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QUALITY RANGE FOR FREE AMINO ACIDS OF CACAO BEANS

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The cocoa and derivatives industries use pH as a biochemical parameter to verify the samples quality and infer their potential for flavour and aroma, without resorting to complex analytical methods. For this reason, we sought to determine a pH range in dry cacao beans (DCB) whose free amino acids (FAAs) response was associated with satisfactory values of the fermentation index (FI). Graphical and statistical techniques were used to project classifying elliptical clustering of FI values onto scatterplots. The overall pH range of 5.79 to 6.05 predicts FAAs contents of DCB with high fermentative quality. The Tyrosine content range from 930.20 to 1252.05 mg kg^{-1} fat free dry material was associated with this pH prediction, being a potential indicator of fermentative quality.

Key words: *Theobroma cacao* L., cacao quality, fermentation process, biochemical prediction, elliptical clustering technique.

Faixa de qualidade de aminoácidos livres de amêndoas de cacau. As indústrias cacau e derivados utilizam o pH como parâmetro bioquímico para verificar a qualidade das amostras e inferir seu potencial em sabor e aroma, sem recorrer a métodos analíticos complexos. Por esse motivo, buscou-se determinar uma faixa de pH em amêndoas de cacau secas (ACS) cuja resposta aos aminoácidos livres (AAL) estivesse associada a valores satisfatórios do índice de fermentação (IF). Técnicas gráficas e estatísticas foram usadas para projetar agrupamentos elípticos classificatórios de valores de IF em gráficos de dispersão. A faixa geral de pH de 5,79 a 6,05 prevê o conteúdo de AAL de ACS com alta qualidade fermentativa. A faixa de conteúdo de tirosina de 930,20 a 1252,05 mg kg^{-1} do material seco isento de gordura foi associada a essa predição de pH, sendo um potencial indicador da qualidade fermentativa.

Palavras-chave: *Theobroma cacao* L., qualidade do cacau, processo de fermentação, predição bioquímica, técnica de agrupamento elíptico.

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Introduction

Dry and well-fermented beans of cacao (*Theobroma cacao* L.) give unique characteristics to the chocolate flavour and aroma through a profile of highlighted biochemical attributes such as free amino acids (FAAs) (Araujo et al., 2021; Brunetto et al., 2020; Deus et al. 2020; Hinneh et al., 2018; Marseglia et al., 2014; Pätzold and Brückner, 2006; Tchouatcheu et al., 2019). Meanwhile, according to Biehl et al. (1985), the high flavour potential does not depend on the maximum release of amino acids or peptides. It is not the amount of FAAs, but the mixture of hydrophobic FAAs and hydrophilic oligopeptides that compounded the typical flavour and aroma of cacao (Rohan and Stewart, 1967; Voigt et al., 1994). Some investigations have tried to reduce the distance between farmers and chocolate manufacturers by determining quality standards through the FAAs characterization of dry cacao beans (DCB) of different origins and genotypes (Araujo et al., 2021; Brunetto et al., 2020; Hinneh et al., 2018; Marseglia et al., 2014; Tchouatcheu et al., 2019). However, the cacao that reaches the industry is often not sufficiently characterized in biochemical terms. For this reason, the cocoa and derivatives manufacturing industry has used simple parameters such as pH to infer cacao quality (Voigt and Biehl, 1995), because it reflects retrospective fermentation and drying processes under ideal conditions in biochemical attributes (Biehl et al., 1985; Ilangantileke et al., 1991; Melo et al., 2021). Therefore, the determination of the pH range in which the FAAs contents of a cacao genotype are associated with a good fermentation condition is very useful information for the industry.

The acidic condition associated with the low pH in the fermentation process allows proteins to be hydrolysed by aspartic endoprotease and carboxypeptidase generating FAAs and peptides (Biehl et al., 1993; Voigt et al., 1994). There is a diversity of FAAs found in DCB (Araujo et al., 2021; Brunetto et al., 2020; Deus et al., 2020; Hinneh et al., 2018; Marseglia et al., 2014; Pätzold and Brückner, 2006; Tchouatcheu et al., 2019) and these can be classified biochemically according to their specific side chain (R group): 1) aliphatic - glycine (Gly), alaline (Ala), valine (Val), leucine (Leu) , isoleucine (Ile), cyclic aliphatic-proline (Pro), and, aliphatic and sulfur-containing-methionine (Met); 2) aromatic-phenylalanine (Phe), tyrosine (Tyr), and, tryptophan (Trp); 3) polar neutral - serine (Ser) and threonine (Thr); 4) amide-asparagine (Asn) and glutamine (Gln); 5) cationic - histidine (His), lysine (Lys), and arginine (Arg); 6) anionic - aspartic acid (Asp) and glutamic acid (Glu). Other non-protein amino acids are also described, such as ã-aminobutyric acid (GABA) (Araujo et al., 2021; Marseglia et al., 2014; Pätzold and Brückner, 2006; Tchouatcheu et al., 2019), citrulline (Cit) and Ornithine (Orn) (Hinneh et al., 2018). Tchouatcheu et al. (2019) have reported an FAA content of 10563.27 (± 2705.162) mg kg^{-1} of fat free dry material (FFDM) in well-fermented dry cacao beans obtained from the identification of "brown colour" using the cut test. Ilangantileke et al. (1991) have observed that the highest values of the fermentation index (FI) (approximately 1.2-1.4) are associated with the "fully brown" colour category. Ilangantileke et al. (1991) have also described an inverse relationship between pH and FI, and direct relations between FAAs contents and FI. The total acidity (TA) or titratable and the FI show a direct relationship with the increase in hours of the fermentation process (Melo et al., 2021). Therefore, pH and FI can indicate the fermentative quality of cacao beans and the FAAs contents that reflect this condition.

FI is a variable measured at the end of the fermentation process (Loureiro et al., 2017; Melo et al., 2021). In DCB, the FI cannot be determined, leaving only the pH or the total acidity as variables capable of predicting the degree of fermentation from the sample observations. The boundary line technique can be useful for obtaining regressions (Walworth et al., 1986). Graphical techniques to obtain class intervals for the x and y- variables can facilitate the selection of boundary regression points (Armstrong et al., 2003; Bivand et al., 2020; ESRI, 2022; Smith, 1986). In turn, the FI values can be used as a categorical variable to project an elliptic cluster with a multivariate normal distribution based on a correlation matrix (Murdoch et al., 2022; Wickham et al., 2020) in the two-dimensional space of the response of the FAAs as function to pH. Therefore, the aim of this study was to determine the pH ranges whose response of the FAAs is related to the highest FI values.

Materials and Methods

Cacao origin, sampling, and post-harvest processes

The materials used consisted of fermented and dry cacao beans from the clone Porto Híbrido 16 (PH-16) from 12 cropping sites in the southeastern region of Bahia, as described by Araujo et al. (2021). The original sample is composed of 36 observations from a completely randomized design, with three replications per cropping site. Fruit collections were carried out in November 2008, during the second annual harvest. The fermentation processes of 168 h (7 days) and the drying of 192 h (8 days) are fully related by Loureiro et al. (2016, 2017).

Fermentation index

The method adapted from Gourieva and Tserevitinov (1979) was used to determine the FI. Samples of 0.5 g of milled fermented cacao beans (FCB) were homogenized with 50 ml of a mixture of CH_4O (methanol) and HCl (hydrochloric acid) in a 97:3 volume ratio. The homogenate was left in a refrigerator at 8°C for 20 hours and then vacuum filtered. The FI of the three replicates of each sample was obtained by calculating the absorbance at 460 nm to absorbance at 530 nm in the spectrophotometer (FEMTO® 600 plus, FEMTO, Brazil).

Moisture, pH and total acidity

The pH and TA of the fermented and cacao beans were determined by methods 970.21-1974 (AOAC International, 2005d) and 942.15-1965 of the Association of Official Analytical Collaboration (AOAC) International Official (AOAC International, 2005c), respectively. The moisture content of DCB was determined according to the AOAC International Official Method 931.04 (2005b).

Protein and free amino acids

Total protein content in DCB was estimated based on the conversion factor was 6.25 of the total nitrogen, which was determined using micro-Kjeldahl assay according to the method 31.1.08 (AOAC International, 2005a). The steps for the analysis and determination of free amino acid contents in DCB by High Performance Liquid Chromatography were described by Araujo et al. (2021).

Data analysis

The statistical procedures used in this study were performed using the R software (R Development Core Team 2021). The samples of each variable were submitted to the Shapiro-Wilks normality test at 5% significance. Outliers were analyzed graphically.

The graphic analysis was performed by classifying continuous variables using intervals generated with an equivalent proportion of the sample standard deviation (ESRI, 2022). Four intervals were determined. The goodness of fit of variance (GVF) and the tabular precision index (TAI) described by Armstrong et al. (2003) were used to assess class intervals through the "classInt" package (Bivand et al., 2020). The extreme points in the scatter plot between the response variable (y-axis) and the independent variable (x-axis) were used to construct the boundary lines (Walworth et al., 1986) of polynomial regressions.

The graphical functions "stat_ellipse" from the "ggplot2" package (Wickham et al., 2020) and "ellipse" from the "ellipse" package (Murdoch et al., 2022) were used to obtain the ellipse area in the scatterplots, assuming a multivariate normal distribution, calculated from the correlation matrix. The "scale" parameter corresponds to the standard deviations and the "centre" to the means of the subgroup of samples represented by the ellipse. Was used a confidence at the 0.95 level of a 95% pairwise confidence region (Murdoch et al., 2022).

Results and Discussion

It was found that 83.33% of the dataset variables have a normal distribution by the Shapiro-Wilks test at 5% significance level (Table 1). Variables pH and TA of the FCB, and moisture, pH, and Lys of the DCB did not show a normal distribution (Table 1). These deviations from normality occur mainly due to the reduction of observations in the sample. Ten observations of the original sample were eliminated according to the following three criteria: 1) missing values of FAAs detection; 2) FI outliers; and 3) extreme outliers clearly related to analytical errors. Due to the low number of observations in the sample ($n = 26$), some outliers outside the interquartile range were not eliminated (Figures 1-5), as they are not outliers from the analytical point of view for FCB or DCB.

Table 1. Sample analysis of biochemical attributes of cacao beans of the Porto Hibrido 16 (PH-16) clone cropping in the southeastern region of Bahia, Brazil

Type	Variable (Abbreviation or acronym, unit)	S-W test[1]		Outliers' detection	Descriptive statistics				Indexes[2]		Scatterplot analysis					
											FI quality range (ellipse cluster)					
											x limits			*y* limits		
		W	p-value	(boxplot)	Minimum	Maximum	Average	Standard deviation	GVF[3]	TAI[4]	*x* variable	*x* min	*x* max	*y* variable	*y* min	*y* max
FCB[5]	Potential of hydrogen (pH_r, dimensionless)	0.92	0.03	1	4.13	5.56	4.64	0.34	0.94	0.70	pH_r	4.25	5.01	FI	0.92	1.23
	Total acidity (TA_r, meq NaOH 100g^{-1})	0.88	<0.01	2	13.40	31.01	25.30	4.38	0.96	0.77	TA_r	23.98	26.49	FI	0.92	1.23
	Fermentation index (FI, dimensionless)	0.95	0.27	0	0.33	1.19	0.75	0.25	0.94	0.76	pH_r	4.49	4.77	TAf	23.98	26.48
	Moisture (Moi, g 100 g^{-1})	0.92	0.04	0	5.47	7.90	7.12	0.64	NA	NA	NA	NA	NA	NA	NA	NA
	Potential of hydrogen (pH_d, dimensionless)	0.86	<0.01	3	5.82	6.57	6.05	0.19	0.94	0.72	pH_d	5.79	6.05	TA_d	11.56	17.30
	Total acidity (TA_d, meq NaOH 100g^{-1})	0.95	0.28	1	10.53	19.24	14.40	2.27	0.90	0.65						
	Total protein (g kg^{-1})	0.97	0.58	0	135.74	199.55	163.12	16.19	NA	NA	NA	NA	NA	NA	NA	NA
	Total free amino acids (mg kg^{-1} FFDM[7])	0.97	0.61	0	10980.40	17276.53	13888.92	1754.92	NA	NA	NA	NA	NA	NA	NA	NA
DCB[6]	Glycine (Gly, mg kg^{-1} FFDM[7])	0.93	0.09	0	63.18	155.81	95.15	25.63	0.96	0.79	pH_d	5.78	6.05	Gly	34.48	156.06
	Alanine (Ala, mg kg^{-1} FFDM[7])	0.98	0.78	0	891.80	1439.70	1192.86	141.47	0.91	0.66	pH_d	5.79	6.04	Ala	747.20	1699.20
	Valine (Val, mg kg^{-1} FFDM[7])	0.93	0.08	2	670.29	1163.60	974.11	118.68	0.96	0.78	pH_d	5.78	6.05	Val	583.34	1448.80
	Leucine (Leu, mg kg^{-1} FFDM[7])	0.98	0.82	1	1576.86	2962.09	2331.36	337.12	0.96	0.84	pH_d	5.79	6.04	Leu	1590.61	3150.48
	Isoleucine (Ile, mg kg^{-1} FFDM[7])	0.97	0.62	1	477.07	871.02	694.01	92.33	0.93	0.74	pH_d	5.78	6.05	Ile	433.53	1015.78
	Phenylalanine (Phe, mg kg^{-1} FFDM[7])	0.96	0.37	0	1270.29	2382.24	1863.07	318.54	0.95	0.79	pH_d	5.79	6.05	Phe	1181.28	2677.53
	Tyrosine (Tyr, mg kg^{-1} FFDM[7])	0.96	0.35	0	735.04	1319.33	998.84	165.20	0.93	0.74	pH_d	5.78	6.06	Tyr	930.20	1252.05
	Tryptophan (Trp, mg kg^{-1} FFDM[7])	0.98	0.78	0	118.19	461.31	269.17	80.15	0.93	0.73	pH_d	5.78	6.05	Trp	138.82	388.20
	Serine (Ser, mg kg^{-1} FFDM[7])	0.97	0.62	0	248.50	476.20	372.74	62.57	0.94	0.73	pH_d	5.78	6.05	Ser	181.29	612.37
	Threonine (Thr, mg kg^{-1} FFDM[7])	0.95	0.29	0	145.09	348.52	221.51	48.49	0.93	0.72	pH_d	5.78	6.06	Thr	101.49	334.53
	Asparagine (Asn, mg kg^{-1} FFDM[7])	0.97	0.71	0	645.82	1474.17	1026.34	219.32	0.92	0.69	pH_d	5.79	6.05	Asn	429.86	1812.98
	Glutamine (Gln, mg kg^{-1} FFDM[7])	0.96	0.49	0	482.04	860.50	647.82	101.32	0.93	0.73	pH_d	5.81	6.03	Gln	319.81	1035.04
	Lysine (Lys, mg kg^{-1} FFDM[7])	0.88	<0.01	1	235.34	1560.64	602.92	285.39	0.93	0.72	pH_d	5.80	6.04	Lys	181.40	1020.21
	Aspartic acid (Asp, mg kg^{-1} FFDM[7])	0.98	0.81	0	301.67	566.20	439.10	71.61	0.91	0.67	pH_d	5.78	6.05	Asp	210.13	671.59
	Glutamic acid (Glu, mg kg^{-1} FFDM[7])	0.97	0.57	1	590.23	1586.58	1203.93	246.67	0.93	0.72	pH_d	5.78	6.05	Glu	868.96	1663.43
	Gamma-aminobutyric acid (GABA, mg kg^{-1} FFDM[7])	0.96	0.40	0	573.29	1317.47	955.99	213.46	0.91	0.69	pH_d	5.78	6.06	GABA	410.74	1580.17

[1]Shapiro-Wilks test at 5% significance level. [2]Indexes for assessing class intervals (Armstrong et al., 2003; Bivand et al., 2020). [3]Goodness of variance fit. [4]Tabular accuracy index. [5]Fermented cacao beans (undried). [6]Dry cacao beans. [7]Fat free dry material.

The two-dimensional space of the scatterplots was subdivided into 4 class intervals for the x- and y-variables (Figures 1-5). The average is a class limit on the x and y axes as shown in Figures 1 to 5; it is a parameter used in the GVF and TAI interval quality assessment indexes (Armstrong et al., 2003). 100% of the variables present GVF values equal to or higher than 0.90 (Table 1). GVF values closer to 1.00 indicate that the method for determining class intervals has a good fit (Smith, 1986). TAI values higher than 0.60 and less than or equal to 0.70 for the variables TA, Ala, Asn, Asp, and GABA, indicate that there was more smoothing or some loss of information in the class intervals in relation to the other variables whose values are equal to or higher than 0.70 (Table 1). 76.19% of the dataset variables have enough internal variability for its classes (Table 1).

The low number of observations in the set of samples can compromise the selection of boundary points and the respective significance of the coefficients of the regression models, as shown in Figure 1b. Therefore, these models will only be valid if their biological or biochemical explanation is coherent. The inverse relations between FI and pH and between FAAs and pH (Ilangantileke et al., 1991) are the information

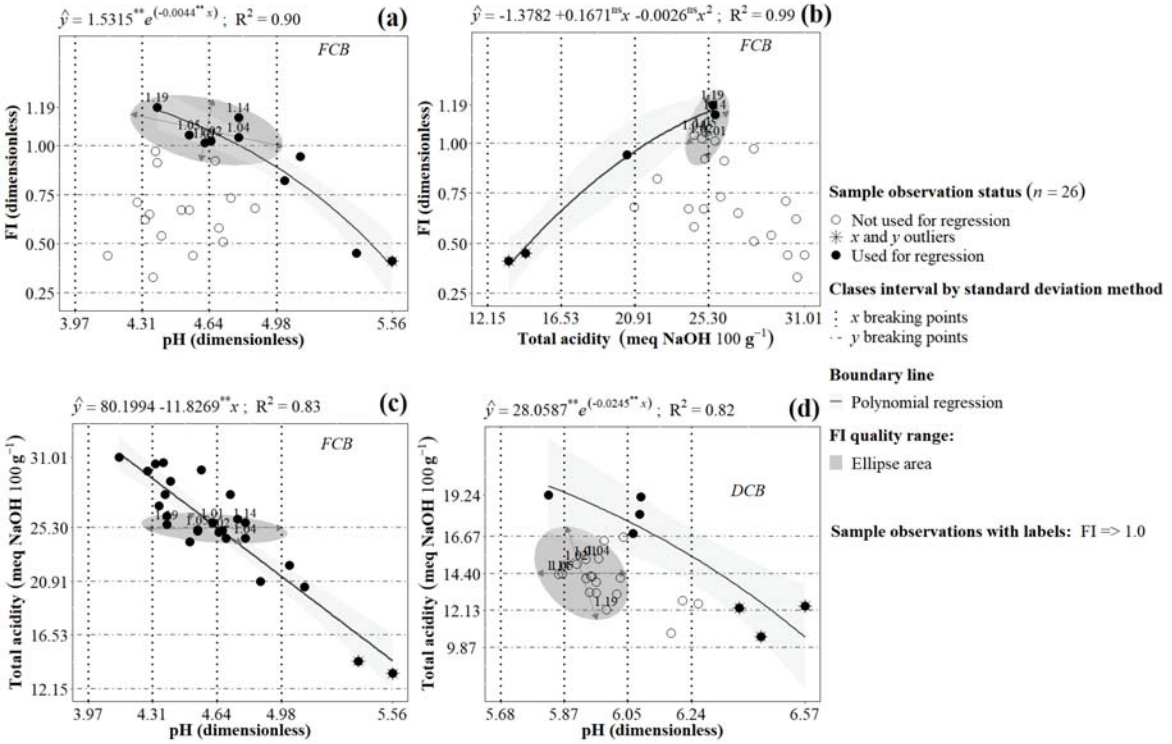

Figure 1. Boundary lines fitting of the polynomial regressions of the pH (a) and total acidity (b) as a function of the fermented index of fermented cacao beans (FCB, undried), and pH (c) as a function of total acidity of the dry cacao beans (DCB). Significance level of regression coefficients by the F test: $p \leq 0.01$ (**), and not significant (^{ns}). Coefficient of determination (R^2). A confidence interval at 95% level. Ellipse area calculated with multivariate normal distribution. Porto Híbrido 16 cacao clone; Humid region of southeastern Bahia, Brazil.

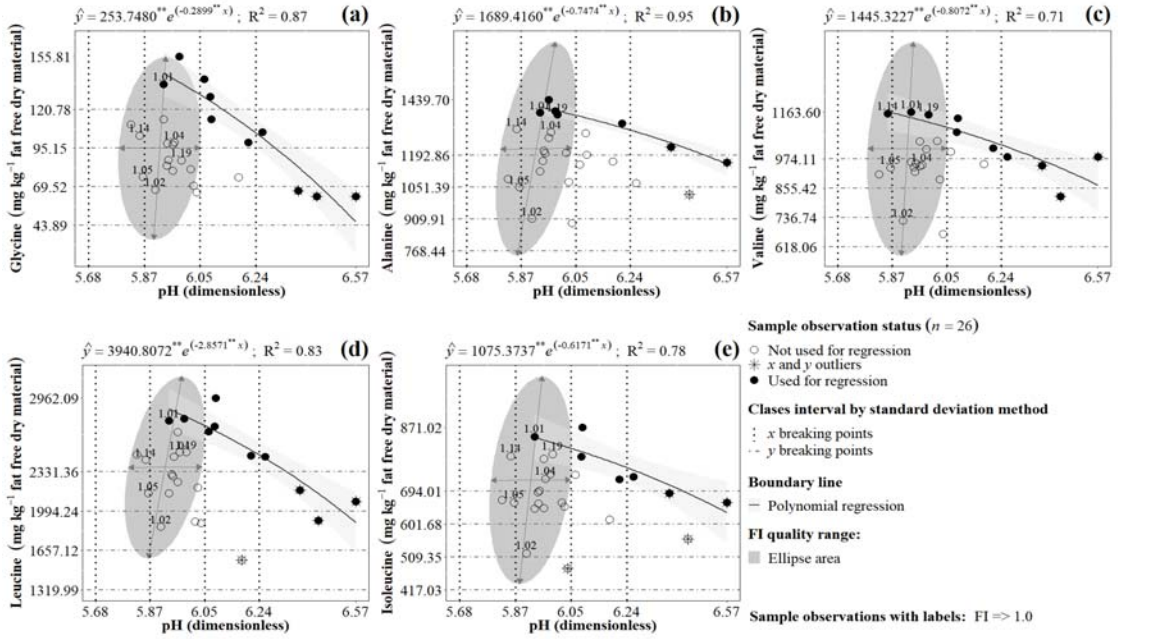

Figure 2. Boundary lines fitting of the polynomial regressions of the free amino acids as a function of pH of dry cacao beans. Amino acids with aliphatic side-chains, glycine (a), alanine (b), valine (c), leucine (d), and isoleucine (e). Significance level of regression coefficients by the F test: $p \leq 0.01$ (**). Coefficient of determination (R^2). A confidence interval at 95% level. Ellipse area calculated with multivariate normal distribution. Porto Híbrido 16 cacao clone; Humid region of southeastern Bahia, Brazil.

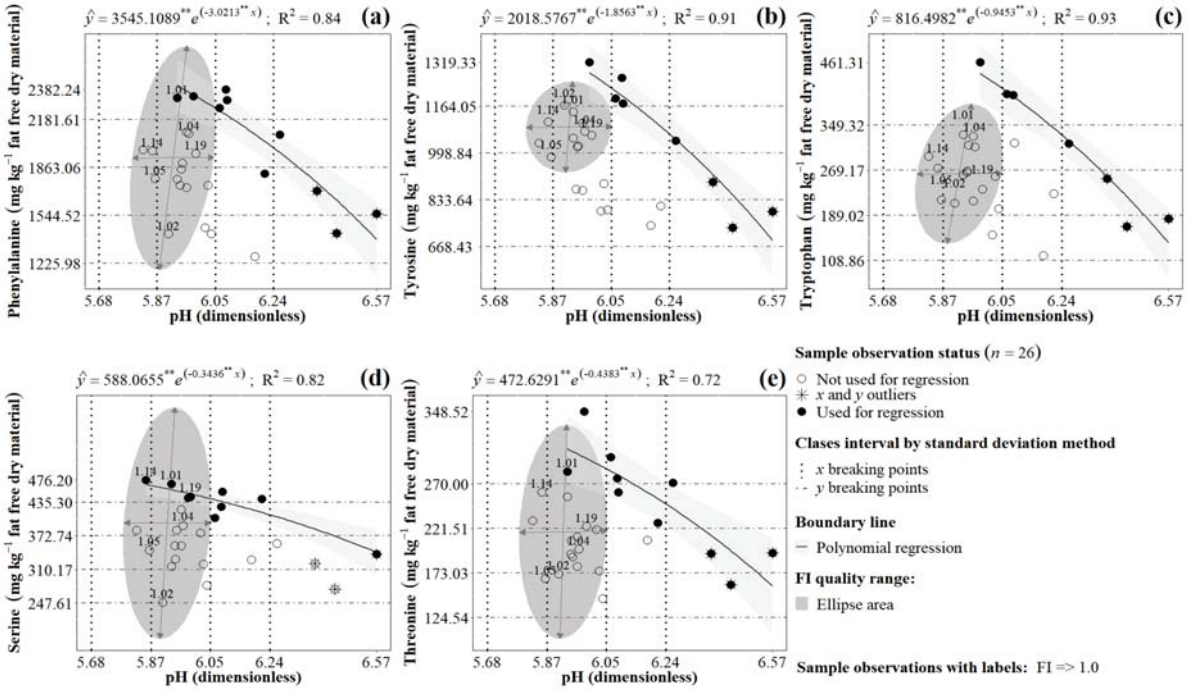

Figure 3. Boundary lines fitting of the polynomial regressions of the free amino acids as a function of pH of dry cacao beans. Amino acids with aromatic side-chains, phenylalanine (a), tyrosine (b), and tryptophan (c), and with polar neutral side-chains, serine (d), and threonine (e). Significance level of regression coefficients by the F test: $p \leq 0.01$ (**). Coefficient of determination (R^2). A confidence interval at 95% level. Ellipse area calculated with multivariate normal distribution. Porto Híbrido 16 cacao clone; Humid region of southeastern Bahia, Brazil.

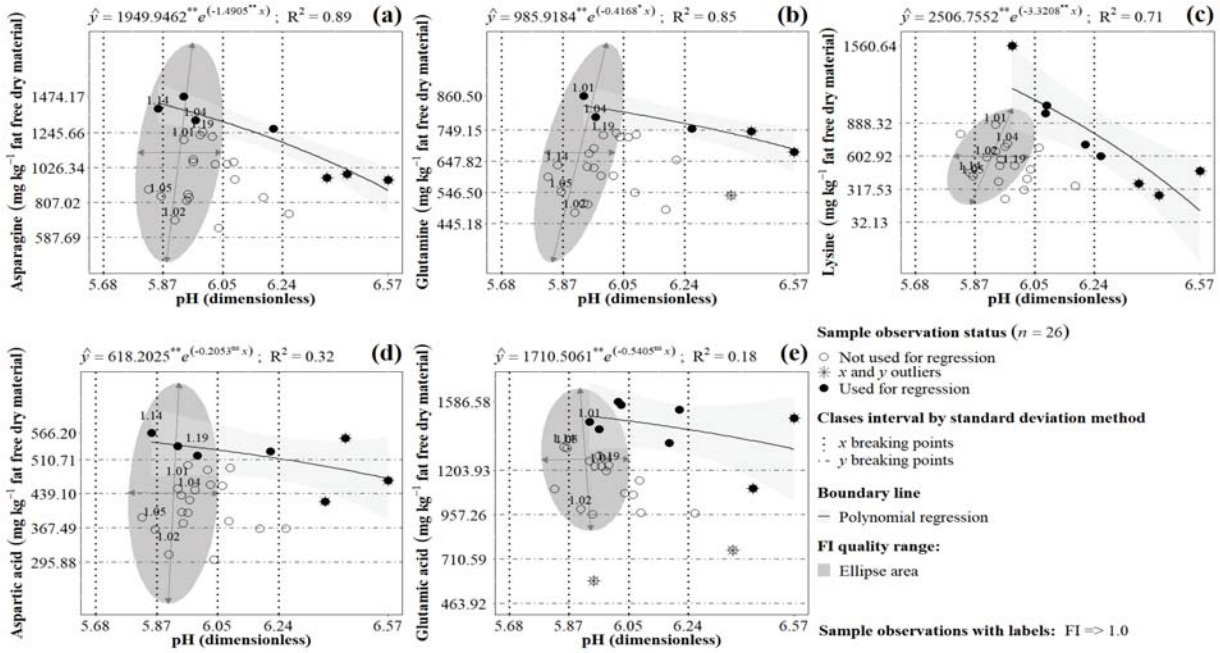

Figure 4. Boundary lines fitting of the polynomial regressions of the free amino acids with as a function of pH of dry cacao beans. Amino acids with amide side-chains, asparagine (a), and glutamine (b), with cationic side-chains, lysine (c), and with anionic side-chains, aspartic acid (d), and glutamic acid (e). Significance level of regression coefficients by the F test: $p \leq 0.01$ (**), $0.01 > p \leq 0.05$ (*), and not significant (^{ns}). Coefficient of determination (R^2). A confidence interval at 95% level. Ellipse area calculated with multivariate normal distribution. Porto Híbrido 16 cacao clone; Humid region of southeastern Bahia, Brazil.

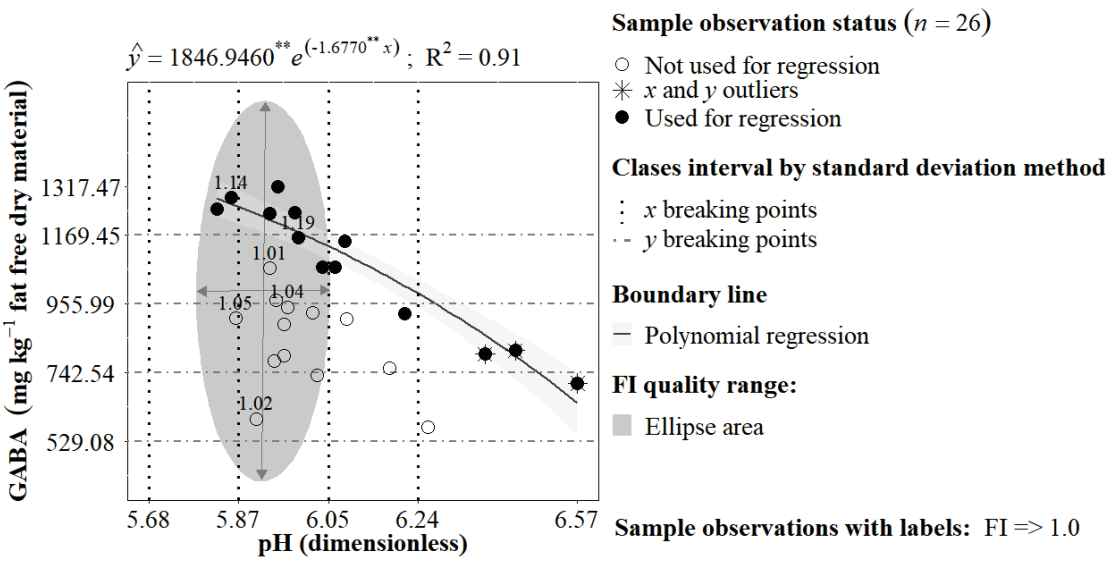

Figure 5. Boundary lines fitting of the polynomial regressions of the gamma-aminobutyric acid (GABA) with as a function of pH of dry cacao beans. Significance level of regression coefficients by the F test: $p \leq 0.01$ (**). Coefficient of determination (R^2). A confidence interval at 95% level. Ellipse area calculated with multivariate normal distribution. Porto Híbrido 16 cacao clone; Humid region of southeastern Bahia, Brazil.

taken as criteria for the selection of the extreme points used to obtain the boundary lines of the first degree and exponential polynomial regressions in Figures 1 to 5. The literature corroborates the behaviours of the response variables as a function of the pH observed in Figures 1 to 5 (Ilangantileke et al., 1991; Melo et al., 2021; Tchouatcheu et al., 2019).

The scatterplots projections of the elliptical clustering (Murdoch et al., 2022; Wickham et al., 2020) of the sample observations whose FI values were equal to or higher than 1.0 (Ilangantileke et al., 1991; Tchouatcheu et al., 2019), as a category of the retrospective fermentation process, allowed the definition of quality ranges for the pH and for the other variables, mainly the FAAs (Figures 1-5). The elliptical clustering of observations with FI values equal to or higher than 1.00 occurred in a pH range between 4.25 to 5.01 (Figure 1a), and in a TA range between 23.98 to 26.49 (Figure 1b). Melo et al. (2021) have observed inverse relations between pH and cacao fermentation time. In 144 h of fermentation, the pH reached an average of 4.72 ± 0.01, TA an average of 26.52 ± 2.52 meq NaOH 100 g^{-1}, and moisture and average of 5.82 ± 0.16 g 100 g^{-1}. After 96 h of fermentation, the FI reached values equal to or higher than 1.0 (1.058-1.157). Ilangantileke et al. (1991) have observed FI values between 1.0 and 1.3, corresponding to pH values close to and below 5.0. These antecedents are corroborated with the pH and TA values of the FCB (Table 1).

Taking all the pH quality ranges for the FAA by the elliptical clusters related to good fermentation in the DCB (Figures 2-5), an overall range of pH of 5.79 to 6.05 was obtained (Table 1). As flavour and aroma precursors of chocolate, FAAs should reflect the fermentative condition in the pH range between 5.5-5.0 (Voigt and Biehl, 1995). Tyrosine (Tyr) proved to be the FAA most sensitive to fermentation, given the delimitation observed in the elliptical clusters (Figure 3b). It has been shown that the oxidation rate of polyphenols can be increased by tyrosinase in crude cacao beans, reducing excessive astringent and bitter taste (Misnawi et al., 2002). Therefore, the formation and degradation of Tyr in cacao beans is a potential indicator of fermentative quality. The average Tyr content was one of the highest detected in the DCB of the PH-16 cacao clone (Table 1; Figure 3b) and is close to that reported by Deus et al. (2020) for the same cacao genotype, and in turn statistically close to the average theory found for the clone Castro Naranjal Collection 51 (CCN-51). However, the FAAs profile of the chocolate samples prepared with the DCBs of clone PH-16 was grouped with the Ipiranga and Pará

genotypes. Deus et al. (2020) have classified Tyr in tasteless and acidic sensory characteristics groups. Variables Trp (Figure 3c), Lys (Figure 4c) and Glu (Figure 4e) also seem to have this potential for discriminant analysis; however, it would be important to verify this behaviour with larger samples (*n* e" 100). The exponential coefficients of the boundary regressions for the variables Asp (Figure 4d) and Glu (Figure 4e) were not significant, therefore, it is assumed that only pH values above 6.57 (sample maximum, Table 1) must be predictive of the decrease in the content of these FAAs.

Once the behaviour of the responses of the FAAs to pH variations has been determined, it has been shown that the elliptical projection of a cluster of sample observations categorized by FI values is a viable exploratory graphic technique to monitor retrospective processes such as the end of the fermentation. The pH range associated with fermentative quality is useful information that can be used to characterize other aspects of cacao quality such as human nutrition.

Conclusion

The overall pH range of 5.79 to 6.05 predicts the FAAs contents related to the fermentative quality of cacao beans of PH-16 cacao clone. Tyr content range from 930.20 to 1252.05 mg kg^{-1} fat free dry material was shown to be more associated with the observations of the sample that correspond to FI values higher than or equal to 1.0. Therefore, it is suggested that this FAA is a potential indicator of fermentative quality.

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