ABS Pomegranate Sterols
Toxicology Data

<table>
<thead>
<tr>
<th>EINECS #: N/A</th>
<th>Name of Study</th>
<th>Type of Study</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermal &amp; Ocular Irritation Tests</td>
<td>In-vitro</td>
<td>Both the dermal and ocular assays reveal that <strong>ABS Pomegranate Sterols</strong> is non-irritating and should not cause any of the aforementioned conditions.</td>
<td></td>
</tr>
<tr>
<td>AMES Test</td>
<td>In-vitro</td>
<td>The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, that <strong>ABS Pomegranate Sterols</strong> was considered to be Non-Mutagenic to Salmonella typhimurium testerstrains TA98, TA100, TA1537, TA1535 and Escherichia coli WP2uvrA.</td>
<td></td>
</tr>
<tr>
<td>OECD TG 442D In-Vitro Skin Sensitization</td>
<td>In-vitro</td>
<td>The results using the ARE-Nrf2 Luciferase Test Method in accordance with UN GHS indicate that <strong>ABS Pomegranate Sterols</strong> was not predicated to be a skin sensitizer</td>
<td></td>
</tr>
<tr>
<td>OECD TG 442C Direct Peptide Reactivity</td>
<td>In-chemico</td>
<td>Based on HPLC-UV analysis <strong>ABS Pomegranate Sterols</strong> was determined as a non-sensitizer and will not cause allergic contact dermatitis.</td>
<td></td>
</tr>
<tr>
<td>OECD 301B Ready Biodegradability</td>
<td>In-chemico</td>
<td>The results of the Modified Sturm Test ensure <strong>ABS Pomegranate Sterols</strong> met method requirements for the Readily Biodegradable classification.</td>
<td></td>
</tr>
<tr>
<td>Name of Study</td>
<td>Type of Study</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>OECD 202 Acute Daphnia</td>
<td>In-vivo</td>
<td>According to the EU Directive 93/67/EEC, ABS Pomegranate Sterols is not classified as harmful to aquatic organisms.</td>
<td></td>
</tr>
<tr>
<td>Phototoxicity</td>
<td>Instrumental</td>
<td>There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that ABS Pomegranate Sterols is not a photoirritant when used at the suggested use levels of 0.5% - 5.0%.</td>
<td></td>
</tr>
</tbody>
</table>
**Sample:** ABS Pomegranate Sterols

**Code:** 10247

**CAS #:** 949109-75-5

**Test Request Form/Submission #:** 320

**Lot #:** SN120618-4

**Sponsor:** Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
**Study Director:** Maureen Danaher
**Principle Investigator:** Jennifer Goodman

**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% CO₂, 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. 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% CO₂ 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 H₂O
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% CO₂ 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% CO₂ 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% CO₂, 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% CO₂, 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% CO₂, 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 (OD₅₇₀) is ≥ 1.0 and ≤ 2.5
   (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).
Dermal and Ocular Irritation Tests

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

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.

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
SAMPLE DESCRIPTION:

<table>
<thead>
<tr>
<th>ACCESSION #:</th>
<th>SAMPLE:</th>
<th>LOT #:</th>
<th>BATCH #:</th>
<th>QTY</th>
</tr>
</thead>
<tbody>
<tr>
<td>528350</td>
<td>ABS Pomegranate Sterols</td>
<td>SN070302-12</td>
<td>Not Specified</td>
<td>50g</td>
</tr>
</tbody>
</table>

TEST PERFORMED:

- Genotoxicity: Bacterial Reverse Mutation Test
- BTS METHOD #: N/A
- REFERENCE: 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 TA1537, and in tryptophan-dependent *Escherichia coli* strain WP2uvrA 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 WP2uvrA. 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 WP2uvrA. 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 WP2uvrA 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 carcogenic 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 histidine 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 (WP2uvrA) 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.

<table>
<thead>
<tr>
<th>Tester Strain</th>
<th>Mutations/Genotypic Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> TA 98</td>
<td>hisD3052, rfa, <em>uvrB</em>, frame shift, pKM101</td>
</tr>
<tr>
<td><em>S. typhimurium</em> TA100</td>
<td>hisG46, rfa, <em>uvrB</em>, missense, pKM101</td>
</tr>
<tr>
<td><em>S. typhimurium</em> TA1535</td>
<td>hisG46, rfa, <em>uvrB</em>, missence</td>
</tr>
<tr>
<td><em>S. typhimurium</em> TA1537</td>
<td>hisC3076, rfa, <em>uvrB</em>, frame shift</td>
</tr>
<tr>
<td><em>E. coli</em> WP2uvrA</td>
<td>trpE65, <em>uvrA</em>, missence</td>
</tr>
</tbody>
</table>

*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 MgCl₂/1.65 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*, WP2uvrA, 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 WP2uvrA, 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 SWI 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 WP2uvrA.
7. 1X Methylmethane-sulfonate (known mutagen) with and without S9 activation with strain W02uvrA.
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 revertants 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 WP2uvrA 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 WP2uvrA 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 WP2uvrA. 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

<table>
<thead>
<tr>
<th>Characteristics (expected)</th>
<th>TA98</th>
<th>TA100</th>
<th>TA1535</th>
<th>TA1537</th>
<th>WP2uvrA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin-TA98 &amp; TA100 = (Resistant) TA1535, TA1537 &amp; WP2uvrA = (Sensitive)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>rfa Mutation; CV (Sensitive)</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NA</td>
</tr>
<tr>
<td>uvrB/uvrA (No Growth)</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>Histidine Requirement; (Growth)</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>NA</td>
</tr>
<tr>
<td>Tryptophan Requirement; (Growth)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>G</td>
</tr>
<tr>
<td>Biotin (No Growth)</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NA</td>
</tr>
<tr>
<td>L-tryptophan (No Growth)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NG</td>
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<tr>
<td>Purity (Pure)</td>
<td>PURE</td>
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<td>PURE</td>
<td>PURE</td>
<td>PURE</td>
</tr>
<tr>
<td>Total Plate Count CFU’s ($10^{-7}$)</td>
<td>68</td>
<td>61</td>
<td>241</td>
<td>18</td>
<td>301</td>
</tr>
<tr>
<td>Mean</td>
<td>50</td>
<td>74</td>
<td>226</td>
<td>19</td>
<td>298</td>
</tr>
<tr>
<td>Titer (Organisms/ml)</td>
<td>$5.0 \times 10^8$</td>
<td>$7.4 \times 10^8$</td>
<td>$2.3 \times 10^9$</td>
<td>$1.9 \times 10^8$</td>
<td>$3.0 \times 10^9$</td>
</tr>
</tbody>
</table>

R = Resistant    S = Sensitive    NG = No Growth    G = Growth    NA = Not Applicable
## Appendix 2 – Spot Plate Inhibition Screen Results

<table>
<thead>
<tr>
<th>Zone of Inhibition (mm)</th>
<th>TA98</th>
<th>TA100</th>
<th>TA1535</th>
<th>TA1537</th>
<th>WP2uvrA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sponsor provided negative control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Test article solution</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dexon positive control</td>
<td>34</td>
<td>39</td>
<td>37</td>
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<td>28</td>
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### Appendix 3 – Standard Plate Incorporation Assay – Reversion Rates For Tester Strains

<table>
<thead>
<tr>
<th></th>
<th>Salmonella typhimurium</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA98</td>
<td>TA100</td>
</tr>
<tr>
<td><strong>CFTP</strong></td>
<td>Mean</td>
<td>CFTP</td>
</tr>
<tr>
<td><strong>Sponsor provided negative control w/o S9</strong></td>
<td>20</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>137</td>
</tr>
<tr>
<td><strong>Sponsor provided negative control w/ S9</strong></td>
<td>28</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>155</td>
</tr>
<tr>
<td><strong>Test article solution w/o S9</strong></td>
<td>28</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>152</td>
</tr>
<tr>
<td><strong>Test article solution w/ S9</strong></td>
<td>28</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>126</td>
</tr>
<tr>
<td><strong>Dexon w/o S9 positive control</strong></td>
<td>1792</td>
<td>1120</td>
</tr>
<tr>
<td></td>
<td>1424</td>
<td>1648</td>
</tr>
<tr>
<td><strong>Dexon w/ S9 positive control</strong></td>
<td>1696</td>
<td>1104</td>
</tr>
<tr>
<td></td>
<td>1344</td>
<td>992</td>
</tr>
<tr>
<td><strong>2-aminofluorene w/o S9 positive control</strong></td>
<td>173</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>192</td>
</tr>
<tr>
<td><strong>2-aminofluorene w/ S9 positive control†</strong></td>
<td>1616</td>
<td>1552</td>
</tr>
<tr>
<td><strong>Sodium azide w/o S9 positive control</strong></td>
<td>2080</td>
<td>3024</td>
</tr>
<tr>
<td></td>
<td>3472</td>
<td>2859</td>
</tr>
<tr>
<td><strong>Sodium azide w/ S9 positive control</strong></td>
<td>2752</td>
<td>3083</td>
</tr>
<tr>
<td></td>
<td>2544</td>
<td>2583</td>
</tr>
<tr>
<td><strong>2-aminoanthracene w/o S9 positive control</strong></td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td><strong>2-aminoanthracene w/ S9 positive control†</strong></td>
<td>576</td>
<td>512</td>
</tr>
<tr>
<td><strong>Methylmethane-Sulfonate w/o S9 positive control</strong></td>
<td>864</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td>640</td>
<td>683</td>
</tr>
<tr>
<td><strong>Methylmethane-Sulfonate w/ S9 positive control</strong></td>
<td>944</td>
<td>880</td>
</tr>
<tr>
<td></td>
<td>576</td>
<td>880</td>
</tr>
</tbody>
</table>

CFTP = Counts from triplicate plates  
Mean = Average of triplicate plates  
= Not Applicable

*Negative control for S9  
†Positive control for S9  

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Wendy Yang, M.S.  
Microbiology Manager
**Tradename:** ABS Pomegranate Sterols

**Code:** 10247

**CAS #:** 949109-75-5

**Test Request Form #:** 2086

**Lot #:** 46090P

**Sponsor:** Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

**Study Director:** Maureen Danaher

**Principle Investigator:** Jennifer Goodman

**Test Performed:**
OECD TG 442D: In Vitro Skin Sensitization ARE-Nrf2 Luciferase Test Method

**Introduction**

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals\(^1\). Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **ABS Pomegranate Sterols** in accordance with the UN GHS.

**Assay Principle**

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

Materials

A. Incubation Conditions: 37°C at 5% CO2 and 95% relative humidity (RH)
B. Equipment: Humidified incubator; Biosafety laminar flow hood; Microplate Reader; Pipettes
C. Cell Line: KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers: Dulbecco’s Modified Eagle Medium (DMEM); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Genetin
E. Culture Plate: Flat bottom 96-well tissue culture treated plates
F. Reagents: Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS)
G. Other: Sterile disposable pipette tips; wash bottles

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluence in DMEM containing 10% FBS and 500μg/mL G418 geneticin. Twelve test concentrations of ABS Pomegranate Sterols were prepared in DMSO with a concentration range from 0.98 - 2000 µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64 µM. The negative control was a 1% test concentration of DMSO.

24 hour post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO2 and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO2. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC50 and IC30 values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega’s ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and EC1.5 and maximum response (Imax) values were obtained.
Data and Reporting

Acceptance Criteria:

1. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
2. The EC1.5 value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
3. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

A KeratinoSens™ prediction is considered positive if the following conditions are met:

1. The Imax is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
2. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the EC1.5 determining concentration)
3. The EC1.5 value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

<table>
<thead>
<tr>
<th>Compound</th>
<th>Classification</th>
<th>EC1.5 (µM)</th>
<th>IC50</th>
<th>Imax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamic aldehyde</td>
<td>Sensitizer</td>
<td>19</td>
<td>289.19 µM</td>
<td>31.52</td>
</tr>
<tr>
<td>DMSO</td>
<td>Non-Sensitizer</td>
<td>No Induction</td>
<td>243.24 µM</td>
<td>0.16</td>
</tr>
<tr>
<td>ABS Pomegranate Sterols</td>
<td>Non-Sensitizer</td>
<td>No Induction</td>
<td>&gt; 1000 µM</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table 1: Overview of KeratinoSens™ Assay Results (Imax equals the average induction values Fig.1)
Discussion

As shown in the results, **ABS Pomegranate Sterols (10247)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **ABS Pomegranate Sterols** can be safely used in cosmetics and personal care products at typical use levels.
Tradename: ABS Pomegranate Sterols

Code: 10247

CAS #: 949109-75-5

Test Request Form #: 2231

Lot #: 45923P

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
Study Director: Maureen Danaher
Principle Investigator: Jennifer Goodman

Test Performed:
OECD TG 442C: In Chemico Skin Sensitization Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis. The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%).

This assay was conducted to determine skin sensitization hazard of ABS Pomegranate Sterols in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an in chemico method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

Materials

A. Equipment: HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance

B. HPLC/Guard Columns: Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5µm; Phenomenex Security Guard C18 4mm x 2mm

C. Chemicals: Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAACAA-COOH); Lysine peptide (Ac-RFAAKAA-COOH); Cinnamic aldehyde

D. Reagents/Buffers: Sodium phosphate buffer (100mM); Ammonium acetate buffer (100mM)

E. Other: Sterile disposable pipette tips

Methods

Solution Preparation:

- 0.667mM Cysteine Peptide in 100mM Phosphate Buffer (pH 7.5)
- 0.667mM Lysine Peptide in 100mM Ammonium Acetate Buffer (pH 10.2)
- 100mM Cinnamic Aldehyde in Acetonitrile
- 100mM* ABS Pomegranate Sterols in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as ABS Pomegranate Sterols, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

Reference Controls:

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

Sample, Reference Control, and Co-Elution Control Preparation:

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

<table>
<thead>
<tr>
<th>1:10 Ratio, Cysteine Peptide</th>
<th>1:50 Ratio, Lysine Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5mM Peptide, 5mM Test Chemical</td>
<td>0.5mM Peptide, 25mM Test Chemical</td>
</tr>
<tr>
<td>750µL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls)</td>
<td>750µL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls)</td>
</tr>
<tr>
<td>200µL Acetonitrile</td>
<td>250µL Test Chemical Solution (or Acetonitrile for Reference Controls)</td>
</tr>
<tr>
<td>50µL Test Chemical Solution (or Acetonitrile for Reference Controls)</td>
<td></td>
</tr>
</tbody>
</table>
Calibration Curve:

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
  - For the Cysteine peptide using the phosphate buffer, pH 7.5
  - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

<table>
<thead>
<tr>
<th>mM Peptide</th>
<th>Standard 1</th>
<th>Standard 2</th>
<th>Standard 3</th>
<th>Standard 4</th>
<th>Standard 5</th>
<th>Standard 6</th>
<th>Standard 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.534</td>
<td>0.267</td>
<td>0.1335</td>
<td>0.0667</td>
<td>0.0334</td>
<td>0.0167</td>
<td>0.000</td>
</tr>
</tbody>
</table>

HPLC Analysis:

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220nm
- Flow Conditions:

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes</td>
<td>0.35 mL/min</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10 minutes</td>
<td>0.35 mL/min</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>11 minutes</td>
<td>0.35 mL/min</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>13 minutes</td>
<td>0.35 mL/min</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>13.5 minutes</td>
<td>0.35 mL/min</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>20 minutes</td>
<td>End Run</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data and Reporting

Acceptance Criteria:

1. The following criteria must be met for a run to be considered valid:
   a. Standard calibration curve should have an r² > 0.99.
   b. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be <14.9 for the percent cysteine depletion and <11.6 for the percent lysine depletion.
   c. Mean peptide concentration of the reference controls A should be 0.50±0.05mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be <15.0%.

2. The following criteria must be met for a test chemical’s results to be considered valid:
   a. Maximum standard deviation should be <14.9 for percent cysteine depletion and <11.6 for percent lysine depletion.
   b. Mean peptide concentration of the three reference control C should be 0.50±0.05mM.
Prediction Model:

<table>
<thead>
<tr>
<th>Cysteine 1:10/Lysine 1:50 Prediction Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Cysteine and Lysine % Depletion</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>0% &lt; Mean % Depletion &lt; 6.38%</td>
</tr>
<tr>
<td>6.38% &lt; Mean % Depletion &lt; 22.62%</td>
</tr>
<tr>
<td>22.62% &lt; Mean % Depletion &lt; 42.47%</td>
</tr>
<tr>
<td>42.47% &lt; Mean % Depletion &lt; 100%</td>
</tr>
</tbody>
</table>

If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

<table>
<thead>
<tr>
<th>Cysteine 1:10 Prediction Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Cysteine and Lysine % Depletion</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>0% &lt; Cys % Depletion &lt; 13.89%</td>
</tr>
<tr>
<td>13.89% &lt; Cys % Depletion &lt; 23.09%</td>
</tr>
<tr>
<td>23.09% &lt; Cys % Depletion &lt; 98.24%</td>
</tr>
<tr>
<td>98.24% &lt; Cys % Depletion &lt; 100%</td>
</tr>
</tbody>
</table>

Therefore the measured values of % depletion in the three separated runs for each peptide depletion assay include:

<table>
<thead>
<tr>
<th>Cysteine 1:10/Lysine 1:50 Prediction Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Cysteine and Lysine % Depletion</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>3.27</td>
</tr>
<tr>
<td>3.26</td>
</tr>
<tr>
<td>3.31</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cysteine 1:10 Prediction Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of Cysteine and Lysine % Depletion</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>3.16</td>
</tr>
<tr>
<td>3.12</td>
</tr>
<tr>
<td>3.18</td>
</tr>
</tbody>
</table>

Results and Discussion

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

Percent peptide depletion is determined by the following equation:

\[
Percent \, Peptide \, Depletion = \left[1 - \left(\frac{Peptide \, Peak \, Area \, in \, Replicate \, Injection}{Mean \, Peptide \, Peak \, Area \, in \, Reference \, Controls}\right)\right] \times 100
\]

Based on HPLC-UV analysis of ABS Pomegranate Sterols (10247) we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.22% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.
OECD 301B Ready Biodegradability Assay

Tradename: ABS Pomegranate Sterols

Code: 10247

CAS #: 949109-75-5

Test Request Form #: 1979

Lot #: 46420P

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
Study Director: Maureen Danaher
Principle Investigator: Jennifer Goodman

Test Performed:
OECD 301 B
Ready Biodegradability: CO₂ Evolution (Modified Sturm Test)

Introduction

A study was conducted to assess the ready biodegradability of ABS Pomegranate Sterols in an aerobic aqueous medium. In the OECD guideline 301 for ready biodegradability, six methods are provided as options. This report uses method B, CO₂ Evolution, also known as a Modified Sturm Test. This method was chosen based on the solubility, volatility, and adsorbing capabilities of the test sample.

Assay Principle

A solution or suspension of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC (Dissolved Organic Carbon) in the test solution due to the inoculum should be kept as low as possible compared to the amount of organic carbon due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blanks with inoculum but without test substance. A reference compound is run in parallel to check the procedures' operation.

In general, degradation is followed by the determination of parameters such as DOC, carbon dioxide production, and oxygen uptake. Measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation.

Normally this test lasts for 28 days, but it may be ended before that time if the biodegradation curve reaches a plateau for at least three determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but the plateau has not yet been reached. In such cases the test substance would not be classified as readily biodegradable.
OECD 301B Ready Biodegradability Assay

The pass levels for ready biodegradability are 70% removal of DOC and 60% of ThOD (Theoretical Oxygen Demand) or ThCO$_2$ (Theoretical Carbon Dioxide) production for respirometric methods. They are lower in the respirometric methods since, as some of the carbon from the test chemical is incorporated into new cells, the percentage of CO$_2$ produced is lower than the percentage of carbon being used. These pass values have to be reached in a 10-day window within the 28-day period of the test. The 10-day window begins when the degree of biodegradation has reached 10% DOC, ThOD, or ThCO$_2$ and must end before day 28 of the test. Test substances which reach the pass levels after the 28-day period are not deemed to be readily biodegradable.

In order to check the procedure, reference compounds which meet the criteria for ready biodegradability are tested by setting up an appropriate vessel in parallel as part of normal test runs. Suitable compounds are freshly distilled aniline, sodium acetate, and sodium benzoate. These compounds all degrade in this method even when no inoculum is deliberately added.

Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate. It is usually found that the larger the concentration of microorganisms initially added to the test medium, the smaller the variation between replicates.

Materials

- Water
  - Deionized or distilled, free from inhibitory concentrations of toxic substances
  - Must contain no more than 10% of the organic carbon content introduced by the test material
  - Use only one batch of water for each series of tests
- Mineral media
  - To prepare the mineral medium, mix 10 mL of solution A with 800 mL water. Then add 1 mL each of solutions B, C, and D and make up to 1 liter with water.
  - Solution A (Dissolve in water and make up to 1 liter; pH 7.4)
    - Potassium dihydrogen orthophosphate, KH$_2$PO$_4$: 8.5g
    - Dipotassium hydrogen orthophosphate, K$_2$HPO$_4$: 21.8g
    - Disodium hydrogen orthophosphate dehydrate, Na$_2$HPO$_4$.2H$_2$O: 33.4g
    - Ammonium chloride, NH$_4$Cl: 0.5g
  - Solution B (Dissolve in water and make up to 1 liter)
    - Calcium chloride, anhydrous, CaCl$_2$: 27.50g
    - Calcium chloride dehydrate, CaCl$_2$.2H$_2$O: 36.40g
  - Solution C (Dissolve in water and make up to 1 liter)
    - Magnesium sulphate heptahydrate, MgSO$_4$.7H$_2$O: 22.50g
  - Solution D (Dissolve in water and make up to 1 liter)
    - Iron (III) chloride hexahydrate, FeCl$_3$.6H$_2$O: 0.25g
OECD 301B Ready Biodegradability Assay

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Methods

I. Preparation of flasks: As an example, the following volumes and weights indicate the values for 5-liter flasks containing 3 liters of suspension. If smaller volumes are used, modify the values accordingly.
   a. To each 5-liter flask, add 2,400 mL mineral medium.
   b. Add an appropriate volume of the prepared activated sludge to give a concentration of suspended solids of not more than 30 mg/L in the final 3 liters of inoculated mixture. Alternatively, first dilute the prepared sludge to give a suspension of 500-1000 mg/L in the mineral medium before adding an aliquot to the contents of the 5-liter flask to attain a concentration of 30 mg/L.
   c. Aerate these inoculated mixtures with CO₂-free air overnight to purge the system of carbon dioxide.
   d. Add the test material and reference compound, separately, as known volumes of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 – 20 mg DOC or TOC per liter. Leave some flasks without addition of chemicals as inoculum controls. Add poorly soluble test substances directly to the flasks on a weight or volume basis. Make up the volumes of suspensions in all flasks to 3 liters by the addition of mineral medium previously aerated with CO₂-free air.
OECD 301B Ready Biodegradability Assay

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e. If required, use one flask to check the possible inhibitory effect of the test substance by adding both the test and reference substances at the same concentrations as present in the other flasks.

f. If required, check whether the test substance is degraded abiotically by using a sterilized uninoculated solution of the chemical. Sterilize by the addition of a toxic substance at an appropriate concentration.

g. If barium hydroxide is used, connect three absorption bottles, each containing 100 mL of 0.0125M barium hydroxide solution, in series to each 5-liter flask. The solution must be free of precipitated sulfate and carbonate and its strength must be determined immediately before use.

h. If sodium hydroxide is used, connect two traps, the second acting as a control to demonstrate that all the carbon dioxide was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 mL 0.05M sodium hydroxide to each bottle. This is sufficient to absorb the total quantity of carbon dioxide evolved when the test substance is completely degraded.

i. In a typical run, the following flasks are used:
   i. Flasks 1 & 2: containing test substance and inoculum (test suspension)
   ii. Flasks 3 & 4: containing only inoculum (inoculum blank)
   iii. Flask 5: containing reference compound and inoculum (procedure control)
   iv. Flask 6: containing test substance and sterilizing agent (abiotic sterile control)
   v. Flask 7: containing test substance, reference compound and inoculum (toxicity control)

II. Start the test by bubbling CO₂-free air through the suspensions at a rate of 30-100 mL/minute.

III. CO₂ Determination
   a. It is mandatory to follow the CO₂ evolution from the test suspensions and inoculum blanks in parallel and it is advisable to do the same for the other test vessels.
   b. During the first ten days it is recommended that analyses of CO₂ should be made every second or third day and then at least every fifth day until the 28th day so that the 10-day window period can be identified. On the days of CO₂ measurement, disconnect the barium hydroxide absorber closest to the test vessel and titrate the hydroxide solution with 0.05M HCl using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the test vessel and place a new absorber containing 100 mL fresh 0.0125M barium hydroxide at the far end of the series. Make titrations are needed (for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly). Alternatively, with NaOH as absorbent, withdraw a sample of the sodium hydroxide solution from the absorber nearest to the test vessel using a syringe. The sample volume needed will depend on the carbon analyzer used, but sampling should not significantly change the absorbent volume over the test period. Inject the sample into the IC part of the carbon analyzer for analysis of evolved carbon dioxide directly. Analyze the contents of the second trap only at the end of the test in order to correct for any carry-over of carbon dioxide.
   c. On the 28th day withdraw samples, optionally, for DOC and/or specific chemical analysis. Add 1 mL of concentrated hydrochloric acid to each test vessel and aerate them overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.
OECD 301B Ready Biodegradability Assay

Data and Reporting

I. Treatment of Results
   a. Data from the test should be entered onto the attached data sheet.
   b. The amount of CO₂ produced is calculated from the amount of base remaining in the absorption bottle. When 0.0125M Ba(OH)₂ is used as the absorbent, the amount remaining is assessed by titrating with 0.05M HCl.
   c. Since 1 mmol of CO₂ is produced for every mol of Ba(OH)₂ reacted to BaCl₂ and 2 mmol of HCl are needed for the titration of the remaining Ba(OH)₂ and given that the molecular weight of CO₂ is 44 g, the weight of CO₂ produced (in mg) is calculated by:

   \[
   \frac{0.05 \times (50 - mL HCl Titrated) \times 44}{2} = 1.1 \times (50 - mL HCl Titrated)
   \]

   Therefore, the factor to convert volume of HCl titrated to mg CO₂ produced is 1.1 in this case. Calculate the weights of CO₂ produced from the inoculum alone and from the inoculum plus test substance using the respective titration values. The difference is the weight of CO₂ produced from the test substance alone.

   d. The percentage biodegradation is calculated from:

   \[
   \% \text{ Degradation} = \frac{mg \text{ CO}_2 \text{ Produced}}{ThCO_2 \times mg \text{ Test Substance Added}} \times 100
   \]

   Or

   \[
   \% \text{ Degradation} = \frac{mg \text{ CO}_2 \text{ Produced}}{mg \text{ TOC Added in Test} \times 3.67} \times 100
   \]

   Where \(3.67\) is the conversion factor \(\frac{44}{12}\) for carbon to carbon dioxide.

   e. When NaOH is used as the absorbent, calculate the amount of CO₂ produced after any time interval from the concentration of inorganic carbon and the volume of absorbent used. Calculate the percentage degradation from:

   \[
   \% \text{ ThCO}_2 = \frac{mg IC \text{ from Test Flask} - mg IC \text{ from Blank}}{mg \text{ TOC Added as Test Substances}} \times 100
   \]

   f. Display the course of degradation graphically and indicate the 10-day window. Calculate and report the percentage removal achieved at the plateau, at the end of the test, and/or at the end of the 10-day window, whichever is appropriate.

   g. When appropriate, calculate DOC removals using the equation given in 301 A paragraph 27.

   h. When an abiotic control is used, calculate the percentage abiotic degradation by:

   \[
   \% \text{ Abiotic Degradation} = \frac{CO_2 \text{ Produced by Sterile Flask After 28 Days (mg)}}{ThCO_2 (mg)} \times 100
   \]
Validity of Tests

I. The IC content of the test substance suspension in the mineral medium at the beginning of the test must be less than 5% of the TC, and the total CO₂ evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/L medium. If values greater than 70 mg CO₂/L are obtained, the data and experimental technique should be examined critically.

Data Sheet

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Active Concepts Tissue Culture Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Start Date</td>
<td>03/23/2016</td>
</tr>
<tr>
<td>Test Substance</td>
<td>ABS Pomegranate Sterols</td>
</tr>
<tr>
<td>Stock Solution Concentration</td>
<td>2 g/L</td>
</tr>
<tr>
<td>Initial Concentration in Medium</td>
<td>20 mg/L</td>
</tr>
<tr>
<td>Inoculum</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>Activated Sludge</td>
</tr>
<tr>
<td>Treatment Given</td>
<td>Centrifugation</td>
</tr>
<tr>
<td>Pre-conditioning</td>
<td>N/A</td>
</tr>
<tr>
<td>Suspended Solids Concentration</td>
<td>4 mg/L</td>
</tr>
<tr>
<td>Reaction Mixture</td>
<td></td>
</tr>
<tr>
<td>Reference Material</td>
<td>Sodium Benzoate</td>
</tr>
<tr>
<td>Concentration</td>
<td>20 mg/L</td>
</tr>
<tr>
<td>CO₂ Production and Degradability</td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td></td>
</tr>
<tr>
<td>Ba(OH)₂</td>
<td>0.0125M</td>
</tr>
<tr>
<td>NaOH</td>
<td>N/A</td>
</tr>
<tr>
<td>Other</td>
<td>N/A</td>
</tr>
<tr>
<td>Total Contact Time</td>
<td>28 Days</td>
</tr>
<tr>
<td>Total CO₂ Evolved Measurements</td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td>2, 4, 11, 17, 23, 28</td>
</tr>
<tr>
<td>Degradation Over Time</td>
<td>85.4% and 92.0% after 28 days</td>
</tr>
<tr>
<td>Remarks</td>
<td>Test material was readily biodegradable</td>
</tr>
<tr>
<td>Conclusion</td>
<td>This test met the criteria for a valid assay</td>
</tr>
</tbody>
</table>

Discussion

Based on the testing conducted in accordance with the specified test method, **ABS Pomegranate Sterols** achieved 88.7% biodegradation after 28 days of testing. The product met method requirements for the Readily Biodegradable classification.
OECD 202 Acute *Daphnia* Assay

**Tradename:** ABS Pomegranate Sterols

**Code:** 10247

**CAS #:** 949109-75-5

**Test Request Form #:** 2412

**Lot #:** 45923P

**Sponsor:** Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

**Study Director:** Maureen Danaher

**Principle Investigator:** Jennifer Goodman

**Test Performed:**
OECD 202  
*Daphnia* spp. Acute Immobilization Test

**Introduction**

The purpose of the present study is to determine the toxicity of *ABS Pomegranate Sterols* by exposing *Daphnia* spp. to the test substance for 48 hours and measuring the immobilization rate against the control. The present study defines an organism as being immobilized when it does not move for 15 seconds after the test vessel is gently shaken.

OECD Guideline 202 on “*Daphnia* spp., Acute Immobilization Test and Reproduction Test”, adopted in 1984, included two parts: Part I – the 24 hour EC$_{50}$ acute immobilization test and Part II – the reproduction test (at least 14 days). Revision of the reproduction test resulted in the adoption and publication of Test Guideline 211 on “*Daphnia magna* Reproduction Test” in September 1998. Consequently, the new version of Guideline 202 is restricted to the acute immobilization test.

**Assay Principle**

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilization is recorded at 24 hours and 48 hours and compared with control values. The results are analyzed in order to calculate the EC$_{50}$ at 48 hours. EC$_{50}$ is the concentration estimated to immobilize 50% of the daphnids within a stated exposure period. Immobilization refers to those animals that are not able to swim within 15 seconds after gentle agitation of the test vessel, even if they can still move their antennae.

The water solubility and vapor pressure of the test substance should be known. A reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should also be available.
A reference substance may be tested for EC\textsubscript{50} as a means of assuring that the test conditions are reliable.

For this assay to be valid, the following performance criteria apply:

- In the control, not more than 10% of the daphnids should have been immobilized.
- The dissolved oxygen concentration at the end of the test should be at least 3 mg/L in control and test vessels.

**Materials**

- Glass Test Tubes and/or Beakers
- Dissolved Oxygen Meter
- pH Meter
- Temperature Control Apparatus
- Total Organic Carbon (TOC) Analyzer
- Chemical Oxygen Demand (COD) Analyzer
- \textit{Daphnia magna} Straus
  - Use organisms less than 24 hours old. Do not use first offspring of parents.
- Water
  - Use water suitable for culturing and testing Daphnia spp. It can be natural water (surface water or groundwater), dechlorinated tap water, or artificially prepared water (Table 1), but must satisfy the conditions listed in Table 2. Do not use Elendt M4 or M7 media or water containing chelating agents for testing metal-containing substances.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate Matter</td>
<td>&lt;20 mg/L</td>
</tr>
<tr>
<td>Total Organic Carbon</td>
<td>&lt;2 mg/L</td>
</tr>
<tr>
<td>Unionized Ammonia</td>
<td>&lt;1 ug/L</td>
</tr>
<tr>
<td>Residual Chlorine</td>
<td>&lt;10 ug/L</td>
</tr>
<tr>
<td>Total Organophosphorus Pesticides</td>
<td>&lt;50 ng/L</td>
</tr>
<tr>
<td>Total Organochlorine Pesticides plus Polychlorinated Biphenyls</td>
<td>&lt;50 ng/L</td>
</tr>
<tr>
<td>Total Organic Chlorine</td>
<td>&lt;25 ng/L</td>
</tr>
</tbody>
</table>

**Table 1: Chemical Characteristics of Suitable Water**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount Added to 1 Liter Water</th>
<th>To prepare the reconstituted water, add the following volumes of stock solutions to 1 liter water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Chloride</td>
<td>11.76 grams</td>
<td>25 mL</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>4.93 grams</td>
<td>25 mL</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>2.59 grams</td>
<td>25 mL</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>0.23 grams</td>
<td>25 mL</td>
</tr>
</tbody>
</table>

**Table 2: Examples of Suitable Reconstituted Test Water**
Methods

Test Conditions

- **Test Method**
  - Test is performed under a static, semi-static, or flow-through condition. If test substance is unstable, a semi-static or flow-through test is recommended.

- **Exposure Period**
  - 48 hours

- **Test Volume**
  - At least 2 milliliters

- **Number of Test Organisms**
  - At least 20 organisms for each test concentration and the control.

- **Test Concentration**
  - Adopt a concentration range of at least 5 concentrations, with the highest concentration inducing 100% immobilization and no effect at the lowest concentration.

- **Culture Method**
  - **Illumination:** The photoperiod is set to 16 hours light and 8 hours dark
  - **Temperature:** The temperature is between 18°C to 22°C
  - **Dissolved Oxygen Concentration:** Must be kept at 3mg/L or higher
  - **Feeding:** Do not feed test organisms

Observation

- Observe mobility of the organisms at least twice (i.e., at 24 and 48 hours after exposure).
- The organisms are considered immobilized when they do not move for 15 seconds after test vessel is gently shaken.

Measurement of Test Substance Concentrations

- At the beginning and end of exposure, measure test substance concentrations at the lowest and highest test concentration groups.
  - For volatile or adsorptive substances, additional measurements are recommended at 24 hours intervals during exposure period.

Test Condition Measurements

- Measure dissolved oxygen in the control and at the highest test concentration at the beginning and end of the exposure period.
- Measure pH in the control and at the highest test concentration at the beginning and end of the exposure period.
- Water temperature should be measured at the beginning and end of the exposure period.
Data and Reporting

I. Data
   a. Data should be summarized in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilization at each observation. The percentages immobilized at 24 and 48 hours are plotted against test concentrations. Data are analyzed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC\textsubscript{50} with 95% confidence limits (p = 0.95).
   b. Where the standard methods of calculating the EC\textsubscript{50} are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100% immobility should be used as an approximation for the EC\textsubscript{50} (this being considered the geometric mean of these two concentrations).

II. Test Report
   a. The test report must include the following:
      i. Test substance:
         1. Physical nature and relevant physical-chemical properties
         2. Chemical identification data, including purity
      ii. Test species:
         1. Source and species of Daphnia, supplier of source (if known), and the culture conditions (including source, kind and amount of food, feeding frequency)
      iii. Test conditions:
         1. Description of test vessels: type and volume of vessels, volume of solution, number of daphnids per test vessel, number of test vessels (replicates) per concentration
         2. Methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used
         3. Details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc); composition of reconstituted water if used
         4. Incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.
      iv. Results:
         1. The nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported
         2. All physical-chemical measurements of temperature, pH and dissolved oxygen made during the test
         3. The EC\textsubscript{50} at 48 hours for immobilization with confidence intervals and graphs of the fitted model used for calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC\textsubscript{50}.  

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Results

General Information:

<table>
<thead>
<tr>
<th>Name of new chemical substance</th>
<th>ABS Pomegranate Sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INCI Nomenclature</strong></td>
<td>Punica granatum Sterols</td>
</tr>
<tr>
<td><strong>CAS number</strong></td>
<td>949109-75-5</td>
</tr>
<tr>
<td><strong>Formulation Method</strong></td>
<td>Fractionalization</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>414.72 Da</td>
</tr>
<tr>
<td><strong>Purity of the new chemical substance used for the test (%)</strong></td>
<td>100%</td>
</tr>
<tr>
<td><strong>Lot number of the new chemical substance used for the test</strong></td>
<td>45923P</td>
</tr>
<tr>
<td><strong>Names and contents of impurities</strong></td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Solubility in water</strong></td>
<td>Insoluble</td>
</tr>
<tr>
<td><strong>Properties at room temperature</strong></td>
<td>White to Very Light Yellow Waxy Paste</td>
</tr>
<tr>
<td><strong>Stability</strong></td>
<td>Stable Under Normal Conditions</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solubility in solvents, etc.</th>
<th>Solvent</th>
<th>Solubility</th>
<th>Stability in solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
### Test Materials and Methods:

<table>
<thead>
<tr>
<th>Items</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test Organisms</strong></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td><em>Daphnia magna</em></td>
</tr>
<tr>
<td>Source</td>
<td>Carolina Biological Supply Company</td>
</tr>
<tr>
<td>Reference substance (EC₅₀)</td>
<td>Potassium dichromate (0.94 mg/L)</td>
</tr>
<tr>
<td><strong>Culture</strong></td>
<td></td>
</tr>
<tr>
<td>Kind of Medium</td>
<td>Elendt Medium M4</td>
</tr>
<tr>
<td>Conditions (Temperature/Photoperiod)</td>
<td>20°C/16 Hour Light-8 Hour Dark</td>
</tr>
<tr>
<td><strong>Test Conditions</strong></td>
<td></td>
</tr>
<tr>
<td>Test Vessel</td>
<td>Glass</td>
</tr>
<tr>
<td>Material Water</td>
<td></td>
</tr>
<tr>
<td>Kind</td>
<td>Elendt Medium M4</td>
</tr>
<tr>
<td>Hardness</td>
<td>250 mg/L</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
<tr>
<td>Date of Exposure</td>
<td>03/07/2016</td>
</tr>
<tr>
<td>Test Concentrations</td>
<td>200, 89.4, 42.3, 19.2, 7.8 mg/L</td>
</tr>
<tr>
<td>Number of organisms</td>
<td>120</td>
</tr>
<tr>
<td>Number of Replicates</td>
<td></td>
</tr>
<tr>
<td>Exposure Group</td>
<td>4</td>
</tr>
<tr>
<td>Control Group</td>
<td>4</td>
</tr>
<tr>
<td>Test Solution Volume</td>
<td>2 mL</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>Use or Not</td>
<td>N/A</td>
</tr>
<tr>
<td>Kind</td>
<td>N/A</td>
</tr>
<tr>
<td>Concentration</td>
<td>N/A</td>
</tr>
<tr>
<td>Number of Replicates</td>
<td>N/A</td>
</tr>
<tr>
<td>Culture Method (Static, Semi-Static, Flow-Through)</td>
<td>Static</td>
</tr>
<tr>
<td>Water Temperature</td>
<td>20°C ± 2°C</td>
</tr>
<tr>
<td>Dissolved Oxygen Concentration (DO)</td>
<td>3 mg/L</td>
</tr>
<tr>
<td>Photoperiod</td>
<td>16 Hour Light-8 Hour Dark</td>
</tr>
<tr>
<td>Statistical Method</td>
<td>Probit Analysis</td>
</tr>
</tbody>
</table>
OECD 202 Acute *Daphnia* Assay

Test Results:

<table>
<thead>
<tr>
<th>Items</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity Value 48hr EC50</td>
<td>771.40 mg/L</td>
</tr>
<tr>
<td>Exposure Concentrations Used for Calculation Nominal Values</td>
<td>200, 89.4, 42.3, 19.2, 7.8 mg/L</td>
</tr>
<tr>
<td>Remarks</td>
<td>Not harmful to aquatic organisms</td>
</tr>
</tbody>
</table>

Discussion

After 48 hours, the EC50 value for **ABS Pomegranate Sterols** was determined to be 771.40 mg/L. The conditions of OECD guideline 202 for the validity of the test were adhered to: The immobility of controls in purified drinking water (dilution water) did not exceed 10%. According to the EU Directive 93/67/EEC, this product is not classified and therefore not harmful to aquatic organisms.
Phototoxicity Assay Analysis

**Tradename:** ABS Pomegranate Sterols

**Code:** 10247

**CAS #:** 949109-75-5

**Test Request Form #:** 2366

**Lot #:** 48147P

**Sponsor:** Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

**Study Director:** Maureen Danaher

**Principle Investigator:** Jennifer Goodman

**Test Performed:**
In Vitro EpiDerm™ Model (EPI-200-SIT) Phototoxicity

**SUMMARY**

In vitro phototoxicity irritation studies were conducted to evaluate whether ABS Pomegranate Sterols would induce phototoxic irritation in the EpiDerm™ model assay.

The product was tested according to the manufacturer's protocol. The test article solution was found to be a non-photoinirritant at concentrations of 0.4%, 1.2%, and 3.5%. Reconstructed human epidermis was incubated in growth media for one hour to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substance was applied to the tissue inserts in four varying concentrations and incubated overnight at 37°C, 5% CO₂, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm² (=6 J/cm²). After substance incubation, irradiation, and washing was completed, the cell viability test was conducted. Cell viability was measured by dehydrogenase conversion of MTT [(3-4,5-dimethyl thiazole 2-yl)], present in the cell mitochondria, into blue formazan salt that was measured after extraction from the tissue. The photoirritation potential of the test chemical was dictated by the reduction in tissue viability of UVA exposed tissues compared to non-UVA exposed tissues.

Under the conditions of this assay, the test article was considered to be non-phototoxic at concentrations of 0.4%, 1.2%, and 3.5%. The negative and positive controls performed as anticipated.
I. Introduction

A. Purpose
In vitro dermal phototoxicity study was conducted to evaluate whether a test article would induce photoirritation in the EpiDerm™ model assay. MatTek Corporation’s reconstructed human epidermal model is becoming a standard in determining the phototoxicity potential of a test substance. This assay is able to discriminate between photoirritants and non-photoirritants at varying concentrations.

II. Materials

A. Incubation Conditions: 37°C at 5% CO₂ and 95% relative humidity
B. Equipment:
   - Forma humidified incubator, ESCO biosafety laminar flow hood,
   - Synergy HT Microplate reader; UVA-vis Irradiation Equipment; UVA meter; Pipettes
C. Media/Buffers:
   - Dulbecco’s Modified Eagle Medium (DMEM) based medium;
   - Dulbecco’s Phosphate-Buffered Saline (DPBS); sterile deionized H₂O
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, and 6-well tissue culture plates
F. Reagents:
   - MTT (3-(4,5-dimethyl thiazole 2-yl) (1.0mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1%)
G. Other:
   - Wash bottle; sterile disposable pipette tips; Parafilm; forceps

III. Test Assay

A. Test System
The reconstructed human epidermal model, EpiDerm™ consists of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis. This model consists of organized basal, spinous, and granular layers, and contains a multilayer stratum corneum containing intercellular lamellar lipid layers. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control
Sterile deionized water is used as the negative controls for the EpiDerm™ Phototoxicity assay.

C. Positive Control
Concentrations of chlorpromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.

D. Data Interpretation Procedure
A photoirritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance and 60 minutes of 6 J/cm² is reduced by 20% compared to the non-irradiated control tissues.
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% CO₂ and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and tissue insert dosing begins.

B. Test Substance Exposure
50µL of the diluted test substance in their respective concentrations are applied to 2 tissue inserts and allowed to incubate for overnight in a humidified incubator (37°C, 5% CO₂, 95% RH).

C. Tissue Irradiation
Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm² at room temperature. The non-irradiated tissue inserts are incubated at room temperature in the dark.

D. Tissue Washing and Post Incubation
After UVA-irradiation and dark incubation is complete the tissue inserts are washed using sterile DPBS and transferred to fresh 6-well plates and media for overnight incubation at 37°C, 5% CO₂, 95% RH.

E. MTT Assay
Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO₂, 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 (OD₅₇₀) is ≥ 0.8.

B. Positive Control
The assay meets the acceptance criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.

C. Standard Deviation
Since the phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

VI. Results

A. Tissue Characteristics
The tissue inserts included in the MatTek EpiDerm™ assay kit were in good condition, intact, and viable.
B. Tissue Viability Assay
The results are summarized in Figure 1. Cell viability is calculated for each tissue as a percentage of the corresponding vehicle control either irradiated or non-irradiated. Tissue viability was not reduced by 20% in the presence of the test substance and UVA-irradiation at concentrations of 0.4%, 1.2%, and 3.5%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

C. Test Validity
The data obtained from this study met criteria for a valid assay. The negative and positive controls performed as anticipated.

VII. Conclusion
Phototoxicity (photoirritation) is defined as an acute toxic response that is elicited after exposure of the skin to certain chemicals and subsequent exposure to light. Under the conditions of this assay, the test article substance was considered to be non-phototoxic at concentrations of 0.4%, 1.2%, and 3.5%. The negative and positive controls performed as anticipated.

There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that ABS Pomegranate Sterols is not a photoirritant when used at the suggested use levels of 0.5% -5.0%.