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Beyond cadmium accumulation: distribution of other trace elements in soils and cacao beans in Ecuador

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Abstract:

Since cacao beans accumulate Cd in high levels and restrictions have been imposed on safe levels of chocolate consumption, concern about whether or not cacao trees store other toxic elements seems to be inevitable. Following a previous study in Ecuador examining Cd content in five cacao varieties collected in pristine areas and in places impacted by oil activities, we present here the concentrations of 11 trace elements (TEs) (As, Ba, Co, Cu, Cr, Mo, Mn, Ni, Pb, V and Zn) in soils, cacao tissues (leaves, pod husks, beans) and cocoa liquor (CL). Several TEs showed concentrations in topsoils above the Ecuadorian limits, and may have a mixed natural and anthropogenic origin. Ba and Mo concentrations in cacao tissues are slightly higher than those reported in other surveys, but this was not the case for toxic elements (As and Pb). TE contents are lower in CL, than in beans, except for Pb and Co, but no risk was identified for human health.

Compared with control areas, Enrichment Factors were below 2 in impacted areas, except for Ba. Transfer factors (from soils to cacao) indicated that cacao does not accumulate TEs. A positive correlation was found between Cd and Zn in topsoils and cacao tissues for the CCN-51 variety, and between Cd and Ni for the Nacional variety. Identifying patterns of TE distribution and potential interactions in order to explain plant internal mechanisms, which is also dependent on the cacao variety, is a difficult task and needs further research.

Keywords: cacao; trace elements; soil-plant uptake; metal-Cd interactions; Ecuador

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1. Introduction

Cocoa production is one of the main incomes for farmers in tropical countries, and is pursued for high yields and premium quality selection as there are no alternative crops or synthetic products with which to make chocolate (Figueroa-Hernández et al., 2019; Utomo et al., 2016). According to Trade Map (ITC, 2020), in 2019 almost 65% of global cacao exportation was cacao beans (whole or broken, raw or roasted), followed by cocoa butter (fat and oil, 16.9%), cocoa powder not containing sugar (16.9%) and cocoa shells, husks, skins and other waste (1.8%).

Cacao is cultivated on tropical lands in West Africa, Central and South America, the West Indies and Asia (Kongor et al., 2016). Cacao from Ecuador is famous for its fine flavor which is found in several varieties, the two most important being “Nacional Fine Flavor”, which is in great demand in Europe for producing the finest chocolates, and the “CCN-51 variety”, which is very productive and more resistant to tropical diseases (Beg et al., 2017; Vargas Jentzsch et al., 2016). Although its pod morphology and tree architecture are distinct from the traditional Nacional genotypes, CCN-51 is sometimes mistaken for being of Nacional lineage (Boza et al., 2014).

In Ecuador, just under 20 ha of cacao plantations (nearly 76% of the total cultivated) are located on small farms, where it is specifically family labor. Up to 90% is managed under traditional systems, especially for fine flavor cocoa, which means a lesser use of chemical fertilizers, machinery and irrigation units compared to CCN-51 farming (Saravia-Matus et al., 2020).

It is now largely admitted that cacao can contain trace elements (TEs) that mainly accumulate in the beans shell (Yanus et al., 2014). Some are required by plants as micronutrients (Mn, Cu, Zn, Ni and Mo) but others are considered toxic for human health, especially As, Cd, Cr and Pb (Kabata-Pendias, 2011; Page and Feller, 2015; Assa et al., 2018). These elements are obtained directly from the soil and/or during the production process (Assa et al., 2018).

The distribution of elements in cacao beans has recently been studied using LA-ICP-MS bio-imaging techniques, which showed that both essential elements (Mg, P, K, Cu and Zn) and the toxic ones (Pb and Cd) were enriched in the seed shell of the processed cacao bean (Thyssen et al., 2018).

Special attention is being paid to Cd, as the European Union has enforced stricter upper limits for its content in cocoa-based products since January 1st, 2019 (European Commission, 2014). In Latin America high levels of Cd have been found in cacao beans from Ecuador (Argüello

et al., 2018; Barraza et al., 2017; Chavez et al., 2015), Honduras (Gramlich et al., 2018) and Peru (Arévalo-Gardini et al., 2017; Zug et al., 2019). Differences in Cd concentrations in the edible parts may be explained by cultivar or genotype differences in its uptake, root-to-shoot translocation and above-ground partitioning (Barraza et al., 2019; Engbersen et al., 2019; Lewis et al., 2018; Mengist et al., 2017; Moore et al., 2020). Cd is highly toxic for human health, but it is certainly not the only element which accumulates in cacao beans, and more investigation is needed regarding other TEs to better understand the potential risks involved for human health and the possible combined effects of the co-accumulation of toxic metals. Arévalo-Gardini et al. (2017) reported that concentrations of Cr, Ni and Pb in cacao beans and leaves in Peru varied between sampling sites and genotypes. High levels of Ni have also been found in raw cacao and in chocolate (7.6 and 4.5 mg kg⁻¹ respectively) made with beans from Ecuador and the Dominican Republic (Kruszewski et al., 2018). In a recent review of exposure to toxic metals, Anyimah-Ackah et al. (2019) reported variable and sometimes high concentrations -compared to international regulations- of As, Hg and Pb in cacao beans and/or cocoa-based food. A health risk assessment conducted in Saudi Arabia concluded that the estimated daily intake of heavy metals (Al, As, Co, Cu, Cd, Cr, Hg, Mn, Ni and Pb) from the consumption of chocolate and cocoa products was below the US EPA safe standards, however, the hazard index for a specific group of cocoa products was almost twice as high as the recommendations, suggesting that frequent consumption may result in adverse non-carcinogenic health effects in consumers (Salama, 2019).

Interactions between Cd and several micronutrients have been observed, notably with Cu, Fe, Mn, Ni, Se and Zn, producing depressing or enhancing effects in plant nutrition and physiology (Kabata-Pendias and Szteke, 2015; Khaliq et al., 2019), and then potential cumulative effects on human health after consumption. The transport of essential TE across membranes at long-distance, and intracellular sequestration, is often carried out by high specific proteins, but when ions have similar chemical properties they can share the same routes. This also applies to non-essential TEs (Andresen et al., 2018; Van der Ent et al., 2013).

There thus seem to be several questions about the amounts of TEs -potentially toxic or not- in cacao beans, motivating researchers to conduct more studies that can generate reliable data about concentrations, sources, transfer and accumulation within the soil-cacao system.

The aims of our study are thus to: (i) determine the distribution of TEs in the soils of more than 30 Ecuadorian cacao small-scale farms, (ii) understand the distribution of TEs in cacao plant tissues (leaves, pod husks and beans) and in cocoa liquor, in specific varieties of cacao,

(iii) determine if these elements are transferred from soil and later bioaccumulated, in order to better assess the possible health implications, and finally (iv) identify the potential interactions between Cd and other TEs.

2. Materials and Methods

2.1 Study Area

Cacao samples and their associated topsoils were collected between 2014 and 2016 as described in Barraza et al. (2017) from a total of 31 small-scale farms (Fig. SI-1, ESI) located in the Ecuadorian provinces of Orellana and Sucumbíos in the northern Amazon region (NAR); in Morona-Santiago, in the southern Amazon (MS); and along the north and south Pacific coast, in Esmeraldas (NPC) and Manabí (MC), respectively.

The choice of these sites was based on the presence or absence of oil facilities (to serve as control areas), as a part of the MONOIL research program (Environmental Monitoring, Health, Society and Oil in Ecuador).

Five cacao varieties were collected: Nacional, CCN-51, Forastero, Zamora and Nativo amazonico (native) (Fig.SI-1, ESI). Two cocoa liquor (also known as cocoa mass) samples were also collected in Pacayacu (Sucumbíos province, NAR) in order to determine the elemental concentrations in the final product. Detailed information about cocoa liquor elaboration is provided in Barraza et al. (2017).

2.2 Soil and cacao sampling and preparation

One mature cacao pod and ten green leaves were selected for each sampled tree (2 or 3 trees for each point) and mixed together. The associated soil samples were collected as composites around each tree, from 0 to 60 cm, and in some sites to 100 cm depth, which corresponds to the natural geochemical background. Note that the present study focused on the topsoil (0-20 cm) as results have been already presented and discussed for the deepest layers in Barraza et al. (2018). It has been observed that most cacao roots are situated at a depth of 0-20 cm (Kummerow et al., 1982; Niether et al., 2019).

Cacao beans were separated from their pod husks, and air-dried for three days before being oven-dried at 40 °C for two days, as were the leaves. All cacao tissues were ground with liquid nitrogen using an agate mortar and pestle. Soil samples were air-dried, sieved at 2 mm, and ground with an agate bench ring mill.

2.3 Soil physico-chemical properties and soil identification

Pedological and physico-chemical properties such as pH, cationic exchange capacity (CEC) and total organic carbon (TOC) were reported for each sampling site in Barraza et al. (2017). Briefly, soils were divided into three categories: inceptisols (NAR, MS), alfisols (MC) and entisols (NPC) according to the USDA classification (USDA, 1999). pH was acidic (between 4 and 6.8) in the NAR and MC regions, and slightly neutral to basic in the NPC and MC areas (between 7.1 and 8). Low CEC (ranging from 3 to 18 cmol kg⁻¹), typical of tropical soils, was related to soil enrichment in secondary minerals such as gibbsite, goethite and kaolinite (Juo and Franzluebbers, 2003). Contents in TOC were variable, with low values (below 1 g kg⁻¹) in the deepest soil layers (up to 80 cm), and higher values (above 40 g kg⁻¹) in upper layers (0-60 cm).

2.4 Determination of trace elements concentration

Eleven trace elements were identified in the collected samples: As, Ba, Co, Cu, Cr, Mo, Mn, Ni, Pb, Zn and V. These elements were chosen because of concerns regarding their environmental effects, and the potential impact of oil activities in Ecuador (led by the French MONOIL research program).

Dried soil and cocoa liquor (CL) samples (0.1 and 0.125 g respectively) were mineralized -in a clean lab- in a high pressure microwave (Mars 5 CEM®) with a solution of 11 ml of aqua regia (9:2) and 3 ml of HF (for soils) or 9 ml HNO₃, 1 ml H₂O₂ and 0.2 ml HF (for cocoa liquor). Reagents were of Suprapur® quality or sub-boiled (HNO₃ and HCl). Solutions were then completely evaporated at 60 °C on a hotplate and the dried residue was resuspended by adding 0.5 ml HNO₃.

Dried plant samples (0.125 g of leaves, pod husks or beans) were digested on a hotplate system (Digiprep, SCP Science®) using 5 ml of HNO₃ and 5 ml of H₂O₂. Digestion solutions were diluted with high purity deionized water (Milli-Q Element®) and filtered through a clean 0.22 µm cellulose acetate membrane.

Mineralized solutions were finally diluted with HNO₃ 0.37N prior to taking concentration measurements by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent® 7500 CE, GET laboratory).

Each batch of samples included a lab blank, certified reference material and some replicates. The limits of detection (LD) and quantification (LQ) were calculated as three and ten times the standard deviation (SD) of lab blanks. Three certified reference materials (from the National Institute of Standards and Technology): SRM 2709 “San Joaquin soil”, SRM 1515

“Apple leaves” and SRM 2384 “Baking chocolate”, were used to validate the analytical methods. Recoveries between 75 to 100% were obtained for these elements (Table SI-1, ESI).

2.5 Global Enrichment and Transfer Factors

The global enrichment factor (GEF), as defined in Schreck et al. (2014), was calculated to determine the degree of metal accumulation in cacao beans grown in areas impacted by oil activities with respect to a control area, according to the following equation :

Equation (1):

$$GEF = \frac{[TE] \text{ cacao beans grown on contaminated sites}}{[TE] \text{ cacao beans from control site}}$$

where [TE] is the concentration of each trace element expressed in mg kg⁻¹.

Trace element concentrations in cacao beans from the control site were calculated as the mean value of Samples 3 and 4, corresponding to the CCN-51 variety, located in areas not affected by extractive activities, in Morona-Santiago (MS) as described by Barraza et al. (2017). This MS area is free from mining or oil production, agriculture is not intensive and crops are not irrigated. Even if the CCN-51 variety differs from the Nacional, the latter is often used as a parent in crosses in the breeding programs in Ecuador (Boza et al., 2014). As previously noted, it is also common to find Nacional and CCN-51 natural hybridization on local farms.

Trace elements uptake and accumulation from soil to plant, known as bioaccumulation or the transfer factor (TF), and described by Nirola et al. (2015) and Schreck et al. (2012), were also calculated (Equation 2) in order to determine a possible enrichment in TE in cacao tissues (leaves, pod husks and beans) relatively to the topsoil (0-20 cm).

Equation (2):

$$TF = \frac{[TE] \text{ cacao tissues}}{[TE] \text{ soil}(0-20 \text{ cm})}$$

TF>1 indicates that cacao tissues are enriched in an element, TF around 1 indicates the relatively indifferent behavior of the plant towards this element, and TF<1 shows that the plant excluded this element from its mineral nutrition and thus from its uptake (Mingorance et al., 2007).

2.6 Statistical analysis

The R® software version 3.1.1 was used for graphics and statistical analyses. As our data did not follow a normal distribution, Spearman’s correlation (r_s) was used to measure the strength

and relationship between soil properties, TE concentrations in the topsoil and in cacao leaves, pod husks and beans. In addition, Cd concentrations for the same type of samples were included and taken from Barraza et al. (2017). For this purpose, only CCN-51 (13 samples) and Nacional varieties (17 samples) were considered, independent of the sampling site.

Two ranges were fixed to evaluate correlations: a moderate correlation for the r_s range between 0.5 and 0.64, and a strong one for r_s up to 0.65.

3. Results and Discussion

3.1 Distribution of Trace Elements in Soils

In all sampling sites, topsoils included at least two elements above the Ecuadorian threshold for TEs (Table SI-2, ESI). In the oil extraction area (NAR), most showed concentrations above the national recommendations for Ba, V, Zn, Cr, Cu and Ni (Co and Mo in a lesser extent). Only V was above the limits according to international guidelines for soil quality in agricultural areas in Canada (Canadian Council of Ministers of the Environment, 2018) and the European Union (The European Commission, 1986) (Table SI-2, ESI).

National regulations have different limits depending on land use (residential, commercial, industrial and agricultural) but none take into account TE mobility in special environments such as plant systems and water corps (Antoniadis et al., 2019). There is no international agreement with respect to the TEs that have to be regulated, as their natural content varies according to soil type, weathering level and, physiographic regions (Da Silva Costa et al., 2017).

The farms with the highest concentrations were located in the NAR, close to oil facilities (e.g. platforms, oil pits). Concentrations of Ba, V and Ni ranged from 31-785, 86-284 and 8.1 to 37 mg kg⁻¹ respectively, whereas their concentrations in the NPC (in the refinery zone) were less variable, with mean values of 337 ± 66 and 144 ± 10 mg kg⁻¹ for Ba and V respectively. Ni showed concentrations between 35 to 157 mg kg⁻¹ in the NPC (Fig. 1, Table SI-2).

Our previous study in the NAR (Barraza et al., 2018) has already discussed the potential sources of trace elements (a combination of parent rock material, volcanic emissions, oil activities, agrochemical products, among others) and explained their high spatial variability, which also seems to be true in the MS and NPC areas. High concentrations of Ba, V and Ni were measured in crude oil samples (133 ± 5 , 305 ± 36 , and 99 ± 1 mg kg⁻¹, respectively), however, it may not only be oil activities behind high levels of some elements recorded in

these areas, especially considering we studied the farms located in MS and MC, free of oil extraction or refining.

It's now well known that a combination of natural and anthropogenic sources can release significant amounts of TEs into the environment. As an example, intensive agriculture -as developed in MC and in some farms in the NAR- using P-fertilizers, herbicides, pesticides and livestock manure, can increase contents of As, Cd, Cu, Cr, Zn in soils (Kabata-Pendias, 2011; Shi et al., 2018). In addition to the intense weathering and leaching observed in the tropics, combined with clay content, we might expect low pH values, low OM and high levels of Al-Fe or even Mn oxides to result in the relatively high mobility and bioavailability of TEs in soils, such as As, Ba, Cd, Co, Cu and Pb (Cappuyns, 2018; Rieuwerts, 2007; Souza et al., 2018).

Arévalo-Gardini et al. (2016) reported a wide range of concentrations for Mn (82 to 1275 mg kg⁻¹), Zn (3.76 to 112 mg kg⁻¹), Cu (2.43 to 54 mg kg⁻¹), Ni (1.64 to 43 mg kg⁻¹) and Pb (5.62 to 22 mg kg⁻¹) in topsoils in cacao plantations in eight provinces in Peru, depending on the cocoa plantation's location. Elements such as Cu and Ni were more concentrated in the deepest soil layers (until 80 cm) unlike Mn, Zn and Pb. It has been already reported that these elements, especially Mn and Pb, are more concentrated in topsoils, rather than leaching down from the upper layers (Da Silva Costa et al., 2017; Kabata-Pendias, 2011) due to their low mobility in the soil column. The concentrations reported by Arévalo-Gardini et al. (2016) in Peru were in the same range as those found in our study, however the levels recorded for Ni and Cu (8-157 and 12-92 mg kg⁻¹ respectively; Fig. 1, Table SI-1) are slightly higher in our study in Ecuador.

3.2 Trace elements concentrations in cacao tissues and cocoa liquor, and potential health implications

The concentration of TEs in cacao tissues (leaves, pod husks and beans) followed the same tendency observed for Cd in our previous study (Barraza et al., 2017): they were highest in leaves, then in pod husks and finally in beans (Fig. 2; Table SI-3). Concentrations of elements such as As, Co, Cr, V and Pb, were below the detection limits in cacao tissues.

Only Co and Pb were detected in cocoa liquor samples (Table 1).

3.2.1 In cacao leaves

Manganese concentrations ranged from 12 (NPC, Nacional variety) to almost 1960 mg kg⁻¹ (NAR, Nacional variety). The mean concentration of Ba (from all sites and all varieties) was

115 \pm 19 mg kg⁻¹, with the highest value reaching 354 mg kg⁻¹ in the NAR for the Nacional variety (Table SI-3).

Crystals of BaSO₄ were observed by environmental scanning electron microscopy with energy dispersive x-ray spectroscopy (ESEM-EDX; results not shown) in washed and unwashed cacao leaves from some sampling sites located near open flares in oil platforms in the NAR. These results could suggest a link between oil activities and Ba concentrations in cacao tissues, either through atmospheric deposition and/or root uptake, as BaSO₄ is used as a weighting agent during drilling (Oskarsson, 2015).

Concentrations of Zn varied between 17 and 263 mg kg⁻¹, with the highest value also recorded for the Nacional variety. Copper and Ni concentrations in cacao leaves were between 0.65 and nearly 40 mg kg⁻¹. The highest concentrations for both elements were found in the NAR for the CCN-51 variety. Molybdenum concentrations ranged from 0.05 (in MS) to 8.44 mg kg⁻¹ (in MC) (Table SI-3).

Concentrations of Zn and Mn were 1.4 to 4.5 times higher in our study than those reported by Arévalo-Gardini et al. (2017), which mainly included CCN-51 genotypes collected in Peru. However, also in Peru, Huamani et al. (2012) recorded Mn and Zn concentrations approximately 2.7 to 5.4 times lower than values found for cacao leaves in Ecuador. Arévalo-Gardini et al. (2017), however, found Cu and Ni mean concentrations similar to those found in our study.

3.2.2 *In pod husks*

Mean manganese concentrations varied from 11 \pm 2 to 136 \pm 26 mg kg⁻¹ in NPC and MS, respectively (Fig. 2). Concentrations of Zn reported by Gramlich et al. (2017 and 2018) in Latin America were of 42.9 \pm 1.6 and 45.8 \pm 1.4 mg kg⁻¹, respectively in Bolivia and Honduras, and similar to the values found in our study, in the NPC (41.5 \pm 5.9 mg kg⁻¹) and MC areas (47 \pm 4.7 mg kg⁻¹).

Barium concentrations varied from 2 (MC) to 98 mg kg⁻¹ (NAR) in the CCN-51 cacao variety. The highest concentration of Cu (24.20 mg kg⁻¹) was found in the NAR, also in the CCN-51 variety (Table SI-3). Mean nickel concentrations ranged from 3.10 to 6.27 mg kg⁻¹. The highest mean Mo concentrations were 0.58 and 0.24 mg kg⁻¹ for Nacional and CCN-51, respectively (Table SI-3).

3.2.3 *In cacao beans*

Mean Zn concentrations in cacao beans varied from 51 to 65 mg kg⁻¹ in the NPC and in the MS, respectively (Fig. 2). The highest concentration of Mn (176 mg kg⁻¹) was found in the NAR for the Nacional variety (Table SI-3) in a specific fruit affected by a disease caused by a fungus (*Moniliophthora roreri*), one of the most serious problems for cacao production in Latin America (Bailey et al., 2018).

Barium concentrations ranged between 1.2 and 45.6 mg kg⁻¹ for the Nacional variety, in MC and in NAR, respectively (Table SI-3). In the NAR, mean concentration of this element was 16 ± 3 mg kg⁻¹ (Fig. 2). Mean Nickel and Cu concentrations for each variety ranged from 1 to 14 and 5 to 37 mg kg⁻¹ respectively, with the highest values in the Nacional variety (Table SI-3).

In Esmeraldas (NPC) and Manabi (MC), Romero-Estévez et al. (2019) found that the highest concentrations of Ni were 8.53 and 6.03 mg kg⁻¹ respectively, also for Nacional, CCN-51 and blended cacao (i.e. Criollo, Forastero and Trinitario) samples. Compared to our results for the same locations and varieties, Ni mean concentrations were 9.81 ± 1.52 and 8.11 ± 1.02 mg kg⁻¹ (calculated from data summarized in Table SI-3). The same authors found non-negligible Pb contents in beans collected in nine provinces of Ecuador (mean 1.43 mg kg⁻¹), while they were all under the LQ in our study.

In a recent study of the elemental composition of cacao beans (Forastero variety), Bertoldi et al. (2016) reported concentrations of Cu and Ni in samples collected in South America close to our values. Ba (22.2 ± 12.6 mg kg⁻¹), Mn (35.4 ± 11.6 mg kg⁻¹) and Zn (73.4 ± 60.8 mg kg⁻¹) concentrations in cacao beans for West Africa and Asia were also similar to our values for NAR and MS sampling areas. Assa et al. (2018) demonstrated Cu concentrations in cacao beans (without shell) of 10 to 19 mg kg⁻¹ for three cacao clones in Indonesia, which is 1.5 times lower than the content found in our study. Kruszewski et al. (2018) showed concentrations of Ni in cacao beans with shell from Ecuador and the Dominican Republic of 2.32 ± 0.45 and 7.62 ± 0.51 mg kg⁻¹, respectively. The Nacional variety had, considering all sampling sites, a similar mean concentration (6.91 ± 3.62 mg kg⁻¹) to those from Dominican Republic, but almost half that of the CCN-51 variety.

In the same way, mean Mo concentration in our study reached 0.37 ± 0.19 mg kg⁻¹ for CCN-51 variety, similar to the values recorded by Bertoldi et al. (2016) in beans from South America for the Forastero variety. Conversely, Yanus et al. (2014) reported very low Mo concentrations of 0.032 ± 0.005 mg kg⁻¹ in beans from an unknown origin imported by Israel.

Based on these results, we cannot confirm that TEs concentration in cacao tissues are strongly related to a variety effect with any certainty, even if some differences have been observed with respect to Cd concentrations, which seem to be higher in the Nacional variety than in CCN-51 (Argüello et al., 2019; Barraza et al., 2017).

3.2.4 In cocoa liquor

Cocoa liquor is a pure cocoa mass in liquid form produced from the cocoa nibs (fermented, cleaned and unshelled roasted cacao beans) through heat generation during the grinding process. It contains both cocoa solids and cocoa butter in roughly equal proportions to the end product (Beg et al., 2017). For didactic purposes, our results are mainly compared to dark chocolate, as cocoa liquor is closer to the final product than the beans presented in the previous section.

Elemental analysis performed in 10 dark chocolate samples (70-75% of cocoa solids) purchased at local markets in Serbia (Mrmošanin et al., 2018) showed mean contents of 23.3, 14.2, 13.4, 5.37 and 3.21 mg kg⁻¹ for Zn, Cu, Mn, Ba and Ni, respectively. Of these elements, only Mn and Ba concentrations were similar in our study, and Zn, Cu and Ni were twice as high in our CL samples. Kruszewski et al. (2018) reported Ni concentrations of 5.72 ± 0.13 and 5.87 ± 0.10 mg kg⁻¹ in cocoa liquor samples from multiple producers (chocolate factories in Poland). These values were in accordance with our sample CL-1, but 1.5 lower than our sample CL-2 (Table 1).

Eight dark chocolate samples (> 60% of cacao solids) elaborated with cacao beans from South America (Bertoldi et al., 2016) presented Mn and Ni concentrations similar to those in our study, whereas Zn and Cu were half as much (25.9 ± 10.2 and 13.3 ± 4.7 mg kg⁻¹ respectively). By contrast, Ba mean concentrations were almost the double those in our samples (7.93 ± 1.78 against 4.9 ± 1.84 mg kg⁻¹). Recently, Vanderschueren et al. (2019) found mean concentrations of 29 ± 8 , 13.6 ± 5.9 , 13 ± 4.2 , 4.9 ± 3.3 , 3.2 ± 1.8 and 0.24 ± 0.1 mg kg⁻¹ for Zn, Cu, Mn, Ba, Ni and Mo, respectively, in chocolate bars from South America (including 34 samples from Ecuador) with a median of 70% cacao solids, which were 2 to 3 times lower than our results, except for Mn (Table 1).

If we calculate mean TE concentrations in raw beans – mixing Nacional and CCN-51 varieties samples in Sucumbíos (farms 13 to 31) which is the same region as where the CL samples were collected – Cu, Mo and Ni showed similar values while Mn, Zn and Ba concentrations were higher in CL compared with raw beans. This is in agreement with Yanus et al. (2014) who found increased Mn, Co and Mo in cocoa powder with respect to raw beans

and less in the cocoa butter. These differences might be explained by the fact that some elements are more concentrated in the shells, which is removed to elaborate cocoa products, than in the bean core itself (Thyssen et al., 2018; Yanus et al., 2014).

Conversely, for Co and Pb -which were not detected and/or were below the LQ in raw beans- the concentrations found in CL samples would suggest either an enrichment or a slight contamination during manufacturing or lab analysis. The Pb concentrations measured in our CL samples (between 6 and 10 $\mu\text{g kg}^{-1}$; Table 1) were a tenth of the mean observed in several chocolate brands consumed, for example, in Israel (Yanus et al., 2014) and 100-fold lower than the Pb concentrations measured in Ecuadorian cocoa beans (Romero-Estévez et al., 2019).

Finally, in terms of toxic element content and associated health risks, there are no current or updated regulations for TEs other than Cd in chocolate, but several limits are proposed in the CODEX Alimentarius. A report of the Codex Committee (FAO and WHO, 1997) indicates that the presence of Pb and Cu should not exceed 1 or 2 and 20 or 35 mg kg^{-1} respectively in food, depending on the content of sweeteners (with vs without) and the percentage of cocoa solids in chocolate. Assuming this scenario, both CL samples had levels above the recommendations for Cu (in chocolate products, with the exception of the unsweetened ones). Co, Mn, Mo and Zn are essential for humans, and knowing that their toxicity (very low in some cases) is related to other relevant exposure pathways (inhalation, water ingestion or dermal contact), none represent a real threat to the health of regular chocolate consumers. Some of the estimated safe or adequate daily dietary intakes (Co: 0.005-0.029 mg; Mn: 2-5 mg; Mo: 0.075-0.250 mg; Zn: 8-11 mg, as reported by Kabata-Pendias and Szeke, 2015) are not exceeded even when consuming a bar of dark chocolate (~110 g) per week.

The results are the same for non-essential elements such as Ba, with an estimated daily dietary intake of 0.75 to 1.33 mg and a tolerable daily intake of 0.2 mg kg^{-1} BW, and Ni with a tolerable daily intake of 12 $\mu\text{g kg}^{-1}$ of BW (Kabata-Pendias and Szeke, 2015), they do not pose any threat to human health.

3.3 Influence of biotic and abiotic factors on TE uptake in cacao

The biochemical cycles of TEs in plants are governed by several external and internal factors including soil properties (mobility and fate), the behavior of elements in soil solution (competition, interactions), plant physiology (transport, genotypes), and the rhizosphere microbiota (Antoniadis et al., 2017; Kabata-Pendias, 2011). Each factor will be discussed in more detail below.

3.3.1 *Plant variety*

The uptake capacity of TEs varies between genotypes of the same plant species as observed in Chinese cabbage (Mi et al., 2019), asparagus (Conversa et al., 2019) and rice (Fahad et al., 2019). Significant differences between genotypes have been found in cacao with regard to physiological traits such as leaf chlorophyll content (Daymond and Hadley, 2004), stomatal conductance (Daymond et al., 2011), biomass and plant growth (Lahive et al., 2019).

As already mentioned in Section 3.2, similar concentrations of TEs have been found in the CCN-51 and Nacional varieties (which represents 88% of the samples), suggesting that this factor might not have any strong effect on TE accumulation in cacao tissues. Correlations between elements and soil parameters could suggest some differences, however, which will be discussed in Section 3.4.

3.3.2 *Influence of anthropogenic activities*

Based on GEF calculations (Fig. 3) that allow comparisons with pristine sites in terms of extractive activities, the cacao beans in our study appear to be mainly enriched in Ba (66% of the samples), Zn (62%), Ni (59%), Mn (28%) and to a minor degree, Cu (3%). None of the samples displayed a GEF up to 1 for Mo.

The highest GEFs (8.2 and 11.8) were found for Ba in cacao beans (Nacional and CCN-51 varieties, respectively) collected in two farms located in the NAR, in areas well-known for the large record of oil spills, oil waste disposal and gas open flares emissions (Sampling Sites 6 and 9 respectively).

The GEF for Cd in the same samples (Barraza et al., 2017) was 4 to 18 times higher than the values for the TEs presented here, explaining among other things, why Cd is of high concern nowadays.

3.3.3 *Effect of soil and elements properties*

The transfer factors for the CCN-51 and Nacional varieties were higher in leaves, and then in pods or beans, except for Cu (beans > pod husks ~ leaves). TFs from soil to leaves for essential elements such as Zn, Cu, Mo and Ni were also lower than those for Mn and Ba, whereas TFs in beans were higher for Mo, Cu and Zn (Table 2).

As the TF depends on both soil properties and plant characteristics, it is closely related to the concept of the bioavailability of TEs, because for a given element, it records only the portion that is able to be uptaken by the plant (Antoniadis et al., 2017).

The TF values also showed that after translocation to the shoots, the redistribution from leaves to pods and then to beans seems to be limited. As discussed by Page and Feller (2015), some elements within shoots can be redistributed from senescing leaves via the phloem to sinks such as growing vegetative parts and maturing fruits. As recently observed for three different cacao cultivars in Honduras, however, a substantial amount of Cd would be directly loaded from stems and branches into the beans, without prior passage through leaves, which implies a substantial xylem-to phloem transfer in the woody parts of cacao trees (Engbersen et al., 2019). Our results therefore also suggest different mechanisms for the transfer of other TE and their distribution within cacao tissues.

Usually Mn, Cu, Mo and Ni are quite mobile in soil and readily taken up by plants, whereas Cd and Zn are very mobile and easily bioaccumulated (Kabata-Pendias, 2011). Cadmium and Zn share very similar chemical properties and it has been shown that a relative deficiency in Zn in the soil may lead to increased Cd uptake as they compete for the same transport membranes (Sarwar et al., 2010).

Our results only found a Zn enrichment in a few samples ($TF_{\text{leaves}} > 2$, both varieties), as Zn could be widely leached in depth in these kinds of tropical soils with low pH values and high weathering processes. Several factors (P compounds, clay fractions, solved organic matter) can also hold Zn quite strongly, reducing its phytoavailability (Kabata-Pendias and Szteke, 2015).

The mobility of Cu decreased at pH 7-8, and its phytoavailability was affected by Cu species in soil solutions rather than by total concentration (Kabata-Pendias and Szteke, 2015). The Cu-TF also remained below 2 (Table 2), indicating that cacao trees only seem to uptake the Cu quantities needed for physiological functions.

Nickel is highly phloem-mobile and can be repeatedly redistributed throughout vegetative growth and the reproductive phase (Page and Feller, 2015), but, in some samples of cacao, it seemed to remain concentrated in leaves and pod husks ($TF = 1.40$ in these two tissues, for both varieties), without reaching higher levels, as in Ni-accumulators (Rascio and Navari-Izzo, 2011). However, contrary to the assumption that Ni availability increases at pH less than 6.5 (Shahzad et al., 2018), our results did not show a clear trend in the increase of Ni concentration in cacao tissues, nor its TFs.

The behavior of Mo in soils has been extensively studied because it holds a unique position among other micronutrients. In soils with $pH < 5.5$, Mo is hardly available to plants, whereas it is readily mobilized in alkaline soils (Kabata-Pendias and Szteke, 2015). These statements

are in agreement with our results: the lowest Mo-TFs were observed in soils with a pH < 5.9, although some high TFs were found in leaves (see Sampling Site 23).

A TF_{leaves} of 14.29 and 5.04 were found for the Nacional variety in the sampling site 11 (NAR) for Mn and Ba respectively, where the soil pH was 4.4. Both elements are readily phytoavailable in acid soils (Abbasi et al., 2016; Kabata-Pendias, 2011). In tropical soils Ba generally occurs in the mobile form of hollandite - associated with Mn (hydro)oxides- and easily available to plants (Cappuyns, 2018; Kabata-Pendias, 2011)

An enrichment of Mn was observed in leaves for one sample affected by the frosty pod disease (Sampling Site 7, Nacional variety). This essential element is known to have positive effects in enhancing plant tolerance against fungal diseases (Eskandari et al., 2018) and on the mitigation of Cd toxicity in the CCN-51 cacao genotype (Oliveira et al., 2020). A continued accumulation of Mn in the oldest leaves, with a limited translocation to young organs, results from the translocation of Mn from the roots in some plants (Kabata-Pendias, 2011; Xu et al., 2006).

Close attention should be paid to the transfer factors calculated here for leaves and pod husks, since concentrations of TEs could also result from dust depositions and not necessarily only from root uptake and redistribution within the plant. The foliar transfer of metals is either not present or in some cases appears as the main pathway for uptake in severe contaminated sites (Shahid et al., 2017). This could be the case for certain cacao farms located close to oil flares, since TE concentrations in air seem to be high for elements such as Ba (Barraza et al., 2020).

3.4 Insights for patterns of distribution and potential interactions between TEs in the soil-cacao system

Synergetic and antagonist effects are common behaviors among TEs in plants where different chemical species of the same element can affect each other, or when one or several elements alter the distribution of the others in plant tissues. The best ways to try to identify these effects in cacao, together with its mineral nutrition status, is through controlled conditions in greenhouse experiments (Blommaert, 2019; Pereira de Araújo et al., 2017) or directly in the field collecting samples from a well-defined study area (Marrocos et al., 2020; Quintino R. de et al., 2020).

In this study, we were confronted with the reality of the field sampling, where crop conditions were not controlled: (i) cacao varieties were classified based on the phenotype information supplied by the farmers, and (ii) TEs concentrations in Ecuadorian tropical soils displayed high spatial variations due to natural or anthropogenic perturbations (Barraza et al., 2018).

Nevertheless, the combination of TF calculations and correlation matrices, demonstrated some differences in the pathways for the uptake of TEs.

3.4.1 Correlations between Ba, Cu, Mn, Mo, Ni and Zn

In the Nacional variety, Ni concentrations in leaves, pods and beans were strongly correlated, and in the CCN-51 variety, Zn concentrations in leaves and beans were strongly correlated (Fig. SI-2, Table SI-4). Both varieties showed strong correlations between Mn and Ba concentrations in leaves, pods and beans; Ba and Zn in pods and leaves; Mn and Zn in pods and between Ba and Cu in beans (Fig. SI-2, Table SI-4). The concentrations of these three elements (Ba, Mn and Zn) in leaves also correlated with physicochemical soil properties: negatively with pH and positively with CEC. Total organic carbon (TOC) was moderately correlated with Mn, Ba and Zn in leaves in the Nacional variety (Fig. SI-2 and Table SI-4). These observations suggest that soil characteristics can play a crucial role in Ba, Mn and Zn absorption, by changing their chemical form and properties, and thus by extension their bioavailability for cacao plants.

Some positive or negative correlations were also observed between TEs in topsoils and cacao tissues:

- In the CCN-51 variety: Zn in topsoil had a positive correlation with Zn in leaves and beans, and Ni in topsoil was also positively correlated with Ni in beans. Zn in topsoil showed a positive correlation with TOC. High levels of soil organic matter and lower pH increase the pool of bioavailable Zn in soils (Gupta et al., 2016). This result would suggest that the soil clearly affected the absorption by diffusion of these two elements. Passive diffusion and active transport are reported as the two main mechanisms responsible for the root uptake of Zn and Ni in plants (Gupta et al., 2016). Low affinity Zn transporters present at the plasma membrane (epidermal cells of roots) are also known to play a key role in root Ni-uptake (Gupta et al., 2016; Shahzad et al., 2018).
- In the Nacional variety: Ba in topsoil showed a negative correlation with Ba in leaves and beans, and Ba in topsoil correlated positively with pH (Fig SI-2 and Table SI-4), highlighting a more specific active mechanism of Ba uptake. The strong ability of plants to absorb Ba could be related to the Ca or K uptake (Ferreira et al., 2019; Myrvang et al., 2016a, 2016b) that occurs during active or passive transport. Nevertheless, a higher pH enhances Ba^{2+} sorption to Al and Fe oxyhydroxide minerals, which are difficult for plants to take up (Lu et al., 2019), while a low CEC has the opposite effect (Myrvang et al.,

2016a). The latter is demonstrated by the strong positive correlation between CEC and Ba in leaves (Fig SI-2 and Table SI-4)

3.4.2 *Potential interactions between Cd and other TEs*

In CCN-51, Cd concentrations in leaves were not only strongly correlated with Ni and Zn in leaves but also with Cd in beans and in topsoil, respectively. In pods, Cd was correlated with Cu in pods and Cd in beans. Cadmium in beans correlated with Zn and Cd in topsoil and with Zn in beans (Fig. SI-2, Table SI-4).

In the Nacional variety, concentrations of Cd in leaves were strongly correlated with Cd in beans, whereas Cd in pods was correlated with Ni and Cd in beans. The Cd content of beans correlated with the Ni contents. The main differences with the CCN-51 variety were: (i) Cd concentrations in topsoil correlated with Mo concentrations in pods; (ii) Cd concentrations in topsoils correlated negatively with Ba contents in pods and Mn contents in beans; and (iii) the correlation between Cd in topsoils and cacao tissues was low ($r < 0.5$) (Fig. SI-2, Table SI-4).

Recent studies dealing with the bioavailability of elements in soils can provide some explanations, especially for Zn and Cd interactions. Cd and Zn have been reported to depress and/or enhance their bioavailability to each other, but synergistic interactions have mostly been observed (Gupta et al., 2019; Kabata-Pendias, 2011), perhaps because both elements could share the same transporters such as ZIP proteins (Clemens et al., 2013).

According to Meter et al. (2019), the results of fewer correlative studies between Zn and Cd levels in soil and cacao beans have so far not increased understanding of the role of Zn in reducing Cd uptake. In fact, Zn fertilization has been proposed as a mitigation technique to reduce Cd in cacao, as observed in other plants (Gray and Wise, 2020; Hamid et al., 2019).

Nevertheless, most studies of cacao have reported positive correlations between Cd and Zn in cacao leaves (Blommaert, 2019; Engbersen, 2017; Gramlich et al., 2018) or between Zn in beans and Cd in leaves (Arévalo-Gardini et al., 2017), suggesting that competition did not play a major role in uptake. Argüello et al. (2019) found that Zn in cacao leaves was within the range of adequate plant nutrition, suggesting that its deficiency was unlikely to be a factor affecting Cd accumulation in cacao beans.

These observations are in agreement with our results, but apparently CCN-51 and Nacional varieties display different mechanisms for Cd uptake. The former showed stronger correlations between Zn and Cd in all cacao tissues (leaves, pod husks and beans) and in

topsoil, while the latter showed correlations mainly with Ni (in cacao tissues) and with Mo in topsoil.

Recent studies of Zn and Cd isotopes in wheat had shown an opposite fractionation caused by the distinct affinities of these elements to functional groups of organic ligands: Cd might be bound to S ligands (glutathione or phytochelatin), whereas Zn is expected to be bound to O and N functional groups (phytosiderophores or nicotianamine) (Wiggenhauser et al., 2016; 2018).

In the case of cacao plants, Cd isotopic fractionation showed either an enrichment of light isotopes (Nacional variety) –similar to Zn in wheat (Wiggenhauser et al., 2018)- or a non-resolvable fractionation (CCN-51 and hybrids) in beans with respect to leaves, suggesting different mechanisms for root retention and/or translocation (Barraza et al., 2019). Conversely, under controlled conditions, cacao clones seem to utilize similar pathways for Cd sequestration but differ in their efficiency, suggesting that NRAMP5 transporters are an important pathway for the uptake of Cd by cacao (Moore et al., 2020).

With respect to the other correlations, Cd and Ni have been reported to compete for the same membrane carriers in plants (Gupta et al., 2019). An interesting observation is that the positive correlation found between these two elements (in the Nacional cacao variety) and the weak correlation between Cd and Zn could be explained by the fact that a reduction in Zn^{2+} uptake is expected when Ni^{2+} is more abundant in the soil solution (Shahzad et al., 2018). However, some authors have reported that the lack of linear correlation between Cd and Ni in cacao beans was simply because each metal has an independent mechanism of absorption (Romero-Estévez et al., 2019).

The uptake of Mo was found to increase under Cd stress, without a clear impact from Cd on the Mo translocation from shoot to root (Ismael et al., 2018). This trend has been observed in other plants, such as yellow monkey flower, where both elements showed a strong positive relationship probably because both elements are tightly linked through Fe-homoeostasis (Lowry et al., 2012).

Finally, this kind of correlation matrices can bring new insights for the understanding of interactions between elements in the cacao mineral nutrition, depending on the cacao genotypes and topsoil characteristics. It is still hard to draw conclusions, however, and further studies are in progress to better identify the role of each element and how they can modulate the global uptake of major and trace elements, and thus the potential contamination risk through bioaccumulation processes.

4. Conclusion

The present study, conducted in a total of 31 small farms located in five different provinces of Ecuador, has provided a special focus to the distribution of a cocktail of trace elements in topsoils and the associated cacao plant tissues. The main results suggest that even if concentrations of As, Ba, Co, Cu, Cr, Mo, Mn, Ni, Pb, V and Zn are relatively high in soils (due to natural occurrence and/or anthropogenic activities), their transfer to cacao leaves, pod husks and beans remains quite low.

Cacao varieties displayed different levels of TEs in each tissue but their effect is clearly modulated in the field by physicochemical soil properties, plant physiology and perhaps also by their proximity to oil facilities in the study area. The TEs concentrations in the cocoa liquor samples –including Pb and Cu, both regulated by the Codex Committee- remained low and safe for human consumption when compared to estimated daily intakes.

Although only two of the most popular cacao varieties cultivated in Ecuador were considered (CCN-51 and Nacional), moderate to strong correlations and some differences have been observed across some parameters, including Cd interactions with Zn and Ni. These results have already been observed in other crops, since elements with the same ionic charges share membrane transporters.

However, the use of isotopic geochemistry tools seems to be an effective method with which to identify the role and the expression of those transporters, and further studies are now in progress to better understand these mechanisms.

Finally, Cd interactions between major elements (N, P, K, Mg, S, Ca and Fe) essential for cacao crops but not addressed in this study, could led to a better interpretation of their interactions. Further research is thus required in order to evaluate the nutritional status of cacao seedlings exposed to different levels of Cd, specifically under controlled conditions or specific fertilizing amendments.

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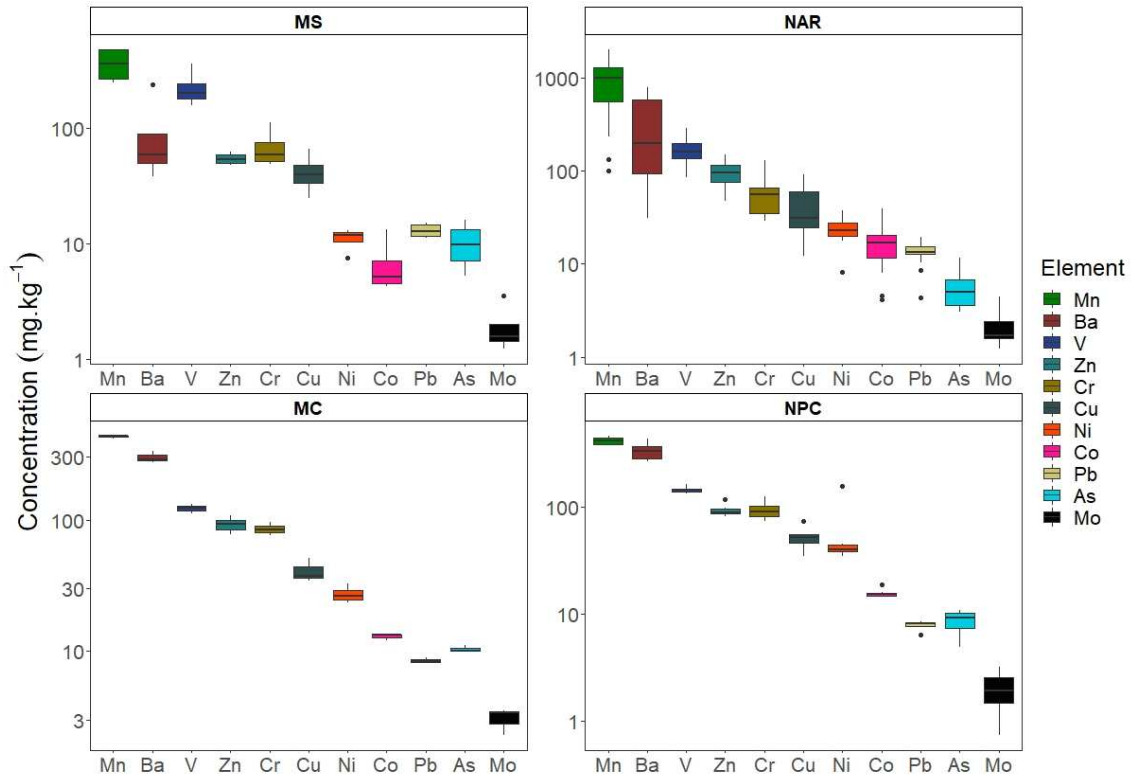


Fig. 1. Mean concentrations (mg kg⁻¹)* of Mn, Ba, V, Zn, Cr, Cu, Ni, Co, Pb, As and Mo in topsoils (0-20 cm) collected in the Ecuadorian provinces of Morona Santiago (MS: control area, free from mining and oil activities), Orellana and Sucumbíos (NAR: oil extraction), Manabí (MC: control area, free from mining activities but with intensive agriculture) and Esmeraldas (NPC: close to the main national oil refinery).

*from analytical replicates

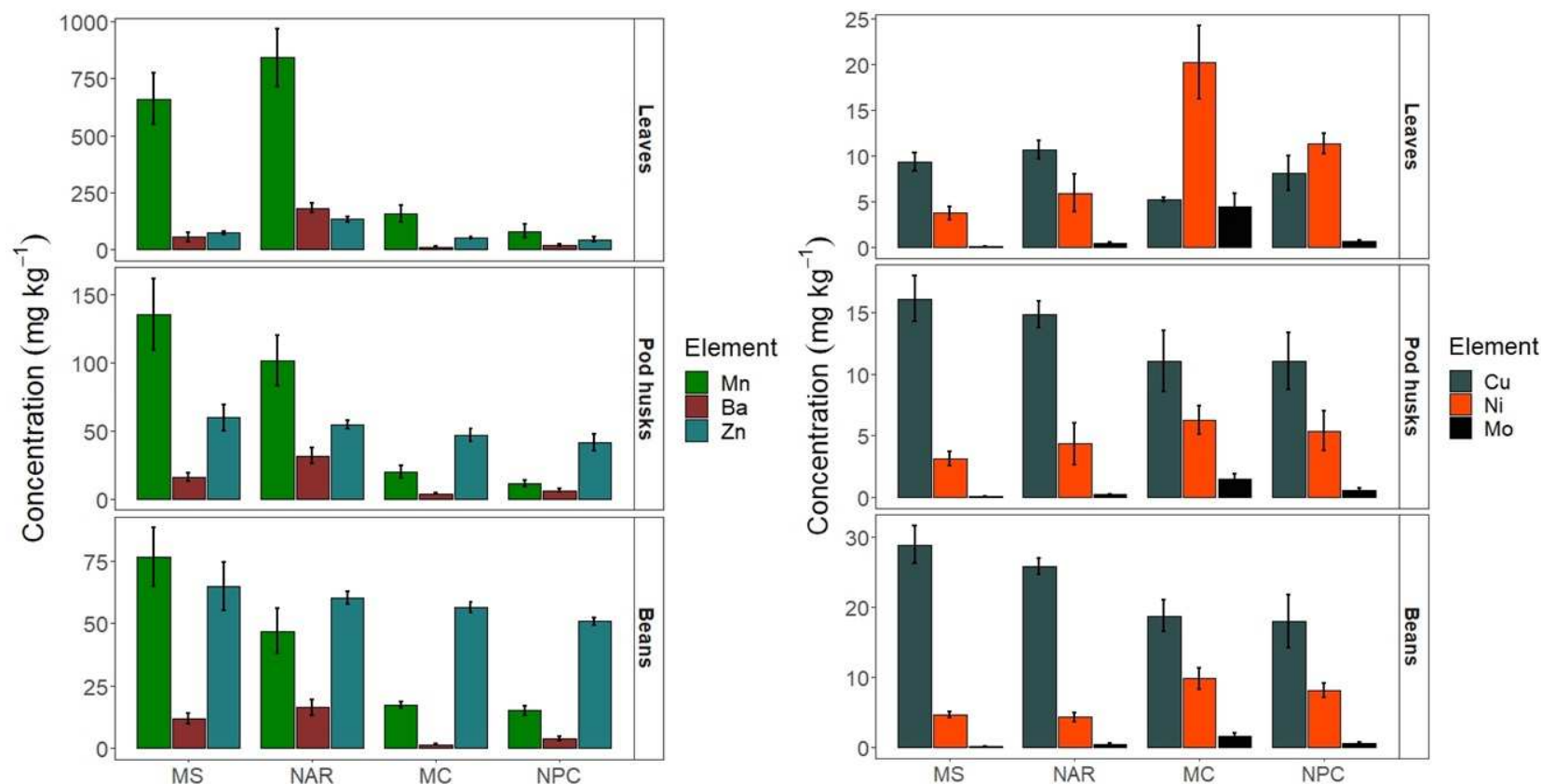


Fig. 2. Mean concentrations (mg kg⁻¹ ± SE) of Mn, Ba, Ni, Cu and Zn in cacao leaves, pod husks and beans from Ecuador for all varieties collected in the field and classified according to sampling area: Morona Santiago (MS: control area, free from mining and oil activities), Orellana and Sucumbíos (NAR: oil extraction), Manabí (MC: control area, free from mining activities but with intensive agriculture) and Esmeraldas (NPC: close to the main national oil refinery).

SE: standard error, calculated from SD and number of samples

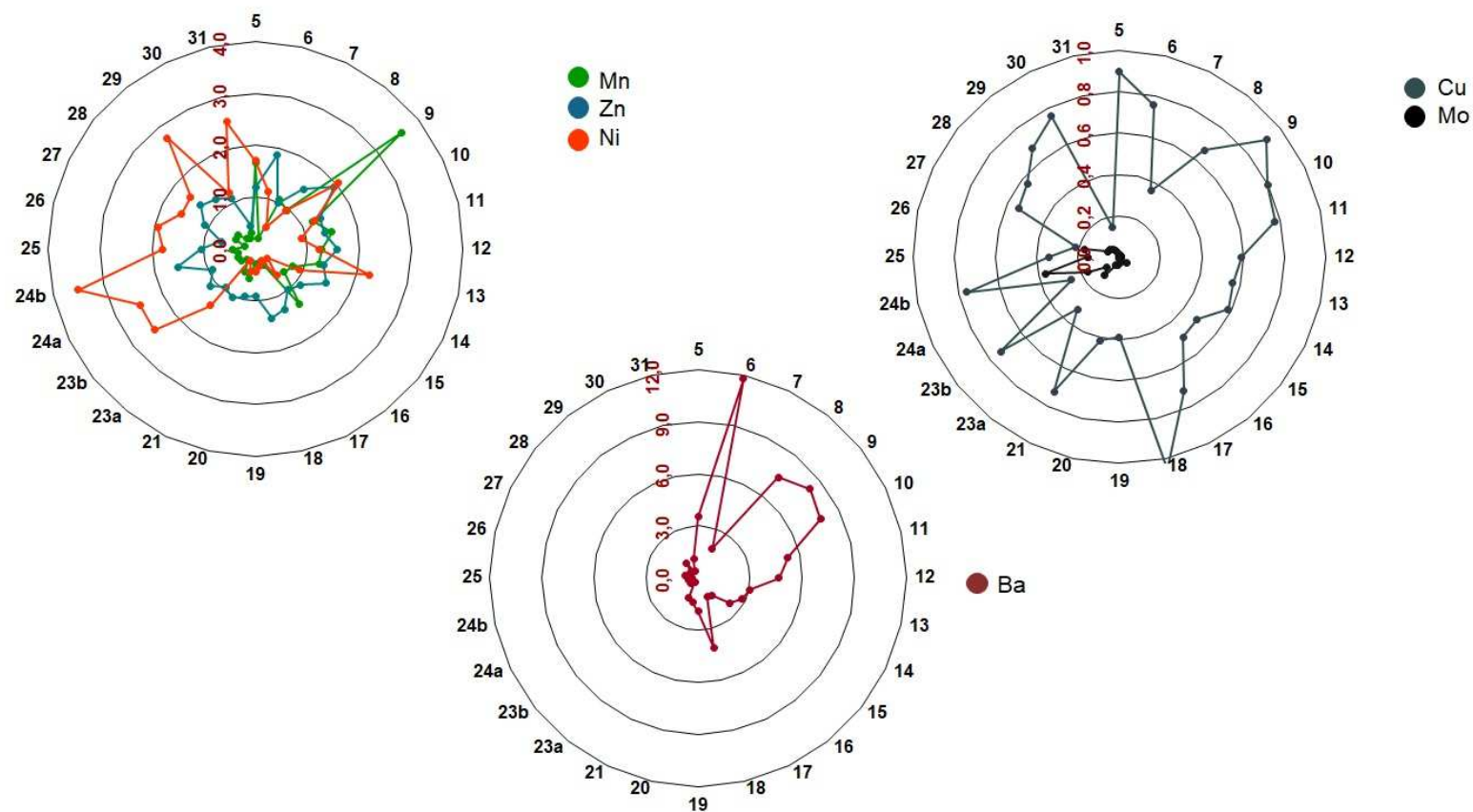


Fig. 3. Global Enrichment Factors (GEF) calculated for Mn, Zn, Cu, Mo, Ni and Ba in Ecuadorian cacao beans. Numbers outside the spider graphic (from 5 to 31) represent the sampling codes for cacao beans (for CCN-51 and Nacional varieties).

Table 1. Concentrations of trace elements (in mg kg⁻¹ ± SD, except for Pb expressed in µg kg⁻¹ ± SD) in the 2 cocoa liquor samples (CL-1 and CL-2) collected in Pacayacu, province of Sucumbíos, in the North Amazon Region (NAR) of Ecuador, compared with the CODEX Alimentarius guidelines.

Samples	Zn	Cu	Mn	Ba	Ni	Mo	Co	Pb [¥]
Codex^a	--	20 [§] ,35 [‡]	--	--	--	--	--	1000 [§] ,2000 [‡]
CL-1^{*,b}	54 ±3	26±0.06	18±0.2	6.68±0.06	4.38±0.02	0.93±0.02	0.55±0.03	6.08±0.30
CL-2^{*,c}	44±5.4	23±0.06	12±0.1	9.19±0.09	7.76±0.03	0.54±0.01	0.38±0.02	10.36±0.52

SD= standard deviation from lab replicates of the same sample

¥ in µg kg⁻¹

^a United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO), CODEX Committee on cocoa products and chocolate (1997)

[§]chocolate, [‡] unsweetened chocolate

*CCN-51 x Nacional hybrids

b,c different years of production

Table 2. Mean, min and max values of Transfer Factor (TF) of trace elements for cacao leaves, pod husks and beans (CCN51 and Nacional varieties, n=30).

Trace elements		Essentials					Non-essential
		Mn	Zn	Cu	Mo	Ni*	Ba
Transfer Factor	Leaves						
	Mean	1.42	1.21	0.26	0.51	0.35	0.96
	Min	0.03	0.17	0.08	0.01	0.03	0.01
	Max	14.29	2.98	0.73	3.65	1.40	5.04
	Pod husks						
	Mean	0.17	0.59	0.38	0.18	0.18	0.17
	Min	0.01	0.23	0.09	0.02	0.02	0.01
	Max	1.37	1.11	1.01	0.86	1.40	1.14
	Beans						
	Mean	0.09	0.68	0.66	0.24	0.21	0.09
	Min	0.01	0.36	0.14	0.02	0.04	<0.01
	Max	0.56	1.28	1.94	1.25	0.45	0.34

*Ni is considered as essential for several plants (Fabiano et al., 2015; Kabata-Pendias and Szteke, 2015)

Mixed- sources of trace elements (TEs)

