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Research Note

Quantification of protein and phosphorus in livestock feed using mobile NMR sensor technology

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Low-field NMR sensor technology is proposed for accurate and operationally simple on-farm or laboratory determination of protein and phosphorus constituents in livestock feed. The total phosphorus content is determined directly on the native sample, while quantification of digestible protein involves enzymatic digestion here adapted to provide the total protein content. Comparison with traditional laboratory reference analysis for the total protein and phosphorus contents of 31 feed samples (including various grains, feed mixtures, and commercial feed products for cattle, pigs, horses, poultry, and sheep) demonstrate the feasibility and good accuracy of the NMR measurements.

1. Introduction

Increasing demands for production of sufficient amounts of food to feed a globally increasing human population while simultaneously reducing the environmental footprint of food production and ensuring animal welfare impose huge demands on improving agricultural production procedures (Tona, 2018). This applies to all parts of the production cycle, where an obvious point of focus could be the enormous amounts of livestock feed consumed in animal production. Correct feeding has direct consequences for production

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output (quality, nutritional and health value, amount of meat, milk etc.), animal health and welfare, and environmental footprint. The same applies to the production profitability influenced by the livestock feeding being the major production cost for modern farmers (Banhazi et al., 2012). Following this line of thinking, an effective, healthy, and more sustainable livestock production/husbandry is in strong need of information-based adaptive control of the animal nutrition to manage correctly dosed feeding. Key to real-time adaptive control and optimization of the nutrition cycle, often involving many sources of feed components, is the availability of reliable analytical methods to assess the composition of the applied feed portions. A major challenge is substantial variation in composition depending on the origin of feed and feed components in feed mixtures calling for regular on-site/real-time analysis.

Two important parameters in optimizing animal growth, while simultaneously minimizing environmental pollution (by leaching of N and P to the environment through animal manure) and approaching sustainable use of finite natural resources (P), are the feed content of protein and phosphorus (Perry et al., 2003). For complex mixtures where information may not be provided from suppliers, farmers may obtain accurate, but typically only representative information, through submission of samples to external laboratories or rely on less accurate results on-site using near infra-red spectroscopy (NIRS) sensor technology (Beć et al., 2021; González-Martín et al., 2006; Modroño et al., 2017; Soldado et al., 2013). Laboratory analysis may be costly and time consuming not only because of the sample submission process, but also through the use of demanding wet-chemistry based Kjeldahl or Dumas methods for quantification of protein estimated from the total-nitrogen content, and harsh acid digestion followed by inductively coupled plasma (ICP) methods for quantification of phosphorus. Such methods can typically not be used on-site farms. This may be achieved using NIRS technology which, however, is less accurate and as an indirect method highly dependent on databases with detailed information from samples of similar composition and origin. Both may be difficult to obtain for the given location or for feeds composed of constituents from local as well as purchased ingredients without precise track of origin. Furthermore, NIRS is known to have difficulties in measuring the content of phosphorous.

In this Communication, we introduce mobile, low-field nuclear magnetic resonance (NMR) sensor technology for precise and reliable on-farm or laboratory analysis of livestock feed with focus on protein and phosphorous determination. We note that evaluation of other parameters such as salts, fat, and moisture may also be feasible using the same technology. NMR relies on detection of the response of specific nuclei with a nuclear spin towards radiofrequency irradiation when subjected to a strong external magnetic field. The method has found widespread application through expensive and highly specialised instrumentation to dedicated NMR laboratories in universities, hospitals (known as magnetic resonance imaging, MRI), and large-industry laboratories. Much less focus has been devoted to low-cost, robust, and non-expert usage in direct production control, as here introduced for farmers livestock feed analysis. Relative to other technologies, NMR has the great advantage that it provides quantitative analysis of the bulk material (not only the surface as applying to optical methods such as NIRS), is a direct method not relying on databases, and does not need time-consuming running calibrations.

Focusing on on-farm analysis, it is of fundamental importance that analytical methods are easy and safe to apply (i.e., does not require harsh chemicals etc., needing safety certification) in a farmer's environment. This is in general also an advantage in a laboratory setup. This demand is particularly easy to meet for phosphorous quantification, which solely amounts to administering a sample with the livestock feed (directly or with addition of minor amounts of water) in a sample tube for ^{31}P analysis in a low-field NMR instrument. For accurate determination of the protein content, we propose an easy protocol involving an initial digestion of the sample with enzymes to cleave proteins into amino acids or small peptides prior to ^{14}N analysis by low-field NMR. This has the additional advantage that information is obtained for the digestible part of the protein in the livestock feed.

2. Material and methods

The proposed method involves a mobile, multinuclear, low-field NMR sensor which earlier has found applications in a variety of contexts, including monitoring of heavy fuel oil onboard ships (Sørensen et al., 2014), laboratory and on-line quantification of nutrients in animal slurry (Jensen et al., 2021; Sørensen et al., 2015), oxygen analysis (Sørensen et al., 2016), benchtop solid-state NMR (Sørensen et al., 2018), and analysis of salt, fat, and protein in food products. The sensor is based on a 1.5 T Halbach-array permanent magnet, a digital field-programmable gate array (FPGA) spectrometer, a fast-tunable probe with bore diameter of 9.2 mm, and acquisition of ^{31}P Carr-Purcell-Meiboom-Gill (CPMG) (Meiboom & Gill, 1958) and quadrupolar CPMG (QCMPG) (Larsen et al., 1997) data (see supplementary material for further details). For comparison the investigated livestock feed samples were analysed in an external laboratory (AGROLAB LUFA GmbH, Kiel, Germany) using Kjeldahl and ICP for protein and phosphorous determination, respectively.

To form the basis for measuring the protein content in digested feed samples, Fig. 1 demonstrates the applicability of low-field NMR to quantitatively assess the nitrogen content in dissolved amino acid/small molecule samples. The figure correlates ^{14}N QCPMG signal intensities (vertical axis) to known nitrogen contents (horizontal axis) for 22 aqueous solutions of amino acids and small nitrogen containing molecules. Reflected by a squared correlation coefficient (R^2) of 0.993, the figure demonstrates excellent match with known nitrogen concentrations for a series of samples including 18 of the most common amino acids (see supplementary material for details).

This result may translate directly into protein content determination by ^{14}N low-field NMR upon cleavage of proteins into amino acids and small peptides through digestion. We note that without cleavage, the ^{14}N NMR signals will be subject to spectral broadening and very fast relaxation due to large ^{14}N quadrupolar coupling interactions and dipole–dipole coupling to protons influencing detection and quantification

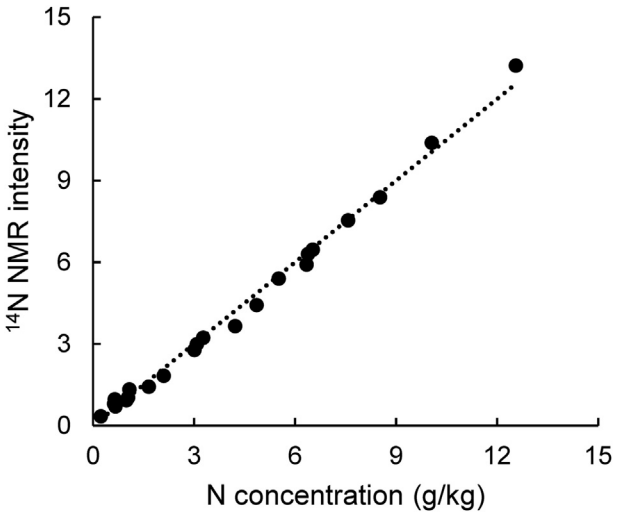

Fig. 1 – Calibrated ^{14}N NMR intensity versus calculated nitrogen concentration for 22 aqueous solutions of small molecule compounds. The samples include 18 distinct amino acids, urea, a dipeptide (GlyGly), and two mixtures of amino acids. The ^{14}N QCPMG signals were recorded at 4.63 MHz using a recycle delay of 30 ms and accumulation over 1 h for each sample. See supplementary material for a list of included samples and details on NMR experiments.

for large molecules where molecular motion is not sufficiently fast to average anisotropic nuclear spin interactions. Protein cleavage may be accomplished using acid hydrolysis, alkaline hydrolysis, or enzymatic hydrolysis (Álvarez et al., 2013; Badadani et al., 2007; Mótyán et al., 2013; Tsugita & Scheffler, 1982). We focus here on the latter approach since this method is relatively easy to realise in practice, is associated with low costs, and most importantly, handling of harsh chemicals is not required. Focusing on an easy work-flow appropriate for farmers and on-site operation, but also advantageous in a laboratory setup, we investigated various approaches to enzymatic digestion. For a total-protein quantification, we found incubation at 50 °C for 24 h with two commercially available enzyme products Protamex® and Flavourzyme® (Novozymes, Bagsværd, Denmark) to work well. These enzymes are easy to handle, stable, and for the proposed doses associated with very low cost in practical applications.

Figure 2 provides a schematic overview of the proposed procedures for low-field NMR livestock feed protein and phosphorus quantification. The procedure involves initial milling/grinding of the sample to improve homogenization and break down macroscopic shells (a simple coffee grinder may apply). Phosphorous analysis may be performed either on the native milled sample loaded directly to the NMR measuring tube, or by adding small amounts of water to hydrate the sample before transferring to the measuring tube. For protein determination the preprocessing step involves adding enzymes and subsequent digestion. In both cases, the final step involves NMR analysis providing the quantitative information for farm production control, statistics, or reporting.

The proposed low-field ^{14}N/^{31}P NMR based quantification of protein and phosphorus was demonstrated for 31 feed samples including various grains, commercial (complete and supplementary) feed products for cattle, pigs, horses, poultry, and sheep as well as prepared mixtures for pigs and dairy cows (see supplementary material for a full list of samples). The feedstuff was obtained from a variety of feed providers present on the Danish market and from local farms. One sample was fresh feed mixed for dairy cows (water content 63%), whereas the rest were dry, milled products, and dried crops (water content 0–17%). To ensure representative subsampling, all dry samples were ground initially to ensure a homogeneous sample. Hereafter, aliquots of each sample were sent for analysis of protein (total nitrogen times 6.25) and phosphorus at an external commercial laboratory (Agrolab LUFA GmbH, Kiel, Germany). As a supplement, the content of protein was also determined at a laboratory at Aarhus University (see supplementary material for details). Similarly, aliquots of each sample were kept for NMR analysis. The single fresh sample were divided in aliquots without prior grinding and sent fresh to laboratory analysis. For the fresh sample, the aliquot for NMR analysis were frozen and ground just before it was prepared for analysis.

For protein quantification, a weighted amount of about 2 g for each feed sample was mixed with 18 ml of enzymes solution (1% Protamex® and 0.625% Flavourzyme®) in 120 ml containers, and thereafter incubated at 50 °C for 24 h. Subsequently, subsamples of 2.1–2.3 ml were loaded to sample tubes of perfluoroalkoxy alkane (PFA) with an inner diameter of 8 mm. Care was taken that possible air (bubbles) were located in the top of the tubes where they have no effect on the measurements. Finally, the samples were analysed in the NMR sensor with the sample volume adjusted to the NMR coil length to avoid influence from precipitation.

For phosphorous quantification, samples were prepared by transferring 0.7–1.4 g of raw ground feedstuff directly to PFA sample tubes noting the actual weight applied for each sample. For measurement, these samples were positioned with the sample volume centered in the NMR coil ensuring that the entire sample is located inside the detection volume (rf coil length 38 mm, about 2 ml sample volume). This procedure enables the obtained NMR intensities to be used relative to the actual amount of analysed feedstuff, which eliminates effects

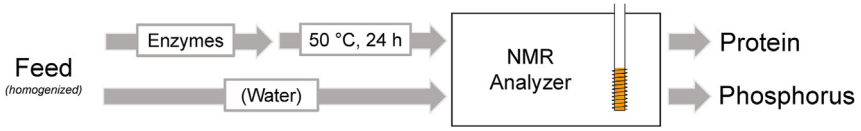

Fig. 2 – Schematic illustration of procedures proposed for low-field NMR quantification of protein (upper track) and phosphorus (lower track) in livestock feed samples.

of different densities for dry samples and inhomogeneity over the individual samples. For validation of the alternative procedure using hydrated samples, a set of samples were also prepared where about 6 g feed (2 g for one sample, see supplementary material) was mixed and stirred with 18 ml demineralised water for each sample, whereafter subsamples were transferred to PFA sample tubes and positioned in the NMR sensor using same volume and position as described above for the samples for protein quantification. The digested samples for protein analysis and the hydrated phosphorus samples were analysed with a measuring time of 1 h for each sample, whereas phosphorus on native, non-hydrated samples were measured for 15 min per sample (see supplementary material for details).

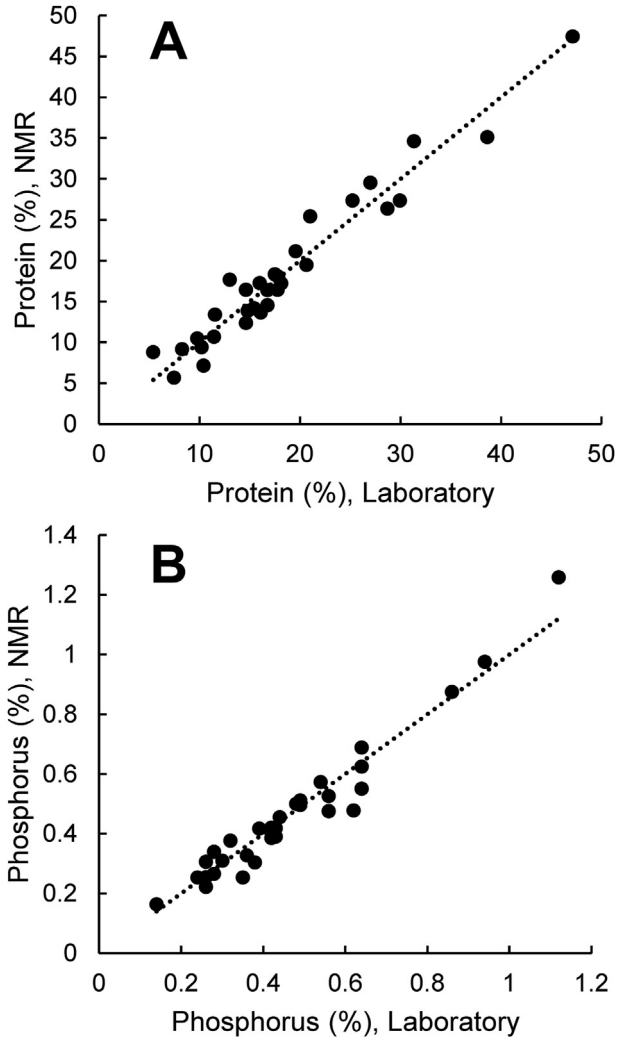

Fig. 3 – Results of NMR measurements versus results from laboratory analysis for 31 feed samples. A) Calibrated ^{14}N NMR results of enzymatically digested samples versus mean of protein analysis (Kjeldahl total nitrogen times 6.25) at two laboratories, and B) calibrated ^{31}P NMR results using native feed samples versus laboratory analysis of total phosphorus. See text and supplementary material for details on samples, laboratory analysis, and NMR experiments.

3. Results and discussion

Figure 3 gives correlation plots correlating protein (A) and phosphorous (B) quantities determined by low-field NMR and laboratory measurements. The correlations are associated with R^2 coefficients for best linear regression of 0.943 for protein and 0.942 for phosphorus reflecting a good match between the mobile on-site possible NMR measurements and traditional laboratory analysis. For phosphorus, the hydrated samples (not shown) show similar good correlation with an R^2 coefficient of 0.921. For these correlations, the NMR measurements were calibrated to the laboratory reference data by a calibration (scaling) factor to provide 1:1 translation between NMR measurements and laboratory measurements as desired for on-site analysis. Comparing measurements on pure reference samples (aqueous solutions of serine for protein and K_2HPO_4 in 10 mM ferric EDTA for phosphorus) with measurements for feed samples reveals that a reduced intensity of the nitrogen/phosphorus signal is observed for the feed samples. This amounts to 58%, 74%, and 93% for protein, phosphorus with native samples, and phosphorus with hydrated samples, respectively, and is ascribed to unfavourable relaxation properties combined with experimental parameters optimised to ensure best signal to noise ratio, while a minor part may also relate to incomplete solubility and possibly incomplete digestion (see supplementary material for details).

We note that, the proposed method for protein analysis may readily be adapted to quantify the actual digestible protein content for specific animal types, rather than the total protein content, if a digestion method mimicking *in vivo* conditions is chosen. Furthermore, NMR has the capability to discriminate various molecular constituents (e.g., based on ^{14}N NMR relaxation times correlating with the molecular mass). With a sufficient signal-to-noise ratio, this may be employed for specific quantification of certain (groups of) amino acids and non-protein nitrogen such as nitrate and ammonium, which may thereby allow for bypassing the Jones factor for direct protein quantification.

4. Conclusions

In this Communication, we have demonstrated quantification of total/digestible protein and total phosphorus in livestock feed using mobile, low-field NMR sensor technology suitable for on-site application at farms or similar. We anticipate that this will provide the basis for a new analytical tool for livestock feed analysis in agriculture, which may also be adapted to other instances in agriculture, the food and pet food industries, and chemical industries with demands for quantification of proteins, amino acids or phosphorus.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Patents submitted in the area. The

authors are affiliated to NanoNord A/S selling the applied NMR sensor for industrial and scientific purposes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biosystemseng.2022.02.004.

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