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Original Article

Development and validation of a gas chromatography with a flame ionization detector method for quantitative analysis of four major fatty acids extracted shark liver oil

[Desarrollo y validación de un método GC-FID para el análisis cuantitativo de los ácidos grasos mayoritarios extraídos del aceite de hígado de tiburón]

Pedro C. Quero-Jiménez[1, 3*], Lester A. Arias Felipe[1, 2], María E. Jorge Rodríguez[3], Reinaldo Molina Ruiz[1], Julio O. Prieto García[2], Jorge B. de la Torre López[2], Osvaldo N. Montenegro[1]

[1]Centro de Bioactivos Químicos. Universidad Central «Marta Abreu» de Las Villas. Carretera a Camajuaní km 5 ½ Santa Clara. CP 54830, Villa Clara, Cuba.
[2]Departamento de Licenciatura en Química. Universidad Central «Marta Abreu» de Las Villas. Carretera a Camajuaní km 5 ½ Santa Clara. CP 54830, Villa Clara, Cuba.
[3]Departamento de Licenciatura en Farmacia. Universidad Central «Marta Abreu» de Las Villas. Carretera a Camajuaní km 5 ½ Santa Clara. CP 54830, Villa Clara, Cuba.
*E-mail: pquero.jimenez@gmail.com

Abstract

Context: The shark liver of the species *Ginglimostoma cirratun*, *Carcharhinus longimanus*, and *Carcharhinus falciformis*, captured in the north-central coast of Cuba are a source of oil, whose content of major fatty acids could be used in its quality control.

Aims: To develop a simple and robust gas chromatography with a flame ionization detector (GC-FID) method that is suitable for routine analysis of four major fatty acids extracted shark liver oil.

Methods: Four major fatty acid content in shark liver oil pool of species *Ginglimostoma cirratun*, *Carcharhinus longimanus*, and *Carcharhinus falciformis*, was analyzed through the gas chromatography with a GC-FID. The fatty acids were analyzed as methyl esters derivatives, using 5% aqueous sulfuric acid in methanol. The method was validated in terms of linearity, precision, accuracy, specificity and limit of detection and quantitation.

Results: Under the optimum analytical conditions, the analysis revealed that each target component was well separated with satisfactory recoveries and reproducibility. The method linearity was found to be high with good determination coefficient values for all target components. The evaluation of the matrix effect, demonstrated, that there is not interference from substances other than analysis. The method was also found to be accurate, precise and reproducible and it was applied to the quantitative determination of the fatty acid content in shark liver oil pool; oleic acid was the most abundant fatty acid (22.69%), followed by palmitic (18.85%), stearic (6.01 %) and myristic acid (0.40 %).

Conclusions: The GC-FID developed method is reliable and suitable for determination of four major fatty acids in shark liver oil pool.

Keywords: fatty acid; GC-FID; shark liver oil pool; validation.

Resumen

Contexto: El hígado de tiburón de las especies *Ginglimostoma cirratun*, *Carcharhinus longimanus* y *Carcharhinus falciformis*, capturados en la costa centro-norte de Cuba, son una fuente de aceite, cuyo contenido de ácidos grasos mayoritarios podría utilizarse en su control de calidad.

Objetivos: Desarrollar un método mediante cromatografía de gases con un detector de ionización de llama (GC-FID) simple y robusto que sea adecuado para el análisis de rutina de cuatro ácidos grasos mayoritarios extraídos del aceite de hígado de tiburón.

Métodos: Se analizaron cuatro ácidos grasos mayoritarios presentes en el aceite de hígado de tiburón de las especies *Ginglimostoma cirratun, Carcharhinus longimanus* y *Carcharhinus falciformis*, mediante GC-FID. Los ácidos grasos se analizaron como derivados de ésteres metílicos, utilizando ácido sulfúrico acuoso al 5% en metanol. El método fue validado en términos de linealidad precisión, exactitud, especificidad y límites de detección y cuantificación.

Resultados: Sobre las condiciones analíticas óptimas, el análisis reveló que cada componente determinado estaba bien separado, con recuperaciones y reproducibilidad satisfactorias. Se encontró que la linealidad del método era alta con buenos valores de coeficiente de determinación para todos los ácidos grasos. La evaluación del efecto matriz demostró que no existe interferencia de sustancias distintas al análisis. El método también resultó ser exacto, preciso y reproducible y se aplicó a la determinación cuantitativa del contenido de ácidos grasos en la reserva de aceite de hígado de tiburón; el ácido oleico fue el ácido graso más abundante (22,69%), seguido del palmítico (18,85%), esteárico (6,01%) y el mirístico (0,40%).

Conclusiones: El método GC-FID es confiable y adecuado para la determinación de cuatro ácidos grasos mayoritarios presentes en la reserva de aceite de hígado de tiburón.

Palabras Clave: aceite de hígado de tiburón; ácido graso; GC-FID; validación.

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AUTHOR INFO
ORCID: 0000-0001-6145-416X (PCQJ)

INTRODUCTION

The oil of marine origin are a source of nutrients such as fatty acids (Innes and Calder, 2020), squalene (Lozano-Grande et al., 2018), alkylglycerols (Deniau et al., 2010; Iannitti and Palmieri, 2010), among others. The fatty acid has a positively effect on the health, which action on the depression and anxiety symptoms (Deane et al., 2019), cholesterol in blood (DiNicolantonio and O'Keefe, 2018; Harris et al., 2018; Oteng and Kersten, 2020; Stonehouse et al., 2020), cardiovascular diseases (Sanders, 2019; Innes and Calder, 2020; Li et al., 2020), vascular effects (Hamadate et al., 2015), diabetes (Chewcharat et al., 2020), memory (Ruhland et al., 2020), among others (Simopoulos, 2020).

The shark liver oil are employee as nutraceutical consumption, however according to Hilleman et al. (2020), there are critical differences between dietary supplement and prescription omega-3 (ω-3) fatty acids. Among ω-3s, docosahexaenoic acid (DHA)/eicosapentaenoic acid (EPA) combination ω-3s should not be substituted for the EPA-only product as they are not therapeutically equivalent and DHA may raise low-density lipoprotein cholesterol.

In Cuba there are specialized centers for the capture of marine species, where the capture of sharks is classified as an incidental and alternative activity, however, there is an average level of history of shark capture in the country that oscillates around 1500 t/year. Although in recent years, the average catch has decreased, in the Empresa Pesquera Industrial de Caibarién (EPICAI) 73.314 t have been caught in 2015; 63.449 t in 2016 and 76.272 t in 2017 (ONEI, 2017). During the fishing campaigns carried out by the fishing center, by-products such as shark liver, which are discarded and constitutes a biological pollutant for the marine ecosystem; that is why new products can be generated from the fishing waste. The use of livers of sharks in the production of oils is not an industrial activity at present and there are not consumption habits in the population, due to their strong fishy smell and taste (García Rodríguez, 2005); however, from these by-products, develops a nutraceutical food.

Generally the gas chromatography (GC) with flame ionization detector (FID) is the simpler analytical technique commonly used for the analysis of fatty acids (Zhang et al., 2015). In the literature, most fatty acids analyses by GC require derivatization due to the high boiling points of fatty acids, which are difficult to evaporate and have a low FID detection response (Laakso et al., 2002). The aim of this study was to develop and validate a simple method for the simultaneous analysis of four fatty acids present in shark liver oil of the species *Ginglimostoma cirratun*, *Carcharhinus longimanus*, and *Carcharhinus falciformis*, captured in the north-central coast of Cuba.

MATERIAL AND METHODS

Extraction of oil

Shark specimens of *Ginglimostoma cirratun*, *Carcharhinus longimanus*, and *Carcharhinus falciformis* were captured in the Caribbean Sea (between 23.40°160′ to 22.82°160′ N, and 81.27°145′ to 78.94°145′ W), near Villa Clara province shore in Cuba in the summer (June) of 2018. Specimens, as well as their livers were weighed for hepatosomatic index (HSI). The material was identified and authenticated by MSc. Yoandry Arencibia. Dissected livers were placed in polyethylene bags and frozen at -20°C for their transportation in coolers from the Empresa Pesquera Industrial de Caibarién (Villa Clara, Cuba). Livers were stored at -80°C, for no more than 2 weeks, until oil extraction. Livers were at thawed at room temperature and homogenized for 2 min using a 14-507-7 M cutter (Fisher Scientific, Pittsburgh, PA). The homogenized liver was heated at 50°C for 20 min with agitation and centrifuged at 7500 rpm for 20 min at room temperature in a centrifuge (model TG16, Yingtai Instrument Co., China), to release solid impurities from liver cells; then the oils were washed three times with hot distilled water (50-60°C). A second centrifugation was performed at 7500 rpm in the oil that was released from the

heavy fats and other impurities; these were clean and transparent with a characteristic light-yellow color.

Performance quantification

It consisted of weighing 1 kg of sample (M); then the heavy portion was subjected to the extraction process, where the volume of oil obtained was quantified. Oil mass (m) was determined by the relative density at 20°C. The percent yield ($\%_R$) was calculated following the equation [1].

$$\%_R = (m/M) * 100 \quad [1]$$

The physicochemical characterization of the extracted oil was reported previously (Quero-Jiménez et al., 2020).

Fatty acid mixture solution

A fatty acid mixture solution was prepared by mixing 8.0 mL of each of the individual fatty acid stock solutions into a 100 mL of volumetric flask, excluding lauric acid, and diluted to volume with sample solvent.

Preparation of fatty acid methyl esters (FAMEs)

The method used in the derivatization of the samples are development by Christie (1993). A 50 mg of shark liver oil pool was weighed, transferred to a volumetric balloon, dissolving it with 1 mL of toluene and 2 mL of 1% sulfuric acid in methanol. It was coupled to a reflux condenser and reflected for 2 hours; subsequently, 5 mL of 5% sodium chloride was added to them, it was transferred to a separatory funnel, and two successive washes were carried out with 5 mL of heptane and it was left to stand until achieving a good separation between the two phases (the aqueous and organic). The aqueous phase was discarded, and the organic phase was added with 4 mL of 2% potassium bicarbonate, recovered and transferred to a test tube with anhydrous sodium sulfate, after leaving it standing, filtered and roto-evaporated at 40°C until dry. Dissolved with heptane to a final volume of 2 mL in a volumetric flask and refrigerated until use.

Gas chromatography analysis

An Agilent Technologies 6890 N gas chromatograph (Germany) was used, equipped with a flame ionization detector and capillary column (30 m, 0.53 mm) (Agilent Technologies 6890 N, Germany), which has as a stationary phase fused silica. The chromatographic conditions were detector temperature 280°C; injector temperature 250°C; initial column temperature 120°C for 1 min, programmed to increase at a rate of 10°C per minute up to 200°C and then at 4°C per minute up to the final temperature of 220°C. Nitrogen and hydrogen for chromatography R, as carrier and auxiliary gas, respectively, a flow rate of 1.3 mL/min. To perform the determination, 1 µL of the derived sample was injected, alternatively with a sample volume/internal standard ratio of 80/20. Fatty acids were identified by comparing the retention times and relative retention times of the standards with those of the samples. The quantification was by internal standardization using the methyl esters of lauric acid as the internal standards. The results obtained in mg/100 g of the sample were calculated according to AOCS methodology (AOCS, 2017).

A certain volume of gas standard was injected into a GC system under optimized analytical conditions. The output signal was monitored using Agilent ChemStation for GC systems, data analysis and A/D converter 35900E. The data were estimated by automated integration of the area under the resolved chromatographic profile.

Statistical analysis

All measurements in this study were made in three repeated samples. All reported data points and spectra denote the means of repetitions. For the statistical processing of the data, R program (version 3.6.3) was used.

Validation of test procedure

GC method for determining fatty acid methyl esters was subjected to validation following recommendations of the International Conference on Harmonization (ICH, 2005). Quantification of individual fatty acids was based on the obtained

peak area, a result was normalized, and no correction factor was used. Criteria used for evaluation of obtained results were established according to literature (AOAC, 2016), including method linearity, limit of detection and limit of quantification, precision (repeatability and reproducibility), accuracy, selectivity, and specificity. Adopting the acceptance criteria: Recovered in interval 97 to 103%.

Linearity

To establish linearity of the proposed method, five series of methyl stearate (50 to 300 µg/mL), methyl oleate (100 to 800 µg/mL), methyl palmitate (150 to 400 µg/mL) and methyl myristate (100 to 250 µg/mL) standard solution were prepared from the stock solutions and analyzed on three consecutive days. Least square regression analyses were done for the obtained data. ANOVA test (one-way) was performed based on the absorbance values observed for each ascorbic acid concentration during the replicate measurement of the standard solutions. The linearity of the calibration process was investigated by means of the Lack-of-fit test (Akritas and Papadatos, 2004; de Souza and Junqueira, 2005; Hsieh and Liu, 2008), the quality coefficient (De Beer et al., 2007; 2012; Tellinghuisen, 2008), correlation coefficient (Van Loco et al., 2002; de Souza and Junqueira, 2005; Asuero et al., 2006; Hsieh and Liu, 2008), determination coefficient (Van Loco et al., 2002), ANOVA (Nunes et al., 2015; de Haro Moreno et al., 2018).

Precision

Precision of the method was checked through the repeatability and reproducibility experiment. Repeatability of the method was evaluated by three preparations of methyl stearate, methyl oleate, methyl palmitate and methyl myristate samples, and each preparation was analyzed in duplicate according to the method. For repeatability, six samples were processed (100% concentration) to which the technique to be validated was applied under homogeneous conditions, by the same analyst and on the same day; while the intermediate precision consists of a similar procedure, with the same concentration, carried out for different days. In both cases, the relative standard deviation (%RSD) of the results is determined. The criteria adopted for its evaluation were as follows: The %RSD for repeatability CV ≤ 2% and for intermediate precision %RSD ≤ 5%. After that, the calculated %RSD´s were compared with theorical relative standard deviation of precision from Horwitz formula (equation [2]) (Linsinger and Josephs, 2006).

$$\text{CV-Hortwitz (\%)} = 2^{(1 - 0.05\log C)} \quad [2]$$

Where C is the concentration of analyte stated in decimal fraction.

Obtained results confirmed precision of the method due to multiple preparation of the sample and preparation by different analysts.

Accurancy

To evaluate the accuracy, the standard addition method was used. The samples (10% concentration) were analyzed in triplicate, applying the sample procedure. To this end, increasing amounts of both standards (methyl stearate: 50, 100, 150 µg/mL; methyl oleate: 50, 100, 150 µg/mL; methyl palmitate: 50, 100, 150 µg/mL; and for methyl myristate were added to a known quantity of the sample (50 µL): 20, 30, 40 µg/mL). In each case it was completed to a volume of 1 mL of heptane. Two replicates of each of the added concentrations were performed. The added and recovered concentrations were represented in a graph and the recovery was evaluated as the value obtained for its slope (Borman and Elder, 2018). In addition, the recovery percentages were calculated for each of the samples analyzed. This parameter was developed in the gas chromatography technique for the four FAMEs standards analyzed in this work. The criteria adopted for its evaluation was: Recovered in the 97 to 103% interval (Borman and Elder, 2018).

Selectivity and specificity

To evaluate the specificity, the retention times of the substances present in the sample and in the standard were compared. The width of the half height was measured, and the peak symmetry factors were calculated in the chromatograms corresponding to ten standard injections and ten

samples; the mean and variance of the standard and the sample were determined, Student's t was found, and Fisher's F was calculated, t and F values were compared with the tabulated ones, in order to rule out possible peak overlaps. For this, the confidence limits for the symmetry factors were calculated by the following the equation [3].

$$\bar{X} \pm (t * s)/\sqrt{n} \quad [3]$$

To compare the peak symmetry factors for the standard and the sample, the confidence intervals for these factors were calculated, so that some intervals include the others.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of fatty acid analyzed by the proposed methods were determined using analytical curves. LOD and LOQ were calculated as shown in equations [4] and [5], respectively (ICH, 2005).

$$LOD = (3.3S_{bl})/b \quad [4]$$

$$LOQ = (10S_{bl})/b \quad [5]$$

where S_{bl} is the standard deviation of y-intercept of regression equation and b is the slope of the calibration curve.

RESULTS

The methyl ester of lauric acid (12:0) (methyl laurate) was used to verify the detector response for a saturated fatty acid with regard and internal standard.

Method validation

Linearity

The linearity of the method was evaluated by analyzing the calibration curve. The results showed a linear model to describe the relationship between absorbance and concentration. The ANOVA analysis showed that the p-value is less than 0.05, existing a statistically significant relationship between the absorbance and the concentration with a confidence level of 95.0% in all cases. The R^2 statistic ($R^2 > 0.98$) indicates that the adjusted model explains 99.71, 99.28, 99.09 and 99.39% of the absorbance variability, for the methyl stearate, methyl oleate, methyl palmitate and methyl myristate respectively (Table 1). The correlation coefficient ($r > 0.990$) is greater to 0.990 for all the fatty acid studied, indicating a relatively strong relationship between the variables (Eurachem Guide, 2014). The value of the intercepts (a) is showed in the Table 1. Confidence limits of the intercept containing to zero in all the cases. The statistical significance of the coefficients is checked by means of a Student's t test where the intercept is shown to be different from zero. Then the slope is highly significant, the calibration curve linearity being demonstrated. Reject the Lack-of-fit test the linear regression model must systematically be at 95% confidence level. This test demonstrates the calibration curve linearity, admitting the hypothesis of linearity, thus, the unexplained variability is due to the variability inherent of the data, and not because the conditioned distributions means of the dependent variable to each value of the independent variable are not on the line.

The proportionality test does not include the zero of the origin of coordinates, hence it is considered in calculations in all the cases studied. These values indicate the possible existence of an error of the systematic type. The standard deviation relative to the slope ($S_{b\ (rel.)} < 3\%$), is less than 3% taken as acceptance criteria for the four methyl of fatty acid analyzed (Jurado et al., 2017), which indicates that the calibration curves analyzed on different days have the same variance, so the slope will not change. As a value of the F test, it is obtained that $F_{cal} > F_{crítica}$, is an additional element that reaffirms that the variability in the curve does not affect the linearity (Eurachem Guide, 2014; Borman and Elder, 2018). Alternatively, the residual graphs provided useful information to validate the chosen regression model. The residuals graphs was used to verify whether the underlying assumptions, such as residue normality and homoscedasticity, are met to evaluate the fit goodness of the regression model (Vandeginste et al., 1998). Finally, the determined quality coefficients, which is a mathematical tool to determine the quality of the calibration line (De Beer et al., 2012), were less than the 5% established as acceptance criteria, and

these reaffirm the linear dependence between these variables, in the interval studied for each case. In the Table 1, shows the parameters evaluated to verify linearity and proportionality.

Precision

This parameter includes the terms repeatability and intermediate precision. For the first case, the technique is repeated without altering the conditions, while in the second the technique is affected on different days. The %RSD obtained for the repeatability of the sample were found to be less than that established for this test when performed on pharmaceutical raw materials (≤2%), which indicates that the technique has good repeatability. On the other hand, in the intermediate precision, a %RSD of less than that established (≤5%) was also obtained for the fatty acid methyl esters analyzed.

The analysis of variance carried out shows that there are no significant differences between the groups when obtaining in the Fischer test an $F_{Cal} < F_{Critica}$ in all cases. Finally, the %RSD between groups turned out to be less than the value obtained for the CV-Horwitz (%), confirming with this the veracity of the rest of the tests carried out. The results obtained (Table 2) in the parameters ensure that the gas chromatography technique for the determination of FAMEs in the shark liver oil pool is precise.

Accurancy

The added concentrations of the methyl esters used as standard and the recoveries obtained are shown in the Table 3. The average recovery percentage for the standards is in accordance with that required for chromatographic techniques. When performing the recovery curves, the value of the slope is within the range obtained in the test, very close to the calculated average recovery value. These values demonstrate a high accuracy in the determinations of methyl esters in the shark liver oil pool samples, with the procedure used.

Selectivty and especificity

As part of determining the limits of detection and quantification, the injection of the blank alone was performed and no response was obtained, which demonstrated the non-existence of interferences to the retention time of the substances present in the sample. If the chromatographic peaks of the standards are compared (methyl stearate, methyl oleate, methyl palmitate and methyl myristate) with the corresponding in the sample under the optimal conditions of analysis (Figs. 1 and 2) where no tails, fuzzy foreheads or shoulders were observed with the naked eye, it can be inferred that there should be no peak overlaps. There were also no significant variations in retention times, which are kept within a range of ± 2%.

These results showed the absence of appreciable interferences that may affect the reliable determination of fatty acids in shark liver oil. However, with a view to ratifying them, the peak symmetry factors of standards and samples for each fatty acid were analyzed. The statistical parameters used to determine the confidence intervals of the symmetry factors are shown in Table 4.

The symmetry factors of the peak for the standard and the sample were found in such a way that some intervals included the others in all the fatty acids analyzed. This result is presented in the Table 5.

Considering that in each fatty acid the interval obtained for the sample is included in the interval obtained for the standard, it can be ruled out that there is overlapping of the peaks and therefore it is a good reason to consider the technique as specific. With the results obtained in the validation parameters, it can be stated that the gas chromatography technique evaluated for the determination of fatty acids in shark liver oil is reliable.

Limits of detection and quantification

Limit of quantification (LOQ) and limit of detection (LOD) of the method were calculated according to literature (ICH, 2005). The calculated value for LOQ was 0.03% and for LOD was 0.01% but values were corrected based on established criteria for mass deviation to 0.1% for LOQ and 0.05% for LOD. The obtained results for LOQ and LOD proved that the method is sensitive enough at low concentrations for its purpose.

Table 1. Parameters evaluated to verify linearity and proportionality, LOD and LOQ results.

Parameter	Acceptance requirements	Methyl stearate	Methyl oleate	Methyl palmitate	Methyl myristate
Data number (n)	-	6	8	6	4
Concentration range (µg/mL)	-	50 to 300	100 to 800	150 to 400	100 to 250
Equation of the line	y = bx + a	y = 0.0053x + 0.0718	y = 0.0053x - 0.0248	y = 0.0077x - 0.1933	y = 0.0862x - 1.526
Intercept	-	0.0718	- 0.0248	- 0.1933	- 1.526
Slope	-	0.0053	0.0053	0.007	0.0862
Linear correlation coefficient (r)	r > 0.990	0.9985	0.9963	0.9952	0.9969
Determination coefficient (R^2)	$R^2 > 0.98$	0.9971	0.9937	0.9905	0.9939
Confidence interval of *a* (95% confidence)	Include the zero	0.0718 ± 0.2105	0.0248 ± 0.3570	- 0.1933 ± 3.52191	- 1.526 ± 32.9808
Coefficient of variation of response factors CV_f (%).	$CV_f \leq 5$	4.77	4.72	4.22	4.18
Standard deviation relative to the slope (%)	$S_{b\,(rel.)} < 3$	0.0034	0.00086	0.0057	0.0184
Ficher test (95% confidence)	$F_{calculated} > F_{critic}$	$3432.65 > 5.10 \times 10^{-14}$	$6649.06 > 1.88 \times 10^{-15}$	$1044.55 > 1.88 \times 10^{-11}$	$3432.66 > 5.09 \times 10^{-14}$
Quality coefficient (%)	Q.C. < 5	0.8565	0.0899	0.4033	0.3994
Sensitivity (%)	97.00-103.00	100.65	101.20	102.25	100.32
LOD	-	7.075	9.616	19.396	7.64
LOQ	-	21.439	29.139	58.774	23.15

Table 2. Precision results.

Parameter	Acceptance requirements	Methyl stearate	Methyl oleate	Methyl palmitate	Methyl myristate
Data number (n)	-	6	6	6	6
Experimental concentration (µg/mL)	-				
RSD (repeatability) (%)	$CV_r \leq 2$	1.542	1.577	1.480	1.52
RSD (intermediate precision) (%)	$CV_R \leq 5$	1.920	1.09	2.02	1.88
Ficher test (95% confidence)	$F_{calculated} > F_{critic}$	1.610 < 3.682	3.557 < 3.682	2.683 < 3.682	6.088 < 4.964
%RSD between groups	-	1.95	1.14	1.185	2.12
CV-Horwitz (%)	-	4.44	3.53	3.83	2.86

Table 3. Accuracy results.

Parameter	Acceptance requirements	Methyl stearate	Methyl oleate	Methyl palmitate	Methyl myristate
Data number (n)	-	3	3	3	3
Experimental concentrations (µg/mL)	-	50 to 150	50 to 150	50 to 150	20 to 40
Equation of the line	y = mx + n	y = 1.0396x – 2.2612	y = 1.01096x + 1.2733	y = 1.0357x - 1.9322	y = 1.037x – 0.284
Slope	-	1.0396	1.01096	1.0357	1.037
Determination coefficient (R^2)	$R^2 > 0.98$	0.9995	0.9992	0.9992	0.999
Average recovery percentage (%)	97.00-103.00	101.24 ± 1.81	101.632 ± 1.96	101.637 ± 1.63	100.95 ± 2.60

Table 4. The symmetry factors of the peak for the standard and the sample.

FAMEs	Sample (µg/mL)	Standard (µg/mL)
Methyl stearate	1.0329 to -0.0223	1.2652 to -0.0951
Methyl oleate	1.1243 to -0.0433	1.2008 to -0.0268
Methyl palmitate	1.1848 to -0.0546	1.1218 to -0.0200
Methyl myristate	1.2336 to -0.0701	1.8215 to -0.1121

Table 5. Statistical parameters used to determine the confidence intervals of the symmetry factors.

Methyl stearate		Methyl oleate		Methyl palmitate		Methyl myristate	
Patron	**Sample**	**Patron**	**Sample**	**Patron**	**Sample**	**Patron**	**Sample**
$\bar{X}$= 1.1701	$\bar{X}$= 1.0106	$\bar{X}$= 1.1740	$\bar{X}$= 1.0810	$\bar{X}$= 1.018	$\bar{X}$= 1.1302	$\bar{X}$= 1.7	$\bar{X}$= 1.16
t = 1.83	t = 1.83	t = 1.83	t = 1.83	t = 1.83	t = 1.83	t = 1.83	t = 1.83
S = 0.291	S = 0.068	S = 0.082	S = 0.1330	S = 0.0610	S = 0.1670	S = 0.2	S = 0.34
n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10	n = 10

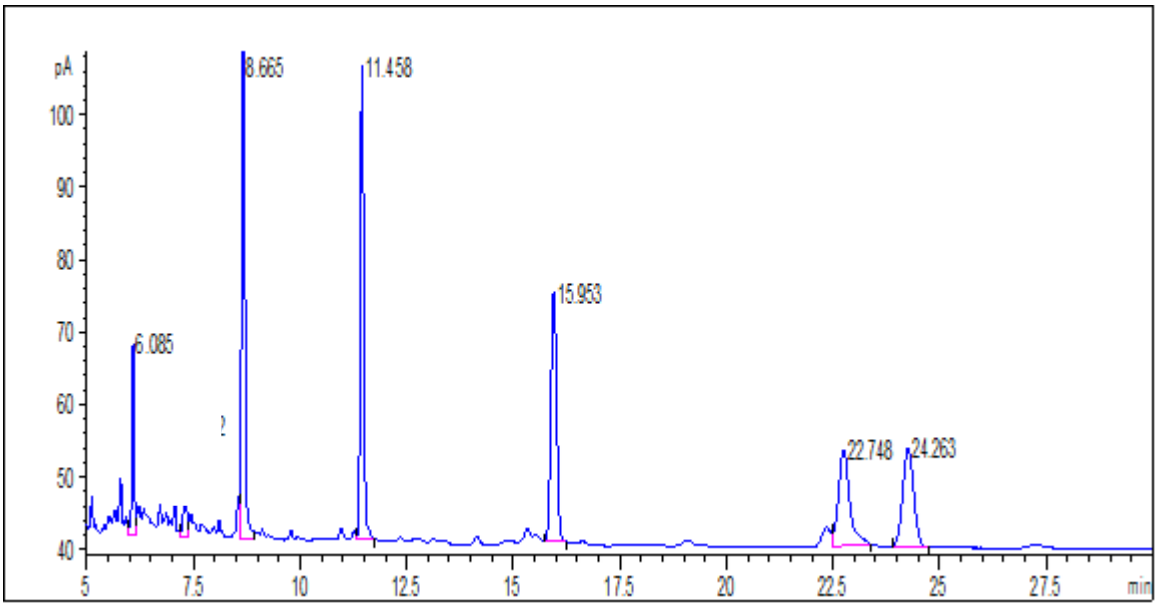

Figure 1. Chromatogram obtained for the mixture of these five standards and the SI. Peak **1**: Methyl behemate; Peak **2**: Methyl laureate (internal standard); Peak **3**: Methyl myristate; Peak **4**: Methyl palmitate; Peak **5**: Methyl oleate; and Peak **6**: Methyl stearate.

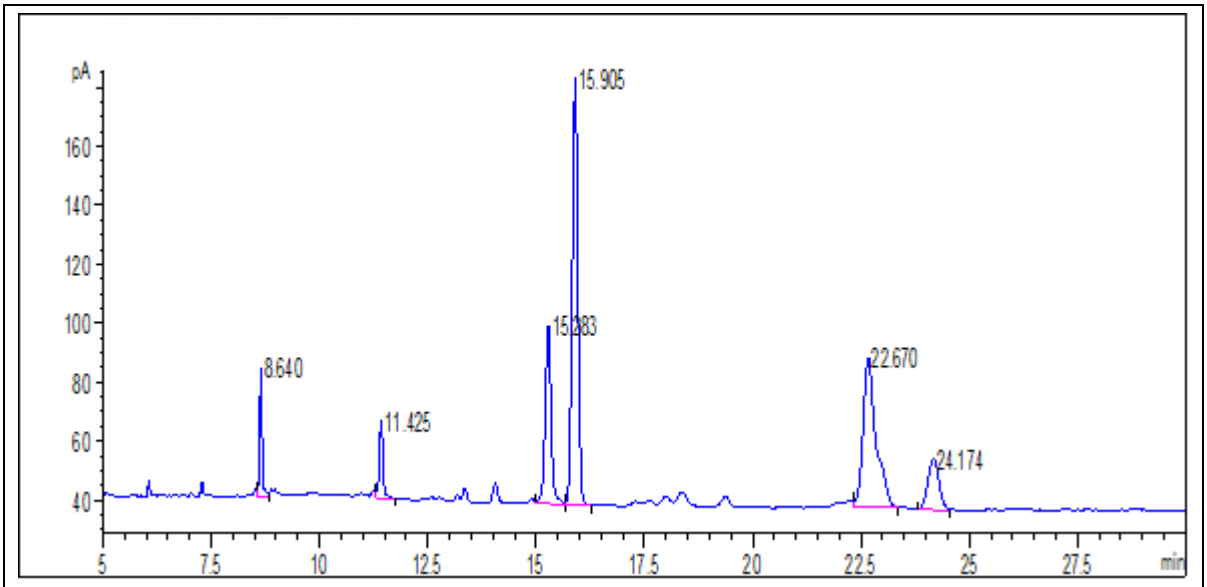

Figure 2. Chromatogram obtained for the sample under the same operating conditions.

Table 6. Contents of fatty acid in shark liver oil pool samples.

Fatty Acid	Content (%)
Myristic	0.40
Palmitic	18.85
Oleic	22.69
Stearic	6.01

Fatty acid content in shark liver oil pool

The chromatographic conditions of the technique used in the present study were optimized and it was demonstrated that the optimal temperature to achieve a good separation of the possible fatty acids present in shark liver oil is 200°C, applying it in isocratic mode.

Taking this into account, and using five methylated fatty acids as standards, a chromatogram with very well resolved peaks was obtained. Fig. 1 shows the chromatogram obtained for the mixture of these five standards, which appear in the following order in the chromatogram: methyl behenate (tr = 6.085 minutes), methyl laureate (tr = 8.665 minutes), methyl myristate (tr = 11.458 minutes), methyl palmitate (tr = 15.953 minutes), followed by unsaturated fatty acid methyl oleate (tr = 22.748 minutes) and finally methyl stearate appears (tr = 24.263 minutes). On the other hand, Fig. 2 shows the chromatogram obtained for the sample under the same operating conditions, observing a total correspondence in the retention times of both chromatograms for the evaluated standards; this result corroborated the presence of these fatty acids in shark liver oil. As observed in the sample chromatogram, a well-defined peak at tr = 15.283 minutes appears in addition to the identified peaks, which could not be identified due to the unavailability of this fatty acid standard. It was decided that the remaining standard (methyl laureate) with a tr = 8.665 minutes was the internal standard (SI) since it fulfilled the conditions to be used for this purpose.

DISCUSSION

In our study, it was confirmed that the fatty acid with the highest concentration is oleic, followed by palmitic, stearic and finally myristic as shown in Table 6. However, according to the work carried out by García et al. (2006) in the shark species *Mustelus antarticus*, *Galeorhinus galeus* and *Squalus acanthias* from the Cuban coast, the contents are totally different, since twenty-one fatty acids were identified in the neutral fraction of the oil and eighteen in the polar fraction, showing that palmitic and stearic acids are the predominant saturated fatty acids in both fractions.

On the other hand García et al. (2014) established that the percentages of palmitic acid, out of the total fatty acids, was between 20 and 40%, with this fatty acid being the predominant one (García et al., 2005; 2006; Cruz-Nuñez et al., 2009), and the one that takes into account for the calculation of the limits of quality specifications, which establishes that the content of this in the samples must be greater than 10.99 %, so our oil meets this criterion. It should be taken into account that the fatty acids present in the shark's liver may vary depending on the geographical location of the shark, and possibly also on the diet and period of the year in which it was caught (Nichols et al., 2001; Kohlmeier, 2015).

In a study carried out by Onyeche and Okaka (2018) the presence of various fatty acids was detected in *Clarias gariepinus*, which are quantified by CG-FID; in these studies melisic acid (24.378), stearic (13.659), lauric (9.569), myristic (9.246), palmitic (9.639), palmitoleic (8.899), EPA (22.944) and linoleic (1.662) were detected. Many of these acids are not observed in the Cuban shark liver oil under study, they are found in much lower percentages, only palmitic acid (18.85%). For his part Venugopal et al. (2016) performed a characterization of the *Echinorhinus brucus* liver oil, where they found that the fatty acid that presented the highest percentage in this was palmitic with 14.79%, followed by oleic (12.13), stearic (8.27) and lastly myristic (2.36), in accordance with previous studies (Lopez-Garcia et al., 2005). Studies carried out by Akpinar et al. (2009) in livers and muscles of *Salmo trutta macrostigma* demonstrated that palmitic acid was found in concentrations similar in our samples (19.0 to 21.6%), followed by stearic acid (5.32 to 11.3%) above the contents of our samples, oleic acid had contents (15.6 to 22.4%) below that found in our study, while myristic acid was not present in the samples studied.

So far, comparisons between fatty acid contents in fish have been made using the GC-FID technique; however, ^{1}H-NMR can also be used, as

demonstrated Bratu et al. (2013). In this study, the concentrations of 37 fatty acid methyl esters were determined in seven fish species, with oleic acid being the one with the highest concentration in all species (27.9 to 16.9%), followed by palmitic acid, with concentrations ranging from 22% and 13%, concentrations that are close to ours. CG-MS can also be used (Truzzi et al., 2017), the determination in *Trematomus bernacchii*, showed the myristic, palmitic, oleic and stearic acid contents are found were 8.38 ± 0.9, 9.10 ± 0.8, 26.7 ± 0.9 and 1.36 ± 0.3%, respectively, which are higher than that of shark liver oil except for palmitic acid. Pravinkumar et al. (2014) made a determination in *Sardinella longiceps* (Valenciennes, 1847) coming from the Muttom coasts, myristic acid is at 6.01% and stearic acid had 9.57% in crude oil, much higher than the content of these in shark liver oil, while palmitic acid had 16% and oleic 14.78% results lower than ours. Prato and Biandolino (2012) studied the lipid composition of important commercial species in the Mediterranean Sea, palmitic acid being the one with the highest concentration in all species with ranged between 32.44 and 27.48%, followed by oleic acid (5.72 to 12.98%), concentrations that found above and below those obtained in our research, the stearic acid content is similar to that reported in our research since it has a range of 5.87 to 7.88%, being the myristic in this research one of the lowest concentration presents (3.63 to 4.97) is higher than that reported for shark liver oil.

The fatty acids have not only been isolated from animal tissues, but have also been identified and quantified in plants. Omeh et al. (2013) quantified the content of fatty acids in seeds of *Irvingia gabonensis*, where they managed to quantify six of these compounds, with the highest concentration being myristic acid (51.85%), followed by lauric acid (35.12%); both in concentrations much higher than the shark liver oil under study, another of the fatty acids present was palmitic (7.95), which is below the concentrations of shark liver oil. They have also been quantified in cultures of the *Spirulina* cyanobacteria where palmitic acid had 49.9 ± 0.6%, stearic 1.2 ± 0.2, oleic 2.7 ± 0.3, results that are contrary to those seen so far (Chaiklahan et al., 2008). In other studies in *Citrus* seeds oils from the Atacama desert in Chile, a high concentration of palmitic acid (>23%), stearic acid (>4.9%), and oleic acid (>22%) have also been verified by Garrido et al. (2019), the researchers applied different methods of extraction, on the other hand, among the extraction techniques, Soxhlet has had a better result in the oil extraction yield compared to novel methods, such as ultrasound-assisted extraction.

CONCLUSIONS

Fatty acid (FA) composition is one of the most important indicators of quality and physicochemical properties of oils. A GC-FID method was developed for simultaneous analysis of major fatty acids identified in shark liver oil pool of species *Ginglimostoma cirratun*, *Carcharhinus longimanus*, and *Carcharhinus falciformis*, captured in the Caribbean Sea near Villa Clara province shore in Cuba. The oil was extracted at a 50°C by 20 min. The fatty acids were analyzed as methyl esters derivatives, using 5% aqueous sulfuric acid in methanol and the methyl laurate was used as internal standard. The method was validated and proved to be specific, precise, and accurate for analysis of four major fatty acids present in shark liver oil.

The results demonstrated that the method had sufficient capability for the accurate quantification of the fatty acids determined in the oil and therefore can be applied in its quality control.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Quero-Jiménez PC	Arias LA	Jorge ME	Molina R	Prieto JO	de la Torre JB	Montenegro ON
Concepts or ideas	x		x	x	x	x	
Design	x						
Definition of intellectual content	x	x					
Literature search	x	x	x	x	x	x	x
Experimental studies	x	x	x				
Data acquisition	x						
Data analysis	x			x	x	x	x
Statistical analysis	x					x	x
Manuscript preparation	x		x	x		x	
Manuscript editing	x		x	x		x	
Manuscript review	x	x	x	x	x	x	x

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