Microchemical Journal 158 (2020) 105149

Contents lists available at ScienceDirect

Microchemical Journal

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

Rotating-disk sorptive extraction for the determination of sex hormones and triclosan in urine by gas chromatography-mass spectrometry: Clean-up integrated steps and improved derivatization

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ARTICLE INFO

Keywords:
Rotating-disk sorptive extraction (RDSE)
Clean-up
Urine
Derivatization
Sex hormones
Triclosan

ABSTRACT

Different clean-up stages were coupled to rotating-disk sorptive extraction (RDSE) of testosterone, progesterone, 17β-estradiol and triclosan in urine samples prior to derivatization and detection by gas chromatography-mass spectrometry (GC–MS). By using Oasis® HLB as the sorptive phase, extraction equilibrium was reached after 60 min at a disk rotation velocity of 2000 rpm.

The factors involved in sample preparation of the urine were comprehensively studied and implemented to minimize matrix effects that were mainly produced by polar pigments in the urine. A 10-fold dilution of the sample was necessary prior to RDSE, followed by a washing step of the sorptive phase with 10% (v/v) methanolic solution and final selective desorption of the analytes with ethyl acetate. Derivatization of the analytes was also studied in detail and implemented prior to GC–MS. The reaction was optimized in terms of derivatizing agent consumption, time and temperature, achieving significant improvements in these factors.

Under the optimized conditions, the matrix effects decreased almost five-fold for all analytes, and the relative recoveries were between 89 and 111% with detection limits in the range of 0.004–0.54 ng mL^{-1}, whereas the precision, expressed as relative standard deviation (RSD), was below 14%.

Analytes were determined in real samples in the presence and absence of enzymatic hydrolysis, assessing both their free and total forms. The free triclosan concentration was only 10% of the total concentration found in the same sample after hydrolysis. Estradiol and testosterone were quantified with high sensitivity at concentrations between 0.11 and 10.45 and 0.20–21.23 ng mL^{-1}, respectively. Progesterone was only quantified in a urine sample from a woman during pregnancy.

1. Introduction

Sex hormones are related to a wide variety of biochemical processes mainly concerning reproductive function and sexual development [1]. Progesterone (Prog) and 17β-estradiol (E2) are the principal female sex hormones and they play an important role in pregnancy, fertility and the menstrual cycle. Analogously, testosterone (Test) is the male sex hormone associated with the production of sperm and the development of physical characteristics. Alterations in these hormones are related to various clinical conditions, such as a reduction in sexual impulse, infertility, depression and decreased energy [2]. It has also been reported that sex hormones are related to eating disorders [3], polycystic ovary syndrome [4], circulating natriuretic peptide [5], benign prostatic hyperplasia [6], hypogonadism [7], breast cancer [8–11], laryngeal cancer [12] and prostate cancer [2,13].

Traditionally, sex hormone testing is performed via serum, in which the reference ranges are well established. In this case the sample collection is relatively simple, although it includes an invasive method. In addition, in some cases, it is not possible to distinguish between bound and free hormones, which may result in misdiagnosis [14], and punctual sampling is not entirely correct since the secretion of hormones is pulsatile throughout the day. Additionally, it is possible to test sex hormones and metabolites in urine to assess their concentrations. For instance, a 24 h collection is frequently used and is the most reliable way to assess the metabolism of steroid hormones [15,16] because urine tests can measure the free and total hormones, reflecting the amount that is bioavailable; moreover, there is the advantage in that the collection of samples is not invasive. In addition, access to high sample volumes allows the preconcentration of analytes, favoring the analytical sensitivity of chemical methodologies. A hormonal profile in

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https://doi.org/10.1016/j.microc.2020.105149
Received 18 May 2020; Received in revised form 8 June 2020; Accepted 9 June 2020
Available online 11 June 2020

urine reflects not only endocrine disorders through the inadequate action of the glands [17] but is also a measure for doping control in athletes to establish possible ingestion of endogenous hormones [1,18].

There have been several studies in which specific alterations associated with the metabolism and secretion of sex hormones are related to the presence of organic pollutants, including polychlorinated biphenyls [19], zearalenone [20], phthalates [21], acrylamide [22] and phenols, parabens and triclocarban [23]. Other pollutants, such as triclosan (TCS), which has been described as an endocrine-disrupting chemical, could also affect the level of sex hormones in urine after continuous exposure. To assess its eventual effects, it is first necessary to develop a simultaneous analytical methodology, such as the one proposed in this work, and then exposure to TCS and its effects on sex hormones could be studied elsewhere.

The simultaneous determination of sex hormones by GC–MS represents some drawbacks in terms of cost, time and ecoefficiency because it requires two methods of derivatization (one for keto groups and another for hydroxy groups) [24,25]. An alternative proposed by some authors is the use of a derivatizing mixture composed of one derivatizing agent, a catalyst and a reducing agent for the determination of sex hormones by the same reaction route [26–31]. Liquid chromatography coupled to mass spectrometry (LC-MS), mainly with MS/MS, is another derivatization-free alternative for the determination of sex hormones in urine [32–34]. However, in the case of estrogens, derivatization reactions have been necessary to improve the sensitivity [35,36]. A comparative study between LC-MS and GC–MS for the analysis of estrogens and their metabolites in urine (both with derivatization) shows that the limits of quantification by GC are 50 times smaller, although LC is faster and less expensive [37].

Sample preparation prior to determination by the mentioned techniques is conditioned by the complexity of each matrix under study. When biological samples are analyzed, different interferences can affect the quality of the results. Therefore, known methods of extraction, including clean-up steps prior to or during the application of the methodology should be implemented. For example, in solid-phase extraction (SPE) of hormones from urine, some authors include a clean-up stage with a methanolic solution to discard unwanted components of the sample [38]. Other researchers have quantified estrogens in urine samples by stir bar sorptive extraction (SBSE) using a sorbent of polydimethylsiloxane (PDMS) and GC–MS [39], and they considered a dilution of approximately three times that of the urine sample in addition to enzymatic hydrolysis prior to extraction to decrease matrix complexity. Similarly, other authors used SBSE combined with HPLC-DAD for the determination of estrogens and progestogens in urine matrices [40]. They refer to the high complexity of the urine matrix; however, studies of matrix effects were not executed in their research to compensate for the low recoveries of some compounds.

Similar to SBSE, rotating-disk sorptive extraction (RDSE) is an equilibrium-based microextraction technique that integrates extraction and rotation in the same device [41,42]. An important advantage of RDSE is its considerable cost savings because the que body of the Teflon extraction device can be reused countless times, easily changing the sorptive phase portion. In addition, the high rotation velocity of the disk (2000–3000 rpm) in most RDSE applications together with the high surface area to volume of the sorptive phase make this microextraction technique highly efficient from a kinetic point of view.

Different strategies for quantification have been described in RDSE applied to different kinds of liquid samples, such as wastewater [41], drinking water [42], river water [24], leachates [43], animal plasma [44] and urine [45,46]. Although matrix effects can be much higher in urine, in previous RDSE applications, only dilution of the sample was required because the pigments contained in the urine were not significantly sorbed onto the nonpolar sorptive phases (e.g., C18). In contrast, in more polar sorptive phases, such as Oasis® HLB, pigments (mainly urobilin) are coextracted together with analytes, which can be transferred to the final extract after elution. A detailed study of the clean-up steps integrated into the RDSE process have been developed to minimize the effects of urobilin interference in the measurement of sex hormones (E2, Test and Prog) and TCS by GC–MS with preoptimized derivatization.

2. Materials and methods

2.1. Reagents

Water from a Simplicity® Water Purification System, Millipore (Darmstadt, Germany) was used throughout the experiment. Testosterone (Test), 17β-estradiol (E2), progesterone (Prog) and triclosan (TCS) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Testosterone-2,3,4-$^{13}C_3$ (Test-$^{13}C_3$) from Sigma-Aldrich (Milwaukee, United States) and triclosan-2,3,5-D_3 (TCS-D_3) from Dr. Ehrenstorfer GmbH were used as surrogate standards. The HPLC grade solvents ethyl acetate (EtAcO), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), and acetic acid/sodium acetate buffer (> 99% purity) were from Merck (Darmstadt, Germany). Standard stock solutions of the analytes (10 mg L^{-1}) were prepared separately in MeOH. Multistandard solutions were prepared between 0.1 and 100 ng mL^{-1}. Nitrogen (99.995% purity) and helium (99.9999% purity) were purchased from Linde (Santiago, Chile) and were used for final extract evaporation and as the chromatographic carrier gas, respectively. Oasis® HLB and PRiME HLB were obtained from Waters Corporation (Milford, United States). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) from Merck and iodotrimethylsilane (TMIS) and 1,4-dithioerythritol (DTE) from Sigma-Aldrich were used for derivatization. β-Glucuronidase from *Helix pomatia* type HP-2 aqueous solution, ≥100,000 units mL^{-1} from Sigma-Aldrich was used for enzymatic hydrolysis. The physicochemical properties of the analytes and solvents mentioned in the text were provided by ChemIDplus by SRC, Inc.

2.2. Instruments

A Thermo Scientific TRACE 1300 gas chromatograph (Milan, Italy) coupled to a Thermo Fisher Scientific ISQ (Austin, TX, United States) mass-selective detector was used for GC–MS determinations. A Restek (Bellefonte, United States) RTX-5MS (30 m × 0.25 mm i.d.; 0.25 μm film thickness) was used as the chromatographic column. Two microliters of sample extract were injected into the gas chromatograph using an injector temperature of 250 °C in splitless mode. The column temperature started at 75 °C (1 min) and then increased to 150 °C at a rate of 20 °C min^{-1} (5 min) and to 300 °C at a rate of 10 °C min^{-1} (5 min). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The solvent delay was 7 min. A dwell time of 0.2 s was used for each *m/z* value. The MS transfer line and ion source were maintained at 250 °C and 200 °C, respectively, and compound quantification was based in selective ion monitoring (SIM) mode.

The vial containing the sample and the rotating disk was placed on an MR 300 multimagnetic stirrer (Heidolph Instruments, Germany). The pH values were measured with a Microprocessor 537A pH meter (WTW, Germany). A KMC-1300 V (Vision Scientific Co., Ltd., Korea) was used as the vortex mixer. Statistical software (Statgraphics Centurion XV for Windows; Manugistics, United States) was used for chemometric designs.

A Nicolet iS5 FTIR (Thermo Fisher Scientific) spectrometer equipped with a KBr/Ge beam splitter, a high-performance deuterated triglycine sulfate detector and a smart iTX-iD7 attenuated total reflectance (ATR) sampling accessory with diamond crystal was used for infrared spectra measurements. It has the all-reflective optics that allow the highest throughput possible without spectral range losses associated with using focusing lenses or elements. The wavenumber range was 4,000 to 400 cm^{-1}. A resolution of 4 cm^{-1} and 16 scans per sample were used. OMNIC 8.0 software (Thermo Fisher Scientific) was used to

Table 1
Experimental conditions associated with RDSE and sample clean-up.

Stage	Experimental conditions
Extraction	20 mL of diluted urine at 2000 rpm for 60 min. The solution was discarded and the disk was washed with deionized water.
Washing	10 mL of 10% MeOH at 2000 rpm for 5 min. The solution was discarded and the disk was washed with deionized water.
Elution	10 mL of EtAcO at 2000 rpm for 20 min. The ethyl acetate extract was transferred to another vial and evaporated to dryness for derivatization and measurement by GC–MS.

resolve the spectra.

2.3. Rotating-disk sorptive extraction (RDSE)

The material of the disks used was Teflon, and they contained an internal magnetic stirrer. The disk contains a cavity where 50 mg of Oasis® HLB was added. The cavity was covered with a fiberglass filter and sealed with a Teflon ring. Preconditioning of the sorptive phase in the disk was performed with 5 mL of ethyl acetate, 5 mL of methanol and 5 mL of water. A 2 mL aliquot of each urine sample was added to an extraction vial, and 18 mL of deionized water (10 × dilution) was added followed by manual stirring. The pH was adjusted to 5.8 with acetate buffer. The extraction procedure is summarized in Table 1.

2.4. Derivatization

The derivatization mixture was previously prepared; 1000 μL of MSTFA was added to 5 mg of DTE, and the mixture was mixed by vortexing until the reducing agent dissolved. Then, 2 μL of TMIS was added and manually homogenized, and the reagent was stored at 4 °C until use. The multistandard or sample extract (after RDSE) was placed in a vial and evaporated to dryness under a N_2 stream. An aliquot of 50 μL of the derivatizing mixture consisting of MSTFA/DTE/TMIS and 50 μL of ethyl acetate was added to the dry vial and mixed on a vortex for 5 min at room temperature. Then, the sample was transferred to a vial insert and was ready to be analyzed by GC–MS. Different organic solvents were studied as part of the derivatization reaction to reduce the volume of the derivatizing mixture. In addition, the ratio of the volume of solvent selected and the volume of derivatizing mixture was tested.

2.5. Validation of the method

The analytical quality of the proposed methodology was verified through figures of merit at 5 ng mL^{-1}. Accuracy was expressed as a function of recovery (%Re), precision was based on percent relative standard deviation (%RSD, n = 6), sensitivity according to detection (3 times the σ blank signal) and quantification (10 times the σ blank signal) limits and matrix effects when comparing the response of the analytes in the organic solvent and the urine matrix. Linearity was verified between 1 and 100 ng mL^{-1} in the urine matrix. The recoveries (eq. (1)) and matrix effects (eq. (2)) were calculated according to the following equations:

$$Re(\%) = (A_{S4} - A_{S3})/A_{S2} \quad (1)$$

$$ME(\%) = (A_{S2} - A_{S3})/A_{S1} \quad (2)$$

where each term corresponds to the relative area (A) of S1: multistandard solution of 5 ng mL^{-1} of each analyte; S2: urine extract obtained using RDSE, which was enriched with 5 ng mL^{-1} analytes just before injection; S3: extract obtained directly from a urine sample (blank); and S4: extract obtained from a urine sample enriched with 5 ng mL^{-1} multistandard since the beginning of the extraction. Using these equations, the recovery value obtained was free of any matrix effects and vice versa.

2.6. Application to real urine samples: Determination of the free and total (enzymatic hydrolysis) concentrations of the analytes

Urine samples from five healthy volunteers were collected in sterile containers and stored at −20 °C. The samples were differentiated according to age and sex, including a woman during pregnancy (5 months), and the samples were taken from the first urine of the day. Prior to analysis, the urine samples were centrifuged at 3500 rpm for 10 min, and in this way, all crystals and solids present were separated. The samples were analyzed in triplicate, for the initial determination of the free form of the analytes.

The same real samples were also analyzed considering the previous step of enzymatic hydrolysis described above [47]. To 2 mL of urine sample in the extraction vial, 2 mL of 1 mol L^{-1} sodium acetate/acetic acid buffer (pH 5.2) was added. The solution was homogenized by vortexing for 10 s, and then 20 μL (2000 U) of the enzyme β-glucuronidase was added and homogenized manually for a few seconds followed by incubation at 37 °C for 24 h. After this time, the optimized RDSE methodology was applied considering the same 10-fold dilution of the sample. The real samples were quantified by matrix-matched calibration using the urine of a child of 5 years old, which is practically free of endogenous hormones. The urine sample was spiked before the dilution involved in the method. All subjects were previously informed about the procedure and the nature of the analytical study. All subjects signed an informed consent form prior to any other action.

3. Results and discussion

3.1. Derivatization studies

Although the MSTFA/DTE/TMIS mixture has been used successfully for the derivatization of some sex hormones in solvents [30], different kinds of waters [48] and bovine serum [29], it has not been previously tested for derivatization in urine samples. This is a silylation reaction where the keto groups of Test and Prog are previously reduced to hydroxyl groups to later form the -$Si(CH3)_3$ species at those positions. TMIS acts as a catalyst and promotes reaction. The hormone E2 and, in general, any hormone that does not have keto groups simply reacts with MSTFA without any structural alterations.

First, using experimental conditions suggested in the literature for the derivatization reagents (1000:2:5), an experimental design (2^k) was made to evaluate the effects of temperature, time and volume of this mixture on the derivatization. Similar to observations by other authors [30], we found that the temperature and time have no significance on the derivatization, verifying the strong derivatizing character of this mixture. In addition, we found that the volume of the derivatizing mixture had a significant effect on the reaction (see Fig. A1, Supplementary Information).

The amount of each reagent in the derivatization mixture was not studied since these reagents were previously optimized to the minimum expression. However, to save on the cost and ecoefficiency of the methodology, dilution of this mixture in different solvents was assessed. As seen in Fig. 1, the use of the derivatizing mixture without dilution (100%) is statistically equivalent to the result obtained when the mixture was diluted (50%) with solvents such as EtAcO, ACN and DCM. EtAcO was selected for dilution of the derivatizing mixture because of

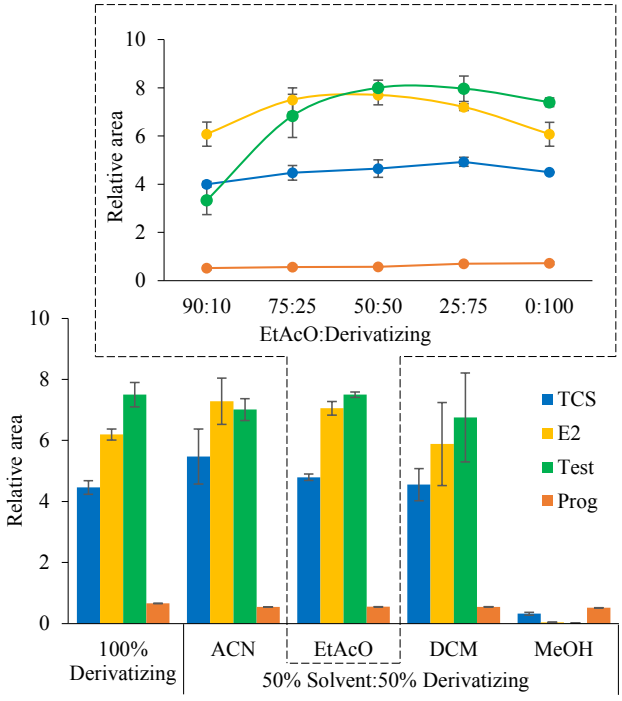

Fig. 1. Effect of the presence and volume of organic solvent on the derivatization of TCS, E2, Test and Prog.

its better compatibility with GC systems. DCM was discarded considering the higher dispersion of the results, and MeOH was also discarded because it reacts with the derivatizing mixture. On the other hand, some tests were carried out at different proportions of the derivatizing mixture and EtAcO as the solvent, starting with 10:90 through 100:0 (Fig. 1, top). Fifty microliters of the derivatizing mixture and 50 μL of EtAcO represent the best relationship between high chromatographic response and low volume of derivatizing mixture. Consequently, the final conditions selected for derivatization were 50 μL of MSTFA/DTE/TMIS and 50 μL of EtAcO at 25 °C for 5 min in a vortex. The derivatization reaction was evaluated in the concentration range of 1–500 ng mL^{-1}, obtaining a linear relationship with an R^2 value of > 0.995. The RSD (n = 6) was lower than 5% for the total analytes in the study. Our proposed method represents a significant improvement with respect to other studies reported in the literature due to the reduction of the consumption of the derivatizing mixture used by 50% [30,48] as well as the time and temperature used [29].

3.2. Gas chromatography-mass spectrometry studies

The chromatographic analysis shows separation of the analytes under study in just 15 min. Table 2 shows the retention times and *m/z* ions used in SIM mode for each analyte by GC–MS.

Fig. 2 shows the elution chromatogram of the compounds attributed to the following products in order of elution: TCS-mono-TMS, E2-di-TMS, Test-di-TMS and Prog-di-TMS. The O-TMS species in the figure highlighted in red came from a hydroxyl group, while those marked in blue were originally ketone groups. Likewise, due to possible double bonds formation after enolization of the ketone group in Prog, two products were formed by performing derivatization with MSTFA/DTE/TMIS as observed previously [30]. Since it is not possible to favor one product with respect to the other, under the same derivation protocol, the same relation of the derived compounds was achieved at concentrations between 1 and 500 ng mL^{-1}; therefore, for quantification, the sum of the areas of all compounds was considered. Surrogate standards TCS-D_3 and Test-$^{13}C_3$ elute at the same retention times as their homologs TCS and Test.

Table 2
GC–MS data.

Analyte	Retention time (min)	Target ion *m/z* value	Qualifier ion *m/z* values
TCS	7.94	345	347, 360
TCS-D_3	7.94	350	365
E2	11.66	416	285
Test	11.69	432	433
Test-$^{13}C_3$	11.69	435	436
Prog	13.52–13.64	458	443

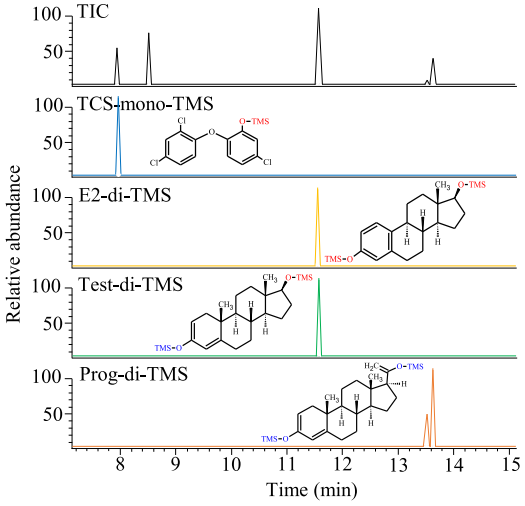

Fig. 2. GC–MS chromatograms of the derivatized analytes in TIC mode (top) and SIM mode for each analyte (*m/z* 345, 416, 432 and 458 for TCS, E2, Test and Prog, respectively).

3.3. Rotating-disk sorptive extraction: Extraction, clean-up, and elution studies

3.3.1. Sorbent phase and pH

RDSE is considered a versatile technique that has been applied to a large number of analytes in different kinds of samples, mainly due to its extraction device allowing the use of sorptive phases with different polarities [24,41,49]. Considering the range of polarity of the analytes under study (log $K_{o/w}$ between 3.32 and 4.76) and their capability to form hydrogen bonds, the lipophilic-hydrophilic Oasis® HLB sorptive phase was selected. Recently, Oasis® HLB PRiME SPE has emerged as a commercial sorptive phase for SPE, which, according to the manufacturer, has the advantages of the absence of conditioning stages as well as improvement in the extractions through a decrease in the matrix effect. Taking this into account, both phases (Oasis® HLB and HLB PRiME) were compared for the extraction of E2, Test, Prog and TCS through RDSE from urine samples, also considering the presence and absence of preconditioning stages. Despite these expectations, the Oasis® HLB phase resulted in better results for this set of analytes, and independent of the sorbent phase used, preconditioning of the phase was mandatory (see Fig. A2, Supplementary Information). Consequently, Oasis® HLB was selected for use in this RDSE application.

A pH study was carried out considering the acid-base properties of both the phase and analytes of interest. The best analytical responses were found at pH values between 6 and 8 (see Fig. A3, Supplementary Information), which is consistent with similar studies done previously for this sorbent phase [42].

3.3.2. Dilution of the sample

The analysis of complex matrices such as urine requires a detailed

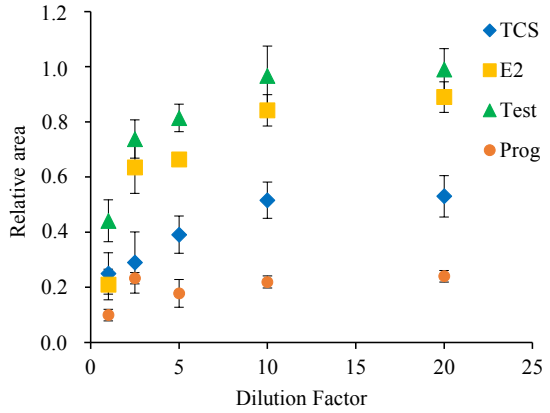

Fig. 3. Effects of the dilution of the urine sample (before RDSE) on the relative response of the analytes.

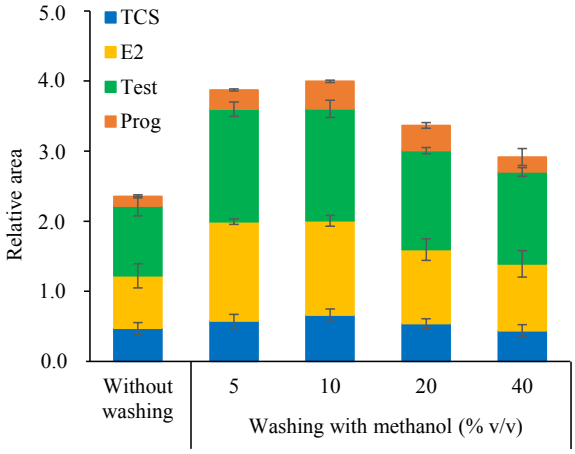

Fig. 4. Effect of the concentration of MeOH used in the clean-up stage (after RDSE) on the relative response of analytes.

study of all experimental conditions. Therefore, a complete study of all the sample preparation steps, such as dilution, extraction, clean-up and desorption, was necessary. First, several authors have suggested that dilution of the urine sample up to 20-fold could improve selectivity in different extraction techniques [46,50,51]. The effect of the dilution factor on the response of the present method is shown in Fig. 3. A positive effect was observed for the responses with increasing dilution of the sample. The effects were no longer significant between dilutions of 10 and 20-fold. Consequently, a 10-fold dilution was considered for the extraction process for a higher preconcentration factor.

3.3.3. Washing step

It should be stressed that even after diluting the urine sample, the method provided low recovery and high matrix effects (approximately 20% and 70%, respectively), mainly due to interferences present in the intense yellow color observed in the final extract. Consequently, the application of a clean-up stage was evaluated to avoid interference from the pigments present in urine, such as urobilin, which are coextracted together with the analytes by HLB on RDSE. Urobilin shows similar behavior to the analytes under study; its molecular mass does not exceed 600 g mol^{-1}, which allows it to easily cross the boundary layer in RDSE, and its high polarity (log $K_{o/w}$ 1.36) allows easy sorption onto the hydrophilic part of the sorbent phase. The use of diluted MeOH (between 20 and 40% in water) has been reported previously [52,53] for clean-up of the extracts in the determination of estrogens from urine because this polar mixture only provides desorption of the pigments. In this study, the use of between 5 and 40% MeOH was studied to desorb interferences from the sorptive phase in the disk prior to desorption of the analytes. Fig. 4 shows that the use of 5 and 10% MeOH improves the response associated with each analyte with respect to extraction without clean-up. In addition, considering that this washing step removed part of the sorbed interferences, the final extract became almost colorless. At higher concentrations of MeOH (over 20%), the signals begin to decay due to concomitant desorption of the analytes. Extraction with 10% MeOH for 5 min at 2000 rpm was selected as the clean-up stage.

3.3.4. Selective elution

After the clean-up with 10% MeOH, part of the pigments remained sorbed onto the disk together with the analytes; consequently, to obtain clean extracts, the selected desorption sorbent should remove only the analytes without desorption of the remaining pigments on the disk. Desorption of the analytes was studied using different solvents: DCM, MeOH, ACN and EtAcO. Using EtAcO as the desorption solvent, a higher response of all analytes (see Fig. A4, Supplementary Information) was obtained. In addition, it was noted that after this, an intense yellow color remained in the filter on the disk, and the final extract became colorless. EtAcO has the lowest polarity of the solvents tested (dielectric constant (ε) of 6.2), which allowed it to more selectively desorb the analytes without interacting with the polar interferences remaining on the phase, obtaining colorless extracts.

Fig. 5-A (left side) shows the comparative results for absolute recovery and matrix effects regarding the three optimized steps: sample dilution, washing with diluted methanol and elution solvent. The first section (C0) reflects the results obtained by applying the RDSE technique without considering sample clean-up or sample dilution and using MeOH as the desorption solvent, as is typical in other RDSE applications [24,41,42]. Under these conditions, it is only possible to extract a maximum of 8% of the analytes; in addition there is a highly negative matrix effect of approximately 90%. By including the different stages studied (C1, C2 and C3), it can be observed that in parallel, the recoveries increase and the matrix effects of each analyte decrease. In the last step (C3), absolute recoveries reach between 46 and 57% and negative matrix effects are between 15 and 22%. In the top of Fig. 5-A, it can be observed how the color intensity in the final extract decreases as the different clean-up stages are applied, starting with a deep orange to a light yellow, almost colorless. Dirty extracts in GC–MS complex matrices can suppress ionization of the analytes [54] or affect the injection process of the sample [55].

Steps C2 and C3 only vary in the solvent used for elution. A comparison between the use of MeOH (C2) and EtAcO (C3) was followed by obtaining the infrared (IR) spectrum of the surface of the disk (Fig. 5-B or right side). In the surface of the disk eluted with EtAcO (IR spectrum in red), double signals at approximately 1634 and 1584 cm^{-1}, particular to C = O and C = N stretching, are observed in addition to the wide signal at approximately 3273 cm^{-1} that is particular to O–H stretching. These signals coincide with those expected for urobilin. The absence of these signals on the surface of the disk eluted with MeOH (blue IR spectrum) is because urobilin was eluted together with analytes, transferring the yellow color to the final extract.

3.3.5. Hydrodynamic variables in RDSE

After optimizing all the clean-up steps, a study of the hydrodynamic variables associated with RDSE was necessary to find the optimal conditions of extraction. Through an experimental design based on a Doehlert [56], the matrix was constructed with coded and real values (see Table A1, Supplementary Information) to evaluate the effects of three factors simultaneously: volume of the diluted sample, rotation velocity of the disk, and extraction time. Fig. 6 shows the response surface diagram, which indicates the following optimal conditions: 2000 rpm, 60 min and 20 mL of diluted sample (2 mL of urine and 18 mL of water) for rotation velocity, extraction time and sample

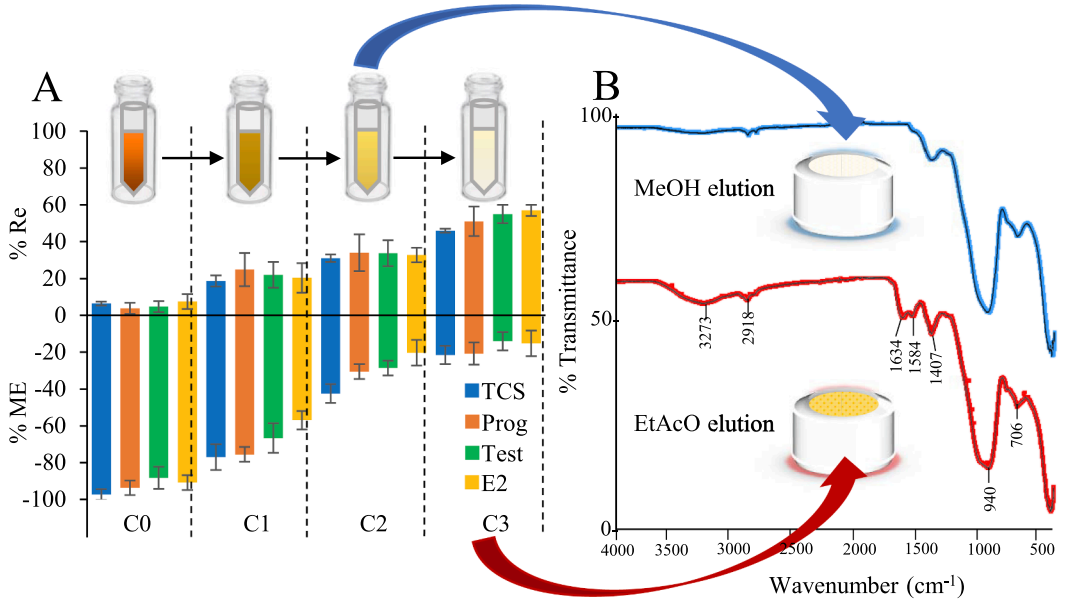

Fig. 5. A. Effect of clean-up stages on the recovery and matrix effects. [C0: Without dilution and without clean-up; C1: with urine dilution (10 ×) and without clean-up; C2: with urine dilution (10 ×) + MeOH (10%) for clean-up; and C3: with urine dilution (10 ×) + MeOH (10%) for clean-up + EtAcO elution. Note: C0, C1 and C2 were eluted with MeOH] B. Infrared spectra of the surface of the rotating disk after the desorption stage with MeOH (blue) and EtAcO (red).

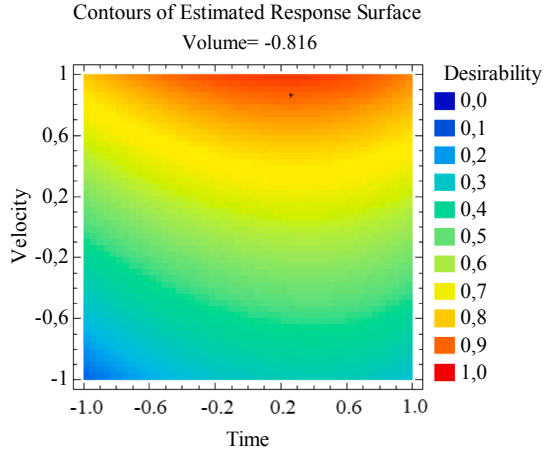

Fig. 6. Estimated response surface contours about the hydrodynamic variables (sample volume, rotation velocity and extraction time) for RDSE in urine samples.

volume, respectively. The overall desirability function for the total number of analytes was 0.8965.

3.4. Figures of merit of the method

Table 3 shows the figures of merit of the method. The accuracy is expressed as a function of the relative recovery (based on TCS-D_3 and Test-$^{13}C_3$) and the precision according to the relative standard deviation (% RSD). RDSE is based on a partition equilibrium; therefore, surrogate standards were used to show the efficiency of the method, obtaining relative recoveries between 89 and 111%. The linear equation for each analyte was built in the urine matrix, and the linearity (R^2) was > 0.959, which is considered satisfactory according to the matrix under study. Except for Prog, the limits of detection (LOD) and quantification (LOQ) showed consistent results, considering that these analytes are usually present in concentrations higher than these limit values. The negative matrix effect between −14 and −22% indicates that there is little suppression of the response of each analyte, and it could be ensured that this remnant found is more possibly due to inhibition of the ionization capacity of the analytes in the gas phase and not due to a possible blockage occurring at the injection port by the sample.

Comparison with previously reported methods by SBSE applied to sex hormones in urine samples denoted that the proposed method is more rapid with an extraction time of 60 min compared with 2–4 h to reach equilibrium in SBSE [40], which mainly occurred because the stir bar does not exceed 750 rpm. Regarding detection limits, the lowest reported limits were 0.03 [39], 0.18 and 0.12 ng mL^{-1} [57] for E2, Test and Prog, respectively, which are higher than those reported in this work (except for Prog). The recoveries previously found by SBSE were 11.1 [40], 21.2 and 49.5% [57] for E2, Test and Prog, respectively, which were considerably lower than those reported in this method. The lower recoveries reported in these studies are since only dilution of the sample was considered to decrease the complexity of the matrix.

In the case of extraction of TCS from urine by SBSE, the reported extraction time was 90 min [58]. The lowest detection limit previously reported was 0.05 ng mL^{-1}, which is slightly higher than that reported in this study. In this same work, as in our case, they found a high ratio of conjugation for TCS metabolites in urine samples, from 86.8 to 96.4% [59].

Table 3
Figures of merit of the method.

Analyte	Sensitivity (mL ng^{-1})	Linearity (R^2)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	% Re (relative)	% RSD (n = 6)	% ME
TCS	0.0152	0.993	0.04	0.14	111	9	−21
E2	0.0383	0.969	0.01	0.04	96	7	−15
Test	0.0456	0.979	0.004	0.01	99	11	−14
Prog	0.0044	0.959	0.54	1.77	89	14	−22

Table 4
Concentrations (± standard deviation) of analytes in the different urine samples studied (ng mL^{-1}).

Analyte	Man < 35	Woman < 35	Man > 50	Woman > 50	Pregnant woman
Without enzymatic hydrolysis					
TCS	< LOD	< LOD	0.23 ± 0.09	< LOD	< LOD
E2	< LOD	0.74 ± 0.18	< LOD	0.11 ± 0.01	0.43 ± 0.11
Test	0.57 ± 0.14	0.20 ± 0.07	0.45 ± 0.28	< LOD	< LOD
Prog	< LOD	< LOD	< LOD	< LOD	4.82 ± 1.48
With enzymatic hydrolysis					
TCS	< LOQ	0.44 ± 0.15	2.32 ± 0.20	0.63 ± 0.03	< LOD
E2	2.77 ± 0.64	8.89 ± 1.02	1.34 ± 0.72	2.10 ± 0.07	10.45 ± 2.07
Test	21.23 ± 3.54	2.62 ± 1.32	17.67 ± 1.83	0.44 ± 0.16	2.70 ± 0.43
Prog	< LOD	< LOD	< LOD	< LOD	11.92 ± 2.03

(Standard deviations were calculated considering a triplicate of each sample).

3.5. Real sample analysis

To assess the applications of the methodology, urine samples from healthy persons of different ages and sexes, including a woman during pregnancy, were analyzed. In addition, enzymatic hydrolysis was alternatively applied to quantify the free and total concentrations of the analytes. Without enzymatic hydrolysis, it was only possible to quantify TCS in the man > 50 years of age, E2 in the women samples, Test in the men samples and in woman < 35 years of age, and Prog in the woman who was pregnant. The use of enzymatic hydrolysis considerably increased the concentration of the analytes in the urine, which is consistent with the fact that these compounds are generally excreted in the urine in the form of their more polar metabolites. This expected result for sex hormones was also found for TCS (an O-glucuronide metabolite), which was similar to that previously found for methylparaben using the same route for enzymatic deconjugation [47]. For the man > 50 years of age, the ratio of the conjugate of TCS in human urine samples was 90%. E2 and Test were found to be approximately 5- and 40-fold more concentrated in their total form, respectively. Normally, Prog increases to levels up to 100 times in women during pregnancy; therefore, Prog could be quantified in this sample, which could not be done in the samples from the other individuals in the study due to the lower sensitivity found for this hormone in the proposed methodology (Table 4).

Saliva testing is other noninvasive alternative for the determination of hormones. Consequently, we will investigate the application of RDSE in the preparation of this kind of sample.

4. Conclusions

The proposed method allowed the determination of sex hormones and TCS in urine samples using RDSE with integrated clean-up, derivatization, and GC–MS quantitation. Regarding derivatization, the experimental conditions were significantly improved by reducing the consumption of the derivatizing mixture by 50% and avoiding the typical use of timer-controlled heating systems, thus achieving a more ecoefficient method than those already proposed according to the premises of green chemistry. The clean-up strategy integrated with RDSE included dilution of the sample (10-fold), washing of the disk with 10% methanolic solution after extraction and desorption of the analytes with ethyl acetate. This improved method considerably reduces the matrix effects exerted by the presence of pigments such as urobilin in urine

Total TCS was successfully quantified in almost all urine samples in the range of 0.44–2.32 ng mL^{-1}, which continues to be a warning signal for the Chilean population considering that this compound has suggested characteristics as an endocrine-disrupting chemical. Furthermore, the ability of this compound to be secreted in urine as a metabolite has been demonstrated; therefore, many of the already reported concentrations of contaminants in biological fluids could be higher.

E2 and Test were quantified with high sensitivity using the proposed technique at concentrations between 0.11 and 10.45 and between 0.20 and 21.23 ng mL^{-1}, respectively. However, determination of progesterone by this route is not entirely satisfactory due to the low sensitivity obtained by GC–MS. Progesterone was only quantified in a woman during pregnancy (4.82 and 11.92 ng mL^{-1} in free and total form, respectively).

It is recommended to apply this method elsewhere to study the effects of triclosan exposure on the relative concentration level of sex hormones.

CRediT authorship contribution statement

Daniel Arismendi: Conceptualization, Supervision, Formal analysis, Validation, Investigation, Writing - original draft. **Karolina Díaz:** Formal analysis, Validation, Investigation. **Natalie Aguilera-Marabolí:** Formal analysis, Validation, Investigation. **Betsabet Sepúlveda:** Conceptualization, Methodology, Resources. **Pablo Richter:** Project administration, Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank FONDECYT, Chile (Regular Project 1180742) and ANID, Chile (National PhD scholarship 21180429) for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105149.

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