Stable isotope ratio analysis of lactose as a possible potential geographical tracer of milk.

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1. Introduction

Milk has always been an important dietary element, for the richness of its composition[1]. Thanks to its numerous, properly balanced and indispensable nutrients, milk is among the more complete foods existing in nature[1]. The quality of milk varies significantly according to the diet of the animal it comes from and the method of production and treatment[2,3]. To protect consumers, the European Union banned the use of the word "milk" associated with vegetable drinks, such as soy drinks and several countries (e.g. France and Italy) decided to introduce the mandatory indication of geographical origin on the milk label. The European Court recently endorsed this possibility "if the existence of a proven link between the quality of the product and its origin is demonstrated" (ECLI:EU:C:2020:763).

In order to guarantee the geographical origin and fight against possible fraudulent practices (e.g. mislabeling or blending with low-quality milks), it is necessary to adopt specific analytical approaches. Several methods have been proposed over the years, including mass spectrometry

(MS)[4], infrared spectroscopy (IR)[5], and gas chromatography (GC)[6] alone or associated with other analytical techniques such as trace elements content determination[7]. In particular, among the MS techniques, isotope ratio mass spectrometry (IRMS) is considered one of the most specific analytical tools for such a purpose. IRMS is a technique based on the analysis of ratios of stable isotopes of elements such as carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), hydrogen ($^{2}H/^{1}H$), oxygen ($^{18}O/^{16}O$) and sulphur ($^{34}S/^{32}S$). A number of studies have investigated the use of stable isotope ratio analysis in determining the geographical origin of milk and whey produced in different regions of Europe[8–13].

Most of these studies are based on the analysis of the isotopic ratio of oxygen (express as $\delta^{18}O$) in milk water, which is closely related to that of the water drunk by the cow. This in turn is characteristic of the geographical area and can be considered a powerful tracer of origin.

In milk samples several fractions were analyzed for their stable isotope ratios (in particular $\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$): water[14], casein[15], fat[16] and, recently, lactose[13].

Among the various isotope analysis techniques, a most specific technology is the Site-specific Natural Isotope Fractionation - Nuclear Magnetic Resonance (SNIF-NMR), which makes it possible to determine the deuterium / hydrogen isotopic ratio, in a site-specific way, in the methyl $(D/H)_I$ and methylen $(D/H)_{II}$ site of the ethanol molecule. The technique has been successfully applied in determining the botanical origin of ethanol of wines and musts, whether from grapes or as fraudulently added sugars. Sugars from plants with a C4 photosynthetic cycle, such as cane and corn as well as beet, have typical $(D/H)_I$ (average 109.5 ppm and 92.5 ppm respectively) values which are completely different from those of grape sugar (between 98.8 and 106 ppm)[17]. The close correlation with the $\delta^{18}O$ parameter of wine water reported by Rossman et al.[18] has shown how the $(D/H)_I$ of ethanol can also be used as a geographic tracer[19]. Both vary from the equator to the poles, according to a gradient that, in the boreal hemisphere, sees higher values in the south and lower in the north of the country[20].

To perform this type of analysis in milk it is necessary to convert its fermentable sugar fraction, mainly represented by lactose, into ethanol, without isotopic fractionation. Other sugars present in milk, such as galactose, are not or just slightly fermentable. Vallet et al. defined an efficient strategy that leads to ethanol, which is isotopically representative of its lactose precursor, highlighting possible sources of isotope fractionation intervening in the preparation of lactose or in fermentation[21].

Masud et al. investigated the correlation between the isotopic composition of the cows diet and the $(D/H)_I$ of milk lactose[22]. The results seem to indicate that the parameter is poorly correlated to other isotope ratios such as carbon. On the contrary, Masud et al. demonstrated that the $(D/H)_I$ value of ethanol from lactose fermentation is more closely correlated with the (D/H) isotopic value of milk water $(D/H)^O$[22]. In turn, the $(D/H)^O$ of water is a parameter closely related to water $\delta^{18}O$ and this makes the $(D/H)_I$ parameter a potential geographical tracer of milk.

In order to demonstrate the discriminating power of the SNIF-NMR analysis applied to ethanol from milk lactose, 120 samples of authentic milk from different geographical areas (Italy, Germany, Austria, France, Spain) were analysed and discussed. Moreover two laboratories (Fondazione Edmund Mach in Italy and Eurofins Analytics in France) tested the method to obtain ethanol from lactose fermentation in terms of repeatability and reproducibility of measurements and investigated the effect of different yeast and sugar content on the isotopic ratios after lactose fermentation of.

2. Materials and Methods

2.1 SAMPLES

120 milk samples (2 L) were collected in different farms located in five countries (Italy, Germany, Austria, France, Spain) in two consecutive years. The date and site of sampling description are reported in Table 1S. The sampling included representative drinking water samples.

Samples of commercial lactose, whey, lactase enzyme, triptone and all reagents and ingredients used for the media formulations were purchased from MERCK KGaA (Darmstadt, Germany).

Bacterial strains (*Kluyveromyces marxianus, Saccharomyces cerevisiae rf. cerevisiae* (CH 101) or *bayanus* (MUT 210) were taken from the private collection of Fondazione Edmund Mach (Trento, Italy).

2.2 Extraction, purification and fermentation of lactose

The protocol has been adapted from the one developed by Vallet et al. for lactose and whey permeate fermentation[21].

In brief, two liters of raw milk were centrifuged for 10 minutes at 3000 g. The lower liquid layer (the whey) was taken and brought under stirring to pH 4.3 with HCl 3M so as to obtain the precipitation of the casein. The whey was heated to 90-95 °C in a microwave oven so as to bring out the whey proteins.

One liter of whey was freeze dried to obtain 60 to 70 g of dry matter (lactose, whey protein and minerals) .

Two media were then prepared[21]: one with the aim to perform yeast growth (Ma) and another for whey lactose fermentation (Mb)

1. Ma - yeast extract of *Kluyveromyces marxianus*: 3.0 g/L, triptone: 5.0 g/L, in distilled water solution adjusted to pH 5.5 by adding 3M HCl.

5 g of whey dry matter (or 3 g of pure lactose) were added to 60 ml of Ma in a 250 ml flask. This medium was immediately sterilized at 121°C for 15 min., inoculated with *Kluyveromyces marxianus* and incubated at 30°C in a shaking water bath (set at 115 rpm). After 24 h the culture reached the expected population of about $1x10^8$CFU/ml (calculated with the plate count method).

2. Mb: (NH4)2SO4: 4.0 g/L, KH2PO4: 3.0 g/L, MgSO4*7H2O: 1.0 g/L, in distilled water solution adjusted to pH 5.5 by adding 3M NaOH.

The remaining part of the whey dry matter (or 50 g of pure lactose) was added to one liter of Mb in a flask. This medium was then sterilized at 121°C for 15 min., inoculated with the activated yeasts prepared as previously described (Ma) and incubated again at 30°C, stirring manually two or three times a day. After 3 days the fermentation was finished, as verified by measuring the weight loss of the fermented solution (normally a weight loss of 20 g).

2.3 Stable isotope analysis

The D/H and $^{13}C/^{12}C$ isotope ratios of the alcohol from milk lactose fermentation were determined, using the official methods for wine (OIV MA-AS-311-05 and MA-AS-312-06), by SNIF-NMR (Site-specific Natural Isotope Fractionation-Nuclear Magnetic Resonance) (FT-NMR AVANCE III 400, Bruker BioSpin GmbH, Karlsruhe, Germany) and IRMS (SIRA II-VG ISOGAS, FISONS, Rodano, Milano, Italy or DeltaV Advantage, Thermo Fisher,

Bremen, Germany) interfaced with an Elemental Analyser (Flash 1112, Thermo Fisher, Bremen, Germany or NA 1500, Carlo Erba, Milano, Italy). The D/H values were measured site-specifically in the methyl and methylenic sites of ethanol [$(D/H)_I$ and $(D/H)_{II}$]. They are expressed in parts per million (ppm).

According to the IUPAC protocol, the $^{13}C/^{12}C$ values are expressed in the delta scale (δ‰) against the international V-PDB (Vienna-Pee Dee Belemnite) standard according to the equation:

$$\delta i\, E = \frac{(i\, R_{SA} - i\, R_{REF})}{i\, R_{REF}}$$

where i is the mass number of the heavier isotope of element E (for example, ^{13}C); RSA is the respective isotope ratio of a sample (such as for C: number of ^{13}C atoms/number of ^{12}C atoms or as approximation $^{13}C/^{12}C$); RREF is the respective isotope ratio of internationally recognized reference material.

The $^{18}O/^{16}O$ ratio of milk water was analysed in the CO_2 gas according to the water equilibration method described for wine in the OIV MA-AS2-12 method, using an IRMS interfaced with a CO_2 equilibration system (Isoprep 18 VG ISOGAS - IRMS SIRA II VG ISOGAS, Rodano, Milano, Italy or Multiflow Elementar, Isoprime, UK).

The $^{18}O/^{16}O$ values are expressed in the delta scale (δ‰) against on the VSMOW/SLAP (Vienna-Standard Mean Ocean Water/Standard Light Antarctic Precipitation) scale.

The isotopic values were calculated against in-house working standards (ethanol and water), calibrated against international reference materials: ethanol BCR 656 (Institute for Reference Materials and Measurements, Geel, Belgium) with a value of -26.91±0.07 ‰ and fuel oil NBS-22 (International Atomic Energy Agency, Vienna, Austria) with a value of -30.03±0.04‰ for $\delta^{13}C$, V-SMOW2 with a value of 0.0±0.02‰ and V-SLAP2 with a value of -55.50±0.02‰ (IAEA) for $\delta^{18}O$ and certified Tetramethylurea 99% (TMU) STA-003m with a value of 153.70 ppm (IRMM) for $(D/H)_I$ and $(D/H)_{II}$.

Analytical uncertainties (calculated as 2 standard deviation) of the measurements were below 0.30 ‰ for $\delta^{18}O$ in feed and milk water, below 0.30 ‰ for $\delta^{13}C$ in ethanol, and below 0.5 ppm for $(D/H)_I$ and 1.0 ppm for $(D/H)_{II}$ ethanol in ethanol.

3. Results and Discussion

3.1 Repeatability and reproducibility of lactose fermentation to ethanol

In order to check the repeatability of the protocol, pure lactose was fermented four times. Tables 1 and 2 indicate the results obtained for the $\delta^{13}C$ and D/H measurements of the resulting ethanol.

Table 1: Deuterium/Hydrogen and $^{13}C/^{12}C$ ratio of ethanol obtained by the fermentation of reference lactose. Repeatability is calculated as 2.8 * Sd

Experiment	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	$\delta^{13}C$ (‰, vs V-PDB)
1	114.5	135.8	-21.03
2	114.2	137.1	-20.56
3	114.6	135.5	-20.87
4	115.0	136.1	-20.61
Mean value	114.6	136.1	-20.77
Standard deviation	0.3	0.7	0.2
Repeatability (r)	0.8	1.9	0.6

The repeatability is consistent with the one reported in literature [21,23].

The inter-laboratory reproducibility of the fermentation has been assessed by submitting three different samples (pure lactose and two different whey samples) to the fermentation process in two different laboratories, Fondazione Edmund Mach (Italy) (FEM) and Eurofins (France).

Table 2a: $(D/H)_I$ values obtained by Eurofins and FEM after fermentation of the same samples.

Sample	$(D/H)_I$ (ppm) Eurofins	$(D/H)_I$(ppm) FEM	Mean	Deviation
Lactose	114.6	114.6	114.6	0.0
Whey 1	112.1	112.4	112.2	0.3
Whey 2	112.8	113.4	113.1	0.6

Table 2b: $(D/H)_{II}$ values obtained by Eurofins and FEM after fermentation of the same samples.

Sample	$(D/H)_{II}$ (ppm) Eurofins	$(D/H)_{II}$(ppm) FEM	Mean	Deviation
Lactose	136.1	135.4	135.8	0.8

Whey 1	134.9	134.6	134.8	0.3
Whey 2	133.9	135.5	134.7	1.6

Table 2c. $\delta^{13}C$ values obtained by Eurofins and FEM after fermentation of the same samples.

Sample	$\delta^{13}C$ (‰, vs V-PDB)	$\delta^{13}C$ (‰, vs V-PDB)	Mean	Deviation
	Eurofins	FEM		
Lactose	-20.77	-20.88	-20.83	0.11
Whey 1	-24.26	-23.88	-24.07	0.38
Whey 2	-25.54	-25.35	-25.45	0.19

The differences found between the values of $(D/H)_I$, $(D/H)_{II}$ and $\delta^{13}C$ measured in the two laboratories are consistent with the reproducibility reported in literature for other fermented products[23,24]. It is therefore possible to take the published reproducibility values also for the method illustrated here.

3.2 Effect of yeast strain and use of lactase enzyme on D/H of ethanol from lactose

Whey samples and solutions containing different mixtures of glucose, fructose and galactose were fermented using different bacterial strains (*Kluyveromyces marxianus, Saccharomyces cerevisiae rf. cerevisiae* (CH 101) or *bayanus* (MUT 210), to evaluate the possible isotopic

fractionation from sugars to alcohol. The effect of lactase (for hydrolyzing lactose into glucose and galactose) with different activity times to favor fermentation with the different yeasts was also evaluated.

Table 3: Fermentation tests with *Kluyveromyces* and *Saccharomyces* in serums and synthetic solutions

	$(D/H)_I$ ppm	$(D/H)_{II}$ ppm	R	$\delta^{13}C$ ‰
Serum TQ Kluyv. marx. (3.24% vol*)	110.3	129.7	2.352	-24.0
Serum + lactase 24 h Kluyv. marx. (3.08%vol*)	110.5	131.0	2.371	-23.9
Serum + lactase 24 h CH 101 (2.95%vol*)	110.8	118.3	2.135	-23.8
Serum + lactase 3 h CH 101 (2.71%vol*)	109.2	117.5	2.153	-23.9
Water sol. GLU 3.5% + FRU 3.5% Kluyv .marx.	100.6	130.1	2.586	-19.1

Water sol. GLU 3.5% + FRU 3.5%	CH 101	99.7	117.7	2.362	-18.7
Water sol. GLU 3.5% + GAL 3.5% Kluyv. marx.		112.5	130.2	2.314	-18.0
Water sol. GLU 5% + GAL 5%	CH 101	112.3	121.6	2.166	-17.7

Legend:

CH 101: Saccharomyces cerevisiae, r. f. cerevisiae: slowly ferments galactose

lactase: b-galactoxidase, MERCK

GLU: glucose

GAL: galactose

FRU: fructose

*: total alcohol

The results in Table 3 show how the *Kluyveromyces marxianus* compared with the *Saccharomyces cerevisiae rf. cerevisiae* (CH 101) produces alcohol with the same $(DH)_I$ values but different $(DH)_{II}$ (on average higher than 12 ppm). The values of $\delta^{13}C$ are not significantly different ($p>0.01$). The same differences in $(DH)_I$ and $(DH)_{II}$ values are found following fermentation with solutions of glucose or galactose (GLU and GAL) (from plants with photosynthetic cycle C4 like cane) and fructose (FRU) (from beet), with different isotopic composition (see Table 3).

It is shown how *Kluyveromyces marxianus* and *Saccharomyces cerevisae* transmit to alcohol the same information about the isotopic ratio of deuterium relative to the non-exchangeable hydrogen in position 1 and 6,6' of glucose (GLU and GAL) and glucose from fructose (FRU), determining the value of (DH)I at about 85%[25]. The two yeasts, on the other hand, have a different transmission of information relating to the methylene part which in turn has a 70% correlation with the isotopic composition of the fermentation water[25].

The use of lactase does not involve significant changes, either in the fermentation process (no change in the total quantity of alcohol) or in the isotope ratios $(DH)_I$ and $(DHI)_{II}$ (variations below the analytical uncertainty) and $\delta^{13}C$ ($p > 0.01$).

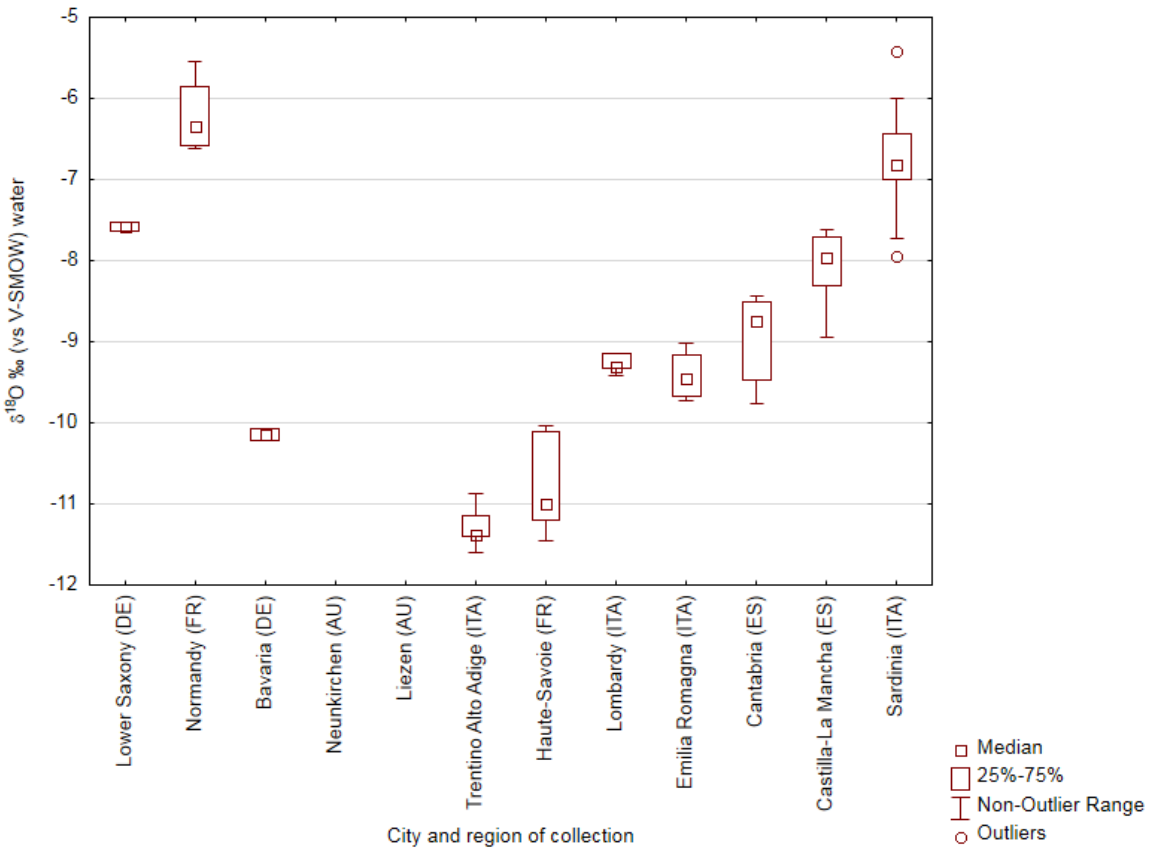
3.3 $^{18}O/^{16}O$ IRMS measurements of milk water and drinking water

There are two main sources of ingested water in herbivores: drinking water and water in plants. The $\delta^{18}O$ value of drinking water is related to the value of meteoric precipitation, which is correlated with the mean annual temperature[26]. Plant water (e.g. grass) is richer in ^{18}O than groundwater, thanks to evapotranspiration. The enrichment is higher in arid areas [27] where this phenomenon is more intense.

The $\delta^{18}O$ values of water are reflected in the milk water, with a metabolically driven isotope shift [28]. This difference between water and milk is not always constant. As reported in Table 1S, comparing the average isotopic $\delta^{18}O$ composition of milk and drinking water samples collected in the same place and on the same date, an enrichment in ^{18}O values higher than 4‰ was observed for Sardinia (Italy), Cantabria and La Mancha (Spain) and Haute-Savoie (France), while for samples from other geographical origins it remained below 3‰. The reason could be that the animals in the first locations are probably on pasture throughout the year (in particular during summer), eating fresh grass containing water enriched in ^{18}O as a result of evapotranspiration, which therefore also increase the $\delta^{18}O$ value in milk.

In the colder seasons, the main source of water is groundwater, whose stable oxygen isotope ratio is rather constant, while in the warm season an additional source of water—fresh grass rich in ^{18}O, is introduced into the bovine diet.

Cattle from other regions (eg northern Italy) eat mainly hay, which being dry pushes the animal to a greater water intake. The isotopic ratio of the milk of these animals is therefore mainly influenced by the drinking water (with lower $\delta^{18}O$ values).



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211 Figure 1: Variation in $\delta^{18}O$ content in drinking water from different geographical regions

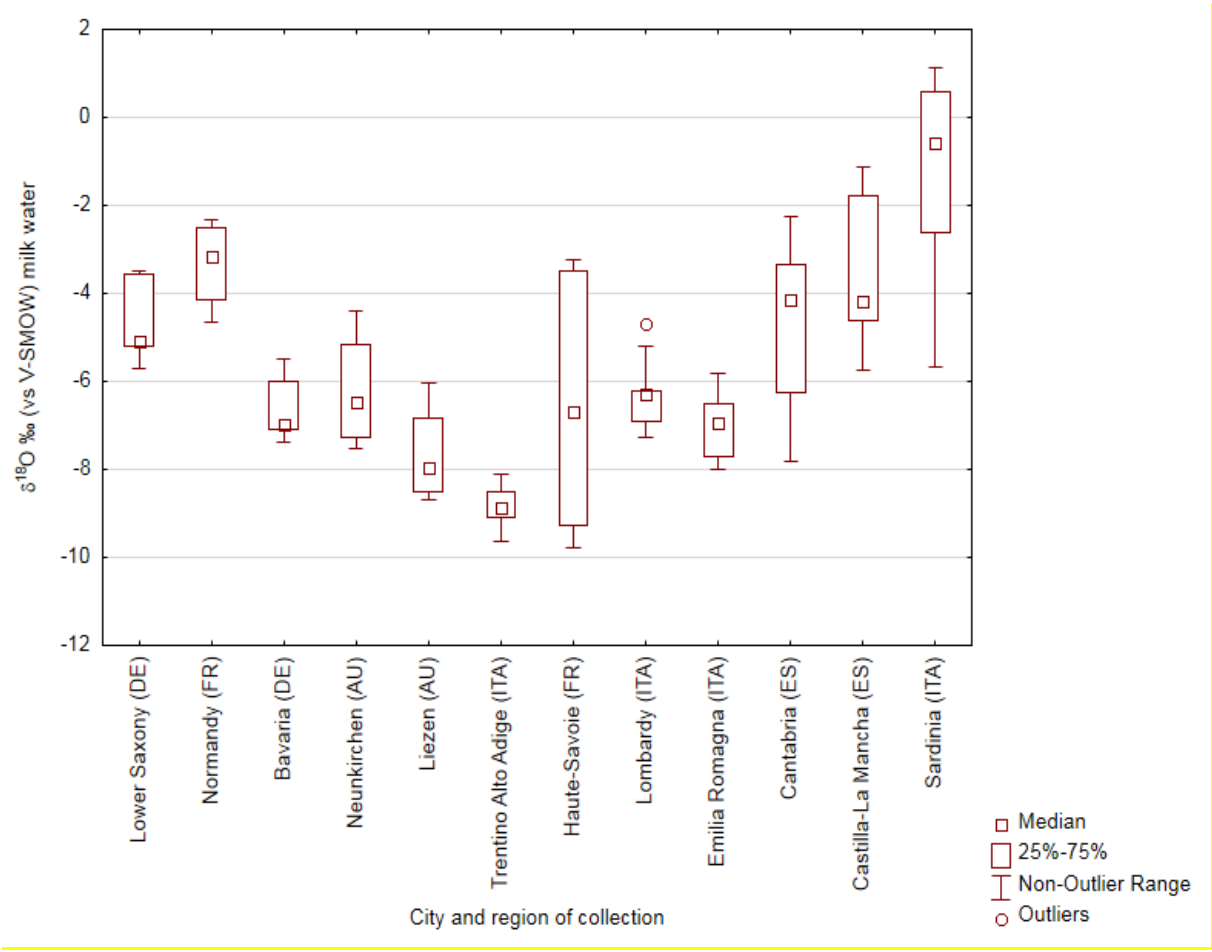


Figure 2: Variation in $\delta^{18}O$ content in milk water from different geographical regions

Milks from Sardinia, Cantabria and Castilla-La Mancha (Spain) and Haute-Savoie (France), present a high dispersion of the $\delta^{18}O$ data during the year. This is probably due to the higher evapotranspiration which takes place in the summer and is closely related to the average temperature.

Earlier investigations already demonstrated the importance of the $\delta^{18}O$ values of water to determine the geographical origin of natural products (e.g. fruit juice, wine and meat) [29–33] and this seems to be confirmed here by the results given in Figure 1, where the samples are sorted by latitude from North (Lower Saxony (53°31′N) to South (Sardinia (40°47′N).

The highest values (Figure 2) were observed in the southernmost regions, such as La Mancha and Cantabria in Spain (average -3.4‰ and -4.7‰) and Sardinia in Italy (average -1.0‰), while the others ranged between -9.8‰ and -3.5‰. Similar low values were reported in

Lithuania[28], Italy[34] and Slovenia[13]while high values were recorded in Taiwan (average -3.7‰)[35] and Ireland (average -3.3‰)[11].

The German Lower Saxony samples (average -4.6‰), despite being the northernmost ones, show the highest central Europe values, together with those of Normandy, France (average -3.3‰) (Figure 2), probably due to the lower altitude (e.g 184 m s.l.m for Normandy vs 1160 m s.l.m for Haute-Savoie) which, as reported in the literature, represents another important factor of the isotopic viability of $\delta^{18}O$ [36].

The $\delta^{18}O$ isotope values found in milk (Figure 2) are compatible with those measured in water, with higher values in the northern than in the southern regions of central Europe. This is due to the fact that milk water is closely related to drinking water and, in turn, $\delta^{18}O$ in water (i.e. ground water) is linked to the geographical location.

Variations are also observed during the year: milk water is remarkably ($p<0.05$) rich in ^{18}O during the summer months compared to the winter months (see Table 1S). This is explained by the fact that in summer, animals generally graze, and the evapotranspiration phenomenon, which leads to ^{18}O concentration in water from plants or from animals, because $H_2{}^{16}O$ evaporates faster than $H_2{}^{18}O$, is more significant [37]. Consequently, milk water is also rich in this isotope. The influence of the season is more noticeable in Haute-Savoie milk (France), probably because the evapotranspiration phenomenon is emphasised in the Alps, due to a wider temperature range between summer and winter, and also because the winter feeding regime in the Alps does not include fresh grass (which supplies part of the water) at all, while it can be part of the Normandy diet. Consequently, when the difference between summer and winter climate and diet is considerable, it might be necessary to know the seasonal origin before using the $\delta^{18}O$ values to verify the geographical location. In some cases, the season could be deduced from the $\delta^{13}C$ analysis, since it is indicative of the feeding regime (see below).

3.4 $^{13}C/^{12}C$ IRMS measurements of ethanol from milk lactose

As it is well known, in ethanol, $\delta^{13}C$ from lactose, as well as from other ingredients (e.g. casein), is related to the feeding regime of the cattle[38]. High $\delta^{13}C$ values result from high content in C4 plants in cattle diets. Indeed, while C3 plants range from -30 to -23‰, C4 plants have higher $\delta^{13}C$ values in their compounds (between -14 and -12‰)[9,39]. The variation is mainly due to the biochemical fractionation due to the plant uptake of CO_2 (C3, C4 and CAM plants and their photosynthesis pathways) that affects the carbon ratio ($^{13}C/^{12}C$)[40]. The stable

carbon isotope composition of plant tissue is determined mainly by discrimination against the heavier isotope ^{13}C during photosynthetic CO_2 fixation.

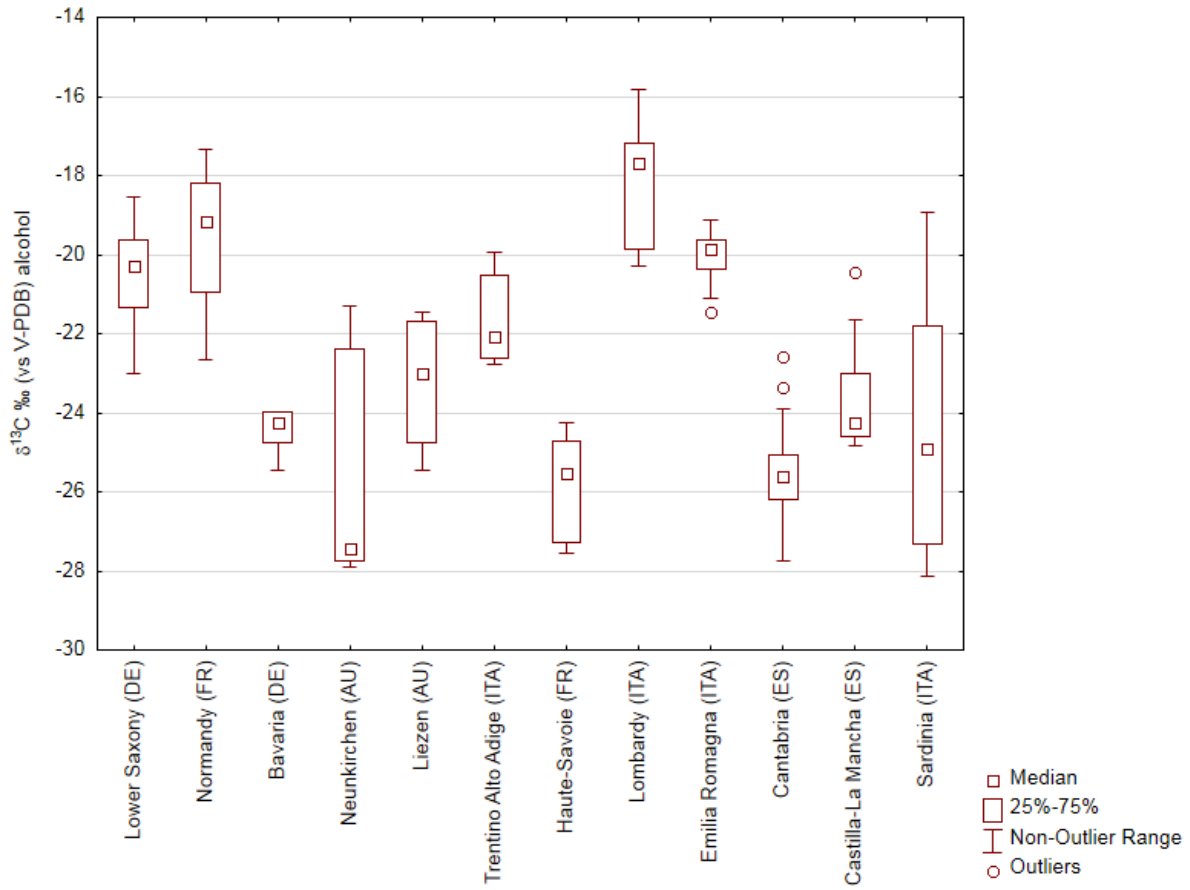


Figure 3: Variation of $\delta^{13}C$ of ethanol from lactose from different geographical regions

The highest $\delta^{13}C$ values showed a high content in C4 plants like corn in the diet in samples from "Mantova, Lombardy" (used for the production of Grana Padano cheese for which the use of this ingredient is mandatory) and from Normandy where, unlike the Haute-Savoie milk used for the production of Reblochon cheese, there are no restrictions on the use of silage and maize silage (Figure 3). This leads to higher $\delta^{13}C$ values, with a decrease in summer corresponding to higher proportions of grass in the diet. On the contrary, samples from Sardinian (IT), Neunchirken (AU) and Cantabria (ES) cattle, all kept on pasture throughout the year, show the lowest $\delta^{13}C$ values. For Haute-Savoie milk, which also shows low values of $\delta^{13}C$, silage is prohibited for the production of Reblochon cheese and the animals are fed only on C3 plants (fresh grass or hay, plus sometimes cereals, like bran and mineral additives) .

The $\delta^{13}C$ values of the milk from northern and southern Germany are clearly discernible (Figure 3). While the $\delta^{13}C$ value of ethanol from lactose in Sellstedt went from -18.5 to -23‰,

the corresponding results for samples from Bavaria are -24 to -25.5‰. In addition, in this case the reason is the different amount of maize being fed in different regions.

The $\delta^{13}C$ levels of milk are different in North and Central Spain. Milk from Santander is poor (average -25.5‰) in heavy isotopes in comparison with milk from Toledo (average -23.6‰). These results could be explained taking into account that in North Spain a relatively high amount of C3 plant grass is used in the total diet.

Carbon isotope ratios for a given substance or subfraction preferably depend on the amount of maize being fed to the animals. For certain regions the continuous feeding of high amounts of maize is the usual practise (e.g. northern Italy, Normandy, northern Germany), while in others the amount of maize varies remarkably over the season depending on the availability of food components (e.g. Austria, Toledo-Spain, Sardinia-Italy), and some regions have only low amounts or even no feeding of maize at all (e.g. certain parts of Bavaria, Trentino, Haute-Savoie, Santander).

3.5 $(D/H)_I$ measurements of ethanol from milk lactose and correlation with the $\delta^{18}O$ of milk

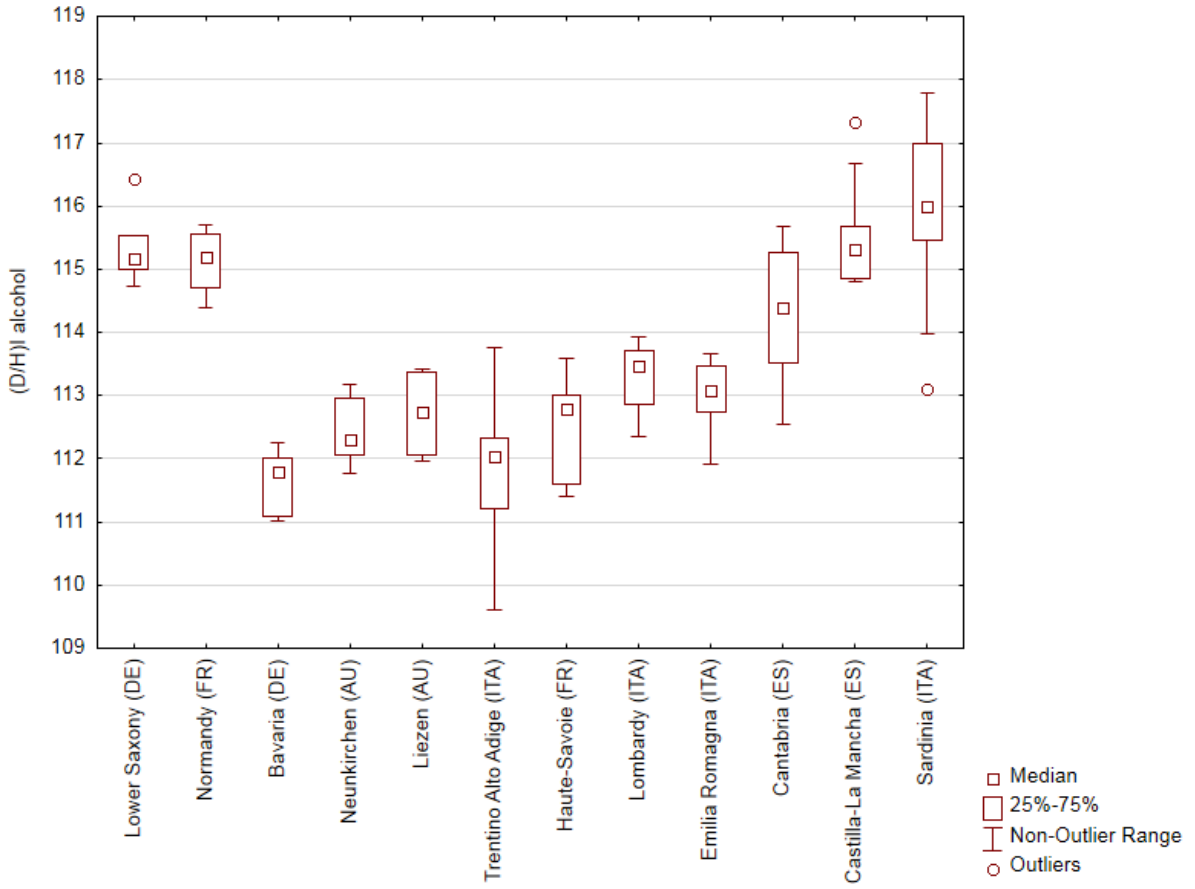


Figure 4: Variation of $(D/H)_I$ of ethanol from different geographical regions

When comparing the mean $(D/H)_I$ values of alcohol from lactose from the different milks (Figure 4), significant differences appear, related to the geographical origin.

$(D/H)_I$ values are highest for the southern European regions with dry and hot climates, such as Sardinia (Italy) or Toledo (Spain) or for warm and humid regions close to the ocean such as Normandy (France), and northern Germany (Sellstedt). The $(D/H)_I$ values are low in cold, wet and continental mountainous regions, such as Trentino (~ 500 m above sea level), Haute-Savoie or southern Bavaria, and they are intermediate in drier continental regions, such as some Austrian locations, in wet and cold regions close to the ocean (e.g. Santander/Spain) or in warm regions supplied with water from nearby mountain areas, as in northern Italy (Mantova, Modena).

A difference in the $(D/H)_I$ values between the 2 years considered was observed only for milk from Trentino (Table 1S). A seasonal variation is also apparent in the $(D/H)_I$ values of milk from Sardinian cows over the year, with higher values evident in the summer (Table 1S).

The $(D/H)_{II}$ isotope value did not show discriminated capacities and, as demonstrated in chapter 3.1, it is affected by a high analytical error (high Sr). The parameter is therefore not treated in this paper.

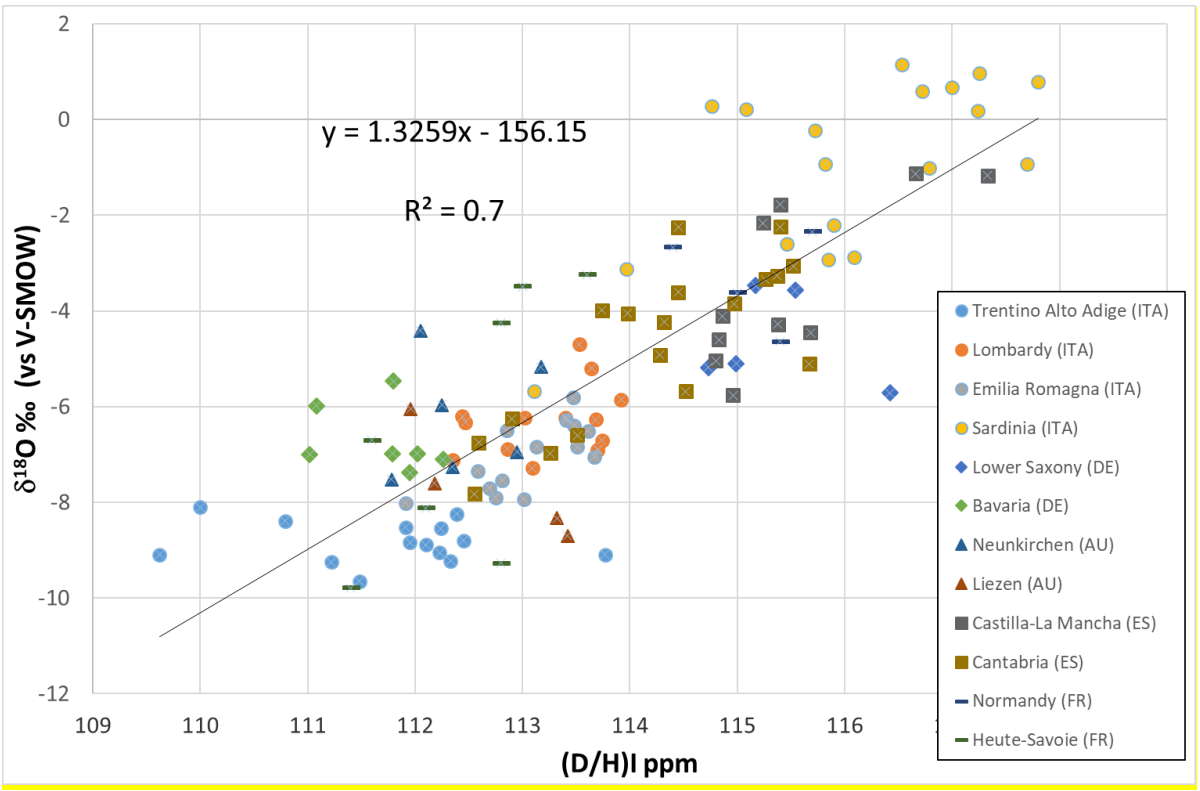


Figure 5: Plot of DH_I of ethanol vs $\delta^{18}O$ of milk water

One aim of this study was to establish whether hydrogen isotopic ratios in the organic compounds present in milk were closely related to the oxygen isotope ratios of the milk water. This is of practical importance, as origin assignment of powdered milk (lactose) has to be done on the basis of organic compounds, because no original water from milk is available for this purpose. Figure 5 represents the distribution of $(DH)_I$ data of ethanol obtained from the fermentation of the milk sugar fraction (lactose) with respect to the $\delta^{18}O$ value of milk water. It was possible to confirm that the $(DH)_I$ value of ethanol derived from milk lactose significantly correlated with the $\delta^{18}O$ value of milk water (R^2=0.70). A reliable prediction capability (approximately 70%) of one value from the other should be emphasised. The score position in Figure 4 supports the use of both parameters in the geographical characterisation of milk, despite the effects of seasonal variations in the kind of feed (summer pasture or winter stable fodder).

4. Conclusions

From a commercial point of view, the development of an analytical method capable of identifying the geographical origin of milk would make it easier to identify fraud. Such a method would be particularly useful in identifying frauds concerning PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) cheeses. The results obtained within this study clearly show that the determinations of provenance through the use of stable isotopes ratio analysis are reliable.

Taking into account the problems of customs authorities dealing with milk and milk products from eastern European countries, the methods can differentiate between milk from eastern and western European countries based on hydrogen and oxygen isotope ratios and oceanic or northern regions milk from central or southern regions milk based on carbon isotope ratio.

The measurement of the $(D/H)_I$ values of ethanol obtained from the fermentation of the sugar fraction (lactose) of milk by 2H-NMR gives similar results to the measurement of the $\delta^{18}O$ content of milk, and it can allow its geographical identification even in powder form where the $\delta^{18}O$ analysis of the water is not applicable. A specific study demonstrated that the use of different bacterial strains during lactose fermentation did not induce significant isotope fractionations of the $(D/H)_I$ ratio.

In general, the application of the stable isotope methodology for determinations of the geographical origins of milk labelled as coming from third countries makes it necessary to provide authentic material from those countries in order to obtain conclusions submissible in court cases.

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