Final Report

Study Title	*Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay with Mesozeaxanthin
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Covance Study No.	7609-100
Genetic Toxicology Assay No.	26471-0-409OECD
Report Issued	03 April 2006
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QUALITY ASSURANCE STATEMENT

Salmonella-Escherichia coli/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay with Mesozeaxanthin

This report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc. and accurately reflects the raw data. The following inspections were conducted and findings reported to the study director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a process inspection program. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study may be included below.

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
29 Sep 2004	29 Sep 2004	Protocol Review	26 Oct 2004
29 Sep 2004	29 Sep 2004	Test Article Administration	07 Dec 2004
21 Dec 2004	21 Dec 2004	Draft Report and Data Review	21 Dec 2004
21 Dec 2004	21 Dec 2004	Protocol Amendment Review	21 Dec 2004
03 Apr 2006	03 Apr 2006	Draft to Final Report Review	03 Apr 2006

Representative
Quality Assurance Unit

0963/2006
Date

STUDY COMPLIANCE AND CERTIFICATION

Except as noted below, the study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice, ENV/MC/CHEM (98) 17, with any applicable amendments. There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria. All test and control results in this report are supported by an experimental data record and the Study Director has reviewed this record.

Exceptions: 1) dosing preparations were not analyzed for stability, homogeneity, or concentration.

Study Director:

Michael S. Mecchi

Michael S. Mecchi, MS
Genetic and Molecular Toxicology
Covance-Vienna

03 APR 2006
Study Completion Date

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ABSTRACT

The objective of this study was to evaluate the test article, Mesozeaxanthin, for its ability to induce reverse mutations either in the presence or absence of mammalian microsomal enzymes at 1) the histidine locus in the genome of several strains of *Salmonella typhimurium* and at 2) the tryptophan locus of *Escherichia coli* tester strain WP2*uvr*A.

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strains TA100 and WP2*uvr*A and ten doses of test article ranging from 6.67 to 5000 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* tester strain WP2*uvr*A. The assay was conducted in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested in the mutagenicity assay with all tester strains in both the presence and absence of S9 mix were 10.0, 33.3, 100, 333, 1000, and 5000 µg per plate. The results of the initial mutagenicity assay were confirmed in an independent experiment.

The results of the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, the test article, Mesozeaxanthin, did not cause a positive increase in the mean number of revertants per plate with any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

STUDY INFORMATION

Sponsor
Howard Foundation (Holdings) Ltd.

Test Article
Sponsor's Identification: Meso-Zeaxanthin
Aztec Marigold Carotenoid Concentrate
Date Received: 24 August 2004
Physical Description: Orange Powder
Storage Conditions: Room Temperature

Assay Information
Type of Assay: *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay
Protocol Number: 409OECD, Edition 2
Covance Study No: 7609-100
Genetic Toxicology Assay No: 26471-0-409OECD

Study Dates
Initiation Date: 25 August 2004
Experimental Start Date: 16 September 2004
Study Start Date: 17 September 2004
Experimental Termination Date: 25 October 2004
Study End Date: 03 April 2006

Supervisory Personnel
Study Director: Michael S. Mecchi, MS
Laboratory Supervisor: Gerasimo Borneo, MS

OBJECTIVE

The objective of this study was to evaluate the test article, Mesozeaxanthin, and/or its metabolites for the ability to induce reverse mutations either in the presence or absence of mammalian microsomal enzymes at 1) the histidine locus in the genome of several strains of *Salmonella typhimurium* and at 2) the tryptophan locus of *Escherichia coli* tester strain WP2*uvr*A. The assay design was based on OECD Guideline 471, updated and adopted 21 July 1997.

TEST SYSTEM RATIONALE

The bacterial reverse mutation assay detects point mutations, both frameshifts and/or base pair substitutions. The strains of *Salmonella typhimurium* and *Escherichia coli* used in this assay are histidine and tryptophan auxotrophs, respectively, by virtue of conditionally lethal mutations in the appropriate operons. When these histidine (*his*–) or tryptophan (*trp*–) dependent cells are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine or tryptophan), only those cells which revert to histidine (*his*+) or tryptophan (*trp*+) independence are able to form colonies. The trace

amount of histidine or tryptophan in the media allows all the plated bacteria to undergo a few cell divisions, which is essential for mutagenesis to be fully expressed. The *his* or *trp* revertants are readily discernable as colonies against the limited background growth of the *his*– or *trp*– cells. By utilizing several different tester strains, base pair substitution mutations and frameshift mutations can be detected. The bacterial reverse mutation assay has been shown to be a sensitive, rapid, and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes.

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames *et al.* (1975) and Green and Muriel (1976). The assay design was based on OECD Guideline 471, updated and adopted 21 July 1997.

Test System

Tester Strains. The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 (Ames *et al.*, 1975) and the *Escherichia coli* tryptophan auxotroph WP2*uvr*A (Green and Muriel, 1976). The specific genotypes of the strains are shown in Table I.

Table I. Tester Strain Genotypes

Tester Strain	*his*/*trp* Mutation	Additional Mutations		Plasmid
		Repair	LPS	
TA98	*his*D3052	*uvr*B	*rfa*	pKM101
TA100	*his*G46	*uvr*B	*rfa*	pKM101
TA1535	*his*G46	*uvr*B	*rfa*	–
TA1537	*his*C3076	*uvr*B	*rfa*	–
WP2*uvr*A	*trp*	*uvr*A	–	–

In addition to a mutation in the histidine or tryptophan operons, the tester strains contain additional mutations that enhance their sensitivity to some mutagenic compounds. A mutation

of the *uvr*A gene (*Escherichia coli*) or the *uvr*B gene (*Salmonella typhimurium*) results in a deficient DNA excision repair system, which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, the *Salmonella typhimurium* tester strains containing this deletion also require the vitamin biotin for growth.

The *Salmonella typhimurium* tester strains also contain the *rfa* wall mutation, which results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (e.g., benzo[a]pyrene) that would otherwise be excluded by a normal intact cell wall.

Strains TA98 and TA100 also contain the pKM101 plasmid, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid

increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strains TA100, TA1535, and WP2*uvr*A are reverted from auxotrophy to prototrophy by base substitution mutagens.

Source of Tester Strains. The *Salmonella typhimurium* tester strains in use at Covance were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley. The *Escherichia coli* tester strain, WP2*uvr*A, was received from The National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom).

Frozen Permanent Stocks. Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 mL per mL of culture) and freezing away appropriately vialed aliquots. Frozen permanent stocks of the tester strains were stored at -60°C to -80°C.

Master Plates. Master plates of the tester strains were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine and biotin or tryptophan, and for strains containing the pKM101 plasmid, ampicillin. Tester strain master plates were stored at >0°C to 10°C.

Preparation of Overnight Cultures

Inoculation. Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in late log phase when density monitoring began.

Harvest. To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture density. Cultures were harvested once a predetermined density was reached which ensured that cultures had reached a density of at least 0.5×10^9 cells/mL and had not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target density was reached and were held at >0°C to 10°C until used in the assay.

Confirmation of Tester Strain Genotype. Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

***rfa* Wall Mutation.** For the *Salmonella* tester strains, the presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity of the culture to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media, and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid. The presence of the pKM101 plasmid was confirmed for cultures of tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media, and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants. The mean number of spontaneous revertants per plate in the vehicle controls that is characteristic of the strains was demonstrated by plating 100 µL aliquots of each culture along with the appropriate vehicle on selective media.

Culturing Broth. The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Minimal Bottom Agar Plates. Bottom agar (25 mL per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (w/v) agar and 0.2% (w/v) glucose.

Top Agar for Selection of Revertants. Top (overlay) agar contained 0.7% (w/v) agar and 0.5% (w/v) NaCl. It was supplemented with 10 mL of 0.5 mM histidine/biotin solution, or 10 mL of 0.5 mM tryptophan solution, per 100 mL agar for selection of histidine or tryptophan revertants, respectively. When S9 mix was required, 2.0 mL of the supplemented top agar was used in the overlay. However, when S9 mix was not required, water was added to supplemented
top agar (0.5 mL of water per 2.0 mL of supplemented top agar), and the resulting 2.5 mL of diluted supplemented top agar was used for the overlay. This dilution ensured that the final top agar and amino acid supplement concentrations remained the same in the presence and absence of S9 mix.

Test Article
The Sponsor was responsible for the identity, strength, purity, stability and uniformity of the test article. A Certificate of Analysis is included in Appendix 1.

Test Article Disposition
The remaining test article was appropriately discarded after issuance of the audited draft report. Disposal of the remaining test article was documented in the study file.

Control Articles
Vehicle Controls. Vehicle controls were plated for all tester strains in the presence and absence of S9 mix. The dimethylsulfoxide (DMSO) vehicle control was plated, using a 100 µL aliquot (equal to the maximum aliquot of test article solution plated), along with a 100 µL aliquot of the appropriate tester strain and a 500 µL aliquot of S9 mix (when necessary), on selective agar.

Positive Controls. The combinations of positive controls, activation conditions, and tester strains plated concurrently with the assay are indicated in Table II.

Table II. Positive Controls

Tester Strain	S9 Mix	Positive Control	Dose (µg/plate)
TA98	+	benzo[a]pyrene	2.5
TA98	–	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	2.5
TA100	–	sodium azide	2.0
TA1535	+	2-aminoanthracene	2.5
TA1535	–	sodium azide	2.0
TA1537	+	2-aminoanthracene	2.5
TA1537	–	ICR-191	2.0
WP2*uvr*A	+	2-aminoanthracene	25.0
WP2*uvr*A	–	4-nitroquinoline-N-oxide	1.0

The sources and grades of positive control articles are as follows:

benzo[a]pyrene (CAS #50-32-8), Sigma Chemical Co., purity ≥98.3%.
2-aminoanthracene (CAS #613-13-8), Aldrich Chemical Co., purity ≥97.3%.
2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., purity ≥98.0%.
sodium azide (CAS #26628-22-8), Sigma Chemical Co., purity ≥99.9%.
ICR-191 (CAS #17070-45-0), Sigma Chemical Co., purity ≥94.0%.
4-nitroquinoline-N-oxide (CAS #56-57-5), Sigma Chemical Co., purity = 100%.

Sterility Controls. The most concentrated test article dilution was checked for sterility by plating a 100 µL aliquot (the same volume used in the assay) on selective agar. The S9 mix was checked for sterility by plating 0.5 mL on selective agar.

S9 Metabolic Activation System
S9 Homogenate. Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Lot Nos. 1698 (37.1 mg protein/mL) and 1718 (39.4 mg protein/mL). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg/mL in corn oil) at 500 mg/kg as described by Ames *et al.,* (1975).

S9 Mix. The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table III.

Table III. S9 Mix Components

Component	Amount
H_2O	0.70 mL
1M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
0.25M Glucose-6-phosphate	0.02 mL
0.10M NADP	0.04 mL
0.825M KCl/0.2M $MgCl_2$	0.04 mL
S9 Homogenate	0.10 mL
	1.00 mL

Dose Rangefinding Study
The growth inhibitory effect (cytotoxicity) of the test article to the test system was determined in order to allow the selection of appropriate doses to be tested in the mutagenicity assay.

Design. The dose rangefinding study was performed using tester strains TA100 and WP2*uvr*A in both the presence and absence of S9 mix. Ten doses of test article were tested at one plate per dose. The test article was checked for cytotoxicity up to a maximum concentration of 5000 µg per plate.

Rationale. Cytotoxicity of test article observed on tester strain TA100 is generally representative of that observed on other *Salmonella typhimurium* tester strains. Because of the comparatively high number of spontaneous revertants per plate observed with this strain, gradations of cytotoxicity can be readily discerned from routine experimental variation. *Escherichia coli* tester strain WP2*uvr*A does not have the *rfa* wall mutation possessed by
Salmonella typhimurium strains; thus, a different range of cytotoxicity may be observed. Also, cytotoxicity induced by a test article in the presence of S9 mix may vary greatly from that observed in the absence of S9 mix. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

Evaluation of the Dose Rangefinding Study. Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn.

Selection of the Maximum Dose for the Mutagenicity Assay. Since no cytotoxicity was observed in the dose rangefinding study, the highest dose level of test article used in the mutagenicity assay was the same dose as that tested in the rangefinding study.

Mutagenicity Assay
Design. The assay was performed using tester strains TA98, TA100, TA1535, TA1537, and WP2*uvr*A in the presence and absence of S9 mix. The doses of test article were selected based on the results of the dose rangefinding study. The results of the initial mutagenicity assay were confirmed in an independent experiment.

Frequency and Route of Administration. The tester strains were exposed to test article via the plate incorporation methodology originally described by Ames *et al.* (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain, and the S9 mix (where appropriate) were combined in molten agar, which was overlaid onto a minimal agar plate. Following incubation, revertant colonies were counted. All doses of the test article, the vehicle controls, and the positive controls were plated in triplicate.

Plating Procedures
Each plate was labeled with a code that identified the test article, test phase, tester strain, activation condition, and dose level. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 µL of tester strain and 100 µL of vehicle or test article dose were added to 2.5 mL of molten selective top agar (maintained at 45 ± 2°C). When S9 mix was required, 500 µL of S9 mix, 100 µL of tester strain and 100 µL of vehicle or test article dose were added to 2.0 mL of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay solidified, the plates were inverted and incubated for 52 ± 4 hours at 37 ± 2°C. Positive control articles were plated using a 50 µL plating aliquot.

Scoring the Plates
Plates which were not evaluated immediately following the incubation period were held at >0°C to 10°C until such time that colony counting and bacterial background lawn evaluation could take place.

Bacterial Background Lawn Evaluation. Condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control and recorded along with the revertant counts for that dose.

Counting Revertant Colonies. Revertant colonies were counted by automated colony counter or by hand.

DATA

Data Presentation
For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables section of this report. The historical control data are presented after the data tables.

Assay Acceptance Criteria
Before assay data were evaluated, the criteria for a valid assay had to be met. The following criteria were used to determine a valid assay:

Tester Strain Integrity.

***rfa* Wall Mutation.** To demonstrate the presence of the *rfa* wall mutation, *Salmonella typhimurium* tester strain cultures exhibited sensitivity to crystal violet.

pKM101 Plasmid. To demonstrate the presence of the pKM101 plasmid, cultures of the appropriate tester strains exhibited resistance to ampicillin.

Characteristic Number of Spontaneous Revertants. To demonstrate the requirement for histidine or tryptophan, the tester strain cultures exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions.

The acceptable ranges for the mean vehicle controls were as follows:

TA98	8	- 60
TA100	60	- 240
TA1535	4	- 45
TA1537	2	- 25
WP2*uvr*A	5	- 40

Tester Strain Culture Density. To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures were greater than or equal to 0.5×10^9 bacteria per mL and/or had reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per mL.

Positive Control Values in the Absence of S9 Mix. To demonstrate that the tester strains were capable of identifying a mutagen, the mean value of a positive control for a respective tester strain exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

Positive Control Values in the Presence of S9 Mix (S9 Mix Integrity). To demonstrate that the S9 mix was capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 mix for a specific strain was evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

Cytotoxicity. A minimum of three non-toxic doses was required to evaluate assay data. Cytotoxicity was detectable as a decrease in the number of revertants colonies per plate and/or by a thinning or disappearance of the bacterial background lawn compared to the appropriate vehicle control. A thinning of the bacterial background lawn that was not accompanied by a reduction in the number of revertants per plate was not evaluated as an indication of cytotoxicity.

Assay Evaluation Criteria
Once the criteria for a valid assay had been met, responses observed in the assay were evaluated as follows:

Tester Strains TA98, TA100, and WP2*uvr*A. For a test article to be considered positive, it had to produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535 and TA1537. For a test article to be considered positive, it had to produce at least a 3-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

RESULTS

Test Article Handling

In solubility testing with deionized water, the test article was observed to form a dark-orange, heterogeneous suspension at approximately 50 and 100 mg per mL. In solubility testing with dimethylsulfoxide (DMSO), the test article was observed to form a dark-orange, heterogeneous suspension at approximately 100 mg per mL and a dark-orange, homogeneous suspension at approximately 50 mg per mL. For this reason, DMSO (Acros Organics, Lot No. A019540701) was selected as the vehicle for this study. At 50 mg per mL, which was the most concentrated stock dilution prepared for the mutagenicity assay, the test article was observed to form an opaque, orange to dark-orange, non-viscous suspension. The test article was observed to dilute to a solution at 0.333 mg per mL and lower concentrations prepared for the mutagenicity assay.

Dose Rangefinding Study

Doses tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted on the test article using tester strains TA100 and WP2*uvr*A in both the presence and absence of S9 mix with one plate per dose. Ten doses of test article ranging from 6.67 to 5000 µg per plate were tested and results are presented in Table 1. These data were generated in Trial 26471-A1. No cytotoxicity was observed with either tester strain in the presence or absence of S9 mix as evidenced by no dose-related decreases in the number of revertants per plate. Bacterial background lawns were observed to be normal up to and including 667 µg per plate. Background lawns were obscured by test article precipitate at ≥1000 µg per plate.

Mutagenicity Assay

Mutagenicity results for Mesozeaxanthin are presented in Tables 2 through 5. These data were generated in Trials 26471-B1 and 26471-C1. Data are presented as individual plate counts (Tables 2 and 4) and as mean revertants per plate ± standard deviation (Tables 3 and 5) for each treatment and control group.

Results of the dose rangefinding study were used to select doses tested in the mutagenicity assay. Doses tested in the mutagenicity assay with all tester strains in both the presence and absence of S9 mix were 10.0, 33.3, 100, 333, 1000, and 5000 µg per plate.

In the initial mutagenicity assay, Trial 26471-B1 (Tables 2 and 3) and in the confirmatory mutagenicity assay, Trial 26471-C1 (Tables 4 and 5), all data were acceptable and no positive increases in the mean number of revertants per plate were observed with any of the tester strains in either the presence or absence of S9 mix.

All criteria for a valid study were met.

CONCLUSION

The results of the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, the test article, Mesozeaxanthin, did not cause a positive increase in the mean number of revertants per plate with any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor™-induced rat liver (S9).

RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol, and the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna, for at least 1 year following submission of the final report to the Sponsor. After the 1-year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna, for an additional period of time or sent to a storage facility designated by the Sponsor.

REFERENCES

Ames, B.N., McCann, J., and Yamasaki, E., "Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test." *Mutation Research, 31*:347-364 (1975).

Green, M.H.L. and Muriel, W.J., "Mutagen testing using *trp*$^+$ reversion in *Escherichia coli*." *Mutation Research, 38*:3-32 (1976).

Maron, D.M. and Ames, B., "Revised Methods for the *Salmonella* Mutagenicity Test." *Mutation Research, 113*:173-215 (1983).

OECD, "Bacterial Reverse Mutation Tst," *OECD Guidelines for Testing of Chemicals*, Section 4, Guideline 471, updated and adopted 21 July 1997.

Vogel, H.J. and Bonner, D.M., "Acetylornithinase of *Escherichia coli*: Partial purification and some properties." *Journal Biological Chemistry, 218*:97-106 (1956).

DATA TABLES

Table 1 : Dose Rangefinding Study

Test Article ID: Mesozeaxanthin

Assay/Study No.: 26471-0-409OECD | Trial No.: A1

Date Plated: 17-Sep-04 | Vehicle: DMSO

Date Counted: 20-Sep-04 | Plating Aliquot: 100

		Revertants per Plate			
	Dose/Plate	TA100	Background Lawn[a]	WP2*uvr*A	Background Lawn[a]
Microsomes: Rat Liver					
Vehicle Control		122	N	18	N
Test Article	6.67 □g	111	N	21	N
	10.0 □g	90	N	14	N
	33.3 □g	103	N	21	N
	66.7 □g	108	N	13	N
	100 □g	107	N	13	N
	333 □g	108	N	17	NP
	667 □g	111	N	20	NP
	1000 □g	104	OP	9	OP
	3330 □g	105	OP	19	OP
	5000 □g	118	OP	12	OP
Microsomes: None					
Vehicle Control		88	N	16	N
Test Article	6.67 □g	106	N	16	N
	10.0 □g	99	N	13	N
	33.3 □g	99	N	14	N
	66.7 □g	89	N	11	N
	100 □g	99	N	14	N
	333 □g	88	NP	12	NP
	667 □g	86	NP	11	NP
	1000 □g	87	OP	13	OP
	3330 □g	71	OP	14	OP
	5000 □g	86	OP	12	OP

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

Table 2 : Mutagenicity Assay Results – Individual Plate Counts

Test Article ID: Mesozeaxanthin

Assay No.: 26471-0-409OECD | Trial No.: B1

Date Plated: 29-Sep-04 | Vehicle: DMSO

Date Counted: 07-Oct-04, 08-Oct-04 | Plating Aliquot: 100 □L

		Revertants Per Plate															Back-ground Lawn[a]
	Dose/Plate	TA98			TA100			TA1535			TA1537			WP2*uvr*A			
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
Microsomes: Rat Liver																	
Vehicle Control		20	13	18	87	96	104	15	8	8	4	11	8	15	C	14	N
Test Article	10.0 µg	21	22	18	116	93	90	13	7	9	6	5	7	14	6	8	N
	33.3 µg	18	19	10	102	71	116	12	17	9	10	6	2	17	17	18	N
	100 µg	29	21	22	108	84	90	13	9	15	4	10	4	12	16	18	N
	333 µg	27	15	12	101	84	107	13	10	9	5	8	5	12	11	15	NP/OP[d]
	1000 µg	19	19	26	79	103	88	12	16	11	9	6	6	22	14	16	OP
	5000 µg	21	22	26	104	101	110	11	12	8	11	17	4	14	15	9	OP
Positive Control[b]		313	408	372	798	626	756	138	128	147	167	150	154	490	495	550	N
Microsomes: None																	
Vehicle Control		10	11	6	67	77	61	14	15	11	3	8	8	9	14	12	N
Test Article	10.0 µg	10	9	11	87	87	69	17	13	19	4	6	5	11	17	15	N
	33.3 µg	6	10	12	53	61	82	14	10	17	6	7	7	22	16	18	N
	100 µg	15	9	11	72	90	71	17	10	12	6	2	9	13	13	24	N
	333 µg	12	6	10	90	70	75	13	6	10	10	6	2	14	20	17	NP
	1000 µg	5	8	11	95	69	73	8	15	8	1	0	7	14	15	10	OP
	5000 µg	5	10	10	68	88	86	14	11	7	2	8	2	14	14	9	OP
Positive Control[c]		391	353	326	1265	1167	1115	592	701	777	844	937	1096	211	281	309	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 □g/plate
TA100	2-	2.5 □g/plate
TA1535	2-	2.5 □g/plate
TA1537	2-	2.5 □g/plate
WP2*uvr*A	2-	25.0

[c]

TA98	2-nitrofluorene	1.0 □g/plate
TA100	sodium azide	2.0 □g/plate
TA1535	sodium azide	2.0 □g/plate
TA1537	ICR-191	2.0 □g/plate
WP2*uvr*A	4-nitroquinoline-N-oxide	1.0 □g/plate

C = No count due to contamination on the plate.

[d] The first entry is the lawn evaluation for tester strains TA98, TA100, TA1535 and TA1537.
The second entry is the lawn evaluation for tester strains WP2*uvr*A.

Table 3 : Mutagenicity Assay Results – Summary

Test Article ID: Mesozeaxanthin

Assay No.: 26471-0-409OECD Trial No.: B1

Date Plated: 29-Sep-04 Vehicle: DMSO

Date Counted: 07-Oct-04, 08-Oct-04 Plating Aliquot: 100 □L

		Mean Revertants Per Plate with Standard Deviation										Back-ground Lawn[a]
	Dose/Plate	TA98		TA100		TA1535		TA1537		WP2*uvr*A		
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Microsomes: Rat Liver												
Vehicle Control		17	4	96	9	10	4	8	4	15	1	N
Test Article	10.0 µg	20	2	100	14	10	3	6	1	9	4	N
	33.3 µg	16	5	96	23	13	4	6	4	17	1	N
	100 µg	24	4	94	12	12	3	6	3	15	3	N
	333 µg	18	8	97	12	11	2	6	2	13	2	NP/OP[d]
	1000 µg	21	4	90	12	13	3	7	2	17	4	OP
	5000 µg	23	3	105	5	10	2	11	7	13	3	OP
Positive Control[b]		364	48	727	90	138	10	157	9	512	33	N
Microsomes: None												
Vehicle Control		9	3	68	8	13	2	6	3	12	3	N
Test Article	10.0 µg	10	1	81	10	16	3	5	1	14	3	N
	33.3 µg	9	3	65	15	14	4	7	1	19	3	N
	100 µg	12	3	78	11	13	4	6	4	17	6	N
	333 µg	9	3	78	10	10	4	6	4	17	3	NP
	1000 µg	8	3	79	14	10	4	3	4	13	3	OP
	5000 µg	8	3	81	11	11	4	4	3	12	3	OP
Positive Control[c]		357	33	1182	76	690	93	959	127	267	50	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 □g/plate
TA100	2-	2.5 □g/plate
TA1535	2-	2.5 □g/plate
TA1537	2-	2.5 □g/plate
WP2*uvr*A	2-	25.0

[c]

TA98	2-nitrofluorene	1.0 □g/plate
TA100	sodium azide	2.0 □g/plate
TA1535	sodium azide	2.0 □g/plate
TA1537	ICR-191	2.0 □g/plate
WP2*uvr*A	4-nitroquinoline-N-oxide	1.0 □g/plate

[d] The first entry is the lawn evaluation for tester strains TA98, TA100, TA1535 and TA1537.
The second entry is the lawn evaluation for tester strains WP2*uvr*A.

Table 4 : Mutagenicity Assay Results – Individual Plate Counts

Test Article ID: Mesozeaxanthin

Assay No.: 26471-0-409OECD | Trial No.: C1

Date Plated: 15-Oct-04 | Vehicle: DMSO

Date Counted: 25-Oct-04 | Plating Aliquot: 100 □L

Dose/Plate		Revertants Per Plate															Background Lawn[a]
		TA98			TA100			TA1535			TA1537			WP2*uvr*A			
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
Microsomes: Rat Liver																	
Vehicle Control		23	22	19	59	67	80	14	8	17	11	9	15	18	24	23	N
Test Article	10.0 µg	18	22	16	132	113	91	8	15	16	7	5	5	16	12	8	N
	33.3 µg	15	12	11	106	98	113	10	10	9	2	9	8	20	12	15	N
	100 µg	18	18	20	117	104	111	5	12	12	6	8	5	23	11	14	N
	333 µg	21	20	11	100	103	68	11	11	11	4	5	3	13	20	16	NP
	1000 µg	14	26	20	80	76	74	11	14	12	8	5	3	19	22	17	OP
	5000 µg	25	25	21	85	108	91	14	8	C	6	8	5	13	17	17	OP
Positive Control[b]		359	356	393	2294	1585	1509	248	172	145	285	229	243	155	203	234	N
Microsomes: None																	
Vehicle Control		12	11	12	91	96	46	18	14	9	8	2	4	17	16	15	N
Test Article	10.0 µg	10	12	9	95	80	80	12	C	19	5	9	5	10	16	10	N
	33.3 µg	15	C	16	67	92	73	20	11	9	5	5	4	19	6	7	N
	100 µg	13	19	7	99	80	94	14	14	12	3	7	4	11	11	11	N
	333 µg	10	10	16	87	81	83	14	18	6	6	5	3	8	13	9	NP
	1000 µg	8	15	17	82	71	76	10	11	13	2	7	6	11	11	10	OP
	5000 µg	14	8	9	82	72	75	12	19	10	3	3	4	11	9	7	OP
Positive Control[c]		256	260	278	1601	1330	1271	992	953	971	612	551	600	72	85	113	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]
TA98	benzo[a]pyrene	2.5 □g/plate
TA100	2-	2.5 □g/plate
TA1535	2-	2.5 □g/plate
TA1537	2-	2.5 □g/plate
WP2*uvr*A	2-	25.0

[c]
TA98	2-nitrofluorene	1.0 □g/plate
TA100	sodium azide	2.0 □g/plate
TA1535	sodium azide	2.0 □g/plate
TA1537	ICR-191	2.0 □g/plate
WP2*uvr*A	4-nitroquinoline-N-oxide	1.0 □g/plate

C = No count due to contamination on the plate.

Table 5 : Mutagenicity Assay Results – Summary

Test Article ID: Mesozeaxanthin

Assay No.: 26471-0-409OECD | Trial No.: C1

Date Plated: 15-Oct-04 | Vehicle: DMSO

Date Counted: 25-Oct-04 | Plating Aliquot: 100 □L

		Mean Revertants Per Plate with Standard Deviation										Back-ground Lawn[a]
	Dose/Plate	TA98		TA100		TA1535		TA1537		WP2*uvr*A		
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Microsomes: Rat Liver												
Vehicle Control		21	2	69	11	13	5	12	3	22	3	N
Test Article	10.0 µg	19	3	112	21	13	4	6	1	12	4	N
	33.3 µg	13	2	106	8	10	1	6	4	16	4	N
	100 µg	19	1	111	7	10	4	6	2	16	6	N
	333 µg	17	6	90	19	11	0	4	1	16	4	NP
	1000 µg	20	6	77	3	12	2	5	3	19	3	OP
	5000 µg	24	2	95	12	11	4	6	2	16	2	OP
Positive Control[b]		369	21	1796	433	188	53	252	29	197	40	N
Microsomes: None												
Vehicle Control		12	1	78	28	14	5	5	3	16	1	N
Test Article	10.0 µg	10	2	85	9	16	5	6	2	12	3	N
	33.3 µg	16	1	77	13	13	6	5	1	11	7	N
	100 µg	13	6	91	10	13	1	5	2	11	0	N
	333 µg	12	3	84	3	13	6	5	2	10	3	NP
	1000 µg	13	5	76	6	11	2	5	3	11	1	OP
	5000 µg	10	3	76	5	14	5	3	1	9	2	OP
Positive Control[c]		265	12	1401	176	972	20	588	32	90	21	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 □g/plate
TA100	2-	2.5 □g/plate
TA1535	2-	2.5 □g/plate
TA1537	2-	2.5 □g/plate
WP2*uvr*A	2-	25.0

[c]

TA98	2-nitrofluorene	1.0 □g/plate
TA100	sodium azide	2.0 □g/plate
TA1535	sodium azide	2.0 □g/plate
TA1537	ICR-191	2.0 □g/plate
WP2*uvr*A	4-nitroquinoline-N-oxide	1.0 □g/plate

HISTORICAL CONTROL DATA FOR BACTERIAL MUTAGENICITY STUDIES

Plate Incorporation Method - Report Period 02D

Vehicle Controls with S9 Mix					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	26.4	91.9	12.9	9.5	17.2
Standard Deviation	6.6	15.9	4.5	3.3	5.8
Maximum	44	149	28	19	37
Minimum	7	52	3	2	3
Count	255	255	214	204	189
Vehicle Controls without S9 Mix					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	15.9	85.4	15.8	7.7	16.4
Standard Deviation	5.2	16.9	6.3	3.9	5.8
Maximum	33	156	45	28	37
Minimum	5	46	2	1	6
Count	243	236	212	227	181
Positive Controls with S9 Mix[a]					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	400.4	706.4	143.4	118.6	595.0
Standard Deviation	95.3	312.3	75.1	80.2	214.8
Maximum	688	2970	691	727	1098
Minimum	202	111	68	52	80
Count	212	220	191	180	173
Positive Controls without S9 Mix[b]					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	238.7	1054.4	749.8	835.6	242.5
Standard Deviation	81.9	191.6	148.5	266.9	113.6
Maximum	691	1515	1291	1485	839
Minimum	53	390	107	97	55
Count	201	199	189	200	170

[a]

TA98	benzo[a]pyrene	2.5 □g/plate
TA100	2-aminoanthracene	2.5 □g/plate
TA1535	2-aminoanthracene	2.5 □g/plate
TA1537	2-aminoanthracene	2.5 □g/plate
WP2*uvr*A	2-aminoanthracene	25.0 □g/plate

[b]

TA98	2-nitrofluorene	1.0 □g/plate
TA100	sodium azide	2.0 □g/plate
TA1535	sodium azide	2.0 □g/plate
TA1537	ICR-191	2.0 □g/plate
WP2*uvr*A	4-nitroquinoline-N-oxide	1.0 □g/plate

APPENDIX 1:
CERTIFICATE OF ANALYSIS

Industrial Orgánica

Safety No.:
26471-001
Aug 24, 2004

26471

CERTIFICATE OF ANALYSIS

Product: Meso Zeaxanthin

Lot No: Sample

Date: Aug 19, 2004.

GUARANTEED ANALYSIS	LOT ANALYSIS
Carotenoid Activity (HPLC)	745,200 ppm
Purity:	
Zeaxanthin (HPLC)	68.2 %
Lutein (HPLC)	30.4 %
Free Xanthophylls, AOAC (%)	99.1 %
Moisture(%)	0.5 %
Particle size: pass Tyler sieve # 35	95.0 %
Appearance:	Orange powder

Sincerely,

M.Sc. Ricardo Montoya Olvera.
Quality Control

Industrial Orgánica, S.A. de C.V.
Ave. Almazán No. 100 Col. Topo Chico 64260 Apdo. Postal 1654 Monterrey, N.L., México
Tel. (81) 83-52-22-90 01-800 926-7000 Fax (81) 83-76-72-14 e-mail: iosa@att.net.mx

Safety No.:
26471-001
Aug 24, 2004

MESO ZEAXANTHIN CONCENTRATE 26471

1. Identity:

The Meso Zeaxanthin concentrate is an orange natural pigment concentrate with a content of Zeaxanthin enantiomers (> 70%) obtained by an isomerization process (U.S.Pat. 5, 523,494) refined and purified (U.S.Pat. 6, 504,067) from a hydrolyzed extract of marigold flowers *Tagetes erecta* (C.F.R. 73.295 Subpart A Foods). Zeaxanthin and Lutein are the main components of this concentrate.

2. Technical name:

Meso Zeaxanthin

***CAS* Reg. No.: [144-68-3]**
M.F. $C_{40}H_{56}O_2$
M.W. 568.85
Common name: β, β`-Carotene-3,3'diol;
(3R, 3S)-meso-all-Trans-anchovyxanthin ;(3R, 3S)-meso-all-Trans-zeaxanthin
(3R, 3R)-all-Trans-anchovyxanthin ;(3R, 3R)-all-Trans-Zeaxanthin.

IUPAC Nomenclature
β-carotene, 1,1`-(3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17 Octadecanonaene-1,18-diyl)bis (2,6,6-trimethyl-4-hidroxycyclohexene).

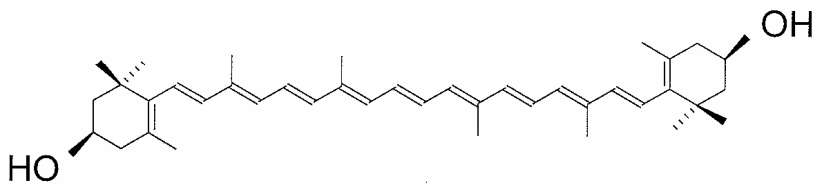
Lutein

***CAS* Reg. No.: [15904-92-4] of plants;**
***CAS* Reg. No.: [127-40-2] all-trans-lutein, all-trans-xanthophyll**
M.F. $C_{40}H_{56}O_2$
M.W. 568.85
Common name: β, ε- Carotene-3, 3´ diol; (3R, 3´R, 6´R)-(all-trans-lutein, all-trans-xanthophyll)
IUPAC Nomenclature
β,ε-carotene,(all-E)-1,3,3-trimethyl-5-hidroxy-2-[3,7,12,16-tetramethyl-18-(2,6,6-trimethyl-4-hidroxy-2-cyclohexen-1-yl)-1,3,5,7,9,11,13,15,17-Octadecanonaenyl] cyclohexene.

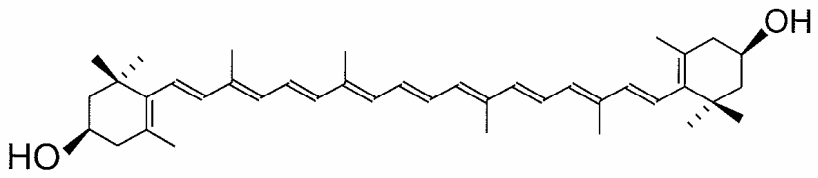
3.- Structural Class:

Carotenoid

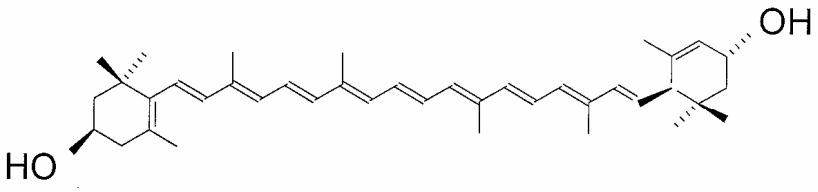
4. Chemical Formula



Meso-Zeaxanthin; (3R,3'S)-β,β-Carotene-3,3'-diol



Zeaxanthin; (3R,3'R)-β,β-Carotene-3,3'-diol



Lutein; (3R,3'R, 6'R)-β,ε-Carotene-3,3'-diol

Safety No.:
26471-001
Aug 24, 2004

26471

5. Specifications:

Test	Typical
Concentration (%p/p)	> 75.0 %
Zeaxanthin (% HPLC)) (3R,3S)-Meso-all-trans-zeaxanthin (Min 75.0 %); (3R,3R)-All-trans-zeaxanthin (Max. 25.0 %)	> 65.0 %
Lutein (%HPLC)	<35.0%
Moisture:	< 1.5%
Waxes (%p/p)	<20.0%,
Ash (%p/p)	< 1.0%,
Hexane	< 25.0 ppm

6.- Physical Chemical Properties:

Physical Properties
Description: Orange crystals.
Solubility: Water insoluble. Slightly soluble in Hexane, Ethanol, Isopropanol, Dimethylformamide, Tetrahydrofurane.

Chemical Properties
The factors that affect the Meso Zeaxanthin Concentrate stability:
TEMPERATURE: The Concentrate is very stable at low temperatures (< 30°C), stored under vacuum and darkness. At higher temperatures a slight degradation occurs.
LIGHT: Like all carotenoids, the Meso Zeaxanthin Concentrate degrades when exposed to light under prolonged periods of time.

The Meso Zeaxanthin Concentrate is very stable when stored in its original vacuum sealed package and is kept away from heat and light.