Novel sample-substrates for the determination of new psychoactive substances in oral fluid by desorption electrospray ionization-high resolution mass spectrometry

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Abstract: A reliable screening and non invasive method based on the use of microextraction by packed sorbent coupled with desorption electrospray ionization-high resolution mass spectrometry was developed and validated for the detection of new psychoactive substances in oral fluid. The role of different sample substrates in enhancing signal intensity and stability was evaluated by testing the performances of two polylactide-based materials, i.e. non-functionalized and functionalized with carbon nanoparticles, and a silica-based material compared to commercially available polytetrafluorethylene supports. The best results were achieved by using the non-functionalized polylactide substrates to efficiently ionize compounds in the positive ionization mode, whereas the silica coating proved to be the best choice for operating in the negative ionization mode. LLOQs in the low µg/L, a good precision with CV% always lower than 16% and RR% in the 83(±4)-120(±2)% proved suitability of the developed method for the determination of the analytes in oral fluid. Finally, the method was applied for screening oral fluid samples for the presence of psychoactive substances during private parties revealing the presence of mephedrone in only one sample out of 40 submitted to analysis.

Opposed Reviewers:

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DIPARTIMENTO DI SCIENZE CHIMICHE, DELLA VITA E DELLA SOSTENIBILITÀ AMBIENTALE

Editorial Office,
Talanta

Parma, 12th December 2018

Dear Editor,

Please find enclosed the manuscript entitled "**Novel sample-substrates for the determination of new psychoactive substances in oral fluid by desorption electrospray ionization-high resolution mass spectrometry**" by F. Bianchi*, S. Agazzi, N. Riboni*, M. Hakkereinen, L. Ilag, L. Anzillotti, R. Andreoli, F. Marezza, F. Moroni, R. Cecchi, M. Careri for publication on Talanta.

Being able to mimic the effects of controlled illicit drugs, new psychoactive substances are considered as a major threat to the public health by both National and International Organisms. In this context, the development of reliable screening methods for the analysis of these substances is of pivotal importance especially to monitor their consumption among young people.

The manuscript proposes advances in the desorption electrospary ionization – high resolution mass spectrometry determination of NPS with the development of new DESI substrates able to enhance signal intensity and stability. Four sample substrates i.e. non functionalized polylactate, polylactate functionalized with oxidized and reduced carbon nanoparticles and a silica-based material were synthetized, characterized and their performances compared with those of commercially available polytetrafluorethylene sampling supports.

Excellent results were obtained in terms of both sensitivity and signal stability by using both the non functionalized polylactate and the silica sample substrates. With respect to commercially available slides remarkable analytical results in terms of sensitivity and signal stability were achieved obtaining detection and quantitation limits in the low µg L^{-1}, thus demonstrating the reliability of the developed method for high throughput monitoring NPS in oral fluid using a non invasive approach.

Supporting material is available to provide more information about the achieved results.

The manuscript has not been published elsewhere and it is not currently under submission procedure to another Journal.

Yours sincerely

Federica Bianchi

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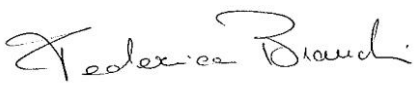
NOVELTY STATEMENT

Parma, 12th December 2018

Dear Editor,

the novelty of the work relies on the development of a reliable desorption electrospray ionization-high resolution mass spectrometry based method for the determination of new psychoactive substances (NPSs) in oral fluid using properly developed sample-substrates able to increase signal intensity and stability of the investigated compounds. Four sample substrates i.e. non functionalized polylactate, polylactate functionalized with oxidized and reduced carbon nanoparticles and a silica-based material, were properly synthetized, characterized and their performances compared with those of commercially available polytetrafluorethylene sampling supports. Excellent results were obtained in terms of both sensitivity and signal stability by using both the non functionalized polylactate and the silica sample substrates, thus allowing to validate a non invasive screening method useful for the determination of analytes belonging to several classes of NPSs in human oral fluid. The main advantages of the developed method relies in the consumption of very low amounts of sample, a very quick and simple sample preparation and shorter analysis times compared to the commonly applied GC-MS and LC-ESI analyses.

Prof. Federica Bianchi



Highlights

Innovative sample-substrates for desorption electrospray ionization mass spectrometry

Remarkable results with the silica-based and the polylactate sample-substrates

Improved detection of new psychoactive substances in oral fluid

Reliable method for high-throughput purposes

Novel sample-substrates for the determination of new psychoactive substances in oral fluid by desorption electrospray ionization-high resolution mass spectrometry

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Abstract

A reliable screening and non invasive method based on the use of microextraction by packed sorbent coupled with desorption electrospray ionization-high resolution mass spectrometry was developed and validated for the detection of new psychoactive substances in oral fluid. The role of different sample substrates in enhancing signal intensity and stability was evaluated by testing the performances of two polylactide-based materials, i.e. non-functionalized and functionalized with carbon nanoparticles, and a silica-based material compared to commercially available polytetrafluorethylene supports. The best results were achieved by using the non-functionalized polylactide substrates to efficiently ionize compounds in the positive ionization mode, whereas the silica coating proved to be the best choice for operating in the negative ionization mode. LLOQs in the low µg/L, a good precision with CV% always lower than 16% and RR% in the 83(±4)-120(±2)% proved suitability of the developed method for the determination of the analytes in oral fluid. Finally, the method was applied for screening oral fluid samples for the presence of psychoactive substances during private parties revealing the presence of mephedrone in only one sample out of 40 submitted to analysis.

Keywords: Sample-substrates; New psychoactive substances; Desorption electrospray ionization; High resolution mass spectrometry

1. Introduction

New psychoactive substances (NPSs) are a wide group of drugs of abuse not yet under international control conventions: being considered as a major threat to public health by both the United Nation Office of Drugs and Crimes and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), great interest is paid toward their monitoring [1]. These compounds are also referred to as *designer drugs*, thus highlighting their nature as synthetic drugs able to mimic the effects of controlled illicit drugs (*recreational drugs*). Most of the NPSs were synthetized and patented between the ‘60s and the ‘70s as anesthetics, antidepressants, antiparkinsonian agents or appetite suppressants [2]. NPSs are obtained through a slight modification of the chemical structure of known illicit substances, and are commonly sold as legal or herbal highs, research chemicals or bath salts. From the toxicological point of view, adverse effects usually include anxiety, paranoia, hallucinations, seizures, hyperthermia and cardiotoxicity [3]. However, these effects are much higher than those exerted by recreational drugs: as an example, the effect of synthetic cannabinoids is approximatively ten times stronger than that produced by tetrahydrocannabinol (THC).

By the end of 2016, EMCDDA monitored more than 670 NPSs on the EU market, 51 of which were detected for the first time in Europe in 2017 [4]. These substances include synthetic cannabinoids, synthetic cathinones, stimulants, opioids, benzodiazepines, ketamine and analogs. Since one of the major threat in drug abuse is driving while impaired by pshycoactive substances, in 2006 the DRUID (Driving Under the Influence of Drugs, Alcohol and Medicines) project was carried out to estimate both the scale and impact of drug driving in Europe [5]. Given the need for a European harmonization of drug analytical protocols (e.g. analytical cut-off limits; use of standardized analytical procedures, methodologies of data collection), more insight into the effects of psychoactive drugs to ensure the development of suitable countermeasures and an improved monitoring of drug use in traffic controls

are some of the final outcomes of the project. Illicit substances are detected in body fluids like blood, and urine. Recently oral fluid has been used as an alternative matrix because of its ease of sampling and non-invasiveness. In this context, a key issue is the use of new sampling techniques to be used during routine control activities, as well as the development of novel screening methods able to assess the presence of the investigated compounds at trace levels. Different portable devices [6] have been reported for the on-site screening of common psychoactive substances such as opioids, cocaine, benzodiazepines, amphetamine, methamphetamine and cannabinoids; however, no official methods for the detection of NPSs are available.

NPSs have been analyzed by using different analytical techniques like gas chromatography (GC) [7-10] and liquid chromatography (LC) coupled to mass spectrometry (MS) [8, 11, 12] or capillary electrophoresis with both UV and MS detection [13, 14]. Immunoassay tests have also been developed to rapidly screen the presence of NPSs in urine [15] and oral fluid [16].

Owing to the demand for high-throughput analysis and identification of NPSs, fast screening methods based on mass spectrometry including ion mobility-high resolution mass spectrometry [17], matrix assisted laser desorption ionization-time of flight mass spectrometry [18] and direct analysis in real time-mass spectrometry [19, 20] have been proposed for the analysis of herbal products.

More recently, the possibility of analyzing samples in their native state without or with minimal sample pretreatment by ambient mass spectrometry (AMS) [21] has generated increasing interest. Ambient ionization techniques are powerful analysis tools for the rapid screening of samples with minimal or without any sample preparation or chromatographic separation prior to analysis, thus reducing analysis time. Consequently, their combination with high resolution mass spectrometry (HRMS) becomes a necessity to solve several matrix related problems rising from the presence of isobaric interferences or sensitivity issues [22]. Therefore, the use of AMS techniques is very attractive for scientific laboratories in order to develop fast and reliable screening methods.

Among the available ambient ionization sources, desorption electrospray ionization mass spectrometry (DESI) has been widely used for pharmaceutical, food and forensics applications [23-25].

DESI source is a high-velocity pneumatically assisted electrospray (ESI) source, generating charged micro-droplets by the application of an appropriate potential on the ESI needle. The jet is directed towards the probe surface where the impact of the primary droplets leads to: i) formation of a micrometer-size thin solvent film, ii) solvation of the sample by a thin layer of solvent at the liquid-solid interface and iii) generation of an electrostatic field. Primary droplets splash secondary droplets containing the dissolved analytes from the solvent by electrostatic repulsions [26]. The desolvated analyte molecules are ionized in the gas-phase as in the traditional ESI process. Finally, ions enter the MS inlet through a heated extended capillary.

Ionization efficiency of the DESI source is strongly affected by the sample substrate as well as by the spray solvent system [27]. The hydrophobicity of the surface, the dielectric constant between the substrate and the spray [28] and the type of interactions between the analyte molecules and the surface at the liquid-solid interface [29] are the most important parameters able to influence both signal intensity and stability. In particular, the latter parameter has proven to be strongly affected by both the deposition of the analytes on the surface and the subsequent desorption/solvation phenomena in the sprayed solvent [30]. Different materials have been tested as DESI sample substrates: slides in polytetrafluorethylene (PTFE), polymethylmethacrylate and porous silica [28, 31] are commercially available, but sol-gel hydrophobic materials [32], nanoporous silicon and ultra-thin layer chromatography plates [33] also have been proposed.

Being part of a research program dealing with the investigation of factors affecting desorption electrospray ionization of low-molecular weight compounds, this study was focused on the development, optimization and validation of a microextraction by packed sorbent (MEPS)-DESI-HRMS screening method for the detection of NPSs in human oral fluid. Owing to the structure of the

investigated compounds, acquisition was performed in both positive-ion mode and negative-ion mode. The performance of both the new polylactide-based materials functionalized with carbon nanoparticles and the new silica-based material used as sample surfaces was evaluated with the final aim of increasing sensitivity for the detection of NPSs. To our knowledge, this is the first study in which the performances of new sample-substrates are tested for screening the presence of NPSs in oral fluid by DESI-HRMS. Finally, a confirmatory GC-MS Selected Ion Monitoring (SIM) method was developed to confirm the presence of the investigated compounds in positive samples.

2. Material and Methods

2.1. Chemicals and materials

Ketamine HCl and mephedrone HCl (each at 1000 mg/L in methanol) were purchased from Lipomed (Arlesheim, Switzerland). UR-144 (100 mg/L in methanol), Spice Cannabinoid Mix (100 mg/L in acetonitrile), Spice Cannabinoid Mix 2 (100 mg/L in acetonitrile), 2-propanol (99.9% purity), acetonitrile (ACN, 99.8% purity), dichloromethane (DCM, > 99% purity), ammonium hydroxide (NH_4OH, 33% in water), ethanol (EtOH, ≥ 99.8% purity), acetic acid (99% purity), nitric acid (HNO_3, 70%), sulphuric acid (H_2SO_4, 95-98%), sodium chloride (NaCl), α-cellulose and caffeic acid (CA, ≥ 98% purity), tetraethyl orthosilicate (TEOS, ≥ 99% purity) and poly(dimethylsiloxane) hydroxy terminated (PDMS) were purchased from Merk (Milan, Italy). Chloroform and ethanol (both 96% purity) were from VWR. Ketamine d_4 HCl (100 mg/L in methanol) and (-)-11-nor-9-carboxy-Δ^9-THC-d_3 (1000 mg/L in methanol) were purchased from LGC (Teddington, UK), whereas methanol (MeOH, > 99.9% purity) was from J.T. Baker (PA, U.S.A.). Deionized water (DI) was obtained by using a MilliQ element A10 System (S. Francisco, CA, USA). MEPS BIN C_{18} and M1 were purchased from LabService Analytica (Bologna, Italy). Poly(L-lactide) (PLLA; 4032D) was obtained from

NatureWorks (Minnesota, USA), whereas Membrane spectra/Por 7 pretreated dialysis tubing (MWCO 1 kDa) was obtained from Spectrum Laboratories (CA, USA).

2.2. Working solutions and sample pretreatment

All the stock solutions were kept in the dark at -18°C. Not contaminated oral fluid taken from 10 volunteers was used as blank matrix for method optimization and validation purposes by spiking the proper amount of NPSs. Deuterated ketamine and deuterated carboxy-THC were used as internal standard (IS) at the concentration of 1 and 5 mg/L, respectively. The spiked oral fluid was treated and diluted with MeOH (1:1) for protein precipitation. Then, the sample was centrifuged at 13000 rpm for 10 min. Finally, the supernatant was collected and centrifuged two more times until complete precipitation of proteins occurred.

2.3. Experimental Design and Optimization of the MEPS Procedure

A commercial e-Vol® device (Trajan Scientific and Medical, Victoria, Australia) equipped with a 50 µL syringe (Trajan Scientific and Medical) and a C_{18} BIN (Trajan Scientific and Medical) was used. Prior to extraction, the BIN was activated by using 10 × 50 µL of MeOH. The experiments were performed on blank oral fluid samples spiked with 10 mg/L of ketamine, mephedrone, UR-144, JWH-250 and JWH-081, used as model compounds.

After protein precipitation, 50 µL of the supernatant were loaded into the BIN. A 2^2 two-level full factorial design (FFD) was carried out by investigating the effects of loading and eluting cycles. In both cases, low and high levels were 5 and 25 cycles, respectively. A mixture of 78:20:2 of DCM:2-propanol:NH_4OH was used as eluting solvent [34]. Four replicates at the center of the experimental

domain were performed in order to evaluate the experimental error. An *F*-test comparing the experimental and calculated responses at the center of the experimental domain was used to evaluate the existence of relevant quadratic effects.

The significance of the loading/eluting effects and of their interactions was evaluated by using the statistical package SPSS Statistics v.23.0 (IBM, Milan, Italy). The best regression models were obtained by a forward search step-wise variable algorithm. Finally, the optimal extraction conditions were calculated by using the multi-criteria method of the desirability functions [35-37].

The final optimal conditions can be summarized as follows: 5 x 50 µL loading cycles and 25 × 50 µL eluting cycles using DCM:2-propanol:NH_4OH as solvent. After extraction, 10 × 50 µL washing cycles were performed to avoid carryover effects. Both fill and injection speeds of 2 arbitrary units were used.

2.4. GC-MS (SIM) analysis

GC-MS analysis was performed using an HP 6890 Series Plus gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a MSD 5973 mass spectrometer (Agilent Technologies). Helium was used as the carrier gas at a constant flow rate of 1 mL/min; the gas chromatograph was operated in splitless mode for 1 min with the programmed temperature vaporizer (PTV) injector (Agilent Technologies) maintained at the temperature of 300 °C and equipped with a 1.5 mm i.d. multi-baffled liner (Agilent Technologies).

Chromatographic separation was performed on a 30 m × 0.25 mm, df 0.25 µm MDN-5S capillary column (Supelco, Bellefonte, PA, USA), using the following temperature program: 120 °C for 1 min, 20 °C/min up to 310°C, 310°C for 15 min.

The transfer line and ion source were maintained at the temperatures of 280 and 150 °C, respectively. Preliminarily, EI spectra in full scan mode were acquired to select diagnostic ions to be monitored in

SIM mode. Mass spectra were acquired under the following conditions: ionization energy: 70 eV; mass range: 45-400 amu; scan time: 3 scan/s; electron multiplier voltage: 2165 V. Signal acquisition and data handling were performed using the HP Chemstation (Agilent Technologies).

The *m/z* ratios of the fragment ions of the investigated compounds are reported in Table S1.

2.5. DESI-substrates

Different sample-substrates for DESI-HRMS analysis were tested, namely polytetrafluoroethylene (PTFE, Prosolia, Inc., Indianapolis, IN, USA), polylactide-based materials (PLLA) and a silica-based coating.

2.5.1. PLLA-based supporting material

2.5.1.1. Nanoparticle preparation

Two different types of nanoparticles were used: nGO (oxidized nanoparticles) and r-nGO (reduced nanoparticles), both derived from carbon spheres obtained from the microwave assisted carbonization of cellulose according to previously reported procedures [38, 39].

The carbonization process of α-cellulose was performed as follows: 2 g of α-cellulose was mixed with H_2SO_4 (0.1 mg/mL) and processed for 2 h at 180 °C excluding the ramp time of 20 min in a Milestone UltraWAVE (Milestone Inc.) under an external pressure of 40 bar. The solid fraction (carbon spheres) was obtained by filtration and dried in vacuum oven at room temperature (RT). nGO nanoparticles were produced by oxidation of the carbon spheres in HNO_3 (1:1, *w:w*) by sonication for 30 min and subsequent heating at 90 °C for 30 min under stirring. Subsequently, the acidic mixture was diluted in

DI water and removed by rotary evaporation. The final product was collected by rinsing with DI water and by freeze drying the dispersion. r-nGO were produced through a reduction process of nGO using microwave technology. nGO and CA powder were mixed (1:3, *w:w*) in DI water and treated according to the same microwave program used for the cellulose processing. The obtained product was dialyzed (MWCO 1 kDa) for 24-48 h to remove excess CA by repeatedly replacing the DI water and then collected by freeze-drying. Both nGO and r-nGO were kept in vacuum oven at RT for at least 5 days.

2.5.1.2. Film processing by solution casting

PLLA (3.6 g) was dissolved in 60 mL of chloroform at 40 °C for 2 h. Four hundred mg of nanoparticles (nGO and r-nGO, 10 % *w:w*) were dispersed in 1 mL of ethanol and sonicated using an ultrasonic bath for 5 min. The nanoparticle dispersion was then dropped into the dissolved PLLA solution while stirring for approximately 1 min. Thereafter, the dispersion was drop-casted into petri dishes (d=18.5 cm) and left to dry under a fume hood. For neat PLLA, 4 g of PLLA were dissolved in 60 mL of chloroform at 40 °C for 2 h. One mL of ethanol was added dropwise into this batch before casting into the petri dish. Films were stored in a vacuum oven at RT and named according to the polymer and nanoparticle type i.e. PLLA, PLLA nGO and PLLA r-nGO.

2.5.2. Silica-based supporting material

Two mL of TEOS were mixed with 200 µL of ethanol and sonicated for 10 min, then 400 µL of acetic acid were added and sonicated for other 10 min; finally, 1750 µL of PDMS were added and the solution was sonicated for 6 h. Subsequently, 160 µL of water were added and the mixture was sonicated for 10-15 min until a slightly opaque solution was obtained. The solution was spin-coated on

glass microscope slides with revolving speed at 2500 rpm. The supports were dried for 8 h at room temperature and then in an oven using the following temperature program: 40°C for 90 min, 1 °C/min to 350°C, 350°C for 120 min.

2.6. Characterization of the DESI substrates

The developed sample substrates (n=3 for each analysis) were characterized by atomic force microscopy (AFM), scanning electron microscopy (SEM), profilometric and contact angle measurements. AFM analyses were performed on a PARK XE-100, Park Systems (Mannheim, Germany). The images were acquired in tapping mode with a scan rate of 0.5 Hz and a 50 × 50 μm window. SEM characterization was performed on a Leica 430i instrument (Leica, Solms, Germany) operated at 25 kV. Profilometric analyses were carried out using a Talysurf CCI optical profiler (Taylor Hobson, PA, U.S.A). A 50× objective was mounted on the instrument, producing an accuracy of < 1nm in the vertical direction and a resolution of 0.4 μm in the horizontal direction. The contact-angle measurements were performed by spotting 2 μl of deionized water (3 replicates) on the tested surfaces. Static contact angle was measured by the sessile drop method and a properly calibrated goniometer to be used after the acquisition of the images of each drop. Pictures processing was carried out with the software ImageJ (National Institute of Health, USA). Finally, the dynamic contact angle was measured using a Cahn Dynamic Contact Angle analyzer (DCA-312, Cahn Instruments Inc, Cerritos, USA).

2.7. DESI-HRMS analysis

All the analyses were performed using a LTQ Orbitrap XL hybrid FTMS instrument (Thermo Finnigan, San Jose, CA, USA), equipped with an Omni Spray^{TM} (Prosolia, Inc., Indianapolis, IN, USA)

DESI source operating in positive ion mode for ketamine, mephedrone, UR-144, JWH-250, JWH-200, JWH-122, JWH-019, AM-2201, JWH-081, and in negative ion mode for HU-211, CP,47-497 and its C_8 homologous. A point-to-point oscillating acquisition mode was used. Data acquisition was performed using a Xcalibur 2.0 software in automatic gain control mode. Maximum ion injection time was 400 ms; each spectrum was recorded at 1 microscan/s. The experimental conditions are reported in Table 1.

Different spraying solvents were tested: i) H_2O:MeOH (1:1 and 3:1) and ii) H_2O:ACN (1:1 and 3:1). The sample plate was positioned on a movable stage with 1-D automatic control movement (x-axis), whereas CCD cameras were mounted on the source to visually monitor the sample position and the spray alignment.

Preliminarily, full-scan accurate mass spectra in the 150–400 amu range were acquired to determine the appropriate masses for each analyte. Identification and quantitation of target compounds was performed using the accurate mass of the analytes within a mass window of 5 ppm. Quantitation was performed by using the extracted ion chromatograms of the *m/z* value corresponding to the protonated ion for the compounds investigated in positive mode and the deprotonated ion for the analytes investigated in negative mode (Table 2).

Signal acquisition and data processing were performed using the Xcalibur 2.0 software (Thermo Finnigan).

2.8. Method Validation

Validation of both DESI-HRMS and GC-MS (SIM) methods was carried out under the optimized conditions to meet the acceptance criteria for bioanalytical method validation [40]. Briefly, LLOQs were calculated as the analyte response ≥ five times the response of the zero calibrator using

independent measurements. In order to meet the previously cited international criteria LLOQs were tested to ensure that accuracy and precision limits are met.

The calibration curves for all the investigated NPSs were constructed at six concentration levels in the LLOQ–10 mg/L range for ketamine, mephedrone, UR-144, JWH-250, JWH-200, JWH-122, JWH-019, AM-2201, JWH-081 and in the LLOQ-5 mg/L range for HU-211, CP47,497 and its C_8 homologous. Lack-of-fit and Mandel's fitting tests were performed to assess the goodness of fit and linearity, whereas the significance of the intercept (significance level 5%) was established by running a Student *t*-test.

Within-run and between-run precision were calculated in terms of CV% on 4 concentration levels, i.e. LLOQ, 1 (low level), 5 (medium level) and 10 mg/L (high level) for all the analytes except for UR-144, which was studied at LLOQ, 2.5 (low level), 5 (medium level) and 10 mg/L (high level), HU-211, CP47,497 and the C8 homologous, which were studied at LLOQ, 1 (low level), 2.5 (medium level) and 5 (high level) mg/L. Six replicate measurements *per* level were performed. Between-run precision was estimated over three days verifying homoscedasticity of data and performing the analysis of variance (ANOVA) at the confidence level of 95%.

Accuracy was calculated in terms of recovery rate (RR%) as follows:

$$RR\% = c_1/c_2 \cdot 100$$

where c_1 is the measured concentration and c_2 is the concentration calculated from the amount spiked into the sample. Recovery rate values were assessed by performing six replicated measurements per level at the same concentration levels used for the evaluation of precision.

Selectivity was evaluated by analyzing blank oral fluid samples taken from 25 males and females volunteers, testing the absence of interferences.

Finally, stability was evaluated in terms of bench-top, freeze-thaw, stock solution and long-term stability by performing 3 replicates at two concentration levels, i.e. LLOQ and 5 mg/L. More precisely,

bench-top stability was studied by maintaining the samples at ambient temperature for 6 h, whereas the long-term stability of real samples was evaluated by maintaining the spiked samples for 15 days at -18°C. The stability of the stock solution was evaluated for 10 months at -18°C, and the freeze-thaw stability was assessed by thawing the samples at ambient temperature for 2h over a 6h period.

2.9. Oral fluid sample analysis

Fourth oral fluid samples were collected anonymously during private parties from young volunteers (19-30 years old), both males and females after having signed an informed consent. All samples were collected anonymously, and only gender and age were recorded. Oral fluid was collected into polypropylene vials and maintained in the dark at -18°C until analysis.

Four hundred µL of sample were transferred into 1.5 mL vials, diluted 1:1 with MeOH and centrifuged 3 times. Fifty µL of the supernatant were submitted to MEPS procedure after the addition of the ISs at the concentration of 1 (ketamine-d_4) and 5 mg/L (carboxy-THC-d_3), respectively.

Finally, 2 µL of the eluate were deposited on the sample substrate for DESI-HRMS analysis. One µL of the extract was used for confirmatory GC-MS analysis.

3. Results and Discussion

Surface chemistry of DESI substrates plays a significant role on ion-formation yield, since it affects the adsorption/solubilization of the analytes, thus influencing the ionization of the investigated compounds and the repeatability of the analytical procedure.

In order to develop and validate a rapid MEPS-DESI-HRMS method for the detection of new psychoactive substances in human oral fluid, the performances of four sample substrates, i.e. silica-

based coating, unmodified PLLA, and PLLA substrates functionalized with 0.1% of oxidized and reduced carbon nanoparticles, respectively were evaluated. These materials were synthetized and characterized in terms of morphology and composition.

Preliminary experiments were carried out to find the best instrumental conditions for the detection of NPSs. Owing to their molecular structure, HU-211, CP,47-497 and CP,47-497 C_8 were ionized in the negative mode, whereas all the other NPSs were analyzed by operating in the positive mode. Additive addition and spray composition proved to be important parameters able to affect the DESI-HRMS responses of the investigated compounds. According to Honarvar and Venter in the case of DESI-MS analysis of proteins [41], it was observed that the addition of NH_4OH in the working solutions (10 mM) of HU-211, CP,47-497 and CP,47-497 C_8 was able to enhance their ionization efficiency in the negative ion mode. Spots were examined with several desorbing spray mixtures, testing four different solvent compositions, i.e. MeOH:H_2O 1:1 and 1:3 and ACN:H_2O 1:1 and 1:3, respectively. As shown in Figure 1**,** the best results were achieved when acetonitrile:water mixture was used at a solvent ratio of 1:1, thus obtaining a significant enhancement of the responses especially for the Spice Cannabinoid Mix 2.

3.1. DESI-HRMS sample substrates

With the aim of exploring sensitivity of DESI-MS method for the analysis of NPS compounds, the performance of the sample substrates previously described was compared with that obtained from commercially available PTFE supports in terms of signal stability and DESI-HRMS responses. PTFE slides are commonly used for DESI applications due to the high chemical inertness and hydrophobicity of the polymer. By using these substrates, weak solvent-surface and analyte-surface interactions usually

take place, thus allowing enhancement of the solubilization/desorption of analytes in the spray solvent with the final result of increasing both the DESI-MS response and signal stability.

PLLA has been recently proposed as bulk polymer for the SALDI detection of drugs [42], obtaining enhanced signal-to-noise ratios when carbon nanoparticles were embedded in the material. Embedding nanoparticles in a bulk polymeric matrix provides a means for preventing instrumental contamination phenomena, making sample preparation easier while ensuring good ionization efficiency for the analyzed drugs. In addition, embedded nanoparticles can affect the surface properties of the final substrate mainly influencing the hydrophobicity of the system. Due to the tunable surface properties, PLLA and carbon nanoparticles proved to be excellent candidates as DESI sample substrates for the analysis of NPSs. Another important advantage of the proposed materials is related to their non-toxic and environmental friendly properties, being obtained from renewable sources.

In order to compare hydrophobicity of the sample substrates tested, solid surface tension was determined from the contact angle measurements. The achieved results showed that the developed substrates were characterized by higher hydrophilicity compared to the PTFE slides since they exhibited small contact angles (< 92°), thus leading to higher wettability by both spotted solutions and DESI spray (Table 3).

AFM analysis of PLLA-based films showed an irregular surface characterized by the presence of large granules, whereas a regular distribution of the silica-based surface was observed with a maximum difference in height equal to 200 (±1) nm (Fig. 2).

SEM analysis of non-functionalized PLLA films confirmed the presence of rough surface characterized by fused polymer grains with an average thickness of 82.4 ± 7.9 μm. By contrast, a very thin coating (8.0 ± 0.5 μm) was observed in the case of silica-based coating (Fig. 3).

Profilometry data analysis evidenced a mean roughness depth of 22.8 ± 3.8 µm in the case of non-functionalized PLLA, whereas a lower value of 291.3 ± 45.3 nm was obtained for the silica-based coating, thus confirming the results achieved by AFM analysis (Fig. S1).

Finally, in order to select the most appropriate substrate for NPSs determination, preliminary experiments were carried out by spotting on each surface 2 µl of a standard solution containing the investigated analytes with concentration of 1 mg/L (Fig. 4).

As shown in the figure, non-functionalized PLLA showed the best performance in terms of both signal intensity and repeteability for ketamine, mephedrone, UR-144, JWH-019, JWH-122, AM-2201 and JWH-081, whereas HU-211, CP,47-497 and CP,47-497 C_8 could be detected only at higher concentration levels. In order to increase ionization efficiency, HU-211, CP,47-497 and CP,47-497 C_8 were detected in negative ion mode by applying the source ion fragmentation: under these conditions the best performance was obtained using the silica sample substrate.

3.2. Optimization of the MEPS Procedure

Taking into account that oral fluid is a complex matrix composed of electrolytes, blood, epithelial cells and proteins, a sample pretreatment step prior to DESI-HRMS analysis was devised in order to enhance detectability of the investigated compounds. In fact, the direct deposition of oral fluid onto the HTC slide was not feasible since the presence of a strong matrix effect did not allow the detection of the analytes.

Since the amount of oral fluid collected during controls could be very low, the MEPS technique was selected for sample clean-up and analyte enrichment using reduced sample amount. According to previous studies [34, 43], preliminary experiments were carried out to evaluate the effects of both sorbent material and eluting solvent on the MEPS extraction of five analytes belonging to different NPS classes, used as model compounds i.e. ketamine, mephedrone, UR-144, JWH-250 and JWH-081.

After extraction, the investigated compounds were analyzed by GC-MS. The achieved results demonstrate that between the two different sorbent materials tested, i.e. C_{18} and M1, the C_{18} sorbent was characterized by the highest extraction capabilities except for mephedrone and to a lesser extent for ketamine (Fig. S2).

Unlike literature studies, no washing step was performed between loading and eluting cycles, since a noteworthy decrease in the responses of the investigated compounds occurred due to the elution of the analytes from the MEPS BIN. Regarding the choice of the eluting solvents, both MeOH containing 50 mM NH_4OH (eluent A) and a mixture 78:20:2 of DCM:2-propanol:NH_4OH (eluent B) were evaluated, obtaining the highest extraction capabilities when using eluent B (Fig. S3).

Optimization of the MEPS conditions in terms of both loading and eluting cycles was carried out by running the experiments of a 2^2 FFD. The experimental domain was defined taking into account that a minimum number of both loading and eluting cycles is required to promote interactions among the analytes, the sorbent material and the eluting solvent. The minimum value of these cycles was set to 5, whereas a value of 25 was selected as maximum level for both sampling and eluting steps, thus ensuring the feasibility of the extraction for screening purposes. As for the fill/injection speed, a value of 2 arbitrary unit was used to avoid the presence of bubbles in both the filled and in the eluted solutions. The experimental error was assessed by performing 4 replicates at the center of the experimental domain. The model obtained for the investigated compounds and single desirability values are reported in Table S2. Finally, the models were used to search for the highest MEPS-GC-MS response by means of the multi-criteria method of desirability functions. The optimal experimental conditions were found in correspondence to a number of loading and eluting cycles equal to 5 and 25, respectively. Since a global desirability D=0.85 and excellent single desirability values were obtained, the developed procedure proved to be suitable for the simultaneous extraction of the investigated NPSs.

These findings demonstrate that a low number of loading cycles were sufficient to promote the adsorption of the analytes on the MEPS sorbent, whereas a greater number of eluting cycles were required to assess the complete recovery of the extracted compounds, highly retained on the C_{18} resin.

3.3. Validation of the MEPS-DESI-HRMS Method

The validation of the MEPS-DESI-HRMS method was performed by operating under the optimized extraction conditions. LLOQ values in the 0.05-0.25 mg/L range were obtained, thus demonstrating that the developed MEPS-DESI-HRMS method can be proposed for screening NPSs at low concentration levels in oral fluid. Good linearity was proved by applying the Mandel's fitting test over one or two order of magnitude for all the investigated compounds (Table 4).

As for method precision (Table 5) satisfactory results were obtained both in terms of within-run and between-run precision with CV always lower than 20%, thus meeting the criteria as described in the guidelines for the validation of bioanalytical methods [40].

According to the requirements of the guidelines for bioanalytical methods validation, accuracy calculated in terms of recovery rate provided the results reported in Table 6. RR% in the 89(±6)-115(±5)% range at the LLOQs and in the 83(±8)-120(±2)% range at the other concentration levels were obtained, thus proving extraction efficiency and accuracy of the developed method.

Good selectivity was also observed, since the analysis of blank saliva samples taken from more than 20 volunteers did not show the presence of interferences.

As for stability, long-term stability of the stock solutions was demonstrated, since ANOVA performed on data obtained by the analysis of standard solutions daily prepared from the stock solutions did not show significant differences (p>0.05) up to 10 months when the stock solutions were stored at -18°C. A similar behavior was observed for the long-term stability of the oral fluid samples: no significant

differences in the responses were obtained for all the investigated NPSs when the spiked samples were maintained for 15 days at -18°C.

Bench-top stability was also proved by analyzing standard solutions maintained at room temperature up to 6 h. By applying the student *t*-test, no significant differences ($p>0.05$) were observed between the mean responses. Finally, as for the freeze-thaw stability, ANOVA did not show significant differences among the calculated mean values ($p>0.05$) by thawing the samples at ambient temperature for 2 h over a 6h period.

In order to investigate reliability of the developed method, 40 oral fluid samples collected during private parties were analyzed. Only in one sample out of 40 the presence of mephedrone was detected at the concentration of 5.81 ± 0.33 mg/L (n=3).

The results achieved were further verified by analyzing the same samples with a MEPS-GC-MS (SIM) method developed and validated (Tables S2-S4) for confirmatory purposes.

Good agreement among the results achieved using the MEPS-DESI-HRMS screening method and the confirmatory method was observed. In the case of the positive sample containing mephedrone, no significance differences between the mean results were obtained ($p>0.05$), thus proving reliability of the devised method for screening and quantification of NPSs in oral fluid.

Conclusions

Advances in the determination of new psychoactive substances in oral fluid are proposed in terms of new sample substrates for DESI-HRMS. The best performances were obtained when using both the silica-based coating and the non-functionalized PLLA**,** thus allowing the rapid screening of the investigated analytes in few minutes. Being a valid alternative to more laborious approaches commonly used in forensic labs**,** the devised method proved to be suitable for high-throughput qualitative and

quantitative purposes. The combination of MEPS as a miniaturized sample treatment technique with DESI-HRMS proved useful for the rapid screening of NPSs in oral fluid. On the other hand, since isomers cannot be resolved by operating under the developed conditions, a further improvement could be represented by the use of mass spectrometers able to combine ion mobility separations with high resolution MS, providing a valuable tool to distinguish overlapped isobaric compounds, thus enhancing method selectivity.

Declaration of interest: none

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Supplementary data

Information regarding the operating conditions, the characterization of the proposed sample surfaces and validation data of the GC-MS (SIM) method are provided in the supporting material.

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Figure captions

Fig. 1. Effect of the spray composition on the DESI-HRMS responses of the investigated NPSs

Fig. 2. AFM images of the non functionalized PLLA (top) and silica-based coating (bottom): optical microscopy view (left) and error-signal images of surface morphology (right)

Fig. 3. SEM micrographs of the non-functionalized PLLA (left) and silica-based coating (right)

Fig. 4. DESI-HRMS responses of NPSs (1 mg/L) spotted on different sample substrates. Positive ionization mode for ketamine, mephedrone, UR-144, JWH-250, JWH-200, JWH-019, JWH-122, AM-2201, JWH-081; negative ionization mode for HU-211, CP,47-497 and CP,47-497 C_8

Table 1

DESI operating conditions

	Positive ion mode	Negative ion mode
solvent flow (μL/min)	1	2
spray voltage (kV)	4	-3.5
tube lens voltage (V)	100	100
capillary voltage (V)	15	15
capillary temperature (°C)	250	250
nitrogen pressure (bar)	8.5	8.5
incident angle (°)	50	50
collection angle (°)	40	40
tip-to-surface distance (mm)	1.7	1.7
inlet-to-surface distance (mm)	0.5	0.5
tip-to-inlet distance (mm)	3.6	3.6
spray tip length (mm)	1	1
source ion fragmentation (V)	-	35
sampling substrate	PLA	TEOS
additive	-	NH_4OH

Table 2

Monitored ions for quantitative analysis and corresponding exact *m/z* ratio values

Compound	Ion	Theoretical *m/z*	Measured *m/z*	Δm *(ppm)*
Ketamine	$[M+H]^+$	238.09959	238.09921	0.62
Ketamine d_4	$[M+H]^+$	242.12443	242.1483	0.66
Mephedrone	$[M+H]^+$	178.12267	178.12267	0.12
UR-144	$[M+H]^+$	312.23257	312.23199	0.23
JWH-250	$[M+H]^+$	336.19666	336.19666	0.25
JWH-200	$[M+H]^+$	385.19073	385.19073	0.28
JWH-122 and JWH-019	$[M+H]^+$	356.20157	356.20157	0.27
AM-2211	$[M+H]^+$	360.17566	360.17566	0.26
JWH-081	$[M+H]^+$	372.19556	372.19556	0.28
HU-211	$[M]^-$	385.28155	385.27451	0.79
CP,47-497	$[M]^-$	317.24751	317.24829	0.78
CP,47-497 C_8	$[M]^-$	331.26316	331.26385	0.69
THC-COOH d_3	$[M]^-$	346.20922	346.20975	0.53

Table 3

Contact angle values (standard deviation in parenthesis)

Material	Contact angle±st.dev. (°)
PTFE	131 (±1)
PLLA	72 (±1)
PLLA nGO	70 (±1)
PLLA r-nGO	83 (±4)
Silica-based coating	92 (±1)

Table 4

LLOQs, linearity and regression coefficients of the MEPS-DESI-HRMS method

Compound	LLOQ (mg/L)	Range (mg/L)	a (±st.dev.$_a$)*
Ketamine	0.05	LLOQ - 10	0.860 (± 0.013)
Mephedrone	0.05	LLOQ - 10	0.819 (±0.010)
UR-144	0.5	LLOQ - 10	1.562 (±0.014)
JWH-250	0.25	LLOQ - 10	2.560 (±0.044)
JWH-200	0.25	LLOQ - 10	0.303 (±0.006)
JWH-019 and JWH-122	0.25	LLOQ - 10	2.612 (±0.055)
AM-2201	0.25	LLOQ - 10	0.709 (±0.015)
JWH-081	0.25	LLOQ - 10	1.724 (±0.022)
CP,47-497	0.25	LLOQ - 5	2.923 (± 0.075)
CP,47-497 C_8	0.25	LLOQ - 5	2.723 (±0.055)
HU-211	0.25	LLOQ- 5	1.174 (±0.023)

*Regression equation: y=ax

Table 5

Within-run and between run precision (CV%) of the MEPS-DESI-HRMS method

Compound	Within-run* (CV%)				Between run* (CV%)			
	LLOQ	Low level	Medium level	High level	LLOQ	Low level	Medium level	High level
Ketamine	3.2	3.0	4.2	3.0	9.6	8.2	9.0	7.3
Mephedrone	10.4	7.2	6.3	4.9	19.0	12.3	7.1	5.1
UR-144	15.9	12.8	10.6	1.5	18.8	15.0	13.2	9.4
JWH-250	5.0	4.1	6.1	1.7	15.6	12.9	10.7	9.3
JWH-200	9.1	11.3	10.7	12	19.4	14.8	12.9	14.5
JWH-019/JWH-122	11.2	6.7	5.3	2.2	19.0	11.8	10.1	9.6
AM-2201	11.3	8.4	6.6	1.2	18.7	10.0	8.4	6.2
JWH-081	1.4	2.8	2.9	3.2	19.0	13.6	10.5	9.4
CP,47-497	8.1	9.4	10.5	9.9	8.8	12.8	9.6	12.3
CP,47-497 C_8	5.5	10.4	6.1	7.3	16.3	10.3	9.9	11.9
HU-211	6.7	9.8	8.3	6.1	19.9	14.7	14.2	13.7

*low level: 1 mg/L, medium level: 5 mg/L, high level: 10 mg/L for all the analytes except for UR-144 (low level: 2.5 mg/L; medium level: 5 mg/L and high level: 10 mg/L), HU-211, CP47,497 and the C8 homologous (low level: 1 mg/L; medium level: 2.5 mg/L; and high level: 5 mg/L).

Table 6

RR% (n=6) of the MEPS-DESI-HRMS method

Compound	Recovery Rate% (±s.d.)			
	LLOQ	Low level	Medium level	High level
Ketamine	108 (±2)	120 (±2)	105 (±4)	99 (±3)
Mephedrone	96 (±3)	102 (±1)	100 (±2)	100 (±5)
UR-144	102 (±2)	101 (±2)	101 (±6)	100 (±1)
JWH-250	95 (±1)	85 (±4)	88 (±2)	100 (±2)
JWH-200	90 (±3)	87 (±2)	91 (±8)	100 (±1)
JWH-019/JWH-122	96 (±2)	101 (±2)	100 (±3)	100 (±2)
AM-2201	89 (±6)	83 (±8)	88 (±3)	100 (±1)
JWH-081	90 (±3)	86 (±1)	93 (±4)	100 (±1)
CP,47-497	107 (±6)	115 (±1)	114 (±2)	105 (±12)
CP,47-497 C_8	115 (±5)	113 (±4)	111 (±3)	99 (±7)
HU-211	94 (±4)	87 (±6)	103 (±8)	100 (±6)

Figure 1

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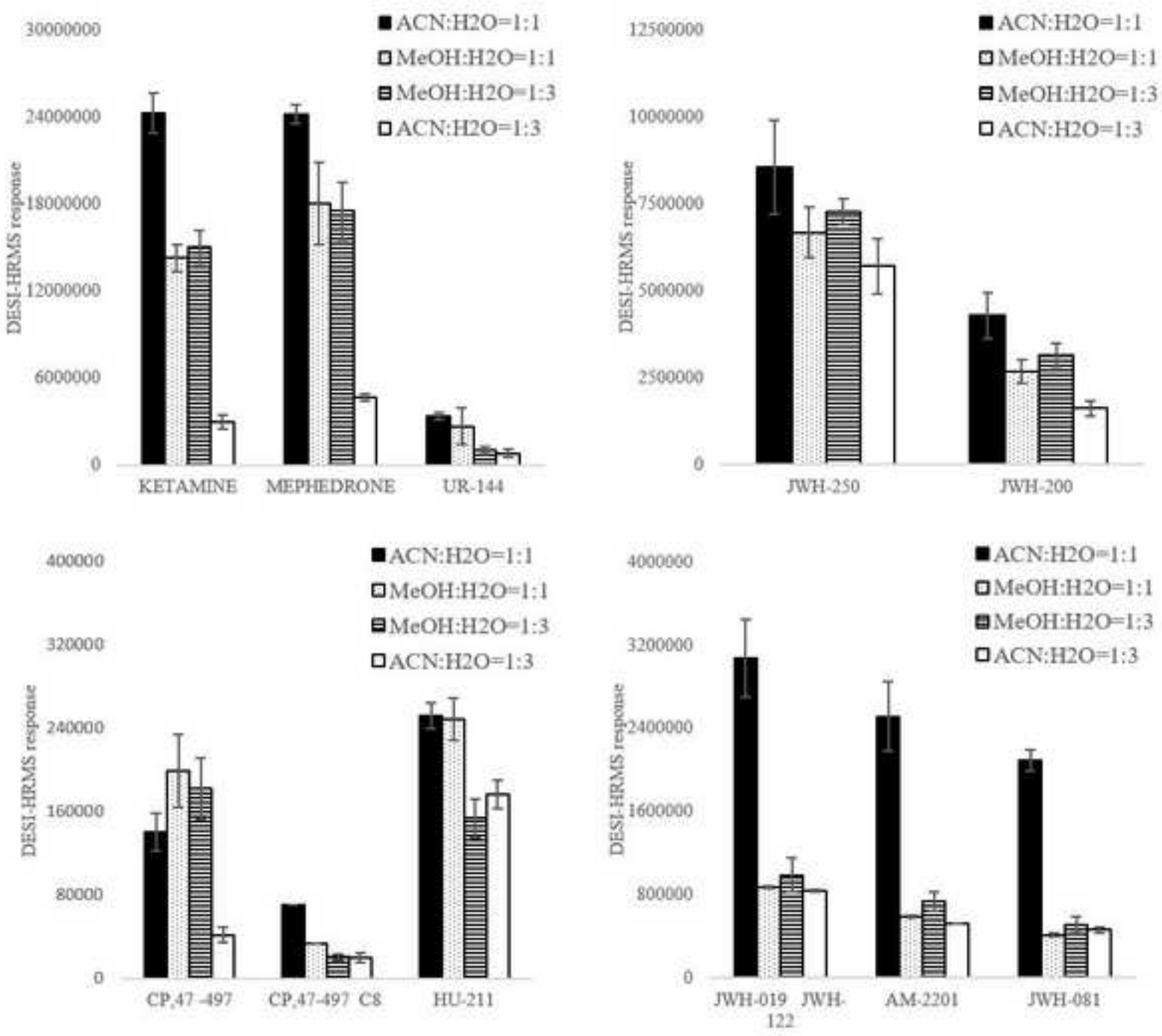

Figure 2

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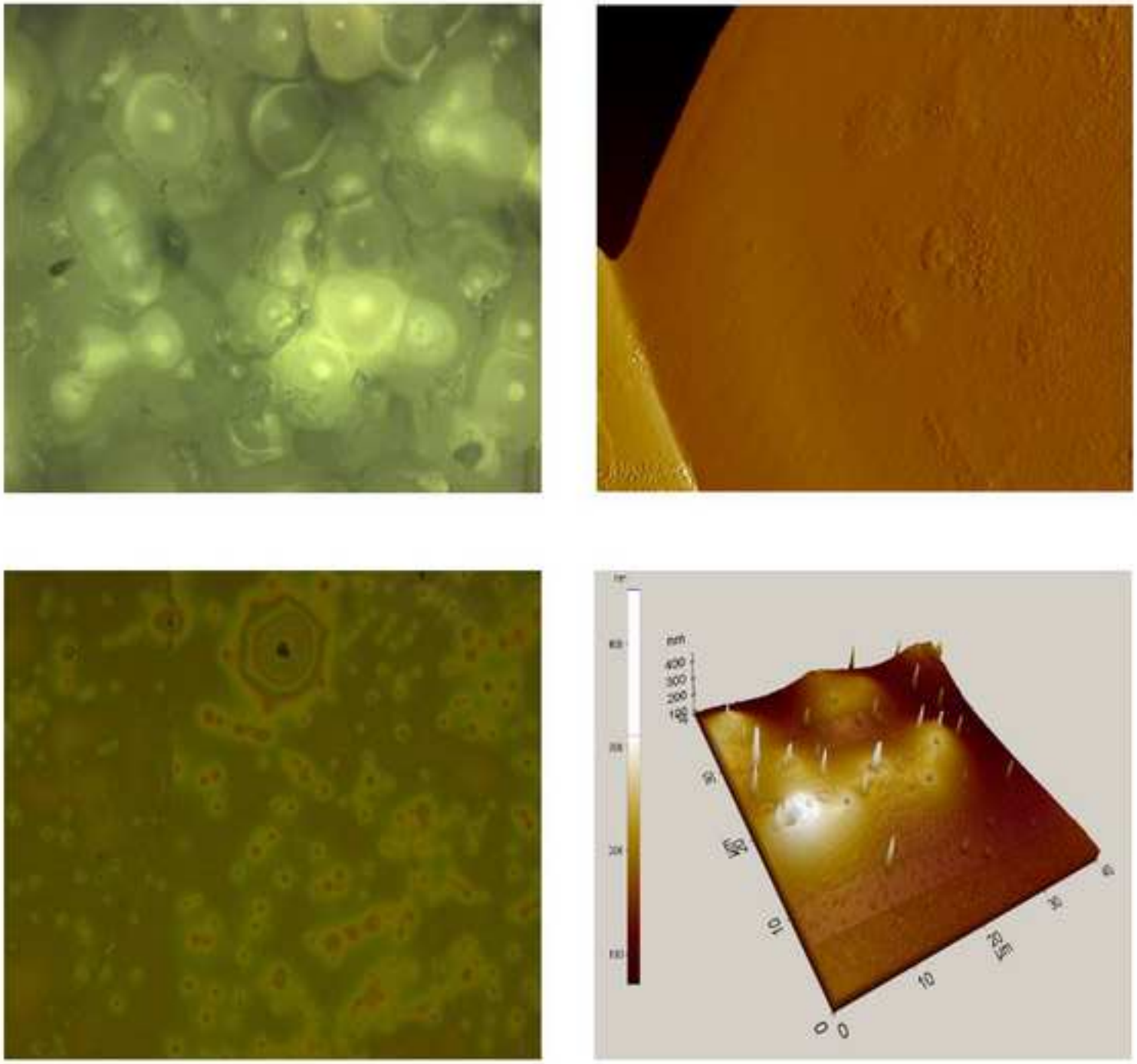

Figure 3

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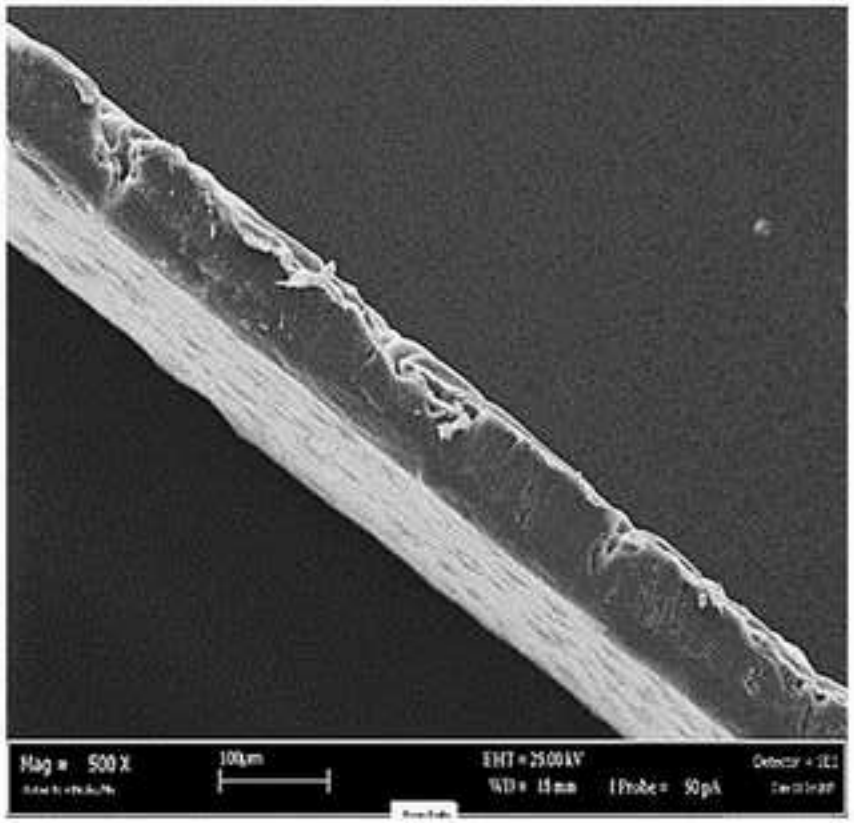

Figure 4

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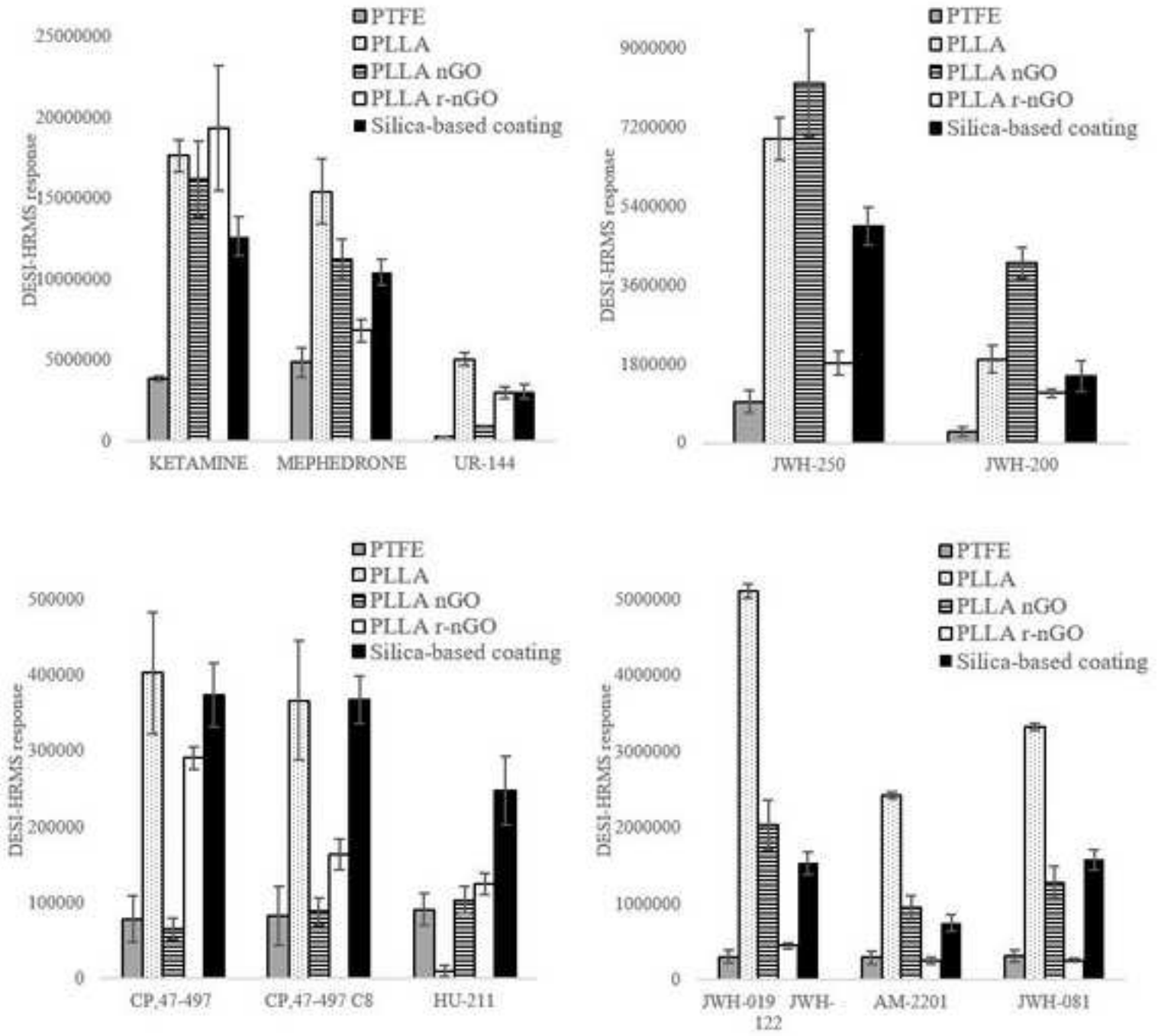

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*List of Three Potential Reviewers

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Supplementary Material

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*Graphical Abstract (for review)

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