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1 **Absolute quantification of uric acid in human urine using surface enhanced Raman**
2 **scattering and standard addition method**

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ABSTRACT

High levels of uric acid in urine and serum can be indicative of hypertension, and the pregnancy related condition, preeclampsia. We have developed a simple, cost-effective, portable surface enhanced Raman scattering (SERS) approach for the routine analysis of uric acid at clinically relevant levels in urine patient samples. This approach, combined with standard additions method (SAM), allows for the absolute quantification of uric acid directly in a complex matrix such as that from human urine. Results are highly comparable and in very good agreement with HPLC results, with an average <9% difference in predictions between the two analytical approaches across all samples analysed, with SERS demonstrating a 60-fold reduction in acquisition time compared with HPLC. For the first time, clinical pre-preeclampsia patient samples have been used for quantitative uric acid detection using a simple, rapid colloidal SERS approach without the need for complex data analysis.

INTRODUCTION

Preeclampsia is a hypertension disorder that can occur in pregnant women and is a primary cause for maternal morbidity and mortality worldwide, mainly in developing countries, with 2-5% of pregnancies affected.[1-3] The cause of preeclampsia remains unknown and even predicting which women are susceptible/have an increased risk of developing the condition is problematic.[4,5] Moreover, when preeclampsia appears in the second or third trimester, there are no treatments available other than premature delivery of the baby,[6] and those babies born are exposed to an increased risk of developing hypertension, heart disease and diabetes[1,7,8]. Currently, diagnosis of preeclampsia is a challenge as it relies on non-specific signs of the disease[9]; commonly associated symptoms include raised blood pressure and elevated protein levels (proteinuria) in the urine and so blood tests and urine protein measurement tests are often performed.[10]

Uric acid has been identified as an important biomarker for various diseases such as cardiovascular diseases, gout, renal diseases and preeclampsia.[11-15] Uric acid is the end product of the metabolic breakdown of purine nucleotides and in normal circumstances, concentrations range from 3.5-7.0 mg/dL in the blood and from 16-100 mg/dL per 24 h in urine.[16] Moreover, elevated uric acid levels (referred to as hyperuricemia) in urine and serum has been associated with preeclampsia, with levels greater than 0.4 mM indicating severe preeclampsia.[17]

Colorimetric enzymatic assays, liquid chromatography and capillary electrophoresis methodologies have been reported for uric acid detection; however, associated disadvantages include that these tests are often only applicable to late-stage preeclampsia, involve time consuming assay based tests, expensive enzymes, sophisticated instrumentation and equipment and often lack sensitivity. Therefore, there is a great need for a rapid, inexpensive, routine, diagnostic test to aid early uric acid detection, thus allowing hyperuricemia conditions to be properly monitored and managed to prevent further health implications.[18,19]

Surface enhanced Raman scattering (SERS) has increasingly been employed for use in quantification of biologically relevant molecules[20-24]. SERS is the dramatic enhancement of Raman signals when an analyte is in close proximity/absorbed onto a nanoscale rough metallic surface, with typical enhancements of 10^4-10^6 observed. It is an attractive approach for disease diagnosis as it yields molecular specific information, is label-free, has the ability to be performed in aqueous environments and has high sensitivity with low analyte concentration detection (down to fM level and single molecule detection).[25-28]

Raman spectroscopy also offers several advantages including its ease-of-use along with portability[29] leading to the ability to develop point-of-care analysis systems for on-site Point-and-Shoot analyses.[30] Recently, there have been several Raman spectroscopy based approaches, mostly utilising electrochemical surface enhanced Raman (E-SERS), to measure uric acid detection. However, these studies have demonstrated problems in establishing reliable, quantitative detection of uric acid at clinically relevant concentrations[31], have

100 employed complex sample make up[32,33] and used complex data analysis[34]. In all cases, the
101 studies were not performed in real-life situations, i.e. biological fluids, but instead were
102 performed in either urine stimulant, synthetic urine or water.[35]

103

104 In this paper, we present an optimised SERS approach for uric acid detection in human urine
105 from clinical pre-preeclamptic patients (urine samples collected between 11-14 weeks
106 gestation) using a portable, easy-to-use Raman instrument. As uric acid is already present in
107 urine, our approach involves using the well-known standard additions method (SAM), as well
108 as HPLC analysis for additional benchmarking (see **supporting information Figure S1**). In
109 SAM, a series of samples are analysed involving spiking in known amounts of uric acid in
110 increasing concentrations whereby the subsequent calibration curve is used to determine the
111 unknown concentration of uric acid in the original (undiluted) sample. We have performed
112 this approach on 21 clinical samples, of which, 11 were performed in triplicate analysis to
113 establish reproducibility, affording direct quantification of the target analyte within the
114 sample thus accounting for the complex sample matrix. To the best of our knowledge, this
115 simple colloidal uric acid detection approach in clinical patient samples is the first study of its
116 kind.

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132 # RESULTS AND DISCUSSION

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134 ## SERS optimisation

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136 We have previously established optimised conditions for uric acid SERS detection within an
137 enzymatic system by varying certain parameters such as colloid type, pH, concentration, time
138 aggregation and aggregating agent.[8] However, uric acid detection in a more complex matrix,
139 such as urine which is a molecular milieu of small molecules, organic acids and ions as well
140 as proteins, required a slightly different set of conditions. Optimised conditions were
141 established using a pooled QC urine sample (from all 58 patient samples), and as before
142 hydroxylamine-reduced silver colloid was the optimum SERS substrate to use, at pH 7.6,
143 without the need for an aggregating agent. The aggregation time was slightly modified, with
144 optimum uric acid detection achieved after 3.5 min (± 1 min) aggregation (see **Figure 1** and
145 **supporting information Figures S2 and S3). Table 1** shows the tentative SERS band
146 assignments for uric acid (see **supporting information Figure S4** for normal Raman spectra
147 of uric acid (solid) which aided in band assignment).[8,13,35]

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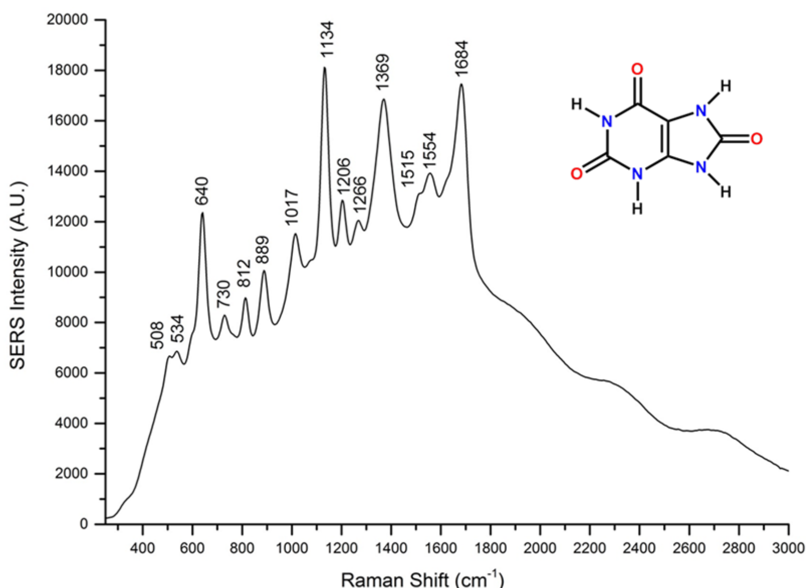

Figure 1 Annotated mean averaged SERS spectra (n = 5) of uric acid. SERS spectra were obtained for 20 s, at 25 µM (dissolved in water) using 200 µL of hydroxylamine reduced silver colloid, potassium phosphate buffer at pH 7.6; measurements were made 3 min after aggregation.

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161 **Table 1:** Band assignments for normal Raman (solid) and tentative SERS (25 µM solution) band assignments for the Uric Acid

Raman Shift (cm^{-1})	SERS Peak (cm^{-1})	Tentative band assignment[8,13,35]
380 (mw)	-	-
-	508 (vwsh)	C-N-C ring vibrations
-	534 (vw)	
626 (w)	640 (s)	Skeletal ring deformation
705 (w)	730 (w)	N-H bending
783 (m)	812 (m)	Ring vibration
884 (w)	889 (m)	N-H bending
998 (s)	1017 (w)	Ring vibrations
1033 (vs)	-	-
1122 (s)	1134 (vs)	C-N
1233 (s)	1206 (m)	N-C-C stretching and bending
1289 (s)	1266 (w)	-
1355 (w)	1369 (s)	C-O
-	1515 (vwsh)	Asymmetric deformation NH_3
1406 (vs)	-	-
1499 (s)	-	-
1596 (m)	1554 (m)	C-N
1679 (vs)	1684 (s)	-

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163 ν –stretching, b-bending, R-ring; trigd-trigonal deformation, s-strong, vs-very strong, m-medium, w-weak, vw-very weak

164

165 A concern for the analysis in human urine was the effect the pH of individual samples would
166 have on the SERS spectrum as well as any potential difference in how the target analyte
167 would interact with the silver surface. After dilution, the pH of the urine samples varied
168 considerably from 4.45 – 9.3, meaning uric acid would be in different ionisation states and
169 consequently interact with the surface differently, generating sub-optimal SERS spectra,
170 ultimately not representing the actual concentration in the sample. To overcome this and to
171 ensure the urine sample had an overall pH that coincided with optimal uric acid detection, the
172 sample environment was carefully modified. A potassium phosphate buffer at pH 7.6 was
173 added to the colloid and urine, as well as the uric acid spiked in to the sample for the standard
174 additions measurements. Therefore, after all constituents were added (i.e. urine, buffer,
175 spiked uric acid and colloid), the overall sample pH for all patients varied from pH 7.2 – 8.

176 This meant that in all analyses uric acid was predominantly in its negative 1 ionisation state
177 and would have the same affinity for the colloidal surface thus affording more reproducible
178 and optimal SERS uric acid detection for all samples (see **supporting information Figure**

179 **S3**). Moreover, the presence of additional buffer (i.e. 75 μL) in the sample make up was
180 necessary as it also acted as an aggregating agent to help stabilise the SERS response,
181 especially for samples where low uric acid (or none, as in the blank) was spiked in.

182

183 **Standard additions method (SAM) approach**

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185 Previously for quantifying uric acid within a mixture (in terms of monitoring
186 biotransformation(s) involving three analytes) we used various chemometric approaches such
187 as partial least squares regression (PLSR) and multivariate curve resolution-alternating least
188 squares (MCR-ALS).[8] However, these data analysis methods are not suitable for quantifying
189 analytes in complex matrices where the sample matrix actually contributes to the analytical
190 signal (i.e., when uric acid is already present in urine). Standard additions method (SAM)
191 works by spiking in known amounts of standard, in this case uric acid, and plotting peak area
192 (of a characteristic peak, *viz.* 1134 cm^{-1}) against the concentration of the standard spiked in,
193 thus yielding a straight line ($y = mx + b$; where m and b are the slope of the line and
194 y-intercept, respectively). From there, the concentration of the analyte can be determined
195 from the point at which the extrapolated line crosses the concentration axis (x) at zero signal
196 (i.e. where $y = 0$ and thus $x = -b/m$ such that the concentration = b/m).[36,37]

197 A prerequisite in order to use SAM is a linear relationship between the concentration and the
198 SERS signal of uric acid. From performing a concentration profile (see **supporting**
199 **information Figure S2a**), the linear concentration range was determined to be between 1 –
200 100 μM). Therefore, for some samples, dilution of the initial urine sample was performed to
201 ensure that on spiking in a known amount of standard, this linear concentration range was
202 retained. Notably, in cases of saturation, comparably lower SERS signals are observed for
203 higher analyte concentrations, as seen beyond 100 μM, and a non-linear relationship is
204 observed. We also had to consider the effect of the concentration-dependence on the
205 orientation of the target molecule on the colloidal surface. We noticed that below 5 μM,
206 although still part of the linear relationship, the SERS spectrum of uric acid changed slightly,
207 in terms of intensity and broadness of certain peaks, indicating the orientation of the analyte
208 molecule on the surface was probably different. Therefore, for this reason, 5 - 100 μM was
209 determined to be the optimum total concentration range of uric acid in the urine sample,
210 taking into account spiking of standard and dilution factors.

211

212 **Sample selection and SAM Analysis**

213

214 Rather than measure all samples, 21 samples from the 58 human urine samples were
215 randomly selected for comparative SERS and HPLC SAM analysis – we ensured that the
216 samples selected covered the entire uric acid concentration range. The characteristic peak for

217 uric acid used in the analysis was at 1134 cm^{-1}, corresponding to the C-N vibration. **Table 2**
218 shows a summary of the results from each analytical approach along with the associated
219 percentage differences. As an example, for sample 18, HPLC results predicted a uric acid
220 concentration of 172 µM, compared to 186 µM predicted from SERS, a difference of 7.5%.
221 Overall, for all samples analysed, the percentage difference between the two analytical
222 approaches ranged from 0.8% (sample 20) to 19.1% (sample 40), with the average percentage
223 difference being 8.4 %. This is very encouraging considering the uric acid concentration
224 range was extensive (starting from around 65 µM to 670 µM) meaning this approach could
225 be extended to target other uric acid related diseases (i.e. those related to *low* concentration of
226 uric acid (hypouricemia) as well as elevated uric acid levels (hyperuricemia) such as in
227 preeclampsia).

228

229 **Table 2:** Summary of results for all samples analysed: HPLC (µM), SERS (µM) and the
230 associated percentage differences between the two analytical approaches

Sample	HPLC (µM)	SERS (µM)	Difference	Sample	HPLC (µM)	SERS (µM)	Difference
QC (*n*=58)	190.3	181.1	4.8%	**26**	215.0	182.9	14.9%
1	274.9	266.6	3.1%	**31**	548.5	601.6	8.8%
4	112.9	111.8	1.0%	**36**	206.9	187.7	10.2%
5	119.4	135.1	11.6%	**40**	219.2	184.0	19.1%
9	64.5	73.2	11.9%	**41**	309.8	294.2	5.3%
12	91.5	88.4	3.4%	**44**	196.6	188.7	4.0%
14	70.1	73.1	4.1%	**47**	183.0	157.5	16.2%
16	511.2	561.6	9.9%	**49**	679.4	665.5	2.1%
18	172.0	186.0	7.5%	**52**	249.5	233.8	6.7%
20	151.9	153.1	0.8%	**53**	126.3	117.4	7.1%
22	234.6	199.3	15.0%	**55**	345.8	301.6	12.8%

231

232 NB Dilution factors taken into account. See 'Preparation of urine for HPLC and SERS analysis' in Supporting
233 Information.

234

235 We next wanted to address the reproducibility of this SERS approach. Eleven of the 21
236 samples were randomly selected for triplicate HPLC and SERS analysis. For the latter, this
237 meant using a different colloidal batch for each replicate performed (see **supporting**
238 **information Figure S5).** In general, batch-to-batch variation and reproducibility of SERS
239 enhancements is a key discussion point within the Raman community.[38-41] Groups report
240 noticeable discrepancies in results due to variations in colloidal batches, colloid
241 concentrations and nanoparticle aggregation – all of which can result in inconsistent
242 enhancements. However, we have demonstrated using these optimised set of conditions for
243 uric acid detection in urine that we can produce consistent, reliable results that are in good

244 agreement and comparable to HPLC results, the 'gold standard' in this case and used for
245 benchmarking **(see Table 3).**

246 As an example, sample 20 was subjected to triplicate analysis (see **supporting information**
247 **Figure S6**). The average percentage difference between the HPLC and SERS results was just
248 over 1.5% across the three replicates. The standard deviation (SD) for SERS results was 4.1
249 (compared to 3.3 for HPLC) and the relative standard deviations (RSD) were 2.1 and 2.7%
250 for HPLC and SERS respectively. These results are excellent in terms of accuracy (difference
251 between HPLC and SERS), precision (SD for these repeat measurements) and overall
252 reproducibility. To emphasise this further, the average percentage difference for triplicate
253 analysis between the two analytical approaches was 7.2%, with the average SD and RSD for
254 SERS being 12.8 and 6.7% and for HPLC, 6.3 and 3.3% respectively.

255

256 **Table 3:** Summary of results for samples involving triplicate analysis including associated
percentage differences between the two analytical approaches, standard deviations (SD)
257 and relative standard deviations (RSD)

Sample	HPLC (µM)	SD (±)	RSD (±)	SERS (µM)	SD (±)	RSD (±)	Difference
1	274.9	13.6	5.0%	266.6	17.4	6.5%	3.1%
4	112.9	3.8	3.4%	111.8	9.6	8.6%	1.0%
5	119.4	1.5	1.3%	135.1	11.1	8.3%	11.6%
12	91.5	6.0	6.5%	88.4	9.2	10.5%	3.4%
20	152.3	3.2	2.1%	153.1	4.1	2.7%	1.5%
36	206.9	5.0	2.4%	187.7	8.4	4.5%	10.2%
40	219.2	1.6	0.7%	184.0	12.5	6.8%	19.1%
41	309.8	10.8	3.5%	294.2	16.8	5.7%	5.3%
47	183.0	6.1	3.3%	157.5	13.4	8.5%	16.2%
49	679.4	9.4	4.2%	665.5	16.5	2.5%	2.1%
52	249.5	8.5	3.4%	233.8	22.1	9.4%	6.7%
Control*	51.0	0.7	1.4%	53.9	4.6	8.5%	7.8%

258

259 *Control is to establish overall errors associated with each technique. 50 µM (i.e. known concentration) was spiked into blank

260 The average difference between the two analytical approaches was 7.2%. The average SD and RSD for SERS was 12.8 and 6.7% and for HPLC, 6.3 and 3.3% respectively.

261

262 To confirm further how excellent the agreement of data are between the two analytical
263 approaches, **Figure 2a)** shows a HPLC *versus* SERS plot for all 21 analyses, reflecting a very
264 good linear trend with an R^2 of 0.9792. In addition, **Figure 2b)** is a plot of percentage
265 difference (from HPLC – SERS predictions) against HPLC predicted concentration for all
266 samples analysed. The 'randomness' of the plot (i.e. both positive and negative differences)
267 indicates that there is no systematic bias in the analysis. Notably, there are less samples
268 analysed at high uric acid levels, and ideally we would want an even spread of samples across

269 the entire uric acid concentration range to demonstrate fully how applicable this SERS
270 approach is. However, these samples are real, clinical pre-preeclampsia samples and hence
271 this is reflected by the uric acid concentrations observe

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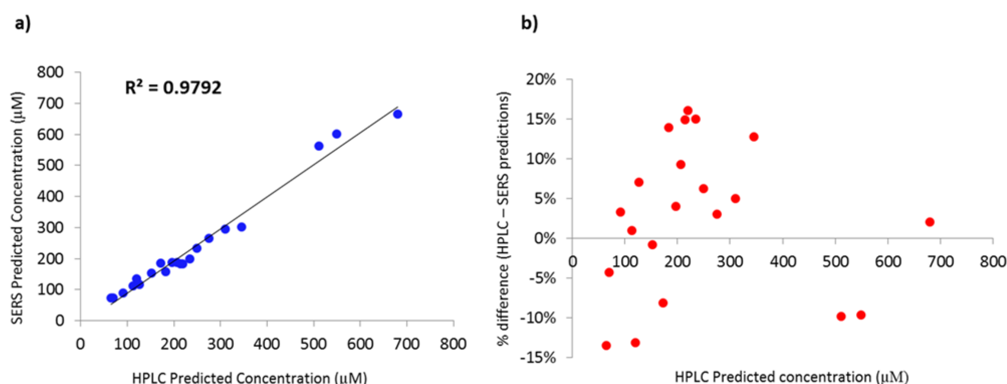

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280 **Figure 2 a)** A plot of predictions from HPLC *vs.* SERS for all samples (including replicates)
281 with an R^2 of 0.9792 indicating results are in very good agreement. **b)** A plot of percentage
difference (from HPLC – SERS predictions) against HPLC predicted concentration for all
282 samples analysed, indicating that there is no systematic bias in the analysis.

283

284 Noticeably, SERS results have slightly higher SD and RSD throughout the samples analysed.
285 However, the reduction in acquisition times for SERS analysis largely compensates and
286 outweighs this. In total, SAM HPLC takes 140 min (7 samples × 20 min HPLC trace) for uric
287 acid prediction for each sample. Comparatively, SAM SERS analysis takes 140 s (7 samples
288 × 20 s SERS acquisition), a 60-fold reduction and yet results are in very good agreement and
289 highly comparable. There is only on average 8.4% difference for all samples analysed. There
290 was on average 7.2% difference for the triplicate analysis indicating that the precision and
291 accuracy were similar. Moreover, if one considers the actual associated error with each
292 analytical approach (see **Table 2**) - for HPLC there are associated SD and RSD of 0.7 and
293 1.4% and for SERS 4.6 and 8.5% respectively - there is overlap and leeway in the results
294 between the two analytical approaches, making this SERS approach a highly attractive
295 alternative that could be used in clinical settings. Finally on this point, the cost of the analysis
296 is comparable, as HPLC apparatus include instrument costs and consumables, which are
297 equivalent to low-cost portable and indeed hand-held Raman spectrometers plus cost of
298 colloid and reagents.

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302 # ONLINE METHODS

303 Full experimental details are provided in the **supporting information** 'Supplementary
304 Methods' section.

305 **Materials.** All chemical reagents were of analytical grade and used with no additional
306 purification unless otherwise stated.

307 **Human urine samples** were kindly donated by Prof B. Thilaganathan from St George's,
308 University of London & St George's University Hospitals NHS Foundation Trust Clinical
309 Sciences Research Centre. Written informed consent was obtained from each study
310 participant and the study conformed to the principles set out in the WMA Declaration of
311 Helsinki and the NIH Belmont report. The study was approved by Wandsworth Local
312 Research Ethics Committee.

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

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

325 **Sample preparation.** 3.5×10^{-4} M uric acid stock solution was prepared in 1M potassium
326 phosphate buffer solution at pH 7.6. Samples for individual analyses were then prepared as
327 follows: For SERS samples, 200 µL of hydroxylamine reduced Ag colloid (HRSC), 75 µL
328 potassium phosphate buffer at pH 7.6 and 125 µL urine was added to a glass vial. The silver
329 colloid was prepared according to the method of Leopold and Lendl.[42] For HPLC samples,
330 the same procedure was followed except water was added instead of colloid. For SAM
331 sample preparation the concentration of uric acid spiked into each urine sample was 0, 8.5,
332 16.7, 24.4, 31.8, 38.9, 45.7 and 55.3 µM. All dilution factors were taken into account when
333 predicting final uric acid concentrations.

334 **Raman Instrumentation.** A DeltaNu Advantage 200A portable Raman spectrometer
335 (DeltaNu, Laramie, WY, USA) was used for collection of spectra using a HeNe 633 nm laser
336 with ~3 mW on the sample.

CONCLUSION

There is a real urgent unmet need for early diagnosis of preeclampsia in pregnant women. We have demonstrated a SERS based approach to measure uric acid in pre-preeclamptic urine samples which yields absolute quantitation. In combination with the well-known SAM approach, uric acid concentrations have been predicted in urine samples from 21 different patients, and benchmarked against HPLC. It is highly notable that there is a 60-fold reduction in acquisition time when employing this SERS-based approach, and yet the average difference between the two analytical approaches is less than 9%. Furthermore, we have demonstrated excellent reproducibility, with 11 of these samples performed in triplicate analysis using different colloidal batches (for each replicate) highlighting its use for applications in rapid, routine detection of uric acid. We believe this new SERS-SAM analytical approach could easily be translated into an on-site, diagnostic tool for early diagnosis of preeclampsia and indeed other disease where quantification of uric acid is needed.

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