

# Evaluation of in situ (trans) esterification of lipids from the green microalga *Chlorella minutissima* for improvement the yield of FAEE.

Pedro, Guilherme A.<sup>1</sup>, Amaral, Mateus S.<sup>1</sup>, Zorn, Savienne M. F. E.<sup>1</sup>, Loures, Carla C. A.<sup>2</sup> e Silva, Messias B.<sup>1,3</sup>

<sup>1</sup>Engineering School of Lorena-  
University of São Paulo-USP  
Lorena, SP, Brazil.

Centro Federal de Educação Tecnológica Celso Suckow da Fonseca  
Angra dos Reis-RJ, Brasil

<sup>3</sup>Engineering Faculty of Guaratingueta  
State University Julio de Mesquita Filho-UNESP,  
Guaratingueta, SP, Brazil.

**Abstract**— The production of biodiesel from microalgae has become promising in view of the exhaustion of non-renewable sources to produce fuels. The cultivation of microalgae has proved advantageous as a raw material to produce biofuels. However, there is a need to explore some factors and methodologies in the production of microalgae biodiesel for the process to be feasible. This paper analyzed the direct transesterification (in situ) reaction of the *Chlorella minutissima* microalgae biomass in two Taguchi L4 Orthogonal Array. The ethanol/biomass, chloroform/biomass and sulfuric acid/biomass ratios were analyzed in the direct process conversion of microalgae lipids. Four ethanol/biomass ratios were studied and separated in the two experimental matrices for the most effective ascertainment of the alcohol effect in the in situ process. The Ethanol/biomass ratio proved to be indispensable to achieve a higher mass yield of ethyl esters in the in situ reaction process. The maximization of the mass yield (% w / w) of Process in situ for the microalga *Chlorella minutissima* occurs with the use of 25 ml/w to 50 ml/w ethanol. The best adjustment of the parameters surpassed the traditional process of solvent extraction of the lipids in 3.9% of mass more. The adjustment reached a percentage of lipid conversion in Fatty Acid Ethyl Ester (FAEE) of about 88% at 95 ° C and in 90 minutes of reactor operation.

**Keywords**— *Transesterification; in situ; FAEE; Microalgae, Chlorella minutissima, Taguchi Orthogonal Array*

## I.

### INTRODUCTION

Global energy consumption in the last century was strongly based on fossil energy consumption which accelerated CO<sub>2</sub> emissions into the atmosphere, accounting for 63% of greenhouse gases [1]. Even so, an increase in the consumption of

renewable energy and natural gas is predicted [2]. Most of this growth belongs to the electric power sector, having the hydric energy as more responsible, however, it is still expected to grow by 3.1% per year in the use of biomass for energy production [2].

In this way, biodiesel plays an important role in the country's energy policies. Not in replacing petroleum diesel, but in bringing positive impacts on the environment and government policies [3]. In Brazil, according to the Ministry of Mines and Energy (MME), biodiesel production increased by 19.2% between 2014 and 2015. [4]. However, the national biodiesel production profile has the dominance of soybean oil as raw material as shown in Figure 1.

Reference [5] indicates basic criteria for the selection of raw materials to produce biofuels: it should not be a food oil; it should not occupy agricultural land for food production; it should not cause deforestation; its cost should not be higher and its yield should not be lower than that of palm oil; and its production must be socially sustainable. Consequently, Brazilian biodiesel production does not meet these criteria, since about 98% of the raw material for biodiesel is grown in food production areas. Reference [5] further states that these criteria are not easily achieved, however, microalgae is a potential candidate for sustainable biofuel production.

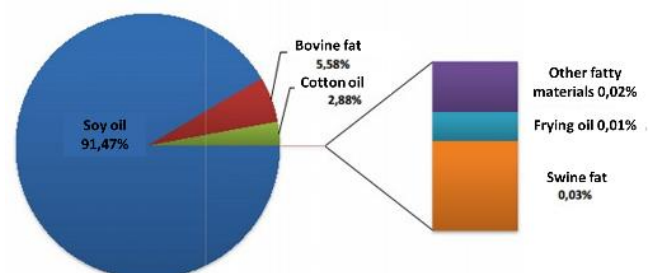


Fig.1 National profile of raw materials for biodiesel production in December 2015. Source: ANP (2016)

Thus, the production of biofuels from microalgae cultivation has become relevant, since microalgae do not compete with food sources such as soy or corn, for example [6], and can be grown in different locations, even in arid regions, occupying relatively little space, with high productivity in biomass [7]. Due to the scarcity of fresh water in several regions of the world, species of marine microalgae have become advantageous, as is the case of *Chlorella minutissima*, because they grow naturally in salt water and can therefore be cultivated with the use of the same sea water [8]. Consequently, the cultivation of this and other similar microalgae becomes advantageous, as a renewable source to produce biofuels.

Chemically, transesterification of oils from renewable sources is one of the most effective ways and one of the most important steps in producing a clean and environmentally friendly fuel [5]. However, the industrial route of production of microalgae biodiesel has its productivity limited by the extraction stage [6], representing about 20 to 30% of the biodiesel production cost [7]. Direct transesterification of microalgae biomass is a potential alternative in reducing production costs by eliminating the extraction stage, saving the high consumption of solvents and labor time [8]. In-situ transesterification, a Latin expression meaning "in situ", is notorious for performing lipid extraction and the transesterification reaction concomitantly, in a single step process. The conversion takes place in acid or base catalyzed medium, however, the microalgae oil is known to have a high acid content [9]. In this way, basic catalysts are avoided so that secondary reactions do not occur, such as the saponification reaction. Therefore, the acid-catalyzed route is the most suitable for the biodiesel synthesis from microalgae. However, acid catalysis still yields relatively low yields for scaling. However, the incorporation of co-solvents, such as hexane or chloroform in the reaction medium, contributes to increase the yield of the reaction, by helping to extract the lipids at the moment of reaction [6], [10]. Reference [11] also mentions that the ultrasonic radiation is effective in the breakdown of the microalgae cell wall.

Nevertheless, reference [12] performed an energy analysis of the process of in situ transesterification and that even its experimentation presenting lower yield than literature, shows that it is energetically feasible to apply this type of process to obtain biodiesel.

In addition, the choice of the type of alcohol in the transesterification may or not favor some steps and factors of the process. Methanol is widely applied due to commercial reasons and its physicochemical characteristic of higher reactivity [13]. In contrast, methanol is known for its high toxicity, while ethanol is well regarded as being a non-toxic, renewable reactant. Although the use of ethanol implies problems in the separation of glycerol from the reaction medium [14], with additional steps being required such as centrifugation with or without addition of co-solvents or surfactants [15], the advantage of the ethyl route, as the reference [13] shows, is in the great offer of this

alcohol throughout the national territory influencing the costs of production of biodiesel, in addition to the environmental point of view of being a sustainable reagent. Thus, the use of ethanol for the Brazilian production of biodiesel is more feasible and recommended to meet the ecological and industrial appeal imposed on the production of biofuels. Therefore, obtaining better yields in the biodiesel synthesis process is of interest to the industrial sector. For the purpose of analysis, the experimental planning is used in the experimental design, since it is a statistical tool that allows the purposeful alteration of the input variables of a process and later analyzes how the system responded to the changes undergone [16]. One of the tools of experiment planning is the orthogonal arrangement of Taguchi's methodology. The Taguchi methodology emphasizes the approach between engineering and quality, while minimizing the variation in the performance of a particular product or process, increasing its quality and therefore customer satisfaction [16].

In this way, this work was dedicated to the exploratory analysis of the transesterification reaction for the microalgae *Chlorella minutissima* in homogeneous systems, in acid and in situ in a Taguchi experimental matrix L4. The amount of Ethanol used in the study was divided into four Ethanol / Biomass ratios: 100, 50, 25 and 10 ml / w. The ratios were organized in two experimental arrangements L4 as shown in Figure 2.

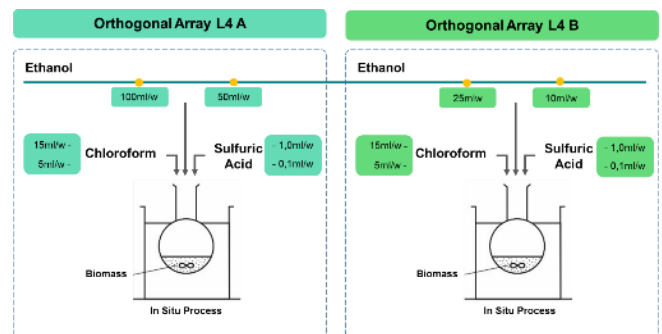


Fig. 2 Graphical Abstract

## II. MATERIALS AND METHODS

### A. Microalgae Cultivation

The inoculum used was a line of the microorganism *Chlorella minutissima*, isolated in Cabo Frio - RJ, belonging to the USP Oceanographic Institute Marine Algae Bank, kindly donated by the Department of Biological Oceanography of the USP Oceanographic Institute, São Paulo. The biomass was obtained from cultures carried out at the Microalgae Engineering Laboratory (Lorena School of Engineering). Three photobioreactors bubble columns were used with a volume of 40 liters each, aeration of 1.33 vvm and illuminated by 40W fluorescent bulbs 24 hours a day for seven days. The Figure 3 shows the photobioreactors used to obtain the biomass.

Cultures were conducted on f / 2 medium without silica [] and biomass harvesting was performed by

flocculation induced by 1N Aluminum Sulphate, followed by vacuum filtration. The wet biomass was placed in an oven at 60 ° C for drying the sample. After 24h of drying, part of the sample was separated and weighed for determination of moisture and solvent-extractable lipid content.



Fig. 3 Photobioreactors used to obtain biomass.

**B. Determination of percentage of extractable lipids**

The extraction consisted of the joint action of organic solvents and ultrasound. About 515 mg of sample was initially placed in a 2: 1 (v / v) methanol: chloroform mixture and then it was introduced into the ultrasonic bath for 25 minutes. This step was repeated with a 1: 2 (v / v) methanol: chloroform mixture and it was reinstalled in an ultrasonic bath for another 25 minutes. The entire mixture was filtered with filter paper for the separation of the residual biomass. The liquid fraction containing the oil was placed in an oven for the solvents evaporation until constant weight.

**C. Experiment Design and Statistical Analysis**

This study aimed to explore the factors for the transesterification in situ from marine microalgae *Chlorella minutissima* biomass. Therefore, it was decided to work with a Taguchi orthogonal matrix L4 (Table I) that allows the analysis of up to three factors in two levels. Table II (A and B) presents the factors studied and respective levels in the reactive extraction process. The response variable was the percentage yield and FAEE content determined by the RMN1H spectrum. The mass yield percent was calculated by the formula:

$$\text{Yield (\% w/w)} = \frac{B}{C \cdot M} \quad (1)$$

Where B is the mass of ethyl esters obtained by the process in situ; C is the percentage of lipids contained in the material (dry mass %); M is the mass of the dry biomass placed in the reactor. Thus, equation (1) relates how much the TEIS process can recover the lipids compared to lipids extracted with solvents only in the traditional extraction method.

TABLE I. TAGUCHI L8 ORTHOGONAL ARRAY

Experiment	Factor		
	A	B	C
1	1	1	1
2	1	2	2
3	2	1	2
4	2	2	1

TABLE II. FACTORS AND LEVELS OF EXPERIMENTAL PLANNING FOR THE ETHANOL / BIOMASS RATIOS 50ML / W AND 100ML / W (ORTHOGONAL ARRAY L4 A)

Coded Factor	Factor	Level 1	Level 2
A	Ethanol/Biomass (ml/w)	50	100
B	Chloroform/Biomass (ml/w)	5	15
C	Sulfuric Acid/Biomass (ml/w)	0,1	1

TABLE III. FACTORS AND LEVELS OF THE EXPERIMENTAL PLANNING FOR THE RATIOS ETHANOL / BIOMASS 10ML / W AND 25ML / W (ORTHOGONAL ARRAY L4 B)

Coded Factor	Factor	Level 1	Level 2
A	Ethanol/Biomass (ml/w)	10	25
B	Chloroform/Biomass (ml/w)	5	15
C	Sulfuric Acid/Biomass (ml/w)	0,1	1

Statistical analysis was performed by Statistica software (version 13) with the factor effects graphs generation and the variance analysis table.

**D. In Situ (Trans)esterification Reaction and Product Separation**

The synthesis procedure was carried out in a round-bottomed glass flask coupled to a reflux condenser. The addition of the reagents was done according to the matrix of experiments. After addition of the reactants, the reaction was conducted with constant stirring and water bath at 90 ° C. The schematic of the experimental apparatus is shown in Figure 4.

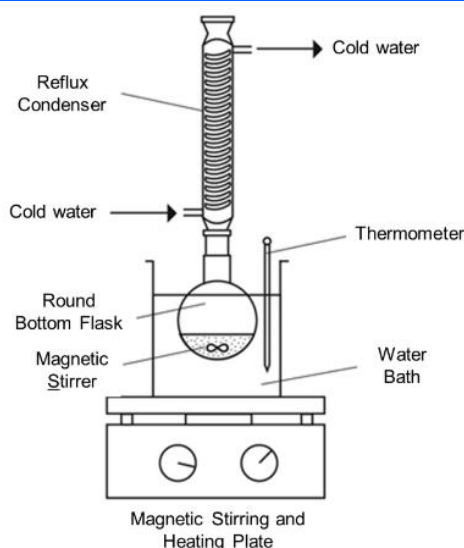


Fig. 4 Experimental apparatus.

The reaction and the steps of the product separation process were based on the references [17] e [18]. At the end of the reaction time the reaction medium was cooled to room temperature. The reaction mixture was then filtered and the biomass residue washed twice with ethyl alcohol for complete product removal. To the filtered solution 0.5 ml of water per ml of filtered solution and 0.5 ml of hexane per ml of filtered solution was added, in order to transfer hydrophilic and hydrophobic compounds to the respective phases. The sample was shaken and placed in a separatory funnel and waited 30 minutes for phase separation. For the organic phase, upper phase, the biodiesel was transferred. For the lower phase, aqueous phase, the excess alcohol, the acid catalyst and the glycerol formed were transferred. After separation, the organic phase was washed twice with water to remove excess acid. Finally, the organic phase was oven-dried at 60 ° C for 24 hours for hexane evaporation. After the period, the biodiesel sample was weighed to determine the mass yield of ethyl esters. Each experimental condition was performed in triplicate. The process yield was determined in grams of ethyl esters per gram of extractable lipids in the microalgae biomass.

#### E. $RMN^1H$ Analysis

Nuclear Magnetic Resonance of Hydrogen ( $RMN^1H$ ) analysis applies to account number of hydrogens present in the sample compound, confirming the conversion of the lipids into FAEE. In this work it was used Varian spectrometer model Mercury-300 MHz. Samples were diluted in deuterated chloroform ( $CDCl_3$ ). The presence of FAEE in a  $RMN^1H$  spectrum is the quartet between 4.05 and 4.35 ppm, characteristic of the ethoxide group formed during the (trans)esterification reaction. According to reference [14], it is possible to determine the proportion of FAEE and unconverted oil in the transesterification reaction relating the fourth peak area to 4.08 ppm and the area of all signals between 4.35 and 4.05 ppm. Samples from each experimental condition of

experiment planning were analyzed ( $RMN^1H$ ) and the respective spectra are presented in [...]. For each spectrum it was used *MestreC* software for calculating the fourth peak area near 4.08 ppm and the range area 4.35 to 4.05 ppm. From the areas it was estimated the percentage of FAEE content in each sample and presented in the table.

### III. RESULTS

#### A. Statistical analysis

Experiments employing Taguchi's methodology simultaneously investigated effects of three variables involved in in situ FAEE synthesis: Ethanol / Biomass Ratio (ml / w), Chloroform / Biomass Ratio (ml / w) and Sulfuric Acid / Biomass Ratio ). The experimental matrix in duplicate and results are shown in Tables. Table \_\_ presents results for the Ethanol / Biomass ratios 100ml / w and 50ml / w. Table \_\_ presents results for the Ethanol / Biomass ratios 25ml / w and 10ml / w. Each experimental condition is presented in table rows and each column refers to a process variable. Table \_\_ shows mean responses for Yield (% w / w) and FAEE content (%) for each experimental condition.

TABLE IV. EXPERIMENTAL MATRIX AND RESULTS OBTAINED IN IN SITU PROCESS FOR ETHANOL / BIOMASS RATIOS 50ML / W AND 100ML / W (ORTHOGONAL ARRAY L4A)

Experiment	Factor			Response Variable	
				Yield (% w/w)	FAEE content (%)
	A	B	C		
1	1	1	1	83,90 ± 2,76	80,00
2	1	2	2	88,70 ± 3,82	72,00
3	2	1	2	77,84 ± 2,27	64,00
4	2	2	1	65,84 ± 3,97	48,00

A – Ethanol; B – Chloroform; C – Sulfuric Acid.

TABLE V. EXPERIMENTAL MATRIX AND RESULTS OBTAINED IN IN SITU PROCESS FOR ETHANOL / BIOMASS RATIOS 10ML / W AND 25ML / W (ORTHOGONAL ARRAY L4B)

Experiment	Factor			Response Variable	
				Yield (% w/w)	FAEE content (%)
	A	B	C		
1	1	1	1	74,38 ± 3,33	48,00
2	1	2	2	72,75 ± 2,30	80,00
3	2	1	2	86,16 ± 4,16	96,00
4	2	2	1	87,18 ± 2,58	56,00

A – Ethanol; B – Chloroform; C – Sulfuric Acid.

TABLE VI. REACTOR LOAD VOLUME AND SULFURIC ACID CONCENTRATION IN EACH EXPERIMENTAL CONDITION

	Experiment	Reactional Volume (mL)	Acid Concentration (mol/L)
L4 A Array	1	55,10	0,03
	2	66,00	0,28
	3	106,00	0,18
	4	115,10	0,16
L4 B Array	1	15,10	0,12
	2	16,00	1,17
	3	31,00	0,61
	4	40,10	0,05

**B. Factors Effects in Yield (%w/w)**

Yields of each experimental condition obtained in situ synthesis presented values ranging from 65% to about 88%. At Matrix L4 A best results occurred when the lowest level of Ethanol / Biomass ratio was used, that is, 50ml / w (experiments 1 and 2). At Matrix L4 B, better results were found when the highest ethanol / biomass ratio was applied, that is, 25 ml / w (experiments 3 and 4). This result indicates that best conditions in situ process are among Ethanol / Biomass ratios 25ml / w to 50ml / w. In the case of the L4 A matrix, analysis of the effect graphs for averages, shown in the Figure 5 it can be observed that ratio Ethanol / Biomass (Factor A) has greatest impact under in situ process, considering amplitude of the line formed between the maximum point and averages of means, that is, the greater the amplitude, the greater the influence of the factor on the response variable. Followed by ratio Sulfuric Acid / Biomass (Factor C), it has showed a large amplitude, therefore, also significant for the process. These facts are confirmed by ANOVA table, where all factors were significant at a 95% confidence level. Thus, adjustments that suggest maximization of response variable are factors A and B at the low level (50ml / w ethanol and 5ml / w chloroform, respectively). Only factor C is suggested at the high level (1ml of H<sub>2</sub>SO<sub>4</sub> per gram of biomass). In coded variable, suggested setting becomes 1,1,2. However, this adjustment does not exist in L4 Taguchi array, so this adjustment has been tested to confirm the best fit.

Following the same experimental conditions: 95 °C and time reaction 90 minutes, confirmatory test was performed in triplicate with 50 ml ethanol, 5 ml chloroform and 1 ml sulfuric acid, as suggested by the effect graphs, resulting in an average of 103.93% ± 3.76. This result confirms that the adjustment suggested by Taguchi method is in fact the best fit for the chosen levels.

This yield in percentage is calculated based on maximum amount of lipids that can be extracted via solvents, being this a control parameter for in situ process. Thus, obtaining values above 100%, as in this case, means that in situ method was more effective than solvent extraction, or it can be explained by conversion of cell membrane phospholipids that are extracted during in situ process, increasing mass yield.

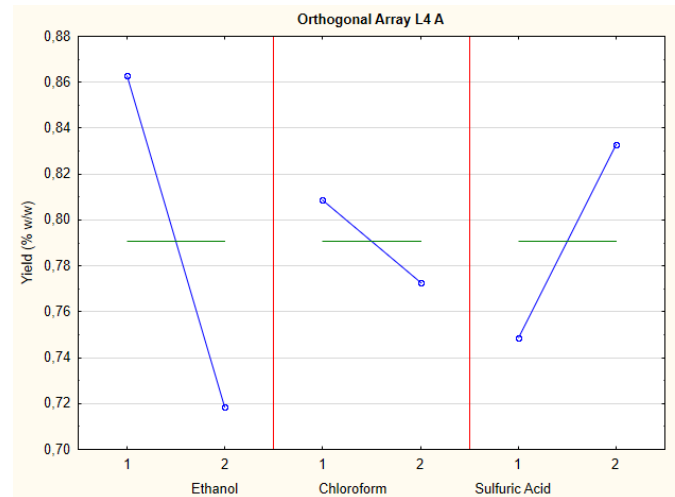


Fig. 5 Main Effects Graphs for Yield Averages at Ethanol/Biomass ratio 50 ml/w and 100 ml/w (Orthogonal Array L4 A)

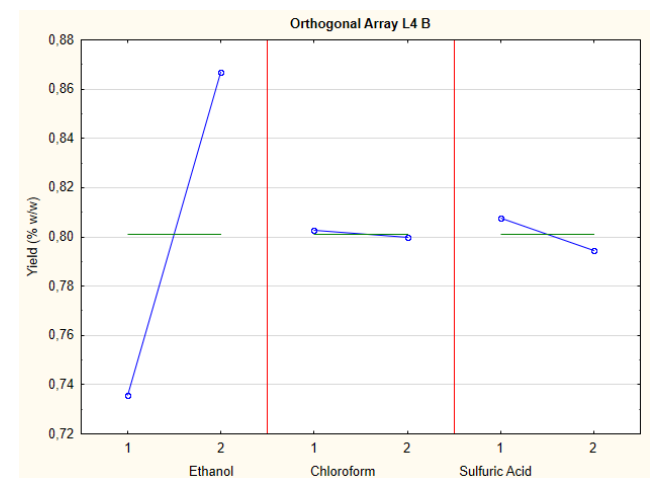


Fig. 6 Main Effects Graphs for Yield Averages at Ethanol/Biomass ratios 10 ml w and 25 ml/w (Orthogonal Array L4 B)

For L4 B matrix (Figure 6), in which the lowest ethanol rates were analyzed in the reaction medium, Ethanol / Biomass ratio was also shown to be factor with the greatest impact on the response variable seen in the effect graph. However, other factors were not significant for the chosen levels. This fact can be confirmed by ANOVA table where only Ethanol / Biomass ratio was significant at a 95% confidence level. Thus, the adjustment that suggests maximization of response variable happens with factor A at high level (25ml / w ethanol). Therefore, no confirmatory test is required.

By analyzing in general two matrices, it is possible to state that in reaction medium with a lot of ethanol (100 ml / w) or little ethanol (10 ml / w) lowest mass yields are obtained. This first situation can be explained by excessive dilution of other reagents in alcohol, that lose their effect in lipid extraction. And the second situation occurs due to ethanol dilution in other reagents.

Thus, it is clear that yield maximization occurs with use of ethanol in ratio 25 or 50 ml / w, an adequate reagents range where excessive reagents dilution effect is not possible, enabling all reagents to have their actions occurring synergistically in the process.

### C. Factors Effects in FAEE content (%)

Conversion percentages estimated by areas RMN1H spectrum range from 48% to about 96%. For L4 A matrix best results also occurred when low level of Ethanol / Biomass ratio was used (experiments 1 and 2). It is noteworthy that as reactor load volume was high, FAEE conversion efficiency was lost. This is due to other reagents dilution by ethanol, in particular Sulfuric Acid, which catalyzes lipid conversion reaction in FAEE.

For matrix L4 B best conversions occurred when adjusting Sulfuric Acid / Biomass ratio level at the high level (experiments 2 and 3), ie 1 ml / w, where acid highest concentrations are present. Again, it is evidenced that FAEE percentage decreases as acid concentration is decreased by reactor final load. Thus, it is important not to carry out in situ process with higher acid concentrations.

In the analysis of effect graphs at experimental matrix L4 A, shown in Figure 7, it is observed that Ethanol / Biomass (factor A) and Chloroform / Biomass (factor B) ratios are factors that have greatest amplitude, therefore they influence FAEE yield. This adjustment suggested at effect graph that maximizes the FAEE content response variable is the same as that suggested for % Yield response variable, that is, factor A and B at the low level (50ml / w ethanol and 5ml / w chloroform respectively). Only factor C is suggested at high level (1ml of H<sub>2</sub>SO<sub>4</sub> per gram of biomass). In coded variable, suggested setting becomes 1,1,2. Such adjustment suggests a condition with lower reactor load, where sulfuric acid concentration is not too low for not efficiently catalyzing FAEE synthesis reaction.

Since this adjustment is the same as that suggested for Yield analysis (% w / w), the same confirmatory test can be used in both. Following same experimental conditions, confirmatory test resulted in an estimated conversion by FAEE RMN1H spectrum in 88%. It is also confirmed, that adjustment suggested by Taguchi method is in fact is the best fit for chosen levels. This indicates that ensuring the sulfuric acid concentration at higher levels in final reactor load is a better way to achieve FAEE higher percentages.

For experimental matrix L4 B, in the effect graph analysis (Figure 8) ratio Sulfuric Acid / Biomass presents the greatest amplitude, therefore the most

influential factor in the FAEE content percentage. Followed by ratio Ethanol / Biomass as a factor of great influence in process. Thus, adjustment that suggests the maximization FAEE variable content (%) happens with factor A at high level (25ml / w ethanol), because at a lower ethanol level acid would be in great concentration, and with factor C (1ml / w sulfuric acid). This suggested adjustment is found at experimental matrix, experiment 3, and no confirmatory experiment is necessary.

In both matrices it is possible to affirm that guarantee of best conversion of lipids in FAEE will always occur when acid catalyst concentration is sufficiently high to guarantee FAEE synthesis. In found adjustments, it was possible to determine that ideal sulfuric acid concentration in the reactor should be about 0.3 mol / L (confirmatory test) at 0.6 mol / L (Experiment 3 L4 B matrix).

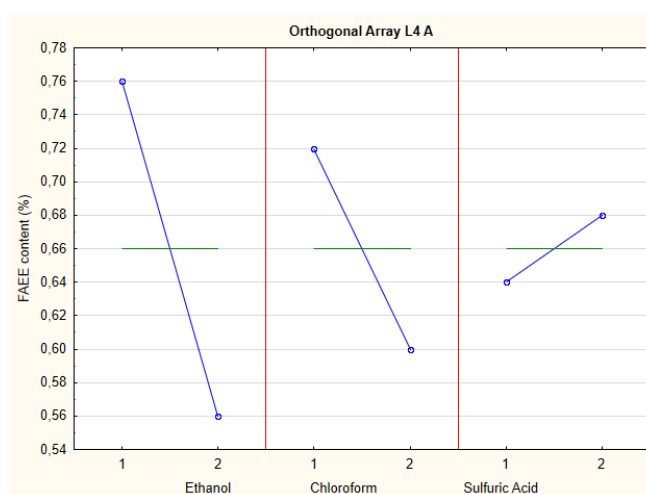


Fig. 7 Main Effects Graphics for FAEE content for Ethanol / Biomass ratio 100 ml / w and 50 ml / w (Orthogonal Array L4 A)

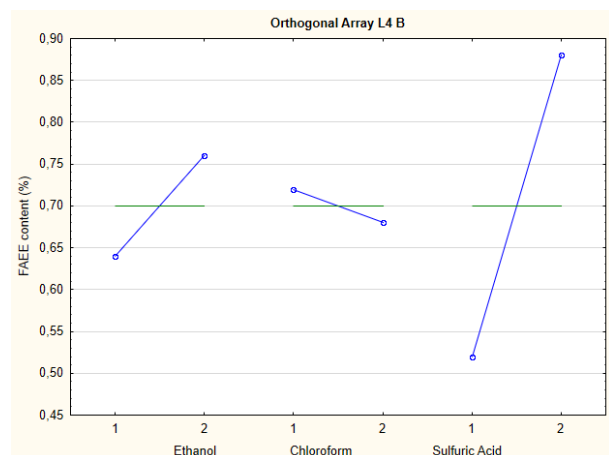


Fig. 8 Main Effects Graphics for FAEE content for Ethanol / Biomass ratio 25 ml / w and 10 ml / w (Orthogonal Array L4 B)

## IV. CONCLUSIONS

This study demonstrates that excessive ethanol volumes reduce in situ process yield. In other studies, excess alcohol is used, therefore, there is loss of lipid yield extracted in in situ process due to dilution of other reagents in process. FAEE yield is strongly dependent

on Ethanol and Sulfuric Acid amount in reaction medium because reaction medium should not overly dilute acid catalyst. Maximal product yield (% w / w) from in situ process for microalgae *Chlorella minutissima* occurs with ethanol 25 ml to 50 ml, quantities that allow the synergistic action of reagents in lipids reactive extraction. Best result was found with 50ml Ethanol, 5ml Chloroform and 1ml Sulfuric Acid. This parameter adjustment overcomes traditional process extraction of lipids with solvents in about 3.9% of extracted lipids. This adjustment achieves a lipid percentage FFAE conversion of about 88%. An important factor in achieving high FFAE conversions is to ensure sulfuric acid concentration in reactor between 0.3 mol / L and 0.6 mol / L.

#### ACKNOWLEDGMENT

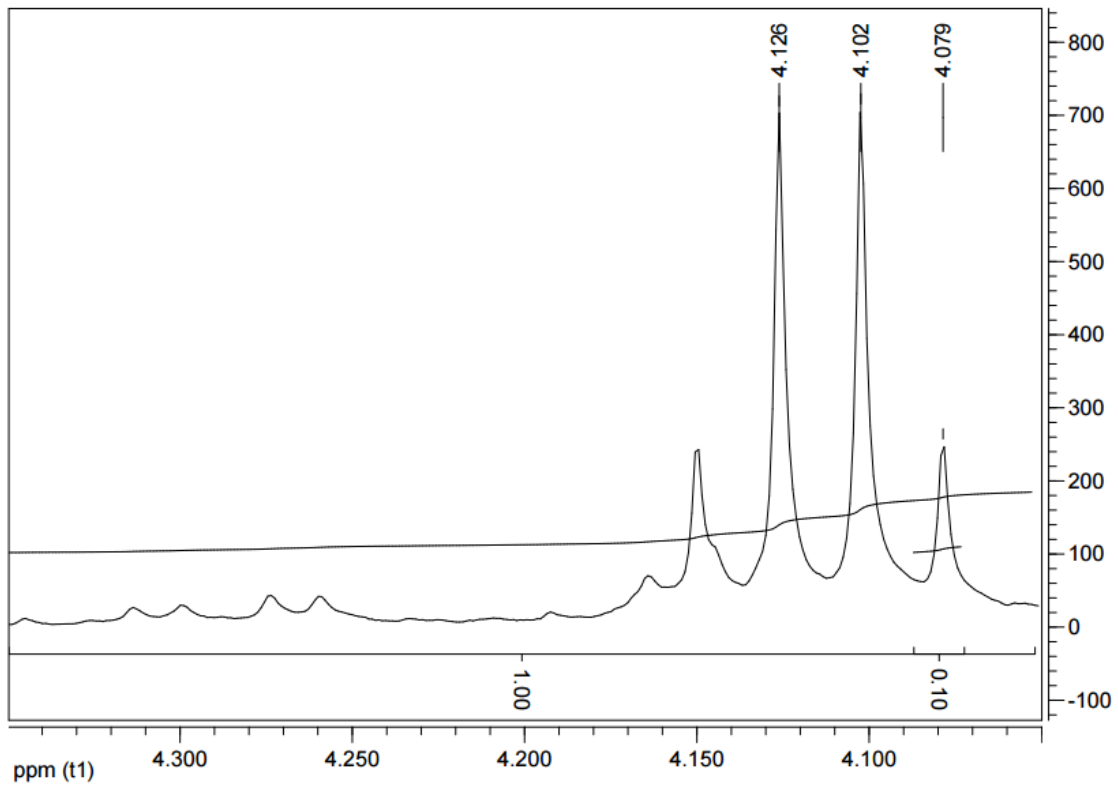
The authors would like to thank the Capes and Oceanographic Institute – USP. We thank Dr. J.C. S. Barboza for <sup>1</sup>H-NMR analysis

#### REFERENCES

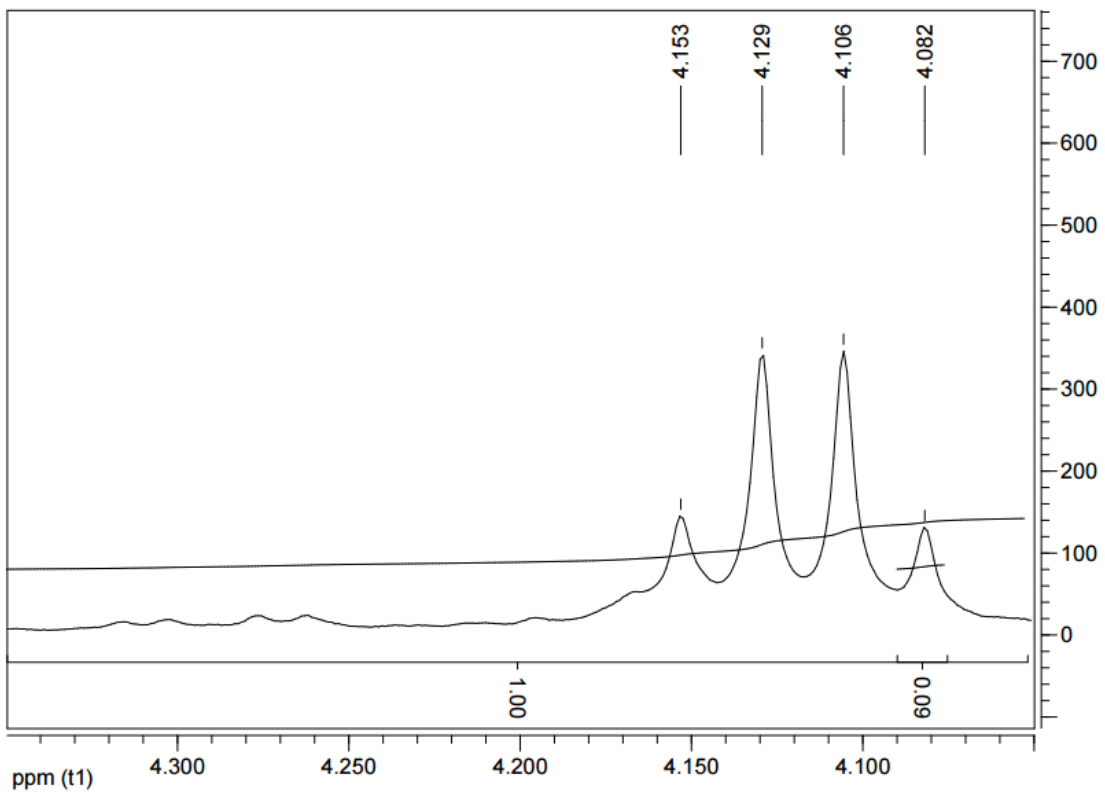
- [1] M. Hattab and A. Ghaly, "Production of Biodiesel from Marine and Freshwater Microalgae: A Review," *Adv. Res.*, vol. 3, no. 2, pp. 107–155, 2015.
- [2] "US Energy Information Administration (EIA)," 2015. [Online]. Available: [http://www.eia.gov/forecasts/aeo/section\\_energycconsump.cfm](http://www.eia.gov/forecasts/aeo/section_energycconsump.cfm). [Accessed: 10-Feb-2016].
- [3] "Biodiesel Benefits - Why Use Biodiesel? - Pacific Biodiesel." [Online]. Available: <http://www.biodiesel.com/biodiesel/benefits/>. [Accessed: 10-Feb-2016].
- [4] M. de M. e Energia, "Produção de biodiesel cresce 10% em um ano," *06/01/2016*, 2016. [Online]. Available: [http://www.mme.gov.br/web/guest/secretarias/petroleo-gas-natural-e-combustiveis-renovaveis/pagina-inicial/spg-destaques/-/asset\\_publisher/hq42wpm81ruP/content/producao-de-biodiesel-cresce-10-em-um-ano;jsessionid=C07E1C6E1C332466E9FD0967DCA21ACC.srv154?red](http://www.mme.gov.br/web/guest/secretarias/petroleo-gas-natural-e-combustiveis-renovaveis/pagina-inicial/spg-destaques/-/asset_publisher/hq42wpm81ruP/content/producao-de-biodiesel-cresce-10-em-um-ano;jsessionid=C07E1C6E1C332466E9FD0967DCA21ACC.srv154?red). [Accessed: 10-Feb-2016].
- [5] T. Mathimani, L. Uma, and D. Prabaharan, "Homogeneous acid catalysed transesterification of marine microalga *Chlorella* sp. BDUG 91771 lipid – An efficient biodiesel yield and its characterization," *Renew. Energy*, vol. 81, pp. 523–533, 2015.
- [6] P. Hidalgo, G. Ciudad, S. Schober, M. Mittelbach, and R. Navia, "Biodiesel synthesis by direct transesterification of microalga *Botryococcus braunii* with continuous methanol reflux," *Bioresour. Technol.*, vol. 181, pp. 32–9, Apr. 2015.
- [7] Y. H. Seo, M. Sung, Y.-K. Oh, and J.-I. Han, "Lipid extraction and esterification for microalgae-based biodiesel production using pyrite (FeS<sub>2</sub>).," *Bioresour. Technol.*, vol. 191, pp. 420–5, Sep. 2015.
- [8] T. Lewis, P. D. Nichols, and T. A. McMeekin, "Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs," *J. Microbiol. Methods*, vol. 43, no. 2, pp. 107–116, Dec. 2000.
- [9] A. L. C. Franco, I. P. Lôbo, R. S. Da Cruz, C. M. L. L. Teixeira, J. A. De Almeida Neto, and R. S. Menezes, "BODIESEL DE MICROALGAS: AVANÇOS E DESAFIOS," *Quimica Nova*, vol. 36, no. 3. pp. 437–448, 2013.
- [10] P. Hidalgo, G. Ciudad, S. Schober, M. Mittelbach, and R. Navia, "Improving the FAME Yield of *in Situ* Transesterification from Microalgal Biomass through Particle Size Reduction and Cosolvent Incorporation," *Energy & Fuels*, p. 150114144614009, 2015.
- [11] D.-Y. Kim, D. Vijayan, R. Praveenkumar, J.-I. Han, K. Lee, J.-Y. Park, W.-S. Chang, J.-S. Lee, and Y.-K. Oh, "Cell-wall disruption and lipid/astaxanthin extraction from microalgae: *Chlorella* and *Haematococcus*," *Bioresour. Technol.*, Aug. 2015.
- [12] R. miguel de Carvalho, "Desenvolvimento e análise energética do processo de obtenção do biodiesel de microalga por metanolise in situ," Universidade Federal do Paraná, 2010.
- [13] M. D. de Á. Völz, "ÓLEOS DE ALTA ACIDEZ: Desenvolvimento de um processo catalítico homogêneo," 2009.
- [14] C. M. Garcia, "Transesterificação de óleos vegetais," Universidade Estadual de Campinas, 2006.
- [15] K. T. Kucek, "Otimização da transesterificação etílica do óleo de soja em meio alcalino," p. 123, 2004.
- [16] R. K. Ranjit, *A primer on the Taguchi Method*, 2<sup>a</sup> Edição. New York: Society of Manufacturing Engineers, 2010.
- [17] E. A. Ehimen, Z. F. Sun, and C. G. Carrington, "Variables affecting the *in situ* transesterification of microalgae lipids," *Fuel*, vol. 89, no. 3, pp. 677–684, Mar. 2010.
- [18] Donald L. Pavia, G. M. Lampaman, G. S. Kriz, and R. G. Engel, *Química Orgânica Experimental - Técnicas de Escala Pequena*, 2<sup>a</sup> Edição. Bookman, 2009.

SUPPLEMENTARY DATA

Experiment 1 Orthogonal Array L4 A

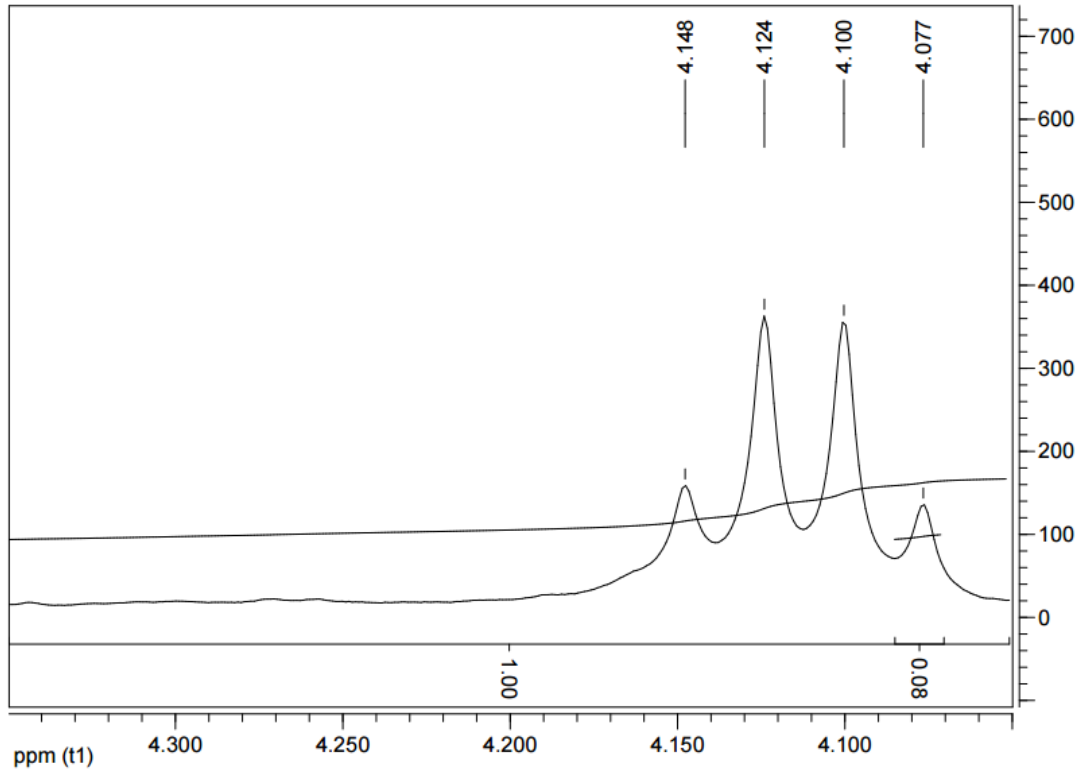


Experiment 2 Orthogonal Array L4 A

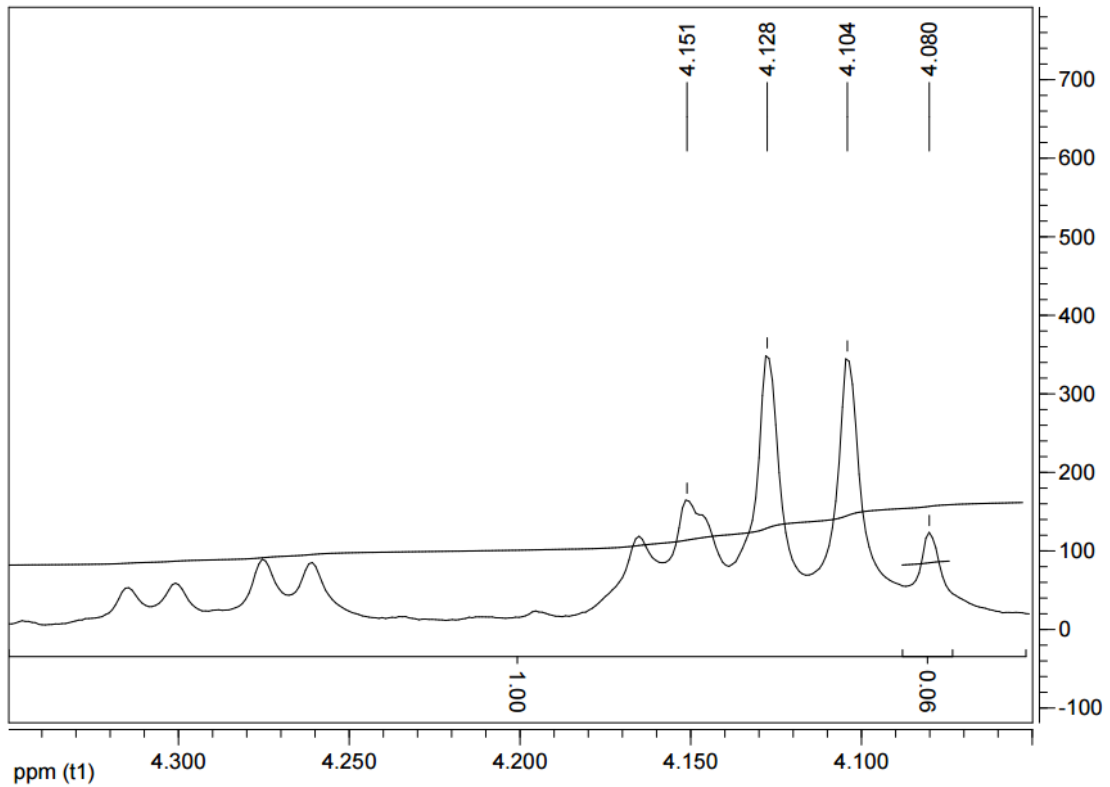




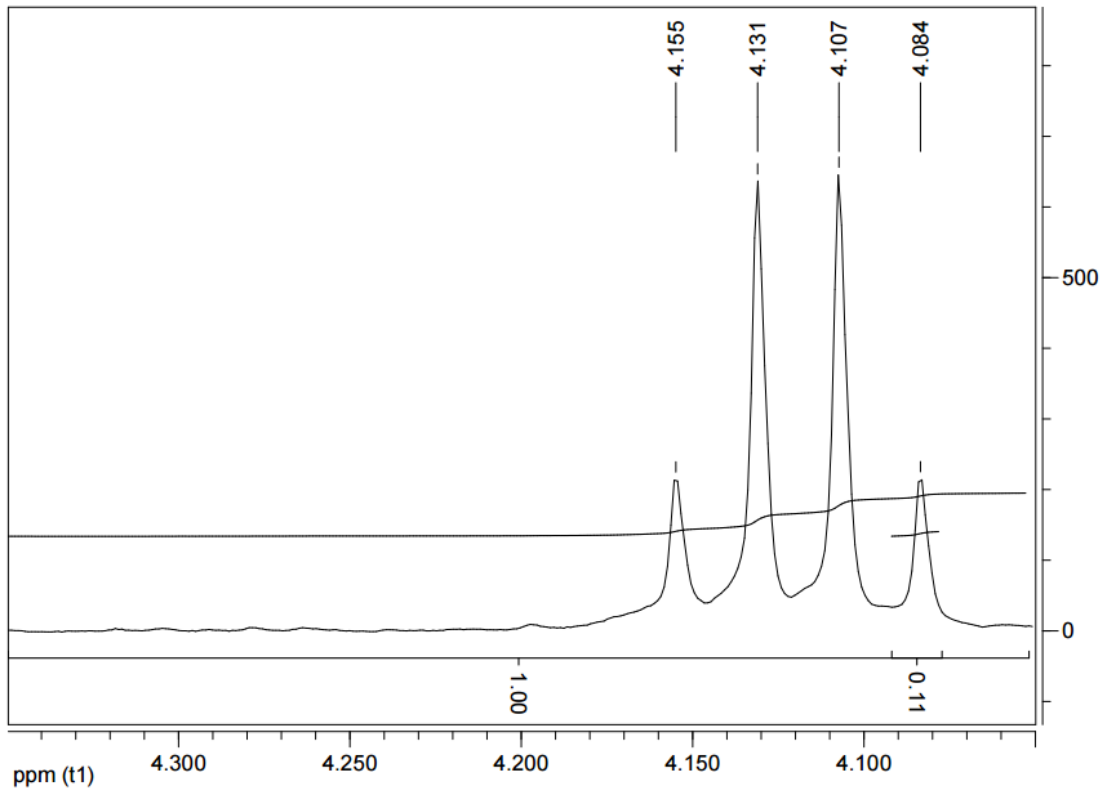
Experiment 3 Orthogonal Array L4 A



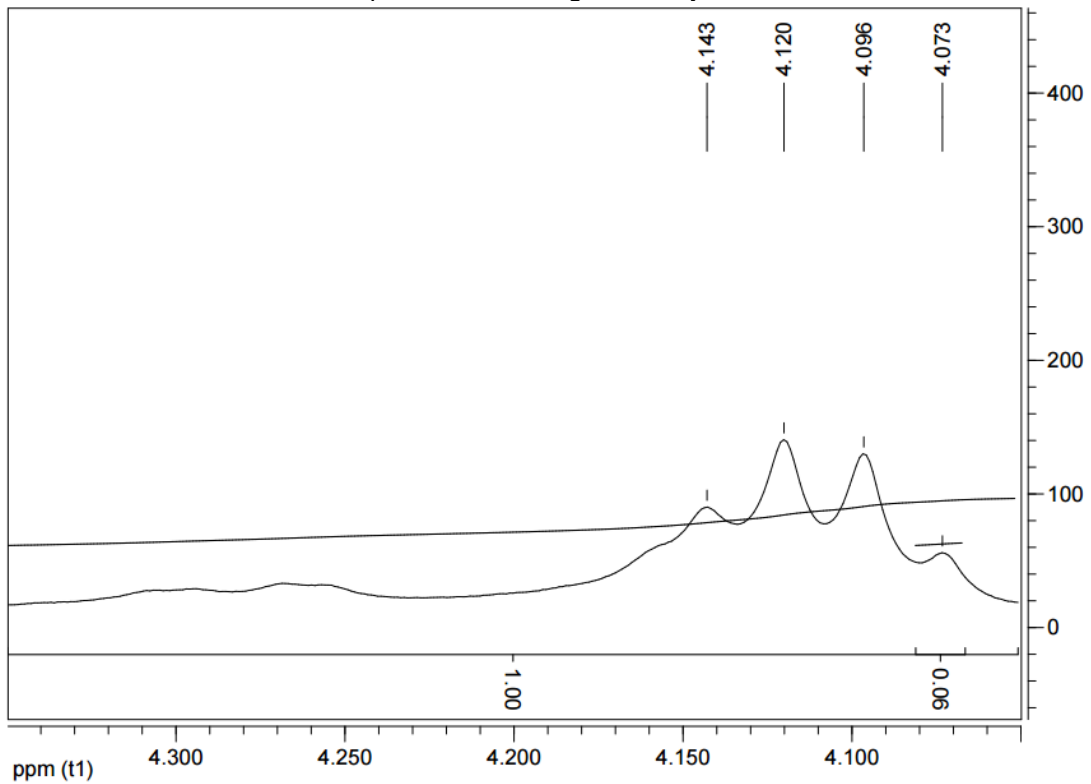
Experiment 4 Orthogonal Array L4 A



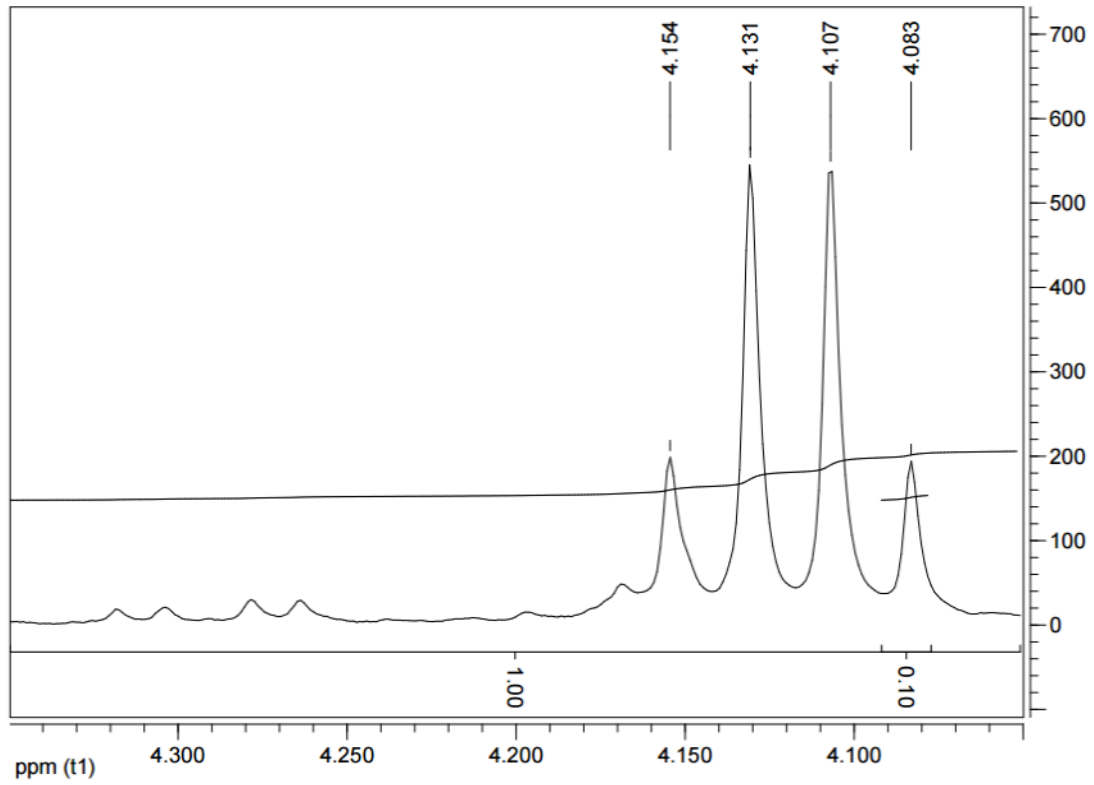
Confirmatory Experiment Orthogonal Array L4 A



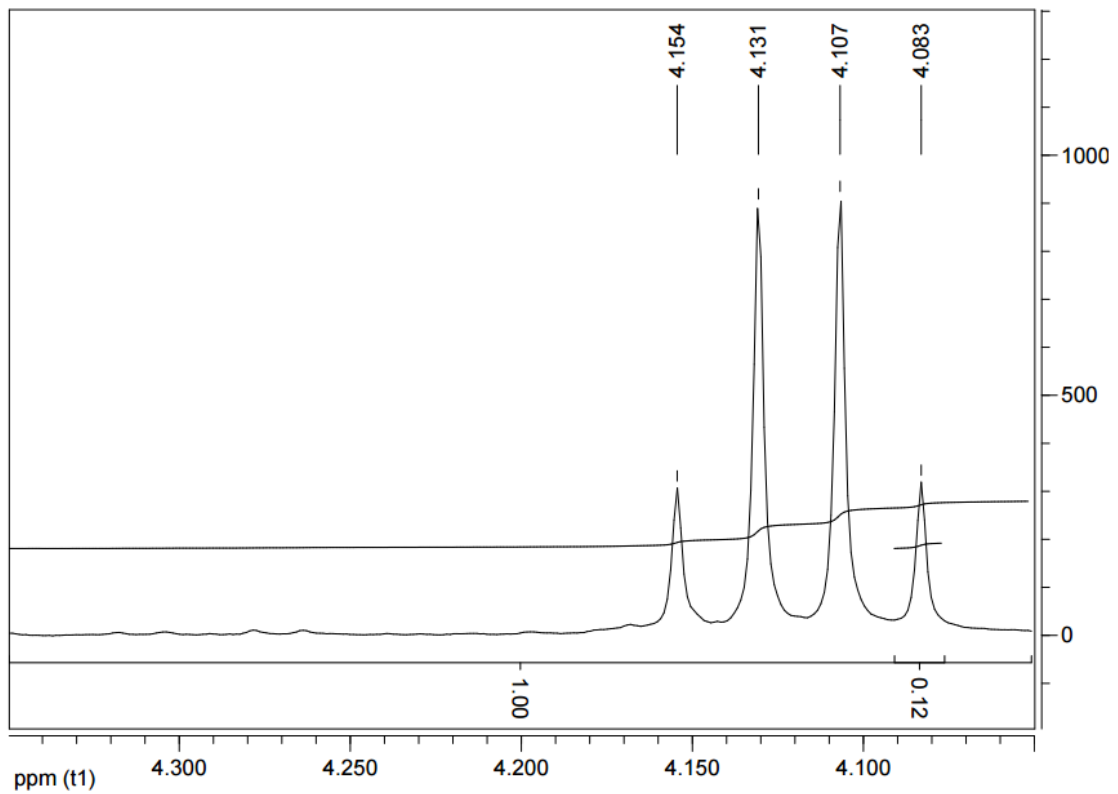
Experiment 1 Orthogonal Array L4 B



Experiment 2 Orthogonal Array L4 B



Experiment 3 Orthogonal Array L4 B



Experiment 4 Orthogonal Array L4 B

