# 嘉南藥理學院專題研究計畫成果報告
# 以螢光分析法檢測奶粉之氧化劣變

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Abstract

Commercial milk powders were stored at 37℃ under air to monitor the changes in intrinsic fluorescence and oxidation parameters. Results were used to investigate the use of intrinsic fluorescence in evaluating oxidative deterioration of milk powders. The front-surface fluorescence of oxidized whole milk powder had an excitation maximum at 365 nm and an emission maximum of between 435 and 440 nm. The fluorescent compounds were soluble in the organic phase of chloroform-methanol, and the solution showed excitation and emission maxima at 360 and 440 nm, respectively. Differentiation between fresh and oxidized whole milk powders could be determined by measuring solid sample fluorescence or solution fluorescence. The latter method yielded results with less variation. The fluorescence intensity in the organic phase increased steadily throughout the 19 week of storage period for whole milk powders, however, the peroxide value and TBA determination at 450 nm were not significantly correlated with storage duration. These results suggest that determination of fluorescence intensity in organic phases is more reliable in evaluating oxidative deterioration in whole milk powders.

Key words: Fluorescence, Milk powder, Oxidation

## INTRODUCTION

The oxidation of unsaturated lipids in foods leads to the formation of free radicals and hydroperoxides, which cause oxidation of other food constituents, and the formation of secondary products, including ketones, aldehydes and acids (Nawar, 1985). The peroxide value and 2-thiobarbituric acid (TBA) value are commonly used as indices of lipid peroxidation (Frankel, 1993). However, these indices are not always correlated quantitatively with reaction extent, because the oxidation products can further react with some food constituents and cause intrinsic fluorescence changes (Hasegawa et al., 1992). In milk powders, lipid oxidation is a major cause of deterioration, which renders milk powders unacceptable or reduces their shelf life (Nielsen et al., 1997). Hence, appropriate and reliable methods for measurement of oxidative deterioration in milk powders are of interest.

The intrinsic fluorescent products resulting from the interaction of oxidizing lipids and proteins have been used for the assessment of food qualities (Pikul and Kummerow, 1990; Liang, 1999). The fluorescent products are either lipid- or water-soluble, or insoluble, and a major portion of the fluorescent compounds in oxidizing biological tissues are extractable into a chloroform-methanol (CM) mixture (Bouzas et al., 1985). The measurement of intrinsic fluorescence is usually carried out using the transmission or reflectance technique (Guilbault, 1989). The transmission technique, which is used for the measurement of solution fluorescence, has been applied in detecting the lipid oxidation products of biological systems (Liang, 1996; Aubourg et al., 1997). A previous study found that the fluorescence intensity of CM extracts increased with time during frozen storage of chicken meat (Pikul et al., 1984). The organic phase of CM extract has also been used for the determination of lipid oxidation in stored freeze-dried meats, and the results showed that the fluorescence intensity at an excitation of 350-360 nm and an emission of 420-430 nm increased with storage time (Kamarei and Karel, 1984). When the lipid oxidation in peanut flour was assayed on the organic layers of CM extracts, the intensity ratio of emission at 440 nm over excitation at 270 nm was a useful indicator for determination of lipid oxidation in peanut flour (Liang, 1996).

The reflectance technique has been used for examining front-surface fluorescence of turbid suspension or solid material (Castelain and Genot, 1996). The oxidative deterioration in a dried fish model system was investigated using a reflectance spectrofluorometer (Hasegawa et al., 1992). The results showed that organic solvent-insoluble fluorescence with a spectrum different from that of lipofuscin increased in stored fish products. Protein-bound fluorescence with excitation at 450 nm and emission at 500 nm was also found in freeze-dried pork and egg yolk during storage (Hasegawa et al., 1993). The reflectance technique also shows much promise in food chemistry, e.g. for the measurement of fluorescence compounds in grain or flour in the solid state (Guilbault, 1989).

In this investigation, commercial milk powders were stored at 37℃ under air to examine the changes in intrinsic fluorescence and oxidation parameters during storage. The feasibility of using intrinsic fluorescence as an indicator of

oxidative deterioration in milk powders was examined.

## MATERIALS AND METHODS

**Materials.** The composition of the canned milk powders used in this study is listed in Table 1. These values were obtained directly from the product formulas. The milk powders were bought from a local market. Ammonium thiocyanate and 2-thiobarbituric acid were purchased from E. Merck (West Germany). Quinine sulfate dihydrate and ferrous chloride anhydrous were supplied by Fluka (Buchs, Switzerland). Other chemicals used were of analytical grade and were purchased from reliable commercial sources.

**Storage and treatment of milk powders**. Eight kinds of fresh milk powders were used. Approximately 100 g of each of the milk powders was spread on dishes (23 cm in diameter), and then stored in an electric oven at 37°C under air in the dark for 20 weeks. Experiments were performed twice and all data were obtained from duplicate measurements. At various time intervals, 0.5 g of each of the samples was withdrawn and extracted with 10 mL chloroform-methanol (2:1,v/v), and then filtered through Toyo # 1 filter paper (Toyo Roshi, Kaisha, Ltd. Japan). The filtrate (the CM extract) was made up to 10 mL with the same solvent. Aliquots of the CM extracts were subjected to peroxide value, TBA value and transmission fluorescence assays.

**Solid sample fluorescence of milk powders.** Measurement of the front-surface fluorescence of milk powders was performed using a Hitachi Model F-2000 spectrofluorometer equipped with a solid sample holder (part no. 650-1376). Milk powder samples of 5 mg were placed into a powder sample cell (part no. 018-0190), and the cell was then attached to the solid sample holder. The excitation light, which was a xenon lamp, was incident at an angle of 37 degrees to lower the reflected light. The excitation spectra of the samples were scanned from 220 to 400 nm with the emission wavelength fixed at 435 nm. The emission spectra were scanned from 400 to 600 nm with the excitation wavelength fixed at 365 nm. The spectra were measured under the following conditions: scan speed, 240 nm/min; response, 0.5 sec; bandpass, 20 nm; photomultiplier voltage, 400 V.

**Solution fluorescence of the CM extracts.** Six milliliters of the CM extract were transferred to a centrifuge tube, mixed well with 2 ml distilled water, and then centrifuged at 1000 × *g* for 10 min. The organic and water phases were collected and used to obtain the fluorescence spectra and fluorescence intensity.

The spectra of the solution fluorescences were determined using a Hitachi F-2000 spectrofluorometer with a quartz cuvette (10 mm pathlength) in the conventional right-angle orientation. The excitation spectra were scanned from 220 to 400 nm with the emission wavelength fixed at 440 nm. The emission spectra were scanned from 400 to 600 nm with the excitation wavelength fixed at 350 nm. The fluorescence intensity was determined at an excitation wavelength of 350 nm and emission wavelength 440 nm. The intensity was expressed as a relative ratio for a standard solution of 1 ppm quinine sulfate in 0.1 N $H_2SO_4$ (Gillespie, 1985).

**Peroxide values.** Peroxide values were determined by the ferric thiocyanate method (Mitsuda et al., 1966). The CM extract (2.0 mL) was diluted with 1.8 mL of chloroform-methanol, to which was added 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 0.02 M ferrous chloride in 0.35 % HCl. The absorbance of the solutions was determined at 500 nm after reacting for 3 min. The peroxide value was expressed as milliequivalents peroxide per kilogram of sample by comparison with standards of ferric ions.

**Thiobarbituric acid-reactive substances.** Thiobarbituric acid values were determined on the CM extracts according to the method of Nair and Turner (1984). TBA values were expressed as absorbance units at 450 nm and 530 nm, respectively.

**Statistical Analyses.** Analysis of variance (ANOVA) was used to interpret the differences of fluorescence intensity among milk powders under different oxidation conditions. When F-values were significant, least significant differences (LSD) were determined at the 5 % level. Correlation analyses were carried out with the comparisons between storage time and oxidation parameters. All statistical procedures were performed using the Statistical Software Package for Windows (SPSS Inc., Chicago, IL, 1994).

## RESULTS AND DISCUSSION

The front-surface fluorescence of whole milk powders were measured by solid sample spectrofluorometry. Oxidized whole milk powder (A8) had an excitation maximum at 365 nm with a small shoulder at 270 nm and an emission maximum between 435 and 440 nm (Fig. 1). In contrast, the fluorescence was negligible for fresh sample (F) and remained low for sample stored under vacuum (V8) as control. The fluorescence spectra of oxidized whole milk powder were quite broad, which may be representative of the sum of several spectra of different intrinsic fluorescent compounds.

The oxidative deterioration of milk powders was evaluated by measuring front-surface fluorescence emission at 435 nm with 365 nm excitation. Table 2 shows the fluorescence intensity (FI) with standard deviation for each treatment. The FI mean values were significantly different ($p < 0.01$) between treatments. The results demonstrate that the oxidation of stale milk powders could be monitored by measuring front-surface fluorescence intensity of solid samples. However, the fluorescence intensity changes greatly depending on the pressure applied to the sample. This uncertainty

is mainly responsible for the high coefficient of variation (23-27 %).

Milk powders with different treatments were extracted with chloroform-methanol (CM), and the CM extracts were assayed by transmission spectrofluorometry. The variations of the excitation and emission spectra of the CM extracts, during storage of whole milk powders, are shown in Fig. 2. Scanning of the CM extract of oxidized milk powder (A8) revealed that excitation at 350nm resulted in a maximum emission at 440nm. The excitation spectra of all samples were measured with the emission wavelength set at 440nm, while the emission spectra of all samples were measured with the excitation wavelength set at 350nm. The excitation spectra of fresh sample (F) showed a single peak with a maximum wavelength of around 270 nm. As the extent of oxidation increased, two fluorescence excitation peaks with wavelength maxima at 270 and 365 nm became evident. In the emission spectra, there was a broad peak at around 440 nm which gradually increased in intensity upon oxidation. At 37°C under vacuum (V8), the intensity at excitation 365 nm and emission 440 nm did not increase significantly with storage time, showing that vacuum is an effective condition for preventing fluorescence development.

The CM extract was separated into organic phase and water phase after the addition of water. Fluorescence products with an excitation of 350 nm and an emission of 440 nm were separated into organic phase, and the fluorescence intensity of these products increased with storage time (Fig. 3). Fluorescence products with an emission of around 520 nm were separated into water phase, as shown in Fig. 4, and the intensity of these products increased during the initial four months (A4) but decreased for longer storage times (A8). These data show that measurement of the fluorescence intensity in the organic phase was more feasible for determining the oxidative deterioration of milk powders than measurement in the water phase. Table 3 shows the fluorescence intensity in the organic phase for whole milk powders stored under different conditions. The FI values were significantly different between samples and increased in proportion to the extent of oxidation. The coefficient of variation was lower than that obtained using the front-surface method.

To investigate the application of solution fluorescence in measuring the oxidative deterioration of milk powders, eight commercial samples were stored at 37°C under air in order to monitor the changes in intrinsic fluorescence, peroxide value, and TBA value. The correlation coefficients of storage time with fluorescence intensity, peroxide value, and TBA value at 450 nm for milk powders stored at 37°Care shown in Table 4. The fluorescence intensity in the organic phase showed a high positive correlation with time for whole milk powders and medium milk powders. However, the fluorescence intensity, as well as peroxide and TBA values, were not suitable for monitoring stale skim milk powders because of poor response and correlation. Oxidative deterioration in whole milk powders were better characterized using fluorescence intensity than peroxide and TBA values. Furthermore, a linear relationship (r=0.928) was observed between storage time and fluorecence intensity in all of the whole milk powders investigated. These observations show that the intrinsic fluorescence in the organic phase of CM extracts might be more appropriate for the evaluation of oxidative deterioration in whole milk powders.

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Table 1. Composition of the milk powders used in the current study.

| Sample | Fat (%) | Total protein (%) | Carbohydrate (%) |
|---|---|---|---|
| Whole milk powder | | | |
| W1 | 28.5 | 27.2 | 35.9 |
| W2 | 28.2 | 26.0 | 37.1 |
| W3 | 28.0 | 26.0 | 37.3 |
| W4 | 28.0 | 26.0 | 37.1 |
| Medium milk powder | | | |
| M1 | 21.5 | 18.0 | 53.3 |
| M2 | 16.9 | 16.0 | |
| Skim milk powder | | | |
| S1 | 0.8 | 37.6 | |
| S2 | 0.8 | 37.0 | |

Data obtained from the product formulas

Table 2. Front-surface fluorescence intensity measured at an excitation of 365 nm and an emission of 435 nm for whole milk powders stored under different conditions.

| Sample[1] | FI[2] | N | CV(%) |
|---|---|---|---|
| F | 230.2 ± 62.4[a] | 6 | 27.08 |
| V8 | 513.5 ± 120.4[b] | 6 | 23.55 |
| A8 | 1094.7 ± 273.0[c] | 6 | 24.94 |

1. F, fresh whole milk powder W4; V8, stored at 37°C under vacuum for eight months; A8, stored at 37°C under air for eight months.
2. FI, fluorescence intensity expressed as mean ± standard deviation; means with different letters are significantly different ($p < 0.01$).
1 ppm quinine sulfate served as a standard solution (FI=1417).

N, number of assays.

CV, coefficient of variation.

Table 3. Fluorescence intensity (ex. 350 nm, em. 440 nm) in the organic phase of CM extracts for whole milk powders stored under different conditions.

| Sample[1] | FI[2] | N | CV(%) |
|---|---|---|---|
| F | 146.7 ± 14.4[a] | 6 | 9.80 |
| V8 | 308.0 ± 12.9[b] | 6 | 4.09 |
| A4 | 949.9 ± 44.0[c] | 6 | 4.63 |
| A8 | 1289.0 ± 110.1[d] | 6 | 9.01 |

1. F, fresh whole milk powder W4; V8, stored at 37°C under vacuum for eight months; A4, stored at 37°C under air for four months; A8, stored at 37°C under air for eight months.
2. FI, fluorescence intensity expressed as mean±standard deviation; means with different letters are significantly different ($p < 0.01$). 1 ppm quinine sulfate served as a standard solution (FI=1417).

N, number of assays.
CV, coefficient of variation.

Table 4. Correlation coefficients of storage time with fluorescence intensity, peroxide value, and TBA value at 450 nm during the storage of milk powders at 37°C in the dark.

| Sample | Fluorescence intensity | Peroxide value | TBA value(450nm)[1] |
|---|---|---|---|
| Whole milk powder | | | |
| W1 | 0.878 | 0.769 | 0.750 |
| W2 | 0.957 | 0.863 | 0.846 |
| W3 | 0.966 | 0.659 | 0.653 |
| W4 | 0.931 | 0.861 | 0.419 |
| Medium milk powder | | | |
| M1 | 0.944 | 0.943 | 0.959 |
| M2 | 0.975 | 0.492 | 0.597 |
| Skim milk powder | | | |
| S1 | 0.332 | 0.593 | 0.161 |
| S2 | 0.155 | 0.371 | 0.658 |

1. Determination at an excitation of 350 nm and an emission of 440 nm in the organic phase of chloroform-methanol extracts.

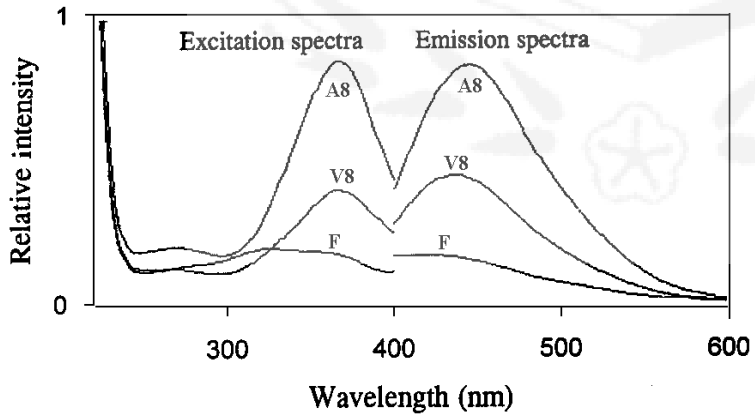


Fig. 1. Front-surface fluorescence spectra of whole milk powders. F, fresh whole milk powder (W4); V8, stored at 37°C under vacuum for eight months; A8, stored at 37°C under air for eight months.

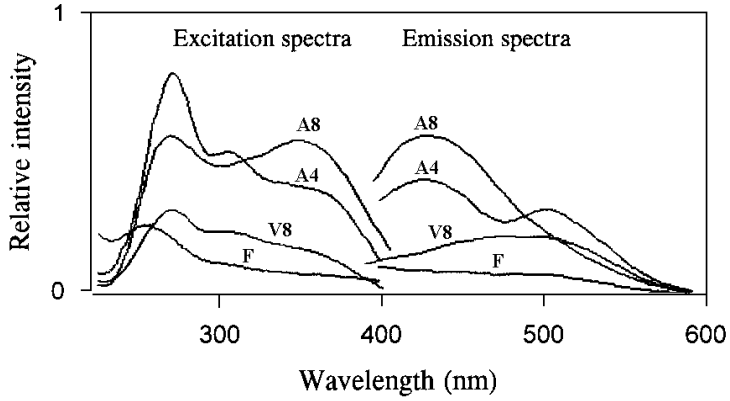


Fig. 2. Fluorescence excitation and emission spectra in chloroform-methanol extractions of whole milk powders. F, fresh whole milk powder W4; V8, stored at 37℃ under vacuum for eight months; A4, stored at 37℃ under air for four months; A8, stored at 37℃ under air for eight months.

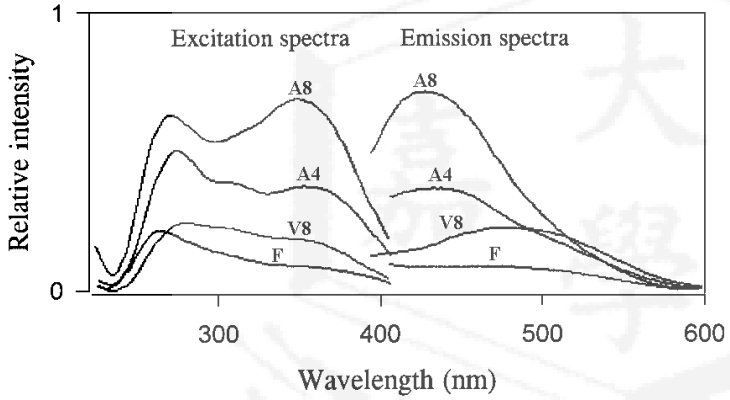


Fig. 3. Fluorescence excitation and emission spectra in the organic phase of chloroform-methanol extractions during the storage of whole milk powders. F, V8, A4, A8, same as in Fig. 2.

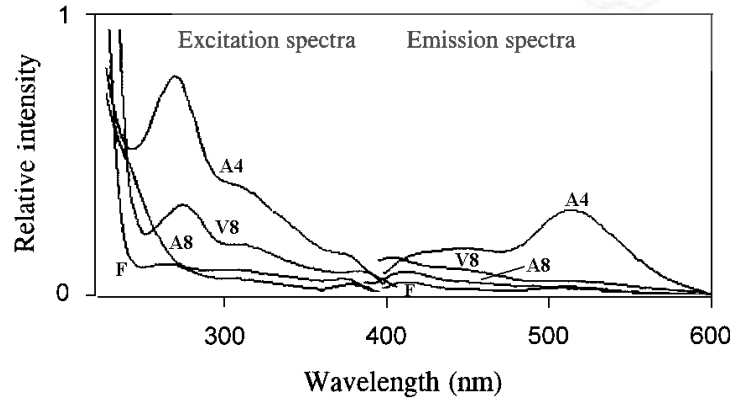


Fig. 4. Fluorescence excitation and emission spectra in the water phase of chloroform-methano extractions during the storage of whole milk powders. F, V8, A4, A8, same as in Fig. 2.