UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias e Ingenierías

Removal of caffeine in artificial water using extracts from *Moringa oleifera* Lam. seeds available in Ecuador

Artículo académico

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Ingeniería Química

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HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

Remoción de cafeína presente en agua artificial mediante extractos de semillas de *Moringa oleifera* Lam. disponibles en el Ecuador

María Fatme Troya Espín

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Resumen

Estudios previos muestran que el extracto de semilla de *Moringa oleifera* Lam. tiene el potencial de remover materia orgánica e inorgánica debido a una proteína catiónica. En este estudio, se desarrollan diferentes procedimientos que evalúan distintos factores con el fin de establecer un método para la remoción de cafeína, ya que es un contaminante orgánico emergente que abunda en las descargas de aguas residuales del Distrito Metropolitano de Quito. Para los distintos procedimientos se analizó: temperatura previa al tratamiento, tipo de agitación, extracción de aceite por el método Soxhlet, tamaño de partícula y extracción proteica utilizando cloruro de sodio, agitación magnética y filtración al vacío. Este último fue establecido como tratamiento definitivo (TD) al tener la mayor eficiencia en la remoción de cafeína con un rango de remoción de 62 a 82% correspondiente a concentraciones iniciales de cafeína de 100 ppm y 25 ppm respectivamente. La remoción alcanzada por el resto de procedimientos se encontró en un rango de 13 a 25% utilizando una concentración inicial de Cafeína de 100ppm. La remoción de cafeína para cada tratamiento se cuantificó usando Cromatografía Líquida de Alto Desempeño (HPLC). Un análisis de Espectroscopia Infrarroja de Transformación de Fourier (FTIR) se llevó a cabo con la finalidad de detectar los grupos funcionales presentes en las semillas de *Moringa oleifera* Lam. y en el aceite extraído de las mismas. Específicamente, se detectó el grupo funcional aminas corroborando de esta manera la existencia de proteínas. Además, se realizó un análisis Kjeldahl para localizar y cuantificar la proteína existente en semillas completas y luego, por separado, en su cáscara y cotiledón.

Palabras clave: *Moringa oleifera* Lam., Cafeina, Remoción, HPLC, Extracción, Proteína

Abstract

Previous studies show that *Moringa oleifera* Lam. seed extract had the potential to remove organic and inorganic material due to cationic proteins. In this study, different procedures evaluating different factors are developed in order to establish a method for caffeine removal, since it is an organic emergent contaminant which is abundant in the waste water discharges of the Quito Metropolitan District. For the different procedures, the following was analyzed: pre-treatment temperature, type of agitation, oil extraction by Soxhlet method, particle size, and protein extraction by using sodium chloride, magnetic stirring and vacuum filtration. The latter was established as the definitive treatment with the highest efficiency in the removal of caffeine with a range of removal of 62 to 82% corresponding to initial caffeine concentrations of 100 ppm and 25 ppm respectively. The removal achieved by the other procedures was found in a range of 13 to 25% using an initial caffeine concentration of 100 ppm. Caffeine removal for each procedure was quantified by using High Performance Liquid Chromatography (HPLC). An additional analysis by using Infrared Fourier Transformation Spectroscopy (FTIR) was carried out with the purpose of detecting the functional groups present in the seeds of *Moringa oleifera* Lam. and in the extracted oil from them. Specifically, the amines functional group was detected thus corroborating the existence of proteins. In addition, a Kjeldahl analysis was performed to locate and quantify the existing protein in whole seeds and then, separately, in its shell and cotyledon.

Key words: Moringa oleifera Lam., Caffeine, Removal, HPLC, Extraction, Protein

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Removal of Caffeine in artificial water using extracts from *Moringa oleifera* Lam. seeds available in Ecuador

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1. Introduction

Currently, the modern world has begun a process of technological development that has generated great advances in the areas of science. However, globalization advances within shooting distance also has brought greater demand of resources and generation of waste which, when combined with rapid population growth, produce an environmental impact in different areas and in key resources for life. This affects the population with a myriad of problems, one of which is the case of emerging contaminants in rivers and seas. The United States Geological Survey defines emerging contaminants as: "any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects" (Raghav, Eden, Mitchell, & Witte, 2013). Some examples of these contaminants are pesticides, pharmaceuticals, antidepressants, drugs, hormones, among others (Usma, Gutiérrez, Gil, & Soto, 2013).

For this reason, it is of fundamental importance to look for economically feasible options that allow us to restore the environment and that are of effective reproduction. One of these options is inspired by the studies involving the use of the tree *Moringa oleifera* Lam. (Wuana, Sha'Ato, & Iorhen, 2015), specifically its seeds (husk and cotyledon). This tree has clotting (Ndabigengesere & Narasiah, 1998), flocculants and adsorbing capabilities (Paula et al., 2016; Santos, Matos, Sousa, & Costa, 2015; Wuana et al., 2015). In addition, even though it is a tree from India, the climatic conditions suitable for its growth can be also found on the coast of Ecuador, since the development temperature of this tree varies between 22 and 35°C (Bruhns, 2011).

Based on studies carried out in Ecuador, a variety of emerging contaminants recognized as organic compounds have been found. According to characterization results taken along the San Pedro Guayllabamba river, high concentrations of different pollutants were found (Voloshenko-Rossin et al., 2015), some of which are caffeine, acesulfame, carbamazepine, estrogens and steroids.

It is for all mentioned that the potential application of *Moringa oleifera* Lam. is viable and extensive in countless industries. Taking into account water treatment, its application is remarkable because of its composition. It contains vitamins A, B and C (ascorbic acid), calcium, potassium, steric acid, palmitic acid and soluble proteins (Sankhyan, Sharma, Attri Seth, Chauhan, & Kulshrestha, 2015). Some studies in this field have been developed and have found the effectiveness of the application of *Moringa oleifera* Lam especially in the removal of heavy metals (Beatriz, Arnal Arnal, A, & Sdenka, 2012; Landázuri, A., Cahuasquí, J., & Lagos, A. 2017; Kwaambwa, Hellsing, & Rennie, 2010). Nevertheless, its application in the field of organic compounds has not been explored in the same way. One study that did explore the application in this area is the study of removal of organic dye by using *Moringa oleifera* Lam. (Beltrán-Heredia, Sánchez-Martín, & Delgado-Regalado, 2009), which was used as a reference for this work. In the above mentioned study, the protein extraction from *Moringa oleifera* Lam seeds took place. For this procedure, sodium chloride (NaCl) was the only reactant that was needed. As a result, this method turned out to be non-aggressive and it did not generate adverse environmental or human health effects. In addition, several studies (Pavankumar, Norén, Singh, & Chandappa, 2014; Vieira Lo Monaco, Matos, & Sarmento, 2010) show that the use of *Moringa oleifera* Lam. is economically viable when using few reagents.

Caffeine is a flavoring agent commonly used in foods and beverages. According to Voloshenko-Rossin et al. study, it corresponds to one of the most abundant organic compounds in one of the largest discharges of the Metropolitan District of Quito. For this reason, Caffeine is the component on which this analysis focuses, being the first reference for the removal of organic compounds by using extracts of *Moringa oleifera* Lam. seeds, taking into account that its concentration in the discharge is approximately 5500 μgL^{-1} (Voloshenko-Rossin et al., 2015).

In the present study, different procedures are performed in order to determine the most appropriate methodology to optimize the removal of Caffeine from artificial water,

taking into account the effects of temperature, particle size, oil removal, kind of agitation (vibrating or magnetic) and protein extraction.

For each treatment a Caffeine removal test was done by placing the treated seeds or their extract in artificial water with different Caffeine concentration, and periodic samples were taken to prove the effectiveness of each treatment in the Caffeine removal. Then each sample was analyzed by using High Performance Liquid Chromatography (HPLC) to quantify the concentration of Caffeine remaining over time.

In addition, in order to test the applicability for organic compounds, the method of Landázuri et al. (2017) developed with *Moringa oleifera* Lam. for removal of metals was replicated.

Finally, Kjeldahl analysis to identify the *Moringa oleifera* Lam. seed part of greater abundance were performed as well as an Infrared Fourier Transformation Spectroscopy analysis (FTIR) to determine the functional groups that are present in three different parts of *Moringa oleifera* Lam. seeds.

2. Experimental procedure

2.1 Materials and Reactants

2.1.1 Reactants:

Moringa oleifera Lam. seeds provided by Ecuamoringa, Caffeine 97% m/m supplied by Quifatex, Caffeine 99%m/ supplied by Aldrich. Sodium Chloride 99% m/m. Hexane supplied by Fisher Scientific. HPLC grade Methanol supplied by Fisher Scientific, and glacial acetic acid supplied by Fisher Scientific. Distilled water to prepare all the solutions in all experiments.

2.1.2 Apparatus

High-performance liquid chromatography HPLC analyses were conducted using a Buck Scientific BLC-10/11 system equipped with a manual injection valve with a 20µL sample loop, and a fixed-wavelength UV detector set at 254 nm.

FTIR analyses were carried out using a Perkin Elmer model Spotlight 200 with a spectrum range of 4000 to 500 cm^{-1}.

2.2 Methodology

The methodology was divided into two parts, one corresponding to the determination of proteins in *Moringa oleifera* Lam. seeds by locating its corresponding functional group through an FTIR analysis, in addition to its quantification through the Kjendahl analysis.

The second part corresponds to the development of five different procedures (treatments) focused on different effects applied to achieve the Caffeine removal. All the treatments begin with the pre-treatment, followed by the procedure development and conclude with the Caffeine removal test and its subsequent quantification using HPLC. Figure 1 shows the general flow diagram for all treatments and the next section details each of them.

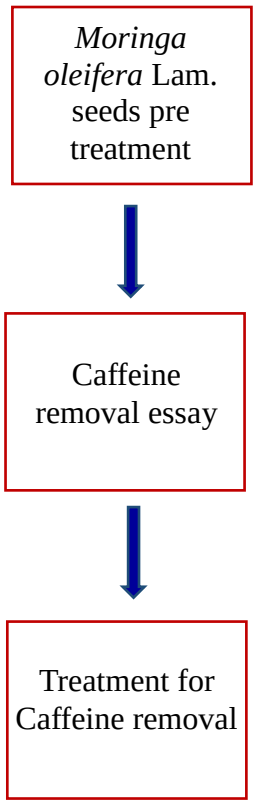

Figure 1. General flux diagram followed for each treatment

2.2.1 FTIR Analysis of Moringa oleifera Lam. *seeds and extracted oil*

First check the electrical connections of the equipment, turn on the power source and place liquid nitrogen in the cooling chamber. The "Spectrum Image" control software is then started. Meanwhile, prepare the sample and place it in the sample holder to be placed in the equipment and perform the analysis. Save the infrared spectrum obtained.

2.2.2 Kjeldahl Analysis of Moringa oleifera Lam. *Seeds*

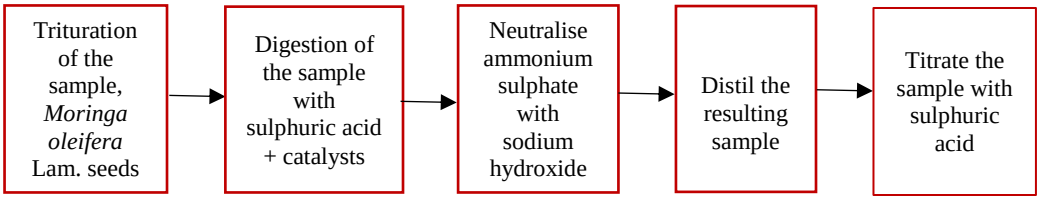

Figure 2. Kjeldahl analysis flux diagram

The Kjeldahl analysis was performed in order to determine the abundance and localization of protein through the determination of the amount of organic nitrogen. The flux digram is shown in Figure 2.

Moringa oleifera Lam. seeds (Figure 3) were analyzed taking into account complete seed (husk and cotyledon) and husk and cotyledon each one separately. Each sample was ground by using mortar and pistil and then 5g of each one were placed in the Digester with sulphuric acid in the presence of catalysts. The result of the digestion was ammonium sulphate that was neutralized with a base and then distilled. Finally, the resulting product was collected in a solution of boric acid to be entitled with standard sulphuric acid.

Figure 3. *Moringa oleifera* Lam. Seeds

2.2.3 Moringa oleifera Lam. *Preparation / pre-treatment:*

Moringa oleifera Lam. dried seeds were supplied by Ecuamoringa, Ecuador. The preparation process starts with the washing of the seeds with distilled water by placing the seeds in a deep bowl and removing impurities by hand (using gloves). Next, water is withdrawn through sing each time. Finally, the seeds are dried at 70ºC for Treatment 1 and 60ºC for Treatments: 2 (T2), 3 (T3), 4 (T4) and Definitive (TD).

2.2.4 Treatments for Caffeine removal:

A summary table of the developed procedures is shown in Table 1 with all the treatments performed in order to achieve the most efficient methodology for Caffeine removal.

Table 1. Summary of treatments for Caffeine removal

Treatment	**Description**	**Amount of *Moringa oleifera* Lam. seeds used [g]**
T1	→ Drying temperature 70ºC → Complete seeds crushed, magnetic stirring for Caffeine removal test	1
T2	→ Drying temperature 60ºC → Complete seeds crushed, magnetic stirring for Caffeine removal test	1
T3	→ *Moringa oleifera* Lam. seeds without husk, cotyledon was cut as orange slices (4 parts)to preserve the protein. Vibrating agitation in Caffeine assay.	1
T4	→ *Moringa oleifera* Lam. After oil removal by means of Soxhlet extraction to increase the action of the exposed protein. Magnetic stirring for Caffeine removal test	1
Val	→ Replication of Landázuri et al. (2017) procedure	1
TD	*Moringa oleifera* Lam. extraction with NaCl: → Drying temperature 60ºC → Vigorous crushing of whole seeds → Stock solution of 1M sodium chloride (5,437g of sodium chloride) in a 100mL gauge balloon with distilled water. → In a second ball with a capacity of 100mL, place the 5g of pre-weighed crushed, using a glass funnel to avoid losses, gauge with distilled water. → Place the solution in a 500mL beaker and stir magnetically for one hour. → Filter the resulting solution twice using filter paper placed in a Buchner funnel connected to a 200mL kitasato. → Magnetic stirring	1, 2.5, 5

2.2.4.1 Treatment 1

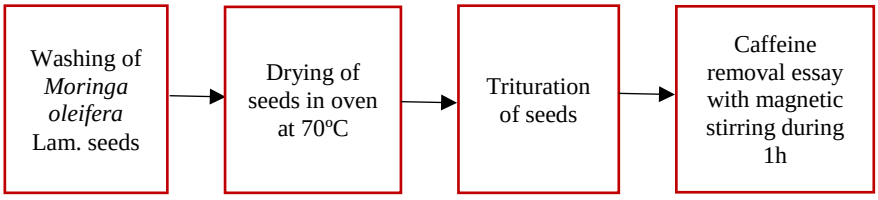

Figure 4. Flux diagram for T1

The process begins with the pretreatment of the seeds, with a drying temperature of 70ºC. Then, *Moringa oleifera* Lam. complete seeds were grounded using mortar and pistil, 1g of the resulting powder was placed in Caffeine sample and interval sampling was taken for one hour to quantify the removal using HPLC. Complete flux digram for Treatment 1 is shown in Figure 4.

2.2.4.2 Treatment 2

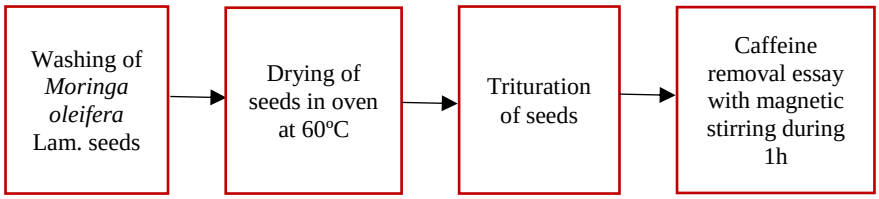

Figure 5. Flux diagram for T2

For Treatment 2 (T2), the drying temperature in the pre-treatment of Moringa oleifera Lam. seeds was decreased to 60ºC in order to avoid the denaturation of the protein and also to generate a more viable treatment without the necessity of a very high drying temperature. After preparation the same procedure of T1 was performed for Caffeine removal test. The complete process is shown in Figure 5.

2.2.3.3 Treatment 3

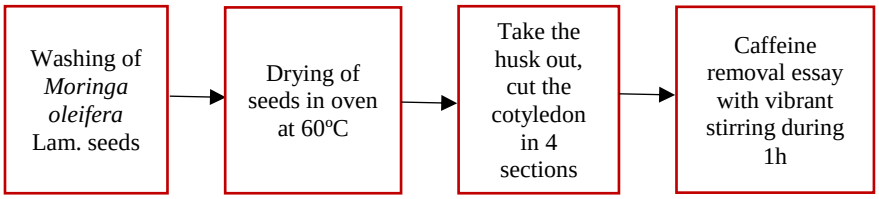

Figure 6. Flux diagram for T3

In Treatment 3 (T3) the variation was in the particle size. The seed was cut with a knife avoiding the damage of the cotyledon, the same that was cut simulating orange slices (cross sections generating 4 pieces) to verify the hypothesis that the conservation of the protein structure could enhance its action and increase the removal. Then, the Caffeine removal essay was carried out using vibrant stirring instead of magnetic to contribute the hypothesis of conservation of the cotyledon, generating the least possible distortion towards the structure. The flux diagram for Treatment 3 is shown in Figure 6.

2.2.4.4 Treatment 4

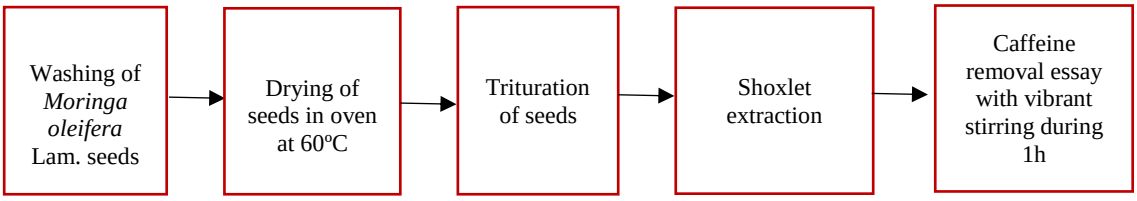

Figure 7. Flux diagram for T4

For Treatment 4 (T4) a Soxhlet extraction was carried out using the complete seeds of *Moringa oleifera* Lam. in order to release the protein from grease wrapper to verify the hypothesis of potentiating its action taking out the grease agent.

For this procedure, the seeds were grounded using mortar and pistil and then placed on filter paper caps inside the automatic Soxhlet extractor. Hexane was used as solvent for the extraction.

At the end of the procedure, remaining seed without oil were taken from the extractor, 1g was placed in Caffeine sample solution to carry on removal test using magnetic stirring.

2.2.4.5 Definitive Treatment

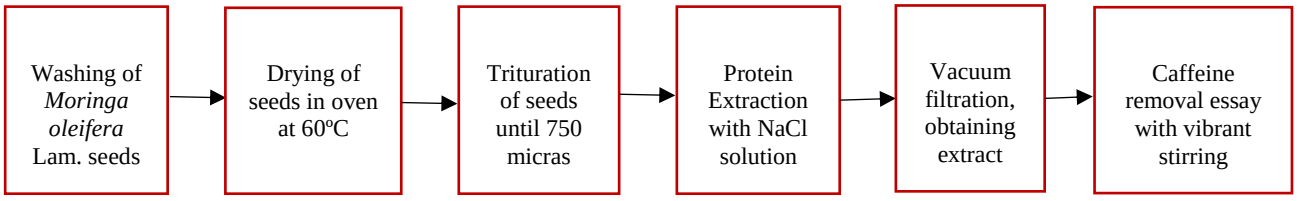

Figure 8. Flux diagram for TD

The procedure was based in reviewed literature (Beltrán-Heredia, Sánchez-Martín, & Delgado-Regalado, 2009) concerning the "Removal of organic dye Carmine indigo using *Moringa oleifera* Lam. seeds". The flux diagram of the process is shown in Figure 8.

The methodology starts by grounding complete *Moringa oleifera* Lam. seeds with mortar and pistil after the preparation treatment. The particle size was restricted until 750 micras to avoid large particles and have a larger contact surface. Then, 5g of this powder was weigh and placed in a volumetric balloon (1) of 100mL.

Then, a sodium chloride solution with concentration 1M (NaCl 99% purity) was prepared placing 5.437g of sodium chloride in a volumetric balloon with capacity of 100mL, completing its volume with distilled water. This was the mother solution used to afore the volumetric balloon 1 with the Moringa oleifera Lam. seeds powder. This was made using a glass funnel to avoid losses.

Finally, the resulting solution was placed into a glass beaker of 500mL and stirred magnetically for one hour at room temperature and then the resulting extract was filtered with a filtering equipment consisting of a Buchner funnel, with filter paper, connected to a 200mL kitasato connected to a vacuum pump.

The resulting solution corresponds to the protein extract contained in the seeds of *Moringa oleifera* Lam. which was used in its entirety for the removal of Caffeine test. The process of definitive treatment is illustrated below (Figure 9).

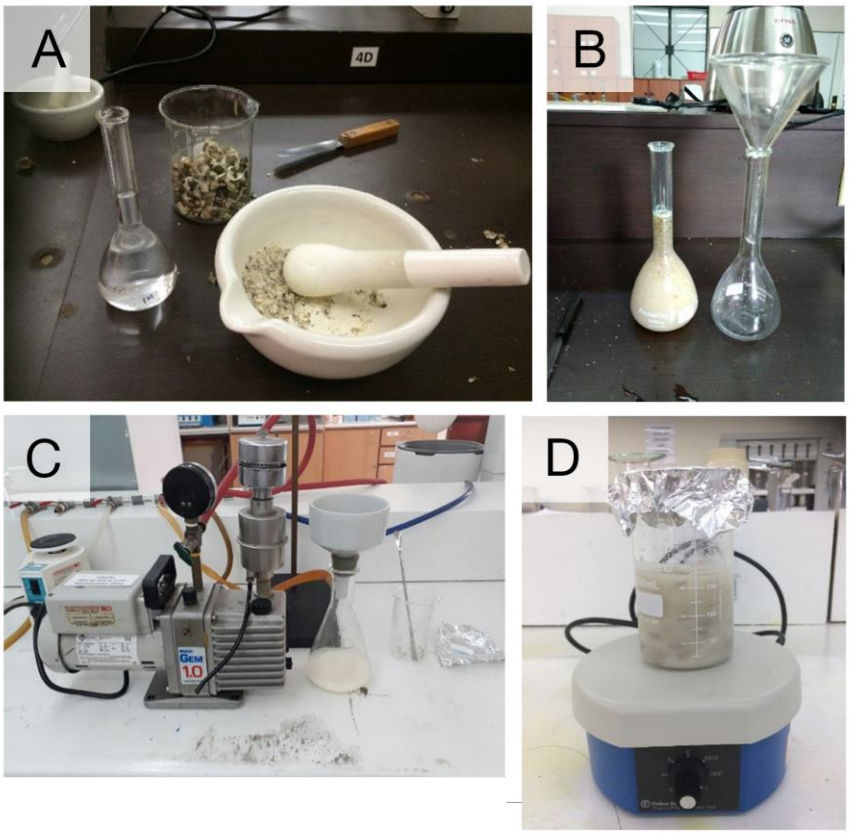

Figure 9. Definitive treatment procedure: **A)** Magnetic stirring of extractive solution and grounded seeds. **B)** Filtration to obtain protein extract**. C)** Trituration of seeds **D)** Seeds with extractive solution

2.2.5 Caffeine standard solutions and removal test

A stock solution of Caffeine was made using 10mg diluted in 100 μg/mL, then, solutions of 5, 10, 50, 100 μg/mL were performed in 100 mL flasks, all of them were degassed for 30 minutes with ultrasonic cleaner (model 5510R-DTH) prior injection in HPLC, for which 3 mL of each solution were filtered with nylon syringe filters (Millipore, Millex-HV) and placed in PCR vials.

For Caffeine removal test, solutions of 100μg/mL were made initially to be performed with preliminary treatments T1, T2, T3 and T4. Then, solutions of 50μg/mL, 30μg/mL and 25μg/mL were also carried out with definitive treatment TD to simulate more realistic cases and practical removal of emerging contaminants (Caffeine) in water.

Removal test consisted on the placement of Caffeine solution in a beaker of 400 mL together with each one of the resulting treatments. Stirring was applied, for magnetic stirring agitator Fisher Scientific model 12OS at speed of 1 (first speed) was used during 1 hour, this applies for treatments (T1, T2, T4, Replication Landázuri et al. (2017). Caffeine removal test for treatment 3 was carried out with vibrant stirring.

During these periods, sampling was performed at different intervals and then stored in PCR vials all night using refrigeration and in an amber color container to avoid contact with light. Every sample was tested the next day using HPLC.

3. Results and discussion

3.1 Kjeldahl analysis Characterization

From previous papers it is known that *Moringa oleifera* Lam. contains protein. This fact was confirmed trough Kjendahl analysis, from which it was determined that most of the protein content resides in the cotyledon (inner part) of the *Moringa oleifera* Lam. seed however the shell also has a number of protein as shown in Table 2.

Table 2. Results from Kjeldahl analysis

Sample	***Description***	***Amount (g)***	***Nitrogen (%)***	***Protein (%)***
1	Complete *Moringa oleifera* Lam. seeds	0.5005	5.34	33.375
3	*Moringa oleifera* Lam. cotiledon	0.5184	6.516	40.725
4	*Moringa oleifera* Lam. shell	0.5059	1.247	7.79375

3.2 Infrared Fourier Transformation Spectroscopy analysis (FTIR)

As part of the characterization of *Moringa oleifera* Lam. seeds, an FTIR analysis was carried out in three different zones of the seed: outer shell, internal husk and cotyledon. In addition, an FTIR analysis was carried put using the extracted oil of the seeds recovered by a Soxhlet extraction.

The following results are taken as an approximation of the composition for *Moringa oleifera* Lam. seeds and oil. For its analysis, the spectral identification tool software Bio-Rad's KnowItAll ID Expert was used to identify the compounds and functional groups of the samples. As a result, the compounds that were identified were those with greater similarity through the software libraries and also those that have been reported in literature and previous studies for *Moringa oleifera* Lam. characterization(Jhosianna et al., 2010; Lalas & Tsaknis, 2002; Okuda, Baes, & Nishijima, 2001).

The results of the FTIR analysis and the identification reached in each of the samples are shown below:

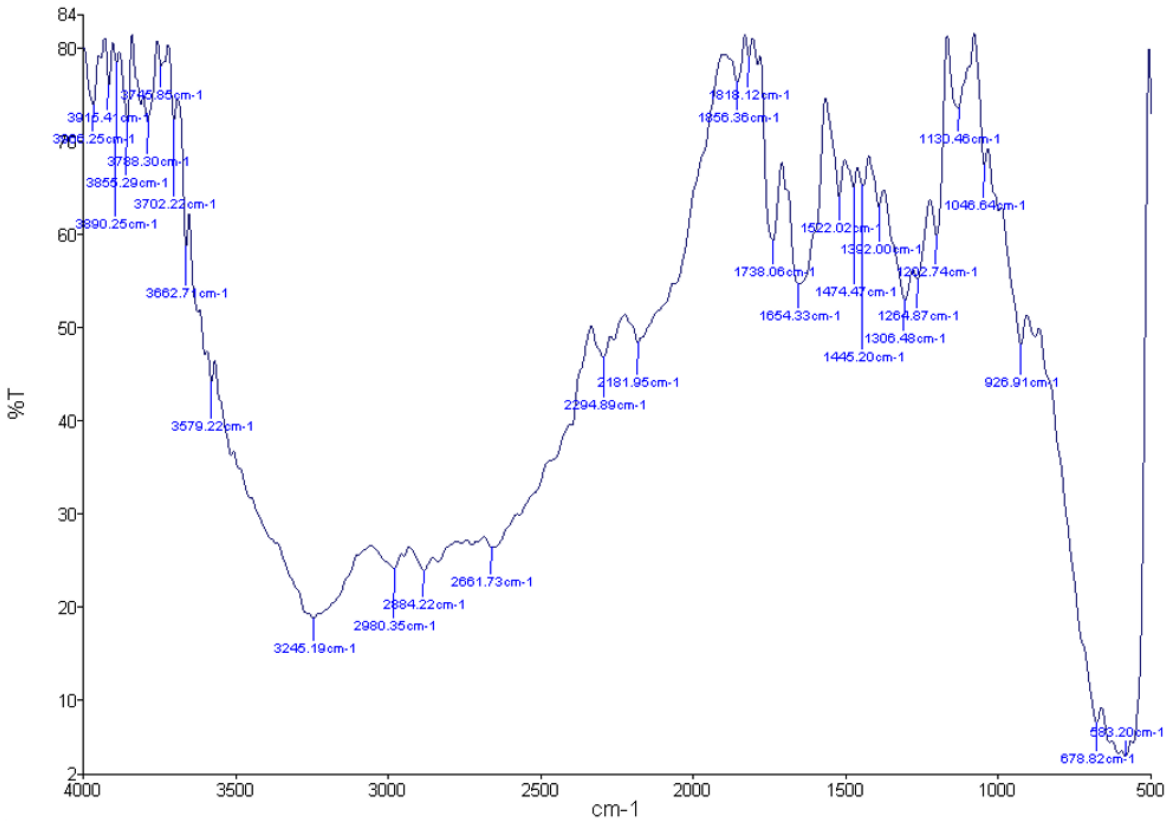

Figure 10. FTIR Analysis, Outer Shell

The spectra for *Moringa oleifera* Lam. Outer shell is shown in Figure 10, the functional groups founded are: alkanes, halogens, amines and oximes. The most relevant compounds detected from the outer shell from FTIR results were minerals such as: iron, potassium, nickel chromate, sodium and calcium. Glyoxime

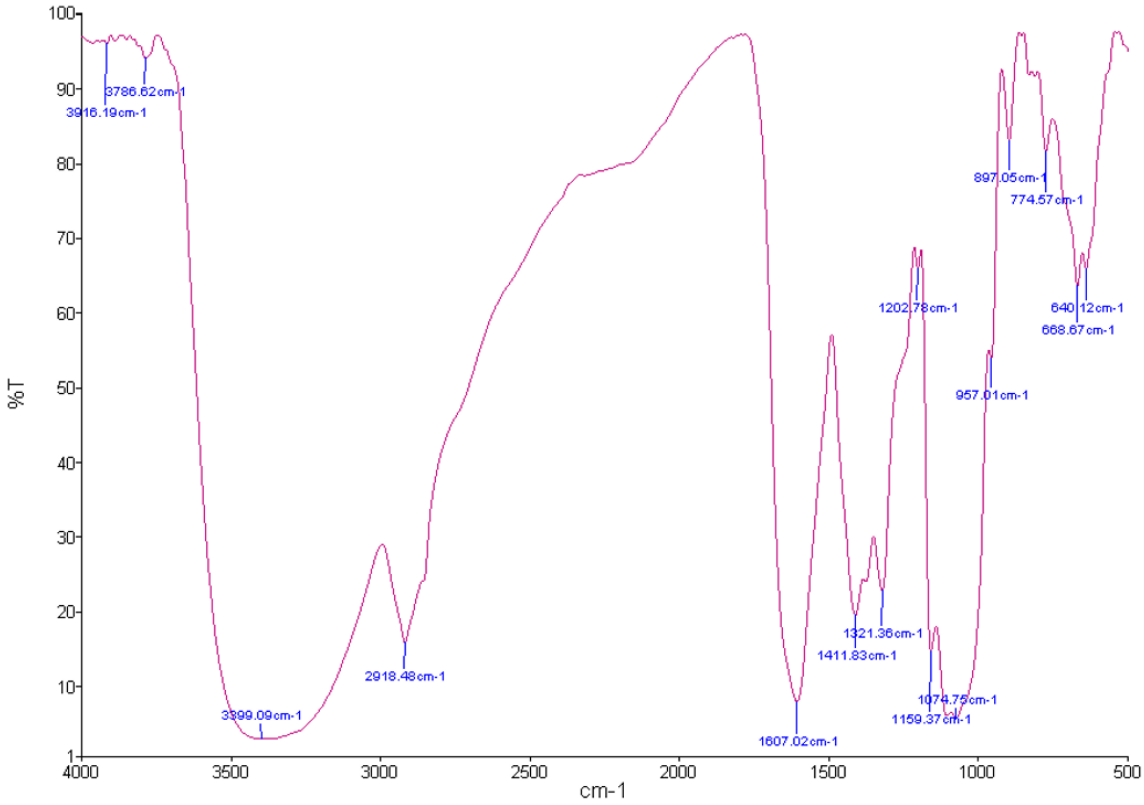

Figure 11. FTIR Analysis, Internal Shell

The spectra for *Moringa oleifera* Lam. Internal shell is shown in Figure 11, from this information, the functional groups founded are: alcohols, carbo-acids, and ketones. Some of the compounds with greater similarity in their spectrum were: polymers, monosaccharides and disaccharides such as alginic acid, chitin, cellulose, ribose, and lactobionic acid.

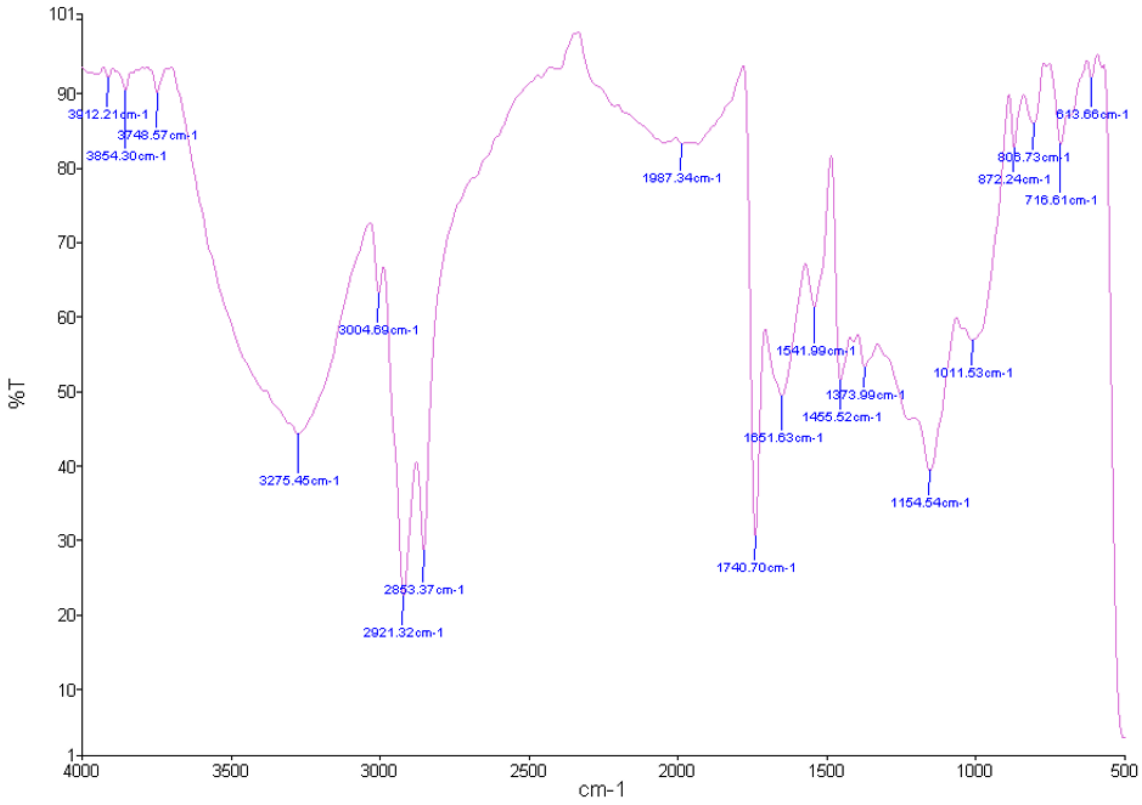

Figure 12. FTIR Analysis, Cotyledon

The spectra for *Moringa oleifera* Lam. seed cotyledon is shown in Figure 12, the functional groups detected are: alkanes, alcohols, esters and amines. Some compounds identified in the cotyledon for this study were: qutina, vitamin A, calcium, iron and some amines such as: 6-amino-1-2dihydro-2-[(3-hidroxipentina)], 1-amino-3-m-tolylguanidine, n-methylacetamide.

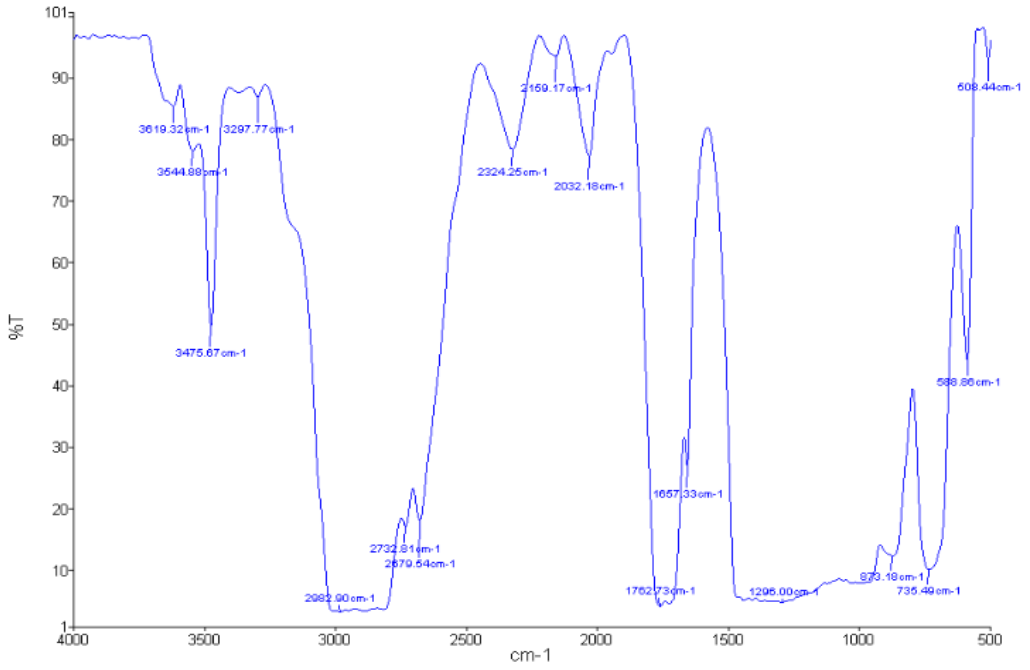

Figure 13. FTIR Analysis, extracted oil from *Moringa oleifera* Lam. Seeds

The spectra for *Moringa oleifera* Lam. extracted oil is shown in Figure 13, the functional groups founded are: esters, halogens, ketones, amines, amines salts.

A summary table of the functional groups for each area can be found below:

Table 3. Summary results from FTIR analysis

Origin of the sample	Functional groups
Outer shell	→ Alkanes → Halogens → Amines → Oximes
Internal shell	→ Alcohols → Carbo-acids → Ketones
Cotyledon	→ Alkanes → Alcohols → Esters → Amines
Extracted oil	→ Esters → Halogens → Ketones → Amines

From FTIR results, amines functional group was founded in the outer shell and cotyledon of Moringa oleifera Lam. seeds, confirming the presence of proteins.

3.3 Caffeine removal treatments results

Previous work referred use *Moringa oleifera* Lam. for water treatment(Beatriz, Arnal Arnal, A, & Sdenka, 2012; Dayal et al., 2013; Pavankumar, Norén, Singh, & Chandappa, 2014). Moreover most of these works have explored its potential for the removal of heavy metals. As an approach to an efficient methodology for organic compounds using Caffeine as a first step some preliminary analysis were needed. As a consequence different variables were taken into account as described in methodology section. For treatments T1, T2, T3 and T4 results are shown in Figures14 and 15.

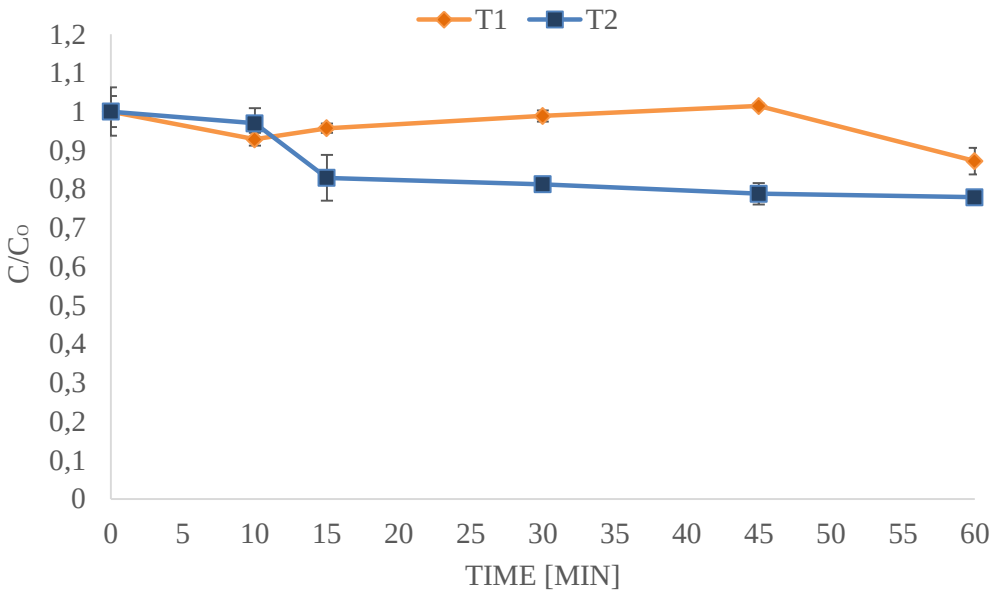

Figure 14. Caffeine essay using T1 and T2

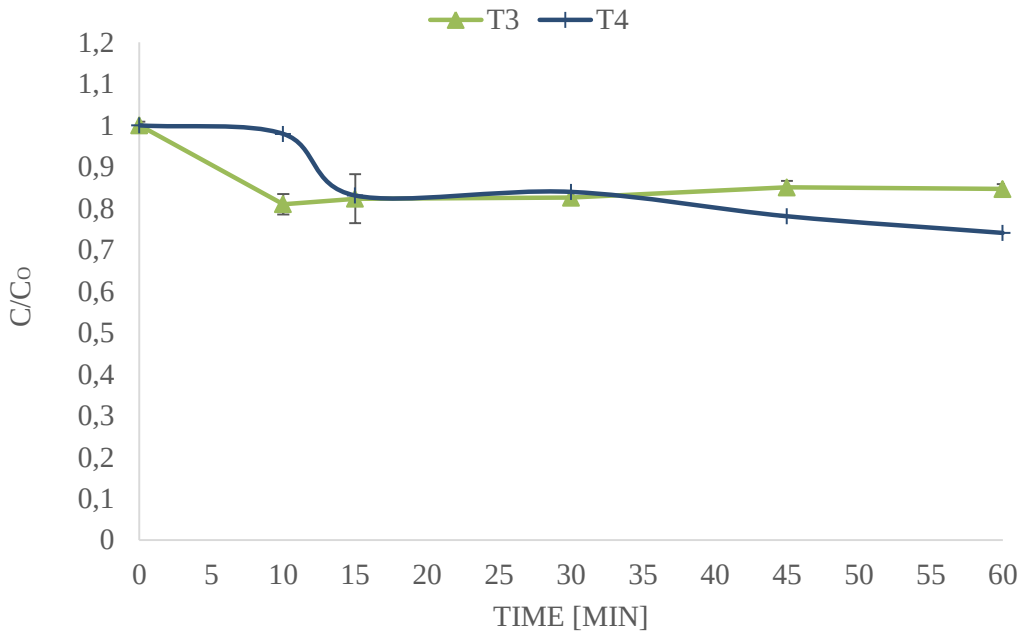

Figure 15. Caffeine essay using T3 (cross sections generating 4 pieces) and T4 (Oil extraction, Soxhlet method)

For treatments T1, T2, T3, T4 and Landázuri et al. (2017) procedure, the Caffeine assay carried out corresponds to a concentration of 100µg/mL. In the case of T1 (Figure.14) the results show a very low clearance. Considering the initial drying temperature of 70ºC for *Moringa oleifera* Lam. seeds it was deciding to decrease it to 60ºC as the temperature do not generate a necessary effect on the seeds components but it can prevent a possible damage to the protein structure. The rest of treatments maintain the drying temperature of 60ºC.

For T2 results an increase in the elimination was observed (Figure.14), although it was a very small percentage, which is why other parameters were modify in the other methods, establishing the fixed temperature of 60ºC.

Complete crushing of the seeds was used for T1 and T2 but for T3 it was sought to preserve the integrity of the seed, as a result the cotyledon was carefully extracted from the outer shell using a knife and then it was cut into four parts. Additionally, the removal Caffeine assay was performed using vibrant agitation, this simulates horizontal waves as agitation took place. Results are presented in Figure 15. The total removal achieved with this treatment was minimal, as a consequence the need to preserve the structure of the seed under treatment and its subsequent application is ruled out.

Based on previous literature (Sankhyan, Sharma, Attri Seth, Chauhan, & Kulshrestha, 2015) it is known that *Moringa oleifera* Lam. seeds contain edible oil (up to 40% by weight) and also water soluble proteins that act as natural coagulants that are used in wastewater treatment. Taking on account this information treatment 4 was developed in order to remove the oil from seed to release the protein and enhance its action. The Soxhlet extraction results in a dry powder that was used in the Caffeine removal assay. Results of T4 are shown in Figure 15. and from this it is understood that the extraction of the oil present in *Moringa oleifera* Lam. seeds did not represent improvements in the removal of Caffeine as the percentage obtained was similar to the previous treatments. Also this extraction is a long procedure that would not be favorable if it is desired to apply *Moringa oleifera* Lam. in a sustainable way, first because it uses hexane as reagent and its subsequent elimination would become an additional problem and second because this study focuses on a reproducible methodology that could be apply in different environments whose need is urgent, for example in affected areas polluted by emerging contaminants.

3.4 Results from Landázurí et al. (2017) procedure replication

Through the repetition of the procedure Landázuri. et al. (2017) it was determined that it is not the appropriate method to be used in the removal of organic compounds, specifically with Caffeine as the removal was minimal compared with the results of the methodology in the case of metals. Results from removal are shown in Figure 16.

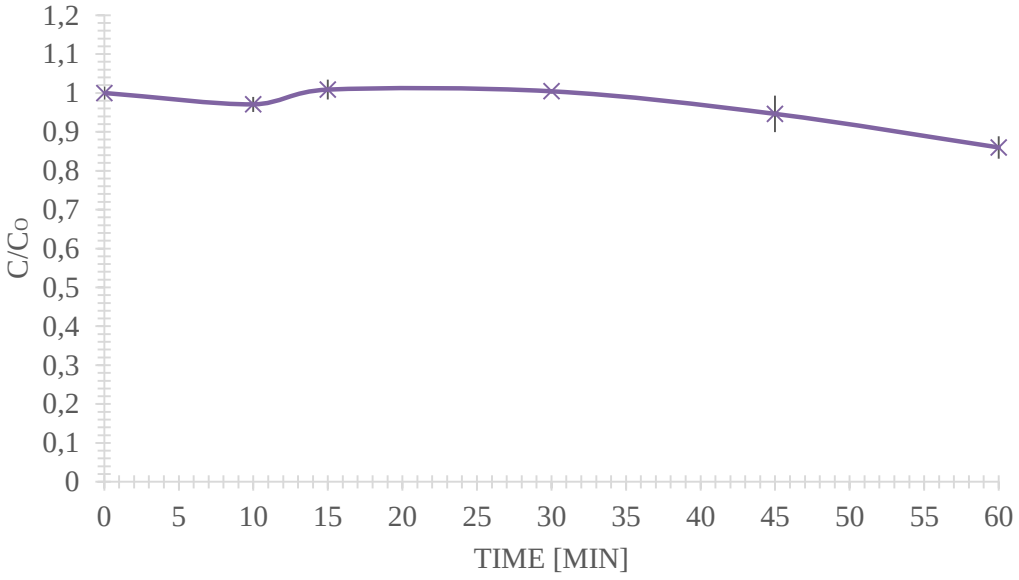

Figure 16. Repetition of Landázuri (2017) procedure

3.5 Definitive Treatment (TD) Results

Definitive treatment product of protein extraction described in experimental section, using sodium chloride (NaCl) presented best performance results. First tested with an essay of Caffeine with concentration of 50 ppm, in contrast with the other treatments in which initial concentration was higher (100ppm). Sampling was performed for an hour using 5g of initial grounded seeds as raw material present a total removal of 65.2% from initial concentration. The results from this treatment can be considered efficient, taking into account that the procedure do not demand complex or excessive reagents, as a consequence, the impact on the environment is minimum.

Results of this first analysis (TD) using 5g of *Moringa oleifera* Lam. ground seeds are observed in Figure 17. Additionally, TD was reproduced reducing the initial amount of seeds of *Moringa oleifera* Lam. to 2.5g (TD1) and 1g (TD2) in order to compare the efficiency of the removal with the amount of raw material initially used for protein extraction (Figure 17). The total Caffeine removal for an initial amount of 2.5g of seeds was 62.23% from initial concentration. This results present a very slight decrease in Caffeine removal compared to the one obtained in TD1 (5g of seeds) considering that raw has decreased by half.

It is important to mention the fact that the definitive treatment was restricted in terms of the size of particle unlike previous treatments. This differentiation has contributed to the process as long as the large particles were crushed to 750 microns to generate the greatest possible contact with extractive solution and to obtain all of its potential in the removal of Caffeine.

In addition, TD was replicated by decreasing the initial concentration of Caffeine for the trial of removal in order to observe the variation of its effectiveness against lower concentrations of Caffeine. For this purpose, two different concentrations were used, 30ppm (TD3) and 25ppm (TD4). The reason for which these concentrations were used was to approach the HPLC detection limit corresponding to 5ppm and also to simulate similar concentrations of this compound found in the environment. The results of TD3 and TD4 are shown in Figure 18.

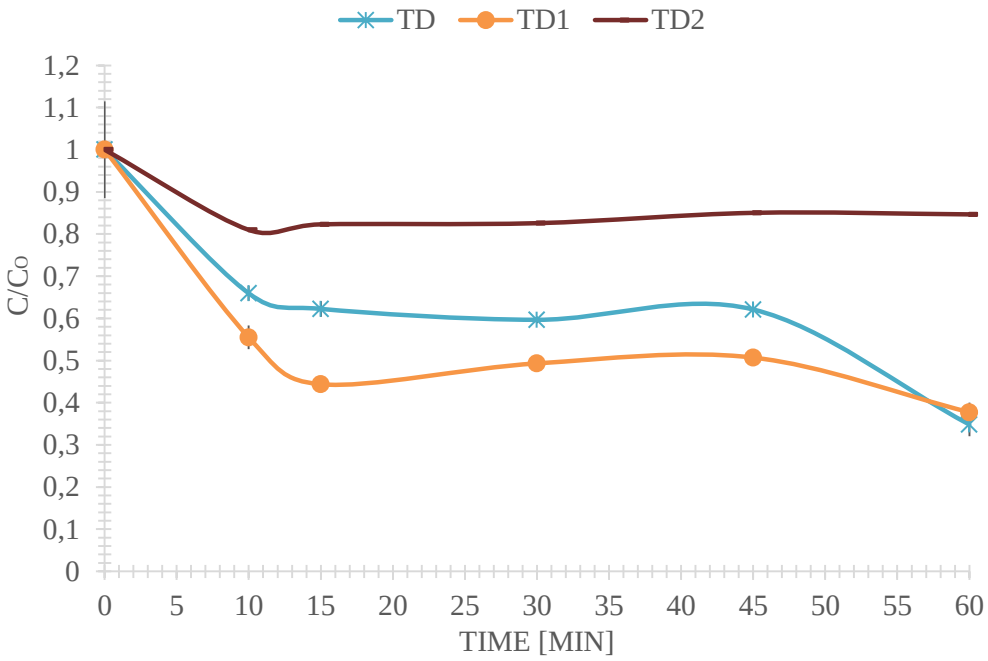

Figure 17. Caffeine essay with TD (Protein Extraction Using NaCl)

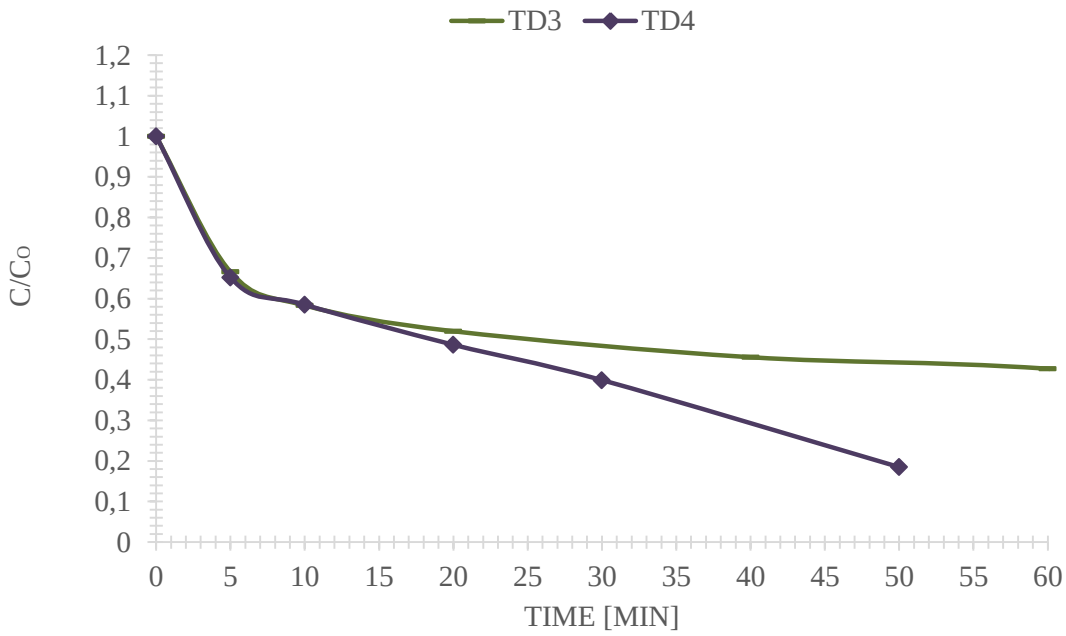

Figure 18. Caffeine essay with TD for different initial concentrations

As it can be seen by means of Figure 18, the maximum removal detected by HPLC analysis, for Definitive treatment, was until a concentration of 4.62ppm in the 50th minute of the Caffeine removal test. After this period, it was no longer possible to detect the peak of Caffeine in the sample. This fact agrees with having reached the limit of detection of the device, for which a total removal of 82% is established for the analysis of Caffeine removal using a concentration of 25 ppm.

In order to determine the mechanism of action of *Moringa oleifera* Lam. In Caffeine removal, a UV analysis was performed based on the Caffeine assay using the Definitive treatment (TD). As a result, UV spectra showed that Caffeine removal takes place with an adsorption process, meaning it is not a degradation of the organic compound. Results are shown below (Figure 19).

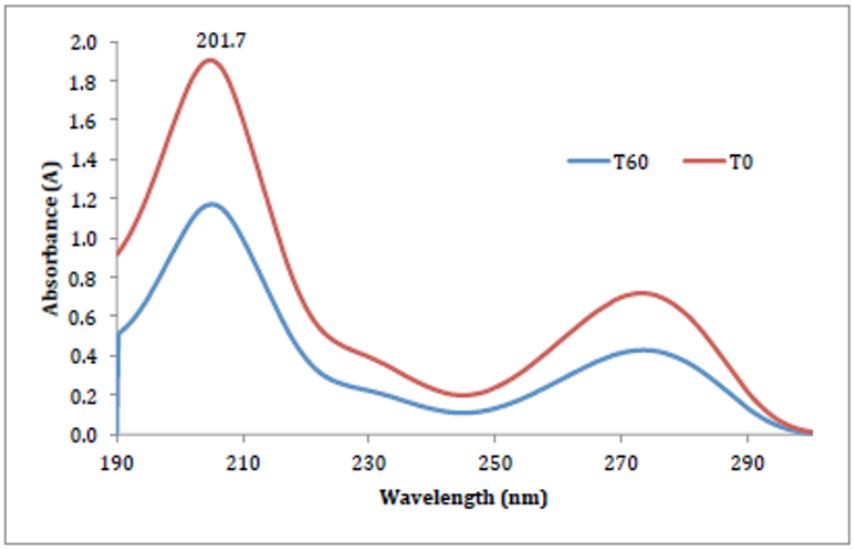

Figure 19. UV spectra of Caffeine removal using *Moringa oleifera* Lam. seeds

As you can see, the shape of the curve remains the same, only its height has decreased, which demonstrates an adsorption process that drops the initial concentration of Caffeine without generating a structural change in the compound.

Finally, a comparison of the preliminary treatments (T1, T2, T3 and T4) and definitive treatment (TD) in their different repetitions is shown in Figure 23. By means of which the difference in the removal efficiency of each treatment is observed. It can be observed that definitive treatment is the most effective in terms of Caffeine removal. These results reveal the importance of the trituration of *Moringa oleifera* Lam. seeds for the release of the compounds that allow the removal of contaminants from the water. This, coupled with the magnetic and vigorous agitation used by TD, makes it possible to discard the fact that the compounds that allow the removal of Caffeine can be affected by an initial aggressive treatment for the seeds, on the contrary, this is fundamental to generate the extraction protein that is later used in the removal.

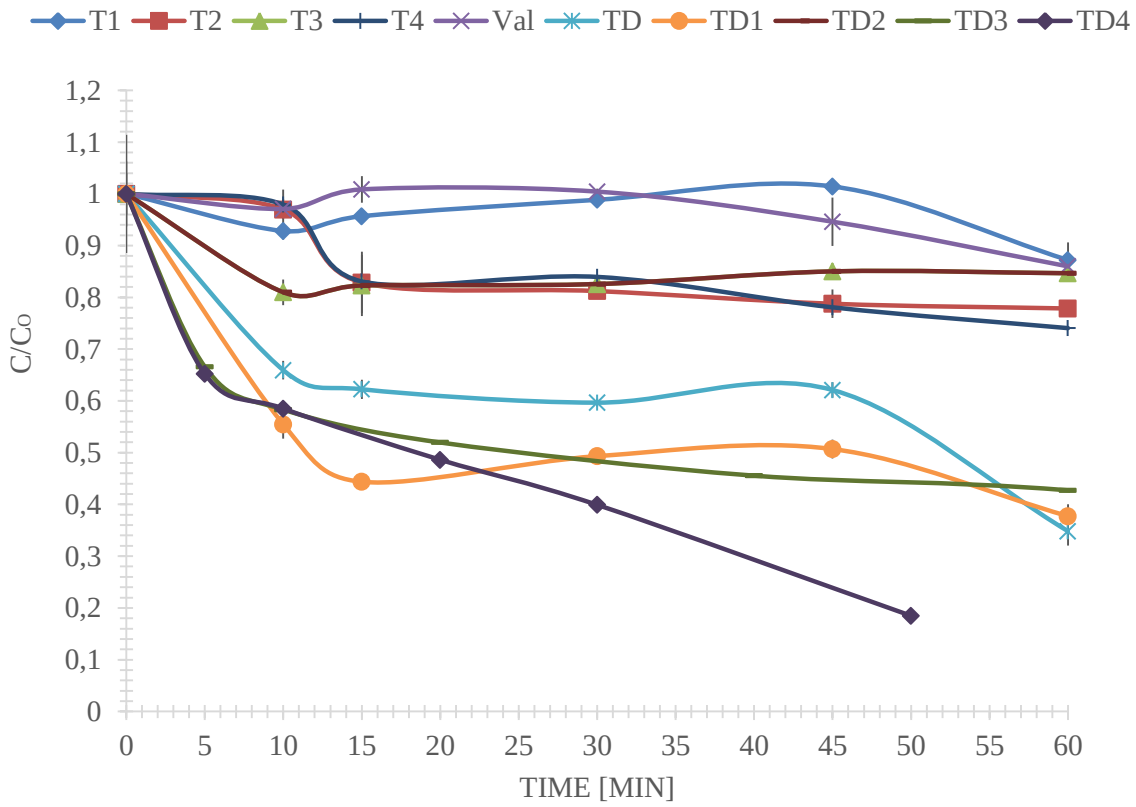

Figure 20. Comparison between treatments

4. Conclusions

This investigation drives to the following conclusions:

- Drying temperature of *Moringa oleifera* Lam. seeds for posterior usage can be 60ºC in oven for 24h. This is sufficient to dry the seeds from humid without damaging the inside protein.
- The contact surface is critical to obtain the potential for removal of the *Moringa oleifera* Lam. protein on its application for waste water treatment and particle size restriction contributes for increasing this condition.
- *Moringa oleifera* Lam. is a highly effective Caffeine removal agent. It presents removal of Caffeine in a range of 62% to 82% depending on the initial concentration.
- *Moringa oleifera* Lam. is a natural coagulant with a high potential for been applied in high scale waste water treatment as the results can be compared with artificial coagulants.

- Magnetic stirring was a fundamental condition in the moment of the protein extraction and in the Caffeine assay.
- The definitive treatment (TD) constitutes an environmental friendly option as it just depends on one reactant that is sodium chloride.
- Definitive treatment is economically viable and easy to reproduce. Two important characteristics to make it a possible alternative to be apply on waste water treatment.

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