A Novel Method For Measuring Of Fat Content In Low-weight Tissue: A NMR Study

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Article ID: WMC001368
Article Type: Research articles
Submitted on: 15-Dec-2010, 02:05:29 PM GMT  Published on: 16-Dec-2010, 02:17:18 PM GMT
Article URL: http://www.webmedcentral.com/article_view/1368
Subject Categories: OBESITY
Keywords: weight bias, meat traits, muscle lipids, data calibration
How to cite the article: Kaerst S, Schmitt A, Brockmann G. A Novel Method For Measuring Of Fat Content In Low-weight Tissue: A NMR Study. WebmedCentral OBESITY 2010;1(12):WMC001368

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Illustration 2
A Novel Method For Measuring Of Fat Content In Low-weight Tissue: A NMR Study

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Abstract

We developed a protocol to accurately determine low fat content in small tissue samples like e.g. in mouse muscles, using nuclear magnetic resonance spectrometry (NMR). Measurements of very low-weight samples (< 0.5 g) tended to yield higher fat values, which was caused by a systematic, sample-mass-dependent distortion. We developed a new protocol and a calibration equation to account for the weight-bias, when the device is used for the analysis of low-weight samples. The correction of the raw data was accomplished by a calibration with a linear regression model. The adjustment based on the analysis of test material in eight dry-mass (0.025 g - 0.500 g) and six fat percent levels (3.04% - 7.63%). Observed NMR results and the results of a chemical analysis were compared. Using the modified protocol and the developed equation, the minor fat differences in tissue samples below 0.500 g dry-mass, down to 0.050 g could be detected accurately. The protocol provides a rapid way to repeatedly measure a high number of low-weight, low-fat samples with a minimum of handling. We demonstrate the application of the new protocol for the determination of differences in the intramuscular fat content between selected mouse strains.

Introduction

In genetic research, appropriate methods for the accurate phenotyping of different organisms are crucial to elucidate the association between genes and traits. Depending on the subject of interest and the available resources, it can be necessary to modify an existing method or to establish completely new methods of analysis. In this work, we present the adjustment of a new protocol for the rapid measurement of crude fat in mouse muscles, using nuclear magnetic resonance (NMR) spectroscopy. This protocol will also provide the determination of fat content in other small tissue samples. We are interested in genes, which influence certain muscle traits including the intramuscular fat content (IMF). Our experiments are performed in mice. The determination of IMF in mouse muscles is a challenge because the fat content is low (about 2% of the moist-mass) and the amount of tissue per animal is limited. Especially in cross-bred experiments (crosses between two different mouse strains), each sample is unique in terms of the genetic composition of the animal and, therefore, sample pooling is not an option. Furthermore, repeated measurements from a given sample are desired for reasons of data quality.

An appropriate method had to be established to rapidly measure the muscle weight and intramuscular fat for a high number of samples. Different methods exist to measure fat content in tissue samples. The gravimetric ether extraction method [1] is referred to as the standard method, but there are also other methods like infrared spectrometry [2], the gas chromatography based Caviezel method [3] or the supercritical CO2 extraction method [4]. In rodents, also the staining of lipids in histological specimen was used to estimate the fat content [5]. However, most methods are time-consuming and expensive. NMR spectrometry is a fast alternative method for the determination of overall fat content [6]. The technology has been applied in medicine, research and in the food industry over the last decades [7; 8; 9]. NMR spectroscopy allows the detection of fat in a given sample by discriminating signals from lipid-bound protons and signals of protons bound in other compounds such as proteins and carbohydrates. The signal volumes scale linearly with concentration, which allows quantification of fat amounts.

We developed a method using NMR spectroscopy to determine the fat content in small tissue samples with varying tissue mass. An additional challenge was that the muscles differed in mass. Depending on the particular type of the muscle and the individual body weight, the muscle mass ranged from 0.1 g to 1.6 g moist-mass, corresponding to 0.025 g to 0.400 g dry-mass, which dramatically influenced the NMR results. We found that measurements of low-weight samples tended to result in distorted high fat content values as compared to heavier samples. Samples with the same fat content yielded different results depending on their weight. This made it impossible to compare the fat contents of muscles between mice of different size. We analyzed the error shift to see, whether the distortion was random or systematic. Using test samples of different masses and fat percentages for measurements of fat content with the
NMR device and chemical analysis, we provided evidence that the distortion was systematic. Therefore, we derived a calibration equation that permits to derive the ‘true’ fat percentage from the raw data by considering the nature of the distortion and the sample dry-mass.

**Methods**

NMR Device. All NMR measurements were performed with the SMART Trac System (CEM, Kamp-Lintfort, Germany). The system consists of three subunits (analyzer, processor and magnet). The analyzer contains the user interface and a microwave with an integrated balance. It is connected with the processor-subunit and the magnet. The permanent magnet is thermally stabilized and has a magnetic field strength of 0.47 Tesla that corresponds to a proton resonance frequency of 20 MHz. The radio frequency generator provides a pulse power of 250 W [13].

Sample Preparation. For the establishment of a gold standard with different fat contents, we generated homogeneous test materials from mixtures of chicken breast filet (low IMF) and pork loins (high IMF). Visible fat was removed, chicken meat and pork were diced into approximately 2.0 cm³ cubes and thoroughly homogenized Homogenates were used pure, as either chicken meat or pork, or mixed in four weight ratios to obtain test samples at six different levels of fat content. The fat contents of these prepared test materials were then measured chemically according to Weibull-Stoldt (Bilacon GmbH, Berlin, Germany). To determine the fat content in dry-mass, the water content in the test samples was measured. Samples were dried either by forced air oven or by freeze-drying (Spearman correlation coefficient between NMR data obtained with both drying methods was r = 0.95). The difference between moist-mass and dry-mass was taken as the water content [14]. Pure chicken breast and pork loin homogenates had 74.68% (±0.20) and 74.73% (±0.10) water contents, respectively. According to the chemical analyses, the six levels had 3.04%, 3.56%, 5.26%, 6.29%, 7.08% and 7.63% fat in the dry-mass, respectively. These data were used as a gold standard for the measurements of the same test materials with the NMR device. For the measurements with the NMR device, compact portions of the test material with the six fat levels were shaped in the respective moist-masses of 0.10 g, 0.20 g, 0.30 g, 0.40 g, 0.50 g, 0.70 g, 1.00 g and 2.00 g (± 0.01 g). Three portions were generated per weight and fat level. Finally, there were 48 groups (with three specimens per group) of dried test material in six fat content levels and eight weight levels with 0.025 - 0.500 g dry-mass.

**NMR Measurements.**

In the system that we used, a low field proton NMR spectrometry is applied for the detection of fat in dried samples. According to the manufacturer’s specifications, 2.0 g to 5.0 g of moist material with low-fat content, e.g. raw meat is required per measurement. In the original standard protocol, samples are initially homogenized, attached to a glass fibre pad, and subsequently dried by an integrated microwave and balance subunit, before they are placed into the NMR subunit via a special plastic tube [10; 11; 12]. This standard protocol had several drawbacks for our application. Firstly, the homogenization of mouse muscles is impractical and would have caused a considerable loss of material. Secondly, repeated measurements of one sample were not possible, since the samples stick irreversibly to glass fibre pads cannot be measured twice in this protocol. We modified the manufacturer’s protocol, which allowed us to operate with non-homogenized low-weight samples and to obtain more than one measurement of the same sample.

These modifications included a) the drying of the sample in an external forced air oven (Heraeus ‘UT 6200’), Hanau, Germany) instead of using the microwave unit in the SMART Trac system, b) weighing of samples with an external balance (Sartorius ‘AC 210 P’, Göttingen, Germany, accuracy ± 0.0001, connected to the SMART Trac system) instead of the balance within the microwave subunit, c) free positioning of the sample inside the trac-tube instead of the fixed attachment at a glass fibre pad, d) repeated measurements of the externally dried samples, and e) a correction of the raw data with an external program, using a regression equation.

The test samples of the 48 groups with three specimens per group were taken out of the forced air drying oven separately in succession, weighed immediately on the external balance and placed into the prepared trac-tube inside of the SMART Trac NMR subunit. The trac-tube was closed at one side, using one half of a glass fibre pad (CEM, Kamp-Lintfort, Germany), wrapped into a plastic film (CEM, Kamp-Lintfort, Germany) and put firmly at one end of the trac-tube to carry the sample during the NMR measurements. This allowed repeated measurements of a sample. The top of the trac-tube was covered with a 7 ml weighing boat (VWR International, Germany). The first NMR measurement of a sample was manually initiated after two minutes of adaptation time. During this time, the sample adapted to the temperature of the magnet (40.8 - 41.0 °C). This NMR...
measurement took 64 seconds. Subsequently, the sample was taken out of the tube, weighed again and the measurement was repeated. The temperature adaptation time for the second measurement was reduced to 20 seconds. All test samples were measured twice to yield information about the method’s precision as technical variability and repeatability. The means were used to derive the equation for the data correction.

Calibration Equation. The raw data of the SMART Trac system NMR measurements below the normal range had to be calibrated for the sample mass. In our case, measurements made with the chemical analysis were regarded as gold standard. In order to derive a calibration equation between the raw NMR measurements for fat content \( y \), the results of the chemical analysis for the fat content of the test material \( x \) and the sample dry-mass \( z \), we set up the following linear model:

\[
y = a + b * x + c / z \quad \text{(equation 1)}
\]

The coefficients \( a \), \( b \) and \( c \) were determined in a regression analysis with the linear model function, lm, from the statistical analysis package R [15].

Animals. We examined muscles from male and female mice of the Berlin Muscle Inbred (BMMI) strains (Department for Crop and Animal Sciences, Humboldt-Universität zu Berlin, Germany) using the modified protocol given above. The BMMI strains have been selected for high muscle mass since 1978 and were inbred for more than 20 generations at the time of sampling. In particular, we used 8 to 26 male and female mice of the strains BMMI806 and BMMI816. All animals were fed with a standard breeding diet ('Altromin breeding diet no. 1314', Lage, Germany) and kept according to the German animal welfare regulations. The mice were sacrificed at the age of ten weeks. Musculus longissimus (ML) and Musculus quadriceps (MQ) were collected and stored at -20 °C. Statistics. NMR measurements of fat content in mouse muscles were corrected according to the calibration equation. Corrected data were analyzed with the procedure 'Mixed' along with the estimation method REML of the SAS software package (SAS System for Windows, Release 9.1.3 and 9.2). Strain, parity, litter size and sex were used as fixed effects. P-values were calculated by Tukey adjustment and the significance level was set to \( \alpha = 0.05 \). Distortion-dependent correlations for the measurements of the test materials were calculated using the SAS procedure ‘Corr’ with Spearman’s rank correlation.

Results

Three test samples of six fat and eight weight levels (48 groups) were measured to analyze the influence of the sample weight on the NMR results. We observed a clear dependence of the NMR results on the sample weights (Spearman’s \( r = -0.68 \), \( p < 0.001 \)). Samples from the same fat level yielded higher fat percentage values if they were from a lower weight class. In particular, sample weights below 0.100 g dry-mass yielded the most distorted results among all weight groups analyzed. To describe the distortion, we rearranged equation 1 as follows:

\[
x = \frac{-a}{b} + \frac{y}{b} - \frac{c}{b * z} \quad \text{(equation 2)}
\]

where \( x \) is the corrected ('true') fat value, \( y \) is the NMR measurement value and \( z \) is the sample dry-mass.

With the data of the chemical analysis as a reference (gold standard), the regression coefficients were determined to be: \( a = 1.214314 \), \( b = 0.80046 \), \( c = 0.198752 \) (adjusted \( R^2 = 0.99 \)). Using equation 2, we could correct the NMR raw data for the sample mass. Hence, the weight-dependent distortion of the measurements was eliminated. Without this data correction, differences in the sample weight would largely bias the fat content measurements in a weight scope below 0.5 g dry-mass (Illustration 1).

To assess the technical and environmental influence on the quality of data, we performed two subsequent NMR measurements of every sample. Precision, defined as repeatability of measurements from the same sample, ranged from 0 to 0.93 percentage-points difference and depended strongly on the sample mass. The precision increased with increasing sample weights (\( r = 0.67 \), \( p < 0.001 \)). The highest loss of precision and accuracy occurred below 0.100 g dry-mass. But, even in the smallest sample weight levels of 0.025 g and 0.050 g dry-mass, the six different fat levels could be distinguished. The analysis of repeated measurements from samples between 0.050 g and 0.500 g dry-weight revealed an average precision of 0.11 percentage-points between two measurements of the same sample, which was sufficient for our tests. This weight scope can be considered a ‘safe range’ for the suggested calibration, whereas for lower mass precision was impaired up to an average range of 0.39 percentage-points for repeated measurements in samples of 0.025 g dry-mass. Corresponding to the repeated measurement results, we found that the variation within the same test material group (same fat and weight level) depended on the sample weight, too. The standard deviations within the 48 test groups, each
consisting of three samples, increased with decreasing sample masses (Spearman’s \( r = -0.36, p < 0.01 \)). This observation could result from the process of the test material preparation, but, it mainly mirrors a lower accuracy in low-weight samples (Illustration 2). When we applied the modified protocol to determine the IMF of two BMMI strains (BMMI806 and BMMI816) and used equation 2 to correct the raw data results, we found significant differences between these two strains. IMF of the M. longissimus (ML) was significantly higher in male BMMI806 (10.56% ± 1.36) compared to BMMI816 males (6.71% ± 1.21, \( p < 0.0001 \)). We found a similar tendency for higher IMF in female BMMI806 (9.79% ± 1.97 vs. 8.28% ± 1.68 in BMMI816) (Illustration 3).

Discussion

NMR spectroscopy is a rapid and convenient new method to measure the total fat content of a high amount of small tissue samples. The advantages are compelling, especially as compared to chemical analysis via ether-extraction. With our method, measurements can be performed repeatedly for the same specimen with a minimum of material, time and handling. However, we observed a systematic, sample-mass-dependent distortion in the measurements of muscle fat content in samples below 0.5 g dry-mass with the used NMR device. A likely explanation for this flaw is the high sensitivity of the NMR system to water molecules. An absorption of water from air humidity by the sample during the short period between the end of the drying process and the measurement could lead to a distortion in the measurement. The water protons’ signals interfere with the signal from the fat protons [16]. This would affect smaller samples to a higher degree, because of a bigger surface per volume, and could explain the curved slope of the distortion. Although the sample weights did not give a hint on water uptake between the two subsequent measurements per sample, we cannot exclude this as a cause for the distortion. Another possible explanation for the distortion could be the device-internal computation of the raw signal. Since less sample-matter emits signals of lower intensities, the relation between noise and signal becomes increasingly disadvantageous with decreasing sample weight. An additional point can be the timing for record of the fat-protons’ signal during the measurement. The early signals from protons of carbohydrates and proteins are filtered and only the signal from the later occurring lipid-bound protons is recorded. The time point, when the signal recording is started is determined by the filter for the different T2 relaxation times of the various component and molecule types. It is also possible that the filter and the pulse time settings are not suitable in the area of lower sample weight. Since the T2 relaxation times depend on spin-spin adjacencies and also strongly on the mobility of the molecules, it is conceivable that the absorption of water from the air humidity has an effect on the mobility and therefore the relaxation behaviour of the molecules. Assuming that this causes a certain signal-alteration, a weight-dependent distortion would occur, because internally the sample mass data is set off against the signal for the data output as fat percentage. The significant differences in the IMF between the two BMMI strains despite identical environmental conditions suggest genetic reasons for variations in this trait. BMMI806 and BMMI816 are wild-type for myostatin, a gene that influences muscle growth and intramuscular fat [17]. Therefore, we assume that additional genetic factors, besides myostatin, are involved in the development of IMF. Those factors could control the storage of lipids directly within the muscle cells or the composition of muscle tissue from different cell types, e.g. by the distribution and the amount of adipocytes [18; 19].

Conclusion(s)

From our study we conclude that NMR spectrometry can be used to determine fat content in non-homogenized samples with small amounts of material, such as mouse muscles or muscle tissue biopsies. Compared to other established methods, the suggested set-up rapidly yields reliable data for a high number of low, but varying, weight, low-fat samples along with a simplified handling. We could reliably detect fat contents of 1.0 - 2.5% in small tissue masses down to 0.20 g moist-mass, corresponding to about 4.0 - 10.0% in the respective 0.05 g dry-mass. Our approach expands the application range of NMR spectrometry for samples with a mass below the usual required minimum. The high data collection rates along with the minimal usage of material make this protocol a very efficient alternative in the field of fat content determination.

Abbreviation(s)

NMR - Nuclear Magnetic Resonance
IMF - Intramuscular Fat Content
Acknowledgement(s)

The project was supported by the Deutsche Forschungsgemeinschaft (Project BR1285/8-1). We acknowledge critical discussion of the technique of NMR spectroscopy with Wolfram Gronwald and Feride Severcan. The authors also wish to thank Carsten Berndt, Annett Kannegießer and Uwe Müller for their support.

References

13. URL: http://www.cem.de/documents/pdf/produkte/englisch/SMARTtrac.PDF
Illustrations

Illustration 1

Correction of the measurement distortion. NMR measurements of the test material (dots) have been related to the results of the chemical analysis (“Gold Standard”) and the sample dry-mass. (a) Accuracy decreased non-linearly with decreasing sample weight. (b) NMR measurements of the test material have been corrected according to equation 2. The distortion is eliminated and accuracy for low-weight sample measurements is improved.
Illustration 2

NMR results for fat percentages in the test material.

<table>
<thead>
<tr>
<th>Fat Level</th>
<th>Fat Percentage (%)</th>
<th>Sample Weight Levels (dry-mass in g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td>1</td>
<td>3.04</td>
<td>11.42</td>
</tr>
<tr>
<td></td>
<td>± 0.50 ± 0.20 ± 0.04 ± 0.07 ± 0.07 ± 0.11 ± 0.12 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.56</td>
<td>12.10</td>
</tr>
<tr>
<td></td>
<td>± 0.09 ± 0.05 ± 0.12 ± 0.11 ± 0.04 ± 0.06 ± 0.09 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.26</td>
<td>12.59</td>
</tr>
<tr>
<td></td>
<td>± 0.33 ± 0.03 ± 0.19 ± 0.08 ± 0.07 ± 0.22 ± 0.04 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.29</td>
<td>14.00</td>
</tr>
<tr>
<td></td>
<td>± 0.71 ± 0.23 ± 0.11 ± 0.35 ± 0.04 ± 0.11 ± 0.17 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.08</td>
<td>14.15</td>
</tr>
<tr>
<td></td>
<td>± 0.34 ± 0.13 ± 0.08 ± 0.06 ± 0.24 ± 0.04 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7.63</td>
<td>15.03</td>
</tr>
<tr>
<td></td>
<td>± 0.40 ± 0.18 ± 0.17 ± 0.28 ± 0.32 ± 0.27 ± 0.38 ± 0.16</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means and standard deviations, * determined by chemical analysis.
Illustration 3

IMF measurements in mouse muscles. NMR raw results vs. corrected values, IMF is shown according to dry-mass (means & standard deviations). Raw data of M. longissimus (ML) and M. quadriceps (MQ) of the BMM806 (a) and BMM816 (b) strain were corrected for sample masses using equation 2 and ML results were compared (c). Asterisks indicate significant differences between the strains (** = p < 0.0001).
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