COMPARISON OF ANALYTICAL METHODS FOR DETERMINING NON-EXCHANGEABLE STABLE HYDROGEN RATIOS IN TEA

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Determining the δ^2H value of organic compounds is challenging due to the presence of moisture traces from ambient humidity, hygroscopic compounds, and exchangeable non-bound hydrogen atoms. Several approaches have been developed to avoid the interference of exchangeable hydrogen. However, these approaches are comparatively difficult to use for the δ^2H analysis of tea samples due to a lack of suitable solid reference materials for two-point calibration and time-consuming procedures. This study adapts and scales the method for lignin analysis, targeting methoxy groups to measure non-exchangeable hydrogen for the analysis of tea samples using gas chromatography coupled with isotope ratio mass spectrometry (GC-IRMS). It aims to compare the mentioned method with bulk δ^2H analysis of tea samples using equilibration techniques that involve different isotopic compositions and laboratory moisture. A total of 45 orthodox black tea samples were collected from three different tea growing regions with varying elevations in Sri Lanka: Nuwara Eliya (n=15), Kandy (n=15), and Ruhuna (n=15), and δ^2H values were measured using three different methods: GC-IRMS analysis of methoxy group, water vapor equilibration (enriched δ^2H) and laboratory moisture equilibration. One-way analysis of variance (ANOVA) was performed, and the results indicated a statistically significant difference in the averages of δ^2H values between the three methods ($p < 0.01$). The GC-IRMS method for analysing δ^2H in the methoxy groups of tea samples was the most reliable and precise method for analysing δ^2H in tea, as it offered consistent δ^2H values with low variability. There was a slight variability due to natural variations in the δ^2H of the non-exchangeable hydrogen in different elevations. It provided a clear separation between regions with minimal overlap. The laboratory moisture equilibrium and water vapor equilibration methods demonstrated high variability and lower precision, which may be due to environmental factors such as temperature and humidity. In addition, these methods were time-consuming compared to the GC-IRMS method for analysing the δ^2H in methoxy groups. Therefore, GC-IRMS methoxy group analysis is a robust method for δ^2H determination in tea with precision and consistency.

Keywords: δ^2H analysis, Methoxy group, non-exchangeable hydrogen, stable isotope ratio, tea authenticity

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INTRODUCTION

Stable hydrogen isotope ratios (δ^2H) have been widely used to identify the geographical origin, address mislabelling, and determine the history of samples in food products such as olive oil and fruit juice (Bandoniene et al., 2018). Determining the δ^2H value of organic compounds is challenging due to the presence of moisture traces from ambient humidity, hygroscopic compounds, and exchangeable non-bound hydrogen atoms (Qi & Coplen, 2011). Although the problematic issues of moisture traces from ambient humidity and hygroscopic compounds are easily overcome by drying, the effect of exchangeable hydrogen must be carefully addressed without compromising the accuracy and reproducibility of the resulting data (Wassenaar & Hobson, 2000).

Several approaches have been developed to avoid the interference of exchangeable hydrogen, including equilibrating exchangeable hydrogen with water of specified isotopic composition at or above 100 °C, equilibration with water of specified isotopic composition at ambient temperature, and equilibration with laboratory moisture at ambient temperature (Qi & Coplen, 2011). However, these approaches are comparatively difficult to use for the δ^2H analysis of tea samples due to the limited availability of matrix-matched reference materials and lengthy equilibration times (approximately 10 days) (Wassenaar & Hobson, 2000).

This study adapts and scales the method proposed by Keppler et al. (2007) for lignin analysis, where methoxy groups are targeted to measure non-exchangeable hydrogen for the analysis of tea samples using gas chromatography coupled with isotope ratio mass spectrometry (GC-IRMS). It aims to compare the mentioned method with bulk δ^2H analysis of tea samples using equilibration techniques that involve different isotopic compositions and laboratory moisture.

METHODOLOGY

1. Sample Collection and Preparation

A total of 45 orthodox black tea samples were collected from three different elevations in Sri Lanka: Nuwara Eliya (n = 15), Kandy (n = 15), and Ruhuna (n = 15). Samples were ground to a fine powder with a laboratory mill, passed through a 400 µm sieve, and dried for two hours at 60 °C. These samples were then subjected to three distinct procedures for determining stable hydrogen ratios (δ^2H), as outlined below.

2. Determining stable hydrogen ratios of tea samples by analysing the methoxy group

Approximately 100 mg of a finely ground tea sample was subjected to acidolysis using 0.1 M HCl at 80 °C for 4 hours to extract lignin methoxy groups. The methyl groups were then derivatized with methyl iodide to form methyl iodide gas. 2 ml of the headspace gas was injected into the GC-IRMS system for δ^2H analysis.

3. Determining stable hydrogen ratios of tea samples by equilibration at ambient temperature with water vapour of different isotopic compositions

Two identical sets of tea samples were weighed in silver capsules. One set was equilibrated in a glass desiccator with water depleted in ^2H and the other in a desiccator with water enriched in ^2H. Each set of tea samples was loosely covered and suspended within a desiccator containing approximately 20 mL of isotopic reference water. The desiccators were evacuated with a rotary mechanical pump for 2 min to remove air. The samples were equilibrated in each desiccator at ambient temperature for 6 days and analysed using EA-IRMS.

4. Determining stable hydrogen ratios of tea samples by ambient temperature equilibration with laboratory moisture

500 ± 10 µg of tea samples were loaded into silver capsules and loosely closed to allow for satisfactory equilibration with atmospheric laboratory moisture and enable satisfactory drying. The samples and reference materials were equilibrated with ambient lab moisture for at least 5 days and analysed using EA-IRMS.

5. Calibration and Quality Control

All δ^2H measurements were calibrated against international standard reference materials, including USGS90, USGS91, USGS42 and USGS43, following a two-point normalization procedure to correct for instrumental drift and bias. The samples were analysed in triplicate, and laboratory blanks were included to monitor potential contamination. One-way analysis of variance (ANOVA) was performed using IBM SPSS Statistics v27. Post-hoc pairwise comparisons (Tukey's test) and Pearson correlation analysis were also conducted to evaluate the relationships between methods.

RESULTS AND DISCUSSION

The stable hydrogen isotope ratios (δ^2H) of black tea samples from different elevations (Ruhuna, Kandy, and Nuwara Eliya) were measured using three distinct methods: GC-IRMS analysis of the methoxy group, water vapor equilibration with depleted and enriched δ^2H water vapor, and laboratory moisture equilibration. The results are summarised in Table 1.

Table 1: Average δ^2H values and standard deviations for each method and region.

Method	Ruhuna Region (n=15)	Kandy Region (n=15)	Nuwara Eliya Region (n=15)
GC-IRMS Analysis of Methoxy Group	-65.20 ± 1.20	-72.40 ± 1.10	-78.60 ± 1.30
Water Vapor Equilibration (Depleted δ^2H)	-5.40 ± 3.50	-5.20 ± 3.40	-5.60 ± 3.60
Water Vapor Equilibration (Enriched δ^2H)	8.20 ± 4.10	8.10 ± 4.20	8.30 ± 3.90
Laboratory Moisture Equilibration	-73.51 ± 6.12	-80.90 ± 4.25	-84.27 ± 7.70

The δ^2H values of GC-IRMS analysis of the methoxy group in tea samples represented a narrow range of variation. The δ^2H values of all the tea samples were –65.20 ± 1.20 ‰ for Ruhuna, –72.40 ± 1.10 ‰ for Kandy, and –78.60 ± 1.30 ‰ for Nuwara Eliya. This method showed a distinct significant decrease in δ^2H values with increasing elevation, representing the isotopic composition of local precipitation and environmental water sources. The narrow standard deviations indicate good precision and reproducibility for this method.

For the water vapour equilibration method, two sets of samples were equilibrated with water vapour enriched in δ^2H and depleted in δ^2H. The average δ^2H value for tea samples equilibrated with depleted δ^2H water and the average value for those equilibrated with enriched δ^2H water were –5.40 ± 3.50‰ and 8.20 ± 4.10‰ for Ruhuna, –5.20 ± 3.40‰ and 8.10 ± 4.20‰ for Kandy, and –5.60 ± 3.60‰ and 8.30 ± 3.90‰ for Nuwara Eliya. This method shows the differences between the two isotopic compositions, although the variability is high across the samples. It may be due to being more sensitive to factors such as ambient humidity, temperature, and equilibration time. The variability in δ^2H values between the regions and the overlaps of values suggest that this method may be less precise in determining the subtle differences in isotopic composition based on elevations.

The average δ^2H values for tea samples equilibrated with ambient laboratory moisture were -73.51 ± 6.12‰ for Ruhuna, –80.90 ± 4.25‰ for Kandy, and –84.27 ± 7.70‰ for Nuwara Eliya. The results represented a significant variability, and the average δ^2H values of the Ruhuna, Kandy, and Nuwara Eliya regions showed a clear trend of decreasing δ^2H with increasing elevation. The δ^2H values for the mid and high-grown regions were significantly lower than those of the low-grown region, which could reflect differences in the local moisture and climate at different elevations. Due to the wide variability, overlaps were observed between the values of the Kandy and Nuwara Eliya regions. In addition, this method showed the highest variability among the three methods for analysing δ^2H value, since the results may be affected by environmental conditions during the equilibration process. Therefore, this method is less reliable for obtaining precise isotopic measurements compared to the other two discussed methods.

One-way analysis of variance (ANOVA) was performed to determine whether there were significant differences between the averages of δ^2H values from samples obtained using three different methods. The results indicated a statistically significant difference in the averages of δ^2H values among the three methods ($p < 0.01$). According to post-hoc pairwise comparisons using Tukey's Multiple test, the difference between δ^2H values of GC-IRMS vs. water vapour equilibration and GC-IRMS vs. laboratory moisture equilibration were significant ($p < 0.01$). A Pearson correlation test was conducted to determine the relationship between the three methods. According to the results, there is a moderate positive correlation between the GC-IRMS and water vapor equilibration methods ($r = 0.67$, $p < 0.01$). It indicates that these two methods produce similar results, although the degree of precision varies. However, the correlation between the GC-IRMS method and laboratory moisture equilibration was weaker ($r = 0.48$, $p < 0.05$).

CONCLUSION

The GC-IRMS method for analysing δ^2H in the methoxy group of tea samples was the most reliable and precise method for analysing δ^2H in tea. It offered a clear separation between regions with minimal overlap. Additionally, this method yielded consistent δ^2H values with low variability. The laboratory moisture equilibrium and water vapour equilibration methods demonstrated high variability and lower precision due to environmental factors such as temperature and humidity. In addition, these methods were time-consuming compared to the GC-IRMS method for analysing the δ^2H in methoxy groups. Therefore, GC-IRMS methoxy group analysis is a robust method for δ^2H determination in tea with precision and consistency.

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