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Effect of relative humidity on lipid oxidation in freeze-dried emulsions

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RESUMEN

Efecto de la humedad relativa sobre la oxidación lipídica en emulsiones liofilizadas.

Se ha estudiado la estabilidad oxidativa en una emulsión liofilizada compuesta de ácido linoleico (LA), Tween-20, sacarosa y maltodextrina en presencia de un catalizador ($FeSO_4$/ácido ascórbico). Los cambios en ácido linoleico remanente (LA) y dienos conjugados en función del tiempo fueron monitorizados a humedades relativas (RH) del 0, 32, 43 y 75%. Basado en análisis por cromatografía gaseosa, la oxidación de LA fue más significativa en la fracción superficial que en la encapsulada. La pérdida de aceite superficial con el almacenamiento puede deberse también al encapsulado parcial. Sin embargo, la más rápida oxidación del aceite superficial fue confirmada por medida de los dienos conjugados. La oxidación fue más rápida a humedades relativas menores (0 y 32%RH) y disminuyó con el aumento de RH. A humedad alta, se observaron modificaciones físicas en las muestras, incluyendo la porosidad reducida, el colapso de la estructura, la reducción del aceite superficial y la coalescencia de las gotas de aceite provocadas por la cristalización de la sacarosa. Estas modificaciones pueden ser responsables de la disminución de la oxidación. La cristalización de la sacarosa a humedades mayores inhibió la oxidación. Además, mientras las muestras con rango de temperatura de transición vitrea similares se comportaron diferentemente, las muestras con rango de transición vitrea diferente mostraron un comportamiento oxidativo similar. Los cambios microestructurales que condujeron al encapsulamiento del aceite y a la coalescencia de las gotas fueron significativos en este caso.

PALABRAS-CLAVE: Emulsión liofilizada - Humedad relativa (efecto de) - Lípido - Oxidación.

SUMMARY

Effect of relative humidity on lipid oxidation in freeze-dried emulsions.

Oxidative stability was studied in a freeze-dried emulsion consisting of linoleic acid (LA), Tween-20, sucrose and maltodextrin in the presence of a catalyst ($FeSO_4$/ascorbic acid). Changes in residual LA and conjugated dienes as a function of time were monitored at 0, 32, 43 and 75% relative humidities (RH). Based on GC analyses, LA oxidation was more significant in the surface fraction than the entrapped. The loss of surface oil upon storage may also be due to partial entrapment. However faster oxidation of the surface oil was confirmed by measurement of diene conjugation. Oxidation was more rapid at the lower relative humidities (0 and 32% RH) and decreased with increasing RH. At high moisture, physical modifications in the sample were observed, including reduced porosity, structural collapse, reduction of the surface oil and coalescence of oil droplets triggered by sucrose crystallization. These may be responsible for the decreased oxidation. Sucrose crystallization at the higher humidities inhibited oxidation. In addition, while samples with similar glass transition temperature (Tg) range behaved differently, samples with different glass transition range showed similar oxidative behaviour. Microstructural changes leading to oil entrapment and oil droplet coalescence were found to be significant, in this case.

KEY-WORDS: Freeze-dried emulsion - Lipid - Oxidation - Relative humidity (effect of).

1. INTRODUCTION

Dehydration is a method of food preservation that extends the shelf life of food products by removal of water, that may otherwise enhance biological, chemical and physical deterioration. Physical properties of food powders, e.g., flowability, dispersibility, porosity and density, can dramatically change upon storage and influence quality depending on temperature and moisture (Konstance *et al.*, 1995; Onwulata *et al.*, 1995; Chuy and Labuza, 1994; Moreyra and Peleg, 1981; Saltmarch and Labuza, 1980).

In certain cases, changes in emulsion stability may significantly influence the amount of oil exposed to oxygen enhancing oxidation (Gejl-Hansen and Flink, 1977; Shimada *et al.*, 1991; Labrousse *et al.*, 1992; Imagi *et al.*, 1992; Rosenberg *et al.*, 1990; Rosenberg and Moreau, 1996; Lin *et al.*, 1995). On the other hand, if the structure is maintained, the lipid substrate would be protected from oxidation for an extended period of time (Shahidi and Han, 1993).

In a hygroscopic system such as one containing small carbohydrates, moisture is quickly sorbed by amorphous components resulting in a decrease in viscosity allowing some crystallization of sugar molecules (Roos and Karel, 1991). Once crystallization starts, the water molecules are expelled from the crystalline lattice resulting in a decrease in moisture content (White and Cakebread, 1966; Iglesias and Chirife, 1978). Therefore, during storage of an amorphous sugar at an intermediate water activity, the amount of water sorbed increases and then decreases when re-crystallization progresses. At low a_w, the viscosity remains relatively high and, therefore, no crystallization takes place.

In the presence of lipids, adsorption of water also occurs followed by sugar crystallization which results in a release of internal lipids to the surface (Fäldt and Bergenstahl, 1996). Thus, one may assume that sugar crystallization normally leads to an increased oxidation (Roos *et al.* 1996).

Structural collapse prior to sugar crystallization can also influence oxidation of trapped lipids by

providing protection from oxygen (Labrousse *et al.* 1992). In addition to the loss in porous structure at higher humidity, physico-chemical changes (such as changes in viscosity, sugar crystallization and collapse) may lead to disruption in the oil/water interfacial stability which in turn affect the oxidative processes. Limited data are available on emulsion stability (e. g. oil droplet size distribution, coalescence, etc.) in such systems.

In encapsulated lipids prepared by spray and freeze drying in sugar containing systems, hydroxyl groups of sugars were reported to have an inhibitory effect on the autoxidation of linoleic acid while carbonyl groups of sugars accelerated oxidation (Yamauchi *et al.,* 1987). Minemoto *et al.* (1997) observed a significant effect of morphologies or microscopic structure of encapsulated lipids on oxidative stability. Unfortunately, in this work other physical changes (collapse, morphology, etc.), emulsion stability, were not measured and the surface and entrapped lipid fractions were not analyzed.

The objective of this work was to monitor these physical changes including collapse, glass transition, sugar crystallization, microscopic changes, and the oxidative behavior of the surface and entrapped lipids.

2. MATERIALS AND METHODS

Maltodextrin (Maltrin M150, Grain Processing Corporation, Muscatine, IA) and sucrose (Mallinckrodt Inc., Paris, KY) were used as encapsulating materials. Reagents were of analytical grade (Sigma, St. Louis, MO). Solvents (hexane, isopropanol and methanol, all HPLC grade) and salts were purchased from Fisher Scientific (Pittsburgh, PA). Polyoxyethylenesorbitanmonolaurate (Tween-20) was obtained from Curtin Matheson Scientific, Inc. (Houston, TX).

Samples of the emulsion were prepared by mixing exact weights of about 2 g of linoleic acid (LA) and 0.2 g of Tween-20 with 200 mL of 0.05 M phosphate buffer (pH 7.4) under nitrogen. The suspension was further emulsified at high shear mixing for three minutes (Waring blender model 700, Waring, New Hartford, CT). To avoid heat development and to exclude oxygen during mixing, a few droplets of liquid nitrogen were added to the blender cup prior to blending. Two hundred mL of a carbohydrate solution (40% maltodextrin w/v, 40% sucrose w/v) and 2 mL, of a catalyst solution (20 mM ascorbic acid, 1 mM $FeSO_4$) were added. The approximate composition of the liquid emulsions was 0.5% w/v linoleic acid, 0.05% w/v Tween-20,20% w/v maltodextrin, 20% w/v sucrose. The liquid emulsion (0. 1-0.3 μm oil droplet diameter) was freeze dried (~0. 5 cm thickness) for 3 days at 100 mTorr vacuum and 15°C shelf temperature (Virtis Co., Gardner, NY). The freeze-dried sample was subsequently ground with an electric mill to 425 μm size (40 mesh) and stored in a desiccator containing P_2O_5. Moisture content of the freeze-dried product ranged from 1 to 3% on dry basis. The particle size of the powder ranged from 30 to 425 μm, with a mean value of 150 μm. The sample contained 43 ± 2% surface and 57 ± 2% entrapped LA.

Storage study. Samples (0.5 g each) were placed in open screw cap scintillation vials (20 mL, 2.5 cm diameter) and incubated at 37°C and 0, 32, 43, 75% RH using saturated salt solutions (Greenspan, 1977) in mini-desiccators (Lang *et al.*, 1981). Samples were analyzed over a 144 h period for lipid oxidation and physical stability, as described below.

Linoleic acid extraction. The lipid material in the powders exists in two fractions, surface and entrapped. The surface oil is that which can be easily removed by washing the powder with hexane. The entrapped fraction requires disruption of the encapsulant matrix before the solvent can reach the oil. The extraction methods are reported below (modified from Coupland *et al.,* 1996).

Surface oil extraction. One-half milliliter of internal standard for chromatographic analysis (heptadecanoic acid, 5 mg/mL in hexane) and 9.5 mL of hexane were added to 0.5 g of the powder in the scintillation vials. The content of the vials was mixed with a vortex mixer and then centrifuged at 11 x 10^2 g for 15 min. The solvent was then removed, saved and the procedure repeated after the addition of 5 mL added solvent. The hexane solutions were then pooled for GC and UV analyses.

Entrapped oil extraction. One-half milliliter of heptadecanoic acid standard solution was added to a powder sample after extraction of the surface oil and the powder dissolved in 5 mL double distilled water. The emulsion was then broken with two drops of 6N HCl. Solvent (isopropanol:hexane 2:1) was added and the mixture centrifuged at 11 x 10^2 G for 15 min. The hexane fraction, containing the entrapped linoleic acid, was collected for lipid oxidation analysis.

A. Oxidation tests. Oxidation of linoleic acid was monitored by gas chromatography and UV spectroscopy. As used here, gas chromatography measures the amount of both non-conjugated and conjugated linoleic acid. The UV method measures the amount of conjugated dienes which may be present in the substrate or in its oxidative decomposition products. Therefore, the two methods complement each other but do not necessarily give parallel results at all times.

Conjugated dienes. A modification of A.O.A.C. method 28.044 a (1970) was used. The extracted fatty acid, was diluted with hexane to an approximate concentration of 0.015 mg/mL. The UV absorbance at 235 mm of a 3mL aliquot was measured by means of Lambda-3 UV/VIS Spectrophotometer (Perkins-Elmer Co., Oak Brook, IL) against a blank of hexane. A calibration curve was obtained over a concentration range of 0-0.03 mg/mL using conjugated linoleic acid standard in hexane. The linear regression equation

for the curve was x = y/74.306 (R^2= 0.989). The results were expressed as mg conjugated LA/g of powder and as mg conjugated LA/mg remaining unoxidized LA, as quantified by GC analysis (method below). Significant differences were assessed by means of a paired t-test (Damon and Harvey, 1987).

Linoleic acid. One half of internal standard (C: 17 fatty acid, approximately 5 mg/mL) was added to 0.5-1.0 mg of linoleic acid. The mixture was then treated with 0.5 mL, of 2N NaOH in methanol at 80°C for 10 min and methylated with 2 mL, of 14% BF_3 in methanol for 10 min at 80°C. At the end of the reaction, 0.5 mL of hexane and 1-2 mL of saturated $NaCl/H_2O$ solution were added and the samples centrifuged for 2 min. The top hexane layer was analyzed in a Varian Model 3700 gas chromatograph (Varian Instrumental Division, Palo Alto, CA) with a Supelcowax™ fused silica capillary column (30 m, 0.20 mm ID, 0.2 μm film thickness, Supelco Inc. Bellefonte, PA) and a SP2470 Integrator (Spectra Physics, San Jose, CA). Helium gas head pressure was fixed at 50 mL/min and the injection port at 250°C in the splitless mode. The FID detector temperature was 300°C and the oven temperature programmed from 150°C to 220°C at 3°C/min. Significant differences were assessed by means of a paired t-test (Damon and Harvey, 1987).

B. Physical tests

Particle size distribution. Fractionation by size (10 to 425 μm range) was performed with an ATM Sonic Sifter separator (ATM Corporation, Milwaukee, WI) at amplitude 7 for 10 min in a dry nitrogen gas atmosphere.

Moisture content. Samples were analyzed following the method presented by Roos and Karel (1991 a). Moisture content was calculated by weight difference, after storing 2 g of the powders for 6 days at room temperature in a desiccator over P_2O_5.

DSC analysis. The samples were adjusted to various moisture contents at 37°C as previously described. Eight to ten milligrams of sample were placed in a stainless steel hermetic pan (Perkin-Elmer, Somerseth, NJ). The samples and an empty reference pan were positioned in the DSC (Seiko Instruments, Torrance, CA) and quench-cooled with liquid N_2 to -80°C. The chamber was then heated at 2°C/min to 150°C, recooled and the sample rerun to 190°C. The instrument was calibrated using indium. The endothermic peaks, indicating melting, were recorded as the initial and final peak temperatures and enthalpy. Percent crystallization was calculated according to the following formula

$$\%\ Crystallization = \left[\frac{H}{H_s}\ x\ Ws\right] x\ 100$$

Where H_s is the enthalpy of the sucrose and H that of the sample expressed in mJ/mg. Ws is the mg of sucrose per mg sample.

Glass transition (observed from a shift in the baseline due to a change in heat capacity) was recorded as the temperature at the beginning, midpoint and end of the shift in baseline. Significant differences were assessed by means of a paired t-test (Damon and Harvey, 1987).

Porosity (ε). Porosity was calculated from true (ρ_s) and bulk (ρ_b) density using the equation (Hicsaszman, 1990),

$$\varepsilon = \frac{(\rho_s - \rho_b)}{\rho_s}$$

True density (ρ_s) was measured by the gas expansion method using a stereopycnometer (SPY-2, Quantachrome, Boynton Beach, FL). Bulk density (ρ_b) was measured by solids displacement technique. Significant differences were assessed by means of paired t-test (Damon and Harvey, 1987). The bulk density was 0.34 ± 0.01 g/mL, the true density 1.46 ± 0.08 g/mL and the porosity 0.770 ± 0.005 for all samples.

Moisture Sorption Characteristics. The powders were equilibrated for 144 h against saturated salt solutions in Proximity Equilibration Cells (PEC) at 37°C (Lang *et al.*, 1981). The equilibrium moisture content at a given %RH was calculated from the initial moisture content and weight gained. The %RH was controlled by using phosphorous pentoxide (0 % RH) and saturated salt solutions of magnesium chloride (32% RH), potassium carbonate (43% RH) or sodium chloride (75% RH) (Greenspan, 1977).

Microscopic observation (Confocal Laser Scanning Microscopy, CLSM). The emulsion for this experiment was produced with the addition of 0.01% (v/v) Nile Red, a fluorescent dye specific for lipids (Sigma, St. Louis, MO). The equilibrated powders (0%, 32%, 43%, 75% RH) were embedded with acrylic resin, LR White Soft (Sigma, St. Louis, MO) and examined with a BioRad MRC600 microscope (Biorad Laboratories, Hercules, CA) equipped with a krypton/argon laser MRC600/1000 (American Laser Corp., Salt Lake City, UT). Thirteen images obtained for each sample were analyzed for oil droplet size using an image analysis software (Mocha TM, Jandel Scientific, San Rafael, CA). Due to the resolution limit of the microscope, the diameters obtained were larger than 0.2 μm. Significant differences were assessed by means of a paired t-test (Damon and Harvey, 1987). The oil droplet size obtained was confirmed by the data from the light scattering technique.

3. RESULTS AND DISCUSSION

Figure 1a shows that, in the surface fraction, the linoleic acid decreased with time and with increasing

RH from 0 to 75% RH. Within 48 h of storage, the surface linoleic acid decreased to about zero in all samples except in the case of the 0% RH where there was a slight initial increase in the first 10 hrs followed by a decrease (Fig. 1a). The surface oil decrease may arise from oxidative loss and/or difficulty in extraction.

At 0 and 32% RH, LA in the entrapped fraction remained relatively unchanged. At 43 and 75% RH, the amount of LA increased within 12-24 h of storage to approximately 14 mg LA/g powder and then remained unchanged (Fig. 1b). The increase of entrapped LA suggests that at these higher RH's, there was an amount of surface LA which perhaps, through some physical changes, became difficult to extract. Total LA, obtained by adding the amounts of the surface and entrapped fractions (Fig. 1c), decreased after 8h at 43 and 75% RH from 16 to 14 mg/g powder, and then remained relatively unchanged. At 32 and 0% RH, the decrease was greater during the first 48 h and more extensive than at the lower relative humidities.

Measurement of the total LA indicated that the samples stored at higher relative humidity (43% and 75% RH) oxidized less than those stored at 0% and 32% RH (Fig.1c).

Conjugated dienes determination. Conjugated dienes are an indicator of early stages of oxidation (Nawar, 1996). Figure 2 shows the fatty acid amounts as a function of time. The results are expressed as mg conjugated linoleic acid (CLA)/g powder. In the surface fraction (Fig. 2a) a large amount of conjugated dienes was formed at 0 and 32% RH. At 0% RH the production of conjugated dienes peaked at 48 h and at 32% RH the production reached a maximum in 24 h of storage. At 43 and 75% RH the production of conjugated dienes was minimal. In the entrapped fraction (Fig. 2b), no significant difference was observed among samples during the first 48 h. At 96 h, however, the samples stored at 32% RH showed 0.6 mg CLA/g powder, which remained constant thereafter. The total (surface plus entrapped) conjugated dienes formed indicated that the more rapid oxidation occurred at 0 and 32% RH (Fig. 2c).

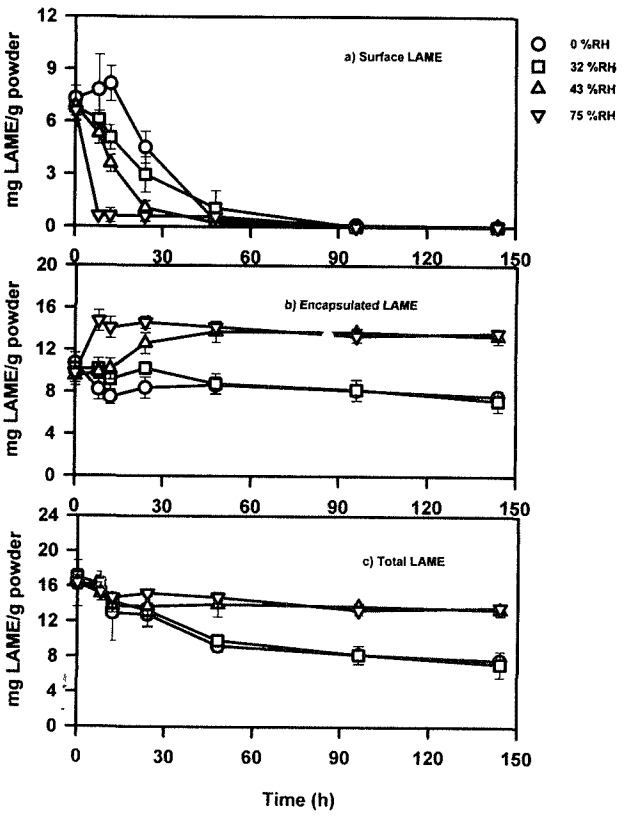

Figure 1
GC determination of surface (a), entrapped (b) and total linoleic acid (LA) (c), for freeze-dried emulsions stored at 37°C and different relative humidities. Total LA was obtained from mass balance. Data points are average of three separate experiments

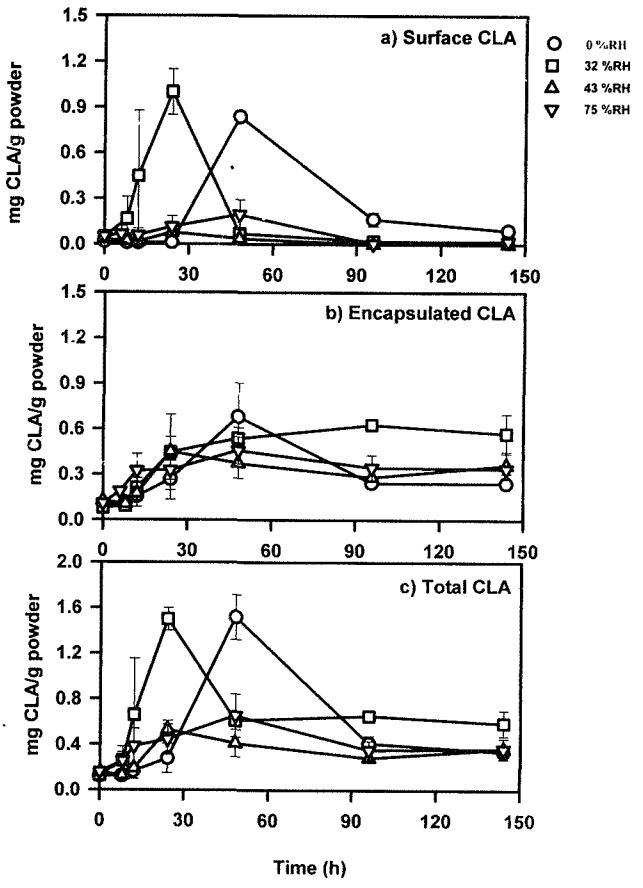

Figure 2
Determination of surface (2a), entrapped (2b) and total (2c) conjugated linoleic acid (CLA) for freeze-dried emulsions stored at 37°C and different relative humidities. Total CLA obtained from mass balance. Data points are average of two separate experiments

Physical properties. *Structural collapse.* Figure 3a shows that samples stored at 0, 32, 43, and 75% RH reached equilibrium moisture contents of 0, 5, 7 and 15%, within 90 h, 24 h, 24 h and 90 h, respectively. The powder stored at 0% RH remained free flowing while that at 32% RH caked over time. At higher % RH the powders turned sticky and started to cake within the first 24 hrs. Changes in porosity are shown in Fig. 4.

Samples stored in a drier environment (0 and 32% RH) did not change significantly in porosity over time. At higher humidities (43 and 75% RH), porosity decreased by more than 50% within 24 h at 43% RH and within 12 h at 75% RH. This structural collapse corresponds to a moisture content of approximately 6% (Fig. 3a).

Glass Transition. Figure 3b presents glass transition ranges for samples stored at the different relative humidities. In general, Tg decreased proportionally with the amount of moisture absorbed (Fig. 3a). At 37°C, the samples stored at 0% RH was glassy, that stored at 75% RH rubbery and those stored at 32 and 43% RH intermediate. The intermediate samples showed similar Tg's at around

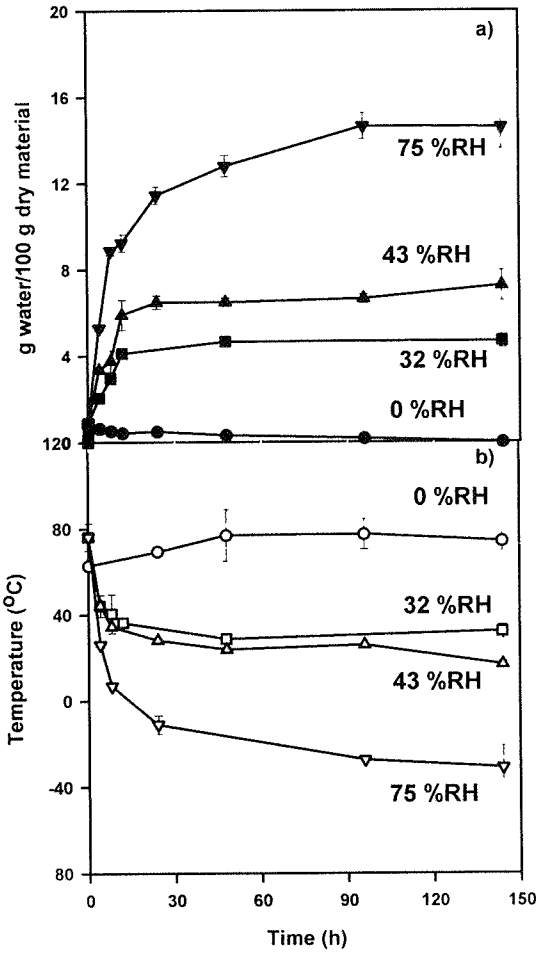

Figure 3
Moisture gain (a) and glass transition (midpoint) temperature (Tg) (b) at 37°C. Data points are the average of duplicated experiments. Transition range was approximately 20°C

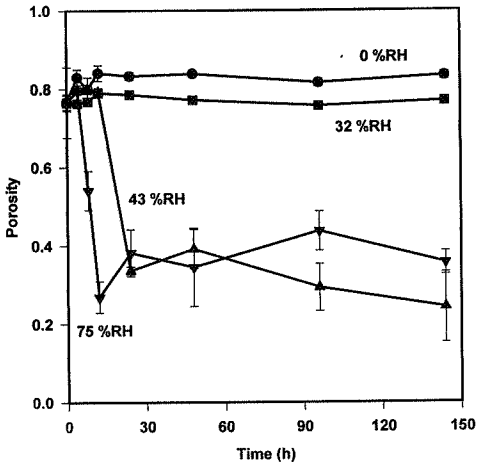

Figure 4
Porosity for freeze-dried emulsions stored at 37°C and different RH

the storage temperature. Plasticization normally leads to collapse of the porous structure but when compared with the porosity data (Fig. 4), samples with similar Tg (32 vs. 43% RH) collapsed differently. Interestingly, they oxidized differently (Fig. 1 and 2). On the other hand, at 43 and 75% RH, the samples' oxidization behaviors were similar although the Tg's were different. Therefore oxidative behavior was more consistent with the degree of collapse (porosity) than with Tg. This is in disagreement with earlier reports (Roos et al., 1996; Shimada *et al.*, 1991) that Tg was the controlling factor. Perhaps this is due to the fact that the earlier reports investigated changes in the rubbery state (T > Tg) while in the present study, samples in both the rubbery and glassy states (T > Tg, T ≅ Tg and T<Tg) were examined. The data reported here indicates that glass transition alone is not a reliable indicator of oxidative stability in freeze-dried emulsions. Data presented below demonstrate that additional factors, e.g. structural collapse and oil droplet size can play a more significant role.

Crystallization. Some authors reported that crystallization in dried emulsions promoted lipid oxidation due to release of the internal lipids to the surface (Shimada *et al.*, 1991, Labrousse *et al.*, 1992). In the present study no evidence of crystallization was found during storage at 0 and 32% RH while at 43% RH, slight crystallization occurred only at 144 h. In the case of 75% RH, crystallization of sucrose was more evident (Table I). Contrary to the earlier reports, sugar crystallization did not lead to a higher amount of surface fat. Therefore, less LA oxidized at 75% RH.

Microscopic Observation. Oil droplets in the powders were observed by Confocal Laser Scanning Microscopy. Six days after equilibration at 0, 32 and 43% RH, the oil droplet size did not change but at

Table I

Percent sucrose crystallization in freeze-dried emulsions stored at various humidities at 37°C

% RH	Time (h)	% Sucrose Crystallization
0	0	0
	144	0
32	0	0
	144	0
43	0	0
	144	1.8
75	0	0
	144	28

75% RH coalescence of oil droplets to larger size was observed (Fig. 5). Frequency of the smaller size (0.20-0.35 μm, chosen as an indicator of coalescence), decreased from 33% to 24% only at 75% RH but remained unchanged at 0-43% RH. This coalescence of oil droplets at 75% RH may be due to the more extensive sucrose crystallization which destabilized the oil/water interface (Vincent, 1984). At 0% RH, the viscosity was high and the oil droplets were trapped in highly viscous matrices. At 32 and 43% RH, the additional moisture sorbed did not allow a viscosity decrease to the level that promoted sucrose crystallization. No oil droplet coalescence was observed in these cases.

4. CONCLUSIONS

Lipid oxidation in low moisture foods may not follow the classical relationship that rate of oxidation goes through a minimum at intermediate water activity (Labuza, 1971). In the present study, LA oxidation was more significant in the surface fraction than in the entrapped. However, oxidation was found less extensive at the higher relative humidities (43 and 75% RH). This was related to structural changes, including reduced porosity (structural collapse), surface LA becoming more entrapped, and sucrose crystallization that triggered coalescence of the oil droplets. The extent of lipid oxidation found here was not directly related to a single event such as glass transition and crystallization since samples with similar Tg range (43 and 32% RH) behaved differently, while samples with different Tg range (e. g.

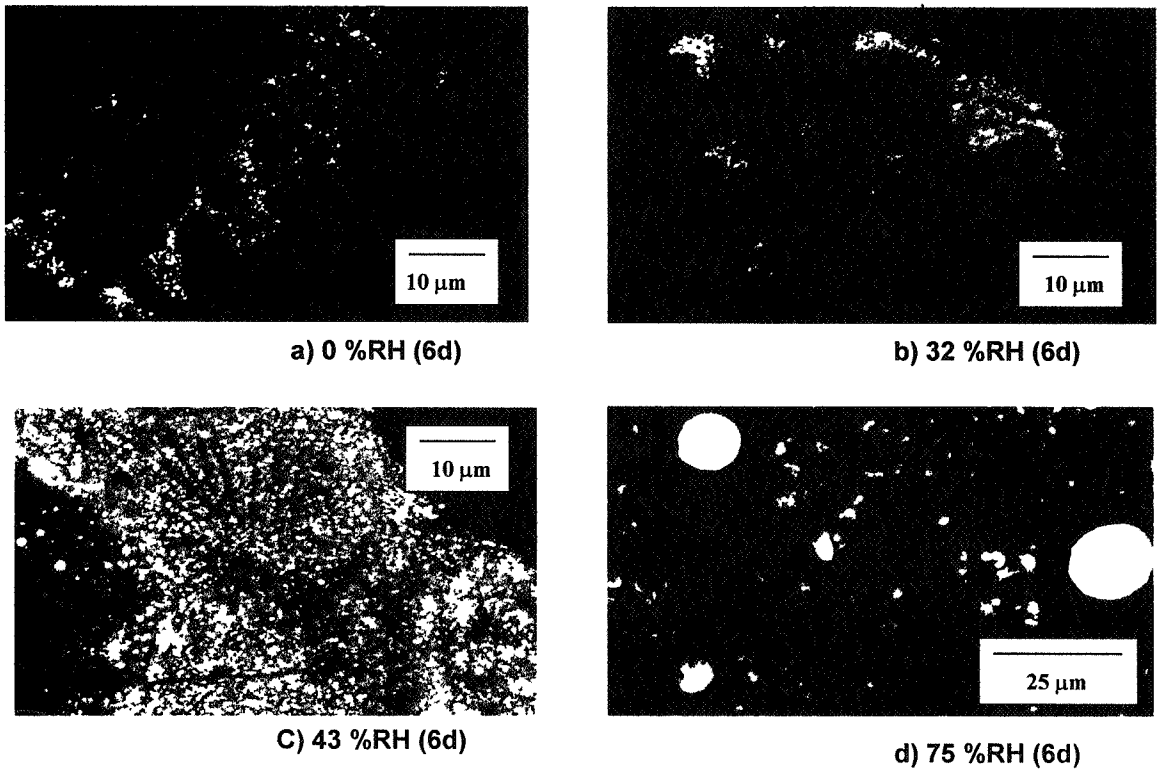

Figure 5
Confocal Laser Scanning Microscopy (CLSM) of freeze-dried emulsions stored for 6 days at 37°C and different relative humidities. Figures 5a and 5b indicate air pockets (black) surrounded by solid matrix containing oil droplets (white). Figure 5c shows a collapsed sample. Figure 5d shows oil droplet coalescence (relatively large white circles)

43 and 75% RH) behaved similarly in their oxidative behavior. At 75% RH, sucrose crystallization led to significant coalescence of the LA droplets but no release of lipids to the surface was observed.

The observations made here may not be applicable to all systems involving dried emulsions. In a recent report (Márquez and Dobarganes, 1997), for example, encapsulated oil was found to oxidize faster than the surface oil. However, the experimental parameters were significantly different. Work in this laboratory is ongoing to study the effects of various physical parameters on the physico-chemical relationships affecting oxidation in dry states.

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