CARGILL
NUTRACEUTICAL DEPARTMENT

15407 McGinty Road West, MS 110
Wayzata, MN 55391-2399
Mail Address: Box 9300, MS 110
Minneapolis, MN 55440-9300
952/742-5246
Fax: 952/742-7573

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November 22, 2000

Dockets Management Branch
HFA-305
Food and Drug Administration,
5630 Fishers Lane, Rm 1061
Rockville, MD 20852

Re: Docket Nos. OOP-1275 and OOP-1276
Plant Sterol/Stanol Esters and Coronary Heart Disease, Interim Final Rule (65 Fed. Reg. 54686, September 8, 2000)

Dear Sir or Madam:

Cargill Incorporated (Cargill) hereby submits comments to the Food and Drug Administration (FDA) on the Interim Final Rule for Plant Sterol/Stanol Esters Health Claim referenced above.

Cargill is a processor of a variety of oilseeds from which plant sterols are isolated. Cargill welcomes this health claim, and hopes it will help introduce an array of products to the marketplace which will benefit consumers.

The effect of sterols in reducing serum cholesterol levels has been known for 50 years. Sterols are an adaptable substance and can be hydrogenated to make stanols and/or esterified to make sterol/stanol esters. Any of these forms can be formulated into products with a range of processing aids such as emulsifiers, thickeners and acidulants. The use of such aids does not diminish the cholesterol reduction ability of the sterol/stanol substances.

There are a variety of phytosterol forms – including free, hydrogenated (stanols), or esterified sterols – that can prevent cholesterol uptake when dispersed in the delivery matrix, regardless of the nature of that matrix. However, it has been shown in the scientific literature that the sterol chemical backbone is the critical element in preventing cholesterol uptake. Cargill urges FDA to acknowledge this range of effective forms and thus to include free sterols in its rule.

The use of plant sterols, in any of their forms, in a properly prepared food matrix should help consumers manage their own cholesterol absorption. It is thus important to facilitate the incorporation of this substance into a range of products. Cargill suggests

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that FDA allow manufacturers to introduce additional food forms, as long as they possess the data to support safety and efficacy.

Finally, it is important to have analytical methods available for the variety of food forms. While Cargill acknowledges the utility of the analytical method included in the Interim Final Rule for sterols, we also suggest that FDA add a range of sample preparation methods for a variety of methods in addition to spreads and dressings. We will be submitting such methods as an addendum in the near future.

Please contact me with further questions.

Sincerely,

Stephen D. McCurry, Ph.D.

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Re: Docket Nos. OOP-1275 and OOP-1276
Plant Sterol/Stanol Esters and Coronary Heart Disease, Interim Final Rule (65 Fed. Reg. 54686, September 8, 2000)

Cargill Incorporated (Cargill) hereby submits comments to the Food and Drug Administration on Plant Sterol/Stanol Esters and Coronary Heart Disease, Interim Final Rule (65 Fed. Reg. 54686, September 8, 2000) (Interim Final Rule). Cargill welcomes this health claim, and hopes it will enable an array of products in the marketplace which will be to the benefit of consumers.

Cargill is a processor of a variety of oilseeds (soy, canola, corn, peanut, and sunflower) from which sterols can be isolated. The dominant sterols in these oils are β-sitosterol, stigmasterol, and campesterol. Cargill also esterifies sterols with fatty acids from vegetable oils, and has a GRAS notification in process for sterol esters (GRAS Notification #48).

INTRODUCTION

The use of sterols and their chemical derivatives to reduce serum cholesterol has been known for 50 years (Peterson, 1951). Pollack (1953) showed that increasing the sterol consumption in the human diet could result in a considerable reduction in cholesterol absorption and in serum cholesterol. The data from this era was summarized in the book Sitosterol (Pollack and Kritchevsky, 1981). Therein, in a summary of 52 human studies Pollack and Kritchevsky showed an average reduction in plasma or serum cholesterol was 20 ± 1.5% (the average number of subjects was 17 ± 3), using an average phytosterol dosage was 1.3 ± 1.1 g/d fed for 27 ± 4 weeks.

Researchers have also investigated more available or more active forms of plant sterols. For example, Mattson et al. (1977) tested for the effects of various fatty acid esters of sitosterol and showed no ester that was more effective than the free sterol. In the same time period Sugano and coworkers (1977) showed that β-sitostanol was a more effective hypocholesterolemic agent than β-sitosterol.

COMMENTS

The FDA has asked for comments at several places in the Interim Final Rule. For the sake of simplicity, Cargill will make its comments in the context of these requests.

Section V.B.: Nature of the Claim:

Exemption of the requirement to adhere to the disqualifying level for fat for spreads and dressings (the only categories where the requested products would be subject to this disqualification level).

FDA's desire to encourage consumers to alter their diets by making exceptions to the fat level in certain products for sterol/stanol esters is laudable. FDA's argument that doing so will help consumers to get used to sterol/stanol esters in their diet makes sense (65 Fed. Reg. 54708). However, the difficulty with the solution offered in the Interim Final Rule is that it makes an exception for some products and not others. Given FDA's argument that this exemption will encourage proper diet by enabling consumer choice, the agency should allow more products, including those that may need such exemptions.

FDA has left open the inclusion of other products by inviting comments and by stating that other products requiring exceptions may be submitted. **We would suggest that FDA further offer a mechanism, simpler and faster that by petition, for the consideration of other products requiring exemptions even after the comment period has ended**. At the very least, we suggest that some sort of nutrient content claim, tied to the health claim (even on products that fall under the disqualification requirements) be allowed. This should apply even on products which the dietary cholesterol allowance is exceeded, since sterols have been shown to block the absorption of endogenously synthesized cholesterol as well as dietary cholesterol.

Setting of the daily intake level of plant sterol esters that qualify for the health claim at 1.3g or more per day

For this conclusion, FDA confined itself to looking at levels of sterols/sterol esters that adequately reduced serum cholesterol in foods that would be permitted to bear the claim (i.e., did not have a disqualifying level of fat, or cholesterol). It would seem that any relevant information concerning the efficacious level should be considered and not just that from those that meet all of the nutrient qualifications. This would make more food formats available, and thus serve the greater purpose of helping consumers integrate these cholesterol lowering substances into their diets.

Setting the level set for plant stanol esters at 3.4 g or more per day.

We believe that the petitioner for stanol esters sought approval for the maximum efficacious level, rather than a level that showed measurable cholesterol reduction. The plant stanol esters have produced the same sort of response as plant sterol esters (Westrate and Meijer, 1998) and therefore the level per day should be set at an analogous amount based on free sterol content.

Section V.C.: Nature of the Substance

Definition of the allowed make-up of the oil, and the determination that only edible oils are to be used as source material.

We agree that vegetable oils are excellent sources of sterols, but we urge the agency to add tall oil from trees (from the paper industry) as another acceptable source of phytosterols. It is discussed as an acceptable stanol source in the Interim Final Rule (65 Fed. Reg. 54706), and GRAS notifications by McNeil and Novartis demonstrate the safety and suitability of this oil source for sterols (which can be hydrogenated to stanols). It is therefore appropriate for FDA to add tall oils from trees as a source for sterols.

Suitability of the method for the measurement of sterol ester content included in the Interim Final Rule

The analytical method included in the Interim Final Rule works well for sterol esters in the food matrices requested by the petitioner. This saponification method of analysis also works well for free sterols, sterol esters, free stanols, and stanol ester samples isolated from any other food or supplement matrix , though the sample preparation has to be adapted to fit the particular matrix.

Appendix 1 of these comments contains the Cargill saponification method. It is essentially the same as the method in the Interim Final Rule, except that in addition to the procedures in that method, Cargill includes a silanization to clean up the chromatogram and to provide better chromatographic separation and peak quantitation.

Cargill also plans to submit sample preparation methods for a variety of matrices other than spreads and dressings with FDA as an addendum to these comments in the coming weeks.

Suitibility of McNeil's methods for analysis of stanol esters.

We reiterate the comments above, that the saponification method will correctly analyze stanols put through this type of analysis.

Section V.D.: Nature of the Food Eligible to Bear the Claim

Food matrices available for sterol esters

FDA allows the sterol ester claim on salad dressings and spreads, as requested by the petitioner. For a sterol to be available for blocking cholesterol absorption (in the intestinal lumen) it must be soluble in the food matrix used for delivery. In addition, is also required to make the food palatable. Through modification of the food formula, such solubility is possible in virtually any product matrix.

We believe that it should be up to the manufacturer to introduce additional product matrices provided that the company has data to support safety and efficacy of the specific matrix.

Level of stanol esters per day

The level of 3.4 g/day suggested by FDA requires too high an amount as the minimum level for the stanol esters. The amount should be the same as for sterol esters (and the same for sterol/stanol materials) – in sterol equivalents.

Disqualifying levels of total fat – exemptions

FDA agrees that other products may apply for exemption. We would request that there be an expedited review process for such requests short of new health claim petition.

Qualifying levels of total nutrient level.

We agree that other exemptions may be sought and may be allowed by FDA. We would request that there be an expedited review process for such requests short of new health claim petition.

Other Issues

As established in the GRAS notification, Cargill's sterol ester substance is substantially equivalent to the Lipton Take Control™ substance. As such, it meets the "safe and lawful" and nutrient requirements for making a health claim, 21 C.F.R. §101.14(c)(ii), should not be an issue.

Cargill has determined that this substance is GRAS and has filed a GRAS Notification (GRAS Notification #48), with attached Expert Panel Reports (Appendices 2 and 3) in support of such safety. Cargill recognizes that a GRAS Notification may not itself automatically satisfy the health claim safety standard. "Food Labeling: General Requirements for Health Claims for Dietary Supplements," 59 Fed. Reg. 395 (1994). However, in the Interim Final Rule, FDA determined that both the Lipton GRAS Notification, submitted January 11, 1999, and the McNeil GRAS Notification, submitted February 24, 1999, meet the safety standard necessary to justify a health claim. The Cargill substance also meets this standard.

Moreover, Cargill's sterol ester substance qualifies as a nutrient under 21 C.F.R.§101.14(b)(3)(i). As noted at 65 Fed. Reg. 54688, plant sterol esters act through an effect on the digestive system, and "nutritive value" "includes assisting in the efficient functioning of classical nutritional processes and other metabolic processes necessary for the normal maintenance of human existence." 59 Fed. Reg. 407. Therefore, just as FDA concluded that Take Control™, so too is Cargill's sterol ester substance.

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Sugano M, Morioka H, Ikeda I. A comparison of hypocholesterolemic activity of β–sitosterol and β–sitostanol in rats. J. Nutr. 107(11):2011-2019 (1977).

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Determination of Sterol Content in Food Matrices by Capillary Gas Chromatography

Nutraceuticals Group, Cargill Inc.
P.O. Box 5702
Minneapolis, MN 55440

A. Scope

This method is applicable to foods containing sterols and sterol esters. In a saponification step, sterol esters are all converted to sterols, which are then analyzed by gas chromatography (GC). It is expected that different food matrices containing these analytes will require different sample preparation steps (e.g. grinding, water extraction, solvent extraction and concentration, etc.). This method does not address those initial sample preparation issues, but focuses on the saponification and GC analysis of the sample.

B. Principle

Sterol esters are first saponified into free sterols with sodium hydroxide in methanol. In the same process, Triacylglycerols (TAG's) are saponified into free fatty acids (FFA's). To permit better chromatographic analysis the free sterols are then silylated with bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS). Separation and component quantitation is then accomplished by gas chromatography with a flame ionization detector (FID). Epicoprostanol (5β-Cholestan-3α-ol) is used as the internal standard. Stigmasterol is used as a calibration standard to determine a response factor for the sterols.

C. Safety

1. This work is done under the local Chemical Hygiene Plan and established safety procedures for handling materials, cleaning up spills and disposing of wastes.
2. Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheets for all chemicals used in this procedure.
3. Avoid contact with reagents and solvents used in this procedure. Toluene, pyridine, and BSTFA should be handled under a hood at all times.

D. Waste Disposal

1. Liquid wastes generated by this procedure must be collected and placed in a fluorocarbon waste container for disposal.

E. Apparatus

1. Hewlett-Packard (HP) 6890 Gas Chromatograph with cool on-column injection and Flame Ionization Detection or equivalent
2. HP 7683 Automated Sampler or equivalent
3. Electronic integrator HP 3396 Series II (Waters Millennium, Version 3.0 or HP ChemStation, Version 6.03) or equivalent
4. DB-5ht capillary column, 15m x 0.25 mm i.d. x 0.1 μm (P/N 122-5711, J&W Scientific, Inc.)
5. Analytical balance, accuracy to 0.0001g
6. 50-ml flat bottom flasks (P/N 09-559F, Fisher Scientific, Inc.) or equivalent
7. Explosion-proof hot plate (P/N 11-496-3, Fisher Scientific, Inc.) or equivalent
8. Air or water cooled condensers
9. Spatulas
10. 500ml Class A volumetric flask
11. 1-ml Class A volumetric pipettes
12. 1-ml Pipetman pipette (P/N P-1000, Rainin, Inc.)
13. 1-ml Pipetman pipette tips (P/N RC-1000, Rainin, Inc.)
14. Autosampler vial Teflon-lined caps (P/N 5181-1210, HP)
15. Crimper
16. Vortex Mixer
17. Transfer pipettes (P/N 13-711-7, Fisher Scientific, Inc.)
18. Glass boiling beads
19. 2-ml amber autosampler vials and caps

F. Reagents

1. Pyridine, (P/N 270013, Regis Technologies, Inc.) or equivalent
2. Bis(Trimethylsilyl) Trifluoroacemide (BSTFA) + 1% Trimethylchlorsilane (TMCS) (P/N T-6381, Sigma Chem. Co.) or equivalent
3. Epicoprostanol, 5β-Cholestan-3α-ol (P/N C-2882, Sigma Chemical Co. or C5050, Steraloids, Inc.)
4. 0.5N Sodium Hydroxide in Methanol. Dissolve 20g of sodium hydroxide (P/N S318-500, Fisher Scientific, Inc.) into a 1 L class A volumetric flask and dilute to volume with methanol, Optima, (P/N A454-4, Fisher Scientific, Inc.)
5. Heptane, Optima (P/N HX00787-1, Fisher Scientific, Inc.)
6. Saturated sodium chloride, reagent grade (P/N S640-3, Fisher Scientific, Inc.). Add excess sodium chloride in warm deionized water.
7. Sodium sulfate, reagent grade, (P/N S415-1, Fisher Scientific, Inc.)

G. Internal Standard Preparation

Before the internal standard is used, the purity and detector response has to be confirmed.

1. Weigh out 20 mg of the internal standard and dissolve it in 5.0 ml toluene.
2. In a 2 ml GC vial, place 20 ul of the internal standard solution, 0.5 ml pyridine and 1 ml BSTFA.
3. Analyze using gas chromatograph conditions given in section J.

4. To determine the purity of the epicoprostanol internal standard, integrate all peaks and calculate the area percent of the epicoprostanol peak. The area percent of the epicoprostanol peak within the internal standard solution chromatogram is defined as the purity of the epicoprostanol. This purity value will be used in the data analysis to calculate sample concentrations.
5. To prepare the internal standard, accurately weigh out 0.25g of Epicoprostanol into a 500 ml Class A volumetric flask. Record exact weight.
6. Fill to 500 ml volume with toluene and allow to dissolve.

H. Sample Preparation

As stated earlier, the scope of this method is not to specify sample preparation steps for all the food matrices that could contain sterols or sterol esters. However, the major concern in sample preparation is that the food matrix be solubilized as well as possible in the saponification reaction. Vegetable oils or any foods containing fats should be melted to obtain a homogeneous solution. If necessary, solid samples should first be ground to a powder and, often, dissolved or well dispersed in water prior to submitting it to the saponification reaction.

I. GC Sample Preparation

1. Add 1.0 ml of Internal Standard with a 1 ml Class A pipette to a 50-ml flat bottom flask, and evaporate to dryness with Nitrogen. Record the amount of internal standard added.
2. Accurately weigh 100 mg of sample into the tarred 50-ml flat bottom flask. Record the exact weight.
3. Add a couple of glass boiling beads, then add 4 ml of NaOH in MeOH to the flask. Attach an air or water-cooled condenser.
4. Boil on an explosion-proof hot plate for 1 hour or until oil is saponified (becomes one phase).
5. Remove from the hotplate and add 5 ml of Heptane. Return to the hot plate and boil for 1 minute.
6. Remove the flask from the hot plate and let cool. Add 30 ml of saturated sodium chloride, mix, and allow the two layers to separate.
7. Add a 100 mg of sodium sulfate to a 2-ml autosampler vial. Transfer 30 ul of the top (Heptane) layer to the GC vial.
8. Add 0.5 ml of pyridine, then 1 ml of BSTFA (1% TMCS) to the vial.
9. Crimp on the Teflon-lined caps, and vortex to mix well. The samples are now ready for chromatographic separation.

J. Gas Chromatograph Conditions

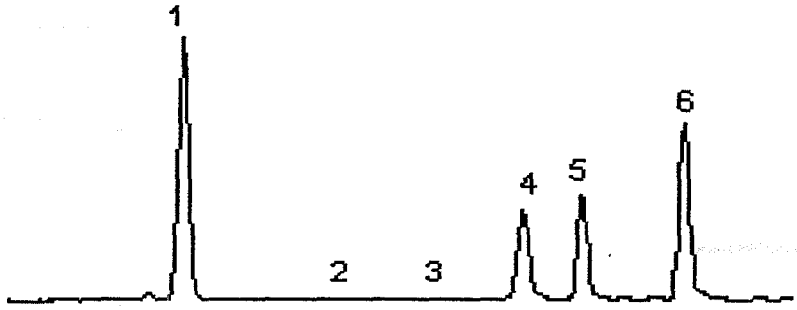
1. Column: 15m x 0.25mm x 0.1 μm , DB-5ht

2. Injector Temperature: Programmed to follow oven temperature (Oven Track)
3. Inlet Pressure: 6.7 psi@ 110 °C
4. Flow Rate: 1.25 ml/min. (Constant Flow Mode)
5. Carrier Gas: Hydrogen
6. Linear Velocity: 50 cm/s
7. Temperature Program: 110 °C initial, increase 30 °C/minute to 140 °C, then 10 °C/minute to 340 °C, hold 10 minutes. Reequilibration time is 1.0 minute.
8. Run Time: 33 min.
9. FID Temperature: 340 °C
10. Injection Volume: 1.0 µl
11. FID AirFlow Rate: 400 ml/min.
12. FID Hydrogen Flow Rate: 40 ml/min.
13. Makeup Gas: Nitrogen
14. Makeup Gas Flow Rate: 50 ml/min.

K. Data Analysis

Below is a typical chromatogram of an extracted sterol ester that has been saponified to its constituent sterols.

Example of Chromatogram



Time →

The following calculations are accomplished within the GC software.

First, a calibration curve is established from the peak areas of the stigmasterol calibration solutions. These peak areas are corrected for slight differences in injection volume or detector response by ratioing with the areas of their corresponding internal standard peaks.

To calculate % sterols in a food, the sterol peak areas are ratioed with the areas of their internal standard peaks and then substituted into the equation:

$$\% \text{ sterols} = 100 * \frac{(\text{sterol peak area}) * (\text{Stigmasterol Response Factor (mg/area)})}{(\text{mg. Sample})}$$

As an example only, the following data was taken from the above chromatogram and used to calculate the given weight % sterols. This profile of sterols is fairly typical of a soy-based sterol source.

Peak	Name	Retention Time (Min.)	Area	Amount (mg)	Wt. %
1	Epiprostanol (Internal Std.)	12.438	23162	2.3700	
2	Cholesterol	12.887	126	0.0001	0.012
3	Brassicasterol	13.148	124	0.0001	0.012
4	Campesterol + Campestanol	13.512	9323	0.0090	0.914
5	Stigmasterol	13.698	9154	0.0090	0.897
6	Sitosterol + Sitostanol	14.023	16631	0.0160	1.630

ADDENDUM TO
THE OPINION OF AN EXPERT PANEL ON THE GENERALLY RECOGNIZED AS SAFE (GRAS) USE OF PHYTOSTEROL ESTERS IN SPREADS AND RELATED PRODUCTS INTENDED TO PROMOTE HEALTHY CHOLESTEROL LEVELS

An independent panel of internationally recognized experts (the Expert Panel), qualified by their scientific and/or medical training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by the Cargill Company to determine the Generally Recognized As Safe (GRAS) status of a phytosterol ester product for use in spreads and related products intended to promote healthy cholesterol levels. That opinion was finalized May 10, 2000 and filed with the FDA as part of the Cargill GRAS Notification (#000048). Through telephone conversations with Dr. Linda Kahl of FDA the Panel became aware of some statements in the Opinion that were less specific than intended and therefore open to misinterpretation.

When the Expert Panel referred to Take Control™ or Benecol™ as evaluated by the FDA, the references were for spreads, and to no other product categories. In addition, when the Expert Panel used the term "affirmed" the meaning was that the other products had been determined to be GRAS by their respective companies' i.e., via the GRAS self-affirmation process.

As stated in the Opinion, the Expert Panel reviewed safety data from numerous sources to reach the opinion that the Cargill product is GRAS. To be more specific, the Expert Panel critically evaluated the identity, characteristic properties, methods of manufacture, batch analyses, proposed levels of use, dietary exposure, and applicable toxicological (safety) studies in animals and in humans. The Expert Panel carefully considered the Cargill GRAS Exemption Claim for the product and the substantial equivalence of this product to currently marketed self determined GRAS products to which the FDA had no objections (viz., Take Control™ or Benecol™).

Hepburn, et al. (1999) reported on a series of toxicity studies on the phytosterol esters present in Take Control™ spread in experimental animals including a 90-day oral toxicity study in rats where the NOAEL was 8.1% in the diet, the highest concentration tested. The Cytellin™ that was marketed by Lilly had a reported composition of beta-sitosterol (80%), sitostanol (10%), campesterol (7%), and campestanol (2 %). The Cargill phytosterol ester product is primarily composed of beta sitosterol (45%), campesterol (25%), and stigmasterol (25%). The NOAEL reported in an 18 month feeding study with beta-sitosterol in rats was 5%, the highest concentration fed. Beagle dogs received Cytellin™ in capsules daily for 18 months and the NOAEL was 1000 mg/kg bw/day. Because phytosterols have been studied extensively, safety considerations are often indirectly addressed (some of these studies are summarized in the Report to the Expert Panel). There were no consistent dose-dependent phytosterol-related significant adverse effects reported in the studies evaluated. The Expert Panel

concluded that the available animal (preclinical) toxicity data did not identify adverse effects attributable to the phytosterols administered orally.

Although there were no reported clinical toxicity studies on the Cargill phytosterol ester product, the available clinical data on Take Control™ and Cytellin™ were critically evaluated since the Cargill phytosterol ester product and the phytosterol esters present in Take Control™ are compositionally (substantially) equivalent and also similar to Cytellin™. Hendriks, et al. (1999) reported that human subjects tolerated 3.24 grams per day of plant sterols in a margarine matrix for 3.5 weeks. Weststrate, et al. (1999) reported that male subjects tolerated 40 grams/day of the Take Control™ spread for 3 weeks and female subjects for 4 weeks. Fourteen volunteers ingested from 20-50 grams of Cytellin™ per day for a mean of 280 days (range was 91-480 days).

Moreover, since the date of filing the notification, FDA has issued an interim final rule (IFR) on a Health Claims for sterol and stanol esters (65 Fed. Reg. 54686, September 8, 2000). In this IFR the FDA also included its analysis of available safety data for some other categories of products containing sterol and stanol esters, in particular salad dressings, snack bars, and other products, concluding:

> The agency notes that authorization of a health claim for a substance should not be interpreted as affirmation that the substance is GRAS. A review of Lipton's January 11, 1999, submission and of its September 24, 1999, letter to the agency, however, reveals significant evidence supporting the safety of the use of plant sterol esters at the levels necessary to justify a health claim. Moreover, FDA is not aware of any evidence that provides a basis to reject the petitioner's position that the use of plant sterol esters in spreads and dressings for salad up to 1.6 g/serving is safe and lawful. As discussed in section V.B of this document, the level of plant sterol esters necessary to justify a claim is 1.3 g per day. Therefore, FDA concludes that the petitioner has satisfied the requirement of §101.14(b)(3)(ii) to demonstrate that the use of plant sterol esters in spreads and dressings for salad at the levels necessary to justify a claim is safe and lawful. P 54689

In the paragraph above, the FDA set the level based on the amount needed for a claim at 1.3g/day or 0.65g/serving with two servings per day. The agency further found that 1.6g/serving is safe and lawful in multiple products.

Stanol esters are chemically very similar to sterol esters and have the same biological effects. With respect to stanol esters:

> The agency notes that authorization of a health claim for a substance should not be interpreted as affirmation that the substance is GRAS. A review of McNeil's February 18, 1999,

> submission, however, reveals significant evidence supporting the safety of the use of plant stanol esters at the levels necessary to justify a health claim. Moreover, FDA is not aware of any evidence that provides a basis to reject the petitioner's position that the use of plant stanol esters in spreads, dressings for salad dressings, snack bars, and other foods is safe and lawful. FDA therefore concludes that the petitioner has satisfied the requirement of §101.14(b)(3)(ii) to demonstrate that the use of plant stanol esters in conventional foods at the levels necessary to justify a claim is safe and lawful.

The level of stanol esters to which FDA's conclusion is addressed is 1.7g/serving (or 3.4 g/day). The agency expects two or more servings/day as stated in interim 21CFR 101.83(C)(2)(G) (p 54718):

> (G) The claim specifies the daily dietary intake of plant sterol or stanol esters that is necessary to reduce the risk of CHD and the contribution one serving of the product makes to the specified daily dietary intake level. Daily dietary intake levels of plant sterol and stanol esters that have been associated with reduced risk of are: (sic)
> (1) 1.3 g or more per day of plant sterol esters.
> (2) 3.4 g or more per day of plant stanol esters.
> (H) The claim specifies that the daily dietary intake of plant sterol or stanol esters should be consumed in two servings eaten at different times of the day with other foods.

Based upon its independent and collective critical evaluations of the information summarized in the previously submitted notification, the Expert Panel concluded that the Cargill phytosterol ester product meeting appropriate food grade specifications and produced in accordance with current Good Manufacturing Practice is GRAS by scientific procedures for use in spreads and related products intended to promote healthy cholesterol levels at a daily intake of at least 1.3g consumed in one or more product servings with meals or snacks. The Expert Panel considers the intake level established as adequate for the Health Claim to be conservative with respect to safety. The panel notes that up to 50g of a related product have been consumed daily without evidence of adverse effects. The panel finds this evidence reassuring that daily phytosterol intakes, in excess of the health claim amount, which could possibly arise from multiple product uses, are safe.

Professor Joseph Borzelleca Medical College of Virginia	Professor Walter Glinsmann Georgetown University and	Professor David Kritchevsky Wistar Scholar

Richmond, VA 23298-0613

President Glinsmann, Inc.
Washington, DC 20024-2201

Wistar Institute
Philadelphia, PA 19104-4268

OPINION OF AN EXPERT PANEL ON THE GENERALLY RECOGNIZED AS SAFE (GRAS) USE OF PHYTOSTEROL ESTERS IN SPREADS AND RELATED PRODUCTS INTENDED TO PROMOTE HEALTHYCHOLESTEROL LEVELS

An independent panel of internationally recognized experts (the Expert Panel), qualified by their scientific and/or medical training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by the Cargill Company to determine the Generally Recognized As Safe (GRAS) status of a phytosterol ester product for use in spreads and related products intended to promote healthy cholesterol levels. The ingredient is intended to be used at levels equal to those in spreads (Take Control™, Benecol™) that have been self determined as GRAS and for which formal GRAS notification was made to the U.S.F.D.A. The Agency did not question the GRAS determination. The ingredient is also intended to be used at levels equal to those in other products (identified as Take Control™ or Benecol™ products) that have been self determined as GRAS but for which formal GRAS notifications to the U.S.F.D.A. have not been made. A comprehensive search to the scientific literature for safety and toxicity information through March 2000 was conducted by Cantox and was made available to the Expert Panel. In addition, the members of the Expert Panel considered other information deemed appropriate or necessary. The information considered essential by the Expert Panel is summarized in the accompanying Final Report to the Expert Panel Regarding the Evaluation of the GRAS Status of a Cargill, Inc. Product Consisting of Esterified phytosterols (Cantox US Inc., 28 April 2000). The members of the Expert Panel independently critically evaluated the available information on phytosterol esters, conferred by telephone several times, and then conferred via conference call with members of Cargill and Cantox U.S., Inc. The members of the Expert Panel then conferred privately to determine the GRAS status of the Cargill phytosterol esters for use in spreads and related products intended to promote healthy cholesterol levels.

To determine the GRAS status of the Cargill phytosterol ester product by scientific procedures, the Expert Panel critically evaluated the identity, characteristic properties, methods of manufacture, batch analyses, proposed levels of use, dietary exposure, and applicable toxicological (safety) studies in animals and in humans.

The Expert Panel carefully considered the GRAS Exemption Cargill Claim for the Product and the substantial equivalence of this product to currently marketed self determined GRAS products to which the U.S.F.D.A. had no objections (viz. Take Control™ or Benecol™). The Expert Panel critically evaluated the analytical chemical data (identity and specifications) provided and concluded that the soy-based Cargill phytosterol ester product to be compositionally equivalent (substantially equivalent) to the phytosterol esters present in a currently marketed Take Control™ spread. The Expert Panel further noted that the phytosterols found in the Cargill phytosterol ester product and in Take Contol™ spread are similar to those naturally occurring in fruits and vegetables. Food-grade specifications for the Cargill phytosterol ester product were provided together with the analysis of five non-sequential batches. The data indicate that food-grade specifications can be consistently met. All raw materials and processing aids used in the production of the Cargill phytosterol ester product are approved for use in food processing.

The Cargill phytosterol ester product is to be used in vegetable oil based spreads and related products to help maintain healthy blood cholesterol levels and is an alternative source of phytosterol esters for food manufacturers. The current recommended daily intake for phytosterol containing products (Take Control™ or Benecol™) is one portion of any product three times a day with meals and snacks. The Expert Panel was not aware of self-limiting uses of the Cargill phytosterol ester product of Take Control™ spread.

Most animal and human studies indicate that the absorption of phytosterols is about 5% or less of dietary intake levels (cholesterol absorption is about 40%). The concentration of phytosterols in serum is a function of dietary intake and may be increased through dietary supplementation. Phytosterols are not endogenously synthesized and are not converted to cholesterol and cholesterol is not converted to phytosterols. Phytosterols are esterified but at a slower rate than cholesterol in the rat. Phytosterols that are absorbed are eliminated through the biliary route. Anaerobic bacteria in the lower intestine may metabolize inabsorbed phytosterols.

Although there were no reported preclinical toxicity studies on the Cargill phytosterol ester product, the available preclinical data on Take Control™ were critically evaluated since the Expert Panel concluded that the products are compositionally (substantially) equivalent. The Expert Panel also critically evaluated the

preclinical and clinical data on Cytellin™, a product marketed by Eli Lilly in the U.S. between 1954 and 1982. This product was a mixture of phytosterols derived from tall oil and was marketed as an anti-hypercholesterolemic agent.

Hepburn, et al. (1999) reported on a series of toxicity studies on the phytosterol esters present in Take Control™ spread in experimental animals including a 90-day oral toxicity study in rats where the NOAEL was 8.1% in the diet, the highest concentration tested. The Cytellin™ that was marketed by Lilly had a reported composition of beta-sitosterol (80%), sitostanol (10%), campesterol (7%), and campestanol (2 %). The Cargill phytosterol ester product is primarily composed of beta sitosterol (45%), campesterol (25%), and stigmasterol (25%). The NOAEL reported in and 18 month feeding study with beta-sitosterol in rats was 5%, the highest concentration fed. Beagle dogs received Cytellin™ in capsules daily for 18 months and the NOAEL was 1000 mg/kg bw/day. Because phytosterols have been studied extensively, safety considerations are often indirectly addressed (some of these studies are summarized in the Report to the Expert Panel). There were no consistent dose-dependent phytosterol-related significant adverse effects reported in the studies evaluated. The Expert Panel concluded that the available animal (preclinical) toxicity data failed to identify adverse effects attributable to the phytosterols administered orally.

Although there were no reported clinical toxicity studies on the Cargill phytosterol ester product, the available clinical data on Take Control™ and Cytellin™ were critically evaluated since the Cargill phytosterol ester product and the phytosterol esters present in Take Control™ are compositionally (substantially) equivalent and also similar to Cytellin™. Hendriks, et al (1999) reported that human subjects tolerated 3.24 grams per day of plant sterols in a margarine matrix for 3.5 weeks. Weststrate, et al (1999) reported that male subjects tolerated 40 grams/day of the Take Control™ spread for 3 weeks and female subjects for 4 weeks. Fourteen volunteers ingested from 20-50 grams of Cytellin™ per day for a mean of 280 days (range was 91-480 days).

Esterified phytosterols could interfere with the absorption of fat-soluble nutrients particularly carotenoids and other fat-soluble materials by the extent of this effect and its impact on human safety could not be determined. The literature bearing on the effects of phytosterol products on plasma levels of alpha- and beta- carotene, lycopene, alpha-tocopherol, and vitamins K1, D, and E was critically evaluated and the data indicate no changes in serum levels of vitamins A, D, E, and K but lower levels of lycopene and

beta-carotene. Other phytosterol products also lower the concentration on plasma carotenoids. The significance of this reduction is not known. Recent studies show that a high intake of phytosterol esters increases the amounts of neutral steroids in the feces but is without increased formation of bile acids or sterol metabolites. The Expert Panel concluded that the potential nutritional impact of increased intake of phytosterol esters is not known but available preclinical and clinical data on the oral intake of high doses of phytosterol esters failed to identify any adverse effect on general health and survivability (of the experimental animals). The Expert Panel further concluded that the available information supports the safety of ingested phytosterol esters at recommended doses of at least 4.5 grams per day although no toxicity was reported at doses up to 50 grams per day.

Based upon its independent and collective critical evaluations of the information summarized in the attached report, the Expert Panel concluded that the Cargill phytosterol ester product meeting appropriate food grade specifications and produced in accordance with current Good Manufacturing Practice in GRAS by scientific procedures for use in spreads and related products intended to promote healthy cholesterol levels at a level of 1.5 grams of phytosteryl esters per serving, and one serving three times a day with meals or snacks. The Expert Panel recognizes that this is a conservative level since up to 50 grams of a related product have been consumed without evidence of an adverse effect.

Professor Joseph F. Borzelleca
David Kritchevsky
Medical College of Virginia
Richmond, VA 23298-0613

Professor

Wistar Scholar
Wistar Institute
Philadelphia, PA

19104-4268

Professor Walter H. Glinsmann
Georgetown University and
President, Glinsmann, Inc.
Washington, DC 20024-2201

GRAS NOTIFICATION

for

VEGETABLE OIL DERIVED PHYTOSTEROLS ESTERIFIED WITH FATTY ACIDS

Submitted by:

Cargill, Incorporated
15407 McGinty Road West
Wayzata, MN 55391

Contacts:

for scientific or technical information:
Stephen D. McCurry Ph.D.
Manager Scientific and Regulatory Affairs
952-742-6462

for Cargill Washington Office:
Linda Thrasher
Director of Public Policy
202-530-8160

for other information:
Shirley R. Boyd
Senior Attorney
952-742-6361

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1.0 INTRODUCTION

1.1 Background

Plant sterols or "phytosterols", which structurally resemble cholesterol but have a much lower absorption rate, are gaining popularity in consumer products. Recently marketed vegetable spreads such as Benecol™ and Take Control™ are two examples of phytosterol-containing products intended to promote healthy cholesterol levels. These two vegetable oil spreads contain up to 20% by weight added fatty acid esterified phytosterols. Phytosterols, which are found in a variety of plant sources, cannot be manufactured by the human body and are obtained exclusively through the diet. Because phytosterols can compete with cholesterol for absorption in the gastrointestinal tract, they are presumed to provide substantial health benefits by helping to maintain healthy blood cholesterol levels.

Benecol™ is a GRAS vegetable oil spread marketed by McNeil Consumer Healthcare. It contains a blend of fatty acid esterified hydrogenated sterols derived from vegetable and from tall oil, which is oil obtained from trees. Hydrogenation of this blend saturates the component sterols into phytostanols. The resultant phytostanol product is composed of campestanol (32%) and sitostanol (62%), as well as approximately 5% unsaturated sterols. Take Control™, which is manufactured by Lipton, contains fatty acid esterified phytosterols derived from vegetable oil such as canola, soybean, and corn oil. It is also considered to be GRAS. The composition of sterols in Take Control™ varies, depending on the oil used, but generally consists of approximately 14% to 23% stigmasterol, 20% to 28% campesterol, 40% to 55% β-sitosterol, and up to 8% of about 20-30 minor sterols. These sterols are commonly found in foods. Esterification of these sterols to vegetable oil fatty acids facilitates their incorporation into the matrix of common food items such as vegetable oil spread.

Both Benecol™ and Take Control™ have been self-affirmed by their respective manufacturers as Generally Recognized as Safe (GRAS). The U.S. Food and Drug Administration (FDA) is aware of and is not in disagreement with this opinion. Cargill Incorporated manufactures a phytosterol ester product with properties virtually identical

to the esterified phytosterols present in Take Control™ and similar to the phytostanols present in Benecol™, the use of which is intended to help maintain a healthy blood cholesterol level. Cargill plans to market this product as an alternative source of vegetable oil phytosterol esters for use in phytosterol-formulated food products currently on the market for which GRAS status has already been established, i.e. primarily in vegetable oil based spreads, salad dressings, bars or yogurt. Accordingly, no change is expected in phytosterol ester intake by individual consumers; the Cargill product simply represents an additional choice of phytosterol ester ingredient supply. Cargill will require that a manufacturer wishing to employ the Cargill phytosterol ester product in a new product application assume the responsibility for establishing the GRAS status or other regulatory status for its proposed product.

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients was requested by Cargill Incorporated to determine the GRAS status of a natural product derived from vegetable oil. The panel of qualified experts determined that the Cargill phytosterol ester product may be considered GRAS based upon its substantial and demonstrable compositional equivalence to phytosterols in currently marketed products, particularly Take Control™, as well as on the basis that these phytosterols:

1) Are commonly found in food products;

2) Are to be consumed at levels occurring in currently marketed products; and

3) Have an expected safety and physiologic activity profile equivalent to currently used phytosterols.

The panel of qualified experts has reviewed the necessary technical, safety, and product information and has determined that the use of Cargill's esterified vegetable oil phytosterols be considered GRAS based on scientific procedures. A written statement to that effect and signed by each member of the expert panel appears in Section 11 of this document.

1.2 History of Safe Use

Phytosterols are abundant in nature, occurring naturally in a variety of fruits and vegetables that are part of the human diet. In the United States, the average intake of phytosterols is approximately 250 mg per day; vegetarians consume nearly twice as much. Research conducted over the past fifty years has indicated that increased phytosterol intake can contribute to the lowering of blood cholesterol levels in humans. Long term clinical studies and studies with dose levels of up to 25,000 mg phytosterols per day (i.e., 100 times the average dietary intake) have been conducted (Pollak & Kritchevsky, 1981; Pollak, 1985). Over 1,800 men, women, adolescents, and children have participated in these studies, some of which date back to the 1950s. Under the conditions of these studies, no significant adverse health effects have been reported (Lees *et al.*, 1977; Oster *et al.*, 1977).

Plant sources rich in phytosterols include rice bran, wheat germ, and oats, which can contain up to 4% phytosterols. Some crude vegetable oils contain 0.1% to 0.5% phytosterols. Reduced fat and low fat spreads currently available in the marketplace may contain background phytosterol levels between 0.3% and 0.4%. Take Control™ and Benecol™ are two previously mentioned phytosterol-containing spreads with up to 20% added esterified phytosterols by weight. Phytosterols are often esterified with C_{12} - C_{18} fatty acids to facilitate their incorporation into the fat matrix of common food items such as vegetable oil spreads.

Three of the most common plant sterols are β-sitosterol, campesterol, and stigmasterol. The predominant phytosterols in Take Control™, by weight, are β-sitosterol (40% to 55%), campesterol (20% to 28%), and stigmasterol (14% to 23%). Up to 8% of this product may be comprised of 20 to 30 different minor sterol components. The phytosterols in Take Control™ are derived from vegetable oils such as soybean, canola, and corn. Consequently, the principal sterol components can vary from batch to batch depending on the type of vegetable oil used to obtain the phytosterols. Benecol™ spread, on the other hand, contains a blend of vegetable oil and tall oil phytosterols. Hydrogenation of this blend saturates the sterols into stanols, resulting in approximately 62% sitostanol and 32% campestanol. The remaining 6% are

comprised primarily of unsaturated sterols. The Cargill phytosterol product is derived from vegetable oils and is intended to be an alternative source of esterified phytosterols for uses similar to Take Control™ and Benecol™.

Take Control™ and Benecol™ are products recently marketed in the United States with the aim of achieving a healthy cholesterol level, although attempts to capitalize upon the recognized benefits of increased phytosterol intake are not new. For example, Cytellin™, a preparation marketed by Eli Lilly between the 1950s and the 1980s, was available in the United States as treatment for hypercholesterolemia. The same product was also available in Canada and sold under the brand name Positol™. Cytellin™ contained phytosterols derived from tall oil, and was composed of approximately 80% β-sitosterol, 10% sitostanol, 7% campesterol and 2% campestanol. Recommended therapeutic levels ranged from 9,000 to 30,000 mg per day. No contraindications or adverse side effects were reported during repeated clinical investigations of Cytellin™ (Best *et al.*, 1955; Joyner and Kuo, 1955; Lesesne *et al.*, 1955; Farquhar *et al.*, 1956; Kuo, 1956; Duncan and Best, 1963).

In summary, the phytosterols found in the products mentioned are similar to those naturally occurring in fruits and vegetables that are part of the human diet. In addition, no significant adverse effects have been associated with consumption of relatively high levels or long term use of phytosterols during the course of clinical trials. Furthermore, the Cargill phytosterols are identical to those present in Take Control™ products and have been determined to be GRAS by a panel of qualified experts.

2.0. BASIS FOR REGULATORY APPROVAL OF GRAS AFFIRMATION

2.1 GRAS Regulatory Status

Per Volume 62, Number 74 of the April 17, 1997 Federal Register, Pages 18937-18964 (Proposed Rules, 21 CFR Parts 170, *et al.*), Cargill Incorporated wishes to notify the U.S. Food and Drug Administration (FDA) that it has determined that the use of its phytosterol ester product as an ingredient in currently marketed phytosterol formulated food products is Generally Recognized as Safe (GRAS). The GRAS determination is

based upon scientific procedures that have been affirmed by a panel of qualified experts who reviewed the technical evidence of safety and determined that there is reasonable certainty that the substance is not harmful under the intended conditions of use. Therefore, this warrants exemption from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act for this product.

The Cargill phytosterol ester product is equivalent in structure and function to the phytosterol esters employed in Lipton's Take Control™ vegetable oil phytosterol spread. It is also similar in nature to those in the Benecol™ hydrogenated vegetable oil / tall oil phytosterol spread, however, unlike Benecol™, it contains no tall oil derived phytosterols and contains only minor amounts of phytostanols. Both Take Control™ and Benecol™ are currently self-determined to have GRAS status by their manufacturers. The Cargill phytosterol ester product is derived primarily from soy. Its chemical composition is essentially identical to the phytosterol esters in Take Control™, which also contains phytosterol esters derived predominantly from soy and which has been self-affirmed by Lipton as GRAS and is currently marketed in the United States, with full U.S. FDA awareness.

In accordance with the specific requirements outlined in 21 CFR Section 170.36(c) of the Proposed Rule, this document provides the FDA with necessary supportive information which, in conjunction with the evaluation by a panel of qualified experts, forms the basis for a conclusion by expert consensus that this product may be considered safe for its intended use. The following table outlines the applicable Sections, Subsections, and specific requirements of the Proposed Rule.

Table 1: Subset of Requirements for GRAS Determination by Scientific Procedures

Proposed Rule 21 CFR Section	Specific Requirements
Identity and Specifications: 170.36(c)(2)	Notice must include detailed information about the identity of the notified substance, including chemical name, structural formula, quantitative composition, method of manufacture, characteristic properties, specifications, etc.
Self-limiting levels of use: 170.36(c)(3)	Notice must include any self-limiting levels of use of the substance.
Technical evidence of safety: 170.36(c)(4)(i)(A)	Notice must include a detailed summary of the basis for determination that use of the substance is GRAS by scientific procedures. Summary should include a comprehensive discussion of, and citations to, generally available and accepted scientific data, information, methods, or principles used to establish safety, as well as consideration of probable consumption and cumulative effect of the substance in the diet.
Basis for concluding expert consensus: 170.36(c)(4)(i)(C)	Notice summary of a scientific procedure GRAS determination must include the basis for concluding that there is a consensus among qualified experts that there is reasonable certainty that the substance is not harmful under the intended conditions of use.

Cargill's scientific and technical data presented herein is in support of a GRAS determination by a panel of qualified experts for the Cargill phytosterol ester product. These supportive materials were obtained from the following sources and are available for further FDA review:

1) A comprehensive review of the scientific literature through March of 2000 for data on the safety of plant sterols was conducted;

2) Other supporting documentation from scientific reference texts, reviews, compendia, etc.;

3) Information from recent GRAS notifications for substantially equivalent products (e.g., Take Control™ and Benecol™).

The determination of GRAS status is based upon affirmation by a panel of qualified experts that the substance is not harmful under the intended conditions of use and that it is substantially equivalent to other GRAS phytosterol-containing products currently marketed in the United States. By meeting the requirements outlined in the

Proposed Rule for substances Generally Recognized as Safe (21 CFR Parts 170 *et al.*) in Volume 62, Number 74 of the April 17, 1997 Federal Register, Pages 18937-18964, it is assumed that the requirements outlined in Parts 201 *et al.* of the Federal Food, Drug, and Cosmetic Act for this product have been met.

2.2 Substantial Equivalence to a GRAS Substance

The Cargill phytosterol ester product is essentially identical in structure and function to the phytosterols contained in the Take Control™ vegetable oil spread and similar in nature to those in the Benecol™ vegetable oil / tall oil hydrogenated phytosterol spread. However, the Cargill phytosterol ester product is derived exclusively from vegetable oil and contains no tall oil phytosterols, as does the Benecol™ product. Chemical analyses of the Cargill phytosterol ester product have determined that its chemical composition and phytosterol profile are essentially identical to those in Take Control™. The intended conditions and method of use, associated levels of intake, considerations of safety, and physiological effect of the Cargill phytosterol ester product are all similar to those of Take Control™. Consequently, because of the similarities between the Cargill phytosterol ester product and the phytosterols found in the GRAS substance Take Control™, a qualified panel of experts has determined that these two products may be considered substantially equivalent and should be treated in the same manner with respect to safety and efficacy.

2.2.1 Composition

The composition of the Cargill phytosterol ester product exhibits a profile of major phytosterol components essentially identical to those in Lipton's product Take Control™ but is different from the phytosterol content of the McNeil product Benecol™, which contains predominantly stanols. Table 2 compares the approximate compositions of the three products. In each product, some natural variation may occur in specific phytosterol component content. The data in Table 2 indicates that levels of the individual component phytosterols in Cargill's phytosterol ester product are comparable

to levels in the Lipton GRAS phytosterol product Take Control™. The variation in constituent phytosterol profile among the three products arises from two main factors: phytosterol source and use of hydrogenation processing. In each product, the phytosterols are esterified to vegetable oil fatty acids whose exact profile varies somewhat according to the choice of vegetable oil from which they are sourced.

2.2.1.1 Source and Hydrogenation

The Cargill phytosterol ester product contains significant levels of β-sitosterol, campesterol, and stigmasterol similar to those occurring in Take Control™. Similarly, the Cargill product and Take Control™ contain only minor quantities of the naturally occurring saturated (stanol) compounds sitostanol and campestanol, found at high levels in Benecol™. In contrast, Benecol™ employs a hydrogenation process to saturate most double bonds present in the sterol components, converting them to stanols, predominantly sitostanol and campestanol.

Since many of the minor components in these products are variously unsaturated congeners of the same saturated structures, hydrogenation may also reduce, somewhat, the diversity of minor components. However, Benecol™ still contains a range of minor phytosterols of up to 6% (ref. Benecol™ GRAS notification). The phytosterols in the Cargill phytosterol ester product and Take Control™ are not hydrogenated and contain up to 4-6% by weight of minor sterol and non-sterol components (see Table 2). Similarly, the Cargill phytosterol product contains the same minor components as Take Control™, primarily representing variation in the position and / or number of double bonds within β-sitosterol (C_{29}) and campesterol (C_{28}) structures. See Section 3 for compositional details. The Cargill phytosterol ester product has compositional elements which are equivalent to those in the Take Control™ GRAS product as well as minor constituents which may be found in both products and which, in aggregate, supports a determination of substantial equivalence as evaluated by a panel of qualified experts.

Table 2: Comparison of Approximate Phytosterol Compositions

Sterol	Cargill Phytosterol Ester Product[1]	Take Control™[2]	Benecol™[3]
β-Sitosterol	45	46	4
Campesterol	25	25	3
Stigmasterol	22	18	
Brassicasterol	1	4	
Sitostanol	2	2	64
Campestanol	1	1	23
Minor Sterols	4	4	6
Total Phytosterols	100	100	100

[1] As determined by Cargill (see Section 3.0);
[2] As determined in the Take Control™ GRAS notification;
[3] As determined in the Benecol™ GRAS notification.

2.2.1.2 Esterification

The phytosterols in the Cargill product exist in an esterified form, similar to those in the two GRAS products. All have been esterified with common vegetable oil fatty acids to enhance their solubility in a vegetable oil product matrix. Esterification does not materially affect the substantial equivalence of the Cargill phytosterol ester product to the other products. The ester forms are rapidly de-esterified *in vivo* through the action of lipase enzymes yielding the active free phytosterols. Esterification does affect quantitative parameters of equivalence. The Take Control™ and Benecol™ products are approximately 60% by weight phytosterols; the remainder being fatty acids. Accordingly, the phytosterol content of the Cargill phytosterol ester product will also be approximately 60% phytosterol by weight and approximately 40% by weight vegetable oil fatty acids.

2.2.2 Intended Use and Intake

The Cargill phytosterol ester product will provide an alternative ingredient source of phytosterol esters for use by manufacturers of products currently marketed with enhanced phytosterol ester content, i.e. primarily vegetable oil based spreads, salad

dressings, bars or yogurt, for which use of phytosterols have been affirmed as GRAS. Accordingly, there will be no change in individual phytosterol ester intake, simply an additional choice of phytosterol ester ingredient supply. Cargill will require that a manufacturer wishing to employ Cargill phytosterol esters in a new product application assume the responsibility for establishing the GRAS or other regulatory status for its proposed product.

2.2.3 Physiologic Properties

The phytosterols in Cargill phytosterol ester product have been determined through scientific review by a panel of qualified experts to be substantially equivalent in physiologic properties to those in Take Control™ with regard to their active form and effect on blood cholesterol parameters, blood phytosterol levels and absorption of vitamins and nutrients.

2.2.3.1 Active Form

Esterified phytosterols are converted *in vivo* to, and are physiologically equivalent to, proportional amounts of free phytosterols and stanols. Evidence indicates that the active form of the sterol esters is the free sterol. Swell (1953) determined that pancreatic cholesterol esterase hydrolyzes both cholesterol esters and phytosterol esters to their free forms. Cholesterol is not absorbed in the esterified form but must first be cleaved before it can be absorbed into the body. Hellman (1953) fed radio labeled cholesterol to rats and observed that the labeled sterol appeared in the lymph in the free fraction before it appeared in the ester fraction. Although the above experiment has not been performed with phytosterol esters, it can be inferred that only phytosterols in the free form are absorbed. Direct comparisons between free phytosterols and esterified phytosterols in the rat found that the esterified forms were less effective as inhibitors of cholesterol absorption (Pollak & Kritchevsky, 1981). Similarly, Mattson (1982) reported lower efficacy of sitosterol oleate than free β-sitosterol in inhibiting absorption of cholesterol in the human and stigmasterol oleate was less effective than

free stigmasterol in decreasing cholesterol absorption in the human (Mattson, 1977). These results indicate that cleavage of the sterol esters controls the availability of phytosterols for interaction with the cholesterol absorption mechanism. It can also be inferred that it is primarily the free phytosterols which interact with the cholesterol absorption mechanism.

2.2.3.2 Effectiveness in Reducing Blood Cholesterol Levels

Phytosterols are effective in lowering blood cholesterol levels when administered orally in animals and humans. A summary of several published clinical studies is provided in Section 10. The maximum lowering of LDL cholesterol observed in human studies with phytosterols is in the range of 13% - 15%. One study by Weststrate (1998) directly compared the Take Control™ product with that of the Benecol™ product in hyper-cholesterolemics over a 25 day treatment interval (Table 3).

Table 3: Comparative Effectiveness of Sterol Products in a Margarine Matrix

Product:	Take Control™	Benecol™
Dosage	3 g per day [1]	2.7 g per day [1]
Δ Total Cholesterol [2]	-8.3%	-7.3%
Δ LDL Cholesterol [2]	-13.0%	-13.0%
Δ HDL Cholesterol [2]	+0.6%	+0.1%

[1] Weststrate (1998).
[2] Values are corrected for the changes occurring in the control group.

In a review by Kritchevsky (1997), the suggested mechanisms of hypocholesterolemic properties of β-sitosterol were proposed. They consisted of: 1) Mixed crystal formation; 2) adsorption aggregation; 3) Decreased solubility of cholesterol micelles; 4) Enhanced cholesterol excretion; 5) Interference with cholesterol synthesis; 6) Competitive esterification; 7) Competition for acceptor sites; and 8) Reduced membrane permeability.

2.2.3.3 Effects on Circulating HDL-Cholesterol

There was no evidence of any significant effect on plasma HDL levels for either of the two currently available GRAS products. Due to the compositional equivalence of the Cargill phytosterol ester product, no effect on circulating HDL cholesterol levels can be reasonably expected to occur.

2.2.3.4 Effects on Circulating Levels of Phytosterols

Phytosterols are a normal circulating constituent in the blood. Phytosterols are not synthesized by the body but are absorbed from plant material in the diet. The major circulating phytosterols are β-sitosterol and campesterol, reflecting the higher content of these sterols in food sources. Ingestion of Take Control™, which has a high content of these phytosterols, results in an increase in their circulating blood levels. Ingestion of Benecol™ has an opposite effect, which can be attributed to its elevated stanol content, as illustrated below in Table 4. Sitostanol, which is particularly poorly absorbed, appears to inhibit not only the absorption of cholesterol but also that of other sterols. The phytosterols in the Cargill phytosterol ester product are likely to have an effect very similar to that displayed by the Lipton Take Control™ product.

Table 4: Effect of Phytosterol Products on Blood Phytosterol Levels

	Take Control™	Benecol™	Benecol™
References	Weststrate *et al.*, 1998	Weststrate *et al.*, 1998	Gylling *et al.*, 1995
β-Sitosterol	+38.8%	-36.2%	-36.1%
Campesterol	+72.6%	-17.1%	-48.2%

2.2.3.5 Effects Upon Vitamin and Nutrient Absorption

The activity of ingested phytosterols with regard to absorption of fat soluble nutrients including vitamins A, E, D and K, has been thoroughly reviewed and discussed in the process of establishing the GRAS status of Take Control™ and Benecol™ (see

Benecol™ and Take Control™ GRAS Notifications). Weststrate (1998) and Hendriks (1999) evaluated sterol ester consumption and observed some decrease in the absorption of carotinoids such as α- and β-carotene and lycopene. Gylling (1996) evaluated phytostanol esters and reported a similar decrease in plasma β-carotene levels. Hendriks (1999) observed no effect on lipid standardized plasma α-tocopherol and lycopene levels or upon plasma concentrations of the fat-soluble vitamins K_1 and D. Hallikainen (2000) observed a dose dependant decrease in serum lycopene levels in women only and a dose dependant decrease in α- and γ-tocopherol levels in men and women when they consumed phytostanol esters in a margarine matrix. When compared to serum total cholesterol concentration, there were no significant differences in α- and γ-tocopherol among the different dose periods. No significant changes were reported in serum retinol, or α- and β- carotene levels.

No other significant impairment of the availability of fat-soluble vitamins has been noted for plant sterol or sterol esters. This included data from the intake of Cytellin™ over the period of its availability. Expert and FDA reviews of the issue have resulted in findings of no expected significant risk and that these phytosterol products may be regarded as GRAS. While the Cargill phytosterol ester product has not been specifically tested in this regard, its substantial compositional equivalence and intended use level compared to Take Control™, assures that it presents no significant risk of adversely altering vitamin and nutrient absorption to any degree materially different from Benecol™ or Take Control™.

2.2.4 Safety

The safety of ingested phytosterols has been thoroughly reviewed and discussed in the process of establishing the respective GRAS status of the Take Control™ and Benecol™ vegetable oil spreads. The development of the Lipton product, Take Control™, has yielded substantial research into the safety of phytosterols, particularly β-sitosterol, campesterol, and stigmasterol as discussed by Baker (1999), Hepburn (1999), and Wallkens-Berendsen (1999). Similarly, the development of the McNeil product, Benecol™, has yielded substantial research into the safety of phytostanols,

particularly sitostanol, campestanol, and stigmastanol as discussed by Slesinski (1999), Turnbull (1999a, b, c), and Whittaker (1999).

The information reviewed in determining the GRAS status of these two products included extensive clinical and non-clinical data. Clinical data included a history of safe intake of these phytosterols as components of everyday foodstuffs. Safe exposure to β-sitosterol has further been demonstrated at levels of up to 30 grams per day for extended periods of use over the course of the marketing of the pharmaceutical agent Cytellin™. Results of direct clinical and nutritional investigations with products containing phytosterols in over 2000 subjects have been evaluated. Non-clinical studies included those designed to assess acute, short term and sub-chronic safety, genetic toxicity, estrogenic activity, developmental and reproductive safety, ADME (absorption, distribution, metabolism, and excretion), and nutrient effects.

Expert and FDA reviews have resulted in findings of a reasonable certainty of no harmful effects and that phytosterols may be regarded as GRAS when used in the Benecol™ and Take Control™ vegetable oil spreads. The safety of the Cargill phytosterol ester product is assured by its substantial compositional equivalence, equivalent level of intake, and use in previously established product matrices to the Lipton GRAS product Take Control™. This has been determined by scientific review of a panel of qualified experts.

3.0 CARGILL PHYTOSTEROL ESTER CHEMISTRY AND COMPOSITION

3.1 Chemical Identity of Major Component Phytosterols

Plant sterols, or phytosterols, were originally thought to be cholesterol isomers. With the advent of modern analytical techniques such as chromatography and mass spectrometry, the empirical formula and chemical structure of many phytosterols, including all the major components of the Cargill phytosterol ester product, have been elucidated. The formulae and structure of many phytosterols found in minute quantities throughout the plant kingdom are still poorly characterized. In large part, phytosterols, are similar to cholesterol at the basic level of skeletal structure. The difference exists on

the aliphatic side chain of the compounds, where phytosterols contain methyl, ethyl, di-methyl, di-ethyl or other groups next to the C_{24} position. (Pollak & Kritchevsky, 1981).

A diagram of the chemical structure of the three major phytosterol constituents found in the Cargill phytosterol ester product and Take Control™ (β-sitosterol, campesterol, and stigmasterol) is provided below in Figure 1. Additionally, a diagram of the cholesterol molecule has been included to better illustrate their similar chemical structures. The precise chemical specifications of the three predominant phytosterol components found in the Cargill phytosterol ester product and constituting approximately 85% of sterol content by weight are provided below, in Table 5.

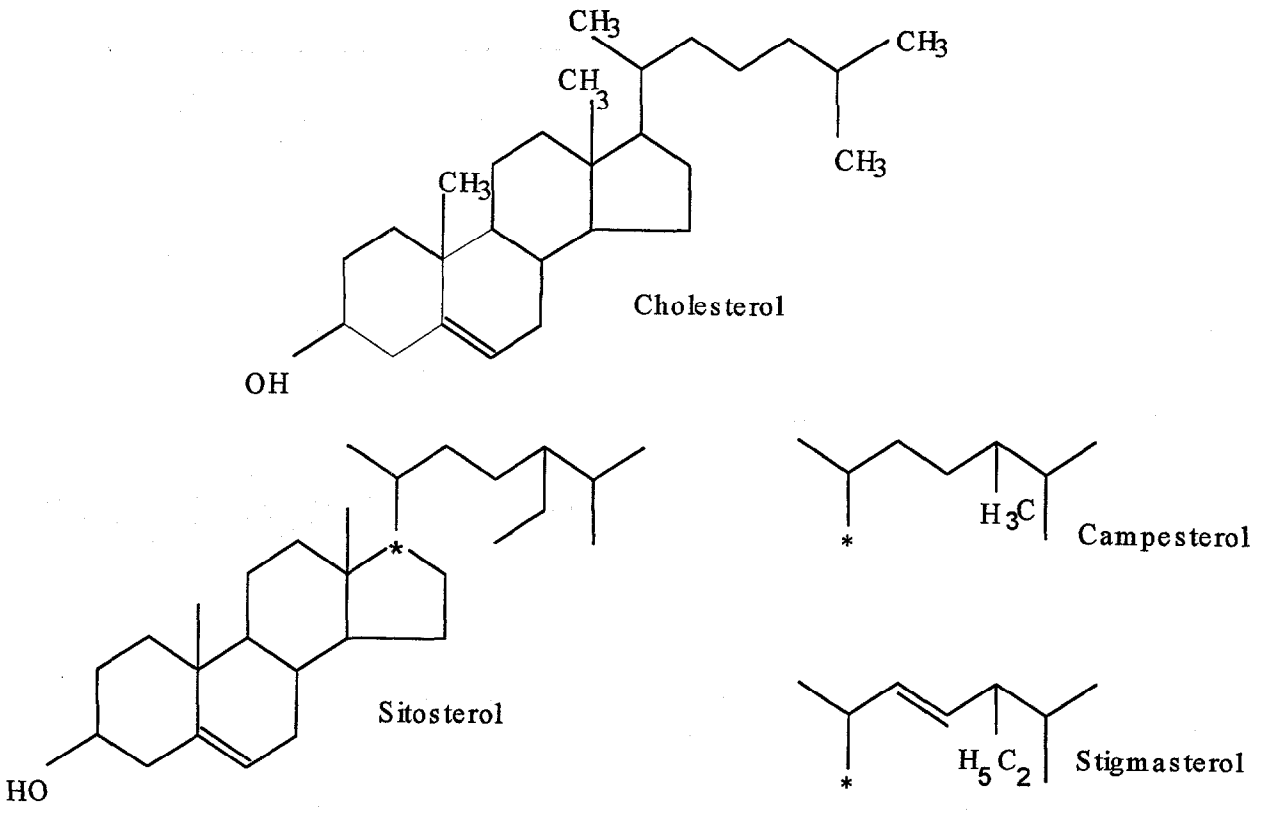

Figure 1: Major Cargill Phytosterol Ester Constituents and Cholesterol

Table 5: Chemical Data for Constituent Cargill Phytosterols

	Chemical Name	Chemical Formula	Molecular Weight	Melting Point	Approximate Percentage
β-Sitosterol	(3β)-Stigmast-5-en-3ol	$C_{29}H_{50}O$	414.72	140° C	40-45%
Campesterol	(3β,24R)-Ergost-5-en-3-ol	$C_{28}H_{48}O$	400.69	157° C	20-25%
Stigmasterol	(3β,22E)-Stigmasta-5,22-dien-3-ol	$C_{29}H_{48}O$	412.7	170° C	20-25%

3.2 Minor Component Phytosterols

The saturation of phytosterols at the 5-α position results in the formation of phytostanols, or simply stanols. These are found in nature at very low concentrations and are present in the Cargill phytosterol ester product at levels between 2-3% of the total phytosterols present. The other minor sterol components comprise approximately 3-5% of the total and are commonly found at low levels in soy and other vegetable oil derived phytosterols. While attribution of their individual identities was not performed, comparative gas chromatograph analysis demonstrated them to be identical in composition to the minor phytosterol components found in Take Control.

3.3 Comparative Analysis

As has been discussed in Section 2.2, the principle reasons the Cargill phytosterol ester product is considered GRAS by a consensus of qualified scientific experts is its substantial chemical and compositional equivalence to the phytosterols found in the Lipton Take Control™ product. In order to quantify this determination, Cargill completed a comparative compositional analysis employing gas chromatography. As illustrated below in Table 6a, a comparison of the major constituent phytosterol concentration by weight using identified peaks in the Cargill product and in Take Control™ reveals them to be virtually identical. Furthermore, Table 6b contains a comparison of the unattributed sterol peaks found in several batches of the Cargill product and Take Control™. Once again, a comparison between the two products indicated that they are virtually identical in composition.

Table 6a: Comparison of Identified Sterol Peaks in Cargill Phytosterols and Take Control™

Peak ID	Take Control™ Sample 1 (wt %)	Take Control™ Sample 2 (wt %)	Cargill Phytosterol Samples Lot# 3023 (wt %)	Lot# 3026 (wt %)	Lot# 3027 (wt %)	Lot# 3028 (wt %)	Lot# 3029 (wt %)
Total Tocopherols	0.38	0.34	0.5	0.44	0.27	0.41	0.48
Cholesterol	0.38	0.34	0.39	0.37	0.38	0.37	0.31
Brassicasterol	2.50	4.08	0.49	0.44	0.50	0.48	0.35
Campesterol	23.44	25.04	23.57	23.47	22.60	24.35	23.91
Campestanol	0.63	0.57	0.88	0.82	0.94	0.89	0.90
Stigmasterol	17.88	15.97	21.36	21.63	26.58	22.63	24.00
delta 7 Campesterol	0.52	0.40	0.66	0.72	0.75	0.71	0.69
β-Sitosterol	45.68	45.99	42.68	42.86	41.54	42.70	41.56
Stigmastanol	1.13	1.06	1.54	1.53	1.50	1.47	1.51
delta 5-Avenasterol	0.86	0.80	0.92	1.04	0.65	0.80	0.67
delta 7 Stigmasterol	0.88	0.75	0.90	0.82	0.62	0.62	0.62
Stigmast-7-en-3β-ol	0.88	0.75	0.90	0.82	0.62	0.62	0.62
delta 7 Avenasterol	0.20	0.14	0.29	0.26	0.19	0.30	0.28
Total %	95.36	96.22	95.09	95.23	97.13	96.36	95.90

Table 6b: Comparison of Unattributed Sterol Peaks in Cargill Phytosterols and Take Control™

Peak ID	Take Control™ Sample 1 (wt %)	Take Control™ Sample 2 (wt %)	Cargill Phytosterol Samples Lot# 3023 (wt %)	Lot# 3026 (wt %)	Lot# 3027 (wt %)	Lot# 3028 (wt %)	Lot# 3029 (wt %)
Unknown 8	0.66	0.52	0.85	0.89	0.12	0.64	0.73
Unknown 9	0.71	0.51	0.31	0.16	0.52	0.43	0.67
Sterol Unknown 2	0.47	0.50	0.58	0.61	0.37	0.55	0.47
Sterol Unknown 5	0.75	0.57	0.66	0.56	0.42	0.75	0.52
Sterol Unknown 6	0.27	0.28	0.31	0.33	0.29	0.00	0.24
Sterol Unknown 7	0.42	0.33	0.54	0.49	0.21	0.46	0.43
Sterol Unknown 8	0.64	0.57	0.54	0.56	0.52	0.44	0.43
Sterol Unknown 10	0.24	0.12	0.27	0.21	0.13	0.00	0.09
Total %	4.17	3.38	4.06	3.82	2.58	3.27	3.58
Grand Total %	**99.53**	**99.60**	**99.15**	**99.05**	**99.71**	**99.63**	**99.48**

Please note that this data is normalized to weight % of all constituents found in the organic extract of the saponification of these products (essentially sterols, stanols, and tocopherols).

3.4 Proposed Food-Grade Specifications

As a food ingredient, the Cargill phytosterol ester product can be incorporated directly into any number of food products by manufacturers seeking to provide their consumers with the potential benefits of elevated phytosterol consumption. However, Cargill will not market their phytosterol esters directly to individual consumers of products containing phytosterols, but rather to manufacturers of products which contain phytosterols. Cargill's phytosterol ester product serves as an alternative source of phytosterols to those manufacturers seeking to incorporate phytosterols into food products. The proposed food grade specifications for the Cargill phytosterol ester product are shown below:

Phytosterol content *	> 95%
β-Sitosterol	40% to 45%
Campesterol	20% to 25%
Stigmasterol	20% to 25%
Total major sterols	> 85%
Arsenic	< 0.1 mg/kg
Lead	< 0.1 mg/kg
Mercury	< 0.1 mg/kg
Cadmium	< 0.1 mg/kg

* The Cargill phytosterol ester product is approximately 60% by weight phytosterols and 40% by weight vegetable oil fatty acids to which they are esterified; percentages refer to non-fatty acid portion.

4.0 PRODUCTION OF STEROL ESTERS

4.1 Background

Cargill presently manufactures sterol esters at a contract manufacturing facility. The following sections provide an overview of the current manufacturing process.

4.2 General Process Description

Sterol esters are produced by reacting vegetable oil derived sterols (mostly soy) with fatty acid methyl esters (FAME), in the presence of sodium methoxide. The fatty acids are from soy, canola, sunflower, or other vegetable oils (as desired in the finished product). Methanol is produced as a by-product of the esterification and is removed during the process. The basic manufacturing process is described in Figure 2:

$$Sterols + FAME \xrightarrow{\text{Sodium Methoxide}} Steryl\ Ester + Methanol \uparrow$$

Figure 2: General Schematic of Sterol Ester Process

In the first step, sterol and FAME are mixed in a reactor and dried under vacuum at an elevated temperature to remove water. The esterification reaction is initiated and catalyzed by the addition of sodium methoxide. The reaction is carried out at >100°C under varying degrees of vacuum. Food grade anti-foam is added as needed to control problem foaming caused by evolution of the methanol. Under these conditions nearly all methanol is removed during the reaction. When the reaction is complete, the product is cooled to < 100°C and water is added to deactivate the catalyst, which is converted irreversibly to sodium hydroxide and methanol. The water contains most of the sodium hydroxide, methanol, and fatty acid soaps and is separated from the product stream by centrifugation.

After the reaction is complete and the catalyst removed, multiple adsorbents are used to remove trace components and color bodies. Trysil™ is used to remove any remaining traces of polar compounds (mainly sodium hydroxide and fatty acid soaps). Bleaching earth and activated carbon are added to remove color bodies and poly-aromatic hydrocarbons, respectively. Diatomatious earth is added solely to aid filtration.

In the final processing step, excess FAME and minor impurities, as well as flavor and odor components are removed by vacuum steam stripping. The product is then filtered to remove any trace foreign material and packaged into drums or totes under nitrogen. Food approved anti-oxidants are added to maintain product stability.

4.3 Raw Materials, By-products, and Processing Aids

The raw materials used in the manufacturing process (i.e., soybean sterols and fatty acid methyl esters or FAME) undergo monitoring at regular intervals to ensure they are free of pesticides, foreign matter (particulates), and heavy metals coming into the process. Any such contaminants that were present would be actively removed by filtration or are eliminated during the process. To date, the levels of these contaminants found in the final product have been below detectable limits. Fatty acid methyl esters are reacted during the process. Unreacted material is removed in the deodorization step.

A number of processing aids, each of which are approved for food contact, are

used during the manufacture of esterified sterols. Most are deactivated or removed during processing. Exceptions include antioxidants (which are included in the label as such), and dimethyl polysiloxane (anti-foam). Dimethyl polysiloxane remains in the product at levels below 10 ppm, consistent with its status as an accepted food additive by the FDA.

4.4 Good Manufacturing Practices (GMP)

The product is produced at a contract manufacturing facility under conditions that are in accordance with Food Good Manufacturing Practices according to 21 CFR Part 110.

5.0 INTENDED USE IN FOOD

Phytosterols are naturally occurring plant sterols which resemble cholesterol in molecular structure but have a much lower absorption rate. Because they compete with cholesterol for absorption in the gastrointestinal tract, phytosterols are gaining popularity in consumer products intended to maintain blood cholesterol levels within acceptable healthy limits, as indicated by the recently marketed vegetable oil base spreads Benecol™ and Take Control™. The human body cannot manufacture phytosterols; they are derived strictly from dietary intake of plant sterol sources such as vegetable oils.

Cargill Incorporated has developed a vegetable oil based phytosterol ester product which has been determined by a qualified panel of experts to be substantially equivalent in composition to those in Lipton's Take Control™. The phytosterols present in the Cargill phytosterol ester product, like those in Take Control™, are derived predominantly from soy, although other vegetable oils may be employed in their manufacture. They have been well described by Cargill Incorporated and are also currently used in other consumer products. As with Take Control™ and Benecol™, the Cargill phytosterol ester product is intended to help maintain healthy blood cholesterol levels. This product contains esterified vegetable oil phytosterols in a formulation intended for use by manufacturers of products currently marketed with enhanced

phytosterol ester content, i.e. primarily in vegetable oil based spreads, salad dressings, bars or yogurt for which use of phytosterols has been affirmed as GRAS. Cargill will require that a manufacturer wishing to employ Cargill phytosterol esters in a new product application assume the responsibility for establishing the GRAS or other regulatory status for its proposed product.

6.0 ANTICIPATED USE AND INTAKE

Cargill will market their phytosterol ester product to manufacturers who employ, or plan to employ, soy based phytosterol esters in their consumer products. The Cargill phytosterol ester product is intended as an alternative source of phytosterol esters that other food manufacturers may choose for the production of food products in categories already marketed with enhanced phytosterol content, i.e., vegetable oil spread, salad dressings, bars and yogurt.

Therefore, no significant change in the intake of phytosterols is anticipated at the individual consumer level. The major constituent phytosterols of the Cargill phytosterol ester product; β-sitosterol, campesterol, and stigmasterol, are already established as GRAS for use in other products such as in Lipton's vegetable oil based Take Control™. Minor phytostanol constituents of the Cargill phytosterol ester product, sitostanol and campestanol; are also established as GRAS in McNeil's Benecol™ product. The Cargill phytosterol ester product represents an additional ingredient source choice for manufacturers seeking to incorporate phytosterol esters into products, such as a vegetable oil spread, in order to foster maintenance of a healthy cholesterol level.

7.0 STRUCTURE-ACTIVITY RELATIONSHIPS

Analysis of structure activity relationships is suggested as a useful approach to correlating the molecular structure of a chemical with its biological activity (Food and Drug Administration, 1982). Due to their low toxic potential, the Cargill vegetable oil phytosterols belong in Structure Category A. However, since the FDA has classified mixtures as belonging to Structure Category B, the Cargill phytosterols mixture must be

placed into Category B. Structure category notwithstanding, phytosterols are structurally similar to cholesterol and the intermediate products of lipid metabolism in humans. It is therefore unlikely that any Cargill vegetable oil phytosterol-containing product would cause any adverse health effect in humans, based on this type of structure activity relationship.

8.0 PHYTOSTEROL ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION (ADME) PROFILE

Phytosterols are plant sterols and are structurally related to cholesterol, however, differences exist in the configuration of their side chains. A wide variety of phytosterol structures exist in nature but the most abundant phytosterols are β-sitosterol, campesterol, and stigmasterol. Less abundant are the saturated phytosterols, or stanols. Sitostanol and campestanol are respectively formed by hydrogenation at the 5-α position of β-sitosterol and campesterol. Sitostanol is also produced by the complete hydrogenation of stigmasterol. Stanols are found in the Cargill phytosterol ester product at only very low levels and are not significant to the ADME profile of the sterols found therein.

Mattson (1977) determined that phytosterol esters are hydrolyzed to free sterols and fatty acids. Mattson (1982) also stated that the hydrolysis of phytosterol esters, possibly via pancreatic carboxylic ester lipase, was essential to their role in the inhibition of cholesterol absorption as only the free sterol can co-precipitate with cholesterol with the consequent decrease in cholesterol absorption. Therefore, literature employing free and esterified phytosterols is relevant to determining the ADME profile of vegetable oil phytosterol esters following ingestion.

8.1 Absorption

Natural variation in the phytosterol occurrence in different plants consumed by humans affect their level of intake by those ingesting plant derived foods. Sources of phytosterols usually come from corn, bean, nuts, and plant oils, which are common components of diet. Cerquira's (1979) research into vegetarian diets confirms that they

contain higher amounts of phytosterols compared to the conventional western diet and that saturated phytosterols are present only in trace amounts in the western diet.

8.1.1 Absorption in Animals

Phytosterols are poorly absorbed from the intestinal tract. Sylven (1969) determined the absorption of tritium labeled β-sitosterol fed to rats. Feeding of 1.5, 50, or 100 μ-moles of β-sitosterol resulted in a transfer to the lymph of 3-6% within the first 24 hours. This was largely independent of the dose fed to the animals. In contrast, 30% of the dose of labeled cholesterol was transferred to the lymph in the first 24 hour period. Most of the studies indicate an absorption rate of approximately 5%. Further investigation revealed that that β-sitosterol had no effect upon lymphatic transport of cholesterol and that the same percentage of each sterol was always absorbed. No mutual interference between each other's absorption was in evidence and it was concluded that the mechanisms for specificity within the intestinal mucosa cells in sterol absorption must lie in the early transport of sterols.

In a study by Ikeda (1978), the rate of dietary absorption of [^{14}C]β-sitosterol was compared to that of [^{14}C]β-sitostanol by examining excretion levels in rats. In that study, 97% of the sitostanol was recovered whereas only 88% of the β-sitosterol was recovered from the feces. Ikeda (1988; 1988) followed this work with a pair of studies in which he determined that two major sites of discrimination existed between absorbable and non-absorbable sterols. Differential uptake occurs in the brush border cell membranes with the uptake of β-sitosterol one eleventh that of cholesterol. There is also a differential sterol affinity dependent on the composition of bile salt micelles. The data suggested that plant sterols displace cholesterol from taurocholate bile salt micelles. Other minor sites of discrimination may exist intracellularly since cholesterol and β-sitosterol are processed differently with respect to esterification and chylomicron incorporation.

8.1.2 Basal Phytosterols Level in Human Plasma and Absorption

Those phytosterols which may be detected in the human body at any particular time were absorbed from dietary sources and that phytosterols are not synthesized endogenously in the body. Salen (1970) determined that in diets devoid of plant sterol content, human plasma and feces became free of β-sitosterol or other phytosterols. Salen also determined the basal level of β-sitosterol in human plasma to range between 0.3 and 1.02 mg/dl in individuals consuming a typical western diet.

Values of β-sitosterol concentration in the blood of two patients were determined by Gould (1969) to be 0.47 and 0.76 mg/dl. This compares favorably with a determination made by Von Bergmann (1998) along with a comparison of basal phytosterol levels between normo- and hypercholesterolemic subjects. Normocholesterolemics were found to have an average basal β-sitosterol concentration of 0.3 mg/dl and 0.42 mg/dl for campesterol. Hypercholesterolemic patients maintained elevated β-sitosterol levels in the average range of 0.55 mg/dl and 0.78 mg/dl campesterol. A comparison between the two groups revealed that the ratio of phytosterol to cholesterol remained similar. In the normal population, the ratio was 2.33 campesterol/cholesterol and 2.08 in hypercholesterolemics. Similarly, the β-sitosterol /cholesterol ratio was 1.7 and 1.54 between normo and hypercholesterolemic groups, respectively.

In humans, the absorption of dietary cholesterol is approximately 40% whereas the dietary absorption of phytosterols is in the range of 5% or less than dietary intake levels, as indicated by Salen (1989) and Miettinen (1990). A review by Pegel (1997) states that campesterol is absorbed at approximately 20% in the human body. Heinemann (1993) conducted a study in 10 healthy subjects using an intestinal perfusion technique wherein absorption of phytosterols was compared over a 50cm segment of the upper jejunum. The respective absorption percentages of β-sitosterol, campesterol, stigmasterol, and campestanol, were 4.2%, 9.6%, 4.8%, and 12.5%. Sitostanol was also examined and was not absorbed to any significant extent. Thus, the absorption of phytosterols is somewhat selective along the intestinal tract.

8.2 Distribution

8.2.1 Distribution in Animals

Bhattacharyya (1979) fed rabbits a low cholesterol diet containing 2% plant sterols from an unidentified source over a 10-week period. The mean plasma levels of β-sitosterol and campesterol were 0.76 and 8.9 mg/100 ml respectively. The blood levels of β-sitosterol and campesterol plateaued after about 5 weeks. The initial blood levels of phytosterols were below 0.01mg/100 ml. In an analysis of distribution, campesterol and β-sitosterol were found to have accumulated in the abdominal organs, liver, intestine, kidney, adrenals and ovary. Campesterol was also found in numerous peripheral tissues. No esterified campesterol or β-sitosterol were found in any of the tissues examined.

Subbiah (1973) reported β-sitosterol was initially taken up by the liver and to a lesser extent by other tissues following injection of [4-^{14}C]cholesterol and β-[22,23-^{3}H]sitosterol. On the basis of tissue weight, adrenals displayed the highest uptake. In liver microsomes of the rat, Leikin (1989) determined that the amount of phytosterols was only 5% that of cholesterol.

Phytosterols are very similar to cholesterol in structure (see Figure 1). Cholesterol interacts with cell membrane phospholipids and affects membrane lipid composition. Similarly, high intakes of dietary phytosterols, when incorporated into rat liver microsomes, have been reported to decrease liver cell membrane fluidity (Leikin, 1989). No changes in phospholipid or total sterol content of the liver cell membranes following elevated phytosterol consumption were noted.

8.2.2 Distribution in Humans

Phytosterols (1 to 42 μmol/L) are found in human serum under normal conditions with dietary intakes of 160-360 mg/day. However, work by investigators such as Connor (1968), Cerquira (1979) and Salen (1970) has shown that plasma levels may increase up to two-fold through dietary supplementation.

The effect on plasma content of orally administered phytosterols is dependent on the nature and composition of the sterols administered. Elevated dietary β-sitosterol

content depresses plasma levels of campesterol and elevates plasma β-sitosterol. Weststrate (1998) observed that Lipton soy derived phytosterol esters which have elevated β-sitosterol and campesterol content, raised the concentration of both phytosterols in the plasma.

Becker (1992, 1993) further confirmed the elevation of plasma levels through phytosterol consumption in hypercholesterolemic children employing phytosterol preparations high in β-sitosterol compared to a low cholesterol diet. In each assay, β-sitosterol was observed at elevated levels compared to the control diets.

Tilvis (1986) determined that as compared to cholesterol, serum free and esterified phytosterols tended to be accumulated in HDL where the phytosterol / cholesterol ratios were almost 40% higher than in LDL and VLDL. The serum phytosterol concentrations, the phytosterol / cholesterol ratios and the fractional absorption of cholesterol were higher in women than in men. However, serum levels of noncholesterol sterols are effectively determined by the absorption which is in turn proportionate to the fractional absorption of cholesterol.

Ling (1995) has stated there is little information available regarding the distribution of phytosterols in various tissues of the body. The liver is the major organ concerning cholesterol homeostasis, however, whole liver and liver microsomes of normal subjects contain only trace levels of phytosterols (Nguyen, 1990). High phytosterol levels were found in the liver microsomes of subjects with sitosterolemia, a condition which is discussed further in the human clinical safety section 10.5.

Bhattacharyya (1983) found that in providing patients with a sterol free diet, β-sitosterol gradually decreased and eventually disappeared all together from the skin as well as the feces. Upon reintroduction of phytosterols into the diet, β-sitosterol reappeared in the skin surface lipids within one week. The results demonstrated clearly that those plant sterols absorbed into the plasma from the diet were distributed into the skin surface lipids.

8.3 Phytosterol Metabolism

Phytosterols are not endogenously synthesized in the body and they are not

converted to cholesterol in humans or other mammals, nor is cholesterol converted to any of the phytosterols. This has been confirmed in a review by Pollak (1987) and through the direct experimentation of Salen (1970).

Subbiah (1973) determined that β-[22,23-^{3}H]sitosterol when injected intravenously into the rat is esterified with fatty acids by a reaction mediated by cholesterol-lecithin acyltransferase. Cholesterol is esterified in the same manner. A review by Ling (1995) stated that the esterification rate of β-sitosterol was slower than that of cholesterol. However, unlike cholesterol, which is metabolized into C_{24} bile acid, as indicated by Subbiah (1973), Skrede (1985), Boberg (1990a), and Lund (1991), β-sitosterol is converted into di- and trihydroxylated C_{21} bile acids in the rat. Boberg (1990b) tentatively identified two of the bile acids as having hydroxyl groups at the C_3 and C_{15} positions. Additionally, one bile acid has a keto group and the other a hydroxyl group, although their exact position on the molecule remains undefined.

8.4 Phytosterol Elimination

The elimination of absorbed phytosterols from the body takes place primarily through the biliary route. This was confirmed by Boberg (1988) in the determination that in humans, β-sitosterol is not metabolized into C_{24} bile acid but rather into a C_{21} Bile acid. This is in keeping with observations Boberg (1990) has made in other mammalian species. Lin (1984) further indicated the possibility of selectively greater excretion of other sterols relative to cholesterol. In an analysis of sterol excretion after prolonged consumption of stigmasterol in rats, Andriamiarina (1989) concluded that stigmasterol induces increased fecal neutral sterol levels in a similar fashion to β-sitosterol. Ling (1995) has stated that there is a correspondingly low pool size of endogenous phytosterols relative to cholesterol. This was due to poor absorption in the intestine and faster excretion via the bile. Once in the anaerobic lumen of the lower intestine, unabsorbed phytosterols may be acted upon by normal bacterial flora and converted to other naturally occurring metabolites.

Weststrate (1999) conducted an evaluation in 12 male and 12 female normolipidemic volunteers. Following run in period, they consumed 40 g of a vegetable

oil spread, Take Control™, containing 8.6 g of vegetable oil phytosterols (46% β-sitosterol, 26% campesterol, 20% stigmastanol by w/w) for 21-days in males and 28-days in females. Vegetable oil spread containing phytosterol esters increased fecal neutral sterol concentrations from 40 mg/g to 190 mg/g, dry weight, where fecal neutral sterol metabolites increased from 30 mg/g to 50 mg/g. Of the total fecal neutral sterol, β-sitosterol, campesterol and stigmasterol comprised 28%, 15%, and 12% respectively. This reflects the composition of the phytosterol ester enriched vegetable oil spread. Major sterol metabolites excreted were predominantly formed by oxidation at the 3-position or saturation at the 5,6-position in a β-configuration. Secondary bile acid was reduced from 7.6 mg/g to 6.0 mg/g in dry feces. Fecal concentrations of the mutagen 4-cholesten-3-one remained low and no sterol oxides could be detected. The study concluded that increased vegetable oil phytosterol ester consumption increased the levels of neutral sterols in the feces but did not result in increased bile acid or sterol metabolite formation.

Ayesh (1999) conducted an assay employing the same substance, at the same dose levels, in the same number of subjects. The test group showed an 18% reduction in total and a 23% reduction in LDL-cholesterol levels, respectively. Fecal lactic acid was also decreased as was serum progesterone in the females, none of whom took oral contraceptives. Within the test group, when compared to baseline measurements, fecal lactic acid concentration and the ratio of acetic acid to total short-chain fatty acids was reduced, as was lactobacilli content. No other significant treatment effects were noted in the test group. None of these findings were considered to be of biological significance. No clinical toxicological endpoints were noted. It was concluded that the daily consumption of the phytosterol esters found in Take Control™ did not affect the bacterial profile or the metabolic activities of the gut microflora. No biologically relevant effects on serum female sex hormone levels resulted from daily consumption of this product and it was well tolerated by both male and female volunteers.

9.0 PRECLINICAL TOXICOLOGY

The safety of this product, as determined by a panel of qualified experts, is based upon its chemical similarity to other phytosterol products for which preclinical safety and GRAS status has been investigated and documented. Therefore, no toxicology studies of the Cargill phytosterol ester product have been conducted.

9.1 Genotoxicity

The phytosterols contained within the Cargill phytosterol ester product do not exhibit any potential for genotoxicity. The lack of genotoxicity has been well documented in the two currently available GRAS dossiers for Benecol™ and Take Control™. Specifically, the phytosterols in the Lipton Take Control™ product, with their similar nature and composition to the Cargill phytosterol ester product demonstrate a lack of genotoxicty. In their dossier are unpublished reports of an Ames assay and a metaphase chromosome analysis of human lymphocytes cultured *in vitro*, both with and without the presence of S9 Aroclor-induced rat liver microsome fraction. In neither study was any evidence of genotoxicity observed.

One incidence of mutagenicity has been observed in the case of β-sitosterol. An investigation by Zaied (1996) reported that subcutaneous injection of β-sitosterol in rats at a low and high dose of 5 and 7 mg/kg, respectively, induced the development of micronucleated erythrocytes in their progeny, which the author attributes as possibly due to a phytoestrogen effect. Newborn rats were affected when either parent was treated individually but particularly, when both parents were injected with β-sitosterol. This study does not take into account the normal metabolic pathway following typical ingestion of phytosterols and has been included for completeness. The topic of potential estrogenic responses following phytosterol consumption is addressed in the reproductive toxicology subsection (9.3).

An assay conducted by Ansari (1982) further supports the lack of genotoxicity of the Cargill phytosterol ester product. In this assay, the mutagenicity of oxidized commercially available sterols was examined via the Ames test. Autoxidized β-sitosterol, stigmasterol, brassicasterol and lanosterol, as well as 38 individual

cholesterol derivatives were all found to be nonmutagenic with and without the presence of the S9 fraction.

Furthermore, an evaluation has been conducted of the genotoxic potential of vegetable oil derived stanol esters, some of which are found at very low concentrations in the Cargill phytosterol product. Turnbull *et al.* (1999b), in an Ames assay performed using *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 with and without the presence of S9 Aroclor-induced rat liver microsome fraction, concluded that the plant stanol esters were not toxic to the cells, nor were they mutagenic. This investigation also reported a chromosome aberration study conducted on Chinese hamster ovary, or CHO cells, and on mouse lymphoma L5178Y cells, also in the presence or absence of S9 Araclor-indiced rat liver microsome fraction. Similarly, no mutagenic potential was observed.

9.2 Subchronic and Chronic Non Clinical Phytosterol Toxicological Safety Assays

Cargill Inc. has not instituted any new toxicological evaluations of its phytosterol product. The safety of the Cargill phytosterol product has been determined by a panel of qualified experts based upon its substantial equivalence to phytosterols present in other GRAS products and for which ample evidence of safety is available in the published literature. This evidence includes toxicological evaluations conducted to assess the potential effects in animals fed elevated levels of β-sitosterol, the primary component of the Cargill phytosterol product. The results of these studies, as discussed below, establishes that elevated intake of β-sitosterol as well as other phytosterols may be reasonably considered safe.

9.2.1 Vegetable Oil Phytosterol Ester Safety Study

A 90-day oral toxicity assay was recently conducted by Hepburn (1999) which is particularly relevant due to the similarity of the material evaluated to Cargill's phytosterol ester product. Hepburn's analysis of the material employed in this toxicity assay revealed a mixture (w/w) with a compositional breakdown of 48.7% β-sitosterol, 25.8%

campesterol and 21.6% stigmasterol. Only 8.4% (w/w) of the phytosterol content existed as free sterols, the remainder were esterified. Conducted in 20 male and 20 female rats per dose group, soy-derived phytosterol esters were supplied through the diet at levels of 0, 0.16, 1.6, 3.2, and 8.1% (w/w). Clinical observations throughout the length of the study did not reveal any changes in behavior, body weight, or water consumption. A slightly elevated level of food consumption was noted, probably due to the lower energy content of the diets due to the phytosterol component. It was not considered to be of toxicological significance. A thorough post mortem examination did not reveal any changes in organ weight, gross macroscopic observation at necropsy, or any histopathological changes of toxicological significance.

9.2.2 Cytellin™ Toxicology Studies

Eli Lilly Research Laboratories marketed a mixture of phytosterols derived from pine tall oil in the United States between 1954 and 1982. It was sold as Cytellin™ in the United States and as Positol™ in Canada. Cytellin™ / Positol™ was available either as a powder or liquid suspension and marketed as an anti-hypercholesterolemic agent. Its reported composition was 80% β-sitosterol, 10% sitostanol, 7% campesterol, and 2% campestanol. The Cargill phytosterol ester is primarily composed of β-sitosterol (45%), campesterol (25%) and stigmasterol (25%). Although Cytellin™ was eventually withdrawn from the market due to business considerations, several toxicology studies had been conducted with the product. In 565 albino mice, a single 5 g/kg β-sitosterol triturated in sesame oil dose was administered by stomach tube. Little or no toxicity following administration of large single oral doses to mice was observed. This correlates with a study by Gupta (1980) in which he determined that the LD_{50} of mice intraperitoneally administered β-sitosterol was greater than 3 g/kg.

Thirty female rats were also administered 1% and 5% β-sitosterol in their diet for a period of 18 months. Both dose groups of rats survived, gained weight comparable to controls, and upon sacrifice showed no visceral or hematopoetic damage and no alterations in serum cholesterol, lipid phosphorus or blood protein fractions. Another study employed female mongrel dogs given capsules of Cytellin™. Four dogs received

500mg/kg/d and 4 dogs 1000mg/kg/d each for 18 months. Dogs that received daily doses of 500mg/kg/d or 1000 mg/kg for 18 months survived, gained weight and had no hematological or visceral damage. No changes in serum cholesterol, calcium, phosphorus, total lipids, lipid phosphorus, vitamin A and blood protein fractions were observed. Total lipid and total cholesterol values in the livers were unchanged.

9.2.3 Other Non-Clinical Assays Employing Phytosterols

Phytosterols have been studied extensively. Many instances in the scientific literature discuss their non-clinical safety indirectly while focusing upon other known or suspected properties. This section seeks to document the general safety of phytosterols by reviewing scientific publications which discuss the safety of phytosterols in general terms.

In a series of evaluations conducted by Shipley (1958), the safety of β-sitosterol was assessed. Groups of rats were fed a mixture of rat chow and 5% β-sitosterol derived from tall oil for periods of 8, 18, and 22 months. Parallel groups received 5% dose levels of β-sitosterol derived from cottonseed oil for 18 and 22 months, respectively. No detectable alterations in growth, blood cell counts, blood urea nitrogen, serum proteins or any gross or microscopic appearance of any organ or tissues were detected. Dogs were also fed β-sitosterol at levels of 0.5 and 1 g/kg for periods of 8 to 22 months. All the dogs maintained weight and had normal values for serum composition and formed blood elements. No gross or microscopic pathological changes attributable to β-sitosterol were observed in any dog. An evaluation was also conducted in New Zealand white rabbits which were fed a mixture of 4% soy β-sitosterol and 3% cottonseed oil, which is also high in phytosterol content. Rabbits were maintained on this diet for as long as 842 days and showed no gross or microscopic pathological abnormality. Furthermore, the total liver and aortic lipid concentration and aorta sterol concentrations were comparable to controls.

In an attempt to determine the effect of β-sitosterol on aorta lipid, cholesterol, and plaque regression Beher (1956) fed 40 female albino rabbits a high cholesterol test diet followed by a high β-sitosterol test diet for 4 months. Incorporating 2.5% β-sitosterol

into the feed of atherosclerotic rabbits slightly increased the regression of plasma and liver cholesterol but had no effect on aorta lipid, cholesterol or plaque regression. No significant toxicological effects were reported. Furthermore, in a companion study by Beher (1957), β-sitosterol was administered to rabbits at 2% of their diet for a period of 7-months. Its administration proved to be non-toxic, nor were any gross lesions observed in aortic or liver tissue.

Sugano (1977) conducted a comparative assay in rats wherein he evaluated the hypocholesterolemic activity of 93% β-sitosterol / 7% campesterol to 93% β-sitostanol / 7% campestanol . Some differences in the hypocholesterolemic activity between the two mixtures were observed. However, no demonstrable negative effect on growth or the weight of visceral tissues was observed in either test group.

In an assay by Laraki (1991) to study the effects of dietary phytosterols on plasma cholesterol, Wistar rats were fed diets containing a cholesterol overload (24 mg/day). To this, phytosterols (72.5% β-sitosterol, 20.5% campesterol, 7% stigmasterol) were added (24 or 96 mg/day). The phytosterol rich diet reduced the effect of cholesterol over-load and no undesirable toxicological effects were reported from elevated phytosterol intake.

Ambrosova (1999) fed a 21 mg/kg diet rich in phytosterols (65% β-sitosterol, 18% stigmastanol, 14% campesterol and 3% campestanol) to two groups of rats, one normal and the other PHHC (an experimental model of hypercholesterolemia) for 60 days. No clinical signs of toxicity were observed in the normal group and a positive effect on the biochemical lipid profile and on endothelial physiology was noted in the PHHC group. However, an increase in free unsaturated fatty acids was noted in both groups indicating a possible advancement in the process of atherogenesis. No causal relationship between phytosterol treatment and development of atherosclerosis could be established.

In a much more recent toxicological analysis, Malini (1990) administered β-sitosterol to rats through subcutaneous injection at dose levels of 2.5 mg/kg/day, 5.0 mg/kg/day and 10.0 mg/kg/day over 60 days. No evidence of gross microscopic lesions in the kidney or liver was observed. β-sitosterol intake was well tolerated and all

biochemical parameters were within normal range with the exception of reduced serum cholesterol.

Iyer (1998) conducted a study in neonatal piglets. Intravenous injection of soy phytosterols at 18 nM per kg per day for 14 days was carried out and the presence of phytosterols in plasma, bile and the liver was examined. Serum bile acid levels increased and a reduction in bile acid-stimulated bile flow were observed. Liver function tests remained normal, as was liver histology.

In an assay to determine if β-sitosterol had any correlation with the fact that a diet rich in vegetables is associated with a reduced risk of colon cancer, Raicht (1980) fed β-sitosterol at 0.2% of the diet to rats. The known carcinogen N-methyl-N-nitrosurea (MNU) was then administered intracolonically. It was demonstrated that over 28 weeks of β-sitosterol feeding, there was a significant decrease in the proportion of tumor-bearing animals. No adverse effects of long term β-sitosterol feeding were noted.

In an assay by Janezic (1992) the effect of dietary phytosterols on the proliferative status of the intestinal epithelium was investigated in inbred C57B1/6J mice. Their diet consisted of 0.%, 1.0% or 2.0% of a phytosterol blend consisting of 60% β-sitosterol, 30% campesterol and 5% stigmasterol administered over 2 weeks. Phytosterol intake significantly reduced the enhanced labeling index and the position of the highest labeled cell in a dose dependant manner. The mitotic index was also reduced but no dose dependant relationship was observed. The overall results indicate that phytosterols influence colonic epithelial cell morphometrics which are important preneoplastic events in colonic carcinogenisis and may therefore contribute to a reduction in the risk of cancer.

9.3 Reproductive Toxicity and Estrogenic Activity

9.3.1 Reproductive Toxicity

Studies were available to evaluate and confirm the reproductive safety and lack of estrogenic activity from feeding elevated levels of phytosterols to animals. In a reproductive study investigating the effects of soy phytosterol esters in the rat, Waalkens-Berendsen (1999) incorporated levels of 0, 1.6, 3.2 and 8.1% (w/w) phytosterol esters into the diet over the course of 2 successive generations. A wide range of reproductive and developmental parameters were then evaluated, including sexual maturation and estrous cycle length. No effect on the reproduction of parental F_0- and F_1-generation Wistar rats or the development of F_1 and F_2 pups were reported, nor were any histopathological effects on selected organs reported. The high dose of 8.1%, determined to be the equivalent of 2.5-9.1 g of phytosterol ester per kilogram per day, was considered to be the no observed adverse effect level following daily oral administration of phytosterol ester over two successive generations.

9.3.2 Estrogenic Activity

A number of reports have suggested that β-sitosterol may have estrogenic properties. Ghannudi (1978; 1979) conducted research in immature male and female rabbits, injecting them with β-sitosterol daily for 30-days. Estrogenic responses were noted in both assays. El Samannoudy (1980) also noted an estrogenic response when β-sitosterol was injected subcutaneously to immature female sheep over a period of 20 days. A pair of articles by MacLatchy (1995; 1997) indicated that exposure to elevated levels of β-sitosterol may be a contributing factor to reproductive dysfunctions observed in goldfish. Burck (1982) even determined that β-sitosteryl sulfate, when released in the uterine horn of female rabbits induced a significant reduction in the number of embryos. β-Sitosterol sulfate, but not β-sitosterol, has an acrosin inhibitory activity, which would reduce the sperm efficiency in ova fertilization. Implantation of silicone rods containing β-sitosterol sulfate into uterine horns of rabbits for 16 days, significantly reduced the

number of embryos present in those horns. This treatment did not effect the number of corpora lutea.

Malini (1991) investigated and noted effects on male rat reproductive tissue by injecting 0.5 mg/kg or 5 mg/kg β-sitosterol subcutaneously for 16, 32, and 48 days. An antifertility effect of β-sitosterol was pronounced only at the high dose but there was significant decrease in testicular weight and sperm concentrations after long-term treatment with the low dose of β-sitosterol. The weights of all accessory sex tissues except caput epididymis increased following low dose β-sitosterol treatment. High dose treatment reduced sperm concentrations as well as the weights of testis and accessory sex tissue in a time-dependant manner. Withdrawal of treatment for 30 days restored the weights of the accessory sex tissue but not the testis. Further study was indicated to elucidate the exact site and mechanism of action.

Malini (1993) also investigated and noted effects on female rat reproductive tissue by subcutaneously injecting 50, 250, or 500 μg/100g body weight of β-sitosterol to ovariectomized rats for 10 days. Uterine growth response to β-sitosterol was found to be dose-dependant and only the high dose produced an increase in uterine weight with massive edema comparable to that of an estradiol positive control. Test results therefore indicate a uterotrophic effect of β-sitosterol in ovariectomized rats and a potential synergism with estradiol.

The relevance of the fish model or the other models which employ subcutaneous or intravenous injection to the normal route of human phytosterol intake may be questionable. The kinetics of phytosterols administered in this manner differ from those of oral consumption. Phytosterols administered orally are poorly absorbed. Those which become absorbed may be incorporated into very low density lipoproteins (VLDL) or chylomicrons before entering the blood stream whereas subcutaneously or intravenously administered phytosterols would more likely be available as free sterols.

In order to more thoroughly evaluate the possible estrogenic effects of phytosterols, Baker (1999) carried out a combination of *in vitro* (2) and *in vivo* (1) assays employing a combination of 47.9% β-sitosterol, 28.8% campesterol and 23.3% stigmasterol. The phytosterols were sourced from a variety of vegetable oil distillates

such as soy or sunflower. The phytosterols employed were then esterified with fatty acids from sunflower oil.

In the first of two *in vitro* assays, competitive binding of the phytosterol esters was compared with [2,4,5,6-^{3}H]estradiol for the ability to bind to immature rat uterine estrogen receptors. As a positive control, β-estradiol was employed. This assesses the ability of the phytosterols to bind to estrogen receptors. The phytosterols were negative in the receptor binding assay and did not compete with [2,4,5,6-^{3}H]estradiol for binding to the estrogen receptors at concentrations of to 10^{-4} M. The β-estradiol positive control demonstrated an EC_{50} value of $1.3X10^{-10}$. This is the concentration required to reduce β-estradiol binding by 50% and is indicative of the low estrogenic response of phytosterols in this assay.

In the second *in vitro* assay, a recombinant yeast strain was used to directly assess the ability of the phytosterol mixture to stimulate the transcriptional activity of human estrogen receptor. In this case, the yeast responded positively to a known phyto-estrogen , coumestrol providing an EC_{50} value of $3.3x10^{-8}$ whereas the β-estradiol positive control yielded 3.2 as an EC_{50} value. By contrast, at $2x10^{-4}$ M, the highest dose tested, neither the phytosterol mixture nor β-sitosterol stimulated the transcriptional activation of human estrogen receptor in yeast.

The third *in vivo* immature rat uterotrophic assay was conducted in order to evaluate the effects of phytosterols on estrogen-responsive tissue. Over a three day period, 0, 5, 50, and 500 mg/kg/day were administered by oral gavage to groups of 10 animals. A weak positive control, the phyto-estrogen coumestrol, was administered to parallel groups at doses of 0, 5, 20, 40, and 80 mg/kg/day. Furthermore, a 0.4 mg/kg/day β-estradiol positive control was administered to one group of animals. The group mean uterus weight in all dose groups treated with the phytosterol ester mixture was unaffected whereas the coumestrol induced a statistically significant dose-response increase in uterine weight in the 20, 40, and 80 mg/kg/day groups. As expected the β-estradiol positive control produced a significant increase in uterine weight when compared to each test substance.

In a study conducted by Awad (1998), the metabolism of testosterone as influenced by dietary phytosterols was evaluated. Testosterone metabolism includes

reduction to more active metabolites or aromatization to estrogen. Elevated levels of estrogens and androgens are risk factors in the development of prostate cancer. After feeding male rats for 22 days a 2% mixture of 56% β-sitosterol, 28% campesterol, 10% stigmasterol and 6% dihydrobrassicasterol, the liver, testes, and prostate were evaluated. No phytosterol effect was noted in the testes but in the liver and prostate, 5-α -reductase activity was reduced by 41% and 33%, respectively. This resulted in a net reduction of 33% in serum testosterone and a 55% decrease in aromatase activity on the prostate. It was concluded that dietary phytosterols feeding may reduce the risk of prostate cancer by lowering the activities of the enzymes associated with testosterone metabolism.

In conclusion, the only evidence of toxicity to animals reported in the literature is for injected phytosterols. The blood levels of phytosterols achieved by this route of administration would be much higher than could be obtained by oral administration, where absorption is quite low.

9.4 Fully Saturated Phytosterol (Stanol) Safety

The safety of fully saturated phytostanol esters has also been extensively studied. Phytostanols are contained within the Cargill phytosterol ester product at very low levels, approaching 3% in aggregate. Therefore, the low phytostanol levels found in the Cargill product, in conjunction with the similarity in sterol - stanol molecular structure, necessitated a brief review to the panel of qualified experts on recent studies in stanol safety.

Turnbull (1999c) carried out a 13-week rat oral toxicity study with both vegetable oil and wood (tall oil) derived stanol esters in dietary concentrations of 0, 0.2, 1, and 5%. Both preparations were well tolerated as evidenced by the absence of clinical changes or major abnormalities in growth, food and water consumption, ophthalmoscopic observation, hematological and clinical chemistry values, renal concentrating ability, organ weight, gross necropsy finding and histopathological evaluation. In each case, a no observed adverse effect level was set at the mid dose level. Plasma levels of vitamins E, K and to a lesser extent, vitamin D, were decreased in males and females fed the high

dose diet. Hepatic levels of vitamin D and E also showed similar changes. Compared to control, male rats fed stanol esters showed slightly lower liver weights and more pronounced glycogen depletion. These hepatic changes were thought to reflect an altered nutritional condition and not a pathological condition.

Turnbull (1999a) also investigated the potential for estrogenic activity in plant stanols (soy or tall oil) and plant stanol esters using the *in vitro* E-screen test, which determines the ability of a compound to cause induction and proliferation of estrogen-responsive human breast MCF-7 adenocarcinoma cells. An *in vivo* assay to determine uterotrophic activity in immature female rats fed the phytostanol and phytostanol ester test substances was also conducted. Under the conditions of testing employed, neither the stanol ester or free stanols displayed any evidence of uterotrophic or estrogenic activity.

Slesinski (1999) carried out a developmental toxicity assay in rats employing a vegetable oil derived stanol fatty acid ester. The test article was administered at concentrations of 0, 1, 2.5 and 5% total stanols in the diet from days 0 to 21 of gestation. A statistically significant increase in food consumption was noted in the high dose group during days 7-21 of gestation. However, there were no statistically significant differences in uterine, placental, or fetal weights. The number of fetuses, implantation sites, corporea lutea and early / late resorptions were similar between test and control groups. Visceral and skeletal examination of the fetuses did not reveal any incidence of malformation, anomaly or variation which could be considered treatment related. Dietary stanol esters at the highest concentrations administered were concluded to have no adverse effect on reproduction or development.

In the fourth and final assay reviewed herein to establish the safety of phytostanols, Whittaker (1999) carried out a two generation reproductive toxicity study employing vegetable oil stanol esters at concentrations of 0, 1, 2.5 and 5% total stanols in the diet. No treatment related adverse effects were observed on the reproductive performance of both male and female rats in any of the dose groups. Increased food consumption was noted in both the F_0 and F_1 high dose males and females and was considered to be a result of the reduced caloric value of the test diet. In the F_1 or F_2 pups, no adverse developmental effects were noted in either the low or mid-dose

groups. Lower body weight and body weight change were observed in the F_1 and F_2 pups of the high-dose group during the later stages of lactation. This was also attributed to reduced caloric intake from the test diet as compared the control diet.

10.0 CLINICAL TOXICOLOGY

The safety of this product, as determined by a panel of qualified experts, is based upon its chemical composition, which is essentially identical to the phytosterol esters in the GRAS product Take Control™. The chemical composition of the Cargill phytosterol ester product has also been determined by the same panel of qualified experts to be substantially similar in nature to those in the GRAS product Benecol™. Furthermore, the safe clinical history of Cytellin™ use further supports the determination of GRAS status, by the qualified panel of experts, for phytosterol consumption. Therefore, no clinical studies of the Cargill phytosterol ester product have been conducted.

10.1 Literature Review of Clinical Studies Employing Lipton's Phytosterol Ester Product Take Control™

The safety of the constituent phytosterols of the Cargill phytosterol ester product has been determined by a panel of qualified experts due to its identical chemical and compositional makeup to the GRAS phytosterol esters in Lipton's Take Control™ product. The compositional comparability of the phytosterol esters in the two products has been established in Section 3 of this document. These recently published studies documenting the safety of the Lipton phytosterol ester product Take Control™ were provided for review by the panel of qualified experts.

In an assay by Weststrate (1998), the effect on plasma total, LDL-, and HDL-cholesterol concentrations was observed in test vegetable oil spreads containing either Lipton phytosterol esters or Benecol™ phytostanol esters. The exact phytosterol percentages were unspecified, however, β-sitosterol followed by campesterol and stigmasterol were the predominant phytosterols by weight. The test was carried out in 100 male and female subjects with plasma total cholesterol levels below 8 mmol/l at entry over the course of 4 periods of 3.5 weeks employing an incomplete Latin square

design. None of the vegetable oil spreads induced any adverse changes in blood chemistry, serum total bile acids or hematology. Compared to control, plasma total and LDL cholesterol concentrations were reduced in subjects consuming spreads containing phytosterol esters and phytostanol esters. No effect on HDL cholesterol concentration occurred. The LDL to HDL cholesterol ratio was reduced by 0.37 and 0.33 units, respectively. It was concluded that a vegetable oil spread containing phytosterol esters was as effective in lowering blood total and LDL-cholesterol as the Benecol™ spread containing primarily phytostanol esters.

Hendriks (1999) conducted an investigation to determine any dose response relationship between cholesterol lowering and three different intake levels of esterified phytosterols in a margarine matrix. The impact of esterified phytosterol intake upon plasma vitamin and nutrient levels was also investigated. Relative phytosterol ester levels were set at (w/w) 3.37%, 6.47%, and 13.06% which provided 0.83, 1.61, and 3.24 g per day of plant sterols. No statistically significant difference between dose levels could be determined in the cholesterol lowering effect. No side effects were reported as determined by measuring the liver enzymes ALP, ALT, AST and gamma-GT. Minor effects on vitamins and nutrient levels were observed and are discussed in greater detail in Section 10.4. No adverse events were reported. It was concluded that a dose level of 1.6 g/day of phytosterols would have a beneficial effect upon plasma cholesterol concentration without any serious impact upon carotenoid concentration in the plasma.

In a study previously discussed in the ADME section of this document (Section 8.4), Weststrate (1999) conducted an evaluation in 12 male and 12 female normolipidemic volunteers who consumed 40 g of the vegetable oil spread Take Control™. Phytosterols levels where comparable to the Cargill phytosterol ester product at 46% β-sitosterol, 26% campesterol, and 20% stigmasterol (w/w). Fecal concentrations of the mutagen 4-cholesten-3-one remained within the normal range indicating that vegetable oil phytosterols would not be expected to increase the risk of colon cancer; no sterol oxides were detected. The study concluded that increased vegetable oil phytosterol ester consumption increased the levels of neutral sterols in the feces but did not result in increased bile acid or sterol metabolite formation. No effects in clinical toxicological endpoints were noted.

Also discussed in the ADME discussion section (Section 8.4), Ayesh (1999) conducted an assay employing Take Control™, at the same dose levels, in the same number of subjects. The test group showed an 18% reduction in total and a 23% reduction in LDL-cholesterol levels, respectively. Fecal lactic acid was also decreased as was serum progesterone in the females, none of whom took oral contraceptives. Within the test group, when compared to baseline measurements, fecal lactic acid concentration and the ratio of acetic acid to total short-chain fatty acids was reduced, as was lactobacilli content. No clinical toxicological endpoints were noted. It was concluded that the daily consumption of the phytosterol esters found in Take Control™ did not affect the bacterial profile or the metabolic activities of the gut microflora. No biologically relevant effects on serum female sex hormone levels resulted from daily consumption of this product and it was well tolerated by both male and female volunteers.

10.2 Literature Review of Other Clinical Studies Employing Phytosterols

Humans are continually exposed to phytosterols in the diet. The average dietary phytosterol intake has been estimated to be about 250 mg per day, while vegetarians may consume twice that amount. There is ample scientific literature on the effects of human exposure to elevated phytosterol intake dating back as far as the 1950's. Pollak and Kritchevsky (1981) published an extensive review of the scientific data available at the time and estimated that clinical data on the cholesterol-lowering action of phytosterols in approximately 1800 subjects was available at the time of their review. They concluded that alimentary use of β-sitosterol could be employed as part of a sensible regimen to reduce elevated plasma cholesterol levels to a mean normal range. Among other more recently conducted reviews of phytosterol safety are those written by Ling (1995), Kritchevsky (1997), Jones (1997a), Jones (1997b), Plat (1998), Jenkins (1999), and Moghadasian (1999). Other peer reviewed scientific analyses also confirm the safety of phytosterols for human consumption.

In two previously mentioned studies conducted by Becker (1992, 1993) children with heterozygous familial hypercholesterolemia where treated over a course of 3-

months with β-sitosterol pastils at 6 g / day. In the 1992 study, the cholesterol lowering drug benzafibrate was then administered separately and concomitantly with β-sitosterol. Combined treatment reduced the necessary dosage of each agent and was as effective as elevated doses of benzafibrate, resulting in an average 40 and 50% lowering of total and LDL cholesterol values, respectively. The combined regimen was then maintained for 24-months as was the lipid lowering effect. Laboratory safety parameters and physical examination revealed no obvious side effects. In the 1993 evaluation, following the 3-month β-sitosterol feeding regimen, sitostanol was then administered for 7 months. In neither case were there any physical or clinical toxicological effects. However, sitostanol was determined to be more effective than β-sitosterol at reducing LDL cholesterol levels.

Vanhanen and Miettinen (1991) conducted an assay wherein 50 g per day of rapeseed oil spread was administered to 24 hypercholesterolemic patients for 6 weeks. Following this β-sitosterol or sitostanol was added to the rapeseed oil spread divided into 3 subgroups, a control, β-sitosterol and sitostanol. This continued for 9 weeks. While differences in the absorption between β-sitosterol and sitostanol where noted, as well as changes in the respective proportions of cholesterol and campesterol, no clinical or toxicological effects where noted.

Miettinen and Vanhanen (1994) also conducted a 9-week study in which 31 hypercholesterolemic men and women were fed rapeseed oil spread in which an equivalent 1 g per day free sterol of β-sitosterol, sitostanol, or sitostanol esters had been dissolved. Results in lowering LDL cholesterol were observed and compared across different apo lipoprotein E phenotypes. In each phytosterol regimen, dietary plant sterols were observed to decrease serum total and LDL cholesterol by a proportional decrease in cholesterol absorption. This was in turn associated with a compensatory increase in cholesterol synthesis. The effects where deemed most consistent in those individuals with the E4 allele and insignificant with the apo E3/3 phenotype. No change in body weight or serum lipid profile were observed.

Pelletier (1995) also evaluated the effect of soy derived phytosterol over the course of a 9-week feeding study in which a phytosterol blend was mixed with butter and ingested by 12 normolipidemic subjects at 740 mg/day. Similar to the other studies

evaluated, a significant lowering of the plasma total and LDL cholesterol was observed. No clinical or toxicological side effects were observed.

In studies conducted by Berges (1995) and Klippel (1997), the effect of β-sitosterol on benign prostatic hyperplasia (BHP) was evaluated. In the Berges study, 200 patients were administered 20 mg capsules of β- sitosterol three times per day for six months. In the Klippel study, 177 patients received 130 mg capsules of β-sitosterol twice per day for six months. In each case, while no clear mechanism of action could be determined, significant improvement in symptoms and urinary flow parameters showed the effectiveness of β-sitosterol in treating BPH. No adverse events were reported in either study.

10.3 Literature Review of Clinical Studies Employing Phytostanols

Although they are only a minor component found in the Cargill phytosterol ester product, it is worth noting that the safety of phytostanol esters has also been extensively evaluated, as evidenced by the GRAS status of Benecol™. Several clinical studies have been conducted using phytostanols, as reflected below in Table 7. This information was also taken into account by the panel of qualified experts in their determination of GRAS status for the Cargill phytosterol ester product.

Table 7: Clinical Evaluations Reporting the Safety of Phytostanol Consumption

Reference	Disease State [1]	Number & Sex	Age Range or Mean (yr)	Phytosterol	Plant Source	Dosage Form [2]	Dosage (g/day)	Duration	Clinical Endpoints	Adverse Events Reported
Heinemann *et al*, 1986	HC and FH	3 M 3 F	27-59	Sitostanol	No Data	Capsule	1.5	4 weeks	- Total cholesterol - Total phytosterol(s) - Triglycerides - HDL cholesterol - LDL cholesterol	None
Vanhanen *et al*, 1993	HC	47 M 20 F	25-60	Sitostanol Ester	No Data	Mayonnaise Suspension	3.4 (n=34)	6 weeks	- Total cholesterol - Total phytosterol(s) - Triglycerides - HDL cholesterol - LDL cholesterol - ApoE phenotype	None
Gylling *et al*, 1994	NIDDM with HC	11 M	57.8±1.9	Sitostanol Ester	Tall Oil	Margarine Suspension	3.0	6 weeks	- Total cholesterol - Total phytosterol(s) - Triglycerides - apolipoproteins A I, A II, & B	None
Vanhanen *et al*, 1994	HC	11 M 4 F	33-60 M 37-55 F	Sitostanol Ester	No Data	Mayonnaise Suspension	0 (n=8) 0.8 (n=7) followed by 2.0 (n=7)	15 weeks 9 weeks followed by 6 weeks	- Total cholesterol - Total phytosterol(s) - - HDL cholesterol - LDL cholesterol	None
Denke, 1995	HC	33 M	31-70	Sitostanol	Tall Oil	Capsules	3.0	90 days	- Total cholesterol - Triglycerides - HDL cholesterol - LDL cholesterol - VLDL cholesterol	None
Miettinen *et al*, 1995	HC	64 M 89 F	25-64	Sitostanol Ester	Tall Oil	Margarine Suspension	0 (n=51) 1.8 (n=51) 2.6 (n=51)	1 year	- Body weight - Total cholesterol - Triglycerides - HDL cholesterol - LDL cholesterol - Total phytosterol(s)	None

[1] M – male; F – female; HC – hypercholesterolemic; NIDDM - non-insulin-dependent diabetes mellitus; FH - familial hypercholesterolemia.

[2] Unless otherwise stated, pretreatment and (or) control diets were also consumed during treatment phases.

10.4 Clinical Vitamin and Nutrient Uptake Levels

Weststrate (1998) and Hendriks (1999) evaluated sterol ester consumption and reported a decrease in the absorption of carotenoids such as β-carotene and lycopene. Gylling (1996) evaluated phytostanol esters and reported a similar decrease in β-carotene levels. As reported by Shipley (1958) β-sitosterol had no effect on vitamin D absorption in rats and dogs. No other significant impairment of the availability of fat-soluble vitamins has been noted for plant sterol or sterol esters.

Hendriks (1999) observed a decrease in plasma concentrations of α- and β-carotene of 12%, 11%, and 19% in three dose levels of phytosterol esters in a vegetable oil spread matrix. Lycopene plasma concentration was decreased by 11-15% although no difference was observed between dose levels. Lycopene concentration per total lipid in plasma was not affected by the consumption of phytosterol esters. Similarly, α-tocopherol plasma concentrations were decreased in the mid- and high dose groups by 6% and 8%, respectively, after consumption of esterified phytosterols. The concentration of α-tocopherol per total lipid in plasma was unaffected. No effect upon plasma concentrations of the fat-soluble vitamins K_1 and D was observed.

Hallikainen (2000) observed no significant changes in serum retinol, α- and β-carotene nor their ratios to serum total cholesterol concentrations over different dose levels of a phytostanol ester administered to a hypercholesterolemic test population. Serum lycopene concentrations differed significantly between men and women. There were no significant differences in the serum lycopene concentrations in the men throughout the study. However, in women, serum lycopene concentrations were significantly lower at the end of all experimental dose periods than at the end of the control period. In addition, serum α-tocopherol concentration was significantly lower at the end of the high dose 3.2 g period than at the end of the 0.8 g low dose period. Serum γ-tocopherol concentration was significantly lower only at the end of the 3.2 g and the 2.4 g dose periods than at the end of the control period. The changes in the α + γ-tocopherol concentration were parallel to the changes in the serum α-tocopherol concentration during the trial. However, after relating the serum α-, γ-, and α + γ-

tocopherol to the serum total cholesterol concentration, there were no significant differences among the different dose periods.

In the comparative analysis of the cholesterol lowering effects between esterified phytosterols in Take Control™ and esterified phytostanols in Benecol™, Weststrate (1998) observed a lowering of plasma lycopene, α-, and β-carotene levels in all individuals consuming the fortified margarines. Lipid standardization of the carotenoid concentrations did reduce the impact of both Take Control™ and Benecol™ and lipid standardization in the Take Control™ phytosterol ester data showed the decrease in lycopene not to be statistically significant.

It is possible that esterified phytosterols may interfere with fat soluble vitamin and nutrient uptake in the intestine. This is primarily true for carotinoids. All of these reports were for esters dissolved in margarine. The effect may depend on incomplete hydrolysis of the fatty acid esters with the ester remaining in the intestine and acting as a reservoir to hold fat-soluble vitamins. The impact on human safety is not clear. These changes are small enough that they could be offset by supplementation of the diet with these vitamins or nutrients.

10.5 Phytosterolemia in Humans

Phytosterolemia, also known as sitosterolemia, is an extremely rare disease involving lipid storage. Berger (1998), in a review of the literature, could only identify 34 cases of individuals with this condition. It is characterized by elevated plant sterol levels and 5α-saturated stanols in plasma and tissue. It is associated with xanthomatosis and premature atherosclerosis. In individuals with this condition, phytosterols account for an average of 13% of the total plasma sterols, compared to about 0.4% in non-phytosterolemic subjects. Salen (1985) measured the plasma sterol composition of 14 subjects with this condition. Elevated plasma β-sitosterol and campesterol levels were observed at 35 ± 16 mg/dl and 16 ± 7 mg/dl, respectively. Normal to moderately high cholesterol levels of 258 ± 96 mg/dl was also noted.

Phytosterolemia is inherited as a recessive trait. Salen (1992) and Cobb (1997) determined that heterozygous individuals are biochemically and clinically normal.

However, the plasma phytosterol levels in some heterozygous individuals may be slightly elevated.

The pathophysiological basis for disease involves abnormalities in both the absorption and excretion of phytosterols. In phytosterolemics, the β-sitosterol absorption rate is very high. In the intestine, the sterol uptake mechanism does not distinguish between phytosterols and cholesterol. Therefore relatively equal proportions of are absorbed. Shellfish sterols are also absorbed in excess.

In addition, Bhattacharyya (1974) observed that less cholesterol is secreted into the bile. Bhattacharyya (1991) confirmed this by determining the β-sitosterol ratio in the bile was very low compared to the plasma, indicating a slow turn-over of β-sitosterol, low excretion of β-sitosterol into the duodenal bile and feces, and low cholesterol synthesis. Shefer (1994) determined that the large quantities of β-sitosterol and cholestanol in the liver of phytosterolemic subjects competitively inhibited cholesterol 7α-hydroxylase mediated bile acid synthesis.

In most cases, a rigorous low-sterol diet to curtail input, combined with bile acid resins to enhance sterol excretion, are the current treatment recommendation for this disorder. Cobb (1997) has identified at least one case wherein a very low β-sitosterol diet to curtail sterol input was of minimal therapeutic benefit. Regardless, in those individuals afflicted with sitosterolemia, consumption of any product containing phytosterol esters would be contraindicated.

11.0 DETERMINATION OF THE GRAS STATUS OF VEGETABLE OIL DERIVED CARGILL PHYTOSTEROL ESTERS

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by Cargill incorporated, to determine the generally recognized as safe (GRAS) status of a natural product derived from vegetable oil. The expert panel statement follows:

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