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High-Throughput and Rapid Screening of Drugs of Abuse in Saliva by Multi-Segment Injection Using Solid-Phase Microextraction-Automated Microfluidic Open Interface-Mass Spectrometry

Wei Zhou,
Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Emir Nazdrajić,
Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Janusz Pawliszyn
Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Abstract

There is great demand for analytical methods capable of providing high-throughput and rapid screening, especially for anti-doping and clinical point-of-care applications. In this work, automated microfluidic open interface-mass spectrometry (MOI-MS) was used for coupling with high-throughput, automated solid-phase microextraction (SPME) to achieve this objective. The design of the MOI-MS interface provides a continuous and stable electrospray fluid flow to the MS without introducing any bubble, a feature that we exploit to introduce the concept of multi-segment injection for the determination of multiple samples in a single MS run. By eliminating the need to start a new MS run between sample assays, the developed approach provides significantly simplified protocols controlled by programmed software and increased reproducibility. Furthermore, the biocompatible SPME device, which utilizes coating consisting of hydrophilic–lipophilic balanced particles embedded in a polyacrylonitrile (PAN) binder, can be directly used for biological sample analysis, as the PAN acts as both a binder and a matrix-compatible barrier, thus enabling the enrichment of small molecules while eliminating

Corresponding Author: **Janusz Pawliszyn** – *Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada*; janusz@uwaterloo.ca.

Author Contributions

The manuscript was composed through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

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Chemicals and materials; methods; MS parameters (Table S1); intra-day and inter-day RSD% (Table S2); the validation data in saliva samples from three different volunteers (Table S3); the photo of the automated SPME system and SPME fiber holder (Figure S1); the design of the MOI-MS interface (Figure S2); the MS spectrograms of multi-segment injection using the automated-MOI-MS system (Figure S3); the influence of desorption solvents to the peak area (Figures S4 and S5); the carryover of analytes with different desorption solvents and the MS intensity of different analytes by direct infusion with different solvents (Figure S6); the influence of desorption time, sample dilution, washing time, and extraction time to the peak area (Figures S7–S10); the calibration curves for the analysis of DOAs in saliva samples (Figures S11–S13) (PDF)

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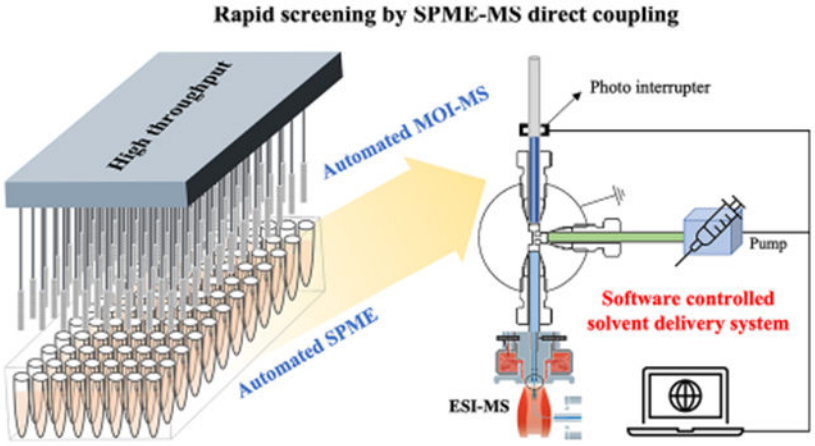
interferences associated with the presence of interfering macromolecules. The above design was employed to develop a fast, quantitative method capable of analyzing drugs of abuse in saliva samples in as little as 75 s per sample. The findings indicate that the developed method provides good analytical performance, with limits of detection ranging between 0.05 and 5 ng/mL for analysis of 16 drugs of abuse, good calibration linear correlation coefficients ($R^2 \geq 0.9957$), accuracy between 81 and 120%, and excellent precision (RSD% < 13%). Finally, a proof-of-concept experiment was performed to demonstrate the method's suitability for real-time analysis in anti-doping applications.

Graphical Abstract



Mass spectrometry (MS) is a powerful analytical tool that is frequently used for bio-sample analysis in the biomedical, clinical, and anti-doping fields, as it provides sensitive detection and highly selective molecular information about target analytes based on their molecular weights and chemical structures.[1–3] In such applications, MS is normally coupled with techniques such as gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) to achieve the separation of the complex bio-samples under study.[4–6] At present, the LC–MS method is the gold standard for therapeutic drug monitoring and metabolism studies.[7] However, chromatography-based MS methods require tedious sample preparation and time-consuming separation processes. As a result, analytical techniques capable of providing high-throughput and rapid screening—and even on-site analysis—remain in high demand for anti-doping and clinical point-of-care (POC) applications.

Recently, researchers have developed direct/ambient MS techniques with the aim of eliminating the tedious and time-consuming chromatographic separation process, thereby enabling sample analysis within mere minutes.[8,9] New direct MS technologies like nanoelectrospray ionization-mass spectrometry (nanoESI-MS), open port probe sampling interface (OPPI), and ambient MS (AMS) technologies such as direct analysis in real time (DART), desorption electrospray ionization (DESI), and laser ablation electrospray ionization (LAESI) have dramatically reduced analytical turnaround times and have become a major research focus in many fields.[10–14] The features of direct/ambient MS, such as fast screening speeds, highly specific analytical results, and on-site capability, make

these technologies suitable for POC analysis during surgery or on-site anti-doping tests during competitions.[15,16] However, the direct analysis of real samples, especially complex biological samples, is often hampered by inadequate sensitivity, high background noise, and cumulative MS contamination.[17,18] As such, the selection of a suitable sample-preparation method is critical, as effective sample preparation can solve the above issues, while also influencing the total speed and throughput of the method.

Solid-phase microextraction (SPME) is a simple and effective sample-pretreatment method that combines sampling, extraction, cleanup, and enrichment into a single step.[19] With biocompatible SPME devices, the extraction phase consists of a small amount of high-capacity sorbent embedded in a polyacrylonitrile binder, which forms a thin film that acts as a barrier that enables the enrichment of small molecules while eliminating interferences due to the presence of cells and macromolecules, thus significantly decreasing matrix effects.[20,21] The direct coupling of SPME and MS has emerged as a good solution to the above problems and has proven effective for analyzing trace amounts of target compounds in complex bio-matrices.[22,23] In the past few years, a variety of SPME-direct MS interfaces have been developed, including SPME-nanoESI-MS, SPME-DART-MS, coated blade spray-MS (CBS-MS), and SPME-microfluidic open interface-MS (MOI-MS).[24–28] Conceptually, SPME-MOI involves flow-isolated desorption followed by the direct injection into a MS with an atmospheric pressure ionization source. MOI design has been shown to exhibit excellent sensitivity due to the small volumes (about 10 μL) of desorption solution required and its ability to prevent electrospray instability by maintaining constant liquid flow for ionization.[27,29]

In this work, we couple a recently developed software-controlled automated MOI-MS with SPME to achieve high-throughput and rapid screening.[30] The Concept 96 SPME system was employed to enable the automatic and simultaneous handling of 96 samples with an average sample-preparation time of about 25 s, while an automated MOI-MS interface, which used programmed software to control the solvent delivery system, was used to directly couple SPME to MS for fast analysis (50 s for each sample). The automated MOI-MS system's ability to provide a continuous and stable liquid flow to the MS without introducing any bubbles was harnessed to develop the new concept of multi-segment injection, which enabled the analysis of multiple samples in one MS run, thus dramatically decreasing the turnaround time and reproducibility of MS detection. These components, along with an extraction phase consisting of biocompatible hydrophilic–lipophilic balance (HLB), were combined to create an SPME-MOI-MS method for simultaneously determining 16 performance-enhancing drugs in saliva samples. Tests were conducted to optimize the experimental parameters and evaluate the method's linearity, reproducibility, sensitivity, precision, and accuracy, and the method was validated using spiked human saliva samples from three different subjects. All obtained results indicated that the developed method provided good analytical performance. Finally, a proof-of-concept experiment demonstrated the method's capability for the real-time detection of threshold levels of the targeted substances in saliva, thus affirming its potential for on-site anti-doping testing during competitions.

EXPERIMENTAL SECTION

Chemicals and Materials.

See the Supporting Information.

Sample Preparation and Concept 96-Fiber-Based SPME.

Stock solutions of all standards and internal standards were made by dissolving them in methanol or acetonitrile at concentrations of 1 mg/mL (standards) and 0.1 mg/mL (internal standards). For method optimization, the artificial saliva was spiked with preselected amounts of standards; for method development, the artificial saliva was spiked with standards and internal standards. All internal standards were spiked at a concentration of 5 ng/mL, except for cocaine-d_3 (1 ng/mL), codeine-d_3 (50 ng/mL), and morphine-d_3 (10 ng/mL). The spiked saliva was diluted three times with PBS solution (pH 7.4). The method validation experiments were conducted using human saliva samples from three health volunteers who had not taken any targeted drugs. The saliva samples were collected by having the volunteers spit into a glass vial. After the samples had been spiked with standards and internal standards, they were incubated for 1 h at 25 °C to allow the analytes to bind with the matrix. No other sample-preparation methods, such as centrifugation or filtration, were required.

For Concept 96-fiber-based SPME, the conditioning, extraction, and washing steps were carried out automatically using 96-well plates. Photos of the system are shown in Figure S1. The sample (1.5 mL) was added to the well plate for extraction, the bio-SPME fibers were immobilized into the Concept 96-fiber holders (made in the University of Waterloo Science Technical Services), and the agitator speed was set to 1500 rpm. The extraction time and washing time used in the SPME procedure were optimized, resulting in the following final conditions: conditioning for 20 min; extraction for 20 min; and washing for 5 s. After the automatic extraction process, the SPME fibers were subjected to the MOI-MS/MS step.

Automated MOI-MS/MS Analysis.

The automated MOI-MS/MS system (Figure 1) was used for the desorption, injection, and MS detection steps. The MOI-MS/MS analysis for each sample consisted of three steps: (1) desorption, (2) injection, and (3) washing. The liquid delivery system was automated and controlled using homemade software programmed with Microsoft Visual Studio. The design and process of the liquid delivery system are detailed in the Results and Discussion section. The fiber with extract analytes was put into the MOI desorption chamber, where it was desorbed for 10 s with a mixture consisting of methanol/acetonitrile/water (v/v/v, 7/2/1) + 0.1% formic acid. After desorption, the solvent was injected into the MS by suction flow for analysis, and the MS peak was used for quantitative analysis. The details about method development for SPME-MOI-MS/MS analysis are shown in the Supporting Information.

RESULTS AND DISCUSSION

High-Throughput SPME with Automated MOI-MS/MS.

Sample preparation is always the most time-consuming step in the analytical workflow. Consequently, there is great demand for high-throughput and automated methods, as such approaches can reduce the total analysis time and increase reproducibility. SPME uses small amounts of solid coating material on different substrates, such as fibers, blades, or mesh, which always have reproducible dimensions and can be easily processed in large numbers with a suitable holder unit. In this work, the SPME procedure was performed using a Concept 96-fiber system (Figure S1) featuring a custom-designed fiber holder and a commercially available 96-well plate, which allowed up to 96 samples to be processed simultaneously. The automated workflow consisted of three steps: (1) conditioning (20 min) (the 96 bio-SPME fibers were conditioned with a methanol/water (v/v, 1/1) solution to activate the extraction phase), (2) extraction (20 min) (due to the biocompatible characteristics of the HLB-PAN coating material and the open-bed extraction nature of SPME, the SPME fibers can be immersed directly in bio-fluidic and/or tissue samples for extraction without any other tedious sample-preparation methods such as centrifugation and/or filtration), and (3) rinsing with water (10 s) (a quick rinse with water to eliminate the nonspecific attachment of salts or other residues on the coating surface). The use of the Concept 96 system allows the above three steps to be performed automatically with an auto arm, resulting in a total analytical time of approximately 25 s for each sample in a high-throughput fashion for 96 samples.

It is much easier to automate SPME-MS direct coupling methods, as the direct/ambient MS interface is always in an open-to-air environment. As can be seen in the MOI-MS interface diagram shown in Figure 1 and Figure S2, the design used in this work employed an inexpensive and simple three-port tee instead of the complex coaxial design used previously.[27] The three ports of the tee were connected to the capillaries of the syringe pump (P1), the ESI source of the MS (P2), and the desorption chamber (P3). The MOI-MS interface works based on the concept of flow-isolated desorption. In this concept, the ESI source employs nebulizing gas to enhance ionization efficiency and to provide constant liquid flow inside the spray capillary via the Venturi effect (flow rate of P2, around 120 μL/min in the present experiment configuration). If the flow rate from the syringe pump (P1) is the same at P2, the liquid level in the desorption chamber will remain constant during desorption; if the flow rate of P1 > P2, the liquid level at P3 will increase (during refilling and washing); if the flow rate of P1 < P2, the liquid in P3 will decrease, and the solvents will be injected to the MS (during injection). Each sample analysis cycle contains three steps. (1) desorption (10 s) (the desorption chamber is filled with solvent; the flow rate of P1 = P2 to ensure that the liquid in the desorption chamber remains constant; and the SPME fiber is inserted into the chamber for desorption), (2) injection (3 s) (the syringe pump is stopped and the desorption solution containing the analytes is quickly injected into the MS due to the suction flow in P2), and (3) washing and refilling (15 s) (when the liquid level has been decreased to the bottom of the chamber, the syringe pump restarts with a high flow rate of 300 μL/min to over-flow in order to wash the desorption chamber and remove any carryover in the three-port tee and connection tube; after the washing step, the flow rate is

decreased to match the flow rate of P2; this ends one sample analysis cycle and indicates the interface's readiness for the following sample). In this study, programmed software was used to control the delivery of solvents into the MOI-MS interface via a programmable syringe pump and the detection of liquid flow by a photo interrupter located at the bottom of the desorption chamber. The photo interrupter can recognize the flow of liquid into the semitransparent PTFE tubing by detecting the adsorption value of the infrared ray (IR) and providing a signal to the data acquisition driver connected to the computer. At the beginning of the analysis, the desorption chamber is filled, and the syringe pump flow rate is equal to the suction flow. After inserting the SPME fiber and pressing the start button, the flow rate was maintained for 10 s and the fiber was then removed and the syringe pump was stopped. When the liquid level in the chamber reached the photo interrupter, the infrared ray adsorption signal decreased. This decrease in the IR light received by the transistor decreased the current produced, which was converted by the data drive to voltage and then read by the computer communication port to the software. Next, the software restarted the syringe pump with a flow rate of 300 μL/min to over-wash the chamber for 15 s and then adjusted it to match the suction flow rate. At this point, the chamber was ready for the next desorption and injection cycle. The software can calculate the suction flow rate, as the volume of the desorption chamber was constant, and the time used for the liquid level going from the top to the bottom chamber was recorded by the computer. Besides being simple and automated, the above design is advantageous because it provides a continuous and stable liquid flow to the MS, thus guaranteeing that no bubbles will be introduced. Including the handling of the SPME fiber and the MOI-MS process, the total analysis time for each sample was about 50 s. The total analysis time for each sample, including sample preparation and MS analysis, required about 75 s per sample. Although the SPME fiber was manually transferred from the holder to the MOI interface in this work, it is possible to fully automate the SPME-MOI-MS workflow by using an auto arm—which has already been commercialized in automated SPME-GC–MS instruments—to transfer the 96-fiber holder unit.[31]

Multi-Segment Injection Using High-Throughput SPME Coupled with Automatic MOI-MS.

In most SPME-MS direct coupling technologies, such as SPME-nanoESI-MS, CBS-MS, and SPME vibrating sharp-edge spray ionization (VSSI)-MS, sample injection is performed separately, as there is no continuous flow and/or signal to the MS.[24,32,33] A new sample run should be started on the MS end for another sample analysis cycle, with voltage only being applied during sample introduction. These operations are tedious and could cause higher RSD%, as MS conditions might shift when the high voltage and/or the gas flow needs to be turned on/off. Taking advantages of the automatic MOI-MS interface, including its ability to provide continuous liquid flow to the MS, its small open-to-air desorption chamber, and its repeatable auto-cycle, a new concept of multi-segment injection that enables the analysis of multiple samples in a single MS run without requiring the MS to be switched on/off was developed. The constant MS environment provided by the ESI-MS interface ensured that the handling of SPME fibers, either manually or using the auto arm, will not influence the constant flow of liquid to the MS. The MS run time was adjusted based on how many samples needed to be analyzed. This new concept, which relies on an automated design, provides excellent reproducibility, as the MS can be run without adjusting conditions such

as voltage, gas flow, and electronic contact. As shown in Figure 2 and Figure S3, the overall RSD% of the peak area of buprenorphine from 9 saliva samples (without calculation via internal standards) was 7%, which is incredible when compared to other SPME-MS or AMS methods. The multi-segment injection concept also improved the method's throughput and simplified its operational flow by eliminating the need to operate the MS instrument during the sample analysis process actively, thus resulting in an average MOI-MS analytical time of around 50 s for each sample.

Application in the Quantitative Analysis of DOAs in Saliva for Anti-Doping Testing.

Despite the variety of analytical protocols available for anti-doping applications, there is still considerable demand for methods capable of providing simple, fast, efficient analysis of alternative matrices and standard ones like blood and urine.[34] As an extracellular fluid, saliva has received particular interest for use in clinical applications, as it reflects the concentration of substances in the blood through passive diffusion and/or the ultrafiltration of chemicals from the blood vessels in the salivary glands to saliva.[35] Saliva is especially appealing for the analysis of drugs of abuse, as it can be collected noninvasively and on-site, in addition to minimizing the potential for adulteration due to the ability to supervise collection without compromising the athlete's privacy.[26] Nevertheless, saliva analysis is challenging, as access to large volumes is somewhat restricted, and its properties (pH, water and mineral content, viscosity, etc.) can vary due to the strong influence of diet, lifestyle, and drugs, among other factors.[34] Numerous sample-preparation methods, including SPME, solid-phase extraction, single-drop microextraction, and dried saliva spot extraction, have been coupled with different separation and detection techniques in an attempt to solve the above issues.[36] In this work, the developed SPME-MOI-MS technique was applied to achieve the high-throughput quantitative analysis of DOAs in saliva samples using a matrix-match calibration curve. The method optimization, development, and validation are described below.

To optimize the method's ability to analyze performance-enhancing drugs in saliva samples, various parameters impacting the SPME and/or MOI-MS procedures were investigated, including the desorption solution, desorption time, saliva dilution, washing time, and extraction time. The results of these tests are shown in Figures S4 to S10. Here, it is important to emphasize several points. First, unlike SPME-LC–MS, the desorption solvent used in MOI-MS also acted as a mobile phase. As such, it influenced not only the desorption efficiency but also the ionization efficiency. As shown in Figure S4, the methanol with 0.1% formic acid provided better desorption efficiency for most of the analytes but worse ionization efficiency when compared with methanol/ACN/water 8/1/1 + 0.1% formic acid as the solvent. In addition, the suction flow rate can be influenced by the solvent's viscosity; for example, the addition of isopropanol, which has a high viscosity, decreased the suction flow rate. Considering the overall peak areas, especially for these low-sensitivity compounds, a desorption solvent consisting of methanol/ACN/water 7/2/1 + 0.1% formic acid was selected. Second, since the viscosity and pH value of saliva can differ widely based on the donor, collection time, and collection method, the dilution of the samples with PBS buffer (pH 7.4) can increase the method's reproducibility while also retaining desirable sensitivity. In this experiment, the saliva samples were diluted three times with

PBS buffer. Third, since the amount of extracted analytes increases alongside the extraction time, the selection of an optimal extraction time depends on the sensitivity required by the application. For example, according to the WADA, the threshold of morphine is 1 μg/mL; with this requirement, a relatively short extraction time of 1 min is enough to provide an acceptable limit of detection value.[37] As shown in Figure S10, the extraction rate for most of the compounds slowed or plateaued after 20 min. Since the method's sensitivity had already been shown to be acceptable at this time point, 20 min was selected as the optimal extraction time for the main experiments. The optimum analytical conditions are described in the Experimental Section.

Matrix-compatible calibration curves for quantitative analysis were developed using artificial saliva spiked with standards and their respective internal standards at different concentrations. The results of these tests are shown in Figure 3 and Figures S11–S13 and Table 1. Each calibration point represents four replicate tests. The LOD was defined as the peak height that was at least three times higher than the blank saliva sample without spiking, and the LOQ was defined as the peak height at least 10 times higher than the blank saliva sample without spiking with the RSD% of the peak area being less than 20%. Good linearity was observed for all compounds, ranging from the LOQs to 100 ng/mL. The exception to this trend was fentanyl (highest point was 50 ng/mL), as the higher concentration level of this compound saturated the MS. The method showed good sensitivity, with LODs ranging from 0.01 to 1 ng/mL, and excellent linearities with R^2 larger than 0.9957. The intra-day and inter-day RSD% are shown in Table S2; the method showed intra-day RSD% (n = 5) ≤12% calculated by the raw peak area and ≤9% calculated by the peak area ratio to IS. The inter-day RSD% (n = 3) was ≤14% calculated by the raw peak area and ≤13% calculated by the peak area ratio to IS. Since saliva is still not a validated matrix for anti-doping tests by the WADA, there are no standard criteria for the detection limits of these drugs of abuse in this matrix. Therefore, we compared our LOQs with the WADA's minimum required performance levels (PRPLs) for the analysis of these substances in urine samples. The sensitivity of our method satisfied these requirements (e.g., WADA, 2 ng/mL fentanyl; our LOQ, 0.05 ng/mL; WADA, 5 ng/mL buprenorphine; our LOQ, 5 ng/mL).[38]

To validate the method, saliva samples collected from three volunteers who had not taken any of the targeted drugs were spiked with the standards at concentrations of 3, 30, and 75 ng/mL. As shown in Table S3, the method provided acceptable accuracy, with recoveries between 81 and 120% and good precision with RSD% less than 13%.

Real-Time Detection of the Threshold Substance in Saliva: Proof-of-Concept and Outlook.

Applications such as anti-doping testing during competitions or pharmacokinetics studies during surgery require real-time analysis with simplified operational and data analysis processes. In the MOI-MS system, the peak width of the target compound depends on the suction flow rate and the volume of the desorption chamber; however, it is not related to the concentration level like in chromatography-based methods. Therefore, with the same instrumental setup and solvent, the peak width should be the same, and the concentration level can be directly inferred from the peak height, which is visible on the real-time MS spectrum without using post-run software. Taking advantage of this feature and the matrix-

compatible SPME method, a visible detection method that does not require data processing in the post-run can be applied for real-time analysis.

Consequently, a proof-of-concept experiment was designed to show the developed method's potential as a rapid anti-doping test. For threshold substances such as morphine (threshold concentration: 1 μg/mL in urine), quickly evaluating whether the athlete is "positive or negative" is more challenging than with non-threshold substances, which only register as "have or not". As shown in Figure 4, we applied the SPME-automated MOI-MS using blank saliva spiked with a threshold concentration of 1 μg/mL as the quality control (QC) sample. The extraction of the test samples and QC sample can be performed simultaneously using the automated SPME system, and they can be analyzed in one single MS run using multi-segment injection. The visible data was shown in real time as the MS spectra with different peak heights. In the future, this strategy could also be investigated for use in the real-time monitoring of therapeutic drug concentrations with in vivo SPME technology, as the operator simply needs to inject the lowest and highest concentration levels in the therapeutic range after SPME extraction at the beginning of the MOI-MS run and analyze the drug concentration later in a sequence during the therapy. The real-time MS signal height can directly reflect whether drug concentration is still in the therapeutic range.

CONCLUSIONS

In this work, a multi-segment injection strategy using the automated MOI-MS system was developed that enables the development of a high-throughput and semi-automated analytical method for the analysis of prohibit drugs in a complex sample saliva matrix using the biocompatible SPME coating. The proposed method can also be extended for the analysis of plasma and urine samples, as the biocompatible SPME coating material can also be used for direct extraction from these matrices without pretreatment. The multi-segment injection strategy allows the detection of multiple samples in a single MS run with constant and stable MS conditions, thereby ensuring good reproducibility. Furthermore, the MOI-MS was coupled with an automated Concept 96-fiber system to create a quantitative method for the fast screening of 16 drugs of abuse in saliva samples that could provide satisfactory analytical performance. Finally, a proof-of-concept experiment demonstrated the proposed method's ability to provide anti-doping testing in real time during competition. Future research could focus on fully automating the SPME-MOI-MS system with an auto arm to precisely control the 96 fibers or to pick up and deliver them individually to the MOI interface. In addition, although the present design's use of a three-port tee is easy and cheap, fabricating the MOI interface with less dead volume using a 3D printer or micromachining could further decrease the washing time and narrow the MS peaks. From an application point of view, more efforts will be dedicated to the application of SPME-MOI-MS for real-time body fluid analysis to monitor the traditional biomarkers of health and/or drug residues during medical procedures using in vivo SPME technology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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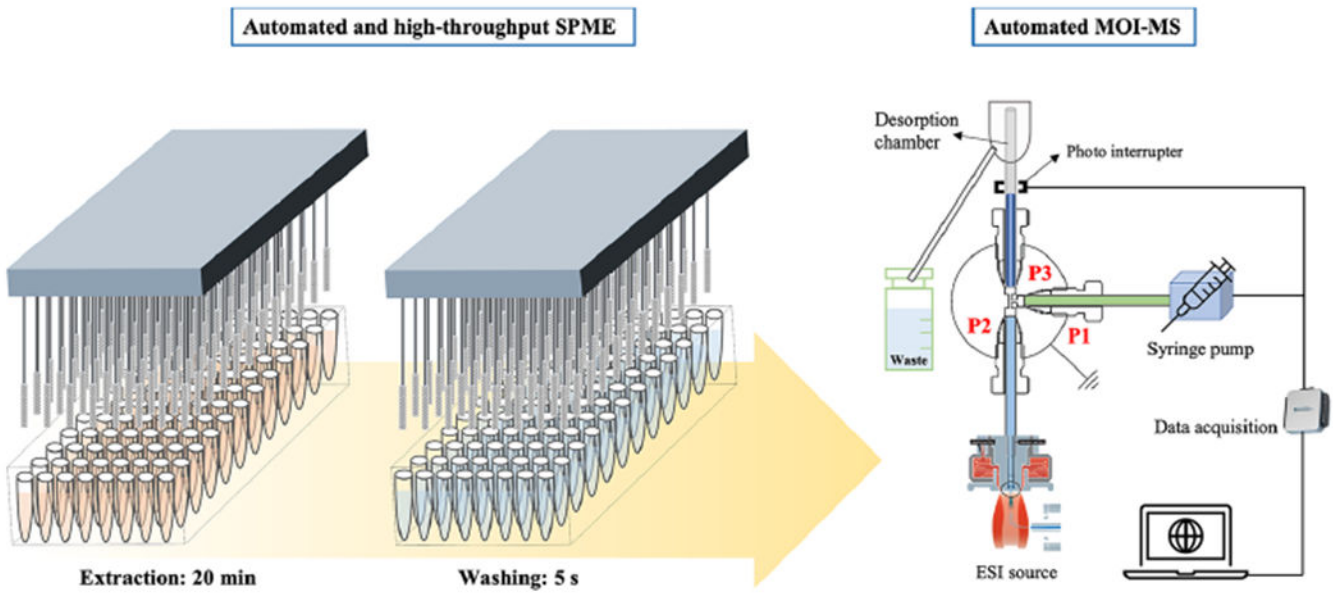

Figure 1.

High-throughput SPME-automated MOI-MS system (preconditioning step not shown; the P1, P2, and P3 represented three different ports of the tee, and the fiber after washing was transferred to the desorption chamber for desorption).

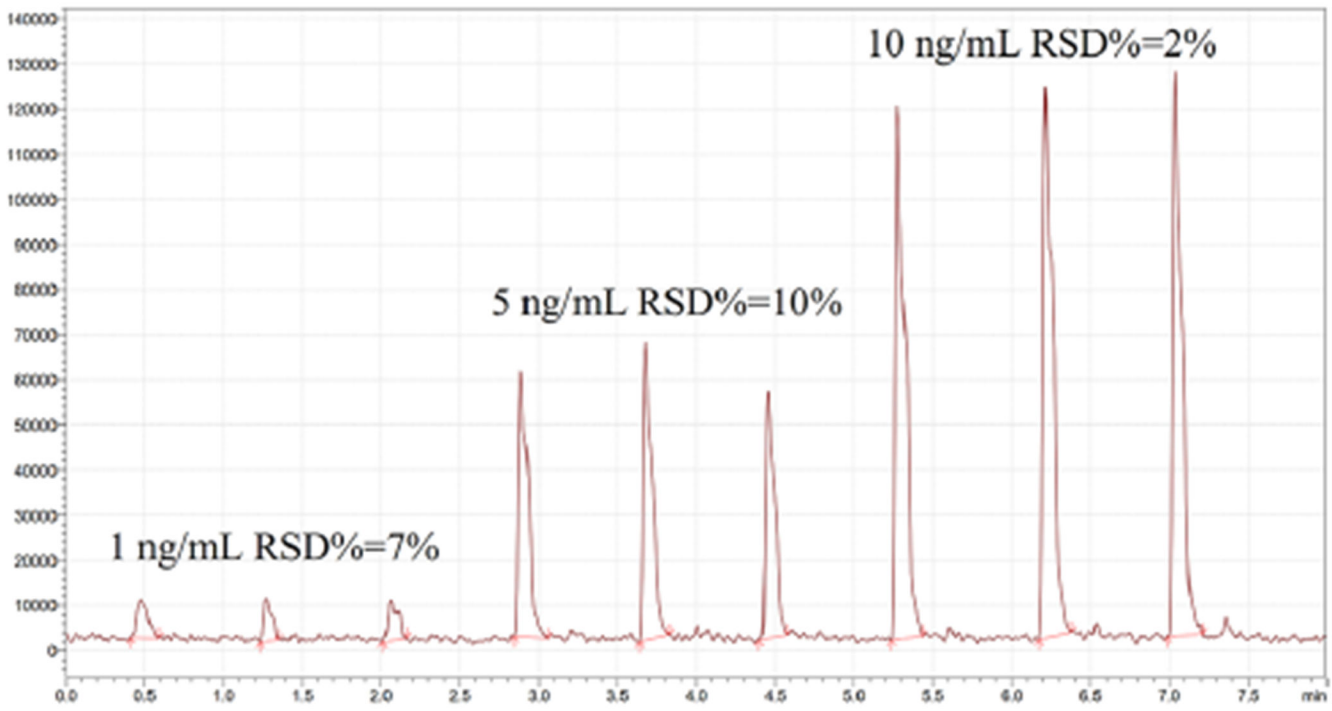

Figure 2.

MS spectra obtained via multi-segment injection using an automated MOI-MS interface for nine saliva samples spiked with buprenorphine at three concentration levels.

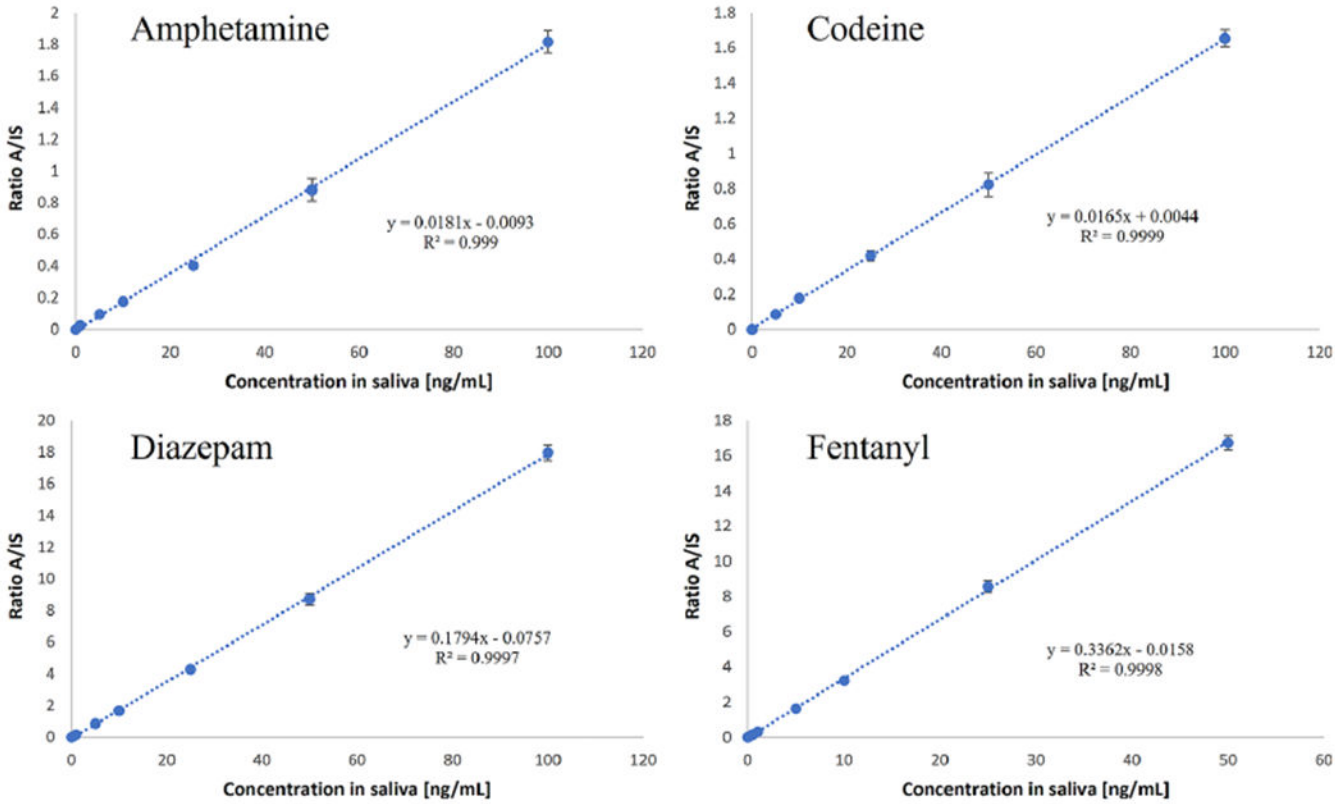

Figure 3.
Calibration curves for the analysis of amphetamine, codeine, diazepam, and fentanyl in saliva samples.

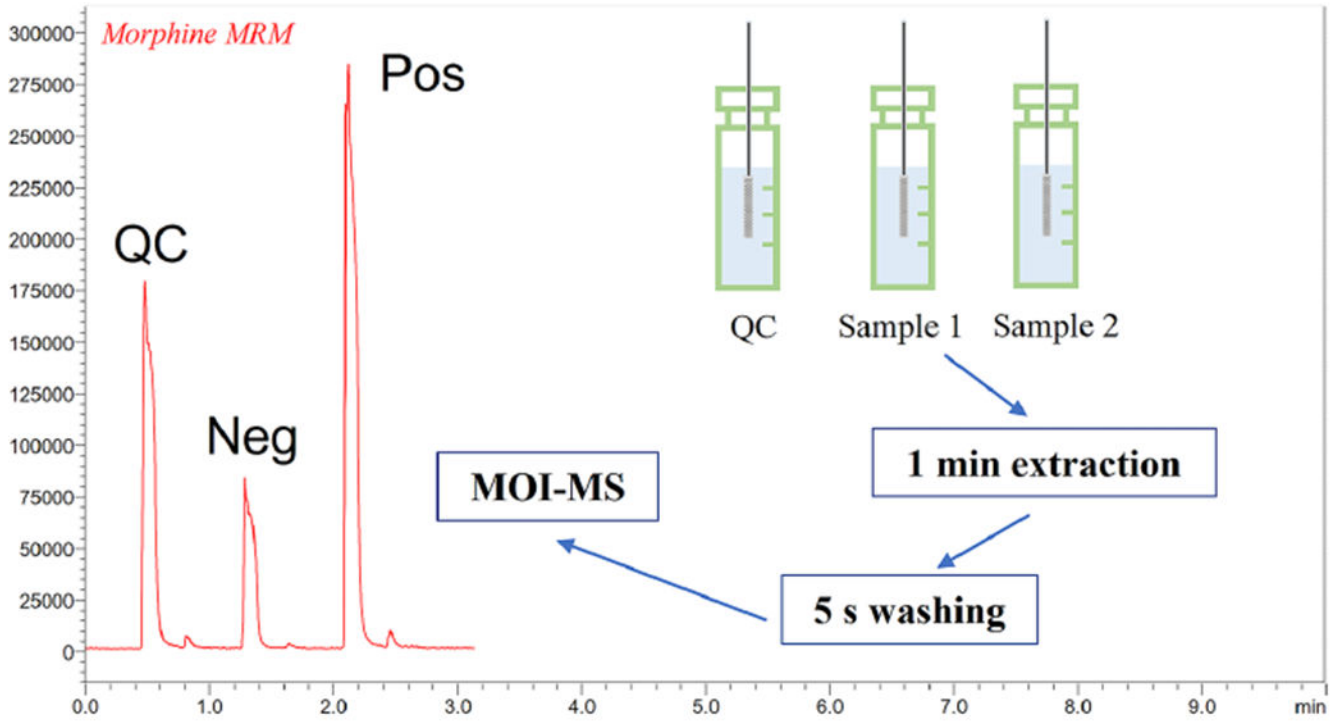

Figure 4.
Real-time visible detection of morphine by SPME-MOI-MS in saliva samples.

Table 1.

Figures of Merit for the Quantitation of Multiple Analytes in Saliva via the SPME-Automated MOI-MS Method

compounds	log *P*	slope	intercept	R^2	LOD[a]	LOQ[a]	linearity range[a]
morphine	0.9	0.0886	0.0194	0.9957	1.00	5.00	5.00–100
codeine	1.2	0.0165	0.0044	0.9999	1.00	5.00	5.00–100
benzoylecgonine	1.3	0.3292	−0.3184	0.9967	1.00	5.00	5.00–100
amphetamine	1.8	0.0264	−0.0005	0.9999	0.25	1.00	1.00–100
strychnine	1.9	0.0430	−0.0088	0.9958	1.00	5.00	5.00–100
cocaine	2.0	0.1730	−0.0003	1.0000	0.05	0.10	0.10–100
methamphetamine	2.1	0.1801	−0.1209	0.9976	0.10	0.25	0.25–100
oxazepam	2.2	0.1867	−0.1462	0.9979	1.00	5.00	5.00–100
clenbuterol	2.3	0.2409	−0.0856	0.9997	0.10	0.25	0.25–100
lorazepam	2.4	0.5784	0.4564	0.9995	1.00	5.00	5.00–200
diazepam	2.6	0.1794	0.0757	0.9997	0.10	0.50	0.50–200
carbamazepine	2.8	0.1731	−0.1016	0.9995	0.50	1.00	1.00–100
nordiazepam	2.9	0.2311	−0.0656	0.9998	0.10	0.50	0.50–100
propranolol	3.0	0.3445	−0.2696	0.9966	0.10	0.25	0.25–100
fentanyl	4.1	0.3362	−0.0158	0.9998	0.01	0.05	0.05–50
buprenorphine	4.5	0.0472	−0.0506	0.9990	1.00	3.00	5.00–100

[a]Unit in ng/mL.