Journal of Magnetic Resonance **147,** 89–94 (2000)
doi:10.1006/jmre.2000.2163, available online at http://www.idealibrary.com on IDEAL®

Relationships between ^{1}H NMR Relaxation Data and Some Technological Parameters of Meat: A Chemometric Approach

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Received February 7, 2000; revised June 12, 2000

In this paper chemometrics (ANOVA and PCR) is used to measure unbiased correlations between NMR spin-echo decays of pork *M. Longissimus dorsi* **obtained through Carr–Purcell–Meiboom–Gill (CPMG) experiments at low frequency (20 MHz) and the values of 14 technological parameters commonly used to assess pork meat quality. On the basis of the ANOVA results, it is also found that the CPMG decays of meat cannot be best interpreted with a "discrete" model (i.e., by expanding the decays in a series of a discrete number of exponential components, each with a different transverse relaxation time), but rather with a "continuous" model, by which a continuous distribution of T_2's is allowed. The latter model also agrees with literature histological results.**

Key Words: **NMR relaxometry; chemometrics; water holding capacity; meat quality; continuous T_2 distribution.**

INTRODUCTION

Water holding capacity (WHC) is a qualitative parameter of primary importance in the meat industry. In fact, the sensory characteristics of this product, as well as suitability for storage and processing, are strictly influenced by water binding in meat. Drip loss represents a relevant commercial damage caused by weight decrease and by partial loss of the nutritional characteristics of the final product, owing to the relatively high concentration of proteins dissolved in the exudate (about $\frac{2}{3}$ of that found in muscle). Moreover, a high drip loss shortens the shelf life of the product, because of the oxidative and hydrolytic processes promoted by the microorganisms which easily develop in the exudate.

From a histological point of view, water loss has been described as a three-step process consisting of (i) loss of water from within myofibrils due to postmortem shrinkage, (ii) relocation of water from intra- to extracellular compartments, and (iii) subsequent flow of this liquid to the surface (*1*).

Since different states of water exist at the end of this process, it is unfortunate that not many studies have investigated the relationships between WHC, as measured by accepted standard methods, and the NMR relaxation properties of meat (*2*). Indeed, the latter spectroscopic technique permits characterization of the different dynamic states of water through the measure of the transverse proton relaxation time (T_2) (*3, 4*). The experimental procedure is easily carried out by means of the well-known Carr–Purcell–Meiboom–Gill (CPMG) spin-echo technique, where a $\pi/2$ RF pulse, followed by a sequence of equally spaced π pulses, is applied to the sample so as to generate an equal number of spin echoes (*5, 6*). If the intensity of each echo is plotted versus the time elapsed between the $\pi/2$ pulse and detection, an exponentially decaying curve is obtained, in the simplest case following Eq. [1]:

$$I(n\tau) = I_0 \cdot \exp(-n\tau/T_2) \quad n = 1 \ldots N, \qquad [1]$$

where I_0 is the intensity of the free induction decay (FID) that would be recorded immediately after the $\pi/2$ pulse, τ is the time between π pulses, $I(n\tau)$ is the intensity of the nth echo (occurring at $n\tau$ s after the first $\pi/2$ pulse), N is the total number of echoes, and T_2 is the transverse relaxation time of the population of protons that gives the signal.

In muscle, water is contained within isolated compartments (*1*) whose diffusional exchange is slow on the NMR time scale. In each compartment three types of water are recognized: "structural" (s), "multilayer" (m), and "bulk" (b) water (*7, 8*). Due to different interactions with the biopolymers at the compartment's surface, their NMR relaxation properties are very different. Further, when the condition of fast exchange on the NMR time scale is achieved, these three water states contribute a single observed transverse relaxation time per compartment ($T_{2,\mathrm{obs}}$), as summarized by Eq. [2]:

$$T_{2,\mathrm{obs}}^{-1} = F_\mathrm{s} T_{2,\mathrm{s}}^{-1} + F_\mathrm{m} T_{2,\mathrm{m}}^{-1} + F_\mathrm{b} T_{2,\mathrm{b}}^{-1}, \qquad [2]$$

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1090-7807/00 $35.00

where $T_{2,s}$, $T_{2,m}$ and $T_{2,b}$ are the transverse relaxation times of structural, multilayer and bulk water, respectively, and F_s, F_m and F_b are their molar fractions, which depend, in turn, on the compartment shape and dimension.

Although a dispersion of compartment dimensions and shapes (and thus of T_2's) is presumable—and indeed found (*9, 10*)—many authors have modeled the spin-echo decays of meat as the summation of a discrete number of exponential functions (the "discrete" model). By this model, a spin-echo relaxation curve is fitted by an equation that comprises exactly M exponential components, each characterized by a different T_2 ($T_{2,i}$) and intensity ($I_{0,i}$):

$$I(n\tau) = \sum_{i=1}^{M} I_{0,i} \cdot \exp(-n\tau/T_{2,i}) \quad n = 1 \ldots N. \qquad [3]$$

For example, three populations of differently relaxing water were proposed, on the basis of a thorough application of the discrete model, in heart muscle of chicken, pork, and beef (*11*). The presence of three components was more recently confirmed in chicken heart muscle by ^{1}H NMR, but not by ^{2}H and ^{17}O NMR spectroscopy, by which "at least two" water components were detected (*12*). Three components were found also in pork *M. Longissimus dorsi,* but one was classified as "expelled water," because of its long T_2 and too small detected population, and not considered any further during discussion (*13*). In another paper (*14*), again, *L. dorsi* muscle was studied and three to four components were considered necessary to fit time domain NMR signals. Finally, in some other cases, i.e., in frog leg muscle (*15*) and in pork *psoas,* cardiac diaphragm, and *L. dorsi* muscles (*16*), only two water fractions were recognized.

It is possible that the limited—and often changing—number of water populations found in the above muscle tissues is but a symptom of an intrinsic unsuitability of fits by Eq. [3] to the task of interpreting the CPMG decays. Indeed, it has been demonstrated that the high covariance of the relaxation rates and amplitudes in a region of the parameter space close to the global minimum can make Eq. [3] fit equally well—although incorrectly representing—synthetic relaxation curves containing a large number of differently relaxing components (100 or more), so as to resemble a continuous distribution of intensities and relaxation times (*17*). On the contrary, in the latter case a "continuous" model would be more appropriate:

$$I(n\tau) = \sum_{i=1}^{M} I_0(T_{2,i}) \cdot \exp(-n\tau/T_{2,i}) \quad n = 1 \ldots N, \qquad [4]$$

where $I_0(T_{2,i})$ provides a distribution of I_0 intensities for each T_2 component, sampled logarithmically in the interval $T_{2,\min} - T_{2,MAX}$ as shown in Eq. [5] (*17, 18*),

$$T_{2,i} = T_{2,\min} \cdot \exp[(i-1)\ln(T_{2,MAX}/T_{2,\min})/(M-1)]. \qquad [5]$$

A more correct approach to the study of the NMR relaxation properties of meat would therefore imply the inspection of the $I_0(T_{2,i})$ distribution calculated by Eq. [4] as the appropriate way to check whether a simpler model is justified. Unfortunately, this key step is not mathematically well conditioned: distributions tend to display more features than required, thus calling for countermeasures like the introduction of empirical smoothing functions that may yield equivocal results (*18, 19*).

Besides the issue of the correctness of the model used to fit the experimental data, it is reasonable to think that any difference in the WHC of meat samples should be detected by NMR to some extent. Therefore, it would be desirable to measure the correlations between NMR and the WHC parameters without the assumption of any prior model for the spin-echo decays (i.e., by using the *raw, time domain,* relaxation data), thus avoiding any related bias. In this paper we get inspiration from some recently published papers (*20–23*) and use a chemometric approach to test the correlations between the NMR relaxation decays of pork *M. L. dorsi* (LD) and the values of 14 technological parameters commonly used to assess meat quality in the pork industry. Based on ANOVA results, we also give evidence that the discrete model fails in describing appropriately the CPMG experimental data measured in meat.

MATERIAL AND METHODS

Animals and slaughter procedures. The animals used were 44 heavy pigs, castrated males, and gilts of average weight equal to 134 kg, 10 months old. Genetic types, feeding phases, and rearing conditions were those admitted by the guidelines of Parma Ham production (*24*). The pigs were slaughtered according to the customary procedures used in a commercial slaughterhouse. The carcass weight was measured 25 min postmortem. At cutting, the various meat quality analyses were made in the dissected LD, at the level of the last two ribs.

Technological and chemical meat analysis. Temperature and pH values were measured with a OHM pH meter (Model HD8602) equipped with a combined electrode (Double Bore, Hamilton) at 45 min (pH_1) and 24 h (pH_2) postmortem. Meat color was determined using a Minolta Cr-300 apparatus, according to the Cielab system (L*, a*, b*). The water holding capacity of LD samples was measured at 24 h postmortem: drip loss was determined according to Honikel (*25*) and by filter paper press experiments (*26*). Cooking loss was measured according to Honikel (*25*). Samples used for the determination of the chemical composition were taken at cutting and stored at −20°C until analysis. Moisture, crude protein (Kjeldhal method), and intramuscular fat (Soxhlet method) content were determined according to standard methods (*27*).

NMR analysis. Three meat cylinders, approximately 30 mm long and 35 mm^2 in cross-sectional area, were excised

from each LD muscle and inserted into 10-mm-diameter NMR tubes in such a way as to arrange the muscle bundles parallel to the external magnetic field. Each sample was thermostatically held at 25°C for 10 min prior to the NMR measurement. The transverse relaxation time (T_2) of water protons was measured in triplicate at a frequency of 20 MHz using the CPMG sequence (*5, 6*) with a Bruker Minispec PC/20 spectrometer. Each measurement comprised 168 points, corresponding to 168 echoes with an interpulse spacing of 3 ms. Forty-nine scans (with a 5-s recycle delay) were accumulated so as to obtain a S/N ratio always between 1000 and 1700. Discrete and continuous curve fittings were carried out, respectively, with the programs EXPON and UPEN (*19*). In the latter case the first 5 echoes were discarded to get rid of instrumental instabilities that may bias the results. Default values for all of the parameters required by the programs were used throughout the calculations.

Chemometric analysis. One-way ANOVA calculations, one for each measured technological parameter of meat, were carried out with the program Statistica for Windows. The normalized spin-echo decays were divided into three groups, depending on the value of the technological parameter (TP) being tested: the first group comprised spin-echo decays of samples whose corresponding TP belonged to the average TP value ± one standard deviation interval; the other two were constituted by samples exceeding the above interval in one or the other direction. Parameters not passing the Shapiro–Wilk test of normality were normalized beforehand, according to accepted procedures (*28*). Principal component analysis (PCA), based on the decomposition of the covariance matrix, was carried out with a Fortran program written for the purpose and based on the subroutine "pca.f" taken from the Statlib repository (http://www.stat.unipg.it/pub/stat/statlib/multi/pca). After PCA, the scores corresponding to samples excised from the same pig were averaged so to yield a single set of scores per animal. A principal component regression (PCR) was then calculated with Statistica for Windows, using an appropriate number of PCs as independent variables and each technological parameter of meat as a dependent variable.

RESULTS AND DISCUSSION

Analysis of Variance

As a preliminary analysis of the NMR data, the ANOVA technique was applied to check whether a discrimination among the spin-echo decays was possible, based on the value of each TP. In this way, portions of the CPMG curves that vary systematically within the three groups (see Material and Methods) give high F values and appear "structured" (*21*). The results are shown in Fig. 1. Although the majority of the plots show points that lie well above the $F = 3.00$ value needed to achieve significance at the 95% level, it is apparent that not all the TPs have the same discriminative power. In fact, at least

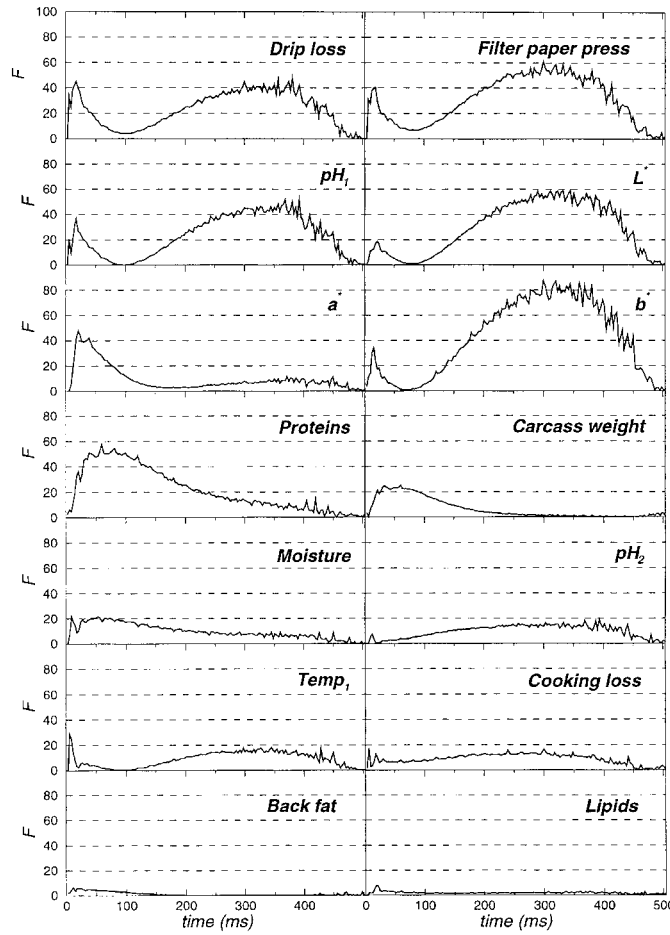

FIG. 1. ANOVA of the CPMG spin-echo decays based on the variation of each of 14 technological parameters of pork *M. Longissimus dorsi.*

two of them (namely lipid content and back fat) seem far less effective than the others, as judged by their almost null ANOVA plot. The remaining plots are much more structured.

The ANOVA plots show broad peaks whose maxima fall in three main regions of the time domain centered at about 20, 60, and 350 ms. It is thus possible to admit the presence of at least three broad populations of water (acting on three different regions of the CPMG decay) which would arise from three main types of water compartments in pork LD muscle. Actually, by applying the discrete model we found three relaxation components, one spanning the 20- to 85-ms T_2 range (hereafter called T_{2b}), with the highest population (65–90%), and two minor components, spanning the ranges 85- to 260-ms (T_{2a}) and 3- to 20-ms (T_{2c}), which share the remaining signal intensity. The first two relaxation components agree with the available literature data about NMR relaxometry of LD (*13*), whereas a T_2 component as short as T_{2c} is often thought of as being due to the effect of nonfreezable water in the hydration shell of the biopolymers such as proteins (*29–31*).

It is difficult to explain all of the features evidenced by the ANOVA plots on the basis of the discrete model only. In fact,

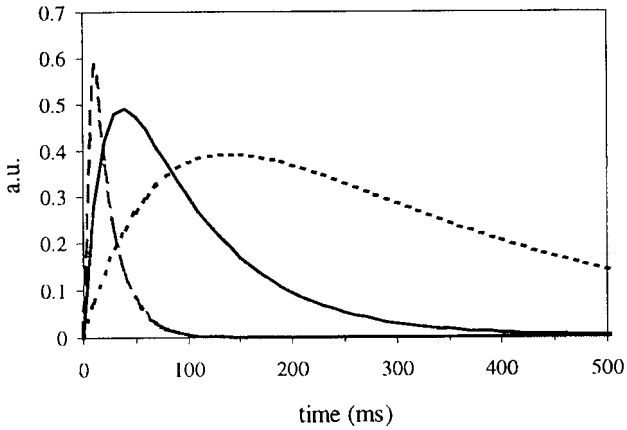

FIG. 2. Plot of the function $\exp(-n\tau/T_{2,\mathrm{MAX}}) - \exp(-n\tau/T_{2,\mathrm{min}})$ (see text) for T_{2a} (dotted, $T_{2,\mathrm{MAX}} = 260$ ms; $T_{2,\mathrm{min}} = 85$ ms), T_{2b} (solid, $T_{2,\mathrm{MAX}} = 85$ ms; $T_{2,\mathrm{min}} = 20$ ms), and T_{2c} (dashed, $T_{2,\mathrm{MAX}} = 20$ ms; $T_{2,\mathrm{min}} = 3$ ms). The curves evidence the only regions of the CPMG decays that can be significantly affected if a linear correlation between T_2 and each TP is assumed.

if some level of interdependence is present between the value of a TP and T_{2a}, T_{2b}, or T_{2c}, high F values should be detected at those points of the ANOVA curve that correspond to CPMG data points which are very much influenced by a change of the T_2 value. These points can be adequately described by the function $\exp(-n\tau/T_{2,\mathrm{MAX}}) - \exp(-n\tau/T_{2,\mathrm{min}})$ (where MAX and min refer to the upper and lower T_2 bounds detected by discrete model analysis), i.e., by the derivative of $\exp(-n\tau/T_2)$, taken with respect to T_2, and subsequently integrated between $T_{2,\mathrm{min}}$ and $T_{2,\mathrm{MAX}}$ (Fig. 2). By comparing Fig. 2 to Fig. 1 it is apparent that the proposed correlation can only account for the shorter times part of the ANOVA plots, the broad feature centred at about 350 ms remaining unexplained. As far as the latter peak is concerned, it is reasonable to relate its presence to the effect of further T_2 components whose decay is slower than T_{2a}. A component of this kind has been actually found by Tornberg *et al.* (*13*), yet in a percentage as low as 1%. Although none of our calculations detected any slow component that could be assigned to "expelled water," the possibility that under our conditions the discrete fitting could overlook this slowly relaxing signal must be taken into account.

Calculations based on the continuous model strengthen this view. In fact, as it appears from the typical continuous T_2 dispersion reported in Fig. 3, each meat sample does not contain three distinct kinds of water, but rather a huge number of them which cluster to yield two broad water populations, i.e., a main one, centered at about the previously calculated T_{2b} and spanning 16–84 ms, and a minor, slowly relaxing one, about one order of magnitude wider. The two types of water are not separated, the slower relaxing one revealing itself as a long tail extending up to a half second at the right side of the main narrower peak. This tail comprises the so-called "extracellular water," usually identified by the longest T_2 component found by discrete analysis only (*11*, *16*) and the expelled water found by Tornberg *et al.* (*13*). Thus, contrary to the discrete model, the continuous model is able to detect water whose T_2's are compatible with the formation of significant features in the longer times region of the ANOVA plots. Interestingly, the only plots that show marked long-times features are the ones related to the TPs that measure WHC. This is another indication of the actual dependence between the NMR relaxation behavior of meat and the state of water within the samples.

It is worth noting that the present interpretation of the CPMG NMR signal of pig LD muscle, based on the continuous model, corroborates the optical microscopy evidences by Offer and Cousins (*9*), who reported that the sizes of the extracellular water compartments have a large dispersion around a mean value and that, in many instances, they can be as small as the intracellular gaps. Therefore, the part of the T_2 distribution corresponding to extracellular water (at higher T_2's) is expected to be broader than the one corresponding to intracellular water (at lower T_2's). This is what we actually observe.

Principal Components Regression

Having found reasons to consider the discrete model oversimplified with respect to the continuous model, a problem is raised about the way of quantifying the correlations between the NMR signal of LD and each of the TPs studied. This is a trivial task when a discrete fitting is performed: in that case zero-order data structures are produced (i.e., numbers: signal intensities and T_2's), which can be immediately tested against the TP of interest (*22*, *32*). If, instead, the NMR properties of a sample are represented by a first-order data structure, as it is the case for a continuous T_2 dispersion or a T_2 spin-echo decay itself, the *whole* data structure must be used in the regression. Principal components analysis of the first-order structures is then particularly useful in that it reduces the structure dimensionality to the minimum required number of numerical elements (the scores) that are necessary to express the information contained into the raw data in terms of the principal compo-

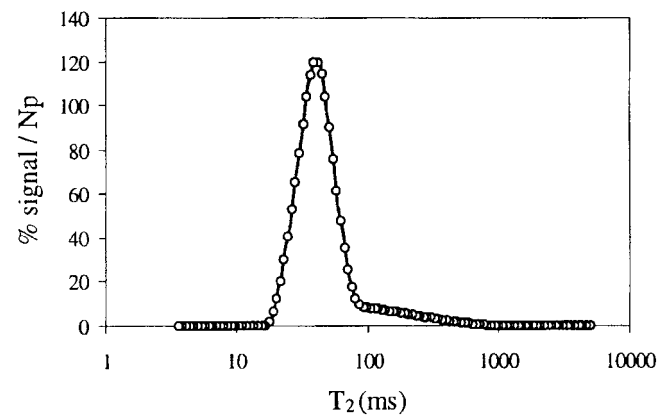

FIG. 3. Continuous T_2 relaxation plot yielded by the program UPEN (*19*). The unit on the *y* axis gives the percentage of NMR signal per Neper, i.e., logarithmic time unit.

TABLE 1

Regression Coefficients (*r*) of the PCR Carried out by Calculating the Linear Regression TP = $a_1 \cdot s_1 + a_2 \cdot s_2$ for 14 Technological Parameters of Meat

Parameter	*r*
Drip loss	0.74***
Filter paper press	0.71***
pH_1	0.71***
Proteins	0.63***
L*	0.61***
b*	0.59***
Cooking loss	0.43*
$Temp_1$	0.39*
a*	0.33
Lipids	0.27
Back fat	0.25
Carcass weight	0.19
pH_2	0.13
Moisture	0.12

Note. Here s_1 and s_2 are the first two scores obtained by PCA (see text). ***Significant at the 99.9% level. *Significant at the 95% level.

nents (the loadings). The scores are eventually used as independent variables in a principal component regression.

If two or more first-order data structures are independently measured representations of the same sample property, it is possible to interchangeably use them in PCR. This is not the case when T_2 spin-echo decays and T_2 dispersions are considered, since the latter derive from the former ones via the continuous fitting procedure and may thus contain additional noise or bias. Raw T_2 spin-echo decays were therefore directly submitted to PCA, thus obtaining two main principal components explaining up to 96% of the total variance (respectively, 82 and 14%). Subsequent PCR yielded the results reported in Table 1.

It is interesting to note that the parameters measuring WHC of meat (i.e., drip loss and filter paper press) are those better correlated to the NMR signal of LD. This confirms our expectations, since the NMR signal is physically related to the state (bound or free) of the water embedded within the meat fibers as are the TPs measuring drip loss. Relevant correlations between NMR signal against pH_1, protein, and chromatic coordinates (L* and b*) are also found: this confirms the well-known relationships among the latter meat parameters during postmortem mechanisms (*33*). The pH fall rate is known to influence the structure of myofibrils in meat, which is of relevant importance for the water holding capacity and the color of the meat (*1*). The paleness of meat is caused by increased light scattering as a consequence of pH decline after death and it is thus inversely proportional to pH_1. Meat color also depends on pigment concentration, amount of intramuscular fat, and oxidation–reduction status (*34*). The poor a* correlation coefficient (compared to the one obtained by a regression against b*) reveals that any CPMG signal variation is much more related to a blue–red than to a yellow–green change of color. Also, the meat protein content is strictly correlated to NMR signal, indicating a clear dependence of the latter on the soluble fraction of meat protein. This fraction is eluted into the exudate lost during storage and it is particularly detrimental to the commercial appearance of meat.

By looking at Table 1 it is also apparent that some of the TPs that had previously displayed structured ANOVA plots lack a significant correlation with the CPMG decays. This evidence should not be taken as proof of the failure of the preliminary analysis of the spin-echo decays based on the ANOVA: those TP could simply lack a *linear* correlation with the CPMG decays, which is the only kind of relationship yielded by PCR. We are currently considering the joint use of chemometrics and neural networks (*35*) to overcome the problem of nonlinearity and to obtain, hopefully, better correlations.

CONCLUSIONS

In this paper it has been made clear, on the basis of one-way ANOVA evidences, that the NMR data of pork *M. L. dorsi* meat obtained through CPMG spin-echo experiments cannot easily be correctly interpreted when analyzed in terms of a summation of a discrete number of exponential components, as it is often found in the literature. A continuous model is more appropriate and clearly shows the presence of two water populations, each spanning a broad range of transverse relaxation times. By resorting to chemometrics it has been possible to obtain an unbiased measure of the correlations between the CPMG data and 14 analytical descriptors of meat commonly used in meat technology.

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