LR $^1$H NMR measurements for determination of internal egg quality

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Summary
Low resolution $^1$H Nuclear Magnetic Resonance (LR $^1$H NMR) spectroscopy was evaluated as an alternative, fast, non invasive and non destructive method for the determination of internal quality of eggs from caged hens of three different breeds in the absence and presence of inoculated microorganisms. For comparison, regular noninoculated eggs were subjected to traditional Haugh units measurement and HPLC analyses of components of the egg white as well as the vitelline membrane. Microbially induced NMR responses were correlated with bacterial numbers by using routine microbiological methods. Eggs were collected at defined hen ages during the laying cycle and investigated under varying storage conditions. Correlations between relaxation time T2(2) and Haugh units were very high (> 0.92) for the various applied storage regimes. There were no significant correlations between T2(2) values and changes in the content of VMOI, VMOII, lysozyme and conalbumin. Using a statistical model it could be shown that there is a dependency of the T2(2) relaxation time upon storage time as well as the laying age of the hens. In contrast, T2(1) depends only upon laying age of the hens and does not change significantly during storage. Characteristic changes in the NMR relaxation times indicated bacterial growth to levels > $10^7 - 10^8$ c.f.u./g of egg content. These changes frequently correlated with optical-olfactorial deviations of the egg contents. The resistance of the eggs against microbial growth decreased with increasing storage time before inoculation. Eggs from younger hens showed a higher resistance than the eggs from older hens. The percentage of 'NMR-positive eggs' increased with decreasing Haugh units and T2(2) relaxation times.

Introduction
Currently, there exist three major physical and chemical methods for the determination of egg quality: (i) The height of the air space is used as a measure for the freshness of eggs as it is increasing during storage caused by diffusion of carbon dioxide and evaporation of water via the egg shell. However, these values are not very reliable as the initial height is varying from egg to egg in dependency upon the laying period of the hens and the prevailing environmental conditions. (ii) The pH of the egg white is another parameter changing in eggs after laying. It is rising from values of about 7.5 to 9.6 during storage in dependency upon time and temperature. Its increase is correlated to liquefaction of the egg white and the decrease of viscosity (Heath, 1977). However, these values vary considerably. (iii) During storage the viscosity of the egg white and the stability of the vitelline membrane are decreasing. These characteristics are applied in different procedures for the determination of the age of eggs, e.g. albumen index (Heiman and Carver, 1936) and Haugh unit (Haugh, 1937). There is also a variety of chemical changes in egg components which can be used as indicators for egg quality. For example, aggregated ovomucin is dissociating into α- and β-ovomucin in the course of storage (Miller et al., 1982) and ovalbumin is transformed into S-ovalbumin (Smith and Back, 1962). All these methods require the destruction of eggs.

An alternative fast, non-invasive and non-destructive method for the determination of internal egg quality could be low resolution $^1$H Nuclear Magnetic Resonance spectroscopy (LR $^1$H NMR). This study was performed to evaluate the potential of LR $^1$H NMR for this purpose. Changes in the egg caused by physicochemical mechanisms leading to alterations in the inner egg quality were investigated by means of LR $^1$H NMR and compared to results obtained by Haugh units (HU) measurement and HPLC analysis. Possible interferences with NMR measurements due to the presence of microorganisms were evaluated with inoculated eggs. In addition, it is demonstrated that
LR $^1$H NMR may be used as a rapid and convenient tool for studying the impact of egg quality parameters on microbial growth within the egg under defined conditions of storage.

**Material and methods**

Hens of *Lohmann Brown* (breed 1), *Lohmann Selectic* (breed 2) and *ISA Warren* (breed 3) were reared under defined conditions and feeding program in conventional cage systems.

Storage of eggs was performed in dependency upon breed, hen age and storage conditions (temperature, humidity, atmosphere, lighting). For each breed eggs were collected at four to five different hen ages. Eggs were delivered at the day of laying, individually numbered and subsequently subjected to the various storage conditions.

Applying the low resolution $^1$H spectrometer mq 10 NMR Analyzer (Bruker, Karlsruhe, Germany) the transversal (spin-spin) relaxation times $T_2(1)$ and $T_2(2)$ of intact eggs were determined at an excitation frequency of 7.5 MHz and a scanning time of 20 sec consisting of four scans. The method implies the insertion of individual eggs into a homogeneous magnetic field using appropriate teflon cuvettes. The hydrogen nuclei in the egg are excited by radio frequency pulses. The resulting relaxation times $T_2(1)$ and $T_2(2)$ reflect the times required for the excited system to return into its relaxed state.

Haugh units (HU) were determined by a Haugh unit meter (TSS-Technical Services and Supplies, York, England). The instrument allows the determination of the egg weight and after breaking the egg the height of the thick egg albumen. Haugh units are automatically calculated.

The egg white proteins lysozyme and conalbumin as well as the vitelline membrane outer proteins VMOI and VMOII were separated by means of HPLC (Beckman System Gold, Munich, Germany) on a C 18 column (Poroshell 300SB-C18 column, Agilent Technologies, Karlsruhe, Germany) and their content estimated via the peak areas.

For microbiological studies eggs were submitted to storage in the dark at 20°C and 60% r.h. for 1, 8, 15 and 22 days before inoculating with a pool of bacterial strains. After inoculation storage was continued under the previously selected conditions for up to 9 days. The inoculation pool consisted of *Serratia marcescens* BAFF-SM19, various *Salmonella* strains relevant to eggs (6-strain pool of *S. Enteritidis*, one strain each of *S. Senftenberg* and *S. Typhimurium*), *Pseudomonas aeruginosa* BAFF-P15 and *Staphylococcus aureus* DSM1104. A suspension of washed bacterial cells in saline (0.1 ml) was injected into the albumen close to the shell at the sharp egg pole. Inoculation densities were 10, 1000, 100 and 100 cfu per egg for *Serratia*, *Salmonella*, *Pseudomonas* and *Staphylococcus*. At least ten eggs were inoculated per storage experiment.

For determination of bacterial growth in eggs the LR $^1$H NMR spectrometer was applied as outlined above. During egg storage, NMR measurements were performed before and after inoculation at regular intervals. Microbial growth in eggs was recorded as the ratio of eggs giving a ‘positive’ NMR response, i.e. a significant deviation of the relaxation times in comparison to non inoculated eggs. Eggs were judged ‘NMR-positive’ if their relaxation times deviated significantly from the negative controls by day 9 after inoculation. At this time positive eggs were broken and subjected to visual-olfactorial evaluation and bacterial count determinations.

**Results and discussion**

LR $^1$H NMR RESPONSES OF NON-INOCULATED EGGS

Determined Haugh units are dependent upon applied storage conditions like temperature, atmosphere and duration. The inner egg quality of egg collectives of the three breeds collected at defined times during the laying cycle are not only influenced by storage time but also by the laying age of the hens.

Changes in the egg, e.g. liquefaction and increase of the pH value are influencing $T_2(2)$ as well as the resulting Haugh units. Correlation coefficients between the relaxation times $T_2(2)$ and the median Haugh units resulting from storage experiments performed under comparable conditions lead to similar values for egg collectives from the three breeds tested (R > 0.92 for all storage experiments at 20°C, 60 % r.h under air).

The content of lysozyme in the egg white as well as in the vitelline membrane is not significantly changing during storage up to 36 days at elevated temperatures of 20 °C and 60 % r.h. for egg collectives of the three breeds. In the case of VMOII there exists in all cases a significant decrease of the content especially during the first two days after laying.
For various egg collectives from hens of the three breeds stored at 20 °C, 60 % r.h. under air in darkness the results of a statistical model applying multiple regression show a correlation between T2(1) and the laying age of the hens, whereas there is no significant change of T2(1) in dependency upon the storage time (Figure 1). Regarding T2(2) values in dependency upon storage time (Figure 1) there can clearly be shown a decline of the relaxation times of the various egg collectives collected at defined times during the laying cycle in the course of storage at 20 °C, 60 % r.h. under air and darkness for the selected three hen breeds. An increase of the T2(2) values of the eggs in dependency upon the age of the hens during the laying cycle of the three breeds can also be observed, which appears to be rather similar expressed.

**LR ^1^H NMR RESPONSES OF INOCULATED EGGS**

The thresholds for detecting microbial growth by LR ^1^H NMR spectroscopy were > 10^7 - 10^8 cfu/g of egg content, i.e. only microbially induced physicochemical changes are detected by LR ^1^H NMR, not the bacteria themselves. These changes in the albumen and yolk lead to dramatic deviations of the NMR relaxation times T2(1) and T2(2) in comparison to regular eggs and frequently correlated with optical-olfactorial defects of the egg contents. *Serratia* caused a fruity odour and red discolorations on the yolk and eggshell membranes, while a fishy smell was indicative for *Salmonella*. With proceeding spoilage the egg contents progressively denatured. Microbial spoilage at 20 °C was strongly dominated by *Serratia* and *Salmonella*. It was accompanied with a continuous increase of T2(1) by up to 30 msec and an transient increase of T2(2) by up to 150 msec. The extent of the deviations depended on the degree of spoilage. These changes are readily detected during routine measurements of inoculated and non-inoculated control eggs. It must be emphasized that this method can not be employed to detect bacterial growth below the threshold levels mentioned above and for determining whether an egg is safe for consumption or not. Nevertheless LR ^1^H NMR spectroscopy is a useful and time saving tool for monitoring microbial growth in intact shell eggs, making conventional microbiological analyses dispensable in all cases where the contaminating microorganisms are able to modify the egg contents to give an NMR response different from the mere aging response. If this is the case, the response of an individual egg to a microbial challenge can be continuously monitored over a prolonged time period without the need for destroying the egg.

Inoculated microorganisms showed a similar growth response in eggs from all three hen breeds. In general, resistance of fresh eggs, i.e. eggs inoculated at day 1 after laying, was higher than of eggs stored for one ore more weeks before inoculation. But, compared to breed 1 and breed 2, egg age dependent resistance against microbial growth in the albumen was less expressed in eggs from *ISA Warren* hens. For eggs from both *Lohmann Brown* and *Lohmann Selectic* layers, the ratio of NMR-positive eggs increased with egg age from about 10-20 % to 80-90 % at 1 and 22 days of pre-storage. For eggs from *ISA Warren* hens the ratio of NMR-positive eggs increased with egg age from about 50 % to 75 % at 1 and 22 days of pre-storage. The differences in NMR response at 20 °C of eggs from breed 1 and breed 2 from different hen ages seems to be due to a hen age dependent influence. In the middle of the laying cycle eggs were apparently more sensitive to microbial challenges than in the early and late phase of the laying cycle. Eggs from hens in living weeks 26 - 31 were less sensitive than eggs from hens in living weeks 37 – 46 and, at hen ages of 59 – 63 weeks resistance of stored eggs to microbial growth was higher than at living weeks 37 – 46. However, such an effect was not observed for breed 3. Here, eggs from 30 weeks old hens were about as sensitive as those from older hens.

Compared to egg age, influences of breed and hen age appeared to be less important. They may, however, at least partly be responsible for the variance in ‘NMR-positive’ eggs at the different egg ages.

While storage dependent influences on microbial stability may be explained by physicochemical changes in the egg, e.g. changes in albumen viscosity, decrease of vitelline membrane stability, state of ovomucin aggregation, conversion of ovalbumin to S-albumin (Heath, 1977; Miller et al., 1982; Smith and Back 1962; Haugh 1937; Heiman and Carver 1936), an influence of breed reflects genetic differences and, hen age may reflect age dependent changes in gene expression. As mentioned above, there is a tendency that eggs from breed 1 and breed 3 were more sensitive than eggs from breed 2, at least in the first week of storage. However, for a definitive answer additional data are needed.
Figure 1  Relaxation times T2(1) and T2(2) determined at 20 °C, 60 r.h. in dependency upon storage time and hen age in the case of shell eggs of breed 1 – Lohmann Brown (above), breed 2 – Lohmann Selectic (middle) and ISA Warren (below).
Microbiological analyses of the inoculated eggs at the end of the incubation period confirmed the results obtained by LR $^1$H NMR. The percentage of ‘growth-positive’ eggs increased with egg age and, for breed 2 the percentage of positive eggs was highest in the middle of the laying cycle. Microbial analyses compared best to LR $^1$H NMR results when only those eggs were considered as positive where the bacterial counts exceeded $10^6$ cfu/g of egg content. If eggs were considered as ‘growth-positive’ already at bacterial cell densities 100 times above the inoculation level, which is too low for a ‘positive’ NMR response, the percentage of growth-positive fresh eggs was on average already higher than 60%.

Correlations between egg age and percentage of NMR-positive eggs 9 days post inoculation suggest that egg defence, i.e. the antimicrobial activity of the albumen, decreases in the order *Lohmann Selectic* > *Lohmann Brown* > *ISA Warren*. The storage effect as described by the slope of the equation was most pronounced for *Lohmann Selectic* and least for *ISA Warren*. For old eggs (22 days) the differences between the breeds were negligible.

The correlations between internal egg quality, represented by Haugh unit, and microbial growth, represented as percentage of NMR-positive eggs 9 days post inoculation, were very high for *Lohmann Brown* and somewhat lower for the other two breeds. When the data from all three breeds were combined, thus neglecting possible influences of the breed, the coefficient of determination as well as the slope of the equation were very close to 1. As already mentioned above the NMR relaxation time $T_2(2)$ is highly correlated with Haugh unit. Therefore, a similar correlation between relaxation times and microbial stability of eggs can be expected.

**Conclusions**

Low resolution $^1$H NMR spectroscopy is capable of detecting storage dependent changes in eggs. Because of the variation from egg to egg, it cannot be used as an absolute indicator for internal quality of individual eggs. However, the method can be applied for this purpose, if relaxation times of larger egg collectives are taken into consideration.

Correlations between Haugh units and $T_2(2)$ values for all storage experiments at 20°C, 60 % r.h under air show R values higher than 0.92. LR $^1$H NMR allows the detection of microbially spoiled eggs and thus offers a possibility for studying microbial growth in intact eggs without the need for destroying the eggs. At the selected storage conditions microbial growth in shell eggs was primarily affected by egg age. Eggs from *Lohmann Brown* hens respond similar to microbial challenges as eggs from *Lohmann Selectic* hens but may be somewhat more sensitive for microbial growth. For both breeds there is evidence that hen age has an impact on microbiological stability of the egg with microbial stability being reduced in the middle of the laying cycle. In comparison, eggs from *ISA Warren* hens seem to be the most sensitive. Results from LR $^1$H NMR, based on microbially induced physicochemical changes of the egg contents, are comparable to those from standard microbiological analysis making LR $^1$H NMR an innovative and rapid tool for studying microbial growth in intact shell eggs. There is a tight correlation between microbial stability (‘egg defence’), Haugh unit and LR $^1$H NMR relaxation time $T_2(2)$.

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**References**