SUPPORTING INFORMATION

Absolute quantification of uric acid in human urine using surface enhanced Raman scattering with the standard addition method

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SUPPLEMENTARY METHODS

REAGENTS AND MATERIALS

All chemical reagents were of analytical grade and were used with no additional purification unless otherwise stated. Uric acid (≥99%), hydroxylamine hydrochloride (99%), and water (HPLC grade) were purchased from Sigma Aldrich Ltd. (Dorset, UK). Potassium dihydrogen phosphate, dipotassium phosphate, sodium hydroxide, silver nitrate, hydrochloric acid and nitric acid were of analytical grade and obtained from Fischer Scientific (Loughborough, UK). Clinical pre-preeclampsia urine samples were kindly donated by Professor B. Thilaganathan from St George's, University of London & St George's University Hospitals NHS Foundation Trust Clinical Sciences Research Centre.

METHODS

Synthesis of Silver Nanoparticles

All glassware used in the synthesis of nanoparticles were pre-cleaned using aqua regia (3:1 hydrochloric acid: nitric acid) and washed thoroughly with deionised water. These glassware were left to dry at 50℃ before use.

The reduction of silver nitrate by hydroxylamine ions (HRSC) followed Leopold and Lendl.[1] Briefly, 180 mL (1.88×10^{-3} M) hydroxylamine hydrochloride and NaOH (3.33×10^{-3} M) were prepared. To this, $AgNO_3$ solution (20 mL, 1×10^{-2} M) was added drop wise and stirred for 15 min. A colour change to orange/brown indicated successful nanoparticle formation. The pH of colloidal solution was pH 7.5.

The nanoparticle size distribution of HRSC was characterized by UV-Vis spectrometry to allow comparison of different batches. To ensure that the absorbance of the nanoparticles was less than 2, the sols were diluted 1 part in 3 parts water. The hydroxylamine-reduced Ag colloid was shown to have a surface plasmon band (λ_{max}) around 408 nm, which was in agreement with the literature[10]. All spectra were background corrected.

The best colloid batch from each set of colloids synthesised was selected to serve as the colloid use in SERS analysis (see **supplementary Figure S5**). This process was repeated each time for each experimental replicate meaning a different colloid batch was used for each of the three replicates.

We previously used transmission electron microscopy (TEM) to determine the morphology and distribution of the nanoparticles from multiple batches and this produced results suggesting the average particle size being around 30± 5 nm, and so it is assumed that the new batches produced behave in a similar manner and particle size.

Preparation of urine for HPLC and SERS analysis

Note, urine samples are pre-preeclamptic (i.e., come from pregnant indivduals who have not developed the condition yet, as samples collected between 11-14 weeks gestation). Certain patients were known to go on and develop the preeclampsia).

Processing of Urine. For quantitative analyses in urine we used a pooled urine stock from 58 different patients (QC *n*=50). For each urine sample, a protein crash method was performed using methanol (at RT). 150 μL urine was aliquoted in Eppendorf tubes. 600 μL methanol was added to this, and centrifuged for 15 min at 13,500 x*g*. 400 μL was removed from the supernatant into a new Eppendorf tube and concentrated for ~ 4 h using an Eppendorf Vacufuge Concentrator 5301 (Eppendorf, UK). Once all methanol had been removed, the sample was re-suspended in water by adding 150 μL and vortexed for 5 s.

Dilution of urine samples. The urine samples were diluted with water based on their uric acid concentration (determined by HPLC). All HPLC urine samples were diluted 1:4 (urine:water) with dilution factors taken into account when predicting uric acid concentrations. For SERS analysis, as a general guideline, uric acid concentrations <70 μM were diluted 1:2, 70 – 320 μM were diluted 1:4 and >320 μM 1:10 (urine:water).

Sample Selection. All urine samples from the 58 patients were processed as described above. For the QC sample used in optimising various parameters for SERS detection, all 58 urine samples were used. The 58 urine samples were screened using HPLC to establish uric acid concentration. 21 of these 58 urine samples were randomly selected to cover the entire uric acid concentration range to remove measurement redundancy. Triplicate analyses were then performed on 11 of these 21 samples to establish reproducibility.

Sample preparation. 3.5×10^{-4} M uric acid stock solution was prepared in 1 M potassium phosphate buffer solution at pH 7.6. Samples for individual analyses were then prepared as follows: For SERS samples, 200 μL of HRSC, 75 μL potassium phosphate buffer at pH 7.6 and 125 μL urine was added to a glass vial. A potassium phosphate buffer was required to ensure samples had an overall pH that coincided with optimum SERS uric acid detection (pH 7.2 – 8) as well as acting as an aggregating agent to help stabilise the SERS response, especially for samples in the absence of spiked uric acid.

For HPLC samples, the same procedure was followed except water was added instead of colloid. For all samples, volume of uric acid spiked into sample (with corresponding concentration): 0, 10, 20, 30, 40, 50, 60 and 75 μL (0, 8.5, 16.7, 24.4, 31.8, 38.9, 45.7 and 55.3 μM). All dilution factors were taken into account when predicting final uric acid concentrations.

Random urine samples, including Sample 49 (identified with the highest uric acid concentration by HPLC) were checked using visible microscopy for uric acid crystals. No crystal of uric acid or any other substance was observed, meaning samples analysed were representative of the actual uric acid concentration present. Moreover, sample pre-treatment, dilution of urine and the use of standard addition methods minimised interference from other components present in urine, thus enabling absolute quantification of uric acid.

INSTRUMENTATION

UV-Vis characterization was carried out using Thermo Biomate 5 (Thermo Fisher Scientific Inc., Massachusetts, USA). 1 mL of the dilute nanoparticle solution was pipetted into a plastic cuvette and inserted into a sample holder. Data were acquired over a wavelength range of 300-800 nm.

SERS analysis was performed using a DeltaNu Advantage 200A portable Raman spectrometer (DeltaNu, Laramie, WY, USA) equipped with a 633 nm HeNe laser excitation, giving a power of 3 mW on the sample. Spectra were acquired for 20 s over a range of 200 – 3400 cm^{-1}; the spectral resolution was 10 cm^{-1}. Solution samples were placed in an 8 mm glass vial, vortexed for 3 s and allowed to aggregate for 3 min, before subjected to laser irradiation once loaded into the sample cell attachment. The instrument was calibrated using toluene to find the ideal distance from laser to sample. All analyses were conducted in five technical replicates. Triplicate analysis, using a different colloid batch for each replicate, was performed on 8/21 samples randomly selected for analysis.

Raman instrumentation for uric acid crystal formation analysis were collected using a Renishaw 2000 Raman microscope (Renishaw, Wotton-under-edge, Gloustershire, UK) with a low power (27 mW) at 633 nm excitation wavelength with power at the sampling point between 2 and 4 mW respectively. The instrument was wavelength calibrated with a silicon wafer focused under a x50 objective and collected as a static spectrum centred at 521 cm^{-1} for 1 s. The GRAMS WIRE software package (Galactic Industries Corp., Salem, NH) running under windows 95 was used for instrument control and data capture. Experimental parameters were three accumulations, extended scans between 200 and 2000 cm^{-1}, and an exposure time of 20 s. Five replicated were obtained.

HPLC separation was conducted using an Agilent 1100 series HPLC system set up for reverse phase consisting of a diode array detector. The column was 250 x 4.6 mm, ACE 5 C18-AR (Reading, Berkshire) with a 5 μm particle size. For each injection, the run time was 25.0 min. The mobile phase was 100% 20 mM potassium phosphate buffer at pH 5.8, pumped at a flow rate of 1.0 mL min^{-1}. 20 μL of each sample was introduced using an auto-injector. UV absorbance detection was measured at 290 nm.

DATA PROCESSING

Raman data analysis

Raman data was exported from the Raman instruments operating software and analysed using Matlab R2013a (The Mathworks, Natick, MA, USA). Spectra were baseline corrected and smoothed using a wavelet smoothing.

SERS data analysis

All SERS data was exported from the DeltaNu Advantage 200A operating software and analysed using Matlab R2013a (The Mathworks, Natick, MA, USA). Using an in house script, the band area under peak at 1134 cm^{-1} was used. Like before, there were 7 analyses for each urine sample. To find the original concentration of the unknown in the urine sample, a plot of peak area against concentration spiked into sample was plotted. We then extrapolated from the regression line to determine the uric acid concentration in the 'blank' i.e. '0 μM spiked', taking into account dilution factors. All analyses were conducted in five technical replicates and for 11/21 samples, three experimental replicates were collected.

HPLC data analysis

The peaks of the target analyte were integrated using ChemStation (Agilent 1100 series). From the standard addition approach, there were 7 analyses for each urine sample. To find the original concentration of the unknown in the urine sample, a plot of peak area against concentration spiked into sample was plotted. Using the classical linear regression of $y = mx + b$ (where m and b are the slope of the line and y-intercept, respectively), we then extrapolated from the regression line to determine the uric acid concentration in the 'blank' i.e. '0 μM spiked', taking into account dilution factors. This is where $y = 0$ and $\therefore x = -b/m$ such that the concentration in the sample = b/m μM.

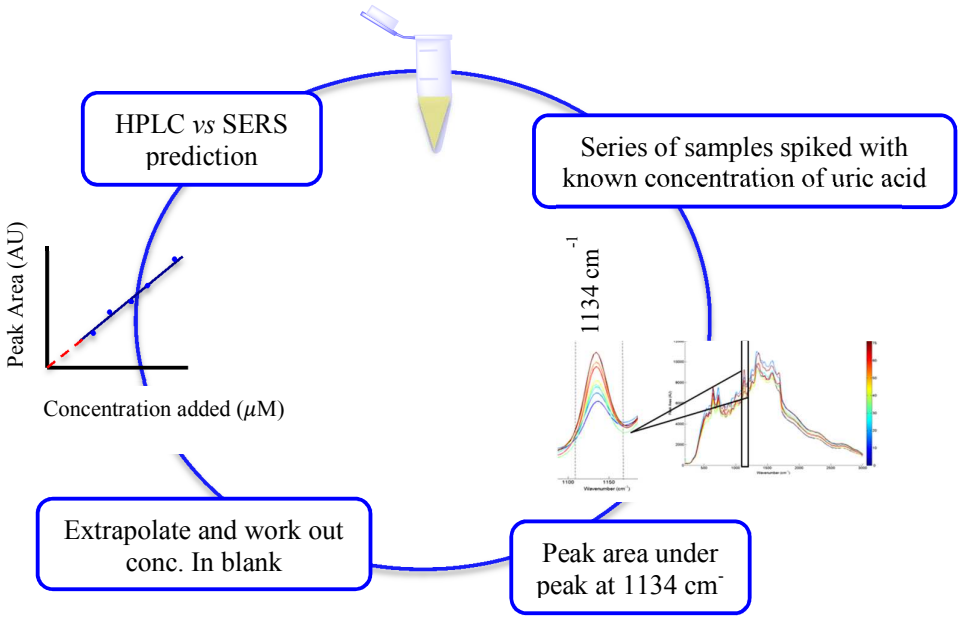


Figure S1: A pictorial representation of the overall process described in this paper. Standard addition method (SAM) is performed whereby a series of urine samples are spiked with known amounts of uric acid. The area under the characteristic peak of 1134 cm^{-1} from C-N vibration found in uric acid is used to extrapolate and work out the original concentration of uric acid in the urine sample. The findings are then compared to the HPLC results to see how well the two approaches agree with one another.

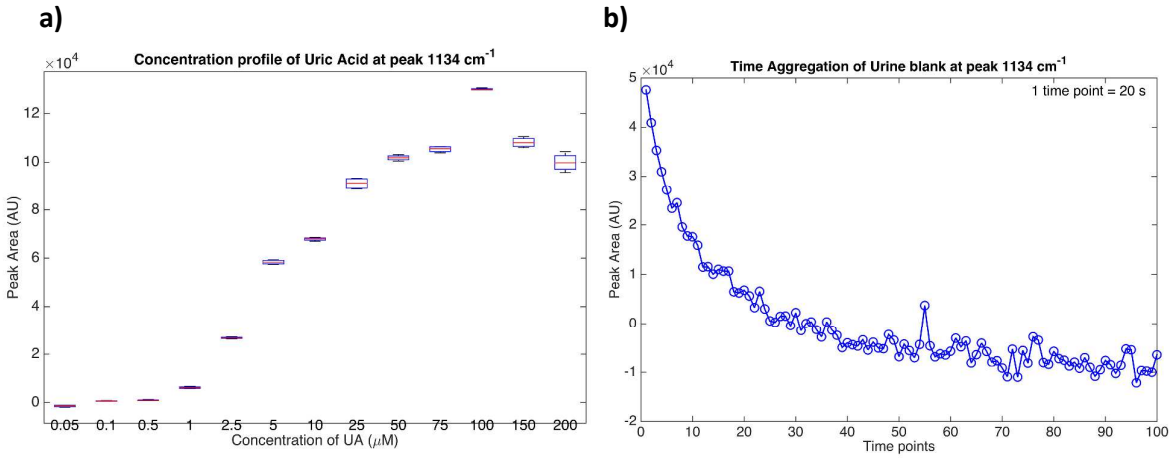


Figure S2: Optimisation involved for improving SERS enhancements focusing on the characteristic peak of uric acid at 1134 cm^{-1}: **a)** concentration profile with the red arrow signifying linear concentration range used in standard addition method approach; **b)** plot of time versus signal showing aggregation of the urine blank with the double headed red arrow signifying the time window in which replicates should be collected. The optimum conditions were 3.5 min (± 1 min) aggregation time, with a linear concentration range for standard addition between 1-100 µM. For all SERS analyses, five technical replicates were collected.

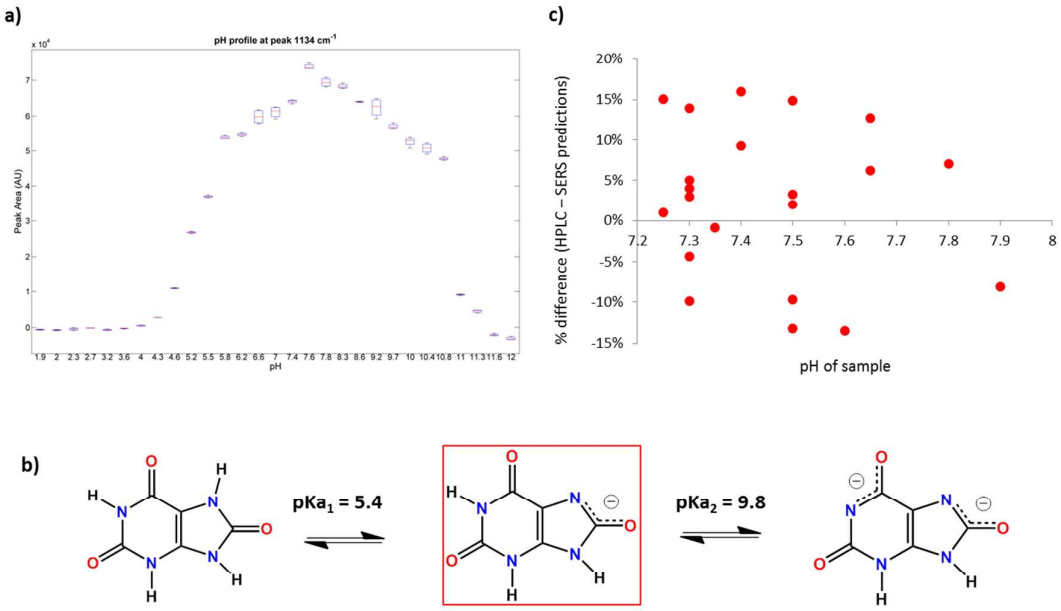


Figure S3: a) pH profile of uric acid SERS spectra focussing on characteristic peak at 1134 cm^{-1}. **b)** Ionisation states and pKa values for uric acid. On screening the urine samples, the pH ranged from 4.45 – 9.3. On sample make up for SERS, the overall pH for all samples was modified using a potassium phosphate buffer at pH 7.6 to between pH 7.2 – 8, ensuring that uric acid is predominantly in -1 ionisation state. c) A plot of percentage difference (from HPLC – SERS predictions) against pH for all samples analysed, indicating that there is no systematic bias in the analysis.

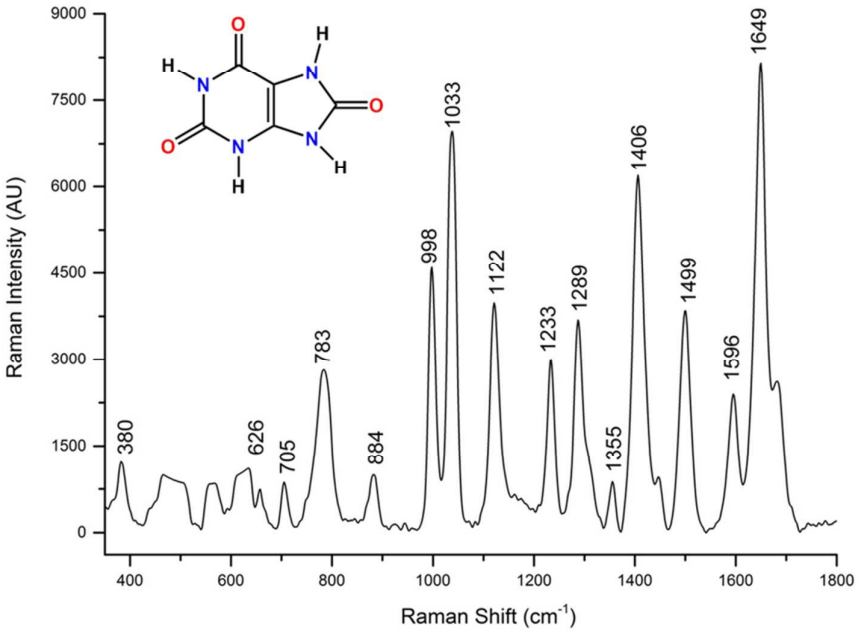


Figure S4: Annotated mean average Raman spectra (n = 5) of uric acid (solid). Raman spectra were obtained for 20 s, 3 accumulations using a Renishaw 2000 Raman microscope (Renishaw, Wotton-under-edge, Gloucestershire, UK). Spectra were baseline corrected and smoothed by wavelet smoothing.

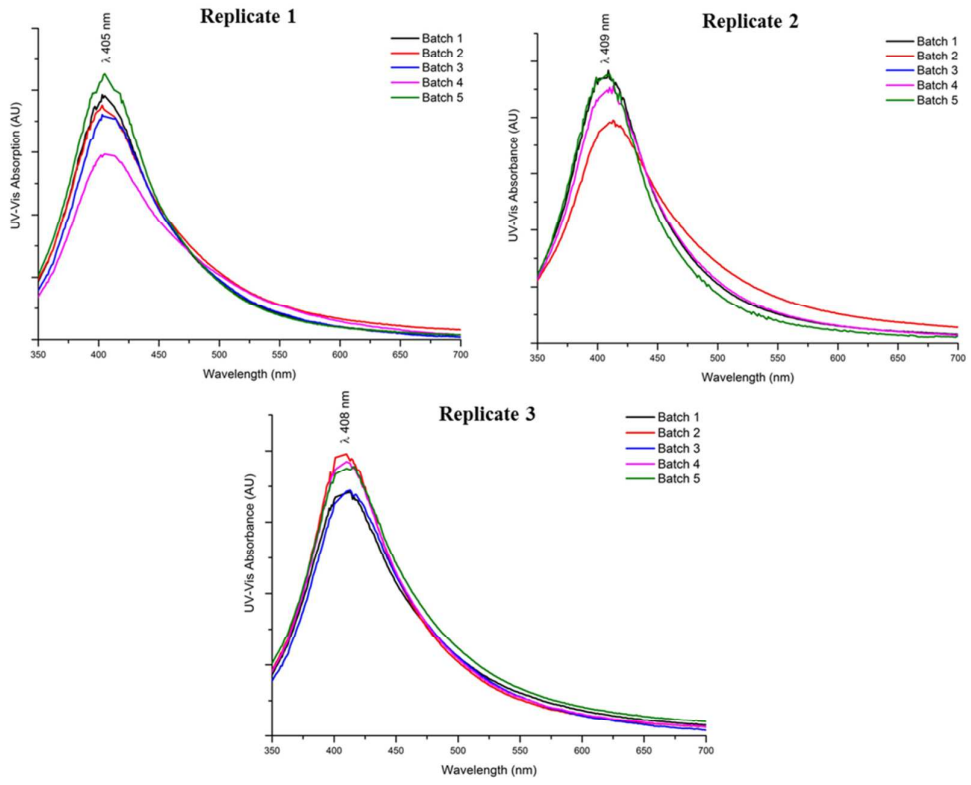
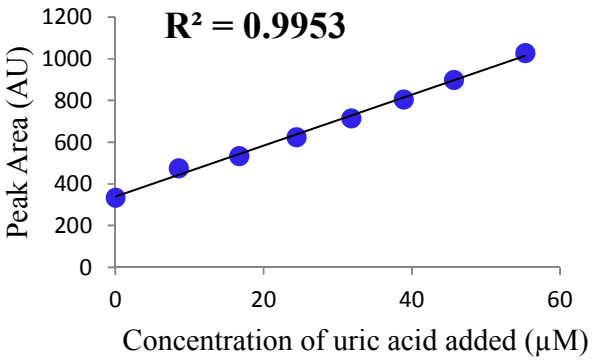
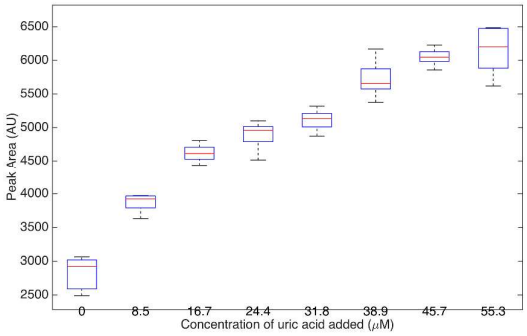


Figure S5: UV absorbance of five different hydroxylamine-reduced silver colloids showing the comparison of λ_{max} between three technical replicates. Moreover, these batches were similar to ones previously synthesised, with an average particle size of around 30±5 nm established using TEM.

a)



b)



c)

	1	2	3	SD	RSD
HPLC (µM)	151.3	155.8	149.5	3.3%	2.1%
SERS (µM)	149.2	157.4	152.9	4.1%	2.7%
Difference	1.4%	1.0%	2.2%		

Figure S6: Sample 20, replicate 1 is shown here as an example of the standard addition approach adopted. **a)** HPLC standard addition with an R^2 of 0.9953 and **b)** SERS standard addition plot using the characteristic peak at 1134 cm^{-1}. Both plots are peak area against volume of uric acid spiked in; the data are then extrapolated and converted to concentrations. **c)** Summary table of both analytical methods across all three replicates with the associated differences and errors. For all SERS analyses, five technical replicates were collected. SD: standard deviation; RSD: relative standard deviation.

REFERENCES

(1) Leopold, N.; Lendl, B. *J. Phys. Chem. B* **2003**, *107*, 5723-5727.