Comparison of Three Methods Based on Electron Spin Resonance Spectrometry for Evaluation of Oxidative Stability of Processed Cheese

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Electron spin resonance (ESR) spectrometry has been adapted to accelerated (light and temperature) storage experiments with processed cheese comparing the spin trapping technique with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as spin trap added to the cheese and the spin labeling technique with 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) as spin label added to the cheese. Both methods showed that light was the more important factor compared to temperature for early stages (up to 11 days) in the formation of radicals in processed cheese. For the spin labeling technique other, unidentified reactions interfered during the early stages of the accelerated storage, indicating that longer reaction times are required for the evaluation, and the spin trapping technique is recommended. As a third method, direct measurement of free radicals formed in processed cheese stored for 15 months and subsequently freeze-dried showed that for longer storage, temperature is more important for the steady-state concentration of radicals than light exposure. In agreement with this result, formation of secondary lipid oxidation products determined as thiobarbituric acid reactive substances was found to correlate with the direct ESR measurement.

Keywords: *Processed cheese; oxidative stability; ESR; spin labeling; spin trapping; radicals*

INTRODUCTION

Lipid oxidation is a major factor affecting the quality of processed dairy products, especially during long periods of storage. Autoxidation of unsaturated lipids has been proposed to involve initiation, propagation, and termination reactions: The initiation of oxidation is the formation of free radicals followed by the formation of hydroperoxides, and eventually secondary lipid oxidation products are formed, which together with the hydroperoxides are non-radical compounds. Hydroproxides are relatively unstable, and, subsequently, various volatile carbonyl compounds, responsible for off-flavors in oxidized products, are formed. Oxidation processes in dairy products may also degrade vitamins and β-carotene and lead to the formation of cholesterol oxides and conjugated linoleic acid (O'Connor and O'Brien, 1995; Shantra and Decker, 1993).

The early stage of lipid oxidation in dairy products is normally followed by the formation of hydroperoxides by HPLC or by evaluation of the peroxide value (Emmons et al., 1986). Secondary oxidation products have been determined by various methods depending on reactions with thiobarbituric acid (TBARS) or static or dynamic headspace GC/MS methods (Christensen and Hølmer, 1996; Lee et al., 1991; Park and Goins, 1992). However, it is becoming of increasing interest to detect the radicals formed prior to formation of peroxides, and recently electron spin resonance (ESR) spectrometry has been used for measurement of the levels of radicals during the progress of oxidation in whole milk powder (Nielsen et al., 1997; Stapelfeldt et al., 1997a,b). Radicals could be detected in the very early stages of oxidation. Furthermore, the relative concentration of radicals measured directly on the powder correlated with the concentration of TBARS measured at 450 nm and correlated (negatively) with the sensory impression of oxidation in the reconstituted milk. Hence, the relative concentration of radicals correlated with secondary oxidation processes and can be used for measurement of the oxidative status of milk powder. ESR spectrometry is often used in more fundamental studies of antioxidants to monitor the early events in oxidation involving radicals and also in biological systems (Thornalley, 1993). In immobilized materials it is possible directly to detect radicals, as the otherwise very reactive radicals are trapped, as in bones, dried fruits, nuts, and spices for which levels of γ-irradiation can be monitored (Stevenson, 1991).

Spin trapping is a technique for nonrigid systems now widely used (Janzen and Haire, 1990). By this technique a diamagnetic compound (the spin trap), typically a nitroso or a nitrone derivative, reacts with the radical (the spin) and forms a stable chemical bond between the two, giving rise to a relatively stable radical, which can be observed by ESR, also in fluid solution, and which in some cases further allows identification of the original radical. For food systems only a limited number of studies so far have used this powerful technique, one example being evaluation of the flavor stability of beer (Uchida et al., 1996).

An alternative technique uses stable radicals such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to probe the formation of free radicals as they will react rapidly with the stable probe radical, turning it into a diamagnetic (ESR silent) compound (Krisna and Samuni, 1994).

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Processed cheese is a product for which oxidation often determines the shelf life, as it is often sold in transparent glass jars. Light exposure is generally known to cause detrimental changes in milk and cheese as it results in the formation of off-flavors and the degradation of vitamins, especially riboflavin and β-carotene (Bekbölet, 1990). Oxidation in cheese has previously been examined by sensory evaluation (Deger and Ashoor, 1987; Kristoffersen et al., 1964; Riddet et al., 1961; Zhang and Mahoney, 1991), determination of TBARS (Hong et al., 1995; Zhang and Mahoney, 1990, 1991), or determination of cholesterol oxides (Nielsen et al., 1995, 1996; Rose-Sallin et al., 1997; Sander et al., 1989) and formation of conjugated linolenic acids (Shantra and Decker, 1993).

The present study explores the potential of the three different ESR methods (direct measurement of free radicals, spin labeling, and spin trapping) for evaluating the influence of light and temperature on the formation of free radicals in processed cheeses. These methods may in turn be alternative methods for prediction at an early stage of the oxidative stability of such products.

MATERIALS AND METHODS

Materials. Processed cream cheese spread (density ~ 1.1 g/mL) with 65% fat in dry matter was obtained from MD Foods, Viby, Denmark. The processed cheese contained the following ingredients: cheese based on cow's milk, starter culture, salt, and emulsifier; the cheese was produced on an industrial dairy plant as part of the standard production. The product was initially filled without any headspace in transparent glass containers, each containing 140 g of cheese, and sealed with a metal lid. The product was received 2 weeks after production. Different batches were used for the accelerated experiment (spin trapping/spin labeling) and the storage experiment.

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 97% (found to be ESR-silent), and TEMPO, 97%, were purchased from Aldrich (Steinheim, Germany) and used without further purification. Sodium azide pro analysi was obtained from Merck (Darmstadt, Germany). Other chemicals were of analytical grade, and water was purified through a Millipore Q-plus purification system (Millipore Corp., Bedford, MA).

Spin Trapping and Spin Labeling. Cheese samples for the storage experiments were prepared as follows: 0.10 M DMPO in processed cheese was obtained by addition of solid DMPO; 50 μM TEMPO in cheese was obtained by addition of an appropriate volume of 5.0 mM stock solution of TEMPO in water. Sodium azide (0.010%) was added to samples using a 1% stock solution to prevent microbial spoilage. Blank samples were produced by addition of sodium azide but omitting addition of DMPO or TEMPO. Samples (1.0 g) of the material were transferred to 16 mL vials with atmospheric air, sealed with a rubber septum, and stored at 5 or 37 °C exposed to or protected from light (by wrapping the samples in aluminum foil) from fluorescent tubes (Phillips TLD 18/83 W) with a light intensity at 2000 lx as measured by a Topcon IM-1 illumination meter (Tokyo Kogaku Kikai K.K., Tokyo, Japan), on the vial surface. For each of the four conditions, five samples in individual vials were used for storage for up to 2 weeks, both for spin trapping (DMPO) and for spin labeling (TEMPO), giving a total of 40 samples in the experiment. During storage, vials were withdrawn and part of the sample was transferred to a tissue cell (Suprasil WG-806-A-Q, Wilmad Glass, Buena, NJ), and the exact amount was determined by weighing (~75 mg). ESR spectra were recorded in an ECS spectrometer (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany), with an ER 4103 TM X-band resonator. Instrument parameters for DMPO experiments were as follows: sweep width, 100 G, and centerfield, 3472 G; modulation frequency, 100 kHz; modulation amplitude, 1.007 G; receiver gain, 5.00 $\times 10^5$; time constant, 81.92 ms; conversion time, 81.92 ms; and microwave power, 20 mW. For the TEMPO experiments the parameter setting was identical to those used for DMPO except for receiver gain, which was 4.00×10^4 or 6.30×10^4 depending on the intensity of the signal, and the microwave power, which was 2.0 mW.

Recording of ESR signals was performed at ambient temperature (20 ± 2 °C). For identification of the radicals formed in the cheese, the hyperfine structure and hyperfine splitting coupling constants of DMPO spin adducts A_N and A_H were calculated from the spectra (Buettner, 1987). For quantitative determination of formation of free radicals in the cheese, the normalized area of the doubly integrated area of the first-derivative ESR signal of the second peak in upfield direction in the DMPO spectrum was divided by the mass of cheese applied in the tissue cell to yield a relative measure of the free radical concentration.

Direct Measurement of Free Radicals in Cheese. Processed cheese was stored in the original sealed glass containers for 15 months at 5, 20, and 37 °C exposed to or protected from light (by wrapping the glass container in aluminum foil) from fluorescent tubes (Phillips TLD 18/83 W) with a light intensity of 2000 lx as measured by a Topcon IM-1 illumination meter (Tokyo Kogaku Kikai K.K.). For each of the six storage conditions the seal was broken from the vial after 15 months of storage, and a 1-cm surface layer of the cheese was collected for analysis and freeze-dried by freezing the cheese smeared on a freeze-drying plate for 2 days at −50 °C and freeze-drying at 0.15 mbar until all ice was evaporated.

The freeze-dried cheese was subsequently gently ground in a mortar until a homogeneous powder was obtained, which was kept in an amber glass container to minimize light exposure until the ESR experiment. The powders were transferred to a cylindrical 702-PQ/ 7 quartz ESR tube with a 5-mm inner diameter (Wilmad Glass Co.). The tubes were filled with sample material (~0.4 g). To secure approximately the same density of all samples, the tubes were tapped against the table until the height of the freeze-dried cheese in the tubes reached 10 cm. Instrument parameters for ESR experiments were as follows: sweep width, 100 G, and centerfield, 3485 G; modulation frequency, 100 kHz; modulation amplitude, 5.66 G; receiver gain, 4.00×10^5; time constant, 655.369 ms; conversion time, 81.92 ms; and microwave power, 20 mW. Recording of ESR signals was performed at ambient temperature (20 ± 2 °C).

TBARS. TBARS were used as a measure of secondary lipid oxidation products using a modified literature method (Sans et al., 1993). To ~6.0 g of cheese, exactly weighted, was added 18.00 mL of 0.67% w/v thiobarbituric acid in 50% v/v aqueous acetic acid, and the resulting mixture was homogenized using an Ultra Turrax homogenizer (Jankel & Kunkel IKA-Labortechnik, Staufen, Germany) until the mixture appeared to be homogeneous. Six milliliters of the suspension was transferred to a Pyrex tube to which 3.5 mL of chloroform was added, followed by gentle mixing for 5 min by a Mixer 440 (Swelab Instrument AB, Stockholm, Sweden). The mixture was centrifuged for 15 min. The aqueous layer was transferred to another test tube, which was placed in a water bath at 100 °C for 10 min, followed by cooling with ice. The absorbance spectrum from 200 to 800 nm was subsequently measured by an HP 8452A diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). The results are expressed as absorbance units at 450 nm, A_{450} per gram of cheese.

Water Content. The water content of the cheese before and after freeze-drying was determined by infrared drying at 105 °C using Sartorius Moisture MA 30 (Göttingen, Germany).

Statistical Analyses. All experiments were performed in triplicate unless otherwise stated. Statistical analyses were performed using the GLM procedure of the SAS version 6.12 software (SAS Institute, Cary, NC).

RESULTS

During autoxidation of lipids, radicals are highly reactive compounds formed in the initiation steps as the

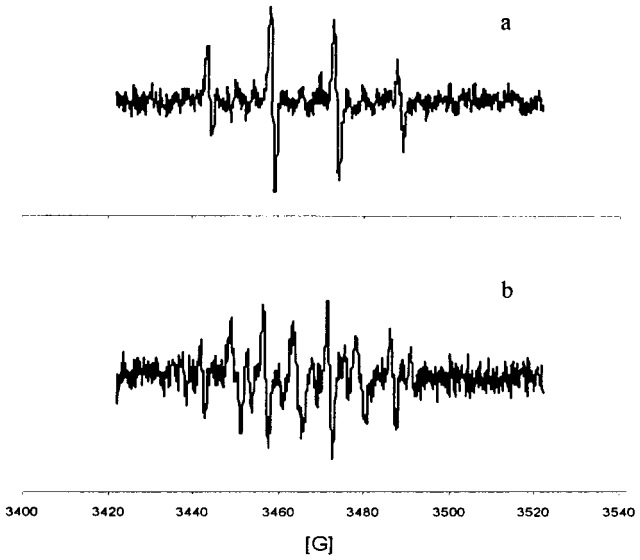

Figure 1. ESR spectrum of radical adducts of DMPO formed in processed cheese stored for 4 days at 5 °C with light exposure from fluorescent tubes (2000 lx) (a) and protected against light (b). DMPO was added to the cheese corresponding to yield 0.10 M DMPO. For instrument parameters, see Materials and Methods.

result of cleavage of peroxides. Measurement of radical concentration requires that the reactivity of the radicals is moderated by lowering of water activity by drying or freezing (direct measurement) or stabilizing by spin trapping or by the capacity of the free radicals to react with other stabilized radicals (spin labels). These three techniques were compared for processed cheese during storage, and the development of free radicals in processed cheese was monitored during storage for up to 15 months. No radicals were detected in cheese without DMPO or TEMPO added and not freeze-dried.

Spin Trapping. In cheese with added DMPO no radicals were detected at the start of the storage experiment. However, by the following day an ESR spectrum could be recorded for the cheeses. In the ESR spectra of all samples withdrawn at day 1, except for the samples stored at 5 °C in the dark, quartets with an intensity ratio of 1:2:2:1 with $A_N = A_H = 14.8$ G were characteristic. This ESR spectrum is typical of the hydroxyl radical adduct of DMPO (•OH-DMPO) (Buettner, 1987), as shown in Figure 1a for 4 days of storage with light exposure. For samples stored at 5 °C in the dark for 1 day, less intensive ESR spectra with several lines of various intensities were obtained. However, these radicals could not be identified. Samples stored at 37 °C in the dark for 1 day also showed these additional lines due to other radicals, and also for these conditions the quartet could be identified in the spectrum. Additional lines were further observed in samples stored in the dark at 5 °C at days 2 and 4 and in samples stored at 37 °C at day 2. An ESR spectrum of a sample stored for 4 days in the dark at 5 °C is shown in Figure 1b. The influence of light and temperature on the formation of DMPO spin adducts other than the hydroxyl radical adduct awaits, however, further experiments. The development in the signal intensity is given as the peak area of line 2 in the upfield direction for storage up to 11 days (Figure 2a). In the beginning of the storage the samples stored in light had the highest concentration of •OH-DMPO spin adducts. No radicals could be detected in samples stored in the dark at 5 °C

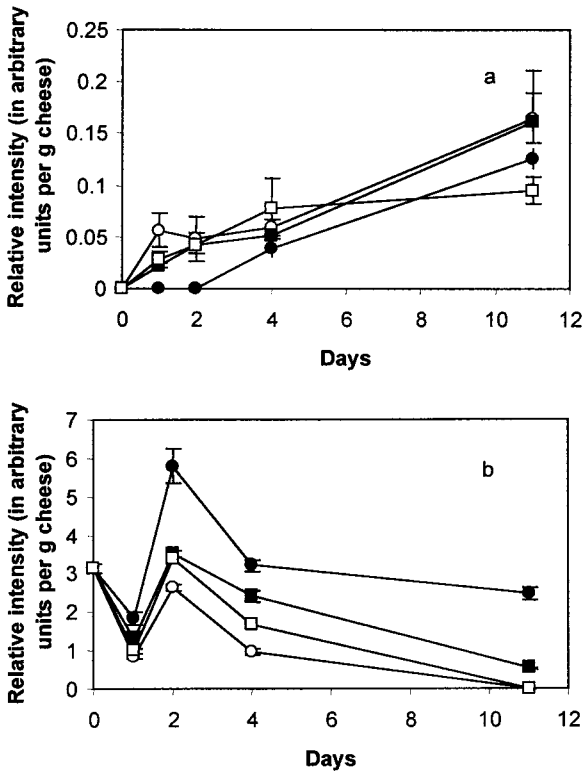

Figure 2. Influence of temperature and light exposure from fluorescent tubes (2000 lx) during storage of processed cheese on intensity (in arbitrary units) on ESR spectrum in spin trapping experiment with DMPO (a) and in spin labeling experiments with TEMPO (b): (●) 5 °C, protected against light; (○) 5 °C, exposed to light; (■) 37 °C, protected against light; (□) 37 °C, exposed to light. Results are means of triplicate determinations; the bars show standard deviations.

until 4 days of storage. After 2 days of storage, no differences were observed between light and dark for samples stored at 37 °C, and no further development in the amount of •OH-DMPO spin adducts was seen after 4 days of storage; at day 11 it even had the lowest intensity. Under these conditions favoring lipid oxidation, secondary reactions involving light and/or radicals may start to degrade the spin trap.

Spin Labeling. Immediately after addition of 50 μM TEMPO to the cheese, the expected triplet of the spin label TEMPO with an intensity of 1:1:1 was detected (data not shown). After 1 day, the signal intensity of the triplet was diminished, most noticeably for samples exposed to light at 5 °C (Figure 2b). At day 2, the signal intensity was unexpectedly higher than the signal intensity at day 1 for all conditions of storage, and for cheese stored at 5 °C in the dark the intensity was even greater than found immediately after the mixing of cheese with TEMPO. At day 4, however, the signal intensity decreased to about the same level as day 1, and with the same differences with regard to light exposure and temperature. After 11 days of storage, signals could be detected only in samples stored in the dark.

Direct Measurement of Free Radicals. Table 1 shows water content in the cheeses before and after freeze-drying. Although the cheese had the same water content before freeze-drying independent of storage conditions, after freeze-drying, the cheese stored at 37 °C had a slightly higher water content compared to that of cheese stored at 5 and 20 °C ($p < 0.01$). This stronger

Table 1. Water Content in Processed Cheese Stored for 15 Months at 5, 20, and 37 °C Exposed to or Protected against Light Exposure (2000 lx) after Freeze-Drying at 0.15 mbar[a]

temp (°C)	illuminaton	freeze-dried[b] (%)
5	light	4.21 ± 0.23
5	dark	4.22 ± 0.08
20	light	4.18 ± 0.17
20	dark	4.31 ± 0.07
37	light	5.40 ± 0.13
37	dark	5.30 ± 0.21

[a] Prior to freeze-drying, the water content of the cheeses was 52.41 ± 0.15%. [b] Results express the means of two determinations.

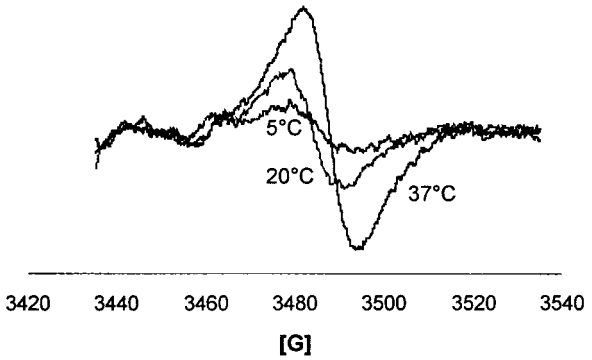

Figure 3. ESR spectrum of processed cheese stored for 15 months at 5, 20, and 37 °C exposed to fluorescent light (2000 lx). To provide a direct measurement of the free radicals formed during storage, the cheese was freeze-dried prior to analysis. Results are means of triplicate determinations; the bars show standard deviations. For instrument parameters, see Materials and Methods.

water absorption may be due to structural changes in the cheese resulting from the higher temperatures giving higher water binding capacities.

In all samples of freeze-dried processed cheese stored for 15 months, radicals could be detected by direct measurement with the exception of cheese stored at 5 °C in the dark. The spectra were broad and structureless (Figure 3). Hence, no information concerning the identity of the radical(s) could be obtained apart from the *g* value of 2.0046, which has been obtained from calibrating against a weak pitch using a *g* value of 2.0028. This *g* value suggests that the radical in the freeze-dried cheese is a carbon-centered radical.

The influence of temperature and exposure to light on the level of radicals (e.g., the area of the double integrated area of the ESR spectra) is shown in Figure 4. A highly significant influence of temperature ($p < 0.0001$), but no influence of light ($p = 0.11$), was noted.

Secondary Lipid Oxidation Products. Applying the TBARS analysis to the processed cheese prior to freeze-drying resulted in formation of a yellow color in the analysis solution with a maximum absorbance at 450 nm, but not in a red color corresponding to absorbance around 530 nm. This is typical for dairy products and is normally ascribed to the high content of saturated and monounsaturated fatty acids in milk fat, resulting in the formation of mainly monounsaturated aldehydes during oxidation (Hoyland and Taylor, 1991). After storage for 15 months, the value of A_{450} per gram of cheese, as depicted in Figure 5, showed differences for the different storage conditions, and as was the case for the determination of radicals in the freeze-dried cheese,

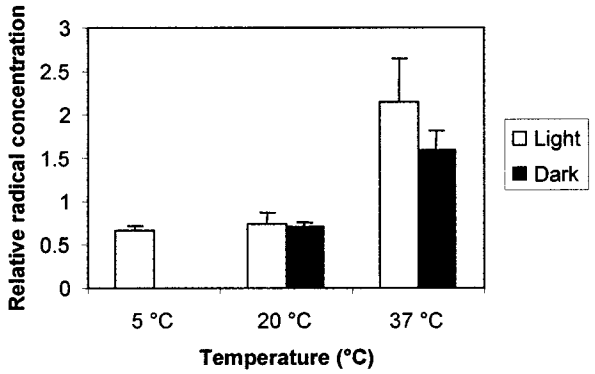

Figure 4. Influence of temperature and of light exposure from fluorescent tubes (2000 lx) on the signal intensity in the ESR spectrum as a measure of free radicals in processed cheese stored for 15 months. Prior to recording the spectra as shown in Figure 3, the cheese was freeze-dried. Results are means of triplicate determinations; the bars show standard deviations.

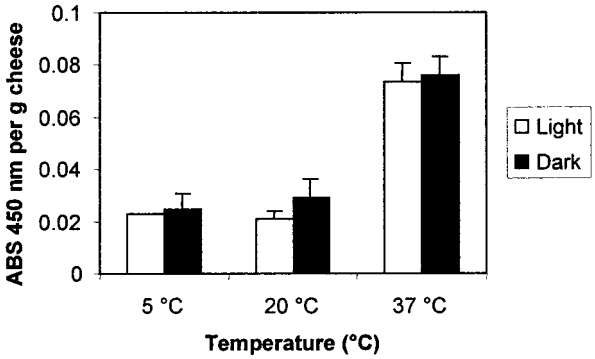

Figure 5. Secondary lipid oxidation products measured as A_{450} per gram of cheese in processed cheese stored for 15 months at 5, 20, and 37 °C with and without light exposure from fluorescent tubes (2000 lx). Results are means of triplicate determinations; the bars show standard deviations.

there was a highly significant influence of temperature ($p < 0.0001$), but no influence of light ($p = 0.26$). Hence, at least a good qualitative correlation between radicals as measured by direct ESR spectrometry, and A_{450} per gram of cheese as a measure of secondary lipid oxidation products, was observed.

DISCUSSION

ESR spectrometry has in the present study been shown to give quantitative information on the formation of free radicals in processed cheese. The three different techniques employed each had advantages, and for each technique certain problems were encountered. However, the overall conclusion is that ESR spectrometry may have a potential for application in quality control in dairy production in relation to accelerated tests and product formulation, as has already been shown for milk powder where direct measurement of free radicals was applied (Stapelfeldt et al., 1997a).

As for the spin trapping technique, the intensity of the DMPO adduct signal increased with time and also showed that formation of radicals increased with increasing temperatures and on light exposure. For the data presented in Figure 2a it is seen that the spin trapping method with DMPO has the potential of being used in accelerated storage tests in which differences in storage conditions are compared. However, it should also be noted that the combination of light exposure and high temperature for longer times leads to secondary

reactions degrading the spin adduct. However, from the results obtained in the early stages of the experiment shown in Figure 2, it can be concluded that after 2 days (or even 1 day), the method allow a clear distinction between the effect on lipid oxidation of 5 and 37 °C storage conditions and of the presence of absence of light exposure.

DMPO has been widely used and for many applications as a spin trap (Buettner, 1987). Davies and Slater (1986) used DMPO to trap alkoxyl and peroxyl radicals as well as unidentified carbon-centered radicals in peroxidizing fatty acids. In peroxidizing methyl esters of sunflower oil, alkoxyl radicals have been detected using the spin trap *N*-*tert*-butyl-α-phenylnitrone (PBN) (Milić et al., 1998). However, in all of the cheese samples in the present study using rather moderate conditions for lipid oxidation, only •OH-DMPO spin adduct could be assigned. Although this was somewhat unexpected, because photolysis of complex samples such as cheese is anticipated to generate different kinds of radicals, it is known that detection of the •OH-DMPO spin adduct does not necessarily mean that a hydroxyl radical actually has been trapped, because secondary reactions are known to be important (Janzen, 1984). However, no further attempt was made to verify if the hydroxyl radical was the sole contributor to the observed generating of •OH-DMPO spin adducts, but future investigations should include other spin traps and also lower temperature conditions to prevent secondary rearrangent reaction of the spin adducts.

The results obtained for the spin labeling technique are less clear. TEMPO certainly reacts with free radicals formed in the cheese during the first day of storage and is thereby being transformed into a nonparamagnetic, ESR-silent species. TEMPO and other nitroxides are known to undergo reductions by a variety of reductants including those present in living cells to the corresponding one-electron-reduced hydroxylamine (Krisna and Samuni, 1994). The hydroxylamine may be oxidized back to the nitroxide by various oxidants depending on factors such as redox potential and oxygen tension. TEMPO is oxidized by superoxide forming the oxoammonium cation, which is rapidly reduced by another molecule of superoxide, in effect resembling superoxide dismutase activity (Janzen, 1984). A combination of such reactions may result in the somewhat unexpected pattern seen in Figure 2b for the first 2 days of storage, although a clear pattern emerges for longer storage. Again, light seems to be more important for the generation of free radicals than high-temperature conditions.

Together these results indicate that oxidation processes in processed cheese can be monitored by application of ESR spectrometry. Spin labeling and spin trapping have demonstrated that apparently the most pronounced changes happen for samples stored in light with less significant influence of temperature. For accelerated tests for shelf-life prediction the spin trapping technique (DMPO) will, however, have to be recommended in favor of the spin labeling technique (TEMPO), as the latter probe showed fluctuations in intensity presumably due to regeneration processes and chemical degradation. For the spin trapping techniques accelerated storage should be limited to a few days to avoid influence of other subsequent reactions. It should be noted that the purpose of the present study was to explore the potential of ESR spectrometry for detection of the tendency of free radicals to form in processed cheese and that the developed method now has to be used in long-term storage experiments combined with the more classical analytical methods for lipid oxidation products.

For cheeses following long-term storage analyzed by the content of free radicals in freeze-dried samples, only temperature was found to be of importance and notably the same conclusions are reached from the TBARS test (Figure 5). An explanation for this discrepancy between the accelerated test and the realistic storage experiment could be that light is most important for initiation of free radicals (early events), whereas progression of oxidation rather depends on temperature.

In conclusion, it seems that ESR spectrometry may find use both in accelerated tests and in real storage experiments. Clearly more detailed studies of reaction mechanism are warranted, and future studies are planned to include other spin traps to be able to identify the free radicals formed in cheese, followed by storage experiments under various conditions.

ABBREVIATIONS USED

ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; TBARS, thiobarbituric acid reactive substances; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl radical.

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