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Edited by

C. Arteaga, C. Franco, J. Silva, D. Terán

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Reviews and surveys on specific topics relevant to the advance of the Mediterranean food industry are particularly welcome.

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INTRODUCTION

The first international congress of Food Science and Biotechnology (CICABI from the Spanish) – 2018 was organized at the Ambato city in Ecuador, between the 25th and 29nd of June of 2018. The aim of the congress was to promote the careers of Food sciences and biotechnology of the Universidad Técnica de Ambato (UTA). Also this event promoted the presentation of the milestones of the different researches that are carried out at the UTA, but also the presentation of senior and young researchers of Food sciences and Biotechnology from different countries.

This international congress had the honour of hold oral presentation from well-known international researchers. Among these international researchers we can mention, Dra. Rosa Lamuela and Yolanda Cajal from the Universidad de Barcelona - España, Dr. Diego Hidalgo from the Universidad de California - Berkeley, Dra. Silvina Drago from the Universidad del Litoral de Argentina and the Msc. Gabriele Bigoni from the Universidad Nacional Autónoma de México. Also this congress had the honour to have the oral presentation from Jhonn Pressing representative de FAO – ECUADOR, Dr. César Paz y Miño and other well-known researchers that shared their knowledge with the participants of this congress.

It is important to mention that CICABI – 2018 had the support of different administrative and technical direction from the UTA. Especially, the Direction of Research and Development (DIDE from the Spanish) and the public company from the UTA (UTA-EP from the Spanish), that provided the economical support for this especial edition.

This special edition is the result of the participation of academics, researchers and students in this international congress. Also this special edition highlights Food sciences and Biotechnology as principal topics for this volume. Also these topics had high relevance and attention for the Central Andean Region in South America. Therefore, this edition highlights researches that show technics developed for the region such as, technics that allows the improvement of food processing and experiments in the search of primary material with higher nutritional components for the development of new food products. In addition, this special edition presents studies that had as base line the analysis of the native microorganisms and application of them in an industrial level.

CICABI – 2018 made a precedent of scientific production not only for Ecuador but also for other countries in the region. Thus, CICABI constitute a space for knowledge interchange between scientists in the field of Food sciences and Biotechnology.

C. Arteaga, C. Franco. J. Silva, D. Terán

SESSION I

“FOOD SCIENCE”

SENSORIAL QUALITY OF BREADS AND COOKIES PREPARED WITH FLOUR FROM THE SHELLS OF TWO VARIETIES OF COCOA IN ECUADOR

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ABSTRACT

This research is carried out as an alternative for the bakery industry when looking for new sources of vegetable flour to be used in the preparation of breads and cookies, because the wheat production in Ecuador is not enough. On the other hand, the cocoa industries in the country produce a high quantity of cocoa shells that are considered as agroindustrial waste, which come from the two main varieties of cocoa, Nacional Arriba and CCN51. That is why, as a product of the grinding of these husks, flour was obtained that was used for the production of breads and biscuits with different dosage percentages based on various bibliographical sources and the authors' own experiences. In the case of the breads, the dosage used was 10% and 20%, while for the cookies a dosage of 70% and 80% was applied. Both the breads and the cookies were evaluated for their sensorial quality, by means of untrained judges using a hedonic scale from 1 to 5. The results confirm a high sensory quality in the cookies compared with the sensory quality obtained in the breads.

Keywords: baked products, cocoa Nacional Arriba, cocoa CCN 51, flour, sensorial characteristics

1. INTRODUCTION

The cocoa sector is one of the most important in the world, according to the information published by The World Cocoa Foundation (WCF, 2013), who confirmed that there are between 5 and 6 million cocoa farmers, in addition to 40-50 million people who depend on the cocoa sector around the world. In Ecuador there are 2 cultivated varieties of cocoa: Nacional Arriba, which is fine or aroma cocoa (native to Ecuador) and clone-type cocoa developed in the country, Castro Naranjal Collection (CCN 51).
The shells of the two varieties of cocoa are considered as agroindustrial waste when they are generated by the manufacture of cocoa byproducts, being that these husks represent approximately 12% of the weight of the roasted cocoa beans according to the data of Cuesta (2008).
Taking into consideration the current trend to change the productive matrix of Ecuador, related to the national plan of good living (2013-2017), one of the main axes is "to encourage exports of new products, from new actors, particularly the popular and solidary economy, or that include greater added value - fresh and processed foods ".
In this way, it is considered that this policy will lead to an increase in the amount of agroindustrial waste, in the form of husks, in the case of cocoa.
On the other hand, the bakery industry in Ecuador is growing, according to data from the Ministry of Industries and Productivity (2013). The Ministry and the National Development Bank (BNF) granted new loans within the Renova Bakery Program, which so far has delivered more than USD 5.5 million to 829 breadmakers. Regarding the cookies industries, according to the data of the Export and Investment Promotion Institute (PRO ECUADOR, 2012) in 2012, the consumption of cookies in Ecuador grew 5.2% to US $ 224 million.
Based on this information, the present research work was carried out in which the shells of both varieties of cocoa were used to make flour, which was dosed with wheat flour for the preparation of breads and cookies. Finally, the sensory quality of both products was analyzed to identify which of them would have better acceptance for their consumption.

2. MATERIALS AND METHODS

2.1. Materials

The shells of the two different varieties of cocoa were taken from 2 cocoa industries in the province of Guayas, the selection criteria of these two factories being the ease and flexibility given by their officials to take the samples, obtaining a variety for each industry.
The samples of each variety of cocoa were taken from five different lots for each industry, making the milling of the husks with an ® Oster mill to obtain the flour. For the elaboration of each bread formula, 3000 g of the flour were separated for the breads and cookies. Each bread or cookie formula was made with three repetitions.
The proportion based on the dosage of the flour from the cocoa shells, with respect to the wheat flour, was carried out with two different percentages (10%, 20% of cocoa shells for bread making and 70%, 80% for cookies making) with three repetitions for each product.
The formulation of the bread were coded as P1 to P4 and as G1 to G4 for the cookies, The formulation: P1, refer to bread made with 20% of flour coming from the cocoa National Arriba shells, P2 refer to the bread made with 10% of flour coming from the cocoa National Arriba shells, P3 refer to the bread made with 20% of flour coming from the cocoa CCN51 shells and P4 refer to the bread made with 10% of flour coming from the cocoa CCN51 shells.

For the cookies, the formulation: G1, refer to cookies made with 80% of flour coming from the cocoa National Arriba shells, G2 refer to the cookies made with 70% of flour coming from the cocoa National Arriba shells, G3 refer to the cookies made with 20% of flour coming from the cocoa CCN51 shells and G4 refer to the cookies made with 10% of flour coming from the cocoa CCN51 shells.
After the preparation of the baked products, the sensory characteristics were analyzed to select the best product.
In order to carry out the sensorial analyzes, determining the organoleptic quality of the bread and cookies made with cocoa shells flour, a panel of 30 untrained people was used (they are not trained judges for the organoleptic analyzes), and a sensory evaluation was applied. Hedonic scale, from "I like a lot" to "dislike a lot" with numerical scale from 1 to 5, where 5 corresponds to "I like a lot" and 1 corresponds to "I dislike a lot".
For the sensory evaluation of the bread, each judge was provided with 4 bread samples, each sample of approximately 60 g, and the judges were asked to evaluate the Color, Flavor, Odor, and Texture of each sample and record their evaluations according to the sensory attributes aforementioned.
The same process was used for the evaluation of the cookies, where each judge was provided with 4 cookies of approximately 15 g each and was asked to evaluate the same attributes mentioned above.
Both the bread samples and the cookies were coded to guarantee the reliability of the processes and their results.
The analysis of the results of the sensory tests was carried out through the determination of the analysis of variance, through the statistical program INFOSTAT student version 2014. The variance coefficients for each attribute were analyzed by comparing the variability between the different formulas of each product and the higher the value, the greater the variance.

3. RESULTS AND DISCUSSION

3.1. Results of the sensory analysis of the breads

As it can be observes in the Table 1, the breads made with the flour dosage of the National Arriba cocoa shells, with a percentage of 20% and codified with the code (P1), there was no evaluation by the judges on the scale of "I dislike a lot" for none of the sensory attributes.
It was also obtained, a single vote for "I dislike moderately" and 14 evaluations for "I am indifferent".
On the other hand, 53% of the evaluations were for the highest category in the questionnaire and 34% for the scale of "I like moderately", 13% of the evaluations were assigned for the other scales.
The results of the sensory analysis of the breads made with the 10% dosage, of the same flour and codified with the code (P2), there were 17 evaluations by the judges in the scales of " I dislike moderately "and another on the "I dislike a lot" scale. However, there were 38 votes on the "I am indifferent" scale. In total, 23% of the evaluations of the four attributes were for the highest scale (I like a lot), 30% for the category "I like moderately" and 47% of the evaluations were assigned for the other scales.
With respect to the sensory analysis of the breads made with the dosage of 20% flour from the CCN 51 cocoa shells (Table 2), codified with the code (P3), there was an evaluation by the judges in the scale of "I dislike moderately", one on the scale of "I dislike a lot" and 21 votes on the "I'm indifferent" scale. In total, 46% of the evaluations of the four attributes

were for the highest scale (I like a lot), 34% for the "I like moderately" scale and 20% of the evaluations were for the other scales.

Table 1. Results of the sensory analysis of the bread made with flour from the National Arriba cocoa shells.

Attribute	I like a lot	I like moderately	I am indifferent	I dislike moderately	I dislike a lot
Bread made with 20% of flour from the National Arriba cocoa shells					
Color	15	12	2		
Flavor	16	7	4	1	
Odor	15	12	5		
Texture	18	10	3		
Total	64	41	14	1	
Bread made with 10% of flour from the National Arriba cocoa shells					
Color	9	7	10	4	
Flavor	4	10	10	6	
Odor	8	9	9	4	
Texture	7	10	9	3	1
Total	28	36	38	17	1

Source: Own elaboration, 2017.

The results of the sensory analysis of the loaves made with the flour from the CCN 51 cocoa shells but with a 10% dosage (Table 2), which were codified with the code (P4), there were 10 evaluations by the judges on the "I dislike moderately" scales, 4 for the "I dislike a lot" scale. In addition, there were 38 evaluations on the scale "I am indifferent", being 11 for color, 8 for flavor, 1 for odor and 9 for texture.

Table 2. Results of the sensory analysis of the bread made with flour from the CCN51 cocoa shells.

Attribute	I like a lot	I like moderately	I am indifferent	I dislike moderately	I dislike a lot
Bread made with 20% of flour from the CCN 51 cocoa shells					
Color	14	9	7		
Flavor	15	11	4		
Odor	14	9	6		1
Texture	13	12	4	1	
Total	56	41	21	1	1
Bread made with 10% of flour from the CCN 51 cocoa shells					
Color	7	7	11	3	1
Flavor	6	13	8	4	1
Odor	7	11	10	1	1
Texture	7	10	9	2	1
Total	27	41	38	10	4

Source: Own elaboration, 2017.

In total, 22% of the evaluations of the four attributes were for the highest scale (I like a lot), 34% for the "I like moderately" scale and 44% for the other scales.
Applying the numerical scale for the four evaluations mentioned above, multiplying the assigned number of each scale by the number of evaluations and adding the results of each formula of the breads, it can be concluded that the breads made with flour from the National Arriba Cocoa shells, with a dosage at 20% (P1) have better sensory acceptance with 528 points, as can be seen in Table 3. The breads made with the same flour with a percentage of 10% (P2) were in fourth place with a total of 433 points and the breads made with CCN 51 cocoa shells with a percentage of 20% (P3) occupied the second place with a total of 509 points.
On the other hand, the loaves made with 10% flour from CCN 51 coca shells (P4) ranked third with a total of 437 points.

Table 3. Total results of the score sum of the sensory evaluation.

Bread	P1	P2	P3	P4
Total score	528	433	509	437

Source: Own elaboration, 2017.

3.2. Analysis of variances between the formulas of the breads

Using the INFOSTAT student version 2014 to analyze the results and verify whether or not there is a significant difference between the different formulas, it is observed that, in relation to the attributes (Color, Flavor, Odor and Texture), for the answer "I like a lot", the coefficient of variation was 15.75%; for the answer "I like moderately" the coefficient of variation was 18.73%; for the answer "I am indifferent", the coefficient of variation was 15.98%; for the answer "I dislike moderately", the coefficient of variation was 27.76%; and for the answer "I dislike a lot", the coefficient of variation that was obtained was 35.09%; therefore, analysis of variance indicates that there is a significant difference between the formulas of the breads, thus confirming the previous data.
Comparing the results obtained from the sensory evaluation of bread made with different formulas with the research carried out by CERÓN *et al*. (2011), where the partial replacement of wheat flour by potato flour (*solanum tuberosum*) is carried out for the preparation of breads, it was observed that those elaborated with a partial substitution of 20% obtained the highest scores in the sensory analysis, which is in agreement with the results of the present investigation.
Contrary to what was reported in the research conducted by AMIR *et al*. (2013) to develop high fiber bread by utilizing the cocoa pod husk with five different percentages, For the overall acceptance, the formulation with 5% of the flour coming from the cocoa pod husk, had the highest mean score among the composite breads.
On other hand, GUERRA and VERANZA (2014) on the preparation of bread with mashua flour (*Tropaeolum tuberosum*), where it was confirmed that the formula with the lowest evaluation was 20% mashua flour.
In the work carried out by ORDOÑEZ and OVIEDO (2010), for the elaboration of bread based on wheat flour, banana and rye, it was concluded that the best formula, in relation to the organoleptic characteristics, was with the dosage 25% banana flour and 5% rye flour. Similar results can be observed in the research conducted by DÍAS and SEVILLA (2011) on the mixture of rice flour and wheat flour for baking, where they reported that the

breads with the best sensory characteristics were those made with 30% rice flour. In both investigations, higher percentages than those reported in the present investigation were obtained to substitute wheat flour.
Likewise, in the research carried out by AROZARENA and MARIN (2014) for the production of bread using flour composed of wheat, cassava and soybean, it was confirmed that the breads made with partial replacement of wheat flour with 5% soybean meal and 10% cassava flour is the best treatment.
According to the results obtained from the work carried out by COBO *et al*. (2013), it was confirmed that the percentage to replace wheat flour with white carrot flour (*Arracacia xanthorrhiza* B.) for bread making, obtaining the best characteristic sensory and physical, was 10%.
This percentage is in agreement with the one reported in the research of bread making with wheat flour and kiwicha flour (*Amaranthus caudatus L*.) made by CHAGMAN and HUMÁN (2010), where they confirmed that the best formula was the one elaborated with 10% of kiwicha flour and 90% wheat flour. In this way, it is observed that the sensory evaluation varies depending on the origin of the vegetable flour to be dosed in the baking processes, which is responsible for significant changes in the organoleptic characteristics of the loaves.

3.3. Results of the sensory analysis of cookies

In relation to the sensory analysis of cookies made with the flour dosage of the National Arriba cocoa shells (Table 4), with a percentage of 80% and codified with the code (G1), there was no evaluation on the "I dislike moderately" scale or on the "I dislike a lot" scale. On the other hand, approximately 83% of the evaluations were for the "I like a lot" scale, the rest of the evaluations for the other scales.
The results of the sensory analysis for the cookies made with the flour from the National Arriba cocoa shells, but with a percentage of 70% (Table 4), and with the coding (G2), there was no evaluation on the scales of "I dislike moderately" or "I dislike a lot" and 78% of the total evaluations are on the highest scale.

Table 4. Results of the sensory analysis of cookies made with flour from the National Arriba cocoa shells.

Attribute	I like a lot	I like moderately	I am indifferent	I dislike moderately	I dislike a lot
Cookies made with 80% flour from the National Arriba cocoa shells					
Color	25	5			
Flavor	25	4			
Odor	26	4			
Texture	26	4	1		
Total	102	17	1		
Cookies made with 70% flour from the National Arriba cocoa shells					
Color	23	4			
Flavor	24	7	1		
Odor	24	7			
Texture	23	6	1		
Total	94	24	1		

Source: Own elaboration, 2017.

With regard to the sensory analysis of cookies made with the dosage of 80% of flour from the CCN 51 cocoa shells, which were coded (G3) it is observed that there is no evaluation for the scale "I dislike a lot," however, there are 15 evaluations for the "I dislike moderately" scale. In total approximately 50% of the evaluations are on the "I like it a lot" scale, the rest of the evaluations correspond to the other scales.
As we can see in Table 5, the sensory analysis of the cookies made with the flour from the CCN 51 cocoa shells and with a dosage of 70%, which were coded (G4), it is observed that there is no evaluation for the scale "I dislike a lot ", nor for the scale "I dislike moderately". In total, approximately 71% of the evaluations were obtained on the "I like a lot" scale, while the rest of the evaluations correspond to the other scales.

Table 5. Results of the sensory analysis of cookies made with flour from the CCN 51 cocoa shells.

Attribute	I like a lot	I like moderately	I am indifferent	I dislike moderately	I dislike a lot
Cookies made with 80% flour from the CCN 51 cocoa shells					
Color	15	9		4	
Flavor	15	8	5	3	
Odor	16	8	4	3	
Texture	15	7	3	5	
Total	61	32	12	15	
Cookies made with 70% flour from the CCN 51 cocoa shells					
Color	21	6	3		
Flavor	22	8	3		
Odor	22	6	1		
Texture	21	6	1		
Total	86	26	8		

Source: Own elaboration, 2017.

Applying the aforementioned numerical scale for the four evaluations, multiplying the assigned number of each scale by the number of the evaluations and adding the results of each formula of the cookies, it can be concluded that those made with flour from the National Ariba cocoa shells 80% (G1) have the best sensory acceptance with 581 points, as can be seen in Table 6. The cookies made with the same flour, with a percentage of 70% (G2), occupied the second place in sensory acceptance, with a total of 572 points. The cookies made with the flour from the CCN 51 cocoa shells, with a percentage of 70% (G4), occupied the third place with a total of 558 points and in the last place are the cookies made with this same flour, with a percentage of dosage of 80% (G3), with a total of 499 points.

Table 6. Total results of the sum of the score of the sensory evaluation of the cookies.

Galletas	G1	G2	G3	G4
Puntaje Total	581	572	499	558

Source: Own elaboration, 2017.

3.4. Analysis of variances between cookie formulas

The INFOSTAT student version (2014) was used again to analyze the results and verify if there is a significant difference between the formulas. The results indicated that, in relation to the attributes (Color, Flavor, Odor and Texture), for the answer "I like a lot", the coefficient of variation is 2.34%, that is, there is no significant difference between the formulas for making cookies; for the answer "I like moderately", the coefficient of variation is 16.33%, that is, there is a significant difference between the cookie-making formulas; for the answer "I am indifferent", the coefficient of variation is 71.79%, which indicates that there is a significant difference between the cookie-making formulas and, finally, for the answer "I dislike moderately", the coefficient of variation is 36.47%, that is, there is a significant difference between the formulas for making cookies. In this way, analysis of variance indicates that there is a significant difference between cookie formulas, confirming the high acceptance for their consumption. Therefore, by analyzing the results, it is concluded that the cookies have higher sensory acceptance than the breads.
Comparing the results obtained from this research with the work done by CEDEÑO *et al*. (2014), for the preparation of cookies with mango rinds and pineapple peels in Ecuador, it was observed that the best formulas for making cookies were those made with 8% pineapple flour and 92% wheat flour and those made with 12% mango husks and 88% wheat flour, which have very low percentages of shell flour as a substitute for wheat flour, what was applied in this investigation.
On the other hand, in the work carried out by GONZÁLEZ and GOMES (2007) in Mexico for the preparation of cookies with orange bagasse flour, different levels of substitution of wheat flour for orange bagasse flour were used (0%, 10%, 20%, 30% and 40%) and with the application of sensory tests it was determined that the best formulas were cookies made with 10% and 20% orange bagasse flour, because the highest levels of This flour caused bitter taste and tougher texture in the cookies. Contrary to the present investigation where the best formula, in relation to the sensory analysis, was the one elaborated with a high flour level of the cocoa shells.
In another work carried out in Peru by Rodríguez (2014), potato peel flour was used with dosages of 30%, 50% and 70%, while the rest of the flour corresponded to wheat flour for the preparation of cookies. These studies confirm, through sensory analysis, that the ideal formula is that which has a percentage of 30% of potato peel flour, being higher than what was reported in the aforementioned research.
In the investigation of MICHELIN *et al.* (2014) to develop cookie recipes using different amounts of guava peel flour (GPF) levels (30%, 50%, and 70%), the cookies containing 50% and 70% GPF received satisfactory acceptance regarding to aroma only. However, the percentage is still lower compared to the dosage of flour from the shells used in the present investigation.
Therefore, the flour originating from the shells of the Nacional Arriba cocoa is the flour with the best sensory quality in the preparation of breads or cookies, likewise that the cookies made with the flour coming from the shells of the National Arriba cocoa have better sensory acceptance compared to breads made with the same flour.

4. CONCLUSIONS

Once the sensory analysis of the bread and cookies, made with flour from the shells of the two analyzed cocoa varieties, it was determined that the flour with the best organoleptic characteristics was obtained from the shells of the National Arriba cocoa, presenting a better sensory quality in the preparation of breads or cookies.

Likewise, it was determined that the biscuits made with the flour coming from the shells of the Cacao Nacional Arriba, with a percentage of 80%, have a better sensorial acceptance compared with the breads made with the same flour.
Finally, through this research it has been possible to verify the importance of using other sources of vegetable flour, in this case from the cocoa shells, to be used in the bakery industry, being an incentive for countries such as Ecuador where the production of wheat is not enough for the demand of the bakery industry.

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KINETICS OF ULTRASONIC OSMOTIC DEHYDRATION OF PHYSALIS

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ABSTRACT

Physalis is an exotic fruit with increasing international market; however there are few studies to obtain added value products with extended shelf life. Physalis slices were osmotically dehydrated (OD) in a sucrose solution of 70°Bx using ultrasounds. The effect of temperature (40, 50, 60°C) on water loss (WL) and solid gain (SG) kinetics were studied and fitted to empirical models. Ultrasounds allowed obtaining final moistures of 25-33% and SG of 7-10% in 150 min. The temperature increase accelerated the WL, SG and diffusivity. Peleg and Page models presented the best fitting for WL and SG with R2 values above 0.98 and 0.87 respectively. Diffusivity and activation energy values for WL and SG were determined. Ultrasound is a suitable technology for obtaining Physalis OD.

Keywords: kinetics, osmotic dehydration, *Physalis Peruviana,* ultrasounds, water loss

1. INTRODUCTION

Physalis peruvuana L. is an exotic fruit considered as a delicatessen in international markets thanks to its sensory and nutritional characteristics. Cultivation in Colombia is steadily increasing to satisfy the growing export demands, ranking it second after banana fruit exports. The fruit is good source of bioactive compounds like provitamin A, minerals, vitamin C, B-complex, dietary fiber and fructose (RAMADAN, 2011). Surplus of fruit not suitable for exporting, reaches 50% of total production, so is important to study methods for obtaining added value products with extended shelf life (CASTRO *et al.*, 2008).
Osmotic dehydration is a widely used method to partially remove water from fruits by immersion of cellular tissue in hypertonic aqueous sugar solutions (HUI *et al.*, 2012; NOWACKA *et al.*, 2014). Ultrasonic osmotic dehydration (UOD) allows higher water loss (*WL*) and solid gain (*SG*) rates by means of mechanical waves that can propagate through a material medium generating a series of rapid and successive compressions, known as "sponge effect", with rates depending on their frequency (FERNANDES and RODRIGUEZ, 2007). This promotes the alteration of physical and chemical properties of food products accelerating moisture removal and solid gain (NOWACKA *et al.*, 2014), while preserving nutritive components, sensorial and functional properties (RASTOGI and RAGHAVARAO, 1997).
The objective of this work was to determine the kinetics of ultrasound osmotic dehydration in *Physalis peruviana* slices and evaluating the fit of the experimental results in terms of *WL* and SG, using pre-established mathematical models.

2. MATERIALS AND METHODS

2.1. Raw material and sample preparation

The study was carried out with using fresh Colombia eco-type *Physalis peruviana* from a farm located in Gualmatán, Nariño. Fruit ripeness was 4-5 in a color scale, and caliber diameter C to D according to the Colombian Technical Standard NTC 4580. The fruit was received and selected by size and quality, then were washed and cut into slices of approximately 2 mm of thick.

2.2. Experimental protocol

The slices were immersed in an ultrasound equipment (Elma S10H®), with a sucrose solution of 70°Bx, using a ratio sample:solution of 1:5. Ultrasound frequency was set at 37 kHz and manual agitation was carried out every 15 minutes, in order to avoid crusting sugar on the surface of samples and accelerating the dehydration process (AYALA *et al.*, 2010). Three temperatures were evaluated: 40, 50 and 60°C (VEGA *et al.*, 2007), with a variation of ±2°C, due to the heating generated by the ultrasound, which was controlled in a similar way to (HUI *et al.*, 2012) by withdrawing hot solution and adding hypertonic solution at temperature of 10°C. Samples were taken at a pre-established time periods of 5 min during 150 min and dried with absorbent paper to remove the excess of solution in the surface. Samples for *SG* determination were washed quickly with distilled water and then dried.

2.3. Response variables methods

Water loss (*WL*) was determined by oven heating at 105°C for 24 hours according to the method used by (LUCHESE *et al*., 2015). Sugar gain (*SG*) was calculated according to the method proposed by (GOMEZ, 2014) by measuring°Brix with a Brixco equipment® (model 3030, Labexco®, Bogotá) with precision of 0.25°Bx. All determinations were made in quintupled.

2.4. Analytical evaluations

SG (%), and *WL* (%) were calculated based on the equations used by NOWACKA *et al*. (2014) (Eq. 1 and 2).

$$WL(\%) = \frac{(w_i * X_{hi} - w_t * X_{ht})}{w_i} * 100 \quad \text{(Eq.1)}$$

$$SG(\%) = \frac{(w_i * X_{si} - w_t * X_{st})}{w_i} * 100 \quad \text{(Eq.2)}$$

Where:
X_{hi}: initial fruit humidity on wet basis,
X_{ht}: fruit humidity at a time t on wet basis,
w_i: initial fruit mass,
w_t: fruit mass at time t of the treatment
X_{si}: initial fraction of soluble solids of fresh fruit,
X_{st}: fraction of fruit solids at time t on wet basis.

2.5. Modeling of kinetic parameters according to the effect of treatment conditions

Mass transfer kinetics was evaluated in terms of *WL*(%) and *SG*(%) and modeled according to the equations of Peleg (Eq.3), Azuara (Eq.4), Weibull (Eq.5), Page (Eq.6) and Magee (Eq.7), which are empirical models used in the phenomenological description of dehydration. The effective diffusivity was determined using the solution of the second law of Fick applied to plate geometry and long times (Eq.8) and (Eq.9). The effect of temperature on the kinetic parameters was analyzed using an Arrhenius type relation (Eq.10).

$$WL(t) = M_i \pm \frac{t}{k_1 + k_2 t} \quad \text{Eq.3} \qquad \frac{t}{WL} = \frac{1}{S_1 * WL_\infty} + \frac{t}{WL_\infty} \quad \text{Eq.4} \qquad MR = \exp\left(\frac{-t}{b}\right) \quad \text{Eq.5}$$

$$MR = \frac{M_t - M_\infty}{M_i - M_\infty} = \exp(-Kt^n) \quad \text{Eq.6} \qquad WL = k * t^{0.5} + a \quad \text{Eq.7}$$

$$MR = \frac{M_t - M_\infty}{M_i - M_\infty} = 1 - \sum_{n=o}^{\infty} \frac{8}{(2n+1)^2 \pi^2} exp\left\{-D_e (2n+1)^2 \frac{\pi^2 t}{4L^2}\right\} \quad \text{Eq.8}$$

$$n\,Y = Ln\frac{8}{\pi^2} - \left\{\frac{D_e \pi^2}{4L^2}\right\} * t \quad \text{Eq.9} \qquad D_e = D_o * \exp\left(\frac{-E_a}{R*T}\right) \quad \text{Eq.10}$$

Where k_1, k_2, *S*, WL_∞, *K*, *n*, *k*, *a*, and *b*, are model coefficients, *MR* is the moisture ratio,
M_t is *WL* or *SG* at a time *t*,
M_i *is WL* or *SG* at $t = 0$;
M_∞ is *WL* or *SG* at equilibrium time.

Diffusivity was determined with data of humidity and solid concentration, where n is the number of data, L is the thickness of the slice, *t* is time, *De* is the effective diffusivity, *Ea* is the activation energy, *T* is the temperature of the medium, and *R* is the universal gas constant.

For mathematical modeling of the experimental data, the software "Statgraphics Centurion XVI ®" was used, where model coefficients were calculated by means of non-linear regression analysis. The quality of the fit between the experimental data and the values predicted by the equations were analyzed through the correlation coefficient R^2_{adj} (Eq.11) and the standard error of prediction (SEP) (Eq.12). Where R^2 is the correlation coefficient, m is the number of regression parameters, Dat_{Exp} is the experimental data Dat_{Pred} is the predicted data with the model, and N is the number of observations data.

$$R^2{}_{adj} = 1 - (1 - R^2) * \frac{N-1}{N-m-1} \quad \text{(Eq.11)}$$

$$SEP = \sqrt{\frac{1}{N} * \sum_{i=1}^{N} (Dat_{Exp} - Dat_{Pred})^2} \quad \text{(Eq.12)}$$

3. RESULTS AND DISCUSSION

Higher temperatures increased final values of *WL* and *SG*, but the effect was only significant at 60ºC (Table 1), in addition the magnitudes of *SG* are lower than those of *WL* because the membrane selectivity, and the molecular size difference between water and solid. Final moistures obtained were 40°C = 34.08%, 50°C = 33.31% and 60°C = 25.91%. As can be seen in Fig 1, the treatments conducted at 40 and 50 ºC had very similar rates of dehydration. Most *WL* occurred in the first 60 min of the process, with an increasing exponential behavior according to temperature increase. After 75 min, time increase had no appreciable effect on *WL*. Previous behavior could be due to the increasing sugar concentration in the superficial layer of the fruit, which acts as a barrier to the mass transfer (MALDONADO *et al.*, 2008).

Table 1. Final values (t = 150 min) of water lost (*WL*) and solids gain (*SG*) in *P. peruviana* dehydrated by UOD at different temperatures.

Parameter	Temperature		
	40°C	50°C	60°C
WL (%)	72.29[a]	72.35[a]	74.40[b]
SG (%)	7.41[a]	7.43[a]	10.04[b]

Values in rows with different letter denote significant differences (P < 0.05).

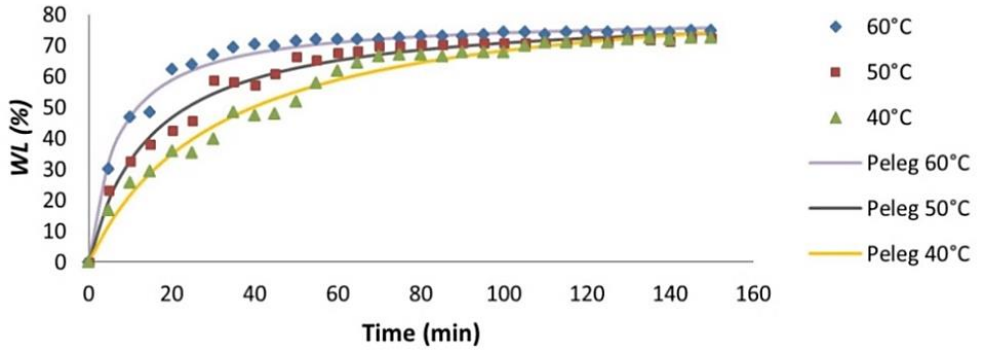

Figure 1. Kinetics and Peleg model fitting for water loss (WL) during the UOD of P. peruviana.

WL final values obtained were higher than that found by LUCHESE *et al.* (2015) in *Physalis*, maybe because in this study fruit was cut in slices, increasing the effective area for mass transfer. Also using ultrasounds improve water loss.
Similar to the behavior observed for *WL*, the effect of temperature on *SG* was only significant at 60 °C, where a sudden increase in SG was observed after 40 min process (Fig. 2). During the first 15 min of UOD at temperatures of 50 and 60°C, a pseudo-equilibrium state was observed, with almost any increase in solid gain, similar to was observed by VEGA *et al.* (2007), but after 15 min process, *SG* increased gradually, as can be seen in Fig 2. This behavior is related with the strong *WL* observed at the early stage of the process. After most water has been delivered from the product, *SG* starts to increase. The *SG* values obtained were similar to those reported by MALDONADO *et al.* (2008) in *Smallanthus sonchifolius*, higher than those obtained by NOWACKA *et al.* (2014) in Kiwifruit and lower that *SG* reported by SHAFIQ *et al.* (2017) in kinnow fruit.

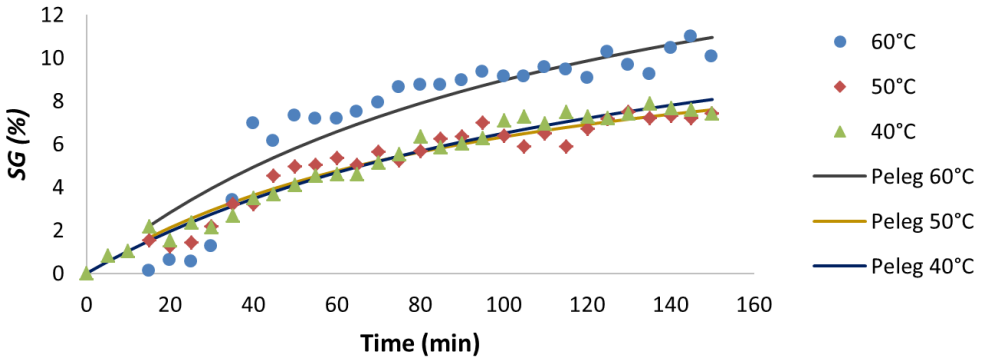

Figure 2. Kinetics and Peleg model fitting for solid gain (*SG*) during the UOD of *P. peruviana.*

Temperature increased *WL*, *SG* and the effective diffusivity of sucrose, due to a reduction in the osmotic solution viscosity and an increase in the velocity of mass transfer (LUCHESE *et al.*, 2015; SHAFIQ *et al.*, 2017). Ultrasounds cause an effect of compression and expansion (sponge effect) generating microscopic channels in the fruits tissue, which increase the effective water diffusivity (SIMAL *et al.*, 1998; FERNANDES *et al.*, 2007).
Table 2 shows R^2_{adj} and SEP values, which allow determining the goodness of fit between the empirical models and the experimental data. For *WL*, Peleg and Azuara models had the same R^2 and SEP values, so both of them could be applied for *WL* behavior analysis

and prediction. Page model presented a better fitting for *WL*, however, the use of Peleg model is recommended due to its versatility and ease of calculation (MOREIRA and MURR, 2004). Peleg model coefficients obtained for *WL* were k_1 (40°C) = 0.350507, k_2 (40°C) = 0.0111363, k_1 (50°C) = 0.182443, k_2 (50°C) = 0.0123054, k_1 (60°C) = 0.0873859, k_2 (60°C) = 0.0126328.

Table 2. Goodness of fit of kinetic models used to predict *WL* and *SG* at evaluated conditions.

Parameter (%)	Models									
	Peleg		Azuara		Magge		Weibull		Page	
	$R_{2\ adj}$	SEP	$R_{2\ adj}$	SEP	$R_{2\ adj}$	SEP	$R_{2\ adj}$	SEP	$R_{2\ adj}$	SEP
$WL_{40°C}$	0.9812	2.5246	0.9812	2.5246	0.9369	4.7835	0.9763	0.0384	0.9840	0.031
$SG_{40°C}$	0.9748	0.3449	0.9785	0.3391	0.9357	0.4474	0.9756	0.0306	0.9784	0.0283
$WL_{50°C}$	0.9832	2.1595	0.9832	2.1595	0.8138	7.1977	0.9703	0.0394	0.9884	0.0242
$SG_{50°C}$	0.925	0.5062	0.925	0.5062	0.8223	0.5736	0.8885	0.0524	0.9133	0.0453
$WL_{60°C}$	0.9851	1.8685	0.9851	1.8685	0.6332	9.2729	0.9791	0.0299	0.9907	0.0196
$SG_{60°C}$	0.84	1.2344	0.84	1.2344	0.8301	1.2721	0.854	0.1002	0.8704	0.0925

Page model presented the best fitting for *SG*, similar to was observed by MOREIRA and MURR (2004) and the coefficients obtained were: *k* (40°C) = 0.0113934, *n* (40°C) = 0.91778; *k* (50°C) = 0.0167055, *n* (50°C) = 0.825085, *k* (60°C) = 0.00430261, *n* (60°C) = 1.2739.

Low fitting obtained with Magge model, was due to this model is only appropriated for short times. Also R^2 and SEP values calculated for Weibull and Page models, should be compared carefully with those of the other models, because they are obtained using dimensionless *WL* and *SG* (OCHOA and AYALA, 2005).

Effective diffusivity values obtained (Table 3), were higher compared with those reported by MOREIRA and MURR (2004), PARK *et al.* (2002), LUCHESE *et al.* (2015) and VEGA *et al.* (2007) in cherry tomatoes, pears, *Physalis peruviana*, and papaya respectively, and are similar to those of ARIAS *et al.* (2017) and GOMEZ (2014) in mango and melon respectively. Using a relationship type Arrhenius (Eq. 10) the activation energy was obtained, with values of 11.707 kJ/mol for *WL* (R^2 = 0.9838) and 14.549 kJ/mol for *SG* (R^2 = 0.9986). Fick diffusional model was appropriate to determine effective diffusivity (*ED*) values of liquids and solids in the matrix of *Physalis peruviana*.

Table 3. Effective diffusivity (*ED*) of water and solids in *Physalis peruviana* at evaluated conditions.

Parameter	Temperature					
	40°C		50°C		60°C	
	ED (x10^{-8})	R^2	*ED* (x10^{-8})	R^2	*ED* (x10^{-8})	R^2
Water	1,8844^a	0.977	2,0902^b	0.9275	2,4556^c	0.8915
Solids	1,6148^a	0.8501	1,9406^b	0.9418	2,2579^c	0.8851

Values in rows with different letter denote significant differences ($P < 0.05$).

ED values increased significantly with temperature, similarly to what was found by PARK *et al*. (2002). This effect was associated with the formation of microscopic channels due to the loss of cellular adhesion which produced large cell interspaces and increasing of

molecular agitation, consequently enhancing diffusion rates with increasing temperature. Another effect is related to the cellular material that is quite sensitive to the temperature. Overheating causes a cell lysis, leading to a decrease of the membrane mass transfer limitation and selectivity (GOULA, 2017).

4. CONCLUSIONS

The effect of the temperature on the kinetics of ultrasound osmotic dehydration of slices of *Physalis peruviana* was studied. The use of ultrasound technology allowed accelerating the process and achieves the major water loss of 74.4% and 10.04% solid gain at 60ºC in around 150 min.
Most models presented a good fit for *WL* and *SG* experimental datas, but, according to the results obtained, Peleg and Azuara models got the best estimation for *WL* and Page model had a better representation for *SG* values, these models can provide good estimations under similar conditions what were studied, to ensure the technique as pre-treatment in the agro industrialization of the fruit.

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ANTIOXIDANT ACTIVITY AND DIFFERENTIATION OF ESSENTIAL OILS OF GUAVIDUCA (*PIPER CARPUNYA* L.) AND SACHA AJO (*MANSOA ALLIACEA* L.)

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ABSTRACT

Essential oils are aromatic substances found in different parts of plants, which can be extracted from leaves, stems, flowers and/or roots. These complex mixtures of hydrocarbons, terpenes, alcohols, carbonyl compounds, aromatic aldehydes and phenols, coming from spices and condiments, are increasingly used in the food and pharmaceutical industry. The present work aim was to differentiate the essential oils levels of Guaviduca (*Piper carpunya* L.) and Sacha Ajo (*Mansoa alliacea* L.) species, from the Ecuadorian Amazon region from fresh and dry leaves, by means the polyphenolic activity trough Folin-Ciocalteau, also total antioxidant activity according to FRAP (Ferric ion reducing antioxidant Power) and ABTS (2,2-azinobis (3-ethylbenzthiazolin)-6-sulphonic acid). The essential oils extraction method used in this investigation was steam distillation, using reflux with a Clevenger trap in order to separate them, taking advantage that oils are lighter than water. The yield for each the essential oils was determined quantitatively from the wet weight of each aromatic plant. A Simplex-Lattice design was used to determine the measuring points of antioxidant activity (essential oils proportions). Higher values are observed, in all determinations, in the essential oils obtained from fresh samples of guaviduca leaves and Sacha Ajo. This behavior might be due to the active compounds degradation with antioxidant activity and to the loss of essential oils by the evaporation process during drying.

Keywords: Guaviduca, Garlic sacha, essential oils, antioxidants

1. INTRODUCTION

The plants were used from the origin of humanity as phytotherapeutics treatment to prevent or heal injuries and diseases. From the year 1649, with the arrival of Christianity in America, the Jesuits studied the first Amazonian plants and their utilities. The results of those investigations were published in Shedula Romana, book where they reported about Cinchona officinallis (quina), from which various phenolic alkaloids have been obtained, including quinine, which has been used for more than three hundred years to cure malaria. In order to make good use of the plants, basic knowledge must be known about the types of plant species, their management and use, the dosage and the method of preparation (QUISPE *et al*., 2017).
The Amazonian environment is an important source of bioactive molecules; there is evidence that Amazonian species contain polyphenols. According GARCÍA-RUIZ *et al*. (2017) Ilex guayusa,on extracts has 14 phenolic compounds with high antioxidant activity.
Among the polyphenols, the bioactive compounds, represent an important group. Every year more studies are presented relate this class of molecules with beneficial functions for human health (DE VARGAS *et al*., 2016).
The most recognized and applied methodology to determine the total content of polyphenols is the Folin-Ciocalteau test (PROESTOS and VARZAKAS, 2017). Among other techniques for the determination of total antioxidant activity, BENZI and STRAIN (1996) reported FRAP (Ferric ion reducing antioxidant Power) and ABTS (2,2-azinobis (3-ethylbenzthiazolin) -6- acid). sulfonic) reported by RE *et al*. (1999).
In the present work, the essential oils differentiation of Guaviduca (*Piper carpunya* L.). and Sacha Ajo (*Mansoa alliacea* L.) from leaves, was determined through the polyphenolic activity by Folin-Ciocalteau and in order to determinate Total antioxidant activity by FRAP and ABTS.

2. MATERIAL AND METHODS

2.1. Location

It was carried out in the Chemistry and Biology laboratories at Amazonica University, located at Km 2 ½ Puyo to Tena road.
This applied research, is based on experimentation. In order to control independent variables Quantitative methods were used such as, numerical calculations and statistical analysis.

2.2. Active principles extraction

The plant material was washed with potable water, dried by oven (Barnstead International, USA) with air recirculation at 45°C, pulverized in a knife mill (Thomas Scientific, USA) and then sieved, in order to guarantee a particle size of less than 0.5 mm, considered adequate for the subsequent extraction of the extracts (Azwanida, 2015, Ph. Eur., 2017). The extracts of the two selected plants were made with the Ultrasound Assisted Extraction (UAE) method (Branson Ultrasonics, E.E.U.U.). For extraction, an ethanol: water mixture was used in a 9: 1 ratio, with a ratio of 250 mL of solvent per 50 g of pulverized sample. The extractions were made in triplicate. The mixture was worked at 35°C for 1 hour and then the mixture was filtered through a Gooch filter and the crude extract obtained was concentrated with a rotary evaporator (Büchi, Germany) at a temperature of 45°C and a reduced pressure of 600 mmHg to a final volume of 50 mL.

2.2. Total phenols determination

For the implementation of the Folin-Ciocalteu test (PROESTOS and VARZAKAS, 2017), a calibration curve was previously constructed by making successive dilutions from a concentrated solution of 1000 mg.L^{-1} of gallic acid (reference standard, Table 1). From this solution, 10 ml of each of the diluted solutions of increasing concentrations of gallic acid between 5 and 25 mg.L^{-1} were prepared. For this determination, 40 µl of the sample was taken in a 10 ml volumetric flask and 500 µl of Folin Ciocalteu reagent was added. It was resting with protected from light for 10 minutes. Once this time has ended, 500 µl of 10% sodium carbonate solution is added. It is homogenized and placed in darkness for 2 hours, to finish with the measurement of the observance at 765 nm against the reagent blank.

Table 1. Preparation of the gallic acid standard curve from a concentrated solution of 1,000 mg.L^{-1}. Final volume 10 mL (distilled water).

Added components	Gallic Acid Concentration (mg.L^{-1})				
	5	10	15	20	25
gallic acid (uL.)	50	100	150	200	250
Reactive Folin-Ciocaltea (uL.)	500	500	500	500	500
Sodium carbonate dilution 10% (uL.)	500	500	500	500	500

2.3. Antioxidant activity determination

FRAP Method (Ferric ion reducing antioxidant Power): The reduction of 2,4,6-Tripiridyltriazine Ferric (TPTZ) to a product colored by the activity of antioxidant compounds is measured (BENZI and STRAIN, 1996).

A calibration curve was constructed by making successive dilutions from a concentrated solution of 1000 mg.L^{-1} of gallic acid (reference standard, Table 2). From this solution, 10 ml of the diluted solutions were used, increasing the gallic acid concentrations between 5 and 25 mg.L^{-1}. For this determination, 80 µl of sample was taken in a 10 ml volumetric flask and 5 ml of FRAP solution was added, and distilled water was added to the mixture. It is allowed to stand, in an oven at 37°C, for 30 minutes and the absorbance is read at a wavelength of 593 nm against white.

For this determination, in a 10 mL flask, where, 80 µL of sample, 5 mL of FRAP solution was added. It was left to rest, in a dark chamber at 37°C, for 30 minutes. To finally measure the absorbance at a wavelength of 593 nm against white.

Table 2. Preparation of the trolox standard curve from a concentrated solution of 1,000 mg.L^{-1}. Final volume 10 mL (distilled water).

Added Components	Gallic Acid Concentration (mg.L^{-1})				
	5	10	15	20	25
Gallic Acid (uL.)	10	20	25	30	35
Disolution FRAP (mL.)	5	5	5	5	5

ABTS method (2,2-azinobis (3-ethylbenzthiazolin) -6-sulphonic acid): This is based on the ability of an antioxidant to stabilize the ABTS colored cation radical, which is previously formed by the oxidation of ABTS (2). , 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) by metamioglobin and hydrogen peroxide. The results are expressed as Trolox equivalents (RE *et al*., 1999).
A calibration curve was constructed by making successive dilutions from a concentrated solution of 1000 mg.L^{-1} of gallic acid Table 3). From this solution, 10 ml of each of the diluted solutions of increasing concentrations of gallic acid between 5 and 25 mg.L^{-1} were prepared.
For this determination, 40 µL of sample was taken and placed in the spectrophotometer cuvette. 2 mL the solution of the radical was added, after 7 minutes. The absorbance reading was performed at a wavelength of 730.0 nm against an ethanol blank.

Table 3. Preparation of the trolox standard curve from a concentrated solution of 1000 mg.L^{-1}. Final volume 10 mL (distilled water).

Added Components	Gallic Acid Concentration (mg.L^{-1})				
	5	10	15	20	25
Gallic Acid (uL.)	20	30	40	50	60
Radical ABTS (mL.)	2	2	2	2	5

2.5. Statistical methods

An experiment was carried out with two factors. These shown in the following table:

Table 4. Factors and levels involved in an experiment.

Factor	Levels
State of leaves	Dry leaves
	Fresh leaves
Analysis techniques	Folin-Ciocalteua method
	FRAP (Ferric ion reducing antioxidant Power)
	ABTS (2,2-azinobis (3-ethylbenzthiazolin) -6-sulphonic acid)

Six treatments were obtained per species with the output variable total polyphenols and antioxidant activity. The analysis techniques used were ANOVA and Tukey test, with the determination of confidence intervals for the population mean.
Analysis techniques: The statistical analysis was developed using IBM SPSS Software Version 21.0.

3. RESULTS AND DISCUSSION

As Table 5 shows, the results of polyphenolic activity by the Folin-Ciocalteau method and total antioxidant activity according to the FRAP and ABTS methods. Higher values are observed, in all determinations, in the essential oils obtained from wet samples of

Guaviduca leaves. This behavior may be due to the degradation of active compounds with antioxidant activity and to the loss of essential oils by the evaporation process during drying.

Table 5. Total polyphenolic activity according to Foli-Ciocalteau and total antioxidant activity by FRAP and ABTS to essential oils of Guaviduca (*Piper carpunya* L.) in fresh and Dry samples.

Essentials Oils	Analysis Technics		
	Folin-Ciocalteau	FRAP	ABTS
	765 nm	593 nm	730 nm
Essentials Oils Fresh sample (mg/L.)	23,72456667	13,26978333	0,314
Essentials Oils dry sample (mg/L.)	19,33716667	9,1035	0,126666667

The Table 6 presents the results of polyphenolic activity by the Folin-Ciocalteau method and total antioxidant activity according to the FRAP and ABTS methods. Higher values are observed in all determinations in the essential oils obtained from wet samples of Sacha Ajo leaves.

Table 6. Total polyphenolic activity according to Foli-Ciocalteau (mg equivalent of gallic acid.L^{-1}) and total antioxidant activity by FRAP and ABTS (mg equivalent Trolox.L^{-1}) to essential oils of Sacha Ajo (*Mansoa alliace*a L.) in wet and dry samples.

Essentials Oils	Analysis Technics		
	Folin-Ciocalteau	FRAP	ABTS
	765 nm	593 nm	730 nm
Essentials Oils Fresh sample (mg/L.)	6,58925	1,866616667	0,171816667
Essentials Oils dry sample (mg/L.)	2,0215	0,60795	0,084166667

Significant difference in the column for the techniques used in the analyzes ($p < 0.05$ according to Tukey).

Next, the total polyphenolic activity is plotted by Folin-Ciocalteua and the antioxidant activity according to FRAP and ABTS, in the essential oils of Guaviduca (*Piper carpunya* L.) in fresh and dry samples (Fig. 1). There is evidence of greater total polyphenolic activity according to Folin-Ciocalteau (mg equivalent of gallic acid.L^{-1}) in essential oils of Guaviduca (*Piper carpunya* L.) in fresh and dry samples. Total antioxidant activity by FRAP showed higher values of activity than the ABTS method.
Fig. 2 shows the total polyphenolic activity and the antioxidant activity, analyzed in the essential oils of Sacha Ajo (*Mansoa alliace* L.) in fresh and dry samples.
In the statistical analysis, the homogeneity of the six treatments was determined, for which an ANOVA hypothesis test was carried out with a significance level of 0.05, the following hypotheses were declared.

H0: The treatments are homogeneous
H1: The treatments are not homogeneous

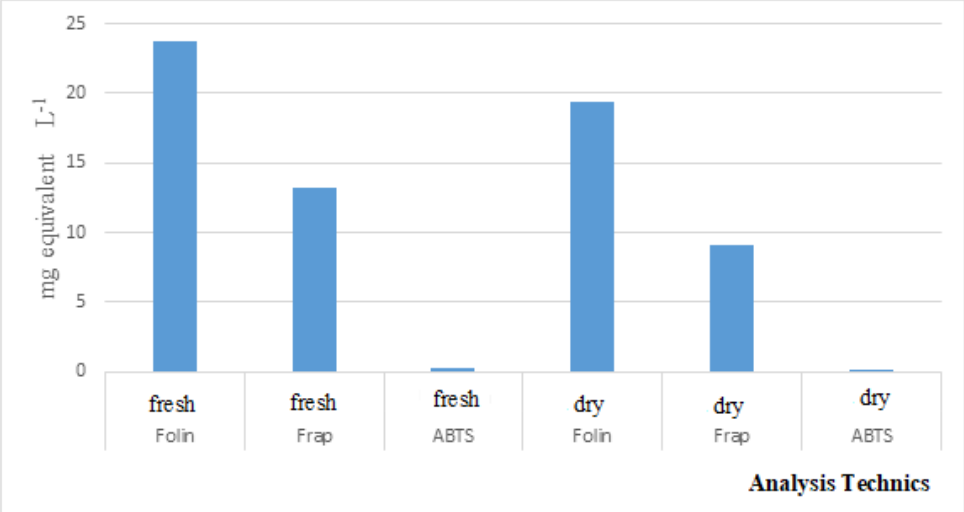

Figure 1. Total polyphenolic activity according to Folin-Ciocalteau (mg equivalent of gallic acid.L^{-1}) and total antioxidant activity by FRAP and ABTS (mg equivalent of TROLOX.L^{-1}) to essential oils of Guaviduca (*Piper carpunya* L.) in fresh and dry samples.

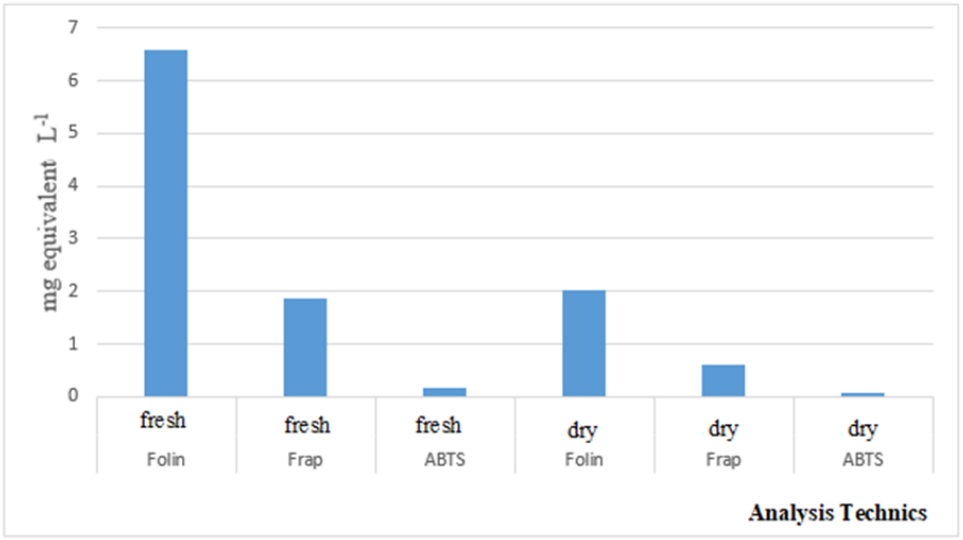

Figure 2. Total polyphenolic activity according to Folin-Ciocalteau (mg equivalent of gallic acid.L^{-1}) and total antioxidant activity by FRAP and ABTS (mg equivalent Trolox.L^{-1}) to essential oils of Sacha Ajo (*Mansoa alliace*a L.) in fresh and dry samples.

ANOVA PARA GUAVIDUCA

	Sum of Squares	gl	Half Quadratic	F	Sig.
Among Groups	2835,534	5	567,107	2737,396	0,000
Inside Groups	6,215	30	0,207		
Total	2841,749	35			

Similarly, all treatments are significantly different from each other. In this case except for dry ABTS and fresh ABTS, fresh ABTS and dry Frap, fresh Frap and dry Folin. It is noteworthy that fresh Folin is significantly different from the other analysis techniques.

Guaviduca

HSD Tukey[a]

Treatments	N	Alfa Subset = 0.05				
		1	2	3	4	5
ABTS dry	6	0,1267				
ABTS fresh	6	0,3140				
Frap dry	6		9,1035			
Frap fresh	6			13,2698		
Folin dry	6				19,3372	
Folin fresh	6					23,7246
Sig.		0,979	1,000	1,000	1,000	1,000

The means for the groups are displayed in the homogeneous subsets.
a. Use the sample size of the harmonic mean = 6,000.

All treatments are significantly different from each other, except dry ABTS and fresh ABTS.

ANOVA SACHA AJO

	Sum of Squares	gl	Half Quadratic	F	Sig.
Among groups	179,746	5	35,949	548,554	0,000
Inside groups	1,966	30	0,066		
Total	181,712	35			

Sacha ajo

HSD Tukey[a]

Treatments	N	Alfa Subset = 0.05			
		1	2	3	4
ABTS dry	6	0,0842			
ABTS fresh	6	0,1718	0,1718		
Frap dry	6		0,6080		
Frap fresh	6			1,8666	
Folin dry	6			2,0215	
Folin fresh	6				6,5893
Sig.		0,991	0,061	0,898	1,000

The means for the groups are displayed in the homogeneous subsets.
a. Use the sample size of the harmonic mean = 6,000.

The level of significance is 0.00, the null hypothesis is rejected, the hypothesis alternative is assumed, the group is not homogeneous. The Tukey test is applied where the treatments shows significant differences.

Likewise, all treatments are significantly different from each other. In this case except for dry ABTS and fresh ABTS, fresh ABTS and dry Frap, fresh Frap and dry Folin. It is noteworthy that fresh Folin is significantly different from the other analysis techniques.
In Ecuador, traditional medicine has been supported by the poorest peoples, who have found less expensive this alternative and more consistent with their worldview. In this context, Guaviduca has been used, for ancestral medicine, for dozens of years in different regions.
Many antioxidant compounds can be found in plants, including phenols, carotenoids, anthocyanins and tocopherols (JAKUBOWSKI and BARTOSZ, 1997). Approximately 20% of the known plants have been used in pharmaceutical studies, which has had a positive impact on the health system, such as the treatment of cancer and other harmful diseases (NACZK and SHAHIDI, 2006). Plants can produce a large number of different bioactive compounds among them polyphenols.
High concentrations of phytochemicals, which can protect against free radical damage, accumulate in fruits, vegetables and plants in general (SufFredini *et al*., 2004). Plants that contain beneficial phytochemicals can complement the needs of human body by acting as natural antioxidants (BOOTS *et al*., 2008). Phenolic compounds such as flavonoids, tannins and lignins, which are found in plants, act as antioxidants with efficacy (SUFFREDINI *et al*., 2004). The consumption of fruits and vegetables has been linked to several health benefits, as a result of medicinal properties and high nutritional value (VALKO *et al*., 2006). Antioxidants control and reduce oxidative damage in foods by delaying or inhibiting oxidation caused by reactive oxygen species, which ultimately increases the shelf life and quality of these foods (AMES *et al*., 1993). Beta-carotene, ascorbic acid and many phenolic compounds play dynamic roles in delaying aging, reducing inflammation and preventing certain cancers (DUTHIE *et al*., 1996).
The Guaviduca, used in the present work was previously investigated by CASTILLO (2014) who verified the anti-ulcer activity of the hydroalcoholic extract of the Guaviduca (*Piper carpunya*). This study was developed using the technique described in the Manual of Research Techniques (CYTED, 1995) through the induction of acute gastric ulcer by absolute 96% ethanol in female rats (Rattus Wistar), with a percentage of inhibition ulcers 50% in the treated group and 46.2% in the control group with which it is concluded that the hydroalcoholic extract of Guaviduca (*Piper carpunya*) has gastroprotective activity, producing a protection and recovery of the gastric mucosa evidencing itself in the macroscopic analysis of the stomachs.
For da SILVA *et al*. (2017) the Piper genus is the most representative within the Piperaceae with 2000 species distributed in the pantropical region. In the Neotropics, their species are represented by herbs and shrubs that are used in traditional medicine through infusions. Its essential oils have high performance and are chemically constituted by a complex mixture where volatile oils predominate. The chemical composition of Piper shows interspecific or intraspecific variations, depending on the site of collection or seasonality. The main volatile compounds identified in Piper's essential oils are monoterpene hydrocarbons, oxygenated monoterpenoids, sesquiterpene hydrocarbons, oxygenated sesquiterpenoids and large quantities of phenylpropanoids. There are many reports of the use of Piper essential oils as antimicrobial agents against fungi and bacteria, antiprotozoals (*Leishmania* spp., *Plasmodium* spp., and *Trypanosoma* spp.), Acetylcholinesterase inhibitor, anti-inflammatory and cytotoxic activity against different tumor cell lines such as breast, leukemia, melanoma, gastric, among others (QUIJANO *et al*., 2006; RAMÍREZ, 2016).
According to MONSERRATE (2014), the Sacha Ajo or *Mansoa alliace*a is a species of Ecuadorian origin, of medicinal and culinary use in the Amazonian tribes, but its use is uncommon in the rest of the country. This author prepared a seasoning for meats from dehydrated leaves of *Mansoa alliace*a, also seeking a quality as a source of antioxidant

agents. This plant, common in tropical areas with rainfall between 1,800 and 3,500 mm / year, temperatures between 20° and 26°C, contains some sulfur components, flavonoid pigments and saponins, as non-sulfur compounds. In addition, the presence of vitamins E and C has been found, as well as the minerals selenium and chromium, the presence of essential oils, alkaloids, sterols.

According to LIZCANO *et al*. (2015) there is a growing interest in identifying natural products derived from plants with antitumor activities. These authors studied the effects of aqueous leaf extracts of the Amazonian species of Vismia and Piper on the toxicity of human hepatocarcinoma cells. The results showed that, depending on the type of cell, the plants showed differential effects; therefore, Vismia baccifera induced the selective death of HepG2, while increasing the cellular growth of PLC-PRF and SK-HEP-1. On the contrary, these last two cell lines were sensitive to toxicity by Piper krukoffii and Piper putumayoense. All the extracts induced cytotoxicity for the rat hepatoma McA-RH7777, but they were innocuous. In all cases, cytotoxicity was accompanied by an intracellular accumulation of reactive oxygen species (ROS). These results provide evidence of anticancer activities of the plants studied in specific cell lines. The results also support the use of Vismia and Piper extracts with opposite effect as a model system to study the mechanisms of antitumor activity against different types of hepatocellular carcinoma.

The genus Mansoa, family Bignoniaceae includes eleven species that are found mainly in the dry and rain forests of Brazil and from Argentina to the southeast of Mexico. These species, present in the Brazilian Amazon region as vine garlic, have leaves with a penetrating smell of garlic when crushed. It has several uses in folk medicine, among them, the most cited are the treatment of fever, pain and inflammation of arthritis and rheumatism. Despite all these uses, it still has little application in herbal medicine compared to garlic (*Allium sativum*). The essential oils of Mansoa spp. show the presence of allyl polysufides that contribute to the characteristic aroma and flavor. The chemical composition of Mansoa's organic extracts includes alkanes, alkanols, triterpenoids, flavonoids, lapachol derivatives and organic sulfur compounds (ZOGHBI *et al*., 2009).

4. CONCLUSIONS

In the present work was to differentiate the essential oils of Guaviduca (*Piper carpunya* L.) and Sacha Ajo (*Mansoa alliacea* L.) species, from the Ecuadorian Amazon region from fresh and dry leaves, by means the polyphenolic activity trough Folin-Ciocalteau, total antioxidant activity according to FRAP (Ferric ion reducing antioxidant Power) and ABTS (2,2-azinobis (3-ethylbenzthiazolin) -6-sulphonic acid). Higher values in total polyphenols and antioxidant activity were observed by FRAP and ABTS in all the determinations in the essential oils obtained from wet samples of Guaviduca leaves, as well as higher values in all determinations in the essential oils obtained from wet samples of leaves of Sacha Ajo. The content of total polyphenols and antioxidant activity in essential oils from the leaves of the Guaviduca, is twice what has been found in the essential oil from the leaves of the Sacha Ajo, so, the use of leaves of Guaviduca as an ingredient for the manufacture of products of interest would have a greater impact on the contribution of antioxidants than the Sacha Ajo.

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CONSUMPTION AND LABELING OF ECOLOGICAL PRODUCTS IN THE ECUADORIAN ANDES

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ABSTRACT

The reality of the ecological market in Ecuador is still being studied, focusing on its production as well as its commercialization and consumption. These products are currently regulated by the food labeling system plan processed by the Ministry of Public Health (MSP).
This study focuses on defining the consumer profile of ecological products through socio-demographic characteristics, purchasing habits and attitudes towards the consumption of these foods. At the same time, packaging labels of non-ecological products that could induce the consumer to consider them as such was studied. To this end, 94 people from the city of Ambato, in the center of the Andean country, were surveyed.
The results depict different places, frequency as well as purchasing and consumption factors that depend on variables such as gender, age or purchasing power. It was also detected that the majority of respondents perceived some products as ecological when in fact they were not, due to the misleading labeling on the package.
Finally, the consumption habits of this type of food is discussed along with the legislative regulations about labeling that were found in products that are not officially ecological.

Keywords: ecologic products, consumers, labeling, packaging, attitudes

1. INTRODUCTION

In recent years, consumers have seen how their decision to purchase can affect the environment and even more so their health. Also, human beings have more and more places to choose what they will buy and what they will not buy. "These factors, among others, have generated an exponential growth of the production and consumption of organic food" (OROZCO ABUNDIS *et al*., 2003).

This all began at the end of the 20th century on the European continent through *alternative stores*. These types of products resulted in ecological consumers, who are people that try to be environmentally-friendly, and therefore try to change their patterns of behavior and consumption in order to be respectful with the environment in which they live.

The consumer is more conscientious of their role in the purchase action, trying to adopt a rational attitude not only about their needs, ability to spend (…), etc., but also adjusting their decision toward aspirations of qualitative improvement of society (JIMÉNEZ HERRERO, 1989).

In recent years, Ecuador has shown significant growth in organic agriculture, determining an important source of economic profits through their sale to countries in North American and Europe. These international markets have been the engines that have dynamized the export of these products and not so much their sale within the country. "Although the productive base in the country is growing, the local market for these products is very limited, due in large part to unawareness (…) and limited availability of products that meet internal demand" (VECO Ecuador, 2008).

Environmental behavior is identified when the individual's concern for the environment is great, and the individual "actively participates in the improvement of it, showing a great environmental responsibility and modifying their habits that are less respectful with the environment, even when they are willing to pay more for less polluting products, such as the case of organic food" (FRAJ, 2003; MARTÍNEZ-CARRASCO PLEITE *et al*., 2008).

In some cases, the products that are considered to be ecological are simply products that pollute the environment in lesser amounts and according to their information, they are presented as a better option that damages the environment less than others. However, they are still damaging in a percentage by the elements that compose them.

This fact can lead to fraudulent advertising when sending messages that lead to deception. Thus, a product would be sold as if it were ecological appealing to concepts and labeled as natural, handmade or healthy.

Within the country there are rules and laws that regulate advertising and defend the consumer (Ley Orgánica de Defensa del Consumidor and Reglamento para la autorización y control de la publicidad y promoción de alimentos procesados, 2013). It seeks to protect the rights of consumers by penalizing all types of advertising that generate error, whether misleading or abusive.

Therefore, its purpose would be to regulate the control and monitoring of advertising and promotion of processed foods for human consumption to guarantee the constitutional right of people to receive accurate and not misleading information about the content and characteristics of processed foods, which would allow the consumer to make the correct choice based on its acquisition and consumption.

Studies such as those carried out by LÓPEZ *et al*. (2015) explain that many of the behavioral actions of ecological consumption by users are the result of the handling of information, which at the same time fulfills the function of forming from the message through the organization and characterization of the missive they detail, as well as the benefits and properties of the use of these products.

The messages that are transmitted, or that are implicit within the products, will be those that directly or indirectly influence the decision to buy a product. This is how the nutritional information, the quantities with which an A or B product is made, or other information will be the indicators for a product to be accepted and bought by consumers.
The labeling of products is one of the elements that make up these messages. It "is the main means of communication between food producers and sellers, as well as their buyers and consumers" (FAO-Organización de las Naciones Unidas para la Alimentación y la Agricultura, 2007).
The Ministry of Public Health (2014) defines this labeling as "any description intended to inform the consumer about the nutritional properties of a food product which includes the declaration of nutrients and complementary nutritional information".
In Ecuador, the "Substitute Sanitary Regulation for Processed Foods for Human Consumption 5103" was created and approved on August 25, 2014 with reforms made on December 16, 2014. Its purpose is:
To regulate and control the labeling of processed foods for human consumption in order to guarantee the constitutional right of people to receive timely, clear, accurate and non-misleading information about the content and characteristics of these food products, allowing the consumer to make a correct choice based on its acquisition and consumption. (Ministry of Public Health, 2014)
So, we can find the Ecuadorian Technical Standard NTE INEN 1 334-1, which must be present in all processed and packaged foods. Within this regulation, there is specific terminology.

Table 1. Regulation INEN – NTE INEN 1 334-1.

Concept	Description
Addition, enrichment and / or fortification	Addition of one or several nutrients to a food product for nutritional purposes of the population, according to current regulations.
Food added, enriched or fortified	It includes natural, processed or artificial food that has had essential amino acids, vitamins, mineral salts, basic fatty acids or other nutrients added in its pure form or as components of some other ingredient.
Modified food	A product that has been partially deprived of some of its components or reinforced in any of the constituent elements of the product.
Nutrition declaration	The normalized enumeration of the nutrient content of a product.
Nutritional labeling	All of the descriptions that are intended to inform the consumer about the nutritional properties of a food product including the declaration of nutrients and complementary nutritional information.
Nutritional complementary information	This facilitates the consumer's understanding of the nutritive value of the food and helps them interpret the declaration about the nutrient.
Nutrients	Chemical substances consumed as components of a food product that provides energy or is necessary for the growth, development and maintenance of life and health.
Portion or portion size	The amount of food consumed by habit and chance (cup, piece, spoon, etc.).

Source: Instituto Ecuatoriano de Normalización (2008: 1). Own elaboration.

In the Sixth Live Food Congress (2016), Javier Guzmán spoke in his conference about the misleading advertising of food products, arguing that what has happened in the last 30 years is a serious health problem, and also arguing that the marketing and advertising

strategies are several of the causes that affect the mind of the consumer, causing them to consider certain products as homemade, natural or ecological. That is how concepts are used to get consumers to buy these products.
One of the organisms responsible for regulating and elaborating the norms for the elaboration, commercialization, labeling and production of organically produced food is the Codex Commission, which has approximately 170 members. This commission created the Codex Alimentarius (2007), which is a compilation of the codes of behavior, rules, recommendations and guidelines expressed by the commission to produce good products, as well as provide necessary and clear information to the consumer.
Among the elements that make up a product is the packaging, which is referred to as "a system of interlaced elements that generate added value to the product, meeting the needs and characteristics of the product to the requirements of the target market, reinforcing the identity of the productive unit (brand)" (CORRADINE, 2014).
The packaging could tempt consumers to buy an 'ecological´ or 'healthy´ product. Depending on the design, several functions have been attributed to the packages whose aim would be to persuade consumers in the buying process.
Formal aesthetic functions refer to those that are perceived visually and psychologically by the user in the case of color, texture, contrast, direction and rhythm. Finally, symbolic functions refer to the indirect meanings of packaging that can only be extracted from their socio-cultural context (BÜRDEK, 1994 cited by MARTÍNEZ REYES, 2016).
There are several products with messages in their packaging which lead to traps. Although it is stated that the product is healthy, if it does not have the explicit regulations, it will not be considered 100% healthy because many brands present products with exaggerations and confusion about the true effects they cause on health (GUZMÁN, 2016).
Another possible confusion could occur with products that are considered to be artisanal or with those labeled as healthy. Thus, we can find how these names can be misused by companies, while consumers also mistakenly associate the idea of healthy with the concept of artisanal. However, for a product to be really healthy or denote this characteristic, it should contain a certified label that indicates this on the packaging.
Regarding organic products, the Andean country resorts to the meaning of organic for appropriate labeling. This explain why a lot of packaging resorts to the ecological expression; it would mean nothing but carries with it the official seal of an organic product.
Under these approaches, this descriptive and exploratory study attempts to clarify the knowledge of consumers regarding ecological products and the publicity of these in their packaging. The hypothesis argues that misleading labeling in non-organic products would cause consumers to recognize them as healthy, and this would influence the acquisition of falsely ecological products.

2. MATERIAL AND METHODS

This paper used some studies as a reference such as the one done by BERTUGLIA and GONZÁLEZ (2016) about ecological products based on a survey conducted on 500 consumers from Granada (Spain).
94 online surveys were carried out during the second month of 2018 with people between the ages of 18 and 62 from the city of Ambato, who were mostly students and university workers.
The survey contained the following blocks of information:

- Socio-demographic characteristics of the interviewee (sex, age, economic condition).

- Habits of purchase of organic food (Place of purchase of food, frequency of consumption).
- Perception of ecological product in 12 images of food packaging with different indicators: artisanal (1-2-3), natural (4-5-6-7-9), organic (8) and healthy (10-11-12).
- Issues such as:

    - Purchasing factors.
    - Perception of organic products.
    - Definition.
    - Advantages and disadvantages.

With the obtained information, the database was prepared and analyzed through the statistical program SPSS (version 23 for Windows®). The online survey can be seen at the following link: www.goo.gl/forms/VYW4KMGUNSPnByAO2

3. RESULTS

51% correspond to women and 49% to men. The mean age is between 20 and 30 years, making up a total of 75.4% of the sample surveyed with ecological consumers being the majority in this group. The economic condition is mostly medium (73.1%), medium - low (13.8%) and medium - high (11.7%).
The frequency of food purchases that are considered to be organic was the following:

-Never: 9.6%
-Once a year: 22.3%.
-Two or three times a month: 8.5%.
-Once a week : 20.2%.
-Two or three times a week : 25.5%.
-Everyday: 13.8%.

The places where this food is often bought are markets (41.9%) and supermarkets (38.7%), mainly in supermarket chains throughout the country such as Supermaxi® or AKI®.
Regarding the 12 images of packaging, the following percentages were obtained from respondents who perceived each food product as an ecological product according to the established categories, emphasizing that only so-called organic (picture 8) can be considered ecological.

-Handmade: Picture 1 (21.3%), Picture 2 (31.9), Picture 3 (53.2%).
-Natural: Picture 4 (1.1%), Picture 5 (34%), Picture 6 (31.9%), Picture 7 (62.8%), Picture 9 (57.4%).
-Organic (ecological): Picture 8 (75.5%).
-Healthy: Picture 10 (33%), Picture 11 (66%), Picture 12 (66%).

The results indicate that most people perceive the product as really ecological (organic, Picture 8). However, more than half confused ecological with other denominations such as homemade, natural and healthy. This means that many people falsely consider certain foods as organic because their labeling leads them to think that they are, when really they are not.

Regarding the purchase factors of this type of food, the following results were obtained based on the type of response (not all respondents answered these questions, so the sum of the percentages does not give 100% for these omissions).

Table 2. Factors of food purchase.

	Nothing important	Somewhat important	Important	Very important
Brand	16%	31.9%	40.4%	5.3%
Price	5.3%	22.3%	50%	14.9%
Geographical origin	21.3%	29.8%	31.9%	10.6%
Quality badge	3.2%	23.4%	38.3%	27.7%
Ease of preparation	23.4%	28.7%	27.7%	13.8%
Nutritional information	5.3%	19.1%	39.4%	30.9%
Flavor	4.3%	20.2%	43.6%	26.6%
Durability	2.1%	27.7%	37.2%	27.7%
Kind of packaging	13.8%	28.7%	31.9%	19.1%

Source: Survey. Own elaboration.

As observed, the factors that matter the most in the purchase of organic products are the distinctive quality, nutritional information, taste and durability, and what matters less is the brand or image, geographical origin and ease of preparation.
With regard to the perception of this type of food, the following results were obtained with the same indication about the total percentage mentioned above, since not all of the participants answered these questions.

Table 3. Perceptions about organic products.

	Totally disagree	Somewhat disagree	Agree	Somewh at agree	Totally agree
Products that have an eco-label favor your purchase decision	3.2%	31.9%	35.1%	13.8%	10.6%
The information received by the media (TV, radio, press, Internet, etc.) affects your decision to purchase organic products	9.6%	36.2%	29.8%	16%	4.3%
Organic products with healthy or natural information means that it is suitable for health	3.2%	25.5%	27.7%	28.7%	10.6%
The information issued by the nutritional traffic light is a determining factor of purchase as an ecological product	7.4%	28.7%	31.9%	17%	11.7%
Advertising information on the use of the term "natural" in organic products is linked to preventive or therapeutic effects	8.5%	36.2%	26.6%	18.1%	5.3%
The promotion or advertising of artisinal products confirms that they are ecological and good for the environment and health	11.7%	34%	31.9%	16%	3.2%

Source: Survey. Own elaboration.

These results show that most people of the respondents confuse healthy or natural information as an appropiate food product for health, while being guided by advertising that does not represent truly organic products. This fact can be explained by observing the majority of definitions of the denomination "ecological product": natural, without chemicals, environmentally-friendly, handmade, healthy, organic, good quality.
This confusion of concepts is also found with the assessment of for and against of this type of food:

- Advantages: improves health, helps the environment, without chemicals, more confidence, more natural, organic.
- Disadvantages: limited expiration time, price, few places to buy them.

An inferential analysis (chi-square) showed that there were no differences between gender in the frequency of consumption, place and factors of purchase, and opinion (advantages and disadvantages). However, many more women than men perceived ($p < 0.05$) the Pictures 2, 3, 7 and 12 as organic products. Also, the only differences observed between the sexes were the brand or image when buying these products; men care more about this aspect than women ($p < 0.05$).
Regarding age, only significant results were obtained where younger people look at the price and expiration date more than older people. Older people pay attention to the nutritional information and they consider the media more when buying this type of product compared to younger people. At the same time, at an older age, the so-called artisanal (handmade) products are considered ecological and good for the environment and health.
Although some people argued as a disadvantage the price of these products, no differences were found in the frequency of consumption depending on the economic condition.
Finally, relating the frequency with other aspects, it was only observed that the higher the consumption, the more they are guided by the factor of the quality of the brand. In turn, there was no difference between the frequency and the perception of ecological product in any of the twelve pictures, so people who consumed the most and those who did not, expressed the same opinion about the twelve foods presented.

4. DISCUSSION

More than 50% of those surveyed say that they consume organic products weekly, but as the data on the recognition of food through the packaging pictures show, the problem is that many consumers do not have a clear definition of what is ecological. They cannot even recognize the official seals as warranty that the food is organic. There continues to be a lot of confusion about terms such as natural, artisanal or healthy, which leads to the consumer having a false perception of an organic diet. The association of these concepts creates the erroneous idea that the consumer buys products that are environmentally-friendly or healthy.
Some of the causes found in the study are the packaging labels and misinformation of the consumer about these labels. However, before blaming the victim, it would be more prudent to talk about the involvement of laws and government when allowing fraudulent denominations as well as not educating the population about the meaning of the labels.
This especially affects the sale of fake organic products, and therefore, the increase of a market encouraged by corporate intrusiveness to want to lure and deceive consumers with false allusions of what is ecological.

The association of healthy with the environment and healthy for the organism is a fundamental error since a nutritional diet is delegated to external factors such as the environment. In this sense, the frequency of consumption of organic products would not guarantee a correct diet, but rather it would be the nutritional values of these foods and above all, the amount ingested of this type of product. That is why false labels appeal to a healthy diet with terms such as natural, healthy, artisanal, and even organic or ecological, when really the health of the consumer is more affected by the variety and amount of food ingested, and of course, of other essential factors such as physical activity, lifestyle habits or psycho-social conditions.

In Ecuador, it is not easy find procedures that adequately sanction misleading advertising. Pérez (2014:10) points out that "this dilemma is evident, the effectiveness of the regulation that protects consumers against prohibited advertising depends on the regulation that can do it from the procedures and the sanctions to advertising".

This bias that still present could be affected in the application of the Organic Law of Consumer Protection that defends and protects the interests of the subjects that may be affected by advertisements of a deceptive or abusive nature. The current constitution regulates misleading advertising in Article 52, which affirms that "people have the right to dispose of goods and services of the highest quality and to choose them freely, as well as accurate and non-misleading information about their contents and characteristics". However, the sanction does not differentiate small companies from large ones. (…) the fine is practically the same for either a multinational company or a small business, so the sanction does not mean the same for what company and its possibilities to pay the fine" (JIMÉNEZ and VAYAS, 2018).

The advertising presented as descriptive information of the products in their messages details that they have natural, artisanal or healthy characteristics. For this, consumers recognize it as information that determines their purchasing action as organic or ecological food. The presence of labels on food packaging with these characteristics in supermarkets and stores almost always shows exaggerated health benefits without considering other factors that determine their effectiveness as mentioned before.

Consistent with the results of previous research, food advertisements are represented by quality, price, product brand and healthy conditions (PEREZ D, *et al*., 2009). This leads to the segmentation of the market of both organic and falsely ecological food.

On the one hand, through the information of the products, people are encouraged to consume foods that have properties that balance or improve their health (body weight, cholesterol, triglycerides). However, the study has proven that consumers do not clearly identify the organic certifications approved by the Ecuadorian government in order to differentiate these products. Most of the advertising for food tends to promise things as little as possible, ensuring that many food products are completely healthy when that is not completely true (ConsumoB: 2018).

Just as the meaning of natural, healthy or artisanal becomes a synonym of organic for consumers, the information that refers to the benefit of health and weight really becomes a persuasive message when considering it as truly ecological. It should be noted that the most widely used strategy to promote food as organic is associated with the use of indigenous images, the product in its "natural" state without being processing, allowing it to generate a symbolic relationship between nature and the ecological, without considering different packaging and preservation processes that the products have had to go through.

All this causes harmful effects on the beliefs of the consumer who look with veracity that the product is organic, in addition to the false interpretation given to the message by ignorance. "Most of the advertising strategies could be classified as deceptive or abusive

because the foods they advertise attribute properties that they could hardly have" (PEREZ *et al*., 2009).
Therefore, it is basic to recognize the activity of advertising as an element of commercial transactions that take place in society. For this reason, this activity must be carried out in a "lawful" manner as provided by the Constitution of the Republic of Ecuador, in an appropriate manner through the various regulations, to avoid the increase of misleading advertising, not only in the food sector but also in the other fields of its application.
In turn, it is really important that consumers are informed about their rights and about what it means to be ecological, natural, artisanal and healthy in order to have optimal quality goods and services and choices according to their needs, demanding accurate information that is not misleading about its content and characteristics.
It is necessary to train the country's citizens from childhood, in schools, to rethink food as a pending issue for all phases of life as well as to establish and promote consumer associations which demand information and education as a right through different channels on the different products and their characteristics, so that in this way they can defend their interests when facing the big food multinationals.

5. CONCLUSION

This study has shown that although there are many consumers in favor of an organic diet, they are hit with a multitude of products that are considered to be healthy, natural, and artisanal or organic, but without certification, presenting in their packaging information alienated to the quality of health, particularly associated with that idea that they are "healthy" and with great properties and seeing them as ecological without considering true information that determines its organic quality. Therefore, it is expected that with this paper, measures will be taken in this regard by the pertinent organizations in the face of a problem that increasingly affects more consumers.

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WATER PH INFLUENCE AND COOKING TIME OVER TEXTURE, GELATINIZATION AND RETROGRADATION FROM ANDEAN TUBERS

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ABSTRACT

The great diversity of tubers in Ecuador are being revalued due to their nutritional content.

It is, however, necessary to study ways to maintain the organoleptic properties of tubers during processing. The influence of water pH and cooking time on the gelatinization, retrogradation and texture of potato, *mashua*, *oca* and *melloco* was analyzed and the results showed that: water pH significantly influences the texture of hardness, independently of cooking time; complete gelatinization was observed; retrogradation did not register significant differences. Meanwhile, an increase in the onset, peak and endset temperatures and a decrease in enthalpy were observed, suggesting increased stability.

Keywords: tubers, pH, hardness, gelatinization, retrogradation

1. INTRODUCTION

Tubers are a fundamental part of both the diet and the industry of the Andean region (MAZA and AGUIRRE, 2002). Some tubers, such as potato (*Solanum tuberosum*), have attained worldwide importance, and others such as *mashua* (*Tropaeolum tuberosum*), *oca* (*Oxalis tuberosa*) and *melloco* (*Ullucus tuberosus*) remain less well known (MEDINA, 2003; GUPTA *et al*., 2015; LIM, 2016 a; LIM, 2016 b). In Ecuador, the production of Andean tubers is largely confined to their natural ecoregions, due to their reliance on appropriate amounts of rain and particular soil conditions (BARRERA *et al*. 2004).

Tubers are very well suited to agribusiness (BARRERA *et al*., 2004), especially since new healthy dietary habits have led consumers to seek out functional foods which are a good source of nutrients, which has increased the potential market for potato, mashua, oca and melloco (VILLACRÉS *et al*., 2013).

Water pH during food processing and cooking time are direct correlation with the physical and chemical changes that occur in tubers (STIPPL, DELGADO and BECKER, 2004; CRESPO, 2012; ZHAO *et al*., 2016). Texture, an essential factor in consumer appreciation of crop quality, is affected directly by the cooking process (GARCÍA-SEGOVIA, ANDRÉS-BELLO, and MARTÍNEZ-MONZÓ, 2007).

In addition, structural changes in tubers such as cooked potatoes are mainly associated with the behavior of the gelatinization and retrogradation of starch and with enzymatic and non-enzymatic changes in pectin (KIM, WIESENBORN and GRANT, 1997; TIAN *et al*., 2016a; TIAN *et al*., 2016b).

The aim of this study was to examine the influence of water pH and cooking time on texture – in terms of hardness – gelatinization and retrogradation of potato, mashua, oca and melloco.

2. MATERIAL AND METHODS

2.1. Materials

The tubers used for this study were potatoes of the *chaucha* variety (*Solanum phureja*), yellow mashua (*Tropaeolum tuberosum*), sweet white oca (*Oxalis tuberosa Mol*) and white melloco (*Ullucus tuberosus Caldas*). All of the tubers were purchased in the same local market (Cajabamba) in Colta Canton, Chimborazo, Ecuador. The tubers were washed with water to remove any dirt and cut into 1 cm slices perpendicularly to the main axis. Using a punch, a cylinder 1 cm in diameter was extracted from the central part of each slice.

2.2. Cooking process

The cooking process was carried out following the procedure indicated by LINARES *et al*. (2005) and ESCOBAR *et al*. (2007) with minor modifications. The cooking times selected were: 3, 5, 7 and 10 minutes. For each cooking time, eight cylinders of each tuber were placed in vessels of boiling water with a pH of 3, 7 and 10 respectively. The cooked samples were immediately cooled under a stream of cold water, drained for one minute, stored in polyethylene bags to prevent moisture loss and placed in refrigeration until the texture test was performed.

2.3. Texture Analysis

The Texture Profile Analysis of the tuber samples was performed using a CT3 Texture Analyzer (Brookfield, US). The samples were placed on the base plate (TA-BT-KIT) and compressed with a cylindrical probe (TA-ATT), using a 10 kg load cell. The speed of the test was 0.3 mm/s and the degree of deformation was of 80% of the original length. The textural parameter of hardness (N) was examined from each curve. Eight repetitions were made for each cooking treatment (LINARES *et al*., 2005).

2.4. Differential Scanning Calorimetry (DSC)

To determine calorimetric parameters, the method reported by KARLSSON and ELIASSON (2003b) was followed, with some modifications. The differential scanning calorimeter DSC 3 Star System (Mettler Toledo, EU) was used. The instrument was calibrated using indium, and an empty pan was used to set the baseline (TAKO *et al*., 2014). The instrument was controlled using STARe software. Portions of approximately 4 mm were extracted from the central part of each cylinder following treatment. The samples were placed in aluminum crucibles and hermetically sealed. The gelatinization analysis was carried out by heating it from 25 to 140°C with a rate of increase of 10° C/min. In order to determine the retrogradation parameter, the pans with samples were stored for seven days at 4° C, then the heating profile was repeated using the same parameters as in the gelatinization test. The transition enthalpy (J/g), onset temperature (T_0), peak temperature (Tp) and endset temperature (Te) were measured, following which the average values for each sample were calculated.

2.5. Statistical analysis

The statistical analysis of the data was performed using STATGRAPHICS® Centurion XVII software (Statgraphics Technologies Inc, USA). A two-factor variance analysis (ANOVA) was used, together with the Tukey test with a significance level of 0.05.

3. RESULTS AND DISCUSSION

3.1. Texture (Hardness)

Figures 1, 2, 3 and 4 show that when the cooking time increases and the pH changes from acid to base, hardness decreases in all of the tubers in the sample.
The interaction of water pH and cooking time on the hardness of the tubers studied presents statistically significant differences. The Tukey test for pH for each tuber indicated that the highest average hardness was obtained at pH 3, this behavior may be due to modifications in cell structure resulting from biochemical changes during treatment (ZHAO *et al*., 2017).
In the four tubers studied, the lowest hardness value was observed at pH 10: alkaline cooking water weakens or dissolves organic tissues, softening the texture. This result is agree with the reports by CORDOVA (2003) and BADUI (2006).

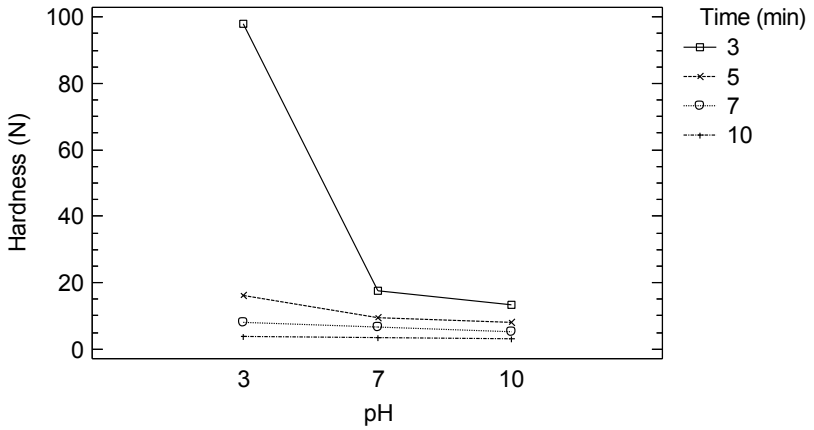

Figure 1. Interaction of pH and cooking time on potato hardness.

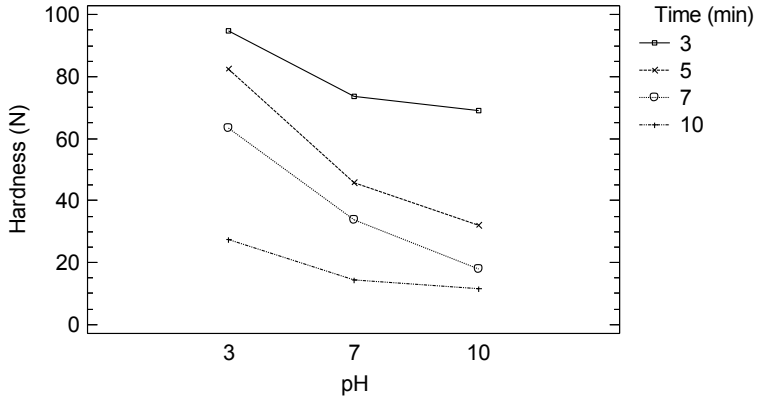

Figure 2. Interactions of pH and cooking time on mashua hardness.

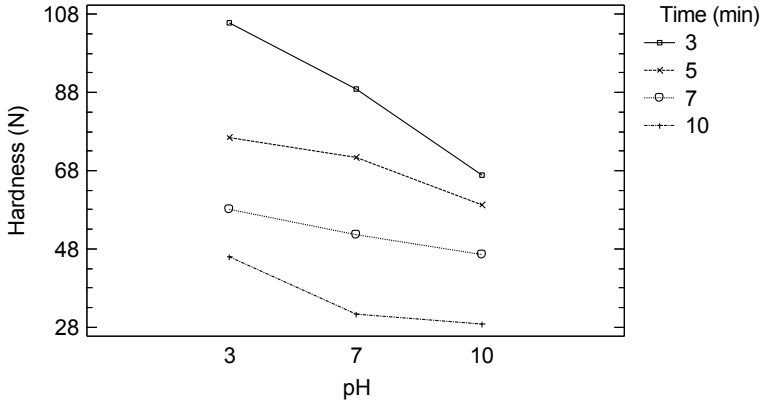

Figure 3. Interaction of pH and cooking time on oca hardness.

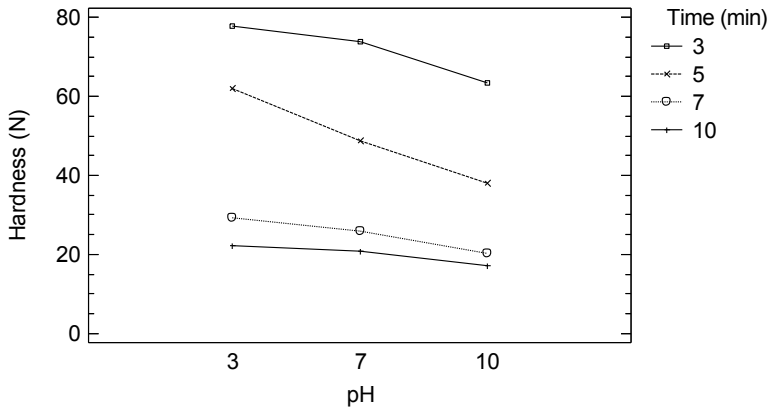

Figure 4. Interaction of pH and cooking time on melloco hardness.

Texture is a fundamental parameter of the quality perceived by consumers (GARCÍA-SEGOVIA *et al*., 2007; CHEN and ROSENTHAL, 2015) and hardness is often used as an indicator of consumer preference (ZHENG *et al*., 2013; MORENO *et al*., 2015; MUÑOZ *et al*., 2017). However, in the food industry there are no international standards for the hardness of products, which makes it difficult to compare the results with other processing methods (KADAM *et al*., 2015; ZHAO *et al*., 2016). Therefore, following the results obtained for hardness, the treatments at pH 3 and 7, with cooking times of 3 and 5 minutes were selected to determine their influence on the gelatinization and retrogradation of potatoes, mashua, oca and melloco, since these values are closest to those reported for potatoes by ŞERBAN *et al*. (2014); KAUR *et al*. (2002).
Precooking in acidulated water greatly improved the texture of the cooked tubers subject to study. These data agree with those of ZHAO *et al*. (2017), whose experiments yielded the highest hardness in cooked potato slices following an immersion pretreatment in 0.8% acetic acid for 18 h. This study provides useful data for the processing of potatoes, mashua, oca and melloco.

3.2. Gelatinization

Table 1 shows the averages obtained for gelatinization of potatoes, mashua, oca and melloco without treatment (raw). (ZHAO, *et al*., 2017), reported similar values of 57.6°C, 60.86°C and 64. 23°C for To, Tp and Te respectively in samples of raw potatoes.

Table 1. Average values of thermal properties of gelatinization of tubers without treatment.

Tubers	To (°C)	Tp (°C)	Te (°C)	ΔH (J/g)
Papa	59,98±0,93	63,51±0,08	66,73±0,47	1,66±0,02
Mashua	61,05±0,13	62,65±0,13	64,37±0,04	0,14±0,01
Oca	56,88±0,08	58,75±0,01	61,12±0,06	0,69±0,01
Melloco	58,42±0,15	61,63±0,61	65,82±0,37	0,14±0,04

The enthalpy of gelatinization was higher in the potato sample (at 1.66 J/g) and lower in the samples of mashua and melloco (at 0.14 J/g for both). As shown by the results obtained by ROMANO *et al*. (2018), enthalpies of gelatinization varied from 0.9 to 3.6-3.8 J/g for different varieties of raw potatoes. In addition, KARLSSON and ELIASSON

(2003a) obtained wholly gelatinized blanched samples by simulating the scalding process in tissue samples in a DSC device with a temperature range of 10 to 74°C; therefore, no peak in non-gelatinized starch was obtained by performing a second scan until a temperature of 120°C, immediately after cooling.

3.3. Retrogradation

Retrogradation is the step following gelatinization and is widely used to refer to the quality of the starch of cooked potatoes (KARLSSON and ELIASSON, 2003b). It commonly occurs during the storage period (ZOBEL and KULP, 1996) and directly influences the texture, acceptability and digestibility of the foodstuff (TAKAHIRO *et al*., 2005). Table 3 indicates the values obtained from the retrogradation of the untreated (raw) potato, mashua, oca and melloco. This is consistent with the results obtained by KARLSSON and ELIASSON (2003b), who found an onset temperature of 57°C when working with raw potato tissue. The data reveal a decrease in retrogradation temperatures compared to gelatinization in untreated samples; ROBLES (2012), observed a similar behavior in his study of potato starch.
The enthalpy of retrogradation in all untreated tubers decreased considerably in comparison with the enthalpy of gelatinization of raw samples. This may be attributed to the lower energy needed to melt the chains of retrograded starch (OAKS, 2012), and consequently, the treatments could be said to be more stable.

Table 2. Average values of thermal retrogradation properties tubers without treatment.

Tubers	To (°C)	Tp (°C)	Te (°C)	ΔH (J/g)
Patato	55,3±0,3	55,6±0,2	56,3±0,2	1,6E-04±1,5E-06
Mashua	52,8±0,1	53,5±0,2	54,1±0,1	3,1E-04±8,2E-07
Oca	48,4±0,3	49,3±0,4	50,1±0,3	3,9E-04±1,1E-05
Melloco	52,3±0,1	52,9±0,0	53,4±0,0	3,3E-04±5,1E-07

Tables 3, 4, 5 and 6 indicate the average values obtained from the retrogradation of potato, mashua, oca and melloco respectively, at different pH and cooking times.

Table 3. Average values of the thermal retrogradation properties in potato at different pH and cooking times.

pH	Cooking time (min)	Potato			
		To (°C)	Tp (°C)	Te (°C)	ΔH (J/g)
3	3	57,4±0,16 [a]	57,64±0,23 [a]	57,89±0,22 [a]	2,31E-05±4,17E-07 [a]
3	5	57,5±0,01 [a]	57,81±0,00 [a]	58,16±0,08 [a]	2,32E-05±7,07E-08 [a]
7	3	57,4±0,18 [a]	57,56±0,12 [a]	57,77±0,21 [a]	2,31E-05±1,41E-07 [a]
7	5	57,14±0,01 [a]	57,48±0,01 [a]	57,66±0,02 [a]	2,33E-05±2,62E-07 [a]

*a - homogenous groups.
p-value ≤ 0.05

Table 4. Average values of the thermal retrogradation properties of mashua at different pH and cooking times.

pH	Cooking time (min)	Mashua			
		To (°C)	Tp (°C)	Te (°C)	ΔH (J/g)
3	3	54,83±0,61 [a]	55,09±0,58 [a]	55,31±0,52 [a]	1,59E-05±3,61E-07 [a]
3	5	55,28±0,04 [a]	55,59±0,12 [a]	55,86±0,12 [a]	1,64E-05±3,54E-07 [a]
7	3	55,44±0,25 [a]	55,69±0,21 [a]	55,96±0,17 [a]	1,58E-05±1,91E-07 [a]
7	5	55,05±0,28 [a]	55,25±0,35 [a]	55,58±0,42 [a]	1,54E-05±3,61E-07 [a]

*a - homogenous groups.
p-value ≤ 0.05

Table 5. Average values of the thermal retrogradation properties of oca at different pH and cooking times.

pH	Cooking time (min)	Oca			
		To (°C)	Tp (°C)	Te (°C)	ΔH (J/g)
3	3	50,66±0,38 [a]	51,09±0,35 [a]	51,64±0,14 [a]	4,87E-05±1,24E-06 [a]
3	5	51,12±0,30 [a]	51,43±0,35 [a]	51,73±0,31 [a]	4,92E-05±1,02E-06 [a]
7	3	50,425±0,01 [a]	51,00±0,00 [a]	51,55±0,10 [a]	4,86E-05±3,75E-07 [a]
7	5	50,62±0,52 [a]	51,01±0,72 [a]	51,51±0,97 [a]	4,84E-05±7,85E-07 [a]

*a - homogenous groups.
p-value ≤ 0.05.

Table 6. Average values of the thermal retrogradation properties of melloco at different pH and cooking times.

pH	Cooking time (min)	Melloco			
		To (°C)	Tp (°C)	Te (°C)	ΔH (J/g)
3	3	55,50±0,08 [a]	55,82±0,01 [a]	56,18±0,10 [a]	4,30E-05±6,01E-07 [a]
3	5	55,09±0,09 [a]	55,50±0,00 [a]	55,79±0,07 [a]	4,26E-05±2,83E-08 [a]
7	3	55,09±0,41 [a]	55,57±0,13 [a]	55,86±0,23 [a]	4,30E-05±5,87E-07 [a]
7	5	55,15±0,42 [a]	55,65±0,23 [a]	56,03±0,08 [a]	4,25E-05±2,12E-08 [a]

*a - homogenous groups.
p-value ≤ 0.05.

The onset (To), peak (Tp) and endset (Te) temperatures of retrogradation in potato, mashua, oca and melloco increased from 1 to 3°C in relation to the retrogradation temperatures of their respective raw samples. However, there was a minimum decrease in enthalpy in all tubers compared to the retrogradation enthalpy of the untreated samples, and a considerable decrease in relation to the enthalpy of gelatinization. In line with results reported by KARLSSON and ELIASSON (2003a), it was determined that, when stored at 6°C, the samples of potato tissue that had previously been scalded experienced starch retrogradation with very low ΔH values. No statistically significant differences of To, Tp, Te and retrograded ΔH in potato, mashua, oca and melloco were observed between treatments. In addition, SANTACRUZ, RUALES and ELIASSON (2003)

determined that pH was not influenced by analyzing the enthalpy and gelatinization temperatures in a pH 3 starch solution.

4. CONCLUSIONS

Our study indicates that the water pH and cooking time have an influence on tuber texture. The highest hardness values were obtained at pH 3 and cooking time 3 minutes, meanwhile, the lowest values were observed at pH 10 and cooking time of 10 minutes. In addition, complete gelatinization of the starch was attained in the samples after treatment and thermal retrogradation properties were recorded at lower values in the treated samples compared to the corresponding raw samples; the lowest value was obtained at pH3 suggesting increased stability.

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INTAKE AND ADAPTATION OF MACRONUTRIENTS IN THE DIET OF INDIGENOUS CHILDREN OF ECUADOR

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ABSTRACT

Adequacy of intake and macronutrients were assessed in the diet of indigenous children under 5 years who were in a feeding program (G1) versus a control group (G2). There was an excess of carbohydrates and proteins' consumption for those within the program (Px <0.05); and a fat deficit in the diet of those who are not in the program. It is concluded that the menus of the two groups are inadequate and caused in G1 overweight and in G2 malnutrition. These results can be improved, if the diet includes: vegetables, meats (fish), fruits, and dairy products.

Keywords: nutrition, food, macronutrients, intake, adequacy, indigenous children

1. INTRODUCTION

Limited access to a diet, which covers the nutritional needs of the human being, is one of the causes of malnutrition. It causes the 35% of deaths in children under five years of age, and 11% of all illnesses in the global scope. Although, chronic child malnutrition is decreasing, there are still 155 million children under the age of five affected by this disorder worldwide. According to the FAO (2017), one in every 12 children under the age of five (52 million) has acute malnutrition, with a higher prevalence in indigenous communities. On the other hand, overweight and obesity increases in children in most regions of the world. In 2016, 41 million children under the age of five were overweight; and the number of people with chronic undernourishment increased to 815 million (compared to 777 million in 2015) (FAO, IFAD, WHO, WFP and UNICEF, 2017). Obese and overweight children tend to remain obese as adults, and are more likely to develop non-communicable diseases, such as diabetes and cardiovascular disease at a younger age, which is a serious problem that must be addressed urgently. According to a study conducted by Imperial College London and the WHO, they determined that by 2025, the number of overweight infants and children will rise to 70 million and in the last four decades, it will multiply by ten in children and adolescents (5-19 years), if the current food trend is maintained. Likewise, 7.2% of children, under 5 are overweight; it is 3.9 million children, of whom 2.5 million live in South America (WHO, PAHO, FAO, 2017). The 2030 Agenda for Sustainable Development and the United Nations Decade of Action on Nutrition (2016-2025) urge all countries and stakeholders to work together to eradicate hunger and prevent any form of malnutrition by 2030. This would prevent the premature death of mothers, infants and young children, poor physical and cerebral development in young people, and an increase in communicable diseases, all of which entail a heavy burden in the form of social and economic consequences, negative for people, families, communities and states (O'DWYER, 2013; ALKON, 2014; WHO, FAO, UN, 2016). Accessibility to safe and nutritious food, incorporating safety and cultural preferences is a right of all human beings (FAO, 2017). Hence, the purpose of the research was to evaluate the pattern of food consumption, the contribution of macronutrients, and percentage of adequacy of the diet of indigenous children under 5 years.

2. MATERIALS AND METHODS

This was a descriptive observational cross-sectional, research, in which participated 180 indigenous children under five, male and female were divided into two study groups. The first group (G1) was consisted of children receiving food (4 meals) through the program provided by the Decentralized Autonomous Government (GAD), and the Ministry of Economic and Social Inclusion (MIES) ECUADOR. The second group (G2) consisted of children who were fed at home, and received midmorning light meals by the government. The study groups G1 and G2 belonged to 18 Salasaka indigenous communities from Aymara origin, of the ancient Inca society. It is located in Tungurahua province at the central area of Ecuador. *Salasaka* has approximately 12,000 hbt, who speak *Kichwa* language and Spanish and its economy is based mainly on craftwork. In the area, there are corn, wheat, barley, beans, potatoes, cabbage, lettuce, beet, cauliflower, apple, pears, peaches, capuli, chochos and vegetables. The most common animals are sheep and cattle small animals such as guinea pigs and rabbits. Milk production is minimal. They have a very traditional drink that they extract from the *penco (Aloe vera)* called *tzawar mishki* which is consumed as an energizing or medicinal drink.

The sample was based on the information provided by the Zonal District N-3, a cluster sampling was used, which included children from the Children's Centres for Good Living (CIBV: n = 6), and children of the Educational Units (EU: n = 2). The informed consent signed by the parents was also considered for inclusion. Based on the above, the following information was obtained:

2.1. Pattern of food consumption

A survey was applied to identify the pattern of Food Consumption in the diet of the investigated subjects, analysed through the frequency of food consumption and number of meals. The 24-hour reminder was considered for children attending the Educational Units (UE) and the analysis of the menus provided by the program in the CIBV. The foods were classified based on the contribution of macronutrients, identifying those with the greatest contribution.

2.2. Macronutrients contribution to the total energy of the diet

The total contribution of the macronutrients consumed by children was evaluated, and distributed in percentage of energy contribution of carbohydrates, fats, and proteins.

2.3. Adequacy of the Diet

In order to know if the diet of the children was adequate, to know if the consumed food that contribute with enough amount of energy and macronutrients according to their age. This consumption was compared with the references established by the FAO (2017) considering the group to which they belong, the gender and age of the children.

2.4. Statistical analysis

Descriptive and inferential statistics were applied to identify consumption frequencies, average values and standard deviations (σ) of consumption, percentage of adequacy of macronutrients, tests of hypothesis testing, making comparisons between group, gender, and age, at a level of 95% confidence.

3. RESULTS AND DISCUSSION

3.1. Pattern of food consumption

Group within the Program - These children receive four meals program, and one in the evening he has at home, altogether five meals distributed as follows:
The breakfast is composed of oatmeal, or pouring sweet with a piece of bread. At midmorning, there is again a pour of sweet or fruit. The lunch has two courses, the soup that can be of vegetables, quinoa, barley, *morocho*, oats, noodle; and the second plate brings always a portion of rice accompanied potato, legumes sometimes as bean or lentil, and less often a small portion of beef or chicken, fruit drink is added. In the middle of the afternoon they take a pour of sweet without milk; home and receive, the fifth meal is often noodle soup, herbal tea with a piece of bread, rice, or colada.
Group fed at home. - This group of children have four meals in total. Breakfast consisting of smoothies or washes for 80% of children. It has a piece of bread or tortilla (prepared with wheat flour without yeast and salt); in a lower percentage consume milk, or an egg.

Mid-morning they receive from the Government's program, vitalized and fortified milk plus a cover with granola or coarse oatmeal that they mix with *panela* (syrup not distilled from sugar cane). Lunch at home has two dishes, the first is always a soup of any farinaceous, sometimes vegetables, and the second dish is rice with legumes, and sometimes sardines or a small portion of meat, in the evening they usually do not eat and those who do so repeat the soup of lunch or drink herb water.
Fruits are not always in the diet, and when they are, they are usually bananas. They do not use oils or margarines; however, they do contain lard in small quantities. Consume sodas or sweet drinks, candies and sweets.
Table 1 shows the most frequently consumed foods, in a 24-hour reminder report for those who are not in the program; and through a weekly menu review for those who are included in the program. Regarding dairy, it was observed that it is not a frequent practice to give milk to children, 50% of 86% consume, take a portion twice a week, only 1% of children consume more than 3 times a week despite having cattle in the community. The parents pointed out that when they drank milk, it caused them "diarrhea", and most likely, they are lactose intolerant. Milk production is intended for sale as a means of economic income.
The cereals and farinaceous constitute the base of the feeding in the diet of the children of Salasaka, its consumption is daily and frequent. This is one of the products of higher consumption, the rice, noodles and maize or wheat flour (> 80%), and the less frequent are oats, corn-starch, barley rice and *morocho*. As sugars and drinks processed consume "brown" sugar the one that has not been refined, the brown colour is due to the presence of molasses, to a lesser extent white sugar or brown sugar is consumed, there is also the presence of gas, and the *tawar misky* obtained from the penco (American Agave) whose consumption is higher in adults. Beef, chicken and eggs are not frequently consumed, 90% consume between one and 3 times per week. Guinea pigs and rabbits are bred for sale, being very liked by mestizos; the indigenous population only in sacred celebrations consumes this meat. The population eat potato is almost every day for over 80% of children, consume seasonal fruits by 50%. Vegetables and roots are not desired products, less than a third of the population consumes only 1.2 or 3 times a week. Vegetables mainly onion is prepared in dishes such as soups or meat). The food nutrition groups are classify in Table 2.
From tables described above it follows that the greatest contribution to energy contribution comes from carbohydrates (approximately 70.8%), followed by proteins (approximately 46.3%) and fat (approximately 1%). They have reduced consumption of meat, dairy and fruit, it is endangering the consumption of micronutrients such as vitamins and minerals especially iron.
There are variations of consumption in proportion according to the child's age and consumption of milk in smaller, it was observed that only 20% of the smallest (2 and 3 years) consume milk, the rest is possible intolerance, the parents indicated that it produced them diarrhoea. Children 4 and 5 years old had fortified milk provided by the government program to the educational units. No fried food consumption was observed; do not use oils or vegetable margarines, nor sausages because their cost is not accessible to them.
Patterns of similar consumption were observed in other studies, for example GONZÁLEZ-GONZÁLEZ *et al*. (2016) studied 1142 schoolchildren; all had four daily meals with a high consumption of cereals. However, low consumption of vegetables and fruits. JIMÉNEZ *et al*. (2018) indicated low consumption of fruits and vegetables, with an average caloric intake of the diet of children under 1 year of 914 kcal, about 50% of children exceed the energy recommendations and presents excessive consumption, while children of 1 to 2 years consume 1052 kcal. The low consumption of minerals, especially iron, also stand out. According to research conducted by the US Centers for Disease Control and Prevention

(CDC) in September 2014, children who are fed juice and sugary drinks before the year are more likely to be obese five years later. Excess carbohydrate intake is seen, being a predisposing factor to obesity from the early stages of life. STAIANO *et al*. (2016) stated that the daily consumption of fruits and vegetables would improve the quality of the diet, by the contribution of antioxidants and fibre, decreasing the risk of overweight and obesity, which at the same time lead to non-communicable diseases.

Table 1. Frequency of food consumption.

Consumed food	Does not consume	3 times or less per week	More than 3 times a week
Milk products (milk, cheese, yogurt)	14.0%	84.9%	1.2%
Cereals and farinaceas (Rice, flours, bread, noodles)	0.0%	17.4%	82,64%
(Panela, black sugar, tawar mischqui, soft drinks, sweets)	33.8%	32.2%	34.1%
Tubers (potatoes)	0.0%	19.2%	80.8%
Red meat, chicken, eggs	2.5%	97.4%	0.2%
Fruits (claudias, capulí, bananas)	0.0%	50.0%	50.0%
Vegetables	84.3%	15.7%	0.0%

Table 2. Food sources with a higher percentage contribution to energy and nutrient consumption.

Carbohydrates	Fats	Proteíns	Iron	Calcium	Vitamin A	Vitamin C
Rice, bread, noodles, coladas, gasosas, packaged juices, tzawar mishki, panela, sweets, candies, cookies, beans, lentils, bananas	Beef, cheese, eggs, chicken, whole milk, lard	Milk, eggs, beef and chicken	Beef, eggs, sardines, cooked cereals, potatoes.	Milk, cheese, yogurt, beans, bananas, eggs.	Pera, capuli, banano, claudia	Claudia, capulí, pera, packaged juices, banana, potatoes

3.2. Contribution of macronutrients

The dietary contribution in macronutrients recorded in children from 2 to 5 years of the *Salasaka* community - Ecuador, does not register significant difference with respect to the group (G1 and G2), gender, and age, evaluated 5% of significance (Px> 0.05). The contribution of macronutrients corresponded to an average energy value of 1066.1 Kcal / d ± 9.2; 688 Kcal / d ± 1 in carbohydrates; 163.7 Kcal / d ± 5.5 in proteins and 227.7 Kcal / d ± 4.2 in fat. The values recommended by FAO and WHO correspond to 976.5 ± 137.7 Kcal / d; 576.1 ± 81.2 Kcal / d; 146.5 ± 20.7 Kcal / d and 292.9 ± 41.3 Kcal / d; in calories, carbohydrates, proteins and fat respectively. This suggests that there is a good contribution of macronutrients compared to the values of recommended macronutrients; except in fat, whose difference is minimal.
In previous studies, deficiency in the consumption of macronutrients, mainly proteins was observed and indigenous populations were vulnerable to malnutrition. There has been a high percentage of malnutrition and excess of carbohydrate consumption, representing a risk for overweight and obesity. Children under five are considered a vulnerable population, since during the first three years 80% of the human brain develops; and any deficiency or excess will affect its growth and development, it is a stage where food habits are formed that will affect the child's future. (WHO-PAHO-FAO, 2017).

3.3. Adjustment of diet

The percentage of adequacy of macronutrients was calculated based on the total amount of consumed nutrients, and total daily intake requirement. Considering a normal range of 90-100, <90 is considered a deficit, and> 100 is considered excess.

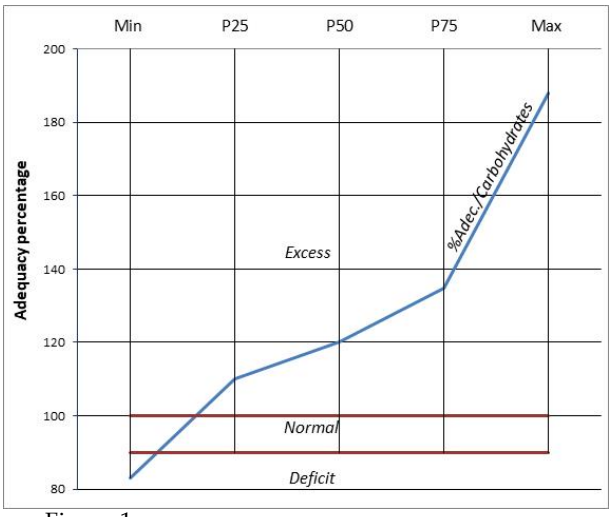

Figure 1.

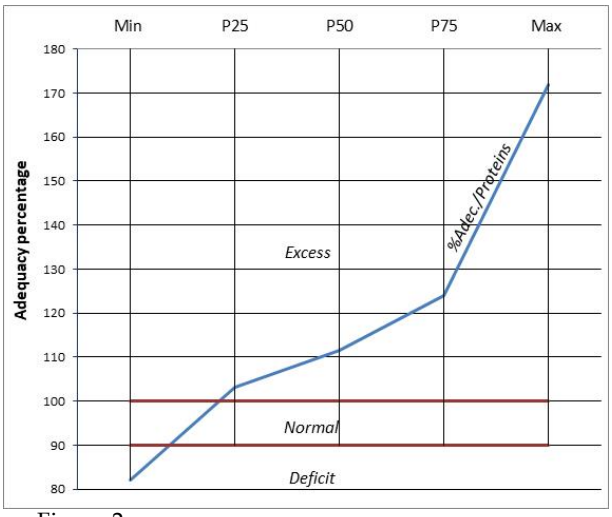

Figure 2.

Figure 1. Deficit and excess of CH in the diet of indigenous children.

Figure 2. Deficit and excess of proteins in the diet of indigenous children.

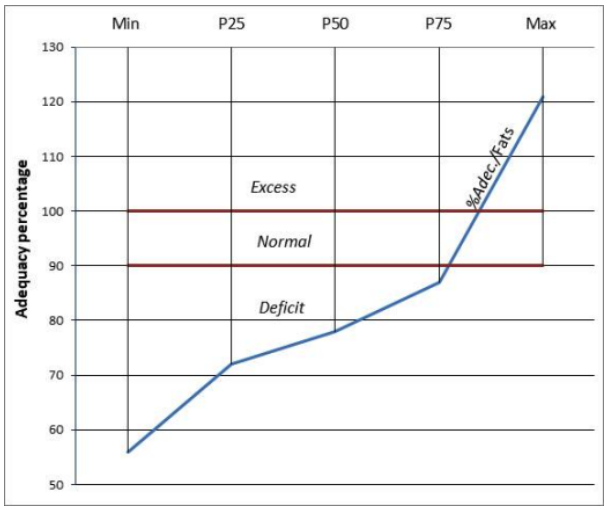

Figure 3.

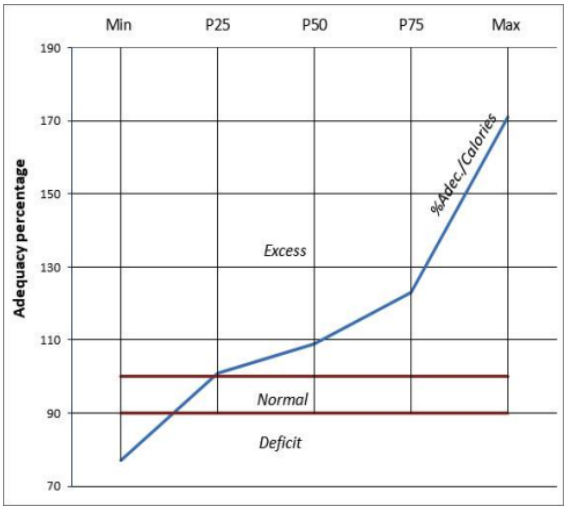

Figure 4.

Figure 3. Deficit and excess of fats in the diet of indigenous children.

Figure 4. Deficit and excess calories in the diet of indigenous children.

In the indigenous community, deficits in fat consumption were detected compared with WHO recommendations. The population does not consume any vegetable oil, and occasionally animal fat in prepared dishes, but does not cover the needs of children. This pattern of non-fat intake not repeated in the studies reviewed for this article.

3.4. Percentage of adaptation according to sex

Adequacy revised percentages of macronutrients, about sex, both children have excess consumption of carbohydrates and protein, but it is higher in girls, Px <0.05 (Table 3).
Fat is deficient in daily food consumption in both boys and girls, the differences between the averages are statistically significant Px <0.05, *Tukey* showes a higher percentage of fat deficiency in children (Table 3).

3.5. Percentage of adequacy with respect to whether they receive the feeding program

There is evidence statistically significant at 5% the percentages adequacy macronutrients differ in children found or not in the program. Although the adequacy percentages for excess CH and protein are found in both groups of children, the *Tukey* range test indicates higher values in the children that are included in the feeding program (Table 4).
The adequacy percentage indicates a fat deficit in the diet of both those who are in the program and those who are not included in it, being higher in children who are not in the program (Table 4).
LLORENS-IVORRA (2017), notes that 60.4% of school menus are adequate, but that some food groups should be included, such as vegetables, meat, fish, dairy products and fruit. SEIQUER I (2016) indicates that according to the recommendations of the program PERSEO (Pilot School Pilot Program for Health and Exercise against Obesity), vegetables

should be included in the children's menu every day of the week, either on the first course or as in a side dish.

Table 3. Analysis of variance for percentages of adaptation of macronutrients a according to sex.

		Sum of squares	gl	Square means	F	Sig.
CARBOHYDRATES_%/Adec	Between groups	1371.187	1	1371.187	4.759	0.03
	Within groups	50999.349	177	288.132		
	Total	52370.536	178			
PROTEINS_%/Adec	Between groups	993.687	1	993.687	4.613	0.033
	Within groups	38129.922	177	215.423		
	Total	39123.609	178			
FATS_%/Adec	Between groups	540.823	1	540.823	4.892	0.028
	Within groups	19566.887	177	110.547		
	Total	20107.709	178			

p-value ≤ 0.05

Table 4. Analysis of variance for percentages of adequacy of macronutrients according to the group to which it belongs.

		Sum of squares	gl	Square means	F	Sig.
CARBOHYDRATES_%/Adec	Between groups	8950.746	1	8950.746	36.296	.000
	Within groups	43895.982	178	246.607		
	Total	52846.728	179			
PROTEINS_%/Adec	Between groups	1572.078	1	1572.078	7.403	.007
	Within groups	37798.872	178	212.353		
	Total	39370.950	179			
FATS_%/Adec	Between groups	1876.647	1	1876.647	18.173	.000
	Within groups	18381.553	178	103.267		
	Total	20258.200	179			

Several studies have shown that vegetables and fruits are among the most frequently rejected foods by children and adolescents. Therefore, public schools in Granada are making a significant contribution in this regard by including these foods in a consistent and varied way the menus offered in their dining rooms. (ARANCETA *et al*., 2004; RUFINO-RIVAS *et al*., 2007; ZULUETA *et al*., 2011; PÉREZ *et al*., 2011; SEIQUER *et al*., 2016)

A school feeding program can provide the ideal framework for children to have a healthy and balanced diet adapted to their nutritional needs, and can be a useful tool in learning and adopting healthy eating habits that can have a positive impact on development and health promotion in later ages (SEIQUER *et al*., 2016).

3.6. Percentage of adequacy with respect to age

Statistically significant evidence of the percentages of adaptation of macronutrients in the daily diet is recorded with respect to age at 5% of significance (Px < 0.05) (Table 5).
On the other hand, Table 6 shows the summary of Tukey ranges. Significative values represent a variable correlation between food groups that used in this study. Table 6 shows some values obtained by Tukey method indicates high and similar values at ages of 2 and 3 years, and a different and lower value at 5 years in carbohydrates. In protein, there are high values for excess of consumption between 2, 3 and 4 years old, and a different and lower value at 5 years. It is in fat consumption where a deficit was identified. Tukey presents three subgroups for alpha = 0.05. The first group where 5 years old children who have lower values than normal, e.g. 68.6%. The second group children between 3 and 4 years old with values are between 77.9 and 80.4%. Finally, the third group with similar and higher values to the previous data, which are, located 2-year-old children and 3-year-old children.
Children under five are considered a vulnerable population, since during the first three years 80% of the human brain develops; and any deficiency or excess will affect its growth and development, it is a stage where food habits are formed that will affect the child's future. (WHO-PAHO-FAO, 2017).

Table 5. Analysis of variance for percentages of adaptation of macronutrients according to age.

		Sum of squares	gl	Square means	F	Sig.
CARBOHYDRATES_%/Adec	Between groups	16191.600	3	5397.200	25.915	.000
	Within groups	36655.127	176	208.268		
	Total	52846.728	179			
PROTEINS_%/Adec	Between groups	6900.742	3	2300.247	12.468	.000
	Within groups	32470.208	176	184.490		
	Total	39370.950	179			
FATS_%/Adec	Between groups	4698.706	3	1566.235	17.716	.000
	Within groups	15559.494	176	88.406		
	Total	20258.200	179			

Table 6. Summary of TUKEY ranges for the percentage of adaptation according to age.

HSD Tukey[a,b]

CARBOHYDRATES_Adequacy percentage				
		Subset for alpha = 0.05		
age_1T_rec	N	1	2	3
5 years	28	102.6786		
4 years	35		117.2286	
3 years	69		125.0435	125.0435
2 years	48			131.4792
PROTEINS_Adequacy percentage				
5 years	28	100.5714		
4 years	35		113.9429	
3 years	69		114.3188	
2 years	48		120.2292	
FATS_Adequacy percentage				
5 years	28	68.6429		
4 years	35		77.9143	
3 years	69		80.4493	80.4493
2 years	48			84.6250

The means for the groups are displayed in the homogeneous subsets

P-value ≤ 0.05

4. CONCLUSIONS

It is concluded that the menus offered inside and outside the program to children from 2 to 5 years can be improved, reducing carbohydrate rations (rice, pasta, flour, potatoes and sweetened beverages) and in turn including vegetables in the diet, meats (fish) and fruits.
It is important to identify the intake of energy and nutrients in the diet of children in the early stages to reverse malnutrition due to deficit or excess and ensure healthy growth and development.
The study indicates that one of the main components in the diet of indigenous children of the *Salasaka* community are farinaceous foods, followed by sugary foods; fruits, dairy products, meats, and eggs are less frequently consumed, and vegetables are almost never eaten.
Although, the school feeding program is especially important in indigenous communities, it must be planned in such a way that it provides the amount of nutrients necessary for the child according to his age. In addition, it needs to be considered the area's food and cultural aspects of preparation; this is something that is not being taken into account. Due the fact, that in this study there is an excess of carbohydrates and proteins in children diet receiving the feeding program by GAD and MIES, the diets could be improved by decreasing food with a high content of sugar and carbohydrates, and including dairy products, fruits and vegetables. Eating a balanced school menu will lead to a healthier eating pattern.
It must be considered; these results cannot be inferred to other indigenous communities. Since, each community has specific cultural and social characteristics. It is important that specific studies must be carried out in the indigenous communities of the four zones in Ecuador where diet, number of meals, rations served, and adequacy percentages should

be analysed, to determine the health implications of children from 2 to 5 years of children under five.

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NUTRITIONAL STATUS AND COGNITIVE DEVELOPMENT FUNCTIONS IN INDIGENOUS CHILDREN 4 TO 5 YEARS OLD

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ABSTRACT

The relationship between nutritional status and development of cognitive functions in children aged 4 to 5 years was determined. Their nutritional status was assessed, and cognitive functions were identified with the Infantile Neuropsychological Maturity questionnaire. The results showed 42% of chronic malnutrition and significant percentages of risk of overweight. The IMC / Age indicator and the Global Development presented a significant difference in children of 5 years, showing that there is a relationship between the variables. The nutritional deficit is a risk factor in brain maturation, which is associated with a lower cognitive development of the infant.

Keywords: nutritional status, cognitive functions, anthropometric indicators, CUMANIN Test

1. INTRODUCTION

Nutritional status is the relationship between intake and physiological adaptation processes, which depends on the degree of satisfaction of nutrients and can be altered by several factors. The assessment of nutritional status shows the current situation of the individual and it is underpinned in preventing possible diseases, which may alter physical growth or cognitive development in both children and adults (CLARK, 2005; PLANAS *et al*., 2010). ROSELLI and MATUTE (2010) argued that appropriate nutrition reflects in brain performance involved in cognitive functions. PAREDES (2015) mentioned that nutrition has an impact on brain development, mainly affecting the verbal abilities of the children; it has a significant impact on brain development especially in chronic malnutrition.

Nutrition is essential for proper brain development in preschool stages, because if there are nutritional deficiencies, it will affect motor skills, cognitive and social functions. Its manifestations will appear in short and long-term periods, due to the immaturity level of the nervous system (RED *et al*., 2015).

The development of cognitive functions will depend on two factors: the growth and cerebral maturation related to reasoning, analyzing and understanding the environment that surrounds it (RUIZ, 2016); at the same time, they will depend on neuronal plasticity and environmental stimulation (ORTEGA *et al*., 2013). Neuropsychological maturity is understood as the level of organization and maturational development that allows a development of cognitive and behavioral functions according to the subject's chronological age (PORTELLANO, 2014).

One of the problems that has caused intense migratory movements in Tungurahua province in Ecuador was the lack of jobs for people in rural areas. It is an example of what has happened in the last twenty years. According to the data of the 2010 Census, it was estimated that migration affects 22.8% of the economically active Ecuadorian population, considering the population over 12 years of age; 68% of migrants are men and 32% are women. In terms of the labor market, 54.24 percent work in construction, 7.56 percent in agriculture, 18.30 percent are developing construction activities in Galapagos and 17 percent as domestic employees, and 5% migrate to Europe. This situation has defined patterns of cultural adaptation that have affected new generations. All these social problems translate into the breakup of entire families, leaving the children to the care of direct family members (LÓPEZ, 2014). The research of the resulting aspects of this complex situation was the motivation to carry out this study.

2. MATERIAL AND METHODS

A descriptive cross-sectional study was carried out; nutritional status variables were associated with cognitive development. Eighty children aged 4 to 5 years from the indigenous communities of Salasaka and Rosario province Tungurahua - Ecuador, after signing the informed consent by the parents were evaluated. Nutritional analysis for weight, height, and demographics such as age and sex were assessed using growth patterns of the World Health Organization 2012 to analyze the nutritional status score Z.

Maturity Neuropsychological Questionnaire for Children "CUMANIN" is an integrated instrument, which allows the detection of potential problems of development was used to assess cognitive development. The application of the test was individual. The duration of the test varies between 30 and 50 minutes and contains 8 main scales, which evaluate: psychomotricity, articulatory language, expressive language, comprehensive language, spatial structuring, visoperception, iconic memory and rhythm (PORTELLANO *et al*., 2000). In addition, five auxiliary scales were also assessed: attention, verbal fluency,

reading, writing and laterality. It focuses on brain maturation related to the acquisition and improvement of cognitive function, according to the child's chronological age (URUZA *et al*., 2010).

3. RESULTS AND DISCUSSION

3.1. Anthropometric evaluation

Figure 1 shows some results of Anthropometric Nutritional Status Indicators. Highlight with the highest value that chronic malnutrition was present in 42% of the reviewed cases. This data is consistent with the study of FREIRE *et al*. (2014) conducted in Ecuador, which indicates that in the rural sector in the highlands approximately one out of two preschoolers have malnutrition problems; it was 38.4% of chronic malnutrition.
Global malnutrition appears in 8% of children. This situation that has decreased in relation to previous periods as confirmed by the Public Health Minister of Ecuador (FREIRE *et al.*, 2014) the study showed 6.4% of malnutrition. ROSIQUE *et al.* (2010) showed that cumulative behavior of chronic malnutrition in relation to age during the period of children's growth affect their development. This may indicate that an adverse food environment could be associated with indigenous life. An increase in chronic malnutrition of 48.4% in children from 0-2 years to 76.6% between 2 and 5 years was shown.
The indicators of Weight/Height and BMI/Age showed a 19% and 13% possibility to increase the risk of overweight because of inadequate feeding. It was characterized by excess of carbohydrates and shortage of protein of high biological value. These data agree with the study by CEBALLOS *et al*. (2012) who determined 24.6% of overweight and obese preschool children, considering that obesity begins in early age and can persist into adulthood, as mentioned PADRON (2002).

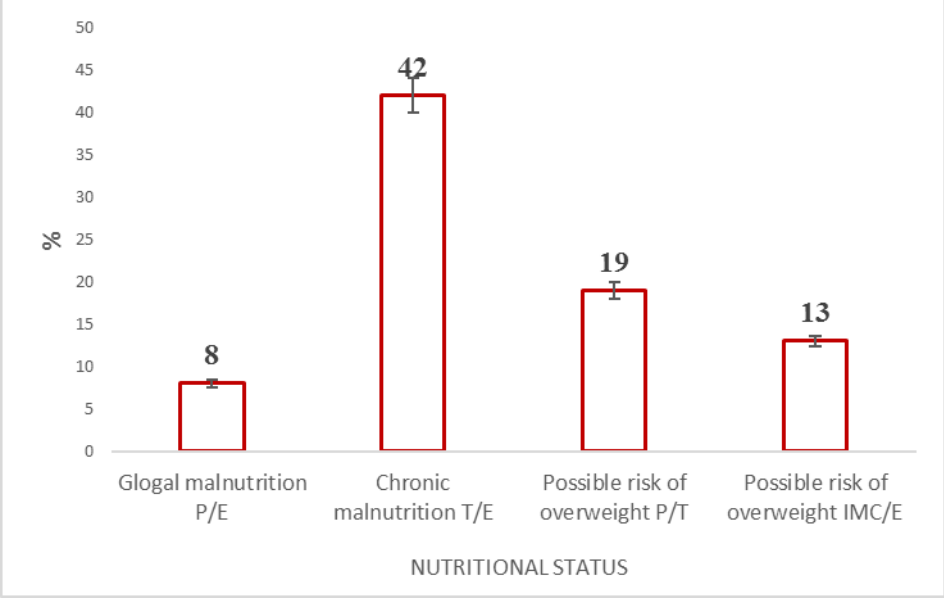

Figure 1. Anthropometric Nutritional Status Indicators.

3.2. Evaluation of Neuropsychological Maturity

3.2.1 Psychomotor

It was determined that 21% of children showed deficits in psychomotor and 18% is in a below average level. This shows motor difficulties associated with movement, coordination and body balance, probably due to inadequate attention and interest from parents.
The remaining 61% of the investigated children are in the ranges of average and higher, because the average benefit and experience mastery, coordination and body control.

3.2.2 Articulatory language

A ninety percent of children were identified deficit and below average that demonstrated inadequate articulation of phonemes that affect communication; as a result, of the absence of stimulus at home to support such learning.

3.2.3 Expressive language

A ninety-six per cent of children presented deficit and below average, showing difficulty in morph syntactic structuring, morph related phrases with subject, verb and complement.

3.2.4 Comprehensive language

A fifty-two per cent of children are in deficit and below average, as a result, it produces difficulties in understanding, remembering, and analyzing the meaning of words.

3.2.5 Spatial Structuring

A forty per cent of children are in deficit and lower than average, because they have not developed the awareness of how body coordinates movement. Meanwhile sixty percent of children are in average higher, as they have developed the ability to represent their body in a spatial context, due to the relationship with the environment they developed.

3.2.6 Visoperception

A seventy six percent of children are in deficit and below average. Children have difficulty to recognize and discriminate the stimuli of their environment. It is related to the oculo-manual coordination showing the lack of fine motor stimuli. Meanwhile, twenty four percent are in average and higher because of attendance at Child Development Centers MIES.

3.2.7 Iconic memory

A twenty four percent of children are in deficit and below average, due the lack of visual stimuli at home that could support the identification of images or objects. As contrast seventy six percent are average and above. Child Development Centers (MIES) provide teaching resources used for identifying images through vision that is a new stimulus for the storage of additional information.

3.2.8 Rhythm

An eight two percent of children are in deficit and below average. They don't have the ability to perform rhythmic series. The movements are more global than specific, this may be since the parents and / or caregivers do not provide audio stimuli and / or ludic activities. The eighteen percent are in average or higher, as they have been stimulated in early life.

3.2.9 Attention

An eighty three percent of children are in deficit and below average due the lack of novel stimuli, and distractors in class time. It causes difficulties in learning, also lack of regulations from parents or caregivers were identified. Only a seventeen percent of children are located on average and above.

3.2.10 Verbal Fluency

An eighty-seven per cent of children are in lower average or deficit due to difficulties to express themself easily, freely and spontaneously. Possibly, due to the limitations and lack of vocabulary. In addition, thirteen were above average; it could be possible due the motivation and interest of parents or caregivers through dialogue by the time they spend with children.

3.2.11 Reading and writing

A sixty six percent of children could not be evaluated as start learning to read and write when they are six years old. However, thirty-four percent are below average, this is a positive finding due the evidence of adequate understanding and expression despite age. In addition, to an adequate graphic representation of the language through traced signs.

3.2.12 Laterality

It is shown that 43 children have homogeneous laterality. They have a predominance of a specific cerebral hemisphere it showed their brain maturity, according to its function; Meanwhile thirty-seven children have atypical laterality, because they use both hemispheres at the same time, which may mean that they are in process of maturity.
In Table 1, below, is presented the results of Ho comparison, by different statistical methods.
According to the results of the X2 test (Chi-square) between the BMI / Age and the Global Development in the two groups of children of 4 years and 5 years. It has been found a significant difference in the first group of children / as 4 years, $Px = 0.013 < 0.05$ showing a relationship between nutritional status and development of cognitive functions. However, in the second group of children of 5 years old, it did not happen.
The results of the first group of children of 4 years old were related to the study of MAMAMI, CHOQUE and ROJAS (2014), which showed that the relationship between nutritional status and IQ is directly proportional. Since, children with early malnutrition obtained in the Goodenough test a low to normal range and a lower index with a bright normal range.
While the results of the second group of children who are 5 years old supports the study conducted by CAMPO, TUESCA and CAMPO (2012), which showed that there is no

relationship between the degree of infant neuropsychological maturity and the height and weight index. The evaluation of anthropometric nutritional status showed that size and weight were appropriate for age. Moreover, the CUMANIN test showed deficits in scales of verbal fluency, comprehensive language, attention and rhythm.

Table 1. Ho comparison test to determine significant difference between BMI/age and overall development.

Age		Value	Gl	Sig. asymptotic (bilateral)	Sig. exact (bilateral)
4 years	Pearson Chi-square	10,560[c]	3	0,014	0,013
	Reasonableness	8,653	3	0,034	0,021
	Statistical exact of Fisher	8,826			0,013
	Linear association by linear	5,817[d]	1	0,016	0,023
	N- of valid cases	43			

4. CONCLUSIONS

The nutritional status of children / as 4 to 5 years of community SalasaKa and Rosario-Pelileo, according to size / age indicator has high percentages of chronic malnutrition and this is reflected in cognitive deficit. However, the BMI / age and weight / height of the population showed a nutritional status within the normal range. It has been identified problems due the risk of overweight, because of excessive carbohydrate feeding.
The degree of development of cognitive functions in children 4 to 5 years old of Salakaca community and Rosario – Pelileo- Ecuador showed that most of its children population reported deficit in the overall development of cognitive functions. According to the result of the articulatory, expressive and empathetic children showed deficits or below average. This situation may be caused by lack of proper management of Spanish language, which fail to fully understand the instructions for each scale test CUMANIN.
The difficulties in language possibly were related to the type of teaching in educational units because the teachers speak and give instructions in *kiwchua* while the tasks to be executed were in Spanish. This may cause confusion in understanding and expression.

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ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF LIMA BEANS (*PHASEOLUS LUNATUS* L.) PROTEIN

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ABSTRACT

Lima Bean is a legume known in Ecuador as "haba pallar" which has high protein content. The aim of this work was to evaluate the biological activities out of proteins concentrates. Protein was isolated by the isoelectric precipitation method at different pH using water as solvent. The antioxidant activity was carried out by the TBARS method while the anti-inflammatory activity was evaluated by the denatured protein method.

Proteins concentrates at pH 5.0 and 6.0 showed the higher antioxidant activity at 1 mg/mL concentration. However, the higher anti-inflammatory activity was observed at 1000 μg/mL of concentration for proteins concentrates at pH 3.0 and pH 5.0.

Obtaining active-biologically proteins concentrates from an industrial point of view is important, since they could be used as natural antioxidant or anti-inflammatory sources in functional products.

Keywords: anti-inflammatory, antioxidant, denaturation, lipid peroxidation, proteins

1. INTRODUCTION

Inflammation is considered as a normal protective response to tissue injury caused by different agents (physical trauma, noxious chemical or microbial agents), this response is part of host defense system (ALHAKMANI *et al.*, 2013; SEN *et al.*, 2015). The body try to inactivate or destroy the invading organisms and different synthetic drugs are used to manage this natural response (CHANDRA *et al.*, 2012a). However long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with several adverse effects like gastric irritation or duodenal ulcer, etc. (AMIR *et al.*, 2010). It is known that denaturation of tissue proteins lead to inflammatory and arthritic diseases (OPIE, 1962). Then, natural products that can prevent protein denaturation can be a good alternative to synthetic drugs, and to find a potent, but effective, yet safe and low-cost anti-inflammatory agents.
On the other hand, different diseases such as inflammation, thrombosis, diabetes, arteriosclerosis, and various types of cancers can be associated to oxidative stress (a serious imbalance between reactive species production and antioxidant defenses). Free radicals are atoms, molecules or ions with unpaired electrons, this condition make them highly unstable and active towards chemical reaction with other molecules. Some reactive oxygen species (ROS) include O_2, HO_2, OH, H_2O_2 and can produce oxidative damage in human body (CAROCHO and FERREIRA, 2013; HALLIWELL, 2007; KHLEBNIKOV *et al.*, 2007). Any substance that delays, prevents of removes oxidative damage to a target molecule is defined as antioxidant according to (HALLIWELL, 2007). In the food industry synthetic antioxidants are added to food to prevent oxidation (principally rancidity), damage caused by various treatments and conditions and at the end to prolong shelf life. Two or the more used synthetic antioxidants are BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) (CAROCHO and FERREIRA, 2013), but due to the possible toxic and carcinogenic effects associated to them (JAYALAKSHMI and SHARMA, 1986; LANIGAN and YAMARIK, 2002) it is necessary to search for natural alternatives.
It has been found antioxidant and anti-inflammatory activities in protein concentrates from different sources (CÁRDENAS *et al.*, 2018; CARRILLO *et al.*, 2017a; CARRILLO *et al.*, 2017b; QUINTEROS *et al.*, 2016). Lima beans (*Phaseolus lunatus* L.) knowing in Ecuador as "haba pallar" is a legume with a high content of protein. There are no studies about antioxidant and anti-inflammatory activity of proteins isolated from this legume which will be a found plus to the well-known quality of this food component which currently is driving a great attraction for conducting studies to obtain products with higher nutritional value as are functional foods which, when consumed, contribute favorably to the health of consumers.
In Ecuador, the National Autonomous Institute of Agricultural Research (INIAP), since 1992, with the purpose of promoting and increasing the production and income of farmers belonging to the Ecuadorian coast, released two improved bean varieties: INIAP PORTOVIEJO- 490 (for winter) and INIAP PORTOVIEJO- 491 (for summer), these two variants, have a high production capacity in rainy and dry seasons, respectively, as well as a high tolerance to diseases, which allows the farmers of the Ecuadorian coast to increase the economic development in marginal sectors, in addition to rescuing a traditional type of crop.
Besides the high content of protein, lima beans are an important source of vitamins, minerals, antioxidants, plant sterols and dietary fiber. Dry seed of the leguminous *Phaseolus lunatus* L. is used as a functional or nutraceutical food presenting astringent, febrifugal and emollient characteristics (POCHETTINO *et al.*, 2012). The phenolic profile and the antioxidant activity of 50 samples of *Phaseolus lunatus* L. grown in a germplasm bank in Brazil have been characterized, finding a wide diversity of polyphenols (total phenolic compounds, flavonoids and condensed tannins) which were strongly related to

the antioxidant activity, the samples that had higher coloration presented high polyphenols content and therefore greater antioxidant activity (AGOSTINI-COSTA *et al.*, 2015).
The aim of this work was the isolation of protein from lima beans cultivated in Ecuador and the study of biological activities such as: antioxidant and anti-inflammatory on those protein concentrate.

2. MATERIALS AND METHODS

2.1. Materials

Dry lima beans were purchased in May 2016 in a market in the Rocafuerte canton of the province of Manabí. The beans were dried at 50 °C for 2 to 3 hours. Then, the beans were ground in the mill PERTEN Laboratory mill 3100 and sieved in sieve ADVANTECH DuraTap™ DT168 with mesh # 70 (0.210mm). The obtained flour was vacuum packed and stored at laboratory temperature.
All reagents for proteins characterization, antioxidant activity and anti-inflammatory assay were analytical grade purchased to Merck®, Emsure®, Invitrogen® o Fisher®.

2.2. Protein concentrates

The isoelectric precipitation technique was used in order to obtain protein concentrates of *Phaseolus lunatus*, as described by Martínez and Añón (1996), but with modifications. Ratio 1:10 (w/v) flour: water was used, and later the pH adjusted to 8.0 with a solution of NaOH at 2M and finally stirring for 1 hour. The suspension was centrifuged, and the supernatant was adjusted at different pH (3.0, 4.0, 5.0 and 6.0 with HCl 2M) to provoke the precipitation, after the whole precipitate was lyophilized and later keep in storage until the corresponding analysis. The protein content in concentrates was determined by the Biuret method (GORNALL *et al.*, 1949).

2.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and NATIVE PAGE

For protein separation, electrophoresis SDS-PAGE was used according to the method described by Laemmli (1970). The protein concentrates at different pH were dissolved in distilled water, mixed with sample buffer (ratio 1:1 v/v) and heated at 80 °C for 10 minutes in a microincubator at 400 rpm. It was used a 4% acrylamide stacking gel and a 12% acrylamide separating gel in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, Life Science, USA). Electrophoresis was conducted at a constant current of 200 V per gel. Polypeptide bands were stained in Coomassie brilliant blue R-250 solution and then faded by washing the gel with a methanolic acid solution.

2.4. Determination of *in-vitro* antioxidant activity

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the potential antioxidant capacity of the protein concentrates. It was carried out following the method described by Carrillo et al. (2017b). Different solutions of protein concentrations at different pH (2.0, 3.0, 4.0, 5.0 and 6.0) were prepared (100, 200, 500 and 1000 μg/mL). The protein solution and oxidized olive oil were mixed in a 1:1 ratio (v/v). The samples were remained at 28 °C for 12 hours in the microincubator with constant agitation at 400 rpm. 1

mL of 1% thiobarbituric acid was added to each sample and heated at 95°C for 60 minutes at 400 rpm. The samples were cooled with ice for 5 minutes and the measurement was made in a Thermo Scientific Evolution 200 spectrophotometer at 532 nm. A commercial antioxidant, Butyl hydroxy toluene (BHT), at the same concentrations was used as positive control. Olive oil without sample was used as negative control. All were prepared by triplicate. The inhibition of lipid peroxidation as percentage of antioxidant activity was calculated with equation 1:

$$(\%)\text{Inhibition of lipid peroxidation} = \left(1 - \frac{T}{C}\right) x\ 100 \qquad \text{Equation 1}$$

Where T is the absorbance of the test sample measured at 532 nm and C is the absorbance of blank (control sample) at the same wavelength.

2.5. Determination of *in-vitro* anti-inflammatory activity

The anti-inflammatory activity of protein concentrates was carried out according to the method described by Chandra *et al.* (2012) and Sen *et al.* (2015) with modifications. 2 mL of protein concentrations at different pH and sodium diclofenac as a standard drug (100, 200, 500 and 1000 µg/mL) were mixed with 2.8 mL of phosphate buffer at pH 6.4 and 0.2 mL of albumin fresh egg. The mixture was incubated at 37 °C for 15 minutes, then the denaturation of the albumin was induced maintaining the samples at 70 °C for 10 minutes. Finally, the absorbance was measured at 660 nm using distilled water as a blank. This assay was performed by triplicate. The percentage inhibition of protein denaturation was calculated by using the equation 2:

$$(\%)\text{Inhibition of albumin denaturation} = \left(1 - \frac{A_s}{A_b}\right) x\ 100 \qquad \text{Equation 2}$$

Where A_s is the absorbance of the test sample measured at 660 nm and A_b is the absorbance of blank tested at the same wavelength.

2.6. Statistical analysis

The results are presented as means ± standard deviation of three repetitions of each experiment. In the case of protein quantification, four replicas were used. The analysis of variance and Tukey test were considered significant at $p<0.05$ using the SPSS software for Windows, to identify significant differences between mean values.

3. RESULTS AND DISCUSSION

3.1. Protein concentrates and protein content

Proteins were isolated from lima bean flour by isoelectric precipitation and using water as solvent. Four different pH (3.0, 4.0, 5.0 and 6.0) for precipitation were probed to obtain the better yield. The best treatment was obtained at pH 6.0 (32.58% ±0.30) while the lower one was observed at pH 3.0 (8.64% ± 0.10). The protein content was evaluated by the Biuret method. Protein concentrate obtained at pH 5.0 was the best treatment (71.33±0.92). All data were reported as the mean of 3 measurements ± the standard deviation.

3.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein concentrates obtained at different pH were analyzed by SDS-PAGE electrophoresis using 2-mercaptoethanol. In the presence of 2-mercaptoethanol, a reductor agent, it was observed several high expressed bands at all pH (Fig. 1). These bands showed molecular weights between 12 and 100 kDa. All the concentrates present the same profile of proteins and most of these bands were identified as globulins and albumins.

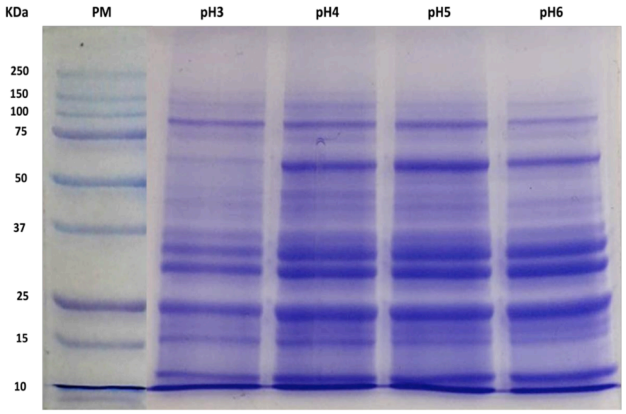

Figure 1. SDS-PAGE electrophoresis of proteins concentrates from lima beans in the presence of 2-mercaptoethanol. PM: molecular weight marker.

3.3. Determination of *in vitro* antioxidant activity by the TBARS method

Antioxidant activity of lima beans protein concentrates at pH 3.0, pH 4.0, pH 5.0 and pH 6.0 were evaluated by the TBARS method using different concentrations (100, 200, 500 and 1000 μg/mL). BHT, a synthetic antioxidant, was used as positive control at the same concentrations. This activity of the protein concentrates showed a dose depend relation, the higher the concentration the higher the antioxidant activity (Table 1).

Table 1. Inhibition of peroxidation lipid (TBARS) of lima beans protein concentrates.

Inhibition (%)	Concentrations			
	100 μg/mL	200 μg/mL	500 μg/mL	1000 μg/mL
BHT	62,74±2,73^{a}	68,10±2,35^{ab}	75,67±6,09^{bc}	80,36±2,34^{c}
pH 3.0	3.17±0.1^{a}	4.52±0.23^{b}	8.61±0.17^{c}	16.48±0.15^{d}
pH 4.0	14.34±0.96^{a}	23.39±1.78^{b}	23.54±0.60^{b}	29.28±0.60^{c}
pH 5.0	16.85±0.84^{a}	43.74±2.63^{b}	45.09±2.72^{b}	64.29±0.63^{c}
pH 6.0	15.18±1.50^{a}	37.52±1.67^{b}	51.78±3.35^{c}	68.00±1.31^{d}

Data are expressed as the mean ± SD (n=3). Values in the same row having different letters differ significantly ($p<0.05$). ANOVA and Tukey's test. SD: Standard deviation. TBARS: Thiobarbituric acid reactive substances. BHT: Butylated hydroxytoluene.

Protein concentrates at pH 5.0 and pH 6.0 presented the higher antioxidant activity at the maximum concentration tested with 64.29% and 68.00% respectively. BHT present high antioxidant activity with values between 62.74%-80.36% of inhibition of lipid peroxidation.

3.4. Determination of *in vitro* anti-inflammatory activity by the protein denaturation method

One of the causes related with inflammatory and arthritic diseases is the denaturation of tissue proteins (CHANDRA *et al.*, 2012b). Alteration of protein structure (hydrophobic, hydrogen and disulphide bonding) can be related to denaturation of protein (SEN *et al.*, 2015). So, the identification of natural agents that can prevent protein denaturation has an important role in food and pharmacology research.
In this study, the *in vitro* anti-inflammatory effect of protein concentrates from lima beans was evaluated against the denaturation of egg albumin. The results summarized in Table 2 showed a concentration dependent inhibition of protein denaturation by the concentrates in a range concentration of 100-1000 μg/mL. Sodium diclofenac used as the reference also exhibited concentration dependent inhibition of albumin denaturation. The effect of lima beans protein concentrates was less in comparison with the drug.
Decreases in the absorbance of test samples with respect to control indicated protection of albumin to denaturation effect induced by the temperature applied. So, the percent inhibition of protein denaturation with respect to control is a measure of protein stabilization (CHANDRA *et al.*, 2012a).
The protein concentrates exhibited membrane stabilizing effect, at 1000 μg/mL, producing 37.55% (pH 3.0) and 32.66% (pH 5.0) protection against albumin denaturation.
The *in vitro* anti-inflammatory effect of protein concentrates may also be induced by secondary metabolites like phenolic compounds. Since these compounds are also soluble in water and many biological properties have been attributed to these natural metabolites (ALHAKMANI *et al.*, 2013; FU *et al.*, 2016; NAM *et al.*, 2012; PADMANABHAN and JANGLE, 2012; TOMÁS-BARBERÁN and ANDRÉS-LACUEVA, 2012). Even though diverse pharmacological actions have been attributed to the presence of secondary plant metabolites such as phenolic compounds, proteins isolated from lima bean flour have also showed a biological anti-inflammatory activity. However, no such high as sodium diclofenac. The protein concentrates showed *in vitro* anti-inflammatory effect against the denaturation of protein. Son it can be used as a source of anti-inflammatory agents.

Table 2. *In* vitro anti-inflammatory activity of lima bean protein concentrates.

Inhibition (%)	Concentrations			
	100 μg/mL	200 μg/mL	500 μg/mL	1000 μg/mL
Sodium Diclofenac	18.31±2.37^{a}	33.60±2.21^{b}	56.15±0.32^{c}	100±3.53^{d}
pH 3.0	11.38±0.72^{a}	24.30±0.08^{b}	36.97±0.52^{c}	37.55±0.94^{c}
pH 4.0	5.55±0.59^{a}	13.01±0.20^{b}	21.27±0.24^{c}	25.22±0.25^{d}
pH 5.0	19.27±0.14^{a}	23.84±0.11^{b}	30.86±0.35^{c}	32.66±0.76^{d}
pH 6.0	7.47±0.51^{a}	7.88±0.26^{a}	12.67±0.17^{b}	17.88±0.17^{c}

Data are expressed as the mean ± SD (n=3). Values in the same row having different letters differ significantly ($p<0.05$). ANOVA and Tukey's test. SD: Standard deviation.

4. CONCLUSIONS

Protein concentrates were isolated from lima beans (haba pallar as named in Ecuador). These concentrates were characterized by SDS-PAGE electrophoresis showing high expressed bands. From these concentrates two biological activities were evaluated. Protein concentrates at pH 5.0 and pH 6.0 presented the higher antioxidant activity at the maximum concentration tested, while protein concentrates at pH 3.0 and pH 5.0 showed the major membrane stabilizing effect, at 1000 $\mu g/mL$. Even though the percentage of antioxidant and anti-inflammatory activities were not such high as the positive control, they were significant. These protein concentrates could be used to increase the nutritional value of food products.

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FOOD HABITS, ANOREXIA AND BULIMIA, IN THE YOUTH POPULATION OF THE CENTER OF ECUADOR

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ABSTRACT

Understanding the eating habits of a society makes it possible to intervene with rigor in the failures that can lead to serious health problems.

This study delves into the eating habits of university students from Ambato. 428 people were surveyed about consume and eating behaviors in order to be able to do a diagnosis about anorexia and bulimia rates.

The results show deficiencies and excesses of certain basic foods in an adequate diet for the young people surveyed. Also, levels of anorexia and bulimia are found in students, especially in women.

It concludes with proposals to improve eating habits in young people.

Keywords: anorexia, bulimia, eating habits, Ecuador, young people

1. INTRODUCTION

According to the National Institute of Statistics and Census (INEC) in the National Health and Nutrition Survey (ENSANUT-ECU, 2014), approximately 40% of the Ecuadorian population between 19 and 29 years old are overweight or obese. Regarding disorders such as anorexia or bulimia, there are no general numbers in this regard, since they are not considered public health problems (elcomercio.com, 2014). Nevertheless, more local research speaks of a high incidence of these disorders among the population, especially in young women (CEDILLO *et al*., 2014; ROMÁN, 2012).

These indices should be considered a public health problem because these people can, in the medium and long term, be carriers of diabetes or hypertensive and cerebrovascular diseases, which are some of the principal causes of death in Ecuador (OPS/OMS 2014), and they can even lead to a chronic disease that can also result in death in cases of eating disorders like anorexia.

In the past decades, the World Health Organization (WHO, 2014) has detected a considerable increase in overweight and obesity in Latin America, which is currently affecting almost half of its population in each country (FAO / PAHO, 2017).

The World Health Assembly (2004), with its approval of the Global Strategy on Diet, Physical Activity and Health, obligated the Andean country to commit to taking measures that ensure both the provision of balanced information to consumers to facilitate their adoption of healthy decisions such as the availability of appropriate programs for health promotion and health education.

There are many laws about this issue, especially concerning the responsibilities of government entities. Thus, the Organic Law of Consumer Protection (2000-2011) in Articles 4 and 6 proposes to ensure that citizens receive adequate, accurate, clear, timely and complete information about the goods and services offered by the market, as well as to protect them from misleading or abusive advertising.

But despite the institutional and legislative efforts, the number of people with overweight and obesity has been continually increasing in recent years (WHO, 2014), making it more and more necessary to get involved in a problem that affects all sectors of the Ecuadorian population.

In addition to these risk factors, there are disorders such as anorexia and bulimia. The fact that there are not exact numbers at the national level is because it is not considered a public health issue, making it even more necessary to analyze this problem and find solutions in this regard. Eating disorders affect more and more people, especially young women (KOHN and GOLDEN, 2001). They are influenced by numerous psychological, socio-cultural factors and more than anything by the mass media (GUERRO-PRADO *al*., 2001).

Obesity is the result of the imbalance between the consumption and the contribution of energy, whose consequences reach catastrophic proportions while representing a high cost for health services (GARCÍA and CREUS, 2016).

There are many causes of this problem but the main ones are the lack of physical activity and the increase of a sedentary lifestyle along with an unhealthy diet (MARTÍNEZ *et al*., 2017).

The most commonly known eating behavior disorders (EBD) are nervous anorexia and nervous bulimia. EBD are serious but they are a curable illness if the person receives treatment from psychologists and specialized doctors. EBD are characterized by a pathological behavior against food intake and an obsession with weight control. They have many causes (biological, psychological, family and socio-cultural) and cause negative consequences for both the physical and mental health of a person (ACAB, 2017).

In the third objective of the National Plan for Good Living (2013-2017), health is discussed from an inter-sectorial perspective, taking into consideration the modes of nutrition and the promotion of physical activity. Based on this, Section 3.6 focuses exclusively on promoting nutritious and healthy eating habits that allow people to enjoy a level of physical, emotional and intellectual development according to their age and physical conditions.
Therefore, the approach of the study is to diagnose, explain and evaluate the eating habits of young people from Ambato as well as to detect possible psycho-social risk behaviors that could result from bad eating practices in this sector of the population.

2. MATERIALS AND METHODS

Some references used for this paper are the questionnaires on eating habits and physical exercise carried out by MEMBIELA and CID (1998), PARDO *et al*. (2004), PINO *et al*. (2011), UNIKEL *et al*. (2004), as well as the EAT-40, EDI and BSQ (BEHAR *et al*., 2011) about anorexia and bulimia.
428 surveys were conducted during the second quarter of 2018 to students between the ages of 17 and 30 with different majors at the Technical University of Ambato and the Technical University of Indoamerica (mean = 22.06). 55.4% were women and 41.6% were men (3% were missing). The questionnaire took 10 minutes to complete and was taken by qualified personnel previously trained and prepared for it. All participants gave their consent and were informed of the voluntary and anonymous nature of the questionnaire. Besides, it was presented and inspected by the bioethic committee of the Faculty, according to the regulations of the University and the pertinent Code of Ethics.
It included three parts. It was assigned a number to the last two ones (1=minumum frequency / 4 and 5=higher frequency) which served lately to get a more accurate and extensive analysis (mean instead of mode) and to make factorarial anaylisis of the main components. It should be mentioned that it could not be possible to evaluate the real intake of each individual due to the fact that this would requiere a harder information survey (daily food record, 24-hour reminder, etc. (and everything handled by qualified personnel)).

-Individual characteristics (sex, age, weight, height, etc).
-Consumption of foo: never or almost never (1), once a week (2), two or three times a week (3), almost every day (4) and several times a day (5).
-Habits and eating disorders: never (1), only a few times (2), sometimes (3) and many times (4).

Once the information was obtained, the database was prepared and analyzed through the statistical program SPSS (version 23 for Windows).

3. RESULTS

Table 1, below, shows the averages of frequency of food consumption. The frequencies of food consumption found were the following, taking into account the attributed values: never or almost never (1), once a week (2), two or three times a week (3), almost every day (4) and several times a day (5). It is showed mean instead of mode to be more precise with data (1=minumum frequency / 5=higher frequency).

Table 1. Averages of frequency of food consumption.

Food	Mean	Food	Mean
Green vegetables (lettuce, cabbage...)	2.97	Yogurt / milkshakes	2.77
Vegetables (tomatoes, peppers)	3.3	Soft drinks with sugar	2.9
Legumes (lentils, beans...)	2.66	Soft drinks without sugar	2.19
Cereals (oats, quinoa...)	2.66	Juices with sugar	3.14
Rice	4.04	Juices without sugar	2.09
Corn (“choclo”, “mote”, toasted...)	2.54	Low alcohol (beer, wine ...)	1.68
Bread	3.45	High alcohol (whiskey, rum, vodka, “puro“...)	1.46
Noodles	2.56	Infusions	2.94
Biscuits	2.33	Chips	2.6
Eggs	3.03	Hot spicy	2.26
Unprocessed meats	2.63	Sweets / cupcakes	2,.4
Processed meats	2.54	Junk food	2.61
Chicken	3.43	Dried fruits	2.02
Red meats (pork, beef, lamb, etc.)	2.89	Snacks	2.55
Fish	1.98	Transgenic foods	1.98
Seafood	1.83	Pre-cooked foods	1.86
Fruit	3.35	Grilled food	2.23
Milk	3.11	Fried foods	2.83
Fresh cheese	2.61	Roasted food (baked)	2.60
Cured or semi-cured cheese	1.73	Barbecued food	2.27

Source: Own elaboration.

The average number of meals eaten per day was 3.40, and the liters of water drunk per day was 1.21. 93% of the respondents said they were not on any type of diet. Those who were on a diet were following diets that are high in protein, low in fat, with few carbohydrates (especially bread and rice), rich in fruits, vegetables, fiber, or they directly practiced vegeterianism or veganism.

An inferential analysis (U of Mann Whitney, $p < .05$) showed that women consumed less water, rice, bread, processed meats, red meat, and alcohol - both in high and low grade - and consumed more vegetables, cereals, fruit, fresh cheese, yogurt, and infusions than men.

Regarding eating habits, a factorial analysis of main components with Varimax rotation (KMO = .794, Bartlett, $p = .000$, Cronbach's alpha = .847) was carried out, assigning the following values to the answers: never (1), only a few times (2), sometimes (3) and many times (4). 16 factors were obtained that explained 61.94% of the variance with eigenvalues that are greater than one.

In Table 2, below, some results are presented for factors 1 to 3. In Table 3, is showed the factorial analysis for factors 4 to 6.

To simplify, the first six components (factors) extracted with eigenvalues greater than 1.5 and whose factor weights were greater than .40 (41.04% of the variance explained) are shown below.

-Factor 1: possible eating disorder
-Factor 2: nervous bulimia
-Factor 3: practices of losing weight
-Factor 4: control of intake and aversion from eating too much
-Factor 5: physical exercise and protein intake (possible vigorexia disorder)
-Factor 6: sedentary lifestyle and eating out

Table 2. Factorial analysis I.

Items	Factor 1	Factor 2	Factor 3
I have used pills to try to lose weight	0.802		
I have taken diuretics (substances to lose water) to try to lose weight	0.738		
I have taken laxatives (substances to facilitate evacuation) to try to lose weight	0.711		
I have vomited after eating to try to lose weight	0.665		
I have visited a food specialist or nutritionist	0.624		
I think my life is not very useful	0.455		
I feel like I've eaten too much; I've gotten stuck in food		0.781	
I have lost control over what I eat (I have the feeling that I cannot stop eating)		0.687	
I keep eating even if I'm not hungry		0.660	
I sample dishes when I am anxious or I lack encouragement		0.482	
I have gone on diets to try to lose weight			0.748
I have exercised to try to lose weight			0.748
When I try to lose weight, I tell everyone around me so that they will support me			0.628
I was worried about getting fat			0.581
I feel people think I'm fat		0.515	0.517
I worry about the amount of calories I consume			0.463
I have fasted (stopped eating for many hours) to try to lose weight			0.437

Source: Own elaboration.

Table 3. Factorial analysis II.

Items	Factor 4	Factor 5	Factor 6
I stop eating even though I'm still hungry	0.719		
I serve small portions	0.570		
When I see a lot of food I feel nauseous	0.564		
I do sports or exercise weekly		0.815	
I consume protein supplements		0.418	
I spend many hours sitting down			0.641
I eat food made outside the home			0.519

Source: Own elaboration.

Contrasting these factors with gender, men scored more in the fifth item (physical exercise and protein intake (possible vigorexia disorder)).
Although there are no gender differences with the other factors, an individualized analysis of the items shows that women do the following actions more than men: weigh themselves, cook their own food, eat slowly, go on diets to try to lose weight, worry about getting fat, serve small portions, be more aware of the calories they take in a day, sampling when they have anxiety or feel low; they also feel that they eat too much or that they have gotten stuck in food more often. On the other hand, the idea that people think they are very thin is more predominant in men.
The six analyzed factors are related (Pearson, $p < .5$) with a mayor or minor frequency of consumption of certain food (+ or -).

Table 4. Foods related to factors.

Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
+Grilled food +Liters of water +Cereals -Rice -Bread -Chicken +Fish +Seafood +Soft drinks without sugar +Juices without sugar +Going on some type of diet	+Hot spicy	-Meals a day +Fresh cheese -Cereals -Low Alcohol -High Alcohol +Going on some type of diet	-Grilled food -Fried food - Seafood -Cured cheese -Chips -Junk food -Baked foods	+Dried fruits + Grilled food +Meals a day +Liters of water +Green vegetables +Vegetables +Legumes +Noodles +Eggs +Unprocessed meat +Fish + Seafood +Fruit +Yogurt +Low alcohol +Baked foods	+Snacks +Rice +Juices with sugar -Juices without sugar +Chips +Junk food

Source: Own elaboration.

Finally, approximately 7% reported that they have suffered from some type of eating disorder, especially anorexia and bulimia in their various types. In addition, to the question of how they would improve their nutrition, most indicated that they would seek the help of a nutritionist, eat more fruits and vegetables and less fat and sugar, do more physical exercise, and drink more water.

4. DISCUSSION AND CONCLUSION

The results show that frequency in the consumption of food could well be related to the fact that the subjects surveyed are young people and students. As shown in factor six, many people are inclined toward a sedentary lifestyle. In turn, 25.9% said that they eat out many times and 37% do so sometimes, so most of the respondents probably have to eat around the university several times a week.
This fact explains why more meat is eaten compared to fish or seafood, why the main carbohydrate is rice, and why more soft drinks and juices are consumed with sugar than without sugar. This also explains the high rates of junk food, snacks and fried foods because these are the basic components of the standard lunches that are usually offered at

establishments around the university at an affordable price for young people. That is why this diet appears as characteristic of factor six which was analyzed.
The differences found between gender reveal that women tend to maintain a lower carbohydrate diet with more healthy foods (vegetables, fruits, etc.) than men. At the same time, while they choose these types of ingredients in order to take care of their diet, they also do more physical exercise as shown by the INEC indexes (2015) and the results by correlating factor five with gender.
In addition, women tend to think that they are fat more, but they consider that people think they are very thin, which explains the differences found in the frequency of consumption of food and physical activity, as women would seek to lose weight by controlling their food, while men would seek to gain more weight by doing sports and consuming protein supplements.
The fact that the factors contain a food pattern shows how those aimed at the control of the diet (factors 1, 2, 3, 4) are related to low diets in alcohol, fats or carbohydrates while the fifth component, which is predominant in men, contains a diet rich in fats, carbohydrates and more calories in general from various foods
As a conclusion, this study has detected how approximately 7% declared to have suffered an eating disorder, a figure that will increase if we are take into account the results of the items related to the risk behaviors derived from the work of UNIKEL *et al*. (2004). In any case, the figures are relevant enough to reflect on the rating of possible eating disorders in the society of Ecuadorian youth.
While solutions to improve nutrition require the commitment of all sectors involved such as the State, the private sector, the civil population, and health professionals (ENSANUT-INEC, 2013), with eating disorders such as anorexia or bulimia, the first big step needs to be taken at the level of government so that they would be considered topics of interest in public health. Until this step is taken, the country will not be able to provide the necessary and adequate prevention and intervention.
Finally, the recommendations should not only be informative. As the suggestions found to improve the diet, most of them know well what the elements are that would improve their diet, so the problem would not be so much the lack of knowledge about eating or having too little information, but rather the lack of habit. Thus, the possible education or media diffusion programs should have less theoretical content and focus more on strategies for changing attitudes and behaviors in a practical and contextual way.
In this sense, and as part of a transversal problem, the consumer's environment should also be affected since, as shown by the results obtained, the diet of young people is mostly determined by what is offered at the places where the food is sold. Likewise, the State must be aware of this reality and promote good eating habits among young people; at the same time they should be involved with the establishments in order to seek joint measures that benefit all consumers. Some of them could be aimed at subsidizing and designing healthy menus, making it expensive to buy or penalizing products that are high in fats or sugars, establishing laws and regulations for the nutritional composition of foods and their advertising in establishments, making voluntary agreements to reduce critical nutrients, implementing food surveillance and control programs as well as health awareness plans at schools and universities, or offering food guides to the population according to the nutritional offer (MINISTRY OF HEALTH - GOVERNMENT OF CHILE, 2015/10/08).
In short, it is expected that this paper has contributed with enough analysis about the issue for the results to be considered, and that it would serve as a reference in the near future to act on an important issue for health as is the case of the diet of citizens.

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PHYSICOCHEMICAL, MICROBIOLOGICAL AND SENSORY EVALUATION OF RABBIT MEATBALLS MARINATED IN TAMARILLO JUICE (*SOLANUM BETACEUM)*

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ABSTRACT

Rabbit meat and tamarillo juice were used to prepare a new type of meatball, considering the effect of tamarillo juice concentration in marinated solution and its impact on physicochemical, sensory and microbiological properties. A factorial design A*B was applied, which involved the percentage of tamarillo juice in the marinated solution and marinade time. Results indicated that the 75% of tamarillo juice had the highest level of acidity. Sensorial analysis showed that the best treatment was the sample, which had 50% of tamarillo juice and 60 minutes of marinade time. Microbiological tests of the product showed the absence of microorganisms such as *Escherichia coli* and aerobic mesophilic bacteria. The study demonstrates the potential of tamarillo juice as a non-conventional ingredient in the marinated solution to improve tenderness, shelf life and sensory characteristics of rabbit meatballs.

Keywords: meatball, marinate solution, marinade time, rabbit meat, tamarillo juice

1. INTRODUCTION

Rabbit meat has a high protein content and a low content of cholesterol, uric acid and purines compared to beef, pork and chicken, which makes it nutritionally better. (GUEVARA *et al.*, 2016; HERNÁNDEZ, 2007; DALLE ZOTTE and SZENDRŐ, 2011; HERNÁNDEZ, 2008). Consumption of rabbit meat is very low, due to the lack of meat supply and unavailability of marketing (PRIYANTI and RAHARJO, 2013). Although there is a habit of consumption rabbit meat in rural or touristic areas, there are few traditional products made from it. Several preserved rabbit products have been developed, such as cured meat, dried meat and sausages (XUE *et al.*, 2017; HUISHI, 2008; LENGKEY and LOBO, 2016; WAMBUI *et al.*, 2017). Despite the demand for products with little time for meal preparation, most rabbit meat worldwide is still sold as a whole and cut-up parts (PETRACCI and CAVANI, 2013). On the other hand, one of the most promising technologies for raw meat materials in order to obtain added-value products is the marinated process (ZEUTHEN and BØGH-SØRENSEN, 2003). Rabbit meat has a particular flavour and a firm texture (MANCINI *et al.*, 2016; TARIQ *et al.*, 2016). A marinated process would allow getting a more pleasant flavour and texture because it improves the relaxation of meat fibers resulting in a tenderer, juicier and more easily chewable product. Marinating increases water retention during cooking, even when there is excessive cooking due to lack of attention, which provides more juiciness and avoids the loss of moisture in the product, added to the fact that salt helps to increase the shelf life (SANTOS and RAMOS, 2018). Modern technologies are using acid fruit juices, such as passion fruit, orange, lemon, among others, as an ingredient in the marinade solution. Acid solutions facilitate processing and improve the sensory properties of marinated meat products. The use of tamarillo, which is acid, can influence functional factors such as softening of fibrillar structures by swelling the meat, increasing proteolysis by cathepsins and conversion of collagen to gelatin at low pH during cooking. (BERGE *et al.*, 2001; OFFER, 1988; SERDAROĞLU *et al.*, 2007; YUSOP *et al.*, 2010). Tamarillo or tomato tree *(Solanum betaceum*) is native from Ecuadorian-Peruvian Andes but is also cultivated in other countries such as New Zeland, Brazil, Bolivia, and Chile (HURTADO *et al.*, 2009). It is recommended for its nutritional qualities as a good source of pro-vitamin A, vitamins C, B6 and E, and iron (VASCO *et al.*, 2009; DE ROSSO and MERCADANTE, 2007). The aim of this research was to evaluate physicochemical, microbiological and sensory properties of rabbit meatballs marinated in tamarillo juice.

2. MATERIALS AND METHODS

2.1. Materials

Rabbit meat was purchased from a local farm and refrigerated at 4°C before processing. *Longissimusdorsi* from rabbits of 6 to 8 months old were used for all marinade process. Tamarillo was obtained from organic crops of a local plantation. Marinade tests were composed by 90% of liquid fraction and 10% of solid fraction (pepper, garlic, marjoram, onion, cumin, salt and curing salts). The liquid fractions were prepared with three different levels of tamarillo juice (50%, 75% and 100%, w/w). All marinated solutions were prepared the day before marination and kept at 4°C until required. All rabbit meat cuts were processed using a Bowl Cutter (Mainca CM14, Spain). The dry-cooking of meatballs was done in a convection oven (Zucchelli Mini Fanton, U.S.A). The pH was determined using a potentiometer (HI 9126 HANNA, Rhode Island, U.S.A). The texture analysis was carried out using a texturometer (CT3 Brookfield texture analyser, USA).

2.2. Meatballs production

Marinade was carried out by means of individual immersion of rabbit meat cuts into different chilled (4°C) solutions in a stainless steel container. During the first 15 minutes, rabbit meat cuts were massaged manually to distribute the solution homogeneously. After marination time, the meat was processed to obtain meatballs around 30 g each and dry-cooked at 150 °C for 18 minutes. Table 1 shows the treatments used which combine concentrations of tamarillo juice and marinade time.

Table 1. Treatments used to produce "rabbit meatballs."

Sample	Tamarillo juice concentration (%)	Marination Time (min)
RNT560	50	60
RNT760	75	60
RNT160	100	60
RNT512	50	120
RNT712	75	120
RNT112	100	120
RNT518	50	180
RNT718	75	180
RNT118	100	180

2.3. Physicochemical and proximal analysis

All physicochemical and proximal analysis was performed under AOAC methods: acidity (AOAC 947.05), ash, (AOAC 923.03), moisture (AOAC 950.46), protein (AOAC 2001.11), fat (2033.06). Carbohydrate content was estimated by difference. All determinations were performed by triplicate.

2.4. Cooking loss

The surface of marinated samples was dried with paper towels, weighed, and then cooked until an internal temperature of 75°C was obtained. After the cooking process, the samples were dried with paper towels and weighed. The cooking loss was calculated according to equation 1:

$$\%\ cooking\ loss = \frac{w1-w2}{w1} * 100 \qquad \text{(Equation 1)}$$

Where w1=weight before cooking, and w2=weight after cooking.

2.5. Sensory evaluation

The sensory characteristics were evaluated using an incomplete block design with 15 semi-trained judges. Attributes as odour, flavour, texture and general acceptability were considered. Three tastings sessions with a structured hedonic scale of 5 points were performed. For attributes such as odour, flavour and acceptability the hedonic scale was 1

for "*dislike*" and 5 for "*like very much*", for texture the scale was 1 for "*hard*" and 5 for "*very soft*".

2.6. Microbiological analysis

Total aerobic mesophilic bacteria and *Escherichia coli* and total coliforms were assessed following the official methods AOAC 991.14 and AOAC 990.12, respectively.

2.7. Texture analysis

A TA 39 probe was used with a speed of 2 mm/s to compress the center of samples. Hardness, cohesiveness, elasticity index and firmness of the product were measured.

2.8. Statistical analysis

A Two-way ANOVA was obtained using the GraphPad Prism 5.0 program (GraphPad Software, San Diego, California, USA). Additionally, statistical comparisons of the mean data values were carried out using the Tukey test with a significance level of $P \leq 0.05$.

3. RESULTS AND DISCUSSIONS

3.1. Physicochemical properties

The proximal composition and pH of raw rabbit meat were as follows: moisture, 74.5±1.1%; protein, 22.5±0.12%; fat, 1.8±1.59%; ash, 1.2±0.2%; and pH, 6.66±0.03. After marination, samples of all treatments were evaluated for pH, acidity, moisture, ash, and cooking loss. The effects of marinade are shown in Table 2. Concerning pH, a difference is evidenced due to marinade time but not to the concentration of tamarillo juice, attributed to the osmosis phenomenon that exists during the marinade with tamarillo juice, which was acid at pH = 3.8. During a marination process, there is a significant increase in the pH values of meat because there is a process of protein degradation, which releases peptides and amino acids (EPLEY, 1992). There is also a release of sodium and calcium ions from the sarcoplasmic reticulum, leading to an increase in the osmotic pressure of the muscle cells and consequently an increase in pH (PALMA and SINDY, 2009). In this study, the maximum marinade time was 180 minutes, time that could not have been enough to produce significant protein degradation. As was expected, the samples that were immersed in a solution with 100% of tamarillo juice had the highest acidity. Likewise, the acidity of the tamarillo juice could hinder the osmotic process between the inside and outside part of meat, making that the moisture content shows a significant difference ($p < 0.05$). In this sense, samples which were immersed in 100% tamarillo juice show the lowest moisture values in comparison with other juice concentrations, probably to the fact that the acidity could alter the outer layer of the samples (YUSOP *et al.*, 2010). These results agree with other researchers who found the lowest moisture content with the highest acidity of the marinade solution (KOMOLTRI and PAKDEECHANUAN, 2012). Ash content shows the highest values in samples that were immersed in the highest concentration of tamarillo juice because more concentration of juice could transfer more components to the rabbit meat.

Table 2. Effect of the marinade on physicochemical properties of rabbit cooked meatballs.

Samples	pH	Acidity (%)*	Moisture (%)	Ash (%)	Cooking loss (%)
RNT560	5.41±0.01^{d}	0.37±0.03^{a}	68.66±0.35^{c}	1.16±0.15^{a}	30.12±0.14^{d}
RNT760	5.40±0.02^{d}	0.44±0.01^{c}	69.28±0.36^{f}	1.67±0.15^{b}	31.43±0.15^{e}
RNT160	5.39±0.02^{d}	0.48±0.01^{d}	69.44±0.40^{h}	1.75±0.26^{c}	32.40±0.27^{e}
RNT512	5.21±0.01^{c}	0.61±0.02^{e}	68.74±0.34^{d}	2.16±0.14^{d}	27.93±0.53^{c}
RNT712	5.19±0.01^{bc}	0.68±0.03^{f}	69.13±0.17^{e}	2.33±0.29^{f}	28.64±0.29^{c}
RNT112	5.17±0.01^{b}	0.71±0.03^{g}	69.36±0.35^{g}	2.25±0.25^{e}	29.96±0.59^{d}
RNT518	5.12±0.02^{a}	0.38±0.02^{a}	67.82±0.37^{a}	2.24±0.25^{e}	20.97±0.27^{a}
RNT718	5.11±0.02^{a}	0.42±0.01^{b}	68.13±0.36^{b}	2.33±0.29^{f}	22.03±0.35^{b}
RNT118	5.10±0.02^{a}	0.45±0.02^{c}	68.74±0.30^{d}	2.66±0.29^{g}	23.02±0.29^{b}

Results are the mean±standard deviation. One-way ANOVA: different letters (a,b..h) in the same column indicate significant differences between samples ($P \leq 0.05$).
*expressed as lactic acid.

3.2. Cooking loss

Cooking loss results are shown in Table 2. Based on the results, samples that were treated in 100% of tamarillo juice solution had the lowest values. In this sense, sample RNT 160 (100 % of tamarillo juice and 60 min of marinade time) has the lowest value. These data could be attributable to the relation and concentration of ingredients in marinating solutions, where the combination of two or more ingredients in marinate solutions result in a lower cooking loss than when ingredients are used singly (SHEARD and TALI, 2004). This effect could also be explained due to the higher number of ions that cause electrostatic repulsion, which enlarges the space between the thin and thick filaments of the muscle fibre; resulting in higher water uptake into the muscle (SAMS, 1999; SHEARD and TALI, 2004). These results are in agreement with a previous study who reported that a combination of NaCl/ sodium tripolyphosphate / citric acid at 4°C for 2 hours was found to be the optimal marinating treatment for low cooking loss of golek chicken (KOMOLTRI and PAKDEECHANUAN, 2012).

3.3. Sensory evaluation

From the sensory assessment of meatballs, it was possible to establish that at a 95% level of confidence there are differences between treatments in all attributes, which were evaluated. The judges evidenced the effect of concentration of tamarillo juice and marinade time in the developing of odour, flavour, texture and acceptability of meatballs. The sensory profile of treatments for the four characteristics showed that the highest acceptability was obtained by the treatment RNT560 (Fig. 1), which had 50 % of tamarillo juice and 60 minutes of marinade. The odour and colour were ranged from like lightly to like, while the texture was between lightly hard to soft. In all attributes, the multiple range test of Tukey HSD established that the RNT560 treatment was the best. The acceptability evaluation showed that same treatment obtained the highest acceptability.

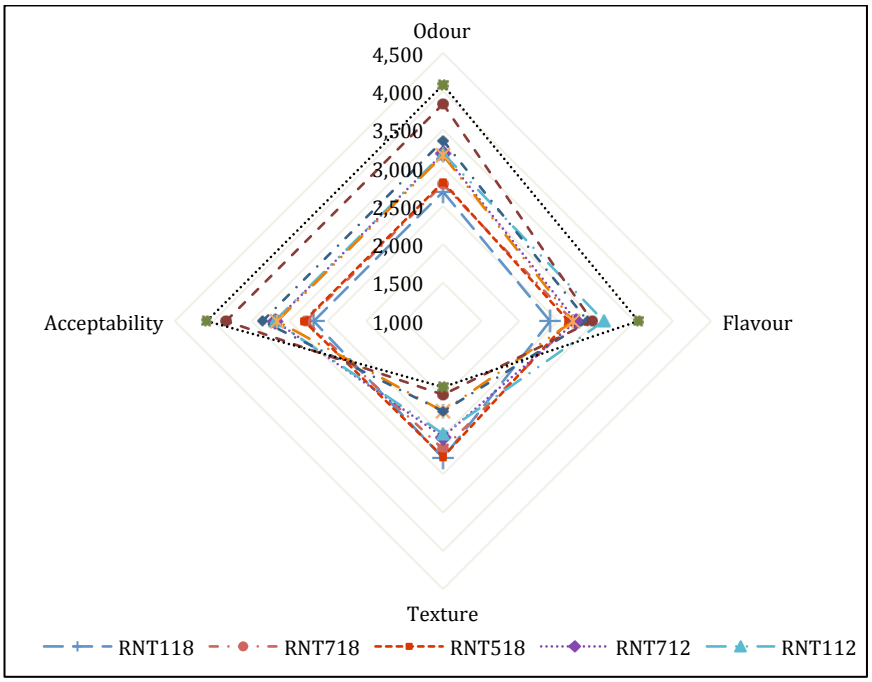

Figure 1. Sensorial evaluation of "rabbit meatballs."

3.4. Proximal composition

Nutritional characteristics were performed for the best treatment (RNT560). Sensory analysis and pH were selected to determine the best treatment. As a rule, meat and meat products must have a pH lower than 5.9. Acidic substances that reduce pH have been reported to play a significant role in the tenderization and flavouring of treated meat, causing meat fibres to swell (YUSOP *et al.*, 2010). Likewise, sensory analysis is a powerful tool in the development of new products because it is essential to know how customers perceive a new product as they are the ones that are going to purchase it (PÉREZ-CACHO *et al.*, 2005). Protein, fat and carbohydrates contents were 15.90%, 3.61%, and 10.66% respectively. These values are in concordance with Ecuadorian food regulation. Moisture content was 68.66%. The ash content of meatballs was 1.16%, this result could be explained by the addition of tamarillo juice to the marinade solution, and the juice concentration could increase ash values at the end of the marination process (SIDIRA *et al.*, 2016).

3.5. Texture profile analysis (TPA)

Only for textural parameters, a control sample (rabbit meat marinated in water and spices) was developed. Texture profile analysis (TPA) results for best treatment RNT560 in comparison with control are shown in Table 3. Significant differences in hardness and firmness were found when comparing treatments ($p<0.05$). Rabbit meatballs from control exhibited the highest hardness while the ones from tamarillo juice concentration (RNT560) had the lowest value; this was due to water retention within the muscle fibers and the effect of acidic marinade on connective tissue (BERGE *et al.*, 2001; CRUZEN *et al.*, 2015; CHAURASIYA *et al.*, 2015). On the other hand, no significant differences were found in cohesiveness and elasticity index ($p>0.05$). Although there are no statistical differences, the mean data show that the lowest cohesiveness is for the marinated sample. This effect

could be due to acidic marinades decrease the mechanical resistance of meat and reduce cohesiveness parameters (KIM, 2018; YUSOP *et al.*, 2010).

Table 3. Effect of the marinade on textural properties of rabbit meatballs.

Samples	Hardness (g)	Elasticity Index (mm)	Firmness (g)	Cohesiveness (mJ)
RNT560	335.10±0.7^{a}	2.89±0.07^{a}	219.33±1.1^{a}	5.93±0.9^{a}
Control	471.67±0.4^{b}	2.74±0.2^{a}	294.67±0.7^{b}	7.97±0.6^{a}

Results are the mean±standard deviation.
One-way ANOVA: different letters (a,b) in the same column indicate significant differences between samples ($P \leq 0.05$).

3.6. Microbiological quality

The microbiological analysis establishes that the processed meatballs comply with the provisions stated by the Ecuadorian technical standard INEN 1338:2012 for aerobic mesophilic bacteria and *Escherichia coli*, which must correspond to less than 10 cfu/g. All the products comply with the normative that request the absence of harmful microorganisms to public health. The shelf life of the best treatment was obtained by linearization of the concentration of *Escherichia coli* (ln (ufc/g) = 0.1095* time + 0.4187; R^2 = 0.92). The shelf life was calculated by the equation and was 3.5 months, which is an excellent value for meatballs with no preservatives added in its formulation.

4. CONCLUSIONS

This research allowed to develop a rabbit meatball marinated in tamarillo juice as a variant to the classic meatballs. The best treatment was obtained using 50% of tamarillo juice and 60 minutes of marinade time, being the same one that obtained the best acceptance test in the sensory evaluation and therefore, could be accepted by potential customers. The pH of this product was 5.41, with a percentage of lactic acid of 0.37 at the end of the marination process. The cooking loss of the product ranges from 23.02% to 32.40%. The meatball contains protein, fat, moisture and ashes according to the current regulations. The microbiological analysis made it possible to establish the absence of pathogenic microorganisms for public health. Finally, the study provides valuable information regarding the use of rabbit meat as a non-conventional raw material that is little used and that could have an interesting opening to the national and international market.

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WALNUT PROTEIN CONCENTRATE (*JUGLANS NEOTROPICA* DIELS), GASTROINTESTINAL DIGESTS AND THEIR ANTIOXIDANT CAPACITY

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ABSTRACT

Walnut Protein Concentrate (WPC) showed eleven bands. WPC at pH 3.0 to pH 6.0 and gastrointestinal digests of WPC showed antioxidant activity using the FRAP, ABTS and ORAC methods. WPC at pH 6.0 showed the highest value using ORAC, 730 μmoL TE/ g protein. TBARS *in vitro* and *in vivo* methods were used to evaluate lipid peroxidation of WPCs and their digests. In vitro TBARS of WPC at pHs 3.0 to pH 6.0 presented values between 84.28% to 86.61% TBARS respectively. *In vivo* TBARS of WPC at pHs 3.0 to pH 6.0 presented values between 65.97% to 72.23% TBARS.

Keywords: walnut, Juglans neotropica Diels, antioxidant activity, walnut protein concentrate, inhibition of lipid peroxidation, zebrafish larvae

1. INTRODUCTION

Food proteins from animal and plants play an important role in human nutrition, but vegetable protein that are the big important, especially in developing countries such Ecuador where protein intake is less than the recommended dose for the Food and Agriculture Organization of the United Nations (FAO). Genus Juglans has around 23 species distributed in different places such as North, Central and South America, Eastern Europe and Asia (MUZAFFER *et al.*, 2018). In the world exist two Junglas species with important economic for the industry (BEIKI *et al.*, 2018). They are the Persian walnut (*Juglans regia*) appreciated for the nutritional and biological value of their nuts (walnuts) and the black walnut (*Juglans nigra*) appreciated for the resistance of its timber (CARRILLO *et al.*, 2017a). In South America, there are distributed other species of genus Juglans know it such Argentinian walnut (*Juglans australis*), Bolivian walnut (*Juglans boliviana*) and black cedar (*Juglans neotropica* Diels) is native crop in Colombia, Venezuela, Peru and Ecuador and known as walnut (Tocte) (VILCACUNDO *et al.*, 2018). It is known that a living system possesses several natural defense mechanisms, such as antioxidants and enzymes. Oxidative stress can be produced for the imbalance of oxidant agents between antioxidant agent homeostasis still leads to the generation of reactive oxygen species (ROS), which cause extensive damage to biomolecules such as lipids, proteins and nucleic acids (TIAN *et al.*, 2011). Particularly, the brain is sensible organ to oxidative stress damage because of its high-energy use, high metabolic demands, high cellular contents of lipids and protein, and low levels of endogenous scavengers (GAO *et al.*, 2012).

Lipid peroxidation in the organism human has been thought to be a toxicological process leading to various diseases such Alzheimer's, cancer and diabetes. Malondialdehyde (MDA) is a product formed from lipid peroxidation of unsaturated lipids that is reacts with thiobarbituric acid (TBA) to produce pink MDA-TBA stable adduct which can be measured at 532 nm (CARRILLO *et al.*, 2017). Linoleic acid is used to determine in vitro lipid peroxidation with TBARS method because is sensible at oxidation process. Lipid peroxidation process is important for the food industry because they need to conserve the vegetables oils, food processed like snacks, biscuits and bakery products with vegetable oils in their formulation are conserved using butylated hydroxyanisole (BHA), butylate hydroxytoluene (BHT), these chemical additives have high cytotoxicity (CACHO *et al.*, 2016). Canada, United States, South Africa, and South America (Ecuador) are some of the countries that permit the use of BHT such additive in foods processed.

Food proteins can be reduced at small fragments named peptides, these bioactive peptides can have most activity than precursor protein. Enzymatic hydrolysates can present more activity that the pattern protein. Different hydrolysates obtained of food proteins have been described with different biological activities such as anti-inflammatory, antimicrobial, antinociceptive, antiproliferative, antiviral, anti-ulcerative, antihypertensive and antioxidant activities (ANADÓN *et al*, 2010; CARRILLO *et al.*, 2014; CARRILLO *et al.*, 2016a; 2016b; CARRILLO *et al.*, 2018a, b; CARDENAS *et al.*, 2018; QUINTEROS *et al.*, 2016; RODÍGUEZ SAINT-JEAN *et al.*, 2013).

The aim of this work was to obtain Walnut Protein Concentrates (WPC) of *Juglans neotropica* Diels to evaluate the WPCs *in vitro* and *in vivo* antioxidant activity and the WPC gastrointestinal digests antioxidant activity.

2. MATERIALS AND METHODS

2.1. Materials

Walnut seeds (*Juglans neotropica* Diels) were obtained of crop from Otavalo, Ecuador. WPC Pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232-468-9), porcine bile extract, fluorescein disodium (FL), were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2′-azobis (2-methylpropionamide)-dihydrochloride (AAPH) and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich (Milwaukee, WI, USA). The rest of chemicals used were of HPLC grade.

2.2. Walnut Protein Concentrate (WPC)

WPC was prepared according to the process described by VILCACUNDO, MARTÍNEZ-VILLALUENGA and HERNÁNDEZ-LEDESMA, (2017b). Defatted walnut flour (DTF) was suspended in water (1:10 w/v) and was adjusted to pH 8.0 with 1 M NaOH. The slurry was centrifuged at 5,000 x g for 30 min at 25°C. The insoluble walnut protein pellet was reslurried with pH adjusted distilled water and centrifuged again. The supernatants mixed together were adjusted at pH 3.0; pH 4.0; pH 5.0 and pH 6.0 and kept for 48 h at 4°C with 1 M HCl and subsequently centrifuged at 5,000 x g for 30 min. The precipitate was neutralized at pH 7.0 with 1 M NaOH, the samples were dialyzed with a membrane of MWCO of 5 KDa Spectra/ Por (Spectrum Labs, USA) to remove residual salts and phenolic compounds, then lyophilized. Protein content was determined with the Dumas method (JUNG *et al.*, 2003).

2.3. *In vitro* gastrointestinal digestion of WPC

The digestive process (10 mg/mL of WPC at pH 5.0) was carried out according to MINEKUS *et al.* (2014). The environment of the stomach and duodenum was reproduced as closely as possible. This study uses harmonized model of in vitro digestion protocol developed by the INFOGEST Cost Action
Gastric digestion: it was used pepsin porcine (Sigma Chemical, Saint Louis, MO, USA) in a concentration of 2,000 U/mL of protein at pH 3.0 in Simulated Gastric Fluid (SGF) (0.35 M NaCl), for 2 h at 37 °C with agitation. The pepsin reaction was stopped with heat at 90 °C, for 10 min.
Duodenal digestion: it was used pancreatin (Sigma Chemical, Saint Louis, MO, USA) in a concentration of 100 U/mL of protein in Simulated Intestinal Fluid (SIF). The composition is a monobasic potassium phosphate solution of pancreatin with bile salt (10 mM) and CaCl2 1.5 M was added. 1 mL of gastric digestion at pH 3.0 was mixed with 1 mL of solution of pancreatin in medium SIF for 3 h at 37 °C with agitation. The reaction was stopped with heat at 90 °C, for 10 min.

2.4. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE electrophoresis of WPC was made using 12% polyacrylamide gels in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue G-250 for 16 h. Relative molecular masses of protein were determined by a comparison to molecular weight markers (10 kDa -250 kDa) (Bio-Rad, Hercules, CA, USA) (ACOSTA *et al.*, 2016; POVEDA *et al.*, 2016).

2.5. Total polyphenols content (TPC)

TPC was determined according to LARA *et al.* (2017). Extraction was made using a solution of 70% (v/v) acetone under magnetic stirring for 45 min, using 0.3-1.0g of dried sample. Mix was centrifuged for 10 min at 3500 rpm and supernatant (raw extract) was recovered. TPC (only a fraction) and the water-soluble compounds (B fraction) were evaluated from the raw extract. A fraction, at 25, 50 and 75 μL of the raw extract in 500 mL of methanol. 500 μL of raw extract were diluted in 3.5 mL of distilled water, 2 mL aliquot of this solution was injected. A and B fractions were measured using the Folin-Ciocalteu method with a UV-VIS spectrophotometer Shimadzu 2200 (Kioto, Japan) at 760 nm, using a standard curve of gallic acid. TPC was expressed as the difference between the A and B fractions as mg of gallic acid equivalents GAE/100g of the weight sample. After extraction of TPC in all WPC samples assayed, TPC were lyophilized and used to evaluate their antioxidant activity with different methods (FRAP, ABTS, ORAC and TBARS methods).

2.6. Radical Scavenging Activity ABTS

ABTS radical scavenging activity was assayed according to the method of BENJAKUL *et al.* (2012). The stock solutions included a 7.4 mM ABTS solution and a 2.6 mM potassium persulphate (K_2SO_8) solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 50 mL of methanol to obtain an absorbance of 1.1±0.02 units at 734 nm using a spectrophotometer (Thermo Fisher Scientific Evolution 200 UV/Vis, Waltham, MA USA). A fresh ABTS solution was prepared for all assays. The sample (150 µL) was mixed with 2.850 µL of ABTS reactive and the mixture was left at room temperature for 2 h in the dark. The blank sample was prepared in the same manner except that methanol was used instead of ABTS. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was calculated after the blank sample subtraction and was expressed as µmoL Trolox equivalents (TE)/ g WPC (VILCACUNDO *et al.*, 2018)

2.7. Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay

The reaction was made at 40°C in 75 mM phosphate buffer (pH 7.4). The final solution mixture (200 mL) contained fluorescein (FL) (70 nM), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) 14 mM, and antioxidant [Trolox (0.2-1.6 nmoL) or samples of the walnut proteins (at different concentrations). The fluorescence was measured for 137 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The instrument was controlled by the FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox solutions were prepared for each assay and FL was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in triplicate and at least three independent runs were performed for each sample. ORAC-FL values were expressed as μmoL of Trolox equivalent (TE)/ g of protein (VILCACUNDO *et al.*, 2018).

2.8. Ferric-reducing antioxidant power assay (FRAP)

1 mL of samples were previously diluted (1:2 and 1:4) and was mixed with 2.5mL of buffer phosphate pH 6.6 and 2.5 mL of the potassium ferrocyanide solution 1% (w:v). The mixture was incubated at 50 °C for 20min. After incubation, 2.5mL of trichloroacetic acid

at 10% (w:v), 2.5 mL distilled water and 0.5 mL of ferric chloride at 1% (w:v), were added. The solution was stained for 30 min at room temperature (BENZIE & STRAIN, 1996).
The activity was measured at 700 nm by UV-VIS spectrophotometry (Shimadzu Spectrophotometer model 2600, Kyoto, Japan) versus a standard Trolox curve. The results were reported as μmoL Trolox Equivalents (TE)/ g sample.

2.9. *In vitro* thiobarbituric acid reactive substances (TBARS)

WPC obtained at pH 3.0; pH 4.0; pH 50 and pH 6.0 were used to evaluate the inhibition of lipid peroxidation (CARRILLO, GUZMÁN and VILCACUNDO *et al.*, 2017b). 0.5 g of linoleic acid was oxidized by heat at 65°C for 8 days. WPCs were added to obtain concentrations of 2.5 mg/mL, being incubated at 30°C for 48 h. Butylhydroxytoluene BHT was used as positive control at concentrations of 0.1 mg/mL of BHT. Distilled water was used as negative control. One milliliter of sample was mixed with one milliliter of the 1% thiobarbituric acid (TBA). The solution was heated at 95°C for 1 h, and cooled down for 15 min. Then, absorbance of the final solution containing WPC and digests was measured at 532 nm using a spectrophotometer (Thermo Scientific Evolution 200). The decrease of absorbance indicates an increase of antioxidant activity.
The values TBARS inhibition were expressed as the percentage of inhibition of lipid peroxidation as follows: % Inhibition of lipid peroxidation = As/Ab x 100, where Ab is the absorbance of blank and As is the absorbance of the sample.

2.10. *In vivo* thiobarbituric acid reactive substances (TBARS)

The TBARS method was used as described by CARRILLO *et al.* (2016a). The group of animals was fed three times a day with food chips for fish. Adult zebrafish were kept for 16 h light and 10 h dark cycles. Embryos were obtained by photo-induced spawning over green plants and cultured at 28°C in a fish tank water. 5 days post fecundation (dpf) larvae were then incubated in 24-well plates, 30 larvae per well, with 2.5 mg/mL of walnut proteins in each well. Lipid peroxidation was initiated by adding 1 mL 1.5% of ethanol and incubated for 8 h at 28°C. Groups of 30 larvae/well in aquarium water were used as controls. BHT was used as positive control and distilled water was used as negative control. Then, ethanol was removed with a micropipette and 500 μL of Tween 0.1% was added. All groups were mixed and homogenized with a T25 Ultra turrax IKA. One milliliter of the solution TBA at 1% was added and subsequently the solution was heated at 95°C for 1 h, then the sample was cooled down during 15 min. Then, absorbance of the solution of zebrafish larvae and sample was measured at 532 nm. The decrease of absorbance indicates an increase of antioxidant activity (TBARS). The values of inhibition TBARS were expressed as the percentage of inhibition of lipid peroxidation as follows: % Inhibition of lipid peroxidation = As/Ab x 100, where Ab is the absorbance of blank and As is the absorbance of the sample.

2.11. Test of toxicity in the zebrafish eggs and larvae model

The zebrafish colony was established in the laboratory, in an environmental growth or glass aquarium, provided with an internal filter and aerator activated carbon for water oxygenation. The population of animals was fed three times a day with food chips (Tetra S.A, Melle, Germany) for fish. The toxicity assay was based on the OECD guideline chemicals for fish embryo toxicity (FET) test N° 236. Adult fish were kept on 16 h light and 8 h dark cycles. Embryos were obtained by photo-induced spawning over green plants and were cultured at 28 °C in fish tank water. 20 embryos (eggs) were incubated with

WPC and duodenal digests at 2.5 mg/mL for 24 and 48 h. Early larvae post-fertilization Zebrafish were maintained according to KIMMEL *et al.* (1995). Larvae of 5 days post fecundation (dpf) were then incubated in 24-well plates, 30 larvae per well for each sample of WPC at 2.5 mg/mL for 24 and 48 h. The volume of fish tank water was 200 µL/well. After 48 h of treatment, the mortality as well as the morphologic changes were assessed. After their respective times, the percentage of larvae death in each dilution was determined. This percentage was plotted versus time. Groups from 30 larvae/well in aquarium water were used as controls. Stereoscopic microscope images were taken to obtain registration expressing the morphological effects on larvae anatomy, as compared to controls. Eggs and larvae were observed at 24 and 48 h under a stereomicroscope (magnification used in the stereomicroscope for observations was 40× using stereomicroscope Motic SMZ8 with camera Moticam 5 MP (Hong Kong, China).

2.12. Statistical analysis

Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The post hoc analysis was performed by the Tukey test. All tests were considered significant at $P<0.05$. Statistical analysis was performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

3. RESULTS AND DISCUSSION

3.1. SDS-PAGE electrophoresis analysis of WPC

In this study, for the first time, the proteins from walnut were characterized with the SDS-PAGE method. WPC showed eleven bands, out of which three bands showed high expression with molecular weights of 20 kDa, 35 kDa and 50 kDa (Fig. 1A). Three groups of bands were clear with molecular weights ranging from 6.5 kDa to 50 kDa. Those three groups of bands were clearly stained with coomassie at reducing conditions. These results of *Juglans neotropica* Diels were similar to the ones reported by MAO and HUA (2012) to Juglans regia L with molecular weights of 40 kDa, 35 kDa, 23 kDa and 20 kDa. Both species have then a similar profile of proteins obtained with the alkaline extraction isoelectric precipitation method. WPC at pH 5.0 presented the highest expression of proteins, as those proteins were strongly stained with coomassie. WPC obtained at pH 5.0 was subjected at *in vitro* gastrointestinal simulation digestion. In the gastric and intestinal phase all proteins were hydrolyzed totally (Fig. 1B). Proteins from walnut *Juglans neotropica* Diels present high *in vitro* digestibility using pepsin and pancreatin enzymes.

3.2. Protein and total polyphenols content of WPC

Protein content of WPC was determined using the Dumas method. At pH 3.0 WPC present 68.84% of protein content, at pH 4.0 WPC present 65.77% of protein content, at pH 5.0 WPC present 73.13% of protein content and at pH 6.0 WPC present 46.77% of protein content. All WPC present high content of protein, being the best percentage the one obtained at pH 5.0 with 73.13% of protein content. Total phenolic content (TPC) was determined for WPC obtained at pH 3.0; pH 4.0; pH 5.0 and pH 6.0. Low levels of total polyphenols have been detected in WPCs. WPC at pH 3.0 present a value of 25.40± 0.24 of mg GAE/ 100 mg of sample, WPC at pH 4.0 present a value of 32.00± 0.31 mg GAE/ 100 g of sample, WPC at pH 5.0 present a value of 34.51± 0.42 mg GAE/100 g of sample and

WPC at pH 6.0 present a value of 40.00± 0.11 mg GAE/ 100 g of sample. These TPC values are lower when are compared to TPC values of kernels walnut. SLATNAR *et al.* (2015) reported high TPC in kernels walnuts (*Juglans regia* L.) of different varieties, (Adams variety 794.6 mg GAE/ 100 g sample), (Fernette variety 728.7 mg GAE/ 100 g sample), (Fernor variety mg 672.5 mg GAE/ 100 g sample) and (Franquette variety with 1092.5 mg GAE/ 100 g sample). PEREIRA *et al.* (2008) reported TPC in walnuts between 589.0 to 951.0 mg GAE/ 100 g of sample. LABUCKAS *et al.* (2008) reported TPC from different varieties of walnuts (*Juglans regia* L.) with values between 23,000 to 49,000 mg GAE/ 100 g of sample. Residual TPC values of WPCs reported in this study are low than the ones reported for kernels walnuts in the literature. In the preparation of WPC using the alkaline extraction followed by the isoelectric precipitation is possible to remove a great part of TPC. Residual TPC was removed of WPCs with a mix of water and acetone solvent.

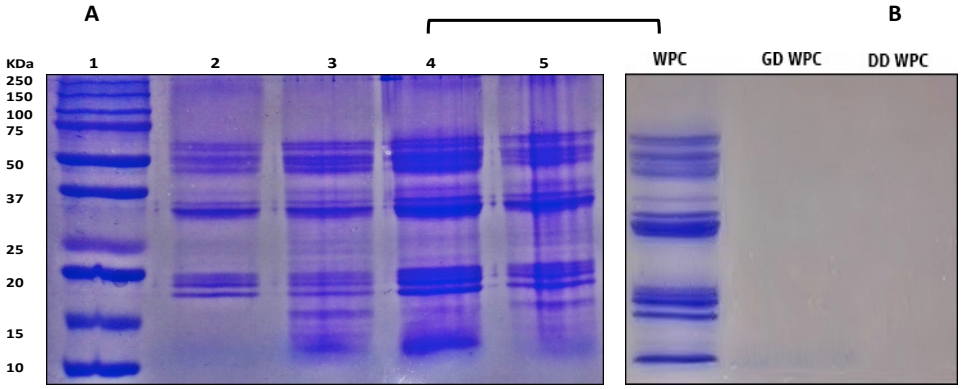

Figure 1. SDS-PAGE electrophoresis analysis of WPC and digests.
A) WPCs at different pHs
Lane 1: molecular weight, lane 2: WPC at pH 3.0, lane 3: WPC at pH 4.0, lane 4: WPC at pH 5.0 and lane 5: WPC at pH 6.0
B) Gastrointestinal digests of WPC
GD WPC: gastric digestion of WPC, DD WPC: gastrointestinal digestion of WPC

3.3. Radical Scavenging Activity ($ABTS^{\cdot}$) of WPC and digests

ABTS radical scavenging activity of different walnut proteins using various pHs of extraction and gastrointestinal digest is presented in Table 1. In general, ABTS radical scavenging activity of WPC was high. In WPC, the extraction pH had an influence on the ABTS radical scavenging activity. Results indicated that WPC obtained at pH 3.0; pH 4.0 and pH 6.0 presented values of 108.2, 99.0 and 74.3 μmoL TE/ g of WPC. These results show then a high activity applying the ABTS radical scavenging method.
WPC obtained at pH 5.0 only presented 33.4 μmoL TE/ g of WPC, being this pH the lowest value (Table 1). The statistical analysis indicated that there are differences between groups of protein isolate at different pHs ($P<0.05$). ABTS (2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) can be oxidized to generate a radical cation, ABTS, that is green in color and can be measured by absorbance at 734 nm. Antioxidants suppress this reaction by electron donation or radical scavenging, thereby inhibiting the formation of the colored ABTS radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation and absorbance at 734 nm (ALCOLEA *et al*., 2002).
WANG *et al*. (2016) reported that walnut protein and hydrolysates of walnut protein from Juglans regia L presented a high antioxidant activity. CHEN *et al*. (2012) reported

hydrolysates and peptides isolate from Juglans regia with high antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. TIRONI and AÑÓN, (2011) reported *Amaranthus mantegazzianus* protein isolates with high antioxidant activity using the ABTS method, the amaranth crop 2006 had a value of IC50 of 93.5 μg protein/mL of sample and the amaranth crop 2007 presented a value of IC50 of 91.3 μg protein/mL in the samples analyzed. ORSINI-DELGADO, TIRONI and AÑÓN, (2011) reported *Amaranthus mantegazzianus* protein isolates at pH 5.0 with high antioxidant activity using the ABTS method with a value IC50 of 10.2 mg protein/ mL of sample.
Gastric digest of WPC present higher ABTS radical scavenging activity than WPCs (Table 1). Gastric digest of WPC presents a value of 224.23±0.29 μmoL TE/ g of WPC. Duodenal digest of WPC presents a value higher than gastric digest of WPC with a value of 315.69±0.54 μmoL TE/ g of WPC. Gastric digest obtained with pepsin and duodenal gastric obtained with pepsin/pancreatin were more active than proteins patterns as peptides increase the antioxidant activity.

3.4. WPC and WPC digests antioxidant activity using the ORAC and FRAP methods

WPC antioxidant activity obtained at pH 3.0, pH 4.0, pH 5.0 and pH 6.0 was evaluated using the ORAC method. Table 1 shows results of antioxidant activity using ORAC method. The best sample was WPC obtained at pH 6.0 with 730.00± μmoL TE/mg of protein. Next best sample was WPC at pH 3.0 with a value of 630.01±0.04 μmoL TE/g of protein (Table 1). ORSINI-DELGADO, TIRONI and AÑÓN, (2011) reported that amaranth protein isolates at pH 5.0 present 0.12 μg TE/μg of sample (crop 2006) and amaranth protein isolate at pH 5.0 present 0.19 μg TE/μg of sample (crop 2007).
WPC gastrointestinal digest obtained with pepsin and pepsin/pancreatin presented antioxidant activity using the ORAC method (Table 1). WPC gastric digest presented a value ORAC of 1084.00±0.26 μmoL TE/g of protein and duodenal digest of 1345.00±0.31 μmoL TE/g of protein. WPC gastric and duodenal digests were more active than WPC without enzymatic hydrolysis.
Antioxidant activity of WPCs and their gastrointestinal digest were evaluated using FRAP method. The best value of WPC was to WPC obtained at pH 6.0 with a value of 165.56 μmoL TE/ g sample. Gastric digest of WPC presents a value of 182.35 μmoL TE/ g sample and duodenal digest of WPC present higher value with 216.29 μmoL TE/ g sample. These results have correlation with results of ORAC method to gastrointestinal digests.

3.5. *In vitro* inhibition of lipid peroxidation of WPC and digests

WPC obtained at pH 3.0, pH 4.0, pH 5.0 and pH 6.0 were used to inhibit the lipid peroxidation of linoleic oil. WPC at pH 3.0 present a value of 84.28% of inhibition TBARS, WPC at pH 4.0 present a value of 83.45% of inhibition TBARS, WPC at pH 5.0 was able of inhibiting lipid peroxidation with a value of 84.33% and WPC at pH 6.0 presented the higher percentage with a value of 86.61% of inhibition TBARS. CARRILLO, GUZMÁN and VILCACUNDO (2017b) reported native casein hydrolysate with a value of 55.55% of inhibition TBARS, heated casein hydrolysate with a value of 58.00% of inhibition TBARS. Whey proteins hydrolysates also were able of inhibiting lipid peroxidation but were less active than casein hydrolysates.
Gastrointestinal digests from WPCs were used to evaluate their capacity to inhibit lipid peroxidation in the in vitro model. WPC gastric digest presents a percentage of inhibition TBARS of 90.59±0.01 % TBARS and WPC duodenal digest presented a percentage of inhibition TBARS of 95.93±0.01 % TBARS both hydrolysates were more active than WPCs proteins patterns assayed in this study.

3.6. *In vivo* inhibition of lipid peroxidation

The values of inhibition of lipid peroxidation indicated that all WPCs and hydrolysates were efficient to inhibit lipid peroxidation in the zebrafish larvae (Table 1).

Table 1. Antioxidant activity of WPCs and their gastrointestinal hydrolysates using FRAP, ABTS, ORAC and TBARS methods.

Sample	TPC (mg GAE/100g sample)±SD	FRAP (μmoL TE/g sample)±SD	ABTS (μmoL TE/g sample)±SD	ORAC (μmoL TE/g protein)±SD	% TBARS *In vitro*±SD	% TBARS *In vivo*±SD
WPC pH 3.0	25.40±0.24^{a}	125.63±0.11^{a}	108.20±0.01^{a}	630.01±0.04^{a}	84.28±0.02^{a}	65.97±0.22^{a}
WPC pH 4.0	32.00±0.31^{b}	136.78±0.36^{a}	99.01±0.03^{a}	290.00±0.00^{b}	83.45±0.02^{b}	68.36±0.44b
WPC pH 5.0	34.51±0.42^{c}	138.94±0.51^{a}	33.40±0.02^{b}	280.00±0.02^{b}	84.33±0.03^{a}	68.00±0.35c
WPC pH 6.0	40.00±0.11^{d}	165.56±0.44^{a}	74.30±0.00^{c}	730.02±0.03^{c}	86.61±0.01^{c}	75.23±0.21d
BHT					94.12±0.01^{d}	92.65±0.01^{d}
GD WPC		182.35±0.31^{d}	224.23±0.29^{d}	1084.00±0.26^{d}	90.59±0.01^{d}	80.23±0.01^{d}
DD WPC		216.29±0.17^{d}	315.69±0.54^{d}	1345.00±0.31^{d}	95.93±0.01^{d}	84.57±0.01^{d}

Results represent the average of three determinations±SD. Data analyzed by one-way ANOVA and followed by Tuckey´s test.
Different letter represents significant differences between sample as $P<0.05$ (n=3).
BHT (Butylhydroxytoluene), GD WPC (gastric digests of WPC) and DD WPC (duodenal digests of WPC).

The values of inhibition of lipid peroxidation ranged from 65.97% to 84.57%. These values were high when compared to the BHT positive control. WPCs and their hydrolysates were highly efficient to inhibit lipid peroxidation using this *in vivo* animal model. As an example, WPC obtained at pH 6.0 presented a result of 75.23% TBARS inhibition at a concentration of 2.5 mg/mL, this activity was high compared to the activity presented in the positive control (BHT) at 0.1 mg/mL with a value of 94.12% of inhibition lipid peroxidation (Table 1). When we compared the results *in vitro* TBARS with *in vivo* TBARS, we can observe that in vitro TBARS percentages of all samples assayed were higher than *in vivo* TBARS. On the other hand, gastrointestinal digests from WPCs were used to determine their capacity to inhibit lipid peroxidation in the zebrafish larvae model. Gastric digest was able of inhibiting lipid peroxidation in zebrafish larvae with a value of 80.23±0.01 % TBARS and duodenal digests from WPC presented a value of 84.57±0.01 % TBARS. Both hydrolysates were less active than TBARS in the *in vitro* model. Both hydrolysates were more active than WPCs without hydrolysis. Zebrafish larvae is a complex model because of the content of *in vivo* metabolism of larvae. VILCACUNDO *et al*. (2017a) reported gastric and duodenal hydrolysates from quinoa (*Chenopodium quinoa* Willd) with capacity to inhibit lipid peroxidation in the zebrafish larvae. Gastric digestion at pH 1.2 present a value of 75.12% and duodenal digestion 82.10% of inhibition TBARS in the zebrafish larvae model. CARRILLO, TUBÓN and VILCACUNDO, (2016c) reported hydrolysates from hen egg white lysozyme with a reduction of lipid peroxidation in the zebrafish larvae model. Hydrolysate of commercial isolate lysozyme at pH 1.2 present a value of 82.0% and 84.0% of inhibition of lipid peroxidation in zebrafish larvae. Lysozyme without hydrolysis only presented 21% and 23% of inhibition TBARS in zebrafish larvae. CARRILLO, GUZMÁN and VILCACUNDO, (2017b) reported native and heated hydrolysates from cow milk with capacity to inhibit lipid peroxidation *in vivo* in the

zebrafish model. Previous studies have reported the ability of peptides derived from different food sources to inhibit lipid peroxidation. Even though the exact mechanism of peptides to act as antioxidant is not clearly known, some aromatic amino acids and histidine are reported to play a vital role in this activity (MOURE, DOMÍNGUEZ and PAJARÓ, 2006; QIAN, JUNG and KIM, 2008). To determine oxidative stress, lipid peroxidation inhibition in the zebrafish larvae model was used to determine cellular damage *in vivo*. Figure 2 presents WPC cytotoxicity assay at a concentration of 2.5 mg/mL in zebrafish eggs and larvae for 24 and 48 h of incubation. This assay confirmed that WPC were not toxic for zebrafish embryos. The percentage of survival of WPC and gastrointestinal digest was 95% of survival after 48 h of incubation. Zebrafish embryos presented a normal aspect after 48 hours of assay. Zebrafish embryos presented normal movements and normal development of body embryos. Head, tail, heart, heartbeat and circulation were normal. Zebrafish eggs presented no coagulation after 48 h of incubation with the samples (Fig. 2). Finally, zebrafish embryos could hatch and go into the larval stage.

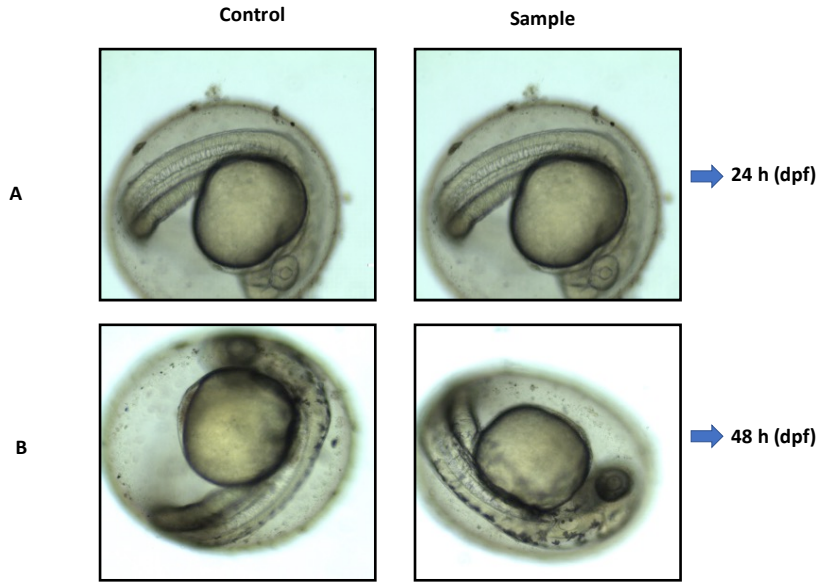

Figure 2. Micrographs of zebrafish eggs incubated with WPC and duodenal digest.
A) Zebrafish eggs incubated with 2.5 mg/mL of WPC at pH6.0 for 24 h and
B) Zebrafish eggs incubated with 2.5 mg/mL of duodenal digest for 48 h.
Control (zebrafish eggs without sample).

When zebrafish larvae were examined, no morphological abnormalities were shown such as crooked bodies, spinal deformities or any other significant effects in the growth of the body (Fig. 3).
This study showed the antioxidant activity of WPC (*Juglans neotropica* Diels) cultivated in Ecuador. This work shows for the first time, the capacity of WPC to inhibit lipid peroxidation in the zebrafish larvae model. Proteins from Walnut (*Juglans neotropica* Diels) are a good source of bioactive components, which can be used in functional foods and in medical components. The hydrolysates obtained from Walnut (*Juglans neotropica* Diels) proteins can be used as functional ingredients. Peptides present in these hydrolysates can be identified and evaluated to be used as functional food ingredient. Also, proteins from walnut can be used to reduce the lipid oxidation in the food industry.

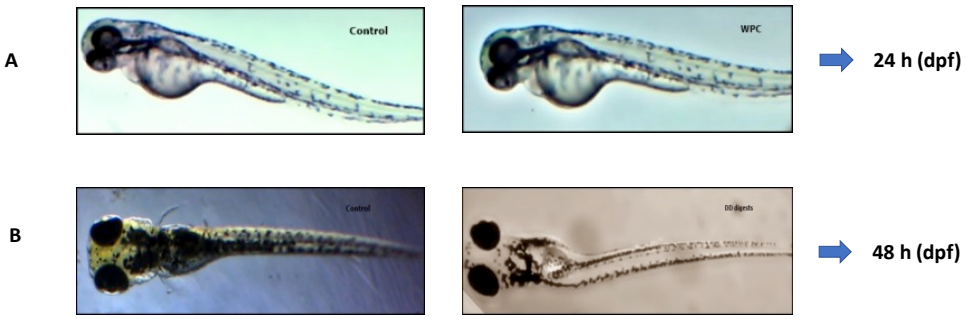

Figure 3. Micrographs of zebrafish larvae incubated with WPC and duodenal digest.
A) Zebrafish larvae incubated with 2.5 mg/mL of WPC at pH6.0 for 24 h and
B) Zebrafish larvae incubated with 2.5 mg/mL of duodenal digest for 48 h.
Control (zebrafish larvae without sample).

4. CONCLUSIONS

This study shows the WPC, (*Juglans neotropica* Diels), antioxidant activity. WPC proteins have a high digestibility under *in vitro* gastrointestinal simulation digestion, using pepsin and pancreatin. WPC gastrointestinal digest present high antioxidant activity. This work shows for the first time, the capacity of WPC and WPC digests to inhibit lipid peroxidation in the zebrafish larvae model. Walnut (*Juglans neotropica* Diels) proteins can be a good source of bioactive components, which can be used in functional foods and in medical components. Walnut proteins can be used to reduce the lipid oxidation in the food industry.

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DRYING KINETICS OF WHEAT, BARLEY AND MAIZE GRAIN

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ABSTRACT

Drying of wheat, barley, and maize grains was studied at 40, 50 and 60°C at an air velocity of 1.1 m/s. When the equilibrium humidity was reached, the physicochemical characteristics were evaluated. The moisture data were adjusted to the mathematical models proposed by Crank and Omoto, which resulted in accurate predictions through the correlation coefficient. In both models, the temperature dependence of the effective diffusivity followed an Arrhenius relationship. Comparisons of the experimental values with the calculated values demonstrated that the two models representing an excellent tool for estimation of the drying parameters, which aim to maintain the quality grain.

Keywords: drying process, effective diffusion, Crank model, Omoto model, Arrhenius

1. INTRODUCTION

In 2017, Ecuador produces over 1.2 million metrics tons of maize, 6746 metrics tons of wheat and 24700 metric tons of barley (TELEGRAFO, 2016, LIDERES, 2018). It is estimated that the annual losses in grain harvesting are approximately 10-25% and are significantly high in developing countries (DALPASQUALE *et al*., 1991, BALA, 2016). The large accumulation of wet grains requires very careful handling to avoid quality deterioration and losses. Grains cannot be stored if they are not dry and is important to determine when the grain is considered as dry. In general, moisture content of 15% could be the limit of dryness. In a country with a cold climate, cereals such as wheat can be stored at 15% humidity, but in a country with a warm climate, the storage humidity should be 12 - 13%. The oleaginous grains must be stored at even lower humidity, 10 to 11%, and even lower (DALPASQUALE *et al*., 1991). Grain drying is an important operation to avoid postharvest losses. During the drying process, some grain losses occur, such as breakage and dust production, which are not usually very high. The most important loss is the overdraft. But over-drying has another extra cost: the higher energy consumption, which increases in greater proportion the lower final moisture content (DALPASQUALE *et al*., 1991). Hence, it is difficult to interpret the data reported in the drying studies regarding the effects of heat and moisture on grain quality characteristics (CETINER *et al*., 2017; SOKHANSANJ *et al*., 1984). The selection of the correct drying technique requires the knowledge of competent specialized personnel, to minimize inconveniences.
The drying of cereal grains has been studied for many years. Grain drying is a nonlinear process with a long delay (LIU *et al*., 2006). Empirical correlations for the prediction of drying rates have been performed, to predict drying behavior and the variables which control the dehydration of thin layer (monolayer) grains (AGUERRE *et al*., 1982). Producing the grain with equilibrium moisture content is an important process control objective. In general, the mechanism of moisture movement in grain drying follows the Fick's law. Moisture transport mechanism governed by a moisture gradient and interpreted mathematically by a Fick's-type law. However, Fick's law commonly used in food and chemical engineering applications might not be fully accurate for modeling fluid transport near glass transition (VILLENEUVE and GÉLINAS, 2007). The most common method used to calculate drying diffusion being the solution of Fick's second law, assuming uniform initial moisture content distribution and negligible external resistance, proposed by Crank (LIU *et al*., 2012).

Mathematical modelling
Crank model

The drying kinetics has been empirically predicted using a first order approach described by Eq. (1)

$$-\frac{dM}{dt} = k(M - M_e) \quad (1)$$

Where, M is the material moisture content (dry basis) during drying (kg water/kg dry solids), Me, is the equilibrium moisture content of dehydrated material (kg water/kg dry solids), k is the drying rate (min^{-1}), and t is the time of drying (min). The drying rate is determined as the slope of the falling rate-drying curve.

The solution of this first order equation, using appropriate initial and equilibrium conditions, is the following Eq. (2):

$$MR = \frac{M-M_e}{M_0-M_e} = C\ exp(-kt) \quad (2)$$

Equation 2, suggested by Lewis in 1921 (SOKHANSANJ and GENKOWSKI, 1988; JAYAS *et al*., 1991; MARINOS-KOURIS and MAROULIS, 1995) is applied for porous hygroscopic materials which are dried in the falling rate period. It is assumed that the layer of the drying material is quite thin, so that the conditions of the drying air (temperature and humidity) are kept constant throughout the material (KARATHANOS, 1999).
Diffusion of water in a sphere can be represented by Eq. (3).

$$\frac{dM}{dt} = D(\frac{\partial^2}{\partial r^2} + \frac{2\partial M}{r\partial r}) \quad (3)$$

Where D is independent of the unit and has dimensions length^2 time^{-1}, r is the radial coordinate (m).
The general solution of Fick's second law in spherical coordinates was given by Crank in 1975 (Equation 4) (SOARES *et al*., 2016; SHIVHARE *et al*., 1994).

$$MR = \frac{M-Me}{Mo-Me} = \frac{6}{\pi^2}\exp -(\frac{Deff\ \pi^2 t}{r^2}) \quad (4)$$

Linearizing, Eq. (5):

$$Ln(MR) = -\frac{1}{2} - \frac{Deff\ *\pi 2*t}{r^2} \quad (5)$$

Where, MR is the moisture ratio (dimensionless), M is the moisture content as a function of time (%, d.b), M_0 is the initial moisture content (%, d.b), M_e is the moisture content at saturation or equilibrium moisture (%, d.b), D_{eff} is the effective diffusivity (m^2/s), t is the time (h), r is the equivalent radius (m).
Temperature is a critical parameter that affects the diffusion of food. The dependence of diffusion on temperature is described by the Arrhenius equation used for the determination of the activation energy (Ea), Eq. (6):

$$D_{eff} = Do\ exp\frac{Ea}{R\ (T+273.15)} \quad (6)$$

Where D_{eff} is the effective diffusivity coefficient, D_0 diffusivity at the reference temperature, Ea the activation energy (KJ/mol), R the gas constant 8.31451 J/mol.K and T temperature (K) (SOARES *et al*., 2016).
Omoto model
The equation 7 represents the variation of the mass water (ϱAV) in the grain over time (t); A, the grain area and NA, the water mass flow ($Kg/m^2.h^{-1}$) (OMOTO *et al*., 2009).

$$\frac{d\rho AV}{dt} = NA \quad (7)$$

Taking into account that the geometry of the grain is spherical, constant volume and the adjustment of the mass flow NA = Ks (ϱeq-ϱA) the equation 8 is obtained:

$$\frac{d(\rho A)}{dt} = -3\frac{Ks}{r_0}(\rho Aeq - \rho A) \quad (8)$$

Where Ks is the diffusivity coefficient ($m.h^{-1}$); r_o, radius and ϱAeq, the mass of water in equilibrium. The Omoto model was developed for hydration of grains, but including a minus sign in the equation 8, could be adapted to a drying process (SOARES *et al*., 2016).
The aim of this research was the study and the modeling of the drying kinetics during the hot-air drying process of maize, wheat and barley, and the analysis of the influence of temperature on the physicochemical properties and the kinetic constants of the proposed models.

2. MATERIALS AND METHODS

2.1. Materials

Grains of white maize (*Zea mays* L), wheat (*Triticum aestivum* L) and barley (*Hordeum vulgare* L) were collected from crops in the growing season of the second semester of 2017 in Cotopaxi and Tungurahua, Ecuador. They were stored at 8°C ± 1°C to maintain a uniform moisture content until the drying test (MARKOWSKI *et al*., 2010).

2.2. Drying kinetics

The drying experiments were carried out at air temperatures of 40, 50 and 60°C, at ~ 6% relative humidity and 1.1 m/s air velocity in a convective air circulation oven (GANDER MTN CD -160, China). The dryer was run without load for 30 min to stabilize the drying conditions. Then the samples were uniformly spread on the basket as a single layer. Each sample used in the experiment weighed about 300 g. The initial moisture content of maize was about 57% ± 0.2 (w/w), for wheat 12% ± 0.2 (w/w) and barley 14% ± 0.2 (w/w). The amount of water removed during the drying process was recorded at 30-min intervals using a digital balance (Mettler Toledo, model L-EQ-5/10, USA), with an accuracy of ± 0.5 g., until constant weight. The product was cooled and packed in low-density polyethylene (LDPE) bags. The experiments were performed by triplicate, and the average of the moisture content at each value was used for calculating. The drying data from the drying tests were then expressed as moisture ratio (MR) versus drying time.

2.3. Physicochemical properties

Moisture content was measured following the method ISO 7971-1:2009 section 2 for maize (ISO 2009a), and ISO 712:2009 for wheat and barley (ISO 2009). The mass of 1000 grains (P1000) was evaluated by ISO 520:2010 method (CETINER *et al*., 2017) and expressed as dry basis. For the measurement of grain size, a Vernier calibrator was used (length and width) in 50 grains of each sample. For the determination of the specific weight and protein content a NIR grain analyzer (PERTEN Inframatic 9500, USA) was used as was described in the ISO 16634-2:2009b Dumas method (MÜLLER, 2017). The water activity measurements were performed with an Aqualab meter (aquaLab, 4TE, USA). All the analysis were performed by triplicate.

2.4. Statistical analysis

For the physicochemical properties, the statistical analyses were performed using GraphPad Prism software (GraphPad, USA). An analysis of variance (ANOVA) including

post hoc comparison Tukey's test, at the probability level $\alpha = 0.05$, to determine significant differences was performed.

3. RESULTS AND DISCUSSION

3.1. Drying kinetics

Figure 1 shows the drying kinetics of maize, wheat, and barley. It can be observed that the higher air temperature, a short time is needed to reach the equilibrium moisture. This fact is related to the air temperature which favors an increase in water evaporation in the grain, and consequently, the loss of humidity becomes faster (VÁZQUEZ-CHÁVEZ and VIZCARRA-MENDOZA, 2008). When the drying air contains less moisture, the absorption of the water contained in the grain will be greater, and therefore the drying process will take less time (LARROSA *et al*., 2016). In each graph, the data exhibit a slope reduction with time. For instance, the time required to reduce the moisture content of maize until 0.35 were about 12, 8 and 6 h for air temperatures of 40, 50 and 60°C, respectively. Other studies reach a moisture content of 0.35 between 1 to 3 h, the differences are related to the initial moisture content of the grain (GINER and MASCHERONI, 2002).
The maize sample reached the equilibrium moisture of 0.102 ± 0.009 at 60°C, 0.082 ± 0.005 at 50°C and 0.135 ± 0.009 at 40°C after 24, 25 and 32 hours respectively (Figure 1A). In wheat samples: 0.053 ± 0.001 at 60°C; 0.073 ± 0.002 at 50°C and 0.061 ± 0.001 at 40°C at 9, 9.5 and 27 hours respectively (Figure 1B). Finally, in the barley samples the equilibrium moisture was 0.054 ± 0.002; 0.069 ± 0.002 and 0.062 ± 0.002 at 60°C, 50°C and 40°C respectively, at 7; 10.5 and 25 hours respectively (Figure 1C). In all cases, that raising the temperature from 40 to 60°C increased the specific drying rate at the beginning of drying. As drying progressed, the drying rates approached closer to each other towards attaining the equilibrium moisture content irrespective of the drying temperatures (GHOSH *et al*., 2007).

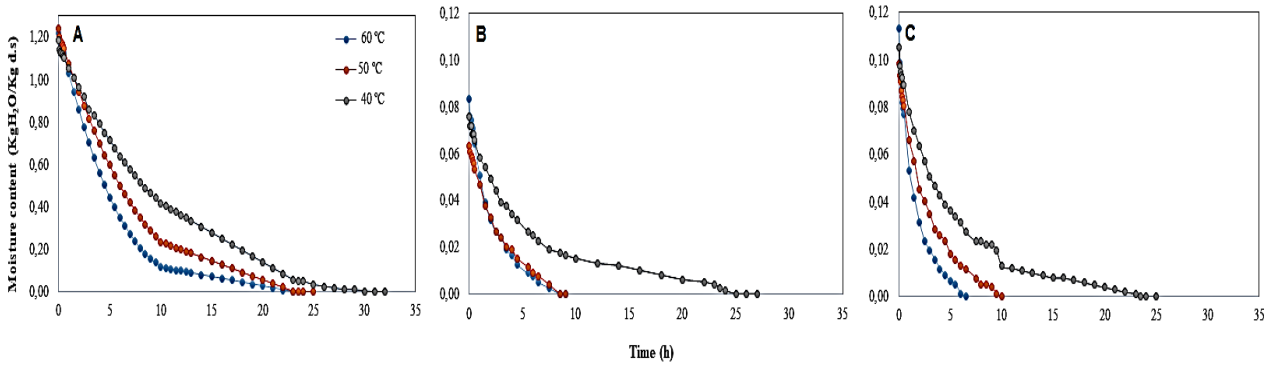

Figure 1. Drying kinetics of maize (A), wheat (B) and barley (C) for the drying process at 40, 50 and 60°C.

3.2. Drying speed curves

As was expected, the drying rate increased (Fig. 2). The surface temperature increase until reaching the dryness state in the grains (SINGH and HELDMAN, 2009). In maize, the

constant speed period was observed but not detected in wheat and barley. It could be related to the diffusion process, which allows a fast movement of moisture from inside the grain, therefore is very small and can be disregarded (ALVARADO, 1996). Drying of most foods is defined only by the falling rate period (KIRANOUDIS *et al*., 1992). Similar results were obtained in other foods matrices such as garlic (MADAMBA *et al*., 1996), red chili (GUPTA *et al*., 2002), kiwi (SIMAL *et al*., 2005) and figs (BABALIS and BELESSIOTIS, 2004).

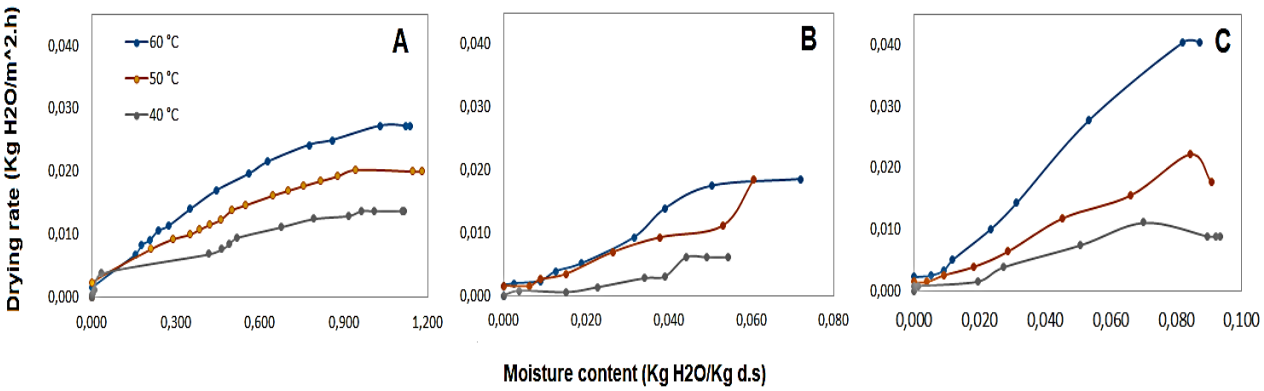

Figure 2. Drying speed curves for wheat (A), maize (B) and barley (C) at 40, 50 and 60°C.

3.3. Drying mathematical models

Related to the selected models, Crank and Omoto, show a great fit. More than 85% of the data were adjustable and gave similar R2 values greater than 0.90 (Table 1). The experimental behavior was like the red pepper drying (VEGA-GÁLVEZ *et al*., 2009). Graph ln (MR) versus drying time (t) was plotted and equations expressed in Table 1.

Table 1. Model equations and coefficient of correlation (R2) at 40, 50 and 60°C drying temperature for maize, wheat, and barley.

Sample	Model	Temperature (°C)					
		40		50		60	
Maize	Crank	Ln (MR) = -0,1507t + 0,2976	R^2 = 0,9104	Ln (MR) = -0,1574t + 0,0278	R^2 = 0,9832	Ln (MR) = -0,1992t - 0,0372	R^2 = 0,983
	Omoto	Ln (ɵ) = -0,1507t + 0,2976	R^2 = 0,9104	Ln (ɵ) = -0,1574t + 0,0278	R^2 = 0,9832	Ln (ɵ) = -0,1992t - 0,0372	R^2 = 0,983
Wheat	Crank	Ln (MR) = -0,1255t - 0,1919	R^2 = 0,9743	Ln (MR) = -0,3383t + 0,0275	R^2 = 0,9881	Ln (MR) = -0,4238t - 0,0311	R^2 = 0,9915
	Omoto	Ln (ɵ) = -0,1255t - 0,1919	R^2 = 0,9743	Ln (ɵ) = -0,3383t + 0,0275	R^2 = 0,9881	Ln (ɵ) = -0,4238t - 0,0311	R^2 = 0,9915
Barley	Crank	Ln (MR) = -0,1653t - 0,1564	R^2 = 0,985	Ln (MR) = -0,343t - 0,0187	R^2 = 0,996	Ln (MR) = -0,5421t - 0,1282	R^2 = 0,9971
	Omoto	Ln (ɵ) = -0,1653t - 0,1564	R^2 = 0,985	Ln (ɵ) = -0,343t - 0,0187	R^2 = 0,996	Ln (ɵ) = -0,5421t - 0,1282	R^2 = 0,9971

The effective diffusivities for different drying temperature are presented in Table 2. They were obtained by nonlinear regression of the mathematical model for the three drying temperatures. The values of effective diffusivity increased with increasing drying temperature. This is due to that a higher drying temperature, and lower relative humidity promotes a greater moisture concentration gradient between the surface and interior structure of the grains. In all cases, drying at 60°C gave the highest effective diffusivity values. The obtained results are in good agreement with values published previously. The D_{eff} for cereal drying found in the literature are in the range from 10^{-13} to 10^{-8} m^2/s. This great variation is ascribed mainly to the type of grain, initial and final moisture content and drying conditions applied. DE OLIVEIRA *et al* [14] reported values between 1.54×10^{-13} to 4.85×10^{-13} m^2/s for maize drying from 40 to 100°C. JANAS *et al*., (2010), found a D_{eff} of 7.14×10^{-11} for maize during fluidized-bed drying at 50°C. The effective moisture diffusivity for barley ranged between 9.56×10^{-11} to 1.56×10^{-10} m^2/s close to 1.99×10^{-11} and 5.31×10^{-11} m^2/s, and $2.20 - 4.52 \times 10^{-11}$ m^2/s and $3.04 - 4.79 \times 10^{-11}$ m^2/s for barley dried in a spouted-bed and in an IR-convection dryer, respectively (MARKOWSKI, 2010). For the wheat, the D_{eff} values ranged from 7.84×10^{-11} to 2.46×10^{-11} m^2/s similar to the 2.97×10^{-11} m^2/s (GASTÓN, 2004; GILI *et al*., 2018) in the order of 10^{-11} m^2/s (GASTÓN, 2002). The difference in the estimated diffusion coefficients obtained in the present work may be due to the different approaches taken to solve the drying model.

Respect to the effective diffusivity (Ks) obtained with the Omoto model, in all cases, the values of Ks increased with increasing drying temperature as was observed with the Crank model. For barley were obtained values of 1.68×10^{-4} at 40°C and 6.06×10^{-4} at 60°C, similar to 1.07×10^{-4} at 40°C and 1.81 10^{-4} at 60°C calculated by SOARES (2016). However, no results were found in the scientific literature for wheat and maize.

Table 2. Estimated diffusivity coefficient from Crank and Omoto models and coefficient of correlation (R^2).

Drying temperature (°C)	Crank model D_{eff} (m^2/s)					
	Maize	R^2	Wheat	R^2	Barley	R^2
40	1.39E-10 ± 0.02E-10a	0.914	2.46E-11 ± 0.03E-11a	0.962	4.30E-11 ± 0.05E-11a	0.985
50	1.63E-10 ± 0.01E-10b	0.902	6.26E-11 ± 0.01E-11b	0.988	9.56E-11 ± 0.04E-11b	0.970
60	1.83E-10 + 0.04E-10c	0.983	7.84E-11 ± 0.01E-11c	0.991	1.56E-10 ± 0.06E-10c	0.966
	Omoto model Ks (m/h)					
	Maize	R^2	Wheat	R^2	Barley	R^2
40	2.87E-04 ± 0.03E-4a	0.910	1.13E-04 ± 0.04E-4a	0.962	1.68E-04 ± 0.06E-4a	0.985
50	3.37E-04 ± 0.02E-4b	0.902	2.89E-04 ± 0.02E-4b	0.988	3.72E-04 ± 0.06E-4b	0.970
60	3.80E-04 ± 0.02E-4c	0.983	3.62E-04 ± 0.01E-4c	0.991	6.06E-04 ± 0.05E-4c	0.966

Different letters (a, b, c) represent significant differences at different drying temperature.

The estimations of the diffusion coefficient obtained were correlated by using an Arrhenius-type relationship. The values of activation energy by Crank (Ea) and Omoto (E) are listed in Table 3. It was found that the Ea of barley was higher than the wheat and maize. The activation energy found for maize of 12.11 kJ/mol, wheat of 50.63 kJ/mol and barley of 55.89 kJ/mol were higher than the values from the literature 17.77, 41.46 and 51.61 kJ/mol for maize, wheat and barley, respectively (ORDOÑEZ *et al*., 2012;

MARKOWSKI *et al*., 2010). Activation energy is related with the work required to remove one mole of moisture from a given material and thus depends on the material–moisture bonding. Ea represents the energy required to break the grain–moisture bonding of a mole water molecule, and to bring the molecule to the surface when will finally evaporate. These values are higher than the 5 kJ/mol for chemically bound moisture (stoichiometric) (STRUMILLO AND KUDRA, 1986). DEBENEDETTI (2003), suggested that hydrogen bond strengths were of the order of 20 kJ/mol, similar to the activation energy found in the present work. In the context of these numbers, an activation energy of 12.11 kJ/mol for maize of, 50.63 kJ/mol for wheat and 55.89 kJ/mol for barley suggests that surface diffusion of water molecules along cellular spaces inside the grain is the actual diffusional process occurring as was explained by BAINI (2007). Similar behavior was observed in E from the Omoto model. However, there are few studies in the literature addressing the determination of this transport property, pointing to the need to expand the studies on drying studies with the Omoto model.

Table 3. Activation energy (Ea, E) values determined using drying constant from Crank and Omoto model, R^2>0.90 in all cases.

Sample	Crank model Ea (KJ/mol)	Omoto model E (K^{-1})
Maize	12.11	1457.10
Wheat	50.63	6089.30
Barley	55.89	6722.70

3.4. Effect of drying on the physicochemical properties

As can be observed in Table 4, the temperature had a significative effect on the physicochemical properties. The decrease in the specific weight is related to the hardness, size and density of the grain. If the temperature increases the grain volume decreases by water extraction (VEGA-GÁLVEZ, DI SCALA *et al*., 2009).

Table 4. Physicochemical properties of maize, wheat and barley at drying temperature of 40, 50 and 60°C.

		Moisture (%)	Water activity	Protein* (%)	Length (cm)	Width (cm)	Specific weight* (Kg/hl)	1000 grains weight*
Maize	Raw sample	56.92 ± 0.31a	0,70 ± 0.01a	n.d	1.28 ± 0.01a	1.07 ± 0.01a	n.d	759.14 ± 13.2a
	40 °C	11.48 ± 0.52b	0.26 ± 0.05b	11.73 ± 0.03a	1.18 ± 0.01b	0.97 ± 0.02b	60.03 ± 0.23a	286.89 ± 7.72b
	50 °C	9.89 ± 0.23c	0.15 ± 0.01c	11.30 ± 0.03b	1.15 ± 0.01c	0.94 ± 0.02c	58.53 ± 0.07b	256.07 ± 2.32c
	60 °C	9.59 ± 0.04d	0.15 ± 0.01c	11.80 ± 0.06a	1.15 ± 0.01c	0.97 ± 0.01b	58.60 ± 0.11b	276.52 ± 1.56d
Wheat	Raw sample	12.02 ± 0.08a	0.56 ± 0.00a	8.17 ± 0.03a	0.67 ± 0.01ab	0.36 ± 0.00a	74.57 ± 0.03a	42.38 ± 0.50a
	40 °C	6.87 ± 0.39b	0.23 ± 0.01b	7.93 ± 0.03b	0.67 ± 0.00a	0.34 ± 0.00b	74.50 ± 0.01b	39.28 ± 0.84b
	50 °C	5.94 ± 0.08c	0.16 ± 0.01c	7.77 ± 0.07c	0.66 ± 0.00b	0.34 ± 0.00b	74.33 ± 0.07c	37.12 ± 1.00c
	60 °C	6.83 ± 0.07d	0.13 ± 0.01d	8.03 ± 0.03d	0.67 ± 0.01ªb	0.36 ± 0.01a	74.40 ± 0.10b	39.18 ± 0.60b
Barley	Raw sample	14.31 ± 0.25a	0.62 ± 0.00a	10.43 ± 0.03a	0.96 ± 0.01a	0.41 ± 0.00a	58.55 ± 0.12a	57.33 ± 0.91a
	40 °C	7.10 ± 0.08b	0.27 ± 0.01b	10.20 ± 0.06b	0.93 ± 0.01b	0.40 ± 0.00b	58.23 ± 0.03b	52.25 ± 1.78b
	50 °C	5.86 ± 0.07c	0.18 ± 0.00c	9.93 ± 0.09c	0.92 ± 0.00b	0.39 ± 0.01b	57.53 ± 0.03c	51.38 ± 0.81b
	60 °C	7.29 ± 0.15d	0.17 ± 0.01c	10.27 ± 0.03b	0.93 ± 0.01b	0.40 ± 0.00b	57.60 ± 0.25d	52.06 0.47b

*expressed as dry basis.

n.d. not detected by the equipment.

Different letters (a, b, c) represent significant differences between the sample at different drying temperature.

Respect to the length and width of the grain, significant differences were observed in maize and wheat. However, no significant differences were obtained in barley by drying; this fact indicates that the temperature did not affect the grain dimension. An increasing relationship was found between 1000 grain mass and moisture content in all samples as was observed by GEZER *et al*. (2002) in apricot.
On the other hand, the protein content shows a slight reduction in each cereal. The drying temperature of 60°C leads to obtaining the highest protein values than 40 or 50°C. It could be due to at high temperatures the drying time is shorter, so the grains will retain more protein content (SOARES *et al*., 2016). As was expected, water activity decreased with reduction in moisture content (CANO-CHAUCA *et al*., 2004). The water activity in the sample dried at 60°C was lower compared with those samples dried at 40°C and 50°C.

4. CONCLUSIONS

The drying temperature of 60°C was the most efficient regarding physicochemical properties and quality of maize, wheat and barley. Both the Crank and Omoto models can be used to describe the drying behavior. The constant drying values for maize were lower than wheat and barley, attributed to the relatively impervious skin of white maize which acts as a barrier to moisture transfer. The diffusivity coefficient for the grains, in the Crank model, varied from 10^{-10} to 10^{-11} m^2/s and, the higher the temperature, the higher the diffusivity. In the Omoto model, Ks values were around 10^{-4} m/h, increased with rising temperature. The study of the drying process of maize, wheat and barley grains is important to optimize the drying process on suitable temperature and time, improving the final quality of the grain. Results obtained from this study were unique and indicated that is necessary to expand the research on drying studies using the Omoto model.

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MICROBIOLOGICAL AND HEAVY METAL RISK IN ALFALFA JUICE (*MEDICAGO SATIVA*), SOLD IN MARKETS

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ABSTRACT

The objective of this study was evaluated the possible health risks associated with the content of heavy metal and microorganisms in the alfalfa (*Medicago sativa* L.) juice. Thirteen samples from 33 different markets of Quito-Ecuador were purchased f and analyzed the commercial quality (soluble solids pH, titulable acidity, relative density and humidity), heavy metal (lead, cadmium, copper, chromium and nickel) and microorganism (*Aerobic mesophiles* and parasites). The results showed that the concentration of lead ranged between 1.40 to 2.49 ppm. These exceeded the maximum limits allowed by CODEX. In addition, in most cases cadmium (0.07 ppm) and copper (0.47 ppm) did not exceed the allowable limits, while chromium (traces) and nickel (0.52 ppm) did not have regulations for their respective comparison. On the other hand, the microbial quality of most of the samples of alfalfa juice showed contamination with mesophilic aerobic microorganism (3.50E+03 UFC/g) and parasites (positive). These finding provide valuable information that can funding food safety decisions, and confirm that the vast majority of vegetables available on Quito's markets present a problem in terms of bacteriological hazards and heavy metal.

Keywords: food safety, mesophilic aerobic, parasites, lead, cadmium

1. INTRODUCTION

Alfalfa (Medicago sativa L.) is a species mostly consumed in the form of grass by animals; however it also presents gastronomic and medicinal uses by humans. This species has been used as antianemic, antirheumatic, hemostaticand other medicinal uses.Its commercialization is common in the form of natural juice or milkshake (RÍOS *et al*., 2007). Alfalfa has a great nutritional value, its leaves have 24 % protein in dry matter, 10 % protein in stems and their roots are nitrogen fixers by symbiosis.
On the other hand, it has been described as a biaccumulative plant, which immobilizes and recovers 80-90 % of the heavy metals in the soil (COYAGO and BONILLA, 2016), mainly lead and cadmium in its roots, which are then transported to the aerial parts. The bioaccumulation of alfalfa makes this species become a vehicle of food contamination.
In addition, with respect to human nutrition, it has been found that the consumption of fresh products carries a high microbiological risk (BEJARANO and CARRILLO, 2007). Pathogens identified as risks on fresh vegetables include *Shigella spp. Listeria monocytogenes*, *Staphylococcus aureus*, *Aeromonas hydrophila* and the spore-formers *Bacillus cereus*, *Clostridium botulinum* and *C. perfringens*. However, the ones implicated in most outbreaks involving fresh fruits and vegetables are Salmonella, Escherichia coli O157:H7 (FAOUR-KLINGBEIL *et al*., 2016). Also, vegetables as alfalfa could participate in the life cycle of important helminths (*Ascaris lumbricoides*, *Fasciola hepatica*) and protozoa (*Entamoeba histolytica* and *Giardia* sp.). The helminth and protozoa infections are among the most common diseases in the world, which can affect the nutritional balance, causing significant complications such as intestinal obstruction and rectal prolapse in humans (CÉLIA et al., 2012).
In general, no bromatological, nutritional and toxicity studies have been found in alfalfa juices used in human food, causing the need to compare certain parameters with regulations applied to juices, pulps, nectars and fruit concentrates (INEN-2337, 2008).
For this reason, the objective of this study was evaluate the possible health risks associated with the content of heavy metal and microorganisms in the consumption of alfalfa juice in the markets of the Metropolitan District of Quito, to raise awareness about food safety.

2. MATERIALS AND METHODS

2.1. Commercial quality

Thirteen samples of randomly selected alfalfa (*Medicago sativa* L.) juice used as an ingredient in milkshakes were purchased from 33 different markets located in the 9 zonal administrations of the Metropolitan District of Quito. The samples were labeled, immediately refrigerated and transferred to the laboratory of Life Sciences of the Salesiana Polytechnic University (Girón-Quito) for the respective analyses. The transit time was 1 to 3 h between the market and the laboratory. The samples from markets were analyzed within 24 h or immediately upon reaching the laboratory.
The bromatological analyses performed in the alfalfa juice were soluble solids, pH, titutalbe acidity, relative density and humidity. The soluble solids (°Brix) were quantified with a Hand-Refractometer RHC-200ATC (Huake, China) as described by AOAC method 932.14 (44.1.04): 2000 (BEMETTI, 2000); the pH with a S40 SevenMulti pHmeter (Mettler Toledo, Belgium); titulable acidity (%) as described by AOAC (Bemetti, 2000a) and INEN (INEN-ISO-2173, 2013); relative density 20°C/ 20°C (%) as described by INEN (INEN-391, 2012); a humidifier HB4 3-S Halogen (Mettler Toledo, Belgium) was used for measure the

humidity and dry matter, while the ash was determined with a muffle Thermo Scientific (Espectrocrom, Ecuador) as described by HARBERS (1998).

2.2. Heavy metal analysis

The 429 samples of alfalfa juice were centrifuged separately by 15 min and the solid phase was separated and dried at 100°C for 24 hours in a KBF 240 air recirculation oven (Binder, Germany). The dried samples were ground in a basic IKA an 11 mill, then stored in glass bottle and hermetically sealed. The sample was treated as described by AOAC-999.10, (2002) with modifications. Approximately, 250 mg of the pulverized sample was mixed in a Teflon digester with 2.5 mL of concentrated nitric acid and 2.5 mL of sulfuric acid. The mixture was digested for 40 min according to specifications of the equipment Varian manual (VARIAN, 1989). The digested mixture was graduated in 25 mL with deionized water.
The calibration curve was built on the same day with concentrations of cadmium, copper, chromium, nickel and lead standards separately from 0.5, 1.0, 2.0 and 4.0 ppm. Quantification was performed on a SpectrAA-55 Atomic Absorption Spectrophotometer (Varian-Agilent Technologies, United States), as described by AOAC-999.10, (2002). The cadmium, copper, chromium, nickel and lead were quantified at 228.8, 324.7, 357.9, 232.0 and 217.0 nm, respectively. All the digested samples were read three times and the concentration expressed in mg/kg dry weight (DW).

2.3. Microbiological and parasitological analysis

The most widely accepted and used techniques are that recommended by WHO for total count of microorganisms in plant materials. According to the methodology of the WHO, 10 g of fresh sample was suspended in 90 mL of sodium chloride-peptone buffer at pH to 7.0. To count total aerobic bacteria's, fresh sample was plated in duplicate and incubated at 30-35°C for 48 h (WHO, 2011).
For the parasitological analysis, dilutions 10^{-1} were made with the extract of the plant and sterile water, centrifuged at 3000 rpm for 5 min and the pellet formed was removed, placed in an Eppendorf tube. After this, in an optical microscope was placed a slide with 0.2 μL of sample and 0.2 μL of violet crystal and the presence of parasites or cysts was observed or not.

2.4. Statistical analysis

Statistical differences were determined by analysis of variance (simple ANOVA). The mean separation was made via a Tukey's test with 0.01 significant differences and correlations by Pearson with 99 % confidence level were employed to estimate the possible significance of the samples. Moreover, Principal Components Analysis (PCA) was applied in order to select the variables that most influence the differences between samples. The INFOSTAT 2009 and STATGRAPHIC CENTURION XVII software were used for statistical analyses.

3. RESULTS AND DISCUSSION

3.1. Measurements performed on foods

In the study of commercial quality, they were considered 33 markets (Table 1) of which 7 did not register the sale of alfalfa juice. Thus, the available samples of alfalfa juice showed

ranges of soluble solids (SS) between 0.0 to 1.57 ºBrix (Puembo market); pH values between 4.77 to 6.47 (El Calzado market); titratable acidity expressed as tartaric acid between 0.07 to 0.92 % (Llano Grande market); relative density between 1.00 to 1.13 (Puembo market) for; dry matter between 0.00 to 2.30 % (Cotocollao market) and 0.12 to 2.01 % (La Magdalena market) for ash. In turn, all the markets showed significant differences with respect to the measurements performed on foods, except for titratable acidity that did not show significant differences. These results were related to other studies that showed that in the alfalfa the predominant acid was the tartaric acid, which provided the acidic flavor to those species (Pico, Gutiérrez, Aragón, Escobar, Ortiz, & Sánchez, 2011). In addition, in this study the reported relative density for alfalfa juices was lower than fibrous juices such as coconut juice enriched with soy (Gálvez, 2015) and mortiño juice (Tupuna, 2012) ; which reported densities of 1.03 and 1.05 respectively. This difference could be due to the fact that the alfalfa juice is more fibrous than the other juices reporting higher relative densities, as suggested RIOS *et al*. (2007). In turn, the alfalfa juice in most cases showed values of dry matter close to zero, this shows that the samples did not present a considerable amount of solids, suggesting that these preparations had in the majority water, this same effect was observed in the ash.

3.2. Heavy metal analysis

The concentration of lead in alfalfa juice showed ranges between 1.40 to 2.80 ppm (Carapungo, Mayorista and Yaruquí market). These values exceeded the maximum limits accepted by the Ecuadorian Technical Standard fixed in 0.5 ppm (INEN-2392, 2007) and 0.3 ppm established by CODEX and the European Union Regulations for heavy metals (Alonso, 2018). This may be due to the fact that alfalfa has the capacity to accumulate heavy metals, without achieving a detoxification as other plants do (Coyago & Bonilla, 2016). In turn, the cadmium concentration ranged between 0.03 to 0.13 ppm (Cumbayá market). The value of cadmium reported in the Cumbayá market exceeded the maximum limits allowed for cadmium in food (0.1 ppm), as established by the INEN regulation (INEN, 2013). The cooper ranges between 0.13 to 3.11 ppm (Carapungo market). The results obtained for Cu did not exceed the permissible limit of 5 ppm, established by the INEN regulations (INEN, 2013). The nickel ranges between 0.11 to 0.97 ppm and traces of chromium were found in all the samples under study. The Cr and Ni, no permissible limits were observed in any of the aforementioned regulations.

On the other hand, at least one market showed statistical differences with respect to the values reported for the remaining markets under study. These data indicated that there is no specific region that accumulates heavy metals, this may be due to the fact that the soils of the sierra region are volcanic and have high amounts of heavy metals. These values becoming a risk to health, since lead can replace calcium accumulating in bone tissues, as suggested by other authors (QAISAR *et al*., 2012).

Table 1. Average value of parameters related to the commercial quality of alfalfa juice (*M. sativa*)[a].

Market	Pb (ppm)	Cd (ppm)	Cu (ppm)	Ni (ppm)	Mesophiles (UFC/g)	Parasites	SS (ºBrix)	pH	TA (%)	$RD_{20\,°C/20\,°C}$	Dry matter (%)	Ash (%)
Alangasí	na	na	na	na	na	na	na	na	na	na	na	na
Amaguaña	na	na	na	na	na	na	na	na	na	na	na	na
América (m4)	2.50 ± 0.00	0.07 ± 0.00	0.24 ± 0.00	0.41 ± 0.00	3.5E+03	Positive	0.97 ± 0.06	5.60 ± 0.00	0.07 ± 0.12	1.00 ± 0.00	0.13 ± 0.06	0.22 ± 0.04
Calderón (m25)	2.50 ± 0.00	0.08 ± 0.00	0.16 ± 0.00	0.57 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	6.10 ± 0.17	0.10 ± 0.17	1.00 ± 0.00	0.07 ± 0.00	0.47 ± 0.02
Carapungo (m26)	2.80 ± 0.00	0.03 ± 0.00	3.11 ± 0.00	0.60 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	6.17 ± 0.06	0.30 ± 0.03	1.00 ± 0.00	0.23 ± 0.01	1.47 ± 0.05
Carcelén (m23)	2.50 ± 0.00	0.07 ± 0.00	0.4 ± 0.00	0.46 ± 0.00	3.5E+03	Positive	0.00 ± 0.00	5.80 ± 0.00	0.40 ± 0.05	1.00 ± 0.00	0.00 ± 0.00	0.71 ± 0.06
Central (m5)	2.50 ± 0.00	0.07 ± 0.00	0.80 ± 0.00	0.46 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	5.70 ± 0.00	0.17 ± 0.03	1.00 ± 0.00	0.07 ± 0.00	0.37 ± 0.06
Chiriyacu (m11)	2.30 ± 0.00	0.08 ± 0.00	0.13 ± 0.00	0.51 ± 0.00	3.5E+03	Positive	0.90 ± 0.00	5.10 ± 0.00	0.10 ± 0.02	1.00 ± 0.00	0.13 ± 0.01	0.22 ± 0.02
Ciudadela Ibarra (m18)	2.50 ± 0.00	0.08 ± 0.00	0.29 ± 0.00	0.59 ± 0.00	3.5E+03	Positive	1.00 ± 0.00	6.00 ± 0.00	0.07 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.27 ± 0.06
Comité del Pueblo (m22)	2.60 ± 0.00	0.07 ± 0.00	0.64 ± 0.00	0.46 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	5.60 ± 0.00	0.33 ± 0.04	1.0 ± 0.0	0.10 ± 0.01	0.56 ± 0.01
Conocoto	na	na	na	na	na	na	na	na	na	na	na	na
Cotocollao (m24)	2.70 ± 0.00	0.07 ± 0.00	0.25 ± 0.00	0.48 ± 0.00	3.5E+03	Positive	1.00 ± 0.00	6.20 ± 0.00	0.27 ± 0.04	1.02 ± 0.00	2.30 ± 0.00	0.72 ± 0.03
Cumbayá (m1)	2.70 ± 0.00	0.13 ± 0.00	0.54 ± 0.00	0.97 ± 0.00	3.5E+03	Positive	0.00 ± 0.00	5.80 ± 0.00	0.07 ± 0.00	1.00 ± 0.00	0.10 ± 0.00	0.34 ± 0.01
El Calzado (m7)	2.50 ± 0.00	0.08 ± 0.00	.23 ± 0.00	0.46 ± 0.00	3.5E+03	Positive	1.00 ± 0.00	6.47 ± 0.06	0.33 ± 0.04	1.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.01
Iñaquito (m30)	2.50 ± 0.00	0.08 ± 0.00	0.35 ± 0.00	0.60 ± 0.00	3.5E+03	Positive	1.00 ± 0.00	6.10 ± 0.00	0.10 ± 0.01	1.03 ± 0.06	0.00 ± 0.00	0.14 ± 0.07
La Ecuatoriana	2.60 ± 0.00	0.08 ± 0.00	0.35 ± 0.00	0.60 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	5.42 ± 0.04	0.09 ± 0.00	1.08 ± 0.02	0.29 ± 0.01	0.12 ± 0.03
La Floresta (m19)	2.60 ± 0.00	0.07 ± 0.00	0.24 ± 0.00	0.63 ± 0.00	3.5E+03	Positive	0.00 ± 0.00	5.87 ± 0.12	0.17 ± 0.03	1.10 ± 0.00	0.00 ± 0.00	0.27 ± 0.04
La Kennedy (m29)	2.40 ± 0.00	0.03 ± 0.00	0.19 ± 0.00	0.41 ± 0.00	3.5E+03	Positive	0.00 ± 0.00	6.20 ± 0.10	0.07 ± 0.00	1.00 ± 0.00	0.17 ± 0.01	0.12 ± 0.03
La Magdalena (m10)	2.60 ± 0.00	0.07 ± 0.00	0.32 ± 0.00	0.62 ± 0.00	3.5E+03	Positive	1.00 ± 0.00	6.13 ± 0.06	0.10 ± 0.0	1.00 ± 0.00	0.03 ± 0.00	2.01 ± 0.10
Las Cuadras (m17)	2.40 ± 0.00	0.08 ± 0.00	0.28 ± 0.00	0.59 ± 0.00	3.5E+03	Positive	0.00 ± 0.00	5.40 ± 0.00	0.43 ± 0.06	1.10 ± 0.00	0.23 ± 0.01	0.19 ± 0.01
Llano Grande (m27)	2.60 ± 0.00	0.03 ± 0.00	1.83 ± 0.00	0.52 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	6.40 ± 0.00	0.93 ± 0.1	1.03 ± 0.06	0.10 ± 0.00	0.57 ± 0.01
Mayorista (m8)	2.80 ± 0.00	0.07 ± 0.00	0.52 ± 0.00	0.44 ± 0.00	3.5E+03	Positive	0.10 ± 0.00	5.53 ± 0.06	0.33 ± 0.04	1.00 ± 0.00	0.00 ± 0.00	0.22 ± 0.01
Nayón	na	na	na	na	na	na	na	na	na	na	na	na
Pomasqui	na	na	na	na	na	na	na	na	na	na	na	na
Puembo (m3)	2.60 ± 0.00	0.10 ± 0.00	0.64 ± 0.00	0.59 ± 0.00	3.5E+03	Positive	1.50 ± 0.00	5.63 ± 0.06	0.10 ± 0.02	1.13 ± 0.15	0.03 ± 0.00	0.27 ± 0.04
Quinche (m15)	2.40 ± 0.00	0.08 ± 0.00	0.14 ± 0.00	0.53 ± 0.00	3.5E+03	Positive	1.00 ± 0.00	5.80 ± 0.20	0.10 ± 0.02	1.00 ± 0.00	0.00 ± 0.00	0.36 ± 0.04
Quito Sur (m16)	na	na	0.23	0.64	na	na	na	na	na	na	na	na

Rumiñahui (m21)	2.50	±	0.00	0.08	±	0.00	0.32	±	0.00	0.52	±	0.00	3.5E+03	Positive	1.00	±	0.00	6.20	±	0.00	0.17	±	0.03	1.00	±	0.00	0.00	±	0.00	0.52	±	0.04
San Antonio de Pich (34)	na			0.08	±	0.00	0.18	±	0.00	0.45	±	0.00	3.5E+03	Positive	1.00	±	0.00	5.90	±	0.00	0.20	±	0.03	1.00	±	0.00	0.10	±	0.00	0.78	±	0.03
San Bartolo	na			na			na			na			na	na	na			na			na			na			na			na		
San Roque (m6)	2.70	±	0.00	0.08	±	0.00	0.20	±	0.00	0.43	±	0.00	3.5E+03	Positive	1.00	±	0.00	4.77	±	0.06	0.23	±	0.03	1.00	±	0.00	0.00	±	0.00	0.20	±	0.02
Santa Clara (m33)	2.60	±	0.00	0.08	±	0.00	0.24	±	0.00	0.59	±	0.00	3.5E+03	Positive	1.00	±	0.00	6.00	±	0.17	0.13	±	0.02	1.03	±	0.06	0.00	±	0.00	0.38	±	0.02
Solanda (m9)	1.40	±	0.00	0.08	±	0.00	0.42	±	0.00	0.11	±	0.00	3.5E+03	Positive	1.00	±	0.00	5.60	±	0.00	0.10	±	0.02	1.00	±	0.00	0.00	±	0.00	0.41	±	0.09
Tumbaco Arenal (m13)	1.60	±	0.00	0.08	±	0.00	0.27	±	0.00	0.37	±	0.00	3.5E+03	Positive	0.00	±	0.00	6.13	±	0.06	0.10	±	0.02	1.00	±	0.00	0.00	±	0.00	0.78	±	0.01
Tumbaco Centro (m2	2.40	±	0.00	0.07	±	0.00	0.41	±	0.00	0.47	±	0.00	3.5E+03	Positive	0.00	±	0.00	5.93	±	0.06	0.07	±	0.01	1.00	±	0.00	0.13	±	0.00	0.19	±	0.01
Yaruquí (m14)	2.80	±	0.00	0.08	±	0.00	0.13	±	0.00	0.61	±	0.00	3.5E+03	Positive	1.40	±	0.00	5.80	±	0.00	0.10	±	0.02	1.00	±	0.00	0.17	±	0.01	0.78	±	0.09
p-value	***			***			***			***			ns		***			***			ns			***			***			***		
Max. value	2.80			0.13			3.11			0.97			3.50E+03		1.50			6.47			0.93			1.13			2.30			2.01		
Min. value	1.40			0.03			0.13			0.11			3.48E+03		0.00			4.77			0.07			1.00			0.00			0.12		
Average	2.48			0.07			0.47			0.52			3.50E+03		0.57			5.83			0.20			1.07			0.15			0.48		

[a]Mean values ± SD (n=13). Significance of differences is given: ns, not significant; *P<0.1; **, P<0.01; ***, P<0.001. na, not available.

3.3. PCA analysis for foods quality

Applied to our data set, PCA revealed that the first principal components explain 32.9 % for alfalfa juice of total variance. A plot of the scores of the first factor (PC1) versus the second principal component (PC2), i.e. the projection of the samples along the directions identified by the first two PCs, is reported in Fig. 1. PC1 was mainly linked to lead in most of the samples under study, all with positive loading values in this component.

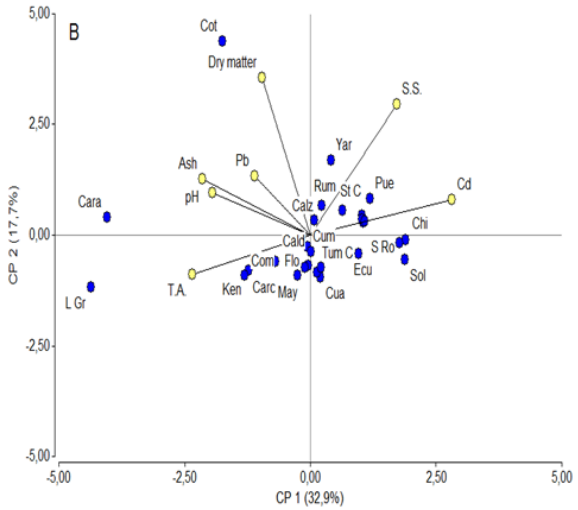

Figure 1. Scores plot of alfalfa juice (*M. sativa*) using the two first principal components obtained by PCA of pH, soluble solids, titulable acidity, dry matter, ash, lead and cadmium.

3.4. Microbiological quality

The average of mesophilic aerobic microorganisms in all samples was 3.5 E+03 UFC/g. The values of alfalfa juice were lower than those found by Fu *et al*. (2001) (7 to 8 log10 CFU per g at 24 hours at alfalfa sprouts). In addition, the concentration of alfalfa juice exceeds the value of 3 log10 CFU/g or $1.0x10^3$ CFU/g established by the (INEN-2337, 2008) regulations that apply in Ecuador. On the other hand, the pH values for alfalfa juice found could explain the high microbial quantity in the different species, as indicated (GONZAGA *et al*., 2012), who points out that when pH is low (presence of acidic substances) the bacteria count may be low, but at neutral or higher pH (5 to 8.5), the level of contamination of the herbal preparations may be higher.
On the other hand, in the present study was evaluated the presence or not of eggs and cysts of parasites in alfalfa juice, they reported positive values in most cases.
In the case of PCA analysis, the statistical analyses were not made because the value of aerobic mesophilic microorganisms was very similar.

4. CONCLUSIONS

In summary, the present study supports the idea that alfalfa juice meets the bromatological, microbiological and heavy metals requirements in the markets of the Metropolitan District of Quito-Ecuador. In general, the markets that presented the highest values in relation to the commercial quality on food were the Puembo market in SS (1.57

ºBrix) and relative density (1.13); El Calzado market in pH (6.47); Llano grande in acidity titulable (0.92 %); Cotocollao in dry matter (2.3 %) and La Magdalena in ash (2.01 %). The Pb in alfalfa juice (average value of 2.48 ppm) exceeds the limits allowed by Ecuadorian regulations, CODEX, and European regulations, while the cadmium (average value 0.07 ppm) and Cu did not exceed the limits established by the different regulations (0.1 ppm for Cd and 5.00 ppm for Cu) except in the Cumbayá market for Cd. In the case of Cr and Ni, no regulatory limits were found, however these metals have been observed to present a risk to health. In addition, the PCA reported that the lead presented greater relevance within the study. On the other hand, according to our microbiological results, it is suggested that the species studied have lower quality hygiene considering *Aerobic mesophiles* and parasites (outside acceptable microbiological limits). The high load of microorganisms could be for the contamintation between the transport of vegetables of the farm to the market or for contaminated water use in the preparation of the juice.

ABBREVIATIONS USED

SS, soluble solids; FW, fresh weight; DW, dry weight.

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NOTES

The authors declare no competing financial interest.

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SESSION II

“BIOTECHNOLOGY”

THE EFFECT OF SUNLIGHT ON THE CONTENT OF THIOCYANATES, SUGARS AND STARCHES IN ACCESSIONS OF *TROPAEOLUM TUBEROSUM* RUÍZ & PAVÓN

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ABSTRACT

The objective of the study was to identify accessions of *T. tuberosum* with highest quantities of thiocyanates, starches and sugars. The study was performed in Cevallos, Ecuador. Six accessions were cultivated in six individual plots and processed. The Yellow, White and Purple accessions were exposed to sunlight for periods of 3, 6 and 9 days. The resulting biomass was analysed: The Purple variety presented the greatest quantity of thiocyanates (663 mg/100g) after three days' exposure. Meanwhile, the Yellow variety showed the highest amount of starches (47%) and total sugars in freshly-harvesting. The results reveal the potential of these accessions as a source of starch and thiocyanate.

Keywords: sunlight, sweeting, thiocyanates, starches and sugars, secondary metabolites

1. INTRODUCTION

Tropaeolum tuberosum (mashua) has been grown in the Andean region for thousands of years (CONDORI *et al*., 2008). It is traditionally cultivated - as a single crop, or together with other Andean tubers, such as the potato, oca and ullucus - between 2,400 and 4,300 metres of altitude (MANRIQUE *et al*., 2014). Despite the importance of *T. tuberosum* as a staple of ancestral communities, in recent decades it has been largely replaced in the diet and agriculture of most rural families (GRAU *et al*., 2003). However, it has established its importance in natural medicine for its therapeutic properties in the treatment of genitourinary disease (MANRIQUE *et al*., 2014) as well. Among the Andean countries there are a great number of varieties, but in Ecuador the cultivable genotypes are limited in number. The yellow variety is most common and may be found in the marketplace (ESPIN *et al*., 2003). Mashua is known for its primary and secondary constituents (isothiocyanates) of interest to both industry and medicine. However, the variability of these in different crops is unknown, as is the effect of sunlight on their levels of concentration.

Preliminary investigations indicate that mashua plants contain, among other substances, isothiocyanates, proteins, starches, and sugars (KING and GERSHOFF, 1987). The principle glucosinolates identified were: 4-Hydroxybenzyl GSL (OHB, Glucosinalbin), Benzyl GSL (B, Glucotropaeolin), and m-Methozybenzyl GSL (MOB, Glucolimnathin) (ORTEGA *et al*., 2006). Isothiocyanates have properties which enhance the prostate treatment in men (AIRE-ARTEZANO *et al*., 2013). Mashua plants contain high levels of glucosinalates (methoxybenzyl glucosilanate and benzyl glucosilanate). These compounds produce isothiocyanates in the presence of the mirosinase enzyme. Benzyl isothiocyanate appears to be most abundant in mashua plants and has been reported to be an inhibitor in the development of breast and stomach cancer (QUIROS and ORTEGA, 2004). Furthermore, mashua plants release glucose, sulphates, and toxic compounds such as nitriles, thiocyanates and oxazolidines, through hydrolysed glucosinalate (BELL *et al*., 2015).

In another study, in Colombian mashua accessions, isothiocyanates were quantified through the method of High-Performance Liquid Chromatography (HPLC). From the four accessions studied, Yellow yielded between 12.96 and 27.69 μmoles/g; Purple 19.32 and 22.54 μmoles/g; Purple-White 11.47 and 20.05 μmoles/g; and Red-White yielded between 4.86 and 45 μmoles/g (ARIAS, 2011). Additionally, there is evidence that polyphenols derived from mashua plants are effective antioxidants, combatting the damage caused by oxidizing processes in biological structures. These properties may be utilized in the food and cosmetics industry (CHIRINOS *et al*., 2008). In Ecuador, the yellow variety known as *zapallo* has been shown to consist of 9.17% protein; 46.92% starch; and 42.81% (ESPÍN, VILLACRÉS and BRITO, 2003).

There are evidence that mashua plants reduce sperm production (CÁRDENAS-VALENCIA *et al*., 2008). Extracts of *T. tuberosum* have been evaluated in the effect on the male reproductive system of mice, narrowing sperm-production parameters without any toxic effects (VÁSQUEZ *et al*., 2012). The use of the plant as an aphrodisiac is thought to derive from these qualities (JONHS *et al*., 1982).

Mashua plants take various forms and colours (MELCHIORRE, 1985). According ancestral knowledge, the darker varieties (purple and black) are considered to have greater medicinal value. However, these are poorly cultivated, so as consequence rarely seen in local markets, and in danger of disappearing (KING and GERSHOFF, 1987). The yield of the mashua crop is generally some 36 t/ha when grown with suitable fertilizers (VALDIVIA *et al*., 1999).

In some indigenous communities, mashua tubers are still consumed once they have been exposed to sunlight (called sweetening). This constituting a part of the alimentary diet with other Andean tubers (GONZALES *et al*., 2003). Furthermore, artisanal agroindustry has developed some mashua by-products for medicinal purposes. Herbalists frequently elaborate mashua extracts or dehydrated mass of tubers for sale.
The object of this study was to identify which accessions of mashua contained the highest levels of thiocyanates, sugars and starches in freshly harvested under exposed to sunlight, in order to better understand the traditional practices of rural areas, and to define the optimum exposure to sunlight that produces the greatest yield of medicinal compounds.

2. MATERIALS AND METHODS

2.1. Materials

Six mashua were recollected from indigenous communities within the provinces of Tungurahua and Chimborazo. All the accessions were sowed in sandy loam and grown in separate plotin in the experimental farm of the Agricultural Sciences Faculty-Technical University of Ambato, Ecuador, located at 2740 metres of altitude. These accessions were named Yellow (A1), White (A2), Purple (A3), Milicia Red (A4) Poza Rondador (A5) and Green Yellow (A6). From the freshly-harvested tubers, samples were taken for an analysis of their thiocyanate, starch and sugar content. In addition, the samples were subjected to phytochemical analysis to evaluated the presence of secondary metabolites based in the method proposed by CASTILLO *et al*. (2014).

2.2. Methods

2.2.1 Natural sweetening

Three of the accessions (A1, A2, and A3) were subjected to the natural sweetening process in nine days. After harvesting the three varieties were washed and exposed to sunlight in an open area with no danger of interference. The levels of solar radiation are expressed in hours of sunlight per days, as follows: 11.6h/ after 3days; 21.1h/ after 6 days and 29.3 h/ after 9 days.
The factors studied were the accessions and the length of time exposure to sunlight. As a control sample (time=0) an unexposed root of the plant was used. The three accessions were subjected to three lengths of exposure to sunlight: T1=3 days; T2=6 days; T3=9 days. The experimental model consisted of 3 accessions in three exposures (3x3) + 1 sample with 3 repetitions, making a total of 30 experimental units. The biomass collected from the experiment was sliced into thin sections and dried at a temperature of 40°C for 72 hours, and then ground into powder for analysis.

2.2.2 Starch content

Starch content was measured using the polarimetry method described by HAROLD *et al.* (1993). In accordance with this method, the starch was treated with a dilute solution of hydrochloric acid in water bath and determine the rotation angle with the polarimeter. The percentage of starch was calculated using the mathematical formula: % starch = (a-b)f, where a = the rotation angle of the sample in degrees, b = the rotation angle of the blank in degrees, and f = the starch factor.

2.2.3 Total sugars

The measurement of total sugars was developed by the method of DUBOIS *et al*. (1956) with modifications. This method consists in performing a hydrolysis of the polysaccharides in a warm acidic medium. The anthrone reacts with the hexoses and the aldopentoses to produce a blue-green complex with a maximum absorbency at 625 mm. The Indian Standards Institute method was used for the measurement of the thiocyanates, as proposed by the FAO. The isothiocyanate contained in the matrix was distilled with water vapour and collected in a solution of silver nitrate. The excess of the solution of $AgNO_3$ was determined by volumetric method with a standard solution of ammonium thiocyanate. The content of isothiocyanate was calculated with the following mathematical formula: isothiocyanate (g/kg) = 9.915(Vo-V1) x N x 10/M, where Vo = the volume of the standard solution of ammonium thiocyanate needed to determine the blank (ml); V1 = the volume of the solution of ammonium thiocyanate needed to establish the sample (ml); N = the normality of the standard solution of ammonium thiocyanate; and M = the weight of the sample.

3. RESULTS AND DISCUSSION

3.1. Analysis of thiocyanates

The analysis of thiocyanates, total sugars and total starch carried out in six samples of recently-harvested mashua accessions show differences in the accessions of the study. In regard to the content of thiocyanates, the sample of Poza Rondador showed the greatest content (574.2 mg/100g). Meanwhile the Yellow accession showed the lowest content (240.9 mg/100 g). On the other hand, the Green accession showed the greatest total sugar content (26.9%) and the White accession the least amount (17.9%). As to total starch content, the Yellow accession showed the greatest content (46.8%) and Red Milicia showed the least (2.5%).
The presence of a low content of thiocyanate and a high content of starch in the Yellow accession could be an excellent option to improve the cultivation and commercialization. The accessions with a high thiocyanate content present unpleasant taste and are little accepted to consumption (ESPINOSA *et al*., 1996). Based in previous studies that mashua can be effective in prostate infections treatment, the Yellow variety has the lowest content of thiocyanates, so it would be the least suitable for pharmaceutical uses. The Poza Rondador and Red Milicia varieties could be the most suitable option due to their high thiocyanate content after harvesting. Table 1 shows the content of thiocyanates per 100 g of dry plat material, sugars and starches are expressed in percentage.

3.2. Screening

The phytochemical screening demonstrated that the Poza Rondador accession had the greatest content of alkaloids, sterols and 2 – deoxy sugars (Table 2). Meanwhile, the Green-Yellow accession shows the lowest ocontent of all secondary metabolites evaluated in this study. The White and Yellow varieties showed a high content of anthocyanins (CHIRINOS *et al*., 2006).

Table 1. Content of thiocyanates, sugars and starches in fresh samples, after harvesting.

Accessions	Thiocyanate* (mg/100 g)	Total sugars (%)	Total starches (%)
Yellow	240.9^{c}	18.838^{de}	46.842^{a}
White	511.5^{b}	17.936^{e}	13.642^{b}
Purple	491.7^{b}	20.188^{d}	14.493^{b}
Red Milicia	504.9^{b}	22.733^{c}	2.525^{d}
Poza Rondador	574.2^{a}	24.528^{b}	3.063^{d}
Green-Yellow	534.6^{ab}	26.908^{a}	9.977^{c}

*Expressed as allyl thiocyanate.

Table 2. Phytochemical screening of various mashua accessions.

	Alkaloids	Sterols	2 – deoxy sugars	Leucoanthocyanins	Flavonoids	Tannins	Anthraquinones	Saponins
White	++	+++	+	+++	+	-	-	+
Poza Rondador	+++	+++	+++	++	+	-	-	+
Purple	++	+	++	+	++	-	-	++
Green-Yellow	+	+	+	+	+	-	-	+
Yellow	++	++	++	+++	+	-	-	+
Red Milicia	+++	++	+++	++	++	-	-	+

3.3. Sunlight on the content of thiocyanates

Regarding the effect of sunlight on the content of thiocyanates, starches and total sugars were analysed. The results show the content of thiocyanate under sunlight increase the first three days, reaching 548.9 mg/100 g and decline after this. In contrast, fresh harvested mashua tubers showed the highest level of starch (24.9%), and by the ninth day of exposure, starch content decreased significantly. Meanwhile sugar content showed a increase over the time of exposure to sunlight (Table 3). The results in general evidence the importance of sunlight exposure in the carbohydrates transformation and explains why rural communities in producing countries prefer to consume these tubers after sweetening process.

Table 3: Variation in thiocyanates, starch and total sugars according to length of exposure to sunlight*.

Parámetros	Unit	Days			
		0	3	6	9
Thiocyanate $^{+}$	mg/100g	414.7	548.9	523.49	481.58
Total starch	%	24.992	18.173	16.472	14.529
Total sugar	%	19	20.57	21	21.7

*Average of three accessions.
+ Expressed as allyl thiocyanate.

3.4. Quantity of thiocyanates per accession

Regarding the behaviour of the quantity of thiocyanates per accession, it was found that the Purple accession, after three days of exposure, showed the highest quantity (663 mg/100 g) of all the accessions studied. After this time, the content of thiocyanate decline. The White accession present a different pattern, with a steady decline during the first three days, and gradually became to its initial thiocyanate content (Fig. 1). Previous studies in Peru, report differences in accessions exposed to sunlight freshly harvesting, where the antioxidant capacity and the content of bioactive compounds changed. Meanwhile the anthocyanins showed a marked decline (CHIRINOS *et al*., 2007). A similar pattern was found in the thiocyanates content of the Purple variety, with a significant decrease over time of exposure to sunlight. In regard to glucosinolates, the Purple varieties have been found to contain between 19.32 and 22.54 mg/100 g (ARIAS, 2011).

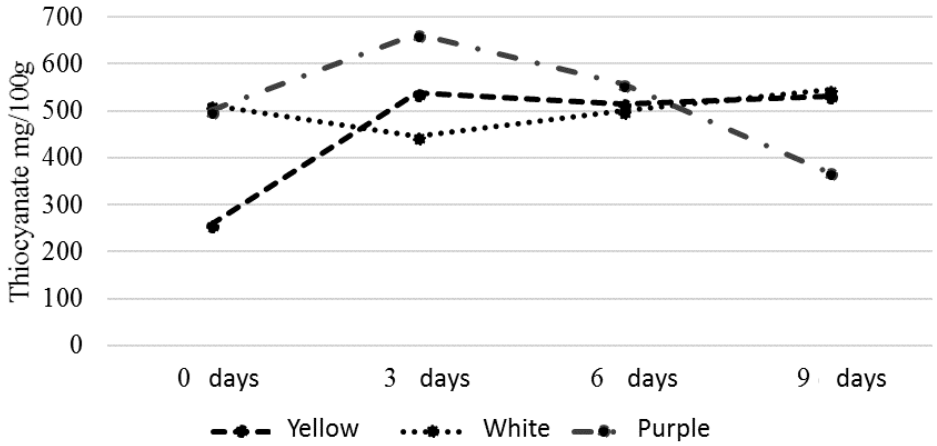

Figure 1. Variation in thiocyanates with time of exposure to sunlight.

3.5. Total sugars

In reference to total sugars, White accession showed the highest content of sugar (23%) after three days of exposure under sunlight. Purple accessions present a slight increase between the first and ninth day (Fig. 2). This suggests, a transformation from starches to sugars through the effect under sunlight. For example, in mashua accessions a 27.44% content of amylose has been observed (VALCÁRCE-YAMANI *et al*., 2013). The sugars in oca (*Oxalis tuberosa*) showed 53% sugar content after 9 days, and 70% after 15 days of exposure, especially in white variety (BRITO *et al*., 2003) as our results in the mashua tubers. So as consequence, we can infer that mashua tubers might reach a greater sugar content with longer exposure to sunlight.

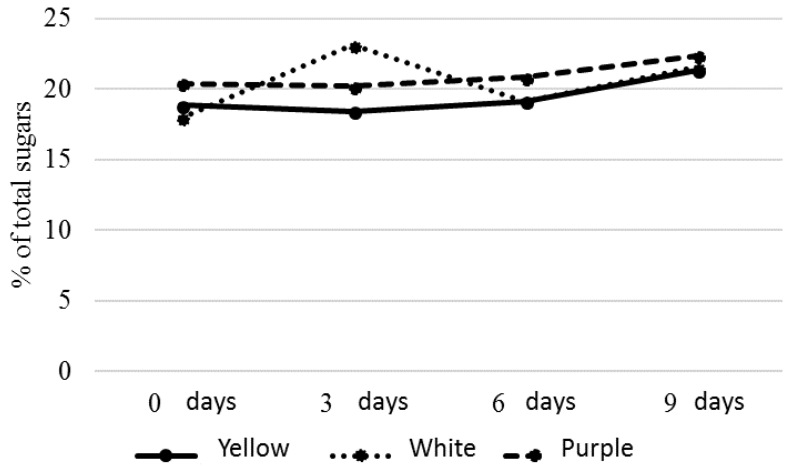

Figure 2. Variation in total sugars with length of exposure to sunlight.

3.6. Total starch

In regard to the total starch, the freshly-harvested Yellow accession present the highest quantity (47%). Following exposure under sunlight, the initial starch content decreased significantly. A similar pattern was observed in the Purple and White accessions (Fig. 3). This suggests that freshly-harvested tubers can be used as the best sugar, specially the Yellow accession is the most suitable in regard of this parameter. The percentage of starch observed in mashua crops in Peru was 3.65% (HERMOSA, 2013).

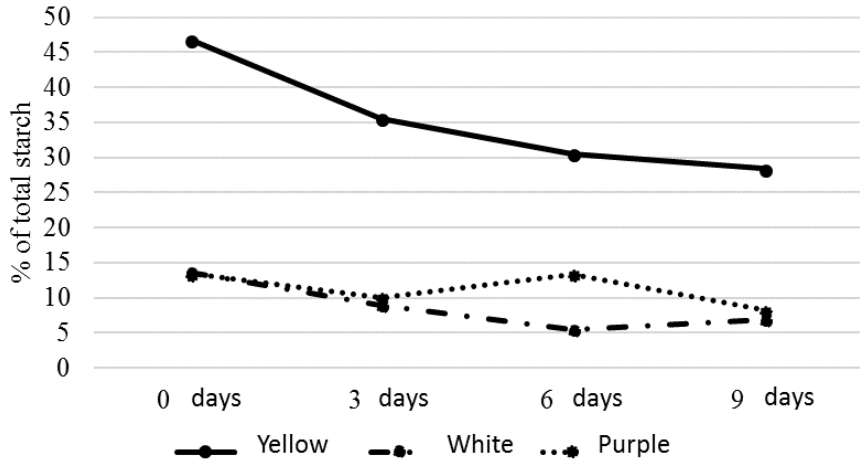

Figure 3. presence of starches according to mashua accessions.

3.7. Variation analysis between the accessions

The variation analysis shows highly significant differences between the accessions, the number of days and the contrast of accession versus days of exposure for thiocyanate,

starch and total sugar content (Table 4). On the other hand, the coefficient of variation is low, which reflects a low variability between data.

Table 4. Analysis of variance in the factors studied.

Source of variation	gl	Thiocyanates	Starch	Sugar
Repetitions	5	1633.60	41.39	1.81
Accession	2	25208.9**	5128.73**	25.17**
Days	3	61877.9**	372.77**	24.49**
Accession*Days	6	82150.3**	79.57**	13.65**
Mean		492.17	18.542	20.58
CV %		7.43	5.3	7.04

** Highly significant at a level of 0.05.

Finally, the Pearson correlation tests demonstrate an inverse relationship between the total starch and thiocyanates (-0.40), and likewise between total sugars and total starches (-0.49). Starches are also known as polysaccharides in nutritional terms and are formed by the linkages of glucose in position α1-4 and branches in position α1-6 (PLAZA-DÍA *et al*., 2013).

4. CONCLUSIONS

The Purple accession, after three days of exposure to sunlight, was most suitable for obtaining thiocyanates. Meanwhile, the Yellow accession is most apt as a food source, due to its high content of starches and sugars, and low concentrations of thiocyanates. The results suggest two industrial applications for mashua plants: on the one hand as a source of starch and sugar nutrients, and on the other hand as a source of thiocyanates for medicinal purposes.

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STUDY OF THE BACTERIAL MICROBIOTA, PRESENT IN THE AIRBORNE BIOLOGICAL PARTICLES FROM LANDFILLS OF THE CENTRAL AREA IN ECUADOR

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ABSTRACT

Limited information about studies on microbiota in atmospheres of areas where operating landfills in the center of Ecuador and the interest to safeguard the health of the population, were the main reasons for this study that aimed at identifying the pathogenic organisms present in the air and their level of toxicity to the human being.

5 landfills, which were identified with the biochemical tests, 16 species of pathogenic bacteria, with a level of 2 and 3 of endangerment according to the National Institute of Security, Health and Welfare at Work (Real Decreto, 664/1997) were analyzed.

Keywords: diseases, level of danger, pathogens, IMViC's tests, turbidity

1. INTRODUCTION

The microflora of landfills air contains a variety of airborne biological particles of different composition. Their survival is due to the location, amount of waste collected and weather conditions (BREZA, 2016; FRĄCZEK and KOZDRÓJ, 2016), isolated microorganisms use the degradation of common solid waste (organic matter) and hospital waste as a source of carbon and nutrients.

A large part of particles isolated from air into landfills, show that many of them are bacteria such as *Salmonella, Escherichia coli, Klebsiella, Enterobacter cloaceae* and fungi such as *Aspergillus fumigatus, Aspergillus spp, Fusarium*, among the most representative, this is given by the conditions of rot that provide these places (HUANG *et al*., 2002; MIAŚKIEWICZ and SZYŁAK, 2015; SÁNCHEZ *et al*., 2006; VÉLEZ and CAMARGO, 2009).

According to the National Institute of Safety and Hygiene at Work (1997) biological agents are classified depending on their level of involvement to human health and propagation, therefore, shows four levels.

Level 1: those who become unlikely that cause disease in man.

Level 2: mention of microorganisms that can cause mild illness in humans, however, are not easy to spread and possess a medical treatment for their resettlement.

Level 3: can cause severe disease in humans and it can spread, however, also can be treated with medication.

Level 4: cause a fatal disease in humans, they have most likely to spread, without any effective treatment for their recovery.

The objective of this study was to compare the microorganisms suspended in the air from landfills of Ambato, Latacunga, Salcedo, Riobamba and Pujili-Saquisili, and to identify the level of endangerment of the microorganism according to Real Decreto 664/1997 (National Institute of Security, Health and Welfare at Work, 1997) (Instituto Nacional de Seguridad Salud y Bienestar en el Trabajo, 1997).

2. MATERIALS AND METHODS

The methodology that will be exposed below was appliedtoeachofthefivelandfills in the cities of Ambato, Latacunga, Riobamba, Salcedo and Pujili-Saquisili.

2.1. Sampling design

Sampling was carried out in two weeks, two daily samplings from Monday to Friday, in th e morning and afternoon hours. Were scheduled 10 sampling points with the following provision, 5 measuring points in the section of common waste with a distance of 100 meters between each point, 5 measuring points in the section around a hospital waste with a distance of 10 meters between each one of the points.

The method of collection of the microflora of the air stayed with total asepcia during the process, equipment and staff that made the collection. The method of collection was based on taking 50 aspirations with a syringe at a height of 1.50 meters, aspirations were inoculated into a flask that with contained 200 ml of infusion brain heart infusion (BHI) culture. Each 10 aspirations shook the flask so that there is interaction between the breeding ground and the air. The flasks were incubated at 37°C for 48 hours, microbial growth was observed by presence of turbidity in the media (FLORES TENA and PARDAVÉ, 2007).

2.2. Microbial quantification

Turbidimetric method was used in order to quantify the microbial population, therefore, it was compared with McFarland standards (table 1). Then, aliquots of 1 ml of each flask were taken for the determination of the absorbance at 600 nm

Table 1. McFarland standards for microbial quantification.

Standards	Barium chloride 1.17% (ml)	Sulfuric acid 1,00% (ml)	Bacterial concentration (UFC/ml)
0	0.05	9.95	1.50E+08
1	0.10	9.90	3.00E+08
2	0.20	9.80	6.00E+08
3	0.30	9.70	9.00E+08
4	0.40	9.60	1.20E+09
5	0.50	9.50	1.50E+09
6	0.60	9.40	1.80E+09
7	0.70	9.30	2.10E+09
8	0.80	9.20	2.40E+09
9	0.90	9.10	2.70E+09
10	1.00	9.00	3.00E+09

2.3 Isolation of microorganisms

2.3.1 Bacterial isolation

Serial dilutions in peptone broth 1% were prepared at the end of the incubation period. For the Tripteina soy agar (CHENG *et al*., 2012) were prepared dilutions from 104 to 106 and for the MacConkey Agar were prepared dilutions from 102 to 104. An aliquot was taken 100 μl of each of them and were inoculated in Petri dishes with the mentioned solid media. Subsequently inoculated boxes were incubated at 37°C for 48 hours.
For the isolation of bacteria, simple striae were made from the colonies that presented different macroscopic characteristics, obtained by plaque diffusion. Stretch marks were made in Tri-Petri boxes containing MacConkey Agar and TSA culture media, respectively. The boxes were incubated under conditions at 37°C and 48 hours. Subsequently, they performed the IMVIC tests.

2.3.2 Fungal isolation

The samples collected in the BHI medium were used, after 48 hours of incubation. Serial dilutions from 10^{1} to 10^{2} were prepared using peptone water 1%. A superficial harvest was carried out with 100 μl of each sample utilizing Malta Agar (MA), glucose sabouraud 4% (SUB) and Ceftriaxona (50 μl/ml) as a supplement. Finally, the Petri dishes were incubated and inoculated at 25°C for 48 hours.

2.4. Biochemical tests

2.4.1 Production of indole

Test tubes containing 5 ml of peptone broth were used to carry out this test. Into each tube each obtained microorganisms was incubated and with the application of 3 drops of reagent of Kovac´s identified the formation of ring of dark red as a positive result.

2.4.2 Methyl red

The same amount of tubes to the indole test was used for this test, however, used the liquid culture medium RM-VP (methyl and Voges-Proskauer red), whereby each bacteria were inoculated for 48 hours at 37°C, after that time was added 3 drops of solution of 0.2% methyl red. The coloration of the stock (red), established the test positivity

2.4.3 Voges-Proskauer

This test used test tubes with RM-VP medium inoculated with each of the microorganisms, these tubes were incubated at 37°C for 48 hours, subsequent to that time was added 2 drops of potassium hydroxide solution 40% and 3 drops of α-naftol to 6 %, stirred, for better interaction with the inoculate medium placed compounds, finally be left to rest for 5 minutes. The formation of reddish ring was identified as a positive result.

2.4.4 Simmons Citrate

For the citrate test were used a microbiological screw cap tubes where the Simmons Citrate solid culture medium was sterilized and allowed it to cool in an inclined position. Retrieved once inclined agar were stretch marks of all bacteria identify, these tubes remained for 48 hours at 37 °c in incubation, subsequently assessed the change of coloration from green to blue bromothymol blue indicator, taking it as a positive result.

3. RESULTS AND DISCUSSION

Five landfills for the central zone of Ecuador were studied. The landfill of the waste management in Ambato and Pujilí-Saquisili, waste disposal site in Latacunga, Salcedo and Riobamba. The determination of microbial concentrations was carried out with the analysis of turbidity samples, using the curve of Mc Farland outlined in Table 1. The values of this analysis are shown in the Table 2, data obtained in the determination of concentration of microorganisms present in each landfill, with their respective meteorological characteristics and the presence of organic matter as a source of nutrients for the development of microorganisms colonies from the area of common solid waste of each sector studied. In the case of the area of hospital waste, the main source of nutrients for the microorganisms were infectious waste generated in the medical area (blood, placentas, materials contaminated with body fluids, among others).
Table 3 shows the different species of microorganisms suspended in the air, obtained from each of the landfill, among which are some of the most dangerous, this categorization based on the classification explained in Real Decreto 664/1997. The presence of *E. coli* and *Salmonella typhi* were predominant in the isolations of the 5 landfills, the microorganisms found have been identified following the methodology of the IMViC's tests.

Table 2. Concentration of microorganisms with their respective characteristics of each sector studied.

Location	a1	a2	T (°C)	RH (%)	OM (%)
Ambato	5.87E+12	5.10E+12	17.50	77.00	46.90
Latacunga	2.23E+09	2.48E+09	15.60	72.50	71.53
Riobamba	1.29E+10	1.17E+10	16.00	60.00	55.00
Salcedo	2.63E+09	2.18E+09	16.30	43.50	63.00
Pujili-Saquisili	3.44E+09	2.50E+09	18.00	64.90	60.00

Note: a1: hospital waste area; a2: common waste area; T: average temperature presented during the sampling period; RH : relative humidity corresponding to the sampling period of each sector; OM: percentage of fermentable organic matter of each landfill.

Table 3. Species of microorganisms and their level of danger according to Real Decreto 664/1997.

Microorganisms	Level of danger	Location				
		Ambato	Latacunga	Riobamba	Salcedo	Pujili-Saquisili
Citrobacter freundii	N.I.	+	+	+	+	+
Citrobacter Koseri	N.I.	+	+	+	+	+
Enterobacter aerogenes	2	+	-	+	+	+
Enterobacter cloacae	2	+	-	+	+	+
Enterobacter cloacae Tipo II	2	-	-	-	+	+
Enterococcus faecalis	2	+	-	+	+	+
Escherichia coli	3	+	+	+	+	+
Hafnia alvei	N.I.	-	-	+	+	+
Klebsiella pneumoniae	2	+	+	+	+	+
Klebsiella oxytoca	2	+	-	+	+	+
Pantoea agglomerans	N.I.	-	-	+	+	+
Salmonella typhi	3	+	+	+	+	+
Shigella flexneri	2	-	+	+	+	+
Staphylococcus aureus	2	+	-	+	+	+
Proteus vulgaris	2	+	-	+	-	-
Proteus mirabilis	2	+	-	+	+	-

Note: + presence of microorganism; - absence of microorganism; NEITHER. unidentified species, that is, in Real Decreto 664/1997 there are no records of the microorganism's danger. (Acosta and Muso, 2017; Andache and Castillo, 2016; Campaña and Navarrete, 2018; Flores, 2017; Solís and Vásconez, 2017).

The microorganisms suspended in the air develop better when the relative humidity exceeds 35%, this parameter is very important because the particles absorb moisture and use it as a shield against radiation, however, an excessive increase in the content of water decreases their survival (JONES and HARRISON, 2004). The temperature as well as the availability of nutrients (organic matter and hospital waste), play a very important role in the development and type of microorganisms found in the analyzed zones, therefore, they can affect the proliferation of microorganisms. In this study, mesophilic microorganisms were identified and their temperature range lies at 25-40°C. In the same way it happens

with the availability of nutrients, if this is scarce, the development of microorganisms will decrease. Taking into account the influence of external factors (temperature, relative humidity, substrates used as nutrients), the concentration of microorganisms found in the 5 localities analyzed was compared and it can be seen that in the Ambato landfill there is a higher concentration of microorganisms suspended in the air.

However, despite the optimal conditions for optimal development of microorganisms, in the study only the presence of microbial organisms was identified, with fungal organisms absent. The absence of these organisms was due to the fact that the surface of the fillings is in constant movement, preventing the development on the surface of these organisms; In addition, bacteria are simpler organisms than fungi, so they are more ubiquitous and can develop in almost all environments. The determination of the bacterial species allowed categorizing them according to their level of danger of affectation and propagation, said categorization was based on the Real Decreto 664/1997. This Real Decreto, does not identify in any level of danger to *Citrobacter freundii, Citrobacter koseri, Hafnia alvei, Pantoea agglomerans;* however, these organisms are cause serious injuries to human health. In the case of *Hafnia alvei,* it has been identified that it can be the cause of nosocomial infections (MUKHERJEE and MISRA, 2008), including gastroenteritis, bacteremia, pneumonia, meningitis, operative wound infection, endophthalmitis and gluteal abscess (GÜNTHARD and PENNEKAMP, 1996).

Pantoea agglomerans is responsible for most nosocomial infections, as well as a major epidemic caused by a perfusion contamination (MAKI and MARTIN, 1975).

Citrobacter freundii is the cause of urinary tract infections, superficial wound infections, brain abscesses, pneumonia in the elderly and hospitalized patients (KATARIA and SAAD, 2015); *Citrobacter Koseri* is the causative agent of neonatal meningitis and brain abscesses (LIU *et al*., 2015).

For the case of the bacterial species identified and categorized in Real Decreto 664/1997, we have caused severe disease, which is why they are categorized in level 2 and 3 respectively, as in the case of: *Enterobacter aerogenes* affects the respiratory tract, urinary, blood or gastrointestinal, in certain cases causes septic shocks against man (REGLI and PAGÉS, 2015). *Enteobacter Cloace*, is the cause of nosocomial infections (CALDERÓN *et al*., 2003; MEZZATESTA *et al*., 2012; REGLI and PAGÉS, 2015).

Escherichia coli is not highly dangerous because it is part of the gastrointestinal tract microbiota of humans, but diseases arise when it evolves and mutates, generating diarrheal diseases with blood, urinary tract infections, peritonitis, colitis, bacteremia and infant mortality (BLOUNT, 2015; PUERTA-GARCÍA and MATEOS-RODRÍGUEZ, 2010).

Escherichia faecalis is responsible for nosocomial infections, infections in the urinary tract, bacteremia and endocarditis (ASPIROZ *et al*., 2000; KAU *et al*., 2005; Murray, 1990).

Klebsiella pneumoniae is the cause of necrotizing pneumonia, it can mimic tuberculosis of pulmonary reactivation (PINTO *et al*., 2012; PUERTA-GARCÍA and MATEOS-RODRÍGUEZ, 2010). *Klebsiella oxytoca* agent responsible for hemorrhagic colitis (CHENG *et al*., 2012).

Salmonella typhi is the cause of acute gastroenteritis, typhoid fever (ELIKA, 2013; JURADO *et al*., 2010).*Staphylococcus aureus*, causes skin lesions and abscesses, in addition to deep infections such as: endocarditis, osteomyelitis (LIU *et al*., 2015).

Shigella Flexneri is the cause of endemic infections (AYALA *et al*., 2006; SHEN *et al*., 2017).

Proteus vulgaris is the cause of serious urinary tract infections and bacteremia (PUERTA-GARCÍA and MATEOS-RODRÍGUEZ, 2010). Proteus mirabilis is responsible for infections in the urinary tract, side pain, hematuria, dysuria, increased frequency of urination and back pain, high fever and chills when the prostate is inflamed (HEALTH, 2016).

4. CONCLUSIONS

The presence of the microorganisms found, regardless of the frequency in which they found in the places analyzed, shows that the type of species is related to the amount of common and hospital solid waste that exists in the sector.
Knowing the diseases that produce these microorganisms and the level of danger of each one of them could be carried out a study that allows to implement preventive and corrective measures to mitigate the effects of these airborne pathogenic microorganisms in the health of nearby populations and workers of the landfills.

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EFFECT OF MIXTURES OF ORGANIC WASTES ON SOIL INDICATORS

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ABSTRACT

With aim of improving properties of a dross impacted soil, mixtures of papermaking sludge, used edible oil and inorganic nitrogen were prepared and maturated, during 60 days. Then, they were applied at soil, resulting on a decreased pH, from strongly alkaline (9.1) to close to neutrality for all systems, while the electrical conductivity was around 1000 μS cm^{-1}. The mixtures incremented the organic matter content (from close to 1% until 4%), soil microbiological respiration (from 35 mgC kg^{-1} h^{-1} to 70 mgC kg^{-1} h^{-1}) and microbial biomass carbon (150 mgC-CO^{2} kg^{-1} day^{-1} to 350 mgC-CO_{2} kg^{-1} day^{-1}).

Keywords: biochemical parameters, compound amendments, degraded soil, metal waste, organic wastes

1. INTRODUCTION

Modern agriculture currently faces several difficulties, such as the effect of anthropogenic pollution, as well as the decrease of arable soils due to the growth of urban areas or desertification (PUTRA and YULIANDO, 2015). In accordance with the above, most of the technological developments in the agrarian field focus on sustainability, since food must be guaranteed to a growing population, without damaging the environment (GODFRAY and GARNETT, 2014).

One of the techniques that can be used to remedy or minimize contamination in a soil is the use of organic amendments (CHEN and JIANG 2014; MAHAR *et al*, 2015). In this sense, using amendments from industrial wastes, sometimes, has replaced the use of fertilizers, insecticides and biostimulants (GARCÍA and RIVERO, 2011).

Steel industry generates large amounts of dross that, if not properly disposed, when coming into contact with the soil modify their physical and chemical properties. Previous studies in soil samples impacted by dross from an old steel industry (located in Tinaquillo, Cojedes State, Venezuela) indicate that their chemical and biochemical properties are affected, and suggest that those one can be used as bioindicators of pollution in a soil (ARMADO-MATUTE *et al*, 2018).

In this work, it is proposed to evaluate the effect using of mixtures of papermaking sludge (PS), used edible oil (UEO) and NH_4Cl as inorganic nitrogen (IN), as an amendment on some chemical and biochemical properties, as the first step in the remediation of soil affected by dross.

2. MATERIALS AND METHODS

2.1. Samples

Soil: Samples of soil contaminated with metals were collected on land belonging to a company dedicated to metallurgical activity, located in Tinaquillo, Cojedes state, Venezuela (9 ° 55 '26.958' 'N; 68 ° 17' 4.405 '' O), following the same scheme described by ARMADO-MATUTE *et al.* (2018). The section most exposed to metallic dross was chosen for the treatment with mixtures of PS, UEO and IN. Ten simple samples of this section were taken, to form a composite sample. The sample comes from 20 cm of the surface horizon of a sandy soil, of pH 9.3 (1:2 in water) and electrical conductivity of 282 μS/cm, a low content of organic matter (0.39%) and a moisture retention of 28.7% (DURAN, 2011). A portion of the sample was sieved and refrigerated to maintain the microbiological activity, while the other portion was left to dry in the open air for 72 hours and passed through a 2 mm sieve to perform the corresponding physicochemical tests.

Mixtures of PS, UEO and IN: three mixtures were prepared, making use of plastic containers, as follows: Mixture 1 (M1): UEO and PS 1:1; Mixture 2 (M2): UEO and PS 1:1, with 1% IN; Mixture 3 (M3): UEO and PS 1:3, with 1% IN. Allowing them to mature for 60 days.

Mixed soil systems: Systems were prepared by adding 5% of the matured mixtures, and allowing them to incubate for 10, 20 and 30 days, to subsequently determine their physicochemical and biochemical properties.

2.2. Determinations analytics

Physicochemical properties: pH and electrical conductivity (EC) were measured in water, using soil: water ratio 1:2 with pH-meter and conductimeter, respectively. Humidity

Retention (HR) was determined following procedure describe by Jackson (1970): 15 grams of air-dried substrate were placed in a funnel with glass wool, previously weighed and labeled, then distilled water was added until observed drip, and let stand at room temperature for 6 hours. Soil texture was determined using the Bouyoucos´s hydrometer method. Soil organic matter (SOM) was determined by difference between initial weight of the sample dried at 105°C, and the weight after calcination at 400 °C overnight (FAITHFULL, 2005).
Biochemical properties: soil microbiological respiration (SR) was estimated using closed incubation systems, by the method of ANDERSON (1982), while microbial biomass carbon (MB) was obtained applying the technique of substrate-induced respiration (ANDERSON and DOMSH, 1978). Both properties were determined by the procedure described by ARMADO-MATUTE et al. (2018).

2.3. Statistical analysis

All the statistical analyzes were done using Microsoft office Excel and PAST3 software. Through a Kruskal-Wallis analysis, it was determined if there were significant differences between the treatments. Spearman´s correlation was used to establish possible relations between the parameters investigated. Also a principal components analysis was carried out, in order to explain the variability of the samples.

3. RESULTS AND CONCLUSIONS

Characterization of the soil under study, evaluating parameters such as texture, pH, CE, HR and SOM, is shown in Table 1.

Table 1. Soil Studied Characteristics.

Properties	Values
HR (%)*	30.3±0.4
SOM (%)*	0.65±0.01
pH*	9.1±0.1
EC (µS/cm)*	282±10
Textural Class	Sandy

*Average ± uncertainties expressed as standard deviation, n=5.

The values obtained are like those reported by DURAN (2011). In the case of the textural class, a change from sandy loam to sandy was observed, probably because Duran used tact qualitative method. However, it is possible that the impact with dross has exerted an effect modifying the texture of the soil. It is advisable to study the texture of the soil to show if there are indeed changes due to contamination.
In general, the values obtained in the physicochemical properties (high pH and low organic matter content) indicate the need to perform a remediation of the soil impacted with metallic dross to improve its quality, to be used in a new function, in turn preserve the sustainability of the ecosystem. Therefore, the SP-UEO-IN mixture was added to the soil samples to improve the properties mentioned before.

The application of the mixtures generated an improvement in soil properties, starting by a pH descent, changing from alkaline (9.1±0.1) to neutral (7.08-7.45), in each system. At these pH values, better availability of macro and micronutrients occurs, besides more suitability for microbial growth. In Table 2 shows the pH descent from initial state for all soil/mixture systems, with a significant different with respect to control sample, but without differences over time though.

Table 2. Physicochemical properties of studied systems.

Incubation Time(Day)	Sample	pH*	EC (µS/cm)*	SOM (%)*
10	M1	7.08±0.08	595±43	2.3±0.2
	M2	7.08±0.07	869±126	2.8±0.7
	M3	7.24±0.09	795±50	2.0±0.6
	control	9.3±0.3	875±32	0.95±0.06
20	M1	7.14±0.03	529±122	1.6±0.2
	M2	7.27±0.03	724±167	2.5±0.5
	M3	7.7±0.04	554±54	1.4±0.8
	control	10.1±0.1	994±115	0.55±0.01
30	M1	7.17±0.05	539±123	1.8±0.6
	M2	7.4±0.1	649±84	1.9±0.5
	M3	7.5±0.2	562±65	1.9±0.9
	control	9.5±0.3	950±43	0.69±0.05

*Average±uncertainties expressed as standard deviation, n=3.

Regarding the EC, it can be seen in Table 2 that both mixtures M2 and M3 initially do not show significant differences with respect to control soil, which is the opposite of what happens with M1; over time the mixtures generate a decrease in EC, the organic matter contained in the mixtures possibly immobilize the ions, decreasing the salinity of the soil.
On the other hand, SOM underwent a significant increase in all systems studied, ranging from a soil with low content of organic matter (<1%) to soil with medium content (> 1% SOM <3%), as evidenced in Table 1. It's known that compounds present in the added organic matter are used by the microbiota as energy sources and molecules precursors for the synthesis of biomolecules necessary for the conformation of cellular structures, enzymes, coenzymes and typical cellular functions (ARMADO-MATUTE *et al.*, 2018).
In the Fig. 1, is viewed the microbiological indicators variation when SP-UEO-IN mixtures are incorporated in the impacted soil.
In SR a significative increment was obtained, during the first 20 days of incubation, then a decrease was observed. This gradual descent of respiration can be ocassionate by the presence of material with high degradation resistant (GARCÍA and RIVERO, 2011). In M1 and M3 mixtures a significant difference with respect to control was observed over time though.
Regarding the carbon of the MB, in the first 10 days of incubation, all soil-mixture systems showed a significant increase compared to the control, attributed basically to the incorporation of biodegradable organic matter, which stimulates the growth native microbial and/or the incorporation of exogenous microorganisms to the soil proper of the added mixture (ROS *et al.*, 2006). After 20 days of incubation, a decrease is observed, possible due to the depletion of easily degraded organic matter. However, for the systems

with the M2 and M3 mixtures the values obtained at 20 and 30 days of incubation are significantly greater than the control. This observed behavior for soil-mixture systems indicates that mixtures containing nitrogen have a positive effect in terms of MB. In summary, M3 mixture positively affected both SR and MB.

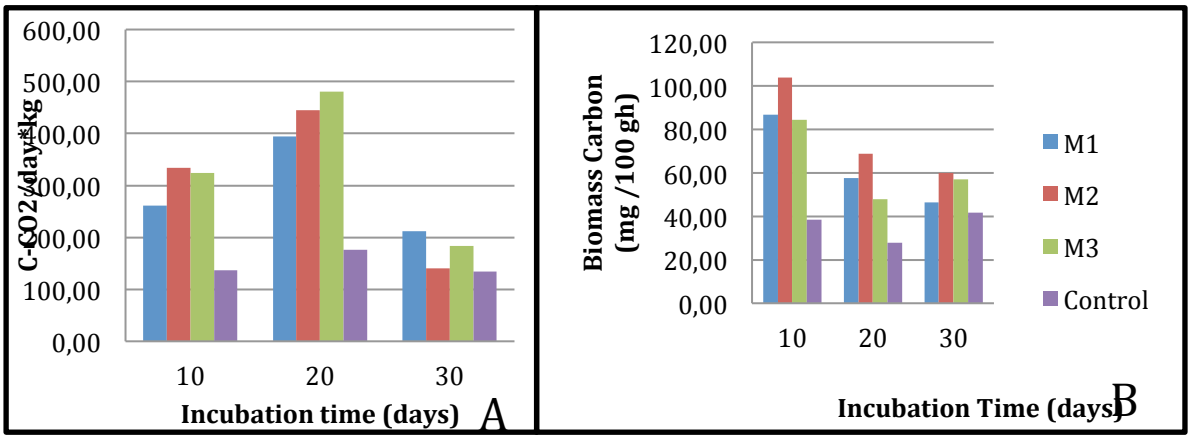

Figure 1. Microbiological parameters at different incubation times.
A. Soil microbiological respiration (SR) B. Microbial biomass carbon (MB).

According to the Spearman coefficient (Table 3), the pH is inversely proportional correlated to the SOM and the two biochemical factors studied (SR and MB). This effect is expected when the mixture is added to the soil, because neutral pH favors the microbial growth conditions. On the other hand, the SOM presented a directly proportional correlation with MB. It is reported that the soil microbiota requires energetic sources to replenish the homeostatic state of the cell (SULMON *et al*., 2015). Consequently, the oxidation of carbonated molecules present in the habitat where organisms proliferate provides energy for their metabolism.

Table 3. Spearman-s correlation for physicochemical and biochemical properties.

	EC	SOM	pH	MB
SR	-0.52448	0.49037	-0.63748	0.53147
	0.080019	0.10555	0.025754	0.075362
EC		-0.22417	0.53941	-0.23776
		0.48367	0.070306	0.4568
SOM			-0.73333	0.9317
			0.0066476	0.0000140
pH				-0.7951
				0.0019935

Probability level $P < 0.05$.

When carrying out principal components analysis (PCA), it was determined that with only two components 99.067% of the variability of the sample can be explained, as shown in Table 4.

Table 4. Principal components analysis.

PC	Eigenvalue	% variance	Acumulate Variance
1	32914.6	72.828	72.828
2	11858.7	26.239	99.067
3	421.397	0.9324	99.9994
4	0.0928234	0.00020539	99.99960539
5	0.0263859	5.84E-05	99.99966377

The Fig. 2 shows the correlation coefficient of each variable with the 2 principal components studied (PC1 and PC2). It is observed that the pH and the EC have the highest correlation with PC1 (explain 72.828% of the variability) and the SR with the PC2. It is clear that pH and conductivity have the greatest effect on the variability of the samples, indicating that they were the most influential parameters in the variations of soil properties, when the mixtures of organic materials were added.
In conclusion, physicochemical and microbiological parameters studied worked adequately as indicators of improvement dross impacted soil when treated with SP-UEO-IN mixtures. In addition, it can be stated that mixtures were effective for the improvement of the physicochemical and biochemical properties evaluated, with pH and EC being the properties for which a greater improvement was observed. It is possible, then, to recommend the application of these mixture types as a first step for remediation of these soils.

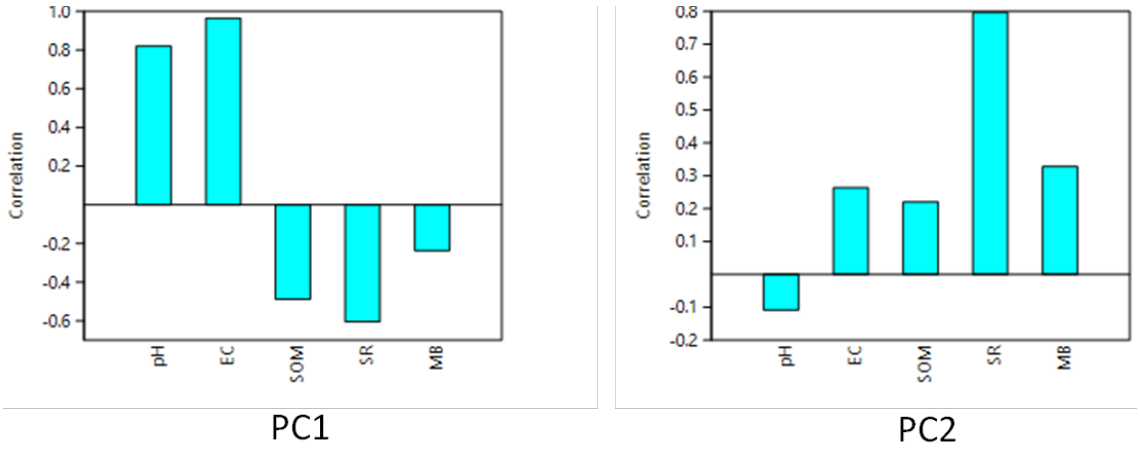

Figure 2. Indicators' correlation with principal components.

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PRODUCTION OF ANTIFUNGAL COMPOUNDS BY ACTINOMYCETES ISOLATED FROM ANDEAN AND ANTARCTIC SOILS

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ABSTRACT

A preliminary screening to determine the production of bioactive compounds to control plant pathogenic fungi that belong to the genera *Alternaria*, *Botrytis*, *Fusarium* and *Phoma* was examined for eight actinomycete strains isolated from Andean and Antarctic soils. Phylogenetic analyses of partial sequences of the 16S rDNA gene showed that five strains were closely related to *Streptomyces fildesensis* GW25-5[T], two to *Streptomyces griseus* ISP 5236[T] and one to *Streptomyces olivochromogenes* DSM 40451[T]. Only one actinomycete strain could inhibit the growth of all four pathogens while the rest had different degrees of control. The best results were obtained for the plant pathogens *Alternaria* and *Phoma* since all the actinomycetes could stop their growth. For *Fusarium*, only the *S. griseus* strains were able to show efficiency in controlling it. This study shows that actinomycetes are a prolific source of bioactive compounds.

Keywords: Streptomyces, antifungal compounds, 16S rDNA gene, fungal plant pathogens

1. INTRODUCTION

Actinomycetes are a diverse group of filamentous bacteria that can produce mycelium and spores (BARKA *et al*., 2016). As part of their growth and development, they produce secondary metabolites with many applications in biotechnology (AL_HUSNAN and ALKAHTANI, 2016, BARKA *et al*., 2016, DE LIMA PROCÓPIO *et al*., 2012). They are present in different ecosystems fulfilling a central role as secondary decomposers of organic matter. Their main reservoir is in the soil, however, in the last decade, a large number of actinomycete new species have been discovered in non-traditional places like marine sediments (DHAKAL *et al*., 2017, DUNCAN *et al*., 2014), plant tissue (ÁLVAREZ-PÉREZ *et al*., 2017, GOLINSKA *et al*., 2015), desert soils (MOHAMMADIPANAH and WINK, 2016) and mangrove mud (AZMAN *et al*., 2015, LEE *et al*., 2014). Often these discoveries have been accompanied with finding novel leads in active metabolic compounds, such as antibiotics (SHEN, 2015, WOHLLEBEN *et al*., 2016). Actinomycete secondary metabolites have been used to develop many drugs that are commercially successful. The primary focus in the discovery of new molecules has been centred in antibiotics to combat the rise of resistant bacteria that causes many lethal human diseases.
Plant pathogenic fungi could cause a total loss in the production of a varied range of crops. Traditionally, the primary tool for control is the use of fungicides that often are not only harmful to the pathogen, but also, they can accumulate in the plant where they could pose a risk to humans and the environment (GEISSEN *et al*., 2010). In developing countries there is an increase of resistance in many plant pathogens like *Botrytis* and *Fusarium*, causing a direct impact on the fragile economy of farmers (HOBBELEN *et al*., 2014). It is evident that new approaches are needed to find molecules that are effective in controlling plant pathogens and are innocuous to humans and the environment.
The main objective of the present study was to determine the ability of actinomycetes isolated from soils collected in the extreme cold environments of Antarctica and the Andes to produce bioactive compounds to control the pathogenic fungi *Alternaria, Botrytis, Fusarium* and *Phoma*. A polyphasic taxonomy approach was also used to determine the taxonomic position of the isolated actinomycete strains within the Actinomycetales. This study tries to establish the foundations towards developing a bioproduct useful in agriculture that will be able to control plant pathogenic fungi that pose a significant risk in the production of many important commercial crops.

2. MATERIALS AND METHODS

2.1. Selective isolation of actinomycetes

2.1.1. Soil samples

Soil samples were obtained during the XIV, XV and XVI Ecuadorian Scientific Summer Expeditions to Antarctica, spanning from 2010 to 2012. Sites were located near the Pedro Vicente Maldonado Research Station, Greenwich Island, in the South Shetland Archipelago. The soil samples were collected from the rhizosphere of the vascular plant *Colobanthus quitensis*. Additionally, in 2014 soil were collected on the slopes of Mount Chimborazo from the rhizosphere of the native Andean plants *Culcitium rufescens* and *Chuquiraga jussieui* (Table 1). At each site, a composite sample was prepared from three samples collected at a depth of 0 - 15 cm and stored at 4°C. Physicochemical characteristics of the soils were determined according to the methods by RODRÍGUEZ *et al*. (2018).

Table 1. Details of collection data of the soil samples.

Soil ID	Origin	Altitude (m.a.s.l)	Collection year	Coordinates
S1	Greenwich Island	15	2010	62°26′57″S 59°44′29″W
S2	Greenwich Island	15	2011	62°26′57″S 59°44′29″W
S3	Greenwich Island	15	2012	62°26′57″S 59°44′29″W
S4	Mount Chimborazo	4000	2014	1°27'22"S 78°50'1"W
S5	Mount Chimborazo	4500	2014	1°27'22"S 78°50'1"W

2.1.2. Isolation and purification of actinomycete strains

Colonies resembling actinomycete morphology were isolated from each of the samples. Ten grams of each soil were added to 90 ml of sterile tap water. Serial dilutions were prepared down to 10^{-3}, and 100 uL from dilutions 10^{-1}, 10^{-2} and 10^{-3} were spread over the surface of two sets of arginine vitamins agar (AVA) and starch casein agar (SCA). All plates containing the different media formulations were supplemented with nystatin (75 ug/mL) and adjusted to pH 7. After the inoculation procedure was completed, the plates were left to dry at room temperature for half an hour and then incubated at 20°C, for three weeks. The colonies growing on the surface of the many media were expressed as colony forming units per dry weight (ufc/g). Seventeen actinomycete colonies were isolated using sterile toothpicks and seeded on glucose yeast extract mal extract agar (GYM) or oatmeal agar (OA) plates and incubated at 20°C. The resultant microbial cultures were examined by eye to discard any contamination. All pure cultures were used to inoculate fresh agar plates and to prepare cell or spore suspensions for long-term preservation in 20% glycerol (w/v) and the phenotypic tests in sterile tap water.

2.2. Phenotypic characterisation

2.2.1. Colour grouping of the isolates

Microbial cultures that were grown on glucose yeast extract agar (GYM) and oatmeal agar (OA) plates were used to determine the colour of the aerial spore mass, substrate mycelium and any diffusible pigments. In both cases, a standard colour chart from the British Standard Specification for Colours for Identification Coding and Special Purposes was used. Strains that shared the same pigments were grouped to form preliminary colour groups.

2.2.2. Microscopic characterisation
Sterile coverslips were inserted in GYM plates that had been previously streaked with a fresh actinomycete inoculum. After a three weeks incubation period at 20°C, the coverslips were taken out of each plate and fixed on the surface of a glass slide. With the help of a light microscope, the spore chain morphology was determined for each actinomycete strain.

2.2.3. Physiological tests

Five microliters of the spore aqueous suspensions were used to inoculate plates with different media formulations and incubation conditions. Plates containing GYM agar were used for the temperature assays. The inoculated plates were incubated at 4°C and 37°C. For

the pH range growth determination, GYM agar plates were prepared at different values from 4.5 up to 8.5. The pH was adjusted using the buffer system proposed by McILVAINE (1921). The inoculated plates were incubated at 20°C. Finally, tolerance to concentrations of 1%, 10% and 15% of NaCl (w/v) were determined. GYM agar plates were supplemented with the corresponding amount of NaCl studied. The inoculated plates were incubated at 20°C. For all physiological tests, after the incubation period, the presence of growth was recorded as 1, and absence as 0.

2.3. Preliminary screening for antifungal compounds production

2.3.1. Test phytopathogenic fungi

Four phytopathogenic fungi that belong to the genera *Alternaria*, *Botrytis*, *Fusarium* and *Phoma* were isolated from necrotic plant tissue. Macroscopic and microscopic characteristics were determined, and comparisons with taxonomic descriptions were made to confirm their identification. The four fungi were grown on potato dextrose agar plates (PDA) at 24°C for two weeks to assure that enough sporulation occurred. A primary spore suspension was prepared by transferring aerial mycelium with spores from the plates to a flask containing tap distilled water. This spore suspension was used to prepare PDA plates inoculated with the fungal pathogens at a concentration of 1×10^3 spores x mL.

2.3.2. Screening to determine the production of antifungal compounds

Potential production of antifungal compounds was determined for the seventeen actinomycete strains using a modified protocol based on the agar plug diffusion method (BALOUIRI *et al*., 2016). Actinomycete strains were grown on GYM agar plates for three weeks at 20°C. After the incubation period, with the help of a sterile cork borer, agar cylinders of 6 mm of diameter were obtained and placed upside down, that is, with the actinomycete mycelium growth in contact with the surface of the PDA test plates that had been inoculated with each of the four phytopathogenic fungi. Five uL of nystatin (75 mg/mL) were also laid on the surface of the plates. The test plates were incubated for 72 hours at 20°C. After the incubation time, the polar and equatorial diameters of any inhibition zone were measured. Finally, a comparison was made with the size of the inhibition zone obtained with the nystatin and reported as a percentage of control by the actinomycete strains on the growth of each phytopathogenic fungi.

2.4. Numerical taxonomy analyses

All the results from the phenotypic characterisation and screening for bioactivity were used to construct a data matrix in binary code. The data were analysed with the NTSYS software suite (ROLF, 1998). Similarities were calculated using the simple matching coefficient (Ssm) and Jaccard's coefficient (SJ). Clustering was achieved by the UPGMA (Unweighted Pair Group Method with Arithmetic Means) algorithm. Phenetic clusters were identified by analysing the results from the two resulting dendrograms. Finally, a principal component analysis (PCA) was performed to establish the phenetic relationships among the clusters in a tridimensional graphics.

2.5. Molecular Identification of the Isolates

2.5.1. Extraction of genomic DNA

The actinomycete strains were grown over cellulose membranes (Ø 0.45 um), that had been placed on the surface of non-sporulating agar. The inoculated plates were incubated for five days at 26°C. In all cases, a loopful of fresh actinomycete biomass (ca. 100 mg) was suspended in 500 uL of extraction buffer (50 mM glucose, 25 mM Tris-HCl -pH 8-, 10mM EDTA; pH8) containing acid-washed glass beads (Ø 106 μm). The preparations were kept at -10°C for 24 hours, vortexed for 15 minutes and centrifuged at 14000 rpm for 20 minutes. Separation of DNA from the cell debris was achieved by mixing each supernatant with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v) followed by centrifugation at 14000 rpm for 15 minutes. DNA from the aqueous phase was precipitated with two volumes of cold ethanol, washed twice with 70% ethanol and centrifuged at 14000 for 15 minutes. The preparations were air-dried for 4 hours at room temperature and each of the dried pellets resuspended in 50 uL of TE buffer (10 mM Tris – HCl, 1mM EDTA, pH 8.0). To ensure that the DNA samples were not excessively sheared and fragmented, 2 uL of each sample were mixed with 2 uL of loading buffer (Invitrogen) and loaded into a 1% agarose gel (Sambrook *et al*., 1989). A 100bp molecular size marker (Invitrogen) was used as a standard. Electrophoresis was carried out in 0.5X TBE running buffer at 100V for forty-five minutes. Genomic DNA was visualised using the ChemiDoc Imagin System (Bio-Rad Laboratories, Hercules, U.S.A.).

2.5.2. 16S rDNA sequencing and data analysis

Genomic DNAs from eight representative actinomycete strains were used as a template for PCR amplification and direct sequencing of 16S rRNA genes as described by KIM *et al*. (1998). The resultant 16S rRNA gene sequences were aligned manually, using the pairwise alignment option and 16S rRNA secondary structural information in the PHYDIT program (CHUN, 1995), against corresponding sequences of representatives of the genus *Streptomyces* retrieved from the GenBank database. Phylogenetic trees were inferred using the least-squares (FITCH and MARGOLIASH, 1967), maximum-likelihood (FELSENSTEIN, 1981), maximum-parsimony (Kluge and Farris, 1969) and neighbour-joining (SAITOU and NEI, 1987) tree-making algorithms from the PHYLIP suite of programs (FELSENSTEIN, 1993). Evolutionary distance matrices for the least-squares and neighbour-joining methods were generated after Jukes and Cantor (1969). The resultant unrooted tree topologies were evaluated by bootstrap analysis (FELSENSTEIN, 1985) of the neighbour-joining dataset based on 1000 resamplings **using the TREECON program (VAN DER PEER, 2001).**

3. RESULTS AND DISCUSSION

3.1. Selective isolation and enumeration of actinomycetes

The physicochemical characteristics of the rhizospheric soils, number of isolates and cfu/g numbers are shown in Table 2. The highest ufc/g count ($1{,}26 \times 10^{4} \pm 5{,}09 \times 10^{3}$) was found for the soil collected in Antarctica in 2010. On the contrary, the lowest count was found in the soil sample from Chimborazo, at 4500 m.a.s.l ($2{,}26 \times 10^{3} \pm 6{,}38 \times 10^{2}$ c.f.u/g). In general, the actinomycete numbers in the samples were similar to other studies from cold environments (WANG *et al*., 2014). It is important to notice that the Antarctic soils yielded

higher c.f.u/g per dry gram weight than the ones from Mount Chimborazo. A possible explanation could be attributed to the water content in the soil samples. If we remove the water content datum from sample S2, there is strong negative correlation coefficient (r = -0,91) between water content in the soil and the actinomycete count (Fig. 1). When the whole set of data is taken into account to perform the correlation analysis, only a moderate negative correlation was found between these variables (r = -0,56). In any case, actinomycetes are likely to form spores when the water content decreases in the soil as a mean of preservation from what could be perceived as a detrimental environmental change. Many selective isolation techniques developed for actinomycete studies usually dry the soils to stimulate spore formation and thus increase the probability to obtain higher actinomycete count and diversity. No other direct correlation was found between the physicochemical properties of the soils and the c.f.u/g per dry weight soil. From the selective isolation plates, seventeen actinomycetes were isolated in pure cultures (Tables 2 and 3). From soil samples S1 and S4 it was possible to isolate the majority of the strains. From sample S3 only one actinomycete was grown as a pure culture despite that in this soil there was a high actinomycete count. Regarding the selective isolation media, the majority of isolated actinomycetes were obtained from the SCA plates (twelve strains). Only five actinomycete cultures were isolated from the AAV plates. Finally, ten strains were isolated from the Antarctic soils while seven were recovered from the Mount Chimborazo soils.

3.2. Phenotypic characterization

Table 4 shows the assignment of the seventeen actinomycete strains to colour groups and the results for the phenotypic characterisation. The three colour groups formed according to the aerial spore mass pigmentation are very homogeneous since for almost all the test carried out, the members in each group show the same results. Only in colour group one, there are small differences that could be explained by the size of the group. It is possible that in a large set of individuals the probability to find a more considerable diversity is higher than if the group is represented only by a few microbial strains. In colour group 1, strain JV-043 showed different phenotypic results since it was the only one to grow at 4°C, pH 4,5 and 8, and NaCl concentrations in the culture media of 10% and 15%. In any case, these results can be used to separate the three groups based on phenotypic characteristics because in all the test performed there are marked differences in the results.
Numerical taxonomy analyses showed that there is strong congruence between the colour grouping of the isolates and the phenetic clusters (Fig. 2). The same topology was found when the similarity was calculated either with the S_{SM} or the Jaccard coefficients. These findings were supported as well for the PCA analysis (Fig. 3). Seven representative strains from the three phenetic clusters were selected for 16S rDNA gene sequencing.

Table 2. Physicochemical characteristics, total actinomycete count numbers and number of isolates obtained from cold rhizospheric soils.

Soil ID	pH	Electrical conductivity (uS/cm)	Water content (%)	Organic matter content (%)	c.f.u per gram dry soil (cfu/g)	Number of isolates
S1	8,63	185	3,37	6,88	$1,26x10^4 \pm 5,09 \times 10^{3\ a}$	6
S2	7,27	204	3,57	3,68	$3,60x10^3 \pm 1,27 \times 10^{3\ a,b}$	3
S3	7,25	192	4,23	5,42	$1,22x10^4 \pm 6,37 \times 10^{2\ a}$	1
S4	8,59	562	35,93	13,68	$6,32x10^3 \pm 1,28 \times 10^{3\ a,b}$	5
S5	8,32	451	32,15	8,89	$2,26x10^3 \pm 6,38 \times 10^{2\ c}$	2

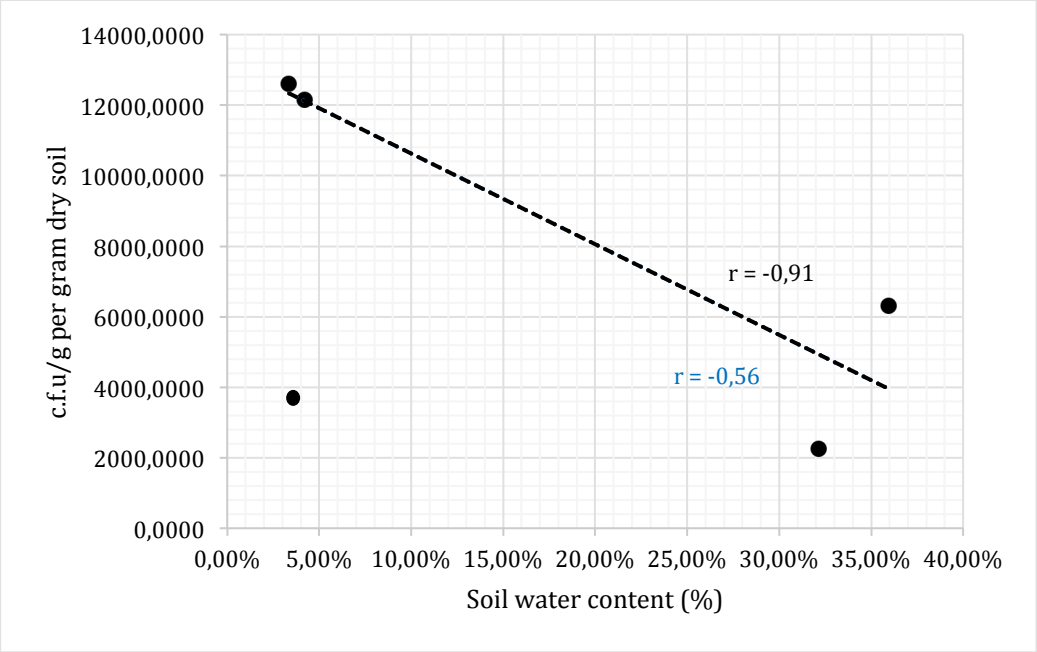

Figure 1. Pearson correlation analyses between soil water content and actinomycete colony forming units per gram dry weight soil.

Table 3. Origin and codification of actinomycetes isolated from Andean and Antarctic rhizospheric soils.

Soil Sample	Actinomycete strains
S1	JV-009, JV-012, JV-013, JV-017, JV-031*, JV-034*
S2	JV-014, JV-015, JV-036*
S3	JV-039*
S4	JV-019, JV-021, JV-022, JV-026, JV-043*
S5	JV-027, JV-029

*Strains isolated from AAV plates. All the rest were isolated from SCA plates.

Table 4. Actinomycete strain assignment to colour groups and frequency of positive characters found in each group.

	Colour groups		
	1	**2**	**3**
Number of strains	**12**	**3**	**2**
ID actinomycete strains	JV-009, JV-012, JV-013, JV-017, JV-019, JV-026, JV-027, JV-029, JV-031, JV-034, JV-036, JV-043	JV-014, JV-015, JV-039	JV-021, JV-022
Aerial spore mass colour			
Olive grey	0	0	2
Grey	0	3	0
White	12	0	0
Diffusible pigment in OA	12	3	0
Spore chain morphology			
Straight	12	0	2
Spirals	0	3	0
Temperature range growth			
4°C	1	0	0
28°C	12	3	2
37°C	0	0	0
pH range growth			
4,5	1	3	2
5,5	12	3	2
6,5	12	3	2
7,5	6	3	2
8,5	1	0	0
NaCl range growth (w/v)			
1%	12	3	2
10%	1	0	0
15%	1	0	0

3.3. Production of antifungal compounds

All seventeen actinomycete strains were able to produce bioactive compounds with various degrees of efficacy against the four phytopathogenic fungi used for this study (Table 5). Strain JV-015 was able to inhibit the growth of all four pathogens whereas strains JV-022 and JV-036 could produce metabolites to control only *Alternaria* and *Botrytis*. The remaining strains were able to stop the growth of three pathogens. Most of them controlled *Alternaria*, *Botrytis* and *Phoma*. Finally, strain JV-039 produced bioactive compounds against *Botrytis*, *Fusarium* and *Alternaria*. Regarding the percentage of strains that produced anti-fungal compounds to control the fungal plant pathogens were as follow: *Alternaria* (94%), *Botrytis* (88%), *Fusarium* (12%) and *Phoma* (100%). It is evident that the majority of actinomycete strains did not possess the metabolic ability to produce antifungal compounds against *Fusarium*.

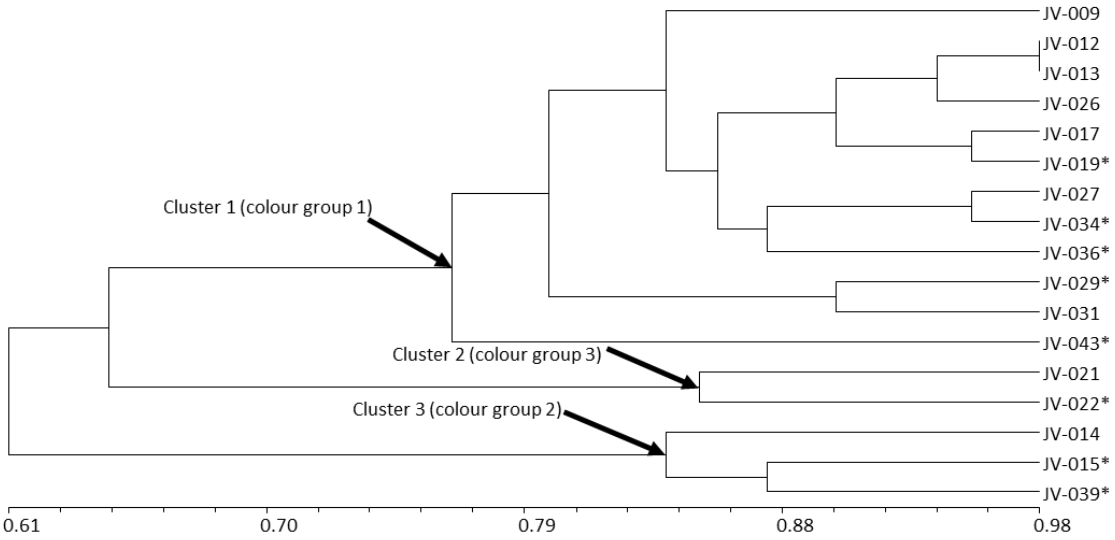

Figure 2. Dendrogram based on the analysis of phenotypic data of seventeen actinomycete strains. Clustering was achieved by the UPGMA (Unweighted Pair Group Method with Arithmetic Means) algorithm from a similarity matrix calculated using the single matching coefficient (SSM). All three phenetic clusters were also recovered using the Jaccard's coefficient (SJ). Asterisks denote representative strains that were selected in each phenetic cluster for 16S rDNA gene sequencing.

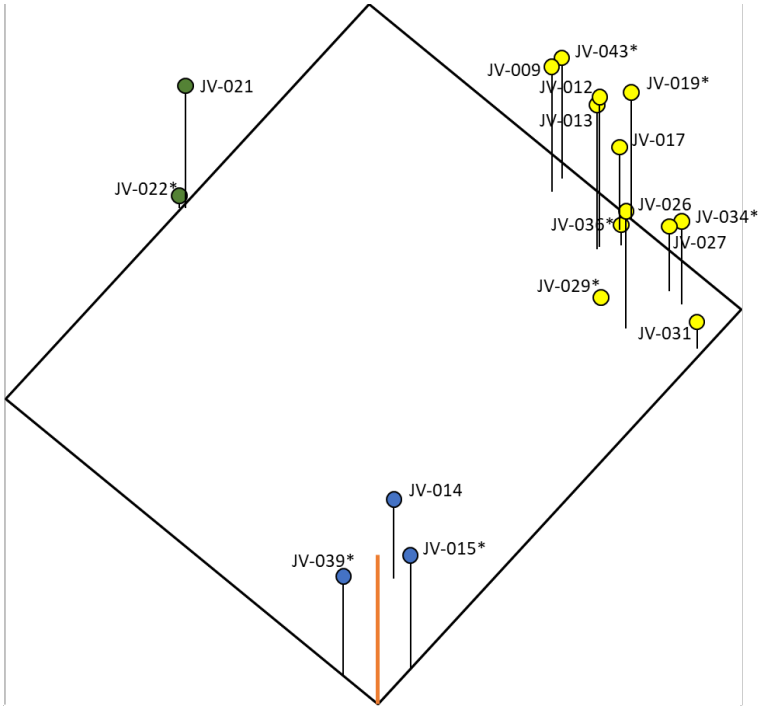

Figure 3. Principal component analysis based on the similarity calculated with the S_{SM} coefficient. Asterisks denote representative strains that were selected in each phenetic cluster for 16S rDNA gene sequencing.

Table 5. Diameter of growth inhibition (mm) produced by the actinomycete isolates in the screening plates inoculated with plant pathogenic fungi.

Strain ID	Phytopathogenic fungi			
	Alternaria	*Botrytis*	*Fusarium*	*Phoma*
JV-009	11	15	0	13,5
JV-012	12	11	0	12,5
JV-013	11	14	0	11
JV-014	10	5	0	11,5
JV-015	11,5	9	8,5	9,5
JV-017	8,5	15	0	13
JV-019	12	11	0	12
JV-021	11	11	0	12
JV-022	10,5	0	0	11,5
JV-026	12	5	0	12,5
JV-027	11,5	8,5	0	11,5
JV-029	10,5	7	0	10
JV-031	9	10	0	10
JV-034	12	10	0	11
JV-036	11	0	0	11
JV-039	0	10	7	10
JV-043	13	15	0	14

It is important to point out that there are not literature reports where they had mentioned the ability of members of these *Streptomyces* species to produce antifungal compounds against the plant pathogenic fungi used for this study. On the other hand, the three streptomycetes are prolific producers of antibacterial compounds. This probably is the first study that has demonstrated that these actinomycetes are able to produce bioactive compounds for a potential use in agriculture.

3.3. Molecular identification of the isolates

Partial 16S rDNA gene sequences were obtained for seven representative actinomycete strains chosen from the phenetic clusters. The length of the partial sequence for each strain is shown in Table 6. Molecular Identification with similar sequences retrieved from the GenBank database (Fig. 4) showed that members of phenetic cluster one were closely related to *Streptomyces fildesensis* (GW25-5^T), phenetic cluster 2 to *Streptomyces olivochromogenes* (DSM 40451^T) and strains from cluster 3 to *Streptomyces griseus* (ISP 5236^T). The clades that were formed with these *Streptomyces* species were supported with bootstrap values higher than 90%, thus showing the robustness of the result. The percentage of similarity and nucleotide differences between these strains are shown in Table 6. It looks like all the strains isolated in this study belong to *Streptomyces* species that have been already discovered and do not belong to new centres of taxonomic variation.

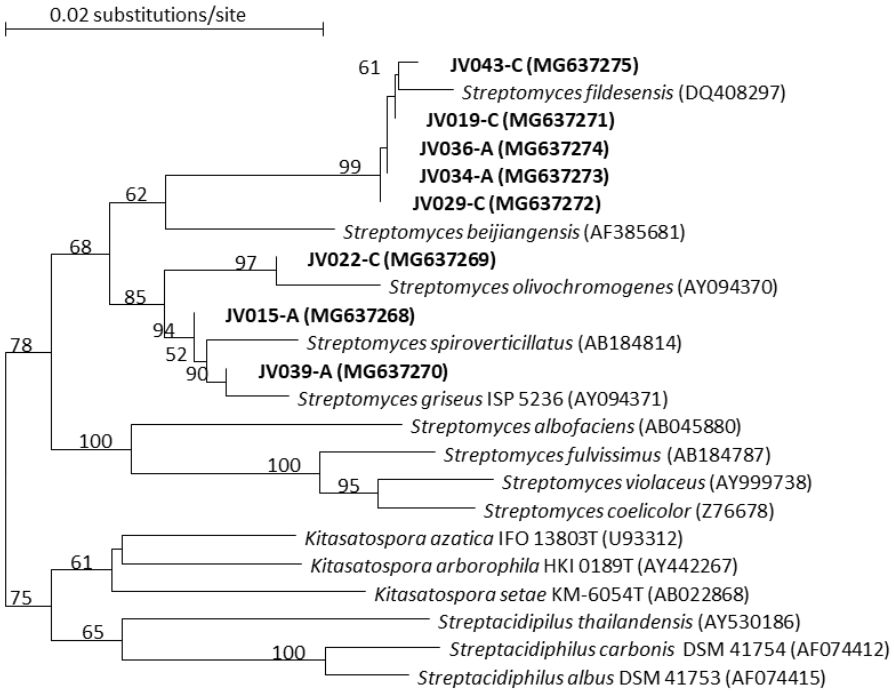

Figure 4. Neighbour-joining tree (Saitou & Nei, 1987) based on partial 16S rRNA gene sequences showing the positions of representative actinomycete strains isolated from Andean and Antarctic rhizospheric soils, closely related *Streptomyces* species and representative members of the genera *Kitasatospora* and *Streptacidiphilus*. The numbers at the nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50% are given. The scale bar indicates 0.1 nucleotide substitutions per nucleotide position. T, type strain.

Table 6. 16S rDNA gene nucleotide length, similarity (%) and the number of different nucleotides of representative actinomycete strains from the phenetic groups and related *Streptomyces* species.

	ID Strain	16S rDNA gene (nt.)	1	2	3	4	5	6	7	8	9	10	11
1	***S. griseus***	1449	---	29	46	3	20	3	22	35	20	36	44
2	***S. olivochromogenes***	1448	97,9	---	51	10	6	15	22	38	19	35	49
3	***S. fildesensis***	1447	96,6	96,2	---	35	33	36	4	5	4	6	6
4	**JV015**	1146	99,7	99,1	96,9	---	13	0	20	31	18	33	32
5	**JV022**	1195	98,3	99,5	97,2	98,8	---	17	21	30	18	27	31
6	**JV039**	1258	99,8	98,8	97,1	100,0	98,6	---	20	32	18	33	34
7	**JV019**	772	97,2	97,2	99,5	97,4	97,3	97,4	---	0	0	0	0
8	**JV029**	1243	97,2	96,9	99,6	97,3	97,5	97,4	100,0	---	0	2	2
9	**JV034**	773	97,4	97,5	99,5	97,7	97,7	97,7	100,0	100,0	---	0	0
10	**JV036**	1080	96,7	96,8	99,4	96,9	97,4	96,9	100,0	99,8	100,0	---	2
11	**JV043**	1305	96,6	96,3	99,5	97,2	97,4	97,3	100,0	99,8	100,0	99,8	---

4. CONCLUSIONS

Members of the genus *Streptomyces* are prolific producers of bioactive compounds. Many studies have demonstrated that they can produce a wide range of antibacterial compounds. However, little is known about the production of metabolites to control fungi. This study has shown that seventeen actinomycetes isolated from rhizospheric soils from Antarctic and Andean soils can control phytopathogenic fungi and therefore they could be used to develop a bioproduct to control these plant pathogens successfully. As part of this study, a polyphasic approach was used, and phenotypic and genotypic tests were performed. The actinomycete strains were divided into three phenetic clusters. Representative strains were chosen from these groups and identified as members of *Streptomyces fildesensis*, *Streptomyces griseus* and *Streptomyces olivochromogenes*. Further studies are needed to explore ways to identify the chemical nature of the bioactive compounds and to successfully express the metabolic pathways responsible for their synthesis towards the development of a bioproduct useful for agriculture.

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LIGNITE AS ORGANIC AMENDMENT IN A DROSS AFFECTED SOILS

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ABSTRACT

Lignite is a mineral with a high content of organic matter. In this work, the potential use of lignite as organic amendment in soils impacted by metallurgical activity was evaluated. The experiments were carried out with contaminated soils spiked with known amounts of lignite, and the content of Fe, Zn, Ni, Pb, Cr and Co in fractions extracted with EDTA was determined. Bioassays of acute toxicity with seeds *Raphanus sativus* did not indicate presence of phytotoxic substances up to 10% of added lignite. Therefore, this proportion could be used as a viable alternative for remediation in soils contaminated by slag.

Keywords: bioassay, bioavailable metal, lignite, slag, remediation

1. INTRODUCTION

Heavy metals are one of the main pollutants of terrestrial and aquatic ecosystems, due to their toxicity. Specifically, in soils, heavy metals can be retained or mobilized by different biological and chemical mechanisms (SOLANO, 2005). Some factors that influence the mobilization of heavy metals in the soil are: soil characteristics (pH, cation exchange capacity, organic matter, texture, among others), nature of the contamination (origin of metals and deposition form) and environmental conditions (acidification, changes in redox conditions, variation of temperature and humidity), and others (JUNG, 2008; OLANIRAN *et al.*, 2013).

The determination of metals in soils is of great importance, and has applications in different fields. For example, in the study of anthropogenic pollution by different industries, to establish remediation processes, and even in environmental legislation for the establishment of basal levels, which allow to establish permissible values in soils for different uses.

The steel industries generate large amounts of metallic dross that, if not properly disposed, when they come into contact with the ground modify its physical and chemical properties. Studies previously conducted on soil samples impacted by dross, from an old steel industry (located in Tinaquillo, Cojedes State, Venezuela) indicate that their physical, chemical and biochemical properties are altered, due to the presence of metals, in concentrations considered permissible by current Venezuelan legislation (ARMADO *et al.*, 2018).

One of the techniques that can be used to remedy or minimize metal contamination in a soil is the use of organic amendments (MAHAR *et al.*, 2016; MAHAR *et al.*, 2015). In this work, the use of lignite is proposed as an organic amendment, firstly to reduce the pH of the soil due to its acidic nature, and, on the other hand, to evaluate the immobilization capacity of metals, and keep them in non-toxic concentrations, because when the pH decreases, the availability of the different polluting metals increases.

2. MATERIALS AND METHODS

2.1. Samples

Soil: Samples of soil contaminated with metals were collected on land belonging to a company dedicated to metallurgical activity, located in Tinaquillo, Cojedes state, Venezuela (9°55'26.958'' N; 68°17'4.405''O). Following the same scheme described by (ARMADO *et al*., 2018), samples were taken from the affected area divided into nine sections (1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 3c) of 4000 m^2 each, and the control area of 20,000 m^2. For the treatment with lignite, sections 1b and 2b were selected, as they show the highest metal content available.

Lignite (L): Lignite that was incorporated as an organic amendment used in this study was Lignite A (LA), previously characterized by (LIZCANO, 2011).

Soil-LA systems: A total of 24 soil-LA incubation systems (microcosms) were prepared, 12 systems for each affected area (1b, 2b). They were placed in anime containers to allow a greater surface area of contact soil-LA. Each system was prepared in quadruplicate with a total mass of 600g, adding quantities of lignite (0, 2, 4, 6, 8 and 10% w/w of lignite in soil). Samples were stored for 2 months at room temperature, verifying the moisture content every 7 days, in order to keep soil moist (between 45 and 50% of its moisture retention capacity). After that time, the corresponding analytical determinations were made.

2.2. Analytic determinations

Physicochemical properties: pH and electrical conductivity (EC) were measured in water, using soil: water ratio 1:2 with pH-meter and conductimeter. Organic matter (OM) was determined by weight difference between the initial weight of the sample (dried at 105°C) and after calcination at 400°C overnight (FAITHFULL, 2005). For determination of cation exchange capacity (CEC), 1M ammonium acetate pH 7 method was used (JARAMILLO, 2002).
Determination of available metals: Methodology proposed by FAITHFULL (2005), which uses EDTA as a complexing agent, was used. The extractant solution contained 0.05 M EDTA, 0.1 M triethanolamine, 0.01 M calcium chloride dihydrate dissolved with water at pH 7. All the reagents were of analytical grade purity or higher. The extracts were subjected to a stage of acid digestion, prior to the determinations, to guarantee total clarification of the extract. The digestion was carried out in the following manner: 60 min at 60°C, then 90 min at 100°C and finally 30 min at 200°C. It was cooled (approximately 6°C) and filtered with quantitative filter paper, then gauging with 0.14 M HNO_3 in a 25 mL flask. Just before use, standard solutions of cobalt (0.5-10 mg/L), chromium (0.2-10 mg/L), iron (0.3-10 mg/L), nickel (0.3-10 mg/L), lead (1.0-10 mg/L) and zinc (0.05-2 mg/L) were prepared daily from their respective monoelemental aqueous standards (Merck) of 1 g/L for every metal. The standards were diluted with 0.14M HNO_3 solution (Merck). The wavelength used for the determination of each metal was Co 240.7 nm, Cr 357.9 nm, Fe 248.3 nm, Ni 232.0 nm, Pb 283.3 nm, Zn 213.9 nm (ZAGAL and SADWAKA, 2007). The available metals concentrations Co, Cr, Fe, Ni, Pb, Zn were quantified in each acid extract by a flame atomic absorption spectrometer (Thermo Electron Corporation, S-Series AA).

2.3. Bioassays with radish seeds (*Rhaphanus sativus*)

Germination index (GI) was used to evaluate phytotoxic potential of the extracts obtained from the prepared systems.
Positive control: once metals were quantified in soil-LA systems, salts of the two that were found in higher concentrations (Fe and Zn) were used to prepare solutions at different concentrations (10%, 5% and 1%).
Negative control: a solution of reconstituted hard water was prepared, containing 0.6 g of $MgSO_4$, 0.96 g of $NaHCO_3$ and 0.04 g of KCl dissolved in 4.75 L of distilled water and 0.6 g of $CaSO_4.2H_2O$ in 0.25 L of distilled water, maintaining the pH at 8 (ESCOBAR and PEREIRA, 2009).
Leachate: 10 g of amended soil sample were weighed in 100 mL of reconstituted water, stirred for 1 hour, left to stand for 24 hours and filtered with quantitative paper Whatman 41 (VALERIO *et al.*, 2007). Then dilutions of the concentrated leachate (10%) were prepared, obtaining the 0.1, 1 and 5% solutions.
Germination systems: A disk of filter paper was placed in Petri dishes previously identified. The filter paper was saturated with 5 mL of the leachate, avoiding the formation of air pockets. Ten seeds were carefully placed, leaving enough space between them to allow elongation of the roots. Petri dishes were left in the dark for 120 h at a temperature of 22±2°C. Replicas were made for each dilution tested. The IG was determinate with TIQUIA (2000) equation.

2.4. Statistical analysis

All statistical analyzes were done using Microsoft office Excel and the program Statgraphics XVI, free version. Through an ANOVA analysis of variance, it was

determined if there are significant differences between the treatments. Pearson´s correlation was used for establishing relations between the parameters studied.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties

Systems soil-LA showed change in the properties. Values obtained in the physicochemical properties for different soil-LA treatments indicate that as the amount of added lignite increased, the content of OM and CEC increased significantly ($p < 0.05$) with respect to the soil system without amendment (data not shown). Organic matter is one of soil components that provides greatest number of change positions, and contributes significantly to the CEC, depending on soil type (KABATA and PENDIAS, 2004; PERIS, 2006).

Evolution of pH in different Soil-LA systems is showed in Fig. 1. As the percentage of added lignite increased, a significant decrease ($p < 0.05$) in the pH values with respect to LA system 0% is observed, for both areas under study. According to the pH values obtained, the systems of zone 1b can be classified as basic soils (pH> 7.3) and neutral soils (pH between 6.5 and 7.3); while systems of zone 2b are considered as acid soils, pH <6.5 (JARAMILLO, 2002).

The decrease in pH may be due to the mineralization of labile compounds present as organic matter in the lignite and/or the addition of humic acids (WANG et al., 2017) and fulvic acids that are part of the organic matter of the amendment. Additionally, when decomposed they release nutritious elements for plants, such as water and CO_2, among others.

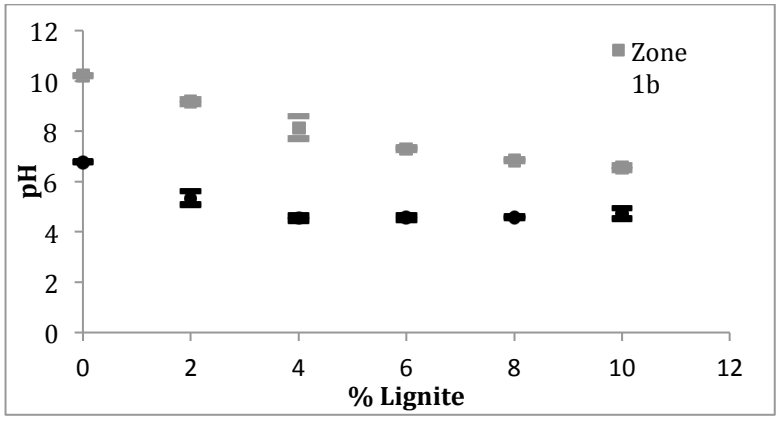

Figure 1. Evolution of the pH found in the system soil-LA after incubation. Uncertainties are expressed as standard deviation.

3.2. Determination of available metals

In this study, the Co, Cr and Pb concentrations in the extracts with EDTA for the soil-LA systems were found to be below the limit of detection (Table 1). Fe, Ni and Zn availability increased proportionally with the amount of lignite added. However, their values do not exceed the permissible limits, in consulted normative.

Table 1. Variation in concentration of available metals from Soil-LA systems after incubation with different percentages of lignite.

System Soil-LA		Metal content (mg extracted/kg soil)					
		0%	2%	4%	6%	8%	10%
Zone 1b	Co	0.7±0.2	<0.10	<0.10	<0.10	<0.10	<0.10
	Cr	1.22±0.09	<0.13	<0.13	<0.13	<0.13	<0.13
	Fe	72.7±8.3	198.0±27.4	191.0±26.2	342.6±34.4	371.4±47.3	364.0±37.7
	Ni	14.6±2.0	27.5±3.5	26.8±3.5	34.4±3.8	7.2±0.7	18.5±2.0
	Pb	2.8±0.6	<0.12	<0.12	<0.12	<0.12	<0.12
	Zn	18.3±1.7	16.3±1.8	15.3±1.0	20.8±2.0	26.0±2.8	28.3±1.9
Zone 2b	Co	0.7±0.2	<0.10	<0.10	<0.10	< 0.10	<0.10
	Cr	1.82±0.04	<0.13	<0.13	<0.13	< 0.13	<0.13
	Fe	68.3±9.2	105.4±13.3	169.2±11.1	173.8±16.1	220.4±17.8	195.2±22.6
	Ni	11.6±1.7	18.7±3.0	7.3±0.9	13.1±1.7	24.9±3.6	11.3±1.6
	Pb	4.3±0.1	<0.12	<0.12	<0.12	<0.12	<0.12
	Zn	25.5±1.9	30.7±2.6	35.7±3.2	33.9±2.8	46.4±1.4	53.0±5.0

Every experiment consists of a total 4 replicates. Data are expressed as average and the uncertainties as standard deviation.

The values obtained show that the content of metals available in the soil-LA systems did not exceed maximum recommended levels. Taking into account metal availability, lignite is a good option as amendment in dross impacted soil.
The knowledge of available content of metals present in soils is valid to help to establish whether or not the toxic levels of certain metals are reached, compared with the maximum permissible concentration of metals in soil, which are published in tables of environmental regulations. Each country proposes maximum permissible concentrations for metals contained in soils. Current Venezuelan regulations (GORBV, 1998, Decree 2635) establish that soil leachates should contain a maximum concentration of 5 mg/L for hexavalent chromium, nickel or lead. Iron, zinc and cobalt values are not specified.

3.3. Bioassays with radish seeds (*Rhaphanus sativus*)

The results obtained, in the positive control, showed that at concentrations higher than 0.2413 and 0.2050% p/v there is lethal toxicity of Fe and Zn, respectively. To inhibit the radicle growth (effective toxicity), concentrations above 0.0553% in Zn and 0.3373% in Fe were needed. Radicle length in presence of Zn was more sensitive than germination due that it was inhibited to lower concentrations, while in the presence of Fe germination was more sensitive than the radicle growth.
On the other hand, according to germination index values obtained (>100%), lignite incorporated as an organic amendment (up to a proportion of 10%) in dross impacted soils does not cause a negative effect on the germination of the selected seeds (radish).

3.4. Correlation between available metals, germination index and physicochemical properties

Table 2 shows the Pearson correlation matrix of the properties studied. Regarding the correlations of the metals. In summary: the content of Fe increases with CEC, LA and MO and decreases with GI ($p < 0.001$) and EC ($p < 0.05$); the concentration of extractable Ni

increases with pH ($p < 0.05$) and decreases with EC ($p < 0.01$); Zn values increase with CEC, EC, LA and decrease with pH ($p < 0.001$).

Table 2. Pearson correlation matrix between the parameters selected for the Soil-LA systems (n = 48).

	CEC	EC	Fe	GI	LA	Ni	OM	pH
CEC	1							
EC	0.3861[b]	1						
Fe	0.4656[a]	-0.4251[b]	1					
GI	NS	0.6200[a]	-0.4683[a]	1				
LA	0.7326[a]	NS	0.7399[a]	-0.2948[c]	1			
Ni	NS	-0.4415[b]	NS	NS	NS	1		
OM	0.5239[a]	NS	0.5195[a]	NS	0.3995[b]	NS	1	
pH	-0.7435[a]	-0.8106[a]	NS	-0.5241[a]	-0.5129[a]	0.3358[c]	-0.3604[c]	1
Zn	0.6542[a]	0.7770[a]	NS	NS	0.5677[a]	-0.3572[c]	NS	-0.8299[a]

NS = not significant. c, b and a = significant at probability level $P < 0.05$, 0.01 and 0.001, respectively.
CEC = cation exchange capacity. EC = electrical conductivity. GI = germination index. LA = Lignite A. OM = organic matter.

Concerning physicochemical properties, pH correlated positively with Ni content ($p < 0.05$) and negatively with LA, CEC, CE, GI, Zn ($p < 0.001$) and MO ($p < 0.05$). MO correlated directly with CEC ($p < 0.001$) LA and Fe content ($p < 0.01$). Likewise, LA presented an inverse relationship with GI ($p < 0.05$) and a direct relationship with CEC and the Fe and Zn contents ($p < 0.001$). As for the GI, it correlated positively with EC and negatively with Fe content ($p < 0.001$).
From the results obtained, we can affirm that pH had a high correlation with the percentage of lignite added. This aspect has great importance on the variation of soil properties, as evidenced by the correlations observed.

4. CONCLUSIONS

The lignite incorporated as organic amendment in soils affected by metallurgical activity decreased the pH and did not cause toxicity or negative effect on the germination of the selected seeds (*Raphanus sativus*) up to 10% of the added lignite. For all the systems studied, the maximum levels allowed in the two environmental regulations consulted were not exceeded (GORBV., 1998; Presidency Ministry, 2005). A significant difference was found between the pH values for the two zones studied, even with the addition of lignite.

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PRELIMINARY STUDY OF LYCOPENE EXTRACTION FROM SOLANUM BETACEUM RESIDUALS

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ABSTRACT

The tree tomato (*Solanum betaceum*) is a shrub native to the Andean region of South America that is grown for its semi-acid tropical fruit, lycopene is the pigment lipophilic that gives it the red color, this has an antioxidant effect. A preliminary study was carried out for the extraction of lycopene from agroindustrial residues, obtaining that with the ratio vegetal material - solvent volume of 1:70 and the extraction time of 30 minutes, the percentage of the extraction efficiency and the mass of lycopene present in one hundred grams of extracted solids were maximized. The antioxidant activity of the extract was demonstrated, so its use in the formulation of a pharmaceutical form would be feasible.

Keywords: antioxidant activity, carotenoids, *Solanum betaceum*

1. INTRODUCTION

The consumption of fruits and vegetables is essential since it provides the body with exogenous antioxidants. The exogenous antioxidants come from the diet, and within this group are included vitamin E, vitamin C and carotenoids. Lycopene is a carotenoid that has an aliphatic chain with thirteen double bonds, of which eleven are conjugated, which give it the peculiarity of being very reactive against oxygen and free radicals. The production of free radicals is a natural event regulated by different metabolic pathways, an imbalance between endogenous antioxidants and free radicals is associated with different diseases or with human aging (CORONADO *et al*., 2015; RODRÍGUEZ *et al*., 2015).

Lycopene is the lipophilic pigment that gives the red color to tomatoes, watermelon and papaya. The fresh tomato provides 90% of the lycopene necessary for the organism, since it is a micronutrient not synthesized by the human body. In epidemiological studies, it was shown that the increase in tomato intake decreased the incidence of prostate cancer (GIOVANNUCCI, 2002).

The concentrations of carotenoids in the tree tomato (*Solanum betaceum*) are the following: lycopenes of 11.99 to 21.70 μg/g, α-carotene of 16.52 to 29.64 μg/g and
β-carotene from 16.87 to 31.04 μg/g (PINCHAO *et al*., 2016).

Tree tomato production in Tungurahua province represents 59.62% of the national total according to the data reported by the National Institute of Statistics and Census (INEC, 2014). The industries of the city of Ambato and the rest of Ecuador, process the tomato by discarding the husks, where their incorporation into human diets could provide protection against oxidative damage in different vital organs. The objective of this work is to determine the ratio influence of the plant material - volume of solvent and time, on the extraction of lycopene from agroindustrial residues of tree tomato (*Solanum betaceum*) and the demonstration of the antioxidant capacity of the extract.

2. MATERIALS AND METHODS

2.1. Materials

Pyrex borosilicate precipitation glasses of 500 mL, micropipettes of 100 - 1000 μL, graduated glass test tubes of 500, 100 mL, magnetic bars, filter paper.

2.2. Reagents

96% Ethanol, Agroindustrial residues of Tomato (*Solanum betaceum*), Distilled Water, Trolox Aldrich S43353, DPPH Aldrich STBC1252V, Methanol 99.8 % Scharlab.

2.3. Equips

Tray dryer GANDER MTN, Citizen MB 200 moisture balance, magnetic stirrer with heating plate THERMO Model SP1359350, mill MICROPULV model MG 1511, UV-VIS spectrophotometer HACH DR 5000, Germany.

2.4. Methods

2.4.1 Obtaining the lycopene extract

The agroindustrial residues obtained from the plant Hortifrutícola Ambato SA, Planhofa C.A. were dried at the temperature of 60°C using a GANDER MTN tray dryer and ground

in the mill MICROPULV. Lycopene extraction was carried out at a temperature of 50 °C using 96% ethanol as a solvent. The ratio vegetal material - solvent volume (MV/E) ratios studied were 1:30, 1:50 and 1:70, for extraction times of 30, 60 and 120 minutes, using a Pyrex 500 mL beaker in a heating plate with stirring.

2.4.2 Analysis of the extracts

2.4.2.1 Determination of the percentage of total soluble solids

The residual moisture of each extract was determined using a Citizen MB 200 moisture balance. The percentage value of extracted solids (% SST) was calculated through the following expression:

$$\% \text{ SST} = 100 - \% \text{ H}$$

Where:
% H: Percent moisture of the extract.
The mass of extracted solids (MSE) was calculated through the following expression (SALOMÓN, 2011):

$$\text{MSE} = (\text{V X} \% \text{ SST}) / 100$$

Where:
V: volume of the extract.
The mass of lycopene in one hundred grams of solids extracted (mL in 100 g) was calculated establishing the relationship with the mass of extracted solids.

$$m_L \text{ en } 100 \text{ g} = (m_L \text{ X } 100) / \text{MSE}$$

2.4.2.2 Determination of the concentration of lycopene

Using the HACH DR 5000 UV-VIS spectrophotometer, the absorbance of each extract (A) was determined at the wavelength of 472 nm (RODRIGUEZ, 2001), and the concentration of lycopene (C) was calculated using an absorptivity value (E) of the ethanol of 3450 (STRATI and OREOPOULOU, 2011) according to the following equation:

$$C = A \text{ X } 10^4 / E$$

The percentage of extraction efficiency (% E.E) was calculated by the ratio of the mass of lycopene present in the extract and the mass of the plant material that was used for the extraction.

2.4.2.3 Determination of the antioxidant capacity of the extract

To quantify the antioxidant activity of the extracts, a methanolic solution of DPPH (2,2-diphenyl-1-picrylhydracil) was used (BOBO-GARCÍA *et al.*, 2015). The absorbance was determined at the wavelength of 515 nm in a Multiskan GO spectrophotometer (Thermo Scientific, Denmark).
The results were expressed as equivalents of μmol of Trolox g^{-1} of plant material from a calibration curve with Trolox (100-700 μmol L^{-1}), with four repetitions for each sample.
The antioxidant capacity of the extract was estimated from the calculation of the percentage of inhibition of DPPH from the absorbance values obtained (Abs) of the sample, by the following expression:

$$\% \text{ inhibition DPPH} = (1 - Abs_{sample} - Abs_{White} / Abs_{control} - Abs_{White}) * 100$$

2.5. Statistical analysis

An analysis of variance was performed using the Statgraphics Centurion XVII software to determine if there are significant influences of the factors: ratio plant material - volume of solvent, extraction time, and their interactions. Optimization was carried out for both variables: percentage of extraction efficiency (% E.E) and lycopene mass in one hundred grams of extracted solids (mL in 100 g) for a p-value <0.05.

3. RESULTS AND DISCUSSION

3.1. Interpretation of the analysis of the extracts

Through an experimental design of response surface 3^2, the obtained values of percentage of extraction efficiency (% E.E) were analyzed to determine the effect caused by the ratio plant material - solvent volume and extraction time.
In Table 1 it can be seen that the P-value for the time factor is greater than 0.05 for a confidence level of 95%, so that the extraction time did not influence the extraction efficiency percentage, while that their quadratic interactions, as well as the factor plant material relation - volume of solvent and their quadratic interactions do influence significantly. The Pareto Diagram shows the graphic representation of the effects for both variables and their interactions (Fig. 1).
In the Main Effects graph it is observed that as the MV/E ratio increases, the extraction efficiency percentage increases to the average value of this factor (1:50). With the time of 60 minutes, the highest percentage value of extraction efficiency was obtained, because of, at higher values of time, this percentage decreases, due to the extraction of other solids from the plant material that are not lycopene (Fig. 2).
The MV/E ratio of 1:50 and the time of 60 minutes optimized the process by generating the highest percentage efficiency values of the extraction (Fig. 3)

Table 1. Analysis of variance for the percentage of extraction efficiency.

Source	Sum of Squares	Degree of Freedom	Square Medium	Reason-F	P Value
A: Ratio MV/E	9,63333E-7	1	9,63333E-7	7,67	0,0170
B: Time	3,E-8	1	3,E-8	0,24	0,6338
AA	7,51111E-7	1	7,51111E-7	5,98	0,0308
BB	0,0000149511	1	0,0000149511	119,08	0,0000
blocks	8,88889E-9	1	8,88889E-9	0,07	0,7947
Total Error	0,00000150667	12	1,25556E-7		
Total (runs)	0,0000182111	17			

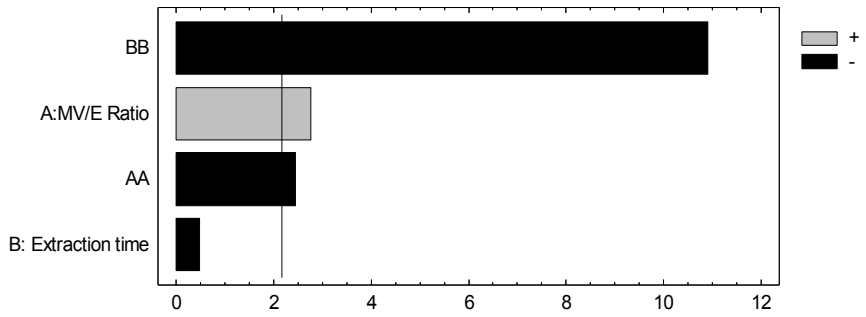

Figure 1. Standardized Pareto Diagram for the percentage of extraction efficiency.

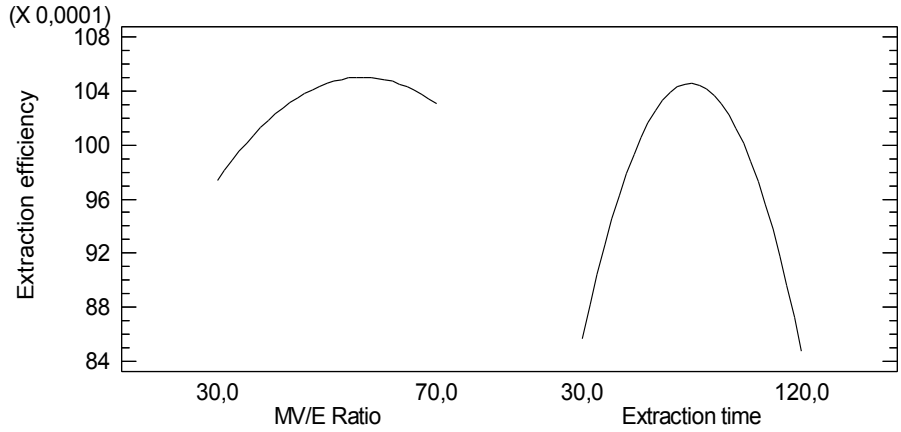

Figure 2. Main Effects for the percentage of extraction efficiency.

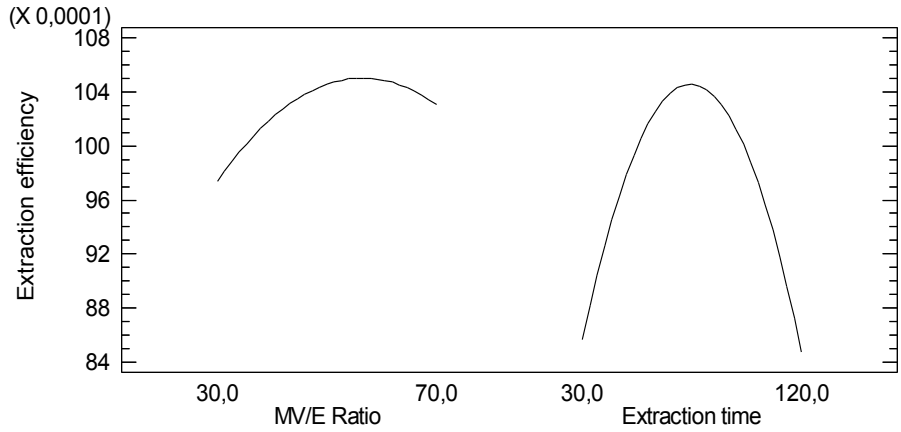

Figure 3. Estimated response surface for the efficiency percentage of the extraction.

When performing the variance analysis to evaluate the effects of the factors MV/E ratio and extraction time on the lycopene mass in one hundred grams of extracted solids, P-values lower than 0.05 were obtained, meaning that there is significant influence of these factors and their quadratic interactions (Table 2).

Table 2. Analysis of variance for the lycopene mass in one hundred grams of extracted solids.

Sources	Square Sum	DF	Square Medium	Reason-F	P Value
A: Ratio MV/E	981,021	1	981,021	30,66	0,0001
B: Time	10668,4	1	10668,4	333,39	0,0000
AA	192,747	1	192,747	6,02	0,0304
BB	1947,75	1	1947,75	60,87	0,0000
blocks	10,4272	1	10,4272	0,33	0,5786
Total error	383,993	12	31,9994		
Total (runs)	14184,3	17			

The Pareto Diagram (Fig. 4) shows the graphical representation of the factors and their quadratic interactions on the lycopene mass in one hundred grams of extracted solids.
In the Main Effects chart, it is observed that as the MV/E ratio increases, the lycopene mass increases, while the lycopene mass decreases as the extraction time increases (Fig. 5). With the graph of optimization of the response, it was obtained that with the MV/E ratio of 1:70 and the time of 30 minutes, the highest lycopene mass was reached in one hundred grams of solids (Fig. 6).

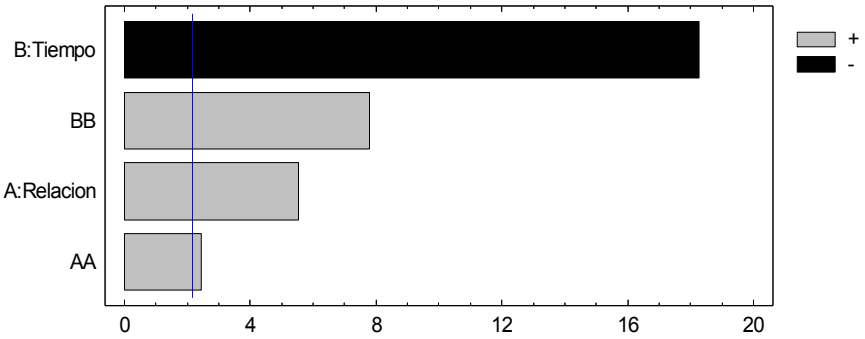

Figure 4. Standardized Pareto diagram for the lycopene mass in one hundred grams of extracted solids.

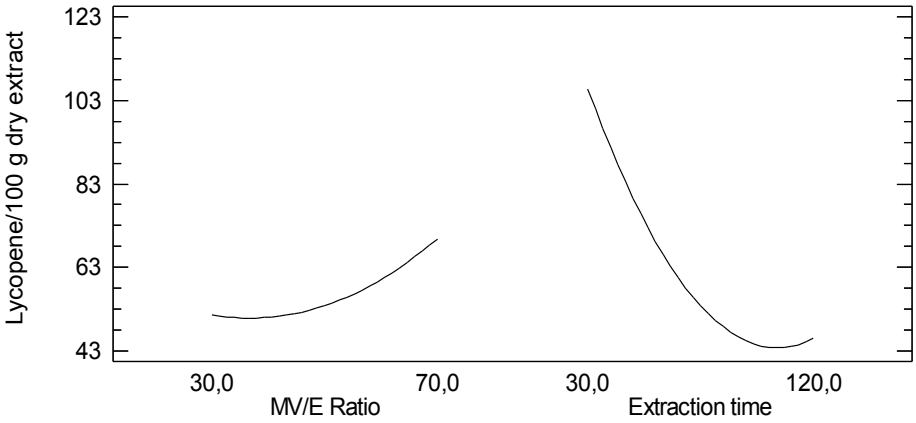

Figure 5. Main effects for the lycopene mass in one hundred grams of extracted solids.

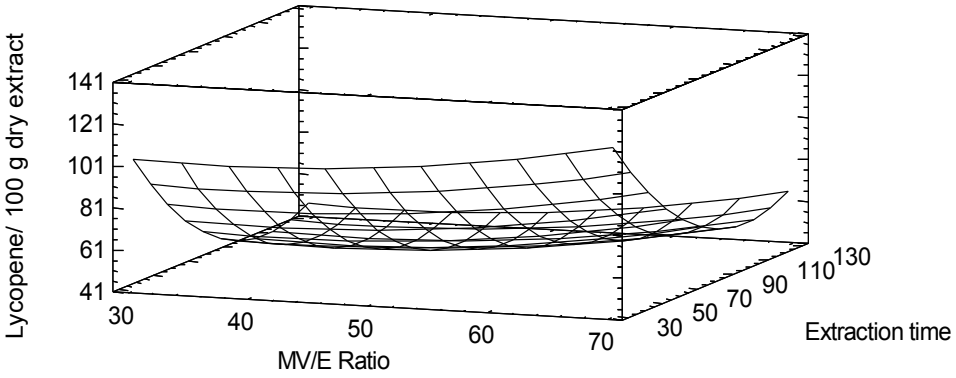

Figure 6. Estimated response surface for the lycopene mass in one hundred grams of extracted solids.

In Fig. 7 it is observed that with an MV / E ratio of 1:70 and an extraction time of 30 minutes, the highest values for the response variables are obtained, obtaining an efficiency percentage of lycopene extraction of 0.0084% lycopene, which represents 84 µg of lycopene / g of plant material, higher than the value reported by PINCHAO *et al.* (2016) from 11.99 to 21.70 µg of lycopene/g of plant material.
In addition, 121.3 mg of lycopene are obtained in 100 g of solids, with this value it can be inferred that ethanol extracts in addition to lycopene other compounds for their high solubility in fatty compounds.

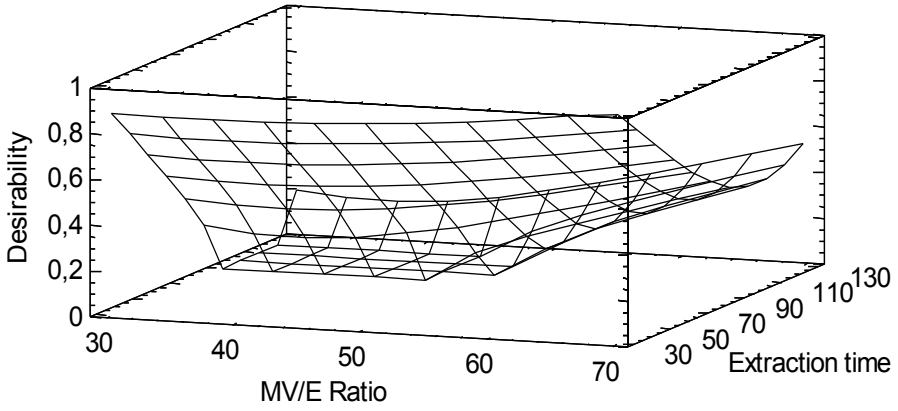

Fig. 7. Estimated response surface for the extraction efficiency percentage and the lycopene mass in one hundred grams of extracted solids.

With the results obtained in this experimental design it can be concluded that the plant material - solvent volume ratio is one of the most important factors in solvent extraction since the mass transfer between the plant material and the solvent depends on it. Also, the more solvent volume exists with respect to the plant material, the more solute will be extracted since the saturation of the solvent will be avoided. These results are in agreement with what was concluded by CARDONA and RÍOS (2006).

3.2. Determination of the antioxidant capacity of the extract

The equation of the line obtained for the Trolox calibration curve was as follows:

$$y = 0.1559X - 2.6548 \qquad R^2 = 0.9992$$

The calculated inhibition percentage was 74.68%, which corresponds to an equivalent Trolox concentration in the 496 μM extract representing 490 μmol of Trolox in 1 g of MSE. This obtained result demonstrates the presence of carotenoids and their antioxidant activity in the agroindustrial residues of Tree Tomato (*Solanum betaceum*) very similar to what was proposed by MENDELOVÁ (2013) and VARZAKAS (2016).

4. CONCLUSIONS

In this work it was obtained that the extraction time did not have significant influence on the percentage of extraction efficiency, however, if it had a significant influence on the mass of lycopene extracted, so that with a time of 30 minutes the highest values were obtained for both variables. The vegetal material - solvent volume ratio factor had a significant influence on the percentage of extraction efficiency and the lycopene mass in one hundred grams of extracted solids, so that with a MV/E ratio of 1:70, both variables were maximized. It was demonstrated that the obtained extract presented antioxidant capacity equivalent to 490 μmol of Trolox in 1 g of mass of extracted solids.

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ANTIMICROBIAL ACTIVITY OF *LENTINULA EDODES* MUSHROOM EXTRACTS AGAINST PATHOGENIC BACTERIA

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ABSTRACT

The resistance of pathogenic bacteria to multiple antimicrobials has become a global threat to health and food security. This research determined the antimicrobial activity of *Lentinula edodes* mushroom extracts, setting as study factors: parts of the mushroom (pileus, stem), type of solvent (chloroform, ethanol, ethyl acetate, ethanol-water (50-50)) and type of bacteria (*Salmonella spp, Listeria spp, E-coli*), isolated from meat. The best results for antimicrobial efficacy were obtained from the ethanolic stem extract that inhibited 83% of the maximum reached by Penicillin, which makes it a promising product in regards to the continuing of this study.

Keywords: antibiogram, pileus, stem, pathogenic bacteria, bacterial sensibility

1. INTRODUCTION

Bacterial resistance to antibiotics has become a global threat to health and food security. Antibiotics have saved millions of lives, however a reality of yet unmeasured dimensions is approaching, the world could soon face a grave situation brought on by multiple lethal bacteria resistant to existing antibiotics, lack of availability of effective antibiotics, and absence of innovation and development of new antibiotics (MAGIORAKOS *et al.*, 2012; LAXMINARAYAN *et al.*, 20013; ALÓS, 2015). This has prompted an increase in the demand of natural-origin antimicrobial agents (derived from vegetables or fungi), as they have proved to be less toxic and have fewer side effects than synthetic agents (MAEDA *et al.*, 1974; GADEA *et al.*, 2017).

Natural compounds with biological activity are usually present in plants, fungi, and many other sources. From a medicinal standpoint, an ample number of fungi, between 270 and 700 species, have exhibited bioactive and pharmacological properties (SMITH *et al.*, 2002; CHANG, 2008).

The use of extraction techniques to obtain substances with bioactive properties is of great importance (SPIGNO and DE FAVERI, 2007). In general, the entire mushroom, the pileus, the stem or the mycelium, fresh or dehydrated, are used in the production of compounds of pharmacological value. These compounds are obtained by contact with solvents through extraction techniques like maceration. In order to accomplish a higher concentration of active ingredients and more effective action, it is necessary to perform various procedures utilizing appropriate solvents selected according to the solubility and stability of the substance to be extracted (WICHTL, 2004). The efficacy of the extraction depends on the polarity and nature of the solvent used (DEVI, *et al.*, 2013).

The sensibility of microorganisms to natural extracts is related to the size of the microbial growth inhibition zone. According to the diameter of inhibition halo, microorganisms are classified in: resistant or not sensible ($d < 8$ mm.), sensible (9 mm. $< d <$ 14 mm.), very sensible (14 mm. $< d <$ 19 mm.) and extremely sensible ($d > 20$ mm.) (HEARST *et al.*, 2009; PONCE *et al.*, 2008).

Within medicinal fungi, Shitake (*Lentinula edodes)* stands out as the second most cultivated edible mushroom and is widely used in the food industry in the global market (VAN BA *et al.*, 2017). This mushroom has high nutritional value and contains several bioactive compounds, including polysaccharides, dietary fiber, ergosterol, vitamin B1, B2 and C, folates, niacin, and minerals, in addition to compounds that give it antimicrobial properties (RAO *et al.*, 2009; JIANG *et al.*, 2014). In this sense, this research focused on the evaluation of the antimicrobial activity of extracts obtained from the *Lentinula edodes* mushroom, by use of the Kirby-Bauer technique against pathogenic bacteria: *Listeria spp*, *Escherichia coli*, and *Salmonella spp* isolated from beef.

2. MATERIALS AND METHODS

The *Lentinula edodes* (L-SSC strain) was cultivated using the method described by RUILOVA et al. (2017a), it was harvested separating its parts (pileus and stem), then lyophilized and pulverized. Samples of the fresh fungus were previously analyzed in the Bromatology laboratory of the Research Department of Bolivar State University (Table 1).

Table 1. Analyses performed on the stem pileus of the *Lentinula edodes* mushroom.

Parameter	Norm used for its determination	References
Humidity	AOAC925.10	AOAC, 1980
Ashes	AOAC923.03	AOAC 990.03, 2005
Fiber	INEN 522	AOAC (2005)
Fat	NTE INEN-ISO11085	AOAC (2005) 922.06
Protein Content in the fungus (PCF)	AOAC*.	AOAC 990.03,2005

*The nitrogen to protein content conversion factor was 4.38 (Yang *et al.,* 2001).

2.1. Experimental design used in the preparation of the extracts

A factorial design considering three study factors was applied: parts of the *Lentinula edodes* mushroom, type of solvent, and type of bacteria as it is shown in Table 2.

Table 2. Factors and studied levels.

Factors	Code	Level
Parts of *Lentinula edodes* mushroom	a	a_1= stem a_2= pileus
Solvents	b	b_1= Chloroform b_2= Ethanol b_3= Ethyl acetate b_4= Ethanol-Water (50-50)
Type of bacteria	c	c_1=*Listeria Spp* c_2=*Salmonella Spp* y c_3=*Escherichia coli*

Generating 24 treatments with 3 repetitions each, a total of 72 experimental units.

2.2. Preparation of the Extracts

Of the pulverized parts of the fungus, 15 grams were weighted and cold macerated adding 100 ml of solvent according to the corresponding treatment (Chloroform, Ethanol, Ethyl acetate, and Ethanol-Water 50-50). The extracts were stored in a place out of direct light for 6 days, then were centrifuged at 8000 rpm. The supernatant was collected and kept at 4 ºC in amber colored glass vials.

2.3. Collection of the inoculum and determination of antimicrobial activity

Three strains of bacteria were used (*E-coli, Listeria spp y salmonella spp*), isolated from samples of bovine meat, porcine meat, and chicken acquired from the marketplace located in the Guaranda - Ecuador city center and preserved in cryovials (Microbank ™ Prolab Diagnostics®, USA) at -80 ºC in the Molecular Biology Laboratory of Bolivar State University (RUILOVA *et al*., 2017b). These strains were reactivated and cultured, *E-coli* in Nutrient Agar (Oxoid® brand) and *Listeria* in Chromogenic Listeria Agar (Acumedia® brand) with Bacto Agar as the gelling agent.
From each bacteria, 4 colonies were selected and transferred to a test tube containing 9 mL of 1% saline solution, then homogenized by vortexing until turbidity equivalent to the Nº 0.5 Mc Farland (1.5 x 10^6 UFC/mL) standard tube was obtained. Bacteria from each

suspension were inoculated in Muller Hilton Agar (7101A, Neogen®, Michigan-USA.), using a sterile cotton swab and then the dishes left to stand for a couple minutes.
Antimicrobial sensitivity was evaluated by employing the Kirby-Bauer technique (BAUER *et al.,* 1966). Sterile filter paper discs of 6 mm in diameter (TSMX 7215, Oxoid®, UK) were imbued with the different extracts on top of the agar surface. Discs with antibiotics (penicillin and ciprofloxacin) were placed in the middle part as positive control. The Petri dishes were incubated at 37° C. Reading of the inhibition halos was conducted after 18 and 24 hours of incubation. All the trials were performed thrice.

2.4. Statistical Analysis

An analysis of variance (ANOVA) was applied to establish differences between treatments. A Fisher test at 5% was conducted to determine differences in the means of the treatments. The program Statgraphic Centurion VII was used to analyze the results.

3. RESULTS AND DISCUSSION

3.1. Characterization of the parts of the *Lentinula edodes* mushroom

The results of the proximate analysis of stem and pileus are presented in Table 3.

Table 3. Average values and standard deviation of the composition of the representative parts of the *Lentinula edodes* mushroom.

Sample	Humidity (%)	Ashes (%)	Protein (%)	Fat (%)	Raw fiber (%)
Stem	10.2	7.9	19.7	1.4	22.8
	(0.91)	(0.79)	(0.98)	(0.12)	(1.20)
Pileus	11	5.3	24.2	2.3	6.7
	(0.82)	(0.37)	(0.44)	(0.16)	(0.21)

The percentages correspond to the average of three repetitions. Values between ( ) represent the standard deviation.

The humidity content in the stem and pileus had a variation inferior to 10%, while protein, ashes, and fat varied between 19-33% in relation to the two parts of the mushroom and fiber was 71%, higher for the stem. BISEN *et al.,* (2010), studied the pileus of this fungus, and found values of 22.8% for protein, 2.3% for ashes, and 8.4% for fiber, values very similar to those encountered by this research, while humidity was lower (4.7%). As for ZHANG *et al.,* (2013) analysis of the stem detected humidity of 5.2%, ashes of 9.8%, protein of 16.2%, fat of 1.37% and fiber of 38%, the highest values found in this work. JIANG *et al.,* (2010) indicate that the parts of a fungus vary greatly in chemical composition and the stem has a larger fraction of insoluble raw fiber (around 38 g/100 g) compared to the pileus (7.2 g/100 g), similarly ZHANG *et al.,* (2013) reported in their work that the protein content of the stem (7.20 g/100 g) was much lower in comparison to the pileus (18.29 g/100 g).

3.2. Final volume of the extracts using different solvents

As result, 24 translucent extracts with no particles in suspension were obtained. The resulting values from the experimental measurements of the fungus (stem and pileus) extract volumes in different solvents are shown in Table 4.

The highest mean belonged to the fungus stem plus chloroform (67.3 mL). The higher volume could be because of the stem's fibrous nature compared to the pileus' spongy one, which has a higher liquid absorption capacity (RUILOVA *et al.*, 2014), and due to the solvent used, since chloroform is miscible with a large number of organic compounds, it is heavier than water so it improves filtering of the solution (ROJAS *et al.*, 2001). The lowest mean corresponded to the pileus plus ethanol-water (50-50), resulting in 52.7 mL, possibly due to the pileus' high absorption and water retention capacity, making filtering more difficult (RUILOVA *et al.*, 2014).

In order to analyze the obtained volumes, an analysis of variance (ANOVA) was conducted using Statgraphic Centurion VII. The results are shown in Table 5.

Table 5 breaks down the volume variability in contributions dependent on the two factors (parts of the mushroom and solvents). The P-Values prove the statistical significance of each factor since they are less than 0.05, these factors have a statistically significant effect on the volume with a 95.0% level of confidence.

The difference observed can be due to the polarity of the solvents and the mushroom's capacity to absorb and retain liquids (RUILOVA *et al.*, 2014). DEVI *et al.*, (2013), evaluated the return rates of the different solvents used to prepare vegetable extracts. They determined that each solvent has different yield characteristics in the extraction process and pointed out said characteristics' dependence on polarity and the nature of the solvents.

Table 4. Volume of the *Lentinula edodes* fungus extract in different treatments.

Extracts	Chloroform (mL)	Ethanol (mL)	Ethyl Acetate (mL)	Ethanol-Water 50% (mL)
Stem	67.3	62.0	64.3	60.3
	(2.49)	(1.63)	(2.87)	(1.25)
Pileus	62.3	57.3	60.0	52.7
	(2.49)	(1.25)	(1.63)	(2.49)

The volumes correspond to the average of three repetitions. Values between ( ) represent the standard deviation.

Table 5. Analysis of variance (ANOVA) of the obtained volumes.

Source	Sum of squares	DF	Mean squares	F-Value	P-Value
Main effects					
A:Parts of the mushroom	176.042	1	176.042	26.57	0.0001
B:Solvents	227.458	3	75.8194	11.44	0.0003
AB interactions	10.4583	3	3.48611	0.53	0.6705
Residues	106.0	16	6.625		
Total (revised)	519.958	23			

All the F-Values are based on the squared mean of the residual error.

3.3. Fisher test to compare means

Once the multiple range test was conducted for the volume, employing Fisher's least significant difference (LSD) procedure, it was determined that the means corresponding to the *parts of the mushroom* factor are significantly different, as shown in Table 6.
The results are detailed in Fig. 1.

3.4. Evaluation of the antimicrobial activity of the obtained extracts

The results of the sensibility of the *Lentinula edodes* mushroom extracts to *listeria spp*, *Salmonella spp* y *Escherichia coli*, are shown in Table 7.
In the case of *Listeria spp*, the highest means for the antimicrobial inhibitory effect corresponded to the stem ethanol extracts (12.7mm), followed by the pileus ethanol extracts (10mm). The results can be attributed to the better solubility of the organic compounds in ethanol, which led to improved extraction (LAPORNIK *et al.*, 2005). The lowest mean corresponded to the pileus + ethyl acetate treatment (3.7 mm), this can be related to the acetate's volatility. In a study by VAN BA *et al.*, (2017), they evaluated the antimicrobial activity of the *Lentinula edodes* on pathogenic bacteria and reported that ethanolic and aqueous extracts inhibit the growth of the *Listeria monocytogenes.* HEARST *et al.*, (2009), also studied the effects of *Lentinula edodes* ethanolic extracts on *listeria monocytogenes* and measured 11mm oh inhibition halo, result smaller to the one found in our research.
Regarding *Salmonella spp* and *E-coli*, the highest means corresponded to the ethanolic stem extract (9.3 and 9 mm) (LAPORNIK *et al.*, 2005). HEARST *et al.*, (2009), used *Lentinula edodes* ethanolic extracts on *Salmonella* and obtained similar results (9 mm of inhibition halo) VAN BA *et al.*, (2017). Similarly, HEARST *et al.*, (2009), evaluated concentrated aqueous extracts of *Lentinula edodes* on *Escherichia coli*, and obtained an inhibition halo of 10 mm.

Table 6. Fisher tests at 95 % for volume of extract

Parts of the mushroom	Cases	LS Mean	LS Sigma	Homogenous groups
Pileus	12	58.0833	0.743023	A
Stem	12	63.5	0.743023	B

Figure 1. Graph of the means for Fisher test at 95% of the volume of extract in relation to parts of the mushroom.

Table 7. Antimicrobial activity of the *Lentinula edodes* mushroom extracts on pathogenic bacteria.

Bacteria	Extracts	Inhibition halos of the bacteria (mm)				+ Control	
		Chloroform	Ethanol	Ethyl A.	Ethanol-Water 50%	P	C
Listeria spp	Stem	5.3 (0.94)	12.7 (0.94)	4 (0)	8.7 (0.47)	15.3 (1.25)	1 (0)
	Pileus	4.7 (0.47)	10 (0)	3.7 (0.47)	8 (0)		
Salmonella spp	Stem	6.0 (1.63)	9.3 (0.47)	4.3 (0.47)	7.0 (0.94)	1.3 (0.47)	24 (0.82)
	Pileus	5.3 (0.94)	7 (0.47)	3.3 (0.47)	6 (0)		
Escherichia coli	Stem	3.3 (0.47)	9.0 (0)	4.3 (0.47)	7.0 (0)	0.3 (0.47)	25 (1.41)
	Pileus	2.7 (0.47)	6.7 (0.94)	4.0 (0)	6.3 (0.47)		

The inhibition halo measurements correspond to the average of three repetitions. Values between ( ) represent the standard deviation.
P: Penicillin; C: Ciprofloxacin

A comparison of the *Lentinula edodes* ethanolic extracts' antimicrobial inhibition effect against commercial antibiotics Ciprofloxacin (C) and Penicillin (P) revealed that Ciproflaxacin does not inhibit growth of the Gram-positive bacteria *Listeria spp*. Its inhibition halo measured only 1 mm, while Penicillin exhibited a halo of 15.3 mm. The ethanolic extracts obtained from the stem of the mushroom inhibited 83% of the maximum obtained with Penicillin, which makes it a promising product in regards to the continuing of natural antibiotic studies.

The Gram-negative bacteria *Salmonella spp* showed greater sensibility to the *Lentinula edodes* stem ethanolic extracts, with an inhibition halo of 9.3 mm. The test showed this bacteria is very sensitive to Ciprofloxacin, with an inhibition halo of 24 mm and as expected, it was not sensitive to Penicillin which only reached 1 mm of inhibition halo. These results, even if demonstrating enough sensibility to be considered inhibitory according to PONCE *et al.,* (2008) and HEARST *et al.,* (2009), represent only 40% of what Ciprofloxacin achieves. A similar situation was evidenced for the Gram-negative bacteria *Escherichia coli.*

In order to analyze antimicrobial activity in relation to the size of inhibition halo, an analysis of variance (ANOVA) was conducted using Statgraphic Centurion VII. The results are shown in Table 8.

Table 8 breaks down halo variability in contributions according to three factors (parts of the mushroom, solvent, and type of bacteria). Except for the AB and ABC interactions, the P-Values prove the statistical significance of each factor since they are less than 0.05, these factors have a statistically significant effect on the volume with a 95.0% level of confidence. LAPORNIK *el al.,* (2005), who compared extracts prepared using different solvents and extraction times, determined that the type of solvent used influences the extraction of active compounds due to the solvents' different capacity to extract substances with antimicrobial activity from the pulverized mushroom, as indicated by VAN BA *et al.,* (2016) who reported that the type of solvent used to make the extracts affects antimicrobial activity significantly.

Conducting Fisher's multiple range test of the inhibition halo revealed that the means for the size of the halo are significantly different, as shown in Table 9.

Table 8. Analysis of variance (ANOVA) of the inhibition halo

Source	Sum of squares	DF	Mean suares	F-Value	P-Value
Main effects					
A:Bacteria	40.4444	2	20.2222	36.95	0.0000
B:Parts of the mushroom	17.0139	1	17.0139	31.09	0.0000
C:Solvents	303.819	3	101.273	185.03	0.0000
Interactions					
AB	0.444444	2	0.222222	0.41	0.6683
AC	46.2222	6	7.7037	14.08	0.0000
BC	5.15278	3	1.71759	3.14	0.0327
ABC	1.55556	6	0.259259	0.44	0.8453
Residues	28.00	48	0.583333		
Total (revised)	442.653	71			

All the F-Values are based on the squared mean of the residual error.

Table 9. Fisher test at 95% of the inhibition halo according to type of bacteria.

Bacteria	Cases	LS Mean	LS Sigma	Homogenous groups
E. Coli	24	5.29167	0.155902	A
Salmonella	24	6.125	0.155902	b
Listeria	24	7.125	0.155902	C

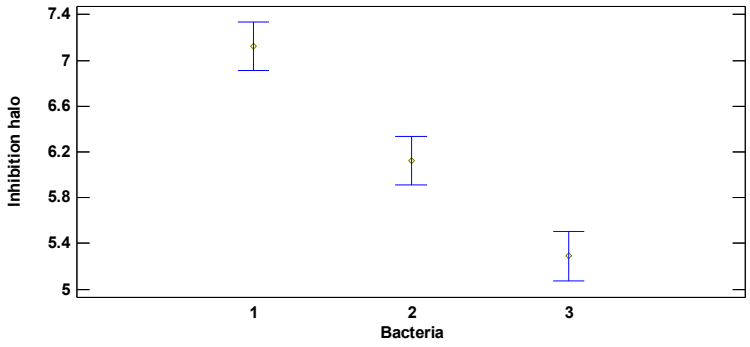

Figure 2. Graph of the means for Fisher LSD test at 95% of the inhibition halo according to type of bacteria.

Results are showed in Fig. 2 taking values from Table 9. There, confirm that *Listeria* is the most sensitive to the fungus extracts while *E-coli* is less sensitive. Table 10 summarizes bacteria sensitivity to the extracts obtained from the *Lentinula edodes* using different solvents and to the positive controls (Penicillin and Ciprofloxacin).

The *Listeria spp*, *Salmonella spp*, and *E-coli* bacteria are sensitive to the ethanolic extract of the *Lentinula edodes* stem and the *Listeria spp* is also sensitive to the ethanolic extract of the pileus. The *Listeria spp* is also very sensitive to Penicillin but not to Ciprofloxacin, while *Salmonella spp* and *E-coli* are extremely sensitive to Ciprofloxacin but resistant to Penicillin. These values are to be considered a baseline that justifies continuing the study in order to isolate, purify and determine the chemical structure of the compounds responsible for the antibacterial effect. The finding of new antibiotic agents, whether they come from natural or synthetic sources has become a necessity for the medical and scientific community.

Table 10. Bacteria sensitivity to the *Lentinula edodes* extracts and + controls.

Extracts	*Listeria spp*	*Salmonella spp*	*E. Coli*
Stem + Cloroform	-	-	-
Stem + Ethanol	+	+	+
Stem + Ethyl acetate	-	-	-
Stem + Ethanol - Water (50-50)	-	-	-
Pileus + Chloroform	-	-	-
Pileus + Ethanol	+	-	-
Pileus + Ethyl acetate	-	-	-
Pileus + Ethanol - Water (50-50)	-	-	-
+ Controls			
Penicillin	++	-	-
Ciprofloxacin	-	+++	+++

(+++) Extremely sensitive (++) Very sensitive (+) Sensitive and (-) Resistant.

4. CONCLUSIONS

Of all the obtained *Lentinula edodes* stem and pileus extracts, using different solvents, the stem+chloroform combination produced the most volume (67 mL) and the pileus+ethyl acetate treatment produced the least volume (52.7 mL). This result is the consequence of the stem's fibrous content compared to the spongy structure of the pileus, giving the second a higher liquid absorption capacity.

The ethanol solvent, out of the four evaluated solvents, exhibited the largest inhibitory effect on the three bacteria strains that were tested, the largest inhibition halo evidenced in when the stem extract (12.7 mm) was used on the *Listeria spp*. The lowest mean corresponded to the chloroform extract, which can be due to this solvent's volatility in the dish.

The *Listeria spp, Salmonella spp*, and *E-coli* bacteria are sensitive to the ethanolic extract from the *Lentinula edodes* stem and the *Listeria spp* bacteria is also sensitive to the pileus ethanolic extract, this bacteria is also very sensitive to Penicillin but not to Ciprofloxacin, on the other hand, *Salmonella spp* and *E-coli* are extremely sensitive to Ciprofloxacin but resistant to Penicillin.

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OBTAINING BIODIESEL IN SUBCRITICAL CONDITIONS THROUGH THE CONVERSION OF RESIDUAL FRYING OIL

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ABSTRACT

The residual frying oils contaminate the water by capturing oxygen, which prevents the subsistence of aquatic organisms. This research was developed to recycle and convert these oils into biodiesel. For this purpose, sub critical reaction conditions were obtained by using a prototype of batch reactor with external recirculation, a completely randomized block design was applied (A, B, C), the variables and factors of study were: A molar ratio oil-methanol 1: 6 and 1: 9; B percentage of 0.5% and 1% sodium hydroxide and C reaction temperatures of 160°C, 180°C and 200°C. We obtained 12 combinations of variables that resulted in triplicate 36 experimental units and the reaction time was 10 minutes. When analyzing the biodiesel complies with the ASTM - D1298 quality standard and the combustion analysis, it was determined that for every 1200 ml of Biodiesel combusted in one hour it will decrease: 11700 (mg/m^3) of CO, 48% CO_2 and 1551 (mg/m^3) SO, contributing in this way to reduce environmental pollution.

Keywords: conversion, frying oil, reactor, biodiesel

1. INTRODUCTION

The residual frying oils contaminate the water producing an absorption of oxygen that prevents the subsistence of aquatic organisms. This type of waste interferes with the exchange of gases between water and the atmosphere, causing low levels of dissolved oxygen, acidification of water and preventing the penetration of sunlight (OKAFOR, 2011). The overheating of the oil forms acrolein, this substance causes gastric irritation, in addition, aromatic compounds such as benzopyrene and benzoanthracene are generated, which are carcinogenic agents (CHINCHILLA et al., 2016). When the oil is reused (it is fried again in the same oil), complex reactions occur that affect the nutritional quality of the food and the formation of toxic compounds, such as polymers, fatty acid monomers and polar compounds that migrate to the oil (ABREU et al., 2017).
For the production of biodiesel the cost of raw materials is 60-75%, the low cost are animal fats, cooking oil and some inedible oils. Using low-cost raw materials can be an alternative to obtain an economic attractiveness for the production of biodiesel (RETNO et al., 2016).
In developed countries, homogenous alkali catalyzed transesterification is used to produce biodiesel, however, this method has the following drawbacks: used oils must contain low amounts of free fatty acid and water to prevent the formation of soap, longer time reaction (more than 60 minutes). lower reaction rates and strict reaction conditions. To improve the production of biodiesel, a new technology called supercritical methanol transesterification has been developed that does not require a catalyst, does not generate wastewater and is produced at higher reaction speeds (FAROBIE et al., 2016).
Subcritical transesterification is a promising method for the production of biodiesel, the flexibility of the raw material with high or low acidity, the efficiency of production and the ecological benefits. The decrease in temperature and pressure (<240° C and <8.0 MPa) synthesis under subcritical conditions but with the help of a heterogeneous catalyst can be the desirable technology (GLISIC and ORLOVIĆ, 2014).
In Ecuador there are possibilities for the development of a biofuels market, using them in propulsion engines for transport, combined with fossil fuels derived from petroleum. The use of biofuels has important environmental advantages, since the life cycle of biofuels is short compared to that of petroleum products, which generates a lower impact on the environment (GOMELSKY, 2013).

2. MATERIALS AND METHODS

2.1. Materials and equipment

Precipitation beakers 500 mL and 1000 mL, test tube 100 mL, Erlenmeyer 500 mL and 1000 mL, weigh filters, spatula, 1000 mL separating funnel, heating plate, steel ring, universal holder, magnetic stirrer and jars of glass with screw cap, hydrometer, analytical balance, a discontinuous reactor prototype coupled to an external recirculation system, BACHARACH® model ECA 450 gas meter and a Kubota® model GL 1200 NB diesel generator.
The methanol used was AppliChem Panreac quality® (purity 99.8%) ACS, ISO. The sodium hydroxide catalyst of the transesterification reaction was of Fisher Chemical quality and the frying oil came from restaurants and fast food outlets in the city of Guaranda.

2.2. Determination of variables for transesterification under subcritical methanol conditions

The variables selected were: temperature, methanol-oil molar ratio and catalyst concentration, considered to be influential in biodiesel yield (MUSA, 2016; BULLA et al., 2014; MEDINA et al., 2012). The catalyst concentration and alcohol-oil molar ratio was based on the experience of ELKADY et al. (2015) and three subcritical reaction temperatures were in accordance with the following factors combination (Table 1).

Table 1. Experimental design of biodiesel obtained in sub-critical conditions.

Study Factors	Description
Factor A: Molar ratio	A1=6:1
	A2=9:1
Factor B: % NaOH	B1:0.5%
	B2:1.0%
Factor C: Reaction temperature	C1=160°C
	C2=180°C
	C3=200°C

AxBxC design, 12 combinations with three replicas giving a total of 36 experimental units.

The r^2 value (coefficient of determination), which is the proportion of the total variance of the variable explained by the regression that reflects the goodness of fit; for which, the following equation was applied:

$$\mathbf{r^2 = 1 - \frac{\sum(Yi - \hat{Y}i)^2}{2\sum(Yi - \hat{Y})^2}} \qquad \text{(Eq.1)}$$

Where: r^2: coefficient of determination; Yi: Observed value; $\hat{Y}i$: Predicted value; and $\hat{Y}$: arithmetic mean

2.3. Description of the process and considerations of reactor operation

Figure 1, shows an illustrative diagram of the reactor used and a process diagram. The reactor consisted of a discontinuous system or by load, to which was added a recirculation system, that is, it consisted of tank, pipe and recirculation pump, the addition of this system to the reactor, facilitated the agitation of the reactants.
In the reactor 600 mL of frying oil plus the methanol solution with sodium hydroxide was added through the valve, the stirring system is switched on and heat was supplied until it reached the desired temperature (160°C, 180°C and 200°C) for a period of 10 minutes. The heat was then removed and cooled keeping the agitation. Once cold the product was removed by the discharge valve, it was received in an Erlenmeyer and then was transferred to a separation funnel in which the glycerin is separated from the biodiesel by decantation.

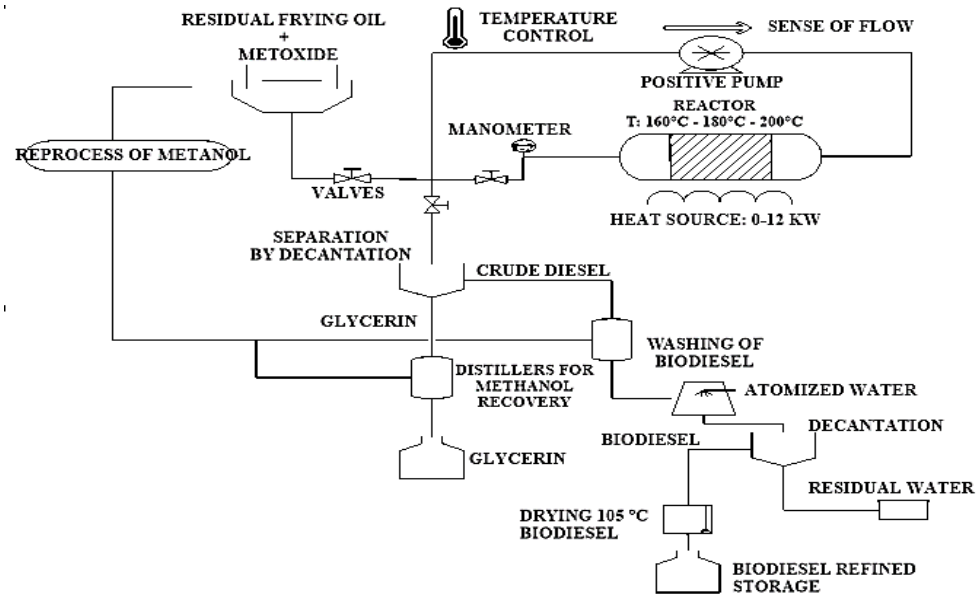

Figure 1. Discontinuous reactor, with external recirculation and process diagram.

Biodiesel is made up of mixtures of methyl esters, but it may also contain traces of: glycerin, soap, glycerides, free fatty acids, water, etc. The presence of these compounds affects to a greater or lesser extent the quality of biodiesel (CASTELLS and BORDAS, 2012). The process performance of biodiesel obtained was determined by the equation 2.

$$Pv\ \% = \frac{Bv}{Ov}\ x\ 100 \qquad \text{(Eq.2)}$$

Where: *Pv%*: performance value *%*; *Bv*: biodiesel volume (product volume) and *Ov*: Oil volume.

3. RESULTS AND DISCUSSION

3.1. Sub critical conditions for obtaining biodiesel

As a result of this experimental work biodiesel was obtained at temperatures of: 160°C, 180°C and 200°C, at molar ratios of 6: 1 and 9: 1 and the reaction time of 10 minutes, which shows significant differences when compared with the experiences of the following authors: GLISIC and ORLOVIĆ, (2014); FAROBIE and MATSUMURA, (2015), who used the subcritical and supercritical method under the following conditions: temperature from 150°C to 400°C, methanol in molar ratio from 12: 1 to 45: 1, and reaction times from 30 minutes to 10 hours. Therefore, it is concluded that this method and the working conditions have advantages in reaction time and in the amount of methanol used.

3.2. Density of biodiesel

The density is fundamental to determine the quality of the fuel (PHANKOSOL et al., 2014), determining values of densities between 0.87 g/mL and 0.89 g/mL to 15°C in the 36 biodiesel samples analyzed. The ASTM - D1298 determines that the allowed density value is between 0.85 to 0.90 g /mL, in the same way the ISO 3675 and ISO 12185 standards

establish values between 0.86 to 0.90 g / mL, With the data of the density of the 36 samples of biodiesel, the statistical analysis was carried out, which is summarized in Table 2.

Table 2. Analysis of the variance (SC type III) for the density of biodiesel.

Source of Variation	SC	GI	CM	F	p-valor
Replicas	3.6E-05	2	1.8E-05	3.10	0.0650 *ns*
Molar ratio	1.6E-05	1	1.6E-05	2.74	0.1120 *ns*
Catalyst	8.1E-05	1	8.1E-05	13.87	0.0012 **
Temperature	2.4E-04	2	1.2E-04	20.42	<0.0001 **
Interactions					
Molar ratio* Catalyst (A*B)	2.2E-05	1	2.2E-05	3.73	0.0664 ns
Molar ratio *Temperature (A*C)	6.2E-06	2	3.1E-06	0.53	0.5970 ns
Catalyst*Temperature (B*C)	5.0E-07	2	2.5E-07	0.04	0.9582 ns
Molar* Catalyst *Temperature (A*B*C)	1.0E-04	2	5.1E-05	8.80	0.0016 **
Experimental error	1.3E-04	22	5.8E-06		
Total	6.8E-04	35			
Coefficient of determination r^2	0.80				
CV	4.39				

Values of P: *, P≤0.05 (Significant statistical difference); ns, statistical difference not significant.

The coefficient of determination (r) was 0.80, consequently, it was concluded that 80% of the variability in biodiesel density depended on the concentration of the catalyst and the reaction temperature. For this type of samples analyzed, the coefficient of variation value was 4.39, this value is considered acceptable because it did not exceed the 7% limit (DANE, 2008).

3.3. Performance of biodiesel

The variables that affect the yield in terms of biodiesel production are: purity of the reagents used, mixing time, reaction temperature, concentration, type of catalyst used and mass ratio of the amount of methanol and oil used (ABREU et al., 2017). Performance values of the biodiesel samples were obtained from 78.78% up to 93.06%, with this data the analysis of variance was made, summarized in Table 3, determining that: the concentration of the catalyst, the reaction temperatures and the double B * C interaction presented a statistically significant difference (P≤0.05).
The triple interaction A * B * C showed a highly significant statistical difference (P≤0.01). However, there was no statistically significant difference in the molar ratio, replications and double interactions A * B (molar ratio * catalyst concentration) and A * C (molar ratio * reaction temperatures). In conclusion, it was demonstrated that the concentration of the catalyst and the reaction temperatures influence the yield of the obtained biodiesel.
The coefficient of determination (r) was 0.70, consequently, it was concluded that 70% of the variability in biodiesel yield depended on the concentration of the catalyst and the reaction temperatures. For this type of samples analyzed, the coefficient of variation value

was 4.39, this value is considered acceptable because it did not exceed the 7% limit (DANE, 2008)

Table 3. Analysis of the variance (SC type III) for the performance of biodiesel in sub critical condition.

Source of variation	SC	GI	CM	F	p-valor
Replicas	67.50	2	33.75	2.42	0.1125 *ns*
Molar ratio	9.87	1	9.87	0.71	0.4095 *ns*
Catalyst	98.37	1	98.37	7.05	0.0145 *
Temperature	139.85	2	69.93	5.01	0.0161 *
Interactions					
Molar ratio * Catalyst (A*B)	23.12	1	23.12	1.66	0.2116 *ns*
Molar ratior* Temperature (A*C)	81.20	2	40.60	2.91	0.0758 *ns*
Catalyst* Temperature (B*C)	133.74	2	66.87	4.79	0.0188 *
Molar*Catalyst* Temperature (A*B*C)	164.48	2	82.24	5.89	0.0089 **
Experimental error	307.20	22	13.96		
Total	1025.30	35			
Coefficient of determination r^2	0.70				
CV	4.39				

Values of P: * ≤0.05 (Statistically significant difference); P **, P≤0.01 (Highly significant statistical difference); ns, statistical difference not significant.

In Fig. 2, it is observed that all the treatments exceeded 78% of yield, by means of the analysis of comparison of means of the treatments, it was observed that there was a significant difference between them, the treatment being: A2B2C2 (molar ratio, 9: 1, catalyst, 1% and reaction temperature, 180°C) showed the highest yield with 93.06%; followed by the A1B1C2 treatment (molar ratio 6: 1, catalyst concentration, 0,5% and reaction temperature, 180°C) with 90.56%. Determining that the appropriate temperature in the reactor is 180°C. In the following figure the means with a common lowercase letter refer to that they were not significantly different (P> 0.05).

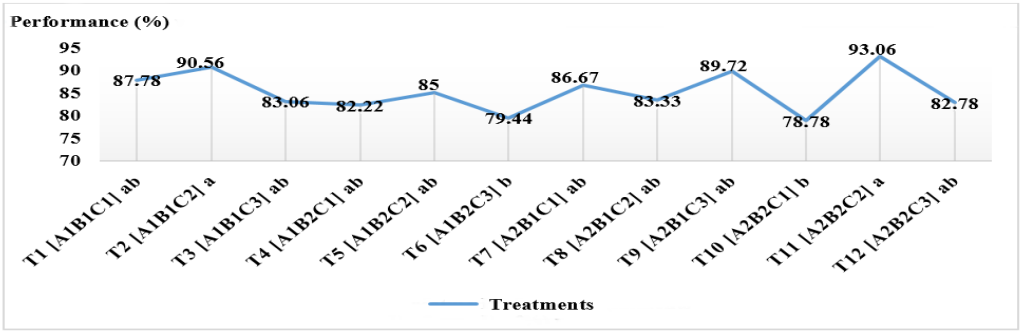

Figure 2. Effect and comparison of means of the 12 reaction processes on the performance of biodiesel.

3.4. Combustion of biodiesel

To measure the combustion gases of biodiesel, a BACHARACH® molelo ECA 450 gas measuring equipment was used, which establishes the percentage of oxygen in 15.4%. The results corresponded to a speed of operation of the generator brand Kubota® to diesel at 1500 rpm, obtaining a greater efficiency of combustion of biodiesel with respect to the diesel of oil in 1.3%. The fuel consumption was 20 cm^3 per minute of operation. The fuel consumption was 20 cm^3 per minute of operation.
The amount of gases obtained from the combustion of one hour (combustion in engine of the generator) of diesel and biodiesel, was determined by the following relationship: If in one minute 20 ml of fuel is consumed, in one-hour 1200 ml will be spent and therefore, there will also be a greater emission of gases to the environment, the values obtained are reported in Table 4.

Table 4. Gases of the combustion of diesel and biodiesel in one hour of fuel consumption.

Gases of combustion	Gases from the combustion of biodiesel in a minute	Gases of diesel combustion in one minute	Gases of diesel combustion in one hour	Gases from the combustion of biodiesel in one hour
C0 (mg/m^3)	704	899	53940	42240
CO_2 (%)	3,2	4	240	192
NO_X (mg/m^3)	42	39	2340	2520
SO_2 (mg/m^3)	12	38	2280	729

When comparing the amount of gases that emit 1200 ml of diesel and biodiesel in one hour in the generator, it was determined that with biodiesel will be decreased 11700 (mg/m^3) of CO, 48% CO_2 and 1551 (mg/m^3) SO_2, contributing in this way to the reduction of environmental pollution.

4. CONCLUSIONS

The reaction time for obtaining biodiesel, using the prototype batch reactor with external recirculation and the reaction conditions applied in this study, was 10 minutes, compared with the traditional method, improved by 85%. The quality of the biodiesel in relation to the density complies with the ASTM-D1298, ISO 3675 and ISO 12185 standards that determine the density value allowed for this fuel. The density of the biodiesel obtained was 0.87 g/L and 0.89 g/L, and in the standards the limit allowed for this type of biofuels is 0.86 to 0.90 g /L. The combustion of diesel versus biodiesel, established that the gases generated by biodiesel are quantitatively lower than the gases generated by diesel. Specifically, in carbon dioxide production, biodiesel presented a generation of 48% less than that generated by diesel.

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ETHANOL PRODUCTION USING LIGNOCELLULOSIC BIOMASS

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ABSTRACT

Lignocellulosic materials such as corn cobs, rice straw, and wheat straw have a high content of cellulose, hemicellulose, and lignin, and can be used to produce ethanol. The process involved the hydrolysis of cellulose in the biomass reducing sugars. The objective of this study was to determinate the better pretreatment to remove hemicellulose (xylans) and lignin. The pretreatments used in this study were organosolv and alkaline methods. The best method was the alkaline using the following conditions: 4% NaOH and 30 min at 121 °C, reducing 61% the content of xylans and 81% the content of lignin on rice straw.

Keywords: biomass, ethanol, lignin, pretreatment, xylans

1. INTRODUCTION

Worldwide the lignocellulosic biomass production is abundant (Sánchez and Cardona, 2008), this biomass is mainly composed of high content of cellulose, hemicellulose, and lignin, and are important sources to produce cellulosic ethanol (HOWARD *et al*., 2003; GALEANO-SUAREZ *et al*., 2014). Lignocelluloses as agricultural, industrial and forest residuals account for the majority of the total biomass present in the world. Bioconversion of the cellulosic components into fermentable sugars is crucial to initiate the production of industrially essential products from cellulosic biomass (KUMAR *et al*., 2008). The ethanol production from lignocellulosic biomass comprises the following main steps: hydrolysis of cellulose and hemicellulose, sugar fermentation, separation of lignin residue and, finally, recovery and purifying the ethanol to meet fuel specifications (ALVIRA *et a*l., 2010).
Pretreatments have been used in the last years, showing that the different methods can be successfully applied to all types of biomass, reflecting the wide range of biomass structures (GALBE and ZACCHI, 2007). The purpose of the pretreatments is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective (SUN and CHENG, 2002).
In this research two pretreatments methods, organosolv and alkaline were used. The organosolv technique is carried out to separate cellulose and lignin by extraction of the lignin from lignocellulosic feedstock using an organic solvent like ethanol due to the capacity for delignification and hemicellulose solubilization (CATETO *et al*., 2011; OBAMA *et al*., 2012; DE LA TORRE *et al*., 2013). For otherwise, the alkaline method is mainly used to digest wood pulps using in some cases sodium hydroxide solution. Its principal advantages are short processing times and the absence of formation of sulfur by-products (VON SCHENCK *et al*., 2013).
The purpose of this research was to evaluate two pretreatment techniques in different biomass and determine the best pretreatment and lignocellulosic biomass for ethanol production.

2. MATERIALS AND METHODS

2.1. Preparation of the biomasses

Corn cobs, rice straw, and wheat straw were provided by the AgroFarm Sánchez-Jaramillo. The material was stored at 4°C in plastic bags. The biomass with 10% of moisture was milled in a Willey type mill to a particle size of < 5mm.

2.2. Pretreatments

In the alkaline pretreatment, the lignocellulosic biomass was autoclaved for 30 min at 121 °C and the content of NaOH was 4% (w/w, based on dry weight), and the total dry weight was 10%. On the other hand, 25 g of the wastes were mixed with aqueous ethanol (50%), the mixture was heated at 190 °C for 90 min with continuous stirring in the organosolv method. After that, the pretreated wastes were washed with distilled water to eliminate the soluble molecules produced during the both pretreatments (RODRÍGUEZ-ZUÑIGA et al., 2015; VÁSQUEZ-VÉLIZ *et al*., 2018).

2.3. Chemical analysis of the lignocellulosic biomass and sugar analysis

A proximal analysis was realized to determine the chemical composition of the lignocellulosic material: cellulose and lignin determined according to the methodology of (GAITÁN-HERNÁNDEZ *et al*., 2006).
Approximately 2 g milled samples were extracted with 95% ethanol/ cyclohexane (1:2 v/v) and water for 6 h in a Soxhlet apparatus. Extracted samples were hydrolyzed with 72% (v/v) sulfuric acid at 45°C for 10 min. The acid was diluted to a final concentration of 3%, and the mixture was autoclaved at 121°C for 30 min. The residual material was filtered through a filter paper (NASCIMENTO *et al*., 2016).
For the structural carbohydrate determination, sugar analysis was performed with a Dionex ICS5000 HPAEC system. The temperature of columns was maintained at 28 °C. Pure water was used as the main eluent for 32 min at 0.250 mL min^{-1}, after that by a washing step with 0.25 M NaOH for 10 min. Subsequently, the initial conditions were restored for 15 min, before a new injection. For the post-column eluent, a solution of 0.2 M NaOH was added at 0.1 mL min^{-1} during each step (RODRÍQUEZ-ZUÑIGA *et al.*, 2015).

2.4. Statistical analysis

In all experiments, a completely randomized design and the results were examined using one-way analysis of variance (ANOVA) to determine the significance of individual differences at $p<0.05$ level, of the chemical composition of the agricultural wastes and the sugar composition. Once the statistical differences were found, the Duncan Test with $\alpha = 0.05$ was applied. The analyses were carried out using Statgraphic ver. 16 statistical software.

3. RESULTS AND DISCUSSION

Pretreatments (organosolv and alkaline) were applied to the lignocellulosic biomasses with dry matter (DM) content at 10%. The chemical composition of the biomasses and the composition of the xylans were measured before and after the pretreatments (Tables 1 and 2). All the pretreated biomasses were hydrolyzed with a cellulolytic cocktail.

3.1. Chemical analysis of the lignocellulosic biomasses

The chemical composition of the lignocellulosic biomasses showed a different chemical structure after using the different methods. Table 1 shows that with the organosolv method, the higher increase of cellulose was presented on rice straw with 68%, while with alkaline method was showed on rice straw with 80%. Regarding to the lignin content, it decreased 59% on corn cobs, 68% on rice straw and 39% on wheat straw using the method organosolv, whereas using the alkaline it decreased 74% on corn cobs, 81% on rice straw and 71% on wheat straw. The wheat straw treated with the alkaline method presented the greatest lignin removal of 81% and an increase in the composition of cellulose of 80% in comparison with the others lignocellulosic materials. Similar results have been published, VÁSQUEZ-VÉLIZ *et al.* (2018) showed using the organosolv method content of cellulose increased 52% on corn cobs and 67% on rice straw and content of lignin decreased 43% on corn cobs and 54% on rice straw, while using the alkaline technique the content of cellulose increased 38% on corn cobs and 45% on rice straw and the content of lignin decreased 59% on corn cobs and 61% on rice straw, whereas (RODRÍQUEZ-ZUÑIGA *et al*.,

2015) presented the chemical composition of sugarcane bagasse treated for the organosolv and alkaline methods, using the organosolv pretreatment removed 61% of the lignin, while with the alkaline treatment provided the greatest lignin removal of 70% in comparison with its native form, and the content of cellulose increased more than 60% using these pretreatment technologies. ZHAO *et al*. (2009) indicated that the organosolv pretreatment cleaves the bonds of hemicellulose–lignin or the hydrolysis of glycosidic bonds in hemicelluloses releasing hemicellulose–lignin fragments, and the cleavage of α-aryl and β-aryl ethers in the native lignin. The alkaline process also cleaves the α-ether and ester linkages in the phenolic polymer and between lignin and polysaccharides (KRISTENSEN *et al*., 2015). KUMAR *et al.* (2009) pointed out that the goal of the pretreatment process is to remove lignin and increase the porosity of the lignocellulosic materials. The modification of the lignin content allows the access of the polysaccharides of the cell wall of the plant to chemical digestion increasing the yield of the fermentable sugars for the production of ethanol (CHEN and DIXON, 2007). The variations in the composition of the lignocellulosic biomass are directly related to the moisture, the waste and the treatment.

Table 1. The chemical composition of corn cobs, rice straw, and wheat straw before and after organosolv and alkaline pretreatment at 10% solids loading.

Samples	Cellulose (%)	Klason Lignin (%)
Native		
Corn cobs	44.2±1.3^{b}	22.3±1.5^{f}
Rice Straw	41.3±0.6^{a}	22.9±0.9^{g}
Wheat straw	42.9±0.1^{a}	20.1±1.8^{e}
Organosolv Pretreatment		
Corn cobs	64.5±1.9^{d}	9.2±0.6^{c}
Rice straw	69.4±1.0^{e}	7.3±0.3^{b}
Wheat straw	54.9±0.1^{c}	12.3±1.1^{d}
Alkaline Pretreatment		
Corn cobs	72.3±2.1^{f}	5.7±0.4^{a}
Rice straw	74.3±1.1^{g}	4.4±0.2^{a}
Wheat straw	65.4±0.2^{d}	5.8±0.5^{a}

*Different letters in each column indicated significant difference among the cellulose and lignin values at level $P<0.05$, according to Duncan test, $n = 3$.
*The values indicate the chemical composition of the different lignocellulosic biomasses before and after of the pretreatments.

3.2. Sugar composition of xylans

The content of xylans in the biomass previously treated in both methods was modified to the raw biomass. Table 2 presents the lignocellulosic materials pretreated with the organosolv method. The higher decrease of xylans was showed on corn cobs with 33%, while with the alkaline was presented on rice straw with 61%. Otherwise, the content of arabinan also decreased using the organosolv and alkaline methods more than 72%, while the content of galactan only was determined in the native material and the content of

mannan was not presented. Rice straw treated with the alkaline technique showed the most significant reduction of xylans of 61% in relation with the others the lignocellulosic materials and with the other method. Authors have reported similar results, VÁSQUEZ-VÉLIZ *et al.* (2018) using the organosolv method the content of xylans decreased 34% on corn cobs and 27% on rice straw, whereas using the alkaline procedure the xylans decreased 62% on corn cobs and 56% on rice straw, while (RODRÍQUEZ-ZUÑIGA *et al*., 2015) showed in their study that the sugar composition changed after the lignocellulosic materials were treated using the organosolv and alkaline methods, the contents of xylans and arabinan decreased in both methods, and the content of galactan only was determined on the raw material, but in relation with the content of mannan was determined in the biomass treated using the organosolv method. JAYAPAL *et al*. (2013) indicated that the hemicellulose fraction is composed of a xylan backbone, several sugars including arabinan, glucose, and glucuronic acid present in different proportions. The sugar composition of xylans vary with the method of isolation from its raw materials (PENG *et al*., 2009).

Table 2. Compositional analysis of corn cobs, rice straw, and wheat straw before and after organosolv and alkaline pretreatment at 10% solids loading.

Samples	Galactan (%)	Xylan (%)	Arabinan (%)	Mannan (%)
Native				
Corn cobs	1.3±0.1^{c}	20.4±0.9^{g}	2.1±0.4^{c}	np
Rice straw	nd	17.7±0.7^{f}	2.8±0.4^{d}	np
Wheat straw	nd	19.2±1.0^{g}	2.2±0.2^{c}	np
Organosolv Pretreatment				
Corn cobs	0.7±0.1^{a}	13.8±0.7^{d}	0.5±0.1^{b}	np
Rice straw	nd	12.8±0.5^{c}	0.5±0.1^{b}	np
Wheat straw	nd	15.2±0.7^{e}	0.6±0.1^{b}	np
Alkaline Pretreatment				
Corn cobs	1.0±0.1^{b}	9.8±0.7^{b}	0.3±0.1^{b}	np
Rice straw	nd	6.9±0.3^{a}	0.2±0.0^{a}	np
Wheat straw	nd	11.7±0.6^{c}	0.2±0.0^{a}	np

*Different letters in each column indicated significant difference among the sugar composition at level $P<0.05$, according to Duncan test, n = 3; *np=not presented.
*The values indicate the composition of the xylans of the different lignocellulosic biomasses before and after of the pretreatments.

4. CONCLUSIONS

The results of this research pointed out that the alkaline method provides lignocellulosic materials (corn cobs, rice straw, and wheat straw) with the higher content of cellulose and lower content of lignin, xylans and other sugars in comparison with the organosolv technique.
Rice straw was the lignocellulosic material with the better chemical composition in comparison with the other lignocellulosic materials used in this study after the using organosolv and alkaline pretreatment methods for ethanol production.

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MICRONUTRIENTS AND *IN VITRO* PROTEIN DIGESTIBILITY OF LIMA BEANS (*PHASEOLUS LUNATUS* L.) GROWN IN ECUADOR

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ABSTRACT

Lima Bean is a tropical legume part of the ancestral culinary culture from the Ecuadorian littoral which has a high protein content. The aim of this work was to isolate and characterize food-proteins and its digestibility, as well as micronutrients contents (Ca, Fe and Mg).

Lima beans were ripped at mature state since it is supposed to improve the parameters of digestibility and other nutrients. Protein was isolated from the flour by isoelectric precipitation and the digestibility was carried out using a two steps method (gastric and duodenal digestion). The micronutrients were evaluated by atomic absorption spectrometry.

The higher yield was found at pH 6, while the higher protein content found at pH 5. Different proteins with weights between 10 and 100 kDa were found, and after digestion some proteins were not hydrolyzed. Regarding the micronutrients, Mg showed the major content.

The geographic location, the incidence of lightning and the quality of the soil provide nutritional qualities that can be associated with the micronutrients observed.

Keywords: digestibility, electrophoresis, lima beans, micronutrients, proteins

1. INTRODUCTION

Seeds are the reserve material that plants use for propagation. When the conditions are favorable all this material is used as a source of nitrogen (storage proteins) and carbon (starch or oil) to achieve the germination or developing seedling. The percentage of proteins in legume seeds (20-25%) is higher than the percentage of protein in cereal grains (10-15%) of dry matter, in average (DERBYSHIRE *et al.*, 1976). Generally, proteins from animal sources are relatively difficult to produce and obtain, and usually they are expensive. So, in the last years the research on vegetable proteins has increased drastically in order to identify the availability sources (CHEL-GUERRERO *et al.*, 2002). So, legume seeds in general are a good food source for human beings and animals as well. Importantly, legumes are the second major food sources as cereal, in developing counties.

It has been reported that the protein quality of legumes is poor regarding the levels of essential amino acids like cysteine and tryptophan. Two of the major classes of protein present in legume seeds are storage proteins (with the major proportion of globulin) and metabolic proteins (CHAVAN *et al.*, 2001b). The amino acid composition varies significantly based on the legume type. However, all legume globulins had a high content of basic amino acids as well as of glutamin acid/glutamine and aspartic acid/asparagine (CARBONARO, 2006b).

Phaseolus vulgaris L (lima bean), known as "haba pallar" in Ecuador, is a well-known specie of bean which are consumed in the world due to its high nutritional value. Overall, beans are excellent sources of protein, starch, polyunsaturated fatty acids, dietary fiber, minerals and vitamins (DU *et al.*, 2014). This legume shows a good nutritional profile due to the high levels of lysine, arginine and phenylalanine, and also the presence of glutamic acid and aspartic acid in a high content (SEIDU *et al.*, 2015). The high quantity of protein makes this legume an excellent potential protein source for several applications in the food industry.

Lima beans is a legume belonging to the Fabaceae family, the common name changes according to the place or origin. This legume requires small amounts of moisture compared to common bean (*Phaseolus vulgaris* L.), due to its high adaptability and tolerance to heat which allows harvesting during periods of drought (ADVÍNCULA *et al.*, 2015).

It is cultivated frequently in high quantities in zones of lower altitude until the 320 msnm, but it can be produced until the 2,030 msnm (BERMEJO, 1992). Lima bean is distributed in many regions worldwide, such as throughout Latin America (Colombia, Venezuela, Peru and Ecuador) and the southern United States and Canada (BETANCUR-ANCONA *et al.*, 2004).

In Ecuador, lima bean is a crop that grow both in the areas of the mountains (high altitude) and on the coast (low altitude), the variation in altitudes ranges between 900 to 2800 m, but its grow is higher at sea level. There are two varieties such as: INIAP 490, which is for winter and INIAP 491, which is for summer (MENDOZA and LIZÁN, 1993). These two varieties are cultivated mainly in the province of Manabí, specifically in the cities of Portoviejo with 18.3 Ha and in greater quantity in Rocafuerte with 236.87 Ha (CARRIÓN and VILLARREAL, 2012), and recently extending towards the province of Santa Elena.

The aim of this work was the isolation of proteins from lima beans by isoelectric precipitation at different pH (2-6) and its characterization by electrophoresis SDS-PAGE. Also, to evaluate gastric and duodenal digestibility of the concentrates, and the content of micronutrients (Ca, Fe and Mg).

2. MATERIALS AND METHODS

2.1. Materials

Dry lima beans were obtained in a market in the Rocafuerte canton of the province of Manabí in May 2016. The beans were dried at 50°C for 2 to 3 hours. Subsequently the beans were previously ground in the mill PERTEN Laboratory mill 3100 and sieved in sieve ADVANTECH™ DuraTap DT168 with mesh #70 (0.210mm). The obtained flour was vacuum packed and stored at room temperature.
All reagents for proteins characterization were analytical grade purchased from Merck®, Emsure®, Invitrogen® o Fisher®. For atomic absorption spectroscopy, concentrated HCl and HNO_3 of TraceMetal grade were purchased from Fisher Scientific™. Calcium, iron and magnesium solutions were prepared daily by diluting commercially available 1 g L^{-1} standards (Ca & Fe from AccuStandard, New Haven, USA and Mg Merck, Darmstadt, Germany) with 0.14 M HNO_3 solutions. Purified water was obtained from a Milli-Q system (Thermo Scientific, England, UK).

2.2. Protein concentrates

Protein concentrates of *Phaseolus lunatus* L. were obtained by isoelectric precipitation, following the method used by Martínez and Añón (1996), with modifications. The flour was mixed with water in 1:10 ratio (w/v), adjusted to pH 8.0 with NaOH (2M) and stirring for 1 hour. Then the suspension was centrifuged and the supernatant was adjusted at pH 3.0, 4.0, 5.0 and 6.0 with HCl (2M) and refrigerated. The precipitate was lyophilized. Each extraction was done by triplicate and equation 1 was used to calculate the yield.

$$\text{Protein yield (\%)} = \frac{W_P\,(\text{g})}{W_F\,(\text{g})} \text{ x } 100 \qquad \text{Equation 1}$$

Where W_P is the weight of protein isolated at different pH and W_F is the weight of flour, both expressed in grams.

2.3. Protein content of the concentrates

The protein content in concentrates was determined with the colorimetric method of Biuret, described by Gornall *et al*. (1949). 10 mg of the protein concentrates, 1 mL of distilled water and 75 µL of NaOH (0.2 M) were kept under stirring for 10 min. Two aliquots of 250 µL were taken and 1.25 mL of the Biuret reagent was added to the sample. The aliquots were centrifuged at 13000 rpm for 30 min. The samples were reading in a Genesis 4001/4 Colorimeter at 540 nm, distilled water was used as blank. The tests were done by duplicate. The protein quantification was obtained using the standard straight-line BSA equation (bovine serum albumin): Abs= 0.0316[C]-0.0015. Finally, the percentage purity of the protein isolates was determined.
Protein content was also determined by the Kjeldahl method. Sample was digested with H_2SO_4 and the catalyst solution until the sample turns blue-green. The sample was cooled and distilled water was added. The digested sample was mixed with NaOH solution and distilled. The distillate was titrated with 0.1 N HCl. A violet color indicates the end point of the titration. Each equivalent of the HCl used corresponds to an equivalent of NH_3 or an equivalent of N in the original sample. It was determined by the equation 2.

$$(\%) = N_{HCl} \frac{V_{acid}}{W_{sample}} \frac{14\ gN}{mol}\ x\ 100 \qquad \text{Equation 2}$$

Where, N_{HCl} is the HCl normality in moles/1000 mL, V_{acid} is the corrected acid volume calculated as the difference of mL of the acid standardized for the sample and the mL of the acid standardized for the blank, W_{sample} is the weight of the sample used in the analysis and 14 is the atomic weight of the nitrogen. It was necessary to use a factor to convert the nitrogen percentage to crude protein percentage, this value can change according to the food group, for vegetables is used 6.25.

2.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and NATIVE PAGE

For protein separation, electrophoresis SDS-PAGE was used according to the method described by Laemmli (1970). The protein concentrates at different pH were dissolved in distilled water, mixed with sample buffer (ratio 1:1 v/v) and heated at 80°C for 10 minutes in a microincubator at 400 rpm. It was used a 4% acrylamide stacking gel and a 12% acrylamide separating gel in a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, Life Science, USA). Electrophoresis was conducted at a constant current of 200 V per gel. Polypeptide bands were stained in Coomassie brilliant blue R-250 solution and then faded by washing the gel with a methanolic acid solution.
Native-PAGE electrophoresis was done as described in the previous section, but without sodium dodecyl sulphate (SDS). Reduction of disulfide bonds was performed with 2-mercaptoethanol by heating at 95°C for 10 min.

2.5. *In vitro* digestibility

Gastric digestion: Lima beans protein concentrate at pH 6.0 was used for *in vitro* gastric digestion simulating human physiological conditions, according to the method described by Jiménez-Saiz *et al.* (2011) and Minekus *et al.* (2014). 10 mg of protein were mixed with 1 mL of simulated gastric fluid (SGF 0.35 M NaCl solution at pH 1.20, 2.00 and 3.20, respectively). To each sample 100 µL of porcine pepsin solution (2000 U/mL; EC 3.4.23.1, 4500 U/mg protein, Sigma-Aldrich) were added; the samples were heated at 37°C for 2 hours at 450 rpm in a Esco micro-incubator, Provolcell Shaking. The digestion was stopped by mixing with sodium bicarbonate (1M, 200 µL) and heating at 80°C for 10 minutes to irreversibly inactivate pepsin.
Duodenal digestion: Gastric digestion at pH 3.20 (before the inactivation of pepsin) and pancreatin solution (100 U/mg, 10 nM bile salts and 1.5 Mm $CaCl_2$), were mixed in 1:1 ratio (v/v). The pH was adjusted to 7.0 and the sample was incubated at 37°C for two hours at 450 rpm. The hydrolysis was stopped by heating at 90°C for 5 minutes.
The hydrolysates were analyzed by SDS-PAGE electrophoresis using a constant voltage of 200 V.

2.6. Analysis of protein concentrates using reversed-phase high-performance liquid choromatography (RP-HPLC)

All protein concentrates were analyzed by RP-HPLC on Agilent 1200 infinity series UHPLC System (Agilent Technologies, Waldbronn, Germany) coupled to a diode array detector and using a EC C18 column (Agilent Poroshell 120, 4.6 x 50 mm x 2.7 μm of particle size). 100 μL of samples were injected and eluted with a mixture of water (solvent

A) and methanol (solvent B) with 0.27% trifuoroacetic acid (TFA). The elution was at 1.0 mL/min in a gradient (0-70% B, 5 min) and linear (70% B, 6 min) mode. The samples were followed at wavelength of 280 nm.

2.7. Determination of minerals by atomic absorption spectroscopy

The flour homogenized (1 to 2 g) was subjected to dry ashing in well cleaned and dried porcelain crucibles at 550°C for 12 hours in a Nabertherm muffle furnace by quintuplicate. The resultant ash was dissolved in 0.5 mL of HCl and 0.25 mL of HNO_3 both concentrated, while heating on a hot plate until brown fumes disappeared. The mineral solution was transferred into a volumetric flask by filtering through a 0.45 μm filter and the volume made to the mark with 0.14 M HNO_3 solution. The micronutrients were determined using the AOAC (2005b) for iron and AOAC (1997a) for calcium and magnesium. The measurements were carried out using a line source atomic absorption spectrometer PG Instruments: AA500 equipped with both flame and graphite furnace atomizers and continuum source background correction.

2.8. Statistical analysis

Results are presented as means±standard deviation (SD) of three repetitions of each experiment. In the case of protein quantification, four replicas were used. The analysis of variance (ANOVA) and Tukey test were considered significant at $p<0.05$ to identify differences between mean values. Statistical analysis was performed using the SPSS software for Windows.

3. RESULTS AND DISCUSSION

3.1. Protein concentrates and protein content

Dry lima beans were used to obtain a fine flour. The isolation of protein was achieved by the isoelectric precipitation method at different pH (3.0, 4.0, 5.0 and 6.0), using water as solvent. The highest yield was obtained at pH 6.0 with 32.58± 0.30% while the lowest one at pH 3.0 with 8.64±0.10%, all concentrates showed significant difference. The protein contents of samples were determined by the Biuret and Kjeldahl methods. The first test used for detecting the presence of peptide bonds by the formation of violet-colored coordination complexes in an alkaline solution due to a copper(II) ion. The second used for determining the organic nitrogen by a titration acid-base. The treatment at pH 5.0 and 3.0 showed the higher percentage of protein with both methods without any statistical differences with $p<0.05$. 71.33±0.92% and 69.96±0.78%, for pH 5.0 and 3.0, respectively was obtained by Biuret method. 42.31±0.58% and 41.19±1.1%, respectively was obtained by Kjeldahl method. The other treatments showed lower protein content (Table 1).
Since the protein content at all pH was lower than 85%, it was obtained protein concentrates (35-80% protein content in dry basis) instead of protein isolates (higher than 85%) (Lara *et al.*, 2017). The best treatment was at pH 5.0 with the higher protein content on a dry basis and it was used to evaluate the digestibility.

Table 1. Percentage of yield and protein content of concentrates of Lima beans.

pH	Yield of protein isolates (%)*	Protein content (%)** Biuret	Crude protein (%)* Kjendahl
3.00	8.64^{a}±0.10	69.96^{c}±0.78	41.19^{c}±1.1
4.00	18.56^{c}±1.14	62.39^{b}±0.35	38.92^{b}±0.14
5.00	12.86^{b}±0.16	71.33^{c}±0.92	42.31^{c}±0.58
6.00	32.58^{d}±0.30	56.80^{a}±1.77	34.36^{a}±0.44

*The values reported represent the mean of 3 measurements±the standard deviation.
**The values reported represent the mean of 4 measurements±the standard deviation.
Different letters indicate statistical differences ($p < 0.05$) between the groups of protein concentrates obtained at different pH of precipitation.

3.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and NATIVE PAGE

The protein concentrates at different pH were analyzed by electrophoresis native and SDS-PAGE methods, to obtain a profile protein in each case. In the polyacrilamyde gel worked in the absence of SDS (Native-PAGE), it was observed one band with a molecular weight around 150 Kda which showed the proteins not denatured, in all pH assayed (Fig. 1).

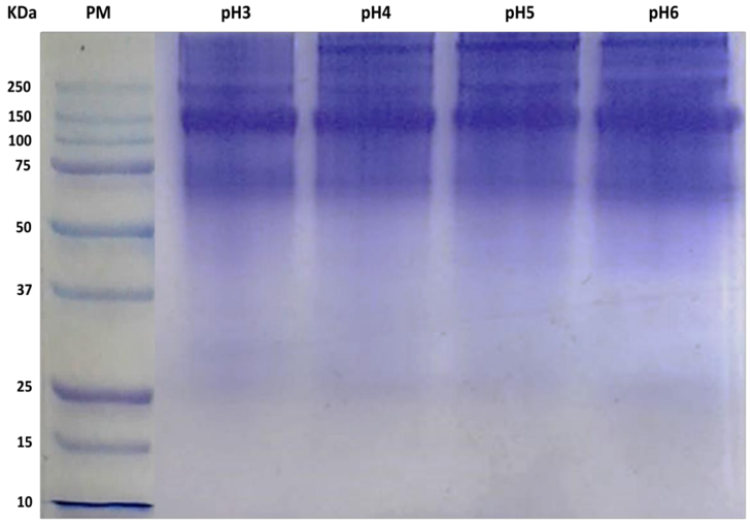

Figure 1. NATIVE-polyacrylamide gel electrophoresis of proteins from lima beans obtained at different pH of precipitation (3.0, 4.0, 5.0 and 6.0). PM: molecular weight marker.

The gel without 2-mercaptoethanol showed two bands between 75-100 kDa (globulins 7S), three bands between 30-37 kDa (globulins 11S acidic subunit), one intense band at 25 kDa (globulins 11S basic subunit); and one band at aprox 10 kDa (albumins 2S) (Fig. 2).

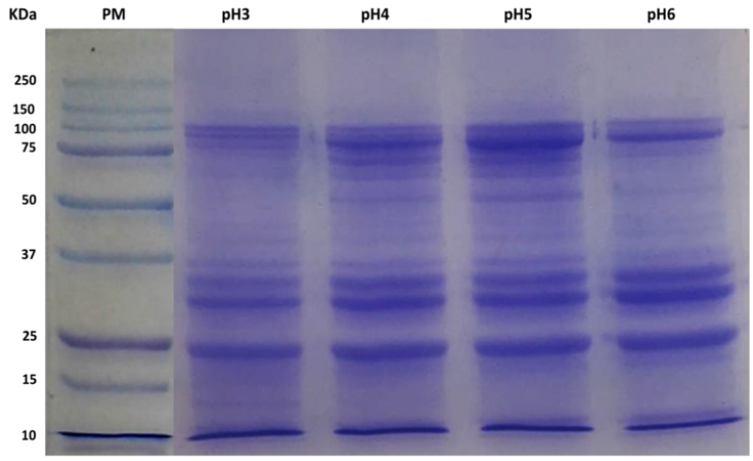

Figure 2. SDS-PAGE electrophoresis of proteins from lima beans obtained at different pH of precipitation (3.0, 4.0, 5.0 and 6.0), without 2-mercaptoethanol (reductor agent). PM: molecular weight marker.

In the presence of 2-mercaptoethanol, it was observed a major number of bands with higher intensities at all pH in comparison to the gel obtained in the absence of this reductor agent which is used to disrupt disulphide bridges (DERBYSHIRE *et al.*, 1976). Thus, showing the presence of disulfide bonds in the isolated proteins (Fig. 3). The gel with 2-mercaptoethanol showed better resolved bands with molecular weights around 100 (globulins 7S), 60, 33, 30 (globulins 11S acidic subunit), 25 (globulins 11S basic subunit), 15 and 12 (albumins 2S) kDa at all pH. However, the band observed at 60 kDa almost was not appreciated at pH 3.0 (Fig. 3).

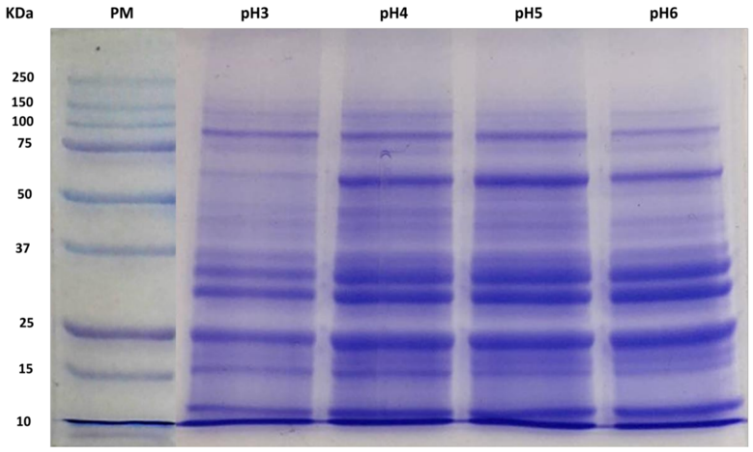

Figure 3. SDS-PAGE electrophoresis of proteins from lima beans obtained at different pH of precipitation (3.0, 4.0, 5.0 and 6.0), in the presence of 2-mercaptoethanol. PM: molecular weight marker.

It is reported that in legume seeds, two of the storage proteins with major contents are 7S and 11S proteins. 7S globulins have molecular weights of 150-200 kDa and they are glycoproteins, while the 11S globulins consist of two opposed hexagonal rings, each containing three pairs of disulfide-linked acidic (29-35 kDa) and basic (18-28 kDa) subunits (ROMERO-ZEPEDA and PAREDES-LOPEZ, 1996). One of the principal legume proteins

stored in the protein bodies of the common bean is the 7S trimeric glycoprotein (phaseolin). According to the sedimentation coefficient, the storage proteins from legume seeds can be classified as 2S, 7S or 11S globulins. 7S globulins extracted form common bean (*Phaseolus vulgris* L.) seeds showed major bands between 43-55 kDa (CARBONARO, 2006b). Legume seed storage globulins proteins, named 7S vicilin and 11S legumin, are oligomeric proteins consisting of three subunits (MW 140 000 – 200 000 Da) and six subunits pairs (MW 300 000 – 400 000 Da), respectively (CARBONARO *et al.*, 2005a). Although 11S and 7S globulins are the major storage proteins of legume seeds, other globulins occur in extracts such as 2S globulins, which are water soluble during isolation. 11S and 7S globulins are found in protein body fractions isolated. However, legumin and vicilin may not all have same protein composition although they both laid down in the same protein bodies. The fact that the relative proportions of the different storage proteins can differ considerably in different legume species, and in different varieties of the same species, suggests that there are possibilities for considerable change in the storage protein without loss of seed viability (DERBYSHIRE *et al.*, 1976). Albumins present low molecular weight with 11 kDa aprox. (LARA *et al.*, 2017; QUINTEROS *et al.*, 2016).
According to the molecular weight of the different bands observed in the electrophoretic gels, it was determined globulins 7S, globulins 11S acidic and basic subunit and at lower molecular weight albumins 2S.

3.3. Simulation of gastrointestinal digestibility *in vitro*

Protein concentrate with the higher protein content (pH 5.0) was chosen to hydrolysis with pepsin at three different pH (1.2, 2.0 and 3.2) to simulate the physiological conditions of a sick adult with gastritis, a wealthy adult and a baby, respectively. It was observed that proteins isolated were resistant to the hydrolysis of enzyme, only the heavy proteins (150-37 kDa) were hydrolyzed by the action of pepsin (Fig. 4). Two different concentrations (172 U/mL and 2000 U/mL) of pepsin were proved to observe the resistance of the proteins. However, no differences were observed in protein hydrolysis as can be observed in Figs. 4 and 5. Regarding to the second step of the digestion model (duodenal digestion) it was observed that proteins with molecular weights below 37 kDa were resistant to hydrolysis with pepsin and pancreatin enzymes. Thus, showing the stability to proteolytic digestion.
It has been reported a low nutritional value of proteins in *P. vulgaris seeds*, due to a limited amount of Sulphur amino acids, poor digestibility and low bioavailability of essential amino acids (CARBONARO, 2006b). Resistant to proteolysis because of structural properties (compact structure, stability conferred by S-S bonds and by the carbohydrate moiety) has been observed in *in vitro* studies on digestibility (CARBONARO, 2006b). So, we cannot conclude in apparently low biological value of legume proteins determined by their poor intestinal digestibility. It would be necessary an *in vivo* assay.

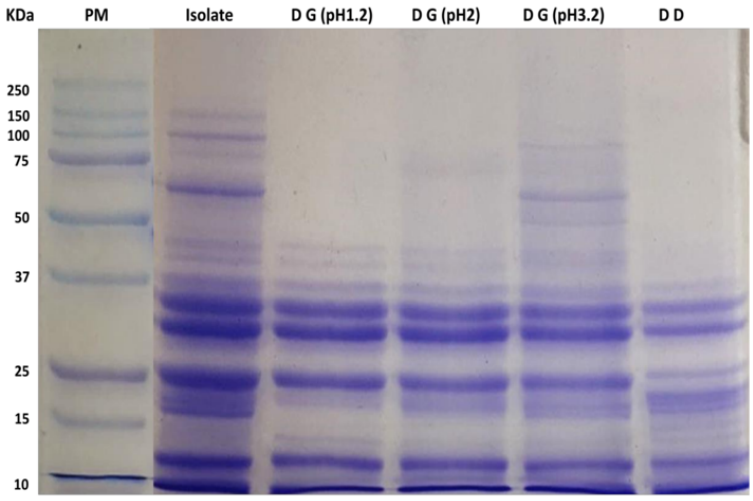

Figure 4. SDS-PAGE electrophoresis of proteins of lima beans protein concentrate of pH 5.0 (best treatment) hydrolyzed with pepsin (gastric digestion at low concentration of pepsin 172 U/mL) more pancreatin (duodenal digestion). PM: molecular weight marker, DG: gastric digestion and DD: duodenal digestion.

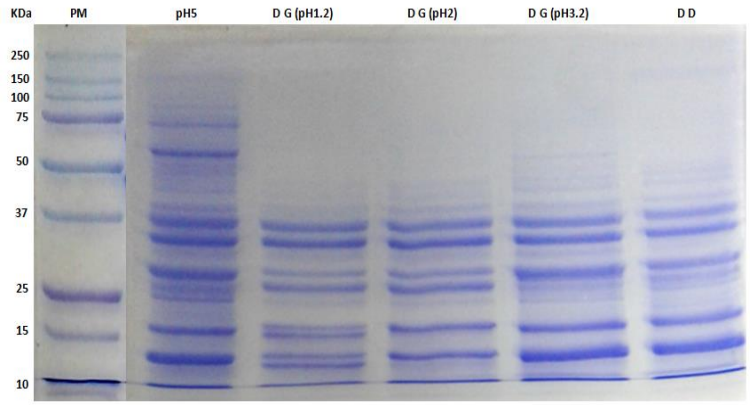

Figure 5. SDS-PAGE electrophoresis of proteins of lima beans protein concentrate of pH 5.0 (best treatment) hydrolyzed with pepsin (gastric digestion at high concentration of pepsin 2000 U/mL) more pancreatin (duodenal digestion). PM: molecular weight marker, DG: gastric digestion and DD: duodenal digestion.

3.4. HPLC measurements

All protein isolates were analyzed by RP-HPLC. The chromatograms obtained show the profile of proteins precipitated at different pH. The first intense peak corresponds to the TFA. The profile of protein concentrates at pH 3.0 and 4.0 showed basically the same peaks and intensity, in the same way the profile of concentrates at pH 5.0 and 6.0. All peaks appear in the first six minutes and show low hydrophobicity. Since the proteins were isolated at low pH, showed an acid character (Fig. 6).

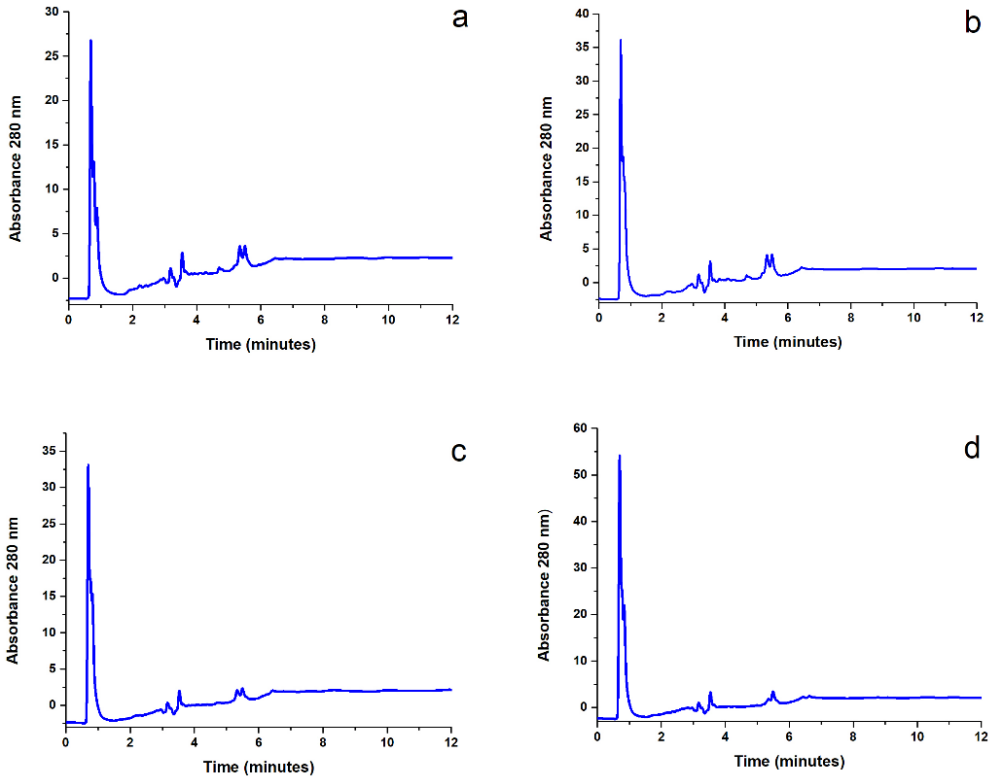

Figure 6. Chromatographyc profile of protein concentrates at (a) pH 3.0; (b) pH 4.0; (c) pH 5.0 and (d) pH 6.0.

3.5. Determination of minerals by atomic absorption spectroscopy

Three minerals were determined in lima beans flour because they interest from a dietetic point of view. Two macroelements (calcium and magnesium) and one microelements (iron). The concentration of elements (Ca, Mg and Fe) was determined using a line source atomic absorption spectrophotometer (spectrometer PG Instruments: AA500). Calibration curves of absorbance values versus concentration of each element at appropriate concentrations (to obey Beer's-Lambert Laws) showed in Table 2, were constructed using their respective standards of 1000 mg/L. The concentration of each element in the sample was calculated as mg/100 g of dry matter.

Table 2. Figures of merit observed for the monitoring of the different elements studied using Flame Atomic Absorption Spectroscopy, FAAS.

Element	Wavelength, nm	Prediction equation	Correlation coefficient	Linear range mg/L
Ca	422.7	Signal = 0.2193X+0.0024	0.9989	0.02-2.0
Fe	248.3	Signal = 0.1076X+0.0012	0.9997	0.03-4.0
Mg	285.2	Signal = 0.5426X+0.0039	0.9992	0.007-0.5

X: concentration of each element expressed in mg/L.

Magnesium showed the higher content with 155.65±7.65 followed by calcium with 14.51±1.41; while the lower content was of iron with 4.75±0.37. Mg and Fe showed similar values found in other legumes like different types of peas (beach pea, green pea, field pea). Nevertheless, the content of Ca is very low in comparison with reported by (CHAVAN *et al.*, 1999a).

4. CONCLUSIONS

Protein concentrates from lima beans (*Phaseolus lunatus* L.) were obtained by isoelectric precipitation at different pH. The best treatment was at pH 5.0 which is nearly close to the pI (isoelectric point) of this legume proteins. All concentrates showed the same protein profile in polyacrylamide gels. It was determined basically globulins and albumins proteins. Regarding to the micronutrients, Mg was observed in a major concentration compared to Ca and Fe. The high protein content makes this food matrix a good source of nutrients that can be used directly or by means any technological transformation. Even though it could use the concentrates in the formulation of a functional product that contributes to the health of the littoral people of Ecuador since the high content of protein showed.

5. ACKNOWLEDGMENTS

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ANTIMICROBIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF PROTEINS OF *PHASEOULUS LUNATUS* (FABACEAE) BABY LIMA BEANS PRODUCED IN ECUADOR

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ABSTRACT

Phaseoulus lunatus L., a variety of baby lima bean, which is produced in the coastal region of Ecuador, is a profitable crop of that country. Various cultivars of this common bean are considered a sources for nutraceutical compounds, such as bioactive peptides. To assess the potential biologic activities of protein isolates and hydrolysates of *P. lunatus* baby lima beans, this study evaluates the proteins antimicrobial, antioxidant and anti-inflammatory activities. Antioxidant activity was measured by the TBARS method. In-vitro anti-inflammatory activity was measured by the inhibition of denatured protein as well as a diffusion method, according with CLSI guidelines by antimicrobial activity. Both fractions (isolate and hydrolysates) showed anti-inflammatory and antioxidant activity. However, protein hydrolysates (pH 5) had a better performance than protein isolates. The same effect was observed in antimicrobial activity, when protein hydrolysates had a broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria. These preliminary studies suggest that *P. lunatus* baby lima beans could have a considerable biological activity for nutraceutical applications.

Keywords: *Phaseoulus lunatus*, protein, baby lima bean, antimicrobial, antioxidant, anti-inflammatory

1. INTRODUCTION

The genus *Phaseolus* (beans), included in the family *Leguminosae*, represent one of the most ancient, common legumes consumed by humans around the world. In developing countries, this legume specie is one of the principal sources of dietary proteins (BROUGHTON *et al*., 2003; JOSHI *and* RAO, 2017). Today, in terms of morphological and genetic diversity, *Phaseolus* are a complex genus with four genetic groups (BITOCCHI *and* NANNI 2012). Previous research has shown that the northwestern region of South America (Colombia, Ecuador and northern Peru) represents the convergence of the two principal genetic *P. vulgaris* varieties (Central American and Andean) (DEBOUCK *et al.* 1993). This convergence shows a clear difference of morphology and molecular characteristics. Considering the current correlation between population growth and bean consumption (BELLUCCI *et al*., 2014; SOZER *et al*., 2017), a detailed analysis of chemical and biological characteristics would help us better understand the properties and differences between common bean varieties.

An increasing amount of literature demonstrates the biological properties of protein isolates of legumes. These properties represent a considerable nutritional role and a source of essential amino acids (BETANCUR-ANCONA *et al*., 2009; CARBONARO *et al*., 2015; GRELA *et al*., 2017). Varieties and cultivars of common beans has been considered as sources of nutraceutical compounds, such as bioactive peptides, polyphenols, and polysaccharides (NGOH *and* GAN 2016; CAMPOS-VEGA *et al.* 2010; BETANCUR-ANCONA *et al.* 2009; BITOCCHI *and* NANNI 2012).

The variety of baby lima bean that is produced in the coastal region of Ecuador is a profitable crop for that country in terms of economic benefits (EL PRODUCTOR 2015). At the moment, no published information about physicochemical and biological characteristics of this Ecuadoran variety of bean. Considering the differences between *Phaseolus* germplasm according their precedence (BERNAL *and* GRAHAM 2001), the differences are expected in chemical composition and biological activities. This study evaluates the antimicrobial, antioxidant and anti-inflammatory activities of protein extracts and protein hydrolisates of *P. lunatus* var. baby lima beans, in order to identify the beans potential source of bioactive compounds for nutraceutical applications.

2. MATERIALS AND METHODS

2.1. Samples

P. lunatus var. baby lima beans were purchased in a local market in Machala, Ecuador. Seeds were dehydrated in a Laboratory Incubator (ESCO, Singapore) at 50°C for 48 hours. Subsequently, dehydrated seeds were grinded into a fine flour powder with a mill MG 1511.

2.2. Protein isolates from baby lima beans

Baby lima bean protein concentrate was obtained according to the methodology of BARRIO *and* AÑÓN, (2010) with minor modifications. Homogenized flour was diluted in a 1:10 solution using distilled water. The pH level was set at 8 by using sodium hydroxide 2 M for 1 hour, shaken at 800-900 rpm. Those samples were centrifuged at 4.400 rpm for 30 minutes. The supernatant was recovered and treated at different pHs (3, 4, 5 and 6) using, if necessary, hydrochloric acid 2 N or sodium hydroxide 2M. The mixture was vigorously homogenized and stored at 4°C for 24 hours. The supernatant was discarded and the

precipitates were freezed at -80°C and lyophilized using a freeze dryer Bench Top Pro BTP-3ES0VW (SP Scientific, Stone Ridge, NY, USA), at -50°C and 0.2 Pa. The extraction performance was calculated with this formula: % performance = (Pf / Po) * 100. Where Po: Initial weight of flour, Pf: Final weight of lyophilized sample. The protein quantification was carried out by Biuret method (NIELSEN, 2017).

2.3. Protein hydrolysates

To obtain protein hydrolysates, physiological human gastric fluids were simulated according to the methodology of MINEKUS *et al.* (2014). Prior to the ejecution, Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared. For SFG, 25 mg of pepsin (2000 U/mg; MP Biomedicals, California, USA) was diluted in 50 mL of sodium chloride 0.35 M pH 2. For SIG, 61.6 mg of sodium hydroxide (Merck Millipore, Darmstadt, Germany) and 680 mg of sodium phosphate monobasic monohydrate (Sigma Aldrich, Missouri, USA) were dissolved in 100 mL of deionized water and adjusted to pH 7. For the gastric digestion, E:S mixture was used: 100 mg of the protein isolate was dissolved in 10 mL of NaCl 0.35 M pH 2.0. A relation 50:50 of diluted protein and simulated gastric fluid (SGF) was made for each sample. Subsequently, the samples were incubated during 2 hours at 37°C and 500 rpm in a water bath (StableTemp, Cole Palmer, Illinois, USA). Immediately, a duodenal digestion was done, using pancreatin (Merck Millipore, Darmstadt, Germany) at a final concentration of 109 U/mL. A relation 1:1 (v/v) of the prior mixture and the simulated intestinal fluid (SIF) was made for each sample. Finally, the samples were incubated for 2 hours at 37°C at 500 rpm in a water bath. Afterwards, the enzymatic reaction was stopped using 200 μL of sodium bicarbonate 1 M (Merck Millipore, Darmstadt, Germany) at 80°C for 10 minutes. The samples were frozen at -80°C and lyophilized. This protocol was carried out three times.

2.4. Antioxidant activities (TBARS assay)

TBARS assay (thiobarbituric acid reactive substances) is one of the most ancient and widely used assays in order to quantify oxidative stress (DASGUPTA *and* KLEIN 2014), which is based in the reaction between malondialdehyde and 2-thiobarbituric acid (TBA) that produces an adduct that shows pink color species, who absorbs at 532-535 nm. For this study, with minor modifications, the method of ROJANO, B. A., GAVIRIA, C. A. *and* SÁEZ (2008) was used. Aliquots of 0.2, 0.4, 1.0 and 2.0 mg of protein isolates or protein hydrolisates were mixed with 2 mL of distilled water (protein solution). Then, 500 μL of olive oil (previously oxidized) was mixed with 500 μL of protein solution. The samples were mixed at 450 rpm in a micro incubator Provocell Shaking (Esco, Singapore) for 13 hours, at 28°C. Then, 1 mL TBA (1%) was placed and immediately incubated for 1 hour, at 95°C. Finally, the samples were chilled at 4°C for 15 minutes in a freezer and then analyzed at 532 nm in a UV Vis spectrophotometer (Thermo Scientific, USA). Butylated hydroxytoluene (BHT) ≥ 99% (Merck, Darmstadt, Germany), as positive control, was diluted in ethanol 96% (Merck, Darmstadt, Germany).
Oxidized olive oil was prepared with a heat treatment for 15 days. During the first eight days, the oil was heated in a laboratory oven (VWR, Pennsylvania, USA) at 70°C. During latter seven days, the oil was heated at 40°C. The results were compared with the positive control, quantifying the antioxidant activity percentage with this formula: % AA=(M–C)/C*100; Where AA: Antioxidant activity, C: Oxidized oil absorbance (532 nm), M: Sample (protein sample + oxidized oil) absorbance (532 nm).

2.5. Anti-inflammatory activity

The anti-inflammatory activity of the protein isolates and hydrolisates was analyzed by following the methodology of PADMANABHAN P (2012), with minor modifications. Sodium diclofenac (25 mg/mL) was used as the positive control. Samples were homogenized, and aliquots of 0.2, 0.4, 1.0 and 2.0 mg were dissolved with 2 mL of deionized water. Then, the suspension was homogenized with 0.2 mL of egg albumin and 2.8 mL of phosphate buffered saline (PBS) pH 6.4. This suspension was mixed gently. The mixtures were incubated at 37°C for 15 minutes, then incubated at 70°C for 10 minutes. The samples were finally chilled in cold water for 10 minutes, and the absorbance was analyzed using a UV-Vis spectrophotometer at 660 nm, with distilled water as a blank. The obtained data was compared with the results of the positive control (sodium diclofenac). The anti-inflammatory activity percentage was determined with this formula: % anti-inflammatory activity = (M-C)/C * 100, where C: Denatured egg albumin absorbance (without sample) and M: Sample absorbance (protein suspension + egg albumin).

2.6. Antimicrobial activity

In order to evaluate the antimicrobial activity, these certified strains were used: *Staphylococcus aureus* (ATCC® 25923), *Escherichia coli* (ATCC® 25922), *Bacillus cereus* (ATCC® 10876), *Listeria monocytogenes* (ATCC® 19115) and *Pseudomonas aeruginosa* (ATCC® 10145). The guidelines of the Clinical and Laboratory Standards Institute (CLSI) were applied, with minor modifications. A culture of each bacteria was inoculated in Tryptic Soy Broth TSB (Merck Millipore, Darmstadt, Germany) until each culture´s optical density was 0.5 Mc Farland units. Then, in Mueller-Hinton agar plates (Oxoid, Basingstoke, Hampshire, UK), the inoculum was struck with a long cotton swab. The protein isolates and hydrolysates were evaluated at five concentrations: 500, 375, 250, 200 and 150 mg/mL, all diluted in distilled water. After the inoculum was struck in the agar, 6mm wells were made with sterile plastic pipette tips (200 μL). 70 μL of protein solution or controls were deposited into the 6mm wells. Gentamicin (500 μg/mL) was used as a positive control and sterile deionized water was used as a negative control. After the plates stood for 30 minutes, they were incubated for 48 hours, at 37°C. The inhibition zones were measured after 24 hours and 48 hours. In order to determine the percentage of antimicrobial inhibition, this formula was used: % Inhibition = (B–A)/(C–A)*100. Where A: Negative control diameter (mm), B: Sample inhibition zone (mm), C: Positive control diameter (mm).

2.7. Statistical analysis

Statistical analysis was done by using Statgraphics Centurion 16.103 and Microsoft Excel® software packages. Simple variance analysis (ANOVA) and Tukey's comparative test at 95 % of confidence were used to evaluate statistical differences between protein extraction conditions and biological activities. Four (antioxidant activity) and three (anti-inflammatory and antimicrobial activities) replicates of each experiment were done, and the results were summarized as a mean and standard deviation.

3. RESULTS AND DISCUSSION

3.1. Preparation of protein isolates and hydrolizates

A proximate analysis of the baby lima bean flour was done at the National Institute of Agriculture Research of Ecuador (INIAP Santa Catalina). The analyzed samples had a mean of 21.15% of protein. This result is similar to the reported by FAO, 2017 (23.0%), TYLER, YOUNGS, *and* SOSULSKI, 1981 (22.8%) and SATHE, 2002 (17.5-28.7%).
Baby lima bean flour protein precipitation was based on isoelectric precipitation (CÁRDENAS *et al.* 2018). The pH was adjusted at four levels (3.0; 4.0, 5.0; 6.0) using HCl solution (2 N) and the precipitates were recovered. The highest percentage of protein was obtained at pH 5.0 (19.56±1.55 %). At pH 6, no precipitation was observed, however the solution was again frozen and lyophilized. The precipitation effect was only present at pH <6. These precipitation effects could be related to the fact that a majority of legumes proteins have their isoelectric points under pH 6 (EL-SAYED M *et al.* 1986; KLUPŠAITĖ *and* JUODEIKIENĖ 2015).
The protein quantification of the isolates was determined by using the Biuret colorimetric method (DOREY *and* DRAVES 1998). At pH 5, protein isolate represents 62.53±0.02 % (dry weight base). The protein isolates were characterized by Native-PAGE and SDS-PAGE (data pending for publication), which showed a predominance of 2S albumins and 11S globulins (Basic and acid subunities). Both gastric and intestinal digestibility were performed while simulating human physiologic conditions. The SDS-PAGE shows five protein bands with sizes between 15 kDa and 37 kDa. These results suggest that the proteins from baby lime beans were not completely hydrolyzed during human digestion. This effect was observed in related research, during which proteins from different legumes had a high persistence during proteases digestion (CARBONARO, GRANT, *and* CAPPELLONI 2005; DESHPANDE *and* DAMODARAN 1989).

3.2. Antioxidant activity (TBARS method)

The antioxidant activity of protein isolates and hydrolysates were evaluated by the TBARS method. The protein isolates obtained at pH 3, 4, 5 and 6; and concentrations of 100, 200, 500 and 1000 μg/mL were evaluated (Table 1). The differences between precipitation pHs, concentrations and antioxidant activity were not statistically different ($p<0.05$; simple variance analysis). However, at pH 5, the highest inhibition of lipid peroxidation was observed in all four of the evaluated concentrations (100, 200, 500, 1000 μg/mL), in relation to the obtained isolates at the pH 3, 4 and 6 levels.

Table 1. *In vitro* antioxidant activity of baby lima bean protein isolate using TBARS method.

Precipitation pH	Concentrations			
	100 μg/mL	200 μg/mL	500 μg/mL	1000 μg/mL
pH 3	10.82±0.006[a]	15.69±0.007[a]	22.30±0.017[a]	29.99±0.012[a]
pH 4	13.97±0.012[a]	19.12±0.011[a]	23.81±0.022[a]	32.69±0.008[a]
pH 5	15.66±0.009[a]	19.65±0.008[a]	24.45±0.016[a]	33.19±0.024[a]
pH 6	13.40±0.006[a]	17.31±0.009[a]	22.60±0.010[a]	30.59±0.027[a]

Data are expressed as the mean±Standard deviation SD (n=4).
Values in the same column having different letters differ significantly ($p<0.05$). ANOVA and Tukey's test.

For this parameter, Butylated hydroxytoluene (BHT) ≥99% was used as a positive control at concentrations of 100, 200, 500 and 1000 μg/mL. Between 70.82±0.042 (100 μg/mL) and 91.73±0.050 % (1000 μg/mL), BHT showed considerable values of antioxidant activity. Fig. 1 shows the relationship between protein isolates and hydrolysates at different pHs and the percentages of lipid peroxidation inhibition. The maximum antioxidant activity in protein isolates was observed at pH 5 and 1000 μg/mL, with a value of 33.19±0.024 %. The protein hydrolysate obtained at pH 5 and 1000 μg/mL was at a value of 77.17±0.029 % of lipid peroxidation inhibition. As expected, in comparison with the evaluated samples, the BHT showed considerable differences in their antioxidant activity.

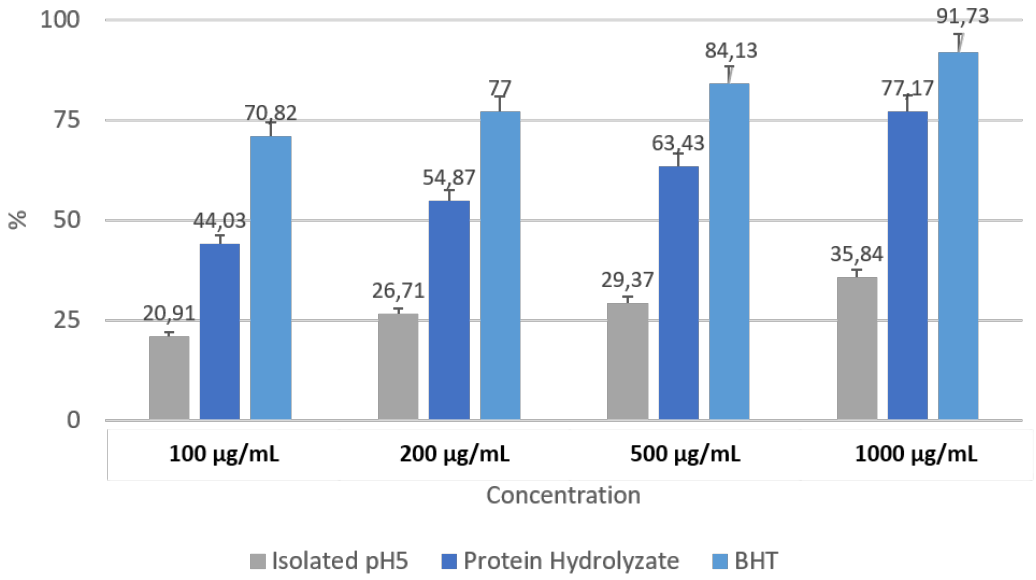

Figure 1. Inhibition of lipid peroxidation (TBARS) of the protein isolate and hydrolysate from baby lima bean at concentrations of 100, 200, 500 and 1000 μg/mL.

Fig. 1 shows clear differences in antioxidant activity values in all of the evaluated concentrations as well as between isolates and hydrolysates while displaying statistical differences ($p<0.05$; simple variance analysis) between concentrations. In comparison with 100, 200 and 500 μg/mL, 1000 μg/mL was the highest observed peroxidation inhibition. This results supports studies which show an inverse correlation between molecular weight and antioxidant activity (ZHAO *et al.* 2012). The effect observed in different legume protein hydrolysates showed considerable antioxidant activity (DO EVANGELHO *et al.* 2016; DURAK *et al.* 2013; ALASHI *et al.* 2014; CARRASCO-CASTILLA *et al.* 2012).

3.3. *In-vitro* anti-inflammatory activities

Table 2 shows the anti-inflammatory activities of protein isolates obtained at four pH levels (3.0, 4.0, 5.0 and 6.0) and hydrolysates (pH 5). Diclofenac sodium (25 mg/mL) was used as a positive control at 100-1000 μg/mL. Statistical analysis do not show differences between anti-inflammatory activities and the precipitation pH of the isolates ($p<0.05$; simple variance analysis). However, for the protein isolates, the maximum activity was observed at pH 5 and 1000 μg/mL, with a value of 21,53±0,39 % when inhibited by denatured protein.

Table 2. *In vitro* anti-inflammatory activity of baby lima bean protein isolate using albumin protein denaturation method.

Precipitation pH	Concentrations			
	100 μg/mL	200 μg/mL	500 μg/mL	1000 μg/mL
pH 3	2.54±0.018[a]	4.00±0.038[a]	8.14±0.046[a]	10.14±0.038[a]
pH 4	4.24±0.030[a]	7.36±0.032[a]	12.55±0.039[a]	17.52±0.056[a]
pH 5	5.82±0.048[a]	11.09±0.054[a]	16.29±0.037[a]	21.53±0.039[a]
pH 6	2.34±0.039[a]	5.42±0.033[a]	10.72±0.040[a]	15.80±0.057[a]

Data are expressed as the mean±Standard deviation SD (n=4).
Values in the same column having different letters differ significantly ($p<0.05$). ANOVA and Tukey's test

Protein hydrolysates (pH 5) showed an improvement in the percentages of inhibition in relation with protein isolates, with 30.62±0.01 % of inhibition of denatured protein at 1000 μg/mL (Fig. 2). Positive controls of diclofenac (100–1000 μg/mL) showed anti-inflammatory activities in the range of 32.14% -100.00 %, as reported in related research (CÁRDENAS *et al.* 2018). Related research showed a clear inhibition of pro-inflammatory mediators of protein hydrolysates and unhydrolysed proteins from legumes (NDIAYE *et al.* 2012; POWNALL, UDENIGWE, *and* ALUKO 2010). Therefore, because baby lima bean proteins and hydrolysates have displayed an interesting anti-inflammatory activity, further research could be considered.

3.4. Antimicrobial activity

Several protein hydrolysates from plant and animal origin, and their peptides, have shown antimicrobial activity against Gram-positive and Gram-negative bacteria. Other findings suggest that certain peptides can alter the cytoplasmic membrane or inhibit nucleic acid synthesis, protein synthesis or enzymatic reactions (BROGDEN, 2005; WANG, 2017). However, the details of the peptides active mechanics have not yet been completely determined due to the vast number of peptides with potential biological activity (DASHPER, LIU, *and* REYNOLDS 2007).

To evaluate the potential antimicrobial activity of *P. lunatus* baby lima bean protein isolate and hydrolysates, the samples were screened against clinical bacterial strains (Fig. 3). In the evaluated bacteria, between 150-500 mg/mL, no inhibition zones were observed in non-hydrolyzed protein samples. However, in four of five pathogenic bacteria evaluated (Table 3), their hydrolysates have shown a considerable antimicrobial activity. Significant differences were found among hydrolysate concentrations ($p< 0.05$; simple variance analysis).

The major percentage of inhibition was observed in 500 mg/mL of protein hydrolysate in Gram-negative *E. coli* ATCC ® 25922, 71.7 % of which was related to the positive control (Gentamicin 500 μg/mL), followed by Gram-positive *L. monocytogenes* ATCC® 19115 with 51.37 %. Gram-negative *P. aeruginosa* ATCC® 10145 shows a considerable inhibition of 33.31 %. A slight inhibition of 15.37% was observed in Gram-positive *B. cereus* ATCC ® 10876. The results were negative with Gram-positive *S. aureus* ATCC ® 25923™. These results suggest that protein hydrolysates from *P. lunatus* baby lima beans possess a broad-spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria.

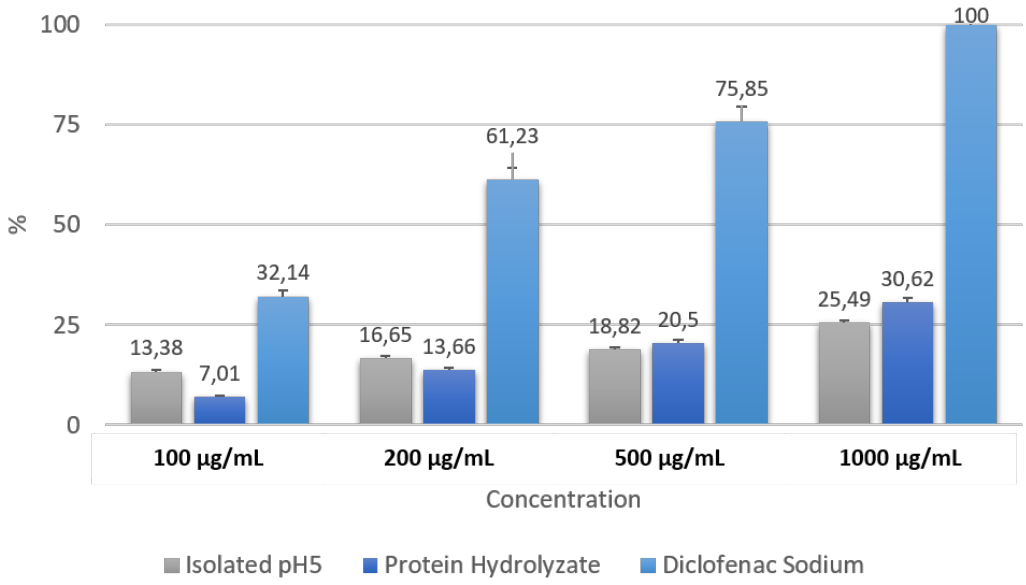

Figure 2. Anti-inflammatory Activity of protein isolate and hydrolysate from baby lima bean at concentrations of 100; 200; 500 and 1000 µg/mL.

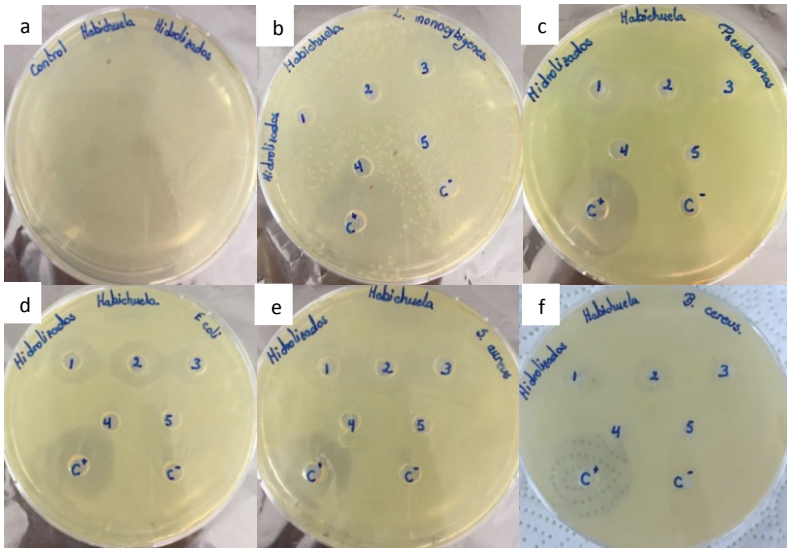

Figure 3. Antimicrobial activity of the protein hydrolisate obtained at pH 5.
A) Protein hydrolysate without bacteria (negative control); B) L. monocytogenes; C) P. aeruginosa; in the D) E. coli; E) S. aureus; F) B. cereus.

Table 3. Bacterial inhibition areas from protein isolates and hydrolysates of *P. lunatus* baby lima beans against certified bacterial strains.

Bacteria	Concentration	Isolate	Hydrolysate		Gentamycin 500 μg/mL (mm)
		Inhibition zone (mm)	Inhibition zone (mm)	Inhibition (%)	
Escherichia coli (ATCC® 25922™)	500 mg/mL	<6	$18.67^{a\pm}0.58$	71.70	23.67±0.58
	375 mg/mL	<6	$15.67^{b\pm}0.58$	54.72	
	250 mg/mL	<6	$12.33^{c\pm}0.58$	35.82	
	200 mg/mL	<6	<6	0	
Pseudomonas aeruginosa (ATCC® 10145)	500 mg/mL	<6	$12.33^{a\pm}0.58$	33.31	25.00±1.00
	375 mg/mL	<6	$10.33^{b\pm}0.58$	22.78	
	250 mg/mL	<6	$8.33^{c\pm}0.58$	12.26	
	200 mg/mL	<6	<6	0	
Listeria monocytogenes (ATCC® 19115™)	500 mg/mL	<6	$18.33^{a\pm}0.58$	51.37	30.00±1.00
	375 mg/mL	<6	$16.33^{b\pm}0.58$	43.04	
	250 mg/mL	<6	$13.67^{c\pm}0.58$	31.96	
	200 mg/mL	<6	<6	0	
Bacillus cereus (ATCC® 10876)	500 mg/mL	<6	$9.33^{a\pm}0.58$	15.37	27.67±0.58
	375 mg/mL	<6	$7.33^{b\pm}0.58$	6.14	
	250 mg/mL	<6	<6	0	
	200 mg/mL	<6	<6	0	
Staphylococcus. aureus (ATCC® 25923™)	500 mg/mL	<6	<6	0	35.00±1.00
	375 mg/mL	<6	<6	0	
	250 mg/mL	<6	<6	0	
	200 mg/mL	<6	<6	0	

The values represent the average of 3 measurements ± the standard deviation.
Different lowercase letters indicate, for each test, significant differences ($p < 0.05$) between the distances of the different concentrations of the protein hydrolyzed bean.
The distances are expressed in millimeters (mm); the positive control was gentamicin and sterile water was used as a negative control.

4. CONCLUSIONS

The *P. lunatus* baby lima bean protein isolates and hydrolysates displayed a considerable *in vitro* biological effect, based on the observed antioxidant, anti-inflammatory and antimicrobial activities. In comparison with protein isolates, protein hydrolysates performed better within the evaluated parameters. However, more in depth research is needed in order to evaluate the effect of bioactive peptides in baby lima bean proteins. Such peptides could be the source for notable biological activity for nutraceutical applications.

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FUNCTIONAL FOODS AS STIMULATORS OF THE IMMUNE SYSTEM OF *LITOPENAEUS VANNAMEI* CULTIVATED IN MACHALA, PROVINCE OF EL ORO, ECUADOR

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ABSTRACT

Functional foods are of great importance in shrimp farming as substitutes of growth promoter antibiotics to increase productivity together with environmental impact minimization in the culture ponds. In this work we studied the behavior of some physical and immunological indicators in *Litopenaeus vannamei* after treatment with a symbiotic functional food composed by a mixture of fructoligosaccharides (FOS) and the yeast *Saccharomyces cerevisae* isolated from the Machala culture ponds (province of El Oro, Ecuador). As a result, a positive influence was observed in physical parameters such as water quality, nitrites, dissolved oxygen, pH and temperature as a result of the symbiotic treatment. An increase in the amount of proteins as well as the superoxide anion and the hemocytes count in the hemolymph was also observed, which indicates positives stimulation in the immune system of the treated animals with this functional food in the pelleteded feed, unlike no – treated controls.

Keywords: FOS, symbiotic, *Saccharomyces cerevisae*, immunology, shrimps, functional foods

1. INTRODUCTION

Aquaculture has an average annual growth rate of 8.6% (FAO, 2016). In Ecuador, shrimp farming is the second non-oil product of economic importance. By the end of 1996, Ecuador was the second largest shrimp exporter worldwide after bananas. This increment in shrimp farms also represented greater foreign exchange and source of labor in the country. However, with the emergence of *white spot syndrome virus* (WSSV) infection, shrimp production faced a decline that affected socially and economically the country. Nowadays, joined to the production increments and intensive culture methods, some problems still arise like decrease in water and food quality, increment of animal stress, together with bacterial, viral or parasite infections and so, all these factors together affects the production costs in almost all the country.

For a long time and up to date, the most common method for dealing with the occurrence of microbial infections in aquaculture, was the administration of antibiotics. However, aquaculture faces serious problems due to various adverse effects of these drugs such as accumulation in the animal tissues, immunosuppression, development of antibiotic resistant bacteria and destruction of environmental microbial flora.The substitution of antibiotic growth promoters by functional foods such as probiotics, prebiotics, symbiotic and other alternative compounds has recently received considerable attention from producers in the country to maintain the health of the ponds in an environmentally friendly manner.

Probiotic term means "for life", derived from Greek words "pro" and "bios" (GISMONDO *et al.*, 1999). The most widely probiotic definition was made by FULLER (1989) indicating that a probiotic is considered as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal balance". Prebiotic, unlike probiotic, is not an organism and has less influence in natural environment. Based on definition of GIBSON and ROBERFROID (1995), prebiotics are a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health. The prebiotics have several advantages, but the main advantage of prebiotics over probiotics is that they are natural feed ingredients, not alive organisms. Their incorporation in the diet does not require particular precautions and their authorization as feed additives may be more easily obtained, in spite of some concerns about their safety and efficacy. Originally, prebiotics were chosen to stimulate bifidobacteria and lactobacilli in human microbiota (GATESOUPE *et al.*, 2005). Few studies, however, have focused directly on the effects of supplementation of the balanced used for shrimp feeding of with functional foods from native yeasts isolated from the culture ponds and fructo-oligosaccharides (FOS) in a joint way place a symbiotic functional food. Considering the immuno-modulating effect of these compounds, we evaluated the effects of autochthonous yeasts, in combination with a prebiotic (FOS), forming a symbiotic product (probiotic+prebiotic) on the immune system of *Litopenaeus vannamei* and in some productive parameters (weight of animals) that influence the good development of shrimp crops.

2. MATERIALS AND METHODS

2.1. Components of the symbiotic (Probiotic+Prebiotic).

Saccharomyces cerevisiae, which constitutes the probiotic part of the symbiotic, was isolated from the shrimp ponds of the continental region of the province El Oro-Ecuador. Fructoligosaccharides (FOS), which constitutes the prebiotic part of the symbiotic, were

obtained with a procedure established in the ESPE (Universidad de las Fuerzas Armadas), following the procedure of cell immobilization described by MARTÍNEZ *et al.* (2014).
Animals used. Litopenaeus vannamei of 8.9 g, were obtained from the Santa Ana shrimp farm, where the bioassay was carried out. Previously, shrimps were acclimated to avoid stress in the animal. Subsequently, 10 shrimp per liter of pool water were placed in 50 L plastic tanks. The bioassay lasted 30 days. Determinations of physical-chemical parameters, microbiological and immunological analyzes were made.

2.2. Diets and treatments.

During the experimental period of 30 days, shrimps were fed with a commercial concentrate without additives (control) and supplemented with different amounts of the functional additive (symbiotic) forming the following treatments where the prebiotic part, constituted by FOS were kept constant at a rate of 0.01 mg/kg of commercial concentrate in all treatments. T1: 50 mg of yeast + FOS, T2: 100 mg of yeast + FOS, T3: 200 mg of yeast + FOS, T4: 400 mg of yeast + FOS. Each treatment had 3 experimental replicas. To the pelleted diets were incorporated the mixtures of the different treatments that were tested in a bioassay using plastic tanks with a volume of 50 L where 10 juvenile shrimp of 8.9 g/L of seawater were incorporated.

2.3. Analysis of immunological parameters.

Determination of the different immunological parameters described were carried out according to the procedures described by CAMPA-CÓRDOBA *et al.* (2002, 2005).

2.4. Statistical analysis.

Numerical data for each sampling were subjected to variance (ANOVA) homogeneity, one way ANOVA analysis and the Tukey test, in both cases with a 5% significance. The results are presented as mean±standard deviation. The statistical analyzes were carried out with the Software SPSS version 18 for Windows.

3. RESULTS AND DISCUSSION

About 100 g of *Saccharomyces cerevisiae,* which constitutes the probiotic part of the symbiotic was obtained in 250 mL shake flask cultures as shown in Fig. 1 (A,B). The yeast growth was carried out at 20ºC, pH 5.0, and 300 rpm as reported by SANTOS *et al.* (2007) for 96 h.
On the other hand, FOS was obtained from sucrose using inmobilized *Pichia pastoris* cells (Fig. 1C) with fructosyltransferase activities, yielding a FOS rich syrup containing more tan 50% of FOS as revealed by HPLC (Fig. 1D) where also were detected as products, glucose, fructose and the remaining sucrose used as substrate.
Preparation steps of the different dosis of the concentrated balanced supplemented with the symbiotic are depicted in Fig. 2 (A, B). Once the different ingredients were mixed, according the final concentrations described in methods, the concentrated food with the symbiotic were added in the pools for further shrimp consumption.

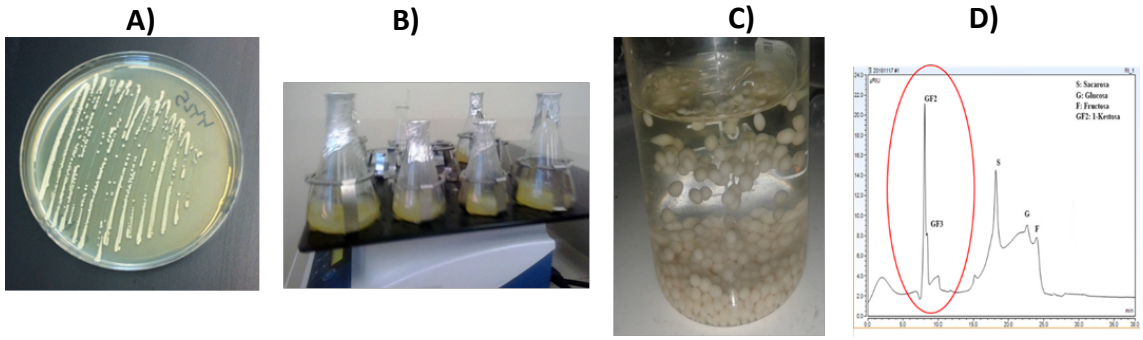

Figure 1. *Saccharomyces cerevisiae* production at the laboratory level.
A) Isolation of pure yeast cultures in solid medium YPG. B) Yeast Growth in orbital shaker. C) Biocatalyst of immobilized cells that produce FOS (GF2-GF3). D) HPLC detection of the components of the mixture present in the obtained syrup. GF2 and GF3 correspond to the FOS fraction. S, G, F correspond to sucrose, glucose, and fructose, respectively.

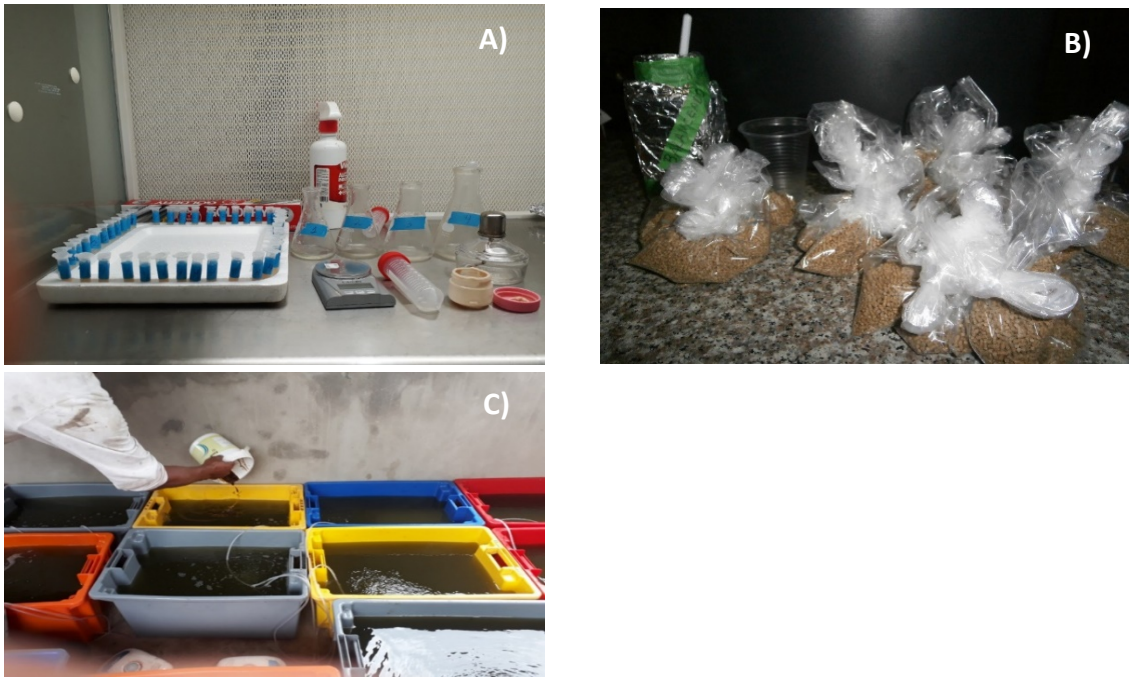

Figure 2. A) Preparation of the symbiotic, yeast + FOS, with different doses. B) Formulation of the balance including the symbiotic ones. C) Application of the product in the different shrimp pools.

As shown in Table 1, after 30 days of experiment the final weight and animals survival in the different treatments were evaluated and compared with controls lacking the symbiotic. There were not significant differences in growth when treatments and control were compared. However, an slight increment in weight and survival rate was observed for treatment 1.
Figs. 3A, 4A and 4B demonstrate that the symbiotic had a positive influence in some inmunological response indicators as hemocytes counting , superoxidase anion and total proteins. These results could be explained due to a rapid shrimp immunological system activation due to a better development and production of cells in the hematopoietic organ, which according to JOHANSSON *et al.* (2000), and JIRAVANICHPAISAL *et al.* (2006), it is already proven that it is the hemocytes producer organ. We have no explanation by now

about why the phagositosys activity was lower in the symbiotic treatment than in the controls according to Fig. 3B.

Table 1. Survival rate and total weight before and after symbiotic treatment. Results correspond to the means of three treatments ± standard deviation.

Treatments	Inicial weight (g)	Final weight (g)	Survival rate (%)
1 (50 mg)	8.9±0.1	11.0±0.2	70
2 (100 mg)	8.9±0.1	9.96±0.1	50
3 (200 mg)	8.9±0.1	9.20±0.2	40
4 (400 mg)	8.9±0.1	9.50±0.1	40
Control	8.9±0.1	10.50±0.1	40

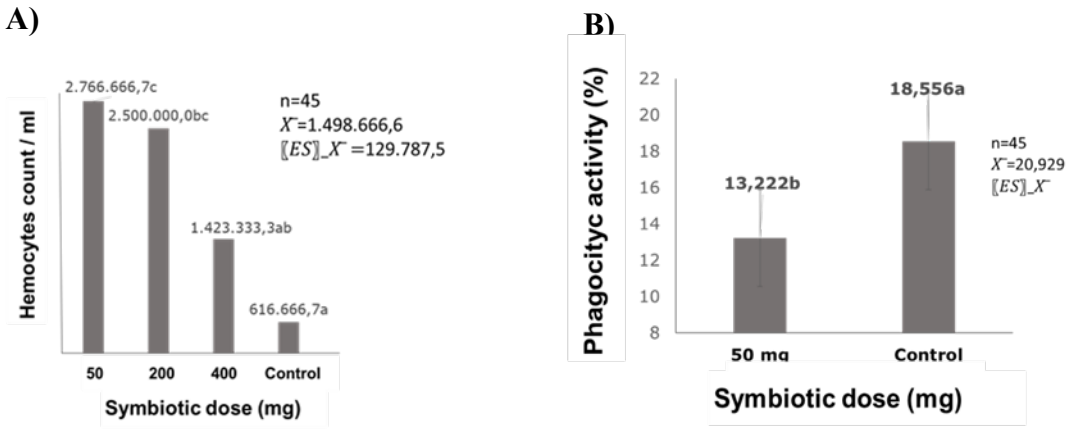

Figure 3. A) Hematocyte counting. The results plotted correspond to the mean of 3 replicates±desv. standard. ANOVA were performed for a probability of $p<0.05$. Different letters indicate significant differences. B) Phagocytosis activity at the end of the experiment. The transformed results plotted correspond to the average of 3 replicates±desv. standard. The ANOVA were performed for a probability of $p<0.05$. Different letters indicate significant differences.

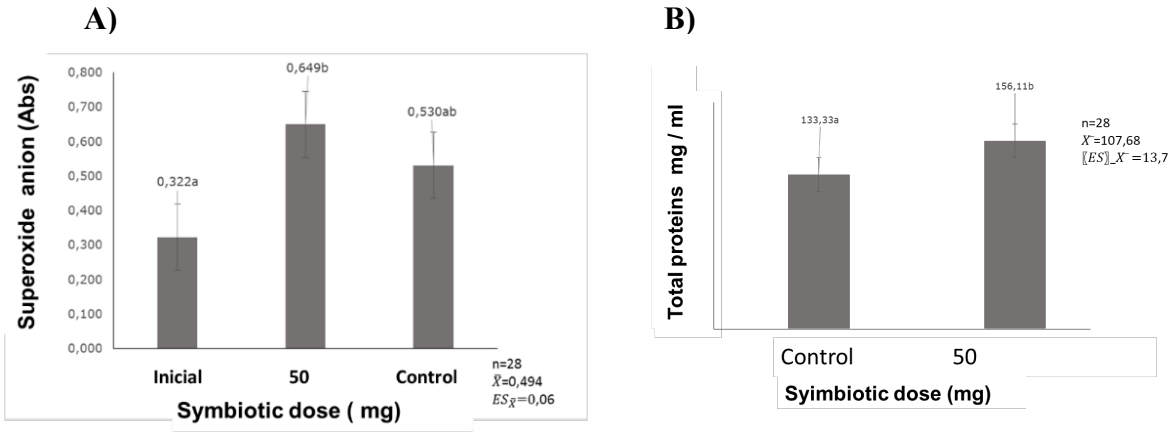

Figure 4. A) Total proteins in the haemolymph. B) Superoxide anion detection. The results plotted in both experiments correspond to the mean of 3 replicates±desv. standard. ANOVAs were performed for a probability of $p<0.05$. Different letters indicate significant differences. The results are the average of 3 replicas±desv. standard. ANOVAs were performed for a probability of $p<0.05$. Different letters indicate significant differences.

The water quality parameters measured during the experiment showed no significant variation ($p<0.05$) between symbiotic treatments compared to controls, which ensures the results obtained with the assayed diets.

4. CONCLUSIONS

According to the results presented in this research the use of functional foods could be useful in order to improve health of shrimps under production in an environmentally friendly manner. The application of symbiotics has shown positive results, but still insufficient evaluation of biological influence of these molecues in natural environment and cost of the material are the main restriction at this time. Following the numerous genome sequencing tools that are currently used, future research effects should involve transcriptome and proteome analysis using high throughput assays. In addition, transcriptome and proteome profiling of gut microbiota should be achievable.

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CHARACTERIZATION OF *ERYTHRINA EDULIS* TRIANA AND OBTAINING PROTEIN ISOLATE

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ABSTRACT

The multipurpose legume *Erythrina edulis* Triana has a wide spectrum of uses, ranging from human and animal food until the recovery of degraded soils. The objective of this study was to characterize *Erythrina edulis* Triana and to obtain protein isolate. Its proximal composition, protein digestibility, protein and amino acid profile were determined in addition to obtaining a protein isolate. The proximal composition was determined according to COVENIN standards, protein digestibility by using a multienzyme complex based on pH change, protein profile in polyacrylamide gels under denaturing conditions and amino acid profile according to the method described by Waters Associates, in addition to obtaining a protein isolated by precipitation at the isoelectric point.

It was obtaining as a result of content: 26.66% of crude protein, 1.00% of crude fat, 68.76% of carbohydrates, 4.58% of ash (on a dry basis) and a protein digestibility of 72.70±0.26%. The approximated molecular weight of several proteins varies between 218.78 and 7.08 kDa. An protein isolated 99% was obtained. Based on the results obtained and amino acid profile, it is considered a complete protein product.

Keywords: Chachafruto, protein isolate, proteins, *Erythrina edulis* Triana, digestibility

1. INTRODUCTION

The legume *Erythrina edulis* Triana, is usually known as nopás bean in Venezuela, chachafruto, balú, baluy or sachaporoto in Colombia, basul or guato in Ecuador and pashuro, pajuro, sachaporoto of the basul or sacha bean in Peru, Bolivia and Argentina. Its distribution is reported from the cordillera of Mérida, Venezuela, to the Bolivia-Argentina border, passing through the Andean mountain ranges of Colombia, Ecuador and Peru (RUSKIN, 1989).
The chachafruto tree can reach an average height of 8 meters, the seed is presented as a large bean, concave-convex, 3.5 to 7 centimeters long and 2 to 3 cm in diameter (BIOCOMERCIO SOSTENIBLE, 2003). *Erythrina edulis* Triana, it is a multipurpose legume with a wide spectrum of uses, ranging from human and animal food to the recovery of degraded soils (BIOCOMERCIO SOSTENIBLE, 2003). It has a high content of proteins (23%), in addition to have an aminogram comparable to the egg, and superior to the beans and peas (BARRERA, 2002).
Currently this legume is a little no widely known and used, however, the essay is trying to take advantage of this legume in a better way considering the high quality of that its protein. The price of this protein is cheaper than the animal one. Moreover, it has been developed it has lower cost compared with an animal, it has developed some improvements in the extraction processes of these vegetable proteins for use in human nutrition. Thus, our objective was to characterize and to obtain a protein isolate from *Erythrina edulis* Triana.

2. MATERIALS AND METHODS

2.1. Materials

We have working with a sample of *Erythrina edulis* Triana obtained in the Merida State of Venezuela

2.2. Obtaining flour

The flour of *Erythrina edulis* Triana was elaborated, according to the method described by PÉREZ *et al*. (2007). Beans were cut into rectangular pieces (≈2 inches diameter), and dehydrated at a temperature of 45°C for a period of 24 hours, inside the tray dehydrator "Mitchell Dryers" model No. 655149.
The dehydrated pieces were crushed in a manual mill and the obtained flour was packed in glass jars.

2.3. Proximal characterization of chachafruto flour

The chemical characterization of *Erythrina edulis* Triana flour was carried out, according to COVENIN standards:

a) humidity-COVENIN 1553-1980;
b) proteins-COVENIN 1195- 1980;
c) fat-COVENIN 1785-1981; d) ash-COVENIN 1783-1981;
e) carbohydrates-estimated by difference.
The COVENIN Standards are based on the AOAC International standards.

2.4. Determination of the protein digestibility of the flour

It was carry out according the proposed method by HSU *et al*. (1977), which is based on the change in pH. For this, a multienzyme system [(8 mg trypsin +15.5 mg chymotrypsin + 6.5 mg peptidase)/5 mL distilled water] it is consisting of pancreatic porcine type IX trypsin, bovine pancreatic chymotrypsin type II and intestinal peptidase porcine was selected. III (Sigma Chemical Co®., St. Louis, MO, USA). A specific amount of each sample, with distilled water, was used to prepare 50 mL of an aqueous suspension of protein (6.25 mg protein/mL), the pH of the suspension was adjusted to 8.0; while stirring in a water bath at 37°C. To each one protein suspension, it maintained at 37°C and under constant agitation, 5 mL of multienzyme solution was added; the pH was recorded at 10 min. The protein digestibility (DP) was calculated with the following equation:

$$DP = 210.46 - 18.10\ X \qquad \text{eq. 1}$$

Where: X = pH recorded 10 min after adding the multienzyme system to the protein solution.

2.5. Determination of molecular weight of *Erythrina edulis* Triana proteins by electropgoresis in polyacrylamide gels

To compare the distribution pattern of *Erythrina edulis* Triana proteins, polyacrylamide-sodium dodecylsulfate gel electrophoresis (SDS-PAGE) was applied according to the method of LAEMMLI (1970). For this, the process used was: the 4% stacking gel containing 0.5 mL of acrylamide: bis-acrylamide (29.2: 0.8); 0.38 mL TRIS / 1M HCl pH 6.8; 30 µL SDS 10%; 30 µL ammonium persulphate (APS) 10%; 3 µL N-N-N'-N'-Tetramethylethylene diamine (TEMED) and 2.1 mL of distilled water. The 12% separation gel contained 4.0 mL of acrylamide / bis-acrylamide (29.2: 0.8); 2.5 mL of TRIS buffer / 1.5 M HCl pH 8, 8; 100 µL of 10% SDS; 100 µL of 10% APS; 4 µL of TEMED and 3.3 mL of distilled water. These concentrations of gels were used according to Rojas et al. (2009) molecular weights of up to 6.5 kDa can be determined.

The samples to introduce inside the gel were placed in an eppendorf tube at a final concentration of approximately 10 mg / mL of proteins for each sample and treated with 4X SDS sample buffer (250 nM Tris, 40% glycerol, 4% SDS, 4 mL β-mercaptoethanol, 2 mg bromophenol blue, taken to 100 mL with distilled water, pH 6.8, and diluted 1: 4 with the sample). The samples were boiled for 5 min and applied in pockets of the stacking gel in the appropriate volumes. The same treatment was given to the broad-range protein standard of BIO-RAD (catalog 161-0318) of known molecular weights, which was applied in the first of the pockets of the gel in a concentration of 20 µL.

The rest of the samples were applied in the same pockets of gel by syringe. Running buffer (25 mM Tris, 1.5% glycine, 0.1% SDS) 1X was placed in the upper and lower part of the electrophoresis chamber and the lid of the chamber was placed. It was connected to a power source and a constant voltage of 30 amps was applied, without allowing the proteins to escape towards the end of the gel (approximately 0.5-1 cm from the end of the gel). At the end the proteins were visualized by staining with silver.

2.6. Extraction of proteins from *Erythrina edulis* Triana

The protein isolate was obtained by alkaline extraction and precipitation at the isoelectric point of the protein, to combine and modifying the methods of SÁNCHEZ-VIOQUE *et al*., (1999) and ADEBOWALE *et al*., (2003).

The flour was dispersed in distilled water (1:10 p / v) and the pH adjusted to 11.0 with 0.1 M NaOH, in order to facilitate the solubilization of the protein. The dispersion was kept under stirring for 1 hour, after that time the dispersion was centrifuged at 2,500 rpm for 20 minutes. The supernatant was separated and adjusted to pH 4 to precipitate the protein, stirred for 1 hour and the protein recovered by centrifugation at 2,500 rpm for 30 minutes. The recovered proteins were lyophilized and frozen for further hydrolysis.

2.7. Obtaining the amino acid profile of *Erythrina edulis* Triana

The amino acid profile of the protein hydrolysates was carried out according to the method described by WATERS ASSOCIATES (1984), as detailed below:

2.7.1. Preparation of the sample.

Samples with 40 mg of protein were hydrolyzed at 110°C for 24 hours with 15 mL of 6 M HCl. Then, 50 mL was adjusted and a 1 mL aliquot was filtered through a Millipore filter. The hydrolysates were derivate using phenylisothiocyanate (PITC by its English name: phenylthiocarbamyl).

2.7.2. Determination of the amino acid profile

It was performed on the Waters 1525 binary pump HPLC, coupled to a Waters 2487 dual-wave UV-Visible detector using a wavelength of 254 nm, with a C18 column of 150 x 3.9 mm (V0 = 1 8 cm^3). The samples were injected using a 50 μL Hamilton syringe, the loop of the injection valve should be 5 μL. The mobile phases used were, A: constituted by an acetate buffer at pH 5.70; B: constituted by a mixture CH3CN-H2O (60-40) at a flow of 1.0 mL / min. Each chromatographic run was carried out in a period of 45 minutes at a temperature of 40°C.

3. RESULTS AND DISCUSSION

3.1. Characterization of *Erythrina edulis* Triana flour

The percentage of dry matter is one of the most important quality factors at the industrial level, since the performance and efficiency percentages obtained during the processing of the *Erythrina edulis* Triana in flour will depend on it. The yield of the cultures in flours is an important fact in the food industry, since this will affect the costs of the finished product. Thus, in the production of Chachafruto flour, the yield was 18.85%, due to its high moisture content in fresh grain (81.5%).
Once obtained the flour from the chachafruto was analyzed and in Table 1 the proximal composition of *Erythrina edulis* Triana flour is reported, where a content of 26.19% of protein in dry base is observed, value that differs from that reported by PÉREZ *et al*., (1979) who reported values between 18 and 21% of protein, while at the National University of Colombia (BIOCOMERCIO, 2003) found that chachafruto seed contains 23% of Protein and DELGADO and ALBARRACÕN (2012) report values of 22.81% protein, 0.7% fat, 5.35% ashes. These differences, can be attributed to many factors such as the extraction method used, the environmental-geographical and agrarian conditions of the crop.

Table 1. Proximate Composition of *Erythrina edulis* Triana flour.

Components	Wet base (%)	Dry base (%)
Humidity	2.04±0,22	0.00
Crude Protein	25.66±0,15	26.19
Crude fat	1.00±0,02	1.02
Carbohydrates	68.76±0,00	70.19
Ash	4.58±0,21	4.68

This table shows the proximal composition of *Erythrina edulis* Triana flour in dry and wet basis.

3.2. Protein digestibility flour

The *in vitro* digestibility of chachafruto flour was determined with equation 1, its was 72.70±0.26%, this value is relatively low compared to casein (92.98%). The low digestibility of *Erythrina edulis* Triana flour may be due to its resistance to proteolytic attack and to the presence of protease inhibiting factors (GÓMEZ-SOTILLO, 1990). In addition, SANGRONIS and MACHADO (2007) indicate that the *in vitro* protein digestibility of pigeon pea and beans increases when reducing anti-nutritional factors such as phytic acid and tannins, which according to ALONSO *et a*l. (2000), are responsible for protein binding to form complexes that prevent enzymatic attack for protein degradation.
It should be noted that HSU *et al*. (1977) report values of protein digestibility in-vivo and *in vitro*, observing that values obtained by the multienzyme method (*in vitro*) are similar to in-vivo digestibility.

3.3. Proteins from the flour of *Erythrina edulis* Triana

In order to determine the molecular weight of the proteins present in *Erythrina edulis* Triana, electrophoresis was used under denaturing conditions (PAGE-SDS). Fig. 1 shows the different protein bands with different intensity and coloration, caused by the type of stain and quantity of proteins present in chachafruto. The approximate molecular weight obtained from several of the proteins that chachafruto has are: 218.78; 77.67; 49.37; 19.95; 16.43; 13.53 and 7.08 kDa, and the name of the protein cannot be established reliably according to the calculated molecular weight. This may be due to: the albumins electrophoretically respond to a very heterogeneous pattern since in SDS-Page there appear 12 molecular weight bands ranging from 8.5 to 95 kDa, with three main polypeptides of 8.5; 11 and 16 kDa (JULIANO, 1980).
Fig. 1 described two columns, where: column 1 represents the molecular weights and the standard sample with its respective protein bands, while column 2 shows the protein bands of the Erythrina edulis Triana sample, where the arrows indicate the protein bands of which determined the approximate molecular weights.
According to JUNG *et al*. (1998) silver staining is a very sensitive method and has the characteristic to produce usually brown or black colorations. However, some proteins have characteristic colors such as lipoproteins that tend to be blue colored and some glycoproteins that appear yellow, brown or red.
The globulins have a characteristic band corresponding to 25-26 kDa polypeptides (KRISHNAN *et al*., 1992, KOMATSU and HIRANO 1992, FURUKAWA *et al*., 2003, KUMAGAI *et al*., 2006) and another at 16-18 kDa (JULIANO 1980; KRISHNAN *et al*., 1992). Prolamines it constitutes heterogeneous polypeptides with molecular masses varying between 10 and 23 kDa (PINCIROLI, 2010) and in legumes the glutelin fraction is

constituted by two alpha and beta polypeptides, both linked by disulfide bridges (UTSUMI, 1992) and by proglutellin, a polypeptide of 55 kDa unprocessed, all these polypeptides would be forming aggregates through disulfide bonds.

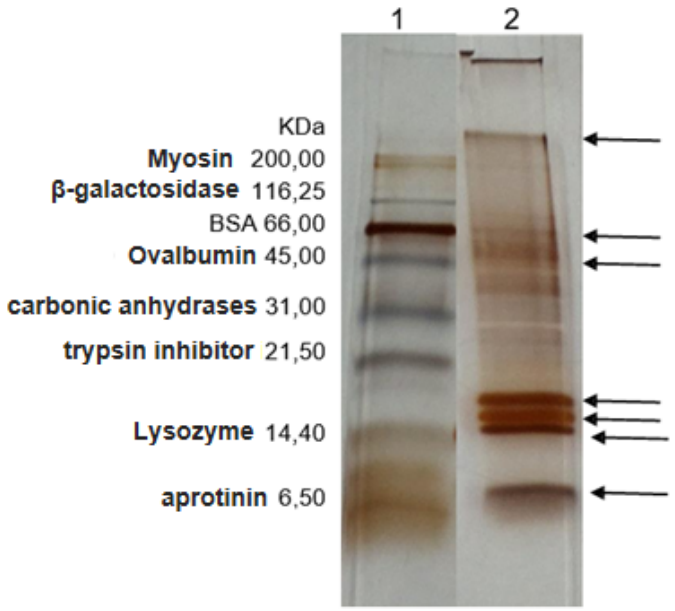

Figure 1. Protein profile of *Erythrina edulis* Triana by page-SDS. 1. Standard.2. *Erythrina edulis* Triana.

3.4. Extraction of *Erythrina edulis* Triana flour proteins by isoelectric point precipitation

The availability of vegetables protein sources, its lower cost compared with protein of animal origin and the tendency to reduce the intake of animal proteins for health reasons, it means that in recent years there has been a great development in the extraction and improvement processes of these plant proteins for use in human nutrition.
Table 2 shows the value at which the protein was taken, content so that the product is considered protein isolate since, according to CODEX STAN 175-1989, it must pose more than 90% protein.

Table 2. Moisture content of the protein isolate of *Erythrina edulis* Triana.

Components	Fresh Basis (%)	Dry Base (%)
Humidity	7.31±0,29	0.00
Crude protein (retentate)	88.99±0,63	96.01

Table 2 shows the values of protein isolate of Erythrina edulis Triana on a dry and wet basis.

Similar studies with small variations were made in obtaining the concentrates, so GONÇALVES *et al*., (1997) performed the extraction at pH 12 of the soluble proteins

followed by an acid precipitation (pH 5) at the isoelectric point, obtaining a protein with 86% protein; SÁNCHEZ-VIOQUE *et al*., (1999) obtained two protein concentrates from chickpea seeds by alkaline extraction at pH 12 and 10.5 and acid precipitation of proteins at the isoelectric point (pI 4.3), recovering to pH 12 and 10.5 the 65.9 and 62.1% of the protein, respectively; while the concentrates contained 78 and 88.1% protein.

3.4 Amino acid profile of AMINO *Erythrina edulis* Triana flour

The amino acid profile allows to determine the amount and type of amino acid present in the sample, in Fig. 2 it is observed that the concentration of AA (amino acids) of the protein isolate is lower than that reported bibliographically (full grain), it can be mentioned that during the process To obtain the isolate, many of the amino acids and even proteins could undergo changes, from the drying of the chachafruto grain, the temperature conditions reached during the hydrolyzate obtaining (40°C), and even pH variations ranging from 2 to 11. In addition, the difference in amino acid content could also be affected by the protein determination method, which in this investigation was performed by the micro-Kjeldahl method, which overestimates the amount of proteins by quantifying nitrogen total (protein and non-protein).

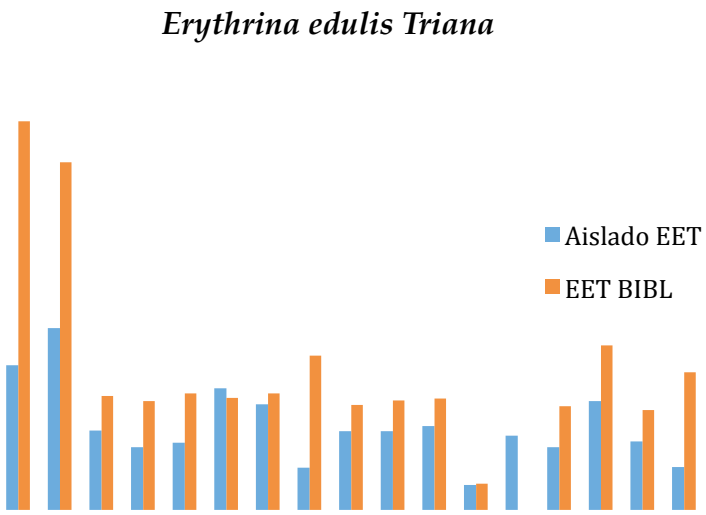

Figure 2. Protein isolate amino acid profile *Erythrina edulis* Triana with bibliographic data (grain).

In Fig. 2 there are two columns for each amino acid. Where the blue column represents the amount of amino acid for each 16 grams of nitrogen of the protein isolate (EET Isolated), while the orange column represents the amount of the amino acid for each 16 grams of nitrogen of Erythrina edulis Triana (EET BIBL) according to bibliographic data.

4. CONCLUSIONS

The flour of *Erythrina edulis* Triana was prepared and characterized, obtaining a low yield. Additionally, it was determined that the *in vitro* digestibility of *Erythrina edulis* Triana flour is lower compared to that of casein, due to possible antinutritional compounds.

It is possible to determine the apparent molecular weights of *Erythrina edulis* Triana proteins by electrophoresis in polyacrylamide gels under denaturing conditions (SDS-PAGE), which allowed to determine that this legume has proteins that are in the range of 6.5 kDa up to greater than 200 kDa.
A protein isolate of *Erythrina edulis* Triana was obtained by precipitation at the isoelectric point, obtaining 96.01% of proteins. Finally, the amino acid profile of the isolate of *Erythrina edulis* Triana was determined, confirming that it is a product that contains all the essential amino acids.

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CHARACTERIZATION AND DRYING KINETICS MODELING FOR ORANGE PEEL TO OBTAIN FOR ALIMENTARY PURPOSES IN BOLIVAR PROVINCE, ECUADOR

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ABSTRACT

In this work, three orange varieties from Bolívar, Ecuador were characterized physically, compositionally and proximally. The influence of the height on the available residual peels was analyzed by regression; In addition, the kinetics of drying in a convection oven was modeled. The results show dietary fiber between 62.4 and 68.7%, which suggests that dry orange peel flour, is suitable for the manufacture of high fiber food products. In relation to the model of drying kinetics, Midilli and Page had the best fit to the observed data with a correlation coefficient of 0.991 and 0.995 respectively.

Keywords: drying kinetics, orange peel drying, proximate analysis, dietary fiber

1. INTRODUCTION

In Ecuador, 16,578 ha (ESPAC, 2016) are dedicated to the cultivation of oranges. According to ULLOA (2012), the Las Naves canton in Bolivar Province has the highest production, 93% corresponding to the Valencia variety, 4% to the Common variety and the remaining 3% to the Lime variety. Valencia is the most widely spread variety because of how well adapted it is to this zone's climate and its resistance to plague and diseases. SPREEN *et al.* (2003) indicate that consumption of processed orange has increased worldwide, which has also increased the generation of residues.

Some fruit residues are now being considered as potential sources of polyphenols, carotenoids, vitamins and dietary fiber (ROBERTA *et al*., 2014). The high content of fiber and phenolic components in citric peels has led to studying them after industrial processing (GORINSTEIN *et al*., 2001). According to OKPALA and AKPU (2014), orange peel (the part surrounding the endocarp) could be regarded as a potential source of fiber and phytochemicals. Orange peel flour has been used as a wheat flour substitute in cooking, because of its higher fiber content (OKPALA and AKPU, 2014). Despite the extensive literature and research being done on oranges and their properties, there are not many data about the physical and nutritional properties of the orange peel, depending on variety. The use of orange peel as an industrial by-product could increase revenue for farmers; however, its use is attached to several difficulties in the sensory aspect as well as insufficient information about quantities and types of processing for these residues.

Among the preservation and processing techniques for these by-products, drying (GIRALDO-ZUNIGA, 2010) is regarded as a simple and economical method that increases the product's preservation time and makes transportation and storage easier. In order to understand the drying process, the variation of moisture in time needs to be analyzed, using models presented in the literature that correlate the moisture radio (MR) as a function of the drying time (KHAWAS *et al*., 2014; MORADI *et al*. 2013; AKPINAR and TORAMAN, 2013; RAYAGURU, 2012; ASMA *et al*., 2012; TAHERI *et al*., 2011, TULEK, 2011), which are frequently used to describe the drying phenomena of agricultural products. Moisture ratio is estimated by the equation (1), where: MR is the dimensionless parameter; ωt, ωe and ωo are the moisture content at any time, initial moisture content and equilibrium moisture content, respectively (VELÁZQUEZ-MARTI *et al*, 2016) .

$$MR = \frac{\omega_t - \omega_e}{\omega_o - \omega_e} \quad (1)$$

In this sense, this research's objective is to conduct a proximate analysis, as well as a complete physical and compositional characterization of three orange varieties from Caluma canton in Bolivar Province-Ecuador, and determine the kinetic model parameters that better fit the drying data of these three varieties, which will enable to understand the drying kinetics and minimum moisture content that can be obtained in set conditions for the processing of this by-product.

2. MATERIALS AND METHODS

2.1. Study Area

Physical and proximate characterization of the peel of the three orange varieties most cultivated in the Canton Las Naves of the province Bolivar was carried out (Lima, Valencia and Common). Canton Las Naves is constituted by 3 different zones. Zone 1 is

between 48 m and 180 m height, with rainfall ranging from 1750 to 2000 mm, average temperatures between 24-27°C. Zone 2 is located between 180 and 500 m height. Landform is more irregular with moderate slopes. Its average annual rainfall is between 1000 and 1400 mm and an average annual temperature of 22-26°C. This zone is the most populated area, small nerkels can be found, such as Las Naves, Buenos Aires, Jerusalén, Bellavista, La Unión, and Voluntad de Dios. Zone 3 is the highest area in the canton, located between 500 and 1500 m. Its landform has strong hillsides and slopes. Climate is sub-tropical with average annual precipitation 2000-2500 mm , and average temperature of 18-20°C.
Fifty fruits of the three varieties (Lima, Valencia and Common) were randomly sampled and collected from the three zones of Canton Las Naves. They were collected during the second harvest season (December 2014).

2.2. Orange characterization from descriptors for citrus

In order to characterize the oranges, studies were conducted on 25 units per variety of orange, analyses were perfomed thrice. Physical and compositional characterization was performed for each sample, according to Descriptors for Citrus of International Plant Genetic Resources Institute (IPGRI, 1999). Table 1 presents the used descriptors with the identifier number. In addition, height (H) and diameter (D) were measured using a digital vernier caliper with a sensitivity of 0.01 mm. Afterwards, the oranges were washed and dried. Peel, juice and saccules were weighted using a digital scale with 0.01 g acuracy, pH and degrees Brix were measured in the orange juice, using a pH meter S220 and HI96801 digital refractometer respectively. Number of seeds was also counted.

Table 1. Citrus descriptors number and characteristics measured/observed.

Descriptor number	Characteristic
7.4.1	Fruiting season
7.4.3	Fruit weight (g)
7.4.4	Fruit diameter (mm)
7.4.5	Fruit length (mm)
7.4.6	Fruit shape
7.4.7	Shape of fruit base
7.4.8	Shape of fruit apex
7.4.9	Fruit (epicarp) color
7.4.11	Width of epicarp at equatorial area (mm)
7.4.12	Fruit surface texture
7.4.13	Adherence of albedo (mesocarp) to pulp (endocarp)
7.4.17	Fruit rind (mesocarp) thickness (mm)
7.4.18	Albedo color
7.6.1	Pulp (flesh) color
7.7.1	Average number of seeds per fruit
8.5.1	Titratable acids content (meq of citric acid/100 ml juice)
8.5.2	pH
8.5.3	Sugar content (%)

Others parameters quantified.
Epicarp and mesocarp weight (g), Juice weight (g), pulp weight (g).

These data were statistically analyzed, using Stathgraphics Centurion, in order to determine the mean difference of the studied parameters, by conducting a multiple range test (LSD), with a significance level of 0.05.

2.3. Proximate Analysis for the orange peel and orange peel flour

After the physical analysis was conducted, 25 oranges of each variety were washed and dried with absorbent paper, peeled and cut in pieces of aproximately 1 cm each, then divided in two groups. The first was directly subjected to the proximate analysis for the orange peel while the second was first used to obtain the orange peel flour, then subjected to the proximate analysis. The procedure to make the flour was as follows: the orange peel was immersed in hot water at a temperature of 60°C for 5 minutes, then subjected to thermal shock in a 100 mg/kg of sodium metabisulphite solution at 20 °C, in order to sterilize the peel. To this fraction of the peel, the moisture was removed through drying in a convection oven, vertical exchanger type for 6 hours at an average temperature of 60°C. The dried peels were ground into flour using a Wiley mill 115V33383L20 with mesh number 60. The analyses were conducted three times. Proximate analysis of the peel and peel flour was carried out using the standard methods listed in Table 2.

Table 2. Proximate analisys parameter and standard methods used.

Nutritional parameter	Standard method
Protein	AOAC 2001.11
Ash	AOAC 923.03
Fat crude	AOAC 2003.06
Moisture	AOAC 925.10
Dietetic Fibre	AOAC 985.29
Crude Fibre	WEENDE

2.4. Determination and modeling of drying curves

The sterilized peel was subjected to a drying process. Before that, they were pressed to eliminated the excess moistures. Initally moisture content ranging was between 69.5 and 73.8%, with an average of 72.1% wet basis. Trays with orange peel pieces of a similar size of approximately 1 cm were dried in a vertical oven using external heated air at a temperature of 60°C which flew through with a speed of 2.5 m/s, until constant moisture content was reached. To measure the evolution moisture content, 5 pieces were randomly taken each hour, crushed and mixed, then had their moisture content measured using the method indicated in Table 2. Moisture was measured thrice.
The test data from the drying process fit the mathematical models available in the literature (KHAWAS *et al*., 2014; MORADI *et al*. 2013; AKPINAR and TORAMAN, 2013; RAYAGURU, 2012; ASMA *et al*., 2012; TAHERI *et al*., 2011, TULEK, 2011), using the TableCurve 3D V4.0 software by the minimization of the sum of the squared errors expressed as the difference between the value determined by the model and the test value. The curve's degree of adjustment was determined by the correlation coefficient.

3. RESULTS AND DISCUSSION

3.1. Orange characterization from descriptors for citrus

Table 3 shows the results for the qualitative characterization of three oranges varieties, based on the descriptor for citrus reference.

Table 3. Results for the qualitative characterization for the oranges varieties studies.

Characteristic	Orange variety		
	Valencia	Lima	Common
Fruiting season	Midseason	Midseason	Midseason
Fruit shape	Spheroid	Spheroid	Spheroid
Shape of fruit base	Truncate	Truncate	Truncate
Shape of fruit apex	Truncate	Truncate	Truncate
Fruit (epicarp) color	Orange	yellow	orange
Fruit surface texture	Rough	Rough	Rough
Adherence of albedo (mesocarp) to pulp (endocarp)	Medium	Medium	Medium
Albedo color	White	White	White
Pulp (flesh) color	Orange	Orange	Orange

According to Table 3 in terms of physical characterization of the three varieties such as Valencia, Lima and Common was observed and most of the parameters analyzed and presented were similar, In the three varieties it can be said that the fructification time was in a mid-season, the spheroid shape with flattening at the base and apex or truncated rough surface texture, an average adhesion between the albedo and endocarp, albedo's color is white, yellow colored flesh and the only parameter that is different is the color of the epicarpio where Valencia and Common varieties have an orange colored one and the Lima is yellowish. According to ALLAN and VERA (2012), the orange fruit is a sphere-shaped, more or less flattened at the poles, an average diameter of 6 to 10 centimeters. Its weight varies from 150 grams to 200 grams without the skin. Their shells called pericarp is colored and is provided with oily vesicles (flavedo tissue). Under the shell smooth or rough depending on the variety a second white skin that surrounds the fruit protecting the pulp or albedo, this last very spongy and appears orange. The Table 4 presents the most important results from the quantitative characterization of the three oranges varieties.

All these results are consistent with some report find out in the bibliography. According to ROUSSOS (2011) indicates that the average value is about 74.43 mm diameter and weight of 197.33 g on two adjacent commercial organic and integrated orange orchards. In the determination of dimensional parameters of four orange varieties (Alanka, Finike, Navel and Shamouti) report TOPUZ *et al*. (2005) a length of 69.22 to 82.60 mm, diameter of 71.57 to 80.14 mm, and orange weight 175.71 to 271.40 g. Regarding the physicochemical parameters determined, the results for acidity, pH and Brix are in the order of those obtained by KEFFORD and CHANDLER (1970) for other varieties of orange. They indicate pH from 3.19 to 3.84, titulable acidity from 0.687 to 1.375, and° Brix values 6-14 for sweet orange and 9-14 for bitter orange.

The variables measured were subjected to a means analysis in order to determine the significance of the differences between them (same letters in Table 4), finding that the Lime and Valencia varieties, which represent 97% of the oranges available, belong to the same group in regards to the height and fruit weight variables. As such, the influence of these variables on the quantity (weight) of the residual peels available was studied, using regression analysis with the help of TableCurve 3D V4.0. The results show that equation 2 peel weight (*PW*) vs. height of the Orange (*h)* is the better representation with a correlation coefficient (R^2) of 0.883368.

This equation can predict the amount of orange peel available at any Valencia and Lime orange plantation in Las Naves – Ecuador, just by measuring the height of the fruit.

$$PW = (35.597489 - 44.225635h^2)/(1 + 36.26251h^2) \quad (2)$$

Table 4. Results for the quantitative characterization for the oranges varieties studies.

Characteristic according to fruit	Orange Variety		
	Valencia	**Lima**	**Common**
Fruit weight (g)	196.64±37.65a	194.56±35.18a	232.56±30.66b
Fruit diameter (mm)	70.72±4.78b	63.36±4.97a	72.24±3.14b
Fruit length (mm)	64.36±4.57a	62.84±4.90a	72.16±4.37b
Fruit rind (epicarp plus mesocarp) thickness (mm)	3.52±0.09b	3.88±0.10c	2.24±0.01a
Average number of seeds per fruit	5.28±2.37a	15.76±7.02b	20.72±4.82c
Titulable acids content (meq of citric acid/100 ml juice)	1.63±0.33c	0.34±0.41a	1.21±0.18b
pH	3.61±0.19a	5.80±0.12c	3.95±0.11b
Sugar content (%)	8.27±0.69ab	7.72±1.05a	8.77±1.31b
Other parameters quantified			
Epicarp plus mesocarp weight (g)	40.36±11.55a	42.24±10.34a	62.40±10.14b
Juice weight (g)	101.00±19.27b	96.31±20.31b	79.03±16.90a
Endocarp weight (g)	41.20±9.789a	35.08±7.94a	58.80±14.80b

Each parameter was evaluated in 25 items, 3 times per item. The data are expressed as average and the uncertainties as standard deviation.
*Different letters indicate significant differences ($p < 0.05$).

3.2. Proximate Analysis for the orange peels and orange peels flour

Primarily the proximate analysis of the orange peels based on wetness in the three varieties (Valencia, Common and Lima). Table 5 shows the results from the analysis.

Table 5. Proximate analysis results for the orange peel and flour from the dryed orange peel.

Proximate analysis	Orange Peel			Orange peel flour		
	Valencia	**Lima**	**Common**	**Valencia**	**Lima**	**Common**
Protein % (n*6.25)	1.92±0.04	1.39±0.04	1.47±0.02	4.52±0.14	5.6±0.02	4.52±0.02
Moisture (%)	73.9±0.15	72.88±1.42	69.45±2.19	8.3±0.38	12.1±0.42	9.4±0.02
Ash %	1.18±0.02	1.22±0.02	1.06±0.01	3.98±0.03	3.76±0.01	3.50±0.02
Crude fat (%)	0.42±0.21	0.47±0.06	0.48±0.12	1.01±0.03	0.64±0.08	0.79±0.23
Dietary fiber (%)	24.7±0.03	25.6±0.05	26.6±0.02	62.4±0.05	68.7±0.04	62.5±0.04

Each experiment consists of 3 replicas. The data are expressed as average and the uncertainties as standard deviation.

As expected, except for moisture, all the factors are superior in orange peel flour as compared to fresh peel, however, the higher proteint content found in the fresh peel of the

Valencia orange was surpassed by the Lime orange when turned into flour. The same happened to moisture, while ash content is higher in the Lime variety for the fresh peel but higher in the Valencia variety for the dried peel. Regarding crude fat and dietary fiber, the highest values belong to the Common variety for the fresh peel, while Valencia has the highest crude fat content and Lime has the highest dietary fiber content for the dried peel. This is reasonable as the Common variety has the least moisture for the fresh peel and is less concentrated than other varieties when dried.

A 5.15% value for protein was reported by NASSAR *et al*. (2008) in their study on the effect of orange flour addition to cookies, these values are among the ones obtained in this study for the analyzed varieties. Very similar values were reported by ZAKER *et al.* (2016). As for the ash content, the values found for the Valencia variety (3.98%), the Common variety (3.50%) and the Lime variety (3.76%) were all higher than the values reported in the literature, 2.53% (ZAKER *et al*. 2016), 2.65% (LUNDBERG *et al*. 2014) and 3.435 (OCEN and XU, 2013).

Regarding the dietary fiber, the value found by ZAKER *et al.* (2016) was 7.3% and superior to the best value found in this study. However, WANG *et al.* (2015) indicate that fiber values of 63.24%, comparable to the ones found in this study, are promising for using citrus fruit peel as an ingredient in high fiber fruit products. The registered value for moisture was 8.3% in the Valencia variety, 9.4% for the Common variety and 12.1% for the Lima variety, similar to the ones reported by NASSAR *et al*. (2008), ROMERO *et al*. (2011) and ZAKER *et al* (2016). In the case of crude fat, 1.01% for the Valencia, 0.79% for the Common and 0.9% for the Lime varieties, the values are inferior to those reported by LUNDBERG *et al*. (2014), who determined a 1.05% value for crude fat.

These results suggest a prospective use of orange peel flour as a dietary fiber source in the manufacture of bakery wares, controlling the proportion of wheat flour substituted by this by-product, without compromising the taste and caloric value.

3.3. Determination and modeling of drying curves

The variation of moisture content was studied and the Fig. 1 shows the variation of average moisture content (ω) versus time. As it can be seen, the initial moisture content was about 72.1% and reached constant value at 8 hours. The minimum moisture content was about 10%. These values were obtained for the Valencia and Lime varieties, showing no statistically significant difference for the mean values. Table 6 shows that the average data fit with different drying models. They let know that the Midili and Page models results with the best fit. From many studies (KHAWAS *et al*., 2014; MORADI *et al*. 2013; AKPINAR and TORAMAN, 2013; RAYAGURU, 2012; ASMA *et al*., 2012; TAHERI *et al*., 2011; TULEK, 2011) considering the drying kinetics of vegetal material, usually the Midilli and Page model shows the best performance to characterize the drying process, at different drying conditions and dryer used.

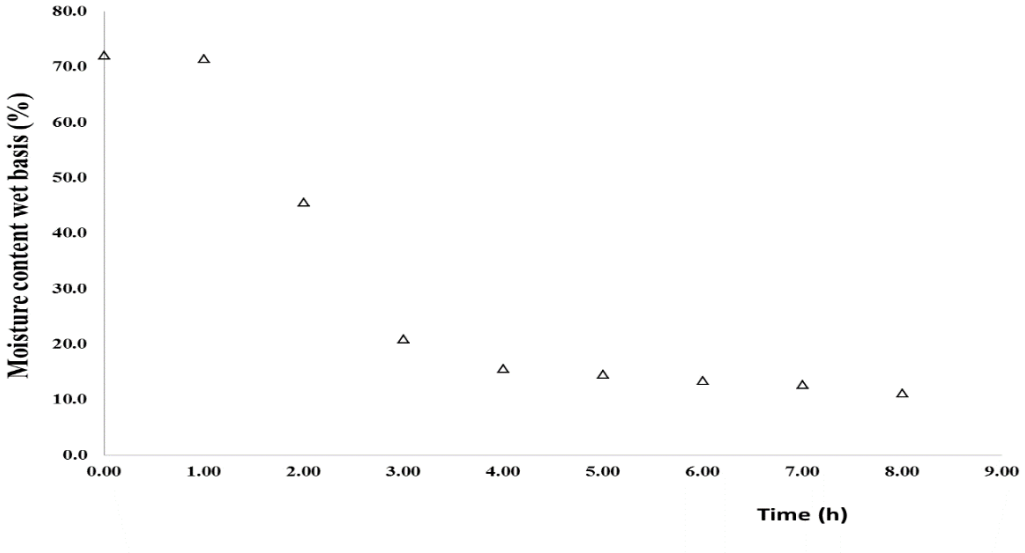

Figure 1. Variation of average moisture content with the drying time for the mixed Lime and Valencia varieties.

Table 6. Drying models tested for determine drying kinetics.

Model name	Model equation	Parameters model	Statistics
Newton (Lewis)	$MR = e^{-k \cdot t}$	k=0.4049408	R^2=0.8826 RMSD=0.0449556
Page	$MR = e^{-k \cdot t^n}$	**k=0.0658905** **n=3.0221099**	**R^2=0.9912** **RMSD=0.0123117**
Henderson and Pabis	$MR = a \cdot e^{-k \cdot t}$	k=0.4487363 a=1.1412666	$R^2$0.9013 RMSD=0.041228
Logaritmic	$MR = a \cdot e^{-k \cdot t^n} + b$	k=0.3454644 a=1.2552761 b=-0.1329415	R^2=0.9688221 RMSD=0.0171092
Midili	$MR = a \cdot e^{-k \cdot t^n} + b \cdot t$	**k=0.0738002** **n=2.9890560** **a=1.0238878** **b=-0.0050326**	**R^2=0.9952** **RMSD=0.0090293**
Diffusional Model	$MR = a \cdot e^{-k_1 \cdot t} + (1-a) \cdot e^{-k_2 \cdot t}$	k1=0.4 k2=-0.3999 a=7608.0632	R^2=0.8826 RMSD=0.044967

4. CONCLUSIONS

It was found that, when dried at determined values, dietary fiber in orange peel increases. The orange peel flour obtained can be used in the manufacture of foodstuff with high fiber content. A correlation to estimate the weight of the orange peel as a function of its height (R^2=0.88) was established, this makes it possible to quantify the orange peel weight available in an orange plantation. The parameters required to modeling the drying kinetics of this by-product and the models that better adjust to the process were found to be Page and Midili, with a correlation coefficient of 0.9912 and 0.9952 respectively. It is necessary to do further research on this by-product in order to determine the percentage of wheat flour that can be substituted for orange peel flour without compromising energy requirements and taste of the final product.

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"NUTRIMENTAL, TECHNOFUNCTIONAL AND BIOFUNCTIONAL STUDY OF BANANA FLOUR (*MUSA PARADISIACA*)"

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ABSTRACT

M. paradisiaca is an alternative as an additive and functional ingredient in the food sector. The objective was to evaluate the nutritional and functional properties of pulp flour, as well as pulp-peel flour of *M. paradisiaca*. The proximal composition; total, resistant and available starch content was determined. The techno-functionality and biofunctionality was determined by assessing the oil and water retention; the capacity of absorption and adsorption of water, the capacity of absorption of organic molecules, the capacity and foaming stability, the solubility and the power of swelling, as well as the antioxidant and antidiabetic activity. Results suggest the use of flours as an additive and functional food ingredient.

Keywords: *Musa paradisiaca*, characterization, nutrimental, functional

1. INTRODUCTION

The banana (*Musa paradisiaca*) is one of the most important crops in Mexican agriculture. It occupies the second place in the production of tropical fruits due to its versatility of use for consumption, low price, pleasant taste, availability and nutritional value (COVECA, 2010). The total consumption and average per capita, from 2000 to 2010 was 2,037,909 and 19.7 kg; the production was 2,114,182 t, of which 96.4% was for the national market in Mexico (García-Mata *et al*., 2013).

The banana is of great socioeconomic importance from the point of view of food security and employment generation. In the marketing process, fresh marketing predominates, although changes in the socio-cultural habits of the population have increased the consumption of processed snack-type based on fried plantain or green plantain chips; the precooked ones like frozen pre-fried "patacón" and "tostones"; semi-processed products such as banana peeled and vacuum-packed, mature frozen and bottled slice and, to a lesser extent, flour production (Quinceno *et al*., 2014).

Several studies show the properties of banana. The pulp is an excellent source of potassium, providing up to 23% of the daily requirement. The banana, is also rich in vitamins A, B6, C and D, providing benefits especially to the bones and muscles of the human body. Since, only one of these fruits contains 41% of the necessary requirement of vitamin B6 per day, recent studies have shown that banana consumption helps improve mood for people with depression and pre-menstrual syndrome due to its high content of vitamins, specifically vitamin B6, composed of pyridoxal, pyridoxine and pyridoxamine, which have an attenuating role in the metabolism of several neurotransmitters (serotonin, norepinephrine, colinergenic system, dopamine and γ-aminobutyric acid) whose deficiency contributes to the development of depression as well as premenstrual syndrome and premenstrual dysphoric disorder. The main by-product of the industrial process of the banana, is the husk which represents approximately 30% of the weight of the fruit. The banana peel is rich in dietary fiber (50g / 100g), proteins, essential amino acids, polyunsaturated fatty acids and potassium. Efforts to use the peel have included proteins, methanol, ethanol, pectins, enzymes, as well as antioxidants such as gallocatechin and dopamine (Blasco López and Gómez Montaño, 2014).

The above shows that not only the fruit but also by-products such as shell, contain compounds of high nutritional value and functional potential, resulting in interest for the food industry the use of them, as raw material for the development of new products, such as flours, with health benefits for their content of proteins, lipids, fiber and antioxidants.

Therefore, the objective of the present study was to evaluate the nutritional and functional properties of pulp flour and, pulp and peel of *M. paradisiaca*.

2. MATERIALS AND METHODS

2.1. Nutritional properties of pulp flour and, pulp-peel of *M. paradisiaca*

2.1.1 Proximal composition

The proximal composition of the flours was determined by the methods of the AOAC (1995). *Humidity* (Method 925.09): by drying the sample in an oven at 105°C until constant weight (4 h). *Ash* (Method 923.03): result of the incineration of the sample at 550°C, until the total loss of organic matter during 4 h. *Raw fat* (Method 920.34): obtained by the soxhlet method, using hexane as solvent. *Raw protein* (Method 954.01): determined by the Kjeldahl method, by acid digestion (sulfuric acid) and alkaline distillation (sodium hydroxide). *Raw*

fiber (Method 962.09): combustible and insoluble organic residue, obtained after an acid and alkaline digestion of the sample. *Nitrogen free extract* (N.F.E.): Obtained by difference to 100 of moisture content, ash, fat, protein and crude fiber.

2.1.2 Total starch (TS)

The content of total starch (TS) was determined by the enzymatic method of Goñi *et al*. (1997). To perform this technique 50 mg of sample were weighed in centrifuge tubes with a capacity of 50 mL. Subsequently, 3 mL of distilled water and 3 mL of KOH 4 M were added; it was mixed and stirred vigorously at room temperature for 30 minutes. After this time, 5.5 mL of HCl 2 M and 3 mL of sodium acetate buffer 0.4 M were added. Then, the pH was adjusted to 4.75 and 60 µL of amyloglucosidase was added; it was mixed and incubated at 60°C for 45 minutes in a bath with shaking, followed by centrifugation for 15 minutes at 3000 x g, recovering the supernatant in 50 mL volumetric flasks. Then 10 mL of distilled water was added and centrifuged again in order to perform at least one sample wash; capacity was carried out (V). Finally, the glucose content was determined with the glucose oxidase/peroxidase reagent (GOD/POD). For this purpose: 500 µL of test sample, glucose standard solution (up to 100 ppm) or water (reagent blank), as well as 1 mL of GOD/POD reagent were transferred into test tubes. The tubes were incubated in a shaking bath for 30 minutes at 37°C and the absorbance at 500 nm was read.

2.1.3 Available starch (AS)

It was determined by the technique of Holm *et al*. (1986). 500 mg of dry sample was weighed in beakers by triplicate. Subsequently, each sample was suspended in 20 mL of distilled water and homogenized by magnetic stirring. A potentiometer was used to maintain the pH of the solution between 6 and 7. 100 µL of α-amylase was added and boiled for 20 minutes with constant stirring. Once this time had elapsed, it was allowed to cool to room temperature and both the magnetic stirrer and the solution were poured into a 100 mL graduated flask. The solution was adjusted and, under constant magnetic stirring, 0.5 mL of the dilution was taken and transferred to test tubes containing 1 mL of sodium acetate buffer. Then, 25 µL of the dilution of amyloglucosidase was added and incubated at 60° C for 30 minutes, stirring every 10 minutes. Once the time was finished, the contents of the tube were transferred to a 10 mL flask, completing up to its capacity. 100 µL of the dilution was mixed with 2 mL of the GOD / POD reagent in a 37°C bath, for 30 minutes, adding 2 mL of 12 N sulfuric acid (H2SO4). Finally, the absorbance at 540 nm was read in a UV-VIS spectrophotometer.
The content of resistant starch (RS) was obtained by difference of the total starch content minus the starch available.

2.2. Technological functional properties of pulp flour and, pulp-peel of *M. paradisiaca*

2.2.1 Water retention

20 mL of distilled water were added to 1 g of sample (d.b.); it was stirred for 1 minute using a vortex. Subsequently, it was centrifuged at 2200 x g for 30 minutes at 25°C. Finally, the volume of the supernatant was measured in 10 mL graduated cylinders. The water retention capacity was expressed as the g of retained water / g of sample (Chau *et al*., 1997).

2.2.2 Oil retention

10 mL of corn oil was added to 1 g of sample (d.b.); it stirred for a minute in a vortex. Subsequently, it was centrifuged at 2200 x g for 30 minutes at 25° C. Finally, the volume of the supernatant was measured in 10 mL graduated cylinders. The oil holding capacity was expressed as the g of oil absorbed / g of sample, considering the oil density of 0.89 g / mL (Chau *et al*., 1997).

2.2.3 Water adsorption capacity

This property was determined according to Chen *et al*., (1984). 1 g of sample (d.b.) was placed in a microenvironment of 98% relative humidity in equilibrium, which was reached by placing 20 ml of saturated potassium sulfate saline in sealed glass jars and placed in a 25 ^{a}C desiccator. The sample was left in that microenvironment until reaching constant weight (which was achieved at 72 hr), with water adsorption capacity being reported, such as g of water adsorbed / g of sample.

2.2.4 Water absorption capacity

It was determined by Method 88-04 proposed by the AACC (1984). 2 g of sample (d.b.) were weighed; water was added to saturation (approx. 40 ml) and centrifuged at 2000 x g for 10 minutes. Subsequently, the excess water was discarded and the sediment was weighed. The approximate water absorption capacity was calculated by dividing the gain in weight of the sample between g sample. Then, the amount of water needed was determined to complete a 15 g weight of the sample (2 g d.b.). To determine the water absorption capacity, the sample was placed in 4 tubes and 1.5 and 0.5 mL of water were added above and below what was determined; they were shaken vigorously in a vortex for 2 minutes; centrifuged at 2000 x g for 10 minutes; the supernatant was discarded and weighed. The g of water absorbed in each tube were calculated and averaged. The water absorption capacity of the sample was calculated as the average of the water absorbed and expressed as the g of absorbed water / g of sample.

2.2.5 Absorption capacity of organic molecules

It was determined by the method of Zambrano *et al*. (2001). 3 g of sample (d.b.) was placed in an excess of corn oil (approximately 10 ml) for 24 h at 25°C. After that time, it was centrifuged at 2000 x g for 15 minutes at 25°C. The ACOM was expressed as the hydrophobic components absorbed and, it was calculated as the gain in weight by the sample (g of oil / g of sample).

2.2.6 Foam capacity and stability

It was determined according to the method indicated by CHAU *et al*. (1997). 100 mL of a 1.5% sample suspension was prepared; the pH was adjusted (2, 4, 6, 8, 10) and mixed at low speed in a blender for 5 minutes. The suspension was transferred to a 250 mL test tube and the volume of foam was recorded after 30 seconds. Foaming capacity was expressed as the percentage increase in foam volume after 30 seconds. It was allowed to stand and, the foam volume was measured after 5, 30 and 120 minutes. The stability of the foam was determined as the remaining foam volume after 5, 30 and 120 minutes.

2.2.7 Solubility and swelling power

The patterns of solubility and swelling power were determined according to Sathe *et al*. (1981). In 50 mL centrifuge tubes previously tared, 40 mL of a 1% (w / v) sample suspension was prepared on a dry basis (P1). The tube was placed in a constant temperature water bath (60, 70, 80 and 90°C) and stirred constantly for 30 minutes. After the time, the tube was removed from the bath, it was centrifuged at 5000 x g for 15 minutes, the supernatant was decanted and the swollen granules were weighed (P2). From the supernatant, 10 mL were taken, which were poured into an aluminum tray; they were weighed and dried at 120° for 4 h; once cold they were weighed again (P3). The calculations were made using the following formulae:

% solubility = (P3 x 400) / P1
Power of swelling: (P2 x 100) / (P1 x 100-% solubility)

Where: P1 = Weight of the sample (d.b.); P2 = Weight of the swollen granules; P3 = Weight of the solubilized starch in 10 mL of solution.

3. RESULTS AND DISCUSSION

3.1. Antioxidant activity

3.1.1 Reducing power of Fe (II)

To 250 µL of sample or standard, 250 µL of phosphate buffer (0.2M, pH 6.6) and 250 µL of potassium ferrocyanide (1%) were added. It was mixed vigorously and incubated at 50°C for 20 minutes. After incubation, 250 µL of trichloroacetic acid (10%) were added and centrifuged at 3000 rpm for 10 minutes. 500 µL of supernatant was taken, 400 µL of distilled water and 100 µL of ferric chloride (0.1%) were added. It was mixed vigorously and incubated at 50°C for 10 minutes. Finally, it was left to rest for 10 minutes and the absorbance was determined at a wavelength of 700 nm. The percentage of reducing power was calculated using the following equation:

$$PR = (\frac{AM - AB}{AM})(100)$$

Where: AM = absorbance in the presence of sample or control; AB = absorbance of the target.

3.1.2 ABTS Assay

It was determined according to the method of Pukalskas *et al*. (2002). A stock solution of cation radical ABTS (2,2-azinobis-6-sulfonic acid, 3-ethylbenzothiazoline) was prepared, dissolving 54.8 mg of ABTS (2 mM) in 50 mL of phosphate-buffered saline (PBS 0.01 M, pH 7.4). The radical cation ABTS•+ originated when 10 mL of the stock solution of ABTS reacted with 40 µL of 70 mM K2S4O8; said solution was prepared 16-17 h before use. To study the antioxidant compounds, 7 mL of the ABTS•+ radical was diluted in 52 mL of PBS buffer until reaching an absorbance of 0.8±0.030, measured at a wavelength of 734 nm. A standard Trolox curve was made, initially dissolving in 2 mL microtubes, 990 µL of diluted ABTS•+ radical and 10 µL of Trolox solution at 0.5, 1, 1.5, 2.5 and 3.5 mM.

Subsequently, said solution was placed in a quartz cell and the absorbance was read at 734 nm for each trolox concentration after 6 minutes. The percentage of inhibition was calculated; the concentration of Trolox vs Inhibition % was plotted and the equation of the trend line (y = mx + b) was obtained, as well as its correlation (r2). The antioxidant activity of the samples was determined by mixing 10 µL of sample, 10 µL of PBS buffer and 990 µL of ABTS•+ radical in 2 mL microtubes, to subsequently read the absorbance at 734 nm after 6 minutes.
The percentage of inhibition of the standards (% IE) and of the sample (% IS) was calculated using the following equations:

$$\%IE = [(AB - AT)/AB] \times 100$$
$$\%IS = [(AB - AM)/AB] \times 100$$

Where: AB = Absorbance of the target at zero time; AT = Absorbance of Trolox after 6 minutes; AM = Absorbance of the sample after 6 minutes.

4. INHIBITORY ACTIVITY OF AMYLOLYTIC ENZYMES

4.1. *In vitro* test of α-amylase inhibition

This trial was carried out according to the methodology cited by Dineshkumar *et al*. (2010). 2 mg of starch were weighed in a tube with 200 µL of TRIS-HCl buffer (0.5M, pH 6.9) and 0.01 M of calcium chloride. The tube was heated for 5 minutes at 100°C; it was allowed to cool to room temperature for 5 minutes and was preincubated at 37°C for 5 minutes. The samples were mixed with dimethyl sulfoxide (0.1%) to obtain concentrations of 10 mg / mL. 200 µL of the sample solution were placed in a tube containing the substrate solution and 0.1 mL of porcine pancreatic amylase dissolved in TRIS-HCl buffer (2U / mL). The reaction was carried out at 37°C for 10 minutes. 500 µL of the DNS-miller reagent was added and allowed to incubate at 100°C for 10 minutes. It was cooled to room temperature and the absorbance was read at a wavelength of 540 nm. The inhibitory activity of α-amylase was calculated with the following formula:

$$\text{Inhibition of } \alpha\text{-amylase} = (AC+) - (AC-) - (As-Ab) / (Ac+-Ac-) \times 100$$

Where Ac +, Ac-, As, Ab are defined as: Ac+, absorbance of 100% the enzymatic activity (only solvent with the enzyme); Ac-, 0% enzymatic activity (only solvent without enzyme); As, sample (with enzyme); Ab, white (sample without enzyme).

4.2. Statistical analysis

All results were expressed as mean±standard deviation of three independent experiments in triplicate. Data were analyzed by one-way analysis of variance (ANOVA) with tukey´s *post hoc test*.

5. RESULTS

5.1. Nutritional properties of pulp flour (PF) and pulp-peel flour (PPF) of *M. paradisiaca*

Table 1 shows the proximal composition of the study flours. Of these, the one constituted by pulp and skin was the one that registered the highest content of ashes (4.12 g), fat (1.11

g), protein (3.17 g) and crude fiber (3.84 g). The moisture content was lower than the maximum value (15%) allowed in flours by NOM-247-SSA1-2008. On the other hand, the energy content of PPF was 265.22 kcal, lower than the registered in PF of 270.55 kcal.

Table 1. Proximal composition of pulp flour (PF) and, pulp-peel (PPF) of *M. paradisiaca.*

Nutritional information /100g	PF (g)	PPF (g)
Humidity	5.26±0.2552[a]	5.19±0.0524[b]
Ashes	3.04±0.1416[b]	4.12±0.1157[a]
Raw fat	0.47±0.5026[b]	1.11±0.1134[a]
Crude protein	2.73±0.1057[b]	3.17±0.0004[a]
Raw fiber	1.57±0.1359[b]	3.84±0.2710[a]
NFE	86.93	82.57

NFE: nitrogen-free elements.

The total, available and resistant starch content of pulp and pulp-peel of *M. paradisiaca* is presented in Fig. 1. The results show that PPF registered lower TS content, compared with PF. However, AS was higher in PPF than in PF and its RS content was lower.

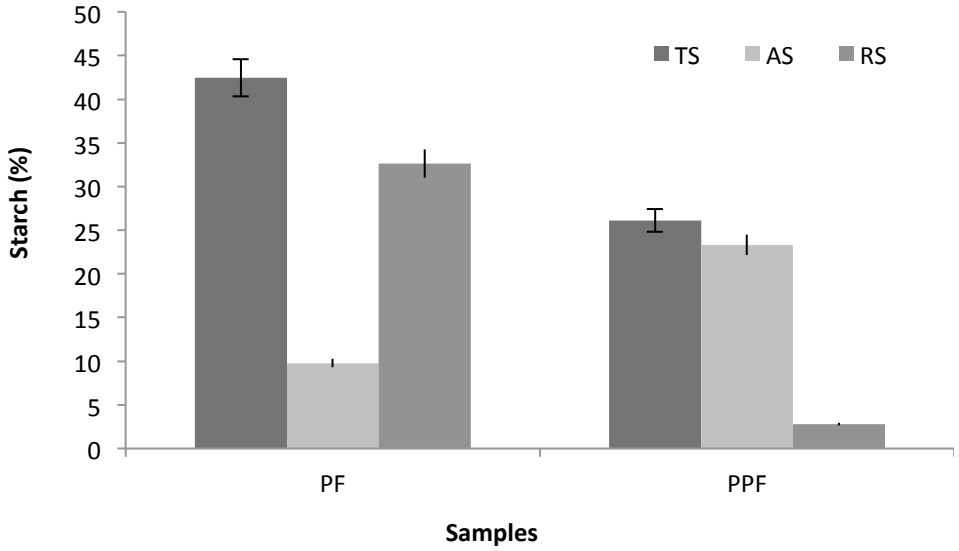

Figure 1. Total starch (TS), available (AS) and resistant (RS) of pulp flour (PF) and, pulp-peel (PPF) of *M. paradisiaca.*

5.2. Techno-functional properties of pulp flour and, pulp-peel of *M. paradisiaca*

Table 2 shows some of the techno-functional properties determined in this study. The water retention of both samples was greater than the oil retention. Thus, both materials retained the water against the centrifugal action, this possibly being comprised of water bound, hydrodynamically and physically trapped, being statistically higher in PF than in PPF. Oil retention is related to the chemical composition, size and surface area of particles such as fiber. In this regard, the higher oil retention capacity of PPF could be related to the

higher fiber content in said flour. On the other hand, pulp-peel flour recorded a higher water adsorption capacity than pulp flour. This property is a surface phenomenon in which samples adsorb water spontaneously when exposed to atmospheres of constant relative humidity. In this regard, the higher content of macromolecules such as proteins in PPF, could contribute to the greater capacity of water adsorption, with respect to PF. The capacity of water absorption of PF and PPF, indicated the greater aptitude of the latter, to imbibe spontaneous water in its structure when putting it in contact with it possibly due to the higher protein content.

Table 2. Technofunctional properties of water retention, oil retention, water adsorption capacity, water absorption capacity and, capacity of absorption of organic molecules of pulp flour (PF) and, pulp-peel (PPF) of *M. paradisiaca.*

Properties	PF (g/g sample)	PPF (g/g sample)
Water retention	18.20±0.2828[a]	17.37±0.2081[b]
Oil retention	7.95±0.0707[b]	8.05±0.0707[a]
Water adsorption capacity	0.2265±0.0932[b]	0.3287±0.0482[a]
Water absorption capacity	0.128±0.0102	0.345±0.0224
Absorption capacity of organic molecules	2.19±0.0109	2.14±0.0192

Fig. 2 shows the foaming capacity at 30 seconds and the stability thereof for 120 minutes of the pulp flour and, pulp-peel flour of *M. paradisiaca* at different pH values (2-10). The highest foaming capacity in PF was recorded at pH 4. However, the highest stability of the foam was recorded at pH 2.

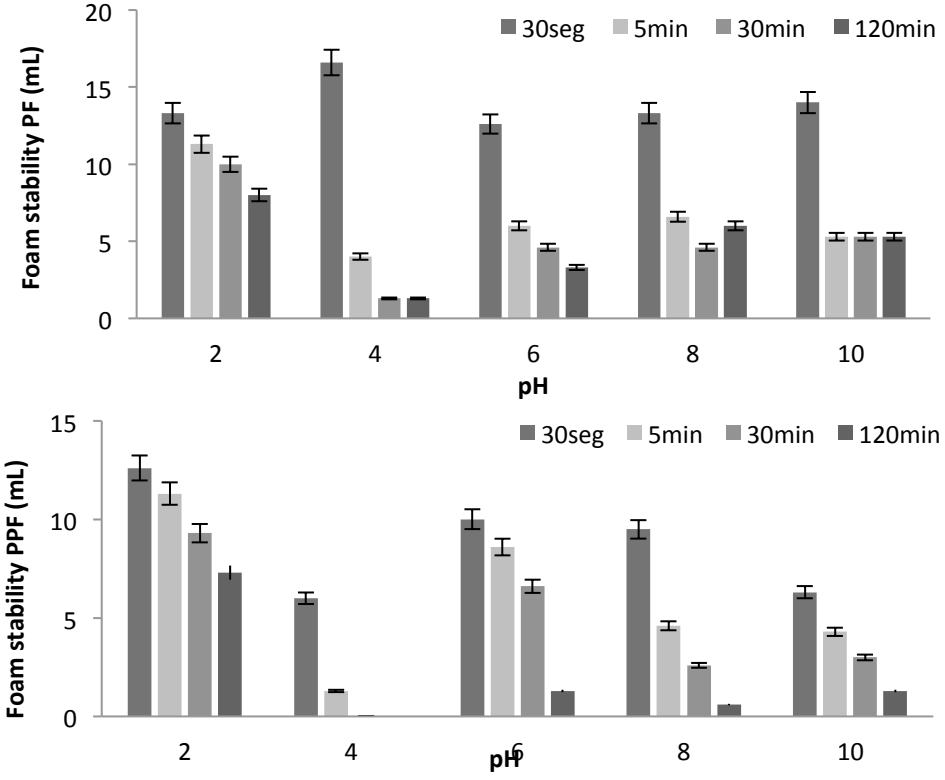

Figure 2. Foaming capacity at 30 seconds and foam stability for 120 min of pulp flour (PF) and pulp-peel (PPF) of *M. paradisiaca*, evaluated at pH 2, 4, 6, 8 and 10.

A similar behavior was observed in PPF, which recorded the highest capacity and stability of the foam at pH 2. Thus, the results show that both PF and PPF showed the highest effectiveness of gas encapsulation and average life of said capsules, at acid pH values.
The solubility and swelling power PF and PPF was temperature dependent being at 90 ºC where the greatest potential technofunctional recorded thereon (Fig. 3). Thus, the solubility and swelling power patterns of PF and PPF showed that, in the range between 70 to 90°C, macromolecules such as proteins, fibrous constipants and starch granules, gradually solubilized and swelled as it was increased the temperature, as a result of the rupture of the intermolecular hydrogen bridges, which allowed a progressive absorption of water. Finally, there was no significant statistical difference between the ACOM of PF and PPF, suggesting that both flours could function efficiently in intestinal trapping of fat, bile acids, cholesterol, drugs and even toxic and carcinogenic compounds.

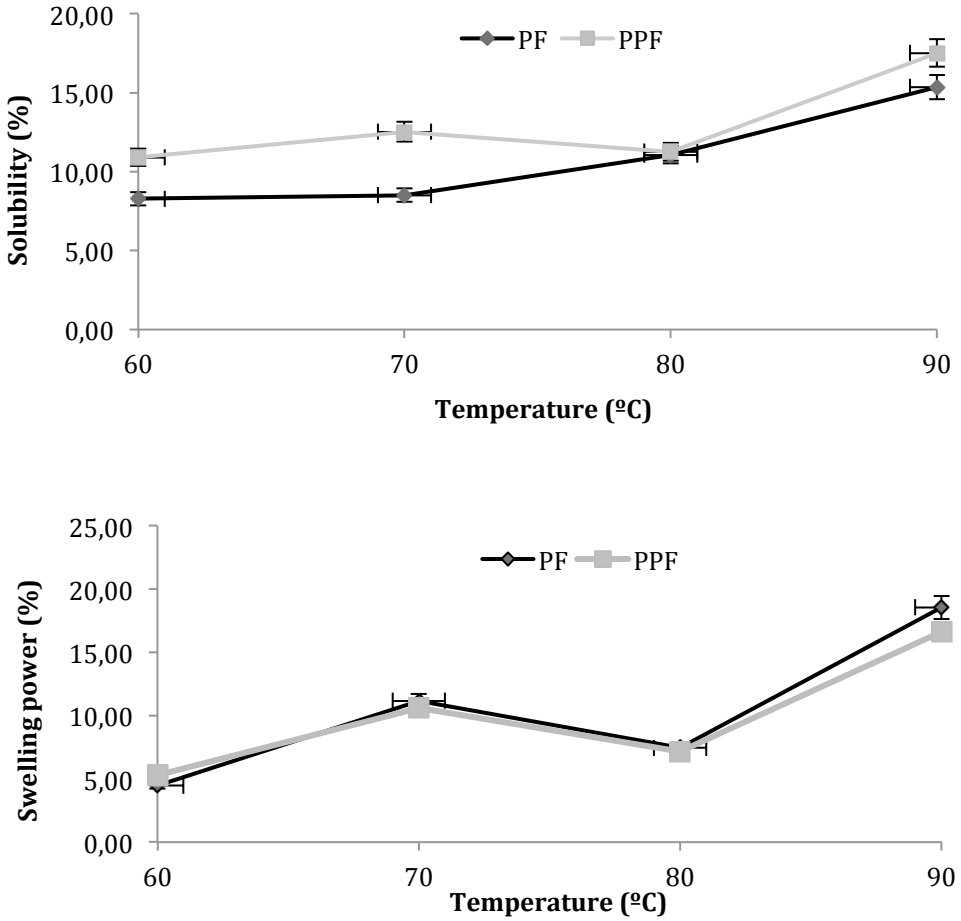

Figure 3. Solubility and swelling power at temperatures of 60, 70, 80 and 90° C, pulp flour (PF) and, pulp-peel (PPF) of *M. paradisiaca*.

5.3. Biofunctional properties of pulp flour and, pulp-peel of *M. paradisiaca*

The functional potential of pulp flour and pulp-peel of *M. paradisiaca*, determined through the study of antioxidant activity and inhibition of amylolitic enzymes showed that, at concentrations of 10 mg / mL, PPF (7.71%) registered higher antioxidant activity than PF (5.24%) in the DPPH assay. However, PF was the flour that registered the highest antioxidant activity at concentrations of 1mg / mL, in the ABTS test (5.08%) and Fe (II)

reducing power (6.11%) compared to PPF which registered percentages of 4.11 and 5.98, respectively. The antidiabetic potential of PF and PPF, determined by the α-amylase inhibition assay, showed the bioactivity of PPF, with 46.06%. However, no activity was observed at the study concentration (1mg / mL) in the pulp of *M. paradisiaca.*

6. CONCLUSIONS

The results show the nutrimental, technological and biofunctional value of pulp flour and pulp-peel of *M. paradisiaca.* However, future research is necessary to suggest the use of these as additives or functional food ingredients as adjuvants in the prevention of diseases.

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SOYBEAN β-AMYLASE AND *BACILLUS ACYDOPULLULYTICUS* PULLULANASE IMMOBILIZED ONTO ACTIVATED SEPHAROSE 4B AND CALCINED BONE TO PRODUCE MALTOSE SYRUPS

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ABSTRACT

High maltose syrup have some potential applications in food, beverage and pharmaceutical industries. Its production requires the utilization of a debranching enzyme besides maltogenic amylases. Syrups industries use mostly soluble enzymes, however, immobilization may improve enzymes stability and let their repeated use. In this study soybean β-amylase (SβA) and *Bacillus acydopullulyticus* pullulanase (BAP) were separately adsorbed onto calcined chicken bone ($calB_{360}$) or covalently bound to Sepharose 4B activated with 1-cyano-4-(dimethylamino) pyridinium tetrafluoroborate. Immobilization yield and activity of the immobilized biocatalysts were assessed by activity determination while its performance in maltose syrup production was evaluated by reducing sugars quantification. Derivatives of SβA and BAP obtained by covalent binding to CDAP-Sepharose 4B expressed twice as high activities than those produced by adsorption onto $calB_{360}$ for both enzymes, probably because of the highly porous nature of Sepharose 4B that facilitated the interaction with the macromolecular substrate. Thermal stability of SβA at 60°C in activity buffer increased by immobilization onto both supports; on the contrary, the resistance of BAP to thermal inactivation remarkably enhanced (36-fold) by its covalent binding only.

Keywords: Calcined bone, cassava starch, immobilization, maltose syrup, Sepharose 4B

1. INTRODUCTION

Beta amylases (EC 3.2.1.2) are exo-hydrolases, which release β-maltose from the non-reducing ends of α-1,4 poly- and oligo-saccharides and are unable to hydrolyze or bypass α-1,6 linkages (REEVE, 1992). In order to produce high maltose syrups (>70% maltose), it is necessary to add a debranching enzyme such as pullulanase (EC 3.2.1.41) to split α-1,6 linkages in amylopectin, thereby permitting the continued action of β-amylase (SINGH *et al*., 2010). Although soluble amylases are generally used to hydrolyze starch, immobilized enzymes may yield shorter reaction times, since immobilization allows the utilization of high enzyme loads. In addition, immobilized enzymes are easier to handle and recover, and tend to be more stable (SIRISHA *et al*., 2016, CHAKRABORTY *et al*., 2014).
Maltogenic amylases have been both separately immobilized (ATIA *et al*., 2003a, NODA *et al*., 2001, VRETBLAD *et al*., 1973, CHANG *et al*., 2005, DAS *et al*., 2018, SRIVASTAVA *et al*., 2015) and co-immobilized (ATIA *et al*., 2003b) onto different supports. Co-immobilization decreases diffusion times of the substrate to the active site of the enzyme, thus improving system efficiency (ATIA *et al*., 2003b, TALEKAR *et al*., 2013). However, determining the optimum ratio of enzyme loads to be applied for co-immobilization has proven complex, while separate immobilization facilitates the combination of different amounts of β-amylase and pullulanase in order to obtain maximal conversion rates.
Immobilization can be carried out by encapsulation, by entrapment within polymeric matrices, by adsorption or by covalent binding to insoluble carriers (DATTA *et al*., 2013, CHAKRABORTY *et al*., 2014). Selection of the immobilization system (using catalytic and non-catalytic components) depends on the particular requirements of each application. The diversity of the process (varying substrates, reaction types, reactor configurations and downstream processes) gives rise to specific immobilized enzyme designs, which satisfy these requirements. The task centers on selecting the appropriate carrier (organic/inorganic, porous/non-porous), immobilization conditions (pH, temperature and medium) and enzymes (source and purity) to obtain a suitable immobilized biocatalyst (CAO, 2005b, DAS *et al*., 2018).
In this study, the individual immobilization of soybean β-amylase (SβA) and *Bacillus acydopullulyticus* pullulanase (BAP) was explored through the methods of adsorption and covalent binding on different supports. The presence of stabilizers, ionic strength, pH and enzyme load added to the matrix were evaluated for their effect on both the amount of enzyme attached to the selected support, and the expressed activity of the resulting derivatives.
The main properties of soluble and immobilized enzymes, including thermal stability at 60°C, were compared. Finally, the study assessed the operational stability of immobilized SβA and BAP, mixed in a ratio previously determined with soluble enzymes, in the batch production of maltose syrups at 55°C.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in the study were obtained from the sources detailed as follows:
Cassava starch isolated by wet milling (BREUNINGER *et al*., 2009). Soybean β-amylase (SβA) S1500 [EC 3.2.1.2] purchased from Nagase (Kyoto, Japan).

Bacillus licheniformis α-amylase Termamyl 120L® [EC 3.2.1.1] and *Bacillus acidopullulyticus* Pullulanase (BAP) Promozyme 400L® [EC 3.2.1.41] supplied by Novo Nordisk A/S (Bagsvaerd, Denmark). Pullulan from *Aerobasidium pullulans*, Sepharose 4B, 1-cyano-4-dimethylaminopiridinium tetrafluoroborate (CDAP), Triethylamine (TEA), 25 % glutaraldehyde solution, chitin from crab shells (practical grade, coarse flakes), 3-aminopropyl triethoxy silane (APTES) all obtained from Sigma Aldrich (USA). Papain [EC. 3.4.22.2] and 3,5 dinitrosalicylic acid (DNS) supplied by Merck KgaA (Darmstadt, Germany). Bovine serum albumin (BSA) fraction V and Coomassie Plus Protein Assay Reagent purchased from Pierce (Rockford, IL, USA). Control pore silica (CPS) obtained from Fluka, and chicken necks from Carnasa (Puembo, Ecuador). Soluble potato starch obtained from Panreac Quimica S.A. (Barcelona, Spain). All other chemicals used were of reagent grade and were used without any treatment. Each experiment was performed a minimum of three times, and the results are presented as their mean value±standard deviation. The maximal experimental error accepted was 10%.

2.2. Activity of free and immobilized SβA and BAP

To determine SβA activity, the enzyme was extracted from the commercial powder using a 0.016 M sodium acetate buffer of pH 4.8 for 1 hour at room temperature (22°C). The resulting slurry was centrifuged at 15,600 g for 15 minutes. The activity of soluble SβA was assayed as described in the Worthington Enzyme Manual (WORTHINGTON, 1993), at 25°C using 5% (w/v) soluble starch solution in the activity buffer. A blank was used, prepared by mixing the SβA solution with DNS before adding the substrate. To determine maltose concentration, a standard curve was created with a 2 mg mL^{-1} maltose stock solution. Free BAP activity was assayed according to the method reported by DÍAZ *et al*. (1995), at 40°C using 0.2% (w/v) pullulan dissolved in the activity buffer.
The activities of immobilized SβA and BAP were assayed by batches. Initial velocities were measured at 25°C by incubating aliquots of suction-dried SβA derivatives in capped tubes with 10 mL of 5% (w/v) starch solution, using a water bath fitted with a reciprocal shaker. Samples of the reaction mixture (0.3 mL) were withdrawn every 3 minutes, and poured on a 0.3 mL DNS solution to stop the reaction. The reducing sugars formed were quantified using the substrate as a blank (MILLER, 1959). The process was repeated at 40°C to determine the activity of immobilized BAP, using as substrate 5 mL of 4% (w/v) pullulan solution.
For SβA, one unit of activity (βU) was defined as the amount of enzyme necessary to release 1 μmol of β–maltose per minute at 25°C and pH 4.8, while one unit of activity for pullulanase (PU) was defined as the amount of enzyme that hydrolyzes pullulan, releasing carbohydrates with a reducing power equivalent to 1 μmol of glucose per minute at 40°C and pH 4.8.

2.3. Protein determination

The protein content in the SβA and BAP solutions, supernatants and washings was determined by the Coomassie blue G-250 dye-binding method using the Coomassie Plus® reagent and BSA as standard (micro assay procedure, Pierce Instructions booklet). The immobilized protein was estimated as the difference between the amount of protein applied to the support, and that recovered in the centrifuged (15,600 g) supernatant and washings.

2.4. Preparation of bone (B_{360}) and calcined bone ($calB_{360}$)

Bone particles were prepared as described by CARPIO *et al*. (2009b) using chicken necks. A matrix with an average particle size of 360 μm (B_{360}) was obtained by sifting and mixing bone fragments retained by standard ASTM sieves, numbers 45, 50 and 60. Calcined bone ($calB_{360}$) was obtained by reducing B_{360} particles to ash at 550±5°C, as described by CARPIO *et al*. (2009a).

2.5. Activation of CPS with glutaraldehyde

Before activation, commercial CPS was silanized as described by WEETALL (1993) with 10% APTES aqueous solution of pH 4 (about 200 mL per 15g of moist CPS at 75°C for 3 hours under intermittent stirring). The reaction mixture was filtered, and the silanized CPS (APTES-CPS) was washed with distilled water and dried at 110°C for 16 hours. Aliquots of APTES-CPS (0.2 g) were soaked in a (2.0 mL) 0.1M sodium phosphate buffer of pH 7 for 1.5 hours. The buffer solution was then drained, and the moist aliquots were mixed with 2 volumes of a 2.5 % glutaraldehyde solution diluted with a phosphate buffer. The reaction was allowed to continue for 45 minutes at room temperature (22°C) under gentle stirring. Finally, the activated matrix (GLUT-APTES-CPS) was washed thoroughly with the same buffer.

2.6. Activation of Sepharose 4B

Sepharose 4B was activated following the process described by GIACOMINI *et al*. (1998) using 1-cyano-4-dimethilaminopyridinium tetrafluoroborate (CDAP) as the activating agent.

2.7. Adsorption of SβA and BAP onto B_{360}, $calB_{360}$ and chitin

The adsorption of SβA and BAP was carried out by equilibrating 0.3 g of the support particles in 3.0 mL of activity buffer (or one of the assayed buffers) for 1.5 hours under stirring. After removing the buffer, the equilibrated support was incubated at 20°C for 20 hours in 6.0 mL of the enzyme solution (with or without previous gel-filtration for BAP adsorption) under gentle agitation. The excess enzyme was removed by filtration and washings of the derivative with the activity buffer, until no more enzyme was released. Enzyme activity and protein content in the supernatant and washings were then quantified. To determine the maximal load capacity (q_{max}) for SβA and BAP adsorption, increasing amounts of SβA (13.6-163.9 mg g^{-1} support) or BAP (3.2-24.8 mg g^{-1} support) in activity buffer were applied to the matrix, at 20°C. The amount of SβA or BAP present on the adsorbent, q^*, was calculated as the difference between the amount of enzyme present at the beginning of the experiment (C_iV) and the amount still in the soluble phase (supernatant) at equilibrium (C*V) (Equation 1) (CHASE, 1984, SORIANO *et al*., 1999).

$$q^* = \frac{(C_i - C^*)V}{W} \qquad (1)$$

where C_i and C^* are the initial and equilibrium protein concentrations in milligrams per milliliters of enzyme solution, V is the immobilization volume in milliliters, and W the weight of the carrier in grams. Finally, q^* versus C^* was plotted.

2.8. Covalent coupling of SβA or BAP to GLUT-APTES-CPS or CDAP-Sepharose 4B

Aliquots of activated carrier (0.3 g) were separately incubated at 20°C with SβA or BAP solutions in a (6 mL) phosphate buffer of pH 7.0 for 20 hours under gentle stirring. The unbound enzyme was removed by filtration and washings of the derivative, first with the coupling buffer and then exhaustively with the activity buffer. The maximal capacity of CDAP-Sepharose 4B for SβA and BAP immobilization was determined by applying increasing loads of SβA (4.3-95.1 mg protein g^{-1} of suction-dried gel) or previously-gelfiltered BAP (2.5-21.8 mg protein g^{-1}) to the matrix as described above. For both adsorption and covalent binding, the immobilization yield was calculated by dividing the total amount of immobilized enzyme by the amount of enzyme initially added to the matrix.

2.9. Determination of kinetic parameters ($K_{M,}$ $K_{M\ app}$, V_{max} and $V_{max\ app}$)

Kinetic and apparent kinetic parameters of soluble and immobilized SβA and BAP were determined by measuring the initial reaction rates with the respective substrate solutions prepared in an activity buffer. Starch concentrations for soluble SβA ranged between 0.6 and 8.2 mg mL^{-1}, or 0.6-41.2 mg mL^{-1} for immobilized SβA. Meanwhile, pullulan concentrations varied from 0.09-0.54 mg mL^{-1} for soluble BAP, or between 0.19-0.95 mg mL^{-1} for immobilized BAP to CDAP-Sepharose 4B, or 5.7-38.1 mg mL^{-1} for BAP adsorbed onto $calB_{360}$. The kinetic parameters were estimated by fitting the experimental data to the Michaelis-Menten equation using Statgraphics plus software version 5.1.

2.10. Effect of pH and temperature on the activity of free and immobilized SβA and BAP

The effect of pH on the activity of soluble and immobilized SβA and BAP was assayed by determining the initial reaction rates for 15 minutes in kinetic mode at 25°C or 40°C respectively. For free enzymes, as well as for SβA and BAP derivatives to CDAP-Sepharose 4B, the substrates were 5% (w/v) soluble starch or 0.2% and 4% (w/v) pullulan solutions in a 0.016 M citrate-phosphate buffer, of a pH range of 2.6-7.0. For SβA and BAP derivatives onto $calB_{360}$, the substrate solutions were prepared in a Gly-HCl buffer (pH 2.2-3.6). Soluble enzymes were diluted in each buffer just before the test.
To assess the effect of temperature on enzyme activity, the initial reaction rates at different temperatures (25-80°C) were determined using the corresponding substrate (in activity buffer) pre-warmed at each of the temperatures tested.

2.11. Thermal stability of SβA and BAP

The thermal stability of soluble and immobilized SβA or BAP was determined by incubating aliquots (1 mL) of enzyme solutions or suction-dried enzyme derivatives in capped tubes containing 6 βU or 5 PU per mL of activity buffer at 60°C. At pre-established intervals, the capped tubes were cooled and the residual activity determined. Inactivation kinetics were monitored for 24 hours for SβA and for 8 hours for BAP. To describe thermal inactivation kinetics, the biexponential decay model (Equations 2 and 3) was tested. Model parameters were determined using Statgraphics Plus software version 5.1.
Bi-exponential decay model:

$$E \xrightarrow{k_1} E_1^{\alpha_1} \xrightarrow{k2} E_2^{\alpha_2} \quad (2)$$

$$A = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (3)$$

where A (%) is the residual activity of SβA or BAP at a specific time t; α_1 (%) and α_2 (%) are the relationships between the activities of the final states E_1 or E_2 to the initial state E. It was assumed that E_2 had no residual activity, so $\alpha_2 = 0$; k_1 (hours^{-1}) and k_2 (hours^{-1}) were the first order inactivation constants, and A_1 and A_2 (Equations 4 and 5) were coefficients described by the following relationships:

$$A_1 = 100 + \left(\frac{\alpha_1 k_1}{k_2 - k_1} \right) \quad (4)$$

$$A_2 = 100 - A_1 \quad (5)$$

The half-lives ($t_{1/2}$, time at which the residual activity is 50%) were calculated by Equation 3 after determining the model parameters.

2.12. High maltose syrup production

2.12.1 Determining the optimal mixture of SβA and BAP for maltose syrup production

The optimal blend of SβA and BAP for maltose syrup production was determined for soluble enzymes using a 3^2 factorial design with two replicates (Table 1). The response variable was starch conversion expressed as a dextrose equivalent (DE). Data were analyzed using the program Statgraphics version 5.1, and mean differences were determined by a Duncan multiple range test (Duncan, $p<0.05$) applied after an analysis of variance (ANOVA).

Table 1. The 3^2 factorial design for determining the optimal blend of Soybean β–amylase (SβA) and *Bacillus acidopullulyticus* pullulanase (BAP) for high maltose syrup production from liquefied cassava starch.

Factor A: amount of SβA (βU g^{-1} maltodextrin)	Factor B: amount of BAP (PU g^{-1} maltodextrin)		
	1 15 PU	2 30 PU	3 45 PU
Coded level 1 12 βU	SβA 1-BAP 1	SβA 1-BAP 2	SβA 1-BAP 3
2 30 βU	SβA 2-BAP 1	SβA 2-BAP 2	SβA 2-BAP 3
3 48 βU	SβA 3-BAP 1	SβA 3-BAP 2	SβA 3-BAP 3

2.12.2 Cassava maltodextrin hydrolysis, batch procedure

Cassava maltodextrin (DE = 20) was prepared as described by CARPIO *et al*. (2009a). The optimal blend of SβA and BAP derivatives was added to 40 g of 30% (w/w) cassava

maltodextrin solution in an activity buffer to produce high-maltose syrups. The hydrolysis was carried out at 55°C for 22.5 hours under stirring. During the reaction, reducing sugars were monitored with DNS solution (MILLER, 1959). The operational stability of biocatalysts was determined by batches by their repeated use for the hydrolysis of cassava maltodextrin. At the end of each batch, the derivatives were separated from the reaction mixture by filtration, washed with tap water, and reused for the next batch. The ratio between substrate and suction-dried derivatives was kept constant for each batch.

3. RESULTS AND DISCUSSION

3.1. Immobilization of soybean β-amylase (SβA) to different supports

Several assays were performed of SβA immobilization to organic and inorganic matrices such as chitin, Sepharose 4B, bone (B_{360}), calcined bone ($calB_{360}$) and controlled pore silica (CPS) in order to select the matrices that rendered the most promising biocatalysts. Adsorption was used to couple SβA onto chitin, B_{360} and $calB_{360}$. Meanwhile, covalent binding was achieved through the formation of Schiff base linkages, or isourea type bonds for SβA immobilization to CPS silanized with 3-aminopropil–triethoxysilane (APTES) and activated with glutaraldehyde (GLUT-APTES-CPS), and to Sepharose 4B activated with 1-cyano-4-dimethyl amino pyridinium tetrafluoroborate (CDAP–Sepharose 4B). Table 2 summarizes the results of SβA immobilization on the above mentioned supports.

Table 2. Immobilization of soybean β-amylase (SβA) on different supports.

Support	Applied enzyme load (βU g^{-1} of support)	Immobilized enzyme (βU g^{-1} of support)	Immobilization yield (%)	Expressed activity (βU g^{-1} of support)	Immobilization efficiency[5] (%)
Chitin	602±10.0	310±6.1[a]	51.5	27±1.4[a]	8.7
B_{360}[1]	614±2.4	324±12.0[ab]	52.8	11±1.3[b]	3.4
$calB_{360}$[2]	607±1.1	572±4.2[c]	94.2	51±3.3[c]	8.9
GLUT-APTES-CPS[3]	633±14.0	625±5.4[d]	98.7	42±0.3[d]	6.7
CDAP-Sepharos 4B[4]	600±6.5	528±6.2[f]	88.0	170±6.7[f]	32.2

Mean of triplicates±standard deviation; different letters within the column mean significantly different results (p<0.05, Duncan test); βU = β-amylase units; B360[1]: bone with an average particle size of 360 μm; calB360[2]: calcined B360; GLUT-APTES-CPS[3]: control pore silica silanized with 3-amino-propiltriethoxysylane and activated with glutaraldehyde; CDAP-Sepharose 4B[4]: Sepharose 4B activated with 1cyano-4-dimethylamino pyridinium tetra-fluoroborate (CDAP).
Immobilization yield[5] = (immobilized enzymex100/applied enzyme); Immobilization efficiency[6] = (Expressed activityx100/Immobilized activity).

Adsorption of SβA onto chitin, B_{360} and $calB_{360}$, showed that $calB_{360}$ had the highest adsorption capacity and rendered the most active derivative (SβA-$calB_{360}$), yet the SβA-$calB_{360}$ biocatalyst is easier to recover from the reaction mixture. On the other hand, covalent immobilization of SβA to CDAP-Sepharose 4B and GLUT-APTES-CPS rendered high

coupling yields of 88 and 99% respectively. Although the highest immobilization yield was attained by the covalent binding to GLUT-APTES-CPS, the expressed activity of the resulting derivative was only 25 % that of SβA biocatalyst to CDAP-Sepharose 4B. Studies on the utilization of glutaraldehyde for enzyme coupling have shown that several enzymes, especially those rich in sulfhydryl groups, are very sensitive to this reagent. As a result, STANLEY *et al*. (1976) observed marked drops in their activity. Activity loss may also be due to the tendency of glutaraldehyde to polymerize (MONSAN, 1978), which in turn produces serious diffusion limitations of starch within the pores of the derivative, also observed for sweet potato immobilized to glutaraldehyde agarose (TAVANO *et al*., 2013). On the other hand, a high degree of activity of SβA-CDAP Sepharose 4B derivatives may be ascribed to the porous nature of Sepharose 4B (JANSON *et al*., 2011) and to the selected immobilization method of isourea type bonding (BATISTA-VIERA *et al*., 2011).
The capabilities of the tested matrices for SβA immobilization may be arranged as follows, based on the expressed activity of SβA derivatives: B_{360} (11 βU g^{-1}) < chitin (27 βU g^{-1}) < GLUT-APTES-CPS (42 βU g^{-1}) < $calB_{360}$ (51 βU g^{-1}) < CDAP-Sepharose 4B (170 βU g^{-1} of filter dry gel). Therefore, $calB_{360}$ and CDAP-Sepharose 4B were selected for SβA immobilization, and were also used for BAP immobilization.

3.2. Effect of selected parameters on the immobilization of SβA and BAP

The effect of parameters such as the presence of stabilizers in commercial BAP, ionic strength, immobilization pH, and enzyme load was assessed on both the amount of enzyme immobilized on the supports, and on the activity of the derivatives (expressed activity) in order to obtain immobilized SβA and BAP biocatalysts with the highest activity.

3.2.1 Influence of ionic strength and immobilization pH on SβA adsorption onto $calB_{360}$

The adsorption of proteins on solid surfaces mainly depend on van der Waals forces, hydrogen bonding, and ionic and hydrophobic interactions. Therefore, SβA adsorption is expected to depend on ionic strength and pH, in addition to enzyme load, and temperature (CAO, 2005a, SRIVASTAVA *et al*., 2015). To determine the effect of ionic strength on adsorption and on the expressed activity of the insoluble SβA, 0.050 and 0.016 M sodium acetate buffers of pH 4.8 were used for SβA immobilization. In the first instance, the amount of SβA adsorbed onto $calB_{360}$ was 753±3.6 βU g^{-1} of support, which was 5 % higher than that obtained with the 0.016 M buffer (714±1.8 βU g^{-1} of support) for an enzyme load of 840 βU g^{-1} of support. However, the expressed activity of the derivatives produced at both ionic strengths showed no significant differences between them (80±4.1– vs 79±3.3 βU g^{-1} of support) and, therefore, for successive immobilization processes 0.016 M was the selected diluent concentration. To test the effect of immobilization pH on SβA adsorption, the enzyme was diluted with 0.016 M solutions of acetic acid of pH 3.2, a sodium acetate buffer of pH 4.8, or sodium acetate of pH 7.0.
Soybean β-amylase adsorption was greater than 98% at every pH value tested for an initial load of approximately 600 βU g^{-1} of $calB_{360}$, and the expressed activity of all these derivatives was similar: 45±2.2 to 49±2.2 βU g^{-1} of support. Therefore, 0.016 M sodium acetate buffer of pH 4.8 was chosen as the activity buffer for subsequent immobilizations onto $calB_{360}$.

3.2.2 Effect of stabilizers on BAP immobilization

The effect of stabilizers (42.4% reducing sugars) of commercial BAP on the immobilization onto $calB_{360}$ and to CDAP-Sepharose 4B, and on the activity of the insoluble BAP biocatalysts was assessed by applying gel-filtered and non-gel-filtered BAP solutions (2,000-2,113 PU g^{-1} of $calB_{360}$ and 575-585 PU g^{-1} of filter-dried CDAP-Sepharose 4B). The results of this study are summarized in Table 3.
BAP adsorption on $calB_{360}$ showed that the sole parameter affected by the utilization of gel-filtered BAP was the amount of adsorbed enzyme, which was 9% lower than that obtained with non-gel-filtered BAP, while the expressed activity was similar for both derivatives (111 vs 112 PU g^{-1} of support; $p>0.05$). Since BAP adsorbed onto $calB_{360}$ (673 PU g^{-1}) was only 33% of the applied activity (2 000 PU g^{-1}), additional experiments were carried out by applying 402 PU of non-gel-filtered BAP g^{-1} of support. The reduction of the initial load allowed the immobilization of 92% of the applied activity (370 PU g^{-1} of support) and produced a 50 % more active derivative (173 PU g^{-1} of support).

Table 3. Effect of stabilizers on the immobilization of *B. acidopullulyticus* pullulanase (BAP) onto calcined bone ($calB_{360}$) and to CDAP activated Sepharose 4B (CDAP-Sepharose 4B).

Derivative	Support particle size (μm)	Applied BAP (PU g^{-1} support)	Immobilized BAP (PU g^{-1} support)	Expressed activity (PU g^{-1} support)
Gel-filtered BAP on $calB_{360}$	360[1]	2,113±125.1	615±30.2	111±7.3[a]
Non-gel-filtered BAP on $calB_{360}$	360[1]	2,000±120.0	673±39.0[a]	112±8.1[a]
Non-gel-filtered BAP on $calB_{360}$	360[1]	402±23.6	370±1.3	173±4.5[b]
Gel-filtered BAP on CDAP-Sepharose 4B	90[2]	575±20.1	570±0.5[c]	119±4.1[a]
Non-gel-filtered BAP to CDAP-Sepharose 4B	90[2]	585±37.5	496±2.3[b]	27±1.7[c]

[1]Data obtained by granulometric analysis; [2]Data from bibliography (JANSON *et al*., 2011). Results are the average values of triplicates±standard deviation. Different letters within the column mean significantly different results ($p < 0.05$, Duncan test). PU = Pulullanase units.

Removal of the reducing sugars from the BAP solution for covalent immobilization to CDAP-Sepharose 4B produced a 15 % increase in the amount of activity bound to CDAP-Sepharose 4B. Moreover, this improved the expressed activity of the derivative by a factor of approximately 4.4 (from 27 to 119 PU g^{-1} support). This increment exceeded expectation due to the higher immobilization yield (15 %). One possible explanation is that the removal of sugars caused a favorable orientation of BAP molecules during immobilization, which in turn improved the accessibility of the active site for the substrate (HERNANDEZ *et al*., 2011). From this study, it can be concluded that gel-filtration may not be omitted when coupling BAP to CDAP-Sepharose 4B, although it is not necessary for BAP adsorption onto calcined bone particles. This may represent a significant advantage for large scale applications.

3.2.3 Effect of the applied enzyme load on the immobilization of SβA and BAP onto calB$_{360}$ and to CDAP-Sepharose 4B

The binding capacity of the matrix, and the enzyme load that produces the most active derivative are critical parameters for determining a cost-effective immobilization of any enzyme. Both of these parameters were obtained by applying increasing enzyme loads to the matrix.

a) Adsorption of SβA and BAP onto calcined bone (calB$_{360}$)

Fig. 1 shows the relationship between the amount of protein adsorbed onto calB$_{360}$ (q^*) and that remaining in the solution (C^*) at equilibrium for SβA and BAP adsorption at 20°C. Both isotherms followed the Langmuir equation (Equation 6) within the range of protein concentration tested for each enzyme (R^2 = 96.8 % for SβA and R^2 = 97.4% for BAP). The model parameters, maximal loading capacity (q_{max}) and the desorption constant (K_d), were 54.9 mg protein g^{-1} of support and 3.4x10^{-1} mg protein mL^{-1} of solution for SβA, while for BAP they were 3.7 mg protein g^{-1} of support and 1.1x10^{-2} mg protein mL^{-1} of solution.

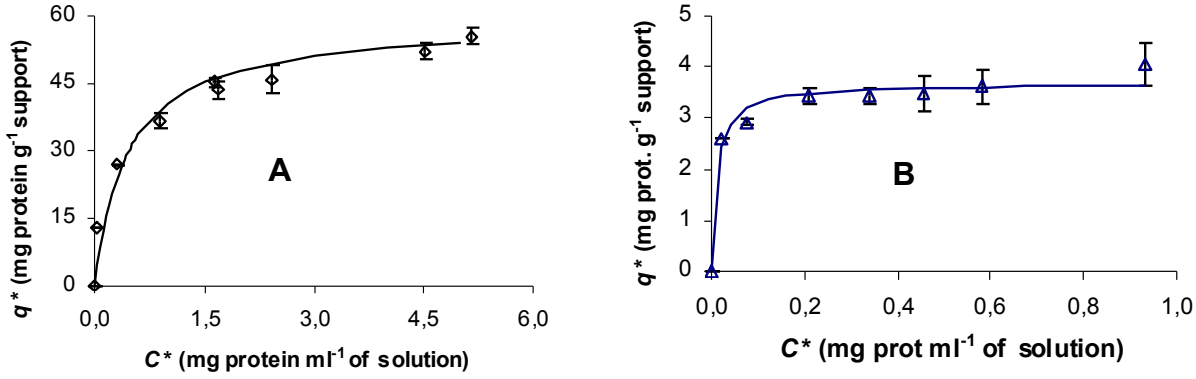

Figure 1. Adsorption isotherms of soybean β-amylase (A) and *B. acidopullulyticus* pullulanase (B) onto calcined bone (calB$_{360}$), determined at 20°C in 0.016 M sodium acetate buffer of pH 4.8.

$$q^* = \frac{q_{max} C^*}{K_d + C^*} \qquad (6)$$

In a previous study related to GA adsorption onto calcined and non-calcined bone (CARPIO *et al*., 2009a), it was determined that the qmax and Kd values changed as a result of the variation in temperature, surface area and availability of charged groups on the adsorbent. However, protein adsorption is influenced not only by the adsorbent's nature (porosity, number and availability of charged groups) and particle size, but also by the characteristics of the protein itself, that is, size, shape and distribution of charged groups on its surface (BLANCO *et al*., 2008 , BATISTA-VIERA *et al*., 2011). The protein content of commercial enzyme preparations (SβA: 78.1 mg protein g^{-1} and BAP: 5.7 mg protein g^{-1}), as well as their molecular weight (SβA: 57 kDa, and BAP: 116 kDa) and distribution of charged groups on the enzyme surface seem to be the determining factors for the differences observed for q_{max} and K_d values. Therefore, the q_{max} of calB$_{360}$ for SβA was 14.8 times

higher compared to that of BAP, while the enzyme affinity for the matrix was 31 times lower for SβA as proved by its higher K_d value.

b) Covalent immobilization of SβA and BAP to CDAP-activated Sepharose 4B.

The amount of protein immobilized to CDAP-Sepharose 4B depended almost linearly on the protein load added to the matrix (up to 35 mg protein g^{-1} of filter-dried support for SβA, and 7.5 mg protein g^{-1} for BAP), and showed a saturation of over 50 mg and 10 mg protein g^{-1} of filter-dried matrix for SβA and BAP respectively (Fig. 2). The maximal load capacity of CDAP-Sepharose 4B was 29.1 mg and 5.4 mg protein g^{-1} of filter-dried matrix for SβA and BAP respectively.

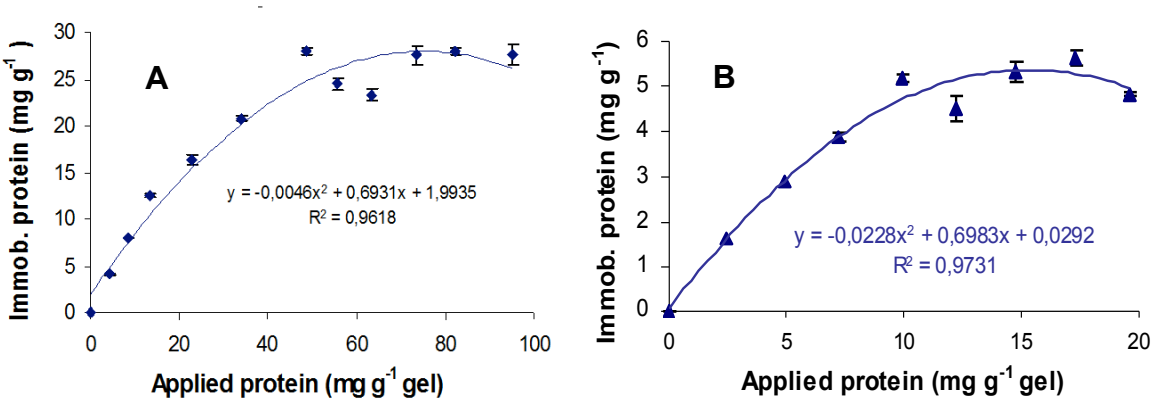

Figure 2. Effect of the applied enzyme loads on the immobilization of: A) soybean β-amylase (4.3-95.1 mg protein g^{-1} of filter-dried gel), and B) *B. acidopullulyticus* pullulanase (2.3-20.1 mg protein g^{-1} of support) to CDAP-activated Sepharose 4B, at 20°C, using 0.1 M sodium phosphate buffer of pH 7.0 as a coupling buffer.

Although Sepharose 4B is a highly porous matrix, the highest amount of SβA bound to this support was 1.9 times lower than that adsorbed onto $calB_{360}$, probably due to the fact that covalent immobilization is not a surface phenomenon, but process-dependent on matrix activation (FRANCO-FRAGUAS *et al.*, 2000, TAVANO *et al.*, 2013). Conversely, the maximal amount of BAP adsorbed onto $calB_{360}$ (3.7 mg protein g^{-1}) was lower than that bound covalently to CDAP-Sepharose 4B (5.4 mg protein g^{-1}). It seems that differences in size, structure and amino acid composition between both enzymes, which determine the availability of amino acid residues needed for immobilization, may have contributed to this result. In addition, in the increasing differences in viscosity between gel-filtered and non-gel-filtered BAP solutions due to stabilizers (sugars), the corresponding increase in sugar concentration may restrict the mobility of BAP molecules to the internal surface of $calB_{360,}$ with a consequent decrease in BAP adsorption.

c) *Effect of the enzyme load on the expressed activity of SβA and BAP derivatives*

SβA derivatives. The expressed activity of SβA-$calB_{360}$ derivatives rose from 53 to 92 βU g^{-1} of support as the initial loads increased from 507 to 1,900 βU g^{-1}. For higher SβA loads (up

to 5,973 βU g^{-1} of support), derivative activity diminished gradually (Fig. 3A), although the amount of immobilized SβA remained approximately constant (1,200 βU g^{-1} of support). An excess of enzyme added to the matrix probably resulted in a poor orientation of SβA molecules during immobilization, thus preventing the easy access of substrate to the SβA active site (CAO, 2005a). However, a loss of activity can also be observed for low SβA loads, as proved by the expressed activity of the insoluble biocatalysts, which did not exceed 10.8% of the immobilized activity. Similar recovered activity (10%) was reported by (NODA *et al*., 2001) for sweet potato β-amylase adsorbed onto porous chitosan beads. Immobilization may have affected the three-dimensional structure of SβA, and deformed its active site (RASOULI *et al*., 2016), impeding the access of the substrate.

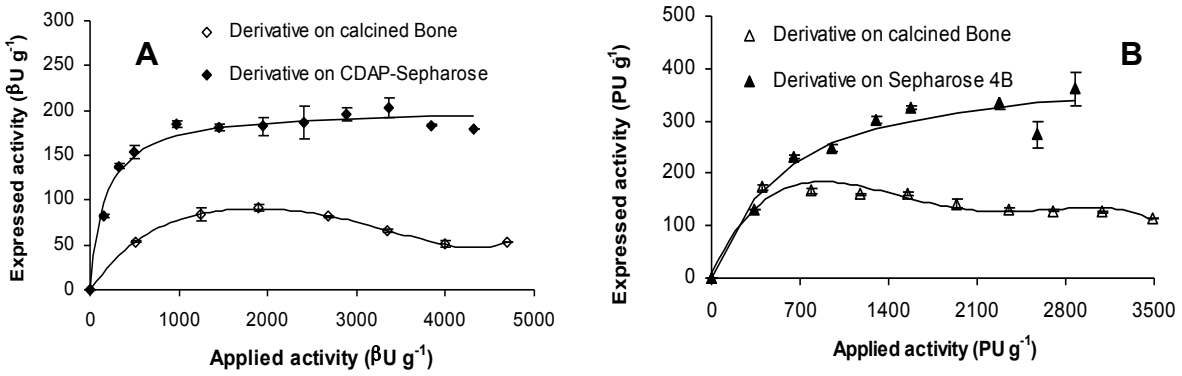

Figure 3. Dependence of the expressed activity of soybean β-amylase (A) and *B. acidopullulyticus* pullulanase (B) derivatives on the applied activity.

The expressed activity of SβA derivatives to CDAP-Sepharose 4B increased from 82 to 185 βU g^{-1} as the SβA load rose from 161 to 974 βU g^{-1}, and then remained relatively constant for loads of up to 3,400 βU g^{-1}, before slowly decreasing for higher loads of up to 4,309 βU g^{-1}, which was the highest load assayed (Fig. 3A) as observed by RASOULI *et al*. (2016) for the immobilization of βA onto $ZnFe_2O_4@SiO_2$-NH_2 nanoparticles. The most active SβA biocatalyst to CDAP-Sepharose 4B expressed 26% of the immobilized activity. This result differs from that obtained by VRETBLAD *et al*. (1973) using the traditional CNBr method for agarose activation, in which inactive biocatalysts were observed. CDAP utilization, in place of CNBr, could prevent enzyme inactivation as reactive groups to CDAP-Sepharose 4B are able to react with proteins under very mild conditions (pH 7) (BATISTA-VIERA *et al*., 2011).

The expressed activity of the optimal SβA-CDAP-Sepharose 4B biocatalyst was twice that of the best SβA-$calB_{360}$ derivative, probably because polymer matrices, having a high degree of hydration, swell markedly and exhibit an enlarged pore size (ATIA *et al*., 2003a). This greatly diminished the diffusion limitations of substrate and products (ROY *et al*., 2004), and may improve the activity shown by this derivative.

From previous studies, optimal conditions for adsorbing SβA on $calB_{360}$ were established as being 20°C and 1,900 βU g^{-1} of support diluted in 0.016 M sodium acetate buffer of pH 4.8 (activity buffer). For covalent binding to CDAP-Sepharose 4B, the optimal enzyme load to be applied was 1,000 βU g^{-1} of suction-dried gel diluted in 0.1 M sodium phosphate buffer of pH 7.0, using 20°C as the immobilization temperature.

3.2.4 BAP derivatives

The effect of applying variable enzyme loads on the expressed activity of BAP derivatives is shown in Fig. 3B. Adsorption onto $calB_{360}$ was carried out under the experimental conditions previously determined for SβA immobilization, using commercial BAP without any treatment, and the coupling process to CDAP-Sepharose 4B was performed at pH 7.0 as described above, using gel-filtered BAP solutions.
The dependence of the expressed activity of BAP and SβA biocatalysts on the initial load applied to CDAP-Sepharose 4B showed similar patterns. Thus, the expressed activity of BAP increased significantly (p-value < 0,05) from 131 to 325 PU g^{-1} of filter-dried gel, as the initial load rose from 332 to 1,570 PU g^{-1}; it then remained relatively constant (between 337 and 361 PU g^{-1}) for higher loads up to 2,875 PU g^{-1}. Similar dependence was reported by DESSOUKI *et al*. (2001) for the expressed activity of *Klebsella pneumoniae* Pullulanase (KPP) immobilized on agarose, activated with epichlorohydrine and triclorotriazine. However, after the initial increment in retained activity with increases in KPP concentration, a decrease in activity was observed showing that the application of very high loads impaired activity of KPP derivatives.
The BAP derivative to CDAP-Sepharose 4B with the highest expressed activity (361 PU g^{-1} of filter-dried gel) was attained using the maximal enzyme load tested (2,875 PU g^{-1} of suction-dried gel). However, immobilized biocatalysts with an average activity equivalent to 90% of the maximum value (325 PU g^{-1} of suction-dried gel) were obtained by applying about half the maximal load (1,572 PU g^{-1} of suction-dried gel). Therefore, for cost-effectiveness, the best initial load would be 1,572 PU g^{-1} of suction-dried gel. The dependence of the expressed activity of BAP-$calB_{360}$ derivatives on the initial load applied followed a different pattern from that observed for CDAP-Sepharose 4B biocatalysts (Fig. 3B). Thus, the lowest enzyme load (400 PU g^{-1}) yielded maximal activity (173 PU g^{-1}), while higher loads (786-3,091 PU g^{-1}) caused it to decrease moderately (from 166 to 126 PU g^{-1}). Although the highest expressed activity of BAP-$calB_{360}$ derivative (173 PU g^{-1}) was almost half (48%) the maximum of the CDAP-Sepharose 4B biocatalyst (361 PU g^{-1} of filter-dried gel), its production required only 400 PU g^{-1} of $calB_{360}$ versus 2,875 PU of gel-filtered BAP solution per gram of suction-dried gel needed to obtain the most active CDAP-Sepharose 4B biocatalyst. In addition, the gel filtration of the enzyme solution increased immobilization costs, thereby favoring $calB_{360}$ in economic terms.

3.3. Properties of free and immobilized soybean β-amylase and Pullulanase

Immobilization often affects properties of free enzymes such as kinetic constants, stability, and optimal pH and temperature, making their assessment necessary for the efficient utilization of insoluble biocatalysts. Table 4 shows the main properties of soluble and immobilized SβA and BAP.

3.3.1 Kinetic constants

Determining the differences in K_M values between native and immobilized enzymes aids understanding of the influence of the microenvironment on enzyme behavior, and facilitates the adjustment of reaction conditions, particularly those related to substrate concentration when this is necessary.
K_M values tend generally to increase through immobilization, as reported by various researchers (DAS *et al*., 2018, TAVANO *et al*., 2013, SRIVASTAVA *et al*., 2014). K_M values of

SβA adsorbed onto $calB_{360,}$ and those of SβA covalently bound to CDAP-Sepharose 4B were observed to increase by 10.5 times and 7.0 times respectively. (TAVANO *et al*., 2013) reported a 15-fold K_M variation for SβA immobilized in glutaraldehyde-agarose. However, RASOULI *et al*. (2016) reported a decrease in K_M by the immobilization of βA to $ZnFe_2O_4@SiO_2$-NH_2 nanoparticles. On the other hand, K_M increments produced after BAP immobilization to CDAP-Sepharose 4B (19-fold) and $calB_{360}$ (850-fold) were higher than those reported by KUSANO *et al*. (1989) and DESSOUKI *et al*. (2001) for pullulanase bound ionically to Amberlite IRC-50 (17-fold), or coupled covalently to agarose activated with epichlorohydrine (7.5-fold) and trichlorotriazine (13-fold).

Table 4. Some characteristics of free and immobilized soybean β-amylase (SβA) and *B. acidopullulyticus* pullulanase (BAP).

Enzyme	$K_M/K_{M\ app}$ (starch[1]/pullulan[2]) (mg mL^{-1})	$V_{máx}$ (mg malt[3]/glu[4] min^{-1} mg^{-1} protein)	Optimal pH	Optimal Temperature (°C)
Free SβA	3.00[1]	13.7	6.0[5]	60
SβA immobilized to CDAP-Sepharose 4B	31.60[1]	2.6	5.7[5]	65
SβA immobilized on calcined bone ($calB_{360}$)	20.70[1]	2.1	3.8[6]	65
Free BAP	0.12[2]	30.3	5.0[5]	60
BAP immobilized to CDAP-Sepharose 4B	2.30[2]	35.3	3.0[5]	55
BAP immobilized on calcined bone ($calB_{360}$)	102.00[2]	11.4	5.6[6]	60

[1]Starch was used for KM or KM app determination; [2]Pullulan was the substrate for KM or KM app determination; 3maltose was the main product of starch hydrolysis; [4]Products of pullulan hydrolysis was expressed as glucosa; [5]Optimal pH was estimated using citrate-phosphate buffer. [6]pH estimation was performed using Gly-HCl buffer.

In general, increments in K_M upon immobilization result from partitioning and/or diffusional effects. In this case, they may be due to conformational changes of enzyme molecules, altering the active site and thus reducing the bonding efficiency with substrates(ATIA et al., 2003b). Additionally, K_M increments for immobilized enzymes are often observed as a consequence of external and/or internal diffusional limitations, particularly when enzymes are immobilized inside porous supports, and are acting on macromolecular substrates, such as starch and pullullan. For both of these enzymes, immobilization to CDAP-Sepharose 4B reduced K_M variations; it is likely that the highly hydrated and porous nature of this matrix enabled the diffusion of substrate to the active site. On the other hand, large K_M increments were observed for SβA and BAP adsorbed onto $calB_{360}$, probably due to the highly ionic character of the calcined bone (mainly hydroxyapatite).

3.3.2 Effect of pH and temperature on the activity of free and immobilized SβA and BAP

Fig. 4 shows the effect of pH on the relative activity of free and immobilized SβA and BAP. The pH activity profiles of the enzymes in both their free form, and after their

immobilization to CDAP-Sepharose 4B were similar. However, the activity of SβA derivative over the pH range 2.6-4.5 was higher than that of the soluble SβA. On the other hand, the pH activity curves for SβA and BAP adsorbed onto $calB_{360}$ were shifted to the acidic side, and their optimum pH value (5.1) decreased by approximately 1 pH unit for SβA-$calB_{360}$ derivative, and 2 pH units for BAP-$calB_{360}$. These variations depend upon the partitioning of protons produced by the presence of ionized groups on the matrix (CAO, 2005a). The pH shift to the acidic side is frequently observed when enzymes are immobilized onto positively charged matrices, as happened for the adsorption of pullulanase on nanoparticles modified with polyethyleneimine (WANG *et al*., 2017). In this case, hydroxyapatite analogue, the main component of calcined bone, has two types of union sites for enzyme attachment: Ca^{2+} ions, and phosphate groups; the utilization of acidic media (acetate buffer of pH 4.8) for SβA and BAP adsorption seems to promote immobilization of these enzymes through the Ca^{2+} moiety. This contrasts with the adsorption mechanism of Ipomoea batata βA onto celite-545 via hydrogen bonds, as reported by KHAN *et al*. (2011), which produced no change in the optimal pH of the immobilized enzyme.

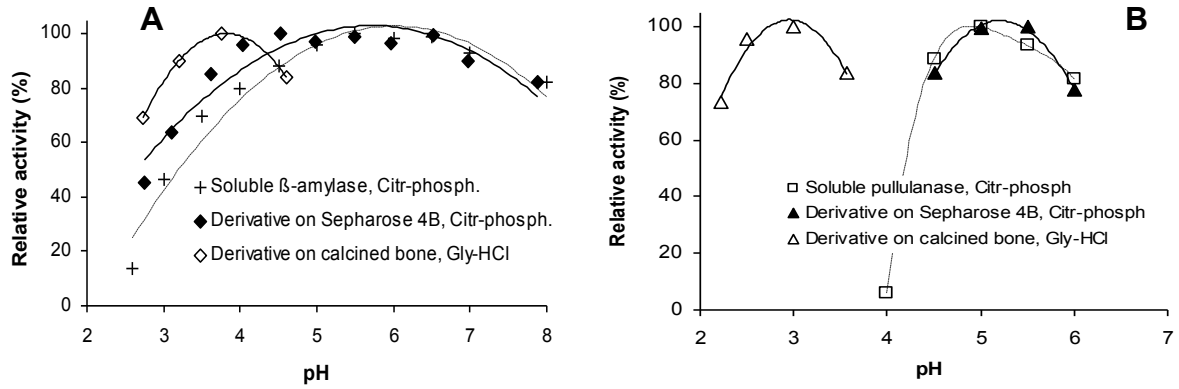

Figure 4. Effect of pH on the relative activity of free and immobilized soybean β–amylase (A) and *B. acidopullulyticus* pullulanase (B) determined at 25°C and 40°C, respectively, using 0.016 M citrate-phosphate buffer for free and covalent immobilized enzymes to CDAP-activated Sepharose 4B, and 0.016 M Glycine–HCl buffer for SβA and BAP adsorbed onto calcined bone ($calB_{360}$).

Maximum activities for SβA immobilized on both supports were within the range of 60–65°C, which is 5°C higher than that of the native enzyme. YOSHIDA *et al*. (1989) found similar variation for SβA immobilized onto chitosan beads. This increment may be due to the establishment of covalent bonds or non-covalent interactions between the enzyme and the supports, which may decrease enzyme flexibility and retard denaturation (ADLERCREUTZ, 1993).

The optimum temperature of BAP-CDAP-Sepharose 4B derivative was marginally higher than that of the native enzyme, while the optimal temperature for the BAP-$calB_{360}$ biocatalyst decreased by approximately 3°C. KUSANO *et al*. (1989) reported similar variations when BAP was immobilized onto porous glass, chitosan beads and Amberlite IIRC-50.

3.3.3 Thermal stability of free and immobilized SβA and BAP

Thermal inactivation of soluble and immobilized SβA and BAP followed the bi-exponential decay model ($R^2 > 0.97$) for a completely deactivated final state E_2 ($\alpha_2 = 0$; Eq. 6). Table 5 shows the corresponding model parameters (k_1, k_2 and α_1) and half-lives ($t_{1/2}$). In the present study, improvement of thermal stability was defined as the increase in $t_{1/2}$ of insoluble biocatalyst in relation to the respective $t_{1/2}$ for the soluble enzyme.

Table 5. Deactivation parameters and half-lives ($t_{1/2}$) of soluble and immobilized soybean β-amylase (SβA) and *B. acidopullulyticus* pullulanase (BAP).

Enzyme	k_1 (hours^{-1})	k_2 (hours^{-1})	α_1 (%)	$t_{1/2}$ (hours)
Free SβA incubated in activity buffer at 55°C	1.18×10^{-1}	2.16×10^{-3}	69.3	159.0
Free SβA, incubated in activity buffer at 60°C	3.77×10^{0}	1.13×10^{-1}	73.1	3.6
SβA immobilized to CDAP Sepharose 4B incubated in activity buffer at 60°C	3.16×10^{-1}	2.19×10^{-2}	66.6	14.9
SβA immobilized onto calcined bone ($calB_{360}$) incubated in activity buffer at 60°C	6.25×10^{-1}	3.41×10^{-2}	52.7	4.7
Free BAP, incubated in activity buffer at 60°C	2.37×10^{0}	2.36×10^{0}	0.0	0.3
BAP immobilized to CDAP-Sepharose 4B incubated in activity buffer at 60°C	3.43×10^{-1}	1.41×10^{-2}	54.8	10.9
BAP immobilized onto calcined bone ($calB_{360}$) incubated in activity buffer at 60°C	2.35×10^{0}	2.34×10^{0}	0.3	0.3

The thermal stability of free SβA at 55°C was very high, as proved by its low inactivation constants k_1 and k_2, and high $t_{1/2}$ (159 hours); therefore, further thermal inactivation tests were performed for soluble and immobilized SβA at 60°C in order to better observe the effect of immobilization in SβA stability.

The resistance of SβA to heat inactivation improved upon immobilization on both matrices (Fig. 5A). However, covalent immobilization to CDAP-Sepharose 4B was 4.1 times more efficient. Results obtained for both supports agree with that reported by TAVANO *et al.* (2013) for the thermostability of sweet potato β-amylase glutaraldehyde-agarose.

Most pullulanases have low thermal stability, and a large portion of their initial activity is lost within a few hours (WANG *et al.*, 2017). Immobilization may improve the thermostability of enzymes: the thermal resistance of soluble BAP was remarkably enhanced (36-fold) as a result of its covalent coupling to CDAP-Sepharose 4B (Table 5), while remaining unchanged after BAP adsorption onto $calB_{360}$ (Fig. 5B).

Studies into the thermal stability of insoluble pullulanases show diverse results, ranging from a reduction to an increment in this property. For instance, the thermostability of *K. pneumoniae* pullulanase (KPP) and BAP immobilized onto chitosan beads by adsorption, or by covalent binding with glutaraldehyde, remained unchanged after immobilization. However, BAP adsorbed onto porous glass (KUSANO *et al.*, 1989), and BAP ionically adsorbed onto Amberlite IRC-50, exhibited a decrease in the former, and a marginally improved stability in free and immobilized forms in the latter (KUROIWA *et al.*, 2005, KUSANO *et al.*, 1989). On the other hand KPP immobilization onto agar gels activated with periodate (KUROIWA *et al.*, 2005), and KPP bound to agarose treated with

epichlorohydryn or activated with trichorotriazine (DESSOUKI *et al*., 2001) improved thermal tolerance.

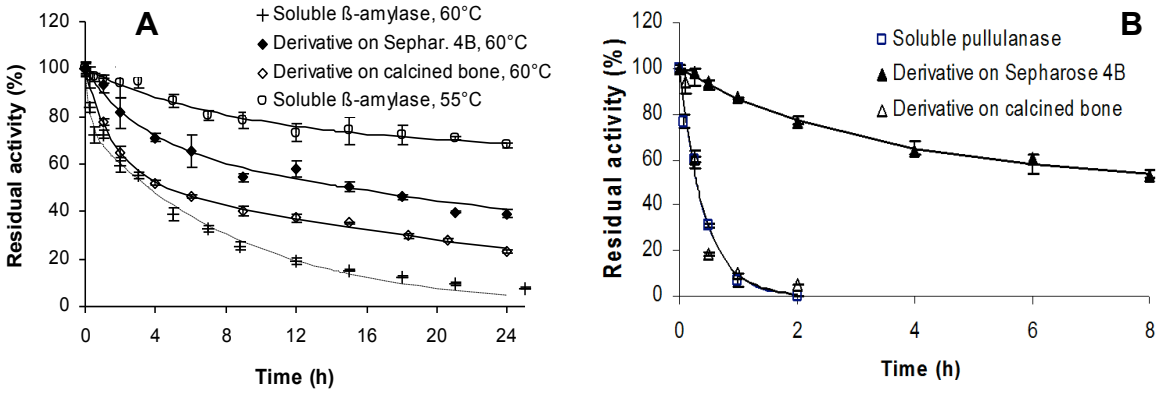

Figure 5. Thermal stability of free and immobilized soybean β-amylase and *B. acidopullulyticus* pullulanase, determined at 60°C for enzyme concentrations of 6.5 βU mL^{-1} and 5.0 PU mL^{-1} of 0.016 M sodium acetate buffer of pH 4.8.

In general, enzymes immobilized by covalent methods become more heat resistant than their soluble counterparts, while most of the enzymes immobilized by adsorption or ionic binding show either no change, or a decreased heat stability (LONG *et al*., 2015). However, it is difficult to predict whether an immobilization method will result in increased thermal resistance, as exemplified by the different results obtained for the thermal stability of GA (CARPIO *et al*., 2009a), SβA and BAP adsorbed onto calcined bone (calB203 or calB360), as well as by the increased resistance to thermal denaturation of BAP and SβA derivatives to CDAP-Sepharose 4B. Greater improvement in thermal stability obtained by covalent immobilization may be due to the strong multipoint attachment of enzyme molecules to the carrier, which reduces their flexibility (ATIA *et al*., 2003b, LONG *et al*., 2015), and produces a certain rigidity of the tertiary structure of enzymes (DAS *et al*., 2018).

3.4. Operational stability of SβA-BAP mixture for the production of high maltose syrups at 55°C

The activity of immobilized-enzyme catalysts decays with time. Therefore, knowing the long term stability of an immobilized system is of paramount importance from a practical point of view, since the main goal of immobilization is enzyme recovery and subsequent reutilization after reaction (HUSAIN, 2017). Operational stability was evaluated in batches by the successive use of the selected SβA-BAP blend of immobilized enzymes, determined by soluble enzymes (Optimal mixture: SβA3 = 48 βU g^{-1} of maltodextrin and BAP2 = 30 PU g^{-1} of maltodextrin).

Operational stability of the SβA-BAP mixture obtained with SβA and BAP derivatives to CDAP-Sepharose 4B was higher than that determined for the blend prepared by adsorbing each enzyme onto $calB_{360}$ (Fig. 6). In the former case, the starch conversion (DE) diminished from 68.5 to 64.2 during the production of 20 batches. The greatest decrease in DE was

observed between batches 1 and 2 for both mixtures. However, the variation for the blend of SβA and BAP derivatives to CDAP-Sepharose 4B (from DE=68.5 to 65) was lower than that produced during the preparation of maltose syrups with the mixture of SβA and BAP biocatalysts onto $calB_{360}$ (from DE 70.8 to 63.6). The conversion attained (DE=63.6) for the second batch remained the same until the seventh batch, and then stayed at 58 for the following four batches (8th-11th); moreover, the final DE in batch 11 did not change even after a reaction time of 48 hours. It is likely that the BAP-$calB_{360}$ derivative was gradually inactivated, and the final DE of 58 would be the result of the hydrolytic activity of SβA, which showed a remarkably high stability at 55°C ($t_{1/2}$=159°hours).

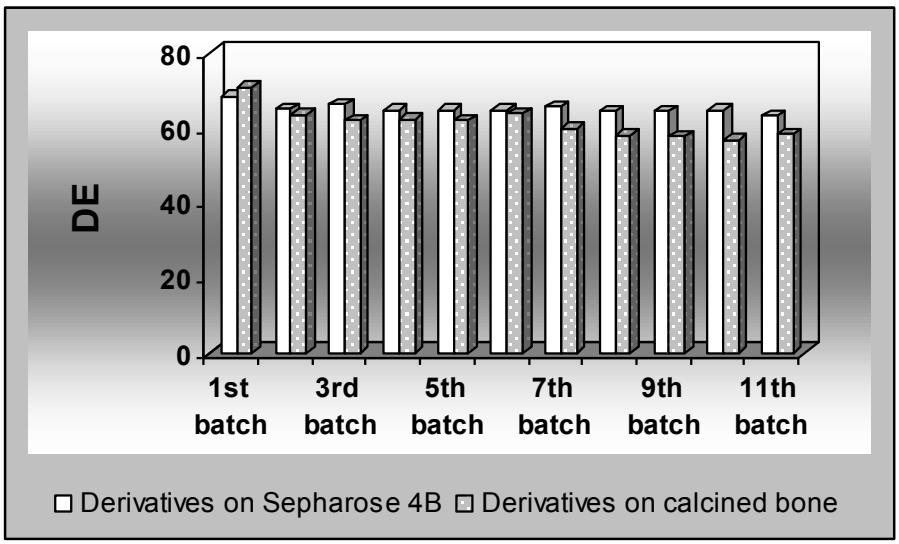

Figure 6. Operational stability of mixtures of soybean β-amylase (48 βU g^{-1} of cassava maltodextrin) and *B. acidopullulyticus* pullulanase (30 PU g^{-1} of cassava maltodextrin) immobilized to CDAP-activated Sepharose 4B and onto calcined bone, determined by batches at laboratory scale at 55°C using a stirred tank reactor.

The results obtained in this study agree with those reported by YOSHIDA *et al.* [33] (1989) and NODA *et al.* [9] (2001), which showed that maltose syrups obtained by the combined action of β-amylase and pullulanase reached higher degrees of conversion (DE) than those syrups lacking pullulanase (control tests). Hence, improved operational stability was obtained by the utilization of the SβA-BAP blend to CDAP-Sepharose 4B, which contained the most stable BAP biocatalyst.

4. CONCLUSIONS

Calcined bone and Sepharose 4B were selected as the most promising matrices among the supports assessed for soybean β-amylase immobilization (chitin, bone, calcined bone, activated control pore silica and Sepharose 4B) based on the expressed activity of their derivatives. From the comparison of the covalent methods for SβA immobilization—formation of Schift bases and isourea linkages—it was determined that processes based on

the establishment of isourea linkages between SβA and CDAP-Sepharose 4B produced the most active derivatives.
The SβA and BAP derivatives with the highest activity were obtained using CDAP-Sepharose 4B. For both enzymes, their expressed activity was twice that of the insoluble biocatalysts onto calcined bone with an average particle size of 360 μm.
Free SβA was very thermostable, and its heat resistance was increased even more by its immobilization to CDAP-Sepharose 4B and calcined bone, while BAP become more heat stable only after covalent immobilization to CDAP-Sepharose 4B. This stabilization was reflected by the high operational stability of the blend SβA-BAP to CDAP-Sepharose 4B for the production of high maltose syrups. However, Sepharose 4B has two important drawbacks compared to calcined bone for commercial BAP immobilization: a) its high price and b) the necessity of enzyme purification (stabilizer elimination) for its binding to CDAP-Sepharose 4B.

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