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Screening of non-polar heterocyclic amines in urine by microextraction in packed sorbent-fluorimetric detection and confirmation by capillary liquid chromatography

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ABSTRACT

A rapid and simple procedure for the direct screening of urine samples is described. The method involves microextraction in a packed sorbent (MEPS) that is on-line coupled to a capillary liquid chromatograph with fluorimetric detection. The overall arrangement works as a screening/confirmatory system for monitoring non-polar heterocyclic aromatic amines (HAAs) in urine samples. This configuration allows the selective retention of HAAs from urine on a C_{18} MEPS cartridge integrated in the needle of a micro-well plate autosampler. Retained HAAs were eluted with methanol/water (90:10, v/v) and directly injected into the fluorimetric detector. This screening method provides a yes/no binary response that may require confirmation. The samples for which the concentration of HAAs was close to or above the established threshold limit (30 ng mL^{-1}) were subjected to capillary liquid chromatography (CLC) for confirmation purposes. A mobile phase of acetonitrile and triethylamine (25 mM) at pH 2.5, through a gradient of composition at a flow rate of 20 μL min^{-1}, resulted in good separations between the analytes in less than 11 min. This confirmation method allowed the determination of the analytes in the 10–100 ng mL^{-1} range for harmane and norharmane and from 20 to 200 ng mL^{-1} for 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b] indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido-[4,3-b] indole (Trp-P-2), 2-amino-9H-pyrido-[2,3-b] indole (AαC) and 2-amino-3-methyl-9H-pyrido-[2,3-b] indole (MeAαC), with relative standard deviation (RSD) values between 2.12% and 3.73%, and limits of detection between 1.6 and 5.6 ng mL^{-1} for all the HAAs.

1. Introduction

Heterocyclic aromatic amines (HAAs) are chemicals that have high carcinogenic potential and are formed when amino acids and creatine react at high temperatures from the cooking of meats such as beef, pork, fowl and fish [1,2]. Free amino acid and sugar levels also influence the content of HAAs. The synthesis of these compounds depends on different cooking conditions such as temperature and time, the presence or absence of enhancers, pH, transport of water and water-soluble substances [3]. At temperatures above 150 °C, concentrations of HAAs become highly significant and the higher the temperature, the higher the level of apolar HAAs generated [1,4].

These compounds have proven to be carcinogenic in rodents and non-human primates [5–10]. Several studies (e.g., European Prospective Investigation into Cancer and Nutrition) have associated an increased risk of developing colorectal, pancreatic and breast cancer to high intakes of well-done, fried, or barbequed meat and fish, although from these studies there is insufficient scientific evidence to support the hypothesis that human cancer risk is specifically due to the greater uptake of heterocyclic aromatic amines [11,12]. However, the importance of other carcinogens, co-carcinogens and anticarcinogens in the diet – and genetic susceptibility to HAAs – should be taken into account. Consequently, HAAs may be considered as human carcinogens and The International Agency for Research

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on Cancer (IARC) recommended a decrease in their consumption [13–15].

The quantification of HAAs in cooked foods and in human urine indicates the continual exposure of humans to low levels of these substances in the diet [16]. HAAs have been isolated and identified, not only in foods [17–21], alcoholic beverages [22] and cooking residues, such as cooking smoke and pan residue extracts [23,24], but also in biological matrices such as plasma, human tissues, hair, urine, bile, human milk [9,25–33] and even in environmental samples such as cigarette smoke [34], river and rain water [35,36] and air [37]. The trace quantities in which HAAs are present and the high level of interference in matrices require selective and sensitive methods to detect and identify these analytes. This requirement makes the extraction and clean-up steps for analyses extremely important. Liquid–liquid extraction, solid-phase extraction (SPE), solid-phase microextraction (SPME), focused microwave-assisted extraction and hollow fiber-supported liquid membrane extraction have been utilised to eliminate interference at the retention times of HAAs in real samples and, in general, large volumes of sample, solvent and time are employed [38–40].

Microextraction in a packed sorbent (MEPS) represents a new technique for miniaturized SPE. In MEPS, about 1 mg of solid packing material is inserted into a syringe (100–250 μL) or integrated into autosampler robotics, i.e. not into a separate column, and this allows an online connection to a GC or LC. MEPS is based on multiple extractions in which the sample flows through a bed of solid extractant, the size (and particle size) of which has to be as small as possible to speed up the transfer of analytes from the sample to the solid phase. The transfer is also facilitated by the close contact between these phases. The packed sorbent can also be reused, more than 100 times for plasma or urine samples and more than 400 times for water samples, whereas a conventional SPE column can only be used once. MEPS can handle volumes from 10 to 1000 μL and is more robust when compared with SPME. This new technique can be used for complex matrices (such as plasma, urine, blood and organic solvents), which is not always the case with SPME, and it is fairly sensitive to the nature of the sample matrix. Moreover, high extraction recoveries can be achieved (>60%) [41,42]. The MEPS technique has been used to extract a wide range of analytes from different matrices (urine, plasma, blood). Hence, several drugs such as local anaesthetics and their metabolites [43–45], neurotransmitters such as dopamine, serotonin [46], methadone [41] and cotinine [47] have been extracted from biological samples such as blood, plasma or urine using the MEPS technique. References regarding the use of MEPS for the extraction of HAAs have not been published to date.

HPLC, GC and capillary electrophoresis (CE) coupled to mass spectrometric (MS), photometric, fluorimetric (FLD), evaporative light scattering (ELSD) and electrochemical detectors (ECD) have been used for the identification and quantification of HAAs in real food and biological samples [38,48,49]. Daily intake and exposure to HAAs can be estimated by their determination in urine samples, which is useful to correlate the uptake of these compounds with cancer risk. In this sense, qualitative analysis provides binary yes/no responses with the least possible delay in order that timely decisions can be made. However, this technique does not provide quantitative data. To date it has been common to identify an analyte or a group of analytes or, in the case of qualitative analysis devoted to sample classification, to make a rapid and reliable classification of samples on the basis of previously established criteria, for example a cut-off concentration fixed by the legislation or client. Consequently, screening methods can reduce purchase and maintenance costs of equipment, diminishing the use of capillary liquid chromatography (CLC) to process only the positive samples tested with the screening system.

The objective of the work described here was to develop a method for the extraction and determination of HAAs in urine samples through the use of a screening system to obtain rapid responses for the presence or absence of these analytes. The use of CLC to identify and quantify the HAAs present in urine samples was markedly reduced because only those samples that gave positive responses in the screening system were analysed. The interferences present in the samples were also reduced by the use of MEPS integrated in the micro-well plate autosampler, thus avoiding time-consuming procedures for sample preparation. Good repeatability of the responses and high sensitivity were achieved. This method was applied to determine six HAAs in urine samples with less reagent consumption and low cost instrumentation.

2. Experimental

2.1. Chemicals, materials and samples

The heterocyclic aromatic amines, 9H-pyrido-[3,4-b] indole (norharman, NH) and 1-methyl-9H-pyrido-[3,4-b] indole (harman, H) standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Amino-9H-pyrido-[2,3-b] indole (AαC), 3-amino-1-methyl-5H-pyrido-[4,3-b] indole (Trp-P-2), 2-amino-3-methyl-9H-pyrido-[2,3-b] indole (MeAαC), and 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b] indole (Trp-P-1) standards were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) (Fig. 1). Acetonitrile (HPLC grade) and phosphoric acid were supplied by Panreac (Barcelona, Spain). Methanol (HPLC grade) and triethylamine (TEA) used to prepare the mobile phase were purchased from Sigma–Aldrich. Water was purified with a Milli-Q filtration system from Millipore (Bedford, MA, USA). All solutions prepared for capillary liquid chromatography were passed through a 0.45 μm nylon filter before use. Microextraction packed sorbents (MEPS) and syringes were purchased from SGE Analytical Science (Ringwood, Australia), C_{18} solid phase extraction cartridges were obtained from Varian (Palo Alto, CA, USA) and Análisis Vínicos (Tomelloso, Spain). Strata-X cartridges were purchased from Phenomenex (Bellefonte, PA, USA) and Sep-Pak solid phase extraction cartridges were obtained from Waters (Milford, MA, USA). Urine samples were donated by volunteers.

2.2. Equipment

Chromatographic analysis of the HAAs was carried out on an Agilent 1200 capillary liquid chromatograph equipped with a binary pump, a degasser, a micro-well plate autosampler and a column heater-cooler. The chromatograph was coupled with an Argos 250B fluorescence detection system from Flux Instruments AG (Basel, Switzerland), equipped with a fluorescence flow-cell directly connected to the excitation and emission light guides. The emitted light was guided along the capillary by total reflection and focused on the emission light guide using a patented light cone. This configuration allowed direct detection at the flow cell. The detection light path was defined by the inner diameter of the capillary. The detector was also equipped with a xenon–mercury (Xe–Hg) lamp as the irradiation source (power 75 W) with a radiation range lying between 185 and 2000 nm, and included a photomultiplier tube (PMT) detection unit working in the 185–700 nm range. The chromatograms were recorded using a program developed in-house and controlled by Labview software (Labview, National Instruments, USA) running on a computer fitted with a multipurpose interface card (NI USB-9161). The analytical column was a capillary polar encapped C_{18} reverse phase Luna® column (250 mm × 500 μm i.d., 5 μm) from Phenomenex.

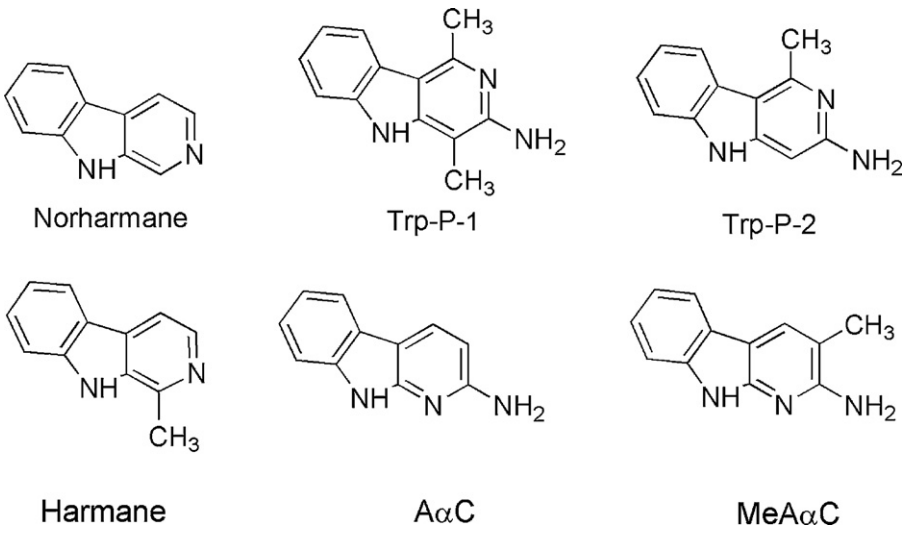

Fig. 1. Chemical structures of the heterocyclic aromatic amines.

2.3. Standard preparation

Individual stock standard solutions were prepared in acetonitrile at a concentration of 100 mg mL^{-1} and stored in the dark at −20 °C prior to use. Fresh standard working solutions were prepared daily by diluting each stock solution with water/methanol (H_2O/MeOH = 10:90, v/v) for the screening system and the mobile phase, which consisted of water/acetonitrile (H_2O/ACN = 95:5, v/v, 25 mM triethylamine, pH 2.5), for the confirmation of positive samples.

2.4. Sample preparation and MEPS conditions

Human urine was filtered through a 0.45 μm nylon filter, adjusted to pH 8, and a 100 μL sample was withdrawn through the solid phase sorbent (1 mg of silica-C_{18}) connected to the autosampler of the CLC system. MEPS were initially conditioned with 250 μL of MeOH followed by 250 μL of H_2O. The sorbent was then washed with 100 μL of 30% MeOH to eliminate the interferences and the analytes were eluted with 100 μL of methanol/water 90:10 (v/v) – all of these steps were carried out by the autosampler. The sorbent was then cleaned and re-conditioned before re-use. For confirmation purposes, all steps (conditioning, clean-up, retention and elution) were carried out manually using a 250 μL syringe as indicated by the MEPS manufacturer [50]. The eluate was evaporated to dryness under a stream of nitrogen, the residue was reconstituted in 100 μL of the CLC mobile phase and 8 μL of this solution was injected into the CLC system. Spiked urine samples were prepared by adding few microliters of the analyte standard solutions to 1 mL of urine.

2.5. Chromatographic conditions

The mobile phase was a binary mixture of 25 mM triethylamine in water and acetonitrile, adjusted to pH 2.5 with phosphoric acid, in a gradient elution mode at a flow rate of 20 μL min^{-1}. Gradient conditions were as follows: from 5% acetonitrile and 95% triethylamine (25 mM at pH 2.5) to 27.5% acetonitrile and 72.5% TEA over 3 min; then to 30% acetonitrile and 70% TEA over 1.5 min; after that, from this composition to 50% acetonitrile and 50% TEA for 0.5 min, then changed to the initial mobile phase composition over 2 min, with these final conditions maintained for 5 additional minutes. Mobile phases were filtered daily through nylon Millipore filters (Millipore, Bedford, MA, USA) before use. The capillary column was maintained at ambient temperature and the photomultiplier was set at 1040 mV. A band-pass filter for excitation in the 240–400 nm range and a long pass filter for emission at 495 nm were employed for detection. UV spectra from 210 to 420 nm were recorded for peak identification.

3. Results and discussion

3.1. Optimal conditions for clean-up and extraction of HAAs

Different commercial solid phase cartridges (C_{18}, Strata-X and Sep-Pak) and MEPS were tested as sorbent materials. In the case of Strata-X cartridges, interferences were detected in the extracts. Different washing solutions of methanol in the range from 5% to 25% were applied in the extraction process. Additionally, different solvents at different ratios were also utilised: a methanol/acetonitrile mixture (1:1) and different methanol solutions in the range from 80% to 100% were also assayed. The recovery values obtained were in the range from 48% (MeAαC) to 87% (Trp-P-1) for C_{18} cartridges and from 50% (NH) to 86% (Trp-P-1) for the Sep-Pak material. Thus, on the basis of the recoveries, the selectivity, the volume of sample and solvents utilised, MEPS was chosen as the optimum material for sample clean-up and extraction of HAAs. Different washing and elution solutions were tested with MEPS. Water, methanol and acetonitrile were utilised in different ratios to obtain extracts without interferences and with high recovery values for the HAAs. A washing solution of 30% methanol in water and an elution solution of water/methanol (10:90) gave high recoveries of HAAs and interferences were not observed for the resulting extract at the retention times of the analytes studied (Fig. 2). To minimize the carryover, the sorbent was washed with 250 μL of methanol and then with 250 μL of water after each extraction procedure.

3.2. Characteristics of the screening method

The screening system was designed to provide a rapid response to the presence or absence of heterocyclic aromatic amines at a threshold limit concentration. In this work, the limit was established by scientific criteria due to the absence of a legal limit in European legislation. This system is based on the fluorimetric detection of extracted HAAs from MEPS. The responses of the analytes were obtained by injection experiments in CLC equipment for the six HAAs. All compounds were injected at the same concentration in 90% methanol in water, at a flow rate of 20 μL min^{-1}.

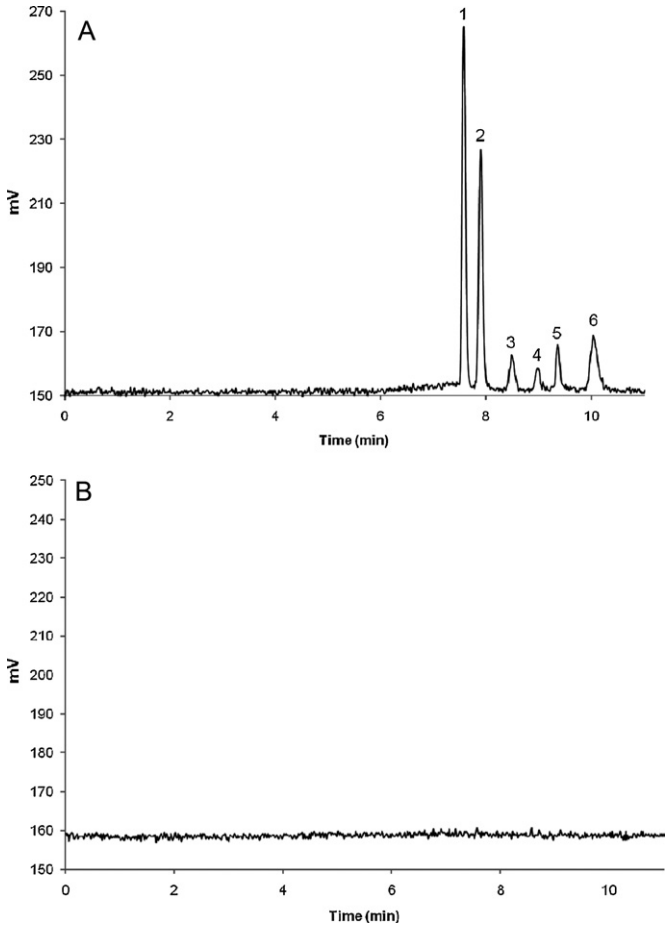

Fig. 2. Fluorescence chromatogram obtained from human urine spiked with 100 ng mL^{-1} of HAAs (A) and from blank human urine (B). Column (C_{18} reverse phase Luna®, 250 mm × 500 μm i.d., 5 μm particle size); mobile phase, triethylamine/acetonitrile (adjusted to pH 2.5 with phosphoric acid, in a gradient elution mode; ionic strength: 25 mM); flow rate, 20 μL min^{-1}; injection volume, 8 μL; ambient temperature. Peaks: norharmane (1), harmane (2), Trp-P-2 (3), Trp-P-1 (4), AαC (5) and Me AαC (6).

Under these conditions, the samples were analysed by triplicate injections. The system was calibrated by carrying out fluorimetric measurements in the flow-cell. A linear calibration graph was obtained in the range 10–500 ng mL^{-1}. Under these conditions, the regression equation obtained was: slope = 2.18 ± 0.03 [HAAs] − 2.37 ± 6.36 (R^2 = 0.999; $S_{y/x}$ = 12.8, n = 8). The precision of the method, expressed as RSD for the determination of 50 ng mL^{-1} of each HAA, was 2.48% (n = 25). The limit of detection (LoD) was 8.75 ng mL^{-1}, calculated as the intercept plus three times its standard deviation (SD), and the limit of quantification, expressed as the intercept plus 10 times its standard deviation, was 29.2 ng mL^{-1}. It is crucial in measuring the total HAAs content of urine samples by fluorescence that all of the components have the same fluorescence properties and, thus, the peak containing the overall response for the total amount of HAAs in the sample can be obtained. In order to establish the unreliability region that defines an interval where 95% of errors (false positive or negative responses) take place, different cut-off values (12.5 and 30 ng mL^{-1}) were supposed. For each cut-off value the probability–concentration graph was established and this enabled the unreliability region to be calculated, including the threshold concentration of the highest and lowest level to warrant the decision on the asssigned attribute (Fig. 3). The probability–concentration graph was calculated by determining the relative proportion (expressed as a percentage) of false positives and negatives through the analysis of 30 spiked urine

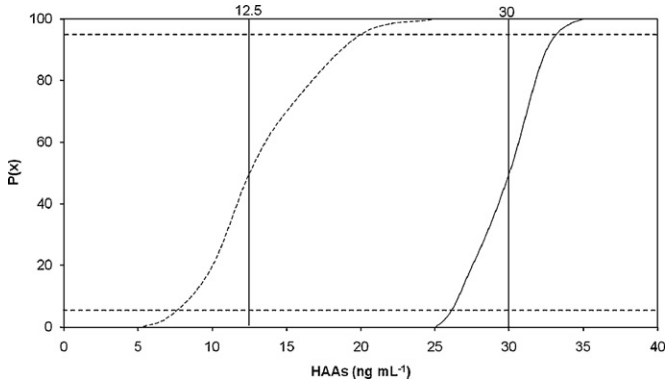

Fig. 3. Real probability–concentration graphs for the screening of HAAs in urine for several values of cut-off concentration.

samples at each concentration level. The binary yes/no response was obtained by transformation of the analytical signal, assuming that analytical signals lower than that obtained at the cut-off concentration, as calculated using the calibration graph, were assigned as negative responses. Consequently, when the analytical signal was higher than that provided by the cut-off concentration a binary positive response was assumed. The associated unreliability region became wider as the cut-off value approached the LoD. Thus, 30 ng mL^{-1} was selected as the most appropiate cut-off value for the screening system. The final decision, however, can be made by subjecting the samples to the CLC–FLD confirmatory method.

3.3. Confirmation CLC–FLD system

In the second part of this study, the positive samples from the screening system were extracted and injected into the CLC–FLD system. The chromatographic separation of the HAAs was accomplished by using the gradient method detailed in Section 2. Prior to the application of the proposed method, optimal values of the parameters that cause variability of the measurements were selected. Thus, the pH and concentration of triethylamine were optimised. Triethylamine/acetonitrile (adjusted to pH 2.5 with phosphoric acid, 25 mM) at a flow rate of 20 μL min^{-1} was selected due to the good chromatographic performances obtained, the optimised fluorescence signals obtained and a short run time of less than 11 min (Fig. 2A). Moreover, impurities did not coelute with these analytes when samples were injected into the CLC system. The mean retention times (min ± SD; n = 10) under the optimum conditions were as follows: 7.53 ± 0.04 (NH), 7.87 ± 0.04 (H), 8.47 ± 0.05 (Trp-P-2), 8.94 ± 0.04 (Trp-P-1), 9.34 ± 0.04 (AαC), 10.04 ± 0.07 (MeAαC). The injection volume was 8 μL and fluorimetric detection was performed as indicated in Section 2.5. The figures of merit for the proposed method are shown in Table 1, namely linear range, intercept and slope of the curve, the regression coefficient, the precision and sensitivity (as LoD and LoQ) for each individual heterocyclic aromatic amine. Analytes were retained in MEPS, eluted with 90% methanol in water, evaporated to dryness under a stream of nitrogen, reconstituted in the appropriate separation mobile phase and injected into the chromatographic system for separation and quantification of individual analytes. Individual calibration graphs were run with standard mixtures of the six HAAs within the linear ranges 10–100 ng mL^{-1} for harmane and norharmane, and 20–200 ng mL^{-1} for Trp-P-1, Trp-P-2, AαC and MeAαC. Each solution was injected in triplicate. The precision of the method, expressed as relative standard deviation (RSD), for the determination of 50 ng mL^{-1} of each analyte was found within the range of 2.12–3.72 (n = 10). The LoDs and LoQs, calculated as indicated in Section 3.2, was calculated for each individual HAA. Thus,

Table 1
Figures of merit for the CLC–FLD confirmation method.

HAAs	Linear range (ng mL^{-1})	$Y=(a\pm S_a)X+(b\pm S_b)$	R^2	$S_{y/x}$	LoD (ng mL^{-1})	LoQ (ng mL^{-1})	RSD (%)
NH	10–100	$Y=(5.15\pm0.05)X+(3.08\pm2.82)$	0.999	3.82	1.6	5.5	2.22
H	10–100	$Y=(3.95\pm0.05)X+(2.01\pm3.03)$	0.999	3.95	2.3	7.7	2.12
Trp-P-2	20–200	$Y=(0.758\pm0.012)X+(2.21\pm1.20)$	0.999	1.79	4.8	16	3.72
Trp-P-1	20–200	$Y=(0.369\pm0.007)X+(2.82\pm0.69)$	0.998	0.948	5.6	18.7	2.87
AαC	20–200	$Y=(0.795\pm0.012)X-(1.83\pm1.20)$	0.999	1.78	4.5	15	2.36
MeAαC	20–200	$Y=(1.70\pm0.02)X+(4.30\pm2.45)$	0.999	3.50	4.3	14.4	2.76

a: slope (mV ng mL^{-1}); S_a: standard deviation of the slope; b: intercept (nA); S_b: standard deviation of the intercept; R: regression coefficient; $S_{y/x}$: standard deviation of residuals; LoD: limit of detection; LoQ: limit of quantification, RSD: relative standard deviation ($n=10$).

Table 2
Determination of HAAs in human urine samples using the CLC–FLD confirmatory method (figures correspond to concentrations in ng mL^{-1}).

Added	Analyte											
	NH		H		Trp-P-2		Trp-P-1		MeAαC		AαC	
	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)
30	24.3	81	24.3	81	D/NQ	NC	D/NQ	NC	27	90	D/NQ	NC
35	31.1	89	24.9	71	D/NQ	NC	D/NQ	NC	28	80	D/NQ	NC
40	28	70	28.8	72	D/NQ	NC	D/NQ	NC	35.2	88	D/NQ	NC
45	31.5	70	33.8	75	D/NQ	NC	D/NQ	NC	33.2	83	D/NQ	NC
50	48	96	41.5	83	43	86	40.5	81	47.5	95	46.5	93
60	42	70	45.6	76	57.6	96	42.6	71	49.2	82	57	95
70	46.2	66	47.6	68	67.2	96	44.8	64	63.7	91	69.3	99
80	38.4	48	41.6	52	62.4	78	68	85	67.2	84	76.8	96
90	57.6	64	66.6	74	83.7	93	74.7	83	87.3	97	75.6	84
100	77	77	80	80	94	94	93	93	89	89	85	85

the LoDs obtained for the proposed method were in the range from 1.6 ng mL^{-1} for norharmane to 5.6 ng mL^{-1} for Trp-P-1, and the LoQs ranged from 5.5 ng mL^{-1} to 18.7 ng mL^{-1} for norharmane and Trp-P-1, respectively.

3.4. Analytical applications

In order to evaluate the applicability of the confirmatory method, recovery studies were carried out on urine samples spiked with HAAs at different concentration levels within the linear region of the analytical curves for the method. All of the cases were positive in the screening method. The absolute recoveries for all the analytes are given in Table 2. Interferences from the matrix were not present in any of the samples. All of the heterocyclic aromatic amines were detected in all of the samples, but Trp-P-1, Trp-P-2 and MeAαC were not quantified in samples at concentrations below 50 ng mL^{-1}. Recovery values ranging from 48% to 99% were obtained.

4. Conclusions

An MEPS procedure for the clean-up of urine samples and the extraction of HAAs has been succesfully applied. A combination of an automatic screening system (fluorimetric) with a confirmatory (identification) technique using CLC–FLD was developed to control HAAs in urine samples. This method has several practical advantages. A rapid and simple classification of a large number of samples as positive or negative through the screening system is possible when required, and only positive samples need to be confirmed using the CLC–FLD method. Thus, a reduction in analysis times and costs in routine studies has been demonstrated.

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