Carotenoid Potential to Protect Cow's Milk Fat Against Oxidative Deterioration

U. Antone, V. Sterna, and J. Zagorska

Abstract—Milk from differently fed cows (supplemented with carotenoids from carrots or palm oil product Carotino CAF 100) was obtained in a conventional dairy farm to assess the carotenoid potential to protect milk fat against oxidation. The extracted anhydrous milk fat (AMF) was tested by peroxide value, and Rancimat tests. Temperature, and light stimulation for reaction acceleration was used. The oxidative stability enhancement by carotenoids was detected in peroxide value test – the strongest effect was observed in palm oil, following by carrot supplemented group, compared to control group, whose feed was unchanged. Rancimat accelerated oxidation test results did not show any superiority of the oxidative stability of the AMF samples from milk of the carotenoid-supplemented cow groups. The average oxidation stability of AMF dark-stored samples was 12.59 ± 0.294 h, and it was significantly ($p < 0.05$) higher than that of AMF light-affected samples, i.e. 2.60 ± 0.191 h.

Keywords—antioxidants, dairy products, forages, lipid aging, peroxide

I. INTRODUCTION

ONE of the basic chemical reactions that can occur in milk starting from the moment of harvesting the raw milk, and proceeding through the manufacture of dairy products until their storage in warehouses, on store shelves, and consumer storages, is the lipid oxidative deterioration, generally resulting in the decrease of sensory, and nutritional quality of food [1], [2]. Therefore the oxidative stability of the milk fat attainable as the result of balance between anti- and prooxidative processes, and components in the food system is important for the quality, and shelf life of milk, and a number of dairy products. Factors such as storage temperature, dissolved oxygen, unsaturation degree of fatty acids, content of antioxidants, and metal prooxidants, etc., have an impact on oxidation intensity [3] – [6]. It is essentially a free-radical chain reaction where unsaturated fatty acids are oxidized to form odorless, tasteless hydroperoxides which, on their part, are unstable, and constitute a degrade towards secondary oxidation products: various carbonyl compounds that, despite minute concentrations, can produce strong off-flavors of food products due to their flavor thresholds [1], [2].

In milk, the concentration of antioxidants as α-tocopherol, and carotenoids is considered important for the oxidative

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stability [7]. However, antioxidants do not reduce the ultimate degree of rancidity; rather they lengthen the induction period in rough proportion to their concentration [8]. It is known that carotenoids are ranked among the strongest natural antioxidants, due to their ability to deactivate reactive chemical species such as singlet oxygen, triplet photochemical sensitizers, and free radicals which would otherwise induce lipid peroxidation [9], [4], [2]. The most important feature that determines their oxidative, and antioxidative properties is the degree of conjugation [10]. The role of carotenoids, as singlet oxygen deactivators in milk, is of high importance. Regarding milk fat constituents, oleic acid, the average content of which in milk fat is 20 – 30 % of total fatty acids [11], is one of the most important reactants in lipid oxidation reactions catalyzed by singlet oxygen in milk [10]. Thus, despite the fact, that milk fat, compared to many other edible fats, has relatively low polyunsaturated fatty acid content, and high proportion of saturated fatty acids [12], it is nevertheless subjected to oxidative deterioration. Moreover, singlet oxygen affects proteins, amino acids, and DNA bases at much higher rates, than hydro peroxides produced from fatty acids [10]. One of the main protective areas of carotenoids is their counteraction against photosensitized reactions. This function is ensured thanks to their ability of quenching both, excited photosensitizers, and singlet oxygen at a diffusion-controlled rate without being consumed in the process, mainly by a harmless energy transfer physical quenching mechanism [10]. Thus, the defensive role of carotenoids in respect of lipids, as well as stability of other milk compounds, cannot be underestimated, as well as their significance in relation to human health through enhancement of nutritional value of milk, and dairy products.

β-Carotene (BC) is the predominant carotenoid in dairy production, comprising approximately 90 % of total carotenoid content [13], and possessing good antioxidative properties [14], [15]. Carotenoids appear in milk as the result of their ingestion by the cow. Usually carotenoid concentrations in milk reflect the nature, and quality of the forage used. They are abundant in plant material; however, the composition of carotenoids, and other natural antioxidant contents is subjected to seasonal variations in the cow feed. Not infrequently carotenoids are easily oxidized, and their concentrations decrease quickly during cow feed storage [16], [17]. Thus, to save, and improve the anti-oxidative potential of dairy products, it is important to avoid oxidative-antioxidative imbalance problems of the milk lipids occurring

due to natural antioxidant shortages in cow feed, and consequently in milk. Crude palm oil, and carrots are known as one of the richest sources of carotenoids containing 0.05 to 0.20 %, and 0.006 to 0.055 % carotenoids, respectively [18], [19], with mainly α-, and β-carotenes present, and having great potential as cow feed supplements providing natural antioxidants. The potential benefits of enrichment of cow diet, and the role of carotenoids in dairy have been investigated by different scientists, but their influence is not so clearly understood yet. For example, in the study of Havemose *et al.,* 2004 [4], higher levels of tocopherols, and BC, lutein, and zeaxanthin in milk did not prevent oxidation of polyunsaturated lipids, and a conjecture was expressed, that carotenoids could possibly delay protein oxidation. However in the further study of Havemose *et al.,* 2006 [20] the influence of carotenoids on protein oxidation was not observed. In the same way, a higher concentration of BC in milk from cows fed grass-clover silage during the study neither delayed, nor reduced the accumulation of lipid peroxides. The authors concluded that the role of α-tocopherol, and β-carotene on the oxidative stability of milk appeared to be less important than the variation in the fatty acid profile. *Our aim in the present study was to investigate the influence on milk fat oxidation stability of cow feed enriched with carotenoids from carrots, and palm oil.*

II. Materials and Methods

A. Experimental Design

Feed of different carotenoid concentrations was administered to 3 groups of cows – one control group (CG), and 2 trial groups (TG1, and TG2) of 5 cows in each that were selected in a conventional dairy farm in Latvia. The selection of the Latvian Brown breed cows for the three groups followed the same pattern: cows with average stage of lactation 4.6 months, and average lactation number 2.3 were selected. Feed supplementation was implemented at the end of the indoor period (April). The basic feed was equal in all groups, i.e. haylage – ad libitum, mixed feed concentrate – 2 kg, hay – 2 kg per cow per day. The amounts of the supplemental cow feed, and the content of total carotenes, and vitamin E in each group's feed are provided in Table I.

B. Milk Sample Collection, and Storage

Individual cow milk samples were obtained from the morning milking on day 25 after the beginning of feed supplementation. Equal amounts of each group's milk (5 l) were pooled together acquiring 1 bulk milk sample per group. After collection, milk samples were transported to the laboratory, where milk was used for the extraction of milk fat.

C. Milk Fat Extraction, and Storage

For better milk fat extraction, milk was warmed up to 40 – 45 °C temperature subsequently separating cream with a conventional milk separator to approx. 30 % fat content.

Cream was placed in the refrigerator for ripening (4 – 6 °C, 20 ± 1 h). After that, cream was churned till formation of pats of butter. The buttermilk was removed, and butter was washed with cold (20 °C) distilled water for 5 times. Subsequently, butter was warmed up to 40 – 50 °C, and centrifuged 13000 rpm, 10 minutes at 40 °C to separate the clear anhydrous milk fat (AMF) layer that had collected into glass beaker. The AMF was carefully split into smaller sub-

TABLE I
COW FEED COMPOSITION

Cow groups	Basic feed, per cow per day	Supplemental feed, per cow per day	Total carotenes	Vitamin E
			mg per cow per day	
TG1		Carrots – 7 kg	387	80
TG2	Haylage – ad libitum, mixed feed concentrate – 2 kg, hay – 2 kg	CAF 100[a] – 400 g	292	200
CG		–	242	80

[a] Animal Feed (Carotino SDN. BHD, J.C. Chang Group, Malaysia) – light-orange colored powder, containing > 99 % palm stearin, rich in carotenes and vitamin E (approximately 120, and 300 ppm, respectively).

samples:

1) For Peroxide value (PV) analyses, AMF sub-samples (20 g) were poured into appropriate number of transparent plastic one-way Petri plates. Thanks to our previous observations of high oxidative stability of milk fat, the light influence was used to hasten the fat aging, i.e. half of the AMF sub-samples were subjected to direct sunlight action for 3 hours at room temperature, while other samples were stored refrigerated at temperature of 4-6 °C in dark. After that light-affected sub-samples, and stored in dark sub-samples were placed into oven at 60 °C temperature;

2) For Rancimat test, AMF subsamples (20 g) were placed into transparent glass containers of 25 ml. Half of the sub-samples were subjected to direct sunlight action for 3 hours at room temperature to hasten the oxidation reactions, while other samples were stored refrigerated at temperature of 4 – 6 °C in dark. After that all samples were stored frozen at temperature of – 20 °C until the start of testing (no longer than 1 month).

D. Peroxide Value (PV) Measurements of the Anhydrous Milk Fat

The AMF dark-stored samples were tested for PV several times within a 25-day period after sample preparation. The AMF light-affected samples were tested for PV several times within a 19-day period after sample preparation. The PV tests were carried out in accordance with iodometric titration method described in [21]. For analysis 1 g of the fat was put in a 100 ml glass flask, and mixed with 6 ml solution, containing chloroform, and glacial acetic acid (2:1). Parallelly blank analysis without fat sample was performed.

Than 1 ml of saturated potassium iodide solution, and 30 ml of distilled water were added. After that flasks were sealed, and carefully shaken for exactly 3 min. Next, 3 – 5 drops of starch solution (1 %) to the reaction mixture were added, followed by the titration against 0.01 M sodium thiosulphate solution. As it is important for the measurements of PV, all reagents were of the highest analytical purity. Equation (1) was used for the calculation of PV:

$$PV = (V_0 - V) \cdot 0.00127 \cdot 100 / m, \qquad (1)$$

Where:

PV – The peroxide value, expressed as % of iodine utilized for the reduction of 100 g fat;

(V_0-V) - The difference between a blank titration volume, and the titration with the fat, ml;

0.00127 – The iodine weight that corresponds to 1 ml of 0.01 M sodium thiosulphate solution, g;

100 – The conversion factor to 100 g amount of fat;

m – The mass of the fat sample, g.

The PV analyses were carried out in the Research Laboratory of Biochemistry and Microbiology of the Research Institute of Biotechnology and Veterinary Medicine "Sigra" of the Latvia University of Agriculture.

E. Rancimat Oxidation Stability Test of the Anhydrous Milk Fat

The Rancimat accelerated aging, and oxidation stability test is a conductometric determination of volatile acid dissociation products (mainly formic, and acetic acid) produced during oxidation, and was chosen as an alternative method [22] consuming less time, and chemicals, compared to PV method, still performed in more rough conditions using Rancimat equipment (Methrom-Herisau AG, Switzerland, model 743). Heating block was hold at 110 °C. Air flow blown through liquid butter oil was adjusted at 10 l/h. Before testing, the frozen samples of AMF were thawed in 30-35 °C temperature, and 3 g of AMF were taken for analysis. The Rancimat oxidative aging test was carried out in the Research Laboratory for Fuel Quality Control of the Institute of Applied Chemistry of the Riga Technical University (Latvia).

F. Statistical Analyses

The results were calculated, analyzed, and graphs were made using MS Office program Excel or Microsoft Windows for SPSS. Data are presented as means ± confidence (95 %) interval. Differences between groups were tested for significance ($p < 0.05$) by ANOVA (SPSS 17.0, SPSS Inc. Chicago, Illinois, USA).

III. RESULTS AND DISCUSSION

A. Milk Lipid Stability as Measured by PV Changes

The observed oxidative stability of anhydrous milk fat stored at the temperature of 60 °C, and measured by PV was relatively high, especially when there was no contact of AMF with light (Fig. 1).

In the case of AMF dark-stored samples within 25 day period only the induction period was seen (Fig. 1), and fast oxidative changes were not observed, also known as the exponential or active phase of peroxidation - the second oxidation period, as described in [23]. PV did not exceed 0.08 %, what is normally for fresh fat accordingly with [21].This proves that lacking light, and other prooxidants milk fat is rather stable against oxidation. There were no significant differences among 3 groups' PV growth intensity when represented as exponential function trend lines ($p > 0.05$).
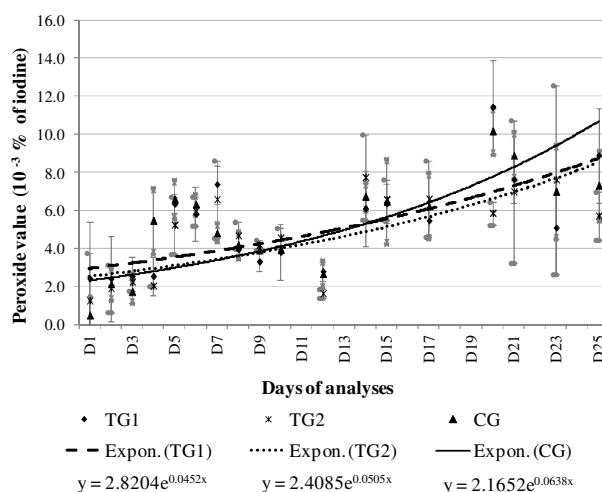

Fig. 1 Peroxide value changes of 3-type dark-stored anhydrous milk fat at 60 °C temperature during 25 day period

In case of fat samples affected by light, on the other hand, much faster oxidation was observed. As seen from Fig. 2, the course of fat oxidation is marked by an induction period of slow oxygen uptake (while the level of initiating free radicals is built up) followed by the period of rapid oxidation, as was expected according to literature [8] statements. Comparing the trend line exponential function coefficients of the PV measurements of 3-type fat samples, as seen from Fig. 2, the oxidative deterioration was more intense for AMF light-affected fat samples obtained from milk of the CG. The impact of antioxidant protection trough the influence of cow's diet, i.e., its antioxidant content, was evident. The exponential coefficient of CG was higher (0.341), compared to coefficients 0.250, and 0.233 for TG1, and TG2, respectively. The strongest defense effect against lipid oxidation was observed in TG2 group where the cow feed was enriched with palm oil supplement. This possibly occurred due to the increased tocopherol concentration in CAF 100, and further in milk, as well as to presumably easier absorption, and assimilation of carotenoids from this supplement in cow's body, compared to that from crude carrots, but this needs to be studied further.

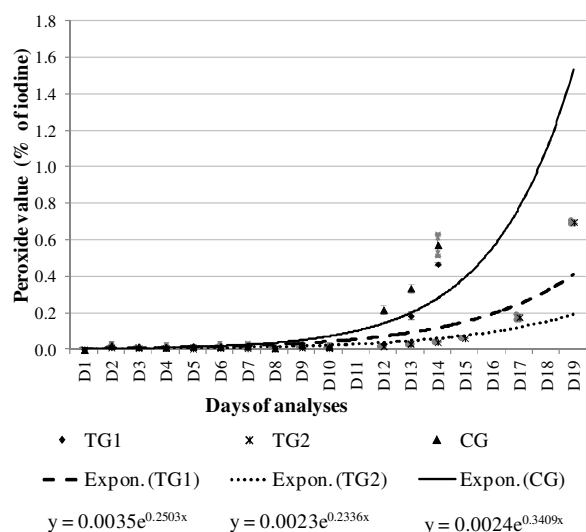$$y = 0.0035e^{0.2503x} \qquad y = 0.0023e^{0.2336x} \qquad y = 0.0024e^{0.3409x}$$

Fig. 2 Peroxide value changes of 3-type light-affected anhydrous milk fat at 60 °C temperature during 19 day period

The improved oxidative stability of the TG2 (palm oil supplemented group's) AMF light-affected samples can be explained not only by the relatively higher tocopherol content in TG2 group's feed, but also by further synergism between carotenoids, and tocopherols in lipid protection, the mechanism of which in a more detailed way is described in [10]. Here we can also notice the other benefits of CAF 100 – the feed supplementation was simpler thanks to easier dosage, storage, and longer shelf life, compared to carrots.

Still, the improved oxidative stability of the TG1 (carrot supplemented group's) AMF light-affected samples, compared to CG, is approvable as well. The advantage of carrots as a carotenoid source lies in their lower price, and better availability as locally grown vegetable products, however their supply from many local farms during the time of this trial (the end of spring) was problematic, their preparation (washing, and cutting) was more time, and resource-consuming, besides, they are more prone to decay.

B. Milk Lipid Stability as Measured by Rancimat Test

Accelerated tests are frequently used in food industry to get more information on changes in the oxidative status of the sample in the future [23]. However, they can not fully imitate the real circumstances of the fat storage or usage, and the advantage of short analysis time should not dominate over time, and effort consuming testing in more realistic circumstances.

The comparison of the results of oxidation stability of dark-stored, and light-affected 3-type anhydrous milk fat samples is given in Fig. 3. The average oxidation stability of AMF dark-stored samples from the three groups was 12.59 ± 0.294 h, and it was significantly ($p < 0.05$) higher than that of AMF light-affected samples, i.e. 2.60 ± 0.191 h. This assures the

notable influence of the light action on lipid deterioration processes. Considerably longer Rancimat (oxidized at 110 °C, air flow 20 l/h) induction period of conventional "extra" (82 % of fat) summer butter – 21.23 ± 0.07 h, and of the same brand winter butter – 26.53 ± 0.04 h, was observed in the study of Gramza-Michalowska et al. [24] that can be related with other unknown antioxidative, and prooxidative factors related to AMF or butter composition or analytical conditions.

Rancimat accelerated oxidation test results did not show any superiority of the oxidative stability of the AMF samples from milk of the carotenoid-supplemented cow groups. Significant differences of oxidation stability among 3 groups' AMF dark-stored or among 3 groups' AMF light-affected samples were not established ($p > 0.05$). Thus we can make a conclusion that in these rather severe test conditions carotenoids could have lost their protective potential to hinder oxidative deterioration. It can be explained by the loss of antioxidant activity of carotenoids due to their instability in high temperatures, and in contact with abundance of air. The average oxidative stability was insignificantly ($p > 0.05$) higher of both – AMF dark-stored, and light-affected samples from control group, compared to samples obtained from trial groups' milk. This slight tendency can be related to the statement of [9] that under high pressure of oxygen carotenoids can express a prooxidative behavior that could very likely happen in case with Rancimat aging procedure due to the impact of air flow blown through liquid butter oil (10 l/h). As explained in [10] carotenoids may be stimulated by heat, and / or mechanical agitation. As a result of stimulation, they undergo isomerization, and oxidative destruction, and may act as prooxidants for co-existing lipids.
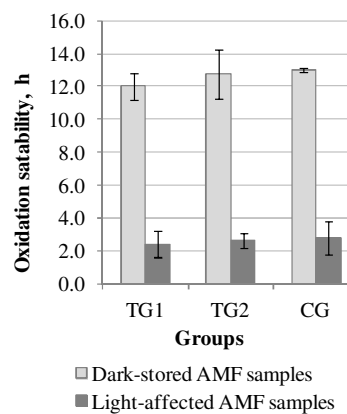

Fig. 3 Rancimat oxidation stability of 3-type dark-stored, and light-affected anhydrous milk fat samples

Since as there can occur many different chemical reactions, due to the influence of severe temperatures, oxygen presence or a lot of other factors, such as the presence of carotenoids, and other compounds, which can change their behavior becoming prooxidative, we can conclude that testing procedures for the determination of oxidative stability of fats,

and oils should be chosen carefully, and accelerated data should always be interpreted cautiously. The Rancimat test results obtained in our investigation yet can give a rough insight in fat deterioration processes in elevated temperatures, and aerated conditions, as, for example, in the production of dry milk powder or in the frying processes.

IV. CONCLUSION

The oxidative stability enhancement of milk fat, by carotenoid supplementation of cow's feed in this study was observed. This possibly is only one issue of the wide field of research related to natural antioxidant protective role, and one benefit of the spectrum of other possible milk quality enhancements, thanks to its potential in cow health strengthening. This is particularly important in milk production nowadays when the dairy farm management intensity is growing incredibly, and when the efforts of cow milk composition changes forward more unsaturated fat is made, influencing milk, and dairy product nutritional, and storage stability features.

ACKNOWLEDGMENTS

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