Implementing time-domain 1H-Nuclear Magnetic Resonance relaxometry to investigate recovery and stability of vegetative oil in bioenergy crops following feedstock preprocessing

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Research

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Implementing time-domain ^{1}H-Nuclear Magnetic Resonance relaxometry to investigate recovery and stability of vegetative oil in bioenergy crops following feedstock preprocessing

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Abstract

Background: The bioenergy crops energycane, miscanthus and, sorghum are being genetically modified using state of the art synthetic biotechnology techniques to accumulate energy-rich molecules such as triacylglycerides (TAGs) in their vegetative cells to enhance their utility for biofuel production. Typically, measuring and analyzing vegetative lipid contents at each step of feedstock preprocessing requires tedious sample preparation and extraction with an organic solvent. In the present study, proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was successfully adapted for non-invasive and rapid quantification of vegetative oil in untreated and pretreated cellulosic biomass.

Results: We show that the establishment of a precise and specific NMR calibration for each biomass with a distinct oil composition is key for accurate quantification of vegetative oil. The values obtained with ^{1}H-NMR were validated using a conventional solvent extraction method and cross-referenced values were within 10 % deviation. ^{1}H-NMR relaxation time distribution provided insight into the proton environment associated with the vegetative oil in the biomass. *T1T2* correlation spectra resolved two distinct populations of proton molecules based on their 'molecular tumbling' rate. The population of protons with short and long relaxation times was characterized as bound and free oil in the biomass sample, respectively. Besides, we show that biomass pretreated with two-staged hydrothermal and mechanical pretreatment can be directly used for NMR analysis unlike dilute acid and alkaline pretreated biomass which needs an additional step for neutralization of sample.

Conclusion: Time-domain ^{1}H-NMR provides a chemical-free and one-step analysis of *in situ* vegetative oil in transgenic cellulosic biomass. *T1T2* correlation spectra facilitated the resolution of the influence of various pretreatment procedures typical of cellulosic bioprocessing on the chemical composition of molecular and local ^{1}H population in each sample, hence yield information on the stability and oil recovery subsequent to each step of feedstock preprocessing.

Keywords

Time-domain ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR), *T1T2* relaxation time, Biofuel, Biodiesel, Vegetative oil, Non-invasive quantification, Bioenergy crops, Energycane, pretreatment

Background

Bioenergy crops like energycane, sugarcane, miscanthus, and sorghum have immense potential for biofuel production. These bioenergy crops primarily produce structural carbohydrates that can be extracted as sugars and further bio-processed to biofuels (especially bioethanol) and value-added bio-products. However, biodiesel production in the U.S.A. is predominantly dependent on oil seeds like soybean and corn, which are also marketed as food and feed. Hence, to expand the supply of biodiesel without impacting food production, cellulosic biomass, especially bioenergy crops, are being genetically modified to accumulate energy-rich triacylglyceride (TAG) and fatty acids, which are rich in short, unbranched, and unsaturated side chains. Andrianov et al (2010) and Sanjaya et al (2013) have successfully achieved an increase in TAG accumulation as high as 20 and 25-fold in *Nicotiana tabacum* and *Arabidopsis thaliana*, respectively [1,2]. Recently, Zale et al (2016) reported a 1.5 to 9.5 fold increase in TAG accumulation in vegetative tissues of sugarcane [3,4]. Energycane is a specially bred high fiber hybrid of sugarcane and is a promising bioenergy crop because it is more tolerant than the later to extreme weather and drought conditions. Research efforts are now underway to engineer energycane to convert solar energy into energy-rich storage chemicals in the form of TAGs in leaf and stem tissues.

Research to develop and learn to process energy crops with *in situ* oil requires rapid and convenient analytical methods to characterize and quantify their oil contents. Sample processing is particularly complicated because new, likely multistep, processes will be needed to recover the oil and optimizing the process necessities measuring oil recoveries and losses at each step. Traditional organic solvent-based methods are too slow and tedious.

Analytical studies with Nuclear Magnetic Resonance (NMR) have been reported in various research fields since mid-1950's [5]. The application of NMR has expanded remarkably since then, specifically in oil chemistry, for quantitative and qualitative analysis of fatty compounds, identification of vegetable oils, determination of fatty acid composition and, other constituents [6–9]. Its application has been extended towards cellulosic biomass conversion to biofuels. NMR is employed to probe the compositions of polysaccharides, crystallinity index of cellulose, porosity, and lignin characterization as a measure of biomass recalcitrance [10–13]. Recently, Berman et al. (2013) proposed NMR based method for the quality assessment of biodiesel [14]. In cellulosic related studies, ^{1}H-NMR has been used to explore water mobility within untreated and pretreated biomasses [15–17]. Therefore, it was of interest to see if it could be used for the measurement of vegetative oil within non-seed biomass- the future of biodiesel production. In this study, besides quantification, ^{1}H-NMR technology has been used to differentiate between bound and free oil present in the biomass and investigate their fate after three distinct feedstock preprocessing i.e., two-staged hydrothermal and mechanical, dilute acid and alkaline pretreatment to confirm their suitability for bioenergy crops with *in situ* oil.

The present study is proof of the above-mentioned concept using ground energycane bagasse soaked in crude corn oil as model biomass. The model biomass was used to investigate the fate of vegetative oil during pretreatment procedures. It helped in analyzing the accuracy of measured values and the correlation between the NMR quantification and relaxometry spectra as the concentration of oil per gram of dry biomass was known. A two-way validation that involved two distinct biomasses (soyhull and transgenic lipidcane- having *in situ* oil) and the conventional oil extraction method i.e., organic solvent extraction was performed to validate ^{1}H-NMR spectroscopy for quantification of vegetative oil in cellulosic biomass. This study aims to

develop an analytical method based on td-^{1}H-NMR spectroscopy for non-invasive, chemical-free, and, rapid quantification of vegetative oil in cellulosic biomass. Moreover, we also investigated proton relaxometry correlation spectra associated with oil to understand the fate of oil in biomass during different pretreatment.

Results

^{1}H-NMR T1T2 relaxometry correlation allocates the bound and free oil in cellulosic biomass

^{1}H-NMR relaxation time distribution of energycane (control), energycane sample soaked in crude corn oil, transgenic lipidcane 1566, and soyhull were analyzed. The samples exhibited the presence of two distinct populations of proton molecules, one with shorter relaxation time and others with longer relaxation time for each *T1* and *T2* relaxation time (Table 1).

Table 1 *T1* (spin-lattice) and *T2* (spin-spin) relaxation times for various concentrations of energycane-oil mixtures, energycane test sample, transgenic lipidcane 1566, and soy hull. Shorter and longer relaxation times correspond to lesser and higher fluidity of molecule and hence, indicates bound and free oil in the biomass sample

Biomass (g oil/ g dry biomass)	***T1* (ms) (1)**	***T1* (ms) (2)**	***T2* (ms) (1)**	***T2* (ms) (2)**
0 (Control)	6 ± 1	80 ± 3	0	0
0.096	25 ± 5	150 ± 10	46 ± 3	192 ± 4
0.198	39 ± 6	200 ± 10	60 ± 1	234 ± 3
0.309	44 ± 6	220 ± 10	73 ± 1	265 ± 3
0.393	48 ± 6	230 ± 10	78 ± 1	277 ± 3
0.501	53 ± 6	240 ± 10	81 ± 1	283 ± 2

Energycane (test sample)[a]	37 ± 6	200 ± 10	60 ± 2	235 ± 3
Transgenic lipidcane 1566	18 ± 4	125 ± 5	11 ± 3	54 ± 9
Soy hull	17 ± 3	108 ± 8	14 ± 2	94 ± 3

Average ± Standard Deviation

[a] Ground energycane bagasse with ~ 20% crude corn oil per g dry biomass. Energycane test sample has been used for all the pretreatment studies.

Shorter relaxation time represented the population of proton molecules in biomass samples with restricted movement likewise, longer relaxation time represented the population of proton molecules with a higher degree of freedom of molecules. As depicted in the schematic diagram (Fig. 1 b), the shorter relaxation times were assigned to the proton molecules associated with bound oil, similarly, longer relaxation times were assigned to the proton molecules associated with free oil in the biomass sample. The existence of bound oil can be attributed to either entrapment of oil molecules in the porous structure of cellulosic biomass or the formation of weak bonds between oil molecules and biomolecules of cellulosic biomass.

Relaxometry analysis of representative/model biomass showed that increasing the concentration of oil in the energycane-oil mixture increases the magnitudes of both *T1* and *T2* relaxation time (Table 1). This implies that variation in the magnitude of relaxation time is associated with the concentration of oil in the biomass provided other respective parameters are kept constant. The lower rate of increase of the magnitude relaxation time associated with bound oil was low as compared to the relaxation time associated with free oil on doubling the concentration of external

oil indicates that entrapment of oil molecules in pores or formation of bonds with cellulosic biomass is a rare event.

Surface chemistry and local environment of sample influence relaxation time distribution

The influence of surface chemistry or local environment on *T1T2* relaxometry correlation spectra of oil in cellulosic biomass was investigated by comparing for two distinct sets of biomasses. Set 1 was comprised of biomass having *in situ* oil, for instance, soy hull and transgenic lipidcane 1566, and Set 2 was comprised of ground energycane bagasse with externally added crude corn oil (Table 1). Ground energycane bagasse without oil served as control. Interestingly, biomasses from Set 1 and Set 2 presented contrasting results. Dry biomass of soy hull exhibited *T1*(1) 17 ± 3 ms and *T1*(2) 108 ± 8 ms, and *T2* (1) 14 ± 2 ms and *T2* (2) 94 ± 3 ms. Likewise, dry biomass of transgenic lipidcane 1566 exhibited *T1* (1) 18 ± 4 ms and *T1* (2) 125 ± 5 ms and *T2* (1) 11 ± 3 ms and *T2* (2) 54 ± 9. On the contrary, ground energycane bagasse with externally added crude corn oil exhibited a reverse trend i.e, higher magnitude of *T2* relaxation times as compared to *T1* relaxation time. Data presented in Table 1 show that increasing the concentration of externally added oil from 0.096 g oil per g dry biomass to 0.501 g oil per g dry biomass increases the magnitude of *T2* (1) and *T2* (2) from 46 ± 3 ms and 192 ± 4 ms to 60 ± 2 ms and 235 ± 3 ms, respectively, while the magnitude of *T1* (1) and *T1* (2 increased from 19 ± 3 ms and 70 ± 10 ms to 31 ± 6 ms and 120 ± 10 ms (after deducting control), respectively. The significant effect of externally added oil on *T2* relaxation time as compared to *T1* relaxation time implies that the dephasing of the spinning electron is influenced by the local environment of the sample to be analyzed. Although the magnitude of *T2* was greater than *T1* for the biomass samples in Set 2,

142 they followed the thumb rule of NMR physics i.e., $T2 \leq 2T1$, thereby, confirming the validity of
143 NMR relaxometry correlation analysis.

144

145 *NMR spectroscopy for quantification of vegetative oil in cellulosic biomass*

146 To this end, having confirmed the population of bound and free oil in the representative
147 cellulosic samples, calibration curves were constructed for the quantification of oil. Specifically,
148 separate curves were constructed for energycane-crude corn oil mixture and soybean hull. The
149 difference in the slopes of NMR calibration curves of crude corn and soybean oil (Fig. 2 a and b)
150 show that crude corn and crude soybean oil has a significantly different percentage of hydrogen
151 molecule which corresponds to a different composition of TAGs and fatty acid profile. NMR
152 calibration for each oil showed excellent predictive capabilities as validated by the very high
153 correlations (r, $> 99.5\%$). Specific calibration curves were used to measure the oil contents of
154 energycane test samples containing corn oil and soy hull pellets. For the energycane test sample
155 made up using 0.20 g crude corn oil per gram dry biomass, the td-^{1}H-NMR method predicted
156 0.182 g oil per g dry biomass, which corresponds to 9 % deviation. Soy hull pellets contained
157 0.017 g oil per g of dry biomass as measured using NMR spectroscopy calibrated with crude
158 soybean oil. The percentage of hydrogen in oil is critical for the absolute quantification of oil
159 using NMR spectroscopy. The hydrogen content in different oil varies significantly depending
160 on their fatty acid profile. Therefore, each transgenic biomass containing oil require a separate
161 calibration based on the composition of TAGs and fatty acid profile [18].
162 The oil contents measured using NMR spectroscopy were further compared with the values
163 obtained using the classical organic solvent extraction gravimetric method for further validation.
164

Comparison of NMR quantification with conventional organic solvent extraction method

Fig. 3 compares the NMR and solvent extraction methods for quantifying oil content in energycane test sample and soy hull pellets. Oil contents measured using both the methods were not significantly different for both the samples ($p \geq 0.05$).

Since feedstock preprocessing is indispensable for biofuel production using lignocellulosic biomass, energycane test sample containing ~0.20 g crude corn oil per g dry biomass was pretreated with two-staged hydrothermal and mechanical milling, dilute acid, and alkaline procedures and examined for oil recovery. A comparison of NMR spectroscopy and organic solvent extraction methods for quantification of total oil content in pretreated energycane test samples are presented in Fig. 4. Comparable numbers for total oil content per g dry biomass were obtained for the biomass samples processed with two-staged hydrothermal and mechanical, and alkaline pretreatment in contrast to results for the dilute-acid pretreated biomass. Control energycane biomass (without oil) also exhibited increased NMR values on dilute acid pretreatment (Additional file, Table S1). The higher NMR reading in dilute acid pretreated biomass can be attributed to an increase in the concentration of hydronium ions during acid pretreatment which did not get completely cleansed even after thorough washing [19,20]. Data in Table 2 presents the percent variance in the measured total oil content of untreated and pretreated biomass between ^{1}H-NMR and the conventional organic solvent method. The percent variance between the two methods is least when the pH of the sample after pretreatment is close to neutral i.e., 7. Dilute acid pretreated sample (pH 1-2) showed maximum variance in measurement i.e., 74.6 %.

The minimum oil content was observed in alkaline pretreated biomass. Most likely this resulted from alkali catalyzed saponification of the oil [21]. The alkali pretreated biomass had a soapy

texture and produced frothing while washing with deionized water (Additional file, Fig. S2). A decline in total oil content subsequent to each type of feedstock preprocessing was observed (Fig. 4). The decline can be ascribed to either extraction of the free oil or release of bound oil during pretreatment and hence, was further investigated using NMR relaxometry study.

Table 2 Comparision of organic solvent extraction and NMR spectroscopy method for total oil content in biomass samples before and after feedstock preprocessing

Feedstock	pH of sample	Organic solvent extraction	NMR Spectroscopy	Percent variance
Untreated (test sample)	6.6-7.2	0.205 ± 0.015	0.182 ± 0.001	11.2
HT	6.1-6.8	0.156 ± 0.011	0.168 ± 0.002	7.6
HT + DM	6.1-6.8	0.099 ± 0.011	0.099 ± 0.0002	0.4
DA[a]	1.0-2.0	0.083 ± 0.003	0.145 ± 0.0003	74.6*
Alkaline[a]	12.5-13.5	0.023 ± 0.004	0.028 ± 0.001	21.7

Average ± Standard Deviation

HT- Hydrothermal pretreatment at 180 °C

HT + DM- Hydrothermal pretreatment at 180 °C followed by disk milling

DA- Dilute acid pretreatment.

[a] The pH of the sample was measured after washing.

* denotes significant difference ($p \leq 0.05$)

Evaluation of the fate of oil during feedstock preprocessing

1. Suitability of different feedstock processing for oil containing cellulosic biomass

T1T2 relaxometry correlation analysis was performed for pretreated biomasses to understand the effectiveness of different feedstock preprocessing for the extraction of oil from cellulosic biomass. Results listed in Table

Table ***3*** show the change in relaxation time distribution which corresponds to change in the fluidity/degree of freedom of proton molecules in the biomasses on various pretreatment procedures. As observed previously (Table 1), the magnitude of relaxation time is also associated with the concentration of oil in the biomass. Therefore, a reduction in the magnitude of *T1* and *T2* relaxation time of the pretreated sample is directly correlated with the extraction of corresponding bound and free oil from the biomass on pretreatment. Interestingly, hydrothermal pretreatment at 180 °C followed by disk milling reduced the magnitude of *T1* and *T2* relaxation time from *T1* (1) 37 ± 6, *T1* (2) 200 ± 10 and *T2* (1) 60 ± 2, *T2* (2) 235 ± 5 to *T1* (1) 19 ± 2, *T1* (2) 110 ± 7 and *T2* (1) 8.9 ± 0.4, *T2* (2) 41.5 ± 0.8, respectively. Acid pretreated biomass also exhibited a loss of bound and free oil i.e., from *T1* (1) 37 ± 6, *T1* (2) 200 ± 10 and *T2* (1) 60 ± 2, *T2* (2) 235 ± 5 to *T1* (1) 26 ± 3, *T1* (2) 151 ± 9 and *T2* (1) 25 ± 1, *T2* (2) 140 ± 3, respectively. However, as mentioned previously, biomass pretreated with dilute acid exhibit a higher proton signal due to the increased concentration of H^+ ions, thus, the magnitudes of *T1* and *T2* might vary for the neutralized sample.

In contrast, pretreatment of biomass containing oil with alkali at high temperature had an adverse effect. Alkali likely reacted with the oil present in biomass to form soap and alcohol [21]. The alteration in biomass composition led to the inconsistency of NMR values (Table

Table ***3***).

2. <u>The fate of vegetative oil during two-stage hydrothermal and mechanical pretreatment</u>

The biomass pretreated with two-staged hydrothermal and mechanical pretreatment showed promising results with ^{1}H-NMR analysis without sample preparation hence, it was studied in detail. Analysis of total oil content (Fig. 5 a and b) and *T1T2* relaxometry correlation spectra (Table

229 Table ***3***) of pretreated biomass provided insight into the stability and percent recovery of bound
230 and free oil during the pretreatment processes. The *T2* relaxometry study presented in Table
231 Table ***3*** indicates that hydrothermal pretreatment at 180 °C resulted in the reduction of
232 approximately 50% of the oil associated fluidity (degree of freedom of molecular movement) of
233 the biomass sample as compared to untreated biomass while coupling hydrothermal pretreatment
234 with disk milling process reduced the fluidity of pretreated biomass by 80%. The mechanical
235 refining by disk milling extracted a considerable amount of oil from the cellulosic biomass
236 sample (reduced the oil associated fluidity of biomass by approximately 65%). The extent of
237 bound and free oil extracted from cellulosic biomass at each step of pretreatment can be
238 interpreted from Fig. 5 a. Free oil in biomass is more accessible than bound oil and hence, was
239 extracted efficiently. comparatively Combination of hydrothermal treatment and disk milling
240 recovered approximately 50% of total oil per g dry biomass. The two-staged pretreatment helped
241 in the enrichment of bound oil by defibrillating the cellulosic matrix that makes the vegetative oil
242 easily extractable from cellulosic biomass. Comparable values of oil recovery were obtained
243 using ^{1}H-NMR and organic solvent method at each step of pretreatment (Fig. 5 b).

244

245 **Discussion**

246 *Implication of T1T2 Relaxometry correlation spectra*

247 In ^{1}H-NMR, the relaxation time of an object represents the time taken by proton molecules to
248 reach the equilibrium state by losing the pulsed energy. In most of the liquid, the relaxation time
249 is inversely proportional to the viscosity. The correlation between viscosity and relaxation time
250 can be explained by the equation

251 $$\tau_c \ll 1/\nu_o \quad (1)$$

where τ_c and ν_o represent correlation time associated with Brownian motion of protons in the sample and Larmor frequency, respectively. *T1* relaxation take place along fluctuation in the magnetic field, most effectively at Larmor frequency (ν_o) indicating that *T1* relaxation is field-dependent, while, *T2* relaxation is induced by fluctuation in any field, mainly due to molecular motion [22]. The schematic diagram in Fig. 1 a illustrates the correlation of relaxation time and viscosity of the liquid sample (adapted from Bloembergen et al, 1947 [23]). Similarly, in solids, the ^{1}H-NMR relaxometry spectra of the sample correlate the degree of freedom of proton molecules in the sample and facilitate the resolution of different populations of proton molecules in samples based on their 'molecular tumbling' rate. Typically, in solid samples, the motion of the molecules is restricted and exhibits a shorter relaxation time. The magnitude of relaxation time of solids can vary significantly from the relaxation time of liquids by a factor of 10's or 100's depending on the viscosity of the liquid [24]. A solid sample containing fluidized elements, for instance, free oil or, high moisture exhibits multiple distinct relaxation times based on the molecular tumbling rate of each population of proton molecules in the sample. Therefore, in the present study, it is convenient to assign the population of proton molecules associated with oil in the biomass sample exhibiting short and long relaxation time as bound and free oil provided the moisture of sample is kept minimum (≤ 2% moisture content was maintained in all the samples). A background proton signal was obtained in dry biomass without oil i.e., control energycane biomass. *T1* analysis of control biomass displayed *T1* (1) 6 ± 1 ms and *T1* (2) 80 ± 3 ms which can be attributed to the spin of proton nuclei of biomolecules including proteins, carbohydrates, and any remaining water molecules. However, a zero value of *T2* in control implies a highly restricted movement of proton molecules with no interference of the local environment (a very solid surface with the least molecular movement).

T1T2 relaxation times of transgenic lipidcane 1566 with *in situ* oil exhibited a higher magnitude of free oil. The analysis was in agreement with the study reported by Parajuli et al (2020) on hyperaccumulation of lipids in the form of droplets inside the vegetative tissues of transgenic lipidcane 1566 [4]. A $T2 \geq T1$ (but $T2 \leq 2T1$— validating the NMR relaxometry spectra) in energycane and crude corn oil mixture provides insight into the impact of local chemistry of sample affecting the spin dephasing of oil-associated proton molecules.

Consequence of bioprocessing on NMR analysis

For the first time, we demonstrated ^{1}H-NMR relaxometry correlation spectra for examining bound and free oil in cellulosic biomass subsequent to pretreatment procedures. Time-domain ^{1}H-NMR spectroscopy measures the percentage of hydrogen present in the sample. A significant variation in hydrogen molecules during pretreatment procedures due to certain chemicals limits the usage of NMR spectroscopy for oil quantification. Data presented in Table 2 and Additional file, Table 1 show that acid pretreated samples exhibit a significantly higher magnitude on NMR analysis as compared to the conventional organic solvent method. The acidic pH of the sample confirmed increased H^{+} molecules after pretreatment. The observation is in agreement with the reports by other research groups that acid treatment interferes with the overall charge of the slurry and needs a neutralization process [25,26]. Moreover, Zhu et al., 2019 reported that the pretreatment liquor from acid pretreated biomass possesses a good catalytic activity that can be recycled to obtain reducing sugars from additional cellulosic biomass [27].

The degradation of oil and inconsistency in relaxometry analysis (Table
Table ***3***) suggest that alkaline pretreatment is unsuitable for biomass containing oil. The main chemical reactions during alkaline pretreatment involve solvation and saponification, resulting in the swelling of the biomass, making the cellular parts susceptible to react with the external agent

298 [28]. Thus, biomass pretreated with dilute acid and alkali cannot be directly used for NMR
299 analysis. Further investigations are required to develop methodologies to analyze acid and alkali
300 pretreated samples by ^{1}H-NMR possibly either by neutralizing or derivatizing the analytes.
301 Although the analysis of oil composition is critical, however, from the NMR relaxometry
302 analysis and corresponding quantification of oil, it can be inferred that hydrothermal
303 pretreatment at 180 °C followed by disk milling maintains the stability and quality of vegetative
304 oil in biomass.

305 *Analyzing the effectiveness of feedstock preprocessing*

306 The assessment of water-associated proton molecules for their mobility in biomass structure has
307 been successfully established using NMR relaxometry correlation spectra. The moisture content
308 of biomass is maintained ≥ 10% to study the water-associated relaxometry spectra. Foston and
309 Ragauskas (2010) used a combination of ^{1}H and ^{2}H NMR techniques to demonstrate the pore
310 expansion of cellulosic fibril bundle on acid pretreatment of *Populus* [17]. Jeoh et al. (2017)
311 performed 2D ^{1}H-NMR on SO_2 catalyzed thermal pretreated Spruce biomass to establish
312 microstructure of the water environment within pretreated biomass [10]. In both cases,
313 improvement in the porosity of biomass on pretreatment corresponds to an increase in *T2*
314 relaxation time of water-associated proton molecules in biomass as it suggests more space for the
315 water molecules in the porous biomass to have a higher molecular tumbling. In contrast, for
316 analysis of oil in cellulosic biomass using ^{1}H-NMR, it is necessary to minimize the contribution
317 of water molecules by keeping the moisture content to a minimum. The pretreatment of biomass
318 containing oil resulted in a decrease in *T2* relaxation time (Fig. 6 and Table 3), unlike the
319 observations for water-associated *T2* relaxation time. In this case, the decrease in the magnitude
320 of *T2* relaxation time signifies a decrease in the concentration of oil-associated proton molecules

which in turn corresponds to the extraction of oil from biomass. The relaxometry correlation holds with the quantification of oil in pretreated biomass. Furthermore, Kiemle et al (2003) have thoroughly demonstrated the absolute quantification of major monosaccharides in the acid hydrolysate of lignocellulosic biomass using ^{1}H-NMR without sample derivatization [12]. Hence, the understanding of NMR relaxometry facilitates concurrent analysis of the effectiveness of biomass pretreatment protocol by investigating the critical parameters such as biomass recalcitrance, sugar, and oil recovery during bioprocessing.

Conclusion

Time-domain ^{1}H-NMR is a useful non-invasive technique for the analysis of complex biological or chemical samples. It is not only responsive towards the chemical nature and functionality of the molecules to be analyzed but also the surface chemistry, chemical connectivity, and local environment. *T1T2* ^{1}H-NMR spectroscopy is a powerful analytical tool for qualitative and quantitative analysis of cellulosic bio-oil. The relaxometry study of the lignocellulosic biomass supported the qualitative reasoning of the consequences of various physical and chemical feedstock preprocessing on the fate of vegetative oil. NMR analysis provides absolute quantification of vegetative oil at each step of two-staged hydrothermal and mechanical pretreatment without sample preparation.

^{1}H-NMR based quantification of oil is quick, convenient, and lends itself to high sample throughput measurements. Sample measurements for oil quantification and relaxometry analysis take as little as 10 minutes. No chemical reagents are required, and the sample can be preserved for further analysis. There is also a negligible background signal even with complex sample matrices such as lignocellulosic biomass. This robustness lends its use to analyzing processed

samples and it is expected the convenience will lend itself to being used for continuous process optimization. However, chemicals that ionize into H^+ ions such as acids, bases, or buffers restrict the absolute quantification using ^{1}H-NMR spectroscopy. Excess H^+ ions interfere with the NMR readings and hence, would require sample preparation (e.g. sample neutralization). Further investigations with lipidcane (genetically modified sugarcane for *in situ* oil production in leaves and stems) are in progress.

Materials and Methods

Feedstock and Chemicals

Energycane UFCP82-1655: Energycane UFCP82-1655 bagasse was obtained from the experimental research station located at the University of Florida, Gainesville, Florida, USA. Energycane juice was extracted and bagasse was dried at 50 °C. Dried energycane bagasse was cut into smaller pieces of 1 to 2 inches with pruning shears and ground in a hammer mill (W-8-H, Schutte-Buffalo Hammermill, Buffalo, NY) equipped with a round hole sieve sized at 2 mm. Ground energycane without vegetative oil served as negative control and backbone material for creating representative biomass.

Representative/Model biomass for NMR studies: Research efforts are still underway for the development of energycane with elevated levels of bio-oil. As a proof of concept for the NMR based analytical method, representative biomass samples containing vegetative oil have been prepared that simulate the oil-producing cane. The ground energycane was soaked in crude corn or crude soybean oil of known concentrations. Crude corn and soybean oil were obtained from One Earth Energy LLC (Gibson City, Illinois, USA) and Incobrasa Industries Limited (Gilman,

366 Illinois, USA), respectively. Oil soaked energycane biomasses were incubated at 32 °C for 1 to 2
367 months. Representative biomass samples having six different oil concentrations were prepared
368 using corn and soybean oils. The final oil concentrations of the soak energy cane samples were 0,
369 0.096, 0.198, 0.309, 0.393, and 0.501 g corn oil per g dry biomass (Additional file 1, Fig. S1 a),
370 and 0, 0.101, 0.216, 0.333, 0.400, and 0.533 g soybean oil per g dry biomass. Experiments were
371 performed using the energycane test sample containing 20% crude corn oil per g dry biomass
372 (Additional file 1, Fig S1 b) unless mentioned otherwise.

373 Soybean hull: Soy hull pellets were obtained from Incobrasa Industries Limited, Gilman, Illinois,
374 USA. Soybean hulls are obtained as the coproduct of soybean meal production (Additional file
375 1, Fig. S1 c). It consists of pelletized soybean seed coats and is mixed with external crude
376 soybean oil to provide higher energy values for ruminant animal rations.

377 Transgenic lipidcane 1566: Transgenic lipidcane 1566 having elevated levels of in situ oil in
378 vegetative tissues obtained from the Center of Advanced Bioenergy and Bioproducts (CABBI),
379 University of Illinois at Urbana-Champaign, IL, USA. Lipidcane stems were processed the same
380 as energycane.

381 *Feedstock preprocessing*

382 Pretreated biomass samples were prepared using three methods (two-staged hydrothermal and
383 mechanical, dilute acid, and alkaline) to determine if pretreated biomass can be analyzed for oil
384 content using NMR spectroscopy. All chemicals were of analytical quality.
385 Two-staged hydrothermal and mechanical pretreatment: A fluidized sand bath (IFB-51 Industrial
386 Fluidized Bath, Techne Inc., Burlington, NJ) was used for the liquid-hot water pretreatment.
387 Energycane test sample was mixed with deionized water at 20% w/w solid loading and loaded in

a capped pipe reactor (316 stainless reactors: 10.478 cm length×1.905 cm outer diameter×0.165 cm wall thickness tubing, SS-T12-S-065–20, Swagelok, Chicago Fluid system Technologies, Chicago, IL, 316 stainless steel caps: SS-1210-C, Swagelok, Chicago Fluid system Technologies, Chicago, IL). The *in situ* reaction temperature during pretreatment was monitored using a thermocouple (Penetration/Immersion Thermocouple Probe Mini Conn (-418 to 1652°F), Mc Master-Carr, Robbinsville, NJ) inserted into one reactor and connected to a data logger (HH306/306A, Datalogger Thermometer, Omega, Stamford, CT). After holding the tubes at 180 ºC for 10 minutes, the reaction was immediately quenched by submerging the pipe reactors into a cold water bath. Liquid hot water pretreatment was followed by three passes of disk milling (Quaker City grinding mill model 4E, Straub Co., Philadelphia, PA) [29]. The biomass samples were filtered after each pretreatment step and solid residues were oven-dried at 50 ºC.

Alkaline pretreatment: The energycane test sample was mixed with 1N NaOH solution to obtain 20% w/w solid loading in stainless steel reactors (same set as used for the hydrothermal pretreatment). The pretreatment reactors were heated in a fluidized sand bath (same as used for the hydrothermal pretreatment) at 100 °C for 30 min [30]. The pretreated biomass was cooled and thoroughly washed with deionized water to remove NaOH. The washed sample was oven-dried at 50 °C.

Dilute acid pretreatment: A low severity dilute acid pretreatment was performed as outlined by Sidhu et al. (2011) [31] with slight modifications. The energycane test sample was mixed with 2.0% w/w H_2SO_4 solution to obtain 20% w/w solid loading in autoclavable glass reactors. The mixture was heated at 121 °C for 60 minutes under 15 psi pressure. The sample was cooled and thoroughly washed with deionized water to remove the acid and dried at 50 °C.

TD- ^{1}*H-Nuclear Magnetic Resonance (NMR) spectroscopy*

411 A time-domain one-dimensional benchtop nuclear magnetic resonance system (Minispec mq20,
412 Bruker, Massachusetts, USA) equipped with an 18 mm thermostat ^{1}H-probe operating at 0.47 T/
413 20 MHz was used for the analysis of *T1T2* relaxation times and quantification of vegetative oil
414 contents. The moisture contents of all the biomass samples were kept consistent and below
415 2%w/w to abate the contribution of proton signals from water molecules. For the consistency of
416 analysis, 1 g of dry biomass was used for all analyses.

417 Analysis of *T1T2* relaxation time (relaxometry study): The spin-spin or transverse (*T2*) relaxation
418 time for the biomass samples were obtained by using Carr-Purcell-Meiboom-Gill (CPMG)
419 application [32] with a 180^{o} pulse separator of 2.00 ms over 800 echoes fitted to a bi-exponential
420 equation of order two. A full decay was obtained for *T2* relaxation time. The spin-lattice or
421 longitudinal (*T1*) relaxation time was analyzed using the inversion recovery method [33]. The
422 inverse recovery method was started after 2 ms (to avoid receiver artifacts) and run over a
423 duration of 800 ms for each data point. A set of 10 data points were obtained for each sample and
424 fitted to a bi-exponential equation of order two.

425 Non-invasive quantification of vegetative oil: NMR was calibrated with specific oil
426 corresponding to each type of biomass for absolute quantification of total oil content. A six-point
427 calibration curve was established for each type of biomass i.e., energycane test sample and soy
428 hull. Typically, a regression value of above 99.5% ($R^2 = 0.995$) is recommended for NMR
429 analysis. The weight of the biomass to be analyzed was recorded for each sample as NMR
430 spectroscopy measures total oil present in the biomass sample and generate a report as a
431 percentage of oil per gram dry biomass.

432 *Organic solvent extraction and quantification of oil*

Total lipid content of untreated and pretreated biomass was extracted using the organic solvent method reported by Huang et al., 2017 [34]. Briefly, 1.00 g of the dry biomass sample was mixed with 10 ml isopropanol and 15 ml hexane in a 50-ml screw-top tube and homogenized 2 x 1 min with a homogenizer (LabGen 700, Cole Parmer, Vernon Hills, IL) at a speed of 5000 rpm. The homogenized mixture was agitated with a wrist action shaker (HB-1000 Hybridizer, UVP LLC, Upland, CA) at room temperature for 10 min. The slurry was mixed with 16 ml of (6.7%, w/v) sodium sulfate solution agitated for 10 min and centrifuged at 200 rpm for 20 min. The top phase was collected in a new tared screw-capped tube and the solvent was evaporated by passing over a gentle stream of nitrogen. Once the solvent was removed, the recovered oil was weighed on an analytical balance. Gravimetric oil measurements were compared with the values obtained using NMR spectroscopy.

Statistical analysis

All the samples were analyzed in triplicate. Standard deviation was calculated to measure the deviation of experimental replicates from the mean value. Regression analysis between NMR intensity and total oil content was performed to determine the accuracy of the NMR calibration. ANOVA (ANalysis Of VAriance) was performed using R statistical software (i386 3.6.2) using values obtained with NMR spectroscopy and the organic solvent method for oil quantification using a significance threshold of $p \leq 0.05$.

List of Symbols/Abbreviations

DA	Dilute acid pretreatment
DM	Disk milling

HT	Hydrothermal pretreatment
HT + DM	Hydrothermal pretreatment followed by disk milling
NMR	Nuclear Magnetic Resonance
td-^{1}H-NMR	Time domain-proton associated Nuclear Magnetic Resonance
T1T2 relaxometry	*T1* relaxation time and *T2* relaxation time

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VS and SM conceived the study. SM performed the experiments and drafted the manuscript. VS, SM, BD, and SL analyzed the data and edited the manuscript. All authors read and approved the final manuscript

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Not applicable

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584

585 **Table**

586 **Table 3** ^{1}H-NMR relaxometry spectra for the evaluation of percent loss of bound and free oil in biomass samples after three distinct
587 feedstock preprocessing i.e., two-staged hydrothermal and mechanical, dilute acid and alkaline procedures.

Feedstock preprocessing	***T1* (ms) Bound oil**	***T1* (ms) Free oil**	***T2* (ms) Bound oil**	***T2* (ms) Free oil**	**% loss of Bound oil (*T1*)**	**% loss of Free oil (*T1*)**	**% loss of Bound oil (*T2*)**	**% loss of Free oil (*T2*)**
Untreated energycane	37 ± 6	200 ± 10	60 ± 2	235 ± 3	00	00	00	00
HT	26 ± 4	147 ± 9	22.9 ± 0.6	125 ± 2	29.72	26.50	61.83	46.80
HT + DM	19 ± 2	110 ± 7	8.9 ± 0.4	41.5 ± 0.8	48.64	45.00	85.16	82.34
DA	26 ± 3	151 ± 9	25 ± 1	140 ± 3	29.72	24.50	58.33	40.43
Alkaline	12 ± 2	86 ± 2	100 ± 300	-	67.56	57.00	ND	0

588 Average ± Standard Deviation

589 HT- Hydrothermal pretreatment at 180 °C

590 HT + DM- Hydrothermal pretreatment at 180 °C followed by disk milling

591 DA- Dilute acid pretreatment

592 ND- Not determined

593

Figure legends

Fig. 1 Schematic diagram illustration of one-dimensional NMR relaxometry correlation spectra. a) Typically relaxation time (*T1T2*) of an object varies inversely proportional to the viscosity of that object i.e., highly viscose liquids have shorter relaxation time [23]. b) Objects with lower relaxation time have a lesser degree of freedom of molecules i.e., tightly packed molecules than the objects with longer relaxation time. In the present study with ^{1}H-NMR, the population of protons with shorter and longer relaxation time (*T1* or *T2*) in each biomass sample represent tightly packed and relatively free proton molecules corresponding to bound and free oil in the sample, respectively.

Fig. 2 Calibration of td ^{1}H-NMR spectroscopy with energycane soaked in a) crude corn oil and, b) crude soybean oil for quantification of vegetative oil in cellulosic biomass sample.

Fig. 3 Validation of vegetative oil measured by td-^{1}H-NMR using hexane extraction. The measured values from both the methods were not significantly different i.e., $p \geq 0.05$.

Fig. 4 Comparison of total oil content measured in biomass samples pretreated with various feedstock preprocessing namely hydrothermal (HT), hydrothermal + disk milling (HT + DM), dilute acid (DA), and alkaline using organic solvent extraction and ^{1}H-NMR spectroscopy.

Fig. 5 a) NMR relaxometry spectra demonstrate the release of the bound and free oil with each step of two-staged hydrothermal and mechanical pretreatment. b) Assessment of an average percentage recovery of oil from the cellulosic sample after each step of pretreatment using organic solvent extraction and ^{1}H-NMR spectroscopy.

614 **Fig. 6** The distribution of *T2* relaxation times of oil within untreated and pretreated energycane
615 biomass. The vertical dashed lines are drawn to demonstrate the shifts in the peak position due to
616 various pretreatment procedures.

Figures

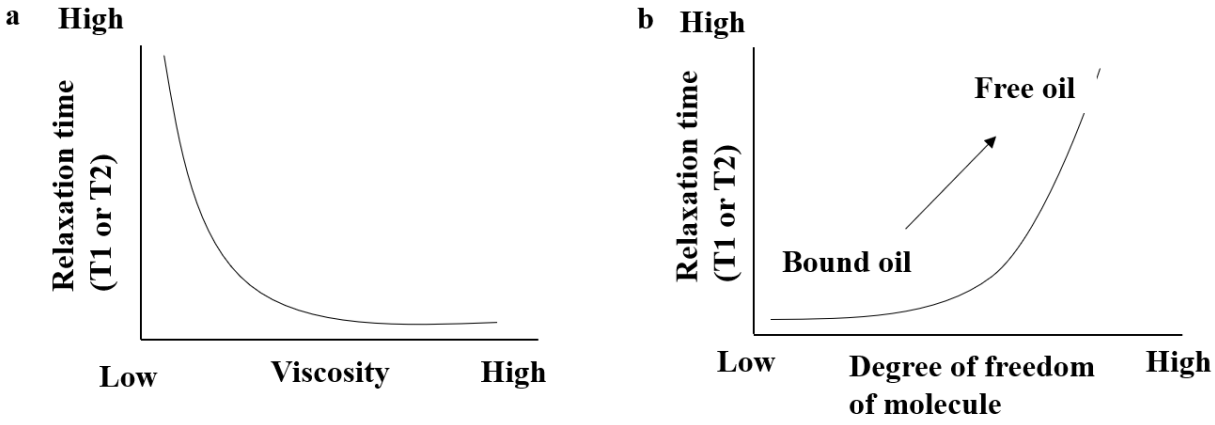

Figure 1

Schematic diagram illustration of one-dimensional NMR relaxometry correlation spectra. a) Typically relaxation time (T1T2) of an object varies inversely proportional to the viscosity of that object i.e., highly viscose liquids have shorter relaxation time [23]. b) Objects with lower relaxation time have a lesser degree of freedom of molecules i.e., tightly packed molecules than the objects with longer relaxation time. In the present study with 1H-NMR, the population of protons with shorter and longer relaxation time (T1 or T2) in each biomass sample represent tightly packed and relatively free proton molecules corresponding to bound and free oil in the sample, respectively.

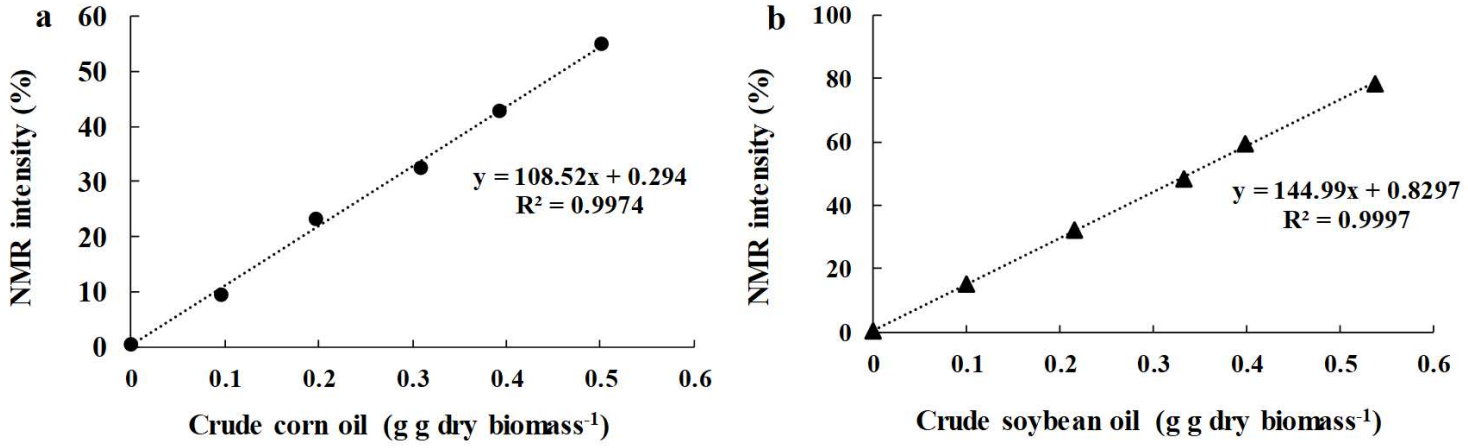

Figure 2

Calibration of td 1H-NMR spectroscopy with energycane soaked in a) crude corn oil and, b) crude soybean oil for quantification of vegetative oil in cellulosic biomass sample.

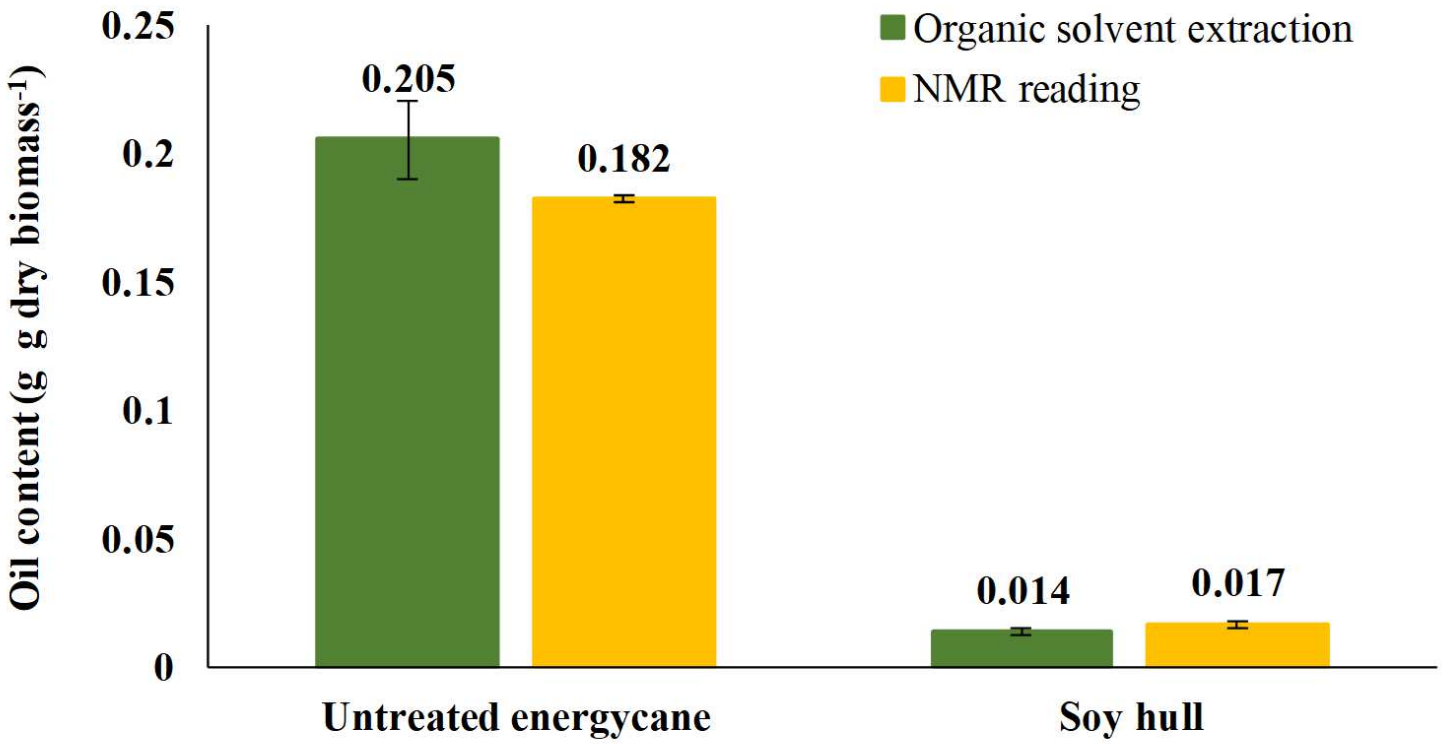

Figure 3

Validation of vegetative oil measured by td-1H-NMR using hexane extraction. The measured values from both the methods were not significantly different i.e., $p \geq 0.05$.

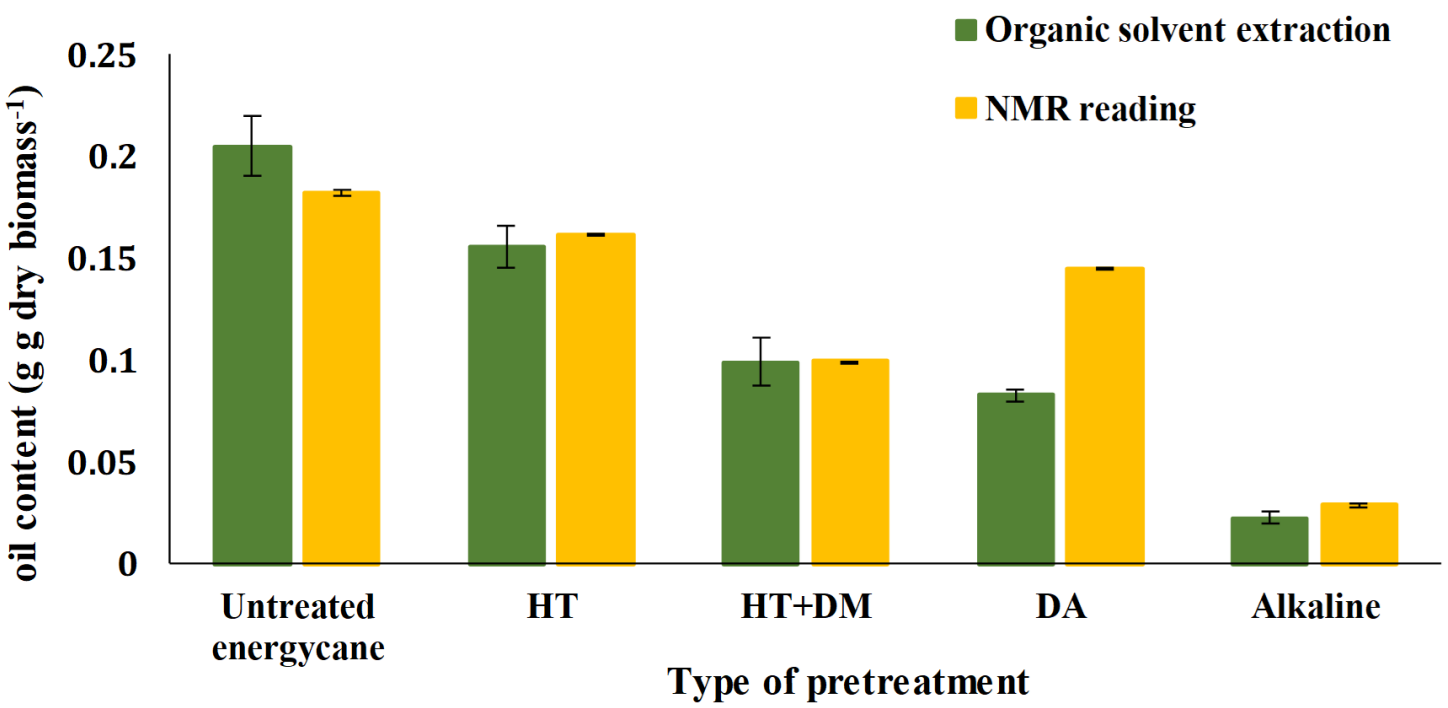

Figure 4

Comparison of total oil content measured in biomass samples pretreated with various feedstock preprocessing namely hydrothermal (HT), hydrothermal + disk milling (HT + DM), dilute acid (DA), and alkaline using organic solvent extraction and 1H-NMR spectroscopy.

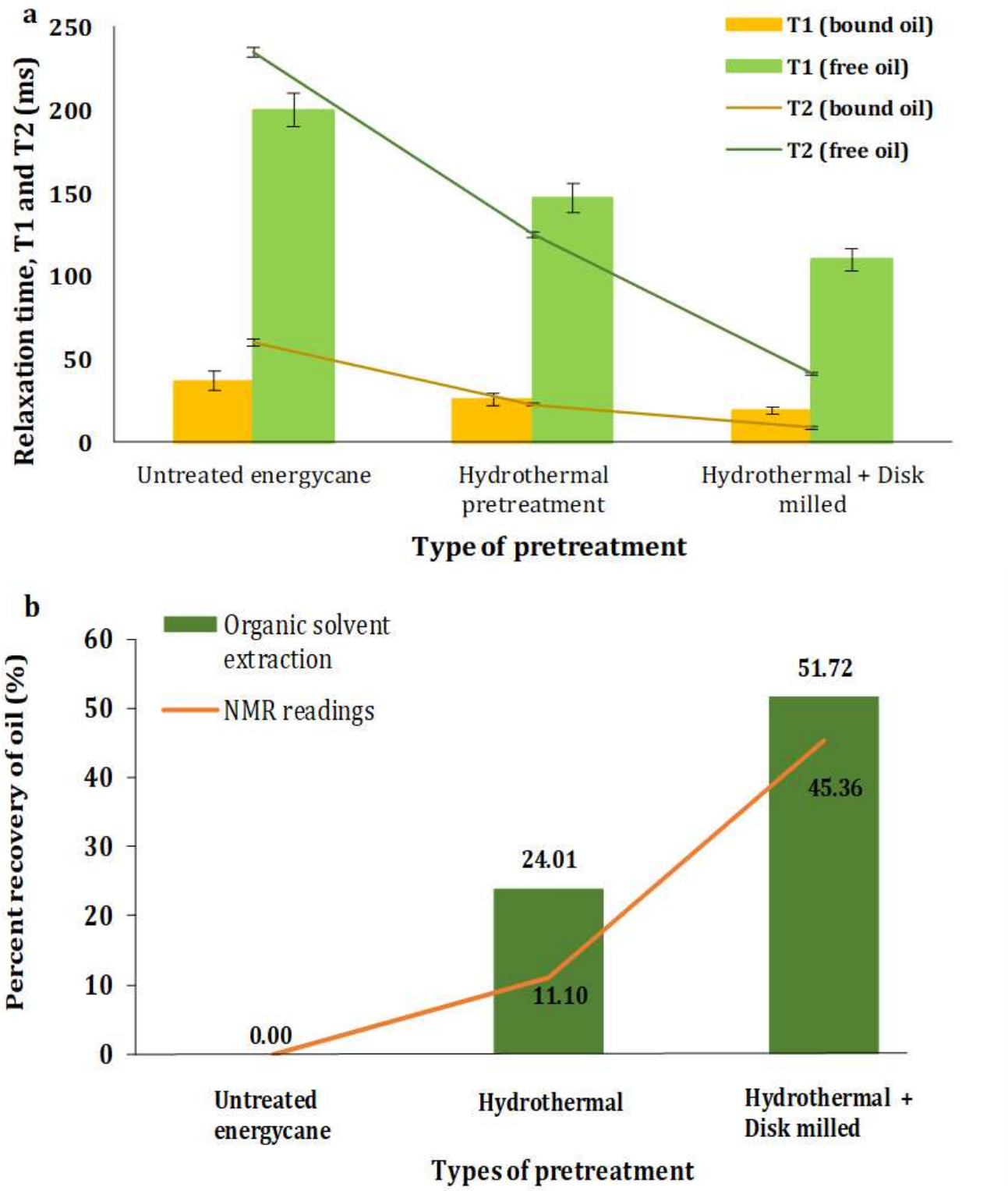

Figure 5

a) NMR relaxometry spectra demonstrate the release of the bound and free oil with each step of two-staged hydrothermal and mechanical pretreatment. b) Assessment of an average percentage recovery of oil from the cellulosic sample after each step of pretreatment using organic solvent extraction and 1H-NMR spectroscopy.

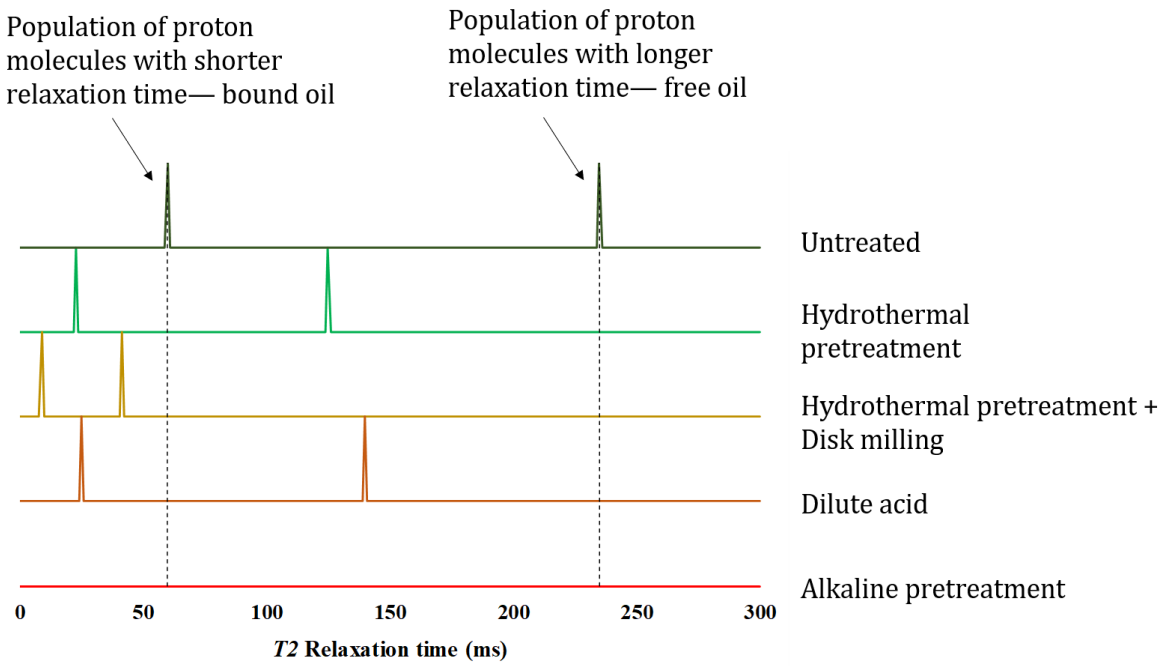

Figure 6

The distribution of T2 relaxation times of oil within untreated and pretreated energycane biomass. The vertical dashed lines are drawn to demonstrate the shifts in the peak position due to various pretreatment procedures.

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- Additionalfile1.pdf