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OXIDATIVE CHANGES OF MILK FAT IN DRY MILK STORED UNDER VARIOUS CONDITIONS

ZMIANY OKSYDACYJNE W TŁUSZCZU MLEKA PROSZKOWANEGO PRZECHOWYWANEGO W RÓŻNYCH WARUNKACH

Abstract: Oxidative changes of milk fat in whole dry milk during 50 days of storage under various conditions were examined. Whole dry milk with 26.47 % of fat and insolubility index 1.27 was taken as a sample. Whole dry milk was manufactured by roller drying in YOG s. r. o. Bojkvice. Whole dry milk was stored in desiccators at temperature 37 ^{o}C in thermoregulator under various water activities (0.23 and 0.82). Water activity was made by 100 cm^3 of saturated salt solution. Water activity 0.23 was made by saturated solution of potassium acetate and water activity 0.82 was made by saturated solution of potassium bromide. The milk powder was stored for 50 days. The sample with water activity 0.82 became dark brown during storage thanks to products of Maillard reaction. The oxidative changes were examined as a content of hydroperoxides, TBARS (*thiobarbituric reactive substances*), peroxide value, neutralization number, content of conjugated dienes and fatty acids composition. The content of hydroperoxides, TBARS and fatty acids, especially unsaturated fatty acids (oleic acid and linoleic acid) decreased during storage. Neutralization number and peroxide value increased during storage. All chemical parametrer were significantly changed during 50 days of storage under various water activities.

Keywords: oxidative changes, milk fat, storage conditions, water activity, TBARS, hydroperoxides

Dairy products are an important group in human nutrition. They are consumed as such or are used in preparation of many food items to provide specific functional properties [1]. Proteins, carbohydrates and lipids in foods or food ingredients undergo inevitable chemical changes during storage, due to interactions amongst themselves [2]. The Maillard reaction between amino acids residues and carbohydrates, and oxidative changes of amino acid residues by lipid peroxides are typical examples concerning proteins changes [3]. Water activity (A_w) is considered to be a principal factor governing these reactions [4]. Milk lipids may undergo chemical and physical changes

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during technological processing such as autooxidation, oxidation and formation of trans fatty acids [5]. Fat oxidation leads to production of low molecular weight substances such as aldehydes, ketones and lactones which can influence the properties of dairy products in inadvisable way for example odour, flavor and color [6]. High content of fat in whole dried milk is one of the major factors which participate in oxidized flavor, other factors are technology of drying, storage conditions above all storage temperature and water activity.

In the present paper, the effect of A_w on the chemical changes in milk fat of whole roller dried milk stored at temperature 37 ^oC was desribed. The oxidative changes were examined as a content of hydroperoxides, TBARS (thiobarbituric reactive substances), peroxide value, neutralization number, content of conjugated dienes and fatty acids composition in dried milk stored under two A_w (0.23 and 0.82).

Materials and methods

Whole roller dried milk was manufactured from pasteurized cow milk with fat content around 3.5 %. Whole dried milk with 26.47 % of fat, insolubility index 1.85, WPNI 3.66 and raw protein 27.31 % was used as a sample. Fat content was made after cold extraction according to Davídek [7], Whey protein nitrogen index (WPNI) according to Niro method No. A 21a and insolubility index according to ČSN ISO 57 0105 [8]. Saturated solutions of following salts were placed in desiccators to adjust the A_w parameter [9, 10]: potassium acetate for A_w = 0.23 and potassium bromide for A_w = 0.82. Twenty grams of whole milk powder were put in Petri dish (Φ 15 cm) and the dish was placed in the desiccators and then stored in incubators at a temperature 37 ^oC. Twenty grams of whole milk powder were packed in polyethylene sachet and then stored as a control sample in refrigerator at 6 ± 2 ^oC. All samples were analyzed under same conditions and were measured three times and compared with control.

Thiobarbiruc acid reactive substances (TBARS)

TBARS were measured by method of King [10] and results are expressed as an absorbance at λ = 450 nm, as measured on a Libra S6 spectrophotometer (Biochrom, Cambridge, England). As a first step 2 grams of whole milk powder were reconstituted in 20 cm^3 of distilled water at 30 ^oC and then the method of King [10] was used. Then 1 cm^3 of trichloracetic acid with a concentration 1 $g \cdot cm^{-3}$ (Lachema, Brno) and 2 cm^3 of ethanol were added to 20 cm^3 reconstituted milk. After 5 minutes the mixture was filtrated through Filtrak 389. Next, 1 cm^3 of TBA solution (Sigma Aldrich, Inc., St Louis, MO, USA), with a concentration of 1.4 g of 2-thiobarbituric acid (TBA) in ethanol to 100 cm^3, was added to 4 cm^3 of clear filtrate. Filtrate with TBA solution was placed in 60 ^oC water bath for 60 minutes and after cooling the absorbance at λ = 450 nm was measured. The water modification was made with 2 cm^3 of TBA, with a concentration of 0.05 M were added to 4 cm^3 of clear filtrate which was prepared same as for ethanol modification. Filtrate with TBA solution was placed in 100 ^oC water

bath for 15 minutes and after cooling the absorbance at λ = 450 nm was measured. Distilled water was used as a blank sample.

Hydroperoxides

Hydroperoxides were measured by method of Ostdal [9] and expressed as an absorbance at λ = 500 nm, as measured on a Libra S6 spectrophotometer (Biochrom, Cambridge, England). 1 gram of whole milk powder was reconstituted in 10 cm^3 of distilled water and after reconstitution the method of Ostdal [9] was applied. 2 cm^3 of reconstituted milk were mixed with 2 cm^3 of methanol and 4 cm^3 of chloroform. Mixture was shaken 30 second and then centrifuged at 1500 xg for 10 minutes using HERMLE Z 300 K (Labortechnik, Wehinaen, Germany). 1 cm^3 of lower chloroform phase was taken and mixed with 1 cm^3 iron(II) thiocyanate solution. Iron(II) thiocyanate solution was prepared by mixing 250 mm^3 of solution I with 250 mm^3 of solution II and adding approximately 25 cm^3 of solution III to yield 25 cm^3 [solution I was prepared by mixing 0.8 % barium chloride dihydrate (Lachema, Brno) with 1 % $FeSO_4 \cdot 7H_2O$ (Lachema, Brno); the solution was filtered and the filtrate was used for the final solution; solution II was 30 % ammonium thiocyanate (Lachema, Brno); solution III was mixture of chloroform and methanol 1:1]. The reaction between chloroform and iron(II) thiocyanate solution run 5 minutes at room temperature and then the absorbance at λ = 500 nm was measured. Distilled water was used as a blank sample.

Extraction of fat by solution of chloroform and methanol

Extraction of fat from the sample was made by the mixture of chloroform and methanol (2:1). The sample was first homogenized with seventeenfold volume of chloroform and methanol for 3 minutes. Suspension was filtered through glass frit S_1 after homogenization. Filtrate was washed by twenty percent of distilled water and water phase was separated. Chloroform phase was washed by mixture of chloroform, methanol and water (3:48:47). Lower chloroform phase was taken to the flask and the phase was evaporated at temperature 37 ^oC.

Neutralization number

10.0000 grams of fat were dissolved in 50 cm^3 of a mixture of ethanol and ether (1:1). Then the mixture of ethanol, ether and oil was titrated by KOH (0.1 mol/dm^3) to turns colour to red [15].

$$k = \frac{a \cdot 5.611}{q}$$

a ... 0.1 mol/dm^3 KOH [cm^3],
q ... weight of fat [g].

Peroxide value

5.0000 grams of fat were dissolved in 50 cm^3 of mixture of acetic acid and chloroform (3:2). After dissolving 2 mm^3 of KI were added. This mixture reacted for 60 seconds and then 100 cm^3 of distilled water and 2 mm^3 of amyloid solution were added. This system was titrated by $Na_2S_2O_3$ (0.01 mol/dm^3) till discolouration. The blank sample, which did not contained fat, was made under the same conditions [15].

$$p = \frac{10 \cdot (b - a)}{q}$$

b ... 0.01 mol/dm^3 $Na_2S_2O_3$ [cm^3],
a ... 0.01 mol/dm^3 $Na_2S_2O_3$ for blank sample [cm^3],
q ... weight of fat [g].

Preparation of fatty acid methyl esters for gas chromatography analysis (GC-MS)

Methyl esters were prepared as described by Davídek [7]. Fat sample extracting from the sample was boiled with 0.5 N methanol solution of NaOH according to Table 1 for 10 minutes in the atmosphere of nitrogen. Then aliquot amount of 12–15 % methanol solution of BF_3 (amount according Table 1) was added and the solution was boiled for 2 minutes. Next, 5 cm^3 of heptane was added and the boiling was other 1 minute. 2 cm^3 of saturated solution of NaCl was added after boiling. Solution was decanted to separator funnel and 15 cm^3 of heptane and 40 cm^3 of saturated solution of NaCl were added. Lower heptane layer was removed and water solution of saturated NaCl was washed by other 15 cm^3 of heptane. Both heptane phases were united and dried by anhydrous Na_2SO_4. Fatty acid methyl esters were determined by gas chromatorgaphy with mass spectroscopy.

Table 1

Conditions for fatty acid methyl esters preparation

Weight of fat [mg]	Amount of NaOH [cm^3]	Amount of BF_3 [cm^3]
100–250	4	5
250–500	6	7
500–750	8	9
750–1000	10	12

Conjugated dienes

0.0250 grams of fat were dissolved in ethanol for UV in 25 cm^3 volumetric flask. Then the absorbance of UV light was measured with UV spectrometr as UV spectrum of dissolved fat.

Results and discussion

Oxidative changes of milk fat during storage under various conditions were examined. Whole roller dried milk was used as a sample. Storage was at temperature 37 ^{o}C and two valued of water activity (0.23 and 0.82). The sample turned dark brown during storage under water activity 0.82 thanks to products of Maillard reaction. The oxidative changes were examined as a content of hydroperoxides, TBARS, peroxide value, neutralization number, content of conjugated dienes and fatty acids composition. Gained results were compared with other similar studies. Initial chemical parameters can be seen from Table 3 and was measured immediately after manufacturing of sample.

Table 2

Initial content of fatty acids in sample without storage

Fatty acid	Content [%]
Caproic acid (C6:0)	1.170 ± 0.125
Caprylic acid (C8:0)	0.900 ± 0.206
Capric acid (C10:0)	2.380 ± 0.266
Lautic acid (C12:0)	3.310 ± 0.308
Myristic acid (C14:0)	12.570 ± 0.885
Palmitic acid (C16:0)	40.330 ± 1.113
Stearic acid (C18:0)	12.390 ± 0.494
Oleic acid (C18:1)	25.540 ± 0.771
Linoleic acid (C18:2)	1.950 ± 0.142

Table 3

Initial values of monitored parameters in sample without storage

Chemical parameters	Value
Hydroperoxides	0.131 ± 0.155
TBARS (ethanol solution)	0.398 ± 0.012
TBARS (water solution)	0.343 ± 0.006
Peroxide value [$\mu gO_2/g$]	1.580 ± 0.004
Neutralization number [mg KOH/g]	0.390 ± 0.0067
Conjugated dienes (absorbance)	0.681 ± 0.173

Hydroperoxides and peroxide value

The influence of water activity on stability of dried milk was main point of many studies of Ostadal [9], Celestino [14] and Hedegaard [13] and but the higher focus was devoted to protein composition of milk. Oxidative stability of dried milk under two different water activities is written in this paper.

As can be seen from Table 4 and 5, the content of hydroperoxides was rapidly increased in desiccators with A_w = 0.23. In sample stored in desicator with A_w = 0.82 has there were not detected any hydroperoxide probably because of the disintegration of present hydroperoxide to another oxidative products. Presented results show that under A_w = 0.82 the creation and disintegration of hydroperoxides is rapidly quick and, therefore, the content of hydroperoxides is immeasurable after 16 days of storage.

Table 4

Chemical parameters of sample after 16 days of storage*

Chemical parameters	Control	A_w 0.23	A_w 0.82
Hydroperoxides	0.101 ± 0.035^a	0.02 ± 0.007^b	no reaction^c
TBARS (ethanol solution)	0.576 ± 0.004^a	0.244 ± 0.005^b	0.282 ± 0.004^c
TBARS (water solution)	0.349 ± 0.002^a	0.355 ± 0.002^b	0.296 ± 0.005^c
Peroxide value [$\mu gO_2/g$]	3.014 ± 0.004^a	20.950 ± 0.004^b	19.160 ± 0.005^c
Neutralization number [mg KOH/g]	1.134 ± 0.001^a	3.180 ± 0.035^b	3.280 ± 0.007^c
Conjugated dienes (absorbance)	0.723 ± 0.007^a	0.924 ± 0.001^b	0.728 ± 0.018^c

* Chemical parameters are presented by mean ± standard deviation. Mean values having the same superscript letter in each line are not significantly different ($p \geq 0.05$).

Table 5

Chemical parameters of sample after 50 days of storage*

Chemical parameters	Control	A_w 0.23	A_w 0.82
Hydroperoxides	0.055 ± 0.017^a	0.135 ± 0.001^b	no reaction^c
TBARS (ethanol solution)	0.613 ± 0.008^a	0.208 ± 0.001^b	0.247 ± 0.008^c
TBARS (water solution)	0.437 ± 0.007^a	0.320 ± 0.015^b	0.359 ± 0.002^c
Peroxide value [$\mu gO_2/g$]	6.650 ± 0.012^a	49.550 ± 0.016^b	34.360 ± 0.005^c
Neutralization number [mg KOH/g]	3.765 ± 0.002^a	6.620 ± 0.008^b	6.220 ± 0.001^c
Conjugated dienes (absorbance)	1.001 ± 0.016^a	0.669 ± 0.003^b	0.567 ± 0.009^c

* Chemical parameters are presented by mean ± standard deviation. Mean values having the same superscript letter in each line are not significantly different ($p \geq 0.05$).

Content of hydroperoxides and peroxide value are in good correlation in spite of the fact that the method of detection of hydroperoxides cannot detect hydroperoxides under A_w = 0.82. This fact can be due to restricted possibilities of used method for detection of hydroperoxides according to Ostdal [9]. Determination of peroxide value is straight reaction between fat and reactionary chemicals and this can caused better detection of lower concentration of hydropexides than reaction according to Ostdal [9].

The results obtained for hydroperoxides content in this study correlate with available literature, eg with Celestino [14] and Hedegaard [13]. Celestino [14] and Hedegaard [13] examined only the influence of storage time but our paper includes the influence of water activity too. Celestino [14] used spray-dried whole milk as a sample. They found the rapid increasing tendency of hydroperoxides content during storage. While the creation of hydroperoxides has close contexture with ways of drying, subsequent fat

changes are influenced by storage time and storage conditions. Hadegaard [13] founded the same development of hydroperoxides content as is reported in this paper. Their paper indicated as a major factor influencing hydroperoxide content storage time. In general, storage time can be considered as a major factor influencing the formation of primary oxidative products. In studies of Celestino [14] and Hedegaard [13] was used spray dried whole milk as a sample while in this paper was used roller dried whole milk.

Changes of hydroperoxides content under water activity are clearer and quicker than those that proceed during storage without the influence of higher water activity. Content of hydroperoxides established as peroxide value and expressed as absorbance was used for comparison with hydroperoxide value. Peroxide value is shown as a better method for the detection of lower hydroperoxides concentration as can be seen from presented data. These data showed significant ($p \geq 0.05$) changes of fat under various A_w.

TBARS

Thiobarbituric acid reactive substances (TBARS) were measured according to King [10] and were expressed as absorbance at $\lambda = 450$ nm. Water and ethanol modifications were used for comparing the better detection of TBARS. Measuring of absorbance at $\lambda = 450$ nm was used because Patton and Kurts [11] and Jenings [12] found the more intensively absorbing yellow pigment at $\lambda = 450$ nm for dairy products.

The content of TBARS in milk powders stored under different A_w was significantly lower than control as can be seen from Table 4 and 5. This difference may be caused by fact that A_w has the influence only on the rise of primary oxidative product. The difference in TBARS content between A_w 0.23 and 0.82 are significant ($p \geq 0.05$) in spite of not very different values of absorbance. Though, TBARS detected under A_w = = 0.82 has higher value of absorbance than those under $A_w = 0.23$. This fact confirmed increasing oxidative changes of lipid in milk powder stored under higher water activity. The same results as for ethanol solution were found for water solution too.

Absorbance value of water solution is higher than ethanol because of different reaction conditions which are used for creation of yellow pigment. Reaction temperature 100 ^{o}C used for water modification can lead to higher absorbance value close to same content of TBARS.

Fatty acids composition and neutralization number

Fatty acids composition is in closed accordance with neutralization number. Neutralization number is an indicator of hydrolysis of triacylglycerols of milk powder fat. Increasing value of neutralization number is an indicator of triacylglycerols decline. Triacylglycerols hydrolysis is influenced by water activity as can be seen from Table 4 and 5. Moreover, there was found difference between two-tested A_w. The neutralization number of milk powder stored under both A_w was twice higher as control.

Content of fatty acids is changed with water activity as can be seen from Table 6. Content of oleic acid (C18:1) and linolenic acid (C18:2) dropped during storage and this changes are closed with increasing values of TBARS, hydroperoxides and decrease of

linolenic acid is oscillated with formation of conjugated dienes. Content of other fatty acids is increased because of triacylglycerols hydrolysis. Slight significant changes of fatty acids were found in comparison with control and milk powder stored under various A_w as can be seen from Table 6.

Table 6

Content of fatty acids after 50 days of storage [%]*

Fatty acid	Control	A_w 0.23	A_w 0.82
Caproic acid (C6:0)	1.591 ± 0.114^a	1.628 ± 0.142^a	1.370 ± 0.120^b
Caprylic acid (C8:0)	1.246 ± 0.144^a	1.279 ± 0.245^a	1.109 ± 0.227^a
Capric acid (C10:0)	3.277 ± 0.276^a	3.125 ± 0.324^a	2.817 ± 0.229^b
Lauric acid (C12:0)	4.606 ± 0.323^a	3.758 ± 0.417^b	4.232 ± 0.185^c
Myristic acid (C14:0)	15.198 ± 0.741^a	14.837 ± 1.246^a	15.338 ± 0.668^a
Palmitic acid (C16:0)	46.967 ± 0.590^a	51.077 ± 2.048^b	47.300 ± 0.700^a
Stearic acid (C18:0)	15.639 ± 0.479^a	13.039 ± 0.542^b	9.847 ± 0.460^c
Oleic acid (C18:1)	16.922 ± 0.834^a	15.010 ± 0.854^b	17.991 ± 0.623^c
Linoleic acid (C18:2)	0 ± 0^a	0 ± 0^a	0 ± 0^a

* Fatty acids are presented by mean ± standard deviation. Mean values having the same superscript letter in each line are not significantly different ($p \geq 0.05$).

Conjugated dienes

Conjugated dienes are formed immediately after peroxides. Conjugated dienes are formed from unsaturated fatty acids especially from linolenic acid (C18:2). Conjugated dienes absorb ultraviolet radiation strongly at $\lambda = 233$ nm. Thus oxidation is following by dissolving the lipid in a suitable organic solvent (ethanol for UV spectroscopy in our case) and measuring the change in its absorbance with UV-visible spectrophotometer.

Milk powder stored under higher Aw has higher content of conjugated dienes than control after 16 days of storage while conjugated dienes detect after 50 days of storage are lower for milk powder stored under higher A_w. These differences are due to the fact that conjugated dienes are later broken down into secondary products, which do not strongly absorb UV-visible light and this leads to a decrease in absorbance. Higher water activity accelerated the oxidative processes and this fact leads to differences between control and samples stored under A_w. Oxidative changes of milk powder stored under standard condition in refrigerator are slower and conjugated dienes in control sample are increased during time of our experiment.

Conclusions

Our research indicates that water activity has influence on oxidative changes of whole milk powder. Whole dried milk is ideal substrate for oxidative changes because of its high fat content. Changes of milk fat stability during storage under various water activities were measured. The oxidative changes were examined as a content of hydroperoxides, TBARS, peroxide value, neutralization number, content of conjugated dienes and fatty acids composition. The content of hydroperoxides, TBARS and fatty

acids especially unsaturated fatty acids (oleic acid and linoleic acid) had decreased during storage. Neutralization number and peroxide value had increased during storage. Significant changes of all monitored chemical parameters were found during 50 days of storage under various water activities.

Research about oxidative changes of milk powder stored under various temperature and water activities is in progress.

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ZMIANY OKSYDACYJNE W TŁUSZCZU MLEKA PROSZKOWANEGO PRZECHOWYWANEGO W RÓŻNYCH WARUNKACH

Abstrakt: Badano zmiany oksydacyjne powstające w tłuszczu mleka suszonego przechowywanego przez 50 dni w różnych warunkach. Próbki przygotowano z mleka o wskaźniku nierozpuszczalności 1.27 i zawartości tłuszczu 26,47 %. Badane mleko zostało wyprodukowane w zakładach YOG Bojkovice. Mleko przechowywano w eksykatorze w temperaturze 37 ^{o}C. Aktywność wody na poziomie 0,23 uzyskano dzięki zastosowaniu nasyconego roztworu octanu potasu, natomiast aktywność wody na poziomie 0,82 uzyskano, stosując nasycony roztwór bromku potasu. Proszek mleczny był przechowywany przez 50 dni. Próbki mleka przechowywane przy aktywności wody 0,82 przybrały kolor ciemnobrązowy w wyniku reakcji Maillarda. Zmiany oksydacyjne zostały zmierzone jako zawartość wodoronadtlenków, TBARS, zawartość nadtlenków, liczba zobojętnienia, zawartość dienów sprzężonych oraz skład kwasów tłuszczowych. Zawartość wodoronadtlenków, TBARS, kwasów tłuszczowych, a w szczególności nienasyconych kwasów tłuszczowych (kwas oleinowy, kwas linolowy) uległy zmniejszeniu w czasie przechowywania. Wartości liczby zobojętnienia i zawartości nadtlenków wzrosły w czasie przechowywania. Wszystkie parametry chemiczne uległy znacznym zmianom w czasie 50 dni przechowywania przy zróżnicowanej aktywności wody.

Słowa kluczowe: zmiany oksydacyjne, tłuszcz mleka, warunki przechowywania, aktywność wody, TBARS, wodoronadtlenki