Multielemental fingerprinting and geographic traceability of Theobroma cacao beans and cocoa products

Multielemental fingerprinting and geographic traceability of Theobroma cacao beans and cocoa products

Bertoldi, D.1, Barbero, A.1, Camin, F.1, Caligiani, A.2, Larcher, R.1

[1] Fondazione Edmund Mach, via E. Mach, 1, 38010 San Michele all'Adige, Italy

[2] Dipartimento di Scienze degli Alimenti, Università di Parma, Parco Area delle Scienze 59A,

43124 Parma, Italy

Abstract

The isotopic profile (δ13C, δ15N, δ18O, δ2H, δ34S) was used to characterise a wide selection of cocoa beans from different renowned production areas (Africa, Asia, Central and South America). The factors most influencing the isotopic signatures of cocoa beans were climate and altitude for δ13C and the isotopic composition of precipitation water for δ18O and δ2H, whereas δ15N and δ34S were primarily affected by geology and fertilisation practises. Multi-isotopic analysis was shown to be sufficiently effective in determining the geographical origin of cocoa beans, and combining it with Canonical Discriminant Analysis led to more than 80% of samples being correctly reclassified

Keywords: cocoa bean, stable isotope, IRMS, geographic origin

Introduction

The cacao tree (Theobroma cacao L.) is a major and stable basis of agricultural income for farmers in a limited tropical zone, corresponding approximately to the tenth parallel to the north and south of the Equator (in particular Central America, South America, Africa and Indonesia). Its fermented and dried beans, designated as raw cocoa, are the main raw material in chocolate manufacturing.[1] In 2012, global production of cocoa beans was around 3945 thousand tonnes and the main world producers were Indonesia and African countries such as the Ivory Coast and Ghana.[2]

Fresh cocoa beans undergo a fermentation and drying process in the countries of origin, where traditional local processes[3] and specific climatic conditions can influence the final chemical composition and flavour of cocoa to a considerable extent,[4] leading to trading of products typical of the country of origin.[5] Three important varieties of cocoa are commonly employed: Forastero, Criollo and Trinitario (the last is a crossbreed between the other two). Forasteromakes up 95% of world cocoa production, but particular varieties (e.g. Criollo or the Forastero variety known as Arriba), grown mainly in certain countries (e.g. Venezuela, Ecuador and Mexico), are renowned for having the finest flavour and command higher prices. In general, cocoa bean prices depend on the variety, seasonal weather conditions, global crop production, the position of cocoa processors and chocolate manufacturers on the market, and last but not least, on geographical provenance.[6] As result of its status as an important global commodity, there is a high risk of commercial fraud. Assessing the provenance of cocoa has become more and more important in the last few years, due to the increasing market for high quality cocoa products and in particular for mono-origin products (e.g. chocolate from Ghana, Ecuador or Venezuela). In this framework of complex economic interests, the development of an effective analytical method capable of establishing the actual geographical provenance of cocoa beans, to detect mislabelling and prevent fraud, is one of the major issues for the chocolate industry. Several analytical techniques and statistical approaches have been used in order to verify the origin of cocoa beans. Determination of nutritional composition (fat content) and fatty acids (FA) has been applied with promising results.[7] Carrillo et al.[8] indicated the content of methylxanthines (particularly caffeine and theobromine/caffeine ratio) as a possible marker to differentiate three cocoa-growing areas in Colombia. Cocoa beans of different origin showed different levels of gamma-aminobutyric acid[9] and different Pb isotope ratios (206Pb/207Pb, 208Pb/207Pb) as well as Pb/Nd, 147Sm/144Nd and 87Sr/86Sr ratios, possibly in relation to the specific impact of soil geology and pollution sources in growing and manufacturing areas.[10] Cocoa beans are rich in polyphenols – including catechins (about 37%), anthocyanins (4%) and proanthocyanidins (58%) – responsible for the antioxidant properties of cocoa.[11] Two different approaches, mass spectrometry[12,13] and HPLC,[14] were used to measure the content of chiral compounds (hydroxy acids, amino acids and polyphenols) in fermented and roasted cocoa beans, with the aim of discriminating products of different origin.

More recently, 1H NMR spectra of traded cocoa beans were used to characterise fermented cocoa beans on the basis of the variety and geographical origin.[15,16] H, C, N, O and S stable isotope ratios measured using Isotope Ratio Mass Spectrometry (IRMS) have been shown to be a reliable tool for establishing the authenticity of a wide variety of food products, such as wine,[17] olive[18] and sesame oils,[19] orange juice,[20] honey,[21] beef,[22] fish,[23,24] shrimps[25] and prawns,[26] ham,[27] milk,[28] rice,[29] lentils.[30] This is due to particular isotopic fractionation processes, which reflect both climatic phenomena(e.g. precipitation, condensation and evaporation) and the geographical setting (e.g. altitude, latitude and continent). In a recent paper,[31] C and N isotope ratios were measured in different tissues and extracts of fermented cocoa beans with different geographical origins. In this work, for the first time, we combined the stable isotope ratios of oxygen (δ18O), hydrogen (δ2H) and sulfur (δ34S) with those of carbon (δ13C) and nitrogen (δ15N), measuring them in bulk samples of a wide selection of cacao beans from different areas (Africa, Asia, Central and South America). The scope of the work was to verify the ability of isotopic profiles to trace the origin of cocoa beans produced in different continents and countries.

Materials and methods

Sampling

53 samples of fermented and dried cocoa beans of known geographic origin and coming from the principal cocoa producing Countries in the world, were kindly provided by Barry Callebaut (Belgium). The sampling is representative of the average world production. The countries and the subcontinental macroareas of provenance are summarized in Table 1 All the samples belonged to Forastero variety except one Mexican sample that belonged to Criollo subspecies. The samples were kept in plastic bags at room temperature in a dry and dark place until preparation and analysis.

Stable isotope analysis

The stable isotope ratios of H, C, N, O and S were measured in the bulk ground cocoa beans.

The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios were measured in one run (around 0.5 mg) using an isotope ratio mass spectrometer (IRMS) (IsoPrime, Isoprime Limited, Germany) following total combustion in an elemental analyser (VARIO CUBE, Isoprime Limited, Germany). The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios were measured in one run (around 0.5 mg) using an IRMS (Finnigan DELTA XP, Thermo Scientific) coupled with a Pyrolyser (Finnigan TC/EA, high temperature conversion elemental analyser, Thermo Scientific).

The values were expressed, according to the IUPAC protocol (32), in $\delta = (R_{sample} - R_{standard})/R_{standard}$, where R is the ratio between the heavier isotope and the lighter one, against international standards (Vienna-Pee Dee Belemnite (V-PDB) for $\delta^{13}C$, Air for $\delta^{15}N$, Cañon Diablo Troilite (V-CDT) for $\delta^{34}S$, Vienna-Standard Mean Ocean Water (V-SMOW) for $\delta^{2}H$ and $\delta^{18}O$). Sample analyses were carried out in duplicate. For $\delta^{13}C$ and $\delta^{15}N$ the isotopic values were calculated against working in-house standards (protein), which were themselves calibrated against international reference materials: fuel oil NBS-22 (IAEA-International Atomic Energy Agency, Vienna, Austria) for $^{13}C/^{12}C$, sugar L-glutamic acid USGS 40 (U.S. Geological Survey, Reston, VA, USA) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ and potassium nitrate IAEA-NO_3 for $\delta^{15}N$.

The $\delta^{2}H$ and $\delta^{18}O$ values were calculated against CBS (Caribou Hoof Standard $\delta^{2}H = -197 \pm 2$ ‰ and $\delta^{18}O = +2.4 \pm 0.1$ ‰) and KHS (Kudu Horn Standard, $\delta^{2}H = -54 \pm 1$ ‰ and $\delta^{18}O = +21.2 \pm 0.2$ ‰) through the creation of a linear equation and adopting the comparative equilibration procedure (33). We used these two keratinous standards because of the absence of any international organic reference material with a similar matrix of ours.

The $\delta^{34}S$ values were calculated against barium sulphates IAEA-SO-5, NBS 127 (IAEA) and a calibrated protein working standard through the creation of a linear equation.

The uncertainty (2 s) of measurements was <0.3‰ for the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ analysis and <1‰ for $\delta^{18}O$, <3‰ for $\delta^{2}H$.

Statistical analysis

All statistical analysis were carried out applying Statistica 9.1. (StatSoft Italia sr, Padua, Italy). Honestly significant difference (HSD) Tukey test for an unequal number of samples was applied in order to identify differences between the isotopic ratios of cocoa beans from various geographic origins. Correlations between parameters were checked using a Pearson correlation test. A Canonical Discriminant Analysis was performed in order to assess the capability of the isotopic profile to discriminate between cocoa beans according to their geographic provenance.

Results and discussion

δ^{13}C

δ13C ranged from -29.7‰ to -25.9‰ (Fig. 1). Similar variability ranges (from -29.4‰ to -25.3‰) were found in the study of Santato et al.[35] for coffee beans cultivated in the same tropical areas. The work of Diomande et al.,[31] focusing on samples from the Ivory Coast (Africa) (37 out of a total of 61 samples), reported a similar but wider range than that found in our study for this country (from -32.7 to -28.6‰vs -29.4 to -28.6‰). As regards other countries common to the study of Diomande et al.,[31] the values for some of them are similar to those found in this study (i.e. Ghana, Venezuela, Peru, Ecuador), whereas in other cases the values reported by Diomande and colleagues were slightly lower (i.e. Dominican Republic, Papua New Guinea and Indonesia). As shown in Fig. 1, δ13C made it possible to discriminate Central America and most of West Africa, located in the northern hemisphere, from the other macro-areas located in the southern hemisphere (South America and East Africa). Moreover, different δ13C values were found within the continent, e.g. between Tanzania and Uganda in East Africa ($P<0.05$). It is worth noting that the particularly high δ13C values in Uganda were also recently described in sesame[19] probably in relation to the geographical characteristics of this country (most of Uganda is a plateau with a height of 1000 - 1200 meters with a mean temperature of 21-23°C with limited changes along the year).

Plants normally exhibit higher δ13C values when high temperatures, low air humidity and a high ground-water deficit lead to narrower stomatal apertures in the leaves of plants.[36,37] In addition, δ13C is very sensitive to soil water deficiency.[38] For the optimum development of cocoa trees a hot and humid atmosphere is essential[6]; indeed in cocoa-producing countries, relative humidity is generally high: often as much as 100% during the day, falling to 70–80% during the night.[39] In our case, as reported in a previous study for different plant species,[39,40] it is more likely that the higher δ13C is correlated with the higher altitude of the cocoa crops. As reported by Körner et al.,[40] the ratio of internal to external partial pressure of CO2 (Pi/Pa) in leaves of high elevation plants is lower than in leaves at low altitude. Despite the small number of samples (not from the Ivory Coast) in the study by Diomande et al.,[31] the same general trend is confirmed, with the lowest values in Central America (Dominican Republic) and the highest in South America

δ^{2}H and δ^{18}O

Hydrogen and oxygen stable isotopes of plant materials arestrongly linked to the climatic conditions (relative humidity, temperature and amount of precipitation) and geographical characteristics (distance from the sea or other evaporation source, altitude and latitude) of the area where the plants grew.[18,29,41–44] East Africa (Uganda) shows significantly higher δ18O (Fig. 1) than the other macro-areas, while Peru and Asia have the lowest. In this case, Central America is not separable from countries in the southern hemisphere. As reported by Carter et al.[45] for coffee, the narrow tropical belt appropriate for growing cocoa trees (10° to the north and south of the Equator) seems not to justify a latitude effect (i.e. the decreasing of δ2H and δ18O values with increasing latitude).[46] In this case δ18O seems to be mainly influenced by the isotopic signature of rainfall precipitation. To evaluate the correlation with this factor, in the absence of direct measurement of the δ18O of rainwater, we used water isotope data fromthe Global Network for Isotopes in

Precipitation (GNIP) database administered by the International Atomic Energy Association and World Meteorological Organization.[47] The data available are monthly weighted average precipitation (δ2Hp and δ18Op) values for sites in all seven continents and islands, spanning from 1960 to the present. As shown in Fig. 2a, where countries are grouped according to the ranges of the weighted annual δ18O of world precipitation of the GNIP database, the δ18O of cocoa is evidently correlated with the δ18O of precipitation. Uganda shows the highest values (δ18O rainfall>-2‰ and δ18O cocoa>+28‰), while Perù has the lowest (δ18O rainfall<-10‰and δ18O cocoa<+21‰, except for one sample). As reported for other plant products, such as olive oil,[18] or orange juice,[20] in cocoa beans δ2H is correlated with δ18O (Fig. 3). Indeed, the δ2H trend in the countries considered is similar to that of δ18O, with the highest value in East Africa (Uganda) and the lowest in South America (Peru) and Asia (Fig. 1). Particularly high δ2H values were previously also reported in sesame oil from Uganda.[19] As shown in Fig. 2b, there is a good correlation ($P<0.01$) between the δ2H of cocoa and the weighted annual δ2H of world precipitation reported in the GNIP database. Santato et al.[35] and Carter et al.[46] found similar values for both the δ18O and δ2H of coffee beans, with the highest values in Africa, coffee being cultivated in the same climatic conditions, countries and at the same altitude as cocoa. As regards the data for South America, it is not possible to compare the results because of the different countries considered within the macro-area.

δ^{15}N and δ^{34}S:

These two parameters are affected by the pedoclimatic and geological conditions of the soil and by the agricultural practices adopted, such as fertilisation.[48,49] Indeed inorganic fertilisers have δ15N values close to that of atmospheric nitrogen (from -6 to +6‰), whereas manure and other organic fertilisers can be substantially enriched (from +1 to +37‰). In the case of cocoa beans, fertiliser is needed for growth because of the poverty of the soil.[51] Very important for δ34S variability in plants, is the so-called sea spray effect, i.e. aerosol particles, formed directly from the ocean and with a high δ34S value (+20‰) that fall along the coasts of the country, making it possible to characterise these areas for higher δ34S values.[51] As reported in Figs. 1, δ15N ranged from +2.6‰ to +7.2‰ and δ34S from -2.4‰ to +13.0‰. Significant differences were found between some macro-areas (e.g. δ34S in West Africa was significantly higher than in South America, East Africa and Asia) and particularly within macro-areas. A similar range for the δ15N of cocoa beans (+1.14 to +7.26‰) was found by Dimande et al.[31] who reported a wider range, as compared with our samples from the Ivory Coast, (+3.6‰to +9.1‰vs +5.2‰to +6.5‰) as observed above for δ13C. As regards countries in common in the studies, other than the Ivory Coast, some presented δ15N values similar to those found in this study (Dominican Republic, Venezuela and Peru) whereas others presented slightly different values (Ghana, Papua New Guinea, Ecuador and Indonesia). In both studies, the lowest values were in Asia, where the average intensity of synthetic fertiliser use is much higher (101 kg/ha in 2002) than elsewhere (e.g. Africa 8 kg/ha).[50] The results are comparable with the range found by Santato et al.[35] in coffee beans (+0.6‰ to 7.1‰). In the case of δ34S, an important contribution may come from the volcanic origin of the soil, which could justify for example the low values in Mexico, Ecuador and Peru (up to -2‰), or from the sea spray effect, which particularly affects the coast of islands such as São Tomé in Africa (up to +13‰).[51]

Combination of isotope composition:

Given that in many cases combining several analytical parameters has previously shown the potential to improve the ability to discriminate food origin,[21,29] multivariate canonical discriminant analysis was applied to the isotopic data determined in cocoa beans, in order to establish whether it is possible to enhance discrimination between different countries. For this approach, we decided to consider only countries with aminimum of four samples. As reported in Table 1, these were the Dominican Republic, Peru, Venezuela, Ghana, Ivory Coast, Nigeria, São Tomé and Principe, Tanzania and Papua New Guinea. Canonical discriminant analysis, CDA (Fig. 4) is a form of statistical analysis that maximises the difference between groups by combining variables, and it was applied to δ13C, δ15N, δ34S, δ18O and δ2H. The canonical variable CAN1 was loaded negatively with δ34S (standardised coefficient: -0.96) and positively with δ15N (0.53), while CAN2 was loaded negatively with δ15N (standardised coefficient: -0.94) and positively with δ13C (0.54). The model was able to effectively discriminate between the samples, with a mean percentage of reclassification of 81%. In particular, typical fertilisation practices (with synthetic fertiliser), and the consequently low δ15N values, justified 100% correct classification of Papua New Guinea samples, whereas high δ34Scombined with low δ15N justified the good discrimination of the island of São Tomé (100% correct classification). Finally, the very low δ34S values made it possible to characterise and differentiate Peru (86% correct classification). To test the predictive discrimination power and the stability of this model a cross validation was used. In detail, three different sets of nine samples, about 20% of the original database were randomly selected, and removed from the dataset. Each time the model was calculated on the remaining cases and was validated with all the samples (including the excluded samples). In all analyses, around 84% of the samples were correctly classified in the right geographical region (ranging from 81% to 88% according to the considered model). The 100% of the right classifications of Papua New Guinea, São Tomé and Peru were confirmed in all the three validation models. The combination of the stable isotope ratios with the trace element profile[52] and/or with the stable isotope ratios of heavy elements would improve origin prediction of cacao beans, as observed for other commodities.[36,53]

References

[1] J. C.Motamayor, P. Lachenaud, J.W. Da Silva EMota, R. Loor, D. N. Kuhn, J. S. Bown, R. J. Schnell. Geographic and genetic population differentiation of the Amazonian chocolate tree (Theobroma cacao L). PLoS One 2008, 3, e3311.

[2] ICCO Quarterly bulletin of cocoa statistics, Vol. XLI, No. 3, Cocoa year 2014/15

[3] V. T. T. Ho, J. Zhao, G. Fleet. Yeasts are essential for cocoa bean fermentation. Int. J. Food Microbiol. 2014, 174, 72.

[4] P. A. Roelofsen. Fermentation, drying, and storage of cacao beans. In Advances in Food Research(E. M. Mrak, G. F. Stewart Eds.) Eds. Academic Press: New York, 1958 225–296.
[5] M. M. Ardhana, G. H. Fleet. The microbial ecology of cocoa bean fermentations in Indonesia. Int. J. Food Microbiol. 2003, 86, 87.
[6] International Cocoa Organization. The cocoa market situation. Economics Committee. EC/4/2. Fourth meeting, London, 2014.
[7] M. Torres-Moreno, E. Torrescasana, J. Salas-Salvado, C. Blanch. Nutritional composition and fatty acids profile in cocoa beans and chocolates with different geographical origin and processing conditions. Food Chem. 2014, 166, 125. DOI:10.1016/j. foodchem.2014.05.141.
[8] L. C. Carrillo, J. Londono-Londono, A. Gil. Comparison of polyphenol methylxanthines and antioxidant activity in Theobroma cacao beans from different cocoa-growing areas in Colombia. Food Res. Int. 2014, 60, 273.
[9] A. Marseglia, G. Palla, A. Caligiani. Presence and variation of gammaaminobutyric acid and other free amino acids in cocoa beans from different geographical origins. Food Res. Int. 2014, 63, 360.
[10] W. I. Manton. Determination of the provenance of cocoa by soil protolith ages and assessment of anthropogenic lead contamination by Pb/Nd and lead isotope ratios. J. Agric. Food Chem. 2010, 58, 713.
[11] P. Cabras, A. Martelli. Chimica Degli Alimenti. Piccin: Padova, 2004.
[12] C. Crews. Authentication of cocoa butter. In Oils and Fats Authentication (M. Jee Ed.) Eds. Blackwell: Oxford, U.K., 2002 66–94.
[13] A. Caligiani, M. Cirlini, G. Palla, R. Ravaglia, M. Arlorio. GC-MS detection of chiral markers in cocoa beans of different quality and geographic origin. Chirality 2007, 19, 329.
[14] J. Oracz, D. Zyzelewicz, E. Nebesny. The content of polyphenolic compounds in cocoa beans (Theobroma cacao L.), depending on variety, growing region and processing operations: a review. Crit. Rev. Food Sci. 2013, 55, 1176.
[15] A. Caligiani, D. Acquotti, M. Cirlini, G. Palla. 1H NMR study of fermented cocoa (Theobroma cacao L.) beans. J. Agr. Food Chem. 2010, 58, 12105.
[16] A. Caligiani, L. Palla, D. Acquotti, A.Marseglia, G. Palla. Application of 1H NMR for characterisation of cocoa beans of different geographical origins and fermentation levels. Food Chem. 2014, 157, 94.
[17] N. Dordevic, R. Wehrens, G. J. Postma, L. M. C. Buydens, F. Camin. Statistical methods for improving verification of claims of origin for Italian wines based on stable isotope ratios. Anal. Chim. Acta 2010, 757, 19.
[18] L. Bontempo, F. Camin, R. Larcher, G. Nicolini, M. Perini, A. Rossmann. Coast and year effect on H, O and C stable isotope ratios of Tyrrhenian and Adriatic Italian olive oils. Rapid Commun. Mass Spectrom. 2009, 23(7), 1043.
[19] M. Horacek, K. Hansel-Hohl, K. Burg, G. Soja, W. Okello-Anyanga, S. Fluch. Control of origin of sesame Oil from various countries by stable isotope analysis and DNA based markers—a pilot study. PLoS One 2015, 10(4), e0123020.
[20] L. Bontempo, R. Caruso, M. Fiorillo, G. L. Gambino, M. Perini, M. Simoni, P. Traulo, R. Wehrens, G. Gagliano, F. Camin. Stable Isotope Ratios of H, C, N and O in Italian citrus juices. J. Mass Spectrom. 2014, 49(9), 785.
[21] A. Schellenberg, S. Chmielus, C. Schlicht, F. Camin, M. Perini, L. Bontempo, K. Heinrich, S. D. Kelly, A. Rossmann, F. Thomas, E. Jamin, M. Horacek. Multielement stable isotope ratios (H, C, N, S) of honey from different European regions. Food Chem. 2010, 121, 770.
[22] O. Schmidt, J. M. Quilter, B. Bahar, A. P. Moloney, C. M. Scrimgeour, I. S. Begley, F. J. Monahan. Inferring the origin and dietary history of beef from C, N and S stable isotope ratio analysis. Food Chem. 2005, 91(3), 545.

[23] M. P. Chaguri, A. L. Maulvault, M. L. Nunes, D. Aparecida-Santiago, J. C. Denadai, F. H. Fogaça, L. S. Sant′Ana, C. Ducatti, N. Bandarra, M. L. Carvalho, A. Marques. Different tools to trace geographic origin and seasonality of croaker (Micropogonias furnieri). LWT-Food Sci. Techno. 2015, 61(1), 194.
[24] H. Kim, K. S. Kumar, K.-H. Shin. Applicability of stable C and N isotope analysis in inferring the geographical origin and authentication of commercial fish (Mackerel, Yellow Croaker and Pollock). Food Chem. 2015, 172, 523.
[25] I. Ortea, J. M. Gallardo. Investigation of production method, geographical origin and species authentication in commercially relevant shrimps using stable isotope ratio and/or multi-element analyses combined with chemometrics: an exploratory analysis. Food Chem. 2015, 170, 145.
[26] J. F. Carter, U. Tinggi, X. Yang, B. Fry. Stable isotope and trace metal compositions of Australian prawns as a guide to authenticity and wholesomeness. Food Chem. 2015, 170, 241.
[27] M. Perini, F. Camin, J. S. Del Pulgar Rico, E. Piasentier. Effect of origin, breeding and processing conditions on the isotope ratios of bioelements in dry-cured ham. Food Chem. 2013, 136(3-4), 1543.
[28] I. M. Chung, I. Park, J. Y. Yoon, Y. S. Yang, S. H. Kim. Determination of organic milk authenticity using carbon and nitrogen natural isotopes. Food Chem. 2014, 160, 214.
[29] T. Korenaga. Traceability studies for analyzing the geographical origin of rice by isotope ratio mass spectrometry. Bunseki Kagaku 2014, 63(3), 233.
[30] F. Longobardi, G. Fasiello, M. Cortese, M. Perini, F. Camin, L. Catucci, A. Agostiano. Discrimination of geographical origin of lentils (Lens culinarisMedik.) using isotope ratio mass spectrometry combined with chemometrics. Food Chem. 2015, 188, 343.
[31] D. Diomande, I. Antheaume, M. Leroux, J. Lalande, S. Balayssac, G. S. Remaud, I. Tea. Multi-element, multi-compound isotope profiling as a means to distinguish the geographical and varietal origin of fermented cocoa (Theobroma cacao L.) beans. Food Chem. 2015, 188, 576.
[32] W. A. Brand, T. B. Coplen, J. Vogl, M. Rosner, T. Prohaska. Assessment of international reference materials for isotope-ratio analysis (IUPAC Technical Report). Pure Appl. Chem. 2014, 86, 425.
[33] L. I. Wassenaar, K. A. Hobson. Comparative equilibration and online technique for determination of non-exchangeable hydrogen of keratins for use in animal migration studies. Isotopes Environ. Health Stud. 2003, 39, 211.
[34] StatSoft, Inc - STATISTICA (version 9.1) “Software”, 2010.
[35] A. Santato, D. Bertoldi, M. Perini, F. Camin, R. Larcher. Using elemental profiles and stable isotopes to trace the origin of green coffee beans on the global market. J. Mass Spectrom. 2012, 47, 1132.
[36] M. M. Barbour, G. D. Farquhar. Relative humidity- and ABA-induced variation in carbon and oxygen isotope ratios of cotton leaves. Plant Cell Environ. 2000, 23, 473.
[37] G. Schleser, M. Winiger, A. Bräuning, H. Gärtner, G. Helle, E. Jansma, B. Neuwirth, K. Treydte. TRACE – Tree Rings in Archaeology, Climatology and Ecology, Vol. 1: Proceedings of the DENDROSYMPOSIUM 2002, April 11th - 13th 2002, Bonn / Jülich, Germany. Schriften des Forschungszentrums Jülich. Reihe Umwelt 2003, 33, 66.
[38] M. Blakeney. Geographical Indications and TRIPIn. In Extending The Protection Of Geographical Indications - Case Studies(M. Blakeney, T. Coulet, G. Mengistie, M. T. Mahop Eds.). Earthscan from Routledge: New York, 2012 7–34.
[39] X. F. Wang, R. Li, X. Li, F. Ma, B. Sun, J. Wu, Y. Wang. Variations in leaf characteristics of three species of angiosperms with changing of altitude in Qilian Mountains and their inland high-altitude pattern. Sci. China Earth Sci. 2014, 57(4), 662.
[40] C. H. Körner, G. D. Farquhar, Z. Roksandic. A global survey of carbon isotope discrimination in plants from high altitude. Oecologia 1988, 74, 623.
[41] A. Hermann, S. Voerkelius. Meteorological impact on oxygen isotope ratios of German wines. Am. J. Enol. Vitic. 2008, 59, 194.

[42] P. Iacumin, L. Bernini, T. Boschetti. Climatic factors influencing the isotope composition of Italian olive oils and geographic characterisation. Rapid Commun. Mass Spectrom. 2009, 23, 448.
[43] D. A. Magdas, G. Cristea, R. Puscas, F. Tusa. The use of isotope ratios in commercial fruit juices authentication. Rom. J. Phys. 2014, 59(3-4), 355.
[44] M. Horacek, J. S. Min, S. C. Heo, G. Soja. Discrimination between ginseng from Korea and China by light stable isotope analysis. Anal. Chim. Acta 2010, 682(1-2), 77.
[45] J. F. Carter, H. S. Yates, U. Tinggi. Isotopic and elemental composition of roasted coffee as a guide to authenticity and origin. J. Agric. Food Chem. 2015, 63, 5771.
[46] H. Craig. H. Isotopic variations inmeteoric waters. Science 1961, 133, 1702.
[47] Global network for isotopes in precipitation (GNIP) database administered by the International Atomic Energy Association and World Meteorological Organization (IAEA/WMO 2001).
[48] A. S. Bateman, S. D. Kelly, T. D. Jickells. Nitrogen isotope relationships between crops and fertilizer: implications for using nitrogen isotope analysis as an indicator of agricultural regime. J. Agric. Food Chem. 2005, 53, 5760.
[49] A. S. Bateman, S. D. Kelly. Fertilizer nitrogen isotope signatures. Isot. Environ. Health Stud. 2007, 43, 237.
[50] M. Morris, D. R. Byerlee, V. A. Kelly, R. J. Kopicki. Fertilizer use in African agriculture : lessons learned and good practice guidelines. In Directions in Development. World Bank: Washington DC, 2007 1–137.
[51] M. P. Richards, B. T. Fuller, M. Sponheimer, T. Robinson, L. Ayliffe. Sulphur isotopes in paleodietary studies: a review and results from a controlled feeding experiment. Int. J. Osteoarcheol. 2003, 13, 37.
[52] D. Bertoldi, A. Barbero, F. Camin, A. Caligiani, R. Larcher. Multielemental fingerprinting and geographic traceability of Theobroma cacao beans and cocoa products. Food Control. 2016, 65, 46.
[53] D. Goitom-Asfaha, C. Quétel, F. Thomas, M. Horacek, B. Wimmer, G. Heiss, C. Dekant, P. Deters-Itzelsberger, S. Hoelzl, S. Rummel, C. Brach-Papa, M. Van-Bocxstaele, E. Jamin, M. Baxter, K. Heinrich, D. Bertoldi, L. Bontempo, F. Camin, R. Larcher, M. Perini, S. Kelly, A. Rossmann, A. Schellenberg, C. Schlicht, H. Froeschl, J. Hoogewerff, H. Ueckermann. Combining isotopic signatures of n(87Sr)/n(86Sr) and light stable elements (C, N, O, S) with multi-elemental profiling for the authentication of provenance of European cereal samples. J. Cereal Sci. 2011, 53, 170.

Figure captions:

Figure 1. Box and whisker plot graphs and the results of the Unequal N Tukey's HSD test on the isotopic values of cacao samples grouped by macro-area (δ13C; δ18O; δ2H; δ15N; δ34S) of cocoa beans collected . Mean values ofmacroareas with different letters are significantly different (P<0.001): □,median; , 25%-75%; , sp. not-outlier; , outlier; extreme.

Figure 2. Box and whisker plot graphs of the isotopic values (a, δ18O; b, δ2H) of cocoa beans collected in different countries, plotted against weighted annual δ18O (a) and δ2H (b) of world precipitation reported in the GNIP database. □, median; ; , 25%-75%; , sp. not-outlier; , outlier; extreme. Group 1 (Peru), 2 (Ecuador, Indonesia, Papua New Guinea), 3 (Santo Domingo, Mexico, Venezuela, Ghana, Ivory Coast, Nigeria, Sierra Leone, São Tomé, Tanzania), 4 (Uganda).

Figure 3. Plot of the distribution of δ2H and δ18O values for cocoa beans.

Figure 4. Scatter plot of the first two canonical variables in stepwise discriminant analysis (cumulative variance explained: 81%). based on the isotopic composition of cocoa beans of different geographical origin

Table 1. Number of samples and geographic provenance of cocoa beans

Macroarea	Country	Cocoa beans
Central America	Dominican Republic	5
	Mexico	2
South America	Ecuador	2
	Perù	7
	Venezuela	4
West Africa	Ghana	4
	Ivory Coast	4
	Nigeria	4
	São Tomé and Principe	6
	Sierra Leone	2
East Africa	Tanzania	5
	Uganda	2
Asia	Indonesia	2
	Papua New Guinea	4

Figure 1 a

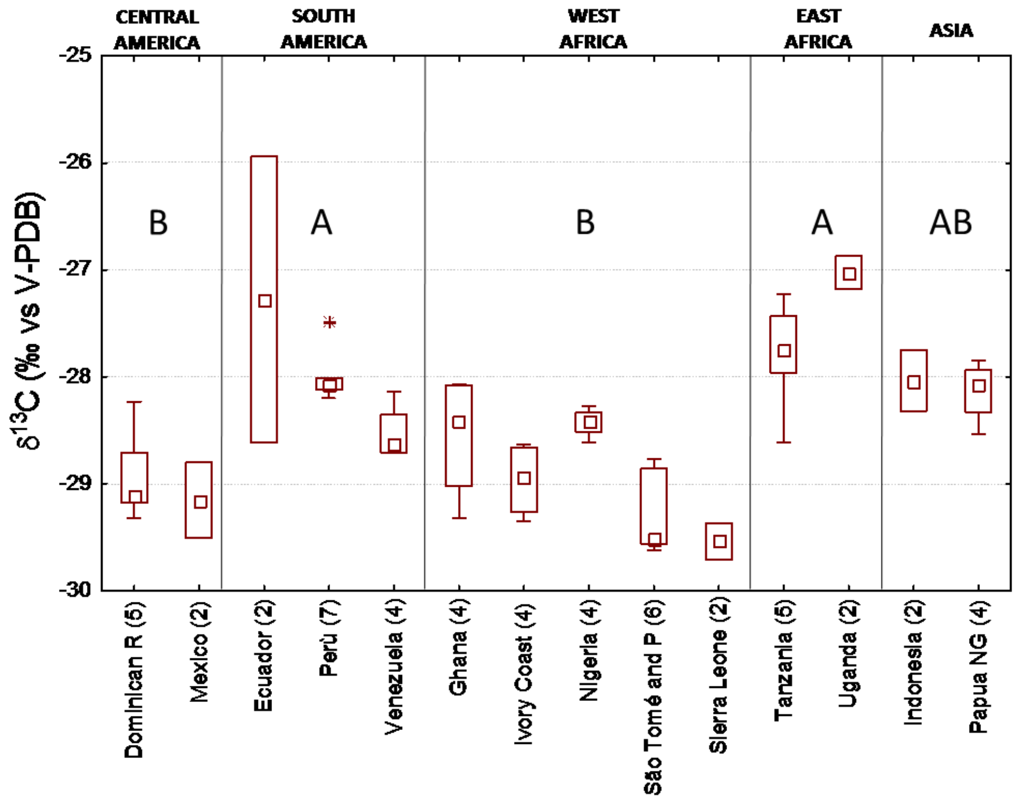


Figure 1b

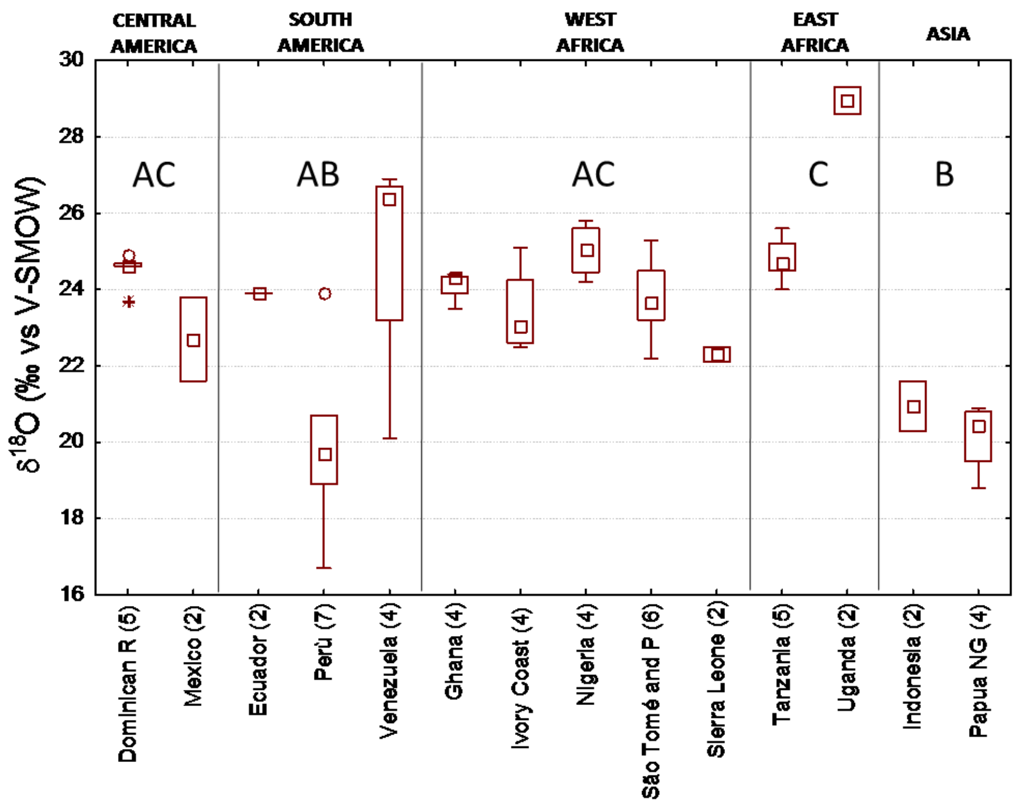


Figure 1c

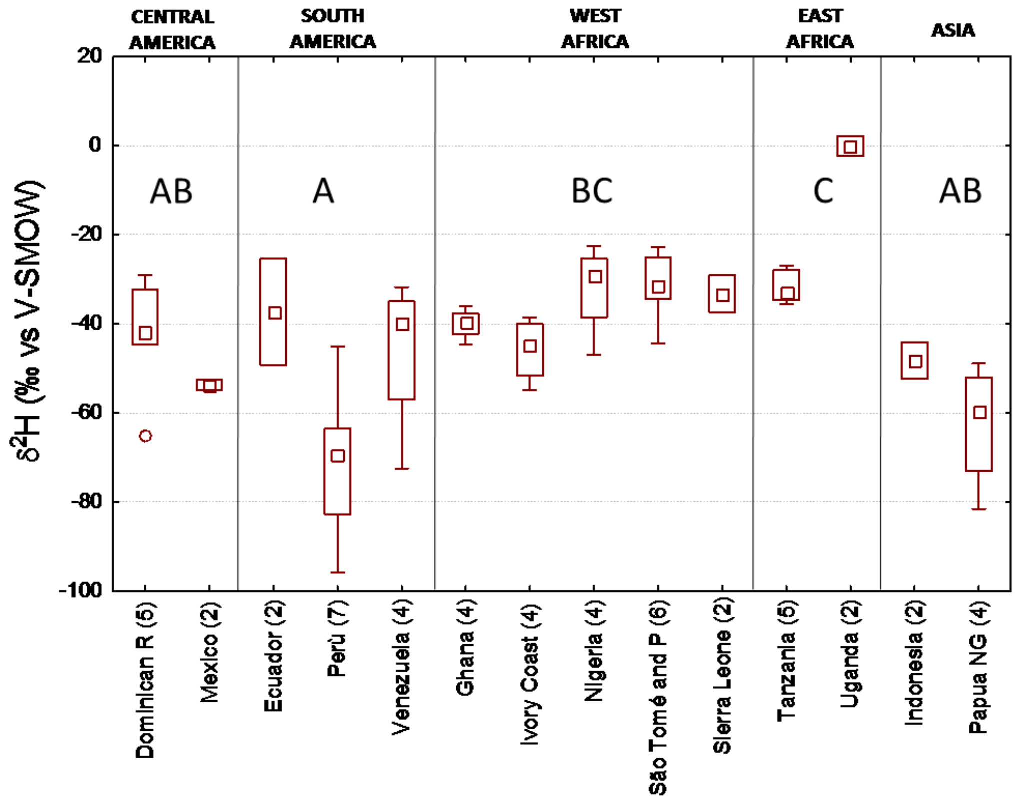


Figure 1d

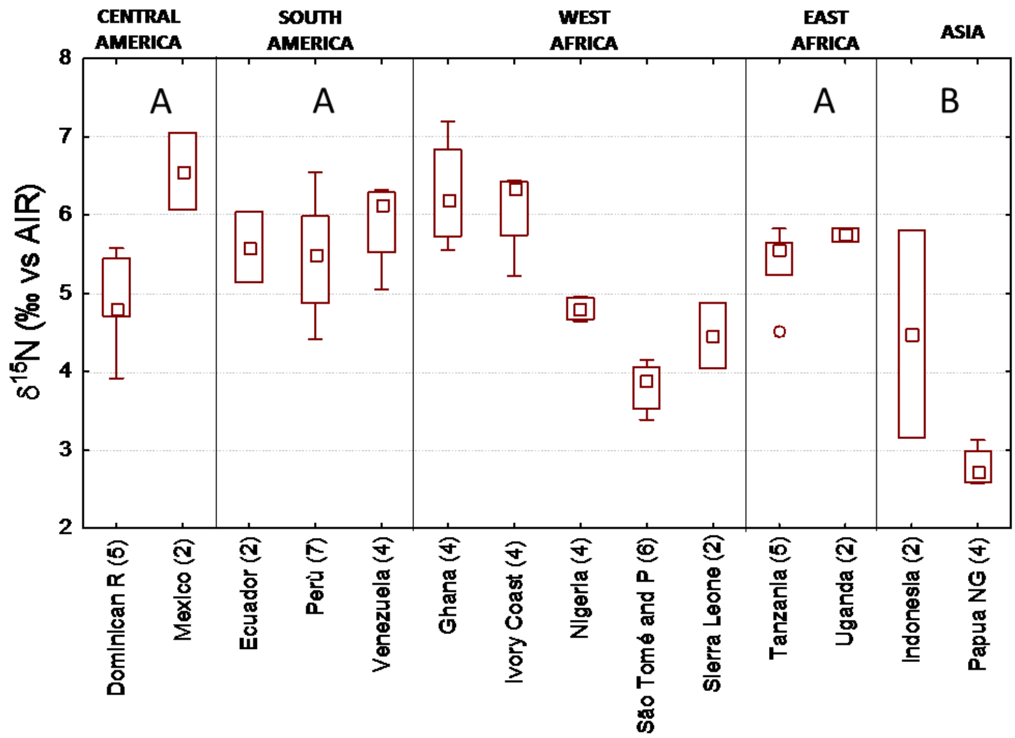


Figure 1e

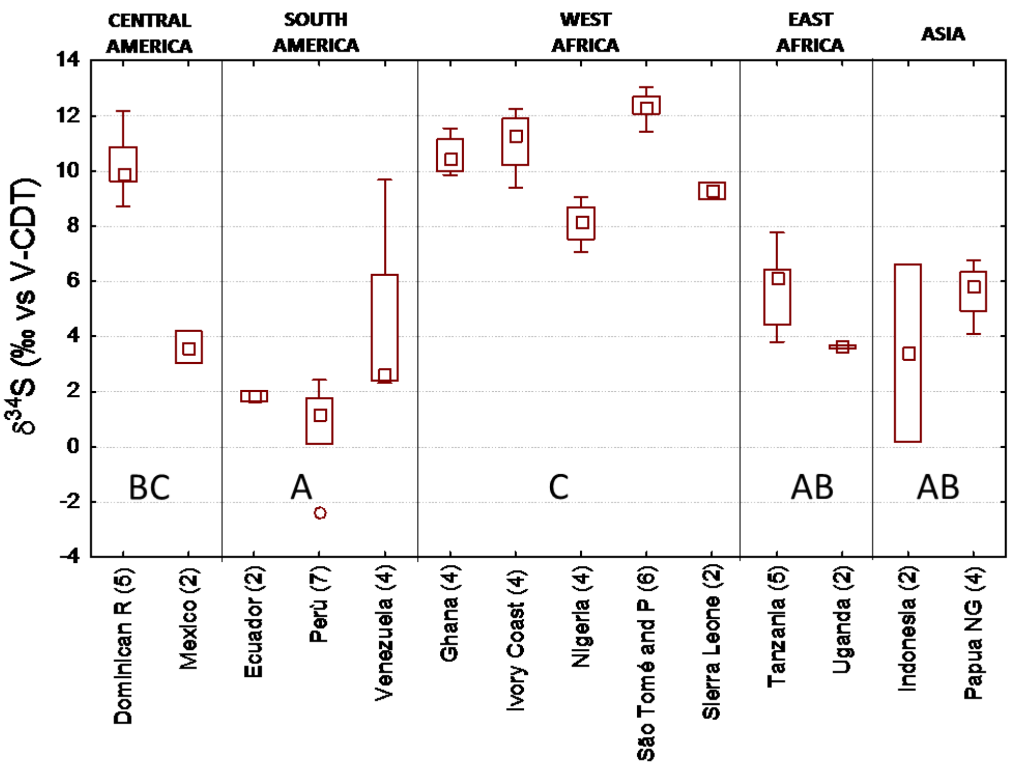

CENTRAL AMERICA
SOUTH AMERICA
WEST AFRICA
EAST AFRICA
ASIA
δ34S (‰ vs V-CDT)
14
12
10
8
6
4
2
0
-2
-4
BC
A
C
AB
AB
Dominican R (5)
Mexico (2)
Ecuador (2)
Perù (7)
Venezuela (4)
Ghana (4)
Ivory Coast (4)
Nigeria (4)
São Tomé and P (6)
Sierra Leone (2)
Tanzania (5)
Uganda (2)
Indonesia (2)
Papua NG (4)

Figure 2a

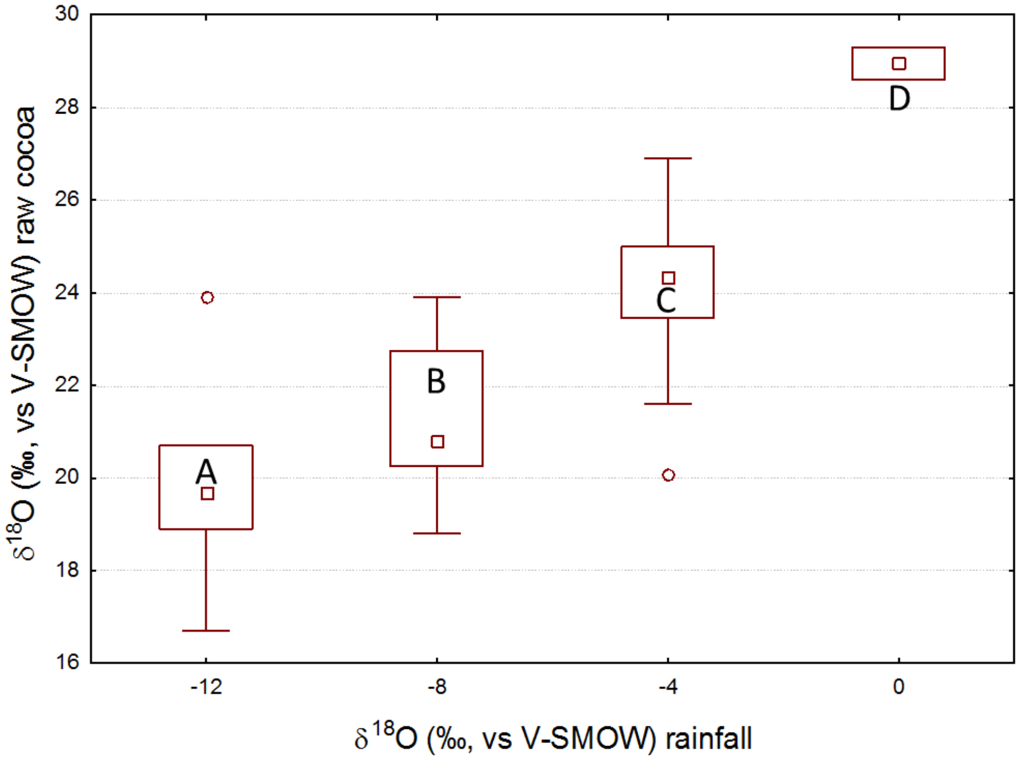


Figure 2b

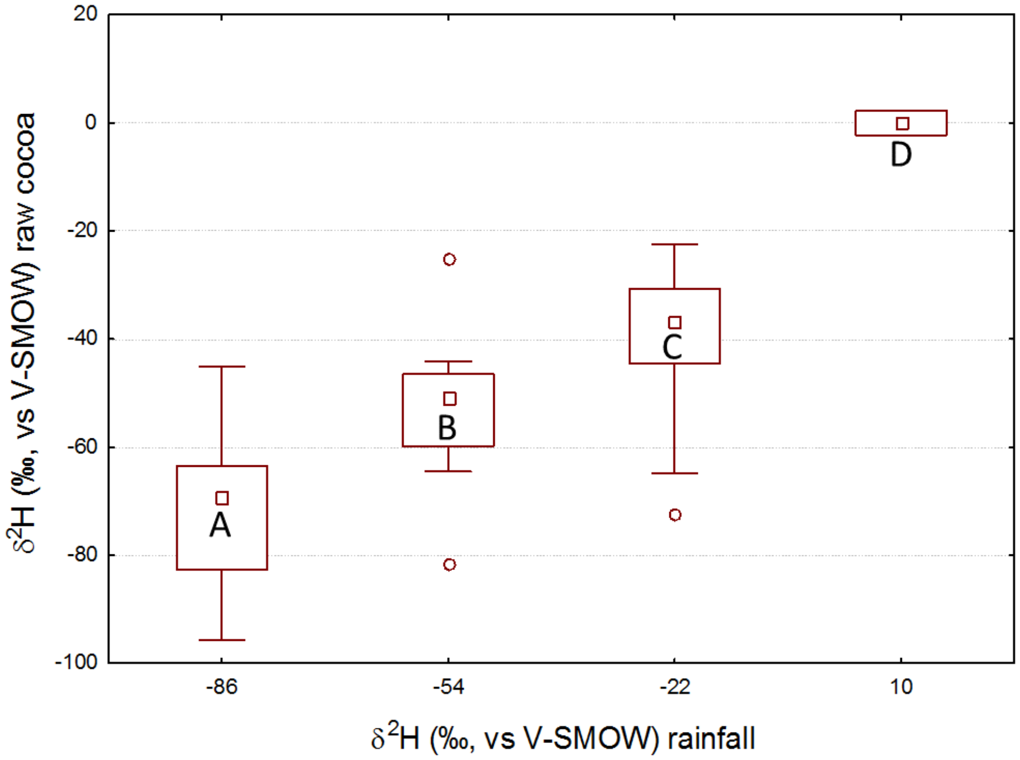


Figure 3

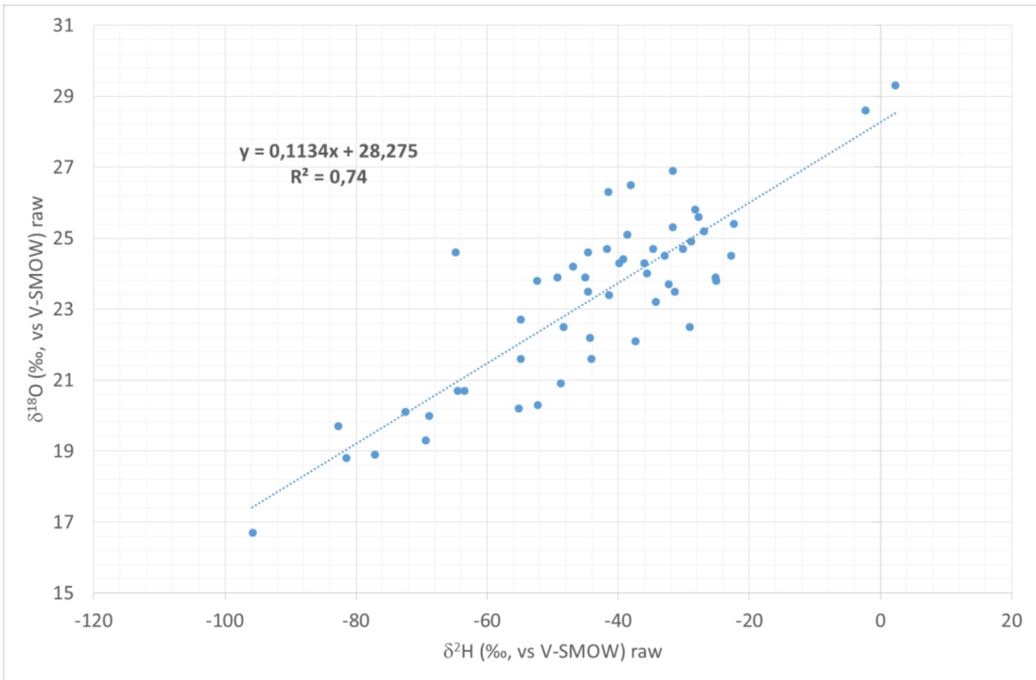
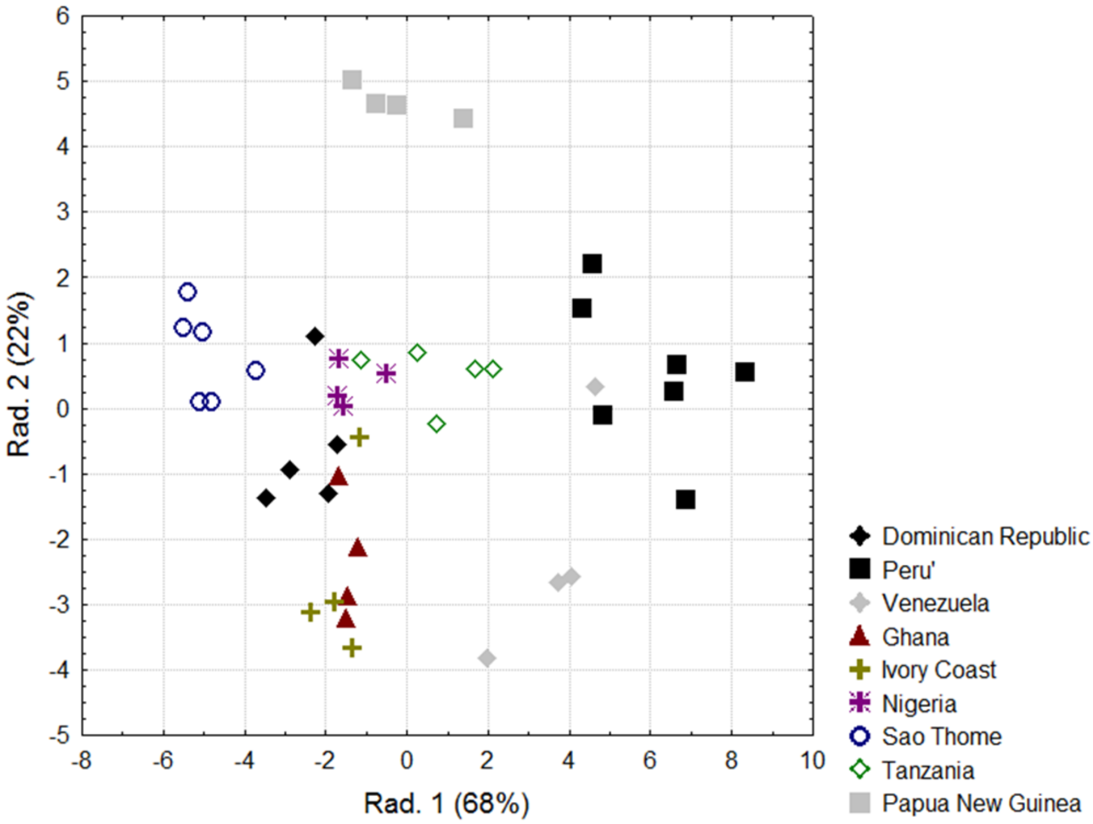

y = 0,1134x + 28,275
R² = 0,74
δ18O (‰, vs V-SMOW) raw
δ2H (‰, vs V-SMOW) raw
31
29
27
25
23
21
19
17
15
-120
-100
-80
-60
-40
-20
0
20

Figure 4


Rad. 2 (22%)
Rad. 1 (68%)
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-8
-6
-4
-2
0
2
4
6
8
10
Dominican Republic
Peru'
Venezuela
Ghana
Ivory Coast
Nigeria
Sao Thome
Tanzania
Papua New Guinea