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ORIGINAL

Miniaturized Analysis of Methylhexanamine in Urine by Gas Chromatography Applying In Situ Derivatization

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Abstract

The use of stimulants by professional and amateur athletes, as well as in the general population, to enhance their athletic performance represents a human health hazard. Methylhexaneamine (MHA) is a stimulant found in numerous dietary supplements and detected frequently in urine samples. The analytical process accepted by most Drug Testing Programs that employ urine use two methods, the first is an initial screening test, followed by a more specific method for the confirmation of presumptive positive urine specimens. For this reason, two specific analytical methods were developed and validated to determine MHA in urine based on its in situ derivatization with isobutyl chloroformate; the first was salting out assisted liquid–liquid extraction (SALLE) with gas chromatography coupled to mass spectrometry (GC–MS) and the second headspace solid-phase microextraction (HS-SPME) followed by gas chromatography with flame ionization detector (GC–FID). Both methods were evaluated for precision (relative standard deviation < 10%), linearity ($r^2 > 0.98$), accuracy at two concentration levels (recovery > 92%) and limit of detection (5–7 ng mL^{-1}). The methods were successfully applied to analyze athletes' urine samples from Comisión Nacional de Cultura Física y Deporte (CONADE, Mexico). The proposed methodologies are sensitive, precise, accurate, fast, and miniaturized, and they are suitable specific methods to analyze MHA in urine for anti-doping or forensic purposes even with an affordable GC–FID system.

Keywords Methylhexaneamine · Urine · SALLE · HS-SPME · Gas chromatography

Introduction

Methylhexanamine (MHA) is an aliphatic primary amine (ESM, Table S1) that mimics the action of epinephrine. It is a central nervous system stimulant and can induce sympathomimetic effects. This compound has been intentionally added to dietary supplements for weight loss and as a stimulant to accelerate many metabolic functions; manufacturers use various names like dimethylamylamine (DMAA), dimethylpentylamine, geranamine, 4-methyl-2-hexaneamine and 2-amino-4-methylhexane, in an attempt to avoid being easily recognized and sometimes this stimulant is not declared on the labels [1]. It was claimed that MHA was non-synthetic and found naturally in *Pelargonium* or *Geranium* species, but recent studies have shown it is not from natural origin [2, 3]. MHA has received much attention because of its high incidence among athletes and the general population and for its numerous harmful effects. It can cause adverse cardiovascular events due to rapid increase in blood pressure as well as shortness of breath, which can lead to sudden death; also, this compound may compromise liver function; because of those safety concerns, MHA was banned by Health Canada in 2011 and by Food and Drugs Administration (FDA) in 2013 [4, 5], but in Mexico, it can still be found in some supplements without any restriction [6]. MHA was listed in the World Anti-Doping Agency (WADA) prohibited list in 2010, and currently, it remains as a specified stimulant (class S6) prohibited in competition [7]. Several athletes in multiple sports tested positive to MHA, and most of them received suspensions even with unintentional use of the banned substance [8, 9]. Also, the use of MHA by the general population was demonstrated by two studies that analyzed anonymous pooled urine samples

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collected from 12 portable street urinals designed for use by men only in central London, UK. The first study analyzed samples taken after urinals were used over a 12-h period and MHA was detected in 9 of 12 urinals tested [10]. In the second study, urinals were sampled once a month over a 6-month period, every month MHA was detected, its lower frequency was 3 and the highest 12 from the 12 urinals sampled [11].

The preferred route of administration of MHA is ingestion, and users report oral doses of MHA from 5 to 150 mg [12]. Most stimulants are determined in urine because despite hepatic metabolism, an important percentage of stimulants are excreted in urine unaltered [13]. Because urine is a complex matrix that can contain salts, proteins, and numerous organic compounds, sample preparation techniques are used for isolation, cleaning, and concentration [14]. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are the most used [13, 16], but they have some limitations. Classical LLE demands large sample and solvent volumes, low selectivity, emulsion formation and is unsuitable for hydrophilic analytes; SPE involves time-consuming procedures, is expensive and sometimes has poor reproducibility [17]. Also, miniaturized techniques like solid-phase microextraction (SPME) and salting-out-assisted liquid–liquid extraction (SALLE) have been used to determine stimulants in urine. SPME integrates sampling and enrichment into a single step and it can be used in headspace and immersion modes. It has been successfully applied to determine amphetamines and cocaine from urine [18–20]. SALLE is another suitable alternative that is more efficient, simple, fast, and greener than LLE. With this technique, extraction and clean-up can be carried out simultaneously and has already been applied for the determination of amphetamines [21] and cannabinoid metabolites [22] in human urine. The most frequent analytical methods for the determination of stimulants in urine include gas chromatography coupled to mass spectrometry (GC–MS) and liquid chromatography coupled to mass spectrometry (LC–MS) [13, 14]. The analysis of stimulants, in which the chemical structure is a primary aliphatic amine, is mainly performed by LC–MS because of the short pretreatment time without the need for derivatization. GC–MS analysis is difficult due to the high volatility, adsorption, and decomposition of the analytes on the column and also have base peaks at low *m/z* values. To overcome these problems, derivatization has been employed to reduce the polarity of the amino group and to increase the number of characteristic ions at higher *m/z* values. For this purpose, silylation, Schiff base, and carbamate formation have been proposed, while the last one has the advantage of being performed in situ in an aqueous medium at room temperature [13, 15, 24]. Also, GC–MS has the advantage of having a lower matrix effect than LC–MS, and the mass spectral library can be used to identify unknown compounds.

This work aimed to develop two miniaturized, selective, and sensitive SALLE-GC–MS and HS-SPME–GC–FID methodologies to determine MHA in urine with in situ derivatization and to demonstrate their suitability as specific confirmatory methods.

Materials and Methods

Chemicals and Materials

Methylhexanamine hydrochloride (racemic mixture of the R and S enantiomers) with a certified purity ≥ 98% was purchased from Sigma-Aldrich (USA). Isobutyl chloroformate (IBCF) with a certified purity > 98% was purchased from Sigma-Aldrich (USA). Ethyl acetate, methanol, sodium chloride and sodium sulfate anhydrous with certified purity ≥ 99% were acquired from J.T. Baker (Mexico). Methyl dodecanoate (≥ 99.0%) and buffer solution (boric acid/potassium chloride/sodium hydroxide), pH 10.00, were obtained from Merck (Germany). Deionized water (18.2 MΩ cm resistivity) was obtained from a Millipore Direct-Q 3 UV (USA).

For SPME, polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers (65 µm-thick coating) were purchased from Supelco (USA). The fibers were conditioned according to instructions provided by the supplier.

A stock solution of methylhexanamine hydrochloride (400 µg mL^{-1}) was prepared by weighing and dissolving the compound in methanol and the solution was stored at 4 °C. Standard solutions were prepared by dilution of stock solution in methanol.

Chromatographic Conditions

Gas chromatography coupled to mass spectrometry (GC–MS) analyses were performed with an Agilent 6890N GC coupled to a 5973 MSD mass selective detector (Agilent Technologies, USA), and data were collected using Agilent ChemStation software (version A.10.01). A Zebron ZB-5 (30 m × 0.25 mm I.D., 0.25 µm F.T) column (Phenomenex, USA). The oven temperature program was as follows: started at 80 °C for 2 min and then programmed at 10 °C min^{-1} to 300 °C where it was held for 5 min. Helium (99.999%, Praxair, Mexico) was used as carrier gas at 1 mL min^{-1}. The split/splitless injector temperature was 250 °C in splitless mode (1 min) with 1 µL as injection volume. The MS ionization potential was 70 eV; the transfer line and ion source temperature were 280 and 230 °C, respectively. Identification of MHA-IBCF derivative was realized with scan mode (50–550 *m/z*), while selected ion monitoring (SIM) was used for quantification; monitoring ions 126, 144 *m/z* were used for derivatized MHA and 87 y 214 *m/z* for methyl

dodecanoate (internal standard). The mass spectra for these compounds are shown in ESM Figs. S1 and S2.

Gas chromatography with flame ionization detector (GC–FID) analyses were carried out using an Agilent 6890N GC equipped with a split/splitless injector (Agilent Technologies, USA), and data were collected using Agilent ChemStation software (version A.10.01). The column and oven temperature program were the same as that of GC–MS. Hydrogen (99.98%, Praxair, Mexico) was used as carrier gas at 1 mL min^{-1}. The split/splitless injector temperature was 250 °C in splitless mode (1 min), and the fiber was allowed to remain in the inlet for 10 min to prepare the fiber for the next analysis and to avoid carryover. Detector temperature was 250 °C and makeup gas flow 30 mL min^{-1}.

Samples

Methods development and validation were done using urine samples (100 mL) collected from three healthy volunteers who declared not consuming any drug and they were not mixed. These samples were used as blanks. Samples were filtered and divided into aliquots which were stored at − 18 °C until their analysis. To apply the proposed methods, two athlete samples previously reported positive for MHA were provided by Comisión Nacional de Cultura Física y Deporte (CONADE, Mexico). The samples were analyzed in triplicate.

SALLE-GC–MS Method

Analytical Procedure

500 µL of urine sample was mixed with 500 µL of buffer solution, pH 10.00 (1:1 v/v), and 332 mg of sodium chloride. The mixture was stirred until complete dissolution of the salt. Then 10 µL of IBCF was added, homogenized for 30 s using a vortex and placed in a water bath at 30 °C. After 2.5 min of reaction, it was vortexed again for 30 s and placed again in a water bath at 30 °C for 2.5 min (reaction time 5 min). After that, the sample was extracted for 1 min with 500 µL of ethyl acetate which contained an internal standard (IS, 1.5 µg mL^{-1} of methyl dodecanoate). The organic phase was separated and passed through a glass column with 150 mg of sodium sulfate anhydrous and, finally, injected into the GC–MS system. Data collected as peak areas and peak area ratio of MHA-IBCF against IS were considered for all the calculations.

Optimization

To optimize MHA derivatization, two parameters were evaluated: type of derivatizing reagent [*N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), *N*-trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA) and isobutyl chloroformate (IBCF)] and its volume (10 and 20 µL).

Optimization of the SALLE method was performed with water spiked at 1 µg mL^{-1}. A preliminary screening investigation was performed with a full factorial design 2^3; the variables were time, temperature and salt addition that influence the derivatization and SALLE procedures. All the experiments were performed in random order to avoid trends, and two replicates were done for all the performed extractions. The area of MHA-IBCF peaks was considered as the response of the system to be studied. Data were analyzed using Statgraphics Centurion version XV software.

The knowledge about the system allowed a more detailed study to be performed to get the optimal conditions, temperature (20, 30, 40, and 50 °C), and salt addition (132, 249, 299, and 332 mg of NaCl) were studied. All experiments were performed in triplicate.

Matrix effect was evaluated by comparing the average relative response of MHA-IBCF ($n = 3$) between water and urine samples, both spiked at 100 ng mL^{-1} and extracted with the optimized conditions.

Internal Standard

For the internal standard four compounds were tested: ethyl octanoate, methyl undecanoate, methyl dodecanoate, and ethyl dodecanoate.

Validation

The following analytical parameters were evaluated: precision (repeatability), accuracy, linearity, limit of detection (LOD), and limit of quantification (LOQ).

Precision and accuracy were assessed by analyzing urine samples spiked at three concentration levels: 20, 100, and 201 ng mL^{-1}. For each concentration three replicates were made; the recoveries and relative standard deviation (RSD) were calculated for each level. The linearity of the method was evaluated using urine sample spiked at five concentration levels (20, 40, 100, 141, and 201 ng mL^{-1}). For each concentration level, a triplicate was made. The determination coefficient (r^2), slope, and *Y*-intercept of the calibration curve were calculated. The LOD and LOQ of each analyte were determined as a signal-to-noise ratio (*S*/*N*) of 3 and 10, respectively. The peak-to-peak noise was determined with the formula $N = I_{max} - I_{min}$, where N is the peak-to-peak noise, I_{max} is the highest (maximum) intensity peak, and I_{min} is the lowest (minimum) intensity peak in the time range (5 min). The value of *S*/*N* was obtained from the ChemStation software. For the signal-to-noise calculation, the ChemStation uses the formula *S*/*N* = height of the peak/noise.

HS-SPME–GC–FID Method

Analytical Procedure

400 µL of urine sample was mixed with 1600 µL of buffer solution, pH 10.00 (1:4 v/v), and 332 mg of sodium chloride in an 11 mL vial. The mixture was stirred until complete dissolution of the salt. Then a magnetic stirring bar (10 × 3 mm) and 1 µL of IBCF were added; the vial was sealed using an open polypropylene cap with a PTFE septum. It was placed in a water bath at 35 °C and the system was equilibrated for 5 min at 1200 rpm; then, headspace (HS) SPME extraction was performed for 30 min at 35 °C using a PDMS–DVB fiber. After the extraction, the fiber was retracted and desorption was carried out in the GC–FID system.

Optimization

Optimization of the HS-SPME method was performed with water spiked at 1 µg mL^{-1}. The PDMS–DVB fiber was used to evaluate derivatizing reagent volume (1 and 10 µL), extraction time (15, 25, and 30 min), and extraction temperature (25, 30, and 35 °C). All experiments were performed in triplicate. Matrix effect was evaluated by comparing the average relative response of MHA-IBCF ($n = 3$) between water and urine samples, both spiked at 100 ng mL^{-1} and extracted with the optimized conditions.

Validation

The following analytical parameters were evaluated: precision (repeatability), accuracy, linearity, limit of detection (LOD), and limit of quantification (LOQ).

Precision and accuracy were assessed by analyzing urine samples spiked at two concentration levels: 20 and 100 ng mL^{-1}. For each concentration, three replicates were made, and the recoveries and relative standard deviation (RSD) were calculated for each level. The linearity of the method was evaluated using urine sample spiked at five concentration levels (20, 41, 102, 143, and 205 ng mL^{-1}). For each concentration level a triplicate was made. The determination coefficient (r^2), slope, and *Y*-intercept of the calibration curve were calculated. LOD and LOQ of each analyte were determined as a signal-to-noise ratio (*S*/*N*) of 3 and 10, respectively. The value of *S*/*N* was obtained from the ChemStation software using the peak-to-peak noise as it was described in the validation section of the SALLE-GC–MS method.

Results and Discussion

SALLE-GC–MS Method

Internal Standard

To compensate for the variability of the method ethyl octanoate, methyl undecanoate, methyl dodecanoate, and ethyl dodecanoate were tested as internal standards. These compounds were selected because they have similar characteristics to MHA-IBCF such as analogous functional groups, length of the aliphatic chain, molecular weight, and are thermally and pH stable (ESM Table S2). They also presented a similar behavior to the MHA-IBCF derivative in the extraction process and the chromatographic analysis. Ethyl octanoate and methyl undecanoate were not selected, the first coeluted with interferent compounds from the matrix and the second had the same retention time of the first peak of MHA-IBCF. The peaks of methyl dodecanoate and ethyl dodecanoate did not interfere with MHA-IBCF, and the second had the highest retention time. Methyl dodecanoate was selected because its molecular weight and polarity were more similar to MHA-IBCF, and the chromatographic peak was more efficient. Three water samples were extracted with the optimum conditions using ethyl acetate containing methyl dodecanoate at 1.5 µg mL^{-1} in ethyl acetate as extractant. The area obtained was compared with a triplicate of the internal standard in ethyl acetate at the same concentration, and no significant difference was obtained in the response. Additionally, an internal standard calibration curve with MHA-IBCF from 100 to 1505 ng mL^{-1} and IS at 1.5 µg mL^{-1} was prepared in duplicate. For this experiment, good linearity was obtained with $r^2 > 0.99$ (ESM Fig. S3) indicating the suitability of methyl dodecanoate as IS. This is significant because the method can have a wide application range. Perrenoud and co-workers reported one study where healthy volunteers ingested a supplement containing 40 mg of MHA, and the compound excreted in the urine reached up to 18 µg mL^{-1} after 4 h [24].

Optimization

Derivatization is required to improve MHA detection in urine by GC–MS. This compound has two chiral centers (ESM Table S1) and as derivative two peaks corresponding to the pair of diastereomers should be observed [23]. It was reported that MHA reacts with cyclohexane to form a Schiff base, but though derivatization considerably improved chromatography, the diastereomer peaks were not completely resolved [24]. For this reason, three different derivatizing reagents were tested: BSTFA, MSTFA, and IBCF. In all the derivatization reactions, the presence of two peaks corresponding to MHA diastereomers was observed and their area was considered as MHA response. For silylation reactions, water spiked at 1 µg mL^{-1} MHA was previously extracted with toluene due to derivative moisture instability and the extract was evaporated to dryness (N_2, 80 °C), followed by derivatization with 50 µL of silylating reagent at 80 °C for 30 min. When using BSTFA, four pairs of chromatographic peaks were observed (ESM Fig. S4), indicating it was not selective and more than one derivative was formed. This is a

common problem when silylating primary amines, because mono- and disubstituted amines can be formed [13]. On the other hand, the reaction with MSTFA was more selective obtaining one derivative (ESM Fig. S5), but it had a low retention time (around 5 min), poor resolution for the diastereomers (Rs < 0.5), and matrix interference coelution. Finally, the reaction with IBCF was selected; it had several advantages such as it was done in aqueous media at low temperature (30 °C), had short reaction time (5 min), one derivative was obtained with retention time around 10.6 min (ESM Fig. S6), and the resolution between the pair of peaks was 1.7. For derivatization, two IBCF volumes were tested (10 and 20 µL), and the experiments were performed in triplicate. There was no significant difference at 95% in the MHA-IBCF response between the two volumes (ESM Fig. S8); for this reason, the lower volume was selected (10 µL).

Several parameters can influence the derivatization and SALLE performance, including salt addition, extraction time, and temperature. To set each variable, some considerations were done considering the chemistry and characteristics of MHA-IBCF (ESM Table S1), as well as the previously reported derivatization conditions using IBCF [25].

A preliminary screening investigation was performed with a full factorial design 2^3 using as variables reaction time, temperature, and salt addition (NaCl) to improve the extraction. The response was the area sum of both peaks of MHA-IBCF. The design matrix of the factorial design is shown in ESM Table S3.

To identify the key main and interaction effects that affect the response, a standardized Pareto plot of effects (ESM Fig. S7) was constructed. The Pareto plot indicates that all the main effects and two two-factor interactions are not statistically significant at 5% significance level. However, the two variables with the most influence in the response were the salt addition and the temperature. As can be seen in the main effects plot (ESM Fig. S7), when the temperature and the salt addition increased to their upper level (30 °C and 300 mg of NaCl, respectively) the response increased.

These two important variables were further studied. For reaction temperature, at 20 °C the derivative response was lower than with 30, 40, and 50 °C, and there was no significant difference at 95% between those three temperatures (ESM Fig. S9). The highest NaCl amount studied, corresponding to saturation in water, gave the most effective salting-out effect with the highest derivative response (ESM Fig. S10). Based on these results, the extraction conditions selected were reaction time 5 min, reaction temperature 30 °C, and NaCl amount 332 mg.

Matrix effect was evaluated by comparing the average relative response of MHA-IBCF extracted from water and urine samples, both spiked at 100 ng mL^{-1}. The derivative response in spiked urine samples decreased around 25% (Table 1); for this reason, matrix-matched calibration was used to overcome the negative matrix effect for quantitation purposes.

Figure 1 shows the ionic chromatograms of blank urine (a), urine spiked at 141 ng mL^{-1} (b), and a real sample (c). No interferences were detected in the analysis of blank by the optimized method, and chromatograms of urine spiked, and real sample showed good resolution, efficiency, and selectivity for the diastereomers, which showed a similar response in the spiked urine.

Validation

The proposed SALLE-GC–MS method for the analysis of MHA-IBCF was validated in terms of precision (repeatability), accuracy, linearity, LOD, and LOQ. Table 2 shows the results of the validation parameters. Method precision and accuracy were evaluated at two concentration levels (20 and 100 ng mL^{-1}) obtaining RSDs ≤ 5% and recoveries between 94 and 106%. Linearity was studied from 20 to 201 ng mL^{-1}, obtaining an r^2 value of 0.988. This interval was comparable with the linear range reported for the determination of MHA in urine by LLE-LC–MS/MS (50–700 ng mL^{-1}) [24]. LOD and LOQ were 5 and 14 ng mL^{-1}, respectively; LOD obtained was comparable with the one reported for MHA in urine analysis with SALLE-LC–TOF/MS (< 50 ng mL^{-1}) [26]. Additionally, the developed method uses five times fewer sample volume and ten times less organic solvent for extraction. Also, the LOQ obtained was below the minimum required performance limit (MRPL) of 100 ng mL^{-1} established by WADA [27].

Table 1 Comparison of the response of MHA-IBCF in samples of water and urine both spiked at 100 ng mL^{-1} to evaluate the matrix effect

Sample	SALLE-GC–MS		HS-SPME–GC–FID	
	Average area[a]	RSD[a]	Average area[a]	RSD[a]
Spiked water	133,379.3	7.6	80.1	1.5
Spiked urine	99,556.0	10.6	48.6	2.6

[a] $n = 3$

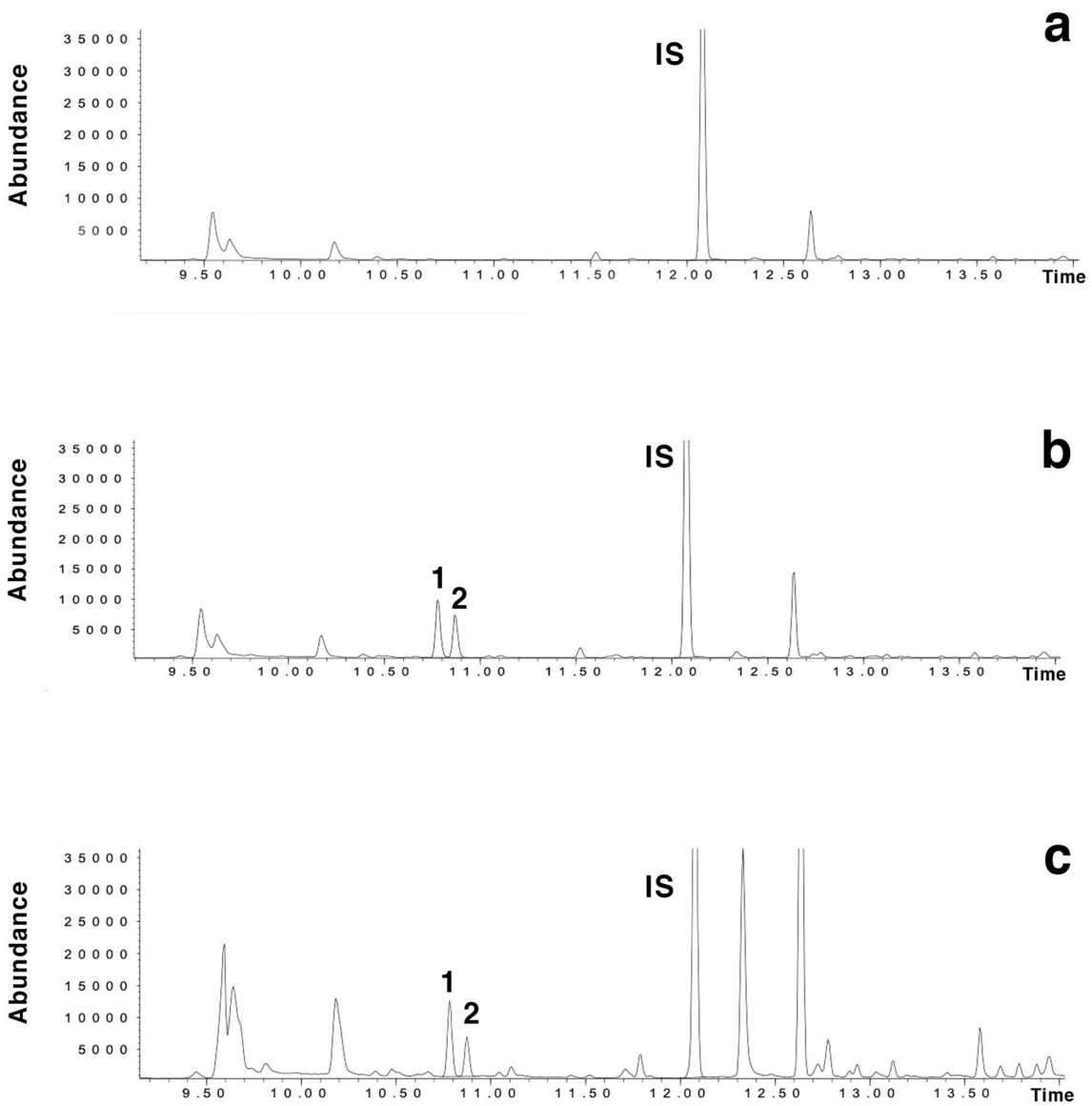

Fig. 1 Analysis of **a** blank urine, **b** urine spiked at 141 ng mL^{-1} and **c** real sample (> 100 ng mL^{-1}) using SALLE-GC–MS in selected ion monitoring mode. Peak identification: 1, 2. MHA; IS. methyl dodecanoate

Table 2 Validation results for developed methods to analyze MHA in urine

Method	Precision RSD[a]		Accuracy % recovery[a]		Linearity[a]			LOD[b]	LOQ[b]
	20[b]	100[b]	20[b]	100[b]	Intercept[c]	Slope[c]	r^2		
SALLE-GC–MS	5	3	94	106	0.007 ± 0.014 20, 40, 100, 141, and 201 ng mL^{-1}	0.0019 ± 0.0001	0.988	5	14
1HS-SPME–GC–FID	3	10	92	110	2.16 ± 5.41 (pA s) 20, 41, 102, 143, and 205 ng mL^{-1}	0.58 ± 0.04 (pA mL ng^{-1} s)	0.984	7	20

[a] $n = 3$

[b] ng mL^{-1}

[c] Confidence interval at 95%

HS-SPME–GC–FID Method

Optimization

HS-SPME conditions were optimized by studying derivatizing reagent volume, extraction time, and temperature. For derivatizing reagent volume, the use of 10 µL damaged the SPME fiber causing low derivative response; to avoid this 1 µL was tested, and with this volume the fiber remained intact and the derivative response increased 300-fold. The extraction time profile for MHA-IBCF showed the equilibrium was not reached even after 30 min (ESM Fig. S11), showing that work in the kinetic interval is more suitable to avoid long analysis time. When extraction temperature increased, the derivative response also increased, and 35 °C gave the highest response with adequate repeatability (ESM Fig. S12). Based on these results, the best conditions were: derivatizing reagent volume of 1 µL, extraction time 30 min, and extraction temperature 35 °C.

Matrix effect was evaluated by comparing the average relative response of MHA-IBCF extracted from water and urine samples, both spiked at 100 ng mL^{-1}. The derivative response in spiked urine samples decreased around 40% (Table 1); for this reason, matrix-matched calibration was used to overcome the negative matrix effect and for quantitation purposes. Figure 2 shows the chromatograms of blank urine (a), urine spiked at 143 ng mL^{-1} (b), and a real sample (c). No interferences were detected in the analysis of blank by the optimized method, and chromatograms of water and urine spiked showed good resolution, efficiency, and selectivity for the diastereomers, which showed a similar response.

Validation

The proposed HS-SPME–GC–FID method for the analysis of MHA-IBCF was validated in terms of precision (repeatability), accuracy, linearity, LOD, and LOQ. Table 2 shows the results of the validation parameters. Adequate precision and accuracy were found at two concentration levels (20 and 100 ng mL^{-1}) obtaining RSDs ≤ 10% and recoveries between 92 and 110%. Linearity was studied from 20 to 205 ng mL^{-1}, obtaining an r^2 value of 0.984. LOD and LOQ were 7 and 20 ng mL^{-1}, respectively. They were comparable with those obtained for SALLE-GC–MS, using a more affordable GC–FID instrument. Also, LOD and LOQ were below the minimum required performance limit (MRPL) of 100 ng mL^{-1} established by WADA [27].

Real Sample Analysis

The validated methods (SALLE-GC–MS and HS-SPME–GC–FID) were applied to analyze two athlete samples provided by CONADE, Mexico. The samples were analyzed in triplicate in two different days to consider the instrumental and environmental variability. The results are presented in Table 3. Samples 1 and 2 were identified as < 50 ng mL^{-1} and > 100 ng mL^{-1}, respectively; this classification was according to their previous analysis in CONADE's laboratory using LLE followed by GC–MS. The results obtained with both validated methods were in agreement with the results provided by CONADE. The concentrations obtained with the method HS-SPME–GC–FID had a higher variability than the method of SALLE-GC–MS. There was no significant difference between the averages of the results obtained with both methods at 95% significance. Also, as expected, the chromatographic profile between urine from healthy volunteers and athletes was different; these last samples showed more co-extracted compounds, but neither of them interfered with the MHA-IBCF peaks (Figs. 1, 2).

Conclusions

Two linear, accurate, precise, selective, and sensitive methodologies were developed for the analysis of MHA in urine samples. The procedures have several advantages: they are miniaturized, simple and MHA can be derivatized directly in the sample. Their applicability was demonstrated with the analysis of two athletes' urine: they are suitable alternatives for doping control because their quantitation limits are below 100 ng mL^{-1} which is the minimum required performance limit established by the World Anti-Doping Agency for stimulants. Both methods can also be suitable for the analysis of other stimulants with primary amines such as amphetamines and can be adequate for other biological matrices like blood and saliva.

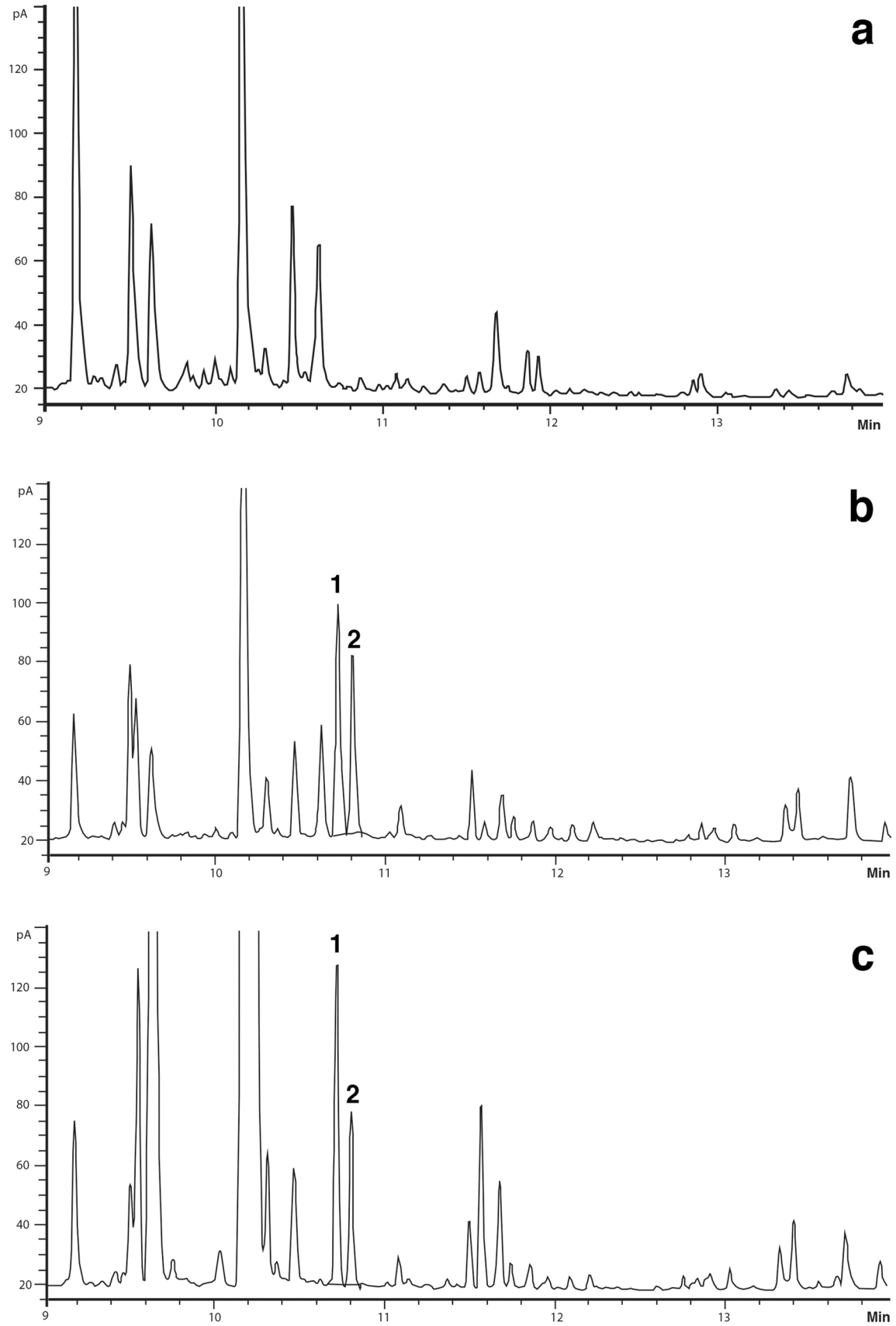

Fig. 2 Analysis of **a** blank urine, **b** urine spiked at 143 ng mL^{-1} and **c** real sample (> 100 ng mL^{-1}) using HS-SPME–GC–FID in selected ion monitoring mode. Peak identification: 1, 2. MHA

Table 3 Results obtained from the analysis of MHA in athletes' urine with the validated methods

Sample	LLE-GC–MS (CONADE)	SALLE-GC–MS		HS-SPME–GC–FID	
	Concentration[a]	Concentration[a]	RSD[b]	Concentration[a]	RSD[b]
1	< 50	36.0	8.3	33.1	8.4
2	> 100	151.5	4.4	147.3	9.2

[a]ng mL^{-1}

[b]$n = 6$

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10337-021-04116-w.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Eichner S, Maguire M, Shea LA, Fete MG (2016) Banned and discouraged-use ingredients found in weight loss supplements. J Am Pharm Assoc 56:538–543. https://doi.org/10.1016/j.japh.2016.03.013
2. ElSohly MA, Gul W, Tolbert C, ElSohly KM, Murphy TP, Avula B, Chittiboyina AG, Wang M, Khan IA, Yang M, Guo D, Zhang WD, Su J (2015) Methylhexanamine is not detectable in Pelargonium or Geranium species and their essential oils: a multi-centre investigation. Drug Test Anal 7:645–654. https://doi.org/10.1002/dta.1726
3. Dos Santos MK, Walber GB, Kreutz T, Soares K, Danielli LJ, Mariotti KC, Ritter M, Jackson GP, Arroyo LE, Limberger RP (2019) Evaluation of the presence of 1,3-dimethylamylamine in pelargonium leaves and essential oils by mass spectrometric and chromatographic methods. Chromatographia 82:875–883. https://doi.org/10.1007/s10337-019-03715-y
4. Denham BE (2017) When contaminated dietary supplements cause positive drug tests: methylhexaneamine as a doping agent in sport. Int J Sport Policy Polit 9:677–689. https://doi.org/10.1080/19406940.2017.1348963
5. Dunn M (2017) Have prohibition policies made the wrong decision? A critical review of studies investigating the effects of DMAA. Int J Drug Policy 40:26–34. https://doi.org/10.1016/j.drugpo.2016.10.005
6. Senado de la República Mexicana (2019) Gaceta: LXIV/1SPO-130/94931. https://www.senado.gob.mx/64/gaceta_del_senado/documento/94931. Accessed 06 Jan 2021
7. World Anti-Doping Agency (2020) Prohibited List. WADA. https://www.wada-ama.org/sites/default/files/wada_2020_english_prohibited_list_0.pdf. Accessed 06 Jan 2021
8. Sobolevsky T, Krotov G, Dikunets M, Nikitina M, Mochalova E, Rodchenkov G (2014) Anti-doping analyses at the Sochi Olympic and Paralympic Games 2014. Drug Test Anal 6:1087–1101. https://doi.org/10.1002/dta.1734
9. Pereira HMG, Sardela VF (2014) Stimulant doping agents used in Brazil: prevalence, detectability, analytical implications, and challenges. Subst Use Misuse 49:1098–1114. https://doi.org/10.3109/10826084.2014.907653
10. Archer JRH, Dargan PI, Hudson S, Wood DM (2013) Analysis of anonymous pooled urine from portable urinals in central London confirms the significant use of novel psychoactive substances. Q J Med 106:147–152. https://doi.org/10.1093/qjmed/hcs219
11. Archer JRH, Dargan PI, Lee HMD, Hudson S, Wood DM (2014) Trend analysis of anonymised pooled urine from portable street urinals in central London identifies variation in the use of novel psychoactive substances. Clin Toxicol 52:160–165. https://doi.org/10.3109/15563650.2014.885982
12. Archer JRH, Dargan PI, Lostia AM, van der Walt J, Henderson K, Drake N, Sharma S, Wood DM, Walker CJ, Kicman AT (2015) Running an unknown risk: a marathon death associated with the use of 1,3 dimethylamylamine (DMAA). Drug Test Anal 7:433–438. https://doi.org/10.1002/dta.1764
13. Deventer K, Roels K, Delbeke FT, Van Eenoo P (2011) Prevalence of legal and illegal stimulating agents in sports. Anal Bioanal Chem 401:421–432. https://doi.org/10.1007/s00216-011-4863-0
14. Müller RK, Grosse J, Thieme D, Lang R, Teske J, Trauer H (1999) Introduction to the application of capillary gas chromatography of performance-enhancing drugs in doping control. J Chromatogr A 843:275–285. https://doi.org/10.1016/s0021-9673(99)00498-7
15. Kataoka H (1996) Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. J Chromatogr A 733:19–34. https://doi.org/10.1016/0021-9673(95)00726-1
16. Domínguez-Romero JC, García-Reyes JF, Molina-Díaz A (2014) Comparative evaluation of seven different sample treatment approaches for large-scale multiclass sport drug testing in urine by liquid chromatography–mass spectrometry. J Chromatogr A 1361:34–42. https://doi.org/10.1016/j.chroma.2014.07.090
17. Zhang J, Wu H, Kim E, El-Shourbagy TA (2009) Salting-out assisted liquid/liquid extraction with acetonitrile: a new high throughput sample preparation technique for good laboratory practice bioanalysis using liquid chromatography–mass spectrometry. Biomed Chromatogr 23:419–425. https://doi.org/10.1002/bmc.1135
18. Snow NH (2000) Solid-phase micro-extraction of drugs from biological matrices. J Chromatogr A 885:445–455. https://doi.org/10.1016/S0021-9673(00)00192-8
19. Alsenedi KA, Morrison C (2018) Determination of amphetamine-type stimulants(ATSs) and synthetic cathinones in urine using solid phase micro-extractionfibre tips and gaschromatography-mass spectrometry. Anal Methods 10:1431–1440. https://doi.org/10.1039/C8AY00041G
20. Song A, Wang J, Lu G, Jia Z, Yang J, Shi E (2018) Oxidized multiwalled carbon nanotubes coated fibers for headspace solid-phase microextraction of amphetamine-type stimulants in human urine. Forensic Sci Int 290:49–55. https://doi.org/10.1016/j.forsciint.2018.06.031

21. Akramipour R, Fattahi N, Pirsaheb M, Gheini S (2016) Combination of counter current salting-out homogenous liquid–liquid extraction and dispersive liquid–liquid microextraction as a novel microextraction of drugs in urine samples. J Chromatogr B 1012–1013:162–168. https://doi.org/10.1016/j.jchromb.2016.01.031
22. Yanes EG, Lovett DP (2012) High-throughput bioanalytical method for analysis of synthetic cannabinoid metabolites in urine using salting-out sample preparation and LC-MS/MS. J Chromatogr B 909:42–50. https://doi.org/10.1016/j.jchromb.2012.10.013
23. Lopez-Avila V, Zorio M (2013) Identification of methylhexaneamine by GC high-resolution TOFMS and soft ionization. Forensic Sci Int 231:113–119. https://doi.org/10.1016/j.forsciint.2013.04.029
24. Perrenoud L, Saugy M, Saudan C (2009) Detection in urine of 4-methyl-2-hexaneamine, a doping agent. J Chromatogr B 877:3767–3770. https://doi.org/10.1016/j.jchromb.2009.09.013
25. Ugland HG, Krogh M, Rasmussen KE (1997) Aqueous alkychloroformate derivatisation and solid-phase microextraction: determination of amphetamines in urine by capillary gas chromatography. J Chromatogr B 701:29–38. https://doi.org/10.1016/s0378-4347(97)00350-2
26. Vonaparti A, Lyris E, Angelis YS, Panderi I, Koupparis M, Tsantili-Kakoulidou A, Peters RJB, Nielen MWF, Georgakopoulos C (2010) Preventive doping control screening analysis of prohibited substances in human urine using rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 24:1595–1609. https://doi.org/10.1002/rcm.4554
27. WADA Laboratory Expert Group (2019) Minimum required performance levels for detection and identification of non-threshold substances. https://www.wada-ama.org/sites/default/files/resources/files/td2019mrpl_eng.pdf. Accessed 06 Jan 2021.

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