

REGION DEPENDENT ^{13}C , ^{15}N , ^{18}O ISOTOPE RATIOS IN THE COW MILK

A. Garbaras ^{a,b}, R. Skipitytė ^a, A. Meliaschenia ^c, T. Senchenko ^c, T. Smoliak ^c, M. Ivanko ^c,
J. Šapolaitė ^a, Ž. Ežerinskis ^a, and V. Remeikis ^a

^aMass Spectrometry Laboratory, Center for Physical Sciences and Technology, Saulėtekio 3, 10257 Vilnius, Lithuania

^bInstitute of Chemical Physics, Vilnius University, Saulėtekio 9, 10222 Vilnius, Lithuania

^cMeat and Dairy Industry Institute, Partizanskiy Prospect 172, BY-220075 Minsk, Republic of Belarus

Email: andrius.garbaras@ftmc.lt

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We present measurements of stable carbon, nitrogen and oxygen isotope ratio values in cow milk, forage and drinking water collected in Belarus. Milk, water and forage were sampled in Brest, Gomel, Grodno, Minsk and Mogilev regions during summer and winter seasons. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in dried milk samples ranged from -30.2 to -20.0‰ and from $+3.63$ to $+5.66\text{‰}$, respectively. The lowest $\delta^{13}\text{C}$ values were obtained in the Mogilev region in summer. $\delta^{18}\text{O}$ values in drinking water were quite constant ($\delta^{18}\text{O} = +9.83 \pm 0.63\text{‰}$), but the $\delta^{18}\text{O}$ pattern in milk water changed across the regions.

Keywords: isotope ratio mass spectrometry, milk isotope analysis, water isotope analysis

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1. Introduction

The stable isotope analysis of light elements is widely used in ecological [1, 2], environmental [3, 4] and archeological studies [5]. The basis of all isotope ratio mass spectrometry applications is that the stable isotope ratio does not change over time, or these changes are governed by physical processes. The isotope ratio change occurs during chemical reactions (photosynthesis, burning, metabolism) or in phase change events (evaporation). The isotope ratio change can be an indicator of the ongoing processes described above.

The stable isotope ratio in milk of cow (and other mammals) is governed by the food, the water isotope pattern as well as by the environmental conditions (temperature, humidity, stress) [6, 7]. Knowing the distribution of the source (food, water) isotopic pattern it is possible to predict the stable isotope signature in milk. The set or database of the stable isotope ratio makes it possi-

ble to distinguish the geographical origin of dairy products. This feature is important for struggling against the trade fraudulence as well as it strengthens dairy producers to confirm the origin of their own production.

The aim of this study is to make the first attempt to create a database of stable isotope ratios in milk collected in Belarus. For this purpose cow milk, drinking water and forage samples were collected across different regions during summer and winter seasons. Carbon and nitrogen isotope ratios were measured in total milk samples, while the oxygen isotope ratio was measured in drinking water and milk water.

2. Materials and methods

2.1. Sampling

Milk, water and forage samples were collected from the farms of Belarus in Brest, Gomel, Grodno, Minsk and Mogilev regions. Most of the samples were

collected during the summer season – 17 milk, 14 drinking water and 2 forage samples were collected and used for the isotope ratio analysis. All samples were frozen immediately after their sampling (at -18°C temperature).

2.2. Sample preparation

Prior to the isotope ratio analysis, the milk samples were defrosted. One part of the samples was freeze dried in a lyophilizer (in order to remove water) prior to the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were measured in the total milk samples possibly containing different amounts of carbohydrates, proteins and lipids. Another part of the milk samples and the drinking water samples was used for the $\delta^{18}\text{O}$ determination. Forage was used for the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis.

2.3. Isotope ratio mass spectrometry

The freeze-dried milk samples were analysed using an elemental analyzer (*Thermo Scientific FlashEA 1112*) connected to an isotope ratio mass spectrometer (*Thermo Scientific Delta V Advantage*) via the ConFlo III interface. The elemental analyzer consists of oxidation and reduction columns (operated at 1020 and 650°C , respectively), a water trap, a chromatographic column PoraPlot Q (*Agilent*, USA) (3 m long, operated at 50°C) and a thermal conductivity detector. The oxidation reactor is filled with chromium oxide and silvered cobaltous/cobaltic oxide, while the reduction reactor consists of reduced copper. Prior to the analysis, the milk samples were weighed and packed into tin capsules.

During the analysis, the autosampler was flushed with He gas at a flow rate of 180 ml/min. Later the samples were dropped into the oxidation column with a helium flow rate of 80 ml/min and an excess of oxygen. After the combustion event, evolved gases passed through the reduction column, the water trap and the chromatographic column. The standard pressed tin capsules with dimensions of 5×3.5 mm were obtained from *Thermo Fisher Scientific*, USA. High purity He 5.0, N_2 5.0, CO_2 5.0 and O_2 4.5 gases were used during the analysis.

The measured stable carbon and nitrogen isotope ratios are reported in the VPDB (Vien-

na-PeeDee Belemnite) scale and in the air- N_2 scale for the carbon and nitrogen isotope ratios, respectively. The VPDB is the internationally accepted isotope ratio scale for reporting relative ^{13}C measurements through the *delta* (δ) notation. The isotopic composition of atmospheric nitrogen (air- N_2) was adopted as the zero point for all nitrogen isotope ratio analyses. The reference material with the known nitrogen and carbon isotopic values (caffeine IAEA 600, $\delta^{13}\text{C} = -27.77\text{‰}$ VPDB, $\delta^{15}\text{N} = 1\text{‰}$ air N_2) was used for the laboratory N_2 and CO_2 tank calibration.

The $\delta^{18}\text{O}$ isotope ratio in water and milk water were analysed using the Gas Bench II system connected to IRMS (*Thermo Scientific V Delta Advantage*). 500 μL of water or milk water was placed in an exetainer and flushed with a He/ CO_2 gas mixture. After 20 h of equilibration, $\delta^{18}\text{O}$ in CO_2 gas was measured with the IRMS system. Water (IAEA WSMOW $\delta^{18}\text{O} = 0\text{‰}$ and IAEA GISP $\delta^{18}\text{O} = -24.76\text{‰}$) was used as the reference material for the CO_2 tank calibration.

The δ notation shows the sample isotope ratio compared to the internationally accepted isotope standard. The δ notation is expressed in parts per thousand. In the case of carbon isotopes, it would be

$$\delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000, \quad (1)$$

where R_{sample} is the carbon isotope ratio in the sample, and R_{standard} is the carbon isotope ratio according to the international standard (defined by the International Atomic Energy Agency IAEA).

3. Results and discussion

Stable carbon isotope ratio values were measured in the total milk samples containing all carbohydrates, proteins and lipids and varied from -30.2 to -20.0‰ . The lowest $\delta^{13}\text{C}$ values of the milk samples were measured in the Mogilev region, while the highest $\delta^{13}\text{C}$ values were obtained mainly in the Brest region samples (Fig. 1) collected in summer. There were only a few milk samples from the winter season, and the Grodno sample had the highest $\delta^{13}\text{C}$ value of -20.0‰ . The nitrogen stable isotope values in the milk samples varied from 3.63 to 5.66‰ and were distributed randomly within the regions.

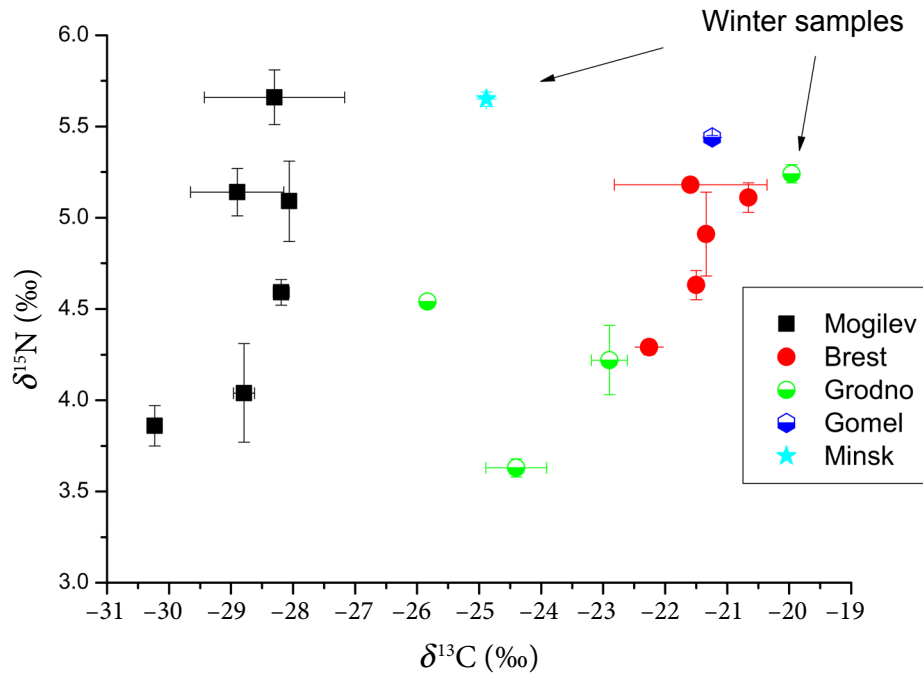


Fig. 1. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the milk samples from different regions in Belarus. All milk samples were collected during the summer periods, except for those indicated by arrows.

The oxygen isotope ratio values in the drinking water varied from -10.54 to -9.16 ‰ (average value $\delta^{18}\text{O} = -9.83 \pm 0.63$ ‰). Meanwhile the $\delta^{18}\text{O}$ values in the milk water varied from -8.67 to -3.87 ‰ (Fig. 2). The highest $\delta^{18}\text{O}$ values were

registered in the Mogilev region. There the largest difference between the oxygen isotope ratio in drinking water and milk water was observed.

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the forage are presented in Table 1.

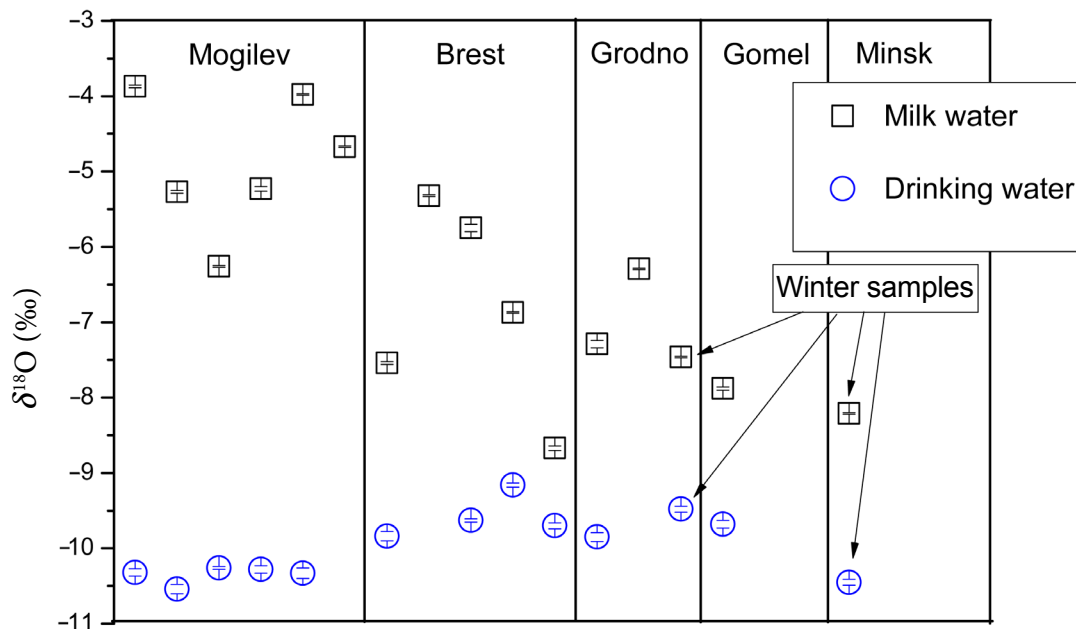


Fig. 2. $\delta^{18}\text{O}$ values in the water and milk water samples from different regions in Belarus. The samples were collected during summer periods, except for those indicated by arrows.

Table 1. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the forage.

	$\delta^{13}\text{C}$, ‰	$\delta^{15}\text{N}$, ‰
Grodno region	from -28.06 to -17.00	$+1.79 \pm 0.2$
Minsk region	from -27.67 to 14.57	$+0.75 \pm 0.2$

The main factor which determines the carbon isotope ratio value in the milk is the diet of animals. Camin et al. (2008) [8] observed that changing the C_3 diet to the C_4 diet the $\delta^{13}\text{C}$ value increased in the milk (casein and lipid). Each 10% increase in the C_4 plant content changes the $\delta^{13}\text{C}$ value of casein by about 1‰. Bahar et al. (2005) [9] showed that the increment of maize percentage in the food changed the $\delta^{13}\text{C}$ values in defatted dry muscles and the lipid fraction. The increase of $\delta^{13}\text{C}$ values in animals compared to the main animal diet was reported by Post (2002) [10] ($0.39 \pm 1.3\%$) and by McCutchan et al. (2003) [11] ($0.4 \pm 0.4\%$). Skipitytė et al. (2017) [12] showed that this increase was different for muscles, feathers, skin and blood. The forage at the examined sites had a broad range of $\delta^{13}\text{C}$ values (Table 1), and it can explain the $\delta^{13}\text{C}$ values in the collected milk. In our study, the cows were fed the forage which contained various amounts of C_4 plants, for example, corn. We can assume that the cow diet had a minimal addition of C_4 plants in the Mogilev region, as the measured $\delta^{13}\text{C}$ values were most negative there. Meanwhile corn was added to the forage in the rest of the regions with some extent. The $\delta^{13}\text{C}$ difference in the summer and winter forage samples from the Grodno region indicates the shift of the forage during the seasons. The highest $\delta^{13}\text{C}$ values in the Grodno region indicate that the forage contained the largest amount of C_4 plants compared to the rest of the samples. It must be noted that $\delta^{13}\text{C}$ values were measured in the total fraction of dried milk and some corrections up to 1‰ can occur due to the fat content in milk, as fat is more depleted in $\delta^{13}\text{C}$ values compared to casein [13]. Nevertheless, these corrections are small and the overall set of $\delta^{13}\text{C}$ values in the milk is correct.

Plant feed is the most important factor in the nitrogen isotope ratio variability in farm animals. $\delta^{15}\text{N}$ values in plants are corrected to those of soils. Nitrogen is transformed from the atmosphere to the soil, and later to a plant via physical processes, where microorganisms are involved.

Nitrogen isotope ratio values do not change during the nitrogen uptake from the soil to the plant, whereas enzymatic reactions affect the isotopic composition. For carnivores, a change of about 3‰ is observed at each trophic level [14]. $\delta^{15}\text{N}$ values in soil are mainly determined by the use of a fertilizer. Naturally occurring fertilizers are isotopically enriched (up to $+25\%$), while synthetic fertilizers are characterized by $\delta^{15}\text{N}$ values ranging from -4 to $+4\%$. Thus, the nitrogen isotope ratio can be an indicator of organic farming. In the measured milk samples the $\delta^{15}\text{N}$ values are not higher than 5.66% and are distributed randomly in the investigated regions. By combining $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values it can be possible to distinguish the region of the milk origin, but a larger set of samples is needed.

The oxygen isotope ratio in the milk reflects the input of drinking water, food and respiration [15]. $\delta^{18}\text{O}$ in water can be predicted according to the geographic location, altitude, and the distance from the ocean [16]. The oxygen isotope ratio in plants depends on the evapotranspiration intensity and the relative humidity leading to oxygen enrichment in a plant. The $\delta^{18}\text{O}$ value in animal water is by about 3‰ enriched compared to the drinking water [6], and depends on the respiration rate, season, and the physiological condition of an animal [17]. A relatively small variation in the milk water $\delta^{18}\text{O}$ values indicates that animals consumed water which had almost a constant $\delta^{18}\text{O}$ value during summer and winter. The $\delta^{18}\text{O}$ value differences between milk and drinking water were observed for the reason described above. It can be noted that the milk water from the Mogilev region was enriched compared to that of the other regions. This enrichment could arise due to the consumption of fresh grass, because water in the grass is highly enriched due to evapotranspiration. Bearing in mind that the carbon isotope ratio in the milk from this region indicates the consumption of fresh grass, this explanation is most reliable.

Even from a small set of the samples we can conclude that relying on the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ data of total milk, drinking water and forage we can characterize different regions with a stable isotope data set. For a more precise characterization of the milk from Belarus (through a stable isotope ratio perspective), additional sampling is needed.

5. Conclusions

The stable isotope ratios of light elements (carbon, nitrogen and oxygen) in Belarussian milk and farm water have been reported for the first time. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the dried milk samples ranged from -30.2 to -20.0‰ and from $+3.63$ to $+5.66\text{‰}$, respectively. The Mogilev region was characterized by the most negative $\delta^{13}\text{C}$ values. It can be related to the pasture type when cows were fed in the field on C_3 type plants. Cows in other regions had some amount of C_4 plants in their diet. It is confirmed by the $\delta^{13}\text{C}$ values of the forage, where $\delta^{13}\text{C}$ values reached -14.57‰ . The nitrogen isotope values showed no visible trend and were scattered across the investigated geographical regions. The $\delta^{18}\text{O}$ values in the drinking water were of similar values, with the mean value of $-9.83 \pm 0.63\text{‰}$. The milk water $\delta^{18}\text{O}$ values were distributed differently across the regions, with the highest ones registered in the Mogilev region. This could be related to the feeding regime when part of the water came to an animal from the grass, which usually had enriched $\delta^{18}\text{O}$ values.

The $\delta^{13}\text{C}$ values in the milk are different for the summer and winter seasons in the same geographical region. It can be related to the change of the diet when the cows were kept in the shelter during the winter season and had a different forage composition compared to that of the summer season.

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^{13}C , ^{15}N , ^{18}O IZOTOPŲ SANTYKIS SKIRTINGŲ REGIONŲ KARVĖS PIENE

A. Garbaras ^{a,b}, R. Skipitytė ^a, A. Meliaschenia ^c, T. Senchenko ^c, T. Smoliak ^c, M. Ivanko ^c, J. Šapolaitė ^a, Ž. Ežerinskis ^a, V. Remeikis ^a

^a *Fizinių ir technologijos mokslų centras, Vilnius, Lietuva*

^b *Vilniaus universiteto Cheminės fizikos institutas, Vilnius, Lietuva*

^c *Mėsos ir pieno pramonės institutas, Minskas, Baltarusija*

Santrauka

Darbe tirta stabilųjų anglies ir azoto izotopų santykio verčių karvės piene ir pašaruose, taip pat deguonies izotopų santykio verčių geriamajame vandenyje ir karvės pieno vandenyje, kaita. Pienas, vanduo ir pašarai buvo renkami vasaros ir žiemos sezonais – Bresto, Gomelio, Gardino, Minsko ir Mogiliovo regionuose (Baltarusija). $\delta^{13}\text{C}$ ir $\delta^{15}\text{N}$ vertės piene kito atitinkamai

nuo $-30,2$ iki $-20,0$ ‰ ir nuo $+3,63$ iki $+5,66$ ‰. Deguonies izotopų santykio vertės geriamajame vandenyje buvo apie $\delta^{18}\text{O} = +9,83 \pm 0,63$ ‰. Mažiausios $\delta^{13}\text{C}$ vertės piene buvo nustatytos vasaros laikotarpiu Mogiliovo regione. Geriamojo vandens $\delta^{18}\text{O}$ vertės kito mažai, o pieno vandens $\delta^{18}\text{O}$ vertės įvairiuose regionuose buvo skirtingos.