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Determination of fatty acid compositions of triacylglycerols by high resolution NMR spectroscopy

Tai-Yow Shiao[1] and Ming-Shi Shiao[2,3]

[1]*Department of Food Processing, National Chia-Yi Institute of Agriculture, Chia-Yi, Taiwan*
[2]*Department of Medical Research, Veterans General Hospital, Shih-Pai, Taipei, Taiwan 11217, Republic of China*

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Abstract. High resolution NMR was applied to determine the fatty acid compositions of triacylglycerols in plant seed oils and animal fats. Structural information by NMR alone was compared with those provided by chromatographic method to evaluate the effectiveness of these two analytical approaches. ^{1}H-NMR was demonstrated to be more rapid and equally informative in the determination of contents and ratio of total saturated to unsaturated fatty acids and the molar percentage of n-3 polyunsaturated fatty acids in naturally occurring triacylglycerols. The ^{13}C-NMR of 6 plant seed oils were found to be characteristic in chemical shift regions of δ 13-40 and δ 128-131. These ^{13}C signal profiles could serve as fingerprint for identification.

Key words: Fatty acid composition; NMR; Plant seed oil; Triacylglycerols.

Introduction

It is well established that hypercholesterolemia is the key risk factor in causing atherosclerosis (Lipid Research Clinics Program, 1984; Steinberg *et al.*, 1985). The calories from fat intake, percentages of saturated and unsaturated fats, and cholesterol in diet are important factors which can profoundly affect body cholesterol homeostasis in human subjects. Excess consumption of saturated fats can promote the resorption of cholesterol and enhance the development of atherosclerosis (Pollak, 1987). In the diet manipulation of hyperlipoproteinemia, n-3 polyunsaturated fatty acids (n-3 PUFA) are widely consumed primarily in the form of fish oils and their concentrated capsules although the molecular basis of effectiveness is still not well characterized (Illingworth *et al.*, 1984; Mehta *et al.*, 1988; Ackman, 1988). Reports have shown that the positional distribution of monoenoic fatty acids and PUFA on C-1, C-2 and C-3 carbons of glycerol moiety of naturally occurring triacylglycerols (TG) is not symmetrical and n-3 PUFA in fish oils are predominantly attached to C-2 (Brockeroff and Hoyle, 1963). Edible oils are mixtures of TG originated from plant seed oils or animal fats. They are different in fatty acid composition and are themselves characteristic in acyl chain length as well as numbers, location, and stereochemistry of olefinic bonds. It remains equally urgent for the development and application of both simple and sophisticated analytical methods to provide structural information of TG in various detail. Traditionally, gas chromatography (GC) is the most widely used method in the determination of fatty acid compositions. Accumulating reports also demonstrated recently that high performance liquid chromatography (HPLC) can also be very successful (Roggero and Coen, 1981; Manku, 1983). The analytical procedure involves tran-

[3]Correspondence should be sent to Ming-Shi Shiao, Department of Medical Research, Veterans General Hospital, Shih-Pai, Taipei, Taiwan 11217, R.O.C.

sesterification or saponification of TG in alkaline condition and subsequent derivatization of released free fatty acids. The clue about positional distribution of specific fatty acid to the C-1, C-2 and C-3 positions of glycerol is unavoidably lost after the saponification step. The asymmetry of distribution of fatty acids in TG mixtures can only be established by a tedious procedure involving stereospecific lipases for differential digestion (Brockeroff, 1965). Furthermore, PUFA can also be severely decomposed and end up with lower estimation of contents because of auto- and photooxidation during the analytical processes. The resolution of nuclear magnetic resonance (NMR) spectroscopy to distinguish subtle difference in structure and stereochemistry of lipid metabolites can not be overlooked (Chapman, 1986). With the advent of many pulse programs, resolution of FT-NMR is greatly enhanced and spectroscopic data can be accumulated within a feasible acquisition time. Recently, FT-NMR is also applied to determine the positional distribution of unsaturated fatty acids to the C-1/C-3 and C-2 positions of glycerol in TG (Ng and Ng, 1983). It is based on the observation in high resolution ^{13}C-NMR that each olefinic carbon signal is well resolved and is dependent on the location of olefinic carbon containing fatty acids to the glycerol C-1/C-3 or C-2 in TG (Ng, 1984). We report in this paper the structural information of TG from 6 seed oils and 2 animal fats that could be directly provided by NMR spectroscopy alone. Additional structural information by NMR techniques which can be supportive or complementary to those obtained by the chromatographic methods is also compared.

Materials and Methods

Tristearin, triolein, fatty acid methyl esters and other related lipid standards were purchased from Sigma (St Louis, MO, USA). Deuterated chlorofrom and 2-bromo-2′-acetonaphthone were obtained from Aldrich (St Louis). Thin layer chromatographic (TLC) plates, 20 × 20 cm, 0.25 mm thickness of Merck silica gel 60 was developed with a mixture of petroleum ether, diethyl ether, acetic acid (85:15:1, v/v) as the elution solvents to check sample purity if commercial plant seed oils and animal fats were used.

NMR measurement was performed on a Varian VXR-300 spectrometer (299.95 MHz for ^{1}H-NMR and 75.43 MHz for ^{13}C-NMR). Samples were dissolved in $CDCl_3$ and spectra were taken at ambient temperature. Spectral data were reported as parts per million downfield from Me_4Si (δ = 0). To secure spectral consistency in ^{1}H-NMR measurement, the following instrumental parameters were commonly applied: spectrum width, 4000 Hz; acquisition time, 2.05 sec; pulse width, 7 μsec; delay time, 1 sec. For broad band ^{1}H-decoupled ^{13}C-NMR measurement, the operational condition was as follows: spectrum width, 18000 Hz; acquisition time, 0.99 sec; pulse width, 8.67 μsec; delay time, 2.00 sec; decoupler pulse width, 17.50 μsec; frequency 9900 Hz.

Results and Discussion

Evidently, NMR techniques alone will not be able to provide detailed information about the molar percentage of any specific fatty acid present in a mixture of triacylglycerols. This compositional information can only be provided by the chromatographic methods. NMR is also less sensitive than the traditional chromatographic approach. It is particularly demanding in ^{13}C-NMR measurement that substantially more material is needed for the acquisition of spectroscopic data in a limited time period. In case that sample availability is not a limiting factor as in this study, we could prove that ^{1}H-NMR was more effective in the rapid analysis of total n-3 PUFA contents in edible oils, including fish oils. In ^{1}H-NMR spectra of TG samples containing n-3 PUFA, an additional characteristic chemical shift at δ 0.97 (t, J = 7 Hz) would appear. It is the proton signals corresponding to the terminal, homoallylic methyl groups of n-3 PUFA. The chemical shift was slightly more downfield than other terminal methyl signals (δ 0.88) which commonly appeared in saturated fatty acids and non n-3 series of PUFA in TG. As illustrated in Fig. 1, the 300 MHz ^{1}H-NMR of concentrated fish oil MaxEPA 300 (commercialized capsules of fish oil containing enriched, 5,8,11,14,17-eicosapentaenoic acid, 20:5(n-3) and 4,7,10,13,16,19-docosahexaenoic acid, 22:6(n-3) as major n-3 PUFA) showed the characteristic signal at δ 0.97 and peak integration gave very good measurement of their gross contents (EPA and DHA were totally 34 mole % in this sample as measured by ^{1}H-NMR. This figure was very close to values obtained by gas chromatography (Ackman, 1987). ^{1}H-NMR spectrum of a commercial mixed vegetable oil was also recorded and is shown in Fig. 2. From GC and other physicochemical data, we conclud-

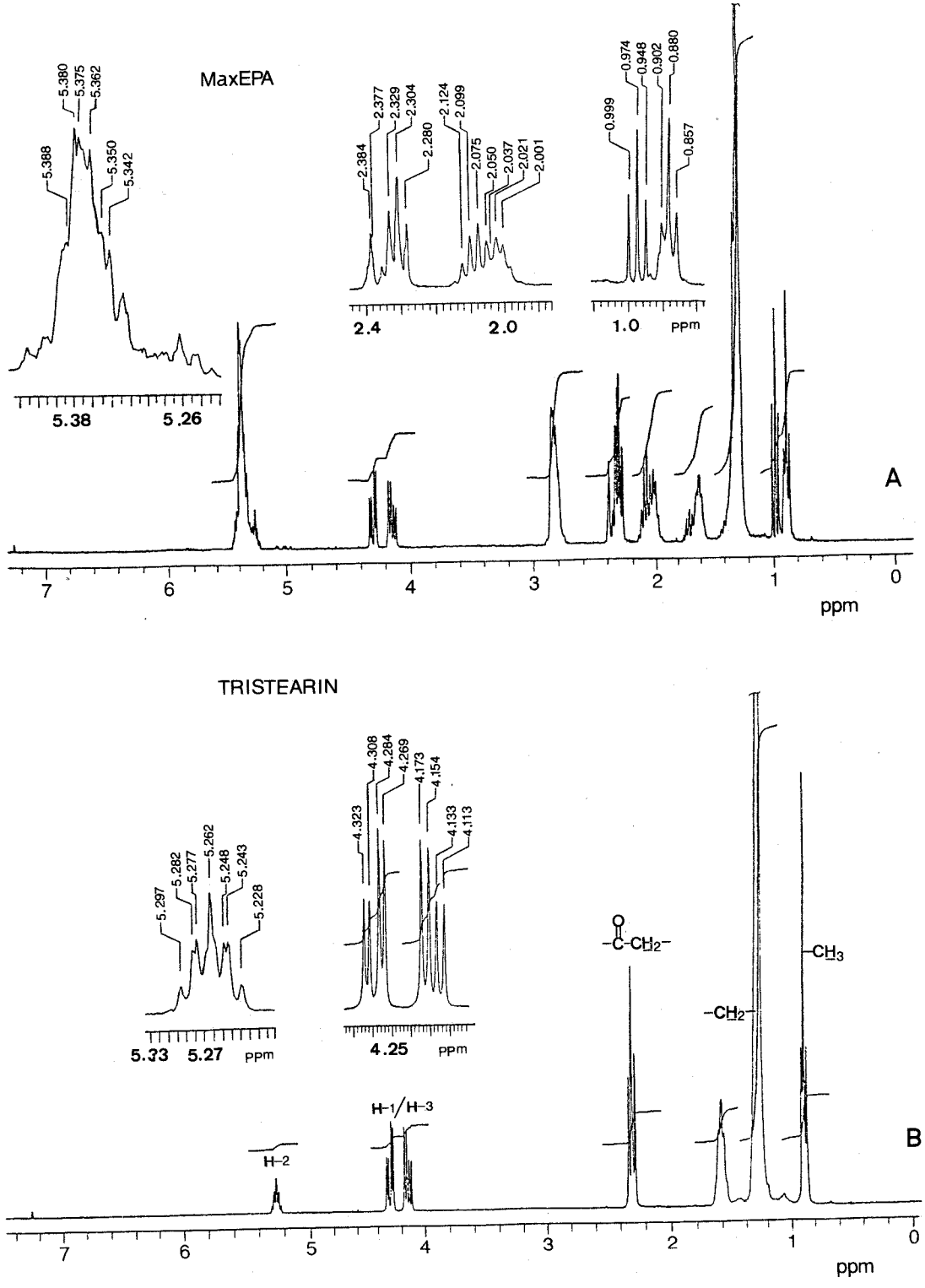

Fig. 1. The 300 MHz ^{1}H-NMR spectra of (A) fish oil (MaxEPA 300) and (B) tristearin standard (TS). Each sample (~80 mg) was dissolved in 0.5 ml $CDCl_3$ and spectra were taken in 5 mm NMR sample tube. In spectrum (A), the chemical shift at δ 0.97 (t) is due to the homoallylic methyl signals of n-3 PUFA in triacylglycerols.

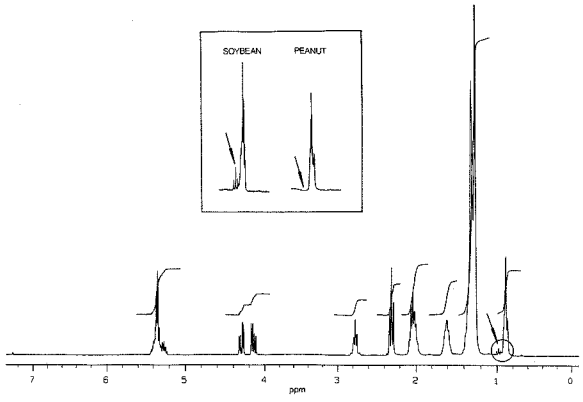

Fig. 2. The ^{1}H-NMR spectrum of a commerciallized vegetable oil (sold as processed peanut oil). ^{1}H-NMR data clearly demonstrate that it was not a pure peanut oil because of the appearance of an additional homoallylic methyl signal at δ 0.97 (circled) which was belonged to n-3 PUFA. This signal due to α-linolenic acid, a n-3 PUFA is not present in pure peanut oil to any significant level (< 1%). This mixed oil was confirmed to be ~1:1 mixture of peanut and soybean oils. The ^{1}H-NMR (upfield region only) of authentic soybean and peanut oils are also shown (upper insert) for comparison.

ed that it was a mixture of peanut and soybean oils. However, the ratio of these two vegetable oils was not certain because the molar percentages of major fatty acids in these two vegetable oils were not very different. It is known that peanut and soybean oils contain significantly different amount of α-linolenic acid (18:3; 9Z, 12Z, 15Z, n-3 PUFA), namely less than 1 molar % in peanut oil and about 7-8 molar % in soybean oil (Padley *et al.*, 1986). The difference in α-linolenic acid could readily serve as basis to determine the ratio of peanut and soybean oils in this mixed vegetable oil. Calculation of the peak areas of terminal methyl protons corresponding to n-3 and non-n-3 signals at δ 0.97 and δ 0.88, respectively, gave a ratio 1:1 of peanut to soybean oils (Fig. 2). The signals at δ 5.2-5.4 corresponded to total olefinic protons of unsaturated fatty acids in TG. This region was masked by one proton signal (δ 5.26, 1H, m) which was belonged to the H-2 chemical shift of glycerol in TG (Fig. 3). However, its contribution to peak area in the region of δ 5.2-5.4 could be accurately subtracted because the remaining 4 protons of glycerol, H-1 and H-3 which resonated at δ 4.1-4.3 as two sets of dd, J=~6Hz, gave good measure-

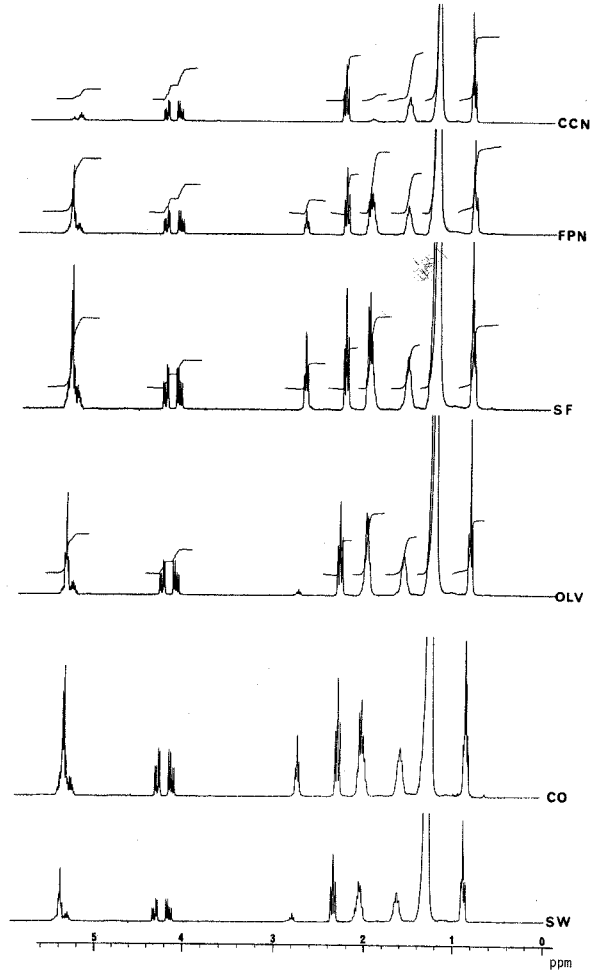

Fig. 3. The ^{1}H-NMR spectra of 6 vegetable oils and animal fats. All NMR data were recorded in identical instrumental conditions (Pulse width 7 μsec; NS 64 or 128; Spec. Width 4000 Hz). Sample identification: CCN: Coconut oil; FPN: Peanut oil from fresh peanuts (Tainan Selection No. 9); SF: Sunflower oil; OLV: Olive oil (Filippo Berio Co., Lucca, Italy); CO: Corn oil; SW: Processed lard (local supplier); BM: TG of *Bombyx mori* (purified by Si gel TLC); PNM: Mixture of peanut and soybean oils (local supplier); Note: Identical abbreviations for samples are used in the subsequent figures of this report.

ment of glycerol H-2 (area ratio was 1:4). We confirmed that the corrected peak area of olefinic region could become a good substitute for the iodine value of TG and could be used to measure the degree of unsatur-

ation in vegetable oils and animal fats. We have obtained a linear relationship between ^{1}H-NMR peak area and iodine values of test samples including coconut, processed lard, olive, sunflower and corn oils (Fig. 4). ^{1}H-NMR signals at δ 2.05 (m) were due to the allylic methylene protons of all unsaturated fatty acids, including monoenoic and polyunsaturated fatty acids. Naturally occurring unsaturated fatty acids in TG of plant and animal origins are predominantly in cis configuration and their double bonds are not conjugated. Since each mole of monoenoic acid or PUFA would give 4 allylic protons (as in $-\underline{CH_2}-CH=CH-\underline{CH_2}-$ and $-\underline{CH_2}-(CH=CH-CH_2)n-CH=CH-\underline{CH_2}-$), we could measure the total molar percentages of unsaturated fatty acids in TG by comparing the peak areas of allylic methylene (δ 2.05) and terminal methyl (δ 0.8-1.0). For TG containing virtually no saturated fatty acids this area ratio should be 12:9 and this figure would become zero in TG containing only saturated fatty acids. Area ratio below 4:3 was a good indication of the presence of saturated fatty acids. We have confirmed this point by using tristearin, triolein, and other TG standards and constructed a calibration curve to measure the molar ratio of saturated and unsaturated fatty acids. The peak areas in these two regions could be accurately measured in ^{1}H-NMR without ambiguity because that there were no overlapping signals in these regions. We have succeeded by using ^{1}H-NMR to measure the total molar percentages of saturated and unsaturated fatty acids for 4 vegetable oils and 2 animal fats. A summary of 4 set of ^{1}H-NMR data and an additional one with reported GC data are listed in Table 1. Results obtained by these two independent methods were amazingly close and were also very compatible with those figures reported in the literature (Padley *et al.*, 1986). Only polyunsaturated fatty acids would give signals at δ 2.78 (m) which corresponded to the chemical shifts of the double-allylic methylene protons. It is known that naturally occurring PUFA in TG contain multiple olefinic bonds which are orderly separated from the adjacent ones by one double-allylic methylene unit. This trend is valid for both n-3 and n-6 series PUFA in TG and phospholipids. Signal intensity of this region provided good estimation of total PUFA in TG. For TG containing linoleic acid as the only n-6 PUFA, the peak intensity at δ 2.78 could give its molar percentage and this situation is valid in many vegetable oils. However, ^{1}H-NMR alone could not give precise molar ratios of each distinct PUFA since the double-allylic methylene signals associated with PUFA containing 2, 3, 4, or even more double bonds were overlapped in this region (Fig. 3). It is worth mentioning that combined information of chemical shifts at δ 0.97 and δ 2.78 could be applied to clearly distinguish the presence of n-3 or n-6 series of PUFA in TG. Since GC or HPLC analyses of positionally isomeric PUFA such as α-linolenic and γ-linolenic acids is also very difficult, ^{1}H-NMR could provide this structural information more effectively. Other supportive information from ^{1}H-NMR was given by signals at δ 2.30 which were due to the methylene protons adjacent to carboxyl carbons of three acyl moieties in TG ($-O-CO-\underline{CH_2}-$). The theoretical area ratio of proton signals at $\delta\sim$0.90,

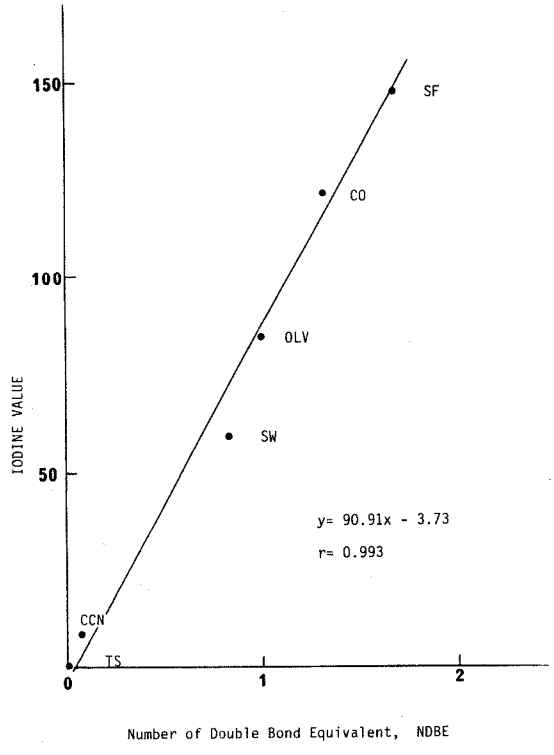

Fig. 4. The calibration curve of iodine value versus number of double bond equivallent (NDBE). NDBE was obtained from ^{1}H-NMR by measuring the corrected peak areas of olefinic regions (δ 5.2-5.4, A_1) and normalized by the peak areas corresponding to terminal methyl signals (δ 0.8-1.0, A_2). Calculation was based on the following equation: NDBE = $(A_1/2) \div (A_2/3)$. Iodine values of samples were obtained by the Wijs method.

Table 1. *NMR measurement of saturated, monoenoic and polyunsaturated fatty acids of triacylglycerols in 4 plant seed oils*

Fatty acid contents in mole % were obtained from peak intensities of relevant regions of chemical shifts in ^{1}H-NMR (300 MHz). Data are expressed as molar percentages (molar %). Values in the parentheses were obtained from chromatographic method.

Sample	Saturated	Monoenoic	Polyunsaturated		
			total	n-3 PUFA	n-6 PUFA
TS*	100	0	0	0	0
CO*	16(16.3)	31(30.5)	50(53)	<1(1.5)	50(51)
OLV*	15	80	5	<1	5
SF*	16	17	67	<1	67
CCN*	93	5	2	—	2

*Abbreviations: TS, tristearin; CO, corn oil; OLV, olive oil; SF, sunflower oil; CCN, coconut oil.

δ~2.30 and δ~4.2 regions was 9:6:4 and this figure could be used to calibrate the instrumental error. It could also serve as normalization factors for precise measurement of other peak areas. After proper correction and normalization, precise measurement was achievable.

Recent reports have shown that high resolution ^{13}C-NMR can distinguish the positional distribution of Δ^9 and $\Delta^{9,12}$ unsaturated fatty acids in TG mixtures (Ng, 1984). It relies on the structural resolution by NMR to give distinct chemical shifts of C-9 and C-10 carbons in oleic acid and C-9, C-10, C-12 and C-13 carbons in linoleic acid which are attached accordingly to the C-1/C-3 or C-2 positions of glycerol. For instance, C-10 and C-12 signals are about 2 ppm more upfield than the corresponding C-13 and C-9 signals in α -linoleic acid. For ^{13}C chemical shift, oleic acid attaching to C-2 of glycerol would have C-9 signal slightly more upfield than that oleic acid attaching to C-1 and C-3 of glycerol. The situation for C-10 chemical shift of oleic acid reported to be in opposite trend. As to C-9 and C-12 chemical shifts, linoleic acid attached to C-2 of glycerol shows ^{13}C signals slightly more upfield than those attached to C-1/C-3 of glycerol. We extended these observations but have also found that ^{13}C-NMR technique was equally time-consuming as the reported lipase enzyme digestion method. For ^{13}C-NMR measurement to be quantitative, the relaxation time T_1 of relevant carbons in TG should be carefully determined. These olefinic carbons in TG normally had T_1 in the range of 1.4-2.8 sec (Ng and Ng, 1983). Application of 5 T_1 for delay time in 90° pulse width would prolong the acquisition of data for ^{13}C-NMR to provide equivalent structural information comparing with those combined stereospecific lipase digestion-chromatography method. Furthermore, ^{13}C-NMR was less likely to resolve the ^{13}C chemical shifts of olefinic carbons of fatty acids attaching to C-1 or C-3 position of glycerol in TG mixtures. It is limited by the nearly pro-chiral character of these two substituents even though different olefinic fatty acids are associated with these two positions. However, ^{13}C-NMR signal profiles was very characteristic and could serve as fingerprint for identification. The upfield region (δ 13-40) of 7 vegetable oils and animal fats are illustrated in Fig. 5. The ^{13}C-NMR were also very characteristic in the olefinic signal region, namely δ 128 to δ 131 (Chapman and Goni, 1986). We have also measured the downfield region of 8 kinds of vegetable oils and animal fats to support this point (Fig. 6). The detection of vegetable oil adulteration by ^{13}C-NMR also deserves further attention (Hwang *et al.*, 1982). To achieve the same goal in even shorter acquisition time, one-dimensional DEPT experiment (distortionless enhancement by polarization transfer) was an even better pulse program than the traditional broad-band ^{1}H-decoupled ^{13}C-NMR spectra. A comparison of the spectra of a fish oil obtained by these two ^{13}C-NMR techniques is shown in Fig. 7 to illustrate this advantage.

Judging by the increasing availability of modern instrumentation in scientific institutions, high resolution NMR could be equally or even more feasible than the chromatographic approach to provide rapidly the structural and compositional characteristics of the

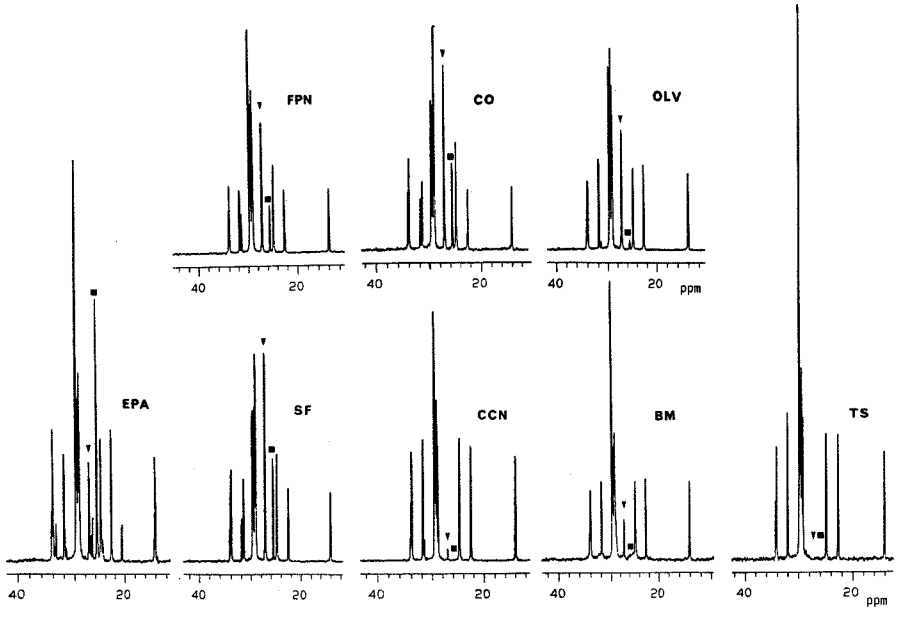

Fig. 5. The upfield region (δ 13-40) of broad-band decoupled ^{13}C-NMR spectra (75.143 MHz) of tristearin and 7 edible oils. The ^{13}C signal profile and relative peak intensity were very characteristic and could serve as fingerprint for sample identification. Peaks marked with "■" and "▼" were ^{13}C signals due to allylic methylene. Their relative intensities were distinct in each sample.

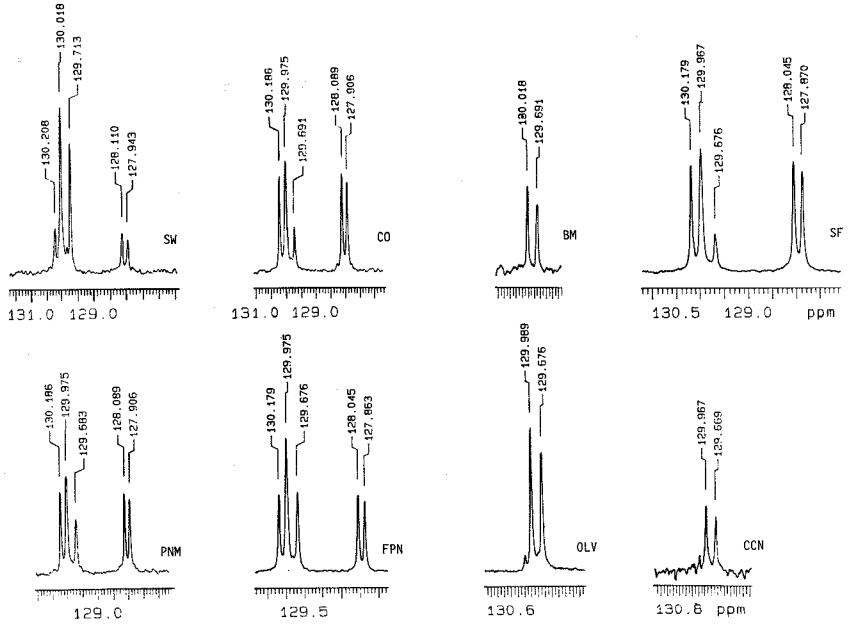

Fig. 6. The chemical shifts of olefinic carbons of fatty acids in 8 edible oils. Samples containing predominantly monoenoic acid gave two broad peaks at ~δ 129.67 and ~129.97. The appearance of additional signals at slightly upfield regions (~δ 127.9 and ~δ 128.1) indicated the presence of polyunsaturated fatty acid. The 8 spectra were expanded in different magnitude and chemical shift was given to each ^{13}C peak to demonstrate this point. In order to give signals of comparable intensities and to keep high signal/noise ratio (S/N $\geqq$ 10), the numbers of scan were set between 256-1024 for different samples.

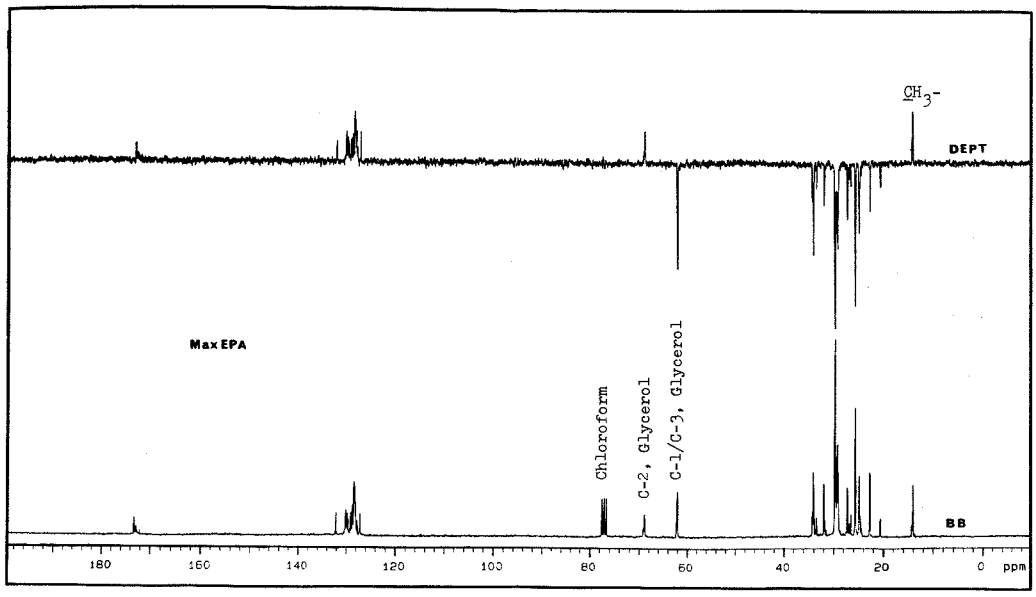

Fig. 7. The DEPT (135°, upper spectrum) and broad-band decoupled ^{13}C-NMR (lower spectrum) of a concentrated fish oil (MaxEPA 300). Equally informative spectrum could be obtained by DEPT experiment in much shorter time. Instrumental conditions: DEPT (135°): Spec. width 18000 Hz; Acq. time 0.99 sec.; Pulse width 13.30 μsec.; Delay time 2.0 sec.; Transients 672. Broad -band decoupled ^{13}C-NMR spectrum: Spec. width 18000 Hz; Acq. time 0.99 sec.; Pulse width 8.67 μsec.; Delay time 2.0 sec.; Transients 1024.

seed oils and animal fats. It is also worth particular mentioning that NMR measurement is non-destructive and completely no sample pretreatment is involved. For measurement of TG in plant seeds and animal fats containing both n-3 and n-6 PUFA and situations that the gross molar percentage of n-3 PUFA is the major concern, NMR spectroscopy is definitively faster and equally informative to the chromatographic methods.

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利用高解相核磁共振法探討三醯甘油的脂酸組成

蕭泰祐[1] 蕭明熙[2]

[1]國立嘉義農專食品加工科 [2]台北榮民總醫院醫學研究部

本報告利用高解相核磁共振光譜法探討植物油所含三醯甘油的構造與組成，並與氣相層析法所獲得資料相比較，以評估兩種方法對獲取結構資料的層次和效率。氫核磁共振法顯然對獲知總飽和與不飽和脂酸比例，總 n-3 型多不飽和脂酸莫耳百分比等各方面較層析法更迅速且有效。不同來源油的碳 -13 核磁共振則在化學位移 δ13-40 及 δ128-131 均有其特異性，可當成指紋區作辨識之用。