Original Article

MITIGATION OF CADMIUM CONCENTRATION IN COCOA KERNELS

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Abstract

Cadmium is a toxic metal for plants, animals and man; its presence in the soil can be of anthropogenic or geogenic origin. This study evaluated a method to mitigate cadmium concentration in cocoa beans through a fast fermentation process using the enzyme pectintranseliminase (PTE). The research was carried out in Quevedo-Ecuador with samples acquired by the APROCANE association of the province of Esmeraldas and analyzed in parallel at the State Technical University of Quevedo and ESPOL. The experimental analysis focused on a completely randomized design, with eleven treatments and three replicates. The analysis of variance and separation of means was carried out with Tukey's test at 5%. Fifteen healthy cocoa pods at maturity were used as a sample. After applying the PTE enzyme, pre-drying, washing, drying and recording variables according to each treatment were carried out. The results showed that the cadmium content in the beans was significantly reduced ($p > 0.05$) when PTE was applied. National cocoa showed lower cadmium content in the T6 treatment (1.07) (complete washing and sieve drying). In CCN-51 cocoa, the lowest level of cadmium was observed in treatment T9 (1.51) (complete washing and drying for 24 hours on a cement sieve). Concerning the control, the commercial cocoa showed the lowest levels compared to the other treatments, which could be related to the process to which it was subjected, treatments and remediation of soils contaminated by heavy metals. It should be noted that the use of the PTE enzyme had a positive effect on the physical and sensory quality of the cocoa beans and the mitigation of cadmium in the cocoa beans.

Keywords: pectintranseliminase, enzyme, fermentation, organoleptic.

Introduction

Cocoa is a tree whose origin comes from the humid tropical regions of North, South and Central America. Cocoa is in wide demand worldwide because its seeds are used for the production of chocolate and other products of commercial interest (Lanza et al., 2016b). Cacao is grown in countries such as Ecuador, Peru, Brazil, Mexico, El Salvador and Venezuela. It is estimated that 90% of fine cocoa production is carried out in traditional and semi-technified systems, while the majority of the CCN-51 variety is grown in technified systems (Acebo-Plaza et al., 2016). Despite the promising future of cocoa, heavy metal contamination is a latent concern.

Toxicity and bioaccumulation of heavy metals are some of the emerging global concerns affecting various forms of life, including plants, animals and humans (Suhani et al., 2020).

Heavy metals are persistent and non-biodegradable; moreover, they can accumulate in different trophic levels of the food chain. The accumulation of Cd in food products represents a threat to the second sustainable development goal of the United Nations "Agenda 2030" related to food security (Maddela et al., 2020; Saini & Dhania, 2020). High Cd concentrations in cocoa negatively influence exports to the European market (Nieves et al., 2019).

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Some works to mitigate Cd include increasing zinc and magnesium levels, increasing pH levels by incorporating calcium and magnesium, avoiding the use of phosphate fertilizers and phosphate rock, using nitrogenous and potassium fertilizers, use of activated carbon by residual biomass of oil palm or cocoa (Soto et al., 2017) inoculation with beneficial bacteria (Aloui et al., 2011; Fite Duressa & Leta, 2015). In this sense, the study on the use of the enzyme pectintranseliminase in the fermentation of cocoa kernels is taken into account for this research due to its effective properties in separating the different components in the cell wall of the plants. It is through this basic principle that the use of this enzyme is proposed to induce the separation of heavy metals; the combination of this enzyme and other components can easily achieve the complete liquefaction process, which is the degradation of the cell wall. Based on the above, the main focus of this work is to decrease the Cd content in cocoa beans by applying proteolytic enzymes (specifically PTE).

MATERIALS AND METHODS

This research was carried out in Quevedo-Ecuador. The Association of Cocoa Producers of Northern Esmeraldas (APROCANE) provided the cocoa cobs with UTM coordinates of X:732642.56 - Y:118276.86 and hermetically transferred them to the soil and plant nutrition laboratory of the Escuela Superior Politécnica del Litoral (ESPOL) for the analysis of Cd content. The physicochemical analyses were carried out at the chemistry laboratory of the Universidad Técnica Estatal de Quevedo (UTEQ) - Campus La María, Ecuador. The province of Esmeraldas has an altitude of more than 3000 m above sea level and is considered to have two different climates: tropical monsoon and tropical humid, with annual rainfall ranging from 500 mm to 700 mm dry, 2000 mm humid and 7000 mm very humid. The average temperature ranges from 21 ºC to 25 ºC.

Experimental Design

The experiment was carried out using two techniques: 1. Analysis of variance and separation of means.

The analysis of variance identified the effects of the two genotypes and 11 postharvest treatments, combined in orthogonal arrangement, in a randomized design with three replications, according to the ADEVA scheme. Orthogonal contrasts identified the partial effects of the factors. 2. Regular analysis according to Taguchi methods. According to Taguchi's methods, regular analysis is one of the quality engineering techniques aimed at improving processes and products. Its approach allows the design of a product or process to determine the optimum levels of factors that enable the best performance of one or more factors. The technique is a multi-stage process that follows a certain sequence of experiments to improve understanding of the product or process. The orthogonal arrangements used are a highly fractional factorial design, on which an ANOVA is used as a mathematical analysis technique, which is based on least squares and allows understanding the influence of each factor on the response. The regular analysis involves four steps: a) Elaboration of the response table, b) Identification of the optimal combination of factors and levels, c) Elaboration of the factorial graphs, and d) Prediction of the maximum response.

Experimental Unit

The cocoa sample consisted of 25 healthy cocoa pods at optimum field maturity. The dry cocoa sample corresponded to the quantity and weight derived from the postharvest process. A total of 66 experimental units were used (2 genotypes x 3 replications x 11 treatments) with 1,650 cocoa pods. In order to make comparisons, the commercial control was included, whose material was obtained from direct sales to producers in the area (Viche Parish, Esmeraldas canton). The treatments are detailed in Table 1.

Cutting Test

The cutting test was performed on the cocoa samples cut longitudinally in two parts to expose the cotyledon. Each sample individually was classified according to its color and the texture of the exposed surface as in the cutting test according to international standards adapted to the following standards (Oliveira et al., 2021).

The variables analyzed after the fermentation and cutting process were: fermentation percentage (well fermented, violet and slaty grains), hydrogen potential (pH), and acidity.

Table 1. Treatments applied to evaluate the reduction of cadmium levels in cocoa using the enzyme pectintranseliminase (PTE).

Treatments	Code	Description
1	A1B1C1D1	PTE 0.10 mL/kg of cocoa in slurry, without washing, without resting in pre-drying and drying on cement pallet.
2	A1B2C2D2	PTE 0.10 mL/kg of cocoa in slurry, with incomplete washing, resting in pre-drying for 24 hours and drying in a canopy.
3	A1B3C3D3	PTE 0.10 mL/kg of cocoa beans in slurry, with complete washing, pre-drying for 48 hours and drying on a splint.
4	A2B1C2D3	PTE 0.20 mL/kg of cocoa in slurry, without washing, with pre-drying for 24 hours and drying on a splint.

5	A2B2C3D1	PTE 0.20 mL/kg of cocoa in slurry, with incomplete washing, resting in pre-drying for 48 hours and drying in cement drying rack.
6	A2B3C1D2	PTE 0.20 mL/kg of cocoa in slurry, with complete washing, without resting in pre-drying and drying in canopy.
7	A3B1C3D2	PTE 0.30 mL/kg of cocoa beans in slurry, unwashed, with 48 hours of pre-drying and drying in a canopy.
8	A3B2C1D3	PTE 0.30 mL/kg of cocoa in slurry, with incomplete washing, without resting in pre-drying and drying in splinting.
9	A3B3C2D1	PTE 0.30 mL/kg of cocoa in slurry, with complete washing, resting in pre-drying for 24 hours and drying in a cement line.
10	Mechanical demixing (ReMe)	Mechanical removal of mucilage, fermentation and drying, according to local process.
11	Commercial Fermentation and Drying (TLP)	Commercial Witness. Local process carried out by farmers in the area.

Note: Code A: Dosage of the enzyme product B: Washing of the fermented cocoa, C: Resting in the tank (Pre-drying), D: Place of cocoa drying, PE enzyme product

Cadmium Analysis

Cd levels were analyzed in the soil and plant nutrition laboratory of ESPOL by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Jiménez Heinert et al., 2020). The pH was determined according to the procedure described in AOAC standard (Al-mentafji, 2005) 970.21. The pH was determined with an OHAUS-Starter 3100 pH meter. The titratable acidity was measured with reference to AOAC (Al-mentafji, 2005) 942.15. The analyses were performed in triplicate on each sample and the results were expressed in ppm.

Sensory Quality Evaluation

In order to obtain a sufficient number of samples to allow the panelists to perform the sensory evaluation in a single session, the samples were grouped using multivariate analysis, in which the sense of taste and smell were used to derive the attributes of flavor and aromas (Vázquez-Ovando et al., 2015). To establish the quality analysis, a small amount of cocoa liquor was taken with the help of a wooden paddle, the same that was placed evenly on the tongue; the tasting was executed individually and before continuing with the following sample was waited for a couple of minutes to lose the flavors of the previous sample, for the evaluation of the taste was used the category scale test method, scale C (Colombian Technical Standard - NTC 3929, 2009) (Vallejo-Torres et al., 2018a).

Statistical Analysis

Descriptive statistics, central tendencies and measures of dispersion, Pearson correlation and multivariate analysis were applied (Esezi Isaac & Eric Chikweru, 2018; Ghasemi-Goojani & Behroozi, 2012). Analysis of variance was used to identify the effects of the two genotypes and 11 postharvest treatments, combined in orthogonal arrangement, in a Randomized Design with three replications, according to the ADEVA scheme. Orthogonal contrasts identified the partial effects of the factors.

Results and discussion

Fermentation of Cocoa Almonds

The analysis showed statistical differences at $p \leq 0.05$ in National cocoa, with the T3 treatment obtaining the highest average in the variable well-fermented almonds with 67.33%, 10.33 of violet almonds and 8.33 of slaty almonds, which would be within limits established by INEN Standard 176. (Ecuadorian Institute of Standardization, 2006). According to the technical standard, the parameters are 65% well-fermented almonds, 15% violet and 9% slaty for fine aroma cocoa of the National ASSS Arriba Superior Summer Selecto (ASSS) variety. On the other hand, the treatment with the lowest statistical values in well-fermented almonds was the commercial control T11 which was used for comparative purposes with 3% and also exceeded the quality of cocoa in violet with 54.33% although, an optimal level in slaty 10.33% Table 2.

In the case of CCN51 cocoa, the T5 treatment shows a statistical difference at $p \leq 0.05$, with 76% of well-fermented almonds, 5% of the violet and 3% of slate; therefore, the T5 treatment maintains acceptable ranges in both violet and slate with low levels, as well as with T4, which maintains its fermentation quality in well-fermented Table 2. The lowest value in the quality of fermentation of cocoa CCN%! was the control T11 in well fermented, which showed a 5% in their results, as well as the highest value in violet and slate; this shows that their processes and treatments are of dubious origin. These results allow us to determine the positive effect of enzyme application, methods, time and type of drying on fermentation for both National cocoa and CCN51. On the other hand, the results of treatment 10 in mucilage removal and local drying process did not show optimal fermentation values but low values in violet and slate.

In this study, the best percentage of fermentation occurred for both Nacional and CCN51 cocoa in treatments T3 and T5, respectively, at 48 hours. The results are comparable to those obtained by (Andrade et al., 2019) for the Ecuadorian varietals in Nacional cocoa with a 77.66 with 72 hours of fermentation; and CCN 51 cocoa with 58.86, with 144 hours of fermentation.

Table 2. Physical analysis of Cacao Nacional and CCN-51 kernels.

		NATIONAL COCOA Fermentation Percentage			CACAO CCN51 Fermentation Percentage		
Source Variation	**n**	**Well Fermented**	**Violet**	**Slaty**	**Well fermented**	**Violet**	**Slates**
T1	3	55 e	9 ab	0,29	13,67 a	14.67 cd	9 ab
T2	3	33,33 c	22 cd	25,67 e	61,33 e	7 ab	8.67 ab
T3	3	67,33 f	10.33 abc	8.33 ab	48,33 d	2,67 a	9.67 ab
T4	3	37 c	17.67 bcd	13 abc	52 of	2,67 a	2,67 a
T5	3	49 of	4,67 a	0,29	76 f	5 a	3 a
T6	3	4,33 a	24,67 d	21.67 of	8,67 a	19 d	6.33 ab
T7	3	36,33 c	22 cd	4,67 a	43 cd	7.33 ab	12,67 b
T8	3	20,67 b	22 cd	19 cde	51.33 of	4,67 a	8.67 ab
T9	3	40.67 cd	11.67 abc	16.67 bcd	32,33 b	12.67 bcd	9 ab
T10	3	15,67 b	25 d	16.67 bcd	36 bc	8 abc	8 ab
T11	3	3 a	54,33 e	10.33 ab	5 a	35,33 e	28 c
p-value		**0,0001	**0,0001	**0,0001	**0,0001	**0,0001	**0,0001
C.V. %		9,11	21,47	27,72	9,11	21,47	27,72

*Significant, ** Highly significant, Coefficient of variation (C.V.), probability (P), number of replicates (n).

Potential of Hydrogen (pH) and Acidity

In the different treatments, it was possible to observe a more significant variation in pH than in acidity in both Nacional and CCN51 Table 5. This is because the acidification of beans allows a series of enzymatic reactions associated with chocolate's final characteristics(Loureiro et al., 2016). According to Amorim Homem de Abreu Loureiro et al. (2017), the optimum pH for quality cocoa should be between 5.0 and 5.4, so a pH lower than 5.0 indicates the presence of volatile acids that are undesirable for the development of cocoa aroma and flavor. The results of this research show that the value close to the optimum was in T10; the other treatments fluctuated between 5.85 and 6.67, indicating a direct incidence with the application of the enzyme. Therefore, pH lower than 5.0 indicates the presence of undesirable volatile acids for the development of the aroma and flavor of cocoa. (Loureiro et al., 2016)..

The values observed in Nacional and CCN51 cocoa fluctuated between 0.03 and 0.06 (Table 3). Excess acidity can be detrimental to the final quality of cocoa beans, while lower total acidity values are related to cocoa quality. The cocoa pulp can be classified as a high acid product ($pH < 4.5$). Therefore, controlling the pH and total acidity of cocoa mucilage are essential factors to consider during the different postharvest stages of cocoa (Amorim Homem de Abreu Loureiro et al., 2017a). High acidity reactions in fermentation can influence the final quality of cocoa beans, compromising chocolate aroma (Loureiro et al., 2016). The pH values were similar to those of Alvarez et al. (2010). Those who obtained an intermediate pH (5.20- 5.49) and lower than those who obtained a total acidity of fermented cocoa beans had average values of 0.88 meq NaOH.

Table 3. Results of chemical parameters in the samples.

	NATIONAL COCOA		CACAO CCN-51	
TREATMENTS	**PH**	**ACIDITY**	**PH**	**ACIDITY**
1	6.15 cd	0,06 b	6,42 e	0,03 a
2	6,67 f	0.04 ab	6,87 f	0.03 ab
3	6,60 f	0,04 a	6,86 f	0.03 ab
4	6,34 e	0,03 a	5,92d	0.03 ab
5	6.31 of	0.04 ab	4,75 a	0.03 ab
6	6,09 c	0,03 a	6,83 f	0.03 ab

7	5,89 b	0,03 a	5,72 c	0,03 a
8	6.15 cd	0,03 a	5,67 c	0.03 ab
9	5,85 b	0,03 a	5,78 c	0.03 ab
10	5,43 a	0,06 b	5,15 b	0,04 b
11	5,74 b	0,03 a	5,71 c	0.03 ab
p-value	**0,0001	0,0001	**0,0001	0,0073
C.V. %	1,01	19,06	0,74	9,42

*Significant, ** Highly significant, Coefficient of variation (C.V.), probability (P), number of replicates (n).

Cadmium content in cocoa

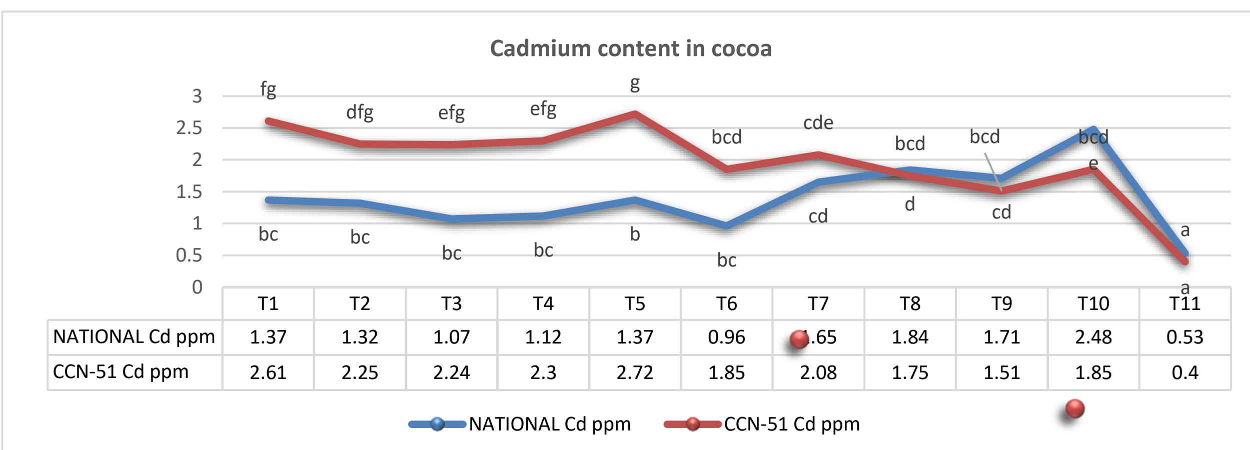
The Cd content in cocoa beans denoted results with statistical differences at $p \leq 0.05$, as shown in Figure 1. The treatments in which the enzyme was applied with the best average were T6 for Nacional cocoa and T9 for CCN51 cocoa Figure 1. The results of the control T11 had a lower incidence of Cd for both Cacao Nacional and CCN-51 and are statistically recommendable. However, the samples were only used for comparison, so they could prevent different farms whose fermentation processes and soil remediation treatment processes are unknown.

Treatments T1, T2 and T5 for National cocoa showed intervals above the critical levels permissible by regulatory agencies (1 ppm). The results of this research are similar to those reported by (Lanza et al., 2016a), who observed ranges between 0.95 and 2.09 mg/kg of cadmium in cocoa. On the other hand, treatment T8 obtained a higher numerical value of Cd concentrations, which implies that T8 was not efficient in the removal of Cd, which opens the possibility of modifying the treatment and the optimal dosage, where complete washing could be an option as well as the fermentation method.

CCN51 cocoa beans at T1, T2, T3, T4, T5, T6, T7, T8 resulted in toxic levels outside the established standards. Biotic and abiotic phenomena can explain the high Cd concentrations of both National and CCN-51 coco; the level of Cd found in traditional systems could be due to the natural concentration in the soil or by the application of phosphate rock and other phosphorus inputs for the nutrition of their crops used by farmers, most of them are of inorganic origin which could cause changes in the properties of the soil (Lopez et al., 2018a).

The concentrations of Cd in the soil in the province of Esmeraldas are currently inaccurate and their remediation treatments of soils contaminated by heavy metals are unknown; however, Assa et al. (2018) state that in Ecuador, they found levels of Cd accumulation in the surface soil which is caused by anthropogenic activities, the sampling sites had a range of Cd in grains that exceeded 0.6 mg/kg, the maximum critical level of Cd established by the European Union. It is also corroborated that the average concentration of cadmium exceeds the limits established by the Codex Alimentarius (2.0 mg/kg) and the European Union (0.8 mg/kg) (Echeverry & Reyes, 2016).

Figure 1. Cadmium content in cocoa National and CCN51



The results obtained for cadmium content in cocoa are lower than those obtained by (Lopez et al., 2018b). However, the regional level in southern Latin American countries also shows that the content of cocoa beans has 2.1 times more Cd in relation to the Cd available in the soil, which demonstrates the bioaccumulation capacity of the cocoa beans (Florida Rofner, 2021).

Biotic and abiotic phenomena can explain the high concentrations of Cd in both National cocoa and CCN-51; the level of cadmium found in the traditional system could be due to the natural concentration in the soil, also farmers apply phosphate rock and other phosphorus inputs for the nutrition of their crops, without taking into account that these inputs contain high amounts of metals mainly cadmium (Lopez et al., 2018a).

Sensory Characteristics of National Cocoa and CCN51

In the cocoa sensory traits, there was a significant correlation between the variables Well fermented and Violet (r= 0.75) and Violet and nut (r= -0.75); however, it was observed that there was a strong correlation between bitter and nut (r= 0.94), similarly but negatively with Well fermented and Nut (r= -0.94) and with significant differences between Slightly violet and Nut (r= -0.86); however, those with the weakest correlation were Slightly violet with Cd and pH with violet (r= 0.27) showing significant differences between the correlation and negatively with pH and Violet (r= -0.26).

Multivariate Analysis of Cocoa Sensory Characteristics

The results of the organoleptic traits allowed determining specific flavors such as astringent, bitter, cocoa, fruity and dark coffee Figure 2; for example, the T7 treatment of Cacao Nacional shows a cocoa flavor reflected in high intensities. On the other hand, the astringent, fruity and bitter flavors remained at low to medium profiles. These results are similar to those reported by Vallejo-Torres et al. (2018b), such as cocoa flavor with values per treatment between 7.44 and 6.59 and bitter flavor with values between 4.15 and 4.61.

Treatment T7 and T10 were qualified as the best cocoa for their natural flavors of bitter cocoa and their physical and sensory characteristic of dark brown color, with certain similarities with significant differences in the bitter flavor T7 (8.67) and T10 (9), cocoa flavor T7 (9) T10 (7.67), dark coffee T7 (8) and T10 (9). The bitter taste T1, T5 and T6 present the same sensory and organoleptic levels with high tastes of (7.67), and T7 and T8 with (8.67) respectively, T3 and T4 both with mean averages of 5.33, which shows the low bitter taste.

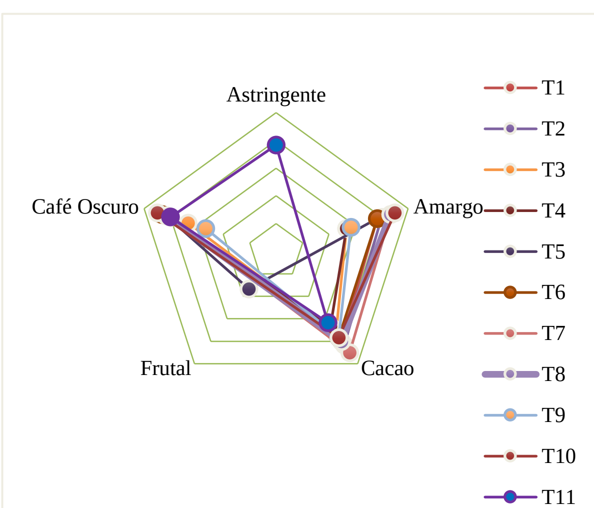
The samples with cocoa flavor showed equality in T1 and T8 with higher averages of (8) and an average similarity with T2 (8.33); this shows a high existence of bitterness in the samples. In addition to this, it was recorded that T5 with value (3.33) indicates that it presents a low vigor to fruity flavor. In addition, it is shown that the control T11 shows high astringency values of 7.67.

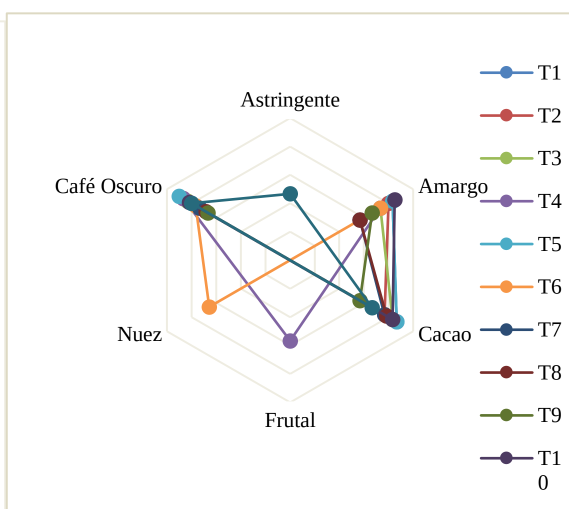
The sensory profiles observed in Figure 6 of CCN-51 Cocoa, both astringent, bitter, cocoa, cocoa, fruity, nutty, and dark brown, denote that T5 shows high intensity in cocoa and bitter flavor and high intensity in dark brown color. Similar results were reported by Moreno-Miranda et al. (2020) in CCN 51 cocoa, who expressed that this material exhibits a moderate amount of cocoa flavor and high levels of bitterness and astringency, consistent with the profile found in this research.

The treatments T5 (8.33), T1 (8.33) and T10 (8.50) were cataloged with a high value for their balanced bitter flavors, having a particular statistical difference with T2 with a percentage of (8,0) and with similar percentages with T1 (8.67), T3 (8.33), T5 (8.67) and T10 (8.33), and its dark brown color T1 (8.0), T2 (8.67), T3 (8.33), T4 (8.67), T10 (8.17), T11 (8.0) which show optimal colors typical of cocoa.

The bitter taste also shows that T3, T4 and T6 with mean values, equal to (7.33), which shows that there is a brief intensity of bitter taste, cocoa flavor with similar values is T2, T7 with (7.67) and T8 (7.83), and dark brown color with similarities of T6 (7.67) and T7 (7.33). The bitter taste with average numerical values is found in T9 and the control T11 (6.67) with equal ranges of flavors, the dark brown color with intervals of (6.83) and (6.67) in T8 and T9, also presenting values (4.67) which shows intensities of astringency, fruity flavor in T4 (5.67) which shows the certain intensity of fruity notes, as well as T6 which presents certain nutty notes. These results are consistent with those reported by Solórzano Chavez et al. (2015), who indicate that cocoa flavor has a great variation in the profiles of (acidity and bitterness); in addition, the sensory profiles of cocoa are unique in their behavior in the different zones of Ecuador (Lopez et al., 2018b).

Figure 2. Sensory analysis of National Cocoa. Figure 3. Sensory analysis of CCN-51 Cocoa





Conclusions

The fermentation processes and sensory characteristics of cocoa beans were favored by the use of the PTE enzyme, which significantly reduced the levels of Cd in the cocoa bean in the postharvest process, both for Cacao Nacional in the T6 treatment and CCN-51 in the T9 treatment. In addition, the physical and sensory quality of cocoa was favored by reducing the levels of cadmium. The reduction of Cd in cocoa complies with the standards allowed by international regulatory agencies for the marketing and consumption of cocoa.

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