Influence of fermentation level and geographical origin on cocoa bean oligopeptide pattern

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Abstract: Peptides and amino acids generated during cocoa bean fermentation are the most important precursors for the development of cocoa aroma. Although fermentation, amino acids and aroma development in cocoa have been extensively studied, the cocoa oligopeptide fraction is under-investigated and studies regarding the identification of peptides in different cocoa beans are completely lacking. In this study we perform a deep investigation on the presence of oligopeptides in well fermented cocoa beans coming from all the main producing countries, in unfermented (slaty) and underfermented (violet) beans. Oligopeptide pattern was determined by UPLC/ESI-MS, 35 low-molecular weight peptides (Mw range 202-1244) were identified, semiquantified and considered for data elaboration. Principal Component Analysis was used as a helpful tool for a better interpretation of dataset. The main factor influencing the peptide content was the fermentation level, however, a variability of peptide pattern was observed among well fermented cocoa samples of different geographic origin.

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Dear Editor of Food Chemistry,

we would submit the manuscript entitled "**Influence of Fermentation Level and Geographical Origin on Cocoa Beans Oligopeptide Pattern"** for the eventual publication in 'Food Chemistry' as a research article.

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We would greatly appreciate Your suggestions about this work.

Thank You in advance,

Yours sincerely

Augusta Caligiani

*Highlights (for review)

- An overview on the peptide pattern of different cocoa samples is provided
- Peptide pattern and amount are strictly related to the fermentation process
- Ratio of peptides from vicilin and from cocoa albumin can be proposed as fermentation marker
- Some peptides are characteristic of specific geographic origin/varieties

Influence of Fermentation Level and Geographical Origin on Cocoa Beans Oligopeptide Pattern

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Short Title: oligopeptides in cocoa beans

Abstract

Peptides and amino acids generated during cocoa bean fermentation are the most important precursors for the development of cocoa aroma. Although fermentation, amino acids and aroma development in cocoa have been extensively studied, the cocoa oligopeptide fraction is under-investigated and studies regarding the identification of peptides in different cocoa beans are completely lacking. In this study we perform a deep investigation on the presence of oligopeptides in well fermented cocoa beans coming from all the main producing countries, in unfermented (slaty) and underfermented (violet) beans. Oligopeptide pattern was determined by UPLC/ESI-MS, 35 low-molecular weight peptides (Mw range 202-1244) were identified, semiquantified and considered for data elaboration. Principal Component Analysis was used as a helpful tool for a better interpretation of dataset. The main factor influencing the peptide content was the fermentation level, however, a variability of peptide pattern was observed among well fermented cocoa samples of different geographic origin.

Keywords

Cocoa beans, oligopeptides, vicilin, 21kDa cocoa seed protein, geographic origin, fermentation level

1. Introduction

Cocoa beans, from the fruit of the cocoa tree (*Theobroma cacao L.*), are transformed into chocolate and other cocoa products by a complex process involving fermentation, drying and roasting. The cocoa bean quality and characteristic aroma are determined primarily by genetic factors related to cocoa tree variety, but agricultural practices and fermentation, both characteristic processes of the origin country, can modify this genetically inherent flavour potential (Lopez & Dimick, 1995). The two major botanical groups of cocoa are Criollo and Forastero. Criollo (native) represents only 5% of world's production, corresponds to the subspecies 'cacao' and represents the original cocoa, it ferments easily and has a pleasant and penetrating aroma, an optimum taste and therefore is considered of excellent quality. The cacao Forastero (foreigner) resulted from dissemination toward the Amazon Valley in Northern Brazil and to African countries, the most important of which are now the Ivory Coast and Nigeria. These countries are now the largest producers of cocoa in the world. Forastero represents about 85% of the world's cocoa production because it is very strong and resistant to disease. The seeds have a strong flavor, not very aromatic and of lower quality. Some Forastero plants growing in particular areas give cocoa of excellent quality (*cru*) such as Arriba, the national cocoa of Ecuador (Caligiani, Marseglia & Palla, 2015).

Fermentation represents a crucial stage in the development of the aromatic precursors and bioactive compounds characteristic of chocolate and cocoa products (Schwan & Wheals, 2004). Cocoa beans are extracted from the fruit together with the surrounding sugary mucilaginous pulp, which is the real substrate for fermentation. Fermentation usually lasts 5–7 days, except for some varieties/hybrids, as for example Criollo and Arriba subjected to shorter fermentation (Fowler, 1999).

Cocoa fermentation is generally a spontaneous phenomenon which takes place without the purpose addition of any starter bacteria. It is operated by a microbial succession of a wide range of yeasts

(Kloeckera and Saccharomyces spp.) and of lactic-acid and acetic-acid bacteria (Lactobacillus, Bacillus, Pediococcus, Acetobacter and Gluconobacter), producing a wide range of metabolic end products, in particular alcohol and organic acids (Schwan et al., 2004). The most important aspects of fermentation, having fundamental consequences on cocoa quality, are the biochemical changes inside the cocoa beans. Acids that are synthesized from pulp sugars move into the beans and lower the internal pH. Acetic acid in particular, diffuses through the beans and causes a breakdown of the cell membranes and the cell contents become mixed, allowing various enzymatic reactions to take place. For example, oxidation of polyphenols and their conversion to insoluble forms due to the reactions with proteins are responsible for the removal of the bitter taste and the change of bean colour, from violet to brown (Jinap, Jamilah, & Nazamid, 2003). In fact, fully fermented cocoa beans have brown colour, while unfermented and underfermented cocoa beans appear respectively slaty and violet in colour, and represent the beans dried without previously being fermented or partly fermented or by using improper procedures. For the manufacturer of chocolate or cocoa powders the degree of fermentation of the beans is the principal quality criterion. In fact, too high contents of unfermented or underfermented beans result in a lack of cocoa flavour in the end-product because they do not develop enough chocolate flavour when roasted. The slaty beans cause a very acid and astringent flavour profile, whereas the violet beans cause a bitter and harsh flavour (Kattenberg & Kemmink, 1993; Puziah, Jinap, Sharifah & Asbi, 1998).

During fermentation, another fundamental biochemical change occurs on the protein fraction. Proteins make up 10-15% of the dry weight of cocoa seeds, the second most abundant constituent after cocoa fat. Cocoa beans contain two main well-characterized proteins fractions, albumin and globulin. Globulins are vicilin-like storage proteins mostly consisting of three subunits with molecular masses of 47 kDa, 31 kDa, and 15 kDa, which come from a common 66-kDa precursor (Spencer & Hodge, 1992). On the other side, the albumin fraction was mostly identified as a 21kDa cocoa seed protein; its primary structure, together with its trypsin inhibitory properties, was reported by Kochhar, Gartenmann & Juillerat (2000). Thus, basically only two proteins constitute most of the protein fractions of cocoa. During fermentation, these proteins are cleaved to hydrophilic and hydrophobic peptides as well as amino acids through autolysis by two endogenous enzymes, aspartic endoprotease and carboxypeptidase (Amin, Jinap, Jamilah, Harikrisna, & Biehl, 2002; Voigt, Biehl, Heinrichs, Kamaruddin, Marsoner, & Hugi, 1994a), which are activated by microbial metabolites (such as acetic acid) produced during fermentation. Aspartic endoprotease was demonstrated active both at pH 5.2 or pH 3.5, while carboxypeptidase esplicates proteolytic activity at pH > 5 (Voigt, Heinrichs, Voigt & Biel, 1994b), so the pH reached during fermentation and the development of organic acids are key factors controlling the proteolysis. Globulin protein fraction is the most degraded during fermentation and amino acids and oligopeptides formed represent the essential precursors for the development of cocoa aroma (Amin, Jinap, & Jamilah, 1997; Voigt, Biehl, Kamaruddin, & Wazir, 1993).

Although the degradation of cocoa proteins into oligopeptides and amino acids is well documented, the specific sequences of peptides formed during cocoa fermentation and autolysis and their role in the development of cocoa flavour, are poorly characterized. Buyukpamukcu, Goodall, Hansen, Keely, Kochhar, & Wille (2001) report the identification of peptides APLSPGDVF and SPGDVF from vicilin, while other authors report HPLC profiles of complex peptide patterns without specific identification and quantification (Rashidah, Jinap, Nazamid, & Jamilah, 2007; Amin et al., 2002; Jinap, Nazamid & Jamilah, 2002; Jinap, Ikrawan, Bakar, Saari & Lioe, 2008). Recently we performed a study aimed at characterizing the oligopeptide fraction in cocoa and we assigned 44 peptides based on their exact molecular mass, mass fragmentation pattern and comparison with vicilin and 21 kDa albumin sequences (Marseglia, Sforza, Faccini, Bencivenni, Palla & Caligiani, 2014). Nevertheless, so far no comprehensive study focusing on the variation of the content and distribution of oligopeptides of the commodity fermented cocoa beans has been made. In the present work, we provide a comprehensive study on the content and pattern of oligopeptides in commercially fermented and dried cocoa beans from different geographical origins and fermentation levels. Results are discussed in respect to fermentation degree and origin specificity. This study represents the first exhaustive dataset on peptide pattern in cocoa.

2. Materials and Methods

2.1 Cocoa samples.

We investigated 48 cocoa beans samples from 22 different geographical origins kindly provided by Barry Callebaut (Lebeke-Wieze, Belgium). The samples analysed were of Forastero varieties, fermented and dried. All fruits were harvested in 2012 and considered well fermented (brown color). Countries of origin and numbers of lots of cocoa beans samples collected are the following: Cuba (1), Mexico (1), Brazil (1), Grenada (1), Perù (6), Dominican Republic (3), Trinidad (1), Venezuela (3), Indonesia (2), Ecuador (3), Sao Thome (4), Ivory Coast (5), Madagascar (1), Sierra Leone (1), Nigeria (1), Tanzania (2), Uganda (1), Ghana (5), Congo (1), Java (1), Malaysia (1), Papua New Guinea (2). A sample of Criollo variety from Mexico was also analysed.

In addition, Forastero cocoa beans with different fermentation level, determined by the visual inspection of internal cocoa bean color (underfermented, violet color, 8 samples; unfermented, slaty color, 5 samples) of the following origins were also considered: Ghana, Ecuador, Ivory Coast, Grenada.

All the collected samples were stored frozen. For chemical analysis, the samples were treated with liquid nitrogen and ground with a coffee mill.

2.2 Chemicals. All solvents and reagents were of HPLC grade and used as commercially available without any further purification; deionized water was obtained by Millipore Alpha Q system (Millipore Corporation, Billerica, MA); formic acid 99% was purchased from ACROS Organics (Fair Lawn, NJ).

2.3 Sample Preparation. Peptides were extracted according to the method previously described (Marseglia et al., 2014). A total of 10 g of finely grinded cocoa sample was suspended in 45 mL of

0.1 N HCl. (L,L)-phenylalanylphenylalanine (Phe-Phe) was added as an internal standard (2.25 mL of a 1mM solution). The suspension was homogeneized for 1.5 min by Ultra Turrax T50 at 4000rpm (Janke and Hunkel Italabortechnik) and then centrifuged at 4000 rpm for 30 min at 4 °C by an ALC 4237R centrifuge. The solution was filtered through paper filters (pore dimensions 15-20 µm) and then extracted four times with 50 mL of ethyl ether. The solution was filtered again with a Millipore 47 mm Steril Aseptic system through 0.45 µm HVLP Millipore filters. A total of 1.5 mL of the resulting solution were mixed with 0.5 mL of a formic acid solution (0.1%). The solution was diafiltered through Sartorius Vivaspin 2 filters (nominal molecular cut-off 10 000 Da) by using an Amicon Micropartition system MPS-1. The filtrate was dried under nitrogen, redissolved in H_2O (0.1% HCOOH), and analyzed by HPLC.

2.4 UPLC/ESI MS (WATERS, Milford, MA) analysis conditions: eluent A: H_2O (0.2% CH_3CN and 0.1% HCOOH); eluent B CH_3CN (0.1% HCOOH); gradient elution was performed according to the following steps: 0–7 min isocratic 100% A, 7–50 min linear gradient from 100% A to 50% A, 50–52 min isocratic 50% A, 53– 58 min from 50% A to 0% A and reconditioning. Column: AQUITY UPLC Beh C18 (1.7 mm, 2.1x150 mm). Flow rate: 0.2 ml/min. MS conditions: ESI, positive ions, single quadrupole analyzer. Capillary voltage: 3.2 kV, cone voltage: 30V, source temperature: 150 °C, desolvation temperature: 300 °C, cone gas flow (N2): 100 L/h, desolvation gas (N_2): 650 L/h, acquisition: 100:2000 m/z. All data were acquired and processed by the software MassLynx 4.0 (Waters, Milford, MA).

2.5 Peptides semi-quantification and statistical analysis

Peptides were semi-quantified by comparison to the internal standard Phe-Phe. For the correct integration of peaks, as the total ion chromatograms (TIC) were crowded, the eXtract Ion Chromatogram (XIC) technique was applied. The oligopeptides were semi-quantified by measuring the ratio between the XIC peptide area and the relative XIC area of Phe-Phe, as previously described (Sforza, Galaverna, Schivazappa, Marchelli, Dossena, & Virgili, 2006), and expressed as mg/kg of cocoa beans. Data are semi-quantitative because do not take into account the different response factors, due to the unavailability of pure compounds for peptides. Data are provided for inter-samples comparison purposes only and they represent the mean of two independent sample extractions and analyses.

An unsupervised learning approach, Principal component analysis (PCA) was used to describe the variation in the dataset. PCA was performed with IBM SPSS statistics (Version 22.0, IBM, New York, USA), based on the correlation matrix, which is equivalent to using the covariance matrix on mean centred and variance scaled variables.

2.6. Determination of organic acids

Lactic, acetic, citric and succinic acids were determined on cocoa aqueous extracts by quantitative ^{1}H NMR , as previously reported (Caligiani, Acquotti, Cirlini & Palla, 2010)

3. Results and Discussion

Typical MS chromatograms (full scan acquisition) of cocoa bean samples of different origins and fermentation levels are shown in Figure 1. The major differences in the peptide patterns were observed among samples of different fermentation levels, but also some differences in samples from different origins could be appreciated.

In our previous study (Marseglia et al. 2014), 44 peptides were identified , many of them belonging to vicilin and to 21 kDa cocoa seed albumin. In the present study, we focused on 35 peptides, among the ones previously identified, showing the most intense signals in our samples. The list of peptides considered, ordinated on the basis of their molecular weight, is reported in Table 1 together with the maximum, minimum and mean values. From the mean values reported in Table 1 it appears well evident that small peptides are quantitatively the most abundant, indicating a general extensive proteolytic activity during cocoa fermentation.

3.1. Total peptide amounts and ratio vicilin/21kDa albumin peptides.

In Figure 2a, the total content of peptides in each cocoa bean sample is reported, considering the sum of all the peptides. Among well fermented beans, results showed that total peptide amount is extremely variable in cocoa from different geographic origin, and the samples with lower peptide content are those from Indonesia. As expected, the other samples containing low amount of peptides are unfermented, slaty beans. Quite surprisingly, supposely underfermented violet beans contain total peptide amounts comparable with well fermented beans.

Well fermented cocoa samples of different geographic origins can be divided according to their peptide content. Low amounts of oligopeptides, in the range of about 50-150 mg kg^{-1} were found in commercial samples from Indonesia that contained values between 43 and 54 mg kg−1, Ecuador (117-121 mg kg^{-1}), Ivory Coast (134-205 mg kg^{-1}), Madagascar (130 mg kg^{-1}), Malaysia (117 mg kg^{-1}), Nigeria (106 mg kg^{-1}), Tanzania (134-160 mg kg^{-1}), Uganda (142 mg kg^{-1}), Venezuela (117-165 mg kg^{-1}). Medium amounts of peptides, from 150 to 250 mg kg-1, were observed in cocoa from Brazil (198 mg kg^{-1}), Ghana (173-211 mg kg^{-1}), Perù (109-225 mg kg^{-1}). High level of oligopeptides (superior to 250 mg kg-1) were showed by all cocoa samples from Central America/Carribean Islands as Mexico, Cuba, Grenada, Santo Domingo, Trinidad, together with African samples from Congo and Sao Thome, Papua New Guinea and also Criollo variety from Mexico.

Intra-country specific variations of total peptides were comparatively low in Ecuador (117-121 mg kg^{-1}), Ghana (173-211 mg kg^{-1}) Papua New Guinea (250-294 mg kg^{-1}) and Santo Domingo (314-324 mg kg^{-1}), whereas large variations were found in cocoa from Perù (109-225 mg kg^{-1}).

Also the sum of the peptides resulting from vicilin and those arising from 21kda was considered, excluding dipeptides because in most cases they were common to both proteins. The ratio vicilin/21kDa peptides, shown in Figure 2b, is generally greater than 1, indicating a quantitative prevalence of peptides from vicilin. The ratio of peptide amounts generated from vicilin and 21kDa cocoa seed protein was variable and in general samples showing low content of peptides (less fermented samples) have a higher ratio, indicating that peptides from vicilin are formed first in the initial steps of fermentation. In fact, a negative correlation (correlation coefficient = -0.76, $p<0.05$) was observed between total peptide amounts and the ratio of peptides from vicilin and 21 kDa cocoa seed albumin. Generally, it was found that fermented cocoas can be differentiated from unfermented cocoas by the higher total amount of oligopeptides and by the lower ratio of vicilin to 21 kDa cocoa seed albumin. So, in the case of genetically identical material, this ratio can be proposed as a suitable indicator for the fermentation level.

3.2. Exploration of cocoa peptide patterns by Principal Component Analysis (PCA)

Semiquantitative amounts of the 35 peptides considered for each cocoa sample are reported in supplementary materials (S1). The organic acids amounts were also calculated for each sample and reported in S1. In order to obtain an easily interpretable quali-quantitative description of the eventual differences present in the peptide pattern of cocoa beans, a PCA was performed on all the bean samples, including all geographic origins and fermentation levels. PCA was performed on the matrix of 35 peptides for 61 cocoa samples, and the principal components were obtained with the correlation method. Figure 3 reports the 3D scatter plot of the scores of PC1 versus PC2 and PC3, explaining more than 70 % of the total system variance, together with the corresponding combinations of PCs in bidimensional plots. The 3D score plot (Figure 3a) shows that samples of cocoa beans are divided into three principal groups, according to the fermentation level; the main group corresponds to the fermented beans of different geographical origins that were considered well fermented by supplier. Unfermented and underfermented beans (slaty and violet color, respectively) are separated each other and from the group of brown, well fermented beans.

The loading values of the variables associated to the first three principal components are reported in supplementary material (S2). PC2 appears as the component that better separates the groups of slaty and violet beans from brown beans (Figure 3b and 3d). The variables with positive values on PC2, that characterize the group of slaty and violet beans, are peptides of higher molecular weight as APLSPGDVF, DEEGNFKIL, Mw1596, DNEWAW, NGTPVIF, while the variables with negative values on PC2, related to the well fermented beans, are low molecular weight peptides, mainly dipeptides as AI, FL(I), AW, FV, VF. This is consistent with the fact that during the course of fermentation an intense proteolytic activity occurs inside the beans degrading longer peptides to shorter ones (Amin, Jinap & Jamilah., 1998).

Slaty beans are well separated from all the other beans based on PC1 (figure 3b and 3c), having the most negative values on this component. The corresponding variables are Mw1244, Mw1596, Mw1090, VLE and TVWRLD. They correspond, except VLE, to high molecular weight peptides. Results of PCA are confirmed in Figure 4 where the general mean (mg/kg) of each peptide in brown, violet and slaty beans was reported. Slaty beans are very different from the others because they are characterized by low amount af almost all peptides except for VLE, TVWRLD and two high Mw peptides (1090, 1244). Brown and violet beans are more similar regarding total peptide content, but generally brown beans contain higher amount of dipeptides, while intermediate Mw peptides as GAGGGGL, KDQPL, SPGDVF characterize the group of violet beans.

Focusing only on well fermented cocoa, no specific grouping was observed according to geographical origin, and generally also in the samples from the same country differences can be observed. Only samples from Perù, Ghana and Ivory Coast showed a larger intra-country similitudine. Sample from Congo seems to be very different from the others. Comparing samples on the left side of PC1-PC2 score plot (Figure 3b) with the samples on the right side, indicated that all the samples on the left, near the group of slaty beans and corresponding to negative values on PC1 and positive on PC2, have low amount of peptides (compare figure 2a): for example Nigeria L6 (106,48 mg/kg), Tanzania L3 (134,43 mg/kg), Ecuador L3 (116,92mg/kg).

On the contrary, the group of sample on the right, corresponding to positive values on PC1 and negative on PC2, have high amount of peptides: Congo (312,20 mg/kg), Criollo (301,29), Papua New Guinea (294,75 mg/kg), S. Domingo L2 (324,12), Sao Thome (302,82 mg/kg), Trinidad (352,50 mg/kg). So it is possible to conclude that moving from negative to positive values on PC1 and from positive to negative on PC2, the degree of fermentation is increasing. As a consequence, peptide pattern and amounts can be considered a very good index of cocoa fermentation.

Samples from Indonesia are in the same group of the slaty beans and showed the same peptide pattern, indicating that they were poorly fermented. They showed also low amount of total peptides and low amount of free amino acids, as previously reported (Marseglia, Palla & Caligiani, 2014b). These data are consistent with the fact that cocoa fermentation in Indonesia is short or even omitted in Sulawesi regions and Indonesian cocoa is of poor commercial value (Rohsius, Matissek, & Lieberei, 2006).

Ecuador samples in the score plot is near the group of slaty beans, and this is compatible with the shorter fermentation times utilized for this cocoa. Ecuador samples showed also low total amount of peptides and high ratios vicilin/21 kDa peptides.

The specific distribution of peptides in some cocoa samples containing respectively low, medium and high amount of peptides was also considered (Figures 5a, 5b and 5c). High-peptide samples show a more uniform distribution of peptides, indicating that for a complete fermentation the peptide profile tend to be similar without specific dependance on cocoa origin. More differences in peptide pattern can be observed in medium- and, even more, low-peptide samples, indicating that the peptide profile is linked to the moment in which fermentation stops. This characteristic makes possible, in most cases, to find specific peptides markers of a particular cocoa origin. For example samples from Ecuador, considered a fine cocoa as Criollo, contain, respect to the other Forastero samples, the highest amount of peptide ASKDQPL and of peptide with Mw 1090. The same peptides characterize Nigeria sample together with Mw1596. Malaysia cocoa is mainly characterized by TVWRLD, IEF and DEEGNFKIL and the last two peptides are high also in Brazil cocoa. APLSPGDVF and SPGDVF are most represented in Ghana samples.

Sample of Criollo variety (origin Mexico) is separated from all the others probably because it represent another variety; it occupies the PCs score plot zone associated with well fermented cocoa, even if Criollo fermentation is generally short. Criollo separation is mainly based on PC3, where it shows the highest negative values (figure 3c and 3d); the associate variables are peptides of different molecular weight as GAGGGGL, RLD, VI, RRSDLD, EVL. All these peptides, except

EVL, derive from 21 kDa albumin. This is confirmed by the specific peptide pattern of criollo compared with the forastero variety of the same region (figure 5d).

In order to better understand the proteolytic activity giving origin to the different oligopeptides pattern observed, a correlation with content of organic acids in cocoa beans was performed. Organic acids (reported in supplementary material) showed slight positive correlations (correlation coefficient 0.6-0.7, data not shown) with cocoa dipeptides. In general, citric acid level is higher in samples having low amount of peptides, while lactic acid and acetic acid are generally more abundant in samples with higher amounts of peptides, as for example Congo, Criollo and Sao Thome. Criollo variety, having a peptide pattern different from other cocoas, showed similar amounts of organic acids.

4. Discussion

Many parameters affect the composition of the fermented cocoa beans: variety, geographical origin, climate during ripening, degree of ripeness, and the processing steps of fermentation and drying. A better knowledge of the biochemical reactions occurring during fermentation, aimed at a better control of cocoa primary processing, is a strategic and challenging issue for cocoa industry. In this context, the knowledge of the structure and cinetic of formation of oligopeptides is very important because proteolysis is the main reaction involved in cocoa aroma formation and, as a consequence, in cocoa quality. The contribute of peptides in generating typical cocoa aroma is currently unknown but it is underlined by a study showing that no cocoa aroma was obtained when synthetic mixtures of amino acids, resembling the spectrum of free amino acids present in fermented cocoa seeds, were roasted in the presence of reducing sugars (Voigt et al., 1994a).

The results in this work clearly showed that peptide amount and profile are strongly influenced by the cocoa fermentation level, which also made possible to appreciate origin-related differences.

PCA demonstrates that the main grouping factor is the fermentation level and this indicates that fermentation influences the cocoa bean composition more than botanical or geographical factors do.

An effect of the botanical variety (Criollo *vs* Forastero) has also been registered, probably because a different cocoa chemical composition influence the modification occurring during fermentation.

Regarding cocoa samples with different fermentation level, lower amounts of peptides were observed in slaty beans, as expected, while similar contents were registered in violet and brown beans, with a slight higher amount of peptides in violet beans. This was a trend observed in all the series of cocoa beans and might be explained considering that progressing fermentation peptides are further hydrolyzed to amino acids. However, in a previous work, we found that also amino acids are higher in violet than in brown beans (Caligiani, Palla, Acquotti, Marseglia & Palla, 2014), indicating that probably the proteolytic activity is higher in the first days of fermentation and then tends to decrease. Biehl & Passern (1982) and Jinap et al. (2008), followed the development of free amino acids during cocoa bean fermentation, reporting that different amino acids reached the maximum value after different days of fermentation. The high content of peptides and amino acids in violet beans suggests that violet beans are able to generate specific cocoa aroma during roasting, but according to Hii et al. (Hii, Law, Cloke & Suzannah, 2009), due to their higher polyphenols content, they probably cause excessive astringency to the final products that masks the chocolate flavour.

Variable amounts of peptides were observed not only in samples with known different fermentation levels, but also in well fermented samples of different geographical origin. In general, different amounts of peptides correspond to different ratios of vicilin/21 kDa peptides and often to different peptides distribution, indicating that sample-specific mechanism/kinetic of proteolysis occur.

Although clearly due to the differences in the fermentation processes and thus on the enzymatic activity brought in by the different microorganisms, the reasons for such differences in peptide content and composition remain largely unclear, since commercial samples usually lack information on fermentation and drying practices as well as planting material used. But, even though sample-specific information is lacking, overall information on the common fermentation practices in use is available for many countries and regions. For example, countries and regions like Ecuador, Tanzania and Sulawesi are known for traditionally short or, in the case of Sulawesi, often omitted fermentation processes. Malaysia normally makes use of beans imported from Indonesia that are usually blend with fully fermented beans, to obtain the desired flavour characteristics and to reduce the excessive astringency and bitterness (Puziah et al., 1998). Ecuadorian cocoa is usually poorly fermented: traditional fermentations of the local Ecuadorian cocoa type Nacional, with its fine flavor, are carried out in boxes and on platforms for a short time (Beckett, 2009; Papalexandratou, Falony, Romanens, Jimenez, Amores, Daniel & De Vuyst, 2011). Accordingly, all the samples analysed from these countries contained low amounts of oligopeptides.

African countries are known to produce bulk cocoa with an average quality through long fermentation times, tipically 3 to 6 days in heaps (Rohsius et al., 2006; Wood & Lass, 1993). High amounts of oligopeptides were found in Congo and Sao Thome samples but not in the other West African countries as Ivory Coast, Ghana, Nigeria. In Ivory Coast, cocoa beans were commonly fermented 4 - 5 days without turning, and fermented cocoa beans were dried by solar drying method (Guehi, Konan, Koffi-Nevry, N'Dri, & Manizan, 2007). Turning ensures uniform fermentation and frequent mixing, at 6–12 hours intervals, produces a higher number of well-fermented beans (Senanayake. Jansz & Buckle, 1997).

In the case of selected cocoa varieties as Criollo, even if fermentation is short, oligopeptide content is one of the highest, so it is possible that the acidification and the induction of proteolysis occurs earlier. Criollo contains more peptides arising from 21 kDa cocoa albumin, respect to all the other cocoa analysed. However, this protein should be more resistant to hydrolysis, since it has been reported that degradation of vicilin-class globulin was about 88% while that of albumin was 47% at the end of fermentation (Amin at al., 1998). Moreover, albumin does not originate specific cocoa aroma, but Criollo is one of the most aromatic cocoa (Voigt et al.,1994b). A previous work showed that no differences were present in HPLC oligopeptide profile obtained by the action of aspartic endoprotease on partially purified vicilin from various cocoa genotypes, comprising Criollo, but no data about peptides from albumin were provided (Amin et al., 2002). It was reported that proteolytic activity in cocoa is higher immediately after the bean death (1-2 days of fermentation) and then tends to decrease (Biehl et al., 1982).

It is well known that formation and degradation of peptides in cocoa is related to the action of two endogenous enzymes, a carboxypeptidase and an aspartic endoprotease, both activated by organic acids of fermentative origin that penetrate cocoa beans lowering the inside pH.

Aspartic endoprotease is able to release peptides but only very low amounts of free amino acids, which were instead formed when globulin peptides were treated with carboxypeptidase, releasing preferentially hydrophobic amino acids (Voigt et al 1994b). The predominant amino acids released from the albumin-derived oligopeptides by carboxypeptidase treatment were instead acidic amino acids such as aspartic acid, glutamic acid and asparagine. Aspartic endoprotease was demonstrated active both at pH 5.2 or pH 3.5, while carboxypeptidase esplicates proteolytic activity at pH > 5 (Voigt et al 1994b). All these data indicates that different peptides patterns can depend on the pH value reached at different steps of fermentation. Amin et al. (1998) showed that the pH during fermentation determines the rate of aspartic endoproteinase and carboxypeptidase activity. The pH range of 5.0-5.5 during fermentation is thus important in producing fermented and dried cocoa beans with high aroma potential, whereas a pH range of 4.0-4.5 will result in low aroma potential (Biehl, Brunner, Passern, Quesnel & Adomako, 1985).

Regarding proteases specificity, it has been found that the cleavage specificity of the cocoa aspartic endoprotease was essential for the formation of the cocoa-specific aroma precursors (Voigt, Voigt, Heinrichs, Wrann, & Biehl, 1994c) while cocoa carboxypeptidases can be substituted by other carboxypeptidases as porcine pancreas carboxypeptidase A (Bytof, Biehl, Heinrichs & Voigt, 1995).

The preferred cleavage sites for cocoa aspartic endoprotease is aspartic acid N-terminal (Biehl et al., 1982), and it cleaves preferentially protein substrates at hydrophobic amino acids residues, although its exact cleavage specificity has not yet been characterized (Watson, Preedy & Zibadi, 2012). Bytof et al (1995) showed that the specificity of carboxypeptidase is influenced by the pH-value, and liberation of acidic amino acids was favoured by low pH-values, but in general peptides with carboxyterminal arginine, lysine or proline residues are resistant against degradation by the cocoa seed carboxypeptidase. The enzyme preferentially liberates hydrophobic amino acids, whereas acidic amino acids are released very slowly. The rate of hydrolysis is not only determined by the carboxyterminal, but is also affected by the neighboring amino acid residue.

Amin et al. (1998) showed that the aspartic endoproteinase levels fell sharply in the first 2 days of fermentation and then increased to 116% of the initial value at 3 days, falling to zero by the end of the fermentation (6 days). The carboxypeptidase activity fell to approximately one third of the initial value during the first 3 days of fermentation and then rose to 157% of the initial value at 4 days and fell to zero by the end of the fermentation (6 days). The changes in both enzyme activities throughout the fermentation could be due to pH changes in the cotyledon during fermentation, with optimum pH values for these enzymes occuring around 72 h for the aspartic endoproteinase and 96 h for the carboxypeptidase. Therefore, the proteolytic activity is higher immediately after the bean death and then tends to decrease, probably due to inhibition by the oxidation compounds of polyhydroxyphenol products which were released from storage cells (Forsyth, 1958; Biehl et al., 1982).

Jinap et al. (2008) followed the development of free amino acids during cocoa bean fermentation, from 0 days (unfermented) to 3 days (underfermented), reporting that some hydrophobic amino acids reached the maximum value after 3 days of fermentation (Tyr, Ile), and others after 2 days (Ala, Leu) or 1 day (Val, Phe). It is possible to infer that a similar behaviour is followed by peptides, and the specific pattern observed in the final cocoa sample is a sort of ‘snapshot’ of the moment in which fermentation and proteolytic activity is stopped. Further work is in progress to confirm these hypothesis by analysing cocoa beans sampled at different days of fermentation

Thus, all the literature data demonstrate that pH values reached during fermentation and their variations are key factors for the activation of proteolytic enzymes and as a consequence for the development of specific peptides and amino acids. The variations in pH of fermented cocoa are the results of different factors: the loss of citric acid concentration in the pulp (Ardhana & Fleet, 2003), the migration of lactic acid, acetic acid and other many organics acids produced by microbial activities from the outside to the inside of cocoa seeds, the loss of volatile acetic acid. Some authors (Guehi, Dadie, Koffi, Dabonne, Ban-Koffi, Kedjebo & Nemlin, 2010) demonstrate that the acidity of fermented cocoa beans varied on the duration and on the method of fermentation. Acidity of cocoa decreased on fermentation duration and partial fermented cocoa beans presented higher acidity than well-fermented, because of volatility of acetic acid. Since the mechanism of fermentation of cocoa beans is very complex in nature, and factors such as temperature mass and aeration affecting the pH during fermentation were not controlled, the correlation between protease activities, fermentation level, peptide pattern with pH and organic acids were not easy to be interpreted. In the present paper, only slight positive correlations were observed among lactic acid and acetic acid with cocoa dipeptides and they are generally more abundant in samples with higher amounts of peptides, while citric acid level is higher in samples having low amount of peptides, a further confirmation that they were poorly fermented. However, the relationship among the production of organic acids during fermentation and the activation of cocoa proteases remain unclear, for example Criollo variety, having a peptide pattern different from other cocoas, showed similar amounts of organic acids.

Another important point that needs to be clarified is the reason why peptides derive only from specific zones of vicilin and 21 kDa cocoa albumin and are not spread in all proteins sequences (Marseglia et al., 2014). This phenomenon is not completely unknown in fermented products, since, for example, only peptides from specific casein regions are usually observed in cheese, and this might be due to a decreased accessibility of certain part of proteins, which upon cleavage become insoluble and aggregate, precluding any further cleavage (Sforza, Cavatorta, Lambertini, Galaverna, Dossena & Marchelli, 2012).

All the data presented in this paper are useful to understand how peptide amount and profile can be used to predict cocoa flavour quality. The data here presented clearly demonstrated this possibility and with larger databases, an effective control, through molecular markers, of environmental factors affecting cocoa quality as geographical origin, harvesting, fermentation and processing can be reached. Moreover, further work is in progress aimed at modeling Maillard reactions on peptide fractions isolated from cocoa sample, in order to identify the contribute of peptides in the development of cocoa aroma. Of particular interest could be to understand the contribute of peptides from 21 kDa cocoa albumin to cocoa aroma, as they seem to be most abundant in Criollo variety, considered a fine flavor cocoa.

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Figure Captions

Figure 1: UPLC-ESI-MS chromatograms of peptide elution zone for different cocoa beans samples: a) Criollo from Mexico, b) Forastero from Mexico, c) Forastero from Ecuador d) Forastero from Ecuador (underfermented, violet beans), e) Forastero from Ecuador (unfermented, slaty beans).

Figure 2: a) Total peptide content (mg/kg) in each cocoa bean sample of different geographical origin and fermentation levels; b) ratio vicilin/21 kDa peptides (light gray: well fermented cocoa beans; dark gray: underfermented beans; black: unfermented beans)

Figure 3: Principal component analysis: (a) 3d-score plot of all cocoa bean samples on PC1, PC2 and PC3 and 2d-score plot on (b) PC1 vs PC2 (c) PC1 vs PC3 and (d) PC2 vs PC3.

Figure 4: mean peptide values of peptides (mg/kg) in cocoa beans with different fermentation levels, calculated on all the cocoa samples

Figure 5: distribution of peptides in some representative cocoa samples having low (a), medium (b) and high (c) total peptide amounts; d) comparison of peptide pattern of Criollo variety from Mexico with a Forastero variety from Mexico

Table 1

Table 1: Maximum, minimum and mean values of each peptide identified in cocoa beans (mg/kg). Values are calculated on all the samples of different origins (well fermented cocoa beans). Peptides are identified by their Mw, sequence and precursor protein (v=vicilin, 21= 21kDa albumin).

Mw	Identification	Protein	Max	Min	Mean
202,2	AI	v	103,78 (Congo)	3,43	37,50
230,2	VI(L)	v/21	39,59	1,51	16,21
264,2	FV	v/21	42,80	2,41	22,75
264,3	VF	v	21,92	2,37	14,15
275,1	AW	21	21,02	0,91	9,28
278,3	FL(I)	v	34,74	2,89	18,05
359,3	VLE	v	5,91	0,37	2,90
359,3	EVL	v	3,65	0,45	1,54
379,2	DVF	v	25,70	0,95	10,57
402,4	RLD	21	11,78	0,06	3,16
407,3	IEF	21	23,26	1,16	9,06
436,3	GDVF	v	4,35	0,01	1,41
474	Mw474		6,83	0,04	1,94
486,3	ANSPV	21	12,84	0,37	6,18
487,3	GAGGGGL	21	21,45	0,12	5,67
533,2	PGDVF	v	9,41	0,04	2,69
599,4	KDQPL	v	13,70	0,17	5,61
620,5	SPGDVF	v	19,43	0,66	7,30
633,3	VSTDVN	21	4,23	0,16	2,01
689,3	NGKGTIT	v	2,88	0,02	0,82
709,4	DEEGNF	v	6,24	0,10	2,66
746,5	NGTPVIF	21	6,91	0,00	2,03
757,4	ASKDQPL	v	18,33	0,95	6,36
760,4	RRSDLD	21	2,27	0,00	0,29
788,6	TVWRLD	21	6,54	0,02	0,62
819,5	DNEWAW	21	1,59	0,01	0,44
837,4	DEEGNFK	v	1,78	0,05	0,62
861,4	SSISGAGGGGL	21	6,33	0,02	1,57
901,6	APLSPGDVF	v	1,61	0,01	0,47
932,6	DSKDDVVR	21	2,51	0,04	0,68
1063,5	DEEGNFKIL	v	5,95	0,05	1,84
1090	Mw1090		8,55	0,00	1,30
1204,5	SNADSKDDVVR	21	5,21	0,06	1,76
1244	Mw1244		3,81	0,02	0,93
1596	Mw1596		13,21	0,01	1,57

Figure1

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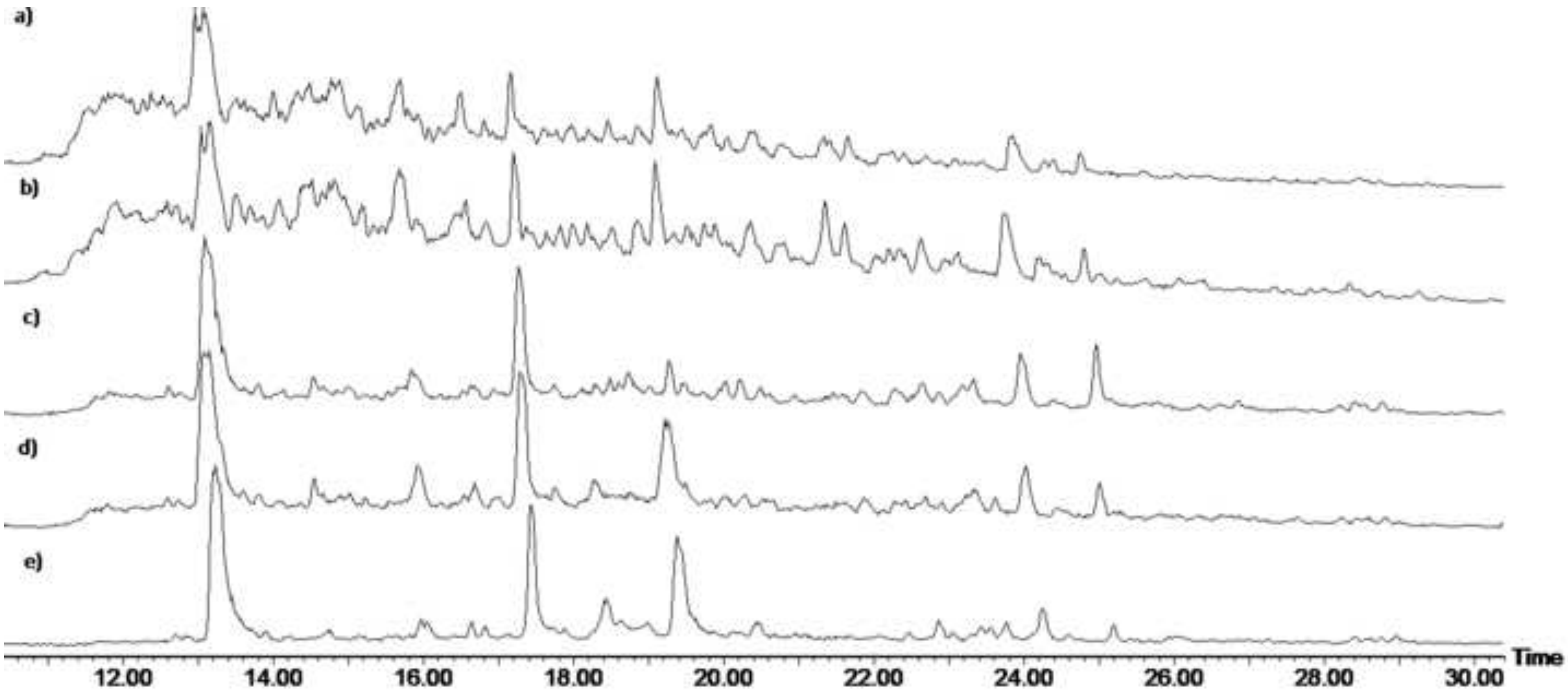
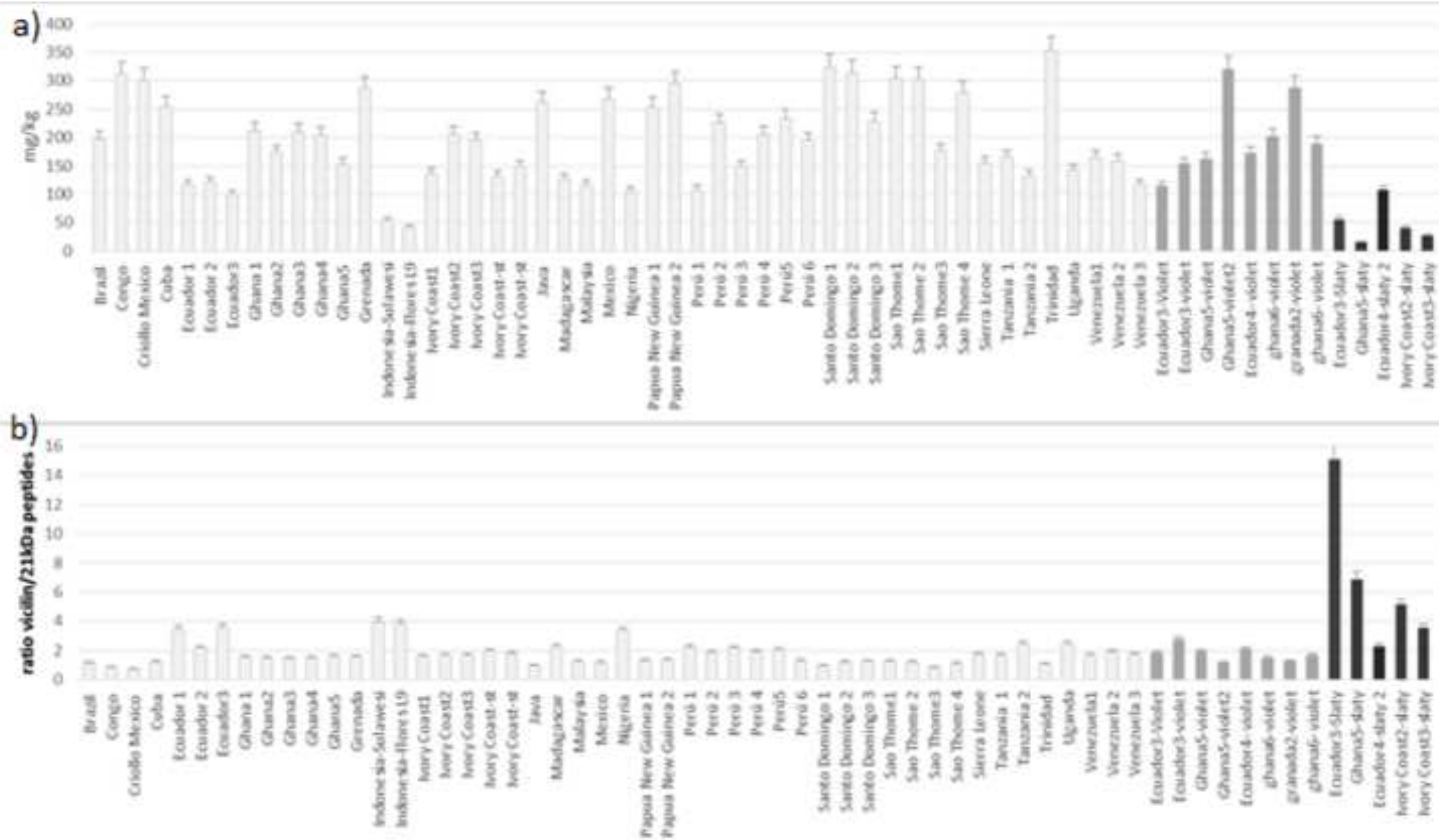


Figure2


a)
mg/kg
0
50
100
150
200
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b)
ratio vicilin/21kDa peptides
0
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Figure3

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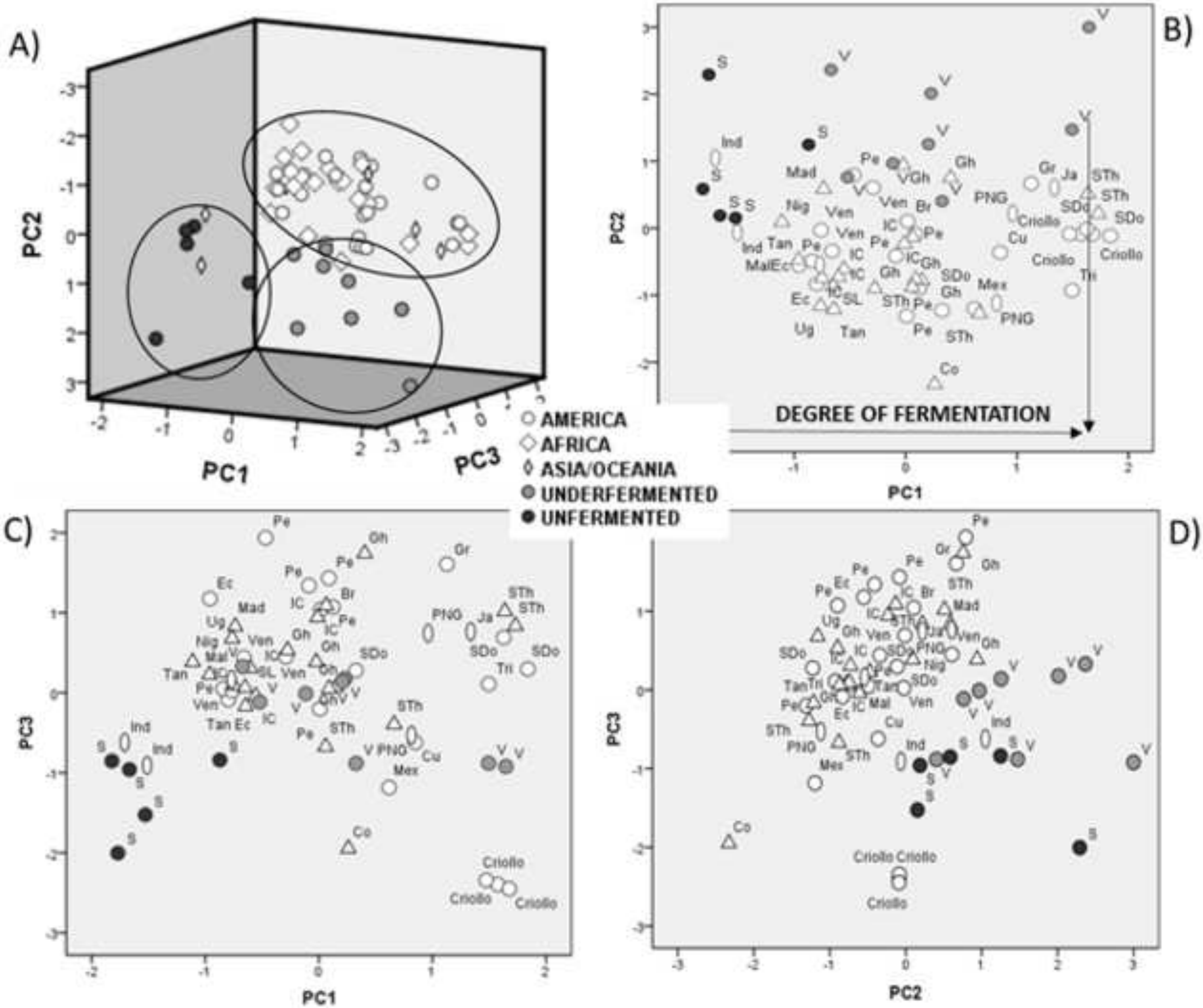

Figure4

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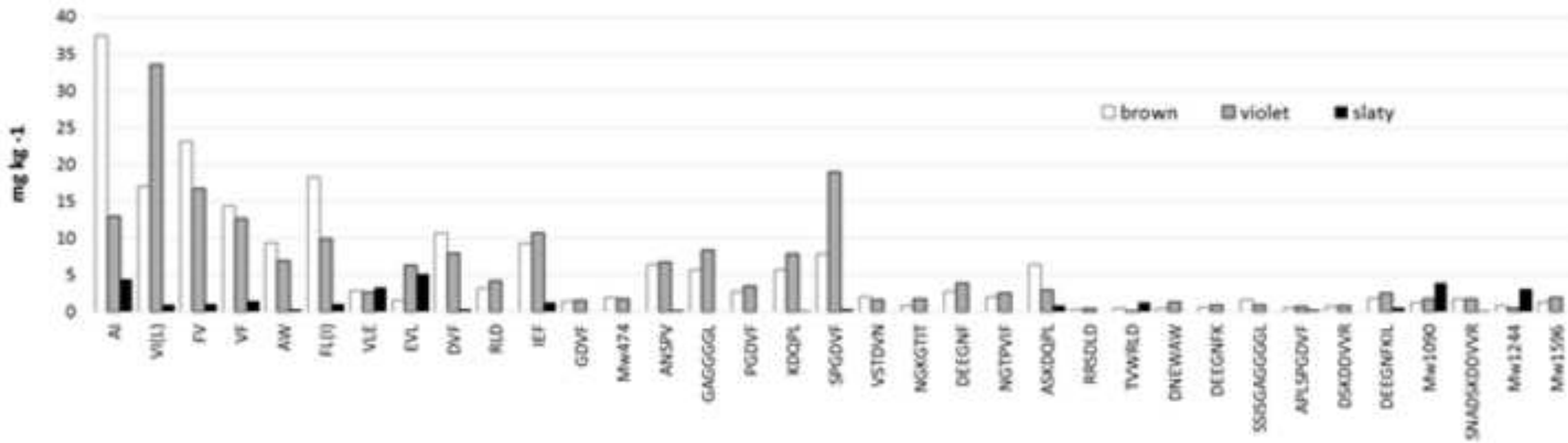

Figure5

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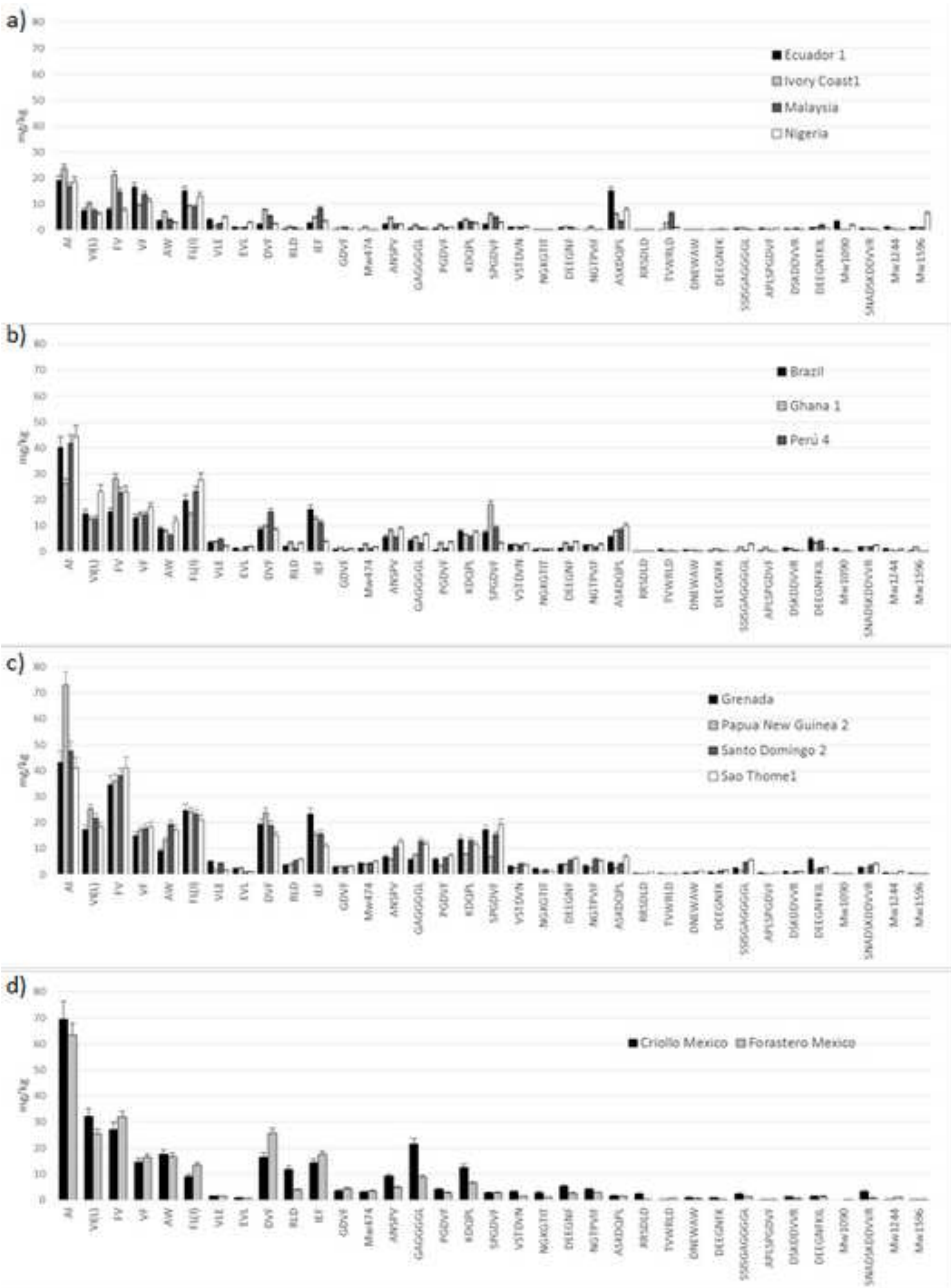

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