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A 43 MHz Low-Field Benchtop ^{1}H Nuclear Magnetic Resonance Method to Discriminate Perilla Oil Authenticity

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Abstract: The aim of this study was to discriminate the authenticity of perilla oils distributed in Korea using their ^{1}H nuclear magnetic resonance (NMR) spectra acquired by a 43 MHz low-field benchtop NMR spectrometer. Significant differences existed in the integration values of all 6 peaks found in the spectrum between authentic and adulterated perilla oil samples. The integration values of 4 peaks that signify the methylene protons present in all fatty acids (FA) and allylic or olefinic protons present in all unsaturated FA were the best variables for establishing perilla oil authenticity. The procedure for applying the range of variables found in authentic perilla oil samples correctly discriminated between the samples of perilla oils with soybean oils added at concentrations of ≥ 6 vol%. The results demonstrated that this NMR procedure is a possible cost-effective alternative to the high-field ^{1}H NMR method for discriminating the authenticity of perilla oils.

Key words: authenticity, economically motivated adulteration, linolenic acid, low-field benchtop ^{1}H NMR, perilla oil

1 INTRODUCTION

Perilla oil is unrefined edible oil obtained by pressing perilla seeds (*Perilla frutescens* var. *frutescens*) after toasting. Perilla oil is an important flavoring agent used frequently in East Asian cooking, particularly in Korea and Japan because it imparts a characteristic nutty, toasted flavor to cooked foods. Korea produced ~31,000 metric tons of perilla seeds annually during 2012-2013 [KMAFRA (http://www.mfds.go.kr)], whereas ~22,000 metric tons of perilla seeds were imported annually during the same period. China has provided the entire quantity of imported perilla since 2011 [KMFDS (http://www.mfds.go.kr)].

Economically motivated adulteration (EMA) of food is the intentional adulteration of food to obtain financial advantage via deception. Perilla oil is a targeted product for EMA in Korea because the retail price of perilla oil is up to 30 times that of other edible oils. Adulterated perilla oils have been produced by blending with relatively inexpensive vegetable oils (e.g., corn and soybean oils) and low-quality ingredients (e.g., extracts from perilla cake). Thus, the development of simple, rapid, cheap, accurate analytical methods that can discriminate the authenticity of perilla oil can help prevent its EMA.

Several published studies have investigated the compositions of fatty acids (FA), triacylglycerols, sterols, tocopherols, phenolic compounds, volatiles, and carbon stable isotopes in perilla oils[1-5]. However, to the best of our knowledge, there have been no published studies attempting to discriminate the authenticity of perilla oil by analyzing the chemical constituents in the oil, although a recent study investigated the patterns of carbon stable isotope ratio and FA composition of Chinese perilla oil with added corn or soybean oil[5].

Several researchers have demonstrated that when compared to conventional chromatographic methods, high-field ^{1}H nuclear magnetic resonance (NMR) spectroscopy is a simpler (i.e., no pretreatment of sample required) and more rapid (i.e., a few minutes of analysis time required) analyti-

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cal technique that can accurately and precisely discriminate the authenticity of high-priced vegetable oils such as extra virgin olive oil and toasted sesame oil[6, 7]. However, there is a limitation in the use of high-field NMR spectroscopy as a practical and routine analytical method for discriminating the authenticity of the oils because the NMR spectrometer is very expensive to purchase and maintain[8].

Since the early 2000s, there has been sufficient development in permanent magnet technology and design to develop much smaller NMR instruments with resolution and sensitivity that are useful in education, research and industrial applications[9]. In the present study, a low-field benchtop ^{1}H NMR spectrometer working at 43 MHz was used as the analytical tool to discriminate the authenticity of perilla oil. This instrument is much less expensive than conventional high-field (e.g., 300-600 MHz) NMR and is compact enough to be used in standard laboratory environments. Furthermore, published studies demonstrated that a 60 MHz low-field benchtop ^{1}H NMR spectroscopy analysis is a possible approach for discriminating vegetable oils or animal fats derived from different sources[8, 10].

Perilla-flavored oil is an edible oil that is used as a replacement for the perilla oil in Korean foods due to its low retail price and similar flavor to perilla oil. This oil is a legal product manufactured by blending lower grades of perilla oil (e.g., extracts from perilla cake) and other cheaper vegetable oils (mainly, soybean oil). Criminal manufacturers produce adulterated perilla oil products in a very similar way. Therefore, we used the commercial perilla-flavored oil as a model of adulterated perilla oil in the present study.

The aim of this study was to discriminate the authenticity of perilla oils distributed in Korea using ^{1}H NMR analysis of the oils acquired by a 43 MHz low-field benchtop NMR spectrometer.

2 EXPERIMENTAL PROCEDURES

2.1 Samples

Twenty seven samples of authentic perilla oils were prepared in our laboratory by extraction from 23 different samples of Korean perilla seeds and 4 samples of Chinese perilla seeds that were obtained from local grocery stores in Korea. Samples of Korean perilla seeds were collected from 6 different provinces [Gyeongbuk (n = 7), Jeonnam (n = 6), Gyeonggi (n = 3), Gangwon (n = 3), Chungnam (n = 3), and Jeonnam (n = 1)] in Korea during the 2015 harvest. Samples of Chinese perilla seeds were imported by the Korea Agro-Fisheries Trade Corporation during 2015. The perilla seeds were toasted in a drum roaster (THDR-01, Taehwan Automation Industry Co., Seoul, Korea) at 200℃ for 30 min. The oil was extracted from the toasted seeds using an oil press (Oil Love, National ENG Co., Goyang, Korea). The extracted oil was centrifuged at 9,600 × g for 10 min to remove the precipitated impurities and then dried completely by flushing with nitrogen gas. Ten samples of adulterated perilla oils consisted of commercial perilla-flavored oils that were obtained from Korean edible oil companies. The samples of authentic and adulterated perilla oils described above were used to determine the best variables for discriminating perilla oil authenticity. Twenty additional samples of authentic and adulterated perilla oils consisted of a sample of authentic perilla oil to which a sample of commercial soybean oil was added at concentrations of 0, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, and 100 vol%. These oil samples were used to evaluate the predictive discrimination power of the integration values of peaks found in the ^{1}H NMR spectrum obtained by the 43 MHz benchtop NMR spectrometer: variables that discriminated the authenticity of perilla oil are described below. All oil samples were kept refrigerated at 4℃ prior to analysis.

2.2 43 MHz benchtop ^{1}H NMR analysis

Each oil sample (100 μL) was dissolved in 500 μL of deuterated chloroform (99.8% atom D) containing 0.03 v/v% tetramethylsilane (TMS) (Sigma-Aldrich, St. Louis, MO, USA) and then placed in a 5-mm outer diameter disposable NMR tube (Wilmad-Lab Glass, Buena, NJ, USA). TMS was used as an internal reference for the chemical shifts. All ^{1}H NMR spectra were acquired using a 43 MHz NMR spectrometer (Spinsolve, Magritek, Wellington, New Zealand). The spectra were obtained after stabilizing the NMR spectrometer using a power shim method that required 41 min to adjust the homogeneity of the magnetic field. After the start of the experiment, shimming was conducted for 2-5 sec using the quick shim method every hour. All NMR experiments were recorded at 25℃ and ^{1}H NMR spectra were acquired at a spectral width of 5000.0 Hz; pulse angle of 45°; acquisition time of 3.2 sec; relaxation delay of 7 sec and 32 scans. The measurement time per sample took 4 to 5 min. A ^{1}H NMR spectrum of perilla oil was additionally obtained using 600 MHz NMR spectrometer (VNS-600, Varian Inc., Palo Alto, CA, USA) to compare it to the ^{1}H NMR spectrum of perilla oil acquired by 43 MHz NMR spectrometer. Sixteen scans were collected with a relaxation delay of 10 sec, a pulse angle of 45°, and an acquisition time of 3.2 sec. The software MestReNova (Mestrelab Research, Santiago de Compostela, Spain) was used for processing the spectra. Phase correction of all spectra was performed, and then baseline correction was automatically performed using the Whittaker Smoother method. In this study, we did not use other methods to enhance the resolution of the spectra. The chemical shift of each peak was adjusted using the TMS peak. The integration value of each peak was calculated using normalization by the absolute integration value of the total peaks (i.e., 6 peaks derived from triacylglycerols, in the present study) found in the ^{1}H NMR spectrum to

reduce the sampling error. Each sample was repeatedly prepared three times and the mean of the measured integration values was used for comparing the authentic and adulterated perilla oils. The calculation of the integration value was performed using the integration method in the MNOVA software.

2.3 Statistical analysis

A two-tailed Student's t-test was used to determine the differences between the authentic and adulterated perilla oil samples ($p<0.05$, 0.01, or 0.001). Pearson's correlation test was performed to determine whether a significant linear relationship existed between the two variables ($p<0.01$). The statistical analysis was performed using IBM SPSS Statistics (version 20) software (SPSS Inc., Chicago, IL, USA).

3 RESULTS AND DISCUSSION

3.1 ^{1}H NMR spectra

Figure 1 shows the ^{1}H NMR spectra of an authentic perilla oil sample acquired using 43 MHz benchtop NMR spectrometer and 600 MHz high-field NMR spectrometer. Six peaks, among many signals, which were signals with strong and distinguishable intensity, were found in the benchtop ^{1}H NMR spectra. Because the spectra of benchtop NMR usually appear as peaks that are broader than those seen in a conventional high field NMR, the signals overlap each other. In benchtop NMR, signals from trace components such as sterols and tocopherols are difficult to identify because they have very small signal intensity. However, even if the signals for triacylglycerols overlap, assignment is possible using the chemical shift of peaks at each position because they are the same in any magnetic field. Descriptions for several of the peaks (6 non-overlapped and large enough peaks) are given in Table 1. The information contains a range of chemical shifts and chemical formula moieties based on previously reported papers[11–13]. Peak no. 1 is divided into two peaks with triplet signals in the 600 MHz NMR spectrum, whereas these were hard to verify with the 43 MHz NMR spectrum. The main peak among the two peaks is derived from the terminal methyl group of linolenic acid (18:3n-3) and the other peak is derived from the terminal methyl group of all FAs except for 18:3 n-3. Peak no. 2 showed an overlapped signal having predominant intensity derived from methylene bridges in all FAs and the acyl group. This peak differs slightly in the position and shape of the signal depending on the content of the FAs in edible oil. Peak no. 3 with a broad shape is derived from methylene protons in position α at the carboxyl end of acylglycerol and allylic protons in unsaturated FA (USFA) with double bonds such as 18:3n-3 and linoleic acid (18:2n-6). Peak no. 4 is derived from bis-allylic protons in USFA such as 18:3n-3 and 18:2n-6. This signal can be used to confirm the presence of USFA in the sample and predict its content. The signals which can be distinguished in the high-field NMR represent overlapped signals which are in the up field derived from 18:2n-6 and in the down field derived from 18:3n-3 as seen with the 43

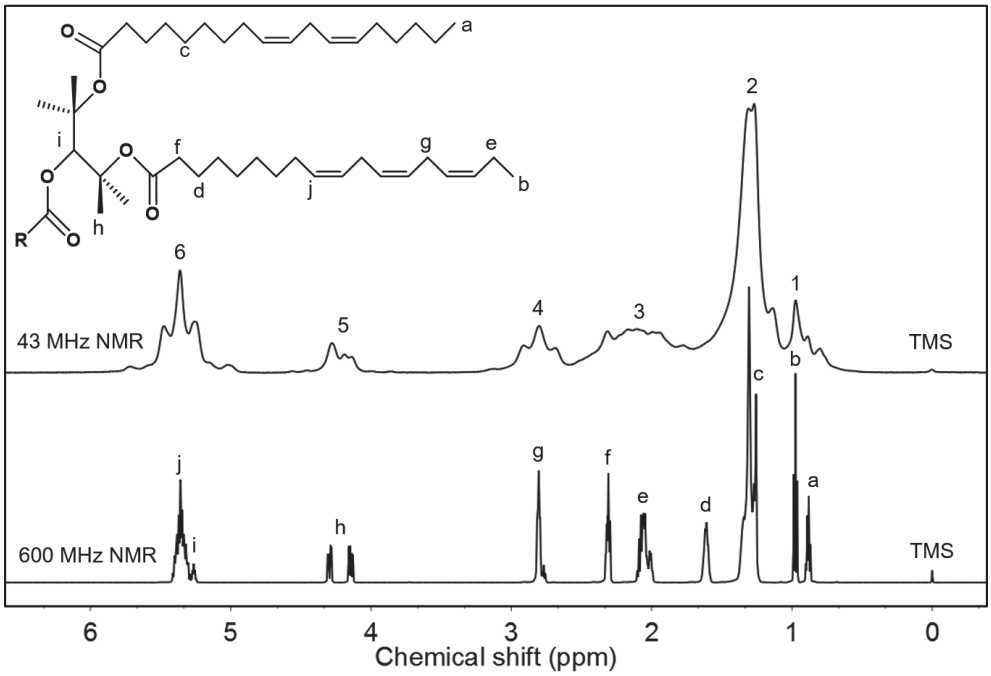

Fig. 1 Comparison of ^{1}H NMR spectra of authentic perilla oil samples acquired by 43 MHz benchtop NMR spectrometer and 600 MHz high resolution NMR spectrometer. Labeled peaks in the 43 MHz ^{1}H NMR spectra are assigned in Table 1.

Table 1 Chemical shifts and peak assignments in the 43 MHz ^{1}H NMR spectra (Fig. 1) of the perilla oil samples.

Peak no.	Chemical shift range (ppm)	Proton	Assignment
1	0.5279–1.0454	-C**H**$_3$	All FA
2	1.0643–1.6640	-(C**H**$_2$)$_n$- / -COOCH$_2$C**H**$_2$-	All FA
3	1.6641–2.5532	-C**H**$_2$-CH = CH- / -COOC**H**$_2$CH$_2$-	All USFA / All FA
4	2.5720–3.1742	-CH = CHC**H**$_2$CH = CH-	Linoleic and linolenic acids
5	4.0046–4.5009	-C**H**$_2$COOCH$_2$CH$_2$-	Glycerol (α- position)
6	4.8631–5.8652	-C**H**COOCH$_2$CH$_2$-/ -C**H** = C**H**-	Glycerol (β- position) / All USFA

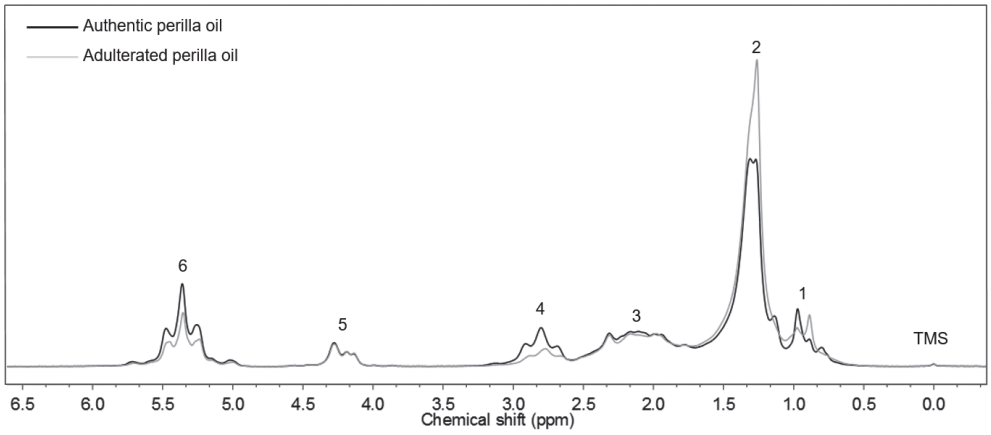

Fig. 2 Overlapped ^{1}H NMR spectra of authentic and adulterated perilla oil samples acquired by 43 MHz benchtop NMR spectrometer. Labeled peaks in the ^{1}H NMR spectra are assigned in Table 1.

MHz NMR spectrometer. When the content of USFA is high, a prominent signal can be observed in this vicinity[14]. Peak no. 5 is derived from the protons attached to carbon atoms at *sn*-1 and *sn*-3 position glyceryl groups and this peak can be used to check the concentration of acylglycerol in the sample. Previous studies using high-field NMR reported that the peak derived from acylglycerols has little difference in peak multiplication or chemical shift. This finding suggests that peak 5 is not useful for distinguishing between various edible oils. Peak no. 6 represents overlapped signals which are derived from the olefinic proton in the USFA and protons from carbon atoms at the *sn*-2 position glyceryl group.

3.2 Integration values for the ^{1}H NMR peaks

^{1}H NMR spectra of the authentic and adulterated perilla oil samples obtained with the 43 MHz benchtop NMR spectrometer are overlapped, as seen in Fig. 2. The figure clearly shows that discernible differences exist in the intensity of peaks nos. 2, 4, and 6 as well as the shape of peak no. 1. In the authentic perilla oil samples, peak no. 1 was observed at around 0.972 ppm, whereas it was observed mostly in the up field position at 0.887 ppm in the adulterated perilla oil samples. Perilla oil is highly unsaturated compared to other conventional vegetable oils, including corn and soybean oils, because of the abundance of 18:3*n*-3 (~60 wt% of total FA; unpublished observations). Thus, these results are in accordance with previous findings employing high-field NMR spectroscopy that showed the presence of a double bond in the USFA positions of the signal for total FA further down field[8, 15]. The integration values for the 6 peaks mentioned above are compared in Table 2. The integration values for all 6 peaks were different between the authentic and adulterated perilla oil samples at the $p = 0.1\%$ level, thereby suggesting that the ^{1}H NMR analysis employing the 43 MHz benchtop NMR spectrometer was an appropriate method for discriminating the authenticity of perilla oil. Particularly, authentic perilla oil samples had a significantly smaller integration value for peak no. 2 (I2 value) than the adulterated perilla oil samples. This is attributed to the fact that authentic perilla oil contains more USFA than adulterated perilla oil, thereby leading to a decrease in the number of methylene protons in the hydrocarbon chains of the FAs from authentic perilla oil. The I2 value was significantly ($p < 0.01$) and negatively correlated with the integration values of peak nos. 3 (I3 value; Pearson's $R = -0.997$), 4 (I4 value; Pearson's $R = -0.999$) and 6 (I6 value; Pearson's $R = -1.000$). These results were due to the fact that the three peaks were partly (for I3 and I6) or totally (for I4) derived from allylic or olefinic protons.

Table 2 Integration values of 43 MHz ^{1}H NMR peaks in authentic and adulterated perilla oil samples.

Variable[a]		Authentic perilla oil[b] (n = 23)	Adulterated perilla oil[c] (n = 10)
I1	Range	779.24-845.87	873.02-909.69
	Mean	817.31	894.57***
I2	Range	4171.38-4489.84	5287.78-5460.91
	Mean	4414.33	5373.04***
I3	Range	2012.08-2088.10	1902.20-1943.46
	Mean	2035.99	1918.25***
I4	Range	791.72-929.77	381.44-456.80
	Mean	824.65	420.35***
I5	Range	363.48-379.87	360.70-365.05
	Mean	374.44	363.29***
I6	Range	1429.01-1577.50	913.59-1001.42
	Mean	1469.03	961.16***

[a] Integration values of ^{1}H NMR peaks. Numbers represent the peak numbers shown in Fig. 1.
[b] Prepared in the laboratory by extraction from perilla seeds after toasting.
[c] Consists of commercial perilla-flavored oils.
*** Significantly different from authentic perilla oil, $p < 0.001$.

3.3 Discrimination of authenticity of perilla oil with added soybean oil

We evaluated the predictive discrimination power of employing the integration values of the 6 peaks as the variables for discriminating the authenticity of perilla oils using 20 samples of perilla oils with soybean oils added at different concentrations (Table 3). We determined the authenticity of the oil samples by applying the range of variables found in the authentic perilla oil samples as follows: if the measured values of the samples lay within the range of the variables listed in Table 2, the sample was an authentic perilla oil, whereas if any of the measured values lay outside this range, the sample had been adulterated. Samples of perilla oils with soybean oils added at concentrations of equal to or more than 6 vol% were correctly discriminated as being adulterated. Among the 6 variables, I2 values contributed most to the discrimination of the authenticity of the oil samples, followed by I6, I4, and I3 values. Conversely, the contribution of I1 and I5 values was relatively low. Consequently, we determined I2, I3, I4, and I6 values were the best variables for discriminating the authenticity of perilla oil.

4 CONCLUSIONS

This study is the first to employ a 43 MHz low-field benchtop ^{1}H NMR machine for discriminating the authenticity of vegetable oils including perilla oil. We demonstrated that the analytical method using the range of integration values for the 6 NMR peaks observed in the authentic perilla oil samples used in this study could discriminate perilla oils adulterated with soybean oil at concentrations of ≥ 6 vol% from authentic perilla oils. Therefore, this study suggests that 43 MHz low-field benchtop ^{1}H NMR spectroscopy can be used as a possible cost-effective analytical method for discriminating the authenticity of perilla oils.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

Table 3 Discrimination of the authenticity of samples of perilla oils with soybean oils added at different concentrations.

Percentage (vol%) of added soybean oil in perilla oil	Variable[a]						Predicted[b]
	I1	I2	I3	I4	I5	I6	
0	779.24	4410.53	2067.49	842.07	374.63	1464.25	Authentic
5	803.83	4473.77	2031.08	806.41	374.30	1448.20	Authentic
6	808.12	**4493.68**[c]	2029.61	797.86	373.89	1435.19	Adulterated
7	803.41	**4500.79**	2033.13	797.88	373.36	**1423.71**	Adulterated
8	794.17	**4514.73**	2038.04	798.83	371.44	**1421.89**	Adulterated
9	832.38	**4523.76**	2019.35	**775.58**	370.10	**1418.44**	Adulterated
10	780.22	**4455.35**	2022.41	**806.49**	**403.89**	**1424.85**	Adulterated
15	793.39	**4526.17**	**2009.72**	**778.76**	**400.35**	**1389.91**	Adulterated
20	788.04	**4565.42**	**2011.90**	**763.59**	**401.19**	**1367.99**	Adulterated
25	790.96	**4614.65**	**2006.05**	**742.60**	**400.77**	**1342.26**	Adulterated
30	816.90	**4719.96**	**2006.36**	**709.39**	368.86	**1319.60**	Adulterated
35	821.53	**4764.80**	**1989.37**	**683.77**	379.41	**1285.44**	Adulterated
40	818.45	**4821.85**	**1987.80**	**668.79**	369.34	**1270.09**	Adulterated
45	799.75	**4825.73**	**1979.53**	**654.09**	**399.68**	**1232.21**	Adulterated
50	808.17	**4864.91**	**1970.75**	**635.19**	**400.46**	**1210.32**	Adulterated
60	814.05	**4989.58**	**1957.81**	**586.21**	**396.30**	**1149.83**	Adulterated
70	813.35	**5075.21**	**1949.86**	**550.79**	**396.64**	**1104.57**	Adulterated
80	832.55	**5171.87**	**1935.91**	**508.35**	**393.99**	**1053.33**	Adulterated
90	**845.90**	**5262.46**	**1921.63**	**468.18**	**390.90**	**1006.99**	Adulterated
100	**850.34**	**5359.95**	**1907.86**	**428.32**	**390.32**	**957.51**	Adulterated

[a] Integration values of ^{1}H NMR peaks. Numbers represent the peak numbers shown in Fig. 1.
[b] If measured values lie within the range of the variables listed in Table 2, then the sample is an authentic perilla oil; whereas, if any of the measured values lies outside this range, the sample has been adulterated.
[c] Entries in boldface type lie outside the range for authentic perilla oils.

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