Development and Application of Stir Bar Sorptive Extraction with Polyurethane Foams for the Determination of Testosterone and Methenolone in Urine Matrices

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Abstract

This work describes the development, validation, and application of a novel methodology for the determination of testosterone and methenolone in urine matrices by stir bar sorptive extraction using polyurethane foams [SBSE(PU)] followed by liquid desorption and high-performance liquid chromatography with diode array detection. The methodology was optimized in terms of extraction time, agitation speed, pH, ionic strength and organic modifier, as well as back-extraction solvent and desorption time. Under optimized experimental conditions, convenient accuracy were achieved with average recoveries of 49.7 ± 8.6% for testosterone and 54.2 ± 4.7% for methenolone. Additionally, the methodology showed good precision (< 9%), excellent linear dynamic ranges (> 0.9963) and convenient detection limits (0.2–0.3 µg/L). When comparing the efficiency obtained by SBSE(PU) and with the conventional polydimethylsiloxane phase [SBSE(PDMS)], yields up to four-fold higher are attained for the former, under the same experimental conditions. The application of the proposed methodology for the analysis of testosterone and methenolone in urine matrices showed negligible matrix effects and good analytical performance.

Introduction

Anabolic androgenic steroids (AAS) are substances related to male sex hormones, which promote muscle growth and development of male sexual characteristics (1–3). Due to these anabolic effects, they are often illegally used by athletes to improve their performance (2,4), which makes the analysis of these substances a priority task in the antidoping control. Since 1976, the use of these chemical agents in sports has been forbidden by the International Olympic Committee, being the World Anti-Doping Agency (WADA) the international organization responsible for the promotion and coordination of the fight against doping in sport in all its forms (3–6). Testosterone (T) and methenolone (Met) are good examples of natural and synthetic hormones, respectively, widely used by athletes and even in animal sports, including horse racing (1,7). The AAS and their metabolites are excreted in urine, as well as a large variety of endogenous compounds, so it is imperative to develop analytical methodologies with enough selectivity and sensitivity to detect these priority substances (5). Gas chromatography coupled to mass spectrometry after derivatization is the technique commonly used to detect AAS in urine samples (1,8–11). Due to the low concentration levels detected for these compounds, liquid–liquid extraction or solid-phase extraction are the sample preparation techniques usually employed (11,12). However, these methods are environmentally unfriendly, due to the use of large amounts of organic solvents and samples (11,13). In recent years, sorptive extraction techniques proved to be interesting alternatives and environmentally friendly approaches in comparison to conventional methodologies. Solid-phase microextraction and more recently, stir bar sorptive extraction (SBSE) are good examples (14–16). In the latter technique, a stir bar coated with a polydimethylsiloxane (PDMS) layer is placed under agitation into the sample in order to promote the extraction of the analytes by the polymeric phase. In several studies, SBSE has proved to have a remarkable correlation between the PDMS–water distribution coefficients and the octanol–water partitioning coefficients ($K_{PDMS/W} \approx K_{O/W}$) at the equilibrium, which are a measure of the polarity of organic compounds and provide a good indication of the extraction yield for a particular compound. However, several parameters must be optimized (i.e., extraction time, agitation speed, matrix characteristics such as pH, polarity and ionic strength and back-extraction conditions, in order to achieve maximum efficiency) (17–24). Although an excellent performance is usually attained (18–23), the stir bars commercially available are only coated with 24–126 µL of PDMS, which is a limitation because the more polar analytes present lower affinities towards this polymeric phase. Recently, it has been demonstrated that sex hormones in urine matrices can be well analyzed by SBSE with PDMS, but this approach did not present enough affinity for some hormones, in particular, the most polar ones (25). To overcome this limitation, several authors have proposed new strategies, such as in situ derivatization (26), the dual-phase

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stir bar (27), alkyl-diol-silica restricted access material (28), among others. Nevertheless, these analytical approaches presented a limited range of applicability. More recently, our group has proposed polyurethane foams (PUs) as alternative polymeric phases for SBSE (29–31), because these polymers present convenient characteristics (i.e., high mechanical and thermal stability, simplicity and "cost-effective") as well as high capacity to extract the more polar analytes (32).

The present contribution aims the development, validation and application of a novel analytical approach by using SBSE with PU and liquid desorption followed by high-performance liquid chromatography-diode array detection [SBSE(PU)–LD/HPLC–DAD], for the analysis of T and Met in urine matrices, whose chemical structures are depicted in Figure 1. The comparison of the data obtained by SBSE(PU) and by SBSE(PDMS) is also addressed.

Materials and Methods

Chemicals, standards, and samples

For the synthesis of the PU foams, two polyols were used, namely, glycerol propoxylate (Sigma-Aldrich, Milwaukee, WI) and trimethylolpropane ethoxylate (Aldrich, Germany), a catalyst, silicone oil (Dow Corning, Midland, MI) as a foam stabilizer, ultra-pure water as foam expander and methylene bisphenyl diisocyanate (BASF, Lupanat, Lemförde, Germany). Methanol (MeOH, 99.9%) and acetonitrile (ACN, 99.9%) of analytical grade, and hydrochloric acid (HCl, 37%) were obtained from Panreac (Barcelona, Spain). Sodium chloride (NaCl, 99.9%) and sodium hydroxide (NaOH, 98.0%) were obtained from AnalaR (BDH Chemicals, England). Phosphoric acid (H_3PO_4, 85 % w/v) was supplied by Riedel-de-Haën (Offenbach, Germany). Ultra-pure water was obtained from Milli-Q (Milipore, Bedford, MA) water purification systems. Stock standard solutions of T and Met (1,000 mg/L) in MeOH were kindly supplied by the National Anti-Doping Laboratory of Portugal. The urine samples were collected in the morning from a healthy 29 year-old-man and were properly stored in a refrigerator (5°C). The samples were filtered and placed under ultrasonic treatment for 20 min before being used.

The synthesis, clean-up, and characteristics of the PU foams used are described in a previous work (30).

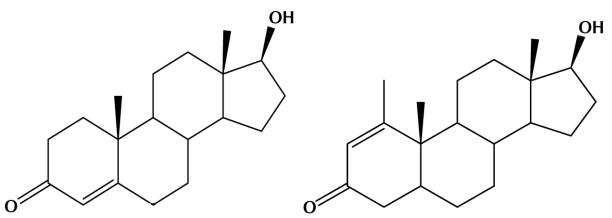

Figure 1. Chemical structures of the steroids under study.

SBSE(PU)-LD assays

In a typical assay, 25 mL of ultra-pure water (pH 7.0, 25°C) spiked at 10 µg/L level of both T and Met and a teflon stir bar coated with the PU were introduced in sampling glass vials (Macherey-Nagel, Düren, Germany), which were sealed using a hand crimper. The assays were performed in a magnetic stirrer with fifteen agitation points (Variomag H+P Labortechnik AG Multipoint 15, Oberschlei Bheim, Germany) at room temperature (25°C). For the optimization studies, parameters such as agitation speed (750, 1000, and 1250 rpm), extraction time (0.5, 1, 2, 4, 6, and 16 h), pH (3.0, 7.0, and 11.0), ionic strength (5, 10, and 15% of NaCl; w/v), and amount of organic modifier (5, 10, and 15% of MeOH; v/v) were systematically studied in triplicate and compared with the controls used to spike the samples. To evaluate the best LD conditions, triplicate assays were performed to test back extraction solvents (MeOH, ACN and an equimolar mixture of both) and time (15, 30, 45, and 60 min). For LD assays, the stir bars were removed with clean tweezers and placed in 10 mL glass vials (Macherey-Nagel, Düren, Germany) with 5 mL of MeOH, ensuring their total immersion prior to ultrasonic treatment (Branson, ultrasonic cleaner, model 3510 E-DTH, Danbury, CT) at a constant temperature (25°C). After back extraction, the stir bar was removed, and the resulting extract was evaporated until dry under a gentle stream of purified nitrogen (> 99.5 %), followed by reconstitution with 200 µL of MeOH, transferred to a 2 mL glass vial, closed with a seal using a hand crimper and placed in the automatic sampler tray for HPLC–DAD analysis. To verify possible losses occurred during the evaporation step, triplicate assays were performed by spiking 5 mL of MeOH at a concentration level of 10 µg/L and evaporating them to dryness followed by HPLC–DAD analysis. The signals obtained for the samples after redissolution and the control were compared. The carry over effect was also evaluated. For validation purposes, triplicate assays were performed under optimized experimental conditions. Blank assays were also performed using the same procedure as described previously, employing ultra-pure water samples without spiking. In order to validate the methodology, assays with different concentration levels (1.5–20.0 µg/L) were performed under optimized experimental conditions.

For real sample assays, the standard addition methodology (SAM) was used to quantify and suppress possible matrix effects. In these assays, 5 mL of urine were diluted with ultra-pure water to 25 mL of sample and spiked with working standards at the desired concentrations (2.0–16.0 µg/L), performing as before, under optimized experimental conditions. Blank assays (zero point), were also performed on real matrices using the same procedure as previously described without spiking. The assays with the commercial stir-bars (Twister; Gerstel, Müllheim a/d Ruhr; Germany) coated with 20 mm in length and 1.0 mm in film thickness of PDMS (126 µL) were performed using the optimized procedure.

HPLC–DAD analysis

HPLC–DAD analyses were carried out on an Agilent 1100 Series LC system (Agilent Technologies, Waldbronn, Germany), equipped with the following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A) and the diode

array detector (G1315B). The data acquisition and instrumental control were performed using the software LC3D ChemStation [version Rev.A.10.02(1757); Agilent Technologies]. A Tracer Excel 120 ODS-A column, 150 mm × 4.0 mm, with 5 µm particle size (Teknokroma, Sant Cugat, Spain) was used. The analyses were performed under isocratic conditions, with a flow of 1 mL/min, using a mobile phase consisting of a mixture of 60% of MeOH and 40% of 0.1% (w/v) phosphoric acid in ultrapure water. The injection volume was 20 µL with a draw speed of 200 µL/min. The detector was set at 245 nm. For identification purposes, the samples were spiked with pure standards, and the identification was based on the retention parameters and UV–vis spectral data obtained from the pure standards. For quantitation purposes, calibration plots using external methodology were performed. For recovery calculations, peak areas obtained from each assay were compared with those of the standard controls used for spiking. For quantitation purposes on real matrices, calibration plots using the standard addition method (SAM) were performed.

Results and Discussion

HPLC–DAD operating conditions

In a first approach, the HPLC–DAD conditions including UV–vis spectral data for the detection of T and Met, as well as retention time characteristics were carefully assessed. It was observed that 245 nm was the wavelength that provided the maximum response for both steroids. Figure 2A exemplifies a chromatogram of a standard mixture of T and Met showing an excellent resolution within a suitable analytical time (< 21 min), under the instrumental conditions used. Instrumental calibration performed with standard solutions, using concentrations ranging from 50.0 to 5,000.0 µg/L, showed excellent linearity with correlation coefficients (r^2) higher than 0.9997 for both steroids. The instrumental sensitivity was checked through the limits of detection (LODs) and quantitation (LOQs), obtained by the injection of diluted standard solutions and calculated with a signal-to-noise ratio (S/N) of 3/1 and 10/1, respectively. The LODs and LOQs obtained were 9.0 and 29.0 µg/L for T, and 5.0 and 17.0 µg/L for Met, respectively. Furthermore, instrumental precision was also evaluated through repeatability injections of standard solutions, resulting in relative standard deviations (RSD) lower that 8%. During the present work, no instrumental carry-over was observed, because the background was always below the LODs achieved.

Optimization of the parameters affecting the SBSE(PU)-LD efficiency

After optimizing the instrumental conditions, it was decided to proceeded to the optimization of the parameters that could affect the SBSE(PU)-LD efficiency. To determinate the best LD conditions that allowed the complete back extraction of the target analytes from the PU, the process started by evaluating the desorption solvent. The solvents studied were MeOH, ACN and equimolar mixtures of both, using standard conditions, that is, 1 h of extraction (1000 rpm), and 15 min for desorption time. In Figure 3A, it can be observed that the different solvents used did not influence drastically the back-extraction yields of the target compounds. However, MeOH presented slightly higher recoveries and, therefore, it was chosen as the LD solvent. After the selection of the most efficient solvent, the effect of desorption time under ultrasonic treatment was studied during 15, 30, 45, and 60 min. The results obtained are depicted in Figure 3B and show that the recovery of T and Met was higher when a LD period of 60 min was used. Consequently, this time was established for the back-extraction process. After LD optimization, the evaporation step under a gentle stream of purified nitrogen was also evaluated. The results demonstrated that the evaporation is not a limiting step for this analytical approach, which was expected because both steroids under study are non-volatiles substances, as demonstrate before (25). Furthermore, the back-extraction of both analytes in a single LD step was confirmed by performing two consecutive LDs. No observable amounts of the target analytes could be further recovered from the PU after the first LD extraction.

According to literature (15–26), the agitation speed is an important parameter that can increase the recovery yield by reducing the extraction time. This parameter can influence very much the mass transfer of the analytes towards to sorbent material during the equilibrium process (15). Different agitation speeds (750, 1000, and 1250 rpm) were evaluated to verify this effect on the recovery yields of both analytes. Figure 3C shows that the differences in the efficiency of T are negligible, although in the case of Met there is a significant increase in the recovery yields when the agitation speed decreased. Therefore, an agitation speed of 750 rpm was chosen for the further assays. The effect of the equilibrium time during the extraction process is

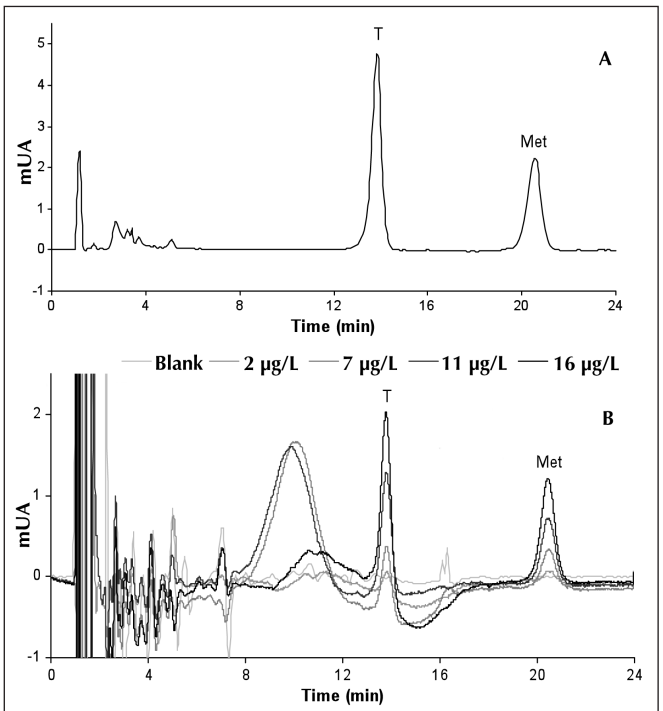

Figure 2. Chromatograms obtained from a standard aqueous solution of T and Met (10.0 µg/L) by HPLC–DAD (A) and after the SAM in urine matrices by SBSE(PU)-LD/HPLC–DAD (B), under experimental optimized conditions.

another important parameter, because it determines the time required for each compound to be quantitatively transferred from the matrix bulk towards the polymeric phase. It is expected that the efficiency of extraction increases with time until it becomes constant, meaning that the equilibrium is attained. Figure 3D illustrates the effect of the equilibrium time (0.5, 1, 2, 4, 6, and 16 h) on the recovery yields. For both T and Met, the equilibrium was achieved after 4 h of extraction, therefore, it was the period used in the subsequent experiments. The characteristics of the aqueous medium were also addressed, particularly the pH, ionic strength and polarity. In order to study the effect of the pH in the SBSE(PU) efficiency, three values (3.0, 7.0, and 11.0) were assessed. This parameter is described to have great relevance for ionisable compounds depending their recoveries on the pH of the matrix (21,25). The results obtained show that the recovery of T is slightly higher at pH 7.0, but no significant differences were observed for Met, so it can be considered that this parameter is not relevant to be taken into account for the particular target compounds involved. In general, an increase of the ionic strength through the addition of a strong electrolyte reduces the solubility of the analytes in the matrix ("salting-out effect"), which will favor their migration towards the polymeric phase, thereby increasing their recoveries (20,30). The influence of the ionic strength was evaluated by the addition of different amounts of NaCl (0, 5, 10, and 15%). Figure 3E shows the effect of the NaCl content where both analytes present higher recoveries in the absence of salt. This phenomenon can be explained through the occupation of the superficial area of PU foam with the salt ions, which diminishes the availability to interact with T and Met. Consequently, further experiments were performed without salt addition. The adsorption of the target analytes onto the vial glass walls is also a phenomenon that can occur ("wall-effect"), giving rise to a decrease of the extraction efficiency. To overcome this problem it is common to add an organic modifier to the aqueous matrix, in order to reduce possible adsorption effects. MeOH is the solvent usually chosen for this purpose (20,30). As depicted in Figure 3F, the progressive addition of MeOH (0, 5, 10, and 15%) reduces significantly the recovery of both analytes and therefore, the following assays were carried out in the absence of MeOH. This may be explained by the fact that the amount of MeOH in solution may increase the solubility of the analytes in the aqueous layer, thus reducing their migration towards the polymeric phase. This effect is thus stronger than the "wall-effect".

Validation of the SBSE(PU)-LD/HPLC–DAD methodology

The parameters were optimized that affect the proposed methodology and then corresponding validation was reached by studying the analytical limits, linear range, and precision associated. In a first approach, assays performed at the 10 µg/L level, under optimized experimental conditions [SBSE(PU): 4 h (750 rpm, pH 7.0); LD: 1 h (MeOH, 60 min)], showed that the proposed methodology presents good performance for T and Met, with average yields of 49.7 ± 8.6 % and 54.2 ± 4.7 %, respectively. The linear range was evaluated using seven levels of concentration between 1.5 and 20.0 µg/L, showing linearity with excellent correlation parameters ($r^2 > 0.9963$; T: a = 4.1675, b = 0.3916; Met: a = 3.3705, b = 0.8296). The analytical limits achieved for T and Met were 0.3 µg/L and 0.2 µg/L concerning the LODs, and 0.8 µg/L and 0.6 µg/L for LOQs, respectively, being determined as before and presenting the same order of magnitude of those achieved in an earlier study using SBSE(PDMS) (25). It must be emphasized that the values achieved with the proposed methodology were much lower than the minimum required performance limits (10 µg/L) demanded by the WADA (33) for the detection of these anabolic agents in laboratorial work, as well as when compared with other analytical systems (11,34). Additionally, the precision of the presented methodology was also evaluated through repeatability assays calculated as RSD of three replicates of samples spiked at the 10 µg/L giving rise to values lower than 9%. Table I summarizes the validation data obtained for T and Met by the proposed methodology under optimized experimental conditions.

It was also addressed the comparison between the recoveries obtained by SBSE(PU) and by SBSE(PDMS), using commercial

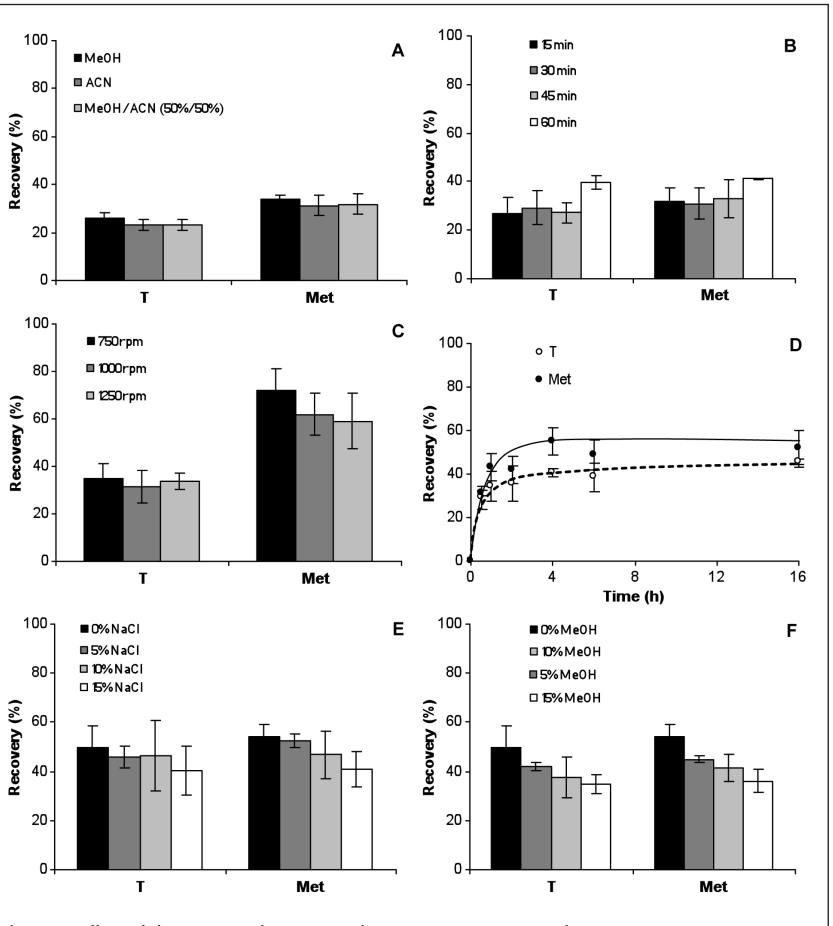

Figure 3. Effect of desorption solvent (A) and time (B), agitation speed (C), extraction time (D), ionic strength (E), and organic modifier (F) in the recovery of T and Met from aqueous samples by SBSE(PU)-LD/HPLC–DAD.

stir bars (126 µL). Figure 4 demonstrates the efficiency attained, where the recovery yields obtained for T and Met with PU are up to four-times higher than with PDMS, under the same experimental conditions. Therefore, it can be stated that SBSE(PU) presents higher advantages to recover analytes with more polar characteristics in aqueous media, that is, log $K_{O/W}$ < 4.0, such as T (log $K_{O/W}$ = 3.27) and Met (log $K_{O/W}$ = 3.69), according to previous reports (30).

Application to real matrices

To demonstrate the analytical ability of the optimized methodology to analyze T and Met in urine matrices, assays were performed in real samples, because AASs and their metabolites are commonly excreted in these biological fluids. The SAM was chosen because it takes into account the possible matrix effects caused by potential interfering compounds, due to the complexity of these types of matrices. Therefore, urine samples were spiked with four levels of concentration of both target analytes ranging from 2.0 to 16.0 µg/L and blank assays were also performed without spiking. The results obtained by SAM showed suitable linearity (r^2 > 0.9884) and negligible matrix effects (T: a = 4.5141, b = 0.3612; Met: a = 3.2241, b = 1.1534), under the optimized experimental conditions established in the previous sections, as can be observed in Figure 5. Figure 2B exemplifies chromatograms obtained from urine matrices through the SAM at different levels of concentration by SBSE(PU)-LD/HPLC–DAD, where very high sensitivity and selectivity can be noticed. The application of this methodology to the urine sample collected from a healthy man showed that the concentration of the target analytes were clearly below the LODs achieved, under optimized experimental conditions. Although T is a natural steroid, the amounts excreted in urine during the day are in general variable, depending on each individual metabolism. In the case of Met, because it is a synthetic steroid, it was expected not to be detected in this particular matrix. In spite of the main goal to prove the remarkable performance attained by the proposed methodology to determine Met and T in the complex urine matrices, the absence of levels detected in the particular sample studied are referred only to the case of free steroid hormones, although in organisms they are mainly in a conjugate form, metabolized by the endocrine glands (25).

Table I. Retention Times, LODs, LOQs, Linear Dynamic Parameters Obtained For T and Met in Aqueous Samples by SBSE(PU)-LD/HPLC–DAD, Under Optimized Experimental Conditions

	RT (min)	LOD (µg/L)	LOQ (µg/L)	Linear range (µg/L)	r^2	a	b
T	13.5	0.3	0.8	1.5–20.0	0.9991	4.1675	0.3916
Met	20.5	0.2	0.6	1.5–20.0	0.9963	3.3705	0.8296

*Determination coefficient (r^2), slope (a), and intercept (b).

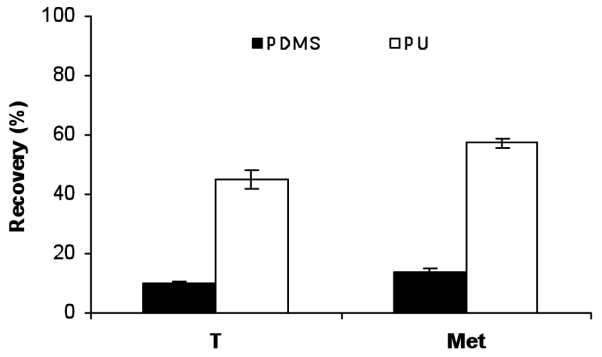

Figure 4. Comparison between the performance of SBSE(PU) and SBSE (PDMS) on the recovery of T and Met from aqueous samples followed by LD/HPLC–DAD, under optimized experimental conditions.

Conclusions

In the present work, SBSE(PU)-LD/HPLC–DAD proved to be simple, easy to work-up, reliable, sensitive and with low sample requirement to analyze T and Met in aqueous media at trace levels. Furthermore the PU foams are easy to synthesize, stable and cost-effective. When comparing SBSE(PU) with the conventional SBSE(PDMS), the former approach presents higher performance with good precision, excellent linear dynamic range and good analytical limits. The application of the proposed methodology to analyze T and Met in urine matrices proved that the polyurethane foam can be used efficiently in more complex matrices than water without matrix effects.

Acknowledgments

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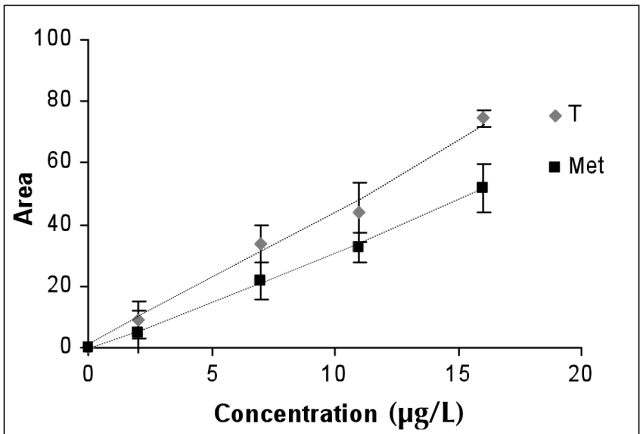

Figure 5. SAM plots obtained for T and Met in urine matrices by SBSE(PU)-LD/HPLC–DAD, under optimized experimental conditions.

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