Oxidative Stability of Milk Influenced by Fatty Acids, Antioxidants, and Copper Derived from Feed

M. S. Havemose,* M. R. Weisbjerg,* W. L. P. Bredie,† H. D. Poulsen,* and J. H. Nielsen*¹

*Danish Institute of Agricultural Sciences, Department of Food Science, Research Centre Foulum, P.O. Box 50, 8830 Tjele, Denmark

†The Royal Veterinary and Agricultural University, Department of Food Science, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

ABSTRACT

Differences in the oxidative stability of milk from cows fed grass-clover silage or hay were examined in relation to fatty acid composition and contents of antioxidants and copper in the milk. The oxidation processes were induced by exposing the milk to fluorescent light. Protein oxidation was measured as an accumulation of dityrosine, whereas lipid oxidation was measured as an accumulation of lipid hydroperoxides as the primary oxidation product, and as the secondary oxidation products, pentanal, hexanal, and heptanal. No differences were found in the protein oxidation of the 2 types of milk measured as accumulation of dityrosine, but there was an increased accumulation of lipid hydroperoxides and hexanal in milk from cows fed grass-clover silage, compared with milk from cows fed hay. The higher degree of lipid oxidation in milk from cows fed grass-clover silage could not be explained from the concentration of α -tocopherol, carotenoids, uric acid, and copper in the milk. However, it was thought to be highly influenced by the significantly higher concentration of linolenic acid present in milk from cows fed grass-clover silage. A larger part of α -tocopherol and β -carotene was transferred from the feed to the milk when cows were fed grass-clover silage than when cows were fed hay as roughage. The significantly higher concentration of polyunsaturated fatty acids in milk from cows fed grass-clover silage may be important for the better transfer of α -tocopherol from the feed to the milk. Other circumstances, as the different conditions in the rumen may also play a role, due to the different types of roughages and their digestibility, or be related to the mechanisms during milk production for the higher yielding cows fed grass-clover silage.

Key words: oxidation, roughage, antioxidant, fatty acid composition

INTRODUCTION

Milk used for consumption or dairy processing should have a high antioxidative capacity to achieve high quality products for the consumers. The oxidative processes have several implications on milk or other dairy products such as short shelf life, development of off-flavor, and deterioration of nutritional quality. The composition of the milk is, to a certain extent, reflected by the composition of the feed given to the dairy cows (AbuGhazeleh et al., 2002; Collomb et al., 2002; Stockdale et al., 2003). Several studies have shown that the composition and concentration of unsaturated fatty acids in milk fat is easily changed by altering the feeding of the dairy cow (Hermansen, 1995; Ramaswamy et al., 2001; Timmons et al., 2001; Havemose et al., 2004), whereas it can be very challenging to influence total protein content and composition of the milk protein through the diet (Barry and Donnelly, 1980; Grandison et al., 1985; Vagnoni and Broderick, 1997; Hermansen et al., 1999; Baer et al., 2001). Antioxidants were shown to be transferred either directly from the feed to the milk (Nalecz-Tarwacka et al., 2003; Havemose et al., 2004), or from supplements added to the cows' diet and to the milk (Schingoethe et al., 1979; Nicholson and St-Laurent, 1991). Transition metal ions are known to propagate lipid oxidation, which can give rise to several secondary oxidation products (Ford et al., 1986; Leland et al., 1987; Rao and Murthy, 1987). The transition metal ions can be derived directly from the feed or from mineral mixtures, which are commonly given to the cows to prevent a depletion of, for example, copper in ruminants particularly during the grazing season (Suttle et al., 1980).

Lipid oxidation of milk is highly influenced by long-chain unsaturated fatty acids, which are particularly susceptible to oxidation, and can give rise to development of off-flavor (Badings, 1970; Ullrich and Grosch, 1987; Timmons et al., 2001; Huang et al., 2004).

In the present study, cows were fed either grass-clover silage or hay. It was expected that milk from cows fed grass-clover would have a higher concentration of unsaturated fatty acids, α -tocopherol, and β -carotene than milk from cows fed hay, and that the higher con-

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¹Corresponding author: jacobh.nielsen@agrsci.dk

centration of antioxidants would have a preventive effect on the oxidation of the milk. Havemose et al. (2004) found that α -tocopherol only had an effect on delaying the dityrosine formation (Type I reaction) but no effect on the lipid oxidation (Type II). Therefore, it was particularly interesting if differences in the oxidation of lipids and formation of dityrosine could be related to the great variation in concentration of antioxidants expected to be found in the 2 types of milk.

MATERIALS AND METHODS

Animals, Design, and Feeding

Eight Holstein cows in a 2×4 double reversal design, in which the cows were divided into 2 treatments comprising 2 different roughages: grass-clover silage and hay. In 3 periods of 4 wk each, cows were fed first one ration, switched to the other, and then switched back to the first ration. The grass-clover silage was rich in white clover (66% of DM), whereas the hay was meadow hay without clover.

Rations were fed ad libitum as a TMR. Grass-clover silage TMR was composed of (% of DM) 49.9% grass-clover silage; 10.8% sugar beet molasses; 39.3% concentrate (concentrate = 25.5% soybean meal; 72.9% barley; 1.6% mineral mixture). Hay TMR was composed of (% of DM) 49.2% hay; 10.7% sugar beet molasses; 40.1% concentrate (concentrate = 24.7% soybean meal; 70.7% barley; 1.1% urea; 1.3% lime; 2.2% mineral mixture). The mineral mixture did not contain α -tocopherol.

Milk Samples

Morning milk was collected at the end of each 4-wk period. Milk was collected once for all the analyses performed in this study except for protein and fat content, which was performed daily for the last 5 d of each feeding period. Milk from the 4 cows fed the same ration within the period was pooled and subsequently pasteurized at 72°C for 15 s.

Milk samples were stored at -20°C for approximately 2 mo until analysis for riboflavin, fatty acids, dityrosine, α -tocopherol, β -carotene, lutein, zeaxanthine, uric acid, and copper were completed. Analysis of volatiles and lipid hydroperoxides began on the day of pasteurization.

Feed Samples

Samples of grass-clover silage and hay were stored at -20°C for approximately 2 mo until analyses for α -tocopherol and β -carotene were completed.

Determination of Fat and Protein

Fat and protein percentage were determined daily for the last 5 d of each feeding period using a Milko-Scan 6000 (Foss Electric, Hillerød, Denmark).

Analysis of Fatty Acid Composition

Before GC separation and quantification, the lipid was extracted by chloroform:methanol and the extracted lipid was transesterified to methyl esters in a sodium methylate solution. 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 (Havemose et al., 2004).

Analysis of α -Tocopherol

Milk samples were mixed with ethanolic ascorbic acid. Saturated potassium hydroxide solution was added and mixed and the solution was placed in a heating chamber for saponification. Water and heptane were added and mixed. The solution was centrifuged, for 3 min at $1,700 \times g$ and 4°C, the supernatant was filtered, and aliquots were injected on an HPLC system. Samples were quantified using external standard curves (Havemose et al., 2004).

Determination of β -Carotene, Lutein, and Zeaxanthine

Milk samples were added to ethanolic butylhydroxytoluene and mixed. Saturated potassium hydroxide solution was added and mixed. The headspace above the samples was replaced with nitrogen, and the samples were placed in a heating chamber for saponification. The samples were subsequently cooled in an ice water bath. The samples were added water and heptane:dichloromethane (90:10, vol:vol). The samples were centrifuged for 3 min at $1,700 \times g$ and 4°C. The extraction was repeated 3 times, and the supernatants were collected. The extracts were evaporated under nitrogen flow until dry and redissolved in a mixture of acetonitrile:methanol:dichloromethane:triethylamine (85:10:5:0.5). Aliquots were injected onto an HPLC system. Samples were quantified using external standards (Havemose et al., 2004).

Determination of Riboflavin

Milk samples were mixed with sodium acetate and acetic acid. The samples were slowly agitated before centrifugation for 10 min at $1,500 \times g$ and 4°C. The supernatant was filtered through a 0.45- μ m filter and the fluorescence was read (Havemose et al., 2004).

Dityrosine Determination

Milk samples were mixed with 0.1 M sulfuric acid in 1 M sodium chloride and 2-propanol. The samples were shaken, pentane was added, and the samples were shaken again. Samples were precipitated by centrifugation for 5 min at $1,500 \times g$ and 4°C . The supernatant was discharged, and the extraction procedure was repeated. The pellet was redissolved in 50 mM phosphate buffer (pH 7.4). The protein was precipitated by adding TCA to a final concentration of 10%. The samples were allowed to stand for 10 min and ethanol was added before centrifugation. The pellet was washed with 1 M hydrochloric acid before an additional centrifugation. The pellet was mixed with 6 M hydrochloric acid, flushed with argon, and hydrolyzed overnight. Samples were neutralized with 6 M sodium hydroxide. Aliquots of the hydrolyzed samples were injected onto an HPLC column. Samples were spiked with a dityrosine standard for identification and quantified by the use of a standard curve made from the same standard (Østdal et al., 2000).

Determination of Lipid Hydroperoxides

Milk samples were mixed with methanol. Chloroform was added and the samples were mixed before centrifugation. The precipitate was added iron-II/thiocyanate. The samples were left to react at room temperature and were measured spectrophotometrically (Østdal et al., 2000).

Analysis of α -Tocopherol in Feed

Feed samples (1 g) were frozen in liquid nitrogen and ground in a mill to sizes of ~ 2 mm in length. The feed samples were added to a mixture of 12 mL of ethanol, 4.5 mL of methanol, 5 mL of 10% ascorbic acid in water, and 3.5 mL of saturated potassium hydroxide. The suspension was saponized for 90 min at 70°C and subsequently cooled in an ice water bath. The liquid phase (2 mL) was extracted quantitatively twice with 2.5 mL of heptane (mixed and centrifuged for 3 min at $1,700 \times g$ at 4°C). Aliquots of 100 μL were injected on a HPLC system, and the separation and identification were the same as described for analysis of α -tocopherol in milk (Havemose et al., 2004).

Determination of β -Carotene in Feed

The extraction procedure was the same as that for determination of α -tocopherol in feed and the analytical separation and quantification followed the procedure for determination of β -carotene in milk as described by Havemose et al. (2004).

Determination of Uric Acid in Milk

Milk samples (1 mL) were mixed with 1 mL of metaphosphoric acid (1.12%) and centrifuged at $1,000 \times g$ at 4°C for 20 min to precipitate the protein. The supernatant was mixed for 1 min with chloroform and centrifuged at $1,000 \times g$ at 4°C for 10 min to remove the fat. The supernatant was filtered and 30- μL aliquots were injected onto an HPLC system (HP 1100, Agilent Technologies, Palo Alto, CA) with UV detection at 290 nm. The C18 column was a Hypersil ODS (4.0×250 mm, 5 μm ; Agilent Technologies) and the mobile phase was 10 mM potassium dihydrogenphosphate, pH 4, with a flow rate of 1 mL/min. Quantification was performed using an external calibration curve (Østdal et al., 2000).

Determination of Copper in Milk

All the glassware used during the copper analysis were soaked for 12 h in 5% nitric acid, followed by 4 rinses with deionized-distilled water. The milk samples were ashed at 450°C for 6 h and the ash was dissolved in a 21.7% nitric acid solution. The concentration of copper was determined by atom absorption spectrophotometry (Unicam SP9, Philips, Germany).

Exposure of Milk to Fluorescent Light

The milk samples (9.0 mL) were transferred to 10-mL glass tubes. The glass tubes were placed on a rotating device in a refrigerator kept at a temperature of 4°C . A fluorescent light was placed (Philips TLD 18W) above the rotating device. The milk was exposed to a light intensity of 2,000 lx at the surface of the glass. Only one sample was taken from each glass tube to secure a constant headspace in all tubes. Samples were withdrawn at different intervals up to 24 h of light exposure.

Determination of Volatile Lipid Oxidation Products in Milk Using Solid-Phase Microextraction

Sodium chloride (0.25 g) was weighed out in an annealed 4-mL vial with screw cap including a small magnet, and 2 mL of milk was added to the vial. A preconditioned solid-phase microextraction fiber (divinylbenzene and carboxen on polydimethylsiloxane; Supelco, Bellefonte, PA) was exposed to the headspace of the milk for 30 min at 43°C to allow adsorption of volatiles to the fiber before introduction to the GC injector port. The fiber was left in the injector port for desorption for approximately 30 to 45 min before introducing the fiber to the headspace of a new sample.

Analysis of Volatiles in Milk by Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GCMS) analyses were performed on a Varian 3400CX gas chromatograph coupled with a Saturn 3D ion trap mass spectrometer (Varian Inc., Walnut Creek, CA). The volatiles were separated on a J&W DB-FFAP column (P/N 122-3232 30 m length, 0.25 mm i.d., and 0.25 μ m film thickness) obtained through Agilent Technologies. Helium was used as carrier gas with a pressure of 1,034 kPa through the column. The injector with splitless injection (splitless for 0.6 min) was kept at a temperature of 250°C. The temperature was programmed at 35°C for 1 min, 3°C/min to 225°C with a hold time of 1 min, 10°C/min to 250°C with a hold time of 5 min. The mass spectrometer operated in the electron impact mode with electron energy of 70 eV, and spectral data from mass range m/z 35 to 300 were obtained. The GCMS transfer line temperature was 275°C, the temperature of the trap was 200°C, and the manifold temperature was 50°C.

Identification of Volatile Compounds

A method including different volatiles analyzed as authentic standards on the GCMS system was set up in SaturnView version 5.40 (Varian Inc.). The compounds in the milk samples were identified by comparing retention times and target ions with those of the authentic standards in the method.

Statistical Analyses

Two-tailed *t*-tests for paired observations were performed to test for significant differences in yield, total fat percentage, total protein percentage, fatty acids, and antioxidants for the 2 types of milk. The same method was also used to test for significant differences in riboflavin concentration for the 2 types of milk before and after 24 h of exposure to fluorescent light.

RESULTS

The cows fed grass-clover silage had a significantly higher milk yield compared with cows fed hay as shown in Table 1. There were no significant differences in the milk fat percentage and milk protein percentage of the 2 milk types.

The cows had an average daily intake of 11.1 kg of DM of grass-clover silage and 9.5 kg of DM of hay. The total daily DMI was 22.3 kg for cows fed grass-clover silage and 19.3 kg for cows fed hay as roughage.

The fatty acid composition of the 2 types of milk is shown in Table 2. The milk from cows fed grass-clover

Table 1. Milk yield and total composition of fat and protein¹

	Grass-clover silage	Hay
Yield, kg/d	22.3 $\pm$ 3.3*	16.5 $\pm$ 1.3
Fat, %	4.14 $\pm$ 0.14	4.07 $\pm$ 0.20
Protein, %	3.79 $\pm$ 0.25	3.85 $\pm$ 0.11

¹Yield, fat, and protein are mean values $\pm$ standard deviation of results from the 3 periods.

*Significant difference, $P < 0.01$.

silage had a significantly higher concentration of the polyunsaturated fatty acid C18:3n-3 (linolenic acid), compared with milk from cows fed hay, whereas milk from cows fed hay had significantly higher concentrations of the monounsaturated fatty acids C16:1n-7 (palmitoleic acid) and C18:1n-9 (oleic acid) compared with milk from cows fed grass-clover silage. No significant differences were found for C18:2n-6 (linoleic acid) between the 2 types of milk.

The concentrations of the antioxidants α -tocopherol, β -carotene, lutein, zeaxanthine, and uric acid in the milk types are shown in Table 3. There were no significant differences between the concentrations of α -tocopherol, lutein and zeaxanthine or uric acid in the 2 types of milk. A mean value for the concentration of β -carotene in milk from cows fed hay was not reported because a decrease in the concentration was observed during the study period by the end of each of the 3 feeding periods. Instead, the highest and the lowest concentrations measured by the end of the feeding periods are listed in Table 3.

The concentrations of both α -tocopherol and β -carotene were analyzed in the 2 types of roughage, and the results are shown in Table 4. The concentration of α -

Table 2. Fatty acid compositions of milk from cows fed grass-clover silage and hay¹

Fatty acid	Grass-clover silage	Hay
C4:0	4.1 $\pm$ 0.4	4.6 $\pm$ 1.0
C6:0	1.7 $\pm$ 0.1	1.5 $\pm$ 0.1
C8:0	1.0 $\pm$ 0.1	0.9 $\pm$ 0.0
C10:0	3.0 $\pm$ 0.2	2.5 $\pm$ 0.2
C12:0	4.3 $\pm$ 0.3	3.5 $\pm$ 0.3
C14:0	13.5 $\pm$ 0.6	13.1 $\pm$ 0.5
C16:0	44.1 $\pm$ 1.1	41.7 $\pm$ 0.3
C16:1n-7*	2.8 $\pm$ 0.1	3.3 $\pm$ 0.0
C18:0	6.4 $\pm$ 0.3	6.6 $\pm$ 0.6
C18:1n-9*	16.1 $\pm$ 0.2	20.0 $\pm$ 1.5
C18:2n-6	2.0 $\pm$ 0.2	1.9 $\pm$ 0.1
C18:3n-3*	0.8 $\pm$ 0.1	0.4 $\pm$ 0.0

¹Results are mean values $\pm$ standard deviation of samples from the 3 periods; units are percentages of the total peak area for all the fatty acids listed.

*Significant differences, $P < 0.01$.

Table 3. Concentration of antioxidants in milk from cows fed grass-clover silage and hay¹

Antioxidant	Grass-clover silage	Hay
α -Tocopherol, $\mu\text{g/L}$	472 $\pm$ 33	504 $\pm$ 48
β -Carotene, $\mu\text{g/L}$	440 $\pm$ 23	445 to 264
Lutein, $\mu\text{g/L}$	9 $\pm$ 2	9 $\pm$ 2
Zeaxanthine, $\mu\text{g/L}$	2 $\pm$ 0	1 $\pm$ 0
Uric acid, mg/L	8.0 $\pm$ 0.3	8.0 $\pm$ 1.1

¹Results are mean values $\pm$ standard deviation of samples from the 3 periods, except for β -carotene in milk from cows fed hay in which the decline in concentration over the feeding periods is shown.

tocopherol in both grass-clover silage and hay was constant over the course of the study. The concentration of β -carotene was constant over the course of the study for grass-clover silage, whereas there was a decline in the concentration of β -carotene in hay, which was also reflected in the milk. However, in all cases, the concentration of β -carotene was higher in hay than in grass-clover silage.

The concentration of copper was determined in the 2 types of milk. Milk from cows fed grass-clover silage contained 0.07 mg/kg of Cu, and milk from cows fed hay contained 0.06 mg/kg of Cu. The differences were not significant, and the copper levels found in the 2 types of milk were neither low nor high compared with other studies (Ford et al., 1986; Anderson, 1992; Sol Morales et al., 2000; Rodríguez Rodríguez et al., 2001; Timmons et al., 2001).

In Figure 1, the degradation of riboflavin is shown as a function of hours exposed to fluorescent light for the 2 types of milk. The riboflavin was measured after 0, 2, 4, 6, and 24 h. Initially ($P < 0.03$) and after 24 h ($P < 0.05$) there was significantly less riboflavin in milk from cows fed hay compared with milk from cows fed grass-clover silage. The absolute decay of riboflavin over the first 24 h was of the same magnitude for the 2 types of milk.

To examine the progress of protein oxidation dityrosine was measured. The accumulation of dityrosine during exposure to fluorescent light at 4°C for 0, 2, 4, 6, and 24 h is shown in Figure 2. There were no significant

Table 4. Concentration of antioxidants in grass-clover silage and hay¹

Antioxidant, mg/kg of DM	Grass-clover silage	Hay
α -Tocopherol	11.1 $\pm$ 2.3	13.8 $\pm$ 0.1
β -Carotene	13.1 $\pm$ 0.1	41.6 to 20.8

¹Results are mean values $\pm$ standard deviation of samples from the 3 periods, except for β -carotene in hay in which the decline in concentration over the feeding periods is shown.

Figure 1. Decay of riboflavin (fluorescence response to excitation at 445 nm and emission at 520 nm) as a function of the time of exposure to fluorescent light (2,000 lx) at 4°C. Mean $\pm$ SD of samples from the 3 periods; milk from cows fed grass-clover silage (■), and from those fed hay (◆).

differences in the accumulation of dityrosine for the 2 types of milk.

The progress of lipid oxidation was examined in the 2 types of milk during exposure to fluorescent light for 24 h. The accumulation of lipid hydroperoxides was measured as well as the secondary lipid oxidation products, pentanal, hexanal, and heptanal.

Figure 2. Accumulation of dityrosine as a function of the time of exposure to fluorescent light (2,000 lx) at 4°C. Mean $\pm$ SD of samples from the 3 periods; milk from cows fed grass-clover silage (■), and from those fed hay (◆).

Figure 3. Accumulation of lipid hydroperoxides (UV absorbance) as a function of the time of exposure to fluorescent light (2,000 lx) at 4°C. Mean $\pm$ SD of samples from the 3 periods; milk from cows fed grass-clover silage (■), and from those fed hay (◆).

The accumulation of lipid hydroperoxides during storage in fluorescent light at 4°C for 0, 2, 4, 6, and 24 h is shown in Figure 3 for the 2 types of milk. The accumulation of lipid hydroperoxides in milk from cows fed grass-clover silage was higher than in milk from cows fed hay. The 2 curves also show differences in the pattern of accumulation. The curve for milk from cows fed hay is more linear ($R^2 = 0.9992$) compared with the curve for milk from cows fed grass-clover silage ($R^2 = 0.9542$). The accumulation of lipid hydroperoxides in milk from cows fed grass-clover silage is higher for the first 4 h than later between 6 and 24 h.

Figure 4 shows the accumulation of hexanal during exposure to fluorescent light at 4°C for 0, 3, 6, 9, and 24 h. The figure shows a higher accumulation of hexanal in milk from cows fed grass-clover silage than in milk from cows fed hay, just as for the lipid hydroperoxides. The pattern of both curves shows a small accumulation of hexanal for the first 6 to 9 h after which there is a steep increase in the accumulation pattern of hexanal for both types of milk.

The levels of pentanal and heptanal were also determined during exposure of the milk to fluorescent light at 4°C for 0, 3, 6, 9, and 24 h in the 2 types of milk. Measurable quantities were only observed after 24 h. The concentrations of both pentanal and heptanal were higher in milk from cows fed grass-clover silage as shown in Table 5.

The degradation of α -tocopherol during exposure to fluorescent light was followed under the same conditions used to follow the lipid hydroperoxide accumulation and dityrosine accumulation. Figure 5 shows the

Figure 4. Accumulation of hexanal as a function of the time of exposure to fluorescent light (2,000 lx) at 4°C. Mean $\pm$ SD of samples from the 3 periods; milk from cows fed grass-clover silage (■), and from those fed hay (◆).

degradation of α -tocopherol exposed to fluorescent light at 4°C for 0, 3, 6, 12, and 24 h in the 2 types of milk; no differences were observed in the degradation. The pattern of the curves shows a steep degradation within the first 3 to 6 h, in which approximately 75% of the α -tocopherol was degraded, followed by only minor or no further degradation up to 24 h of exposure to fluorescent light.

DISCUSSION

Antioxidants in the Feed and Transfer to the Milk

Higher concentrations of α -tocopherol and β -carotene were expected in grass-clover silage compared with hay, although great variations are reported in the literature. Grass-clover silage is reported to contain 10 to 150 mg/kg of DM of α -tocopherol and 30 to 150 mg/kg of DM of β -carotene, whereas hay is reported to contain 15 to 65 mg/kg of DM of α -tocopherol and 0 to 90 mg/kg of DM of β -carotene (Jensen, 2003). Hay was found to have a higher concentration of β -carotene than the grass-

Table 5. Accumulation of pentanal and heptanal in milk from cows fed grass-clover silage and hay after 24 h of exposure to fluorescent light¹

Area count of single ion	Grass-clover silage	Hay
Pentanal (ion 69)	701 $\pm$ 117	193 $\pm$ 1
Heptanal (ion 70)	701 $\pm$ 78	402 $\pm$ 6

¹Results are mean values $\pm$ standard deviation of samples from the 3 periods.

Figure 5. Degradation of α -tocopherol as a function of the time of exposure to fluorescent light (2,000 lx) at 4°C. Mean $\pm$ SD of samples from the 3 periods; milk from cows fed grass-clover silage (■), and from those fed hay (◆).

clover silage, which was not anticipated, but it is in agreement with the large variations found in hay by Jensen (2003). The generally low concentrations of both α -tocopherol and β -carotene in grass-clover silage and hay in this study may be due to both the quality of the roughages and to the extraction method used.

The total daily intake of α -tocopherol through roughage was calculated to be 123 mg of α -tocopherol for cows fed grass-clover silage, and 131 mg of α -tocopherol for cows fed hay.

The total daily output from milk was calculated to be 10.6 mg of α -tocopherol for cows fed grass-clover silage, and 8.4 mg of α -tocopherol for cows fed hay. The calculations are based on the yields determined by the end of each feeding period.

The total daily intake of β -carotene through the roughage was calculated to be 145 mg of β -carotene for cows fed grass-clover silage, whereas there was a decline from 395 to 198 mg of β -carotene for cows fed hay. The total daily output of β -carotene in milk from cows fed grass-clover silage was calculated to be 9.8 mg of β -carotene, and the total daily output in milk from cows fed hay declined from 7.3 to 4.4 mg of β -carotene. The decline in the concentration of β -carotene in milk from cows fed hay over the course of the study was related to the decline in the concentration of β -carotene analyzed in the hay.

From the above, it can be calculated that 8.6% α -tocopherol and 6.8% β -carotene are transferred from the feed to the milk when cows are fed grass-clover

silage, and 6.4% α -tocopherol and 1.8 to 2.2% β -carotene are transferred from the feed to the milk when cows are fed hay. These calculations are based on the assumption that α -tocopherol and β -carotene are derived solely from the roughage. From determinations of α -tocopherol from the feed intake and α -tocopherol in the milk in a study by Charmley et al. (1993), the degree of transfer of α -tocopherol from the feed to the milk was calculated to be 16% for cows fed alfalfa silage as roughage (51% alfalfa silage of TMR). This is almost double the transfer compared to that found in this study when cows were fed grass-clover silage, and more than twice the transfer found when cows were fed hay.

Charmley and Nicholson (1994) found that the degree of transfer to the milk of α -tocopherol supplements increased as the proportions of C18:2n-6 and C18:3n-3 increased as a result of feeding micronized soybeans compared with soybean meal. These findings were supported by studies by Atwal et al. (1990) and Goering et al. (1976), who found that increasing proportions of C18:2n-3 increased the transfer of α -tocopherol to the milk. The significantly higher concentration of C18:3n-3 in milk from cows fed grass-clover silage compared with milk from cows fed hay is therefore thought to be important for the better transfer of α -tocopherol from the feed to the milk.

The fatty acid composition of milk from cows fed grass-clover silage in this study was very similar to that of milk from cows fed alfalfa silage (Whiting et al., 2004), and the concentrations of C18:2n-6 and C18:3n-3 cannot explain the higher transfer of α -tocopherol from the feed to the milk in the study by Charmley et al. (1993) compared with this study. As well as differences in the concentration of the fatty acids C18:2n-6 and C18:3n-3 in milk from cows fed grass-clover silage and hay in this study, other circumstances may be important for the better transfer of α -tocopherol from the feed to the milk when cows were fed grass-clover silage. Jensen et al. (1999) found that the transfer of α -tocopherol and β -carotene from plasma to milk was independent of milk yield and milk fat content. This means that the differences in milk yield found for cows fed grass-clover silage and hay would not be expected to influence the transfer of α -tocopherol and β -carotene from plasma to milk differently in the 2 types of milk. It was suggested by Yeargan et al. (1979) that there is a physiological upper limit for the transfer of α -tocopherol into the milk of approximately 45 μ g of α -tocopherol/g of milk fat. Milk from cows fed grass-clover silage and hay contain approximately 11 to 12 μ g of α -tocopherol/g of milk fat, which indicates that both types of milk were below the physiological upper limit suggested by Yeargan et al. (1979).

Transfer of α -tocopherol and β -carotene from plasma to milk was influenced by genetic background and stage of lactation (Jensen et al., 1999), but because this study was designed to eliminate these effects, they are supposed to be of minor relevance in this study.

Other circumstances that can be suggested to be related to the differences in transfer of both α -tocopherol and β -carotene, are different conditions in the rumen, due to the different types of roughages and their digestibility, or be related to the mechanisms during milk production for the higher yielding cows fed grass-clover silage.

Influence of Fatty Acid Composition and Antioxidants on the Lipid Oxidation of Milk

The fatty acid composition is known to influence oxidative stability (Barrefors et al., 1995; Timmons et al., 2001; Havemose et al., 2004). The degree of unsaturation of the fatty acids also influences the accumulation of lipid hydroperoxides (Korycka-Dahl and Richardson, 1980; Richardson and Korycka-Dahl, 1983).

The higher concentration of C16:1n-7 (palmitoleic acid) and C18:1n-9 (oleic acid) in milk from cows fed hay did not result in a higher accumulation of lipid hydroperoxides. This indicates that the more unsaturated C18:3n-3 (linolenic acid) is important for the higher degree of lipid hydroperoxide formation occurring in milk from cows fed grass-clover silage as was also reported by Havemose et al. (2004). It can be explained by singlet oxygen attacking the double bonds of the unsaturated fatty acids, in which the number of double bonds are determining for the higher amount of lipid hydroperoxides that can be formed (Korycka-Dahl and Richardson, 1980; Richardson and Korycka-Dahl, 1983). Autoxidation will also take place along with the light-induced oxidation, and again the larger number of double bonds will give rise to a larger amount of lipid hydroperoxides formed because of the larger number of H-atoms allylic to the double bonds, which can be abstracted (Frankel, 1985; Belitz et al., 2004).

The higher concentration of β -carotene over the course of the study in milk from cows fed grass-clover silage did not result in a delay or a lower accumulation rate of dityrosine, meaning that higher concentrations of β -carotene in milk from cows fed grass-clover silage had a minor antioxidative effect.

There was a higher accumulation of lipid hydroperoxides observed in milk from cows fed grass-clover silage compared with the accumulation observed in milk from cows fed hay. Also, the pattern of accumulation of lipid hydroperoxides in milk from cows fed either grass-clover silage or hay tended to be different. The higher concentration of β -carotene in milk from cows fed grass-

clover silage during the study neither delayed nor reduced the accumulation of lipid hydroperoxides.

It is possible that the effect of β -carotene as antioxidant is part of the explanation for the less steep accumulation of lipid hydroperoxides reflected on the curve for milk from cows fed grass-clover silage in Figure 3 between 6 and 24 h of exposure to light, but it may more likely be caused by the limitations in riboflavin concentrations due to the ongoing degradation of riboflavin. Singlet oxygen is produced during exposure to light through the type II reaction (Bradley and Min, 1992; Kristensen et al., 2002), and because riboflavin also reacts with the formed singlet oxygen at a very fast rate (Huang et al., 2004), it can explain the easy degradation of riboflavin.

The concentration of riboflavin left to induce oxidation is slightly higher in milk from cows fed grass-clover silage compared with milk from cows fed hay, but the higher accumulation of hexanal is expected to be of greater importance, as argued by looking at the accumulation patterns of both lipid hydroperoxides (Figure 3) and hexanal (Figure 4). In Figure 4, the highest accumulation of hexanal occurs after 6 to 24 h, which correlates well with the data shown in Figure 3, where there is a less steep accumulation of lipid hydroperoxides after 6 h of light exposure. This could indicate that the reaction rate for formation of lipid hydroperoxides from activated riboflavin was lower than the reaction rate for the further formation of hexanal from lipid hydroperoxides, when the milk is exposed to fluorescent light for more than 6 to 9 h. The less steep accumulation of lipid hydroperoxide after 6 h, which is shown in Figure 3 for milk from cows fed grass-clover silage, can also be influenced by the effect of copper, which can catalyze the formation of the very reactive ($\text{LO}\bullet$) lipid oxyl radical from lipid hydroperoxides (Hill et al., 1977). But because the concentration of copper does not vary significantly in the 2 types of milk, the less steep accumulation of lipid hydroperoxides may be caused by the ongoing oxidation in general, which gives rise to the formation of an array of secondary oxidation products (Ford et al., 1986; Leland et al., 1987; Rao and Murthy, 1987).

After 6 h of light exposure only very little or no α -tocopherol was further degraded, which could indicate that when concentrations of α -tocopherol were too low (50 to 100 $\mu\text{g/L}$), it has no or limited effect as a chain-breaking antioxidant, scavenger of radicals, or as a quencher of singlet oxygen. On the other hand, the lower levels of α -tocopherol in the 2 types of milk did not give rise to sudden increases in lipid hydroperoxide accumulation. This indicates again that the limiting levels of riboflavin and the formation of secondary oxidation products may have a larger impact on the lower

accumulation of lipid hydroperoxides than the concentration of α -tocopherol.

In a previous feeding study (Havemose et al., 2004), no effect of the α -tocopherol on the prevention of lipid hydroperoxide formation in milk from cows fed grass silage was observed even though the initial concentration of α -tocopherol was higher than in the present study. This indicates that the antioxidative effect of α -tocopherol may be of lesser importance than the effect related to the composition of the unsaturated fatty acids. The mechanisms behind the antioxidative effect of α -tocopherol in emulsions as milk are appreciably different from the reaction mechanisms in bulk lipids (Frankel et al., 1994; Huang et al., 1996; McClements and Decker, 2000). It is known that the activity of α -tocopherol is influenced by the polarity, viscosity, and pH of the medium (Huang et al., 1996; Nenadis et al., 2003), and that the presence of synergists (e.g., other phenols, ascorbic acid, carotenes, amines, amino acids) is expected to increase the antioxidative potency of α -tocopherol by regeneration or metal chelation (Böhm et al., 1997; Zhang and Omaye, 2000).

Influence of Antioxidants on Protein Oxidation

In a study by Havemose et al. (2004), the higher levels of α -tocopherol, β -carotene, lutein, and zeaxanthine in milk from cows fed grass silage compared with milk from cows fed corn silage was reported to be of importance in delaying protein oxidation. In this study we cannot determine if there is an effect on protein oxidation because the antioxidants are present in the same concentrations in the 2 types of milk.

Antioxidative Effect of α -Tocopherol on the Oxidative Stability of Milk

The effect of α -tocopherol on the oxidative stability of milk can be questioned because several studies show contradicting effects depending on concentration in milk and the diet of the cow. The studies below are not related to the effect of α -tocopherol in preventing light-induced oxidation, but are related to development of oxidized flavor. Studies have been conducted by Schingoethe et al. (1979) and Charmley and Nicholson (1994), in which supplemental dietary α -tocopherol was fed to the cows and the concentrations in the milk reached 890 and approximately 600 $\mu\text{g/L}$, respectively. These levels of α -tocopherol in the milk were not effective in improving the oxidative stability of the milk. Studies by Goering et al. (1976) and Focant et al. (1998) showed improved oxidative stability of the milk when cows were fed supplements of α -tocopherol to the diet, and the concentration of α -tocopherol in the milk reached 2,100

and 2,670 $\mu\text{g/L}$, respectively, in those 2 studies. This indicates that concentrations of α -tocopherol in the 2 types of milk in this study needed to be approximately 5 times higher to improve the oxidative stability measured as oxidized flavor.

The effect of supplementing the diet with α -tocopherol is also dependent on the type of roughage. Nicholson and St-Laurent (1991) found that oxidized flavor in milk from cows fed corn silage could be eliminated within 1 wk of supplementation with α -tocopherol, whereas α -tocopherol supplementation had no influence on the oxidized flavor for the first 3 wk of supplementation on milk from cows fed alfalfa silage. The differences observed in the oxidative stability can be related to differences in the fatty acid composition of the milk from cows fed corn silage and alfalfa silage, respectively, because milk from cows fed corn silage generally contain less linolenic acid than milk from cows fed alfalfa silage (Havemose et al., 2004; Whiting et al., 2004).

In conclusion, the composition of the lipids in milk, especially the concentration of linolenic acid, seems to be an important factor for formation of lipid oxidation products in milk. The ratios of fatty acids are influenced by the type of feeding and thus influence the oxidative stability of the milk. The higher transfer of α -tocopherol may be related to the higher uptake of polyunsaturated fatty acids in milk from cows fed grass-clover silage. However, the role of α -tocopherol and β -carotene on the oxidative stability of the milk appears to be less important than the variation in the fatty acid profile, and because the concentration of α -tocopherol in the 2 types of milk were very similar, it could not be determined if the α -tocopherol showed different antioxidative effects against lipid oxidation (Type II) and dityrosine formation (Type I).

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