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Research Article

Content of 17β-Estradiol in Raw Milk in Ukraine

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Abstract: The main source of estrogen supply of steroid origin in the organism of consumers is milk and dairy products, which consume 60 to 80% of these hormones. Excess estrogen, including 17β-estradiol in the serum, has been linked to female and male genital cancers. The purpose of the search was to determine the amount of 17β-estradiol in milk on various farms in Ukraine and during its processing at the dairy plant. The determination of 17β-estradiol in cow's milk samples was performed by enzyme-linked immunosorbent assay. It was found that the average value of 17β-estradiol content in raw milk obtained during the day on one farm does not depend on the time of milk production. At the same time, the content of 17β-estradiol in whole milk from one herd changes significantly during the year of its receipt. Mixing whole milk from different farms at the processing plant does not lead to a significant difference in 17β-estradiol content compared to milk produced on a particular farm in the region. It was found that whole milk with a higher mass fraction of milk fat contains more 17β-estradiol. Separating milk influences the distribution of 17β-estradiol between cream and skim milk. The amount of 17β-estradiol in skim milk was almost 5-7 times less than in cream. Therefore, to possibly reduce the daily intake of 17β-estradiol and dairy products during consumption, it is necessary to reduce the mass fraction of fat in them.

Keywords: *17β-estradiol, Whole milk, Skim milk, Cream*

Ukrayna'da Çiğ Sütte 17β-Östradiol İçeriği

Öz: Tüketicilerin bünyesindeki östrojen orijinli steroidin temel kaynağını süt ve süt ürünleri oluşturur ve bu hormonlar %60-80 oranında alınmaktadır. Serumdaki 17β-östradiol de dahil olmak üzere aşırı östrojen, kadın ve erkek genital kanserleriyle ilişkilendirilmiştir. Bu çalışmanın amacı, Ukrayna'daki çeşitli çiftliklerde ve süt fabrikasında işlenme esnasındaki sütte 17β-östradiol miktarının belirlenmesiydi. İnek süt örneklerinde 17β-östradiol miktarı, enzyme-linked immunosorbent assay ile analiz edildi. Bir çiftlikte gün boyunca elde edilen çiğ sütteki ortalama 17β-östradiol miktarının süt sağım zamanına bağlı olmadığı tespit edildi. Aynı zamanda, bir sürüden alınan tam yağlı sütteki 17β-östradiol içeriği, alındığı yıl boyunca önemli ölçüde değişmekteydi. Farklı çiftliklerden gelen tam yağlı süt karışımının 17β-estradiol içeriği, bölgedeki belirli bir çiftlikte üretilen sütteki içerik ile karşılaştırıldığında önemli bir farklılık saptanmadı. Yüksek kütle oranına sahip tam yağlı sütün daha fazla 17β-östradiol içerdiği saptandı. Sütün, krema ve yağsız süt olarak ayıklanması 17β-östradiol dağılımını etkilemekteydi. Yağsız sütteki 17β-östradiol miktarı kremadakinden neredeyse 5-7 kat daha azdı. Bu nedenle, tüketim sırasında 17β-östradiolün günlük alımını azaltmak için, süt ürünlerinin içeriklerindeki yağın kütle fraksiyonunu azaltmak gerekmektedir.

Anahtar sözcükler: *17β-östradiol, Tam yağlı süt, Yağsız süt, Kaymak*

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Introduction

It is well known that milk and dairy products are considered essential sources of nutrients and are the basis of the ration for most of the population [1,2]. Milk contains the optimal ratio of calcium and phosphorus for their assimilation, milk proteins are complete, and raw milk is the basis for producing many dairy products [3-5]. However, the usefulness of milk and dairy products can be only if they meet the quality and safety indicators. Contaminants of milk can be various xenobiotics, including antibiotics and detergents and disinfectants [6,7], heavy metal salts [8], pesticides, hormones, etc. [9,10]. Recently our research has shown that beef containing the synthetic hormone zeranol, which is forbidden for use as a stimulant of animal weight gain, has been processed [11]. Therefore, the research of products of animal origin, in particular milk, for the presence of steroid hormones, which are considered stimulants to increase production, is constantly relevant, despite the fact that the use of hormones is officially prohibited.

Milk contains a group of estrogenic hormones of natural origin (17β-estradiol, 17α-estradiol, estriol, and estrone), which have an influence on several vital functions in the organism (protein synthesis, signaling transmission between receptors, etc.), including the regulation of reproduction [12]. Estrogen steroid hormones of natural origin are present in milk as a result of secretion of internal glands, respectively, a certain amount of them will always be present in raw milk [13]. Hormones of artificial origin are present in milk in the treatment of the reproductive system of animals, as well as in intended use to increase production [14,15]. However, modern methods for determining 17β-estradiol in milk and dairy products cannot identify its origin. Therefore, the research aims to establish a safe content of 17β-estradiol in milk and dairy products, namely the amount of hormone synthesized by animals under normal physiological conditions [12]. However, there are cases when animals are given 17β-estradiol to increase milk production [15,16], in which case the amount of this hormone rises significantly in milk. High levels of 17β-estradiol in milk and dairy products are a concern among scientists, as the use of these products leads to an increase in estrogen levels in the blood of consumers, which is considered dangerous because these hormones are responsible for various cancers (breast, uterine, uterine, ovaries, testes, prostate) [17-20]. In addition, the intake of significant amounts of estrogen with food in the organism of prepubertal children causes disorders of the development of genitourinary and central nervous system [21,22].

It is reported that the content of 17β-estradiol in whole milk of healthy cows depends on many factors related to the physiological state of the organism (gestation period, estrus), from the composition of feed ration, breed, and age of animals [23]. The researchers found in drinking milk which was selected from different regions of Iran from 75.5 pg/mL to 922.3 pg/mL of 17β-estradiol, with an average of 330 pg/mL [24]. Another search [25] reported an amount of 17β-estradiol in pasteurized and raw milk in the range of 5.6-51 pg/mL. In the research of samples of pasteurized and sterilized skim milk, it was found that the average level of 17β-estradiol was 8.2±0.59 pg/mL [26]. According to Malekinejad and Rezabakhsh [12] and Janowski et al.[27], the content of 17β-estradiol in drinking milk averaged 20 mg/mL.

According to the requirements of the Codex Alimentarius Commission, the maximum amount of external (synthetic) estradiol entering the organism together with food should not exceed 50.000 pg/kg/day [28]. The analysis of Ukrainian normative documents revealed that the control of raw milk coming for processing on the content of 17β-estradiol is not provided.

Due to the significant influence of high estrogen concentrations on the organism of consumers to date, the safe quantitative levels of 17β-estradiol in milk and dairy products are not entirely justified, given the intensive technology of milk production. Therefore, conducting systematic research, including the influence of the maximum number of factors (fat content, period of lactation, seasonality, feeding, breed) on the level of 17β-estradiol in raw milk, is promising and relevant.

The research aimed to determine the amount of 17β-estradiol in milk on various farms in Ukraine and during its processing at the dairy plant.

Material and Methods

A milk sampling for research was conducted on three dairy farms in Ukraine in the Western and Central regions and on one milk processing plant. Milk is got on these farms from cows of the Ukrainian Black-Spotted breed. Milk samples were taken and delivered to the laboratory in a refrigerated state at a temperature of +2±1°C. The research was conducted in State Scientific and Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise (Kyiv).

Quantitative determination of 17β-estradiol in cow's milk samples was performed by enzyme-linked immunosorbent assay using a test system RIDASCREEN®17β-őstradiol Art. No. R2301 (manufactured by firm Art-Biopharm / R-Biopharm, Darmstadt, Germany). Before use, the test system lasted 20-30 min. at a temperature of 20-25°C, the reagents were prepared according to the manufacturer's protocol. Standard solutions of 17β-estradiol with concentrations of 0; 50; 200; 800; 3200; 12800 pg/mL were used to construct the calibration curve.

Before the research, the milk samples were heated in a thermostat to a temperature of 20-25°C and homogenized using an IKA homogenizer (T 18 Basic) with nozzles (S 18 N-10 G), to ensure homogeneity. 20 μL of standard solutions, test samples, 50 μL of a diluted antibody preparation, and 17β-estradiol conjugate were added to the wells of the microtiter plate sensitized to antibodies to 17β-estradiol. The tablet was incubated for 2 h at a temperature of 20-25°C. Then the device for washing tablets (BIORAD PW 40) washed the wells of the tablet with distilled water. 50 μL of substrate and chromogen solution was added to each well and incubated for 30 minutes at 20-25°C. After incubation, 100 μL of stop reagent was added to the wells. The optical density was measured on an enzyme-linked immunosorbent reader, Sunrise (Austria), at a wavelength of 450 nm. Specialized RIDA®Soft software was used for computer processing of measurement results.

Statistical analysis was performed using disperse analysis with Fisher's criteria (ANOVA). The data are presented as x ± SD (mean ± standard error). Significance of the obtained data was evaluated according to F-criterion with the significance levels of $P<0.05$, $P<0.01$, $P<0.001$ (taking into account Bonferroni correction) by SAS (Version 9.2, 2009).

Results

At the first stage of the research, the amount of 17β-estradiol in the milk of different milking (morning, lunch, evening) was determined on three dairy farms. Milk samples were taken after milking from the cooler tank (common milking) three times a day to get milk for morning, lunch, and evening milking. The experiment was conducted for two months in February-March. The research results are given in *Table 1*.

The searches show in *Table 1* that a significant difference between the average content of 17β-estradiol in raw milk obtained at different periods of the day was not found, both within one farm and on three other farms. The amount of hormone ranged from 439.8±41.8 pg/mL to 585.5±61.2 pg/mL in morning milk, from 497.6±46.5 pg/mL to 605.7±71.0 pg/mL in lunch milk, and from 543.3±53.2 pg/mL to 641.3±62.8 pg/mL in evening milk. At the same time, significant fluctuations of the content of 17β-estradiol in raw milk were found during milking, both at one and in different periods of the day. In particular, on farm number №1, the difference between the minimum and maximum amount of hormone was from 3.0 to 3.7 times ($P<0.01$); on the second farm, this difference was the smallest from 2.3 to 2.5 times ($P<0.01$) and the largest difference was found on the third farm between the minimum and maximum amount of hormone from 3.4 to 4.5 times ($P<0.01$).

Therefore, the difference between the maximum and minimum amount of 17β-estradiol in collected raw milk obtained during the day on one farm fluctuates significantly, but the average value does not depend on the milk production time.

In order to determine the influence of various factors, particularly the year, on the change in the content of 17β-estradiol in raw milk, the amount of this hormone was determined during the year. Milk samples were taken at these three farms twice a month. The results are given in *Table 2*.

Table 1. *Content of 17ß-estradiol in raw milk on three farms in Ukraine, pg/mL, (x ± SD; n = 90)*

Farm Number	Milk Sampling Time	Number of Tested Samples	Minimum Quantity	Maximum Number	Average Number, x ± SE
№1	1	n=10	257.3	851.6*	514.9±66.5
	2	n=10	242.5	918.3*	605.7±71.0
	3	n=10	268.2	797.4*	583.2±55.3
№2	1	n=10	324.7	819.9*	439.8±41.81
	2	n=10	348.5	833.7*	497.6±46.5
	3	n=10	371.7	875.1*	543.3±53.2
№3	1	n=10	198.6	903,4*	585.5±61.2
	2	n=10	206.5	747.1*	604.8±59.4
	3	n=10	238.8	825.5*	641.3±62.8

1- milk of morning milking; 2 - milk of lunch milking; 3 - milk of evening milking; P<0.01 - compared to the minimal content of 17ß-estradiol*

Table 2. *The amount of 17ß-estradiol in raw milk during the year, pg/mL, (x±SD; n=48)*

Months of Research	Number of Tested Samples	Minimum Quantity	Maximum Number	The Average Number of x ± SE
January – February	12	582.3	1.243	836.9±79.2*
April – May	12	247.7	739.5	404.5±40.6
July – August	12	358.4	912.1	512.4±47.6
October – November	12	436.5	875.8	571.7±54.3

** P<0.05 - compared with the content of 17ß-estradiol in milk taken in other months*

As can be seen from *Table 2*, we see that the highest amount of 17β-estradiol was found in milk samples taken on farms in January and February - 836.9±79.2 pg/mL. The amount of 17β-estradiol in this period was 2.0 times higher (P<0.05) compared with the content in milk selected in April and 1.6-1.5 times (P<0.05) higher than in July-August and September-October, respectively. The increase in the amount of 17β-estradiol in milk in January-February can be explained by the fact that probably in this period, the most significant number of cows in the third trimester of pregnancy physiologically produce milk with high levels of this hormone [29,30]. The lowest number of pregnant cows giving milk was in April and May.

Thus, searches indicate that the content of 17β-estradiol in whole milk from one herd undergoes significant changes during the year of its receipt.

Due to the fact that whole milk after delivery to the processing plant can be mixed with milk from other farms and reserved in large tanks for processing, we determined the amount of 17β-estradiol in whole milk before pasteurization. The search was performed in October and November *(Table 3)*.

Table 3 shows that the content of 17β-estradiol in whole milk collected at the processing plant generally reflects the amount of the hormone in milk on farms. The average amount of 17β-estradiol in October ranged from 423.4±40.3 pg/mL to 518.7±52.5 pg/mL, i.e., fluctuations within100 pg/mL. In November, the content of 17β-estradiol increased to an average of 578.7±52.8 pg/mL.

Therefore, the researches show that mixing processing plant of whole milk from different farms does not lead to a significant difference in 17β-estradiol content compared to milk produced on a particular farm in a given region.

The next step was to determine and compare the content of 17β-estradiol in the whole milk, depending on the content of mass fraction of fat. It is known that the steroid hormone 17β-estradiol belongs to the lipophilic ones, which are more concentrated in the milk fraction. The results of the research are given in *Table 4*.

As can be seen from *Table 4* we can see that a significant difference between the content of 17β-estradiol in raw milk with a mass fraction of fat from 2.8% to 3.5% is not observed. However, in whole milk with a fat content of 3.6% to 4.0%, the amount of 17β-estradiol was, on average, 20% higher than in milk with a fat content of 2.8% - 3.0%. In milk with a fat content of 4.1% to 4.5%, the amount of 17β-estradiol was 704.8±58.6 pg/mL, which is 27.8% (P<0.05) more compared to milk with the lowest milk fat content.

Therefore, the data indicate that whole milk with a higher mass fraction of milk fat contains more 17β-estradiol.

Considering that the level of steroid hormones in milk depends on the concentration of milk fat, the influence of skimming on the distribution of 17β-estradiol by fractions (cream, skim milk) was studied. Milk with different initial content of 17β-estradiol was taken into the experiment *(Fig. 1)*.

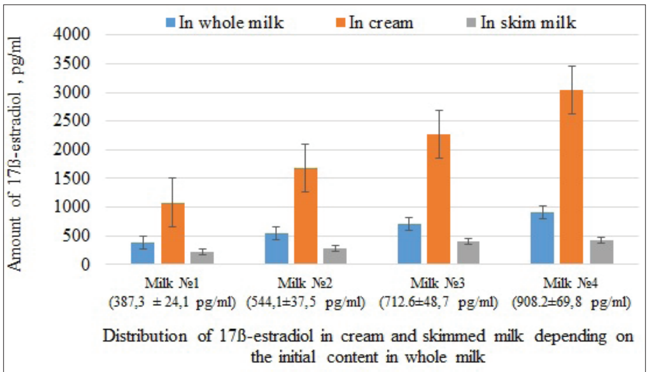

Fig 1. The influence of skimming whole milk on 17β-estradiol content

Table 3. *Content of 17β-estradiol in raw milk at the dairy processing plant of Ukraine, pg/mL, (x±SD; n = 30)*

Months of Research	Research Days	Investigated Samples	Minimum Quantity	Maximum Number	The Average Number of x ± SE
October	1	n=5	302.5	627.2	423.4±40.3
	15	n=5	323.2	716.4	518.7±52.5
	30	n=5	281.6	519.3	437.5±42.7
November	40	n=5	311.8	775.1	514.2±45.1
	50	n=5	353.2	807.9	564.1±50.3
	60	n=5	430.7	756.4	578.7±52.8

Table 4. *The amount of 17β-estradiol in raw milk, depending on the mass fraction of fat, (x±SD; n = 40)*

Milk with Fat Content, %	Number of Tested Samples	Minimum Quantity	Maximum Number	The Average Number of x ± SE
2.8 – 3.0	n=10	327.1	689.3	551.4±51.7
3.1 – 3.5	n=10	373.3	707.6	565.7±54.5
3.6 – 4.0	n=10	395.2	819.5	663.5±57.3
4.1 – 4.5	n=10	398.8	925.6	704.8±58.6*

** P<0.05 - compared with the content of 17β-estradiol in milk with a fat content of 2.8-3.0%*

The results of *Fig. 1* show that the milk separation process influences the distribution of 17β-estradiol between cream and skim milk. In particular, the highest level of 17β-estradiol was found in the cream. The content of this hormone was 2.8-3.3 times (P<0.01) higher in cream than in whole milk. At the same time, skimmed milk contained 1.7 to 2.1 times (P<0.05) less 17β-estradiol than whole milk. The results of this investigation indicate that fat-containing dairy products will be a much greater source of intake of steroid hormone 17β-estradiol than low-fat. As the amount of 17β-estradiol in skim milk was practically 5-7 times (P<0.001) less than in cream, it is evident that reducing the daily intake of 17β-estradiol and dairy products during consumption is necessary to reduce the mass fraction of fat in them.

Discussion

It is believed that the main source of estrogen of steroid origin in the organism of consumers is milk and dairy products, which consume 60% to 80% of these hormones [25,26]. The presence of excessive amounts of estrogen, in particular, 17β-estradiol, in the serum has been associated with the development of female and male genital cancer [17-20]. Therefore, the importance of milk and dairy products in the development of these pathologies today is considered twofold. Some authors confirm the connection between the consumption of dairy products and the emergence of oncology [31-33], while others point to the ambiguity of this issue, and the correlation is probable [34-36]. Therefore, our research aimed at determining the amount of 17β-estradiol in milk on various farms in Ukraine and processing it at the dairy plant. After all, no study has been conducted to determine this hormone in Ukraine, and available foreign data indicate that the amount of 17β-estradiol in milk is very variable and depends on many factors.

When determining 17β-estradiol on three farms, we found that the amount of hormone ranged from 439.8±41.8 pg/mL to 585.5±61.2 pg/mL in morning milking, from 497.6±46.5 to 605.7±71.0 pg/mL in lunch milking and from 543.3±53.2 pg/mL to 641.3±62.8 pg/mL in evening milking. At the same time, significant fluctuations of the content of 17β-estradiol in raw milk got during milking were found, both at one and in different periods of the day. In particular, the difference between the minimum and maximum amount of the hormone ranged from 3.0 to 4.5 times (P<0.01). Thus, our data are consistent with the results of researches by Iranian scientists on fluctuations in the content of 17β-estradiol from 75.5 pg/mL to 922.3 pg/mL of milk [24]. However, they differ from other researchers, who found significantly lower amounts of 17β-estradiol in whole cow's milk - from 5 pg/mL to 51 pg/mL [25-27]. We also found that the highest amount of 17β-estradiol was found in milk samples taken from farms in January and February - 836.9±79.2 pg/mL. The amount of 17β-estradiol in this period was 2.0 times higher (P<0.05), compared with the content in milk taken in April and May and 1.6-1.5 times (P<0.05) higher than in July-August and September-October, respectively. Seasonal increase in the amount of 17β-estradiol in raw milk, we explain the presence on the farm in this period a significant number of cows in the second and third trimesters of pregnancy, which according to scientists [29,30] in this period of pregnancy produce milk with 17β- estradiol is 26-33 times more than non-pregnant cows [25,29,37].

Research of raw milk at a milk processing plant revealed that mixing milk from different farms did not lead to a significant difference in 17β-estradiol content compared to milk obtained on a particular farm in the region. This gives reason to believe that the range of 17β-estradiol in whole milk from one herd undergoes significant changes during production during the year.

In a research of the content of 17β-estradiol, depending on the mass fraction of fat in raw milk, it was found that a significant difference between the content of the hormone in milk with a mass fraction of fat from 2.8% to 3.5% is not observed. Changes were detected in milk with a fat content of 3.6% to 4.0%, in particular, the amount of 17β-estradiol was, on average, 20% higher than in milk with a fat content of 2.8% - 3.0%. In milk with a fat content of 4.1% - 4.5%, the amount of 17β-estradiol was 27.8% (P<0.05) higher compared with milk with a fat content of up to 3.0%. The obtained results confirm researchers' data [26] on the lipophilicity of 17β-estradiol, as a result, milk with a higher mass fraction of fat contained a higher content of this hormone. It was reported [38] that in pasteurized milk with a fat content of 1.0% and 2.0% fat, the amount of 17β-estradiol was 1.8 and 1.3 times (P<0.05) less than in whole milk.

Our results indicate the lipophilic nature and redistribution of 17β-estradiol by separation technology. Thus, it was found that the process of separation of milk influences the distribution of 17β-estradiol between cream and skim milk. In particular, the amount of 17β-estradiol in cream was 2.8-3.3 times (P<0.01) higher than in whole milk. At the same time, skimmed milk contained 1.7 to 2.1 times (P<0.05) less 17β-estradiol than whole milk. In searches [38], the hormone content in cream was 3.2 time (P<0.01) higher than in whole milk and 15 times higher than in skim milk. Therefore, we believe that foods fortified with milk fat are also a significant source of 17β-estradiol for consumers.

So, summarizing the study, we can note the following. Significant fluctuations in 17β-estradiol in cows' milk may be mainly due to the presence of different levels of fat in the herd of cows at different stages of pregnancy, milk production with different fat content, or dietary influence, as phytoestrogens of the green feed in summer, may increase steroids hormones in the blood [23,39]. All these factors must be investigated in a specific experiment. Also, it is likely that significant differences in the content of 17β-estradiol in milk may be due to the use of different methods for its determination (enzyme-linked immunosorbent assay, radioimmunoassay, chromatographic). In addition, considering that 17β-estradiol is a lipophilic hormone whose solubility is better in fats, it is advisable to indicate the mass fraction of milk fat when comparing its content in dairy products. This will allow a more objective assessment of the level of 17β-estradiol in milk and the calculation of possible daily consumption.

It was found that the average value of 17β-estradiol in raw milk obtained during the day on one farm does not depend on the time of milk production. At the same time, the content of 17β-estradiol in whole milk from one herd changes significantly during the year of its receipt. Mixing whole milk from different farms at the processing plant does not lead to a significant difference in 17β-estradiol content compared to milk produced on a particular farm in the region.

It was found that whole milk with a higher mass fraction of milk fat contains more 17β-estradiol. The process of separating milk affects the distribution of 17β-estradiol between cream and skim milk.

The amount of 17β-estradiol in skim milk was almost 5 - 7 times less than in cream. Therefore, to possibly reduce the daily intake of 17β-estradiol and dairy products during consumption, it is necessary to reduce the mass fraction of fat in them.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding author (Y. Horiuk).

Financial Support

No funding was received.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Statement

Ethics committee approval is not required for this study because of performing *in vitro* in the laboratory.

Author Contributions

MK, VS and GK conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. ZM, KM, YH and OP collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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