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Article

Synthesis and Characterization of Molecularly Imprinted Polymers for the Determination of Cocaine in Urine Using Microextraction in Packed Sorvent

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Among stimulant drugs, cocaine deserves attention due to its high rates of seizures worldwide. This work presents the synthesis and characterization of hybrid molecularly imprinted polymers (MIPs) for use in preparing biological samples using a homemade microextraction in a packed sorbent device to extract cocaine. The MIPs synthesized using caffeine and cocaine as templates have been compared. Caffeine was used due to its low value and be easier to obtain than cocaine. Additionally, restricted access molecularly imprinted polymers (RAMIPs) were also produced for comparison purposes. The polymeric materials were characterized using scanning electron microscopy, textural analysis, Fourier transform infrared spectroscopy, and cross polarization/magic angle spinning ^{13}C nuclear magnetic resonance. The method optimization was performed using linear ion trap mass spectrometer to evaluate the effects of sample pH, type of eluent, washing solvent, adsorption cycles, and eluent volume. In the optimized method, RAMIPs indicated better cocaine extraction compared to MIPs. The quantitative study demonstrated that the developed method was able to accurately quantify cocaine in urine samples with values close to actual concentrations.

Keywords: cocaine, molecularly imprinted polymers, urine

Introduction

According to the United Nations Office on Drugs and Crime (UNODC),[1] which offers an overview of the supply and demand for various types of drugs and their health impacts, the production of cocaine is continuing at unprecedented levels. Although global production decreased by 25% from 2006 to 2013, it more than doubled from 2013 to 2017, and between 2016 and 2017, it increased by 25% to reach 1,976 tons (expressed in 100% purity).[1]

Following consumption by users, cocaine and its metabolites remain present in the body and can be detected and quantified in biological fluids such as urine, saliva, and blood.[2] Analysis of biological samples is useful for conducting toxicological tests, tracking drug trafficking routes, chemical dependency treatment programs, and anti-doping tests, among other applications.

However, many analyses require treating these samples to pre-concentrate the analyte, which is typically present in low concentrations, and to eliminate interferences for chromatographic analysis, such as high-performance liquid chromatography (HPLC). A sample preparation technique widely used for this purpose is solid phase extraction, which employs a solid phase, known as adsorbent, to extract the analyte through sorption mechanisms. To use smaller amounts of solvents and samples, miniaturized versions of this technique, such as microextraction by packed sorbent (MEPS), have been employed.[3] Therefore, the search for increasingly selective adsorbents has led to the development of molecular imprinted, which involves forming selective

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sites using a template into a polymeric network. These materials have been called molecularly imprinted polymers (MIP), which can be applied in various areas.

Another widely studied type of adsorbent is restricted access materials (RAM), which exclude macromolecules from the matrix, allowing smaller molecules to be retained. They are extremely useful in analyzing biological fluids, which contain significant amounts of macromolecules, such as proteins, polysaccharides, and lipids.[4]

The isolated use of MIP or RAM adsorbents may have some limitations. For instance, MIP, despite their high selectivity, are used in biological samples that have undergone a treatment to remove proteins, as these macromolecules can interfere with the selective cavities of the MIP. On the other hand, although RAM adsorbents have several advantages, they are less selective for smaller molecules. Therefore, it is worthwhile to consider the combination of the benefits of both materials to create a hybrid adsorbent called restricted access molecularly imprinted polymer (RAMIP), which has a special coating that prevents macromolecules from interfering with its high selectivity.[4,5]

In this sense, this work aimed to synthesize different MIP and RAMIP, in which cocaine or caffeine were used as templates. Other synthesis reagents were kept the same, such as methacrylic acid (MAA) as a monomer, trimethylolpropane trimethacrylate (TRIM) as a cross-linker, and 2,2'-azobisisobutyronitrile (AIBN) as radical initiator. These materials were properly characterized using scanning electron microscopy (SEM), textural analysis, Fourier transform infrared spectroscopy (FTIR), and cross polarization/magic angle spinning ^{13}C nuclear magnetic resonance (NMR). The MIP and RAMIP were used as adsorbent in MEPS to extract cocaine, and its adulterants in urine samples. This miniaturized technique aims to reduce the required amount of the sample and consequently minimize the usage of solvents, which contributes to the principles of Green Chemistry analyses.

Experimental

Samples and reagents

To synthesize the polymers, the following materials were used: caffeine (pharmacy and handling Espírito Santo, Brazil), 0.2 mol L^{-1} solution of AIBN in toluene, MAA, and TRIM, which were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA), acetic acid (P.A.) and methanol were purchased from Dinâmica (Indaiatuba, São Paulo, Brazil), and cocaine isolated from samples seized by the Civil Police of Espírito Santo, technical cooperation agreement, Process No. 23068.022157/2020-69, (Figure S1, Supplementary Information (SI) section). Phenacetin (Sigma-Aldrich, St. Louis, USA), benzocaine (pharmacy and handling Espírito Santo, Brazil), procaine (pharmacy and handling Espírito Santo, Brazil), and lidocaine (Sigma-Aldrich, St. Louis, USA) were used in selective studies. The urine sample was collected from a healthy volunteer. The sample was collected following the ethics principles and had been approved by the Universidade Federal do Espírito Santo' ethical committee (No. 29141520.1.0000.5542).

Equipment

The SEM analysis was performed using a scanning electron microscope (Jeol, Tokyo, Japan), model JSM-6610LV, operated at an acceleration voltage of 20 kV. Prior to SEM analysis, the polymers were coated with gold using the Desk V Denton Vacuum (Morristown, New Jersey, USA).

The textural analysis was conducted using the Quantachrome (Florida, USA) Autosorb-1 texture analyzer, with nitrogen adsorption at 77 K. Prior to the measurement, the samples were subjected to vacuum purging at 120 °C for 1 h. The specific surface area was determined using the multipoint Brunauer-Emmett-Teller (BET) method with relative pressures ranging from 0.05 to 0.31. The isotherm was acquired at seven points. The determination of pore volume and size distribution was carried out using the Non-Local Density Functional Theory (NLDFT) method, assuming slit or cylindrical pore geometry. The isotherms were measured using 54 adsorption points and 10 desorption points, and the samples were subjected to vacuum purging at 120 °C for approximately 3 h.

FTIR analyses were conducted, model Cary 630 (Agilent Technologies, Santa Clara, USA), in the range of 4000 to 400 cm^{-1}, employing attenuated total reflection (ATR) mode with 128 scans and 2 cm^{-1} resolution. In addition to the MIPs, the substances involved in the polymer synthesis were also analyzed.

^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (^{13}C-CP/MAS NMR) (Varian-Agilent Technologies, Santa Clara, USA) was performed using a Varian-Agilent 400 MHz (9.4 T) NMR equipment, employing 4 mm diameter rotors, 1200 transients acquisition, 3.6 µs 90-degree (^{1}H) pulse, 1 ms contact time, 5 s repetition time, 20.48 ms acquisition time, 2048 data points, 50 kHz spectral window, 10 kHz MAS frequency, and SPINAL decoupling.

The separations were carried out using a high-performance liquid chromatography with diode array detection (HPLC-DAD), model 1260 (Agilent Technologies,

Palo Alto, USA), equipped with a quaternary pump (G1311C), DAD detector (G1260D), and automatic injection (G1329B). The data were collected and analyzed using Agilent OpenLab Control Panel software. The separations were performed using a C_{18} column (Phenomenex®, Gemini, 250 mm × 4.6 mm, 5 µm).

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), 9.4 T Solarix mass spectrometer (Bruker, Bremen, Germany), analyses were performed. The solutions were directly infused at a flow rate of 7 µL h^{-1} in the positive mode of the electrospray ionization (ESI) source. The acquisition was conducted in a region of *m/z* 154 to 1500. The conditions of the ESI(+) source were as follows: nebulizer gas pressure of 1.5 bar, a capillary voltage of 3.8 kV, and an ion accumulation time of 0.015 s. Each spectrum was acquired by accumulating 16 scans. The linear ion trap mass spectrometer (LTQ-MS) (Thermo Fisher Scientific, San Jose, USA) analyses were conducted. The following parameters were used: ESI(+) voltage: 4 kV, capillary temperature: 275 °C, tube lens voltage: 109 V. The analyses were performed using a heated electrospray ionization (HESI) source at temperature of 50 °C.

Synthesis of MIPs and NIPs

Cocaine was weighed (100.8 mg) using an analytical balance and solubilized in acetonitrile using an ultrasonic bath. The ratio of cocaine, MAA, and TRIM, respectively, in terms of mole/mole/mole, was 1:4:20. Following the in-bulk polymerization procedure, these substances were added to the solution along with the AIBN. After a 10 min ultrasonic bath, the solution in the amber flask was placed in an oven at 60 °C for 24 h.[6]

The step of template removal, known as the washing step, was carried out as follows: after synthesis, the polymer was weighed on a filter paper that was used as an envelope adapted for ultrasonic baths of 30 min with immersion in a solution of methanol:acetic acid (7:3, v/v). About 25 to 30 washes were performed, followed by an additional 5 washes with methanol only. The first, intermediate, and final wash fractions were collected and analyzed using a HPLC-DAD to confirm the removal of template. After drying in an oven at 60 °C, the polymers were ground using a mortar and pestle and stored in Falcon tubes at room temperature (25 ± 3 °C).

The previously procedure described was also conducted for MIPs of caffeine and non-imprinted polymers (NIP), which were synthesized without the addition of template. The resulting MIP is named poly(methacrylic acid-trimethylolpropane trimethacrylate) due to the substances utilized in its synthesis.[7]

The RAMIP was obtained as follows: (*i*) coat with hydrophilic monomers (HM), the template, MAA, and TRIM were dissolved in acetonitrile and the synthesis was carried out as it was for MIP, but for only 1 h; After this moment, 1.0 g of 2-hydroxyethyl methacrylate and 0.11 g of glycerol dimethacrylate dissolved in 35 mL of chloroform were added into the synthesis flask, and the polymerization was carried out for 23 h more. This material was dried in an oven at 60 °C for 24 h; (*ii*) the coating with bovine serum albumin (BSA) was performed as follow: the dried material obtained from the previous step was weighed (1.0 g) and then 20 mL of 1% (m/v) BSA solution in 0.05 mol L^{-1} phosphate buffer pH 6 were added. This solution was vortexed at 3000 rpm for 1 min. After 30 min, the supernatant was discarded. Then, 5.0 mL of 25% glutaraldehyde solution were added and vortexed for 1 min at 3000 rpm. After 5 h of rest, the supernatant was discarded. To the material, 10 mL of 1% (m/v) sodium borohydride were added and vortexed for 1 min at 3000 rpm. After 30 min, the supernatant was removed, and the material was dried at 60 °C in an oven for 24 h. The resulting RAMIP, which was coated with HM and BSA, was washed with ultrapure water to remove any excess synthesis, and then dried again.[2]

Optimization of extraction

Optimization of washing step

After developing the method using a mobile phase of water and methanol acidified with 0.1% (v/v) acetic acid, and verifying the chromatographic parameters, optimization of the extraction step was carried out. The first step was to evaluate the washing solvents, ultrapure water and acetonitrile, following the scheme represented in Figure S2 (SI section). Before the process, a urine sample underwent a treatment process: 25 mL of urine sample were added to a volumetric flask, and 350 µL of 1 mol L^{-1} hydrochloric acid solution were added. The solution was left in a water bath at 65 °C for 1 h, then centrifuged. The pH of the supernatant was adjusted to 6 with 1 mol L^{-1} NaOH.[8]

Tests with an aqueous solution

After adjustments to the developed chromatographic method, tests were performed using an aqueous solution containing 5 µg mL^{-1} of cocaine and benzoylecgonine (BE) and 2 µg mL^{-1} of caffeine.

Tests 1 and 2 were conducted using 3 mg of the cocaine-based restricted access molecularly imprinted polymer (RAMIPcoc) adsorbent material, 250 µL of ultrapure water for conditioning, 250 µL of the aqueous solution with the analytes, and 250 µL of acetonitrile for washing. For

elution, in Test 1, a 250 µL solution of 10% (v/v) acetic acid in methanol was used, while in Test 2, only methanol was used. For Test 3, the analyte concentration was doubled (10 µg mL^{-1}) and a larger volume was used for all steps: 300 µL. The same washing and elution solvents from Test 2 were used.

The tests were analyzed by HPLC using a gradient mobile phase composed of ultrapure water (A) and acetonitrile (B), at a flow rate of 0.75 mL min^{-1}, and detection at 233 nm. Additionally, the tests with the aqueous solution were analyzed using LTQ-MS under the previously described conditions.

Percolation and elution step

Extraction assays were conducted to assess the impact of acidity and alkalinity of the percolation and elution solutions, and each stage was analyzed via LTQ-MS. For these solutions, 1 mol L^{-1} aqueous solutions of formic acid (HCOOH) or ammonium hydroxide (NH_4OH) were added. Conditioning was carried out with 250 µL of ultrapure water, and sample percolation was conducted with 250 µL of an aqueous solution of cocaine and BE with a concentration of 5 µg mL^{-1}. No washing step was performed, and elution was performed using 250 µL of methanol. The pH adjustments of the samples, in the percolation and elution steps, followed those described in Table S1 (SI section).

In some studies,[9,10] it has been reported that the concentration of BE found in urine samples is at least twice that of cocaine. Therefore, in the following steps, a higher concentration of BE was added to the urine sample to spike it. Prior to fortification, the urine sample, which initially had a pH of 5.35, was adjusted to pH 4.01 using a 1 mol L^{-1} solution of formic acid.

Washing step

After adopting the condition that showed the best result, an analysis was conducted on the washing step of the extraction material. The study examined the effectiveness of using ultrapure water and acetonitrile as washing solvents. The other conditions for the study were as follows: 250 µL of ultrapure water for conditioning, 250 µL of urine pH 4 fortified with cocaine (5 µg mL^{-1}) and BE (10 µg mL^{-1}), 250 µL of the applicable washing solvent, and 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution) for elution.

Concurrently, cocaine (5 µg mL^{-1}) and BE (10 µg mL^{-1}) were added to 250 µL of ultrapure water adjusted to pH 4. To the elution solution, 250 µL of ultrapure water were added, while 250 µL of methanol were added to the conditioning, sample percolation, and washing solutions. The acetonitrile washing solution was left to evaporate, and then 250 µL of ultrapure water and 250 µL of methanol were added. Finally, the solutions were analyzed by LTQ-MS.

Adsorption cycles

With the optimized results from the previous stage, the next optimization test was carried out, taking into consideration the number of times the sample passed through the sorbent. Therefore, the number of adsorption cycles was analyzed: 1, 2, or 3 times. The analysis was conducted as follows: 3 mg of RAMIPcoc, 250 µL of urine sample at pH 4 spiked with cocaine (5 µg mL^{-1}) and BE (10 µg mL^{-1}), 250 µL of ultrapure water for washing, and 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution) for elution. Additionally, 250 µL of methanol were added to the conditioning, percolation, and washing solutions, and 250 µL of ultrapure water were added to the elution solution. The solutions were then analyzed by LTQ-MS.

Eluent volume

With the optimized conditions, a test was conducted using lower concentrations of cocaine and BE (2 and 4 µg mL^{-1}, respectively). Following the final elution, a second and third elution was performed by passing 250 µL of methanol through the MIP and collecting it in separate vials. This was done to determine whether any analytes remained for desorption or if 250 µL of methanol would be adequate for elution.

Selectivity test

A selectivity test was carried out under the following conditions: (*i*) conditioning: 250 µL of ultrapure water, (*ii*) percolation: 250 µL of urine pH 4 spiked with cocaine (5 µg mL^{-1}), BE (10 µg mL^{-1}), caffeine, phenacetin, benzocaine, procaine, and lidocaine (Sigma-Aldrich, USA) (3 µg mL^{-1}), (*iii*) washing: 250 µL of ultrapure water, and (*iv*) elution: 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution). The LTQ-MS analyses were conducted using the same conditions as those used in the optimization stage.

Comparison between MIPs by MEPS

Extractions were carried out with the optimized conditions using the materials RAMIPcoc, caffeine-based restricted access molecularly imprinted polymer (RAMIPcaf), restricted access non-imprinted polymer (RANIP), molecularly imprinted polymer based on cocaine (MIPcoc), molecularly imprinted polymer based

on caffeine (MIPcaf), and non-imprinted polymer (NIP). Three milligrams of sorbent, 250 µL of ultrapure water for conditioning, 250 µL of urine at pH 4 spiked with cocaine (2 µg mL^{-1}) and BE (4 µg mL^{-1}) (3×), 250 µL of ultrapure water for washing, and 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution) for elution were used. The LTQ-MS analyses were performed under the same conditions as the optimization stage.

Efficiency of extraction

To analyze the efficiency of RAMIPcoc and RAMIPcaf, a triplicate study was conducted using 250 µL of ultrapure water for conditioning, 250 µL of urine with pH 4 spiked with cocaine (2 µg mL^{-1}) and BE (4 µg mL^{-1}), 250 µL of ultrapure water for washing, and 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution) for elution. The eluates were allowed to evaporate. Concurrently, solutions containing 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution) at the same concentration used in sample fortification were also prepared in triplicate and subjected to natural evaporation in the fume hood. Upon complete evaporation, 250 µL of methanol:ultrapure water (1:1, v/v) were added to the 9 vials, and the analysis was performed on LTQ-MS. The LTQ-MS analyses were conducted under the same conditions as in the optimization step.

Quantitative study

A quantitative study of the developed method was carried out. The optimized extraction assays were performed using 250 µL of ultrapure water (conditioning); 250 µL of the spiked sample (percolation, 3×); 250 µL of ultrapure water (washing); 250 µL of methanol (with the addition of 1 mol L^{-1} NH_4OH solution) for elution. In the preparation of the calibration curve, both analytes were used: cocaine (0.2 to 2.4 µg mL^{-1}) and BE (0.4 to 4.6 µg mL^{-1}).

Triplicate stock solutions containing cocaine and BE at concentrations of 20 and 40 µg mL^{-1}, respectively, were prepared. Working solutions in methanol were prepared from each stock solution with appropriate concentrations of cocaine and BE for each point on the calibration curve. For each point, 50 µL of the working solution were added to 200 µL of the matrix to prepare the samples. Extraction assays were performed on the prepared samples.

The eluates were allowed to evaporate naturally in a fume hood. Then, 250 µL of a mixture of methanol:ultrapure water (1:1, v/v) were added. The prepared samples were then analyzed by LTQ-MS under the same conditions as the optimization step. The analytical curve was obtained by plotting the signal intensity of the most abundant fragment, using the sum of the total current of ions obtained during 1 min, against the analyte concentration. The theoretical limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the data of the analytical curve using the expressions 3B/m and 10B/m, respectively, where B represents the standard deviation of the blank and m is the slope of the trendline.[11]

Results and Discussion

Characterization

SEM

The surface characteristics of the materials are demonstrated through the images obtained via SEM (Figure 1). At a magnification of 250× it is possible to observe that the formed polymers consist of heterogeneous and agglomerated particles, specifically consistent with the chosen polymerization type for the synthesis. Bulk polymerization results in rigid and heterogeneous polymers, which necessitates a milling process before their utilization.[12] At magnification of 5000×, it is possible to verify a difference in the surface of uncoated materials compared to coated materials. RAMIP showed a less rough

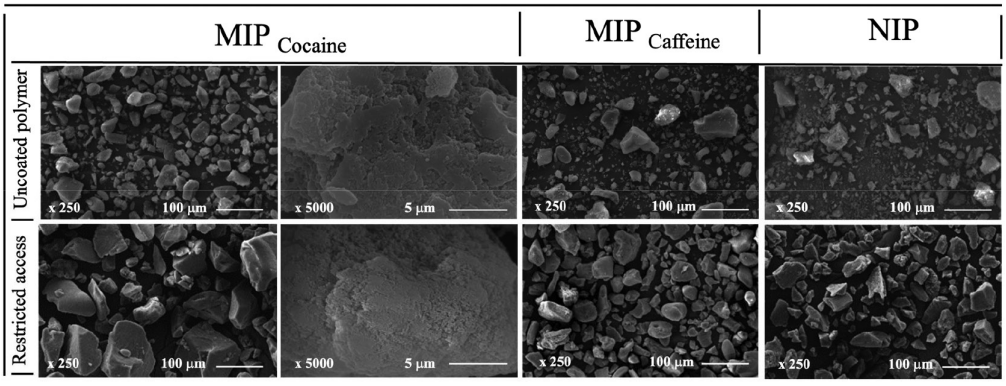

Figure 1. SEM images of the MIPs synthesized from cocaine and caffeine template molecules and NIPs. SEM images of the RAMIPs are also shown.

surface in comparison with uncoated polymers. MIPcoc and MIPcaf did not present difference in its surface.

Textural analysis

Each region of the adsorption isotherm can provide information about the porosity of the analyzed material. In the case of specific surface area analysis using the BET multipoint method, the region of the isotherm with relative pressures (P/Po) between 0.05 and 0.35 is typically analyzed, as most materials exhibit a linear relationship between relative pressure and the expression 1/(W((Po/P)-1)), where W is the weight of gas adsorbed. This allows the slope of the line and the point of intersection with the Y axis to be used to calculate the volume of gas adsorbed on the material surface and, thus, its specific area.[13] In Figure 2, the linearized versions of part of the adsorption isotherms can be visualized. It is noteworthy that all of them exhibit good linearity.

Table S2 (SI section) shows the specific surface area for each analyzed polymer. It can be observed that the NIP had a higher specific surface area (S_{BET}) than their corresponding MIP. The difference between MIP and NIP, besides their specific sites produced from the template in the synthesis, is the washing step with acetic acid and methanol to remove the template, which can change the structure of polymer if it was too aggressive.

RAMIP exhibited a lower S_{BET} than their corresponding MIP, which may have been due to the coating with the protein BSA.[8] The S_{BET} of the MIPs was similar to what has been previously reported in the literature.[14] In general, the higher the specific surface area of the material, the greater its adsorptive capacity due to the greater contact between its surface and the analyte.[8]

The complete nitrogen adsorption isotherms of porous materials can be classified into six different types according to their porosity. Figure S3 presents the complete nitrogen adsorption isotherms of the MIPcaf, MIPcoc and NIP. It can be observed that they resemble the type IV isotherm, which

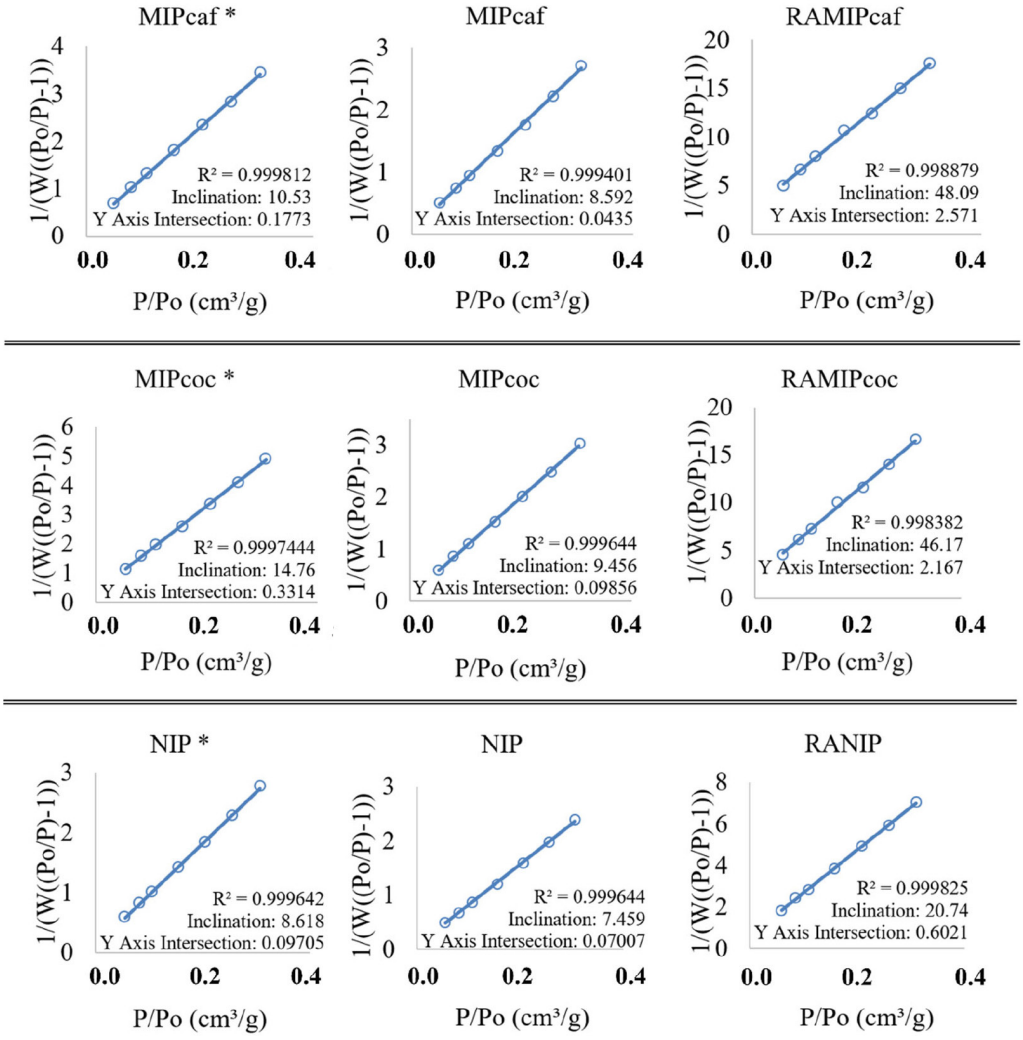

Figure 2. Analysis through the 7-point BET method of the adsorption curves for each polymer. *Without washing.

is characteristic of mesoporous materials that also possess micropores in their structure. In mesoporous materials, multilayer adsorption usually occurs, which means that in addition to the superficial adsorption on the adsorbent (monolayer adsorption), there is also an accommodation of more layers of molecules.[15]

The lower branch of the isotherm indicates the amount of gas adsorbed as the relative pressure of the process increases, while the upper branch indicates the amount desorbed as the relative pressure decreases. When the adsorption and desorption processes occur through different paths, usually due to capillary condensation, hysteresis occurs, resulting in a "spacing" effect in the graph. Moreover, hysteresis becomes more pronounced as the pore size distribution increases.[13]

The pore size distribution is depicted in Figure 3, where it can be observed that both micropores and mesopores are present in the polymers. Table S3 shows that most size pores found by the NLDFT method in MIPcaf, MIPcoc, and NIP polymers are mesopores,[15] with a percentage greater than 90%.

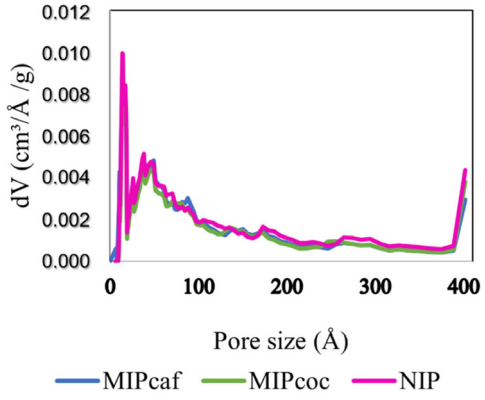

Figure 3. Pore size distribution of MIPcaf, MIPcoc, and NIP obtained by NLDFT.

In Table S4, the pore volume found for each polymer after washing can be observed. Just as the pore size distribution is similar among the polymers, the pore volume was also similar among the polymers analyzed. The values obtained are significantly higher than those reported in the literature.[16]

FTIR

Sequentially, the reagents used in the synthesis, as well as the MIP and RAMIP, were analyzed by FTIR, as shown in Figure S4 (SI section). The FTIR spectra indicate that some bands present in the analysis of the reagents used are also present in the MIP spectra, such as the band at 1638 cm^{-1} observed in the spectrum of MAA and TRIM, as well as bands at 1721, 1460, and 1144 cm^{-1} present in the TRIM spectrum, indicating successful synthesis.

The band at 1638 cm^{-1}, attributed to the stretching of the C=C bond, is less intense in the MIP spectra than in the spectra of MAA and TRIM before the reaction. This indicates that there was a consumption of double bonds during the synthesis, demonstrating that polymerization occurred.[17]

In the overlaid spectra of MIPcaf, MIPcoc, and NIP (Figure 4), spectral similarity can be observed since the same reagents (MAA and TRIM) were used for the three materials. The band at 2958 cm^{-1} can be related to the stretching of C–H bonds in $–CH_2$ and $–CH_3$ groups, present in the polymer chain, and at 1721 cm^{-1} can be related to the C=O stretching of ester (2-hydroxyethyl methacrylate and glycerol dimethacrylate) and carboxylic (MAA) groups.[2] At 1142 and 1261 cm^{-1}, the bands can be related to axial deformations of the C–O group of the ester and carboxylic acid, respectively.[16,17] The band at 1388 cm^{-1} can be attributed to the out-of-plane bending of methyl groups.[18] The signal at 1461 cm^{-1} can be attributed to the bending vibration of $–CH_2$ groups.[19] The band observed at 1638 cm^{-1} can be attributed to the stretching of C=C double bonds in vinyl groups.[16] Both these bands, as well as the observed at 969 cm^{-1}, which are attributed to out-of-plane angular deformation of C–H bonds, are proportionally related to the unreacted double bonds and the degree of crosslinking of the polymer.[20,21]

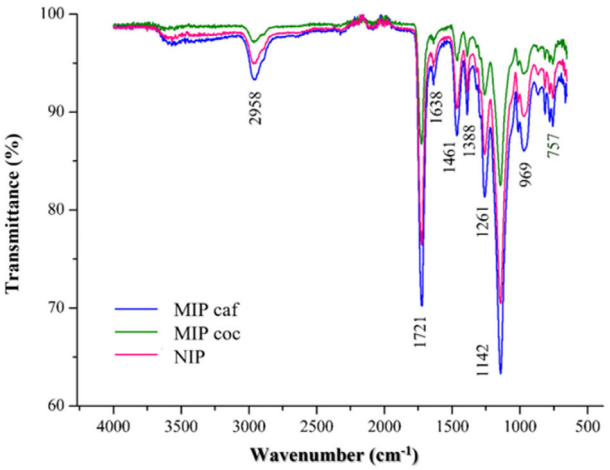

Figure 4. Infrared spectra (ATR) of the MIP synthesized from caffeine (MIPcaf), the MIP synthesized from cocaine (MIPcoc), and the non-imprinted polymer (NIP).

Figure S5 (SI section) presents spectra referring to RAMIPs. In addition to the bands already observed in Figure 4, other bands can also be observed. The band at 3284 cm^{-1} can be attributed to the –NH stretching of the BSA protein.[22,23] At 2949 cm^{-1}, the band is attributed to the asymmetric stretching of C–H in CH_2 and CH_3 groups of the polymer chain.[23]

NMR

The ^{13}C CP-MAS NMR spectra (Figure S6, SI section) also suggest that the studied polymeric solids have the same carbon structure. The signal at 168 ppm can be attributed to the C=O adjacent to C=C (unsaturated carbonyl), and at 177 ppm the signal can be attributed to the saturated carbonyl.[21,24] The signals at 138 and 129 ppm can be attributed to carbons in C=C bonds, and at 67 ppm the signal refers to carbons in RO–CH_2–R groups.[25,26] The signals at 42 ppm is attributed to the quaternary carbon of TRIM,[26] and at 8 ppm can be attributed to carbon in –CH_3 groups.[26,27]

Analysis of MS and development of chromatographic method

Figure S7 (SI section) shows the ESI(+)FT-ICR MS of the analytes used in the development of the method. It is possible to observe signals corresponding to the protonated molecules of the analytes, as well as sodium adducts, as in the case of cocaine, BE, and benzocaine. Dimer signals are also visible in almost all spectra. In general, the ESI(+) FT-ICR MS technique confirms the purity of the respective analyzed standards.

The ESI(+)FT-ICR mass spectrum of BE shows a signal corresponding to the protonated molecule ($[M + H]^+$, *m/z* 290.13870, error −0.06 ppm) and with sodium adduct ($[M + Na]^+$, *m/z* 312.12065, error −0.06 ppm), as well as in the form of dimers ($[2M + H]^+$, *m/z* 601.25204, error −0.20 ppm). Similarly, this occurs for the other analytes, which can be seen in more detail in Table S5 (SI section).

Sequentially, different chromatographic methods were evaluated using the HPLC-DAD technique, as shown in Figures S8a-S8d (SI section). In the methods using acetonitrile and buffer solution,[28] the best chromatogram generated was obtained using a mobile phase composed of 20% acetonitrile and 80% buffer solution: 0.05 mol L^{-1} ammonium acetate pH 3.1 (adjusted with acetic acid) in an isocratic mode and at a flow rate of 2.5 mL min^{-1}, operating within the pH, temperature, and pressure specifications of the chromatographic column manufacturer.[29] A stationary phase based on C_{18} (250 mm × 4.6 mm, 5 µm) has been used, but there were still broad and unresolved peaks.

When another mobile phase composition was used, using acidified water and methanol, the chromatogram with the best separation (Figure S8b) between the analytes was generated under the following conditions: isocratic mode using 70% (A) ultrapure water with 0.1% (v/v) acetic acid and 30% (B) methanol with 0.1% (v/v) acetic acid; C_{18} column (250 mm × 4.6 mm, 5 µm); flow rate at 1.0 mL min^{-1}; injection volume of 5 µL; and temperature at 25 ºC.

As there were still poorly separated peaks and a large time range without elution of any compound, gradient modes of mobile phase composition were tested and the one that presented the best result was with the following conditions: (A) ultrapure water with 0.1% (v/v) acetic acid; (B) methanol with 0.1% (v/v) acetic acid; C_{18} column (250 mm × 4.6 mm, 5 µm); flow rate at 1.0 mL min^{-1}; injection volume of 10 µL; and temperature at 22 ºC (Figure S8c).

As the peaks were more separated, the chromatographic parameters of the method were calculated, which can be viewed in Table S6 (SI section). However, the number of theoretical plates referring to the efficiency of the column in the separation of procaine was below the minimum considered the limit.[30]

Thus, a new mixture of standards was made in methanol without the presence of lidocaine, considering that its peak was very close to both procaine and cocaine. The chromatogram of this mixture in the same method is shown in Figure S8d, and the chromatographic parameters of the separation are presented in Table S7 (SI section).

As the parameters were within the expected range, the optimization step was followed. However, the results were not satisfactory. Thus, a new standard mixture was prepared in methanol without the presence of procaine, and the wavelength was adjusted so that the intensity of the cocaine and BE peaks increased. In addition, solvents were used without acidification, and an improvement in the chromatogram was observed. Furthermore, methanol was replaced by acetonitrile in the mobile phase, and the flow rate was adjusted. Thus, the method that generated the best chromatogram was with the following conditions: Mobile phase: ultrapure water (A) and acetonitrile (B), flow rate at 0.75 mL min^{-1} in gradient mode as described in Table S8 (SI section).

The chromatogram generated under these conditions can be seen in Figure 5.

The term reverse phase chromatography refers to the use of a mobile phase that is more polar than the stationary phase. Analytes with higher affinity to the mobile phase will be eluted faster, while those with lower polarity will have longer retention times due to their greater interaction with the stationary phase. The gradient used with ultrapure water and acetonitrile increased the proportion of acetonitrile throughout the chromatographic run so that the more polar components were eluted initially, followed by the more apolar analytes that were strongly bound to the stationary phase.[31,32]

The obtained chromatographic parameters were within the expected range. In addition to the parameters analyzed previously, the peak symmetry was also evaluated,

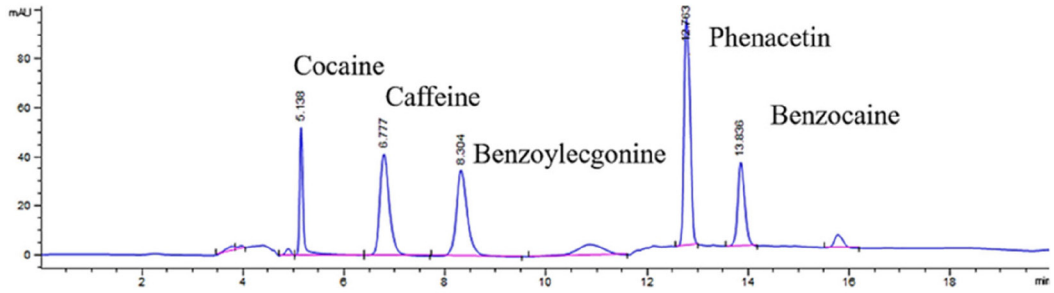

Figure 5. Chromatogram referring to the analysis of cocaine, caffeine, benzoylecgonine, phenacetin, and benzocaine using a C_{18} column (250 mm × 4.6 mm, 5 µm), wavelength at 233 nm, mobile phase composed of ultrapure water (A) and acetonitrile (B), flow rate at 0.75 mL min^{-1} in gradient mode, as indicated in Table S8.

which showed data within the expected range.[30,33] The chromatographic parameters for the cited method can be viewed in Table 1.

Extraction optimization

HPLC analysis

It was noticed that in both the washing and elution steps, the BE peak (t_R 11.23 min) is not detected (Figure S9a, SI section). The peaks corresponding to caffeine (t_R 9.99 min), phenacetin (t_R 22.5 min), and benzocaine (t_R 25.49 min) are detected in the elution step but not in the washing step. However, at 2.9 min (t_R corresponding to cocaine), there are more intense peaks that interfere with its visualization.

On the other hand, in Figure S9b, the chromatograms of washing with acetonitrile are presented. The interferents that elute in this washing step are caffeine (t_R 9.99 min), phenacetin (t_R 22.5 min), and benzocaine (t_R 25.49 min). However, the BE peak (t_R 11.2 min) did not appear in this step (washing) or the elution of the analytes. The visualization of the cocaine peak (t_R 2.9 min) is also compromised due to the appearance of peaks at similar retention times. Thus, it was noticed that there was a problem in visualizing the main analytes (cocaine and BE), and therefore, the chromatographic method was adjusted to the conditions mentioned earlier, using a gradient mode of ultrapure water and acetonitrile and a wavelength closer to the maximum absorbance of these analytes in the ultraviolet region, 233 nm,[33] Figure 5. Afterward, tests were performed with an aqueous solution of the analytes, that is, without the presence of the matrix.

Tests with an aqueous solution

The tests using aqueous solutions generated chromatograms similar to those shown in Figures S10a-S10c (SI section), in which there were also more intense peaks at a retention time similar to that of cocaine (t_R 5.14 min), impairing its visualization. It was possible to perceive that there was a loss of analytes in the percolation step of the solution. In the elution step, it was not possible to visualize peaks of cocaine and BE. Given the limitation of detecting cocaine and BE peaks by HPLC after extraction, the samples were analyzed by LTQ-MS.

Analysis by LTQ-MS

In the MS analyses, in addition to the protonated signals of cocaine and BE, *m/z* 304 and 290, respectively, their fragment signals were also analyzed. For cocaine, the most abundant fragment was *m/z* 182, and for BE the signal was at *m/z* 168.[34-36]

The MS spectra of the aqueous solutions that had been analyzed by HPLC indicated that the cocaine molecule was

Table 1. Chromatographic parameters of the method using a C18 column (250 mm × 4.6 mm, 5 µm), wavelength at 233 nm, mobile phase composed of ultrapure water (A) and acetonitrile (B), flow rate at 0.75 mL min^{-1} in gradient mode

Analyte	Retention time (t_R) / min	Separation factor (α)	Tail factor (T)	Number of theoretical plates (N)	Resolution (Rs)
Cocaine	5.14	1.15	1.45	36,848.3	
Caffeine	6.78	3.47	1.31	6,691.4	7.50
Benzoylecgonine	8.30	1.12	1.22	8,341.7	4.41
Phenacetin	12.8	2.02	1.28	51,016.7	15.2
Benzocaine	13.8	1.13	1.20	49,079.1	4.52
Limits suggested by the FDA[30]		> 1	< 2	> 2.000	> 2

FDA: U.S. Food and Drug Administration.

not being retained in the sorbent (RAMIPcoc), as it was already eluted in the sample percolation and washing step, as shown in Figure S11a (SI section).

Based on the absolute intensities of the signals of cocaine and BE and their main fragments, as shown in Figure S11b, it is possible to observe the minimal intensity of the signal of these analytes in the elution step.

Percolation and elution step

The evaluation of the influence of the pH of the cocaine and BE solution and the pH of the eluent solvent can be seen in Figures 6a-6b. The intensities of the signals corresponding to the protonated cocaine molecule ($[C_{16}H_{19}NO_4 + H]^+$ (*m/z* 304.17)) were analyzed as well as its fragment at *m/z* 182.08. In the case of BE, the signal of its protonated molecule (*m/z* 290.17) and its fragment at *m/z* 168.08 were also analyzed.

The results indicated that Test 1 (sample pH at pH 4 and elution solvent of methanol with the addition of 1 mol L^{-1} NH_4OH solution) provided better results, with low intensity of the signal referring to cocaine in the sample percolation step and higher intensity in the elution step. An improvement in the extraction by using the sample in an acidic environment and a basic elution was also observed in the works of Sánchez-González *et al*.,[9,10] in which the sample pH at 5.5 and the elution solvent with the addition of ammonium hydroxide favored the extraction of cocaine and metabolites from urine samples. Therefore, in the following tests, the sample at pH 4 and the elution solvent (methanol with the addition of 1 mol L^{-1} NH_4OH solution) were used.

Washing step, adsorption cycles, and eluent volume

In the washing step, whose purpose is to remove possible interferents without eluting the analyte,[37]

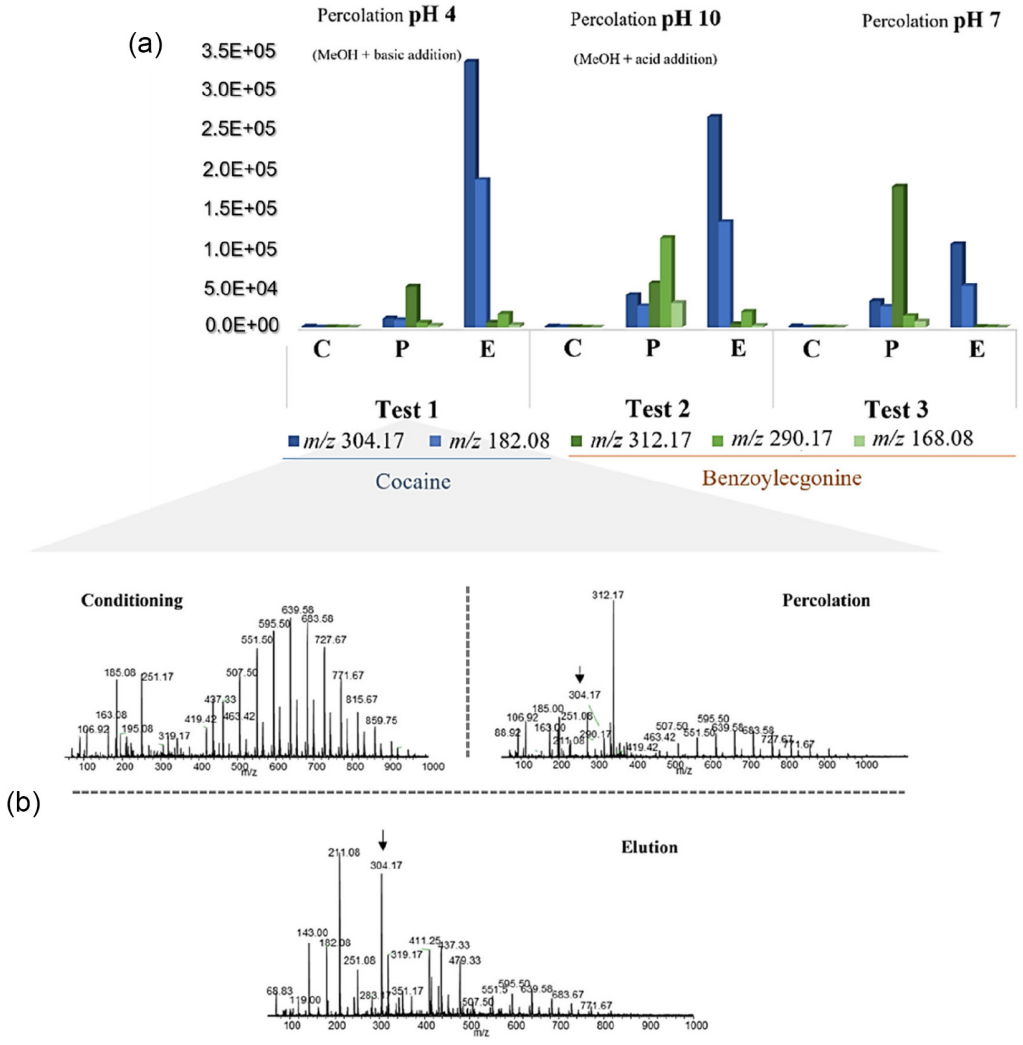

Figure 6. (a) Absolute intensity of signals related to the analytes in each extraction step: conditioning (C), percolation (P), and elution (E); (b) ESI(+)LTQ-MS spectra of the conditioning, percolation using the sample at pH 4 and elution using methanol (with the addition of 1 mol L^{-1} NH_4OH solution).

acetonitrile, and ultrapure water were evaluated. Water is a solvent often used in the washing step for urine sample preparation using MIP[2] because, in addition to improving extraction, it is a cheap, non-toxic solvent that does not harm the environment.[38] On the other hand, acetonitrile, due to its affinity for eluting organic compounds (possible interferents), can also be used as a washing solvent for cocaine extractions.[34]

The analyses showed (Figure S12a, SI section) that the use of acetonitrile as a washing solvent indicated a possible loss of analyte in this step. On the other hand, the intensity of the signals referring to the analytes was more intense in the elution when ultrapure water was used in the washing step. Thus, in the following analyses, ultrapure water was used as the washing solvent.

Regarding the adsorption cycles, an improvement in the extraction was observed by placing the sample in contact with the MIP more times, as shown in Figure S12b. Particularly in Test 3, using three adsorption cycles, there was an increase in the intensity of the analyte signals. Thus, the sample percolation was performed three times in each assay.

The optimization of sample preparation using MEPS involves the use of reduced volumes, typically ranging from 10 µL up to approximately 250 µL.[39] The results of this optimization step indicated that 250 µL of eluent was sufficient to elute most of the analytes, with no increase in average or high intensities when more eluent was in contact with the sorbent, as shown in Figure S12c.

The optimization of the extraction with the chosen parameters for analysis generated results that suggest losses in the percolation and washing steps. However, this was the best scenario achieved with much higher intensities in the elution step, which had not been obtained with the initial study conditions.

Test of selectivity

The selectivity of the MIP is associated with its molecular recognition of the analyte that was used as the template in the material synthesis.[40] Therefore, the formation of selective cavities provides the MIP with the ability to extract the analyte in the presence of possible interferents.

For this selectivity test, possible interferents of cocaine were evaluated. The signals of the analytes observed can be visualized in Table S9 and Figure S13 (SI section). The *m/z* 202, referring to phenacetin, presented less interference, while the signals of *m/z* 235, 237, and 195, referring to lidocaine, procaine, and caffeine, respectively, were shown to be more intense interfering. Among the investigated molecules, phenacetin is the only one that does not have a tertiary amine in its chemical structure. On the other hand, the interfering procaine and caffeine, have, in addition to the tertiary amine, the ester function (in procaine), and bicyclic (caffeine). However, *m/z* 235 is observed as the most intense ion, possibly because it is more liposoluble than procaine (for example). Cocaine is also fat soluble, and this shared affinity between lidocaine and cocaine may explain their greater chemical similarity and affinity.[41]

Comparison between MIPs

In the extraction assay comparisons for the MIPs (Figure 7), it was observed that all the synthesized MIPs and NIPs were able to extract cocaine. Both the cocaine MIPs and the caffeine MIPs were capable of such extraction. This shows that they were able to act as adsorbents, but not necessarily the cocaine MIP was able to act as a selective sorbent.

MIPcaf and NIP generated very similar intensities of signals related to cocaine and its fragment (*m/z* 304 and 182), while MIPcoc showed slightly higher intensities of these signals. The use of analogues as a template (dummy template) does not always bring good results, as small differences in the interaction sites can interfere with the recovery of the analytes from the matrix. These strategies should be explored for analytes whose standards are difficult to achieve, as is the case with cocaine. As expected, the MIP using cocaine as template showed better results (ca. 65% better), as it presents the specific sites for cocaine. Meanwhile, the RAMIPs, in all cases, showed higher intensities than their corresponding MIPs, which was expected, since these materials prevent macromolecules present in urine from interfering with adsorption.[3]

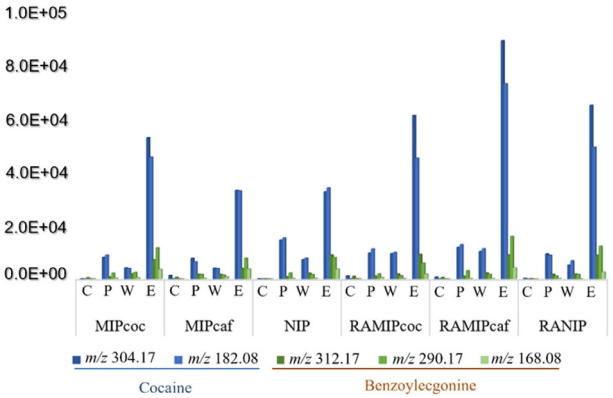

Figure 7. Absolute intensity of signals referring to cocaine, benzoylecgonine, and their fragments in the conditioning (C), percolation (P), washing (W), and elution (E) steps.

Table 2. Data from the quantitative study conducted: coefficient of determination, linear range, RSD of the slope of the calibration curves, theoretical LOD and LOQ

Equation	R^2	Linear range / (μg mL^{-1})	RSD / %	LOD / (μg mL^{-1})	LOQ / (μg mL^{-1})
$y = 4641x + 1438$	0.9981	0.4-2.4	9.7	0.025	0.082

RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantitation; R^2: coefficient of determination.

Efficiency of extraction

As the RAMIPcaf and RAMIPcoc showed the highest intensities in the previous step, they were chosen to evaluate their extraction efficiencies. Studies involving MIP for cocaine extraction generally show efficiencies above 80% using solid phase extraction (conventional technique)[9,42,43] however, as can be seen in Figure S14 (SI section), both showed efficiencies around 30-45%. The low efficiency may have been due to the formation of dispersed interaction sites and/or cavities with low affinity for the analyte,[42] but these are also results that are expected in miniaturized techniques of sample preparation, such as the MEPS that was used in this work.[44-46]

Quantitative study

The most abundant fragment of the cocaine molecule (*m/z* 182) was used for the construction of the analytical curve. The linearity data can be seen in Table 2, as well LOD and LOQ. The results obtained for LOD and LOQ were close to those that used conventional techniques, LOD 0.025-0.061 μg mL^{-1}and LOQ around 0.2 μg mL^{-1}.[33,42,43]

In Table 3, precision, recovery, and relative error data for concentrations at three levels (low, medium, and high: 0.8, 1.6, and 2.4 μg mL^{-1}) are presented. The relative standard deviation (RSD) obtained from the analysis of precision between samples was < 10%. Regarding accuracy, which can be seen in the recovery and relative error data, a maximum relative error of 5.86% was obtained for all cases. The concentrations obtained from the curve were around 99.6 to 105.7% of the true value considered.

Table 3. Data from the quantitative study performed: precision and accuracy

The concentration of cocaine / (μg mL^{-1})	Precision RSD / %	Recovery / %	Relative error / %
0.8	5.8	105.7	5.86
1.6	8.6	99.6	–0.37
2.4	5.9	101.7	1.72

RSD: relative standard deviation.

Conclusions

The synthesized MIPs by the bulk polymerization method were properly characterized. The specific surface area, pore volume and pore size were similar to those reported in the literature. From the FTIR and NMR data, it was possible to observe that the MIPs and NIPs have a similar structure. With the optimized LTQ-MS and MEPS conditions, it was possible to note that the MIPs were capable of extracting cocaine molecules from urine samples. The recoveries for cocaine and BE from urine samples were around 30-40% using RAMIP, achieving high capacity for extracting analytes using low amount of solvents and sample. The quantitative study indicated that was possible to evaluate the concentrations of cocaine and BE from urine samples, obtaining values close to the real concentrations. Thus, for future work, the validation of the method is suggested for further application in real samples from users.

Supplementary Information

Supplementary information with structure of the main molecules used for synthesis, tables with information on methods used, spectra and other figures are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Juliana B. Ferreira was responsible for data curation, formal analysis, investigation, methodology, validation, writing original draft; Nayara

A. dos Santos for writing original draft, review and editing; Keyller B. Borges for validation, visualization, writing original draft; Nathália S. Conceição for conceptualization, data curation, resources, software, visualization, writing original draft; Clara S. D. Baptista for conceptualization, validation, writing original draft, review and editing; Hildegardo S. França for supervision, visualization, writing original draft; Wanderson Romão for data curation, writing original draft, review and editing.

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