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^{1}H and ^{13}C Magic-Angle Spinning Nuclear Magnetic Resonance Studies of the Chicken Eggshell

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ABSTRACT: The chicken eggshell, a product of biomineralization, contains inorganic and organic substances whose content changes during the incubation process. Bloch-decay (BD) ^{1}H, ^{13}C, and cross-polarization (CP) ^{13}C nuclear magnetic resonance (NMR) spectra of chicken eggshells were acquired under magic-angle spinning (MAS). Variable contact time ^{13}C CP MAS NMR experiments revealed the signals of carbonyl groups from organic and inorganic compounds. In the ^{13}C BD NMR spectra, a single peak at 168.1 ppm was detected, whereas in the ^{1}H BD spectra, the signals from water and the bicarbonate ion were assigned. A simultaneous decrease of the water signal in the ^{1}H MAS NMR spectra and an increase of the carbonate ion signal in the ^{13}C CP MAS NMR spectra of eggshells collected during the incubation period indicate the substitution of calcium ions by hydrogen ions in the calcium carbonate crystalline phase during the incubation of an egg.

KEYWORDS: *Solid-state NMR, ^{13}C NMR, cross-polarization, eggshell, calcite*

1. INTRODUCTION

The application of solid-state nuclear magnetic resonance (NMR) is widely used in food[1,2] and agricultural[3] science. This technique was shown also to be an excellent method for analyzing insoluble or multicomponent biological systems, such as plant cell walls,[4] mycelium,[5] and hard tissues, such as bones and cartilage.[6−9] An interesting object from that group of materials is the chicken eggshell. Bone tissue is mainly composed of phosphates, whereas the avian eggshell is mostly made up of carbonates. The chicken eggshell is composed of a foamy layer of cuticle, a calcite or calcium carbonate layer, and two shell membranes.[10] The avian eggshell is sometimes referred to as a "natural composite bioceramic" containing organic (3.5%) and inorganic (95%) phases. It is composed of 1.0% (w/w) matrix proteins in addition to calcium carbonate (95%, w/w).[11] The fatty acid content in the insoluble eggshell layers (after decalcification) is in the range of 2−4%.[12]

The eggshell is a product of the physiological process called biomineralization, which takes place on the egg membrane in an acellular medium. The uterine fluid contains inorganic minerals and precursors of the organic matrix.[13] The structure of the eggshell results from the deposition of calcium carbonate on the membranes, concomitantly with an organic matrix.[14] The matrix proteins control the process of calcite crystal growth,[15] thereby affecting the texture and biomechanical properties of the eggshell.[16] In the case of chicken eggshells, approximately 5 g of $CaCO_3$ is deposited within 22 h in an acellular medium as the egg passes through the oviduct. This makes avian eggshells one of the fastest mineralized hard tissues in biological systems.[17]

However, eggshells from the fertilized eggs change their composition during the process of egg incubation, which typically lasts 21 days.[18] During the incubation period, chemical processes occur and some elements are absorbed by the growing chicken embryo.[19] Although these changes have not been fully recognized, it is suggested that one of the products is calcium bicarbonate $[Ca(HCO_3)_2]$,[20] which is chemically unstable and does not exist in solid state.[21]

According to previous reports,[22] the chicken eggshell is a good source of calcium (contains 95% $CaCO_3$) and strontium, more easily dissolved with gastric juice because of its porous structure. This is the reason why it can be used as a dietary supplement in the prevention of osteoporosis. The diet fortified with eggshell powder containing only 0.10% phosphorus would help restore the proper dietary balance between calcium and phosphorus.[23]

The changes in its composition and processes that take place during the incubation were followed using solid-state NMR spectroscopy. To the best of our knowledge, this is the first application of a magic-angle spinning (MAS) NMR technique to the study of the chicken eggshell.

The aim of this study was to analyze the chicken eggshell during the process of incubation. Understanding this subject is not only interesting from the scientific point of view but also important because of the increasing role of the *in ovo* injections in the poultry immunoprophylaxis, in which efficiency also depends upon the strength and structure of the eggshell[24] and possibilities of application of an eggshell as a dietary calcium supplement.

2. MATERIALS AND METHODS

Samples of chicken (*Gallus gallus domesticus*) eggshells were collected on the 1st, 14th, 18th, and 21st days of incubation at a farm localized in Mazovian Province, Poland. The samples were washed with water; the membranes were removed; and the eggshells were dried in air and powdered.

Solid-state MAS NMR spectra were recorded at 298 K on a Bruker Avance DSX 400 WB spectrometer in the magnetic field of 9.4 T at 400.13 MHz (^{1}H) and 100.62 MHz (^{13}C). The samples were spun at 10 kHz in a 4.0 mm zirconia rotor and at 35 kHz in a 2.5 mm zirconia rotor for ^{13}C and ^{1}H MAS NMR experiments, respectively.

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Standard ^{13}C cross-polarization (CP) MAS spectra were obtained using a ^{1}H 90° pulse length of 2.0 μs, with continuous wave (CW) proton decoupling, a contact time of 2−20 ms, and a repetition time of 10.0 s. Chemical shifts were calibrated indirectly through the glycine CO signal recorded at 176.0 ppm (^{13}C spectra) and the adamantane signal at 1.78 ppm (^{1}H spectra), relative to tetramethylsilane (TMS). The conventional $^{1}H-^{13}C$ CP pulse sequence with reversal of the spin temperature in the rotating frame was applied with high-power proton decoupling during signal acquisition. The variable-contact time CP experiments were performed using contact times ranging from 0.05 to 20.0 ms. For the direct polarization [DP, Bloch decay (BD)] experiments, the single pulse (a 90° pulse of 3.4 μs) sequence with high-power decoupling and a repetition time of 300 s was employed. Deconvolution of the NMR spectra was performed with a MestReNova program (MestreLab, Inc.). CP kinetics for selected signals was analyzed using the non-classical (I−I*−S) model.[25,26]

The CP kinetics describe the dependence of the intensity of the signal in the spectra from the contact time [t (ms)]. With a short contact time, the signal in the spectra increases because of the polarization transfer from hydrogen atoms. With the elongation of the contact time, the intensity of the signal is decreasing because of the relaxation process. Within the non-classical I−I*−S model, CP kinetics is described by the following equation:

$$I(t) = I_0 \exp(-t/T_{1\rho}^{I})[1 - \lambda \exp(-t/T_{df}) - (1 - \lambda)\exp(-1.5t/T_{df})\exp(-0.5t^2/T_2{}^2)]$$

Analysis of the CP kinetic parameters provides information about the molecular environment. T_{df} is the time constant describing proton spin diffusion from bulk protons to protons of the spin cluster, in which CP begins. The parameter $T_{1\rho}^{I}$ is the relaxation time in the rotating frame and describes the magnetization decay under the spin-lock condition. Both of these parameters depend upon the number of hydrogen atoms in a functional group and their mobility and decrease with lower mobility or higher content of hydrogen in the spin cluster. Parameter I_o is an absolute amplitude of a signal, and T_2 is the time constant that depends upon the dipolar interaction between the carbon atom and neighboring hydrogen atoms. Parameter λ describes how the energy is shared between the spins in the spin cluster and is approximate to the value $1/(n + 1)$, where n is the number of hydrogen atoms in the spin cluster.[25]

3. RESULTS AND DISCUSSION

A series of NMR spectra was recorded for powdered eggshell samples collected before incubation as well as during the incubation process. In the standard ^{13}C CP MAS NMR spectra of the eggshell, numerous signals can be observed in the region of 10−60 ppm, which were assigned to organic compounds, mainly proteins and lipids (Figure.1). Two signals were observed in the carbonyl region, at 168.1 and 172.3 ppm. In the spectra

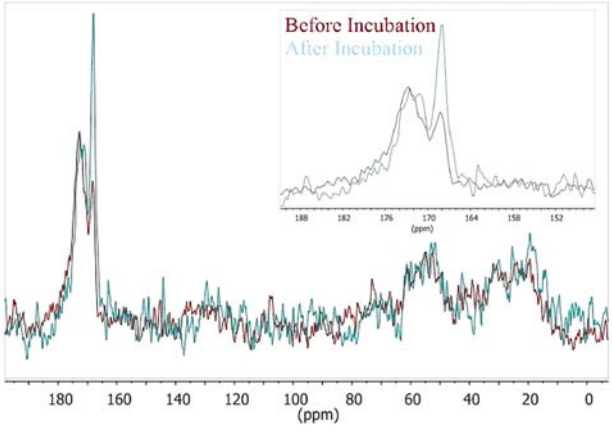

Figure 1. Comparison of the ^{13}C CP MAS NMR spectra of chicken eggshell before incubation (red) and after incubation (blue). Spectra were acquired with the contact time of 10 ms and 2000 scans (expanded carbonyl region).

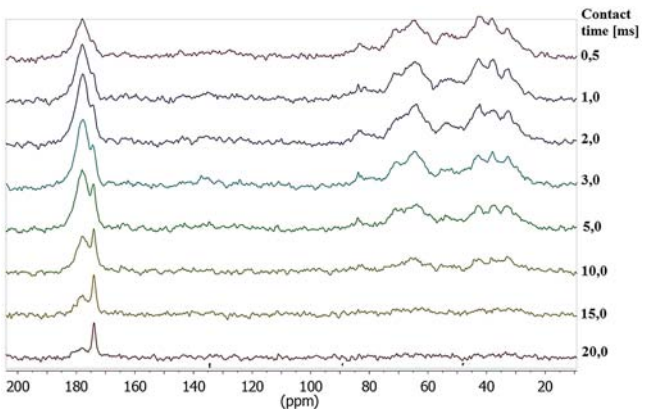

Figure 2. ^{13}C CP MAS NMR spectra of chicken eggshell (before incubation) recorded with different contact times (0.5−20 ms) and 2000 scans.

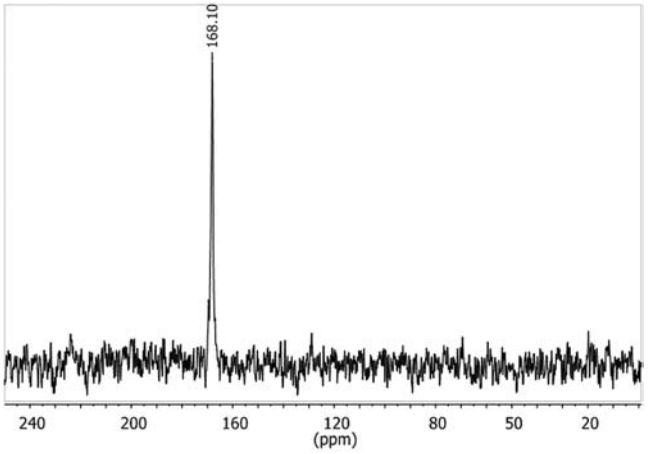

Figure 3. ^{13}C BD MAS NMR spectrum of chicken eggshell (before incubation).

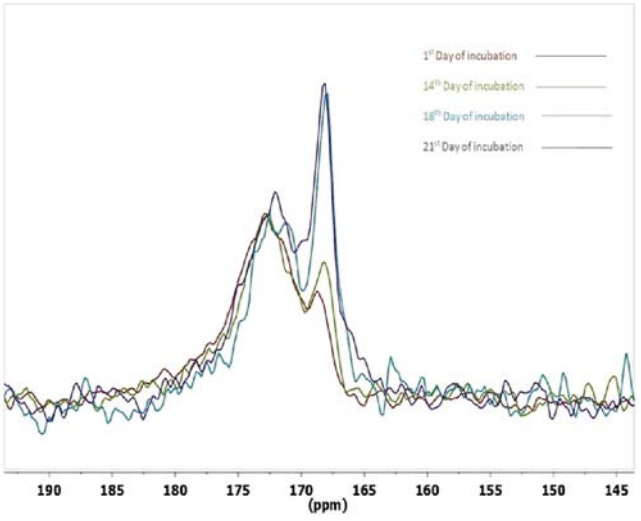

Figure 4. Superimposed ^{13}C CP MAS NMR spectra of chicken eggshells collected during the incubation process (expanded carbonyl carbon region).

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Table 1. Parameters of the $^{1}H-^{13}C$ CP Kinetics for Carbonyl Groups of Chicken Eggshell, Calculated Using the Non-classical Kinetic Model

eggshell sample		before incubation	after incubation	before incubation	after incubation
chemical shift		168.1 ppm	168.1 ppm	172.3 ppm	172.3 ppm
non-classical (I–I*–S)	T_2	0.014	0.014	0.014	0.014
	$T^{I}_{1\rho}$	436.1	163.6	9.16	8.59
	I_0	134	102	332	263
	T_{df}	15.49	3.52	1.12	1.33
	λ	0.89	0.91	0.9	0.9

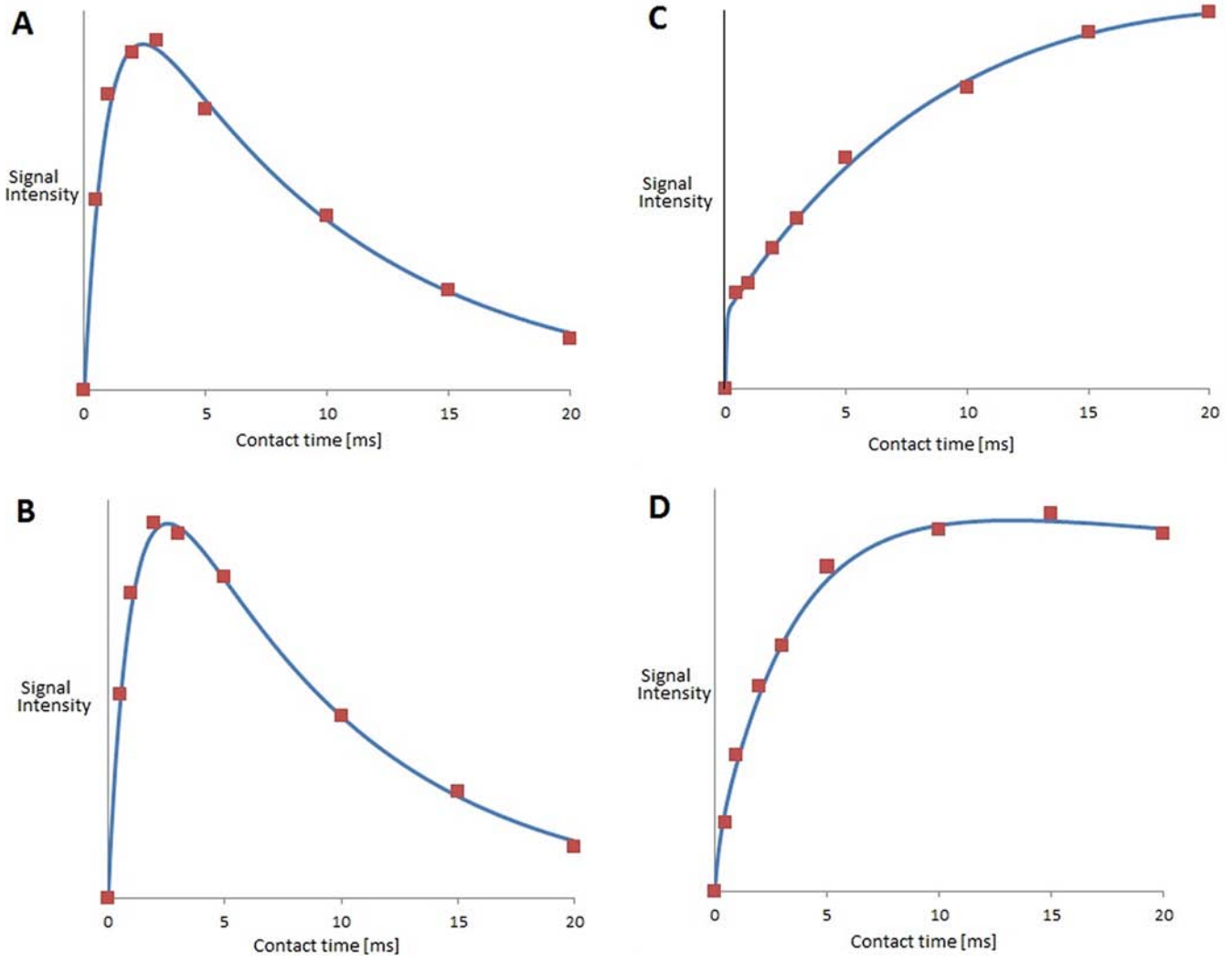

Figure 5. Fitting of the I–I*–S non-classical CP kinetic model to the experimental data. Panels A and B present the kinetics of the carbonyl groups signal at 172.3 ppm (A) before and (B) after incubation. Panels C and D present the kinetics of the carbonate ions signal at 168.1 ppm (C) before and (D) after incubation.

recorded with the contact time increasing from 0.5 to 5 ms, the dominating signal was at 172.3 ppm. However, with the extension of the contact time, a considerable rise of the intensity of the carbonyl signal at 168.1 ppm was observed (Figure 2). One of those signals supposedly came from the calcite carbonate group, and the other came from carbonyl groups of proteins.

To differentiate those signals, the direct polarization (BD) ^{13}C spectra were recorded, which showed only one signal at 168.1 ppm (Figure 3). This signal was assigned to the inorganic carbonate ion from $CaCO_3$ because the chicken eggshell is mainly (95%) composed of calcite. Moreover, the observed chemical shift for the inorganic carbonate ion is very similar to literature values reported for the calcite polymorph of $CaCO_3$.[27] Another characteristic feature is an extremely long T_{1C} time. The experiments with repetition time showed that the intensity increased until the time reached as long as 250 s. Therefore, a repetition time of 300 s was necessary to avoid the saturation effect. The signal at 172.3 ppm was assigned to the carbonyls of the peptide bonds.

For the eggshell samples from the 1st, 14th, 18th, and 21st days of incubation, the ^{13}C CP MAS spectra were recorded with the 10 ms contact time, 10 s repetition time, and 2000 scans. In those spectra, both carbonyl signals could be observed. The increase of the intensity of the signal from the carbonate

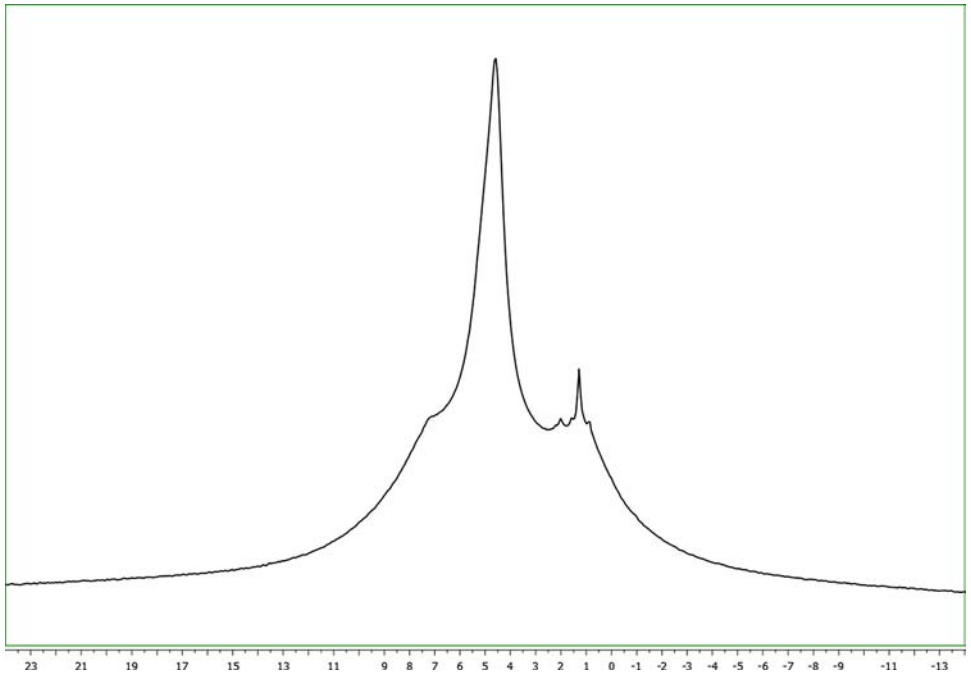

Figure 6. ^{1}H BD NMR spectrum of chicken eggshell after incubation.

ion (168.1 ppm) occurs during the incubation process, and no major changes for signals from organic compounds were observed (Figures 1 and 4).

In the ^{13}C CP NMR spectra of pure calcium carbonate in the form of calcite recorded by Feng et al.,[27] the authors did not observe any signal from the carbonate ion. The signals observed in the ^{13}C MAS NMR spectra of chicken eggshell suggest that not only the calcite form of calcium carbonate is present in the studied material but also hydrogen atoms from other compounds in the chicken eggshell can serve as donors of magnetization for the carbon nuclei in the carbonate ions.

As two signals in the carbonyl region of CP MAS spectra were partially overlapped, a deconvolution of the spectra had to be performed. This allowed for the separation of the signal arising from the inorganic carbonate ion and one from the organic carbonyl group. The calculation of the respective signal areas showed that, during the process of incubation, the intensity ratio of the inorganic carbonate/organic carbonyl increased about 2 times. This might be the result of an increase in the amount of carbonate ions in the eggshell. However, it seems very unlikely because of the very high (95%) concentration of calcium carbonate before incubation. Another possibility is that those signals undergo distinctly different CP. Therefore, the CP kinetics should be studied in detail.

The ^{13}C CP MAS spectra with different contact times were recorded for the samples from the 1st and 21st days of incubation to estimate the CP kinetics for both carbonyl signals. The ^{1}H $\rightarrow$ ^{13}C CP kinetics was fitted to the non-classical I−I*−S model. The CP kinetics parameters are collected in Table 1, and the experimental data fitting are shown in Figure 5. Panels A and B of Figure 5 present the kinetics of the carbonyl groups signal at 172.3 ppm (A) before and (B) after incubation. Panels C and D of Figure 5 present the kinetics of the carbonate ions signal at 168.1 ppm (C) before and (D) after incubation.

There is no significant difference in the kinetic parameters for the organic carbonyl groups before and after incubation, suggesting that the protein fraction does not undergo any changes during the incubation process.

Significantly shorter $T_{1\rho}^{H}$ relaxation times (ca. 9 ms) were obtained for the organic carbonyl group (panels A and B of Figure 5) than for the carbonate ions (160−890 ms; panels C and D of Figure 5), probably because of the larger number of hydrogen atoms located near the protein carbonyl groups.

Inspection of the carbonate ion CP kinetics, measured for the samples after incubation, indicates that the values of $T_{1\rho}^{H}$ and T_{df} decreased, suggesting the increase of the amount of hydrogen atoms in the inorganic matrix.

The λ parameter depends upon the heteronuclear dipolar couplings, and any weakening of the ^{1}H−^{13}C dipolar couplings, caused by either molecular dynamics or a decreased number of hydrogens in the vicinity of a given carbon, should increase the λ parameter.

Looking at the chemical composition of the eggshell, one can find two possible donors of magnetization for the carbonate ions: water molecules and hydrogen ions. To confirm this assumption and to find the sources of magnetization, the ^{1}H MAS NMR spectra were recorded. In the ^{1}H MAS NMR spectrum, a strong signal of water appears at 4.8 ppm (Figure 6). The intensity of this signal decreases rapidly in the spectra recorded during the period of incubation; the largest difference occurs between the 1st and 14th days (Figure 7). This may result from the evaporation of weakly bound water molecules incorporated into the eggshell during its formation in the uterine fluid. However, the decrease of the water concentration in the chicken eggshell does not explain the increase of the carbonate ion signal in the ^{13}C CP MAS spectra. If the water molecules were really donors of magnetization for the inorganic carbonate group, the intensity of the carbonate signal should be the highest in the spectra of the eggshell from the 1st day of incubation. However, our results were completely opposite; the highest intensity of the carbonate signal was after incubation when the amount of water in the eggshell was the lowest, which neglects the assumption of water molecules being the donors of magnetization for carbonate groups.

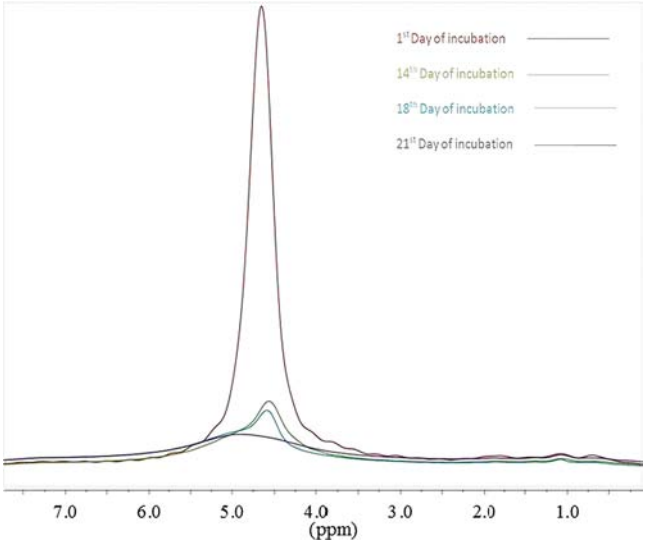

Figure 7. Superimposed ^{1}H BD NMR spectra of chicken eggshells collected during the incubation period.

Another possibility is that the hydrogen ions that are formed during the process of incubation as a result of the physiological process of chicken embryo development can be incorporated into the carbonate group. It is worth mentioning here that no signal appears around 14 ppm, characteristic for the hydrogen ions present in acid salts, such as $KHCO_3$ and $NaHCO_3$, which, in opposition to $Ca(HCO_3)_2$, are stable in solid state. Instead, the signal at 7.2 ppm can be found, which has the similar chemical shift to the signal of calcium bicarbonate in the spectra recorded by Nebel et al.[28] For this reason, we have assumed that the increase of the intensity of the carbonate signal was the result of substitution of the Ca^{2+} ion by the H^+ ion during the egg incubation, according to the reaction $2H^+ + 2CaCO_3 \rightarrow Ca(HCO_3)_2 + Ca^{2+}$. This confirms the statement first expressed in 1974[19] of possible calcium liberation in the eggshell during the process of incubation. As a result, the calcium ion is liberated. NMR data, such as CP parameters, confirm the hypothesis of the acid–base mechanism. The Ca^{2+} ions needed by the growing chicken embryo are gained from calcium carbonate, $CaCO_3$.

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Notes

The authors declare no competing financial interest.

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