

D. G. CORNELL  
Eastern Regional Research Center<sup>1</sup>  
Philadelphia, PA 19118

#### ABSTRACT

Mathematical models for the prediction of antioxidant distribution in whole milk have been derived and verified. These distribution equations allow prediction of the division of added antioxidant between the oil and water phases and the amount bound to the proteins of milk. Simple considerations of oil/water partitioning and protein binding are involved in the predictions. For systems where the oil is in the form of a continuous bulk phase, the agreement between experiment and theory is good. The protein binding exerts a marked influence on the distribution of antioxidants between the aqueous and oil phases. For emulsions, where the lipid is finely divided globules, the experimental results show higher than anticipated antioxidant content in the oil phase, suggesting sorption of the antioxidant at the greatly expanded oil/water interface. The results are consistent with the known concentration dependence of sorption phenomena and the size of the antioxidant molecule. The nature of the interfacial complex was not determined. Agreement between theory and experiment for antioxidant in the fat phase of whole milk powder was reasonable. The extent of antioxidant sorption at the fat/solid matrix interface of whole milk powders was not determined.

#### INTRODUCTION

Stabilization of the initial flavor of whole milk powders produced by various spray (9) and vacuum (12) drying techniques has been

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<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

one of the objectives of research in the Dairy Laboratory for some time. Packaging in inert gas (13) or heat treatment of the milk before drying (14) enhance storage stability, but objections arise to the economics of the inert gas packaging and to the cooked flavor developed in powders from milk heated in excess of pasturization requirements. Attempts have been made to avoid these problems with antioxidants but with limited success (15).

Many antioxidants work well when added to single component foods such as cooking oils but perform poorly in heterogeneous systems such as whole milk powder. The antioxidant presumably should reside either in the fat or at the fat globule surface for adequate protection of the powder. Although many of the commonly used antioxidants exhibit a marked preference for the oil phase (3), binding to the proteins of milk also occurs (4), which would cause some degree of retention of the antioxidant in the aqueous phase. Simple mathematical relations characterize these interactions (3, 4), suggesting the possibility of predicting the distribution of antioxidants in whole milk. The problem of predicting the distribution of additives in heterogeneous systems is a general one of which antioxidants in milk is but a special case. Accordingly, the exposition and proof of validity of the mathematical model describing the distribution of gallic acid esters and butylated hydroxyanisole (BHA) in fluid and dry whole milk are below in detail. Derivations of the equations are in an appendix.

#### MATERIALS AND METHODS

The antioxidants in this work have been described (3, 4). Laurylgallate (LG) was synthesized from carbon-14 labeled dodecyl alcohol and gallic acid according to previous procedures (3). This dialysis tubing was from Arthur H. Thomas Company.<sup>2</sup>

Reagents used for liquid scintillation analysis, 2,5-diphenyloxazole (PPO), 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP), Triton X-100, and Soluene 100, were from the

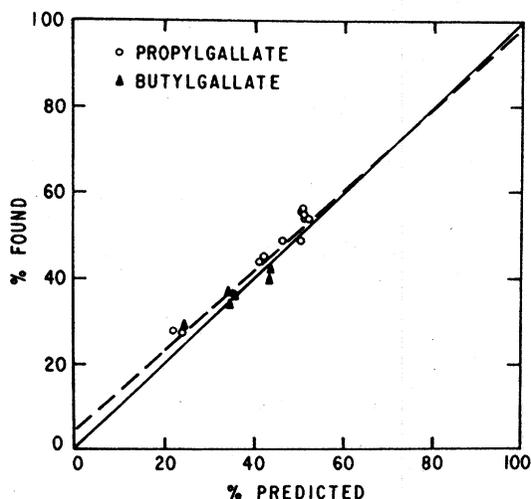


Figure 1. Antioxidant in aqueous phase of model milk. Values experimentally observed vs values predicted from equation 3.

Packard Instrument Company.

Model milk systems were composed of skim milk, cream, and butteroil prepared the usual way. Cottonseed oil was purchased from a Washington, DC area supermarket. Most model systems contained naturally occurring lactose, but for the systems requiring extended equilibration at 40 C (Figure 1) lactose was removed by dialysis in the cold against Jenness and Koops buffer (10). Holstein cows' milk was from a herd at the Beltsville Agriculture Research Center.

Procedures of AOAC were used for the determination of total nitrogen, fat, and moisture (2). A factor of 6.38 was used to convert nitrogen to protein. Inclusion of nonprotein nitrogen was estimated to have a negligibly small effect on the distribution calculations.

Procedures for liquid scintillation analysis varied according to the system to be analyzed, with the following typical directions, primary scintillation solvent, one volume Triton X-100 plus three volumes toluene (5.5 g PPO and .3 g dimethyl POPOP per liter toluene); cocktail for aqueous phase analysis, 15 ml primary solvent plus 1 ml aqueous phase; cocktail for analysis of heterogeneous phases, 2 ml Soluene 100, .5 ml or .5 g heterogeneous phase, heat 50 C 5 min, add 2 drops  $H_2O_2$ , heat 1 min, add sufficient acetic acid (5 to 6 drops) to neutralize the

mixture, then add 15 ml primary solvent. All cocktails were cooled to 5 C prior to counting. The Soluene was used to disperse the heterogeneous phases to clear solutions, the  $H_2O_2$  to decolorize the solutions, and acetic acid to minimize chemiluminescence. In questionable cases, the cocktails were dark-adapted overnight before counting. Cocktails for standard and blanks were made up identical to the unknowns except for the presence or absence of labeled antioxidant. Counting efficiency was not critically dependent on the amount of protein or fat; hence, some variation in cocktail make-up was permitted. Data were taken as counts per minute and used directly in all calculations without rectification to disintegrations per minute.

Antioxidant was added to the aqueous phase of the systems unless otherwise mentioned. Addition was from a stock aqueous solution or by direct weighing of the solid material followed by heating and agitation in the few cases where the antioxidant was added to the oil phase. Values for  $V_o$ ,  $V_w$ , and  $g$  (equations 1 to 3 below) were calculated from results of the AOAC procedures. Cottonseed oil/water partition coefficients were determined according to procedures of (3).

Systems of bulk oil/skim milk (Figure 1, Table 3) were stirred slowly to prevent emulsification of the skim milk with the overlying oil phase. Equilibration times were 1 to several days. Emulsions (Table 4) were prepared from unhomogenized cream or cottonseed oil homogenized with skim milk in a single stage Manton Gaulin laboratory homogenizer at 70 kg/cm<sup>2</sup>. Equilibration was for 1 day with stirring at the appropriate temperature. Surface areas of the oil/water interface were calculated from Coulter analysis (5) of the fat globule size distribution of samples taken at the end of the runs. The high fat model powders were of cream and skim milk concentrate made up to about 25% total solids and equilibrated with stirring for 2 h at 50 C after addition of the antioxidant and prior to freeze drying. The simulated "real" milks were prepared from butteroil and skim milk solids with the antioxidant added to the aqueous or heated oil phase. Homogenization at 250 kg/cm<sup>2</sup> and equilibration with stirring for 1 h at 50 C were followed by freeze drying.

Separation of the aqueous, protein, and lipid

components for antioxidant assay was effected by equilibrium dialysis and low speed centrifugation. Determination of protein bound antioxidant was by difference between skim milk and aqueous assays. Analysis was by liquid scintillation counting of labeled isotopes as given above except for the data in Figure 1, where analysis of the aqueous phase for unlabeled antioxidant was by ultraviolet absorption spectrophotometry (3, 4). Analysis of the lipid phase for labeled isotope was on aliquots of the bulk oil or samples of cream plug obtained by low speed centrifugation (15 min at a few hundred g). The cream plugs were analyzed for fat, protein, and water, and corrections were subtracted from the total assay to account for antioxidant associated with the protein and water components. Corrections amounted to at most a few percent of the total count. The fat phase of milk powders was extracted with 98/2 vol/vol decalin/isopropanol and analyzed by liquid scintillation counting to determine the gallate content. The BHA was analyzed by the method of Mahon and Chapman (11) with hexane as the extraction solvent. The solvent-powder suspension was agitated for 20 to 30 min, the suspended matter was removed by filtration, and an aliquot of the extract was analyzed for fat content by the AOAC method (2). The results were calculated as antioxidant content per gram of fat, the extracted lipid was assumed to be a representative sample, and the values for antioxidant in the fat phase (Table 5) were based on the total fat content of the powders. The degree of extraction, which was up to 90% in the high fat powders, seemed to have

little effect on the final values calculated for percent antioxidant in the fat phase. Control samples of freeze-dried skim milk powder containing either propylgallate (PG), hexylgallate (HG), or BHA showed that a negligible amount of antioxidant was extracted when lipid was absent.

## RESULTS

The distribution equations developed during the course of this work are derived in the appendix. The final equations are:

% Antioxidant in oil =

$$PV_o (100)/(PV_o + bg + V_w) \quad [1]$$

% Antioxidant bound to protein =

$$bg 100/(PV_o + bg + V_w) \quad [2]$$

% Antioxidant in water (free) =

$$V_w (100)/(PV_o + bg + V_w) \quad [3]$$

where  $V_o, V_w$  = volume in liters of oil and water phases, respectively;  $g$  = total grams of protein in the system;  $P$  = oil/water partition coefficient of the antioxidant;  $b$  = binding coefficient of the antioxidant.

For brevity, only a few systems, selected to illustrate the range of compositions employed are in Table 1. System compositions were chosen to give an appreciable fraction of antioxidant associated with each of the compon-

TABLE 1. Composition of model systems.

	Fluid			Powder	
	1	3	5	8	12 <sup>b</sup>
	System No.				
	Oil <sup>a</sup>				
	C	C	B	B	B
% Composition					
Oil	25.4	17.7	29.5	64.8	25.6
Protein	1.93	4.3	1.74	20.2	26.4
Water	68.5	77.9	67.6	....	....

<sup>a</sup>B = butteroil, C = cottonseed oil.

<sup>b</sup>Compositions of systems no. 11 and 13 (Table 5) are similar.

TABLE 2. Binding and oil/water partition coefficients (pH 6.5).

Antioxidant	Binding coefficient (b)	Partition coefficient (P)		
		Butteroil (40 C)	Cottonseed oil (40 C) (10 C)	
Propylgallate	.040	.84	.64	.87
Butylgallate	.084	3.9	....	....
Hexylgallate	.36	44.8	29	88
BHA	.04	825	....	....

ents oil, protein, and water to provide the most rigorous test of the distribution equations. Three of the model milk systems were made up to simulate the composition of powdered whole milk. Table 2 shows the binding coefficients and the butteroil/water and cottonseed oil/water partition coefficients of the antioxidants in this work.

The binding and butteroil/water partition coefficients were determined (3, 4); the cottonseed oil/water partition coefficients were determined according to procedures in (3). Figure 1 shows close correlation between predicted (equation 3) and experimentally determined antioxidant in the aqueous phase of bulk oil/skim milk systems, where the dashed line represents best fit with  $R = .966$ . The oil/water/protein distribution of antioxidant added to bulk cottonseed oil/skim milk systems is in Table 3, which shows general agreement between predicted and experimental values. Antioxidants added to emulsions showed a higher than predicted preference for the oil phase, as

in Table 4. The discrepancy was assumed to be due to oil/water interfacial sorption, and estimates of the average interfacial area per sorbed antioxidant molecule are given. Figure 2 shows the calculated distribution of hexylgallate between the oil, water, and protein components of milk with typical solids composition and varying water content. Table 5 gives the comparison between theory and experiment for freeze dried powders produced from concentrated emulsions with antioxidant added either to the aqueous or oil phase.

#### DISCUSSION

Experimentally observed antioxidant content of the aqueous phase and that predicted from distribution equation 3 agreed. These bulk oil/skim systems required equilibration times of one day or longer at 40 C and resulted in a high proportion of the runs being discarded because of souring of the solutions. Bacteriostats were only marginally successful in preventing degra-

TABLE 3. Antioxidant distribution in bulk cottonseed oil/skim milk model systems at 10 C.

	System No.					
	1 Propylgallate		2 Hexylgallate		3 Hexylgallate	
	% Distribution					
	Calculated	Found	Calculated	Found	Calculated	Found
Oil	14.6	14.9	72.6	68.1	48.5	40.6
Bound	43.6	41.5	23.1	23.0	49.2	51.0
Water	41.8	43.2	4.3	6.1	2.3	2.7
Recovery	....	99.6%	....	97.2%	....	94.3%

TABLE 4. Antioxidant distribution in high fat model emulsions.

	Cottonseed oil (10 C)		Butterfat oil (cream) (40 C)					
	System no. 4		System no. 5		System no. 6		System No. 7	
	Hexylgallate		Propylgallate		Hexylgallate		Hexylgallate	
	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found
% Distribution								
Oil	35.5	54.2(2.7) <sup>a</sup>	16.3	29.4(.5) <sup>a</sup>	37.1	52.6	28.1	47.2
Bound	59.6	44.7(2.6) <sup>a</sup>	42.5	34.4(1.8) <sup>a</sup>	56.1	39.3	62.6	45.3
Water	4.8	5.0(.3) <sup>a</sup>	41.2	33.5(1.0) <sup>a</sup>	6.9	4.6	9.2	7.3
Recovery	....	103.9%	....	97.3%	....	96.5%	....	99.8%
Antiox. conc. in water M		$7.5 \times 10^{-5}$		$7.4 \times 10^{-4}$		$1.6 \times 10^{-5}$		$7.9 \times 10^{-5}$
A <sup>2</sup> /molecule <sup>c</sup>		155(22) <sup>a</sup>		309(16) <sup>a</sup>		399(25) <sup>b</sup>		535(25) <sup>b</sup>
% at O/W interface		19		16		25		22

<sup>a</sup>Mean deviation of duplicates.

<sup>b</sup>Estimate of probably error of single determination.

<sup>c</sup>Average interfacial area (sq angstroms) per antioxidant molecule sorbed.

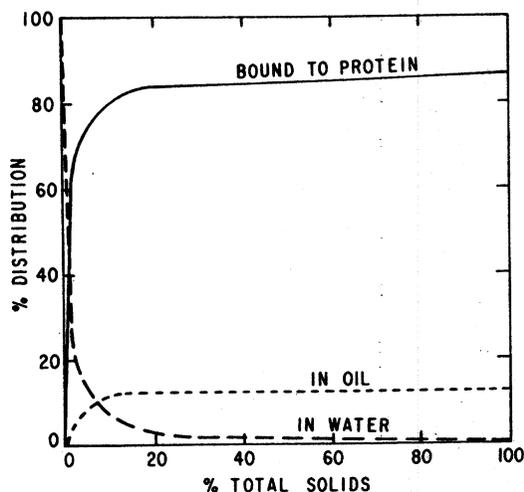


Figure 2. Hexylgallate distribution in milk as a function of solids content, calculated from equations 1 to 3.

ation. Use of cottonseed oil as the lipid phase permitted equilibration at 10 C while keeping the oil phase in the molten state necessary for partitioning and virtually eliminated the degradation problem. Use of carbon-14 labeled compounds facilitated assay of antioxidant associated with all three components, fat, protein, and water. The results in Figure 1 and Table 3 indicate agreement between the observed antioxidant distribution and values calculated from equations 1 to 3 for bulk oil/protein/water systems. Lactose, absent from the systems in Figure 1 but present in all other cases,

had little if any effect on the antioxidant distribution.

The antioxidants in emulsions, in contrast to those in systems where the oil formed a continuous bulk phase, exhibited a higher than predicted preference for the fat phase (Table 4). This increased preference is thought to be due to sorption of the propylgallate (PG) and hexylgallate (HG) at the oil/water interface as discussed below.

The difference between nonhomogenized systems and emulsions lies in the greatly expanded oil/water interface of the latter, which provides for greater absorption of surface active species, such as protein, which are known to be effective film forming agents (8). The esters of gallic acid are surface active (6) and would be expected to concentrate at the oil water interface, very likely forming a mixed film with the absorbed proteins. Mixed films involving proteins can be stable and have been subjects for much interesting research (8).

To calculate the antioxidant concentration at the oil/water interface we assume that for systems at equilibrium the distribution (partition) law must hold (7). Making the usual simplifying assumption that for dilute solutions, concentrations can be used in place of activities, and, assuming transport across the oil/water interface, we can use the partition coefficients given in Table 2 to calculate the antioxidant concentration in the interior of the fat globules of an emulsion. Subtracting this value from the observed concentration of antioxidant associated with the fat phase (from Table 4) gives the "excess" antioxidant which we assume

TABLE 5. Antioxidant content of fat phase in freeze dried milk powders.

	High fat model powders			Simulated "real" powders				
				System no.				
	8	9	10	11A	11B	12A	12B	13
	Antioxidant added to							
	Water PG <sup>a</sup>	Water PG	Water HG	Oil HG	Water HG	Oil LG	Water LG	Oil BHA
% In fat (calculated)	5.2	29.6	57.5	12.1	12.1	...	...	94.7
% In fat (found)	2.7	42.0	58.4	17.7	13.7	27.8	27.0	73.5

<sup>a</sup>PG = propylgallate; HG = hexylgallate; LG = laurylgallate; BHA = butylated hydroxyanisole.

to be sorbed at the oil/water interface. The aqueous and protein components also will contribute antioxidant to the interface. The interfacial excess in general will be higher than the quantity [~~found~~, calculated] (oil) derived from Table 4, where interfacial sorption was ignored in arriving at the calculated value. Surface area of the oil phase as determined from Coulter Counter particle size analysis (5) was  $1.41 \text{ m}^2/\text{cm}^3$  fat for cream and  $2.4 \text{ m}^2/\text{cm}^3$  fat for the cottonseed oil emulsions. These data are summarized in the form of the average oil/water interfacial area occupied per antioxidant molecule (Table 4). The data are consistent with the picture of surface sorption since higher concentrations of antioxidant are associated with increased surface excess (lower area/molecule). In all cases the surface coverage by the antioxidant molecules was much less than one monolayer, as seen from molecular models and surface tension-Gibbs law calculations (1) which gave a surface area per molecule of  $30 \text{ \AA}^2$  and  $60$  to  $80 \text{ \AA}^2$  for molecules on edge and lying flat, respectively (6).

The excess antioxidant in the lipid phase truly is associated in some way with the expanded interface and not due to antioxidant carried into the cream plug upon centrifugation by protein and water. Corrections for the latter were applied to the total count in calculating the "found" antioxidant in the lipid phase (Table 4).

An interesting question remains as to what happens to the antioxidant distribution upon rapid dehydration during spray drying or rapid solidification during freeze drying where the antioxidant might be precipitated out of the aqueous or oil phases. Equations 1 to 3 were used to calculate the distribution of hexylgallate as a function of the water content of the system. Typical values for the solids of Holstein cows' milk (16) were chosen and remained invariable; only the water content ( $V_w$ ) was changed. The calculated curves in Figure 2 show that as the water content of a system at hypothetical infinite dilution (zero total solids) is reduced, equilibrium in the distribution of HG between the fat and protein components is expected to be established at relatively low total solids and remain constant up to total dryness. This must be only an approximation since both interfacial sorption and changing activity of the water during "drying" were ig-

nored. Comparisons between the calculated and observed antioxidant content of the fat phase of freeze dried model milks (Table 5) suggests that the approximations are fairly reasonable for the gallates but relatively poor for butylated hydroxyanisole (BHA). Both the calculated and experimental results show the same order of preference of the antioxidants for the fat phase, namely,  $\text{PG} < \text{HG} < \text{BHA}$ . The higher than expected antioxidant content of the fat phase in two cases may be related to interfacial sorption, but the results are variable, and the undoubtedly complex nature of interactions at the fat/nonfat solid-solid interface precludes detailed discussion on this point. These results suggest that with the aid of distribution equations 1 to 3 and a few straightforward binding (4) and partitioning (3) determinations, a reasonable description of the antioxidant distribution in milk powders may be obtained. Results for laurylgallate in "real" powder, for which no predicted values are available, are included in Table 5. Comparison of these values with the previously postulated distribution (3), PG 20% in the fat phase, HG 90%, and BHA 99%, based on oil-water partitioning alone, shows the effect of protein binding on antioxidant distribution. It appears that BHA should be the most favorably distributed antioxidant for protection of milk lipids against oxidative deterioration. The PG and HG apparently concentrate at the oil/water interface in fluid milk, but the situation in dry milk is much less clear. Further, it is not certain that antioxidants concentrated at the oil/solid matrix interface would be effective in protecting the lipid phase from oxidation.

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#### APPENDIX

For fluid milk containing added antioxidant we consider the antioxidant as distributed between three components fat, protein, and water. Further, we assume that antioxidant in the aqueous phase will be in equilibrium with both antioxidant dissolved in the oil phase and bound to protein. We use concentrations instead of activities and make a further simplifying assumption that the activity of water is a constant independent of the solids content of

the systems under study.

The fraction of antioxidant in the oil phase is the amount in the oil phase divided by the total amount of antioxidant in the system; we write:

$$\begin{aligned} \text{\% Antioxidant in oil} = \\ A_o (100)/(A_o + A_p + A_w) \end{aligned} \quad [a]$$

where  $A_o$ ,  $A_p$ ,  $A_w$  = moles of antioxidant dissolved in oil, bound to protein, and dissolved (free) in water, respectively.

The following relations also hold:

$$A_o = C_o V_o \quad [b]$$

$$A_w = C_w V_w \quad [c]$$

$$A_p = M g \quad [d]$$

where  $C_o$ ,  $C_w$  = molar concentration of antioxidant in oil and water, respectively;  $M$  = moles of antioxidant bound to 1g of protein;  $g$  = total grams of protein in the system; and  $V_o$ ,  $V_w$  = volume in liters of oil and water phases, respectively. <sup>3,4</sup>

Previous work (<sup>6,7</sup>) has shown that the partitioning and binding of antioxidants follow the linear relations:

$$C_o = P C_w \quad [e]$$

$$M = b C_w \quad [f]$$

where  $P$  = oil/water partition coefficient, and  $b$  = binding coefficient.

Substitution of [e] and [f] into equations [b] and [d], respectively, yields:

$$A_o = P V_o C_w \quad [g]$$

$$A_p = b g C_w \quad [h]$$

Now substituting the right hand side of [c], [g], and [h] into [a] and cancelling the  $C_w$  term yields:

$$\begin{aligned} \text{\% Antioxidant in oil} = \\ P V_o (100)/(P V_o + b g + V_w) \end{aligned} \quad [1]$$

Distribution equations [2] and [3] can be derived

in a similar manner.

Equations 1 to 3 show the antioxidant distribution is independent of concentration. This is due to the linear relation between concentration, binding, and partitioning, equations [e] and [f]. For nonlinear relations between binding or partitioning the same general approach, equations [a to d] can be used, but the resulting distribution equations will show the antioxidant distribution is concentration dependent.

#### REFERENCES

- 1 Adamson, Arthur W. 1976. Page 68 in *The physical chemistry of surfaces*. 3rd ed. Interscience, New York.
- 2 Association of Official Agricultural Chemists. Page 266 in *Official methods of analysis*. 11th ed. AOAC, Washington, DC.
- 3 Cornell, D. G., E. D. DeVilbiss, and M. J. Pallansch. 1970. Partition coefficients of some antioxidants in butteroil-water model systems. *J. Dairy Sci.* 53:529.
- 4 Cornell, D. G., E. D. DeVilbiss, and M. J. Pallansch. 1971. Binding of antioxidants by milk proteins. *J. Dairy Sci.* 54:634.
- 5 Cornell, D. G., and M. J. Pallansch. 1966. Counting and sizing fat globules electronically. *J. Dairy Sci.* 49:1371.
- 6 Cornell, D. G. Unpublished observation.
- 7 Glasstone, S. 1946. Page 738 in *Textbook of physical chemistry*. 2nd. ed. D. Van Nostrand Company, New York.
- 8 Goddard, E. D., ed. 1975. *Monolayers*. Adv. Chem. Series 144., Amer. Chem. Soc., Washington, DC.
- 9 Hanrahan, F. P., A. Tamsma, K. K. Fox, and M. J. Pallansch. 1962. Production and properties of spray-dried whole milk foam. *J. Dairy Sci.* 45:27.
- 10 Jenness, r., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk Dairy J.* 16:153.
- 11 Mahon, J. H., and R. A. Chapman. 1951. Butylated hydroxyanisole in lard and shortening. *Anal. Chem.* 23:1120.
- 12 Sinnamon, H. J., N. C. Aceto., R. K. Eskew, and E. F. Schoppet. 1957. Dry whole milk. I. A new physical form. *J. Dairy Sci.* 40:1036.
- 13 Tamsma, A., M. J. Pallansch, T. J. Mucha, and W. I. Patterson. 1961. Factors related to the flavor stability of foam-dried whole milk. I. Effect of oxygen level. *J. Dairy Sci.* 44:1644.
- 14 Tamsma, A., T. J. Mucha, and M. J. Pallansch. 1962. Factors related to the flavor stability during storage of foam-dried milk. II. Effect of heating milk prior to drying. *J. Dairy Sci.* 45:1435.
- 15 Tamsma, A., T. J. Mucha, and M. J. Pallansch. 1963. Factors related to the flavor stability during storage of foam-dried whole milk. III. Effect of antioxidants. *J. Dairy Sci.* 46:144.
- 16 Webb, B. H., A. H. Johnson, and J. A. Alford. 1974. Page 9 in *Fundamentals of dairy chemistry*. 2nd ed. Avi Publishing Company, Westport, CT.