Shelf-Life Prediction of an Infant Formula Using an Accelerated Stability Test (Rancimat)

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The purpose of this study was to predict the shelf life of an infant formula using accelerated stability tests to save time. The stability of two infant formulas (a commercially available infant formula and the same infant formula supplemented with microencapsulated fish oil) was followed throughout 18 months of storage at room temperature and for 20 days stored at 60 °C by measuring the peroxide value. Stability was also measured using an accelerated stability test (Rancimat) at different temperatures (60, 80, 90, 100, 110, 120, or 130 °C). An equation has been derived by which shelf life can be predicted on the basis of the results of accelerated stability tests and therefore saving time.

Keywords: *Shelf life; oxidative stability; Rancimat; infant formula*

INTRODUCTION

The main cause of deterioration of lipids and lipid-containing foodstuffs is lipid peroxidation. The degree of lipid oxidation can be measured by chemical and/or physical methods as well as stability tests, which measure the stability of an oil under conditions that attempt to accelerate the normal oxidation process (Gray, 1978). Oxidation curves thus obtained usually comprise an induction period. The induction period is measured as the time required to reach an end point of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation. For practical purposes, however, predictions of oxidative stability in foods and oils based on the measurement of the induction period should be related to measured product shelf life (Frankel, 1993). Metrohm Rancimat was developed as a rapid automated method, which agrees well with the active oxygen method (Läubli and Bruttel, 1986). This method differs from ambient storage conditions by using a flow of air and high temperatures to accelerate oxidation. The products, measured by the change in electrical conductivity, include volatile dicarboxylic acids (de Man *et al.*, 1987), while under normal storage conditions hydroperoxides are the most important products formed and detected in the traditional peroxide test. The aim of this study is to predict the shelf life of an infant formula with a reformulated fat fraction using an accelerated stability method (Rancimat) and so avoid long-term studies. Human milk has a fatty acid composition that perfectly meets the requirements of the neonate for polyunsaturated fatty acids (PUFA) and long-chain polyunsaturated fatty acids (LCPs), which are of major importance during this period of life in which the brain and retina are developing and will therefore have an influence upon visual acuity and learning abilities (Innis, 1991; Crawford *et al.*, 1993; Clandinin *et al.*, 1994; Decsi and Koletzko, 1994). Due to the importance of LCPs and to the fact that commercial infant formulas are devoid of these fatty acids, there has been an increasing interest in the supplementation of infant formulas with LC-PUFA. In this study a commercial infant formula (nonsupplemented infant formula, NSIF) has been supplemented with microencapsulated fish oil (dry $\omega-3$) (Danochemo, Denmark) containing similar levels of C22:$6n-3$ docosahexaenoic acid and C20:$5n-3$ eicosapentaenoic acid (15:15) to obtain an experimental product (supplemented infant formula, SIF).

MATERIALS AND METHODS

Materials. A commercial powder infant formula (nonsupplemented infant formula, NSIF) (ORDESA S.L., Barcelona, Spain) and an experimental infant formula obtained by the addition of 2% microencapsulated fish oil (25% w/w oil) (dry $\omega-3$, Danochemo) to the commercial infant formula (supplemented infant formula, SIF) rich in long-chain polyunsaturated fatty acids (LCPs) were selected for investigation. Fat contents of both formulas were similar (28 g/100 g of powder).

Storage Conditions. The SIF and NSIF were packed in the usual containers under nitrogen atmosphere and stored at 25 °C for 18 months. Samples were analyzed after 1, 3, 6, 9, 12, and 18 months and at the beginning of the storage term (control). Both formulas were also stored at 60 °C, and samples were removed for analysis after 0.5, 1, 2, 3, 4, 6, 10, 13, and 20 days of storage. The SIF and NSIF were periodically manufactured and stored to repeat the long-term storage study and for sensory analysis.

Fat Extraction. The NSIF fat fraction in long-term studies was extracted using dichloromethane–methanol (2:1 v/v) according to a modification of the method proposed by Folch *et al.* (1957). Dichloromethane was chosen instead of chloroform due to its lower toxicity and equal extracting capacity (Chen *et al.*, 1981; Christie, 1989). The SIF fat extraction followed a procedure proposed by the manufacturer of the microencapsulated product (Danochemo) to liberate the oil contained in the coated product as well as the fat in the formula. Approximately 20 g of infant formula was weighed into a 500 mL erlenmeyer flask with a glass stopper, and 100 mL of distilled water and 4 mL of Alkalase 2,4 L FG (declared enzyme activity 2,4 AU/G, Novo Nordisk, Denmark, for research) were added. The sample was placed in a water bath

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at 35 °C for 30 min, with regular stirring. Absolute ethanol (100 mL) was added while the flask was swinging to avoid protein precipitation. Hexane (500 mL) was added, and the mixture was then shaken vigorously for 2 min. Furthermore, 3 × 30 mL of absolute ethanol was added. The protein was then precipitated. The organic phase was filtered through anhydrous granulated Na_2SO_4, and hexane was removed by rotatory evaporator.

Fat was extracted from the control SIF and NSIF for Rancimat analysis following the procedure proposed for the SIF in long-term studies to avoid possible error caused by different fat extraction procedures. Fat was kept in dark vials at the end of both extracting procedures. Vials were flushed with nitrogen, capped tightly, and stored at −20 °C until analysis. Exposure to high temperatures and bright light were avoided throughout the entire process because these factors may induce oxidative decay of polyunsaturated fatty acids. Fat extraction was repeated twice for each sample.

Fatty Acid Composition. Fatty acid methyl esters (FAME) were prepared with methanolic BF_3 and dissolved in hexane according to the method of Morrison and Smith (1964). Separation was performed on a Model 5890 A gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and split−splitless injector. A 60 m × 0.25 mm i.d. fused-silica capillary column SP-2380 (Supelco, Bellofonte, PA) with a 0.20 μm film thickness was used under the following conditions for the infant formula analysis: the initial oven temperature was 90 °C with a hold of 5 min and then a rise of 10 °C/min to 185 °C with a hold of 10 min followed by a rise of 7 °C/min to 235 °C with a hold of 20 min. The run time was 40 min. Two FAME were prepared for each sample and injected twice. Chromatographic conditions for dry ω−3 identification were as follows: initial temperature, 160 °C, with a hold of 2 min and then a rise of 2 °C/min to 235 °C with a hold of 20 min. Peak identification was based on relative retention times of two external standards, PUFA 1 and PUFA 2, as well as individual standards (Supelco). Fatty acid quantification was calculated by internal normalization.

Peroxide value (PV) determination was made according to the Spanish standard methods (AENOR, 1973).

Stability tests were performed according to the Rancimat method. The Model 679 RANCIMAT (Methrom, Herisau, Switzerland) equipped with an electric heating block was used without modification. Air flow rates were set at 20 L/h for all determinations. Temperature was set at 60, 80, 90, 100, 110, 120, or 130 °C. Three grams of fat was introduced into each reaction cell. Rancimat was linked to a microprocessor, and the experimental curves were printed out automatically.

Sensory analysis was performed initially (at 0 time, control), and after 9 and 18 months storage at room temperature. Comparison among three different storage periods was achieved because the NSIF and SIF were manufactured periodically and stored at room temperature.

Sensory judges were trained for duo-trio test and to recognize the taste and smell of oxidized fat using several infant formulas, which had been stored under different conditions and for different intervals. Nine students were retained for the taste panel on the basis of their ability to consistently select the oxidized samples in preliminary pair tests.

In the duo-trio test the subject is presented with three products; the first is identified as the reference (or control) and the other two are coded. The subject's task was to indicate which product was more similar to the reference. The chance probability associated with the duo-trio test was identical to that of the other two product tests, $p = 1/2$.

The pair comparison test was a two-product test, and the subject's task was to indicate, by circling or by some similar means, the one product that had more of a designated characteristic, which was identified before the test and stated on the scoreboard (Stone and Sidel, 1993).

In an initial, informal evaluation of the formulas, the flavor of SIF appeared to differ from that of the NSIF. To test for significant differences in flavor, duo-trio tests were conducted between the two formulas initially (at 0 time) and after 9 and 18 months of storage. In addition, to test whether significant differences in flavor developed during storage for each formula, the control was compared in duo-trio test with the samples stored for 9 and 18 months and between the 9 and 18 month storage treatments.

To compare storage time and formulas for differences in oxidized flavor, the same comparisons were performed using a pair test for intensity of oxidized flavor.

Each duo-trio test and each pair test were performed in triplicate by the nine judges. The results were interpreted statistically by one-tailed binomial test, where $p = 1/2$ (O'Mahoney, 1986).

SIF and NSIF were reconstituted with water at 45 °C following the manufacturer's instructions, and 75 mL was presented to the judges in coded, odorless, opaque plastic cups. For both duo-trio and pair tests, 9−10 comparisons were presented at each session.

Table 1. Fatty Acid Composition of the Microencapsulated Fish Oil

fatty acid	% (w/w)	fatty acid	% (w/w)
C12:0	0.12	C20:1*n*−11	1.42
C14:0	8.58	C20:4*n*−6	1.32
C15:0	0.90	C20:5*n*−3	14.43
C16:0	20.69	C22:4*n*−6	0.68
C16:1*n*−7	8.00	C22:5*n*−3	2.36
C17:0	0.71	C22:6*n*−3	15.36
C18:0	3.90	SFA[a]	34.90
C18:1*n*−9/*n*−7	15.38	MUFA[a]	24.80
C18:2*n*−6	1.80	PUFA[a]	40.30
C18:3*n*−6	0.17	LSP[a]	34.15
C18:3*n*−3	1.45		
C18:4*n*−3	2.73		

[a] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCP, long-chain polyunsaturated fatty acids.

Table 2. Fatty Acid Composition (Percent)[a] of the SIF and the NSIF

fatty acid	SIF	NSIF	fatty acid	SIF	NSIF
C4:0	0.72	0.74	C18:2*n*−6	11.97	12.15
C6:0	0.92	0.96	C20:0	0.23	0.25
C8:0	0.66	0.68	C18:3*n*−3	0.67	0.66
C10:0	1.66	1.68	C20:1*n*−11	0.60	0.64
C12:0	4.33	4.50	C20:4*n*−6	0.19	0.17
C14:0	6.40	6.33	C20:5*n*−3	0.27	nd[c]
C15:0	0.56	0.50	C22:4*n*−6	0.05	nd
C15:1	0.76	0.77	C22:5*n*−3	0.09	nd
C16:0	27.92	28.02	C22:6*n*−3	0.27	nd
C16:1*n*−7	1.34	1.26	SFA[b]	52.0	52.34
C17:0	0.51	0.50	MUFA[b]	34.49	34.68
C17:1*n*−7	0.29	0.30	PUFA[b]	13.51	12.98
C18:0	8.09	8.18	LSPs[b]	0.87	0.17
C18:1*n*−9/*n*−7	31.50	31.71			

[a] Mean of duplicate analyses. [b] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LCPs, long-chain polyunsaturated fatty acids. [c] nd, not detected.

RESULTS AND DISCUSSION

The coated product added represented 2% of the infant formula. Table 1 shows the fatty acid composition of the microencapsulated fish oil. This new fat formulation increased the polyunsaturated fatty acid content (PUFA) from 12.98% to 13.51% and the LCP content from 0.17% to 0.87%. Table 2 shows the fatty acid composition of the SIF and NSIF. The PV is a good guide to the quality of a fat. Freshly refined fats should have PVs of less than 1 mequiv/kg. Fats that have been stored for some time after refining may be found to have PVs up to 10 mequiv/kg before undue off-flavor is encountered (Rossell, 1989). The limiting peroxide value specified by Joint FAO/WHO (1989) standards for refined oil is 10 mequiv/kg. This study showed values of 0.76 and 0.75 mequiv/kg for the SIF and NSIF at the

Table 3. Test for Significant Differences in Flavor Using Duo-trio Differences between the Two Formulas Initially (at 0 Time) and after 9 and 18 Months of Storage

storage period (months)	SIF vs NSIF[a]
0/0	*
9/9	***
18/18	****

Test for Significant Differences in Flavor Using Duo-trio Differences during Storage

storage period (months)	SIF[a]	NSIF[a]
0/9	***	**
0/18	****	***
9/18	***	***

[a] SIF, supplemented infant formula; NSIF, nonsupplemented infant formula. * $p < 0.05$; ** $p < 0.03$; *** $p < 0.02$; **** $p < 0.0001$.

Table 4. Test for Significant Differences in Oxidized Flavor Using Pair Comparison Test between Both Formulas Initially (at 0 Time) and after 9 and 18 Months of Storage

storage period (months)	SIF vs NSIF[a]
0/0	nd
9/9	nd
18/18	***

Test for Significant Differences in Oxidized Flavor Using Pair Comparison Test during Storage

storage period (months)	SIF[a]	NSIF[a]
0/9	nd	nd
0/18	****	nd
9/18	***	nd

[a] SIF, supplemented infant formula; NSIF, nonsupplemented infant formula. * $p < 0.05$; ** $p < 0.03$; *** $p < 0.02$; **** $p < 0.0001$.

beginning, which were satisfactory. After 18 months of storage, the PVs were 9.05 and 6.64 mequiv of O_2/kg for the SIF and NSIF. The PV for the SIF is very close to 10, and although it seems that off-flavor was not yet perceptible, sensory analysis reported a distinctive oxidized flavor for the SIF. Sensory analysis to test for significant differences in flavor using duo-trio differences showed significant differences between the two formulas initially (control) and after 9 and 18 months of storage (Table 3). Significant differences were also found using duo-trio differences, when the control was compared with the samples stored for 9 and 18 months and between the 9 and 18 month storage treatments (Table 3). Results for oxidized flavor using the pair test showed that the SIF was found to have an oxidized flavor after 18 months of storage at room temperature (Table 4). The limiting PV used to estimate shelf life in long-term storage in our study was 9. Table 5. shows the evolution of PV throughout a storage period of 18 months at 25 °C and the evolution of PV at 60 °C for 20 days for the SIF and NSIF. Peroxide value evolution at 25 and 60 °C followed an exponential curve for the SIF and NSIF. Shelf life was predicted using long-term storage studies, based on PV at 25 and 60 °C for the SIF and NSIF. Induction periods (IP) were calculated by extrapolation from the linear equation obtained by plotting storage period against the logarithm of PV. Linear equations showed a high degree of correlation with coefficients of 0.986 and 0.976 for the SIF and NSIF, respectively, at 25 °C and of 0.906 and 0.915, respectively, when stored at 60 °C. The SIF shelf life was 18 months (547 days), 5 days, and 5 h at 25 and 60 °C respectively. Likewise, the shelf life obtained for the NSIF was 22 months and 23 days (691 days), 5 days, and 13 h at 25 and 60 °C, respectively.

Table 5. Evolution of Peroxide Value for the SIF and the NSIF at 25 and 60 °C

storage term (months)	PV[a] at 25 °C		storage term (days)	PV[a] at 60 °C	
	NSIF	SIF		NSIF	SIF
0	0.75	0.76	0.5	0.99	1.09
1	0.80	0.96	1	1.08	1.22
3	1.09	1.20	2	1.49	1.57
6	1.22	1.44	3	1.80	1.95
9	1.92	2.14	4	6.26	7.36
12	2.00	2.93	6	0.40	0.90
18	6.64	9.05	10	0.20	0.68
			13	0.00	0.40
			20	0.00	0.00

[a] PV, peroxide value; mequiv of O_2/kg. NSIF, nonsupplemented infant formula; SIF, supplemented infant formula. Mean of $n = 4$ analyses. Standard deviations ranged from 0.03 to 0.18.

Table 6. Rancimat OSI Values for the Control SIF[a] and NSIF[b]

temp (°C)	OSI(h)[c] SIF ($n = 12$)	OSI(h) NSIF ($n = 12$)
60	133.00	199.00
80	46.35	48.83
90	30.00	32.70
100	13.23	15.96
110	8.76	9.53
120	5.31	6.80
130	1.55	2.02

[a] Supplemented infant formula. [b] Nonsupplemented infant formula. [c] Oil stability index.

Rancimat is an automated instrument test that measures the conductivity of low molecular weight fatty acids (e.g. formic acid) produced during autoxidation of fats at 100 °C or above. This method requires a somewhat higher level of oxidation (PV > 100) than other methods to obtain measurable results (de Man and de Man, 1984). Several studies have found that the activation energy associated with the induction time for oxidation increases at temperatures below 90 °C (Cash *et al.*, 1988). This implies that the shelf-life at room temperature is overestimated with the currently used accelerated aging techniques (Toro *et al.*, 1993). Another study reported by Kaya *et al.* (1993) found that induction periods of two different oil samples, estimated with an accelerated method and long-term storage results based on peroxide values, showed that the accelerated method led to underpredictions or overpredictions depending on the type of oil. The SIF and NSIF were subjected to seven temperatures in the Rancimat method (60, 80, 90, 100, 110, 120, or 130 °C). Temperatures above or below this range did not show good results. Table 6 shows the OSI (oxidative stability index) values obtained for the control SIF and NSIF. Reproducibility among experimental runs was also good, showing standard deviations of 0.16 and 0.05 and ranges of 0.42 and 0.15 for the NSIF and SIF, respectively.

A curve is obtained when the different temperatures are plotted versus log OSI. The equation that best fit the curve, taking into account that at low temperatures OSI values increase rapidly, was

$$(T + T_{ref}) = A \exp[-B(\log(\mathrm{OSI})] \quad (1)$$

where T is temperature (°C), T_{ref} is a constant parameter that was determined for different values (the best

numeric results were found for a T_{ref} value of 20), and A and B were calculated using a linear regression equation which was deduced from the above-mentioned curve equation when neperian logarithm was applied:

$$\mathrm{Ln}(T + T_{ref}) = \mathrm{Ln}(A) - B \log(\mathrm{OSI}) \quad (2)$$

The curve equation will be as follows:

$$t = [A/(T + T_{ref})]^{2,30258/B} \quad (3)$$

The results obtained from the accelerated oxidative stability test (Rancimat) and the shelf life calculated using long-term studies at 25 and 60 °C based on the peroxide value evolution as well as the sensory analysis results were used to obtain a linear equation for the SIF when $\mathrm{Ln}(T + T_{ref})$ was plotted versus $\mathrm{Ln}(A) - B \log(\mathrm{OSI})$. A and B values were then calculated and substituted in the curve equation as well as $(T + T_{ref})$ by (25 °C + 20), and a shelf-life prediction of 12.624 h (526 days) was obtained for the SIF.

If the shelf life was calculated using only the results obtained with the accelerated stability test (Rancimat method), its value was 489.62 days. The difference between SIF shelf-life predictions was 6.91%.

When the NSIF shelf life was calculated, the difference between both predictions using either long-term studies or the accelerated stability test was 13.9% at room temperature. The differences in shelf-life prediction at 60 °C were 2.2% and 6% for the SIF and NSIF, respectively.

Therefore, this mathematical equation seems to be suitable to predict the shelf life of an infant formula using accelerated oxidative stability tests (Rancimat) without error, therefore saving a lot of time, which is very precious in control laboratories as well as research and development laboratories. In our case the addition of the LCP decreased the stability of the experimental formula which has to be taken into account because of the potential danger of oxidized fatty acid consumption by the newborn infant.

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