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A high-throughput magnetic-based pipette tip microextraction as an alternative to conventional pipette tip strategies: Determination of testosterone in human saliva as a proof-of-concept

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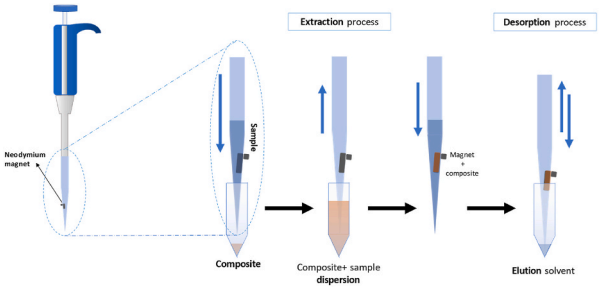
HIGHLIGHTS

- A high-throughput dispersive pipette tip microextraction approach is presented.
- The use of a magnetic sorbent reduces significantly manipulation and analysis time.
- Good results are obtained when compared with other pipette tip-based strategies.
- The sample throughput is further increased with a multichannel pipette.
- Salivary testosterone is selected as proof-of-concept to test the proposed approach.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, a lab-made and affordable high-throughput pipette tip microextraction strategy is presented. In this approach, a magnetic sorbent is quickly dispersed in the sample by means of a disperser solvent and the resulting dispersion is immediately aspirated into a pipette tip containing a small neodymium magnet inside, which entraps the magnetic sorbent containing the analytes. The sample is then discarded, and the subsequent cleaning and desorption steps are conducted by aspirating/dispensing the suitable solvents. This methodology provides satisfactory results when compared with previous pipette tip extraction strategies (i.e., pipette-tip solid-phase extraction and dispersive pipette extraction). Furthermore, it requires a minimum number of aspirating/dispensing cycles, thus benefiting the ergonomics for the operator, and it is not commercially dependent. This new approach has been tested by measuring testosterone levels in saliva of volunteers as a proof-of-concept. In this regard, a magnetic composite made of $CoFe_2O_4$ magnetic nanoparticles embedded in octadecyl bonded silica microparticles was employed as sorbent material. Testosterone was quantified by liquid chromatography coupled to tandem mass spectrometry. Under the optimized conditions, low limits of detection and quantification (7.5 ng L^{-1} and 24.8 ng L^{-1}, respectively), good repeatability (RSD <9%) and relative recoveries between 82 and 106% were obtained with just one adsorption and two desorption cycles, which requires few seconds per sample. In order to increase even more the sample throughput, a multichannel pipette was also evaluated.

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1. Introduction

Sample preparation is usually considered the most limiting step of an analytical process, since it usually requires long times and high sample manipulation. This becomes especially critical when trace analysis and complicated matrices converge, given that high efforts are needed to isolate the target compounds from the matrix and to concentrate them [1]. Classical extraction techniques like liquid-liquid extraction (LLE) and solid-phase extraction (SPE), despite being extensively used in the past, are being replaced by microextraction techniques, respectively [2–4].

These miniaturized sample preparation techniques are aligned with some of the principles of the so-called Green Sample Preparation (GSP) [5], since they require less amounts of solvent and sorbent, thus generating less waste. Furthermore, those approaches based on sample flow through the acceptor phase (e.g., pipette-tip solid-phase extraction (PT-SPE) or microextraction by packed sorbents (MEPS)), and those based on dispersing the acceptor phase into the sample (e.g., dispersive liquid-liquid microextraction (DLLME) or dispersive solid-phase extraction (DSPE)), allow to accelerate the extraction kinetics [6,7] and therefore to reduce the extraction times. Nevertheless, some of the static approaches such as, for example, solid-phase microextraction (SPME) or thin-film microextraction (TFME), enable a high level of automation that counteracts the low sample throughput obtained. In this sense, the complete retention-elution-measurement procedure can be performed by means of robotized autosamplers [8]. The implementation of multi-well (usually 96-well) format workstations notably increases the sample treatment throughput [9]. The 96-well format has been also implemented for miniaturized SPE [10] and PT-SPE [11] by using pipetting workstations.

Leaving aside robotic workstations, which are not affordable for most of the laboratories, it should be emphasized the (semi)automation degree achieved when PT-SPE is used, since the aspirating/dispensing operations are conducted easily by using a manual pipette [12]. Moreover, PT-SPE allows on-site sampling and the tips are disposable, thus reducing carryover and cross-contamination [13]. However, tens of aspirating/dispensing cycles are needed in order to achieve a high extraction/elution efficiency, which may cause upper limb disorders. Furthermore, problems associated with clogging usually appear [14]. In this sense, monoliths presenting high permeability [15], paper-based sorbents rolled inside the tip [16] or, more recently, a sorbent packing-free approach based on functionalized-inner wall pipette tips [17], have been proposed to alleviate clogging problems, however they still require long extraction times.

The so-called disposable pipette extraction (DPX) [18], later rebranded as dispersive pipette extraction [19], can be considered as a hybrid technique between DSPE and PT-SPE. Unlike PT-SPE, the sorbent is not packed between two frits but it is placed loosely, in such a way when the sample is aspirated the sorbent is dispersed like in DSPE. All this increases the extraction kinetics thus increasing the sample throughput compared to PT-SPE at the same time it avoids the problems associated with clogging. However, it has been observed that the aspiration rate is not high enough to achieve an efficient dispersion, and different pipette-tip designs have been proposed to improve it. Based on this, commercially-available tips can be found incorporating a baffle system inside that is shaped to disrupt the flow of aspirated sample and thus force the dispersion [19]. It should be emphasized that the lab-made manufacture of these tips is not easy, and in any case, the design is under patent [20], which makes the commercial dependence indisputable. Besides this commercial dependence and high cost, the amount of extracting phases commercially available is limited [18].

Changing the subject to other issues, it should be emphasized that the use of magnetic (nano)materials in microextraction techniques, especially in DSPE, has been extensively exploited due to their easy retrieval by using an external magnet [21]. If the magnet is placed inside the sample and acts as stirring engine and sorbent retrieval, as occurs in stir bar sorptive dispersive microextraction (SBSDME) [22], both the retrieval and sample handling are further facilitated.

All this inspired us to propose a high-throughput, operator-friendly and cost-effective approach, based on the (semi)automated character of pipettes and the advantages of magnetic (nano)materials. To this regard, a conventional pipette tip with a small neodymium magnet inside is proposed as an alternative to PT-SPE and DPX tips. First, a magnetic sorbent is quickly dispersed in the sample in a 1.5 mL microcentrifuge tube by means of a disperser solvent and, immediately, the resulting dispersion is aspirated into the tip, in such a way the magnetic sorbent entrapping the analytes is retained by the magnet. Then, the sample is discarded, and finally, a small amount of desorption solvent is aspirated/dispensed to desorb the analytes, thus providing an extract ready to be measured.

A previous work combining a magnetic sorbent with a pipette tip was published as clean-up method [23]. In this work, an annular magnet was surrounding the pipette tip, in such a way it was manually moved up and down continuously for a defined period of time (2 min) to ensure the contact between the sorbent and the solution. However, in this approach the sorbent is not efficiently dispersed and the manual intervention by the operator is unavoidable, thus decreasing the degree of (semi)automatitation provided by the employment of pipette-tip strategies, such as PT-SPE, DPX and the presented approach.

To test the proposed approach, a magnetic composite made of $CoFe_2O_4$ magnetic nanoparticles (MNPs) embedded on octadecyl silica (C18) microparticles (i.e., $CoFe_2O_4$–C18) has been selected for the determination of testosterone in saliva as a proof-of-concept. Testosterone levels can be used as a biomarker of different diseases [24–26]. In addition, testosterone is widely employed by professional athletes in doping [27]. Saliva samples have the advantage of not being as stressful and invasive as serum or urine [28], and it has been demonstrated that salivary testosterone, despite being quite lower than in serum, presents good correlation [28]. To overcome the issue of low concentration levels, a sensitive technique like liquid chromatography-tandem mass spectrometry (LC-MS/MS) is proposed as measurement technique.

2. Experimental

2.1. Reagents

All reagents were acquired from major suppliers. Testosterone (purity ≥99% (HPLC)) and 16,16,17-d_3-testosterone (testosterone-d3) were obtained from Sigma Aldrich (Steinheim, Germany).

For the preparation of the cobalt ferrite ($CoFe_2O_4$) magnetic nanoparticles (MNPs), cobalt (II) chloride hexahydrate ($CoCl_2{\cdot}6H_2O$) and iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) were obtained from Acros Organics (New Jersey, NJ, USA), and ethanol was purchased from Panreac (Barcelona, Spain). These MNPs were embedded in commercial octadecyl silica (C18) microparticles (50 μm average diameter) from Sigma Aldrich (Steinheim, Germany) using toluene from Fischer Scientific (Loughborough, United Kingdom).

Deionized water was obtained from a Connect water purification system provided by Adrona (Riga, Latvia).

To prepare the chromatographic mobile phase, LC-MS grade methanol (MeOH) from Honeywell (Seelze, Germany), LC-MS grade water from Panreac (Barcelona, Spain) and formic acid 98% (for mass spectrometry) from Fluka (Steinheim, Germany) were used. 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).

For the preparation of synthetic saliva, sodium chloride (NaCl) from Fisher Scientific (Loughborough, United Kingdom), potassium chloride (KCl), calcium chloride ($CaCl_2{\cdot}H_2O$), potassium thiocyanate (KSCN) and di-sodium hydrogen phosphate ($Na_2HPO_4{\cdot}H_2O$) from Panreac (Barcelona, Spain), sodium sulfide (Na_2S) from Scharlau (Barcelona, Spain)

and urea from VWR Chemicals (Fontenay-sous-Bois, France) were used.

2.2. Sample collection and pre-treatment

Passive drooling was employed to collect the samples, since commercial sampling devices may affect the concentration of testosterone in saliva [29].

In this sense, three samples from different volunteers (one female and two male) were analysed and used for recovery studies. Other four samples (two female and two male) were collected before and after physical exercise. All samples were first centrifuged (5 min, 6000 rpm) to separate saliva from leftovers and/or phlegm, and the supernatant was frozen at −20 °C to break the polysaccharides. To be analysed, samples were thawed at room temperature and centrifuged again (5 min, 18000 rpm).

2.3. Apparatus and materials

An Ultrasons-HD ultrasonic bath from J.P. Selecta (Barcelona, Spain) and a EBA 21 centrifuge (provided with a rotor of 15 cm radius for centrifuge tubes and a rotor of 9.7 cm radius for microcentrifuge tubes) were used.

For the extraction devices, a single-channel EHP pipette from VWR (Radnor, PA, USA) and a multichannel pipette P8X1200L from Gilson (Middleton, WI, USA), and neodymium magnets (6 mm length x 2 mm diameter, 3 mm × 1 mm, and 1 × 1 mm) from Supermagnete (Uster, Switzerland) were acquired. DPX XTR tips were kindly provided by DPX technologies (Columbia, SC, USA).

Those instruments employed for characterization of the sorbent material are listed in Supplementary Material.

2.4. Preparation of synthetic saliva

For the validation of the method, synthetic saliva was prepared according to an adapted protocol [30]. In this sense, an aqueous solution of ultrapure water (250 mL) containing NaCl (400 mg L^{-1}), KCl (400 mg L^{-1}), $CaCl_2{\cdot}H_2O$ (795 mg L^{-1}), $Na_2HPO_4{\cdot}H_2O$ (690 mg L^{-1}), KSCN (300 mg L^{-1}), Na_2S (5 mg L^{-1}) and urea (1000 mg L^{-1}) was prepared.

2.5. Synthesis of $CoFe_2O_4$–C18 magnetic sorbent

The synthesis of $CoFe_2O_4$ MNPs was performed by wet chemical co-precipitation following an adapted protocol [31]. In this sense, 100 mL of a 0.4 M aqueous solution of $FeCl_3$ and 100 mL of a 0.2 M aqueous solution of $CoCl_2$ were mixed. Afterwards, 100 mL of a sodium hydroxide 3 M solution was slowly added under vigorous stirring at 80 °C and the mixture was kept stirring at this temperature for 1 h. The resultant MNPs were isolated by magnetic decantation and washed twice with ultrapure water and once with ethanol. Finally, MNPs were dried overnight at 80 °C.

For the preparation of the $CoFe_2O_4$–C18 composite, 0.04 g of the previous synthesized MNPs were mixed with 0.4 g of C18 microparticles suspended in 10 mL of toluene, and the mixture was stirred for 2 days at room temperature. The yield obtained in this part was 70%, obtaining ca. 0.3 g for each synthesis, allowing to perform over 300 analyses per synthesis.

2.6. Preparation of standard and sample solutions

Firstly, stock solutions of testosterone at 500 μg mL^{-1} and testosterone-d_3 (surrogate) at 1000 μg mL^{-1} were separately prepared in MeOH. These stock solutions proved to be stable at least for one month.

Then, an intermediate solution at 2 μg L^{-1} of testosterone was prepared in synthetic saliva (see Section 2.4), whereas a surrogate solution at 5 μg L^{-1} was prepared by proper dilution of its stock solution in ultrapure water.

The working standard solutions (N = 7) between 25 and 2000 ng L^{-1} were prepared directly in a 96-well plate, employing the proper volume of the testosterone intermediate solution (2 μg L^{-1}) and adding the corresponding volume of synthetic saliva up to 400 μL. Finally, 100 μL of the surrogate intermediate solution were added (i.e., 1 μg L^{-1} in the final solution).

Saliva samples were also prepared in a 96-well plate, similar to the working standard solutions, i.e., 400 μL of sample were mixed with 100 μL of the surrogate intermediate solution.

2.7. Scheme of the microextraction device

For the preparation of the single-channel microextraction device, a neodymium magnet (6 mm length x 2 mm diameter) was placed inside a 1 mL pipette tip. Then, a smaller magnet (1× 1 mm) was placed outside the tip in order to attach the inner magnet to the wall of the tip and avoid its movement. Otherwise, the magnet would fall plugging the orifice of the pipette tip, and thus hindering the flow of solutions. Fig. 1a shows a scheme of this device. Likewise, a picture of the system is shown in Fig. 1c.

On the other hand, a multichannel device was also tested by employing a 12-channel pipette. In this case, as can be seen in Fig. 1b and d, no external magnet is employed, since the inner magnets themselves interact in pairs between consecutive pipette tips. In this way, they are suspended on the side of the wall of the pipette tip, allowing the correct flow of the solutions. For this second system, the magnets employed were smaller (3× 1 mm) than those in the single-channel approach (as discussed in Section 3.2.2).

2.8. Extraction procedure

To carry out the extraction, 1 mg of $CoFe_2O_4$–C18 magnetic sorbent was first dispersed in 35 μL of MeOH by 10 s of sonication. 500 μL of each working standard or sample solution (prepared as described in Section *2.6.*) were aspirated into the pipette tip of the extraction device, and they were dispensed over the dispersed sorbent. The resultant dispersion was immediately aspirated into the pipette tip where the sorbent was retained by the inner magnet, and the remaining solution was discarded. Later, ca. 500 μL of ultrapure water were aspirated and discarded as a clean-up step to eliminate the leftovers of saliva inside the tip. Finally, testosterone was desorbed by aspirating/dispensing 35 μL of MeOH twice, and the extract was transferred into a 250 μL insert placed inside a 2-mL injection vial.

Fig. 2 shows a scheme of the extraction procedure, whereas Supplementary Video S1 shows the steps of dispersion and the retention of the magnetic material by the magnet.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2022.340117.

2.9. LC-MS/MS analysis

An Agilent 1100 Series chromatography system comprised of a degasser, a programmable pump, an autosampler and a thermostatic column oven with a Zorbax SB-C18 (50 mm length, 2.1 mm I.D., 1.8 μm) column, coupled to an Agilent 6410B Triple Quad MS/MS was employed throughout the study.

For the LC-MS/MS analysis, each extract was injected (10 μL) into the chromatographic system. The mobile phase was composed of 0.1% formic acid and 2 mM ammonium acetate aqueous solution and MeOH containing 0.1% formic acid with isocratic elution at a mixing ratio of 15:85% (v/v) employing a flow rate of 0.2 mL min^{-1}. The column temperature was set constant at 35 °C.

The triple quadrupole MS detector operated in electrospray ionization mode (ESI), by multiple reaction monitoring (MRM). Specifically, positive polarity (ESI^+, capillary voltage at 5 kV) was used to measure testosterone and testosterone-d_3. The other conditions were: gas

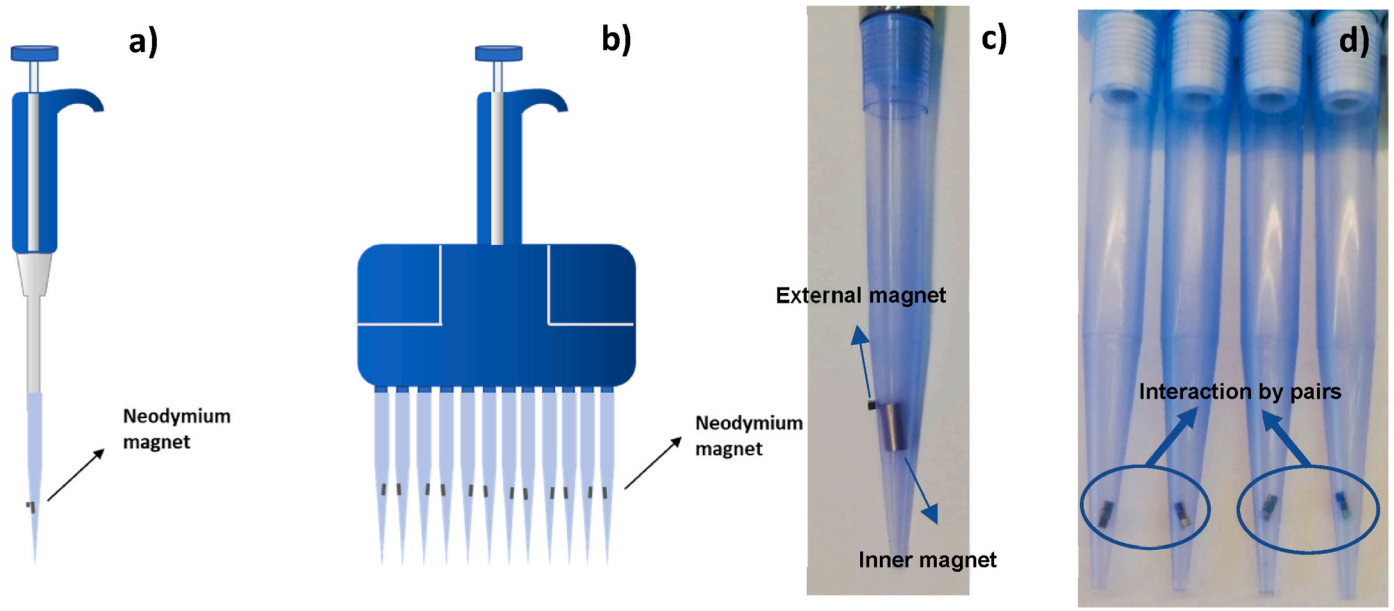

Fig. 1. Scheme of the extraction devices: a) single-channel device, b) multichannel device, c) detail of the tip for the single-channel device with both the external and the inner magnets, and d) detail of the tip for the multichannel device showing the magnetic interaction between adjacent magnets.

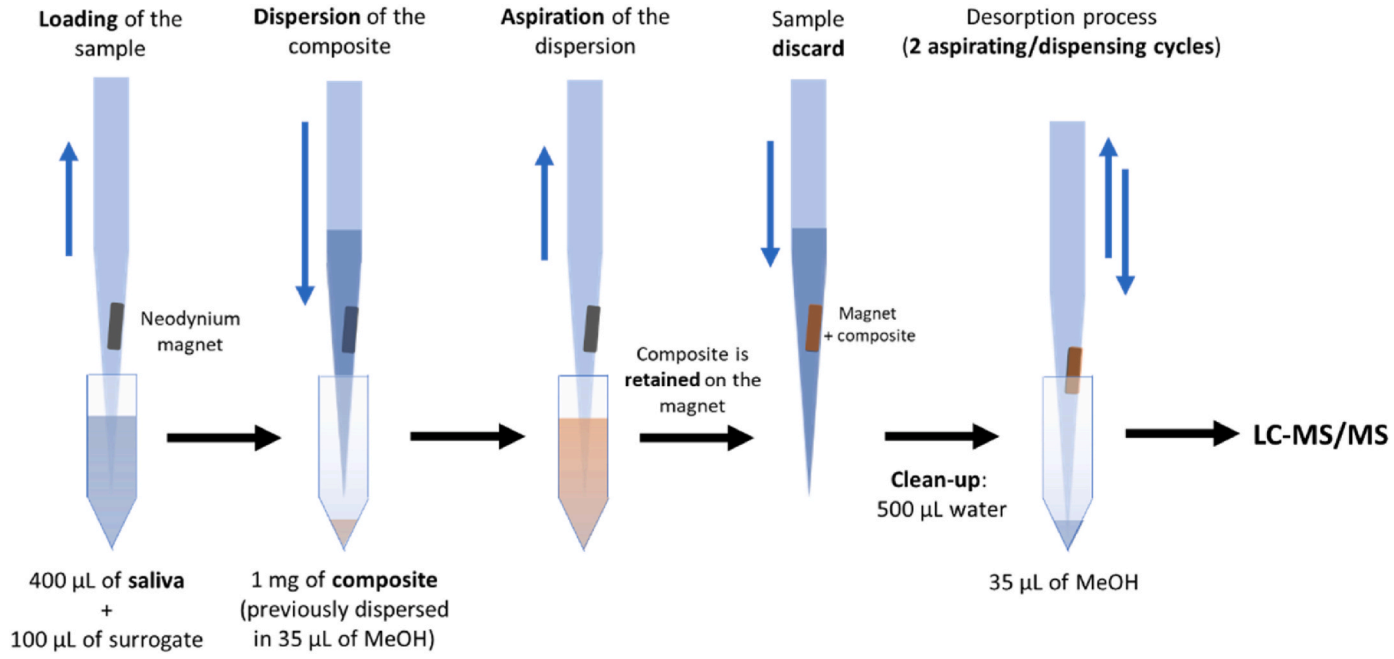

Fig. 2. Scheme of the proposed analytical method.

temperature at 230 °C, nebulizer gas flow rate at 13 L min^{-1}, nebulizer gas pressure at 35 psi, collision energy at 21 V, fragmentor at 132 V, and dwell time at 200 s. The *m/z* precursor → product ion transitions for quantification and identification of testosterone were 289 → 97 and 289 → 102, respectively. For testosterone-d_3, only the transition 292 → 97 was selected. Total run time was 2.5 min.

3. Results and discussion

3.1. Selection and characterization of $CoFe_2O_4$–C18 magnetic sorbent

As one of the purposes of this work was the comparison with commercial pipette tip-based techniques, it was necessary to employ a common material, which is limited due to the lack of variety of sorbents that commercial brands present. Among the few available sorbents, octadecyl (C18) was selected, since it allows strong hydrophobic interactions with highly hydrophobic compounds like testosterone (Log $K_{o/w}$ = 3.3). To provide the magnetism needed to be used in the presented approach, a magnetic composite was prepared (as described in Section 2.5) by embedding $CoFe_2O_4$ MNPs on the pores of silica C18 end-capped microparticles by non-covalent bonds.

Different properties of the synthesized $CoFe_2O_4$–C18 magnetic composite such as magnetization, morphology, specific surface area, pore diameter, surface charge, and thermogravimetry stability were studied. The inter-batch comparison and the extraction performance of the composite material and its individual components were also

evaluated. All these results are presented in Supplementary Material.

3.2. Selection of the microextraction device

Two different devices, i.e., the single-channel and the multichannel, have been tested. Despite their similarity, they present some differences that are commented below.

3.2.1. Single-channel device

The single-channel device employs an external magnet to fix the height of the inner magnet and thus avoiding the obstruction of the pipette tip. Since the inner and the external magnet are very close, the magnetic interaction is so strong that the magnets do not move during the sample aspiration/dispensing processes.

The height of the inner magnet can be easily modified by just displacing the external magnet according to the analysis requirements. The recommended height was the minimum to avoid flow obstruction of the pipette tip. If the inner magnet is placed too high, the magnetic sorbent containing the analytes would not be retrieved and it would be discarded along with the sample.

However, sometimes it could be useful to change the height of the magnet. This is especially important when it is necessary more than one cycle to obtain a proper dispersion, as it could happen for viscous samples. In these cases, the inner magnet can be placed in the upper part of the pipette tip to not disturb during the extraction process, otherwise the magnet would retrieve all the magnetic sorbent during the first aspiration of the dispersion provoking that during the further aspiration/dispensing cycles the system would work as PT-SPE. Once the extraction step is over, the magnet could be easily moved back to the bottom of the pipette tip for the subsequent steps.

3.2.2. Multichannel device

Compared to the single-channel device, the multichannel system has the advantage of treating multiple samples at the same time, which allows to significantly reduce the total analysis time. In this device, the external magnet is not needed since the inner magnet is held by the closest magnet from the adjacent pipette tip. In this case, during the aspiration of the sample, the magnets could be slightly displaced to an upper position, where the distance between both magnets is lower. In this case, as said before, it would be necessary to increase the volume of the desorption solvent. The use of external magnets like in the single-channel mode is not possible since they would hit the wall of the 96-well plate and they would be displaced to higher place resulting in the same described problem. To solve it, the employment of square-shape magnets (3 mm length x 1 mm width) provided satisfactory results. With this new configuration, the interaction between adjacent magnets is lower and they remain at the bottom of the pipette tip. Then, when the sample is aspirated, their square form does not obstruct the flow, but the magnets are dragged by the sample up to a determined height where they increase the interaction and thus the ascension is stopped. When the aspiration is stopped, the magnets fall down to the original position allowing to retrieve the magnetic material. A video showing this behaviour is shown in Supplementary Video S2. It should be said that this movement of the magnets only occurs during the sample aspiration. During the desorption process, where small volumes are employed (i.e., 25 μL), the magnets remain on their original positions. A great advantage of this system is that since the magnets are smaller, it is necessary less volume of organic solvent during the desorption process. Nevertheless, the magnetic interactions with sorbent employing these small magnets are weaker, causing that not all the magnetic material is retrieved during the desorption step. In this case, it would be necessary a centrifugation step to avoid the introduction of solid particles in the chromatographic system. In order to evade the centrifugation step, the multichannel device will be improved for future applications.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2022.340117.

Taking into account these observations, single-channel system was selected for further experiments.

3.3. Study of the extraction and desorption variables

The amount of composite, the extraction time, the number of dispensing cycles, the disperser solvent volume, the ionic strength (% NaCl (w/v)) and the sample dispensing speed were evaluated as critical variables during the microextraction process using the single-channel device. A univariate optimization was selected in order to study each variable independently. Considering that human saliva is mainly composted of water (ca. 99%), each experiment was performed by triplicate extracting 500 μL of aqueous standard solution with 1 μg L^{-1} of testosterone during the same working session. Discussion of these results are presented in the Supporting Information. The selected conditions were 1 mg of composite, no additional extraction time, one dispensing cycle, 35 μL of disperser solvent and 0.2% of NaCl. Regarding the sample dispensing speed, it was used as rapid as possible to increase the dispersion of the sorbent.

Moreover, the desorption variables, such as the elution solvent volume and the number of aspirating/dispensing cycles were examined. Discussion of these results are included in Supporting Information. The selected desorption conditions were two aspirating/dispensing cycles and 35 μL of elution solvent.

3.4. Analytical performance

Different parameters were evaluated in order to validate the proposed method.

3.4.1. Selectivity

As it was stablished in Section 3.1, the strong hydrophobicity of C18 allows to separate non-polar trace compounds (such as testosterone), while the major components present in saliva (saccharides, salts, urea, proteins, etc.) are not retained by this composite due to their high hydrophilicity and they are discarded with the rest of the sample. In this sense, the biological matrix is removed and the analytes can be introduced into the MS without harming it or affecting the signal [32]. Furthermore, the subsequent LC-MS/MS analysis allows to separate testosterone from other possible interferences that could be extracted. The testosterone identity was stablished in terms of the retention time and the *m/z* transitions compared with a standard. According to the chromatogram obtained (shown in Fig. S9) after the application of the method to a real sample, no additional peaks besides the target analyte and surrogate were observed, proving the good selectivity of the overall proposed method.

3.4.2. Linearity

High levels of linearity were obtained up to 5 μg L^{-1}. However, according to the usual levels of testosterone, the working range was set from 25 to 2000 ng L^{-1}. The obtained calibration curve was $A/A_{sur} = (1.534 \pm 0.022)\cdot C + (0.110 \pm 0.019)$ with a regression coefficient (R^2) of 0.9993 for $N = 7$, where A is the peak area of the testosterone, A_{sur} is the peak area of the surrogate and C is the testosterone concentration in ng L^{-1}. Moreover, the difference between calculated and experimental values (residuals) was less than 10%.

The heteroscedasticity was also tested with the Breusch-Pagan test [33], the tabulated value for χ_0^2 for 1° of freedom and a significance level of 0.05 was 3.84, and the calculated value for χ^2 was 2.98. As $\chi^2 < \chi_0^2$, it can be concluded that the regression is homoscedastic.

3.4.3. Limit of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were estimated with the 3 and 10 signal-to-noise ratio (S/N) criteria respectively, by measuring different concentrations after the extraction in order to find which one provides the closest S/N to 3 (LOD) and to 10

(LOQ), respectively. Values for LOD and LOQ were 7.5 ± 0.4 and 24.8 ± 1.3 ng L^{-1} respectively.

3.4.4. Repeatability

For the study of repeatability of the proposed method, five independent replicate standard solutions containing the analyte at three concentration levels (50, 250 and 1000 ng L^{-1}) were measured during the same day (intra-day repeatability) obtaining RSD values of 7.8, 6.0 and 4.6% respectively, and five independent replicates were measured on different days (inter-day repeatability) with RSD values of 8.5, 7.8 and 6.5% respectively.

3.4.5. Enrichment factor and extraction efficiency

The enrichment factor (EF) was calculated as the ratio of the signal obtained before and after the extraction of a 250 ng L^{-1} solution, obtaining a value of 4.3 ± 0.4.

The extraction efficiency is related with EF, in such a way it was calculated as the product of the EF and the ratio between final (i.e., 35 μL) and initial volume (i.e., 400 μL) in %. The obtained value was 37 ± 3%.

3.5. Comparison with other pipette tip-based analytical strategies

In order to study if this new pipette tip-based approach provides advantages over PT-SPE and DPX strategies, the extraction of a 1 μg L^{-1} aqueous solution of testosterone was performed using these three strategies.

For comparative purposes, all processes employed the same amounts of composite (i.e., 1 mg) and desorption solvent (i.e., 35 μL), and the same number of aspirating/dispensing cycles for the extraction (i.e., one cycle), for the clean-up (i.e., one cycle) and for the desorption (i.e., two cycles) steps. The detailed processes for PT-SPE and DPX are described in Supplementary Material.

As it can be observed in Fig. 3, similar results were obtained employing DPX and the presented approach. However, for PT-SPE the signal obtained was quite lower, thus demonstrating the higher extraction efficiency of the dispersion approaches versus the static one.

Additionally, a comparison of some of the crucial aspects of the approaches (i.e., analytical performances, prices and ergonomics for the operator) is presented in Table 1. As it was discussed before, the performance of DPX and the presented approach was similar. Nevertheless, the price of the commercialized DPX pipette tips is quite higher.

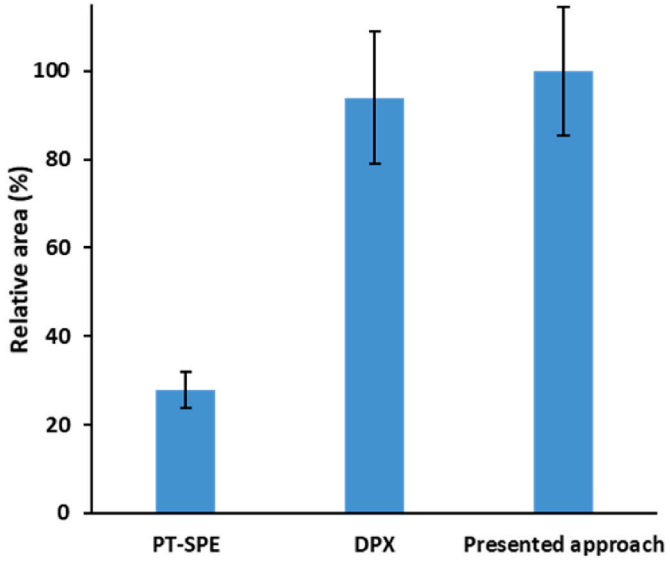

Fig. 3. Comparison between the analytical performance of PT-SPE, DPX and the presented approach. Errors bars show the standard deviation (N = 3).

Table 1
Comparison between PT-SPME, DPX and the presented method.

Extraction technique	EF	Price per 100 analysis (€)[b]	Ergonomics
PT-SPE[a]	1.2	1–2	+
DPX	4.0	100–160	+++
This approach	4.3	3–4	+++

[a] Home-made PT-SPE tips.
[b] Prices do not include shipment charges.

Moreover, DPX required more volume of organic solvent (i.e., 300 μL of MeOH) for preconditioning the sorbent. In terms of comfort and ergonomics, both are quite grateful with the operator, unlike PT-SPE that needs more cycles to achieve the same extraction efficiency as the others. On the other side, the lab-made PT-SPE is the cheapest method, but its analytical performance was lower than the other competitors, also experimenting some of the problems mentioned in the bibliography [16] such as clogging, material loses, etc. It is true that part of these complications would be diminished with commercial tips; however, the prices of each pipette tip would be increased.

According to these aspects, it can be concluded that the presented technique is the best alternative due to its low cost, simplicity, reduced consumption of organic solvents and non-commercial dependence compared with the other two approaches.

Additionally, another advantage of the presented approach over those employing commercial tips is the possibility of using a wide variety of sorbents tailored to the analytes, unlike the commercial ones that present a lack of variety of available sorbents, which significantly reduces their versatility.

3.6. Accuracy and analysis of real samples

For the study of the accuracy, the matrix effects were evaluated. For this purpose, three samples from three different volunteers (a female and two males) were analysed before and after being spiked at three levels of concentration (50, 250 and 1000 ng L^{-1}). As shown in Table 2, good relative recoveries (between 82 and 106%) were obtained, thus proving matrix effects were not significant and, therefore, standard addition calibration was not necessary. The employment of a deuterated internal standard (i.e., testosterone-d_3) that has the same behaviour as the target analyte during the extraction process and the measurement, corrects the matrix effects.

Just as a proof-of-concept, saliva from four volunteers (two females and two males) were analysed before and after physical exercise to see if there was an enhance of the salivary testosterone levels. As expected, an increase in the levels of salivary testosterone after the activity was observed for all volunteers. The growth was higher for females (26 and 40%) than for males (around 10%). Results are presented in Table 3.

Table 2
Relative recoveries of the proposed method.

Volunteer[a]	Amount spiked (ng L^{-1})	Amount found (ng L^{-1})	Relative recovery (%)
1 (M)	0	83 ± 3	–
	50	127 ± 2	94 ± 10
	250	308 ± 20	90 ± 8
	1000	1090 ± 40	92 ± 4
2 (M)	0	98 ± 5	–
	50	144 ± 5	82 ± 3
	250	360 ± 10	88 ± 5
	1000	1140 ± 60	103 ± 6
3 (F)	0	60 ± 5	–
	50	110 ± 7	101 ± 14
	250	324 ± 10	106 ± 4
	1000	1100 ± 20	105 ± 3

[a] **(M):** Male; **(F)**: Female.

Table 3
Comparison of levels of testosterone before and after physical exercise.

Volunteer[a]	Testosterone before training (ng L^{-1})	Testosterone after training (ng L^{-1})	Increase (%)
4 (F)	46 ± 6	58 ± 4	26
5 (F)	33 ± 2	41 ± 3	40
6 (M)	89 ± 1	99 ± 2	11
7 (M)	66 ± 2	75 ± 4	14

[a] **(M)**: Male; **(F)**: Female.

4. Conclusions

In this paper, a high-throughput approach that combines the quickness of dispersive solid phase extraction, the easy retrieval of the magnetic sorbents, and the simplicity of pipette tip-based microextraction techniques has been presented. The preparation of the extraction device just requires the incorporation of small neodymium magnets inside pipette tips. In order to test the viability of this new approach, the determination of salivary testosterone was selected obtaining good analytical features in terms of linearity, enrichment factor, LODs and LOQs, repeatability, and accuracy. Compared with other pipette tip-based microextraction techniques, this one using lab-made tips allows to obtain similar extraction and analysis times than those using expensive commercialized pipette tips that also present a lack of sorbent versatility. Moreover, it requires less amounts of organic solvents per analysis than other pipette tip-based approaches, being in compliance with the principles of Green Sample Preparation (e.g., low volumes of non-toxic organic solvents, minimization of waste, etc). Additionally, a multichannel pipette was tested to increase, even more, the sample throughput, reducing the analysis time. However, this version needs some adjustments in order to avoid centrifugation and it will be improved for future applications. The obtained results prove the excellent properties of this new approach as a low-cost and user-friendly alternative over conventional pipette tip-based strategies for the on-site analysis of small volumes of samples.

CRediT authorship contribution statement

José Grau: Investigation, Data treatment, Writing – original draft. **Juan L. Benedé:** Methodology, Writing – review & editing. **Alberto Chisvert:** Conceptualization, Supervision, Methodology, Project organization, Funding acquisition, Writing – review & editing. **Amparo Salvador:** Supervision, Methodology, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.340117.

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