohorts. Our study of the Exposome begins by searching for targeted contaminants in the urine. This method is that it allows the detection and quantification of certain targeted environmental contaminants but also it could be used for the simultaneous screening for metabolites, degradation products or other non-targeted compounds. Indeed, as the method was developed on crude urine and on a LC-ToF instrument, a more comprehensive approach and screening are the next steps in the study of the Exposome to detect and quantify metabolites and degradation products of known or even unknown contaminants. Future studies will focus on the comparison of urine mapping from different cohorts with the aim of further studying the Exposome and upgrading the databases.

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