SORBITAN FATTY ACID ESTER EMULSIFIERS IN MILK FAT AND TRISTEARIN-WATER EMULSIONS

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To my daughters

AMANDA and KRISTINE:

may they learn that the importance of knowledge is understanding.

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CHAPTER I

INTRODUCTION

It is difficult to overstate the importance of emulsifiers in the formulation of cosmetics, soaps, paints, and petroleum products. Furthermore, the economic benefit of emulsifiers has been demonstrated by the increased number of new products which have appeared in aerosol cans, including such products as hair sprays, insecticides, shaving creams, cheese spreads, and whipped toppings. In fact, natural or artificial emulsifiers are used in nearly all processed foods. These compounds are largely responsible for the smoother textured ice creams and breads which have appeared in recent years, and the development of many "instant" powdered products may be attributed to improved emulsifier technology. The more successful of such products include instant breakfasts, powdered cheese dips, instant powdered milk, coffee creamers, dessert toppings, meat sauces, and instantized potatoes.

The selection of emulsifiers for particular products, however, has been primarily a matter of "trial and error." Unfortunately, this time-consuming approach has been necessary because of the limited knowledge concerning emulsifier mechanisms. For example, it has been reported that two or more emulsifiers together work better than one at the same usage level, but the mechanism of this positive synergistic effect is not understood. Such knowledge could lead to better utilization of present emulsifiers and contribute to the development of new ones.

The Food and Drug Administration has established maximum usage levels for edible emulsifiers in many food products, but little information is available describing the separation of an emulsifier from an oil-emulsifier mixture. Therefore, development of a procedure to measure the quantity of emulsifier in a food shortening would enhance more rigid enforcement of government regulations and allow better product control by the food manufacturer.

In summary, emulsifiers are important to many industries, but their use in the food field has been complicated by limited knowledge. Additional knowledge about the measurement of emulsifier effectiveness, conditions of synergism, and the isolation of these compounds from a fat-emulsifier mixture could contribute to a better understanding of emulsifiers and increase their usefulness.

Thus, the objectives of this research were: (a) to develop a procedure for measuring the stability of emulsified oil-water emulsions, (b) to measure the magnitude of synergism when a single emulsifier is replaced by a binary system, (c) to develop a procedure for the separation and measurement of an emulsifier from an oil-emulsifier mixture, and (d) to compare emulsifier concentrations at an oil-water interface.

CHAPTER II

LITERATURE REVIEW

Emulsion Terminology

The definition of an emulsion varies among authors (2, 24), but most definitions are based on the concept of "stability" between two or more dispersible phases. In this study, Sutheim's (24) term "emulsion stability" will be used to designate the ability of an oil phase and a water phase to remain dispersed within each other. An emulsifier will include any compound which promotes emulsion stability and has the property of being soluble in both phases of an oil-water mixture (2, 24). The region existing between any two phases (oil, water, or air) will be designated as an interface (2).

A large number of substances possess emulsifying properties, but only a few compounds are approved for food use by the Food and Drug Administration (5, 16). Buddemeyer et al. (5) reported that the most widely used compounds today are monoglycerides, lactated monoglycerides, sorbitan monosterate, polyoxyethylene sorbitan monostearate, and stearyl-2-lactylic acid. The fatty acid ester of these compounds can be varied to change the solubility characteristics of the parent emulsifier type (1). In this way, a sorbitan parent type may be made more lipophilic by increasing the proportion of saturated long chain fatty acid. Conversely, decreasing the carbon length of the side chain would increase the molecule's water solubility. Another variable is the degree of satura-

tion in the side chain ester (1), where saturated side chains have been recommended for saturated oil phases of an emulsion and unsaturated side chains for systems containing unsaturated oils (1).

According to Griffin (12), the solubility of an emulsifier molecule in water and oil simultaneously may be expressed as its HLB (Hydrophile-Lipophile Balance). This measurement ranges from 1-20, depending on the ratio of the solubility of the molecule's water soluble portion to its fat soluble portion (1). Emulsifiers having essentially equal hydrophilic and lipophilic portions would possess HLB numbers between 9.0 and 11.0. On this same 20-point scale, an emulsifier's HLB rating would increase from 11.0 toward 20.0 as its hydrophilic portion increased. In a similar manner, the HLB value for an emulsifier would decrease from 9.0 toward zero as its lipophilic portion increased. According to the manufacturer (1), an HLB range of 4.0 to 9.0 is recommended for promoting water in oil (w/o) emulsions that exist in many food systems. Similarly, an HLB range of 11.0 to 18.0 has been recommended for foods having an oil in water (o/w) emulsion system (1).

Until recently, the selection of the best HLB number for a particular fat-water food system had been primarily by trial and error. In 1968, Titus et al. reported a simplified procedure for selecting the emulsifier HLB to obtain maximum emulsifier efficiency in a food emulsion system. This research was based on the stability of model oil-water emulsions containing emulsifiers with HLB values between 5.0 and 13.0. The results demonstrated the importance of matching HLB value and emulsifier amount with the particular fat-water ratio of the food in question. The practicality of the work was shown by the observation that white cakes made with milk-fat shortenings had the largest volumes when

their optimum emulsifier HLB and usage level were determined using this emulsion stability data.

The combination of a low and a high HLB emulsifier will produce a mixture with an intermediate HLB value representing the proportion at which the individual compounds were used. Similarly, the use of two or more emulsifiers is suggested by most workers in order to obtain a superior emulsifier system (1, 16). Buddemeyer et al. (5) reported that emulsifier combinations yielded cake volumes greater than volumes predicted by adding the single effects of each emulsifier separately. It is important, however, to account for the influence of several other factors when reporting synergistic results obtained by mixing different emulsifier types. For example, mixtures of sorbitan esters with mono- or diglycerides or lactated glycerides have been compared to one another with little or no attention being given to the resulting HLB or the total proportion of fat present (5). Other considerations, such as how the number, length, and degree of saturation of fatty acid side chains influence the emulsifier's performance in a particular product, have been reported by Knightly and Klis (14).

In addition to surface-active agents, other factors like temperature, micelle size, and viscosity directly influence the stability of an emulsion (2, 24). These observations support Becher's (3) report that emulsion stability is the result of three factors: (a) reduction of interfacial tension between the phases; (b) formation of rigid interfacial films; and (c) formation of an electrical double layer. Yet, some researchers do not accept this as an adequate explanation for reported increases in emulsion stability when mixtures of two or more emulsifier agents are used at the same concentration as a single com-

pound (positive synergistic effect) in food products. For example, in 1961, Davies and Rideal (8) proposed that water molecules on the aqueous side of a lipid-aqueous interface could form a rather rigid layer approximately 10 Å thick. They described the layer as "soft ice" having the consistency of toffee or butter. In 1964, Davies (7) suggested that this layer was composed of water molecules oriented about polar lipid heads situated at the interfacial region. Therefore, the stability of an o/w emulsion could be promoted by strongly hydrated compounds like emulsifiers. This was due to adsorption of the non-polar portion of the emulsifier molecule into the lipid phase, which increased the fraction of interface covered by the polar heads. The resulting layer of hydration formed about the polar heads would inhibit coalescing by preventing interfacial contact among the fat globules (micelles). But Black (4) attributed the emulsifier synergism (such as that observed in cake volumes) to be the formation of smaller fat micelle size rather than the formation of a more stable interfacial film. Another hypothesis was advanced by Schulman and Cockbain (23), who suggested that stereochemical arrangements permit tighter molecular packing at the interface, thus increasing the number of polar groups available for hydration ("condensing effect" hypothesis). This explanation may be supported by the observations of many researchers on liquid-air interfaces formed in a Langmuir Trough (10, 11, 12). In this manner, Dervichian (9) observed a reduction in the total area (condensing effect) covered by a one-molecule-thick lecithin film when cholesterol was added. This work suggested that inter-molecular association caused a more compact molecular structure in a lecithin-cholesterol film than had existed for lecithin alone.

Emulsifier Quantitation

In 1969, Sahasrabudhe and Chadha (19) reported the identification and quantitative estimation of individual mono- and di- fatty acid esters (palmitic, stearic, and oleic acids) of sorbitol, 1,4-sorbitan and isosorbide. This work is part of an uncompleted scheme for the identification and quantitation of mono- and diglycerides (21), propylene glycol esters (20), and polyglycerol esters (18, 22) used in many food shortening systems. The scheme utilized liquid partition column chromatography (LPCC) run on hydrated silicic acid packing (22) for the fractionation of a lipid-emulsifier mixture. Fractions containing the sorbitan emulsifiers were identified by gas liquid chromatography (GLC) of methyl ester derivatives, according to the procedure of Lemieux and McInnes (15). A similar procedure has been reported by Wetterau et al. (26), who used ethanol extraction followed by silicic acid fractionation and identification by paper chromatography or GLC for the determination of sorbitan monostearate in cakes. As pointed out by Wetterau et al. (26), this procedure is time-consuming and requires the development of appropriate skills. On the other hand, Carroll (6) demonstrated the advantages of LPCC conducted with Florisil (magnesium oxide 15.5 ± 0.5% and sodium sulfate 0.5%) packing. In all instances the stationary phase for silicic acid or Florisil packing has been distilled water (6, 19, 26). The mobile phase varies among experimenters, but the general pattern is sequential elution by solvents of increasing polarity. Sahasrabudhe and Chadha (19) used benzene initially, while Wetterau et al. (26) used heptane and Carroll (6) used hexane. For each researcher the final solvent was ethanol or methanol.

To date, no researcher has reported a satisfactory procedure to

separate and quantitate a commercial sorbitan monostearate stock from a polyethylene sorbitan monostearate stock in a shortening system without making derivatives of the fractions for GLC identification. Development of a procedure that would quantitate and identify un-degraded emulsifier fractions would be desirable for studying possible interfacial concentration changes of the emulsifier molecule.

CHAPTER III

EXPERIMENTAL PROCEDURE

Measurement of Stability Index

In order to measure and compare the variables associated with emulsion stability, an original procedure was developed in 1966 by the author and reported in 1968 (25). Predetermined quantities of an emulsifier system and fat were combined in a 100 ml breaker and warmed to 46-48 C on a steam table. The emulsifier-fat mixture was poured into an assembled hand homogenizer previously heated to approximately 46 C, and the appropriate amount of distilled water, preheated to 46-48 C, was pipetted into the hot homogenizer. The water-fat-emulsifier mixture was then pumped through the homogenizer and collected in the 100 ml beaker which originally contained the fat. This step was repeated three times and each time the emulsion was poured back into the homogenizer, a rubber policeman was used to remove any residual material from the beaker. After the third homogenization, a 5 ml sample was transferred into a tared, screw-cap culture tube to be analyzed later for fat content. Then a 10 ml sample of the same emulsion was pipetted into a conventional test tube, which was stoppered and held in a 37 C water bath for six hours without agitation. Identical homogenization and sampling were repeated for each sample in the series.

When the 10 ml sample had been in the water bath for six hours, a 10 ml pipette, with the top sealed by the forefinger to prevent any of

the emulsion from entering, was carefully lowered to the bottom of the test tube. Then the bottom 5 ml of the sample was slowly drawn into the pipette and transferred to a tared, screw-cap culture test tube. The initial sample and the sample taken after six hours were stored at -17 C until the amount of fat could be analyzed using a modification of the Mojonnier fat extraction procedure. The screw-cap test tubes containing the emulsion samples were thawed in a 37 C water bath and weighed at room temperature without the caps.

Twenty milliliters of a fat extraction solution containing 4.35 parts petroleum ether, 4.35 parts diethyl ether, and 1.3 parts 95% alcohol were added to each test tube containing an emulsion sample, along with one drop of phenol red to clearly define the phases. The phenol red colored the lower aqueous phase, while the fat was extracted into the upper colorless diethyl ether phase. The caps were tightly secured and the test tubes shaken vigorously for 30 seconds. They were then centrifuged for five minutes to promote separation of the phases, and the diethyl ether-fat phase was pipetted to the assigned Mojonnier fat pan. The extraction process, from the addition of 20 ml of extraction solution through removal of the clear layer and rinsing of the pipette, was repeated three times or until the colored aqueous phase of the tube was free of opaqueness. The solvents in the fat pan were evaporated on a hot plate and the pans placed in a vacuum oven at 135 C under 25 inches of mercury for 15 minutes. After cooling at 22-24 C in a desiccator, the weight of each pan was recorded, and the weight of fat in each sample was calculated.

The Stability Index (S.I.) was calculated by dividing the per cent fat in the six-hour sample (a) by the per cent fat in the initial sample

(b) and multiplying by 100. Algebraically, this is expressed as: S.I. = (a ÷ b) 100. Under the conditions of this procedure, the S.I. of every emulsion would range between 0-100. On this scale, a maximum index of 100 would indicate that no separation of the oil and water phases had occurred; conversely, an index of 0 would indicate complete oil and water separation.

Measurement of Emulsifier Performance

Emulsifier performance represents the ability of an emulsifier to promote stability in an emulsion. This was measured by the S.I. procedure given above for emulsion systems containing anhydrous milk fat[1] having a titratable acidity of less than 0.1 meq./ml (calculated as lactic) combined with the following emulsifier[2] systems:

a) Span 60 (sorbitan monostearate - HLB 4.7[3]).

b) Tween 60 (polyoxyethylene sorbitan monostearate - HLB 14.9[3]).

1 Tween 61 (polyoxyethylene sorbitan monostearate - HLB 9.6[3]).

2 Tween 81 (polyoxyethylene sorbitan monooleate - HLB 10.0[3]).

3 Span 60 plus Tween 60 to give a calculated HLB value of 9.5.

4 Span 60 plus Tween 60 to give a calculated HLB value of 9.75.

5 Span 60 plus Tween 60 to give a calculated HLB value of 10.0.

6 Tween 61 plus Tween 80 to give a calculated HLB value of 9.75.

HLB values for emulsifier systems 3, 4, 5, and 6 (above) were calculated according to the manufacturer's recommended procedure (1) for

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[1] Odel Concession Specialties Company, Caldwell, Idaho.

[2] Donated by Atlas Chemical Industries, Inc., Wilmington, Delaware.

[3] Manufacturer's published value.

blending emulsifier stocks, where:

$$\% \text{ emulsifier A} = \frac{100\ (X - HLB_B)}{HLB_A - HLB_B};\ X = \text{desired HLB value.}$$

$$\% \text{ emulsifier B} = 100 - \% \text{ emulsifier A}$$

Calculated proportions of each emulsifier, sufficient for the total study, were combined in a glass test tube, stoppered, and heated in flowing steam until they liquified. After repeated inverting to assure complete mixing, the samples were quickly solidified at -17 C and held at that temperature until needed. Each of the six emulsifier systems (1 to 6 of previous listing) was added to the milk fat at levels of 0.5, 1.0, 1.5, and 2.0%.

The emulsions were prepared with a hand homogenizer by adding deionized water to each fat-emulsifier mixture to give a total sample weight of 50 gm containing 25% fat. A factional arrangement of treatments was laid out in a split-plot design by grouping the emulsifier systems (1 to 6) into each 0.5-2.0% emulsifier usage level. As shown in Table I, each of the emulsifier systems was duplicated to give a total of 48 samples per trial (main plot). The sequence in which each emulsion system and its duplicate were assembled and sampled was randomly determined for each usage level, as was the order in which each of the four usage levels was run. The total trial was repeated after re-randomization and the S.I. for each sample was determined by the procedure described previously. An analysis of variance was used to detect significant effects due to trial, emulsifier usage level, emulsifier system, and HLB values.

A second experiment compared the S.I. of emulsions containing a single emulsifier to emulsions containing a binary mixture of emulsifiers. In this single trial all the emulsifiers were a monostearic acid

ester of sorbitan having an HLB value of 9.6. Twenty-five per cent milk fat emulsions were prepared with emulsifier usage levels of 0.5, 1.0, 1.5, and 2.0% of the fat. These data were analyzed as a split-plot, in which the main plots (emulsifier usage level) were run as a completely randomized design. Each emulsion preparation was randomly run in triplicate at each emulsifier usage level, and an analysis of variance was used to detect possible significant effects.

A third experiment was designed to study the effect of binary emulsifier systems composed of a saturated fatty acid ester (monostearate) of sorbitan, Tween 60 plus Span 60; and the unsaturated fatty acid ester (monooleate) of sorbitan, Tween 80 plus Span 80. An HLB of 9.6 for each emulsifier system was added at usage levels of 0.5, 1.0, 1.5, and 2.0% of the fat, and each emulsion was made up to 25% milk fat. The data of this experiment were statistically analyzed in the same manner as experiment two.

A final experiment was run replacing milk fat with tristearin[4] for emulsions containing only Tween 61 and a combination of Span 60 plus Tween 60 at 1.0% of the fat. Twenty-five per cent fat emulsions were assembled and run in duplicate for each emulsifier system, and the means were compared by analyzing the data as a completely randomized design.

Florisil Chromatography

Quantitative separation of sorbitan monostearate (Span 60) and polyoxyethylene sorbitan monostearate (Tween 60) stocks from each other and from an oil has been accomplished by the following procedure using liquid

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[4]Baker Grade, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

partition column chromatography (LPCC). A chromatography column was constructed from 1 cm inside diameter glass tubing cut to a length of 30 cm. The bottom 4 cm was drawn out to form a tapering tip with a 0.25 cm inside diameter. The tapered end was loosely stuffed with glass wool[5] and topped with a 1 cm diameter disc cut from Whatman No. 42 filter paper. One hundred grams of 60-100 mesh Florisil[6] was placed in a 50 ml Erlenmeyer flask and hydrated with 7 ml of distilled water (6). The flask was stoppered and held overnight to reach equilibrium. Twelve grams of the hydrated Florisil was poured into the prepared glass column to give a bed length of 18-19 cm. Sequential washing of the column with 100 ml benzene[7], 100 ml methanol[7]:diethyl ether[7] (1:7 - v/v), and 100 ml acetone[7]:dioxane[7]:methanol[7] (1:1:1 - v/v/v) at approximately one drop per second removed unwanted fines and possible contamination.

The sample was prepared for chromatography by adding an equal part of chloroform[7] and gently heating until dissolved. Then approximately 0.2 ml of the chloroform-sample mixture was pipetted onto the Florisil bed surface and washed into the stationary packing with benzene. The sample material was sequentially eluted with 40 ml of each reagent above and collected in 5 ml portions numbered 1 to 120. Detection of the eluted material was determined gravimetrically after the 5 ml portions had been transferred, with a minimum of rinse solvent, into tared fat pans. This was done by taking the pans to dryness on a hot plate and holding at 135 C under vacuum for 15 minutes. The weights of the

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[5]Fisher Scientific Company, Houston, Texas.

[6]Floridin Company, Hancock, West Virginia.

[7]A.C.S. Reagent Grade.

collected material were calculated after cooling to 22-24 C in a desiccator. The recovered fractions were tentatively identified by reference compounds run on the column singly. Total recovery yields were gravimetrically calculated from sample volumes, identical to those applied on the column, after taking to dryness under vacuum. Control samples were prepared from tristearin or milk fat plus Span 60 and Tween 60, all in equal parts.

Liquid Scintillation Counting

Liquid Scintillation Counting (LSC) was performed on a Packard Tricarb Liquid Scintillation Spectrometer, Model 3320, to determine the radioactivity of tritiated (^{3}H) labeled emulsifiers. Using a tritium standard, one channel was adjusted to 64.3% gain with 25-1000 window openings. At these settings a maximum counting efficiency of 55.2-55.5% was obtained. The LSC fluid ("cocktail") was prepared from 0.1 gm 1,4-bis-2-(5-Phenyloxazolyl)-benzene[8](POPOP) plus 4.0 gm 2,5-Diphenyloxozole[8] (PPO) made with sulfur-free toluene to a 1-liter volume. Just before counting, 15.0 ml of the "cocktail" was added to each LSC sample vial. All samples were counted for one minute and the results reported as counts per minute (cpm) from which the specific activity (cpm/mg) was calculated and recorded.

Radioactive emulsifiers for this study were tritiated by custom labeling[9] unpurified samples of Span 80 (sorbitan monooleate) and Tween 80 (polyoxythylene sorbitan monooleate) with ^{3}H, presumably without the

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[8]Packard Instrument Company, Inc., Downers Grove, Illinois.

[9]Amersham/Searle Corporation, 2000 Nuclear Drive, Des Plaines, Illinois.

formation of degradation products, at the unsaturated double bond of the oleic acid side chain. Such hydrogenation would result in formation of a saturated stearic acid side chain on the sorbitan parents, corresponding to Span 60 and Tween 60 respectively. Fifty milligrams of ^{3}H-Span 60 in ethanol and 75 mg of ^{3}H-Tween 60 in an aqueous solution having a radioactivity of approximately 65 millicures each were received from the supplier in separate vacuum-sealed vials containing approximately 4.5 ml of solution each. The radioactivity of each ^{3}H-emulsifier was checked by counting approximately 0.1 ml portions according to the LSC procedure described earlier and reported as cpm/ml.

The ^{3}H-Span material was purified by chromatographing a 0.2 ml aliquot on a Florisil column according to the LPCC procedure previously described. Forty 5 ml portions were collected for each of the three solvent systems, and tube numbers 11 and 12, representing the first 10 ml of the methanol:diethyl ether solvent system, were combined. In a like manner, 0.2 ml of the unpurified ^{3}H-Tween material was chromatographed on a Florisil column, but in this instance, tube numbers 18, 19, and 20 were combined, corresponding to the first 15 ml of the acetone:dioxane: methanol solvent system. Both of these fractions were set aside for later preparation of ^{3}H-emulsifier stock mixtures to be used in this study.

Location of ^{3}H-Span and ^{3}H-Tween peaks by LPCC was done with purified ^{3}H-emulsifier stocks. The ^{3}H-Span stock was prepared by taking 4.8 ml of the purified fractions (number 11 and 12 above) to dryness at 70-80 C under vacuum in a LSC vial. To this was added 5.0090 gm of unlabeled Span 60 emulsifier; this mixture was then heated to 80 C and shaken to assure uniformity and immediately placed at -17 C until needed.

The ^{3}H-Tween stock was similarly prepared by drying 10.0 ml of the purified fractions, mixing with 1.5113 gm of unlabeled Tween 60 at 80 C and immediately placed at -17 C until needed. The specific activity of each emulsifier stock was determined by weighing out a known quantity (1-2 mg) into a LSC vial and counting by the procedure previously described. The specific activity was reported as cpm/mg for each emulsifier stock.

Isotope location of the ^{3}H-Span emulsifier peak was determined by column chromatography on Florisil packing, according to the procedure given earlier, using 0.2 ml of 0.0043 gm ^{3}H-Span stock dissolved in 0.6 ml of chloroform. Five milliliter portions were collected in LSC vials and taken to dryness under vacuum at 70-80 C. Each vial was counted by LSC and its total cpm recorded. The radioactive elution pattern was established by plotting cpm vs. 5 ml of solvent collected. An identical 0.2 ml of the ^{3}H-Span-chloroform solution corresponding to that applied on the column was dried and counted along with the 5 ml fraction vials and used for a total cpm standard.

In a similar manner, the ^{3}H-Tween emulsifier peak was located, but in this instance, 0.2 ml of 6.9 mg ^{3}H-Tween stock dissolved in 1.0 ml of chloroform was applied to the column. Likewise, another 0.2 ml of this solution was dried and counted along with the 5 ml vial fractions and used for a total cpm standard. The results were recorded in cpm and the elution pattern was established by plotting cpm vs. 5 ml of solvent collected. Then the radioactivity distribution pattern for each of the ^{3}H-Span and ^{3}H-Tween stocks for each 5.0 ml tube was compared to the previous fat-unlabeled emulsifier elution patterns determined gravimetrically. In this way, the gravimetric LPCC procedure for the identification of a single emulsifier from another or from a fat was checked.

The emulsifier concentration at a lipid-aqueous interface was determined for three different mixtures of a triglyceride plus ^{3}H-emulsifier stocks prepared as: (a) tristearin containing 1.0% ^{3}H-Span stock, (b) tristearin containing 1.0% ^{3}H-Tween stock, and (c) tristearin containing 0.5% ^{3}H-Span stock plus 0.5% ^{3}H-Tween stock. Each mixture was assembled by adding 4.0 gm of tristearin to its respective amount of ^{3}H-emulsifier stock in a LSC vial. Each lipid-^{3}H-emulsifier was used to form three separate interface preparations on the surface of distilled water. This was done by heating the lipid-^{3}H-emulsifier mixture and 25 ml of distilled water (in a 50 ml stoppered flask), together with a 10 ml pipette and a clean 100 ml beaker, to 80 C in an oven[10]. After preheating one hour at 80 C, 8 ml of the distilled water was pipetted into the 100 ml beaker. Immediately afterwards, the lipid-^{3}H-emulsifier mixture was gently poured down the beaker wall onto the water surface without mixing the two phases. After standing another 60 minutes at 80 C, the system was placed at -17 C for approximately 10 minutes until the fat phase had solidified. The intact solidified fat phase was removed with a spatula and placed upside down on a frozen metal block. The exposed surface that had been between the fat and water phases was carefully scraped with a microspatula. The thinnest possible layer of interface material (approximately 4.2 mg) was collected in a tared LSC vial. This sample was taken to dryness under vacuum at 70-80 C and the weight recorded. Then 0.5 ml of chloroform was added to dissolve the sample. A 0.2 ml aliquot of this solution was applied to a Florisil column and eluted according to the LPCC procedure given earlier. Twenty milliliter

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[10]Blue M Electric Corporation, Blue Island, Illinois.

fractions were collected in LSC vials and taken to dryness for a radioactivity assay. This procedure was repeated for each of the interfaces formed from the three different lipid-^{3}H-emulsifier mixtures. Results are reported as cpm/mg of dried interfacial material. From these data, it was possible to compare the molar concentration of a single emulsifier (Span 60 or Tween 60) and the molar concentration of a binary emulsifier mixture (Span 60 and Tween 60 combined) at a tristearin-water interface.

CHAPTER IV

RESULTS AND DISCUSSION

Stability Index Measurements of Emulsifier Performance

Stability Index measurements for 25% milk fat-water emulsions containing emulsifier systems with HLB values of 9.5, 9.75, and 10.0 added at usage levels of 0.5, 1.0, 1.5, and 2.0% of the fat are shown in Table I. The S.I. values ranged from 33 to 78 on a maximum scale of 100. Analysis of variance (Table II) for the 96 total observations showed that the variation among duplicates within trial 2 was much higher than in trial 1. The probable cause of this large variation was that observations were conducted by two different workers, a laboratory technician, and the author. A comparison of the Error Mean Square (EMS) for duplicates within usage level indicated differences in their techniques. The EMS for trial 1 was 39.40, which was lower than the 62.75 for trial 2, indicating that the variation in personnel techniques during trial 2 was much greater. A check of the records verified that trial 1 had been performed by one worker and trial 2 by both workers.

Regardless of these technique differences, the emulsifier usage level was a significant ($P < 0.01$) factor in the stability of these fat-water emulsions, which agrees with the work of other researchers (1, 2, 25). Testing for the effect of HLB values on emulsion stability showed no significant difference ($P > 0.05$), which indicated that there were no differences between the 9.5, 9.75, or 10.0 HLB values of the emulsifier

systems used in this study. However, values outside the HLB range of 9.5 to 10.0 previously have been shown to significantly affect emulsion stability, as reported by Titus et al. (25). The type of emulsifier system was not significant ($P > 0.05$), indicating that none of the emulsifier systems promoted more emulsion stability than any of the others.

To detect possible differences in efficiency between a single emulsifier and a binary mixture of emulsifiers, a second experiment was performed on 25% milk fat-water emulsions containing emulsifier systems with identical saturated fatty acid side chains (monostearate). Both systems were prepared so that the HLB values were identical at each emulsifier usage level. An analysis of variance (Table IV) of the S.I. measurements in Table III shows the emulsifier usage level to be significant ($P < 0.1$), which agrees with literature reports that the stability of oil-water emulsions is enhanced by increased levels of emulsifier usage (1, 2). However, no significant increase in emulsion stability was found for either emulsifier system ($P > 0.05$). This demonstrated that the replacement of a single component emulsifier system with a chemically similar binary emulsifier mixture having an identical HLB and usage level did not improve an emulsion's stability; i.e., there was no synergism. The interaction between types of emulsifier system x usage level was not significant ($P > 0.05$).

A third experiment compared the effect of binary emulsifier systems composed of different fatty acid side chains. In this instance, one system had a saturated monostearate fatty acid side chain and the other an unsaturated monooleate fatty acid side chain. The HLB value for each system was 9.6, and identical usage levels in 25% milk fat-water emulsion

were used. An analysis of variance (Table VI) of the data in Table V again shows that emulsifier usage level was significant ($P < 0.01$). No significance ($P > 0.05$) was found for the effects of different emulsifier systems, which indicates that the stability of a milk fat-water emulsion is not enhanced by an emulsifier having a saturated fatty acid side chain as compared to an unsaturated one. The emulsifier x usage level interaction also was not significant ($P > 0.05$).

The fourth experiment was conducted on 25% fat-water emulsions prepared with tristearin. Two separate emulsifier systems having an HLB of 9.6 were used at a level of 1.0% of the tristearin. One system was a single emulsifier with a monostearate fatty acid side chain and the other a binary mixture of two emulsifiers, each having a monostearate fatty acid side chain. The S.I. observations and the calculated means for each emulsifier system are presented in Table VII. The single emulsifier system produced tristearin-water emulsions having a S.I. mean of 45.45, while the binary emulsifier system had a S.I. mean of 45.85. The 0.4 difference in S.I. means was not thought to be important when compared to the range of S.I. values observed for each emulsion system; i.e., a 43.24 to 47.80 range for the single emulsifier system and 43.30 to 49.22 range for the binary emulsifier system.

Measurement of HLB

The preparation of samples and data reported in this study has been based upon the manufacturer's published HLB value for the specific emulsifiers used. However, to date there has been very little research published concerning an accurate procedure for measuring HLB values. Consequently, some researchers have questioned the reliability of a manu-

facturer's HLB rating for a particular emulsifier lot. The most promising procedure reported for determining HLB values appears to be GLC (gas-liquid chromatography) analysis. Mickle et al. (17) reported reproducible HLB determinations by GLC when using Chromosorb G (60-70 mesh) packing coated with 5% emulsifier. Satisfactory resolution of retention times corresponding to small changes in HLB were obtained when using isoamyl alcohol elution. A comparison of the manufacturer's HLB values and those obtained by the procedure of Mickle et al. (17), for the emulsifiers used in this study, is presented in Table VIII.

Separation of Fat-Emulsifier Mixtures by Florisil Chromatography

Column chromatography using silicic acid (22) or Florisil packing has been reported to be satisfactory for the separation of lipids. However, the author observed the advantages demonstrated by Carroll (6) that Florisil, as opposed to silicic acid, was very simple to pack and the relatively coarse mesh permitted rapid flow rates.

Table IX presents the gravimetric (mg) recoveries collected per 5 ml tube when individual samples of milk fat, tristearin, Span 60, and Tween 60 were eluted. Also presented are the milligrams of sample recovered in each 5 ml tube when two separate mixtures of tristearin : Span 60 : Tween 60 (1:1:1) were eluted from the column. Table IX also shows the total weight as per cent recovery for each sample. Of particular interest is the 100.8% and 103.5% total recoveries obtained from the elution of tristearin : Span 60 : Tween 60 mixtures. A plot of the milligrams recovered for each 5 ml tube number demonstrates the individual elution patterns for tristearin (Figure 1), Span 60 (Figure 2), and Tween 60 (Figure 3). The single component peaks in Figures 1, 2, and 3

were compared to Figure 4 for tentative identification of the three distinct peaks obtained during elution of a tristearin : Span 60 : Tween 60 mixture. In this manner, peaks A, B, and C in Figure 4 were tentatively identified as tristearin, Span 60, and Tween 60 respectively. The per cent recovery of tristearin, Span 60, and Tween 60 is presented in Table X. It is likely that variation in the percent recovery obtained for tristearin (100.98% and 86.71%) represents incomplete elution of the material and that the difference in recovery of Tween 60 (95.59% and 114.69%) is due to weighing errors often associated with gravimetric-type determinations. Reasonable duplication of 105.65% and 108.97% recovery for Span 60 was obtained, but such "synthesis" seems improbable. Therefore, these values likely represent inclusion of some tristearin, Tween 60, or both.

Liquid Scintillation Counting of Tritiated Emulsifiers

As received from the supplier, the tritiated Span 80 and Tween 80 had radioactivity levels of 4.320×10^9 cpm/ml and 8.973×10^9 cpm/ml respectively. After a 0.0926 ml aliquot of this ^{3}H-Span material was diluted with 5.0 gm unlabeled Span 60, the specific activity was 4.69×10^5 cpm/mg. Likewise, 0.1 ml of the ^{3}H-Tween was diluted with 5.0 gm Tween 60 to give a mixture having 4.64×10^5 cpm/mg. A 10 mg portion of each diluted mixture was placed on separate Florisil columns, and the cpm obtained for each 5 ml of solvent are presented in Table XI. These raw data demonstrated that the unpurified preparation of each emulsifier stock as received from the manufacturer contained peaks for tubes numbered 2, 3 and 4; 11, 12 and 13; 19 and 20.

Purified ^{3}H-Span 80 material from tubes numbered 11 and 12 was used

to prepare a ^{3}H-Span emulsifier stock having a specific activity of 2.33 x 10^{4} cpm/mg. This was chromatographed on Florisil and the cpm for each 5 ml of solvent recorded (Table XII). An 88.6% recovery of the total amount of radioactivity was observed. Of the recovered material, 94.17% was eluted by the methanol:diethyl ether (1:7) solvent system and 5.7% by the acetone:dioxane:methanol (1:1:1) solvent system. Similar chromatography of a purified Tween stock having 5.02 x 10^{4} cpm/mg is shown in Table XIII. A total of only 62.8% of this sample's radioactivity was recovered from the Florisil column. This incomplete elution of the ^{3}H-Tween material has been supported by recent work showing similar recoveries. In addition it was found that the per cent of emulsifier recovery could be increased by replacing the acetone:dioxane:methanol solvent with methanol. Of the ^{3}H-Tween material recovered, 0.5% was eluted by the benzene, 11.0% by the methanol:diethyl ether, and 88.42% by the acetone:dioxane:methanol solvent systems. An elution pattern of cpm per 5 ml tube for the ^{3}H-Span (Table XII) and ^{3}H-Tween (Table XIII) stocks has been graphed in Figures 5 and 6 respectively. A comparison of the major single peak eluted for each ^{3}H-emulsifier with the elution pattern obtained from the gravimetrically determined chromatography of tristearin:Span 60:Tween 60 (1:1:1) in Figure 4 provides further evidence that identities of peaks B and C are Span 60 and Tween 60 respectively.

^{3}H-Span and ^{3}H-Tween Emulsifier Concentrations at a Tristearin-Water Interface

A preparation of tristearin containing 1% of the ^{3}H-Span stock had a specific activity of 473 cpm/mg. This value, together with the mol. wt. of 430.5 for Span 60, was used to calculate an emulsifier concentra-

tion of 0.0454 x 10^{-6} M/mg in the tristearin-^{3}H-Span mixture. Material recovered from the interface formed between this same tristearin-^{3}H-Span mixture and distilled water was found to have 1,290 cpm/mg, equivalent to a Span emulsifier concentration of 0.124 x 10^{-6} M/mg of interfacial material (Table XIV). This concentration value represents a 2.73-fold increase in the Span concentration at the interface region as compared to the concentration in the original tristearin-emulsifier mixture. Such a large increase of 273% would not normally be anticipated for an emulsifier like Span 60 which possesses a lipophilic favorable HLB rating of 4.7 (indicating that 75% of the molecule is fat soluble). One possible explanation for this large increase in concentration may be the adherence of the small hydrophilic portion of the molecule to the water phase once a Span molecule in the fat phase happens to touch the interfacial region. In this way, a concentration of the Span molecules could eventually build up at the tristearin-water interface. The relatively small size of a Span molecule (molecular weight of 430.5) could allow movement within the tristearin phase due to convection currents produced by the 80 C temperature at which the interface was formed. This would promote the frequency of contact between a larger number of the Span molecules and the interfacial region.

Florisil chromatography of the tristearin-^{3}H-Span-water interfacial material showed that 0.30% of the radioactivity was eluted with benzene, 96.38% with methanol:diethyl ether, and 3.33% with the acetone:dioxane:methanol solvent systems. Of the 2,067 cpm applied to the column, a total of only 1,352 cpm were eluted. This gave a 65.14% total recovery, which again indicated incomplete elution.

A second preparation containing tristearin plus 1% ^{3}H-Tween emulsi-

fier stock was assembled having 1,056 cpm/mg, indicating a ^{3}H-Tween emulsifier concentration of 6.847×10^{-6} M/mg. This same tristearin-^{3}H-Tween mixture and distilled water was used to form an interface that had 1,534 cpm/mg, or a Tween emulsifier concentration of 9.946×10^{-6} M/mg (Table XIV). This represented a 1.45-fold increase in Tween concentration at the tristearin-water interface. Such an increase would be expected for a Tween emulsifier since it has an HLB value of 14.9 (approximately 75% of the molecule is water soluble). Unexpectedly, on the basis of relative water soluability, the interfacial concentration increase of 2.73-fold for the lipid-preferring Span was larger than this 1.45-fold increase for the water-preferring Tween molecules. This could indicate that under similar convection current conditions, the frequency of molecular contact at the interfacial region was much greater for the smaller Span molecules (mol. wt. = 430.5) than for the larger Tween molecules (mol. wt. = 3070.5).

Florisil chromatography of the tristearin-^{3}H-Tween-water interfacial material demonstrated that 0.96% of the radioactivity was eluted with benzene, 42.91% with methanol:diethyl ether and 56.12% with the acetone:dioxane:methanol solvent systems. The relatively large 42.91% portion of counts eluted by the methanol:diethyl ether did not agree with previous elution data in which only 11.0% was recovered with this solvent system for ^{3}H-Tween emulsifier stock (Table XIII). A repeat study with a new interfacial sample confirmed that almost half the ^{3}H-Tween emulsifier was eluted by the methanol:diethyl ether solvent system tentatively associated with ^{3}H-Span emulsifier elution. This could indicate possible degradative reduction of oxyethylene side chains from the ^{3}H-Tween molecules during the study, resulting in a sorbitan monostearate

structure, corresponding to a Span 60 molecule. Possible reduction of the remaining side chain from the sorbitan parent would release a free stearic acid, which should be eluted by the non-polar benzene solvent during Florisil chromatography (6). The higher 0.96% recovery obtained with the ^{3}H-Tween interface sample supports the possibility of such degradation when compared to the lower 0.57% of the total radioactivity obtained by the benzene elution of the ^{3}H-Tween emulsifier stock (Table XIII).

A third system was prepared using tristearin plus 1% of a 40 mg emulsifier mixture containing equal weights of ^{3}H-Span and ^{3}H-Tween stocks. This emulsifier mixture contained 4.49×10^{-5} M of the Span emulsifier and 4.514×10^{-3} M of the Tween emulsifier, a 1:145 Molar ratio of Span:Tween. The radioactivity of this emulsifier totaled 147.168×10^{4} cpm/mg, representing 46.708×10^{4} cpm from the Span and 100.460×10^{4} cpm from the Tween, or a Span:Tween cpm ratio of 1:2.151. Based on these ratios, a total emulsifier concentration of 4.6569×10^{-6} M/mg (0.0319×10^{-6} M of Span + 4.625×10^{-6} M of Tween) was represented by an observed specific activity of 1,045 cpm/mg for this tristearin-mixed ^{3}H-emulsifier preparation (Table XIV).

Material recovered from the interface between the same tristearin-mixed ^{3}H-emulsifier preparation and distilled water had 231 cpm/mg. When 1,062 cpm of this material was chromatographed on Florisil, a total of 1,300 cpm was eluted, giving a recovery of 122.4%. This large recovery indicated a possible difference between the amount of counting standard and the amount of material applied to the Florisil column. But of the 1,300 cpm recovered, 0.31% was eluted by benzene, 33.46% by methanol:diethyl ether and 66.21% by the acetone:dioxane:methanol solvent

system. Based upon the three different solvent recovery percentages obtained from the elution of each singly ^{3}H-emulsified interface, this mixed emulsifier interfacial sample was found to have a Span:Tween Molar ratio of 186:1. When this ratio was compared to the Span:Tween Molar ratio of 1:145 existing in the original tristearin-^{3}H-mixed emulsifier system, the larger water-attracting property of Span was clearly demonstrated. The total emulsifier concentration of Span and Tween at the tristearin-distilled water interface was 6.542×10^{-6} M/mg. Data in Table XIV show that an interface formed with only Span-emulsified tristearin contained 0.124×10^{-6} M/mg of Tween. But in an interface formed from tristearin containing equal weights of Span and Tween, an intermediate emulsifier concentration of 6.542×10^{-6} M/mg was observed. This observation could explain the earlier results demonstrating that no distinguishable improvement in emulsion stability was observed when a single emulsifier system was replaced by a binary mixture having the same HLB value and usage level because the total number of emulsifier molecules at the fat-water interface had not been increased. In addition, this binary emulsifier concentration data for a tristearin-water interface definitely demonstrated an absence of stereochemical molecular packing to give a total emulsifier concentration greater than could possibly have existed for either emulsifier, which has been a widely proposed explanation for emulsifier synergism ("condensing effect" hypothesis). It could also suggest that reported positive emulsifier synergism resulting from mixing emulsifiers may be in reality a reflection of many other factors contributing to an emulsion's stability. For example, workers reporting increased emulsion stability by blending emulsifiers may have failed to consider the resulting HLB value of the

final mixture or proper matching of HLB values to the fat-water ratio of the system in which it is used, or perhaps stability comparisons were made between an emulsifier mixture and its constituents at non-identical usage levels--all of which are highly important factors as mentioned or demonstrated in this presentation.

TABLE I

STABILITY INDICES FOR 25% MILK FAT-WATER EMULSIONS CONTAINING VARIOUS EMULSIFIER SYSTEMS

HLB	Emulsifier System	Trial 1 Emulsifier Usage Level (% of Fat) 0.5	1.0	1.5	2.0	Trial 2 Emulsifier Usage Level (% of Fat) 0.5	1.0	1.5	2.0
9.5	Tween 61[a]	37	54	59	64	44	53	62	52
		39	55	65	66	66	60	54	76
	Span 60[b] plus Tween 60[c]	44	64	63	61	51	61	57	64
		40	55	66	66	61	57	63	70
9.75	Tween 61 plus Tween 81	33	49	63	69	42	56	59	62
		42	59	61	69	59	53	59	66
	Span 60 plus Tween 60	40	54	41	67	62	40	57	63
		63	41	61	60	36	47	54	57
10.0	Tween 81[d]	39	32	59	69	45	59	57	69
		33	41	54	57	47	49	55	78
	Span 60 plus Tween 60	43	58	59	65	44	57	59	64
		49	62	56	71	63	57	63	63

[a] Tween 61 (polyoxyethylene sorbitan monostearate).

[b] Span 60 (sorbitan monostearate).

[c] Tween 60 (polyoxyethylene sorbitan monostearate).

[d] Tween 81 (polyoxyethylene sorbitan monooleate).

TABLE II

ANALYSIS OF VARIANCE OF COMBINED TRIALS REPRESENTING THE STABILITY INDICES FOR 25% MILK FAT-WATER EMULSIONS CONTAINING VARIOUS EMULSIFIER SYSTEMS

Source	df	SS	MS	F
Total	95	9,726.49		
Trial (A)	1	189.84	189.84[a]	3.72[d]
Usage level (B)	3	4,513.87	1,504.62[b]	10.77[c]
AxB Interaction	3	419.03	139.68[a]	2.73[d]
Emulsifier system (C)	5	582.05	116.41[b]	2.17[d]
(HLB within C)	(2)	(186.33)	93.17[b]	1.74[d]
AxC Interaction	5	267.98	53.60[a]	1.05[d]
BxC Interaction	15	767.82	51.19[b]	1.43[d]
AxBxC Interaction	15	534.88	35.63[a]	0.69[d]
Error (duplicate within usage level)	48	2,451.00	51.02	
Trial 1	24	945.00	39.40	
Trial 2	24	1,506.00	62.75	

[a] Tested by 'Error for duplicate within usage level'.

[b] Tested by following interaction term (a).

[c] $P < 0.01$.

[d] Not significant; $P > 0.05$.

TABLE III

STABILITY INDICES FOR 25% MILK FAT-WATER EMULSIONS CONTAINING SINGLE OR BINARY EMULSIFIER SYSTEMS HAVING A MONOSTEARATE SIDE CHAIN

Emulsifier System[a] (HLB = 9.6)	Emulsifier Usage Level (% of Fat)							
	0.5		1.0		1.5		2.0	
—	62.1	60.9	66.8	67.7	51.8	70.7	73.2	61.5
Tween 61	59.6	58.0	59.6	64.4	50.1	64.4	72.0	74.4
	58.2	65.3	60.3	61.8	79.3	59.8	75.0	76.9
Span 60	62.5	60.9	59.5	68.0	59.6	60.1	79.7	65.5
plus	58.1	64.2	65.7	65.7	62.0	66.7	75.8	64.8
Tween 60	55.0	62.2	62.4	71.3	60.0	69.8	65.2	67.3

[a]See Table I for description.

TABLE IV

ANALYSIS OF VARIANCE OF STABILITY INDICES FOR 25% MILK FAT-WATER EMULSIONS CONTAINING SINGLE OR BINARY EMULSIFIER SYSTEM HAVING A MONOSTEARATE SIDE CHAIN

Source	df	SS	MS	F
Total	47	2,053.81		
Usage level (A)	3	712.36	237.45[a]	4.357[c]
Replicates in usage level	4	218.01	54.50	
Emulsifier system (B)	1	0.06	0.06[b]	0.0006[d]
AxB Interaction	3	30.64	10.21[b]	0.100[d]
Replicates x B in usage level	4	406.98	101.75	
Duplicates in A x B	32	685.76	21.43	

[a] Tested by 'Replicates in usage level' MS.

[b] Tested by 'Replicates x B in usage level' MS.

[c] $P < 0.1$.

[d] Not significant; $P > 0.05$.

TABLE V

STABILITY INDICES FOR 25% MILK FAT-WATER EMULSIONS PREPARED WITH EMULSIFIER SYSTEMS HAVING A MONOSTEARATE OR MONOOLEATE SIDE CHAIN

Emulsifier System[a] (HLB = 9.6)	Emulsifier Usage Level (% of Fat)							
	0.5		1.0		1.5		2.0	
Span 60	36.17	35.38	56.46	61.66	62.98	60.62	66.57	67.45
plus	40.54	39.68	56.27	54.59	59.67	53.46	68.07	68.50
Tween 60	41.00	36.02	54.85	59.31	61.70	61.80	65.41	70.08
Span 80[b]	32.64	33.71	58.54	55.56	58.91	61.22	65.63	66.25
plus	39.42	33.70	58.68	59.97	56.92	60.18	68.33	59.96
Tween 80[c]	35.89[d]	39.84	57.01	51.55	59.82	58.39	64.91	69.01

[a] See Table I for description.

[b] Span 80 (sorbitan monooleate).

[c] Tween 80 (polyoxyethylene sorbitan monooleate).

[d] Missing value estimated for statistical analysis.

TABLE VI

ANALYSIS OF VARIANCE OF STABILITY INDICES FOR 25% MILK FAT-WATER EMULSIONS PREPARED WITH EMULSIFIER SYSTEMS HAVING A MONOSTEARATE OR MONOOLEATE SIDE CHAIN

Source	df	SS	MS	F
Total	47	6,162.72		
Usage level (A)	3	5,834.44	1,944.81[a]	1,185.86[c]
Replicates in usage level	4	6.55	1.64	
Emulsifier system (B)	1	21.64	21.64[b]	2.01[d]
A x B Interation	3	7.99	2.66[b]	0.24[d]
Replicates x B in usage level	4	42.99	10.75	
Duplicates in A x B	32	249.11	7.78	

[a]Tested by 'Replicates in usage level' MS.

[b]Tested by 'Replicates x B in usage level' MS.

[c]$P < 0.01$.

[d]Not significant; $P > 0.05$.

TABLE VII

STABILITY INDICES FOR 25% TRISTEARIN-WATER EMULSIONS CONTAINING SINGLE OR BINARY EMULSIFIER SYSTEMS HAVING A MONOSTEARATE SIDE CHAIN

	Emulsifier System[a] (1.0% of the fat)	
	Tween 61 ——— HLB 9.6	Span 60 + Tween 60
	——[b]	49.22
	43.24	45.79
	45.30	43.30
	47.80	45.09
Total	136.34	183.40
Mean	45.45	45.85

[a]See Table I for description.

[b]Missing data; tristearin solidified during analysis.

TABLE VIII

MANUFACTURER'S AND GAS LIQUID CHROMATOGRAPHY DETERMINED HYDROPHILE-LIPOPHILE BALANCE VALUES FOR VARIOUS EMULSIFIER SYSTEMS

Emulsifier System	Manufacturer HLB	GLC[c] HLB[d]
Span 60	4.7[a]	4.40
Tween 60	14.9[a]	14.90
Tween 61	9.6[a]	10.40
Tween 81	10.0[a]	10.80
Span 60 plus Tween 60	9.5[b]	10.15[e]
Span 60 plus Tween 60	9.6[b]	9.73
Span 60 plus Tween 60	9.75[b]	9.58
Span 60 plus Tween 60	10.0[b]	9.30[e]
Span 80 plus Tween 80	9.6[b]	9.70
Tween 61 plus Tween 81	9.75[b]	10.68

[a]HLB according to manufacturer's label.

[b]HLB calculated using manufacturer's algebraic formula.

[c]See reference; Mickle et al. (17).

[d]Average of 2 determinations.

[e]Possibly reversed during analysis procedure; would not affect results in Table II, III, V or VII ($P > 0.05$).

TABLE IX

RECOVERIES FROM FLORISIL CHROMATOGRAPHY

Solvent System	Tube Number (5 ml.)	Sample Material: Anhydrous Milk Fat	Tristearin	Span 60	Tween 60	Tristearin: Span 60: Tween 60 (1:1:1)	
		mg (dry)					
Benzene	1	0.7	0.0	0.0	0.0	0.1	0.0
	2	17.9	9.6	0.0	0.3	1.3	3.0
	3	53.8	40.9	0.0	0.1	17.5	19.4
	4	13.1	8.0	0.9	0.9	13.3	6.6
	5	3.3	1.4	0.5	0.5	5.7	2.1
	6	1.4	0.6	0.9	0.3	1.8	1.0
	7	1.75[a]	0.3[a]	1.05[a]	0.05[a]	0.7[a]	1.3[a]
	8	1.75[b]	0.3[b]	1.05[b]	0.05[b]	0.7[b]	1.3[b]
Methanol:Diethyl Ether (1:7-v/v)	9			1.6	0.1	0.0	2.3
	10			5.1	5.7	0.0	5.3
	11			49.3	13.5	28.0	29.5
	12			16.1	2.4	9.9	10.6
	13			3.4	1.9	0.9	2.2
	14			2.0	1.1	1.8	1.8
	15			0.7[a]	0.9[a]	1.2[a]	0.75[a]
	16			0.7[b]	0.9[b]	1.2[b]	0.75[b]
Acetone:Dioxane:Methanol (1:1:1-v/v/v)	17				3.4	0.0	0.5
	18				4.0	2.3	4.7
	19				21.9	12.7	12.3
	20				17.4	7.7	7.9
	21				6.3	7.4	6.9
	22				6.4	3.2	5.9

TABLE IX (Continued)

	Tube Number (5 ml.)	Sample Material: Anhydrous Milk Fat	Tristearin	Span 60	Tween 60	Tristearin: Span 60: Tween 60 (1:1:1)	
		mg (dry)					
	23				5.3	2.8[a]	3.85[a]
	24				4.4	2.8[b]	3.85[b]
(A)	Total recovery (mg)	91.5	61.1	78.9	108.0	123.0	124.2
(B)	Applied to column (mg)	95.2	69.8	82.2	88.5	122.0	120.0
Total % recovery[c]		96.11	87.54	96.22	110.50	100.80	103.50

[ab] Collected together as a 10 ml. fraction.

[c] % Recovery = $\frac{A}{B}$ (100) .

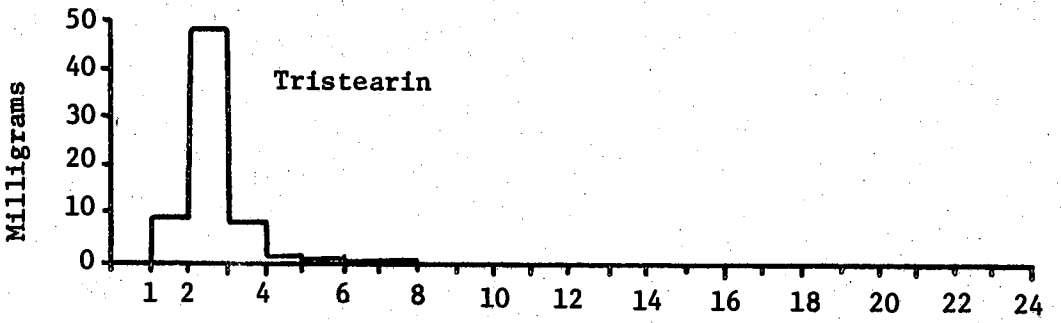

Figure 1. Florisil Chromatography of Tristearin

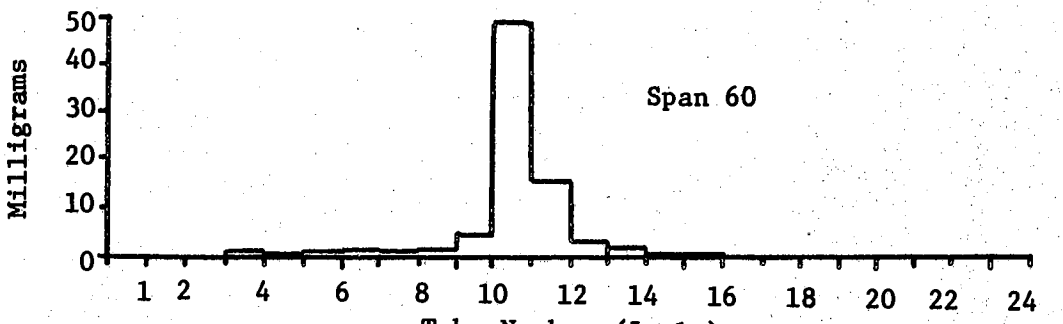

Figure 2. Florisil Chromatography of Span 60

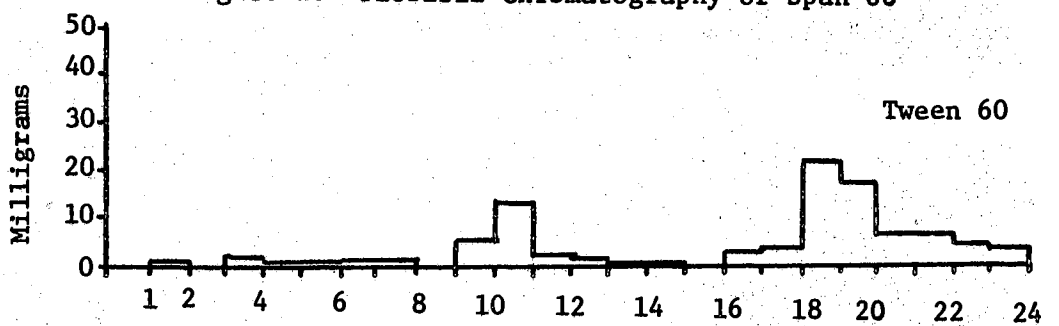

Figure 3. Florisil Chromatography of Tween 60

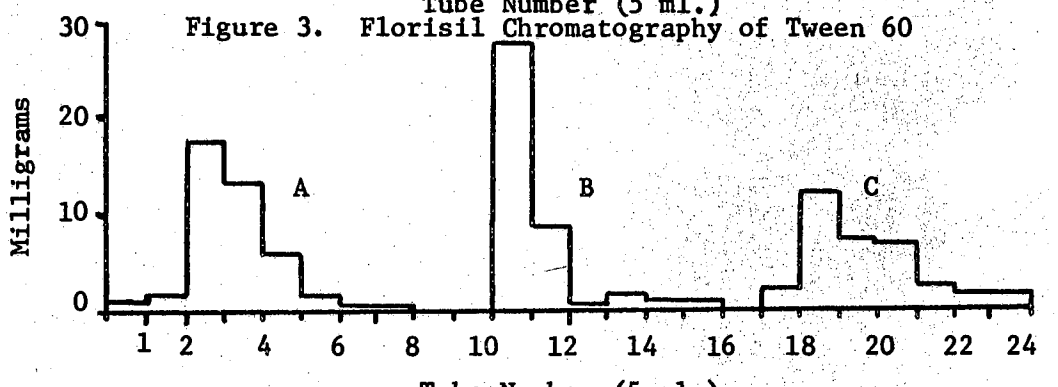

Figure 4. Florisil Chromatography of Tristearin: Span 60:Tween 60 (1:1:1)

TABLE X

PER CENT RECOVERY OF TRISTEARIN, SPAN 60, AND TWEEN 60 MIXTURES FROM FLORISIL CHROMATOGRAPHY

Peak[a]	Mixture 1 Theoretical	Mixture 1 Observed	Mixture 1 %[b]	Mixture 2 Theoretical	Mixture 2 Observed	Mixture 2 %[b]
	mg			mg		
A (Trestearin)	40.7	41.1	100.98	40.02	34.70	86.71
B (Span 60)	40.7	43.0	105.65	40.01	43.60	108.97
C (Tween 60)	40.6	38.9	95.59	39.97	45.90	114.69
Total	122.0	123.0		120.00	124.20	
% Total Recovery		100.8[c]			103.5[d]	

[a] See Figure 4.

[b] % Peak Recovery = [(Observed for peak) ÷ (Theoretical for peak)]100.

[c] % Total Recovery = (123 mg ÷ 122 mg) 100.

[d] % Total Recovery = (124 mg ÷ 120 mg) 100.

TABLE XI

LIQUID SCINTILLATION COUNTS FROM FLORISIL CHROMATOGRAPHY OF UNPURIFIED RADIOACTIVE SPAN AND TWEEN

Solvent System	Tube Number (5 ml.)	^{3}H-Span Observed CPM[a]	^{3}H-Tween Observed CPM[a]
		1×10^{-4}	
Benzene	1	0.0218	0.0051
	2	0.1464	0.4638
	3	0.4155	0.7891
	4	0.1157	0.3054
	5	0.0584	0.1466
	6	0.0500	0.1220
	7	0.0372	0.1088
	8	0.0359	0.0942
Methonol:Diethyl ether (1:7-v/v)	9	0.0424	0.0720
	10	0.0454	0.1237
	11	4.3914	5.7571
	12	5.0535	5.1580
	13	1.7474	1.1795
	14	0.5524	0.3861
	15	0.2942	0.3606
	16	0.2177	0.2386
Acetone:Dioxane: methanol (1:1:1-v/v/v)	17	0.2161	0.3256
	18	0.2232	3.2611
	19	2.0676	6.2156
	20	1.2632	3.3289
	21	0.9317	2.4175
	22	0.7490	1.7983
	23	0.6121	1.4428
	24	0.5343	1.1295

[a]CPM not corrected for machine efficiency.

TABLE XII

RADIOACTIVITY OF PURIFIED ^{3}H-SPAN STOCK FROM FLORISIL CHROMATOGRAPHY

Solvent System	Tube number (5 ml. each)	(A) Observed CPM	(B) Solvent background CPM	Total CPM[a]	Total CPM eluted per solvent	% of observed CPM
			1×10^{-3}			
Benzene	1	0.067	0.050	0.031		
	2	0.044		0.000		
	3	0.039		0.000		
	4	0.037		0.000		
	5	0.029		0.000		
	6	0.046		0.000		
	7	0.053		0.000		
	8	0.041		0.000	0.031	0.04
Methanol:Diethyl Ether (1:7-v/v/v)	9	0.181	0.051	0.236		
	10	0.196		0.261		
	11	23.174		41.671		
	12	8.628		15.457		
	13	2.576		4.550		
	14	1.140		1.963		
	15	0.623		1.031		
	16	0.426		0.676	65.845	94.17
Acetone:Dioxane: Methanol (1:1:1-v/v/v)	17	0.281	0.083	0.414		
	18	0.628		0.982		
	19	0.661		1.042		
	20	0.385		0.544		
	21	0.277		0.350		
	22	0.246		0.294		
	23	0.175		0.166		
	24	0.224		0.254	4.046	5.79
			Total	69.922		100.00
	Control	43.787		78.910		
			% Recovery	88.61		

[a]Total CPM = (A - B) ÷ C; where C = counting efficiency of 0.5549.

TABLE XIII

RADIOACTIVITY OF PURIFIED ^{3}H-TWEEN STOCK FROM FLORISIL CHROMATOGRAPHY

Solvent System	Tube number (5 ml. each)	(A) Observed CPM	(B) Solvent background CPM	Total CPM[a]	Total CPM eluted per solvent	% of Observed CMP
			1×10^{-3}			
Benzene	1	0.243	0.116	0.229		
	2	0.176		0.108		
	3	0.123		0.013		
	4	0.086		0.000		
	5	0.142		0.047		
	6	0.125		0.016		
	7	0.127		0.020		
	8	0.112		0.000	0.433	0.57
Methanol:Diethyl Ether (1:7-v/v)	9	0.100	0.135	0.000		
	10	0.113		0.000		
	11	2.426		4.129		
	12	1.337		2.167		
	13	0.555		0.757		
	14	0.408		0.492		
	15	0.380		0.360		
	16	0.348		0.384	8.289	11.00
Acetone:Dioxane: Methanol (1:1:1-v/v/v)	17	0.381	0.124	0.443		
	18	5.337		9.396		
	19	15.235		27.237		
	20	7.495		13.286		
	21	4.049		7.075		
	22	2.508		4.297		
	23	1.652		2.754		
	24	1.207		2.132	66.619	88.42
			Total	75.342		99.99
	Control	66.557		119.966		
			% Recovery	62.80		

[a]Total CPM = (A - B) ÷ C; where C = counting efficiency of 0.5548.

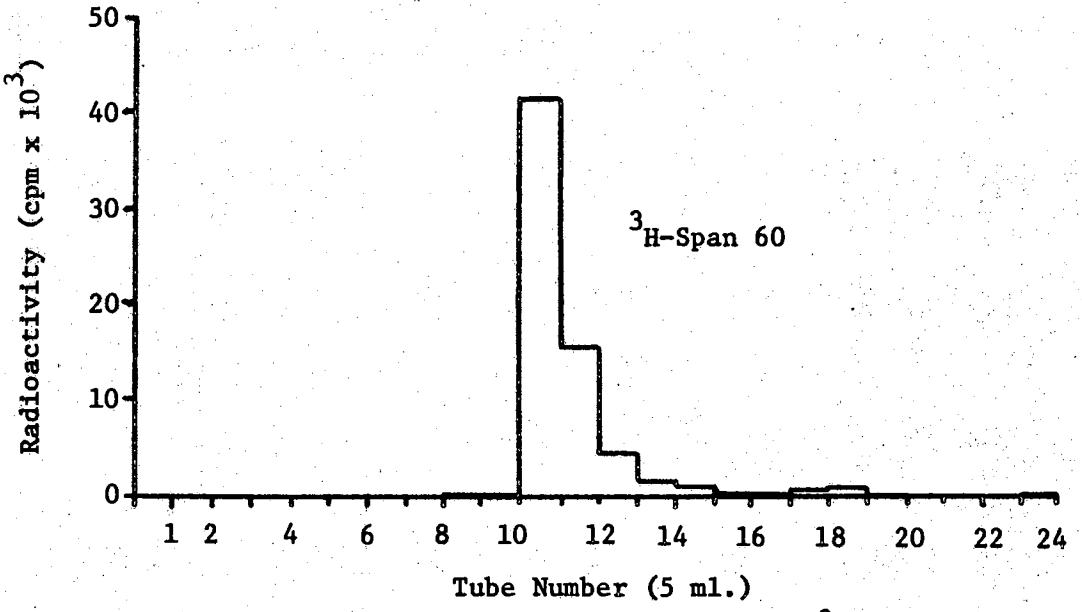

Figure 5. Florisil Chromatography of ^{3}H-Span 60

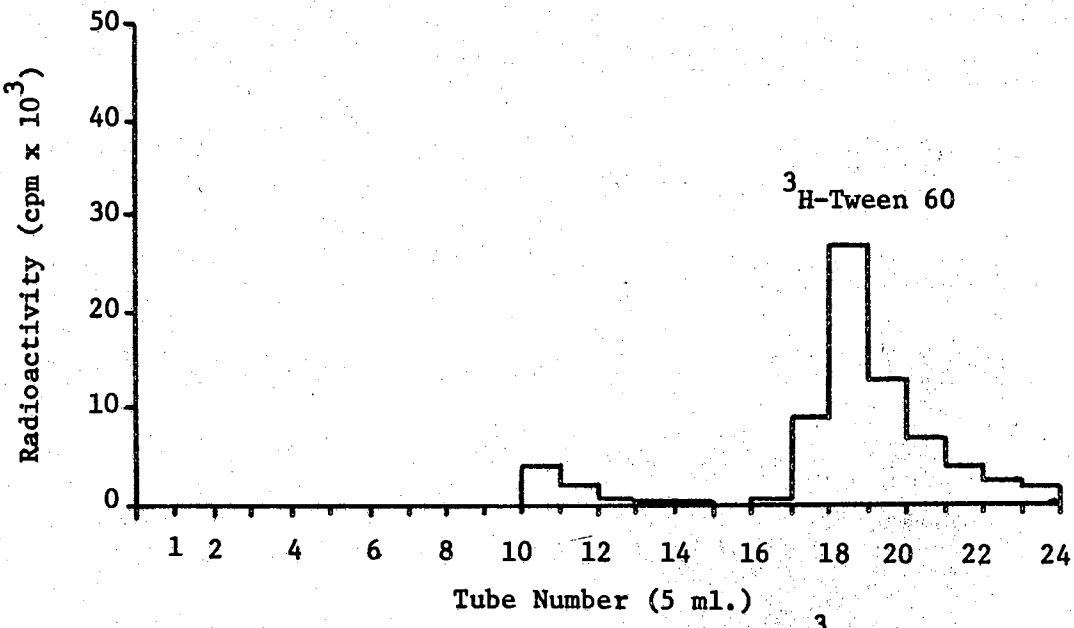

Figure 6. Florisil Chromatography of ^{3}H-Tween 60

TABLE XIV

CONCENTRATION OF SPAN AND TWEEN EMULSIFIERS AT A TRISTEARIN-WATER[a] INTERFACE

Emulsifier system[b]	Specific Activity		Emulsifier Concentration		
	Tristearin plus ^{3}H-emulsifier stock	Tristearin-water Interface	Tristearin plus ^{3}H-emulsifier stock	Tristearin-water Interface	Change at Interface
	cpm/mg		M/mg		
^{3}H-Span	473	1,290	0.0454×10^{-6}[c]	0.124×10^{-6}	+2.73
^{3}H-Tween	1,056	1,534	6.847×10^{-6}[d]	9.946×10^{-6}	+1.45
^{3}H-Span plus ^{3}H-Tween	1,045	231	4.6569×10^{6}	6.542×10^{-6}	+1.41

[a] Distilled water.

[b] 1.0% usage level.

[c] Calculation of span concentration: Specific activity of ^{3}H-span stock = 2.3354×10^{4} cpm/mg; mol. wt. span 60 = 430.5; or 1.0 mg = 0.2245×10^{-5} M.

$$\text{Mole Span/mg mixture} = \frac{(0.2245 \times 10^{-5}\ \text{M/mg})(4.73 \times 10^{2}\ \text{cpm/mg})}{2.3354 \times 10^{4}\ \text{cpm/mg}} = 0.0454 \times 10^{-6}\ \text{M/mg.}$$

[d] Calculation of Tween concentration; like above but used mol. wt. of 3070.5.

CHAPTER V

SUMMARY AND CONCLUSIONS

An "F" test indicated that emulsifier usage level had a significant effect upon the stability of a 25% milk fat-water emulsion ($P < 0.01$). The other variables: whether emulsifier HLB values of 9.5 to 10.0 were used, whether an emulsifier system was a single component or a binary mixture, and whether the emulsifier contained a saturated or unsaturated fatty acid side chain had no significant effect on the stability of a 25% milk fat-water emulsion ($P > 0.05$). Like the milk fat emulsions, the stability of a tristearin-water emulsion was not appreciably affected by replacing a single component emulsifier system with a binary mixture.

Florisil chromatography of a tristearin:Tween 60:Span 60 (1:1:1) mixture showed that the tristearin was eluted by benzene, Span 60 by methanol:diethyl ether (1:7) and Tween 60 by acetone:dioxane:methanol (1:1:1) to give total gravimetric recoveries of 100.8% and 103.5% for two separate columns. A reduction in total radioactivity recovered was observed for mixtures containing ^{3}H-Span and ^{3}H-Tween, but the elution sequence of these radioactive compounds was used to confirm the identity of the emulsifier peaks observed gravimetrically. Radioactivity measurements of tristearin having 1.0% levels of purified ^{3}H-Span or ^{3}H-Tween emulsifier stocks showed that the concentration of Span 60 at a tristearin-water interface was 2.73-fold greater than it was in the tristearin before the interface was formed. Correspondingly, a 1.45-fold

increase in Tween 60 concentration was observed at a tristearin-water interface when compared to the original tristearin-Span 60 mixture. Similar radioactive measurements at a tristearin-water interface containing: (a) Span 60 only had an emulsifier concentration of 0.124 x 10^{-6} M/mg, (b) Tween 60 only had an emulsifier concentration of 9.946 x 10^{-6} M/mg, and (c) an equal weight mixture of Span 60:Tween 60 had a total emulsifier concentration of 6.524 x 10^{-6} M/mg of dried interfacial material. Therefore, no increase in the total concentration of emulsifier molecules (stereochemical packing) was evident at a tristearin-water interface when a single emulsifier was replaced by a binary mixture of identical chemical type, HLB value, and usage level.

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