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Authentication of the Effect of Freezing/Thawing of Pork by Quantitative Magnetic Resonance Imaging

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Magnetic resonance imaging analysis of longitudinal relaxation times (T_1 values), transverse relaxation times (T_2 values), magnetization transfer (MT) rates and apparent water diffusion coefficients (D) was used to authenticate the effect of freezing/thawing in *longissimus dorsi* pig muscle; these NMR parameters were compared with the gravimetric moisture content values obtained independently by oven drying. A significant increase in the MT rate was observed between fresh and frozen/thawed pork prepared from the same animal; however, measurements of other parameters showed little or less significant changes. The significant increase in the MT rate is associated with both the decrease of moisture content in pork after freezing/thawing and with the denaturation of myofibrillar proteins in frozen/thawed pork. On that basis, the present NMR results imply that the duration of the freezing period (from 2 weeks to 2 months at −18 °C) did not significantly enhance the denaturation of the meat proteins.

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INTRODUCTION

Prompted by the present difficulties of food producers and retailers in authenticating by instrumental methods the quality of fresh and frozen/thawed pork, we have explored the possibility of using quantitative magnetic resonance imaging (MRI) with this objective in mind.[1] It is already known that poor freezing/thawing conditions result in enhanced water drip and also induce a decrease in the water-holding capacity (WHC) of the meat;[2,3] these exudates are accompanied by a loss of weight and nutrients, such as vitamins and soluble proteins, and thus a decrease in the economical value of the meat.[2]

Meat is organized in bundles of muscle fibres surrounded by connective tissues made of collagen. The perceived quality of meat varies considerably depending on the conditions of preparation and storage;[3] in particular, the palatability, tenderness and juiciness of the meat are often associated with its WHC, which relates inversely to the amount of expressible juice.[3] The final quality of the pork is sensitive to the conditions of the animal before slaughter, the animal exhaustion and stress, the methodology of stunning and execution and the subsequent rate of chilling the carcass. For pork which is frozen prior to use, the rate of freezing/thawing, which may involve protein denaturation,[2] and the growth of ice crystals during the subsequent storage period are responsible for some damage of the cell membranes.[4] All of these causes can induce significant water drip from the meat[5] and at present it is difficult to evaluate their separate effects on and relative importance to the final quality of the meat; consequently, it is of considerable interest and potential importance to develop analytical procedures which can provide further insight into these complex problems.

In this paper, attention is focused particularly on the evaluation of quantitative magnetic resonance imaging (MRI) measurements to study the effects of freezing/thawing on the structure of fresh pork. NMR is a non-destructive technique, which is a major advantage for food analysis. Furthermore, with the advent of MRI, NMR relaxometry can be used to map various NMR parameters of water protons such as the total water proton content (M_0), the longitudinal relaxation time (T_1), the transverse relaxation time (T_2), the magnetization transfer rate (k) and the diffusion coefficients of water in pork. Ultimately, 'whole-body' MRI scanners currently used for studies of adult humans offer a potentially unique opportunity for the analysis of meat samples of large dimensions.

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Objectives of the study

This paper describes an inter-comparison study of M_0, T_1, T_2, k and diffusion coefficient values obtained by MRI and 'bulk' NMR in fresh pork samples that were successively frozen and thawed.

Advantages of MRI. The advantages of using MRI in this study are twofold:

1. MRI provides spatial resolution in samples that are susceptible to containing heterogeneities at a macroscopic scale (fat and connective tissues). The acquisition of MR images and the determination of the NMR parameters within a region of interest allows meaningful comparison of NMR results between different samples and the statistical analysis of their spatial distribution.
2. Many samples can be analysed simultaneously, along with a reference sample. There are numerous advantages to the approach used here, in particular a potential gain of time and measurement consistency for routine comparative analysis.

Introduction to quantitative MRI analysis. In the past decade, MRI has been used to measure the spatial distribution of T_1 [6] and T_2 values[7] in the human body and living animals. The systematic errors associated with such MRI protocols have already been evaluated for heterogeneous model systems, by comparison with 'bulk' T_1 measurements.[8] Furthermore, T_2 and diffusion ^{1}H MRI[9] have also been used to show the effect of brine distribution in salt-cured rabbit muscle and tumbled ham. In addition, the development of magnetization transfer (MT) contrast MRI has made available a novel form of image contrast related to the rate of proton exchange between muscle proteins and water molecules.[10,11] The concept of T_1^{sat} measurements using proton saturation transfer has been introduced and already explained,[11] where T_1^{sat} is the longitudinal relaxation time of water protons weighted by the magnetization transfer rate (or apparent proton exchange rate, k) of those protons at the exchange sites of the macromolecule.[12]

Some limitations of quantitative MRI. The quantitative MRI protocols used for the present study have already been described.[1,12] As explained in a previous paper,[12] usually only a limited number of informative MR images can be acquired to generate quantitative maps of MR parameters such as T_2 maps, mainly because quantitative MRI is so time consuming. Thus T_1, T_1^{sat} and diffusion mapping involve the acquisition of such a limited number of individual MR images[1] as to reduce the delineation of the relaxation curves for each image pixel that the data have to be fitted to a mono-exponential model. Although this is not a major restriction for these parameters, the transverse relaxation curves associated with each pixel of T_2 MR images have to be fitted to a mono-exponential model, despite the fact that the transverse relaxation behaviour in *post-rigor* muscle is generally recognized as bi-exponential.[9,13,14] Hence, as explained in a previous paper,[12] there is concern about the consistency between mono-exponential T_2 values obtained by MRI and those obtained from standard 'bulk' NMR measurements, for which T_2 values are usually reported with two exponential components. In this study, to ensure the validity of T_2 data obtained by MRI for fresh and frozen/thawed pork, 'bulk' T_2 values obtained from both mono- and bi-exponential fits of transverse relaxation curves were first compared; in this way, the average T_2 values obtained by MRI could be associated with one or the other of the bi-exponential T_2 components. The T_2 data obtained by MRI in fresh and frozen/thawed pork were then analysed on the basis of that preliminary evaluation.

Objectives. Three series of quantitative MRI experiments were performed, with the following aims:

i. to measure MRI parameters in pork analysed as fresh (A);
ii. to compare fresh pork (A) with pork obtained from the same animal but successively/frozen/thawed/frozen and thawed (B);
iii. to compare and discriminate fresh pork (A) from frozen/thawed pork (B) and frozen/stored/thawed pork (C).

EXPERIMENTAL

Sample preparation

Nine pork samples of *longissimus dorsi* muscle [400 g each, 76% (w/w) water content, 1% (w/w) fat, pH 5.62] were supplied from one 4-month-old Large White Cross Landrace breed pig carcass maintained above 0 °C (Chipping Campden, Gloucs., UK). Five days after slaughter, all the samples were deboned and skin and fat were removed. Three fresh pork samples were then vacuum-packed and preserved in a chiller at 5 °C until the beginning of the MRI measurements (*ca.* 2 days), when a small sample (50 g) was extracted and its moisture content was measured. Moisture content was determined by gravimetric analysis[15] after leaving the samples overnight in a drying oven stabilized at 103 °C.

Freezing/thawing experiment I. Three fresh pork samples (400 g each) were placed for 2.5 h in a Phillips fan-assisted freezer operating at −30 °C; these samples were then transferred into a pre-chilled polystyrene box and stored for 14 days in a commercial freezer at −18 °C. The frozen pork was then thawed until totally defrosted ($T^0 \approx 5$ °C) and then re-frozen at −30 °C for 2 h and stored in a commercial freezer for a further period of 2 weeks. The samples were then defrosted once again at room temperature, vacuum-packed and preserved in a chiller at about 5 °C until the beginning of the MRI experiments (*ca.* 2 days). Before the MRI experiments, a small sample (50 g) was extracted from each frozen/thawed sample and its moisture content was measured.

Freezing/thawing experiment II. The same procedure as described in Experiment I was followed although the three meat samples for this experiment were not defrosted, but instead stored in a freezer (−18 °C) for 12 weeks. The samples were then thawed, vacuum-packed and stored in a chiller until the MRI experiments were performed (*ca.* 2 days). Before the MRI experiments, a small sample (50 g) was extracted from each frozen/stored sample and its moisture content was measured.

NMR experiments

NMR hardware. ^{1}H MR images were acquired using a 2.35 T, 31 cm horizontal bore superconducting magnet (Oxford Instruments, Oxford, UK) connected to a Bruker BMT (Bruker Medzintechnik Biospec II) imaging console (Bruker, Karlsruhe, Germany). ^{1}H NMR diffusion measurements were made with a Biospec I imaging spectrometer (Oxford Research Systems, Coventry, UK) connected to a 2 T, 31 cm horizontal bore superconducting magnet (Oxford Instruments). A 16 cm i.d. gradient set that could produce maximum magnetic field gradients of 0.15 T m^{-1} was used for the MRI experiments.

Imaging parameters. MR images of 256 × 128 pixels were acquired using a spin-warp protocol[16] with an inter-echo time (TE) of 20 ms, a slice thickness of 4 mm, a 120 μm per pixel resolution (field of view 3 cm) and two scans. The TE values for the Carr–Purcell–Meiboom–Gill (CPMG) experiments used for T_2 measurements ranged linearly from 0.02 to 0.16 s (eight T_2-weighted images in total). Variable delay lists for series of T_1- and T_1^{sat}-weighted images were set up on the basis of a logarithmic scale;[12] in these experiments, the recovery delay (RD) was varied within the range 0.148, 0.227, 0.306, 0.622, 1.017, 1.886, 3.308 and 7.077 s and the saturation delay (SD) within the range 0.001, 0.120, 0.180, 0.280, 0.420, 0.650, 0.980 and 1.500 s, and a total of 22 images were acquired in *ca.* 6 h. Results from three samples of fresh pork and three samples of frozen/thawed pork obtained from the same animal were also obtained using the same protocol.

Measurement of 'bulk' T_2 values and water diffusion coefficients. A CPMG sequence was used to produce transverse relaxation decay curves of either 256 or 128 points, with TE of 1 and 20 ms, respectively. A version of the alternating pulsed field gradient stimulated echo (APGSTE) sequence[17] implemented in-house by A. J. Lucas *et al.*[18] was used for 'bulk' water diffusion measurements. In these experiments, diffusion encoding gradients were incrementally increased from 0 to a maximum magnetic field gradient of 0.35 T m^{-1}, with a total number of 16 increments, a phase cycling procedure of 16 steps per gradient increment and an RD of 6 s. The duration of the diffusion encoding gradient (δ) and the τ-spacing were constant for all experiments, i.e. $\delta = 2$ ms and $\tau = 2$ ms. Three different storage delays (Δ) corresponding to evolution delays of 40, 200 and 500 ms (corresponding to a mean diffusion path of 9, 20 and 32 μm, respectively) were chosen to investigate the dependence of the apparent water diffusion coefficient on the presence of potential barriers to diffusion, such as cell membranes and skeletal proteins. The diffusion measurements were made in both the z and x directions of the magnet, i.e. for the present experiments, along and across the meat fibres, respectively. Prior to the diffusion experiments with fresh and frozen/thawed pork samples, systematic measurements were performed with a 0.1 mM $MnCl_2$ aqueous solution where 'free' water was chosen as the reference for the determination of apparent diffusion coefficients[19] ($D_{H_2O} = 2.05 \times 10^{-9}$ m^2 s^{-1} at 20 °C).

Data analysis. All the experimental data were transferred from the spectrometer console to a network of Unix workstations, and the image display and numerical analysis of sample regions were performed with imaging software (Cmrview) written-in house by N. J. Herrod. T_1, T_2 and T_1^{sat} maps were generated by fitting the NMR relaxation curves for each pixel of the MR images, using curve-fitting software (Cmrfit) written in-house by J. J. Attard *et al.*[8] Bulk water T_2 values and diffusion coefficients were obtained by fitting the NMR data to mono- or bi-exponential functions, also using Cmrfit. Comparative statistical analyses between NMR parameters were performed with Student's t-test.[20]

RESULTS AND DISCUSSION

T_2 measurements by both bulk NMR and MRI protocols using fresh pork as a control sample (Table 1) were first performed to validate the T_2 values measured by MRI. The effects of freezing/thawing on the MRI parameters of fresh pork were then investigated using

Table 1. Transverse relaxation times measured in fresh port at 2.35 T, using mono- and bi-exponential curve-fitting procedures

Curve-fitting procedure	T_2^A (ms)	T_2^B (ms)	P_A (%)[a]	P_B (%)[a]
Mono-exponential	—	40.0 ± 0.1	—	—
Bi-exponential	35.1 ± 0.1	134.1 ± 2.7	91.4 ± 0.1	8.6 ± 0.1

[a] P_A and P_B are the populations for the first and second relaxation components, respectively.

Table 2. Moisture contents and MRI results (T_1, T_2, M_0, M_{sat}/M_0, T_1^{sat}, k) obtained for fresh pork and frozen/thawed pork samples (Experiments I and II)[a]

Parameter	Meat sample: Fresh pork 76.0 ± 1.0%[b]	Frozen/thawed pork (I) 74.3 ± 1.3%[b]	Frozen/stored pork (II) 71.1 ± 3.0%[b]
Longitudinal relaxation time, T_1 (s)	1.08 ± 0.05	0.99 ± 0.05	0.94 ± 0.03
Transverse relaxation time, T_2 (ms)	41.2 ± 1.6	36.4 ± 3.0	39.0 ± 3.9
Liquid proton density ratio, M_0 (%)	76.4 ± 7.1	70.2 ± 3.6	68.4 ± 4.3
Magnetization transfer ratio, M_{sat}/M_0	0.22 ± 0.01	0.20 ± 0.01	0.20 ± 0.02
Longitudinal relaxation time, T_1^{sat} (s)	0.22 ± 0.02	0.19 ± 0.02	0.17 ± 0.01
Magnetization transfer rate, k (s^{-1})	3.48 ± 0.20	4.44 ± 0.35	4.77 ± 0.48

[a] Mean values and standard deviations were estimated from the results of three quantitative MRI experiments. In each of the experiments, three samples obtained from the same animal were imaged together with a reference solution (0.1 mM $MnCl_2$ aqueous solution). For each sample, an average value was obtained from the region of interest of that sample (cf. Fig. 1). Apparent diffusion coefficients are given at 20 °C for a diffusion evolution delay of 40 ms.
[b] Water content (%, w/w).

two different freezing/thawing protocols; the resultant MRI values (T_1, T_2, M_0, M_{sat}/M_0, T_1^{sat} and k) are summarized in Table 2, along with the moisture contents in fresh, frozen/defrosted/frozen/thawed (I) and frozen/stored/thawed (II) pork. MR images obtained for a typical frozen/thawed sample of pork along with a control sample (0.1 mM $MnCl_2$ aqueous solution) are displayed in Fig. 1. The diffusion NMR results are sum-

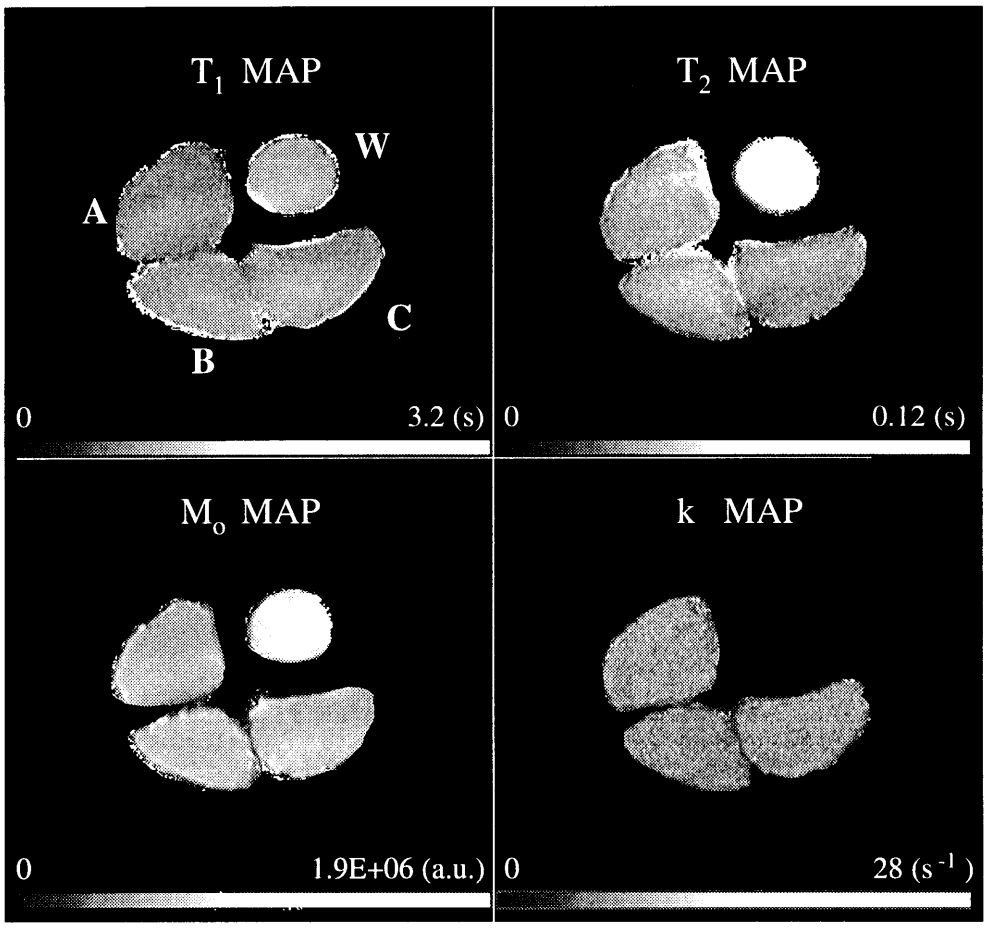

Figure 1. T_1, T_2, M_0 and k (MT rate) images (256 × 128 pixels) of a 0.1 mM $MnCl_2$ aqueous solution (W) and three sub-samples of pork (A, B and C) which are the frozen/stored/thawed pork samples of Experiment II. The average of the values for each area of each sub-sample is used to produce the MRI result for T_1, T_2 and k.

Table 3. Moisture contents and apparent diffusion coefficients in fresh and frozen/thawed pork (Experiments I and II), along and across the meat fibres, for a diffusion evolution delay of 40, 200 and 500 ms at 20 °C

		Apparent diffusion coefficient, D ($\times 10^9$ m^2 s^{-1})		
Gradient[b] position	Δ[c]	Fresh pork 76.0 ± 1.0%[a]	Frozen/thawed pork (I) 74.3 ± 1.3%[a]	Frozen/stored pork (II) 71.1 ± 3.0%[a]
∥	40	1.05 ± 0.05	1.08 ± 0.09	1.07 ± 0.01
∥	200	0.95 ± 0.03	1.04 ± 0.02	1.10 ± 0.01
∥	500	0.98 ± 0.03	1.09 ± 0.02	1.03 ± 0.01
⊥	40	1.03 ± 0.01	1.02 ± 0.04	1.10 ± 0.01
⊥	200	0.96 ± 0.02	0.99 ± 0.04	1.09 ± 0.01
⊥	500	0.94 ± 0.04	1.01 ± 0.04	1.01 ± 0.01

[a] Water content (%, w/w).
[b] Position of the diffusion gradient as compared with the orientation of the meat fibres.
[c] Δ is the time delay between pulsed field gradients as described in the APGSTE diffusion experiment.[17]

marized for each pork sample in Table 3, and the Student's *t*-test probabilities for T_2, magnetization transfer rate, apparent diffusion coefficient and diffusion anisotropy between fresh and frozen/thawed (types I and II) are described in Table 4.

Validity of water T_2 values of fresh pork as measured by MRI

The results from fitting the 'bulk' transverse relaxation curves from fresh pork with one or two components are given in Table 1. The 'bulk' T_2 values obtained confirm that the decay of transverse magnetization in *post-rigor* muscle is best fitted to a bi-exponential model,[13,14] with a short T_2 value of *ca.* 35 ms (*ca.* 90% of the signal) and a long T_2 value of *ca.* 135 ms (*ca.* 10% of the signal). As a result of the great predominance of the former species, the agreement of the average value (*ca.* 40 ms) obtained by mono-exponential fitting coincides with the short T_2 decay component. As explained earlier, this result is important in the context of MRI since it demonstrates that for these specimens mono-exponential fitting under the conditions of the T_2 CPMG imaging experiment provides a good estimate of the average T_2 value in fresh pork (*ca.* 40 ms at 2.35 T); given the similarity of the water contents for frozen/thawed pork, it is reasonable to assume that the same is also true for frozen/thawed pork.

Comparative MRI study of fresh and frozen/thawed pork (Experiment I)

The freezing/thawing procedure (Experiment I) was found to be accompanied by significant decreases in T_1, T_2 and liquid proton density (M_0) values, as shown in Table 2. On the basis of previous NMR studies of meat,[9,13,14] it is reasonable to expect that the T_1 and T_2 values along with the M_0 values for water protons will all decrease as a function of water content. In the present study, the additional water drip consecutive to repeated freezing/thawing cycles [Experiment I: water drip −2% (w/w); Table 2] would contribute primarily

Table 4. *T*-Test probability of the T_1 values, T_2 values, MT rates and apparent water diffusion coefficients along and across the meat fibres in frozen/thawed pork (Experiments I and II) *vs.* fresh pork[a]

	T-Test probability	
Parameter	Frozen/thawed pork (I)	Frozen/stored pork (II)
Transverse relaxation time	<0.001 (9)	0.020 (9)
Magnetization transfer rate	<0.001 (9)	<0.001 (9)
Apparent diffusion coefficient (Δ = 40 ms)		
⊥	0.675 (3)	0.515 (3)
∥	0.654 (3)	<0.001 (3)

[a] Results are considered to be significantly different for *t*-test probability ⩽ 0.05. The numbers of samples are given in parentheses.

to the decrease in the values of those NMR parameters after freezing/thawing (less than 10% decrease on average). In contrast, a significant decrease in the T_2 values was observed after freezing/thawing (see Table 4), despite substantial errors in the T_2 imaging measurements (sometimes as large as 10%, Table 2).

The apparent diffusion coefficients of water were also compared between fresh and frozen/thawed pork (Table 3); the effects of the evolution decay Δ (40, 200 and 500 ms, corresponding to a mean free diffusion path of 9, 20 and 32 μm, respectively) and of the orientation of the fibres (parallel and perpendicular to the diffusion direction) were also investigated. The results summarized in Table 3 show that the water diffusion coefficients generally increased after freezing thawing, probably because the water content decreased as a result of the freezing/thawing protocol used in this study. Although it was expected that the diffusion coefficient would be higher in frozen/thawed pork (Experiment I) than in fresh pork owing to the damage of the cell membranes, this increase was not considered to be statistically significant (Table 4).

Similarly, increasing the mean free diffusion path had little effect on the apparent diffusion coefficient of water in *post-rigor* muscle. It is suggested that, since the range of diffusion paths used in the present experiments (from 9 to 32 μm) is long compared with the size of the myofibrils (*ca.* 1 μm), the main source of restrictions to water diffusion should be observed on a much smaller scale, i.e. that of the myofibrils.

In addition, diffusion anisotropy was not found to be very significant in either fresh pork or frozen/thawed pork (Table 5), even though anisotropy has been clearly observed in living tissues by several workers[21,22] and in brined muscle during onset of *rigor*.[9] Two reasons may explain the results presented here: first, the APGSTE sequence used here is less sensitive to background gradients, and thus to diffusion anisotropy artefacts, than the pulsed field gradient echo (PGSE) sequence used previously;[17] second, diffusion anisotropy may be enhanced by physiological activity in living tissues, which is absent in *post-rigor* muscle. Finally, since the mean free diffusion paths studied here were long compared with the size of the myofibrils, it is possible that the orientation of the meat fibres has a smaller impact on the apparent diffusion coefficient measured at the longer scale of the muscle fibres, rather than at the scale of the myofibrils.

Table 5. *T*-Test probability of the apparent diffusion anisotropy with respect to the meat fibre direction in frozen/thawed pork (Experiments I and II) *vs.* fresh pork[a]

		T-Test probability:	
	Fresh pork	Frozen/thawed pork (I)	Frozen/stored pork (II)
Diffusion anisotropy:			
$\perp$ *vs.* $\parallel$	0.415 (3)	0.323 (3)	0.024 (3)

[a] Results are considered to be significantly different for *t*-test probability $\ll 0.05$. The numbers of samples are given in parentheses.

Attention is now directed to the substantial increase in the MT rate following freezing/thawing (Experiment I, + 28%; Table 2), which indicates that the MT rate is sensitive to some effects of freezing/thawing of pork. By analogy with the effect observed on T_1 and T_2, it is important to note that the increase in the MT rate is inversely related to water content.[23] However, the relative difference between the MT rates of fresh and frozen/thawed pork (Experiment I) is much higher than the difference in moisture content between fresh and frozen/thawed pork (Table 2). It is therefore suggested that the substantial increase in the apparent MT rate in frozen/thawed pork is also enhanced by protein denaturation. Certainly, the hypothesis that the structure of some meat proteins becomes more 'rigid' as a result of protein aggregation is consistent with the known fact that the mechanism of cross-relaxation of water with the macromolecules becomes more efficient as their molecular motion is restricted.[24–26] It is also possible that protein denaturation might expose proton-exchangeable groups, which would further enhance the rate of proton exchange between water molecules and meat proteins. Regardless of the precise mechanisms involved, it is an important fact that the MT rate results obtained in this study allow unequivocal discrimination between pork which is fresh and pork that has already been frozen and thawed.

Comparative MRI study of frozen/thawed pork (Experiments I and II)

Comparisons were also made between MRI results from frozen/thawed pork samples prepared according to the protocols of Experiment I and those of Experiment II. Interestingly, the water content was lower in frozen/stored/thawed pork [Experiment II: water drip −7% (w/w); Table 2] than in frozen/defrosted/frozen/thawed pork [Experiment I: water drip − 2% (w/w); Table 2]. However, the longer period of frozen storage in Experiment II (frozen storage period 12 weeks; MT rate 4.77 s^{-1}) did not induce a significant increase in the proton exchange rate, as demonstrated by the fact that the value of the MT rate in Experiment I was only slightly different (frozen storage period 2 weeks; MT rate 4.44 s^{-1}). This implies that protein denaturation was not significantly enhanced during the 12 week frozen storage period (Experiment II) as compared with that during a 2 week period of frozen storage (Experiment I). Furthermore, no statistically significant increase in the apparent diffusion coefficient was observed after a longer freezing period (Tables 3 and 4).

CONCLUSIONS

This study has demonstrated the routine use of MRI to map T_1 and T_2 values and the apparent MT rate for samples of both fresh and frozen/thawed pork. Comparative analyses of fresh and frozen/thawed pork showed

that both the transverse relaxation time (T_2) and the apparent magnetization transfer (MT) rate of water were sensitive to the damaging effects of repeated freezing and thawing cycles. In particular, the results obtained for fresh and frozen/thawed pork showed that the proton magnetization transfer rate increased significantly after freezing and thawing. It was also found that a longer frozen-storage period did not induce a dramatic change in the NMR parameters, even though some additional water drip loss was observed during that period. Although the water drip losses which accompany freezing/thawing of pork would be expected to induce a decrease in T_2 and an increase in the MT rate, the observed differences are probably enhanced by protein denaturation induced freezing and thawing. Future studies should extend these quantitative MRI analyses to the study of freezing/thawing of other meats, poultry, fish and shell-fish.

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