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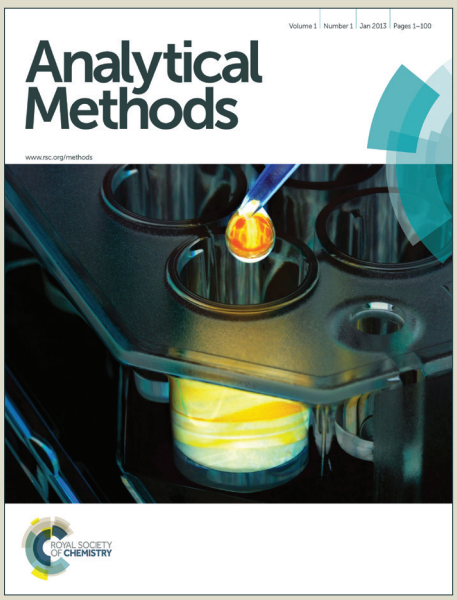

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PAPER

The carbon isotopic ($^{13}C/^{12}C$) signature of sugar cane bioethanol: certifying the major source of renewable fuel from Brazil.

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Ethanol has been increasingly used worldwide as a major renewable fuel. Since it can be produced from several matrixes such as sugar cane, corn, wheat, grape and beet, it has become important for certification purposes to verify the geographical origin and the different raw materials used for ethanol production. In this work, isotope ratio mass spectrometry, coupled to gas chromatography was used to measure the carbon isotopic signature ($\delta^{13}C_{V\text{-}PDB}$) of the Brazilian sugar cane bioethanol. Statistical data analysis was also applied to establish a probabilistic profile for isotopic signature of major renewable Brazilian fuel. Other bioethanol produced from different raw materials in USA and France were also analysed, for comparison.

Introduction

The global energy demand based mostly in petro fuel has become a most important field of concern for science and political actions. [1] The drawbacks of using crude oil as an energy source have been exhaustive debated, and the major limitations are related to its non-renewable nature and severe pollution effects.[2] The search for new renewable, as well as economically viable, energy sources which could compete with crude oil and be more environmentally friendly is a very active area of scientific investigation, known as green chemistry. [3]

Brazil is a large country with a quite adequate environment and geographic characteristics for agriculture. Currently, ethanol seems to be one of the most attractive renewable fuels and its production and use is increasingly worldwide. [4] Brazil is the world's largest ethanol producers and its ethanol is made primarily from sugar cane. [5] The country started its bioethanol program (Proácool) in 1975, and nowadays, nearly half of Brazil's energy comes from ethanol and a few other renewable sources. Brazil has also replaced ca. 40 % of its gasoline needs with sugarcane ethanol, which has been shown therefore to be an environmentally and economically viable matrix for ethanol. [6]

The use of sugar cane ethanol has also reduced the emission of carbon dioxide in Brazil by as much as 189 million tons since 2003. [7] The low-carbon benefits of sugar cane also benefit from other products such as cellulosic ethanol, bio plastics and bio hydrocarbons, bringing diverse social-economic benefits. [8]

Although sugar cane is practically the only matrix used in Brazil, bioethanol can, however, be produced from several other matrixes such as beet, corn, wheat and rice. [6] The certification of "green" bioethanol aims the determination of its geographical origin and the raw-material used for its production. For that, isotopic signatures are the key parameter. Isotope ratio mass spectrometry (IRMS) is the technique used to measure such signatures.[9,10,11] For carbon, differences in measured isotope amount ratios of its stable isotopes ($^{13}C/^{12}C$) are commonly reported as $\delta^{13}C$ values, and when it is traceable to the international zero point of carbon isotopic values – the *Vienna Pee Dee Belemite* (V-PDB), it is referred as $\delta^{13}C_{V\text{-}PDB}$.[12] The $\delta^{13}C$ values of bioethanol are known to be directly correlated with the mechanisms of CO_2 fixation during photosynthesis.[13,14] Values of $\delta^{13}C$ from -32 e -23 ‰ are observed for plants with C3 photosynthesis (grape, rice, barley) whereas C4 (corn, sugar cane) plants display higher $\delta^{13}C$ values from -15 to -9 ‰.[15] Sugarcane is a C4 plant [16] but the range of $\delta^{13}C$ values of bioethanol produced in Brazil from sugarcane has not been systematically determined yet.

In this work, we have used GC-IRMS to measure the carbon isotopic ratios ($^{13}C/^{12}C$) of sugarcane bioethanol from Brazil to determine its $\delta^{13}C$ values and to establish its variation profile as a function of geographical origin of the sugar cane in Brazil. To assess the validity of the measurement techniques used as well for a subtle comparison, other bioethanol samples from different raw materials, e.g. corn, wheat, grape and beet from USA and France were also analysed.

Experimental

Samples. In total, 31 samples of bioethanol from sugar cane produced in different geographic regions of Brazil were analysed. (See Supp. Information, S1). All these samples as well as the bioethanol samples from different raw materials, e.g. corn, wheat, grape and beet from USA and France were analysed without any previous treatment and stored as received at 4 ºC.

Instrumental. The GC/IRMS analyses of bioethanol were performed using a Delta V Advantage mass spectrometer with a

CG IsoLink combustion reactor interface coupled to a Trace gas chromatograph. Samples were introduced by using an AS3000 auto sampler (all from Thermo Scientific, Bremen, Germany).

Methods. The chromatographic separation of bioethanol samples was performed in a CP-WAX (Varian, 30 m x, 0.25 mm i.d. x 0.25 µm film thickness), which is recommended due to its high selectivity for polar compounds such as ethanol. Pure ethanol samples (1 µL) were injected in a split ratio of 1:300. The injector temperature was set to 200 °C. The oven temperature program was as follows: 90 °C to 120 °C at 10 °C min^{-1}, held for 3.5 min. Helium was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. The samples were analysed in 6 replicates. The auto sampler syringe was rinsed with ultrapure waters 10 times before and after every sample injection to eliminate any cross-contamination and rinsed 5 times with the sample before the injection.

The isotope ratio mass spectrometer was operated at an accelerating voltage of 3.005 kV. The ion source was held at a pressure of 2.3 x 10^{-6} mbar and ions were generated by electron ionization (EI) at 124 eV. Three Faraday cup detectors monitored simultaneously and continuously the CO_2^+ signals for the three major isotopologue ions of *m/z* 44, *m/z* 45 and *m/z* 46. For ethanol analysis, three pulses of CO_2 reference gas were admitted into the inlet system for about 20 s with a backflush time of 180 s and a total run time of 450 s. The temperature of the combustion reactor was set and kept at 1030 °C.

The $\delta^{13}C$ of CO_2 reference gas in the GC/IRMS system was determined via a calibration procedure in the same conditions as described above, using a certified isotopic reference material of ethanol from rum (C4 plant origin produced by Indiana University, Bloomington, IN, USA). This standard was injected 10 times and the peak corresponding to the ethanol with a reference value of $\delta^{13}C_{V\text{-}PDB}$= (-10.98 ± 0.02 ‰) was considered the isotopic reference value for CO_2 $\delta^{13}C$ calculation. The mean value of the $\delta^{13}C_{V\text{-}PDB}$ values obtained for CO_2 was therefore used as the reference isotopic value.

To account for variations during analysis, all the measured raw $\delta^{13}C$ values were submitted to the bracketing normalization method. Bracketing requires two isotopic reference materials: one with a value above and the other with a value below that of the unknown sample. The C4 ethanol was used ($\delta^{13}C_{V\text{-}PDB}$ = -10.98 ± 0.02 ‰) and isotopic reference material of ethanol from a C3 plant ($\delta^{13}C_{V\text{-}PDB}$ = -27.53 ± 0.02 ‰) was also used as reference. Both reference standards were injected in the same batch of the correspondent samples. The results of the two isotopic reference materials were used for linear interpolation.[17,18]

Data analysis and statistics. First, an exploratory data analysis through graphs and descriptive measurements was conducted to compare the δ^{13} $C_{V\text{-}PDB}$ behaviour as a function of raw material (cane sugar, corn, wheat, beet and grapes); the metabolism (C3 or C4); and the geographical origin of the samples (Brazil – national states, France and the United States). Following, to establish the profile of Brazilian bioethanol, an inferential analysis on the carbon isotope value of Brazilian sugar cane samples was also carried out. The classical Levene's test [19] was applied to verify the homogeneity of variances of each sample (with six replicates) as well as the "QQ-plot" [20], a visual test, to investigate the hypothesis of data normality. Finally, to describe the expected range of variation of the $\delta^{13}C_{V\text{-}PDB}$ values, a probability density function (PDF) was estimated, by a kernel density model for replicated values [21], with associated mode (point estimator) and sample quantiles. The confidence intervals for each estimator were estimated using the bootstrap technique [22], and an expected range for new samples of cane sugar was proposed. The confidence level considered for all statistical tests was 95% and all statistical analyses were performed using the R Software [23], a free open source environment for statistical computing and graphics.

Results and Discussion

The measures of $\delta^{13}C_{V\text{-}PDB}$ from the 37 bioethanol samples were done with six replicates each, and Table S1 summarizes the mean values obtained. An exploratory data analysis was done to try to find correlations between influence factors or stratification variables that could point to either general or specific characteristics of the δ $^{13}C_{V\text{-}PDB}$ values on Brazilian or international scenarios. An inferential statistical analysis of the sugar cane data was also performed aiming primarily to estimate a point value and a confidence interval that would best represents the data set.

Exploratory analysis of the δ^{13} $C_{V\text{-}PDB}$ value

To summarize the characteristics of the samples, the $\delta^{13}C_{V\text{-}PDB}$ values were stratified by the site of production, raw material and metabolism. The scatterplot in Figure 1 illustrates the distribution of data according to the stratification variables or factors.

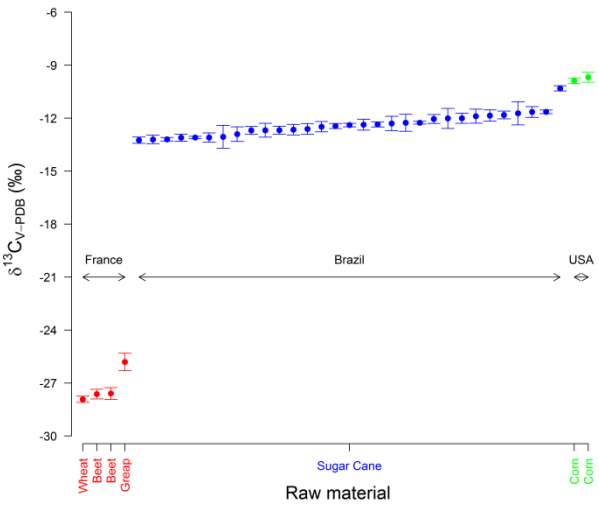

Fig. 1 Distribution of bioethanol sample data according to the stratification variables: raw material, metabolism and geographical origin (site of production).

Table 1 summarizes the frequency distribution by the nation in which the bioethanol sample was produced and by raw material.

Table 1 Sample frequencies by location/nationality.

Nationality	Local (State/Country)	Raw material	Frequency	Percentage (%)
Brazil (31 samples)	São Paulo	Sugar Cane	13	41.94
	Rio de Janeiro		3	9.68
	Pernambuco		1	3.22
	Mato Grosso		1	3.22
	Not Avaliable		13	41.94
	Total		**31**	100
Other countries (6 samples)	France	Grape, Wheat, Beet	4	66.67
	United States	Corn	2	33.33
	Total		**6**	100

Table 2 shows the $\delta^{13}C_{V\text{-}PDB}$ obtained and the type of metabolism and raw material. Note the very unique values for sugar cane bioethanol of around -12.39 ‰. Corn bioethanol was the only sample that displayed a relatively close value of - 9.78 ‰, but all the other bioethanols displayed quite contrasting values (Figure 1).

Table 2. Summary of $\delta^{13}C_{V\text{-}PDB}$ measurements by metabolism and raw material.

	Metabolism		Raw Material		
Statistic	C3	C4	Sugar Cane	Corn	Grape,Wheat, Beet
Sample size	4	33	31	2	4
Mean (‰)	-27.24	-12.23	-12.39	-9.78	-27.24
Median (‰)	-27.61	-12.37	-12.39	-9.78	-27.61
Minimum (‰)	-27.92	-13.25	-13.25	-9.88	-27.92
Maximum (‰)	-25.81	-9.68	-10.32	-9.68	-25.81
SD (‰)	0.97	0.88	0.63	0.14	0.97
CV (%)	-0.04	-0.07	-0.05	-0.01	-0.04

SD = Standard Deviation; CV = Coefficient of Variation.

The $\delta^{13}C_{V\text{-}PDB}$ values from Table 1 were also compared via Figure 2, in which the box plot [20] is shown. This plot is useful to evaluate the empirical distribution of an univariate parameter by observing variability (box width), symmetry (distribution around the median) and potential outliers (dots falling outside the box).

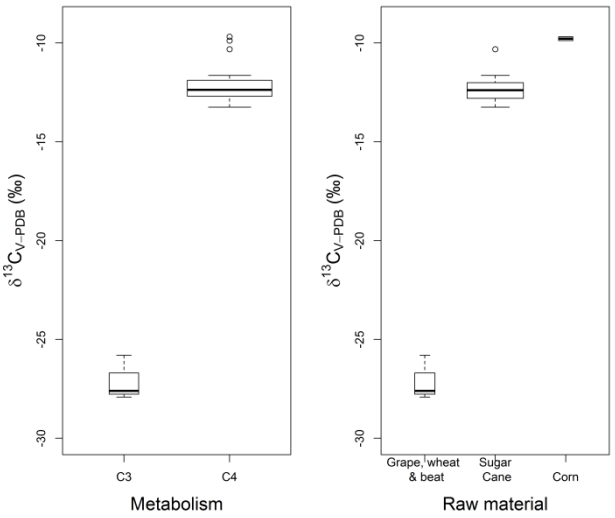

Fig. 2 Box plot diagram for $\delta^{13}C_{V\text{-}PDB}$, stratified by metabolism (C3 and C4) and raw material. The box width represents the variability whereas the dots are potential outliers.

Table 2 summarizes the coefficients of variation (CV, %) for the three types or groups of raw material, The CVs were very similar: 0.05 % for sugar cane, 0.01 % for corn and 0.04 % for the remaining samples. These small deviations from the average, suggests good congruence between samples from different producers and the high accuracy and precision of the $\delta^{13}C_{V\text{-}PDB}$ measurements.

Inferential statistics: the bioethanol profile in Brazil

The goal of the inferential statistical analysis is to infer properties of a variable or a characteristic of interest from a representative population sample. The purpose of such analysis was therefore to estimate a point value for $\delta^{13}C_{V\text{-}PDB}$ that best represents the probability distribution for the 31 samples of Brazilian sugar cane biothanol analyzed (Figure 3).

For the other raw material, such analysis was not performed due to insufficient number of samples, but their values were simply described in an explanatory way.

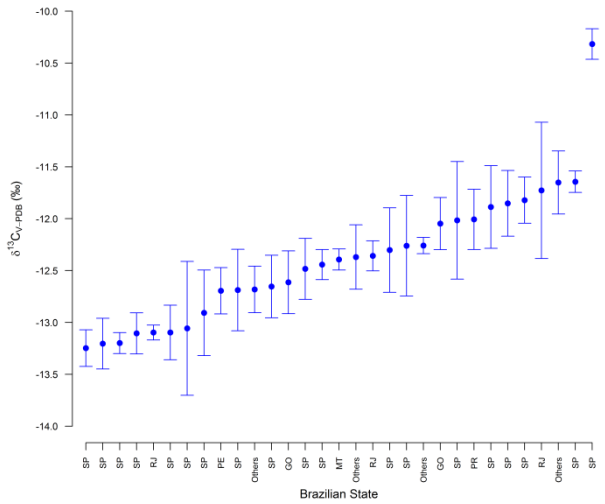

Fig. 3 Distribution of sugar cane sample data.

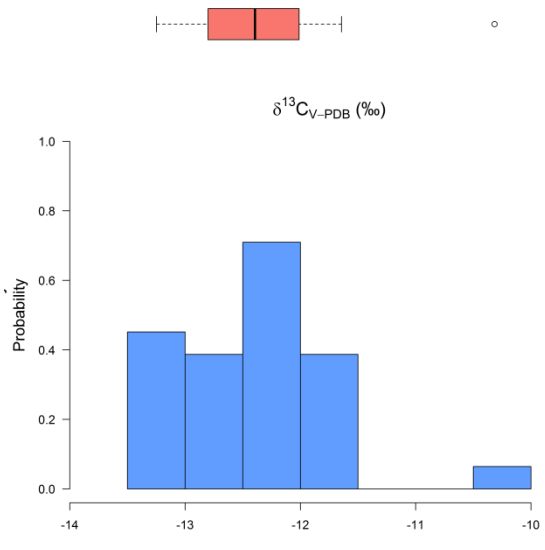

Fig. 4 Histogram and Boxplot of $\delta^{13}C_{V\text{-}PDB}$ for sugar cane bioethanol data.

Figure 3 suggests a heteroscedasticity for the $\delta^{13}C_{V\text{-}PDB}$ data. The classic Levene's test [24] was therefore applied to assess the presence of statistically significant difference among the replicates variances of the 31 sugar cane bioethanol samples. Being the statistic of the test given by F = 2.39 and the associated p-value equals to 0.0035 at 95 % confidence, the null hypothesis of equal variances was rejected, confirming the heteroscedasticity between the samples. This information will be used below to estimate the PDF of $\delta^{13}C_{V\text{-}PDB}$.

For the sugar cane bioethanol data, the Pearson's skewness coefficient [22] was calculated. The value of A = 1.0 > 0, indicates positive skewness on the distribution of data. The *Pearson's kurtosis coefficient* [25], of k = 4.89, indicates a leptokurtic distribution i.e., more peaked relative to the Gaussian distribution. For better viewing of these characteristics, Figure 4 shows the histogram and the boxplot of the data (means of each six replicates), whereas Figure 5 shows the quantile-quantile plot (Q–Q plot). [26]

The histogram of Figure 4 indicates a slight positive skewness, which might have been caused by some samples with abnormal higher values, as seen in Figures 1 and 3. Note that there is only one peak of frequency in the histogram around $\delta^{13}C_{V\text{-}PDB}$ = -12.30, suggesting unimodal distribution.

The QQ plot, which compares the sample empirical quantiles to its equivalents from normal distribution, provides evidence that the samples do not fit the hypothesis of normality. At 95 % confidence level, one point on the graph remained outside of the confidence region within which it is expected that, given the null hypothesis of normality, the data would fit optimally. To confirm the opposition to the normality hypothesis, the Shapiro-Wilk test [27] was applied whose statistic w = 0.91968 and its associated *p*-value = 0.1946 confirmed what was observed in the Q-Q plot.

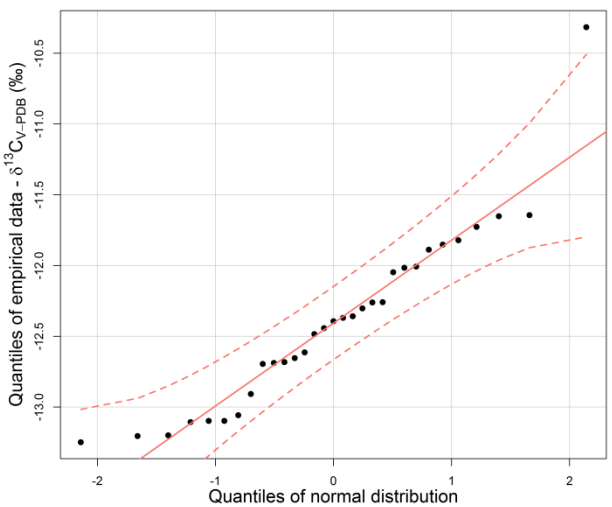

Fig. 5 Quantile-quantile plot (Q–Q plot) for carbon isotopic value from sugar cane bioethanol data.

Point and interval estimation of $\delta^{13}C_{V\text{-}PDB}$

Using data from Table 1, the value that best represent the $\delta^{13}C_{V\text{-}PDB}$ for the unique bioethanol from sugar cane could be proposed. This could be done by a single value (point estimator) or by a range under which it is believed that the true value lies with a given confidence level (interval estimator).

Due to the observed skewness, [24] it is to use robust methods [28] that are less affected by outliers of the distribution. After rejecting the hypothesis of normality, a kernel density model [29,30] was estimated, which is a nonparametric estimation of a probability distribution. To accommodate the information in the replicates, kernel density was considered with measurements errors [31] following a Gaussian distribution with different variances, based on the heteroscedasticity assumption confirmed in the previous section. Choosing the Gaussian kernel, and determining the bandwidth (h) by a rule of thumb bandwidth selection in deconvolution problems [32] based in the mean integrated squared error (MISE), h was equal to 0.2457. All the calculations were done with decon [33] package in R and Figure 6 (a) shows the kernel density estimated for the bioethanol sugar cane samples.

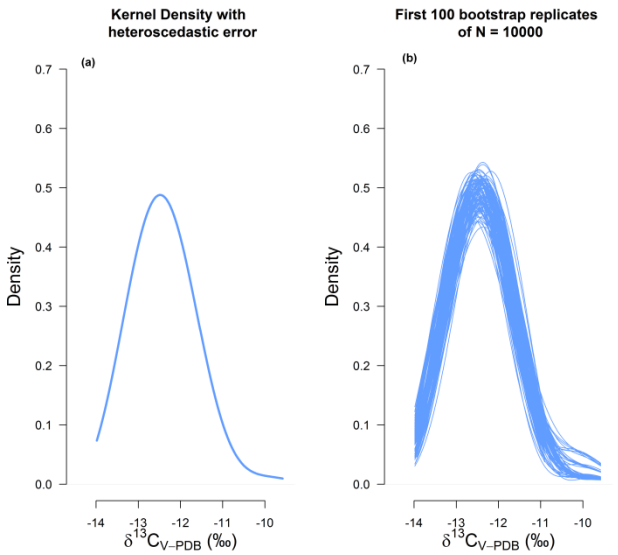

Fig. 6 (a) The kernel density estimate for $\delta^{13}C_{V\text{-}PDB}$ value from sugar cane bioethanol and (b) the first 100 kernel density estimates from Bootstrap samples.

Figure 6b shows the first 100 kernel density estimates from Bootstrap samples. This resampling process [34] is used to estimate the sampling distribution of the point estimator (in this case, the mode), thus inferring features that do not have analytical expression, as the variance of the estimator, for example. It consists of removing a random sub sample with replacement of the same size (n) from the original sample data, and then calculating the statistic of interest, such as the mode and median. By repeating the procedure many times (N), the N provides an estimate for the estimator, with which the confidence interval calculation (interval estimator) of the mode will be possible.

The point estimator with higher associated probability was $\delta^{13}C_{V\text{-}PDB}$ = -12.48 ‰. The bootstrap confidence interval was estimated according to the percentile method [35], with which it was estimated the sample quantiles $Q_{0.025}$ e $Q_{0.975}$ from the bootstrap sample of size N, which will be the lower and upper limits, respectively, of the confidence interval (CI) of the estimator, since contains 95 % probability. Using the data, it was estimated that the probability of the confidence interval for $\delta^{13}C_{V\text{-}PDB}$ from -12.68 up to -12.28 contains the true value is 95 %.

An expected interval for new samples of cane sugar

The estimated kernel density plot can be viewed as a probabilistic profile of $\delta^{13}C_{V\text{-}PDB}$, since this is an estimated probability distribution of this random variable, based on the collected sample. Aiming to set up an expected variability range for $\delta^{13}C_{V\text{-}PDB}$ for new samples of Brazilian bioethanol, the sample quantiles, centered on the point estimate (mode) between which concentrates 95 % probability, should be estimated. It followed that it varied from $Q_{0.025}$ = -13.22 up to $Q_{0.975}$ = -11.31.

Note, however, that the above interval reflects the variability of the sample in question. By inferring up interval estimates for each quantiles, by using the same bootstrap method, it was estimated that the confidence interval for the $Q_{0.025}$ estimator was $^{13}C_{V\text{-}PDB}$ = (-13.27 ‰, -13.10 ‰) and for $Q_{0.975}$ = (-11.35, -10.30) (-11.80 ‰, -10.32 ‰). Table 3 shows these estimators of population parameters.

Table 3 Estimates of mode and quantiles $Q_{0.025}$ and $Q_{0.975}$ from sample $\delta^{13}C_{V\text{-}PDB}$ data.

Estimator	Estimate	Bootstrap Confidence Interval
Mode (‰)	-12.48	(-12.68, -12.28)
Q0.025 (‰)	-13.22	(-13.27, -13.10)
Q0.975 (‰)	-11.31	(-11.80, -10.32)

Although it is not possible to estimate a CI for the isotopic carbon value, it is reasonable to assume that the lower limit of the CI for $Q_{0.025}$ and the upper limit of the CI for $Q_{0.975}$, that is, the interval (-13.27 ‰, -10.32 ‰) is a good estimate for the quality of the data obtained for sugar cane bioethanol samples, being expected that 95 % of the samples average values will lie in this range (Figure 7).

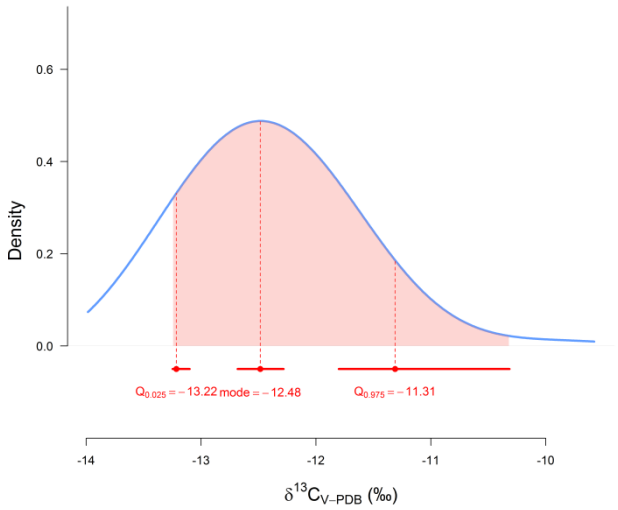

Fig.7 Kernel density plot for bioethanol data. The shadow represents the expected interval for measurements of new samples, being an expected range of measures of carbon isotopic value from sugar cane.

In all, the ability to δ^{13} $C_{V\text{-}PDB}$ to differentiate ethanol samples according to their metabolism (C3 *versus* C4) has been clearly demonstrated (Tables 1 and 2). Corn was the only raw material used for ethanol production that could eventually be overlapped with sugar cane bioethanol. United States and Brazil, are currently the major bioethanol producers contributing to as much as ca. 70% of the ethanol produced in the world. USA bioethanol production employs corn as principal raw material, while sugar cane is produced mainly in Brazil.[36] According to previous data [13,36] the measured $\delta^{13}C_{V\text{-}PDB}$ values of ethanol from corn were ranged between $\delta^{13}C_{V\text{-}PDB}$ = -11.02‰ and -10.40‰. The two samples of ethanol from corn analysed herein showed values just slightly out of this range (mean $\delta^{13}C_{V\text{-}PDB}$ = -9.78‰). Only one sample among the 31 sugar cane bioethanol samples that overlapped with the $\delta^{13}C_{V\text{-}PDB}$ from corn bioethanol.

Conclusions

A $\delta^{13}C_{V\text{-}PDB}$ profile for Brazilian bioethanol has been determined and 95% of the values ranged from -12.68 up to -12.28, by analyzing the carbon isotopic value of 31 bioethanol samples using GC/IRMS. Good congruence between samples from different producers and a high accuracy and precision for the measured values could be achieved.

A probabilistic profile for the sugar cane bioethanol $\delta^{13}C_{V\text{-}PDB}$ was also established. PDF analysis indicated a slightly positive data skewness and the most probable value was determined to be $\delta^{13}C_{V\text{-}PDB}$ = -12.48, with an associated confidence interval of $\delta^{13}C_{V\text{-}PDB}$ = (-12.68 ‰, -12.28 ‰)

The expected variability range for new samples of $\delta^{13}C_{V\text{-}PDB}$ was calculated to lie in the interval $\delta^{13}C_{V\text{-}PDB}$ = (-13.27 ‰, -10.32 ‰) with 95 % of probability. This relatively wide range of variability showed that sugar cane bioethanol profile in Brazil is not as behaved as expected, probably due to the use of different subspecies of sugar canes, farming conditions and geographical climate characteristics. Sugar cane abd corn are both C4 plants and indeed their $\delta^{13}C_{V\text{-}PDB}$ were the closest ones. These two types of bioethanols may show overlapping $\delta^{13}C_{V\text{-}PDB}$ values, but the other raw materials investigated displayed quite contrasting values.

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† Electronic Supplementary Information (ESI) available: [Table S1]. See DOI: 10.1039/b000000x/

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