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Simultaneous Quantification of Oil and Protein in Cottonseed by Low-Field Time-Domain Nuclear Magnetic Resonance

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Abstract Modification of cottonseed quality traits is likely to be achieved through a combination of genetic modification, manipulation of nutrient allocation, and selective breeding. Oil and protein stores account for the majority of mass of cottonseed embryos. A more comprehensive understanding of the relationship between lint quality, lint yield, and embryo reserve accumulation will assist breeders in their efforts to improve seed value. Here we report the development of a rapid, nondestructive, simultaneous method for quantifying oil and protein levels within cottonseed by low-field ^1H time-domain nuclear magnetic resonance (TD-NMR). This approach is suitable for a minimal amount of seed and represents an accurate (oil $R^2 = 0.998$, protein $R^2 = 0.95$), noninvasive alternative to conventional, time-consuming chemical extractions. We demonstrate the value of this approach by surveying seed reserve content, identifying extremes of either protein and/or oil, in two sets of diverse germplasm.

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Introduction

Development of high-quality cottonseed through breeding programs and applied research is essential to the improvement of desired seed traits that directly affect utility and economic value. Currently the cotton industry is in a state of recovery from a recent worldwide economic downturn and increasing emphasis on the production of soybean, corn, and grains in the USA. However, the acreage allotted to cotton production in the USA entering 2011 is expected to increase from 12.40 million bales in 2009/2010 to 15.48 million bales in 2010/2011 [1]. While fiber yield and fiber quality are still the most profitable attributes, there is increasing emphasis on understanding the impact of seed reserve contents (oil, protein, carbohydrates, gossypol, etc.) and subsequent modification through breeding programs and genetic modification.

Protein and oil within oilseeds have traditionally been quantified through destructive, time-consuming, chemical-extraction-based protocols. The two most recognized methods for protein quantification, Kjeldahl [2] and Dumas

[3], determine the protein content of food and feeds through quantification of total available nitrogen. The Kjeldahl method uses sulfuric acid in the presence of catalysts to digest the protein contents through a series of reactions ultimately producing a source of ammonium ions that can be used to calculate the total amount of nitrogen and predicted amount of protein (Protein = $N \times 6.25$) [2]. The Dumas method, also known as the generic combustion method of crude protein, relies on the combustion of protein contents at very high temperatures (~ 900 °C) to liberate gaseous nitrogen that can be detected and used to calculate total protein content (Protein = $N \times 6.25$) [3]. Oil quantification through organic-based total lipid extraction followed by gravimetric analysis, the Soxhlet method, or fatty acid methyl ester analysis is labor intensive [4], involves large amounts of organic solvent to completely extract all lipid contents, and has quantitative limitations.

The development of specialized spectroscopic instruments including near-infrared spectroscopy (NIRS) and nuclear magnetic resonance (NMR) has made it possible to quantify seed reserve contents nondestructively with precision and accuracy as high as traditional chemical measurements. Although NIRS, based on the absorption of electromagnetic energy through molecular vibrations, has successfully been used to quantify several oilseed components including protein, oil, fiber, and moisture [5], this technique has very limited penetration depth, especially for dark samples such as cottonseed. As a result, the chemical composition predicted by NIRS provides information regarding the surface or near-surface fraction of the seeds. NMR is based on the absorption of energy by an atomic nucleus changing its spin orientation in a magnetic field [6]. Manipulation of all the hydrogen nuclei within the entire bulk of the oilseed samples using radiofrequency pulse sequences [time-domain nuclear magnetic resonance (TD-NMR), also known as low-field NMR] produces characteristic relaxation signals for protein, carbohydrate, moisture, and oil [7]. This technique is not affected by the color or opacity of the samples and thus provides true bulk estimation of compositional information even for large samples. Oil and moisture contents have been previously determined for several oilseeds by pulsed-field NMR [8, 9] including the oil content in cottonseed with very high accuracy [10]. The prediction of protein content has been more difficult due to the quick relaxation times of protons in the solid crystalline protein state and orientation effects of asymmetric seed structure. However, protein content has been predicted with moderate accuracy for soybean and rapeseed [9] as well as in fish feed [11] using pulsed-field NMR sequences.

Herein we describe the development of a procedure for nondestructive, simultaneous quantification of oil and protein contents in cottonseed using TD-NMR. Previous research has established a weak negative correlation between protein and oil for several cotton (*Gossypium* spp.) genotypes [12–14] with weak correlations between fiber yield and oil content or fiber yield and protein content [15, 16]. Here we demonstrate the value of this approach by surveying seed reserve content in two sets of diverse cotton germplasm. This method should prove valuable as a rapid screening tool for routine, nondestructive estimation of seed protein and oil in cottonseed, coproducts of lint production with potential for improvement. Ongoing examination of the correlations underlying seed quality parameters will provide breeders with valuable information for future germplasm development [17].

Experimental Procedures

Cottonseed Material

Two sets of germplasm yield trials were conducted to provide cottonseed samples for protein and oil analyses. The first trial was conducted in 2008 at the Clemson University Pee Dee Research and Education Center near Florence, SC as part of the Regional Breeder Testing Network (RBTN) trial (http://cottonrbtn.com/). Annually, the RBTN trial is conducted to evaluate the agronomic performance of experimental germplasm lines developed in public breeding programs representative of the entire US cotton production area. In 2008, the RBTN trial included 31 germplasm lines representing nine public US breeding programs covering the majority of the US cotton production region in addition to three commercial cultivars. The 34 entries were evaluated in a four-replicate randomized complete block design. The second trial was conducted in 2005 at the Clemson University Edisto Research and Education Center near Blackville, SC. This trial was part of an experiment to evaluate the genetic properties of the diverse, 70-year-old USDA-ARS Pee Dee breeding program [18]. The trial included 82 Pee Dee germplasm lines and cultivars representing the history of the Pee Dee breeding program with the addition of six commercial cultivars. The 88 entries were evaluated in a three-replicate alpha-lattice design containing 22 incomplete blocks of size four. In both trials, plots were two rows, 10.6 m × 76 cm. Each plot was harvested using a spindle-type mechanical cotton picker, and total cottonseed weight was recorded. A 25-boll sample was hand-harvested from each plot prior to harvest to determine lint percent and to provide a cottonseed sample. All boll samples from both trials were ginned on a common 10-saw laboratory gin, and lint percent was determined by dividing the weight of the lint sample after ginning by the weight of the cottonseed

sample before ginning. Lint yield was calculated by multiplying the lint percent by the cottonseed yield.

Instrument Setup

NMR signals were recorded on a Bruker minispec mq20 pulsed NMR analyzer (Bruker Corporation, The Woodlands, TX). The instrument operated at 20 MHz proton frequency, with probe dead time of approximately 40 μs, magnet temperature of 40 °C, 16.66 μs 90° pulse length, and 33 μs 180° pulse length. Pooled representative fuzzy cottonseed samples (approximately 1.5 or 3.0 g) were equilibrated to 40 °C in specially designed 18-mm-diameter NMR glass tubes in a forced air oven (Techne Hybridizer HB-1D, UK). Samples were packed down to occupy minimal volume and maximize sample homogeneity. For some experiments, where noted, a probe with shorter dead times was tested for use in the mq20 instrument.

Hahn Spin-Echo Application

Initial total oil content for the germplasm surveys was obtained with the Hahn spin-echo [19] application that used pulse separation of 3.5 ms, receiver gain of 61 dB, and recycle delay of 2 s. Three grams of a commercial delinted variety with known high oil content was used to adjust the instrument gain for optimal sensitivity. The signal intensity was averaged over 15 scans (for a total sample measurement time of 30 s) to reduce noise and then compared against a calibration curve. A univariate standard curve was built by acquiring the signal intensities for a range of oil quantities (100–1,000 mg) using purified, bleached, deodorized cottonseed oil soaked on rolled-up bibulous paper (Thermo Fisher Scientific).

Seed Calibration Set

Preliminary measurements of total oil content using the spin-echo application for several lines in the Pee Dee and RBTN germplasms provided a range of oil values from which a representative subset of these lines were chosen (in addition to available commercial varieties) to build a seed calibration set for other applications (Supplementary Table 1). Representative fuzzy seeds from each line chosen were acid-delinted and dried overnight at room temperature to minimize lint yield impact on seed weights. The spin-echo oil values acquired for these delinted seeds were used as reference oil values for future models. The total nitrogen content (Protein = $6.25 \times N$) of these samples were quantified independently by POS Pilot Plant (Saskatoon, Canada) or Eurofins (Des Moines, IA) through the Dumas method (AOCS Official Method Ba 4d-90 [2], also known

as AOAC 990.03 or AOCS Ba 4e-93) after acquiring their signals from intact delinted seed using the modified combination relaxation described below. Samples (at least 1.5 g representative seed) were ground to approximately 1-mm pieces in a standard coffee grinder or ground at POS Pilot Plant, prior to chemical analysis.

Modified Combined Relaxation (CRelax) Application

The modified CRelax application consisted of a solid echo signal followed by a series of longitudinal relaxation weighted Carr–Purcell–Meiboom–Gill (CPMG) sequences [20, 21]. The CRelax application lasted 40 s with the FID solid echo accounting for a small fraction of that time (solid-echo duration of approximately 0.1 ms within a 5-ms FID signal) and the majority attributed to the CPMG series of pulses. The original combined relaxation pulse sequence, without the solid-echo part, was patented in 2008 [22]. Two full modified CRelax scans for each sample were used for signal averaging. Typical scanning conditions consisted of gain of 60 and recycle delay of 2 s. A chemometric multivariate model was built through a partial least squares-based optimization algorithm [23] using Bruker OPUS software (version 5.5). Varying amounts of delinted cottonseed from approximately 1.5 to 3.0 g were randomly chosen from each genotype of the calibration seed set to cover a wide range of protein and oil values. The TD-NMR signals acquired for each sample and reference grams of protein (through total nitrogen measurements) and oil (through spin-echo application) were used as training data in a cross-correlational matrix. OPUS-processed chemometric models generated an optimal coefficient matrix that, when multiplied by the acquired TD-NMR signal, produces a final gram output of protein and oil content that can be used to back-calculate the percentage within a given amount of seed.

Heating and Orientation Reproducibility Experiments

For heating profiles, samples were placed in the NMR probe (held at 40 °C, the temperature of the magnet) and measurements were taken every few minutes for 1 h 20 min without removing the sample. After temperature equilibration, samples were measured 10 times to assess signal reproducibility. Evaluation of orientation reproducibility was carried out by measuring a sample three times and then rotating the sample tube approximately 45° each time, covering 360°.

Statistical Analysis

All statistical analysis was carried out using Microsoft Excel. Correlations and the square of Pearson's

product-moment correlation coefficient (R^2) were calculated by considering the mean protein, oil, or lint yield values of each germplasm line as single data points.

Results and Discussion

Hahn Spin-Echo Oil Quantification

The development of the Hahn spin-echo signal (Supplementary Fig. 1) [19, 24] established the groundwork for the standardization of low-field NMR for quick, nondestructive quantification of seed oils [24]. Indeed, internationally recognized methods exist for quantifying seed oil content by continuous wide-line [25] and pulsed NMR [26]. The refocusing 180° pulse produces a characteristic peak with high signal-to-noise ratio, being directly proportional to the amount of oil (liquid state) of the sample (Fig. 1a). The spin-echo signal was calibrated with purified, bleached, deodorized cottonseed oil that closely resembles the composition and state of oil within mature seeds. A strong positive correlation ($R^2 = 0.998$) existed between signal intensity and purified oil for a wide range of oil values (Fig. 1b). The probe size in our mq20 restricts the maximum amount of fuzzy seed to approximately 3 g. Larger sample sizes can be measured using TD-NMR technology in a similar fashion with the help of magnets with larger air

gaps and corresponding wider probes. On the other hand, smaller air-gap magnets and probe sizes facilitate analysis of individual seeds with high confidence if desired.

Inhomogeneous samples have significant signal variability that could adversely affect quantification. This variation was minimized by uniformly heating samples (Fig. 1c) at least 30 min prior to data collection to the temperature of the magnet (40 °C). Once equilibrated, the signal variability within repeated measurements ($n = 10$), each averaging 15 spectrum scans, was extremely low (Fig. 1d). However, the orientation of the packing of the cottonseeds (delinted or fuzzy) increased the signal variation by about 1–2 times that of temperature equilibration (Fig. 1e). These results demonstrate the reliability of nondestructively quantifying total oil content of intact cottonseed (both delinted and fuzzy seed) by applying the Hahn spin-echo pulse sequence.

Modified Combined Relaxation Protein and Oil Quantification

The Hahn spin-echo signal has no information that can be used to predict seed protein content directly or indirectly from oil measurements (recent literature has shown there is at best a weak negative correlation between protein and oil in cottonseed [12, 13]). Other studies have used the FID,
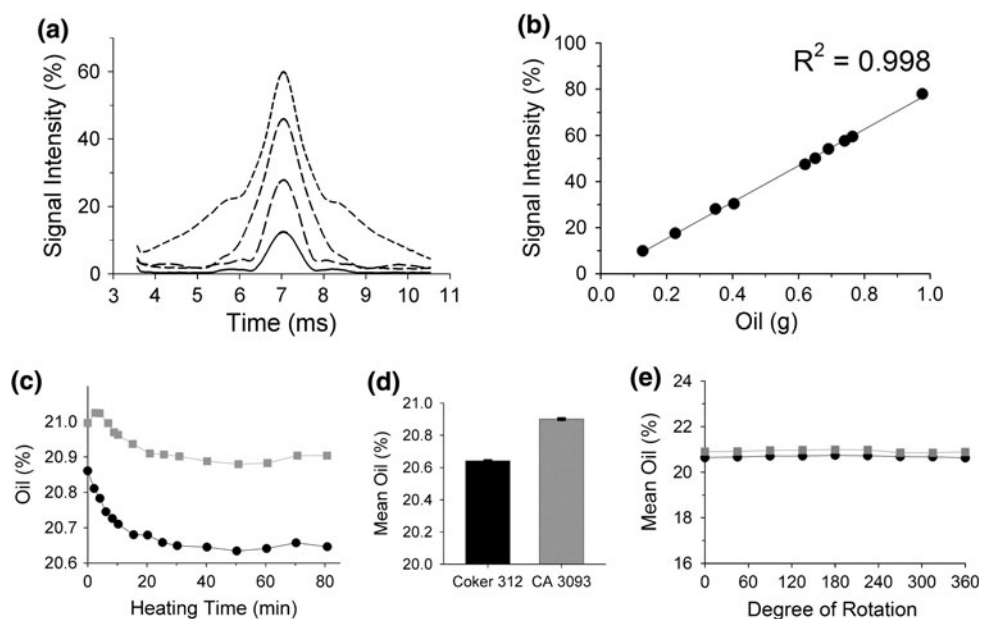

Fig. 1 Spin-echo cottonseed oil quantification validation and parameter variability. **a** Spin-echo profiles of increasing amounts of purified cottonseed oil intensities (*solid line* 70 mg; *long dash* = 150 mg; *medium dash* = 250 mg; *short dash* = 330 mg). **b** Signal intensities for spin echo are directly correlated to the amount of oil sampled (10 total oil samples). **c** Total oil content of delinted (*grey*, cv. CA 3093)

and fuzzy seeds (*black*, cv. Coker 312) as a function of heating time at constant 40 °C. **d** Instrument reproducibility of oil content in CA 3093 and Coker 312 seeds measured after heating equilibration. *Error bars* represent standard deviation of 10 consecutive measurements for each set. **e** Sample orientation reproducibility of oil content in (*grey*) CA 3093 and (*black*) Coker 312 seeds ($n = 3$)

FID-CPMG or CPMG signal regions to develop prediction models to measure seed reserve content [7–9]. Initially we attempted to use CPMG-based models for seed protein and oil quantification using a reference set of values from transgenic cottonseed lines (Bn*FAD2*) [27] that covered a broad range of protein and oil values. Due to the complexity of the signal, a chemometric algorithm was necessary to simultaneously quantify both protein and oil in cottonseed. This was satisfactory for these transgenic lines, which had a significant positive relationship between oil and protein and were all from a Coker 312 genetic background. Unfortunately this algorithm did not predict seed protein content well across diverse genotypes (data not shown). Consequently, we developed the new methodology described here for robust estimation of seed protein and oil content by a modified combined relaxation (CRelax) application for a broad range of cottonseed germplasm (and *Gossypium* spp.).

The modified CRelax application (Fig. 2) detects a complex series of signals utilizing solid echo followed by a longitudinal relaxation weighted CPMG pulse sequence [20, 21]. We found in our experiments that all three components (FID, solid echo, and CPMG) were important to consider for accurate protein prediction. CPMG signals correlated well with total oil content (data not shown), the FID component contained significant additive information for both oil (liquid) and protein (solid) content (Supplementary Fig. 2), and the solid echo provided additional signal from the protein (solid) component, especially for small sample sizes of 10–15 seeds.

Signals were acquired for a representative calibration set (42 genotypes) of cottonseed from a diverse germplasm and available commercial varieties (Supplementary Table 1). Reference seed oil values were quantified using the previously optimized Hahn spin-echo application.
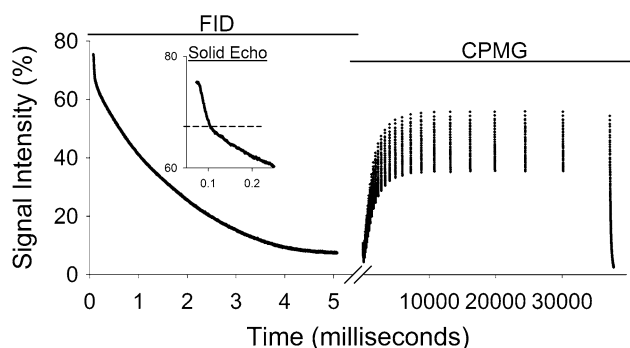
Reference (nitrogen) protein values were quantified through the Dumas method on ground delinted cottonseed. The genotypes within the reference set were selected based on covering a wide range of oil values available in the germplasm surveyed (which was surveyed for oil content initially) and other, readily available genotypes. The seed (nitrogen) protein values covered a small range of values from approximately 17.8% to 28.4% protein with a standard deviation ranging from 0.3% to 1.7% per replicate.

After acquiring TD-NMR signals and associated reference values by alternate methods, chemometric models were built for oil and protein quantification using the entire modified CRelax signal. A very strong prediction for oil (Fig. 3a, $R^2 = 0.998$) was heavily dependent on the CPMG signal region; however, using the entire signal led to unsatisfactory results when predicting protein content (Fig. 3b, $R^2 = 0.66$). Based on experiments focused on sample orientation it was determined that the latter portion of the FID signal region was highly variable (Supplementary Fig. 3) and negatively impacted chemometric algorithms. Elimination of a portion of the FID region (Supplementary Fig. 3) for prediction consideration (i.e., signal acquired but manually removed for consideration with chemometric software) significantly improved protein
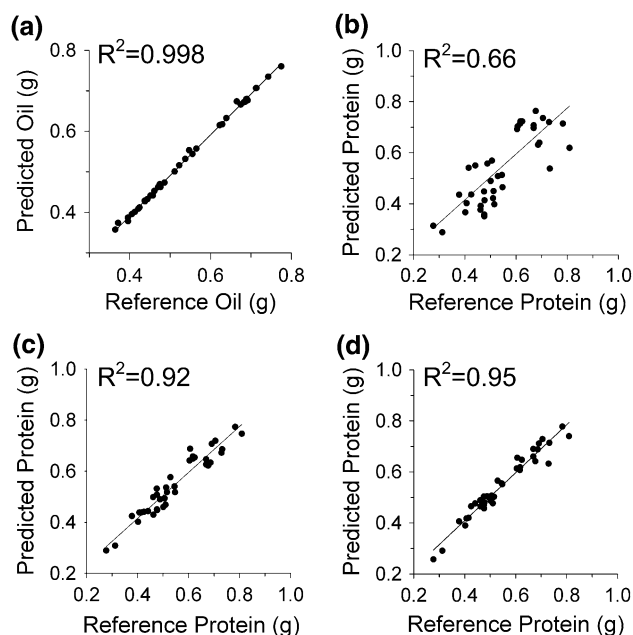
Fig. 2 Modified CRelax signal profile for simultaneous protein and oil quantification: Representative signal profile of the combined relaxation (CRelax) application that combines free induction decay (FID) with an initial solid echo (inset, *dotted line* marks end of solid echo) followed by a longitudinal relaxation weighted Carr–Purcell–Meiboom–Gill (CPMG) sequence of pulses. Each point represents a signal value acquired and used for the calculation of protein and oil

Fig. 3 Chemometric model and validation of oil and protein quantification: Modified CRelax validation of predicted NMR values for **a** oil prediction using full modified CRelax signal versus reference oil values obtained by spin echo, **b** protein prediction using full modified CRelax signal versus reference protein values obtained by nitrogen combustion, **c** protein prediction eliminating variable FID region (Supplementary Fig. 3), and **d** protein prediction eliminating variable FID region plus alternative probe temporarily installed in mq20 minispec that has a shorter dead time acquiring more solid-echo signal

prediction (Fig. 3c, $R^2 = 0.92$). Implementation of a probe (PA-247) with shorter dead time (more information from solid-echo signal region) improved the protein prediction model (Fig. 3d; $R^2 = 0.95$).

Samples not equilibrated to 40 °C had significantly higher protein values (and variation within those values) as a result of signals that were significantly different from those established with the reference set. This variation was minimized by heating samples for at least 45 min to 40 °C
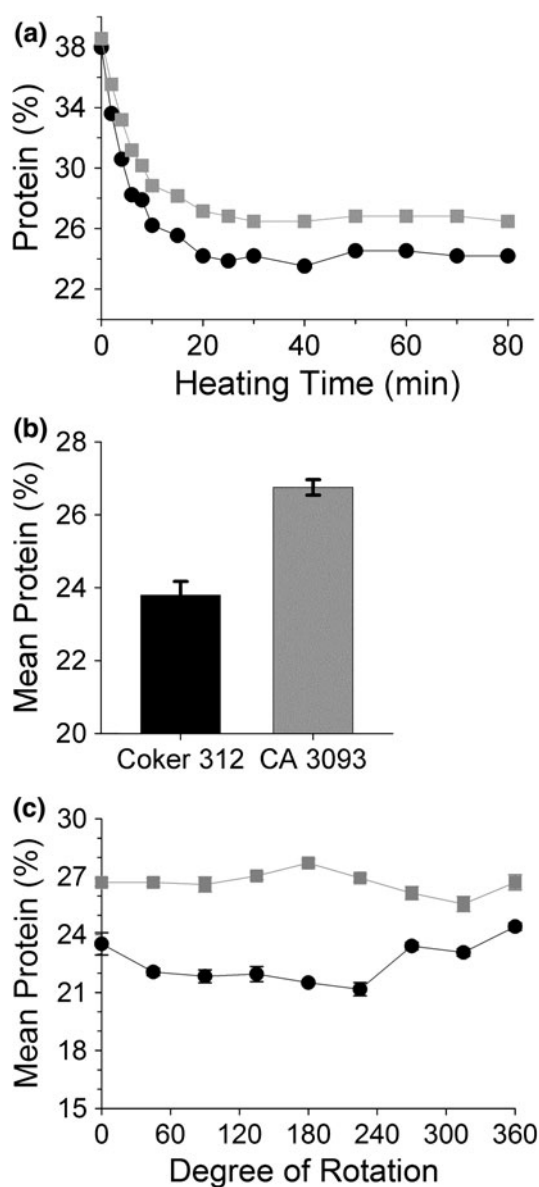

Fig. 4 Modified CRelax signal profile variability. **a** Protein quantification of delinted (*grey*, cv. CA 3093) and ginned seeds (*black*, cv. Coker 312) as a function of heating time at constant 40 °C. **b** Instrument reproducibility of protein content in CA 3093 and Coker 312 seeds measured after heating equilibration. *Error bars* represent standard deviation of 10 consecutive measurements. **c** Sample orientation reproducibility of protein content in (*grey*) CA 3093 and (*black*) Coker 312 seeds ($n = 3$ at each orientation)

(Fig. 4a). Once equilibrated, the signal variability within repeated measurements ($n = 10$), each averaging two spectrum scans, was relatively low (Fig. 4b). The orientation of the packed cottonseeds (delinted or fuzzy seed) increased the signal variation, likely due to their asymmetric structure (Fig. 4c); however, this might be reduced with increased signal acquisition time or by modification of the chemometric model in the future, incorporating the TD-NMR signals from a variety of sample orientations. These results demonstrate the development of chemometric models using the modified CRelax application that are capable of accurately quantifying both seed protein and oil simultaneously.

Germplasm Surveys of Seed Reserve Content

One goal of developing the methodology for quantifying protein and oil using TD-NMR was to be able to rapidly survey diverse germplasm for seed reserve content. Two surveys were conducted, representing germplasm sampled from US public breeding programs (RBTN) and germplasm from a diverse breeding program (Pee Dee). Representative ginned samples of RBTN and Pee Dee germplasm were quantified for both oil (through both spin-echo and combined relaxation) and protein content by TD-NMR, and compared for lint yield. Individual genotypes within both
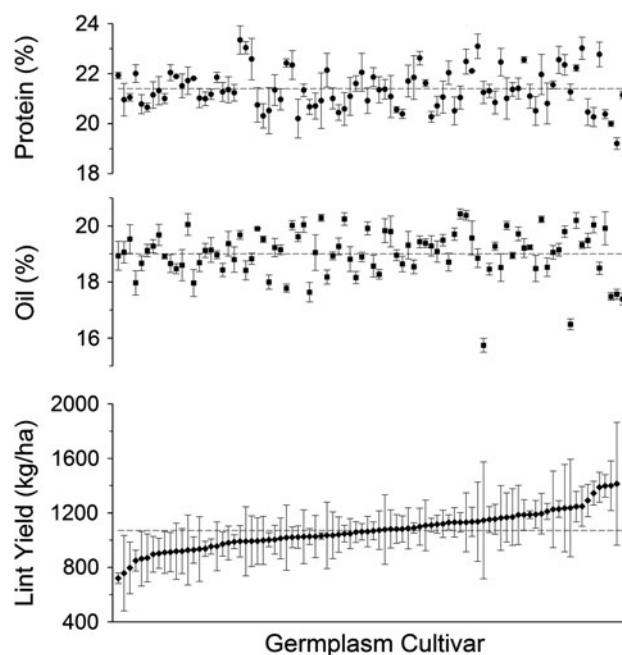

Fig. 5 Protein and oil quantification of Pee Dee germplasm: Quantification of protein, oil, and lint yield of individual genotypes in the Pee Dee germplasm representing a diverse breeding program. *Error bars* represent the standard deviation of three sample replicates. The *dashed grey line* represents the average value for protein, oil, or lint yield, respectively. Genotypes are sorted according to increasing lint yield

Fig. 6 Protein and oil quantification of RBTN germplasm: Quantification of protein, oil, and lint yield of individual genotypes in the RBTN germplasm representing a broad sample of US public breeding programs. *Error bars* represent the standard deviation of three sample replicates. The *dashed grey line* represents the average value for protein, oil, or lint yield, respectively. Genotypes are sorted according to increasing lint yield
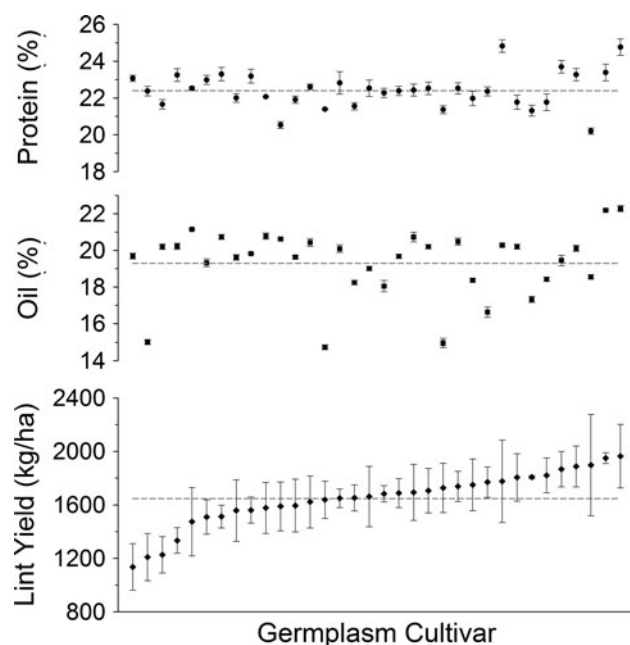
Pee Dee (Fig. 5) and RBTN (Fig. 6) showed considerable variability in protein, oil, and lint yield content.

Qualitative comparison of the two sets showed that they were relatively similar in terms of protein and oil content. The mean protein content of the RBTN set (22.4 ± 1.0%) was slightly higher overall than the Pee Dee set (21.4 ± 0.8%), which is supported by a higher range of protein values. Mean oil content was similar between the two sets, but the RBTN set had a wider range of oil values (14.7–22.3%) relative to the Pee Dee set (15.7–20.4%). Although similar in embryo reserve content, the overall lint yields of these two surveys were considerably different.

Results of both the RBTN and Pee Dee germplasms, and the reference set of samples showed weak relationships between protein and oil, protein and lint yield, and oil and lint yield (Table 1), agreeing with previous research for

several cotton germplasm surveys [12, 13, 15, 16]. The reference set had a slightly negative relationship between protein and oil (correlation = −0.25, $R^2 = 0.06$) that was chemometrically modeled. The RBTN set showed a weak positive relationship between protein and oil content (correlation = 0.34, $R^2 = 0.12$), in contrast to the Pee Dee germplasm set which showed essentially no relationship (correlation = −0.02, $R^2 = 0.00$).

Rapid, noninvasive quantification of protein and oil content permits sampling of large collections of breeding materials or seeds harvested from field trials. This should prove useful for selecting germplasm with particular seed quality traits or for evaluating production parameters that are important for optimal crop growth and production of seed components; for example, in Tables 2 and 3, samples with the greatest variation in seed protein and oil content were selected from genotypes in each survey that we completed (data for all genotypes included in Supplementary Tables 2 and 3). These extremes in seed composition highlight the variation that exists in these characteristics and suggest that breeders might find opportunities to select for material enriched in one seed component over another. It is worth pointing out in the Pee Dee set that the two highest fiber yielding lines identified as low-protein, low-oil by NMR were elite genotypes (DP555BR and DP-491) included as commercial checks. In any case, while additional refinements to this technology may be made, the new tools reported herein for analyzing seed reserves nondestructively (TD-NMR and chemometric analysis) should find broad application in the cotton community.

Conclusions

While a few reports on simultaneous quantification of oil and protein in other oilseeds have appeared in recent literature, to our knowledge there have been no reports of the application of TD-NMR to simultaneous quantification of oil and protein in cottonseed on a broad scale. The modified CRelax application, which used a FID solid-echo plus longitudinal relaxation weighted CPMG signal sequence, demonstrated sufficient accuracy for quantifying both protein and oil content in small quantities of seed. Results

Table 1 Correlation among protein, oil, and lint yield for germplasm survey

	RBTN germplasm		Pee Dee germplasm		Calibration set	
	Correl.[a]	R^2	Correl.	R^2	Correl.	R^2
Protein:oil	0.34	0.12	−0.02	0.00	−0.25	0.06
Protein:lint yield	−0.03	0.00	−0.13	0.02	−0.17	0.03
Oil:lint yield	0.08	0.01	−0.01	0.00	0.00	0.00

[a] *Correl.* correlation between two variables, R^2 square of the Pearson product-moment correlation coefficient

Table 2 RBTN germplasm candidates for extremes of protein and oil content

	Sample ID	Protein (%) [rank][a]	Oil (%) [rank]	Lint yield (kg/ha) [rank]
High-protein, High-oil	(1) Ark0012-03-08	24.8 [2]	22.3 [1]	1964 [1]
	(2) Ark0001-01-03	23.4 [4]	22.2 [2]	1950 [2]
	(3) Ark0002-03-02	24.8 [1]	20.3 [10]	1776 [9]
Low-protein, low-oil	(1) AU-5367	21.4 [30]	14.7 [34]	1638 [21]
	(2) AU-5491	21.4 [31]	15.0 [33]	1727 [13]
	(3) GA2004230	21.3 [32]	17.3 [30]	1808 [7]
High-protein, low-oil	(1) Acala 1517-99	23.1 [9]	19.7 [18]	1136 [34]
	(2) LA05307061	23.2 [8]	19.8 [17]	1560 [26]
	(3) 04PST-250	23.7 [10]	19.4 [23]	1510 [29]
Low-protein, high-oil	(1) PD99041	20.5 [33]	20.6 [7]	1589 [24]
	(2) LA05307029	21.7 [26]	20.2 [12]	1805 [8]
	(3) 0020-31 ne	22.1 [22]	20.8 [4]	1577 [25]

[a] Rank of seed reserve content or lint yield in ascending order of 34 samples in germplasm

Table 3 Pee Dee germplasm candidates for extremes of protein and oil content

	Sample ID	Protein (%) [rank][a]	Oil (%) [rank]	Lint yield (kg/ha) [rank]
High-protein, high-oil	(1) PD2165	22.5 [10]	20.4 [2]	1131 [27]
	(2) PD6132	23.3 [1]	19.7 [20]	991 [66]
	(3) PD6044	22.2 [15]	20.2 [6]	1246 [8]
Low-protein, low-oil	(1) PD0683	20.4 [81]	18.6 [64]	1082 [38]
	(2) PD93007	20.5 [76]	18.0 [79]	1002 [61]
	(3) PD0753	20.7 [72]	17.6 [83]	1026 [54]
High-protein, low-oil	(1) PD93019	23.0 [3]	18.4 [75]	991 [65]
	(2) PD5286	22.8 [5]	18.5 [70]	1387 [4]
	(3) PD0756	22.4 [12]	17.8 [82]	1018 [58]
Low-protein, high-oil	(1) FM-958	20.3 [85]	20.0 [9]	1344 [5]
	(3) AC235(9)	20.6 [74]	20.2 [4]	1047 [48]
	(3) PD3246	20.9 [64]	20.3 [3]	1031 [52]

[a] Rank of seed reserve content or lint yield in ascending order of 88 samples in germplasm

from surveys of cottonseed oil and protein variation reported herein are generally consistent with other research that showed a slight negative relationship between seed protein and oil content. Here, however, several advantages are now also evident with this method: (1) nondestructive sampling means that seeds can be grown out immediately after analysis as opposed to being sacrificed for chemical analyses; (2) small sample sizes mean that valuable collections can be subsampled for seed oil and protein content without harming seed collections; (3) simultaneous measurements of oil and protein allow for two potentially important seed traits to be estimated by the same instrument at the same time; and (4) quantification is rapid, requires minimal sample handling, and no time-consuming, potentially hazardous chemical extractions.

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