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New challenges in sample preparation: Miniaturized stir bar sorptive dispersive microextraction as a high-throughput and feasible approach for low-availability sample preparation

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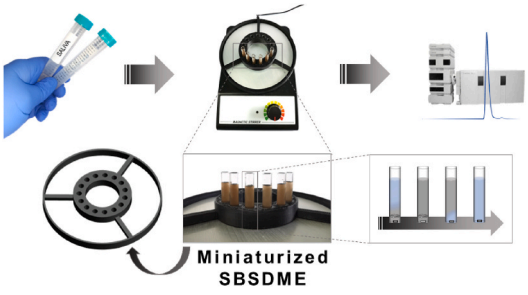
HIGHLIGHTS

- Further miniaturization of stir bar sorptive dispersive microextraction is achieved.
- An affordable extraction assembly based on a 3D-printed support is designed.
- High-throughput method for simultaneous extraction of up to 15 samples is proposed.
- A green sample preparation for bioanalysis of barely available samples is presented.
- Determination of salivary cortisone and cortisol was performed as proof-of-concept.

GRAPHICAL ABSTRACT



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ABSTRACT

The miniaturization of stir bar sorptive dispersive microextraction (mSBSDME) for the analysis of low-availability samples is presented. This new methodology is based on the principles of stir bar sorptive dispersive microextraction, but the amount of sorbent and, most importantly, the amount of sample are considerably reduced to a tiny amount and a few microliters, respectively. Thus, affordable 400-μL flat-base glass inserts and minute bar-shape neodymium magnets (3 mm length x 2 mm diameter) were used as extraction devices hold by a specifically designed multiextraction assembly, which comprises a high-rate stirring plate and a 3D-printed support to treat 15 samples simultaneously. This new approach allows a fast, affordable, portable, and high-throughput analysis of low-volume samples, expanding the potential of the technique. The same extraction device is used along the different stages, thus avoiding transfers, which reduces sample handling. Besides, the reduction in the sample, sorbent and organic solvent amounts allows a considerable decrease of the waste generation, and thus pursues a green sample preparation for bioanalysis. As a proof-of-concept of this new methodology, cortisone and cortisol were determined in human saliva using a composite material made of a reversed phase polymer (Strata™-X-RP) and $CoFe_2O_4$ magnetic nanoparticles. Liquid chromatography coupled to tandem mass spectrometry was used to measure both analytes obtaining good analytical features in terms of linearity ($R^2 > 0.997$), method limits of detection and quantification (22.6 and 75.5 ng L^{-1} for cortisone, and 19.3 and 64.3 ng L^{-1} for cortisol, respectively), repeatability (RSD ≤11%) and relative recoveries (78–134%).

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1. Introduction

Sample amount may be a challenge when the analysis of biological matrices is conducted. Large volumes of these samples are often not available either because of the nature of the sample itself (e.g., saliva, semen, follicular fluid, cerebrospinal fluid) or because of the characteristics of the person under study (e.g., newborns, immunosuppressed or anemic patients, etc.). This drawback gets worse if the target compounds are at trace level. Furthermore, biological matrices usually comprise many compounds with very diverse natures that make them really complex samples to deal with. As a consequence, these matrices require more or less time-consuming sample pretreatments, contradicting the principles of Green Analytical Chemistry [1], which stand for direct analysis. In this sense, principles of Green Sample Preparation [2], in which the need of a sample preparation stage is assumed, pursue the reduction of sample size, chemical usage and waste generation, among other principles. Besides, bioanalysis requires rapid and affordable methods that enable the analysis of a vast number of samples in clinical laboratories every day. Therefore, the development of sample treatment procedures that minimize the amount of sample needed for the analysis is interesting and of paramount importance in the context of bioanalysis.

Since their introduction in the 90s, microextraction techniques have evolved and adapted to the trends and needs of the moment [3], and their application in bioanalysis is not an exception. However, the scientific community has mainly focused on looking for safer and greener extraction phases [4] and/or on reducing their amounts [5,6] while the efforts on reducing sample amount are still scarce [7]. Furthermore, the reduction of both the sample and the acceptor phase could lead to an effective miniaturization of the extraction device, which implies a reduction in physical space. This permits the development of parallel extraction devices in which a higher number of samples can be treated simultaneously.

All these considerations led us to our objective: to contribute to the development of new feasible and high-throughput approaches that allow the routine sample treatment of low-availability biological samples and, moreover, simultaneously. This is in line with different microextraction workstations that have been previously developed, mainly based on the 96-well format: 96-blade solid-phase microextraction (SPME) [8,9], single drop microextraction (SDME) [10], hollow-fiber liquid-phase microextraction (HF-LPME) [11] and electromembrane extraction (EME) [12,13]. However, although it is true that these systems allow to analyze many samples simultaneously, the extraction phase remains static, causing the extraction kinetics to be slow and, consequently, the extraction times are long, even hours. On the other hand, as previously demonstrated [14], the extraction efficiency increases when smaller particles of an extraction phase are used due to the large surface area and shorter diffusion route of the analytes, resulting not only in improved extraction efficiency but also in faster extraction kinetics. Additionally, the dispersion of the extraction phase also enables a higher contact area between the donor phase and the extractant, which accelerates the mass transfer between both phases [15]. For this reason, dispersive techniques boast of faster kinetics compared when non-dispersive ones [16].

From this perspective, a new multiextraction assembly has been tailor-designed in this article, consisting of a 3D-printed support mounted on a conventional high-rate stirring plate to hold 15 miniaturized extraction devices, each one containing few microliters of sample (Fig. 1a). Each extraction device is based on a miniaturization of the stir bar sorptive-dispersive microextraction (SBSDME) approach, developed by our research group few years ago [17]. In this technique, a magnetic sorbent, initially attracted to the surface of a neodymium magnetic bar, is dispersed into the donor solution contained in a 40-mL extraction vial. When stirring is over, the magnetic bar rapidly retrieves the magnetic sorbent containing the extracted analytes for subsequent desorption and injection into a chromatographic system. The fundamentals of SBSDME, and an overview of the published papers using this technique have been recently compiled in a tutorial review [18]. As exposed in this review, SBSDME has been successfully applied to different matrices such as environmental samples, cosmetic products, urine or food, with very good analytical features. However, in all these applications, relatively high amounts of sample solution (i.e., $\geq$5 mL, usually ca. 25 mL) were needed, thus not being possible to analyze low sample volumes as is required in those scenarios mentioned above. In the proposed miniaturized SBSDME (mSBSDME) the amount of sorbent and, most importantly, the amount of sample are considerably reduced. So, 40-mL vials and 10 mm length x 3 mm diameter magnets, typically used in SBSDME, have been replaced by 400-μL flat-base glass inserts and 3 $\times$ 2 mm magnets, respectively (Fig. 1b). Herein, we present mSBSDME for the first time, as a novel contribution to the miniaturization of sample preparation techniques for bioanalysis with important advantages over SBSDME as a consequence of this miniaturization: 1) the volume of sample is considerably reduced to only a few microliters; 2) both the amount of sorbent and volume of elution solvent are substantially decreased and thus the generation of waste is diminished; 3) up to 15 low-volume samples can be simultaneously treated, thus increasing the sample throughput and reducing analysis cost and time consumption; and 4) the new multiextraction assembly is easily portable to any location to carry out on site sample treatment with the only requisite of a power supply, which could be solved with the use of low consumption batteries. This multiextraction assembly can be used in any laboratory in an affordable and "ready-to-use" manner since it is easy to set up with glass inserts and magnets readily available in laboratories, beyond fabricating the support by the 3D-printing technology, highly affordable to anyone nowadays.

In order to present this approach and to show its potential, the determination of cortisone and cortisol in human saliva has been selected as a proof-of-concept. These salivary biomarkers are related to Cushing's syndrome [19], stress [20], and type 2 diabetes mellitus [21], among other diseases, and therefore, the determination of both compounds in human saliva is of great interest and different methods have been published [22–30]. For this purpose, a previously described magnetic composite, made of cobalt ferrite ($CoFe_2O_4$) magnetic nanoparticles (MNPs) embedded into a commercial pyrrolidone-modified styrene-divinylbenzene copolymer (i.e., Strata™-X-RP), was used as

Fig. 1. (a) Multiextraction assembly. (b) Comparison of the extraction device between SBSDME and mSBSDME.

magnetic sorbent in mSBSDME prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. This composite material has shown good extraction performance for these analytes despite its low selectivity [30].

2. Experimental

2.1. Reagents and instrumentation

All reagents and solvents were obtained from major suppliers. Cortisone (99%) and prednisolone (≥99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Cortisol (98%) was provided by Acros Organics (New Jersey, USA). For the synthesis of $CoFe_2O_4$ MNPs, cobalt (II) chloride hexahydrate ($CoCl_2{\cdot}6H_2O$) and iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) were purchased from Acros Organics (New Jersey, USA), sodium hydroxide was purchased from Scharlau (Barcelona, Spain), and ethanol was purchased from Panreac (Barcelona, Spain). For the synthesis of the composite, Strata™-X 33 μm Polymeric Reversed Phase SPE cartridges (500 mg/6 mL) were obtained from Phenomenex (Torrance, USA). For the preparation of synthetic saliva, sodium chloride (NaCl) from Fisher Scientific (Loughborough, United Kingdom), potassium chloride (KCl), calcium chloride monohydrate ($CaCl_2{\cdot}H_2O$), potassium thiocyanate (KSCN) and urea from Panreac, and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) from Scharlau were used.

Ultrapure water was obtained from a Connect water purification system provided by Adrona (Riga, Latvia). LC-MS grade methanol from Honeywell (Seelze, Germany), LC-MS grade water from Panreac, and ammonium fluoride (98%) from Acros Organics were used to prepare the chromatographic mobile phase. Nitrogen used as nebulizer and curtain gas in the MS/MS ion source was obtained by a NiGen LCMS 40–1 nitrogen generator from Claind S. r.l. (Lenno, Italy). Extra pure nitrogen (>99.999%), used as collision gas in the MS/MS collision cell, was provided by Praxair (Madrid, Spain). An EBA 21 centrifuge from Hettich® (Tuttlingem, Germany), provided with a rotor of 15 cm radius for centrifuge tubes and a rotor of 9.7 cm radius for microcentrifuge tubes, was used.

2.2. Multiextraction assembly

Miniaturized extraction devices to carry out the mSBSDME consist of 400-μL flat-base glass inserts (31 mm heigh x 4 mm i. d.) from Labbox (Barcelona, Spain) and cylindrical neodymium magnets (3 mm length x 2 mm diameter, 45 MGO) from Supermagnete (Gottmadingen, Germany). For the design of the extraction assembly to hold the extraction devices, a magnetic stirrer MX-3K (18 W, 0–3000 rpm) from Anzeser (Frankfurt am Main, Germany) with a 3D-printed support for 15 flat-based glass inserts was used. The 3D-printed support was designed with FreeCAD software (version 0.19 from Jürgen Riegel, Werner Mayer and Yorik van Havre) and printed by Impresión 3D LowCost (Girona, Spain) with black poly(lactic acid) (PLA) and 55% infill density.

2.3. mSBSDME procedure

First, to prepare the extraction devices, 0.5 mg of the $CoFe_2O_4$-Strata™-X-RP composite (prepared as described in Supplementary Material) were weighted into a flat-base glass insert and a neodymium magnet was introduced. Subsequently, 5 μL of methanol were added to assist dispersion of the material and facilitate the introduction of the aqueous standard and sample solutions in the narrow flat-base glass inserts by reducing the surface tension of water. To carry out the extraction stage, working standard solutions of cortisone (1–20 ng mL^{-1}) and cortisol (0.1–5 ng mL^{-1}) were directly prepared into the extraction device by adding the appropriate volume of 35-ng mL^{-1} aqueous standard solutions of cortisol and cortisone, 50 μL of 35-mg mL^{-1} aqueous standard solution of prednisolone and then diluted up to 350 μL with synthetic saliva (see Supplementary Material). Regarding sample preparation, the same procedure of standards was followed, but introducing an appropriate aliquot of pretreated saliva (as described in Supplementary Material) instead of cortisone and cortisol standard solutions. In any case, a minimum dilution of 1:1 v/v with synthetic saliva was necessary to reduce viscosity of saliva samples and ensure the dispersion of the extraction material (as discussed later in Section 3.5.).

They were stirred for 1 min to achieve total dispersion of the composite within the solution. Afterwards, the stirring was stopped, and the extraction devices were left to rest so that the magnetic composite was retrieved by the magnetic field and recoated the stir bar again. Next, the solution was taken carefully with the aid of a gel loading pipette tip from Fisher Scientific (Loughborough, United Kingdom) and discarded. Then, 350 μL of ultrapure water were added to the device to wash the sorbent, which were also discarded. In order to carry out the liquid desorption of the analytes, 20 μL of methanol were introduced into the extraction device. After 30 s of vigorous stirring, the sorbent was left to sediment on the magnet, and the methanolic extract containing the analytes was transferred to a conical glass insert inside of an injection vial. Then, 15 μL of ultrapure water were subsequently added to ensure good chromatographic performance. Finally, prior to LC-MS/MS analysis, the vial was centrifuged in a test tube at 6000 rpm for 5 min to ensure any particle that could be present was settled and prevent it to enter the chromatographic system. Fig. 2 shows a schematic diagram of the workflow of the proposed method.

2.4. LC-MS/MS analysis

An Agilent 1100 Series chromatographic system comprised of a degasser, a binary pump, an autosampler and a thermostatic column oven, coupled to an Agilent 6410B Triple Quad MS/MS was employed. Separations were carried out in a Zorbax SB-C18 (50 mm length, 2.1 mm i. d., 1.8 μm) column from Agilent Technologies (Palo Alto, USA). Ten microliters of each solution were injected into the chromatographic system. Mobile phase consisted of solvent A (H_2O, 0.5 mM ammonium fluoride) and solvent B (methanol), by isocratic elution at a mixing ratio of 50:50% (v/v). The flow rate was 0.25 mL min^{-1} and the column temperature was kept constant at 35 °C. The run time was 5 min.

The triple quadrupole MS detector operated in positive electrospray ionization mode (ESI^+, capillary voltage at 5 kV), by multiple reaction monitoring (MRM). The rest of conditions were gas temperature at 350 °C, nebulizer gas flow rate at 11 L min^{-1} and nebulizer gas pressure at 50 psi.

The *m/z* precursor → product ion transitions (collision energy; fragmentor; dwell time) for quantification and for identification were, respectively, 361 → 163 (21 V; 140 V; 120 ms) and 361 → 105 (21 V; 140 V; 50 ms) for cortisone; 363 → 121 (26 V; 155 V; 120 ms) and 363 → 105 (26 V; 155 V; 50 ms) for cortisol; and 343 → 325 (0 V; 135 V; 120 ms) and 361 → 163 (0 V; 135 V; 50 ms) for prednisolone. Fig. S1 in Supplementary Material shows a chromatogram of a standard solution containing cortisone and cortisol at 10 ng mL^{-1} and 2 ng mL^{-1}, respectively, and 5 ng mL^{-1} of prednisolone (as surrogate) obtained after applying the mSBSDME-LC-MS/MS approach.

3. Results and discussion

3.1. Concept and design of the multiextraction assembly

As previously mentioned, the objective of this work was to present a new feasible methodology to analyze low-availability samples, either for having a low amount/volume or for being hard to obtain. For this purpose, the SBSDME approach, developed by our group in 2014 [17], was considered as a starting point due to its great proven benefits [18], but it was necessary to make some important modifications in the methodology.

In this sense, we chose to carry out the extraction directly in low-

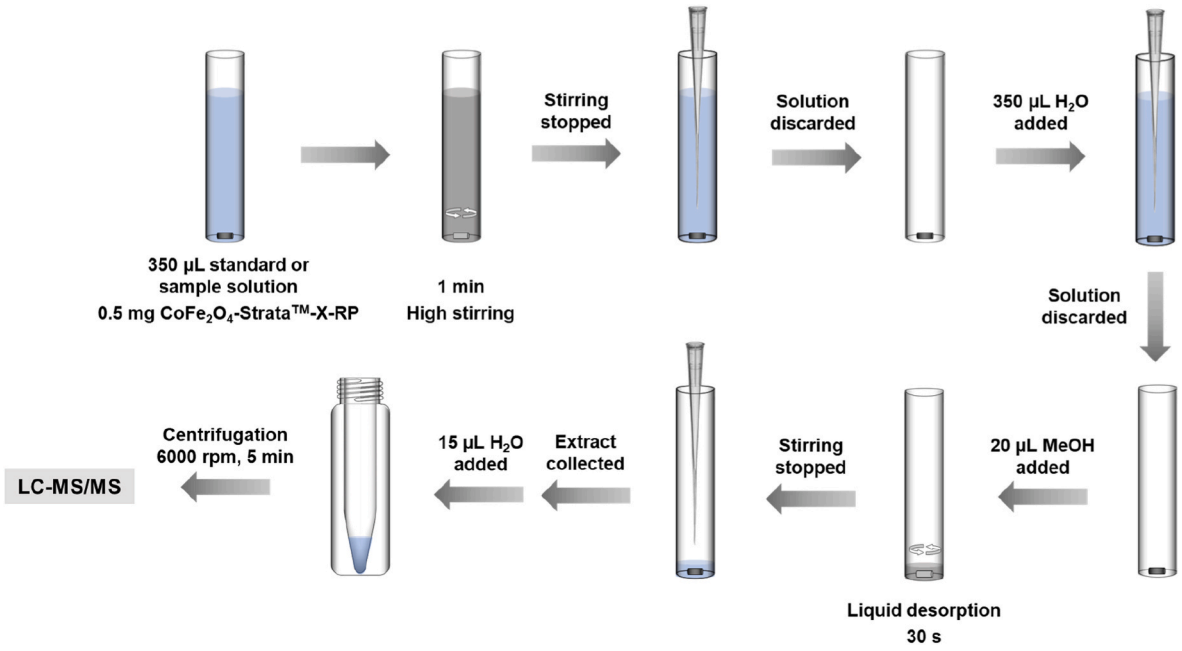

Fig. 2. Schematic diagram of the proposed mSBSDME-LC-MS/MS method.

volume inserts (up to ca. 400 µL capacity) to considerably reduce the sample volume, unlike the conventional SBSDME in which the extraction is typically carried out in 40-mL vials (Fig. 1b). As a direct consequence of their reduced diameter, the size of the stir bars was also reduced from 6x3-mm magnets used in previous works [31–33], to 3x2-mm magnets to fit the inserts. It is important to note that the inserts had to have a flat base, and not the classical conical ones, so that the stirring of the bar-shape magnets could be done correctly.

Once the new requirements of both the extraction vessel and stir bar were stablished, the new multiextraction assembly was designed. The main objective was to develop a methodology that could be applied in the routine analysis in a clinical laboratory, where a large number of samples need to be processed per day. Simultaneous analysis of several samples reduces energy and time consumption and, therefore, the analysis cost. Different configurations were explored in order to maximize the sample throughput, i.e., to allow the extraction of the maximum number of samples at the same time.

Most stirring plates work based on a magnet rod attached to an engine to make it rotate. This magnet rod interacts with the bar-shape magnet, generating its rotation for stirring. In this case, the magnet rod of the stirring plate was able to interact with more than one of the small bar-shape magnets, therefore allowing to stir multiple samples at a time. To ensure equal stirring of all samples, they should be in a circular arrangement on the stirring plate, so that they were at the same distance from the center, hence, the magnetic field is equivalent at each position. As the perimeter of the circumference depends on its radius ($C = 2\pi r$), a higher radius permits to place more extraction devices in a circular disposition. In this sense, different radius from the center of the stirring plate were tested at the maximum stirring rate (3000 rpm) by extracting 400 µL of a standard solution prepared in synthetic saliva containing 10 ng mL^{-1} of cortisone and cortisol. At low radius (i.e., 1 cm), samples were vigorously stirred because the magnetic field from the magnet rod was strong, but only 6 extraction devices could be stirred simultaneously. At high radius (i.e., ≥4 cm), up to 20 extraction devices could be stirred, but the magnetic field was not strong enough to completely disperse the material in the solution, thus causing a reduction in the extraction efficiency as shown in Fig. 3. In this regard, there should be a trade-off between the maximum dispersion of material (and therefore maximum extraction efficiency) and the simultaneous extraction of a high number of samples. Thus, an intermediate radius (i.e., 2.5 cm) was

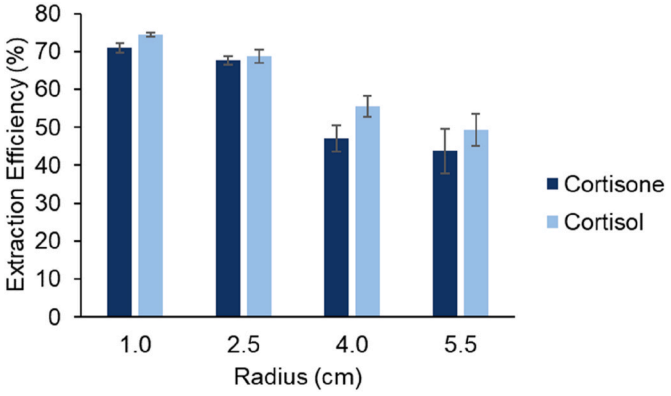

Fig. 3. Influence of radius of the multiextraction assembly.

selected so that a maximum volume of 350 µL was correctly stirred (as illustratively shown in the Supplementary Video). This arrangement allows the stirring of 15 extraction devices simultaneously.

Finally, in order to hold the extraction devices during the whole extraction procedure, a specific support with an inner ring with capacity for 15 extraction devices was tailor-designed to perfectly attach it to the stirring plate, by using 3D-printing technology, as shown in Fig. 4.

In order to confirm the equal performance of all positions, a same aqueous standard solution containing 10 ng mL^{-1} of cortisone, cortisol and prednisolone was extracted in the different positions. From a practical point of view, 0.5 mg of magnetic sorbent were weighted in each one of 15 extraction devices and 350 µL of the standard solution were extracted during 1 min in the 15 positions simultaneously. After removing the aqueous solutions and washing with 350 µL of water, the analytes were desorbed for 30 s by adding 20 µL of methanol. Subsequently, they were injected into the LC-MS/MS system. The relative standard deviation (RSD) of the 15 measures was below 7%, therefore proving the extraction is equivalent at any position of the assembly.

3.2. *Optimization of the extraction variables in mSBSDME*

A response surface methodology (RSM) [34] based on a three-factor

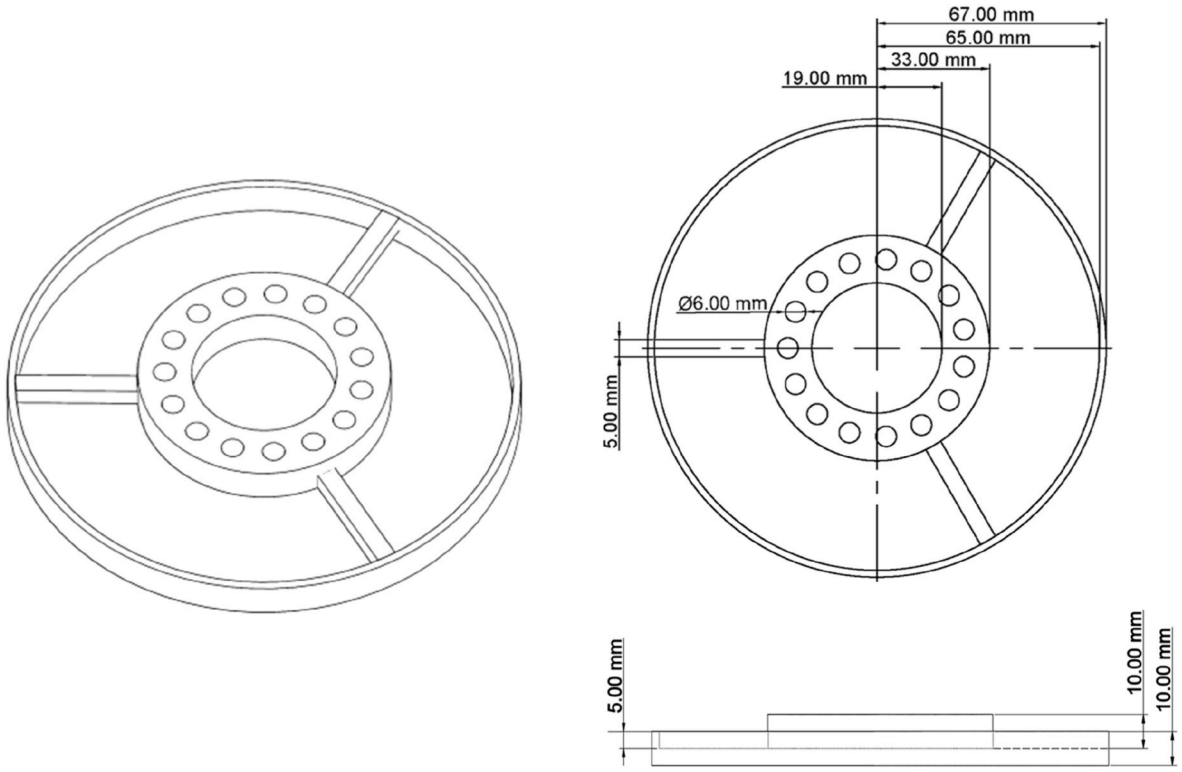

Fig. 4. Tridimensional view and dimension sketches (top and lateral views) of the 3D-printed support.

three-level Box-Behnken design was performed to establish the optimal values of the variables involved in the extraction procedure. The studied variables and ranges were: (i) sorbent amount (0.5–2.5 mg), (ii) extraction time (1–10 min), and (iii) ionic strength (1–10% NaCl (w/v)). As analytes do not present ionizable groups, pH was not studied.

Since human saliva is mainly composed of water (ca. 99%), 350 μL of aqueous standard solution containing 10 ng mL^{-1} of cortisone and cortisol were used to perform the experiments. After the extraction and washing, the analytes were desorbed in 20 μL of methanol for 30 s. The selected analytical responses were the peak area of each analyte. The description of the statistics of the Box-Behnken design are included in Supplementary Material. The 15 experiments required, including 3 replicates of the central point, were performed by duplicate, thus a total of 30 experiments were carried out. StatGraphics Centurion XVI (Stat Point Inc. Herndon, VA, USA) software was employed for the statistical analysis.

Fig. 5 shows response surfaces of desirability function, estimated as described in Supplementary Material. As can be seen in Fig. 5a, higher sorbent amounts had a negative effect on extraction efficiency. It can be caused by the worse dispersion of high amounts of magnetic composite, producing less contact area between the sorbent and the solution, and therefore, the minimum amount of sorbent (i.e., 0.5 mg) was selected. Smaller quantities were not tested due to the inability to accurately weigh them. Regarding the extraction time and ionic strength, as can be seen in Fig. 5b, none of these variables significantly affected the extraction, and therefore, the ionic strength no needs to be adjusted and the minimum extraction time (i.e., 1 min) was selected to reduce time consumption. Likewise, shorter times were not tested to allow enough

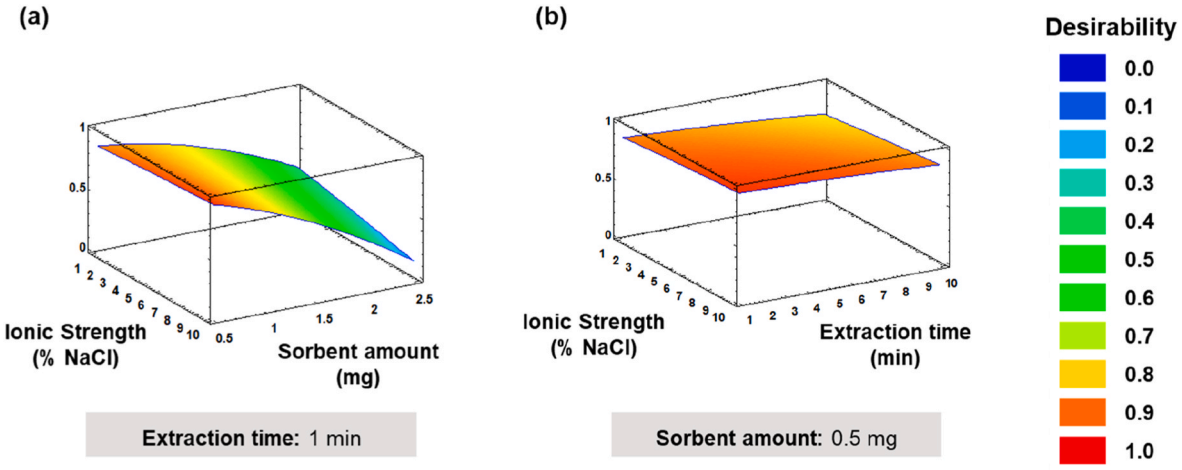

Fig. 5. Response surface of the desirability function representing the relation between the studied variables in the extraction stage: (a) sorbent amount vs ionic strength, and (b) extraction time vs ionic strength.

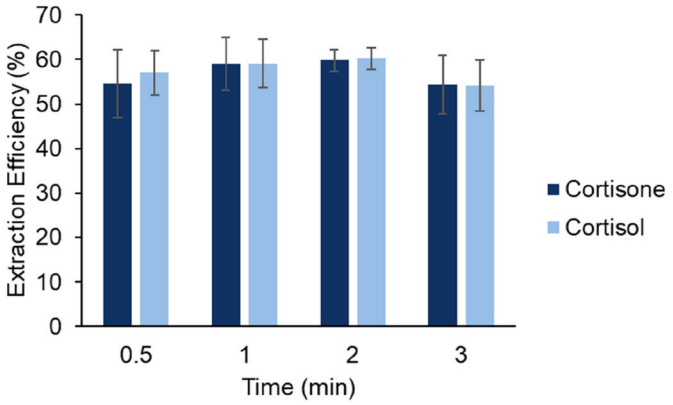

Fig. 6. Optimization of the desorption time. Extraction conditions: sample volume, 350 μL; sorbent amount, 0.5 mg; extraction time, 1 min; ionic strength, not adjusted. Desorption conditions: 20 μL of methanol.

time for all the material to completely disperse in the solution.

3.3. Optimization of the desorption variables in mSBSDME

After the optimization of the extraction process, the variables involved in the desorption step were also studied using a univariate approach. Methanol was selected as desorption solvent because in previous studies no significant differences in signals were found among the tested solvents (methanol, acetonitrile), but methanol provided the best chromatographic profiles. The volume was fixed at 20 μL to ensure the solvent covers the bar-shape magnet with the minimum possible amount to maximize the preconcentration of the analytes. The composite coating the surface of the magnet was stirred with 20 μL of methanol for 0.5, 1, 2 and 3 min. Fig. 6 shows negligible differences in extraction efficiency at different desorption times, so the minimum time (i.e., 30 s) was selected.

3.4. Analytical performance of the proposed method

The quality parameters of the proposed method, such as linearity, enrichment factor, limits of detection and quantification, and intra- and inter-day precision were evaluated under the optimized conditions.

The linearity was studied by applying the proposed method to standard solutions at different concentrations prepared in synthetic saliva, all of them containing 5 ng mL^{-1} of prednisolone as surrogate. Calibration plots were built using the ratio of the peak area of each target analyte to the surrogate (A_i/A_s) versus the analyte concentration. Regarding the levels expected in real samples, working ranges were fixed from 1 to 20 ng mL^{-1} for cortisone and from 0.1 to 5 ng mL^{-1} for cortisol although the linearity reached at least 20 ng mL^{-1} for both analytes. The determination coefficients reveal good linearity ($R^2 > 0.997$).

The EF values were estimated as the ratio of the signal obtained after the extraction of a standard solution containing 10 ng mL^{-1} of cortisone and 2 ng mL^{-1} of cortisol prepared in synthetic saliva and the signal of a standard solution of the same concentration prepared in MeOH without extraction. They were 5.9 ± 0.3 for cortisone and 6.2 ± 0.3 for cortisol.

The limits of detection (LOD) and quantification (LOQ) were calculated as 3 times and 10 times, respectively, the signal-to-noise ratio of a standard solution prepared in synthetic saliva and subjected to the proposed method. The LOD and LOQ values were 11.3 and 37.7 ng L^{-1} for cortisone, and 9.6 and 32.1 ng L^{-1} for cortisol, respectively. The method limit of detection (MLOD) and quantification (MLOQ) were obtained from the previous limits considering the sample pretreatment (i.e., the sample amount and minimum dilution), and were 22.6 and 75.5 ng L^{-1} for cortisone, and 19.3 and 64.3 ng L^{-1} for cortisol, respectively, allowing the determination of both analytes in human saliva.

The precision of the method was evaluated applying the proposed method to different standard solutions prepared in synthetic saliva containing the target analytes at three levels of concentration (i.e., 1, 10 and 20 ng mL^{-1} for cortisone and 0.1, 2 and 5 ng mL^{-1} for cortisol), all of them containing 5 ng mL^{-1} of prednisolone (as surrogate). The results, expressed as RSD (%) of the concentration obtained for five replicates during the same working session (intra-day precision) or in different working sessions (inter-day precision), are presented in Table 1 and revealed that good precision was achieved for the target analytes.

3.5. Analysis of real human saliva samples

Four saliva samples from volunteers (two male and two female) were collected as described in Supplementary Material. In a first attempt, it was tried to extract 350 μL of a saliva sample directly, without any dilution. However, due to the slight viscosity of pretreated saliva, it was found that not all the material was dispersed homogeneously between different saliva samples from different volunteers, which could hinder the reproducibility and accuracy of the extraction. Therefore, the effect of dilution was first tested. For this purpose, the saliva samples were spiked at two concentration levels (i.e., 1, and 10 ng mL^{-1} for cortisone, and 0.1 and 2 ng mL^{-1} for cortisol) and diluted to two ratios (i.e., 1:1 and 1:3 v/v) with synthetic saliva to evaluate the matrix effects by means of the relative recovery (% RR) values (eq (1)), calculated as follows:

$$\%\ RR = \frac{\text{Concentration}_{\text{spiked sample}} - \text{Concentration}_{\text{sample}}}{\text{Spiked amount}} \cdot 100 \quad (1)$$

These results are presented in Table 2. Both dilution levels showed similar recovery values between 78 and 134%, thus proving a 1:1 dilution was enough to avoid matrix effects and then external calibration was suitable for quantification of cortisone and cortisol in saliva samples.

Then, these saliva samples were treated by the proposed mSBSDME approach and measured by LC-MS/MS. The nominal concentration of cortisone and cortisol are presented in Table 3, showing the suitability of the method to measure salivary levels of these two biomarkers. As expected, cortisone levels were higher than cortisol since the latter is rapidly converted to cortisone as the salivary glands exhibit high levels of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) [35].

Table 1
Precision of the proposed method.

Analyte	Concentration (ng mL^{-1})	Intra-day precision (% RSD)[a]	Inter-day precision (% RSD)[a]
Cortisone	1	7.5	6.1
	10	3.4	11.0
	20	1.7	7.4
Cortisol	0.1	3.1	6.8
	2	1.4	7.6
	5	3.1	7.1

[a] RSD: relative standard deviation (n = 5).

Table 2
Relative recoveries obtained from real samples at different saliva dilutions.

Sample[a]	Dilution ratio	Spiked amount (ng mL^{-1})		Relative recovery (%)[b]	
		Cortisone	Cortisol	Cortisone	Cortisol
A	1:1	1	0.1	95 ± 4	95 ± 7
		10	2	85 ± 1	91 ± 7
	1:3	1	0.1	96 ± 3	89 ± 8
		10	2	91 ± 3	93 ± 2
B	1:1	1	0.1	95 ± 3	91 ± 8
		10	2	88 ± 1	92 ± 3
	1:3	1	0.1	94 ± 4	92 ± 6
		10	2	94 ± 6	96 ± 3
C	1:1	1	0.1	129 ± 5	78 ± 2
		10	2	134 ± 4	92 ± 2
	1:3	1	0.1	117 ± 9	98 ± 8
		10	2	124 ± 1	103 ± 3
D	1:1	1	0.1	125 ± 5	81 ± 6
		10	2	110 ± 4	87 ± 1
	1:3	1	0.1	93 ± 5	84 ± 2
		10	2	100 ± 2	89 ± 3

[a] Sample A and B: female; Sample C and D: male.
[b] Mean of three replicates ± standard deviation.

Table 3
Concentration of real saliva samples.

Sample[a]	Found amount (ng mL^{-1})[b]	
	Cortisone	Cortisol
A	1.31 ± 0.06	0.092 ± 0.003
B	3.27 ± 0.16	0.248 ± 0.010
C	3.82 ± 0.16	0.28 ± 0.04
D	5.90 ± 0.08	0.61 ± 0.02

[a] Sample A and B: female; Sample C and D: male.
[b] Mean of three replicates ± standard deviation.

3.6. Comparison of mSBSDME with previously reported approaches

Being the miniaturization of a microextraction technique, the volume of sample, sorbent material and waste are considerably reduced in mSBSDME. Furthermore, the possibility to extract up to 15 samples simultaneously with the designed extraction assembly is also one of its main virtues, since after the extraction time (i.e., 1 min), a sample throughput lower than 7 s per sample is achieved. Even though it is true that 96-well approaches previously described [8–13] can extract many more samples simultaneously, extraction times are considerably longer due to their static nature, thus resulting in higher times per sample. Future research will be focused on increasing the number of devices using the 96-well format and the possibility of partial automation. The portability of the assembly, only requiring an external power supply, allows its use in any laboratory in an affordable and "ready-to-use" manner. To do this, it would only be necessary to previously weigh the material inside the inserts of the extraction device.

Compared to conventional SBSDME, the mSBSDME provides a series of advantages. On the one hand, the handling is easier since all the stages (i.e., extraction, clean up, and desorption) are carried out in the same vessel (i.e., the flat-base insert). Moreover, the amount of sorbent material obtained in the same synthesis allows a greater number of extractions to be performed, since less than 1 mg is used per extraction. In contrast to its advantages, the tiny amount of material used in the approach (i.e., 0.5 mg) is difficult, if not impossible, to recover from the magnet after the process and, therefore, its reusage is not feasible. In any case, it is important to note that mSBSDME is proposed to analyze samples with a low availability, but not for those in which the availability is greater (e.g., environmental samples, urine, or extracts of other types of samples such as cosmetics). In fact, by using a larger volume of sample, higher preconcentration factors may be obtained, especially relevant when determining traces at the ng L^{-1} level. Therefore, conventional SBSDME is recommended for larger volumes of sample.

Additionally, the proposed approach has been compared with previous reported extraction-based approaches for the determination of cortisone and cortisol in saliva samples (Table 4). The proposed method presents comparable or better analytical features in terms of EF and MLODs than other reported methods. Besides, a considerably reduced volume of sample is used and, as well as the rest of dispersive-based techniques, the extraction time is low. The greenness of these methods was evaluated by estimating their AGREEprep score [36], a new metric tool for the assessment of analytical sample preparation. AGREEprep establishes a scale from 0 to 1 for each of the ten principles of Green Sample Preparation [2], considering environmental and health impact factors, such as the use of reagents, the energy consumption, sample size and generated wastes. As can be seen in Table 4, classical liquid-liquid extraction (LLE) provided the lowest score largely due to the use of dichloromethane as extraction solvent. Solid-phase extraction (SPE) and dispersive liquid-liquid microextraction (DLLME) provided similar score. Although the latter is a miniaturized technique, the fact of using a larger sample volume (up to 1.5 mL) harmed the result. The two DSPE-based methods (i.e., magnetic-based solvent-assisted-DSPE (M-SA-DSPE), and the proposed mSBSDME) presented higher scores, mainly due to the low consumption of solvents as mentioned above. The methods based on on-line SPE present good AGREEprep scores because of the high degree of automatization and the low consumption of solvents and waste generation. Nonetheless, one of the reported methods includes other previous steps (i.e., LLE and derivatization) that, despite achieving very low MLODs, significantly reduced the greenness of the method.

Table 4
Comparison of mSBSDME with other extraction-based approaches for the determination of cortisone and cortisol in saliva.

Extraction technique[a]	Analytical technique[b]	Sample volume (μL)	Extraction time (min)	EF[d]	MLOD[e] (ng L^{-1})	Handling	Throughput	Waste	AGREEprep Score	Reference
On-line SPE	LC-MS/MS	100	n.r.[c]	n.r.	141.4–281.2	+++	+++	+++	0.79	[22]
SPE	LC-MS/MS	300	n.r.	n.r.	72.5–108.1	+++	++	++	0.49	[23]
LLE	LC-MS/MS	50	5	n.r.	39.1	++	+	+	0.40	[24]
LLE+derivatization+on-line SPE	LC-MS/MS	250	4	n.r.	2–5	++	+	+	0.54	[25]
DLLME	LC-UV/Vis	1000–1500	2	5.0–6.3	110–160	++	+	++	0.48	[29]
M-SA-DSPE	LC-MS/MS	1000	1	5.2–5.6	18–29	++	+++	++	0.56	[30]
mSBSDME	LC-MS/MS	175	1	5.9–6.2	19.3–22.6	++	+++	+++	0.60	This work

[a] DLLME: Dispersive liquid-liquid microextraction; LLE: Liquid-liquid extraction; M-SA-DSPE: Magnetic-based solvent-assisted dispersive solid-phase extraction; mSBSDME: miniaturized stir bar sorptive dispersive microextraction; SPE: Solid-phase extraction.
[b] LC: Liquid chromatography; MS/MS: tandem mass spectrometry; UV/Vis: ultraviolet–visible spectroscopy.
[c] n. r.: not reported.
[d] Enrichment factor.
[e] Method limit of detection.

4. Conclusions

In this work, a miniaturization of stir bar sorptive dispersive microextraction (mSBSDME) is presented for the first time for the feasible high-throughput analysis of low-availability samples in bioanalysis. The dispersion of the extraction material allows a high extraction kinetics that, together with the possibility of simultaneously analyzing of up to 15 samples, offers promising enhancements with respect to previously presented multi-extraction workstations.

This new methodology allows a rapid determination of target analytes employing few microliters of sample, organic solvents and sorbent, permitting the analysis of barely available samples, broadening the applications of the technique and getting closer to the current trends in Analytical Chemistry and the principles of Green Sample Preparation.

As proof-of-concept of this new methodology, the determination of salivary cortisone and cortisol was selected. Good analytical features were obtained for both analytes. The successful application to saliva samples from different volunteers proves its potential as new sample preparation technique for the analysis of biomarkers in saliva.

Despite the achievement of the simultaneous extraction of several samples, it is still below the previous (semi)automated approaches based on 96-well plates. Future research will be focused on expanding the possibilities of this new miniaturized methodology to treat a larger number of samples by adapting it to the 96-well format and by attaining a higher degree of (semi)automation.

CRediT authorship contribution statement

Cristian Azorín: Methodology, Validation, Investigation, Data curation, Writing – original draft. **Juan L. Benedé:** Methodology, Writing – review & editing, Supervision. **Alberto Chisvert:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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