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Review

NMR Determination of Free Fatty Acids in Vegetable Oils

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Abstract: The identification and quantification of free fatty acids (FFA) in edible and non-edible vegetable oils, including waste cooking oils, is a crucial index to assess their quality and drives their use in different application fields. NMR spectroscopy represents an alternative tool to conventional methods for the determination of FFA content, providing us with interesting advantages. Here the approaches reported in the literature based on ^{1}H, ^{13}C and ^{31}P NMR are illustrated and compared, highlighting the pros and cons of the suggested strategies.

Keywords: nuclear magnetic resonance; free fatty acids; acid value; NMR quantification; waste cooking oils; waste oil characterization

1. Introduction

Free fatty acids (FFA) are hydrolysis products of triglycerides (TG) in vegetable oils. In edible oils, their formation occurs primarily during the production and the storage procedures of the oil and, in general, during handling of the raw material. Deterioration processes in lipids are additional sources of FFA. For instance, short-chain FFA can arise from the secondary oxidation of unsaturated aldehydes as well as from the cleavage of lipid hydroperoxides [1,2]. The FFA concentration in vegetable oils depends on multiple factors, namely the quality and variety of raw material, collecting conditions, processing, storage, the age of the oil and deterioration status [3,4]. The amount of FFA is even higher in waste cooking oils (WCO), since high temperature and exposure to air occurring during frying promote the hydrolysis and oxidation of triglycerides and increase the content of FFA in the oil [5–8].

An established method to measure the content of FFA is the so-called "acid value" (AV), referred to as the amount of potassium hydroxide (milligrams of KOH) required to neutralize the acidic fraction in one gram of sample (mg(KOH)/g(oil)). Practically speaking, the AV is a volumetric method for the determination of FFA in lipids (fatty oils and waxes), that consists in the titration of the sample with a standardized ethanolic solution of KOH using phenolphthalein as an endpoint indicator [9].

The AV is an important parameter for quality assessment in the determination of FFA in lipids. Indeed, the content of FFA in vegetable oils affects their properties and has implications in many application fields. In edible oils, FFA are undesirable, as high FFA content results in increased losses during refining, poor flavour quality and stability of the finished edible oil and rancidity of the oil [10–12]. At an industrial level, FFA are usually removed from crude oil through a refining procedure, but the process is not 100% efficient. Hence, the quantitative determination of the AV plays a central role as a quality index in production, trading storage, and marketing of edible oils, and in official food and pharmaceutical control [1,3,11]. Both the European Pharmacopoeia and the Codex Alimentarius establish maximum values for AV in pharmaceutical products and edible oils.

The FFA content is also important for edible and non-edible vegetable oils as well as WCO, with applications in biodiesel production [5,8]. Biodiesel is composed of methyl/ethyl esters containing long chain fatty acids and can be obtained directly by a transesterification reaction of raw materials having a low content of FFA. In the case of oils with a high content of FFA, the transesterification reaction can give a saponification reaction, causing deactivation of the catalytic species and a decrease in biodiesel yield [8]. It has been reported that, for FFA levels higher than 5%, the separation between the biodiesel and glycerol is hindered by the presence of soap, with a consequent decrease in the yield of the final product [5]. Thus, the FFA content of the feed stocks is a crucial quality parameter. A maximum acid value of 0.5 mg(KOH)/g is fixed by European norms for biodiesel.

The identification and quantification of FFA is also a main target in the development of new sustainable processes for WCO recycling and management. These used vegetable oils can be transformed into valuable goods and employed as bio-lubricants, animal feed, additives for bio-asphalts and bio-concrete, or in the field of energy production both by direct burning and for biofuel synthesis. Regardless of the specific application, a pre-treatment step is needed, which changes the composition and, consequently, also the FFA content [13–15]. The determination of the composition of waste oils, including the analytical measurement of acidity, is then crucial to assess its quality [16] and thus to determine how to better exploit the raw material.

Unfortunately, the classical AV method has a number of drawbacks. It usually depends on a visual endpoint, so that its accuracy can be compromised, for instance, in the case of a strongly coloured sample. Although the introduction of technologies such as potentiometric endpoint determination significantly improved the method, it still suffers from several disadvantages: it is usually slow, requires a large sample size and large amounts of organic solvents and harmful chemicals, and lacks specificity [2,12].

Alternative techniques have been proposed [17], which give good correlations with the classical AV, such as pH-metry [18], gas chromatography [19–22], HPLC [11,23], FTIR [10,24,25], near IR [26,27], colorimetry [28–30], or flow injection analysis (FIA) [31–33].

Moreover, NMR spectroscopy (^{1}H, ^{13}C, ^{31}P) has been proposed as an alternative method for FFA determination in the analysis of oils and fats [2,34]. As strong points of the technique, one can mention that NMR spectroscopy is a non-destructive, non-invasive methodology; it is usually quick and straightforward, and yields information on the composition of a mixture in a single spectrum, without the need for derivatization or pre-treatment of the sample. Moreover, it usually requires negligible sample preparation and small quantities of organic solvents or reagents. Given that the experimental setup complies with specific requirements, NMR analysis is quantitative, meaning that the integral of a signal is directly proportional to the number of the corresponding nuclei. This allows us to directly obtain the molar concentration from the NMR spectrum. Moreover, the NMR approach is also useful for samples that cannot be analysed by chromatographic techniques due to heat sensitivity or other issues. Over the years, NMR has stood out as an efficient technique in fatty acid characterization, to derive the composition of fatty acids (the so-called fatty acid profile) and the ratio between different acyl groups, to classify the quality of edible oils, to study and monitor the oxidation and deterioration process, to assess the purity and authenticity or detect adulteration. Many reviews and book chapters are available in the literature on this wide topic [2,34–38]. Particularly important in fatty acid characterization is the distinction between saturated and unsaturated acyl moieties, as well as the determination of the degree of unsaturation (through parameters such as the iodine value (IV), an empirical number related to the total number of unsaturations). The usefulness of NMR spectroscopy in this respect has also been widely demonstrated [39,40].

In this minireview, the focus will be the application of NMR methodology to the determination of the content of FFA in vegetable oils. The reviewed methods are applicable to edible and non-edible oils, as well as WCO, where the determination of the FFA content requires more and more attention.

2. Discussion

2.1. ^{1}H NMR

Thanks to its favourable properties (spin number I = 1/2, high gyromagnetic ratio equal to 26.75×10^7 rad T^{-1} s^{-1} and natural abundance of 99.99%), the ^{1}H nucleus is the most sensitive NMR probe and is suitable for the identification and quantification of even minor components within a short experimental time [2]. ^{1}H NMR spectroscopy has been widely used to obtain insights into lipid classes, fatty acid composition, levels of unsaturation, and several minor compounds [2,41,42]. Vegetable oils are mainly made up of mixtures of triglycerides, with different substitution patterns according to the length, degree and type of unsaturation of the acyl chains, and by minor components including mono- and di-glycerides, fatty acids, sterols, vitamins, and others [43]. The ^{1}H chemical shifts of the triglycerides are well known and minor oil components can be detected by ^{1}H NMR when their signals are not overlapped with those of the main components. Unfortunately, because of the chemical similarity between the free fatty acids and the glyceride esters in the oil, the ^{1}H NMR signals resonate close together and build clusters [42]. Hence, the ^{1}H NMR spectrum of vegetable oils have a small number of characteristic peaks so that the quantification of the individual components in mixtures can be challenging. To get an idea, the ^{1}H chemical shifts of the main FFA found in WCO (Figure 1) are given in Table 1 [8,16,44].

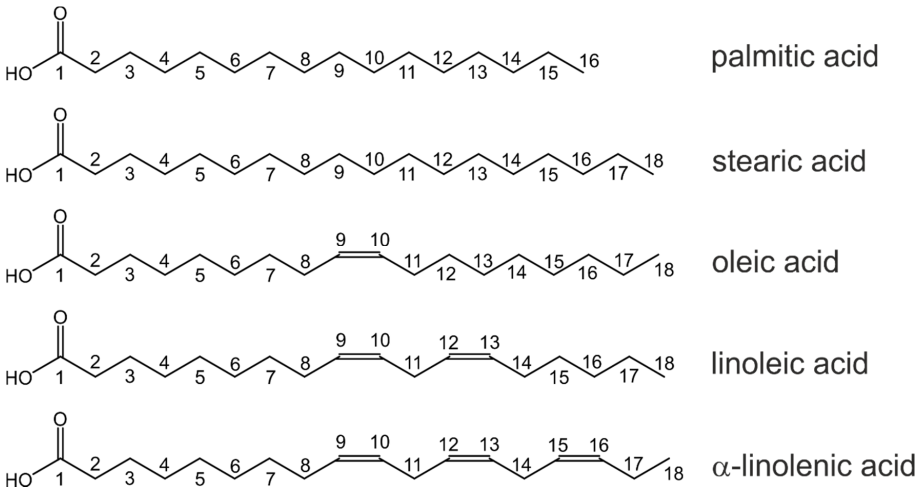

Figure 1. Structures of selected fatty acids present in waste cooking oils (WCO).

Table 1. ^{1}H chemical shifts of selected fatty acids present in WCO [44,45].

Proton Signal	Palmitic Acid (16:0)	Stearic Acid (18:0)	Oleic Acid (18:1 Δ^9)	Linoleic Acid (18:2 $\Delta^{9,12}$)	α-Linolenic Acid (18:3 $\Delta^{9,12,15}$)
=C*H*-			5.36 (H9, H10)	5.37 (H9, H10, H12, H13)	5.36 (H9, H10, H12, H13, H15, H16)
=CH-C*H*$_2$-CH=				2.78 (H11)	2.80 (H11, H14)
-C*H*$_2$COOH	2.36 (H2)	2.35 (H2)	2.36 (H2)	2.36 (H2)	2.35 (H2)
=CH-C*H*$_2$-			2.03 (H8, H11)	2.06 (H8, H14)	2.04 (H8, H17)
-C*H*$_2$CH$_2$COOH	1.64 (H3)	1.63 (H3)	1.64 (H3)	1.64 (H3)	1.61 (H3)
-C*H*$_2$-	1.24 (H4–H15)	1.25 (H4–H17)	1.30 (H4–H7, H12–H17)	1.35 (H4–H7, H15–H17)	1.31 (H4–H7)
-C*H*$_3$	0.89 (H16)	0.88 (H18)	0.89 (H18)	0.90 (H18)	0.98 (H18)

Satyarthi et al. developed a ^{1}H NMR method to quantitatively determine FFA in vegetable oils, animal fats and biodiesel [46]. It is based on the integration of the signal corresponding to the α-carbonyl methylene protons of FFA (the methylene group directly adjacent to the COOH group) and the α -carbonyl-CH_2 signal of esterified fatty acids. In vegetable oil and biodiesel, α-CH_2 peaks of fatty acids appear at chemical shift (δ) values higher than those of the ester, as a consequence of the stronger deshielding effect of the carboxylic group with respect to the ester group. Hence, one of the peaks of the triplet of FFA is visible outside the α-CH_2 region of the ester, while the other two peaks overlap with those due to the ester (Figure 2). This means that a sample of vegetable oil or biodiesel containing both FFA and ester shows a pseudo-quartet signal in the α-CH_2 region of the proton NMR spectrum and that the intensity of the peaks depends on the content of FFA in esters. The FFA content can be calculated from the unmerged peak of the FFA triplet using the following equation [46]:

$$\% \text{ FFA} = \frac{4 \cdot I_{\text{unmerged}-\alpha-\text{CH}_2-\text{FFA}}}{I_{\text{total}-\alpha-\text{CH}_2-\text{FFA}+\text{ester}}} \cdot 100 \quad (1)$$

where $I_{\text{unmerged}-\alpha-\text{CH}_2-\text{FFA}}$ is the area of the unmerged FFA peak and $I_{\text{total}-\alpha-\text{CH}_2-\text{FFA}+\text{ester}}$ the total area of the α-CH_2 of both FFA and ester. The pre-factor 4 accounts for the fact that the triplet of the α-CH_2 group of the FFA has an intensity ratio of 1:2:1, so that the total area is four times the area of the single unmerged FFA peak.

Another option is to deconvolute the peaks of the ester and the acid, so that the FFA content can be determined according to the following equation [46]:

$$\% \text{ FFA} = \frac{I_{\alpha-\text{CH}_2-\text{FFA}}}{I_{\text{total}-\alpha-\text{CH}_2-\text{FFA}+\text{ester}}} \cdot 100 \quad (2)$$

with $I_{\alpha-\text{CH}_2-\text{FFA}}$ being the area of the triplet corresponding to the α-CH_2 of FFA.

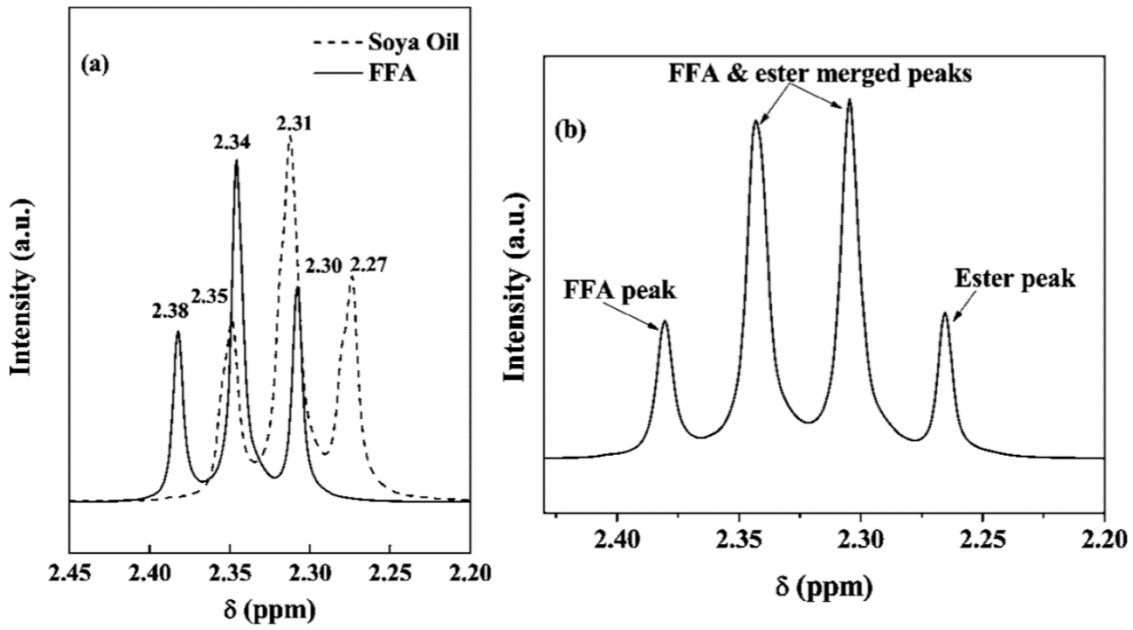

Figure 2. ^{1}H NMR spectrum of α-CH_2 region of (**a**) soybean oil and the free fatty acid (FFA) oleic acid, and (**b**) a mixture of oleic acid and its methyl ester. The sample for ^{1}H NMR was prepared by dissolving 20–25 mg in 0.6 mL of $CDCl_3$. The spectrum was acquired on a Bruker AV 200 MHz spectrometer at 298 K, using 30 scans, with an acquisition time of 3.9 s, a 30° flip angle and a relaxation delay of 1 s. Reprinted with permission from *Energy & Fuels*, Vol. 23, J. K. Satyarthi, D. Srinivas, P. Ratnasamy, "Estimation of Free Fatty Acid Content in Oils, Fats, and Biodiesel by ^{1}H NMR Spectroscopy" (pages 2273–2277). Copyright (2009) American Chemical Society.

The method was validated against conventional titration by measuring the FFA content in standard solutions of soybean oil and biodiesel added with known amounts of oleic acid. A good correlation of the calibration curves was observed, even though an intrinsic error of approximately 1% was reported [46]. To further test the performance of the ^{1}H NMR method versus titrimetric methods, the decrease in the oleic acid content was followed and quantified during its esterification reaction with methanol. Again, a good correlation in terms of oleic acid content at different intervals was observed [46]. A peculiar advantage of the proposed ^{1}H NMR method over the titrimetric method emerged in the quantification of FFA content in non-edible oils containing acidic impurities other than FFA. For instance, the ^{1}H NMR technique detected FFA content in karanja oil equal to 4.5 wt%, while a higher value (5.3 wt%) was estimated by titration. After transesterification into biodiesel, no FFA resulted by ^{1}H NMR analysis, whereas a residual acidity (0.79 wt%) was detected by the titrimetric method. These outcomes indicate that ^{1}H NMR spectroscopy is even more accurate than titration, since it detects exclusively the FFA content instead of the total acid value given by FFA and other acidic entities.

The main limitation of the approach by Satyarthi et al. is that it is designed for non-edible lipids and biodiesel with significant FFA amounts and may not be sensitive enough to detect small FFA contents present in commercially available edible oils or in pharmaceutical products.

Skiera et al. described an alternative rapid and simple ^{1}H NMR method that overcomes this limitation, which exploits the integration of the carboxyl group signal of FFA located in the downfield region of the spectrum [3]. The lipid sample is dissolved in a $CDCl_3$/DMSO-d_6 mixture (5:1 *v*/*v*) with tetramethylsilane as an internal reference and then dried over a molecular sieve. This sample preparation is necessary to reduce the fast proton exchange processes that occur in pure $CDCl_3$, which would cause line broadening or even the loss of the COOH signal. The quantification of FFA is based on the integration of the signal corresponding to the carboxyl proton at 11–12 ppm and the α-carbonyl CH_2 protons at 2.2–2.4 ppm (Figure 3). By normalising the α-carbonyl CH_2 signal to 6000, the number of those protons per TG unit, one obtains the FFA content in mmol/mol TG.

The efficacy and accuracy of the proposed ^{1}H NMR method was validated against AV on a wide range of oil varieties [1,3]. First, analysing by both techniques some standard solutions of rapeseed oil with known amount of palmitic acid, the authors derived a mathematical equation to convert the molar FFA amount determined by NMR into a parameter AV_{NMR} that is compared to the classical AV [3]. Then, more than 400 samples of different oil varieties (around 20) were analysed by both methods in two different studies and the results were similar for the majority of the samples [1,3]. Systematic deviations were observed for the pumpkin seed oils, with the classical AV method giving slightly higher values [3]. This is likely due to the strong colour of this oil, which complicates the visual endpoint determination in the AV method. The suitability for coloured oils represents, then, a strength of the ^{1}H NMR method compared to the classical AV method. Additional advantages of the ^{1}H NMR method are that it offers the opportunity to evaluate authenticity parameters out of the same ^{1}H NMR spectrum, such as the amount of hydroperoxides and aldehydes [3].

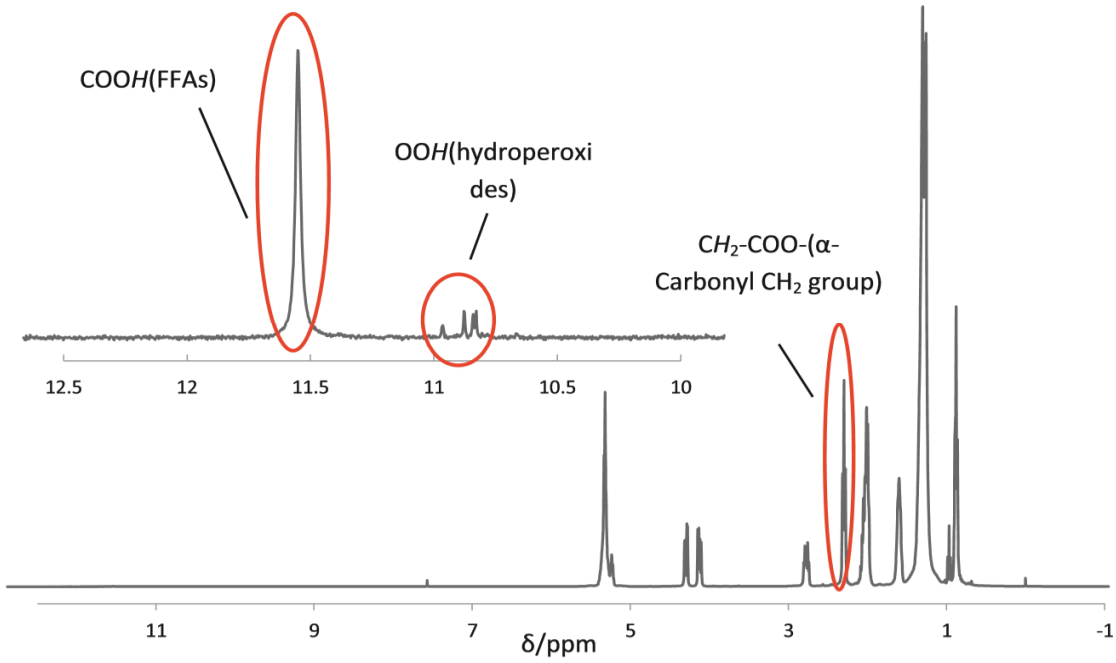

Figure 3. ^{1}H NMR spectrum of a rapeseed oil dissolved in a mixture of $CDCl_3$ and DMSO-d_6 (5:1, *v*/*v*), with enlargement of the carboxylic region. The spectrum was acquired on a Bruker AVANCE 400 MHz spectrometer at 300 K, using 128 scans, with an acquisition time of 7.97 s, a 30° flip angle and a relaxation delay of 1 s. Reprinted from the *Journal of Pharmaceutical and Biomedical Analysis*, Vol 93, C. Skiera, P. Steliopoulos, T. Kuballa, B. Diehl, U. Holzgrabe, "Determination of free fatty acids in pharmaceutical lipids by ^{1}H NMR and comparison with the classical acid value" (pages 43–50), copyright (2014), with permission from Elsevier.

However, the ^{1}H NMR method proposed by Skiera et al. presented some limitations in two cases. For hard fat samples, the AV_{NMR} values were significantly smaller than the AV ones [1]. This discrepancy was ascribed to the smaller average molecular weight of hard fat with respect to that of rapeseed oil, which was used for the development of the model equation. An adjustment of the equation was then made, including the integral of the COOH signal of an internal reference (1,2,4,5-tetrachloro-3-nitrobenzene, TCNB), leading to a good agreement with the classical AV method. Issues emerged also in the case of castor oil, where no COOH signal was detected in the ^{1}H NMR spectrum [1]. The effect here was due to the high content of a fatty acid with a hydroxyl functional group, the ricinoleic acid, whose easily exchangeable OH protons affected the fast proton exchange of the carboxylic protons. As a result, the amount of DMSO-d_6 used in the original sample preparation procedure was not enough to slow down proton exchange. To solve the issue, the sample preparation protocol for castor oil was modified by using a mixture of $CDCl_3$/DMSO-d_6 with a molar ratio of 2:1 *v*/*v*, obtaining a good correlation with the standard AV method.

Despite the potential spectral overlap, the ^{1}H NMR method found several applications. It was exploited, for instance, to estimate the FFA content in non-edible oils extracted from thirteen seeds from India [47], to follow the change in the amount of FFA and other components in different developmental stages of a non-edible oilseed, *Jatropha curcas* L. [48], or to detect and quantify all the different constituents, including FFA, coming from the hydrolysis of triglycerides during lipolysis [49]. More recently ^{1}H NMR was also applied to assess the quality especially in terms of the FFA content of biodiesel produced by transesterification of oil from *J. curcas* L. seeds [50].

To maximize the separation of the signals, very recently, San Martín et al. carried out a systematic investigation of different solvent mixtures to be used for the ^{1}H NMR analysis of edible fats and oils [51]. Among the mixtures tested, the best results were obtained using CCl_4/DMSO-d_6 or CS_2/DMSO-d_6/$CHCl_3$ as solvents. In these samples, an excellent separation of the signals of minor

compounds in several edible oils (olive oil, sunflower oil, corn oil, sesame oil, peanut oil) and fats (butter, walnuts, salmon, dry sausage) was possible. An accurate detection and quantification of FFA was achieved with a good correlation between the FFA content estimated by NMR and that calculated by standard titration. As an additional benefit, these solvent mixtures allow the separation of signals with a minimum amount of deuterated solvents, which also means that they can be used in cases of lock and shim automation.

2.2. ^{13}C NMR

The main drawback of ^{1}H NMR spectroscopy in oil analysis is related to the small spectral width covered by protons. ^{13}C NMR spectra as an alternative to ^{1}H NMR offers some advantages [36]. First, ^{13}C NMR provides a large number of signals covering a wide range of chemical shifts, which makes the ^{13}C spectrum very informative. As an example, the ^{13}C chemical shifts of the main FFA found in WCO (Figure 1) are given in Table 2 [44]. Moreover, the low gyromagnetic ratio of the ^{13}C nucleus (6.728×10^{7} rad T^{-1} s^{-1}), its low natural abundance (1.07%) and low receptivity compared to ^{1}H (1.7×10^{-4}), are balanced by the low possibility of ^{13}C-^{13}C scalar coupling and by broadband heteronuclear decoupling. This results in sharp singlets for all ^{13}C absorptions, making it possible to measure small chemical shift differences [2]. Using the inverse gated decoupling technique to remove the nuclear Overhauser proton–carbon enhancement, and long pulse delays to ensure the complete relaxation of ^{13}C nuclei with long spin-lattice relaxation time, it is also possible to acquire a ^{13}C NMR spectrum under quantitative conditions, as in the case of ^{1}H NMR [36]. The main drawback is that ^{13}C NMR experiments typically need long measurement times to obtain a spectrum with proper signal-to-noise ratio. In order to shorten the total experimental time, it is possible to add relaxation reagents (typically chromium (III) acetylacetonate [$Cr(acac)_3$]), which are paramagnetic substances able to significantly reduce the relaxation times of ^{13}C nuclei without inducing relevant shifts.

Table 2. ^{13}C chemical shifts of fatty acids mainly present in WCO [44,45].

Carbon Signal	Palmitic Acid (16:0)	Stearic Acid (18:0)	Oleic Acid (18:1 Δ^{9})	Linoleic Acid (18:2 $\Delta^{9,12}$)	α-Linolenic Acid (18:3 $\Delta^{9,12,15}$)
-COOH	182.62 (C1)	182.78 (C1)	180.50 (C1)	180.16 (C1)	180.10 (C1)
=CH-			130 (C9, C10)	128-130 (C9, C10, C12, C13)	127-131 (C9, C10, C12, C13, C15, C16)
-CH_2COOH	36.68 (C2)	36.70 (C2)	33.96 (C2)	34.01 (C2)	33.95 (C2)
-CH_2-	32-34 (C4-C14)	29-32 (C4-C16)	29-31 (C4-C7, C12-C16)	29-31 (C4-C7, C15-C16)	29 (C4-C7)
=CH-CH_2-			27.12 (C8, C11)	27.25 (C8, C14)	27.22 (C8)
=CH-CH_2-CH=				25.65 (C11)	25-26 (C11, C14)
-CH_2CH_2COOH	27.33 (C3)	27.33 (C3)	24.59 (C3)	24.70 (C3)	24.56 (C3)
-CH_2CH_3	25.36 (C15)	25.36 (C17)	22.52 (C17)	22.54 (C17)	20.58 (C17)
-CH_3	16.78 (C16)	16.79 (C18)	14.07 (C18)	14.06 (C18)	14.27 (C18)

In the field of lipid chemistry and technology, high resolution ^{13}C NMR has established itself as a versatile technique to determine the composition of mixtures of fatty acids and lipid molecules, control the purity and reveal adulteration, authenticate the geographical origin and assess quality, quantitatively determine fatty acid profiles, evaluate oxidation products, analyse the phenolic fraction and determinate iodine value [2,37,38,52].

^{13}C NMR has also been applied in the determination of the free acidity in vegetable oils—that is, their FFA content. Indeed, the structure of the carbonyl region shows distinct signals for different

mono-, di- and triglycerides and FFA in the mixture [35]. As shown in Figure 4, for a sample of virgin olive oil, the free carbonyls resonate at 176–178 ppm, while the esterified carbonyls resonate in the region 171–174 ppm [52]. By comparing the signal intensity of the two regions, the free acidity (% mole fraction) can be easily calculated by ^{13}C NMR as follows:

$$\% \text{ FFA} = \frac{I_{\text{FFA}}}{I_{\text{FFA}} + I_{\text{EC}}} \cdot 100 \quad (3)$$

where I_{FFA} is the integral of the FFA region and I_{EC} is the integral of the esterified carbonyl region.

However, attention must be paid in order to obtain an accurate quantification, since carboxyl and carbonyl carbon atoms have significantly longer relaxation times than protons. Note, for instance, that T_1 relaxation times of 10–20 s were calculated for the carbonyl carbons of acyl groups of the different components present in palm oil (in $CDCl_3$ at 301 K) [53]. To obtain quantitative data, the relaxation issue can be bypassed by adding a relaxation reagent, e.g., [Cr(acac)$_3$], or by running ^{13}C NMR spectra with sufficiently long relaxation delay [2]. Although a validation test of the quantitative measurements run on virgin olive oil is not provided [52], the accuracy of the method was previously tested on samples of fish oils, which show a similar pattern in the carbonyl region [54]. The total FFA content of seven samples of tuna lipids was determined using ^{13}C NMR and compared to the values obtained using a UV method based on the formation of a complex with $(AcO)_2$Cu-pyridine. The good linearity observed between the two methods was then assumed to be a validation of the ^{13}C NMR approach, which was applied also to vegetable oils.

The strong discriminating power of the ^{13}C NMR method in the carbonyl region was demonstrated, for instance, in the estimation of the amount of FFA in non-edible oils extracted from various seeds from India [47], or in oil extracted from commercial samples of grated mullet roe (bottarga) [55].

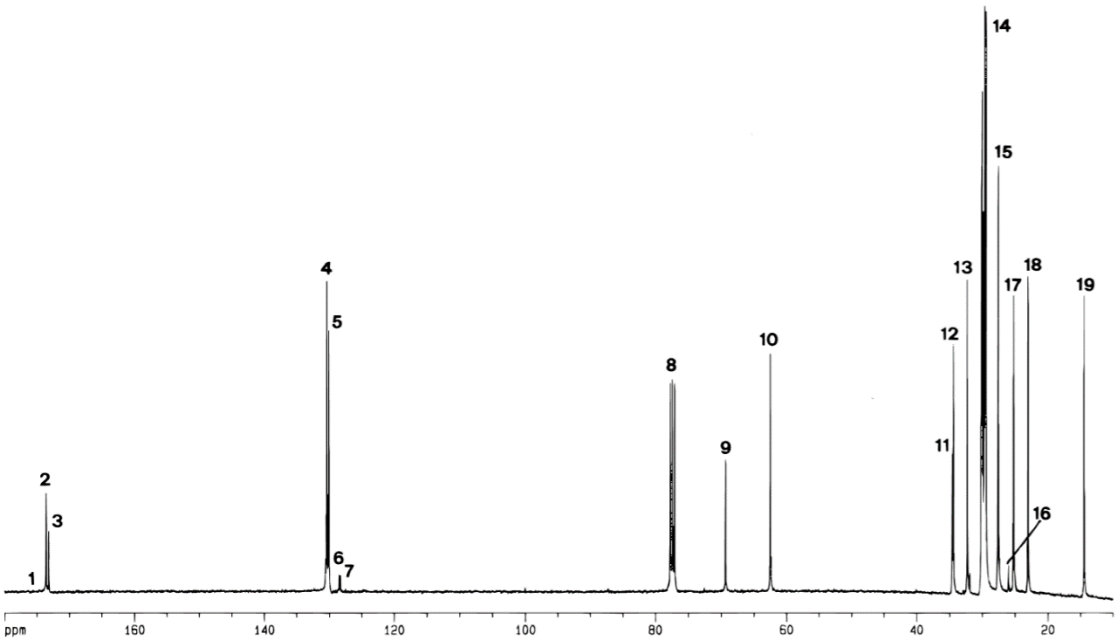

Figure 4. ^{13}C NMR spectrum of a virgin olive oil in $CDCl_3$. Target peaks 1 to 3 in the carbonyl region correspond respectively to: C1 of free fatty acids (174–176 ppm), C1 in 1,3-*sn* position of triacylglycerols (173.26 ppm) and C1 in *sn*-2 position of triacylglycerols (172.81 ppm). For full assignment, see Table 2 of [52]. The spectrum was acquired with broadband proton decoupling on a Bruker AM 400 MHz spectrometer at 303 K, using 16 K data points, with an acquisition time of 0.37 s, a 45° flip angle and a relaxation delay of 5 s. Reprinted from *Magnetic Resonance in Chemistry*, Vol 35, R. Sacchi, F. Addeo, L. Paolillo, "^{1}H and ^{13}C NMR of Virgin Olive Oil. An Overview" (pages S133–S145), copyright (1997), with permission from John Wiley and Sons.

As an alternative method, the region corresponding to aliphatic carbons has been also used for the detection and quantitative determination of FFA in palm oil by ^{13}C NMR spectroscopy [53]. Indeed, the peak for the C3 of an FFA is located at 24.67 ppm, which is 0.16 ppm lower than the peak for C3 of 1,3-acyl groups of the TG in palm oil (Figure 5). In this way, it was possible to easily detect and quantify the composition of FFA in palm oil, as the total integral of the signal at 24.69 ppm represents one FFA chain, whereas the complex band for C3 centred at 24.85 ppm represents three acyl chains of the TG in the oil [53]. The assignment was confirmed by the T_1 value of 0.90 s, measured in $CDCl_3$ solution at 301 K for the C3 in the FFA. Note that the shorter T_1 relaxation times of aliphatic carbons compared to carbonyl carbons makes this method advantageous for the quantitative measurements. Even if the method was developed for palm oil, it can be, in principle, extended to any other vegetable oil.

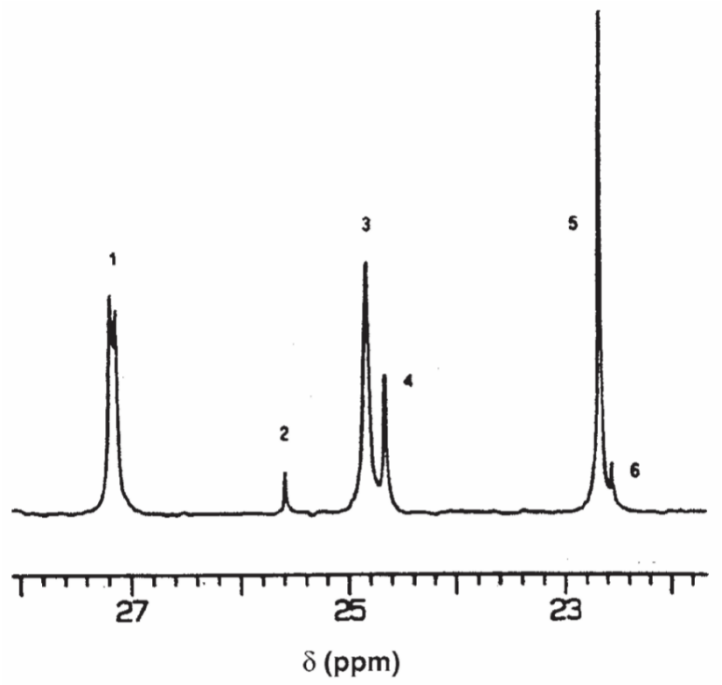

Figure 5. ^{13}C NMR spectrum of the aliphatic carbons in palm oil containing FFA, in $CDCl_3$ solution (concentration 1:3, *v*/*v*). Peak 1: allylic carbons of oleic and linoleic acyls in both triglyceride (TG) and FFA. Peak 2: C11 of linoleic acid. Peak 3: C3 of all acyl chains in TG. Peak 4: C3 of FFA. Peak 5: C15 of palmitic, C17 of stearic, and C17 of oleic acids in TG. Peak 6: C17 of linoleic acid in TG. The spectrum was acquired with broadband proton decoupling on a JEOL FX100 spectrometer at 301 K, using 6000 scans, with an acquisition time of 1.517 s, a 25° flip angle and a relaxation delay of 3 s. Reprinted from the *Journal of the American Oil Chemists' Society*, Vol 77, S. Ng, "Quantitative Analysis of Partial Acylglycerols and Free Fatty Acids in Palm Oil by ^{13}C Nuclear Magnetic Resonance Spectroscopy" (pages 749–755), copyright (2000), with permission from John Wiley and Sons.

2.3. ^{31}P NMR

Edible, waste and recycled vegetable oils are, in principle, phosphorus-free. This is because the crude vegetable oil is subjected to a degumming process, aimed to remove gums, waxes and phospholipids [56]. Nevertheless, ^{31}P NMR spectroscopy has been used as a complementary or alternative tool to ^{1}H NMR and ^{13}C NMR, especially when the analysis is complicated by strong signal overlap in ^{1}H NMR spectra or long relaxation times in ^{13}C NMR spectra [41]. In vegetable oils, alcohols or carboxylic acids constitute many of the minor components of interest. Hence, the strategy is to convert these species into derivatives with the ^{31}P NMR active nucleus. Basically, a phosphitylating reagent, 2-chloro-4,4,5,5-tetramethyldioxaphospholane, is used, which reacts quickly and quantitatively with the hydroxyl and/or carboxyl groups under mild conditions. In detail, according to the protocol originally reported by Dais et al. [57], in order to obtain the oil samples for the ^{31}P NMR analysis,

a stock solution (10 mL) is first prepared mixing pyridine and $CDCl_3$ in a 1.6:1.0 volume ratio and adding 0.6 mg of chromium acetylacetonate [$Cr(acac)_3$] and 13.5 mg of cyclohexanol. Then, 150 mg of the oil sample is weighted directly in a 5 mm NMR tube, before adding 0.4 mL of the stock solution and 5–30 µL of the phosphitylating reagent. The mixture is left to react for 20–30 min at room temperature. In this way, the labile hydrogen atoms of oil constituents bearing hydroxyl and/or carboxyl groups are derivatized, then ^{31}P chemical shifts are used to identify the labile centres [57].

Given the high gyromagnetic ratio (10.839×10^7 rad T^{-1} s^{-1}), the wide range of chemical shifts ensuring a good separation of signals located in different environments, the 100% natural abundance of the ^{31}P nucleus, and its high sensitivity (only ~15 times less than that of ^1H), ^{31}P NMR experiments represent a reliable analytical tool able to detect very low concentrations [2,57]. Moreover, the resonance frequency of ^{31}P is very sensitive to the chemical surroundings within the molecule. Furthermore, by introducing, in the reaction mixture, an internal standard, one can obtain the concentration of the product, and then the concentration of the original compound bearing the functional group [57]. In the original method by Dais and co-workers [57], cyclohexanol is used as internal standard. Alternatively, ^{31}P NMR chemical shifts of the phospholane derivatives can be referenced against an internal standard solution of benzoic acid in $CDCl_3$ [4]. The chemical shift of benzoic acid is, in turn, determined using, as an external standard, phosphoric acid at 85% (0.0 ppm). The solvent is sealed in a capillary tube and inserted into the NMR tube. However, it should be noted that to obtain quantitative ^{31}P spectra it is necessary to use the inverse gated decoupling technique together with a spin relaxation agent such as the paramagnetic chemical shift reagent [$Cr(acac)_3$], which lowers the relaxation times of ^{31}P nuclei, and hence shortens the duration of the measurements significantly [2,57,58].

Following this methodology, minor constituents of olive oil, including FFA, monoglycerides (MGs), diglycerides (DGs) and sterols, have been derivatized into phosphorus-containing dioxaphospholane compounds, in order to be identified and quantified by ^{31}P NMR. The acidity can be calculated by integrating the signal of the phosphitylated free fatty acids centred at 134.8 ppm (Figure 6) [59].

It has been demonstrated that, when ^{31}P NMR is applied to the measurement of various major and minor constituents in olive oil, it gives comparable results to conventional analytical methods. Indeed, 1-MGs, 2-MGs, 1,2-DGs, 1,3-DGs, sterols, polyphenols and FFA present in olive oil samples can be unambiguously detected and reliably quantified [41,57–61]. For instance, in a comparative study, Dais et al. measured the free acidity of 137 olive oil samples by titration, following the European Communities official method, and by ^{31}P NMR [41]. Linear regression analyses based on two different methods showed strong agreement between ^{31}P NMR and conventional methods for free acidity, with a correlation coefficient of 0.994 and 96.4% of the measurements (132 out of 137) within the limits of agreement. Only three measurements (2.2%) were near or at the limits, and only two outliers (1.5%) were detected well outside the limits. In another study performed on coconut oil, Dayrit et al. compared the FFA content estimated by ^{31}P NMR with that obtained by standard titrimetry, obtaining comparable results [4].

The main drawback of the ^{31}P approach is clearly the need for the derivatisation of FFA prior to ^{31}P analysis, and hence the destruction of the analytes. However, the methodology is much faster than the corresponding conventional titrimetric methods [41] and can offer some advantages with respect to ^1H or ^{13}C NMR methods. The higher sensitivity of ^{31}P NMR with respect to ^1H NMR was, for instance, demonstrated in a study of the degradation process of olive oil [62]. Seven olive oil samples were subjected to conventional heating and microwave heating and NMR spectroscopy was applied to the analysis of fresh or degraded olive oils. Despite the fact that the ^1H NMR spectra gave evidence of the formation of secondary products after thermal treatment, the ^{31}P NMR protocol was preferred in terms of sensitivity [62]. The FFA quantification via the corresponding signal at 134.8 ppm indicated that the FFA content did not increase significantly after microwave heating, whereas it increased markedly after conventional heating.

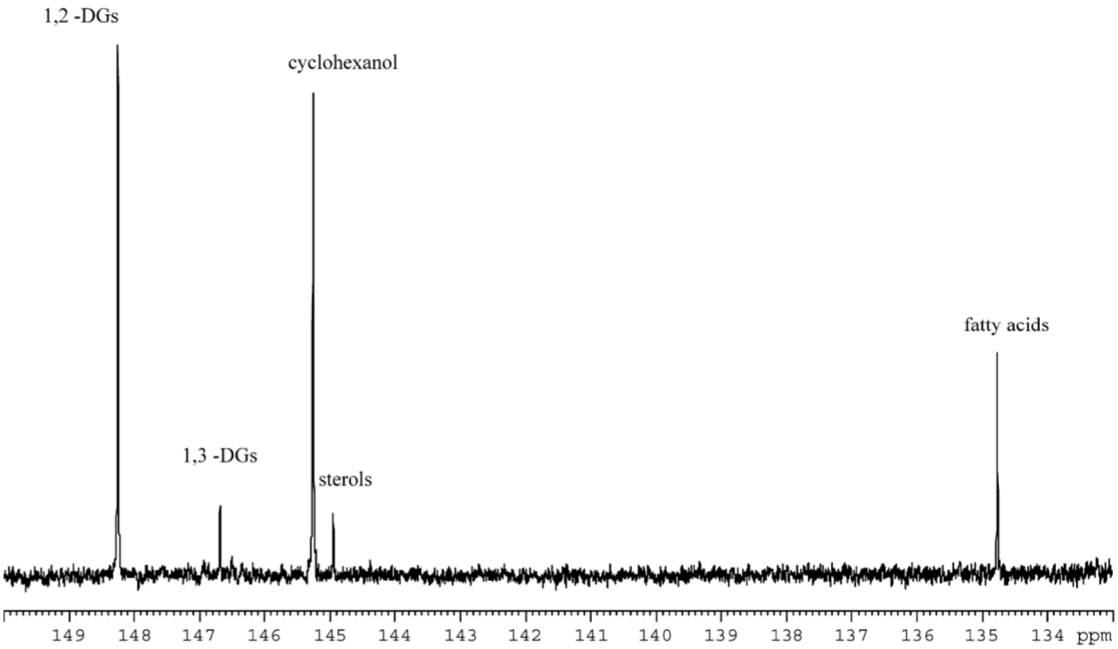

Figure 6. ^{31}P NMR spectrum of virgin olive oil in pyridine/$CDCl_3$, corresponding to the region of phosphitylated total sterols, diglycerides, and free fatty acids. The phosphitylated cyclohexanol is used as internal standard. The spectrum was acquired with inverse-gated decoupling on a Bruker AMX500 spectrometer at room temperature, using 32 scans and 16K data points, with a 90° pulse and a relaxation delay of 30 s. Reprinted with permission from the *Journal of Agricultural and Food Chemistry*, Vol. 51, G. Vigli, A. Philippidis, A. Spyros, P. Dais, "Classification of Edible Oils by Employing ^{31}P and ^{1}H NMR Spectroscopy in Combination with Multivariate Statistical Analysis. A Proposal for the Detection of Seed Oil Adulteration in Virgin Olive Oils" (pages 5715–5722). Copyright (2003) American Chemical Society.

3. Conclusions

Different strategies reported in the literature based on ^{1}H, ^{13}C and ^{31}P NMR spectroscopy for the determination of free fatty acids in vegetable oils have been reviewed and compared. Two ^{1}H NMR methods, based on the integration of the α-carbonyl methylene protons or of the carboxyl proton signal of FFA were reported. The main drawbacks are related to (i) the narrow spectral width and the following risk of signal overlap, (ii) sensitivity issues in the first method, and (iii) the need for some effort in the sample preparation in the second method. In cases of severe overlap in the proton spectrum, ^{13}C methods may be useful, as they rely on the identification and quantification of signals in the carbonyl or the aliphatic region. The main limitation of ^{13}C NMR spectroscopy is the long acquisition time required to obtain a spectrum with a proper signal-to-noise ratio. To solve the problem and shorten the experimental time, it is possible to add relaxation reagents to the samples. An additional method is represented by the ^{31}P NMR of phosphitylated free fatty acids. The need for the derivatisation of FFA prior to ^{31}P analysis represents the main downside of the ^{31}P approach.

All NMR approaches give reliable results in agreement with conventional methods, and can represent, therefore, a non-invasive, non-destructive and quantitative analytical toolbox for the determination of free acidity in vegetable oils, including waste cooking oils.

Typical limitations of all NMR-based techniques are their sensitivity and limits of detection, which could be relevant issues, especially for commercial edible oils or pharmaceutical products. However, impressive progress was made in this respect over the years and, nowadays, the availability of ultra-high magnetic fields and new generation probeheads make it possible to incredibly reduce the noise and push the NMR detection limits in terms of absolute sensitivity.

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