Fatty acid profile evaluation of Isochrysis galbana through the use of acid and alkaline transesterification methods

Evaluación del Perfil de ácidos grasos de Isochrysis galbana mediante el uso de métodos ácidos y alcalinos de transesterificación

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Received: 01-07-2018 Accepted: 12-03-2019

How to quote: Medina, Elena; Ruíz-Domínguez, Maria; Morales, Juan; Cerezal, Pedro (2019). Fatty acid profile evaluation of Isochrysis galbana through the use of acid and alkaline transesterification methods. *Informador Técnico*, 83(1), 68-77.
doi: https://doi.org/10.23850/22565035.1574

Resumen

Isochrysis galbana es una microalga marina destacada por contener una gran diversidad de biomoléculas de interés antioxidante. Su alto contenido en lípidos permite su uso en acuicultura o como fuente de biocombustible, por su perfil de ácidos grasos rico en poliinsaturados como Ácido Docosahexaenoico (DHA) o por su alto contenido en fucoxantina (carotenoide). Desde hace un siglo esta microalga es conocida como alimento para bivalvos, larvas de peces y/o moluscos. Por otra parte, la transesterificación es la reacción necesaria para poder derivatizar los ácidos grasos en Ácidos Grasos Metilados (AGM) y poder identificarlos y cuantificarlos; este es un paso clave para optimizar y conocer la mejora del perfil de ácidos grasos obtenido desde la biomasa de estudio. Por consiguiente, este trabajo presentó la diferencia significativa del perfil de ácidos grasos y contenido de los mismos de *I. galbana* a partir de 8 Métodos de Transesterificación Directa e Indirecta (MTD y MTI, respectivamente), además del uso de catalizadores ácidos y alcalinos (AC1, AC2 y AL1 y AL2). Los resultados arrojaron mejores contenidos de ácidos grasos metilados respecto a la biomasa seca en el método MTD- AL1 con un ~6 % y de una abundancia relativa de DHA del ~12 % en el método MTI-AL2. Asimismo, el perfil de ácidos grasos más abundante presente en la microalga se destacó en MTD-AL2 con un 57,66 % en poliinsaturado. Por otro lado, la adición de un patrón interno en las experiencias llevadas a cabo, pudo identificar que los métodos MTD-AL1 y MTI-AC1 obtuvieron mayor eficiencia en la transesterificación con un ~93 % y ~87 %, respectivamente. Por consiguiente, el método que se seleccione para la lectura correcta de ácidos grasos presentes en cualquier biomasa es relevante para observar un perfil más rico en insaturaciones como se ha comprobado con la microalga *I. galbana.*

Palabras clave: lípidos; ácidos grasos; ácido docosahexaeoico; ácidos grasos poliinsaturados; ingredientes funcionales; antioxidantes.

Abstract

Isochrysis galbana is a marine microalga that highlights by containing a great diversity of antioxidant biomolecules. It shows a high content of lipids that can be used in aquaculture and biofuel, for its high polyunsaturated fatty acid profile such as DHA (docosahexaenoic acid) or for having high fucoxanthin content (carotenoid group). For a century, this microalga is very well known as feed for bivalves, the larva of fish, crustaceans, and mollusks. Transesterification is the necessary reaction to be able to derivative the fatty acids in methylated fatty acids (AGM) and thus be able to identify and quantify them. It is a key step to optimize and therefore know the improvement of the obtained fatty acid profile obtained from the studied biomass. This work presents the significant difference of eight methods for obtaining fatty acids from the direct and indirect transesterification reaction (MTD and MTI, respectively). Moreover, acidic and alkaline catalysts were also used (AC1, AC2 and AL1 and AL2). The results presented better contents of AGMs (methylated fatty acids) respecting dry biomass in the MTD-AL1 method with ~ 6% and a relative abundance of DHA of ~ 12 % in MTI-AL2. In addition, the most abundant fatty acid profile was MTD-AL2 with 57.66 % in polyunsaturated. On the other hand, thanks to the addition of an internal standard in the experiences, it was possible to identify that the MTD-AL1 and MTI-AC1 methods were the most efficient in the transesterification obtained with ~ 93 % and ~ 87 %, respectively. Therefore, the method that is selected for the correct reading of fatty acids present in any biomass is relevant to detect a profile with more unsaturation as has been purchased with the microalga *I. galbana.*

Keywords: lipids; fatty acids; docosahexaenoic acid (DHA); PUFAs; functional ingredients; antioxidants.

Introduction

In recent years, interest in producing functional foods has increased in the grocery industry. This term was introduced since the 80s in Japan, where the ability of certain foods to minimize the incidence of chronic diseases from a given diet is shown (Aronson, 2017). Thus, improvements in the immune system, endocrine, nervous, circulatory and/or digestive system have been described (Arai, 1996; Aronson, 2017). The search for new bioactive compounds has grown in recent years due to the growing demand for consumer interest. In particular, Polyunsaturated Fatty Acids (PUFA) are one of the families defined as compounds of interest and/or bioactive compounds, which have taken on more relevance in the food area (Molino *et al*., 2019). This is because its multiple benefits for human health have been demonstrated, such as blood pressure regulation, thrombosis prevention and/or glucose regulation in patients with diabetes (Lemahieu *et al*., 2015; Shahidi; Ambigaipalan, 2018). Within this family of PUFA, Docosahexaenoic Acid (DHA) is described by its bioactive properties as well as its ability to enhance the development of memory and learning in children, as well as reducing cardiovascular problems in older people (Kuda *et al*., 2016; Tang; Qin; Wang; Li; Tian, 2011).

The traditional source of DHA is fish oil, but it may be insufficient for the food production demanded by the market (Sprague; Dick; Tocher, 2016; Tang *et al*., 2011). For this reason, other alternative sources such as microalgae, described among others, have been sought because they contain between 20 and 50% of their dry lipid weight intracellularly. Even under stress conditions, they can reach up to 85% of their production (Chisti, 2007; Santos-Sánchez *et al*., 2016). It is described in some species that between 37 and 45% of its weight may be exclusive content of DHA (Santos-Sanchez et al., 2016). In this way, microalgae in recent years have been the subject of study for their bioactive properties and their potential biotechnological applications (Mendes, Reis, Vasconcelos; Guerra; da Silva, 2009). The microalgae species *Isochrysis galbana* is known to have several positive properties for human health (Kim; Kang; Kwon; Chung; Pan, 2012), in addition to being a species widely used as food in aquaculture, precisely because of its antioxidant potential (Mishra; Mishra, 2018). Recent studies demonstrate the possible use of *I. galbana* as a source of several important biomolecules, such as polysaccharides, fatty acids, carotenoids, vitamins, among others, that improve nutritional value, not only in feed used in aquaculture but also for human consumption. (Guedes; Amaro; Malcata, 2011; Mishra; Mishra, 2018), such as in the production of DHA (Molina; Sánchez; García; Fernández; Acién, 1994; Qi *et al*., 2002). Particularly, in order

to carry out the extraction and quantification of Fatty Acids (AG), a transesterification reaction thereof is carried out. This procedure is very specific and expensive, especially in those destined for the production of biodiesel, transesterification is the most important process (Chen; Lee, 2018). Figure 1 shows the general diagram where organic solvents (alcohols) and both acidic and basic catalysts are used, which improve the extraction of AGs from biomass (oils) (Fukuda; Kondo; Noda, 2001; Sung; Han, 2016). Finally, the so-called AGM Methylated Fatty Acids can be quantified by gas chromatography (Sung; Han, 2016), plus a residue that is glycerol as a coproduct. In the case of acid transesterification, the reagents mostly described are sulfuric, phosphoric and hydrochloric acid, which describe a slower, but more convenient catalysis for glycerols that have free AGs with a higher aqueous content (Chen; Lee, 2018; Fukuda *et al*., 2001; Silitonga; Masjuki; Ong; Mahlia; Kusumo, 2017). However, in basic transesterification carbonates, sodium and potassium hydroxides are commonly used, where catalysis can be up to 4000 times faster than acid, although glycerols and alcohols must be anhydrous, to avoid saponification and reduce transesterification efficiency (Fukuda *et al*., 2001; González; Gallego, 2011).

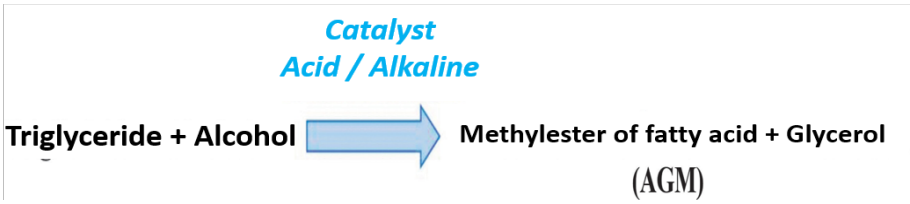

Figure 1. Reacción del proceso de Transesterificación
Source: Self made.

On the other hand, transesterification can be classified as direct or indirect (Figure 2), where the main difference is that the first one works with the biomass directly (either wet or dry), and the second one from a lipid extraction of the biomass (González; Gallego, 2011; Silitonga *et al*., 2017).

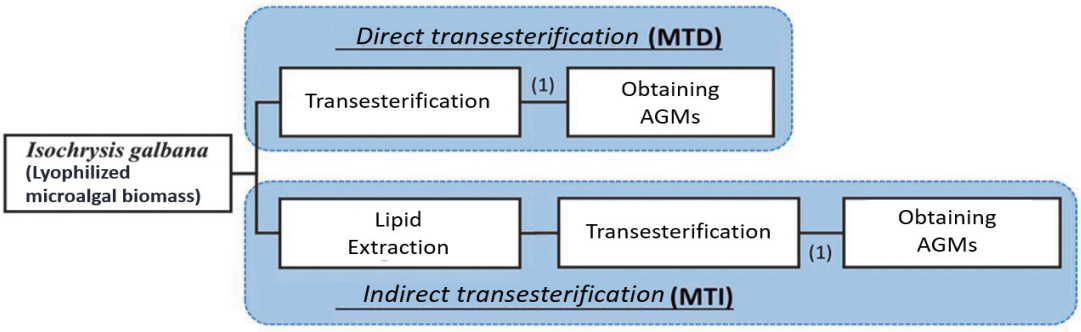

Figure 2. Comparison of direct and indirect fatty acid extraction methods carried out from microalgae *I. galbana*. (1) glycerol and water
Source: self-made.

The work presented a comparison of the various fatty acid profiles resulting from I. galbana from a selection of 8 transesterification methods described in materials and methods. This experience was carried out in two groups that are included as direct and indirect methods called MTD and MTI, respectively, and with four types of catalysts grouped as acids and alkalis (AC1 - AC2 and AL1 - AL2, respectively) in order to determine the influence on the fatty acid profile and particularly on the percentage obtained from DHA, according to the methodology used of *I. galbana*.

Materials and methods

Study material and reagents

The biomass used in this study was the I*sochrysis galbana* microalgae, obtained from the microalgae collection of the Laboratory of Microalgae and Bioactive Compounds (University of Antofagasta, Chile). In all cases, the microalgae was harvested in its exponential phase of growth and lyophilized in a dry frozen system (Labconco Freezone 2.5L Benchtop Freeze Dry System, USA). The reagents used for all tests were of chromatographic quality. Finally, the external standard used in GC-FID gas chromatography equipment (Shimadzu 2010, Japan) was SUPELCO's standard AGM Mix C4-C24 and internal standard C15: 0 (TAG, tripentadecanoic> 99%, NU-CHEK PRE, INC), using a concentration per sample of 10 ppm.

Total lipid extraction and quantification

Total lipid extraction was performed from approximately 20 mg of lyophilized biomass of *I. galbana*, according to the method of extraction chloroform: methanol (2: 1, v / v) modified by Axelsson and Gentili (2014), with continuous stirring. The quantification of total lipids was performed gravimetrically using the following formula 1:

$$L\ (\%) = [(P2-P1)/(Biomasa)\ ]\ x10 \qquad (1)$$

Where L is the percentage of total lipids, P2 is the weight in grams of the vial plus the lipid residue obtained from the lyophilized biomass extraction of *I. galbana* by the method described above, P1 is the weight in grams of the dry vial without sample and Biomass is the lyophilized biomass used in grams of *I. galbana*, for each extraction. Subsequently, the residue obtained from lipids was kept under atmosphere rich in N2 and under darkness to continue with the process of *extracting fatty acids called indirect transesterification.*

Fatty Acid Extraction

The extraction of fatty acids was carried out from the lyophilized biomass of *I. galbana*, (Direct Transesterification, MTD) or from the lipid fraction explained in the previous section (Indirect Transesterification, MTI). In addition, in both cases a comparison was made using an acidic or alkaline/basic catalyst (AC and AL, respectively), presenting the various methods summarized in Figure 3 along with their references. In all cases, AGMs were recovered in n-hexane to proceed with their analysis by gas chromatography - GC-FID.

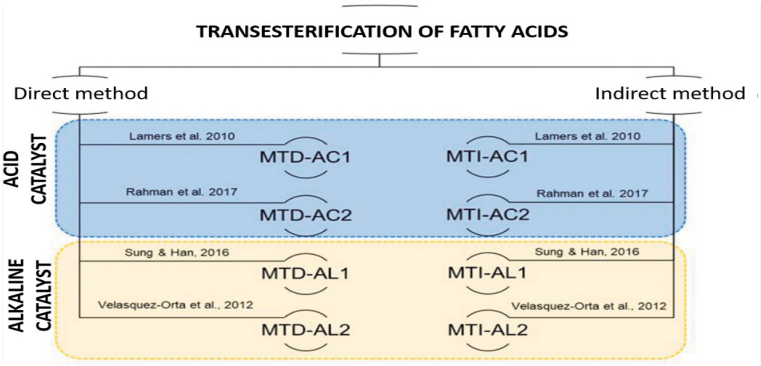

Figure 3. Summary of the methods used for transesterification of AG from lyophilized biomass of *Isochrysis galbana*
Direct Transesterification Methods (MTD): MTD- AC1 (catalyst 10% H_2SO_4 : MeOH* (v/v), (Lamers *et al.*, 2010)), MTD-AC2 (catalyst 1 % H_2SO_4: MeOH (v/v) (Rahman; Aziz; Al-khulaidi; Sakib; Islam, 2017)), MTD-AL1 (catalyst 2,5 M NaOH en MeOH, (Sung; Han, 2016)) and MTD-AL2 (1 % K_2CO_3 : MeOH (p/v) (Velásquez-Orta; Lee; Harvey, 2012)).
Indirect Transesterification Methods (MTI): MTI- AC1 (catalyst 10 % H_2SO_4 : MeOH (v/v) (Lamers *et al.*, 2010)), MTI-AC2 (catalyst 1 % H_2SO_4 : MeOH (v/v) (Rahman *et al.*, 2017)), MTI-AL1 (catalyst 2,5 M NaOH en MeOH, (Sung; Han, 2016)) and MTI-AL2 (1 % K_2CO_3 : MeOH (p/v) (Velásquez-Orta *et al.*, 2012)*MeOH: metanol.
Source: self-made.

Quantification of fatty acids by gas chromatography - GC-FID

Quantification of AGMs: The gas chromatograph (GC Shimadzu 2010, Japan) equipped with a Flame Ionization Detector (DIL) and with a split / splitless injector, was used to analyze the composition of AGMs. In all cases, the samples were injected into a RESTEK capillary column (30 m, 0.32 mm ID, 0.25 μm thickness). The injector temperature was maintained at 250 °C in split mode, with a 4.5: 1 ratio and the carrier gas was nitrogen at a constant flow of 11.25 mL • min − 1. The oven temperature was 80 °C for 5 min, subsequently increased to 165 ° °C at 4 °C • min – 1 for 2 min, continued to increase to 180 °C at 2 °C • min – 1 for 5 min. It was heated with a gradient of 2 °C • min – 1 at 200 °C for 2 min. Then, again it was heated with a speed of 4 °C • min – 1 at 230 °C for 2 min and finally that temperature was maintained for 2 min, reaching 250 °C at 2 °C • min – 1, while the temperature of the detector was 280 °C. Individually, the AGMs were identified by comparing their retention time with the standard AGM mix (AGM Mix C4-C24, Supelco Analytical) and were quantified, according to the comparison of the area thrown under the peaks of the mix set using LabSolutions version 5 software compatible with Windows.

Transesterification Efficiency: to know the efficiency in the transesterification reaction of each of the methods used, an internal standard amount of 100 ppm per sample (Tripentadecanoic> 99%, NU-CHEK PRE, INC) was added from the beginning of the reaction, where Transesterification efficiency was calculated using the following formula 2:

$$\eta TR, (\%) = (IS2/(IS1) \times 100 \qquad (2)$$

Where ηTR is the efficiency of transesterification in percentage, IS2 is the concentration of the internal standard calculated in a gas chromatograph (GC-FID) and IS1 is the actual concentration added per sample of the internal standard (~ 100 ppm).

Statistic analysis

All samples were made in triplicate and, in addition, in the case of an internal standard for calculations of transesterification efficiency, one more sample without biomass was added, only with the standard. The acceptance criteria for each of the validation parameters were calculated using the STATGRAPHICS Centurion XVI software, version 16.1.18.

Results and Discussion

The profile of major fatty acids present in *I. galbana* are shown in Table 1, according to the acid and alkaline transesterification method used. It is observed that, between direct and indirect extraction methods, the latter manage to extract a more varied profile of AG. Lipid extraction was carried out with the solvent mixture described above, chloroform: methanol, 2: 1 (v / v) (Axelsson; Gentili, 2014), which was demonstrated in several studies as the most efficient solvent mixture in lipid extraction (D'Oca *et al*., 2011; Sheng; Vannela; Rittmann, 2011). On the other hand, it can be seen that, among the acid and alkaline catalysts, the latter was the most effective in obtaining PUFA, but not Monounsaturated Fatty Acids (AGMI), which have a high interest for the manufacture of functional foods (Xu; Qian, 2014). It should be noted that both catalyze show palmitic acid (C16: 0), palmitoleic acid (C16: 1), oleic acid (C18: 1), and linolenic acid (C18: 3) as more abundant. According to Velásquez *et al*., (2012), an exclusive AG for alkaline catalysis is linolenic acid (C18: 3), and for acid catalysis, myristic acid (C14: 0) and myristoleic acid (C14: 1), which were also observed in the profile of *I. galbana.*

Table 1.
Transesterification Methods

	Direct Method (MTD)				Indirect Method (MTI)			
Fatty acids	AC1	AC2	AL1	AL2	AC1	AC2	AL1	AL2
C4:0	-	-	-	5,37	1,27	0,23	1,39	8,43
C14:0	20,44	18,73	13,76	2,17	20,68	20,51	19,16	19,41
C16:0	33,22	35,42	21,99	4,94	41,91	38,37	27,45	-
C18:0	-	-	-	-	0,81	2,97	-	-
∑ Saturated	53,66	54,15	35,75	12,48	64,67	62,08	48	27,84
C14:1	1,37	-	-	-	-	-	2,85	-
C16:1	11,25	9,43	9,78	2,15	-	9,17	9,61	-
C18:1	33,08	35,13	34,19	-	3,95	25,46	23,40	12,17
C24:1	-	-	-	-	-	-	-	4,25
∑ Monounsaturated	45,7	44,56	43,97	2,15	3,95	34,63	35,86	16,42
C18:2	-	-	-	12,24	3,38	-	-	-
C18:3	-	-	15,80	40,28	0,97	-	13,99	10,62
C20:4	-	-	-	-	-	0,83	-	10,11
C20:3	-	-	-	5,04	-	1,35	-	-
DHA (C22:6)	0,64	1,29	0,60	0,10	2,20	1,11	2,15	12,32
∑ Polyunsaturated	0,64	1,29	16,4	57,66	6,55	3,29	16,14	33,05
Others	-	-	3,88	27,71	24,82	-	-	22,69

Profile of major fatty acids present in I. galbana, according to the transesterification methods used (% area, n = 3, ± SD was not represented in the table, but was less than 5%)
Source: self-made.

Regarding the abundance obtained from DHA, Table 1 shows that a greater quantity was obtained through the MTI-AL2 method (12.32%) followed by MTI-AC1 (2.20%), which reaffirms what has continuous previously where the lipid phase that involves indirect extraction would enhance the obtaining of DHA from the microalgae, in order to generate functional foods with multiple benefits for human health (Kim *et al*., 2012).

Figure 4 shows the content of AGMs obtained from the *I. galbana* microalgae, according to the transesterification method used concerning biomass and lipid quantification. The MTD-AL1 method was the one with the highest content of AGM concerning biomass and lipids, which showed 59.60 and 124.15 mg / g, respectively, which makes it possible to establish that the lipid phase carried out in indirect extraction does not have a greater influence on the content obtained as total AGM. These results show that since direct extraction is the most effective way to extract a greater amount of AG from the microalgae, the process is faster and cheaper in terms of solvent use. However, as described by Fukuda *et al*., (2001); Santos-Sánchez *et al*., (2016), alkaline transesterification is faster than acidic. However, the MTD-AL2 and MTI-AL2 methods were the ones with the lowest AGM concentrations, although the proportion of DHA was 12% in the case of MTI-AL2. Although it was the case with the greatest abundance of all, it did not imply the fact that it was the one that obtained the greatest amount, since there was 0.28% AGM for dry biomass. In this way, the MTD-AL1 method was not only the one that obtained the best result from AGM but also the best concerning the relative abundance of DHA, being inferior to that described by Santos-Sánchez *et al*. (2016), reaching 5.9% of DHA.

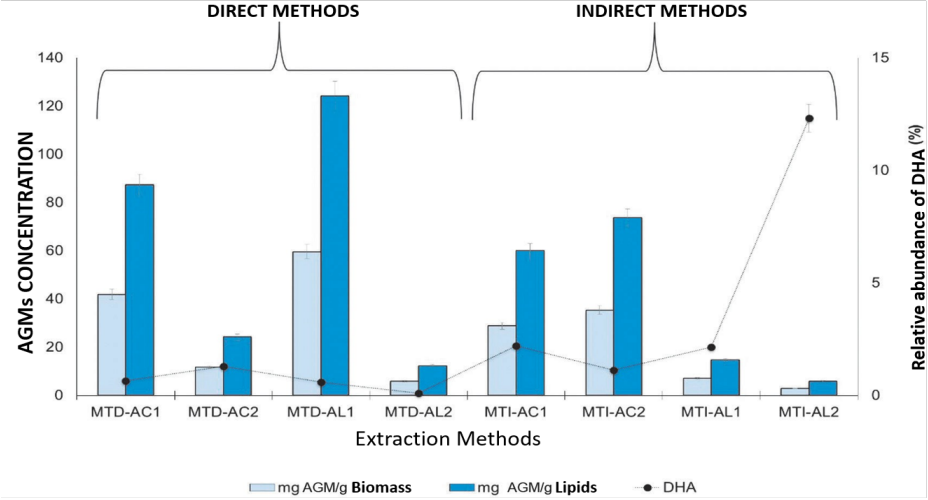

Figure 4. AGM content with respect to biomass and lipids of the microalgae *I. galbana* and relative abundance of DHA (%)
Source: self-made.

Figure 5 shows the effectiveness of each transesterification method calculated from the internal standard added since the beginning of the reaction.

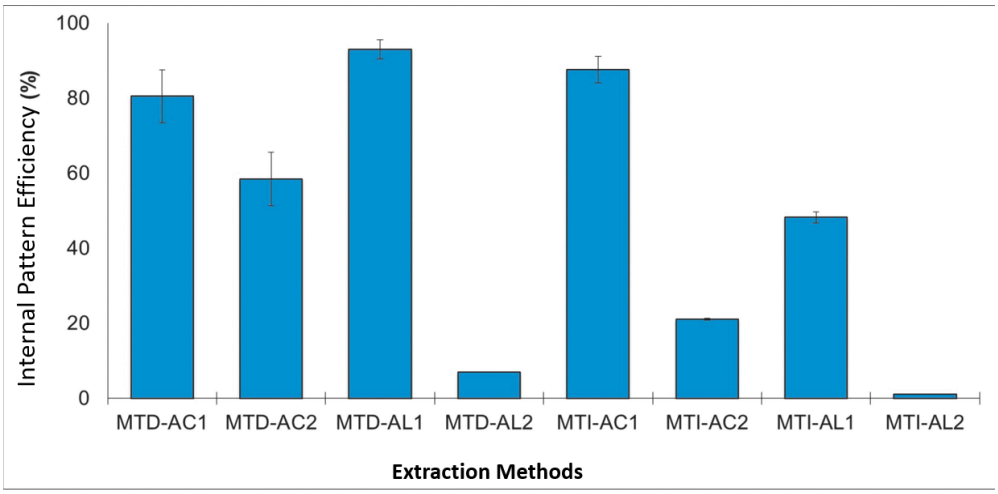

Figure 5. Efficacy of direct and indirect transesterification methods
Source: self-made.

The MTD-AL1 method presented the highest efficacy with 93.03% followed by MTI-AC1 with 87.61%. However, the MTI-AC2 method was one of the lowest (~ 21%), its the one shown in Figure 4 with an average content of AGM regarding the biomass (35.33 mg / g), that according to other authors, it could have happened because in itself the acid catalysis is slow, and a number of fatty acids present in the biomass could have degraded (Fukuda *et al*., 2001). Finally, the methods MTI-AL2 and MTD-AL2 were the ones that had less efficiency (~ 1.0-7.0%), coinciding with the fact that they were the ones with the lowest concentrations of AGM with respect to biomass and lipids (Figure 4). These results coincide in part with what was described by Chen and Lee (2018), in the study of acid transesterification optimization fatty microalgae *Monoraphidium* sp as direct methods. The work concludes with an improvement in AGM performance, using acid transesterification instead of alkaline from both wet and dry biomass. In addition, it proposes a continuous transesterification of catalysts, acids and

basic giving better results a combination of both, which could be raised for further research. Similar results were observed in the work developed by Chamola, Khan, Raj, Verma and Jain (2019), from a Box Behnken design (Narula; Thakur; Uniyal; Kalra; Jain, 2017), whose factors were the percentage of methanol, of catalyst (acid or alkaline) and the concentration, thereof their study reflects an improvement in the performance of AGM under acid catalysis, which presents data very often with alkaline (89.5 and 87.4% of AGM performance, respectively). Although our study highlights MTD-AL1 (2.5M alkaline NaOH catalyst), it is the only case, since in general, both direct and indirect methods resulted in more efficient reactions with acid catalysts (H_2SO_4) and a higher percentage (10% v / v).

Conclusions

Among the eight extraction methods used, the most efficient for the extraction of DHA was MTI-AL2 with more than 12% relative abundance of DHA. However, due to the low content of fatty acids, the productivity of DHA is not consistent. On the other hand, MTD-AL1 was the process with the richest mono and polyunsaturated profile (43.97% and 16.4%, respectively, in addition to containing a higher content of AGM than the biomass of ~ 6%, which implies approximately 12.4% of the lipid content of *I. galbana*. If we make a comparison between the direct and indirect extraction methods, the direct methods were able to extract a greater amount of DHA than the indirect ones, so the extraction process was carried out more quickly, which prevents any AG from degrading. Finally, it was observed in all cases that the *I. galbana* microalgae are marine microalgae with a profile rich in fatty acids useful in food applications, in addition to containing DHA, an important functional ingredient, which makes *I. galbana* can be a potential candidate as a natural source of bioactive compounds.

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