TECHNICAL REPORT
NATICK/TR-88/074

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EXTENSION OF STORAGE STABILITY IN ENERGY-DENSE ENCAPSULATED SYSTEMS BY MINIMIZATION OF LIPID OXIDATION

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JANUARY 1988

FINAL REPORT
OCTOBER 1984 - SEPTEMBER 1987

APPROVED FOR PUBLIC RELEASE;
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UNITED STATES ARMY NATICK
RESEARCH, DEVELOPMENT AND ENGINEERING CENTER
NATICK, MASSACHUSETTS 01760-5000

SCIENCE AND ADVANCED TECHNOLOGY DIRECTORATE

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704 0188
Exp Date Jun 30 1986

1a REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S) NATICK/TR-88/074	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION Biochem Br, BioSD, SATD 6b OFFICE SYMBOL (If applicable) STRNC-YMB	7a NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Kansas St. Natick, MA 01760-5020	7b ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code)	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 11.161102 / PROJECT NO: 01 / TASK NO: 03 / WORK UNIT ACCESSION NO: 020

11 TITLE (Include Security Classification)
Extension of Storage Stability in Energy-Dense Encapsulated Systems by Minimization of Lipid Oxidation (U)

12 PERSONAL AUTHOR(S)
WILLIAM L. Porter, Edward D. Black and Landa Hoke

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final	FROM Oct 84 TO Sep 87	1988 January	40

16 SUPPLEMENTARY NOTATION

17 COSATI CODES		
FIELD	GROUP	SUB-GROUP

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FLUORESCENCE, AUTOXIDATION, ASCORBIC ACID, MAILLARD REACTION, ANTIOXIDANTS, SOLID SAMPLE FLUORESCENCE, GALLATED, HYDROPHILIA, LIPOPHILIA (Cont.)

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Measurement of quality loss through oxidative or Maillard cross-linking of phospholipids or proteins was accomplished using an encapsulated, energy-dense model system simulating a generic calorie-dense ration component. Measurement methods developed were rapid, reliable, and sensitive. They assay cross-linking which occurs as the second or sensible stage of both the degradative processes and correlates with color, odor, and toughness.

Using these methods, it was shown that at an atypically high concentration the synthetic antioxidant tertiary-butyl hydroquinone (TBHQ) was the most potent single antioxidant tested in uncompressed dry and moist systems. The higher alkyl gallates, which have a very low acute and chronic toxicity, and rosemary extract (Herbalox(R)) are nearly as effective. The activity of these substances in dispersed systems and relative inactivity in dry vegetable oils is consistent with the polar paradox rationale developed by the senior author. This states that, other things being equal, polar antioxidants will be more effective in (Cont.)

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION	
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED	
22a NAME OF RESPONSIBLE INDIVIDUAL William L. Porter	22b TELEPHONE (Include Area Code) (508)651-4310	22c OFFICE SYMBOL STRNC-YMB

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

18. SUBJECT TERMS (Continued):

TERTIARY-BUTYL HYDROQUINONE
ANTIOXIDANT CHOICE RATIONALE
LIPOPHILIC ANTIOXIDANTS
ROSEMARY EXTRACT

19. ABSTRACT (Continued):

nonpolar bulk oils and nonpolar antioxidants more effective in dispersed polar lipids like membranes or emulsions.

It was shown that compression of these model systems is a powerful antioxygenic measure and that it has a synergistic effect with dodecyl gallate (DG). By contrast, ascorbic acid produced little to no effect in the compressed system.

DG, a lipophile, was more than twice as effective as propyl gallate (PG), a relative hydrophile, in the dried, dairy-based system, whether compressed or noncompressed. This also is consistent with the polar paradox rationale for appropriate antioxidant choice in dispersed versus bulk systems.

PREFACE

The work reported here was performed under Project No. IL161102AH5203020, "Degradative Mechanisms in Dry, Moist and Fluid High Density Ration Components, Shelf-life Prediction and Extension". William L. Porter was the Project Officer and Principal Investigator. John Kapsalis and Thomas Martin had administrative supervision.

The editorial assistance of Samuel Cohen, Science and Advanced Technology Directorate and Norman Harris, Food Engineering Directorate, is gratefully acknowledged, as is the timely assistance of Patricia Crawford in preparing and organizing the manuscript for publication. The generous assistance of Ron Segars in compression of model systems is also acknowledged.

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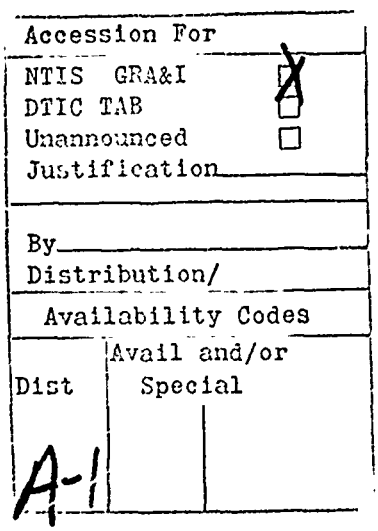

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INTRODUCTION

Individual combat rations contemplated for Army 21 — the year 2000 and beyond -- have many novel requirements, but those relevant to this report are high energy density and very high storage stability under complex and extreme environmental conditions. The latter can be simplified since competent packaging keeps light, water vapor, and oxygen transfer very low. Cyclic induced high temperatures of food in storage remain as the environmental parameter.

Moderate energy density (volume basis) can be achieved by drying and subsequent compression, techniques long ago perfected at U. S. Army Natick Research, Development and Engineering Center.[1] Further advances in energy density demand the substitution of lipid for either protein or carbohydrate, with over twice the caloric density on a weight basis (although less on a volume basis, because of the lower density of lipid). However, because of incipient oxidation, lipid is probably the most fragile major nutrient.

The two major quality loss contributors in rations are lipid oxidation and Maillard, or reducing sugar-amine reactions.[2] The latter, at the relatively low storage rather than high processing temperatures, proceed more slowly than lipid oxidation, particularly under the very dry conditions achieved in energy-dense systems. Thus, one may expect Maillard losses to predominate under processing (e.g., spray-drying) conditions, while oxidation will take over under storage conditions when the product is dry. For this reason, and because lipid predominates on a mass basis in

energy-dense rations, control of oxidation is paramount for long-term storage stability in very dry systems.

Rationale of Lipid Oxidation Control. Control of lipid oxidation may be achieved by oxygen restriction (vacuum or inert atmosphere coupled with impermeable packaging), light restriction (again, impermeable packaging), metal restriction by chelating agents, oxygen scavenging (palladium catalyst with low concentrations of hydrogen), and finally by certain antioxidant hydrogen-donating compounds which have the capability of stabilizing the antioxidant free radical resulting from hydrogen donation.

Oxygen and light restriction have been significantly improved by current vacuum packaging. Indeed, results reported below confirm that another factor, compression, can be strongly antioxygenic. Chelating agents have been less emphasized in current energy-dense candidate ration models, and, again, results reported below suggest a large benefit from their use. Catalytic oxygen scavenging with palladium in a 5% hydrogen atmosphere has been found too expensive for large-scale commercial application, but might well be reconsidered, in the light of the criticality of these rations. Moisture-dependent oxygen scavenging (ascorbic acid, glucose oxidase) is relatively unavailable because of the low water activity.

For extremely long term storage stability under oxidative conditions, then, one is left with the hydrogen-donating antioxidants, which, together with certain resuscitant synergists like ascorbyl palmitate, can be highly effective if the antioxidant-synergist couple is carefully selected for appropriateness to the type of lipid display in the food.

Appropriateness of Antioxidants. One of the authors[3] has proposed a rationale for appropriate choice of antioxidants (the so-called polar paradox) which regularizes much, although not all, of the welter of effectiveness data generated since the 1930s. The rationale is related to the two basic displays of lipid that are of research and practical interest: bulk oils and fats versus dispersions like emulsions, micelles and membranes, such as occur in prepared foods and in natural whole tissues. The dichotomy is more sharply drawn if one contrasts lipids in low surface-to-volume displays (bulk oils and fats, animal or vegetable) with those in high surface-to-volume situations (near-colloidal dimensions). In these two situations, polar, hydrophilic, or high HLB (hydrophilic/lipophilic balance) compounds tend to be much more effective in the low surface-to-volume display, while nonpolar lipophiles or amphiphiles with low HLB are much more effective in high surface-to-volume dispersions.[3]

The contrast in effectiveness is particularly marked between the extremes of dry vegetable oils versus emulsions and membranes with a low concentration of dispersed phase and a high aqueous concentration.

Lipophiles like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or Ethoxyquin(R) are virtually without activity in bulk safflower oil,[4] whereas they are extremely effective in emulsions similar to those in salad dressing.[5] On the other hand, gallic acid, the ubiquitous hydrophile from tea, cocoa, and many vegetables and fruits, is vastly more effective than BHA in vegetable oils,[4] but very low in activity in lecithin dispersions.[6] Nature abounds in the polar materials like gallic acid and, if used in very high concentrations, they can be

moderately effective in dispersions. However, at these concentrations they tend to be bitter and astringent, produce color with trace metals, act as substrates in enzymatic browning, and compromise essential amino acids and protein quality and texture.[7] Indeed, in some vegetable protein processing, it is considered essential to get rid of compounds like gallic and chlorogenic acids.[8]

There are, of course, modifying circumstances. BHA and BHT are quite volatile and may be partially lost in freeze-drying. On the other hand, their volatility is put to use in dry cereal by inclusion in the packaging material, where once in place, they seem to permeate the cereal to trap free radicals.[9] The low molecular weight polyhydric antioxidants like propyl gallate (PG, a synthetic and more lipophilic form of gallic acid) are destroyed at high temperatures, particularly when accompanied by alkaline conditions, as in baking.[10] This fragility seems to disappear for the higher gallate esters, as does the slight bitterness and tendency to color with metals.[10]

Availability. Dependable supply of these compounds hinges on two factors, among others: threat of regulatory proscription, and price.

The standard "Big Four" of FDA-approved antioxidants, BHA, BHT, tertiary butylhydroquinone (TBHQ), and PG, have a regulated status by which they may be used, in general, at 0.02% oil basis, in most foods other than whole meats. This status has rested on nearly a generation of acute and chronic toxicity testing.[10] The status was heavily compromised recently by the finding of carcinogenic potential at extremely high feed concentrations in rats for BHA and BHT, but not the gallates, in spite of the fact that at usual dietary intakes, there are indications of a strong

anti-carcinogenic action.[11] As is well known, the so-called Delaney Anti-cancer Clause, part of the 1958 Food Additives Amendment, mandates the proscription of any food additive, which at any dose level is found to induce cancer when ingested by experimental animals or humans.[12,13] Thus, the black-and-white nature of the Clause clashes with most benefit/risk assessments. The outcome is currently in doubt, considering the current litigious climate in the U. S.

These considerations have placed new emphasis on so-called GRAS or Generally Regarded As Safe, substances. These are substances, such as cornstarch, which have a history of safe use and have been deemed permissible to use in any amount commensurate with the technological objective. Such substances are the tocopherols (Vitamin E, or alpha, as well as beta, gamma, and delta -- all occurring in vegetable oils), ascorbic acid (Vitamin C), spices like rosemary and their oleoresin extracts, sesame oil, and similar materials. Included also are natural chelating agents like citric acid and phosphates.

Synthetic materials like the chelator ethylene diamine tetraacetic acid (EDTA), and the antioxidants BHA, BHT, and TBHQ, very often enjoy a substantial price advantage over natural competitors. It is certainly true that the tocopherols, ascorbic acid and its derivatives are expensive. However, a sensitized public, some individuals of which eventually become soldiers, may soon be demanding more of the natural materials, rightly or wrongly.

Synthetic Versus Natural. A consequence of the renewed attention on GRAS substance is, by their nature, a reopening of the synthetic versus natural controversy in food additives. The public has been alerted,

perhaps overly so, to the dangers of some man-made or xenobiotic (strange-to-nature) substances. Some faddists even suspect synthetic Vitamin C, which is identical to the natural product. Many, but not all GRAS substances, are natural extractives like the tocopherols, citric acid, and rosemary extract, but ascorbyl palmitate, for example, is synthetic, as is most commercially available ascorbic acid.

Nature of the Energy-Dense, Encapsulated Ration Display of Lipid. Appropriate choice of antioxidants depends, among other things, on knowledge of the state of dispersion of lipid. Energy-dense, encapsulated rations usually contain highly dispersed lipid, whether before drying and compression, as in dairy-derived or lecithin-suspended products, or after infusion into extruded nonlipid matrices. In general, the lipid "cells" of the latter are larger than micelles or emulsion droplets (e.g., salad dressing or whole milk) of dairy-derived or lecithin-suspended products. However, the infused products probably have a heterogeneous lipid cell size distribution.

Antioxidants for dispersed systems like this, as noted above, should be nonpolar lipophiles or amphiphiles of low HLB. This is in sharp contrast to the antioxidants needed to protect salad oil, cooking oil, butter, or margarine. However, as lipid cell size becomes larger, more polar antioxidants become more effective.

Antioxygenic Measures Recommended. We have used an energy-dense, encapsulated system designed as a general model for the current and possible future dried, compressed and extruded-infused ration candidates. We have also used rapid assay methods developed in this work unit to detect diagnostic quality losses due to cross-linking, whether from Maillard or oxidation reactions.

With this system and these objective methods, we have shown the following to be highly effective, available, antioxygenic measures to supplement the BHA, tocopherol, and ascorbic acid now in use: (a) physical -- compression, (b) chemical -- TBHQ, long chain alkyl gallates, and rosemary extract (Herbalox$^{(R)}$), (c) chelation -- EDTA. The alkyl gallates have very low toxicity, little flavor, low coloration and low antinutritional potential. EDTA is a GRAS substance. Rosemary extract shares these traits and is a GRAS substance. This report describes tests validating the above statements.

The Model System and Kinetic Measurements. The candidate systems that result from designing energy-dense rations are, by nature of the demands placed on them complex in composition, semisolid, relatively dry and, most important, subject to change in real time as nutrition, acceptance and performance demands dictate. Values of kinetic parameters developed in such heterogeneous, unmixed, immobile systems (as opposed to classic studies with homogeneous, continuously mixed gases or liquids or continuously agitated dispersions) are of necessity quasi or apparent factors. Kinetic studies here embrace only quality loss rates with time and the dependence of such rates on temperature, since mass transfer of ambient oxygen and water vapor is precluded by the packaging used. The work of the past two years in this area has shown that quality loss processes like oxidation and Maillard reactions interact in such a way that the predominant early process tends to affect the later process in very complicated ways. For example, in suitable systems (containing reducing sugars and lysine, for example), Maillard compounds have an antioxidant potential that inhibits subsequent oxidation.[14] Similarly, if a

substance is oxidized early, the amine groups that normally catalyze and participate in the Maillard reaction are lost to fluorescent, yellow polymers so that subsequent browning would be inhibited. In other words, systems vary with each new ingredient, and shelf-life prediction takes on an ad hoc nature when dealing with multicomponent, changing systems. This does not, however, mean that substantial progress in prediction is impossible.

Thus, our primary function in shelf-life prediction is one of developing plausible, simple, but representative ration models which can stand as paradigms in order to develop very rapid, reliable objective measurements of quality loss. Using these methods, many comparative accelerated storage life tests at various temperatures may be quickly run on a new system and quickly related to nonaccelerated conditions by means of Q_{10} measurements on an ad hoc, model-specific basis. Labuza[15] has stressed that it is much less important to know the reaction order in quality loss -- the form of the quality loss/time function -- than it is to measure accurate Q_{10}'s, the dependence of rate on temperature. This is because acceptance or rejection depends on early stages of quality loss at which order of reaction differs little from zero.

The initial stages of either the Maillard reaction or lipid autoxidation result in odorless, colorless, nearly tasteless compounds. For the Maillard reaction, these are the Amadori compounds, which nevertheless result in irreversible compromise of some essential nutritional amino acids of proteins.[16] For lipid oxidation, the first stage is lipid hydroperoxides, which, like the Amadori compounds, are

irreversibly altered monomers.[17] Although with little to no effect on acceptance, these cause major diminution of essential fatty acids.

In the work reported here, which focuses on acceptance, we have chosen to monitor the second stage in lipid oxidation, conversion to volatile carbonyls and dicarbonyls, some of which, notably malonaldehyde, initiate cross-linking of proteins. In the Maillard reaction, the cross-linking is also initiated by carbonyls, but nonvolatile ones.

In either Maillard reaction or lipid autoxidation, this stage is very sensitively indexed by fluorescence, which for the Maillard reaction, is closely, but not obligately correlated with brown color.[13] In lipid oxidation, the associated color is yellow, and much less prominent.[18] At this stage also, in both reactions, volatile odor-producing compounds are formed,[19] which prejudice acceptance as does the texture loss (toughening) associated with the cross-linking. Thus, fluorescence, brown or yellow color, cross-linking (increase of molecular weight of proteins) and flavor changes are all closely correlated, although nutritional loss can occur well before these changes.

For monitoring fluorescence of autoxidizing lipids, we have settled on four methods, the latter three of which are classed as rapid assay: (1) pronase digestion of protein, filtration and measurement of filtrate fluorescence; (2) chloroform-methanol or hexane extraction, followed by centrifugation and measurement of the fluorescence or absorbance of the chloroform or hexane layer, respectively; (3) front-face fluorescence of the acid-precipitated protein in controlled, packed slurry; and (4) measurement of compounds formed in the vapor phase over oxidizing lipids on the surface of polyamide powders supported in thin layers on terephthalate

plastic or glass. The first three methods are more suitable for current status measurement, while polyamide fluorescence is more applicable to accelerated storage tests, like comparative evaluation of antioxidants. It can also be used as an in-package detector of oxidation.

Fluorescence has the advantage of being rapid, reliable, and an order of magnitude more sensitive than absorption spectrophotometry or chemical tests. It is thus very suitable to early detection of small changes, which is what is needed in the rapid, repetitive determination of Q_{10}'s required for multicomponent, semisolid, rations with changeable components. Q_{10}, it will be recalled, is an approximate measure of the dependence of reaction rate on temperature and is essential for estimates of comparative shelf life.

MATERIALS AND METHODS FOR TESTING ANTIOXYGENIC MEASURES

The model systems and most of the rapid assay methods for quality loss measurement have been described in Technical Report NATICK/TR-86/063, 1986.[18] A brief recapitulation will be made here.

Model Systems. Two types of systems have been designed: a soybean oil, egg-yolk dispersed system and a corn oil, soybean lecithin dispersed system. Either of these may be thrown into dry, moist, or fluid states, depending on the water activity over which they are equilibrated: approximately zero, 0.5, or approximately 1.0. Table 1 shows the prescribed compositions of the two systems.

TABLE 1. Encapsulated Energy-dense Model Systems

TYPE	COMPONENT	PERCENTAGE
Corn oil, soy lecithin	Stripped corn oil	35
	Stripped soy lecithin	1.5
	Sodium caseinate	15
	Lactose	35
	Starch	5
	Assorted additives	8.5
Soybean oil, egg yolk	Soybean oil	35
	Fresh egg yolk	5
	Sodium caseinate	12
	Lactose	14
	Starch	4
	Water	30

Details of preparation of the corn oil-lecithin system which was used for the tests reported here, are contained in Technical Report, NATICK/TR-86/063.[18] Either system may be freeze-dried and subsequently reconstituted to desired water content by equilibration over appropriate water activity, or dispersion in deionized water. The systems were designed to be poised with both a high Maillard and oxidative reactivity, either or both of which can be activated by suitable manipulation.

Measurement Methods. Details of the four measurement methods have been reported in Technical Report, NATICK/TR-86/063.[18] They are briefly summarized below:

1. Pronase digestion: 100 mg of a relatively dry (and preferably hexane or chloroform-methanol extracted) oxidized sample is comminuted in a mortar and added to 4 mL (0.1 M) ammonium bicarbonate solution, pH 7.8, containing 2 mg Pronase(R) (Calbiochem., Inc.) in a centrifuge tube. The tube is stoppered and kept in a 37°C water bath (± 3°C) for 24 hours. If the sample has not been pre-extracted, 2 mL of chloroform is added, the

tube is mixed one min on a Vortex mixer, and centrifuged for 1 hr on a clinical centrifuge. The aqueous supernatant is used for absorption spectrophotometry at 420 nm without dilution and is appropriately diluted for fluorescence spectrophotometry using excitation at 392 nm. Care is taken to avoid quenching. If turbidity develops, samples are filtered through a 0.22-μm Millex-GV or 0.45-μm Millex-HV unit (Millipore Corp.) to produce a clear solution. Gel filtration on P-2 or P-6 gels (BioRad, Inc.) may be an optional additional step, using deionized water as eluent.

2. Chloroform-methanol extraction: 100 mg of a relatively dry oxidized sample is comminuted in an agate mortar and added to a separatory funnel containing 4 mL of deionized water and 20 mL of a chloroform-methanol solution (2/1, v/v, spectral grade, Burdick and Jackson, Inc.), the water and organic solvent being initially in two layers. The separatory funnel is stoppered and shaken a timed 5 minutes. The contents are decanted into a centrifuge tube and centrifuged on a clinical centrifuge for a timed 5 minutes. Ca 4 mL of the clear lower layer (92% $CHCL_3$/8% MeOH) are carefully transferred by a Pasteur disposable pipette into a 5-mL test tube, stoppered with aluminum foil. A measured 100 mg of anhydrous sodium sulphate is added and stirred by careful plunger action of a glass rod. An initial colloidal opalescence usually clears immediately, and the fluorescence of the solution is measured at an excitation wavelength of 380 nm, emission being in the 450 to 460 range. This method is about ten times as fast as the enzymatic method, is reproducible and less labor intensive, being applicable even in the frequent cases where turbidity renders the enzymatic method unsuitable. The method has been automated using the Soxtec System HT, an

accelerated Soxhlet extraction method involving 15 min of immersion in boiling solvent and 30 min of reflux rinse. Six samples may be analyzed in two to four hours by this method.

3. Front-face fluorescence of acid-precipitated casein slurry: (Applicable to any Precipitable protein). A measured 600 mg of a relatively dry oxidized sample is extracted with about 50 mL spectral grade hexane in the Soxtec HT using 15 min boil and 30 min rinse. The oil residue remaining in the cups may be diluted with spectral grade hexane and its absorption spectrum read at 233 and 268 nm on a Cary Model 15 Ultraviolet and Visual Spectrophotometer. This gives a supplementary index of oxidation. The powder remaining in the thimbles is weighed and placed in 10 mL deionized water. It is titrated to pH 4.6 with 0.1 N H_2SO_4. The precipitated is centrifuged and washed with 10 mL of deionized water three times, thus removing the fluorophore riboflavin. (Vitamin A, another interfering fluorophore, is removed by the hexane wash, as are such ultraviolet active compounds as vanillin or antioxidants).

To a 10-mm x 4-cm silica cell is added 0.5 mL of a ca 15% slurry of the dispersed powder in water. By pressing a 9-mm x 4-cm quartz wedge into the cell, a 1-mm thick slab of compressed slurry is forced up into the 1-mm-thick void space, nearly filling one face of the fluorescence cell. The compressed slurry shows little to no settling and its fluorescence can be read by front-face viewing on the Spex Fluorolog Spectrofluorometer, at an angle of 22^{c}. Typical excitation wavelength is 360 nm, initial emission of little-oxidized samples being at 445 nm. Slit widths are typically excitation 2.0 mm, emission 1.0 mm, with a 1-nm increment and integration time of 0.1 second. The instrument is set in the DC (nonphoton counting)

mode. A 390-nm cutoff filter is placed in the emission path, and the spectrum is scanned through the residual of the excitation peak at 360 nm. The 360-nm peak can then be used as an internal reference. This permits a normalizing procedure called the fluorescence index, by dividing fluorescence intensity at the emission maximum by intensity at the residual, filtered 360 peak. In heavily scattering samples like these, the fluorescence index so derived is a dependable normalization method when only event marking (induction period) or relative rates are required. The index also has a built-in amplifying factor, in that, as oxidation progresses and fluorophores accumulate on the precipitated protein, the intensity of scattering is diminished because light energy is absorbed. Thus the rate of rise of the index is greater than the rise of the non-normalized emission intensity.

Although the wavelength of maximum emission of little-oxidized protein is about 445 nm, with the progress of oxidation, this shifts toward the red, often reaching 478 nm. At the same time the protein becomes visually yellow (but rarely brown as in the Maillard reaction).

4. Oxidative polyamide fluorescence: We have shown [20] that the vapors from oxidizing lipids, when in contact with polyamide powders, produce characteristic fluorescent compounds on the polyamide surface, similar to those shown for amino-imino-propene compounds by Chio and Tappel.[21] Solid sample methods for measuring this fluorescence have been reported.[20] The method has been applied to the present energy-dense, encapsulated model systems in three modes -- dry, moist, and fluid -- for the purpose of comparative accelerated storage life tests of antioxidants and antioxygenic measures.

Comparative Accelerated Storage Stability Tests. In the typical test, 2 x 3 cm terephthalate plates coated with a 250 μ polyamide layer are suspended powder face down on aluminum mesh screen over open 5 cm Petri dish bottoms containing a carefully weighed 600 mg of dry model system comminuted powder. The Petri dish, in turn, is supported on an overturned 25-mL beaker standing in a half-pint Ball jar containing approximately 20 mL of saturated sodium bromide or about 20 g of Drierite desiccant. If high water activity conditions are desired, the powder is dispersed in 4.4 mL deionized water (13%). The screw-top lid of the Ball jar is carefully lined with aluminum foil, since it has been occasionally found that the rubber seal ring contributes oxidative fluorescence. The jars are equilibrated overnight in the dark at room temperature after sealing, and are placed in a 100°C draft oven for various storage times. If greater acceleration is desired in the liquid exposure, 6 mg of cobalt chloride hexahydrate in 0.1 mL deionized water is added immediately prior to oven storage. The polyamide indicator plate is read before commencement of oven storage and at appropriate intervals, on a solid sample holder in a Baird-Atomic Fluorescence Spectrometer, using excitation wavelength 360 nm. Coarse gain is typically 10, fine gain 0, with entrance slit fine, exit slit medium. Emission begins at 425 nm and undergoes a red shift as oxidation proceeds.

Antioxidant Testing. In the the use of the comparative accelerated storage stability test for antioxidant evaluation, an amount of antioxidant equivalent to 1% (w/w) of the lipid is dissolved in 1 mL of 95% ethanol and added with stirring to the corn oil before preparation of the freeze-dried model system. This is tested against untreated control.

Replicate sample jars containing no model system are stored both at test temperature and at room temperature, the polyamide indicator plates being read at the time of reading the oven sample plates.

In the special cases of water soluble or dispersible materials like ethylene diamine tetracetic acid, disodium salt (EDTA) or rosemary extract (Herbalox$^{(R)}$), the materials were added with stirring and overnight equilibration to the deionized water and tested only in the fluid state (a_w 1.0). In these two cases, only 0.6 mg (0.1%, w/w dry system) cobalt chloride was added as accelerant and 9 mL of deionized water was employed as dispersant. Disodium EDTA was employed at 1% (w/w) lipid basis, and rosemary extract at 4%.

Field Storage Condition Testing. For replicating the relatively low temperatures of field storage, samples of 600-mg model system were prepared in 1/2-pint Ball jars containing both polyamide indicator plates and controlled water activity medium (desiccant Drierite, 15 g/jar, or saturated NaBr solution). The sample statistical design is shown in Table 2.

It was planned to withdraw samples at 3, 6, and 9 months. The samples are to be tested by chloroform/methanol extract fluorescence, front-face slurry fluorescence, and oxidative polyamide fluorescence of the in-package indicator plate. Results of the three-month withdrawal will be reported below.

TABLE 2. Field Storage Condition Test

Sample Description	Water Activity Control	Temperature and Time of Storage* -10°C	24°C	37.7°C	51.7°C
Control with polyamide plate only	Drierite	-	+	+	+
Control with polyamide plate only	$NaBr_{sat}$	-	+	+	+
Sample	Drierite	+	3-6-9*	3-6-9	3-6-9
Sample	$NaBr_{sat}$	+	3-6-9	3-6-9	3-6-9
Sample + TBHQ (1%)	Drierite	+	3-6-9	3-6-9	3-6-9
Sample + TBHQ (1%)	$NaBr_{sat}$	+	3-6-9	3-6-9	3-6-9

*Times of storage - months.

Compression Testing. To determine the effect of compression on oxidation 1-g samples of freeze-dried whole milk containing 1 mg/mL cobalt chloride were compressed using an Instron Universal Testing Instrument under a force of 388 kg/cm^2 (5510 lb/in^2) to disks of area 5.15 cm^2 and thickness 0.20 cm. They were enclosed in 10-cm diameter covered Petri dishes and stored at 65°C. A polyamide plate was secured to the Petri dish cover with double-stick adhesive tape, powder face down facing the compressed disk. At intervals, the plate was removed for fluorescence measurements.

Uncompressed samples of 1 g were stored at 65°C with a polyamide plate secured powder face down to the Petri dish cover. They were measured at the same intervals as compressed samples.

Materials and Instruments. Stripped corn oil for the model system was procured from Eastman Kodak Co., Rochester, New York. Most, but not all, of the tocopherols have been removed by molecular distillation. Sodium caseinate was obtained from Express Foods Co., Louisville, Kentucky. Soy lecithin used was Arlec(R) (acetone-stripped granules) obtained from Archer Daniels Midland Co. We have shown it contains very little of the tocopherols. Lactose was Baker's C. P. Analyzed, J. F. Baker Chemical Co. All were used as received.

Pronase(R) was procured from Calbiochem., Inc. Chloroform and methanol were spectral grade procured from Burdick & Jackson, Inc.

Polyamide plates (2 x 3 cm) are cut from standard 20 x 20 cm polyamide-terephthalate plates used for thin-layer chromatography (TLC). They are Polygram(R) Polyamide-6 UV_{254}, procured from Macherey-Nagel and Co. through Brinkmann Instruments, Inc., Westbury, New York. Polyamide coated on glass plates may be procured from Schleicher and Schuell, Inc., Keene, New Hampshire. They were cut with a standard window-glass cutter.

Antioxidant purity was checked by melting point and by TLC in two solvent systems (chloroform, and chloroform-methanol on heat-activated silica gel). TBHQ was procured from Eastman Chemical Products, Kingsport, Tennessee, Inc.

Octyl (OG) and dodecyl gallate (DG) came from NIPA Laboratories, Ltd., Mid Glamorgan, Wales. They were a gift. Disodium EDTA dihydrate (EDTA) was purchased from Aldrich Chemical Co., Midland, Michigan. Rosemary

extract (Herbalox$^{(R)}$) was obtained as a gift from Kalsec$^{(R)}$, Inc., Kalamazoo, Michigan. Type W, with added lecithin, was used because it is water-dispersible.

Visual and ultraviolet absorption spectrophotometry was performed on a Cary Model 15 Spectrophotometer. Fluorescence measurements were made either on a Baird-Atomic Fluorescence Spectrometer or a Spex Fluorolog Spectrofluorometer. Compression was performed on an Instron Universal Testing Instrument. Automated lipid extractions were performed on a Soxtec System HT, Tecator AB, Hoganas, Sweden.

Whole-milk samples for compression testing were from Garelick Farms, Pasteurized and Homogenized, with Vitamin D added. For accelerated testing of oxidation under compression they were freeze-dried with 1 mg cobaltous chloride hexahydrate per 1 mL milk (0.77% dry weight).

RESULTS AND DISCUSSION

Antioxygenic Measures In Energy-Dense Model System. Figures 1, 2, and 3 show for dodocylgallate the course of development of polyamide oxidative fluorescence in dry, moist, and fluid conditions, typical of the curves used to prepare Table 3 which shows relative effectiveness of antioxidants. Noteworthy is the pronounced increase of induction period and the decrease in rate and maximum fluorescence index shown at the higher water activities.

This reduction with increasing water activity, in rate of fluorescence development and maximum fluorescence index may be due to either a Maillard reaction production of antioxidants or the known inhibitory effect of increasing water activity on autoxidation. However, in general, one expects autoxidation to be greater under liquid water conditions (a_w 1.0) than under intermediate water activities, unlike the present case.

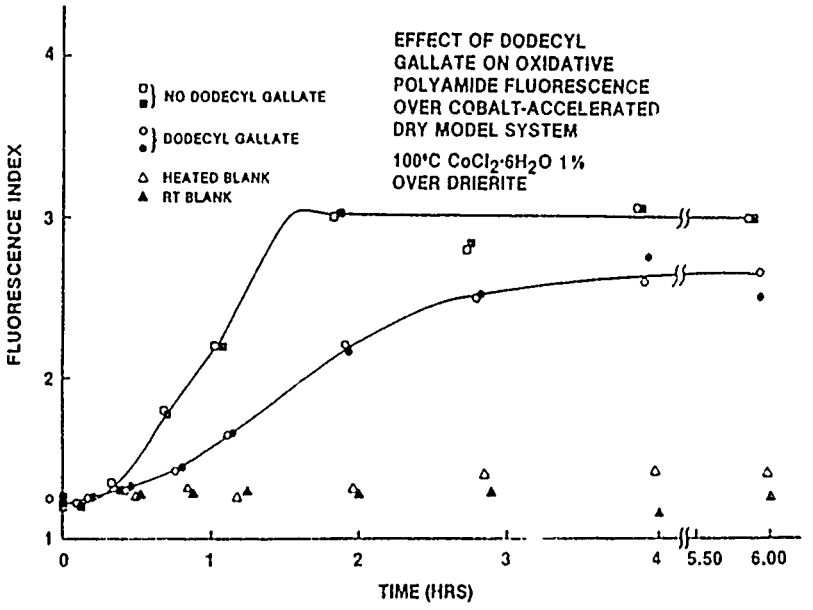

Figure 1. Effect of dodecyl gallate on oxidative polyamide fluorescence over cobalt-accelerated dry model system.

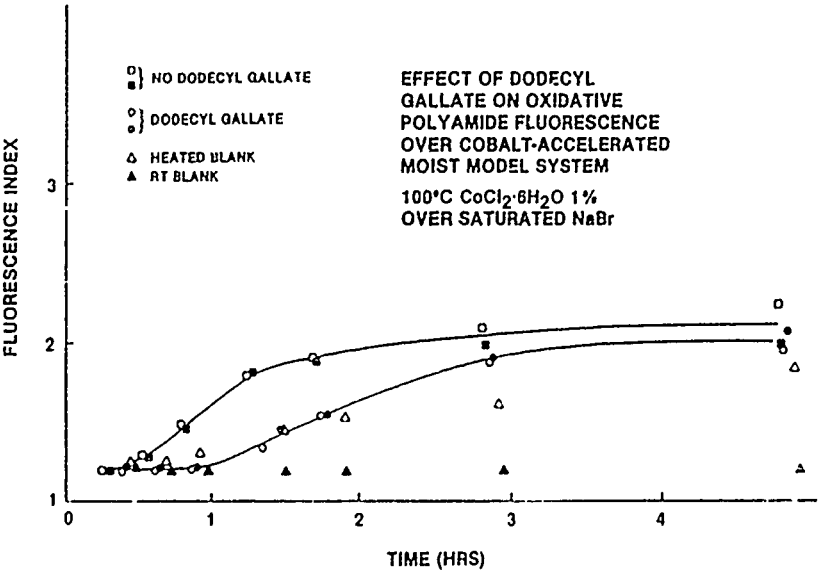

Figure 2. Effect of dodecyl gallate on oxidative polyamide fluorescence over cobalt-accelerated moist model system.

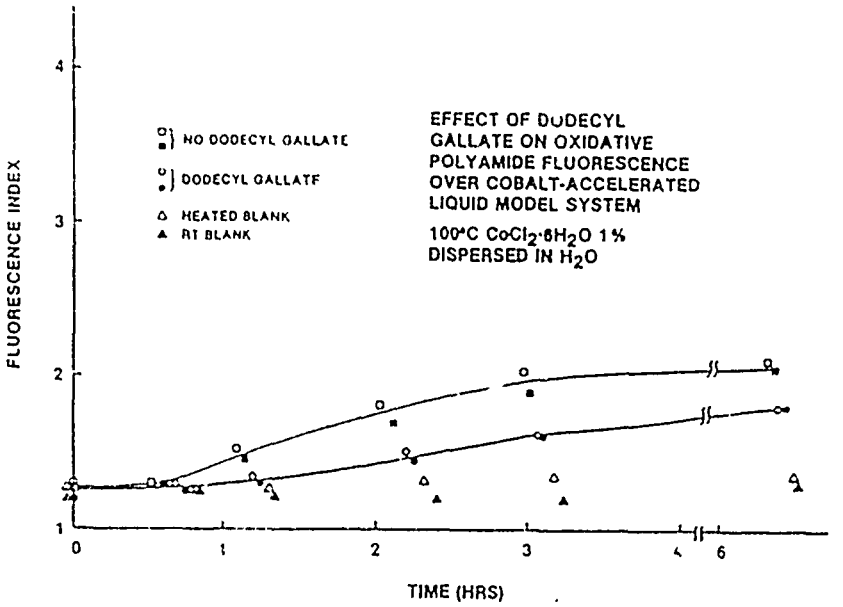

Figure 3. Effect of dodecyl gallate on oxidative polyamide fluorescence over cobalt-accelerated liquid model system.

TABLE 3. Relative Effectiveness (REFF*) of Antioxygenic Treatments Measured by Polyamide Fluorescence in Corn Oil/Soy Lecithin Model System Stored at 100°C with Cobalt Chloride Acceleration at Various Moisture Levels

ANTIOXYGENIC TREATMENT	CONCENTRATION (PER CENT DRY WEIGHT OF LIPID)	RELATIVE EFFECTIVENESS AT MOISTURE LEVEL SHOWN		
		DRY	MOIST	FLUID
TBHQ	1	60	5.0	--
OG	1	2.2	2.6	5.5
DG	1	1.9	2.9	2.3
EDTA	1	--	--	4.0
ROSEMARY EXTRACT	4	--	--	3.4

*REFF = $\frac{\text{Induction period with treatment}}{\text{Induction period without treatment}}$. 1.0 = no activity of antioxidant

Induction period commences at Δ F.I. (sample minus heated blank) = 0.25

As a general rule, if the model system without cobalt is stored at temperatures favoring the Maillard reaction (75-100°C), rapid phase oxidation with rapid polyamide fluorescence and rancidity development does not occur, even in dry systems. At lower temperatures, however, both of the latter usually occur readily after sufficient time. Cobalt invariably produces both effects.

This is strong evidence for production of Maillard antioxidants. However, we have not yet been able to demonstrate unequivocally a completely reliable inhibitory effect of preheating on autoxidation, although as noted, we have observed many cases of drastic reduction in oxidation rate.

Table 3 shows the relative effectiveness of three synthetic antioxidants, one naturally occurring antioxidant extract, and one synthetic chelating substance in inhibiting the development of oxidative polyamide fluorescence over the model system stored at 100°C and also accelerated with cobalt chloride at 1% dry weight. Antioxidants and EDTA are present at 1% concentration (lipid basis), while rosemary extract is at 4%.

At this unusually high concentration, all of the materials were very effective. Conspicuous was the extreme activity of TBHQ in the dry system, its high activity in the moist system, and the high activity of octyl gallate and rosemary extract in the fluid system.

Effects of Compression on Energy-Dense Model System. Figure 4 shows, for a cobalt-accelerated system stored at 65°C, the pronounced increase in induction period and decrease in rate and maximum oxidative polyamide fluorescence produced by compression of a freeze-dried whole milk system.

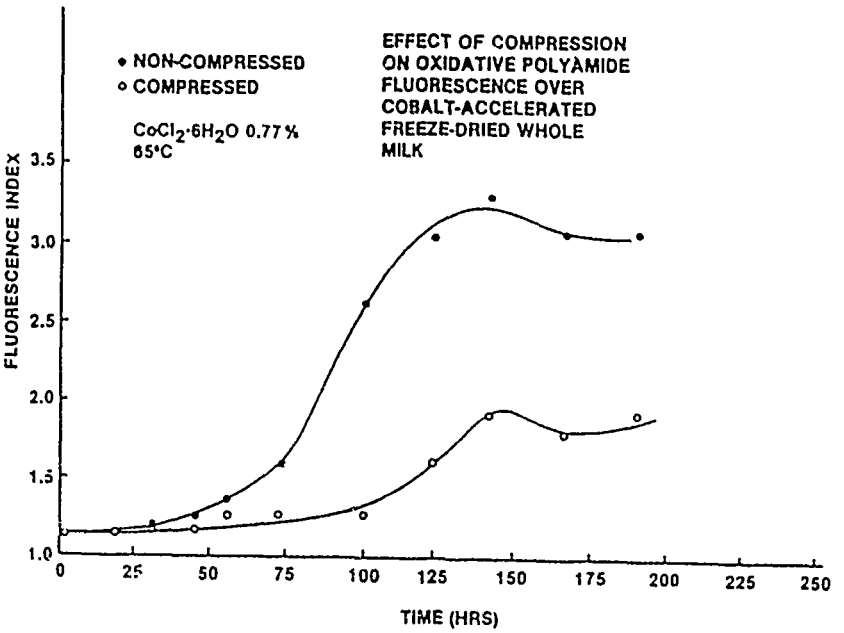

Figure 4. Effect of compression on oxidative polyamide fluorescence over cobalt-accelerated freeze-dried whole milk.

Table 4 shows for compressed and uncompressed systems, the relative effectiveness of the antioxidants propyl gallate (PG) and dodecyl gallate (DG) and the electron-donating, antioxidant-regenerating synergist, ascorbic acid. It is clear that compression approximately doubles the induction period of the untreated and the PG and DG treated systems. However, ascorbic acid, either alone or in combination with PG and DG shows little effect except in isolation in the uncompressed system. In the compressed system, it is even slightly pro-oxidant with PG and DG and shows no enhancement of the ascorbic acid effect observed in the uncompressed system. Compression clearly mechanically de-aerates a product.[22]

TABLE 4. Relative Effectiveness (REFF*) of Antioxygenic Measures in Suppressing Polyamide Oxidative Fluorescence Over Freeze-Dried Whole Milk. 65°C. Cobalt Chloride 0.77%.

ANTIOXYGENIC TREATMENT	CONCENTRATION (PER CENT DRY WEIGHT OF LIPID)	RELATIVE EFFECTIVENESS UNCOMPRESSED	COMPRESSED
UNTREATED CONTROL	0.00	1.0	1.8 - 2.6
PG	0.02	1.9	3.4
DG	0.02	3.5	8.0
AA	0.10	1.9	2.0
PG + AA	0.02 + 0.1	2.1	2.8
DG + AA	0.02 + 0.1	4.4	7.2

*REFF = $\frac{\text{Induction period with treatment}}{\text{Induction period without treatment}}$. 1.0 = no activity of antioxidant

Induction period commences at Δ F.I. (sample minus heated blank) = 0.25

Since ascorbic acid and its palmitate are each used at about 0.1% (dry weight basis) in current candidate dried-compressed, energy-dense, dairy-bar ration components, the finding of very low antioxygenic activity of ascorbic acid in compressed systems is significant. It also stands in contrast to the known strong synergistic activity of ascorbic acid in vegetable oils containing tocopherols and of ascorbyl palmitate in aqueous lipid dispersions of high surface to volume ratio, containing residual tocopherol.

It seems possible that the low activity of ascorbic acid in compressed systems is related to its necessarily low mobility. It cannot be simply

the nonaqueous environment, since ascorbic acid is very potent in dry vegetable oils containing tocopherols.

Noteworthy also in Table 4 is the high activity of the lipophilic DG compared to the much more polar PG. This is to be expected by the rationale for appropriate choice of antioxidants, the so-called polar paradox mentioned above, since the encapsulated, high-lipid model system is freeze-dried from a dilute dispersion and hence has high surface to volume ratio. The lipophilic DG would be expected to surpass the more polar PG in activity.

Noteworthy also is the strong synergism displayed between the effects of DG and compression. The combination of these two treatments produces a system extremely stable to oxidation. The addition of a chelator like EDTA might further enhance this stability.

Application of Rapid Measurement Methods for Oxidation and Maillard Quality Loss to the Encapsulated Model System Stored Under Field Simulating Conditions. As outlined under Measurement Methods above, samples of the encapsulated, energy-dense model system were stored under simulated field storage temperatures at 70, 100, and 125°F (24, 37.7, and 51.7°C), the total storage to be 9 months. Results of the 3 month analysis are shown in Figures 5 to 8.

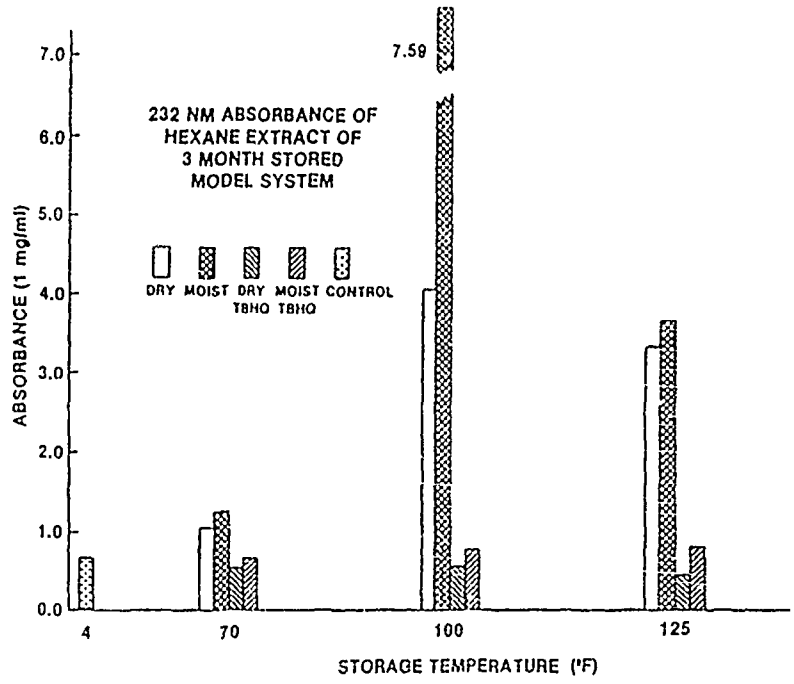

Figure 5. The 232-nm absorbance of hexane extract of 3-month stored model system.

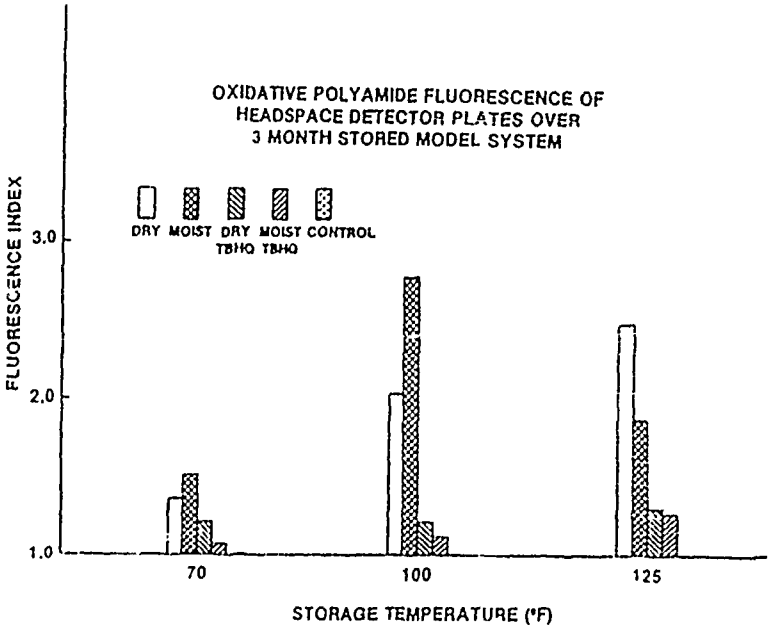

Figure 6. Oxidative polyamide fluorescence of headspace detector plates over 3-month stored model system.

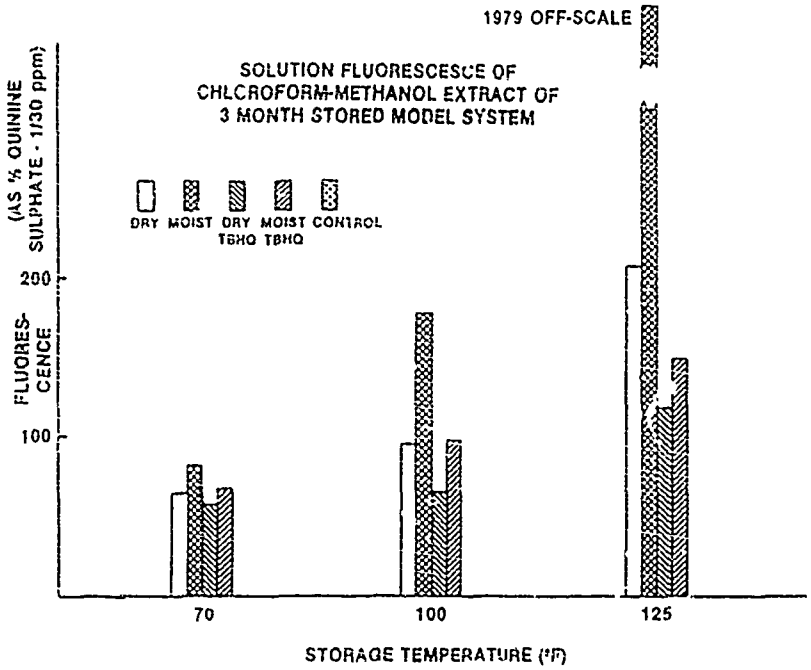

Figure 7. Solution fluorescence of chloroform-methanol extract of 3-month stored model system.

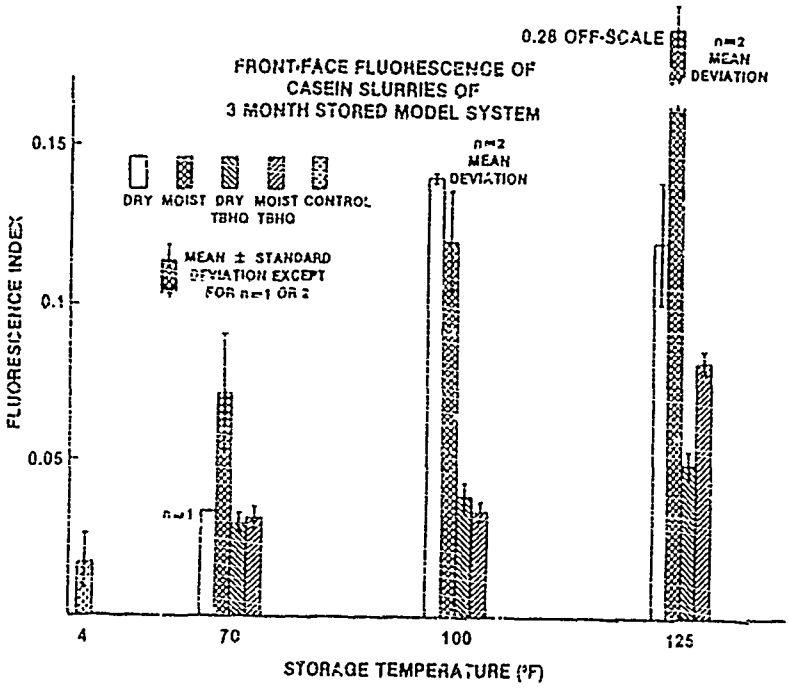

Figure 8 Front-face fluorescence of casein slurries of 3-month stored model system.

Absorbance at 232 nm (reflecting % conjugated diene) of hexane extracts (Fig. 5) is usually linearly related to peroxide value at low (field storage) temperatures. Figure 5 shows that absorbance at 232 nm increased rapidly at 100 and 125°F in the first 3 months for the dry, untreated sample and more rapidly for the moist, untreated samples. Antioxidant-treated samples remained similar to control. As is frequent, 232-nm absorbance peaks later at medium temperatures (100°F) and declines after an early peak at higher temperatures (125°F).

Oxidative polyamide fluorescence developed on detector plates in the headspace (Fig. 6) shows much the same story. Volatile carbonyls (which presumably produce the fluorescence) appeared in the unprotected samples, but not in the antioxidant-treated ones, at the higher storage temperatures. The moist sample was again more oxidized than the dry, the lower index value at 125°F deriving from a shift of most of the total fluorescence towards the red wavelengths as a shoulder, in advanced levels of oxidation, although the intensity at the wavelength of the maximum declined somewhat.

Solution fluorescence of chloroform-methanol extracts (Fig. 7) shows the same rise at higher storage temperatures for the untreated samples (again, greater for the moist sample). However, this fluorescence measurement method does not respond exclusively to oxidation, since it measures both the oxidative cross-linking of phosphatidyl ethanolamine and phosphatidyl serine residues, and also the fluorescence from Maillard reaction cross-linking of the same residues. Thus the rise of the fluorescence in the antioxidant-treated samples stored at 125°F represents

very probably Maillard cross-linking at this temperature, since Figs. 5 and 6 show little oxidation in these samples.

Figure 8, showing the front-face fluorescence of acid-precipitated casein protein from the samples reveals much the same story. Here, the cross-linking is from bound protein, predominantly ε-amino-lysine residues. Oxidation is undoubtedly revealed by the fluorescence of the dry and moist untreated samples, the latter again greater, but the 125°F antioxidant-treated samples show an undeniable rise. Here, again, as in Fig. 7, Maillard cross-linking is probably the culprit in samples shown by Figs. 5 and 6 to be essentially oxidation-free.

The rapid evaluation methods for oxidation and Maillard reaction shown above are simple, sensitive, adaptable, and sufficiently reliable, when coupled with analysis of variance, to give ready, rapid, real-time assessment of cross-linking quality loss with time and temperature. Thus quick comparative assessments of different formulations and additives can be made by fast accelerated storage life tests, keeping up with the fast pace of formulation change as it reflects new nutritional, acceptance, and performance demands.

CONCLUSIONS AND RECOMMENDATIONS

Using an encapsulated, energy-dense model system simulating a generic calorie-dense ration component, we have developed rapid, reliable, sensitive measurement methods for quality loss through oxidative or Maillard cross-linking of phospholipids or proteins. Cross-linking occurs as the second, or sensible stage of both of these degradative processes and correlates with color, odor, and toughness.

Using these methods we have shown that at an atypically high concentration the synthetic antioxidant TBHQ is the most potent single antioxidant tested in uncompressed dry and moist systems. Since TBHQ shares, although in lesser measure than BHA and BHT, forestomach tumor promotion at very high dose levels in rats,[23] we have shown that the higher alkyl gallates, which have a very low acute and chronic toxicity,[24] are nearly as effective. They are partially hydrolyzed in the gut of mammals to harmless compounds, ubiquitous in any diet containing vegetables and fruits. Condensed rosemary extract (Herbalox$^{(R)}$) containing the active ingredient carnosol, a lipophilic species, is as effective, and is a GRAS substance. The high activity of this carnosol-based extract in dispersed systems and its known inactivity in dry vegetable oil is predicted by the polar paradox rationale detailed above.

We have shown that compression of dried dairy-based systems is a powerful antioxygenic measure and that it has a synergistic effect with DG. The combination of compression and DG produced the longest induction period of any measures tested in the dairy-based, dried system. It is to be expected that chelators like EDTA will further extend this protection, to approach the extreme stability mandated for energy-dense, encapsulated systems for Army 21.

By contrast, ascorbic acid produced little to no effect in the compressed system other than a slight pro-oxidant action when combined with the gallates. This is of significance to ascorbic acid fortification, if antioxidant action is expected in addition to nutritional gains.

DG, a lipophile, was more than twice as effective as PG, a relative hydrophile, in the dried, dairy-based system, whether compressed or noncompressed. This would have been predicted by the polar paradox rationale for appropriate antioxidant choice in dispersed lipid systems.

This document reports research undertaken at the US Army Natick Research, Development and Engineering Center and has been assigned No. NATICK/TR-85/014 in the series of reports approved for publication.

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