Trace Detection of Tetrahydrocannabinol in Body Fluid via Surface-Enhanced Raman Scattering and Principal Component Analysis

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ABSTRACT: Tetrahydrocannabinol (THC) is the main active component in marijuana and the rapid detection of THC in human body fluid plays a critical role in forensic analysis and public health. Surface-enhanced Raman scattering (SERS) sensing has been increasingly used to detect illicit drugs; however, only limited SERS sensing results of THC in methanol solution have been reported, while its presence in body fluids, such as saliva or plasma, has yet to be investigated. In this article, we demonstrate the trace detection of THC in human plasma and saliva solution using a SERS-active substrate formed by in situ growth of silver nanoparticles (Ag NPs) on diatom frustules. THC at extremely low concentration of 1 pM in plasma and purified saliva solutions were adequately distinguished with good reproducibility. The SERS peak at 1603 cm\(^{-1}\) with standard deviation of 3.4 cm\(^{-1}\) was used for the evaluation of THC concentration in a methanol solution. Our SERS measurement also shows that this signature peak experiences a noticeable wavenumber shift and a slightly wider variation in the plasma and saliva solution. Additionally, we observed that THC in plasma or saliva samples produces a strong SERS peak at 1621 cm\(^{-1}\) due to the stretching mode of O—C==O, which is related to the metabolic change of THC structures in body fluid. To conduct a quantitative analysis, principal component analysis (PCA) was applied to analyze the SERS spectra of 1 pM THC in methanol solution, plasma, and purified saliva samples. The maximum variability of the first three principal components was achieved at 71%, which clearly denotes the impact of different biological background signals. Similarly, the SERS spectra of THC in raw saliva solution under various metabolic times were studied using PCA and 98% of the variability is accounted for in the first three principal components. The clear separation of samples measured at different THC resident times can provide time-dependent information on the THC metabolic process in body fluids. A linear regression model was used to estimate the metabolic rate of THC in raw saliva and the predicted metabolic time in the testing data set matched well with the training data set. In summary, the hybrid plasmonic-biosilica SERS substrate can achieve ultrasensitive, near-quantitative detection of trace levels of THC in complex body fluids, which can potentially transform forensic sensing techniques to detect marijuana abuse.

KEYWORDS: tetrahydrocannabinol, surface-enhanced Raman scattering, plasmonic nanoparticles, diatoms, drug abuse

The abuse of psychoactive drugs has caused serious health problems and a rise in criminal activity in recent years.\(^1\)–\(^4\) Cannabis, also known as marijuana, is one of the most commonly used psychoactive drugs for medical or recreational purposes.\(^3\)–\(^7\) The most common short-term physical and neurological effects of cannabis include increased heart rate, increased appetite, reduced blood pressure, impairment of short-term and working memory, psychomotor coordination, and concentration.\(^3\)–\(^8\)–\(^14\) Cannabis contains more than 60 psychoactive cannabinoids,\(^3\)–\(^8\)–\(^9\) including the most controversial component, \(\Delta^2\)-tetrahydrocannabinol (THC), which is responsible for hallucinogenic effects.\(^3\)–\(^5\)–\(^8\)–\(^13\),\(^15\),\(^16\) In human body fluid, THC is rapidly oxidized into a hydroxyl metabolite (OH-THC) and a carboxylic acid metabolite (THC-COOH), which are nonpsychoactive but can be present in blood and urine for multiple days after the use of cannabis.\(^3\)–\(^5\)–\(^8\)–\(^13\),\(^15\),\(^16\)

Therefore, trace detection of THC and its metabolic products in body fluid is critical to prevent drug abuse and improve forensic investigation of driving under the influence (DUI) or other criminal offenses.

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trace detection of target species at the single-molecule level. Enhanced Raman scattering (SERS) is a promising tool for the sensitive method for the detection of THC in complex samples. Various molecules via silver nanoparticles (Ag NPs) coupled to hotspots remains in demand for detection of THC in complex samples, which is useful in the accurate identification of THC in body fluids. Many illicit or recreational drugs can be detected using advanced chemistry analysis techniques such as high-performance liquid chromatography, gas chromatography in tandem with mass spectrometry, and capillary electrophoresis. Although these methods have excellent sensitivity, they require a sophisticated protocol for sample preparation and suffer long data processing times. Together with high cost, these sensing techniques are inappropriate for quick detection of THC in complex solutions. A simple, rapid, cost-effective, and ultra-sensitive method for the detection of THC in complex biofluids, including blood, urine, and saliva, will therefore play a significant role in various forensic investigations. Surface-enhanced Raman scattering (SERS) is a promising tool for the trace detection of target species at the single-molecule level. With extreme sensitivity and selectivity, it detects these species via inelastic light scattering from the target molecule. Only a few reports have been published on the detection of THC using SERS from regular plasmonic NPs, however, the available SERS reports on THC sensing have been limited to methanol solution for SERS investigations with concentration down to 1 nM. No examination has been undertaken related to the SERS sensing of THC in body fluids (i.e., saliva or plasma) though the detection of THC in body fluids is a major concern in most drug abuse tests. In particular, THC degrades rapidly in aqueous environments but can be observed in the form of THC metabolite in body fluids. Furthermore, SERS examination of THC metabolite in body fluids would be of significant value for expedited field drug tests. However, the detection of THC or THC metabolite in complex solutions is a challenge when using the SERS platform. Specifically, complex solutions contain multiple biomolecules, which may interfere considerably with the THC SERS signals in lower-concentration detections. Consequently, chemometrics methods, such as principal component analysis (PCA) and the linear regression models, have been applied to classify complex spectral data, which is useful in the accurate identification of THC in body fluids. Additionally, the formation of high-density SERS hotspots remains in demand for detection of THC in complex solutions.

In our previous work, we demonstrated the SERS sensing of various molecules via silver nanoparticles (Ag NPs) coupled with diatom biosilica surfaces. Metallic NPs deposited near or inside diatom pores form hybrid plasmonic-plasmonic modes, which can generate a strong local electromagnetic field and enhance SERS signals. In our current study, we present an ultrasensitive hybrid plasmonic-bios silica SERS substrate using high density in situ growth Ag NPs on diatom frustule surface for THC detection in a complex solution. Using this substrate, we achieve THC detection at extremely low concentrations, down to 1 pM, which is 1000× lower than the previously best reported results. THC metabolite changes in the body fluid were also analyzed by monitoring the time-dependent SERS spectra. The PCA method was applied to differentiate SERS spectra of THC (1 pM) in methanol, plasma, and purified saliva samples, which proved that the PCA method is necessary to suppress the interference from biofluid background signals. Furthermore, THC (10−5 M) in raw saliva solution under various metabolic times was also studied using the PCA method and the linear regression model to estimate the metabolic rate of THC in raw saliva.

### EXPERIMENTAL SECTION

#### Preparation of SERS-Active Substrate.

Diatoms were prepared according to a previously published method. The as-prepared diatom substrates were then used for the growth of NPs. In situ synthesized Ag NPs in diatom photonic biosilica were prepared according to our previously published method. The optimized Ag NP growth concentration, as reported in our previous work, was used to increase the density of NPs in the diatom pores. To increase the density of Ag NPs on diatoms, the NP growth time was increased to 30 min. In brief, the diatom biosilica was first immersed in a solution containing tin(II) chloride (SnCl2) (20 mM) and hydrochloric acid (HCl) (20 mM) for 30 min to deposit nucleation sites of Sn2+ on the diatom surface, before being washed with water and acetone and dried with nitrogen. It was then immersed in a 20 mM aqueous solution of silver nitrate (AgNO3) for 30 min to grow Ag seeds on the diatom surface. The Ag seeds deposited on the diatom surface were immersed in 1.5 mL of the final growth medium (1 mL of 5 mM AgNO3 and 0.5 mL of 50 mM ascorbic acid) for 30 min. Impurities were then removed using water and acetone and the substrate was dried with nitrogen. The morphology of Ag NPs on the diatom surface was characterized using field-emission scanning electron microscopy (FE-SEM).

#### Tetrahydrocannabinol SERS Measurements.

The substrates were soaked in 1 mL of various dilutions of the target probe, Rhodamine 6G (R6G) or THC, for 10 min before being dried in air.
Human plasma solution was obtained from Sigma-Aldrich. THC at various concentrations was mixed with the plasma solution and subjected to SERS detection. Saliva samples were collected from healthy volunteers using an oral swab. The swab wiped the inner mouth for 2 min, collecting ~1 mL of saliva, which is denoted as “raw saliva”. The gathered raw saliva (~1 mL) was mixed with an equal volume of water and centrifuged for 15 min to remove any food particles from the saliva sample. 500 μL of the centrifuged sample, which was denoted as “purified saliva”, was used for SERS measurements. Various concentrations of THC were added to the raw and purified saliva for quantitative SERS sensing and a study of the metabolic process. Saliva or plasma solutions containing THC were loaded directly onto our hybrid plasmonic-biosilica SERS substrate for measurement, which was conducted using a Raman microscope (Horiba Jobin Yvon Lab Ram HR8000). All samples were excited using a 532 nm laser through a 50× objective lens with a laser spot size of 2 μm in diameter. Raman spectra were acquired with an integration time of 10 s in the Raman spectral range of 400 to 1800 cm⁻¹.

**Multivariate Analysis.** Multivariate statistical methods such as the PCA method and partial least-squares regression (PLSR) were used to extract the key features and to explore the spectral variations for the analysis of SERS spectra. In this manuscript, all spectral analysis was performed using MATLAB software. First, all Raman spectra were smoothed using the Z-score method and the background was removed via baseline correction. Three sets of Raman spectra (i.e., pure THC, plasma THC, and purified saliva THC, each with 25 data set) were used for PCA to identify the maximum variation between each data set. The first three PCs (i.e., PC1, PC2, and PC3) were selected as the primary parameters. Similarly, time-dependent THC in raw saliva spectra were analyzed using the first three PCs. PLSR was then applied to predict the outcome at different situations of time-dependent THC concentration in raw saliva.

## RESULTS AND DISCUSSION

### Characterization of Hybrid Plasmonic-Biosilica Substrate

**Figure 1(a)** shows the experimental procedure of the in situ growth of Ag NPs on a diatom surface. The diatom biosilica consists of two-dimensional pores with an average diameter of 200 nm, as shown in **Figure 1(b)**. When the diatom biosilica was immersed in aqueous solution containing a SnCl₂ and HCl mixture, nucleation sites of Sn²⁺ ions were deposited on the pore surface of the diatom biosilica surface.

For Ag seed deposition, the Sn²⁺-deposited diatom surface was immersed in AgNO₃ solution. The reduction of Ag on the diatom surface was achieved using a reduction reaction between Sn²⁺ and two Ag⁺ ions, which produced large Ag seeds on the diatom surface and pore walls, as illustrated in **Figure 1(a)**. The uniform-sized Ag NPs were formed from Ag seeds deposited on the diatom surface with the final growth medium (i.e., a mixture of AgNO₃ and ascorbic acid). The Ag NP-populated diatom is shown in **Figure 1(c)**, with i-iii representing various magnifications. The Ag NPs on the diatom surface were spherical in shape and the particle size distribution was obtained from the FE-SEM image. The Ag NPs have average diameters of 40 ± 5 nm as detailed in the Supporting Information (SI). With a growth time of 30 min, high density Ag NPs were formed inside the pores on the diatom surface, generating strong local fields to enhance SERS signals. Strong extinction peaks at 422 and 556 nm are observed, which are correlated to the localized surface plasmonic resonances of Ag NPs-on-diatoms and guided-mode-resonances of the photonic crystal-like diatom frustule, respectively, as shown in the SI. SERS spectra of 10⁻⁶ M R6G from Ag NPs-on-diatom obtained by 532 and 785 nm laser are shown in the SI and it was found that the 532 nm source produced better SERS spectra. The superiority of the SERS spectra from 532 nm laser can be partially attributed to the good spectral overlap between the interrogation wavelength and the photonic structural resonances, allowing for greater optical field enhancement. SERS spectra of various concentrations of R6G on hybrid-plasmonic biosilica SERS substrate obtained by the 532 nm Raman laser are shown in the SI. The strongest characteristic SERS peak of R6G appeared at 1362 cm⁻¹, which was attributed to the C=C stretching modes.

**THC SERS Detection in Methanol Using the Hybrid Plasmonic–Biosilica Substrate.** The chemical structure of THC is shown in the inset of **Figure 2(a)**. A stock solution of THC is depicted in the Figure 2(a). Right axis: Raman peak shift of I_SERS versus concentration of THC; highlighted inset spectra shows the three SERS bands at 1500–1700 cm⁻¹. The gathered raw saliva (~1 mL) was mixed with an equal volume of water and centrifuged for 15 min to remove any food particles from the saliva sample. 500 μL of the centrifuged sample, which was denoted as “purified saliva”, was used for SERS measurements. Various concentrations of THC were added to the raw and purified saliva for quantitative SERS sensing and a study of the metabolic process. Saliva or plasma solutions containing THC were loaded directly onto our hybrid plasmonic-biosilica SERS substrate for measurement, which was conducted using a Raman microscope (Horiba Jobin Yvon Lab Ram HR8000). All samples were excited using a 532 nm laser through a 50× objective lens with a laser spot size of 2 μm in diameter. Raman spectra were acquired with an integration time of 10 s in the Raman spectral range of 400 to 1800 cm⁻¹.

**Figure 2.** SERS spectra of THC molecules (10⁻⁹, 10⁻⁸, 10⁻⁸, 10⁻⁹, and 10⁻¹² M) on the plasmonic-biosilica SERS substrate and the inset shows THC molecules chemical structures (a). Left axis: Relationship of I_SERS (log I) versus concentration of THC molecules (log C), using I_SERS at 1603 cm⁻¹. Right axis: Raman peak shift of I_SERS at 1603 cm⁻¹ versus concentration of THC; highlighted inset spectra shows the three SERS bands at 1500–1700 cm⁻¹ (b).

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THC (1 mg/mL) in methanol was used to prepare various concentrations of THC, using serial dilution. Hybrid-plasmonic biosilica SERS substrates were soaked in 1 mL of the dilutions of THC in methanol solution for 10 min before being air-dried. The THC solution with concentrations of $10^{-3}$ to $10^{-12}$ M on the hybrid plasmonic-biosilica substrates were measured and the results are shown in Figure 2(a). The SERS results show that SERS peaks appeared at 1000, 1030, 1201, 1275, 1323, 1370, 1450, 1556, 1580, and 1603 cm$^{-1}$ as listed in Table S1 in the SI.27−29,46,47 The strongest characteristic peak of THC solution appeared at 1603 cm$^{-1}$, which is attributed to the C=C stretching modes.27−29,46,47 Moreover, the other two major SERS bands at 1556 and 1580 cm$^{-1}$ can be used to distinguish THC, which are also attributed to the C=C stretching modes. THC activity is based mainly on the formation of two stereoisomers, i.e., (−)-trans-isomer and (+)-trans-isomer, which generate the strong C=C stretching mode under Raman scattering. Our SERS results are similar to the reported THC spectra.27−29 Slight shifting of the SERS band to lower and higher wavenumbers was observed at each concentration of THC. Furthermore, the three major SERS peaks of THC at 1556, 1580, and 1603 cm$^{-1}$ were shifted with standard deviation of 3.4, 2.7, and 3.4 cm$^{-1}$, respectively, as shown in the inset of Figure 2(b). Figure 2(b) and Figure S6 (SI) show the quantitative relationship between the integrated SERS intensity ($I$) versus concentration of THC molecules ($C$) in plasma (b) and purified saliva solution (d), using $I_{\text{SERS}}$ at 1595 cm$^{-1}$. The right axis of (b) and (d) shows the Raman peak shift of THC at 1595 cm$^{-1}$ versus concentration of THC; highlighted inset spectra shows the SERS bands at 1500−1700 cm$^{-1}$ (b,d).

Trace Detection of THC in Complex Solutions. Twenty microliters of plasma solution was added to 20 μL of THC solution at various concentrations under vigorous stirring at room temperature to obtain a homogeneous solution. As a comparison, the Ag NPs on a glass substrate had a THC detection limit of only $10^{-7}$ M. We attribute the ultrahigh SERS sensitivity to the high density hot spots between Ag NPs in diatom pores, which are also coupled with the photonic crystal resonance of diatom frustules to achieve even higher enhancement factors.
Figure 3(a), the SERS spectrum of THC in plasma solution (10^{-3} to 10^{-12} M) exhibits a strong peak at 1595 cm^{-1}, which is assigned to the C=C stretching mode. For the THC in plasma or saliva samples, the SERS spectra significantly changed as a result of rapid oxidization of THC and formation of THC-OH and THC-COOH, which can be used to detect THC in plasma or saliva samples after several hours or even days of intake. The new THC SERS peaks appeared at 1621 cm^{-1}, which were attributed to O=C=O and indicated the formation of a carboxylic acid metabolite (THC-COOH). Thus, the strong C=C stretching modes and O=C=O at 1595 and 1621 cm^{-1} can be used to distinguish lower concentrations of THC in plasma solution. The interference from the plasma background signals reduced the THC SERS intensity and a small SERS shift was obtained at 1595 and 1621 cm^{-1} with standard deviation of 2.2 and 4.8 cm^{-1}, respectively, as highlighted in the inset of Figure 3(b). The strong characteristic peaks of THC at 1595 and 1621 cm^{-1} reveal a linear log-scale correlation to the concentration between 10^{-3} M and 10^{-12} M. THC concentration was quantified using the linear equation with R^2 of 0.998 for 1595 cm^{-1} for 0.99775 and 0.961 for 1621 cm^{-1} were achieved, as shown in Figure 3(b) and Figure S6 (SI). THC in purified saliva sample was also measured by SERS using the hybrid plasmonic-biosilica SERS substrate. Relatively strong SERS bands at 1595 and 1621 cm^{-1} were obtained, as shown in Figure 3(c), and can be attributed to the C=C stretching mode and O=C=O. The Ag NPs on the diatom substrate exhibited strong SERS intensities even down to 1 pM concentration. The concentration of THC in the purified saliva sample was quantified using the linear equation with R^2 of 0.971 for 1595 cm^{-1} and 0.983 for 1621 cm^{-1} as shown in Figure 3(d) and Figure S6 (SI). The linear relationship can be used to determine an unknown concentration of THC in plasma or saliva. A small SERS shift was observed at 1595 and 1621 cm^{-1} with standard deviation of 2.7 and 1 cm^{-1}, respectively, as highlighted in the inset of Figure 3(d) due to saliva sample’s background signals. In the present work, the detection limit for THC (10^{-12} M) is much better than previously reported values (listed in Table S2). Thus,

\[ \log(I) = a \log(C) + b \] (1)
our hybrid plasmonic-biosilica SERS substrate is an effective platform for the detection of THC in a complex solution.

**Hybrid Plasmonic-Biosilica Substrate for Trace Detection of THC in Raw Saliva Solution.** SERS spectra of THC in raw saliva solution (denoted as saliva solution-0 (SS0)) with various concentrations (10⁻³ to 10⁻⁹ M) were also measured using the same setup as earlier. The strong SERS characteristic peaks of THC in raw saliva appeared at 1603 and 1651 cm⁻¹, as shown in Figure 4(a), which are attributed to the C=C stretching mode and O−C=O, respectively. The O−C=O band obtained at 1651 cm⁻¹ could be linked to the THC−COOH because of the strong interaction between proteins or amino groups in raw saliva solution.27−29,46,47 The raw saliva THC SERS characteristic peaks shifted slightly between each concentration of THC, which is associated with the raw saliva Raman signal interference, as shown in Figure 4(b). The concentration of THC in raw saliva solution was quantified using the linear equation with $R^2$ of 0.9558 for 1603 cm⁻¹, as shown in Figure 4(b), which can be used to determine the unknown concentration of THC in raw saliva. These results showed that the lower concentration of THC (1 nM) in raw saliva was distinguished satisfactorily using the hybrid plasmonic-biosilica SERS substrate.

To evaluate the effect of variations among individual saliva samples, we studied THC in saliva from three additional volunteers (denoted as saliva sample-1 (SS1), saliva sample-2 (SS2), and saliva sample-3 (SS3)). Saliva samples were collected from healthy volunteers at different times of the day using an oral swab. THC at various concentrations was added to the raw saliva solutions. The results are shown in Figure 5(a−c). A linear regression was performed to correlate the SERS peak intensity at 1603 cm⁻¹ and the THC concentration. $R^2$ values were achieved of 0.853, 0.849, and 0.881 for SS1, SS2, and SS3, respectively, and is shown in Figure 5(d−f). Furthermore, the Raman peak shift is shown for each saliva sample at various concentrations. Using the THC concentration of 10⁻³ M, we compared the three new saliva samples (SS1−3) with the saliva sample (SS0) used originally. There is a certain level of variation among the SERS spectra from each saliva sample in the 900−1500 cm⁻¹ region, which is due to the difference of the saliva sample, as would be expected. However, the characteristic peak at 1603 cm⁻¹ is distinguishable and remains relatively constant. The intensity and Raman shift of the characteristic peaks from the C=C stretching for the four saliva samples were compared and a relatively large intensity variation occurred between SS0 and SS1−3. This is most likely caused by the difference of the SERS substrates. The difference of the Raman peak wavelength number among all samples, however, is very minimal and even smaller than the standard deviation of each sample. This proves that the variation of THC signals in the same type of biofluidic sample from different people is acceptable.

We also investigated the THC metabolite changes at various time periods (i.e., 0, 3, 6, and 9 h) with a concentration of 10⁻⁵ M THC in raw saliva solution. SERS characteristic peaks of THC were greatly influenced by the time-dependent SERS measurement, as shown in Figure 4(c,d). Notable THC metabolite changes are highly present in real body fluids (raw saliva) after several hours of THC intake. Figure 4(c,d) shows the progression of time of THC in raw saliva from 0 to 9 h, and the formation of the strong O−C=O band at 1651 cm⁻¹ due to significant changes in the THC structure. Moreover, the THC SERS characteristic peak intensity of 1603 cm⁻¹ was notably reduced while increasing the THC time in raw saliva. Thus, the THC metabolite changes could be a better indicator to detect THC in body fluids using SERS sensing.

**PCA and PLSR Analysis.** Two independent PCA processes were applied for different types of analysis. The first PCA process was conducted by extracting the principal components of the SERS spectra of the three samples at 10⁻¹² M concentration of THC in methanol, plasma, and purified saliva. The SERS spectral region of interest is between 400 and 1800 cm⁻¹ for all samples to identify the maximum variations of each data set. The first three principal components (PCs) at 51.49% (PC1), 14.24% (PC2), and 5% (PC3) of the variance were obtained and plotted in Figure 5(a). The three separate clusters clearly show...
the separation of THC in various solutions, reflecting the impact of different biological background signals.

The second PCA process was conducted by extracting the principal components among SERS spectra of THC in saliva at different times to account for the metabolic decomposition and oxidation of THC. The metabolic times of 0, 3, 6, and 9 h of $10^{-5}$ M THC in raw saliva samples were analyzed. The first three PCs of the SERS spectra account for 98% of the variance for different THC metabolic time, which are 79.79% (PC1), 10.92% (PC2), and 8.64% (PC3) as shown in Figure 5(b). At the metabolic time of 0 h, the spectra in the PC space are densely packed, forming a tight cluster. As the structure of THC changes into hydroxyl or carboxylic acids due to the metabolic process, the resulting SERS spectra spread into narrow clusters along distinct directions. These discrete narrow clusters, seen in Figure 5(b), allow for clear identification of individual characteristics of time-dependent THC metabolic changes in raw saliva samples. This can be useful for semiquantitative classification to determine the extent of THC metabolic process. Similarly, the time-dependent nature of SERS spectra for $10^{-5}$ M THC in phosphate buffered saline (PBS) solution was investigated and the results are shown in Figure S9(a,b) in the SI. This analysis shows the time dependence of THC hydroxylation in PBS solution, which is different from the metabolic process in raw saliva. At the beginning, THC in PBS solution generated the typical SERS signature peak at 1595 cm$^{-1}$. However, as time progresses, the THC molecule is altered due to hydrolysis in the PBS solution. The hydrolysis process produces a new SERS peak at 1616 cm$^{-1}$, which is different than the SERS peak at 1651 cm$^{-1}$ due to the stretching mode of O−C==O, which results from the metabolic change of THC structures in body fluids after several hours of THC intake. The maximum variability of the first three principal components was achieved at 71% for THC in methanol, plasma, and purified saliva solution. A linear regressionary model was then used to estimate the metabolic rate of THC in raw saliva. The hybrid plasmonic-biosilica SERS substrate has the potential to monitor THC or THC metabolic changes in body fluids after different times of drug use.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssens.9b00476.

Further description of the SERS characterization of plasmonic-biosilica substrate and SERS characterization of THC in complex solution (PDF)

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Notes

The authors declare no competing financial interest.

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