JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 10197−10204 **10197**

Active Photosensitizers in Butter Detected by Fluorescence Spectroscopy and Multivariate Curve Resolution

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In this study, fluorescence excitation and emission matrices and multivariate curve resolution (PARAFAC) were used to detect and characterize active photosensitizers spectrally in butter. Butter samples were packed under high (air) and low oxygen (<0.05%) atmospheres and exposed to violet, green, or red light. Six photosensitizers were found: riboflavin, protoporphyrin, hematoporphyrin, a chlorophyll *a*-like molecule, and two unidentified tetrapyrrols. By estimation of relative concentrations, we could follow how each sensitizer was photodegraded as function of wavelength, oxygen level, and time. The degradation rate of protoporphyrin, hematoporphyrin, chlorophyll *a*, and one of the tetrapyrrols correlated well (0.83−0.91) with the formation of sensory measured oxidation. The results suggest that mainly type I photoreactions were responsible for the degradation of photosensitizers in both high and low oxygen atmosphere. Type II photoreactions (generation of singlet oxygen) were involved in the oxidation of butter stored in air. The study shows that PARAFAC modeling of fluorescence landscapes is an excellent tool for studying photooxidation in complex systems.

KEYWORDS: Photooxidation; photosensitizer; fluorescence spectroscopy; PARAFAC; butter

INTRODUCTION

Dairy products are in general susceptible to photooxidation due to natural contents of photosensitizers. The best protection against photooxidation and subsequent quality degradation is to shield the products from all ultraviolet radiation and visible light. This might conflict with market demands; consumers usually want to see the product. Black or other non-transparent packaging materials are, therefore, not always desired from a marketing point of view. To develop some kind of transparent packaging with the minimum of adverse effects, it is necessary to have detailed knowledge of the presence and properties of the photosensitizers in the product (*1*).

Riboflavin has been regarded as the active photosensitizer in dairy products (*2*). When riboflavin is exposed to UV radiation or visible light up to about 500 nm, it can initiate photooxidation processes of types I and II (*3*). The violet and blue parts of visible light have therefore been regarded as the most harmful. Recent results, however, have demonstrated that dairy products have natural contents of effective visible photosensitizers, such as protoporphyrin and chlorophyll-like molecules (*4*). These molecules also absorb light in the UV and violet region, with absorption peaks around 410 nm (the Soret band). In addition, they absorb light throughout the visible region, weak in the green and yellow, but pronounced in the red above 600 nm. Recently, it was shown that the sensory measured photooxidation in cheese induced by red light did not differ significantly from that induced by blue light (*5*). The greatest quality degradation was caused by violet light, while green and yellow light (500−600 nm) gave less effects. Similar results have been obtained for milk (*6*). These results support the hypothesis that porphyrins and chlorophylls, not only riboflavin, are active photosensitizers in cheese.

The natural occurrence of porphyrins and chlorins in dairy products was discovered by the use of front face fluorescence spectroscopy. This method enables rapid, nondestructive, and simultaneous measurement of riboflavin, porphyrins, and chlorophylls and offers the possibility to monitor in detail how these molecules are degraded by light exposure. The method is very sensitive; photodegradation can be detected after minutes of light exposure and can, therefore, be used to study the initiation of photooxidation. High correlations between fluorescence spectra and sensory measured attributes such as oxidized, acidic, and sunlight flavor have been established (*4*, *5*, *7*), indicating that the method is relevant for rapid recording of quality related to photooxidation.

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10.1021/jf0621166 CCC: $33.50
Published on Web 11/24/2006

In the previously mentioned studies, it became clear that photodegradation of protoporphyrin and chlorophylls was more closely related to photooxidation than degradation of riboflavin. This is also supported by light exposure experiments on Havarti cheese (*8*, *9*) where it was noticed that exposure to yellowish light (without violet and blue light) gave comparable formation of secondary oxidation products as exposure to white light. In those data, it was observed that a porphyrin fluorescence peak co-varied well with the formation of secondary oxidation products.

Presently, we assume that there are at least five different light sensitizers present in dairy products: riboflavin, chlorophyll *a* and *b*, protoporphyrin, and a second porphyrin, which might be hematoporphyrin (*4*). We do know the basic spectral properties of these molecules in the product and how they act as photosensitizers. Much of this knowledge can be found in medical related literature since similar chemicals are used as agents in photodynamic cancer therapy (PDT) (*10*). It is not clear whether all or only a few of them are active in the photooxidation process in dairy products. It is important to understand the role of the different photosensitizers to be able to develop the best possible product protection.

In this study, we use multivariate curve resolution (MCR) to study the effect of light exposure on each particular photosensitizer in butter. MCR deals with the problem of extracting pure spectra from measurements of mixtures. This is an inherently difficult problem for both mathematical and chemical reasons. First of all, most curve resolution methods assume that the Beers law is valid. That is, the spectrum of each chemical analyte must have the same shape in different mixtures, and the response measured must be the sum of such single analyte contributions. Also, it is mostly required that the signal is linearly related to the concentration, although this is less critical. Traditional curve resolution deals with so-called two-way data (i.e., each sample gives rise to one spectrum, and for a set of samples, a matrix (two-way data) is obtained (*11*). Apart from the chemical assumptions, there are also additional mathematical aspects that restrict the use of two-way curve resolution. To be able to resolve underlying spectra from two-way data, it is necessary that some wavelengths are selective (*12*) or that similar special conditions are satisfied. Otherwise, it is not possible to obtain unambiguous results. Three-way curve solution overcomes the mathematical problems of two-way curve resolution. In three-way curve resolution, each sample gives rise to a matrix, and data from several such samples can be concatenated in a box of data, a three-way data structure. From such data, unambiguous curve resolution is possible through the use of the so-called PARAFAC (parallel factor analysis) model (*13*, *14*). The underlying assumptions for PARAFAC are similar to the ones in two-way curve resolution, but there are no additional mathematical assumptions needed such as selectivity, etc. Fluorescence data in the form of excitation emission matrices (EEMs) have been shown to follow the PARAFAC model (*15*, *16*). From sets of EEMs of mixtures, it is therefore possible to determine the pure excitation and emission spectra as well as the relative concentrations of the chemical analytes measured.

In this paper, we present an approach that effectively can point out active light sensitizers in dairy products or other complex mixtures. It is based on four steps: (i) a designed light exposure experiment involving different colors of light, variation in oxygen availability, and time of exposure, (ii) measurement of fluorescence EEMs and sensory evaluation of the samples, (iii) separation of pure spectral components belonging to the photosensitizers by MCR, and (iv) relating the estimated concentrations of these pure components to the sensory responses. Butter was chosen as product in this study because of its widespread use and its relatively high concentrations of all actual photosensitizers.

Table 1. Light Exposure Design[a]

	high oxygen				low oxygen			
exposure time (h)	violet	green	red	dark	violet	green	red	dark
6	F	F	F		F + S	F	F + S	
12	F + S	F + S	F + S		F + S	2F + S	F + S	
18	F	F	F		F + S	F	F + S	
24	F	F + S	F + S		2F + S	2F	2F + S	
30	2F	F	F		F	F	F	
36	F + S	F + S	F		2F	F + S	2F	
42	F	2F	F		F	F	F	
48	F	F + S	F + S	2F + S	F	F	F	2F + S

[a] F: fluorescence measurement and S: sensory evaluation. 2F means that fluorescence was measured on two different samples at this design point.

MATERIALS AND METHODS

Materials. Commercially produced butter was purchased (TINE BA, Oslo, Norway) in 500 g block packages. The packages were from two different production batches. The blocks were sliced into sample bars of size 9 cm × 12 cm × 0.8 cm, packed according to the design described next, and stored in a dark and cool room (4 °C) before light exposure.

Experimental Design. The butter samples were stored according to the light exposure design summarized in **Table 1**. The purpose of this design was to expose and photodegrade the sensitizers more or less selectively, in such a way that the variation in the concentration of each photosensitizer differed as much as possible from the others. This would result in spectral variation, enabling separation of the different fluorescence peaks by use of MCR. At the same time, it was important that the exposed samples spanned a reasonable range of sensory attributes related to photooxidation. After preliminary experiments, we arrived at a design exploiting different colors of light, different exposure times, and different oxygen levels.

Three colors (violet, green, and red) were obtained by using plastic films manufactured by Rosco (Rosco, Stamford, CT) with different spectral properties. The films used were 19 Super Fire red, 89 Moss green, and 357 Royal Lavendel (violet). These filters were chosen because of their relatively narrow transmission bands in the visible spectral region.

Two oxygen concentration levels were chosen. The samples were packed in a Polimoon tray-packaging machine model 511VG. Low oxygen packages were flushed with 100% nitrogen, giving a residual oxygen level not exceeding 0.05% in the headspace. The high oxygen samples were packed the same way but in an aerated atmosphere. The sample packages were covered with the colored films and stored in light from two broadband 575 W metal halide lamps (OSRAM HMI 575 W/SE, Osram GmbH, München, Germany) in a refrigeration room at 4 °C. In addition to the colored filters, a UV filter (3114 UV Tough filter) was used to block UV contributions transmitted through the red and green filters. No UV block was used together with the violet filter. **Figure 1** shows the three resulting exposure spectra measured at sample distances from the light source. The fluence rate at the sample surface was approximately 1.1, 1.1, and 1.9 W/m^2 for violet, green, and red light, respectively. Fluence rates were measured by a calibrated spectrometer (Apogee Spectroradiometer, Apogee Instruments Inc., Logan, UT) and integrated in the 300−800 nm region.

Samples were put into light at different times so that the exposures ended simultaneously. Two butter bars were stored in each package: one for sensory analysis, and one for fluorescence analysis.

Sensory Analysis. The sensory evaluation was performed by a sensory panel consisting of 11 selected and independent assessors. A descriptive test (*17*) was carried out. Prior to the analysis, the panel was trained in the definition and intensities of the chosen attributes

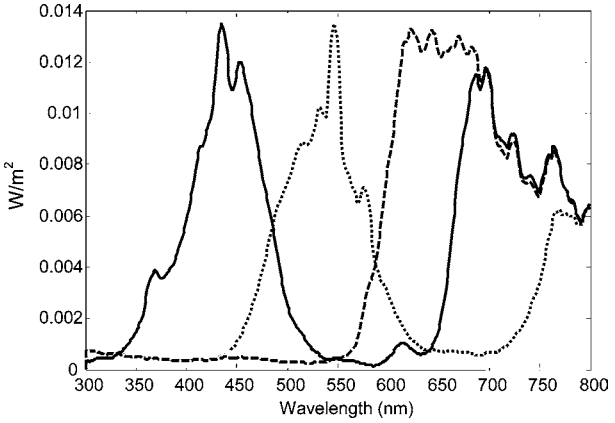

Figure 1. Spectral profiles of exposure light measured at sample positions. The profiles are a result of light source and color filters. Violet (solid), green (dotted), and red (dashed) light.

using butter with varying sensory properties (light exposed and non-exposed). The attributes oxidized flavor and odor, sunlight flavor and odor (related to oxidized proteins, typical training reference is milk exposed to sunlight), and acidic flavor and odor (fruity acids, fresh/acid/sweet); also, whiteness, color tone, color intensity, butter odor and flavor, sweet flavor, bitter flavor, and salty flavor were measured. Each assessor was served each butter sample on a cardboard plate. The serving order was randomized according to sample and assessor. Water and crackers were provided to cleanse the palate between samples. A continuous non-structured scale was used for the evaluation of sensory attributes ranging from the lowest intensity of each attribute with a span from 1.0 to the highest intensity of 9.0. Each judge evaluated the samples at individual speeds on a computer system for direct recording of data (CSA, Compusense, Ver. 4.2, Guelph, ON, Canada). The sensory scores for each sample of butter were obtained by averaging the individual scores for the 11 subsamples. Twenty-one samples were evaluated, and the measurements had to be carried out over 2 days due to capacity limitation. The butter bars were cut into 11 3 × 3 square centimeter subsamples. Each judge replicated the analysis of each sample. Two replicates were used. The replicates were from two separate samples stored under the same conditions. All sensory evaluated butter samples belonged to the same production batch.

Fluorescence Spectroscopy. Fluorescence EEMs were recorded with a Perkin-Elmer LS50B (Norwalk, CT) luminescence spectrometer with a Hamamatsu PMT detector (model R3896, Japan). This detector is particularly sensitive in the red spectral region. The instrument was equipped with a front face solid sample accessory with 60° sampling geometry. The emission side was scanned with a 0.5 nm step size from 580 to 720 nm, and excitation was scanned with a 3 nm step size from 350 to 452 nm. The excitation slit width was 10 nm, and the emission slit width was 6 nm. A cutoff filter at 515 nm was used in front of the detector. The butter samples were placed in a circular cuvette ($D = 15$ mm) and covered by quartz glass. The sample temperature was approximately 6 °C. The total scanning time per sample was approximately 15 min. For illustrative purposes, we recorded excitation spectra for non-exposed butter in the region 250−500 nm for the emission wavelengths of 530 and 670 nm, as well as emission spectra in the range of 450−720 nm for excitation at 380 nm. For comparison, similar measurements were done on a sample of Jarlsberg cheese (Swiss-like).

Single emission spectra were also collected by an optical bench system thoroughly described elsewhere (*4*, *5*). This system was used because of the high signal-to-noise ratio in the most interesting spectral region (the red). In this paper, these emission spectra were used just for illustrative purposes in **Figures 4** and **7**. The emission spectra were measured directly on the light exposed surface of circular ($D = 5$ cm) samples of the butter.

Recording of Pure Component Fluorescence Spectra. To evaluate the estimated pure profiles extracted by PARAFAC, we recorded butter samples spiked with either protoporphyrin IX ($C_{34}H_{34}N_4O_4$, Sigma CAS 553-12-8), hematoporphyrin ($C_{34}H_{38}N_4O_6$, Sigma CAS 14459-29-1), chlorophyll *a* ($C_{55}H_{72}N_4O_5Mg$, Sigma C6144), or chlorophyll *b* ($C_{55}H_{70}MgN_4O_6$, Fluka EC No. 2082724), all obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany). The pure chemicals were simply stirred into butter at 21 °C. The concentration of the added chemicals equaled 0.04 mg/kg (0.08 mg/kg for hematoporphyrin). The samples were subsequently cooled to 4 °C. Fluorescence EEMs of the spiked samples as well as non-spiked butter samples were measured as described previously. Pure spectra were obtained by subtracting the spectrum of a non-spiked sample from the spiked. The reason for not measuring the chemicals in the pure state (not added in butter) is that the spectral profiles can shift with wavelength depending on, for instance, the matrix pH.

Data Modeling. PARAFAC decomposes the fluorescence landscapes into a number of trilinear factors, *f*. The principle behind the PARAFAC decomposition is to minimize the sum of squares of the residuals e_{ijk}, according to the equation

$$\chi_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk}$$

$$j = 1,..., J;\ i = 1,..., I;\ k = 1,..., K;\ f = 1,..., F$$

The element χ_{ijk} represents the fluorescence intensity for sample i, at excitation wavelength j and emission wavelength k. The fluorescence landscapes are thus decomposed into sample scores, a_{if}, excitation loadings b_{jf}, and emission loadings c_{kf}, for each factor f, also called PARAFAC components. The residual e_{ijk} contains the variation not explained by the PARAFAC model.

Two types of model validation were used: (i) the estimated excitation and emission loadings were visually judged and compared to spectral measurements of the expected true pure components and (ii) the model was validated by split-half analysis (*15*), where the strategy is to split the data set into two halves and make a PARAFAC model on each half. Because of the uniqueness of the PARAFAC solution, one should obtain the same result on the two subsets as on the complete set in case the right number of components is chosen.

PARAFAC analysis was performed on a total data set of 60 fluorescence landscapes based on the design in **Table 1**. Initial modeling showed that the variation in some components was of too little scale and confounded so that the estimated spectra got mixed up and modeled as one component by PARAFAC. To avoid this, we added one extra sample, a butter sample spiked with protoporphyrin IX. This was acceptable since we were certain that protoporphyrin IX was present in the butter. In the split-half validation procedure, the spiked sample was used in both subsets.

The PARAFAC analysis was performed in Matlab ver. 7.1.0 (The Mathworks Inc., Natick, MA) by use of the PLS_Toolbox (Eigenvector Research Inc., Wenatchee, WA).

RESULTS AND DISCUSSION

Sensory Analysis. Of the sensory attributes, we have chosen to present results for oxidized flavor, sunlight flavor, and acidic flavor since these span the main sensory variation and are closely related to photooxidation. The other sensory attributes either correlated closely with these (like butter flavor, sweet flavor, and bitter flavor) or little variation was measured (as for salty flavor and color tone whiteness). **Figure 2** shows the main sensory properties of the exposed butter samples. A wide range in the sensory attributes was obtained. For oxidized flavor, the whole range from 1 to 9 was applied by the sensory panel, indicating that severe oxidation had taken place in some of the samples. Violet light induced the most pronounced quality degradation, followed by red light and then green light. This is in accordance with previously reported results on cheese (*4*, *5*). Hansen et al. (*6*), who evaluated the effect of light exposure from differently colored light on oxidation in milk, also found that green light had the least adverse effect. They

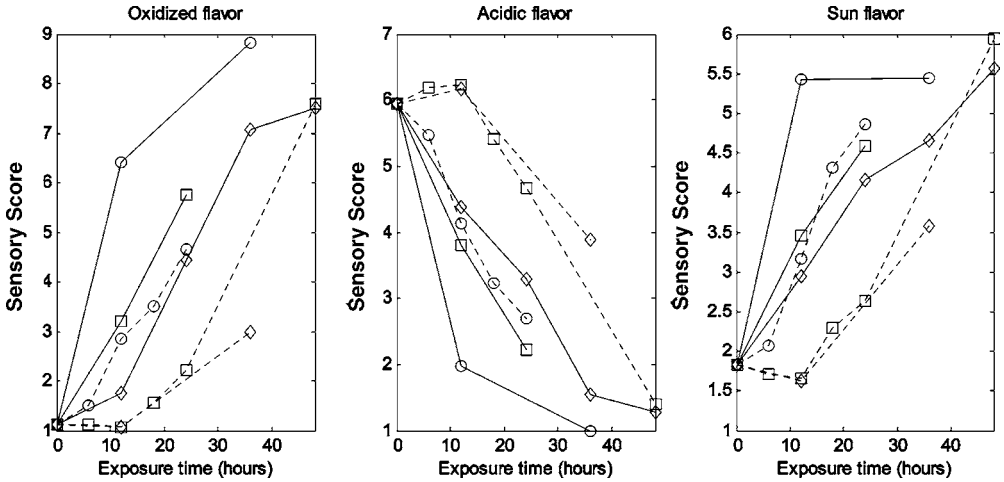

Figure 2. Sensory responses from light exposed butter. Solid lines: storage in air and dotted lines: storage in nitrogen. Exposure to violet (○), red (□), or green light (◇). Start value at time 0 is a sensory value for the sample stored in the dark. Mean standard deviation for each mean value is 1.2 for oxidized flavor, 1.5 for sunlight flavor, and 1.3 for acidic flavor.

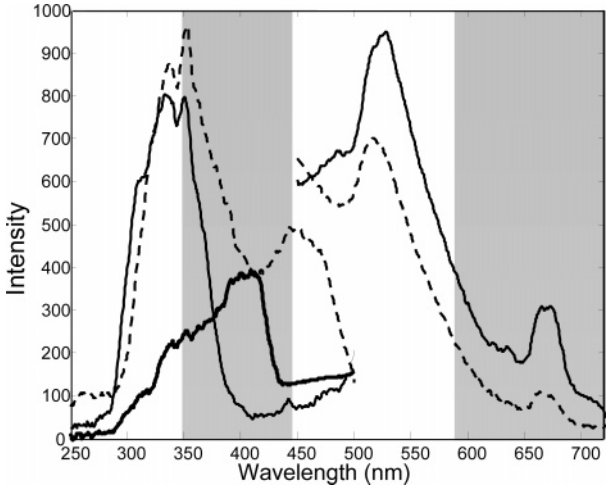

Figure 3. Overview of fluorescence excitation (at emission at 530 nm) and emission spectra (at excitation at 380 nm) for butter (solid line) and Swiss cheese (dotted line). Solid bold line is the excitation spectrum for butter at emission 670 nm. The shaded regions indicate the ranges where EEMs were recorded.

found that pink light was slightly worse, while white fluorescent light (which included violet) gave the most rapid formation of off-flavors.

The rather rapid changes in sensory properties have been observed in similar studies on cheese. Odor changes in sliced Havarti cheese and Swiss-like cheese already after 4−6 h of exposure to soft white fluorescent light have been reported (*5*, *9*). Significant flavor changes in milk after exposure to fluorescent light were observed after 2−6 h (*6*, *18*, *19*). Note that there was no difference in sunlight flavor for air packed samples exposed to violet light for 12 and 36 h. The sunlight flavor after 36 h exposure was probably suppressed by the strong oxidized flavor. This has been seen in similar studies on butter (*20*). It seems that the presence of oxygen promoted the oxidation process significantly, regardless of the color of the light. However, in spite of very low oxygen contamination of the nitrogen atmosphere, a pronounced oxidized flavor was registered also in these samples. This is discussed next in connection with the spectroscopic analysis.

Fluorescence Spectroscopy. Figure 3 shows an overview of excitation and emission spectra from butter and cheese. The riboflavin emission peak at 520−530 nm is clear in both butter and cheese. The concentration of porphyrins and chlorophyll (peaks between 620 and 680 nm) in butter appears to be higher than in the cheese due to a higher fat content. This is in accordance with previous findings (*4*). The excitation spectrum for cheese for emission at 530 nm shows the two characteristic broad riboflavin peaks at approximately 340 and 440 nm (*21*). The corresponding excitation spectrum for butter appears to lack the peak at 440 nm. This was surprising and might be a matrix effect caused by butter containing only about 14% water as compared to approximately 46% in the cheese. The butter excitation spectrum for emission at 670 nm had a different profile with a broad peak around 410 nm, the Soret absorption band typical for porphyrins and chlorophyll. The shaded regions in the figure indicate the ranges where EEMs were recorded. These regions contain distinct features for the light sensitizers of interest in this study.

Figure 4 shows the main features of the fluorescence emission spectra measured with the optical bench system. There are six evident peaks. The broad peak around 530 nm stems from riboflavin. The peak at about 620 nm is a porphyrin, possibly hematoporphyrin. The two peaks at 635 and 705 nm belong to protoporphyrin, and the double peak at 661 and 672 nm probably originates from chlorophylls (*4*). All the peaks were degraded more or less according to exposure time, color of light, and oxygen availability. For violet light, there was a prominent decrease of all peaks. Protoporphyrin was almost completely degraded. This agrees with the absorption properties of the sensitizers; they all absorb in the violet region. Note that the sensitizers are less degraded in air than in nitrogen. Green light also degraded all the peaks but at a slower rate as compared to violet light. Especially the porphyrins were now degraded more quickly in nitrogen. Red light induced just a negligible reduction of riboflavin but a very clear degradation of the porphyrins and chlorophylls, especially in low oxygen atmosphere. This agrees with the absorption properties of the molecules; riboflavin does not absorb red light, while the others do.

PARAFAC Analysis. In advance, we expected to obtain a PARAFAC solution with five components, one for each of the light sensitizers we assumed was present in butter. It turned out that seven components were required for a sound solution. The same solution was obtained for both subsets of EEMs. **Figure 5** shows the excitation and emission loadings for six of the seven components. The seventh factor, which is not shown, was related to temperature variation. During the 15 min it took

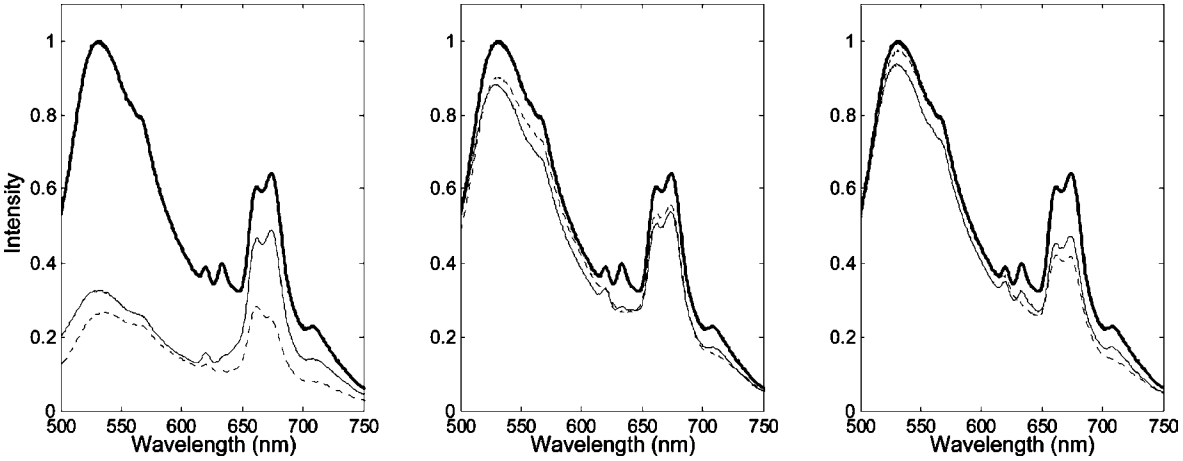

Figure 4. Emission spectra of butter exposed for 36 h to violet (left), green (middle), and red light (right). Bold solid line: sample stored in dark; solid line: stored in air; and dotted line: stored in nitrogen.

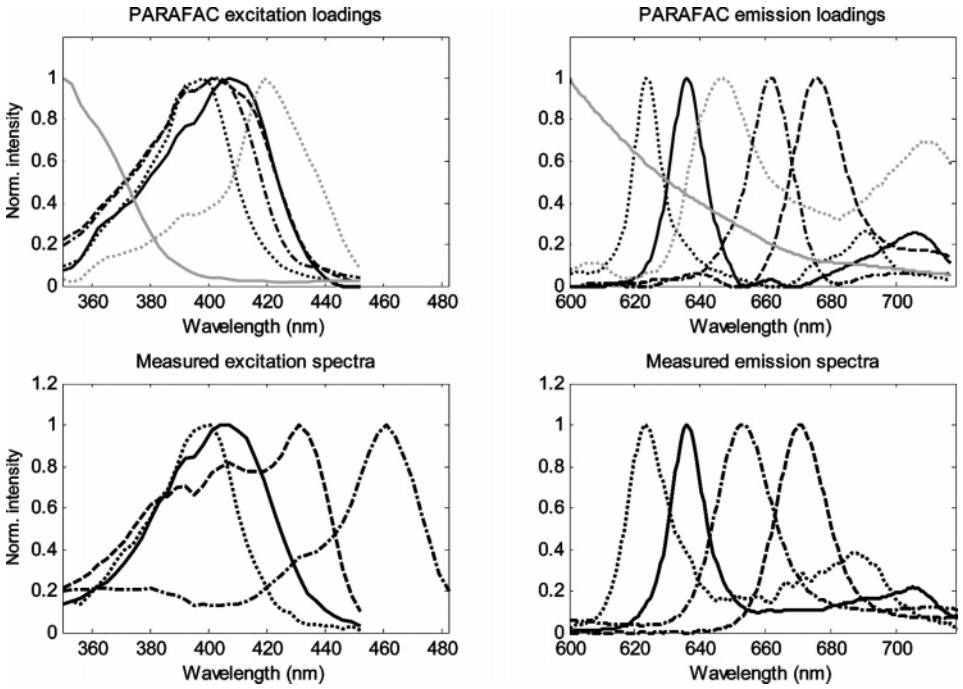

Figure 5. Upper panel: pure excitation and emission components estimated by PARAFAC. Hematoporphyrin (dotted line), protoporphyrin IX (solid line), chlorophyll *a*-like compound (dashed line), compound X1 (dashed and dotted line), compound X2 (dotted gray line), and riboflavin (solid gray line). Lower panel: measured pure excitation and emission spectra in butter, hematoporphyrin (dotted line), protoporphyrin IX (solid line), chlorophyll *a* (dashed line), and cholorphyll *b* (dashed and dotted line). All spectra are normalized to maximum intensity = 1.0.

to record each EEM, the temperature in the butter sample increased slightly. When the temperature increases, the fluorescence intensity drops. This systematic variation was picked up by the model. It is preferable to keep temperature stable during recording to avoid this. The other factors described chemical constituents. The emission and excitation loadings for two of the components were very similar to the measured pure spectra of protoporphyrin and hematoporphyrin. The emission loading with peak at 672 nm closely resembled that of the measured chlorophyll *a*. However, the excitation loading for the same component differed from that of chlorophyll *a*. This means that the detected compound may be some kind of derivative of chlorophyll *a*, with comparable photosensitizing properties. The component with emission peak at 661 nm is clearly not chlorophyll *b*, as assumed. Chlorophyll *b* had an emission peak around 653 nm and an excitation peak at 460 nm. This illustrates that interpretation of only emission spectra, as was done by Wold et al. (*4*), might be misleading. In this paper, we will refer to the 661 nm emission peak as X1. The component with the long tail decreasing toward longer wavelengths for both emission and excitation is riboflavin. A surprising result was the discovery of a sixth component with an excitation peak at 420 nm and emission peaks at about 655 and 710 nm. The spectral properties are typical for tetrapyrrols and very similar to, for instance, tetra (3-hydroxyphenyl) porphyrin, which has been found to be an efficient photosensitizer (*22*). A close visual inspection of the fluorescence landscapes revealed this peak, which was very small as compared to the others. We do not know the exact properties of this compound and will in this paper refer to it as X2. The excitation spectra of the different components are extremely overlapping. It is, therefore, satisfactory that the PARAFAC model was able to separate them.

Since the PARAFAC solution seems sound and meaningful, it is of interest to study the third set of loadings representing the relative concentrations of the components. To show these

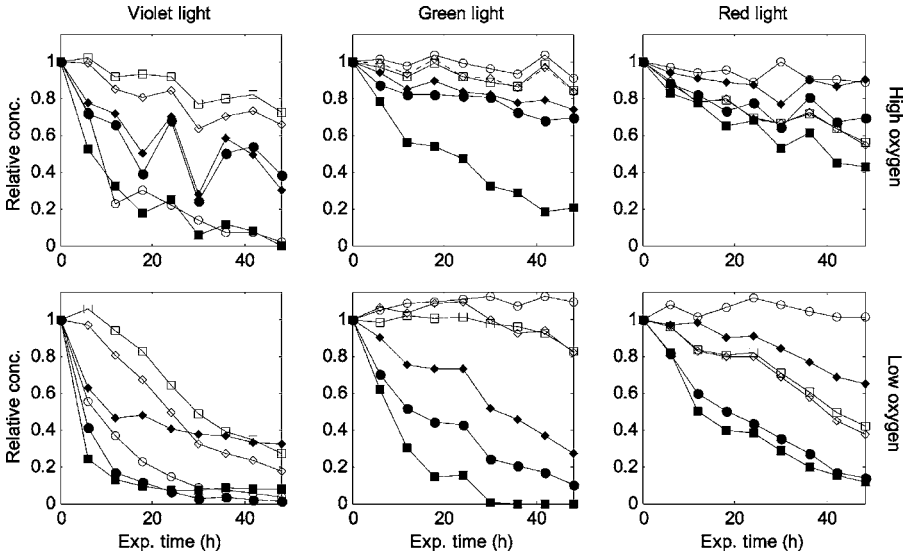

Figure 6. Estimated relative concentrations (normalized) of photosensitizers in light exposed butter according to storage conditions. Riboflavin (○), protoporphyrin (■), hematoporphyrin (◆), chlorophyll *a* (◇), compound X1 (□), and compound X2 (●).

in one figure, the concentrations for each component were normalized with regard to the concentration of a non-exposed sample (i.e., all components have a start concentration of 1.0). The two butter batches were normalized separately since the start concentrations were different. **Figure 6** shows how each sensitizer was photodegraded under different conditions. The trends judged previously by simple visual inspection of the spectra are verified: all sensitizers were degraded during storage in light. Surprisingly, the degradation was generally more rapid in low oxygen atmosphere. Riboflavin was degraded only by violet light, which corresponds well to its absorption properties. All the other sensitizers were also degraded by violet light since they all have their major absorption band in the violet. For exposure to green light, the picture is different. In samples stored in air, protoporphyrin was degraded the fastest, about 80% reduction after 48 h. With a low oxygen concentration, protoporphyrin was completely degraded after 30 h, and hematoporphyrin and X2 were degraded by 70−90% after 48 h. This makes sense since both protoporphyrin and hematoporphyrin absorb throughout the visible region, although less in the green than in the violet and red. Red light degraded all sensitizers, except riboflavin. This is reasonable since tetrapyrroles have absorption peaks in the red region.

There are a few irregularities in the results presented in **Figure 6**. Some of the degradation curves are not continuously decreasing during storage. Some values even exceed 1.0, the value chosen for a sample stored in the dark. We regard these errors as smaller inaccuracies mainly in the experimental measurements.

General Discussion. Photooxidation mechanisms and degradation of photosensitizers during light exposure are complex. It is beyond the scope of this work to explain in detail all results. However, a brief discussion is possible based on our findings. **Figure 2** shows that samples stored in air were sensory degraded more rapidly than samples packed under nitrogen atmosphere. This was probably caused by the formation of singlet oxygen (type II photoreactions). Luby et al. (*23*) studied the oxidation products formed during light exposure of butter and found that cholesterol undergoes oxidation via singlet oxygen attack, as well as by free radical mechanisms. Veberg et al. (*20*) showed that both type I and II reactions are active in butter. They added β-carotene to samples of butter stored in air and measured a pronounced inhibition of photooxidation as compared to samples without β-carotene. β-Carotene is an excellent singlet oxygen quencher (*24*).

Most photosensitizers are photolabile. 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 (*25*). The fact that porphyrins and chlorophylls are less degraded in the presence of oxygen indicates that two different processes take place. When much oxygen is present, singlet oxygen is generated (type II photoreactions). With little oxygen present, the light energy absorbed is used to directly decompose the sensitizers, and free radicals are formed (type I photoreactions). In most cases, it is difficult to determine the relative significance of type I and II partitioning reactions. In the present case, however, it seems that type I reactions were the mechanism responsible for degradation of the photosensitizers, as well as for the main photooxidation process in nitrogen stored butter. For butter stored in air, type I reactions were probably still the main sensitizer breakdown mechanism, while singlet oxygen (type II) attacked unsaturated fatty acids and other molecules in the butter. The arguments for this hypothesis are as follows: the photosensitizers were degraded generally more in nitrogen atmosphere than they were in air. At light exposure in air, singlet oxygen will be formed and instantly react with surrounding molecules. Type I degradation will result in spectrally dependent degradation according to the absorption properties of the photosensitizers. If only one sensitizer is present, as it usually is in PDT related studies, the action spectrum (the breakdown of sensitizers as function of exposure wavelength) would be similar for type I and II reactions. In our case, we have a mixture of different photosensitizers. When singlet oxygen attacks the sensitizers, we would therefore expect a degradation more independent of the color of light. A singlet oxygen molecule generated by, for instance, protoporphyrin is likely to react with, for instance, riboflavin. The fact that degradation of the sensitizers in air follows their absorption properties suggests that type I reactions are dominant.

In studies related to PDT, similar photodegradation of the photosensitizers has been observed (*26*, *27*). The degradation is generally stronger with an increasing amount of oxygen in the system (*28*). In our case, however, it is the opposite. One explanation might be that lipid molecules in butter are relatively more available and susceptible to singlet oxygen oxidation than

Table 2. Correlations between Sensory Measured Oxidation and Photodegradation of Light Sensitizers in Light Exposed Butter

	oxidized flavor score	
light sensitizer	stored in air ($n = 10$)	stored in nitrogen ($n = 11$)
riboflavin	0.57	0.62
protoporphyrin	0.90	0.73
hematoporphyrin	0.78	0.86
chlorophyll *a*	0.72	0.83
tetrapyrrol X1	0.61	0.63
tetrapyrrol X2	0.91	0.83

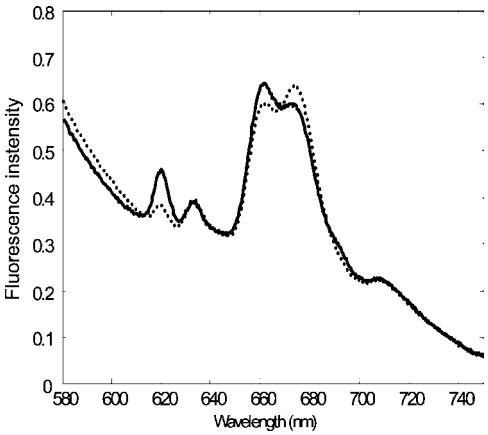

Figure 7. Fluorescence emission spectra from non-exposed butter samples from the two different batches.

are living cells and tissue. Singlet oxygen might therefore react more easily with the lipids than with the photosensitizers. Another reason is that the concentrations of photosensitizers in butter are much lower (below 0.1 ppm) than what is used during PDT.

When relating the breakdown of photosensitizers with sensory properties, it is reasonable to treat samples stored under the two oxygen levels separately since conditions for the photoreactions were different. **Table 2** summarizes these relations by simple correlations. For samples stored in air, the degradation of protoporphyrin, hematoporphyrin, and X2 correlated well with oxidized flavor. For samples stored in nitrogen, the highest correlations were obtained for X2, hematoporphyrin, and chlorophyll *a*. These correlations do not prove that some photosensitizers are more active than others, but they indicate candidates and potential markers that are better suited than others. It is not the actual concentration of sensitizers that is informative but the degree of degradation. Initial concentrations will vary from batch to batch, illustrated by the two production batches of butter used in this study (**Figure 7**).

This study has shown that PARAFAC modeling of fluorescence EEMs is an excellent tool for studying photooxidation in dairy products. The method can, guided by a priori knowledge and control measurements, identify photosensitizers in a complex product with a high degree of certainty. The relative concentrations of these sensitizers, and consequently the photodegradation, can be monitored in detail. The PARAFAC model requires sufficient variation in the spectral data to make possible the extraction of the pure components. This variation can be obtained by systematic light exposure experiments, natural sample variation, and, if possible, inclusion of known, pure spectra. An established PARAFAC model has predictive abilities; it can be used to estimate the concentrations of a number of chromophores in unknown samples, as demonstrated by, for instance, Moberg et al. (*29*). The model can therefore be used as a valuable and effective tool in future work to study how the different photosensitizers are affected by different light, oxygen levels, storage time, etc. If the spectral profiles of the photosensitizers are the same in other dairy products (cheese, milk, sour cream, etc.), the same model might also be used on these products.

The photosensitizers detected in this study are typical not only for butter. They are probably present in most dairy products since the same fluorescence peaks can be observed in milk, cream, cheese, etc. (*4*). The degree of activation of the different sensitizers might vary from product to product. In practice, for the development of protective packaging materials, it is important to be aware of these sensitizers. Limited light exposure experiments with colored light and subsequent sensory analysis will serve as good guidance. More exact knowledge about the photooxidative effect of different spectral regions can be obtained by using, for instance, the approach outlined in this paper. A rough conclusion is, however, that of the visible light, green and yellow light give the less adverse effects.

ACKNOWLEDGMENT

Fruitful discussions with our colleague Gjermund Vogt are gratefully acknowledged.

LITERATURE CITED

(1) Skibsted, L. H. Light-induced changes in dairy products. *Bull. Int. Dairy Fed.* **2000**, *346*, 4−9.

(2) Borlet, F.; Sieber, R.; Bosset, J. O. Photooxidation and photoprotection of foods, with particular reference to dairy products. An update of a review article (1993−2000). *Sci. Aliments* **2001**, *21*, 571−590.

(3) Aurand, L. W.; Boone, N. H.; Giddings, G. G. Superoxide and singlet oxygen in milk lipid peroxidation. *J. Dairy Sci.* **1977**, *60*, 363−367.

(4) Wold, J. P.; Veberg, A.; Nilsen, A. N.; Juzenas, P.; Iani, V.; Moan, J. The role of naturally occurring chlorophyll and porphyrins in light induced oxidation of dairy products. A study based on fluorescence spectroscopy and sensory analysis. *Int. Dairy J.* **2005**, *15*, 343−353.

(5) Wold, J. P.; Veberg, A.; Lundby, F.; Nilsen, A. N.; Moan, J. Influence of storage time and color of light on photooxidation in cheese. A study based on sensory analysis and fluorescence spectroscopy. *Int. Dairy J.* **2006**, *16*, 1218−1226.

(6) Hansen, A. P.; Turner, T. L.; Aurand, W. L. Fluorescent light-activated flavor in milk. *J. Milk Food Technol.* **1975**, *38*, 388−392.

(7) Wold, J. P.; Jørgensen, K.; Lundby, F. Nondestructive measurement of light-induced oxidation in dairy products by fluorescence spectroscopy and imaging. *J. Dairy Sci.* **2002**, *85*, 1693−1704.

(8) Andersen, C. M.; Wold, J. P.; Mortensen, G. Light-induced changes in semi-hard cheese determined by fluorescence spectroscopy and chemometrics. *Int. Dairy J.* **2006**, in press.

(9) Mortensen, G.; Sørensen, J.; Stapelfeldt, H. Effect of modified atmosphere packaging and storage conditions on photooxidation of sliced Havarti cheese. *Eur. Food Res. Technol.* **2003**, *216*, 57−62.

(10) Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. Photodynamic therapy. *J. Natl. Cancer Inst.* **1998**, *90*, 889−905.

(11) Lawton, W. H.; Sylvestre, E. A. Self modeling curve resolution. *Technometrics* **1971**, *13*, 617−633.

(12) Manne, R. On the resolution problem in hyphenated chromatography. *Chemom. Intell. Lab. Syst.* **1995**, *27*, 89−94.

(13) Harshman, R. A.; Lundy, M. E. PARAFAC: parallel factor analysis. *Comput. Stat. Data Anal.* **1994**, *18*, 39−72.

(14) Bro, R. PARAFAC. Tutorial and applications. *Chemom. Intell. Lab. Syst.* **1997**, *38*, 149−171.

(15) Andersen, C. M.; Bro, R. Practical aspects of PARAFAC modeling of fluorescence excitation−emission data. *J. Chemom.* **2003**, *17*, 200−215.
(16) Lee, C.; Kim, K.; Ross, R. T. Trilinear analysis for the resolution of overlapping fluoresecence spectra. *Korean Biochem. J.* **1991**, *24*, 374−379.
(17) ISO. *Sensory analysis−methodology−flavor profile methods*, *6564*; International Organization for Standardization: Geneva, 1985.
(18) deMan, J. M. Possibilities of prevention of light-induced quality loss of milk. *J. Inst. Can. Sci. Technol. Aliment* **1978**, *11*, 152−154.
(19) Whited, L. J.; Hammond, B. H.; Chapman, K. W.; Boor, K. J. Vitamin A degradation and light-oxidized flavor defects in milk. *J. Dairy Sci.* **2002**, *85*, 351−354.
(20) Veberg, A.; Olsen, E.; Nilsen, A. N.; Wold, J. P. Front face fluorescence measurement of photosensitizers and oxidation products during photooxidation in butter. *J. Dairy Sci.*, submitted.
(21) Kotaki, A.; Yagi, K. Fluorescence properties of flavins in various solvents. *J. Biochem.* **1968**, 509−616.
(22) Moan, J.; Peng, Q.; Evensen, J. F.; Berg, K.; Western, A.; Rimington, C. Photosensitizing efficiencies, tumors, and cellular uptake of different photosensitizing drugs relevant for photodynamic therapy of cancer. *Photochem. Photobiol.* **1987**, *46*, 713−721.
(23) Luby, J. M.; Gray, J. I.; Harte, B. R.; Ryan, T. C. Photooxidation of cholesterol in butter. *J. Food Sci.* **1986**, *51*, 904−907.
(24) Min, D. B.; Boff, J. M. Chemistry and reactions of singlet oxygen in foods. *Compr. Rev. Food Sci. Safety* **2002**, *1*, 58−72.
(25) Juzeniene, A.; Nielsen, K. P.; Moan, J. Biophysical aspects of photodynamic therapy. *J. Environ. Pathol., Toxicol., Oncol.* **2006**, *25*, 7−28.
(26) Moan, J. Effect of bleaching of porphyrin sensitizers during photodynamic therapy. *Cancer Lett.* **1986**, *33*, 45−53.
(27) Moan, J.; Kessel, D. Photoproducts formed from photofrin II in cells. *J. Photochem. Photobiol., B* **1988**, *1*, 429−436.
(28) Moan, J.; Sommer, S. Oxygen dependence of the photosensitizing effect of hematoporphyrin derivative in NHIK 3025 cells. *Cancer Res.* **1985**, *45*, 1608−1610.
(29) Moberg, L.; Robertsson, G.; Karlberg, B. Spectrofluorimetric determination of chlorophylls and pheopigments using parallel factor analysis. *Talanta* **2001**, *54*, 161−170.

Received for review July 26, 2006. Revised manuscript received October 4, 2006. Accepted October 6, 2006. This work was supported by The Research Council of Norway.

JF0621166