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ARTICLE

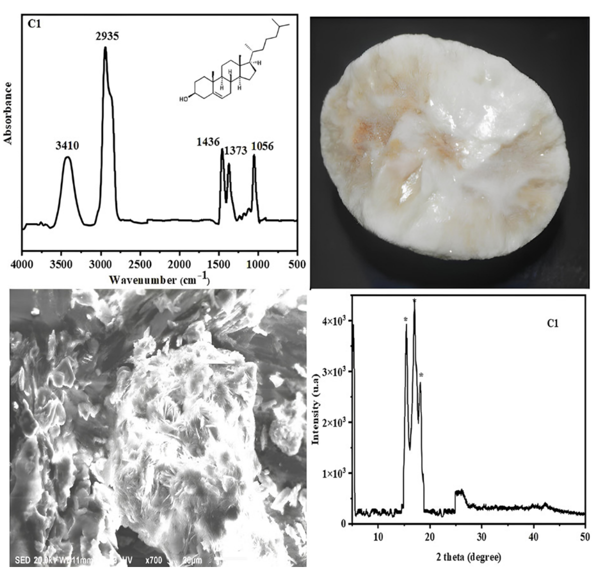
Chemical Composition Analysis of Human Gallstones in Beni Mellal-Khenifra, Morocco

Mohamed Bouha[1*], Malika Echajia[1], Latifa Amini[1], Mohamed Mbarki[1], Najat Elhadiri[2], Mustapha Oubenali[1], Mohamed Berkani[3]

[1]Laboratory of Engineering in Chemistry and Physics of Matter, Faculty of Science and Technics, Sultan Moulay Slimane University, Beni Mellal, 23000, Morocco

[2]Laboratory of Materials Science and Optimization, Cadi Ayyad University, Science Faculty Semlalia, Marrakech, 40000, Morocco

[3]Laboratory of Food Engineering and Technologies (LITA), Faculty of Science and Technology, Sultan Moulay Slimane University, Beni Mellal, 23000, Morocco



This study examines the relationship between the characteristics of patients and the composition of gallstones in Beni Mellal, Morocco. A total of 226 patients with gallstones (198 women and 28 men) from the Beni Mellal Regional Hospital Center took part in the study in 2018. Techniques such as scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD) were utilized to analyze the gallstone samples. Additionally, participants completed questionnaires regarding their demographic data and dietary habits. Among the cohort, 20 gallstones were selected for detailed analysis based on distinct morphological characteristics, such as color, size, and shape. These samples underwent thorough characterization. To represent the different composition profiles observed among the analyzed samples, six representative spectra were selected for detailed presentation in this article, aiming to avoid redundancies, as several samples exhibited similar spectral characteristics. Mixed composition gallstones were identified in 95% of patients, reflecting findings consistent with other regions. Fourier-transform infrared spectroscopy was favored for composition analysis due to its accuracy, sensitivity, and minimal sample preparation requirements. A significant association was observed between patient characteristics (age, gender, geographic origin) and the main components of the stones. Positive correlations emerged between the presence of cholesterol stones, calcium bilirubinate, calcium carbonate, and calcium phosphate, and a high consumption of protein- and calcium-rich foods (such as meat and dairy products) commonly found in the diet of Beni Mellal. The frequent consumption of cholesterol-rich products suggests a potential link with the prevalence of cholesterol gallstones in this region.

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INTRODUCTION

The link between cholesterol gallstones and metabolic syndrome markers suggests a crucial role for insulin resistance. Insulin resistance can disrupt bile cholesterol homeostasis and affect gallbladder motility. This highlights the interplay of various factors during cholesterol gallstone pathogenesis. Gallstones are a prevalent health concern, affecting 10-15% of adults.[1] Their formation results from complex interactions within bile, the digestive fluid stored in the gallbladder. While cholesterol plays a significant role, the exact causes of gallstone crystallization remain under investigation.

Previous studies reveal conflicting results regarding the chemical composition of gallstones, highlighting the complexity of this pathology. For instance, a study conducted in Saudi Arabia found that 54.3% of the analyzed stones were composed of pure cholesterol, while 43.5% were mixed stones, indicating a high prevalence of cholesterol in this population.[2] In contrast, a comparative analysis between Western and Asian countries showed that about 75% of stones in Western populations contained more than 50% cholesterol, while pigment stones containing less than 30% cholesterol were also significant.[3] The structural formula of cholesterol is shown in Figure 1.

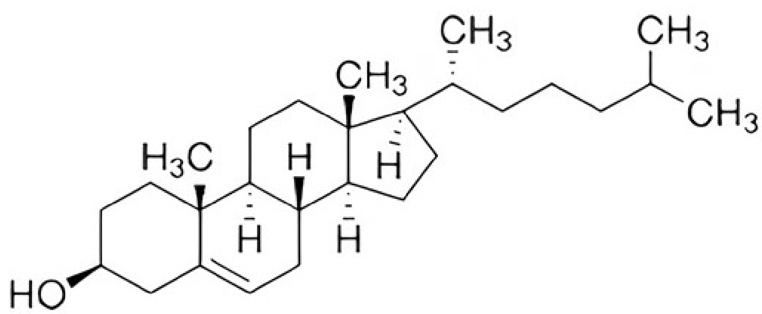

Figure 1. Molecular Structure of Cholesterol.

Additionally, an investigation using Fourier-transform infrared spectroscopy (FTIR) on Korean patients revealed that 50% of the stones were primarily composed of cholesterol; however, the morphological classification differed from the chemical composition analysis, underscoring the influence of clinical and demographic factors.[4] These discrepancies may stem from variations in the studied populations, analysis methods, and classification systems, emphasizing the need for a consensus on gallstone classification to enhance our understanding of their etiology.

Cholesterol gallstones are often linked to excess cholesterol in bile.[5] Reduced solubility of cholesterol leads to its precipitation as solid crystals (cholesterol monohydrate). This phenomenon is explained by the equilibrium phase diagram of the cholesterol-phospholipid (lecithin)–bile salt mixture.[6] Bile itself is a complex mixture of various chemical components, often considered a ternary system with four potential crystalline phases. Each phase region favors the crystallization of a specific type of molecule, although co-existence within the mixture is possible. Several factors influence gallstone formation, including age, gender (female predominance), dietary fat, metabolic syndrome, and a sedentary lifestyle. Additionally, obesity, Diet rich in simple sugars, Glucose intolerance, oral contraceptives, and diabetes are known risk factors.[1,2,4]

The link between cholesterol gallstones and metabolic syndrome markers suggests a crucial role for insulin resistance. Insulin resistance can disrupt bile cholesterol homeostasis and affect gallbladder motility. This highlights the interplay of various factors during cholesterol gallstone pathogenesis. Pigment gallstones, on the other hand, are believed to reflect abnormalities in bilirubin metabolism within the gut-liver axis. Poor bilirubin conjugation leads to the formation of calcium bilirubinate.[5,7,8] This figure illustrates the equilibrium phases and their corresponding crystallization zones. This retrospective epidemiological study analyzes gallstones from patients in Beni Mellal, Morocco, to investigate possible associations between gallstone composition and the risk factors studied.

MATERIALS AND METHODS

This work was carried out at the Department of Pathological Anatomy, Regional Hospital Center of Beni Mellal, Morocco, in collaboration with the Laboratory of Engineering in Chemistry and Physics of Matter, Faculty of Science and Technics, Sultan Moulay Slimane University, Beni Mellal, Morocco. The gallstones samples were provided by the Regional Hospital Center of Beni Mellal as part of a research partnership between the two institutions. All procedures were conducted in accordance with ethical standards and safety recommendations for handling biological materials.

Sample Collection

Two hundred and twenty-six gallstones were collected from patients in Beni Mellal, Morocco, for this analysis. Most of the patients were women. The surgery department of the Regional Hospital Center of Beni Mellal provided the gallstones in 2018. Each patient filled out a form with their information. The gallstones were cleaned, dried, and stored for further examination. All gallstones were ground to create a uniform mixture for testing.

Instrumentation

Analyses were performed to determine the structure and chemical composition of the samples. A scanning electron microscope (Quattro ESEM, FST, Beni Mellal, Morocco) was used to examine the shape and location of the different components within each gallstone. An infrared spectrometer (Nicolet iS50, FSS, Marrakech, Morocco) was used for measurements in the mid-frequency range (4000-400 cm^{-1}) with a resolution of 4 cm^{-1}. The X-ray diffraction technique was applied to create a pattern using a PANalytical diffractometer (X'Pert HighScore, FST, Beni Mellal, Morocco) with a copper tube. This pattern was compared to standard patterns from a reliable source to identify the materials present in the gallstone sample. The intensity of the X-rays was recorded from 5° to 25°. Of the 20 stones analyzed using the aforementioned techniques, we chose to present the results of 6 representative samples. This selection was made to illustrate the diversity of the types of gallstones encountered in our studied population while ensuring a thorough analysis. The data collected from the three analytical techniques were carefully recorded and processed to provide a comprehensive understanding of the structural and chemical characteristics of the gallstones. The potential limitations of each method, such as variability in results due to sample size and the heterogeneous nature of the stones, were considered during the interpretation of the results.

RESULTS AND DISCUSSION

We conducted a chemical composition analysis of gallstones collected from a sample of 20 patients. Although our findings provide valuable insights into the composition of the stones, the limited sample size restricts our ability to establish significant correlations with factors such as diet, body weight, and obesity, particularly within the broader population of 226 patients. To enhance our understanding of the underlying mechanisms involved in gallstone formation, it is crucial to conduct further studies with a larger and more diverse cohort. Future research will broaden perspectives on the relationships between the chemical composition of gallstones and associated risk factors.

The results of this study indicate that the majority of analyzed gallstones were predominantly composed of cholesterol, with a notably higher prevalence in women compared to men. This trend may be associated with factors such as obesity, which is more frequently observed in women, particularly those of advanced age, where sedentary lifestyles and low levels of physical activity are common. Furthermore, the elevated presence of cholesterol in the stones may be influenced by geographical factors, specifically the dietary patterns in the Beni Mellal region, which are characterized by a high intake of cholesterol-rich foods.

The dietary habits of the residents, including significant consumption of animal products and saturated fats, likely contribute to the formation of these stones. This phenomenon underscores the importance of nutrition and physical activity in maintaining biliary health, particularly among at-risk populations. In this study, the identified risk factors for gallstone disease included age and gender, with a marked predominance of women. The 226 patients from the Beni Mellal area had an average age of 45 years.

Women constituted 87.6% (n=198) of the study population, while men accounted for 12.4% (n=28). Regarding residential status, 31% of patients resided in urban areas, while 69% were from rural areas. The sizes of the studied gallstones ranged from 4 mm to 2 cm (Figure 2).

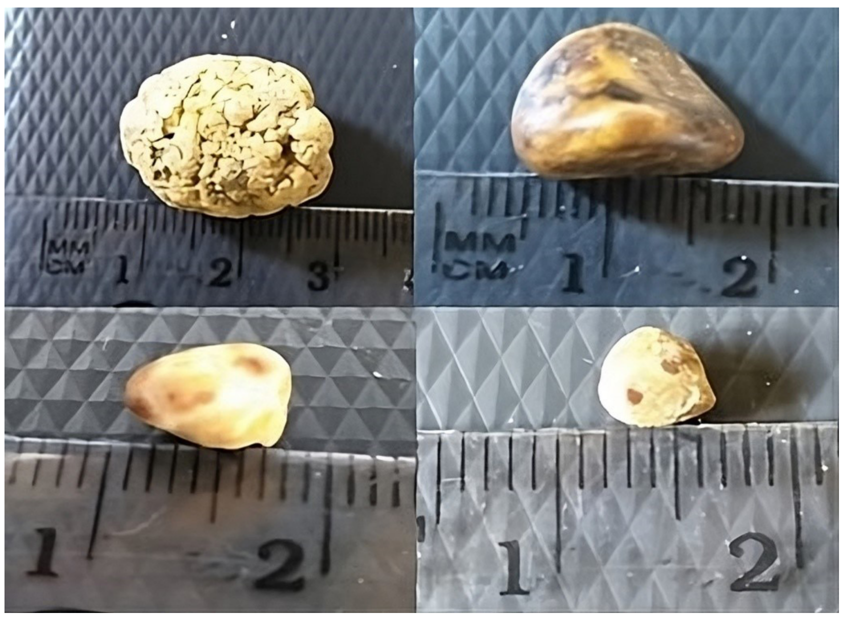

Figure 2. Distribution of gallstone sizes in patients from Beni Mellal.

The morphological analysis indicated that 5% of the gallstones were classified as pure cholesterol stones, whereas 95% exhibited a composition consisting of cholesterol mixed with other bile components. This classification is consistent with prior research but underscores that morphological characteristic may not always accurately reflect the underlying chemical composition, as evidenced by the discrepancies observed in other studies. Therefore, while morphological analysis serves as a valuable initial classification method, it is imperative to augment it with chemical analyses to obtain a more comprehensive understanding of gallstone composition.

Cholesterol stones were characterized by a soft texture, smooth surface, and central radiations on transverse sections Figure 3 (C1). Mixed stones, in contrast, had a rough surface with layered (or laminated) appearances on transverse sections Figure 3 (C2, C3 and C4). Gallstone variations in shape, size, and color suggest a link to their composition and formation process. Factors such as age, gender, diet, and lifestyle might also influence gallstone size. As expected, cholesterol and bilirubin were the primary components identified within the gallstones. Mixed stones contained higher levels of calcium carbonate, calcium phosphate, and calcium bilirubinate, potentially playing a role in them development. However, further exploration is required to fully understand the mechanisms involved.[2,3,4]

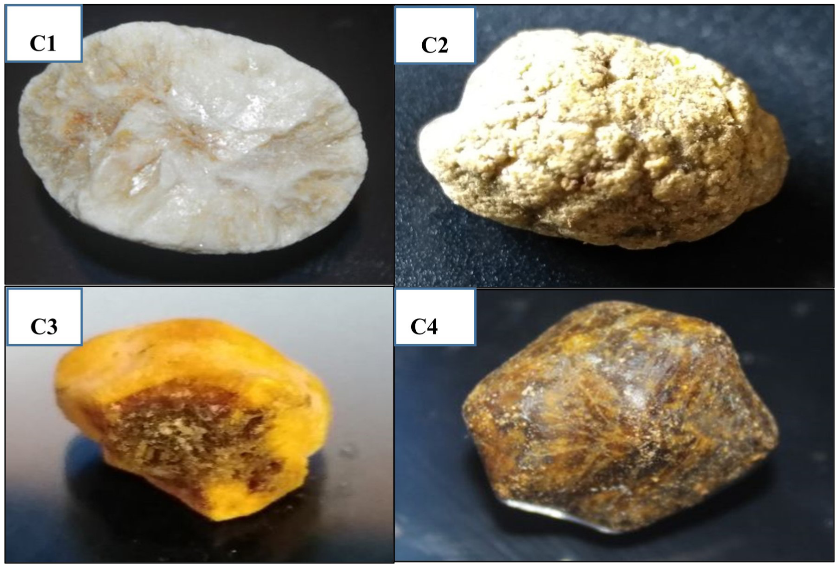

Figure 3. Morphology of gallstones in patients from Beni Mellal: (C1) smooth, radiating pure cholesterol (C2, C3 and C4) rough, laminated mixed cholesterol.

Figure 4 shows the SEM analysis of gallstones from the Beni-Mellal region of Morocco. Sample (C1), composed of pure cholesterol, displayed primarily lamellar and plate-like crystals, with the latter being more abundant. In contrast, samples (C2, C3, and C4), composed of mixed cholesterol, contained both lamellar and needle-like crystals. Consistent with previous studies.[9,10] SEM analysis confirmed that the pure cholesterol gallstones were primarily composed of plates or lamellae, indicating a specific structural organization. Plate-shaped crystals are thought to represent the main phase of monohydrated cholesterol.[8,9] Interestingly, mixed gallstones displayed alternating layers of cholesterol and bilirubin. Calcium carbonate and calcium phosphate were identified as minor components through FTIR, which revealed their characteristic spectral peaks. Notably, these minerals were found to be more prevalent in mixed gallstones compared to pure cholesterol gallstones, despite exhibiting similar textural properties.[9]

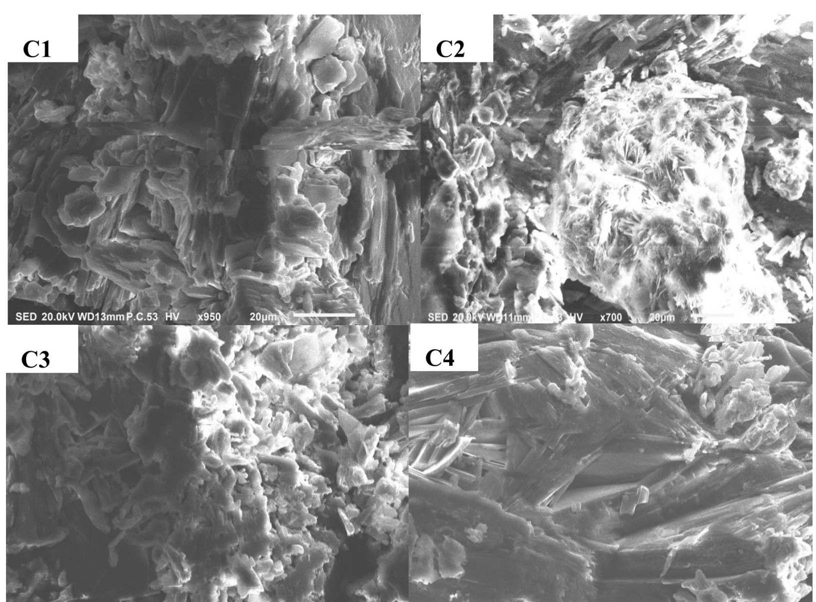

Figure 4. Scanning electron microscopy images of gallstones in patients from Beni Mellal: (C1) pure cholesterol; (C2, C3, and C4) mixed cholesterol.

The FTIR spectra of representative gallstone samples are shown in Figures 5 and 6. These figures show distinct spectral bands associated with various components in the different types of gallstones. In particular, the spectral assignments reveal the presence of salts such as calcium bilirubinate, calcium carbonate, and calcium phosphate, commonly found in mixed gallstone samples (C2, C3, C4, C5, and C6). The FTIR spectrum of a pure cholesterol gallstone (Figure 5: sample C1) displays a distinct profile compared to the mixed gallstones, indicating differing compositions. Chemical analysis of the mixed gallstones (samples C2–C6) also confirmed the presence of these same salts, further supporting the heterogeneous composition observed in their cross-sectional morphology (Table I). The identifiers C2–C6 were assigned to facilitate the distinction between the different types of mixed gallstones analyzed. The attribution of FTIR spectral peaks to the different gallstone samples was based on comparisons with previous studies.[5,11-17]

Table I summarizes the main chemical components identified in gallstones from 20 patients in Beni Mellal, Morocco, as revealed by FTIR analysis. The analysis showed that two main types of gallstones, with four subtypes, were identified, and most gallstones (95%, n = 19) were composed of multiple chemical compounds, while only one gallstone (5%, n = 1) contained a single component.

The absorption peaks at 3421, 3410, 2935, 2934, 1463, 1461, 1459, 1377, 1373, 1056, 1052, and 1050 cm^{-1} were taken as a reference for cholesterol,[5,9-15,17-20] while the absorption peaks at 1649, 1648, 1638, 1637, 1555, and 1251 cm^{-1} were used to detect calcium bilirubinate.[5,11-14,17] The presence of three small peaks at 600, 607, and 612 cm^{-1} has been used to identify calcium phosphate.[5,12,16,21] The absorption peaks at 946, 835, 829, 822, and 711 cm^{-1} have been identified for the detection of calcium carbonate, as referenced in sources.[14,15] The FTIR spectrum of a cholesterol gallstone displays the following absorption bands: an asymmetric stretching band for CH_2 at 2935 cm^{-1}, a bending (δ) band for CH_2 at 1459 cm^{-1}, and a symmetric bending band at 1373 cm^{-1}, which should be clarified as being characteristic of the δ–CH_3 (umbrella) deformation instead. Additionally, the presence of the CH_2 asymmetric stretching band around 2853 cm^{-1}, currently visible as a shoulder, should be noted. The C-C stretching is observed at 1056 cm^{-1}.[15, 17] A broad absorption band in the OH stretching mode is centered at 3410 cm^{-1}, indicating a strong deformation band typically associated with H-O-H vibrations, which is commonly observed around 1630 cm^{-1} in aqueous environments.[15]

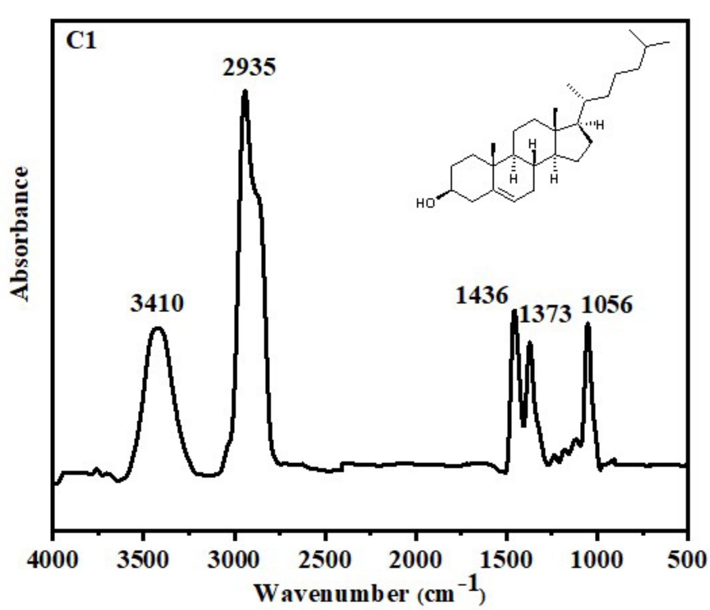

Figure 5. Fourier transform infrared spectroscopy spectrum of a pure cholesterol gallstone.

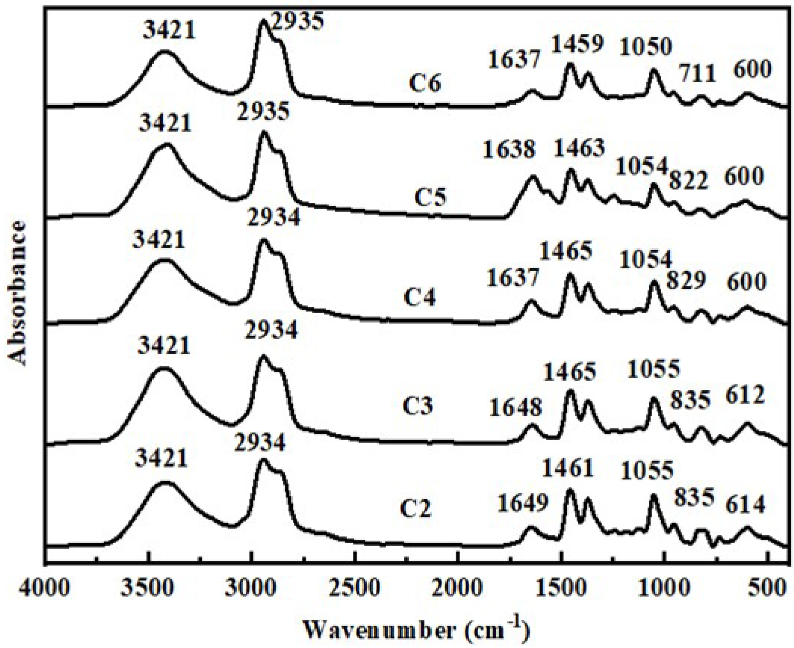

Figure 6. Fourier transform infrared spectroscopy spectra of mixed cholesterol gallstones illustrating the presence of cholesterol, calcium bilirubinate, calcium carbonate, and calcium phosphate.

Moreover, the prominent peaks at 1052 cm^{-1} and 835 cm^{-1} suggest a significant presence of cholesterol and calcium carbonate in the composition of the analyzed mixed gallstones. The band at 835 cm^{-1} corresponds to the bending vibration of the CO_3^{2-} ion in calcium carbonate. The FTIR analysis suggests a significant presence of cholesterol in the patients' gallstones, which is similar to the high cholesterol content found in pure cholesterol stones.[22-24] The precipitation of excess cholesterol from bile in the form of solid crystals is a prerequisite for cholesterol gallstone formation.

Applying FTIR to mixed gallstones reveals strong indications of significant cholesterol and calcium carbonate content. This suggests high levels of these substances within the examined gallstones. This finding underscores the crucial role of cholesterol and calcium carbonate in both the formation and composition of gallstones, potentially acting as key contributors to their development. Notably, the high cholesterol levels align with existing knowledge about cholesterol gallstone formation, highlighting the importance of these findings for understanding the disease process. Furthermore, the analysis revealed that most gallstones are a mixture of various chemicals. This observation suggests that gallstone formation is a complex process involving different salts and organic materials precipitating from bile. The existence of gallstones composed solely of a single component reinforces the concept of diverse gallstone compositions. This diversity underscores the need for tailored approaches in diagnosing and managing different gallstone types.

Table I. Chemical composition of different types of gallstones in patients from Beni Mellal

Type of gallstone	Representative principal chemical components (FTIR spectral results as wave number, in cm^{-1}, corresponding to the peak on the spectrum)	(Frequency %, Number)
Pure cholesterol	Cholesterol (3421, 3410, 2935, 2934, 1463, 1461, 1459, 1377, 1373, 1056, 1052 and 1050 cm^{-1})	(5%, n=1)
Mixed cholesterol	Cholesterol, Calcium bilirubinate (1649, 1648, 1638, 1637, 1555 and 1251 cm^{-1}) Calcium carbonate (946, 835, 829, 822 and 711 cm^{-1}) Calcium Phosphate (600, 607 and 612 cm^{-1})	(95%, n=19)

XRD analysis was conducted to identify the crystalline phases present in both pure cholesterol and mixed gallstones. The gallstone corresponding to sample C1, as depicted in Figure 7, exhibited a sharp peak at a 2θ angle of 16,88° (d-spacing of 5,24 Å), corresponding to the (001) plane of crystallized cholesterol. However, the diffraction pattern also revealed significant peak overlap, as illustrated in Figure 8. The obtained d-spacing values were systematically compared with the reference pattern for cholesterol from the Joint Committee on Powder Diffraction Standards (JCPDS) database (file No. 00-007-0742), as presented in Figure 8 and Table II. The XRD analysis of the pure cholesterol stone yielded d-spacing values consistent with the standard pattern, with notable diffraction peaks observed at 5,34° ((100) plane), 14,12° ((101) plane), 15,32° ((102) plane), 16,88° ((001) plane), 18,09° ((200) plane), 19,34° ((201) plane), and 21,14° ((211) plane). These Miller indices confirm the crystalline nature of cholesterol in the analyzed samples. Additionally, we have reviewed the correspondence of our data with the standard pattern presented in Figure 8, acknowledging some discrepancies in the intensity ratios (%I).

This mismatch may be attributed to factors such as sample heterogeneity and instrumental variations, which are discussed to provide context for these differences. Importantly, no diffraction peaks associated with calcium bilirubinate were detected, reaffirming the crystalline structure observed in the cholesterol samples. The reference data for cholesterol crystals in the JCPDS database indicates a triclinic crystal system characterized by the following unit cell parameters: a = 14,10 Å, b = 33,75 Å, c = 10,46 Å, α = 94,60°, β = 90,00°, and γ = 95,72°. This information has been integrated into our analysis to further substantiate the crystalline characteristics observed in the samples.

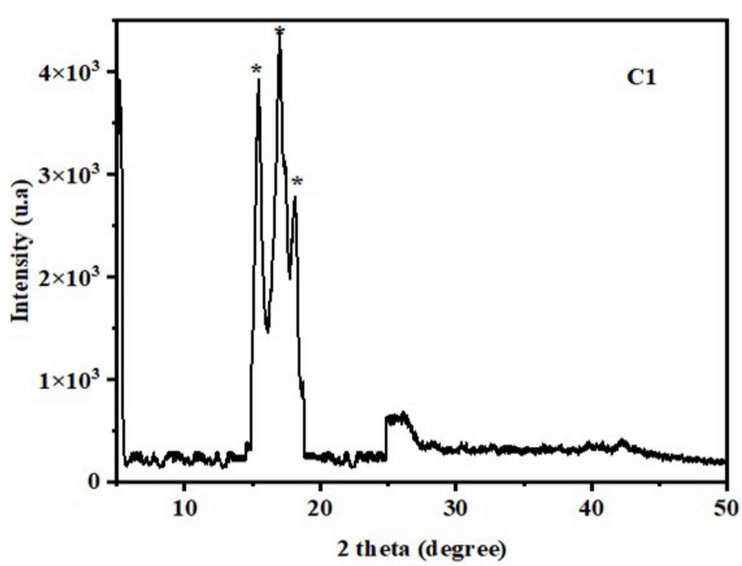

Figure 7. X-ray diffraction pattern of a pure cholesterol gallstone (*): identifying crystalline phases.

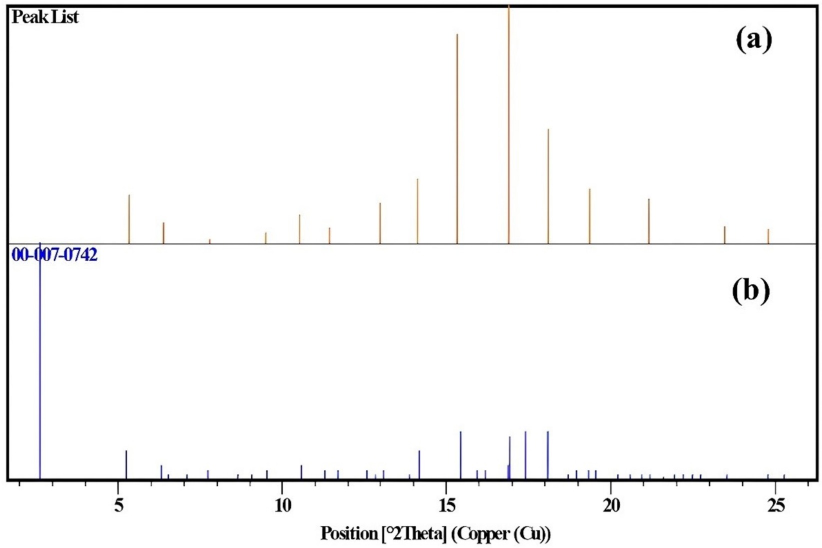

Figure 8. X-ray diffraction spectra of cholesterol: comparing pure gallstone (a) and reference standard (b).

Table II. Comparison of X-ray diffraction data: pure cholesterol gallstone (c1) standard cholesterol (Joint Committee on Powder Diffraction Standards)

Standard cholesterol (Joint Committee on Powder Diffraction Standards)						Gallstone sample		
h	k	l	d-spacing [Å]	Pos. [°2Th.]	Rel. Int. [%]	d-spacing [Å]	Pos. [°2Th.]	Rel. Int. [%]
0	1	0	33,600	2,627	100,0	16,529	5,341	20,33
0	2	0	16,800	5,256	12,0	13,819	6,390	8,43
1	0	0	13,970	6,322	6,0	11,346	7,785	1,60
1	-1	0	13,510	6,537	2,0	9,309	9,492	4,30
1	1	0	12,460	7,089	2,0	8,413	10,506	12,11
1	-2	0	11,430	7,729	4,0	7,734	11,430	6,58
1	2	0	10,230	8,637	2,0	6,813	12,983	16,67
0	1	1	9,740	9,072	2,0	6,263	14,128	26,97
1	-3	0	9,280	9,523	4,0	5,777	15,324	87,85
0	4	0	8,360	10,574	6,0	5,246	16,884	100,00
1	-2	1	7,830	11,292	4,0	4,899	18,092	47,84
1	-4	0	7,560	11,696	4,0	4,583	19,348	22,67
2	0	0	7,040	12,563	4,0	4,199	21,140	18,42
1	4	0	6,880	12,857	2,0	3,788	23,463	7,18
2	1	0	6,750	13,106	4,0	3,587	24,801	5,61

Figure 9 displays the XRD patterns obtained for mixed gallstones. These patterns reveal intense reflection lines for cholesterol compared to other components identified in the study. The d-spacing values derived from the XRD analysis confirm the presence of crystalline cholesterol, calcium carbonate, and calcium phosphate. Notably, XRD serves as a valuable tool for identifying the crystalline phases present in gallstones, allowing for both chemical and mineralogical characterization. [25,26] Importantly, the XRD results corroborated the presence of all other chemical compounds previously identified by FTIR analysis.

This analysis also highlights the diverse composition of gallstones. The presence of both pure cholesterol and mixed gallstones suggests potentially different formation pathways. Additionally, the presence of bilirubin and calcium salts in mixed stones suggests a more intricate interplay of factors influencing their development. This underscores the need for further exploration of how various chemicals interact and contribute to gallstone formation.

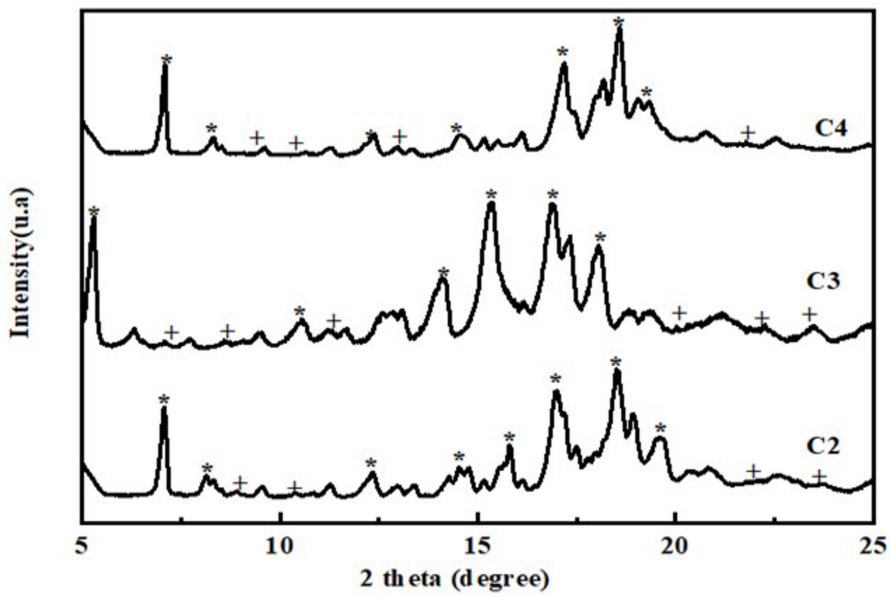

Figure 9. X-ray diffraction spectra of gallstones in patients from Beni Mellal: ∗ cholesterol, + calcium salts (calcium bilirubinate, calcium carbonate and calcium phosphate).

CONCLUSIONS

This study examined the composition of gallstones from patients in Beni Mellal-Khenifra, Morocco. Techniques like scanning electron microscopy, Fourier transform infrared spectroscopy, and X-ray diffraction were used. The results confirm that mixed cholesterol gallstones are the most common type in this region. Age appears to be a significant risk factor, with women more likely to be affected. Additionally, the findings suggest a possible link between geographic location, diet, and gallstone formation. The analysis focused on quantifying the composition of 20 gallstones using diffraction data from patients at the Beni Mellal regional hospital. The gallstones were categorized into two main types: pure cholesterol and mixed cholesterol. The analysis revealed a variety of shapes, sizes, colors, and surface textures. Notably, a high concentration of cholesterol was observed throughout the gallstones, from the center to the outer regions. Furthermore, the presence of various calcium salts, including calcium bilirubinate, calcium carbonate, and calcium phosphate, was confirmed within the mixed stones. These findings highlight the complexity of gallstone formation, likely influenced by a combination of factors beyond just cholesterol levels. Further work is necessary to understand the interplay between various contributing elements, such as geographic variations, dietary habits, and specific genetic predispositions, in the development of different gallstone types in Beni Mellal-Khenifra. This knowledge can pave the way for the development of more targeted diagnostic and therapeutic approaches for managing gallstone disease.

Conflicts of interest

The authors declare that there are no conflicts of interest or funding involved in this work.

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