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Development and validation of a method for the detection and confirmation of biomarkers of exposure in human urine by means of restricted access material-liquid chromatography–tandem mass spectrometry

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ABSTRACT

The present article describes the development and validation of a LC–MS/MS method for the determination and confirmation of biomarkers of exposure to different types of xenobiotics in human urine. The method combines the use of a restricted access material (RAM) coupled on-line to a LC–IT-MS system; in this way, a rapid and efficient matrix cleanup was achieved, reducing manual sample preparation to freezing and sample filtration. The ion trap (IT) mass spectrometry detector provided the selectivity, sensitivity and ruggedness needed for confirmatory purposes. The on-line RAM-LC–MS/MS method developed here has been validated as a quantitative confirmatory method according to the European Union (EU) Decision 2002/657/EC. The validation steps included the verification of linearity, repeatability, specificity, trueness/recovery, reproducibility, stability and ruggedness in fortified urine samples. Repeatability and within-laboratory reproducibility, measured as intraday and interday precisions, were evaluated at two concentration levels, being 12.7% or below at the concentration corresponding to the quantification limits. Matrix effects and non-targeted qualitative analyses were also evaluated in fortified urine samples. Decision limits (CC_α) and detection capabilities (CC_β) were in the range of 3.6–16.5 and 6.0–28.1 ng mL^{-1} respectively. The results of the validation process revealed that the proposed method is suitable for reliable quantification and confirmation of biomarkers of exposure to xenobiotics in human urine at low ng mL^{-1} levels. In addition, working in Data-Dependent Scan mode the proposed method can be used for the screening of these compounds in urine samples.

1. Introduction

Human health is affected by all the activities of an individual, who is subject to a continuum of chemical exposures in the external environment, including air, water, soil and food. Biological monitoring involves the measurement and evaluation of chemical compounds or their metabolites (biomarkers of exposure) in biological fluids as a method for assessing the risk to health deriving from exposure to a toxic agent [1]. Biomonitoring may be used to assess the exposure (amount absorbed or internal dose) and the effects of chemicals and the susceptibility of individuals, and it may be applied regardless of whether such exposure has been from dietary, environmental or occupational sources. The data gleaned from biological monitoring studies can be used in conjugation with other data in clinical diagnosis, health risk assessment, and for risk management programs. Although many methods for measuring pesticides and industrial chemicals in human matrices have been reported, most of them refer to a limited number of compounds or analytes belonging to the same chemical group. There is an important need for validated methods that will allow the unequivocal identification and quantification of an important variety of xenobiotics of different chemical natures for application in the context of biomonitoring [2].

The analytical methodology used in the biological monitoring of exposure to pesticides and other chemicals is mainly based on chromatographic techniques. The application of liquid chromatography–mass spectrometry (LC–MS) in occupational and environmental toxicology has proved to be a very useful tool in the determination of biomarkers of exposure as well as in metabolism studies aimed at investigating new biomarkers [3,4].

In this context, tandem mass spectrometry (MS/MS) seems to be the most efficient technique for quantification and identification. The MS/MS detector most widely used is the triple quadrupole (QqQ), used in multiple reaction monitoring (MRM) mode [5–7]. However, in MRM mode the qualitative information necessary for full confirmation of analytes is lost at low concentration levels owing to the low sensitivity of the confirmation transition. Since confirmation of identification is insufficient with a single MS/MS

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transition, it is necessary to perform a second injection to confirm the positive samples [8,9]. Use of an ion trap (IT) has great potential as a confirmatory method because it allows a complete spectrum of the product ions to be obtained. Nevertheless, there are few applications in this field [10,11] owing to the low sensitivity of ITs when the number of analytes to be determined is high and to their poor reliability in complex matrices [12]. One good approach for addressing these limitations is by coupling the chromatographic system to an on-line preconcentration step [13]. This coupling also automates prior sample treatment, which in many cases is the limiting step in the analysis time.

Restricted access materials (RAMs) can be satisfactorily used for the on-line pretreatment of biological fluids in the analysis of low-molecular weight substances since they permit liquid–solid extraction and the concentration of small molecules. These materials act by limiting the access of macromolecules to the sorbent by means of a porous membrane [14], thereby achieving a highly efficient cleanup of the biological matrix. Souverain et al. [15] have reviewed a large number of applications in which endogenous and pharmaceutical compounds were purified in different biological matrices, such as plasma, urine, saliva and milk.

The aim of the present work was to develop and validate a sensitive and specific multiresidue method based on LC–IT-MS for the detection and confirmation of a broad variety of biomarkers of exposure to xenobiotics in human urine. The xenobiotics and their respective biomarkers of exposure used were selected on the basis of their importance in environmental studies: several pesticides, such as Carbaryl, one of the most popular domestic insecticides [16] and its metabolite, 1-naphthol; 2,4,5-T and 2,4,5-TP phenoxyacids, which are the herbicides most used domestically [17]; chlortoluron and diuron, which are phenylureas and are designated "priority hazardous substances" by the EU [18] and one of their metabolites, 3-chloro-4-methylphenylurea [19]. Several xenobiotics of industrial origin were also included, such as bisphenol-A and bisphenol-F, which are widely used in the preparation of epoxy resins and polycarbonates [20], trichlorophenol, associated with the always dangerous dioxins [21], and the already mentioned 1-naphthol, a metabolite of polycyclic aromatic hydrocarbons (PAHs) [3]. All these analytes and their metabolites are well known to be excreted in urine after environmental exposure [22–26].

To accomplish this, here we propose a combination of RAMs for a fast on-line sample treatment of the urine samples with the high selectivity of IT-MS, thus affording a rapid, automatic and sensitive multi-residue method that will permit the determination of a broad range of biomarkers of exposure to pesticides and industrial compounds, all fulfilling the requisites concerning full validation and identification legislated by the European Union [27].

In addition, a Data-Dependent Scan procedure for non-targeted qualitative analysis was developed. To our knowledge, this methodology has not been applied to the multiresidue analysis of biomarkers of exposure of very different chemical natures.

2. Experimental

2.1. Chemicals

Analytical standards of *chlortoluron* (CLT), 3-(3-chloro-4-methyl)-1,1-dimethylurea, CAS RN [15545-48-9]; *CMPU* (CMPU), *1*-(3-chloro-4-methylphenyl)urea, CAS RN [13142-64-8]; *diuron* (DIN), 3-(3,4-dichlorophenyl)-1,1-dimethylurea, CAS RN [330-54-1] and *1-naphthol* (1NPL), 1-hydroxynaphthalene, CAS RN [90-15-3] were purchased from Dr. Ehrenstorfer (Augsburg, Germany). *Bisphenol-A* (BPA), 2,2-bis(4-hydroxyphenyl)propane, CAS RN [80-05-7]; *bisphenol-F* (BPF), bis-(4-hydroxyphenyl)methane, CAS RN [620-92-8]; *trichlorophenol* (TCPL), 2,4,5-trichlorophenol, CAS RN [95-95-4]; *2*,4,5-T (245T), 2,4,5-trichlorophenoxyacetic acid, CAS RN [93-76-5] and 2,4,5-TP (245TP), 2-(2,4,5-trichlorophenoxy)propionic acid, CAS RN [93-72-1] were obtained from Sigma–Aldrich (Steinheim, Germany).

The organic solvents—acetonitrile (ACN) and methanol (MeOH)—were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Ultra-high quality (UHQ) water was obtained with an Elgastat UHQ water purification system.

All chemicals used for the preparation of the buffer and all other chemicals were of analytical reagent grade.

2.2. Instrumentation

HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany) equipped with a binary pump, an additional isocratic pump, a membrane degasser, an autosampler (equipped with a 1500-μL capillary seat), a six-port valve, and a diode-array detector (DAD). The system was controlled by a HP ChemStation which also performed data collection from the mass spectrometer and quantitative measurements. The restricted access material (RAM) used was a LiChroCART 25-4LiChrospher RP4 ADS (25 μm, 25 mm × 4 mm) from Merck (Darmstadt, Germany). The analytical column was a 150 × 4.60 mm Luna PFP(2) packed with 3 μm particles (Phenomenex, Torrance, CA, USA).

The clean-up isocratic mobile phase, impelled by the isocratic pump, consisted of a 2.5 mM ammonium formate buffer (pH 2.9) with 10% ACN. The separation mobile phase, impelled by the binary pump, consisted of an unbuffered UHQ water (solvent A) and methanol (Solvent B) gradient from 70% to 0% of A. The analytical column was thermostated at 25 °C.

2.2.1. Mass spectrometry

The LC/MSD Trap XCT ion trap mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray (ESI) source with a nebulizer spacer. The ESI settings were a capillary voltage of 3500 V; a drying gas flow of 10 L min^{-1} at a temperature of 350 °C, and a nebulizer pressure of 50 psi. Optimization of the ionization and fragmentation parameters was achieved manually while injecting standard solutions of each analyte (5 μg mL^{-1}) with a syringe pump at a flow rate of 1 mL/h; these solutions were mixed with the mobile phase at 0.3 mL/min by means of a T piece. The trap parameters were set at a smart target of 50,000–100,000 and a maximum accumulation time of 200 ms at an m/z range from 60 to 400 u. A narrow isolation width of 4u was selected. The optimized parameters and retention times for each analyte are listed in Table 1.

2.3. Sample collection

Urine samples collected from two healthy volunteers were used for method development and the preparation of calibration standards. Urine samples were collected in 250-mL brown glass bottles and frozen immediately until analysis. Before use, the samples were thawed at room temperature. An appropriate amount of urine was spiked with dilute analytical standards daily. Samples were filtered through 0.45 μm filters to remove precipitated proteins. Creatinine was determined in order to normalize the results with respect to the concentration and to rule out overdiluted or overconcentrated samples, the concentration values proving to be 87.7 and 126.8 mg dL^{-1}.

2.4. On-line sample preparation and HPLC separation

The experimental setup for RAM-LC–MS/MS is shown in Fig. 1, and the schedule for clean-up and chromatographic separation is shown in Table 2. First, a predetermined volume of urine was

Table 1
Ion trap tandem mass spectrometer parameters optimized for the studied biomarkers of exposure.

Analyte	t_R (min)	Quantification transition	Confirmation transition	Fragmentation amplitude (V)	ESI mode	Window
245T	19.8	253 ⇒ 195	255 ⇒ 197	0.70	–	18–25 min
245TP	23.4	267 ⇒ 195	269 ⇒ 197	0.64	–	18–25 min
CMPU	26.4	185 ⇒ 142	185 ⇒ 168	0.98	+	25–27 min
BPF	27.9	199 ⇒ 93	199 ⇒ 123	1.10	–	27–29.5 min
CLT	30.7	211 ⇒ 166	211 ⇒ 140	0.95	–	29.5–33 min
BPA	31.3	227 ⇒ 212	227 ⇒ 133	0.95	–	29.5–33 min
1NPL	32.3	143[a]	–[a]	–[a]	–	29.5–33 min
DIN	33.8	233 ⇒ 72	–[b]	0.70	+	33–34 min
TCPL	36.1	195 ⇒ 159	197 ⇒ 161	0.95	–	34–39 min

[a] No satisfactory fragmentation was found for 1NPL.
[b] Only one satisfactory fragmentation was found for DIN.

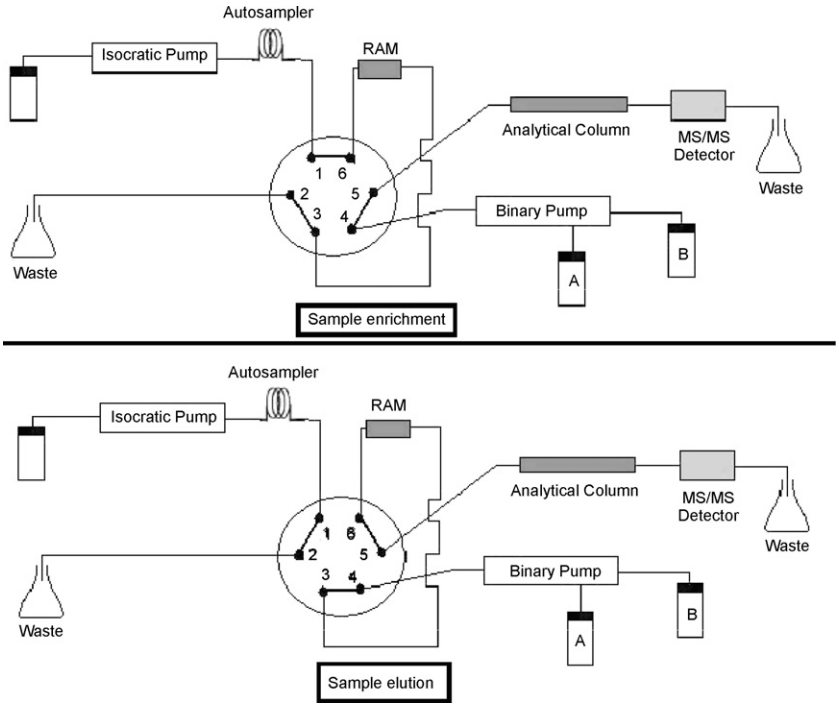

Fig. 1. RAM-LC–MS/MS instrumental setup. Top: switching valve in "sample enrichment" position. Bottom: valve in "sample elution" position (backflush mode).

injected with the autosampler and the isocratic pump was immediately started to pump the clean-up mobile phase at 1 mL min^{-1} for 10 min with the system in the "sample enrichment" position. While the matrix components of the urine were washed to waste, the xenobiotic compounds studied were withheld in the RAM. At 10 min, the system setup was changed to "sample elution" position and the separation gradient (binary pump), shown in Table 2, eluted the analytes at a flow rate of 0.8 mL min^{-1} in backflush mode to the analytical column, where they were separated and finally detected by the mass spectrometer. During this time, the isocratic pump changed the flow rate to 0.3 mL min^{-1} in order to save solvent until the next injection. At 37 min, the separation ended and the gradient was returned to the initial conditions. The flow rate of the isocratic pump was changed to 1 mL min^{-1}. At 39 min, the system was switched to "sample enrichment" mode and a 3-min post-run program was started, keeping the system in the initial conditions, in order to equilibrate the analytical column for the next analysis.

Table 2
Time procedure and valve events of RAM-LC–MS/MS method.

Time (min)	Isocractic pump[a] flow (mL min^{-1})	Binary pump[b,c] % A	Six-port valve position	Event
0–10	1	70	Enrichment	RAM charging and cleanup
10–11	1 ⇒ 0.3	70	Elution	Analyte transfer
11–12	0.3	70 ⇒ 50	Elution	Analyte separation
12–19	0.3	50	Elution	Analyte separation
19–30	0.3	50 ⇒ 30	Elution	Analyte separation
30–35	0.3	30 ⇒ 0	Elution	Analyte separation
35–37	0.3	0	Elution	Analyte separation
37–39	0.3 ⇒ 1	0 ⇒ 70	Elution	Re-equilibrating
39–42	1	70	Enrichment	Equilibrating

[a] Mobile phase: ammonium formate buffer 2.5 mM (pH 2.9) with 10% ACN.
[b] Flow rate: 0.8 mL min^{-1}.
[c] Mobile phase: A: UHQ water B: MeOH.

2.5. Standard preparation and calibration procedure

Individual stock solutions of the analytical standards, at 500 μg mL^{-1}, were prepared by dissolving 12.5 mg of each analyte in 25 mL of acetonitrile. These stock solutions were stored at 4 °C in brown glass bottles. Matrix-matched standards were prepared by adding the appropriate amount of each stock solution to urine. Calibration standards for quantification were prepared in the 10–200 ng mL^{-1} range. The standards were filtered before analysis with a Cameo (0.45 μm Nylon) filter. Calibration curves were obtained by plotting the peak areas of the analytes *versus* concentration using matrix-matched standards.

2.6. Method validation

The method was validated, according to the pertinent legislation [27], by evaluating the following parameters:

- Specificity: by calculating ion suppressions, comparing patterns with matrix-matched samples, and developing a non-targeted analysis.
- Determination of the statistical parameters and the quality of the linear regression, using the transition confirmation. The matrix-matched calibrations (five calibration points) were obtained in the 30–150 ng mL^{-1} range. The decision limit (CC_α) and the detection capability (CC_β) were also calculated.
- Repeatability and within-laboratory reproducibility were determined as intraday and interday precisions by analyzing urine samples at two concentrations levels (10 ng g^{-1} and at the respective limits of quantification).
- The recoveries and RSD% were determined at two concentration levels (25 and 12.5 ng mL^{-1}).
- Ruggedness: evaluating sample preparation by analyzing spiked urine samples before and after freezing and the possible memory effect of the system by analyzing blanks after high-concentration samples.
- Stability: by controlling the storage conditions of the analytes and samples, keeping them in the range in which stability is guaranteed by the manufacturer.

3. Results and discussion

Chromatographic separation was optimized, with the achievement of a satisfactory separation in 27 min on using a water/MeOH gradient. This mobile phase was chosen for the proposed method (Table 2) because it showed greater compatibility with the mass detector [28] than water/ACN mixtures. Additionally, no buffer was employed, since the presence of salts negatively affects the ionization of some analytes in the ESI [29].

3.1. Study of the behavior of the RAM coupled on-line to LC–MS/MS

Liquid chromatography analysis of complex matrices (urine, plasma, etc.) usually requires a previous sample treatment step to eliminate large amounts of interferents from the matrices, which positively affects selectivity and sensitivity and at the same time prolongs columns life and preserves the integrity of the instrumental setup. This step is especially important when mass spectrometry with electrospray (ESI) is used as detector since this source of ionization undergoes severe losses of sensitivity (ion suppression) owing to the presence of interferents. Among the different strategies described to minimize such suppression [30] the following are important: modification of the ionization conditions; the use of a suitable internal standard, usually a stable isotope-labeled ana-

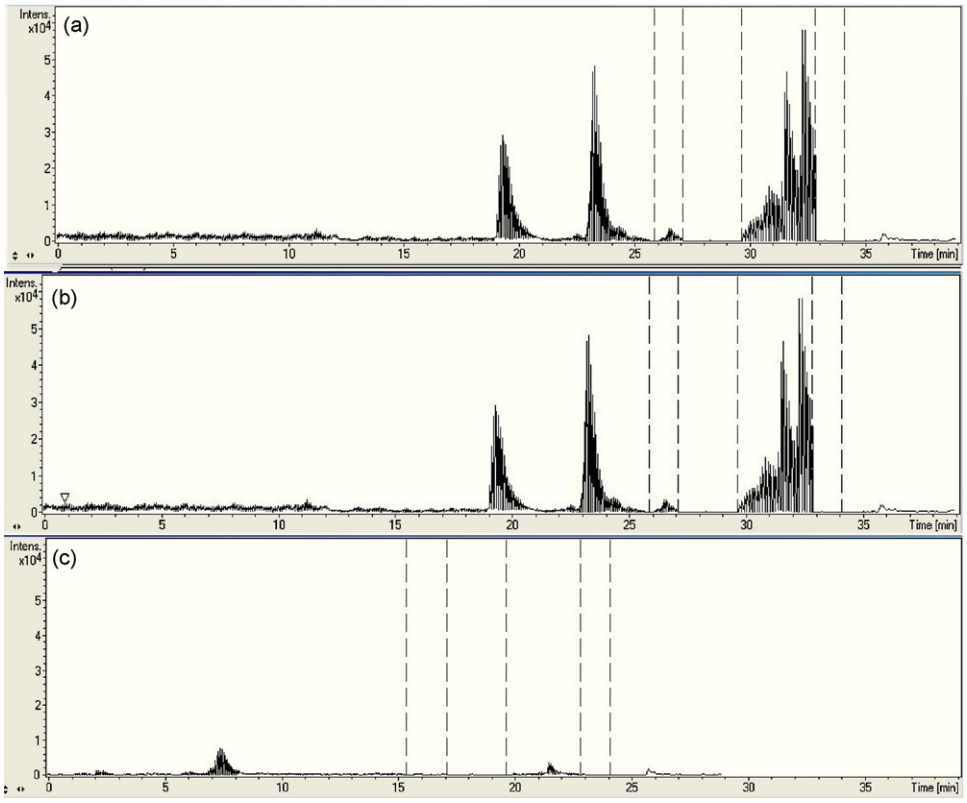

Fig. 2. Total ion chromatograms (TICs) of UHQ standard and urine samples spiked with 100 ng mL^{-1}. (a) UHQ standard, (b) urine injected through the RAM, (c) urine injected directly in the LC–MS/MS system. Dashed lines represent the windows for IT-MS/MS detection.

logue; strong dilution of the sample, or a clean-up step. In our case, we propose a clean-up step using a RAM. Attending to the different chemical natures of the target compounds, the optimization of the behaviour of the RAM column is an important step in the development of the method. The efficiency of the RAM as a clean-up step is mainly affected by the solvent used to eliminate the matrix interferences. The mobile phase impelled by the isocratic pump acts as a washing solvent and at the same time transports the sample through the RAM. The composition of the mobile phase is thus a decisive factor for achieving an efficient washing of the sample matrix with no elimination of the compounds of interest. Different washing solutions based on ammonium formate buffer—ACN mixtures were assayed. It was decided to use one with a composition of 2.5 mM ammonium formate buffer (pH 2.9) –10% ACN, since this was the one offering the best cleaning without producing appreciable losses of the target compounds.

Fig. 2 shows the total ion chromatograms (TICs), the result of summing the signals corresponding to all the analytes, for a standard of 100 ng mL^{-1} in UHQ (Fig. 2a.) and for samples of urine spiked with 100 ng mL^{-1}, injected through the RAM according to the proposed methodology (Fig. 2b) or directly into the analytical columns (Fig. 2c). It may be seen that the signals of the standard in UHQ and the urine sample injected through the RAM are very similar both in shape and in signal intensity. However, when direct injection of urine sample was carried out the signals of all the analytes underwent a strong decrease owing to interferences from the matrix. These observations point to the effectiveness of the proposed clean-up step.

The use of the RAM coupled on-line with the chromatographic system serves not only as a clean-up method but has also been described to be a valid preconcentration step [31]. Accordingly, in order to increase sensitivity, a study was made of the possibility of injecting volumes up to 1500 μL. Fig. 3 shows the normalized signals obtained for three of the analytes (245TP, BPA and TCPL) upon injecting increasing volumes of samples spiked at different concentrations (400, 100 and 10 ng mL^{-1}). It may be seen that the signal becomes saturated upon increasing the volume injected, this effect being especially pronounced in the samples with higher concentrations. Therefore, an injection volume of 100 μL was selected since this was the greatest volume for which there was no signal saturation in the concentration range studied (3–200 ng mL^{-1}). So, to study the possible preconcentration achieved for each analyte upon injecting 100 μL, UHQ water samples spiked with 100 and 20 ng mL^{-1} were injected through the RAM and also injected directly into the LC–MS/MS system. At both levels, the peaks areas of the analytes obtained through RAM and directly were not significantly different, indicating that no analyte preconcentration was occurring in the RAM. This was probably due to the low sample volume injected (100 μL).

3.2. Evaluation of matrix effects

Quantitative determinations using ESI-MS/MS for detection may be affected by the ion suppression that occurs in the electrospray device, mainly due to the co-elution of matrix interferents with the analytes. This suppression can be calculated via equation [13]: ion suppression (%) = $A_s - (A_{su} - A_{usu})/A_s$, where A_s is the analyte peak area in spiked UHQ water; A_{su} is the analyte peak area in urine, and A_{usu} is the analyte peak area, if present, in unspiked urine. Ion suppression was calculated for each analyte, both in direct injection mode and with the proposed instrumental configuration, RAM-LC–MS/MS (Table 3), obtaining values from 77.7% to 99.6% for direct injection and between 13% and 89% for the proposed method. Very high degrees of ion suppression were observed in the case of direct injection of urine samples, which to a large extent was corrected with the use of the RAM. The trend shown

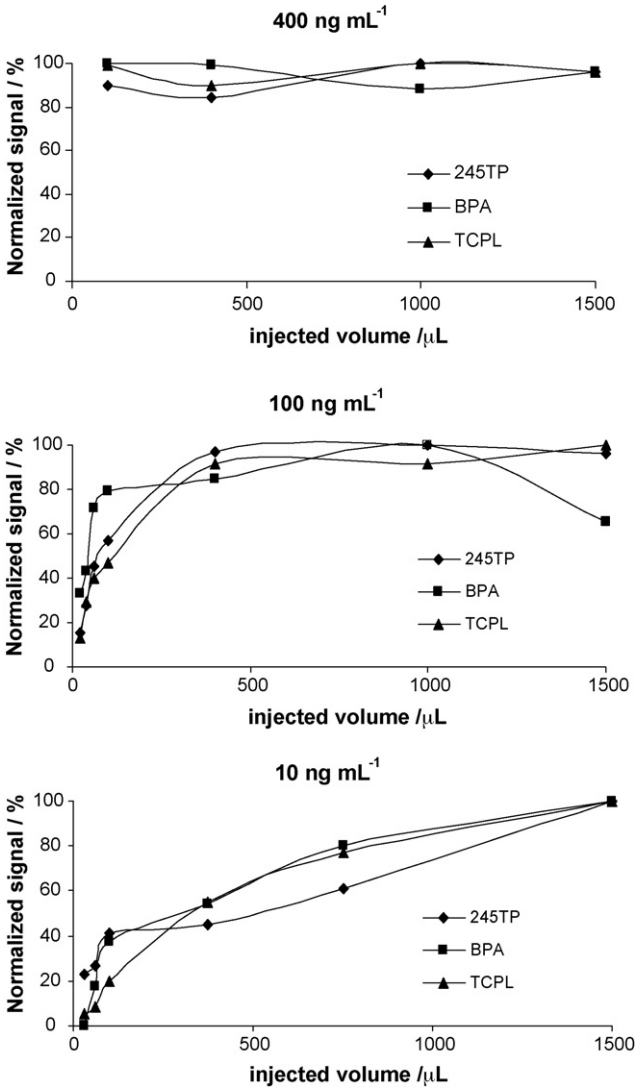

Fig. 3. Influence of the injected urine on the RAM-LC–MS/MS. Analyte response expressed as the signal normalized to the largest peak (%). Urine samples spiked at 400, 100 and 10 ng mL^{-1}, from top to bottom. Analyte identification as seen from Section 2.1.

by these values does not seem to be related to the retention times and hence to the polarity of the analytes; this relationship has been described for other matrices [13]. The use of the RAM-LC–MS/MS configuration afforded a marked decrease in ion suppression but it still persisted, rendering quantification via an external standard inadequate. Therefore, calibration was performed with the matrix-matched method. Fig. 4 compares the calibrations carried out with an external standard in UHQ water and in matrix-matched mode. It may be seen that in fortified urine, sensitivity was decreased, but that this did not affect linearity.

3.3. Limits of detection (LODs) and quantification (LOQs) of the RAM-LC–MS/MS method

Starting out from the optimized parameters, a study was made of the analytical characteristics of the method. Linear relationships were established between the peak areas and the concentration of the analytes for the range studied (10–200 ng mL^{-1}). Table 4 shows the analytical characteristics of the proposed method.

The limits of detection were determined working with the ion trap (IT) in Multiple Reaction Monitoring (MRM) mode, isolat-

Table 3
Ion suppressions for the biomarkers studied obtained by analyzing spiked urine samples.

	Analyte	245T	245TP	CMPU	BPF	CLT	BPA	1NPL	DIN	TCPL
Ion suppression (%)	Directly in LC–MS/MS	88.5	99.6	97.7	77.7	82.7	97.8	–[a]	90.1	72.2
	RAM-LC–MS/MS	40.5	27.3	89.4	43.3	16.4	64.3	36.3	59.1	12.7

[a] No signal was found for 1NPL when urine was directly injected.

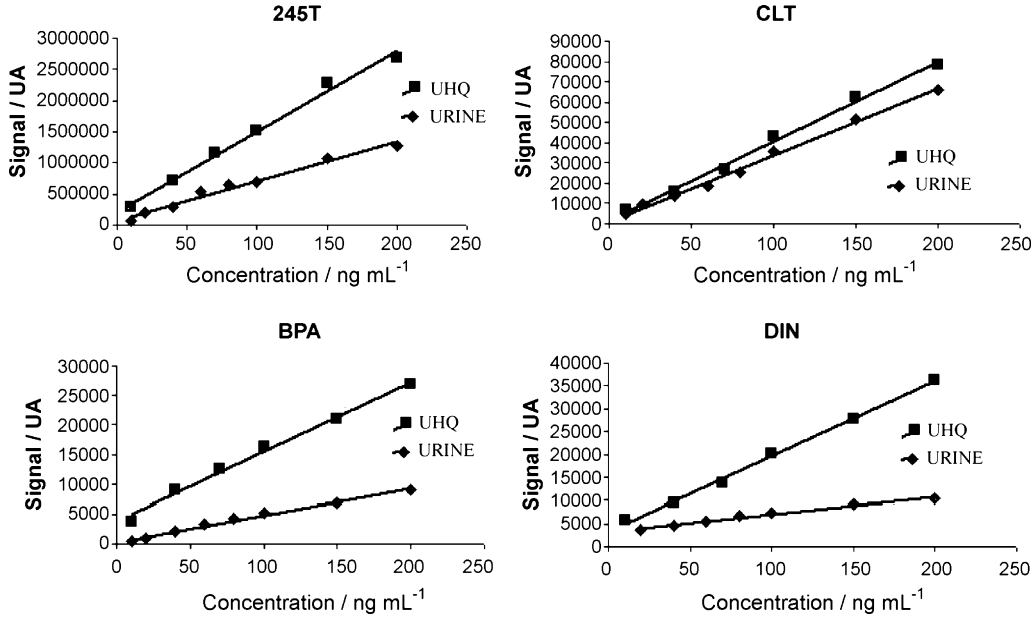

Fig. 4. Comparison of calibration curves for UHQ standards and fortified urine samples. Analyte identification as seen from Section 2.1.

ing the most abundant fragmented ion (quantification transition) and setting six different windows, as shown in Table 1. The chromatograms of the urine samples spiked with 10 ng mL^{-1} and the noise generated by an unspiked urine sample are shown in Fig. 5. The limits of detection were calculated as the concentration for which a signal-to-noise ratio of 3 would be obtained in a real sample. The values thus obtained for all the analytes are shown in Table 4, and they range between 0.2 ng mL^{-1} for 245T and 245TP, and 3.2 ng mL^{-1} for BPF, with the exception of DIN, which had a limit of detection of 15.5 ng mL^{-1}, probably due to the important difference in the mass/charge ratio between the precursor ion and the product ion (233 ⇒ 72). Likewise, the limits of quantification were calculated, in real samples, as the concentration for which a signal-to-noise ratio of 10 would be obtained (Table 4). In this case, values between 3 and 10 ng mL^{-1} were obtained, again with the exception of DIN which had a limit of 50 ng mL^{-1}.

3.4. Method validation

As a confirmatory criterion, the EU Decision suggests the use of at least three points of identification [27]. One of the greatest advantages of tandem mass spectrometry is that it is possible to record a complete spectrum of product ions, thus being able to analyze both the quantification transition and that of confirmation from a single injection, which ensures the presence of at least one precursor and two product ions. With the proposed methodology, four identification points were achieved (one precursor and two products) for CMPU, BPF, CLT and BPA, and five (two precursors and two products) for 245T, 245TP and TCPL. However, for DIN and 1NPL it was not possible to find more than 2.5 (one precursor and one product) and 1 points of identification (one precursor), respectively, such that they were removed from the validation step. This is due to the poor fragmentation that these analytes present by collision-inducted dissociation (CID) in the ion trap.

Table 4
Analytical characteristics of RAM-LC–MS/MS method calculated by the analysis of fortified urine samples.

Analyte	Intercept (area units (au))	Slope (au ng^{-1} mL)	LOD[c] (ng mL^{-1})	LOQ[d] (ng mL^{-1})
245T[a]	$(8.1 \pm 8.5) \times 10^4$	$(6.3 \pm 0.8) \times 10^3$	0.2	3
245TP[a]	$(0.2 \pm 1.3) \times 10^5$	$(1.2 \pm 0.1) \times 10^4$	0.2	3
CMPU[a]	$(2.6 \pm 2.1) \times 10^2$	(28 ± 2)	3.0	10
BPF[a]	$(-0.8 \pm 1.4) \times 10^2$	(19 ± 1)	3.2	10
CLT[a]	$(1.2 \pm 2.6) \times 10^3$	$(3.3 \pm 0.3) \times 10^2$	2.0	10
BPA[a]	$(3.6 \pm 3.9) \times 10^2$	(44 ± 4)	1.4	5
1NPL[a]	$(1.1 \pm 1.0) \times 10^5$	$(8.6 \pm 1.0) \times 10^3$	0.7	5
DIN[b]	$(3.4 \pm 0.5) \times 10^3$	(37 ± 5)	15.5	50
TCPL[a]	$(2.4 \pm 2.9) \times 10^3$	$(4.1 \pm 0.5) \times 10^2$	2.6	10

[a] Concentration range from 10 to 200 ng mL^{-1}.
[b] Concentration range from 20 to 200 ng mL^{-1}.
[c] Limit of detection (LOD) for a signal-to-noise ratio of 3 from quantification transition in MRM mode.
[d] Limit of quantification (LOQ) for a signal-to-noise ratio of 10 from quantification transition in MRM mode.

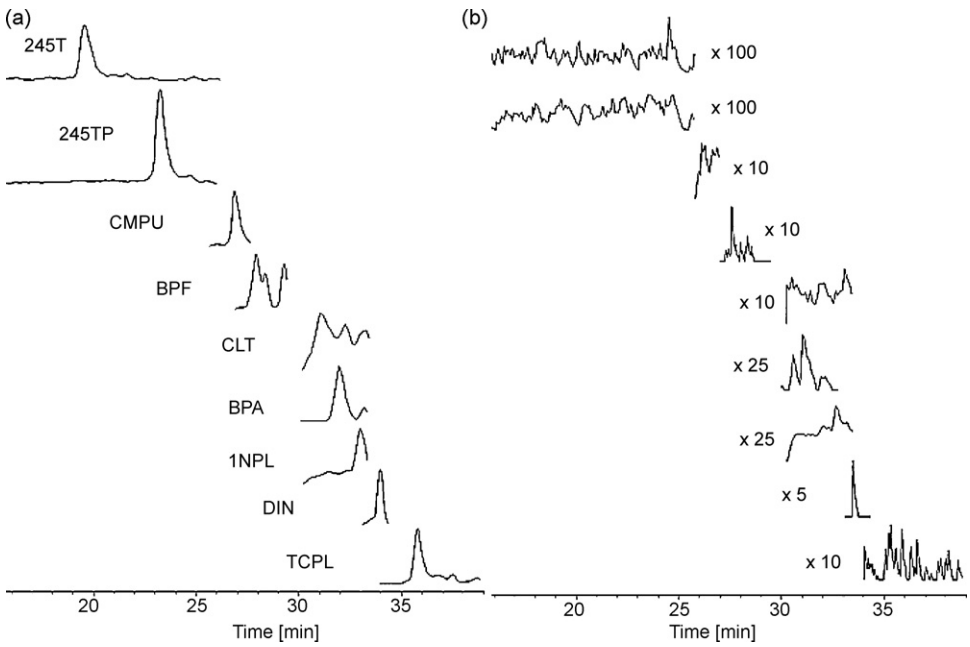

Fig. 5. LC–MS/MS chromatograms of: (a) a urine sample spiked at 10 ng mL^{-1} (except from DIN at 20 ng mL^{-1}) and (b) a blank urine sample (magnified). Analyte identification as seen from Section 2.1.

3.4.1. Calibration curves, decision limits and detection capabilities

Two analytical limits are recommended in the European Decision 657/2002/EC: the decision limit (CC_α), which is defined as "the lowest concentration level of the analyte that can be detected in a sample with a chance of 1% of a false positive decision", and the detection capability (CC_β), which is "the smallest content of the analyte that can be detected in a sample with a chance of 5% of a false negative decision" [27].

To determine these limits, urine samples were studied in MRM mode, recording the full spectrum of product ions. Then, after verifying the existence of both transitions (quantification and confirmation transitions) and ratios according to the EU criteria, an extracted ion chromatogram (EIC) was made of the ion that was the product of the confirmation transition. Thus a calibration of five points ranging between 30 and 150 ng mL^{-1} was built; its characteristics are shown in Table 6. CC_α and CC_β were estimated using the calculations described by Verdon et al. [32]. The values calculated are shown in Table 6. It may be seen that the CC_α values varied between 3.6 ng mL^{-1} for 245T and 16.5 ng mL^{-1} for BPA, and the CC_β values between 6.0 ng mL^{-1} for 245T and 28.1 ng mL^{-1} for BPA.

3.4.2. Repeatability and reproducibility

Keeping the same instrumental configuration as that used to calculate the LODs, the repeatability and reproducibility of the proposed method were evaluated. To accomplish this, urine samples spiked with two different concentrations were analyzed: first at 10 ng mL^{-1} of each analyte, and then with each analyte spiked at its limit of quantification (LOQs, Table 4). Repeatability, as intraday precision, was assessed with eight injections performed on the same day and reproducibility, as interday precision, was determined by analyzing injections (eight each day) carried out over three consecutive days. The values of the different precisions are shown in Table 5. The lowest values were obtained for the sample spiked with 10 ng mL^{-1} in the intraday assay, with precisions between 1.0% for 245T and 5.1% for CLT. The highest values were found upon evaluating the interday precision in samples spiked at the limit of quantification, values of between 7.1% for CMPU and 11.5% for BPA being recorded.

3.4.3. Recoveries

As an additional analysis to validate the method, urine samples that had not been used previously in the method were analyzed. These samples were spiked at two levels: 12.5 and 25 ng mL^{-1}. The signal obtained for each of the analytes in MRM mode by measuring the quantification transition was introduced into the corresponding calibration. For both samples, the results shown in Table 6 were obtained. In the case of urine spiked with 25 ng mL^{-1}, the precisions varied between 7.7% for CLT and 12.5% for 245TP. For the urine spiked with 12.5 ng mL^{-1} they varied between 8.3% for 245T and 18.2% for CLT. In all cases, satisfactory recoveries were obtained.

3.4.4. Ruggedness

Only sample pretreatment was investigated because the LC–MS device was regularly subjected to checks and maintenance, ensuring its ruggedness [33]. Additionally, the method developed is characterized by involving minimum sample pretreatment, such that it is only necessary to evaluate the spiking of the samples

Table 5
Repeatability, evaluated as intraday precision, and within-laboratory reproducibility, evaluated as interday precision, of developed RAM-LC–MS/MS method obtained by analyzing spiking urine samples.

Analyte	RSD[a] (%)			
	Intraday[b] at 10 ng mL^{-1}	Interday[c] at 10 ng mL^{-1}	Intraday[b] at LOQ	Interday[c] at LOQ
245T	1.0	3.1	5.2	9.6
245TP	1.1	4.2	6.3	8.8
CMPU	2.5	7.1	2.5	7.1
BPF	4.9	11.3	4.9	11.3
CLT	5.1	10.4	5.1	10.4
BPA	4.0	8.4	5.3	11.5
TCPL	3.9	10.1	3.9	10.1

[a] RSD, relative standard deviation.
[b] Intraday precision (repeatability) was determined by eight injections.
[c] Interday precision (reproducibility) was determined in three consecutive days (eight injections each day).

Table 6
Statistic and performance characteristics of the proposed RAM-LC–MS/MS method obtained by analyzing fortified urine samples[a].

Analyte	Intercept (au)	Slope (au ng^{-1} mL)	r^2	CC_α (ng mL^{-1})	CC_β (ng mL^{-1})	Recoveries	
						At 25 ng mL^{-1}	At 12.5 ng mL^{-1}
245T	$(-0.09 \pm 3.02) \times 10^4$	$(4.61 \pm 0.03) \times 10^3$	0.999	3.6	6.0	25 ± 3	12 ± 1
245TP	$(-0.2 \pm 1.1) \times 10^5$	$(1.1 \pm 0.1) \times 10^4$	0.999	5.0	8.6	24 ± 3	11 ± 2
CMPU	$(1.8 \pm 2.2) \times 10^2$	(8.3 ± 2.3)	0.996	14.4	24.5	23 ± 2	12 ± 2
BPF	$(-0.7 \pm 1.6) \times 10^2$	(8.6 ± 1.7)	0.998	10.3	17.5	27 ± 3	12 ± 2
CLT	$(5.7 \pm 4.4) \times 10^3$	$(1.6 \pm 0.5) \times 10^2$	0.996	15.2	25.9	26 ± 2	11 ± 2
BPA	$(-0.4 \pm 5.2) \times 10^2$	(17.1 ± 5.4)	0.995	16.5	28.1	25 ± 3	14 ± 2
TCPL	$(-0.3 \pm 4.5) \times 10^3$	$(2.7 \pm 0.5) \times 10^2$	0.998	9.0	15.4	24 ± 3	13 ± 2

[a]Concentration range from 30 to 150 ng mL^{-1} (five calibration points).

because this is performed after freezing and must be compared with samples spiked prior to freezing, in the sense that this latter situation would correspond to a real contaminated sample.

Two urine samples spiked at 100 ng mL^{-1} were prepared and subjected to freezing before and after spiking, respectively. The signal of each of the analytes was analyzed, and it was concluded that there were no significant differences in any case, such that it can be affirmed that spiking before or after freezing does not affect the results of the method.

One of the disadvantages of the use of on-line preconcentration with the same sorbent for different samples is possible contamination among them. This may be especially relevant when RAMs are used, because they have a long half-life. In order to study such a possibility, a high (1500 μL) volume of a high-concentration (200 ng mL^{-1}) sample of urine was analyzed. Following this, a blank of UHQ water was injected and no signal was observed for any of the analytes. Accordingly, the washing to which the RAM had been subjected was effective, since even under the most unfavourable conditions the system did not display carryover.

3.5. Non-targeted screening analysis

All the studies performed using the proposed method led to the presetting of the most suitable parameters for the quantification of each analyte (Table 1). In order to check that the method can be used for screening purposes, it is necessary to check its ability to detect any other type of analyte that has not been preset in the chromatographic program. To accomplish this, the Data-Dependent Scan mode (called "Auto Ms(n)" in the software used [34]) was implemented. This mode does not require the presetting of any type of parameter characteristics of the analytes (transitions, windows, etc.). The detector performs a mass scan from 60 to 400 u and, when the signal surpasses a given threshold, it starts the fragmentation, recording the whole MS/MS spectrum. The optimum fragmentation amplitude was determined empirically in an automatic way as the voltage, between 0.3 and 2.0 V, that reduced the signal of the precursor ion to 10%. Working in this mode, with a signal threshold of 150,000 ua, samples were analyzed in UHQ water—from 10 to 200 ng mL^{-1}—and in real matrices using urine spiked at 0–1000 ng mL^{-1}. In all cases we analyzed whether each of the analytes was detected, taking as a criterion: (i) whether fragmentation would begin or not and (ii) whether the characteristic MS/MS spectrum of the analyte would match the home-made library reference, (iii) observing a signal/noise ratio greater than 3. In that conditions a minimum of four identification points was assured. Fig. 6 shows the chromatogram and the spectrum obtained for a urine sample spiked with 100 ng mL^{-1} of 245T (Fig. 6b) and the corresponding chromatogram and spectrum of the standard (Fig. 6a). All the results thus obtained are shown in Table 7. It may be seen that there are no false negatives and that the identification was correct for all the analytes up to a concentration level of 40 ng mL^{-1} in UHQ and 60 ng mL^{-1} in urine; these limits were improved in the case of 245T and 245TP up to levels of 10 and 20 ng mL^{-1} respectively. Such results suggest the use of Data-Dependent Scan as a general screening method for the detection of low-molecular weight xenobiotics in urine.

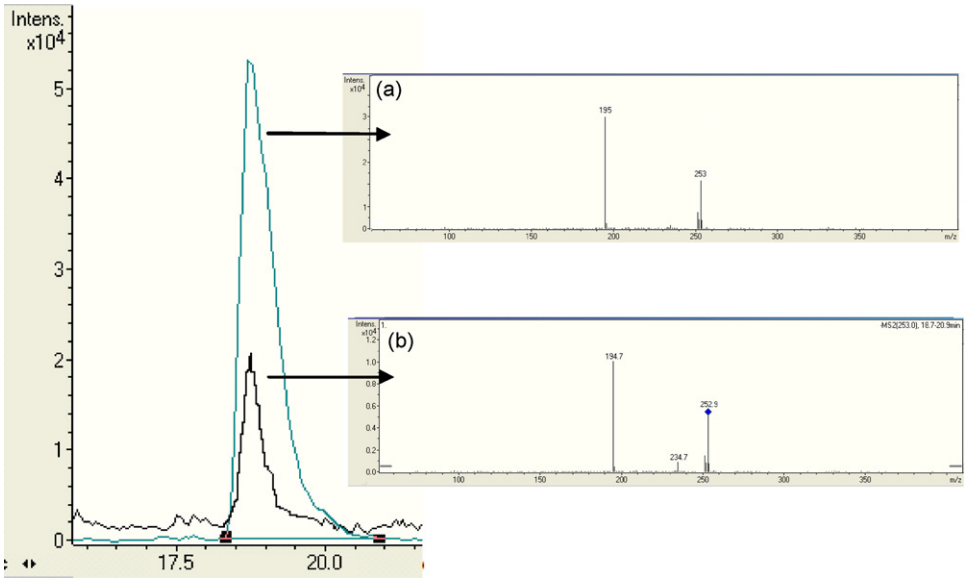

Fig. 6. Non-targeted qualitative analysis: 245T MS/MS chromatograms and spectra obtained from (a) an UHQ standard used as home-made library and (b) a urine sample spiked at 100 ng mL^{-1}.

Table 7
Performance of proposed non-targeted screening analysis for fortified UHQ water and urine samples[a].

Analyte	245T	245TP	CMPU	BPF	CLT	BPA	1NPL	DIN	TCPL
Precursor ion	253	267	185	199	211	227	143	233	195
Product ion	195	195	142	93	166	212		72	159
UHQ ($ng\,mL^{-1}$)									
10	♦	♦	▽	▽	▽	▽	▽	▽	▽
40	♦	♦	♦	♦	♦	♦	▽	▽	♦
100	♦	♦	♦	♦	♦	♦	▽	♦	♦
200	♦	♦	♦	♦	♦	♦	▽	♦	♦
Urine ($ng\,mL^{-1}$)									
Blank	▽	▽	▽	▽	▽	▽	▽	▽	▽
10	▽	▽	▽	▽	▽	▽	▽	▽	▽
20	♦	♦	▽	▽	▽	▽	▽	▽	▽
40	♦	♦	▽	♦	♦	▽	▽	▽	♦
60	♦	♦	♦	♦	♦	♦	▽	♦	♦
80	♦	♦	♦	♦	♦	♦	▽	♦	♦
100	♦	♦	♦	♦	♦	♦	▽	♦	♦
150	♦	♦	♦	♦	♦	♦	▽	♦	♦
200	♦	♦	♦	♦	♦	♦	▽	♦	♦
1000	♦	♦	♦	♦	♦	♦	♦	♦	♦

[a] ♦: Identified analyte; ▽: unidentified analyte.

4. Conclusions

The on-line configuration developed, incorporating a RAM, means that sample treatment is minimum (freezing and filtration); this has a positive effect on precision and affords a shorter analysis time. The configuration has advantages such as automation, high sensitivity and an important reduction in ion suppression. Another advantage is cost, since the half-life of the RAM allowed the injection of at least 190 samples of urine, involving a total volume 20.7 mL of urine. The method described, RAM-LC–MS/MS, is a sensitive, selective, and precise automated tool for the determination and confirmation of several different biomarkers of exposure to xenobiotic compounds in a complex matrix such as human urine. The method has been validated as a quantitative confirmatory method in accordance with the Commission Decision 2002/657/EC. Additionally, IT-MS/MS detection provided high sensitivity and selectivity, allowing limits of detection to be achieved at the low $ng\,mL^{-1}$ level. The method is therefore appropriate for application within the context of the biological monitoring of these biomarkers and as a general screening method.

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