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Rapid and reliable method for composition of concentric layers of kidney stones by fourier transform infrared spectroscopy with KSLS-13

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Abstract: Present study is aimed to explore the rapid and reliable method of analyzing the composition of concentric layers of stones by fourier transform infrared spectroscopy with Kidney Stone Library Software (KSLS-13). Total of 69 kidney stones recovered from kidney stone patients (33 males and 36 females, mean age ranges of males and females were 10.1 to 37.3 & 15.2 to 54.4 respectively) were analyzed by fourier transform infrared spectroscopy. Composition of central, middle, peripheral layers of each kidney stone and whole stone were analyzed in the research laboratory of Institute of Biochemistry. Spectra of layers of kidney stones were collected and compared with kidney stone library software (KSLS-13). Among 69 kidney stones, 25 (36.2%) were pure stones (23.2% pure Calcium oxalate mono hydrate (COM), 10% pure carbonate apatite (CA), 3% pure magnesium ammonium phosphate (MAP)) and 44 (63.7%) mixed stones. Among 69 kidney stones, most prevalent were pure calcium oxalate stones (23.2%) and calcium oxalate mono hydrate stones mixed with carbonate apatite and ammonium hydrogen urate (AHU) (18.8%). The IR bands were compared with KSLS-13 as well as with standards. Calcium oxalate mono hydrate, carbonate apatite and uric acid were significantly increased in middle layer, but ammonium hydrogen urate and calcium oxalate dihydrate (COD) were increased in periphery. Whereas, reverse was true for magnesium ammonium phosphate in central layer. In conclusion, KSLS-13 by FTIR is found to be the most rapid and reliable method to study composition of concentric layers of kidney stones.

Keywords: Mixed kidney stones, calcium carbonate stones, fourier transform infrared spectroscopy, kidney stone library software, calcium oxalate stones.

INTRODUCTION

The world is facing kidney stones as a major health concern especially it destroys Pakistan economy with increased medical expenses and decreased productivity (Hussain *et al.,* 2013). Kidney stones consist of a center that consists of crystal-like substances, and a surrounding region that is composed of layers (middle and periphery). More than one crystalline component may be present in kidney stones. Stone components may be mineral, organic, or both (Bhattacharyya *et al*., 2014; Choo-Kang E, 2008). More than 65 different molecules (including 25 of exogenous origin) have been found in kidney stones. Many investigators have reported a classification of kidney stones in seven distinctive types and twenty-one subtypes, including monohydrate (whewellite) and dihydrate (weddelite) calcium oxalates, phosphates, uric acid, urates, protein, and cystine (amino acid) calculi (Choo-Kang E, 2008; Bibliash *et al*., 2010). Kidney stone constituents can be determined using various techniques such as X-ray diffraction, crystallographic methods, Infrared spectroscopy, Fourier transform infrared spectroscopy and thermal analysis. Fourier transform infrared spectroscopy gives accurate information regarding the stone composition as it helps in quantification of each stone constituent without using any chemicals or solvent systems. Therefore fourier transform infrared spectroscopy is considered as the most suitable technique for stone analysis (Basiri *et al*., 2012; Khaskheli *et al.,* 2012). The information of chemical composition of layers of stones is essential for knowing their etiology and seems to be helpful in making some preventive measures against the disease (Pandeya *et al*., 2010). Up to date there is no reported work regarding study of layers of kidney stones for knowing their etiology have been carried out so far. So we have introduced a method of study of layers of kidney stones by Kidney Stone Library Software with Fourier transform infrared spectroscopy analysis in order to have a better understanding of the disease etiology and its further treatments.

MATERIALS AND METHODS

Collection of kidney Stones

We analyzed the composition of central, middle and peripheral layer along with the whole stone by using fourier transform infrared spectroscopy. Spectra were measured and compared with a library of kidney stone software (version 13.1).

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The analysis of 69 kidney stones recovered from kidney stone patients after got signed informed consent form (33 males and 36 females, mean age range 10 to 37 & 15 to 54) by Fourier transform infrared spectroscopy of the central, middle and peripheral layer along with whole stone of each kidney stone was carried out in the research laboratory of Institute of Biochemistry, University of Sindh, Jamshoro. The ethical approval was achieved from Institutional Ethical Committee prior to start sampling.

Sample preparation

Kidney stones removed during surgery were first washed carefully with double distilled deionized water (to remove blood and debris) and dried over silica gel for one week in dark cabinet. Then morphological feature such as color, number of stones, shape etc. were noted. Single kidney stone from each patient was cut into half using a jeweler saw. The powder collected during cutting into equal halves represented the whole stone sample. Peripheral layer was collected with the help of dissection needle, and then the middle and central layer were collected by scratching carefully in a precise manner. Each part of the sample ground with a pestle and mortar until a fine homogenous powder formed, which was then stored in a sample tube with proper labeling, kept over silica gel in dark until analyzed by Fourier transform infrared spectroscopy.

Analysis of kidney stones by FTIR

The composition of central, middle and peripheral layer along with whole stone was determined for each sample by FTIR spectrometer (Nicollet Avatar 380 FTIR spectrophotometer by thermo electronic corporation) in the frequency range of 4000-600 cm^{-1}. The homogenous fine powder of sample was directly mounted on ZnSe crystal of the smart accessory of FTIR. Each spectrum for the layers and whole stone was obtained after 64 scans with the help of EZ Omnic (7.0 version) software which perform the entire process automatically.

Kidney stone library software, version 13.1 (KSLS-13) and analysis kit

After saving spectra, they were compared and identified with Library of kidney stone software (version 13.1). The KSLS-13 and Analysis Kit was created by spectroscopists and medical doctors to allow analysis of kidney stones using Nicolet FTIR spectrometer with OMNIC software. It contains an algorithm to work with; and the kidney stone guide containing additional analysis information (Kesner *et al.,* 2000). As we have a spectral library of real kidney stones in KSLS-13. So, we compared the unknown renal stone samples to a number of library spectra. A match value close to 100% indicated that the sample consists of the same components in about the same ratio. The stones which match <90% were excluded from the study.

STATISTICAL ANALYSIS

Omnic software collected the spectra, whereas kidney KSLS version 13 analyzed the stones. The percentages, mean and standard deviation were calculated by MS Excel 2010. The student's t-test was calculated for comparison. p value less than 0.05 was kept the level of significance.

RESULTS

Sixty nine (69) kidney stone samples were analyzed by FTIR spectroscopy. The spectra of samples were then compared with kidney stone software version 13.1, which are revealed from fig. 1 and 2, which are illustrated as typical FTIR spectrum of pure and mixed kidney stone samples respectively.

Mainly the kidney stone spectra were matched with standards and were confirmed from the kidney stone software which gave more than 90% matching. The diagnostic bands identified for pure COM stones in present as well as reported in other studies were strong bands around 777.32,1314.81 and 1615.99 cm^{-1} (these bands were due to deformation of OH-group and c=o group) (Bhattacharyya *et al*., 2014; Khaskheli *et al*., 2012). Similarly diagnostic bands for pure CA stones were around 1026.32 and 1636.10 cm^{-1} (these bands were due to stretching vibration of $(PO_4)^{-3}$ and carbonate group). Bhattacharyya *et al*. in 2014 also reported the 1000 to 1100cm^{-1}range of peaks for appetite stones (Bhattacharyya *et al*., 2014).Diagnostic bands for COM stones mixed with CA and AHU were around 2341.86, 2360.14 and 668.87 cm^{-1}, the bands in this region are due to deformation of $(PO_4)^{-3}$ group and the presence of these components (i.e. COM, AHU and CA) was confirmed by matching these peaks with standards also.

Table 1 shows the composition of 69 kidney stones analyzed, among them majority of kidney stones were of mixed type (63.7%), whereas, only 36.2% were pure stones. Of those pure stones majority of the pure stones was of COM. While among mixed stones we found highest percentage (49.27%) of COM mixed with CA, AHU and/or COD in different proportions. Whereas other mixed stones were Of the 4 (5.79%) CA mixed stones, 2(2.89%) stone was CA mixed with MAP and remaining 2.89% were CA, MAP and UA mixed with different components in different proportions.

The composition of concentric layers of renal stones as revealed from table 2 in kidney stones in three layers is significantly varied ($p<0.05$) in all components except the MAP. The concentration of COM, UA and CA are significantly increased in middle layer as compared to other layers, whereas significantly decreased concentration of MAP was noted in central layer while AHU and COD were found increased in periphery as compared to other layers.

DISCUSSION

The study of composition of concentric layers of kidney stones gives us awareness about the role stage wise growth of kidney stones. Moreover, proper management of the disease and prevention of its recurrence is also possible by knowledge of its components present in its central and peripheral layer (Singh and Rai, 2014).

FTIR has been suggested as the most efficient method for

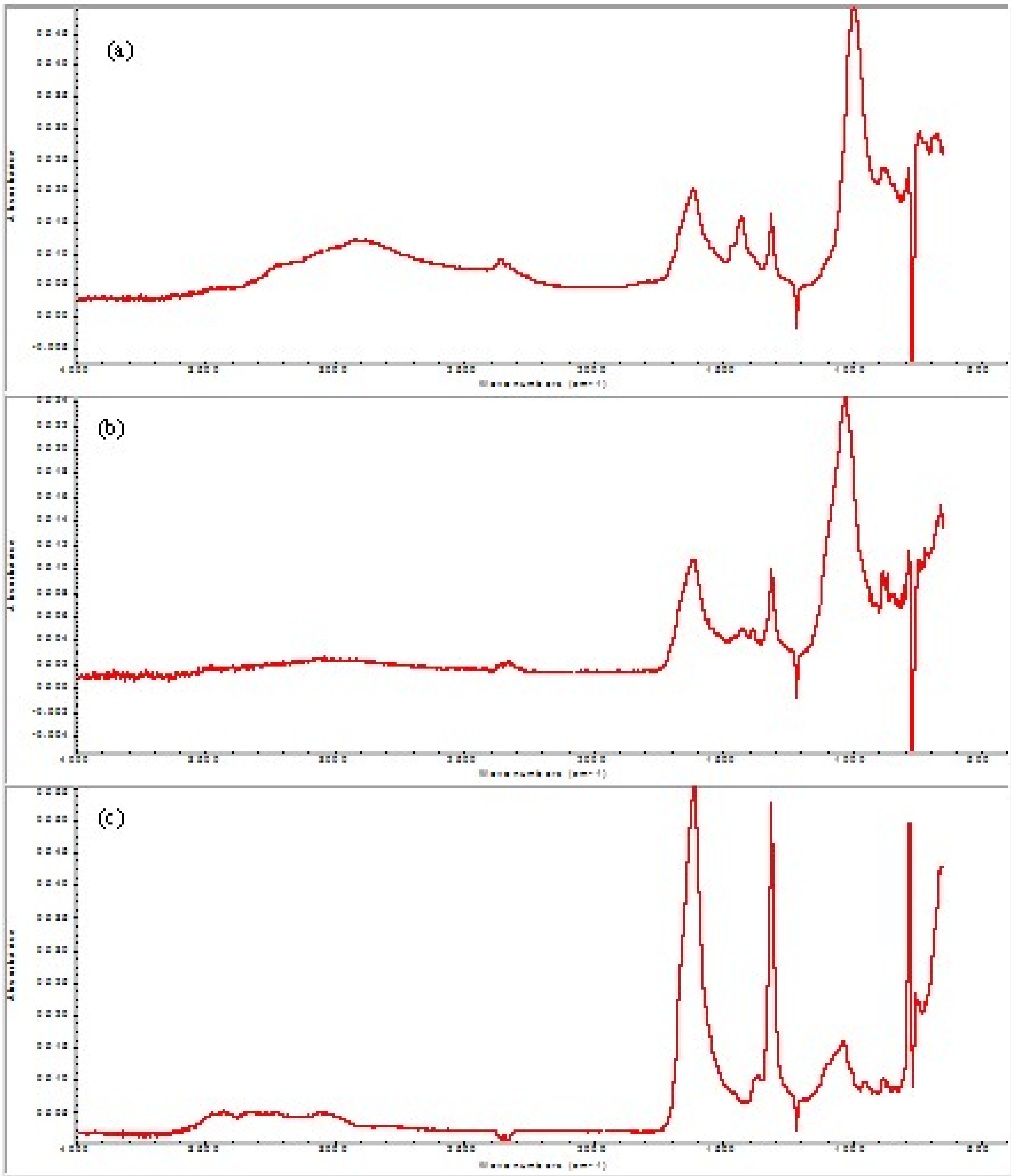


Fig. 1: Typical FTIR spectrum of (a) pure magesium ammonium phosphate stone (b) pure calcium oxalate monohydrate stone (c) pure carbonate apatite stone

the quantitative and qualitative determination of the urinary stones (Al-Ali *et al*., 2014). As it helps to differentiate the stone components as monohydrate and dihydrate forms of calcium oxalate, hydroxyl and carboxyl form of calcium phosphate quantitatively. Also the amount of sample required (10 to 100mg) is very low as compared to other available techniques. Moreover large reference libraries are available that help in quick determination of the exact quantitative stone composition (Bernhardt *et al*., 2014).

In present study, most of the stones (63.7%) recovered from urolithiasis patients were of mixed type rather than the pure stones (table 1). Interesting thing to note was the

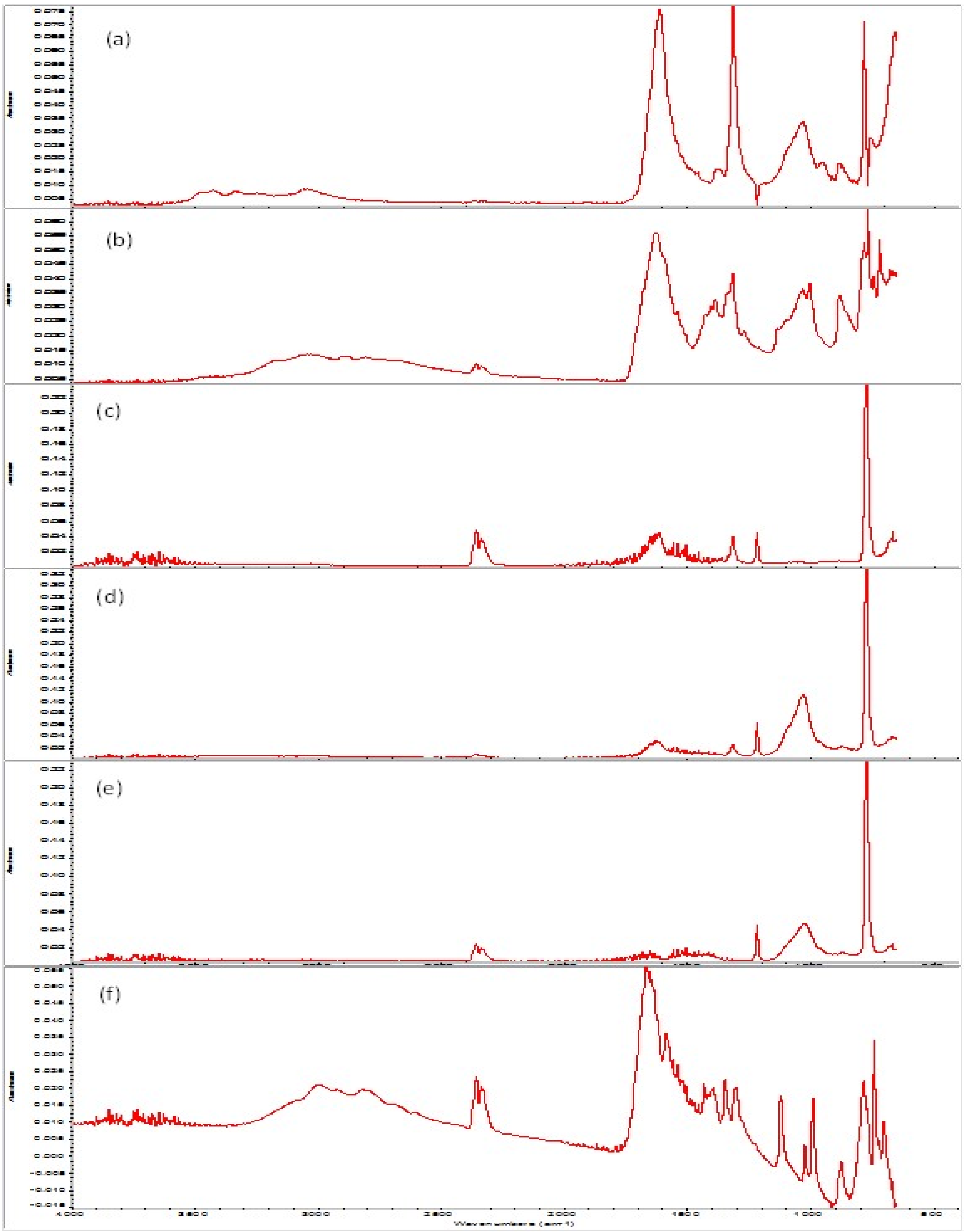
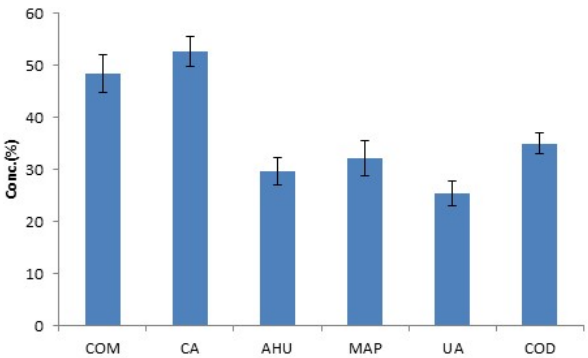


Fig. 2: Typical FTIR spectrum as identified by kidney stone software of (a) Calcium oxalate monohydrate mixed with Carbonate Apatite (b) Calcium oxalate monohydrate mixed with Carbonate Apatite & Ammonium Hydrogen Urate (c) Calcium oxalate monohydrate mixed with Ammonium Hydrogen Urate (d) Carbonate Apatite mixed with Magnesium Ammonium Phosphate & Ammonium Hydrogen Urate (e) Magnesium Ammonium Phosphate mixed with Ammonium Hydrogen Urate (f). Uric Acid mixed with Ammonium Hydrogen Urate

predominant occurrence of COM in both pure stones as well as mixed ones. This highest occurrence of calcium oxalate stones is mainly due to dietary factor, as the patients in present study were great consumers of oxalate rich diet (i.e. leafy vegetables e.g spinach, saag) and tea which results in hyperoxaluria and hypercalciurea that may result from renal tubular leaking, increased GIT absorption of calcium, hyperparathyroidism. So, the urine calcium concentration and urine oxalate concentration affects oxalate super saturation thus finally results in calcium oxalate stone formation (Phillip, 2009; Penniston, 2014).



COM=Calcium oxalate monohydrate, CA= Carbonate Apatite, AHU= Ammonium Hydrogen Urate
MAP= Magnesium Ammonium Phosphate, UA= Uric Acid, COD= Calcium oxalate Dihydrate

Fig. 3: Composition of whole kidney stones.

As Far as the highest percentage of COM mixed stones with carbonate apatite & ammonium hydrogen urate (table 1) is concerned, it may be due to infection (in case of ammonium hydrogen urate deposition over calcium oxalate central layer), or due to hypercalciurea, hyperuricosuria, dysfunction of parathyroid hormone and calcitonin or osteoporosis which causes the release of apatite from bones that helps in Randall plaque formation over which calcium oxalate stones grow (Rizvi *et al*., 2007; Taylor and Stoller, 2015).

In a recent study reported by Bernhardt *et al*. (2014), out of 200 kidney stone samples, pure COM stones were only 21.5% but the 69.5% stones were mixed COM stones. Although, the sample size was quite large, yet these results were similar to our study (Bernhardt *et al*., 2014). In another study, reported by Bhattacharyya *et al*. (2014), both calcium oxalate monohydrate and dehydrate stones were found in combination (i.e. 42.3%) while pure COM stones were only 15.3% (Bhattacharyya *et al*., 2014). Bangash *et al.* (2011) also reported COM as the most common stone component (i.e. 87.5%) followed by uric acid (6.5%), struvite (4.3%) and calcium phosphate (1.29%) (Bangash *et al*., 2011). Khaskheli *et al*. (2012) also carried out a similar study but not on the layers, they reported 29.4% calcium oxalate, 29.4% uric acid, 29.4% calcium oxalate (CaOx)-uric acid, 5.9% phosphate, and 5.9% calcium oxalate-phosphate stones. Although this

Table 1: Composition of kidney stones as analyzed by kidney stone software (KSLS-13)

S. No.	Composition	No. of Samples (n = 69)	Stones (%)
(1)	Pure stones	n = 25	36.20
a)	Magnesium Ammonium Phosphate(MAP)	2	02.89
b)	Calcium Oxalate (Monohydrate)	16	23.11
c)	Carbonate apatite.	7	10.14
(2)	Mixed stones.	n=44	63.73
i)	COM mixed stones.	n=34	49.27
a)	COM mixed with CA.	10	14.49
b)	COM mixed with CA and AHU.	13	18.80
c)	COM mixed with CA and Uric Acid.	5	07.24
d)	COM mixed with COD.	2	02.89
e)	COM mixed with COD and AHU.	2	02.89
f)	COM mixed with AHU.	2	02.89
ii)	CA mixed stones.	n = 4	05.79
a)	CA mixed with MAP	2	02.89
b)	CA mixed with MAP and AHU	2	02.89
iii)	MAP mixed stones:	n = 2	02.89
a)	MAP mixed stone with AHU	2	02.89
iv)	Uric Acid mixed stones.	n = 4	05.79
a)	Uric Acid mixed with AHU.	2	02.89
b)	Uric Acid dihydrate mixed with AHU.	2	02.89

Table 2: Composition of concentric layers of kidney stones by kidney stone software (KSLS-13)

	Central layer Mean ±SEM (Conc. in %)	Middle Layer Mean ±SEM (Conc. in %)	Periphery Mean ±SEM (Conc. in %)	p-value (<0.05)
COM	47.41±3.46	50.92±4.36	44.28±3.335	0.011
CA	56.00±3.29	64.84±2.74	46.97±3.026	0.001
AHU	34.51±2.88	46.31±2.95	47.77±2.195	0.012
MAP	19.23±1.149	31.57±2.81	32.63±3.806	0.062
UA	22.14±2.508	35.33±4.88	17.14±1.288	0.013
COD	0	0	23.33±1.043	--

COM=Calcium oxalate monohydrate, CA= Carbonate Apatite, AHU= Ammonium Hydrogen Urate
MAP= Magnesium Ammonium Phosphate, UA= Uric Acid , COD= Calcium oxalate Dihydrate

study was also carried out in Hyderabad region of Pakistan but their sample size was quite smaller and these results were somewhat different than present study.

Composition Analysis of concentric layers of renal stones revealed that the concentration of COM, UA and CA are significantly increased in middle layer as compared to other layers. This Significant increase indicates the strong involvement of these components in stone formation. In situations of massive calcium oxalate precipitation in urine which is caused by hyperoxaluria, hypercalciuria, hyperuricosuria, increased GIT absorption of calcium, & increased reabsorption of oxalate in large intestine. First plaque forms in the thin limb Basement membrane and extends to the sub urothelial space and COM then deposits over the organic coating of apatite particles (Miller *et al*., 2009).CA mostly helps in formation of stone when there is idiopathic hypercalciuria, hyper parathyroidsm and osteoporosis. Moreover this calculus is also associated with infection &hyperphosphaturia (Andrew, 2010). As far as the uric acid stone formation is concerned, it may occur due to low urinary PH, low urine volume and diets high in purine (meat, fish etc) (Sakhaee, 2014). AHU being the component of uric acid stone can also be seen in high concentration in all the three layers (table 2). MAP was significantly decreased in central layer and COD was only seen in periphery. This relatively less prevalence of these two components in stone layers may be a reason for low occurrence of struvite and COD stones in patients of present study.

Just like the middle layer composition, whole stone composition also revealed highest concentration of CA followed by COM as compared to other stone components (Figure 3). The occurrence of urolithiasis is directly related to dietary habits of patients (Bhattacharyya *et al*., 2014). Also, the results of stone layers composition revealed that patients of present study mostly had a high prevalence of COM, CA stones and COM mixed with CA and AHU, and these results correlate with their consumption of oxalate rich diet, low intake of fruits and fluids. So, the study of layers of kidney stones gives a better understanding of disease pathogenesis, and we can determine the concentration of specific components in stone peripheral, middle and central layers individually. Proper dietary modifications can be carried out with the help of analysis of layers of kidney stones and thus the morbidity and cost associated with recurrent disease can be decreased considerably.

CONCLUSION

KSLS-13 by Fourier transform infrared Spectroscopy is found to be the most rapid and reliable method to study composition of concentric layers of kidney stones. We found calcium oxalate containing stones followed by carbonate apatite more prevalent in patients with urolithiasis. The presence of calcium oxalate monohydrate, carbonate apatite, ammonium hydrogen urate and uric acid in all three layers of kidney stones gives a better understanding of the etiology of kidney stones. Moreover, the novelty of present study lies in the finding that COM, CA and Uric Acid may help in development of middle layer, AHU and COD in peripheral layer while, MAP may promote the development of central layer. This information is helpful in understanding the etiology of kidney stone formation. It may be recommended that software like KSLS-13 can also be developed for analyzing gallstone composition, which may become rapid and reliable method for analysis of gallstones.

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