Assessment of the action spectrum for photooxidation in full fat bovine milk

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Abstract

The action spectrum for photooxidation in full fat bovine milk was measured. Samples of milk with air or argon in headspace were exposed to narrow wavelength bands of light in the range 400-700 nm. Photooxidation in terms of off-flavors was measured by a sensory panel, volatile compounds by headspace solid phase micro extraction (SPME)-GC-MS, and photobleaching of photosensitizers in milk (riboflavin, protoporphyrin IX and a chlorophyllic compound) by front face fluorescence spectroscopy. The action spectrum deviated significantly from the absorption spectrum of milk. Significant oxidation was induced by wavelengths around 400 nm and 500-650 nm in milk with air in headspace. Argon in headspace gave significant oxidation also at 700 nm. It is suggested that protoporphyrin IX and chlorophyll are responsible for oxidation induced by wavelengths > 500 nm, and that also riboflavin is contributing from 400 - 500 nm.

Key words:
Action spectrum, photooxidation, bovine milk, sensory analysis, photosensitizers
Highlights

- An action spectrum for photooxidation in full fat bovine milk has been obtained
- The action spectrum deviates significantly from the absorption spectrum of milk
- The action spectrum is based on sensory analysis and measured volatile compounds
- Violet, yellow, orange and red light induces most oxidation in full fat milk
- Protoporphyrin IX, a chlorophyll compound and riboflavin are responsible photosensitizers
1. **Introduction**

Light induced oxidation is one of the main factors limiting shelf life of milk. Exposure to visible light leads to off-flavors related to oxidation of proteins and lipids due to excitation of photosensitizers among which riboflavin has been recognized to play a major role (Bradley, Lee and Min, 2003). Riboflavin and beta-carotene are the two most prominent light absorbers in milk. They are present in full fat cow milk (typically 3.5 % fat) at the approximate concentrations 141μg/100g and 20μg/100g, respectively (Lindmark-Månsson, Fondén, & Petterson, 2003), and consequently they absorb light at about the same level in the violet and blue region (400-500 nm) of the visible spectrum (Airado-Rodríguez, Intawiwat, Skaret, & Wold, 2011). Of the two absorbers, only riboflavin is a photosensitizer contributing to photochemical reactions leading to photooxidation. Beta-carotene absorbs light in the same region as riboflavin, and it has therefore been suggested to protect against photooxidation since less light then reaches riboflavin (Skibsted, 2000; Airado et al., 2011). Beta-carotene also works as a quencher of the highly reactive singlet oxygen (Foote, 1968).

During the recent years it has been reported that naturally occurring residues of tetrapyrroles in milk play an important role in photooxidation of dairy products. This was first reported for cheese and butter (Wold, Veberg, Nilsen, Iani, Juzenas, & Moan, 2005) and later for milk (Intawiwat et al., 2010; Airado et al., 2011). The exact identification of these tetrapyrroles remains, but protoporphyrin IX (PpIX) is one certain photosensitizer with notable contribution. In addition, there are at least four more photoactive compounds, most likely chlorophyll derivatives (Wold et al., 2006). The concentrations of some of these compounds have been tentatively determined in butter by front face fluorescence spectroscopy (Wold & Lundby, 2007) and are very low (0.02ppm for PpIX). The compounds
are fat soluble and when the concentrations for fat in butter are used for milk with 3.5% fat, the concentrations are in the range 0.8 ppb, about 250 times less than the concentration of riboflavin. All tetrapyrroles absorb strongly in the violet region (the Soret band), and then weaker in the blue to red region. Since riboflavin is not photoactive for wavelengths longer than about 500 nm, photooxidation in milk induced by longer wavelengths has so far been ascribed to these tetrapyrroles (Airado et al., 2011).

Riboflavin is typically a type I photo sensitizer, thus generating radicals either by abstraction of an H-atom or donation of an electron through a direct reaction with double bonds in proteins and lipids (Foote, 1968; Foote, 1976; Huvaere, Cardoso, Homem-de-Mello, Westermann, & Skibsted, 2010), whereas e.g. chlorophylls act primarily as type II sensitizer with the generation of the highly reactive singlet oxygen as a result (Foote, 1968). Singlet oxygen has also been detected after riboflavin induced photooxidation in skim milk (Bradley, 2003), indicating that Type I and II photoreactions are competing with each other.

Abundance of oxygen might favor photoreactions of Type II, while low concentrations of oxygen can lead to domination of Type I reactions. For milk, this is relevant to consider since it can be packed with different levels of oxygen in headspace. The two reaction types might result in different oxidation products, and thereby different volatile compounds and sensory off-flavors (Lee & Min 2009; Dalsgaard et al., 2010; Huvaere et al., 2011; Airado et al., 2011). An action spectrum is defined as the efficiency with which electromagnetic radiation produces a photochemical reaction plotted as a function of the wavelength of the radiation. The action spectrum of a material is usually quite similar to its absorption spectrum, but not always. It depends on the absorption spectrum of the photoactive compounds, but will also
be influenced by other absorbing compounds, light scattering properties, as well as how the photoactive compounds are distributed in the microstructure of the material. The action spectrum can be used as a basis to explain the underlying photoreactions and to develop antioxidants and packaging materials with optimal protective properties.

The objective of the work presented in this article was to experimentally obtain the action spectrum in the visible range for photooxidation in full fat bovine milk. As a response for photooxidation we used sensory analysis and headspace SPME-GC-MS. Photobleaching of the photosensitizers riboflavin, protoporphyrin IX and a chlorophyllic compound was monitored by front face fluorescence spectroscopy. The results are presented followed by a discussion considering factors such as the effects of different light absorbing compounds, light scattering properties, and likely photoreactions in the microstructure of milk.

2. MATERIALS and METHODS

2.1 Overview

Three different light exposure experiments were conducted. In the first, milk samples in different atmospheres were exposed to two broad regions of the visible spectrum (blue and orange). These samples were then analyzed by SPME-GC-MS. In the second and third experiment milk samples were exposed to light of narrower wavelength bands separated by 50 nm. After light exposure, milk samples were analyzed by SPME GC-MS, profiled by the sensory panel and analyzed by front face fluorescence spectroscopy.
2.2 Samples and light exposure conditions

Commercially produced, homogenized, pasteurized bovine milk with 3.5% fat content, packed in gable-top cartons, was obtained from a local dairy company (Tine, Oslo, Norway). The milk for each experiment was obtained from a single batch and stored at 4 °C in the dark before being repacked in plastic trays. Milk from all cartons was mixed before samples were made.

0.4 L milk was filled in transparent, high-density polyethylene (HDPE) trays (5*8.5*13 cm)(Promens AS, Kristiansand, Norway). A magnet for stirring was put into each tray. Each of these trays was placed in the middle of black polyethylene trays (14.5x20.5x7.5 cm) that were sealed with a top web consisting of PET/PE/ethylene vinyl alcohol/PE (Wipak) using a 511VG tray-sealing machine (Polimoon, Kristiansand, Norway). The surface of the milk samples was 117 cm². Two broadband 575 W metal Halide lamps (Osram HMI 575W/SE, Osram, Munchen, Germany), which have a relatively flat emission spectrum in the visible region, were used as light source. The light intensity was measured and adjusted according to a calibrated spectrometer (Apogee Spectroradiometer, Apogee Instruments Inc., Roseville, CA). All light intensity adjustments and light exposure experiments were carried out in a cold-storage chamber at 4 °C.

In the first experiment (exposure to blue and orange light) the milk samples were packed with air, Ar or N₂ in headspace. The packages were covered with two types of colored plastic filters; a blue filter transmitting light between 375 and 550 nm (“69 Super Brilliant Blue”, manufactured by Rosco, Stamford, CT), and an orange filter transmitting light from about 530 to 750 nm (Orange transparent film based on PET (Ciba Specialty Inc., Basel,
Switzerland). The filters were thoroughly described by Airado et al. (2011). Two samples were covered with blue, two with orange and two samples were stored in the dark. This was done for samples in Ar, N\textsubscript{2} and air, a total number of samples of 18. Light intensity at surface of exposed samples was 1.6 W/m\textsuperscript{2}. Exposure time was 20 h. These samples were analyzed for volatile oxidation products by SPME-GC-MS. The colored plastic filters allowed light exposure of the entire surface of the milk samples.

In the second experiment the gas in the headspace was air or argon. The sealed black trays were covered on top with black carton with a 5 cm diameter circular whole in the middle. Over this hole, optical filters were placed to generate light of different wavelengths. Circular (D=5 cm) interference filters with bandwidth 40 nm and center wavelengths at 400, 450, 500, 550, 600, 650 and 700 nm (Filter set 03IFS008, Melles Griot, CA, USA) were used. Forty nm bandwidth means that a filter transmits a band of 40 nm around the center wavelength. For instance the 500 nm filter transmits light in the region 480-520 nm. The filters transmitted about the same share of light, and the combination of the exposure lamps and filters resulted in a light intensity at the milk surface of approximately 1.0 W/m\textsuperscript{2}. With this setup only 20 cm\textsuperscript{2} of the sample surface was exposed. During storage time, the milk was stirred every 6 hour to circulate the milk. The exposure time was 22 h. The samples were analyzed by the sensory panel and fluorescence spectra were measured immediately after light exposure, while samples for SPME-GC-MS were frozen at -80 °C and shipped at dry ice overnight and stored again at -80° C until analysis.

The storage experiment was run over two days. First day the following samples were run (number indicates wavelength, capital letter indicates atmosphere, Argon/aiR): 400A, 450R,

A third similar light exposure experiment similar to the second was repeated after two months, but exposure time was increased to 72 h, and only samples with argon in headspace were included. These samples were analyzed with SPME-GC-MS and front face fluorescence spectroscopy. Samples stored in air were not measured due to limited resources.

2.3 Sensory analysis

The milk samples were evaluated by a trained sensory panel at Nofima AS (Ås, Norway) using a modified quantitative method as described in ISO standard 6564 (ISO, 1985). The panel consisted of ten trained people. The panelists were selected and trained according to the recommendations in ISO standard 8586-1 (ISO, 1993). The sensory laboratory was designed according to guidelines in ISO standard 8589 (ISO, 1988) with separate booths and electronic data registration (CSA, Compusense Five, version 4.80, Guelph, ON, Canada). Prior to the assessments, the panel went through a training session with three samples, two fresh controls stored in the dark and one sample exposed to 650 nm light for 20 h, to agree on the definition of each attribute and variation in attribute intensity on the scale. Six attributes were selected to describe the sensory properties of the stored milk: acidulous odor and flavor (high intensity in these attributes indicates freshness), sunlight odor and flavor, which are related to oxidation of proteins, and rancid odor and flavor, including all odors and
flavors associated with rancidity (grass, hay, candle, and paint), as described in ISO standard 22935-2 (ISO, 2009). Odor is obtained by smelling the samples, flavor is obtained by tasting.

Samples (20 mL aliquots) were served in plastic cups (tested to be free from interfering odors and flavors), and all samples were served at room temperature (20°C). Unsalted crackers and lukewarm water were available for rinsing the palate between samples. The coded samples were served in a randomized order by sample, assessors, and replicate. The samples were evaluated for all six attributes by each assessor. Each assessor was allowed to work at an individual pace. The panelists recorded their results on a 15 cm, non-structured, continuous scale, with the left side of the scale corresponding to the lowest intensity and the right side of the scale corresponding to the highest intensity. The computer transformed the responses into numbers between 1.0 (low intensity) and 9.0 (high intensity). The sensory evaluation was completed within two consecutive days. First day included training and evaluation of first half of the samples. Second day included profiling of the second part of the sample set.

2.4 Analysis of volatile compounds

Immediately after light exposure, 4 subsamples of 10 mL were taken from each milk sample and filled on flasks and sealed. They were frozen at -80°C before they were shipped overnight from Norway to Aarhus University in Denmark for analysis. One sample from each treatment was used for global analysis of volatiles operating the MS in scan mode and the next three samples were run in single ion monitoring (SIM) mode, monitoring specific ions referring to pentanal, pentanol, and hexanal according to Dalsgaard et al. (2010) with some modifications.
Four mL of milk were transferred to a 10 mL vial, added of deionized (18.2 MΩ) filtered water (0.22 µm) with an isotopic hexanal D12 (50 ng) from Fluka (Steinham, Germany). The samples were sealed with teflon coated lids before the headspace was analysed for volatile compounds using a Carboxen/PDMS SPME fibre with a film thickness of 30 µm from Supelco (Bellefonte PA, USA), which was incubated at 50 °C for 30 min in the headspace of each sample. Desorption of the sample from the fibre was performed into the inlet of a GC 7890A from Agilent Technologies (Waldbonn, Germany) equipped with a HPS-MS column from Agilent J&W Scientific (Folsom, CA, USA) and coated with a non-metal 5%-phenyl 95%-dimethylpolysiloxane phase with the dimensions: 0.25 mm i.d., 0.25 µm, 30 m. Helium was used as carrier gas with a constant flow of 1.2 mL/m in. The splitless injector was kept at 250 °C. An SPME injection sleeve liner from Supleco, (Bellefonte, USA) with an inner diameter of 0.75 mm was applied. The column temperature was programmed to stay at 40 °C for 4 min followed by an increase from 40 to 120°C with a rate of 5 °C/min, a hold time of 5 min, and a subsequent temperature gradient from 120 to 300 °C with a rate of 20 °C/min. Mass spectral analysis was performed in SIM mode according to the ions determined by the use of a standard for each compound on a quadrupole MSD 5975 (Agilent Technologies, Germany) with a quadrupole temperature of 150 °C and a fragmentation voltage of 70 eV. The ion source temperature was 230 °C, and the interface was 280 °C. Quantification was performed relatively using and external standard curve with concentrations of 1-500 ng/mL bovine milk of each compound including the isotopic hexanal to avoid in-between-sample variation on individual compounds. Samples were measured in triplicates.
2.5 Measurement of sensitizer photobleaching

Front face fluorescence spectroscopy was used to monitor the photo degradation of tetrapyrroles and riboflavin. Fluorescence emission spectra were measured on intact milk samples using a spectroscopic system previously described by Wold et al. (2005). Aliquots (15 mL) of each sample were filled into sample cuvettes, which exposed a circular surface with a diameter of 5 cm for measurement. The fluorescence emission spectra were measured in the region 500-750 nm for excitation at 410 nm (10 nm bandwidth interference filter, Oriel 59285), using cutoff filter at 475 nm (Melles Griot 03FCG065). Excitation at 410 nm was used to maximize fluorescence from tetrapyrroles. Riboflavin has excitation maxima at 370 and 450 nm; however, the emission for excitation at 410 nm is also strong. Exposure time was 1 s for all measurements.

To ease interpretation and analysis of the fluorescence spectra with regard to protoporphyrin IX and chlorophyllic compounds, an iterative mathematical algorithm was applied to remove the large fluorescence signal from riboflavin. This was done by polynomial fitting, a routine originally introduced to remove background fluorescence from Raman spectra (Lieber & Mahadevan-Jansen, 2003). In the present study a polynomial degree of 3 was chosen and an iteration number of 50 were used for the fitting procedure. The algorithm was applied on the 550-750 nm region of the emission spectra.

2.6 Statistical Analysis

Significance testing of the sensory analysis was performed by General Analysis of Variance (General AOV/AOCV) using Statistic 9 (Analytical Software, Tallahassee, FL) to establish significant differences, followed by Tukey’s multiple-comparisons test.
3. Results

3.1 Milk exposed to blue and orange light

In the first experiment milk was stored in different atmospheres and exposed to light in the violet-blue (<375-550 nm) and orange-red (530-750 nm) regions. A scan of all milk samples screening for all volatile secondary oxidation products only showed 1-pentanol, pentanal and hexanal increasing in some samples exposed to light. Heptanal was detected but did not increase after light exposure, whereas the two protein oxidation products as dimethyl disulfide (DMDS) and benzaldehyde were not detected at all. The levels of pentanal and 1-pentanol were higher in milk exposed to orange versus blue light (Fig. 1A-B). Exposure to blue light gave no increase in these two volatiles compared to the milk kept in dark. Note also that formation of pentanal under orange light was higher in nitrogen and argon compared to air. The level of hexanal was highest in milk exposed to blue light. The samples kept in air and exposed to blue light showed higher level of hexanal than the samples kept in argon or nitrogen, and the samples with nitrogen and exposed to blue light showed higher hexanal concentration than those kept in argon, showing a higher dependence on oxygen than the generation of pentanal and 1-pentanol in that region. The samples exposed to orange light had higher levels of hexanal than the dark control, but no difference was observed between oxygen, nitrogen or argon atmosphere. This indicates a need for a more in-depth analysis of flavor/off-flavor in different region of the visible spectra.

3.2 Generation of an action spectrum

An action spectrum was measured after light exposure at certain wavelengths throughout the visible region (400, 450, 500, 550, 600, 650, and 700 nm) in terms of sensory responses
and volatiles oxidation products. Sensory responses for both air and argon atmosphere are shown in Fig. 2. Milk has a natural acidulous flavor (Fig. 2B) and it is clear that light exposure with 400, 550, 600, 650, and 700 nm reduced the natural occurring acidulous flavor in the milk and induced sunlight flavor the most (Fig. 2A). For milk stored in argon, all these wavelengths gave significantly higher scores for sunlight flavor (or oppositely lower scores for acidulous flavor) compared to samples exposed to 450 nm and 500 nm, as well as those stored in the dark. There was a similar trend for milk stored in air, but in this case exposure to 650 nm gave significantly different scores from the control (dark) but not from samples exposed to 450 nm and 500 nm (not shown). It is noteworthy that the sensory responses to 450 and 500 nm were not significantly different from the controls, neither in milk exposed to light with air or argon in head space. This might be an effect of short storage time (22 h), and it is reasonable to think that they would differ significantly after prolonged light exposure time. However, from the present data it is clear that light of 450 and 500 nm did not damage the milk much. Fig. 2 C and D show sensory scores for sunlight and acidulous odor. The trends are the same as for the flavors; high scores for sunlight odor are obtained for 400 nm and in the 550-700 nm range. Especially in the region 600-700 nm, the sunlight odor scores are higher for samples stored in argon compared to those stored in air. Only at 650 nm the score was significantly higher for argon. Much the same was the case for acidulous odor; at 650 nm the acidulous score was significantly lower for the sample stored in argon. Results for rancid odor and flavor are not shown since these scores were very low, and no significant differences between storage conditions were found. Milk treated the same way was subjected to analysis by SPME/GC-MS but very low signals were obtained and no significant differences were found between exposed samples and the
dark controls. This was probably due to the narrow wavelength range of the light and thus less intense light than in the first experiment and lack of the required sensitivity of the SPME/GC-MS, which could not compete with human organoleptic analysis in sensitivity. The exposure time was therefore increased to 72 hours (third experiment) to obtain results from the GC-MS analysis. The levels of 1-pentanol showed a clear curvature with high levels at 400, 550, and 600 nm whereas the levels at 450-500 nm were the same for the dark control (Fig. 3A). The levels were also lower at wavelengths >600 nm. The generation of 1-pentanol thus resembles the generation of sunlight flavor whereas the levels of hexanal were lower with higher standard deviations at all wavelengths and the curvature was less clear (Fig. 3B).

3.3 Photobleaching of photosensitizers

When photosensitizers are involved in photoreactions, either as part of type I reactions or when reacting with singlet oxygen after type II reactions, the photosensitizers are degraded, also called photobleaching. It has been shown that the photoactive fraction of photosensitizers is often identical with the fluorescent one; that is, when there is no longer any fluorescence, the sensitizer is deactivated (Juženiene, Nielsen, Moan, 2006). Fluorescence spectroscopy therefore enables the indirect measurement of the initiation and extent of photooxidation. Fig. 4 shows the fluorescence emission spectra of three milk samples stored in argon. The background fluorescence from riboflavin has been subtracted from the spectrum, leaving only the spectral contributions from PpIX and Chl. PpIX has an emission peak at about 635 nm, and the Chl peak is at 677 nm. It is clear that compared to the sample stored in the dark, the light exposed samples contained less of these two photosensitizers. The loss of Chl was most pronounced in milk exposed to light at 650 nm. Less degradation occurred with light exposure at 400 nm. For PpIX the pattern was slightly
different; it was more degraded at 400 nm and less at 650 nm. The decomposition of the two photosensitizers in milk stored in air or argon was followed as function of wavelength and illustrated relatively to the content in the dark controls (Fig. 5A-B). Loss of Chl was observed for all investigated wavelengths but more at 600-650 nm than at 400-550 nm and at 700 nm. PpIX loss was highest at 400 nm and lowest at 700 nm. The photobleaching of riboflavin was also measured by fluorescence at 530 nm (Fig. 5C). Riboflavin absorbs in the 400-500 nm region (in the visible), and a slight reduction compared to the controls was observed after exposure to the wavelengths 400 and 450 nm, especially with air in headspace. Note, however, that the percentage changes in the riboflavin peak were very small compared to the two other sensitizers, and the significance of the variation is less certain.

Figures 2, 3 and 5 reveal some common trends in variation between sensory properties, volatile compounds and the degradation of photosensitizers. The simple correlations between 1-pentanol and sunlight and acidulous flavor were 0.88 and -0.88, respectively (for samples stored in argon). It can be assumed that both PpIX and Chl contributed to photooxidation and it is therefore reasonable to estimate correlations based on a combined effect of the two sensitizers. When we use the average of the values of Chl and PpIX concentrations for each sample in Fig. 5A-B, correlations with sunlight and acidulous flavor were 0.84 and 0.85, respectively, for all samples stored in air and argon. The corresponding correlations were higher for samples stored only in argon, 0.90 and 0.91, respectively, and consequently lower for samples stored in air (0.79 and 0.81). Concentrations of the sensitizers correlated well (-0.88) also with 1-pentanol (only for argon).

The measured concentration of riboflavin in the different samples correlated poorly with 1-pentanol (-0.22), acidulous flavor (0.12) and sunlight flavor (-0.08).
4. Discussion

The aim of the present study was to generate an action spectrum for photooxidation in milk. The combination of sensory analysis, front face fluorescence spectroscopy and SPME-GC-MS gave a good estimate of this spectrum, and there was good agreement between the measurements. Josephson (1946) did systematic light exposure experiments with milk and concluded that light in the 590-630 nm region induced the strongest sunlight flavor. This is in agreement with our results. Airado et al. (2011) showed that milk exposed to orange light (550-700 nm) induced significantly higher sensory off-flavor scores than what blue light (400-530 nm) did at the same intensity. They also observed a higher score for sunlight and rancid flavor in milk exposed to orange light packed with nitrogen (N\textsubscript{2}) in headspace than milk exposed to blue light. Also Intawiwat (2010) observed high sensory scores of sunlight flavor for milk exposed to red and orange light. In the present study, the sensory scores for sunlight flavor and odor at 550-650 nm were not higher than at 400 nm. But the results explain why a broad-banded orange filter results in higher off-flavor scores than a broad-banded blue filter (as in Airado et al., 2011); all wavelengths within the orange filter contribute significantly to photooxidation, while a large share of the wavelengths transmitted by a broad banded blue filter (450-500 nm) induces less photooxidation.

The generation of 1-pentanol at different wavelengths corresponds well with the observed sunlight flavor and odor measured by the sensory panel, and thereby supports the obtained sensory spectra. Higher levels of pentanal in milk packed in argon compared to air (in orange
light) (Fig. 1) might also support the sensory finding that the scores for sunlight and acidulous attributes were different for argon and air around 650 nm.

Hexanal formed in all samples but was found significantly higher in sample with air than with Ar or N₂ when exposed to blue light (300 - 580 nm). This is consistent with previous results obtained by Webster et al. (2011) also finding the blue region most pronounced when focusing on hexanal generation. Highest formation of hexanal in the blue region indicates a significant effect of a riboflavin sensitized reaction, which is in accordance with previous findings after addition of riboflavin to milk (Lee & Min, 2009).

Pentanal and 1-pentanol were generated only in milk exposed to orange light (530-700 nm), and it is reasonable to conclude that they are generated through another photochemical reaction mechanism than hexanal, a reaction that involves PpIX and/or Chl. It has previously been suggested that pentanal could be favored by a type I mechanism and hexanal could be formed primarily through a type II mechanism (Dalsgaard et al., 2010; Lee & Min, 2009; Yang, Lee, Lee, Lee, 2007). As riboflavin has been suggested primarily to be a type I sensitizer (Huvaere et al., 2010), PpIX and Chl may be of importance in the blue region as well, also corresponding very well with bleaching of these two sensitizers in this region.

Bleaching of a photosensitizer is an indication of its activation, and is used as a marker within photodynamic cancer therapy to control the effect of treatment (Dysart & Patterson, 2006). In this study it is clear that the wavelengths 450 nm and 500 nm caused least photobleaching of the sensitizers PpIX and Chl, which is in agreement with their low absorption in this region (Fig. 6B). This also harmonizes with less photooxidation initiated at these wavelengths. The high correlations between the degradation of photosensitizers and the sensory responses, suggest that these sensitizers are the main contributors to the photosensitized oxidation in
milk when exposed to light in the region 400-700 nm. The pronounced oxidation due to light around 550-650 nm corresponds well with the strong bleaching of Chl in the same wavelength region.

Milk is a system where the action spectrum is very different from the absorption spectrum (Fig. 6A). The absorption spectrum is dominated by a broad peak from 400-500 nm caused by riboflavin and beta-carotene. The absorption spectrum for milk in Fig. 6 is measured in reflectance mode and is also affected by light scattering, thereby the offset level from 550-700 nm. The difference between the absorption and action spectrum does not support the earlier accepted idea that riboflavin is the major active photosensitizer in milk (Bradley & Min, 1992). Especially the oxidation induced by wavelengths longer than 500 nm is difficult to explain based on riboflavin sensitized oxidation.

The action spectrum is a result of the present light absorbing compounds. Fig. 6B shows the absorption spectra of the apparently most important light absorbers in milk with respect to photooxidation; riboflavin, PpIX, chlorophyll a and beta-carotene. It is not clear whether the chlorophyllic compound in milk is chlorophyll a or a chlorophyll breakdown product, for instance pheophorbide a. The absorption spectra for the two are anyway quite similar. The absorption spectrum of chlorophyll a fits quite well with the degradation of Chl shown in Fig.5A. Strong degradation in the 600-650 nm range corresponds with absorption peaks in the same region. The degradation at 400 nm was less than what would be expected from the large absorption around this wavelength. PpIX absorbs strongly at 409 nm and then throughout the visible region up to 645 nm. This also fits well with the breakdown shown in Fig. 5B, except that a stronger degradation at 400-450 nm could be expected. The main reason for less photobleaching of PpIX and Chl at 400-500 nm is most likely the strong
absorption by beta-carotene and riboflavin in this region. Since the concentrations of these are much higher than those of the tetrapyrroles, the share of light absorbed by the tetrapyrroles will probably be reduced. Around 400 nm the absorption of both beta-carotene and riboflavin is half of their absorption peak at 450 nm. It is then reasonable to suggest that strong photooxidation induced by light around 400 nm is due to the sensitizers PpIX, Chl and riboflavin. The limited oxidation by 450-500 nm might be due to a protective inner-filter effect of beta-carotene. The strong photooxidation from 550 nm to 650 nm is induced mainly by PpIX and Chl, and this region is not protected by beta-carotene.

The light absorption properties alone would make whole milk look orange due to beta-carotene and riboflavin (Frisvad, Christensen, & Jensen, 2007). The white appearance is due to the strong light scattering of all wavelengths caused by fat the globules and protein micelles. The tetrapyrroles are fat soluble and most likely located in the fat globules, while riboflavin is water soluble and located in the water phase. This gives a rather heterogeneous system, which might favor certain photochemical pathways. It is likely that a major part of photoreactions with tetrapyrroles will involve lipids, while riboflavin sensitized oxidation is more prone to involve proteins. This might be a reason why riboflavin apparently has lower impact on photooxidation than what would be expected based on the concentration. In this study, no volatile protein oxidation products like dimethyl disulfide was observed, maybe due to lower detection limit for that volatile compound. Radical transfer between lipid and proteins and vice versa can occur (Schaich, 1980; Dalsgaard et al., 2010) so even though one photosensitizer may favor one substrate over another, flavor compounds will not exclusively be due to oxidative changes in that substrate.
The optical scattering properties of milk has been studied thoroughly (Frisvad et al., 2007), and it is shown that the scattering coefficient increases exponentially from long wavelengths in the red to the shorter towards the violet. This means that more of the violet and blue light will be reflected at the surface of the milk compared to longer wavelengths. A smaller share of blue and violet light will therefore take part in photochemical reactions. It also means that red light will penetrate deeper into the milk, and the probability of photoreactions for these wavelengths will be high.

The action spectrum obtained in this study is for whole milk with 3.5 % fat. For skim milk and low fat milk it will probably look quite different. Less fat means correspondingly lower concentrations of beta-carotene and tetrapyrroles. Riboflavin will then probably be more dominating in the photochemical reactions, and the action spectrum might more closely resemble the absorption spectrum of riboflavin.

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6. References


Figure captions

**Fig. 1** Generation of secondary lipid oxidation products: pentanal A), 1-pentanol B), and hexanal C) in milk samples exposed to blue (300 - 580 nm) and orange (520 - 750 nm) light with \( \text{N}_2, \text{O}_2 \) (air) or \( \text{Ar} \) in headspace during light exposure of full fat milk. Error bars indicate standard deviation.

**Fig. 2** Upper panel: Sensory scores for in milk exposed to light of different wavelengths. Upper panel: Sunlight flavor and acidulous flavor for milk stored with argon in headspace. The vertical bars at each value indicate the minimum significant difference for the values to be statistically significant. Scores that have bars that do not overlap along the \( y \)-axis are significantly different. Lower panel: Sunlight odor and acidulous odor in milk exposed to light of different wavelengths. Filled black symbols: Milk stored with argon in headspace. White symbols: Milk stored with air in headspace.

**Fig. 3** Generation of 1-pentanol A) and hexanal B) in milk exposed to light at different wavelength for 72 h. Interference filters with bandwidth 40 nm and center wavelengths at 400, 450, 500, 550, 600, 650 and 700 nm were applied during light exposure of full fat milk with argon in headspace.

**Fig. 4** Fluorescence spectra from milk with argon in headspace stored in the dark (solid line), exposed to 400 nm (dashed line) and 650 nm (dotted line). Peak at 635 nm is protoporphyrin IX, peak at 678 nm is a chlorophyllic substance.
Fig. 5 Bars indicate concentration of light sensitizing compounds after light exposure at different wavelengths and under headspace of argon or air. Bars are normalized with respect to concentration in samples stored in the dark.

Fig. 6 A) Absorption spectrum for full fat bovine milk measured in reflectance (grey curve) and action spectrum for milk (sunlight odor for milk with argon in headspace) (dark blue curve). B) Absorption spectra for β-carotene, riboflavin, protoporphyrin IX, chlorophyll a and pheophorbide a reported as typical concentrations in whole milk. Note that the scale of the A-axis for tetrapyrroles is zoomed.