

## The Agreement Between Current Stone Analysis Techniques and SEM-EDAX in Urolithiasis

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**Purpose:** Nowadays, there are many physical and chemical methods available for urinary stone analysis. According to the latest guidelines, infrared spectroscopy (IR) or x-ray diffraction (XRD) are the two preferred methods in this issue. Therefore, we decided to do a practical comparison between the two above-mentioned techniques with a reference method in order to set up a proper analysis method in our clinical laboratories.

**Materials and Methods:** A total of 60 kidney stones were obtained at Labbafinejad hospital through open surgery or percutaneous nephrolithotomy. Then stone analysis techniques included both a morphological examination by SEM (Scanning Electron Microscopy) and internal structure analysis by EDAX (Elemental distribution analysis X-ray), XRD, IR and wet chemical analysis. SEM together with EDAX (SEM-EDAX) was considered as reference methods.

**Results:** The results of XRD had the highest agreement with SEM-EDAX analysis (93%), while the total agreement of FTIR and wet chemical analysis was 81% and 71% respectively. The agreement of FTIR for calcium oxalate stones was acceptable (90%), but for uric acid and cystine stones was challenging (65% and 76% respectively).

**Conclusion:** Our results revealed that XRD is more reliable than FTIR; but considering cost issues, FTIR is more suitable for routine clinical laboratory. Moreover, wet chemical analysis, which is routinely used in our laboratories is insufficient for stone analysis and it is mandatory to be replaced by techniques that are more accurate.

**Keywords:** agreement; analysis methods; infrared spectroscopy; urolithiasis; x-ray diffraction

### INTRODUCTION

Urolithiasis is a recurrent condition<sup>(1)</sup>, with the recurrence rate as high as 10–23% per year and might reach 50% within the first five years after treatment, in case the patient does not have an appropriate work-up and proper follow-up.<sup>(2)</sup> Therefore, in addition to focusing on suitable urological interventions for removing urinary stone, the institution of further prophylactic measurements to prevent recurrences including a thorough metabolic work-up and an accurate quantitative stone analysis is of great importance.<sup>(3)</sup> The first purpose of stone analysis is the extensive qualitative differentiation of all stone components, and identification of each component quantitatively is in the second order of importance.<sup>(4,5)</sup> The most common in vitro techniques for stone analysis are x-ray diffraction (XRD), infrared spectroscopy (IR), polarization microscopy or chemical analysis.<sup>(6,7)</sup> XRD identifies the crystalline components of stone material. In this technique non-refractive amorphous materials that are mixed with crystalline component cannot be detected, so can cause problems when amorphous calcium phosphate and non-mineralogical components (such as protein and matrix) are present in the stone composition.<sup>(8)</sup> IR is a rapid and specific method that is based on the interaction of the infrared light with the molecules in the stone components.<sup>(4)</sup> Kasidas et al. revealed that unlike XRD, IR can also identify non-crystalline materials therefore, it is useful for the identification of organic stone components particularly purines and drug metabolites.<sup>(8)</sup> In the analysis by polarization microscopy, the stone is fractured to reveal its internal structure. Then the illumination of each sample is investigated by optical microscopy using polarized light. Therefore, the small amounts of crystalline material in mixed stone component cannot be identified which limits the use of this technique. Wet chemical analytic techniques detect the individual ions of stone material. This technique is still widely applied in routine hospital laboratories in many countries<sup>(8,9)</sup>, however it is obsolete by the majority of laboratories due to very high proportion of errors that has been occurred with wet chemical analysis method (6.5% to 94%).<sup>(10)</sup> Since wet chemical analysis is the only method in

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**Table 1.** The agreement between the results of XRD, FTIR and wet chemical analysis techniques with SEM-EDAX results as the reference method.

SEM-EDAX <sup>a</sup> Analysis Report	Total Agreement	The Agreement between XRD <sup>b</sup> and SEM- EDAX results	The Agreement between FTIR <sup>c</sup> and SEM-EDAX results	The Agreement between Wet Chemical Analysis and SEM-EDAX results
All 60	84%	93%	81%	71%
G1 11	88%	100%	90%	59%
G2 4	82%	89%	65%	92%
G3 2	82%	82%	76%	65%
G4 18	92%	98%	78%	91%
G5 13	81%	95%	44%	91%
G6 7	86%	96%	70%	77%
G7 3	93%	92%	71%	100%
G8 2	67%	82%	0%*	89%

G1 (Group 1): Calcium oxalate, G2 (Group 2): Uric acid, G3 (Group 3): Cystine, G4 (Group 4): Calcium oxalate + Calcium phosphate, G5 (Group 5): Calcium stone + Uric acid, G6 (Group 6): Calcium stone + Cystine, G7 (Group 7): Calcium stone + Mg-NH<sub>4</sub>-P stone, G8 (Group 8): Uric acid + Cystine.

a SEM-EDAX: Scanning Electron Microscopic with Elemental distribution analysis x-ray.

b XRD: X-ray diffraction

c FTIR: Fourier Transform Infrared Spectroscopy

our routine laboratories, we decided to do this study to make a practical comparison between XRD and IR analysis methods in order to set up a proper stone analysis method in our clinical laboratories. According to previous studies SEM can produce very high-resolution images of a sample surface<sup>(11)</sup> and, EDAX shows the internal elemental structure of a sample with high precision<sup>(12)</sup>, therefore we applied SEM together with EDAX (SEM- EDAX) as a reference method<sup>(12)</sup> for evaluating the agreement of XRD, IR and wet chemical analysis results in different groups of urinary calculi.

## MATERIALS AND METHODS

### Study population

Sixty kidney stones were obtained from patients who were treated at Labbafinejad medical center, consecutively. All of the stone fragments that were removed from each patient, were stored in plastic bags without using any solution for analysis during 2-3 weeks later. The procedures were in accordance with the ethical standard of the responsible committee of Urology Nephrology Research Center (UNRC) and informed consent was obtained from the participants. In this study, the stones considered as pure if only one composition was identified and, mixed stone if there were other stones components from different chemical groups.<sup>(13)</sup>

### Procedures

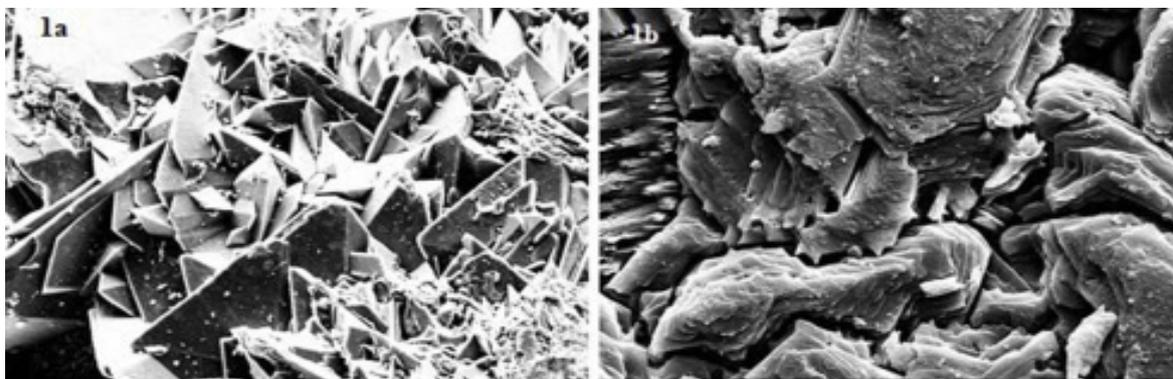
Prior to the internal structural analysis, all of the stone samples were carefully washed in order to remove any remaining blood clots or tissue, dried and then were analyzed by the following techniques:

Scanning Electron Microscopy (SEM). Before the examination, the samples were made conductive by gold sputtering in order to be prepared for morphological analysis by SEM (Tescan, Vega3 series, USA). By this technique the morphology of all crystalline component of the stone samples were recorded through taking photographs at different magnifications.

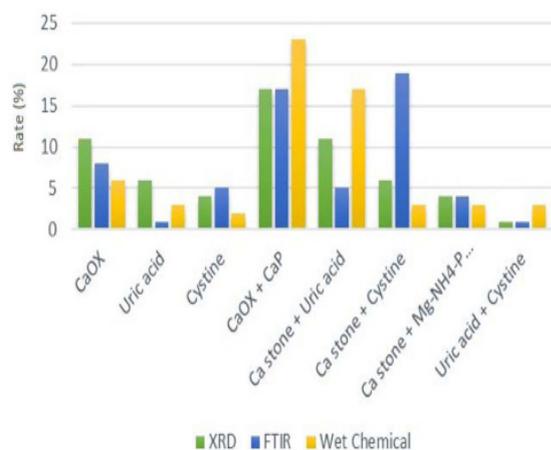
Then the stone samples were powdered, homogenized and divided into four aliquots. Representative fragments of each stone were sent to the laboratories committed to the analysis method, separately (EDAX, XRD, FTIR and wet chemical analysis methods).

Elemental distribution analysis X-ray (EDAX). Elemental analysis was carried out by EDAX probe (Tescan, Vega3 series, USA), which was pointed to the different areas of the sample. Then through the analysis of the produced wave, the components of the crystals were recognized.

Scanning Electron Microscopic with Elemental distribution analysis X-ray (SEM-EDAX). This is the combination of high-resolution images of crystal morphology by SEM together with precise elemental analysis through EDAX probe that we used as a reference method in this study<sup>(12)</sup>.



**Figure 1.** Scanning Electron Micrograph of two kidney stones. a. Calcium oxalate stone. b. Cystine stone.



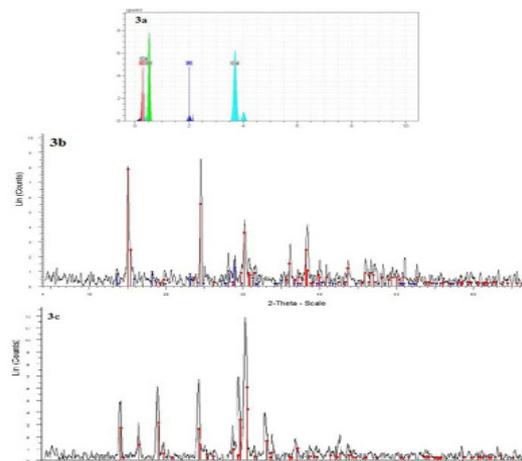
**Figure 2.** The results of X-ray diffraction, Fourier Transform Infrared Spectroscopy and wet chemical stone analysis techniques in this study.

X-Ray Diffraction (XRD). In this technique through monochromatic X-ray penetration to the sample, the crystalline atoms of stone composition produce a beam of x-rays that diffract into many specific directions. A crystallographer by measuring these diffracted beams, can produce a three-dimensional picture of electron within the crystal that is characteristic for that crystal composition. Then, according to the searched match analysis performed on D4 endeavor x-ray diffractometer (Bruker, Germany) using a database, all crystalline components of kidney stones were identified.

Fourier Transform Infrared Spectroscopy (FTIR). The stone powders were homogenized and mixed with potassium bromide and placed in the IR beam of the spectrometer (Bruker, Germany). The infrared radiation, stimulates atomic vibration in the stone sample and then energy absorption, which results in absorption bands in the infrared spectrum that is characteristic for their structure through comparison with a reference library. Wet Chemical Analysis. This technique was performed with commercial Kit (Darmankav Company, Isfahan, Iran). The samples were analyzed for the presence or absence of carbonate, oxalate, calcium, magnesium, urate, cystine, ammonium and phosphate. The results were interpreted according to the instruction of the kit manufacturer.

#### Statistical Analysis

The assessment of the frequency of pure and mixed stone components using each stone analysis techniques was done by KALPHA SPSS macro software. The inter-coder agreement of the stone type was assessed by Krippendorff's Alpha. The Krippendorff's Alpha values range is between from 0 to 1 which, 0 is perfect disagreement and 1 is perfect agreement. Krippendorff suggests: "it is conventional to require  $\alpha \geq .800$ , the lowest conceivable limit that tentative conclusions are still acceptable is where  $\alpha \geq .667$ . This method takes into account both observed and expected agreement as well as the frequency of each category of pure and mixed stone components.



**Figure 3.** Analysis results of three kidney stones. a. mixed composition of calcium oxalate and calcium phosphate (Elemental distribution analysis X-ray). b. Uric acid stone (X-ray diffraction). c. Mixed composition of calcium oxalate and uric acid (X-ray diffraction).

## RESULTS

Among the 60 patients, 17 were women and 43 were men, the mean age of the patients was 47.16 (SD: 11.82, range: 18-79). Eight stones were extracted by anatomic nephrolithotomy and 52 remained samples were obtained by percutaneous nephrolithotomy.

Using SEM with suitable magnification, the morphology of the surface crystals was evaluated, the images of two samples are indicated in **Figure 1 (a, b)**. According to EDAX analysis, the main elements identified were calcium, carbon, phosphorus, oxygen, nitrogen, magnesium and sulfur. The presence of calcium, phosphorus and oxygen showed that the sample was a mixed composition of both calcium oxalate and calcium phosphate.<sup>(11)</sup> A prominent peak for sulfur in EDAX analysis is pertaining to cystine composition,<sup>(14)</sup> which was found in 11 stones. In addition, we found a peak of nitrogen in 18 stones and magnesium in seven stones. The presence of nitrogen with sulfur is related to cystine component while co-existence of nitrogen with oxygen is referred to the uric acid composition. Finally, magnesium is included in the composition of magnesium ammonium phosphate or magnesium hydrogen phosphate kidney stones.

By considering the SEM-EDAX as a reference method, the results of analysis by three other methods are illustrated in **Figure 2**. Based on XRD analysis, 21 (35%) stones had pure composition: calcium oxalate- 11 (18.3%), uric acid- 6 (10%) and cystine- 4 (6.7%). The diagrams related to analysis of three samples is shown in **Figure 3 (a, b)**. According to the FTIR analysis, the percentage of pure stones were lower than XRD technique (23.3%). As it is clear in **Figure 2**, FTIR was weak in the detection of pure uric acid stones and falsely reported more mixed stones of calcium oxalate and cystine composition comparing to XRD analysis. The wet chemical analysis identified only 11 pure samples; calcium oxalate- 6 (54.5%), uric acid- 3 (27.2%), cystine-2 (18.1%). In wet chemical analysis, false positive detection of calcium phosphate and mixed composition of uric acid and calcium stones are noticeable.

According to the classification of pure and mixed

stones, we had eight groups in our results that are summarized in Table 1. The agreement between results of analysis by XRD, FTIR and wet chemical techniques for detection of pure and mixed stone components versus SEM-EDAX results (as the reference method) were indicated in **Table 1**.

## DISCUSSION

The urinary stones may have the mineral composition, organic or both. It is noteworthy that the same chemical components may crystallize in different forms. Therefore, a proper stone analysis technique should be able to identify all chemical constituents in the calculus with their exact crystalline form.<sup>(11)</sup> The SEM-EDAX ability in the detection of the surface and internal structure of stone samples is very high, but due to its high cost (the cost of SEM together with EDAX analysis per sample is 102.3 \$), it is not recommended as a routine technique in clinical laboratories. The comparison between the total agreement of XRD and FTIR analysis revealed that the highest agreement was between XRD and SEM-EDAX results (93%). The total agreement of FTIR with SEM-EDAX results were lower (81%), the main reason for this decline is the problem of the differentiation of uric acid from cystine stones according to this study.

Determination of stone composition is very important for understanding its etiology since the composition of calculus not only specifies the lithogenic conditions involved in its formation, but also reflects long-term or transient urinary or metabolic disorders. Thus, precise determination of stone composition can have an important role in choosing suitable medical treatment for prevention of stone recurrence.<sup>(2-4)</sup> In the nineties, there was a progressive increase in the use of physical techniques (IR and XRD) rather than chemical methods for analyzing the urinary stones, as far as chemical methods have decreased and are considered to be obsolete in some countries.<sup>(9)</sup> SEM-EDAX indicates the stone structure on the surface and interior clearly with very low theoretical detection limits (according to Reed<sup>(15)</sup> about 0.08 percent of stone weight) which makes it a good technique to be used as a reference method<sup>(12)</sup>. Giannossi et al.<sup>(16)</sup> showed that SEM and petrographic thin section analysis of calcium oxalate kidney stones led to the identification of more cores in the same stones. Also they reported that not only the central zone in some stones, but also the point of attachment to the kidney wall can be represented as a core. But this method is much more expensive to be utilized as a routine technique for stone analysis,<sup>(11)</sup> so that is now applied just in modern research laboratories.

According to previous studies, XRD is a reliable and accurate method when is used for qualitative and quantitative analysis of the kidney stones.<sup>(17)</sup> Hidas et al. revealed that x-ray diffraction is the most accurate technique among the three most common methods (XRD, FTIR and polarization microscopy). Therefore, they compared the *in vivo* dual-energy CT findings of their patient's renal stone composition by XRD analysis as standard reference technique.<sup>(6)</sup> In the study by Uvarof et al.<sup>(13)</sup>, 278 kidney stones from the patients were analyzed by XRD, 64.1% were pure and, 35.9% of stones had mixed components. In another study in Japan, on 1816 urinary tract calculi using infrared spectroscopy<sup>(18)</sup>, 58.4% of kidney stones had pure composition

and remained 41.6% were mixed stones consisted of calcium oxalate with other components. In our study according to XRD analysis, 35% and 65% of stones had pure and mixed components, respectively.

Previous studies showed that the infrared spectroscopy technique is able to characterize nearly all stone samples. In addition, in cases that the peak size will directly match with the computerized reference library, the quantity of stone composition can be identified.<sup>(3,11,19)</sup> However, Singh et al. revealed that quantification of all components of mixed stones with various amount of oxalate, phosphate and urate is problematic, and only semi-quantitative results can be obtained.<sup>(3)</sup> In another study, Charafi et al. revealed that the detection of minor components by FTIR can be done when their amount were not less than one tenth of the magnitude of the major component.<sup>(20)</sup>

In the present study, since the library of the FTIR method was limited we could not have a proper qualitative and quantitative analysis of all components in kidney stone samples. The most important problem in our results was the inappropriate differentiation of uric acid from cystine. For example, we identified four pure uric acid stones by SEM-EDAX, which only one of them reported as the same component by FTIR technique. One samples reported a combination of uric acid with cystine and, the other two samples reported as pure cystine composition. In addition, among mixed stone results by SEM-EDAX which composed of calcium oxalate together with uric acid, FTIR results were reported the combination of calcium oxalate and cystine. Fazil et al. stated that recognizing cystine in mixture stones by FTIR method is problematic, due to the similarity of wavelengths of cystine composition with calcium oxalate and uric acid component.<sup>(14)</sup> Our results showed that the correlation of FTIR with SEM-EDAX results in detection of cystine and uric acid components was 76% and 65%, respectively. While, the correlation of FTIR with SEM-EDAX results in detection of calcium oxalate was 90%. It is noteworthy that the differentiation of calcium oxalate dihydrate from calcium oxalate monohydrate was not easy by FTIR technique and to achieve this purpose, use of another combination method such as XRD or polarizing microscopy (depending on the cost of the combined methods for routine practice in clinical laboratories) is recommended. It is noteworthy that the cost of each analysis by infrared spectroscopy and x-ray diffraction is about 18.1 and 31.4 \$, respectively.

In our study, the cumulative correlation of wet chemical analysis with SEM-EDAX was 71%, with the most correlation in mixed calcium stones and pure uric acid composition. In addition, the lowest correlation was between the detection of pure calcium oxalate and cystine stones (59% and 65% respectively). The most components that were reported as false positive were calcium phosphate and magnesium phosphate in comparison with SEM-EDAX results. Another problem of wet chemical analysis technique in our study was false negative report of cystine in mixed stones.

Two external quality assurance scheme (EQA schemes) in 1998 and 1993<sup>(21)</sup> showed relatively poor performance for wet chemical analysis methods with high false positive and negative results. The study by Kasidas et al.<sup>(8)</sup> demonstrated that among different used methods in studied laboratories, XRD and FTIR techniques gave

more acceptable results and should be used in routine laboratories for kidney stone analysis. As seen in Table 1, the best agreement was between XRD results and SEM-EDAX technique with 93% correlation following by FTIR analysis method (81%).

The main limitation of the study is referred to non-familiarity of laboratory staff with infrared spectroscopy in order to analyzing the composition of kidney stones. In addition, due to the limited library of infrared spectroscopy device, the proper qualitative and quantitative analysis of all components of our samples were not possible.

## CONCLUSIONS

No single method is sufficient to provide all the clinically essential information for determination of kidney stone composition and structure.<sup>(11)</sup> In our study the results of XRD had more agreements with SEM-EDAX than other used methods. Although considering the economy, Infrared spectroscopy is less costly if there is a proper reference library. To avoid missing detection of rare stone components with Infrared spectroscopy, it is necessary for clinicians to be in communication with the laboratory staff that perform the analysis in order to alert them when needed.<sup>(22)</sup>

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## CONFLICT OF INTEREST

None.

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