Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Dairy Journal 14 (2004) 563–570
INTERNATIONAL DAIRY JOURNAL
www.elsevier.com/locate/idairyj

Influence of feeding different types of roughage on the oxidative stability of milk

M.S. Havemose[a], Martin Riis Weisbjerg[a], Wender L.P. Bredie[b], J.H. Nielsen[a,*]

[a] Research Centre Foulum, Department of Food Science, Danish Institute of Agricultural Sciences, PO Box 50, 8830 Tjele, Denmark
[b] Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

Received 30 June 2003; accepted 12 November 2003

Abstract

The variation in susceptibility to light-induced lipid and protein oxidation was studied in relation to the concentration of riboflavin, fatty acid composition, α-, γ-, and δ-tocopherol and β-carotene, lutein and zeaxanthine in milk from cows fed diets with roughage containing either grass silage or corn silage mixed with concentrates. The results show that milk from cows fed grass silage had a higher concentration of the antioxidants measured. The contents of unsaturated C18 fatty acids were significantly different in the two types of milk, showing that the compositions of antioxidants and fatty acids were influenced by the feeding. Despite a higher antioxidative capacity in the milk from cows fed grass silage, lipid oxidation was higher in this milk compared to milk from cows fed corn silage. The results indicate that quenching of singlet oxygen by the antioxidants did not prevent lipid oxidation. The degree of unsaturation in linolenic acid, C18:3, is proposed to be important for the higher accumulation of lipid hydroperoxides in milk from cows fed grass silage. In contrast to milk from cows fed grass silage being more vulnerable to lipid oxidation, milk from cows fed corn silage is more vulnerable to protein oxidation. The results indicate that protein oxidation occurs independently of lipid oxidation. It is concluded that the higher concentration of antioxidants in milk from cows fed grass silage is able to extend the lag phase of protein oxidation and delay the formation of dityrosine.

Keywords: Lipid oxidation; Protein oxidation; Grass silage; Corn silage; Light; Tocopherols; Carotenoids

1. Introduction

The oxidative stability of milk and dairy products is of concern to the dairy industry. The oxidation processes in milk can result in strong off-flavors and in deterioration of the nutritional quality of milk, making the oxidized milk unacceptable to consumers (Dunckley, Frankel, & Pangborn, 1962; Dimick & Kilara, 1983; Marsili, 1999). The shelf-life of milk and dairy products is the result of a delicate balance between the anti- and pro-oxidative processes in milk influenced by factors such as degree of unsaturation, content of transition metal ions, content of antioxidants as tocopherols and carotenoids (Barrefors, Granelli, Appelqvist, & Bjoerck, 1995; Morales, Palmquist, & Weiss, 2000). To a certain extent the composition of milk reflects the composition of the feed given to the dairy cows (Bugaud, Buchin, Coulon, Hauwuy, & Dupont, 2001; Ramaswamy et al., 2001). Consequently, certain feeding regimes can increase the content of polyunsaturated lipids and make the milk more vulnerable to oxidation (Barrefors, Granelli, Appelqvist, & Bjoerck, 1995; Charmley & Nicholson, 1995; Hermansen, 1995; Focant et al., 1998; Morales, Palmquist, & Weiss, 2000; Bugaud et al., 2001; Timmons, Weiss, Palmquist, & Harper, 2001). Moreover, numerous studies have shown that antioxidants as tocopherols and carotenoids can be transferred from the feed to the milk and thereby improve the oxidative stability of milk (Barrefors, Granelli, Appelqvist, & Bjoerck, 1995; Focant et al., 1998; Granelli, Barrefors, Bjoerck, & Appelqvist, 1998). Other studies have shown no effect of supplementing the diet with vitamin E (Schingoethe, Parsons, Ludens, Schaffer, & Shave, 1979; Charmley & Nicholson, 1995).

Exposure of milk to light can take place at several stages from milking to the consumers and initiate the sensibilization of riboflavin and result in oxidation of milk (Patton, 1953; Allen & Parks, 1975; Jung, Yoon, Lee, & Min, 1998). The light-activated oxidation

*Corresponding author. Tel.: +45-8999-1163; fax: +45-8999-1564.
E-mail address: jacobh.nielsen@agrsci.dk (J.H. Nielsen).

0958-6946/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.idairyj.2003.11.005

products result in very pronounced off-flavors, which have an adverse effect on consumer acceptance (Patton, 1953; Allen & Parks, 1975; Hansen, Turner, & Aurand, 1975; Dimick & Kilara, 1983; Kim & Morr, 1996). The light-induced off-flavors that become noticeable relatively early are derived from protein oxidation while later in the oxidation progress off-flavors derived from lipids become dominating (Dunckley, Frankel, & Pangborn, 1962; Dimick & Kilara, 1983; Marsili, 1999).

Factors influencing the potential for oxidized flavor development can be manipulated by changing the cow's diet. The aim of the present investigation was to study the influence of feeding grass silage and corn silage on the oxidative stability of milk. The antioxidative capacity of the two types of milk was determined initially along with the fatty acid composition and the total fat and protein content.

The two types of milk were exposed to light for 24 h; the accumulation of lipid hydroperoxides was used as a marker for lipid oxidation, and the accumulation of dityrosine was used as a marker for protein oxidation. The degradation of riboflavin, α-tocopherol and β-carotene was determined during light exposure. The antioxidative effect of the two types of milk on lipid oxidation and protein oxidation was evaluated.

2. Materials and methods

2.1. Milk samples

Morning milk was collected from four Holstein cows fed roughage of grass silage (67% grass and 33% clover) mixed with concentrates and from four Holstein cows fed roughage of corn silage mixed with concentrates. Total mixed rations (TMRs) were fed ad libitum. Grass silage TMR was composed of (% of dry matter) 67.8% grass silage, 21.0% barley, 11.0% soybean meal and 0.2% mineral mixture. Corn silage TMR was composed of (% of dry matter) 67.4% corn silage, 19.6% barley, 10.2% soybean meal, 1.0% urea, 0.9% mineral mixture, 0.6% lime and 0.3% sodium sulfate.

The study was designed as a triple crossover, where the cows were fed the rations for 4 weeks before the samples were taken. Milk from the four cows fed the same ration was mixed and pasteurized at 72°C for 15 s. Milk from cows fed grass silage contained 4.9% fat, 3.9% protein, 4.7% lactose, 0.16% citric acid, and the average yield was 25.0 kg per day. Milk from cows fed corn silage contained 4.7% fat, 3.7% protein, 4.7% lactose, 0.18% citric acid, and the average yield was 25.3 kg per day.

Milk samples were stored at −20°C until analysis for riboflavin, fatty acids, lipid hydroperoxides, dityrosine, α-, γ-, and δ-tocopherol and β-carotene, lutein and zeaxanthine.

2.2. Exposure of milk to fluorescent light

The milk samples were transferred to glass tubes. The glass tubes were placed on a rotating device in a refrigerator kept at a temperature of 4°C. A fluorescent light (Philips TLD 18W) was placed above the rotating device.

2.3. Determination of riboflavin

Milk samples (5 mL) were mixed with 0.5 mL of 2 M sodium acetate and 1.5 mL of 2 M acetic acid. The samples were slowly agitated for 5 min before centrifugation at 1500*g* for 10 min. The supernatant was filtered through a 0.45 μm filter and the fluorescence was read (excitation: 445 nm; emission: 520 nm) using a Perkin-Elmer LS 50B fluorescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, Bucks, UK). All analytical procedures were conducted using glassware wrapped in aluminum foil to avoid light exposure resulting in additional riboflavin degradation during sample preparation.

2.4. Analysis of fatty acid composition

Prior to GC separation and quantification, the lipid was extracted by chloroform:methanol (2:1 v v^{-1}) twice and the extracted lipid was transesterified to methyl esters in a sodium methylate solution (2 g L^{-1} methanol). Analysis of fatty acid methyl esters was carried out with a GC (Agilent Technologies, Palo Alto, CA) using an FFAP column (polyethylene glycol TPA 25 m × 200 μm × 0.30 μm, Agilent Technologies, Palo Alto, CA) and helium as the carrier gas and a flame ionization detector. Injection was splitless with an injector temperature of 250°C. The detector temperature was 300°C. The initial column temperature was 40°C, which was held for 4 min. The temperature was then raised at 10°C min^{-1} to 240°C, which was held for 1 min. Quantification was based on area of the individual fatty acid peaks and given as percentages of the total peak area for the selected fatty acids.

2.5. Determination of lipid hydroxyperoxides

Milk samples (2 mL) were mixed with 2 mL methanol. Chloroform (4 mL) was added and the samples were mixed for 30 s before centrifugation at 1500*g* for 10 min. The precipitate (1 mL) was added to 1 mL of iron-II/thiocyanate solution (Solution I: 0.4 g barium chloride, dihydrate in 50 mL water is mixed with 0.5 g iron-II sulfate, heptahydrate in 50 mL water, the solution is filtered and the filtrate is used. Solution II: 3 g ammonium thiocyanate in 10 mL water. Solution III: 50 mL chloroform mixed with 50 mL methanol. Iron-II/thiocyanate solution: 250 μL solution I and 250 μL

solution II made up to 25 mL with solution III). The samples were left to react for 5 min at room temperature and were measured spectrophotometrically (HP 8453 UV–Visible Spectrophotometer, Agilent Technologies, Palo Alto, CA) at 500 nm.

2.6. Dityrosine determination

Milk samples (3 mL) were mixed with 3 mL 0.1 M sulfuric acid in 1 M sodium chloride and 12 mL 2-propanol. The samples were shaken for 3 min, 9 mL pentane was added, and the samples were shaken for an additional 3 min. Samples were precipitated by centrifugation (1500*g* for 5 min). The supernatant was discharged, and the extraction procedure was repeated with 1 mL 0.1 M sulfuric acid in 1 M sodium chloride, 12 mL 2-propanol and 9 mL pentane. The pellet was redissolved in 4 mL of 50 mM phosphate buffer (pH 7.4; $I = 0.16$). The protein was precipitated by adding trichloroacetic acid to a final concentration of 10%. The samples were allowed to stand for 10 min and 4 mL of ethanol was added before centrifugation (1500*g* for 10 min). The pellet was washed with 4 mL of 1 M hydrochloric acid before an additional centrifugation (1500*g* for 10 min). The pellet was mixed with 1 mL of 6 M hydrochloric acid, and flushed with argon and hydrolyzed overnight. Samples were neutralized with 6 M sodium hydroxide. The hydrolyzed sample (20 μL) was injected onto an HPLC column (Microsorb 100-5 C-18, 250 × 4.6, Varian, Walnut Creek, CA), which was equilibrated with 4% acetonitrile in aqueous 0.10 M citric acid (pH 2.55), with a flow of 1 mL min^{-1}. Chromatographic separation was performed on an HPLC system consisting of a Varian 9012 HPLC pump connected to a Varian 9100 autosampler and a Varian 9075 fluorescence detector (ex = 284 nm, em = 410 nm) (Varian Chromatographic Systems, Walnut Creek, CA). Samples were spiked with a dityrosine standard for identification and quantified by the use of a standard curve made from the same standard.

2.7. Analysis of α-, γ-, and δ-tocopherol

Milk samples (2 mL) were mixed for 10 s with 2 mL 1% ethanolic ascorbic acid. Saturated potassium hydroxide solution (0.3 mL) was added and mixed for 10 s, and the solution was placed in a heating chamber at 70°C for 60 min and subsequently cooled in an ice water bath. Water (1 mL) and heptane (3 mL) were added and mixed for 1 min. The solution was centrifuged for 3 min at 1700*g*, the supernatant was filtered, and aliquots of 30 μL were injected on an HPLC system (HP 1100, Agilent Technologies, Palo Alto, CA) with fluorescence detection (ex = 295 nm, em = 330 nm). Mobile phase: 98% hexane mixed with 2% 2-propanol, flow 0.6 mL min^{-1}. Column: Hypersil SI (Agilent Technologies, Palo Alto, CA). Samples were quantified using external standard curves.

2.8. Determination of β-carotene, lutein and zeaxanthine

Milk samples (2 mL) were added to 2 mL 1% ethanolic butylhydroxytoluene and mixed for 10 s. Saturated potassium hydroxide solution (0.5 mL) was added and mixed for 10 s. The headspace above the samples was replaced with nitrogen, and the samples were placed in a heating chamber at 70°C for 60 min. The samples were subsequently cooled in an ice water bath. The samples were added to 1 mL water and 3 mL of heptane:dichloromethane (90:10 v v^{-1}) and centrifuged for 3 min at 1700*g*. The extraction was repeated three times, and the supernatants were collected. The extracts were evaporated under nitrogen flow at 45°C until dryness and redissolved in a mixture of acetonitrile:methanol:dichloromethane:triethylamine (85:10:5:0.5 v v^{-1}). Aliquots of 100 μL were injected onto an HPLC system (HP 1100, Agilent Technologies, Palo Alto, CA) with DAD (diode array detector) detection at 448 nm (lutein) and 455 nm (β-carotene and zeaxanthine). The gradient flow was 1.6 mL min^{-1} for the first 6 min then increased to 2.2 mL min^{-1} to a final runtime of 22 min. The primary column was a Vydac Protein Peptide column (The Separations Group, Inc., Hesperia, CA), and the secondary column was a Hypersil ODS (octadecyl silane) column (Agilent Technologies, Palo Alto, CA). The mobile phase was similar to the solution used to redissolve the extract. Samples were quantified using external standards.

2.9. Statistical analyses

Two-tailed *t*-tests for paired observations were performed.

3. Results

The concentrations of antioxidants of the two types of milk were examined by measuring the concentration of α-, γ- and δ-tocopherol, β-carotene, and the xanthophylls lutein and zeaxanthine. Table 1 illustrates the concentrations of typical antioxidants in milk from cows fed grass silage and corn silage, respectively, when the milk was not exposed to fluorescent light. Milk from cows fed grass silage had a higher concentration of all the measured antioxidants than the milk from cows fed corn silage. The concentration of α-tocopherol in milk from cows fed grass silage was more than two-fold higher than the concentration of α-tocopherol in milk from cows fed corn silage. The same trend was seen for the concentration of γ-tocopherol. β-Carotene, lutein and zeaxanthine are expected to have antioxidant

Table 1
Concentrations of antioxidants in milk, which are not exposed to fluorescent light, from cows fed grass silage and corn silage

Antioxidant ($\mu g\,L^{-1}$)	Grass silage	Corn silage
α-Tocopherol	854±31	375±12
γ-Tocopherol	31±4	11±3
δ-Tocopherol	2±1	1±1
β-Carotene	697±76	223±25
Lutein	19±2	3±1
Zeaxanthine	4±1	1±0

Note: Results are means±standard deviation of three independent experiments.

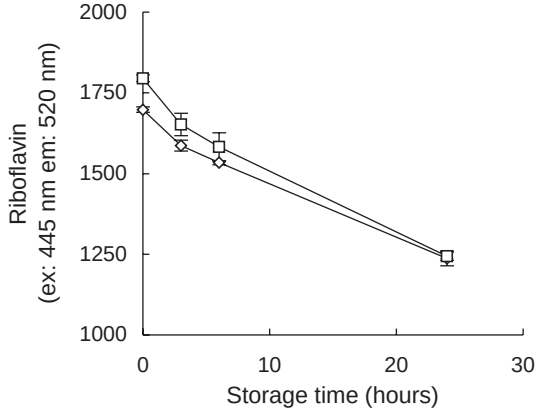

Fig. 1. Decay of riboflavin as a function of the time of exposure to fluorescent light (2000 lux) at 4°C. Means±standard deviation of three independent experiments. ◇ grass silage, □ corn silage.

properties. The concentration of β-carotene in milk from cows fed grass silage is three-fold higher than the concentration in milk from cows fed corn silage, while the concentrations of zeaxanthine and lutein were three- and six-fold higher, respectively, in milk from cows fed grass silage than the concentration in milk from cows fed corn silage.

During storage of milk in light, riboflavin acts as a photosensitizer able to initiate oxidation. The excited sensitizer can initiate oxidation either through direct reaction of the excited riboflavin with the substrate (lipids, proteins or antioxidants) (Type I reaction), or through reaction with oxygen giving rise to the active singlet oxygen (Type II reaction). During the Type II reaction some of the singlet oxygen will not escape from the riboflavin but react with riboflavin and degrade the sensitizer. Fig. 1 shows the decay of riboflavin as a function of storage time where the milk was exposed to fluorescent light. The riboflavin in the two types of milk follows the same pattern of degradation. The concentration of riboflavin at the start of the incubation was slightly higher in milk from cows fed corn silage, but after 24 h of light exposure there were no significant differences between the concentration of riboflavin in the two types of milk. These results clearly indicate that the activation and degradation of riboflavin were similar in both types of milk.

Table 2
Fatty acid compositions for milk from cows fed grass silage and corn silage

	Grass silage	Corn silage
C4	4.0±0.4	3.8±0.2
C6	1.9±0.1	1.8±0.1
C8	1.5±0.1	1.3±0.1
C10	4.0±0.4	3.4±0.1
C12	5.1±0.5	4.2±0.1
C14	14.0±0.7	13.5±0.6
C16	42.4±2.2	40.8±1.2
C16:1	2.3±0.1	2.3±0.1
C18:0	7.6±0.6	8.1±0.3
C18:1*	14.8±0.3	18.6± 0.5
C18:2*	1.9±0.1	2.1±0.1
C18:3*	0.7±0.0	0.2±0.0

*Significant differences, $P<0.05$.
Note: Results are means±standard deviation of three independent experiments.
The unit is given as percentages of the total peak area for all the fatty acids listed in Table 2.

The fatty acid composition, and the concentration of unsaturated fatty acids in particular, influences the susceptibility to lipid oxidation (Barrefors, Granelli, Appelqvist, & Bjoerck, 1995; Timmons, Weiss, Palmquist, & Harper, 2001). Table 2 shows that milk from cows fed grass silage has significantly lower ratios of C18:1 and C18:2, $P<0.05$, whereas it has significantly higher ratios of C18:3, $P<0.05$, than milk from cows fed corn silage. Quantitatively, C18:1 acid was much higher in milk from cows fed corn silage, but the relatively small, but higher level of C18:3 in milk from cows fed grass silage, may have a greater impact on the formation of lipid derived oxidation products. No other significant differences between the other fatty acids in the two types of milk were detected.

In order to follow oxidation in milk produced by dairy cows fed grass silage or corn silage accumulation of typical lipid and protein oxidation products was followed in milk stored at 4°C and simultaneously exposed to fluorescent light.

The concentration of lipid hydroperoxides in milk was used as a measure of the light-induced oxidative stability of lipids in the milk. Fig. 2 shows the development of lipid hydroperoxides in the two types of milk as a consequence of oxidation during storage at 4°C for 24 h while exposed to fluorescent light. The development of lipid hydroperoxides in the two types of milk tends to follow two different patterns. The development of lipid hydroperoxides in milk from cows fed grass silage showed a steep increase for the latter 4 h of storage followed by a lower accumulation of lipid hydroperoxides for the latter 20 h of storage. Accumulation of

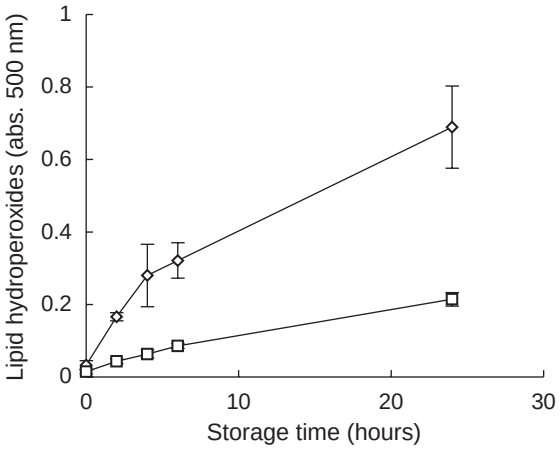

Fig. 2. Accumulation of lipid hydroperoxides as a function of the time of exposure to fluorescent light (2000 lux) at 4°C. Means ± standard deviation of three independent experiments. ◇ grass silage, □ corn silage.

lipid hydroperoxides in milk from cows fed corn silage was not as extreme and seemed more linear than the accumulation of lipid hydroperoxides in milk from cows fed grass silage. The result clearly shows that more lipid hydroperoxides are accumulated in milk from cows fed grass silage than in milk from cows fed corn silage throughout the storage period.

The concentration of dityrosine was used as a marker for light-induced oxidation products of proteins in the milk. The data in Fig. 3 show the accumulation of dityrosine in the two types of milk stored at 4°C and exposed to fluorescent light. The accumulation of dityrosine was similar in the two types of milk for the first 6 h of storage. After 24 h, the concentration of dityrosine was significantly higher in milk from cows fed corn silage than in milk from cows fed grass silage.

The concentrations of α-tocopherol and β-carotene in the two types of milk were followed during exposure to fluorescent light to examine the degradation of the antioxidants as a measure of the interaction of the antioxidants in the oxidative process. The results in Fig. 4 show that the initial concentration of α-tocopherol in milk from cows fed grass silage was two-fold the concentration found in milk from cows fed corn silage. After 24 h of light exposure, the level of α-tocopherol in both types of milk had significantly decreased ($P<0.01$). However, the degradation of α-tocopherol was found to be more pronounced in the milk from cows fed grass silage than the degradation of α-tocopherol in the milk from cows fed corn silage. Despite the more pronounced degradation of α-tocopherol in the milk from cows fed grass silage there were still significant ($P<0.02$) differences in the concentrations of α-tocopherol in the two different types of milk after 24 h of storage.

The other important antioxidant in relation to light-induced oxidation is β-carotene. Fig. 5 shows that the initial concentration of β-carotene was three-fold higher

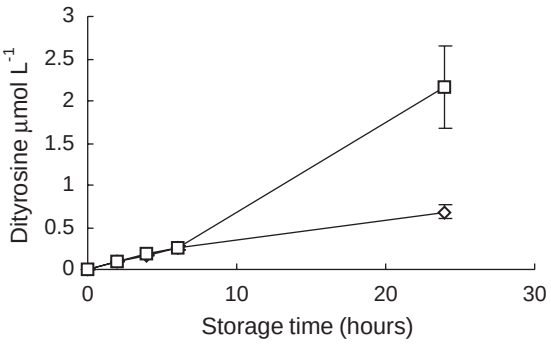

Fig. 3. Accumulation of dityrosine (μmol L^{-1}) as a function of the time of exposure to fluorescent light (2000 lux) at 4°C. Means ± standard deviation of three independent experiments. ◇ grass silage, □ corn silage.

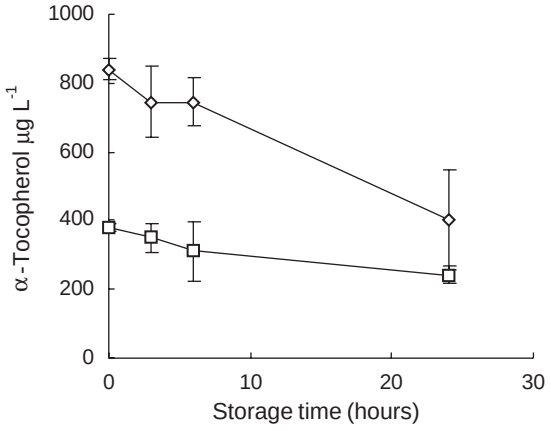

Fig. 4. Degradation of α-tocopherol (μg L^{-1}) as a function of the time of exposure to fluorescent light (2000 lux) at 4°C. Means ± standard deviation of three independent experiments. ◇ grass silage, □ corn silage.

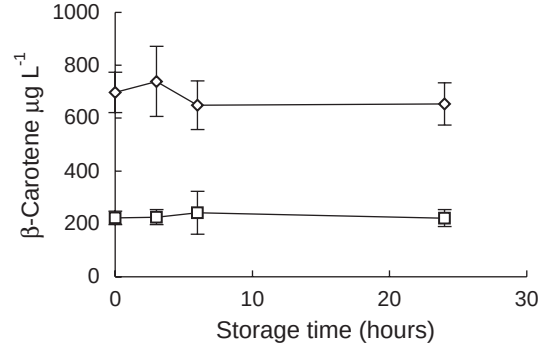

Fig. 5. Degradation of β-carotene (μg L^{-1}) as a function of the time of exposure to fluorescent light (2000 lux) at 4°C. Means ± standard deviation of three independent experiments. ◇ grass silage, □ corn silage.

in milk from cows fed grass silage than in milk from cows fed corn silage. After 24 h of light exposure, the concentration of β-carotene in milk from cows fed grass silage was still three-fold higher than the concentration

in milk from cows fed corn silage. There were no significant differences between the concentration of β-carotene at time zero and after 24 h for the two types of milk, suggesting that there was no degradation of β-carotene in the two types of milk during storage. The concentrations of lutein and zeaxanthine were also determined in the two types of milk. For both lutein and zeaxanthine the concentrations were higher in milk from cows fed grass silage. There was no noticeable decrease of lutein and zeaxanthine during exposure to fluorescent light for either of the two types of milk.

4. Discussion

The concentrations of tocopherols and carotenoids shown in Table 1 and the fatty acid composition shown in Table 2 clearly show that the type of roughage used for feeding dairy cows affects the content of important pro- and antioxidants in milk. These changes are believed to influence the oxidative stability and shelf-life of milk and processed dairy products. Light-induced oxidation in different milk types is used as a model for the oxidative stability of milk and dairy products.

Light-induced oxidation is initiated by sensibilization of riboflavin. From Fig. 1 it was seen that the decay of riboflavin was similar in the two types of milk exposed to fluorescent light. This indicates that the sensibilization and degradation of riboflavin occur similarly in the two types of milk. Several studies show that carotenoids can act as light filters reducing the light intensity that can sensibilize riboflavin (Sattar, Deman, & Alexander, 1976; Fakourelis, Lee, & Min, 1987). The concentration of β-carotene, lutein and zeaxanthine (Table 1) was significantly higher in milk from cows fed grass silage than in milk from cows fed corn silage. However, the higher concentration of β-carotene, lutein and zeaxanthine in the milk from cows fed grass silage could not inhibit the degradation of riboflavin.

The accumulation of lipid oxidation products expressed as the concentration of lipid hydroperoxides was higher in milk from cows fed grass. Furthermore, it was found that there was an accumulation of lipid hydroperoxides from the start of the light exposure indicating that there was no typical lag phase despite the higher concentration of tocopherols and β-carotene, lutein and zeaxanthine in the milk from cows fed grass silage. The formation of lipid hydroperoxides in milk exposed to fluorescent light is proposed to be a direct reaction of singlet oxygen formed by sensibilization of riboflavin and the unsaturated lipids as described by Frankel (1998):

$$LH + {}^1O_2 \rightarrow LOOH. \quad (1)$$

Singlet oxygen will react with the carbon atom participating directly in the double binding (Frankel, 1998). The degree of unsaturation is proposed to be important for the higher accumulation of lipid hydroperoxides in milk from cows fed grass silage, which contained a relatively high level of C18:3.

Carotenoids and tocopherols are known to act as antioxidants through reaction with singlet oxygen by either physical or chemical quenching. The rate of physical quenching of singlet oxygen is described to be several orders of magnitude higher than the chemical quenching (Foote, 1976, 1979; Fragata & Bellemare, 1980). However, tocopherols are poor quenchers compared to carotenoids (Cadenas, 1989; Krinsky, 1992). Tocopherols and carotenoids can react as physical quenchers several times without being destroyed (Bowry & Stocker, 1993), while tocopherols and carotenoids are destroyed after chemical quenching by singlet oxygen (Foote, 1976, 1979; Fragata, & Bellemare, 1980). In the present study, the initial concentration of α-tocopherol was $0.854\,mg\,L^{-1}$ in milk from cows fed grass silage compared to $0.375\,mg\,L^{-1}$ α-tocopherol in the milk from cows fed corn silage and the content of β-carotene was two- to three-fold higher in milk from cows fed grass silage than in milk from cows fed corn silage. Despite the higher concentration of antioxidants able to react directly with singlet oxygen formed by activated riboflavin in the milk from cows fed grass silage, the accumulation of lipid hydroperoxides was significantly higher in the milk from cows fed grass silage than in milk from cows fed corn silage. This indicates that the concentration of β-carotene, lutein, zeaxanthine and tocopherols had no impact on the formation of lipid hydroperoxides and that quenching of singlet oxygen by carotenoids and tocopherols was not able to diminish lipid oxidation.

Carotenoids are also known to act as free radical scavengers, and Krinsky and Deneke (1982) have demonstrated that β-carotene is able to inhibit free radical-induced oxidation of liposomal lipids. β-Carotene exerts antioxidative activity in which chain-propagating radicals (e.g. peroxyl radicals) conjugate with β-carotene:

$$LOO^{\bullet} + \beta\text{-car} \rightarrow LOO\beta - \text{car}. \quad (2)$$

Conjugation of free radicals and β-carotene is proposed to contribute to the degradation of β-carotene in the milk. However, no measurable consumption of β-carotene was found in the two types of milk during the storage period in light, which indicates that β-carotene has no impact as a chain-breaking antioxidant in the two types of milk exposed to fluorescent light.

α-Tocopherol is known as a traditional chain-breaking antioxidant where it donates a hydrogen ion to the oxyradicals (Nagaoka et al., 1992). The formed tocopheryl radical can be reduced back to α-tocopherol by a reducing agent as ascorbic acid or oxidized further to tocopheryl radicals (Kamal-Eldin & Appelqvist, 1996;

Nielsen, Østdal, & Andersen, 2002, Chapter 9) which results in breakdown of α-tocopherols. The present data support this theory, since significant decreases of α-tocopherol were observed during storage of both types of milk. The degradation of α-tocopherol was substantial, especially in the milk from the cows fed grass silage, which may be due to a higher turn-over of lipid hydroperoxides and reaction of oxyradicals with α-tocopherol. Furthermore, Peers and Coxon (1983) found that α-tocopherol in model systems led to increased levels of lipid hydroperoxides due to the ability of α-tocopherol to act as a hydrogen donor to the peroxyl radical. This mechanism might also be partly responsible for the accumulation of lipid hydroperoxides in milk from cows fed grass silage.

The progress in protein oxidation measured as accumulation of dityrosine in the light-exposed milk was found to be identical in the two types of milk for the first 6 h of storage. However, after 24 h of storage the accumulation of dityrosine in milk from cows fed corn silage had increased in an exponential way, indicating that the progress in tyrosine oxidation had passed the lag phase, while the accumulation of dityrosine in the milk from cows fed grass silage was still in its lag phase. Milk from cows fed corn silage appears to be more vulnerable to light-induced protein oxidation than milk from cows fed corn silage. This is in contrast to what was seen for the progress in lipid oxidation. Østdal, Bjerrum, Pedersen, and Andersen (2000) have shown that dityrosine accumulates in milk as a result of light-induced oxidation and former studies by Silva and Godoy (1994) show that the formation of dityrosine in a model system of riboflavin and tyrosine appears to be a Type I reaction where activated riboflavin reacts directly with tyrosine. The results from the present study indicate that formation of dityrosine was independent of both the lipid oxidation and of the higher degree of unsaturation of the milk from cows fed grass silage. In contrast to the formation of lipid hydroperoxides in the milk from cows fed grass silage, where the high content of β-carotene, lutein and zeaxanthine and tocopherols was not capable of restraining lipid oxidation, the antioxidants seem to be able to inhibit the formation of dityrosine and to extend the lag phase. The consumption of α-tocopherol and other potential antioxidants in the milk from cows fed corn silage seems to have passed a level where it is not able to restrain protein oxidation. Several studies (Dunckley, Frankel, & Pangborn, 1962; Dimick & Kilara, 1983; Marsili, 1999) indicate that protein oxidation is responsible for the early developed off-flavor perceived in milk exposed to fluorescent light. The present study shows that milk from cows fed grass silage was more stable in relation to development of protein oxidation products measured as the accumulation of dityrosine. The antioxidative potential is suggested to be related to the higher content of α-tocopherol in this type of milk.

In conclusion, this study has shown that different types of roughage affect the oxidative stability of milk exposed to fluorescent light. The mechanisms and interaction between pro- and antioxidants seem to be complex. The interrelation between progress of lipid oxidation and protein oxidation needs to be further investigated. High levels of tocopherols and β-carotene, lutein and zeaxanthine in milk do not prevent oxidation of polyunsaturated lipids, but can delay protein oxidation.

Acknowledgements

The authors would like to thank Rita Albrechtsen and Ivan Nielsen for excellent technical assistance and the Danish Dairy Research Foundation (Danish Dairy Board) and The Danish Research and Development Programme for Food Technology (FØTEK) for financial support.

References

Allen, C., & Parks, O. W. (1975). Evidence for methional in skim milk exposed to sunlight. *Journal of Dairy Science*, *58*(11), 1609–1611.

Barrefors, P., Granelli, K., Appelqvist, L.-A., & Bjoerck, L. (1995). Chemical characterization of raw milk samples with and without oxidative off-flavor. *Journal of Dairy Science*, *78*(12), 2691–2699.

Bowry, V. W., & Stocker, R. (1993). Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *Journal of the American Chemical Society*, *115*(14), 6029–6044.

Bugaud, C., Buchin, S., Coulon, J.-B., Hauwuy, A., & Dupont, D. (2001). Influence of the nature of alpine pastures on plasmin activity, fatty acid and volatile compound composition of milk. *Le Lait*, *81*(3), 401–414.

Cadenas, E. (1989). Biochemistry of oxygen toxicity. A review. *Biochemistry*, *58*, 79–110.

Charmley, E., & Nicholson, J. W. G. (1995). Influence of dietary fat source on oxidative stability and fatty acid composition of milk from cows receiving a low or high level of dietary vitamin E. *Canadian Journal of Animal Science*, *74*(4), 657–664.

Dimick, P. S., & Kilara, A. (1983). Photooxidative changes in milk proteins and amino acids. *Kieler Milchwirtschaftliche Forschungsberichte*, *35*(3), 289–299.

Dunckley, W. L., Frankel, D. J., & Pangborn, R. M. (1962). Effects of fluorescent light on flavor, ascorbic acid, and riboflavin in milk. *Food Technology*, *16*, 112–118.

Fakourelis, N., Lee, E. C., & Min, D. B. (1987). Effects of chlorophyll and β-carotene on the oxidative stability of olive oil. *Journal of Food science*, *52*(1), 234–235.

Focant, M., Mignolet, E., Marique, M., Clabots, F., Breyne, T., Dalemans, D., & Larondelle, Y. (1998). The effect of vitamin E supplementation of cow diets containing rapeseed and linseed on the prevention of milk fat oxidation. *Journal of Dairy Science*, *81*(4), 1095–1101.

Foote, C. S. (1976). Photosensitized oxidations and singlet oxygen: Consequences in biological systems. In W. A. Pryor (Ed.), *Free*

radicals in biological systems, Vol. 2 (p. 85). New York: Academic Press.

Foote, C. S. (1979). Quenching of singlet oxygen. In: H. H. Wasserman, & R. W. Murray (Eds.), *Singlet oxygen* (pp. 39–171).

Fragata, M., & Bellemare, F. (1980). Model of singlet oxygen scavenging by α-tocopherol in biomembranes. *Chemistry and Physics of Lipids*, *27*, 93–99.

Frankel, E. (1998). *Lipid oxidation*. Dundee, Scotland: The Oily Press Ltd.

Granelli, K., Barrefors, P., Bjoerck, L., & Appelqvist, L.-A. (1998). Further studies on lipid composition of bovine milk in relation to spontaneous oxidised flavour. *Journal of the Science of Food and Agriculture*, *77*(2), 161–171.

Hansen, A. P., Turner, L. G., & Aurand, L. W. (1975). Fluorescent light-activated flavor in milk. *Journal of Milk, Food and Technology*, *38*(7), 388–392.

Hermansen, J. E. (1995). Prediction of milk fatty acid profile in dairy cows fed dietary fat differing in fatty acid composition. *Journal of Dairy Science*, *78*(4), 872–879.

Jung, M. Y., Yoon, S. H., Lee, H. O., & Min, D. B. (1998). Singlet oxygen and ascorbic acid effects on dimethyl disulfide and off-flavor in skim milk exposed to light. *Journal of Food Science*, *63*(3), 408–412.

Kamal-Eldin, A., & Appelqvist, L.-A. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids*, *31*(7), 671–701.

Kim, Y. D., & Morr, C. V. (1996). Dynamic headspace analysis of light activated flavor in milk. *International Dairy Journal*, *6*, 185–193.

Krinsky, N. I. (1992). Mechanism of action of biological antioxidants. *Proceedings of the Society for Experimental Biology and Medicine*, *200*, 248–254.

Krinsky, N. I., & Deneke, S. M. (1982). Interaction of oxygen and oxy-radicals with carotenoids. *Journal National Cancer Institute*, *69*(1), 205–210.

Marsili, R. T. (1999). Comparison of solid-phase microextraction and dynamic headspace methods for the gas chromatographic-mass spectrometric analysis of light-induced lipid oxidation products in milk. *Journal of Chromatographic Science*, *37*(1), 17–23.

Morales, M. S., Palmquist, D. L., & Weiss, W. P. (2000). Milk fat composition of Holstein and Jersey cows with control or depleted copper status and fed whole soybeans or tallow. *Journal of Dairy Science*, *83*(9), 2112–2119.

Nagaoka, S., Sawada, K., Fukumoto, Y., Nagashima, U., Katsumata, S., & Mukai, K. (1992). Mechanism of prooxidant reaction of vitamin E. Kinetic, Spectroscopic, and ab initio study of proton-transfer reactions. *The Journal of Physical Chemistry*, *96*(16), 6663–6668.

Nielsen, J. H., Østdal, H., & Andersen, H. J. (2002). The influence of ascorbic acid and uric acid on the oxidative stability of raw and pasteurized milk. In *Free radicals in foods: Chemistry, nutrition, and health effects*. American Chemical Society Symposium Series, San Francisco, 807, (pp. 126–137).

Østdal, H., Bjerrum, M. J., Pedersen, J. A., & Andersen, H. J. (2000). Lactoperoxidase-induced protein oxidation in milk. *Journal of Agriculture and Food Chemistry*, *48*, 3939–3944.

Patton, S. (1953). The mechanism of sunlight flavor formation in milk with special reference to methionine and riboflavin. *Journal of Dairy Science*, *37*, 446–452.

Peers, K. E., & Coxon, D. T. (1983). Controlled synthesis of monohydroperoxides by α-tocopherol inhibited autoxidation of polyunsaturated lipids. *Chemistry and Physics of Lipids*, *32*, 49–56.

Ramaswamy, R., Baer, R. J., Schingoethe, D. J., Hippen, A. R., Kasperson, K. M., & Whitlock, L. A. (2001). Composition and flavor of milk and butter from cows fed fish oil, extruded soybeans, or their combination. *Journal of Dairy Science*, *84*(10), 2144–2151.

Sattar, A., Deman, J. M., & Alexander, J. C. (1976). Stability of edible oils and fats to fluorescent light. *Journal of the American Oil Chemists' Society*, *53*, 473–477.

Schingoethe, D. J., Parsons, J. G., Ludens, F. C., Schaffer, L. V., & Shave, H. J. (1979). Response of lactating cows to 300 mg of supplemental vitamin E daily. *Journal of Dairy Science*, *62*(2), 333–338.

Silva, E., & Godoy, J. (1994). Riboflavin sensitized photooxidation of tyrosine. *International Journal for Vitamin and Nutrition Research*, *64*, 253–256.

Timmons, J. S., Weiss, W. P., Palmquist, D. L., & Harper, W. J. (2001). Relationships among dietary roasted soybeans, milk components, and spontaneous oxidized flavor of milk. *Journal of Dairy Science*, *84*, 2440–2449.