

ANNEX 5

Technical reports on the stable isotopes analysis

Final report ITTO Africa Project - Technical report on the stable isotopes analysis for sapelli

Gareth Rees, The Food and Environment Research Agency, York; United Kingdom

Phone: +44 (0) 1904 462000/Fax: +44 (0) 1904 462111

Email: info@fera.co.uk

1 Materials and methods

1.1 Sample receipt and preparation

Reference and blind test samples (n=210 and n=20 respectively) of Sapele (*Entandrophragma cylindricum*) were received at FERA (the Food and Environment Research Agency, UK) and logged into the laboratory information management system, (LIMS) where they were each assigned a unique identifier. Reference samples, as well as 10 of the blind test samples consisted of timber shavings of inner cambium, as technical difficulties meant collectors could not take timber cores from many of the sampling locations. For the reference samples, collection teams took information on timber genus and GPS co-ordinates to aid digital mapping processes (ARC-GIS).

Half of the blind test samples (n=10) consisted of offcuts of sawn timber. For these samples, a hand drill was used to extract powder across the growth rings. Freeze-drying of the timber shavings and powder from the blind test samples was carried out in order to remove any excess moisture and to aid cellulose purification. Following freeze-drying, reference samples (including half the blind test samples) were broken into small pieces enabling transfer in small amounts to an IKA handheld analytical mill where they were pulverised to a fine powder.

1.1.1 Cellulose purification

To reduce the influence of tree morphology (lignin:cellulose ratio) upon the isotope distribution of the raw timber, isotope measurements were standardised by performing the analysis of $\delta^2\text{H}\text{‰}$, $\delta^{13}\text{C}\text{‰}$ and $\delta^{18}\text{O}\text{‰}$ isotopic ratios on the isolated alpha cellulose. For this, the method by Brendel *et al.* (2000) was applied in the preparation of the samples.

1.2 Analysis of carbon isotopes in cellulose

Following cellulose purification, 1 mg of cellulose were weighed in duplicate into tin capsules (3.5 x 5 mm i.d.) and sealed before transfer into a 96 position sample tray prior to analysis. Where analysis did not take place on the same day, sample trays were stored in a desiccator. Sealed tin capsules containing the purified cellulose and standards were placed in the autosampler of the elemental analyser (Fisons, Milan, Italy) and purged with helium, before dropping into a vertical quartz tube maintained at a temperature of 1020 °C (Kelly *et al.*, 2006). The carrier gas stream was temporarily enriched with oxygen and the sample and tin capsule oxidised in a 'flash' combustion reaction. Quantitative combustion was achieved by passing the gas mixture over two catalyst layers of chromium oxide and silvered copper or cobaltous oxide (Isoprime, Cheadle, UK). The combustion gases were passed first over elemental copper at a temperature of 650 °C to remove residual oxygen and second through a chromatographic column (Porapak PQS, SS, 2 m, 6 x 5 mm) heated at 35 °C. Residual Water was removed from the gas stream before entering the IRMS by a trap containing anhydrous magnesium perchlorate. During the measurement a portion of the effluent from the elemental analyser (ca. 0.5 ml/min) was transferred into the IRMS (Isoprime, Cheadle, UK) using helium carrier gas (ca. 85 mL/min). The signal from ions at m/z 44, m/z 45 and m/z 46 for CO₂ were monitored. The $\delta^{13}\text{C}$ values (‰) vs V-PDB) of the samples were determined by calibration against certified reference materials, IAEA CH3 cellulose and IAEA CH6 sucrose. The IAEA Cellulose and sucrose standards had $\delta^{13}\text{C}$ values of -24.72‰ and -10.38‰ respectively, which allowed for a stretch correction calculation to be applied to the sample data.

1.3 Analysis of hydrogen and oxygen isotopes in cellulose

Following cellulose purification, 1 mg of cellulose were weighed in quadruplicate into tin capsules (3.5 x 5 mm i.d.) and sealed before transfer into a 96 position sample tray prior to analysis. Sealed tin capsules containing purified cellulose and standards were placed in the autosampler of a Pyrocube elemental analyser (Elementar, Hanau, Germany), purged with helium, and dropped into a vertical glassy carbon tube with quartz liner maintained at a temperature of 1450°C. The products of thermal decomposition (gaseous H₂ and CO) were passed through a water trap containing sodium hydroxide/phosphorus pentoxide to remove residual moisture. A carbon monoxide trap retained CO while enabling free transfer of H₂ into the IRMS for measurement. Following H₂ analysis the CO trap was heated rapidly, transferring desorbed CO into the IRMS for measurement. During the measurement a portion of the effluent from the elemental analyser (ca. 0.5 ml/min) was transferred into the IRMS (Isoprime, Cheadle, UK) using helium carrier gas (ca. 135 mL/min). The signal from ions at m/z 2, m/z 3 for H₂ and m/z 28, m/z 30 for CO were monitored. The lack of suitable matrix matched certified standards posed a challenge for hydrogen measurements. Ratios of ²H/¹H, the δ²H values (‰) vs V-SMOW) were determined by applying a single point calibration with certified reference material IAEA CH7 Polyethylene foil (δ²H‰ -100.3‰). δ¹⁸O values were determined by calibration against certified reference materials of benzoic acid, IAEA 601 and 602 (δ¹⁸O‰ +23.3‰ and +71.4‰). The benzoic acid standards have very different values, thus allowing for a stretch correction calculation to be applied to the data.

1.4 Blind test samples

Hypothesis testing of the blind test samples was performed using chi-square statistical analysis of the CHO isotopes in the following steps:

- 1.4.1 For each of the principal components, a local regression model (“loess” as implemented in R version 3.0.2 for Windows) was fitted so that it predicts the isotope ratio from the geographical coordinates (including a latitude:longitude interaction). The smoothing (bandwidth) parameter (referred to as “span” in loess terminology) was optimized using leave-one-out cross validation.
- 1.4.2 Chi-square statistics were calculated based on the difference between the actual value of the principal component and the value predicted by the model. For the reference data, the predicted value was based on leave-one-out cross validation while for the blind test data, the prediction was based on the nearest point in a 101*101 grid covering the geographical coordinates of the reference data. This was necessary because “loess” can’t always produce predictions for an irregular set of coordinates (such as the reference data).
- 1.4.3 The Chi-square statistics for the blind test data were cut off at the empirical 95 percentile of the chi-square statistic for the reference data. This corresponded roughly to the 97% percentile of the theoretical chi-square distribution, indicating that the residuals are slightly more heavy-tailed than the normal distribution.

2 Results of the reference and blind test data

2.1 Reference samples

The stable isotope ratios of cellulose from timber samples originating from different geographical origins were determined by IRMS. Initially, the stable isotope data was processed by CDA to determine if data could provide sufficient discrimination between the respective countries of origin. The multivariate model achieved a correct classification rate of 60%, with ¹⁸O and ¹³C providing the most discrimination. Fig. 1 shows a cross plot of functions derived from the multivariate model using the CHO stable isotopes variables used in the country of origin assignments.

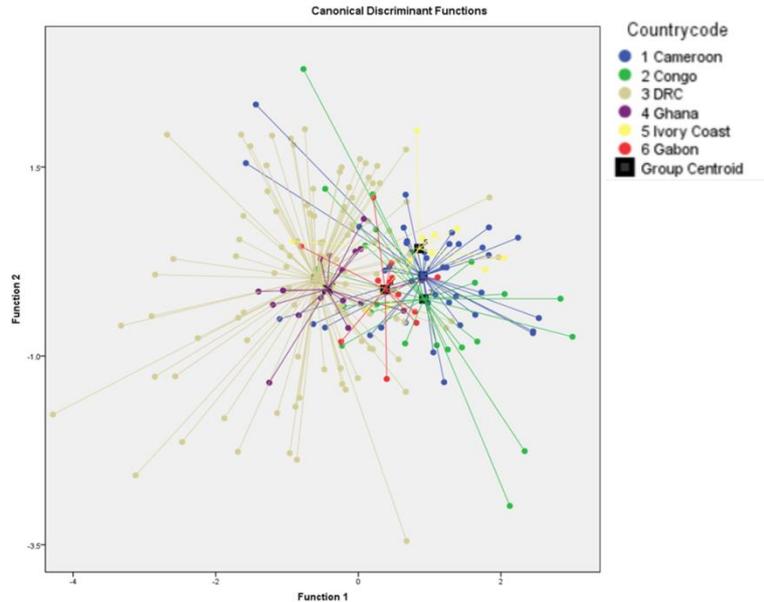


Fig. 1 Functions derived from the statistical analysis of stable isotope variables (^{18}O , ^{13}C and ^2H) for the respective country groups.

2.2 Results of the blind test exercise

2.2.1 Claims from blind test

For the country level claims of origin, the chi-squares statistical analysis of the CHO isotope data, acquired from the analysis of reference and blind test samples, was not able to reject the hypothesis that the claims of county origin for the timber were incorrect (Table 1.1). For regional level claims of origin, the chi squares analysis rejected the labelling claim of four of the twenty samples: BT_2014_573, BT_2014_557, G2S_O_E6, G2S_O_E22.

Table 1.1 FERA's country and regional claim for the blind test samples.

ID-number	species	declared country of origin	declared region of origin [GPS and Radius]	Y/N result concerning the declaration of origin: COUNTRY	Y/N result concerning the declaration of origin: REGION
BT_2014_552	Sapelli	DRC	N0.45; E25.8 (200 km Radius)	correct	correct
BT_2014_577	Sapelli	Cameroon	N2.8; E12 (40 km Radius)	correct	correct
BT_2014_510	Sapelli	Ghana	N6.4; W1.2 (70 km Radius)	correct	correct
BT_2014_592	Sapelli	Congo Brazzaville	N0.51544; E16.72308 (120 km Radius)	correct	correct
BT_2014_573	Sapelli	DRC	S4.77; E16.9 (120 km Radius)	correct	incorrect
BT_2014_557	Sapelli	DRC	S4; E19 (300 km Radius)	correct	incorrect
BT_2014_580	Sapelli	Cameroon	N3.04; E14.5 (90 km Radius)	correct	correct
BT_2014_525	Sapelli	Ghana	N6.3; E0 (75 km Radius)	correct	correct
BT_2014_534	Sapelli	Kongo Brazzaville	S3; E15 (100 km Radius)	correct	correct
BT_2014_522	Sapelli	DRC	N1.55115; E21.07416 (300 km Radius)	correct	correct
G2S_O_E4	Sapelli	Ghana		correct	N/A
G2S_O_E6	Sapelli	DRC	South - west (Bandundu province)	correct	incorrect
G2S_O_E8	Sapelli	Cameroon	South (Sangmelima)	correct	correct
G2S_O_E11	Sapelli	Congo	North Region	correct	correct
G2S_O_E13	Sapelli	Congo	North Region	correct	correct
G2S_O_E14	Sapelli	Congo	North Region	correct	correct
G2S_O_E18	Sapelli	DRC	North-West (Equateur province)	correct	correct
G2S_O_E20	Sapelli	DRC	North-West (Equateur province)	correct	correct
G2S_O_E22	Sapelli	DRC	South-West (bandundu province)	correct	incorrect
G2S_O_E24	Sapelli	DRC	North East region (orientale region, Kisangan	correct	correct

2.2.2 Outcome of blind test

Following submission of the blind test results to the WWF and G2S, the real country and regional origin of the samples were revealed enabling assessment of FERA's performance in the blind test exercise (Table 1.2)

Table 1.2 FERA's performance in blind test exercise.

Laboratory	wood species	Blind test partner	Evaluation in blind test (Country claim)			Total samples able to be analysed in blind test (%)	Success rate in blind test (%)	Adjusted success rate in blind test (samples not-analysed excluded) from analysis (%)
			Right	Wrong	No Answer			
FERA	<i>Entandrophragma cylindricum</i> (Sapele)	WWF	7	3	0	100	80	80
		G2S	9	1	0			

3 Conclusions

Canonical discriminant analysis of data acquired from the analysis of $\delta^2\text{H}$, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ stable isotope ratios in cellulose enabled a 60% country classification rate. The viability of these data to achieve a high degree of country of origin discrimination was promising, and therefore deemed suitable to undergo assessment in the blind test exercise. As shown in Table 1.2, FERA was able to analyse 100% (20/20) of the samples supplied in the blind test, and achieved a 70% success rate for the country claims on the blind test samples supplied by WWF (wood shavings). For the samples received from G2S (sawn timber), a 90% success rate was achieved. The higher performance achieved with the G2S samples may be linked to the presence of a greater number of growth rings. The inclusion of additional isotopes in the statistical model such as ^{15}N , ^{34}S and ^{87}Sr may improve the resolution and predictive power, as may the use of better matrix and delta matched and standards for the calibration of hydrogen data.

Final report ITTO Africa Project - Technical report on the stable isotopes analysis for Iroko

Markus Boner, Agrolsolab, Jülich, Germany

Email: m.boner@agroisolab.de

Background

The stable isotope method is one of the most promising tools in forensic science as it offers the opportunity to verify the authenticity of many materials [Meier-Augenstein 2010]. Today there is a wide array of publications available about the use of stable isotope analysis to determine the provenance of various products such as: wheat, rice, olive oil and animal products such as milk and beef [Kelly 2005]. Stable isotope analysis is an established method to study animal migration [Hobson 1999] and is used in criminal investigations where hair samples are used to predict the provenance of perpetrators [Ehleringer 2008].

The most predominant use of stable isotope analysis is within the food sector where it is regularly used to re-trace and verify the origin of food. The stable isotope method has been incorporated into European regulation: 2729/2000 as the method to authenticate the origin of wine and control mislabelling. Considering that stable isotope analysis is a well-established and robust method widely used in traceability, it is surprising that that it has not yet been applied in the timber industry even though its basic scientific principles dealing with the correlation of cellulose and water were published in the mid-70s [Epstein 1977].

Water plays an essential role in stable isotope traceability. The mean isotopic ratios of hydrogen and oxygen in precipitation are primarily dependent on the annual temperature of specific locations [Dansgaard 1964] and secondarily on other less influential factors such as altitude, latitude and the continental effect [Araguas 2000]. Consequently there is great variability in isotopic patterns in groundwater geographically [Bowen 2002]. Hydrogen and oxygen isotopes in tree cellulose reflect the isotopic patterns observed in groundwater with some modifications, the signature is primarily influenced by the evaporation effects of water [Flanagan 1991a] and secondarily by biochemical fractionation in the anabolism of cellulose [Sternberg 1986, Luo 1992] e.g. with a $\delta^{18}\text{O}$ shift of approximately +27‰. The result is that different shifts of $\delta^{18}\text{O}$ and δH are observable in tree celluloses. Nevertheless, correlation of the two ratios is still applicable and is implemented in paleoclimatic studies [Burk 1981, Yapp 1982]. Furthermore a progress could be an analysis of lignin methoxy group to achieve a higher correlation with the groundwater [Keppler 2007].

The ratio of carbon isotopes in timber is primarily dictated by the photosynthetic pathway the plant uses [O'Leary 1988], this aspect is not geographically distinct therefore it is not relevant in terms of tracking the origin of timber. On the other hand, there is a strong fractionation in carbon ratios that is dependent on stomata conductance and photosynthetic assimilation [Farquhar 1982]; both are influenced by environmental factors such as humidity, light and temperature. Therefore the carbon ratio in timber reflects the local climate of the area in which it grew and is suitable as an additional parameter to add resolution to provenancing using stable isotopes. This has been utilised where measurements of carbon ratios in tree rings were used as a code in conjunction with climate data to decipher the origin of the wood with a resolution of 114-304km [Kagawa & Leavitt 2009]. However, this application of the stable isotope method is costly and time-consuming.

Another strategy is to analyse the average isotopic ratio of carbon in many tree rings with an adapted sampling and preparation procedure to include additional stable isotopic parameters. Historically, sulphur [Thode 1991] and strontium [Capo 1998] have been used as further parameters to decipher the geographical origin of wood.

The stable isotopes of the bio-elements show the highest fractionation effects in nature with respect to their light mass versions. Natural fractionation systems, such as the global water cycle, produce geographically distinct patterns. With the exception of Strontium, stable isotopes of the heavy elements have no relevance in provenancing. Heavy strontium ^{87}Sr is formed in nature by the radioactive decay of the long-lived rubidium isotope ^{87}Rb . As a result, ^{87}Sr can also reflect the age of the geology. Besides this, geological parameters such as ^{87}Sr can provide further information about product adulteration and geographical provenance [Rummel 2010, Voerkelius 2010].

Ratios of $\delta^{15}\text{N}$ in agricultural soils are indicative of the fertilisation method used [Bateman 2007] which result in positive stable isotope ratios, these are notably high in soils fertilised using organic fertilisers such as manure. Positive nitrogen ratios in soil and wood are indicative of forest influenced by fertilisers. On the other hand natural forest tends to have very depleted nitrogen isotope ratios which can be as low as -6‰ [Yoneyama 1990] due to the impact of nitrogen fallout.

The resolution of the origin is increased when combinations of isotope signatures are used, this has been demonstrated in the tracking of larch wood [Horacek 2009] where $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ were used to discriminate Siberian from European larch. In a ground-breaking study from 2008 to 2010, six stable isotopes (COHNS and Sr) were used to build a database Tectonia grandis (teak) and Mahogany genus Swietenia (consisting of *S. macrophylla*, *S. mahagoni* and *S. humilis*) species with over 1,000 samples from 18 different countries [Boner 2011]. The developed database was independently tested by WWF using blind test samples (origin and species of samples not declared) to confirm the reliability of the application. 13 out of 15 samples were addressed correctly with respect to provenance.

In timber, the stable isotope method is most accurate when used in conjunction with additional parameters such as genetics. This was demonstrated in the German GIZ project (GIZ 2010) where samples of Iroko and Sapelli species from Cameroon were mapped using the stable isotopes of the bio elements (COHNS) and genetic parameters, and tested against blind test samples. Alone, the stable isotope method addressed 3 samples incorrectly from 16. In combination with genetic parameters, only 1 sample was addressed incorrectly with both methods.

Material and Methods

Mostly recommended for check of the origin are the stable isotopes of the light bioelements (COHNS): hydrogen, oxygen, carbon, nitrogen, sulphur. Additionally the higher isotope of strontium was included in the project as well.

Sample preparation:

Regularly the wood chips are milled into a fine powder using a ball-mill apparatus. After that the powder is extracted in a Soxhlet apparatus over 6 hours with methylene chloride and methanol. The powder is dried in a laboratory-type drying cabinet for at least 1 hour.

Finally the samples are stored in air tight sample vials and could be weighed in for analysis.

To avoid any equilibration or humidity effect the weight in samples for oxygen and hydrogen analysis are equilibrated overnight in a desiccators with a defined humidity of 10.6 %. Afterwards the samples are vacuum dried for at least 2 hours.

Furthermore the strontium needs an additional preparation. Round about 2 to 4g of wood powder are burned in a combustion furnace at a temperature of 750°C. The ash is transferred in a micro wave heater with round about 10ml nitric acid and 2ml hydrogen peroxide. Digestion temperature: >180°C for >15min. Depending on the samples a purification is necessary to avoid isobaric influence. Therefore purification with Sr resin (Sr-C20-A, Eichrom) is performed.

Equipment:

1. D/H, $^{18}\text{O}/^{16}\text{O}$ measurement:

The high temperature application uses HT-PyrOH with silicium carbide tube (Hekatech) filled with glassy carbon chips and coal powder. Working temperature for pyrolysis of >1550°C. To gain a higher precision the isotopes are measured in a master / slave configuration with two IRMS (Isoprime, Elementar). Each IRMS is only measuring one isotope: D/H or $^{18}\text{O}/^{16}\text{O}$

2. $^{13}\text{C}/^{12}\text{C}$ measurement

EA (Carlor Erba, NA1500) in combination with IRMS (Horizon, NU-Instruments). Working temperature: 1021°C (oxidation), 600°C (reduction)

3. $^{15}\text{N}/^{14}\text{N}$ measurement

EA (Carlor Erba, NA1500) in combination with IRMS (Horizon, NU-Instruments). A further addition of a packed column for CO separation is used to get rid of isobaric effect. Working temperature: 1021°C (oxidation), 600°C (reduction)

4. $^{34}\text{S}/^{32}\text{S}$ measurement:

EA (Hekatech) with IRMS (Optima, VG Instruments). A one tube combustion (oxidation and reduction in one tube) is used to solve any SO₃ problem. Furthermore combustion water is directly trapped with magnesium perchlorate at the end of the tube. Working temperature: 1000°C.

5. $^{87}\text{Sr}/^{86}\text{Sr}$: ICP-MS (Elan 6100) was used. For correction the isotopic standard: NIST SRM 987 was used.

Results

The German isotopic laboratory Agroislab was responsible for development of the iroko (Milicia excelsa) database. In total 474 reference samples from seven African countries (Cameroon, Democratic Republik of Congo, Gabon, Ghana, Ivory Coast, Kenya and the Republic of the Congo) were analyzed in the project time. Most of the results will be included in the international GTTN database.

Regularly the water isotopes (D/H and $^{18}\text{O}/^{16}\text{O}$) represent the main differentiation parameter for origin check. Unfortunately the mapped countries are more or less on the equator line so it could be expected that water isotopes are in tendency very similar. Therefore reference samples from the

costal countries Cameroon, Ghana, Gabon and the Republic of Congo show similar $^{18}\text{O}/^{16}\text{O}$ and D/H ratios in range of +23.8 to +24.3 ‰ and -48 to -51 ‰ respectively. In contrast the continental country Democratic Republic of Congo shows a tendency to increased $^{18}\text{O}/^{16}\text{O}$ and D/H ratios of +25,1 ‰ ($^{18}\text{O}/^{16}\text{O}$) and -45,4 ‰ (D/H). Nevertheless the most increased ratios could be detected in Kenya with an average $^{18}\text{O}/^{16}\text{O}$ ratio of 26.3 ‰ and an average D/H ratio of -40.4 ‰. These significant increased ratios are in well agreement with the known stable isotopic water situation in these regions [Bowen 2012].

Because of the similarities of the water isotopes the geological stable isotopes ($^{34}\text{S}/^{32}\text{S}$, $^{15}\text{N}/^{14}\text{N}$, $^{87}\text{Sr}/^{86}\text{Sr}$) are getting more relevant for discrimination. Therefore the sulphur ratios are useful to distinguish samples from Ghana to Gabon or Cameroon (figure 2).

Only the combination of all six stable isotopes enables a differentiation of various countries (figure 3). Still yet timber from Gabon and RCB are hardly to discriminate and have broad overlapping with several countries. Therefore further parameters are needed to improve the discrimination. A solution is still the combination of stable isotopes with genetic, rare elements or near infrared data.

Figure 1: Isoscapes of the $^{18}\text{O}/^{16}\text{O}$ ratios in timber (*Milicia excelsa*)

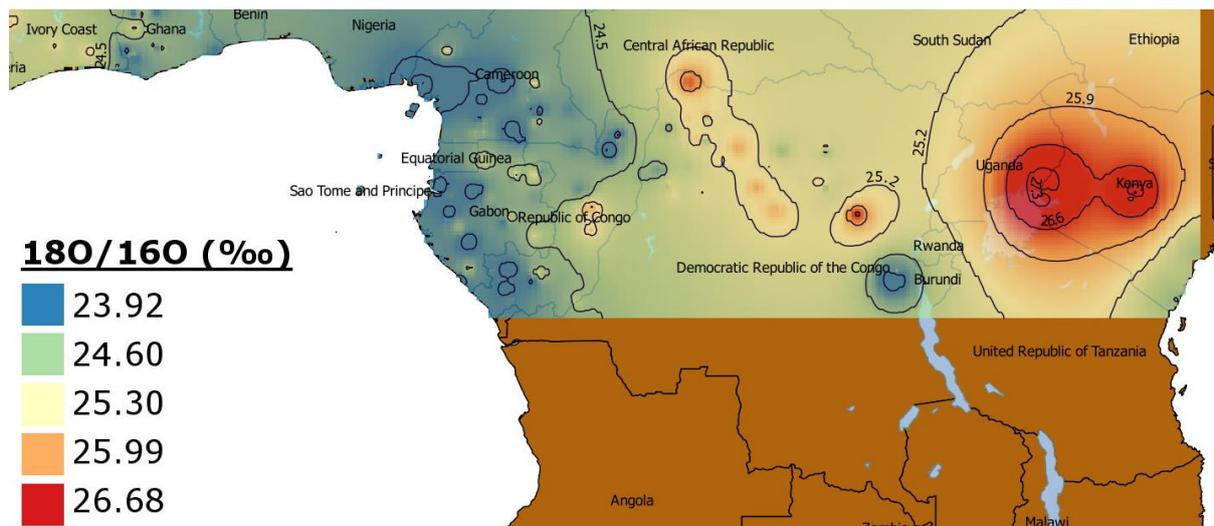


Figure 2: Boxplots of the stable isotopic sulphur ratios of 7 African countries

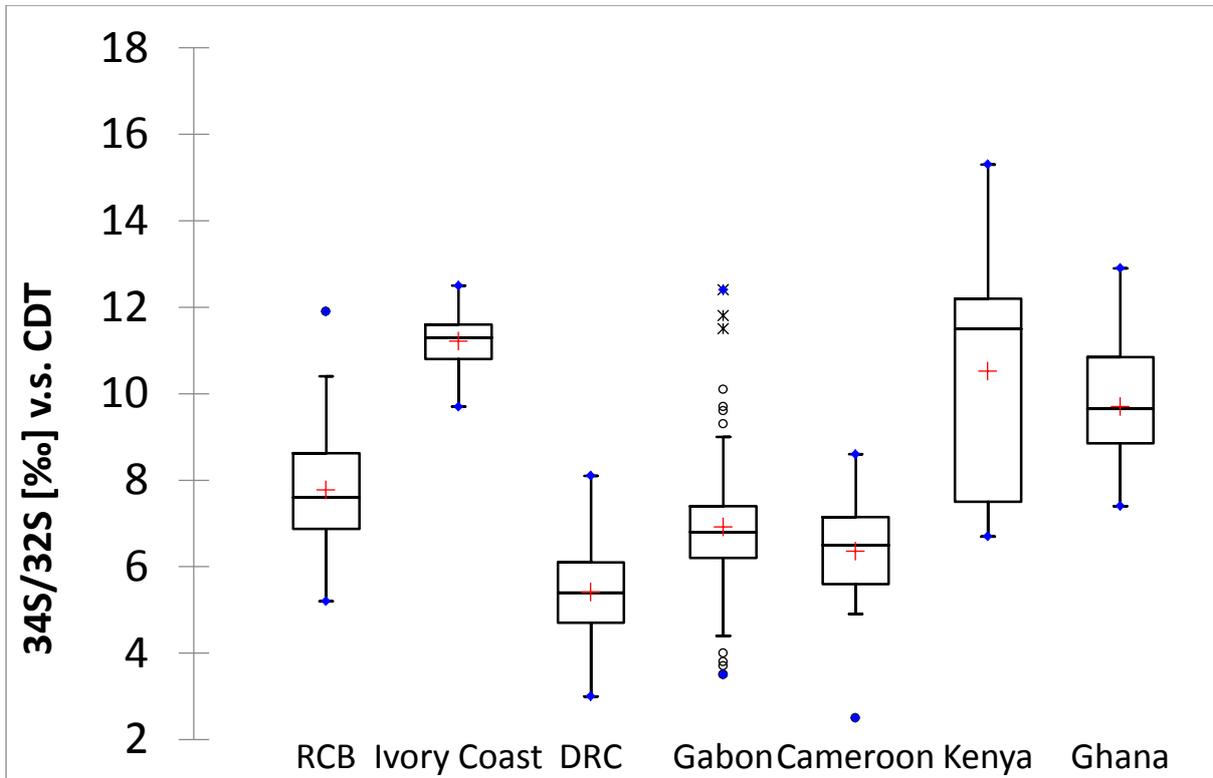
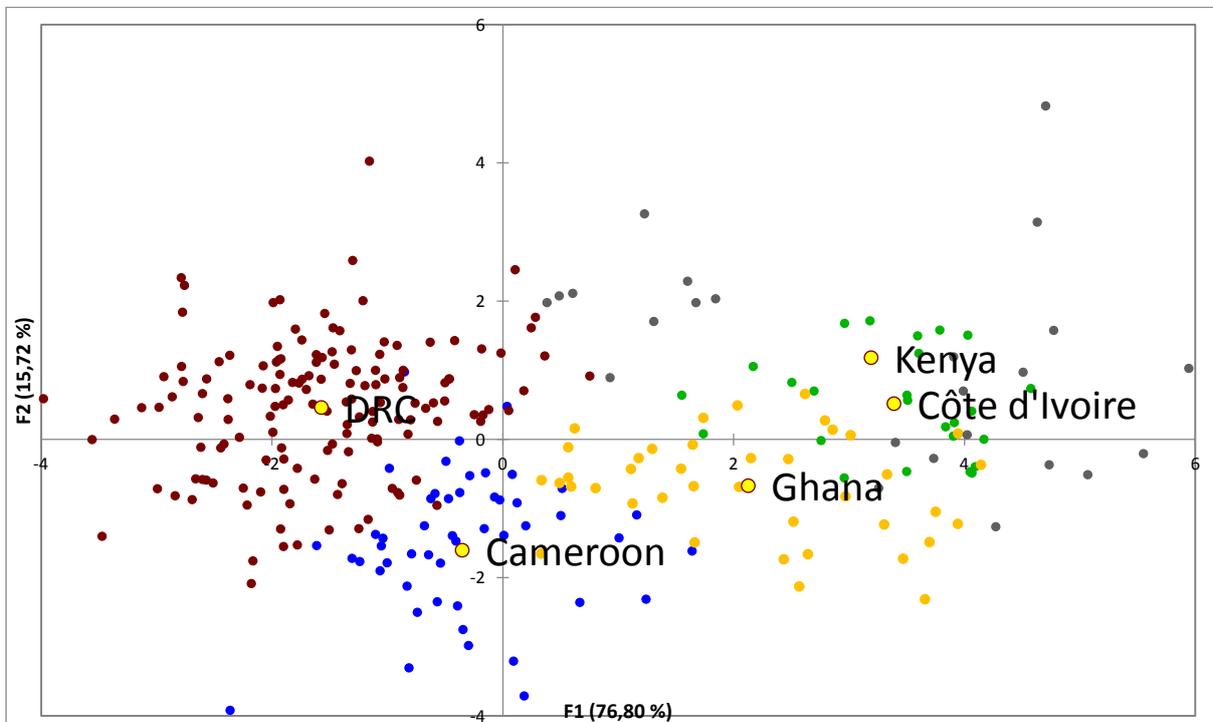


Figure 3: Discriminate analysis (DA) of 5 using stable isotope of bioelements (COHNS) and Strontium



References

1. Araguas-Araguas L.; Froehlich K.; Rozanski K. (2000) Deuterium and oxygen-18 isotope composition of precipitation and atmospheric moisture. *Hydrol. Process.* 14, 1341-1355.
2. Bateman A.S.; et al (2007) Nitrogen Isotope Composition of Organically and Conventionally Grown Crops. *J. Agric. Food Chem.* 55, 2664-2670.
3. Bowen G.J.; Wilkinson B. (2002) Spatial distribution of $d^{18}O$ in meteoric precipitation. *Geology*, 315-318.
4. Burk. R.L.; Stuiver M. (1981) Oxygen isotope ratios in trees reflect mean annual temperature and humidity. *Science* 211, 1417-1419.
5. Boner M. et al (2011) Fighting illegal logging through the introduction of a combination of the isotope method for identifying the origins of timber and DNA analysis for differentiation of tree species, DBU-report [German Federal Foundation for the Environment], AZ 26452/31.
6. Dansgaard W. (1964) Stable isotopes in precipitation. *Tellus* 16, 436-467.
7. Ehleringer J. R. et al. (2008) Hydrogen and oxygen isotope ratios in human air are related to geography. *PNAS*, 105, 2788-2793
8. Epstein S., Thompson, P., and Yapp, B.J (1977) Oxygen and Hydrogen Isotopic ratios in plant cellulose. *Science* 198, 1209-1214.
9. Farquhar G.D.; O'Leary M.H. Berry J.A. (1982) On the relationship between carbon isotope discrimination and intercellular carbon dioxide concentration in leaves. *Aust. J. Plant Physiol.* 9, 121-137.
10. Horacek M. et al. (2009) Control of origin of larch wood: discrimination between European (Austrian) and Siberian origin by stable isotope analysis. *Rapid Com. In Mass Spectr.* 23, 3688-3692.
11. Flanagan L.B. et al. (1991a) Comparison of modelled and observed environmental influences on the stable oxygen and hydrogen isotopes composition of leaf water in *Phaseolus vulgaris* L. *Plant Physiol.* 96, 588-596.
12. GIZ report (2010) Genetic and isotopic fingerprinting methods – practical tools to verify the declared origin of wood. GIZ Division 47.
13. Hobson A. (1999) Tracing origins and migration of wildlife using stable isotopes: A review. *Oecologia* 120, 314-326.
14. Kagawa A.; Leavitt S. W. (2009) Stable carbon isotope of tree rings as a tool to pinpoint the geographic origin of timber. *J. Wood Sci.* 56, Issue 3, 175-183.
15. Keppler F. et al (2007) Stable hydrogen isotope ratios of lignin methoxyl groups as a paleoclimate proxy and constraint of the geographical origin of wood. *New Phytol.* 176, 600-609.
16. Capo R. C. et al. (1998) Strontium isotopes as tracers of ecosystem processes: theory and methods. *Geoderma.* 82, 197-225.
17. Kelly S., Heaton K., Hoogewerff J. (2005) Tracing the geographical origin of food: The application of multi-element and multi-isotope analysis. *Trends in Food Science & Tech.* Vol. 16, Iss. 12, 555-567.

18. Luo Y.H. Ehleringer J.R. (1992) Hydrogen and oxygen isotope fractionation during heterotrophic cellulose synthesis. *Journal Exp. Bot.* 43, 47-50.
19. Meier-Augenstein W. (2010) *Stable isotope forensics*. Wiley-Blackwell, ISBN 978-0-470-51705-5.
20. O'Leary M.H. (1988) Carbon isotopes in photosynthesis. *Bioscience*. 38, 328-336.
21. Roden J.S. et al. (2000) A mechanistic model for the interpretation of hydrogen and oxygen isotope ratios in tree-ring cellulose. *Geochim. Cosmochim. Acta* 64, 21-35.
22. Rummel S. et al. (2010) The combination of stable isotope abundance ratios of H, C, N and S with $^{87}\text{Sr}/^{86}\text{Sr}$ for geographical origin assignment of orange juices. *Food chemistry*, 118, 890-900.
23. Sternberg L.S.L; DeNiro M.J. Savidge R.A. (1986) Oxygen isotope exchange between metabolites and water during biochemical reactions leading to cellulose synthesis. *Plant Physiol.* 82, 423-427.
24. Thode H.G. (1991) Sulphur isotopes in nature and the environment: An overview. In: *Stable Isotopes in the Assessment of Natural and Anthropogenic Sulphur in the Environment*, Scope 42. Wiley, New York, 1-26.
25. Voerkelius S. et al. (2010) Strontium isotopic signatures of natural mineral waters, the reference to a simple geological map its potential for authentication of food. *Food Chemistry*. 118, 933-940.
26. Yapp C.; Epstein S. (1982) Climatic significance of the hydrogen isotope ratios in tree cellulose. *Nature* 297, 636-639.
27. Boner et al. (2011) Fighting illegal logging through the introduction of a combination of the isotope method for identifying the origins of timber and DNA analysis for differentiation of tree species. Report, German Federal Foundation for the Environment, AZ 26452/31.
28. Yoneyama T. et al. (1990) Variation of natural ^{15}N abundance of crops and soils in Japan with special reference to the effect of soil conditions and fertilizer application. *Soil Sci. Plant Nutr.* 36, 667-675.

Final report ITTO Africa Project - Technical report on the stable isotopes analysis for ayous

Micha Horacek, HBLFA Francisco-Josephinum, Wieselburg, Austria

E-Mail: micha.horacek@josephinum.at

Materials and Methods

Sample preparation:

Sample preparation started with close inspection and cleaning of the samples. Afterwards they were homogenized in a ball mill (Retsch MM250, Haan, Germany) and weighed into tin (for carbon and nitrogen analyses) and silver (for hydrogen and oxygen analyses) capsules. Before measurement the silver capsules were stored in a desiccator for equilibration. For sulphur isotope measurement a sulphur pre-concentration step had to be performed. The so processed samples were then afterwards also weighed into tin capsules.

Measurements:

For hydrogen and oxygen measurements the silver capsules were introduced into the autosampler of a high-temperature elemental analyser (Flash HT, Thermo Scientific, Bremen, Germany). Pyrolysis at 1450°C converts the samples into gaseous state and a continuous helium flow transports the gas via a ConFLO (Thermo Scientific) to the Delta V isotope ratio mass spectrometer (Thermo Scientific). There the samples are analysed for the isotope composition.



Fig. 1: EA - IRMS (Thermo Scientific)

For carbon, nitrogen and sulphur isotope measurements the tin capsules were introduced into an elemental analyser (EA) via the autosampler. In contrast to the hydrogen and oxygen measurement a thermal combustion (at $T=1020^{\circ}\text{C}$) takes place in the EA. The further procedure is identical with the procedure for hydrogen and oxygen isotope measurements.

All results are expressed in the conventional δ notation in permil [‰] versus international standards: V-SMOW (Vienna Standard Mean Ocean Water) for hydrogen and oxygen, V-PDB (Vienna Pee Dee Belemnite) for carbon, N_{air} for nitrogen and V-CDT (Vienna Canyon Diabolo Troilite) for sulphur isotope measurements. The results are calibrated against certified standards. For carbon a protein standard (IVA) is used ($\delta^{13}C = -26,98‰$) and hydrogen and oxygen results are calibrated against a cellulose standard IAEA CH-3 ($\delta^{18}O = 32,5‰$, $\delta^2H = -35,5‰$). The maximum standard deviation is about $\pm 0,2‰$ for carbon and nitrogen, $\pm 3‰$ for hydrogen and $\pm 0,3‰$ for oxygen and sulphur isotope ratios.

Results

Carbon isotope ratios: For carbon isotopes the average as well as the range are rather similar for all of the countries. For Cameroon the $\delta^{13}C$ values range from -30,3 to -26,5‰, for the Republic of Congo from -31,3 to -28,1‰, for Cote d'Ivoire from -30,5 to -26,5‰, for the Democratic Republic of Congo from -33,3 to -27,9‰ and for Ghana from -29,3 to -26,77‰.

Hydrogen isotope ratios: The δ^2H values for Cameroon, the Republic of Congo, Cote d'Ivoire, the Democratic Republic of Congo and Ghana are between -54 and -32‰, -51 and -32‰, -55 and -34‰, -47 and -18‰ and between -56 and -30‰, respectively.

Oxygen isotope ratios: Oxygen isotopes show values between 22,5 and 25,3‰ for Cameroon, 19,4 and 22,7‰ for the Republic of Congo (due to different sample material, see below), 22,9 and 25,1‰ for Cote d'Ivoire, 23,3 and 27,0‰ for the Democratic Republic of Congo and values between 22,7 and 25,1‰ for Ghana.

Additionally also the isotope range for N-, S- and Sr-isotopes have been analysed.

Discussion

Generally, the isotope measurement results are quite good for distinguishing between the investigated countries. In the blind test 13 out of 19 samples were judged correctly.

Significant for the results was the sample material analysed and which varied between countries of origin with respect to the reference samples. Samples from Ghana and Cameroon were wood cores, samples from the Democratic Republic of Congo wood shavings and the samples from the Republic of Congo bark material. These differences in sample material had a significant influence on the results. The oxygen isotope values of the samples of the Republic of Congo were significantly lower than the values of the other investigated countries. As the samples from the countries neighbouring the Republic of Congo do not give similar results, the material is most probably responsible for this difference. The results of the other measured element isotopes of the Republic of Congo are not conspicuous and explainable and no influence due to the sample material obvious.

Wood cores are supposed to provide the most trustworthy results because an average over the annual rings is produced. This is important because isotopic values depend on weather and climate, which can change over the lifetime of a tree. Therefore, small differences between the annual rings are possible. Wood shavings give an offset for the nitrogen isotope

values with respect to the neighbouring countries wood core samples. For the other element isotopes no offset is obvious.

Some of the blind-test samples were located in provinces and regions without available reference data. Some of the declared geographic origins of the blind samples were actually situated hundreds of kilometres away from the next reference samples. This is problematic as comparability is not possible or of only limited use. Different influences of actual geographic origin (for example micro – climate, soil type and thickness, water availability, geology, etc..) can differ from the regions which were investigated.

Outlook

A larger amount of samples will increase the reliability and accuracy of the interpretations. Especially for distinguishing between provinces of the investigated countries of origin the current amount of samples is small as in some of the countries the provinces are just represented by two or three reference samples, and sometimes there is no reference sample at all. The isotope analysis of the elements H, C, O, N, S, Sr is necessary for an optimal interpretation. Also the combination of different methods (isotopes and genetics) certainly will be an important improvement.