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Multivariate Analysis Aided Surface-Enhanced Raman Spectroscopy (MVA-SERS) Multiplex Quantitative Detection of Trace Fentanyl in Illicit Drug Mixtures Using a Handheld Raman Spectrometer

Ling Wang[1], Mario O. Vendrell-Dones[1], Chiara Deriu[1], Sevde Doğruer[1], Peter de B. Harrington[2], and Bruce McCord[1]

Abstract

Recently there has been upsurge in reports that illicit seizures of cocaine and heroin have been adulterated with fentanyl. Surface-enhanced Raman spectroscopy (SERS) provides a useful alternative to current screening procedures that permits detection of trace levels of fentanyl in mixtures. Samples are solubilized and allowed to interact with aggregated colloidal nanostars to produce a rapid and sensitive assay. In this study, we present the quantitative determination of fentanyl in heroin and cocaine using SERS, using a point-and-shoot handheld Raman system. Our protocol is optimized to detect pure fentanyl down to 0.20 ± 0.06 ng/mL and can also distinguish pure cocaine and heroin at ng/mL levels. Multiplex analysis of mixtures is enabled by combining SERS detection with principal component analysis and super partial least squares regression discriminate analysis (SPLS-DA), which allow for the determination of fentanyl as low as 0.05% in simulated seized heroin and 0.10% in simulated seized cocaine samples.

Keywords

Fentanyl, heroin, cocaine, surface-enhanced Raman spectroscopy, SERS, chemometrics, super partial least squares regression discriminate analysis, SPLS-DA

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Introduction

The opioid crisis has been a decades-long issue in the U.S., causing approximately 450 000 overdose deaths[1] and around 46 000 deaths in 2018.[2–4] This epidemic affects the users of both prescription opioid and streets drugs. Furthermore, synthetic opioids, such as fentanyl and its analogs, are also increasingly being used as lacing agents in common street drugs, or as cheaper alternatives in the manufacture of counterfeit pills.[5] Whether intentional or unintentional, the combined use of fentanyl and heroin,[6] as well as fentanyl and cocaine,[7] has become increasingly popular. These combinations are extremely dangerous because fentanyl is 50 to 100 times more potent than morphine,[8] and even trace quantities can cause overdose, especially in unsuspecting and naïve users.[9–11] Especially for fentanyl-laced cocaine, the risk of overdosing is further heightened by the inherent health hazard of mixing stimulants and opioids.[12]

Fentanyl and its analogs have been increasingly reported in both drug seizures and toxicological reports in the past four years.[7,13] In the third quarter of 2019 alone, the Drug Enforcement Administration (DEA) has reported that 84% of opioid seizures were fentanyl related, 53% of which contained at least another substance mixed with fentanyl, such as heroin (34% of fentanyl-laced seizures).[14] Based on this continuing level of seizures and reports, on 6 February

[1]Department of Chemistry and Biochemistry, Florida International University, Miami, FL USA
[2]Department of Chemistry and Biochemistry, Ohio University, Athens, OH, USA

Corresponding author:
Bruce McCord, Department of Chemistry, Florida International University, 11200 SW 8th St, CP304, Miami, FL 33199, USA.
Email: mccordb@fiu.edu

2020, the U.S. Congress extended the DEA temporary placement of fentanyl-related compounds as Schedule I substances by passing the Temporary Reauthorization and Study of the Emergency Scheduling of Fentanyl Analogues Act.[15–17] The use of fentanyl-laced drugs has been linked to the growth in fatal overdoses, and is a continuing threat to public health and safety.[4,18] Therefore, it is important to develop cost-effective, sensitive, and reliable methods to detect trace amounts of fentanyl in mixtures of commonly seized street drugs. Published methods for the detection of fentanyl are various and include spot test,[19] immunoassays,[20] ion mobility spectrometry,[21] chromatography–mass spectrometry,[19,22–24] electrochemical sensing,[25,26] and vibrational spectroscopy, such as Fourier transform infrared (FT-IR),[24,27] and more recently, surface-enhanced Raman spectroscopy (SERS).[28–32]

Surface-enhanced Raman spectroscopy embodies the advantages of conventional Raman spectroscopy by providing fingerprint information on analytes, while concomitantly avoiding its greatest shortcoming, poor sensitivity.[33] SERS utilizes metallic nanoparticles sustaining localized surface plasmon resonance to enhance the Raman signal from adsorbed analytes, greatly improving sensitivity.[34] Because of this enhancement, SERS has a strong potential to detect and characterize analytes at trace levels (ng to sub-ng/mL) making it a promising analytical technique for the detection of lacing agents in seized drugs, as well as an alternative to current toxicological screening methods.[35] For example, SERS has been recently applied to the duplex detection of fentanyl in binary mixtures of heroin[29,36] and cocaine,[31] utilizing benchtop Raman instrumentation and an excitation source at 532 nm. For both heroin and cocaine, Haddad et al.[29,31] utilized planar substances obtained by loading Ag nanospheres on filter paper as enhancing platforms, while Salemmilani et al.[36] utilized colloidal Ag nanospheres and a microfluidic device to separate fentanyl from heroin. To the authors' knowledge, no work has been published yet on the use of a handheld Raman spectrometer for the detection of fentanyl in binary mixtures, and only two reports have to date been published utilizing portable equipment. Shende et al.[30] detected fentanyl in codeine utilizing an in-house build filed-usable Raman spectrometer, and Wang et al.[37] detected fentanyl in heroin and other opiates utilizing a portable Raman fitted with a microscope and 96-well plate sample holder. The method optimized by Shende et al. takes advantage of Au nanoparticle-loaded pads that work as both collection devices and planar enhancing substrates, while the one reported by Wang et al. utilizes traditional citrate-reduced Au nanospheres. A summary of these works with their respective detection limits is reported in Table I.

Besides the obvious advantage of being transportable, handheld and portable Raman instruments are much cheaper than benchtop equipment. Cost-effectiveness is fundamental to the implementation of methods that ultimately aim at addressing drug epidemics and their societal

Table I. Summary of previously published SERS methods for fentanyl mixture detection.

SERS-active substrate	Target drugs	Raman instrument	λ_{exc} (nm)	LOD and data analysis approach		References
Ag nanoparticles obtained by microwave-assisted reduction loaded on filter paper	Fentanyl/heroin	Benchtop	532	0.1% (10 ng) (fentanyl mass/total mass)	Univariate analysis band height ratios of fentanyl 1005 cm^{-1} to heroin 628 cm^{-1}	Haddad et al.[29]
Citrate-reduced Au nanospheres	Fentanyl/heroin	Portable fitted with a microscope	785	0.05% fentanyl in heroin	Multivariate analysis, PCA, and PLS-DA	Wang et al.[37]
Ag nanospheres-decorated SiO_2 nanoparticles	Fentanyl/heroin	Benchtop	532	1:10,000 (mol/mol) fentanyl:heroin	Univariate analysis	Salemmilani et al.[36]
Au nanoparticles loaded on glass fiber pads	Fentanyl/codeine	Portable	785	5 ng/mL codeine 6 ng/mL or 40 pg fentanyl	Univariate analysis, codeine 1435 cm^{-1} fentanyl 1000 cm^{-1}	Shende et al.[30]
Ag nanoparticles obtained by microwave-assisted reduction loaded on filter paper	Fentanyl/cocaine	Benchtop	532	5% (500 ng) (fentanyl mass/total mass)	Univariate analysis	Haddad et al.[31]

impact, as lower costs enable a larger number of laboratories to establish testing at the local level, allowing local communities to enact prompt responses to mitigate the presence of illicit drugs. To date, the main limitations of portable and handheld Raman systems when used with SERS involve poor reproducibility of band intensities and lack of standardization.[38] Fortunately, newly developed chemometric approaches permit raw SERS spectra to be processed, improving the utility of data obtained from portable Raman systems.[39]

Chemometric approaches are particularly useful for both qualitative and quantitative analysis of mixtures.[35,39] The challenges associated with multiplex detection by SERS are intrinsic to the mechanism by which signal is enabled and enhanced. When multiple species are present in an analyte, competition for adsorption sites on the enhancing substrate occurs, affecting the signal.[35,39] Thus, it is critical to select marker bands which are unaffected by the background and baseline-resolved from one another. This can be achieved by standard univariate data treatment (i.e., calibration with band height or band area versus known concentration).[39] Although prior extraction or separation of mixture components can be one choice to mitigate this problem,[36,40] multivariate analysis (MVA) is a more rapid alternative to improve the analysis. This procedure takes advantage of the complete array of spectral data. MVA methods based on partial least squares regression (PLSR) have been utilized to qualitatively and quantitatively elucidate SERS spectra in biological samples, such as the determination of nicotine with its major metabolites.[41,42] As for binary drug mixtures, principal component analysis (PCA) and traditional partial least squares discriminant analysis (PLS-DA) were recently used to classify opiates from fentanyl and its analogs, including fentanyl in heroin.[37]

In this work, we present a method utilizing a gold–silver nanostars (Au–Ag NS) based on the SERS method coupled with a handheld Raman analyzer for the detection of fentanyl in binary mixtures of both heroin and cocaine. Quantitative interpretation of mixtures is demonstrated statistically using MVA approaches, namely PCA[43] and super partial least square discriminant analysis (SPLS-DA).[44,45] Our method combines the sensitivity of anisotropic nanomaterials with cost-friendly portable equipment and MVA quantification approaches. It enables trace detection of fentanyl from mixtures of heroin and cocaine, thus addressing the current need for cheap, sensitive, and portable methods to characterize fentanyl-laced drugs.

Experimental

Reagents and Materials

Tetrachloroauric (III) acid trihydrate was purchased from Acros Organics (Waltham, MA). Silver nitrate, L-ascorbic acid (L-AA), trisodium citrate dihydrate, magnesium chloride, hydrochloric acid, sodium hydroxide, methanol, and acetonitrile were purchased from Fisher Chemical (Pittsburgh, PA). Sodium carbonate monohydrate was purchased from Spectrum Chemical (New Brunswick, NJ). Fentanyl and heroin were purchased from Cayman Chemical (Ann Arbor, MI). Cocaine was purchased from Sigma (St Louis, MO).

Synthesis of SERS Substrates

Bimetallic (Au–Ag) colloidal nanoparticles, gold/silver nanospheres (Au–Ag NP), and gold/silver nanostars (Au–Ag NS) were prepared based on a modified version of He's method,[46] as previously published by our group.[32] Briefly, 1.0 mL of water was mixed with 36 μL of $HAuCl_4$ 10 mM and 2.0 μL of $AgNO_3$ 10 mM and vortexed for 10 s. Then, 6.0 μL of L-AA 100 mM were added to the solution and vortexed for another 20 s to create the Au–Ag nanostars. Subsequently, 1.0 μL of Na_2CO_3 1.0 M was added to stabilize the nanoparticles and vortexed for 5 s. The prepared Au–Ag nanostars displayed a light greenish-blue color and had a pH of 6.0.

Sample Preparation of Analytes

Fentanyl and cocaine were dissolved in methanol as 1.0 mg/mL standard solutions. Heroin was dissolved in acetonitrile as 1.0 mg/mL standard solution. Then, a series of dilutions for standard solutions were used in the detection limit for single sample, as well as a series of mixtures were prepared by adding fentanyl to heroin or cocaine as 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, and 100% (v/v) solutions

Sample Preparation for SERS Measurements (Handheld Raman Spectrometer)

For the SERS measurements, a volume of 2.5 μL of magnesium chloride ($MgCl_2$) was mixed with 245 μL of colloidal nanoparticles and allowed to aggregate for 5 min. Then, 2.5 μL of the drug solution was added to the aggregated colloidal solution. This mixture was incubated for another 5 min and then transferred to a quartz Suprasil cuvette (Hellma Analytics). The spectra were recorded by a small handheld Raman spectrometer (FirstDefender, Thermo Scientific) with a 785 nm excitation laser and a solution of 7–10.5 cm^{-1} full width half-maximum (FWHM). The data were recorded in the range from –97 to 2901 cm^{-1} using an auto-exposure and auto-accumulation operation mode and approximately 250 mW laser power. All spectra displayed in this paper were baseline corrected and normalized, except for the spectra utilized for the detection limits of analytes in mixtures, which were treated by chemometric approaches (see Quantitative Evaluation of Mixtures section). The samples utilized for the

determination of detection limits in mixtures consisted of five replicates, those for the reproducibility study consisted of five replicates for the intra-day tests and 10 for inter-day tests. The remainder of the samples was tested in triplicate. All concentrations listed in this work correspond to the final concentration in the solutions.

Optimization of SERS Conditions

To optimize SERS conditions, the SERS signal intensity was used as the criteria to evaluate experimental factors, including aggregating agent and pH of solutions. These parameters were specifically optimized for fentanyl detections, and they were also suitable for heroin and cocaine.

Quantitative Evaluation of Mixtures

Mixtures of standard solutions, 0, 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, 1, and 100% v/v of fentanyl in cocaine or fentanyl in heroin, were measured five times in two days, utilizing the same batch of Au–Ag NS. Data analysis was performed with Matlab v.R2019b (The MathWorks Inc.). In this part, Savitzky–Golay secondary derivative[44] of two points, cubic polynomial filter was used to eliminate baseline issues and each spectrum was then normalized to unit vector length, following with PCA and SPLS-DA[45] to build detection models of mixtures. PCA is an unsupervised approach that uses orthogonal transformation to reduce the dimensionality of the data, and it can provide unbiased visualizations of the true variability of the spectra.[46] SPLS-DA is an automated supervised approached that uses an internal bootstrapped Latin partition (BLP) to optimize the fitting of the model.[47,48] SPLS-DA uses 10 bootstraps and two Latin partitions of the calibration spectra. Both PCA and SPLS-DA were utilized to establish models to predict the sensitivity of our SERS method for the detection of fentanyl in binary mixtures.

Results and Discussion

Optimization of SERS Method

We have previously reported using $MgCl_2$-aggregated Au–Ag nanostars to detect fentanyl and six of its analogs with a benchtop Raman instrument. To ensure a successful translation of our method from the benchtop to the handheld Raman system, we re-optimized the detection conditions in terms of aggregating agent concentration and pH. In brief, five different concentrations of $MgCl_2$ (8 mM, 12 mM, 16.7 mM, 20 mM, and 24 mM) were examined utilizing 10 ug/mL fentanyl as the model target analyte, and a concentration of 16.7 mM produced the strongest SERS signal (Fig. S1a, Supplemental Material). To determine the optimal pH for selected SERS substrate, fentanyl, cocaine, and heroin were tested with 16.7 mM $MgCl_2$ at different pH values (i.e., 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0). The results indicated that a pH of 6.0 was optimal (Fig. S1b). Due to instrumental limitations and the unavailability of drug solutions of sufficiently high concentration, no normal Raman spectra were collected, thus no enhancement factor was calculated.

SERS Study of Individual Drugs Using Univariate Analysis

The calibration curves for the quantitative analysis of fentanyl, heroin, and cocaine were generated from the selected characteristic band from SERS spectra of each analyte. According to our previous work, fentanyl has two characteristic bands: the strongest at 1004 cm^{-1}, and the second of medium intensity at 1030 cm^{-1}. Both bands were assigned to the $\nu(C=C)$ of the two monosubstituted aromatic rings.[32] Figure 1a demonstrates that the spectra obtained using a handheld Raman system provided the same spectra profiles as produced by the benchtop instrument. Fentanyl at a concentration of 10 ug/mL exhibited the strongest band at 1002 cm^{-1}, as well as moderately intense bands at 1206, 1030, 830, and 747 cm^{-1}. As expected, band intensities decreased with dilution (1000, 100, 10, and 1 ng/mL). When the concentration was lower than 1 ng/mL, only the two $\nu(C=C)$ bands at 1030 and 1002 cm^{-1} were still clearly identified; the band at 1002 cm^{-1} was observed even at a concentration of fentanyl as low as 0.25 ng/mL. Therefore, the limit of detection (LOD) was calculated using the intensity of the band at 1002 cm^{-1}. As Fig. 1b shows, the data plots fit a Langmuir function in the 1–10 000 ng/mL concentration range and show a linear response to concentration from 0 to 1 ng/mL. The linear regression produced a sensitivity of 0.027 cts·mL/ng and intercept of 0.009 cts, with a coefficient of determination (R^2) of 0.97. The LOD was calculated as three times the standard deviation of the peak divided by the slope, which resulted in a value of 0.20 ± 0.06 ng/mL (95% confidence interval, or CI).

The SERS parameters optimized for fentanyl, although still adequate, were less sensitive for the detection of heroin and cocaine. Figure 2a is a SERS spectrum of heroin at a concentration of 10 ug/mL, which displayed a strong band at 625 cm^{-1} and less intense bands at 1353, 1243, 1208, 666, and 533 cm^{-1}. When heroin was diluted to 0.75 ug/mL, however, only the band at 625 cm^{-1} was apparent. Interestingly, the relationship between band intensity and concentration did not display the Langmuir behavior that was observed for fentanyl; on the contrary, in the range of 0–10 ug/mL, the plots showed a linear relationship with a sensitivity of 0.014 cts·mL/ng, an intercept of 0.008 cts, and an R^2 of 0.98. The LOD was calculated as 170 ± 30 ng/mL (95% CI).

In Fig. 2b, the SERS spectrum of cocaine at 10 ug/mL had the strongest band at 1002 cm^{-1} ($\nu(C=C)$ of the aromatic ring)[31] and moderately intense bands at 1181, 1021,

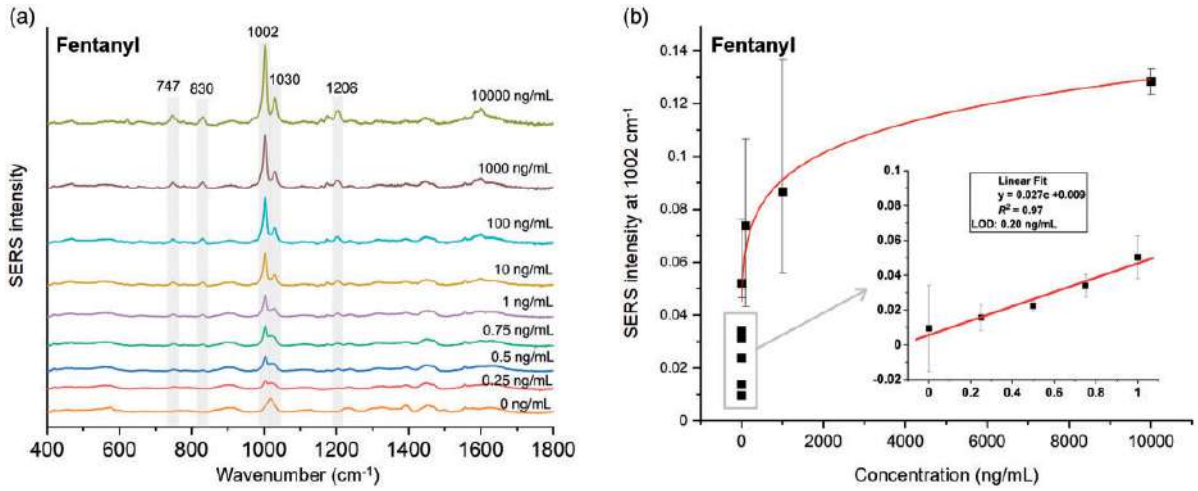

Figure 1. (a) SER spectra of decreasing concentrations of fentanyl with labeled characteristic bands (b) fentanyl intensities at 1002 cm^{-1}. The SERS intensity follows a Langmuir isotherm over the 0–10,000 ng/mL concentration range, with dynamic range over 0–1 ng/mL (inset).

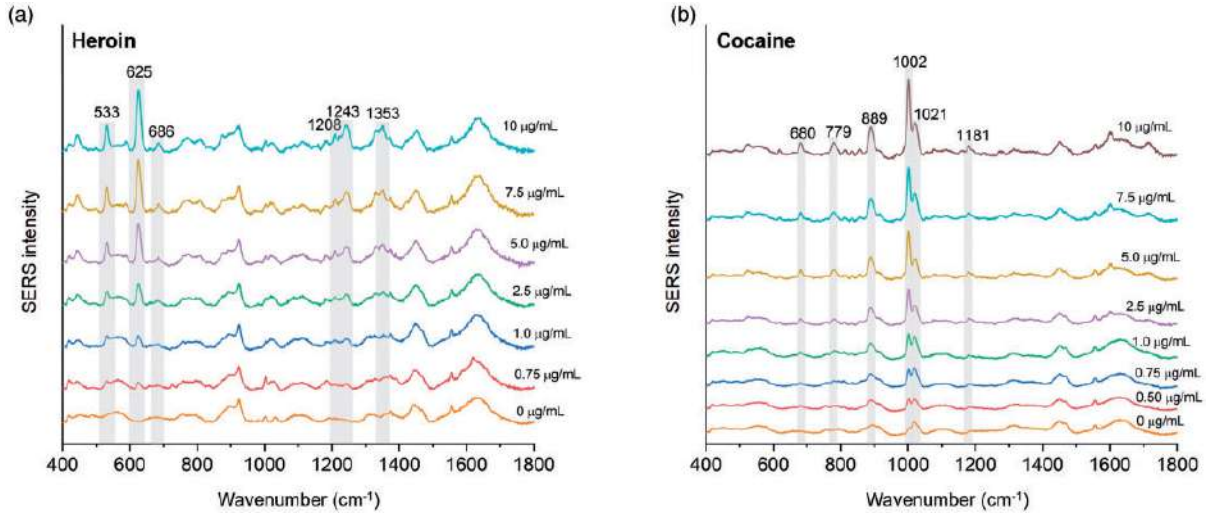

Figure 2. (a) SERS spectra of decreasing concentrations of heroin and labeled characteristic bands, (b) SERS spectra of decreasing concentrations of cocaine and labeled characteristic bands.

889, 779, and 680 cm^{-1}. Only two characteristic bands at 1021 and 1002 cm^{-1} were observable at lower concentrations. Because the band at 1021 cm^{-1} was shared with the background signal of the aggregated colloidal substrate, it was unreliable for the detection of cocaine at the lower concentrations, and therefore the band at 1002 cm^{-1} was selected as a marker for cocaine. When using the intensity of the 1002 cm^{-1} band for quantification, two linear dynamic ranges were observed, one at concentrations below 1 ug/mL and another set at concentrations above 1 ug/mL. Therefore, two calibration curves were plotted, the first for concentrations in the 0–1 ug/mL range, displayed a linear relationship with a sensitivity of 0.043 cts·mL/ng, an intercept of 0.007, and an R^2 of 0.95, the second for concentrations in the 1–10 ug/mL, displayed a linear relationship with a sensitivity of 0.012 cts·mL/ng, an intercept of 0.044, and an R^2 of 0.96. The calculated LOD for cocaine is 100 ± 40 ng/mL (95% CI).

Overall, this Au–Ag NS-based SERS method can detect fentanyl, heroin, and cocaine from 0.2 to 1 ug/mL. This range covers the concentrations of these analytes commonly found in solubilized aliquots of seized drugs. While other excipients may also be present in such mixtures, this paper provides a roadmap for determination. Furthermore, the LOD of fentanyl is in the sub-ng/mL range, indicating this method is also suitable to detect trace levels of fentanyl, such as in the case of trace levels in seized drug mixtures, or toxicological samples.

Quantitative analysis utilizing SERS with a portable Raman can be affected by the presence of auto-exposure and auto-accumulation algorithms. For example in these analyses, the baseline of the collected spectra was highly affected by the environment. To improve detection, the reproducibility of the SERS spectra was calculated using band intensities from 400 to 1800 cm^{-1}. The percent relative standard deviation (%RSD) of fentanyl, heroin, and cocaine were determined individually using raw data, normalized data, as well as with baseline corrected plus normalized data. The intra-day reproducibility of SERS spectra was determined using five spectra for each drug, obtained

Table II. Percent RSD for fentanyl, cocaine, and heroin from spectra recorded using a handheld Raman spectrometer.

		RSD (%)			
Data treatment		Fentanyl	Cocaine	Heroin	Average
Intra-day ($n = 5$)	Raw spectra	23	14	5.8	14
	Normalized spectra	3.6	2.6	3.9	3.3
Inter-day ($n = 10$)	Raw spectra	85	34	44	54
	Normalized spectra	53	40	21	38
	Baseline corrected and normalized spectra	39	24	18	27

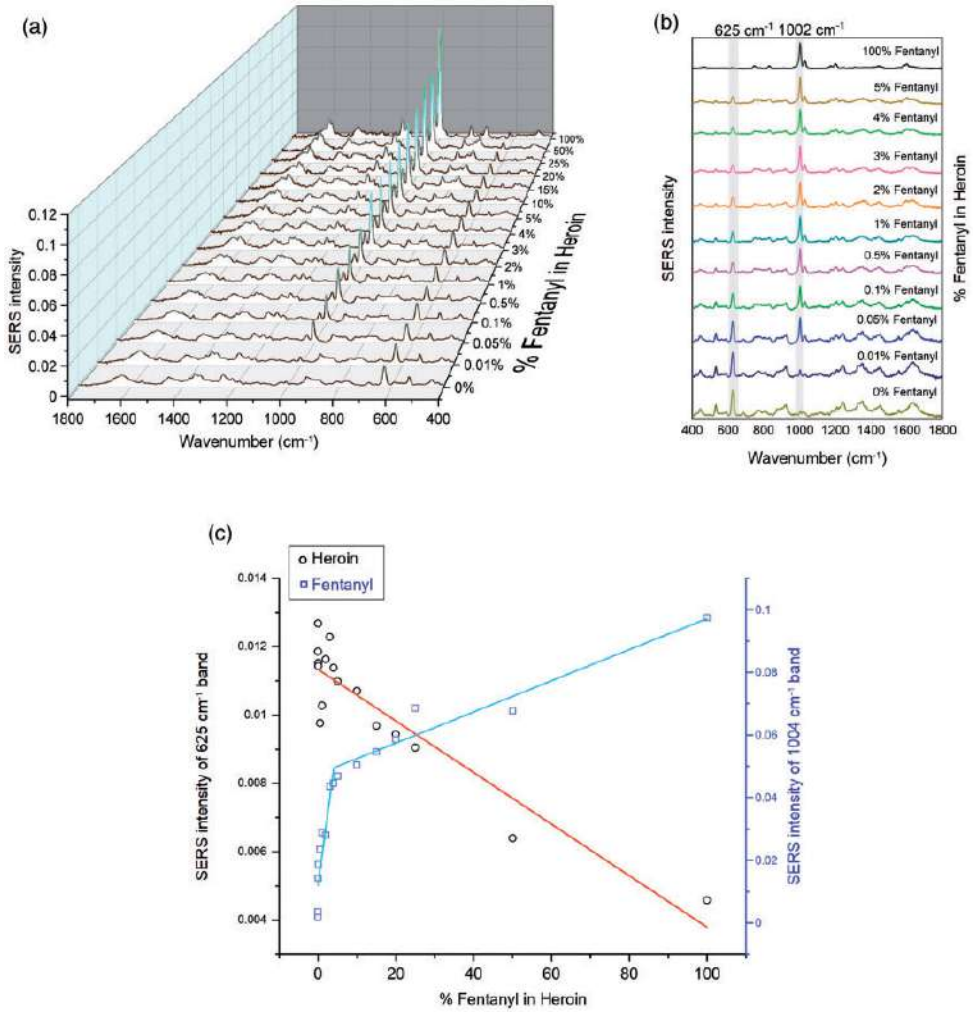

Figure 3. Handheld Raman detection of fentanyl in heroin. (a, b) Different mixture ratios and their characteristic peaks at 625 cm^{-1} (heroin) and 1002 cm^{-1} (fentanyl). (c) Quantitation of fentanyl in mixtures using specific peaks.

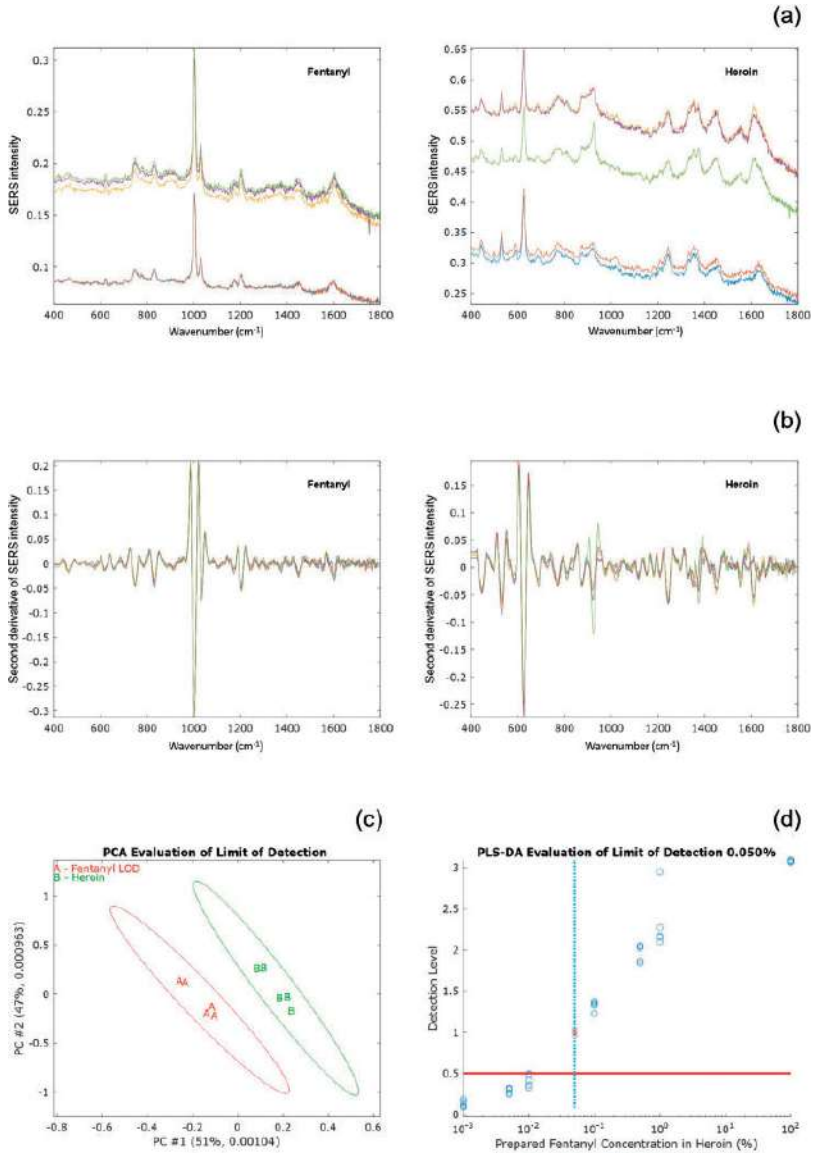

Figure 4. (a) Raw SER spectra of fentanyl and heroin, respectively. (b) Savitzky–Golay second derivatives of fentanyl and heroin. (c) PCA plots from SERS spectra (*n* of 5) of fentanyl (a, left) and heroin (b, right) were used to determine the sensitivity on a handheld spectrometer, while 0.050% fentanyl was the LOD of the mixtures of fentanyl in heroin. (d) SPLS-DA model of 0.050% LOD for low percentages of fentanyl in heroin.

with the same batch of Au–Ag NS. The inter-day reproducibility was calculated using 10 spectra obtained across seven days, by three different operators, with five different batches of Au–Ag NS. As mentioned above, the handheld Raman system utilized in this study operates under an auto-exposure and auto-accumulation mode, and the spectra were affected by the environment during the tests. The intra-day average %RSD was 14%, and it decreased four-fold after normalization. The inter-day average %RSD was 54%. This decreased 1.4-fold after normalization and two-fold after baseline correction and normalization. These values are listed in Table II. The %RSD values show that the intra-day collected spectra were fairly reproducible, while the inter-day spectra required calibration in order to be used for quantification purposes.

SERS Study of Fentanyl/Heroin Mixtures

When using SERS for the analysis of mixtures, the situation becomes more complex. As reported in the introduction, fentanyl is a much cheaper alternative to common illicit opioids such as heroin, and because of this fentanyl has been used as a lacing agent. Due to its high potency, amounts of fentanyl as low as 0.25 mg can cause fatalities,[2] even for experienced drug users. Therefore, it is of

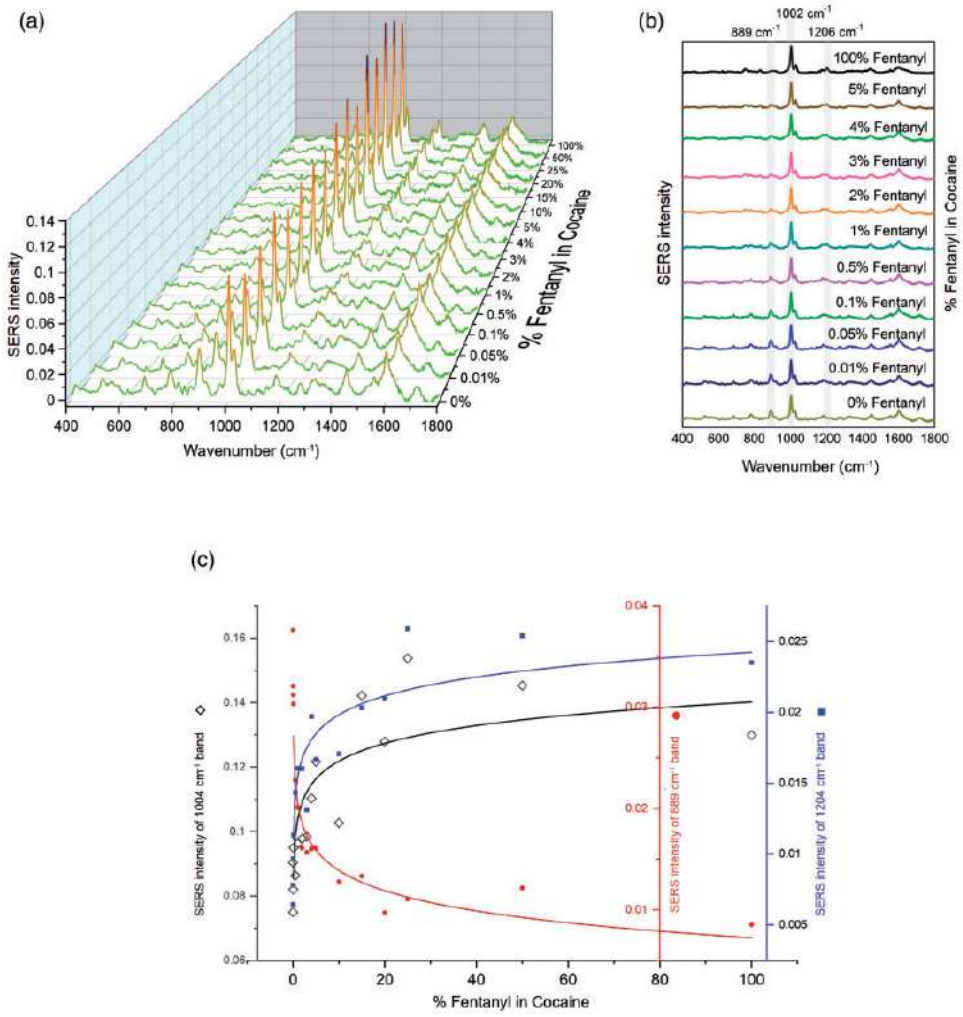

Figure 5. Handheld Raman detection of fentanyl in cocaine. (a, b) Different mixture ratios and their characteristic peaks at 889, 1002, and 1206 cm^{-1}. (c) Characteristic peak approach to quantitation of drugs in mixtures. A nonlinear relationship is observed.

paramount importance to identify trace levels of fentanyl in heroin samples.

In Figs. 1 and 2a, the intensities of the characteristic bands of the two compounds, 1002 cm^{-1} for fentanyl and 625 cm^{-1} for heroin, individually displayed a change according to their dilution which was highlighted in the SERS studies of individual drugs as standard solutions. When fentanyl was present as 0.01% in the mixture samples with heroin, its characteristic band at 1002 cm^{-1} was very weak, but still observable. However, when the amount of the fentanyl was increased to 5%, the intensity sharply increased (Figs. 3a and 3b). When the percentage of fentanyl in the mixtures increased to 10% or higher, the intensity of the band 1002 cm^{-1} had a slower increase, following the Langmuir behavior observed for the individual drug. As for heroin, its band at 625 cm^{-1} decreased with the decreasing heroin content in the mixtures. It must be noted that the band at 1002 cm^{-1} is present only with fentanyl, making it an excellent marker for its identification in heroin mixtures.

However, when regressing both characteristic bands across the range of mixture concentrations of fentanyl in heroin (Fig. 3c), a poor linear relationship was observed for heroin at 625^{-1} at higher concentrations of the mixture (95–100%), while two linear trends were displayed for the fentanyl marker band 1002 cm^{-1}. This finding is similar to the results reported by Inscore et al.[50] While this single-marker band approach allowed for the successful quantitation of the target drugs as single-analyte systems, it did not permit a simple and straightforward way to quantitate these drugs in mixtures. Therefore, to discriminate between different percentages of fentanyl in heroin, we developed a multivariate regression approach which takes advantages of the whole spectrum of a mixture instead of single marker bands.

To distinguish fentanyl from heroin using PCA and SPLS-DA, a two-day test procedure was performed on a single

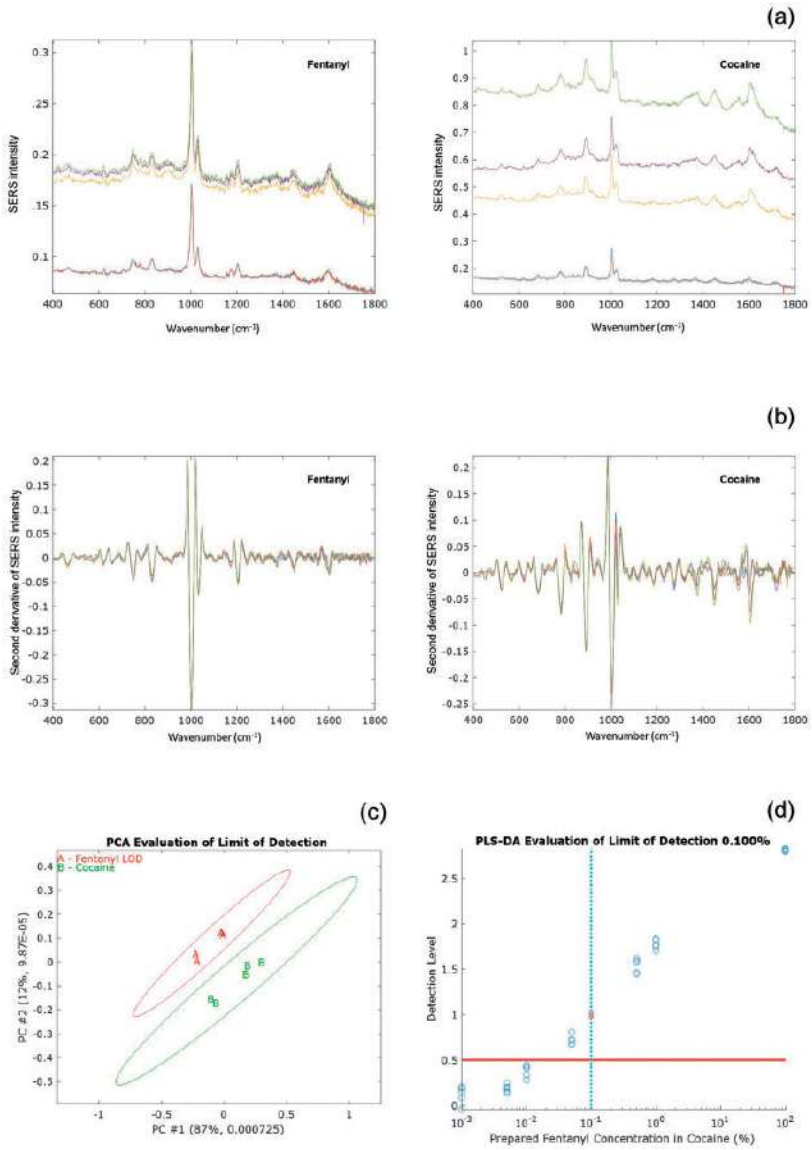

Figure 6. (a) Raw SER spectra of fentanyl and cocaine, respectively. (b) Savitzky–Golay second derivatives of fentanyl and cocaine. (c) The PCA plots from SERS spectra ($n = 5$) of fentanyl (a, red) and cocaine (b, green) were used to determine the sensitivity on a handheld spectrometer, while 0.050% fentanyl was the LOD of the mixtures of fentanyl in cocaine. (d) SPLS-DA model of 0.100% LOD for low percentages fentanyl in cocaine.

Table III. Detection limits of fentanyl in binary mixtures based on different statistical approaches.

	PCA		SPLS-DA	
	Solution	Solid	solution	Solid
Fentanyl/heroin mixture	0.050% fentanyl w/ 99.950% Heroin	5.0 ng fentanyl in 10.0 ug total	0.050% fentanyl w/ 99.950% Heroin	5.0 ng fentanyl in 10.0 ug total
Fentanyl/cocaine mixture	0.100% fentanyl w/ 99.900% Cocaine	10.0 ng fentanyl in 10.0 ug total	0.050% fentanyl w/ 99.950% Cocaine	5.0 ng fentanyl/ 10.0 ug total

batch of Au–Ag NS and five SERS spectra were collected for each sample (fentanyl, heroin, and their mixtures at 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, and 1.00% fentanyl in heroin). Each spectrum of the mixed samples had different baseline offsets across a range of 400 to 1800 cm^{-1} (Fig. 4a). Therefore, the heroin and fentanyl spectra were modified using Savitzky–Golay second derivatives (Fig. 4b), and PCA was performed on these spectra. In Fig. 4c, fentanyl and heroin were well discriminated at 0.050% at a 95% CI, in which PC1 represented 51% and PC2 as 47% of the total variance. Both PC1 and PC2 contributed to the discrimination between fentanyl and heroin with a 95% CI. When fentanyl was 0.001%, 0.005%, and 0.010% in heroin, PC1 represented 89% or higher variances, but the mixture was indistinguishable from the scores of the pure heroin spectra. As the fentanyl concentration increased above 0.050%, the separation between the scores of the spectra improved. This PCA method of validating the detection limit using the 95% confidence intervals is a very conservative measurement in that fentanyl can be reliably detected with confidence as opposed to the IUPAC method of using three times the standard deviation divided by the sensitivity.

A second chemometric approach, SPLS-DA, was also used to study mixtures of fentanyl in heroin. The SPLS-DA model in Fig. 4d was built using pure heroin and a 0.05% mixture, with higher and lower concentrations made as predictions. If the naïve detection criterion of 0.5 on the *y*-axis is lowered, then concentrations of 0.010% and 0.005% may also be detected.

SERS Study of the Fentanyl/Cocaine Mixture

Cocaine is a central nervous system stimulant, and as such, its pharmacological effects are the opposite to fentanyl.[51] There is a common misconception that mixing cocaine with opioids can cancel out or balance the negative effects of opioids.[52] Mixing cocaine with opioids is a popular combination (i.e., speedballs) because the sedative effect of the opioids reduces the anxiety-inducing effect of cocaine. Furthermore, it is a pathway to opioid addiction as tolerance increases, and users find themselves suffering from opioid withdrawal symptoms. The concomitant use of cocaine and fentanyl can pose sever health hazards, including death by respiratory failure.[52] Therefore, we also examined mixtures of fentanyl and cocaine.

Both fentanyl and cocaine exhibited a strong band at 1002 cm^{-1} (Figs. 5a and 5b). When pure cocaine was examined, the band at 1002 cm^{-1} had the lowest intensity," with increasing fentanyl content in cocaine, the intensity of this band, as well as the selected band, 889 cm^{-1} of cocaine and 1204 cm^{-1} of fentanyl increased nonlinearly (Fig. 5c). As previously described for fentanyl/heroin mixtures, five SERS spectra (Fig. 6a) of each cocaine and fentanyl mixture were examined at 0.001, 0.005, 0.010, 0.050, 0.100, 0.500, and 1.000% and processed (Fig. 6b) in the same manner as the heroin–fentanyl mixtures. The principal component scores of fentanyl and cocaine differed significantly when the fentanyl concentration was 0.100% with respect to the 95% CI in Fig. 6c. PC1 and PC2 represented 87% and 12% of the relative variance, respectively. As expected, fentanyl/cocaine mixtures were not discriminated as well as fentanyl–heroin mixtures because both fentanyl and cocaine have strong characteristic peaks at 1002 cm^{-1} and 1028 cm^{-1}, and their SERS spectra only displayed differences in terms of weak and moderately intense bands from 1180 to 1380 cm^{-1} and 850 to 950 cm^{-1}. In contrast, fentanyl and heroin have intense bands from 950 to 1050 cm^{-1} and 600 to 650 cm^{-1}, respectively. The SPLS-DA model also displayed a linear response for lower percentages of fentanyl in cocaine. The 0.100% mixture of fentanyl in cocaine and pure cocaine were used for constructing the SPLS-DA model and the 0.050% predictions appeared above the 0.5 naïve detection criterion.

Conclusion

A gold–silver nanostar-based SERS method has been optimized utilizing a handheld Raman spectrometer, and it was successfully applied to detect fentanyl, heroin, and cocaine, in both single-drug systems and binary mixture systems. In the single-drug system, the quantitation was achieved via a traditional univariate analysis, that is, utilizing a marker band approach. The detection limits for fentanyl were in the sub-nanogram per milliliter range (0.20 ± 0.06 ng/mL), indicating strong potential for future applications in forensic toxicology. For the detection and quantitation of fentanyl in simulated seizures of laced heroin or cocaine, two multivariate analysis approached were used, PCA and SPLS-DA. These chemometric methods utilized the whole spectral range of the fingerprint region (400–1800 cm^{-1}) as opposed to the intensity of selected characteristic bands, significantly improving the detection of fentanyl in the concomitant presence of heroin and cocaine. Detection limits were in the nanogram to microwave level, indicating the method is sensitive enough for applications involving the detection of trance levels of fentanyl in seized drugs.

Declaration of Conflicting Interests

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ORCID iD

Bruce McCord https://orcid.org/0000-0002-8366-1925

Supplemental material

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