Development of ^1H NMR spectroscopic technique for the study of matrix mobility in hen egg
yolk

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Abstract

Three experiments were conducted in developing a proton nuclear magnetic resonance (¹H NMR) spectroscopic technique to study matrix mobility in fresh and freeze-thaw gelled yolk. The Carr-Purcell-Meiboom-Gill (CPMG) sequence was used to measure spin-spin relaxation times of proton pools representing major yolk constituents. A component identification test distinguished 3–4 pools. The least mobile pool was assigned to proteins, protein-lipid and protein-water interactions, and the most mobile to unbound water. The remaining pools were assigned to lipids, lipid-protein and lipid-water interactions. A stability test indicated that yolk had varied matrix mobility within the same sample across five days of refrigeration storage. A reproducibility test demonstrated high repeatability of fresh yolk measurements, but significant differences ($p < 0.05$) were found within gelled yolk samples. This research determined that ¹H NMR spectroscopy, a non-destructive technique, can identify yolk components and detect changes in the matrix.

Keywords: egg yolk; freeze-thawed gelled yolk; low-resolution ¹H NMR; matrix mobility;

1. Introduction

Hen egg yolk is comprised of approximately 50% water, 30% lipids, and 16% proteins. Carbohydrates and minerals are minor constituents that make up the rest of the egg yolk composition (Anton, 2007). The major yolk components and their interactions within the system contribute to the complexity of yolk, which may be studied using nuclear magnetic resonance (NMR) spectroscopy.

High-resolution proton (^1H) NMR spectroscopy is used to study liquids and solutions. In contrast, low-resolution ^1H NMR spectroscopy is used to study food matrices since most foods are solid or more solid-like than liquid (Hills, 2006).

Low resolution ^1H NMR spectroscopy has been used to study molecular mobility in numerous food systems, many of which are simpler than egg yolk in structure and matrix. Typically they are ingredients, simple model systems of food products, or foods with extensively-studied matrices, such as starch gels and starch-based foods such as bread and cake (Le Grand, Cambert, & Mariette, 2007; Lu & Seetharaman, 2013; Luyts et al., 2013; Wang, Choi, & Kerr, 2004). Water mobility is usually analyzed in the aforementioned studies. Simple water-based gels have also been modeled by mixed systems to study water mobility (Huang, Davies, & Lillford, 2011; Shapiro, 2011). Though the results of these experiments cannot be compared to data from ^1H NMR studies of egg yolk because of the vast difference in matrix composition of the studied materials, the principles of proton relaxation and molecular mobility can still be applied. These principal steps include the collection of relative spin-spin relaxation times of proteins, lipids, and water; assignment of proton pools to protons of specific constituents; and interpretation of data in regards to the identification of exchanging and non-exchanging protons.

Few studies utilizing low resolution ^1H NMR spectroscopy have been conducted on hen's egg yolk. Some researchers have studied the molecular mobility of products containing egg yolk (Le Grand, Cambert, & Mariette, 2007; Luyts et al., 2013), but their results may not be used to compare to T_2 data of yolk. In those experiments, yolk was studied as a part of a larger food system with various other molecular interactions, so the obtained CPMG spectra cannot be equated to the spectra obtained in the study of yolk itself. To date, there has been only one study on raw egg yolk (Hills, Benamira, Marigheto, & Wright, 2004). Three proton pools were distinguished and assigned, in order of increasing mobility, to 1) protons of lipids, 2) protons of proteins, and 3) protons of water.

It is difficult to compare CPMG data collected from food products and its model systems among studies because T_2 and proton populations (PP) are affected by different experimental parameters such as resonance frequency, pulse spacing, number of scans, and recycle delay time (Luyts et al., 2013). In addition, there have been no ^1H NMR studies on egg yolk that utilize equivalent instrumentation or sample material so results cannot be directly compared. It is essential that a ^1H NMR spectroscopic technique be developed for research on yolk using experimental parameters that we have defined and determined to obtain reliable and interpretable data. Future research on egg yolks may utilize this method to continue studies on yolk matrix mobility with the assurance that the technique gives reliable T_2 and PP data.

The study of yolk via low-resolution ^1H NMR spectroscopy is important because this technique may reveal matrix changes that occurred during processing or storage that have not yet been observed. A phenomenon referred to as gelation, induced by freezing and thawing of yolk, transforms the yolk matrix and produces a very viscous and gelled material. Gelled egg yolk is undesirable to food processors because: 1) its increase in viscosity causes difficulties in mixing

the yolk with other ingredients, and 2) physicochemical changes in the gelled yolk result in reduced functionality in food products (Powrie, Little, & Lopez, 1963). NMR spectroscopy can be used to observe differences in sample structure due to changes in the internal chemical environment of the food, such as those resulting from freezing and thawing. Furthermore, ^1H NMR spectroscopy is a non-destructive method which can be used to examine the entire yolk with all of its intact, naturally-present components.

The objective of this research was to develop a ^1H NMR spectroscopic technique to study the matrix mobility of fresh and freeze-thawed hen egg yolk, and thus determine if NMR can distinguish the differences in structure and matrix mobility between fresh and gelled yolk. A complex system such as egg yolk is difficult to study, but this technique has been proven in this study to be useful because it can show molecular mobility of several components in yolk.

^1H NMR spin-lattice relaxation time (T_1) has been related to molecular mobility, but spin-spin relaxation time (T_2) measurements are primarily used to study matrix mobility in food (Luyts et al., 2013; Schmidt, 2004). Spin-spin relaxation time is affected by changes in the internal environment arising from chemical exchanges such as those involved in molecular mobility (James, 1998). Single-pulse ^1H NMR experiments are used to study low-mobility proton pools with T_2 of 10–1000 μs . The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence is used to measure protons pools with higher mobility and T_2 of 0.1–1000 ms (Luyts et al., 2013). Yolk constituents corresponding to proton pools detected by ^1H NMR spectroscopy were all expected to fall within T_2 range of the CPMG pulse sequence due to the high water content (50%) of egg yolk. Therefore, only measurements from the CPMG pulse sequence were made in this study.

In this study, three experiments were conducted in the development of a low-resolution ^1H NMR spectroscopic technique to study yolk matrix mobility. The first experiment was designed to identify the components in yolk which were detected in preliminary CPMG measurements. Lipid and water content in a fresh yolk system was varied in order to observe the effects of changes in yolk composition on T_2 spectra. The second experiment was a 5-d stability test using fresh and gelled yolk to determine yolk system stability as well as optimal measurement time and conditions. The main objective of this experiment was to study both yolk systems to determine if changes in the yolk matrix occurred throughout a storage period of 5 d. The third experiment was a reproducibility test using fresh and gelled yolk to determine instrument sensitivity and robustness, thus validating this method of measuring T_2 and PP for the study of yolk matrix mobility. The methodology developed in this research holds promise for studying the changes that occur in hen egg yolk as a result of processing or storage, such as freeze-thaw gelation and may be applied to other foods systems.

2. Materials and Methods

2.1. Materials

Large grade A white shell eggs were purchased from retailers in Ames, IA. The shell eggs were distributed by farms located within 300 mi of the Ames retailers. Eggs were purchased at least 14 d before the sell-by date, i.e., 30 d after the packing date. They were held for no more than 16 d in refrigeration temperature (4°C) during transportation as well as storage at the retailer and at the research laboratory.

Analytical grade di-isopropyl ether and 1-butanol were purchased from Fisher Scientific (Pittsburg, PA). Lecithin with claimed purity near 100% was purchased from Q.P. Corporation (Tokyo, Japan) and kept at -20°C until use.

2.2. Egg yolk separation

Yolk samples were prepared from the fresh, raw eggs no more than 16 d after the packing date. Yolks were separated using a modified version of the method by Powrie, Little, and Lopez (1963). Yolks were separated from the albumen, and the chalazae of each yolk were carefully removed. The albumen and chalazae were discarded. The yolk with intact vitelline membrane was washed carefully in shallow basin of Milli-Q water. Milli-Q, or ultrapure water, was obtained from deionized water purified with Milli-Q® Reagent Water System (EMD Millipore, Darmstadt, Germany). Each yolk was rolled on a sheet of paper towel to remove remaining water and albumen. Once clean, the vitelline membrane was pierced and yolk was drained into a beaker. After completing the yolk harvest for all eggs, the yolks in the beaker were stirred slowly with a stir rod for 1 min to ensure sample homogeneity. Yolks were frozen immediately for the tests requiring gelled yolk samples.

2.3. Yolk freezing and thawing

For samples requiring freezing and thawing, 50 mL of yolk was poured in 10-mL portions into five 25-mL polystyrene vials with snap-on lids. Strips of plastic paraffin film, Parafilm (Beemis Company, Inc., Oshkosh, WI), were wrapped over the lid around the circumference of the vial. Vials were vacuum sealed in a vacuum bag with a FoodSaver® V222 vacuum sealing system (SunBeam Products, Inc., Jarden Consumer Solutions, Boca Raton, FL).

The vacuum-sealed bags of yolk vials were submerged in the reservoir of a Haake SC 100 refrigerated/heated bath circulator (Thermo Fisher Scientific, Waltham, MA) filled with 1:1 ethylene glycol:Milli-Q water at 0°C. The bath was then set to –20°C. After the samples reached –20°C at a cooling rate of 0.3–0.5°C/min, the yolks were held in the –20°C bath for 3 h. The frozen yolks were then stored in a $-20 \pm 2^\circ\text{C}$ upright freezer for 7 d. Yolks were then thawed for 1 h in a 25°C Neslab GP-300 water bath (Neslab Instruments, Inc., Portsmouth, NH). Gelled yolks were analyzed immediately after thawing. Fresh yolks were analyzed no later than 6 h after yolk separation, and if storage was required the yolks were kept at 4°C until analysis.

2.4. Sample preparation for component identification

Seven samples of varying compositions were prepared for the component identification experiment: fresh yolk (F); lyophilized yolk reconstituted to 25 (L-25), 50 (L-50), and 75% (L-75) water (wet basis, wb); and delipidated yolk with 0 (D), 10 (D-10), and 30% (D-30) added lecithin (wb). The natural amount of water and lipid found in yolk is about 50 and 30% (wb), respectively. Fresh yolks were freeze-dried, or lyophilized, in plastic beakers with a Labconco FreeZone 2.5-L benchtop freeze dry system (Labconco, Kansas City, MO). The lyophilized material was collected in polypropylene tubes, capped, and stored in a –20°C freezer until use. Lyophilized yolk was reconstituted with Milli-Q water, mixed thoroughly, and stored at 4°C for 2 h before filling the NMR tubes. Fresh yolk was defatted using a 1:1.5 (v/v) 1-butanol:di-isopropyl ether solvent system as described in the method of Cham and Knowles (1976). The delipidated yolk was collected in polypropylene tubes, capped, and stored in 4°C until use. Egg lecithin was added to two portions of the defatted yolk to yield a sample of 10% and 30% added lecithin each.

171 2.5. *Sample preparation for yolk system stability study*

172 Fresh and gelled yolk were prepared in triplicate per treatment. The same three tubes of
173 each sample were used to obtain measurements throughout the 5-d study. Proton relaxation
174 measurements were performed at approximately the same time each of the five days. Between
175 daily measurements, tubes were stored at 4°C.

177 2.6. *Sample preparation for method reproducibility study*

178 Eggs were purchased from Ames retailers on three separate occasions at least 7 d apart.
179 Yolks were separated, and gelled samples were stored frozen for 7 d and thawed according to the
180 method described in Section 2.3. Fresh and gelled samples were prepared for three repeated
181 trials. Each trial utilized a different batch of eggs purchased at different times but originating
182 from the same farm.

184 2.7. *¹H NMR spectroscopy measurements*

185 For all tests, flat-bottom glass NMR tubes (10 mm diameter, 180 mm length) were
186 prepared in triplicate for each sample. Tubes were filled with yolk samples to a height of 4 cm
187 and capped. Proton relaxation measurements were performed on a Bruker minispec mq-20 low-
188 resolution spectrometer (Bruker, Biospin Corporation, Billerica, MA, USA) at a resonance
189 frequency of 20 MHz. Immediately before measurement, capped samples were held at 20°C in a
190 Duratech TCON 2000 high precision dry bath system (Duratech, Carmel, IN) for 60–70 min.
191 The probe head was regulated at a constant temperature of 20°C by a Julabo F25-ED
192 refrigerated/heated circulator (Julabo GmbH, Seelbach, Germany) connected to the minispec.

Temperature regulation ensured that temperature fluctuations during measurement would not be a factor contributing to possible differences observed in proton relaxation of the samples.

The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to measure T_2 between 0.2 and 1000 ms. Separation between the 90° and 180° pulses was 2.28 and 4.98 μ s, respectively, and 600 data points were collected. For CPMG measurements, 8 scans were made with a 15.0-second recycle delay. Gain was adjusted for each sample to ensure collection of good T_2 data, where the highest signal point was close to 80%. Relaxation curves were fitted to a continuous distribution of exponentials using the Inverse Laplace Transformation (ILT) algorithm (Bruker software, Bruker, Biospin Corporation, Billerica, MA, USA), also referred as the CONTIN algorithm of Provencher (1982). T_2 is reported for each peak in ms. Proton populations (PP), or areas of the peaks, are proportional to the relative quantities of yolk component and are expressed in percentage (%) normalized for each sample. Proton populations were normalized by transforming the peak areas into percentages where the sum of all peaks was 100%.

2.8 Statistical analysis

Statistical analysis was carried out with JMP Pro 11, statistical software from Statistical Analysis System (SAS) Institute Inc. (Cary, NC). One-way analysis of variance (ANOVA) tests were conducted, and Tukey's HSD (honest significant difference) test was applied for multiple-pairwise comparison of samples. Significant differences were defined as having a p -value < 0.05 .

3. Results and Discussion

3.1. Identification of yolk components

Hen egg yolk is comprised primarily of water (48–52%), lipid (30–33%), and protein (16–18%) (Powrie & Nakai, 1986; Vadehra, Nath, & Forsythe, 1973). Three proton pools or peaks correlating to these three major components were expected in the CPMG spectra of yolk. Because large biomolecules possess faster spin-spin relaxation times than smaller molecules such as water (Bloembergen, Purcell, & Pound, 1948), the peak with the smallest T_2 , indicating the fastest relaxing and least mobile components, could be attributed to the yolk proteins. Lipid molecules, which are smaller than proteins but larger than water molecules, could be assigned to the second peak. The third peak indicating the slowest relaxing, most mobile components could assume to be water. However, experimental data showed that up to four peaks were detected by low-resolution NMR. The identification and assignment of yolk components to T_2 peaks was necessary. Based on the results of the first experiment, yolk components were attributed to each proton pool, though they are labeled “Peak 1”, “Peak 2”, “Peak 3”, and “Peak 4” in the entirety of this study and not with the name of each component (i.e., “Proteins”, “Lipids,” or “Water”). This is done for ease of discussion and primarily because the identity of proton pools had not yet been confirmed. Fig. 1 depicts the expected and actual CPMG spectra of fresh yolk.

It is important to note that the fourth peak was consistently present in approximately half of the 18 measurements of fresh yolk, designated by F^* in all figures and tables. When three peaks were detected in fresh yolk, the third peak with highest T_2 was very broad. When four peaks were detected, the two peaks with the largest T_2 were narrower compared to the pool of most mobile protons that was present when there were only three peaks. The spectrometer may only be able to distinguish the two most mobile proton pools some of the time due to the

complexity of egg yolk structure. In contrast, it is possible that in fact egg yolk is very heterogeneous presenting variations in matrix mobility characteristics that can be detected among different samples.

To assign proton pools or peaks to the appropriate yolk components, it was necessary to observe the effect of changes in the yolk composition. Water and lipid content were manipulated since their protons possess longer relaxation times compared to the protons of proteins (Bloembergen et al., 1948; Todt, Guthausen, Burk, Schmalbein, & Kamlowski, 2006), making it easier to detect with CPMG and the resulting T_2 range of 0.2–1000 ms.

The spin-spin relaxation times of Peak 1 were unchanged in all yolk samples ($p > 0.05$) except the lyophilized yolk reconstituted to 75% moisture (L-75) which had an average T_2 of 1.82 ms compared to 0.54–0.71 ms of the other samples (Fig. 2A). This suggests that the T_2 of this proton pool was nonexchangeable and unaffected by compositional changes until a large amount of water was added to the yolk system, leading to increased mobility. Peak 1 PPs were different amongst samples, with the largest differences seen in 25% moisture yolk (L-25) and delipidated yolk with 0 (D), 10 (D-10) and 30% (D-30) lecithin compared to fresh yolk (F). The removal of water and lipids in L-25 and D, respectively, increased the proton populations of proteins that had the fastest relaxation times (Peak 1 PP, Fig. 2B). These results, along with the knowledge that larger molecules and nonexchangeable protons belong to protein protons (Bloembergen et al., 1948; Luyts et al., 2013), led to the assignment of Peak 1 to yolk protein. Peak 1 may also contain a small population of protons participating in protein-lipid or protein-water interactions.

The addition of increasingly larger amounts of water to lyophilized yolk affected all other proton pools. CPMG spectra of fresh yolk (F) and lyophilized yolk reconstituted to 50% water

(L-50) were similar when comparing T_2 and PP across all peaks (Fig. 2). This confirmed that the matrix of fresh yolk was comparable to that of freeze-dried yolk reconstituted with water to its natural moisture content. The spectra of yolk samples with 25% (L-25) and 75% (L-75) water require more in-depth analysis to determine the effect of water on matrix mobility. Peak 2 corresponding to L-25 and L-75 had lower T_2 and PP than F or L-50. Both L-25 and L-75 also had a lower Peak 3 T_2 and a higher Peak 4 T_2 compared to fresh yolk. However, Peak 3 PP was lower for L-25 and higher for L-75 compared to fresh. Peak 4 T_2 of both samples were higher than that of fresh yolk. The shifts in T_2 illustrate that water affected exchangeability or mobility of all proton pools, having a particularly large effect on the slower-relaxing pools, Peaks 3 and 4. Consequently, Peak 4 was assigned the exchangeable proton pool of unbound water.

It should be noted that Peak 4 cannot be regarded as bulk water, as bulk water is characterized by a much greater T_2 of 2.0–3.0 s independent of measurement conditions (Bryar & Knight, 2008; Hills, Tackacs, & Belton, 1990; Luyts et al., 2013). Peak 4 may contain exchanging protons from lipids and proteins via the interaction of lipids, protein, and water. Peak 4 T_2 of L-75 was extremely high compared to Peak 4 of all other samples, and the next highest T_2 was Peak 4 of the delipidated yolk (D) (Fig. 2A). Since delipidation removes lipids, including polar phospholipids, water molecules which normally interact with polar lipid molecules became free and more mobile after defatting. This explains the presence of the fourth, most mobile peak in delipidated yolk that was only sometimes detected in fresh yolk. Thus, the interaction of lipid and water, as well as the effects of increased or decreased quantities of these components, were observed in Peak 3 and 4 T_2 and PP.

The removal of lipids and addition of egg lecithin determined the effect of lipid (in the form of lecithin) on matrix mobility of egg yolk. Peak 2, 3, and 4 T_2 of yolks delipidated with a

butanol-di-isopropyl ether solvent mixture were significantly different ($p < 0.05$) from all other yolk samples (Fig. 2A). Defatted yolk with added lecithin (D-10 and D-30) had a significant decrease ($p < 0.05$) in Peak 2 T_2 as lecithin content increased (Fig. 2A). The same trend in decreasing T_2 with increasing lecithin content was observed in Peak 3, and differences were statistically significant ($p < 0.05$) (Fig. 2A). The addition of lecithin in increasing quantities contributed to the binding of previously mobile water molecules by lecithin, which explains the decrease in T_2 of Peak 2 and 3 as lecithin content increased. Peaks 2 and 3 were assigned the yolk lipids, though both likely contain protons of lipid-water interactions.

A notable difference in the spectra of D-10 vs the spectra of D-30 was that the latter had four peaks while the former had three. Data indicated that Peak 2 as seen in D-10 differentiated into two peaks (Peak 2 and 3) for D-30. T_2 of Peak 2 and 3 of D-30 were 8.6 and 20.0 ms, respectively. D-10 had a Peak 2 T_2 of 12.8 ms, which fell between Peak 2 and 3 T_2 of D-30. Additionally, Peak 4 T_2 of D-30 (71 ms) was similar to Peak 3 T_2 of D-10 (67 ms) (Fig. 2A). The proposal that Peak 2 of the D-10 was detected as two different peaks for D-30 was confirmed by the proton populations. Peak 2 PP of D-10 was close to the sum of Peaks 2 and 3 PP of D-30 (Fig. 2B). These results confirm the assignment of Peaks 2 and 3 as protons of lipid and/or lipid-water interactions.

Assignment of proton pools to yolk components has seldom been attempted due to the complexity of egg yolk, and results were conflicting. Hills et al. (2004) studied raw egg whites and egg yolks with low-resolution 2D ^1H NMR spectroscopy, which correlates T_1 and T_2 . Two and three peaks were detected in T_2 measurements of whites and yolks, respectively. Comparisons between the peaks detected in whites and yolks led to the assignment of the water, protein, and lipid pools. Ordered from lowest to highest mobility, the yolk components were

lipid, protein, and water. Conversely, Luyts et al. (2013) assigned only protein and lipid to a yolk-water model system created as a part of a pound cake study, with the lipid proton pool having higher relaxation time. Five peaks were detected in the yolk-water system, but the other peaks were not identified. The number of detected peaks and their corresponding T_2 and PP are dependent on the instrument, running conditions, and type of sample (Luyts et al., 2013). It was crucial for yolk components to be identified based on the spectrometer, sample, and parameters used in this experiment in order to interpret data properly in matrix mobility studies of fresh and gelled yolk. Furthermore, this work can be used as a model for conducting studies in other food systems.

3.2. Yolk matrix stability

Preliminary data on matrix mobility showed fluctuations in the T_2 spectra when yolks were held in refrigeration storage (4°C) and measured after 1 or 2 d under the same conditions, suggesting that the yolk matrix needed time to equilibrate in refrigeration storage before measurement. Thus, experiments were carried out to determine yolk system stability as well as optimal measurement time and conditions. Fig. 3 shows the representative spectra of fresh (Fig. 3A) and gelled (Fig. 3B) yolk samples measured over the span of five days. Peaks 1, 2, 3, and 4 are not indicated in the figure but are assigned in order of increasing mobility of proton pools: Peak 1 with the lowest T_2 to Peak 4 with the highest T_2 . As previously discussed, three peaks were detected for approximately half of the measurements of fresh yolk, while four peaks were detected for the other half. This only occurred on the first day of testing. On days 2 and 3, the spectra of fresh yolk consistently showed three peaks, similar to the spectra of fresh yolk on the first day (Fig. 3A). However, T_2 shifts and peak-broadening were apparent in Peaks 1 and 2 on

the second and third day (Fig. 3A), though PP was unchanged (S Fig. 2). Additionally, the T_2 shifts were minor in Peak 2 of fresh yolk. On the fourth and fifth days, four well-defined peaks were detected by the spectrometer. In comparing the general shape of the spectra of fresh yolks throughout the five days, the samples were relatively more stable when kept in refrigeration storage (4°C) for the first three days than when storage was extended up to five days. However, the changes in T_2 on the second and third day suggest that optimal measurement time for the matrix mobility study of fresh yolks was the first day, immediately after yolk separation and NMR sample preparation.

Gelled yolk measurements were more stable than fresh yolks throughout the 5-d study. Four peaks were detected each of the five days (Fig. 3B), but the spectra of the fourth day changed drastically compared to days 1-3 and 5. While T_2 were unchanged (S Fig. 1), the proton pool with the greatest PP switched from Peak 2 to Peak 1 on the fourth day (Fig. 3B). On day 5, the PP of all peaks of the gelled yolk returned to the same values as observed in the first three days. It is possible that the fourth-day change in CPMG spectra was a result of operator error or, less likely, changes occurring within the gelled yolk matrix during refrigeration storage. In general, the matrix of gelled yolks as detected by low-resolution ^1H NMR spectroscopy was stable throughout five days of storage.

3.3. Method reproducibility study

The reproducibility of the three repeated trials conducted on fresh and gelled yolk from three separate egg purchases was acceptable based on the number of peaks detected as well as the general shape of the spectra (Fig. 4). Three peaks were detected in fresh yolk for approximately half of the measurements for each trial, while four peaks were detected in the

other half. Four peaks were detected in the gelled yolk spectra of all three trials. However, detailed analysis showed that while peak shapes looked similar within fresh yolk samples and within trials, inconsistencies were observed in gelled yolk samples among trials. Significant differences ($p < 0.05$) were found primarily among the T_2 of all four gelled yolk peaks, though some differences were found in the PP of the gelled yolk as well (Table 1). There were no significant differences for the majority of T_2 and PP of the fresh yolk among trials.

In fresh yolk samples, significant differences were found only in Peak 2 T_2 and Peaks 2 and 3 PP (Table 1). However, the differences between the three trials of Peak 2 T_2 (average 17.0 ms) are minor and are between 0.4 to 0.6 ms. Differences between the three trials of Peaks 2 and 3 PP are also minor, ranging 1.0–2.5% and 0.8–1.8%, respectively. While the values are considered statistically different ($p < 0.05$) it is unclear whether the values are practically different. Instrument sensitivity was high in detecting proton pools belonging to different components, so the high sensitivity may have contributed to the detection of yolk components that were only slightly different from batch to batch. The matrix of the yolk may display subtle changes macroscopically yet will translate to large changes nanoscopically as detected by the highly-sensitive spectrometer. Therefore, the differences observed in fresh yolk matrix across trials may not actually be significant.

The results observed in gelled yolk are more complex. Peak 1 T_2 of gelled yolk between trials was significantly different. The p -value was less than 0.001, so differences were less likely attributed to chance. Similarly, differences found in Peaks 2 and 3 T_2 , as well as Peaks 1 and 4 PP, have a p -value less than 0.0001 and were even less likely to be attributed to chance. Therefore, differences between the trials of gelled yolk samples were real.

Presumably the differences arose from sample preparation. The lack of significant differences among trials of the fresh yolk indicated that there were no differences between the three batches of egg yolks. Sources of error could be the freezing and thawing preparation and the NMR tube sample preparation. Freezing and thawing rates may be different between batches, affecting matrix interaction. It is imperative that freezing, thawing, and NMR tube preparations, as well as sample measurement conditions, are completely standardized and executed in the same manner each time in order to yield the same reproducible results.

Some differences may also have been due to biological variability among different batches of eggs. The differences may not have been statistically significant in the fresh yolk trials, but they may have become more apparent in the gelled yolk trials. To determine whether biological variability from different batches of eggs had an effect on the T_2 and PP, three additional trials of gelled yolk were completed. The samples were prepared as described in Section 2.3 but were obtained from the same batch of eggs rather than three different batches per trial. Supplementary Table 1 showed that there were no significant differences in Peaks 2, 3, and 4 T_2 and Peaks 3 and 4 PP. Significant differences ($p < 0.05$) were found in Peak 1 T_2 and Peaks 1 and 2 PP, but these differences were not as significant as those in Table 1 where yolks were prepared from three different batches. Thus, biological variability of eggs, sample preparation, and instrument sensitivity all had an effect on the T_2 and PP of the fresh and gelled yolk samples.

Differences between the fresh and gelled yolk spectra were observed in the number of peaks detected as well as the T_2 measurements (Table 1 and Fig. 4). In fresh yolk, three peaks were consistently detected in half of the measurements while four peaks were detected in the other half (Table 1). In gelled yolk, four peaks were detected by NMR spectroscopy. Further differences were seen in the T_2 of fresh and gelled yolk, as shown in Supplementary Table 2,

where the T_2 of fresh yolk (with three detected peaks) was significantly higher ($p < 0.05$) than gelled yolk. The most notable difference between the T_2 of fresh and gelled yolk was observed in Peaks 3 and 4, where gelled yolk was significantly higher ($p < 0.05$) than fresh yolk (S Table 2). These differences between the peak numbers and T_2 confirm that NMR spectroscopy was able to distinguish between fresh and gelled yolk. The changes in the system, which was identical in composition but differed in structure and matrix mobility after freezing-thawing, was evident in differences in the NMR spectra. The changes observed after freeze-thaw were likely a result of conformational changes of yolk constituents due to ice crystal formation (Kurisaki, Kaminogawa, & Yamauchi, 1980; Telis & Kieckbusch, 1997).

Changes in the water structure and rearrangement of the yolk components would presumably lead to changes in matrix mobility which correspond to changes in T_2 and overall spectra. As mentioned previously, the most mobile proton pools (Peak 4) represent unbound water and/or exchangeable protons of protein or lipid. It has been hypothesized that upon freezing-thawing yolk components such as low-density lipoproteins (LDL), high-density lipoproteins (HDL), and apolipoproteins aggregate due to protein-protein interaction among the disrupted apolipoproteins caused by slow freezing. Thus, it is probable that water molecules which were previously interacting with the matrix components were released from their interaction becoming free or more mobile and therefore contributing to the increase of T_2 values.

3.4. Summary

Three ^1H NMR experiments were conducted in the development of a nuclear magnetic resonance spectroscopic technique to study the matrix mobility of fresh and gelled egg yolk. The first study showed the effects of composition changes in yolk on the ^1H NMR spectra and was

carried out in order to identify the peaks using CPMG sequence. Changes in water and lipid components cause clear modifications in the ^1H NMR spectra. With moisture removal by freeze-drying, lipids that previously interacted with water molecules were freed, resulting in increased mobility of the peak corresponding to protons of lipids and lipid-water interactions. The addition of water increased the mobility of all proton pools. Four proton populations were successfully assigned to different components in yolk. The least mobile proton pool, Peak 1, was assigned to protein or interactions between protein, lipid, and water. The most mobile proton pool, Peak 4, was assigned to unbound water or exchangeable protein or lipid protons. Peaks 2 and 3 were assigned lipid and lipid-water interactions.

The second experiment studied system stability of fresh and gelled yolks stored in tubes at 4°C over a period of five days. Preliminary data showed that samples may not have been stable enough to run measurements on samples stored for longer than three days, and that there was an optimal measurement time. Experimental results indicated that fresh yolks should be measured within the first 24 h after yolk separation and NMR tube preparation. In general, gelled yolks were stable during the 5 d in refrigeration storage. For consistency, all samples should be measured on the same day of preparation for fresh yolk and immediately after thawing for gelled yolk.

The robustness of the instrument and methodology was determined with a third experiment. Three trials conducted on three different batches of fresh and gelled yolk samples each showed that ^1H NMR measurements of fresh yolk were consistent and highly reproducible. However, spectra of gelled yolk were inconsistent among trials and were not as reproducible as the trials conducted on fresh yolk samples. It should be noted that T_2 and PP of gelled yolk samples prepared from the same batch of eggs were more reproducible than samples prepared

from different batches, indicating that some differences can be attributed to biological variability of eggs. It is extremely important that conditions are highly regulated and that tests are consistently executed so that any differences found in the T_2 or PP of the samples is a result only of changes in the sample matrix. Despite the differences attributed to biological variability, ^1H NMR spectroscopy was able to distinguish between fresh and gelled yolk matrices.

4. Conclusions

The use of ^1H NMR spectroscopy to study fresh and gelled hen egg yolk is a novel approach. ^1H NMR spectroscopy is a non-destructive technique and was shown to have the ability to confirm the identities of yolk components, detect changes in the matrix, and distinguish between two systems with the same composition but different matrix structure. The method developed in this research was sensitive, and repeatable measurements were obtained. Therefore, these experiments may offer added value to the study of yolk and changes in yolk matrix mobility occurring as a result of processing and storage, such as gelation after freezing and thawing.

Note: The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at...

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Figure Captions

Fig. 1. Expected and actual CPMG spectra of yolk. Peaks of actual spectra correspond to components which had not been identified until the completion of this study.

Fig. 2. T_2 (A) and PP (B) of fresh yolk and yolk with varying compositions. Abbreviations are F, fresh yolk; L-25, lyophilized yolk reconstituted to 25% water (wb); L-50, lyophilized yolk reconstituted to 50% water (wb); L-75, lyophilized yolk reconstituted to 75% water (wb); D, delipidated yolk; D-10, delipidated yolk with 10% added lecithin (wb); D-30, delipidated yolk with 30% added lecithin (wb). * denotes a sample in which a different number of peaks was observed in approximately half of the measurements. Three peaks were observed in half the measurements of fresh yolk (F), and four peaks were observed in the other half (F*). Values with different letters within peaks are significantly different ($p < 0.05$).

Fig. 3. Representative CPMG spectra of fresh (A) and gelled yolk (B) obtained at each of five days of refrigeration storage. Abbreviations are F, fresh yolk; G, gelled yolk. Numbers following F and G (1-5) denote day of storage at 4°C. * denotes a sample in which a different number of peaks was observed in approximately half of the measurements. Three peaks were observed in half the measurements of 1 d fresh yolk (F1), and four peaks were observed in the other half (F*1).

Fig. 4. Representative CPMG spectra of fresh and gelled yolk of three repeated trials. Abbreviations are F, fresh yolk; G, gelled yolk. Numbers following F and G (1-5) denote day of storage at 4°C. * denotes a sample in which a different number of peaks was observed in

approximately half of the measurements. Three peaks were observed in half the measurements of fresh yolk (F1, F2, and F3), and four peaks were observed in the other half (F*1, F*2, and F*3).

Supplementary Fig. 1. T_2 of Peak 1 (A), 2 (B), 3 (C), and 4 (D) of fresh and gelled yolk over five days of refrigeration storage. Abbreviations are F, fresh yolk; G, gelled yolk. * denotes a sample in which a different number of peaks was observed in approximately half of the measurements. Three peaks were observed in half the measurements of fresh yolk (F), and four peaks were observed in the other half (F*).

Supplementary Fig. 2. PP of Peak 1 (A), 2 (B), 3 (C), and 4 (D) of fresh and gelled yolk over five days of refrigeration storage. Abbreviations are F, fresh yolk; G, gelled yolk. * denotes a sample in which a different number of peaks was observed in approximately half of the measurements. Three peaks were observed in half the measurements of fresh yolk (F), and four peaks were observed in the other half (F*).

Table 1T₂ and PP of fresh and gelled yolk of three repeated trials from the same batch of eggs.^a

	Trial	T ₂ ^b (ms)				PP ^c (%)			
		Peak 1	Peak 2	Peak 3	Peak 4	Peak 1	Peak 2	Peak 3	Peak 4
F ^d	1	0.71 ± 0.10	16.6 ± 0.3 a	82 ± 5	-	4.2 ± 2.2	75.2 ± 2.8 a	20.7 ± 1.2 a	-
	2	0.77 ± 0.25	17.0 ± 0.4 b	87 ± 3	-	3.9 ± 1.1	76.2 ± 1.2 ab	19.9 ± 0.8 ab	-
	3	0.73 ± 0.19	17.2 ± 0.3 b	85 ± 4	-	3.5 ± 0.7	77.7 ± 1.0 b	18.9 ± 1.0 b	-
	Mean	0.73 ± 0.03	17.0 ± 0.3	85 ± 3	-	3.9 ± 0.4	76.3 ± 1.3	19.8 ± 0.9	-
	CV (%)	3.9	1.9	3.0	-	9.5	1.6	4.5	-
	p	> 0.05	< 0.05	> 0.05		> 0.05	< 0.05	> 0.05	
F* ^e	1	0.55 ± 0.20	16.0 ± 0.2	43 ± 4	98 ± 4	6.2 ± 1.5	66.3 ± 2.8	13.4 ± 2.3	14.1 ± 1.3
	2	0.55 ± 0.20	16.4 ± 0.5	43 ± 9	98 ± 9	5.6 ± 1.8	65.8 ± 2.8	13.3 ± 4.1	15.2 ± 2.8
	3	0.55 ± 0.10	16.3 ± 0.4	37 ± 4	97 ± 7	5.0 ± 0.6	65.1 ± 3.8	14.2 ± 4.4	16.7 ± 1.7
	Mean	0.55 ± 0.00	16.2 ± 0.2	41 ± 3	98 ± 1	5.6 ± 0.6	65.7 ± 0.6	13.7 ± 0.5	15.0 ± 0.8
	CV (%)	0.4	1.2	8.3	0.8	11.1	0.9	3.7	5.4
	p	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
G ^f	1	0.55 ± 0.01 b	17.2 ± 0.2 a	66 ± 5 a	109 ± 25 b	9.5 ± 0.5 a	69.1 ± 0.8	13.9 ± 1.5	7.5 ± 1.3 a
	2	0.67 ± 0.17 a	16.2 ± 0.3 b	55 ± 6 b	126 ± 11 a	4.8 ± 0.4 b	68.4 ± 1.7	15.1 ± 2.4	11.7 ± 2.1 b
	3	0.55 ± 0.02 b	17.0 ± 0.5 a	49 ± 6 c	111 ± 10 ab	5.6 ± 1.7 b	68.3 ± 2.9	13.3 ± 3.2	12.9 ± 1.7 b
	Mean	0.59 ± 0.07	16.8 ± 0.5	57 ± 9	116 ± 9	6.7 ± 2.5	68.6 ± 0.4	14.1 ± 0.9	10.7 ± 2.8
	CV (%)	12.4	3.1	15.8	8.1	37.6	0.6	6.6	26.6
	p	< 0.001	< 0.0001	< 0.0001	< 0.05	< 0.0001	> 0.05	> 0.05	< 0.0001

^a Mean and standard deviation of T₂ and PP were calculated from the mean measurements of the three replicated trials, where the mean of each trial was treated as a single replicate. ^b T₂, spin-spin relaxation time. ^c PP, proton population. ^{d, e} F, fresh yolk. ^f G, gelled yolk. *, a sample in which a different number of peaks was observed in half of the measurements. Three peaks were observed in half the measurements of fresh yolk (F1, F2, and F3), and four peaks were observed in the other half (F*1, F*2, and F*3). Values with different letters within groups (F, F*, and G) and peaks (Peak 1, 2, 3, and 4) are significantly different (*p* < 0.05).

Supplementary Table 1T₂ and PP of gelled yolk of three additional repeated trials from the same batch of eggs. ^a

Trial	T ₂ ^b (ms)				PP ^c (%)			
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 1	Peak 2	Peak 3	Peak 4
1	0.55 ± 0.02 b	15.2 ± 2.3	48 ± 4	116 ± 8	4.4 ± 1.0 a	68.2 ± 3.1 ab	14.5 ± 2.8	12.8 ± 1.6
2	0.55 ± 0.02 b	15.7 ± 0.2	44 ± 4	115 ± 7	4.3 ± 0.8 ab	67.6 ± 1.3 b	14.5 ± 2.1	13.6 ± 1.3
G2 ^d 3	0.62 ± 0.11 a	15.7 ± 0.2	48 ± 5	116 ± 10	3.5 ± 0.6 b	71.0 ± 4.8 a	13.5 ± 3.8	13.7 ± 1.8
Mean	0.57 ± 0.04	15.5 ± 0.3	47 ± 2	115 ± 0	4.1 ± 0.5	69.0 ± 1.8	14.2 ± 0.6	13.4 ± 0.5
CV (%)	7.0	1.7	2.1	0.4	12.5	1.8	4.0	3.4
<i>p</i>	< 0.05	> 0.05	> 0.05	> 0.05	< 0.05	< 0.05	> 0.05	> 0.05

^a Mean and standard deviation of T₂ and PP were calculated from the mean measurements of the three replicated trials, where the mean of each trial was treated as a single replicate. ^b T₂, spin-spin relaxation time. ^c PP, proton population. ^d G2, additional trials of gelled yolk. Values with different letters within peaks (Peak 1, 2, 3, and 4) are significantly different (*p* < 0.05).

Supplementary Table 2Average T₂ and PP of fresh and gelled yolk of three repeated trials from the same batch of eggs.

	T ₂ ^a (ms)				PP ^b (%)			
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 1	Peak 2	Peak 3	Peak 4
F ^c	0.73 ± 0.03 a	17.0 ± 0.3 a	85 ± 3 a	-	3.9 ± 0.4 a	76.3 ± 1.3 a	19.8 ± 0.9 a	-
F* ^d	0.55 ± 0.00 b	16.2 ± 0.2 ab	41 ± 3 c	98 ± 1 b	5.6 ± 0.6 a	65.7 ± 0.6 c	13.7 ± 0.5 b	15.0 ± 0.8 a
G ^e	0.59 ± 0.07 b	16.8 ± 0.5 a	57 ± 9 b	116 ± 9 a	6.7 ± 2.5 a	68.6 ± 0.4 bc	14.1 ± 0.9 b	10.7 ± 2.8 ab
G2 ^f	0.57 ± 0.04 b	15.5 ± 0.3 b	47 ± 2 bc	115 ± 0 a	4.1 ± 0.5 a	69.0 ± 1.8 b	14.2 ± 0.6 b	13.4 ± 0.5 b

^a T₂, spin-spin relaxation time. ^b PP, proton population. ^{c, d} F, fresh yolk. ^e G, gelled yolk. ^f G2, additional trials of gelled yolk. *, a sample in which a different number of peaks was observed in half of the measurements. Three peaks were observed in half the measurements of fresh yolk (F), and four peaks were observed in the other half (F*). Values with different letters within peaks (Peak 1, 2, 3, and 4) are significantly different (*p* < 0.05).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

EXPERIMENT		
1 - Yolk Component ID	2- Yolk Matrix Stability	3 - Method Reproducibility
METHOD		
Yolk composition modified to observe changes in NMR peaks and thus identify yolk components.	Fresh and gelled yolks stored at 4°C for 5 d and studied for system stability to determine optimal measurement time.	Three trials of three different egg batches conducted on fresh and gelled yolk to determine robustness of instrument and methodology.
MAIN FINDINGS		
<p>Four proton populations (peaks) assigned to different yolk components.</p> <p>Least to most mobile:</p> <p><i>Peak 1</i>: protein or interactions between protein, lipid, and water</p> <p><i>Peaks 2 & 3</i>: lipid and lipid-water interactions</p> <p><i>Peak 4</i>: unbound water or exchangeable protein or lipid protons</p>	<p>Fresh yolks should be measured within 24 h after yolk separation and NMR tube prep.</p> <p>Gelled yolks stable during 5 d storage.</p> <p>For consistency, all samples should be measured on same day of prep.</p>	<p>Fresh yolk spectra consistent and highly reproducible.</p> <p>Gelled yolk spectra not as reproducible. Some differences attributed to biological variability of eggs.</p> <p>Despite variations in gelled yolk batches, NMR spec able to distinguish between fresh and gelled yolks.</p>