FLUORESCENCE REDUCTION IN RAMAN SPECTROSCOPY BY CHEMICAL BLEACHING ON RENAL STONES

M. Kocademir [1], M. Kumru [3*], K. Gölcük [2], R. Suarez-Ibarrola [3], A. Miernik [3]

[1] Istanbul University, Science Faculty, Physics Department, Vezneciler, 34134 Istanbul, Turkey
[2] Institute of Experimental Epileptology and Cognition Research, University of Bonn Medical Center Life and Brain, 53127 Bonn, Germany
[3] University of Freiburg – Medical Center, Faculty of Medicine, Department of Urology, 79106 Freiburg, Germany

In this study, a hydrogen peroxide-based chemical bleaching technique was applied on two different types of renal stones. The characterization was achieved after the bleaching process. They were identified as calcium phosphate and calcium oxalate monohydrate. The samples were analyzed using dispersive Raman spectroscopy with a 532 nm excitation laser. To compare the results, the samples were measured using both FT-IR and FT-Raman spectroscopy. Consequently, the mineral/matrix ratio of Raman bands changed for both samples, but without any noticeable frequency shifts in the Raman spectra.

Keywords: *Raman spectroscopy, fluorescence reduction, chemical bleaching, renal stone, calcium phosphate, calcium oxalate.*

ОСЛАБЛЕНИЕ ФЛУОРЕСЦЕНЦИИ ПОЧЕЧНЫХ КАМНЕЙ В СПЕКТРОСКОПИИ КОМБИНАЦИОННОГО РАССЕЯНИЯ СВЕТА С ПОМОЩЬЮ ХИМИЧЕСКОГО ОТБЕЛИВАНИЯ

M. Kocademir [1], M. Kumru [3*], K. Gölcük [2], R. Suarez-Ibarrola [3], A. Miernik [3]

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[1] Стамбульский университет, Везнеджилер, 34134, Стамбул, Турция
[2] Институт экспериментальной эпилептологии и исследований познания, Боннский университет, 53127, Бонн, Германия
[3] Фрайбургский университет, медицинский центр, 79106, Фрайбург, Германия

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Метод химического отбеливания с помощью пероксида водорода применен к почечным камням двух различных типов. После процесса отбеливания осуществлена характеризация. Камни идентифицированы как фосфат кальция и моногидрат оксалата кальция. Образцы проанализированы с помощью дисперсионной КР-спектроскопии с $\lambda_{возб}$ = 532 нм. Для сравнения результатов образцы измерены с помощью ИК-Фурье- и КР-Фурье-спектроскопии. Отношение минерал/матрица для полос КР изменилось для обоих образцов, однако без каких-либо заметных сдвигов частоты в спектрах КР.

Ключевые слова: *спектроскопия комбинационного рассеяния света, уменьшение флуоресценции, химическое отбеливание, почечный камень, фосфат кальция, оксалат кальция.*

Introduction. Raman spectroscopy is a vibrational spectroscopic technique. It has numerous applications, for example, for evaluating the molecular structure of compounds. Raman spectroscopy is a prevalent

technique for biological sample analysis since water molecules do not affect the Raman spectrum. Rapidness, cost-effectiveness, and non-invasiveness are among its advantages.

The Raman method is based on inelastic photon scattering and conventionally works with monochromatic laser light located in the UV, visible, or near-infrared range. The Raman phenomenon occurs when the molecules are excited by photons to virtual energy states. After this interaction, energy transfers between photons and vibrational motions occur. Photons gain (anti-Stokes Raman) or loss (Stokes Raman) energy, and its transfer emerges as frequency shifts for the initial monochromatic light. Hence, different frequency shift patterns appear as a fingerprint for any compound. Raman interaction indicates only polarizable molecules and also depends on vibrational motions. However, Raman scattering is a rare process because most of the photons are exposed to Rayleigh scattering, though this difficulty can be eliminated with efficient filters and sensitive detectors.

There are certain limitations to Raman spectroscopy, but the most significant is background fluorescence. The fluorescence phenomenon acts as a catastrophic effect for Raman spectroscopy because it can overlap the entire spectrum. If fluorescence occurs, the background is usually significant, and Raman bands may become indistinguishable [1]. Fluorescence and Raman scattering are similar processes but have different mechanisms.

Normally, most of the molecules exist in the ground electronic state (especially the lowest vibrational levels) at room temperature. After interacting with electromagnetic radiation, some molecules gain energy and occupy higher vibrational levels of the excited electronic states. After this, the molecules immediately lose their energy by collisions and go to the lowest vibrational level of the excited state. Finally, the molecules may fall to any of the vibrational levels of the ground state, so the energy differences between both electronic states arise as a form of fluorescence radiation. Fluorescence is the most important disadvantage of Raman spectroscopy, especially for biological sample investigations [2–4].

After the specimen and laser beam interaction, Raman signals are nearly inexistent ($\leq 10^{-15}$ s) because there is no excited state transition [5]. However, fluorescence is a relatively slow process and needs more time than the Raman phenomena; it occurs at time scales of 10^{-9} to 10^{-7} s [6]. Usually the most important source of fluorescence is sample impurities, such as hemoglobin, collagen, lipids, and proteins [7]. Fluorescence intensity could be as great as 10^4 compared to the Raman signal [8, 9]. This means that Raman peaks are overshadowed by a strong and broad fluorescence spectrum. Therefore, the utilization of Raman spectroscopy is limited as a result of the fluorescence blockade. The occurrence of this situation is prevalent and depends on the laser wavelength excitation.

Several techniques, such as experimental, computational, photo, or chemical bleaching, can be used in order to avoid fluorescence background from Raman spectra [7, 10, 11]. In order to avoid fluorescence, choosing the correct excitation laser wavelength is also crucial. The probability of fluorescence emission could be diminished by low laser frequencies [12]. For this reason, most of the applications do not use visible wavelengths. The use of higher wavelengths in near-infrared regions is helpful for reducing the fluorescence effect, or laser frequencies can be chosen lower than the electronic transition levels.

Various studies have been performed in order to reduce fluorescence from mineralized tissues. Golcuk et al. investigated the photo-bleaching effect on background fluorescence using bone tissue as the specimen [13]. Using this method, fluorescence was reduced from the photo-induced reaction of the used chemicals with fluorophores [14]. Penel et al. studied the bone tissue and 30% hydrogen peroxide interaction and reported that the sample´s characteristic Raman frequencies had not changed after this application [15].

Hydrogen peroxide (HP) and acetone were used as chemical agents for the bleaching process of renal stones. HP is a strong oxidizer and one of the most prevalent bleaching agents. Its chemical formula is (H_2O_2) and its structural formula H-O-O-H; it contains an oxygen-oxygen single bond. H-O radicals appear readily after the oxygen atom bond is broken. HP reacts with a large number of organic forms, such as fungi, spores, bacteria, and viruses. Moreover, it has mechanical purifying action since it generates oxygen bubbles when it comes into contact with tissues and debris [16].

It possesses both oxidizing and reducing properties, which are pH dependant. Notably, the HOO^- ion is the active agent of HP bleaching, and it is originated by the ionization process. This reaction is reversible. Since the front reaction produces H^+, high pH will increase the HOO^- ion. Hence, this ion selectively reacts with the organic matter, thereby removing organic impurities.

In recent years, Raman spectroscopy has been applied to investigate mineralized tissues and bio-apatite, such as bones, teeth, and renal stones [17–26]. Although Raman spectroscopy has been widely used in determining the composition of urinary calculi, no study in the literature has yet reported on fluorescence re-

duction by chemical bleaching [27–31]. To our best knowledge, this is the first study where hydrogen peroxide is used for chemical bleaching-based fluorescence reduction in Raman spectroscopy for calcium oxalate and calcium phosphate renal stones.

Experimental details. Two different renal stone samples (labeled as sample 1 and sample 2) were transected using a diamond saw. A part from each was kept as the control and the rest utilized for understanding the effects of chemical bleaching. Calcium phosphate (apatite) and calcium oxalate monohydrate (COM) crystals were identified by an FT-IR spectrometer for sample 1 and sample 2, respectively. Hydrogen peroxide (HP) and acetone were used as chemical agents for the bleaching process.

The bleaching process started with the interaction between the 30% hydrogen peroxide solution and renal stone samples, and the process was finished after using acetone to clean pollutants such as fats, proteins, and other residual biological particles. Each sample was then exposed for 30 min, 2 h, and 24 h to the chemical bleaching procedure. Micro-Raman spectroscopic measurements were performed afterwards.

The samples were analyzed with a micro-Raman spectrometer in the 200–3500 cm^{-1} range at room temperature. A Thermo Scientific Dispersive Raman (DXR) spectrometer was equipped with an optical spatial resolution of the ×10 magnification microscope. A 532 nm green excitation laser was used. The ideal laser power was 5 mW, which was determined by testing values between 0.5 to 7 mW on the same area of the sample. Rayleigh scattering photons were eliminated with an edge filter. The spectral resolution was 2 cm^{-1}, the aperture a 25 μm slit, and the grating 900 lines/mm. Finally, the nonbleached parts of the samples were measured with an FT-Raman spectrometer to determine any Raman spectral shifts.

Results and discussion. We used kidney stone samples for FT-IR spectroscopy. Figure 1 shows the FT-IR spectrum of both samples. Sample 1 arises as a calcium phosphate (apatite) stone. Bending and stretching vibrations of phosphate (PO_4^{-3}) ions are normally observed in the 300–600 and 900–1200 cm^{-1} range, respectively [32, 33]. In this study, PO_4^{-3} bending vibration was recorded at 555 and 601 cm^{-1}. A weak peak at 871 cm^{-1} was observed for the CO_3 vibration of apatite [34]. A strong absorption peak of 1040 cm^{-1} reflects P-O stretching, and a weak absorption band of 1645 cm^{-1} O-H deformation [35]. These peaks were observed at 1020 and 1645 cm^{-1}, respectively.

The peak at 511 cm^{-1} marks the presence of O-C-O in the plane bending vibration [36]. Bands at 779 and 665 cm^{-1} correspond to C-C stretching and O-H out-of-plane bending, respectively [37]. We observed these bands at 778 and 657 cm^{-1}. The bands near 885 cm^{-1} correspond to the C-C stretching of COM [35]. A weak peak near 953 or 945 cm^{-1} indicates C-O stretching [38, 39]. In this study, the C-O stretching peak was recorded at 949 cm^{-1}. The weak band near 1370 cm^{-1} refers to CO_3^{-2} stretching [39]. It was found at 1374 cm^{-1} for sample 2. An intense peak arises at 1319 cm^{-1} as a result of C-O stretching [40]. We identified this band at 1313 cm^{-1}. Finally, a broad and intense band near 1615 cm^{-1} indicates the C=O vibration [35]. This peak was observed at 1609 cm^{-1}. The tentative assignments and vibrational frequencies of the samples are given in Table 1.

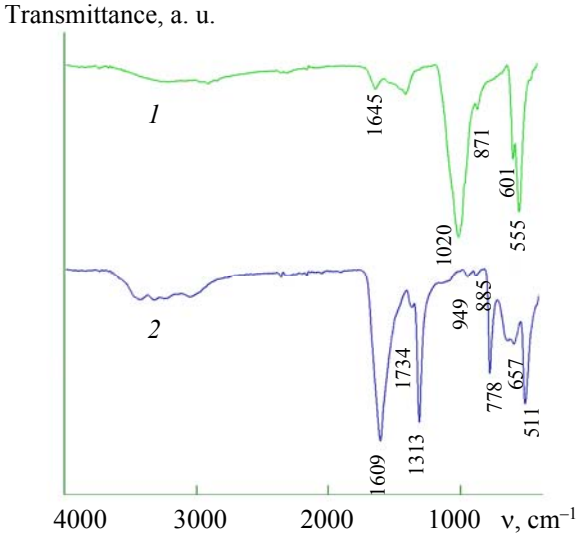

Fig. 1. FT-IR spectra of samples 1 (1) and 2 (2), calcium phosphate (apatite) and calcium oxalate monohydrate (COM).

TABLE 1. FT-IR Frequencies (cm^{-1}), Tentative Assignments and Compound Names of Sample 1 and Sample 2 before the Bleaching Process

S1	S2	Tentative Assignments	Compound
–	511	O-C-O in plane bending	COM
555	–	PO_4^{-3} bending	Apatite
601	–	PO_4^{-3} bending	Apatite
–	657	Out of plane O-H bending	COM
–	778	C-C stretching	COM
871	–	Vibration of CO_3	Apatite
–	885	C-C stretching	COM
–	949	C-O stretching	COM
1020	–	P-O stretching	Apatite
–	1313	Vibration of C-O	COM
–	1374	CO_3^{-2}	COM
–	1609	Vibration of C=O	COM
1645	–	O-H deformation	Apatite

In previous studies, a similar bleaching procedure was applied to urinary stones without achieving fluorescence background reduction. None of these procedures are fully applicable to kidney stones. For example, 360 min of bleaching is applied to other bio-minerals, but this level is not enough to eliminate background fluorescence in renal stones. We determined the most appropriate bleaching procedure after trying different approaches. In this procedure, we extended the chemical bleaching time to 24 h and as a result observed decreases in the background fluorescence intensity. We noted that hydrogen peroxide has a serious impact on the sample's spectra. On the other hand, acetone did not show a significant effect on the sample's spectroscopic results. It is more useful to utilize acetone for cleaning the surface at the end of the hydrogen peroxide bleaching procedure.

It should be emphasized that different points on the sample surface have to be measured in order to record an adequate spectrum since urinary stones have a heterogenic structure [41, 42]. They are composites, made up of an inorganic and organic matrix phase, such as lipids and proteins [43, 44]. To ensure homogeneity, the powder form of the samples was prepared in a mortar and subjected to chemical bleaching with hydrogen peroxide in a tube for six hours. However, contrary to our expectations the background fluorescence increased. As a result, we can conclude that the most appropriate way to bleach a renal stone is by cross-sectioning with a diamond saw and measuring several points on the surface.

Figure 2 shows the changes of fluorescence intensity, which depend on the bleaching time for sample 1 and sample 2, respectively. Since there was too much fluorescence intensity in the nonbleached samples, it is impossible to get a spectroscopic measurement from them. The red spectra illustrate normal (before bleaching) Raman spectra of both samples in Fig. 3. Half an hour followed by two hours bleaching procedures were applied to the samples´ cross-sections with hydrogen peroxide and acetone, but the background fluorescence was not sufficiently reduced.

The chemical bleaching time was consequently extended to 24 h. Fluorescence reduction was achieved for both of them with the 24 h procedure, and Raman spectra of both samples were recorded. Figure 3 show dispersive Raman spectra of sample 1 and 2 at the end of the bleaching process. The most intense and characteristic Raman peaks were determined for the samples. The most intense peak was located at 962 cm^{-1} for sample 1, corresponding to apatite P-O stretching [45]. Additionally, an intense peak appeared after the same process for sample 2 at 1462 cm^{-1}. This peak is characteristic for calcium oxalate monohydrate and assigned to C=O vibration [31].

TABLE 2. Raman Frequencies and Compound Names for Sample 1 and Sample 2 before and after Bleaching

Raman frequencies, cm^{-1}			
Sample	Before bleaching	After bleaching	Compound
S1	962	962	Apatite
S2	1462	1463	COM

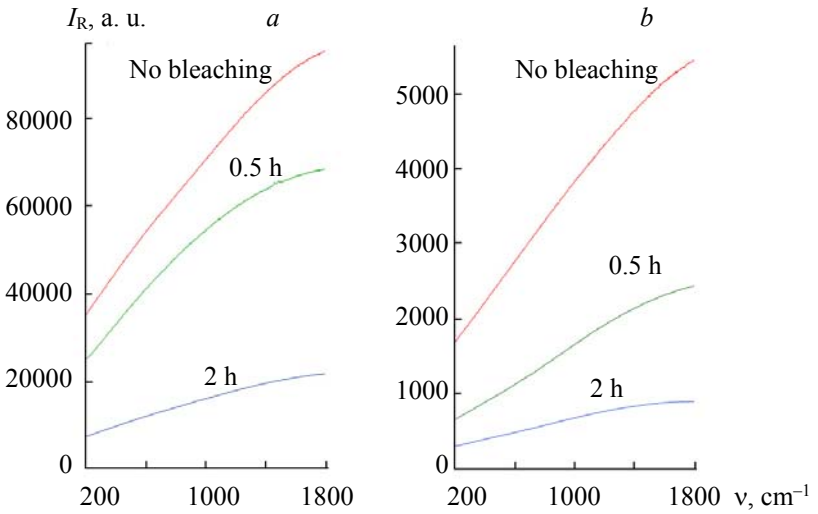

Fig. 2. Fluorescence intensity for different times of the bleaching process for (a) sample 1 and (b) sample 2.

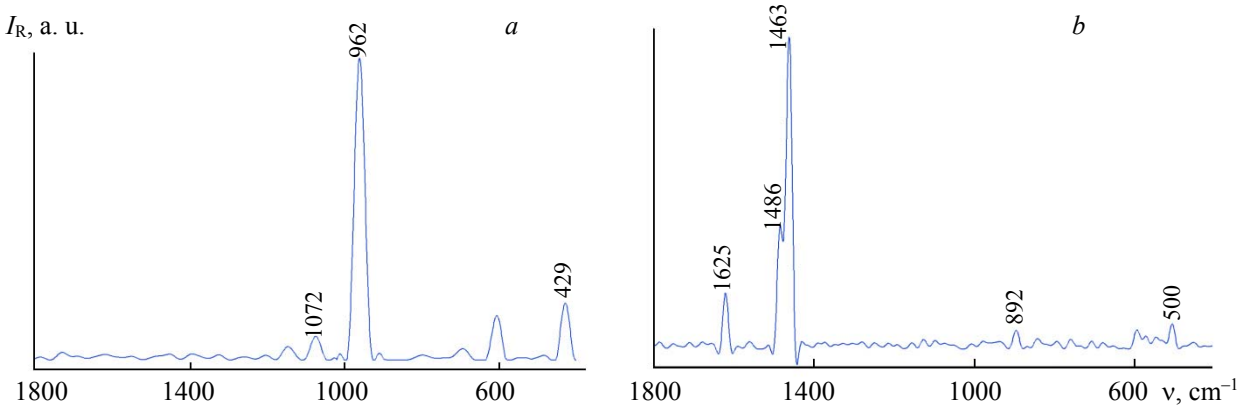

Fig. 3. Dispersive Raman spectra of (a) sample 1 and of (b) sample 2 after the fluorescence reduction process by hydrogen peroxide chemical bleaching.

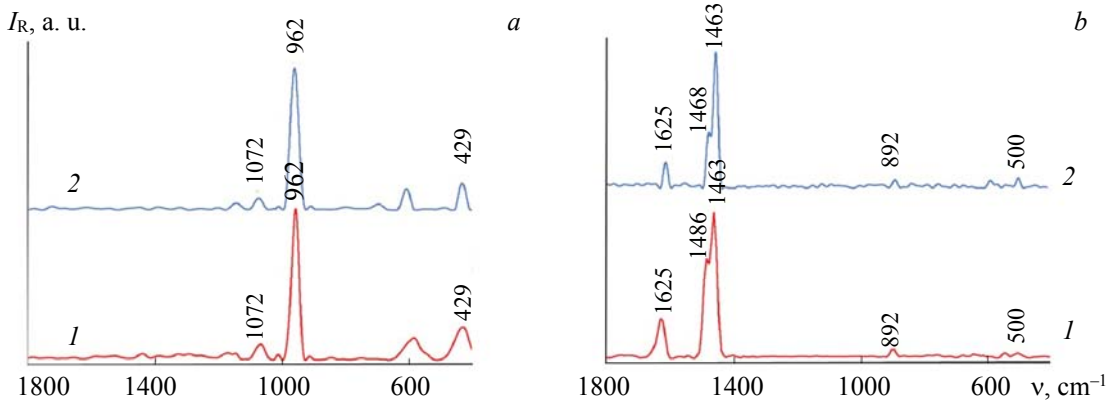

Fig. 4. Spectra of (a) sample 1 and (b) sample 2 before bleaching (1) and after bleaching (2).

We can understand from the results that the 24 h bleaching procedure leads to sufficient fluorescence reduction. Although the mineral/matrix ratio changes for both samples, there are no noticeable frequency shifts in Raman spectra. Hence, we can say that this method is suitable for quantitative studies of kidney stones.

To compare our results, we investigated nonbleached (before bleaching) samples with FT-Raman spectrometry, and the same frequencies were observed. The FT-Raman spectra of the samples are given in Fig. 4. The most characteristic frequency of sample 1 (apatite) is measured at 962 and 1463 cm^{-1} for sample 2 (calcium oxalate monohydrate). FT-Raman results also highlight the accuracy of the chemical bleaching method. The Raman spectral results of samples 1 and 2, before and after 24 h bleaching are shown on Table 2.

Conclusions. Our work shows that chemical bleaching with HP reduces background fluorescence in the Raman spectrum analysis of renal stones; 5 mW laser power should ideally be used for an optimal signal fluorescence ratio. The minimum bleaching time should be approximately 24 h for the two different renal stone types. Fluorescence reduction was achieved for both samples. The most intense peak was located at 962 cm^{-1} for sample 1 and at 1462 cm^{-1} for sample 2. These peaks are indicatory signs for apatite and calcium oxalate monohydrate for sample 1 and 2, respectively. These results coincide with those determined by FT-IR and FT-Raman spectra. Importantly, multiple areas on the sample's surface need to be measured in order to record an adequate spectrum. Although the mineral/matrix ratio changed for both samples, there were no noticeable frequency shifts in the Raman spectra. Consequently, this method can be utilized for the Raman spectrum analysis of urinary stones.

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