GREEN

Safety Assessment of Phytosterols as Used in Cosmetics

Status:	Draft Report for Panel Review
Release Date:	August 16, 2013
Panel Meeting Date:	September 9-10, 2013

The 2013 Cosmetic Ingredient Review Expert Panel members are: Chairman, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; Ronald A Hill, Ph.D. James G. Marks, Jr., M.D.; Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is Lillian J. Gill, D.P.A. This report was prepared by Lillian C. Becker, Scientific Analyst/Writer.

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MEMORANDUM

To: CIR Expert Panel and Liaisons

From: Lillian C. Becker, MS
Scientific Analyst and Writer

Date August 16, 2013

Subject: Draft Report of the Safety Assessment of Phytosterols as Used in Cosmetics

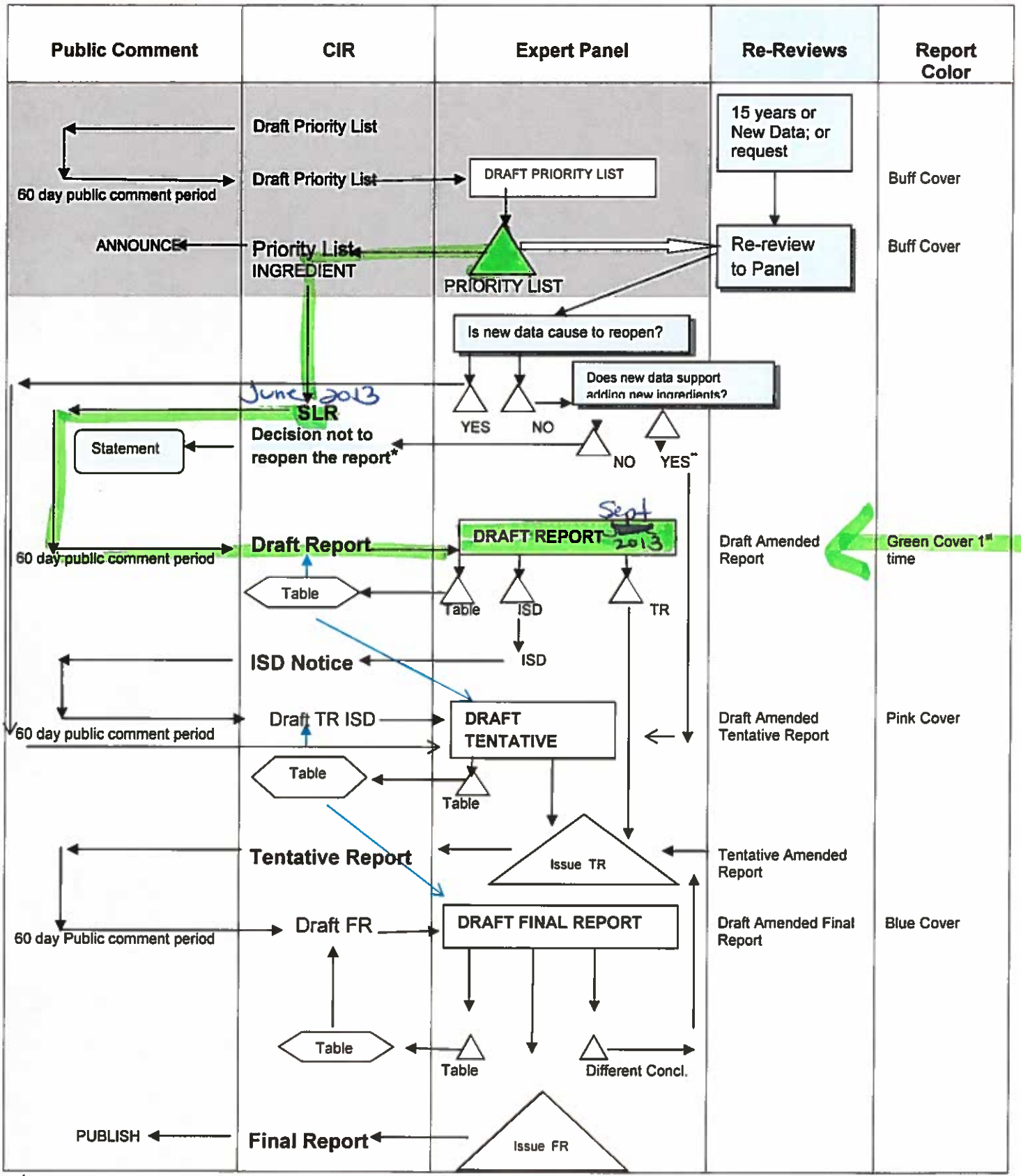
Attached, please find the Draft Report of Phytosterols as used in Cosmetics. The Scientific Literature Review was posted for public comment in May of 2013. Comments from the Personal Care Products Council have been addressed. No other comments were submitted.

Since phytosterols are ubiquitous in plants and are a normal part of the diet, this report concentrates on the potential for reproductive toxicity, genotoxicity, carcinogenicity, irritation, and sensitization. Oral toxicity was not addressed because exposure to phytosterols through food far exceeds any exposure through cosmetic use. Relevant information from the PEG soy sterols 2004 safety assessment is summarized and the report is included in this package.

The Panel is to review the report and decide if there is sufficient data to come to a safety conclusion. If not, then the Panel is to issue an insufficient data conclusion and indicate the additional data that are needed. If the data are sufficient, the Panel is to develop language for the Discussion and issue a Tentative Report.

Phytosterols

SAFETY ASSESSMENT FLOW CHART



*The CIR Staff notifies of the public of the decision not to re-open the report and prepares a draft statement for review by the Panel. After Panel review, the statement is issued to the Public.

**If Draft Amended Report (DAR) is available, the Panel may choose to review; if not, CIR staff prepares DAR for Panel Review.

△ Expert Panel Decision

History of Phytosterols

May, 2013 – SLR was posted for public comment.

September, 2013 – Panel examines Draft Report.

Phytosterol Data Profile for September, 2013. Writer - Lillian Becker																		
	ADME			Acute toxicity			Repeated dose toxicity			Irritation			Sensitization					
	Dermal Penetration	Log K_{ow}	Use	Oral	Dermal	Inhale	Oral	Dermal	Inhale	Ocular Irritation	Dermal Irr. Animal	Dermal Irr Human	Sensitization Animal	Sensitization Human	Repro/Devel toxicity	Genotoxicity	Carcinogenicity	Phototoxicity
Brassica campestris (rapeseed) sterols			X															
Canola Sterols																		
C10-40 isoalkyl acid phytosterol esters																		
Dihydrophyto-steryl octyldecanoate																		
Diosgenin																		
Euterpe oleracea sterols			X															
Glycine soja (soybean) sterols			X							X	X			X				
Persea gratissima (avocado) sterols			X															
Phytosterols			X		X					X	X		X	X	X	X		
Phytosteryl butyrate																		
Phytosteryl canolate			X															
Phytosteryl caprylate/caprate																		
Phytosteryl hydroxystearate																		
Phytosteryl isostearate			X															
Phytosteryl linoleate																		
Phytosteryl linoleate/ linolenate																		
Phytosteryl macadamiate			X															
Phytosteryl nonanoate																		
Phytosteryl oleate			X															
Phytosteryl rice branate			X															
Phytosteryl ricinoleate																		
Phytosteryl sunflowerseedate																		
Punica granatum sterols			X							X	X	X		X		X		
Beta-sitosterol			X															
Beta-sitosteryl acetate																		
Soy sterol acetate			X															
Tall oil sterol			X															
OTHER phytosterols										X	X					X		

Search Strategy for Phytosterols

SciFinder – Searched “phytosterols” for 35 possible hits. 15 were useful.

Internet search – Phytosterols. Located European scientific opinions.

Safety Assessment of Phytosterols as Used in Cosmetics

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INTRODUCTION

This report reviews the available scientific information relevant to the safety of a group of 27 phytosterols and steryl alkanoates as used in cosmetics. The functions of these ingredients include: skin-conditioning agents, hair conditioning agents, viscosity increasing agents, skin protectants, antioxidants, and fragrances (Table 1).[1] The ingredients in this report are:

- brassica campestris (rapeseed) sterols
- canola sterols
- C10-40 isoalkyl acid phytosterol esters
- dihydrophytosteryl octyldecanoate
- diosgenin
- euterpe oleracea sterols
- glycine soja (soybean) sterols
- persea gratissima (avocado) sterols
- phytosterols
- phytosteryl butyrate
- phytosteryl canolate
- phytosteryl caprylate/caprate
- phytosteryl hydroxystearate
- phytosteryl isostearate
- phytosteryl linoleate
- phytosteryl linoleate/linolenate
- phytosteryl macadamiate
- phytosteryl nonanoate
- phytosteryl oleate
- phytosteryl rice branate
- phytosteryl ricinoleate
- phytosteryl sunflowerseedate
- punica granatum sterols
- beta-sitosterol
- beta-sitosteryl acetate
- soy sterol acetate
- tall oil sterol

Plant sterols, or phytosterols, occur naturally as free alcohols and as fatty acid esters (i.e., naturally occurring steryl alkanoates). The ingredients in this report are sterol alcohols or esters (in some cases mixtures of both) extracted from plants, some of which have been saponified to the free alcohols and then esterified with plant-derived fatty acids. These resultant ester-derivatized phytosterols (i.e., steryl alkanoates) share a great deal of structural overlap with the naturally occurring phytosterol esters. Indeed, most of these derived esters are likely to be exact synthetic copies of the components of the naturally occurring phytosterol esters. Accordingly, since there is expected to be a great deal of component overlap between the ingredients in this group, and reviewing them separately would involve a large amount of redundancy, it is more efficient to review them as an ingredient family and employ read-across. The similar chemical structures/components, physicochemical properties, and functions and concentrations in cosmetics enable grouping these ingredients and reading across the available toxicological data to support the safety assessment of the entire group. Table 2 lists these component chemicals and notes whether they are cosmetic ingredients, have been reviewed by the Cosmetic Ingredient Review (CIR) Expert Panel (Panel), and, if so, the Panel's conclusions. All of the reviewed component cosmetic ingredients were found to be safe as used. Butyric acid, caprylic acid/capric acid, and linoleic acid/linolenic acid have not been reviewed. Octyldecanoic acid is not a cosmetic ingredient.

In 2000, the Panel found the data on PEG-5, -10, -16, -25, -30, and -40 soy sterols to be insufficient to support the safety of these ingrdients.[2] In 2004, the Panel found these PEG soy sterols to be safe as used in an amended safety assessment that included data on phytosterols and phytosterol esters.[3] The Panel's approach in these safety assessments was to review the safety of PEGs and phytosterols/soy sterols, as well as the conjugated polyethers, and assessed the safety of the PEG phytosterols from those data. Because the data on the phytosterols/soy sterols are relevant for this safety assessment, summaries of the data from these two safety asessments are provided below in the appropriate sections below.

In 2004, the Panel concluded that dioscorea villosa (wild yam) root extract (with a diosgenin, an ingredient in this report, content of 3.5%) was safe as used.[4] The maximum reported concentration of use was 15%.

Many of the phytosterols in this study are from edible plant sources. Exposure to these phytosterols from consuming foods results in much greater systemic doses than could result from the use of cosmetic products. It was noted in the PEG soy sterol reports that phytosterols and phytosterol esters are not significantly absorbed after oral exposure, and thus, did not result in systemic exposure.[2,3] Therefore, acute and repeated dose oral toxicity potential of these phytosterols as cosmetic ingredients will not be addressed again in depth in this report. The focus of this report is on other end points: reproductive toxicity, genotoxicity, carcinogenicity, irritation, and sensitization. Pertinent data from the PEG soy sterol safety assessment are summarized below in the appropriate sections.

CHEMISTRY

Definition, Structure, and Composition

The definitions and functions of the ingredients in this report are presented in Table 1.

The phytosterol ingredient group is comprised of the plant-derived, free sterols and their esters, the steryl alkanoates. β-Sitosterol is an example of a discreet, free phytosterol ingredient.

To generate a steryl alkanoate with an ester at the 3-position of the sterol, the hydroxyl group at the 3-position of the cyclopentenophenanthrene backbone is esterified, with an alkyl acid or acid chloride (Figure 1). β-Sitosteryl Acetate is an

example of a steryl alkanoate (Figure 2).

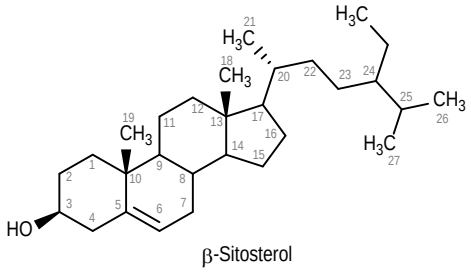


Figure 1. β-Sitosterol

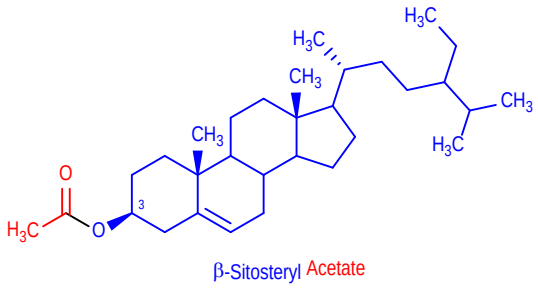


Figure 2. β-Sitosteryl Acetate

Phytosterols occur in plants in the free alcohol, steryl alkanoate, or glycoside forms (e.g., diosgenin). The free phytosterols are characteristic components of the non-saponifiable fractions of plant oils.[5] The steryl alkanoate and glycoside forms, however, are broken down to the free phytosterol form (and respective acid or sugar) under saponification conditions. The majority of the ingredients in this report are mixtures of either sterols or steryl alkanoates, with component concentrations that vary with growth and extraction conditions.

Soybean oil that had been alkali-refined typically contained 0.446 mg/100 mg oil total sterols and 0.287 mg/100 mg oil free sterol. The ratio of esterified to free sterol was 0.55.[6]

Refined plant sterols are reported to contain ~ 88% total sterol content. Of that percentage, 56% is γ-sitosterol, 28% is campesterol, and 4% is stigmasterol. Other compounds isolated from the phytosterols are 4% - 6% sterol hydrocarbons and cholesterol, and 4% - 6% triterpene alcohols, keto-steroids, and other steroid-like substances.[7]

PEGS SOY STEROL REPORT

The chemical characterization of a plant sterol material is provided in Table 3. The distribution of phytosterols in common vegetable oils are provided in Table 4.

In an analysis of another source of phytosterols (source not provided), it was reported that the principal phytosterols were present as follows: β-sitosterol, 47.9%; campesterol, 28.8%; and stigmasterol, 23.3%. No impurities were found. In an analysis of phytosterol esters, it was reported that the principal phytosterols were present as fatty acid esters: β-sitosterol, 47.3%; campesterol, 28.1 %; and stigmasterol, 24.5%. The distribution of the fatty acid chain lengths was consistent with fatty acids derived from sunflower oil.[8,9]

Physical and Chemical Properties

Physical and chemical properties of representative phytosterols in the form of vegetable oil sterols and tall oil sterols/stanols are provided in Table 5.

Phytosterols and their fatty acid esters are thermally stable and only degrade at high temperatures (>100°C) in the presence of oxygen.[10]

Method of Manufacture

Free phytosterol alcohols and phytosterol alkanoates are characteristic components of plant oils; saponification of these oils is the primary means of producting free phytosterol alcohols for commercial use.[5]

Soy sterol is isolated from soybean oil distillates in a saponification process in which the phytosterol alcohols are separated from the fatty acids by extraction with a fat solvent.[2] The phytosterols in the resulting extract are separated from

the tocopherols in the mother liquor, and then purified and/or separated into constituent sterols.

Tall oil sterol, an example of a phytosterol mixture, is obtained from tall oil soap in a multi-step process.[5] The production process involves fractional distillation of the tall oil soap to remove volatile compounds. The resulting residue (tall oil pitch) containing esterified sterols (i.e., steryl alkanoates) is treated with alkali (saponified) to release the free sterol alcohols. After neutralization, the material is subjected to a two-stage distillation process. The distillate is then dissolved in methanol/methylethylketone solvent and the sterols crystallizing from this solution are obtained by filtration, washed with solvent and dried. This procedure results in a lower stanol and a higher sterol content of the phytosterol mixture. Conifers that have naturally lower stanol content are now used as the primary source of the tall oil soap. Stanols (obtained by catalytical hydrogenation of the phytosterol mixture) are added before the crystallization step in order to maintain the original stanol/sterol ratio. The phytosterol composition of the tall oils produced from the two processes is provided in Table 6.

Steryl alkanoates are produced from free sterols by classical esterification methods, via free acids or acid chlorides. Sterol alkanoates may be derived from neutralized, refined, bleached and deodorized (N/RBD) soybean distillates.[11,12] Crude soybean oil is degummed, neutralized, bleached and deodorized to yield N/RBD soybean oil and distillates. The deodorized distillate undergoes further processing (crystallization and/or distillation), resulting in a sterol mixture. This sterol mixture is then crystallized and esterified with fatty acids (from food grade vegetable oils such as rapeseed or sunflower oil), washed, bleached and deodorized to give the final plant steryl alkanoates.

A manufacturer reported that for the manufacture of glycine soja (soybean) sterols, and punica granatum sterols, the raw materials are tested for acceptable qualifications (not specified) before they are cold pressed for oil.[13,14] The oil is then tested for quality (not specified) before the oil is fractionated to isolate the sterols. Pomegranate sterols are heat sterilized at 100°C before fractionation.[15]

Impurities

In assessing the data on soybean oil sterols, the Scientific Panel on Dietetic Products, Nutrition and Allergies noted that there are limited analytical data of sufficient sensitivity and reliability regarding the possible residual allergen (protein) content of phytosterols.[11] The limited analytical data regarding the protein (allergen) content of N/RBD soybean oil-derived plant stanol esters were insufficient to predict the likelihood of adverse reactions in soybean allergic individuals. This Panel concluded that since the starting material is refined soybean oil and there is an adequate subsequent production process, it is not very likely that this product will cause a severe allergic reaction in the majority of soybean allergic individuals.

When selected phytosterol samples (a phytosterol blend and a phytosterol blend spiked with reference protein) were analyzed for residual soybean protein using ELISA (enzyme-linked immunosorbent assay), soy protein was not detectable at or above the 10-20 µg/g detection limit.[16]

Tall oil sterols/stanols was reported to contain < 0.1 mg/kg lead.[10] Vegetable oil sterols, in general, are reported to have < 2.0 mg/kg impurities (mercury, < 0.1%; lead, < 0.1%; cadmium, < 0.1%; and arsenic, < 0.1%). Both contain < 2 ppb PAHs and < 1.5 ng-TEQ/kg dioxins and dioxin-like PCBs. No pesticides were detected.

In an analysis of euterpe oleracea sterols, glycine soja (soybean) sterols, and punica granatum sterols, none of these ingredients contained detectable levels of multiple allergens, including: amyl cinnamal, benzyl alcohol, citronellol, coumarin, linalool, and farnesol (Table 7). Another analysis did not detect several pesticides, including: DDT (detection level 1.00 mg/kg), methidathion (0.20 mg/kg), and pyrethrins (3.00 mg/kg).[17-19]

PEGS SOY STEROL REPORT

Analyses of various lots of soy sterols for pesticide residues were negative for a number of pesticides, including PCB, DDE, DDT, malathion, and β-hexachloride.[20]

PRECURSORS

The final protein content of N/RBD soybean oils (the source of soy phytosterols) depends on the quality and efficiency of purification steps.[11] The protein content of N/RBD oils may be reduced to low levels within the 0.02-0.44 µg/kg range.[21]

When two samples of edible soy oil (crude virgin and deodorized) were analyzed for proteins (by heat-extracted with PBS and BCA assay), 1.89 µg/mL and 0.32 µg/mL of proteins were present, respectively. [22]

USE

Cosmetic

Data on ingredient usage are provided to the Food and Drug Administration (FDA) Voluntary Cosmetic Registration Program (VCRP; Table 8).[23] A survey was conducted by the Personal Care Products Council (Council) of the maximum use concentrations for ingredients in this group.[24]

Data were available from both the VCRP and the Council for the following ingredients:

- Brassica campestris (rapeseed) sterols was reported to be used in 50 leave-on products up to 7% (the highest amount in lipstick) and 7 rinse-off products up to 0.13%.
- Glycine soja (soybean) sterols was reported to be used in 194 leave-on products (mostly skin care and makeup

products) up to 1% (the highest concentration in eye lotion, cuticle softeners, and other skin preparations) 45 rinse-off products up to 4.1% (the highest concentration in skin cleansing products) and one bath product. It is used in tonics, dressings and other hair grooming aids, including an aerosol and a pump at 0.000001%.

- Phytosterols was reported to be used in 177 leave-on products up to 5% including lipsticks (up to 5%) deodorants (up to 0.06%), and eye makeup (up to 0.006%). It is also used in 215 rinse-off products up to 0.5% including hair products (up to 0.5%), bath soaps and detergents (up to 0.005%), and indoor tanning preparations (up to 0.0001%). It is reported to be used in face powders up to 0.05%.
- Phytosteryl isostearate was reported to be used in 15 leave-on products up to 3% and one rinse-off product up to 0.5%. It is used in lipsticks up to 3% and in eye makeup up to 0.5%.
- Phytosteryl [phytosterol] macadamiate was reported to be used in 181 leave-on products up to 8%. It is reported to be used in two rinse-off products up to 1%. It is used in 100 lipsticks up to 7% and in moisturizing products up to 8%.
- Phytosteryl oleate was reported to be used in 20 leave-on products up to 3%. It was reported to be used in 6 paste masks/mud packs (no concentrations of use reported).
- Phytosteryl rice branate was reported to be used in an eye makeup and a moisturizing product. The Council reported that it was used in eye lotions up to 1%, foundations up to 0.5%, and face and neck products up to 0.5%.
- Punica granatum sterols was reported to be used in 29 rinse-off products up to 5% (including 15 lipsticks). It was also reported to be used in two rinse-off products (no concentration of use reported).
- Beta-sitosteryl was reported to be used in 46 leave-on products up to 0.06% and in two rinse-off products (no concentration of use reported).
- Tall oil sterol was reported to be used in 7 leave-on products up to 0.0046%. It is also reported to be used in skin cleansing products up to 0.0006%.

Data were only available on the frequency of use (VCRP) for the following ingredients:

- Euterpe oleracea sterols was reported to be used in one lipstick and one foundation.
- Soy sterol acetate was reported to be used in one moisturizing product.

Data were only available on use concentration (Council) for the following ingredients:

- Persea gratissima (avocado) sterols was reported to be used in eye lotion up to 1%, lipstick up to 0.65%, and face and neck products up to 0.1%.
- Phytosteryl canolate was reported to be used in eye shadow up to 0.06%.

There were no use or concentration of use data reported for:

- Canola sterols
- C10-40 isoalkyl acid phytosterol esters
- Dihydrophytosteryl octyldecanoate
- Diosgenin
- Phytosteryl butyrate
- Phytosteryl caprylate/caprate
- Phytosteryl hydroxystearate
- Phytosteryl linoleate
- Phytosteryl linoleate/linolenate
- Phytosteryl nonanoate
- Phytosteryl ricinoleate
- Phytosteryl sunflowerseedate
- Punica granatum sterols
- Beta-sitosteryl acetate

As noted above, glycine soja (soybean) sterols was reported to be used in propellant and pump spray tonics, dressings and other hair grooming aids up to 0.000001% and phytosterols are reported to be used in face powders up to 0.05%. In practice, 95% to 99% of the droplets/particles released from cosmetic sprays have aerodynamic equivalent diameters >10 µm; with propellant sprays yielding a greater fraction of droplets/particles below 10 µm compared with pump sprays. Therefore, most droplets/particles incidentally inhaled from cosmetic sprays would be deposited in the nasopharyngeal and bronchial regions and would not be respirable (i.e., they would not enter the lungs) to any appreciable amount.[25-30]

Non-Cosmetic

Phytosterols (stigmasterol-rich plant sterols: stigmasterol, >85%; brassicasterol, 1.7%; β-sitosterol, 3%; campesterol, 1.7%) are used in ready-to-freeze alcoholic beverages as a stabilizer.[31]

Phytosterols and phytostanols are commonly used in food products for their properties that reduce absorption of cholesterol in the gut and lower cholesterol in food products.[10] The optimal daily dose for this purpose is 2 - 3 g. For example, in Europe, phytosterol esters are added to margarines and low fat spreads (3.4 g/30 g), yogurts (1.25 g/125 mL), yogurt drinks (3.4 g/100mL), and milk (5 g/L).

The Scientific Committee on Food (SCF) and European Food Safety Authority (EFSA) concluded that phytosterols, phytostanols and their esters are approved for use in various foods (i.e., yellow fat spreads, soya drinks, salad dressings, rye

bread) within the EU at levels resulting in intake of up to 3 g/day.[11,12,16,32-43]

TOXICOKINETICS

Absorption, Distribution, Metabolism, and Excretion

No published dermal or inhalation ADME studies were discovered and no unpublished data were submitted.

Oral

The Western diet consists of ~160-360 mg/d phytosterols consisting of ~80% β-sitosterol. The diet also includes some campesterol and stigmasterol with small amounts of brassicasterol and trace amounts of δ-5-saturated plant stanols.[44]

Less than 5% of dietary phytosterols, phytostanols, and their esters are absorbed in the gastrointestinal tracts of rats and humans.[31] Following absorption, phytosterols/phytostanols are transported in the serum via HDLs in rats and LDLs in humans to various organs and tissues, mostly to the liver. In the liver, phytosterols may be converted to bile acids. Absorbed phytosterols/phytostanols are predominantly excreted as such or as bile acids by the biliary route into the feces. The metabolic fate of phytosterols, phytostanols, and their esters is similar between rats and humans. The individual plant sterols are metabolized in a similar manner to each other. The phytosterols that are not absorbed in the gastrointestinal tract enter the colon intact and are rapidly excreted in the feces.[44-47]

In an oral study (n = 10 healthy men) the intestinal absorption of phytosterols were: campesterol, 9.6%; stigmasterol, 4.8%; and sitosterol, 4.2%.[48] The authors noted that these results were consistent with the results of animal studies showing that increasing the side chain length of cholesterol reduced the absorbability of the sterol with the exception of campersterol. The 5α-campersterol-saturated had greater absorbability than campersterol. Absorption was measured by an intestinal perfusion technique over a 50-cm segment of the upper jejunum.

In male subjects, the biliary secretion rate of β-sitosterol was faster (1.23 mg/h) than that of campesterol (0.76 mg/h).[49]

Plant sterols, including stigmasterol and stanols (34 g/kg in feed), were able to cross the blood-brain barrier in a 90-day feeding study of Watanabe heritable hyperlipidemic rabbits.[50]

Cytotoxicity

β-sitosterol (200 µg/mL in ethanol) and β-sitosterol/campesterol (50%/40%; 200 µg/mL in ethanol) were cytotoxic to mouse macrophages (strain C57BL/6).[51] Cytotoxicity was demonstrated through cell viability, lipid uptake, lactate dehydrogenase (LDH) leakage, cellular protein content, and a 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay.

Phytosterols (0.01 – 40 mM; derived from pomegranate) were not cytotoxic in a Neutral Red Cytotoxicity assay.[52]

PEG SOY STEROL REPORT

β-Sitosterol (l00 µg/ml; 5% in DMSO and saline) was cytotoxic to seven cancer cell lines.

ANIMAL TOXICOLOGY

Many of the phytosterols in this report are from edible sources and exposure to these phytosterols from food would result in a much larger systemic dose than that resulting from use in cosmetic products. A summary of toxicity data on phytosterols, including oral data, from the PEG soy sterol report is presented below for information purposes. However, this report does not address their oral toxicity potential but is focused on the potential for reproductive toxicity, genotoxicity, carcinogenicity, irritation and sensitization. A summary of toxicity data on phytosterols, including oral data, from the PEG soy sterol report is presented below for background information on toxicity.

Dermal - Non-Human

The dermal LD_{50} of two mixtures of phytosterol esters was reported to be > 2000 mg/kg.[53] A wood-derived mixture (a stanol composition of ~94% β-sitostanol and ~6% campestanol in corn oil; WDPSE) and a vegetable oil–derived mixture of phytostanol esters (~68% β-sitostanol and ~32% campestanol in corn oil; VODPSE) were administered dermally to rats (n = 5/sex) for 24 h according to the Organization for Economic Co-operation and Development (OECD) Test Guideline 404. No deaths or clinical signs of toxicity were observed after application of WDPSE. One male rat in the VODPSE group died of unrelated causes during the 14-day observation period.

Peg Soy Sterol Report

Wistar rats administered a basal diet supplemented with cholesterol and maize phytosterols (72.5% β-sitosterol, 0.5% campesterol, and 7% stigmasterol) had decreased hepatic cholesterol concentrations.[54] Rats given the high dose of cholesterol and phytosterols had decreased malic enzyme and acetylCoA carboxylase activities, and had hypotriglyceridemia.

Wistar rats administered subcutaneous injections of 250 to 500 µg/100 g β-sitosterol for 60 days had no gross or microscopic lesions of the liver or kidneys.[55] Rats administered 1000 µg/100 g had mild fibroblastic proliferation around the

hepatic lobules and mild microscopic lesions of the kidney. Serum cholesterol was reduced in a dose-dependent manner, and serum protein was markedly reduced in rats of the high dose group.

In a 90-day oral toxicity study in female Wistar rats (n = 4), diets containing plant phytosterol esters up to 8.1 % were well tolerated.[56] Some small hematology and blood chemistry variations from the controls were observed. No treatment related effects were observed with organ weights and histological examination and there was no evidence of systemic toxicity. Absent any organ effects, the small hematology and blood chemistry variations were not considered of toxicological significance.

Thirteen dogs fed a basic diet supplemented with 0.5 to 1.0 g/kg/day of β-sitosterol had no gross or microscopic changes after 8 to 22 months of treatment. Weight gains and clinical parameters did not differ from controls.[20]

No adverse effects or gross or microscopic abnormalities were observed in six New Zealand white rabbits of both sexes that were given feed containing 3% cottonseed sterols and 4% soy sterols for 70-212 days.[20]

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

In a two-generation feeding study, the no observed adverse effect level (NOAEL) for phytosterol esters was 8.1% in the diet.[57,58] Wistar rats, F_0 generation, (n = 28/sex) were administered phytosterol esters (0, 1.6%, 3.2%, 8.1%) in feed for 10 weeks before mating, and continuing through gestation and weaning. The F_1 generation (n = 28/sex) were fed the same diet as their F_0 parents and mated after 10 weeks. The analysis of the phytosterols revealed the following breakdown: brassicasterol (2.9%), campesterol (26.7%), stigmasterol (17.7%), β-sitosterol (51.0%), cholesterol (0.2%), and unknowns (1.5%).

There were no maternal or teratogenic effects attributed to the test substance. There were no effects on fertility and reproductive parameters, including sexual maturity, estrous cycle length, precoital time, and the histopathology of reproductive tissues in either generation. There were no developmental or reproductive effects observed in either generation. Necropsies were unremarkable.

The NOEL (8.1%) is equivalent to 3.3-6.5 g phytosterol esters/kg/d during the 10-week pre-mating period (~ 2.1-4.1 g phytosterols/kg/d or 400-900 mg stigmasterol/kg/d) and 2.5-9.1 g phytosterol esters/kg/d during gestation (~1.4-5.7 g/kg/d or 300-1200 mg stigmasterol/kg/d). The authors concluded that 2.5-9.1 g phytosterol esters/kg/d and 1.54-5.62 g phytosterols/kg/d (~ 335-1219 mg stigmasterol/kg/d), dependent on the phase of the study, was the NOAEL of daily oral administration of phytosterol esters for two successive generations.[57,58]

There were no signs of reproductive toxicity to American minks (n = 70/sex) orally administered β-sitosterol (at 0, 5, 10 or 50 mg/kg/d) for 10 months.[59] In the second part of the study, after 7 months of exposure, males (n = 10–11) were mated with 4–5 females each. There were no differences in number of pregnant females, litter and kit numbers, postnatal mortality and development and there were no treatment-related changes. After 3 months of exposure, 15 males/group were killed and investigated for organ weights and hematological and clinical chemistry parameters. Males exhibiting low quality fur were selected for this part of the study. There were differences in body fat masses (omental, mesenteric, retroperitoneal, intra-abdominal fat) reported, but increases in fat masses were not dose dependent. There were increased blood hemoglobin and serum high-density lipoprotein cholesterol concentrations observed.

Subcutaneous injections of β-sitosterol (5 mg/kg/d) for 16 to 48 days reduced sperm concentrations and fertility, and decreased testis and accessory sex tissue weights in a time-dependent manner in male Wistar rats.[60] Rats administered 0.5 mg/kg/d had a decrease in sperm concentration of the caput epididymis after 48 days of treatment, but no reduction in fertility. The observed decreases in sperm concentration persisted after termination of treatment, and appeared to be due to a reduction in the rate of spermatogenesis.

ESTROGENIC EFFECTS

In Vitro

There were no signs of estrogenic activity of phytosterols and phytosterol esters in an in vitro competitive estrogen receptor binding assay (up to 1 x 10-4 mol/L) and a recombinant yeast assay (2×10^{-4} mol/L).[61] The phytosterols tested consisted of a mixture of β-sitosterol (47.9%), campesterol (28.8%), and stigmasterol (23.3%) and were sourced from a variety of edible vegetable oil distillates (e.g., sunflower, soya bean and rapeseed oils). The esters were phytosterols esterified with fatty acids from sunflower oil. The competitive estrogen receptor binding assay used a preparation of estrogen receptors isolated from 10-week-old Wistar rat uteri and measured the concentration-dependent substitution of [2,4,5,6-3H]estradiol at the estrogen receptor.

The hormonal activity of the pure substances β-sitosterol, stigmasterol, and their purified chlorine dioxide oxidation products showed estrogenic activity in an estrogen receptor binding assay.[62] In an androgen receptor binding assay, the phytosterols and their oxidation products showed a small but measurable activity.

Four phytostanol mixtures (0, 1, 10 or 100 μmol/L) showed no estrogenic activity in human mammary adenocarcinoma (MCF-7) cells.[53] Estrogenic activity was measured as the ability to induce proliferation of these cells. Proliferation was measured by staining the cells with the protein stain sulforhodamine B and measuring optical density. The MCF-7 cells were cultured for 6 days. 17β-Estradiol was used as a positive control. The percentage of β-sitostanol in the phytostanols, derived from vegetable oil, ranged from 58% - 67%, and campestanol ranged from 29% - 32%. The

phytosterol content was < 4%. Precipitation and slight cytotoxicity were observed at the highest test concentration with all mixtures. No cell proliferation was observable in cells treated with phytostanols. Under the conditions of this study, the phytostanol mixtures tested showed no estrogenic activity.

In Vivo

Neither WDPSE nor VODPSE administered in feed (0, 8.3%) for 4 days influenced the uterine weights of female Wistar rats (n = 10; 17-day-old) in a Teicco assay.[53] Diethylstilbestrol (5, 10 or 20 µg/kg) in the diet was used as positive control. Uterine weight was used as an indicator of estrogenic activity. No treatment-related effects on general condition, body weight or food consumption were observed.

β-Sitosterol, stigmasterol, and their oxidation products were inactive in a 28-day mosquito fish masculinization assay at concentrations up to 100 µg/L.[62]

There were no signs of estrogenic activity for phytosterols and phytosterol esters tested in an in vivo immature rat uterotrophic assay (n = 10; up to 500 mg/kg).[61] The phytosterols tested consisted of a mixture of β-sitosterol (47.9%), campesterol (28.8%), and stigmasterol (23.3%) and were sourced from a variety of edible vegetable oil distillates (e.g. sunflower, soya bean and rapeseed oils). The phytosterol esters were prepared by esterifying these phytosterols with fatty acids from sunflower oil.

Absolute and relative uterine weights were unaffected in an immature rat uterotropic assay of a mixture of phytosterols and phytostanols (0, 500, 1000, 2500 mg/kg) administered twice daily for 4 days when compared with the negative control.[53] The mixture of phytosterols and phytostanols used in this study was derived by solvent extraction (~40–55% β-sitosterol, 16–31% β-sitostanol, 11–15% campesterol and 2–11% campestanol; MPSS-SE) was assessed using female, Crl:CD (SD)IGS BR VAF/Plus, 19-day-old rats (n = 10). Ethinyl estradiol was used as a positive control. Body weight gains of animals in the 2000 and 5000 mg/kg groups were reduced.

PEG SOY STEROL REPORT

Dose-dependent uterotrophic effects of β-sitosterol in ovariectomized rats and its synergism with estradiol could be due to the phytosterol's intrinsic estrogenic properties, and that the effects of β-sitosterol could be inhibited by progesterone.[55]

β-Sitosterol was an effective estrogen-like agonist in exerting vaginal cornification and caused uterine weight gain in adult, ovariectomized Wistar rats.[63] Subcutaneous injections of the sterol caused dose-related increases in uterine glycogen concentration after 10 days.

Progesterone treatment partially suppressed the phytosterol-induced elevation of glycogen concentration when administered in combination with the median and high phytosterol doses. β-Sitosterol also stimulated glucose-6-phosphate dehydrogenase, phosphohexose isomerase, and total lactate dehydrogenase activities.

In a related study, uterine RNA, DNA, and protein concentrations were increased by treatment with β-sitosterol.[55]

Other studies of well-characterized phytosterols and phytosterol esters demonstrated no effect in an estrogen-binding study, a recombinant yeast assay for estrogen or estrogen-like activity, or a juvenile rat uterotrophic assay for estrogen or estrogen-like activity.[55,61,64]

Sulfates of β-sitosterol act as abortifacients in female rats and Dutch-belted rabbits via estrogenic effects. They also exhibit spermicidal effects. β-Sitosterol itself had anti-estrogenic, anti-progestational, gonadotrophic, anti-gonadotrophic, and anti-androgenic effects.[44,65,66]

GENOTOXICITY

In multiple in vitro (up to 5000 µg/plate) and in vivo (up to 2000 mg/kg) assays, phytosterols and phytosterol esters were negative for genotoxicity (Table 8). These tests included reverse mutation, chromosomal aberrations, gene mutation, clastogenicity, sister chromatid exchange (mice), micronucleus induction (rats and mice), and unscheduled DNA synthesis assays (rats).[67-70]

PEG SOY STEROL REPORT

Phytosterols and phytosterol esters were not genotoxic, with or without metabolic activation, in the Ames assay, a human lymphocyte chromosome damage assay, an unscheduled DNA synthesis assay, or a rat bone marrow micronucleus assay.[55,71-77]

CARCINOGENICITY

No new published carcinogenicity studies were discovered and no unpublished data were submitted.

PEG SOY STEROL REPORT

Sitosterol inhibited the tumor-promoting activity of TPA in the skin of female ICR mice after initiation with DMBA. The percent reduction in the average number of tumors at week 18 was 40% in mice given TPA, DMBA, and sitosterol. Sitosterol applied topically before treatment with TPA inhibited TPA-induced epidermal ODC activity; ODC induction can

be representative of the effects of phorbol esters with strong tumor promoting activity. Additionally, dermal inflammation caused by a single application of TPA was slightly inhibited by sitosterol and stigmasterol.[44,78]

Male Fischer CD rats coadministered the direct-acting carcinogen N -methylnitrosourea (by cannulation on days 1, 4, 7, 10) and β-sitosterol (95% pure, with 4% campesterol and 1 % stigmasterol; 0.2% in feed for 28 weeks) had significantly fewer colonic tumors (benign or benign and malignant) compared to rats given the carcinogen alone after 28 weeks.[79] Of rats given the carcinogen alone, 54% had tumors. Of rats given both the carcinogen and sitosterol, 33% had tumors. The incidence of rats with malignant colonic neoplasms increased after coadministration of the phytosterols; 15% (7/48) had invasive carcinomas in the sterol plus carcinogen group compared to 7% (5171) of rats given the carcinogen alone.

The phytosterols decreased epithelial cell proliferation of the colon in mice (0.1 % in feed) and rats (0.2% in feed after induction with N-methyl-N-nitrosourea), and were cytotoxic for human epidermoid carcinoma of the nasopharynx (> 20 μg/ml).[80,81]

IRRITATION AND SENSITIZATION

Irritation

Dermal – Non-Human

WDPSE (2000 mg/kg) administered to the clipped skin of male albino rabbits (n = 3) for 4 h under semi-occlusion was not irritating.[53] VODPSE caused very slight erythema after 1 h of treatment, which was completely reversed within 24 h after treatment. Skin irritation/corrosion was tested with rabbits in according to OECD Test Guideline 404.

Dermal – Human

Phytosterols (100%; 1 mL; derived from pomegranate) were not irritating to scarified skin in a repeat irritation assay (n = 10).[82] The test site was scratched with a 30-guage needle. The test material was administered to the same scarified location on the forearm, using a chamber, for 24 h for three consecutive days. The site was examined 30 min after removal and before the next treatment.

In-Vitro

In an EpiDerm™ assay, phytosterols (100%) from three sources (derived from pomegranate, soybean, and acai) were not predicted to be dermal irritants.[83-85]

Ocular

There was no irritation potential revealed for WDPSE and VODPSE in a chicken enucleated eye assay.[53]

WDPSE and VODPSE (concentration not provided; assumed 100%) were considered minimally irritating in a Draize assay using albino rabbits (n not provided).[53] The assay was conducted in accordance to OECD Test Guideline 405. WDPSE and VODPSE (concentration not provided; assumed 100%) caused slight and slight or moderate discharge, respectively, which was reversible within 24 h after treatment.

In an EpiOcular™ assay, phytosterols (100%) from three sources (derived from pomegranate, soybean, and acai) were not predicted to be ocular irritants.[83-85]

Sensitization

Non-Human

Neither WDPSE nor VODPSE (concentration not provided) caused signs of skin sensitization after administration to male guinea pigs (n = 10) in a maximization assay conducted in accordance with OECD Test Guideline 406.[53]

Human

There were no signs of irritation or sensitization in a human repeat insult patch test (HRIPT; n = 50) of sterols (100%; 0.2 mL; 0.2 g; derived from pomegranate).[86] The test material was heated to liquefy it, then it was applied to an occlusive, hypoallergenic patch. The patch was applied to the infrascapular regions of the back for nine treatments. The same concentration and amount of the test substance used in the challenge phase.

None of the subjects with confirmed soy allergies (n = 29) had a positive reaction to a skin prick test of plant stanol ester.[12] An open challenge with plant stanol ester within four weeks of the HRIPT (cumulative dose 5.55g) was negative in 26 of 33 (the original 29 + 4 more) subjects. Positive reactions consisted of itching of the throat in three participants, cutaneous symptoms in three, and loose stools in one subject. The reactions were observed after the final cumulative dose of plant stanol ester; all symptoms resolved without treatment.

A follow-up double-blind placebo controlled food challenge (DBPCFC) study with plant stanol ester performed on 6 of the subjects with positive reactions in the skin prick test had negative results. The DBPCFC with plant stanol ester in the remaining seventh subject (female) was interpreted as negative, although she reported loose stools the morning after the last challenge, which contained plant stanol ester. In view of the cumulative oil intake, a nonimmune-mediated reaction may be considered.[12]

Of 22 subjects that had positive reactions to a commercial soy extract in a skin prick test, 16 had a positive reaction to soy isolate and 6 to soy.[16] None had a reaction to phytosterols.

In Vitro

CONSTITUENTS

In an immunoblotting assay for soybean proteins using polyclonal, soybean-specific antiserum from rabbits (RBiopharm) and sera from nine soybean-allergic subjects, no soy protein or other protein was detected.[16] Oleosin was added as a control; the oleosin fraction was shown to be a minor IgE-binding constituent of the total soybean protein. The limit of detection was 50 ng of the reference soybean extract and 100 ng of oleosin.

All hydrophilic extracts of vegetable oil deodorized distillate (VOD) samples (n = 9) analyzed by immunoblotting with soy-specific antiserum from rabbits and by IgE-immunoblotting with a pooled human serum detected no soy protein or other protein. There was no IgE binding with the VOD or the phytosterol samples using either the pooled human serum or the serum of one subject who had experienced mild oral allergy syndrome after a DBPCFC with phytosterols. The authors concluded that no IgE-binding proteins were present in the VOD and phytosterol samples at or above 1 and 10 μg/g, respectively.[16]

Refined soybean oils exhibited no detectable IgE binding activity using immunoblotting and enzyme allergosorbent test (EAST) inhibition assays.[87]

CLINICAL USE

Case Studies

A female subject excreted increasing amounts of β-sitosterol, campesterol and stigmasterol through the skin as oral intake of phytosterols increased over sustained periods of time.[88] When phytosterols were removed from the diet, the amount of β-sitosterol in the skin decreased from 6 mg/d to 0.08 mg/d within 83 days and finally became undetectable. Similar results were reported for the other two phytosterols. Twenty days after the administration of 30 g/d phytosterols, β-sitosterol, as well as campesterol and stigmasterol, reappeared in the skin and was excreted at 5 mg/d by 6 weeks.

SUMMARY

A total of 27 phytosterols and steryl alkanoates are described for use in cosmetics. These ingredients are sterols derived from plants, many of which are then esterified with plant-derived fatty acids. These ingredients are reported to function as skin-conditioning agents, hair conditioning agents, viscosity increasing agents, skin protectants, antioxidants, drug astringents, and fragrances.

The Panel concluded that PEG-5, -10, -16, -25, -30, and -40 soy sterols to be safe as used in a prior amended safety assessment. The component chemicals that are cosmetic ingredients that have been reviewed by the Panel were all found to be safe as used. Butyric acid, caprylic acid/capric acid, and linoleic acid/linolenic acid have not been reviewed. Octyldecanoic acid is not a cosmetic ingredient.

Phytosterols are from edible plant sources and exposure to these phytosterols in food results in a much greater systemic exposure than that resulting from use in cosmetic products containing these ingredients. It was noted in the PEG soy sterol report that phytosterols and phytosterol esters are not significantly absorbed after oral exposure. Therefore, acute and repeated dose oral toxicity potential of these phytosterols was not be addressed in this report and the focus is on the potential for reproduction toxicity, genotoxicity, carcinogenicity, irritation, and sensitization.

Protein content of phytosterol blends was not detectable at the detection limits of 10-20 μg/g.

The phytosterols are used in all cosmetic categories except for baby products. They are used at maximum concentrations ranging from 0.000001% - 8%.

Phytosterols are used in food products at up to 5 g/L. The Western diet contains ~160-360 mg/d phytosterols consisting of ~80% β-sitosterol.

Less than 5% of dietary phytosterols, phytostanols, and their esters are absorbed in the gastrointestinal tract of rats and humans.

β-sitosterol (200 μg/mL in ethanol) and β-sitosterol/campesterol (50%/40%; 200 μg/mL in ethanol) were cytotoxic to mouse macrophages in vitro.

The LD_{50} of two mixtures of phytosterol esters was reported to be > 2000 mg/kg.

There were no maternal or teratogenic effects attributed to phytosterol esters administered in the feed of rats in a two generation study. The NOAEL was ≥8.1%, the highest concentration tested. There were no signs of reproductive toxicity to male and female American minks orally administered β-sitosterol up to 50 mg/kg/d for 10 months.

Subcutaneous injections of β-sitosterol at 5 mg/kg/d for 16 to 48 days reduced sperm concentrations and fertility, and decreased testis and accessory sex tissue weights in a time-dependent manner in male rats.

In multiple in vitro (up to 5000 μg/plate) and in vivo (up to 2000 mg/kg) genotoxicity assays, phytosterols and phytosterol esters were negative. These tests included reverse mutation, chromosomal aberration, gene mutation, clastogenicity, micronucleus induction, and unscheduled DNA synthesis assays.

A phytosterol mixture was not irritating to albino rabbits at 2000 mg/kg.

Two phytosterol mixtures were minimally irritating to albino rabbits.

Phytosterols derived from pomegranate at 100% were not irritating to scarified skin in a human repeat irritation assay.

Two phytosterol mixtures were not sensitizing to guinea pigs. Phytosterols derived from pomegranate were not sensitizing in and HRIPT at 100%. None of 29 subjects with confirmed soy allergies had a positive reaction to a skin prick test with plant stanol ester. Of 22 subjects that had positive reactions to a commercial soy extract in a skin prick test, none had a reaction to phytosterols.

There were no IgE-binding proteins detected in multiple hydrophilic extracts of vegetable oils samples using immunoblotting or an EAST inhibition assays.

There was little or no estrogenic activity detected in phytosterols using in vitro estrogen binding assays. Two phytosterol ester mixes administered in feed at 8.3% for 4 days did not affect the uterus weights of 17-day-old rats in a Teicco assay.

There were no signs of estrogenic activity in phytosterol mixtures up to 2500 mg/kg in immature rat uterotrophic assays.

DISCUSSION

The CIR Expert Panel will develop the Discussion at the September Panel meeting.

CONCLUSION

The CIR Expert Panel will develop the Conclusion at the September Panel meeting.

TABLES

Table 1. Definitions and functions of the phytosterols in this safety assessment.[1] Descriptions provided below in *italics* have been generated by CIR staff.

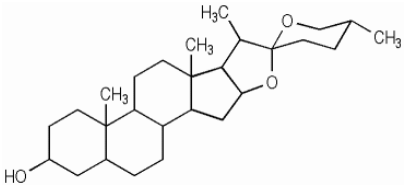
Ingredient CAS No.	Definition	Function
Brassica campestris (rapeseed) sterols	A mixture of sterols obtained from *Brassica campestris* (rapeseed) Seed Oil. *Rapeseed oil is known to contain brassicasterol, poriferasterol, and campesterol.*[5]	Skin-conditioning agent – emollient
Canola Sterols	A mixture of sterols obtained from the seeds of the canola plant.	Skin-conditioning agent – emollient
C10-40 isoalkyl acid phytosterol esters	A complex mixture of esters of phytosterol and C10-40 isoalkyl acid.	Hair conditioning agent; skin-conditioning agent – emollient; viscosity increasing agent – nonaqueous
Dihydrophytosteryl octyldecanoate	The ester of dihydrophytosterol and branched chain octyldecanoic acid.	Skin conditioning agent – occlusive
Diosgenin 512-04-9	The organic compound that conforms to the formula below. *Diosgenin is a glycoside form phytosterol found in wild yams.* 	Skin conditioning agent – miscellaneous
Euterpe oleracea sterols	The sterol fraction isolated from the whole plant of *Euterpe oleracea.*	Skin conditioning agent – miscellaneous
Glycine soja (soybean) sterols	A mixture of phytosterols obtained from the soybean, *Glycine soya. Soybean is known to contain stigmasterol.*[5]	Skin-conditioning agent – emollient
Persea gratissima (avocado) sterols	A mixture of sterols obtained from *Persea gratissima* (avocado) oil.	Skin-conditioning agent – emollient
Phytosterols	A mixture of sterols obtained from higher plants.	Skin conditioning agent – miscellaneous
Phytosteryl butyrate	The ester of phytosterols and butyric acid.	Hair conditioning agent; skin-conditioning agent – miscellaneous
Phytosteryl canolate	The ester of phytosterols and the fatty acids derived from canola oil.	Skin protectant; skin-conditioning agent – emollient; viscosity increasing agent – nonaqueous
Phytosteryl caprylate/caprate	The ester of phytosterols with a mixture of caprylic acid and capric acid.	Hair conditioning agent; skin-conditioning agent – occlusive
Phytosteryl hydroxystearate	The ester of phytosterols and hydroxystearic acid.	Skin-conditioning agent – emollient
Phytosteryl isostearate	The ester of phytosterols and isostearic acid.	Hair conditioning agent; skin-conditioning agent – occlusive
Phytosteryl linoleate	The ester of phytosterols with linoleic acid.	Antioxidant
Phytosteryl linoleate/linolenate	The ester of phytosterols with a mixture of linoleic acid and linoleic acid.	Antioxidant
Phytosteryl macadamiate	The ester of phytosterols and the fatty acids derived macadamia seed oil.	Hair conditioning agent; skin-conditioning agent – miscellaneous
Phytosteryl nonanoate	The ester of phytosterols and nonanoic acid.	Hair conditioning agent; skin-conditioning agent – miscellaneous
Phytosteryl oleate	The ester of phytosterols and oleic acid.	Hair conditioning agent; skin-conditioning agent – miscellaneous
Phytosteryl rice branate	The ester of phytosterols and rice bran acid.	Drug astringent – skin protectant drug; hair conditioning agent; humectant; skin protectant; skin-conditioning agent – emollient
Phytosteryl ricinoleate	The ester of phytosterols and ricinoleic acid.	Hair conditioning agent; skin-conditioning agent – miscellaneous
Phytosteryl sunflowerseedate	The ester formed by the reaction of sunflower seed acid with phytosterols.	Skin-conditioning agent – miscellaneous
Punica granatum sterols	A mixture of sterols obtained from *Punica granatum* seed oil.	Hair conditioning agent; ; skin-conditioning agent – emollient; skin-conditioning agent – occlusive
Beta-sitosterol 83-46-5	*A sterol that is found in most plant oils and conforms to the structure in Figure 1.*[5]	Fragrance ingredient; Skin-conditioning agent – miscellaneous
Beta-sitosteryl acetate 915-05-9	The ester of beta-sitosterol and acetic acid *that conforms to the structure in Figure 2.*	Skin-conditioning agent – miscellaneous
Soy sterol acetate	The acetic acid esters of soy sterol.	Skin-conditioning agent – occlusive
Tall oil sterol	The complex mixture of phytosterols (polycyclic polyterpenes, complex monohydric alcohols and their esters) recovered from fractions of tall oil.	Skin-conditioning agent – miscellaneous

Table 2. CIR safety assessments of constituents of phytosterol ingredients.

Constituent	Conclusion	Maximum concentration of use reported	Reference
PEG-5, -10, -16, -25, -30, and -40 soy sterol	Insufficient; Safe as used.	2%	[2,3]
Diosgenin	Safe as used. *As a constituent of dioscorea villosa (wild yam) root extract (3.5%).*	0.525%	[4]
Plant-derived fatty acid oils	Safe as used.	100%	[89]
C10-40 isoalkyl acid	*As C10-40 isoalkyl acid octylodecanol esters, C4-5 isoalkyl cocoate, C32-36 isoalkyl stearate, and ethylhexyl C10-40 isoalkyl acidate.* Safe in the present practices of use and concentration described in this safety assessment when formulated to be non-irritating.	78%	[90]
Octyldecanoic acid	Not a cosmetic ingredient.	-	
Butyric acid	Not reviewed.	-	
Caprylic acid/capric acid	Not reviewed.	-	
Hydroxystearic acid	Safe as used.	10%	[91]
Isostearic acid	Safe as used.	26%	[92,93]
Linoleic acid/linoleic acid	Not reviewed.	-	
Nonanoic acid	*As pelargonic acid.* Safe as used.	74%	[94]
Oleic acid	Safe as used.	43%	[95,96]
Rice bran acid	Safe as used.	100%	[89,97]
Ricinoleic acid	Safe as used.	69%	[98]
Sunflower seed acid	Safe as used.	100%	[89]
Acetic acid	Safe as used.	0.4%	[99]
Tall oil acid	Safe as used.	8%	[100]

Table 3. Chemical characterization of a single sample and multiple samples of plant sterol material (source plant not provided).[3,8,9]

	Distribution of phytosterols (%)	
Phytosterol	Single sample	Five samples from five batches
Brassicasterol	1.1	2.7-3.1
Campesterol	25.8	26.5-27.0
Stigmasterol	21.6	17.4-18.1
B-Sitosterol	48.7	50.8-51.2
B-Sitostanal	1.8	Not provided
Cholesterol	0.4	0.2-0.3
Other sterols	0.8	1.2-1.7

Table 4. Percent distribution of phytosterols from common vegetable oils.[3,6]

Oil source	Brassicasterol	Campesterol	Stigmasterol	B-Sitosterol	Δ7 Stigmastenol	Unknown
Cocoa butter		8-11	24-31	59-62		
Coconut	2	6-9	18-19	69-75		
Corn		10-20	Trace-6	74-89		1
Cottonseed	Trace-1	8		89-91		
Linseed	2	28	10	53	4	
Olive		1-3	2	80-97		18
Palm		20-21	12-13	62-67		
Peanut	1	10-19	6-12	70-76		
Rapeseed	5-19	22-37		52-62		
Rice bran		14-33	3-6	55-63		
Safflower		8-13	4-9	52-57		23
Soybean		15-21	10-24	57-72		1
Sunflower		11-12	8-12	62-75	20	

Table 5. Chemical and physical properties of representative sterols.

Property	Value	Reference
Vegetable oil sterols		
Physical Form	Crystalline waxy powder or prills	10
	Waxy, free-flowing granular powder	16
Color	White to off white	10
Odor	Vegetable oil-like	16
Melting Point °C	138-158	10
Water Solubility g/L @	< 0.01	10
Other Solubility		10
Fat at ambient temperature	2.5%	
Acetone	Soluble	
Ethyl acetate	Soluble	
Isopropanol	Soluble	
Tall oil sterols/stanols		
Physical Form	Crystalline waxy powder or prills	10
Color	White to off white	10
Melting Point °C	138-158	10
Water Solubility g/L @ °C & pH	< 0.01	10
Other Solubility		10
Fat at ambient temperature	2.5%	
Acetone	Soluble	
Ethyl acetate	Soluble	
Isopropanol	Soluble	

Table 6. Comparison of phytosterol content of tall oil extracted by simpler saponification process and a more complicated, multi-step processes.[2,5]

Phytosterol	Saponification process (%)	Multi-step process (%)
Total phytosterols	98.1	99.7
Major phytosterols	88.7	92.7
β-Sitosterol	49.1	59.8
β-Sitostanol	19.9	23.2
Campesterol	15.0	6.5
Stigmasterol	< 1%	< 1%
Other phytosterols	9.3 (including stigmasterol)	7.0 (including stigmasterol)

Table 7. Allergens not detected in phytosterols derived from acai, soybean, and pomegranate.[17-19]

Alpha-isomethyl ionone	Amyl cinnamal	Anise alcohol
Benzyl alcohol	Benzyl benzoate	Benzyl cinnamate
Benzyl salicylate	Butylphenyl methylpropional	Cinnamal
Cinnamyl alcohol	Citral	Citronellol
Coumarin	Eugenol	Fanesol
Geraniol	Hexyl Cinnamal	Hydroxycetronellal
Hydroxymethylpentyl 3-cyclohexene carboxaldehyde	Isoeugenol	Limonene
Linalool	Methyl 2 octynoate	Evernia prunastri
Evernia furfuracea	Amylcinnamyl alcohol	

Table 8. Frequency of use according to duration and exposure of phytosterols.[23,24]

Use type	Uses	Maximum Concentration (%)	Uses	Maximum Concentration (%)	Uses	Maximum Concentration (%)	Uses	Maximum Concentration (%)
	Brassica campestris (rapeseed) sterols		**Euterpe oleracea sterols**		**Glycine soja (soybean) sterols**		**Persea gratissima (avocado) sterols**	
Total/range	**57**	**0.0008-7**	**2**	**NR**	**240**	**0.000001-4.1**	**NR**	**0.1-1**
Duration of use								
Leave-on	50	0.0008-7	2	NR	194	0.000001-1	NR	0.1-1
Rinse-off	7	0.0055-0.13		NR	45	0.000001-4.1	NR	NR
Diluted for (bath) use	NR	NR	NR	NR	1	NR	NR	NR
Exposure type								
Eye area	3	0.005	NR	NR	17	0.001-1	NR	1
Incidental ingestion	2	0.0008-7	1	NR	3	0.1-1	NR	0.65
Incidental Inhalation-sprays	2	NR	NR	NR	6	0.00000-0.001	NR	NR
Incidental inhalation-powders	NR	NR	NR	NR	1	0.001-0.1	NR	NR
Dermal contact	54	0.0055-0.5	1	NR	193	0.001-4.1	NR	0.1-1
Deodorant (underarm)	NR	NR	NR	NR	NR	NR	NR	NR
Hair-noncoloring	NR	0.13	NR	NR	43	0.000001-0.018	NR	NR
Hair-coloring	NR	NR	NR	NR	NR		NR	NR
Nail	1	NR	NR	NR	NR	1	NR	NR
Mucous Membrane	4	0.0008-7	1	NR	10	0.01-1	NR	0.65
Baby	NR	NR	NR	NR	NR	NR	NR	NR

	Phytosterols		**Phytosteryl canolate**		**Phytosteryl isostearate**		**Phytosteryl [phytosterol] macadamiate**	
Total/range	**403**	**0.0001-5**	**NR**	**0.06**	**16**	**0.003-3**	**183**	**0.001-8**
Duration of use								
Leave-on	177	0.0001-5	NR	0.06	15	0.003-3	181	0.001-8
Rinse-off	215	0.00018-0.5	NR	NR	1	0.5	2	0.01-1
Diluted for (bath) use	11	NR	NR	NR	NR	NR	NR	NR
Exposure type								
Eye area	5	0.00018-2	NR	0.06	4	0.003-0.5	2	0.01-3
Incidental ingestion	63	0.01-5	NR	NR	8	2.8-3	100	4.1-7
Incidental Inhalation-sprays	2	0.0001	NR	NR	NR	NR	NR	NR
Incidental inhalation-powders	1	0.05	NR	NR	NR	NR	NR	0.001
Dermal contact	338	0.0001-3.2	NR	0.06	8	0.003-1	82	0.001-8
Deodorant (underarm)	NR	0.06	NR	NR	NR	NR	NR	NR
Hair-noncoloring	2	0.5-2.4	NR	NR	NR	0.1	1	0.01-1
Hair-coloring	NR	NR	NR	NR	NR	NR	NR	
Nail	NR	NR	NR	NR	NR	NR	NR	0.01
Mucous Membrane	280	0.0002-5	NR	NR	8	2.8-3	100	4.1-7
Baby	NR	NR	NR	NR	NR	NR	NR	NR

Table 8. Frequency of use according to duration and exposure of phytosterols.[23,24]

Use type	Uses	Maximum Concentration (%)	Uses	Maximum Concentration (%)	Uses	Maximum Concentration (%)	Uses	Maximum Concentration (%)
	Phytosteryl oleate		**Phytosteryl rice branate**		**Punica granatum sterols**		**Beta-sitosteryl**	
Total/range	**26**	**1.5-3**	**2**	**0.5-1**	**31**	**0.001-5**	**48**	**0.00007-0.06**
Duration of use								
Leave-on	20	1.5-3	NR	0.5-1	29	0.1-5	46	0.00007-0.06
Rinse-off	6	NR	NR	NR	2	NR	2	NR
Diluted for (bath) use	NR	NR	NR	NR	NR	0.001	NR	NR
Exposure type								
Eye area	1	NR	1	1	3	NR	3	
Incidental ingestion	NR	1.5	NR	NR	14	0.1-5	1	0.00007-0.0008
Incidental Inhalation-sprays	NR	NR	NR	NR	NR	NR	4	NR
Incidental inhalation-powders	NR	NR	NR	NR	NR	NR	NR	0.0021
Dermal contact	26	3	2	0.5-1	15	0.001-0.5	47	0.0004-0.06
Deodorant (underarm)	NR	NR	NR	NR	NR	NR	NR	NR
Hair-noncoloring	NR	NR	NR	NR	2	NR	NR	NR
Hair-coloring	NR	NR	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	1.5	NR	NR	15	0.001-5	1	0.00007-0.0008
Baby	NR	NR	NR	NR	NR	NR	NR	NR

	Soy sterol acetate		**Tall oil sterol**					
Total/range	**1**	NR	**7**	**0.0006-0.0046**				
Duration of use								
Leave-on	1	NR	7	0.0045-0.0046				
Rinse-off	NR	NR	NR	0.0006				
Diluted for (bath) use	NR	NR	NR	NR				
Exposure type								
Eye area	NR	NR	NR	NR				
Incidental ingestion	NR	NR	NR	NR				
Incidental Inhalation-sprays	NR	NR	NR	NR				
Incidental inhalation-powders	NR	NR	NR	NR				
Dermal contact	1	NR	7	0.0006-0.0046				
Deodorant (underarm)	NR	NR	NR	NR				
Hair-noncoloring	NR	NR	NR	NR				
Hair-coloring	NR	NR	NR	NR				
Nail	NR	NR	NR	NR				
Mucous Membrane	NR	NR	NR	NR				
Baby	NR	NR	NR	NR				

NR = Not Reported; NS = Not Surveyed; Totals = Rinse-off + Leave-on Product Uses.
Note: Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure type uses may not equal the sum total uses.

Table 8. Genotoxicity assays of phytosterols.

Assay	Test material(s) (concentration)	Results	Reference
In vitro			
Reverse mutation *Salmonella typhimurium* (strains TA98, TA100, TA102)	7-ketositosterol (up to 5% in acetone/tween80, 3:1 v/v), 7β-OH-sitosterol (up to 5%), 7α-OH-sitosterol (up to 1%), 6α-OH-3-keto-/6β-OH-3-ketositosterol (ratio 4:3; up to 2.5%) and a mixture (up to 10%)	Negative with and without metabolic activation	67
Reverse mutation S. *typhimurium* (TA98, TA100, TA1535 and TA1537)	Phytosterol mixture[a] (5–5000 µg/plate)	Negative with and without metabolic activation	70
Reverse mutation; *S. typhimurium* (TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 uvrA (pKM101)	Phytosterol esters[a] (50-5000 µg/plate)	Negative with and without metabolic activation	70
Reverse mutation *S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537)	Phytosterol oxide concentrate from vegetable oil distillates (1.6-5000 µg/plate)	Negative with and without metabolic activation	68
Reverse mutation *S. typhimurium* (TA98, TA100, TA1535 and TA1537); *E. coli* WP2 uvrA	MPSS-SE[c] (104-1667 µg/plate)	Negative with and without metabolic activation	53
Reverse mutation *S. typhimurium* (TA98, TA100, TA1535, and TA1537); *E. coli* WP2 uvrA	MPSS-VD[d] (16-1000 µg/plate)	Negative with and without metabolic activation	53
Reverse mutation S. *typhimurium* (TA98, TA100, TA1535 and TA1537)	WDPSE[e] (62-5000 µg/plate)	Negative with and without metabolic activation	53
Reverse mutation S. *typhimurium* (TA98, TA100, TA1535 and TA1537)	VODPSE[f] (62-5000 µg/plate)	Negative with and without metabolic activation	53
Reverse mutations histidine-dependent *S. typhimurium* (TA98, TA100, TA1535, TA1537); tryptophan-dependent *E. coli* (WP2uvrA)	Pomeganate sterols (50 mg/mL; 0.1 mL)	Negative with and without metabolic activation	101
Chromosomal aberration; Human peripheral blood lymphocytes	Phytosterol mixture[a] (40-160 µg/mL)	Negative with and without metabolic activation	70
Chromosomal aberration; Human peripheral blood lymphocytes	Phytosterol esters[a] (25-200 µg/mL)	Negative with and without metabolic activation	70
Chromosomal aberration; Human peripheral blood lymphocytes	Phytosterol oxide concentrate[g] (131.1-500 µg/mL)	Negative with and without metabolic activation	70
Chromosomal aberration; Human peripheral blood lymphocytes	MPSS-SE (100-1200 µg/mL)	Negative with and without metabolic activation	53
Chromosomal aberration; Human peripheral blood lymphocytes	MPSS-VD (31.3-1000 µg/mL)	Negative with and without metabolic activation	53
Chromosomal aberration; Chinese hamster ovary cells	WDPSE (up to 500 µg/ml)	Negative with and without metabolic activation	53
Chromosomal aberration; Chinese hamster ovary cells	VODPSE (up to 2000 µg/ml)	Negative with and without metabolic activation	53
Gene mutation; Mouse lymphoma L5178Y cells, *Tk*+/- locus	Phytosterol esters[a] (5-80 µg/mL)	Negative with and without metabolic activation	68
Gene mutation; Mouse lymphoma L5178Y cells, *Tk*+/- locus	MPSS-SE (5-167 µg/mL)	Negative with and without metabolic activation	53
Gene mutation; Mouse lymphoma L5178Y cells, *Tk*+/- locus	WDPSE (20-500 µg/ml)	Negative with and without metabolic activation	53
Gene mutation; Mouse lymphoma L5178Y cells, *Tk*+/- locus	VODPSE (125-3000 µg/ml)	Negative with and without metabolic activation	53
Clastogenicity (micronucleus induction); Human peripheral blood lymphocytes	Phytosterol oxide concentrate[g] (up to 625 µg/mL)	Negative with and without metabolic activation	68
In vivo			
Micronucleus induction; male rats, bone marrow	Phytosterol esters[b] (500-2000 mg/kg/d) for 2 days	Negative	70
Micronucleus induction; male and female rats, bone marrow	MPSS-SE (50, 500, 2000 mg/kg)	Negative	53
Unscheduled DNA synthesis; male rats, liver	Phytosterol esters[b] (800, 2000 mg/kg)	Negative	70
Micronucleus induction; male mice, blood	Triols (up to 9.4 mg/kg) and epoxides of a mixture of β-sitostrol and campesterol (67 mg/kg)	Negative	102
Sister chromatid exchange; male NIH mice (8 weeks old)	β-sitostrol (200, 400, 600, 1000 mg/kg)	Negative	69
Cellular proliferation kinetics; male NIH mice (8 weeks old)	β-sitostrol (200, 400, 600, 1000 mg/kg)	Negative	69
Mitotic index; male NIH mice (8 weeks old)	β-sitostrol (200, 400, 600, 1000 mg/kg)	Negative	69
Micronucleated polychromatic erythrocytes; male NIH mice (8 weeks old)	β-sitostrol (200, 400, 600, 1000 mg/kg)	Negative	69

[a] Phytosterol composition: campesterol (26.7%), stigmasterol (17.7%), β-sitosterol (51%).
[b] Phytosterol composition: campesterol (28.1%), stigmasterol (18.7%), β-sitosterol (45.5%)
[c] MPSS-SE = Mixture of phytosterols and phytostanols derived from solvent extraction, which consisted of ~40– 55% β-sitosterol, ~16–31% β-sitostanol, ~11–15% campesterol, and ~2–11% campestanol.
[d] MPSS-VD = Mixture derived from vacuum distillation which consisted of ~63.5% β-sitosterol, ~21.7% β-sitostanol,~ 6.5% campesterol and ~2.8% campestanol.

Table 8. Genotoxicity assays of phytosterols.

Assay	Test material(s) (concentration)	Results	Reference

[e] WDPSE = A wood-derived stanol mixture which consisted of ~94% β-sitostanol and ~6% campestanol.
[f] VODPSE = A vegetable oil–derived mixture of phytostanol esters which consisted of ~68% β-sitostanol and ~32% campestanol.
[g] Phytosterol oxide concentrate = ~ 30% phytosterol oxides.

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Cosmetic Ingredient Review

Commitment & Credibility since 1976

August 16, 2013

MEMORANDUM

To: CIR Expert Panel and Liaisons

From: Lillian C. Becker, MS
Scientific Analyst and Writer

Subject: Draft Report of the Safety Assessment of Phytosterols as Used in Cosmetics

Unpublished data were submitted by the Personal Care Products Council. These data include:

1) Concentration of use.

2) Safety studies of phytosterol ingredients. These include:

    a. Bacterial reverse mutation assay of pomegranate sterols
    b. HRIPT of pomegranate sterols
    c. Human dermal irritation test on scarified skin
    d. Summary of EpiDermal and EpiOcular assays of pomegranate sterols
    e. Summary of a neutral red cytotoxicity assay of pomegranate sterols
    f. Summary of EpiDermal and EpiOcular assays of soybean sterols
    g. Summary of EpiDermal and EpiOcular assays of acai sterols

3) Impurity and manufacture information on acai, soybean, and pomegranate sterols.

To inform the Panel on data that has already been considered, the 2004 safety assessment of PEG soy sterols is also included.

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: F. Alan Andersen, Ph.D.
Director - COSMETIC INGREDIENT REVIEW (CIR)

FROM: Halyna Breslawec, Ph.D.
Industry Liaison to the CIR Expert Panel

DATE: March 21, 2013

SUBJECT: Concentration of Use by FDA Product Category: Plant Sterols

1101 17th Street, N.W., Suite 300 | Washington, D.C. 20036-4702 | 202.331.1770 | 202.331.1969 (fax) | www.personalcarecouncil.org

Concentration of Use by FDA Product Category - Plant Sterols*

Brassica Campestris (Rapeseed) Sterols
Canola Sterols
C10-40 Isoalkyl Acid Phytosterol Esters
Crambe Abyssinica Seed Oil Phytosterol Esters
Macadamia Ternifolia Seed Oil Phytosterol Esters
Dihydrophytosteryl Octyldecanoate
Jojoba Oil/Macadamia Seed Oil Phytosteryl Esters
Diosgenin
Euterpe Oleracea Sterols
Glycine Soja (Soybean) Sterols
Persea Gratissima (Avocado) Sterols
Phytosterols
Phytosteryl Butyrate
Phytosteryl Canolate
Phytosteryl Caprylate/Caprate
Phytosteryl Hydroxystearate
Phytosteryl Isostearate
Phytosteryl Linoleate
Phytosteryl Linoleate/Linolenate
Phytosteryl Macadamiate
Phytosteryl Nonanoate
Phytosteryl Oleate
Phytosteryl Rice Branate
Phytosteryl Ricinoleate
Phytosteryl Sunflowerseedate
Punica Granatum Sterols
Beta-Sitosterol
Beta-Sitosteryl Acetate
Soy Sterol Acetate
Tall Oil Sterol

Ingredient	FDA Code†	Product Category	Maximum Concentration of Use
Brassica Campestris (Rapeseed) Sterols	03D	Eye lotion	0.0055%
Brassica Campestris (Rapeseed) Sterols	05A	Hair conditioners	0.13%
Brassica Campestris (Rapeseed) Sterols	07E	Lipstick	0.0008-7%
Brassica Campestris (Rapeseed) Sterols	12C	Face and neck products not spray	0.01%
Brassica Campestris (Rapeseed) Sterols	12D	Body and hand products not spray	0.5%
Brassica Campestris (Rapeseed) Sterols	12H	Paste masks and mud packs	0.0055%
Glycine Soja (Soybean) Sterols	03C	Eye shadow	0.01-0.5%
Glycine Soja (Soybean) Sterols	03D	Eye lotion	0.01-1%
Glycine Soja (Soybean) Sterols	03F	Mascara	0.001%
Glycine Soja (Soybean) Sterols	05A	Hair conditioners	0.000001-0.018%
Glycine Soja (Soybean) Sterols	05F	Shampoos (noncoloring)	0.000001-0.0035%
Glycine Soja (Soybean) Sterols	05G	Tonics, dressings and other hair grooming aids	0.000001%

		aerosol pump spray	0.000001% 0.000001%
Glycine Soja (Soybean) Sterols	05I	Other hair preparations (noncoloring)	0.005%
Glycine Soja (Soybean) Sterols	07A	Blushers (all types)	0.001-0.2%
Glycine Soja (Soybean) Sterols	07B	Face powders	0.001-0.1%
Glycine Soja (Soybean) Sterols	07C	Foundations	0.001-0.2%
Glycine Soja (Soybean) Sterols	07E	Lipstick	0.1-1%
Glycine Soja (Soybean) Sterols	08B	Cuticle softeners	1%
Glycine Soja (Soybean) Sterols	10E	Other personal cleanliness products	0.54%
Glycine Soja (Soybean) Sterols	11E	Shaving cream (aerosol, brushless and lather)	0.2%
Glycine Soja (Soybean) Sterols	12A	Skin cleansing (cold cream, cleansing lotions, liquids and pads)	0.01-4.1%
Glycine Soja (Soybean) Sterols	12C	Face and neck products not spray	0.05-1%
Glycine Soja (Soybean) Sterols	12D	Body and hand products not spray	0.005-0.9%
Glycine Soja (Soybean) Sterols	12F	Moisturizing products not spray	0.075-1%
Glycine Soja (Soybean) Sterols	12G	Night products not spray	0.1-0.23%
Glycine Soja (Soybean) Sterols	12J	Other skin care preparations	1%
Glycine Soja (Soybean) Sterols	13A	Suntan gels, creams and liquids not spray	0.1%
Glycine Soja (Soybean) Sterols	13B	Indoor tanning preparations	0.001%
Persea Gratissima (Avocado) Sterols	03D	Eye lotion	1%
Persea Gratissima (Avocado) Sterols	07E	Lipstick	0.65%
Persea Gratissima (Avocado)	12C	Face and neck products	

Sterols		not spray	0.1%
Phytosterols	03C	Eye shadow	0.00018%
Phytosterols	03D	Eye lotion	0.18-2%
Phytosterols	03F	Mascara	0.006%
Phytosterols	03G	Other eye makeup preparations	0.0018%
Phytosterols	05A	Hair conditioners	0.5%
Phytosterols	05F	Shampoos (noncoloring)	0.5%
Phytosterols	05G	Tonics, dressings and other hair grooming aids	2.4%
Phytosterols	07A	Blushers (all types)	3.2%
Phytosterols	07B	Face powders	0.05%
Phytosterols	07C	Foundations	0.0018%
Phytosterols	07E	Lipstick	0.01-5%
Phytosterols	10A	Bath soaps and detergents	0.0002-0.005%
Phytosterols	10B	Deodorants (underarm) not spray	0.06%
Phytosterols	12A	Skin cleansing (cold creams, cleansing lotions, liquids and pads)	0.00018%
Phytosterols	12C	Face and neck products not spray	0.4-2%
Phytosterols	12D	Body and hand products not spray	0.004-0.04%
Phytosterols	12H	Paste masks and mud packs	0.18%
Phytosterols	13B	Indoor tanning preparations	0.0001%
Phytosteryl Canolate	03C	Eye shadow	0.06%
Phytosteryl Isostearate	03B	Eye liner	0.5%
Phytosteryl Isostearate	03G	Other eye makeup preparations	0.003%
Phytosteryl Isostearate	05G	Tonics, dressings and other hair grooming aids	0.1%

Phytosteryl Isostearate	07E	Lipstick	2.8-3%
Phytosteryl Isostearate	12C	Face and neck products not spray	0.2-1%
Phytosteryl Isostearate	12H	Paste masks and mud packs	0.5%
Phytosteryl Macadamiate	03A	Eyebrow pencil	0.01%
Phytosteryl Macadamiate	03B	Eye liner	0.01%
Phytosteryl Macadamiate	03C	Eye shadow	0.1-1.7%
Phytosteryl Macadamiate	03D	Eye lotion	3%
Phytosteryl Macadamiate	03F	Mascara	0.01%
Phytosteryl Macadamiate	05A	Hair conditioners	1%
Phytosteryl Macadamiate	05E	Rinses (noncoloring)	0.04%
Phytosteryl Macadamiate	05F	Shampoos (noncoloring)	0.01%
Phytosteryl Macadamiate	05G	Tonics, dressings and other hair grooming aids	0.01%
Phytosteryl Macadamiate	07A	Blushers (all types)	1%
Phytosteryl Macadamiate	07B	Face powders	0.001%
Phytosteryl Macadamiate	07C	Foundations	0.5%
Phytosteryl Macadamiate	07E	Lipstick	4.1-7%
Phytosteryl Macadamiate	08E	Nail polish and enamel	0.01%
Phytosteryl Macadamiate	08G	Other manicuring preparations	0.01%
Phytosteryl Macadamiate	12A	Skin cleansing (cold creams, cleansing lotions, liquids and pads)	0.5%
Phytosteryl Macadamiate	12C	Face and neck products not spray	0.72-2%
Phytosteryl Macadamiate	12D	Body and hand products not spray	0.5-1%
Phytosteryl Macadamiate	12F	Moisturizing products not spray	3-8%
Phytosteryl Oleate	07E	Lipstick	1.5%
Phytosteryl Oleate	07G	Rouges	3%

Phytosteryl Rice Branate	03D	Eye lotion	1%
Phytosteryl Rice Branate	07C	Foundations	0.5%
Phytosteryl Rice Branate	12C	Face and neck products not spray	0.5%
Punica Granatum Sterols	02D	Other bath preparations	0.001%
Punica Granatum Sterols	07C	Foundations	0.1%
Punica Granatum Sterols	07E	Lipstick	0.1-5%
Punica Granatum Sterols	12D	Body and hand products not spray	0.25%
Punica Granatum Sterols	12G	Night products not spray	0.5%
Beta-Sitosterol	07B	Face powders	0.0021%
Beta-Sitosterol	07C	Foundations	0.0004-0.05%
Beta-Sitosterol	07E	Lipstick	0.00007-0.0008%
Beta-Sitosterol	12D	Body and hand products not spray	0.01%
Beta-Sitosterol	12F	Moisturizing products not spray	0.06%
Tall Oil Sterol	12A	Skin cleansing (cold creams, cleansing, lotions, liquids and pads)	0.0006%
Tall Oil Sterol	12C	Face and neck products not spray	0.0046%
Tall Oil Sterol	12D	Body and hand products not spray	0.0045%

*Ingredients included in the title of the table but not found in the table were included in the concentration of use survey, but no uses were reported.
†Product category codes used by FDA

Information collected in 2013
Table prepared March 21, 2013

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Lillian Gill, Ph.D.
Director - COSMETIC INGREDIENT REVIEW (CIR)

FROM: Halyna Breslawec, Ph.D.
Industry Liaison to the CIR Expert Panel

DATE: July 8, 2013

SUBJECT: Safety Studies Phytosterol Ingredients

BioScreen Testing Services, Inc. 2007. Bacterial reverse mutation tests ABS Pomegranate Sterols.

AMA Laboratories, Inc. 2006. 50 Human subject repeat insult patch test skin irritation/sensitization evaluation (occlusive patch) ABS Pomegranate Sterols.

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BioScreen® Testing Services, Inc.

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MICROBIOLOGICAL REPORT

Active Concepts, LLC.
Attn: Sarah Jindal
121 Ethel Road West Suite 3
Piscataway, NJ 08854

Report Date:	05/22/07
Date Received:	03/29/07
Date Completed:	05/16/07
Project #:	528350
P.O. #:	Not Specified

SAMPLE DESCRIPTION:

ACCESSION #:	SAMPLE:	LOT #:	BATCH #:	QTY
528350	ABS Pomegranate Sterols	SN070302-12	Not Specified	50g

TEST PERFORMED:	BTS METHOD #:	REFERENCE:
Genotoxicity: Bacterial Reverse Mutation Test	N/A	OECD471/ISO10993.Part 3

SUMMARY

A *Salmonella typhimurium* and *Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution of ABS Pomegranate Sterols, Lot: SN070302-12, would cause mutagenic changes in the average number of revertants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA 1537, and in tryptophan-dependent *Escherichia coli* strain WP2*uvr*A in the presence and absence of S9 metabolic activation. This study was conducted to satisfy, in part, the genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was diluted with the sponsor provided solvent, Ceraphyl® 368, to a concentration of 50 mg/ml for testing. The test article solution was found to be noninhibitory to growth of tester strains TA98, TA100, TA1535, TA1537, and WP2*uvrA*. Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *S. typhimurium* strains and with tryptophan for the *E. coli* strain were inoculated with 0.1 ml of culture for each of five tester strains, and 0.1 ml of the test article solution. A 0.5 ml aliquot of sterile Water for Injection or S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across triplicate Minimal E plates. Parallel testing was also conducted with a negative control and five positive controls. The mean number of revertants of the triplicate test plates was compared to the mean number of revertants of the triplicate negative control plates for each of the five tester strains employed. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be nonmutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and to *Escherichia coli* strain WP2*uvrA*. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 10993, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

I. Introduction

A. Purpose

A *Salmonella typhimurium* and *Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* tester strain WP2*uvrA* in the presence and absence of S9 metabolic activation. This test was conducted to satisfy, in part, the requirements of the International Organization for Standardization (ISO) 10993, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

II. Materials

A. Storage Conditions: Room Temperature

B. Sponsor Provided Vehicle: Ceraphyl 368®

C. Preparation: A 50 mg/ml solution was prepared with the sponsor provided vehicle, Ceraphyl® 368. A negative control (vehicle without test material) was similarly prepared.

III. Test System

A. Test System

Each *Salmonella typhimurium* tester strain contains a specific mutation in the histidic operon and other mutations that increase their ability to detect mutagens. In addition, the *Escherichia coli* contains a specific mutation in the tryptophan operon, and a deletion in the *uvrA* gene. These genetically altered *S. typhimurium* strains (TA98, TA100, TA1535, and TA1537) and *E. coli* strain (WP2*uvrA*) cannot grow in the absence of histidine or tryptophan, respectively. When placed in a histidine-free (for *S. typhimurium*) or tryptophan-free (for *E. coli*) medium, only those cells which mutate spontaneously back to their wild type state (histidine independent by manufacturing their own histidine, or tryptophan independent by manufacturing their own tryptophan) are able to form colonies. The spontaneous mutation rate (or reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

Tester Strain	Mutations/Genotypic Relevance
S. typhimurium TA 98	hisD3052, *rfa, uvrB*, frame shift, pKM101
S. typhimurium TA100	hisG46, *rfa, uvrB*, missense, pKM101
S. typhimurium TA1535	hisG46, rfa, uvr*B*, missence
S. typhimurium TA1537	hisC3076, *rfa, uvrB*, frame shift
E. coli WP2*uvrA*	trpE65, *uvrA*, missence

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules (i.e., crystal violet inhibition)
uvrB or *uvrA*	=	deficient DNA excision - repair system (i.e., ultraviolet sensitivity)
frameshift	=	base-pair addition/deletion
missense	=	base-pair substitution
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens

B. Metabolic Activation

Aroclor 1254 - induced rat liver (S9 homogenate) was used as metabolic activation. The S9 homogenate is prepared from male, Sprague Dawley rats. The rats are induced with one intraperitoneal injection of Aroclor 1254 (500 mg/ml) 5 days prior to sacrifice. Just prior to use, the S9 homogenate was mixed with a buffer containing 0.4 M $MgCl_2$/1.65 M KCl, 1.0 M Glucose-6-phosphate, 0.1 M NADP, 0.2 M sodium phosphate buffer, and Sterile Water for Injection.

C. Preparation of Tester Strains

Cultures of *Salmonella typhimurium*, TA98, TA100, TA1535 and TA1537, and *Escherichia coli*, WP2*uvrA*, were inoculated to individual Erlenmeyer flasks containing oxoid broth. The inoculated broth cultures were incubated at 37 ± 2°C in an incubator shaker operating at 115-125 rpm for 10-12 hours.

D. Sponsor Provided Negative Control
Ceraphyl® 368 was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of revertant colonies that developed in each test plate were compared to determine whether the test article had significant mutagenic properties.

E. Positive Control
A known mutagen, Dexon (paradimethylaminobenzene diazosulfonic acid sodium salt), was used as a positive control to demonstrate that tester strains TA98, TA100, and TA1537 were sensitive to mutation to the wild type state. For tester strain TA1535, sodium azide was used as a positive control. For tester strain TA100, 2-aminofluorene was also used as a positive control. For tester strain WP2*uvrA*, 2-aminoanthracene and methyl methane-sulfonate were used as positive controls. Although metabolic activation was only required with 2-aminofluorene and 2-aminoanthracene to induce mutagenic results, all positive controls were tested with and without S9 homogenate.

F. Strain Characteristics and Strain Standard Plate Counts
Strain characteristics were verified and viable counts were determined.

G. Spot Plate Inhibition Screen
The test article solution was evaluated by a spot plate technique, modeled after the ant microbial zone of inhibition test. This screen was used to evaluate the toxicity of the solution to determine whether dilution of the solution was required to provide a solution no inhibitory to the *Salmonella typhimurium* or to the *Escherichia coli.*

Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* and tryptophan for the *Escherichia coli* were inoculated with 0.1 ml of culture for each of the five tester strains. After mixing, the agar was poured across the surface of separate Minimal E plates labeled with lab number and appropriate tester strain. Once the agar solidified, sterile filter discs were placed in the center of the plates. A 0.1 ml aliquot of the rest of the test article solution was added to the filter discs on each of the labeled plates. Parallel testing was conducted with a negative control, and to demonstrate a positive zone of inhibition, 10X Dexon was utilized.

The plates were incubated at 37°C for 2 days. Following the incubation period, the zone of growth inhibition was observed and recorded. Only solutions that were no inhibitory to the tester strains were tested by the standard plate incorporation method.

IV. Method

A. Standard Plate Incorporation Assay
Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* or with tryptophan for the *Escherichia coli* were inoculated with 0.1 ml of culture for each of the five tester strains and 0.1 ml of the test article solution. A 0.5 ml aliquot of SW1 or S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across triplicate Minimal E plates labeled with lab number, appropriate tester strain, and S9 metabolic activation (when applicable). Parallel testing was also conducted with a negative control and five positive controls.

Histidine-free media plates (for *S. typhimurium*) and tryptophan-free media plates (for *E. coli*) were prepared in triplicate as follows:

1. Test article solution with and without S9 activation
2. Negative control with and without S9 activation
3. 1X Dexon (known mutagen) with and without S9 activation with strains TA98, TA100, and TA1537
4. 1X 2-Aminofluorene (known mutagen) with and without S9 activation with strain TA100
5. 1X Sodium azide (known mutagen) with and without S9 activation with strain TA1535
6. 1X 2-Aminoanthracene (known mutagen) with and without S9 activation with strain WP2*uvrA*.
7. 1X Methylmethane-sulfonate (known mutagen) with and without S9 activation with strain W02*uvrA*.

The plates were incubated at 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of revertants was determined. The mean number of revert ants of the test plates were compared to the mean number of revertants of the negative control for each of the five tester strains employed.

V. Evaluation

For the test article solution to be evaluated as a test failure or "potential mutagen", there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all five tester strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* tester strain employed, and at least a 2-fold increase over the respective negative control mean of the *E. coli* tester strain. Exceptions included conditions not intended to provoke a mutagenic response (e.g. 2-aminoanthracene and 2-aminofluorene without metabolic activation). The negative control results of each tester strain with the exception of tester strain WP2uvrA in the absence of metabolic activation exhibited a characteristic number of spontaneous revertants based on historical data collected at BioScreen.

VI. Results

A. Strain Characteristics and Strain Standard Plate Count

Salmonella typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2*uvrA* exhibited appropriate genetic characteristics pertaining to this assay (see Appendix 1).

B. Spot Plate Inhibition Screen

No significant inhibition was observed (see Appendix 2).

C. Standard Plate Incorporation Assay

The results are summarized in Appendix 3. In no case was there a 2-fold or greater increase in the mean number of revertants of tester strains TA98, TA100, TA1535, and WP2*uvrA* in the presence of the test article solution. Each positive control mean exhibited at least a 3-fold increase over the respective mean of the *S. typhimurium* tester strain employed and at least a 2-fold increase over the respective mean of the *E. coli* tester strain.

1. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article solution was considered to be nonmutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and to *Escherichia coli* strain WP2*uvrA*. The negative and positive controls performed as anticipated. This test was conducted to satisfy, in part, the requirements of the International Organization for Standardization (ISO) 10993, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

Appendix 1 – Strain Characteristics And Strain Standard Plate Counts

Characteristics (expected)	Tester Strains				
	TA98	TA100	TA1535	TA1537	WP2*uvrA*
Ampicilin-TA98 & TA100 = (Resistant) TA1535, TA1537 & WP2*uvrA* = (Sensitive)	R	R	S	S	S
rfa Mutation; CV (Sensitive)	S	S	S	S	NA
uvrB/uvrA (No Growth)	NG	NG	NG	NG	NG
Histidine Requirement; (Growth)	G	G	G	G	NA
Tryptophan Requirement; (Growth)	NA	NA	NA	NA	G
Biotin (No Growth)	NG	NG	NG	NG	NA
L-tryptophan (No Growth)	NA	NA	NA	NA	NG
Purity (Pure)	PURE	PURE	PURE	PURE	PURE
Total Plate Count CFU's (10^{-7})	68 32	61 87	241 211	18 20	301 294
Mean	50	74	226	19	298
Titer (Organisms/ml)	5.0×10^8	7.4×10^8	2.3×10^9	1.9×10^8	3.0×10^9

R = Resistant S = Sensitive NG = No Growth G = Growth NA = Not Applicable

Appendix 2 – Spot Plate Inhibition Screen Results

	Zone of Inhibition (mm)				
	TA98	TA100	TA1535	TA1537	WP2*uvrA*
Sponsor provided negative control	0	0	0	0	0
Test article solution	0	0	0	0	0
Dexon positive control	34	39	37	31	28

Appendix 3 – Standard Plate Incorporation Assay – Reversion Rates For Tester Strains

	Salmonella typhimurium								*Escherichia coli*	
	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
	CFTP	Mean	CFTP	Mean	CFTP	Mean	CFTP	Mean	CFTP	Mean
Sponsor provided negative control w/o S9	20 23 17	20	153 137 134	141	19 15 12	15	19 12 14	15	15 22 23	20
Sponsor provided negative control w/ S9	24 28 31	28	151 155 137	148	15 12 16	14	12 13 9	11	25 22 19	22
Test article solution w/o S9	28 20 18	22	138 152 167	152	20 31 25	25	12 15 10	12	25 20 19	21
Test article solution w/ S9	28 26 23	26	166 126 162	151	20 20 16	19	6 15 5	9	18 31 17	22
Dexon w/o S9 positive control	1792 1424 1824	1680	1120 1648 1312	1360			832 976 1056	955		
Dexon w/ S9 positive control	1696 1344 960	1333	1104 992 832	976			912 768 1072	917		
2-aminofluorene w/o S9 positive control*			173 180 192	182						
2-aminofluroene w/ S9 positive control†			1616 1552 1792	1653						
Sodium azide w/o S9 positive control					2080 3024 3472	2859				
Sodium azide w/ S9 positive control					2752 2544 3952	3083				
2-aminoanthracene w/o S9 positive control*									13 15 15	14
2-aminoanthracene w/ S9 positive control†									576 352 512	480
Methylmethane-Sulfonate w/o S9 positive control									864 640 544	683
Methylmethane-Sulfonate w/ S9 positive control									944 576 880	800

CFTP = Counts from triplicate plates Mean = Average of triplicate plates = Not Applicable
*Negative control for S9 †Positive control for S9

Wendy Yang, M.S.
Microbiology Manager

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50 HUMAN SUBJECT REPEAT INSULT PATCH TEST SKIN IRRITATION/SENSITIZATION EVALUATION (Occlusive Patch)

Date: October 13, 2006

AMA Ref. No.: MS06.RIPT.K9345O.50.ACTC

Sponsor: Active Concepts, LLC
121 Ethel Road West Suite 3
Piscataway, New Jersey 08854

1.0 Objective: Consumer products or raw materials designed for consistent reapplication to areas of the skin may, under proper conditions, prove to be contact sensitizers or irritants in certain individuals. It is the intention of a Repeat Insult Patch Test (RIPT) to provide a basis for evaluation of this irritation/ sensitization potential if such exists.

2.0 Test Material:

2.1 Test Material Description:

On August 9, 2006 one test sample labeled ABS Pomegranate Sterols, Code #10247, Lot #SN060706-5 was received from Active Concepts, LLC and assigned AMA Lab No. K-9345.

2.2 Handling:

Upon arrival at AMA Laboratories, Inc., the test material is assigned a unique laboratory code number and entered into a daily log identifying the lot number, sample description, sponsor, date received and tests requested.

Samples are retained for a period of three months beyond submission of final report unless otherwise specified by the sponsor or, if sample is known to be in support of governmental applications, representative retained samples are kept two years beyond final report submission. Sample disposition is conducted in compliance with appropriate federal, state and local ordinances.

2.3 Test Material Evaluation Prerequisite:

Prior to induction of a human test panel, toxicology, microbiology or in-vitro performance spectra may be required to assess the feasibility of commencement as dictated by an Institutional Review Board (IRB) described in Section 4.0.

2.31 Sponsor purports that prior to sample submission the following tests were conducted with no adverse results and that the test data are on file on their premises and have not been made available to AMA personnel:

- USP or CTFA Preservative Efficacy Test or equivalent
- 90 Day Accelerated Stability and Container Compatibility Study

3.0 Institutional Review Board:

Reference: CFR Title 21 Part 56, Subparts A, B, C, and D. The IRB of AMA Laboratories, Inc., consists of five or more individuals, chosen from within the company for technical expertise and from the local community for lay interaction. The list of IRB members is kept on file at AMA Laboratories, Inc. and is available for inspection during the hours of operation.

4.0 Panel Selection:

4.1 Standards for Inclusion in a Study:

-Individuals who are not currently under a doctor's care.
-Individuals free of any dermatological or systemic disorder which would interfere with the results, at the discretion of the Investigator.
-Individuals free of any acute or chronic disease that might interfere with or increase the risk of study participation.
-Individuals who will complete a preliminary medical history form mandated by AMA Laboratories, Inc. and are in general good health.
-Individuals who will read, understand and sign an informed consent document relating to the specific type of study they are subscribing. Consent forms are kept on file and are available for examination on the premises of AMA Laboratories, Inc. only.
-Individuals able to cooperate with the Investigator and research staff, be willing to have test materials applied according to the protocol, and complete the full course of the study.

4.2 Standards for Exclusion from a Study:

-Individuals under 18 years of age.
-Individuals who are currently under a doctor's care.
-Individuals who are currently taking any medication (topical or systemic) that may mask or interfere with the test results.
-Subjects with a history of any acute or chronic disease that might interfere with or increase the risk associated with study participation.
-Individuals diagnosed with chronic skin allergies.
-Female volunteers who indicate that they are pregnant or lactating.

4.3 Recruitment:

Panel selection is accomplished by advertisements in local periodicals, community bulletin boards, phone solicitation, electronic media or any combination thereof.

4.4 Informed Consent and Medical History Forms:

An informed consent was obtained from each volunteer prior to initiating the study describing reasons for the study, possible adverse effects, associated risks and potential benefits of the treatment and their limits of liability. Panelists signed and dated the informed consent document to indicate their authorization to proceed and acknowledge their understanding of the contents. Each subject was assigned a permanent identification number and completed an extensive medical history form. These forms along with the signed consent forms, are available for inspection on the premises of AMA Laboratories, Inc. only. Reference 21 CFR Ch. 1 Part 50, Subpart B.

The parties agree to comply with applicable state and federal privacy laws for the use and disclosure of a subject's personal health information by taking reasonable steps to protect the confidentiality of this information. This obligation shall survive the termination or expiration of this Agreement.

5.0 Population Demographics:

Number of subjects enrolled		52
Number of subjects completing study		50
Age Range		18-65
Sex	Male	7
	Female	45
Race	Caucasian	45
	Hispanic	7
	Asian	0

6.0 Equipment:

-Patch Description: Parke-Davis Hypoallergenic Readi Bandages or the equivalent.
-1ml volumetric syringe without a needle.

7.0 Procedure:

-Subjects are requested to bathe or wash as usual before arrival at the facility.
-As per client request the test material K-9345 was heated in a warm bath to liquefy.
-0.2 ml or 0.2g of the test material is dispensed onto the occlusive, hypoallergenic patch.
-The patch is then applied directly to the skin of the infrascapular regions of the back, to the right or left of the midline and the subject is dismissed with instructions not to wet or expose the test area to direct sunlight.
-After 24 hours the patch is removed by the panelist at home.
-This procedure is repeated until a series of nine consecutive 24 hour exposures have been made for every Monday, Wednesday, and Friday for three consecutive weeks.
-In the event of an adverse reaction, the area of erythema and edema is measured. The edema is estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Reactions are scored just before applications two through nine and the next test date following application nine. In most instances this is approximately 24 hours after patch removal. Clients are notified immediately in the case of adverse reaction and determination is made as to treatment program if necessary.
-Subjects are then given a 10 - 14 day rest period after which a challenge or retest dose is applied once to a previously unexposed test site. The retest dose is equivalent to any one of the original nine exposures. Reactions are scored 24 and 48 hours after application.
-Comparison is made between the nine inductive responses and the retest dose.

8.0 Results:

Please refer to attached Table.

9.0 Observations:

No adverse reactions of any kind were noted during the course of this study.

10.0 Archiving:

All original samples, raw data sheets, technician's notebooks, correspondence files and copies of final reports and remaining specimens are maintained on premises of AMA Laboratories, Inc. in limited access storage files marked "Archive". A duplicate disk copy of final reports is separately archived in a bank safe deposit vault.

11.0 Reference:

Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics, published by The Association of Food and Drug Officials of The United States, 1965 (modified).

12.0 Conclusions:

The test material (AMA Lab. No.: K-9345; Client No.: ABS Pomegranate Sterols, Code #10247, Lot #SN060706-5) when tested under occlusion as described herein, may be considered as a **NON-PRIMARY IRRITANT** and **NON-PRIMARY SENSITIZER** to the skin according to the reference.

Mayya Tatsene, M.D.
Study Director

Ilona Lapkisa, Pharm.D.
Technician

David R. Winne, B.S.
Technical Director

1/13/16
Date

TABLE
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: K-9345
Client No.: ABS Pomegranate Sterols, Code #10247, Lot #SN060706-5

No.	Subject ID	RACE	SEX	Response 1	2	3	4	5	6	7	8	9	Chall. 24 HR	Chall. 48 HR	Score
1	00 0002	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
2	25 0215	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
3	28 0971	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
4	32 3068	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
5	34 3421	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
6	34 8917	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
7	36 1827	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
8	36 3693	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
9	36 7970	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
10	38 0044	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
11	38 1583	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
12	40 2487	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
13	40 4371	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
14	42 0442	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
15	42 1837	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
16	44 6021	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
17	44 7823	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
18	44 8248	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0
19	44 9258	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
20	46 4172	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
21	46 5776	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
22	46 8520	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
23	46 8676	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
24	48 1605	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
25	50 1729	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
26	50 6005	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
27	50 9982	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
28	52 2712	C	F	0	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
29	52 3942	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
30	52 4898	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
31	52 5549	C	F	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
32	54 0763	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0

TABLE (CONT'D)
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: K-9345
Client No.: ABS Pomegranate Sterols, Code #10247, Lot #SN060706-5

No.	Subject ID	RACE	SEX	Response 1	2	3	4	5	6	7	8	9	Chall. 24 HR	48 HR	Score
33	54 9327	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
34	54 9626	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
35	56 3659	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
36	58 7412	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
37	58 9750	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
38	60 9426	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
39	62 4500	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
40	62 6182	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
41	62 7431	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
42	64 5370	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
43	64 6653	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
44	64 8003	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
45	66 4641	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
46	68 1557	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
47	68 4299	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
48	70 5391	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
49	70 6130	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
50	72 5343	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
51	76 2801	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
52	88 4232	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0

Evaluation Period:

This study was conducted from September 6, 2006 through October 12, 2006.

Scoring Scale and Definition of Symbols Shown in Table:

0 - No evidence of any effect

? - (Barely perceptible) minimal faint (light pink) uniform or spotty erythema

1 - (Mild) pink uniform erythema covering most of contact site

2 - (Moderate) pink\red erythema visibly uniform in entire contact area

3 - (Marked) bright red erythema with accompanying edema, petechiae or papules

4 - (Severe) deep red erythema with vesiculation or weeping with or without edema

D - Patch eliminated due to reaction

Dc - Discontinued due to absence of subject on application date

M - Patch applied to an adjacent site after strong test reaction

NA - Score is not calculated for subjects discontinued before challenge

S - Skin stained from pigment in product

T - Tan

NOTE: All technical employees of AMA LABORATORIES, INC. are required to take and pass a visual discrimination examination conducted by a Board Certified Ophthalmologist using the Farnsworth-Munsell 100 Hue Test as published; which determines a person's ability to discern color against a black background. This test was additionally modified to include a flesh tone background more nearly approaching actual use conditions, wherein erythematous skin is graded according to intensity.

13.0 Quality Assurance Statement:

This study was inspected in accordance with the Standard Operating Procedures of AMA Laboratories, Inc.
To assure compliance with the study protocol, the Quality Assurance Unit completed an audit of the study records and report.

Report reviewed by:

Polina Elistratova, M.A.
Quality Assurance Supervisor

Date

PRODUCT INVESTIGATIONS, INC
151 East Tenth Avenue
Conshohocken, PA 19428
610-825-5855 • fax 610-825-7288

Report No. PII 21175

Evaluation of the Skin-irritating Propensities of ABS Pomegranate Sterols on Scarified Skin

Performed for:

Active Concepts
Research and Development Center
121 Ethel Road West
Suite 3
Piscataway, New Jersey 08854

22 August 2014

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Evaluation of the Skin-irritating Propensities of ABS Pomegranate Sterols on Scarified Skin

Objectives:

1. To determine what, if any, locally manifested adverse effects can be elicited by the test material during, or as a consequence of, occluded contact on uncleansed, but scarified areas of the skin of human volunteer subjects.

2. To obtain an Index of Irritation for the test material.

Sponsor: Active Concepts
121 Ethel Road West
Suite 3
Piscataway, New Jersey 08854

Project Director: Kristen Potts, Technical Marketing

Authorization: Letter from K. Potts dated 9 August, 2006

Method: Chamber Scarification Test (1)

Study Sample: ABS Pomegranate Sterols

Received: 8/10/06
Tested As: as supplied

Control Material: Identification: Physiological Saline, Negative Control

Testing Dates: Start Date: 15 August 2006 Completion Date: 18 August 2006

Site of Study:	Product Investigations, Inc 151 East Tenth Avenue Conshohocken, Pennsylvania 19428
Investigator:	Joseph E. Nicholson III
Medical Director:	Morris V. Shelanski, MD FACP
Laboratory Director:	Samuel D. Shelanski
Quality Assurance:	Samuel J. Charles, III
Panel No.:	06198
Equipment:	
Chambers	Prepared by Product Investigations, Inc Chambers were stamped from sheet stainless steel 0.3 mm thick. Each chamber has a well 12 mm in diameter and 1.7 mm deep. Overall diameter is 18 mm, affording a flange 3 mm wide encircling the well.
Calibration Liquid	Physiological Saline, Sterile
Skin Tape	Scanpor (R) Surgical Tape
Functions of Investigator:	1. Selection and preparation of test sites 2. Scarification of contact sites 3. Preparation of chambers 4. Application of test and control materials 5. Removal of chambers 6. Examination and grading of responses 7. Recording of responses. 8. Interpret the results and formulate the conclusions

Selection of Subjects:

Individuals seeking to take part in this study were informed in detail of the purpose of the evaluation, what was required of them, and the foreseeable risks to their health and comfort which might occur as a consequence of their participation.

Inclusion Criteria:

Each volunteer who met these requirements was considered as a prospective participant:

1. Has reached or passed the age of majority in Pennsylvania (18 years).
2. Has expressed willingness to cooperate with the investigator and to comply with the regimen.
3. Has provided the investigator that they are dependable and will persevere despite the inconvenience and discomfort associated with the procedure.
4. Has demonstrated the ability to understand the purpose of the procedure and what was required of them in regard to attendance and compliance.
5. Has demonstrated the ability to understand the risks involved and to make a decision to participate which was not influenced solely by financial considerations.
6. Has demonstrated the ability to read and understand the items in the consent form.

Exclusion Criteria:

A history of past and present health status and medication intake was obtained from, and a brief physical examination was performed on, each prospective participant to detect such abnormalities which would constitute cause for exclusion, to wit:

1. Systemic illness which contra-indicated participation.
2. Skin disease with manifestations which might be misconstrued as an irritant effect of the test material.
3. Ongoing intake of medications which could either enhance or suppress the adverse propensities of the test material.
4. Grossly visible skin pathology in the areas of the body which were designated for contact with the test sample.

Informed Consent:

Each candidate was required to read a prepared request for her/his consent. Only after signing this consent form was any candidate engaged to participate in this evaluation.

Panel Composition: Ten (10) qualified adults were engaged to participate in this study as members of Panel No. 06198.

Panel Dedication: This was an exclusive panel, i.e. the subjects were not engaged in the evaluation of materials submitted by sponsors other than Estee Lauder Companies or their suppliers.

Preparatory Procedures:

Preparation of Test Material: The test material was tested as supplied.

Preparation of Test Patches: Clean webril material was clumped out into pledgets suitable for the chamber well. For liquids, the pads were moistened with 0.1 ml of the appropriate test material immediately prior to application. For sprays, lotions, creams, or powders, the materials were applied directly to the skin and covered with a pledget.

Preparation of Control Patches: Clean webril material was clumped out into pledgets suitable for the chamber well. These were moistened with 0.1 ml of the appropriate control material immediately prior to application.

Site Delineator: A distinct site on the volar surface of the forearm of each individual was selected and designated for contact with the test and control materials.

Instructions to Subjects: Each subject was given these instructions before the study was begun and after each application:

1. Keep your daily appointment at the hour specified.

2. Do not disturb the chambers after they are applied, unless you experience difficulty. If that happens,

   a. Remove the chamber if it is bothering you.
   b. Wash the site thoroughly with mild soap and warm water.
   c. Rinse thoroughly and gently blot dry.
   d. Call the investigator and tell him of the emergency.

3. If a chamber is loose but still in its proper place, reinforce it.

4. If a chamber falls off or is out of place, remove it, make a note of the time, and return it to the laboratory.

Baseline Determination: A chamber containing webril pads, which had been moistened with 0.1 ml of sterile physiological saline, was placed on each scarified skin site for four (4) hours. After four (4) hours, the chambers were removed and the sites were examined. This enabled the investigator to establish a baseline value for the site.

Procedure Design: The procedure was a double-blind non-randomized evaluation in a group of volunteers selected from the local population.

Outline of Test Procedure: The test material was applied on Day 1, Day 2, and Day 3 on the same scarified skin site on the forearm of each participant. After each twenty-four hours of contact, the test material was removed, e.g. on Day 2, Day 3, and Day 4. Thirty minutes after each removal, the skin site was examined and graded. An identical procedure was conducted concurrently using physiological saline on an additional site.

Procedure: Daily Regimen for Each Panelist - (All Subjects)

Day 1:

The skin of the volar surface of the forearm of each panelist was examined and nine sites devoid of any visible pathology were selected for each of the test and control materials.

The skin was cleansed with alcohol and each site was outlined. The skin of each site was scratched with the sharp bevelled edge of a 30-gauge needle. A newly opened sterile needle was used for each participant. Three (3) strokes were made horizontally and three (3) vertically in cross-hatch formation. Just enough pressure was applied to the needle to cleave the epidermis without eliciting frank capillary bleeding. Each site was then covered with a chamber containing webril pads which had been moistened with 0.1 ml of sterile physiological saline. The chambers were secured lightly to the sites with white strips of Hypalix®. Tape. After four (4) hours, the chambers were removed and the scarified sites were examined and graded for adverse changes. This established whether or not inter-site differences existed and, if so, provided a means to compensate for the differences in the calculation of indices of irritation.

After the baseline value of each site had been established, approximately 0.1 ml of a liquid test/control material was placed on the webril pledget of a chamber device and mounted on its respectively designated site. Approximately 0.1 ml of a lotion or cream was applied directly to the skin, covered with a webril pledget as chamber device, and mounted to its respective site. Pressed powders and paper tablets were also applied directly to the skin and covered with a chamber.

Day 2 & Day 3:

As each panelist returned, the arms were examined to determine whether any chambers had been lost or loosened. All chambers were removed by the project scientist to expose the scarified contact sites for examination and grading.

The test or control materials on each test site which had received a grade of ≤ 2 were reapplied. Material was added to the webril pad of a patching device which was then positioned on its assigned site; materials that were not prone to run were applied directly to a site and covered with a patching device. The test or control materials on each test site which had received a grade of ≥ 3 were terminated.

Day 4:

As each panelist returned, all chambers were removed by the project scientist to expose the scarified contact sites for final examination and grading. Each site was examined and graded. The severity of the damage present at that time was evaluated by the investigator to determine whether therapeutic measures were deemed necessary.

This completed the scheduled portion of the study. Each panelist was instructed to return to the clinic if damage persisted or became worse.

Alternate Procedures:

None were necessary.

Criteria for Grading of Responses:

The responses were assigned numerical grades to indicate the intensities of the adverse effects in accordance with these criteria:

0	No scarification marks or erythema visible.
0.5	Scarification marks visible but no erythema present.
1	Faint erythema along the scarification marks
2	Moderate to intense erythema adjacent to the scratch marks, areas between scratch marks unaffected.
3	Confluent, severe erythema filling areas between scratch marks.
4	Severe erythema associated with pustules or blisters.

Methods for Interpreting Results:

1. Categories of Irritating Potentialities: The following is a modification of the scheme presented by Frosch & Kligman in their publication

Mean Score of Irritation Index	Category of Irritating Potentialities
0.0 to 0.49	Very Low
0.5 to 1.49	Low
1.5 to 2.49	Moderate
2.5 to 4.0	High

2. Significance of Differences Between Mean Scores: In the grading of responses in this procedure, we have found that differences having values less than 0.5 cannot be ascertained consistently. Therefore, we believe that the following are justifiable.

   a. When the mean scores differ by less than 0.5, the difference between two test materials is not clinically significant.

   b. When the mean scores differ by greater than 0.5 and less than 1.0, the difference between two test materials is marginally significant.

   c. When the mean scores differ by greater than 1.0, the difference between two test materials is clinically significant.

   d. Whether a difference is significant or not, ranking is useful in indicating what changes in formulation may yield a milder product.

Results: Compilation of Responses and Calculations

Physiological Saline

Subject Number	Bs* Baseline	a 24 Hour	b 48 Hour	c 72 Hour
1	0.5	0.5	0.5	0.5
2	0.5	0.5	0.5	0.5
3	0.5	0.5	0.5	0.5
4	0.5	0.5	0.5	0.5
5	0.5	0.5	0.5	0.5
6	0.5	0.5	0.5	0.5
7	0.5	0.5	0.5	0.5
8	0.5	0.5	1	0.5
9	0.5	0.5	0.5	0.5
10	0.5	0.5	0.5	0.5
Ln 1. Mean Scores Control:	0.50	0.50	0. 55	0.50

XBS Pomegranate Sterols Site 10

Subject Number	Bs* Baseline	a 24 Hour	b 48 Hour	c 72 Hour
1	0.5	0.5	0.5	0.5
2	0.5	0.5	0.5	0.5
3	0.5	0.5	0.5	0.5
4	0.5	0.5	0.5	0.5
5	0.5	0.5	0.5	0.5
6	0.5	0.5	0.5	0.5
7	0.5	0.5	0.5	0.5
8	0.5	0.5	0.5	0.5
9	0.5	0.5	0.5	0.5
10	0.5	0.5	0.5	0.5
Ln 2. Mean Scores, Test Material:	0.50	0.50	0.50	0.50
Ln 3. Adjustment Value: (Bs Ln 1 - Bs Ln 2)	0	0	0	0
Ln 4. Adjusted Value: (Ln 2 + Ln 3)	0.50	0.50	0.50	0.50

* Physiological Saline

Report #1 2115
Date: 21 August 2006

Results:

Comparative Mildness Ranking

Test Material Identification	Irr. Index	Irritation Potential
Saline Control	0.50	Low
ABS Pomegranate Seeds	0.50	Low

Conclusion:

At the end of Seventy Two hours, the effects of Sample ABS Pomegranate Seeds on scarified skin were equal to those of the saline control.

PRODUCT INVESTIGATIONS, INC.

8/22/06
Date

Joseph E. Nicholson III
Investigator

8

Statement of Compliance with Quality Assurance Practices

I have periodically monitored the conduct and progress of this study and have observed neither protocol violations nor deficiencies in the manner in which it was conducted. Further, I have audited the data presented in this report and believe that, to the best of my knowledge, they are accurate transcriptions of the raw data generated during the course of this study.

Statement of Compliance with Good Clinical Practices:

This study was conducted in compliance with the standards of good clinical practices generally applicable for the protection of the privileges and well-being of individuals who participate in patch test procedures.

Samuel J. Charles III
Director, Quality Assurance

References:

1 The Chamber-Scarification Test for Irritancy
Frosch, P.J. and Kligman, A.M. CONTACT DERMATITIS, 1976; 2 pp 314-324

PRODUCT INVESTIGATIONS, INC.
SCARIFICATION TEST RESULTS

Panel No.: 06198

Sponsor: Active Concepts **Dates: Start: 08/15/2006** **End: 08/16/2006**

Subject #	Inits	Age	Sex	Race	Site: 9 Sample: Saline Control B/L	24hr	48hr	72hr	Site: 10 Sample: ABS Pomegranate sterols B/L	24hr	48hr	72hr									
1.	JVB	48	E	C	½	½			½	½											
2.	A-F	42	F	C	½	½			½	½											
3.	AR	68	F	C	½	½			½	½											
4.	DO	47	F	C	½	½			½	½											
5.	L-S	49	F	C	½	½			½	½											
6.	PLP	52	F	C	½	½			½	½											
7.	RIE	58	F	C	½	½			½	½											
8.	AEL	65	F	C	½	½			½	½											
9.	JJP	45	M	C	½	½			½	½											
10.	JSD	60	F	C	½	½			½	½											
				Daily Mean:	0.5	0.5			0.5	0.5											
				72 hr. mean score:																	
					Adjusted Mean:				**Adjusted Mean:**												

Criteria for Grading responses on scarified skin sites

Score	erythema	location and area of involvement	Mean Score	Category of Irritating Potentialities
½	absent	only non-irritated scratches are visible. No irritation		
1	faint or slight	scarification only, normal skin of interstitial spaces not involved	0.0 – 0.49	Very Low
2	moderatete	scarification only, normal skin of interstitial spaces not involved	0.5 – 1.49	Low
3	moderate to intense	scarified and normal skin of interstitial spaces involved	1.5 – 2.49	Moderate
4	moderate to intense	entire contact surface if accompanied by erosion or blistering	2.5 – 4.0	High

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September 11, 2006

To Whom It May Concern:

Sample lot SNO60506-1 of ABS Pomegranate Sterols was submitted for safety testing. The sample was supplied neat.

Best Regards,

Christopher Murphy
Technical Director

This is an Electronically Generated Document

Dermal and Ocular Irritation Tests

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Sample: ABS Pomegranate Sterols

Code: 10247

CAS #: 84961-57-9

Test Request Form/Submission #: 320

Lot #: SN120618-4

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Meghan Darley*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ABS Pomegranate Sterols** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances.

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They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for

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60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

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The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

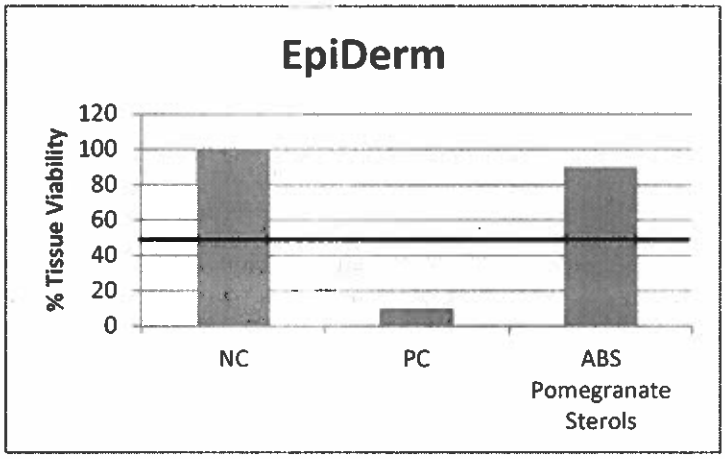

Figure 1: EpiDerm tissue viability

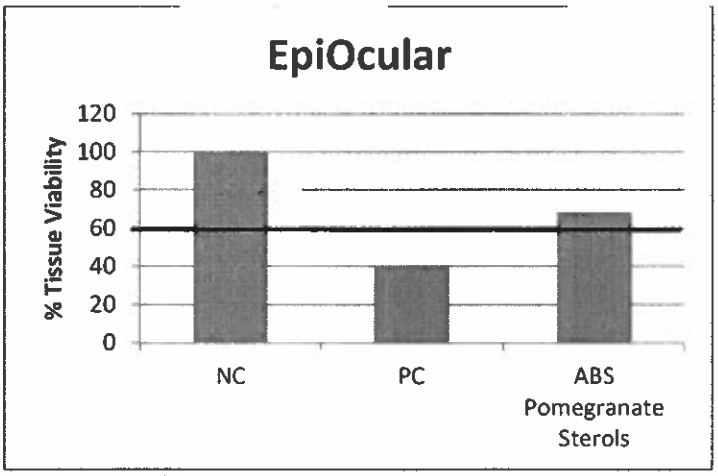

Figure 2: EpiOcular tissue viability

Neutral Red Cytotoxicity Assay for ABS Pomegranate Sterols

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Abstract:
To determine the potential toxicity of ABS Pomegranate Sterols a neutral red cytotoxicity assay was performed using HepG2 cells. Neutral Red (NR) is a weak cationic dye that is capable of penetrating cellular membranes via non-ionic diffusion and it accumulates in lysosomes where it binds at the anionic regions in the lysosomal matrix. Cellular viability is determined by the amount of NR that penetrates the cells, as dying and dead cells have decreased or no NR uptake.

Materials and Methods:
Microtitre tissue culture plates with 96 wells were used to grow the HepG2 cells to 60% confluence. The HepG2 cells were maintained in 0.1% trypsin and 0.04% EDTA, the medium was then replaced with varying concentrations of of ABS Pomegranate Sterols in DMSO ranging from 0.01 to 40μM. As a control a well was filled with medium, but not HepG2 cells. Following 24 hours of incubation, the medium was replaced with 0.33% Neutral Red Solution (Sigma N2889). After 3 additional hours of incubation the cells were washed and fixed with Neutral Red Assay Fixative (Sigma N4270), this removes the dye from damaged or dead cells which have lost their ability to retain NR. The fixative was then replaced with Neutral Red Assay Solubilization Solution (Sigma N4395) to extract the dye from the intact, viable cells. The plate was then left at room temperature for 15 minutes before it was placed in a microplate shaker for an additional 30 minutes. A spectrophotometer with a 540nm filter was used to measure the absorbance of solubilized dye.

Results:

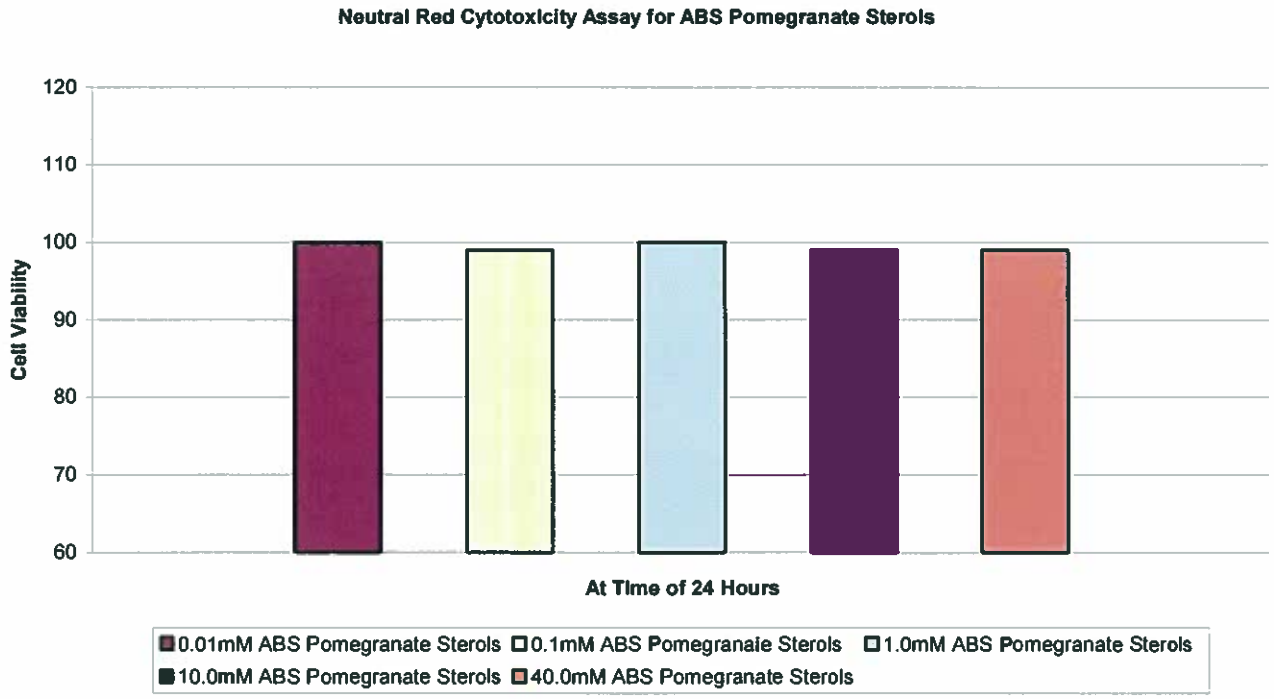

Figure 1. Results for Neutral Red Cytotoxicity Assay.

Discussion:
The results of the neutral red cytotoxicity assay indicate that ABS Pomegranate Sterols do not exhibit cytotoxic effect on HepG2 cells grown to 60% confluence.

Version#1/09-12-06

Dermal and Ocular Irritation Tests

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Sample: AC Soybean Sterols

Code: 10607

CAS #: 8001-22-7

Test Request Form/Submission #: 492

Lot #: NC130319-D

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Meghan Darley*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **AC Soybean Sterols** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be a **non-irritant**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37 °C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can

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differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for

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60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

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Dermal and Ocular Irritation Tests

A. Tissue Characteristics
The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay
The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity
The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

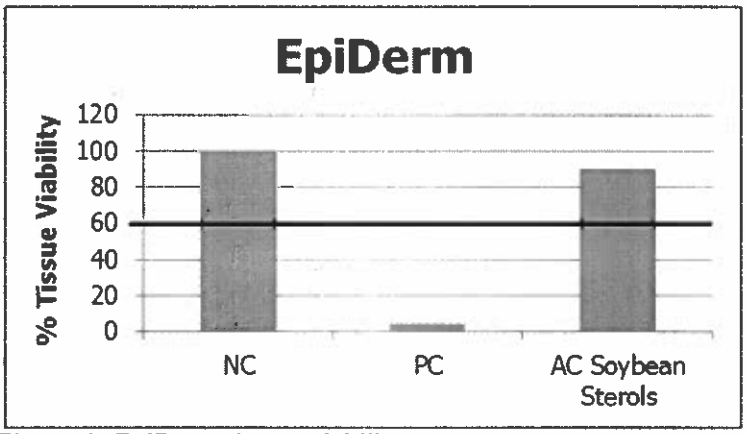

Figure 1: EpiDerm tissue viability

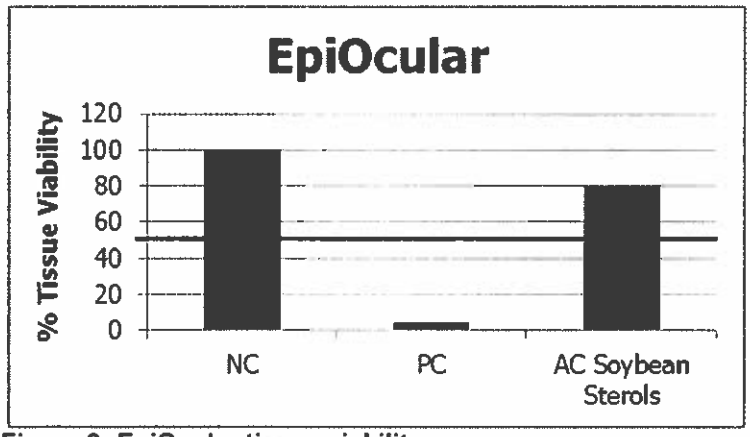

Figure 2: EpiOcular tissue viability

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Dermal and Ocular Irritation Tests

Sample: ABS Acai Sterols

Code: 10408

CAS #: 999999-99-4

Test Request Form/Submission #: 491

Lot #: 29332

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Meghan Darley*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ABS Acai Sterols** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be a **non-irritant**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37 °C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of **MTT** [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

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Dermal and Ocular Irritation Tests

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

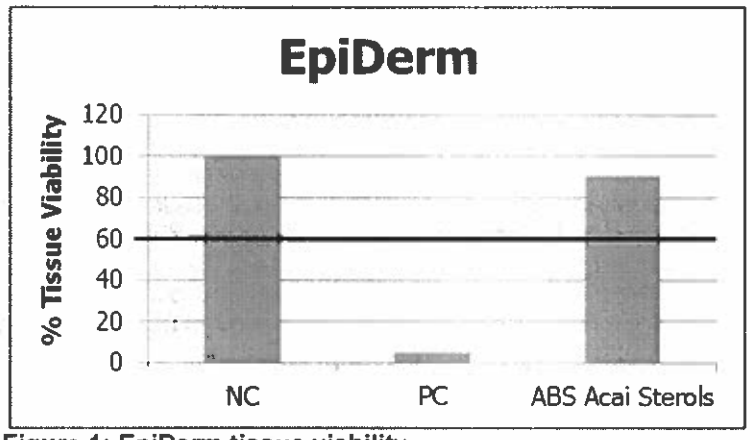

Figure 1: EpiDerm tissue viability

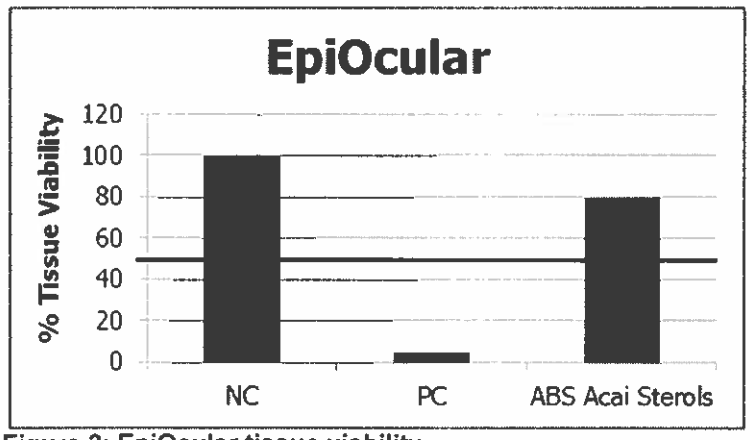

Figure 2: EpiOcular tissue viability

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Lillian Gill, Ph.D.
Director - COSMETIC INGREDIENT REVIEW (CIR)

FROM: Halyna Breslawec, Ph.D.
Industry Liaison to the CIR Expert Panel

DATE: July 9, 2013

SUBJECT: Impurities, Method of Manufacture Euterpe Oleracea Sterols, Glycine Soja (Soybean) Sterols and Punica Granatum Sterols (materials tested in studies provided with plant phytosterols memo 2)

Active Concepts. 2013. Compositional breakdown ABS Acai Sterols (Euterpe Oleracea Sterols).

Active Concepts. 2009. ABS Acai Sterols manufacturing flow chart.

Active Concepts. 2013. Compositional breakdown AC Soybean Sterols (Glycine Soja (Soybean) Sterols).

Active Concepts. 2013. ABS Soybean Sterols manufacturing flow chart.

Active Concepts. 2006. Compositional breakdown ABS Pomegranate Sterols (Punica Granatum Sterols).

Active Concepts. 2010. ABS Pomegranate Sterols manufacturing flow chart.

1101 17th Street, N.W., Suite 300 | Washington, D.C. 20036-4702 | 202.331.1770 | 202.331.1969 (fax) | www.personalcarecouncil.org

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Compositional Breakdown

ABS Acai Sterols
Code: 10408

Compositional Breakdown:

Ingredient	%
Euterpe Oleracea Sterols	100.00

*** To our knowledge the above material is free of materials classified as CMR in accordance with the Directive 2004/93 of 21 September 2004.**

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Compositional Breakdown

This is to certify that the following allergens were not detected in ABS Acai Sterols:

ALLERGENS Dir 2003 15 CEE	
INCI NAME	CAS NUMBER
Alpha-IsoMethyl Ionone	127-51-5
Amyl Cinnamal	122-40-7
Anise Alcohol	105-13-5
Benzyl Alcohol	100-51-69
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Butylphenyl Methylpropional	80-54-6
Cinnamal	104-55-2
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9
Coumarin	91-64-5
Eugenol	97-53-0
Farnesol	4602-84-0
Geraniol	106-24-1
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Hydroxymethylpentyl 3-Cyclohexene carboxaldehyde	31906-04-4
Isoeugenol	97-54-1
Limonene	5989-27-5
Linalool	78-70-6
Methyl 2 Octynoate	111-12-6
Evernia prunastri	90028-68-5
Evernia furfuracea	90028-67-4
Amylcinnamyl Alcohol	101-85-9

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Compositional Breakdown

This is to certify that ABS Acai Sterols does not contain pesticide levels exceeding the following:

EPA Pesticide Levels	
INCI NAME	LIMIT (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin	0.05
Azinphos-methyl	1.00
Bromopropylate	3.00
Chlordane(cis and trans)	0.05
Chlorfenvinphos	0.50
Chlorpyrifos	0.20
Chlorpyrifos-methyl	0.10
Cypermethrin	1.00
DDT	1.00
Deltamethrin	0.50
Diazinon	0.50
Dichlorvos	1.00
Dithiocarbamates	2.00
Endosulfan	3.00
Endrin	0.05
Ethion	2.00
Fenitrothion	0.50
Fenvalerate	1.50
Fonofos	0.05
Heptachlor	0.05
Hexachlorobenzene	0.10
Hexachlorocyclohexane	0.30
Lindane	0.60
Malathion	1.00
Methidathion	0.20
Parathion	0.50
Parathion-methyl	0.20

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Compositional Breakdown

Permethrin	1.00
Phosalone	0.10
Piperonyl butoxide	3.00
Pirimiphos-methyl	4.00
Pyrethrins	3.00
Quintozene(sum of 3 items)	1.00

Active Concepts

10408-ABS Acai Sterols- Manufacturing Flow Chart

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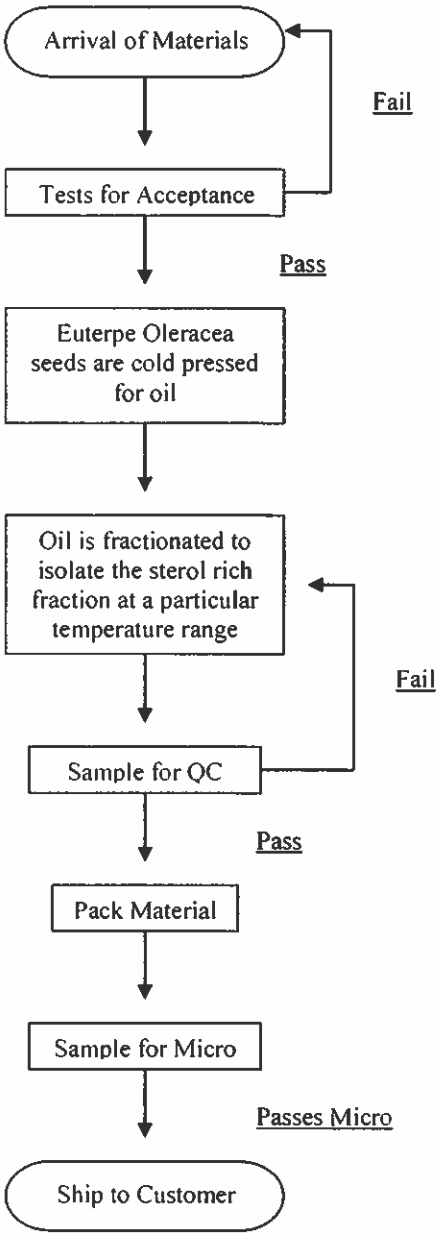

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Compositional Breakdown

AC Soybean Sterols
Code: 10607

Compositional Breakdown:

Ingredient	%
Glycine Soja (Soybean) Sterols	100.00

*** To our knowledge the above material is free of materials classified as CMR in accordance with the Directive 2004/93 of 21 September 2004.**

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Compositional Breakdown

This is to certify that the following allergens were not detected in AC Soybean Sterols:

ALLERGENS Dir 2003 15 CEE	
INCI NAME	CAS NUMBER
Alpha-IsoMethyl Ionone	127-51-5
Amyl Cinnamal	122-40-7
Anise Alcohol	105-13-5
Benzyl Alcohol	100-51-69
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Butylphenyl Methylpropional	80-54-6
Cinnamal	104-55-2
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9
Coumarin	91-64-5
Eugenol	97-53-0
Farnesol	4602-84-0
Geraniol	106-24-1
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Hydroxymethylpentyl 3-Cyclohexene carboxaldehyde	31906-04-4
Isoeugenol	97-54-1
Limonene	5989-27-5
Linalool	78-70-6
Methyl 2 Octynoate	111-12-6
Evernia prunastri	90028-68-5
Evernia furfuracea	90028-67-4
Amylcinnamyl Alcohol	101-85-9

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Compositional Breakdown

This is to certify that AC Soybean Sterols does not contain pesticide levels exceeding the following:

EPA Pesticide Levels	
INCI NAME	LIMIT (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin	0.05
Azinphos-methyl	1.00
Bromopropylate	3.00
Chlordane(cis and trans)	0.05
Chlorfenvinphos	0.50
Chlorpyrifos	0.20
Chlorpyrifos-methyl	0.10
Cypermethrin	1.00
DDT	1.00
Deltamethrin	0.50
Diazinon	0.50
Dichlorvos	1.00
Dithiocarbamates	2.00
Endosulfan	3.00
Endrin	0.05
Ethion	2.00
Fenitrothion	0.50
Fenvalerate	1.50
Fonofos	0.05
Heptachlor	0.05
Hexachlorobenzene	0.10
Hexachlorocyclohexane	0.30
Lindane	0.60
Malathion	1.00
Methidathion	0.20
Parathion	0.50
Parathion-methyl	0.20

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Compositional Breakdown

Permethrin	1.00
Phosalone	0.10
Piperonyl butoxide	3.00
Pirimiphos-methyl	4.00
Pyrethrins	3.00
Quintozene(sum of 3 items)	1.00

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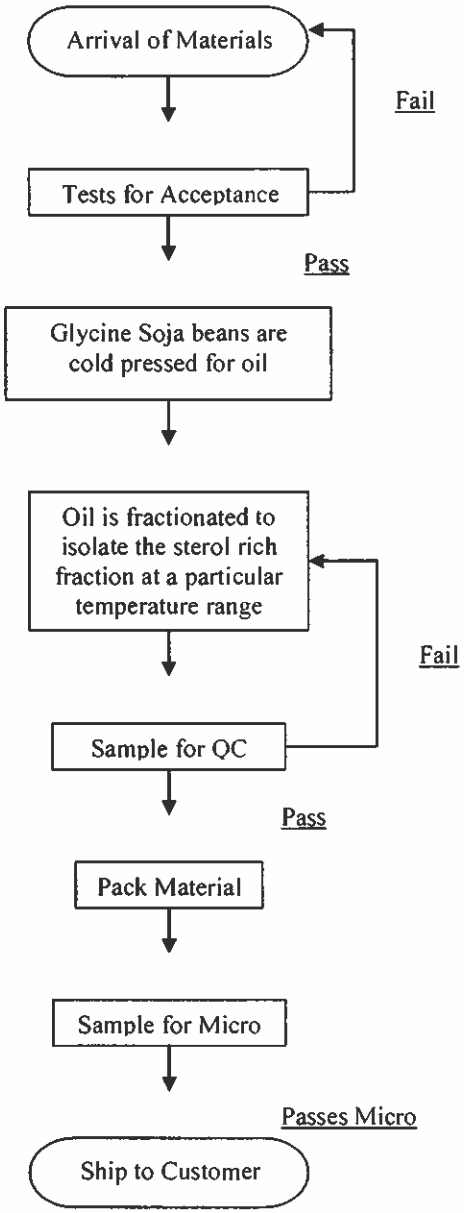
10607-ABS Soybean Sterols- Manufacturing Flow Chart



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Compositional Breakdown

ABS Pomegranate Sterols
Code: 10247

Compositional Breakdown:

Ingredient	%
Punica Granatum Sterols	100.00

*** To our knowledge the above material is free of materials classified as CMR in accordance with the Directive 2004/93 of 21 September 2004.**

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Compositional Breakdown

This is to certify that the following allergens were not detected in ABS Pomegranate Sterols:

ALLERGENS Dir 2003 15 CEE	
INCI NAME	CAS NUMBER
Alpha-IsoMethyl Ionone	127-51-5
Amyl Cinnamal	122-40-7
Anise Alcohol	105-13-5
Benzyl Alcohol	100-51-69
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Butylphenyl Methylpropional	80-54-6
Cinnamal	104-55-2
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9
Coumarin	91-64-5
Eugenol	97-53-0
Farnesol	4602-84-0
Geraniol	106-24-1
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Hydroxymethylpentyl 3-Cyclohexene carboxaldehyde	31906-04-4
Isoeugenol	97-54-1
Limonene	5989-27-5
Linalool	78-70-6
Methyl 2 Octynoate	111-12-6
Evernia prunastri	90028-68-5
Evernia furfuracea	90028-67-4
Amylcinnamyl Alcohol	101-85-9

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Compositional Breakdown

This is to certify that ABS Pomegranate Sterols does not contain pesticide levels exceeding the following:

EPA Pesticide Levels	
INCI NAME	LIMIT (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin	0.05
Azinphos-methyl	1.00
Bromopropylate	3.00
Chlordane(cis and trans)	0.05
Chlorfenvinphos	0.50
Chlorpyrifos	0.20
Chlorpyrifos-methyl	0.10
Cypermethrin	1.00
DDT	1.00
Deltamethrin	0.50
Diazinon	0.50
Dichlorvos	1.00
Dithiocarbamates	2.00
Endosulfan	3.00
Endrin	0.05
Ethion	2.00
Fenitrothion	0.50
Fenvalerate	1.50
Fonofos	0.05
Heptachlor	0.05
Hexachlorobenzene	0.10
Hexachlorocyclohexane	0.30
Lindane	0.60
Malathion	1.00
Methidathion	0.20
Parathion	0.50
Parathion-methyl	0.20

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Compositional Breakdown

Permethrin	1.00
Phosalone	0.10
Piperonyl butoxide	3.00
Pirimiphos-methyl	4.00
Pyrethrins	3.00
Quintozene(sum of 3 items)	1.00

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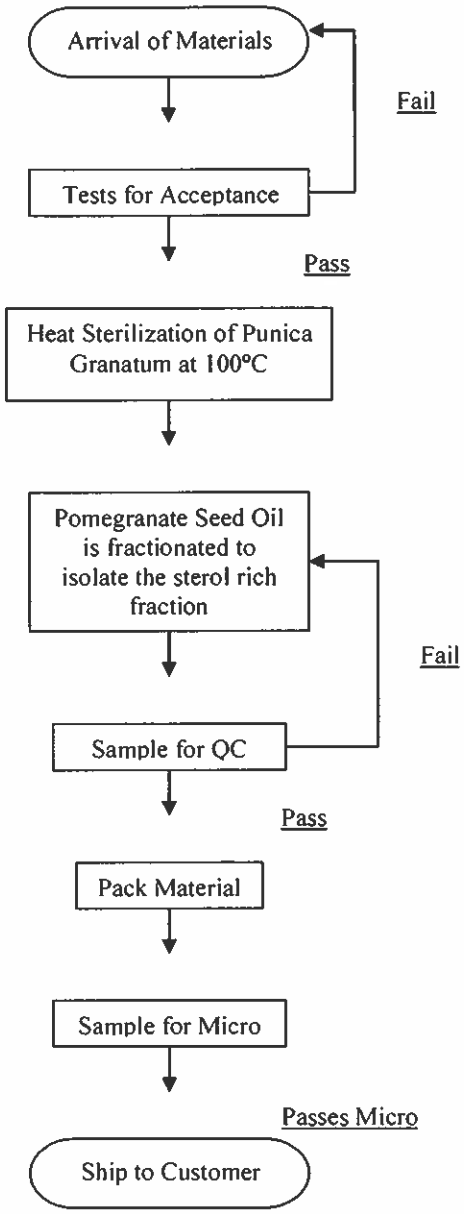
10247-ABS Pomegranate Sterols- Manufacturing Flow Chart



Final Report of the Amended Safety Assessment of PEG-5, -10, -16, -25, -30, and -40 Soy Sterol[1]

PEGs Soy Sterol are polyethylene glycol (PEG) derivatives of soybean oil sterols used in a variety of cosmetic formulations as surfactants and emulsifying agents, skin-conditioning agents, and cleansing and solubilizing agents. When the safety of these ingredients were first reviewed, the available data were insufficient to support safety. New data have since been received and the safety of these ingredients in cosmetics has been substantiated. Current concentration of use ranges from a low of 0.05% in makeup preparations to 2% in moisturizers and several other products. PEGs Soy Sterol are produced by the reaction of the soy sterol hydroxyl with ethylene oxide. In general, ethoxylated fatty acids can contain 1,4-dioxane as a byproduct of ethoxylation. The soy sterols include campesterol, stigmasterol, and β-sitosterol. The distribution of sterols found in oils derived from common plants is similar, with β-sitosterol comprising a major component. Impurities include sterol hydrocarbons and cholesterol (4% to 6%) and triterpine alcohols, keto-steroids, and other steroid-like substances (4% to 6%). No pesticide residues were detected. PEGS: Because PEGs are an underlying structure in PEGs Soy Sterols, the previous assessment of PEGs was considered. It is generally recognized that the PEG monomer, ethylene glycol, and certain of its monoalkyl ethers are reproductive and developmental toxins. Given the methods of manufacture of PEGs Soy Sterol, there is no likelihood of ethylene glycol or its alkyl ethers being present. Also, the soybean oil sterol ethers in this ingredient are chemically different from the ethylene glycol alkyl ethers of concern. PEGs are not carcinogenic, although sensitization and nephrotoxicity were observed in burn patients treated with a PEG-based cream. No evidence of systemic toxicity or sensitization was found in studies with intact skin. Plant Phytosterols: Intestinal absorption of ingested plant phytosterols is on the order of 5%, with 95% of the material entering the colon. Absorbed plant phytosterols are transported to the blood. Although there are some data suggesting that sulfates of β-sitosterol can act as abortifacients in rats and rabbits, other studies of well-characterized plant phytosterols and phytosterol esters demonstrated no effect in an estrogen-binding study, a recombinant yeast assay for estrogen or estrogen-like activity, or a juvenile rat uterotrophic assay for estrogen or estrogen-like activity. In a two-generation reproduction study using rats, plant phytosterol esters in the diet had no effect on any parameter of reproduction or fertility. Subcutaneous injections of β-sitosterol did reduce sperm concentrations and fertility in rats. Sitosterol inhibited tumor promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice after initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA), and reduced the tumors produced by *N*-methylnitrosourea in rats. Phytosterols were not genotoxic in several bacterial, mammalian, and in vitro assay systems. Phytosterols decreased epithelial cell proliferation in the colon of mice and rats, and were cytotoxic for human epidermoid carcinoma of the nasopharynx. PEGs Soy Sterols: The acute oral LD_{50} in rats of PEG-5–25 Soy Sterol was >10 g/kg. The acute dermal LD_{50} of a liquid eyeliner containing 2% PEG-5 Soy Sterol was >2 g/kg in rabbits. PEG-5–25 Soy Sterol was not a primary irritant in rabbits when applied undiluted. Undiluted PEG-5 Soy Sterol did not cause sensitization in guinea pigs. PEGs Soy Sterol did not produce ocular toxicity in rabbits. PEG-5 Soy Sterol was negative in the Ames mutagenicity test, with or without metabolic activation. PEG-5 Soy Sterol, at concentrations up to 2% in formulation, did not cause dermal or ocular irritation, dermal sensitization, or photosensitization in clinical studies. Because of the possible presence of 1,4-dioxane reaction product and unreacted ethylene oxide residues, it was considered necessary to use appropriate procedures to remove these from PEGs Soy Sterol before blending them into cosmetic formulations. Based on the systemic toxicity and sensitization seen with PEGs applied to damaged skin, it was recommended that PEGs Soy Sterol should not be used in cosmetic products applied to damaged skin. Although no dermal absorption data were available, oral studies demonstrate that phytosterols and phytosterol esters are not significantly absorbed and do not result in significant systemic exposure. Some small amounts did appear in the ovaries, however. This raises a concern about the potential presence of free phytosterols and β-Sitosterol, which could have antiestrogenic, antiprogestational, gonadotrophic, antigonadotrophic, and antiandrogenic effects in PEG sterols. These concerns are alleviated by the extensive data showing that well-defined phytosterols and phytosterol esters are not estrogenic and do not pose a hazard to reproduction. Likewise, the absence of impurities in plant phytosterols and phytosterol esters and extensive data demonstrating the absence of any genotoxicity in bacterial and mammalian systems mitigate against the possibility of any carcinogenic effect with those same well-characterized materials. The Cosmetic Ingredient Review (CIR) Expert Panel concluded that the PEGs Soy Sterol are safe as used in cosmetic products.

INTRODUCTION

PEG-5, -10, -16-, -25, -30, and -40 Soy Sterol are polyethylene glycol (PEG) derivatives of soybean oil sterols that function as non-ionic surfactants and emulsifying agents in cosmetic formulations. The safety of these ingredients was first reviewed by the Cosmetic Ingredient Review (CIR) Expert Panel in a series of meetings leading to a conclusion in 1997 that the

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available data were not sufficient to support the safety of this ingredient in cosmetic products (CIR 1997). One concern was the presence of free phytosterols in the soy sterol component of the PEGs Soy Sterol group of ingredients and the potential reproductive toxicity of β-sitosterol, in particular. The Expert Panel also sought impurities information and genotoxicity data.

New information has been provided that characterizes phytosterols and phytosterol fatty acid esters in general, including analyses of impurities. In addition, results of several genotoxicity assays using the characterized materials were provided, along with absorption, distribution, metabolism, and excretion data. Data from a two-generation reproductive toxicity study were also provided.

The CIR Expert Panel considered that because PEGs Soy Sterol may be broken down to PEGs and Soy Sterols, the safety test data on the PEGs and Soy Sterols are directly relevant to the safety assessment of PEGs Soy Sterol.

Polyethylene Glycol of various chain lengths (PEGs) has been reviewed previously by the CIR Expert Panel and the Final Report has been published. The following conclusion was reached:

> PEG -6, -8, -32, -75, 150, -14M, and -20M are safe for use at the concentrations reflected in the Cosmetic Use section and in the product formulation safety test data included in the Final Report. The Expert Panel recommends that cosmetic formulations containing these PEGs not be used on damaged skin (Andersen, 1993).

Data on plant phytosterols, to the extent that they are representative of soy phytosterols, are also relevant to this safety assessment and have been included in this amended safety assessment.

It was considered unlikely that there would be any toxicity of PEGs Soy Sterol esters that was not present in the two constituents.

CHEMISTRY

Definition and Structure

PEG-*n* Soy Sterol is a polyethylene glycol derivative of sterols found in soybean oil where *n* is the average number of moles of ethylene oxide used in synthesis (Wenninger et al. 2000). These phytosterols (generic term) are structurally similar to cholesterol and mainly consist of sitosterol ($C_{29}H_{50}O$: molecular weight [mw] 414.69), campesterol ($C_{28}H_{48}O$: mw 400.66), and stigmasterol ($C_{29}H_{48}O$: mw 412.67) (Applewhite 1985; Budavari 1989; Tyle and Frank 1991).

Campesterol and sitosterol are structurally identical to cholesterol except for side chain substitution of a methyl or ethyl group at the C24 position, respectively. Stigmasterol has an additional double bond at C22 (Heinemann, Axtmann, and von Bergmann 1993). The general structure of cholesterol, campesterol, sitosterol (β and γ), and stigmasterol are depicted in Figure 1.

PEG-*n* Soy Sterol is also known as PEG-*n* Soya Sterol (Pepe, Wenninger, and McEwen 2002) or Polyoxyethylene (*n*) Soya Sterol. A synonym for PEG-16 Soy Sterol is Soyasterole-PEG-16-Ether (Baade and Mueller-Goymann 1994).

Additionally, PEG-10 and -40 Soy Sterol go by the names Polyethylene Glycol 500- and -2000 Soya Sterol, respectively (Pepe, Wenninger, and McEwen 2002).

Chemical and Physical Properties

Physical and chemical properties of PEG Soy Sterol are summarized in Table 1.

PEG-5 and -10 Soy Sterol are soft, amber-colored, waxy solids with little or no odor (Lundmark, Chun, and Melby 1976; Nikitakis and McEwen 1990). PEG-16 and -25 Soy Sterol are ivory-colored, hard waxes (Lundmark, Chun, and Melby 1976). The compounds are more hydrophilic as the degree of ethoxylation increases, although the melting points (Tyle and Frank 1991) and interfacial tensions decrease linearly. The polyethylene chains in the PEG Soy Sterols form conical micelles with the base of the cone at the exterior of the micelle (Lundmark, Chun, and Melby 1976).

PEG-5 Soy Sterol is soluble in ethyl alcohol and hot isopropyl myristate. It is also dispersible in water. PEG-5 Soy Sterol melts in the range of 74°C to 88°C, and has a pH of 5.0 to 7.0 in a 1% aqueous dispersion at 25°C. The compound has an 80 to 110 hydroxyl value (Nikitakis and McEwen 1990).

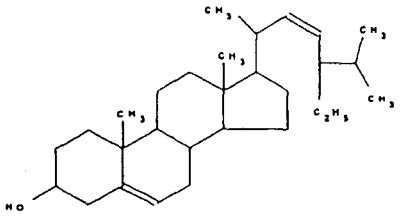
The apparent hydrophile-lipophile balance (HLB) is 5 (Lundmark, Chun, and Melby 1976). The HLB illustrates the simultaneous relative attraction of the compound for both water and oil, and identifies PEG-5 Soy Sterol as being oil-dispersible (Balsam and Sagarin 1974). The HLB for PEG-10 Soy Sterol is 12 (Lundmark, Chun, and Melby 1976); PEG-10 Soy Sterol is dispersible in mineral and vegetable oils at high temperatures, soluble in isopropyl myristate (Lundmark, Chun, and Melby 1976), and, in water, forms a translucent dispersion (Balsam and Sagarin 1974; Lundmark, Chun, and Melby 1976).

PEG-10 Soy Sterol has reported melting ranges of 73°C to 80°C and 55°C to 58°C, a pH of 4.5 to 7.0 (in 1% aq. dispersion at standard temperature), and a hydroxyl value of 60 to 90 (Lundmark, Chun, and Melby 1976; Nikitakis and McEwen 1990).

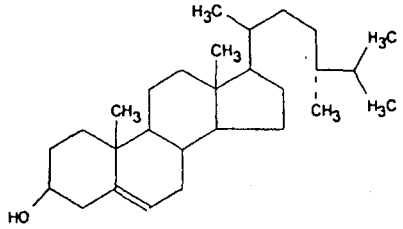
PEG-16 Soy Sterol melts at 46°C to 50°C and has an HLB of 15 (Lundmark et al. 1976). Its hydroxyl value is 70. The critical micelle concentration (CMC) for this compound is 0.22%. PEG-16 Soy Sterol contains enough ethylene oxide adducts to be soluble in water (Lundmark, Chun, and Melby 1976).

PEG-25 Soy Sterol melts at 44°C to 48°C (Lundmark, Chun, and Melby 1976; Tyle and Frank 1991) and has an HLB of 17 (Lundmark, Chun, and Melby 1976). The hydroxyl value of the compound is 55. PEG-25 Soy Sterol has a CMC of 0.46%. Like PEG-16 Soy Sterol, this compound is water-soluble (Lundmark, Chun, and Melby 1976).

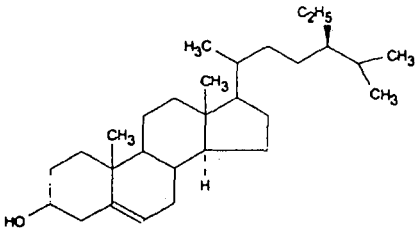
Stigmasterol (Budavari, 1989)



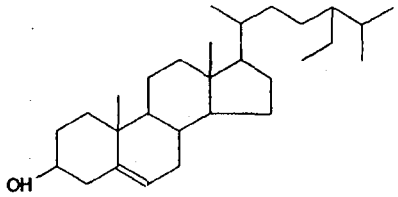
Campesterol (Budavari, 1989)



γ-Sitosterol (Budavari, 1989)



β-Sitosterol (Lewis, 1997)



Cholesterol (Lundmark et al., 1976)

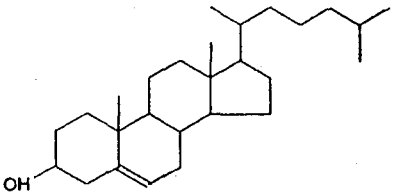


FIGURE 1
The general structure of cholesterol, campesterol, sitosterol (β and γ), and stigmasterol.

Method of Manufacture

Phytosterols

Soy Sterol is isolated from soybean oil distillates in a saponification process in which the phytosterols are separated from the fatty acids by extraction with a fat solvent. The phytosterols in the resulting extract are separated from the tocopherols in the mother liquor, and then purified and/or separated into the constituent sterols.

PEGs Soy Sterol

PEG-*n* Soy Sterol is formed from the reaction of the soy sterol hydroxyl with *n* moles of ethylene oxide (Lundmark, Chun, and Melby 1976).

Analytical Methods

The PEGs Soy Sterol can be determined by nuclear magnetic resonance and infrared spectroscopy. In the presence of an amphoteric surfactant, stabilized oil-in-water emulsions are observed, with the association complexes appearing to form liquid crystalline phases at the oil-water interfaces when viewed by polarized light and freeze-fracture electron microscopy (Tyle and Frank 1990). The temperature at which the liquid crystals form was inversely proportional to the degree of hydrophilicity of the phytosterol in question (Tyle and Frank 1991).

Baade and Mueller-Goymann (1994) separated PEG-16 Soy Sterol from the surface active local drug lidocaine using gel

TABLE 1
Physical and chemical characteristics of PEGs soy sterol

Characteristics	PEG-5 Soy Sterol	PEG-10 Soy Sterol	PEG-16 Soy Sterol	PEG-25 Soy Sterol	Reference
Physical properties	Soft, waxy solid; little or no odor; light to medium amber	Soft, waxy solid; little or no odor; light to medium amber	Hard wax; ivory-colored	Hard wax; ivory-colored	Lundmark et al. 1976; Nikitakis and McEwen 1990
Solubility	Soluble in ethyl alcohol and hot isopropyl myristate; dispersible in water	Dispersible in mineral and vegetable oils; soluble in isopropyl myristate; forms translucent dispersion in water	Water-soluble	Water-soluble	Lundmark et al. 1976; Nikitakis and McEwen 1990
Melting point	74–88°C	73–80°C (55–58°C)	46–50°C	44–48°C	Lundmark et al. 1976; Nikitakis and McEwen 1990; Tyle and Frank 1991
Hydrophile-lipophile balance	5	12	15	17	Lundmark et al. 1976
pH (1% aqueous dispersion at 25°C)	5.0–7.0	4.5–7.0	—	—	Lundmark et al. 1976; Nikitakis and McEwen 1990
Hydroxyl value	80–110	60–90	70	55	Lundmark et al. 1976; Nikitakis and McEwen 1990
Critical micelle concentration	—	—	0.22%	0.46%	Lundmark et al. 1976

permeation chromatography, ultraviolet (UV) spectroscopy, and nuclear magnetic resonance spectroscopy. A 40% PEG-16 Soy Sterol aqueous solution is a highly viscous liquid that is isotropic in polarized light microscopy. The critical micelle concentration of PEG-16 Soy Sterol is approximately 2 mg/L. When analyzed by UV microscopy, the compound absorbs at 270 nm and 293 nm in a ratio of ~1.73.

Impurities

PEGs

Silverstein et al. (1984) reported that PEG-6 may contain small amounts of monomer and dimers. The amounts were not quantified.

Peroxides, formed as a result of autoxidation, are found in PEG-32 and PEG-75 (Hamburger, Azaz, and Donbrow 1975). The amount of peroxide in PEGs is dependent upon the molecular weight of the PEG and its age. The older the compound, the greater the concentration of peroxides. In a colorimetric assay used to determine the peroxide concentrations in several production lots of PEGs, PEG-6 and PEG-8 were each added to acidified potassium iodide solution, and the iodine liberated was titrated against a standard thiosulfate solution. PEG-6 had peroxide concentrations ranging from 1.4 to 9.3 μEq thiosulfate/ml glycol. PEG-8 had concentrations ranging from 3.24 to 5.7 μEq thiosulfate/ml glycol. The specific peroxides present in the PEGs were not determined, but they were thought to be organic peroxides rather than hydrogen peroxide (McGinity, Hill, and La Via 1975).

Ethoxylated surfactants may also contain 1,4-dioxane, a byproduct of ethoxylation (Robinson and Ciurczak 1980). 1,4-Dioxane is a known animal carcinogen (Kociba et al. 1974; Hoch-Ligeti, Argus, and Arcos 1970; Argus, Arcos, and Hoch-Ligeti 1965). In the CIR safety assessment of the PEGs Stearate, the cosmetic industry reported that it is aware that 1,4-dioxane may be an impurity in PEGs and, thus, uses additional purification steps to remove it from the ingredient before blending into cosmetic formulations (Elder 1983).

Soy Phytosterols

In a review of the use of soy sterols, Lundmark, Chun, and Melby (1976) reported that refined soy sterols contain approximately 88% total sterol. Of that percentage, 56% is sitosterol, 28% is campesterol, and 4% is stigmasterol. Other compounds isolated with the phytosterols are 4% to 6% sterol hydrocarbons and cholesterol and 4% to 6% triterpene alcohols, keto-steroids, and other steroid-like substances (Lundmark, Chun, and Melby 1976).

Swern (1979) summarized the results of analyses of the total (phytosterol esters + free phytosterols), free, and the ester/free ratio in soybean preparations. In 100 mg of alkali-refined soybean oil, there was 0.446 mg total sterol and 0.287 mg free sterol. By subtraction, the phytosterol esters would be 0.159 mg and the ratio of esterified to free sterol was 0.55. In another alkali-refined soybean oil, there was 0.481 mg total sterol and 0.333 mg free sterol. By subtraction, the phytosterol esters would be 0.148 mg and the ratio of esterified to free sterol was 0.44.

Analyses of various lots of soy sterols for pesticide residues were negative for a number of pesticides, including PCB, DDE, DDT, malathion, and β-hexachloride (General Mills, Inc. 1979).

Phytosterols

Unilever (1998a 1998b) reported on the chemical characterization and stability of a single sample of plant sterols and from samples from five different batches of a single production process. The plant from which these phytosterols were derived was not stated. High-performance liquid chromatography was used to analyze fatty acid composition, gas chromatography/mass spectrometry for sterols, and Fourier transform infrared analysis as a check that the chemical bonds seen were consistent with the chemical species identified. The results presented in Table 2 show that the distribution of specific phytosterols, etc. are remarkably consistent. Reanalysis indicated the samples to be stable for a period of at least 10 months under refrigerated conditions. The fatty acid content of all samples was around 38% and the phytosterols was 62%.

TABLE 2
Chemical characterization of plant sterol material (Unilever 1998a 1998b)

	Distribution of phytosterols (%)	
Phytosterols	Single sample	Five samples from five batches
Brassicasterol	1.1	2.7–3.1
Campesterol	25.8	26.5–27.0
Stigmasterol	21.6	17.4–18.1
β-Sitosterol	48.7	50.8–51.2
β-Sitostanal	1.8	Not given
Cholesterol	0.4	0.2–0.3
Other sterols	0.8	1.2–1.7

In an analysis of another source of phytosterols using the same techniques described above, Unilever (1996a) reported that the principal phytosterols were present as follows: β-sitosterol, 47.9%; campesterol, 28.8%; and stigmasterol, 23.3%. No impurities were found. In an analysis of phytosterol esters, Unilever (1996b) reported that the principal phytosterols were present as fatty acid esters: β-sitosterol, 47.3%; campesterol, 28.1%; and stigmasterol, 24.5%. The distribution of the fatty acid chain lengths was consistent with fatty acids derived from sunflower oil.

Comparison of Phytosterol Compositions from Different Plants

The argument has been made that the available data on phytosterols is relevant to evaluating the safety of the soy phytosterol component of PEGs Soy Sterol (Brock 2000) because of similarity in structure of the phytosterols that have been studied and soy phytosterols. The relevance of phytosterol characterizations as seen above and the safety test data that will follow to soy sterols is a function of the similarity between phyto-sterols, regardless of source. In one case above, for example, the implication is that the source of the phytosterol esters undergoing analysis was sunflower seed oil and a question should be asked about the relevance of those data to soy sterols. Swern (1979) has compiled data from the literature on the percent distribution of phytosterols from 16 common oils. These data are shown in Table 3.

Clearly, campesterol and stigmasterol are present in significant proportions in most of these oils and β-sitosterol is present at a high percentage in all of them. The chemical characterization data in the preceding section shows a distribution of campesterol, stigmasterol, and β-sitosterol that is not substantially different from that shown in Table 3 for soy.

In the safety test data that follows, sections will be included that describe results of studies on phytosterols without reference to the plant source. The implication is that these data are likely relevant to the assessment of the campesterol, stigmasterol, and β-sitosterol components that principally comprise soy phytosterols based on the comparisons in Table 3.

USE

Cosmetic

PEGs 5-40 Soy Sterol serve as surfactants and emulsifying agents in cosmetic formulations. PEG-5 and -10 Soy Sterol function as skin-conditioning agents and PEG-40 Soy Sterol is used as a cleansing and solubilizing agent (Pepe, Wenninger, and McEwen 2002). These compounds also are used as appearance and consistency modifiers, emollients, viscosity control agents, and pigment dispersion agents (Lundmark, Chun, and Melby 1977).

Table 4 is a summary of the product formulation data submitted to the Food and Drug Administration (FDA) in 1996. PEG-5, -10, -16, -25, and -40 Soya Sterol were used in 41, 35, 15, 5, and

TABLE 3
Percent distribution of phytosterols from 16 common vegetable oils (Swern 1979)

	Phytosterol composition					
Oil source	Brassicasterol	Campesterol	Stigmasterol	β-Sitosterol	Δ^7 Stigmastenol	Unknown
Cocoa butter		8–11	24–31	59–62		
Coconut	2	6–9	18–19	69–75		
Corn		10–20	Trace-6	74–89		1
Cottonseed	Trace–1	8		89–91		
Linseed	2	28	10	53	4	
Olive		1–3	2	80–97		18
Palm		20–21	12–13	62–67		
Peanut	1	10–19	6–12	70–76		
Rapeseed	5–19	22–37		52–62		
Rice bran		14–33	3–6	55–63		
Safflower		8–13	4–9	52–57		23
Soybean		15–21	10–24	57–72		1
Sunflower		11–12	8–12	62–75	20	

1 cosmetic formulations, respectively. PEG-30 Soya Sterol was not reported to be used (FDA 1996).

Concentration of use data are no longer required to be submitted to the FDA by the cosmetics industry, but historical data stated that PEG-5 and -10 Soy Sterol were used at concentrations up to 5%, whereas the maximum concentration of use for both PEG-16 and -25 was 1% (FDA 1984). Current concentration of use information was provided by the industry through the Cosmetic, Toiletry, and Fragrance Association (CTFA). PEG-5 Soy Sterol was used at concentrations of 2% in mascara and eyeliner. In addition, 0.75% PEG-5 Soy Sterol and 1.5% PEG-25 Soy Sterol were reported used in a liquid makeup foundation

TABLE 4
Product formulation data for PEGs Soy Sterol (FDA 1996)

		Total no. of formulations containing ingredient				
Product category	Total no. of formulations in category	PEG-5 Soy Sterol	PEG-10 Soy Sterol	PEG-16 Soy Sterol	PEG-25 Soy Sterol	PEG-40 Soy Sterol
Eyeliner	533	1				
Mascara	218	5				
Other eye makeup preparations	136		1	3		
Hair conditioners (noncoloring)	715	3	1			
Shampoos (noncoloring)	972		1			
Blushers (all types)	277	1	1			
Foundations	355	1	1	1	2	
Rouges	30		1			
Aftershave lotion	268	1	1			
Cleansing	820	1	14	3		1
Face and neck (excluding shaving)	300	5				
Body and hand (excluding shaving)	1012	4	3			
Moisturizing	942	8	5	1		
Night	226		1			
Paste masks (mud packs)	300	1	2		3	
Other skin care preparations	810	8				
Suntan gels, creams, and lotions	196	2	3			
1996 totals		41	35	8	5	1

TABLE 5
Current concentration of use of PEGs Soy Sterol (CTFA 2000)

Product category	Concentration			
	PEG-5 Soy Sterol (%)	PEG-10 Soy Sterol (%)	PEG-16 Soy Sterol (%)	PEG-25 Soy Sterol (%)
Eye lotion	2		0.2	
Mascara	2	2		
Makeup bases		1		
Foundations		0.8		2
Other eye makeup preparations		0.1		
Hair conditioners (noncoloring)		2		
Shampoos (noncoloring)			0.5	0.5
Tonics, dressings, and other hair-grooming aids		0.4	0.5	
Other makeup preparations		0.05		
Shaving cream		2		
Other shaving preparation products		2		
Face and neck creams, lotions, powders, and sprays (excluding shaving preparations)				0.5
Body and hand creams, lotions, powders, and sprays (excluding shaving preparations)	0.2–2	2		
Moisturizing creams, lotions, powders, and sprays (excluding shaving preparations)		2	0.2	
Paste masks (mud packs)				0.5
Skin fresheners		2		

(CTFA 1997). CTFA (2000) updated those data with the information contained in Table 5.

Noncosmetic

In 1982, polyoxyethylene adducts of mixed phytosterols (with 5 to 26 moles average polyoxyethylene content) were exempted from tolerance requirements by the Environmental Protection Agency (EPA) when used as surfactants or related surfactant adjuvants in pesticide formulations applied to growing crops (Rothschild 1990; EPA 2002).

GENERAL BIOLOGY

Campesterol, sitosterol, and stigmasterol are the most frequently occurring plant sterols (Kallianos et al. 1963). Approximately 0.25 to 0.5 g of plant sterols are consumed each day in a typical diet (Sabine 1977; Heinemann, Axtmann, and von Bergmann 1993). As a comparison, up to 1.2 g cholesterol, which is derived from animal fat (Tso and Fujimoto 1991), was consumed (Sabine 1977). Plant sterols account for 20% to 25% of total dietary sterols (Heinemann, Axtmann, and von Bergmann 1993). These sterols are also found in cigarette and tobacco smoke as free sterols (Kallianos et al. 1963).

Phytosterols affect plant membrane structure and water permeability (Hennessey 1992). Membrane fluidity is inversely related to the amount of sterol found in the membrane (Sabine 1977). Phytosterols are commonly found in animal cell membranes following dietary uptake. In general, sterols intercalate into membrane bilayers and align themselves perpendicularly to the plane of the membrane with the 3′ OH facing the water interface. The aliphatic side chain extends into the hydrophobic core to interact with the fatty acid side chains of phospholipids and integral membrane proteins. The phytosterols are less water soluble than cholesterol. Sitosterol and campesterol order bilayer acyl chains most effectively, followed by cholesterol and stigmasterol (Hennessey 1992).

Absorption, Metabolism, Distribution, and Excretion

PEGs

Gastrointestinal absorption of PEGs is dependent on the molecular weight of the compound. In general, the greater the molecular weight of the PEG compound, the lesser the absorption that occurs. In both oral and intravenous studies, no metabolism was observed and the PEGs were rapidly eliminated unchanged in the urine and feces. In a study with human burn patients, monomeric ethylene glycol was isolated in the serum following topical exposure to a PEG-based antimicrobial cream, indicating that PEGs are readily absorbed through damaged skin (Andersen 1993).

Phytosterols

In general, ingested sterols are emulsified in the stomach, where lipid material from lipoprotein complexes is released. The coarse emulsion enters the duodenum of the small intestine, and the emulsion is solubilized with digestive enzymes and bile. Bile salts become conjugated with fatty acids, monoglycerides, dissolved sterols, and other molecules in the jejunum to form mixed micelles. Sterol ester bonds become hydrolyzed until only free sterol remains. Micellar solutions of lipids are very rapidly absorbed, and represent the major pathway of absorption for sterols and other fats (Sabine 1977). For example, incorporation of sterols into cholic acid micelles was 34%, 30%, 23%, and 15% for campesterol, sitosterol, cholesterol, and stigmasterol, respectively (Hennessey 1992). Micelles release sterols to the cells of the intestinal wall. Absorbed sterols are mixed with cholesterol synthesized within the intestinal cells. Before release from mucosal cells, the sterols are esterified. The resulting esters are transported from the intestine via the lymph (Sabine 1977).

Once consumed, phytosterols only enter the body via intestinal absorption. As the absorption rate for the plant sterols is usually less than 5% of dietary concentrations in humans (Sabine 1977; General Mills, Inc. 1979; Ling and Jones 1995), approximately 95% of dietary phytosterols enters the colon (Ling and Jones 1995). Saturated sterols are virtually not absorbed (Vanhanen and Miettinen 1992).

When cholesterol and phytosterols were simultaneously administered, only cholesterol could be isolated from the lymph duct, demonstrating that the phytosterols had not been significantly absorbed. Phytosterols experimentally injected subcutaneously into dogs were not esterified or metabolized. Instead, the plant sterols were treated like inert, foreign materials. Cholesterol, when similarly injected, was readily esterified (Lange 1950). In other studies, sitosterols did not accumulate or deposit in tissues (Gould 1955; Gould et al. 1969). Freshly absorbed sterols are transported into plasma (Sabine 1977).

Dietary supplementation with phytosterols can increase their serum concentrations until the sterols represent 10% of total serum sterols (Hennessey 1992; Heinemann, Axtmann, and von Bergmann 1993). Ling and Jones (1995) reported that 0.3 to 1.7 mg/dl of phytosterols were found in human serum under normal conditions after daily phytosterol consumption of 160 to 360 mg/day.

During metabolism and excretion, the sterol rings generally remain intact; double bonds, constituent groups, and side chains are often added, removed, or modified. The largest proportion of sterol in the body is converted to bile acids (Sabine 1977). In feeding studies, approximately 20% of absorbed β-sitosterol was converted to bile acids (cholic and chenodeoxycholic) in humans (General Mills, Inc. 1979). Boberg et al. (1990b) reported that C21 bile acids were major metabolites of sitosterol in mammals. Sitosterol was apparently not converted into C24 bile acids in humans (Boberg, Einarsson, and Björkhem 1990a). Conversion of campesterol into bile acids was reported in rats (Boberg et al. 1990b). Absorbed phytosterols not converted to normal bile acids were excreted as the free sterol (General Mills, Inc. 1979).

5,6-Epoxides were formed in the liver from β-Sitosterol. β-Sitosterol was metabolized to cortisol by the adrenal glands and to various steroid hormones by the testes. In rats, up to 5% of adrenal gland sterols can be of plant origin, and other tissues can contain large amounts of plant sterols (Sabine 1977).

Phytosterols can act as plant hormone and hormone precursors (Hennessey 1992). Other metabolites include steroid hormones (minor) and vitamin D compounds (Sabine 1977).

Sterols are typically eliminated via feces, urine, milk, and from the skin surface. Skin surface lipids contain 2% to 20% total sterols (Sabine 1977). Ling and Jones (1995) reported that phytosterol elimination via the biliary route appeared to be more rapid than that of cholesterol. The endogenous phytosterol pool size was low compared to cholesterol, due to poor intestinal absorption and faster excretion (Ling and Jones 1995). The excretion rate of sitosterol from bile was ten times greater than that of cholesterol (Gould 1955; American Cyanamid Co. 1957).

Nearly complete recovery of administered phytosterols in mammals was made from the feces (Lange 1950; General Mills, Inc. 1979). Unabsorbed sterols (unspecified) were degraded in the intestinal tract to varying degrees, depending on the species: 5% in rats, 25% in humans, and 65% in monkeys (strain not given) and baboons (Sabine 1977).

Unilever (1997a) traced the fate of radioactively labeled β-sitosterol and β-sitosterol linoleate administered by gavage to male rats. Twenty young, adult male Charles River CD rats (146 to 172 g) were individually housed. Ten animals received the β-sitosterol and 10 received the ester. The vehicle was sunflower oil and delivery was by gavage. Urine and feces were collected and individual CO_2 absorbers filtered the air which was drawn through at a rate of 1 L/min. At 4, 8, 24, 72, and 96 h, one rat from each group was weighed and euthanized for whole body autoradiography. At 96 h, heart blood was collected from the remaining animals and they were euthanized for analysis of radioactivity in internal organs.

The absorption, distribution, metabolism, and excretion of β-sitosterol and β-sitosterol linoleate were similar. Over 90% of the dose was excreted in the feces; less than 0.1% was excreted in the urine. Absorption from the gut was low. What was absorbed ended up in the intestinal lining, liver, lung, and adrenal gland. The highest concentration as well as persistence of the radioactive label was in the adrenal gland. In the gut, the β-sitosterol linoleate was hydrolyzed to release free sterol, but esterification with fatty acids present in the gut with free sterol also occurred.

In another study of the fate of radioactively labeled β-sitosterol and β-sitosterol linoleate, Unilever (1997b) evaluated the impact of a sunflower oil vehicle versus a coconut oil vehicle. In the sunflower oil part of the study, six young male and six young female Charles River CD rats (152 to 174 g) were dosed by gavage. At 24, 48, and 96 h, one animal of each sex was weighed and euthanized for whole body autoradiography. Urine and feces were collected. In the coconut oil portion of the

study, five young male and five young female Charles River CD rats (163 to 193 g) were also individually housed. At 24 h, the rats were weighed and euthanized for analysis of radioactivity in internal organs.

As in the previous study, β-sitosterol and β-sitosterol linoleate were poorly absorbed. The radioactive label was slightly higher in females. The adrenal gland showed the greatest amount and retention of radioactive label, but the ovaries, bone marrow, liver, intestinal lining, and spleen were also target organs for what radioactive label was absorbed. The testes in male rats was not a target organ. There was extensive hydrolysis of the ester and there was esterification of which varied as a function of the vehicle; there was more esterification with the sunflower oil than with the coconut oil. β-Sitosterol fatty acid esters with fatty acid components found only in coconut oil and not in the diet confirmed that esterification of free β-sitosterol.

Many of the results from the above Unilever (1996a, 1996b, 1997a, 1997b) studies were combined with other data on the influence on absorption of specific phytosterols (cholesterol, β-sitosterol, β-sitostanol, campesterol, or stigmasterol) on absorption, distribution, metabolism, and excretion in the rat in a report by Sanders et al. (2000). Using similar methods, the essential findings from above were repeated: the total absorption of phytosterols is low (cholesterol by contrast was well absorbed); female animals absorb more than males; and the largest amout of radioactivity from labeled phytosterols ends up in the adrenal gland, although the ovaries and intestinal epithelia also have significant levels.

Effects on Cholesterol Absorption and Metabolism

The plant sterols are effective inhibitors of cholesterol absorption in the small intestine, producing a hypocholesterolemic effect when the sterols are simultaneously ingested (Sabine 1977; Heinemann et al. 1991, 1993; Tvrzická et al. 1991) in rabbits, chickens, rats, and humans (Peterson 1951; Laraki et al. 1993; Ling and Jones 1995). Phytosterols also interfere with the absorption of structurally different, unsaturated plant sterols. Plant sterol-induced decreases of sterol absorption may be directly related to the absorption efficiency of sterols (Vanhanen and Miettinen 1992).

Heinemann et al. (1991) reported that 20% to 70% of the 750 to 3000 mg/day total cholesterol (dietary and biliary) that entered the intestinal tract was absorbed after solubilization in mixed micelles containing bile salts, mono- and diglycerides, fatty acids, and lysolecithin. Simultaneous high-dose infusion of cholesterol and sitosterol decreased the overall absorption of cholesterol by 25% to 65% in an intestinal perfusion study using nine subjects. The reduction in cholesterol absorption was due to competition with cholesterol uptake in the micelles (Heinemann et al. 1991).

In a second intestinal perfusion study using 10 subjects, Heinemann, Axtmann, and von Bergmann (1993) reported that an inverse relationship existed between absorbability of plant sterols and their inhibition of cholesterol absorption. Phytosterol absorbability was reduced by hydrogenization of the 5α nucleus double bond or by an increase in the side chain length. Sterol absorption rates were 33% for cholesterol, 4.2% for sitosterol, 4.8% for stigmasterol, and 12.5% for campesterol.

A positive correlation between the absorption rate of cholesterol and campesterol was established; a negative correlation was reported between the ratio of sitosterol to cholesterol and the mass of cholesterol absorption. Generally, the sterol absorption rate depended on micellar solubility. Cholesterol solubility was approximately three times greater than that of sitosterol. In addition, the binding of sitosterol to trihydroxy bile salt micelles was energetically favored over the binding of cholesterol. Hydrogenization of the delta-5-nucleus double bond caused a moderate enhancement of sterol hydrophobicity. The authors suggested that hydrophobic plant sterols with a high affinity and low capacity for micellar binding could have effectively displaced cholesterol from micellar binding (Heinemann, Axtmann, and von Bergmann 1993).

In a study by Sato et al. (1995), cultured in the presence of plasma lipoproteins, cells acquired cholesterol through receptor-mediated endocytosis of low-density lipoprotein (LDL). LDL-derived cholesterol esters in lysosome were hydrolyzed, freeing unesterified cholesterol. Free cholesterol crossed the lysosomal membrane and was transported to other intracellular organelles. The rate of esterification of the plant sterols could have been the factor limiting their absorption.

Esterification of endocytosed phytosterols in the endoplasmic reticulum was extremely low. Campesterol esterification was 20% that of cholesterol, and both sitosterol and stigmasterol were not esterified appreciably. When added to cell cultures, sitosterol and stigmasterol did not appear to be transported to intracellular membranes and, therefore, could not substitute for cholesterol to support cell growth. Instead, endocytosed plant sterols accumulated in the phagolysosomes of the cells.

Phytosterol accumulation was not a consequence of the cell's inability to esterify sterols in the endoplasmic reticulum as cholesterol did not accumulate when its esterification was blocked. The side chain structure of sterols was critical for the efflux of sterols from lysosomes. Plant sterols were distinguished from cholesterol at the level of the intestinal mucosal cell, but the mechanisms have yet to be determined. The observations made in this study suggested that cultured macrophages were able to differentiate sterols that differed only by a methyl or ethyl group at the C24 position at their lysosomal compartment (Sato et al. 1995).

The conversion of sterols to bile acids is inefficient; phytosterols and other sterols can inhibit the synthesis of bile acids from cholesterol. Unmetabolized sterols secreted into bile were generally less soluble than cholesterol and can precipitate out if bile salt concentrations were reduced (Clayton et al. 1993).

Miscellaneous Effects of Phytosterols

Exposure to 0.7 mmol/L (incorporated into liposomes) sitosterol for 72 h caused contraction of human umbilical vein

endothelial cells in vitro and an increased release of intracellular lactate dehydrogenase. At 96 h, partial detachment from the substrate was observed. In addition, 0.35 mmol/L of sitosterol caused pertubation of the endothelial cells at 96 h (Boberg, Pettersen, and Prydz 1991).

Results of an in vitro study by Chiang et al. (1991) indicated that β-sitosterol at a concentration of 100 μg/ml (5% in DMSO and saline) was cytotoxic against seven cancer cell lines: Colo-205 (colon), Hep-2 (laryngeal epidermoid), HeLa (uterine cervix), KB (nasopharynx), H1477 (melanoma), HA22T (hepatoma), and GBM8401/TSGH (glioma). A β-sitosterol isolate had antiatherogenic effects through inhibition of platelet aggregation (Pollak 1985). β-Sitosterol isolated from piper betle leaves (concentration not given) inhibited human platelet aggregation induced by arachidonic acid, platelet-activating factor, and adenosine diphosphate (ADP) (Saeed et al. 1993). Sitosterol can have potent anti-inflammatory, antibacterial, and antifungal activities (Padmaja, Thankamany, and Hisham 1993).

A concentration of 25 μg/ml of phytosterols slightly decreased beat rates of fetal rat heart cells whereas similar additions of cholesterol increased beat rates (Hennessey 1992). Phytosterols found in human tumors, particularly breast cancers have osteolytic activity, and increase mobilization and excretion of bone calcium (Sabine 1977).

ANIMAL TOXICOLOGY

Acute Toxicity

PEGs Soy Sterol

Young, male Sprague-Dawley rats were given 10 g/kg doses (50%; in feed) of PEG-5, -10, -16, or -25 Soy Sterol. No adverse effects were reported and the acute oral LD_{50} for each compound was >10 g/kg (Warf Institute, Inc. 1974; Henkel Corp. 1995).

North American Science Associates, Inc. (1987a) evaluated the dermal toxicity of a liquid eyeliner containing 2% PEG-5 Soy Sterol using ten New Zealand white rabbits. Each rabbit weighed between 2.5 to 3.1 kg. The test material (2 g/kg) was applied to the clipped upper back of each rabbit, at both intact and abraded skin sites. After application, the trunk of each rabbit was wrapped with polyethylene plastic that was taped in place, thus forming a reservoir over the test site. The rabbits were then fitted with collars and returned to their respective cages. The wrappings were removed after 24 h, and the collars at test termination. The rabbits were observed for signs of toxicity immediately after treatment, at 4 h, and daily for 14 days. Dermal reactions were scored daily for erythema and edema. On day 14, the skin sites were washed with tap water to remove any remaining residue of the test material. The rabbits were euthanized for necropsy.

No animals died prior to test termination. Three of 10 rabbits had transient diarrhea, and one rabbit "appeared thin" during the last 6 days of the study. Dermal observations were slight redness (9/10), swelling (2/10) that diminished by day 6, and apparent pustules at the test site (1/10). Four rabbits lost between 0.4 and 0.6 kg of weight. No macroscopic changes of the viscera were observed at necropsy. The acute dermal LD_{50} was >2 g/kg, and the eyeliner was considered dermally nontoxic for the rabbit (North American Science Associates, Inc. 1987a).

PEGs

Toxicity studies using rats, rabbits, and dogs indicate that PEGs have low oral and dermal toxicity. In general, the larger molecular weight PEGs appear to be less toxic than the smaller molecular weight PEGs in oral studies. Acute oral LD_{50} values for PEGs in rabbits were 17.3 g/kg (100% PEG-6) and 76 g/kg (100% PEG-75). In acute dermal toxicity studies, no deaths were reported in groups of rabbits dosed with 20 ml/kg of undiluted PEG-6 or 40% PEG-20M (Andersen 1993).

Short-Term Toxicity

PEGs

Andersen (1993) reported that there was no evidence of toxicity, with the exception of transient, mild erythema, in rabbits that received daily topical applications of PEG-75 or PEG-20M (0.8 g/kg/day) for 30 days.

Evidence of systemic toxicity was found, however, in a study designed as an animal model to study the effects of PEG-based antimicrobial creams in burn patients. Elevated total serum calcium, elevated osmolality gap, high anion gap metabolic acidosis, and renal failure resulted in rabbits that received repeated dermal applications of an antimicrobial cream containing 63% PEG-6, 5% PEG-20, and 32% PEG-75 to two paravertebral skin excisions (2.5 × 15 cm), followed by wound dressings (changed every 12 h) for 7 days (Andersen 1993).

Phytosterols

Laraki et al. (1993) studied the effects of phytosterols in the diet of rats. Male adult Wistar rats weighing 215 ± 12 g were randomly assigned to eight dietary groups with 12 rats/group. Rats of each group were fed 22 g/day basal diet with or without supplementation by cholesterol or maize phytosterols (72.5% β-sitosterol, 20.5% campesterol, and 7% stigmasterol) for 3 weeks (see Table 6).

The basal diet contained 16% casein, 68% cornstarch, 8% butter, 4% cellulose, 3% mineral mix, and 1% vitamin mix. Water was available ad libitum. Feed consumption did not differ between treatment groups; mean consumption was 21.4 ± 0.1 g/day. The rats were euthanized and the livers sampled post mortem, washed, and frozen. Enzymatic activities of acetyl–coenzyme A (CoA) carboxylase, malic enzyme, and glucose-6-phosphate dehydrogenase were determined on microsomes purified from 1 g liver homogenized in 10 ml 0.25 M sucrose. Hepatic lipid and fatty acid compositions were determined.

Table 6 presents enzyme activities determined in 12 rats in each treatment group. Acetyl-CoA carboxylase activity was significantly increased in group 4 (12 mg/day cholesterol and 48 mg/day phytosterol) compared to group 1 (12 mg/day cholesterol only); and decreased in groups 6, 7, and 8 (24 mg/day

TABLE 6
Diet composition in a rat short-term feeding study (Laraki et al. 1993)

	Group							
	1	2	3	4	5	6	7	8
Diet								
Cholesterol (mg/day)	12	12	12	12	24	24	24	24
Phytosterol (mg/day)	—	12	24	48	—	24	48	96
Phytosterol: Cholesterol Ratio	0	1	2	4	0	1	2	4
Liver enzyme activities (means ± SEM)[a]								
Acetyl-CoA carboxylase	538 ± 109	536 ± 43	522 ± 154	664 ± 123*	741 ± 199	235 ± 59**	225 ± 92***	233 ± 99***
Malic enzyme	0.39 ± 0.16	0.39 ± 0.07	0.30 ± 0.06	0.32 ± 0.06	0.44 ± 0.13	0.16 ± 0.04*	0.16 ± 0.04*	0.16 ± 0.03*
Glucose-6-phosphate dehydrogenase	1.33 ± 0.20	1.32 ± 0.20	1.26 ± 0.15	1.48 ± 0.26	2.39 ± 1.23	0.46 ± 0.19	0.57 ± 0.19	0.62 ± 0.15
Liver fatty acids and sterols (means [wet weight] ± SEM)[a]								
Fatty acids (g/100 g)	3.1 ± 0.3	3.5 ± 0.3	3.6 ± 0.3	3.0 ± 0.2	7.8 ± 2.8	2.8 ± 0.3***	2.7 ± 0.3***	3.0 ± 0.3***
Cholesterol (mg/100 g)	216.4 ± 1.5	218.7 ± 3.1	210 ± 2.8*	199.9 ± 1.9***	301.0 ± 13.3	239.4 ± 9.0***	209.4 ± 6.1***	203.4 ± 7.7***
Campesterol (mg/100 g)	0.81 ± 0.04	1.86 ± 0.17***	1.96 ± 0.15***	2.19 ± 0.16***	0.87 ± 0.07	4.68 ± 0.51***	5.03 ± 0.50***	8.00 ± 0.66
β-Sitosterol (mg/100 g)	2.95 ± 0.20	3.96 ± 0.23*	3.74 ± 0.16***	3.88 ± 0.24***	2.92 ± 0.06	8.84 ± 0.25***	10.90 ± 0.27***	12.86 ± 0.75***

[a]Student's t test significance (*$p < .05$; **$p < .01$; ***$p < .001$) versus the group receiving the same cholesterol supplementation without cholesterol.

cholesterol with 24, 48, or 96 mg/day phytosterol, respectively), compared to group 5 (24 mg/day cholesterol only). Malic enzyme activity was decreased in groups 6, 7, and 8 compared to group 5. Glucose-6-phosphate dehydrogenase activity was not decreased significantly in any phytosterol treatment groups.

Analysis of liver fatty acid and sterol contents (see Table 6) demonstrated increases in fatty acids in groups 6, 7, and 8 compared to group 5 (see Table 6). Cholesterol decreases in the liver were seen in groups 3 and 4 compared to group 1; and in groups 6, 7, and 8 compared to group 5. Campesterol increases were seen in groups 2, 3, and 4 compared to group 1; and in groups 6 and 7 (but **not** in group 8, although the value reported in Table 6 would appear to be significant) compared to group 5. β-Sitosterol increases were seen in groups 2, 3, and 4 compared to group 1; and in groups 6, 7, and 8 compared to group 5.

The authors noted (see Table 6) that liver fatty acids increased by a factor of 2.5 in group 5 (24 mg/day cholesterol) compared to group 1 (12 mg/day cholesterol) and that addition of phytosterol to the high cholesterol diet (groups 6, 7, and 8) reduced the liver fatty acid levels to that seen in group 1 (Laraki et al. 1993).

Wistar albino rats (10 per sex per group) were given subcutaneous injections of 250, 500, or 1000 μg/100 g/day β-sitosterol in olive oil for 60 days. No mortality was observed throughout the study. Treated rats given the lower doses had no gross or microscopic lesions of either the liver or kidneys. Mild fibroblastic proliferation around the hepatic lobules and microscopic lesions of the kidney were observed in animals given the high dose. These lesions were of very mild degree and had only a few heterophilic cell infiltrations in the medullary tract. All clinical biochemical parameters (hemoglobin, blood glucose, serum protein, serum bilirubin, aspartate aminotransferase, and alanine transaminase) were in the normal range with the exceptions of serum cholesterol and serum protein. Serum cholesterol was markedly depleted in a dose-dependent manner in both sexes. Serum protein was markedly reduced in the rats treated with 1000 μg/100 g/day of β-sitosterol (Malini and Vanithakumari 1990).

Subchronic Toxicity

PEGs

In subchronic, 90-day oral toxicity studies involving groups of albino rats, the largest (PEG-20M) and smallest (PEG-6) molecular weight PEGs tested did not induce toxicity or death when administered daily at concentrations of 4% or less; PEG-20M was administered in the diet and, PEG-6, in drinking water. In a dermal toxicity study, no evidence of toxicity was observed in a group of rabbits that received daily applications of PEG-6 5 days per week (2 ml/kg/day) for 18 weeks (Andersen 1993).

Phytosterol Esters

Hepburn, Horner, and Smith (1999) presented the results of a 90-day oral toxicity study of phytosterol esters. Groups of 20 male and 20 female Wistar derived rats were fed diets containing phytosterol esters (62% phytosterols and 38% fatty acids). Composition data were presented earlier in Unilever (1998a) and the mix of phytosterols shown in the first column of Table 2. Fatty acids were linoleic acid (64.6%), oleic acid (21.6%), stearic acid (4.1%), and palmitic acid (9.6%). Five dose groups received 0%, 0.16%, 1.6%, 3.2%, and 8.1% for 90 consecutive days. Clinical observations, body weights, and food and water consumption were determined during the exposure. At the end of the exposure period, the animals were euthanized; cardiac blood samples were taken, organs (adrenal gland, brain, epididymides, heart, kidney, liver, spleen, testes, thymus) were weighed, and a standard comprehensive set of tissues taken for histological examination.

Diets containing plant phytosterol esters at all levels were well tolerated. During the exposure, there were no changes in body weight gain, food and water consumption, or clinical signs. There were slightly reduced platelet counts and a small decrease in prothrombin time in female rats at all dose levels compared to controls. There was a small increase in the activated partial thromboplastin time in male rats at the 3.2% and 8.1% dietary levels. Other small increases in plasma albumin, phosphorus or magnesium levels, and certain serum enzymes were reported for males and/or females at the 1.6%, 3.2%, and 8.1% dietary levels. No treatment-related effect was seen with organ weights and histological examination revealed no evidence of systemic toxicity. Absent any organ effects, the small hematology and blood chemistry variations were not considered of toxicological significance (Hepburn, Horner, and Smith 1999).

Chronic Toxicity

PEGs

Toxic effects also were not observed in groups of dogs that received PEG-8, PEG-32, and PEG-75 at concentrations of 2% in the diet for 1 year (Andersen 1993).

Phytosterols

Thirteen dogs fed a basic diet supplemented with 0.5 to 1.0 g/kg/day of β-sitosterol had no gross or microscopic changes after 8 to 22 months of treatment. Weight gains and clinical parameters did not differ from controls (General Mills, Inc. 1979).

No adverse effects or gross or microscopic abnormalities were observed in six New Zealand white rabbits of both sexes that were given feed containing 3% cottonseed sterols and 4% soy sterols for 70–212 days (General Mills, Inc. 1979).

Dermal Irritation and Sensitization

PEGs Soy Sterol

Undiluted PEG-5, -10, -16, and -25 Soy Sterol were administered to the clipped back and flanks of albino rabbits (intact and abraded skin), six per group. The treated areas were covered with gauze patches, which were secured with tape. The patches were removed 24 h later, and the treated sites were scored at that time and at 72 h. The primary irritation index (PII) score was

0.50 for PEG-10 Soy Sterol and 0 for PEG-5, -16, and -25 Soy Sterol (Warf Institute, Inc. 1974; Henkel Corp. 1995).

PEG-5 Soy Sterol at induction and challenge concentration of 100% was a nonsensitizer in a study using Pirbright white guinea pigs (number of animals not stated) (Henkel Corp. 1995).

The primary skin irritancy of a liquid eyeliner containing 2% PEG-5 Soy Sterol was evaluated using six New Zealand white rabbits (North American Science Associates, Inc. 1987b). A 0.5-ml volume of the test sample was applied under a double gauze layer to both intact and abraded skin sites on 1 × 1-inch areas of the back. The patches were covered with nonreactive tape and the entire test site was wrapped with a binder. The binders and patches were removed after 24 h, and the skin sites were rinsed with tap water. The sites were evaluated at 24 and 72 h after application using Draize criteria. The PII was 0.96 (barely perceptible), and the investigators concluded that the eyeliner was not a primary skin irritant.

PEGs

The PEGs were not irritating to the skin of rabbits or guinea pigs, and PEG-75 was not a sensitizer. In skin irritation tests, undiluted PEG-6 was applied to the skin of rabbits for 4 h and 50% PEG-75 was applied to guinea pigs for 4 days and to rabbits over a 13-week period. In the guinea pig skin sensitization test, PEG-75 was tested at a concentration of 0.1% (Andersen 1993).

Ocular Irritation

PEGs Soy Sterol

Undiluted PEG-5, -10, -16, and -25 Soy Sterol were instilled into the conjunctival sac of New Zealand white rabbits, six per group. The PEGs Soy Sterol did not induce ocular toxicity in any of the rabbits treated, and the ocular irritation score for each was zero (Warf Institute, Inc. 1974; Henkel Corp. 1995).

A 0.1-ml volume of a liquid eyeliner containing 2% PEG-5 Soy Sterol was instilled into the conjunctival sac of six New Zealand white rabbits. Prior to instillation, the eyes were treated with fluorescein stain, flushed with saline, and observed in a darkened room under UV light to detect or confirm preexisting corneal injury. Ocular reactions were evaluated using Draize and Federal Hazardous Substances Act (FHSA) scoring criteria at 24, 48, and 72 h after instillation. Minimal conjunctival redness was observed in 1/6 test eyes, but was not considered significant. The investigators concluded that the liquid eyeliner was not an ocular irritant (North American Science Associates, Inc. 1987c).

PEGs

PEG-6 and -75 did not cause corneal injuries when instilled (undiluted, 0.5 ml) into the conjunctival sac of rabbits. PEG-8 (35% solution, 0.1 ml) and PEG-32 (melted in water bath, 0.1 ml) induced mild ocular irritation in rabbits (Andersen 1993).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Ethylene Glycol and its Ethers

It is generally recognized that the PEG monomer, ethylene glycol, and certain of its monoalkyl ethers (e.g., methoxyethanol, a.k.a. ethylene glycol monomethyl ether) are reproductive and developmental toxins. The CIR Expert Panel undertook a separate, limited scope review of these compounds in order to assess the possibility that PEG-derived cosmetic ingredients could present similar concerns (CIR 1996). In summary, this report concluded that the ethylene glycol monoalkyl ethers are not themselves toxic, but rather that one or more alcohol or aldehyde dehydrogenase metabolites are toxic. From the available data, the report also concluded that the toxicity of the monoalkyl ethers is inversely proportional to the length of the alkyl chain (methyl is more toxic than ethyl than propyl than butyl, etc.).

Given the methods of manufacture of the PEGs Soy Sterol, there is no likelihood of methoxyethanol, ethoxyethanol, etc. being present as an impurity. Further, the PEGs Soy Sterol are ethers of soybean oil sterols such as stigmasterol, γ-sitosterol, and campesterol and, as such, are chemically different from alkyl ethers.

Estrogenic Effects of Phytosterols and Phytosterol Esters

Malini and Vanithakumari (1992) reported that β-sitosterol acted as an effective estrogen-like agonist in producing vaginal cornification and caused uterine weight gain in adult, ovariectomized Wistar rats. In a short-term study, subcutaneous administration of β-sitosterol (50 μg/kg/day to 5.0 mg/kg/day) resulted in significant dose-related increases in uterine glycogen concentration after 10 days. At the highest dose, the glycogen concentration increase was equivalent to that produced by 50 μg/kg/day of estradiol.

When β-sitosterol was given in combination with estradiol, the estradiol-induced glycogen concentration was slightly enhanced. Progesterone partially suppressed the phytosterol-induced elevation of glycogen concentration after the hormone (20 mg/kg/day) was administered in combination with median and high doses of β-sitosterol (2.5 and 5.0 mg/kg/day). In addition, treatment with β-sitosterol (all doses) stimulated glucose-6-phosphate dehydrogenase, phosphohexose isomerase, and total lactate dehydrogenase activities (Malini and Vanithakumari 1992).

In a later study, Malini and Vanithakumari (1993) administered β-sitosterol alone or in combination with estradiol for 10 days caused a marked increase in the uterine weight of ovariectomized animals. When β-sitosterol was coadministered with progesterone, a twofold increase in uterine weight was observed. Estradiol administration resulted in a threefold increase. β-Sitosterol (50 to 500 μg/100 g/day) given alone caused a progressive dose-dependent increase in uterine weight with maximal increments occurring at the mid- and high-doses (250 and 500 μg/100 g/day). Other effects reported were increased uterine RNA, DNA, and protein concentrations.

These effects varied with coadministration of estradiol or progesterone. Estrogen and β-sitosterol induced DNA synthesis in the uterine luminal epithelium. Progesterone by itself stimulated a very small increase in DNA concentration; when coadministered with β-sitosterol, progesterone inhibited the growth-promoting effect of the phytosterol. Epithelial DNA concentration increased by two-, three-, and sevenfold for the low, mid-, and high doses, respectively; however, the increases were only significant at the two latter doses.

The investigators concluded that the dose-dependent uterotrophic effect of β-sitosterol in ovariectomized rats and its synergism with estradiol could be due to the phytosterol's intrinsic estrogenic properties, and that the effects of β-sitosterol could be inhibited by progesterone (Malini and Vanithakumari 1993).

In a study on the content and estrogen receptor of phytoestrogens in various foods, herbs, and spices, Zava, Dollbaum, and Blen (1998) reported 8 μg of estradiol equivalents per 200 cm^3 of soy milk extract in an estrogen receptor binding assay. Activity was extracted from soy milk with ethanol/water (50:50) for 2 days. These authors also report the results of ingestion of 200 cm^3 of soy milk on the estrogen receptor binding activity of saliva of four volunteers. Saliva was extracted with diethylether, dried to a precipitate, and reconstituted in growth medium for the binding assay. At least one individual had residual estrogen receptor binding 9 to 10 h after ingestion of the soy milk (all had estrogen receptor binding in saliva 1 to 4 h after ingestion).

Baker et al. (1999), however, found no evidence of estrogenic activity of phytosterols in a competitive estrogen-binding assay, a recombinant yeast assay, and in a juvenile rat uterotrophic assay. The phytosterols (see Table 2) used were described earlier (Unilever 1996a).

In the estrogen-binding assay, estrogen receptors isolated from 10-week-old female Wistar rats were prepared and incubated with estradiol (positive control) or test substances. Estradiol produced a concentration dependent (from 10^{-13} M to 10^{-6} M) binding, whereas phytosterols had no binding at concentrations of 10^{-7} M to 10^{-4} M.

In the recombinant yeast assay, the DNA of the human estrogen receptor and plasmids with the gene for the β-galactocidase enzyme controlled by estrogen responsive sequences are incorporated into yeast cells. In the presence of estrogens, the enzyme is produced by the yeast cells and its activity measured. Coumestrol, a known weak phytoestrogen, was included as a test substance to calibrate the sensitivity of the assay. Estradiol was used as a positive control and the activity of a phytosterol mix (see Table 2) and β-sitosterol alone. No enzyme activity was induced by the phytosterol mix ($\sim 10^{-10}$ M to 10^{-4} M) or β-sitosterol ($\sim 10^{-7}$ M to 10^{-4} M) alone. Both estradiol (maximum induction at $\sim 10^{-9}$ M) and coumestrol (maximum induction at $\sim 10^{-7}$ M) were active.

The juvenile rat uterotrophic assay utilized 21- to 22-day-old Wistar rats. Test materials were dissolved or suspended in arachis oil. Groups of 10 animals received a single dose of 0, 5, 50, or 500 mg/kg in a dosing volume of 10 ml/kg on each of 3 successive days by gavage. Estradiol was the positive control. Daily clinical observations were made over the period of dosing and body weights recorded at the same time. Animals were euthanized 24 h after the last dosing and the absolute uterine weight determined as the utertrophic endpoint. Arachis oil (vehicle), phytosterols, phytosterol esters, cholesterol, and cholesterol palmitate failed to produce any increase in uterine weight. The positive control produced a significant increase when compared to these test materials. Coumestrol was less effective in producing increased uterine weight compared to estradiol, but it did produce a clear dose-related increase.

The authors concluded that the well-defined phytosterol materials used in these studies were not estrogenic. They suggested that estrogenic activity seen in other studies may relate to actual phytoestrogens present in crude extracts (Baker et al. 1999).

Reproduction Studies

PEGs

Andersen (1993) described an oral toxicity study in which PEG-75 was given to rats (in drinking water) for 90 days. Doses above 0.23 g/kg/day were associated with testicular tubule degeneration, and scant or degenerated sperm, but historical controls using this strain (strain not given) reportedly had similar effects.

In a chronic oral study, PEG-75 was given to Wistar albino rats in drinking water at doses up to 0.062 g/kg/day and PEG-6–32 was given to a separate group at doses up to 1.69 g/kg/day. Animals were allowed to breed during the study and records were kept of the F_1 and F_2 generations. No changes or adverse responses to either compound occurred in the three generations (Andersen 1993).

Phytosterols

In a study by Malini and Vanithakumari (1991), male Wistar rats were injected subcutaneously with 0.5 to 5 mg/kg/day β-sitosterol for 16, 32, or 48 days. They found reduced sperm concentrations, testis weights, and accessory sex tissue weights in a time-dependent manner. Within 30 days of withdrawal from treatment, the weight of accessory sex tissues were restored to near-normal conditions.

In the same study, half of the long-term-treated rats were mated with unexposed females. Mated females were laparotomised on day 10 of pregnancy and the number of implantation sites was determined. Rats given 5 mg/kg/day of β-sitosterol had reduced fertility. Treatment with 0.5 mg/kg/day did not reduce the fertility of male rats, although a small (significant) decrease in sperm concentration of the caput epididymis was observed after 48 days of sitosterol treatment.

Sperm concentration was defined as the sperm count $\times 10^6$/ml epididymal plasma. The control sperm concentration was 500 ± 42, and the sperm concentration of rats given the phytosterol was 366 ± 28. This decrease persisted, even after withdrawal of treatment, and appeared to be due to a reduction in the rate

of spermatogenesis. Rats of the control groups received 0.5 or 5 mg/kg/day olive oil (Malini and Vanithakumari 1991).

Sulfates of β-sitosterol act as abortifacients in female rats and Dutch-belted rabbits via estrogenic and spermicidal effects. β-Sitosterol itself had antiestrogenic, antiprogestational, gonadotrophic, antigonadotrophic, and antiandrogenic effects (Malini and Vanithakumari 1990; Burck, Thakkar, and Zimmerman 1982; Ling and Jones 1995).

Phytosterol Esters

Noting the estrogenic activity of phytosterols reported by Malini and Vanithakumari (1993) above, as well as by Mellanen et al. (1996) and Rosenblum et al. (1993), Waalkens-Berendsen et al. (1999) conducted a two-generation reproduction study in rats receiving phytosterol esters in their diet. Four groups of Wistar outbred rats (28 animals of each sex in each group) were fed diets with 0%, 1.6%, 3.2%, or 8.1% phytosterol esters. The composition of the phytosterol esters was 62% phytosterol and 38% fatty acid. The distribution of phytosterols is as shown in the first column of Table 2.

Fatty acids were linoleic acid (64.6%), oleic acid (21.6%), stearic acid (4.1%), and palmitic acid (9.6%). At the end of a 10-week premating period, one male and one female (from the same exposure group) were housed together and the female checked each morning for evidence of mating. Mated F_0 females were caged separately. The morning after birth was considered postnatal day 1. On postnatal day 21, F_1 pups were weaned and 28 males and 28 females selected at random. These animals (~5 weeks old) began a premating period and the mating was again performed as above to produce the F_2 generation. Throughout the study, animals were observed daily for clinical signs and abnormal behavior. Body weights were determined weekly. Food consumption was measured weekly.

Reproductive performance included the calculated indicies of mating, fertility, fecundity, gestation, live births, postnatal viability, and lactation, and the sex ratio of pups was determined. Animals not utilized in the ongoing reproductive study were euthanized and subjected to thorough necropsy, including examination of the reproductive organs of the F_0 and F_1 males that failed to sire. The reproductive organs of the nonmated and nonpregnant females of the low- and mid-dose groups were also examined. At study termination, all surviving parental animals in the F_0 and F_1 generations were also euthanized and subjected to thorough necropsy.

There were no treatment-related clinical observations. Body weights of male rats of the F_0 and F_1 generations in high-exposure groups were decreased (compared to control males) in weeks 2 to 7 but not consistently at other times. Weight decreases in the low-exposure group occurred at different times. No body weight differences were seen in females. Food consumption was likewise not consistently different between control and exposed groups. There were no treatment-related organ weight changes, nor were there any macroscopic or histological evidence of systemic toxicity in those organs, including reproductive organs. There was no effect of phytosterol esters on fertility, reproductive performance, sexual maturation, oestrous cycle length, or precoital time. Pup mortality on a per litter basis was not affected by exposure to phytosterol esters in the diet. There were no differences in pre/postimplantation loss, stillborn pups, and litter size and weight. Overall, the authors concluded that dietary phytosterol esters up to 8.1% had no effect on any parameter of reproduction or fertility (Waalkens-Berendsen et al. 1999).

GENOTOXICITY

PEGs Soy Sterol

PEG-5 Soy Sterol at 8-5000 μg/plate (in Tween 80/bidist water) was not cytotoxic to *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, or TA1538. The test compound, with or without metabolic (S9) activation, did not induce reverse mutations in those bacterial strains (Henkel Corp. 1995).

PEGs

PEG-8 was negative in the Chinese hamster ovary cell mutation test and the sister-chromatid exchange test; the maximum test concentration in both studies was 1%. In the unscheduled DNA synthesis assay, a statistically significant increase in radioactive thymidine incorporation into rat hepatocyte nuclei was noted only at the highest concentration tested (0.1% PEG-8). PEG-150 was not mutagenic in the mouse lymphoma forward mutation assay when tested at concentrations up to 150 g/L (Andersen 1993).

Phytosterols and Phytosterol Esters

Huntington Life Sciences Ltd. (1996a, 1996b) evaluated the mutagenicity of phytosterols in *S. typhimurium* strains TA1535, TA1537, TA98, and TA100 with and without metabolic activation. The phytosterol preparation was described earlier (Unilever 1996a). No toxicity at concentrations up to and including 5000 μg/plate, so that level was used in the mutagenicity assay. S9 liver preparations from Aroclor 1254–induced rats was used for metabolic activation. In the absence of S9 activation, *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine was used as a positive control for TA1535 and TA100, 9-aminoacridine for TA1537, and 2-nitrofluorene for TA98. With S9 activation, 2-aminoanthracene was used as the positive control. Tetrahydrofuran was used to dissolve the test substances and served as the negative control. No significant increases in mutations with or without metabolic activation was seen with the phytosterol preparation used.

Likewise, no significant increases in mutations with or without metabolic activation was seen with the phytosterol ester preparation (as described by Unilever 1996b).

This same laboratory evaluated the mutagenicity of plant sterols using the same assay described above (Huntington Life Sciences Ltd. 1998). The plant phytosterol preparation was described earlier (Unilever 1998a). No significant increases in mutations with or without metabolic activation was seen with the plant sterols preparation used.

A metaphase chromosome analysis of human lymphocytes in vitro exposed to phytosterols and phytosterol esters was conducted by Huntington Life Sciences Ltd. (1997a, 1997b). The phytosterols and phytosterol esters were characterized earlier (Unilever 1996a, 1996b). Two tests were done with each test material. In the first test, a range of concentrations of test materials were added to one set of duplicate cultures. Acetone was used as a solvent and control. Mitomycin C was added. The second set of cultures received S9 liver fraction and the same concentrations of test materials and control as in the first set (adjusted to accommodate the extra volume of the S9 mix).

Exposure was 3 h for this second set, at which point the cells were washed and resuspended in fresh medium and incubated for 18 h. The first set of cultures was incubated for 21 h with the test material. Cells were harvested, Colcemid was added to arrest mitotic activity, the cells further incubated for 2 h and then washed and treated prior to placement on microscope slides for visual examination. Metaphase figures were examined (~100 from each culture) at 1000× magnification. In the second test, the procedure was similar, except that some cultures were harvested at 21 h and others allowed to continue for 45 h. The concentrations of phytosterols and phytosterol esters tested in each regimen with notes regarding formation of precipitate are shown in Table 7.

No significant incidence of chromosome aberrations or change in mitotic index was seen in any of the tests with either phytosterols or phytosterol esters in this test at any concentration, with or without metabolic activation (Huntington Life Sciences Ltd. 1997a, 1997b).

Covance Laboratories Ltd. (1999a) determined unscheduled DNA synthesis in the liver of Han Wistar rats dosed orally with plant sterol esters. In each of two studies, groups of five male rats (196 to 231 g) received corn oil (vehicle control), 800 or 2000 mg/kg plant sterol esters, or positive control. In one study 2-acetamidofluorene was the positive control and dimethylnitrosamine in the other. The composition of the plant sterol ester was described earlier (Unilever 1998b). In all cases, the dose volume was 10 ml/kg delivered by oral gavage.

In one study, animals were euthanized 10 to 14 h post dosing and in the other at 2 to 4 h. The liver of each rat was prepared by washing it free of blood and treating with collagenase. The liver was harvested and hepatocytes separated out. Hepatocyte suspensions were placed into wells containing a glass coverslip, incubated at 37°C for at least 90 min to allow adhesion to the coverslip. Adhered cells were washed with serum free medium and then incubated with the same medium with ^{3}H-thymidine added for 4 h. The cells were again washed and incubated overnight in the serum free medium. Incorporation of radioactivity in the cells was determined by counting grains in autoradiographic images of the cells. No evidence of unscheduled DNA synthesis in rat liver hepatocytes exposed in vivo to plant sterol esters was found (Covance Laboratories Ltd. 1999a).

This same laboratory (Covance Laboratories Ltd. 1999b) examined the induction of bone marrow micronuclei in outbred

TABLE 7
Concentrations of phytosterols and phytosterol esters used in chromosome analysis (Huntington Life Sciences Ltd. 1997a, 1997b)

	Concentration (μl/ml)					
	Without metabolic activation			With metabolic activation		
	First test	Second test		First test	Second test	
Test agent	21 h	21 h	45 h	21 h	21 h	45 h
Untreated	—	—	—	—	—	—
Acetone	10	10	10	10	10	10
Phytosterols	1.3			1.3		
	2.5			2.5		
	5			5		
	10			10		
	20			20		
	40[a]	40[b]	40[b]	40	40[b]	40[b]
	80[b]	80[a]	80[a]	80[a]	80[b]	80[b]
	160[b]	160[a]	160[a]	160[a]	160[a]	160[a]
Phytosterol esters	.78			.78		
	1.56			1.56		
	3.13			3.13		
	6.25			6.25		
	12.5			12.5		
	25	25		25	25	
	50[a]	50[a]	50[a]	50	50	
	100[a]	100[b]	100[b]	100[a]	100[a]	100[a]

[a]Precipitate on dosing, still apparent at end of treatment.
[b]Precipitate on dosing, not apparent at end of treatment.

Crl:HanWist male rats given plant sterol esters by oral gavage. Groups of eight rats were treated with corn oil (vehicle control), 500, 1000, or 2000 mg/kg of plant sterol esters, or cyclophosphamide (positive control). The composition of the plant sterol ester was described earlier (Unilever 1998b). In all cases, the dose volume was 10 ml/kg, given once daily on each of 2 consecutive days. Animals were euthanized 24 h after the second administration. A single femur of each animal was removed, cleaned, prepared, and the bone marrow flushed out with fetal bovine serum (2 ml). Bone marrow cells were concentrated by centrifugation and smeared on microscope slides. The slides were allowed to air dry, then fixed and rinsed. A second fixation and rinsing was done before final drying.

Polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were determined in at least 1000 cells. Continued counting of PCEs was done until a total of 2000 PCEs were counted. All PCEs containing micronuclei were tabulated. Cells from rats dosed with plant sterol esters had the same PCE/NCE ratio as the vehicle control. The frequencies of micronucleated PCEs (group means) were similar to controls. The authors concluded that plant sterol esters at doses up to 2000 mg/kg did not

induce micronuclei in rat bone marrow cells (Covance Laboratories Ltd. 1999b).

CARCINOGENICITY

PEGs

PEG-8 was used as a solvent control in a number of oral studies intended to evaluate the carcinogenicity of other chemicals. In those studies, no tumors were observed when the solvent was administered orally to mice (30 weeks of dosing), intraperitoneally to rats (6 months of dosing), subcutaneously (20 weeks of dosing—rats; 1 yr of dosing—mice), or when injected into the gastric antrum of guinea pigs over a period of 6 months (Andersen 1993).

Antitumorigenic Effects

Phytosterols

Ling and Jones (1995) reported that evidence is available to suggest that phytosterols inhibit the induction of tumors in animals.

Yasukawa et al. (1991) demonstrated that sitosterol had an inhibitory effect on the tumor-promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin following initiation by 7,12-dimethylbenz[*a*]anthracene (DMBA). DMBA was administered as a single topical application of 50 μg to the shaved backs of female, 7-week-old ICR mice (20 per group). One week after initiation, promotion was begun with the twice weekly applications of 2.5 μg TPA. Thirty to 40 min after each TPA treatment, 5 μmol/100 μl doses of sitosterol or the vehicle, acetone-DMSO (9:1, 100 μl) were given. The backs of the treated mice were shaved weekly throughout the 18-week experiment.

The first tumor in mice given TPA and DMSO without sitosterol was observed at week 5; all mice in this treatment group had skin tumors by week 10 (average = 21.1 tumors per mouse at week 18). In the group given sitosterol, 80% had skin tumors (average = 11.2 tumors per mouse at week 18). The percent reduction in the average number of tumors at week 18 was 40% in mice given TPA and DMSO plus sitosterol (Yasukawa et al. 1991).

Yasukawa et al. (1991) also hypothesized that epidermal ornithine decarboxylase (ODC) activity induction is a characteristic biochemical alteration elicited by TPA, and can be representative of the effects of phorbol esters with strong tumor-promoting activity. Sitosterol applied topically 30 min prior to treatment with TPA inhibited TPA-induced ODC activity in five female ICR mice. In this study, 5 μmol of sitosterol was dissolved in 200 μl chloroform and applied to the shaved area of the skin. TPA (5 μg) was dissolved in acetone. Four hours after TPA administration, the mice were euthanized by cervical dislocation. The epidermis of the treated skin was separated by brief heat treatment, and ODC activity was determined in the soluble epidermal supernant by measuring the release of $^{14}CO_2$ from [1-^{14}C]ornithine. The results were expressed as nmol CO_2/30 min/mg protein.

ODC activity was inhibited by 65% compared to the vehicle control (control, 2.8 $\pm$ 0.23; sitosterol, 1.3 $\pm$ 0:18). The researchers also reported that dermal inflammation caused by a single topical application of 1 μg TPA in mice was slightly reduced by sitosterol and stigmasterol (Yasukawa et al. 1991).

Male Fischer CD rats coadminsitered the direct-acting carcinogen *N*-methyl-*N*-nitrosourea and β-sitosterol had significantly fewer colon tumors (benign or benign and malignant) compared to rats given the carcinogen alone (Raicht et al. 1980). The phytosterol was 95% pure, with 4% campesterol and 1% stigmasterol, and was given at a concentration of 0.2% in feed. The carcinogen (0.5 ml, in 0.9% saline; 2 mg/dose/rat) was administered by cannulation into the colon on days 1, 4, 7, and 10. Control rats (10 per group) received either control chow and intracolonic saline or control chow plus β-sitosterol and saline.

No deaths occurred during 28-week experiment, and no adverse effects were observed after feeding of the phytosterol. No tumors were detected in rats of either control group. Of rats given the carcinogen alone, 54% had tumors (1.1 tumors/rat and 2.1 tumors/tumor-bearing rat), and 78 tumors were detected. Thirty-three percent of rats that received the sterol-supplemented diet had tumors (0.44 tumors/rat and 1.3 tumors/tumor-bearing rat), and 21 tumors were found. Most lesions were adenomatous polyps.

The incidence of malignant colonic neoplasms increased in rats given the phytosterol, however; 7 of 48 rats (15%) had invasive carcinomas in the sterol plus carcinogen group compared to 5 of 71 rats (7%) in the carcinogen alone group (Raicht et al. 1980).

Cell Proliferation Effects

Dietary addition of phytosterols (60% β-sitosterol, 30% campesterol, 5% stigmasterol) had a dose-related effect on colonic mucosal cell proliferation in female C57B1/6J mice (Janezic and Rao 1992). Six mice per group received 0.1% cholic acid with or without dietary supplementation of 0.1%, 0.3%, or 1.0% phytosterols. The mice were euthanized after two weeks, after being injected with colchicine and [^{3}H]thymidine to determine the number of cells in the colonic crypts undergoing metaphase and DNA synthesis, respectively. Subsequently, the rate of colonic cell proliferation was determined.

Dietary cholic acid significantly increased colonic epithelial cell proliferation and the highest labeled cell position by 92% and 35%, respectively. The mitotic index was 119%. A 1% concentration of dietary phytosterol did not significantly affect cell proliferation compared to the control group, but the mitotic index indicated a significant decrease in the number of cells in mitosis after 0.1% phytosterol was given (Janezic and Rao 1992).

Phytosterol can decrease epithelial cell proliferation by suppressing the bacterial metabolism of cholesterol and/or secretory bile acids in the colon and by increasing excretion of cholesterol.

Dietary phytosterols appear to inhibit colonic cancer development prior to adenoma formation (Ling and Jones 1995).

At concentrations of 10^{-3} to 10^{-6} M, stigmasterol and campesterol from stinging nettle root extracts inhibited the membrane Na^+/K^+-ATPase activity of benign prostatic hyperplasia by 23% to 67%. This inhibition could have subsequently suppressed prostate cell metabolism and growth (Hirano, Homma, and Oka 1994).

A mixture of β-sitosterol, stigmasterol, campesterol, and ducitol isolated from *Gymnosporia trilocularis* was cytotoxic ($ED_{50} \geq 20$ μg/ml) for a human cell line of an epidermoid carcinoma of the nasopharynx (KB) test system (Ling et al. 1981).

Dietary β-sitosterol at a concentration of 0.2% decreased the rate of colonic epithelial cell proliferation and compressed the crypt's proliferative compartment, thus suppressing expression of the altered genome, in Fischer rats induced with *N*-methyl-*N*-nitrosourea. Six rats were treated with the phytosterol alone, three were given the carcinogen (by cannulation), and five were given both the sterol and the carcinogen. Six rats served as control and were given stock feed only. Cell proliferation was reduced in rats given *N*-methyl-*N*-nitrosourea plus β-sitosterol as early as 3 days after initiation of feeding, as compared to rats given the carcinogen alone, and at 28 weeks, when the rats were euthanized. The mean number of radioactive cells per crypt column was 3.3 for rats coadministered β-sitosterol and 5.4 in rats given *N*-methyl-*N*-nitrosourea (Deschner, Cohen, and Raicht 1982).

CLINICAL ASSESSMENT OF SAFETY

Dermal Irritation and Sensitization

PEGs Soy Sterol

Biosearch Incorporated (1992a) patch-tested 12 volunteers, aged 27 to 54 years, with a mascara containing 2% PEG-5 Soy Sterol in a modified Draize repeat-insult patch test (RIPT) to determine irritation potential. On a Friday, 0.75 × 0.75-inch gauze pads (evenly covered with the test material) were applied to skin sites on the back. The occlusive patches were removed at 24 h. The procedure was repeated on the following Monday, Tuesday, and Wednesday. The skin sites were evaluated 48 h after the final application. All subjects completed the study.

No evidence of skin irritation was observed.

Biosearch Incorporated (1992b) performed a different modified Draize RIPT using the same formulation and 84 subjects, 75 of whom completed the study. Approximately 0.01 g of the test material was applied to cover the gauze pad of an occlusive patch. The patch was applied to the back and removed after 24 h. The procedure was repeated 3 alternate days/week until nine applications had been made. Fourteen days after the last induction patching, a challenge patch was applied to an adjacent skin site (untreated). The patch was removed at 24 h, and the site was examined at 24 h, 48 h, and 72 h. No evidence of irritation or sensitization were observed.

PEGs

In clinical studies, PEG-6 and PEG-8 induced mild sensitization in 9% and 4% of 23 male subjects tested, respectively. However, later production lots of PEG-6, as well as PEG-75, did not cause reactions in any of the 100 male and 100 female subjects tested. A product formulation containing 3% PEG-8 induced minimal to mild irritation (induction phase) in over 75% of 90 volunteers participating in a skin irritation and sensitization study. Responses (not classified) were noted in 22 subjects at the 24 h challenge reading. Cases of systemic toxicity and contact dermatitis in burn patients were attributed to PEG-based topical ointments. The ointment that induced systemic toxicity contained 63% PEG-6, 5% PEG-20, and 32% PEG-75 (Andersen 1993).

Photosensitization

PEGs Soy Sterol

Biosearch Incorporated (1987) used a Schwartz-Peck Prophetic Patch Test to determine primary skin irritation of a liquid eyeliner containing 2% PEG-5 Soy Sterol. A group of 101 female subjects, aged 16 to 63 years, was used in this study. Approximately 0.2 g of the test formulation was applied to the upper back using a 0.75-inch-diameter Lintine disc. The patch was then covered with cloth tape and left on the skin for 48 h. Simultaneously, ~0.1 g of the material was applied to the volar forearm as an open patch. The exposed skin sites were examined 48 h after application. Fourteen days later, this procedure was repeated. In addition, the site on the back was exposed to UV light (wave length = 3650 Å) at a distance of 12 inches for 1 min. After the second insult application, the closed patch site was irradiated. The application sites were reexamined after 48 h to determine if photosensitization had occurred. No signs of primary skin irritation or photosensitization were observed during this study.

A Draize-Shelanski RIPT was performed using a liquid foundation containing 0.75% PEG-5 Soy Sterol and 1.5% PEG-25 Soy Sterol (Biosearch Incorporated 1991). Eighty-eight subjects (16 to 55 years old) were enrolled in the study, and 77 completed it. A 0.2-ml volume of the foundation was applied (on a 0.75-inch-diameter Lintine disc) to a skin site on the back. The patch was covered with cloth tape. At the same time, 0.2 ml of the test material was applied to the volar forearm as an open patch. At 48 h, the patches were removed and the skin sites were examined. This procedure was repeated three times a week for three and a half weeks. The application site was divided into quadrants such that three were used for induction patching and one for challenge. The first quadrant received the first, fourth, seventh, and tenth applications; the second received the second, fifth, and eighth applications; and the third received the third, sixth, and ninth applications. Fourteen days later, identical open and closed patches were applied and evaluated at 48 h. In addition, the closed patch was irradiated for 1 min using a UV light source (3650 Å) after the first, fourth, seventh, tenth, and

eleventh applications. The subjects were examined 48 h later to determine if photosensitization had occurred. During this study, no signs of primary irritation, sensitization, or photosensitization were observed.

Ten female volunteers (25 to 47 years old) were used in a phototoxicity study of a mascara containing 2% PEG-5 Soy Sterol (Biosearch Incorporated 1992c). A filtered (150 W) UV solar simulator was used to provide a continuous output in the UVB and UVA region (290 to 400 nm). For exposure to UVA only, a filter was used to eliminate UVB wavelengths (290 to 320 nm). The shutter of the solar simulator was controlled to be closed upon completion of 14 J/cm^2. Prior to the start of the study, each subject's minimum erythemal dose (MED) was determined using skin sites on the back. For the study, the test material (20 μl) was applied to two sites on the back (diameter = 1.5 cm). The third site was untreated and served as an irradiation control site. One treated site and the control site were irradiated 30 to 60 min after application with 0.5 MED of UVB and UVA, and then with 14 J/cm^2 of UVA. The sites were evaluated at 24 h, 48 h, and 72 h. No reactions were observed at either of the treated sites.

The photosensitization potential of the same formulation was determined in a study using 30 subjects, aged 16 to 50 years (Biosearch Incorporated 1992d). Twenty-nine subjects completed the study. In this study, the shutter closed when 4 J/cm^2 was delivered. Approximately 0.1 g of the mascara formulation was applied to the gauze pad of an occlusive patch, which was then affixed to the skin of the back. The patches were applied on Mondays and Wednesdays of three consecutive weeks, and remained in place for 24 h each time. After patch removal, the sites were exposed to 2.0 MEDs of UVB irradiation and 4 J/cm^2 of UVA irradiation. On day 18 after the last induction exposure, the subjects were patched at two separate adjacent untreated sites. The patches were removed at 24 h, and the sites were examined. One site was exposed to 0.5 MED of UVB and 4 J/cm^2 of UVA. One untreated site was similarly irradiated. The challenge sites were evaluated at 24 h, 48 h, and 72 h after irradiation. No evidence of photosensitization was observed.

Comedogenicity

PEGs Soy Sterol

The Educational & Research Foundation, Inc. (1991a) used 62 female subjects to evaluate the comedogenicity of SPF 15 liquid foundation containing 0.75% PEG-5 Soy Sterol and 1.5% PEG-25 Soy Sterol. Fifty-nine of the panelists completed the study. The subjects were between the ages of 14 and 40. Prior to the study applications, a dermatologist examined each subject for irritation and evaluated all noninflammatory lesions (closed and open comedones) and inflammatory lesions (papules, pustules, and nodules).

Thirty subjects had mild acne (>10 noninflammatory and inflammatory lesions), and 32 had no acne (<10 lesions). The subjects used the foundation twice a day for 49 consecutive days. On days 7 and 28, a clinical laboratory technician examined the panelists for tolerance of the product and participant cooperation with testing instructions. The dermatologist examined the subjects at study termination. Several subjects reported stinging and burning sensations, but erythema and scaling were not observed by the dermatologist or technician.

The total scores for subjects with acne decreased from 557 to 397 during the study, and the scores for non-acne subjects remained the same (56 to 55). The foundation was "well tolerated" by the panel, and did not cause comedogenicity in either the acne-prone or non-acne groups (Educational & Research Foundation, Inc. 1991a).

In a second (45-day) study, the Educational & Research Foundation, Inc. (1991b) evaluated an SPF 15 liquid foundation (0.75% PEG-5 Soy Sterol, 1.5% PEG-25 Soy Sterol) using 56 female panelists, 52 of whom completed the study.

Twenty-seven of the panelists had mild acne and 29 had no acne. The subjects used the foundation twice a day (minimum) for 45 consecutive days. On days 28 and 45, the dermatologist examined all subjects for comedones, papules, pustules, and nodules, and a clinical laboratory technician examined them to determine tolerance to the product and participant cooperation with testing instructions. Several of the panelists reported itching during the course of the study. One of the subjects had itching and a face rash at the final visit; at subsequent patch testing, no signs of allergenicity were observed.

All subjects had reductions in acne scores, and with the exception of the subject with the rash, none had signs of irritation. No allergic reactions were observed (Educational & Research Foundation, Inc. 1991b).

Ocular Irritation

PEGs Soy Sterol

North Cliff Consultants, Inc. (1992) evaluated the ocular irritancy of a mascara containing 2% PEG-5 Soy Sterol using 60 female subjects. Each subject was examined by an ophthalmologist prior to the study. Each subject used the mascara twice daily for 4 weeks, and was examined weekly by a trained clinical technician and the ophthalmologist. Four subjects did not complete the study.

Several subjects reported sensations of irritation, but this could not be correlated with the evaluations made by the technician. Eighteen subjects, nine of whom were contact lens wearers, reported itching and/or irritation at least once during the study. Clinical signs of irritation (slight erythema) were observed around the eyes of 23 subjects, 11 of whom were contact wearers (North Cliff Consultants, Inc. 1992).

Biosearch Incorporated (1988) evaluated the cutaneous effects of an eyeliner containing 2% PEG-5 Soy Sterol during a clinical usage study using 60 female subjects between the ages of 18 and 35. Of the subjects, 26 wore contact lenses. Two contact lens wearers and one other subject did not complete the study. The subjects were examined by a dermatologist and

ophthalmologist prior to the start of the study. The subjects applied the eyeliner twice daily for 28 consecutive days. They were examined by a clinical technician weekly, and were reexamined by a dermatologist and an ophthalmologist at the conclusion of the study.

Of the non-contact lens wearers, one subject had a moderate amount of mucoid discharge with blue particles on both eyes, but was asymptomatic. Two subjects complained of burning sensations. Five subjects had inert "chunks" of the product in their conjunctiva that were not noticed by the subjects. None of the non-contact lens wearers had visual findings indicative of ocular irritation at the final examination. Several contact lens wearers had signs of ocular irritation, but irritation is not uncommon among individuals wearing contact lenses. Six subjects had changes from their baseline examinations: five subjects had changes that were "exclusively related to their contact lenses," and one had smeary lenses to which adhered numerous fine particles (Biosearch Incorporated 1988).

Miscellaneous Effects

Phytosterols

The phytosterols are often used to lower cholesterol by blocking its absorption (Pollak 1953; Shipley et al. 1958). Patients with hypercholesteremia given daily doses of sitosterols for periods exceeding 4 years had no signs of adverse effects (Shipley et al. 1958). Upon cessation of phytosterol intake, hemocholesterols returned to the original concentration (Pollak 1953).

Subjects were continuously administered sitosterols in the diet for periods exceeding 4 years, during which the total amount of sitosterol consumed was greater than 50% of the patient's body weight. Kidney and liver function, blood and urine composition, electrocardiogram, and gall bladder visualization were not different from controls. Treatment with sitosterol also did not contribute to the formation or progression of vascular lesions (General Mills, Inc. 1979).

Phytosterolemia is the impaired lipid metabolism characterized by the accumulation of free and esterified plant sterols and cholesterol in blood and tissues (Tvrzická et al. 1991) due to excessive intestinal absorption of sterols. Patients with hereditary phytosterolemia develop xanthomata, thrombocytopenia, hemolytic anemia, and premature atherosclerosis (Clayton et al. 1993).

SUMMARY

PEG-5, -10, -16, -25, -30, and -40 Soy Sterol are PEG derivatives of soybean oil sterols. They function as nonionic surfactants, and as emulsifying, skin-conditioning, cleansing, and solubilizing agents. The PEGs Soy Sterol are also used as appearance and consistency modifiers, emollients, viscosity-control agents, and pigment-dispersion agents. PEGs Soy Sterols are used in a wide variety of cosmetic products. Recent data supplied by industry indicated that PEG-5 Soy Sterol was used at concentrations up to 2%.

Few data were avaliable on the PEGs Soy Sterol. Because these chemicals may easily be hydrolyzed into the component PEGs and soy sterols, data on the PEGs, soy sterols, and phytosterols in general were included because they were considered relevant to the safety assessment of the PEGs Soy Sterol.

The PEGs Soy Sterol are produced by the reaction of the soy sterol hydroxyl with n moles of ethylene oxide. The soy sterols (phytosterols) include campesterol, stigmasterol, and β-sitosterol. The distribution of sterols found in oils derived from common plants is similar, with β-sitosterol comprising the major component. Analysis of production batches of phytosterols demonstrated consistency among batches and stability over time.

PEGs can contain small amounts of monomer and dimers, as well as peroxides formed during autoxidation. Ethoxylated surfactants can contain 1,4-dioxane, which is removed during purification from cosmetic ingredients prior to blending into cosmetic formulations. Refined plant sterols consist of approximately 88% total soy sterol, of which 56% is γ-sitosterol, 28% is campesterol, and 4% is stigmasterol. Impurities of the phytosterols are 4% to 6% sterol hydrocarbons and cholesterol, and 4% to 6% triterpene alcohols, keto-steroids, and other steroid-like substances.

The phytosterols are structurally similar to cholesterol. Approximately 0.25 to 0.5 g of plant sterols are consumed in a typical daily diet (20% to 25% of total dietary sterols). The phytosterols affect plant membrane structure and water permeability, and are commonly found in animal cell membranes after dietary intake. Phytosterols are less water soluble than cholesterol, but sitosterol and campesterol order bilayer acyl chains more effectively than cholesterol and stigmasterol.

Gastrointestinal absorption of PEGs was dependent on the molecular weight of the compound. The greater the molecular weight, the less the absorption. No metabolism was observed during oral and intravenous studies, and the PEGs were rapidly eliminated unchanged in the urine and feces. PEGs were readily absorbed through damaged skin.

Once consumed, phytosterols only entered the body via intestinal absorption. The absorption rate for the plant sterols was usually less than 5% of dietary concentrations in humans. Saturated sterols were virtually not absorbed, and approximately 95% of dietary phytosterols entered the colon. During subcutaneous injection studies using dogs, the phytosterols were not esterified or metabolized, and were treated like inert, foreign materials. Cholesterol, when similarly injected, was readily esterified. In other studies, sitosterol did not accumulate in tissues. β-Sitosterol and the ester, β-Sitosterol linoleate, in particular, have been shown to be poorly absorbed in the gut. A feature of the dynamics in the gut is that the ester may be hydrolyzed and that free sterol may be esterified with linoleate or any other fatty acid present in a dynamic equilibrium.

Freshly absorbed sterols were transported into plasma. The serum concentrations of phytosterols after dietary supplementation increased to 10% of total serum sterols. During metabolism

and excretion, the sterol rings generally remained intact, whereas double bonds, constituent groups, and side chains were often added, removed, or modified. The largest proportion of sterol (in humans, 20% of absorbed β-sitosterol) in the body was converted to bile acids. Absorbed phytosterols not converted to normal bile acids were excreted as free sterol. β-Sitosterol can be metabolized to 5,6-epoxides in the liver, cortisol in the adrenal glands, and various steroid hormones in the testes. In general, phytosterols can act as plant hormone and hormone precursors, and can be metabolized to minor steroid hormones and vitamin D compounds.

Sterols were typically eliminated via feces, urine, milk, and from the skin surface. The excretion rate of sitosterol from bile was 10 times greater than that of cholesterol. Nearly complete recovery of administered phytosterols in mammals was made from the feces. Unabsorbed sterols were degraded in the intestinal tract to varying degrees, depending on the species: 5% in rats, 25% in humans, and 65% in monkeys and baboons.

β-Sitosterol inhibited human platelet aggregation induced by arachidonic acid, platelet-activating factor, and ADP. A dose of 25 μg/ml phytosterols slightly decreased beat rates of fetal rat heart cells, whereas similar doses of cholesterol increased beat rates. Phytosterols in human neoplasms (particularly breast cancers) had osteolytic activity, and increased mobilization and excretion of bone calcium. Human umbilical vein endothelial cells contracted in vitro after being exposed to 0.7 mmol/L sitosterol for 72 h, and an increased release of intracellular lactate dehydrogenase was observed. At 96 h, the cells were partially detached from the substrate, and pertubation of the endothelial cells occurred after exposure to 0.35 mmol/L. Sitosterol had potent anti-inflammatory, antibacterial, and antifungal activities, and β-sitosterol had antiatherogenic effects through the inhibition of platelet aggregation. β-Sitosterol (100 μg/ml; 5% in DMSO and saline) was cytotoxic against seven cancer cell lines.

The acute oral LD_{50} in rats of PEG-5–25 Soy Sterol was >10 g/kg (50% in feed). The acute dermal LD_{50} of a liquid eyeliner containing 2% PEG-5 Soy Sterol was >2 g/kg. The acute oral LD_{50} values of the PEGs in rabbits were 17.3 g/kg and 76 g/kg for undiluted PEG-6 and PEG-75, respectively. No deaths were reported when groups of rabbits were treated topically with 20 ml/kg undiluted PEG-6 or 40% PEG-20M.

In an animal model designed to study the potential toxicity of repeated applications of a PEG-based antimicrobial cream to burn patients, renal failure was observed in rabbits that received repeated applications of an antimicrobial cream that contained 63% PEG-6, 5% PEG-20, and 32% PEG-75 to excised skin sites for 7 days. These data were consistent with the clinical picture in which burn patients that received PEG-based topical antimicrobial ointments had renal toxicity and contact dermatitis.

Wistar rats that received a basal diet supplemented with cholesterol and maize phytosterols (72.5% β-sitosterol, 20.5% campesterol, and 7% stigmasterol) had decreased hepatic cholesterol concentrations. Rats given the high dose of cholesterol and phytosterols had decreased malic enzyme and acetyl-CoA carboxylase activities, and had hypotriglyceridemia.

Wistar rats given subcutaneous injections of 250 to 500 μg/100 g β-sitosterol for 60 days had no gross or microscopic lesions of the liver or kidneys. Rats given 1000 μg/100 g had mild fibroblastic proliferation around the hepatic lobules and mild microscopic lesions of the kidney. Serum cholesterol was depleted in a dose-dependent manner, and serum protein was markedly reduced in rats of the high dose group.

PEG-20M and PEG-6 (≤4% in feed and drinking water, respectively) did not cause death or induce other evidence of toxicity in albino rats dosed daily for 90 days. Rabbits that received daily topical applications of PEG-6 5 days per week for 18 weeks had no signs of toxicity.

In a 90-day oral toxicity study in rats, diets containing plant phytosterol esters at all levels (maximum 8.1%) were well tolerated. Some small hematology and blood chemistry variations from the controls were noted. No treatment related effect was seen with organ weights and histological examination revealed no evidence of systemic toxicity. Absent any organ effects, the small hematology and blood chemistry variations were not considered of toxicological significance.

Dogs given PEG-8, PEG-32, and PEG-75 at concentrations of 2% in the diet for 1 year had no signs of toxicity. Dogs given feed containing 0.5 to 1.0 g/kg/day β-sitosterol had no gross or microscopic lesions after 8 to 22 months of treatment, and weight gain and clinical parameters did not differ from controls. Rabbits fed a diet containing 3% cottonseed sterols and 4% soy sterols for 70 to 212 days had neither clinical signs of toxicity nor gross and microscopic abnormalities.

PEG-5–25 Soy Sterol were not primary irritants when applied undiluted or up to 2% in formulation to intact and abraded skin of six rabbits per group. Undiluted PEG-5 Soy Sterol did not cause sensitization in a maximization study using Pirbright white guinea pigs. PEG-6 and -8 were not irritating to the skin of rabbits or guinea pigs, and PEG-75 was nonsensitizing to the skin of guinea pigs. Transient, mild erythema was observed when rabbits were given daily topical applications of 0.8 g/kg/day PEG-75 or PEG-20M for 30 days.

In clinical studies, PEG-5 Soy Sterol at concentrations up to 2% in formulation did not cause dermal or ocular irritation, dermal sensitization, or photosensitization. A formulation containing PEG-5 Soy Sterol and PEG-25 Soy Sterol (at concentrations of 0.75% and 1.5%, respectively) was tested for comedogenicity and sensitization; no increase in comedones and no signs of irritation or allergic reactions were observed. PEG-6 and PEG-8 were mild sensitizers during a clinical study, but later production lots of PEG-6 and PEG-75 did not cause sensitization. A product formulation containing 3% PEG-8 was a minimal to mild skin irritant.

Undiluted PEG-5, -10, -16, and -25 Soy Sterol and 2% PEG-5 Soy Sterol in formulation did not induce ocular toxicity when instilled into the conjunctival sac of six New Zealand white rabbits. Undiluted PEG-6 and PEG-75 did not cause corneal

injuries when instilled into the conjunctival sac of rabbits. A 35% solution of PEG-8 and PEG-32 (melted in a water bath) induced mild ocular irritation in rabbits.

No adverse reproductive effects occurred during subchronic (90 days) and chronic (2 years) oral studies of 0.062 to 1.69 g/kg/day PEG-6-32 and PEG-75.

Although monoalkyl ethers of ethylene glycol are reproductive toxins and teratogenic agents, given the methods of manufacture of the PEGs Soy Sterol, there is no likelihood of methoxyethanol, ethoxyethanol, etc being present as an impurity. Further, the PEGs Soy Sterol are ethers of soybean oil sterols, and as such, are chemically different from alkyl ethers. It is considered unlikely that there would be monoalkyl ethers of ethylene glycol in PEGs Soy Sterol which would cause reproductive or developmental effects based on their structural characteristics. β-Sitosterol was an effective estrogen-like agonist in exerting vaginal cornification and caused uterine weight gain in adult, ovariectomized Wistar rats. Subcutaneous injections of the sterol caused dose-related increases in uterine glycogen concentration after 10 days.

Progesterone treatment partially suppressed the phytosterol-induced elevation of glycogen concentration when administered in combination with the median and high phytosterol doses. β-Sitosterol also stimulated glucose-6-phosphate dehydrogenase, phosphohexose isomerase, and total lactate dehydrogenase activities. In a related study, uterine RNA, DNA, and protein concentrations were increased by treatment with β-sitosterol. Other studies of well-characterized phytosterols and phytosterol esters demonstrated no effect in an estrogen-binding study, a recombinant yeast assay for estrogen or estrogen-like activity, or a juvenile rat uterotrophic assay for estrogen or estrogen-like activity. Sulfates of β-sitosterol acted as abortifacients in female rats and Dutch-belted rabbits via estrogenic and spermicidal effects. β-Sitosterol itself had antiestrogenic, antiprogestational, gonadotrophic, antigonadotrophic, and antiandrogenic effects.

Subcutaneous injections of 5 mg/kg/day of β-sitosterol for 16 to 48 days reduced sperm concentrations and fertility, and decreased testis and accessory sex tissue weights (time-dependent) in male Wistar rats. Rats given 0.5 mg/kg/day had a significant decrease in sperm concentration of the caput epididymis after 48 days of treatment, but no reduction in fertility. The observed decreases in sperm concentration persisted after withdrawal of treatment, and appeared to be due to a reduction of the rate of spermatogenesis. A two-generation reproduction study of phytosterol esters in the diet of rats (maximum 8.1%) failed to produce any treatment-related adverse effects.

PEG-5 Soy Sterol was not cytotoxic or mutagenic to five strains of *S. typhimurium*, with or without S9 activation. PEG-8 (up to 1%) was negative in the Chinese hamster ovary cell mutation test and the sister chromatid exchange test. At 0.1%, the highest concentration tested, a statistically significant increase in radioactive thymidine incorporation into rat hepatocyte nuclei was noted in the unscheduled DNA synthesis assay. PEG-150 was not mutagenic in the mouse lymphoma forward mutation assay when tested at concentrations up to 150 g/L. Phytosterols and phytosterol esters were not genotoxic, with or without metabolic activation, in the Ames assay, a human lymphocyte chromosome damage assay, an unscheduled DNA synthesis assay, or a rat bone marrow micronucleus assay.

PEG-8, as a solvent control in studies of suspected carcinogenic agents, was not carcinogenic when administered orally to mice (30 weeks), intraperitoneally to rats (6 months), subcutaneously to mice (1 year) and rats (20 weeks), or when injected into the gastric antrum of guinea pigs over a period of 6 months. Such short duration studies are of utility in assessing the carcinogenicity of only the most potent carcinogens.

Sitosterol inhibited the tumor-promoting activity of TPA in the skin of female ICR mice after initiation with DMBA. The percent reduction in the average number of tumors at week 18 was 40% in mice given TPA, DMBA, and sitosterol. Sitosterol applied topically before treatment with TPA inhibited TPA-induced epidermal ODC activity; ODC induction can be representative of the effects of phorbol esters with strong tumor-promoting activity. Additionally, dermal inflammation caused by a single application of TPA was slightly inhibited by sitosterol and stigmasterol.

Male Fischer CD rats coadministered the direct-acting carcinogen *N*-methylnitrosourea (by cannulation) and ß-sitosterol (95% pure, with 4% campesterol and 1% stigmasterol; 0.2% in feed) had significantly fewer colonic tumors (benign or benign and malignant) compared to rats given the carcinogen alone after 28 weeks. Of rats given the carcinogen alone, 54% had tumors. Of rats given both the carcinogen and sitosterol, 33% had tumors. The incidence of rats with malignant colonic neoplasms increased after coadministration of the phytosterols; 15% (7/48) had invasive carcinomas in the sterol plus carcinogen group compared to 7% (5/71) of rats given the carcinogen alone.

The phytosterols decreased epithelial cell proliferation of the colon in mice (0.1% in feed) and rats (0.2% in feed after induction with *N*-methyl-*N*-nitrosourea), and were cytotoxic for human epidermoid carcinoma of the nasopharynx (>20 μg/ml).

DISCUSSION

The CIR Expert Panel considers that the safety of PEGs Soy Sterol ingredients can be evaluated by reviewing the data available on the ingredients themselves, supplemented by the available data in a previous review of PEGs and the available data on soy phytosterols. In addition, because of the similarities between the composition of soy phytosterols and plant phytosterols in general, the available test data on plant phytosterols is also relevant. Also, no particular toxicity, not seen with the two constituents, would be expected from the PEGs Soy Sterol esters.

Although no dermal absorption data were available, oral studies demonstrate that phytosterols and phytosterol esters are not significantly absorbed and do not result in systemic exposure. Some small amounts did appear in the ovaries, however. One of the Panel's concerns in the original safety assessment of

these ingredients was the potential presence of free phytosterols and β-sitosterol, which could have antiestrogenic, antiprogestational, gonadotrophic, antigonadotrophic, and antiandrogenic effects in PEG sterols. These concerns have been alleviated by the extensive data showing that well-defined phytosterols and phytosterol esters are not estrogenic and do not pose a hazard to reproduction. Likewise, the Panel noted the absence of impurities in plant phytosterols and phytosterol esters and extensive data demonstrating the absence of any genotoxicity in bacterial and mammalian systems, and mitigating against the possibility of any carcinogenic effect with those same well-characterized materials.

The Panel noted that, in clinical tests, PEGs Soy Sterol were not irritating, did not sensitize or photosensitize, produced transient ocular irritation, and were not comedogenic. The CIR Expert Panel, however, was concerned about the sensitization potential of the PEG-5, -10, -16, -25, and -40 Soy Sterol when applied to damaged skin. This concern arose because of positive patch tests and incidences of nephrotoxicity in burn patients treated with an antimicrobial cream that contained PEG-6, PEG-20, and PEG-75. PEGs were determined to be the causitive agents in both animal and human studies. No evidence of systemic toxicity or sensitization was found in studies with intact skin. Because PEGs Soy Sterol ingredients may be hydrolyzed to release PEGs, these data were of concern for this safety assessment. The cosmetic industry is advised, therefore, to avoid using PEGs Soy Sterol in cosmetic formulations that may be used on damaged skin.

The Panel members stressed that the cosmetic industry should continue to use the necessary purification procedures to remove possible 1,4-dioxane and ethylene oxide impurities from the ingredients before blending them into cosmetic formulations.

CONCLUSION

On the basis of the available information in this report the CIR Expert Panel concludes that the PEG-5, -10, -16, -25, and -40 Soy Sterol are safe as used in cosmetic products.

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Personal Care Products Council
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Memorandum

TO: Lillian Gill, Ph.D.
Director - COSMETIC INGREDIENT REVIEW (CIR)

FROM: Halyna Breslawec, Ph.D.
Industry Liaison to the CIR Expert Panel

DATE: July 9, 2013

SUBJECT: Comments on the Scientific Literature Review: Safety Assessment of Phytosterols as Used in Cosmetics

The Council does not have any suppliers listed for:
Canola Sterols
Persea Gratissima (Avocado) Sterols
Phytosteryl Caprylate/Caprate
Soy Sterol Acetate

This report has no page numbers. Comments are provided by section headings.

Key Issue

If Diosgenin is included in the report, the CIR report on Dioscorea Villosa (Wild Yam) Root Extract (which contains Diosgenin) needs to be added to the Introduction. The CIR Expert Panel concluded that the Dioscorea Villosa (Wild Yam) Root Extract reviewed (Diosgenin content 3.5%) was safe as used. The maximum concentration of the Extract reported was 15%.

Additional Comments

Introduction - Please correct the first sentence of the report. It currently states: "This report reviews that available scientific information"

Introduction, Use - It is not necessary to list every function of each ingredient in the Introduction. The most common reported functions would be sufficient for the Introduction. Without additional information, stating that these ingredients function as "drug astringents" is misleading. Only one ingredient, Phytosteryl Rice Branate has "Drug Astringents - Skin Protectant Drugs", and this listing comes with the caveat: "See Reported Ingredient Functions-The Cosmetic Drug Distinction, in Regulatory and Ingredient Use Information, Volume I, Part A." that explains although the function may be considered a drug function in the United States, it may not be a drug function in other countries.

Introduction - Please correct "value you may be added with the potential for read-across"

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Introduction - From the information provided in the Introduction, it is not clear if oral exposure studies concerning reproductive toxicity, genotoxicity and carcinogenicity data are included in this report.

PEGs Soy Sterol Report - What was the source of phytosterols that are described in the second paragraph? Based on the heading, perhaps they are soy sterols?

Impurities - The first paragraph concerning the composition of soybean oil should not be in the impurities section. Sterols are a normal component of soybean oil not an impurity. The last two paragraphs also do not belong in the Impurities section. It does not make sense to state "Refined plant sterols are reported to contain ~88% total soy sterol content." Other plants will not contain "soy" sterols.

Absorption, Distribution, Metabolism and Excretion - Please provide references to the animals studies that show that increasing the side chain length of cholesterol decreased absorption. In which species were these studies completed? It should be noted if this statement refers only to the *in situ* stucy e.g., intestinal perfusion studies, results. What doses were used in the 90-day feeding study in rabbits (reference 43)?

Animal Toxicology - The first sentence of this section says: "Many of the phytosterols in this study..." - the study to which this refers is not clear.

PEG Soy Sterol Report - How many rats were used in the 90-day oral study in female Wistar rats?

Reproductive and Developmental Toxicity - It is not clear what is meant by "fraternal effects". How was estrogenic activity measured (references 49, 50)? Were any hematological or clinical chemistry effects observed in the mink study? How long did the effects persist after treatment (reference 52)?

Reproductive and Developmental Toxicity/PEG Soy Sterol Report - The uterotrophic assay from the PEG Soy Sterol Report that is presented in the Reproductive and Developmental Toxicity section should be moved to the estrogenic activity section.

Estrogenic Effects/In Vitro - Please correct "when exposed to of human"

Genotoxicity - Please indicate the species tested in the *in vivo* studies. It would be helpful if the genotoxicity summary could state the compounds that were tested.

Carcinogenicity/PEG Soy Sterol Report - The studies from the PEG Soy Sterol Report in the Carcinogenicity section concern the prevention of carcinogenic effect and should not be presented in the Carcinogenicity section.

Irritation/Ocular - What concentrations of VDPSE and VODPSE were tested in the chicken eye assay?

In Vitro/Constituents - The studies in this section do not appear to be relevant. At a minimum, these studies should be in the Chemistry section as they concern identifying protein in soybean oil.

Summary - The third paragraph of the Summary does not make sense. The first sentence says that systemic exposure from foods is greater than from use in cosmetics. The second sentence says that oral exposure does not result in systemic exposure. It is not clear what is meant by "fraternal effects". In the Summary, it is misleading state that "there were not IgE-binding proteins detected in multiple phytosterol samples". It was not phytosterol samples that were tested for proteins. Earlier in the report it says that hydrophilic extracts of vegetable oils and refined soybean oils that were tested.

Table 2 - The meaning of the column heading "Maximum concentration" is not clear. Presuming that this means Maximum Reported Concentration of Use in Cosmetic Products - it just does not make sense that Acetic Acid is used at 100%. If you look at the CIR report included Acetic Acid. the maximum use concentration reported was 0.4% in other hair coloring preparations. The CIR report on Dioscorea Villosa (Wild Yam) Root Extract should be included in Table 2.

Table 3, Table 4 - As requested by the CIR Expert Panel, specific information, such as the information in these tables, needs to be cited to the original references rather than the PEG Soy Sterol CIR report.

Table 6- Please add a footnote to this table that describes the differences between the old and the new extraction processes.

Table 7 - In the Phytosterols section in the Incidental Inhalation - sprays row, the 0.000 concentration needs to be corrected.

Table 8 - When not indicated, please provide the source of the phtyosterols tested in each study. In footnote a, please correct "compotition"