Technical note: Evaluating nuclear magnetic resonance spectroscopy for determining body composition in Holstein dairy calves using deuterium oxide dilution methods

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ABSTRACT

Deuterium oxide (D₂O) dilution methods have been used to assess body composition in live animals. Estimated body water content can be used to predict body fat and protein, and thus, the amount of energy reserves. It is an alternative method to direct chemical analysis and considered a noninvasive technique that is economical and repeatable. Deuterium oxide use is considered easy, safe, and accurate; however, the traditional methods of analyzing D₂O are expensive, tedious, and time consuming. The objective of this study was to evaluate the potential for using nuclear magnetic resonance spectroscopy (NMR) to determine body composition in Holstein dairy heifers. Nuclear magnetic resonance is less expensive and requires minutes to calculate the percentage of D₂O in the blood. This study used 24 newborn dairy heifer calves blocked by birth and randomly assigned to 1 of 3 treatments: (1) 446 g dry matter (DM) of a conventional milk replacer (MR) [CON; 20% crude protein (CP), 20% fat], (2) 669 g DM of a moderately high protein MR (MOD; 26% CP, 18% fat), or (3) 892 g DM of a moderately high protein MR (aggressive, AGG; 26% CP, 18% fat). All calves had free-choice access to starter and water. Both MR and starter were medicated with decoquinat. During weaning (d 43 to 49), the morning MR feeding ceased. On d 50, all MR feedings ended but starter and water intakes were continuously recorded until d 56. When calves were 50 d of age, a baseline blood sample was taken followed by injection of 300 mg of D₂O/kg of body weight in sterile physiological saline (0.9%). The syringes containing the D₂O in physiological saline were weighed before and after administration to record

the actual dose of D₂O injected gravimetrically. After injection, the D₂O was allowed to equilibrate with body water for 1 h. Six blood samples were taken over 6 d (1/d) at 1630 h to estimate the dilution of the tracer. The plasma was aspirated and stored at –20°C until further D₂O analysis. This new method was validated using 4 calf plasma samples that were sent to an outside laboratory for measurement using an independent validation method. We detected no differences in total body water, protein, fat, or mineral content in calves fed CON, MOD, or AGG; however, results demonstrated that the D₂O dilution technique and analysis by NMR is an appropriate and easy method to estimate water, protein, ash, and fat in young heifers.

Key words: deuterium oxide, nuclear magnetic resonance, body composition, heifer

Technical Note

The composition of lean body mass is relatively constant across species, and knowing body water content can be used to understand the composition of body fat, protein, and minerals (Byers, 1979). Water makes up about 73% of the lean body; it is the largest component and has become the choice for measuring body composition; however, measurement of the size and kinetics of aqueous pools in the digesta of ruminants is complicated by unknown contributions of variable magnitude from the digestive tract (Byers, 1979). Deuterium oxide (D₂O) methods have been used to differentiate these pools such that other body compartments can be estimated for growth (Byers, 1979).

Traditionally, the most accurate way of determining body composition was to slaughter the animal and then grind and analyze the entirety of the animal's tissues (Jesse et al., 1976). This method is precise but precludes measurement of further growth. In order to measure changes in composition over time, studies have conducted serial slaughter techniques where a large population of similar animals are allocated to treat-

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ments with a representative group on each treatment slaughtered at desired intervals of time or size (Byers, 1979). However, this method assumes that all animals are similar in body composition and that all respond similarly to treatments, making this method less accurate or valid. It is necessary to develop methods to precisely estimate body composition and composition of growth as it occurs in the live animal.

Numerous indirect methods have been used to determine total body water and are based on the dilution principle of water-soluble tracers such as antipyrine or urea, but they give rise to errors because tracers are bound to proteins, are rapidly metabolized and excreted, or are slowly and incompletely distributed to all the body water components (Novak, 1967; Schoeller et al., 1980). The best and most frequently used tracers have been water labeled with either tritium or deuterium (Novak, 1967; Schoeller et al., 1980); however, the use of deuterium has the disadvantage of tedious and time-consuming analysis, whereas the use of tritium involves a radiation hazard (Schoeller et al., 1980).

Deuterium oxide dilution methods can be used to predict estimated body water content, which can then be used to predict body fat and protein (Panaretto and Till, 1963). Deuterium oxide dilution methods for estimation of total body water have been used for assessment of body composition for many species including pigs, beef steers, dairy cows, dairy heifers, sheep, and goats (Novak, 1967; Shipley and Clark, 1972; Byers, 1979; Schoeller et al., 1980; Shields et al., 1983; Andrew et al., 1995; Auchtung et al., 2002; Rius et al., 2005) and are considered noninvasive techniques that are economical and repeatable. The D₂O dilution method measures the body water turnover rate and is safe, nonradioactive, and accurate. It was the method used to measure body water turnover in doubly labeled water studies of long-term metabolic rate (Schoeller et al., 1980). Estimated body water content can be used to predict body fat and protein, and thus, the amount of energy reserves (Panaretto and Till, 1963). Panaretto and Till (1963) found that total body fat and total body water were inversely correlated in mature castrated male goats ($r = -0.97$). Rius et al. (2005) compared D₂O methods and direct chemical analysis to assess body composition at 5 and 7 mo of age. In 5-mo-old heifers, correlations between estimates of body protein, water, and mineral contents as determined by D₂O dilution methods and direct chemical analysis of body tissue were 0.86, 0.85, and 0.76, respectively, but fat values were not correlated ($r = -0.068$). Those authors were unable to accurately estimate body composition using the D₂O dilution method for 7-mo-old heifers and attributed this to the gastrointestinal tract in young animals not being fully developed.

Studies have demonstrated that feeding calves a milk replacer (**MR**) with >25% CP at an increasing rate with age up to 900 to 1,200 g of DM/d can facilitate rapid and efficient early lean growth and allow for increased body protein deposition and growth rate without excessive fattening (Diaz et al., 2001; Tikofsky et al., 2001; Blome et al., 2003; Bartlett et al., 2006). However, in these studies, calves were slaughtered. Using D₂O might be an easy, inexpensive, and valid method for determining the composition of growth in calves fed using these aggressive MR feeding programs instead of direct chemical analysis. Deuterium oxide dilution methods will also allow for measurement of further growth. To date, nuclear magnetic resonance spectroscopy (**NMR**) has not been used to estimate body composition through D₂O dilution methods in animals. An NMR-based method has been used to determine total body water in humans (Khaled et al., 1987). Those authors validated the method against a standard infrared absorption procedure using a tracer dose of D₂O and concluded that the NMR method was fast, accurate, needed only a small dose of D₂O, and did not require any sample preparation. The objective of the current study was to evaluate the potential for using NMR to determine body composition in Holstein dairy heifers.

Twenty-four Holstein heifer calves (initial BW, mean $\pm$ SD) of 41.6 ± 4.8 kg were blocked by birth date and randomly assigned at birth to 1 of 3 treatments in a randomized complete block design. The treatments were (1) 446 g DM of a conventional MR (**CON**; 20% CP, 20% fat), (2) 669 g DM of a moderately high protein MR (**MOD**; 26% CP, 18% fat), or (3) 892 g DM of a moderately high protein MR (aggressive, **AGG**; 26% CP, 18% fat). Both MR products were provided by Provimi North America (Brookville, OH) and medicated with decoquinat. The 56-d study was conducted from August 2013 to March 2014. Calves were fed a 20.7% CP textured starter (Provimi North America) and water ad libitum starting at 2 d of age and continuing for the entire 56-d trial. Initial BW and skeletal measurements were taken before calves started MR treatment. Calves on CON were fed 227 g of 20:20 (CP:fat) MR reconstituted to 10.7% solids (at 0800 and 1700 h) from d 2 to 42 (1.89 L twice daily). Calves on MOD were fed 340 g of 26:18 (CP:fat) MR reconstituted to 13% solids (at 0800 and 1700 h) from d 2 to 42 (2.27 L twice daily). Calves on AGG were fed 340 g of 26:18 (CP:fat) MR reconstituted to 13% solids (at 0800 and 1700 h) from d 2 to 6 (2.27 L twice daily) and then 454 g of 26:18 (CP:fat) MR reconstituted to 13% solids (at 0800 and 1700 h) from d 7 to 42 (3.03 L twice daily). On d 43 to 49, calves on all treatments were fed once daily at 0800 h. Calves were fed MR and

water in pails. On d 50, all milk feedings ceased. Calves remained on trial for 7 d after weaning for a total of 56 d. Results from the intake, growth, digestibility, and N efficiency are reported in Chapman et al. (2017).

When calves turned 50 d of age, calves were weighed on a platform scale (VS-2000, A and A Scales LLC, Prospect Park, NJ) to determine the required dose of D₂O (99.9 atom % D, Sigma-Aldrich Corp., St. Louis, MO). Blood samples were collected via jugular venipuncture using 10-mL tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin using 22-gauge needles. A baseline blood sample was immediately followed by injection of 300 mg of D₂O/kg of BW in sterile physiological saline (0.9%). The syringes containing the D₂O in physiological saline were weighed before and after administration to record the actual dose of D₂O injected gravimetrically. After injection, the D₂O was allowed to equilibrate with body water for 1 h. Six blood samples were taken over 6 d (1/d) at 1630 h to estimate the dilution of the tracer. Blood samples were centrifuged at $1,278 \times g$ at 4°C for 20 min (5430R, Eppendorf, Hamburg, Germany). The plasma was aspirated and stored at -20°C until D₂O analysis.

For analysis of D₂O, plasma samples were thawed and aliquots (750 µL) of plasma were transferred to 5-mm borosilicate glass NMR tubes (Wilma LabGlass, Vineland, NJ). All ²H NMR spectra were acquired on a Varian Unity INOVA 500 NMR spectroscope (Varian, Palo Alto, CA) equipped with a broadband probe and tuned to the deuterium (²H) resonance frequency (76.71 MHz). Quantitative experimental parameters included a 1.7-s relaxation delay, 4.3-s acquisition time, and a fixed receiver gain for all data sets. Standards of 0, 0.229, 0.446, and 0.901 mg/mL D₂O were prepared and analyzed by NMR before the calf plasma samples to generate a standard curve. The deuterium peak of the D₂O was reference to 4.79 ppm (Fulmer et al., 2010). Data processing and analysis were performed using Mnova NMR processing software (Mestrelab Research, Escondido, CA). The deuterium resonance of D₂O at 4.79 ppm was integrated and the absolute integration values reported. Chemical shifts are reported in parts per million relative to D₂O at 25°C and the deuterium chemical shifts scale was assumed to be identical to proton.

The percentage of D₂O found in the calf plasma was calculated from the standard curve. Baseline plasma samples taken before injection of D₂O had traces of D₂O; these were subtracted from each of the plasma samples taken after D₂O injection to account for background. The natural logarithm of each calf's plasma sample D₂O concentration was then plotted to determine the best-fit line of the D₂O dilution curve (Figure

Figure 1. Example of calf deuterium (²H) plasma concentration versus time after deuterium oxide (D₂O) injection.

1). Total body water was calculated using the amount of D₂O injected into the calf and dividing it by the y intercept. Body water percentage was then calculated by dividing the total body water by the mass of the calf. Determinations of total body protein and total body fat were made using the equations described by Panaretto and Till (1963).

Estimates of body of water, fat, CP, and ash were analyzed as a randomized complete block design using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ij} = \mu + B_i + T_j + E_{ij}$$

where Y_{ij} = the dependent variable; μ = the overall mean; B_i = the random effect of block i ($i = 1, \dots, 8$); T_j = the effect of MR type or amount, the j th treatment (CON, MOD, or AGG); E_{ij} = the residual error $\sim N(0, \sigma_e^2)$, and σ^2 is the population standard deviation and e is Euler's number, the base of the natural logarithm.

Degrees of freedom were calculated using the Kenward-Roger approximation option of the MIXED procedure. Least squares means were determined for each treatment and the Tukey's option of SAS was selected to differentiate least squares means among treatments. Significant treatment effects were determined at a probability of $P \leq 0.05$ and trends were defined as: $0.05 < P \leq 0.10$.

Results are shown in Table 1. We detected no differences in total body water, protein, fat, or mineral content in calves fed CON, MOD, or AGG. Four calf samples were sent to Metabolic Solutions Inc. (Nashua, NH) to validate the NMR analysis of D₂O in calf plasma samples by cavity ring-down spectroscopy (CRD). Plasma samples were analyzed for deuterium enrichment by CRD spectroscopy using a liquid water

isotope analyzer with automated injection system, version 2 upgrade (Los Gatos Research, Mountain View, CA). Before analysis, plasma proteins were removed by adding approximately 5 mg of zinc sulfate monohydrate to 25 to 50 μ L of plasma in a microcentrifuge tube. Samples were mixed and centrifuged at $4,700 \times g$ to precipitate the proteins. The plasma protein-free supernatant was injected 6 times and the average of the last 3 measurements used for data analysis. A standard curve was run before and after samples to calculate deuterium enrichment as delta per mil (parts per thousand) relative to Vienna Standard Mean Ocean Water (VSMOW). Intra-run precision was less than 2 delta per mil (parts per thousand) and inter-run precision was less than 3.5 delta per mil. The 4 calf D₂O plasma data from CRD analysis showed total body water percentages of 76.5, 75.2, 77.8, and 70.7%, and the results for the current study using NMR were 67.5, 74.5, 77.7, and 77.0%, respectively. Figure 2 shows the validation of the NMR method used in the current study versus the CRD method. This figure shows an R^2 of 0.947, signifying a strong correlation between the 2 methods for analyzing deuterium in calf plasma. Figure 3 shows the residuals of the CRD from the current NMR method used in this study. The majority of data points observed are below zero, which indicates that the NMR method in the current study was more sensitive than CRD at detecting deuterium in calf plasma samples. This is a valid method for estimating body composition of dairy calves as it involves only one step (thus, lowering the error) and accounts for background deuterium occurring naturally in the environment.

Many studies have shown that body composition followed a similar pattern when D₂O dilution methods were compared with direct chemical analysis. Rius et al. (2005) observed correlations between estimates of

Figure 2. Quantity of deuterium (^2H) in 31 calf plasma samples comparing cavity ring-down spectroscopy (CRD) and nuclear magnetic resonance spectroscopy (NMR).

Figure 3. Residuals of deuterium (^2H) in 31 calf plasma samples [cavity ring-down spectroscopy (CRD) – nuclear magnetic resonance spectroscopy (NMR)]. The y-axis shows the quantity of ^2H in milligrams per milliliter from CRD minus quantity of ^2H in milligrams per milliliter from NMR.

body protein, water, and mineral contents. Auchtung et al. (2002) used D₂O dilution methods to determine milk intake in calves and found that a smaller number of samples was sufficient to obtain an accurate D₂O dilution curve compared with Woodford et al. (1984), who collected blood samples every 10 min for the first 4 h after injection of D₂O. Auchtung et al. (2002) only collected 2 blood samples after the D₂O was allowed to equilibrate with body water for 2 h. This was attributed to the gastrointestinal tract in young animals not being fully developed.

Commonly, the analysis of D₂O in plasma consists of detection of D₂O by infrared spectroscopy using the procedure of Byers (1979) as well as other methods; however, these methods are laborious and can be costly. Nuclear magnetic resonance spectroscopy is a viable and easy option to quantify D₂O in plasma for body

Table 1. Body composition of Holstein calves fed 1 of 3 milk replacer (MR) programs¹ estimated using deuterium oxide dilution methods and analyzed with nuclear magnetic resonance spectroscopy

Variable	Treatment			SEM	P-value
	CON	MOD	AGG		
Water, %	69.5	68.4	71.6	1.22	0.19
Ash, %	3.67	3.91	3.12	0.28	0.15
CP, %	17.7	17.4	18.3	0.32	0.16
Fat, %	9.15	10.2	6.95	1.25	0.19
BW, kg	67.6	69.8	73.8	3.06	0.38
Water, kg	47.9	47.4	52.2	2.37	0.31
Ash, kg	2.47	2.81	2.27	0.26	0.31
CP, kg	12.2	12.1	13.4	0.63	0.31
Fat, kg	6.11	7.56	5.04	1.04	0.24

¹Treatments: CON (control) = 446 g (DM) of a conventional MR (20% CP, 20% fat), MOD (moderate) = 669 g (DM) of a moderately high protein MR (26% CP, 18% fat), and AGG (aggressive) = 892 g (DM) of a moderately high protein MR (26% CP, 18% fat).

composition. This method can be used in studies feeding greater amounts of protein or more milk to calves to accurately determine the effects of these feeding programs on body composition while using a small number of animals and eliminating the need for direct chemical analysis. Using D₂O may be an easy, inexpensive, and valid method for determining the composition of growth in calves fed these aggressive MR feeding programs. The D₂O dilution method with analysis by NMR is an appropriate and easy method to estimate water, protein, ash, and fat, in young heifers.

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