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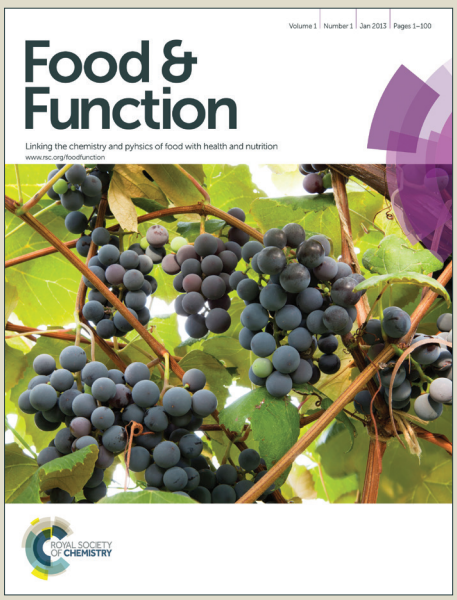


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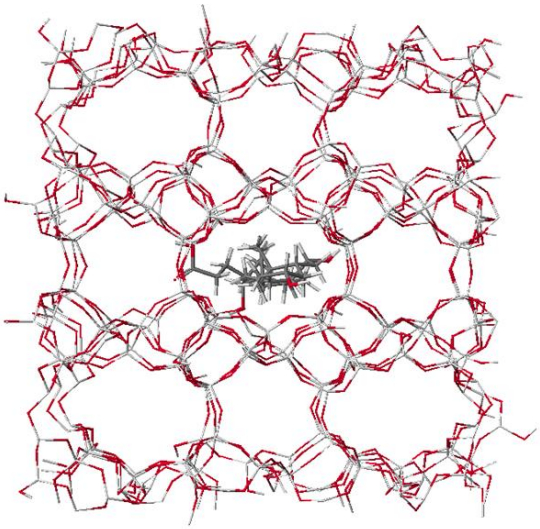
*Bile was sequestered by clinoptilolite under simulated digestion of a high-fat meal. Molecular docking modeling indicates the most electronegative parts of the bile acids (here: cholic acid) docking at an electropositive region of the clinoptilolite matrix.*



Title: Bile sequestration potential of an edible mineral (clinoptilolite) under simulated digestion of a high-fat meal; an *in vitro* investigation.

Running title: Bile sequestration by edible clinoptilolite

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## Abstract

Bile, important for cholesterol homeostasis, is a potential target of hypercholesterolemia management. Bile sequestration by orally administered resins, while mostly effective in reducing blood cholesterol, presents several side effects and disadvantages. Thus, widely available natural edible minerals such as clinoptilolite with adsorptive properties offer an alternative for bile sequestration. In an experimental setting mimicking the physiological conditions of digestion/absorption

(pH, temperature, retention times) with a series of assessment methods, scanning electron microscopy-energy dispersion X-ray (SEM-EDX), X-ray diffraction (XRD), Furier transform infrared (FT-IR), thermogravimetric differential thermal analysis (TG-DTA), and molecular docking modeling, the ability of natural unmodified clinoptilolite to retain bile, while mixed with a simulated high-fat meal, was investigated. Our results demonstrate that clinoptilolite sequesters bile via adsorption of macromicelles at 75.4% efficiency, when the former is administered at a reasonable dose of 4% (w/w) of a meal's weight. This work opens the possibility of clinoptilolite utilization as a bile-sequestering/cholesterol-reducing agent.

**Keywords:** Bile, Cholesterol, Clinoptilolite, Geophagia, Hypercholesterolemia, Zeolite

## Introduction

Bile is an aqueous solution constituted mainly of bile acids and a small fraction of phospholipids, free cholesterol and other minor constituents. Bile acids are 22-28 carbon carboxylic acids, that can either be directly derived from their precursor cholesterol in the liver (primary bile acids: cholic and chenodeoxycholic acids) or synthesized by metabolic action of colonic bacterial flora on the primary bile acids forming secondary bile acids with the most abundant being deoxycholic and lithocholic acids. After their synthesis, the primary bile acids are conjugated with glycine or taurine. The most abundant bile acids in human bile are chenodeoxycholic acid (45%) and cholic acid (31%). Following its secretion by the liver, bile is stored and concentrated in the gallbladder, with a final composition of 92% water, 6% bile salts ($Na^+$ and $K^+$ salts), and the remainder comprised of bilirubin, various lipids such as cholesterol, fatty acids and lecithin, as well as traces of inorganic salts (200 mEq/L).[1,2] In response to fat presence in the small intestine, bile is discharged via gallbladder contraction into the duodenum, the first section of the small intestine. In the jejunum, the middle section of the small intestine, bile acts as a potent digestive surfactant with the amphipathic bile constituents emulsifying fat and creating mixed micelles, thus facilitating the activity of pancreatic lipase and ultimately the absorption of fat and fat-soluble compounds by the enterocytes of the small intestine. Upon completion of fat uptake, at least 95% of bile is re-absorbed in the last section of the small intestine (ileum) via intestinal bile acid transporters (IBAT) and returns to the liver in a continuous cycle (entero-hepatic circulation), while the unabsorbed fraction is eliminated through the colon and into the feces.[3]

In addition to fat digestion/absorption, bile is critical for maintaining cholesterol homeostasis in the human body since cholesterol elimination occurs mainly by conversion to bile acids. Bile acids represent the primary pathway for cholesterol catabolism and account for ~50% of the daily turnover of cholesterol. Excess blood plasma cholesterol plays central role in atherogenesis, the underlying cause of the majority of cardiovascular diseases. In addition to diet and lifestyle changes, cholesterol-lowering drugs and bile acid sequestration resins are used for managing

hypercholesterolemia.[1] Oral administration of non-absorbable resins (eg. cholestyramine, colestipol, colesevelam) that sequester bile acids in the intestine, thus removing them from the enterohepatic cycle, is an effective treatment for reducing plasma cholesterol with the liver further diverting cholesterol in bile acid synthesis in order to maintain an adequate bile acid pool.[4] In addition, glycemic control is another potential target of bile acid sequestrants, with an emerging critical role of bile acids in glucose homeostasis.[5] These observations extend potential for improving Type II Diabetes Mellitus.

While bile sequestration in the intestine is an effective way of reducing blood cholesterol in hypercholesterolemic individuals, the current drugs used for this purpose present several drawbacks. Due to low water solubility and poor access of bile acids to the binding sites, these anion-exchange resins are required in large and often multiple doses (8-12 g/day) leading to adverse effects such as constipation, nausea, meteorism, "sandy taste", and in turn intolerance and poor patient compliance.[4] Furthermore, due to large and frequent dosing, in addition to the actual chemical composition cost, the overall financial cost to the patient cannot be neglected. Hence, the search for non-pharmacological means of reducing bile acids in the small intestine is worth pursuing. Previous research has reported the bile adsorption capacity of various zeolites indicating zeolite potential use as a bile sequestrant.[6,7]

Zeolites are chemically similar to clay minerals, but differ in their molecular bonding conformation. Zeolites are microporous crystalline aluminosilicates containing alkaline metals. Their primary structural units are the silica and alumina tetrahedrals, assembled into secondary polyhedral building units that form a rigid and highly stable final three-dimensional framework containing large cavities and channels.[8] The negative charges developed due to isomorphous substitution of $Si^{+4}$ by $Al^{+3}$ are balanced by cations such as $Na^{+}$, $K^{+}$, $Ca^{+2}$ and $Mg^{+2}$. The extra-framework cations are ion exchangeable and give rise to the rich ion-exchange chemistry of zeolites. By virtue of their unique structure, zeolites exhibit high specific surface areas and can sorb large amounts of molecules both in gas and liquid phases, facilitate ion exchange and act as molecular sieves with long-term chemical and biological stability.[9-16]

Natural zeolites are non-toxic and safe materials with various applications in human and veterinary medicine, such as their use as biomaterials, adjuvants in cancer therapy, drug-delivery systems or anti-diarrheic agents.[17-23]

Geophagia, the intentional and repeated ingestion of clay, soil or other geological materials including minerals such as zeolites, has been practiced for centuries across different populations throughout the world and has been known to have health consequences for humans, although not completely delineated.

Clinoptilolite, a common natural zeolite is an aluminium silicate mineral complex $\{(Na,K,Ca)_{2\text{-}3}Al_3(Al,Si)_2Si_{13}O_{36}\cdot 12H_2O\}$ arranged in a three-dimensional rigid (non-expanded) crystalline structure of interconnected channels and cavities (Fig. 1). As an edible mineral, clinoptilolite is documented to promote animal growth and performance[24], protect against oxidative stress and cancer[25] and modulate immunity.[26] With regards to bile sequestration, $K^{+}$ and $Ca^{+2}$ modified Na-X exchange zeolites with a Si/Al=1 ratio were shown to adsorb both bile acids and phospholipids when contacted with solutions of ox-bile.[6] In addition, modified cancrinite-type zeolites (Ca-, Na-, K-cancrinite) were reported to adsorb components of healthy human bile (bile acids, phospholipids and bilirubin) and compared with cholestyramine, Ca-cancrinite had a higher selectivity to bile acids and thus adsorbed bile acids more effectively.[7] Of note, in both aforementioned studies, the experiments were performed

at 25 °C, at neutral pH and in the absence of any dietary constituents.
The present study investigated for the first time to our knowledge, whether and to what extend unprocessed, unmodified natural clinoptilolite added in a high-fat diet mixture can adsorb bile in an experimental setting resembling the process of human digestion. To this end, a diet mixture resembling a meal containing the three categories of macronutrients (carbohydrate, fat and protein) in a composition mimicking a high-fat meal (Western-type diet) was used under conditions simulating gastric and intestinal digestion (pH 2.5 - 6.7, 37 °C, 15-120 min, motility). Clinoptilolite is an inert and inexpensive, widely available natural mineral, thus it could be a good alternative to bile sequestration agents if proven effective as a bile-binding agent.

## Materials and methods

Natural clinoptilolite ($Ca_{1.5}K_{1.4}Mg_{0.6}Na_{0.5}Al_{6.2}Si_{29.8}O_{72}\bullet 20H_2O$) from Petrota area of the Evros region (Thrace) in Northeastern Greece with cation exchange capacity (CEC) at 226 meq/100g as characterized by Filippidis et al.,[16] in powdered form was mixed with simulated chyme. More specifically, five identical mixtures, clinoptilolite plus diet mixture (clinoptilolite+diet) were prepared by mixing 10 g of clinoptilolite with 240 g of a diet mixture resembling a high-fat meal (Table 1) to form a final amount of 250 g of chyme. This amount of clinoptilolite (4% of the total meal weight) constitutes a reasonable and realistic dose with regards to practicality and palatability as demonstrated by *in vivo* studies.[27] The simulated high-fat meal consisting of 25 g lipids, 44 g carbohydrate and 24 g protein, approx. 45%, 35% and 20% Energy, respectively, was prepared by mixing appropriate amounts of commercially available diet ingredients, and the final weight (240 g) was adjusted by adding distilled water (Table 1). Olive oil was selected as a representative mixture of different types of dietary lipids (saturated, monounsaturated, polyunsaturated fatty acids) and plant sterols.[28] Carbohydrates, (disaccharide, commercial table sugar) and protein (Six Star-Pro Nutrition Whey Protein isolate, a commercially available brand), where chosen in their simple chemical form and an easily digested form, respectively, to mimic to the extent possible, the products of digestion in the small intestine, since no digestion enzymes were used in the present setting. In order to clearly determine the potential adsorption of the sulfur-containing bile constituent taurine, i.e. 2-aminoethanesulfonic acid, a whey protein isolate containing negligible quantities of sulfur-containing amino acids (according to the manufacturer) was selected for this study. Dietary fiber is known to bind bile salts in the intestinal lumen[29], hence no fiber was added to the diet mix aiming to isolate the binding effect of clinoptilolite and derive a more conservative estimate of dosing and effect.
The pH was brought to 2.5 with HCl 6N to simulate the acidity of the stomach. The clinoptilolite+diet mixtures were steadily stirred semi-vigorously at 37 $^{o}$C for various time intervals (15, 30, 60, 90 and 120 min respectively) resembling gastric motility and emptying.[30] At the end of these time periods, the pH was brought to 6.7 with NaOH 0.1N to represent conditions at the duodenum section of the small intestine, and 5 g of dried purified (>95%) ox-bile, equivalent of 40 g of fresh bile (Sigma-Aldrich, 70168 FLUKA) were introduced to each mixture, an amount of bile approximating the gallbladder capacity.[31]
Each of the final mixture (clinoptilolite+diet+bile) was stirred mildly at 37 $^{o}$C for 15, 30, 60, 90 and 120 min respectively. After the end of the stirring times, mixtures were

collected, centrifuged and dried in a drying oven at 180 $^{o}$C for 3.5 h and stored at 4 $^{o}$C in a desiccating chamber until the analyses. The following analyses were performed on these final samples: Fourier transform infrared (FT-IR) to test the sequestration of bile by the clinoptilolite matrix, thermogravimetric differential thermal analysis (TG-DTA) to test the thermic stability of the materials and perform the adsorption kinetics, X-ray diffraction (XRD) to study the crystalline phase and scanning electron microscopy-energy dispersion X-ray spectrometer (SEM-EDX) to study the structure of the clinoptilolite-bile system and perform a semi-quantitative assessment of bile sequestration by clinoptilolite.

*FT-IR:* FT-IR system comprised of a spectrophotometer Nicolet IR 200 by Thermo Electron Corporation, an analogue-to-digital signal transformer Spectra System SN4000 by Thermo Electron Corporation. Spectra were recorded within a bandwidth of 4000-5000cm$^{-1}$. The sample was mixed thoroughly with KBr, lyophilized and compressed into a pellet. The EZOMNIC software was used to derive the data.

*TG-DTA:* Analysis was performed using a Perkin Elmer STA 6000 Thermal Analyzer. Nitrogen (99.999% $N_2$) was used as the inert gas, and 10% $O_2$ in $N_2$ was used as the oxidizing gas. The PYRIS software was used to derive the data.

*SEM-EDX:* A Scanning Electron Microscope (JEOL JSM-840) connected to an Energy Dispersion X-ray Spectrometer-EDX (LINK-AN 10000) was employed to identify the micelles of bile salts and cholesterol (the main bile constituents) on the clinoptilolite surface. Acceleration potential was 10 KV. Computational analyses were conducted using the JEOL and SwiftED-1.ipj softwares. Sample amounts and procedures were followed according to manufacturer instructions.

*XRD:* A Seifert XRD 3003TT diffractometer equipped with a PW1710/00 controller was used to perform the XRD analyses with CuKα radiation (λ=1.5418) and a Ni (0.0170 mm) filter. The samples were scanned over an interval 5-90$^{o}$, 2θ=0.05$^{o}$ at 4 sec. Sample amounts and procedures were followed according to manufacturer instructions.

*Computational details for molecular docking simulations*

The optimized geometries of lithocholic acid, cheneodeoxycholic acid, deoxycholic acid and cholic acid (the main bile acids) were obtained using B88PW91/DZVP and B3LYP/6-31G*, respectively, within the framework of Density Functional Theory.[32] From the computed molecular wave functions the molecular electrostatic potential of each bile acid was calculated. Furthermore, their interactions with clinoptilolite were determined through molecular docking techniques.

## Results and discussion

The results obtained from samples at different time intervals were similar; hence only results from the final sample at 120 min are presented and discussed herein with this sample resembling the most the physiological conditions during digestion and absorption of a high-fat meal in the gastrointestinal (GI) tract. In Fig. 1 we show clinoptilolite's basic unit (Fig. 1.a) and the crystalline framework (Fig. 1.b) as produced by means of molecular simulation.

*Solid phase characterization (FT-IR)*

The presence of functional groups and purity of the solid phase were assessed via FT-IR in the individual solid materials and in the dried and centrifuged matter. Characteristic spectra for each of the solid materials (protein, disaccharide, clinoptilolite, bile) are shown in Figure 2.a in a superimposed fashion.

In the protein spectrum four characteristic peaks are identified. At 3000 $cm^{-1}$ there is absorption attributed to the N-H bonds, while the peaks identified at 1660 $cm^{-1}$, 1548 $cm^{-1}$ and 1320 $cm^{-1}$ are assigned to the amides I, II and III respectively (Fig. 2.a). The disaccharide ($C_{12}H_{22}O_{11}$) displays a wide range of peaks in the 1500 – 4000 $cm^{-1}$ area, which is attributed to the bending vibrations of the C-H bonds and the tension and bending vibrations between plain bonds that connect the methylene group (Fig. 2.a). Further, the peaks observed in the 1300 – 950 $cm^{-1}$ range can be attributed to the tension vibrations of the C-O-C configuration while the peak at 3600 $cm^{-1}$ is due to tension vibration of hydroxyl groups (-OH). The clinoptilolite spectrum displays a peak at 1640 $cm^{-1}$ attributed to the $H_2O$ molecules found in the material structure whereas the peaks detected in the range of 1095 – 440 $cm^{-1}$ are attributed to the Si-O-Al bonds[7] (Fig. 2.a). The bile spectrum exhibits characteristic peaks at 2925 and 2850 $cm^{-1}$, attributed to the aliphatic chain of bile acids. Sulfur bonds are identified through the typical peaks detected at 1250, 1125 and 1080 $cm^{-1}$ (Fig. 2.b), indicating taurine (2-aminoethanesulfonic acid) conjugated to bile.

The IR spectrum of the assessed material derived from the contact of 10 g clinoptilolite and 5 g of dried bile with the total of 240 g of dietary ingredients for 120 min at appropriate pH adjustments displays several characteristic peaks. The peaks seen at 2925, 2853 and 1070 $cm^{-1}$ are attributed to bile; the first two to the aliphatic chain while the third to the sulfuric bonds. The peaks detected at 1640, 1440 and 600 $cm^{-1}$ are also characteristic and attributed to clinoptilolite, while more specifically they constitute identifiers of the $H_2O$ molecules part of the clinoptilolite structure, the nitric anions and the Si-O-Al bonds respectively. Finally, the peak detected at 1540 $cm^{-1}$ is attributed to the amide II class of the protein.

*Crystalline phase assessment (XRD)*

In order to verify the presence of bile into the clinoptilolite matrix in the final sample and to study the crystalline structure XRD studies were performed. The diffractograms produced, verified the high crystalline nature of clinoptilolite with major peaks at $2\theta = 9.88^o$, $2\theta = 16.4^o$, $2\theta = 22.3^o$, $2\theta = 29.3^o$ and $2\theta = 31.5^o$ in line with the literature.[15] Bile on the other hand, exhibits relatively low intensity peaks at angle values of $2\theta = 14^o$, $2\theta = 30.5^o$, $2\theta = 45.6^o$ and $2\theta = 63.3^o$. The diffractogram of the final sample verifies the presence of bile in the clinoptilolite structure, with clinoptilolite maintaining its crystalline structure (Fig. 3). Indeed, the angle values of the sample at $2\theta = 14^o$, $2\theta = 30.5^o$, $2\theta = 45.6^o$ and $2\theta = 63.3^o$ correspond to bile, while the rest of the values represent clinoptilolite peaks (Fig. 3).

*Structure of the clinoptilolite-bile system: a semi-quantitative assessment of bile sequestration by clinoptilolite (SEM-EDX)*

The final sample was assessed via SEM-EDX to further verify the sequestration of bile by clinoptilolite, infer a potential mechanism of sequestration, study the structure of the clinoptilolite-bile system, and perform via the EDX system a semi-quantitative assessment of the degree/efficiency of bile sequestration by clinoptilolite. The SEM images obtained (Fig. 4) demonstrate that the clinoptilolite in the final sample exhibits granular heterogeneity, is comprised of crystals and its particle size is at the order of a

few micrometers. Bile in the final sample on the other hand, exhibits an open type network/frame composed of spherical and disc-shaped micelles (typical micellar configuration). The SEM picture and the elemental analyses by EDX reveal that bile is sequestered on the clinoptilolite exterior part, as observed by the significant presence of micelles as well as the detection of significant sulfur amounts, attributable to taurine conjugated to bile.

*Semi-quantitative assessment of bile sequestration by clinoptilolite*

A semi-quantitative assessment of bile sequestration by clinoptilolite by means of sulfur presence determination and quantification was conducted via EDX analysis. The overall quantity of the final sample received after centrifugation and drying was 12.84 g. The EDX analyses (*N*=3 samples, each sample had a standard rectangle area 9mm$^2$ scanned) demonstrated the presence of sulfur in all patterns taken. An indicative and representative pattern of the final sample is given in Fig. 5c, while patterns of clinoptilolite and bile are presented in Fig. 5a and 5b, respectively. According to EDX measurements 2.15% S (average value with 8% range) was detected in the final sample. This represents a sulfur quantity of 0.276 g (12.84 × 2.15/100). This sulfur quantity can be attributed only to bile since no other ingredient of the mixture clinoptilolite+diet+bile contains sulfur. At the same time the sulfur content of the bile used in this study is up to 22% (as $SO_4$) (supplier's information), while the amount of dried bile into the final mixture clinoptilolite+diet+bile is 5 g. This quantity is equal to 0.366 g of S (5 × 22/100 × 32/96) and is the total sulfur quantity contained in the final sample. Accepting that S is distributed equally within the bile, according to the semi-quantitative assessment there is up to 75.4% [(0.276/0.366) × 100)] efficiency in the sequestration of bile by clinoptilolite, in our experiments. The 75.4% sequestration efficiency has a binding range of: ± 6 percent units as derived based on the 8% S determination value range.

*Further verification of bile sequestration by clinoptilolite and interaction stability*

In separate experiments, the sequestration of bile by clinoptilolite was further verified via TG-DTA analyses, as well as the integrity and stability of the clinoptilolite-bile complex (Fig. 6). Furthermore, the thermal stability of clinoptilolite and bile was assessed. According to the TG-DTA analyses the dehydration of clinoptilolite amounting to 12% of the total initial weight, is conducted in two phases. During the first phase 6.8% dehydration is achieved in a temperature range of 50 – 320 $^oC$ whereas the remaining 5.2% is achieved in a temperature range of 320 – 660 $^oC$. During the first phase of dehydration the intact $H_2O$ molecules are released whereas during the second phase of dehydration hydroxyl groups (-OH), the second form of $H_2O$ in clinoptilolite is released. Intact $H_2O$ molecules are released at temperatures lower than 100 $^oC$ while hydroxyl groups are released at temperatures higher than 400 $^oC$. According to clinoptilolite's chemical analysis total $H_2O$ (both forms) is at 13.56%.[16] This suggests that there is still a 1.56% of $H_2O$ remaining in the clinoptilolite structure after dehydration. TG-DTA analyses on bile show that bile exhibits a relatively high thermal stability since degradation begins at about 210 $^oC$ while a marked weight reduction is demonstrated in the temperature range of 360 – 500 $^oC$, with the final weight reduction at 800 $^oC$. There is a cumulative 59% of weight reduction of which 12% refers to the $H_2O$ loss from clinoptilolite and the remaining 47% originates from the bile. A graphical representation of the bile adsorption reaction kinetics is provided in the supplementary figure (refer to the manuscript's supplementary section). While bile retention is favored by the low $H_2O$

amount in clinoptilolite, it is highly unlikely for water-soluble components (carbohydrate and protein/amino acids) to be sequestered by clinoptilolite. These results taken together clearly suggest that under physiological conditions (i.e.: temperature of ≈ 37 $^{o}$C) the clinoptilolite-bile complex maintains its integrity and is very stable.

*Molecular docking simulation of bile sequestration mechanism by clinoptilolite*
To gain a theoretical insight regarding the bile to clinoptilolite adsorption profile Density Functional Theory calculations on the main bile acids were performed. Their optimized geometries are shown in Fig. 7. The calculated energies are -1239.54 Hartrees for the chenodeoxycholic acid, -1239.52 Hartrees for the deoxycholic acid, -1164.32 Hartrees for the lithocholic acid and -1314,73 Hartrees for the cholic acid. In the same figure (Fig. 7) the calculated electrostatic potential on the molecular surface of the respective bile acids is also displayed in kcal/mol. These values associated with the contours correspond to the interaction energies of a positive point charge with the charge distribution of the molecule. A strongly negative region ($V_{min}$ in red color) is characteristically related to electronegative atoms. In chenodeoxycholic acid the potential reaches its most negative value (-132.77 kcal/mol) near the carbonylic oxygen, in the deoxycholic acid the most negative value (-141.89 kcal/mol) is located near the carbonylic oxygen as well, in the lithocholic acid the most negative value (-131.60 kcal/mol) is found near the carbonylic and the carboxylic oxygen while the cholic acid appears to exhibit the most negative value (-140.38 kcal/mol). These values indicate the most favorable paths of approach of an electrophile to the molecules. As shown in Fig. 8, the most electronegative parts of the bile acids will seek to dock at an electropositive region of the clinoptilolite surface. In conclusion, the molecular docking simulation analyses demonstrate that docking of bile acids (sequestration) to clinoptilolite is thermodynamically favored, suggesting that docking of bile micelles to clinoptilolite occurs via the bile acid side.
In this study we aimed to investigate the potential and properties of clinoptilolite in the context of its use as a simple, natural/unmodified, economical and abundant edible mineral bile-sequestrant to function in hypercholesterolemia treatment. In our experimental setting we simulated the co-administration of a high-fat meal and a clinoptilolite dose, and bile secretion at pH, times and temperature physiologically relevant. Under these conditions we demonstrated that clinoptilolite adsorbs bile in the form of micelles at a maximum of 75.4% efficiency.
We detected characteristic functional groups and evaluated purity of the solid phase via FT-IR in the individual solid materials and in the dried and centrifuged matter. Our results show that there has been retention of bile by clinoptilolite as typical sulfur peaks were detected indicative of bile conjugate taurine. These sulfur peaks cannot be attributed to the other solid ingredients: dietary disaccharide or protein.
Protein retention by clinoptilolite is not favored under the simulated physiological digestion and absorption conditions of the GI track since the low pH causes the protein degradation and partial dissociation, while for protein adsorption to clinoptilolite a special modification of the latter is needed and a pH value much lower than that of protein's pI. In the absence of any enzymatic hydrolysis it is highly unlikely that any amino acids are available in our experimental setup, rendering unlikely amino acid interaction with clinoptilolite. In addition, protein (amino acid) adsorption by zeolite is not anticipated according to previous research whereby up to 5% (per diet weight) of edible zeolite administered in pigs did not seem to affect biochemical indices of protein status, indicating that protein/AA retention to zeolite, if

any, is not sustained and amino acid systemic absorption is not inhibited by zeolite (total protein, albumin, urea nitrogen, creatinine).[27] Finally, in our system, protein contacted clinoptilolite earlier than bile, but the possibility that amino acids are adsorbed in the zeolite surface is excluded, since prior amino acid adsorption would have inhibited bile adsorption into the clinoptilolite's surface, while bile complexation with clinoptilolite is attested by micellar formation as observed by SEM images. Under physiological conditions, partial digestion of proteins in the stomach would produce peptide chains of size unlikely to be absorbed by the clinoptilolite. Following the main digestion of protein in the small intestine, i.e. peptide chains hydrolyzed by intestinal enzymes to individual amino acids, the latter are expected to be quickly absorbed by the enterocytes, allowing minimal opportunity of interaction with clinoptilolite or bile in the intestinal lumen.

XRD analyses of the final sample verify the existence of bile in the clinoptilolite structure, with clinoptilolite maintaining its crystalline structure.

In addition, the SEM pictures clearly delineate the sequestration of bile in the form of micelles by clinoptilolite. A macro-micellar formation mechanism was proposed by Linares et al., to explain bile interaction with the surface of a synthesized nitrated cancrinite-type zeolite.[7] The sulfur present in the clinoptilolite-bile complex is most likely derived from the bile acid conjugates (taurine). Since no other constituent of the final sample could contribute sulfur, bile-derived sulfur (in the form of taurine) demonstrates sequestration of bile by clinoptilolite. There is no sulfur in the carbohydrate and lipids, while the whey protein isolate used in our design, the only other potential contributor of sulfur, contains less than 2.5% sulfur amino acids (according to the manufacturer). Finally, according to the manufacturer the ox-bile used in our experiments contains up to 22% sulfur in the form of $SO_4$. Hence, the sulfur detected in the bile-clinoptilolite complex can be safely attributed to the bile whereas any other potential contribution of sulfur can be deemed negligible. Using the sulfur (EDX-detected) as a surrogate (since in practice the source of sulfur in our sample originates from bile's taurine) our semi-quantitative assessment of clinoptilolite's efficiency in sequestering bile calculated a maximum 75.4% retention efficiency. The clinoptilolite used, although presenting adequate CEC, herein is in its natural unprocessed and unmodified form, hence there is still margin for further improvement of its sequestration capacity should it be enriched. In our study, the clinoptilolite added was at 4% of the total weight of the diet mixture, a level indicated to be well tolerated in long-term animal dietary studies.[27] There is possibility for further dose reduction should the clinoptilolite be modified, as indicated by Linares' previous work reporting adsorption of bile acids by modified cancrinite, although it should be noted that no dietary ingredients were included in the reaction mixture in their experimental setting.[7] However, even this degree of efficiency may prove more than adequate at the physiological state. Sequestration refers to the bile used, which was in the powdered dried form. According to the manufacturer 1 g of dried bile is equivalent to 8 g of fresh bile. Hence the 5 g of dried bile used (added in the flask) correspond to 40 g of fresh bile. In this context, 10 g of clinoptilolite sequestered the equivalent of 40 g of fresh bile (four times clinoptilolite's weight/amount).

Our TG-DTA data indicate a stable clinoptilolite-bile complex under physiological conditions and reveal a very low amount of $H_2O$ detected in the clinoptilolite. Hence it is highly unlikely that any water-soluble dietary components are sequestered (such as carbohydrate and protein).

The possibility cannot be excluded that complex molecules such as carbohydrate and fiber if present in the diet mixture may interact with clinoptilolite. However, the

neutral charge of such molecules would prohibit interactions of electrostatic nature and thus adsorption to the clinoptilolite surface, while their large size would prohibit absorption into the small pores or channels of clinoptilolite.
As indicated in the literature, a temperature of 50 °C is needed to separate oligosaccharides in zeolite fixed bed columns utilizing synthetic zeolites of larger pore size (7-10 Å).[33] Thus, clinoptilolite with smaller pore size (3-7 Å) under physiological temperatures (37 °C) is not expected to exhibit significant interactions if at all with dietary polysaccharides or fiber.
In addition, digestible polysaccharides are processed rather quickly in the GI tract, with their digestion starting while in the oral cavity, minimal digestion and rapid transit in the stomach, and final digestion to monosaccharides taking place in the small intestine. Therefore, the concentration of polysaccharides is progressively diminished in the small intestine, and time-wise there is only a small window of opportunity for digestible polysaccharides to interact with clinoptilolite in the GI tract.
Regarding the mechanism of bile adsorption by clinoptilolite our evidence suggest that bile retention is achieved at the surface level of clinoptilolite, based on our SEM images, but also on the fact that the channel size of clinoptilolite (typically 3 – 7 Å), is much smaller that the molecules comprising bile (molecular weight of bile salts is at the order of 400) or the diet ingredients. In addition, sorption of bile from clinoptilolite cannot occur via cation exchange because at neutral pH bile salts occur as conjugated bases ($R\text{-}COO^-$), a form that prevents cation exchange. This study and the previous work discussed herein suggest that a potential mechanism of bile sequestration by clinoptilolite involves the hydrophilic character of clinoptilolite, which facilitates interactions of electrostatic nature between bases. These interactions result in the coating of clinoptilolite's surface with biliary micelles, leading to the sequestration of the adsorbed bile by clinoptilolite in the form of micelles.
Furthermore, the results from the molecular docking simulation testing demonstrate that the adsorption of bile to clinoptilolite is thermodynamically favored, thus lending further theoretical support to the notion of bile sequestration by clinoptilolite (Fig. 8).
Potential taurine deconjugation by colonic bacteria under physiological conditions is not expected to influence the stability of bile sequestration by the clinoptilolite, since bile micelles dock to clinoptilolite via the bile acid side as indicated by molecular docking simulations in our experiments.
Bile sequestered by clinoptilolite in the small intestine is expected to pass from the large intestine in relatively short time, reducing the potential of taurine deconjugation by colon microbiota as clinoptilolite increases bulk and thus decreases transit time in the large intestine.

## Conclusions

This work aimed to study the sequestration of bile from clinoptilolite in a GI tract-simulated environment (digestion/absorption) with the physiological health implications of blood cholesterol reduction. With the use of a variety of techniques we demonstrate and verify in multiple ways that clinoptilolite, a natural highly crystalline mineral complex, adsorbs on its surface bile in the form of macromicelles. The structure and the sequestration ability of clinoptilolite were not affected by pH, the diet mixture, or the bile, suggesting that the mineral can be utilized in such biological system. The efficiency of untreated, not enriched and unmodified natural clinoptilolite for the sequestration of bile reaches 75.4% under simulated condition of

digestion/absorption of a high-fat meal. Further improvement is possible if clinoptilolite undergoes modification, while other zeolites with higher CEC can also be tested.
The present *in vitro* study suggests the potential of unprocessed, unmodified natural clinoptilolite to sequester bile, while further animal feeding studies are necessary to determine the specific interactions between clinoptilolite and bile acids under conditions of physiological digestion with varying diet compositions and metabolic conditions related to cholesterol and bile acid metabolism. In this context such work illustrates potential biomedical applications of earth materials such as zeolites.

**Conflict of interest**
The authors have no conflict of interest to declare. The protein mix was chosen solely for convenience with no intention of advertisement.

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**Table 1.** Composition of diet mixture resembling a high-fat meal.

| Meal ingredients | Weight (g) | Fat (g) | Carbohydrate (g) | Protein (g) |
|---|---|---|---|---|
| Olive oil[1] | 25 | 25 | 0 | 0 |
| Table sugar[1] | 40 | 0 | 40 | 0 |
| Whey protein isolate[2] | 30 | 0 | 4 | 24 |
| Distilled Water | 145 | 0 | 0 | 0 |
| Total weight (g) | 240 | 25 | 44 | 24 |
| Total energy (kcal) | 497 | 225 | 176 | 96 |
| %Energy[3] | - | **45.3** | **35.4** | **19.3** |

[1] commercially available brand; [2] commercially available brand (Six Star Pro Nutrition); [3] 1g fat = 9kcal, 1g carbohydrate or protein = 4kcal (Atwater system).

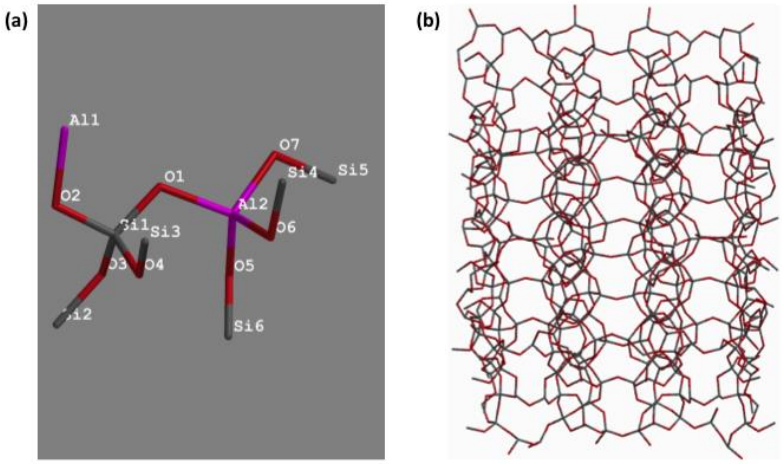



Figure 1. Optimized structure of clinoptilolite: (a) basic unit, (b) crystal framework.

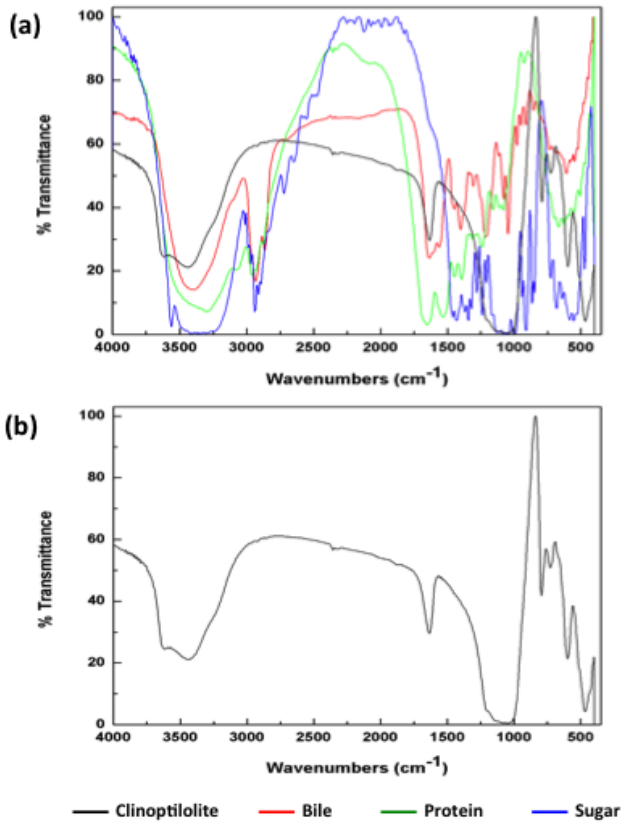



Figure 2. Superimposed FT-IR spectra of: (a) clinoptilolite, bile, protein and sugar; (b) clinoptilolite.

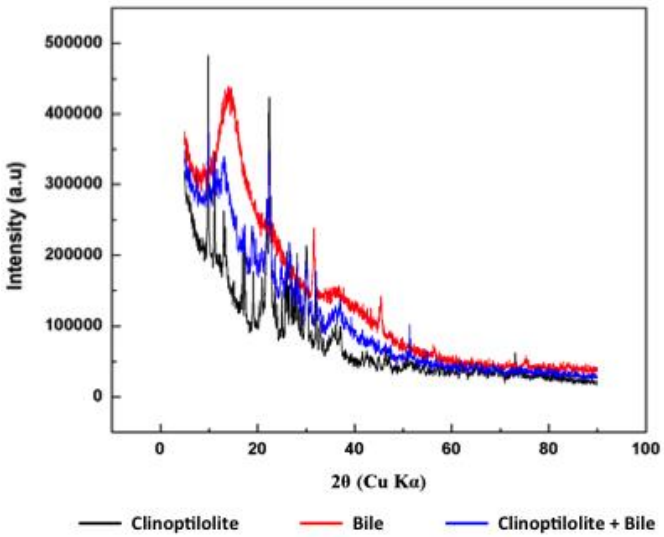



Figure 3. XRD pattern of: clinoptilolite, bile and clinoptilolite+bile.

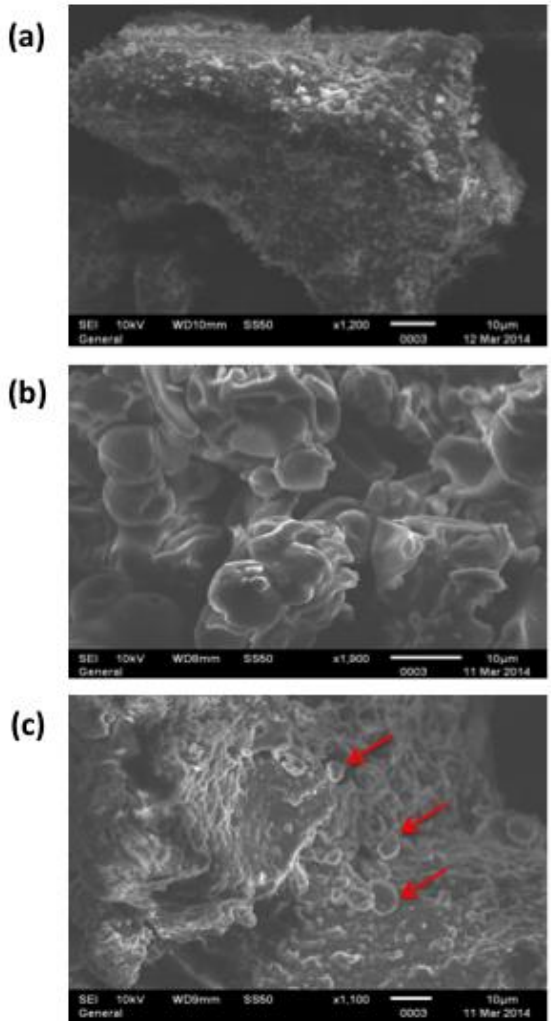



Figure 4. SEM images of: (a) clinoptilolite, (b) bile and (c) clinoptilolite+bile. Arrows (in red) indicate bile micelles.

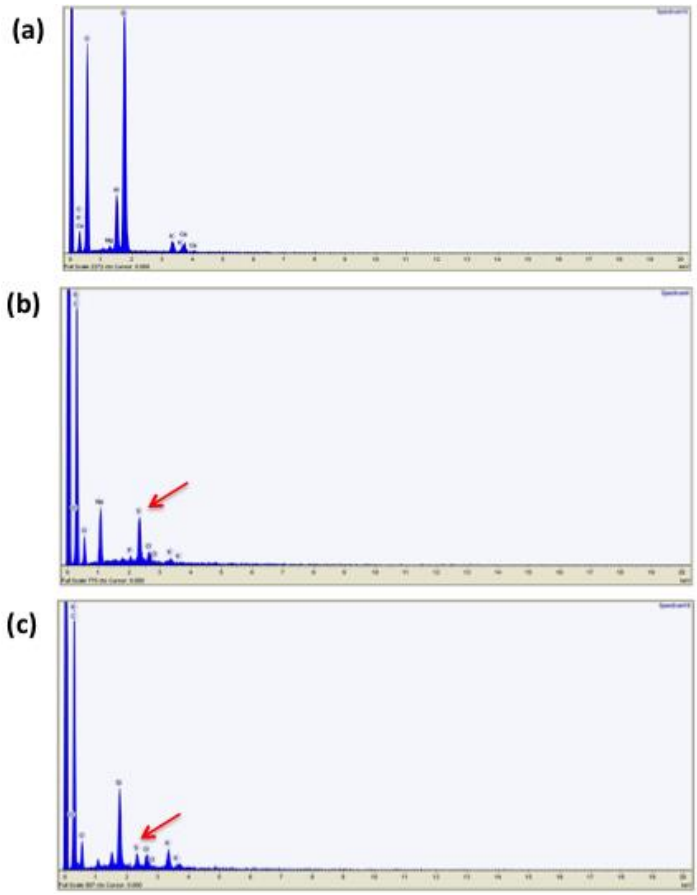



Figure 5. EDX elemental analysis of: (a) clinoptilolite, (b) bile and (c) clinoptilolite+bile. Arrows (in red) indicate characteristic sulfur peaks.

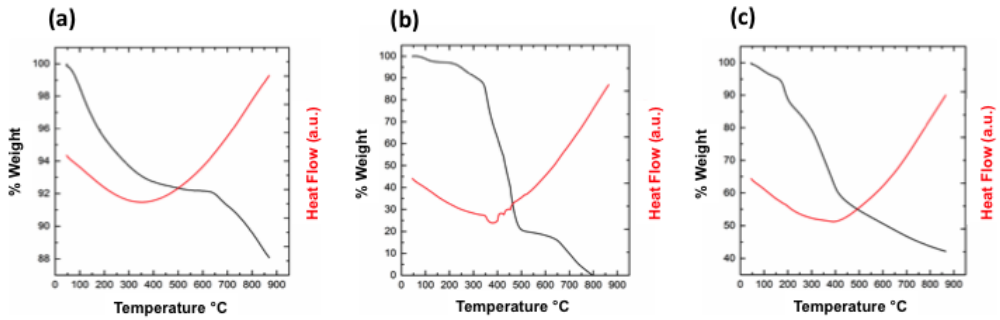



Figure 6. TG-DTA graph of: (a) clinoptilolite, (b) bile and (c) clinoptilolite+bile.

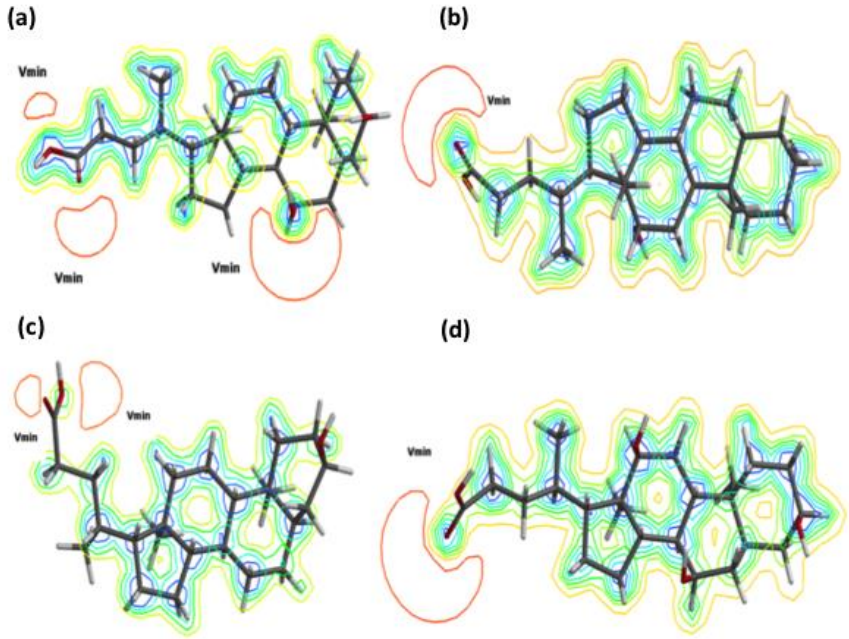



Figure 7. Calculated electrostatic potential for the optimized structure of: (a) chenodeoxycholic acid, (b) deoxycholic acid, (c) lithocholic acid and (d) cholic acid.

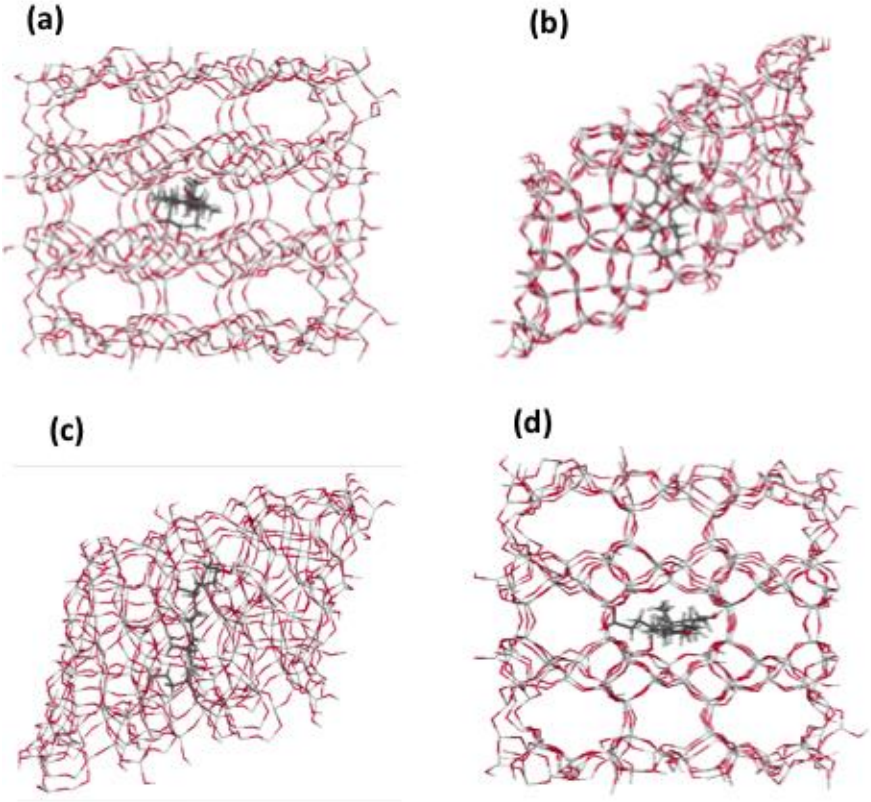
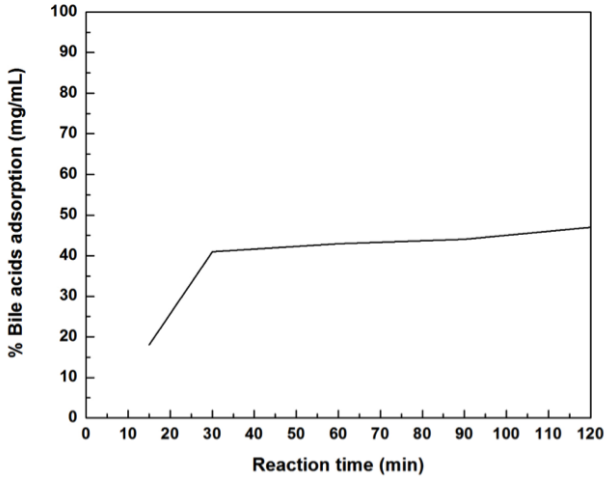



Figure 8. Molecular interactions of clinoptilolite with: (a) chenodeoxycholic acid, (b) deoxycholic acid, (c) lithocholic acid and (d) cholic acid.




Supplementary Figure: Graphical representation of the bile adsorption reaction kinetics.