

LIBS-Raman: an effective complementary approach to analyze renal-calculi

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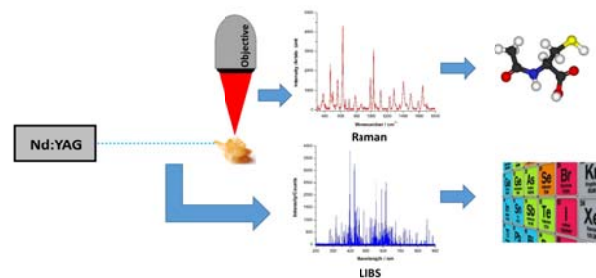
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Presence of renal calculi (kidney stones) in human urethra is being increasingly diagnosed over the last decade and is considered as one of the most painful urological disorders. Accurate analysis of such stones plays a vital role in the evaluation of urolithiasis patients and in turn helps the clinicians toward exact etiologies. Two highly complementary laser-based analytical techniques; Laser Induced Breakdown Spectroscopy (LIBS) and micro-Raman spectroscopy have been used to identify the chemical composition of different types of renal calculi. LIBS explores elemental characteristics while Raman spectroscopy provides molecular details of the sample. This complete information on the sample composition might help clinicians to identify the key aspects of the formation of kidney stones, hence assist in therapeutic management and to prevent recurrence. The complementarity of both techniques has been emphasized and discussed.



LIBS-Raman analysis of renal calculi.

LIBS spectra of different types of stones suggest the probable composition of it by virtue of the major, minor and trace elements detected from the sample. However, it failed to differentiate the crystalline form of different hydrates of calcium oxalate stone. This lacuna was overcome by the use of Raman spectroscopy and these results are compared with conventional chemical analysis.

1. Introduction

Stone formation in the kidney or urinary tract (nephrolithiasis/urolithiasis) is emerging as common diseases throughout the world. Kidney stone (renal calculi) is a hard lumpy deposit made up with tiny crystalline minerals and acid salts in the kidney or urethra. It commonly occurs in both sexes regardless of age. Multiple factors such as environment, genetics, diet, water intake, and occupation are contributing to the formation of kidney stone even though the exact mechanism behind the formation is not yet completely understood¹. This grave health complication may cause severe pain, pus in the urine, obstruction, infection of urinary tract, and in the worst case it can lead to total renal damage². Though kidney stones have different organic and inorganic compounds, most of the stones

have a major single characteristic component. There are four major types of stones. Among this, 80% are calcium based stones, in which calcium is combines with oxalate or phosphate. Remaining 20% are devoid of calcium such as cysteine-type, uric acid-type and struvite/infection type stones^{3, 4}. Mixtures of these chemical compositions on a single stone are also common. Identification and differentiation of renal calculi remains one of the biggest challenges in the medical community because long-term follow-up studies are showing that the chance of recurrence is greater than 50% in 5 years. Only proper medication can prevent this scenario. The treatment or diet to prevent the recurrence of kidney stone is completely dependent on the composition of the stone. It is therefore vital to have a simple, fast, direct and accurate method for the analysis of kidney stones.

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Kidney stones are highly complex matrices and primarily heterogeneous. Hence, exact identification and classification are not easy and straight forward. Multiple physical and chemical methods are available for the identification of kidney stone composition. However, none of the said methods is proven to be satisfactory. Many physical methods such as X-ray diffraction (XRD), X-ray excited fluorescence (XRF), Infrared spectroscopy (IR), Scanning electron microscopy (SEM), Raman, Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS), Laser-Induced Breakdown Spectroscopy (LIBS) and polarization microscopy have been attempted for the identification of kidney stones⁴⁻¹². Among this, XRD and IR are widely used laboratory techniques for identifying the chemical composition of renal calculi. Even though, these techniques need significant quantity of samples for the successive identification of chemical constituents. In the case of XRF and ICP-MS, it is difficult to detect the lighter elements such as C, H, N and O. These deficiencies can be overcome by the use of LIBS technique. Nevertheless, two different techniques are often advisable for the successful identification of complex samples to remove the ambiguity. Among these techniques, LIBS and Raman spectroscopy are the most promising tools as it can identify these complex samples by looking at the atomic emissions and molecular vibrations from the same sample. LIBS and Raman are highly complementary spectroscopic techniques, being used extensively for the identification of minerals, pigments, in the field of archeology, environmental monitoring, geological analysis, space explorations etc.¹³⁻¹⁸. Compared to other techniques, these two techniques are simple, rapid and highly sensitive. Moreover, these techniques need a little amount of sample for the analysis and require no/minimal sample preparation. Since the sample is pristine after measurements, it can also be used for further studies. These advantages make LIBS-Raman techniques an emerging tool for identification of complex samples such as renal calculi. This very fact is of considerable contemporary interest in the context of this study due to the following reason. In recent past, Shock Wave Lithotripsy (SWL) or laser breaking methods are preferred for the kidney stone treatments and as a result minimal amount of sample is available for the analysis.

LIBS employs a high energy pulsed laser focused onto the sample surface, which ablate a small portion, resulting in the formation of a micro-plasma. In most cases, the irradiance is around 1 GW/cm² to breakdown the molecules and excite the atoms to generate microplasma¹⁹. The elemental identification and their chemical abundance can be achieved by detecting the light from the micro-plasma while it's cooling. This technique provides qualitative as well as quantitative information from many major, minor and trace elements²⁰. LIBS can be considered as a micro-destructive technique because only very little amount of sample (μg or ng) is consumed for atomization which

causes little/no damage to the sample. The main advantages of LIBS are; simultaneous multi-elemental detection and the capability for depth profiling.

On the other hand, Raman spectroscopy mainly deals with the spectral analysis of inelastically scattered light from samples irradiated with a monochromatic light source, normally continuous or pulsed lasers. This yields chemical and structural information of materials in any physical phase^{17, 21}. It is a highly sensitive and non-destructive technique and is generally consider as a more popular technique than LIBS for detecting the organic compounds. This is because, it can easily distinguish between various hydrocarbons such as benzene, naphthalene, methane and their various chemical isomers, and different kind of organic molecules such as protein, lipids, amino acids, complex molecules such as pigments, ink, etc. But compared to LIBS, trace analysis is difficult with Raman technique as the Raman signal is proportional to the number of molecules excited by the laser²². Usually, low power lasers are used for the Raman measurements to avoid the photo/thermal degradation or modification of the sample. One of the key considerations in Raman spectroscopy for a particular study is the selection of excitation wavelength. This is because Raman signal from the sample is often swamped by the fluorescence emission induced by a wrongly chosen excitation wavelength¹⁷.

Our group has done some preliminary investigations on the complementarity of LIBS-Raman techniques for the identification of two different hydrates of calcium oxalate stones²². The main objective of the work presented here is to explore the potential of these techniques for the routine identification of different types of kidney stones having complex compositions. We have also demonstrated the bi-directional use of LIBS-Raman technique. In most cases, Raman is used for identifying the sample composition, whereas LIBS gives the major, minor, and trace elemental information. However, Raman scattering could not be detected from every sample due to the weak Raman cross section or the interference of fluorescence emission from the sample. In certain such cases, LIBS provided valuable information and helped to identify the stone composition. On the other hand, LIBS alone cannot be used to differentiate the hydrates of the minerals; whereas Raman can easily detect and differentiate the hydrates by looking at the vibrational spectra. This shows that the potential limitations of both spectroscopic techniques for the identification of complex samples can be tackled by the complementary nature of these two techniques.

2. Materials and Methods

2.1 Sample

Forty-two different types of kidney stones were collected from Department of Urology, Kasturba Medical College (KMC), Manipal, India. All these stones had different size, shape, color, and brittleness. Collected kidney stones were washed properly using saline solution followed by distilled water for removing blood and other possible contaminants. Raman and LIBS measurements have been carried out separately on each of these stones. It is inferred later that all these forty-two samples are mainly falling into the category of seven known types of kidney stones and hence numbered sequentially as $S_1 - S_7$ shown in Figure 1.

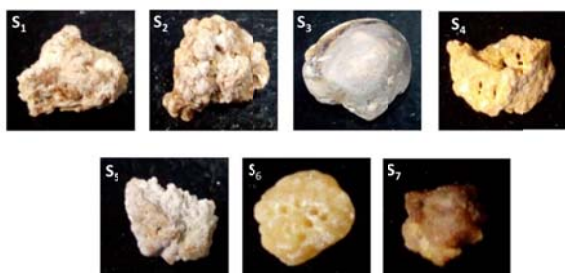


Figure 1 Photographs of kidney stone samples used for LIBS-Raman experiments

2.2 Micro-Raman set-up

The experimental (LIBS and micro-Raman) setup used for identifying different renal calculi has been described elsewhere²³. In brief, Raman measurements were carried out by focusing a 785 nm diode laser (Starbright Diode Laser, Torsana Laser Tech, Denmark) using a 60X microscope objective placed inside an inverted microscope (Nikon Eclipse Ti-U, Japan). 785 nm was chosen to reduce interference of unwanted fluorescent emission from the sample surface. The inelastically scattered light from the sample is collected using a backscattering configuration and directly focused onto the entrance slit of the spectrograph. Rayleigh scattered radiation is rejected using an edge filter. Raman spectra were recorded in the spectral range of 300-2000 cm^{-1} using a Horiba JobinYvon iHR320 spectrograph with a grating of 1200 grooves/mm, coupled to a liquid-nitrogen-cooled charge-coupled device (CCD) (Symphony CCD-1024x256-OPEN-1LS). The spectral resolution of the system is about 5.7 cm^{-1} (for 997 cm^{-1} Raman band of polystyrene bead at 100 μm slit width) as seen from the half-widths of the sharp bands.

Raman spectra were recorded from different sites of each kidney stone by utilizing a maximum laser power of 1~2 mW. The raw Raman spectra were smoothed using 9-point moving averages (Savitzky-Golay filter) followed by multipoint baseline correction using Grams software (Thermo Scientific Inc., Rockford, IL) to eliminate the background noise²⁴.

2.3 LIBS set-up

For LIBS measurements, a nanosecond Q-switched Nd:YAG laser (Spectra Physics PRO 230-10; 355 nm, 6 ns, 10 Hz and 14 mJ) is focused onto the sample surface using a bi-convex lens of focal length $f_l = +200$ mm. The spot size at the focal point was about 100 μm and irradiance $\sim 2.5 \times 10^9$ W/cm^2 . The light emitted from the plasma plume is collected with a UV grade quartz lenses/mirror collection system kept at 45° angle and fed to the echelle spectrometer coupled with thermoelectrically cooled intensified charge coupled device (ICCD) (Andor Mechelle ME5000-DH734, Andor, Ireland) through a 50 μm single fiber cable. Echelle spectrograph provides simultaneous broad spectral range 250-850 nm and very high resolution of 0.05 nm at a fixed slit width of 10 (width) x 50 (height) μm . LIBS measurements were carried out by keeping the samples on an X-Y translation stage which was translated during any measurement to expose different points and to prevent drilling of the sample. LIBS measurements were carried out by recording emission spectrum from 100 laser pulses in a single exposure. Gate delay and gate width of 700 ns and 6 μs respectively were used to minimize interference from early continuum and get optimum emission from plasma plume.

Several precautions were taken in recording LIBS and Raman spectra in order to minimize any site-dependent differences. These included:

1. In each sample, sections which appeared physically (colour, crystalline appearance) uniform over a relatively large area were used for both LIBS and Raman.
2. As mentioned above, LIBS spectra were recorded by moving sample continuously across a cross sectional area to avoid extensive sample damage. Under these conditions, only micron thickness sample is lost from the surface and sample remains unchanged for all practical purposes.
3. Raman spectra were recorded from the same surface at several points.

2.4 Chemical analysis

After Raman and LIBS measurements, chemical analysis of the samples²⁵ were performed as discussed in the Supplementary Information.

3. Results and discussion

3.1 Calcium stones ($S_1 - S_3$)

Different kidney stones are analyzed by LIBS and Raman spectroscopy to extract maximum chemical information. The atomic emission lines observed from

the LIBS spectra were identified using the National Institute of Standards and Technology (NIST) database²⁶. Figure 2 displays the LIBS spectrum of S_1 stone over two different spectral ranges 370-400 nm and 420-450 nm respectively. The spectrum manifest the presence of high intense calcium emission lines observed at 315.8, 317.93, 373.69, 393.36, 396.85 nm etc. Other major emission lines observed from the spectrum are Sr at 393.5, 397, 431.9 nm; Mn at 349.58, 404.87, 423.51 nm; Fe at 392.79, 396.74, 422.54, 527.03 nm; Ti at 395.63, 429.86, 429.96, 439.5 nm; Si at 390.55, 595.75 nm; Na at 541.45, 588.99 nm; Zn at 636.23 nm; O at 615.59, 615.81 nm and Cu at 458.69 nm. Abundant high intense calcium lines observed in the LIBS measurements presumes that the major composition of stone is Ca. Calcium based stones are the most common type of kidney stones and commonly idiopathic in nature. Generally, it can occur in two major forms; calcium oxalate and calcium phosphate. Among this calcium oxalate stones are most common (63%) and occurs in two different crystalline forms such as calcium oxalate monohydrate (COM, whewellite) and calcium oxalate dihydrates (COD, weddellite). The absence of P in the LIBS spectrum confirms the fact that the stone composition is calcium oxalate. But looking at the LIBS spectrum alone, one cannot differentiate the hydrates of the oxalate minerals. Contrary to the report from Oztopark et al.,⁸ a medium intense Cu emission line is observed from the LIBS spectra. This supports the observations of Sing et al.,⁴ who reported the presence of strong Cu lines in calcium oxalate stones. A similar observation has been made by Atakan et al., by stating that Fe and Cu act as promoters for the formation of calcium oxalate stones²⁷.

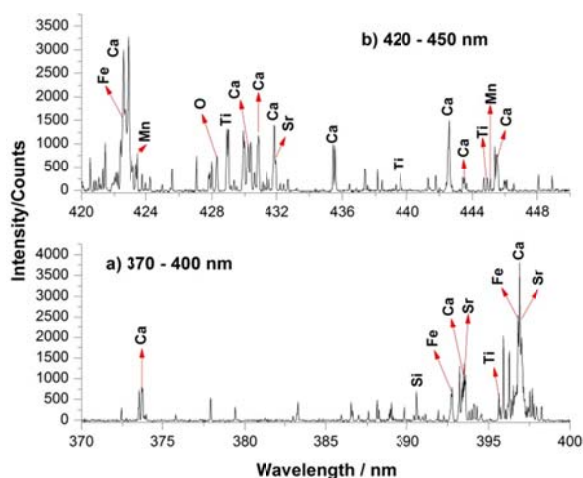


Figure 2 LIBS spectrum of S_1 stone

Figure 3 shows the micro-Raman spectra of S_1 stone, which has an intense Raman line at 1476 cm^{-1} . This is the characteristic band of calcium oxalate dihydrate (weddelite, $\text{Ca}(\text{C}_2\text{O}_4)\cdot 2\text{H}_2\text{O}$) due to C-O symmetric stretching vibration⁹. A weak C-O asymmetric

stretching frequency band at 1629 cm^{-1} is also clearly visible in the spectrum. Other two intense bands at 508 and 909 cm^{-1} were arguably due to O-C-O in-plane bending and C-C stretching vibrations respectively⁹. Raman spectra of S_1 stone confirmed the fact that the major composition of stone is calcium oxalate dihydrate (COD). The result also proves the complementarity of LIBS-Raman technique, since LIBS alone couldn't differentiate the hydrates of minerals. However, Raman can easily identify the hydrates and polymorphs of the minerals through the vibrational (lattice/internal) modes. On the other hand, LIBS can detect the metal elements such as Mn, Ti, and Sr along with impurities and trace elements such as Fe, Cu etc, which is difficult to identify using Raman measurements.

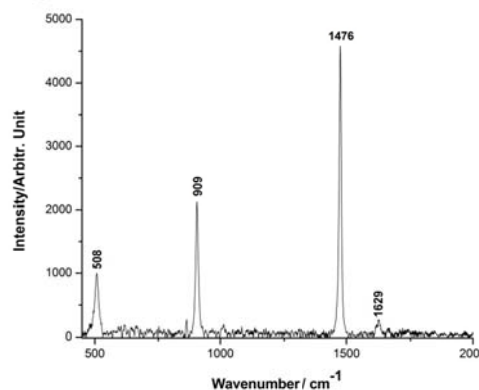


Figure 3 Raman spectrum of S_1 stone

Figure 4 shows the typical LIBS spectrum of the second type of stone (S_2) for our regions of interest. This spectrum shows many intense characteristic emission lines of Ca which confirms the abundance of this element similar to the previous case (S_1). Unlike the case of S_1 sample, additional medium intense Zn lines at 334.5, 589.43, 636.23 nm; weak K lines at 397.25, 404.14; feeble Si lines at 504.1, 549.64 nm (not shown in the figure); moderate Mg lines at 383.23, 389.33 nm and Cu lines at 406.29, 458.69, 647.01 nm were also observed in the spectrum. It thus logical to classify these types of stones as calcium oxalate because of the shreds evidence of large Ca lines observed in the LIBS spectra.

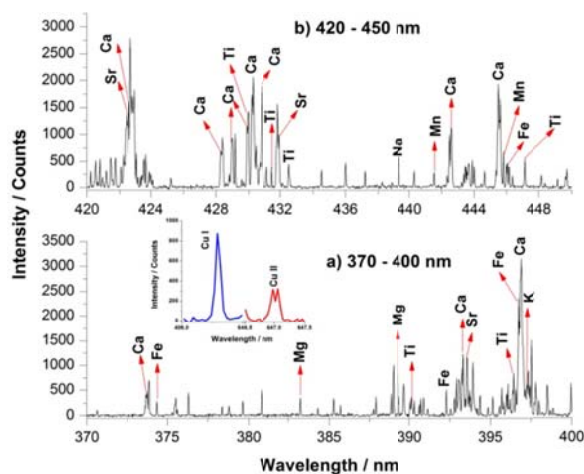


Figure 4 LIBS spectrum of S_2 stone

Figure 5 shows the Raman spectra from different sites of S_2 stone. Figure 5 (a) shows an intense band at 1462 cm^{-1} and two medium intense bands at 894 and 1488 cm^{-1} respectively. Both 1462 and 1488 cm^{-1} are characteristic bands of calcium oxalate monohydrate (whewellite, $\text{Ca}(\text{C}_2\text{O}_4)\cdot\text{H}_2\text{O}$) due to the C-O symmetric vibrations^{1, 9}. Other medium intense vibrational modes observed at 504 and 894 cm^{-1} are probably due to the O-C-O in-plane bending and C-C stretching respectively. A weak C-O asymmetric stretching band at 1625 cm^{-1} also observed from the spectrum. From these, we can infer that the stone is made up of calcium oxalate monohydrate. However, Figure 5 (b) shows the Raman spectra of the same stone recorded from another spot. Here the spectral features are different from that of 5 (a). The spectrum displays characteristic bands of calcium oxalate dihydrate (weddellite) as well as monohydrate at 1476 and 1462 cm^{-1} respectively. All other bands at 506 , 909 and 1625 cm^{-1} are well matched with weddellite type as shown in Figure 3. An additional band at 1665 cm^{-1} also observed in the spectrum compared to Figures 3 and 5 (a), assignment of which is not clear at present, though possibly it could be C-O asymmetric stretch in a different crystal environment. This suggests that the stone could be a mixture of whewellite and weddellite, showing the possible heterogeneous nature of the stone. The presence of both hydrates of oxalate minerals gives an indication that, during the initial stages of stone formation it had weddellite type structure and subsequently transformed into whewellite as it aged resulting in the dual nature⁹. This result also shows the feasibility of micro-Raman analysis for the identification of site to site changes in the crystal even from a minor alteration in the composition, whereas all other standard procedure/techniques can only give the average composition. Point-wise analysis can be useful for the diagnostic and monitoring therapy applications since therapy is dependent on urine chemistry, which one may

be able to understand (as a function of time) by analyzing layer-wise composition of the stone as well as the analysis of stones passed over a period of time.

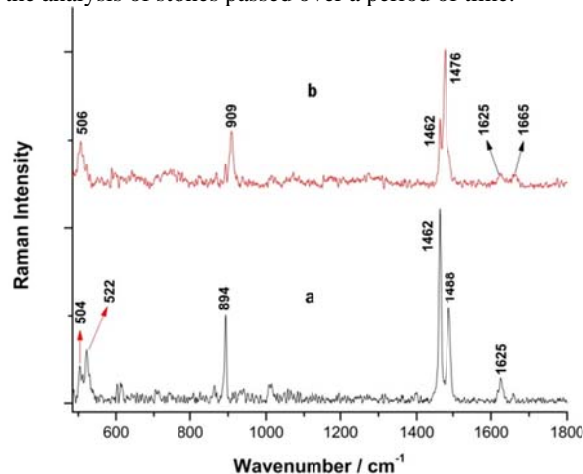


Figure 5 Raman spectra of S_2 ; (a) COM and (b) mixture of COM and COD

Raman spectrum was not obtained from S_3 sample probably due to its shiny black surface. Figure 6 shows the LIBS spectrum of S_3 sample, which has intense Ca, Sr, and P emission lines. Presence of P lines (458.804 , 458.98 , 558.33 , 608.78 and 616.56 nm) along with Ca lines in the LIBS spectra corroborates that the stone belongs to calcium phosphate (CaP) group. Trace elements are generally not considered to be distinguishable markers for differentiating different types of kidney stones except in the case of CaP type. Apart from these major elemental lines, a medium intense Cu line at 458.69 nm is also observed in the spectrum. This is supported by a report from Mayer and Angino wherein it is mentioned that the presence of Cu affects the crystal growth of CaP based stones but not calcium oxalate stones²⁸. Another perspective of Atakan et al., is that Zn and Mg have an inhibitory effect on the crystal growth of calcium oxalate but Cu and Fe act as promoters for the same stone formation²⁹. Abundant Sr lines present in the spectrum suggesting that certain amount of Ca might have been replaced by Sr during the biomineralization. A recent report from Batschko et al., supports this observation by mentioning that Sr is presented as strontium apatite in all calcium based stones³⁰. The strong atomic emission lines of Sr along with the Ca and P ensuring the fact that the probable category of the stone is calcium phosphate. However, authors do agree with the fact that by looking at the major and trace atomic emission lines alone it is impossible to differentiate different types of calcium phosphate stones such as calcium orthophosphate (CaPO_4), calcium hydrogen phosphate dihydrate (brushite $\text{CaHPO}_4\cdot 2\text{H}_2\text{O}$), calcium phosphate hydroxide (hydroxyl apatite) and calcium phosphate mono/dia/tri-basic. In a nut shell,

even when Raman measurements are not successful, a general classification of the stone type is possible with LIBS as in this case.

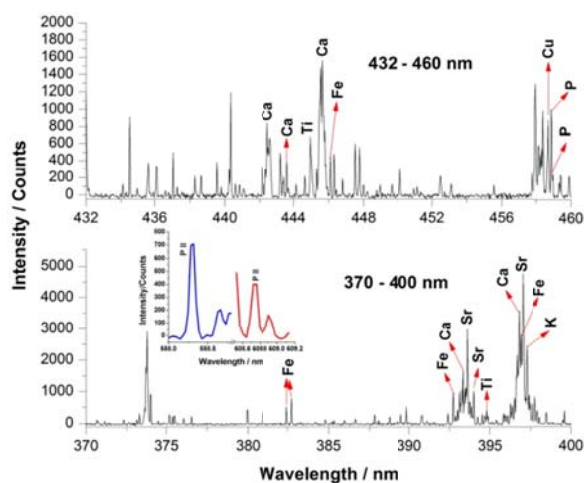


Figure 6 LIBS spectrum of S_3 sample

3.2 Uric acid stones (S_4 & S_5)

In the case of S_4 , the LIBS spectrum (Figure 7) showed medium intensity Ca line at 396.85. Figure 8 shows the Raman spectrum of S_4 stone, which is similar to that reported by Kodati et al.¹¹. Spectra from various parts of the stone surface showed similar Raman bands, indicating that the stone is uric acid type. Uric acid is the final product of the purine nucleotides metabolism and recently identified as a biomarker for several abnormalities such as renal diseases, gout, cardiovascular diseases and preeclampsia²⁹.

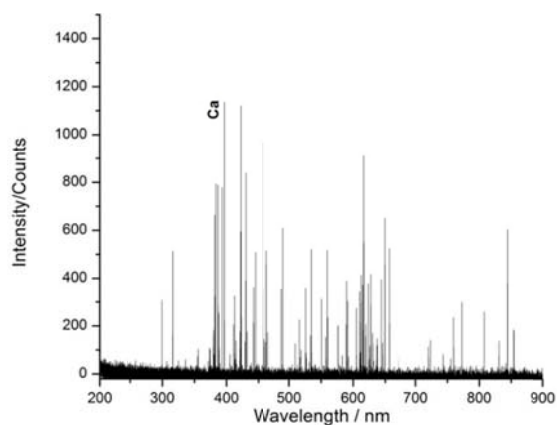


Figure 7 LIBS spectrum of S_4 sample

Uric acid stones have non-salt type of organic compounds, which show a very high intense Raman band at 624 cm^{-1} corresponds to skeletal ring deformation and also a ring vibration mode at 1032 cm^{-1} . The Raman bands at 470 and 501 cm^{-1} are probably due to the C-N-C vibration in the ring. Spectrum also shows a medium intense Raman band at 1645 cm^{-1} and a very weak band at 881 cm^{-1} due to the carbonyl group stretching vibration and N-H in-plane bending vibrations respectively¹¹. Medium intense Ca line observed in the LIBS spectrum probably arises from the calcium contents present in urine or impurity, because the Raman spectrum does not show characteristic bands of calcium based stones. The assignments of the Raman Bands for uric acid stone are tabulated in Table 1.

Table 1 Raman band assignments for uric acid

Obs. peaks	Tentative band assignments ^{I, II, 29}
470	C-N-C vibration in the ring/skeletal ring deformation
501	C-N-C vibration in the ring
559	Ring breathing mode
624	Skeletal ring deformation
656	Ring breathing mode
703	N-H bending
780	Ring vibration
881	N-H bending
992	Multiple bands including ring vibration, C-O, C-C, C-N stretching, and N-C-C stretching and bending vibration
1032	
1119	
1231	
1494	CC stretching
1589	C-N stretching

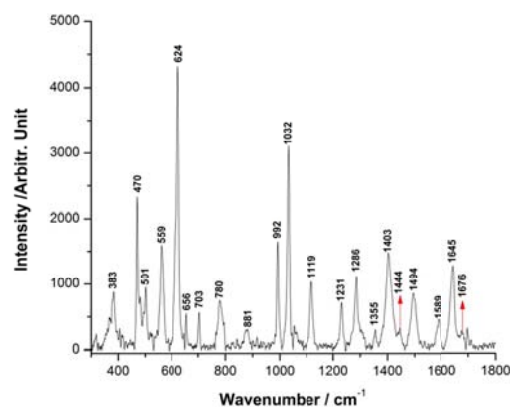


Figure 8 Raman spectrum from S_4 sample

Figure 9 depicts the atomic emission spectra of S_5 sample. As one can observe the spectrum is dominated with large number of Na lines at 315.95, 316.12, 317.91, 430.88, 445.52 nm etc and high intense Ca lines (393.36, 396.85 nm etc.). Other major lines observed in the spectrum are: Fe at 396.74 and 396.93 nm; N at 616.77 nm; O at 394.73, 397.21, 397.48, 428.29, 430.31 nm and C at 589 nm. This highlights one of the main advantages of LIBS over other analytical techniques like XRF in detecting the light elements such as C, H, O and N. Considerably large amount of Na and Ca emission lines in the LIBS spectrum suggest that the main composition of stones could be a mixture of calcium oxalate and sodium urate.

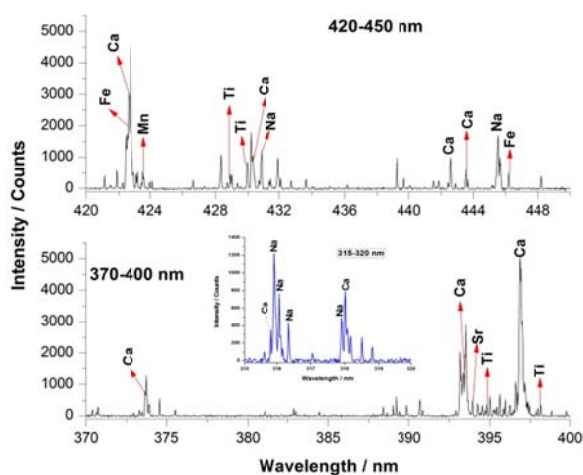


Figure 9 LIBS spectrum of S_5 stone

Figure 10 shows the Raman spectrum of the same stone (S_5). The Raman spectrum (Figure 10 (a)) matches with the earlier reports by Kodati et al.¹¹ for similar type of stones. This spectrum reveals that the main composition of stone is monosodium urate, which is the common form of crystallized urate. Raman spectrum of monosodium urate and uric acid looks similar in appearance but minor difference in wavenumber for each of its band. Figure 10 (b) shows the Raman spectrum recorded from another part of the same stone, which indicate the signatures of whewellite. This complements the results from LIBS and confirms its analytical prediction capability by looking at the atomic emission lines.

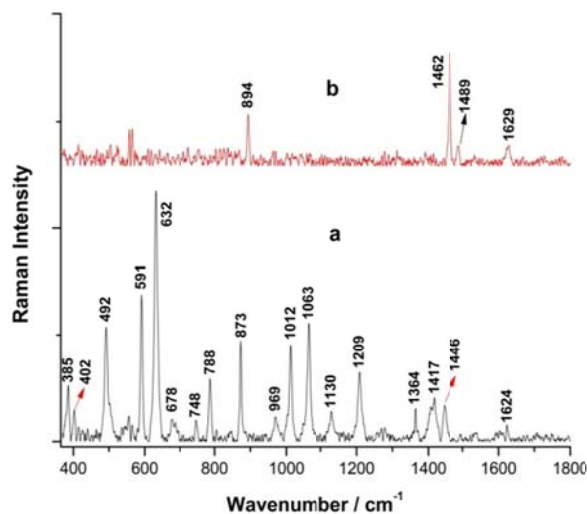


Figure 10 Raman spectra from S_5 stone; (a) monosodium urate (b) calcium oxalate monohydrate

3.3 Cystine stones (S_6)

LIBS could not be recorded from the S_6 sample. The reasons behind this are not clear at present. Figure 11 shows the Raman spectra of the S_6 sample, in which an intense characteristic Raman band of cystine is observed at 498 cm^{-1} due to the S-S stretching vibration¹². Weak and medium intense mode of C-S stretching vibrations is also observed at 617 and 678 cm^{-1} respectively. This confirms the composition of the stone. Spectra also manifest the clear signatures of a medium intense C-C stretching mode at 1339 cm^{-1} , weak C-H bending mode at 1382 cm^{-1} , and mixed vibrational modes of C-H bending and CO stretch of hydrogen bonded COO group in the crystal at 1405 cm^{-1} .

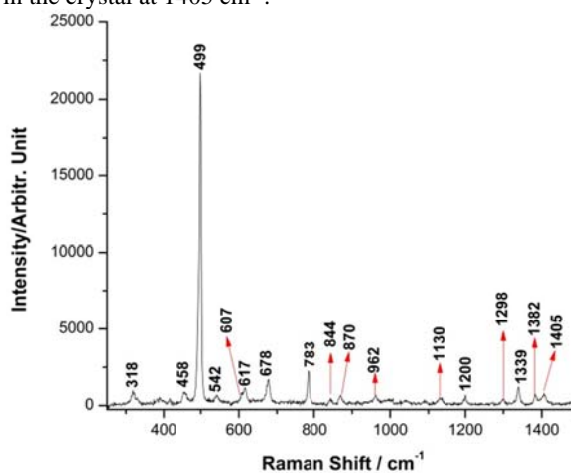


Figure 11 Raman spectrum of Cystine type stone (S_6)

3.4 Mixed stones (S_7)

LIBS spectrum of S_7 sample (Figure 12) shows the presence of abundant calcium lines. Other major emission lines observed from this spectrum are from P at 616.55 nm; Sr at 393.5, 397, 422.46 nm; Mg at 279.55, 285.23, 382.93, 389.33 nm; Mn at 404.13, 408.29, 423.5 nm; Na at 288.11, 430.88, 445.52 nm; Cu at 458.69 nm; C at 588.95 nm and O at 428.29 nm. Intense Ca and P lines observed in the LIBS spectrum suggest that stone is made up of calcium oxalate or calcium phosphate. The other possible stone composition is struvite (magnesium ammonium phosphate hexahydrate) because of the presence of the medium intense Mg and P lines.

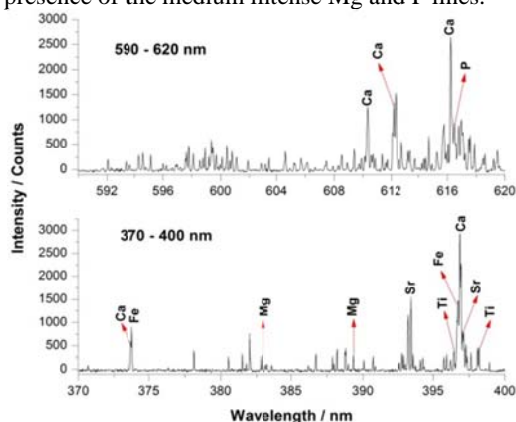


Figure 12 LIBS spectrum of mixed stone S_7

Raman spectrum of S_7 stone (Figure 13) shows a low and medium intense bands at 1461 and 1489 cm^{-1} respectively. These two bands are characteristics of whewellite stone⁹. Other Raman bands of whewellite, due to the C-C stretching and C-O asymmetric stretching at 894 and 1624 cm^{-1} are also observed in the spectrum. At the same time, another medium intense peak observed at 968 cm^{-1} corresponds to the Raman signature of calcium orthophosphate ($\text{Ca}_3(\text{PO}_4)_2$) due to the symmetric stretching mode of phosphate group¹⁰. The spectrum also shows a weak Raman band at 942 cm^{-1} which is the characteristic mode of struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) due to the symmetric stretching vibration of phosphate group.¹⁰ All these information gives a clear idea that the stone is made up of different minerals such as calcium oxalate monohydrate, calcium orthophosphate and struvite circumventing the heterogeneous nature of the kidney stone. Presence of the intense P, Ca and Mg emission lines from the LIBS spectrum strongly supports the observation of Raman spectra.

In order to cross validate all the above discussed results, all the 7 type of stones were also analyzed by conventional chemical method. Table 2 provides the comparison of chemical analysis and LIBS-Raman data from seven types of kidney stones.

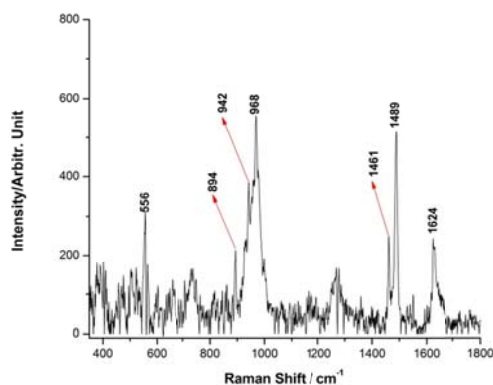


Figure 13 Raman spectrum of mixed stone S_7

The major limitations of chemical analysis are; elaborated sample preparation methodologies, requirement of large sample quantities and time consumption (few days). LIBS-Raman results of four kidney stone samples (S_1 , S_2 , S_4 , and S_7) are well matches with the chemical analysis. Differentiating the two crystalline forms of the calcium oxalate crystals, as in the case of S_1 and S_2 samples, is not feasible with the chemical analysis. On the other hand, Raman spectra of S_1 and S_2 sample shows clear discrimination between hydrates of the calcium oxalate minerals whereas chemical analysis could only provide a brief idea about the average composition (see Table 2). Chemical analysis of S_1 and S_2 also shows the presence of additional ammonium and carbonate minerals. In the case of S_3 sample, a moderate level of uric acid is predicted by chemical analysis, which is not observed from the Raman measurements. Raman measurement failed to gives information of S_3 sample, whereas LIBS gives clear information that the stone could be CaP type due to the presence of intense Ca and P lines. Similarly, chemical analysis of S_5 sample shows the presence of Ca and oxalate along with uric acid. This implies that this stone is a mixture of calcium oxalate and uric acid. LIBS and Raman spectra of the same stone shows a better classification, by differentiating both uric acid and monosodium urate. The large number of Na lines from LIBS spectra and the characteristic bands of monosodium urate from the Raman spectra suggest the possibility of sample being monosodium urate. Raman spectrum also shows the evidence of calcium oxalate monohydrates on the same sample, which is supported by chemical analysis as the sample is a mixture of two different minerals. This manifests another advantage of LIBS-Raman technique over chemical analysis, as it can easily differentiate the uric acid and monosodium urate by analyzing the characteristic atomic lines/molecular bands respectively. Raman spectrum from S_6 stone shows the characteristic bands of cysteine. This is in contrast to the chemical analysis which reports the presence of calcium carbonate only. This is because of

the fact that, when the concentration of an element in the stone is less (as the case of Mg in S₇ in this particular study), it may not be detected by chemical methods, which are basically qualitative, color forming tests. However, LIBS technique is very sensitive to detect these elements even at trace levels. Compared to micro-Raman measurements, chemical analysis gives average values of components present in the sample, and thus, in the case of mixed stones it failed to identify minor components. It's a well-known fact that kidney stone samples are complex in nature with varying chemical composition from surface to core. Chemical analysis provides information about the whole sample whereas LIBS-Raman spectrum can give spatial information at micron levels. This could be one of the reasons for the contrary results obtained from these techniques for certain samples. Nevertheless, efforts are on to set-up a hybrid LIBS-Raman system and study the surface morphology, depth profile and subsequent chemical compositions of renal calculi.

Table 2 Comparison of LIBS and Raman measurements with chemical analysis of kidney stones

Sample	Raman	LIBS	Chemical analysis
S ₁	COD	Ca, Mn, Sr, Fe, Ti, Cu	CO ₃ ²⁻ , NH ₃ , oxalate, Ca, PO ₄ ³⁻ , Mg
S ₂	COM+COD	Ca, Na, K, Sr, Fe, Si, Cu, Mg, Ti, O	CO ₃ ²⁻ , NH ₃ , oxalate, Ca, PO ₄ ³⁻ , Mg
S ₃	No signal	Ca, P, Sr, K, Ti, Cu, O	Uric acid, oxalate, Ca, PO ₄ ³⁻ , Mg
S ₄	Uric acid	Ca	Uric acid
S ₅	Monosodium urate + COM	Na, Ca, Fe, C, N, O	Uric acid, oxalate, Ca, PO ₄ ³⁻ , Mg
S ₆	Cystine	No signal	CO ₃ ²⁻ , Ca
S ₇	COM + Calcium orthophosphate+Struvite	Ca, P, Sr, Mn, Mg, C, N	NH ₃ , oxalate, Ca, PO ₄ ³⁻

4. Conclusion

The complexities involved in performing precise chemical analysis of renal calculi make it one of the most challenging problems currently in the medical fraternity. This problem has considerable significance owing to the fact that treatment methodologies are often decided by the nature of the stones. In view of this, the complementarity nature of two spectroscopic techniques, LIBS and Raman has been effectively utilized for identifying and differentiating different types of kidney stones. The dual analytical capability provides comprehensive information of the complex samples and help to obviate the ambiguity. Most of these stones are identified using either Raman or LIBS by collecting its molecular finger prints as well as characteristic major, minor and trace elements present in the sample. Raman spectroscopy successfully classified different crystalline form of calcium oxalate, which was not possible with LIBS. The evidences of minor elements such as Fe and Cu diagnosed by LIBS are found to be the promoter for the formation of COD stones. Large amount of intense Sr lines in calcium stones is due to the replacement of Ca by Sr during the biomineralization.

In short, advantages of LIBS-Raman over conventional chemical analysis for the successful classification of kidney stones have been demonstrated by a comparison of these three techniques. LIBS-Raman technique has demonstrated its capability to discriminate different hydrates and urate samples. The technique is thus of great importance, from the bio-medical point of applications, because the complete elemental and molecular information can be very useful to understand the mechanism behind the initiation and formation of kidney stones thus providing means for more effective preventive, curative and recurrence-elimination therapy.

Supporting Information

Procedure A:

1. The calculus was pulverized in a test tube using a glass rod. Approximately 10-20 mg of this was transferred to another test tube. Always some crushed calculus was kept in reserve. Whenever the stones were small, immediately carried on with the procedure B.
2. 10-20 mg of calculus was warmed in 1 ml of 0.1 N NaOH at 60° C for 5 minutes, by with shaking the tube 2-3 times in between. The tube was centrifuged and the supernatant was decanted into another tube and reserved for the tests for uric acid, cysteine and ammonia. The specimen in the tube was washed with 1 ml water and centrifuged again. The supernatant was discarded and the washing procedure was repeated once more and the residue was saved for further analysis.

3. Test for uric acid

2 drops each of NaOH extract, 14% sodium carbonate and phosphotungstic acid were mixed in a tube. Development of deep blue color indicated the presence of uric acid. (Development of a faint blue color considered as insignificant)

4. Test for cystine

2 drops each of NaOH extract, ammonia and NaOH were mixed in a tube. 2 drops of sodiumnitroprusside solution was added to it after 5 minutes. Development of a deep red color indicated the presence of cystine.

5. Test for NH₃

0.5 ml each of NaOH extract, phenol color reagent and alkaline hypochlorite solution were mixed in test tube. Development of blue color after 10 minutes indicated the presence of ammonia.

6. Test for xanthine

0.2 ml NaOH extract was diluted with 3 ml water. Its pH was adjusted to 8.0 by adding 7% KH₂PO₄ in a drop-wise manner. An absorption curve of this solution was taken. A major peak at 270 nm and a minor peak at about 240 nm indicated the presence of xanthine.

7. Test for carbonate

To the tube containing residue (step 2), a drop of concentrated HCl was added along the sides of the tube. A momentary, but copious evolution of gas bubbles indicated the presence of carbonate.

An additional 0.2 ml of concentrated HCl and 0.5 ml of water mixed in the same tube. The mixture was boiled for a minute and centrifuged after cooling.

8. Test for oxalate

0.3 ml of the supernatant from step 7 was taken in a tube and its pH was adjusted to 3-4. Appearance of a precipitate or a strong turbidity indicated the presence of calcium oxalate. The tube was centrifuged again.

9. Test for calcium

To the supernatant from step 8, 3 drops of potassium oxalate and about 0.3 ml of water is added. Appearance of turbidity after standing for 10 minutes, indicated the presence of calcium other than oxalate. The tube was centrifuged again.

10. Test for phosphorus

To the 4 drops of supernatant from step 9, 0.3 ml of iron TCA reagent and 2 drops of ammonium molybdate was added. Development of blue color indicated the presence of phosphate.

11. Test for magnesium

The remainder of supernatant from step 9, was mixed with 2 drops of titan yellow and 0.5 ml of 20% NaOH. Appearance of a red color indicated the presence of magnesium.

Procedure B:

1. To the fragment taken in a micro centrifuge tube, 0.2 ml of 0.1 N NaOH was added. It was warmed for 5 minutes in 60 °C water bath. After centrifugation,

the supernatant obtained was used for uric acid and cystine tests.

2. The residue remained in the step 1, was washed twice with 0.2 ml water. After centrifugation the supernatant obtained was discarded.
3. 1 drop of concentrated HCl was added to the residue in the step 2 along the sides of the tube. An effervescence was observed when the acid made contact with the residue. To this 0.4 ml of water was added and heated gently for 5 seconds over a micro burner. It was centrifuged again.
4. The supernatant obtained was decanted into a 13X100 mm test tube. The tests described in procedure A were carried out using this supernatant.

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Graphical Abstract for Table of Contents

Text: Presence of renal calculi (kidney stones) in human urethra has been increasingly diagnosed over the last decade and is considered as one of the most painful urological disorders. Two highly complementary analytical techniques; LIBS and Raman have been used to identify the chemical composition of different types of renal-calculi. Simultaneous elemental and molecular information is useful for the medical community to understand the mechanism behind the formation of kidney stones and to prevent its recurrence.

Image:

