

Phytofuse Rejuvenate® Toxicology Data

Code: 16882
INCI Name: Salvia Hispanica Seed Extract
CAS #: 93384-40-8
EINECS #: 297-250-8

Name of Study	Type of Study	Results
Dermal & Ocular Irritation Tests	<i>In-vitro</i>	Both the dermal and ocular assays reveal that Phytofuse Rejuvenate® is non-irritating and should not cause any of the aforementioned conditions.
AMES Test	<i>In-vitro</i>	The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, that Phytofuse Rejuvenate® was considered to be Non-Mutagenicto Salmonella typhimurium testerstrains TA98, TA100, TA1537, TA1535 and Escherichia coli WP2uvrA.
OECD TG 442D In-Vitro Skin Sensitization	<i>In-vitro</i>	The results using the ARE-Nrf2 Luciferase Test Method in accordance with UN GHS indicate that Phytofuse Rejuvenate® was not predicated to be a skin sensitizer.
OECD TG 442C Direct Peptide Reactivity	<i>In-chemico</i>	Based on HPLC-UV analysis Phytofuse Rejuvenate® was determined as a non-sensitizer and will not cause allergic contact dermatitis.
OECD 301B Ready Biodegradability	<i>In-chemico</i>	The results of the Modified Sturm Test ensure Phytofuse Rejuvenate® met method requirements for the Readily Biodegradable classification.

Name of Study	Type of Study	Results
OECD 202 Acute Daphnia	<i>In-vivo</i>	According to the EU Directive 93/67/EEC, Phytofuse Rejuvenate® is not classified as harmful to aquatic organisms.
Phototoxicity Assay Analysis	<i>In-vitro</i>	



Dermal and Ocular Irritation Tests

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Sample: Phytofuse Rejuvenate®

Code: 16882

CAS #: 93384-40-8

Test Request Form/Submission #: 1570

Lot #: NC150408-A

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 **Phytofuse Rejuvenate®** 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.

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Dermal and Ocular Irritation Tests

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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.



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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% 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™).

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 $\leq 20\%$.

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 Figure 1. In no case was the tissue viability $\leq 50\%$ for EpiDerm™ or $\leq 60\%$ 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.

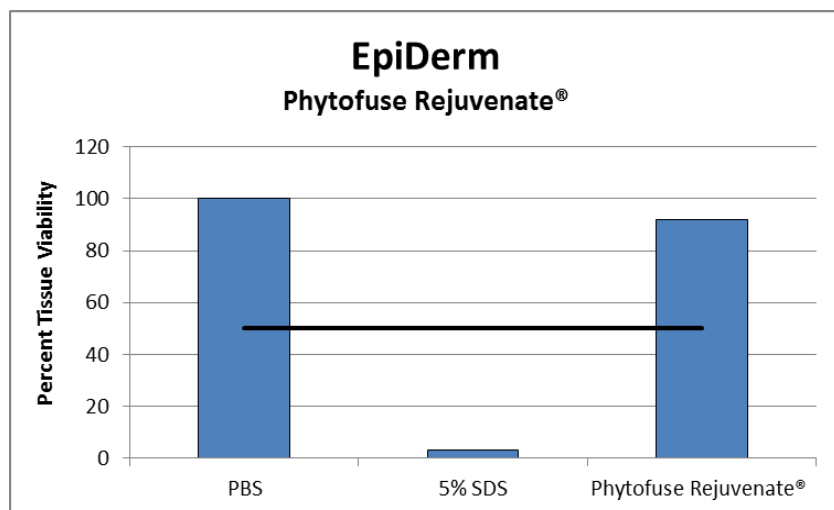


Figure 1: EpiDerm tissue viability

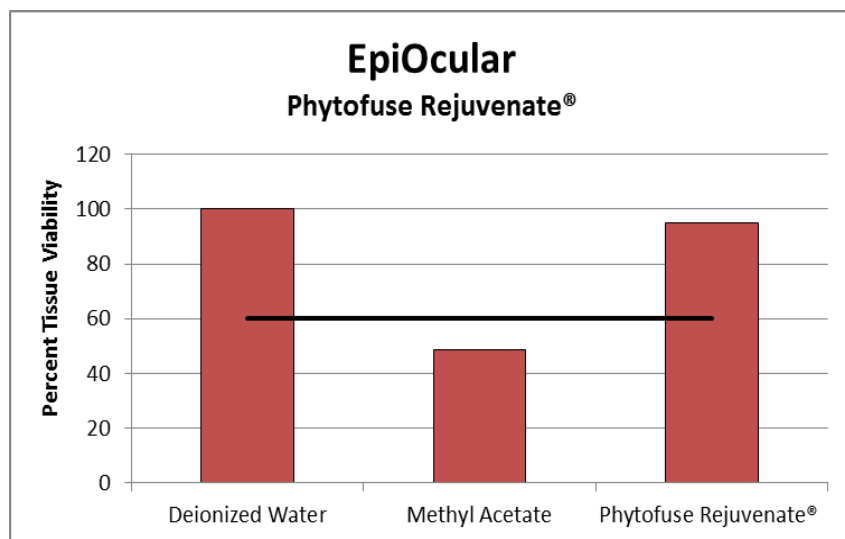


Figure 2: EpiOcular tissue viability

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Bacterial Reverse Mutation Test

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Test Article: Phytofuse Rejuvenate®

Code Number: 16882

CAS #: 93384-40-8

Sponsor:

Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Monica Beltran

Test Performed:

Genotoxicity: Bacterial Reverse Mutation Test

Reference:

OECD471/ISO10993.Part 3

Test Request Number: 1705

SUMMARY

A *Salmonella typhimurium*/*Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article solution **Phytofuse Rejuvenate®** would cause mutagenic changes in the average number of revertants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2uvrA in the presence and absence of Aroclor-induced rat liver S9. 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 tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2uvrA after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45°C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37°C. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control plates for each of the strains tested. 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 Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester 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 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.



Bacterial Reverse Mutation Test

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I. Introduction

A. Purpose

A *Salmonella typhimurium*/*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, TA1537, TA1535 and *Escherichia coli* WP2uvrA in the presence and absences of the S9 metabolic activation. 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 (23-25C).
- B. **Vehicle:** Sterile DI Water.
- C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
- D. **Solubility/Stability:** 100% Soluble and Stable.
- E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System

Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa*), the deletion of *uvrB* gene and the deletion in the *uvrA* gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2uvrA) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states 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.

<u>Tester strain</u>	<u>Mutations/Genotypic Relevance</u>
TA98	hisD3052, Dgal chlD bio <i>uvrB rfa</i> pKM101
TA100	hisG46, Dgal chlD BIO <i>uvrB rfa</i> pKM101
TA1537	hisC3076, <i>rfa</i> , Dgal chlD bio <i>uvrB</i>
TA 1535	hisG46, Dgal chlD bio <i>uvrB rfa</i>
WP2uvrA	trpE, <i>uvrA</i>

<i>rfa</i>	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
<i>uvrB</i>	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
<i>uvrA</i>	=	All possible transitions and transversions, small deletions.

B. Metabolic Activation

Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male Sprague Dawley rats. Material is supplied by MOLTOX, Molecular Toxicology, Inc.

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C. Preparation of Tester strains

Cultures of *Salmonella typhimurium* TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA were inoculated to individual flasks containing Oxoid broth No.2. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.

D. Negative Control

Sterile DI water (vehicle without test material) 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 material had significant mutagenic properties.

E. Positive Control

A known mutagen for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

F. Titer of the Strain Cultures:

Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to 2×10^9 /ml.

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* and tryptophan for *Escherichia coli* were inoculated with 100 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-). When plating the positive controls, the test article aliquot was replaced by 50 µl aliquot of appropriate positive control. The test was conducted per duplicate. The plates were incubated for 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 numbers of revertants of the test plates were compared to the mean number of revertants of the negative control of each strain used.

V. Evaluation

For the test 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 strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* and *Escherichia coli* tester strain used.

VI. Results and Discussion

A. Solubility:

Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

B. Dose levels tested:

The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):

5×10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2×10^9 UFC/ml.

C. Standard Plate Incorporation Assay

In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2uvrA in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

VII. Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* 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 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

Appendix 2:

Bacterial Mutation Assay Plate Incorporation Assay Results

	Concentration µg per Plate	TA98		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	72	69	71
	1500	65	86	76
	500	104	68	86
	150	45	83	64
	50	54	76	65
	15	64	94	79
	5.0	98	64	81
	1.5	60	87	74
Test Solution w/o S9	5000	98	80	89
	1500	42	74	58
	500	76	81	79
	150	86	62	74
	50	80	48	64
	15	73	60	67
	5.0	66	71	69
	1.5	67	73	70
DI Water w/S9		60	67	64
DI Water w/o S9		81	86	84
2-aminoanthracen w/ S9		396	346	371
2-nitrofluorene w/o S9		156	171	164
Historical Count Positive w/S9		43-1893		
Historical Count Positive w/o S9		39-1871		
Historical Count Negative w/S9		4-69		
Historical Count Negative w/o S9		3-59		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates



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	Concentration µg per Plate	TA100		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	135	231	183
	1500	146	166	156
	500	108	152	130
	150	85	161	123
	50	118	118	118
	15	176	188	182
	5.0	148	140	144
	1.5	100	180	140
Test Solution w/o S9	5000	233	249	241
	1500	396	356	376
	500	212	208	210
	150	128	124	126
	50	162	206	184
	15	156	160	158
	5.0	160	144	152
	1.5	98	106	102
DI Water w/S9		140	148	144
DI Water w/o S9		104	106	105
2-aminoanthracen w/ S9		814	816	815
Sodium azide w/o S9		710	639	674
Historical Count Positive w/S9		224-3206		
Historical Count Positive w/o S9		226-1837		
Historical Count Negative w/S9		55-268		
Historical Count Negative w/o S9		47-250		

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	Concentration µg per Plate	TA1537		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	13	13	13
	1500	15	8	12
	500	12	10	11
	150	10	14	12
	50	8	4	6
	15	10	13	12
	5.0	8	3	6
	1.5	10	8	9
Test Solution w/o S9	5000	30	20	26
	1500	12	10	11
	500	8	15	12
	150	13	6	10
	50	9	10	10
	15	14	9	11
	5.0	15	8	12
	1.5	8	13	11
DI Water w/S9		198	190	194
DI Water w/o S9		130	154	142
2-aminoanthracen w/ S9		55	65	65
2-aminoacridine w/o S9		520	792	656
Historical Count Positive w/S9		13-1934		
Historical Count Positive w/o S9		17-4814		
Historical Count Negative w/S9		0-41		
Historical Count Negative w/o S9		0-29		

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	Concentration µg per Plate	TA1535		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	12	20	16
	1500	20	19	20
	500	13	21	16
	150	15	12	14
	50	20	18	19
	15	7	9	8
	5.0	16	13	15
	1.5	7	20	14
Test Solution w/o S9	5000	11	10	11
	1500	17	12	15
	500	12	8	10
	150	12	10	1
	50	7	12	10
	15	11	16	14
	5.0	12	13	13
	1.5	12	9	11
DI Water w/S9		20	25	23
DI Water w/o S9		16	22	19
2-aminoanthracen w/ S9		176	266	226
Sodium azide w/o S9		550	486	518
Historical Count Positive w/S9		22-1216		
Historical Count Positive w/o S9		47-1409		
Historical Count Negative w/S9		1-50		
Historical Count Negative w/o S9		1-45		

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	Concentration µg per Plate	WP2uvrA		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	46	62	54
	1500	63	71	67
	500	69	70	70
	150	41	50	46
	50	57	39	48
	15	56	75	66
	5.0	76	75	76
	1.5	53	70	52
Test Solution w/o S9	5000	35	60	48
	1500	65	66	66
	500	47	47	47
	150	64	48	56
	50	72	52	62
	15	75	71	73
	5.0	75	58	67
	1.5	67	77	72
DI Water w/S9		37	70	54
DI Water w/o S9		71	74	73
2-aminoanthracen w/ S9		596	686	646
Methylmethanesulfonate w/o S9		256	217	237
Historical Count Positive w/S9		44-1118		
Historical Count Positive w/o S9		42-1796		
Historical Count Negative w/S9		8-80		
Historical Count Negative w/o S9		8-84		

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OECD TG 442D: *In Vitro* Skin Sensitization

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Tradename: Phytofuse Rejuvenate®

Code: 16882

CAS #: 93384-40-8

Test Request Form #: 1737

Lot #: NC140813-H

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

Study Director: Erica Segura

Principle Investigator: Maureen Danaher

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 Chemicals¹. 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 **Phytofuse Rejuvenate®** 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.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013

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Materials

- | | |
|----------------------------------|--|
| A. Incubation Conditions: | 37°C at 5% CO ₂ 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); Geneticin |
| 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% confluency in DMEM containing 10% FBS and 500µg/mL G418 geneticin. Twelve test concentrations of **Phytofuse Rejuvenate®** 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 EC_{1.5} and maximum response (I_{max}) 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 EC_{1.5} 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 I_{max} 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 EC_{1.5} determining concentration)
3. The EC_{1.5} value is less than 1000 μM (or < 200 $\mu\text{g}/\text{mL}$ for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

Compound	Classification	EC _{1.5} (μM)	IC ₅₀ (μM)	I _{max}
Cinnamic aldehyde	Sensitizer	19	289.19	31.9
DMSO	Non-Sensitizer	No Induction	243.24	1.3
Phytofuse Rejuvenate®	Non-Sensitizer	No Induction	> 1000	0.5

Table 1: Overview of KeratinoSens™ Assay Results

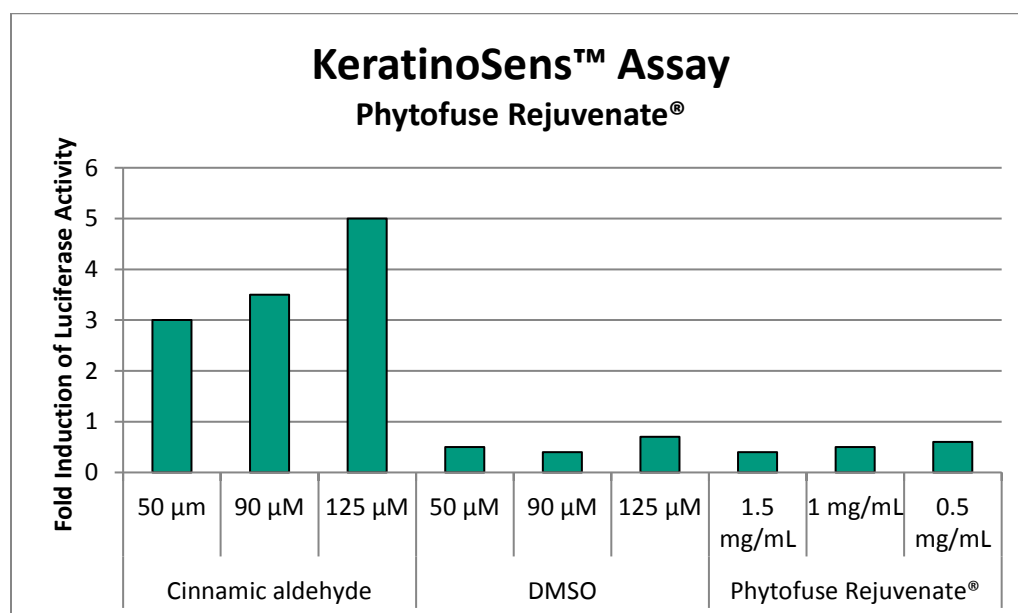


Figure 1: Fold Induction of Luciferase

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Discussion

As shown in the results, **Phytofuse Rejuvenate® (code 16882)** 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 **Phytofuse Rejuvenate®** can be safely used in cosmetics and personal care products at typical use levels.



Tradename: Phytofuse Rejuvenate®

Code: 16882

CAS #: 93384-40-8

Test Request Form #: 1731

Lot #: NC140813-H

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

Study Director: Erica Segura

Principle Investigator: Maureen Danaher

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 contact¹. 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 dermatitis². 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%)³.

This assay was conducted to determine skin sensitization hazard of **Phytofuse Rejuvenate®** 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.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

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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 **Phytofuse Rejuvenate®** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **AC Dermal Respiratory Factor Advanced PF**, 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.

1:10 Ratio, Cysteine Peptide 0.5mM Peptide, 5mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5mM Peptide, 25mM Test Chemical
<ul style="list-style-type: none"> • 750µL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls) • 200µL Acetonitrile • 50µL Test Chemical Solution (or Acetonitrile for Reference Controls) 	<ul style="list-style-type: none"> • 750µL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls) • 250µL Test Chemical Solution (or Acetonitrile for Reference Controls)

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

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

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:

Time	Flow	%A	%B
0 minutes	0.35 mL/min	90	10
10 minutes	0.35 mL/min	75	25
11 minutes	0.35 mL/min	10	90
13 minutes	0.35 mL/min	10	90
13.5 minutes	0.35 mL/min	90	10
20 minutes	End Run		

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 $r^2 > 0.99$.
 - 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 reference controls A should be 0.50 ± 0.05 mM 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 ± 0.05 mM.

Prediction Model:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	Reactivity Class	Prediction
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

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

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	Reactivity Class	Prediction
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

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:

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

Based on HPLC-UV analysis of **Phytofuse Rejuvenate® (code 16882)** we can determine that this product is not a sensitizer and will not cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 1.89% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.



OECD 301B Ready Biodegradability Assay

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Tradename: Phytofuse Rejuvenate®

Code: 16882

CAS #: 93384-40-8

Test Request Form #: 1725

Lot #: NC140813-H

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

Study Director: Erica Segura

Principle Investigator: Maureen Danaher

Test Performed:

OECD 301 B

Ready Biodegradability: CO₂ Evolution (Modified Sturm Test)

Introduction

A study was conducted to assess the readily biodegradability of **Phytofuse Rejuvenate®** 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.



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The pass levels for ready biodegradability are 70% removal of DOC and 60% of ThOD (Theoretical Oxygen Demand) or ThCO₂ (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 CO₂ 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 ThCO₂ 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, KH₂PO₄.....8.5g
 - Dipotassium hydrogen orthophosphate, K₂HPO₄.....21.8g
 - Disodium hydrogen orthophosphate dehydrate, Na₂HPO₄·2H₂O.....33.4g
 - Ammonium chloride, NH₄Cl.....0.5g
 - Solution B (Dissolve in water and make up to 1 liter)
 - Calcium chloride, anhydrous, CaCl₂.....27.50g
 - Or
 - Calcium chloride dehydrate, CaCl₂·2H₂O.....36.40g
 - Solution C (Dissolve in water and make up to 1 liter)
 - Magnesium sulphate heptahydrate, MgSO₄·7H₂O..... 22.50g
 - Solution D (Dissolve in water and make up to 1 liter.)
 - Iron (III) chloride hexahydrate, FeCl₃·6H₂O.....0.25g



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- Flasks, 2-5 liters each, fitted with aeration tubes reaching nearly to the bottoms of the vessels and an outlet
- Magnetic stirrers
- Gas absorption bottles
- Device for controlling and measuring air flow
- Apparatus for carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO₂-free oxygen and CO₂-free nitrogen from gas cylinders in the correct proportions (20% O₂ : 80% N₂)
- Device for determination of carbon dioxide, either titrimetrically or by some form of inorganic carbon analyzer
- Stock solutions of test substances
 - When solubility of the substance exceeds 1 g/L, dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 liter. Otherwise, prepare stock solutions in mineral medium or add the chemical directly to the mineral medium, making sure it
- Inoculum
 - The inoculum may be derived from the following sources
 - Activated sludge
 - Sewage effluents
 - Surface waters
 - Soils
 - Or from a mixture of these.
 - Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning consists of aerating activated sludge in mineral medium or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test method by reducing blank values.

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.
 - 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.



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- 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.

Data and Reporting

I. Treatment of Results

- a. Data from the test should be entered onto the data sheet below.
- 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:

$$\% \text{ Degradation} = \frac{mg\ CO_2\ Produced}{ThCO_2 \times mg\ Test\ Substance\ Added} \times 100$$

Or

$$\% \text{ Degradation} = \frac{mg\ CO_2\ Produced}{mg\ TOC\ Added\ in\ Test \times 3.67} \times 100$$

Where 3.67 is the conversion factor $\left(\frac{44}{12}\right)$ 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:

$$\% ThCO_2 = \frac{mg\ IC\ from\ Test\ Flask - mg\ IC\ from\ Blank}{mg\ TOC\ Added\ as\ Test\ Substances} \times 100$$

- 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:

$$\% \text{ Abiotic Degradation} = \frac{CO_2\ Produced\ by\ Sterile\ Flask\ After\ 28\ Days\ (mg)}{ThCO_2\ (mg)} \times 100$$



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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

Laboratory	Active Concepts Tissue Culture Laboratory		
Test Start Date	12/10/2015		
Test Substance	Name	Phytofuse Rejuvenate®	
	Stock Solution Concentration	2 g/L	
	Initial Concentration in Medium	20 mg/L	
Inoculum	Source	Activated Sludge	
	Treatment Given	Centrifugation	
	Pre-conditioning	N/A	
	Suspended Solids Concentration in Reaction Mixture	4 mg/L	
Reference Material	Sodium Benzoate	Concentration	20 mg/L
CO ₂ Production and Degradability	Method	Ba(OH) ₂	0.0125M
		NaOH	N/A
		Other	N/A
Total Contact Time	28 Days		
Total CO ₂ Evolved Measurements	Days	2, 4, 11, 17, 23, 28	
Degradation Over Time	87.8% and 93.9% after 28 days		
Remarks	Test material was readily biodegradable		
Conclusion	This test met the criteria for a valid assay		

Discussion

Based on the testing conducted in accordance with the specified test method, **Phytofuse Rejuvenate®** achieved 90.9% biodegradation after 28 days of testing. The product met method requirements for the Readily Biodegradable classification.



OECD 202 Acute *Daphnia* Assay

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Tradename: Phytofuse Rejuvenate®

Code: 16882

CAS #: 93384-40-8

Test Request Form #: 1719

Lot #: NC140813-H

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

Study Director: Erica Segura

Principle Investigator: Maureen Danaher

Test Performed:

OECD 202

Daphnia spp. Acute Immobilization Test

Introduction

The purpose of the present study is to determine the toxicity of **Phytofuse Rejuvenate®** 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 EC₅₀ 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 EC₅₀ at 48 hours. EC₅₀ 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.

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A reference substance may be tested for EC₅₀ 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
- *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.

Substance	Concentration
Particulate Matter	<20 mg/L
Total Organic Carbon	<2 mg/L
Unionized Ammonia	<1 ug/L
Residual Chlorine	<10 ug/L
Total Organophosphorus Pesticides	<50 ng/L
Total Organochlorine Pesticides plus Polychlorinated Biphenyls	<50 ng/L
Total Organic Chlorine	<25 ng/L

Table 1: Chemical Characteristics of Suitable Water

Substance	Amount Added to 1 Liter Water	To prepare the reconstituted water, add the following volumes of stock solutions to 1 liter water
Calcium Chloride	11.76 grams	25 mL
Magnesium Sulfate	4.93 grams	25 mL
Sodium Bicarbonate	2.59 grams	25 mL
Potassium Chloride	0.23 grams	25 mL

Table 2: Examples of Suitable Reconstituted Test Water

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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 EC₅₀ with 95% confidence limits ($p = 0.95$).
- b. Where the standard methods of calculating the EC₅₀ 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 EC₅₀ (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 EC₅₀ 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 EC₅₀.

Results

General Information:

Name of new chemical substance	Phytofuse Rejuvenate®		
INCI Nomenclature	<i>Salvia Hispanica Seed Extract</i>		
CAS number	93384-40-8		
Formulation Method	Botanical: <i>Salvia Hispanica Seed</i>		
Molecular weight	1583.9 Daltons		
Purity of the new chemical substance used for the test (%)	100%		
Lot number of the new chemical substance used for the test	NC140813-H		
Names and contents of impurities	n/a		
Solubility in water	100%		
Properties at room temperature	Light Yellow to Amber, Hazy Viscous Gel		
Stability	Stable Under Normal Conditions		
Solubility in solvents, etc.	Solvent	Solubility	Stability in solvent
	n/a	n/a	n/a

Test Materials and Methods:

Items			Contents
Test Organisms	Species		<i>Daphnia magna</i>
	Source		Carolina Biological Supply Company
	Reference substance (EC ₅₀)		Potassium dichromate (0.94 mg/L)
Culture	Kind of Medium		Elendt Medium M4
	Conditions (Temperature/Photoperiod)		20°C/16 Hour Light-8 Hour Dark
Test Conditions	Test Vessel		Glass
	Material Water	Kind	Elendt Medium M4
		Hardness	250 mg/L
		pH	7.4
	Date of Exposure		11/16/2015
	Test Concentrations		200, 89.4, 42.3, 19.2, 7.8 mg/L
	Number of organisms		120
	Number of Replicates	Exposure Group	4
		Control Group	4
	Test Solution Volume		2 mL
	Vehicle	Use or Not	N/A
		Kind	N/A
		Concentration	N/A
		Number of Replicates	N/A
	Culture Method (Static, Semi-Static, Flow-Through)		Static
	Water Temperature		20°C ± 2°C
	Dissolved Oxygen Concentration (DO)		3 mg/L
	Photoperiod		16 Hour Light-8 Hour Dark
	Statistical Method		Probit Analysis



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Test Results:

Items		Contents
Toxicity Value	48hr EC50	133.7 mg/L
Exposure Concentrations Used for Calculation	Nominal Values	200, 89.4, 42.3, 19.2, 7.8 mg/L
Remarks		Not harmful to aquatic organisms

Discussion

After 48 hours, the EC50 value for **Phytofuse Rejuvenate®** was determined to be 133.7 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.