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Characterization of Cod Liver Oil by Spectroscopic Techniques. New Approaches for the Determination of Compositional Parameters, Acyl Groups, and Cholesterol from ^{1}H Nuclear Magnetic Resonance and Fourier Transform Infrared Spectral Data

María D. Guillén,* Izaskun Carton, Encarnación Goicoechea, and Patricia S. Uriarte

Food Technology, Faculty of Pharmacy, University of the Basque Country (UPV-EHU), Paseo de la Universidad No. 7, 01006 Vitoria-Gasteiz, Spain

Six samples of cod liver oil were studied using Fourier transform infrared (FTIR) spectroscopy and ^{1}H nuclear magnetic resonance (^{1}H NMR). These techniques provide information simply and rapidly about the global features of the cod liver oil main components, showing their potential as routine techniques for evaluating certain parameters of the quality of the cod liver oil. FTIR spectroscopy provides information about the molar percentage of polyunsaturated acyl groups in the sample and also about the ratio between unsaturated and saturated structures. ^{1}H NMR provides information about the proportions or concentrations of certain acyl groups and also of some minor compounds such as cholesterol. Both techniques are simple and fast. New approaches are presented to evaluate the molar proportions or concentrations of some acyl groups such as the molar percentages of ω-3, docosahexaenoic, and eicosapentaenoic acyl groups; furthermore, some novel approaches for evaluating the molar percentages of unsaturated and saturated acyl groups are also given. Results obtained from both spectroscopic techniques are in total agreement.

KEYWORDS: Cod liver oil; cholesterol; docosahexaenoic (DHA) acyl groups; eicosapentaenoic (EPA) acyl groups; Fourier transform infrared spectroscopy (FTIR); ^{1}H nuclear magnetic resonance (^{1}H NMR); saturated acyl groups; unsaturated acyl groups; cholesterol

INTRODUCTION

Cod liver oil has been widely used for centuries to treat and protect children from rickets, and currently, it is recommended as a nutritional supplement. This oil contains important proportions of ω-3 acyl groups, mainly docosahexaenoic (DHA) and eicosapentaenoic (EPA) acyl groups, and, in addition, significant amounts of A and D vitamins. Its intake has been associated with beneficial effects on heart, bone, brain, skin, hair, and nails, and it has been recommended against diseases such as cardiovascular diseases, autoimmune disorders, mental illnesses, and cancer, among others (*1*−*5*).

However, the excessive consumption of cod liver oil raises problems associated with A and D vitamin overdose and with the intake of toxic compounds that it might contain. Among these, latter polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), or heavy metals, such as mercury, cadmium, and lead, have been cited (*6*−*8*).

Cod liver oil quality depends both on the quality of the raw material from which it comes and on the extraction process used, as well as on its subsequent purification and storage conditions. Oil quality is defined by its composition and by some parameters, such as ω-3 acyl group proportions and the concentration of A and D vitamins. Its safety to consume is usually measured by the absence or a low level of contaminants, such as heavy metals and PCBs. Furthermore, the oxidation level of the oil relates to both its quality and its safety.

In this context, the study of six samples of cod liver oil was carried out by nondestructive techniques such as Fourier transform infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (^{1}H NMR) to test their ability to provide information about the oil composition, which may be useful to evaluate its quality. The interest of this study is reinforced by the current trend to supplement the diet directly with cod liver oil or indirectly through food enriched with this oil.

MATERIALS AND METHODS

Samples. The study was carried out on six commercial cod liver oil samples, namely, CLO1, CLO2, CLO3, CLO4, CLO5, and CLO6, acquired in European supermarkets; these oils were intended for human consumption and comply with the European regulations (*9*).

* To whom correspondence should be addressed. Tel: 34-945-013081. Fax: 34-945-013014. E-mail: mariadolores.guillen@ehu.es.

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Table 1. Main Bands of the FTIR Spectra of Cod Liver Oil, Together with the Tentatively Assigned Functional Group, the Mode of Vibration, and the Approximate Intensity (*I*)[a]

band	F (cm^{-1}) CLO1	functional group	mode of vibration	I[b]
a	3470.75 ± 0.02	C═O (ester)	overtone	w
c	3011.22 ± 0.02	═C−H (*cis*)	stretching	m
e	2925.28 ± 0.02	−C−H (CH_2)	stretching (asym)	vst
f	2854.20 ± 0.01	−C−H (CH_2)	stretching (sym)	vst
i	1746.15 ± 003	−C═O (ester)	stretching	vst
k	1653.72 ± 0.31	−C═C− (*cis*)	stretching	vw
m	1464.06 ± 0.06	−C−H (CH_2, CH_3)	bending (scissoring)	m
n	1418.68 ± 0.11	═C−H (*cis*)	bending (rocking)	w
p	1377.20 ± 0.07	−C−H (CH_3)	bending (sym)	m
r	1233.56 ± 0.11	−C−O, $-CH_2-$	stretching, bending	m
s	1161.49 ± 0.01	−C−O, $-CH_2$	stretching, bending	st
t	1118.43 ± 0.02	−C−O	stretching	m
u	1098.87 ± 0.05	−C−O	stretching	m
x	968.36 ± 0.05	−HC═CH− (*trans*)	bending out of plane	w
y	916.19 ± 0.09		bending out of plane	vw
z	721.86 ± 0.08	$-(CH_2)_n-$, −HC═CH− (*cis*)	rocking, bending out	m

[a] The names of the bands agree with those given in **Figure 1**. Frequency values (F) in cm^{-1} correspond to the CLO1 sample. [b] vst, very strong; st, strong; m, medium; w, weak; and vw, very weak.

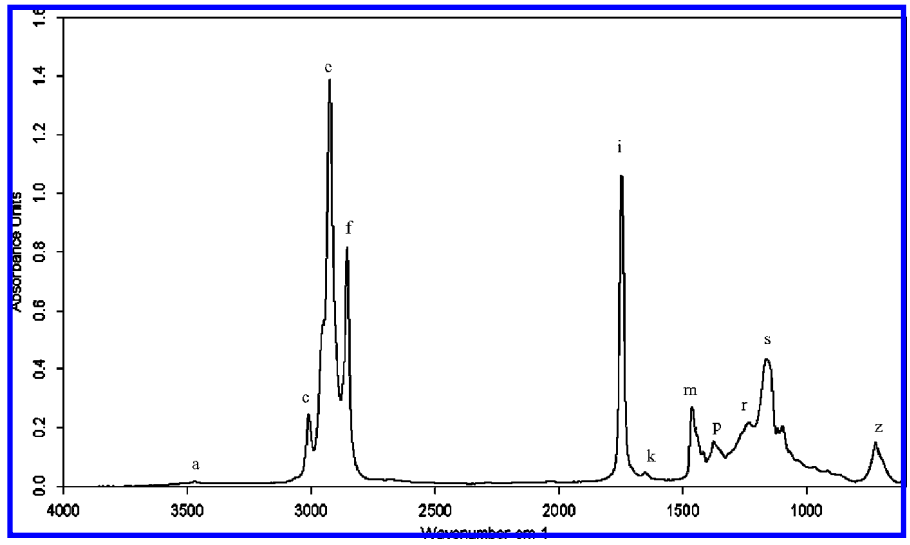

Figure 1. Fourier transform infrared spectrum of the CLO1 sample.

Table 2. Some Parameters of the Several Cod Liver Oil Samples Obtained from FTIR Data

parameter	CLO5	CLO3	CLO6	CLO4	CLO2	CLO1
$F_{band\ c}$ (cm^{-1})	3011.94 ± 0.02	3011.75 ± 0.01	3011.63 ± 0.02	3011.60 ± 0.01	3011.41 ± 0.02	3011.22 ± 0.02
A_i/A_c	2.68 ± 0.04	3.05 ± 0.02	3.16 ± 0.04	3.22 ± 0.01	3.01 ± 0.01	3.35 ± 0.02
$F_{band\ e}$ (cm^{-1})	2925.46 ± 0.03	2925.38 ± 0.01	2925.36 ± 0.03	2925.36 ± 0.00	2925.41 ± 0.01	2925.28 ± 0.02
$F_{band\ f}$ (cm^{-1})	2854.27 ± 0.02	2854.26 ± 0.01	2854.24 ± 0.00	2854.24 ± 0.01	2854.26 ± 0.02	2854.20 ± 0.01

Acquisition of the FTIR Spectra. The infrared spectra were recorded on a FTIR Bruker Vector 33 (Bruker Optic Gmbh) interfaced to a personal computer operating under Opus NT software (version 2.0). As in previous edible oil studies (*10*−*16*), a film of a small amount of sample (approximately 2 μL) was deposited between two disks of KBr avoiding the presence of air, and the screws of the sample holder were tightened up to the limit, so that the path length was constant for all of the samples. All spectra were recorded from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. For each spectrum, 32 interferograms were coadded before Fourier transformation and zero-filled to give a data point spacing of approximately 1.9 cm^{-1}. The measurement accuracy in the frequency data was better than 0.01 cm^{-1} due to the laser He−Ne internal reference of the instrument. The frequency value for each band and the absorbance of some bands were obtained automatically by the equipment software. The FTIR spectrum of each oil was obtained at least three times, in three different experiments, and the derived data are given in **Tables 1** and **2**, as average values together with their standard deviations. It should be mentioned that as the cod liver oil samples are totally homogeneous, and as the registration of the spectrum does not require any modification or manipulation of the sample, the spectra obtained and the derived data are very similar in the several experiments, being that the standard deviations obtained are very small.

Acquisition of the ^{1}H NMR Spectra. The ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. Each oil sample (200 μL) was mixed with 400 μL of deuterated chloroform, which contains 0.2% of nondeuterated chloroform and a small proportion of tetramethylsilane (TMS) used as an internal reference (both chloroform and TMS were acquired from Cortec, Paris, France). This mixture was introduced into a 5 mm diameter tube. As in previous oil studies (*17*−*20*), the acquisition parameters were as follows: spectral width, 5000 Hz; relaxation delay, 3 s; number of scans, 64; acquisition time, 3.744 s; pulse width, 90°; and total acquisition time, 12 min and 54 s. The experiment was carried out at 25 °C. At least three ^{1}H NMR spectra of each cod liver oil were obtained from different tubes, and the derived data are given in **Table 4** as average

Table 3. Assignment of the Signals of the ^{1}H NMR Spectra of Cod Liver Oil[a]

signal	chemical shift (ppm)	functional group	intensity[b]
1	0.66−0.69	cholesterol−C**H**$_3$ (position 18)	s
2	0.84−0.94	−C**H**$_3$ (saturated, monounsaturated ω-9 and ω-7, diunsaturated ω-6 acyl groups)	m
3	0.94−1.00	−C**H**$_3$ (polyunsaturated ω-3 acyl group)	v
4	1.19−1.43	−(C**H**$_2$)$_n$− (acyl group)	l
5	1.54−1.67	−OCO−CH$_2$−C**H**$_2$− (acyl group except for DHA and EPA acyl groups)	m
6	1.67−1.74	−OCO−CH$_2$−C**H**$_2$− (EPA acyl group)	s
7	1.92−2.15	−C**H**$_2$−CH=CH− (acyl group except for −CH$_2$− of DHA acyl group in β position in relation to carbonyl group)	m
8	2.25−2.36	−OCO−C**H**$_2$− (acyl group except for DHA acyl group)	m
9	2.36−2.42	−OCO−C**H**$_2$−C**H**$_2$− (DHA acyl group)	m
10	2.72−2.90	=HC−C**H**$_2$−CH= (acyl group)	m
11	4.10−4.34	−C**H**$_2$OCOR (glyceryl group)	m
12	5.23−5.30	−C**H**OCOR (glyceryl group)	s
13	5.31−5.47	−C**H**=C**H**− (acyl group)	m

[a] The signal number agrees with that in **Figure 2**. [b] Key: l, large; m, medium; s, small; and v, variable.

Table 4. Some Compositional Parameters of the Several Cod Liver Oil Samples Obtained from ^{1}H NMR Data[a]

parameter	equation used	CLO5	CLO3	CLO6	CLO4	CLO2	CLO1
C/T (mmol/mol)	1	18.1 ± 0.0	19.7 ± 0.0	24.9 ± 1.3	24.3 ± 0.9	10.7 ± 0.0	14.7 ± 0.0
C (mg/mL of oil)		8.5 ± 0.0	12.7 ± 1.0	12.4 ± 0.7	12.8 ± 0.9	4.9 ± 0.1	7.4 ± 0.2
ω-3 (%)	2	28.7 ± 0.2	27.4 ± 0.5	26.4 ± 0.4	25.9 ± 0.3	24.5 ± 0.2	22.6 ± 0.3
SMDU (%)	3	71.3 ± 0.2	72.6 ± 0.5	73.6 ± 0.4	74.1 ± 0.3	75.5 ± 0.2	77.4 ± 0.3
DHA (%)	6	9.8 ± 0.2	11.3 ± 0.1	10.0 ± 0.4	10.0 ± 0.0	10.6 ± 0.1	8.9 ± 0.0
DHA (mg/mL of oil)		115.3 ± 0.8	166.8 ± 3.1	128.8 ± 6.7	134.8 ± 7.9	123.0 ± 5.6	106.7 ± 3.4
EPA (%)	8	14.7 ± 0.2	11.3 ± 0.0	11.3 ± 0.9	11.3 ± 0.2	9.4 ± 0.1	9.7 ± 0.1
EPA (mg/mL of oil)		170.6 ± 0.7	158.4 ± 6.1	139.3 ± 3.8	144.9 ± 7.8	104.1 ± 3.9	109.6 ± 5.3
U (%)	10	75.4 ± 0.4	78.9 ± 0.5	79.9 ± 1.2	79.2 ± 0.2	82.4 ± 0.4	78.1 ± 0.5
S (%)	10	24.6 ± 0.4	21.0 ± 0.5	20.1 ± 1.2	20.8 ± 0.2	17.6 ± 0.4	21.9 ± 0.5
MDU (%)	3,10	46.7 ± 0.4	51.5 ± 0.5	53.5 ± 1.2	53.3 ± 0.3	59.7 ± 0.4	55.5 ± 0.5
Rs/o	11	7.27 ± 0.01	7.88 ± 0.03	8.07 ± 0.00	8.16 ± 0.05	7.92 ± 0.01	8.47 ± 0.02

[a] C, cholesterol; T, triglyceride; ω-3, ω-3 acyl groups; SMDU, saturated, mono- and diunsaturated acyl groups; U, unsaturated acyl groups; OPUω-3, other polyunsaturated ω-3 acyl groups; MDU, mono- and diunsaturated acyl groups; S, saturated acyl groups; and Rs/o, saturated/olefinic protons ratio.

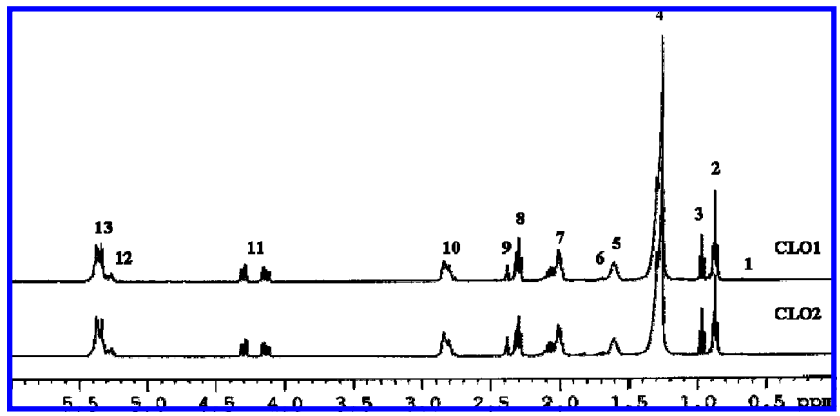

Figure 2. Region between 0 and 6 ppm of the ^{1}H NMR spectra of CLO1 and CLO2 samples.

values together with their standard deviations. The figures of ^{1}H NMR spectra were plotted at a fixed value of absolute intensity to be valid for comparative purposes. The nondeuterated chloroform, contained in the 400 μL, gives the corresponding signal at 7.29 ppm; this compound can be used as standard for quantitative purposes. It should be noted that as the cod liver oil samples are totally homogeneous and because the registration of the spectrum does not require any modification or manipulation of the sample, the spectra obtained and the derived data are very similar in the several experiments, being that the standard deviations obtained are very small.

Statistical Analysis. The results given in **Tables 2** and **4** are based at least on triple determinations and are presented as average values together with their standard deviations. In addition, the fit to linear equations of some sets of data, coming from both spectroscopic techniques, was carried out using the SPSS statistical software package.

RESULTS AND DISCUSSION

Characterization by FTIR. The FTIR spectrum provides information about the nature and proportions of the sample functional groups if they are in significant concentrations. The spectrum of the cod liver oil is similar to that of other edible fats and oils, because all of these mainly consist of triglycerides; however, it shows some differences either in the exact frequency and absorbance of the bands or in the presence or absence of some bands due to differences in length and unsaturation degree and proportions of the acyl groups of their triglycerides and in the oxidation degree of the oil.

Figure 1 shows, as an example, the FTIR spectrum of the cod liver oil sample CLO1. The infrared spectra of the other

five cod liver oil samples are very similar to this, with small differences in the absorbance and in the frequency of some bands. The assignment of the bands to the specific functional group vibration modes can be made by comparison with previous studies of edible fats and oils (*10*) and of salmon lipids (*16*). **Table 1** shows the main common features of the spectra of several cod oil liver samples. These very briefly are as follows: a weak band a, near 3470 cm^{-1}, associated in nonoxidized oil with the overtone of the glyceride ester carbonyl absorption; a shoulder, near 3025 cm^{-1}, due to the stretching vibration of the *trans*-olefinic CH double bonds; a band of medium intensity c due to the stretching vibration of the *cis*-olefinic CH double bonds; two intense bands e and f, due to the methylene asymmetrical and symmetrical stretching vibrations, with their maximum absorption near 2925 cm^{-1} and near 2854 cm^{-1}, respectively; a very intense stretching vibration band i at approximately at 1746 cm^{-1} due basically to, in nonoxidized oils, the C═O group of triglycerides; a small band k, near 1654 cm^{-1}, associated with disubstituted *cis*-C═C of unsaturated acyl groups; the band m near 1463 cm^{-1} (near 1465 cm^{-1} in vegetable oils) due to the bending vibrations of the CH_2 and CH_3 aliphatic groups; a weak band n near 1419 cm^{-1} (near 1418 cm^{-1} in vegetable oils) tentatively assigned to rocking vibrations of CH bonds of *cis*-disubstituted olefins; a band p near 1377 cm^{-1} due to symmetrical bending vibrations of CH_3 groups; two bands r and s, near 1233 (1238−9 cm^{-1} in vegetable oils) and 1160 cm^{-1}, respectively (1163 cm^{-1} in vegetable oils and near 1170 cm^{-1} in lard), both associated with the stretching vibration of the C−O ester groups and with the bending vibration of the CH_2 group; another two bands t and u at approximately 1118 cm^{-1} (near 1119−1120 cm^{-1} in vegetable oils and near 1113 cm^{-1} in lard) and at approximately 1098 cm^{-1} associated with the stretching vibration of the C−O group in esters; two weak bands x and y near 968 and 916 cm^{-1}, the first being assigned to the bending vibration out of plane of isolated *trans*-olefins and the second being difficult to assign; and finally, a band z at approximately 721 cm^{-1} (723 cm^{-1} in vegetable oils and 720 cm^{-1} in lard) of medium intensity, due to overlapping of the methylene rocking vibration of straight chain paraffins of the acyl groups with the out-of-plane vibration of *cis*-disubstituted olefins.

Previous FTIR studies of edible oils have proved the existence of relationships between frequency and absorbance values of certain bands of the oil FTIR spectrum and the oil composition as well as between some of these spectroscopic parameters and the oil oxidation level (*10*−*15*). In spite of the above-mentioned similarity between the FTIR spectra of several cod liver oil samples, some differences can also be found.

One of the most significant differences is in the frequency of the band c. As has been said above, this is due to the stretching vibration of the *cis*-olefinic CH double bonds. **Table 2** gives the values of the frequency of this band for the six cod liver oil samples, which are fairly higher than that of vegetable oils (near 3008 cm^{-1} in sunflower oil and near 3005 cm^{-1} in olive oil) (*10*). It has been proved in previous studies on edible oil that the frequency value of this band c is related, in a direct way, to the unsaturation of the sample expressed as a percentage of polyunsaturated acyl groups. For this reason, it can be inferred from the frequency value of this band that the percentage of polyunsaturated acyl groups in the studied cod liver oil samples decreases in the order CLO5 (3011.94 ± 0.02 cm^{-1}) > CLO3 (3011.75 ± 0.01 cm^{-1}) > CLO6 (3011.62 ± 0.02 cm^{-1}) > CLO4 (3011.60 ± 0.01 cm^{-1}) > CLO2 (3011.41 ± 0.02 cm^{-1}) > CLO1 (3011.22 ± 0.02 cm^{-1}). In addition, taking into account that in this kind of oil, polyunsaturated acyl groups are composed mainly of ω-3 acyl groups, the frequency value of this band c permits one to order the cod oil liver samples according to their percentage or content in ω-3 acyl groups.

Likewise, it has been proved in previous studies that as the oxidation of the edible oils evolves, the frequency of band c decreases, due to the degradation of *cis*-olefinic CH double bonds (*13*); for this reason, the smaller value of the frequency of band c in the spectrum of these oil samples could also be attributed to a higher oxidation level or to a lower polyunsaturation degree or to both factors.

From these results, it is inferred that the quality order of the oils studied is CLO5 > CLO3 > CLO6 > CLO4 > CLO2 > CLO1. This conclusion is reached because, as mentioned above, the proportion of polyunsaturated acyl groups, mainly ω-3 acyl groups, of fish oil is considered to be a quality parameter and the opposite is true for oxidation level. For these reasons, the fact that the frequency value of band c provides information, in a direct way, about these important characteristics of the oil should be considered of great interest because the sample preparation and the acquisition of the FTIR spectrum take only a few minutes and do not require either solvents, reactive agents, or sample modification.

Furthermore, some authors have proved that the ratio of the absorbance (A) of the bands f (or e) and c can also be taken as a measure of the total saturation degree of edible oils because it represents the ratio between the number or concentration of methylene groups (saturation) and the number or concentration of the *cis* double bonds (unsaturation) in the sample multiplied by a constant attending to the Lambert−Beer law (*11, 21, 22*); likewise, the inverse of this ratio can be taken as a measure of the total unsaturation degree. In **Table 2**, the ratios A_f/A_c for the six cod liver oil samples studied are given. It can be observed that the order in total unsaturation degree, expressed in this way, is CLO5 > CLO2 > CLO3 > CLO6 > CLO4 > CLO1. From these results, it can be inferred that, in spite of the CLO2 sample having a lower percentage of polyunsatured acyl groups than samples CLO3, CLO6, and CLO4, its proportion of total unsaturated *cis* double bonds in relation to that of total methylene groups is of a similar order to that of sample CLO3 and higher than those of the CLO6 and CLO4 samples. This may be possible because all *cis* double bonds in the sample contribute to the absorbance of band c. They can be in monounsaturated, diunsaturated, or polyunsaturated acyl groups, among these latter acyl groups with six or less number of *cis* double bonds being possible.

Moreover, it is worth mentioning that it has also been proved (*14*) that the value of the ratio A_f (or A_e)/A_c in an oil is greater as its oxidation level is higher due to the loss of *cis* double bonds; for this reason, the value of this parameter could also be due to either an incipient oxidation process or the unsaturation degree of the sample or to both.

In addition to the above, it has also been shown in previous studies (*10*) that inverse relationships exist between the frequency value of certain bands, such as those due to the methylene asymmetrical and symmetrical stretching vibrations (bands e and f, near 2925 cm^{-1} and near 2854 cm^{-1}, respectively) and the saturation degree of the oil sample; in agreement with this, as **Table 2** shows, the inverse of the frequency values of bands e or f order the oil samples like the ratio A_e (or A_f)/A_c does.

In short, the observation and analysis of the Fourier transform infrared spectral data of the cod liver oil samples permit one to order these in function of their percentages of polyunsaturated

acyl groups, that is to say, in function of their content in ω-3 acyl groups, based exclusively on the value of the frequency of band c; in addition, the ratio between the absorbance of certain bands [A_e (or A_f)/A_c] or the inverse of the frequency values of bands e or f permit one to order the oil samples in function of their total unsaturation (or saturation) degree. Both parameters, the percentage of ω-3 acyl groups and the total unsaturation degree of the cod liver oil sample, are of great interest in the evaluation of its quality. These results are reached in a few minutes, without using solvents or reactives, and in a very fast and simple way, because the frequency and absorbance values of the bands are given automatically by the equipment software. It should be added that, to the best of our knowledge, this is the first time that the usefulness of FTIR in evaluating some important compositional aspects of cod liver oil has been shown.

Study by ^{1}H NMR Spectroscopy. The ^{1}H NMR spectra of the several cod liver oil samples show the same signals with differences in their intensities. **Figure 2** shows, as an example, the ^{1}H NMR spectra of CLO1 and COL2 oil samples. As can be observed, these spectra have 13 main signals, due to protons of the cod liver oil main components. It is well-known that cod liver oil is composed mainly of triglycerides, which have saturated, monounsaturated, diunsaturated, and polyunsaturated (EPA, DHA, and other ω-3 acyl groups) acyl groups (*23*) and also contain cholesterol. The assignments of these signals are given in **Table 3**.

Signal 1, between 0.66 and 0.69 ppm, is a singlet due to the methylic protons on the C_{18} carbon atom of the cholesterol. Signal 2, between 0.84 and 0.94 ppm, is due to the overlapping of the triplets of methylic protons of the saturated, ω-7 and/or ω-9 monounsaturated acyl groups, and that of the ω-6 diunsaturated acyl groups. Signal 3, between 0.94 and 1.00 ppm, is due to the triplet of methylic protons of the ω-3 polyunsaturated acyl groups. Signal 4, between 1.19 and 1.43 ppm, is due to methylenic protons either in position β, or further in relation to double bonds, or in position γ, or further in relation to the carbonyl group in the different acyl groups; obviously, DHA and EPA acyl groups make no contribution to this signal. Signal 5, between 1.54 and 1.67 ppm is due to methylenic protons in the β-position in relation to the carbonyl group, except those of EPA and DHA acyl groups. Signal 6, between 1.67 and 1.74 ppm, is due to methylenic protons in the β-position in relation to the carbonyl group of EPA acyl groups; this latter signal is typical of fish lipids and is absent in the ^{1}H NMR spectra of vegetable oils. Signal 7, between 1.92 and 2.15 ppm, is due to the overlapping of the various signals of allylic protons, that is to say, of α-methylenic protons in relation to a single double bond in the different acyl groups, except those of DHA acyl group being also in β-position in relation to the carbonyl group. Signal 8, between 2.25 and 2.36 ppm, is due to methylenic protons in the α-position in relation to the carbonyl group, except those of DHA acyl group (*24*). Signal 9, between 2.36 and 2.42 ppm, is due to methylenic protons in α- and β-positions in relation to the carbonyl group of DHA acyl group; this signal is typical of fish lipids and is absent in the ^{1}H NMR spectra of vegetable oils. Signal 10, between 2.72 and 2.90 ppm, is due to the overlapping of several triplets of the methylenic protons in the α-position in relation to two double bonds of the different acyl groups, also named bis-allylic protons. Signal 11 at 4.10−4.34 ppm and signal 12 at 5.23−5.30 ppm are due, respectively, to the protons on 1 and 3 carbon atoms of the glyceryl group and to the proton on the carbon atom 2 of the same chemical structure. The latter signal overlaps slightly with signal 13, at 5.31−5.47 ppm, due to olefinic protons of the different acyl groups.

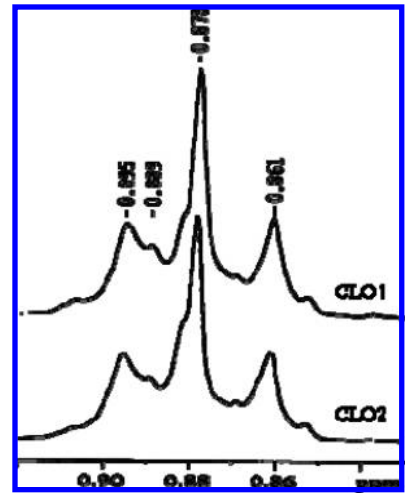

Figure 3. Enlargement of the ^{1}H NMR spectral region between 0.83 and 0.92 ppm of the CLO1 and CLO2 samples.

From the simple observation of the enlarged ^{1}H NMR spectral region between 0 and 0.7 ppm, it can be said that all cod liver oil samples contain cholesterol. This is evidenced by the signal between 0.66 and 0.69 ppm (signal 1) due to the methylic protons on the C-18 carbon atom of this molecule. In addition, from the area of signals 1 (A_1) and 11 (A_{11}), the number of mol of cholesterol per mol of triglyceride (C/T) in each oil can be determined. This is possible because in ^{1}H NMR, the value of the area of the different signals is proportional to the number of protons that generate the signal. For this reason, eq 1 permits the determination of the number of mol of cholesterol per mol of triglyceride.

$$C/T = 4A_1/3A_{11} \quad (1)$$

The results thus obtained are given in **Table 4**, as mmol of cholesterol per mol of triglyceride; as can be observed in this table, the concentration of cholesterol, expressed in this way, varies in the several studied cod liver oil samples between 10.7 ± 0.0 in CLO2 and 24.9 ± 1.3 in CLO6. Furthermore, it is also possible to determine the concentration of cholesterol in these samples; the approach used to this aim is based on knowing the amount of nondeuterated chloroform present in the resonance tube, the area of the ^{1}H NMR signal of nondeuterated chloroform, and the area of signal 1 (A_1). The concentration of cholesterol C (given in mg/mL of oil) determined using this latter approach is also given in **Table 4**. The results are in agreement with the results above commented on, and they are, in general, of a similar order to those found by other authors in cod liver oils (*25*−*27*).

Furthermore, from the simple observation of the enlargement of signal 2, other valuable information related to the composition of these oils can be extracted. **Figure 3** shows, as an example, the enlargement of signal 2 for samples CLO1 and CLO2, those of the other cod liver oils studied being similar to these. From the observation of this signal, it is evident that all cod liver oil samples studied have a much smaller proportion of ω-6 diunsaturated acyl groups than of saturated plus ω-7 and ω-9 monounsaturated acyl groups, as to be expected in fish oil, in agreement with previous studies (*20*). This is observed in **Figure 3** by the much smaller intensity of the triplet centered at 0.889 ppm than that of the triplet centered at 0.878; the first is due to methylic protons of diunsaturated ω-6 acyl groups, and the

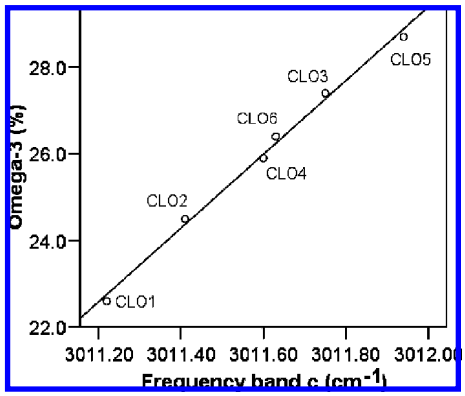

Figure 4. ω-3% of several samples determined from ^{1}H NMR data vs the frequency of band c of their FTIR spectra and the line obtained by fitting of both sets of data.

second is due to methylic protons of saturated, ω-7, and ω-9 monounsaturated acyl groups (*19*).

Likewise, from the simple observation of the ^{1}H NMR spectra, conclusions related to the unsaturation degree of the several oil samples can also be reached. Thus, from observation of some signals in **Figure 2**, it can be concluded that the acyl groups of cod liver oil CLO1 have a higher saturation degree than those of CLO2. This can be inferred because the intensity of the signals, in **Figure 2**, due to double bonds protons (signal 13), to *bis*-allylic protons (signal 10), to methylenic protons of DHA in α- and β-position in relation to the carbonilic group (signal 9), and to methylic protons of ω-3 polyunsaturated acyl groups (signal 3), is higher in the spectrum of CLO2 sample than in the spectrum of CLO1. The same conclusion is obtained from the observation of proton signals associated with more saturated groups. Thus, the intensity of the signals due to methylic protons of saturated and monounsaturated acyl groups (signal 2) and to methylenic protons either in position β or further, in relation to double bonds, or in position γ or further, in relation to the carbonyl group in the different acyl groups (signal 4), is higher in the spectrum of sample CLO1 than in the spectrum of CLO2, showing the higher unsaturation degree of this latter sample, which is in agreement with the above conclusions.

Moreover, quantitative data can also be obtained. So, from the area of signals 2 (A_2) and 3 (A_3), the percentages of ω-3 polyunsaturated acyl groups (ω-3%) and of saturated plus monounsaturated and diunsaturated acyl groups (SMDU %) can be obtained from eqs 2 and 3, respectively.

$$\omega\text{-}3\% = 100A_3/(A_2 + A_3) \quad (2)$$

$$\text{SMDU } \% = 100A_2/(A_2 + A_3) \quad (3)$$

In agreement with previous studies (*20, 28*−*30*), the proportions of these two kind of acyl groups, calculated as above, are given in **Table 4** and show that the percentage of ω-3 acyl groups decreases in the studied samples in the order CLO5 (28.7 ± 0.2%) > CLO3 (27.4 ± 0.5%) > CLO6 (26.4 ± 04%) > CLO4 (25.9 ± 0.3%) > CLO2 (24.5 ± 0.2%) > CLO1 (22.6 ± 0.3%) in total agreement with the order inferred from FTIR frequency values of band c also given in **Table 2**. In fact, both kinds of data, frequency values of band c, coming from FTIR spectra, and percentages of ω-3 acyl groups determined from ^{1}H NMR data, fit to a linear equation (ω-3% = −25578.51 + $8.50F_{\text{band c}}$) with a correlation coefficient of 0.9974. **Figure 4** gives the line corresponding to this equation and the points coming from both sets of data. As mentioned above, the percentage of ω-3-acyl group is considered to be an important parameter of cod liver oil quality.

Other possible approaches to determine the percentages of ω-3 and of the rest of acyl groups (SMDU) are given by eqs 4 and 5; these were deduced assuming that the amount of free fatty acids and of di- and monoglycerides, as well as of phospholipids, is very small in cod liver oil.

$$\omega\text{-}3\% = 100\ 4A_3/9A_{11} \quad (4)$$

$$\text{SMDU } \% = 100\ 4A_2/9A_{11} \quad (5)$$

The results obtained with these equations are slightly higher than those obtained by eqs 2 and 3, the increase being of the same order in all cases. As an example, using eq 4, CLO5 also has the highest percentage values (ω-3% = 29.8 ± 0.4%) and CLO1 the lowest (ω-3% = 23.4 ± 0.2%). The same is also true for molar percentage values of SMDU obtained using data derived from eq 3 or eq 5.

Likewise, from the area of signal 9 (A_9) due to methylenic protons in α- and β-positions in relation to the carbonyl group of DHA acyl groups and from the area of signal 8 (A_8) due to the other methylenic protons in the α-position in relation to the carbonyl group apart from DHA acyl group, the percentage of DHA acyl groups can be determined from eq 6, in agreement with previous studies (*20*).

$$\text{DHA } \% = 100\ A_9/(A_9 + 2A_8) \quad (6)$$

The percentages of the DHA acyl groups, obtained using this equation, are given in **Table 4**, and they indicate that sample CLO3 (11.3 ± 0.1%) is the richest in this kind of ω-3 acyl groups and sample CLO1 (8.9 ± 0.0%) is the poorest.

Another approach to determine the percentage of DHA acyl groups in function of the area of signals 9 and 11 is also possible, as has been indicated in previous studies (*20*); assuming that the amount of free fatty acids and of di- and monoglycerides, as well as of phospholipids, is very small, eq 7 can be used to this aim.

$$\text{DHA } \% = 100A_9/3A_{11} \quad (7)$$

The results obtained using this equation are practically the same as those obtained using eq 6, showing that both approaches are in total agreement.

Furthermore, the concentration of DHA in the oil can be determined taking into account the relationship between the area of the signal of nondeuterated chloroform and its amount in the resonance tube, as described above in the case of cholesterol. Data obtained in this way, expressed as mg of DHA per mL of oil, are given in **Table 4**; they are in agreement with the results above and are of a similar order to those found in other cod liver oils by other authors (*25, 31, 32*).

In addition to DHA, EPA acyl groups can also be determined from the area of the signal 6 (A_6), due to methylenic protons in the β-position in relation to the carbonyl group of EPA acyl groups, from the area of signal 5 (A_5), due to methylenic protons in the β-position in relation to the carbonyl group except those of EPA and DHA acyl groups, and from the area of signal 9 (A_9), by means of eq 8.

$$\text{EPA } \% = 100(2A_6)/(A_9 + 2A_5 + 2A_6) \quad (8)$$

The percentages of EPA acyl groups obtained using eq 8 for the six cod liver oil samples, given in **Table 4**, show that sample CLO2 (9.4 ± 0.1%) is the poorest in this type of acyl groups and that sample CLO5 (14.7 ± 0.2%) is the richest.

The percentage of EPA acyl groups can also be determined from the areas of signals 6 (A_6) and 11 (A_{11}) as eq 9 indicates, assuming, as above, that the amount of free fatty acids and of di- and monoglycerides, as well as of phospholipids, is very small.

$$\text{EPA } \% = 100\ 2A_6/3A_{11} \quad (9)$$

The results obtained with this equation are slightly higher than those obtained using eq 8, the increase being of the same order in all cases and showing sample CLO5 as the highest value and sample CLO2 as the lowest.

Furthermore, the concentration of EPA in the oil can be determined taking into account the relationship between the area of the signal of nondeuterated chloroform and its amount in the resonance tube as before for cholesterol and DHA. Data so obtained, expressed as mg of EPA per mL of oil, are given in **Table 4**; they are in agreement with the results above and are of a similar order to those found by other authors in cod liver oils (*31, 32*).

In addition, the ratios found between the proportion of these kinds of acyl groups [DHA (mg)/EPA (mg) = 0.7 in CLO5, 1.2 in CLO2, and 0.9 in CLO1] agree with the ratios derived from data given by the producers [DHA (mg)/EPA (mg) = 0.6 in CLO5, 1.5 in CLO2, and 0.9 in CLO1]; these latter data had been determined by conventional methods including transformation of triglycerides into methyl esters and quantification of these by gas chromatography (*26*).

Regardless of the approach used to determine the percentages of EPA and DHA acyl groups, it is clear that the percentage of both acyl groups is smaller than the total percentage of ω-3 acyl groups. This strongly suggests the presence in these oils of other ω-3 polyunsaturated acyl groups different from the two already mentioned; the percentage of these other polyunsaturated ω-3 acyl groups (OPUω-3) can be determined by difference, for example, from data obtained using eqs 2, 6, and 8. The occurrence of other polyunsaturated ω-3 acyl groups different from DHA and EPA in cod liver oil is known, and the values obtained for the several cod liver oil samples, using the equations above-mentioned, are the following: CLO5, 4.2%; CLO3, 4.8%; CLO6, 5.1%; CLO4, 4.6%; CLO2, 4.5%; and CLO1, 4.0%. These values are similar to those found in other cod liver oils studies (*23, 32*).

The percentage of unsaturated acyl groups can be determined given that signal 7 is due to allylic protons (or to protons of methylenic groups in the α-position in relation to one single double bond) except those belonging to the DHA acyl groups. Taking into account this fact, the percentage of total unsaturated acyl groups (U) can be determined by eq 10.

$$\text{U } \% = 100(2A_7 + A_9)/(6A_{11}) \quad (10)$$

This approach has been deduced assuming that the amount of free fatty acids and of di- and monoglycerides, as well as of phospholipids, is very small. The results, obtained using eq 10, indicate that, among the samples studied, CLO2 (82.4%) has the highest molar percentage of unsaturated acyl groups of all of the samples studied and that CLO5 (75.4%) has the lowest. The fact that CLO2 sample has the highest molar percentage of unsaturated acyl groups (U %) of all of the samples studied explains its low saturation degree shown by the A_f/A_c ratio obtained from IRTF (see **Table 2**). It should be taken into account that U includes mono-, di-, and poly unsaturated acyl groups. Likewise, the molar percentage of saturated acyl groups (S %), as well as of monounsaturated plus diunsaturated acyl groups (MD %), can be determined by difference with data obtained using previous equations.

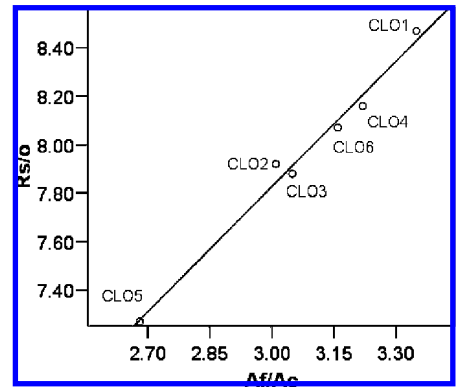

Figure 5. Ratio, Rs/o, of several cod liver oil samples determined from ^{1}H NMR data vs their A_f/A_c ratio determined from FTIR spectra and the line obtained by the fitting of both sets of data.

Finally, the ratio between the area of protons bonded to saturated structures ($A_2 + A_3 + A_4 + A_5 + A_6 + A_7 + A_8 + A_9 + A_{10}$) and the olefinic protons signal area (A_{13}) in the several acyl groups can be taken as a measure of the saturation degree of these samples. This approach has been used by other authors (*33, 34*) and can be named saturated/olefinic protons ratio (Rs/o), being also related to the iodo index.

$$\text{Rs/o} = (A_2 + A_3 + A_4 + A_5 + A_6 + A_7 + A_8 + A_9 + A_{10})/A_{13} \quad (11)$$

The Rs/o values thus calculated represent the ratio between the number of hydrogen atoms bonded to saturated structures and those bonded to unsaturated structures in each sample; **Table 4** shows that the highest value has been found in sample CLO1, and the lowest value has been found in sample CLO5, in agreement with the other parameters coming from FTIR data. The different meaning of the molar percentage of saturated acyl groups S and of the Rs/o parameter should be noted; this latter is closely related to A_f/A_c derived from FTIR spectra. Both sets of data fit to a linear equation [Rs/o = 2.66 + 1.72 (A_f/A_c), R = 0.9925], which is represented in **Figure 5**. This parameter Rs/o provides, like A_f/A_c and frequency value of bands e and f, additional information on the unsaturation of the sample, which is not included in any of the other parameters given in **Table 4**.

To the best of our knowledge, this is the first time that most of the approaches above considered have been proposed to determine the percentages of acyl groups and concentrations of DHA, EPA, and cholesterol in cod liver oil and in general in fish oil; exceptions are the approaches involved in eqs 2 and 3, which have been previously proposed and used by other authors (*20, 28−30*).

In summary, ^{1}H NMR provides important and detailed information about the percentage of several acyl groups and the concentrations of DHA, EPA, and cholesterol in cod liver oil. These data are very useful when evaluating cod liver oil quality and can be obtained in a few minutes in a simple and very fast way.

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