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# Simultaneous Determination of the Saponification Value, Acid Value, Ester Value, and Iodine Value in Commercially Available Red Fruit Oil (*Pandanus conoideus*, Lam.) Using ^{1}H qNMR Spectroscopy

Liling Triyasmono[1,2] · Curd Schollmayer[1] · Jens Schmitz[1] · Emilie Hovah[1] · Cristian Lombo[1] · Sebastian Schmidt[1] · Ulrike Holzgrabe[1]

## Abstract

Red fruit oil (RFO) can be extracted from fruits of *Pandanus conoideus,* Lam., an endogenous plant of Papua, Indonesia. It is a commonly used essential original traditional medicine. By applying a newly developed quantitative ^{1}H NMR (qNMR) spectroscopy method for quality assessment, a simultaneous determination of the saponification value (SV), acid value (AV), ester value (EV), and iodine value (IV) in RFO was possible. Dimethyl sulfone ($DMSO_2$) was used as an internal standard. Optimization of NMR parameters, such as NMR pulse sequence, relaxation delay time, and receiver gain, finally established the ^{1}H NMR-based quantification approach. Diagnostic signals of the internal standard at $\delta = 2.98$ ppm, SV at $\delta = 2.37$–2.20 ppm, AV at $\delta = 2.27$–2.20 ppm, EV at $\delta = 2.37$–2.27 ppm, and IV at $\delta = 5.37$–5.27 ppm, respectively, were used for quantitative analysis. The method was validated concerning linearity ($R^2 = 0.999$), precision (less than 0.83%), and repeatability in the range 99.17–101.17%. Furthermore, this method was successfully applied to crude RFO, crude RFO with palmitic and oleic acid addition, and nine commercial products. The qNMR results for the respective fat values are in accordance with the results of standard methods, as can be seen from the *F*- and *t*-test (< 1.65 and < 1.66, respectively). The fundamental advantages of qNMR, such as its rapidity and simplicity, make it a feasible and existing alternative to titration for the quality control of RFO.

**Keywords** Quantitative ^{1}H NMR · Saponification Value · Acid Value · Ester Value · Iodine Value · Red Fruit Oil

## Introduction

The demand for speed and effectiveness of analytical methods, thereby resulting in high accuracy and precision, has increasingly become priority in recent years. One of the most promising methods for overcoming these challenges is quantitative nuclear magnetic resonance (qNMR) (Yu et al. 2018). The guidance on the use of qNMR and its application for quantitative purposes was reviewed by Holzgrabe (2010) and Beyer et al. (2010a) by depicting the quantitative

analysis of oversulfated chondroitin sulfate and dermatan sulfate in heparin glycosaminoglycans. This has become the required method in the USP, as well as the use of qNMR for purity control of pharmaceutical grade L-alanine and determination of several lipid parameters with internal calibration for iodine, peroxide, and acid values.

Although the cost of NMR equipment is relatively high and requires operator experience, the qNMR method has many outstanding advantages. In addition to the ^{1}H NMR's ability to provide structural information, the proportionality of signal intensity with the number of cores allows quantification if recorded with the proper experimental NMR parameters (Beyer et al. 2010a; Holzgrabe 2010). Furthermore, a reliable non-destructive analysis enabling a rapid, simple, and simultaneous analysis of different analytes in one sample is possible (Hollis 1963; Jungnickel and Forbes 1963). Of note, it is not even necessary to have a reference substance (Holzgrabe 2010). Therefore, these inherent advantages make ^{1}H NMR a powerful tool for

✉ Ulrike Holzgrabe
ulrike.holzgrabe@uni-wuerzburg.de

1 Institute for Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany

2 Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, 70713 Banjar Baru, Indonesia

quantification. Between 1991 and 2015, qNMR was used in more than 1750 publications in the field of food science (Lachenmeier et al. 2016) which indicates its potential.

Conventionally, the saponification value (SV) and acid value (AV) are determined by an acid–base titration method; the ester value (EV) is calculated from both these values. Such titration methods are dependent on observing the visual endpoint, which might be challenging, especially in the case of red fruit oil (RFO) where the solution is already color red. The iodine value (IV) is generally used to estimate the degree of unsaturation of oil and fat. This determination is based on the reaction of double bonds within fatty acids and monoiodine bromide. This reaction consists of several steps and is also time-consuming. Potentiometric pH metrics, chromatography, and FTIR have been used to overcome these problems (Bernárdez et al. 2005; Triyasmono et al. 2013; Tubino and Aricetti 2013). However, some of these methods still have weaknesses because they require chemical modification of the sample for analysis, as described by Guillen et al. (2003). However, some qNMR methods have recently been reported for the characterization and quality assessment of lipids and oils (Guillén and Ruiz 2003a, b; Skiera et al. 2014; Hafer et al. 2020).

Red fruit oil (RFO) is extracted from the fruit of the *Pandanus conoideus*, Lam. plant. This fruit is red, is 68 to 110 cm long, is 10 to 10 cm in diameter, and contains large oil. The plant is endogenous in Papua, Indonesia, and is a commonly used traditional medicine. The oil has large quantities of monounsaturated fatty acids, mainly oleic acid (60–70%) (Rohman et al. 2012), which supposedly account for beneficial impacts on human well-being, for example, forestalling cardiovascular infections, decreasing plasma triacylglycerol (TAG), or expanding cholesterol levels of high-density lipoprotein (HDL) levels (García-González et al. 2008). Testimonies of the effectiveness of RFO have been published, among others, inhibiting tumor growth and killing cancer cells could be observed (Khiong et al. 2009).

The substantial pharmacological potential makes RFO a promising candidate for herbal products or functional food. Today, various RFO products are already available on the market in Indonesia and abroad. However, multiple factors, such as geographical region, harvest time, and processing method, cause this red fruit's oil content and composition (Sarungallo et al. 2015). Therefore, the required RFO quality control is carried out at all stages of the production cycle, including incoming raw materials, during processing stages, and control of product output (Kleymenova et al. 2021). The quality assurance of RFO has to be ensured by the determination of SV, AV, EV, and IV (Endo Y 2018), which are given in a certificate so that certified RFO products will be guaranteed quality and increase the competitiveness of their products.

This study aimed to develop a qNMR method for simultaneously determining the SV, AV, EV, and IV of RFO. Experimental NMR conditions were systematically optimized, including relaxation delay time, pulse angle, and receiver gain. Method validation includes linearity, precision, repeatability, limit of detection (LOD), and limit of quantitation (LOQ) based on the guidelines of the International Conference on Harmonization (ICH) (ICH 2005). Furthermore, the results obtained by the qNMR method were compared with the compendial methods (titration) of the European Pharmacopoeia (Ph. Eur. 10 2020).

## Material and Methods

### Chemicals

Deuterated chloroform (CDCl$_3$, 99.8% D) was purchased from Eurisotop (Saarbrücken, Germany). Tetramethylsilane (TMS) and hexa deuterium dimethyl sulfoxide (DMSO-d$_6$, 99.9% D) from Deutero (Kastellaun, Germany), dimethyl sulfone (DMSO$_2$, TraceCERT®, 99.99%) internal standard for quantitative NMR grade, palmitic acid, and oleic acid standards from Merck (Darmstadt, Germany). Furthermore, for titration, 0.1 M NaOH, 0.1 M sodium thiosulfate, 0.5 M HCl, and 0.5 M ethanolic KOH were purchased from VWR (BDH Chemicals) (Darmstadt, Germany). Ethanol, petroleum ether, chloroform, iodine monobromide, KI, and starch were purchased from Merck (Darmstadt, Germany); they all were of analytical grade and complied with the requirements of the international standard ISO 660:2009.

### Apparatus

Quantitative $^1$H NMR experiments were performed by using a Bruker AVANCE III 400 MHz spectrometer operating at 400.13 MHz (Bruker BioSpin GmbH, Rheinstetten, Germany); using an inverse probe NMR tube Boro 400–5-7 (Deutero, Kastellaun, Germany). The analytical balances AT21 Comparator (FACT) and AB204-S (Mettler Toledo, Gießen, Germany) were used. Titrations were carried out using a Titroline 6000/7000 instrument (SI Analytics, Mainz, Germany); lithium chloride was applied to the ethanol electrode and pH electrode (SI Analytics N6480 Eth, Mainz, Germany).

### Sample Extraction

The fruits of *Pandanus conoideus,* Lam. were collected from different regions (Nabire and Jayawijaya) of Papua, Indonesia. Furthermore, the RFO was obtained using the solvent extraction method by Sarungallo et al. (2015). Briefly, the fruits were cut into small pieces and subsequently subjected

to a commercial blender containing ethanol and water (1:1, w/v). Next, approximately 12 g of the pulp of the red fruit was macerated with 80 ml of a solvent mixture of chloroform and methanol (2:1, v/v) and stirred at room temperature for 1 h. The resulting solution was filtered and evaporated, 16 ml of a 0.88% aqueous NaCl solution was added, and then the aqueous and the organic layers were separated. The organic layer will remain red, and the aqueous layer will be colorless and slightly cloudy. Finally, the organic layer was evaporated at 40 °C, fined in dark bottles, dried with nitrogen gas, and stored at − 20 °C until analysis.

## Commercial Products Collection

Nine samples from different manufacturers of commercial products of RFO were purchased from a traditional herbal market in Jakarta, Indonesia, including one sample of BMOP (Griya An-Nur/Exp date: 03.2023), Golden Red (Basmallah Food/Exp date: 11.2022), MBM (PRIMA SOLUSI/Exp date: 10.2022), Pro Jep (HERBAL 21/Exp date: 06.2022), Red Oil Papua (FIRA HERBALINDO/Exp date: 07.2022), REDOTEN (SERIBU PULAU INDONESIA/Exp date: 07.2022), Redwin (Natures/Exp date: 03.2023), Sari Buah Merah (athaku Herbalife/Exp date: 12.2021), and Sari Buah Merah (Loh Jinawi/Exp date: 10.2022).

## NMR Experiments

833.33 mg of each RFO sample and 3.33 mg of $DMSO_2$ were dissolved in a solvent mixture $CDCl_3$ and $DMSO-d_6$ (5:1, v/v) containing 0.1% TMS and were diluted to 2.0 ml. After mixing for 1 min, 600 µL of each sample was analyzed by NMR spectroscopy in triplicate.

The $^1H$ NMR experiments were measured at $300.11 \pm 0.10$ K with a 30° flip angle, 32 scans, no rotation, and an acquisition time of 6.81 s, followed by a relaxation delay of 3 s. The receiver gain was set to 4, and for processing, a line broadening factor of 0.3 Hz was applied. The resulting digital resolution was 0.15 Hz with a spectral width of 30.00 ppm (time domain size 163 k). The phase and baseline corrections were performed manually with TopSpin version 4.0 (Bruker BioSpin GmbH, Rheinstetten, Germany). All offset signals are referenced to the TMS signal ($\delta = 0.00$ ppm).

## Longitudinal Relaxation Time (T1) Determination

Two hundred fifty milligram of each RFO sample and 1.0 mg $DMSO_2$ were dissolved in a 600 µL of a mixture of $CDCl_3$ and $DMSO-d_6$ (5:1, v/v). After mixing for 1 min, 600 µL of each sample was analyzed by NMR spectroscopy. The relaxation delays of all of these protons were determined by the inversion recovery pulse sequence method, using the T1

cal Bruker program. An arrayed experiment was set with different values of relaxation delay, ranging from 0.05 to 17 s.

## Determination of SV, AV, EV, and IV by qNMR

The following signals were used for quantitative analysis: $DMSO_2$ ($\delta = 2.98$ ppm), SV ($\delta = 2.37–2.20$ ppm), AV ($\delta = 2.27–2.20$ ppm), EV ($\delta = 2.37–2.27$ ppm), and IV ($\delta = 5.37–5.27$ ppm). The acquisition was carried out under the conditions mentioned above. Based on the calculation formula of quantitative NMR discussed by Holzgrabe (2010) and Bharti and Roy (2012) and the development by Skiera et al. (2014), furthermore, the results are calculated according to the equation below:

$$SV_{NMR} = \frac{M_{KOH}}{m_s} \cdot \frac{m_{DMSO2} \cdot P_{DMSO2}}{M_{DMSO2}} \cdot \frac{N_{DMSO2}}{N_s (2)} \cdot \frac{I_{\alpha\text{-}CH2\,(total)}\,(2.37\text{-}2.20\ ppm)}{I_{DMSO2}\,(2.98\ ppm)} \cdot 1000 \quad (1)$$

$$AV_{NMR} = \frac{M_{KOH}}{m_s} \cdot \frac{m_{DMSO2} \cdot P_{DMSO2}}{M_{DMSO2}} \cdot \frac{N_{DMSO2}}{N_s (2)} \cdot \frac{I_{\alpha\text{-}CH2\,(acid)}\,(2.27\text{-}2.20\ ppm)}{I_{DMSO2}\,(2.98\ ppm)} \cdot 1000 \quad (2)$$

$$EV_{NMR} = \frac{M_{KOH}}{m_s} \cdot \frac{m_{DMSO2} \cdot P_{DMSO2}}{M_{DMSO2}} \cdot \frac{N_{DMSO2}}{N_s (2)} \cdot \frac{I_{\alpha\text{-}CH2\,(ester)}\,(2.37\text{-}2.27\ ppm)}{I_{DMSO2}\,(2.98\ ppm)} \cdot 1000 \quad (3)$$

$$IV_{NMR} = \frac{M_{Iod}}{m_s} \cdot \frac{m_{DMSO2} \cdot P_{DMSO2}}{M_{DMSO2}} \cdot \frac{N_{DMSO2}}{N_s (2)} \cdot \frac{I_{CH=CH\text{-}}\,(5.37\text{-}5.27\ ppm)}{I_{DMSO2}\,(2.98\ ppm)} \cdot 100 \quad (4)$$

where $m$ denotes the sample weight in mg, $P$ denotes the purity, $M$ is the molecular weight in g/mol, $N_s$ is the number of protons, and $I$ is the $^1H$ NMR integral area according to Skiera et al. (2014).

## Method Validation

The validation process requires testing for linearity, precision, accuracy (repeatability), LOD, and LOQ according to the International Conference on Harmonization (ICH) guidelines (ICH 2005). For determining the linearity, precision, and accuracy of the method, five solutions containing 50, 100, 150, 200, and 250 mg of RFO and 1.0 mg of $DMSO_2$, respectively, were prepared in 600 µl of a solvent mixture of $CDCl_3$ and $DMSO-d_6$ (5:1, v/v) containing 0.1% of TMS. For the determination of LOD and LOQ, a six-series limited concentration solution containing 0, 2, 4, 6, 8, and 10 mg of RFO and 1.0 mg of $DMSO_2$ was prepared and dissolved in 600 µl of solvent mixture $CDCl_3$ and $DMSO-d_6$ (5:1, v/v) containing 0.1% of TMS. Each final solution was analyzed by NMR spectroscopy in triplicate.

**Linearity** Linearity was assessed by measuring five different concentration solutions of RFO, as described above. The regression curve is presented $y = a + bx$, with the mass ratio representing and the integral values, respectively. The correlation coefficients of quantitative protons were quantified

at $\delta = 2.37$–$2.20$ ppm, at $\delta = 2.27$–$2.20$ ppm, at $\delta = 2.37$–$2.27$ ppm, and at $\delta = 5.37$–$5.27$ ppm.

**Precision** The RSD of repeatability expressed precision. As described above, the repeatability was tested using five different concentration solutions which were measured in triplicate. In addition, the multivariate test was carried out to see all selected quantitative proton contributions of the RFO.

**Accuracy** The accuracy of qNMR was evaluated by a recovery; using five different concentration solutions was determined in triplicate, as described above. Accuracy is calculated by means of the following equation: Recovery (%) = [($m_x$-$m_0$/$m_s$] × 100%, where $m_x$ is the weight of the calculated sample, $m_0$ is the weight of the calculated blank sample, and $m_s$ is the weight of the sample taken.

**Limit of detection and limit of quantitation** LOD and LOQ were calculated based on the standard deviation of the $y$-intercept response and the slope of the calibration curve. A linear calibration curve is assessed by measuring six concentrations of RFO limited of range, as described above. It can be expressed in a model such as $y = a + bx$. This model is used to compute the sensitivity $b$ and the LOD and LOQ. Therefore, LOD and LOQ can be expressed as LOD = $3.3 S_a/b$ and LOQ = $10 S_a/b$, respectively, with $S_a$ being the standard deviation of $y$-intercepts of the response and $b$ being the slope of the calibration curve.

## Determination of SV, AV, EV, and IV by Titration

The SV was determined according to the Ph. Eur. 10.0 (2020). In brief, 2.0 g of RFO was dissolved in 25.0 ml of 0.5 M ethanolic potassium and refluxed for 30 min. The hot solution has to be titrated immediately with 0.5 M aqueous hydrochloric acid (HCl) solution using a potentiometric endpoint detection. A blank test was carried out. The SV was calculated using the equation SV = [28.05 x ($n_2$-$n_1$)]/$m$, with $m$ being the sample weight, $n_2$ being the volume of 0.5 M aqueous HCl solution used for titration of the blank samples, and $n_1$ being the volume of 0.5 M aqueous HCl solution used for titration of the sample. The presumed SV for RFO is 200–300 mg KOH/g.

The AV was determined according to the Ph. Eur. 10.0 (2020). In brief, 250 mg of RFO was dissolved in 50 ml of a mixture of ethanol and diethyl ether (1:1, v/v) and titrated with an aqueous 0.1 M potassium hydroxide solution using potentiometric endpoint detection. The AV was calculated using the equation AV = ($5.610 × n$)/$m$, with $m$ being the sample weight and $n$ being the volume of 0.1 M potassium hydroxide solution used for titration of the sample.

The EV was determined according to Ph. Eur. 10.0 (2020). In brief, the EV was calculated according to the equation EV = SV-AV.

The IV was determined according to the Ph. Eur. 10.0 (2020). In brief, 0.25 g RFO was placed in a dry 250-ml iodine flask. 15.0 ml of chloroform was added, followed by a slow addition of 25.0 ml of iodine monobromide solution; the flask was closed. The solution was allowed to stand in the dark for 30 min, shaking frequently. Then, 10.0 ml of 100 g/l potassium iodide solution and 100 ml of water were added, and the solution was titrated with 0.1 M sodium thiosulphate, using the starch solution as an indicator, which was added towards the end of the titration. A blank test was carried out. IV was calculated using the equation IV = [1.269 x ($n_2$-$n_1$)]/$m$, with $m$ being the sample weight, $n_2$ being the volume of 0.1 M sodium thiosulphate solution used for titration blank sample, and $n_1$ being the volume of 0.1 M sodium thiosulphate solution used for titration of the sample. The presumed IV for RFO is 60–100 g $I_2$/100 g.

## Comparison of the Results with Titration Methods

To compare the qNMR and titration methods, an F test, t-test, and a regression test were applied. The F test was used to assess the same precision and the t-test to assess the consistency between the two methods. A regression test was considered to evaluate the correlation and accuracy of qNMR with the titration method. The data was processed using Microsoft® Excel® 2019 MSO (Version 2204 Build 16.0.15128.20158) 64-bit software.

## Results and Discussion

The main components of RFO are mixed triglycerides formed from different fatty acids. Minor components are mono- and di-glycerides, sterols, vitamins, fatty acids, and others (Rohman et al. 2012; Sarungallo et al. 2015). In general, the RFO NMR spectra have a pattern similar to vegetable oils (Beyer et al. 2010a). The assignment of the $^1$H NMR spectra can be seen in Fig. 1.

## Selection of Solvents

A prerequisite for quantitative NMR spectroscopy is an unambiguous assignment of separated signals; hence, choosing an appropriate solvent is important and was adopted from Skiera et al (2014). A good signal separation was achieved using a mixture of CDCl and DMSO-$d_6$ (5:1, v/v), because the specific protons of the methylene α-CH$_2$ group at $\delta = 2.37$–$2.20$ ppm (F1 and F2) are clearly visible. The beneficial effect of adding DMSO-$d_6$ to CDCl$_3$
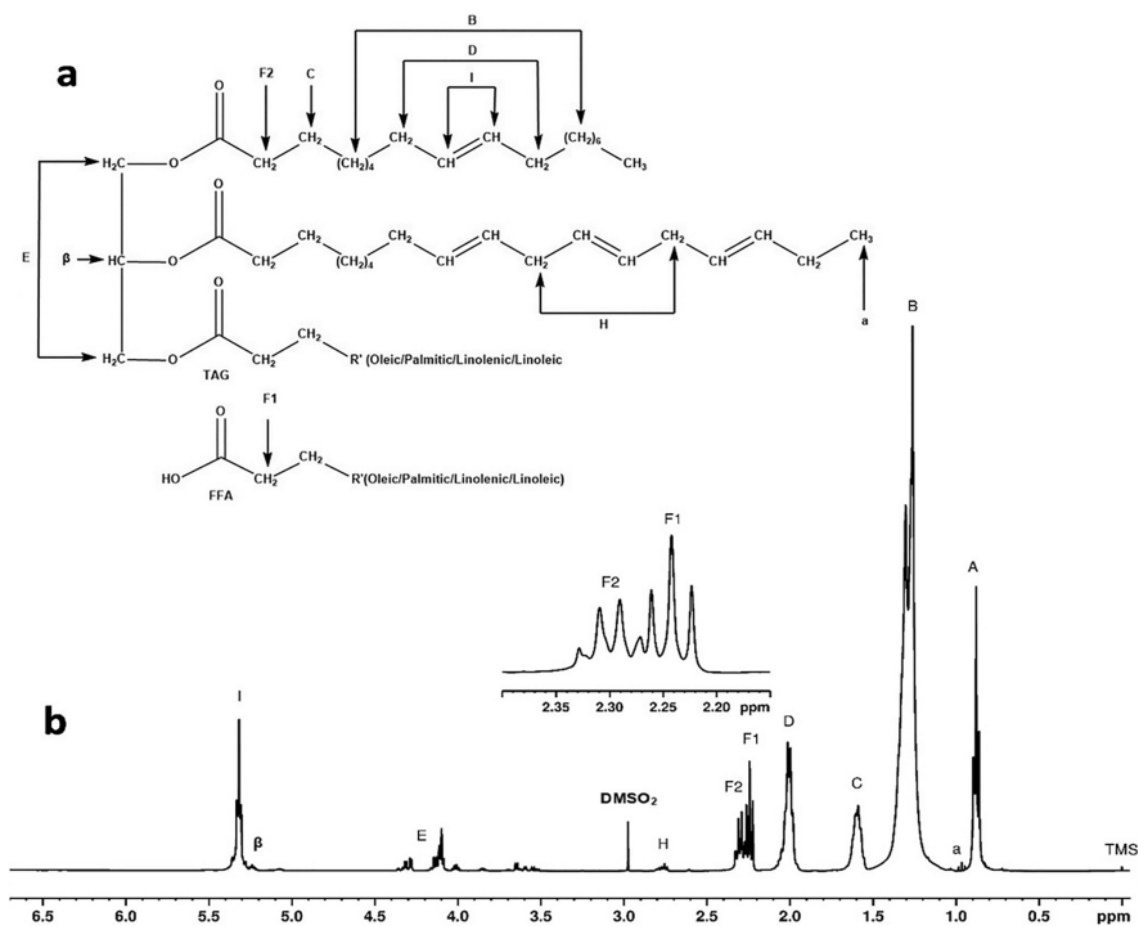
**Fig. 1** **a** Representative structures of triacylglyceride (TAG) and free fatty acid (FFA) and **b** $^1$H NMR spectrum of RFO dissolved in a mixture of CDCl$_3$ and DMSO-d$_6$ (5:1 v/v) containing TMS 0.1% with enlargement signal at δ = 2.20–2.37 ppm (F1 and F2)

is due to the NMR complex formation between DMSO and the fatty acid moiety (Abraham et al. 2006; Beyer et al. 2010b).

### Selection of an Appropriate Internal Standard

Selecting a proper internal standard is of great significance in qNMR experiments. DMSO$_2$ was used in this procedure because its signal at δ = 2.98 ppm does not overlap with sample and/or solvent components (see Fig. 1b). It is close to the analyte's resonance, thus minimizing the impact of pulse resonance (Fulmer et al. 2010; Giraudeau et al. 2014). Furthermore, DMSO$_2$ can be easily obtained with high purity and has good stability and solubility in the solvent system (Wells et al. 2004).

### Assignment of the $^1$H NMR Spectra

The $^1$H NMR spectrum of RFO consists of eleven signal groups appearing in spectral regions between δ = 0.50 and 5.50 ppm (Guillén and Ruiz 2003a, 2003b; Beyer et al. 2010a) (see Fig. 1). The signals are divided into eleven groups (A, a, B, C, D, E, F1, F2, H, I, and β) and are shown in Table. 1.

The α-CH$_2$ of both RFO and the FFA at δ = 2.27–2.20 ppm (F1) and δ = 2.37–2.20 ppm (F2) are of special interest in addition to F1/2 the glyceride protons at δ = 4.32–4.10 ppm.

### Selection of Quantitative Signals

Acidic hydrolysis using aqueous sulfuric acid was performed with RFO sample to confirm the assignment of signals F1

**Table 1** Assignment of signals of ¹H NMR spectra from RFO

| Signal | Functional group | Chemical shift (ppm) | |
|---|---|---|---|
| | | TAG | FFA |
| A | (-CH₃) saturated, oleic and linoleic acyl chains | 0.93–0.83 | 0.93–0.83 |
| a | (-CH₃) linolenic acyl chains | 1.03–0.93 | 1.03–0.93 |
| B | (-(CH₂)n-) methylene groups | 1.42–1.22 | 1.42–1.22 |
| C | (—O-CH₂-CH₂-) β-methylene protons | 1.70–1.52 | 1.70–1.52 |
| D | (—CH₂-CH=C) allyl methylene protons | 2.14–1.94 | 2.14–1.94 |
| E | (—CH₂OCOR) methylene protons in the glyceryl group | 4.32–4.10 | - |
| F1 | (—OCO-CH₂-) α-methylene protons | - | 2.27–2.20 |
| F2 | (—CO-CH₂-) α-methylene protons | 2.37–2.27 | - |
| H | (=C-CH₂-CH=) divinyl methylene protons | 2.84–2.70 | 2.84–2.70 |
| β | (-CHOCOR) methine proton at C2 of glyceride | 5.26–5.20 | 5.26–5.20 |
| I | (—CH=CH-) olefinic protons | 5.37–5.27 | 5.37–5.27 |

Signal number are given in Fig. 1; TAG δ = 0.83–5.50 ppm, FFA δ = 0.83–5.50 ppm

and F2 which were used to assess the SV, AV, and EV. Hydrolyzed RFO yields free fatty acids (FFA) (Salimon et al. 2011). As can be seen in Fig. 2b, upon hydrolysis, α-CH₂ (F2) of the TAG disappears, whereas the α-CH₂ (F1) of the FFA increases. The E signal of the methylene protons TAG at δ = 4.32–4.10 ppm disappeared after hydrolysis. Interestingly, the signals of the free glycerol are not visible because acidic conditions can catalyze the dehydration reaction of glycerol to form acrolein and other products, such as acrylic acid (Chai et al. 2007). Furthermore, the acrolein proton signal will resonate in the downfield region, CHO signal at δ = 9.51 ppm, CH₂=group at δ = 6.26 ppm, and δ = 6.11 ppm relative to TMS. This signal moved slightly, depending on the solvent and pH used (De las Heras et al. 2020). As happened in the hydrolyzed RFO spectra, acrolein gave a signal at δ = 6.52 ppm and δ = 6.37 ppm from protons of the CH₂=group with the enlarged spectra of these regions (see Fig. 2b), while CHO signal overlaps with other signals at δ = 9.00 ppm.

The triplets of the α-CH₂ signals of the FFA are slightly high field shifted in comparison to the corresponding signal of TAG which is a multiplet. This is in accordance with the data reported by Nieva-Echevarría et al. (2014) and Kan et al. (1964).

The comparison of ¹H NMR spectra between RFO, oleic acid, and palmitic acid standards was carried out to confirm the signal I (-CH=CH-) assignment to IV calculation because there is a linear relation between IV and the number of olefinic protons (Miyake et al. 1998). The -CH=CH- signals resonate at δ = 5.37–5.27 ppm in both RFO and oleic acid (see Fig. 2a and d).

The integrals of the signals F1 and F2, corresponding to α-CH₂ of both FFA and TAG at δ = 2.37–2.20 ppm, can be used for the quantification of SV: F1 correlated to α-CH₂ FFA at δ = 2.27–2.20 ppm for the determination of AV, F2 corresponding to α-CH₂ TAG at δ = 2.37–2.27 ppm for the

quantification of EV, and signal I correlated to -CH=CH- signals at δ = 5.37–5.27 ppm can be used for the determination of IV, respectively.

## Optimization of the Measuring and Processing Parameters

It is indispensable to know the relaxation time T1 for each signal when quantifying because of a complete relaxation of all signals to achieve more than 99.3% of the equilibrium magnetization is required (Holzgrabe 2010). An inversion recovery experiment revealed T1 times as follows: DMSO₂ proton 2.748 s (the longest T1), α-CH2 FFA 0.524 s, α-CH2 TAG 0.287 s, and -CH=CH- 1.583 s (see Fig. 3). All the T1 signals of RFO measured are similar to T1 triolein and other edible oils (Miyake et al. 1998). Hence, for 90° flip angle, a relaxation delay of 13 s is reasonable. To shorten the analysis time, a flip angle of 30° was applied, resulting in a delay of 9 s.

Choosing an appropriate NMR receiver gain (RG) can maximize the signal-to-noise ratio. Hence, the RG was varied between 4, 5, 5.5, and 6.35 (Torres and Price 2016). Figure 4 shows that the S/N value of the selected signal has a value of 1000, indicating that the sensitivity is acceptable (Holzgrabe 2010). However, the optimal S/N of each signal is appearing within the range of RG 4 to 5.

Furthermore, a suitable processing of the spectrum is essential to ensure reproducibility and traceability. The phase correction was done manually, and the baseline correction was carried out by the polynomial ABSG resulting in a narrow full width at half maximum (FWHM) value for the selected signal (TMS: 0.81 ± 0.07 Hz; DMSO₂: 0.84 ± 0.09 Hz). Therefore, the spectra appear to have sharp and symmetrical signals as desired (Deborde et al. 2019).
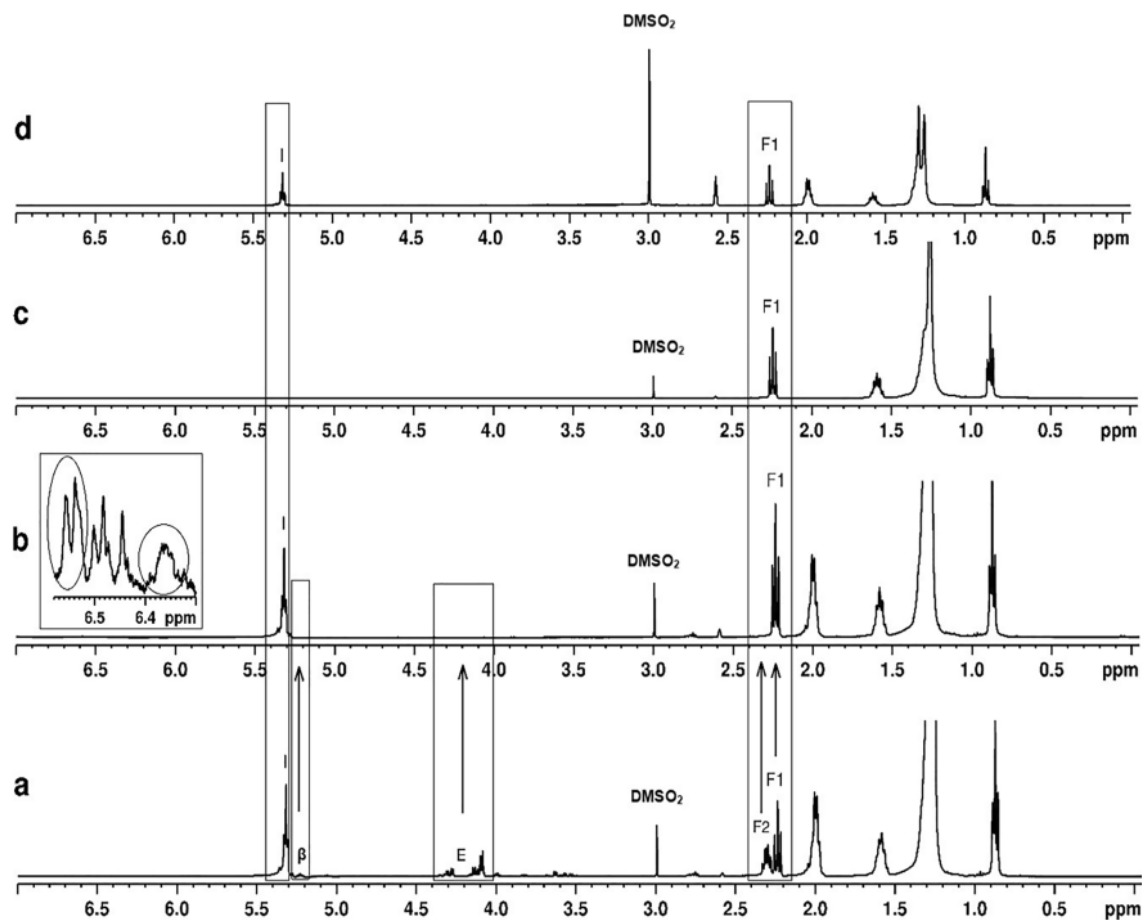
**Fig. 2** Stacked plot ¹H NMR Spectra at δ = 0.00–7.00 ppm (from bottom to top) of **a** RFO (100 mg), **b** hydrolyzed RFO (85 mg) with enlargement region of δ = 6.20–6.80 ppm, **c** palmitic Acid standard, and **d** oleic acid standard

## Method Validation

The linearity was examined with the obtained integrals of the signals at δ = 2.37–2.30 ppm; δ = 2.27–2.20 ppm; δ = 2.37–2.27 ppm; and δ = 5.37–5.27 ppm. They were plotted versus the series of RFO concentrations. Linear regression was processed using Microsoft® Excel® 2019 MSO (Version 2204 Build 16.0.15128.2015) 4-bit software. As shown in Table 2, linearity is represented by the linear regression equation and its coefficient of determination ($R^2$) being 0.999 for all selected signals.

A multivariate test was carried out to see all selected integral contributions of the RFO signal to the precision and repeatability of the chosen measurement method. The principal component regression (PCR) test indicates that the reference concentration of the RFO sample was proportional to the RFO determined by ¹H NMR, indicated by $R^2 > 0.999$ during calibration and 0.999 during validation. Additionally, precision was also demonstrated by small root mean square error (RMSE) values, RMSE calibration of 0.77, and RMSE validation of 0.90. The results of the model prediction test also show a linear relationship between the concentration measured by ¹H NMR and the prediction indicated by $R^2 > 0.999$ and RMSE prediction 0.78. As shown in Table 3, the mean recoveries of the five samples are in the range of 99.17–101.17%, with RSD% less than 0.83% (González et al. 2010). The recovery calculation is based on the principle of an external standard method. Taken together, the NMR method can be regarded as precise and accurate.

LOD and LOQ were determined by calculating the standard deviation of the $y$-intercept response and the slope of the calibration curve of six limited concentrations of RFO (ICH 2005). Furthermore, as shown in Table 4, the LOD is in the same range of 0.35–0.38 mg for all selected signals, and the LOQ for all selected signals has a similar value of
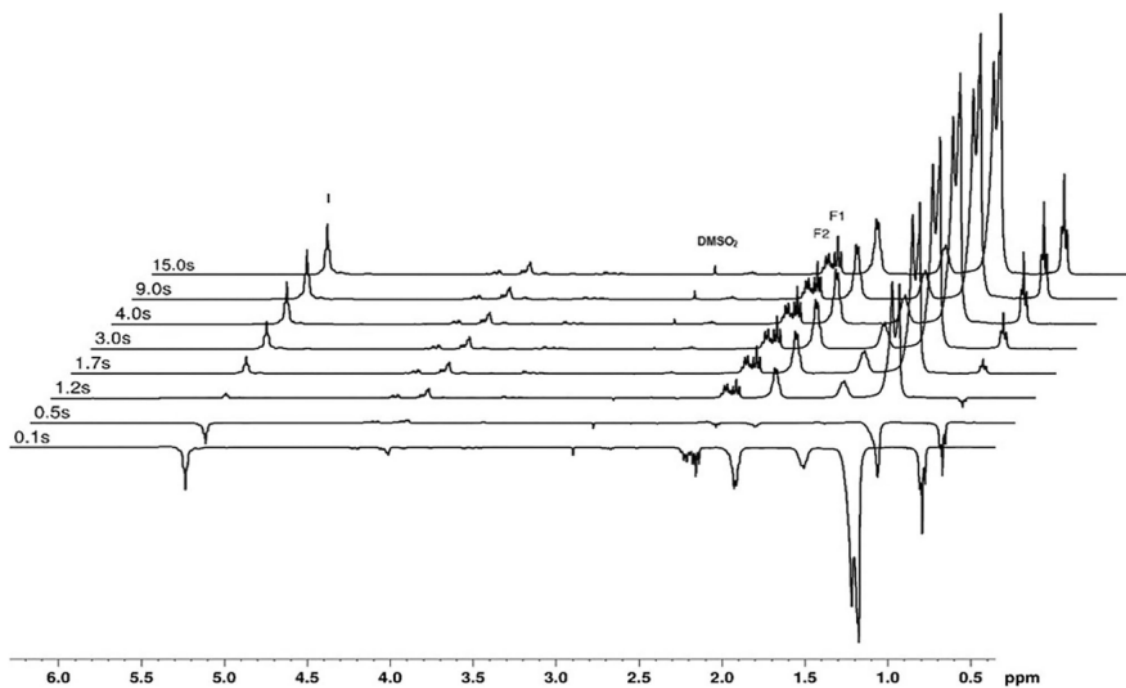
**Fig. 3** 400 MHz $^1$H NMR; an inversion-recovery pulse sequence of experiments used to measure the values of T1 for the protons of RFO in CDCl$_3$: DMSO-d$_6$ (5:1, v/v), flip angle $180^0$—$\tau$—$90^0$ was applied

1.37–1.58 mg, except for signals at $\delta = 2.27$–2.20 ppm that are below 4.78 mg, respectively. All LOQ values are comparable to the minimum S/N of 150 for achieving an RSD of less than 1% (Ph. Eur. 10.0. 2020).

These data demonstrated that the established qNMR approach was precise, accurate, and sensitive enough for the simultaneous quantitative determination of SV, AV, EV, and IV.

**Fig. 4** Relationship between receiver gain and S/N
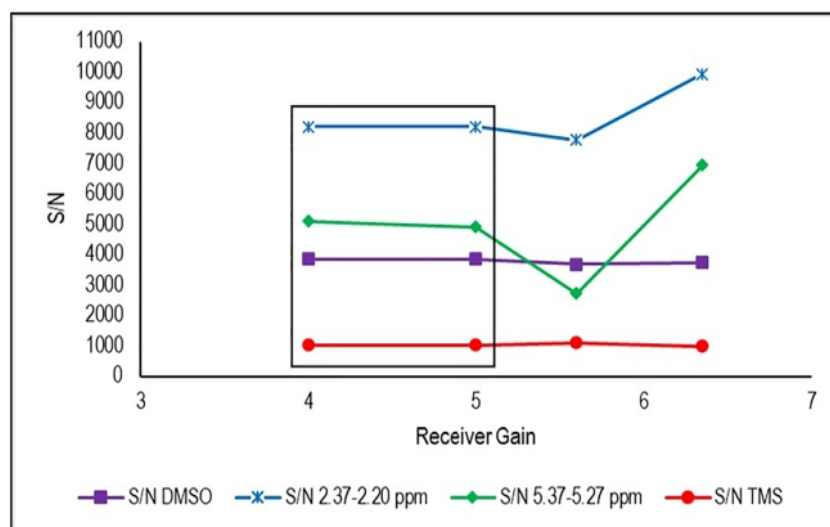
**Table 2** Linearity test results of the qNMR method

| Signal NMR (ppm) | Linear regression | | RSD (%) |
|---|---|---|---|
| | Equation | $R^2$ | |
| 2.37–2.20 | $y = 0.11x—0.02$ | 0.999 | 0.52 |
| 2.27–2.20 | $y = 0.06x + 0.08$ | 0.999 | 0.69 |
| 2.37–2.27 | $y = 0.05x—0.11$ | 0.999 | 0.60 |
| 5.37–5.27 | $y = 0.10x + 0.05$ | 0.999 | 0.47 |

**Table 3** Precision and recovery of five serial amounts of RFO

| Weight taken (mg) | Recovery (%) | RSD (%) | Average recovery (%) | Average RSD (%) |
|---|---|---|---|---|
| 50 | 100.91 | 0.64 | 101.17 | 0.83 |
| | 101.50 | 1.05 | | |
| | 101.12 | 0.79 | | |
| 100 | 99.12 | 0.63 | 99.17 | 0.59 |
| | 98.82 | 0.84 | | |
| | 99.56 | 0.31 | | |
| 150 | 99.51 | 0.35 | 99.92 | 0.34 |
| | 99.64 | 0.25 | | |
| | 100.60 | 0.42 | | |
| 200 | 100.53 | 0.38 | 100.23 | 0.16 |
| | 100.13 | 0.09 | | |
| | 100.04 | 0.03 | | |
| 250 | 100.43 | 0.30 | 99.96 | 0.23 |
| | 99.97 | 0.02 | | |
| | 99.49 | 0.36 | | |

## Quantification of SV, AV, EV, and IV of RFO with Palmitic Acid and Oleic Acid Addition

One of the principal methods that can be used to obtain absolute quantitative data is the standard addition method (Beyer et al. 2010a; Holzgrabe 2010). For this purpose, the standard addition method was carried out by adding palmitic acid and oleic acid to RFO, respectively. Furthermore, the difference of the selected signal integral and its application to quantify SV, AV, EV, and IV can be assessed.

Figure 5a displays the palmitic acid addition effect of linear increase integral signal at $\delta = 2.37–2.20$ ppm ($R^2$ 0.994) and $\delta = 2.27–2.20$ ppm ($R^2$ 0.998); meanwhile, the integral signals at $\delta = 2.37–2.27$ ppm and $\delta = 5.37–5.27$ ppm are constant. Figure 5b shows a linear increase in SV ($R^2$ 0.993; RSD 0.61) and AV ($R^2$ 0.996; RSD 0.16) upon adding palmitic acid, while EV and IV remain.

Upon addition of oleic acid, a linear increase in SV ($R^2$ 0.958; RSD 0.43), AV ($R^2$ 0.964; RSD 0.56), and IV ($R^2$ 0.970; RSD 0.38) of the sample in comparison to RFO was observed when using the integral increase in the RFO signal at $\delta = 2.27–2.20$ ppm and $\delta = 5.37–5.27$ ppm (see Fig. 5c and d). These results prove that adding oleic acid an unsaturated fatty acid affects SV, AV, and IV due to an increase in the number of $\alpha\text{-}CH_2$ signals of FFA and the signal of double bonds -CH=CH- in RFO. As expected, the calculated EV remains constant. Accordingly, this result also proves that the EV calculation can be directly read from the $^1$H NMR RFO spectra on the $\alpha\text{-}CH_2$ TAG signal at $\delta = 2.37–2.27$ ppm. This is in stark contrast to the standard titration approach where it is calculated by EV=SV-AV. Taken together, the standard addition method gives reliable results for SV, AV, EV, and IV using the qNMR method.

### Comparison with the Titration Method

SV, AV, EV, and IV were determined for 17 RFO samples. This sample series contains 4 crude RFO with palmitic acid (10, 40, 80, and 120 mg), 4 crude RFO with oleic acid (10, 20, 40, and 80 mg), and 9 commercial product samples of RFO. Subsequently, SV, AV, EV, and IV were determined using both the standard titration method (Ph. Eur. 10.0) and the qNMR method. An F-test and a Student's *t*-test were applied to evaluate significant differences between the two methods. The results displayed in Table 5 are similar for both analysis methods. The values determined for commercials RFO products differ significantly: AV (9–100), EV (94–107), and IV (66–80), respectively, indicating different qualities. Especially the broad range of the AV limit is an indicator for ongoing hydrolysis processes. Interestingly, the SV is similar (194–198) for all products. These SV results indicate that the fatty acids in

**Table 4** LOD and LOQ based on the calibration curve of SV, AV, EV and IV, respectively

| Signal NMR (ppm) | Signal correlation | Range (mg) | Calibration curve | | LOD (mg) | LOQ (mg) |
|---|---|---|---|---|---|---|
| | | | $R^2$ | Equation | | |
| 2.37–2.20 | SV | 0–10 | 0.995 | $y = 90.88x + 5.93$ | 0.37 | 1.58 |
| 2.27–2.20 | AV | | 0.995 | $y = 90.88x + 5.93$ | 0.37 | 1.58 |
| 2.37–2.27 | EV | | 0.995 | $y = 31.12x + 1.23$ | 1.46 | 4.78 |
| 5.37–5.27 | IV | | 0.996 | $y = 108.98x—0.27$ | 0.35 | 1.37 |
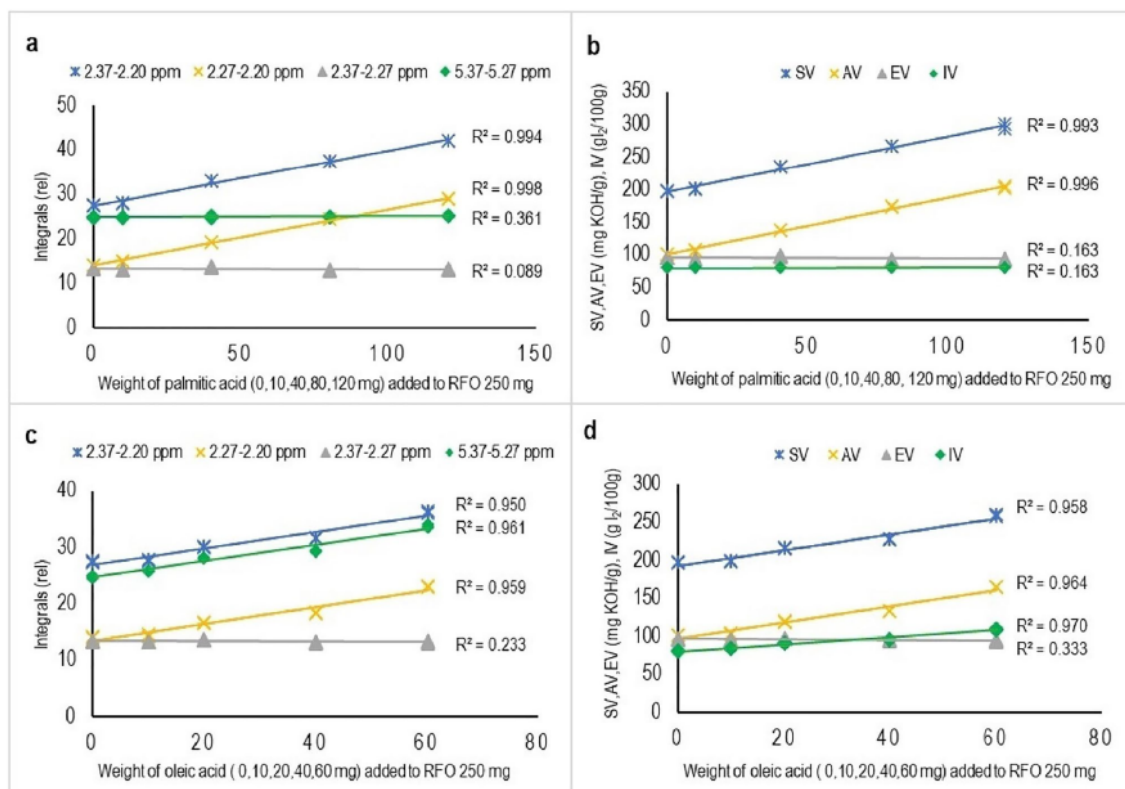
**Fig. 5** **a** Correlation between RFO with palmitic acid addition versus Integral of $^1$H NMR RFO ($\delta = 5.37$–$5.27$ ppm, $\delta = 2.37$–$2.27$ ppm, $\delta = 2.27$–$2.20$ ppm, and $\delta = 2.37$–$2.20$ ppm) and **b** correlation between RFO with palmitic acid addition versus SV, AV, EV, and IV by qNMR calculation. **c** Correlation between RFO with oleic acid addition versus integral of $^1$H NMR RFO ($\delta = 5.37$–$5.27$ ppm, $\delta = 2.37$–$2.27$ ppm, $\delta = 2.27$–$2.20$ ppm, and $\delta = 2.37$–$2.20$ ppm) and **d** correlation between RFO with oleic acid addition versus SV, AV, EV, and IV by qNMR calculation

commercial samples of RFO have a similar mean molecular weight. The range of 190 to 200 points towards a substantial amount of oleic, stearic, and palmitic acid, which is typical for RFO. Several SV values from the NMR calculation do not precisely match if compared with summarizing $AV_{NMR}$ plus $EV_{NMR}$. A random error may cause this condition of the integration technique (Torres et al. 2017). However, the differences are not significant (RSD $< 0.66$).

As can be seen from Table 6, the $t$-test shows the consistency of both methods, and the $F$-test states that both methods are of similar precision. Considering that both methods produce almost identical results for SV, AV, EV, and IV, a regression correlation was applied to calculated SV, AV, EV, and IV directly from $^1$H NMR. The following equation was obtained for calculation of SV ($y = 0.986x + 2.426$); AV ($y = 0.989x + 0.270$); EV

($y = 0.987 + 2.170$); and IV ($y = 0.994x + 0.782$). Based on this result, it can be stated that qNMR could develop into a method for determining SV, AV, EV, and IV parallel to the conventional methods.

## Conclusions

In this work, a qNMR method using the internal standard $DMSO_2$ with optimized conditions was developed (solvent $CDCl_3$ and DMSO-$d_6$ (5:1 v/v) containing 0.1% TMS; acquisition parameters: 163 K, SW 30.00 ppm, AQ 6.81 s, digital resolution 0.15 Hz, d1 9 s, and pulse angle 30°) and successfully demonstrated the advantages of feasible detection speed, selectivity, linearity, precision, and accuracy in the quantitative analysis of four simultaneous oil quality parameters (SV, AV, EV, and IV) in crude RFO, a

**Table 5** The SV, AV, EV, and IV of RFO samples determined by qNMR and titration methods (n = 3)

| Sample[a] | SV (mg KOH/g) | | AV (mg KOH/g) | | EV (mg KOH/g) | | IV (g $I_2$/100 g) | |
|---|---|---|---|---|---|---|---|---|
| | qNMR | Titration[34] | qNMR | Titration | qNMR | Titration (SV-AV)[34] | qNMR | Titration |
| 1 | 194.71 ± 0.56 | 194.50 ± 0.58 | 100.29 ± 0.71 | 100.54 ± 0.80 | 94.42 ± 0.20 | 93.96 ± 0.66 | 76.33 ± 0.83 | 75.53 ± 0.33 |
| 2 | 198.96 ± 1.38 | 199.69 ± 0.30 | 61.93 ± 0.48 | 61.65 ± 0.67 | 137.02 ± 0.90 | 138.03 ± 0.37 | 66.46 ± 0.37 | 66.37 ± 0.43 |
| 3 | 199.46 ± 0.41 | 199.76 ± 0.23 | 45.72 ± 0.59 | 45.30 ± 0.23 | 155.62 ± 0.96 | 154.46 ± 0.29 | 69.58 ± 0.79 | 70.08 ± 0.37 |
| 4 | 191.11 ± 0.76 | 191.63 ± 0.42 | 83.87 ± 0.83 | 84.00 ± 0.21 | 107.24 ± 0.18 | 107.63 ± 0.22 | 77.35 ± 0.35 | 77.50 ± 0.32 |
| 5 | 197.32 ± 0.33 | 197.51 ± 0.32 | 71.23 ± 0.80 | 73.40 ± 0.10 | 126.11 ± 0.78 | 124.11 ± 0.22 | 82.84 ± 0.25 | 81.44 ± 0.68 |
| 6 | 191.19 ± 0.47 | 191.92 ± 0.70 | 60.48 ± 0.51 | 60.87 ± 0.73 | 130.72 ± 0.13 | 131.05 ± 0.04 | 79.24 ± 0.96 | 79.67 ± 0.94 |
| 7 | 187.60 ± 0.03 | 186.86 ± 0.01 | 9.05 ± 0.09 | 9.36 ± 0.03 | 178.55 ± 0.12 | 177.51 ± 0.04 | 89.01 ± 0.30 | 86.99 ± 0.77 |
| 8 | 192.64 ± 0.35 | 191.40 ± 0.23 | 84.22 ± 0.30 | 83.02 ± 0.10 | 107.40 ± 1.63 | 108.38 ± 0.24 | 77.19 ± 0.49 | 77.26 ± 0.19 |
| 9 | 196.81 ± 1.16 | 196.75 ± 0.45 | 100.82 ± 0.57 | 101.19 ± 0.44 | 95.99 ± 0.64 | 95.55 ± 0.55 | 80.59 ± 0.53 | 80.39 ± 0.43 |
| 10 | 201.62 ± 0.97 | 202.12 ± 0.59 | 107.16 ± 0.96 | 110.34 ± 0.03 | 93.97 ± 0.72 | 91.78 ± 0.62 | 80.66 ± 0.41 | 80.37 ± 0.28 |
| 11 | 235.56 ± 0.69 | 231.46 ± 0.06 | 137.42 ± 0.08 | 137.75 ± 0.06 | 98.14 ± 0.77 | 93.71 ± 0.00 | 81.20 ± 0.56 | 80.62 ± 0.48 |
| 12 | 266.93 ± 0.56 | 268.42 ± 0.20 | 174.12 ± 0.20 | 175.52 ± 0.25 | 92.82 ± 0.36 | 92.89 ± 0.05 | 80.41 ± 0.09 | 81.06 ± 0.64 |
| 13 | 299.59 ± 1.07 | 300.07 ± 0.31 | 205.32 ± 0.08 | 202.46 ± 1.34 | 95.90 ± 1.16 | 97.62 ± 1.04 | 81.70 ± 0.35 | 80.79 ± 0.65 |
| 14 | 198.50 ± 1.15 | 200.70 ± 0.53 | 103.02 ± 1.17 | 105.35 ± 0.98 | 95.48 ± 0.04 | 95.35 ± 1.21 | 83.87 ± 0.13 | 82.84 ± 0.25 |
| 15 | 216.09 ± 0.76 | 216.91 ± 0.52 | 118.68 ± 0.80 | 118.32 ± 0.79 | 97.41 ± 0.18 | 98.59 ± 1.31 | 91.23 ± 0.33 | 91.94 ± 0.21 |
| 16 | 228.05 ± 0.73 | 231.23 ± 0.40 | 132.88 ± 0.21 | 135.16 ± 0.74 | 95.18 ± 0.52 | 96.06 ± 1.02 | 95.80 ± 0.02 | 96.83 ± 0.21 |
| 17 | 259.56 ± 0.54 | 258.86 ± 0.79 | 164.83 ± 0.46 | 169.12 ± 0.39 | 94.40 ± 1.34 | 89.74 ± 0.59 | 109.23 ± 0.81 | 108.90 ± 0.55 |

[a]1–8 are commercial product RFO; 9 is crude RFO; 10–13 are crude RFO with palmitic acid (10, 40, 80, and 120 mg); 14–17 are crude RFO with oleic acid (10, 20, 40, and 80 mg)

**Table 6** Statistics *F*-test, Student's *t*-test, and RSD results for four quality parameters of RFO calculated from qNMR method versus titration method

| Parameter | *F*-test | *F*-critical value | $P(T \leq t)$ one-tail | *t* critical one-tail | $P(T \leq t)$ two-tail | *t* critical two-tail | RSD (%) |
|---|---|---|---|---|---|---|---|
| SV | 0.47 | 1.65 | 0.47 | 1.66 | 0.94 | 1.99 | 1.74 |
| AV | 0.47 | | 0.47 | | 0.93 | | 1.65 |
| EV | 0.48 | | 0.44 | | 0.89 | | 2.15 |
| IV | 0.50 | | 0.46 | | 0.92 | | 0.90 |

mixture of crude RFO with palmitic and oleic acid addition and its commercial products.

The NMR results were in good correlation with those determined by the compendial titration method. Furthermore, SV, AV, EV, and IV successfully can be determined directly from $^1$H NMR spectra. In addition, the quantitative $^1$H NMR method is simple and rapid, demands for less chemical reagents, and does not require complex preparation steps. Therefore, it represents an interesting alternative for routine quality control of RFO and commercial products.

**Data Availability** The authors declare that all data supporting the findings of this study are available within the article.

## Declarations

**Competing interests** The authors declare no competing interests.

**Ethics Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of Interest** Liling Triyasmono declares that he has no conflict of interest. Curd Schollmayer declares that he has no conflict of interest. Jens Schmitz declares that he has no conflict of interest. Emilie Hovah declares that she has no conflict of interest. Cristian Lombo declares that he has no conflict of interest. Sebastian Schmidt declares that he has no conflict of interest. Ulrike Holzgrabe that she has no conflict of interest.

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