CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

CHEMOMETRIC APPROACH BASED ON FATTY ACID COMPOSITION AND $\delta^{13}C$ ANALYSIS FOR VERIFICATION OF ORGANIC RAW MILK FROM COWS WITH DIFFERENT DIET

Ya. F. Zhukova[1], P. I. Petrov[1]*, L. Yu. Klimenko[2], Yu. M. Demikhov[3]

[1]*Institute of Food Resources, Kyiv, Ukraine*
[2]*National University of Pharmacy, Kharkiv, Ukraine*
[3]*Institute of Environmental Geochemistry, Kyiv, Ukraine*
**philip.i.petrov@gmail.com*

https://doi.org/10.34302/crpjfst/2019.11.2.1

Article history:
Received:
4 August 2018
Accepted:
22 January 2019

Keywords:
Organic milk;
Grass cattle diet;
Fatty acid composition;
Carbon stable isotopes;
Chemometry.

ABSTRACT

The growing interest in organic farming, which involves a significant percentage of "green grass" and hay in the animal diet and increasing the output of milk labeled "100% grass-fed," induces an urgent necessity for authentication such value-added products. The aim of this study was to develop the integrated approach for discrimination of organic milk from farms with silage-haylage and grass-hay cow diet. The research was based on the characterization of fatty acid composition and carbon stable isotopes ratios (δ13C), and included data processing with chemometric approach.

It has been shown that absolute value ranges of most fatty acids didn't allow to discriminate the milk from different organic farms, although the difference in certain fatty acids content between separate seasons has been proven to be statistically significant. This integrated approach with application of the principal components analysis envisaged that the analyzed parameters were normalized by the maximum value and the data matrix consisted of not only absolute values of the fatty acids content, but also additional derivative parameters (the sum of cis-, trans-isomers etc.). The approach proved the significance of content values of C16:1 trans-9, C18:1 trans-11, C18:3 cis-9,12,15, C18:2 trans-9,12 and conjugates of linoleic acid for milk samples discrimination. Thus, the analysis of the total data set of absolute and derivative normalized parameters by the method of principal components analysis allows to distinguish completely the organic milk with different share of green grass and hay in cows diet both the stalling and pasture periods.

1.Introduction

Increasing of consumers' interest in organic products leads to the growth of their producing. According to the Organic Milk Suppliers Cooperative (www.omsco.co.uk) the world production of organic milk will amount to ≈$13 billion by the end of 2019. Also, the interest in the so-called "pasture dairy management", which involves cows grazing on pastures in summer and feeding by hay with minimum usage of concentrates in winter, is renewed.

The certification requirements for organic dairy farms are different. Thus, the requirements for feedstuff on organic farms range from simple recommendations for pasturing (Japanese Organic Standard) to the certain values of the roughage percentages in the feed dry matter – from 60% (EU regulations, "Bioland") to 90% ("Biosuisse", Switzerland). Meanwhile, in the United States, the world biggest organic products market (Organic Milk Market Report, 2017) a number of organic manufactures has

approved own animal grass-fed cattle standard under the auspices of the American Grassfed Association. It establishes such requirements for the production of dairy and meat raw materials, so that the labelling of "100 % grassfed" of the corresponding dairy and meat products is legitimate. This certification exists in parallel with the additional certification for organic producers – "Pennsylvania Certified Organic 100% Grassfed certification" (www.paorganic.org/grassfed).

Besides, in the United Kingdom market (www.arlafoods.co.uk) organic milk from cows with grazing for more than 200 days per year is labelled as "free range" and in the Netherlands market organic milk and dairy products labelled as "meadow milk" from cows grazing at least for 120 days per year and 6 hours per day are available (www.weidemelk.nl). Accordingly, the price of such type of milk is much higher than the price of both organic and conventional ones. Thereby it is necessary to develop the approaches for authentication of such dairy products.

Cattle diet is one of the decisive factors which affects physical, chemical and biochemical parameters of milk, especially the milk fat composition. It is known that the higher percentage of fresh green grass and hay in the cow's diet leads to higher concentration of polyunsaturated fatty acids, in particular ω-3-acids and conjugates of linoleic acid (CLA) (Średnicka-Tober, 2016; Adler, 2013; Butler, 2008). However, it is usually difficult to distinguish the feed base of milk origin and dairy management type by the absolute values of fatty acids content in milk. Therefore, the combination of methods has recently been used to solve this problem.

Thus, the processing of triglyceride database of milk fat allowed to distinguish organic milk from farms certified according to European standards (organic, biodynamic) from conventional milk without labelling. However, the effectiveness of this approach decreased from ≈90% to 72% when analysing the samples has taken in early spring and summer (Capuano, 2013). Also, it was noted the complexity of identification of organic milk, "pasture" milk and milk from farms with biodynamic type of management only by triglyceride composition (Capuano, 2013).

The principal components analysis (PCA) of milk fatty acid composition discriminated the samples from farms with different types of cattle diet ("fresh-cut green grass", "pasture", organic, biodynamic and conventional). Reliable differences were obtained in summer for conventional and "fresh-cut green grass" milk. However, the differences in winter were less reliable (Capuano, 2014). Also, it was possible to discriminate organic and biodynamic milk from conventional by fatty acid composition. However, it was impossible to distinguish organic milk from biodynamic one by fatty acid composition both in summer and winter (Capuano, 2014).

It has also been shown that comparing the data of fatty acid and triglyceride composition gives the possibility to distinguish retail organic milk, conventional milk and retail milk from farms with the grazing duration at least 120 days per year. At the same time, this approach has not established the significant difference between the last and conventional types (Capuano, 2015). Thus, the absolute values of fatty acids content in milk could indicate the proportion of fresh green grass and hay in the cows' diet, but only in certain periods of the year in the cases of significant differences in feeding.

It is also possible to determine the composition of cattle diet by the ratio of stable isotopes $^{13}C/^{12}C$ ($\delta^{13}C$) in milk. Grass and hay on the one hand, and corn silage and grains on the other hand, are different in the type of plant photosynthesis (C_3 and C_4 respectively) and, accordingly, in the carbon isotopes profiles, which affects $\delta^{13}C$ values in milk. There is a positive correlation between the $\delta^{13}C$ values and the percentage of corn silage in the cows' diet (Camin, 2008; Zhukova, 2017). As a result, the $\delta^{13}C$ values in milk, as well as features of fatty acid composition, could reflect the proportion of grass in the cows' diet.

In addition, the lipids of green grass are characterized by the high content of unsaturated

fatty acids, which are localized in cells chloroplasts, so their composition depends on the type of photosynthesis. At low seasonal temperatures the plants with C_3-type photosynthesis dominate on pasture, and 50 – 75% of all fatty acids of green grass are linolenic acid and CLA (Elgersma, 2006; Elgersma, 2003; Dhiman, 1999, Kochubey, 1996). At the same time, in plants with C_4-type photosynthesis, which are predominantly distributed in warm climate, linolenic acid is less than 40% of all fatty acids (White, 2001). Also, in the pastures C14:0 and C16:0 acids dominate in the grass at the end of summer, and the C18:3*cis*-9,12,15(ω-3) and CLA content decreases as compared to the spring period and the first weeks of summer (Loor, 2002).

Thus, the analysis of correlation of fatty acid composition with $\delta^{13}C$ values could be the effective method for the authentication of dairy products and milk obtained from cows with a high content of grass in the diet. At present, there is a shortage of similar studies. The available results showed that the content of C18:3*cis*-9,12,15(ω3), which minimal value in organic milk products was 0.50%, correlated with $\delta^{13}C$ values, which maximal value was –26.5‰ (Molkentin, 2013). It was also noted that such an approach should take into account the region, feed, climatic conditions etc. (Molkentin, 2009; Petrov, 2016).

The purposes of our study were: 1) analysis of the fatty acid composition and ratios of carbon stable isotopes $^{13}C/^{12}C$ in milk from organic farms with different volumes of green grass and hay in the cattle diet; 2) multivariate analysis of milk fatty acid composition and $\delta^{13}C$ values database; 3) development of the integrated approach to the authentication of milk from grass-fed farms throughout the year.

2. Materials and methods

2.1. Sampling

The organic milk samples were taken from 2 organic farms in Zhytomyr region with silage- and haylage-based cattle diet (n = 30) (O1) and from 2 organic farms in Chernihiv region with grass- and hay-based cattle diet (O2) certified according to Council Regulation (EC) №834/2007 and Commission Regulation (EC) №889/2008 (n = 24). The research was carried out during the indoor period (November – April) and outdoor period (May – October) in 2015 – 2017. The samples of milk were taken from a tank on farms, transported in plastic bottles with a screw cap at +4ºC. Data on cow's feeding diet and daily consumption of dry matter (kg/cow) were received from farmers.

2.2. Instrumental analysis

For the milk fat fraction extraction, the milk sample was heated to 35°C in a water bath ("RVO-400", «Ingos, s.r.o. Czech Republic), stirred and cooled to 20±2°C. 100 ml of milk were mixed in a separating funnel, then were added 80 ml of ethanol, 20 ml of ammonium aqueous solution (14.0 mol/dm^3), 100 ml of diethyl ether, mixed vigorously for 1 min, then was added 100 ml of petroleum ether, gently mixed, then the liquid fraction was poured. Then was added 100 ml of 10% solution of sodium sulfate to the liquid fraction, stirred, filtered through a filter paper and evaporated on a rotary evaporator ("RVO-400", «Ingos, s.r.o. Czech Republic). This method was applied according to ISO 14156:2005.

The mixture of methyl esters of fatty acids were prepared by methanolysis of glycerides. 0.1 g of fat was transferred to a test tube, 5.0 ml of hexane was added, mixed thoroughly. 0.2 ml of sodium methylate solution (2 mol / dm 3) was added to the test tube, carefully stirred for 2 min, and filtered through a paper filter with added $Na_2HSO_4 \times H_2O$. This method was applied according to ISO 15884/IDF 182:2008

The fatty acid composition was analysed using the gas chromatograph Crystallux ("Analityka", Ukraine) with the flame ionization detector, SP 2556 column (Supelco, USA), 100 m × 0.25 mm I.D., 0.20 μm film layer, with the help of "Analityka" software (SPC "Analityka", Ukraine) for system monitoring and data processing. The mixture of 37 methyl esters of fatty acids FAME ("Sigma-Aldrich", USA) was used as the standard.

Parameters of the measurement: the initial temperature of the column was 60°C; isothermal period - 15 minutes; temperature rise to 186°C with rate of 10°C/min; isothermal period - 20 min; the temperature rise to the final column temperature - 220°C, with rate of 5 °C/min. The total analysis time was 120 minutes. The temperature of the detector was 260°C, the temperature of the vaporizer was 250°C. This method was applied according to ISO 15885/IDF 184:2008.

The ratio of $^{13}C/^{12}C$ ($\delta^{13}C$) isotopes was analysed in milk fatty phase using mass spectrometer "MI-1201SG", ("Electron", Ukraine) at the Isotope Geochemistry Lab of the Institute of Environmental Geochemistry. The measurements were made by using the international standard polyethylene foil (PEF-1) and transferred to the international standard Vienna Pee Dee Belemnite (VPDB) according to Gerstenberg and Herrman, 1983. The isotopes ratio is given in ‰ by δ scale and calculated by equation (1):

$$\delta C = \frac{R_1 - R_2}{R_2} \cdot 1000‰ \text{ , (1)}$$

where C – Carbon, R_1 – $^{13}C/^{12}C$ ratio in test sample, R_2 – $^{13}C/^{12}C$ ratio in internal standard PEF-1.

2.3. Statistical analysis

The data statistical processing was carried out using the univariate dispersion analysis in MS Excel 2010. The multivariate data analysis was performed using PCA in PAST software (Hammer et al., 2001) at the Analytical Chemistry Department of the National University of Pharmacy.

3.Results and discussions

3.1. Analysis of cattle diet

Organic dairy farms regulate the composition of cattle diet according to the herd size, the possibilities of fodder procurement, the planned milk productivity and other factors. The analysis of livestock feeding diets has shown the differences depending on farming type (Table 1).

Table 1. Cows' diets on organic farms

Parameter	Organic farms with silage-haylage diet (O1)		Organic farms with grass-hay diet (O2)	
	Outdoor period	Indoor period	Outdoor period	Indoor period
Milk productivity of cows, kg/day*	22.0-23.0	22.0-23.0	11.0-12.0	8.0-9.0
Feed dry matter intake (DMI) per day, kg/cow*	18.1-24.0	20.3-25.4	8.0-9.0	13.5-14.0
Diet composition, % DMI*				
Green fodder (grass on pasture, fresh-cut grass)	11.9-20.5	-	88.0-93.0	-
Roughage (hay, straw)	14.3-23.0	8.5-23.7	-	21.8-24.3
Juicy feed, % DMI*				
- haylage	23.2-33.4	34.4-43.1	-	55.0-63.2
- corn silage	16.4-22.8	10.6-16.0	-	-
Concentrated feed (grains of cereals and beans; sunflower cake)	30.3-34.2	31.3-32.5	7.0-10.0	15.0-18.0

* data is presented in the form of range of values obtained during 2015 – 2017.

Thus, during the outdoor period, the diet on O1 farms with silage and haylage ration and herd size near 400 heads, was characterized by a greater feed variety. In structure of feed dry matter intake (DMI) a significant part was occupied by haylage, silage and hay, that meets the requirements of organic certification standard – not less than 60 % of DMI.

At the same time, the concentrated feed accounts for about one-third of DMI. On O2 farms with the grass and hay diet of cows and with the herd size of ≈115 heads the consumption of DMI comprises more than 90% of fresh grass of annual and perennial plants and concentrated fodder without corn silage addition.

During the indoor period on O1 farms the feed structure differed from the outdoor period by increasing the proportion of haylage and reducing the proportion of roughage. At the same time the values of other groups of feed were without significant differences. Rough, juicy and concentrated feeds without corn silage were used on O2 farms during the indoor period.

In addition, local climatic peculiarities of the farms affect diet forming and herd size. The farms with silage and haylage feeding type are located in Zhytomyr region, where the average temperature during the outdoor period is 21.7°C with the absolute maximum of 36.2°C, however, the rainfall is minor – 74.6 mm. The farms with grass and hay feeding type were located in Chernihiv region, where the average temperature during the outdoor period equals 22.3°C with the absolute maximum of 41.1°C, the rainfall is 66.3 mm. For comparison, in Switzerland and Germany during grazing period the average temperature is 25.3°C and 20.9°C respectively with the absolute maximum of 37.8°C and 36.0°C, however, rainfall is 183.2 mm and 86.2 mm, that causes the greater possibilities for cows feeding with fresh grass.

3.2. Fatty Acid Analysis

The analysis of fatty acids composition of milk from the farms during outdoor period has shown that absolute values of individual short chain fatty acids varied slightly (Table 2). However, the average annual content of C4:0 – C12:0 in milk from O1 farms was higher by 17.3% than in milk from O2 farms, C14:0 and C16:0 content – by 17.9% and 21.5% respectively. The average annual content of C18:0, on the contrary, was higher by 33.7% in milk from O2 farms than from O1 farms.

Variations between indoor and outdoor periods for milk from O1 and O2 farms within each farm were 1.9% and 5.5% for C4:0 – C12:0, 5.0% and 1.8% for C14:0, 4.5% and 7.5% for C16:0, respectively. Variations in C18:0 content between indoor and outdoor periods were 2.9% and 11.7% for O1 and O2 milk respectively (Table 2).

More significant differences have been revealed between O1 and O2 milk fatty acid composition. The content of C4:0 – C12:0 was higher by 21.1% and 14.8% for O1 milk in outdoor and indoor periods than for O2 milk; the C14:0 content was higher by 15.4% and 20.9% respectively, the C16:0 content was higher by 23.7% and 21.5% respectively than for milk from O2 farm (Table 2).

However, the C18:0 content in milk from O1 farms was lower by 24.5% and 43.4% respectively in outdoor and indoor periods than in milk from O2 farm.

Such differences can be explained by peculiar properties of fatty acids metabolism in ruminants. The C4:0 – C14:0 fatty acids are synthesized *de novo* in mammary gland, the synthesis of C16:0 occurs both *de novo* and from fatty acids of feed in the process of digestion in cows' small intestine, and the content of long chain acids depends on their content in feed (Mitani, 2016; Palmquist, 2006).

Table 2. Fatty acid composition of milk fat from cows with different diets, g/100g of fat

Fatty acid	Organic farms with silage-haylage diet (O1)						Organic farms with grass-hay diet (O2)						P-value
	Outdoor period		Indoor period		Average annual		Outdoor period		Indoor period		Average annual		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
C 4:0	4.000	0.228	3.947	0.419	3.961	0.321	3.779	0.401	4.123	0.398	3.988	0.426	>0.05
C 6:0	2.508	0.148	2.410	0.215	2.465	0.184	1.972	0.287	2.237	0.254	2.134	0.293	<0.00001
C 8:0	1.480	0.104	1.446	0.171	1.456	0.137	1.099	0.207	1.257	0.208	1.195	0.218	<0.00001
C 10:0	3.219	0.262	3.142	0.209	3.165	0.245	2.338	0.570	2.266	0.421	2.294	0.474	<0.00001
C 10:1	0.312	0.053	0.332	0.051	0.316	0.052	0.260	0.061	0.253	0.054	0.256	0.056	<0.001
C 11:0	0.057	0.029	0.048	0.027	0.053	0.028	0.038	0.024	0.035	0.011	0.037	0.017	<0.05
C 12: 0	3.542	0.303	3.521	0.198	3.521	0.260	2.451	0.535	2.443	0.454	2.446	0.475	<0.00001
C 13:0	0.189	0.038	0.199	0.020	0.194	0.032	0.218	0.039	0.245	0.051	0.234	0.047	<0.001
C 14:0	10.477	0.867	11.00	0.615	10.68	0.794	8.868	1.671	8.703	1.488	8.768	1.527	<0.00001
Σ *trans* C14:1	0.583	0.057	0.622	0.036	0.602	0.053	1.121	0.245	0.927	0.271	1.003	0.273	<0.00001
C 14:1 *cis*	0.985	0.095	0.932	0.057	0.959	0.086	0.819	0.300	0.845	0.169	0.835	0.223	<0.01
C 15:0	1.062	0.087	1.193	0.060	1.120	0.101	1.280	0.184	1.097	0.245	1.169	0.237	>0.05
C 15:1	0.366	0.046	0.397	0.036	0.379	0.044	0.448	0.054	0.436	0.105	0.441	0.087	<0.01
C 16:0	28.059	1.166	29.31	1.656	28.51	1.473	21.40	3.586	23.00	2.799	22.381	3.153	<0.00001
Σ *trans* C16:1	0.377	0.113	0.336	0.055	0.357	0.084	0.069	0.024	0.067	0.030	0.046	0.027	<0.00001
Σ *cis* C16:1	2.178	0.343	1.824	0.729	2.001	0.536	2.025	0.750	2.010	0.392	2.018	0.571	<0.01
C 17:0	0.697		0.740	0.054	0.716	0.081	0.779	0.102	0.743	0.236	0.757	0.193	>0.05
C 17:1 *cis*	0.240	0.093	0.233	0.021	0.237	0.019	0.224	0.076	0.254	0.054	0.242	0.064	>0.05
C 18:0	9.807	0.018	9.515	1.178	9.783	1.163	12.21	2.947	13.64	2.278	13.082	2.595	<0.00001
C 18:1n (1-9) t	0.531	1.187	0.479	0.128	0.511	0.114	0.612	0.211	0.638	0.182	0.627	0.189	<0.01
C 18:1n (10-13) t	1.394	0.102	1.369	0.334	1.394	0.324	3.255	0.701	1.941	0.633	2.455	0.919	<0.00001
C 18:1n9 *cis*	21.791	0.326	20.89	1.209	21.45	1.227	22.70	2.934	25.71	3.387	24.532	3.488	<0.0001
C 18:2n6 *trans*	0.351	1.162	0.276	0.077	0.324	0.113	0.610	0.184	0.321	0.136	0.434	0.210	<0.05
C 18:2n6 *cis*	2.502	0.129	2.251	0.226	2.403	0.250	2.194	1.485	2.132	0.459	2.156	0.963	>0.05
C 18:3n6	0.208	0.225	0.203	0.018	0.207	0.028	0.241	0.048	0.380	0.148	0.326	0.137	<0.0001
C 18:3n3	0.715	0.034	0.782	0.142	0.745	0.155	1.468	0.236	1.354	0.343	1.398	0.305	<0.00001
C20:0	0.155	0.162	0.155	0.021	0.155	0.019	0.178	0.015	0.240	0.030	0.209	0.023	<0.00001
Σ CLA	0.387	0.011	0.400	0.050	0.394	0.044	1.871	0.523	0.822	0.372	1.347	0.448	<0.00001
C 20:1	0.270	0.038	0.280	0.045	0.275	0.051	0.551	0.056	0.553	0.095	0.552	0.076	<0.05

C 22:0	0.037	0.056	0.041	0.021	0.039	0.018	0.036	0.021	0.049	0.022	0.043	0.022	>0.05
C20:3n6	0.021	0.014	0.033	0.018	0.027	0.017	0.021	0.015	0.037	0.011	0.029	0.013	>0.05
C22:1n9	0.051	0.015	0.074	0.026	0.063	0.024	0.048	0.036	0.066	0.035	0.057	0.036	>0.05
C23:0	0019	0.022	0.024	0.012	0.022	0.012	0.056	0.031	0.054	0.029	0.055	0.030	>0.05
C20:4n6	0.032	0.011	0.038	0.017	0.035	0.013	0.074	0.036	0.088	0.044	0.081	0.040	<0.05
C20:5n3	0.018	0.009	0.025	0.011	0.022	0.011	0.072	0.056	0.059	0.032	0.066	0.044	>0.05
C22:6n3	0.041	0.011	0.032	0.012	0.037	0.017	0.061	0.032	0.056	0.026	0.059	0.029	>0.05
Σ SFA	65.307		66.70		65.85		56.71		60.14		58.799		<0.00001
Σ UFA	32.769		31.19		32.17		37.62		38.02		37.862		<0.00001
Σ MUFA	29.077		27.77		28.58		32.13		33.70		33.084		<0.00001
Σ PUFA	4.275		4.041		4.193		6.611		5.249		5.781		<0.01
Σ n/i*	1.924		2.108		1.971		5.669		1.835		3.338		
Σ omega-3	0.774		0.839		0.804		1.601		1.469		1.523		<0.00001
Σ omega -6	3.501		3.202		3.389		5.010		3.780		4.258		<0.001
Omega 3/6	0.221		0.262		0.237		0.320		0.388		0.358		<0.00001

Σ SFA - the sum of saturated fatty acid; Σ UFA - the sum of unsaturated fatty acid; Σ MUFA - the sum of monounsaturated fatty acid; Σ PUFA - the sum of polyunsaturated fatty acid; Σ n/i – the sum of non-identified peaks.

Table 3. Ratio of carbon stable isotopes in organic milk fat phase from cows with different diets

Parameter	Values of $\delta^{13}C$ in milk fat phase, ‰												P-value
	Outdoor period				Indoor period				Average annual				
	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD	
Milk fat from cows with silage-haylage diet (O1)	-24,53	-28,00	-22,30	1,76	-24,85	-26,70	-22,90	1,32	-24,70	-28,00	-22,30	1,57	<0,00001
Milk fat from cows with grass-hay diet (O2)	-31,58	-32,90	-29,80	1,08	-29,31	-29,80	-28,60	0,33	-30,20	-32,90	-28,60	1,33	

In our study significantly differences ($P < 0.00001$) of the C16:0 content reflected the share of green grass and hay in the cattle diet. The diet based only on fresh grass and hay feeding leads to the energy feed deficiency and increasing the role of fat reserve and decreasing *de novo* synthesis. Correspondingly, this may reflected in the decreasing of the C16:0 content (Roca Fernandez, 2012). Thus, the C16:0 content can be considered as a marker for determination of the share of green grass and hay in cows' diet.

Our studies have shown that the total PUFA content was higher by 30.8% and 15.4% in outdoor and indoor periods respectively in milk from O2 farms than in milk from O1 farm. In particular, the average annual content of ω3-fatty acids was higher by 89.4% and ω6-fatty acids was lower by 25.64% in milk from O2 farms. The average annual ratio of ω3- and ω6-fatty acids was higher by 51.0% in milk from O2 farms than in milk from O1 farms.

The intensity of transformation processes of long chain fatty acids in cow body directly depends on intensity of feed biohydrogenation in rumen and amount of long chain fatty acids, especially PUFA, in livestock feed (Jenkins, T. C., 1993).

In mammary gland stearic acid (C18:0), which is the final product of biohydrogenation of feed PUFA, is the substrate for C18:1*trans*-11 *de novo* synthesis and its following transformation to C18:2*cis*-9,12 and CLA with the help of Δ9-desaturase. From 64% to 97% of CLA content in milk fat comes from C18:1*trans*-acids (Roca Fernandez et al., 2012; Palmquist, 2006; Kemp & Lander, 1984; Kemp et al., 1984). Thus, the large share of feed such as fresh grass on pasture or fresh-cut grass with high level of PUFA leads to increasing of vaccenic acid (C18:1*trans*-11) and conjugates of linoleic acid content in milk fat (Palmquist, 2006).

The average content of ΣC18:1*trans*-acids was 1.6 times higher in O2 milk compared to O1 milk. The differences between O2 and O1 milk in outdoor and indoor periods were 2.0 times and 1.4 times, respectively.

According to our results the average annual CLA content in O2 milk was 3.4 times higher as compared to O1 milk. The difference in CLA content between O1 and O2 milk during the indoor period was equal to 2.0 times, while during the outdoor period – 4.8 times.

The annual content of C18:2*cis*-9,12 was lower by 10.3% in O2 milk than in O1 milk; during indoor and outdoor period – by 5.3% and 12.3% respectively.

However, despite the high statistically significant difference between individual fatty acids of milk from different farms, the analysis of the absolute values ranges of the most of fatty acids does not allow to identify the milk origin. For example, the annual values of CLA content ranged from 0.327 g / 100 g to 0.506 g / 100 g of fat in milk from O1 farms and from 0.478 g / 100 g to 2.523 g / 100 g of fat in milk from O2 farms. During the indoor period the characteristic range of CLA for milk from O1 farms was 0.329 – 0.506 g / 100 g of fat, and for milk from O2 farms is 0.478 – 1.535 g / 100 g of fat. The most significant variations were observed during the outdoor period: for O1 milk the values ranged from 0.327 g / 100 g to 0.441 g / 100 g of fat, and for O2 milk – from 1.035 g / 100 g of fat to 2.523 g / 100 g of fat. It is possible that the CLA content of more than 1.000 g / 100 g of fat is distinctive only for organic milk from cows with the grass-fed diet.

Overlapping of the annual values for ΣC14:1*trans* has been detected: for O1 milk it was 0.489–0.670 g / 100 g of fat, and for O2 milk it was 0.663 – 1.650 g / 100 g of fat. During indoor period the values were 0.557 – 0.670 g / 100 g for O1 and 0.663 – 1.631 g / 100 g of fat for O2. However, during outdoor period they are clearly distinguished – 0.489 – 0.670 g / 100 g of fat for O1 milk and 0.811 – 1.650 g / 100 g of fat for O2 milk.

Annual ranges of the total content of C16:1*trans*-acids were not overlapped: in O1 and O2 milk were ranged 0.157 – 0.585 g / 100 g and 0.017 – 0.112 g / 100 g of fat respectively. It is also possible to suggest a hypothesis that the total C16:1*trans*-acids

content, which is not higher than 0.134 g / 100 g of fat, may be characteristic only for organic milk from farms with the grass-fed diet.

The ranges of annual values of the C18:3*cis*-6,9,12 content were overlapped: for O1 milk was 0.163 – 0.268 g / 100 g of fat and O2 milk was 0.174 – 0.857 g / 100 g of fat. However, during the indoor period the possibility for samples discriminate ion was higher: the ranges were 0.174 – 0.231 g / 100 g for O1 and 0.195 – 0.857 g / 100 g of fat O2 milk.

The similar situation was observed for the C6:0 during the outdoor period. Its values ranged from 2.251 to 2.870 g / 100 g for O1 milk and from 1.365 to 2.393 g / 100 g of fat for O2 milk. Also, the C8:0 content ranged from 1.171 to 1.733 g / 100 g in O1 milk and from 0.693 to 1.461 g / 100 g of fat O2 milk during the outdoor period.

3.3. $\delta^{13}C$ analysis and chemometric data processing

For the complete discrimination of organic milk it was proposed the integrated approach based on the application of PCA to obtained multidimensional data of fatty acid composition and stable isotopes ratios $^{13}C/^{12}C$ ($\delta^{13}C$) in milk fat. Such an integrated chemometric approach has allowed to discriminate the milk obtained from organic farms with silage/haylage and grass/hay feeding diets (Figure 1).

The analysis of $\delta^{13}C$ values in the fatty phase of milk has revealed statistically significant differences between the samples from the farms of both types (Table 3).

PCA has been carried out in several stages and in different variants. Thus, Figure 1a shows the data distribution plot by the first two principal components when analysing only absolute values of fatty acid composition of the milk samples. Such an analysis didn't allow to reliably distinguish milk from O1 and O2 farms. It was shown that the largest contribution into the first two principal components (which explained ≈92% of data variations) was ensured by the content of palmitic (C16:0) (0.69 in the loadings of PC1), oleic (C18:1*cis*-9) (-0.44 in the loadings of PC1), stearic (C18:0) (-0.45 in the loadings of PC1) and myristic (C14:0) (0.26 in the loadings score of PC1) acids. It should be noted that the influence of these parameters could not be considered as characteristic. This is explained by positively correlation of their relatively high content in milk fat and contribution significance PC1 and PC2. Contingently, they can be attributed to macroparameters of fatty acid composition.

The introduction of absolute $\delta^{13}C$ values into the data matrix (Figure 1b) greatly improved the samples discrimination – the zones overlapping on the plot become minimal. The loadings analysis has shown that this parameter has the largest contribution to PC1 (0.43) and PC2 (0.64). Unlike the previous distribution the significance of C16:0 (-0.64 in the loadings of PC1), C18:0 (0.40 in the loadings of PC1) and C14:0 (-0.23 in the loadings of PC1) content decreased, but C18:1*cis*-9 content increased (0.37 in the loadings of PC1).

At the second stage the data of fatty acid composition and $\delta^{13}C$ values in fatty phase were normalized (by the maximum value for each parameter).

Such an approach allowed to minimize the main problem of PCA – the scale, i. e., to equalize the impact significance of macroindicators and minor components on the results of chemometric analysis. As a result of such actions the distribution picture has been improved, but not completely (Figure 1c). Also, the loadings analysis has shown another contribution of parameters to the principal components structure. While PC1 explained only ≈40% of data variations, PC2 and PC3 had the equal value of 10%.

It should be noted that using just this approach the significance of such acids as C16:1*trans*-9 (-0.42 in the loadings of PC1), C18:1*trans*-11 (0.28 in the loadings of PC1), C18:3*cis*-9,12,15 (0.30 in the loadings of PC1), C18:2*trans*-9,12 (0.20 in the loadings of PC1) and CLA (0.31 in the loadings of PC1) were established. These acids are characteristic for milk fat composition and especially for milk from cows with grass and hay diet (Elgersma, 2006). The effect of other UFA content on the

possibility of distinguishing the milk samples from different farms was also quite high. At the same time the significance of $\delta^{13}C$ influence decreased (0.14 in the loadings of PC1).

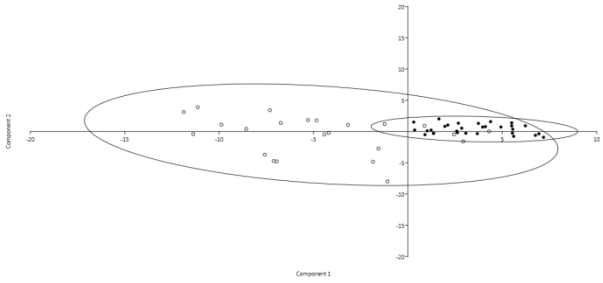

Figure 1a. PC1 and PC2, fatty acids composition, absolute values

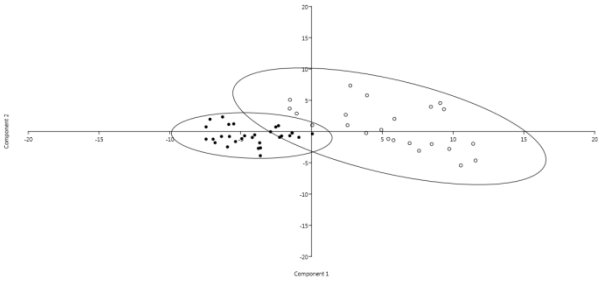

Figure 1b. PC1 and PC2, fatty acids composition and $\delta^{13}C$, absolute values

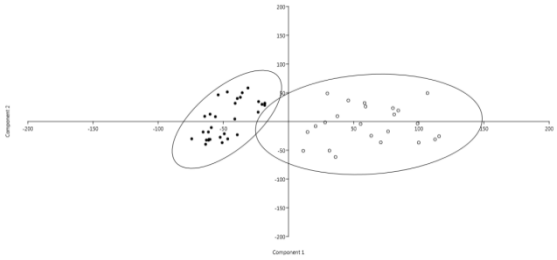

Figure 1c. PC1 and PC2, fatty acids composition and $\delta^{13}C$, absolute normalized values

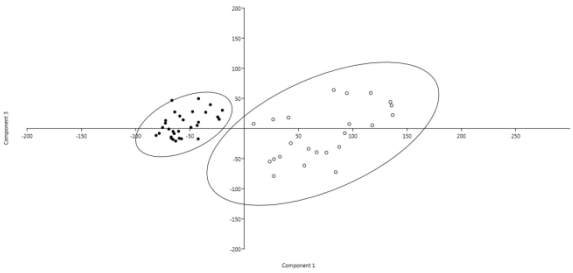

Figure 1d. PC1 and PC3, fatty acids composition and $\delta^{13}C$, absolute and derivative indicators, normalized values

Figure 1. The effect of the integrated approach on discrimination of organic milk from farms with different cows' diets by PCA: ○ – farms with silage-haylage diet; ● – farms with grass- hay diet.

Taking into account the results of chemometric analysis of normalized data we have introduced the additional derivative parameters describing the milk samples composition. Such parameters are the total *cis*-isomers and *trans*-isomers of UFA content, total UFA content, total MUFA content, total PUFA content, total dienes and trienes content, C18:0 content, total C18 UFA content, the ratio of ΣC18 UFA and Σ*trans*-isomers, the ratio of ΣC18 UFA and Σtrienes; the ratio of ΣC18 UFA and CLA content.

At the third stage the calculated data set of derived parameters has been added to the matrix of absolute values of fatty acid composition and $\delta^{13}C$. Then the matrix normalization has been done (in the same way as at the previous stage) followed by its processing by PCA. This analysis of such complex database completely divided two data groups on the plot (Figure 1d). While PC1 explains only ≈45% of variations, and PC2 and PC3 still have an equal value of 10%.

It should be noted that it is rather problematic to allocate the parameters with the greatest contribution to the principal components in the loadings matrix. The most of UFA have high contributions, however, the contribution of C16:1*trans*-9 (-0.35 in the loadings of PC1), vaccenic (C18:1*trans*-11) (0.25 in the loadings of PC1), linolenic (C18:3*cis*-9,12,15) (0.27 in the loadings of PC1), linolalidine (C18:2*trans*-9,12) (0.17 in the loadings of PC1) acids and CLA(0.28 in the loadings of PC1) remained maximal.

Among calculated derivative parameters the contribution into the structure of the first, second

and third components is maximal for such parameters as the total *trans*-isomers content (0.18 in the loadings of PC1), total trienes content (0.23 in the loadings of PC1), total unsaturated C18 UFA (0.11 in the loadings of PC1), the ratio of ΣC18 UFA/CLA content (-0.29 in the loadings of PC1) and far exceeds the contribution significance of $\delta^{13}C$ values (0.12 in the loadings of PC1).

4. Conclusions

The method of milk examination, including the organic one, to detect the presence of grass share in cows' diet has been proposed. This method is based on the integrated approach, which includes the analysis of fatty acid composition, the ratios of carbon stable isotopes in milk fat phase followed by the calculation of the set of derivative parameters, the normalization of the absolute values of these parameters (by the maximum value) and the processing of obtained data matrix by PCA. Among the calculated derivative parameters the contribution into the structure of the first, second and third components is maximal for such parameters as the total *trans*-isomers content, total trienes content, total unsaturated C18 UFA, the ratio of ΣC18 UFA and CLA content. The proposed approach has allowed to distinguish organic milk from farms with different percentage of green grass and hay in cattle diet during indoor and outdoor periods. It could be a potential tool for confirming organic milk authenticity, especially labelled as "grass fed".

5. References

Adler, S. A., Dahl, A. V., Jensen, S. K., Thuen, E., Gustavsson, A. M., & Steinshamn, H. (2013). Fatty acid composition, fat-soluble vitamin concentrations and oxidative stability in bovine milk produced on two pastures with different botanical composition. *Livestock Science, 154*(1-3), 93-102.

Butler, G., Nielsen, J. H., Slots, T., Seal, C., Eyre, M. D., Sanderson, R., & Leifert, C. (2008). Fatty acid and fat-soluble antioxidant concentrations in milk from high-and low-input conventional and organic systems: seasonal variation. *Journal of the Science of Food and Agriculture, 88*(8), 1431-1441.

Camin, F., Perini, M., Colombari, G., Bontempo, L., Versini, G. (2008). Influence of dietary composition on the carbon, nitrogen, oxygen and hydrogen stable isotope ratios of milk. Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute. *Research in Mass Spectrometry, 22*(11), 1690-1696.

Capuano, E., Boerrigter-Eenling, R., Elgersma, A., van Ruth, S. M. (2013). Effect of fresh grass feeding, pasture grazing and organic/biodynamic farming on bovine milk triglyceride profile and implications for authentication. *European Food Research and Technology, 238*(4), 573-580.

Capuano, E., Gravink, R., Boerrigter-Eenling, R., van Ruth, S. M. (2015). Fatty acid and triglycerides profiling of retail organic, conventional and pasture milk: Implications for health and authenticity. *International Dairy Journal*, 42, 58-63.

Capuano, E., Van der Veer, G., Boerrigter-Eenling, R., Elgersma, A., Rademaker, J., Sterian, A., Van Ruth, S. M. (2014). Verification of fresh grass feeding, pasture grazing and organic farming by cows farm milk fatty acid profile. *Food Chemistry*, 164, 234-241.

Dhiman, T. R., Anand, G. R., Satter, L. D., Pariza, M. W. (1999). Conjugated Linoleic Acid Content of Milk from Cows Fed Different Diets. *Journal of Dairy Science*, 82(10), 2146-2156.

Elgersma, A., Ellen, G., Dekker, P. R., Van der Horst, H., Boer, H., Tamminga, S. (2003). Effects of perennial ryegrass (Loliumperenne) cultivars with different linolenic acid contents on milk fatty acid composition. *Aspects of Applied Biology*, *(70)*, 107-114.

Elgersma, A., Tamminga, S. E. E. R. P., Dijkstra, J. (2006). Lipids in herbage. Their

fate in the rumen of dairy cows and implications for milk quality. In book: Fresh Herbage for Dairy Cattle (pp.175-194), Springer.

Gerstenberg, H., Herrman, H. (1983). Report on the Interecomparison for the Isotope Standards KH-2 and PEF-1. *Akademieder Wissenschaftender DDR, Leipzig. Zentral institute fuer Isotopen – und Strahlenforschung*, 67-83.

Hammer, Ø., Harper, D. A. T., Ryan, P. D. (2001). PAST-Palaeontological statistics. www. uv. es/~ pardomv/ pe/2001_1/past/pastprog/past. pdf, acessadoem, 25(07), 2009.

Jenkins, T. C. (1993). Lipid metabolism in the rumen. *Journal of Dairy Science*, *76*(12), 3851-3863.

Kemp, P., Lander, D. J. (1984). Hydrogenation in vitro of α-linolenic acid to stearic acid by mixed cultures of pure strains of rumen bacteria. *Microbiology*, *130* (3), 527-533.

Kemp, P., Lander, D. J., & Gunstone, F. D. (1984). The hydrogenation of some cis-and trans-octadecenoic acids to stearic acid by a rumen Fusocillus sp. *British Journal of Nutrition*, *52*(1), 165-170.

Kochubey, S. M., & Zhukova, Y. F. (1996). Membrane protein phosphorylation in pea chloroplasts from plants grown under unfavourable irradiance or temperature: Heterogenity in photosystem 1. *Indian Journal of Experimental Biology*, *34*(1), 71-77.

Loor, J. J., Herbein, J. H., & Polan, C. E. (2002). Tans18: 1 and 18: 2 Isomers in Blood Plasma and Milk Fat of Grazing Cows Fed a Grain Supplement Containing Solvent-Extracted or Mechanically Extracted Soybean Meal. *Journal of Dairy Science*, *85*(5), 1197-1207.

Mitani, T., Kobayashi, K., Ueda, K., & Kondo, S. (2016). Discrimination of "grazing milk" using milk fatty acid profile in the grassland dairy area in Hokkaido. *Animal Science Journal*, *87*(2), 233-241.

Molkentin, J. (2009). Authentication of organic milk using $\delta^{13}C$ and the α-linolenic acid content of milk fat. *Journal of Agricultural and Food Chemistry*, *57*(3), 785-790.

Molkentin, J. (2013). Applicability of organic milk indicators to the authentication of processed products. *Food Chemistry*, *137*(1-4), 25-30.

Organic Milk Suppliers Cooperative (2017). Organic Milk Market Report. 26 p. Worle, UK.

Palmquist, D. L. (2006). Milk fat: Origin of fatty acids and influence of nutritional factors thereon. In *Advanced Dairy Chemistry Volume 2 Lipids* (pp. 43-92). Springer, Boston, MA.

Petrov, P., Zhukova, Y., & Demikhov Yu. (2016). The Effects of Dairy Management on Milk Quality Characteristics. *Turkish Journal of Agriculture-Food Science and Technology*, *4*(9), 782-786.

Roca Fernandez, F. A., & Gonzalez, R. A. (2012). Effect of dietary and animal factors on milk fatty acids composition of grazing dairy cows: a review. *Iranian Journal of Applied Animal Science*, 2(2), 97-109.

Średnicka-Tober, D., Barański, M., Seal, C. J., Sanderson, R., Benbrook, C., Steinshamn, H., ... & Cozzi, G. (2016). Higher PUFA and n-3 PUFA, conjugated linoleic acid, α-tocopherol and iron, but lower iodine and selenium concentrations in organic milk: a systematic literature review and meta-and redundancy analyses. *British Journal of Nutrition*, *115*(6), 1043-1060.

White, S. L., Bertrand, J. A., Wade, M. R., Washburn, S. P., Green, J. T., & Jenkins, T. C. (2001). Comparison of fatty acid content of milk from Jersey and Holstein cows consuming pasture or a total mixed ration. *Journal of Dairy Science*, *84*(10), 2295-2301.

Zhukova, Y., Petrov, P., Demikhov, Y., Mason, A., & Korostynska, O. (2017). Milk Urea Content and $\delta^{13}C$ as Potential Tool for Differentiation of Milk from Organic and Conventional Low-and High-Input Farming Systems. *Turkish Journal of Agriculture-Food Science and Technology*, *5*(9), 1044-1050.