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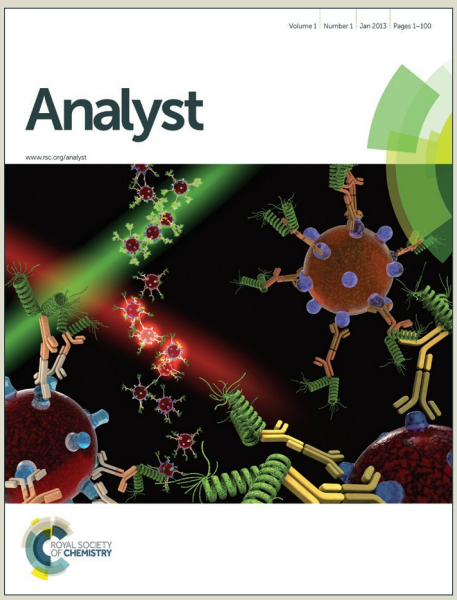

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Detection and quantification of the opioid tramadol in urine using surface enhanced Raman scattering.

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1 Abstract

There is an on going requirement for the detection and quantification of illicit substances. This is in particular the case for law enforcement where portable screening methods are needed and there has been recent interest in breath tests for a range of narcotics. In this study we first developed surface enhanced Raman scattering (SERS) for the detection of tramadol in water and establish a robust and reproducible methods based on silver hydroxylamine colloid. We used 0.5 M NaCl as the aggregating agent, with the pH was ~7.0 and SERS data were collected immediately (i.e., the analyte association and colloid aggregation times were zero). The limit of detection was rather high and calculated to be 5×10^{-4} M which would not be practical in the field. Undeterred we continued with spiking tramadol in artificial urine and found that no aggregating agent or modification of pH was necessary. Indeed aggregation occurred spontaneously due to the complexity of the medium which is rich in multiple salts, which are commonly used for SERS. We estimated the limit of detection in artificial urine to be 2.5×10^{-6} M which is equivalent to 657.5 ng/mL and very close to the levels typically found in individuals who use tramadol for pain relief. We believe this opens up opportunities for testing SERS in real world samples and this will be an area of future study.

2 Introduction

2.1 General

Tramadol hydrochloride is a pain-relieving drug and is available either as a liquid injectable formulation for immediate release or in tablet form for extended release.[1] Tramadol hydrochloride was launched in 1977 in Germany (Grünenthal Aachen, Germany) who specialized in the production of medications for treating pain (see Supplementary Information (SI) for the tramadol synthetic scheme), and these are commercialised in more than 80 countries.[2 3]

Tramadol hydrochloride is a non-narcotic medication which belongs to the opioid group. The mechanism of action is not yet fully understood: some suggest that tramadol, or one of its metabolites, binds to μ-opioid receptors;[4] while others indicate that it inhibits the reuptake of norepinephrine and serotonin neurotransmitter.[5] Unfortunately some people abuse it for its euphoric effects and may become physically dependent on it.[6, 7] Hence, several countries have now band tramadol and it is only made available to individuals under regulated prescriptions.[8-10]

It is therefore important to measure the levels of tramadol in human body fluids and this is currently achieved using liquid chromatography-mass spectrometry (LC-MS).[11-13] Whilst LC-MS is a very powerful analytical approach it does not lend itself to field use. By contrast the vibrational spectroscopic technique of Raman spectroscopy is readily portable and the Raman spectrum of a compound provides a molecularly specific fingerprint that can be used for the identification of the compound and its quantification. Moreover, Raman spectroscopy is particularly attractive for the analysis of biofluids as water is a very weak Raman scatterer. However the Raman effect is generally very weak and the signature needs to be enhanced. This is typically achieved using surface enhanced Raman scattering (SERS), and both Raman and SERS have been used to measure a number of illicit substances and legal highs.[14-17]

The aim of this study was to investigate whether it was possible to develop a SERS based portable assay for tramadol at concentrations found in urine after its administration. In addition, artificial urine was spiked with tramadol to test whether this assay could be used to measure tramadol *in situ*.

3 Experimental

3.1 Tramadol and sample preparation

Tramadol is a Class C agent in the UK and was purchased as tramadol hydrochloride (>99.0%) from Sigma-Aldrich (Sigma-Aldrich Dorset, UK).

Initial experiments were conducted to generate conventional Raman spectra of tramadol and 2-3 μg of powdered tramadol was placed on a small calcium fluoride disc and Raman spectra recorded using excitation at 633 nm (see SI for details of the conventional Raman measurements).

For quantitative SERS analyses once the SERS substrate conditions was optimised tramadol was dissolved in water and diluted to between 1×10^{-2} to 5×10^{-5} M. For quantitative analyses in urine we used artificial urine[18] and this was prepared in 1 L of distilled water as follows (g): peptone L37 (1), yeast extract (0.005), lactic acid (0.1), citric acid (0.4), sodium bicarbonate (2.1), urea (10), uric acid (0.07), creatine (0.8), $CaCl_2{\cdot}2H_2O$ (0.37), NaCl (5.2), $FeSO_4.7H_2O$ (0.0012), $MgSO_4.7H_2O$ (0.49), $Na_2SO_4.10H_2O$ (3.2), KH_2PO_4 (0.95), K_2HPO_4 (1.2), NH_4Cl (1.3). The pH adjusted to 6.5 as this is typical of healthy urine.[19]

3.2 SERS

For all SERS analyses Raman spectra were obtained using a DeltaNu® Advantage 200A portable Raman probe (DeltaNu Inc, Laramie, Wyoming, WY, USA) and this spectrometer was controlled using DeltaNu, NuSpec™ instrument control software. The Raman probe utilised a diode laser operating at 633nm; the output power of the laser was *ca*. 60 mW at laser head and ~30 mW on the sample. Daily calibration of the instrument was achieved by obtaining the Raman spectrum of polystyrene using the calibration routine built into the software. For all analyses the spectral range is 2000 to 200 cm^{-1} with a spectral resolution of 8 cm^{-1}.

For SERS investigations initial experiments were conducted to optimise the SERS conditions. Four different stock colloids were prepared, and these included silver citrate, silver hydroxylamine, silver borohydride and gold citrate. Full details of colloid syntheses are provided in the SI, along with the UV-visible conditions used to characterise these colloids. The aggregating agent used (NaCl or KNO_3) and its concentration were also optimised, as

were the association times of tramadol with the metal nanoparticles, the time for optimal aggregation to occur and the best pH was also assessed. These are discussed in the Results and Discussion section.

4 Results and Discussion

Initial measurements were conducted using Raman spectroscopy using 633 nm excitation. A typical raw spectrum from powdered tramadol hydrochloride collected on a Raman microscope is provided in Figure S3, and Table S1 provides tentative assignments of the major bands. Due to the low scattering efficiencies of the normal Raman effect it is unlikely that Raman spectra would be able to detect low tramadol levels in samples that are forensically important.

4.1 SERS Optimization

SERS is a method that can be used to enhance the relatively weak Raman signal using roughened metal surfaces, most notably of silver or gold. In order for SERS to be reproducible the SERS process has to be carefully optimised and this includes: (i) the type of metal nanoparticles for colloidal-based SERS; (ii) the time that the analyte (tramadol in our case) needs to associate with the metal surface and this can include modification of the pH of the analyte-colloid suspension; (iii) the choice of the most appropriate aggregating agent and its concentration; and (iv) the time needed after aggregation for the most reproducible SERS signal to be generated.

The above four variables above were assessed in an iterative fashion. Initial experiments explored the most appropriate metal surface for SERS and we synthesised multiple batches of silver citrate, silver borohydride, silver hydroxylamine, and gold citrate colloids. The particle size distributions were assessed using UV-visible spectrometry and full details of this and all syntheses are provided in the SI (see text and Figure S1). Initial experiments used tramadol at a concentration of 0.01 M and repeat spectra from the different batches were assessed by eye for reproducibility and information content. Whilst all four SERS substrates showed evidence of Raman bands from tramadol those from the silver hydroxylamine (with NaCl as the aggregating agent; and see below) were the most reproducible as well as containing features (Figure S2). Therefore silver hydroxylamine was used for all further tramadol analyses and three batches of this colloid were synthesised.

Now that the most appropriate colloid was selected the next stage was to observe the effect of analyte-colloid association time on the SERS signal. 0.01 M tramadol was mixed with silver hydroxylamine colloid and left under static conditions at room temperature to associate for between 0 to 60 min and samples were analysed every 5 min during this period. Figure 1A shows plots of the raw SERS spectra over this time course and the most prominent SERS peak at 993 cm^{-1} was used to assess the effect of time on SERS intensity. Figure 1B is a plot of this ring breathing vibration at 993 cm^{-1} against time and shows that the analyte associates very quickly with the metal surface and there was no signal difference in the first 10 min of association. However, after 10 min there is an initial quick decline in signal that then levels out after 25 min. This suggests that the colloid may be saturated with tramadol molecules and that this may somehow affect the aggregation using NaCl; although this is an interesting observation we shall not investigate this point further. For all subsequent analyses we set the associate time to 0, that is to say the addition of the aggregating agent occurred immediately after the tramadol was added to the colloid.

The next stage was to assess the aggregating agent itself and we tested NaCl and KNO_3 initially at 0, 0.5 and 1.0 M concentration. As NaCl produced a stronger and more reproducible SERS response this was then assessed from 0.2 M to 1.0 M, and we discovered that 0.5 M was optimal. Often the aggregation process occurs slowly [20] and it is therefore important to decide on the most appropriate time to start acquiring data after the addition of 0.5 M NaCl. Figure 2A show the raw SERS data of 0.01 M tramadol taken every 30 s after the aggregating agent was added, and Figure 2B the temporal response of the peak at 993 cm^{-1}. It is clear from this figure that the aggregation has been almost instantaneous and the loss of signal at longer time points is due to the colloid crashing out of suspension as it precipitates. Therefore we collected data immediately after the aggregating agent was added to the analyte-colloid mixtures.

Finally we changed the pH of the tramadol-colloid mixtures and assessed the effect on SERS. The pH was varied from 2.5 – 10. 5 using citric acid and NaOH. SERS data were acquired immediately (Figure S4). The effect on signal strength has again been assessed using the peak area under 993 cm^{-1}, from five repeat analyses, and the highest signal intensity occurs at pH 7.0. Inspection of the box-whiskers shows a tight distribution of this peak area (and the ones in that pH vicinity) which highlights that these conditions are reproducible. It is worth noting that pH 7.0 is the natural pH of tramadol hydrochloride when it is dissolved in water, and that this molecule has a pKa of 9.41 [21] which explains the drop in signal when the pH > 9.5. The

drop in signal under acidic conditions can be explained by the acid interaction with hydroxylamine which has a pKa of 5.95.[22]

4.2 Limit of detection

The optimised SERS conditions for the analysis of tramadol were therefore established: silver hydroxylamine colloid was used with 0.5 M NaCl as the aggregating agent, the pH was ~7.0 and SERS data were collected immediately (i.e., the association and aggregation times were zero). The next stage was to assess the limit of detection of tramadol using these conditions and the peak area for 993 cm^{-1} from the ring breathing vibration in tramadol.

As 10^{-2} M tramadol was used for the above SERS optimisation this stock solution was diluted in water down to 5×10^{-5} M. SERS spectra were collected from six different analyte concentrations (5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3} and 1×10^{-2} M tramadol) and these are shown in Figure 4A where it is clear that the peak at 993 cm^{-1} is concentration dependant, as this peak starts to be observed at 5×10^{-4} M and increases up to the maximum tramadol level analysed (1×10^{-2} M). Having established that there was a concentration dependent peak in order to mimic the forensic situation we also conducted a dilution of tramadol from 8.75×10^{-3} to 1.25×10^{-6} M in artificial urine (see Table S2 for information) and a subset of these SERS spectra are shown in Figure 4B.

It is important to note that on addition of the silver colloid to the artificial urine (with and without tramadol) that aggregation occurred 'spontaneously' and this is likely to be due to the additional salts and other organic acids present in this medium. It is however also clear from the SERS spectra in Figure 4B that the ring breathing mode at 993 cm^{-1} can be used to quantify the level of tramadol in artificial urine.

The next stage was to establish the limit of detection of tramadol in both water and artificial urine and this involved plotting the area of the 993 cm^{-1} against the know tramadol concentration (Figure 5). Figure 5A is a plot of peak area at 993 cm^{-1} *versus* concentration of tramadol and it is clear that in this range there is an excellent relationship between peak area and drug concentration and a very good straight line fit of R=0.965 was calculated for this region. This was repeated for tramadol in artificial urine over a wider range from 10^{-2} M to 10^{-6} M (Figure 5B) where it is clear that the signal is linear between *ca.* 10^{-3} – 10^{-4} M, and certainly below 10^{-4} M there is very little signal from the tramadol at all. Finally we

calculated the limit of detection (LoD), based on 3 times the standard deviation in the blank [23], for tramadol in water as 5×10^{-4} M and in artificial urine as 2.5×10^{-6} M.

We were a little surprised that the limit of detection for tramadol in artificial urine was significantly lower that in was in water. On reflection we think that this may be due to increased colloid aggregation and better association of tramadol with the silver surface due to the additional chemical species found in the artificial urine. As detailed in the Experimental section this medium contains in it (amongst other things) a range of organic acids (*viz*. lactic acid, citric acid, uric acid) as well as sodium bicarbonate, urea, and creatine. In addition this artificial urine is rich in salts, which are commonly used as aggregating agents and these include NaCl (which we indeed used in isolation at 0.5 M for the tramadol in water optimisation experiments), $CaCl_2{\cdot}2H_2O$, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $Na_2SO_4.10H_2O$, KH_2PO_4, K_2HPO_4 and NH_4Cl, and so we believe that many of these components may act in combination as additional aggregating agents. This opens up the rather interesting idea of using combinations of salts for increasing SERS signals and this will be explored in the future.

5 Conclusion

This study has developed a simple robust SERS-based method for the measurement of the opioid tramadol. As of March 2014 tramadol has been classified as a Schedule 3 Controlled agent in the UK, and so exempt from safety custody regulations[24,25, 26]. It is however a controlled substance in the Kingdom of Saudi Arabia where its use as a narcotic is increasing[27], and thus there is a need for its detection in human biofluids.

After careful optimisation of the SERS processes we found that silver hydroxylamine colloid gave the most reproducible data when NaCl was employed as the aggregating agent under neutral pH conditions. However, surprisingly we found that there was increased sensitivity when we conducted the same experiments in artificial urine and this allow us to effect detection limits in artificial urine of 2.5×10^{-6} M which is equivalent to 657.5 ng/mL.

In drug users the typical level for tramadol found in urine ranges from 200-3000 ng/mL[28] which is *ca*. 8×10^{-7} to 1×10^{-5} M, and this has been established using GC-MS.[28] Our method is within this range which his highly encouraging for a portable technique. We believe that with further optimisation we shall be able to push the detection limit lower and this could be achieved by concentrating the sample prior to analysis.

In conclusion, the study demonstrated the efficacy of colloid-based SERS for the detection and quantification of tramadol in artificial urine. Moreover, as we used a small Raman spectrometer this opens up the possibility of on-site testing due to the portability of the technique. Future would involved more complex matrices such as human urine and human blood and this will hopefully pave the way forward for the deployment of SERS for the quantitative detection of tramadol and its major metabolites in biological matrices.

6 Acknowledgements

OA thanks forensic and toxicology centre and ministry of health in Saudi Arabia for funding. YX thanks Cancer Research UK for funding and RG is indebted to UK BBSRC for financial support of Raman spectroscopy (BB/L014823/1).

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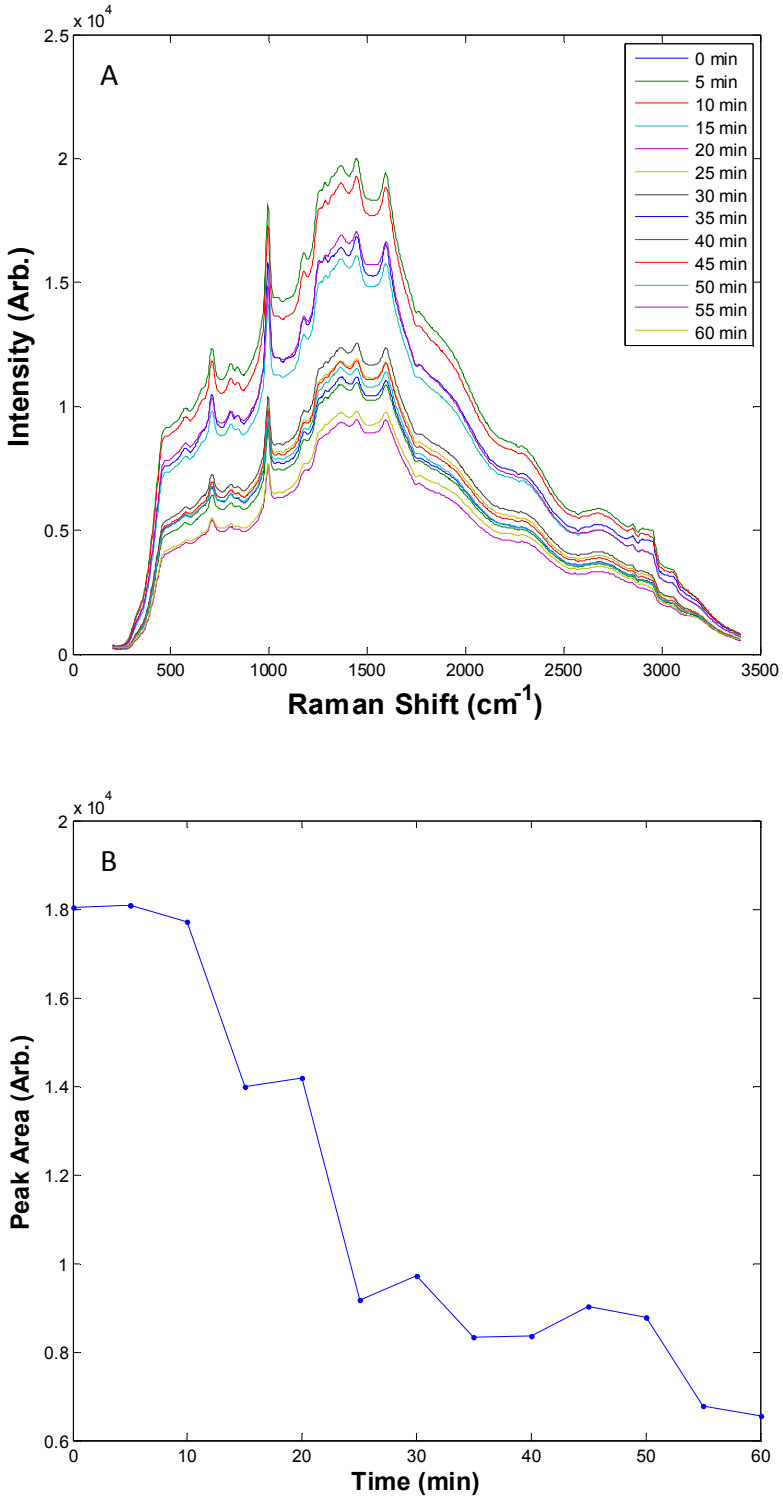

Figure 1: Investigation of SER-analyte association time: (A) Raw SERS spectra of tramadol mixed with silver hydroxlamine colloid and left for intervals of 5 min prior to the addition of 0.5 M NaCl as the aggregating agent; (B) Plot of the area under the tramadol SERS peak (933 cm^{-1}) *versus* the association time (0-60 min).

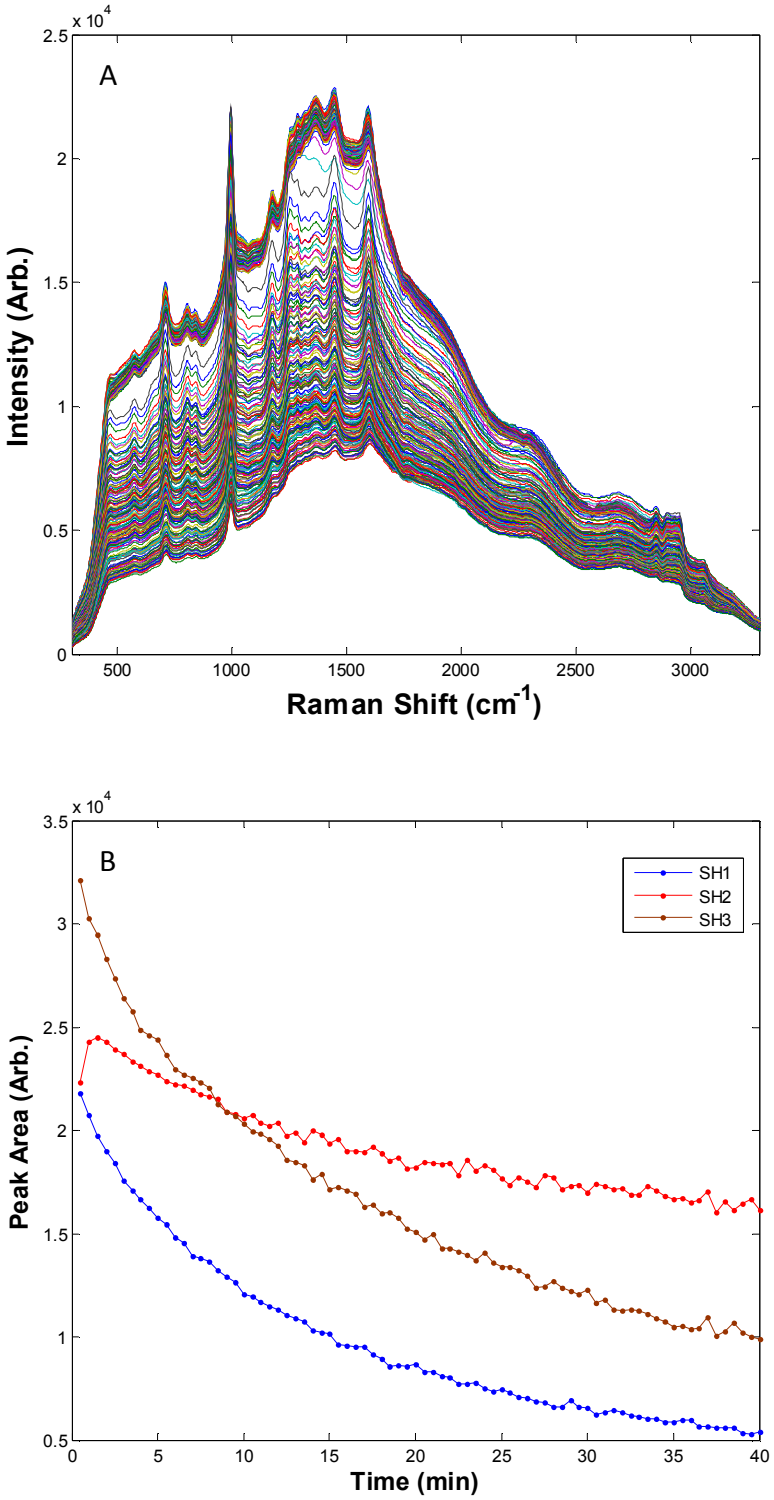

Figure 2: Investigation of the SERS aggregation dynamic: (A) SERS spectra collected for 80 readings after addition of 0.5 M NaCl as aggregating agent; (B) Plot of the peak area under 993 cm^{-1} against time for the 80 readings. In these experiments three batches of silver hydroxylamine colloids were assessed.

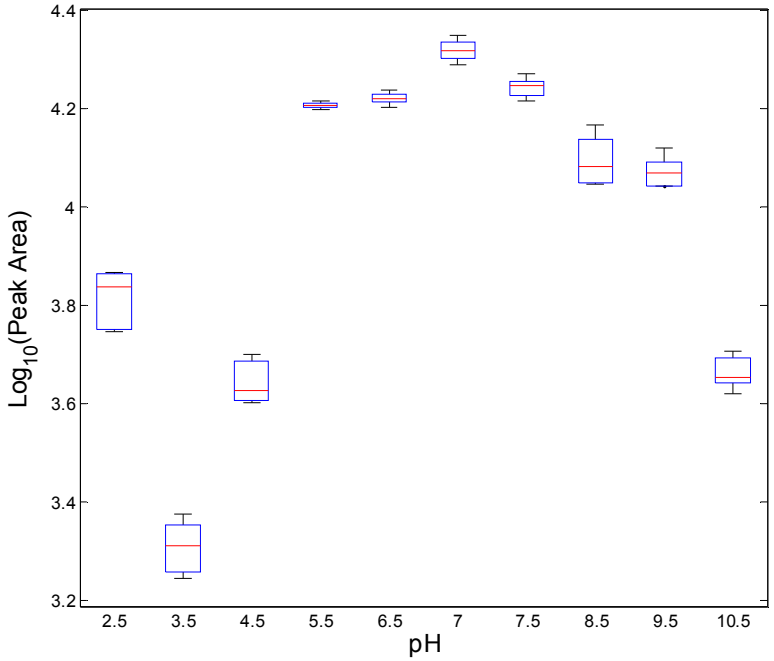

Figure 3: Effect of pH on SERS: Plot of the area under the peak at 993 cm^{-1} *versus* pH from2.5 – 10.5. All analyses were performed 5 times and the plots show box-whiskers where the red line is the median intensity, the top and bottom of the boxes are the 25th and 75th percentiles; the size of the box is the interquartile range (IQR); the whiskers extend to the most extreme data points which are not considered as outliers, defined as no more than 1.5×IQR outside of the IQR.

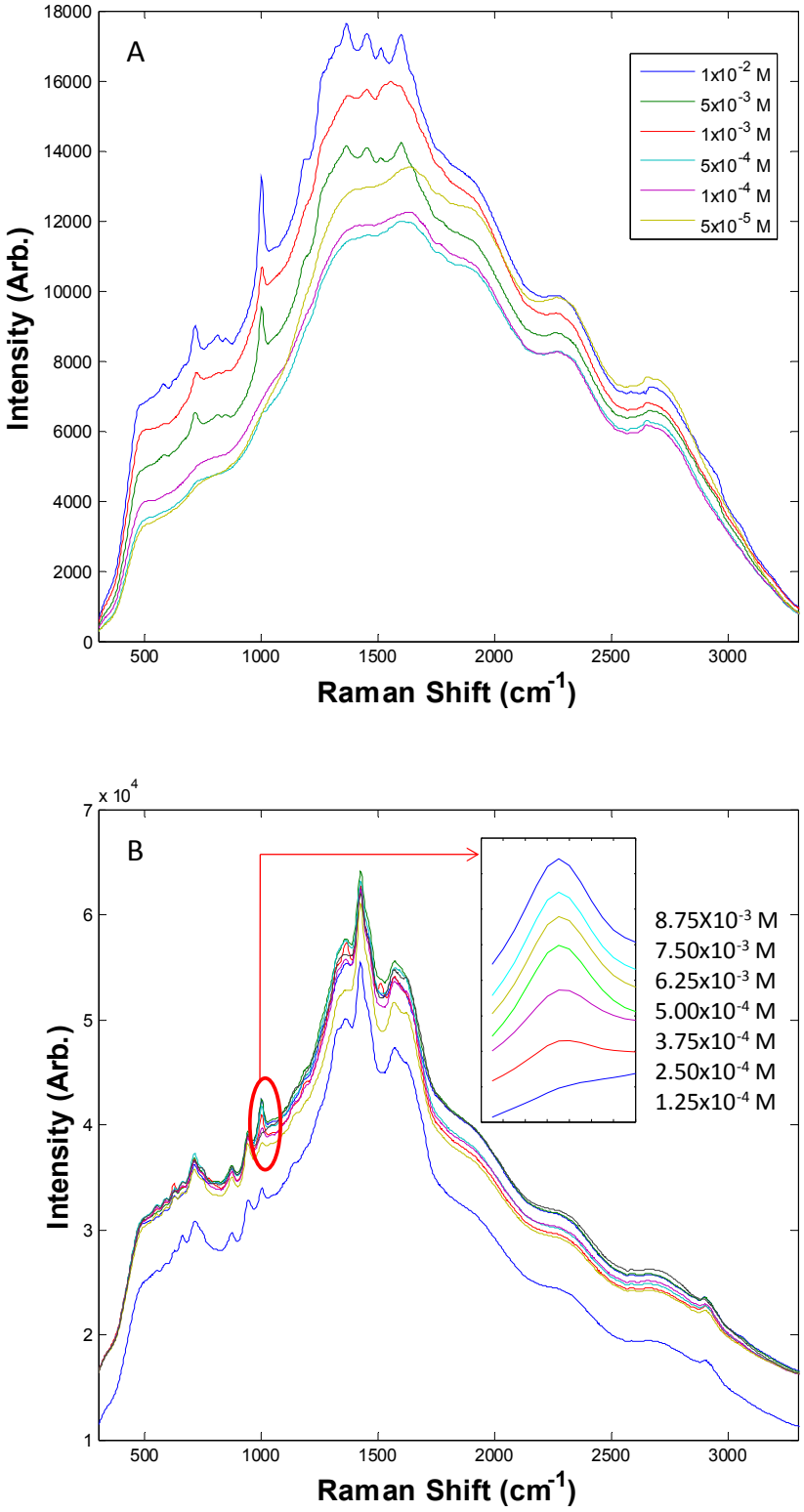

Figure 4: Raw SERS spectra showing quantification of tramadol in (A) water and (B) artificial urine. In B the inset shows the peak at 933 cm^{-1} is correlated with concentration.

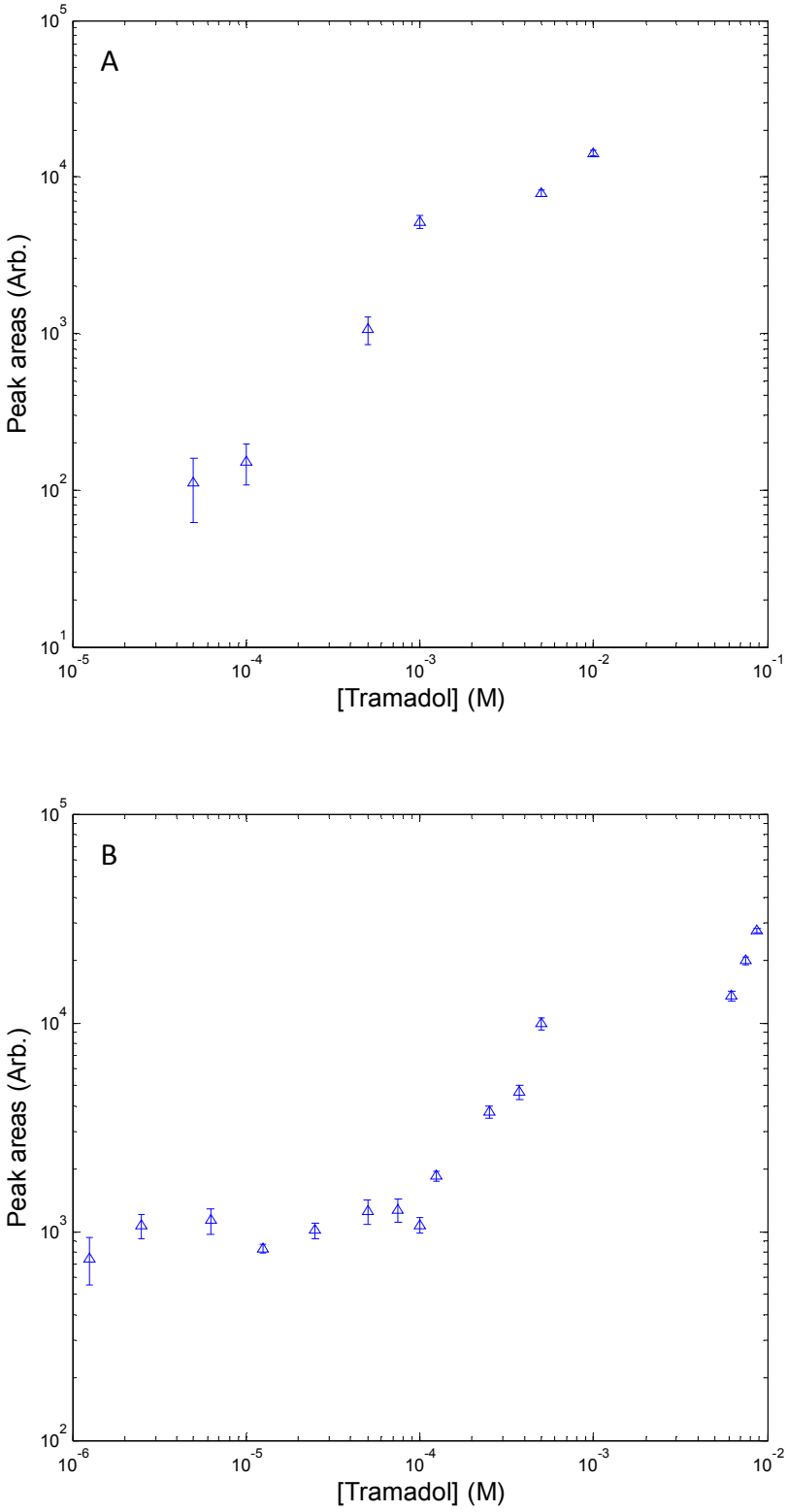

Figure 5: Peak areas plotted against tramadol concentration in (A) water and (B) artificial urine. The data are the mean of 5 measurements and standard deviation error bars are show.