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Comparison of ^{19}F and ^{1}H NMR spectroscopy with conventional methods for the detection of extra virgin olive oil adulteration

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SUMMARY: This paper reports the comparison of determination methods for extra virgin olive oil (EVOO) adulteration with two kinds of oils, refined olive oil (ROO) and soybean oil by ^{19}FNMR, ^{1}H NMR and chemical titration. The determination of adulteration of EVOO with ROO by ^{19}F NMR was comparable to the conventional method. The contents of oleic, linoleic and linolenic acids of different oil samples can be determined by both ^{1}H NMR and GC-MS. The results obtained from the two methods showed little differences. The adulteration of EVOO with soybean oil is detected by ^{1}H NMR, although the limit of detection of the adulteration level is not less than 4.5%. The research demonstrates that ^{19}F NMR can be a fast and convenient method to detect EVOO if it is adulterated with ROO and ^{1}H NMR can be a fast and convenient method to detect EVOO if it is adulterated with seed oils.

***KEYWORDS**: ^{19}F and ^{1}H NMR; Adulteration; Diglycerides; GC-MS; Olive oil*

RESUMEN: ***Comparación de las espectroscopías de ^{19}F y ^{1}H NMR con métodos convencionales para la detección de la adulteración del aceite de oliva virgen extra***. Este artículo trata sobre la comparación de métodos para determinar la adulteración de aceites de oliva virgen extra (AOVE) con dos tipos de aceites, aceite de oliva refinado (ROO) y aceite de soja, mediante ^{19}F NMR, ^{1}H RMN y valoración química. La determinación de la adulteración de AOVE con ROO mediante ^{19}F RMN fue comparable al método convencional. El contenido de ácidos oleico, linoleico y linolénico de diferentes muestras de aceites puede determinarse por ^{1}H NMR y GC-MS. Los resultados obtenidos por los dos métodos mostraron pequeñas diferencias. La adulteración de los AOVE con aceite de soja se detecta mediante ^{1}H RMN, el límite de detección de la adulteración no es menor a 4.5%. Esta investigación demuestra que la ^{19}F RMN puede ser un método rápido y conveniente para detectar EVOO si está adulterado con ROO y la ^{1}H RMN puede ser un método rápido y conveniente para detectar EVOO si está adulterado con aceites de semillas.

***PALABRAS CLAVE**: ^{19}F y ^{1}H RMN; Aceite de oliva; Adulteración; Diglicéridos; GC-MS*

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1. INTRODUCTION

Olive oil is a vegetable oil produced in the Mediterranean Basin and is well-known for its nutritional value and health benefits (Pereira, 2013). A number of previous studies suggested that olive oil exerts a protective effect against cardiovascular diseases, neurological disorders and certain malignant tumors (i.e. breast, prostate, endometrium, digestive tract) due to its well-balanced fatty acids, some trace nutritional components (squalene, phyto-sterols and so on) and natural antioxidants (Ruiz-Canela *et al.,* 2011; Amel *et al*., 2016; Escrich *et al.*, 2013). Extra virgin olive oil (EVOO) is considered to be the highest quality olive oil, in that it does not undergo any treatment other than washing, decantation, centrifugation and filtration. Also, it has an acidity level which is expressed as oleic acid and does not exceed 0.8 % (Fragaki *et al*., 2005).

In recent years, the adulteration of olive oil in China has become rampant since more Chinese people started using olive oil for cooking purposes (Tu *et al.*, 2014). Over the past two decades, NMR spectroscopy, especially ^{1}H and ^{13}C NMR spectroscopies have been widely used to analyze the composition of fatty acids and other minor components in olive oils (Sacchi *et al.*, 1997). The application of ^{31}P NMR spectroscopy for the detection of monoglycerides, diglycerides, phenols and sterols in olive oils has also been studied (Fronimaki *et al.*, 2002). But in China, the deriving reagent for ^{31}P NMR has been banned since 2008 (Zhou *et al*., 2015). The most common method for the determination of fatty acids in olive oils is gas chromatography, but it may be time-consuming and often require the initial methylation of samples (Aparicio *et al.*, 2000).

The present study is divided into two groups: one to detect EVOO blended with low-grade olive oil, especially ROO, while the other is to detect cheap vegetable oils (i.e. corn, soybean, rapeseed…) added into EVOO (Fragaki *et al.*, 2005; Jafari *et al.*, 2009). In this study, we combined the two groups, using ^{19}F and ^{1}H NMR compared with traditional methods (titration and GC-MS) to detect the refined olive oil (ROO) and soybean oil mixed into EVOO. ^{19}F NMR can be a convenient and fast way to detect olive oil adulteration after large-scale promotion.

2. MATERIALS AND METHODS

2.1. Oil samples

A total of 15 oil samples were purchased from local supermarkets, samples 1 to 6 were labeled as EVOO (3 samples imported from Spain, 3 samples imported from Italy). Samples 7 to 10 were labeled as ROO. Samples 11 to 15 were labeled as different seed oils (SO), such as peanut oil, soybean oil, rapeseed oil, corn oil and blended oil. All of the samples were kept in dark glass bottles and stored at room temperature.

For the investigation of olive oil adulteration, fresh EVOO samples were mixed with ROO and soybean oil samples. Two sets of mixtures of 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 80% (w/w) for ROO or soybean oil adulterant in EVOO were prepared. These mixtures were analyzed immediately after preparation.

2.2. Chemicals

NMR. All solvents were of reagent or analytical grade: Hexafluorobenzene (99%) was purchased from Alfa Aesar (Tianjin, China). The deriving reagent (4-fluorobenzoyl chloride, purity: 98%) and 4-tert-butylphenol were purchased from Sigma-Aldrich (Shanghai, China). Pyridine and Chloroform-d were purchased from Macklin (Shanghai, China).

Titration. Acetic anhydride, potassium hydrogen phthalate, n-butyl alcohol, ethanol, potassium hydroxide, phenolphthalein, sodium hydrogen sulfate, diethyl ether were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

GC-MS. Analytical grade trimethylpentane, potassium hydroxide, methyl alcohol, and sodium hydrogen sulfate monohydrate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3. Sample preparation for NMR analysis

A stock solution (100 mL) composed of $CDCl_3$ and pyridine in 1.5:1.0 (v/v), with 0.1 mL hexafluorobenzene and 150 mg 4-tert-butylphenol was prepared. 4-Tert-butylphenol was used as an internal standard for quantification purposes and hexafluorobenzene was used as an internal standard for ^{19}F NMR chemical shift at δ-164.90 ppm. 400 mg olive oil sample were mixed with the stock solution (2 mL) in a 4 mL centrifuge tube. The required volume of the mixed solution (0.5 mL) and the reagent (30 μL) were added into a NMR tube with 5 mm diameter. The reaction mixture was left in the NMR tube to react for 30 minutes at room temperature. Upon completion of the reaction, the ^{19}F NMR spectra of the solution were determined immediately.

120 mg of olive oil sample were dissolved in 0.5 mL chloroform-d, which contained 0.03 % trimethylsilane (TMS). The resulting solution was placed in a 5 mm NMR tube and then the ^{1}H NMR spectra were recorded.

2.4. NMR experiments

All NMR spectra were obtained on a Bruker AMX500 spectrometer, operating at 470 and 500.1 MHz for fluorine-19 and proton nuclei, respectively,

at 26 ±1 °C. Typical ^{19}F spectral parameters for this study were as follows: 90° pulse width, 19.3μs; sweep width, 100 kHz; relaxation delay, 1s; memory size, 64K. 64Ttransients were accumulated for each spectrum. For all FIDs, line broadening of 0.3Hz was applied and drift correction was performed prior to Fourier transformation. A polynomial fifth-order base-line correction was performed before integration. All ^{19}F chemical shifts were reported relative to hexafluorobenzene, which gave a sharp signal in pyridine/$CDCl_3$ at δ-164.90. High-resolution ^{1}H NMR spectra were acquired with the following acquisition parameters: time domain, 32K; 90° pulse width, 9.3 μs; spectral width, 20.7ppm; relaxation delay, 1 s; acquisition time, 3.2 s. 32 scans were accumulated. A long delay time ensures the return of the excited nuclei to their thermal equilibrium prior to the next pulse, which is an imperative condition for a quantitative measurement. Base-line correction was performed carefully by applying a polynomial fourth-order function in order to achieve a quantitative evaluation of all signals of interest. The ^{1}H NMR chemical shift signals in $CDCl_3$ were referenced to TMS at δ 0.00.

2.5. Determination of hydroxyl value and acidity

These parameters were determined by employing the official methods of titration (AOCS method Cd 13-60, ISO 660:1996).

2.6. Methyl esterification

A solution of about 60 mg of oil dissolved in 4 mL trimethylpentane was made up in a test tube with glass stopper. After the addition of 200 μL of 0.2 M solution of potassium hydroxide in methanol, the tube was shaken for 30 s, and left until the phases separated. One gram of sodium hydrogen sulfate monohydrate was added into the solution in order to neutralize potassium hydroxide. It was then stored in a refrigerator until subjected to GC-MS (ISO 5509:2000, ISO 5508:1990).

2.7. GC-MS instrument and analytical conditions

All GC-MS analyses were performed on Shimadzu GC2010A (Kyoto, Japan) gas chromatography instrument coupled with a GCMS-QP2010 quadrupole mass spectrometer (Shimadzu). In the gas chromatographic system, an Rtx®-Wax capillary column, 30 m length, 0.25 mm i.d., and 0.25 μm film, consisting of cross bond polyethylene glycol (Restek) was used. The column temperature was programmed from 140 to 250 °C at the rate of 4 °C/min, held for 1 min at 140 °C and then held for 6 min at 250 °C. The injection temperature was kept at 220 °C, the carrier gas was nitrogen, and the column flow (nitrogen flow rate) was 1.36 mL/min. A sample of 1 μL was injected with a split ratio of 30:1.

Mass Spectroscopy Conditions: The ion source temperature was 230 °C, and the interface temperature was 280 °C. Ionization voltage was 0.2 kv.

The relative content was calculated by using the peak area normalization method.

3. RESULTS AND DISCUSSION

3.1. Determination of DGs and acidity

The content in DGs of the various EVOO, ROO and SO were detected by ^{19}F NMR. This method is based on the derivatization of the labile hydrogens of the hydroxyl groups of the DGs with 4-fluorobenzoyl chloride according to the reaction shown in Figure 1 and the integration of the appropriate peaks in the ^{19}F NMR spectrum. However, the acidity cannot be detected by ^{19}F NMR properly, because the fatty acids were not shown to react with the deriving regent quantitatively (Zhou *et al.*, 2015). Therefore, the conventional method was applied to the acidity determination.

Table 1 contains the percentage contents of the 1,2-DGs, 1,3-DGs, TDGs, the ratio D (1,2-DGs/TDGs), acidity and $D_{T/A}$ [(TDGs/620)/(acidity/282)]. These parameters appear to differentiate the oil samples. The origin of EVOO is divided into two countries, Spain and Italy. The EVOO samples from Italy show the higher content of TDGs, with acidity and $D_{T/A,}$ present in a lower D ratio. EVOO is characterized by low values of TDGs and high values of ratio D compared to ROO and SO. The TDGs in EVOO range from 1.6 to 2.2%. The level of TDGs is higher in ROO, which range from 5.0 to 8.0 %. The ratio D in all EVOO samples is equal to or greater than 0.4. Moreover, the ratio D of EVOO is much higher than ROO and SO. It has been suggested that the ratio D is a useful index of the quality of olive oils because of the fact that the isomerization of 1,2-DGs to 1,3-DGs usually occurs during olive oil storage and the refinement. Previous research on the ratio D of virgin olive oils freshly extracted from olives of normal ripeness should be close to 1 according to Vigli *et al.* (2003). However, due to the fact that all the samples used in our work were stored for half a year, then the ratio D is closer to 0.5, although it is still much higher than ROO and SO. As for the acidity, EVOO ranges

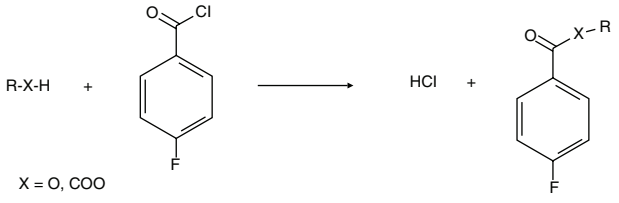

FIGURE 1. Reaction of the active hydrogen of compounds with 4-fluorobenzoyl chloride.

Table 1. Compositional Parameters of Extra Virgin olive oil (EVOO), Refined olive oil (ROO) and Seed oils (SO) determined by ^{19}F NMR Spectroscopy and Conventional method (average value ± standard deviation, n=3).

Sample[a]	Country	1,2-DG$_S$ (%)	1,3-DG$_S$ (%)	TDG$_S$ (%)	D	Acidity	D$_{T/A}$
EVOO							
1	Spain	0.78 ± 0.001	0.86 ± 0.003	1.64 ± 0.002	0.48 ± 0.002	0.265 ± 0.010	2.81 ± 0.004
2	Spain	0.74 ± 0.039	0.96 ± 0.005	1.70 ± 0.048	0.44 ± 0.010	0.28 ± 0.004	2.76 ± 0.012
3	Spain	0.99 ± 0.019	0.94 ± 0.006	1.93 ± 0.010	0.51 ± 0.009	0.285 ± 0.003	3.08 ± 0.010
4	Spain	0.79 ± 0.033	1.10 ± 0.021	1.89 ± 0.023	0.42 ± 0.027	0.281 ± 0.033	3.05 ± 0.021
5	Italy	1.02 ± 0.024	1.13 ± 0.010	2.13 ± 0.017	0.48 ± 0.014	0.292 ± 0.026	3.31 ± 0.018
6	Italy	0.70 ± 0.069	1.07 ± 0.034	1.77 ± 0.055	0.40 ± 0.036	0.276 ± 0.021	2.91 ± 0.025
ROO							
7	Italy	2.30 ± 0.022	5.20 ± 0.063	7.50 ± 0.057	0.31 ± 0.017	0.137 ± 0.031	24.86 ± 0.020
8	Italy	1.43 ± 0.015	3.80 ± 0.020	5.20 ± 0.013	0.28 ± 0.019	0.110 ± 0.015	21.47 ± 0.011
9	Italy	1.99 ± 0.006	4.51 ± 0.005	6.50 ± 0.007	0.31 ± 0.010	0.065 ± 0.027	45.41 ± 0.007
10	Spain	2.22 ± 0.020	5.61 ± 0.070	7.83 ± 0.010	0.31 ± 0.012	0.073 ± 0.012	48.71 ± 0.010
SO							
11	Peanut oil	0.65 ± 0.011	1.38 ± 0.016	2.03 ± 0.016	0.32 ± 0.023	0.334 ± 0.030	2.76 ± 0.019
12	Soybean oil	0.02 ± 0.021	0.07 ± 0.017	0.09 ± 0.023	0.27 ± 0.013	0.028 ± 0.020	1.46 ± 0.020
13	Rapeseed oil	0.32 ± 0.045	1.07 ± 0.024	1.39 ± 0.063	0.23 ± 0.061	0.687 ± 0.023	0.92 ± 0.050
14	Corn oil	1.59 ± 0.043	3.00 ± 0.050	4.59 ± 0.048	0.35 ± 0.052	0.046 ± 0.012	45.32 ± 0.021
15	Blend oil	0.74 ± 0.033	1.62 ± 0.035	2.36 ± 0.031	0.31 ± 0.027	0.082 ± 0.030	13.07 ± 0..030

[a]Data were expressed as mean ± standard deviation (n=3)

from 0.25 to 0.30, and a good linear concentration (r = 0.81) was obtained between acidity and TDGs. According to Fronimaki *et al.* (2002), a much better correlation (r = 0.89) is observed between the acidity and the amount of 1,3-DG. Therefore, there are some differences. The acidity of ROO and SO was much lower than EVOO because ROO and SO had been refined.

Theoretically, every triglyceride molecule is hydrolyzed to form one DG molecule and one free fatty acid (FFA) molecule. However, it was shown that the ratio (D$_{T/A}$) of molecular number of TDGs to that of FFAs was in the range of 2.81~3.31 in EVOO in Table 2. This means that the mole content of DGs is much higher than that of FFA. The question is: where are the FFAs going? The answer is that more FFAs are removed than DGs from EVOO during the water washing process because FFAs are more polar than DGs. Low quality olive oils are mainly pomace olive oil and lampant olive oil. Both of them have high acid values and TDGs and are not edible in their crude form. They become editable and are called ROO after refining, together or alone. The FFAs are removed almost completely, but little DGs are lost. So ROO samples have very high TDGs and low acidity, hence their D$_{T/A}$s are large (21.47~48.71) and much larger than those of EVOOs (Table 2). Definitely, D$_{T/A}$ could be used as a very important parameter to detect whether EVOO was adulterated with ROO.

3.2. Adulteration of EVOO with ROO

ROO, as it is much cheaper than EVOO, is usually added into EVOO by some unscrupulous traders because both of them have the same components, especially the same fatty acid composition if they are from same olive trees. The addition of ROO to EVOO is expected to deteriorate the antioxidant properties and organoleptic characteristics of EVOO. Also, most of the nutritional minor components in ROO are lost due to de-acidification, decoloration and deodorization under high temperatures, and high vacuum with absorbents. Several studies have suggested that the radio D (1,2-DG / TDGs) can distinguish different grades of olive oil (Sacchi *et al*., 1997; Zhou *et al*., 2015). Therefore, using ^{19}F NMR as the method to detect the adulteration of EVOO with ROO is acceptable.

In figure 2, it can be seen how, with the increase in adulteration level, the content of 1,2-DG, 1,3-DG TDGs increased. However, the radio D decreased. A good correlation (r = 0.96) was observed between the radio D and the adulteration level.

The hydroxyl value (OHV) is defined as the number of milligrams KOH equivalent to the hydroxyl groups found in one gram of the sample and is expressed in mg of KOH/g (Srk *et al*., 2013). In olive oil, DGs contain the most hydroxyl groups. In Figure 2, good linear correlations are obtained (r = 0.99) both in (a) and (b). The slope of TDGs to adulteration level is 6.7, which is close to the slope

TABLE 2. The fatty acid composition and squalene content of Extra Virgin olive oil (EVOO), Refined olive oil (ROO) and Seed oils (SO) determined by GC-MS and ^{1}H NMR Spectroscopy (average value ± standard deviation, n=3).

Sample	Country	palmitic[a]	stearic[a]	SFAs[b]	palmitoleic[a]	linolenic[a]	linolenic[b]	linoleic[a]	linoleic[b]	oleic[a]	oleic[b]	squalene[b]
EVOO												
1	Spain	10.64 ± 0.04	4.45 ± 0.11	14.55 ± 1.62	0.59 ± 0.21	0.70 ± 0.05	1.02 ± 0.14	6.59 ± 0.07	3.29 ± 0.30	77.03 ± 0.22	81.14 ± 1.17	0.84 ± 0.04
2	Spain	10.09 ± 0.10	4.82 ± 0.16	14.22 ± 1.84	0.53 ± 0.02	0.61 ± 0.24	0.84 ± 0.03	5.39 ± 0.27	3.29 ± 0.03	78.00 ± 0.32	81.65 ± 1.38	0.64 ± 0.04
3	Spain	11.49 ± 0.12	4.79 ± 0.17	16.35 ± 1.82	0.74 ± 0.04	0.63 ± 0.29	0.90 ± 0.01	5.74 ± 0.30	3.46 ± 0.04	76.61 ± 0.12	79.29 ± 2.11	0.67 ± 0.02
4	Spain	12.09 ± 0.23	3.55 ± 0.29	15.32 ± 1.81	1.00 ± 0.15	0.64 ± 0.32	1.11 ± 0.12	8.83 ± 0.20	5.50 ± 0.06	73.08 ± 0.25	78.07 ± 1.96	0.71 ± 0.06
5	Italy	11.09 ± 0.15	4.41 ± 0.30	15.77 ± 1.70	0.68 ± 0.07	0.62 ± 0.27	0.95 ± 0.01	5.87 ± 0.36	3.60 ± 0.07	77.34 ± 0.34	79.68 ± 1.02	0.91 ± 0.01
6	Italy	11.90 ± 0.24	2.97 ± 0.34	12.67 ± 1.67	0.63 ± 0.21	0.77 ± 0.15	1.19 ± 0.03	7.33 ± 0.38	3.06 ± 0.07	76.40 ± 0.38	83.08 ± 2.18	0.88 ± 0.01
ROO												
7	Italy	12.40 ± 0.42	3.36 ± 0.28	16.83 ± 1.62	0.84 ± 0.05	0.60 ± 0.26	1.54 ± 0.02	11.13 ± 0.29	6.57 ± 0.12	71.61 ± 0.23	75.06 ± 1.82	0.49 ± 0.03
8	Italy	12.17 ± 0.29	3.27 ± 0.05	14.14 ± 1.15	0.86 ± 0.02	0.51 ± 0.18	1.57 ± 0.05	11.70 ± 0.39	7.11 ± 0.11	70.92 ± 0.27	77.18 ± 1.28	0.62 ± 0.07
9	Italy	12.06 ± 0.36	3.18 ± 0.13	13.32 ± 1.37	0.80 ± 0.10	0.61 ± 0.17	1.54 ± 0.07	11.28 ± 0.27	6.76 ± 0.12	72.07 ± 0.33	78.38 ± 1.82	0.35 ± 0.01
10	Spain	12.12 ± 0.24	3.54 ± 0.23	13.90 ± 1.16	0.84 ± 0.27	0.72 ± 0.21	1.48 ± 0.09	11.70 ± 0.22	6.52 ± 0.17	71.08 ± 0.26	78.10 ± 1.44	0.42 ± 0.02
SO												
11	Peanut oil	11.62 ± 0.37	4.20 ± 0.36	20.61 ± 1.75	0.05 ± 0.01	1.12 ± 0.34	0.48 ± 0.03	34.85 ± 0.28	33.64 ± 0.08	42.35 ± 0.19	45.27 ± 2.24	0.04 ± 0.01
12	Soybean oil	10.95 ± 0.08	4.56 ± 0.45	17.39 ± 1.44	0.08 ± 0.02	9.18 ± 0.25	7.76 ± 0.01	50.86 ± 0.29	50.64 ± 0.05	23.88 ± 0.15	24.21 ± 2.10	0.00
13	Rapeseed oil	4.38 ± 0.13	2.01 ± 0.22	6.95 ± 1.52	0.19 ± 0.0.01	7.3 ± 0.22	10.15 ± 0.09	20.25 ± 0.45	16.74 ± 0.16	57.11 ± 0.16	66.16 ± 1.74	0.00
14	Corn oil	12.89 ± 0.04	1.90 ± 0.34	17.03 ± 1.20	0.10 ± 0.02	0.47 ± 0.32	1.39 ± 0.04	49.42 ± 0.23	51.45 ± 0.03	30.85 ± 0.11	30.13 ± 1.06	0.28 ± 0.03
15	Blend oil	10.74 ± 0.13	3.95 ± 0.21	17.35±0.34	0.00	3.99 ± 0.02	4.60±0.03	49.69 ± 0.34	48.35±0.01	40.00 ± 0.12	35.21±1.23	0.00

[a]Determined by GC-MS;
[b]Determined by ^{1}H NMR.

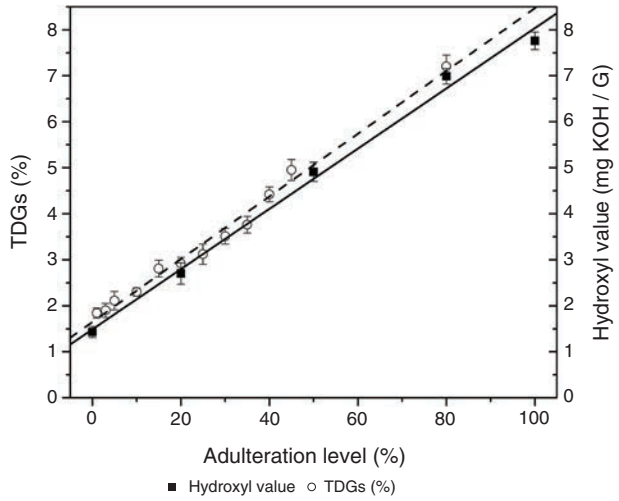

FIGURE 2. Total diglycerides (TDGs) and hydroxyl value for different adulteration levels (average value ± standard deviation, n=3).

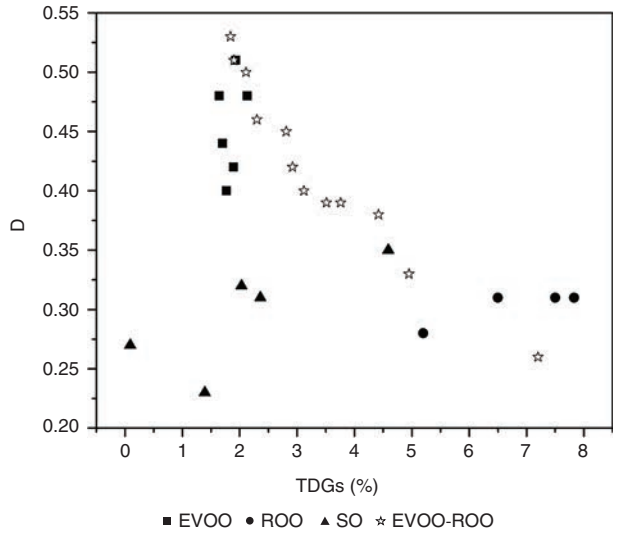

FIGURE 3. Plot of the ratio D against the TDGs (%) for extra virgin olive oils (EVOO), refined olive oils (ROO), seed oils (SO) and for the EVOO mixtures with ROO (EVOO-ROO).

of the hydroxyl value (6.5). The result demonstrates that ^{19}F NMR is useful in detecting TDGs.

OHV cannot discriminate the OH group in hydroxyl fatty acids (ricinoleic acid) or the OH group in glycerol bases of MGs and DGs, but our ^{19}F NMR method can. It can even discriminate 1,2-diglycerides and 1,3-diglycerides clearly (Zhou *et al.*, 2015).

The discrimination of EVOO with respect to the other oils can be seen in Figure. 3, where the ratio D is plotted against the TDGs. It is seen clearly that the EVOO samples are clustered in the upper part of the graph, whereas ROO and SO are dispersed in the lower part of the graph. What is most interesting in this graph is the observation that adulterated EVOO samples with ROO lie between the group of EVOO and ROO. Along with the increase in the adulteration of low price oil, the plots move to the lower right.

In summary, TDGs and $D_{T/A}$ could be used as two key parameters to determine whether EVOO is adulterated with ROO or not. If the TDGs of EVOO is less than 2.5 and sometimes its $D_{T/A}$ is less than 4, it could be concluded that EVOO is not adulterated with ROO. D is only a parameter to indicate the freshness of EVOO. The larger D is, the fresher the EVOO is.

3.3. Determination of fatty acid composition

The compositions of the unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid) and saturated fatty acids (SFAs) in different oils were calculated according to the various signal intensities in the ^{1}H NMR spectra (Vigli *et al.*, 2003; Sacchi *et al.*,1996; Sacchi *et al.*, 1997).

There is one intense peak at δ = 1.68 in the ^{1}H NMR spectrum of virgin olive oils. This peak was identified as the methyl protons of the CH_3-17 and CH_3-29 of squalene when compared with the ^{1}H NMR spectrum of squalene standard substance, which has been reported previously (Mannina *et al.*, 2009). Because the two methyl groups in squalene are equivalent, the weight percentage of squalene can be calculated by a similar method to the determination of fatty acid composition according to Formula 1, but their different molar weights are also considered. In Formula 1, A_S is the area of integration of the signal of the two methyl groups of squalene at 1.68 ppm. The chemical shift of all the methyl protons of fatty acids is at 0.88 ppm (signal J) except linolenic (signal I) whose chemical shift is 0.97 ppm. A_I and A_J are the areas of integration of signals I and J, respectively. 410 is the molar weight of squalene and 296 is the molar weight of methyl oleate which is about 1/3 the molar weight of a triglyceride in olive oil.

$$\text{Squalene}\% = \frac{(A_s/2)\times 410}{(A_I + A_J)\times 296 + (A_s/2)\times 410}\times 100\%$$

Formula 1

The percentages of the unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid) saturated fatty acids (SFAs) and squalene in different oils obtained by ^{1}H NMR spectroscopy are listed in Table 2. Careful analysis of these data reveals some interesting trends as follows:

The percentages of linolenic acid in all olive oil samples ranged from 0.8 to 1.6 %. The linoleic content exit in EVOO samples ranged from 3.0 to 5.5 %, which is lower than ROO (about 7 %) and

SO (16~51 %). The oleic acid percentages in all olive oils are relatively stable and range from 75 to 82%, which is much higher than SO. The percentages of SFA (total saturated fatty acids) in all the oil samples were relatively stable. As for squalene, which exits in olive oils in higher amounts, it can be a very important index to distinguish between olive oils and seed oils.

The content of fatty acid of the oil samples was also detected by the GC-MS, which is a traditional method to detect fatty acids (Capote *et al*., 2007). 6 different kinds of fatty acids (palmitic acid, palmitoleic acid, stearic acid, linolenic acid, linoleic acid, oleic acid) were identified by GC-MS.

In Table 2, it is clearly shown that the sum of the palmitic acid content and the stearic acid content detected by GC-MS was very close to the content of SFAs detected by ^{1}H NMR. However, GC-MS can differentiate palmitic acid from stearic acid easily, while ^{1}H NMR cannot. The contents of oleic, linoleic and linolenic acids can be determined by ^{1}H NMR as well as GC-MS and both methods get close results. Comparing the three parameters (oleic acid, linoleic acid, linolenic acid) detected by ^{1}H NMR and GC-MS showed that the D-value of linolenic acid ranged from 0.2 to 1. As for the linoleic acid, it was 1.2 to 4.6. However, the D-value of oleic acid ranged from 3 to 7. This indicates that the detection of linolenic acid by these two methods is relatively accurate. It is also clearly shown that EVOO and ROO have very similar fatty compositions but ROO contains lower squalene contents than EVOO. Compared to other vegetable oils, the content of squalene is richer in olive oil. So squalene content may be a very important parameter for the determination of EVOO if adulterated with other seed oils.

3.4. Adulteration of EVOO with soybean oil

Soybean oil is a typical additive for the adulteration of olive oils due to its low price. Five parameters (linolenic acid, linoleic acid, oleic acid, SFAs, squalene) of the adulteration of EVOO with soybean oils were determined by ^{1}H NMR. In Figure 4, except for SFAs, the other four parameters, oleic acid, linolenic acid, linoleic acid, squalene are all in good relationship with the adulteration level. With the increase in the adulteration level, the contents of linoleic acid and linolenic acid increased; although the opposite occurred with the contents of oleic acid and squalene. According to Table 2, the content of linolenic acid in EVOO cannot exceed 1.3%. On the basis of the linear equation y = 0.0745x + 0.6636, the limit of detection of the adulteration level was 4.5% by ^{1}H NMR method.

Squalene content and fatty acid composition can indicate whether EVOO is adulterated with SO or not. Squalene content in EVOO is larger 0.6% (w/w) and oleic acid content is larger than 65%.

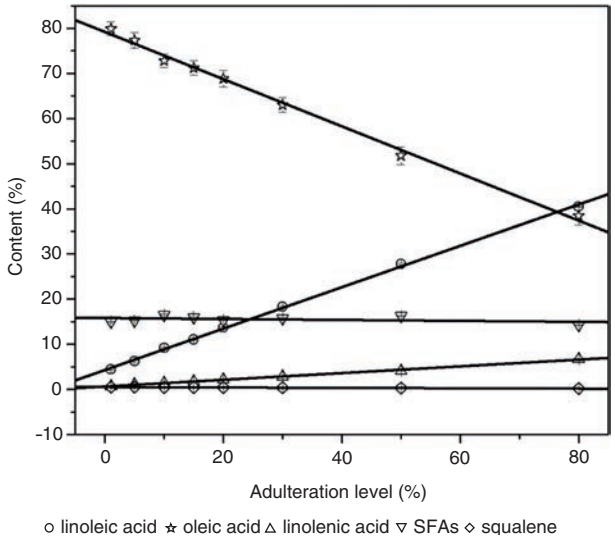

FIGURE 4. Five parameters (the contents of linolenic acid, linoleic acid, oleic acid, SFAs, squalene) of the adulteration of EVOO with soybean oils determined by ^{1}H NMR (average value ± standard deviation, n=3).

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