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ANALYTICAL STUDY OF GALLSTONES IN PATIENTS FROM TRANSYLVANIA, ROMANIA

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ABSTRACT. Here we report the vibrational spectroscopy characterization on human gallstones from patients in Transylvania, Romania. A number of 93 gallstones resulted after surgical intervention were preliminary classified in 6 groups after provenance, shape and color aspect and investigated using optical microscopy and FT-(micro)-Raman spectroscopy in conjunction with the FT-IR. Optical microscopy of the gallstones cross sections revealed concentric layers growing depositions alternating with axial crystalline agglomerated biomaterial. Two distinct type of crystallinity on one hand and different spatial crystals distribution, on the other hand were optically observed. The studied specimens randomly showed very narrow amorphous zones. The microstructure, chemical composition, the nucleation and growing mechanism of gallstones were correlated with the vibrational spectroscopy data. Vibrational assignment allowed identifying cholesterol in all the samples. Other weak bands attributable to bilirubinate salts were randomly observed with different extent in all the groups. Brown, yellowish-brown and yellow gallstones showed higher content of bilirubinate as revealed by the relative intensity ratio of the bilirubinate band at 1620 cm^{-1} versus cholesterol band at 1464 cm^{-1} with values between 0.57 and 0.875. FT-vibrational techniques accurately provided the proof of the cholesterol specificity in the choletitiasis prevalence in the area and allowed getting additional insight into their growing mechanism.

Keywords: *Human gallstones, Transylvania, FT-Raman spectroscopy, FT-IR, cholesterol*

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INTRODUCTION

Gallstone or choletitiasis appearance in the gall bladder is one of the most common gastroenterology disease and constitutes a major health problem worldwide requiring the highest budget in the gastroenterology treatment. Gallstones are clumps of solid biomaterial that are formed in the gall bladder or in the choledoch duct as a result of bile concentration associated with metabolic syndrome and gastric disease. The main function of the gall bladder is to concentrate bile by the absorption of water and sodium. Traditionally, gallstones were classified as cholesterol stones, pigment stones, or mixed stones (a combination of cholesterol and pigment stones) based on their composition, which can only be determined reliably after their removal. Cholesterol stones are composed primarily of cholesterol. Pigment stones are composed of bilirubinate and other substances such as calcium, which are found in the bile. The occurrence of gallstone disease is 2-3 times more common in women than in men [1].

Gallstones and their medical consequences represent a relevant cost factor in Western countries healthcare systems. The incidence of gallstones is 15% in America, 5.9~21.9% in Europe, 4~15% in Asia and 3~11% in China [2, 3]. .According to Sachiorafas et al [2] about 10-20% of inhabitants in the most western countries developed gallstones while the percentage of asymptomatic patients being 50-70% at the diagnosis moment. In Germany, 10.5–24.5% of the female and 4.9–13.1% of the male population are estimated to carry gallstones, and about 170,000 cholecystectomies are performed annually [2-5]. A very recent exhaustive study [3] showed that the gallbladder stones were classified into 8 types and more than ten subtypes, including cholesterol stones, pigment stones, calcium carbonate stones, phosphate stones, calcium stearate stones, protein stones, cystine stones and mixed stones. Current research suggests that different types of gallstones have different pathogenesis [6–9]. Research on the systematic classification of gallbladder stones may help to reveal the formation mechanism of different types of gallstones.

Major types of stones observed in patients are a) white b) black and c) brown stones. This classification based on the color, was proposed at the NIH workshop [11]. Earlier FT-IR and FT-Raman studies suggested an additional category called mixed stones having different proportions of cholesterol and bilirubin [10]. Black and brown color stones contain bilirubin in large amounts in addition to small quantities of cholesterol. The pigmented stones can be further

sub-categorized on the basis of minor variations in chemical composition, such as the presence of calcium carbonate. Three main lipids found in the bile are bile acids, cholesterol and phospholipids [12]. According to the current opinion in the field, gallstones formation is a complex bioprocess where the physical, metabolic, genetic and geographical factors compete to the formation of the precipitated agglomeration of insoluble biomaterial in the gallbladder and even in the choledoch duct [13,14].

In recent years there has been an increasing trend in the number of reported cases. The formation of gallstones *in vivo* takes years and it is quite difficult to monitor such events from nucleation to the consolidation and final diagnostic [15]. Gallstone formation is therefore very poorly understood. Surprisingly, in the last few decades there has been significant rise in gallstone disease among children [15-18]. Removal of the gallbladder by surgical methods is the only solution available to the gallstone disease today and therefore, the disease has a strong impact [19-21]. Earlier reports [19] suggested that biochemical features of a human 42-kDa biliary glycoprotein shows concentration-dependent cholesterol crystallization-promoting activity. Other results showed that the insoluble materials of gallstones are mainly composed of bilirubinate salts and proteins [22].

Since the risk factors of the choletitiasis disease may vary from one region to another, being influenced by the dietary habits, genetic factors, medical history of the patient, race, geographical zone, as well as by other diseases, the literature reports revealed various analytical data on chemical composition of gallstones [3, 6, 10, 20, 22]. To the best of our knowledge, in Romania, such studies were absent, although the risk of gallstones becoming symptomatic was evaluated for medical purpose [23].

Vibrational spectroscopy techniques could provide valuable information on the nature and chemical composition of the gallstones, thus, the specific factors competing in the gallstones formation could be properly managed for prevention. Although the gastroenterology clinics report their own research results on the statistical aspects or surgical removal success [23, 24], the nature of the surgical resulted biomaterial is unknown. On the other hand, the mechanism of gallstone formation and consequently their control and growing inhibition is poorly understood. It should be noted that in spite of the recent pharmaceutical development, a proper formulation for dissolving and eliminating the gallstones is absent. The aim of this work was to explore the capability of the vibrational spectroscopy techniques for assessing the nature and the growing mechanism of the gallstones collected from patients in Transylvania, Romania.

RESULTS AND DISCUSSION

Fourier Transform (FT)-Raman and FT-IR were used as the main analytical techniques for the determination of human gallstone structural composition. These techniques could provide rapid, qualitative and quantitative information about their structure. The gallstones biomaterial taken under study is illustrated in the Fig. 1 and the physical properties of the stones and their origin are described in the Table 1.

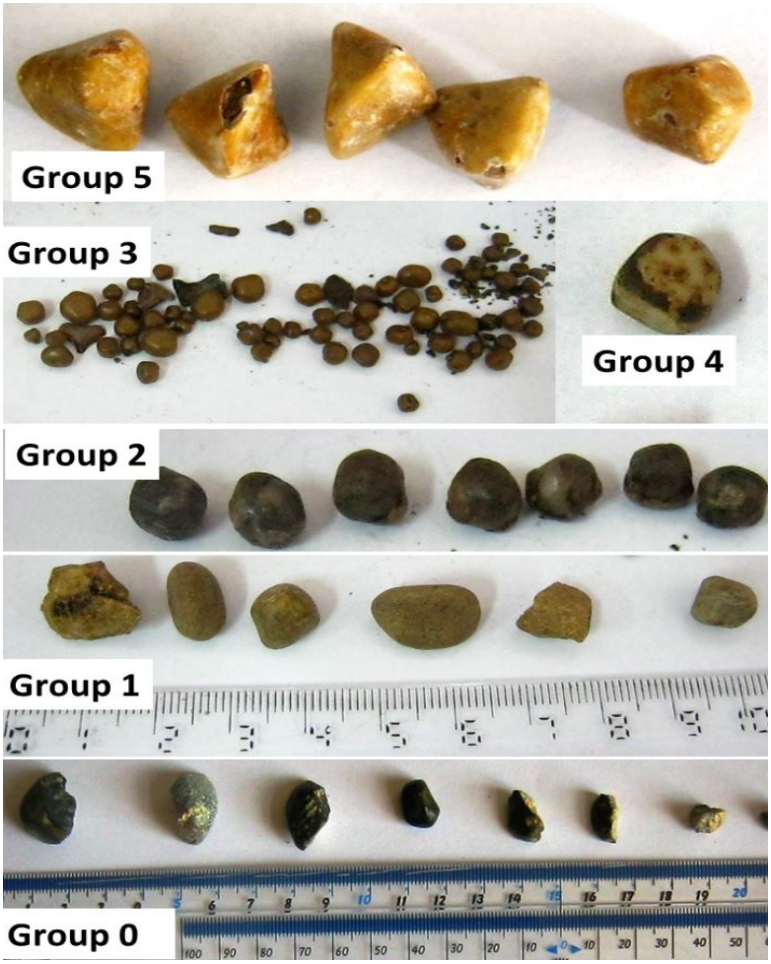

Figure 1. Gallstones from patients in Transylvania, Romania, taken under study.

Table 1. Sample groups classification according to their physical properties and origin.

Group	Provenience	Color	Physical Appearance/Size	Observed specific gravity
0	9 samples of a group of 55 pieces resulted from a surgery intervention in 3-rd Medical Clinics Cluj-Napoca from one patient (woman)	Grey-black	Lustrous grey colored particles up between 0.2-2cm diameter; some layering apparent on fragmentation; brown granules apparent	Low
1	6 samples from a group 0f 12 pieces resulted from a surgery intervention in 3-rd Medical Clinic Cluj-Napoca from a male patient	Yellowish brown	Stone size ranging between 0.5-1.5 cm present abrasive and harsh structure	Low
2	7 samples resulted from a surgery intervention in 3-rd Medical Clinic Cluj-Napoca from male patients	Black	Stone size ranging between 1-1.5 cm, present abrasive surface	Low
3	65 samples resulted from surgery interventions in 4-th Medical Clinic Cluj-Napoca from many patients (male and female)	Brown	Stone size ranging between 0.2-1 cm present polish surface	Low
4	1 sample resulted from a laparoscopic intervention in 3-rd Medical Clinic Cluj-Napoca	Yellow-brown	Stone size ranging 1.4 cm present abrasive surface and some layering apparent on fragmentation; brown granules apparent	Low
5	4 samples resulted from a surgery intervention in Clinical Hospital Blaj, Alba County, Transylvania	Yellowish White	Stone size ranging between 1.5-2 cm present highly polished surface from all of the samples	Low

FT-Raman spectroscopy of gallstones

Raman spectroscopy is currently a widely used method based on inelastic scattering of the light on materials. It is an advanced technique

that allows to acquire rich information about the chemical composition by measuring vibrational frequencies of the chemical bonds of the investigated structure with high spatial resolution within a unique non-destructive manner. FT-Raman technique prevails over the dispersive Raman techniques in the case of materials with high fluorescence such as gallstones, because of its incorporated NIR laser for excitation which could significantly diminished the fluorescence of the colored materials and for the capability to provide the whole spectral range at once.

Representative raw FT-Raman spectra collected from different sample groups numbered from 0 to 5 are showed in the Fig. 2. For each sample group the spectra were acquired from the external side (crust), internal layers observed in the gallstone cross section and nucleation center.

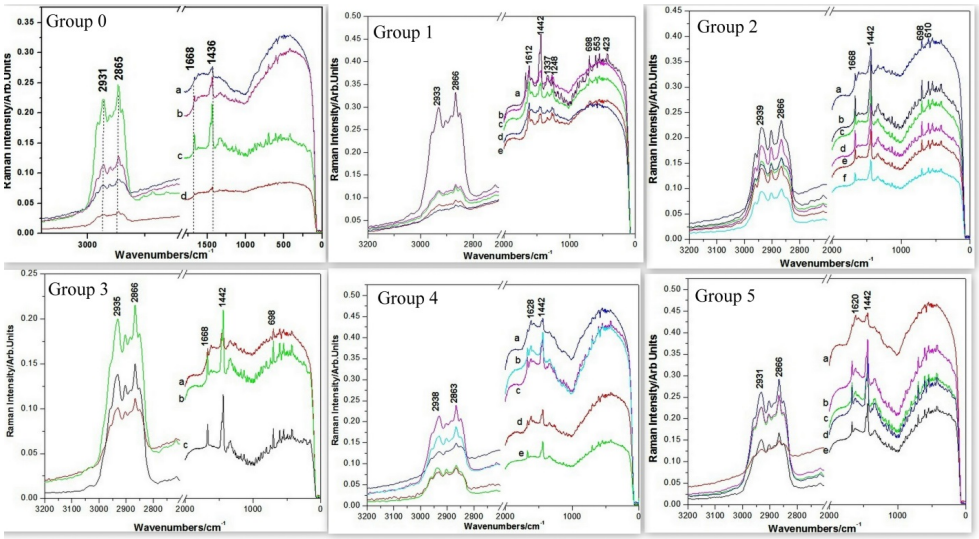

Figure 2. Representative raw FT-Raman spectra collected from various parts of the gallstones from the six groups. Group 0: (a) external part,(b) edges,(c) yellow pigment, (d) middle section; Group 1: (a) external part, (b) edges, (c) yellowish brown pigment, (d) middle, (e) nuclear center; Group 2: (a) external part, (b,c) external part brown, (d) pigment part inside of the gallstone, (e) middle, (f) nucleation center; Group 3: (a) external part, (b) edges, (c) white part inside of the gallstone; Group 4 (a) external part, (b) edges, (c) yellow pigment, (d) middle, (e) nuclear center; Group 5: 4 (a) external part, (b) edges, (c) yellowish white pigment, (d) middle, (e) nucleation center. Excitation: 1064 nm, 350 mW.

The core of one sample from the Group 1 was isolated and kept in ethanol for 24 hours. The treatment allowed recording high quality FT-Raman spectrum with substantially decreased background as shown in the Fig. 3. Ethanol extraction highlighted the bile pigments responsible for high intensity Raman background even for the NIR laser line excitation. FT-Raman spectrum of pure cholesterol (99%, Sigma-Aldrich) is shown for comparison. The gallstone core clearly revealed all the bands attributable to cholesterol and additionally a weak, broaden band at 1620 cm^{-1}.

Table 2. FT-Raman vibrational data (cm^{-1}) of gallstones and their proposed assignment.

Group 0	Group1	Group2	Group3	Group4	Group5	Literature values / cm^{-1} [3, 22, 26]	Assignment	Molecular species
Raman bands/ cm^{-1}								
2931	2938	2933	2939	2935	2931	2964s,2935s, 2904s	$\nu_{as}(CH_2,CH_3)$	Cholesterol
2865	2863	2866	2866	2866	2866	2886s,2850s, 2807s	$\nu_s(CH_2,CH_3)$	
1668	1668	1668	1668	1668	-	1671m	ν(C=C,C=O)	
-	1620	1620	1612	-	1620	1618s	ν(C=C,C=O), δ((N-H)	Bilirubinate salts
1436	1442	1442	1442	1442	1442	1461m,1438m	$\delta(CH_2,CH3)$	Cholesterol
-	-	1337	-	-	-	1341m	$\delta(CH_2,CH3)$, ring stretching	
-	-	1248	-	-	-		ν (C-C)	
-	-	698	698	698	-		Bending modes	Cholesterol
-	-	-	610	-	-			
-	-	553	-	-	-			
-	-	423	-	-	-			

Abbreviations: ν- stretching, δ-bending.

In the raw FT-Raman spectra of all the samples (Fig. 2), the cholesterol bands were clearly identified, without other sample treatment. This fact confirms that the dominant species is cholesterol in all the analyzed samples, allowing to conclude that the FT-Raman technique showed effectiveness in rapid chemical composition assessment of such complex bioprobes. The main

bands observed are summarized in the Table 2 together with the proposed assignments.

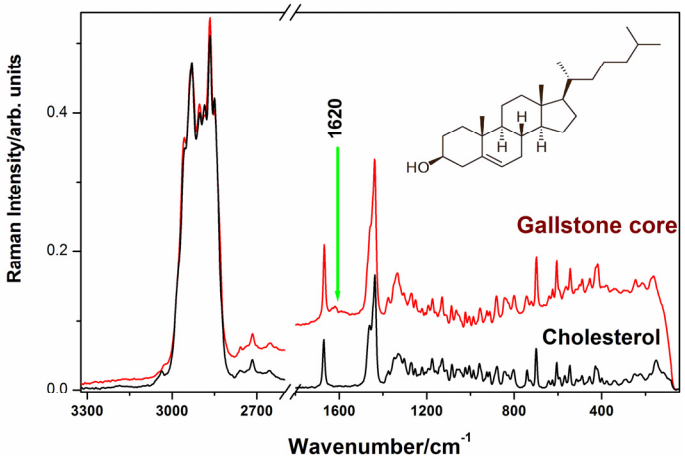

Figure 3. FT-Raman signal collected from cholesterol powder (99%, Sigma-Aldrich), compared to that of the gallstone core belonging to the Group 0. The molecular structure of cholesterol is inserted. Excitation: 1064 nm, 350 mW.

As shown by the Figure 2 the summarized display of the FT-Raman spectral feature of gallstones collection revealed some change in the relative intensity of the bands on passing from exterior through the inner layers to the gallstone center. Each sample was measured on the exterior side and in the interior layers of the cross section. Each group of samples showed similar spectral feature both on the exterior side and in the interior layers. The cross sections of several samples were carefully investigated using FT-micro-Raman spectroscopy in order to extract as much information as possible with high spatial resolution. Although the micro-Raman signal is almost 10 times weaker because of the excitation energy loss along the optical fiber to the Raman microscope and back, the cross sections revealed similar bands attributable to the cholesterol. The difference between adjacent inner layers of gallstones was mainly based on the different background intensity, the dark layers exhibiting the highest background in detriment of signal to noise ratio. Therefore, the micro-Raman spectra were considered not relevant for additional information and are not given here. Since most of the spectra collected from each group were similar, a selection of Raman data collected from the external, internal, edges, nucleation center and different pigmented areas (yellow pigment, yellowish white, yellowish green, brown) from the cross

sections are summarized in the Figure 2 and Table 2. The observed Raman bands at 2931, 2865, 1668, 1436-1442, 1336, 1271, 699, 604, 544, 417, 247 cm^{-1} and crystalline lattice vibration at 162 cm^{-1} were unambiguously assigned to cholesterol, but other bands characteristic of bilirubinate salts that were not overlapped by those of cholesterol, in the 1612-1620 cm^{-1} range were clearly observed with weak to medium intensity in all the sample groups. The complete vibrational characterization of the bilirubin was earlier reported [27]. The theoretically predicted 1567 cm^{-1} backbone stretching mode [27] has a corresponding band in the Raman spectra of acidic forms of bilirubin derivatives. This band was observed at 1564 cm^{-1} with weak intensity on the overall background in all the present sample groups Raman spectra (Figs. 3, 4) while cholesterol spectrum revealed no band on that position. Another specific band free of cholesterol overlap was observed at 1620 cm^{-1}. We also noticed the highest intensity fluorescence background in the spectra collected from the dark edge of the samples (black or brown).

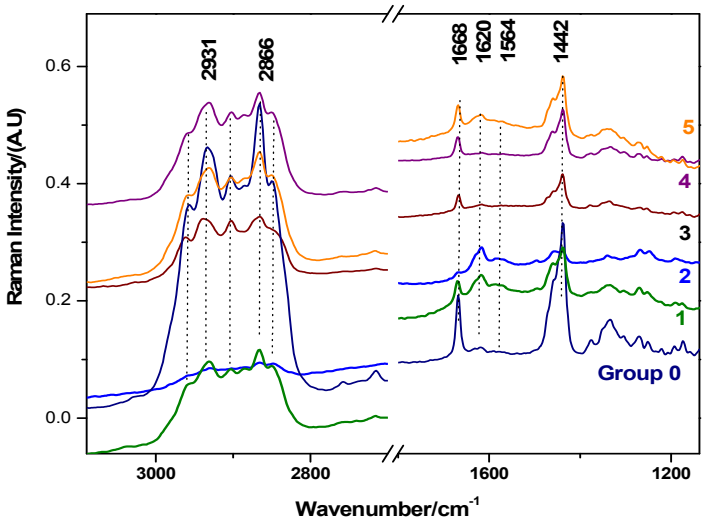

Figure. 4. Comparison of the representative FT-Raman spectra of the five gallstone groups. Spectra were collected from the lighter exterior area of the largest sample from each group. The clear differences are displayed in the 1668-1620 cm^{-1} range, where the balance between cholesterol and bilirubinate salts contribution respectively, is highlighted. In the high wavenumber range, the cholesterol bands are dominant in all the groups. Excitation: 1064 nm, 350 mW. Offset was applied for clarity and due to the fluorescence background extent the high wavenumber range displays changes in spectral ordering.

The highlighted Raman spectral differences between the five groups are shown in the Fig. 4, where the Raman spectra were collected from the lighter area on the external side of the largest sample from each group. Analysis of the FT-Raman results showed that the Group 1, 2 and 5 of brown, yellowish brown and yellow gallstones exhibit visible Raman contribution from both cholesterol and bilirubinate salts with relative intensity extent and could be classified as mixed gallstones, whereas the rest of samples were likely to be cholesterol dominant, although the bilirubinate pigments responsible for the dark color were present but with lower extent.

FT-IR analysis of gallstones

ATR-FT-IR spectra from the nucleation center, exterior surface and inner layers of each gallstone groups have been recorded in the 4000-650 cm^{-1} range. A selection of representative absorbance spectra collected from each gallstones group is displayed in the Fig. 5 for both the exterior side (upper) and the core (lower).

The FT-IR spectral feature showed much similarity, a direct evidence of the differences between groups being reflected in the relative intensity of the weak bands of bilirubinate contribution. The subtle change in the relative intensity of the absorbance bands In the 1600-1700 cm-1 range can be considered relevant for identification of the bilirubinate salts in the gallstones since cholesterol does not exhibit IR bands in this range [22'].

Cholesterol exhibits IR bands at 2933, 2865, 1466, 1365 and 1057 cm^{-1} [21]. The calcium bilirubinate shows characteristic very strong IR bands at 1620, 1696, 1663 and strong to medium intensity band at 1572, and 1250 cm^{-1} which were assigned to (C=C, C=N, C=O) stretching vibration of lactam, (C-O) stretching of COOH; (C-O, C–N, C-C) stretching, asymmetric stretching υ_{as}(COOH), and (C–O) stretching or C–N stretching coupled with NH deformation υ(C–N), δ(NH), respectively [3, 22, 25]. Assigning the IR spectra collected from the gallstones (Fig. 5) the cholesterol bands were dominant in all the recorded spectra, indicating the main compound both in the exterior layers and the gallstones core. The vibrational FT-IR data are summarized in the Table 3 with the proposed assignment. Calculating the relative intensity ratio of the bilirubinate band at 1620 cm^{-1} versus cholesterol band at 1464 cm^{-1}, the values range between 0.57 and 0.875, with the highest values distributed on specimens from group 1, 2 and 5, suggesting the higher bilirubinate content in the samples from these groups. These findings are in agreement with the Raman results, allowing to conclude that all the studied gallstones were cholesterol-based biomaterials with different extent of bilirubinate content. Although the color can be related to the pigment content,

the presence of other cations from the physiological salts can contribute to the color change, as previously reported for cupper bilirubinate gallstones of black color [28]. Complementary methods like energy-dispersive X-ray spectroscopy could bring additional insight into this issue.

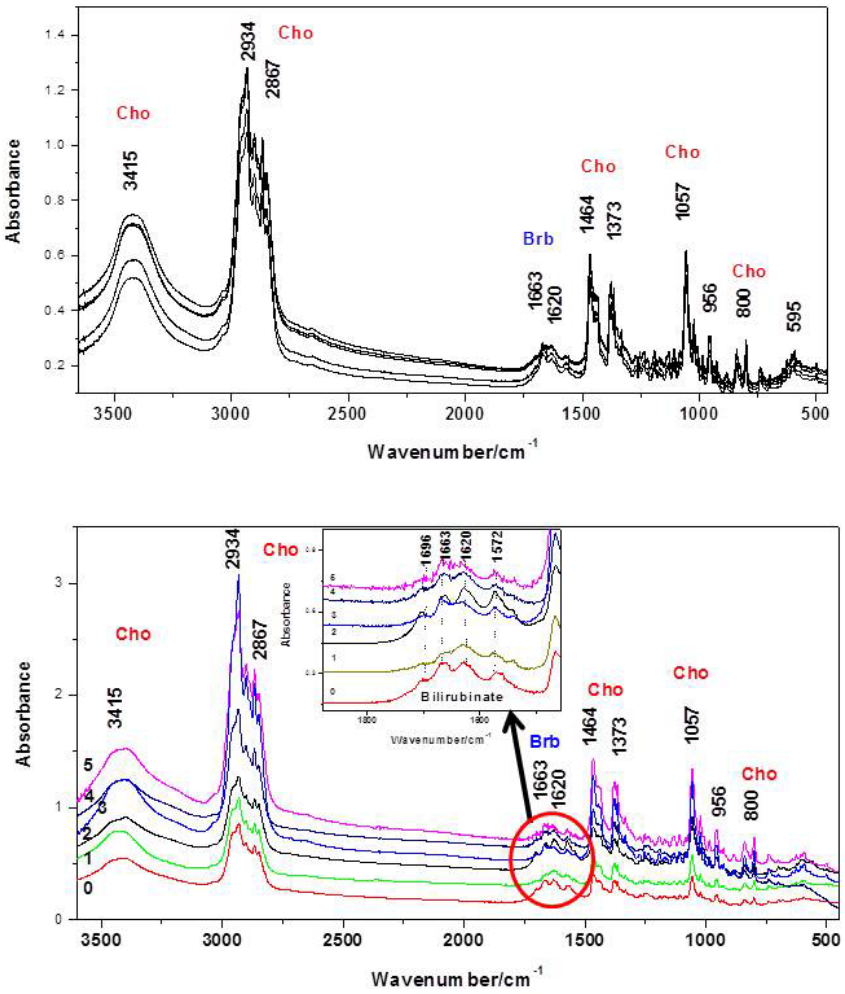

Figure 5. Representative ATR-FT-IR spectra collected from the exterior (upper) and gallstone core area (lower) from each of the five groups from 0 to 5. Insertion: Spectral range from 1800-1600 cm^{-1} where the bilirubinate (Brb) contributions are distinctly shown is highlighted. Cholesterol (Cho) does not exhibit IR bands in this range [22]. Central area (gallstone core) showed clear differences concerning bilirubinate content, highest rates being observed in group 1 followed by 2.

Table 3. FT-IR vibrational data (cm^{-1}) of gallstones and their proposed assignment.

FT-IR bands/ cm^{-1}	Assignments	Literature values [3, 22,25]
Bands due to Cholesterol		
3405	OH stretching	3401
2936	CH asymmetric stretching of CH_3	2725
2902	CH symmetric stretching of CH_3	2860
2868	CH symmetric stretching of CH_2	2860
1465	CH bending of CH_2	1460
1375	CH bending of CH_3	1380
1056	C-C stretching	1050
Bands due to Bilirubin		
1663	OC=O stretching	1670
1630	C=O carbonyl stretching	1626
1573	C=C stretching	1575
1251	C-O-C stretching	1244
1019	C-H in plane bending	1021
957	C-C ring stretching	923
877	C-H out of plane bending	879
838	C-H out of plane bending	832

The vibrational FT-Raman complemented by the FT-IR data suggested a clear nucleation process followed by the additional growing layers of crystalized cholesterol. No distinctive layers of bilirubinate were found in any of the studied samples. The gallstone formation starts when the amorphous cholesterol is organized as small balls which later crystallized and forms the core of gallstone. The core represents the crystallization support/center for the next layers of cholesterol resulting polished stones surfaces when agglomeration occurs in limited biological space.

This study also demonstrated that the chemical composition cannot be strictly related to the color classification criteria. FT-Vibrational techniques accurately provided the proof of the cholesterol specificity in the choletitiasis prelevance in patients from Transylvania, although the pure cholesterol gallstones were not observed.

CONCLUSIONS

This study presents the first analytical study of the gallstones composition and structure from patients in Transylvania, Romania. Although the cholesterol was the main compound found in all the studied samples and according to the current classifications criteria the gallstones would be cholesterol-type, however, cholesterol-bilirubinate mixed stones were considered dominant in Transylvania, based on the Raman and IR spectroscopy data. Cholesterol-bilirubinate stones found in Romanian patients appeared brownish yellow, amber, grey, yellowish brown or black-brownish and were spherical or polyhedron in shape. They were of different sizes, soft, and the surfaces were smooth and glossy or rough. Raman spectra of gallstones exhibit huge fluorescence background for all the visible laser lines, even the NIR line at 1064 nm, which could be removed by the ethanol extraction procedure. The resulted material resembled well the crystalline cholesterol spectra.

The formation, layer-by layer growing mechanism and crystallization mode was described based on vibrational spectroscopy data.

Analytical studies involving complementary techniques, such us X-ray diffraction and thermal analyses are in due course in order to assess the crystalline properties and possible inorganic phases that were not visible through the vibrational techniques. Such complementary approach will certainly contribute to understand cholelitiasis pathogenesis and hence stones growing inhibition and prevention.

EXPERIMENTAL SECTION

Instrumentation

ATR-FT-IR- spectra were recorded using an FT-IR (Bruker) Equinox 55 spectrometer with an ATR Miracle module with ZnSe crystal. FT-Raman and FT-micro Raman spectra were recorded using the integrated FRA 106S Raman module coupled with the Equinox 55 Bruker spectrometer. A fiber optic coupled Ramanscope II Raman microscope with an Olympus 10x objective was used to acquire micro-Raman spectra. A Nd:YAG laser operating at 1064 nm, with an output power of 350 mW, was used for excitation. The number of scans co-added was differed from one sample to another, depending on the fluorescence background extent of each case. Detection was accomplished with a Ge detector operating at liquid nitrogen temperature.

An attenuated total reflectance module with ZnSe crystal for sample contact was used to record absorbance spectra in the 4000-650 cm^{-1} range. The largest samples from each group were cross-sectioned for achieving an optimal crystal contact. The spectral resolution was 4 cm^{-1}.

Materials

Gallstones biomaterial were obtained from volunteer patients that underwent surgery intervention in medical clinics from Cluj-Napoca, Romania. The biomaterial was preliminary classified into 6 groups, numbered from 0 to 5 (Fig. 1), as follows: Group 0: 9 gray samples were selected from a total of 55 pieces of gray color ranging from 2 cm to 1 mm diameter, from a single patient (woman, 60 years old); Group 1 consisted of 6 yellowish-brown samples from a total of 12 pieces ranging from 1.7 cm to 0.5 cm in diameter from a male patient; Group 2 comprised 7 black-brownish samples of about 1 cm diameter; Group 3 comprised 65 brown samples ranging from 0.5 cm to 1 mm diameter from 4 different patients, Group 4 consisted of 1 laparoscopy broken sample of yellowish brown color; and Group 5 comprised the largest 5 yellow samples resulted from a surgery intervention suffered by a woman (45) in the Clinical Municipal Hospital Blaj, Alba County in Transylvania. Before analysis, gallstone samples were cleaned with ethanol and placed in different sterile opaque containers.

Cholesterol powder (≥99%) was acquired from Sigma-Aldrich and stored at -20^{0}C up to the measurement time.

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