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Enhancing Doping Detection of Trenbolone Acetate Using SERS Combined with Drop-Coating Deposition Raman Spectroscopy

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Abstract: ***This study investigates the combined use of surface-enhanced Raman spectroscopy (SERS) and drop-coating deposition Raman (DCDR) for detecting trenbolone acetate, a banned doping agent, at sub-ppb levels. Silver nanoparticles were synthesized and evaluated under 532nm and 785nm laser excitations to identify optimal conditions for spectral enhancement. Results revealed a 60-fold signal enhancement when SERS was combined with DCDR, compared to 11-fold when SERS was used alone. Signal acquisition at the coffee ring edge provided improved intensity, and regression analysis confirmed a detection limit of 9ppb, consistent with World Anti-Doping Agency guidelines. These findings highlight the value of integrating DCDR with SERS to enhance sensitivity in anti-doping applications.***

Keywords: Raman spectroscopy, SERS, DCDR, trenbolone acetate, doping detection

1. Introduction

Conventional Raman spectroscopy is an optical technique that has attracted notable interest in biomedical applications for, among other benefits, being label-free [1] and possessing the ability for in vivo detection of analytes [2]. The unique vibrational modes of any characterized chemical bonds in the molecules allow the optical technique to provide a fingerprint signal for identifying substances [3]. However, the main challenge for its application in biomolecule detection and quantification is its low detection sensitivity [4]. This limitation is even more evident when detecting samples in complex media where the analyte of interest is masked by other features of little or no analytical relevance [5]. Consequently, diverse approaches such as surface-enhanced Raman spectroscopy (SERS), singly or jointly with Drop Coating Deposition Raman (DCDR) spectroscopies have been adopted to solve such limitations [6]. This research aims to evaluate the effectiveness of combining surface-enhanced Raman spectroscopy (SERS) with drop-coating deposition Raman (DCDR) to enhance signal intensity and lower detection limits for trenbolone acetate in anti-doping applications.

Drop coating deposition Raman (DCDR) spectroscopy is a simple and easily accessible technique used to study biomolecules and other complex mixtures [7]. The approach is often based on drying an aliquot of the target analyte on a hydrophobic surface. The drying process introduces and accumulates the target analytes in the 'coffee ring' from where the Raman spectra are obtained [8] which helps to achieve higher sensitivity as compared to the normal Raman spectroscopy technique [9].

Surface-enhanced Raman spectroscopy, on the other hand, benefits from the local field enhancement using a localized surface plasmon resonance (LSPR) that improves the Raman scattering signal [10]. The selection of the most feasible SERS substrate plays an important role in defining the enhancement behaviour. Silver nanoparticles are often selected as possible substrates due to their unique physical, electrochemical, and optical properties that make them useful as substrates in analytical techniques [11]. The characteristics of silver nanoparticles also allow single-molecule detection [12] achieving low detection limits. Although SERS is often employed to achieve signal enhancement, challenges emerge when samples are to be analyzed in liquid form. Kuižová [7] notes that one of the challenges of using SERS is the difficulty in controlling the nanoscale hotspots as well as the problem of placing the target molecules in required hot spots.

Sample preparation of the surface of a SERS substrate is important in achieving the desired efficiency. Methods that have been used to achieve better adsorption of the target chemical species include immersing the SERS substrate in analyte solution before analysis [13], drop-casting the analyte on a surface and letting them dry before analysis [14], [15], mixing both analyte and nanofluid for a specific concentration and then using a sample holder for direct analysis or using drop-casting approach [16]. Combining drop-coating deposition and surface-enhanced Raman spectroscopy emerges as the most feasible approach to improve the detection limit in many optical analytical studies. Cheong et al., [17] employed drop coating deposition surface-enhanced Raman scattering approach to achieve a label-free identification of antibiotic-resistant strains. The approach showed that it was possible to achieve better sensitivity as compared to when SERS is used alone.

By combining SERS and DCDR, the advantages of both optical techniques can be harnessed, resulting in a synergistic enhancement of the Raman signal. As shown in Figures 1 and 2, respectively, while SERS provides electromagnetic and chemical enhancements through the metallic nanostructures, DCDR concentrates the analyte molecules, increasing their density on the substrate [18]. Their combination helps to achieve high signal enhancement and improved detection limits than in conventional Raman.

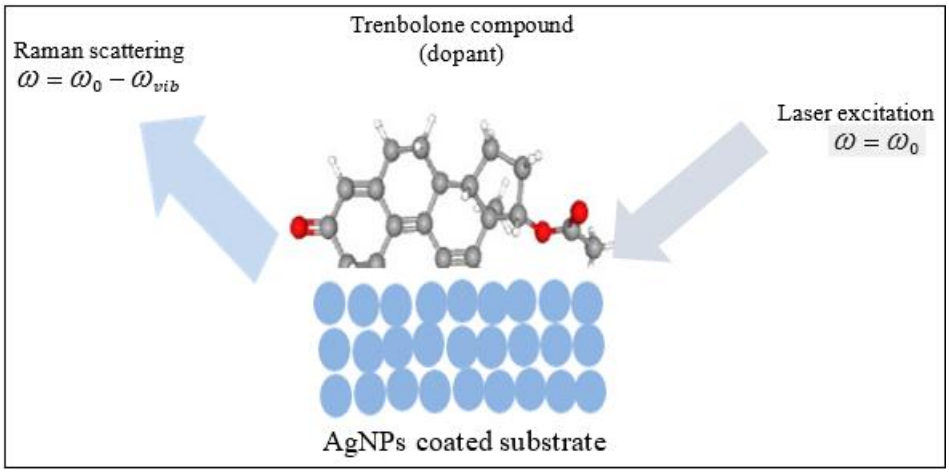

Figure 1: SERS mechanism using pristine silver nanoparticles as substrates.

The enhancement mechanism is given by;

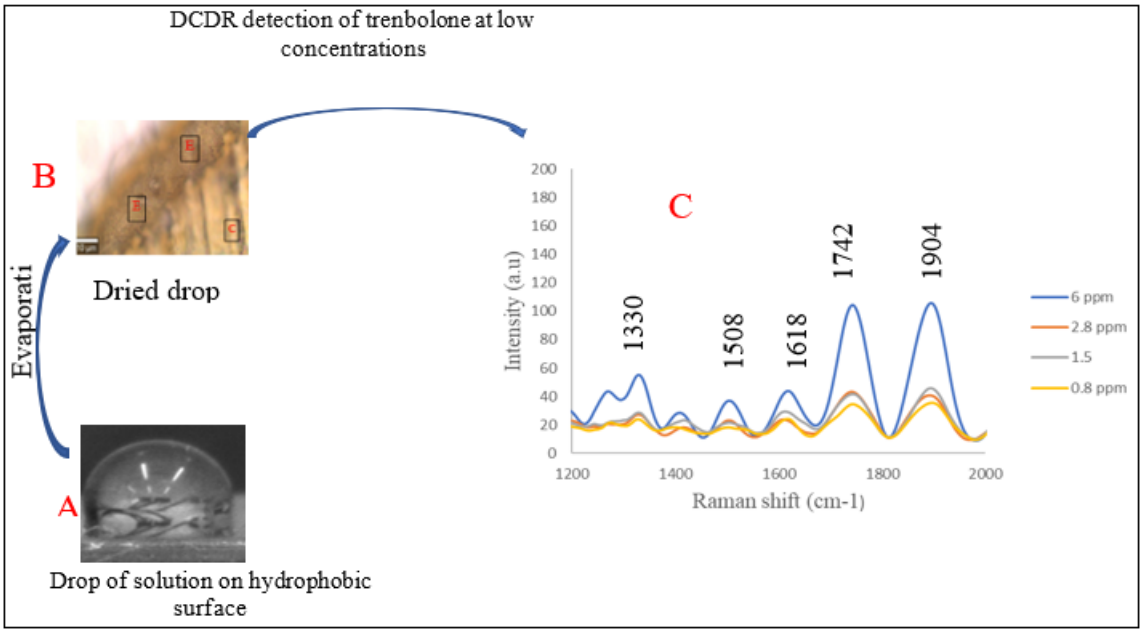

Figure 2: Dried Coating Deposition Raman (DCDR) spectroscopy approach showing a 2µl aliquot drop on Al foil at A; coffee ring effect of the dried drop with the edge and the center of the ring displayed at B; and spectra acquired from the edge of the ring shown at C

$$\mu = \alpha * \varepsilon \quad (1)$$

where α is the chemical enhancement behaviour while ε is the electrical enhancement behaviour. SERS benefits from this enhancement behaviour since the silver nanoparticles provide a localized electric field when placed close to trenbolone acetate. On the other hand, the signal enhancement factor (SEF) is given as;

$$SEF = \frac{\sigma_{SERS}}{\sigma_{Raman}} \quad (2)$$

Where σ_{SERS} is the intensity of the enhanced signal when trenbolone acetate is adsorbed on the silver nanoparticles, while σ_{Raman} is the intensity of the unenhanced signal when silver is used without the nanoparticles. The laser wavelength is an important parameter in obtaining the SERS spectra. Babaei and Savaloni [19] argue that the wavelength used has to excite the collective motions of the plasmons of the SERS substrate as dictated by the electromagnetic theory of the employed substrate that accounts for the desired signal enhancement. Unfortunately, in some instances, resonance Raman can occur with the laser wavelength being in resonance with the electronic transitions of the sample being analysed. Consequently, enhancement of unwanted components may occur at the expense of the target analyte. In this work, both 532nm laser and 785nm laser were used to characterize silver nanoparticles to delineate the wavelength that provides better spectral features of the SERS substrate with minimal interference.

This combined approach maximizes the signal enhancement, thereby enhancing detection of trace-level analytes and weak Raman scatterers. Hu et al., [20] noted that integrating SERS and DCDR enables the extraction of multiparameter information that enables analysis of body fluids. The significance of this study lies in its potential to improve anti-doping efforts through a non-destructive, label-free, and highly sensitive detection approach for steroidal compounds at trace levels.

2. Materials and Methods

2.1 Preparation of Colloidal Silver Nanoparticles

99.99% analytical grade silver granules were obtained from Sigma Aldrich, while Aluminium foil was obtained from the local supermarket. The silver nanoparticles were prepared as described by [21] and [22]. 2µl aliquot volume of the analyte was drop-cast on the substrate and allowed to air dry before characterization.

2.2 Raman spectral characterization

The process of optimizing the type of laser was done using a standard stand-alone confocal laser Raman spectrometer (STR Raman Spectrum System, Seki Technotron Corp-Japan). The system is equipped with a 300mm imaging triple grating monochromator spectrograph and two lasers emitting at 532nm and 785nm. The 300mm imaging spectrometer had 600,1200 and 1800 lines/mm gratings. A sample focusing on the coffee ring edges was done using a motorized stage. The system was equipped with STR software and a camera attached to the microscope, making it easy to visualize the region of interest. The HeNe excitation laser power was optimized at 25mW and x50 objective (NA 0.5, Olympus. The laser focus was maintained at 1µm. Five approximately equidistant positions were randomly selected on the edge of the "coffee ring", and at each point, six spectra were taken. A total of thirty spectra were taken for each sample to compensate for possible inhomogeneity in the drop coating deposition Raman.

3. Results and Discussion

3.1 Characterizing Silver Nanoparticles using 532nm and 785nm Laser

The strength of the laser plays an important role in determining the efficiency of signal acquisition of surface-enhanced Raman spectra [23]. Better characterization is achieved with a laser that has less background interference and provides better signal information. Different peaks were observed within the 150cm^{-1} to 1800cm^{-1} spectral range. The

Some of the peaks that were observed were 204cm^{-1} for the 785nm laser, which appeared at 199cm^{-1} when using the 532nm laser. An intense band at 415cm^{-1} was visible for the 785nm laser and almost invisible for the 532nm laser. The small band at 521cm^{-1} was observed when using 785nm and shifted to 505cm^{-1} when using a 532nm laser. The peak at 791cm^{-1} was the most intense band when using a 532nm laser. This band was way low when using a 785nm laser. 910cm^{-1} and 1164cm^{-1} were only evident when using the 785nm laser, while the bands at 1391cm^{-1} and 1578cm^{-1} were only sharper and more enhanced using the 785nm laser as compared to the 532nm laser.

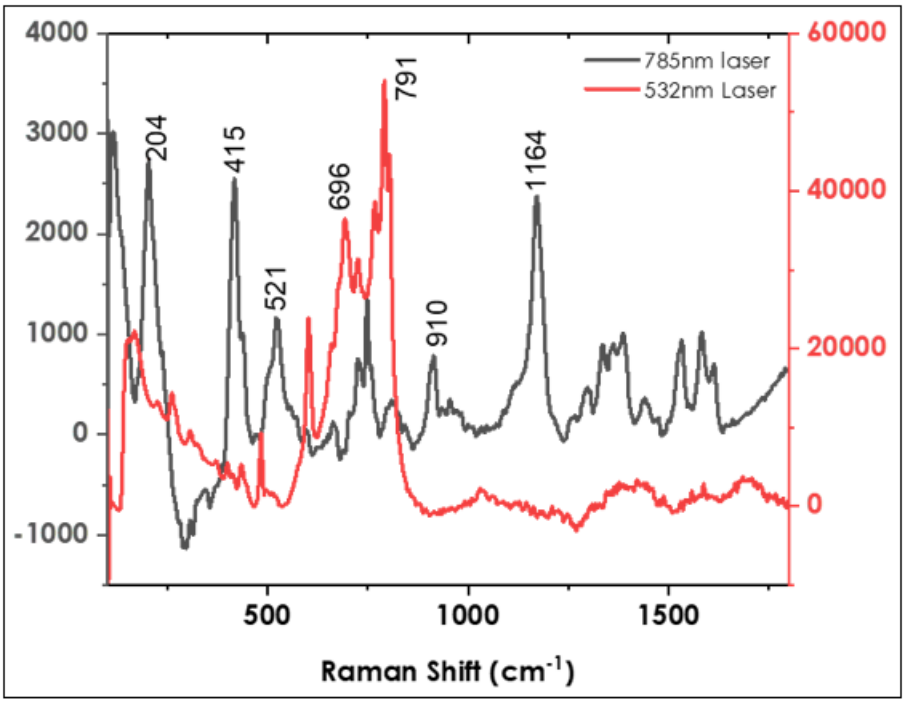

Figure 3: Characterizing silver nanoparticles using 785nm and 532nm Lasers using 25mW laser power, x50 objective lens and 10 S exposure time accumulated 5 times.

The significant spectral variations of the 785nm and 532nm lasers when characterizing silver nanoparticles paint a better picture of subsequent SERS characterization. 785nm laser is more feasible for such characterization since it provides more spectral features and improved signal intensity. The prepared silver nanoparticles had a plasmon resonance band around 411nm (the green region). Since the 532nm laser is closer to this region, it benefits from the strong plasmonic effects that eventually result in significant enhancement of the signals in the UV region which is between 530cm^{-1} and 795cm^{-1}. Therefore, to properly characterize silver nanoparticles, a 785nm laser can be used since it reveals the peaks that are masked by the high fluorescence 532nm laser.

The lack of more spectral information within the medium frequency region of 1000cm^{-1} and 1800cm^{-1} when using

532nm laser suggests that subsequent characterization when the probe is adsorbed to the nanoparticles would probably be explained better using 532nm laser since any Raman scatter bands within this region would easily be attributed to the probe. This is unlike when using a 785nm laser where more silver nanoparticle bands would easily overlap with the signals from a probe that has Raman active modes within the same region.

3.2 Signal Intensity and Signal Distribution in DCDR

When the drops of a prepared mixture of colloid and trenbolone acetate probe were deposited on Aluminium foil, the drying effect was such that the mixture moved on the edge of the ring during the evaporation process. Petra et al., [24] noted that SERS spectra are always influenced by fluctuations in signal intensity and distribution. Therefore, a suitable place often has to be used for an optimal signal intensity to be found. The optical microscope bright field image of the dried drop of the mixture is shown in Figure 4.

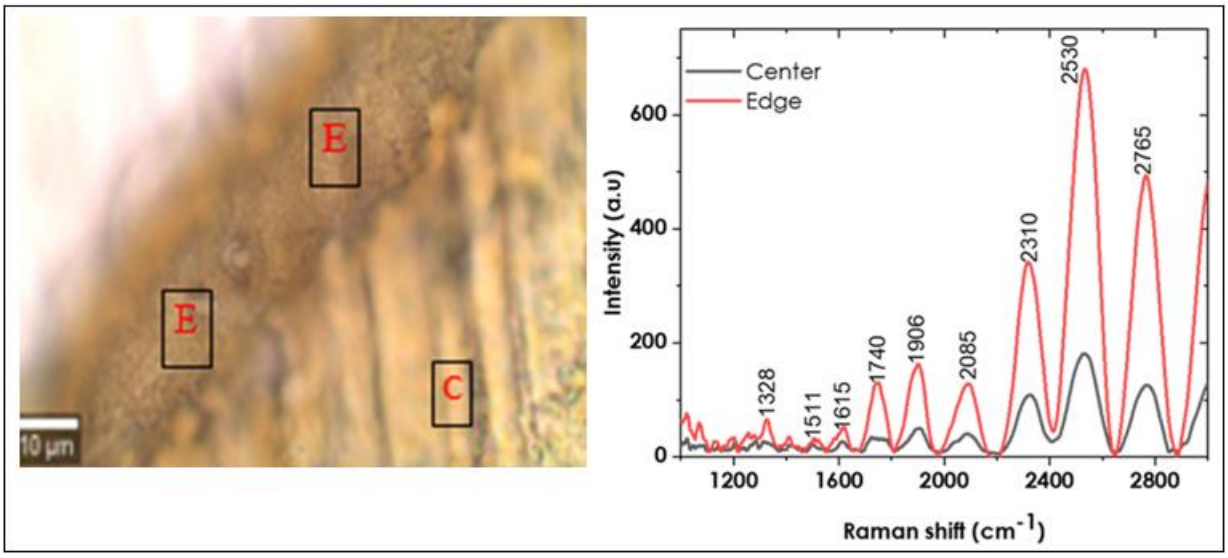

Figure 4: Optical image of a dried coffee ring of a probe mixed with silver nanoparticles with E depicting the edge of the ring and C depicts the center. On the right is the signal distribution at the center and edge of the ring

The signal of the SERS analyte was measured at the edge and centre of the dried drop. The results showed a lack of homogeneity in terms of how the probe concentrations are distributed. The signal obtained at the edge reported a threefold higher enhancement factor than the spectra obtained at the centre of the ring. During spectral acquisition, it was noted that even on the edge of the ring, intensity variation could be observed. The spectra reported in Fig. 4 are an average of thirty spectra taken at the edge as well as at the centre of the ring. The results reported in this work were similar to [24] who argued that the ring region gave a higher signal intensity and more Raman features than the central region. Therefore, as an optical instrument, Raman spectroscopy is sensitive to the analyte distribution and as such, when using drop coating deposition, acquiring the spectra at the edges of the ring would give better signals than when taking the spectra at the centre of the coffee ring. The 1196cm

3.2 SERS and drop coating Deposition Raman Spectroscopy Analysis of Trenbolone Acetate

Trenbolone acetate was characterized with the help of conventional Raman, SERS (by mixing Tren Ac with silver nanoparticles and characterizing directly in liquid) and by drying the prepared mixture on Aluminium foil. As shown in Figure 4, drop casting the SERS-prepared trenbolone acetate sample gave the highest signal intensity as compared to when SERS was employed alone and way better than when conventional Raman was employed. The signal enhancement can be obtained as given by [25] and as described in Equation 2.

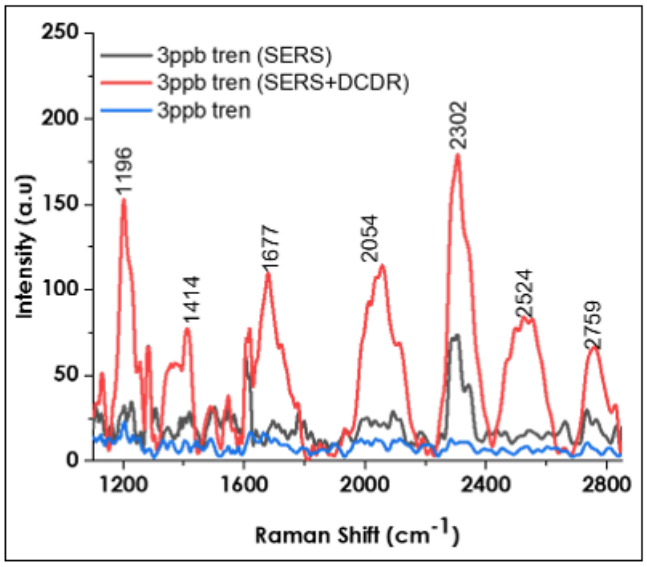

Figure 5: Raman spectra of 3ppb Trenbolone for SERS and SERS when combined with drop coating deposition Raman

The 1196 cm-1 Raman scatter was used to calculate the enhancement factor. The enhancement factor for SERS+DCDR was obtained to be 60-fold, while for SERS in liquid, the enhancement factor was noted to be 11-fold. Therefore, combining SERS and drop coating deposition helped to significantly improve the signal that can be

leveraged in analytical detection for sub-ppm analyte concentrations.

The 1196cm^{-1} band was assigned to the C-O stretching vibration that may as well be associated with the ether linkage that is often associated with any steroid. The band at 1414cm^{-1} can be assigned to the CH_3 rocking and bending vibrations. 1677cm^{-1} is assigned to the C=O stretching vibration, which is often due to ketone functional groups associated with steroids [26]. The other bands above 2000cm^{-1} can also be assigned to C-H stretching vibrations from the aliphatic hydrocarbon chains.

The sensitivity of surface-enhanced Raman/dried coating deposition Raman optical approach in detecting trenbolone acetate was evaluated using regression analysis for a set of eight concentrations. The analysis of variance for these concentrations showed that the band at 1617cm^{-1} was the most responsive to changes in analyte concentrations at sub-ppm levels. Figure 6 shows that the concentrations adhere to the Beer-Lambert law with a 95% correlation as given by the R^2 value.

From the calibration sensitivity curve;

$$LOD = 3.3(Sy/S) \quad (3)$$

From equation 3, the limit of detection was found to be 9ppb, which falls within the World Anti-Doping Agency (WADA) recommended limits. Therefore, combining SERS and dried coating deposition Raman not only enhances the signal but also lowers the detection limit for the target analyte.

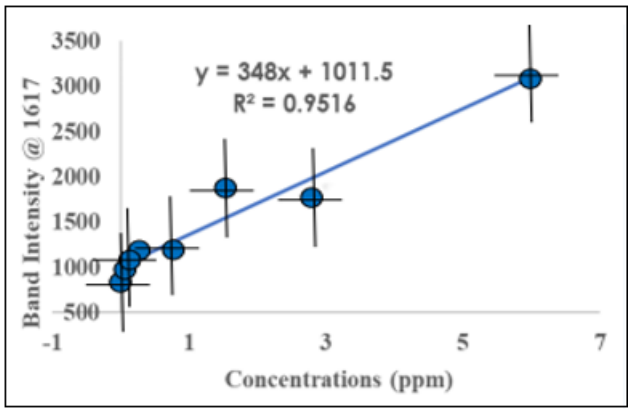

Figure 6: Calibration curve Analysis for trenbolone acetate concentrations.

4. Conclusion

In conclusion, we have shown that both SERS and drop coating deposition Raman (DCDR) can be used as complementary techniques to improve the signal intensity of a probe at a sub-ppb concentration level. The enhanced signal can help to achieve better sensitivity when detecting dopants like trenbolone acetate in complex matrices as is often the case in anti-doping tests. While DCDR provides a way to preconcentrate the probe and consequently improve signal detection, the optimization process is shown to be critical in achieving desired results. The DCDR leveraged the hydrophobic nature of aluminium foil to preconcentrate the trenbolone acetate/silver nanoparticle complex. The work has shown that taking spectra on the edge of the coffee ring helps to achieve a better signal intensity than when spectra are taken at the centre of the ring. The type of laser strength also showed to be important in improving the characterization of silver nanoparticles before they are subsequently applied in SERS. 785nm laser was more reliable in providing spectra with more spectral features and with minimal background interference. Optimizing the laser wavelength and spectral strength distribution on the dried drop and using aluminium foil as a hydrophobic surface was key in achieving the efficiency of drop coating deposition Raman. Therefore, this work has shown that drop-coating deposition Raman can complement surface-enhanced Raman spectroscopy in detecting analytes in sub-ppm concentrations in complex matrices like blood which is often the case in routine doping analysis.

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