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Analytical Methods

Spatial distribution of light-induced lipid oxidation in semi-hard yellow cheese as detected by confocal microscopy

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ABSTRACT

The migration of oxidative damage in semi-hard yellow cheese resulting from surface exposure to 436 nm monochromatic light with an intensity of 2.5×10^{17} quanta min^{-1} cm^{-2} for 0, 5, 10, and 15 min was followed by confocal laser scanning microscopy using labelling with the lipophilic fluorescence ratio probe C_{11}-Bodipy (581/591). From $5 \times 5 \times 5$ mm cubes of cheese exposed to light on one plane 35 μm was sliced off and fluorescence of oxidised and non-oxidised probe recorded simultaneously on the surface of the slice reaching 250 μm inwards from the exposed surface. From the emission of the fluorescence ratio probe light-induced lipid oxidation could be followed with time of exposure to light. The penetration of light inwards in the cheese with time expressed as quanta cm^{-2}, as calculated from light transmittance at 436 nm determined spectrophotometrically for cheese slices of up to 300 μm thickness, together with light intensity as determined by actinometry, could alone account for the inwards migration of oxidation. Diffusion of radicals seems of less importance for spatial dispersion of light-induced oxidation in semi-hard cheese.

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1. Introduction

Cheeses are known to develop off-flavours when exposed to light during storage and retail display (Mortensen, Sørensen, Danielsen, & Stapelfeldt, 2003). The compounds responsible for off-flavours in cheese have been identified as protein and lipid oxidation products for which various analytical methods are available (Mortensen, Bertelsen, Mortensen, & Stapelfeldt, 2004). Photosensitised reactions rather than direct photooxidation of lipid and proteins have been found to be the most important following exposure to light from light sources with the wavelength distribution normally used for retail display (Skibsted, 2000). Riboflavin have often been considered to be the active photosensitizer in dairy products, but degradation products from chlorophyll now also seem to be of importance (Wold, Veberg, Lundby, Nilsen, & Moan, 2006). Photosensitised oxidation involves initial formation of singlet oxygen or depends on the direct formation of free radicals capable of initiating free radical chain reactions (Choe, Huang, & Min, 2005).

In contrast to liquid dairy products where oxidation products may rapidly distribute throughout the product, photooxidation in cheese and other solid foods will locate on the product surface. For cheese, front face fluorescence spectroscopy has been used for detection of oxidation on the product surface, and a correlation between riboflavin degradation and development of oxidised flavour has been demonstrated (Wold et al., 2006). An important question remains, however, to be addressed; to what degree, will oxidation once initiated on the surface of a cheese by light exposure migrate inwards in the product? Confocal laser scanning microscopy is now a well established technique for investigating structural and micro-structural properties of non-transparent dairy products due to the possibilities of optical sectioning with a theoretical working depth of up to 100 μm (Lopez, Camier, & Gassi, 2007; Sheppard & Shotton, 1997). Labelling with specific fluorescent dyes moreover enables detection of specific components (Sheppard & Shotton, 1997). Fluorescent dyes have recently been introduced for detecting lipid oxidation and antioxidant capacity in living cells as well as in liposome systems. Among these, a fluorescent probe, C_{11}-Bodipy (581/591), has proven most valuable regarding microscopical detection of oxidative stress in two or three dimensions in biological systems (Drummen, van Liebergen, Op den Kamp, & Post, 2002). C_{11}-Bodipy (581/591) is a fatty acid analogue resembling phosphatidyl choline and will locate at the interface between lipid and water phases in biological systems. Once oxidised the probe changes the fluorescence emission maximum from 595 nm towards an emission maximum at 520 nm (Naguib, 1998; Pap et al., 1999). This probe was considered for the study of migration of oxidation in a semi-hard yellow cheese.

The aim of the present study was accordingly to explore whether the probe, which previously was found to indicate lipid oxidation in cream cheese, also could be used to follow changes with time (Westermann, Brüggemann, Olsen, & Skibsted, accepted for publication). It was further explored whether this migration of

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oxidation could be accounted for solely by penetration of light or whether other mechanisms such as diffusion of radicals are of importance.

2. Materials and methods

2.1. Materials

Semi-hard yellow cheese (25% fat and 22% protein) was provided by Arla Foods amba (Viby J., Denmark). The fluorescence probe C_{11}-Bodipy (581/591) (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undercanoic acid) was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Potassium ferrioxalate, phenanthrolin, sulphuric acid (97%), and sodium acetate were purchased from Merck (Darmstadt, Germany). Sodium chloride, potassium chloride, sodium hydrogen phosphate, and potassium dihydrogen phosphate were purchased from Sigma–Aldrich (Steinheim, Germany), and were all of analytical grade. Deionised water (Milli Q-water purification system, Millipore Corp., Bedford, MA, USA) was used for all aqueous solutions.

2.2. Irradiation

Cubes of cheese ($5 \times 5 \times 5$ mm) were illuminated on one plane fixed in a holder in an optical train with light from a mercury lamp (HBO 200 w/4 mercury short ARC, photo optic lamp, Osram, Augsburg, Germany). Samples were exposed at room temperature to monochromatic light of 436 nm selected through an interference filter (Linos, formerly Spindler und Hoyer, Göttingen, Germany). A water-filled quartz cuvette (5 cm) in the light pathway served as a heat filter. The light intensity (I_0) was determined by ferrioxalate actinometry and had the value 2.5×10^{17} quanta min^{-1} cm^{-2} with minor day-to-day variations (Hatchard & Parker, 1956). The cheese cubes were exposed on the *xz*-plane to light from the *y*-direction for 0, 5, 10, and 15 min as shown in Fig. 1, each exposure time in duplicate experiments with two cubes of cheese simultaneously exposed. The illuminated cheese cubes were immediately, after illumination, frozen in liquid nitrogen and temperature adjusted to −18 °C before the cubes were sliced at a microtome (2800 Frigocut, LeicaBiosystems (former Reichert-Jung), Nussloch, Germany) in the *y*-direction to give 35 μm thick sections as seen in Fig. 1. Such sections of cheese, each positioned on an object glass, were subsequently labelled with C_{11}-Bodipy (581/591). The C_{11}-Bodipy (581/591) solution (2.52×10^{-5} M in aqueous phosphate buffered saline) was added on top of each slice of cheese, the excess was removed after 15 s and a cover glass was positioned on top of the slices.

2.3. Microscopy

The samples were visualised with a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) for an area of 3.1×10^5 μm^2. Channel 1 (oxidised state) was excited at 488 nm and the emission was recorded from 489 to 540 nm; channel 2 (non-oxidised state) was excited at 543 nm while the emission spectrum ranged from 546 to 625 nm. An oil immersion objective (×40, N.A. 1.25) was used for imaging. Image resolution was set to 1024×1024 pixels. For image formation both channels and a true image of the cheese were recorded simultaneously and each represented line was an average of two repeated scans. The initial settings of acousto optical tuneable filter (AOTF) and photomultiplier parameters were defined on a non-illuminated piece of cheese in order to match the intensity of channel 2 to the optimum according to the Leica glow over/under standards. The settings of AOFT were 56% and 52% and the setting for the photomultiplier gain were 550 V and 600 V for channel 1 (oxidised state) and channel 2 (non-oxidised state), respectively. Fluorescence intensities were determined for each pixel for both channels by using the computer program LeicaLite (Leica Microsystems, Wetzlar, Germany).

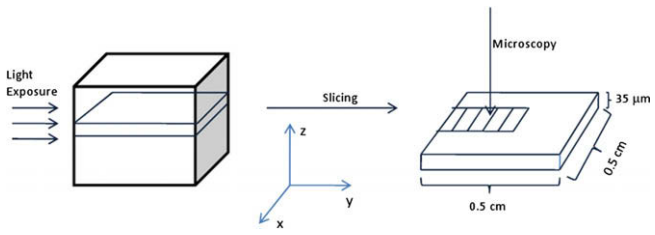

Fig. 1. Light exposure, slicing and microscopy of cubes ($5 \times 5 \times 5$ mm) of semi-hard yellow cheese.

2.4. Light transmittance

Cubes of cheese were frozen in liquid nitrogen, adjusted to −18 °C, and sectionalised on a microtome (2800 Frigocut, LeicaBiosystems (former Reichert-Jung), Nussloch, Germany). Sections with a thickness of 30, 50, and 60 μm were placed between quartz sheets and the light transmittance was measured through one or several slices to give an optical path length of 30, 60, 120, 180, 240, and 300 μm cheese using a UV–vis spectrophotometer (Cintra 40 – UV–visible spectrophotometer, GCB Scientific Equipment, Victoria, Australia). Transmittance was measured from 300 nm to 700 nm with a scan speed of 200 nm/min and a slit width of 0.2 nm.

3. Results

The intensity of light, *I*, penetrating a material like cheese is expected to follow Beers Law:

$$dI/dl = -kI \tag{1}$$

where *l* is the depth of light penetration and *k* is a material dependent constant. Light intensity is accordingly expected to decrease exponentially:

$$I = I_0 \cdot e^{-kl} \tag{2}$$

as was found for cream cheese (Westermann et al., accepted for publication). For moderate length of light penetration, the light intensity may be expected to decrease linearly as a fair approximation based on a Taylor expansion of Eq. (2):

$$I = I_0 \cdot (1 - k \cdot l) \tag{3}$$

$$T = 100\% \cdot (1 - k \cdot l) \tag{4}$$

Light penetration in semi-hard yellow cheese was found to depend linearly on *l* for up to 300 μm of penetration of 436 nm monochromatic light as may be seen in Fig. 2 for transmittance, *T*.

Following illumination of the cheese cubes with 436 nm monochromatic light and penetration of light according to Eq. (4), photons were absorbed by the cheese and oxidation progress depended on the time of illumination. Confocal microscopy with scan in the *xy*-plane clearly showed that oxidation was progressing both with respect to exposure time and distance from the exposed product surface. For non-exposed cheese the non-oxidised probe (channel 2) completely dominated the picture generated (Fig. 3A), while for 5 min of light exposure an emerging intensity of the oxidised probe is seen at the exposed surface and close to the exposed surface (Fig. 3B). For 10 min of exposure, the oxidised probe is seen with increased intensity and further inwards while

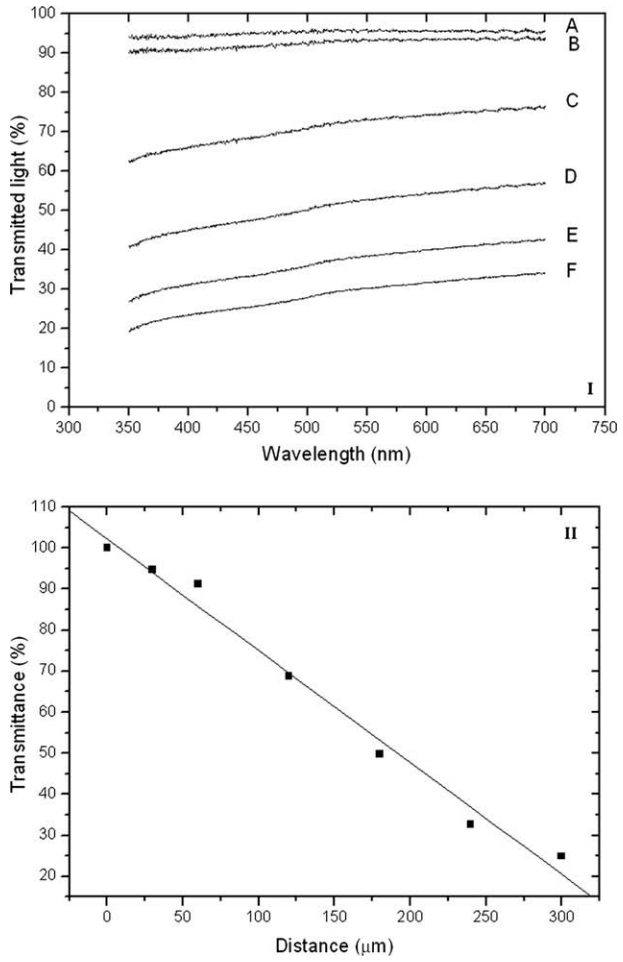

Fig. 2. Light transmission for layers of semi-hard yellow cheese (I) with a thickness of (A) 30 μm, (B) 60 μm, (C) 120 μm, (D) 180 μm (E) 240 μm, and (F) 300 μm. Transmittance of monochromatic light of 436 nm penetrating layers of semi-hard yellow cheese is seen in (II) to be approximated by a linear decrease for up to 300 μm.

the non-oxidised probe is seen with less intensity (Fig. 3C), and this tendency continues further for 15 min of light exposure (Fig. 3D).

The two channels giving rise to the pictures of Fig. 3 were scanned simultaneously in order to reduce photobleaching of the probe as the oxidised form was especially sensitive to degradation (Westermann et al., accepted for publication). The migration of oxidation was evaluated by both a subjective method and an objective method. Fig. 4A shows the result of a subjective evaluation of the moving border of the oxidised probe with time (Fig. 3A–D left row of pictures). Fig. 4B shows the fraction of the probe in the oxidised form in all 5 areas as shown in Fig. 1. Oxidation is increasing in the area subjected to microscopy with exposure time as is evident from Fig. 4B and the border of oxidation is moving inwards as is seen from Fig. 4A.

The area depicted in Fig. 3 was subsequently divided into 5 subareas as illustrated in Fig. 1, each reaching 50 μm inwards from the surface. The calculated fraction of the probe in the oxidised form is shown in Fig. 5 for the 5 subareas and the 4 exposure times. A clear picture of oxidation migration inwards with time is seen from Fig. 5. It is, however, noted that a background oxidation is seen after 5 min of light exposure. We have no ready explanation for this apparent oxidation of approximately 5% of the probe, but note that the inward migration of oxidation follows a similar pattern for different exposure times and it is getting constant at approximately 200 μm.

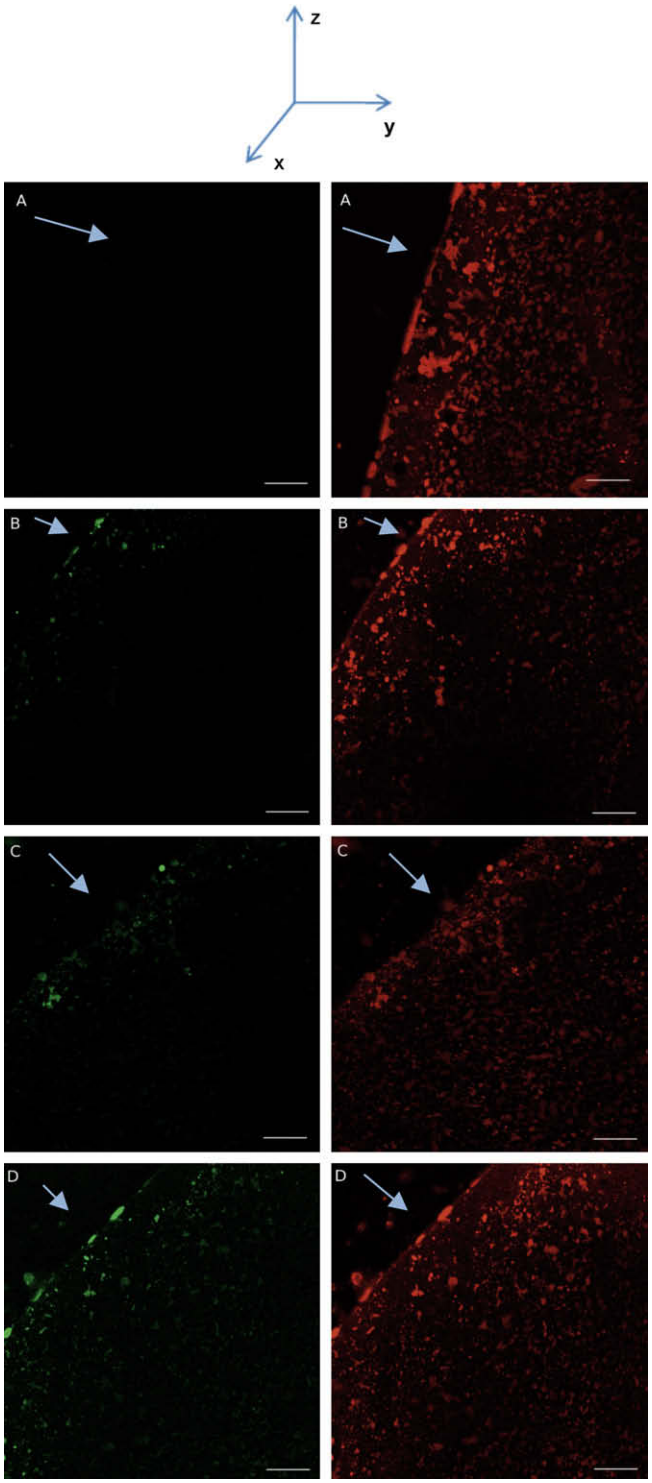

Fig. 3. Light-induced oxidation of cheese exposed to monochromatic light at 436 nm in the *y*-direction as seen in the Fig. 1 and followed by the fluorescence ration probe C_{11}-Bodipy (581/591) by confocal microscopy in the *z*-direction. Pictures are in the *xy*-plane and to the left channel 1 fluorescence (oxidised probe) is shown, while in pictures to the right channel 2 fluorescence (non-oxidised probe) is shown. The bar represents 75 μm. (A): 0 min, (B): 5 min, (C): 10 min, and (D): 15 min. The arrows in the *y*-direction show the light exposed cheese cube plane.

In a final comparison of migration of oxidation inwards in the cheese, the moving border of oxidation as evaluated subjectively from Fig. 3 is correlated with the total number of photons reaching the product surface (Fig. 6). A linear relationship is apparent, which

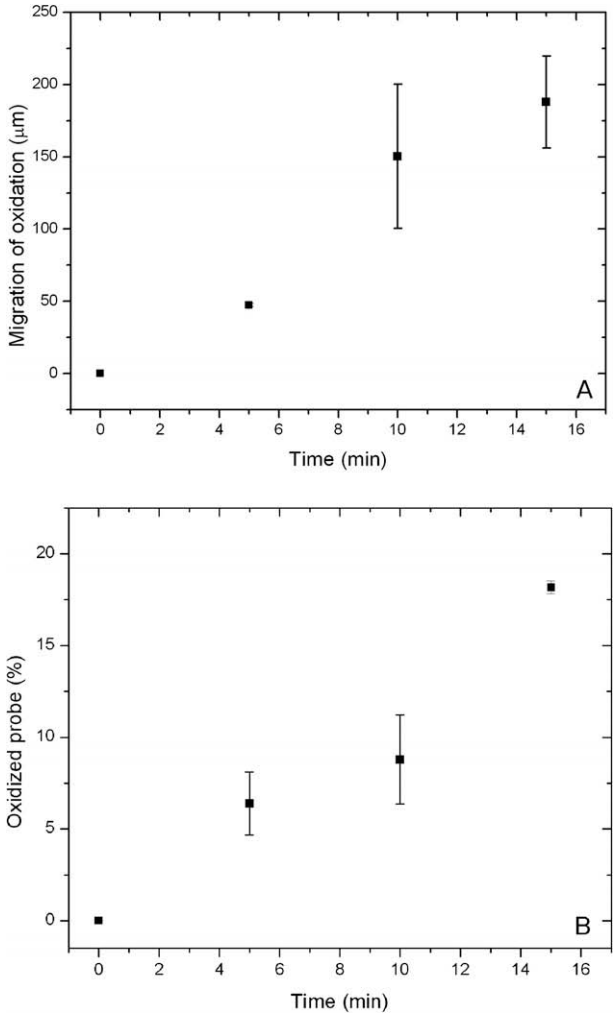

Fig. 4. Oxidation in C_{11}-Bodipy (581/591) labelled semi-hard cheese exposed to monochromatic light of 436 nm in the *y*-direction and measured by confocal microscopy in the *xy*-plane. A: Distance from the exposed surface to a significant amount of oxidised probe as detected visually from the pictures in Fig. 3 (subjective method) as function of exposure time. B: Fraction of oxidised probe in total picture as determined by pixel intensities of the fluorescence of oxidised and non-oxidised probe for increasing exposure time (objective method).

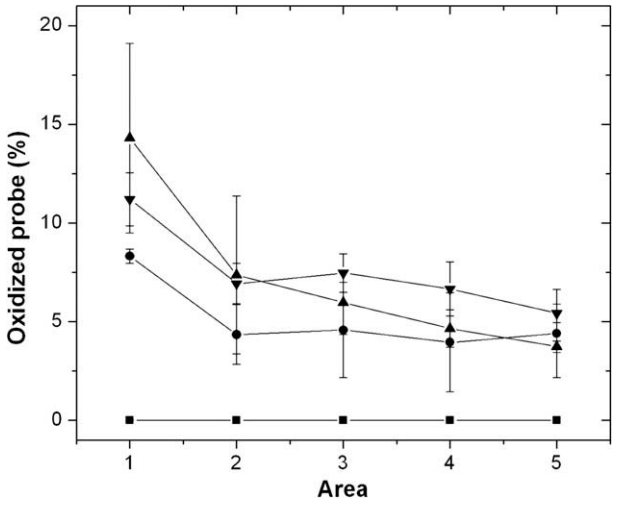

Fig. 5. Fraction of oxidised C_{11}-Bodipy (581/591) probe (%) in 5 areas each reaching 50 μm further inwards in the cheese exposed to 436 nm monochromatic light of 2.5×10^{17} quanta min^{-1} cm^{-2} intensity for 0 min (■), 5 min (●), 10 min (▲), and 15 min (▼). Each area corresponded to further 50 μm inwards from the cheese surface.

together with the linear dependency of light penetration seen in Fig. 2 seems to indicate that the penetration of light determines the position of the moving border of oxidation. A quantitative calculation to substantiate the conclusion is at present not possible since it will depend on a conversion of the fraction of oxidised probe to oxidation events in the cheese matrix (knowledge of sensitivity of the probe to different oxidation products and possible oxygen concentration gradients). On the contrary, a free diffusion of radicals generated at the border of photooxidation would create a non-linear relationship between quanta absorbed by the surface of the cheese and the border of oxidation inwards.

4. Discussion

Confocal laser scanning microscopy on cheese exposed to light followed by labelling with C_{11}-Bodipy (581/591) showed an increased lipid oxidation with increasing illumination time. The actual working depth in cheese turned out to be approximately 20 μm, and it was not possible to monitor the oxidation products by confocal sectioning alone since around 75% of the light is transmitted through 100 μm of cheese. The lack of working depth was caused by spherical aberrations, light scattering, and too low a fat content to perfectly match an oil lens. In microscopy cheese as a specimen contains too little water and fat to match either a water lens or an oil lens perfectly. Sectionalizing the cheese cubes in the *z*-direction on a microtome eliminated the importance of the working depth on the microscope and secured a more uniform labelling and distribution of the probe and furthermore, images of transmitted light could be recorded on the microscope. These images were found helpful when identifying the exact position of the edge of the cheese on the image.

Labelling the samples after illumination reduces the total amount of light affecting the probe. Earlier studies have shown that C_{11}-Bodipy (581/591) suffers photobleaching when exposed to light (Song, Hennink, Young, & Tanke, 1995). During image recording, the amount of light affecting the probe is reduced by simultaneous measurements of the two states of the probe (channels 1 and 2). Measurements of the non-oxidised probe (channel 2) allows for continuous verification of the labelling quality ensuring that appearance or non-appearance of the oxidised probe was caused by exposure to light and not insufficient labelling.

C_{11}-Bodipy (581/591) is capable of detecting lipid oxidation already taken place in the cheese due to presence of reactive oxygen species. Upon light exposure resulting in oxidation, the probe shows how lipid oxidation with prolonged time of illumination increases inwards in the cheese. Results obtained on the microscope from visualising the C_{11}-Bodipy (581/591) labelled cheese showed that lipid oxidation in the cheese increased inwards with illumination time. The distribution of oxidation products was determined by measuring the average distance from the surface to the border of the area of the oxidised probe inwards in the cheese and calculation of average pixel count. The advantage of a technique including measurements of the distances, is that the entire border is taken into account, introducing only limited errors for pixel outliers. On the other hand it is a subjective measurement and needs to be compared to another type of measurement. Pixels of the oxidised and non-oxidised state of the probe were counted within an area being identical for all images. The two types of measurements were based on calculating the amount of lipid oxidation by penetration depth (μm) and as oxidised probe (%), respectively. Calculation of the number of pixels of the oxidised probe compared

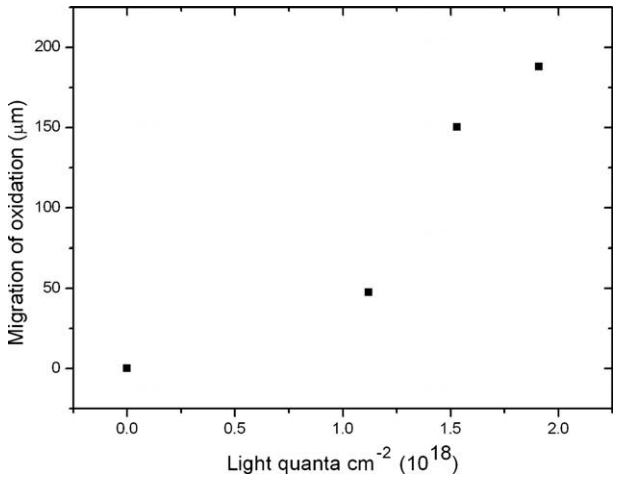

Fig. 6. Migration of oxidation inwards in the cheese as a result of exposure to 436 nm monochromatic light quantified as quanta cm^{-2}. The migration of oxidation shown is based on a subjective evaluation of Fig. 3.

to the number of non-oxidised pixels in the chosen area yields the highest errors between the double determinations probably caused by the fact that odd pixel numbers highly contributed to elevate the standard deviations.

Dividing the area mentioned earlier into five sub areas, the amount of oxidised probe can be followed inwards in the cheese which is shown in Fig. 5. As expected longer exposure times yields more oxidation products. Furthermore the degree of oxidation decreases inwards in the cheese. Formation of oxidation products inwards in the cheese as a consequence of exposure to light can be correlated to the measurements of light transmittance and the number of light quanta min^{-1} cm^{-2} reaching the cheeses surface. The correlation shows that initiation of oxidation requires more light in order to progress inwards in the cheese pointing towards light penetration as the cause of oxidation. Progression of oxidation inwards in the cheese corresponded to the penetration of light strongly suggesting that light penetration and not migration of radicals is the direct cause of lipid oxidation in the cheese. Photooxidation in cheese has been considered to be of great practical importance for consumer appreciation of quality (Mortensen et al., 2004; Wold et al., 2006). The new method to follow migration of oxidation inwards in cheese holds the potential for further development as a tool for design of better packaging systems for cheese (see Fig. 6).

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References

Choe, E., Huang, R. M., & Min, D. B. (2005). Chemical reactions and stability of riboflavin in foods. *Journal of Food Science, 70*(1), R28–R36.

Drummen, G. P. C., van Liebergen, L. C. M., Op den Kamp, J. A. F., & Post, J. A. (2002). C_{11}-BODIPY581/591, an oxidation-sensitive fluorescent lipid peroxidation probe: (micro) Spectroscopic characterisation and validation of methodology. *Free Radical Biology and Medicine, 33*(4), 473–490.

Hatchard, C. G., & Parker, C. A. (1956). A new sensitive chemical actinometer; II Potassium ferrioxalate as a standard actinometer. *Proceedings of the Royal Society of London, 235*, 518–534.

Lopez, C., Camier, B., & Gassi, J. Y. (2007). Development of the milk fat microstructure during the manufacture and ripening of Emmental cheese observed by confocal laser scanning microscopy. *International Dairy Journal, 17*(3), 235–247.

Mortensen, G., Bertelsen, G., Mortensen, B. K., & Stapelfeldt, H. (2004). Light-induced changes in packaged cheeses – A review. *International Dairy Journal, 14*(2), 85–102.

Mortensen, G., Sørensen, J., Danielsen, B., & Stapelfeldt, H. (2003). Effect of specific wavelengths on light-induced quality changes in Havarti cheese. *Journal of Dairy Research, 70*, 413–421.

Naguib, Y. M. A. (1998). A fluorometric method for measurement of peroxyl radical scavenging activities of lipophilic antioxidants. *Analytical Biochemistry, 265*(2), 290–298.

Pap, E. H. W., Drummen, G. P. C., Winter, V. J., Kooij, T. W. A., Rijken, P., Wirtz, K. W. A., et al. (1999). Ratio-fluorescence microscopy of lipid oxidation in living cells using C_{11}-BODIPY581/591. *FEBS Letters, 453*(3), 278–282.

Sheppard, C. J. R., & Shotton, D. M. (1997). *Confocal laser scanning microscopy*. New York, US: Taylor and Francis Group.

Skibsted, L. H. (2000). Light-induced changes in dairy products. *IDF Bulletin, 346*, 4–9.

Song, L. L., Hennink, E. J., Young, I. T., & Tanke, H. J. (1995). Photobleaching kinetics of fluorescein in quantitative fluorescence microscopy. *Biophysical Journal, 68*(6), 2588–2600.

Westermann, S., Brüggemann, D., Olsen, K., & Skibsted, L. H. (accepted for publication). Light-induced formation of free radicals in cream cheese. *Food Chemistry*.

Wold, J. P., Veberg, A., Lundby, F., Nilsen, A. N., & Moan, J. (2006). Influence of storage time and colour of light on photooxidation in cheese: A study based on sensory analysis and fluorescence spectroscopy. *International Dairy Journal, 16*(10), 1218–1226.