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ORIGINAL PAPER

Large-Scale Screening of Intact Castor Seeds by Viscosity Using Time-Domain NMR and Chemometrics

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Abstract Castor is one of the most promising non-edible oil crops, due to its high oil content and since it can be grown on marginal lands and in a semi-arid climate. However, the high content of ricinoleic acid results in an extremely high viscosity of castor-based biodiesel. In this study, we report on the development of a rapid and non-destructive method for large-scale screening of intact castor seeds according to their viscosity by time domain NMR and chemometrics. A qualitative principal component analysis model was constructed, where each observation was assigned to a different viscosity group. This model straightforwardly detects desirable outliers, and can also be applied for detection of other transgenic oilseeds, especially those containing small levels of hydroxylated fatty acid.

Keywords Time-domain NMR · Castor oil · Biodiesel · PCA · Chemometrics

Introduction

Biodiesel offers a viable alternative to petroleum-based diesel fuel. However, large-scale production of biodiesel from edible-grade oils (>95%) can lead to imbalance in the global food market [1]. Land availability is another core limitation. Therefore, other oilseeds are currently being researched as alternative feedstocks.

Castor (*Ricinus communis* L.) is one of the most promising non-edible oil crops, with an annual seed production of 1.14 Mt and an average seed yield of 902 kg/ha [2]. Compared to other conventional oil crops, castor plants can grow on marginal lands and in a semi-arid climate. Castor oil consists mainly of ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid). The high content of this hydroxylated fatty acid (FA) gives castor-based biodiesel unique physical properties such as a favorably high calorific value and cetane number and a low iodine number of less than 90. On the other hand, castor oil is adversely characterized by high hygroscopicity [3]. Nevertheless, extremely high viscosity remains the main barrier for its use as a fuel. Neat castor biodiesel viscosity was found to reach 13.75 mm^2/s, much larger than the ASTM limit of 6 mm^2/s [3].

Genetic breeding programs have introduced new alternatives for the biodiesel industry. These programs aim at increasing the oil content of the seeds and modifying their FA composition to gain high quality and profitable yields [4]. In the case of castor oil, reduced levels of ricinoleic acid will lead to reduced biodiesel viscosity [5], possibly meeting the ASTM limits. Rojas-Barros et al. [6] were able to identify a natural castor mutant with very high oleic acid (18.9%) and low ricinoleic acid content (71.4%). This exceptional mutant, showing reduced viscosity, might be more suitable as a biodiesel feedstock than conventional castor seeds. Screening of oilseeds, as in the Rojas-Barros study, was conducted by standard analytical methods, which are considered exhaustive and environmentally unfriendly. Screening thousands of samples as required for genetic

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breeding programs with these methods is not realistic. An alternative rapid method is therefore required.

TD-NMR is a rapid and non-destructive technology that can be implemented for large-scale screening of intact oilseeds. TD-NMR experiments involve excitation of hydrogen protons in the presence of a constant magnetic field, and acquisition of signal intensities as these relax back to equilibrium. One typical pulse sequence, called CPMG [7, 8], consists of a single 90° pulse followed by multiple consecutive 180° pulses, which allows transverse relaxation to take place. This test can be applied for measuring some qualitative aspects of a sample, since the shape of the signal acquired through time is characteristic of its physical state. The CPMG sequence involves acquisition of thousands of data points per sample. Analysis of these huge datasets requires advanced statistical tools, known as chemometrics. Chemometrics include the application of multivariate statistics, mathematics, and computational methods to chemical measurements to enhance the productivity of chemical experimentation.

Recently, Prestes et al. [9] predicted the viscosity of different oilseeds using TD-NMR and partial least square analysis (a common chemometric tool). Yet castor was excluded from this model due to its unique FA composition. Quantitative prediction of viscosity, however, fails in portraying relations between observations. Pedersen et al. [10] constructed a 2D scatter plot using principle component analysis (PCA), which distinguished between rape and mustard seeds and between dried and fresh seeds from each family. This graphical representation can offer insights otherwise overlooked, especially for large-scale screening, including detection of outliers and groupings [11]. PCA provides a map-like representation, where all observations are scaled according to one (line plot) or two (scatter plot) axes. Classification with this method, however, can only be determined intuitively according to one's judgment. K-means, a clustering algorithm [12], complements this method to achieve impartial sorting.

In this study, a qualitative chemometric model is suggested for rapid large-scale screening of castor seeds according to viscosity. Viscosity was calculated from the FA composition using GC, assuming varieties showing reduced levels of ricinoleic acid will also show reduced castor-based biodiesel viscosity. Lower viscosity will lead to a measurable change in decay rates relative to the position of a sample in a PCA plot. Classification of observations into three viscosity clusters was performed using k-means. The model was first developed for mustard seeds, which show high natural FA variability, and was later applied to the screening of castor seeds.

Experimental Procedure

Materials

Chemicals

All chemicals and reagents used in this study were analytical grade. GC standard rapeseed oil was purchased from Supelco (Bellefonte, PA, USA).

Plant Material

Twenty varieties of mustard seed (M1–M20) were purchased from local suppliers in Israel. Castor seeds were obtained from the international seed bank (US) in 2009, and included 175 varieties from all over the world. Thirty seeds were randomly chosen for building the screening model (C1–C30).

Methods

TD-NMR Measurements

Prior to measurement, the seeds were oven dried at 70 °C for 72 h to remove excess moisture. TD-NMR experiments were performed on a Maran bench-top pulsed NMR analyzer (Resonance Instruments, Witney, United Kingdom), equipped with a permanent magnet and a 18-mm probe head, operating at 23.4 MHz. The seeds were equilibrated at 40 °C for 30 min before measurement. The CPMG sequence was performed using a 90° pulse width of 6.2 μs, echo time (τ) of 0.1 ms, recycle delay of 1 s, and 4 scans. For each sample, 8,000 echoes were acquired.

Oil Extraction

About 3 g of seeds were crushed manually in a mortar and incubated overnight with 100 mL of hexane in an orbital shaker at 25 °C. The oil and hexane solution was then evaporated under a mild vacuum with a rotary evaporator. In cases where larger volumes of oil were required (>10 mL), a cold-pressed extruder was used (Komet CA 59G, Mönchengladbach, Germany).

Viscosity Calculations

The FA composition of the seeds was determined by GC on a Varian 3400 apparatus (Palo Alto, CA, USA), equipped with a flame ionization detector and a capillary column (RESTEK, Bellefonte, PA, USA; Dimensions: 15 m × 0.32 mm × 0.25 μm). Results were normalized to 100% with less than 3% unidentified FAs. The composition was then used to calculate the kinematic viscosity, as described

Table 1 Kinematic viscosities of neat FA methyl esters [14]

Structure	Systematic name	Kinematic viscosity (mm^2/s)	Structure (cont.)	Systematic name	Kinematic viscosity (mm^2/s)
14:0	Myristic acid	3.23	20:0	Arachidic acid	5.85[a]
16:0	Palmitic acid	4.38	20:1	Eicosenoic acid	5.77
16:1	Palmitoleic acid	3.67	22:0	Behenic acid	5.85[a]
18:0	Stearic acid	5.85	22:1	Erucic acid	7.33
18:1	Oleic acid	4.51	24:0	Lignoceric acid	5.85[a]
18:2	Linoleic acid	3.65	24:1	Nervonic acid	8.8[b]
18:3	Linolenic acid	3.14	18:1-OH	Ricinoleic acid	15.44

[a] Allen et al. (1999) [13]

[b] Through personal correspondence with Dr. G. Knothe; data not published

by Allen et al. [13] using Eq. 1. μ_i and μ_0 are the kinematic viscosities at 40 °C of each FA and biodiesel, respectively; y_i is the mass fraction of each FA. Neat FA viscosities, given as fatty acid methyl esters (FAMEs), were taken from [14], and are listed in Table 1.

$$\ln \mu_0 = \sum_{i=1}^{n} y_i \ln \mu_i. \quad (1)$$

Mustard and castor oil contain minor levels of arachidic (20:0), behenic (22:0), and lignoceric (24:0) FAs, which are solid at 40 °C. Their neat viscosities were assumed to have values similar to those of stearic acid (18:0), as suggested by Allen et al. [13]. Nervonic (24:1) acid viscosity could not be found in the literature, thus was extrapolated (8.8 mm^2/s; see Table 1 remarks). The accuracy of the method, following these assumptions, was validated using the ASTM D445 standard method (performed at ICT laboratories, Israel) by comparing measured and calculated viscosities. A 20-mL specimen of the oil were transesterified as described by Dorado et al. [15] for mustard oil and Meneghetti et al. [16] for castor oil. Measured and calculated viscosities were identical for mustard oil (5.53 mm^2/s) and accurate for castor oil (12.68 and 12.78 mm^2/s, respectively), confirming the use of the FAME profile to calculate viscosity. Calculated viscosities are given as triplicates in Table 2 for mustard seeds and Table 3 for castor (STD was less than 0.1 and 0.3, respectively).

Table 2 Average T_2, w_0 and viscosity of 20 mustard seeds

Sample	T_2 (ms)	M_0	Viscosity (mm^2/s)	Cluster[a]
M1	155.46	826.14	4.59	L
M2	131.77	803.86	5.53	H
M3	146.02	796.50	4.82	L
M4	133.97	821.18	5.48	H
M5	135.93	850.46	5.39	H
M6	134.93	824.24	5.54	H
M7	137.69	831.14	5.44	H
M8	137.45	853.90	5.37	H
M9	153.17	804.85	4.59	L
M10	146.09	808.86	5.15	M
M11	147.29	829.37	5.18	M
M12	138.79	846.72	5.30	M
M13	134.90	837.37	5.50	H
M14	135.71	851.52	5.45	H
M15	135.70	825.76	5.47	H
M16	138.35	830.14	5.48	H
M17	153.03	824.18	4.83	L
M18	134.36	836.70	5.55	H
M19	137.07	828.08	5.41	H
M20	133.47	856.19	5.64	H

[a] The three categories were defined by k-means as: $5.08 > L$; $5.08 \leq M \geq 5.32$; $H > 5.32$ mm^2/s

Chemometrics

All chemometric calculations were performed using STATISTICA (version 8.0, StatSoft). PCA is a common chemometric tool, mainly used to reduce data dimensionality [17]. The algorithm maximizes the original variance using a minimal number of principal components (PCs). The PCs are mutually orthogonal and their extraction is such that the first PC holds the maximum variance, the second holds the second-maximum variance, and so on. The general mathematical model can be described by Eq. 2, where **X** is the transverse TD-NMR relaxation data ($I \times J$), **P** contains the underlying profiles ($J \times N$; called loadings), and **T** is the contributing amplitude (I × N; called scores).

$$\mathbf{X} = \mathbf{T} \cdot \mathbf{P}^{\mathrm{T}} + \mathbf{E}. \quad (2)$$

The scalar N is the number of PCs resolved and **E** ($I \times J$) holds the residual unexplained variation. PCA was

Table 3 Average viscosity of 21 castor seeds used for building the screening model and 9 seeds used for validation

Sample	Viscosity (mm^2/s)	Cluster[a]	Sample (cont.)	Viscosity (mm^2/s)	Cluster[a]	Sample (cont.)	Viscosity (mm^2/s)	Cluster[a]
C1	12.39	H	C11	12.06	M	C21[b]	12.56	H
C2	12.12	M	C12[b]	12.08	M	C22	11.96	M
C3	12.00	M	C13	12.43	H	C23[b]	11.87	L
C4	12.26	H	C14[b]	12.36	H	C24	11.72	L
C5	11.85	L	C15	11.85	L	C25	11.90	L
C6	11.71	L	C16	12.23	H	C26	11.80	L
C7[b]	12.05	M	C17	12.33	H	C27	11.97	M
C8	12.34	H	C18[b]	12.38	H	C28[b]	11.83	L
C9	12.06	M	C19[b]	11.87	L	C29	11.67	L
C10	12.30	H	C20	12.37	H	C30[b]	12.13	M

[a] The three categories were defined by k-means as: $11.96 > L$; $11.96 \leq M \leq 12.20$; $H > 12.20$ mm^2/s

[b] Used for validation

applied on the normalized CPMG data using the NIPALS algorithm. Normalization was performed by dividing the spectrum of each sample by its first (and highest) intensity and mean-centering it (covariance matrix). Only the scores of the first two PCs were extracted.

K-means is a fast iterative algorithm that has been extensively used for classification [12]. Defining three clusters yielded high, medium, and low viscosity clusters (H, M, and L, respectively). Clustering was applied to the calculated viscosities (Tables 2 and 3 for mustard and castor seeds, respectively) and the scores of PC_1 and/or PC_2. Observation assignment according to the calculated viscosity was considered the proper grouping.

T_2 Measurements

Assuming an exponential decay of the acquired CPMG data, a fixed number of pre-exponential weighting factors (w_i) and relaxation decay constants (T_{2i}) are extracted. T_{2i}s have been found to characterize and distinguish between populations (such as water and oil), and the pre-exponential weighting factors represent a quantitative measurement of each T_2 population [18].

The CPMG raw data were exponentially fitted using SPSS software (version 15.0, SPSS Inc.) and Eq. 3. w_i is the amplitude of the ith exponential, indicating the concentration of the ith component, and T_{2i} is the characteristic relaxation time constant.

$$W = \sum_{i=0}^{N} w_i \exp(-t/T_{2,i}) \quad (3)$$

The viscosity of oils is one order of magnitude greater than that of biodiesel [5], as can be realized from exploring their decay rates. T_2 of castor oil was approx. 40 ms, whereas for the corresponding biodiesel this was 230 ms. Typically, the transverse relaxation time of solids is rapid and that of liquids is slow [18]. Triacylglycerols have lower mobility due to their more rigid structure, whereas biodiesels, which consist of individual FAMEs, have greater freedom of movement. Previously published findings about the inverse relation of T_2 and viscosity strengthen this result [9].

The oil in oil-containing materials usually follows a bi-exponential behavior, possibly due to the presence of saturated and unsaturated oil phases [19]. Given that viscosity is a property that stems from all of the oil components, only a single exponential was used in this study. T_2 and w_0 of mustard seeds are shown in Table 2 as triplicates. The broader range of T_2s compared to castor (133–156 and 43–46 ms, respectively; data not shown for castor) emphasizes the initial assumption on the broad FA profile natural diversity in mustard.

Results and Discussion

Screening of Mustard Seeds

The score scatter plot of PC_1 (score of PC_1) and PC_2, holding 100% of the original variance, is shown in Fig. 1. Clusters were drawn according to the groupings assigned by k-means, using both scores. Ascription of observations to the appropriate cluster was not in good agreement with the real viscosity categories listed in Table 2. For example, M10 and M20 were clustered together though they were assigned to different calculated viscosity categories (M and H, respectively). Repeating k-means calculations using only PC_2 as the active input yielded similar groupings as the reference data (Fig. 2), with only two misclassifications (M3 and M13). This indicates PC_2 holds information about

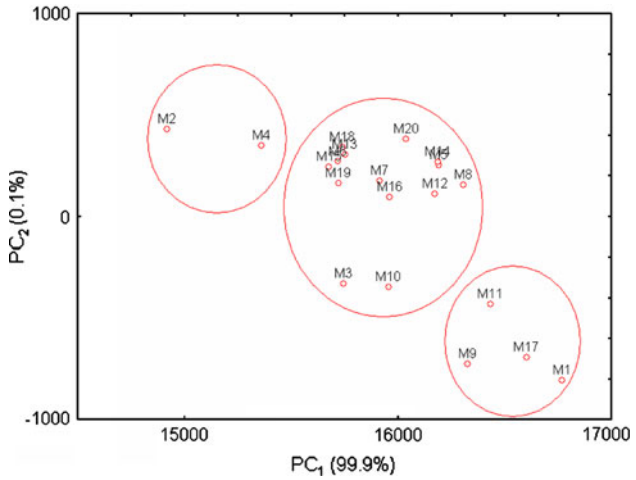

Fig. 1 PCA score scatter plot of 20 mustard seeds, clustered to three groups by 2D k-means. The clusters were not correlated to viscosity

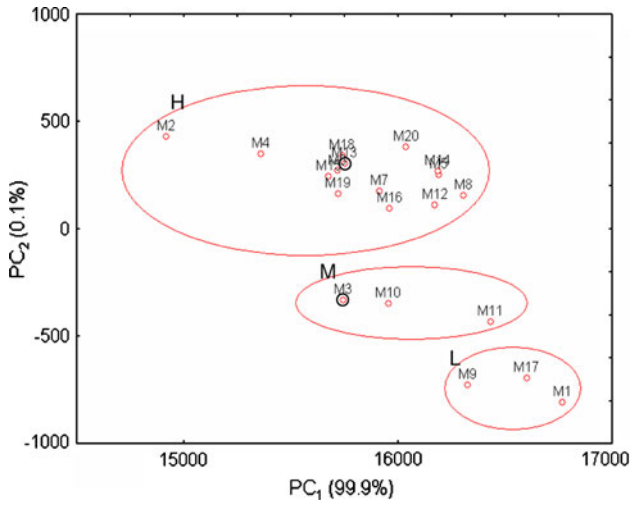

Fig. 2 PCA score scatter plot of 20 mustard seeds, clustered to three groups by 1D k-means with PC_2 as a single input. The new clusters were highly correlated to viscosity, with only two misclassifications (*circled* cases M3 and M13)

viscosity, meaning high score represents high viscosity and vice versa; whereas PC_1, though holding most of the variance (99.9%), was immaterial for screening by viscosity. A possible explanation for the two misclassifications may be their greater water content relative to the other seeds, which has been shown to lead to longer decay rates [10, 19].

Despite the fact that T_2 is extracted using exponential fitting while PC_2 is strictly a linear transformation of the data with no model constrains, they were found to be closely linked ($R^2 = 0.99$; Fig. 3a). Another interesting finding was the correlation of PC_1 to the pre-exponential coefficient w_0 according to each viscosity group, as shown in Fig. 3b ($R^2 = 0.88$, 0.99, and 0.95 for H, M, and L categories, respectively). w_0 was previously found to correlate to quantitative aspects such as oil content [20], when divided by the weight. This interpretation was true only when scaling the raw NMR data of each observation by its first value. Therefore, it is our assumption that by scaling the NMR data by the first value, PC_2 can be used as a viscosity marker.

Since PC_1 was found to only interfere with data interpretation, as previously suggested, observations were plotted against PC_2 in a 1D line plot (Fig. 4). This is a different representation of the same scores, leading to identical viscosity clusters as in Fig. 3. The same three clusters are now separated by linear thresholds as defined by k-means ($L \leq -520$; $-520 < M \leq -148$; and $H > -148$). Mapping of observations using a line plot coupled with k-means was chosen as the preferred screening model. The same methodology was then applied on castor seeds.

Screening of Castor Seeds

The thirty castor seeds that were chosen to represent high, medium, and low viscosity in order to develop a similar screening model are shown in Table 3. This batch was further divided into 21 and 9 samples for constructing and validating the model, respectively. The three viscosity regions ($-40 > L$; $-40 \leq M \leq 60$; and $H > 60$) as defined by k-means with PC_2 as classification criteria are shown in Fig. 5. Only one misclassification was detected (C22; 11.96 mm^2/s), probably since it lies on the border between clusters L and M in Table 3. The performance of

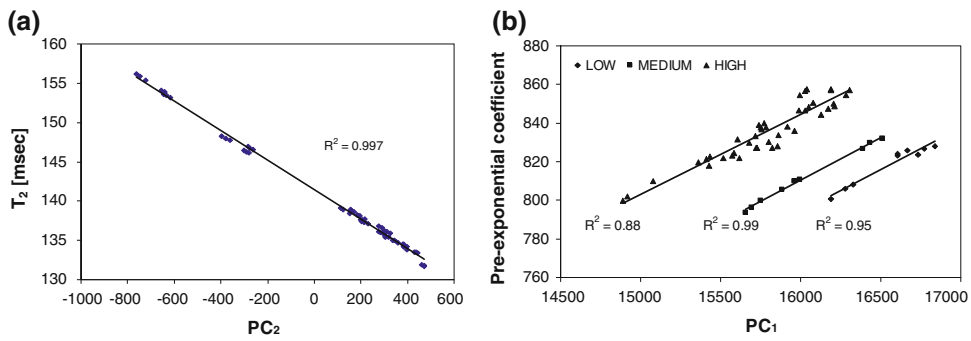

Fig. 3 A strong linear correlation was found between **a** PC_2 and T_2, and **b** PC_1 and M_0, for each viscosity cluster. Results are presented for each measurement and not as triplicates

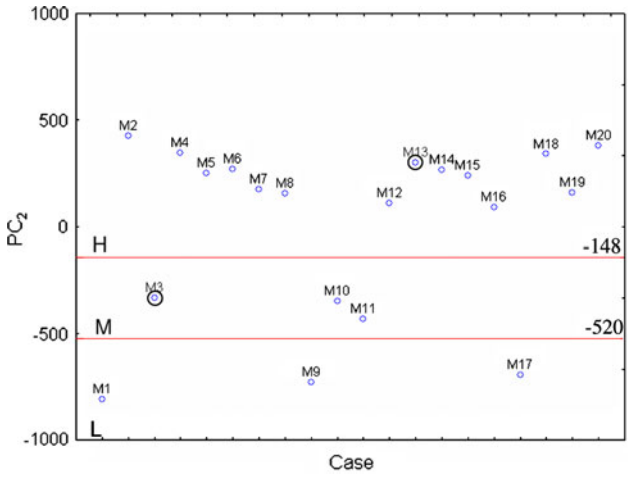

Fig. 4 Line plot of mustard seeds divided into three viscosity regions (L, M, H) by PC_2. Only two misclassifications were found (*circled* cases M3 and M13)

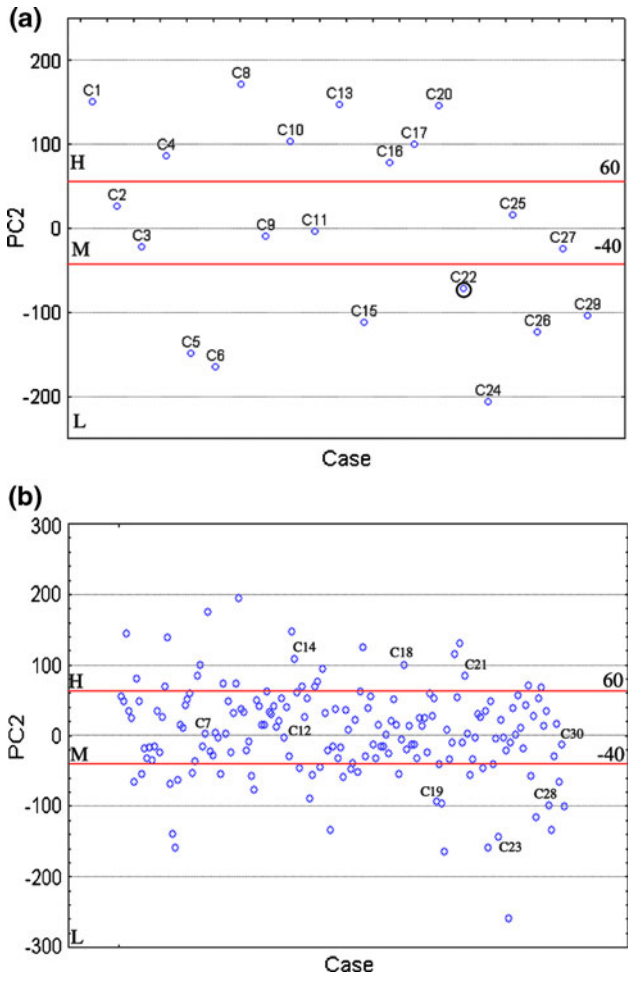

Fig. 5 Line plot of **a** 21 castor seeds divided into three viscosity regions (L, M, H) by PC_2. Only a single misclassification was detected (*circled* case C22); **b** 175 seeds used in this study, with nine samples used for validation, without misclassifications

the model was validated using the remaining nine samples without any misclassifications (marked in Fig. 5b, which shows all of the seeds), emphasizing its accuracy. As in the case of mustard, T_2 and PC_2 were highly correlated ($R^2 = 0.99$; data not shown), indicating that the interaction between these parameters is not limited to a specific family of oilseeds.

According to the screening model proposed here, unique castor seeds with low viscosity will be easily observed at the lowest end of this line plot—the lower its PC_2 score, the lower its viscosity. Unfortunately, among the 175 types of castor seeds from all over the world, no samples were found to comply with the standard viscosity specification.

Mustard and Castor Mixed Model

The FA composition and calculated viscosity of three characteristic seeds, one for each viscosity cluster, from each variety, along with a mixture of these seeds (CM2, CM4, and CM6) are shown in Table 4. CM samples consist of 2 g mustard seeds with varying quantities of castor seeds, i.e. CM4 consists of four castor seeds and 2 g of mustard seeds. Since we were not able to obtain low viscosity castor seeds, measuring this type of mixture provided a simple simulation of reduced ricinoleic acid content. The relation between the level of the dominant FA content and viscosity is also pointed out in Table 4. The main FAs in castor and mustard seeds are ricinoleic and erucic acids, respectively. The greater their relative content the greater the viscosity. This is due to their increased viscosities in relation to all other FAs (Table 1). When exploring each type of seed it can be observed that a decrease of 6.7% in ricinoleic acid content in castor seeds reduces viscosity by 7% (C24 and C21, respectively), while the same 7% reduction of viscosity in mustard seeds (M10 and M18, respectively) is achieved only by decreasing the erucic acid levels by 35%. This can be explained by examining the neat FA viscosities of ricinoleic and erucic acids (15.44 and 7.33 mm^2/s, respectively; Table 1). Therefore, a moderate decrease in ricinoleic acid will lead to a sharp decrease in viscosity, which will be easily detected by the model.

A PCA line plot of all mustard, castor, and mixture samples is shown in Fig. 6. Here CM samples were positioned far below the neat castor seed array, confirming the feasibility of this method in easily detecting outliers with reduced viscosity. Furthermore, neat castor and mustard seed clustering remained unchanged compared to Figs. 5a and 6. These results emphasize the need for genetically modified castor seeds, since their FA composition's natural variability is quite low compared to that of mustard.

The model suggested here can also be used for screening transgenic seeds from established crops, containing elevated

Table 4 Characteristic FA composition of C, M, and CM from each viscosity category

Sample	Category[b]	FA composition[a] (%)								Average viscosity (mm^2/s)
		16:0	18:0	18:1	18:1-OH	18:2	18:3	20:1	22:1	
M1	L	3.48	0.87	23.52	–	29.69	12.58	9.23	18.34	4.59
M10	M	3.15	1.24	11.00	–	19.59	15.19	7.07	37.77	5.15
M18	H	2.26	0.75	8.57	–	16.66	10.74	5.92	50.89	5.55
C24	L	2.12	2.04	8.10	78.86	7.37	0.72	0.78	–	11.72
C11	M	1.97	2.03	5.97	80.97	7.85	0.63	0.58	–	12.06
C21	H	1.64	1.51	2.99	84.13	7.60	1.07	0.53	–	12.56
CM2		2.79	1.53	10.34	11.81	12.55	9.16	6.27	42.78	6.28
CM4		2.29	1.34	8.29	26.32	10.34	7.00	5.14	35.97	7.34
CM6		2.26	1.58	7.93	40.08	9.68	5.94	3.99	26.18	8.22

[a] Only major FAs are shown

[b] According to the corresponding line plots

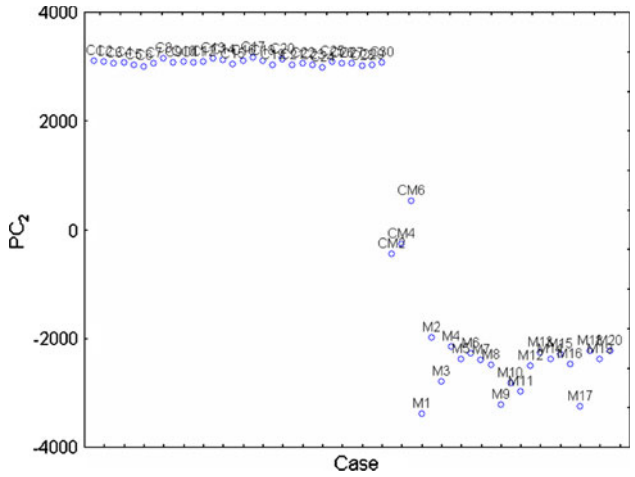

Fig. 6 Castor and mustard seeds from the previous models are plotted in a line plot along with CM mixed samples, simulating seeds with reduced ricinoleic acid content. Outliers with reduced viscosity are easily detected for a large batch of seeds

levels of hydroxylated FAs. Due to strict environmental regulations, sulfur content in petrodiesel has been significantly reduced. This has led to reduced fuel lubricity, which can cause severe damage to an engine [5]. Recent studies have shown that a blend of high oleic vegetable oil and castor oil (with 10–15% ricinoleic acid content) is an excellent feedstock for a variety of lubricant applications [21]. Developing transgenic oilseeds with moderate levels of hydroxylated FAs may answer this industry's need. Detection of transgenic seeds with elevated levels of hydroxylated FAs (and thus increased viscosity) can be easily achieved with the model proposed herein.

The main drawback of the suggested large-scale screening procedure is the extended pre-drying phase (72 h), given the contribution of water components to the acquired relaxation signal [9, 19]. One possible solution would be to employ a pulse sequence consisting of a combination of pulsed field gradient (PFG) and CPMG [22], thus eliminating contributions from water protons without the need for drying.

Conclusions

Rapid large-scale screening of intact castor seeds can be easily achieved using a combined TD-NMR and chemometrics approach. The model suggested in this work is superior to conventional analytical methods when screening thousands of seeds, since unique outliers showing reduced viscosity can be detected straightforwardly. The same approach can also be applied to screening within other types of oilseeds which differ in their FA profiles.

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