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November 14, 2003

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Via Federal Express

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OFFICE OF
FOOD ADDITIVE SAFETY

Dr. Laura Tarantino
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: GRAS Notification for Cassia Gum

Dear Dr. Tarantino:

Pursuant to proposed 21 C.F.R. § 170.36(c) and on behalf of our client, Noveon, Inc., we hereby notify the Agency of our determination on the basis of scientific procedures that cassia gum (tradename Diagum SR™) is generally recognized as safe (GRAS) when used as a thickening agent, texturizer and water retention aid in specified foods. Three copies of Noveon's GRAS notification for cassia gum are enclosed.

We trust you will find the enclosed notification acceptable. Should any questions arise during the review process, please do not hesitate to contact us, preferably by telephone, so that we may respond as quickly as possible.

Very truly yours,

(b)(6)

Melvin S. Drozen

Enclosures

cc: Robert Hinderer

Before the
FOOD AND DRUG ADMINISTRATION
Department of Health and Human Services
Washington, D.C.

GRAS NOTIFICATION

Name of Notifier:	Noveon, Inc.
Post Office Address:	All communications on this matter are to be sent in care of Counsel for the Notifier, Melvin S. Drozen, Keller and Heckman LLP, 1001 G Street, N.W., Suite 500 West, Washington, D.C. 20001. Telephone: (202) 434-4222.
Name of Substance and Intended Use:	Cassia Gum for use in selected foods as a thickener, to improve texture and for water retention.
Dated:	November 14, 2003

Melvin S. Drozen
Counsel for Noveon.

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1. Introduction

Plant-derived polysaccharides are excellent stabilizing and thickening agents and are common components of processed foods. For example, locust (carob) bean gum is listed as generally recognized as safe (GRAS) for use in food up to a maximum level of 0.75% (21 C.F.R. § 184.1343). It is also approved for use as a thickening agent in Europe (EEC No. E410). Guar gum is listed as GRAS for use in food up to a maximum level of 2% (21 C.F.R. § 184.1339). Both are used in various foods including baked goods, cheese, and jams/jellies.

Cassia gum (tradename Diagum SR™), flour from the endosperm of *Cassia tora* and *Cassia obtusifolia*, is well suited for use in the manufacture of gels, frequently in combination with other colloids, and has several food applications. It is used to achieve a variety of functional effects, including thickening, emulsification, foam stabilization, moisture retention, and texture improvement. Besides the aforementioned technical applications, the main application of cassia gum is likely to be to create a range of soft to firm gels in synergism with kappa-carrageenan or xanthan. Cassia gum is used at concentrations comparable to locust (carob) bean gum.

The toxicological profile of cassia gum, including the potential for constituents of its source seeds to cause muscle toxicity when consumed at high levels, and other data were assessed by an Expert Panel to determine GRAS status for use in food. The lack of toxicity of cassia gum demonstrated in animals—via a wide range of toxicological studies, including short-term, subchronic, reproductive, teratogenic, and genetic toxicity studies—and the safe use of related gums indicates that cassia gum meeting finished product specifications may be safely utilized in food and is GRAS.

More specifically, the notifier's conclusion that the intended use of cassia gum is GRAS based on scientific procedures relies upon the collective weight of (1) the various published safety data on similar polysaccharide gums; (2) the published review of the animal feeding studies conducted on cassia gum and the safety data on other GRAS gums (Hallagan, *et al.*, 1997); and (3) the published feeding study on the toxicity of the anthraquinone impurities in cassia seeds (Crawford and Friedman 1990). The Crawford and Friedman study provides published data establishing the safety—*vis-à-vis* the muscle toxicity associated with certain

Cassia species—of the maximum theoretical dietary intake of anthraquinones from the intended use of cassia gum.[1]

Also, certain anthraquinones have been found to be animal carcinogens. Although none of those anthraquinones have been identified in cassia gum (only one anthraquinone has been detected in Diagum SR), because of the carcinogenicity of certain anthraquinones, we have conducted a cancer risk assessment to determine the theoretical risk from the maximum amount of anthraquinones that might be present in the gum, assuming that these anthraquinones were carcinogenic. As discussed in more detail in Section 6.3.6 of the notification, we have determined that the upper-bound calculated risk associated with exposure to the anthraquinones in cassia gum is 7.2×10^{-9}, which is equivalent to eight in one billion. This level of risk is so low as to clearly establish the safety of anthraquinones that may be present in cassia gum with regard to carcinogenicity, particularly since this risk level is lower than the risk presented by anthraquinones from natural sources in the diet.

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[1] As discussed later in this notification, although Noveon's cassia gum (Diagum SR™) has a 10 ppm specification for anthraquinones, only one anthraquinone, physcion, has been found in the gum by HPLC analyses with a detection limit of 0.5 ppm.

1.1 Notice of GRAS Exemption Claim

Cassia gum is exempt from premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because it has been determined to be GRAS under conditions of intended use by experts qualified by scientific training and experience.

i) Notifier:

Noveon, Inc.
9911 Brecksville Road
Cleveland, Ohio 44141

Contact Person:

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
Telephone: (202) 434-4222

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ii) Common/usual name:

Cassia gum (Diagum SR™)

iii) Applicable conditions of use:

As discussed further below, based on the extant data and experts' conclusions, cassia gum is deemed by Noveon to be GRAS for use in the applications set forth in the table below.

Foods	Maximum Concentration (ppm)	Intended Uses
Baked Goods—Cheese-crème filling in pastries and other cheese-crème filled desserts	5,000	Water retention
Soups, Sauces, Seasonings—Dry soup mixes only	2,000 - 5,000	Thickening agent
Salad Dressings—Selected oil-free dressings only	3,000 - 5,000	Thickening agent
Meat and Poultry Products—Sausages, corned beef and canned poultry meats only	3,000	Texture improvement and water retention

iv) The basis of this GRAS determination is through scientific procedures. This determination is supported by an expert panel review of the relevant toxicological data, as evidenced by the conclusions set forth in Appendix I.

v) The data and information that form the basis of the GRAS determination are available for the FDA's review and copying. All references are available through the contact person named above.

Respectfully submitted,
NOVEON

By: _____

Melvin S. Drozen
Keller and Heckman, LLP
COUNSEL FOR THE NOTIFIER

2. Chemical Identity

2.1 Natural Sources of Cassia Gum

Cassia gum (CAS No. 11078-30-1) (trade name Diagum SR™) is the flour from the purified endosperm of the seeds of *Cassia tora* (*C. tora*) and *Cassia obtusifolia* (*C. obtusifolia*), which belong to the *leguminosae* family. *C. tora* and *C. obtusifolia* closely resemble one another but have been classified by some botanists as distinct species. *C. tora* and *C. obtusifolia* grow wild in subtropical regions of the world, and *C. tora* is occasionally cultivated. *C. tora* and *C. obtusifolia* are annual ruderal plants that ripen after approximately 100 days.

2.2 Impact of Admixture of *C. obtusifolia* with *C. tora*

The raw seed material used for Diagum SR™ typically will contain some *C. obtusifolia* along with *C. tora.* The *tora* and *obtusifolia* seeds grow in the same sites and are difficult to distinguish visually; they are therefore harvested together. Poethke *et al.* (1968) concluded that Vietnamese *C. tora* is identical to *C. obtusifolia*. Other investigations have shown that the two are remarkably similar if not interchangeable (Crawford *et al.*, 1990). But there are some detectable chemical differences, in both amino acids and anthraquinone content (Upadhyaya and Singh, 1986). Koshioka and Takino (1978) and Upadhyaya and Singh (1986) suggest that *C. tora* is a phytochemically distinct species derived from *C. obtusifolia*. This is also the conclusion of Randell (1995), who discussed the taxonomy and evolution of the two species. However, regional differences in *C. obtusifolia* and *C. tora* cultivars may easily obscure the minor differences between these closely related species. The close similarity in seed chemistry, morphology and ecological requirements between the two plants are far more significant than the relatively minor differences between them.

2.3 Chemical Structure and Physical Properties of Cassia Gum

Cassia gum is comprised of at least 75% polysaccharide consisting primarily of a linear chain of 1,4-β-D-mannopyranose units with 1,6 linked α-D galactopyranose units. The ratio of mannose to galactose is 5:1. The following composition was revealed in the sugar analysis conducted using HPLC: mannose (77.2-78.9%), galactose (15.7-14.7%) and glucose (7.1-6.3%).

Like most polysaccharides, the following formula applies: $(C_6H_{10}O_5)_n$, H_20. The structure, ratio of mannose to galactose units, and molecular weight of Cassia gum are outlined

in Table 2.1 below. For comparison purposes, Table 2.1 also contains structural properties of several other gums that are commonly used in food.

Table 2.1
Structural Properties of Cassia Gum (Diagum SR™) and Related Substances

Substance	Structure	Mannose:Galactose	Molecular Weight
Cassia gum (CAS No. 11078-30-1)[2]	1,4-β-D-mannopyranose units with 1,6-α-D galactopyranose units attached to every fifth mannose.	5:1	200,000-300,000
Guar gum[3] (CAS No. 9000-30-0)	1,4-β-D-mannopyranose units with 1,6-α-D-galactopyranose units attached to every alternate mannose.	2:1	220,000-250,000
Locust (carob) bean gum[4] (CAS No. 9000-40-2)	1,4-β-D-mannopyranose units with 1,6-α-D-galactopyranose units attached to every fourth or fifth mannose.	4:1	310,000
Tara gum[5] (CAS No. 39300-88-4)	1,4-β-D-mannopyranose units with side chains of 1,6-α-D-galactopyranose attached to approximately every third unit.	Approximately 3:1	Not Reported

Typical physical properties of cassia gum are set forth in Table 2.2 below.

Table 2.2
Physical Properties of Cassia Gum (Diagum SR™)[6]

Appearance	Off-white to pal beige powder without lumps
Odor	Neutral to slightly fruity
Bulk density	0.6 kg/l
Particle size (sieve analysis)	<250 μm (average) not less than 100% 100 μm (average) not less than 60%
Solubility	Disperses well in cold water and forms a colloidal solution
pH (1%)	6.5-8.5

2 CAS Registry Number for "galactomannan".

3 Klose and Glicksman, 1968.

4 Klose and Glicksman, 1968.

5 NTP, 1982b.

6 Leimgruber and Marbot 1991.

1% Viscosity	NLT 250 mPas
Gel-strength	NLT 155 g

2.4 Method of Manufacture

Cassia tora and *obtusifolia* are harvested when the plants are ripe (e.g., November-January) in India. The seeds that are used in the production of cassia gum are generally picked in the wild. *C. occidentalis,* another species of *Cassia* which has been associated with muscle toxicity,[7] does not grow in conjunction with *C. tora* or *C. obtusifolia* and hence, even by oversight, is unlikely to be collected together. The *C. occidentalis* pods look distinctly different from *C. tora* and *C. obtusifolia* and collectors are educated not to collect *C. occidentalis*. Furthermore, Noveon has a specification to limit the presence of *C. occidentalis* in the cassia seeds that are the raw material used to produce Diagum SR™. The specification limits *C. occidentalis* as well as black splits and foreign matter to a combined level of 2%. A copy of a Raw Material Specification Sheet that Noveon will apply to Diagum SR™ together with the specific test standard for *C. occidentalis* (Test No. NC 02602) is included in Appendix II at Tab 1. This specification applies to seeds prior to their undergoing an extensive cleaning process. After cleaning, the level of *C. occidentalis*, if any, would be substantially lower.[8]

Once the pods are harvested, the seeds are subject to a cleaning process before splitting. The seed purification includes mechanical steps to remove stones, dirt and seeds other than *C. tora* and *C. obtusifolia*. Specifically, after removal of dust and coarse impurities with a round sieve, the seeds pass two separators and finally a gravity separator. At this stage, stones and seeds with a similar size but different weight compared to *C. tora* or *C. obtusifolia* seeds are removed.

The production of cassia splits follows the well-known production procedure of the related guar gum splits. The seeds are roasted under such conditions that the endosperm (split)

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[7] Muscle toxicity has also been associated with components of *C. obtusifolia*, although *C. occidentalis* appears to be somewhat more toxic. Steps taken to ensure that Diagum SR™ will not contain harmful levels of muscle toxins from *C. obtusifolia* or *C. tora* are discussed later in this section.

[8] Each shipment of seeds is analyzed for compliance with the raw material specifications upon delivery. In the 1999/2000 collection season, for example, on average only 0.02% *C. occidentalis* seeds were found. Based on testing of 325 samples, <1% of *C. occidentalis* seeds was detected in 47 deliveries that season.

remains intact and flexible, while husk and germ, which are more sensitive to heat, become brittle. Physical stress crushes husk and germ into powder, which is removed from the intact split by sieving. Brushing and polishing steps finally remove last traces of husk and germ from the endosperm. Each split lot is subject to raw material control according to SOP XX 003 (see App. II, Tab 2). For additional details on the production of cassia splits, see the report by Technical Manager Ferdinand Utz of Noveon on the Production of Food Grade Cassia Splits (App. II, Tab 3).

Noveon produces several grades of cassia gum from the splits to be used in different applications. The two grades that are relevant to this notification are Diagum SR™, which is the subject of this notification, and Diagum CS™, which used to be Noveon's top grade of cassia gum before the development of Diagum SR™. Diagum SR™ and Diagum CS™ are produced via exactly the same manufacturing process, which is discussed below, except that Diagum SR™ is further purified and improved by a final soaking in water and extraction using isopropyl alcohol and water. The water treatment improves the performance properties of the gum and the alcohol/water extraction step allows Diagum SR™ to achieve lower impurity levels than Diagum CS™, including total anthraquinone content of no more than 10 parts per million. Because Diagum SR™ was only developed in the last year, there are necessarily numerous references to Diagum CS™ in the notification, such as in the discussion of toxicity tests performed on Noveon's cassia gum in the 1980s and early 1990s. Noveon intends to market only Diagum SR™ for use in human food applications. Diagum CS™ is marketed only for pet food use.

The production of Diagum SR™ from cassia splits is described in SOP-IP XX 004 (App. II, Tab 3)—other than the recently implemented final step of soaking the cassia flour in water and extracting it with a mixture of isopropyl alcohol water extraction. The overall production process involves four main steps: (1) final cleaning of the splits; (2) washing, soaking, squeezing and drying of the flakes; (3) milling; and (4) blending of a batch and final pack. At the completion of these steps (other than final pack) the gum is subjected to an extraction step in which moisturized cassia is dispersed in isopropanol/water mixtures, followed by filtration. More specifically, the extraction step proceeds as follows: cassia gum flour is soaked in water, which causes the gum to swell. The swollen gum is introduced into a mixture of water and isopropyl alcohol while stirring. The wet gum is then separated from the isopropyl alcohol/water solution by filtration, dried and packaged. The drying process drives off residual isopropyl

alcohol in the gum along with water (isopropyl alcohol boils at approximately 180°F), allowing Noveon to establish a suitably low specification for isopropyl alcohol of 0.075%, which is the same level as is allowed in several food additive clearances for gums. Certificates of Analysis for five batches of Diagum SR™ demonstrating compliance with the isopropyl alcohol specification are included in Appendix II at Tab 5.

2.5 Specifications for Cassia Gum and Related Galactomannans

Cassia gum is related to locust (carob) bean gum and guar gum in terms of structure and chemical properties, although in terms of functionality cassia is more comparable to locust bean gum than to guar gum. The specifications and purity of cassia gum and related gums are listed in Table 2.3.

Cassia galactomannan can be differentiated from other galactomannans by electrophoresis. Based on the molecular weights of the structurally related polygalactomannans, guar gum and locust (carob) bean gum (Klose and Glicksman, 1968; Leung and Foster, 1996), the molecular weight of cassia gum is estimated to be between 200,000 and 300,000 (Denkler, 1997). The specifications of cassia gum are reported in Table 2.3.

Table 2.3
Specifications and Purity of Cassia Gum and Related Gums for Use in Foods

	Cassia Gum	Locust (Carob) Bean Gum[9]	Guar Gum[10]
	Proposed Limits	FCC Limits	FCC Limits
Galactomannan	≥70%	≥75%	≥70%
Moisture	≤12%	NR[11]	NR
Ash	≤1.2%	≤1.2%	≤1.5%
Proteins	≤7%	≤7%	≤10%
Fat	≤2%	NR	NR
Total anthraquinones	max. 10 ppm	NR	NR
Heavy Metals:	≤0.002%	≤0.002%	≤0.002%
Lead	≤5 mg Pb/kg	≤5 mg Pb/kg	≤5 mg Pb/kg
Arsenic	≤3 mg As/kg	≤3 mg As/kg	≤3 mg As/kg

9 Food Chemicals Codex, 1996.

10 Food Chemicals Codex, 1996.

11 "NR" means not reported.

Isopropyl alcohol	max. 0.075%[12]	NR	NR

2.6 Anthraquinone Impurities in Cassia Gum

As indicated above, the primary impurities of concern in cassia gum are anthraquinones, which occur naturally in the seeds from which cassia gum is produced. Information on the levels at which anthraquinones typically are present in the whole seed and in the finished gum is provided in the following sections.

2.6.1 Levels of anthraquinones in *Cassia obtusifolia* seed

Anthraquinones and related compounds have been known for years to occur in many *Cassia* species (Takahashi *et al.*, 1978; Takido, 1958a, b 1960). In 1978, Koshioka and Takino developed a quantitative method for measuring anthraquinones in cassia seeds based on optical absorbance at 500 nm (Koshioka and Takino, 1978).[13] Using this method, Japanese and Formosan samples of *C. obtusifolia* and *C. tora* seeds were found to contain anthraquinones at levels ranging from 0.80% to 1.10%. These authors also measured free, bound, and total anthraquinones in 14 samples of powdered seeds of *C. obtusifolia* or *C. tora* used as crude drugs from Korea, China, Japan, Formosa and Vietnam. The results ranged from 0.01 to 0.04% for free anthraquinones, 1.01 to 1.29% for bound anthraquinones, and 1.04 to 1.31 % for total anthraquinones.

It is well established that most of the anthraquinones in cassia are bound to sugars in the form of glycosides (with the aglycon occurring usually in a reduced form, e.g., anthrones, dianthrones, or oxanthrone). Using thin-layer chromatography, Crawford *et al.*, showed that sicklepod seeds contained 1-2% by weight of total anthraquinones. No alkaloids or saponins were present. The major portion of the anthraquinones exist as the di- or triglycosides. These include rhein, physcion, obtusifolin, emodin, questin, lesser amounts of others, and chrysophanic acid. Chrysophanic acid made up virtually 50% of the total anthraquinones in the seed samples (Crawford *et al.*, 1990). Several authors have confirmed the 1990 work by Crawford *et al.*

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[12] This specification is identical to the isopropyl alcohol limit in the food additive regulations for xanthan gum (21 C.F.R. § 172.695) and gellan gum (21 C.F.R. § 172.665).

[13] Koshioka M. and Takino Y. (1978) Studies on the evaluation of crude drug I. Quantitative estimation of anthraquinones in cassia seeds. *Chem. Pharm. Bull.* 25(5) 1345-49.

2.6.2 Levels of anthraquinones in Diagum SR™

The bulk of the anthraquinone content in cassia seeds is located in the outer husk. In the Diagum SR™ production process, the endosperm is separated from the husk and germ and purified in a series of steps culminating in an isopropanol/water extraction step. The de-husking that occurs early in the production process separates and discards the outer husks from the endosperm, resulting in a major reduction in the anthraquinone content. Both the concentration and number of anthraquinones present are reduced. The isopropanol/water extraction at the end of the production process further reduces the anthraquinone content of the gum. While the average anthraquinone content in whole seeds is in the range of at least 10,000 ppm, that in the Diagum SR™ as determined by the current HPLC method is no more than 10 ppm, as discussed below.

Prior to adoption of the HPLC method, Noveon determined total anthraquinone levels in cassia gum via the optical color analytical method of Koshioka and Takino (1978) for total anthraquinones. This method is based on the optical absorbance of the sample at a wavelength of 500 nm. Because the cassia complex mixture may include substances in addition to anthraquinones that absorb at 500 nm, the colorimetric procedure tends to exaggerate the actual anthraquinone content.

The maximum specification for anthraquinones in finished Diagum SR™ is now established at 10 ppm. This reduced maximum level reflects recent improvements in purification techniques as well as analytical methodology. The primary reason Noveon is able to achieve anthraquinone levels of 10 ppm is due to utilization of its novel isopropanol/water extraction process at the completion of the process for making pet food grade cassia gum (Diagum CS™). A key factor in documenting the reductions in anthraquinone levels is the use of a new analytical procedure based on HPLC analysis conducted by Dr. W. Dekant at the University of Würzburg, Germany.

The new method allows for the chromatographic separation and quantification of the individual anthraquinones present in the sample. Based upon testing employing the new method with a detection limit of 0.5 ppm, the only anthraquinone detected in Diagum SR™ is physcion (1,8-dihydroxy-3-methyl-6-methoxyanthraquinone), at less than 10 ppm. Furthermore, in most HPLC analyses at a 0.5 ppm detection limit, no anthraquinone was detected. Physcion was detected in two HPLC analyses of a single sample of Diagum SR™ (before and after acid

hydrolysis) performed by Dr. Dekant in July 2003. These analyses of this sample found 5.20 ppm physcion without acid hydrolysis and 8.59 ppm after hydrolysis. See "Comparison of Diagum CS and Diagum SR", App. II, Tab 9. Following these results, Noveon incorporated an additional washing step into the preparation of samples of Diagum SR™ as well as into the process to be used for the commercial production of the gum. Dr. Dekant analyzed five samples of the additionally cleaned gum post-acid hydrolysis using the same procedure and analytical sensitivity as in the July tests and found no detectable anthraquinones at a detection limit of 0.5 ppm. See Dekant, "Identification and Quantification of Anthraquinones in Cassia Gum" (Dekant 2003), App. II, Tab 7. Collectively, these analyses demonstrate that the current purification process effectively removes virtually all of the anthraquinone content initially present in cassia seeds.

When the new analytical method is applied to the *C. obtusifolia* seeds—rather than the purified product Diagum SR™—the full complement of anthraquinones (chrysophanic acid, emodin, questin, obtusifolin, etc.) are observed in amounts consistent with Crawford *et al.*, 1990. (App. II, Tab 7.) This confirms the sensitivity of the analytical method to the full range of anthraquinones that are potentially present based on their having been detected in *C. obtusifolia* seeds.

Included with this notification are certificates of analysis for six batches of Diagum SR™ confirming compliance with the 10 ppm specification for finished cassia gum (App. II, Tab 6) based upon testing using the HPLC method.[14] Copies of the HPLC method and method validation for measurement of anthraquinones in Diagum SR™ are also attached (App. II, Tab 1) as well as the aforementioned report of Dr. Dekant on his analytical work to identify and quantify the anthraquinones in Diagum SR™ as well as Diagum CS™ (App. II, Tab 7).

3. Intended Use

Plant-derived colloidal polysaccharides are excellent stabilizing and thickening agents and are common components of processed foods. Cassia gum swells in water and forms high-viscosity aqueous colloids after it is boiled. When it is used in aqueous solution, potentially in

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[14] Although, as explained above, the laboratory samples of Diagum SR™ produced and purified according to current procedures have shown no detectable anthraquinones at a 0.5 ppm detection limit, Noveon has established a specification of 10 ppm for total anthraquinones to account for the possibility of variations in anthraquinone content following scale-up.

combination with other gums, it results in the formation of an aqueous gel that has useful food applications. It is used as a thickening and gelling agent in pet food. The applications for which cassia gum is deemed by Noveon to be GRAS are listed in the table set forth in Section 1.1 of this notification. These applications include the use of cassia gum to improve water retention in certain baked goods (including cheese-crème filling in pastries and other cheese-crème filled desserts); as a thickening agent for dry soup mixes and selected oil-free salad dressings; and to improve texture and water retention in sausages, corned beef, and canned poultry meats. Cassia gum will be used in these applications at levels ranging from 2000 ppm to 5000 ppm.

4. Evidence of Self-Limiting Levels of Use

This notification asserts the GRAS status of cassia gum for use in food subject to the maximum concentrations shown in the table set forth in Section 1.1 above. Therefore, information on self-limiting levels of use is not necessary.

5. Estimated Daily Intake (EDI)

5.1 EDI for Cassia Gum

To assess the risk associated with long-term exposure to a food additive or contaminant, FDA recommends use of multiple-day food consumption data.[15] FDA assumes that data on consumption of a variety of foods over a multi-day period better reflects the probable eating pattern of a population over a long period of time. This procedure is particularly important when the food containing the additive is consumed infrequently in specific foods as is the case with cassia gum. The currently available database from the USDA's Continuing Survey of Food Intakes by Individuals (CSFII) 1994-96, 98 includes detailed food consumption data collected on two non-consecutive days and provides estimates of daily intake that thus represent "2-day averages." For example, if an individual reported consumption of 100 grams of a specific food on day 1 and 0 grams of this food on day 2, the 2-day average consumption for this individual would be 50 grams [that is, (100+0) ÷ 2]. For a food that is likely to be eaten only once or twice a month, this 2-day average can give a highly inflated estimate of lifetime daily intake. It is well known that food consumption data collected over longer periods of time (e.g., 14 days as in the

[15] FDA Guidance for Estimating Exposure to Direct Additives and Chemical Contaminants in the Diet, U.S. FDA, Center for Food Safety and Applied Nutrition, OPA, November, 1995.

now unavailable MRCA[16] consumer surveys) yield estimates of daily intake that may be significantly lower than 2-day averages.

For this reason, the Notifier arranged for a more accurate estimate of long-term intake to be determined by Exponent (formerly Novigen Sciences) using the the National Health and Nutrition Examination Survey (NHANES III) and a list of food products in which Diagum SR™ is intended to be used. The NHANES III is a 30-day survey that includes food frequency data (FF) for 60 categories of foods. The Exponent report on cassia gum intake is attached. (See Appendix III.)

The data set forth in Table 4 of the Exponent report are reproduced in Table 5.1 below. The table compares average total daily intake of cassia gum, both for consumers only and for the entire population (i.e., *per capita*), from all the selected foods, using three different methods of estimation. Using the CSFII data alone, the 2-day average daily intake for consumers of cassia gum, from all foods, is 1.7 mg/kg bw/day at the mean, and 3.7 mg/kg bw/day at the 90th percentile. Upon incorporating the food frequency data from NHANES III, the 30-day average daily intake for consumers drops to 1.0 mg/kg bw/day at the mean and 2.1 mg/kg bw/day at the 90th percentile. The latter values are 1.7 times and 1.8 times lower than using CSFII survey data alone for the mean and 90th percentile, respectively.

A third approach to estimating intake includes a correction factor to account for the overestimation in frequency of consumption of specific foods caused by using frequency data for the relatively broad categories included in the NHANES database. Based on the minimum frequency overestimation (corresponding to a correction factor of 3.5, as explained in Appendix III), the 30-day average daily intake for consumers is further reduced to 0.3 mg/kg bw/day at the mean and 0.6 mg/kg bw/day at the 90th percentile.

Based on this analysis, as shown in Table 5.1 below, it is reasonable to conclude that the long-term average daily intake of cassia gum from the specific foods of interest is lower then the 2-day average for the broad categories by a minimum factor of 6 times. Note that we have assumed 100% market share for the proposed uses of this ingredient. In reality, lifetime market share would be much lower. Thus, long-term consumption of cassia gum is expected to be well below the values calculated here.

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16 MRCA stands for Market Research Corporation of America.

Table 5.1 Estimated Total Daily Intake of Gum from Selected Foods (mg/kg bw/day)						
	2-Day Average Intake (Using CSFII Only)		Adjusted for NHANES 30-day average daily intake[17]		Adjusted for NHANES 30-day average daily intake and minimum overestimation based on food categories [18]	
	Per Capita	Per User	Per Capita	Per User	Per Capita	Per User
Mean	0.4	1.7	0.2	1.0	0.06	0.3
90th percentile	1.3	3.7	0.7	2.1	0.2	0.6

5.2 EDI for Anthraquinones

As reported above, the maximum concentration of total anthraquinones allowed under the current specification is 10 ppm, or 10×10^{-6} mg anthraquinone per mg of Diagum SR™. On this basis, intake of anthraquinones resulting from consumption of cassia gum may be conservatively calculated for the mean consumer as follows:

$$(0.3 \text{ mg gum/kg bw/day}) \times (10 \times 10^{-6} \text{ mg AQ/mg gum}) = 3 \times 10^{-6} \text{ mg AQ/kg bw/day}$$

Similarly, intake of anthraquinones by the 90th percentile consumer is calculated as follows:

$$(0.6 \text{ mg gum/kg bw/day}) \times (10 \times 10^{-6} \text{ mg AQ/mg gum}) = 6 \times 10^{-6} \text{ mg AQ/kg bw/day}$$

The safety of this exposure to the anthraquinone impurities in cassia gum is discussed in Sections 6.2 and 6.3 below.

6. Detailed Summary of Basis for Notifier's GRAS Determination

The safety of cassia gum as an ingredient in food has been established by a wide range of toxicological studies, including short-term, subchronic, reproductive, teratogenic, and genetic toxicity studies. These studies are discussed in detail below.

As discussed previously, cassia gum may be characterized as a polysaccharide consisting primarily of a linear chain of 1,4-β-D-mannopyranose units with 1,6 linked α-D galactopyranose units. The ratio of mannose to galactose is 5:1. Cassia gum is closely related in structure and

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17 See Tables 1 and 2 of Exponent report (Appendix III).

18 See Table 3 of Exponent report.

composition to several gums that are used in food, including locust (carob) bean gum, tara gum, and guar gum, all of which are galactomannans. The gum most closely related in structure to cassia gum is locust (carob) bean, which has a mannose-to-galactose ratio of 4:1.

Locust (carob) bean gum has been affirmed by the Food and Drug Administration to be generally recognized as safe for use in food up to a maximum level of 0.75% (21 C.F.R. § 184.1343). Guar gum also is affirmed GRAS by FDA for use in food up to a maximum level of 2% (21 C.F.R. § 184.1339). Both gums are used in various foods including baked goods, cheese, and jams/jellies. It is also worth noting that gum arabic, gum ghatti, guar gum, locust bean gum, sterculia gum, and gum tragacanth all are considered GRAS by FDA for use in animal feeds (21 C.F.R. § 582).

Locust (carob) bean gum is also approved for use as a thickening agent in Europe (EEC No. E 410). The establishment of acceptable daily intakes (ADIs) for locust (carob) bean gum and guar gum was deemed unnecessary by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) because the available data indicate that locust (carob) bean gum and guar gum do not represent a health hazard. As for cassia gum, itself, an application has also been submitted for the authorization of cassia gum as a food additive in the European Union.

Considering its close structural relationship to several gums that have been affirmed GRAS by FDA, there is no basis upon which to expect that the polysaccharide in cassia gum would have a toxicological profile any different from that of these GRAS gums. The testing that has been conducted previously on such gums as guar, tara, and locust (carob) bean gum and that formed the basis of the previous safety assessments for these ingredients also supports the safety of the galactomannan that comprises cassia gum. (Hallagan *et al.*, 1997.) The toxicological studies on cassia gum described below further corroborate the safety of cassia gum as a food ingredient. (Hallagan *et al.*, 1997.[19])

In addition, a large amount of data exists in the public, peer-reviewed literature with regard to the toxicity of *C. obtusifolia*, the seeds of which plant are used to produce cassia gum. As discussed more fully in Section 6.2 below, these studies allow for the determination of a no-observed adverse effect level for the toxic principle in cassia and, in turn, the calculation of an

[19] With the exception of recently conducted Ames tests, all of the toxicological studies on cassia gum discussed herein were evaluated in the peer-reviewed literature. See Hallagan *et al.*, 1997. The Hallagan article does not address the issues of the muscle toxicity or potential carcinogenicity of impurities in cassia gum.

acceptable daily intake for the same. The safety assessment presented below demonstrates a wide margin of safety based on the published toxicity data.

Finally, a quantitative risk assessment is presented here with regard to the anthraquinone impurities potentially present in cassia gum.[20] The risk assessment is based on the results of published carcinogenicity bioassays on 9,10-anthraquinone and on three hydroxy-substituted anthraquinones. The calculations presented in Section 6.3 below demonstrate that the anthraquinones found in cassia gum present only a negligible risk and therefore may be considered safe to a reasonable certainty.

FDA's GRAS notification proposal states that a GRAS substance is distinguished from a food additive on the basis of the common knowledge about the safety of the substance for its intended use. The Agency has stated further that there are two aspects to this common knowledge. First, the data and information relied on to establish safety must be generally available. Second, there must be a basis on which to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The Notifier has concluded that both of these elements of "common knowledge" are satisfied with respect to cassia gum. The general availability, or publication, element is clearly met by the published data regarding *C. obtusifolia* and the anthraquinone impurities in cassia, as well as the widely available information on the closely related GRAS gums. Moreover, the evaluations by JECFA of these closely related gums provide a strong basis on which to conclude that there is a consensus among qualified experts about the safety of such substances for the intended uses. This consensus is further suggested by the European approval for locust (carob) bean gum.

Based on the entirety of the available information, the Notifier has concluded that cassia gum is generally recognized as safe for the intended use described here.

The detailed safety assessment for cassia gum is set forth below.

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[20] As noted above (section 2.6.2), physcion is the only anthraquinone actually detected in any analyses of Diagum SR™. Other anthraquinones potentially present in Diagum SR™ below the 0.5 ppm detection limit of the analyses of the gum would be those that have been identified in *C. obtusifolia* seeds, i.e., rhein, obtusifolin, emodin, questin, chrysophanic acid and lesser amounts of others (Crawford *et al.*, 1990).

6.1 Biological Studies on Cassia Gum

The initial series of laboratory studies conducted on Diagum CS™ by the Noveon Company (then BF Goodrich), prior to the development of Diagum SR™, were designed to determine the toxicological properties of the gum and were not focused on potential impurities. At the time, a less refined analytical technique indicated that anthraquinones were present in the gum at 150 ppm.[21] Testing by the more recent HPLC method later showed that no more than 70 ppm anthraquinones were present in the gum used in the feeding studies. At the time, anthraquinones were not considered toxic at the levels present and the studies did not detect any toxicity due to them. Subsequently, attention has been given to the toxicity and potential carcinogenicity of the anthraquinones themselves. The studies on cassia gum (Diagum CS™), which were conducted in the 1980s and 1990s, are discussed in this section. The following sections will address the safety of the anthraquinone impurities. Specifically, Section 6.2 will discuss muscle toxicity and Section 6.3 will discuss the potential for mutagenicity and carcinogenicity of the anthraquinone impurities in cassia gum as currently produced (Diagum SR™).

6.1.1 Cassia gum test sample

The toxicological studies described below were conducted on Diagum CS™ test samples. These samples contain approximately 70 ppm total anthraquinones. As noted under Section 2.4, Method of Manufacture, Noveon has recently implemented an isopropanol extraction purification step for Diagum SR™ that substantially reduces the total anthraquinones compared to the Diagum CS™ product fed in the animal studies, i.e., anthraquinones are reduced from 70 ppm in Diagum CS™ to 10 ppm in Diagum SR™—well below the level at which anthraquinones have been found to induce muscle toxicity in animal studies (see discussion of muscle toxicity in Section 6.2). Because the addition of this purification step improved the quality of the finished product, the test sample is believed to represent the worst case for toxicological assessment purposes.

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[21] As discussed in section 2.6.2, prior to adoption of the HPLC method, Noveon determined total anthraquinone levels in cassia gum via the optical color analytical method of Koshioka and Takino (1978). This colorimetric method tends to exaggerate anthraquinone content because the method is based on the optical absorbance of the sample at a wavelength of 500 nm and substances other than anthraquinones absorb at that wavelength.

combination with other gums, it results in the formation of an aqueous gel that has useful food applications. It is used as a thickening and gelling agent in pet food. The applications for which cassia gum is deemed by Noveon to be GRAS are listed in the table set forth in Section 1.1 of this notification. These applications include the use of cassia gum to improve water retention in certain baked goods (including cheese-crème filling in pastries and other cheese-crème filled desserts); as a thickening agent for dry soup mixes and selected oil-free salad dressings; and to improve texture and water retention in sausages, corned beef, and canned poultry meats. Cassia gum will be used in these applications at levels ranging from 2000 ppm to 5000 ppm.

4. Evidence of Self-Limiting Levels of Use

This notification asserts the GRAS status of cassia gum for use in food subject to the maximum concentrations shown in the table set forth in Section 1.1 above. Therefore, information on self-limiting levels of use is not necessary.

5. Estimated Daily Intake (EDI)

5.1 EDI for Cassia Gum

To assess the risk associated with long-term exposure to a food additive or contaminant, FDA recommends use of multiple-day food consumption data.[15] FDA assumes that data on consumption of a variety of foods over a multi-day period better reflects the probable eating pattern of a population over a long period of time. This procedure is particularly important when the food containing the additive is consumed infrequently in specific foods as is the case with cassia gum. The currently available database from the USDA's Continuing Survey of Food Intakes by Individuals (CSFII) 1994-96, 98 includes detailed food consumption data collected on two non-consecutive days and provides estimates of daily intake that thus represent "2-day averages." For example, if an individual reported consumption of 100 grams of a specific food on day 1 and 0 grams of this food on day 2, the 2-day average consumption for this individual would be 50 grams [that is, $(100+0) \div 2$]. For a food that is likely to be eaten only once or twice a month, this 2-day average can give a highly inflated estimate of lifetime daily intake. It is well known that food consumption data collected over longer periods of time (e.g., 14 days as in the

[15] FDA Guidance for Estimating Exposure to Direct Additives and Chemical Contaminants in the Diet, U.S. FDA, Center for Food Safety and Applied Nutrition, OPA, November, 1995.

now unavailable MRCA[16] consumer surveys) yield estimates of daily intake that may be significantly lower than 2-day averages.

For this reason, the Notifier arranged for a more accurate estimate of long-term intake to be determined by Exponent (formerly Novigen Sciences) using the the National Health and Nutrition Examination Survey (NHANES III) and a list of food products in which Diagum SR™ is intended to be used. The NHANES III is a 30-day survey that includes food frequency data (FF) for 60 categories of foods. The Exponent report on cassia gum intake is attached. (See Appendix III.)

The data set forth in Table 4 of the Exponent report are reproduced in Table 5.1 below. The table compares average total daily intake of cassia gum, both for consumers only and for the entire population (i.e., *per capita*), from all the selected foods, using three different methods of estimation. Using the CSFII data alone, the 2-day average daily intake for consumers of cassia gum, from all foods, is 1.7 mg/kg bw/day at the mean, and 3.7 mg/kg bw/day at the 90th percentile. Upon incorporating the food frequency data from NHANES III, the 30-day average daily intake for consumers drops to 1.0 mg/kg bw/day at the mean and 2.1 mg/kg bw/day at the 90th percentile. The latter values are 1.7 times and 1.8 times lower than using CSFII survey data alone for the mean and 90th percentile, respectively.

A third approach to estimating intake includes a correction factor to account for the overestimation in frequency of consumption of specific foods caused by using frequency data for the relatively broad categories included in the NHANES database. Based on the minimum frequency overestimation (corresponding to a correction factor of 3.5, as explained in Appendix III), the 30-day average daily intake for consumers is further reduced to 0.3 mg/kg bw/day at the mean and 0.6 mg/kg bw/day at the 90th percentile.

Based on this analysis, as shown in Table 5.1 below, it is reasonable to conclude that the long-term average daily intake of cassia gum from the specific foods of interest is lower then the 2-day average for the broad categories by a minimum factor of 6 times. Note that we have assumed 100% market share for the proposed uses of this ingredient. In reality, lifetime market share would be much lower. Thus, long-term consumption of cassia gum is expected to be well below the values calculated here.

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16 MRCA stands for Market Research Corporation of America.

Table 5.1 Estimated Total Daily Intake of Gum from Selected Foods (mg/kg bw/day)						
	2-Day Average Intake (Using CSFII Only)		Adjusted for NHANES 30-day average daily intake[17]		Adjusted for NHANES 30-day average daily intake and minimum overestimation based on food categories [18]	
	Per Capita	Per User	Per Capita	Per User	Per Capita	Per User
Mean	0.4	1.7	0.2	1.0	0.06	0.3
90th percentile	1.3	3.7	0.7	2.1	0.2	0.6

5.2 EDI for Anthraquinones

As reported above, the maximum concentration of total anthraquinones allowed under the current specification is 10 ppm, or 10×10^{-6} mg anthraquinone per mg of Diagum SR™. On this basis, intake of anthraquinones resulting from consumption of cassia gum may be conservatively calculated for the mean consumer as follows:

$$(0.3 \text{ mg gum/kg bw/day}) \times (10 \times 10^{-6} \text{ mg AQ/mg gum}) = 3 \times 10^{-6} \text{ mg AQ/kg bw/day}$$

Similarly, intake of anthraquinones by the 90th percentile consumer is calculated as follows:

$$(0.6 \text{ mg gum/kg bw/day}) \times (10 \times 10^{-6} \text{ mg AQ/mg gum}) = 6 \times 10^{-6} \text{ mg AQ/kg bw/day}$$

The safety of this exposure to the anthraquinone impurities in cassia gum is discussed in Sections 6.2 and 6.3 below.

6. Detailed Summary of Basis for Notifier's GRAS Determination

The safety of cassia gum as an ingredient in food has been established by a wide range of toxicological studies, including short-term, subchronic, reproductive, teratogenic, and genetic toxicity studies. These studies are discussed in detail below.

As discussed previously, cassia gum may be characterized as a polysaccharide consisting primarily of a linear chain of 1,4-β-D-mannopyranose units with 1,6 linked α-D galactopyranose units. The ratio of mannose to galactose is 5:1. Cassia gum is closely related in structure and

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17 See Tables 1 and 2 of Exponent report (Appendix III).

18 See Table 3 of Exponent report.

composition to several gums that are used in food, including locust (carob) bean gum, tara gum, and guar gum, all of which are galactomannans. The gum most closely related in structure to cassia gum is locust (carob) bean, which has a mannose-to-galactose ratio of 4:1.

Locust (carob) bean gum has been affirmed by the Food and Drug Administration to be generally recognized as safe for use in food up to a maximum level of 0.75% (21 C.F.R. § 184.1343). Guar gum also is affirmed GRAS by FDA for use in food up to a maximum level of 2% (21 C.F.R. § 184.1339). Both gums are used in various foods including baked goods, cheese, and jams/jellies. It is also worth noting that gum arabic, gum ghatti, guar gum, locust bean gum, sterculia gum, and gum tragacanth all are considered GRAS by FDA for use in animal feeds (21 C.F.R. § 582).

Locust (carob) bean gum is also approved for use as a thickening agent in Europe (EEC No. E 410). The establishment of acceptable daily intakes (ADIs) for locust (carob) bean gum and guar gum was deemed unnecessary by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) because the available data indicate that locust (carob) bean gum and guar gum do not represent a health hazard. As for cassia gum, itself, an application has also been submitted for the authorization of cassia gum as a food additive in the European Union.

Considering its close structural relationship to several gums that have been affirmed GRAS by FDA, there is no basis upon which to expect that the polysaccharide in cassia gum would have a toxicological profile any different from that of these GRAS gums. The testing that has been conducted previously on such gums as guar, tara, and locust (carob) bean gum and that formed the basis of the previous safety assessments for these ingredients also supports the safety of the galactomannan that comprises cassia gum. (Hallagan *et al.*, 1997.) The toxicological studies on cassia gum described below further corroborate the safety of cassia gum as a food ingredient. (Hallagan *et al.*, 1997.[19])

In addition, a large amount of data exists in the public, peer-reviewed literature with regard to the toxicity of *C. obtusifolia*, the seeds of which plant are used to produce cassia gum. As discussed more fully in Section 6.2 below, these studies allow for the determination of a no-observed adverse effect level for the toxic principle in cassia and, in turn, the calculation of an

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[19] With the exception of recently conducted Ames tests, all of the toxicological studies on cassia gum discussed herein were evaluated in the peer-reviewed literature. See Hallagan *et al.*, 1997. The Hallagan article does not address the issues of the muscle toxicity or potential carcinogenicity of impurities in cassia gum.

acceptable daily intake for the same. The safety assessment presented below demonstrates a wide margin of safety based on the published toxicity data.

Finally, a quantitative risk assessment is presented here with regard to the anthraquinone impurities potentially present in cassia gum.[20] The risk assessment is based on the results of published carcinogenicity bioassays on 9,10-anthraquinone and on three hydroxy-substituted anthraquinones. The calculations presented in Section 6.3 below demonstrate that the anthraquinones found in cassia gum present only a negligible risk and therefore may be considered safe to a reasonable certainty.

FDA's GRAS notification proposal states that a GRAS substance is distinguished from a food additive on the basis of the common knowledge about the safety of the substance for its intended use. The Agency has stated further that there are two aspects to this common knowledge. First, the data and information relied on to establish safety must be generally available. Second, there must be a basis on which to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The Notifier has concluded that both of these elements of "common knowledge" are satisfied with respect to cassia gum. The general availability, or publication, element is clearly met by the published data regarding *C. obtusifolia* and the anthraquinone impurities in cassia, as well as the widely available information on the closely related GRAS gums. Moreover, the evaluations by JECFA of these closely related gums provide a strong basis on which to conclude that there is a consensus among qualified experts about the safety of such substances for the intended uses. This consensus is further suggested by the European approval for locust (carob) bean gum.

Based on the entirety of the available information, the Notifier has concluded that cassia gum is generally recognized as safe for the intended use described here.

The detailed safety assessment for cassia gum is set forth below.

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[20] As noted above (section 2.6.2), physcion is the only anthraquinone actually detected in any analyses of Diagum SR™. Other anthraquinones potentially present in Diagum SR™ below the 0.5 ppm detection limit of the analyses of the gum would be those that have been identified in *C. obtusifolia* seeds, i.e., rhein, obtusifolin, emodin, questin, chrysophanic acid and lesser amounts of others (Crawford *et al.*, 1990).

6.1 Biological Studies on Cassia Gum

The initial series of laboratory studies conducted on Diagum CS™ by the Noveon Company (then BF Goodrich), prior to the development of Diagum SR™, were designed to determine the toxicological properties of the gum and were not focused on potential impurities. At the time, a less refined analytical technique indicated that anthraquinones were present in the gum at 150 ppm.[21] Testing by the more recent HPLC method later showed that no more than 70 ppm anthraquinones were present in the gum used in the feeding studies. At the time, anthraquinones were not considered toxic at the levels present and the studies did not detect any toxicity due to them. Subsequently, attention has been given to the toxicity and potential carcinogenicity of the anthraquinones themselves. The studies on cassia gum (Diagum CS™), which were conducted in the 1980s and 1990s, are discussed in this section. The following sections will address the safety of the anthraquinone impurities. Specifically, Section 6.2 will discuss muscle toxicity and Section 6.3 will discuss the potential for mutagenicity and carcinogenicity of the anthraquinone impurities in cassia gum as currently produced (Diagum SR™).

6.1.1 Cassia gum test sample

The toxicological studies described below were conducted on Diagum CS™ test samples. These samples contain approximately 70 ppm total anthraquinones. As noted under Section 2.4, Method of Manufacture, Noveon has recently implemented an isopropanol extraction purification step for Diagum SR™ that substantially reduces the total anthraquinones compared to the Diagum CS™ product fed in the animal studies, i.e., anthraquinones are reduced from 70 ppm in Diagum CS™ to 10 ppm in Diagum SR™—well below the level at which anthraquinones have been found to induce muscle toxicity in animal studies (see discussion of muscle toxicity in Section 6.2). Because the addition of this purification step improved the quality of the finished product, the test sample is believed to represent the worst case for toxicological assessment purposes.

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[21] As discussed in section 2.6.2, prior to adoption of the HPLC method, Noveon determined total anthraquinone levels in cassia gum via the optical color analytical method of Koshioka and Takino (1978). This colorimetric method tends to exaggerate anthraquinone content because the method is based on the optical absorbance of the sample at a wavelength of 500 nm and substances other than anthraquinones absorb at that wavelength.

6.1.2 Absorption, distribution, metabolism and excretion

As indicated in Section 2.0, Chemical Identity, cassia gum is comprised of at least 75% polysaccharide, consisting primarily of a linear chain of 1,4-β-D-mannopyranose units with 1,6-linked α-D linked galactopyranose units. The chemical and structural properties of cassia gum, compared to guar gum, locust (carob) bean gum, and tara gum, are presented in Table 2.1 of Section 2.3. The major structural difference between the gums is the ratio of mannose to galactose in the galactomannan. The mannose:galactose ratio increases from 2:1 for guar gum, to 3:1 for tara gum, to 4:1 for carob, to 5:1 for cassia gum. The molecular weight for each of the galactomannans is similar, in the range of 200,000 –310,000 daltons. It is difficult to speculate even theoretically why there might be any difference in the animal or human disposition of these very closely related gums. Galactomannans are recognized as components of dietary fiber and are resistant to digestive enzymes in the gastrointestinal tract. This does not depend upon the specific mannose:galactose ratio (Trowell *et al.*, 1976). Galactomannans were shown to be stable in artificial gastric juice (Nurnberg and Bleimuller, 1981).

The biodisposition of cassia gum has not been studied, but is expected to be similar to structurally related galactomannans, including locust (carob) bean gum and guar gum, for which studies have been reported. The differences among the mannose:galactose ratio in cassia and other gums alter the physical properties, e.g., viscosity, and electrophoretic mobility, gel forming ability, of the gums, but do not measurably affect their digestibility and toxicology. Several nutritional and bioavailability and toxicology studies have been conducted in animals using carob bean gum, the closest in structure to cassia gum.

In vitro tests with human enzyme preparations show little utilization of carob gum by the gut (Semenza, 1975). In a bioavailable calorie assay, groups of weanling rats were given 5 g of basal diet plus sucrose or carob gum for 10 days (Robaislek, 1974). Comparison of the carcass weight gain showed that carob bean gum was not a source of bioavailable calories. The same observations were made with guar gum and tara gum (Robaislek, 1974). A digestibility study in rats showed that 85-100% of the mannose fed as 1% carob bean gum for 18 hours was excreted in the feces (Tsai and Whistler, 1975). The effects noted with carob, guar, and tara in feeding trials in animals at high doses (~5%) are those expected of a non-metabolized polymeric substance acting as a bulking agent. JECFA has also reviewed the available work in several reports on carob bean gum, tara gum and guar gum extending back to 1969 (JECFA, 1981).

In a digestibility study, groups of five male and five female rats (Purdue strain) were maintained on a mannose-free diet and provided 1% locust (carob) bean gum. Eighty-five to 100% of the dose was excreted as mannose (Tsai and Whistler, 1975). No evidence of hydrolysis was observed when locust (carob) bean gum (in solution or suspension) was incubated with human gastric juice, duodenal juice + bile, pancreatic juice, and *Succus entericus* with or without added rabbit small gut membrane enzymes (Semenza, 1975).

Ten percent (w/w) aqueous solution of guar gum was shown to decrease the rate of gastric emptying of rats (Shah *et al.*, 1986). It was suggested that 5% aqueous solution guar gum decreased the efficiency of nutrient digestion and absorption, and increased secretion of digestive juices in rats (Ikegami *et al.*, 1990). Guar gum at a dose of 100 g/kg was fed to rats as part of a basal diet. Only 4% was excreted in the feces (primarily as rhamnose and arabanose) indicating that guar gum was almost completely fermented (Nyman and Asp, 1982).

It is expected that cassia gum would be excreted unchanged. Fermentation of cassia gum by gut microflora may occur to a small extent (Nyman and Asp, 1982). Any hydrolyzed material would be absorbed and metabolized in normal biochemical pathways (Mathews and van Holde, 1990).

Nutrient interaction - A single study suggests that prolonged consumption of very high doses (>5%) of indigestible polysaccharides may lead to significant adaptive changes in the digestive system. The doses have to be high enough to increase the bulk and viscosity of the intestinal contents to the point that effective interaction of substrates and enzymes with mucosal surfaces is hindered. (Ikegami *et al.*, 1990.) JECFA has not limited the intake levels on any of the related gums for use in food (1981-TRS-653 JECFA 25/28; 1986 -TRS 751-JECFA 30/27; 1975- NMRS 55/TRS 576-JECFA 19/14). In the present instance, the dose of cassia gum proposed is orders of magnitude below the reported adaptive effects and would contribute only a fraction of the total intake of gums.

6.1.3 Acute oral toxicity

Cassia gum exhibits very low acute toxicity. The oral (gavage) LD_{50} value of cassia gum was reported to be >5,000 mg/kg bw in male rats (Schering, 1986). No adverse effects were reported when five volunteers ingested 1 gram of the structurally related substance guar flour in a capsule per day for 10 days (Krantz *et al.*, 1948).

6.1.4 Repeated dose toxicity

Cassia gum as a dietary admixture is well tolerated in animals and did not produce evidence of toxicity in three repeated dose studies in dogs, cats and rats. A decrease in food consumption was commonly observed and is attributed to the absorption of water by cassia gum within the gastrointestinal tract and subsequent increase in volume and bulk. The three studies summarized below were conducted according to the Organization for Economic Cooperation and Development Principles of Good Laboratory Practices (OECD, 1981) and were inspected by a Quality Assurance Unit.

Table 6.1
Summary of Repeated Dose Toxicity Tests Performed on Cassia Gum (Diagum CS™)

Assay	Cassia Gum Test Dose(s)	Result(s)	Reference
Short-term (28 days) repeated dose in Rats	0,125, 500, 1250 and 2,500 mg/kg bw/d as dietary admixture and 1000 mg/kg bw/d by gavage	NOAEL: 2,500 mg/kg bw/day oral (dietary) or 1,000 mg/kg bw/d (gavage)	Zühlke, 1990
Subchronic (90 days) repeated dose in Cats	0, 250 and 1,250 mg/kg bw/d as dietary admixture	NOAEL: 1,250 mg/kg bw/d	Virat, 1984
Subchronic (90 days) repeated dose in Beagle Dogs	1,000 and 3,500 mg/kg bw/d as dietary admixture	NOAEL: 3,500 mg/kg bw/d	Schuh, 1990

6.1.4.1 Short-term study on cassia gum in rats

In a 28-day study, groups of five male and five female Sprague-Dawley rats were administered cassia gum as part of a powdered diet at concentrations of 0, 0.25%, 1.0%, 2.5%, or 5.0% (Zühlke, 1990). The dose levels were calculated to be equivalent to 0, 125, 500, 1250, or 2500 mg/kg bw/day.[22] A sixth group, which was fed an untreated powdered diet, was treated by

[22] Where dosage levels are given only as concentrations in the diet, we have converted these levels to the estimated corresponding daily intake in mg/kg bw/day based on standard conversion factors set forth in *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*, published by the Association of Food and Drug Officials of the United States (1959). In particular, for older rats, 1 ppm in the diet is stated to be equivalent to 0.05 mg/kg bw/day. Thus, for example, the 0.25% dietary dose is converted as follows: 0.25% (= 2500 ppm) x 0.05 mg/kg bw/day = 125 mg/kg bw/day.

gavage with cassia gum as a suspension in distilled water, two times a day at a dose of 1000 mg/kg bw/day.

Clinical, hematological, urinary, and biochemical parameters were evaluated. Generally, all hematological parameters examined were considered to be within the normal range of the background data for the species tested. The following minor changes were observed but were not considered treatment-related because they did not occur in the high oral dose group: a statistically significant decrease in the red blood cell count and packed cell volume of males and females at the 500 and 1250 mg/kg bw/d levels; a statistically significant decrease in mean hemoglobin values of females in the 500 and 1250 mg/kg bw/d groups; and a statistically significant increase in white blood cell count for males in the 500, 1250 and 2500 mg/kg bw/d levels.

Clinical chemistry findings included statistically significant increases in mean glucose concentrations of males and females in the 500 and 1250 mg/kg bw/d groups, and mean triglyceride concentrations of males only in the 500 and 1250 mg/kg bw/d groups. These changes are not considered dose-related because they were not observed at the highest dose. A decrease in blood urea nitrogen was statistically significant but not dose-dependent for males in the 500, 1250 and 2500 mg/kg bw/d groups, but was not statistically significant for females in the 1250 and 2500 mg/kg bw/d groups. Statistically significant, dose-dependent decreases in sodium were observed in males in the 500, 1250 and 2500 mg/kg bw/d groups, and the 1000 mg/kg bw/d intragrastic suspension group, and females at 1250 and 2500 mg/kg bw/d only. A statistically significant, dose-dependent increase in group mean triglyceride concentrations was reported in females in the 500 and 1250 mg/kg bw/d groups. Statistically significant but not dose-dependent reduced mean total protein (6.2%) and albumin levels (7.7%) of males in the 2500 mg/kg bw/d group, statistically significant elevated potassium levels (13.0%) of males in the 1250 mg/kg bw/d group, and decreased chloride levels (6.8%) of males treated 1000 mg/kg bw/d intragastrically were also observed. Body weight gain was statistically significantly reduced (20%) in males in the 2500 mg/kg bw/d group and in females (17% each) in groups fed 500 and 1250 mg/kg bw/d, and 1000 mg/kg bw/d intragastrically (gavage). These weight decrements have previously been observed when high dose levels of carbohydrate-based gums are administered to rats (Takahashi *et al.*, 1994) and are not considered to be biologically significant.

All animals were necropsied and all tissues and organs were weighed and examined macroscopically. Histological examination was performed on major organs for animals in the control group, high dose group, and group treated intragastrically. Group mean absolute kidney weights were statistically significantly decreased in males fed 500 mg/kg bw/d (8.0%), 2500 mg/kg bw/d (15.3%), and 1000 mg/kg bw/d (6.9%) intragastrically. Group mean relative kidney weights were statistically significantly increased in females fed 2500 mg/kg bw/d (11.3%). A dose-relationship was not reported. These changes in the kidney weights were not considered treatment-related and/or toxicologically significant since there were no changes in relative weight in males and in absolute weight in females and no histopathology was seen (Zühlke, 1990).

6.1.4.2 Subchronic study on cassia gum in dogs

Two groups of four male and four female Beagle dogs were provided with either 0.75% or 2.5% (w/w) cassia gum as part of a canned dog-food diet for 90 days. The average dose levels calculated on the basis of food consumption data were approximately 1000 and 3500 mg/kg bw/d (>1000-times the anticipated daily dietary exposure levels, respectively). A control group received a similar diet containing 0.23% (w/w) carob (crushed locust beans) (Schuh, 1990).

Clinical, biochemical, hematological and urinary parameters were evaluated. Slight but statistically significant changes in some biochemical and hematological parameters were reported periodically in males and females in both dose groups. Since most of the changes were not dose- or time-dependent as compared to controls, they were not considered to be compound related. Bone marrow examinations, urinalysis, coagulation studies, and gross pathology including organ weights were conducted in all animals. Microscopic examination was performed on all major organs for all animals in the control and high dose groups. For animals in the low dose group, histological examination was limited to the liver, kidney, heart, and macroscopic findings. Survival was 100%. A dose-dependent increase in water consumption was observed in test animals and was most likely associated with water retention in the gastrointestinal tract by cassia gum. No compound-related adverse effects were reported (Schuh, 1990).

6.1.4.3 Subchronic study on cassia gum in cats

Three groups of five male and five female cats were provided with 0, 0.5% or 2.5% (w/w) cassia gum as part of a standard canned-food diet for 13 weeks. Clinical, hematological, urinary, and biochemical parameters were evaluated and were in the normal range. No consistent treatment related dose-dependent adverse effects were reported. Histological examination was performed on all major organs in all animals. There were no adverse or compound-related histopathological findings. Survival was 100%. (Virat, 1984.)

6.1.4.4 Toxicity data on related gums

The structurally related galactomannan, guar gum, did not produce evidence of compound-related toxicity when groups of 10 male and 10 female rats (strain not provided) were provided 0, 1%, 2%, or 5% guar flour as a dietary admixture for 90 days (Til *et al.*, 1974); when two groups of 15 male and 15 female rats were provided either 0 or 5% guar flour in their diet for 24 months (Krantz *et al.*, 1948); or when groups of 5 male and 5 female Sprague-Dawley rats were administered partially hydrolyzed guar gum by gavage for 28 days at 0, 500, or 2500 mg/kg bw/d (Takahashi *et al.*, 1994). When rats were provided guar gum in the diet for 91 days at levels of 1%, 2%, 4%, 7.5%, or 15%, a decrease in weight gain in females of all dose groups and males at the two highest levels was reported. Bone-marrow cellularity was reduced at the 15% level. A dose-related decrease in the absolute kidney weights of males was reported, but relative kidney weights were normal and no histopathology was seen (Graham *et al.*, 1981).

No compound-related toxicological effects were reported when four groups of 5 male and 5 female Beagle dogs were fed 0, 1%, 5%, or 10% of a precooked mixture containing guar and locust (carob) bean gum for 30 weeks (Cox *et al.*, 1974).

When two monkeys received 1 g of guar flour in their diet daily, growth, hematology, and overall health were normal. One monkey died after 16 months, and the other was sacrificed at 24 months. No abnormalities were noted upon gross examination and histopathology (Krantz *et al.*, 1948).

Similarly, the structurally related galactomannan, locust (carob) bean gum, did not produce evidence of compound-related toxicity in groups of 10 male and 10 female rats given locust (carob) bean gum in the diet at levels of 0, 1%, 2% or 5% for 90 days (Til *et al.*, 1974).

6.1.5 Carcinogenicity studies

There are no carcinogenicity studies on cassia gum. However, other gums including locust (carob) bean, guar, and tara were not carcinogenic when fed to rats and mice and were not mutagenic in appropriate test systems. When the chemically related galactomannan locust (carob) bean gum was provided to F344/N rats or B6C3F1 mice as a dietary admixture at concentrations of 25,000 or 50,000 ppm (≈1250 or 2500 mg/kg bw/day) for 103 weeks, it did not induce cancer (NTP, 1981). Nor is locust bean gum mutagenic (Maxwell and Newell, 1972). When guar gum was provided to F344/N rats or B6C3F1 mice as a dietary admixture at concentrations of 25,000 or 50,000 ppm (≈1250 mg/kg bw/d or 2500 mg/kg bw/d) for 103 weeks, it did not induce cancer (NTP, 1982a). F344/N rats fed diets containing 0, 25,000 or 50,000 ppm (0, 1250 or 2500 mg/kg bw/day) tara gum for two years exhibited no tumorigenic effects related to the test material (NTP, 1982b; Borzelleca *et al.*, 1993).

6.1.6 Reproductive and developmental toxicology

Four reproductive or developmental toxicology studies have been conducted in which cassia gum was presented as a dietary admixture or administered by gavage. These include a one-generation study in cats, a two-generation study in rats, and teratology studies in rats and rabbits. Cassia gum did not produce any evidence of embryotoxicity or teratogenicity, as evidenced by the summary of these studies set forth in Table 6.2, below.

Table 6.2
Summary of Reproductive and Developmental (Teratological) Tests Performed on Cassia Gum (Diagum CS™)

Assay	Doses mg/kg bw/day	Result(s)	Reference
One-generation Reproduction in Cats	0, 375, 1250	Transient effects on biochemical parameters at 1,250 mg/kg bw/d. No unequivocal evidence of reproductive toxicity at highest dose level. NOAEL: 375 mg/kg bw/d.	Virat, 1989
Two-generation Reproduction in Rats	0, 250, 1000, 2500	No effects on gonadal function, mating behavior, conception, parturition, weaning and the growth and development of offspring. NOAEL: 2,500 mg/kg bw/d.	McIntyre, 1990
Teratology in Rats	0, 350, 1000	No compound related adverse effects for clinical observations, mortality, necropsy findings, pregnancy incidence, implantation, post-implantation loss, or fetal defects.	Müller, 1989

		NOAEL: 1000 mg/kg bw/d.	
Teratology in Rabbits	0, 350, 1000	No compound related adverse effects for clinical observations, mortality, necropsy findings, pregnancy incidence, implantation, post-implantation loss, or fetal defects. NOAEL: 1000 mg/kg bw/d.	Müller, 1989

The four studies summarized above, and discussed further below, were conducted according to OECD Principles of Good Laboratory Practices (1981) and were inspected by a Quality Assurance Unit.

6.1.6.1 One-generation reproduction in cats

In a one-generation reproductive study, three parental groups (F_0 generation) of 10 male and 20 female cats were provided cassia gum as part of the diet at concentrations of 0, 0.75%, or 2.5% (equivalent to about 375 and 1250 mg/kg bw/day) for at least 83 weeks. After at least 36 weeks, one male was mated with two females of the same dose group. At day 4 post-partum, all kittens in excess of 4 per litter were culled. The F_1 generation was terminated after at least 13 weeks and the F_0 generation was terminated after the 83-week treatment period (Virat, 1989).

Clinical, hematological, urinary, and biochemical parameters were evaluated. No treatment related effects on hematology parameters were noted. A slight, statistically significant decrease in activated pro-thrombin time (APTT) was reported in treated parental females in both groups at week 26, but was considered to have no biological significance because the changes were transient and within normal range. After 13 weeks of dosing, a statistically significant increase in bilirubin was noted in males and females in the 1250 mg/kg bw/d group, and females in the 375 mg/kg bw/d group. From week 26 onwards, this change was only observed in males and females in the 1250 mg/kg bw/d group. The values were transient, never reached a biologically significant range, and did not correlate with morphological alterations. Therefore, the biological significance was considered unclear, but minor with respect to toxicity. Slight, statistically significant increases in clinical chemistry parameters were observed but were not dose-related and were considered to have no biological significance.

Histological examination was performed on all tissues for all F_0 generation cats in the control and high dose groups, and on two kittens from each litter of the control and high dose groups. X-ray skeletal examination was performed on all kittens (Virat, 1989).

High mortality occurred in the control group after 2-3 weeks postpartum resulting in several total litter losses in this group. The mortality was preceded by several clinical signs but no consistent pattern of pathology was observed. Although treatment groups were unaffected, appropriate comparisons between control and treatment groups were impaired (Virat, 1989).

Some effects were observed in both generations, but were not clearly associated with the test compound. For example, females in the high-dose F_0 group exhibited a decrease in food consumption during late gestation and a slight increase in absolute and relative ovarian weights. The latter was not accompanied by evidence of histopathology. In the F_1 generation, the combined incidence of stillborn births and neonatal deaths was significantly higher in the high-dose group. No other findings were noted. The authors concluded that while there was no unequivocal evidence of reproductive toxicity at the highest dose level, the lower dose of 375 mg/kg bw/d cassia gum is considered to be the no observed adverse effect level (NOAEL) (Virat, 1989). While the questionable biological significance of the changes in biochemical parameters suggest that the NOAEL should be 1275 mg/kg bw, we defer to the author's conservative approach and use 375 mg/kg bw/day as the NOAEL.

6.1.6.2 Two-generation reproduction in rats

In a two-generation reproductive study using Sprague-Dawley rats, five groups of 25 males and 25 females were provided with 0, 5,000, 20,000, or 50,000 ppm of cassia gum, or 50,000 ppm of cassia gum-AW[23] as part of the diet. All parental rats (P generation) were treated for approximately 10 weeks prior to mating, during mating, gestation, and lactation. The dose levels are equivalent to 0, 250, 1000, or 2500 mg/kg bw/d. Pregnant females were allowed to rear their offspring (F_{1a}) to weaning. Rats in both 2500 mg/kg bw/d dose groups exhibited low pregnancy rates and were mated again with the same males. They were allowed to litter, and the subsequent offspring (F_{1b}) were terminated on days 5-7 postpartum. Selected F_{1a} offspring were treated for a 10-week period of maturation, and during mating, gestation, and lactation. Pregnant F_{1a} females were allowed to rear their offspring (F_2) to weaning (McIntyre, 1990).

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[23] In order to evaluate the potential for adverse effects associated with vegetable by-products that may be present in cassia endosperm flour, cassia gum-AW was highly purified in preparation for testing by a treatment similar to that employed with Diagum SR™, i.e., thoroughly washing cassia endosperm flour with isopropanol followed by careful drying.

All P and F_1 animals were necropsied. Histopathological examination was performed on gross lesions and on the reproductive organs for all controls and animals fed 2500 mg/kg bw/d cassia gum. No compound related adverse effects were noted. The pregnancy rate of P females was slightly decreased at the first mating in the two 2500 mg/kg bw/d dose groups, but a subsequent mating revealed no evidence of a treatment-related effect on fertility. No adverse effects related to treatment with cassia gum were reported on gonadal function, mating behavior, conception, parturition, weaning and the growth and development of the offspring. There was a slight, but not statistically significant, decrease in the mean body weights of F_{1a} and F_2 pups in the group that received cassia gum-AW (McIntyre, 1990).

6.1.6.3 Teratology in rats

In a teratology study, three groups of 28 pregnant Sprague-Dawley rats were administered cassia gum in distilled water by gavage at concentrations of 0, 17.5, or 50 mg/ml (0, 350, or 1000 mg/kg bw/d) for 14 days from days 6 to 19 post-coitum (inclusive). The control animals received the vehicle (distilled water) alone. A fourth group containing 29 pregnant rats was administered cassia gum-AW by gavage in distilled water at a concentration of 50 mg/ml daily (1000 mg/kg bw/d) over the same period. All animals were sacrificed and examined on day 20 post-coitum (Müller, 1989). No compound-related adverse effects were reported for pregnancy incidence, implantation, post-implantation loss, fetal defects upon necropsy. A statistically significant reduction in mean daily food consumption and mean body weight gain was observed in the pregnant animals dosed at 1000 mg/kg bw/d of cassia gum or cassia gum-AW, and was considered to be a treatment related effect. However, these effects were not accompanied by any evidence of embryotoxicity or teratogenicity (Müller, 1989).

6.1.6.4 Teratology in rabbits

In a related teratology study, three groups of 20 pregnant New Zealand White rabbits were administered cassia gum in distilled water by gavage at concentrations of 0, 17.5 or 50 mg/ml (0, 350 or 1000 mg/kg bw/d) for 22 days from days 6 to 27 post-coitum (inclusive). The control animals received the vehicle (distilled water) alone. A fourth group of 20 pregnant rabbits was administered 1000 mg/kg bw/d cassia gum-AW by gavage in distilled water over the same period. All animals were sacrificed and examined on day 28 post-coitum (Müller, 1989).

No compound-related adverse effects were reported in pregnant animals for clinical observations, mortality, necropsy findings, pregnancy incidence, implantation, post-implantation loss, or fetal defects. A reduction in mean daily food consumption was reported in animals administered 1000 mg/kg bw/d of cassia gum, and was accompanied by a reduction in mean fetal weights. However, these effects were not statistically significant. No indication of teratogenicity was reported (Müller, 1989).

6.1.7 Mutagenicity and genotoxicity

A battery of *in vitro* studies have been conducted to evaluate the genotoxic potential of cassia gum. These include bacterial reverse mutation assays (Ames tests and *Escherichia coli* (*E. coli*) reverse mutation assays), a chromosome aberration assay, and an *in vitro* mammalian cell gene mutation assay. All *in vitro* studies were conducted in compliance with the OECD Principles of Good Laboratory Practice (OECD, 1981) and were inspected by a Quality Assurance Unit.

6.1.7.1 Ames test

Cassia gum (Digum CS™) was tested, with and without metabolic activation, in the *Salmonella typhimurium* (S. *typhimurium*) reverse mutation assay (Ames test) with strains TA1535, TA1537, and TA98 at concentrations of 4, 16, 62.5, and 1000 µg/plate. Cassia gum precipitated on the plates at concentrations of 62.5 µg/plate and higher. Increases in the number of revertant colonies in strains TA1535, TA1537, and TA98 were seen only at precipitating concentrations and were within the historical control data range. Strain TA100 was tested at concentrations of 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate. Increases in the number of revertant colonies were dose-related and outside the historical control data range, but were seen only at precipitating concentrations (Verspeek-Rip *et al.*, 1998a).

A second experiment in the study described above involved testing cassia gum (Diagum CS™) at concentrations of 1.6, 8, 40, 200, and 1000 µg/plate in the same *S. typhimurium* strains and in *E. coli* strain WP_2uvrA. Cassia gum precipitated on the plates at concentrations of 200 µg/plate and higher. The results were negative (i.e., no evidence of mutagenic activity) in strains TA1535, TA98, and WP_2uvrA. Dose-related increases in the number of revertant colonies were observed in strains TA1537 and TA100, but again only at precipitating concentrations (Verspeek-Rip *et al.*, 1998a). Only the results in TA100 were outside the range of historical

controls. Based on the results from the two experiments, it was concluded that cassia gum tested positive for mutagenic activity in *S. typhimurium* strain TA100, and was negative for mutagenicity in strains TA1535, TA1537, TA98, and *E. coli* strain WP_2uvrA (Verspeek-Rip *et al.*, 1998a). The presence of precipitation in the assay and the negativity of cassia gum in all other mutagenic assays (see below) except the single TA 100 strain left considerable uncertainty in the interpretation of the results.

It was decided to conduct a new series of Ames tests to attempt to avoid the solubility/precipitation problem. In addition, the purification method used to prepare the cassia gum was enhanced to reduce potential impurities (notably anthraquinones) with the addition of an extraction step with isopropyl alcohol.[24] The Ames mutagenicity tests were redone on the more stringently purified samples (Diagum SR™) which are representative of the current production method.

The second series of studies was conducted in the same laboratory by the same principal investigator to more fully assess the mutagenic potential of cassia gum using the current, more highly refined product, Diagum SR™ and using the most current international guidelines. The same tester strains were used as described above for the first assay. The main part of the assay was conducted using water as the solvent in an effort to minimize precipitation. A bridging study with DMSO, which was previously used as the solvent in the first Ames test, was conducted at higher precipitating concentrations (100, 300, 1000, 3330, and 5000 μg/plate) to enable comparisons with the first Ames test results, which were previously positive in strain TA 100 as noted above.

Following the current OECD guidelines for selection of concentrations, the concentrations in the main part of the assay were established so that the top doses were limited to two concentrations that caused precipitation (33 and 100 μg/plate). The concentrations in this part of the study were 0.3, 1.3, 10, 33, and 100 μg/plate both with and without activation. Cassia gum did not increase the number revertants in any of the four Ames tester strains nor in the *E. coli* WP_2uvrA test strain both in the presence and absence of S9-metabolic activation. These findings were confirmed in an independently repeated experiment.

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[24] The isopropyl treated samples are denoted as Diagum SR.

Based on the results of these studies, the principal investigator concluded that cassia gum, as prepared by the current production method, is not mutagenic in the Ames (*S. typhimurium*) test. The detailed reports of these three studies are attached. (App. II, Tab 8.)

A full assessment of the safety of the anthraquinone impurities at the 10 ppm maximum specified level is presented in Section 6.3 below. This evaluation includes a worst-case quantitative cancer risk assessment based on two conservative assumptions: (1) that despite detecting only one anthraquinone (physcion) in any sample of Diagum SR™, other anthraquinones could conceivably be present below the detection limit of 0.5 ppm, and (2) all anthraquinones present or potentially present in cassia gum may be carcinogenic despite the negative findings in mutagenicity tests.

6.1.7.2 Chromosome aberration assay

The ability of cassia gum to induce chromosome aberrations in cultured peripheral human lymphocytes was evaluated at concentrations of 1, 3, and 10 µg/ml, with and without metabolic activation. Cassia gum did not induce a statistically or biologically significant increase in the number of cells with chromosome aberrations (Bertens *et al.*, 1998).

6.1.7.3 Mouse lymphoma assay

The effect of cassia gum on the induction of forward mutations at the thymidine-kinase locus in L5178Y mouse lymphoma cells, with and without metabolic activation, was tested at concentrations up to 10 µg/ml. Cassia gum did not induce a dose-related increase in the mutant frequency, therefore, it was concluded that it is not mutagenic in the mouse lymphoma L5178Y test system (Verspeek-Rip *et al.*, 1998b).

6.1.8 Hypersensitivity

Ten albino guinea pigs were intradermally injected with 0.2% concentration and epidermally exposed to a 50% concentration of cassia gum. Approximately 24 hours prior to epidermal exposure, each animal was challenged with 10% sodium dodecyl sulfate. Five control animals were similarly treated with the vehicle control (corn oil). Two weeks after the epidermal application all animals were challenged with a 50% cassia gum concentration and the vehicle. Skin reactions varying between grade 1 and 3 were observed in all experimental animals but were not evident in the control animals. These observations were indicative of sensitization by cassia gum (Busschers and Aigner 1998).

Skin prick tests performed on 125 pharmaceutical workers previously exposed to *Senna* pods (*Senna obtusifolia* is a synonym for *C. obtusifolia*) revealed that 15.3% were allergic to *Senna*. None of the 20 unexposed pharmaceutical workers also tested had a positive skin test to *Senna* (Marks *et al.*, 1991).

The allergenicity of four different species of *Cassia* (*auriculata, occidentalis, roxburghii,* and *siamea*), which were found to be dominant in India, was evaluated in 140 patients with a history of respiratory allergy. Intradermal skin testing was performed by injecting the antigenic extraction of the four *Cassia* species into the patients' forearms. Twelve percent (12%) had a positive skin reaction (Satheesh *et al.*, 1994).

A study on workers health was conducted on 62 volunteers at the Noveon (formerly BF Goodrich) Diamalt Inc., plant in Munich, Germany. The purpose of the study was to determine the risk to workers' health during the production of thickening agents, including ground cassia, with emphasis on pulmonary function and predisposition towards allergic reactions. The study program included: 1) a detailed medical history, 2) a physical examination, 3) clinico-chemical laboratory tests, 4) a pulmonary function test, 5) several allergy tests, and 6) dust measurements from different production areas. In the medical reports 54.8 % reported irritation of the eyes, nose, lungs, or skin and 37% were suffering from acute cold symptoms. While several positive results and diagnoses were made, there was no statistical evidence of correlation between symptoms and occupational factors (including number of days exposure to cassia powder) or personal factors (such as age, gender, occupational non-related previous diseases). However, participants who displayed sensitization to at least one common allergen were also more frequently sensitized to cassia powder and/or store mites. Also, the exposure to powder dust exceeded the limit value that is currently set for the inhalable dust fraction. The investigators recommended that preventive health protection measures be implemented in the workplace (Steget *et al.*, 1999). Such measures have been implemented at the Indiamalt plant where cassia gum is now produced.

The results of the first three sensitization studies for cassia bear no effect on the use of cassia gum in human foods, and in some cases the *Cassia* species tested are not those used in the manufacture of Diagum SR™. In the fourth study, although positive reactions were observed in humans, there was no evidence of correlation between exposure to the cassia powder and

incidence of allergic reactions. Moreover, this is an occupational health issue and is not directly relevant to the safety of the finished product in food.

6.1.9 Calculation of ADI for Diagum SR™

Short-term and subchronic studies on cassia gum in rats, dogs, and cats did not produce treatment-related signs of toxicity at the maximum doses fed. The highest dose in the 90-day dog study was 3.5 g/kg bw/day (Schuh, 1990). The highest dose in the 13-week cat study was 1.25 g/kg bw/day (Virat, 1984). The highest dose in the 28-day rat study was 2.5 g/kg bw/day. All of these levels represent minimal no-effect levels in these studies. The NOAELs for the various reproductive and development studies performed on cassia gum are listed in Table 6.2. The lowest NOAEL observed in any of these studies, was the conservative 375 mg/kg bw/day NOAEL noted above (Section 6.1.6.1) from a one-generation reproductive study in cats (Virat, 1989). Although the NOELs in the other reproductive and development studies and in the 90-day studies were at least three times higher, for conservatism we will use the NOEL from the one-generation cat study to calculate an acceptable daily intake (ADI) for cassia gum. Thus, applying a 100-fold safety factor to the lowest NOAEL of 375 mg/kg bw/day, the ADI is conservatively estimated to be 3.75 mg/kg bw/day. Again, the available toxicity data strongly suggest that a significantly higher dietary intake level would be acceptable.

6.2 Biological Studies on Cassia Impurities: Anthraquinones and Muscle Toxicity

As discussed previously, muscle toxicity has been associated with components of *C. obtusifolia*, from which the seeds used in the production of Diagum SR™ cassia gum are harvested, as well as with another species of cassia, *C. occidentalis*, which is not used as a source for Diagum SR™. The toxic principle is considered to be the anthraquinones, which are present as naturally occurring impurities in cassia.

The toxicological studies discussed in Section 6.1 were carried out using purified cassia gum (Diagum CS™, the predecessor to Diagum SR™), which contains only low levels of anthraquinone impurities. None of the studies on cassia gum showed any evidence of muscle toxicity, suggesting that muscle toxicity will be absent provided the anthraquinone level is below the NOAEL for muscle toxicity. To establish an adequate tolerance for anthraquinone muscle

toxicity, and thus to ensure the adequacy of the 10 ppm specification for anthraquinone content, we review below the literature on the subject and calculate an appropriate tolerance.

6.2.1 Early literature on muscle toxicity from "sicklepod"

Henson *et al.* (1965) reported widespread skeletal myodegeneration in cattle apparently caused by grazing on *C. occidentalis* or *C. obtusifolia.* Both plants appear to be capable of causing the disease, but *C. obtusifolia* (which is one of the two *Cassia* species from which Noveon's cassia gum is produced, the other being *C. tora*) is reported to be slightly less toxic than *C. occidentalis*. All parts of the plant are toxic, especially the seed (McCormack and Neisler, 1980). Nicholson *et al.* (1977) reported toxic myopathy and death in cattle ingesting foliage heavily contaminated with immature sicklepod (*C. obtusifolia).* Poisoned cattle are weak, become recumbent, unable to rise, and their urine is often darkened due to the presence of the muscle pigment, myoglobin. Intake of sicklepod was estimated at 12-25 lbs per cow for 8 to 12 days. Large amounts of sicklepod appear to be required to produce sicklepod toxicosis. The type of sicklepod and the toxic principle, i.e., leaves, pod, seed or stems, were not indicated in the Nicholson article. However, assuming all of the consumed amount was toxic, a crude, short-term intake level from this study would be 5 kg /500 kg bw = 10 g/kg bw/day.

Dairy cows were reported to have died as a result of eating feed that was approximately 50% leaves, stems and seeds of sicklepod (McCormack and Neisler, 1980). The signature lesions of the disease are cardiac and skeletal muscle degeneration, e.g., rhabdomyoltic intoxication (rhabdomylotic refers to striated muscle fibers) (Hensen *et al.*, 1965; McCormack and Neisler, 1980; Nicholson *et al.*, 1977). These toxic myopathies are characterized by impairment of mitochondrial function, including swelling, loss of mitochondrial matrix, fragmented cristae and glycogen depletion (O'Hara and Pierce, 1974a, b; Graziano *et al.*, 1983). A spectrum of acute toxic effects including: anorexia, diarrhea, hyperpnea, tachycardia and progressive muscle incapacitation and recumbency, typically develop over a 5-7 day period. Such effects were observed in both cattle and goats at approximately the same dietary concentration from 2-12 g seed /kg bw/day.

Clinical and laboratory data established that sicklepod produces the same or a very similar disease in chickens, rabbits and cattle (Nicholson *et al.*,1986; Hebert *et al.*, 1983). Loung and McCoy (1992) found muscle lesions in rabbits after feeding large doses of the aerial portions of the plant. Because they fed as high as 100% plant, some (but not all) of their results may be

due to nutritional deficiencies. The lesions in striated muscle resembled those in cattle poisoned with *C. obtusifolia* and those in rabbits poisoned with *C. occidentalis.* In addition to these effects, the liver appears vulnerable to large dietary doses of *Cassia* species. *C. occidentalis* produces liver lesions in chickens (Graziano *et al.*, 1983) and *C. obtusifolia* may produce enlarged livers in rats (Crawford and Friedman, 1990).

The earlier scientific papers on *C. obtusifolia* or *occidentalis* poisoning in farm animals speculated that the toxic principle in *C. occidentalis* might be a toxic albumen, a toxic alkaloid or an anthraquinone derivative. Hebert *et al.* (1983) extracted seeds of *C. occidentalis* and found that the aqueous extract contained the toxic principle typically bound to particulate matter in the extract. They did not characterize the toxic agent, but speculated that its water solubility meant that it was a polar molecule and suggested that it could be proteinaceous.

A literature search has identified no reports of muscle or any other toxicity associated with *C. tora.* In the absence of any published specific toxicity data on *C. tora*, it is reasonable to conclude that while the pattern of anthraquinones may be slightly different in the two seeds, the total anthraquinone levels are approximately the same, and thus toxicity of *C. tora* can be assumed to be approximately the same or less than that of *C. obtusifolia.*

6.2.2 Controlled studies on cassia seeds and extracts

Additional information on the muscle toxicity of cassia is provided by the controlled laboratory studies discussed below.

6.2.2.1 Crawford and Friedman

Crawford and Friedman (1990) showed that despite their toxicity to rats and to range animals at high doses, sicklepod weed seeds do not produce any discernible sign of liver toxicity when fed to rats at 0.5% of ground seeds in the basal diet. This study, as well as some of the others discussed below, allows a quantitative estimate of the toxicity of the anthraquinones in cassia seeds. Specifically, if we assume an anthraquinone content in the seeds of approximately 1%,[25] a dietary level of 0.5% ground seeds corresponds to 50 ppm of anthraquinones in the diet.[26] This is roughly equivalent to an anthraquinone intake of 5 mg/kg bw/day.[27] Based on the

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[25] Koshioka and Takino, 1978; Crawford *et al.*, 1990; see discussion in Section 2.6.1 above.

[26] 5000 ppm (0.5%) seed x 1% anthraquinones (AQ) in seed = 50 ppm AQ in diet.

absence of adverse effects, this dietary intake level for anthraquinones represents a NOAEL for this study.

6.2.2.2 Putnam *et al.*

Groups of 5 bull calves received *C. obtusifolia* seed with their daily ration at doses of 0, 0.45, and 1.81 kg/day for a period of 9 days (Putnam *et al.*, 1988). Effects reported included intermittent feed refusal, diarrhea, and decreased weight gain in some animals. Average daily weight gains for the control, low dose, and high dose groups were 0.76, 0.53, and 0.31 kg/day, respectively. The average daily feed consumptions for the low and high dose groups were 3.45 kg and 2.54 kg; control animals consumed the entire daily ration of 3.63 kg . No change was observed in serum aspartate aminotransferase (AST) creatinine phosphokinase (CPK) activity, lactic dehydrogenase activity (LDH), and histologic examination showed no evidence of muscle necrosis.

Average daily consumption of *C. obtusifolia* seed was calculated as 0.36 and 0.70 kg/day, or 2.4 and 4.7 g/kg bw/day. When a substantial amount of the daily quantity of seed presented was eaten, the toxic effects were transient diarrhea and anorexia. The authors concluded that at these average levels of *C. obtusifolia* seed consumption, the most serious effect, the decrease in weight gain, was probably due to impalatability rather than toxicity. The NOAEL for *C. obtusifolia* seed from this study was therefore 4.7 g/kg bw/day. Again assuming 1% anthraquinones in the seed, the NOAEL for anthraquinone toxicity from this study would be approximately 47 mg/kg bw/day.

6.2.2.3 Lewis and Shibamoto

Adult male Sprague-Dawley rats were fed an extract of ground *C. obtusifolia* seeds for three days, after which control diet was given (Lewis and Shibamoto, 1989). The anthraquinone extract was present in powdered rat chow at a concentration of 0.3%. CPK was significantly increased in the treated rats and returned to normal after the control diet was reintroduced. There

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[27] This is based on a standard conversion factor for younger rats of 1 ppm = 0.1 mg/kg bw/day. Thus: 50 ppm x 0.1 mg/kg bw/day = 5 mg/kg bw/day.

was also a temporary anorexia and loss in weight gain. The dosing level corresponds to an effect level for the anthraquinones of 202 mg/kg bw/day.[28]

6.2.2.4 Dugan and Gumbmann

Diets containing sicklepod at concentrations of 1, 2, 4, 8, 16 and 32% were fed to groups of 3–5 Sprague-Dawley rats in a series of short-term (8- to 9-day) toxicity studies (Dugan and Gumbmann, 1990). In addition, groups of five female Sprague-Dawley rats were fed the same dosages to compare the effects on body weight, feed and water consumption. There was 100% mortality in the 32% group and one death in the female 16% group. Assuming a dose of 20% was lethal to one-half of the test population, a rough LD_{50} for short-term exposure to sicklepod is 10 g/kg bw/day.[29]

Sicklepod fed at 8% or less did not result in adverse clinical signs. A marked dose-related weight gain decrement was significant at 4% but barely detectable at the 2% dose. The testes of rats fed 8% or more sicklepod seed exhibited a significant incidence of hypospermia. Histological evaluation of the liver did not reveal any pathology, and liver weight was not increased. Despite significant weight loss and the detection of testicular lesions, there was no mention of muscle degeneration in rats. CPK levels were not measured, and glutamic-oxalacetic transaminase (GOT) levels increased only at the 16% dose. The seed in these studies was milled, and there is some evidence that the potency of the ground seed can decrease with storage time (O'Hara and Pierce, 1974a). The lowest NOAEL for any toxic effect was 2%, the NOAEL for weight less, equivalent to 1 g/kg bw/day.[30] If anthraquinones are present in the seed at approximately 1.0%, the NOAEL for the anthraquinones is 10 mg/kg bw/day.

6.2.3 Mechanism of action

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[28] Average feed consumption was reported as 26 g/day (26,000 mg/day), and the initial mean body weight as 387 g (0.387 kg). Thus (0.3% AQ extract in feed) x (26,000 mg feed/day) ÷ (0.387 kg bw) = 202 mg/kg bw/day.

[29] Calculated using conversion factor for older rats. Thus, 20% sicklepod in diet (200,000 ppm) x 0.05 mg/kg bw/day) = 10,000 mg (or 10 g) sicklepod/kg bw/day.

[30] (2%, or 20,000 ppm sicklepod in diet) x (0.05 mg/kg bw/day) = 1000 mg (or 1 g) sicklepod/kg bw/day.

Anthraquinones and related compounds have been known for years to occur in many *Cassia* species. More recent evidence indicates that the toxic principle in cassia is the anthraquinones. The relevant studies are discussed below.

Lewis and Shibamoto (1989) found that the extracts of *C. obtusifolia*, as well as the specific anthraquinone emodin, inhibited NADH:cytochrome C oxidoreductase activity of bovine heart mitochondrial particles. This study provides very strong evidence that the anthraquinones in *C. obtusifolia* can cause the muscle toxicity found in cows.

This study involved two experiments which investigated the myotoxic effects of cassia both *in vivo* and *in vitro*. First, an *in vivo* study was carried out in which rats were given ground *C. obtusifolia* seeds mixed with the diet. Due to profound anorexia, this testing was discontinued and replaced with testing in which rats received either an aqueous suspension or organic extract of *C. obtusifolia* seeds by gastric intubation. Both test materials caused a significant elevation in plasma CPK activity 12 hours after dosing. Elevation of plasma CPK activity is a widely used and accepted indication of rhabdomylosis-degeneration of striated muscle. Elevated CPK activities were obtained with all three dosing regimes (i.e., ground seed extract in the diet, an aqueous suspension by gavage, or an organic extract by gavage). In the *in vitro* testing, both the organic seed extract and individual anthraquinones inhibited NADH: cytochrome c oxidoreductase activity of bovine heart mitochondrial particles with comparable potency and in a dose-dependent manner. Morgan-Hughes *et al.* (1979) have reported myopathy due to a genetic deficiency of mitochondrial NADH:CoQ oxidoreductase activity.

Based on the presence of the toxic principle in both the aqueous suspension and organic extract of cassia seeds, Lewis and Shibamoto concluded that the toxin was not a protein, as had been suggested by Hebert *et al.*, but a more lipophilic compound that is bound strongly to plant proteins. The myotoxic organic extract was found to consist of a mixture of nine anthraquinones and three anthrones. Chrysophanic acid was one of the major anthraquinones identified. This study provides clear evidence that the active principle in *C. obtusifolia* muscle toxicity is a mixture of anthraquinones.

Muscle toxicity from exposure to anthraquinones has been shown to occur within a matter of days. Studies with anthraquinones indicate that the short time to occurrence is the result of a biochemical mechanism involving the inhibition of NADH:cytochrome C oxidoreductase activity in the mitochondria. These biochemical interactions ultimately result in

symptoms such as anorexia and weakness and characteristic changes in the muscle tissue such as swelling and loss of the mitochondrial matrix.

Data from the literature show that the muscle toxicity is also reduced by roasting. Suliman and Shommein (1985) observed that the ability of cassia to produce muscle toxicity in goats was virtually abolished when cassia beans were fed at levels of 2.5 g/kg bw/day before and after roasting. Roasting had less effect on reducing the toxicity when the goats were given doses as high as 5 g/kg bw/day. The observation of thermal degradation of the toxic principle in cassia was made on *C. occidentalis* rather than on *C. obtusifolia*. However, the toxic symptoms seen in *C. occidentalis*-fed goats were similar to that observed in cattle fed *C. obtusifolia* (Putnam *et al.*, 1988), indicating that the toxic agents responsible are the same or similar for the plant species.

6.2.4 Determination of NOAEL for anthraquinone-induced muscle toxicity

Table 6.3 summarizes the results from the various field and laboratory controlled studies discussed above from which it is possible to derive NOAEL values. The effect data from Lewis and Shibamoto are also shown for comparison.

Table 6.3
CONTROLLED FIELD AND TOXICITY STUDIES ON *C. OBTUSIFOLIA* SEEDS OR EXTRACTS
NOAEL, mg/kg bw/day (Anthraquinone Basis)[31]

Study	NOAEL	Duration of Study	Toxic Effect	Animal
Crawford and Friedman	5.0	Unstated	No effect	Sprague-Dawley rat
Putnam *et al.*	47	9 days on ration	Weight loss	Holstein calves
Lewis and Shibamoto	202 (LOAEL) [32]	3 days on diet	Muscle toxicity	Sprague-Dawley rat
Dugan and Gumbmann	10	8-9 days on diet	Weight loss	Sprague-Dawley rat
Noveon studies (NOAEL = 2.5%)[33]	0.03	83 weeks	Reproduction	Cat (Virat,1989)

[31] The nominal composition of 1% anthraquinones in seed was used to convert seed-based level to an anthraquinone-based level.

[32] The LOAEL is the lowest observed effect level.

[33] In the laboratory studies conducted by the Notifier, the lowest NOAEL was 0.75% from the one generation reproduction study in cats. (See Section 6.1 above.) Assuming a NOAEL of 0.75% in the diet for purposes of calculation, and the previous maximum anthraquinone level of

6.2.5 Safety assessment and characterization (muscle toxicity)

As discussed in Section 5 above, the 90th percentile EDI of cassia gum is 0.6 mg/kg bw/day, or 36 mg/p/d for a 60-kg individual. Assuming a 10 ppm anthraquinone content in the processed Diagum SR™, the corresponding EDI for the anthraquinone impurities is 6×10^{-6} mg/kg bw/day. This is equivalent to 3.6×10^{-4} mg/day (0.36 µg/day) for a 60-kilogram individual.

The lowest approximate NOAEL for anthraquinones is that derived from Crawford and Friedman, in which ground sicklepod (*C. obtusifolia*) seeds were fed in the basal diet of rats at 0.5% (5000 ppm). As shown above, the NOAEL for the anthraquinones is about 5 mg/kg bw/day. This is close to the NOAEL that may be determined from the Dugan and Gumbmann study. Neither of these two studies reported muscle toxicity at these doses.

Using a 1000-fold safety factor, an ADI for anthraquinones based on muscle toxicity may be estimated from the NOAEL determined in the Crawford and Friedman study. Specifically, the ADI equals: 5 mg/kg bw/day ÷ 1000 = 5 µg/kg bw/day. This is equivalent to 300 µg/day based on a 60-kg body weight. The ADI is therefore approximately 800 times the EDI (300/0.36 = 833). These calculations verify the safety of cassia gum containing levels of anthraquinones of no more than 10 ppm, when used at the anticipated levels in the foods specified in the intended use table in section 1.0, above.

6.3 Biological Studies on Cassia Impurities: Anthraquinones and Carcinogenicity

6.3.1 Mutagenicity of anthraquinones

A high percentage of natural hydroxyanthraquinones are mutagenic in the Ames test, including *S. typhimurium* tester strains TA1537, TA1538, and TA98. The hydroxyl substituents have been theorized to be necessary for the mutagenicity of anthraquinones, with the optimal substitutions being 1-3 hydroxy groups per molecule (Tikkanen *et al.,* 1983). The plasmid-modified strain TA2637 is considered to be particularly sensitive and suitable for testing the mutagenicity of anthraquinones. Chrysazin, emodin, islandicin, alizarin, chrysophanol, 2-

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70 ppm in the cassia gum, the diet therefore contained 0.53 ppm anthraquinones (0.0075 x 70 ppm = 0.53 ppm or 0.03 mg/kg bw/day). This dose was too low to produce a muscle toxicity effect, and also well below the capacity of anthraquinones to produce weight loss.

hydroxyanthraquinone and emodic acid were strong mutagens in strain TA2637 with metabolic activation (Tikkanen *et al.,*1983). The phenolic anthraquinones exhibit broadly based frame shift mutagenicity in *Salmonella* and DNA damage in *Bacillus subtilis* with few requirements for exogenous mammalian activation. A clear DNA damaging potential was demonstrated for 1,8-hydroxyanthraquinones (Mueller *et al.,* 1996). There are few obvious structural correlations with mutagenic potency (Brown, 1979).

Methanol extracts of sicklepod were found to be mutagenic in the Ames test without activation by S9, with TA98 and TA2637 being the most sensitive tested strains (Friedman and Henika, 1991). The extracts required hydrolysis to be maximally effective (a 15-fold increase in activity vs. unhydrolyzed extract). Emodin, an important anthraquinone in sicklepod, is also present in rhubarb extracts and partially responsible for the mutagenicity of these extracts (Van der Hoeven *et al.*, 1983). The extracts of many commonly consumed fruits and vegetables are mutagenic (NAS, 1996).

Emodin, aloe-emodin, and danthron (1,8-dihydroxyanthraquinone) have been found to be genotoxic in mammalian cells without exogenous activation (Mueller *et al.,* 1996; Krivobok *et al.,* 1992). Emodin and danthron are genotoxic in mouse lymphoma L5158Y cells (Mueller *et al.,* 1998).

Phenolic anthraquinones have been shown to bind to calf thymus DNA (Swanbeck *et al.*, 1966). Several phenolic anthraquinones are reported to damage DNA in the *Bacillus subtilis* rec M45 assay (Kada *et al.*, 1978). On the other hand, mutagenic anthraquinones have been found not to bind covalently with rat liver DNA (Bosch *et al.*, 1987). According to Mueller *et al.*, anthraquinone-induced mutagenicity is, at least in part, mediated by non-covalent DNA binding (Mueller *et al.,* 1998).

6.3.2 Anti-mutagenicity and anti-carcinogenicity of anthraquinones

Several studies have shown that water extracts of the seeds of *C. tora* possess anti-mutagenic effects on mutagens in food in both the Ames test and the comet assay (Wu *et al.*, 2001). Moreover, three of the anthraquinones in the water extract of *C. tora*—chrysophanol, emodin, and rhein—possess anti-genotoxic activity that suppresses DNA damage induced by benzo[a]pyrene (B[a]P) in cells (Wu *et al.,* 2001). All 19 tested anthraquinones inhibited mutagenicity induced by 2-amino-3-methylimidazo[4,5-f]quinoline in *S. typhimurium* TA 98 (Edenharder *et al.*, 1998). Most phenolic anthraquinones, with the exception of anthraquinone

itself, were potent inhibitors of mutagenicity induced by 2-nitroflourene, 3-nitrofluoranthene, and 1-nitropyrene in *S. typhimurium* TA 98 (Edenharder and Tang, 1997). There are several other reports of anti-carcinogenicity, anti-mutagenicity, enzyme inhibition of carcinogen-activating enzymes and DNA damage suppression by natural anthraquinones (Takahashi *et al.*, 2001; Marczylo *et al.*, 1999; Choi *et al.*, 1997; Sun *et al.*, 2000).

6.3.3 Significance of mutagenic, anti-mutagenic, and anti-carcinogenic activity of anthraquinones

These conflicting studies make it difficult to predict the potential carcinogenicity of the anthraquinones potentially present in cassia gum. Given the combination of both mutagenic and anti-mutagenic properties, it is unlikely that the mutagenicity or carcinogenicity of a mixture of anthraquinones will be purely additive. It is more likely that there will be inhibition of activity, in keeping with the reports above. Indeed, in a micronucleus assay by Mueller *et al.*, the addition of unfiltered lettuce extract completely abolished the micronucleus frequency induced by danthron, which served as a positive control for the assay (Mueller *et al.,* 1999). The authors stated that, based on these data, and "together with the strong epidemiological evidence for a cancer protective effect of vegetable consumption, it could be postulated that mutagenicity from substances taken up with vegetables is more than compensated by protective effects" (Mueller *et al.,* 1999).

It should also be noted that the mutagenic activity of many anthraquinones may not be a reliable indicator of their likely carcinogenicity. For example, danthron and emodin were found to be mutagenic in *Salmonella* strain TA1537 with or without metabolic activation (Krivobok *et al.*, 1992). Danthron and emodin were also found to be mutagenic in mammalian cells (Mueller *et al.*, 1996). Yet emodin is not carcinogenic in rats or mice, while danthron is clearly carcinogenic (see Section 6.3.4 below). Therefore, positive mutagenicity data on anthraquinones do not necessarily mean that such compounds are carcinogenic.

6.3.4 Carcinogenicity of the class of anthraquinones

Only three of the known phenolic anthraquinones, in addition to 9,10-anthraquinone, *per se*, have been tested in cancer bioassays.[34] From these sparse results, it appears that some of the dihydroxy anthraquinones are weak carcinogens. The available data are discussed below.

6.3.4.1 Emodin

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) was recently tested in NTP bioassays in rats and mice (NTP, 2001). Emodin was fed to groups of male and female F344/N rats at dietary levels of 280, 830 and 2500 ppm (equivalent to average daily doses of 110, 320, or 1000 mg/kg in males and 120, 370, or 1100 mg/kg in females) for 105 weeks. Emodin was fed to groups of $B6C3F_1$ mice at 160, 312 and 625 ppm in males (equivalent to average daily doses of 15, 35, or 70 mg/kg) and at 312, 625, and 1250 ppm in females (equivalent to average daily doses of 30, 60, or 120 mg/kg). No evidence of carcinogenic activity for emodin was found in either male rats or female mice. Equivocal evidence was reported in female rats based on a marginal increase in the incidence of Zymbal's gland carcinoma. Equivocal evidence also was found in male mice based on a low incidence of uncommon renal tubule neoplasms.

Notably, negative trends were observed in the incidences of mononuclear cell leukemia in male and female rats. In females exposed to 2,500 ppm, the incidence was below the historical control range; the incidence in males exposed to 2,500 ppm was at the lower end of the historical control range.

When no higher level of evidence than "equivocal" is found in its testing, NTP classifies such studies as negative. This is evidenced by the fact that NTP includes emodin on a list of compounds posted on its website which it describes as "Positive in *in vitro* (Salmonella) tests and negative in both rodent species in the 2-year Carcinogenesis Study."[35]

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[34] NTP has conducted testing on several other anthraquinone compounds, including 1-amino-2,4-dibromoanthraquinone; 2-methyl-1-nitroanthraquinone; 1-amino-2-methylanthraquinone; 2-aminoanthraquinone; and 1,4,5,8-tetraaminoanthraquinone. Because of the presence of substituent groups that are not present in the naturally occurring anthraquinones in cassia, the data on these compounds are not directly relevant to the safety assessment of cassia gum.

[35] This list is available on-line at the NTP website at the following address: http://ntp-server.niehs.nih.gov/cgi/iH_Indexes/ALL_SRCH/iH_ALL_SRCH_Frames.html.

Based on the results of the NTP bioassay, it is reasonable to conclude that even under the extreme conditions of the tests (doses as high as 2,500 ppm for a lifetime) there is no reliable evidence for the carcinogenicity of emodin.

6.3.4.2 Anthraquinone

Anthraquinone also was recently tested by NTP in bioassays in rats and mice. The results of these studies were peer-reviewed by the NTP Technical Reports Review Subcommittee on May 21, 1999, but a final report has yet to be released (NTP, 1999).

The NTP study yielded the following findings: There was *some* evidence of carcinogenicity in male rats based on increased incidences of tumors in the kidney and urinary bladder; in addition, hepatocellular neoplasms may have been related to exposure to anthraquinone. There was *clear* evidence of carcinogenic activity of anthraquinone in female rats based on increased incidences of tumors of the kidney, urinary bladder, and liver. There was *clear* evidence of carcinogenic activity in male and female mice based on increased incidences of liver neoplasms. Thyroid gland follicular cell neoplasms in male and female mice may have been related to anthraquinone exposure.

Exposure to anthraquinone was associated with increases in the incidences of non-neoplastic lesions of the kidney, liver, spleen, and bone marrow in male and female rats, the liver, urinary bladder, and spleen in male and female mice, and the thyroid gland and kidney in male mice. Notably, decreases in the incidences of mononuclear cell leukemia in male and female rats were attributed to exposure to anthraquinone.

It is worth noting that the anthraquinone used in the NTP bioassay has been reported to have contained 9-nitroanthracene, a highly mutagenic polycyclic aromatic hydrocarbon (PAH), as well as other potentially genotoxic impurities (Butterworth *et al.*, 2001). The presence of the nitrated PAH in the test compound may have contributed significantly towards the observed increases in tumor incidences. Indeed, the same authors noted that anthraquinone produced by a process that does not yield 9-nitroanthracene as an impurity was found to be non-mutagenic (Butterworth *et al.*, 2001); this finding supports the theory that the nitroanthracene may have been the carcinogenic agent in the NTP study. For the sake of conservatism, the safety assessment presented here is based on the assumption that the tumor incidences observed in the NTP bioassay were exclusively due to exposure to anthraquinone.

6.3.4.3 1,8-dihydroxyanthraquinone

1,8-dihydroxyanthraquinone (also called chrysazin, or danthron) has been examined for carcinogenic activity in rats (Mori *et al.*, 1986a). Mori *et al.* found that chrysazin produced intestinal tumors in rats when fed at 1% in their diet for a lifetime. Chrysazin also produced tumors in the large intestine and livers of mice (Mori *et al.*, 1986b).

6.3.4.4 1-hydroxyanthraquinone

A cancer bioassay on 1-hydroxyanthraquinone (HA) was also conducted by Mori *et al,* (1990). Exposure to HA produced neoplasms of the colon, liver, and stomach when fed at 1% in the diet for a lifetime. The incidence of intestinal tumors in the HA study exceeded that observed with chrysazin given to rats of the same strain at the same dietary concentration. In addition, HA induced hepatocellular and stomach neoplasms, which were not obtained with chrysazin, suggesting the carcinogenic potential of HA is greater and of a wider spectrum (Mori *et al.,* 1990).

6.3.5 Anthraquinones present in Diagum SR™

The early studies by Crawford *et al.* and by Lewis and Shibamoto reported as many as 15-20 analytical peaks tentatively identified as specific anthraquinones in the raw seeds of *C. obtusifolia* or extracts of seeds before any processing. As discussed previously, the processing involved in producing Diagum SR™ from cassia seeds results in the removal of virtually all of the anthraquinones. (Anthraquinone levels decrease from about 1%, or 10,000 ppm, to no more than 10 ppm, a three-order-of-magnitude reduction in quantity.) The sole anthraquinone detected in Diagum SR™ is physcion (App. II, Tab 7). No known carcinogenic anthraquinones have been found in Diagum SR™ with a method sensitivity of 0.5 ppm. Nonetheless, recognizing that two of the three phenolic anthraquinones that have been tested, as well as possibly anthraquinone itself, have been found to be animal carcinogens, and considering the structural similarities among the various compounds in question, it is reasonable to deem any untested anthraquinones in cassia as suspect. These include physcion and any other anthraquinones that may be present below the 0.5 detection limit. This cautious approach is also dictated by the finding that several anthraquinones are mutagenic, including physcion, although as discussed above, the mutagenicity of the anthraquinones is not a reliable indicator of their carcinogenicity.

6.3.6 Calculation of hypothetical carcinogenic potency

It is possible to calculate individual carcinogenic potencies from the positive cancer studies on the three individual anthraquinones. The calculated potencies are linearized potencies, i.e., each potency is the slope of the dose-response curve from the origin to the lowest reliable dose-response point. This potency is corrected for background response where necessary.

The studies by Mori on 1-hydroxyanthraquinone and 1,8-dihydroxyanthraquinone were conducted using a single dose and, therefore, can provide only crude dose-response information. The NTP studies on the parent anthraquinone involved testing at several doses in both rats and mice and, thus, may provide a more accurate indication of potency. However, the anthraquinone parent compound (containing no hydroxy groups) is the least structurally related to the anthraquinones found in *C. tora* or *obtusifolia*. Moreover, as noted above, the carcinogenic potency of anthraquinone may have been confounded by the presence of genotoxic impurities in the test compound. Given the uncertainties inherent in interpreting the available data, we respectfully submit that the most reasonable approach is to calculate the carcinogenic potencies of each of the three compounds, and use the mean of the three potencies to represent a hypothetical, upper-bound carcinogenic potency of the anthraquinone mixture present in Diagum SR™.

6.3.6.1 Potency of 1-Hydroxyanthraquinone

Sixty male ACI/N rats, 6 weeks old at the commencement, were divided into two groups. Thirty rats were fed HA at a concentration of 1% in the diet for 480 days; the remaining rats were fed basal diet and served as control. Of 29 effective animals fed HA, 25 developed adenomas or adenocarcinomas in the cecum or upper portion of the colon. In addition, liver neoplasms (neoplastic nodules and hepatocellular carcinomas) were observed in 12 rats, while benign stomach tumors were observed in 5 rats. No rats in the control group developed any of these tumor types. The incidences of the large bowel, liver and stomach tumors were all significant compared to control, clearly indicating that HA is carcinogenic in rats.

A dose of 1% or 10,000 ppm is equivalent to 500 mg/kg bw in older rats, based on standard conversion factors. The potency is the ratio of the tumor incidence to the dose. On this basis, the unit risk factor (URF) for each site at which an increased incidence of tumors was reported may be calculated as follows:

$$URF_{colon} = (25/29) \div 500 \text{ mg/kg bw/day} = 0.0017 \text{ (mg/kg/day)}^{-1}$$

$$URF_{liver} = (12/29) \div 500 \text{ mg/kg bw/day} = 0.00083 \text{ (mg/kg/day)}^{-1}$$

$$URF_{stomach} = (5/29) \div 500 \text{ mg/kg bw/day} = 0.00035 \text{ (mg/kg/day)}^{-1}$$

The total carcinogenic potency of HA is then calculated as the total of the unit risk factors for the individual target organs, as follows:

$$URF_{total} = URF_{colon} + URF_{liver} + URF_{stomach} = 0.00172 + 0.00083 + 0.00035$$
$$= 0.0029 \text{ (mg/kg bw/day)}^{-1}$$

The virtually safe dose (VSD), the dose corresponding to an upper-bound lifetime risk of one in one million, is the risk (10^{-6}) divided by the potency, as follows: $10^{-6} \div [0.0029 \text{ (mg/kg bw)}^{-1}] = 3.4 \times 10^{-4}$ mg/kg/day.

6.3.6.2 Potency of 1,8-dihydroxyanthraquinone

Twenty C3H/HeN mice were fed 0.2% chrysazin in the diet for 500 days. All of the 17 effective mice developed adenomatous hyperplasia of the cecum. In addition to the intestinal lesions, 9 mice developed hepatocellular adenoma or carcinoma, as compared to 5 mice in the controls. Both types of lesions were statistically significant. In an earlier paper (Mori *et al.*, 1985), chrysazin was also tested in male ACI rats. Eighteen rats were fed 1% chrysazin for 16 months before the experiment was terminated. Seven of 12 effective animals developed tumors (adenomas or carcinomas) of the colon or cecum compared to none in the controls.

A dose of 0.2% or 2,000 ppm is equivalent to 300 mg/kg bw in older mice (2,000 x 0.15). The unit risk factor for chrysazin in mice may be calculated as follows, correcting for the incidence found in controls:

$$URF_{mice} = (9/17 - 5/17) \div 300 \text{ mg/kg bw/day} = 0.0008 \text{ (mg/kg/day)}^{-1}$$

A dose of 1% chrysazin in rats is equivalent to 500 mg/kg bw/day. The unit risk factor for chrysazin in rats is calculated thus:

$$URF_{rats} = (7/12) \div 500 \text{ mg/kg bw/day} = 0.0012 \text{ (mg/kg/day)}^{-1}$$

The higher potency thus is that calculated for rats. The VSD is calculated as: $10^{-6} \div [0.0012 \text{ (mg/kg bw)}^{-1}] = 8.3 \times 10^{-4}$ mg/kg/day.

6.3.6.3 Potency of anthraquinone

In the NTP study, groups of 50 male and female F344/N rats were fed anthraquinone at dietary levels of 469, 938, 1875, or 3750 ppm (equivalent to average daily doses of 20, 45, 90, and 180 mg/kg in males and 25, 50, 100, and 200 mg/kg in females). Groups of 50 male and female $B6C3F_1$ mice were fed diets containing 833, 2500, or 7500 ppm anthraquinone

(equivalent to average daily doses of 90, 265, or 825 mg/kg in males and 80, 235, or 745 mg/kg in females).

There was clear evidence of carcinogenic activity in male and female mice based on evidence of increased incidence of liver neoplasms. Incidences of liver neoplasms in males (carcinoma, blastoma and adenoma combined) were 26/50, 35/50, 43/50, 48/49. Although the tumor incidence in the controls was high, there is an apparent dose-response. Incidences of liver neoplasms in females (adenoma or carcinoma combined) were 6/49, 30/50, 30/50, 41/49. The liver neoplasms in mice were the most sensitive findings, with females apparently more sensitive than males, although the difference may be due more to variability in the control incidence.

Using the liver tumor incidence in female mice, the URF for anthraquinone is calculated as follows:

$$URF_{female} = (30/50 - 6/49) \div 80 \text{ mg/kg bw/day} = 0.006 \text{ (mg/kg/day)}^{-1}$$

The URF for anthraquinone in male mice may also be calculated for comparison purposes; here, because of the high incidence of liver neoplasms among controls (26/50, or 52%), a simple Abbot's correction for background tumor response is appropriate. The correction to the tumor incidence among low-dose males is shown below (note that the reported tumor incidence, 35/50, is expressed as 70%):

$$[0.7-0.52]/ [1-0.52] = 0.18/0.48 = 0.38$$

Using the corrected tumor incidence, the potency is calculated as follows:

$$URF_{male} = 0.38 \div 90 \text{ mg/kg bw/day} = 0.0042 \text{ (mg/kg/day)}^{-1}$$

Thus, the URF in female mice is confirmed to be greater than that in males.

The potency in female mice corresponds to a VSD of $10^{-6} \div [0.006 \text{ (mg/kg bw)}^{-1}] = 1.7 \times 10^{-4}$ mg/kg/day.

6.3.6.4 Risk assessment for anthraquinones

The potencies (unit risk factors) and VSDs calculated above for those anthraquinones that have been the subject of carcinogenicity bioassays are set forth in Table 6.3 below.

Table 6.3
Potencies and Virtually Safe Doses for Anthraquinones

Compound	Potency $(mg/kg\ bw)^{-1}$	Virtually Safe Dose (mg/kg bw)

Chrysazin	1.2×10^{-3}	8.3×10^{-4}
1-Hydroxyanthraquinone	2.9×10^{-3}	3.4×10^{-4}
Anthraquinone	6.0×10^{-3}	1.7×10^{-4}
Emodin	not carcinogenic	--

As shown above, the VSDs for the anthraquinones range from 1.7×10^{-4} to 8.3×10^{-4} mg/kg bw/day. As chrysasin (1,8-dihydroxyanthraquinone) is the tested compound that is most closely related in structure to the physcion and the other anthraquinones potentially present in cassia (meaning Diagum SR™), the data on this compound would appear to be the most relevant for present purposes.

The 90th percentile estimated daily intake for anthraquinone impurities in cassia gum calculated in Section 5 above is 6×10^{-6} mg/kg bw/day. Based on the risk assessment for chrysazin, the upper-bound calculated risk associated with exposure to the anthraquinones in cassia is calculated as follows:

$$\text{Risk} = 0.0012\ (\text{mg/kg/day})^{-1} \times 6 \times 10^{-6}\ \text{mg/kg bw/day} = 7.2 \times 10^{-9}$$

This risk is equivalent to 8 in one billion. The worst-case risk is extremely small and is insignificant on both an absolute basis and when compared to the risk that would be associated with consumption of anthraquinones from natural sources in the diet.

A somewhat more exaggerated approach to the risk assessment, rather than relying solely on the data pertaining to chrysazin, would be to compute the carcinogenic risk based on the mean of the potencies calculated above for the three anthraquinones. The average of the unit risk factors is 3.4×10^{-3}. The average of the three VSDs is 4.5×10^{-4} mg/kg bw/day. Even using this more conservative approach, the calculated EDI of 6×10^{-6} mg/kg bw/day for the anthraquinones in cassia is still almost two orders of magnitude below the VSD.

The risk assessment we conduct above is based on the calculated linear cancer potencies of the three anthraquinones found to be animal carcinogens. We assumed for this purpose that the anthraquinones present or possibly present in Diagum SR™ are carcinogenic and that, on average, they possess the same potency as the known carcinogenic anthraquinones. These are conservative assumptions for several reasons:

(1) No anthraquinone detected in *C. obtusifolia* seeds, Diagum SR™ or the less purified Diagum CS™ is a known carcinogen. We are assuming the carcinogenicity of physcion and that of any undetected anthraquinones[36] for purposes of the risk assessment.

(2) Emodin, which is present in *C. obtusifolia,* is not carcinogenic in rodents, and emodin is closer in structure to physcion and the other anthraquinones potentially present in Diagum SR™ than to the three anthraquinones known to be carcinogenic.[37] This suggests that many of the anthraquinones theoretically present in cassia, in addition to emodin, are not likely to be carcinogenic. It is therefore very conservative to assume that the anthraquinones possibly present in Diagum SR™, i.e., the entire 10 ppm in the mixture, are carcinogens.

(3) While some of the anthraquinones in *C. obtusifolia* are mutagenic, others are anti-mutagens, i.e., they possess anti-mutagenic activity against known mutagens. By extension, while some of the anthraquinones in *C. obtusifolia* may be carcinogenic, it is equally likely that some are anti-carcinogenic. This hypothesis is supported by the decreased incidences of mononuclear cell leukemia in male and female rats fed either emodin or anthraquinone in the NTP bioassays. Thus, it is unlikely that the potencies of the individual carcinogenic anthraquinones would be additive as assumed above. In other words, the overall potency of the mixture of anthraquinones in cassia, if any of the anthraquinones are carcinogenic, is very likely to be less than the sum of their individual potencies.

(4) The linear cancer potency we have calculated for the anthraquinones assumes that anthraquinones are carcinogenic initiators and that there is some risk at all doses, even at very low levels. But there is scientific literature that indicates that the anthraquinones are more likely to be promoters than initiators. This is also implied by their weak carcinogenicity and low potencies. If the carcinogenic anthraquinones are in fact

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[36] As noted in section 2.6.2, physcion is the only anthraquinone actually detected in Diagum SR™. Other anthraquinones potentially present in Diagum SR™ below the 0.5 ppm detection limit of the analyses of the gum would be those that have been identified in *C. obtusifolia* seeds, i.e., rhein, obtusifolin, emodin, questin, chrysophanic acid and lesser amounts of others (Crawford *et al.*, 1990).

[37] Emodin contains hydroxy groups at the 1, 3, and 8 locations as well as a 6-methyl group. Both chrysophanic acid and physcion also contain hydroxy groups at the 1 and 8 locations in addition to a 3-methyl group. (Physcion additionally contains a 6-methoxy group.)

promoters, high doses are essential to their effect, and the risk of carcinogenic promotion at low dose would be minimal or zero. Promotional effects are typically seen in cancer bioassays at doses high enough to produce signs of toxicity, orders of magnitude above the level at which anthraquinones may be consumed in cassia gum.

(5) We believe the calculations set forth above are so exaggerative that, for all practical purposes, the risk from ingestion of anthraquinones in Diagum SR™ is *de minimis*. Making any one of several assumptions less conservative would readily reduce the calculated risk to 10^{-10} or below; for example, assuming that not all the anthraquinone impurities in Diagum SR™ are carcinogenic, or assuming that the anthraquinone mixture is inhibitory, as is quite possible.

6.3.7 Other exposures to anthraquinones

Hydroxyanthraquinones have been reported to occur in several species of plants (Bombardellii and Pifferi, 1988), suggesting that human exposure to anthraquinones due to the consumption of vegetables and herbs is widespread. Emodin, chrysophanic acid (chrystophanol), and physcion were detected in several vegetables and herbs in concentrations up to 36 ppm, with physcion being the most abundant anthraquinone present (Mueller *et al.*, 1999). Emodin, chrystophanol and physcion (as free anthraquinones) were found in lettuce, green beans and peas (*id.*). On a dry basis, the levels of total anthraquinones in the vegetables ranged from 21 ppm to 342 ppm. Based on the highest concentrations detected, a serving of 100 g of beans would contain approximately 4 mg of anthraquinones. By comparison to these levels, the EDI for total anthraquinones (free and glycosidic) from the estimated intake of Diagum SR™ is (0.3 mg gum/kg bw/day) x (10×10^{-6} mg AQ/mg gum) = 3×10^{-6} mg AQ/kg bw/day, or 0.3 μg/p/d. It is apparent that the background exposure to anthraquinones that occur naturally in food is many times the anticipated intake from Diagum SR™.

It is also useful to note that anthraquinones are used in phytotherapeutic drugs (laxatives, sedatives, and anti-kidneystone and anti-bladderstone agents). They are also used in food and cosmetics as coloring agents (Simi *et al.*, 1995). Hydroxyanthraquinones: emodine, aloe-emodin and rhein are contaminants in laxatives produced from senna *(C. senna)*, frangula bark (*Rhamnus frangula*), and aloe (Thompson, 1986). According to Kabelitz and Reif, 1994, a maximum of 4 mg of anthraquinones per day may be ingested from senna drugs. For the herbs tested, the anthraquinones were present below 1.0 ppm levels. The liquors that contained rhubarb root

extracts as a part of their base contained the three anthraquinones, but typically less than 1.0 ppm in all. The authors noted, however, that their method measured only free non-glycosidic anthraquinones. Anthraquinone glycosides could be present at up to 10-fold higher concentrations as determined for emodin in senna (Grimminger and Witthohn, 1993).

The current dietary exposure to the anthraquinones, i.e., the chronic intake level, is far lower than these acute drug exposures and, while not precisely known, can be approximated. From the data in Mueller *et al.*, the concentration of anthraquinones is probably less than 10 ppm in most anthraquinone-containing vegetables (free and glycosidic anthraquinones, wet basis) (Mueller *et al.*, 1999). Since consumption of vegetables averages less than 200 g/p/d in the U.S. diet and anthraquinone-containing vegetables are reasonably less than 20% of this, or 40 g/p/d, a reasonable estimate of the average dietary intake of anthraquinones would be calculated as:

10 mg AQ/kg vegetable x 0.04 kg vegetable/day = 0.4 mg/p/d.

Thus, 0.4 mg/p/d is the calculated background intake of anthraquinones from the diet. By contrast, the estimated increment from Diagum SR™ is just 0.36 μg/p/d, three orders of magnitude less than the background level. Clearly, this represents an insignficant incremental increase in the cumulative intake of anthraquinones and in the corresponding toxicological risk.

* * *

The foregoing and attached information considered, it is respectfully submitted that, under the conditions of intended use cassia gum is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because it is generally recognized as safe.

7.0 REFERENCES

Bertens A.M.C., Aigner A., and Frijters C.W. (1998) Evaluation of the ability of Diagum CS to induce chromosome aberrations in cultured peripheral human lymphocytes. Unpublished report.

Bombardelli E. and Pifferi G. (1988) Naturally occurring anthraquinone derivatives. *Bull. Chim. Farm.* **127**: 3-12.

Borzelleca J.F., LaDu B. N., Senti F. R., and Egle J. L., Jr. (1993) Evaluation of the safety of tara gum as a food ingredient: A review of the literature. *J. of the Am. Coll. of Toxicol.* **12**:81-89.

Bosch *et al.* (1987) Investigations on DNA binding in rat liver and in Salmonella and on mutagenicity in the Ames test by emodin, a natural anthraquinone. *Mutat. Res.* **188**:161-168.

Busschers M. and Aigner A. (1998) Assessment of contact hypersensitivity to Diagum CS in the albino guinea pig (maximization test). Unpublished report.

Butterworth, B.E., *et al.* (2001) The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene. *Mutagenesis* **16**:169-77.

Brown J.P. (1980) A review of the genetic effects of naturally occurring flavanoids, anthraquinones and related compounds. *Mutat. Res.* **75**:243-277.

Choi J.S., Lee H.J., Park K.Y., Ha J.O. and Kang S.S. (1997) *In vitro* antimutagenic effects of anthraquinone aglycones and napthohpyrone glycosides from *Cassia tora. Planta Med.* **63**:11-14.

Cox G. E., Baily D. E., and Morgareidge K. (1974) Subacute feeding in dogs with a pre-cooked gum blend. Unpublished report from the Food and Drugs Labs, Inc. Submitted to the World Health Organization by Hercules BV.

Crawford L. and Friedman M. (1990) The effects of low levels of dietary toxic weed seeds (jimson weed) *Datura stramonium* and (sicklepod) *Cassia obtusifolia*, on the relative size of rat liver and levels and function of cytochrome P-450. *Toxicol. Letters* **54**: 175-181.

Crawford L., McDonald G. M., and Friedman M. (1990) Composition of sicklepod (*Cassia obtusifolia*) toxic weed seeds. *J. Agric. Food Chem.* **38** (12):2169-2175.

Dekant W. (2002) Identification and Quantitation of Anthraquinones in Cassia Gum. Unpublished report from the Julius-Maximilians-Universistät Würzburg.

Denkler M. (1997) Based on U.S. Pat. No. 4 743 659; Derivates of Cassia. Freedom Chemical Diamalt GmbH. Private communication, January 31.

Dugan G.M. and Gumbmann M.R. (1990) Toxicological evaluation of sicklepod and black nightshade seeds in short term feeding studies in rats. *Food Chem. Toxicol.* **28** (2):101-7.

Edenharder and Tang (1997) *Food & Chem. Toxicol.* **35**:357-372.

Edenharder R., Speth C., Decker M. and Platt K.L. (1998) The inhibition of napthoquinones anthraquinones of 2-amino-3-methylimidazo4'5-f]quinoline metabolic activation to a mutagen: a structure activity relationship study. *Z. Lebensm.. Unters. Forsch.* **307**:464-471.

Food and Drug Administration (FDA) (1993) Priority-based assessment of food additives (PAFA) database. Center for Food Safety and Applied Nutrition. p. 58.

Food Chemicals Codex. 1996. Fourth Edition. National Academy Press, Washington, D.C.

Friedman, M. and Henika P.R. (1991) Mutagenicity of toxic weed seeds in the Ames test: Jimson Weed (*datura stramonium*), velvetleaf (*abutilon theophrasti*), morning glory (*ipomoea spp,*) and sicklepod (*Cassia obtusifolia*). *J. Agric. Food Chem.* **39**(3), 494-501.

Graham S. L., Arnold A., Kasza L. *et al.* (1981) Subchronic effects of guar gum in rats. *Food Chem. Toxicol.* **19**:287.

Graziano M.J., Flory W., Seger C.L., Hebert C.D. (1983) Effects of a *Cassia occidentalis* extract in the domestic chicken (*Gallus domesticus*). *Am. J. Vet. Res.* **44**(7), 1238-1244.

Grimminger W and Witthohn K (1993) Analytics of senna drugs with regard to the toxicologic discussion of anthroids. *Pharmacol.* **47**:(Supp. 1) 98-101.

Hallagan J.B., La Du B.N., Pariza M.W., Putnam J.M., and Borzelleca J.F. (1997) Assessment of Cassia gum. *Food Chem. Toxicol.* **35**:625-632.

Hebert C.D., Flory W., Seger C., Blanchard R.E. (1983) Preliminary isolation of a myodegenerative toxic principle from *Cassia occidentalis. Am. J. Vet. Res.* **44**(7), 1370-1374.

Henson J.B., Dollahite, J.W., Brigdes, C.H. and Rao (1965) Myodegeration in cattle grazing *Cassia* species. *J. Am. Vet. Med. Ass'n* **147**:142-147.

Ikegami S., Tsuchihashi F., Harada H., Tsuchihashi N., Nishide E., and Innami S. (1990) Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats. *J. of Nutr.* **120**:353-360.

JECFA (1981) WHO/FAO Reviews of Carob (locust) bean gum and Guar gum Listed in Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives, INS 410, 1981 and INS 412, 1975.

Kabelitz and Reif (1994) Anthranoide in Sennesdrogen. *Deutsch Apothesker Zeitung* **134**:5085-5088.

Kada T.Y., Sadaie T.Y., and Hara M. (1978) Analysis of mutagen–antimutagen reactions in food and food additives by the rec assay and reversion-assay procedures. *Mutat. Res.* **53**: 206-207.

Klose R.E. and Glicksman, M. (1968) Gums. In *Handbook of Food Additives*. Thomas E. Furia, editor. pp. 335-337. The Chemical Rubber Co., Ohio.

Koshioka M. and Takino Y. (1978) Studies on the evaluation of crude drug I. Quantitative estimation of anthraquinones in *Cassia* seeds. *Chem. Pharm. Bull.* **25**:(5) 1345-1349.

Krantz J. C., Jr., Carr C. J., and de Farson C. B. (1948) Guar polysaccharide as a precursor of glycogen. *J. Am. Diet. Ass'n* **24**:212.

Krivobok S., Seigle-Murandi F., Steiman R., Marzin D.R., and Betina V. (1992) Mutagenicity of substituted anthraquinones in the Ames/Salmonella microsome system. *Mutat. Res.* **279**:1-8.

Leimgruber R. and Marbot H. (1991) Application for the GRAS evaluation of Cassia gum. Registration and Consulting Company, Ltd., Basel, Switzerland. RCC Project 250424, September 2.

Letter from Dr. George Graber, Center for Veterinary Medicine, to John Hallagan (January 19, 1999).

Leung A. Y. and Foster S. (1996) Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics. John Wiley and Sons, Inc., New York.

Lewis, D.C. and Shibamoto, T. (1989) Effects of *Cassia obtusifolia* (sicklepod) extracts and anthraquinones on muscle mitochondrial function. *Toxicon.* **27**:(5), 519-529.

Loung R.-Z. and McCoy C.P. (1992) Experimentally induced *Cassia obtusifolia* poisoning in rabbits. *J. Clin. Soc. Vet. Sci.* **18**(4) 199-208.

Marczylo T., Hayatsu T., Arimoto-Kobahshi S., Tada M., Fujita T., Kamataki T., Nakayama K., Hayatsu H (1999) Protection against the bacterial mutagenicity of hetrocyclic amines burpurin, a natural anthrquinone pigment. *Mutat Res* **444**:*451-46.*

Marks G.B., Salome C.M., and Woolcock A.J. (1991) Asthma and allergy associated with occupational exposure to Ispaghula and Senna products in a pharmaceutical work force. *Am. Rev. Respir. Dis.* **144**:1065-69.

Mathews C.K. and van Holde K.E. (1990) *Biochemistry.* The Benjamin/Cummings Publishing Company, Inc., New York.

Maxwell W.A., and Newell G.W. (1972) Study of the mutagenic effects of FDA-71-12 (Locust bean gum). Unpublished report from the Stanford Research Institute submitted to the World Health Organization by Hercules BV.

McCormack J. and Neisler W. (1980) *Cassia obtusifolia* (sicklepod) toxicity in a dairy herd. *Vet. Med. Small Animal Clinic* **75**:849.

McIntyre M.D. (1990) Two generation oral (dietary administration) reproduction toxicity study in the rat. Hazelton France Report No. 710791. Unpublished, June 26.

Morgan-Hughes J.A. *et al.* (1979) Mitochondrial myopathy with a deficiency of respiratory-chain NADH-CoQ reductase activity. *J. Neur. Sci.* **43**:27.

Mori H., Sugie S., Niwa K., Takahashi M. and Kawai K.. (1986a) Induction of intestinal tumors in rats by chrysazin. *Br. J. Cancer* **52**:781-783.

Mori H., Sugie S., Niwa K., Yoshimi N., Tanaka T., and Hirono I. (1986b) Carcinogenicity of chrysazin in large intestine and liver of mice. *Jpn. J. Cancer Res.* **77**:871-876.

Mori H., Yoshimi N., Iwata H., Mori Y., Hara A., Tanaka T., and Kawai K. (1990) Carcinogenicity of naturally occurring 1-hydroxyanthraquinone in rats: induction of large bowel, liver and stomach neoplasms. *Carcinogenesis* **11**:799-802.

Müller W. (1989) Oral (gavage) teratogenicity study in the rat. Hazelton Laboratories Report No. 725-14/50, Unpublished, November 23.

Mueller S.O., Eckert I., Lutz W.K., and Stopper H. Genotoxicity of the laxative components emodin, aloe-emodin and danthron in mallian cells, topoisomerase II mediated? (1996) *Mutat. Res.* **371**:165-167.

Mueller S.O., Lutz W.K., and Stopper H.. (1998) Factors affecting the genotoxic potency ranking of natural anthraquinones in mammalian cell culture systems. *Mutat. Res.* **414**: 125-129.

Mueller, S.O., Schmitt, Dekant W., Stopper H., Schlatter J., Schreier P., and Lutz W.K. (1999) Occurrence of emodin, chrysophanol and physcion in vegetables, herbs and liquors. Genotoxicity and anti-genotoxicity of the anthraquinones and of the whole plants. *Food Chem. Toxicol.* **37**:481-91.

NAS (1996) *Carcinogens and Anticarcinogens in the Human Diet.* Committee on Comparative Toxicity of Naturally Occurring Carcinogens, National Research Council, National Academy Press, Washington, D.C.

National Toxicology Program (NTP; 1981) Carcinogenesis bioassay of locust bean gum in F344/N rats and B6C3F1 mice (feed study). NTP-80-66.

NTP (1982a) Carcinogenesis bioassay of guar gum in F344/N rats and B6C3F1 mice (feed study).

NTP (1982b) Carcinogenesis bioassay of tara gum. NTP-TR-224.

NTP (1999) Carcinogenesis studies of anthraquinone (CAS No. 84-65-1) in F344/N rats and $B6C3F_1$ mice (feed studies). TR-494. (Draft report dated May 1999.)

NTP (2001) Toxicology and Carcinogenesis Studies of Emodin (CAS NO. 518-82-1) Feed Studies in F344/N Rats and $B6C3F_1$ Mice. TR-493. (Final report dated June 2001).

Nicholson S.S., Flory W., and Ruhr L.P. (1986) *Cassia obtusifolia* poisoning in cattle: a new development. *La. Agric.* **29**:18-19.

Nicholson S.S., Thornton J.T., Rimes A.J., Jr. (1977) Toxic myopathy in dairy cattle caused by *Cassia obtusifolia* in greenchop. *Bovine Pract.* **12**:120.

Nurnberg E. and Bleimuller G. (1981) Development of a galactomannan product for tablets. *Pharmazeutische Industrie* **43**:1238-42.

Nyman M. and Asp N. (1982) Fermentation of dietary fibre components in the rat intestinal tract. *British Journal of Nutrition* **47**:357-66.

OECD (1981) Principles of Good Laboratory Practice C (81) 30 (Final), 12 May, Organisation for Economic Cooperation and Development, Paris.

O'Hara P. and Pierce K. (1974a) A toxic cardiomyopathy caused by *Cassia occidentalis*. 1 Morphological studies in poisoned rabbits. *Vet. Path.* **11**:97

O'Hara P. and Pierce K. (1974b) A Toxic Cardiomyopathy caused by *Cassia occidentalis.* II. Biochemical Studies in Poisoned Rabbits. *Vet. Path.* **11**:110-24.

Poethke W., Rao D.A., and Loscher K.D. (1968) *Pharm. Zenthralh.* **107**:571.

Putnam M. R., Boosinger T., Spano J., Wright, J., and Wiggins A. (1988) Evaluation of *Cassia obtusifolia* (sicklepod) Seed consumption in Holstein Calves. *Vet. Hum. Toxicol.* **30**(4), 316-18.

Randell B.R. (1995) Taxonomy and evolution of *Senna obtusifolia* and *S. tora. J. Adelaide Bot. Gard.* **16**:55-58.

Robaislek E (1974) Bioavailable calorie assay of Guar gum, Unpublished report from WARF Institute, Inc. Submitted to WHO by Institute Européen des Industries de la Gomme de Caroube, cited by JECFA 19th Report Techn. Rep. Ser. 1975, No. 55.

Satheesh R., Prakashkumar R., Jose J.C., Nair P.K.K., and Rao G.R. (1994) Studies on Cassia pollen grains of India. *Int. Arch. Allergy Immunol.* **103**:280-285.

Schering A.G. (1986) Acute toxicity of Mucigel X-18-H in male rats. Unpublished report.

Schuh W. (1990) Systemic tolerance study in Beagle-dogs after daily oral (dietary) administration over a period of 90 days. Schering Study No. TX 88.404. Unpublished report, February 1.

Semenza G. (1975) Report on the possible digestion of locust bean gum in the stomach and/or in the small intestine in an *in vitro* study. Unpublished report from the Eidgenssische Technische Hochschule Zurich submitted to the World Health Organization by the Institut European des Industries de la Gomme de Caroube.

Shah N., Mahoney R. R., and Pellett P. (1986) Effect of guar gum. lignin and pectin on proteolytic enzyme levels in the gastrointestinal tract of the rat: a time-based study. *J. of Nutr.* **116**:786-94.

Simi S., Morelle S., Gervasi P.G. and Rainaldi G. (1995) Clastogenicity of anthraquinones in V79 and in three derived cell lines expressing P450 enzymes. *Mutat. Res.* **347**:151-156.

Steget A., Pethran A., Radon K., Praml G., and Nowak D. (1999) Studies on the risk to workers' health during the production of thickening agents made from natural products; including ground cassia with special regard for pulmonary function and allergic diathesis. Institute and Clinic for Industrial and Environmental Medicine at the University of Munich. Confidential Final Report 07.09.99.

Suliman H.B. and Shommein A.M. (1986) Toxic effects of roasted and unroasted beans of *Cassia occidentalis* in goats. *Vet. Hum. Toxicol.* **28**(1):6-11.

Sun M., Sakakibara H., Ashida H., Danno G., and Kanazawa K. (2000) Cytochrome P4501A1-inhibitory action of antimutagenic anthrquinones in medicinal plants and the structure-activity relationship. *Biosci. Biochem.* **64**(7):1373-1378.

Swanbeck G. (1966) Interaction between deoxyribonucleic acid and some anthracene and anthraquinone derivatives. *Biochem. Biophys. Acta.* **123**:630- 683.

Takahashi *et al.* (1978) Formation of anthraquinones by the tissue culture of *Cassia obtusifolia* *Planta Med.* **33**:389.

Takahashi H., Yang S. I., Fujiki M., Kim M., Yamamoto T., and Greenberg N. A. (1994) Toxicity studies of partially hydrolyzed guar gum. *J. of the Am. Coll. of Toxicol.* **13**:273-78.

Takahashi E., Marczylo T.H., Wantanabe T., Nagai S., Hayatsu H. and Negish T. (2001) Preventive effects of anthraquinone food pigments on the DNA damage induced by carcinogens in Drosophila. *Mutat. Res.* **480-481**:139-145.

Takido M. (1958a) Studies on the constituents of the seeds of *Cassia obtusifolia* L 1. The structure of obtusifolin. *Chem. Pharm. Bull.* **6**:397.

Takido M. (1958b) Studies on the constituents of the seeds of *Cassia obtusifolia* L 2. The structure of obtusin, chryso-obtusin, and aurianto-obtusin. *Chem. Pharm. Bull.* **8**:246.

Thompson, R.H. (1986) *Naturally Occurring Quinones III Recent Advances.* Chapman and Hall London.

Tikkanen L., Matsushima T. and Natori S. (1983) Mutagenicity of anthraquinones in the Salmonella preincubation test. *Mutat. Res.* **116**:297-304.

Til H. P., Spanjers M., and de Groot A. P. (1974) Sub-chronic toxicity study with locust bean gum in rats. Unpublished report from Centraal Instituut voor Voedingsondertoek TNO submitted to the World Health Organization by Hercules BV and Instituut Europeen des Industries de la Gamme de Caroube.

Trowell H., Southgate D.A.T., Wolever T.M.S., Leeds A.R., Gassull M.A., and Jenkins D.J.A. (1976) Dietary fibre redefined. *The Lancet* p. 967, May 1.

Tsai L.B. and Whistler R.L. (1975) Digestibility of galactomannans. Unpublished report submitted to the World Health Organization by Professor H. Neukom, Chairman of the Technical Committee of Institute of Europe des Industries de la Gomme de Caroube.

Upadhyaya S.K. and Singh V. (1986) Phytochemical evaluation of *Cassia obtusifolia* and *Cassia tora. Proc. Indian Acad. Sci., Plant Sci.* **96**:321-26.

Van der Hoeven J.C., Lagerwij W.J., Bruggeman I.M., Voragen F.J., Koeman J. (1983) Mutagenicity of extracts of some vegetables commonly consumed in the Netherlands. *J. Agric. Food Chem.* **31**:1020-26.

Verspeek-Rip C.M., Aigner A., and Giepmans N.P. (1998a) Evaluation of the mutagenic activity of Diagum CS in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat). Unpublished report.

Verspeek-Rip C.M., Aigner A., and van Oort G. (1998b) Evaluation of the mutagenic activity of Diagum CS in an *in vitro* mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat). Unpublished report.

Virat M. (1984) Thirteen week toxicity study in the cat by the oral route. Institut Francais de Toxicologie Report No. 411233, Unpublished, November 21.

Virat M. (1989) One-generation reproductive toxicology and subchronic toxicity study in cats. Hazelton France Report No. 702586, Unpublished Draft Report, November 20.

Wu Chi-Hao, Hsieh C., Song, Yen G. (2001) Inhibitory effects of *Cassia Tora* L. on Benzo[a]-pyrene-mediated DNA Damage toward HepG2 cells. *J. Agri. Good Chem.* **49**:2579-2586.

Zühlke U. (1990) Twenty-eight day oral (dietary administration and gavage) range-finding subchronic toxicity study in the rat. Hazelton Laboratories Report No. 711-14/48, Unpublished, April 25.

Appendix 1

APPENDIX I

CONCLUSIONS OF EXPERT PANEL

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized in the Notification for cassia gum and conclude that cassia gum produced in accordance with current good manufacturing practices and meeting the food grade specifications described therein is GRAS by scientific procedures for use as a thickening agent, to improve texture and for water retention in the specified foods.

(b)(6)

14 November 2003

Joseph F. Borzelleca, Ph.D.
Professor, Medical College of Virginia

Michael Pariza, Ph.D.
Professor, University of Wisconsin-Madison

APPENDIX I

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Appendix-2

APPENDIX II

SPECIFICATIONS, ANALYTICAL METHODS,

CERTIFICATES OF ANALYSIS

1

Specifications for Diagum SR Tab 1

SPECIFICATION

DIAGUM SR

1. Generic Name	**Cassia Gum**
Origin	**Endosperm of Cassia tora and Cassia obtusifolia**
Chem. Identity	**high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TESTMETHOD*	*TEST-No*
2. Material Description			
Appearance	fine powder	visually	SR 00201
Consistency	Dry, free flowing	Visually	SR 00202
Colour	Off-white to beige	visually	SR 00301
Odour	Neutral to slightly fruity	sensorically	SR 00302
Taste	neutral	sensorically	SR 00303
3. Identity	Synegistic gel with xanthan	Gelling performance	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	Kjeldahl method	SR 014
Fat content	NMT 1%	Butt-type Extraction	SR 015
Ash (total	NMT 1.2%	FCC IV	SR 017
Loss on drying	NMT 12 %	105 °C / 4 h	SR 00502
pH-Value	5,5 - 8,5	Calibrated pH-meter	SR 00602
Gel-strength	NLT 155 g	FIRA-Jelly Tester	SR 021
Viscosity (1%)	NLT 250 mPas	Brookfield RVT	SR 04201
Particle size <250µ	NLT 99 %	sieve DIN 3310	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	HPLC Method	SR 02402
Isopropyl Alcohol	NMT 0.075 %	FCC IV p 438	SR 008
Heavy Metals	NMT 0.002%	FCC IV pp 760-61	SR 010
Lead	NMT 5 mg Pb/kg	FCC IV pp. 762 ff.	SR 011
Arsenic	NMT 3 mg As/kg	FCC IV pp. 755 ff.	SR 012

Specification approved:

16/07/05 (b)(6)

000079

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)		Quality Assurance Test.No.: SR 002 ff Version: 01 Author: Dr.Utz Issued: 04.07.03
	APPEARANCE AND CONSISTENCY	page 1 of 3

CONTENTS

I Principle

II Definition

III Equipment

IV Procedure

V Visual designation
a) appearance (SR 00201)
b) consistency (SR 00202)

000080

Test method SR 002ff V01	Date: 07/07/03	Approved: (b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 002 ff Version: 01 Author: Dr.Utz Issued: 04.07.03
APPEARANCE AND CONSISTENCY	page 2 of 3

I PRINCIPLE

Visual method

II DEFINITION

A visual designation is descriptive only and is not to be regarded as a standard of purity for a particular lot of an article.

III EQUIPMENT

1	white paper sheet	e.g DIN A 3
2	spatula	e.g. broad spatula
3	graduated cylinder	100 ml e.g. Duran®

IV PROCEDURE

Note:
use a good vented hood to avoid dust

000081

1 substance to be tested / 100 ml
pour onto white paper sheet

Test method SR 002ff V01	Date: 07/07/03	Approved: (b)(6)

2	spread / plane surface	broad spatula
3	test / structure / flowing / lumps / mechanical impurities	

V ViSUAL DESIGNATION

a) Appearance (SR00201)

1 *Test result:*
fine powder,without lumps and mechanical impurities

b) Consistency (SR 00202)

1 *Test result:*
dry,free flowing

Requirement see specification

000082

Test method	Date:	Approved:
SR 002ff V01	07/07/03	(b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)		Quality Assurance Test.No.: SR 003 ff Version: 030704 Author: Dr.Utz Issued: 04.07.03
	COLOUR,ODOUR AND TASTE	page 1 of 3

CONTENTS

I Principle

II Definition

III Equipment

IV Procedure

V Sensory designation
a) colour (SR 00301)
b) odour (SR 00302)
c) taste (SR 00303)

000083

Test method	Date:	Approved:
SR 003 ff V01	07/07/03	(b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 003 ff Version: 030704 Author: Dr.Utz Issued: 04.07.03
COLOUR,ODOUR AND TASTE	page 2 of 3

I PRINCIPLE

Sensory method

II DEFINITION

A sensory designation is descriptive only and is not to be regarded as a standard of purity for a particular lot of an article.

III EQUIPMENT

1	open glass dish	e.g. (Kristallisierschale) Duran ®, ∅ 95mm;height: 55 mm
2	analytical balaSRe	e.g. Mettler PM 4000, readings 0,01g

IV PROCEDURE

1 substance to be tested / 25 g
pour into open glass dish

000084

Test method	Date:	Approved:
SR 003 ff V01	07/07/03	(b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)		Quality Assurance Test.No.: SR 003 ff Version: 030704 Author: Dr.Utz Issued: 04.07.03
	COLOUR,ODOUR AND TASTE	page 3 of 3

V SENSORY DESIGNATION

a) Colour (SR 00301)

1 *Test result:*
off-white

b) Taste (SR 00302)

1 *Test result:*
neutral

b) Odour (SR 00303)

1 *Test result:*
neutral to slightly fruity

1 *Note:*
terms such as "fruity odour" apply to examination,after exposure to the air for 15 min,of a freshly opened package (see also USP XXII,page 7)

Requirement see specification

000085

Test method	Date:	Approved:
SR 003 ff V01	07/07/03	(b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 013 Version: 01 Author: Utz Issued: 08/07/2003
Identity	Page 1 of 1

The identity test is according the test for Xanthan FCC IV, p. 438 with the change, that cassia gum is used instead of LBG:

To 300 ml of water, previously heated to 80°C and stirred rapidly with a mechanical stirrer in a 400 ml beaker, add, at the point of maximum agitation, a dry blend of 1.5 g Xanthan and 1.5 g sample to be tested. Stir until the the mixture goes into solution, and then continue stirring for 30min longer. Do not allow the water temperature to drop below 60°C during stirring.
Discontinue stirring and allow the mixture to cool at room temperature for at least 2 hours. A firm, viscoelastic gel forms after the temperature drops below 40°C, but no such gel forms in a 1% control solution of both the sample and xanthan prepared in the same manner but ommitting the second hydrocolloid.

The related LBG shows the similar gelling properties with xanthan. However, the LBG/Xanthan gels provide at least 20% lower gel strength.

000086

Test method	Date:	Approved:
SR 013 V01	08.07.03	(b)(6)

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 00502 Version: 01 Author: Dr.Utz Issued: 04.07.03
LOSS ON DRYING	page 1 of 4

CONTENTS

I Principle

II Definition

III Equipment

IV Procedure

V Calculation

000092

Test method	Date:	Approved:
SR 00502 V01	07/07/03	(b)(6)

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 00602 Version: 01 Author: Dr.Utz Issued: 04.07.03
pH-Value	page 1 of 5

CONTENTS

I Principle

II Definition

III Equipment

IV Reagents

V Test-solution

VI Set up pH-meter

VII pH-measuring

VIII Calculation

IX Remarks

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TESTING STANDARD

	DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 021 Version: 01 Author: Utz Issued: 08/07/2003
	Gel Strength	Page 1 of 10

CONTENTS

I Principle

II Definition

III Equipment

IV Reagents

V Buffer-solution

VI Testing conditions

VII Making the jelly solution

VIII Gel-testing

IX Calculation

X FIRA jelly tester

000101

Test method	Date:	Approved:
SR 021 V01	08.07.03	(b)(6)

I PRINCIPLE

Cassia gum is forming a gel with carrageenan in a phasphate buffer in the presence of potassium chloride.The resistance of this gel to rupture is measured on the FIRA jelly tester by an immersed paddle which is rotated by 30°.

II DEFINITION

The gel strength is defined as the weight of grams of water required to give a 30° deflection on the FIRA jelly tester.

III EQUIPMENT

1	analytical balance	e.g. Mettler PM 4600 , readings 0.01 g
2	plastic weighing dish	e.g. flexible material / 80 x 80 x 20 mm
3	glass beaker,low form	1000 ml , graduated; e.g. Duran®,
4	magnetic stirring bar	e.g. PTFE coated ,length 50 mm
5	magnetic stirrer with heater	e.g. IKAMAG RET; RT...+300°C; 0...1100 rpm
6	magnetic stirrer without heater	e.g. IKAMAG REO; 0... 1100 rpm
7	hair dryer	e.g. neoLab® hot air blower

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8	thermostatic chamber (or water bath)	e.g. precision incubator Memmert BKE 30; 0...+60°C ;thermostability: 20±0,1 °C
9	pH-meter glass electrode calibration buffer	e.g. Metrohm pH-meter 632 e.g. Metrohm 6.203.100 pH = 7: e.g. Metrohm 6.2307.010 pH = 9: e.g: Metrohm 6.2307.020
10	2" square jelly box	plexiglass (wall-thickness: 4mm ;internal dimensions 50x50x60 mm customer made by: Clark & Smith Manufacturing Melbourne House,Melbourne Road, Wallington,Surrey SM6 8SD,UK Tel. 0181 669 4411
11	FIRA-jelly tester equipped with flow meter	H.A. Gaydon &Co Ltd Clyde Works;Clyde Road, Wallington, Surrey SM6 8PZ / England e.g.Fischer & Porter , type D10A3137N (with needle valve)
12	measuring flask	1000 ml ,with ring mark (class a); grounded joint and polystopper; e.g. Duran® (DIN 12664 T1)
13	laboratory timer	
14	thermometer	20°C,accuracy 0,1°C

Test method	Date:	Approved: (b)(6)
SR 021 V01	08.07.03	

IV REAGENTS

1 distilled/de-ionised water

2 sodium dihydrogen phosphate dihydrate ($NaH_2PO_4x2H_2O$); e.g. Merck 106345 (reinst DAB;BP;USP; / 98,0-100,5 %)

3 anhydrous disodium hydrogen phosphate (Na_2HPO_4); e.g. Merck 106586 (p.a. / min. 99 %)

4 anhydrous potassium chloride (KCl); e.g. Merck 104936 (p.a. / min. 99,5%)

5 standard semi refined carrageenan; Danagel PF 8263
FMC GmbH,Food Ingredients Division,
Hahnstr. 38
60528 Frankfurt

6 5 M sodium hydroxide solution

000104

V BUFFER SOLUTION (pH = 6,60)

1 sodium dihydrogen phosphate dihydrate ($NaH_2PO_4x2H_2O$) / 8,00 g
weigh into 1000 ml measuring flask

2 anhydrous disodium hydrogen phosphate (Na_2HPO_4)/ 5,00 g
add

3 anhydrous potassium chloride (KCl) / 3,00 g
add

4 dist./de-ionised water
add,dissolve,fill up to ringmark

6 check pH pH-meter
pH = 6,60 ± 0,05

NOTE: if pH is outside range,adjust using 5 M sodium hydroxide;if adjusting is not possible,the buffer must be discarded and a fresh batch made up

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VI TESTING CONDITIONS

1	0,08 M phoshate buffer with 0,04 M KCL:	pH= 6,6
2	total gel concentration (cassia gum = 0,3 % ; carrageenan = 0,3%)	0,6 %
3	boiling time (95-100°C)	5 min
4	gel setting time (room temperature :4 hours ; 20°C: 1 hour)	min. 5 hours
5	gel strength determination	FIRA jelly tester

000106

Test method	Date:	Approved: (b)(6)
SR 021 V01	08.07.03	

VII MAKING THE JELLY SOLUTION

NOTE: all safety procedures associated with the apparatus and operations described below are observed on every occasion; use safety goggles and gloves when working with hot solutions.

1 buffer solution (pH=6,60) / 497,00 g
weigh into 1000 ml beaker

2 place beaker on magnetic stirrer and add magnetic stirring bar

3 sample to be tested (Diagum CS) / 1,50 g
weigh into plastic weighing dish

4 standard carrageenan / 1,50 g
add plastic weighing dish

5 switch on stirrer and add slowly gel powder from weighing dish into cold buffer solution

NOTE: add the powder slowly enough to eliminate the possible formation of lumps in the solution

6 determine total weight of [beaker +magnetic stirring-bar+ buffer solution + gel powder]

7 ***Partial measuring result:***
$m_{total} = m_{beaker} + m_{magnetic\ stirring\ bar} + m_{buffer} + m_{gel\ powder}$ in [gram]

000107

Test method	Date:	Approved:
SR 021 V01	08.07.03	(b)(6)

8	switch on heater and bring solution to boil	stirrer with heating unit

NOTE: when boiling a sudden froth might be formed on the surface; to avoid a boiling over,blow gentle with a hot air dryer on the surface of the solution;this will keep down the froth.

9	when temperatur has risen to boiling (95-100°C) start timer and boil for 5 min
10	remove beaker from heating unit and place on cold stirrer
11	stir for 5 min at room temperature
12	place beaker on balance and fill up to total weight with cold buffer solution to compensate for loss on evaparotion
13	stir test solution
14	fill up 3 jelly boxes with test solution while still hot
15	allow the jelly boxes to stand undisturbed for at least 4 hours at room temperature (room temperatur must be below 30°C)
16	place jelly boxes in an incubator at 20±0,1°C for another 1 hour
17	gel ready for gel-strength measurement

000108

Test method	Date:	Approved:
SR 021 V01	08.07.03	(b)(6)

VIII GEL TESTING

NOTE: set up FIRA jelly tester acc. to manual
- allow water to flow through FIRA jelly tester for 5 min before testing
- run water through the tester into the bucket for 2 min and discard
- check flow rate: 100±5 ml / min

1 place FIRA jelly tester bucket on balance and re-set the tara weight;

2 attach bucket on FIRA jelly tester and couterpoise

3 Set scale to zero with the damping brake on SET ZERO

4 place jelly box on the FIRA jelly tester

5 raise platform until paddle penetrates the jelly as far as the lower mark on the shaft of the paddle

NOTE: in penetrating the jelly the paddle might turn slightly and cause a change in zero setting of scale,but this is unimportant.The initial scale reading is noted.

6 release damping brake from SET ZERO to TEST

7 depress water valve key and allow water to flow into bucket

8 stop water flow into bucket immediately scale passes the 30° deflection

000109

9 lock damping brake

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10 detach bucket from tester and place on balance

11 note weight of water

12 ***Measuring result:***
gel strength = weight of water in gram

IX CALCULATION

1 Calculate the average value for gel strength for the 3 jelly boxes.
If the range exceeds 5 g, repeat the test.

X FIRA JELLY TESTER

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)		Quality Assurance Test.No.: SR 04201 Version: 01 Author: Dr.Utz Issued: 04.07.03
	1% Viscosity	page 1 of 7

CONTENTS

I Principle

II Definition

III Equipment

IV Reagents

V Test solution

VI Determination of viscosity
a) qualitative
b) viscometer
c) calculation

000111

Test method	Date:	Approved:
SR 04201 V01	07/07/03	(b)(6)

I PRINCIPLE

The solubility of Cassia gum is tested by dispersing the gum in hot water. After cooling an appreciable increase in viscosity is produced.

The increase in viscosity after boiling is due to the complete hydration and solubilisation of the high molecular Cassia hydrocolloid.

II DEFINITION

The solubility is expressed by the increase in viscosity.

The viscosity can be determined:
qualitative: visual
or quantitative with a viscometer.

III EQUIPMENT

000112

Test method	Date:	Approved:
SR 04201 V01	07/07/03	(b)(6)

1	Porcelain beaker	e.g. 500 ml / (Haldenwanger Berlin / type no 42H-Oa top-∅:100 mm / bottom-∅: 75 mm / height: 130 mm wall thickness: 5 mm
2	stirring rod	e.g. V2A-steel / IKA; type R1363 / rod-∅: 8 mm / rod-length: 320 mm / stirrer (cage-type)-∅: 40 mm
3	high shear mixer	e.g. Heidolph-Rührwerk RZR 1 / 280-2200 rpm / 77W
4	balance	e.g. Mettler PC 4400 , readings 0.01 g
5	heating bath (150°C)	e.g. Universal-Heizbad "UH-EL" / Nr 2025-3101 / temperature range up to 250°C
6	cooling water bath (20°C)	e.g. type Kühlmobil® / Fa. Peter v.d.Heijden, Dörentrop
7	timer	e.g type Junghans
8	wood spatula	e.g. 5 x 25 x 50 mm
9	plastic weighing dish	e.g flexible material / 80 x 80 x 20 mm
10	viscometer	Brookfield RVT, Brookfield - Stoughton (Mass.) USA

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IV REAGENTS

1 Distilled Water

V TEST SOLUTION (1%)

1		place beaker on balance	
2		put stirring rod into beaker	
3		note tara weight of beaker with stirrer	
4	tap water / 297,0 g	weigh	beaker
5	substance to be tested / 3,00 g	weigh	weighing dish

SET UP WATER BATH

1 Pre-heat a water bath to 98 +/- 2°C

---

6 place beaker into water bath

7 start stirrer

NOTE: the stirrer is placed 1 cm off-center and 1 cm above bottom of beaker to obtain maximum turbulance of the lower portion of vortex

000114

Test method	Date:	Approved:
SR 04201 V01	07/07/03	(b)(6)

8 add substance to be tested

note: the thickener should be sifted carefully onto the upper wall of the vortex to avoid lumps.the addition of powder should be completed in about 15 sec

9 heat up 90°C

10 Kepp temperature +/- 2°C for 5 min

11 remove beaker with stirring rod

12 place beaker with stirring rod on balance

13 replace water lost by evaporation

14 stirr and homogenize

15 place in water bath (20°C)

16 allow to stand for 2 hours

NOTE: while cooling stirr in between to avoid skim forming on the surface of the solution

---

VI VISCOSITY

a) Qualitative

1 stirr colloidal solution by hand

2 ***Measuring result:***

Test method SR 04201 V01	Date: 07/07/03	Approved: (b)(6)

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solution is viscous

Requirements: solubility = solution is viscous

---

b) Viscometer

NOTE: set up Brookfield viscometer RVT according to manual / adjust speed control knob to 20 rpm / check bubble level / use appropiate spindle

1	Test solution (1 %) / 20°	remove from water bath
2		stirr with wood spatula 5-10 times
3		wait for 1-2 minutes
4		insert spindle No. 2 in test solution to groove cut
5		start Brookfield viscometer RVT with 20 rpm
6		wait 5-10 revolution of dial or until pointer has stabilized
7		note pointer's position on dial (between 0 and 100% full scale)
8	***First measuring result:*** dial readings S_1	
9		stop viscometer / wait 1- 2 min / repeat viscosity measurement from

Pers. Haftende Gesellschafterin: Noveon Verwaltungs GmbH, Amtsgericht Traunstein, HRB 14245 · Geschäftsführung: Patrick Poty, Dr. Oliver Bolzern
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pos. 2

10 ***Second measuring result:*** dial readings S_2

11 ***Partial measuring result:***
Average value V: $V = (S_1 + S_2) / 2$

---

c) Calculation of viscosity

1 ***Measuring result:***
Viscosity η ***= V x F [mPas]***
V *= average value of two dial readings*
F *= factor for appropiate spindle-# at 20 rpm)*
= 2000 for spindle-# 7 at 20 rpm:

Requirements: see specification

Pers. Haftende Gesellschafterin: Noveon Verwaltungs GmbH, Amtsgericht Traunstein, HRB 14245 · Geschäftsführung: Patrick Poty, Dr. Oliver Bolzern
Noveon Pharma GmbH & Co. KG · Postfach 1151 D-83060 Raubling, · Rosenheimer Str. 43 · D-83064 Raubling
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2

Raw Material Specification Sheet for Cassia Splits, SOP XX 003 Tab 2

INDIAMALT Private Limited P.O.Manjusar Ta. Savli Dist. Vadodara - 391 775	SOP - IP XX 003	12.12.1997 Version:01	page 1 of 3 SOP03.DOC

SOP

GENERAL WORKING ORDER

Title:	Raw Material Handling
Valid since:	01.10.1997
This SOP replaces:	
Policy:	To describe the general process of the receipt of raw material, sampling, storage.
Responsibility:	General Manager, Production Manager, Senior Officer Quality Control
Changes:	
distribution:	General Manager Production Office

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written by: (b)(6) Technical consulting	reviewed by: (b)(6) Quality Control	approved by: (b)(6) Managing Director

1. Policy:

This SOP describes the steps to be followed at the receipt of raw material in regard of handling, taking samples, and storage.

2. Area and Responsibility:

Loading platform, warehouse, laboratory

Responsible: General Manager
Production Manager
Administration Manager
Lab in charge

3. Regulations

3.1. Handling of the delivering truck

3.1.1. The truck has to be positioned at the forward platform, the canvas to be opened only under the roof during monsoon time to prevent rain water wetting of the gunny bags.

3.1.2. The freight bills have to be checked by the office to ascertain the conformity of the delivery with the raw material orders.

3.1.3 The delivery is labeled with a unique batch number as per usual standard to allow the verification of the material throughout the whole production.

3 1.4. The warehouse officer allots a suitable warehouse space and takes care of orderly storage, labeling, etc. during unloading.

3.1.5. The samples for quality control are taken by the lab in charge during unloading by statistical method, it is advised to take samples of the square root of bag numbers plus 1.
(That is for example at 180 bags (|√180| + 1)= 14 + 1 = 15, so samples from 15 bags, e.g. every 12th bag.
This samples are mixed, to get one statistic sample of the whole delivery.

For the analysis methods applicable refer to :

Quality Control Manual, Analysis Method NC 02602

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3.2. Documentation

3.2.1. The delivery is entered into the stock register as per customs requirements.

3.2 2. The batch is also entered into the production stock register with all quality parameters for the purpose of production planning, blending of different qualities, etc.

The production stock register is to be seen also as the data source of supplies quality supervision

This register has to be kept on absolutely actual condition on a daily basis for incoming and outgoing quantities and qualities.

3 3 Raw Material Release

According to the content of Cassia Occidentalis, foreign matter and chrysophanic acid, the quality is classified into three different groups (see raw material specifications):

3 3.1 **Super Grade Splits:**

Raw material of this quality , spec. nr. 0150383, is used for the production of food and pet food grade DIAGUM CS

3.3 2. **First Grade Splits:**

Raw material of this quality, spec. nr. 0150478, is used for the production of Cassia Powder.
This Cassia Powder can be released as food or pet food grade DIAGUM CS (sampling and analytical testing see SOP IP XX 004). If the analytical results are in accordance with the specified values of the DIAGUM CS specification.
If the analytical results are not in accordance with the DIAGUM CS specification, the Cassia Powder is determined for use in technical applications, i.e. textile and paper industry.

3.3 3 **Technical Grade Splits:**

Raw material of this quality will be used for production of Cassia Powder for technical applications, i e. textile printing and seizing,only

written by: (b)(6) Technical consulting	reviewed by: (b)(6) Quality Control	approved by (b)(6) Managing Director

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TESTING STANDARD

CASSIA TORA/OBTUSIFOLIA SEEDS	Quality assurance Test.No.: NC 02602 Version: 980501 Author: Dr.Kt Issued: 20.05.98
CONTAMINANT: CASSIA OCCIDENTALIS	page 1 of 6

CONTENTS

I Principle

II Definition

III Equipment

IV Sampling

V Test procedure

VI Calculation

VII Documentation

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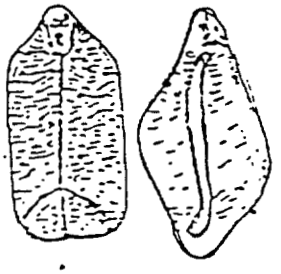
I PRINCIPLE

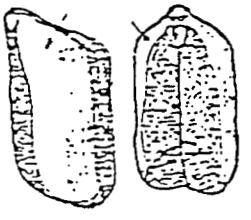
The seeds of Cassia tora / obtusifolia and of Cassia occidentalis are quite different in shape and size.They can be easily segregated by hand and the amount of C.occidentalis is determined gravimetrically.

II DEFINITION OF CASSIA SEEDS

C.obtusifolia



C.tora



Cassia obtusifolia is defined by a narrow and S-shaped aureole,while C.tora forms a broad and almost straight aurole.

C.occidentalis

C.occidentalis is a 4-5 mm long flat,brownisch disk

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III EQUIPMENT

1 sampler

2 lab spoon (e.g. 150 mm)

3 weighing boat

4 bend pincer

5 magnifying glass

6 white sheet

7 balance (readings: 0,001 g)

8 calculator

9 plastic bags

10 sheet welder

IV SAMPLING

1 Select ($\sqrt{n} + 1$) of the bags at random from which 100 g - samples are drawn.These are thoroughly mixed to a representative sample and analysed for Cassia occidentalis,foreign matter and black seeds.

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V TEST PROCEDURE

1 weigh 100 g of seeds and make thereof 10 groups with each 10.00 g (the numbering of groups is $G_1, G_2 .... G_{10}$)

2 pour 10 g of seeds onto a white sheet

3 segragate the sample by hand under a magnifying glass into following categories:

Cassia tora/obtusifolia
Cassia occidentalis
foreign matter
black seeds

4 weigh the separated categories

5 ***Partial measuring result:***

weight Cassia occidentalis	$= W_{co}$ [g]
weight foreign matter	$= W_{fm}$ [g]
weight black seeds	$= W_{bs}$ [g]

6 repeat step 2-5 for each 10 g - group ($G_1 ... G_{10}$)

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VI Calculation

1 sum up each category of the ten groups ($G_1 ... G_{10}$) analysed

2 ***Partial measuring result:***

weight Cassia occidentalis:

$$\Sigma(W_{co}) = G_1(W_{co}) + G_2(W_{co}) \ldots G_{10}(W_{co}) \text{ [g]}$$

weight foreign matter

$$\Sigma(W_{fm}) = G_1(W_{fm}) + G_2(W_{fm}) \ldots G_{10}(W_{fm}) \text{ [g]}$$

weight black seeds

$$\Sigma(W_{bs}) = G_1(W_{bs}) + G_2(W_{bs}) \ldots G_{10}(W_{bs}) \text{ [g]}$$

3 ***Content Cassia occidentalis in percent***

$$\text{Percent Cassia occidentalis} = \frac{\Sigma(W_{co}) \text{ [g]} \times 100}{100 \text{ [g]}} \text{ [\%]}$$

4 ***Content foreign matter in percent***

$$\text{Percent Foreign Matter} = \frac{\Sigma(W_{fm}) \text{ [g]} \times 100}{100 \text{ [g]}} \text{ [\%]}$$

5 ***Content black seeds in percent***

$$\text{Percent black seeds} = \frac{\Sigma(W_{bs}) \text{ [g]} \times 100}{100 \text{ [g]}} \text{ [\%]}$$

6 ***Requirements:*** see specification

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VII Documentation

1 document results in lab journal

2 put segregated categories (c. occidentalis/foreign matter/black seeds) of all ten groups together and seal them in plastic bags

3

Report on Production of Food Grade Cassia Splits Tab 3

August 8, 2001

Dr. Ferdinand Utz
Technical Manager

BFGoodrich Diamalt GmbH
Georg-Reismueller-Str 32
D-80999 Munich
T +49 (89) 8106 452 F +49 (89) 8106 313
Ferdinand utz@noveoninc com

Memo

Date: August 5, 2001

To: Documentation Files

From: Ferdinand Utz

Subject: **Report on the Production of Food Grade Cassia Splits**

Date: July 21, 2001 – July 24. 2001

Location: Bardoli Agro Industries Pvt. Ltd.
Surat Dhulia Road
Ten Naka
Bardoli 391601
Gujarat, INDIA

Summary

Four different master batches with different batch sizes (Batch no. FTR 1 through FTR 4) were produced starting on July 21, 2001. In addition to the regular petfood grade split production, the food grade splits were cleaned twice. The final qualities in all batches met the analytical requirements of food grade cassia split.

Definition: Cassia split = purified endosperm of cassia tora and obtusifolia seed. The split mainly contains polysaccharides, which are the energy

reservoir of the seed. As well husk and germ are removed from the seed.

1. Manufacturing procedure

The production of Cassia tora/obtusifolia split follows a procedure as shown in appendix 1.
The seeds came from the warehouse Dal Godwin No 2 (fresh crop from the seed collection season 2000/2001) and were packed in jute sacks.

The seed was cleaned in a five step purification process in order to remove foreign material like sand, small stones, litter. and seeds with a size different to Cassia tora and obtusifolia. The cleaned seeds were temporarily stored in open silos for further processing.

The splitting process started with roasting of the cleaned seed at a roasting temperature of 200 - 300°C. The temperature of the roasted seed is documented in a batch report and 50 g samples of the cleaned seed were gathered hourly from the pipe to the roasting oven. Both batch report and seed samples are part of each delivery to Indiamalt Pvt. Ldt.. The temperature of the product maintained a temperature of more than 100°C during all further processing steps.

The dehusking and cleaning process starts with two dehusking steps (DH-1/DH-3, resp. DH-2/DH-4), followed by a round sieve (RS-6) and a separator (SE-5). After conveying to a different hall, the product passes a round sieve (RS-5) again, followed by two dehusking mashines (DH5 and DH6). The split is purified by round sieving (RS-4), a separator (SE-6), two stone polishers (P1 and P2), and a brusher (B1).

For the current production the splits, after four times of dehusking and cleaning were packed into bags directly after the brusher B1, the production was interrupted, and the purification starting with round sieve RS-5 was repeated.

The final split was conveyed by air into a separate building, where the split was allowed to cool down over night for final packing into jute bags with PE inliner.

It was not possible to determine the yield from seed to split.

Batch		FTR 1	FTR 2	FTR 3	FTR 4
Seed feeding rate to the roaster		380 kg/h	380 kg/h	320 kg/h	350 kg/h
Split Quantity	4-times dehusking	3417	6097	3834	5245
[kg]	6-times dehusking	2600	4817	2990	4225
	Difference	-24%	-21%	-22%	-19%

2. Quality

As to an agreement in between BAPL and Indiamalt the final quality control is exclusively carried out at Indiamalt. However, BAPL carries out IPCs by visual judgement of the split in terms of the split color and completeness of dehusking.

2.1 Seeds

As per appendix 2 as well the cassia occidentalis content and the ratio in between tora and obtusifolia is not evenly distributed in the product

Batch No BAPL	Average Seeds size [mg]	Cassia Tora [%]	Cassia Obtusifolia [%]	Cassia Occidentalis [%]	Other Seeds [%]	Foreign Matter [%]
FTR 1	15,40	79,93	20,07	0,025	0,050	0,732
FTR 2	15,33	83,06	16,94	0,175	0,030	0,933
FTR 3	15,60	73,67	26,33	0,192	0,050	1,115
FTR 4	15,09	78,17	21,83	0,057	0,070	0,960
Average of all seed collection 2000/2001	19.58	74,6	25,4	0,040	0,090	3 50

The share of Cassia tora in the seed used for the master batches was relatively high and the seed size through the high content of Cassia tora lower than average.

2.2 Split

Contrary to regular split production the split was cleaned again through passing the equipment a second time starting with elevator E 16. The efficiency of the additional cleaning is shown below:

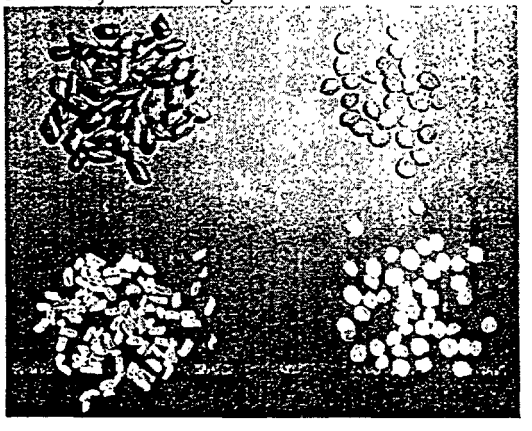
Samples for analysis were taken directly from the production and differ therefore to some extent from the final split analysis:

Batch		FTR 1	FTR 2	FTR 3	FTR 4	Average
Colour Value (anthraquinones) [ppm]	4-times dehusking	156	145	156	191	
	6-times dehusking	122	129	115	149	
	Difference	-22%	-11%	-26%	-22%	-20,3%
Chrysophanic acid [ppm]	4-times dehusking	4,0	4,0	3,2	3,7	
	6-times dehusking	3,4	3,1	2,2	2,7	
	Difference	-15%	-23%	-31%	-27%	-23,9%
Gel strength [g]	4-times dehusking	161	172	166	173	
	6-times dehusking	167	172	169	173	
	Difference	4%	0%	2%	0%	1,4%

2.3 Cassia tora/obtusifolia and Cassia occidentalis

It was observed through comparison of the seed analysis with the split produced out of these seeds, that nearly all types of foreign seeds were destroyed and milled into powder during the roasting and splitting process. The powder is separated along with husk and germ powder of Cassia Tora / Obtusifolia into the so called "Cassia Meal" (Appendix 3). Cassia meal from roasted seeds is traditionally used in the India as an additive to Cattle Feed in concentrations up to 4 %.

Cassia occidentalis seeds survive to some extent the roasting and splitting process and are splitted themselves. As well the different types of Cassia Seeds and the splits of Cassia Tora / Obtusifolia and Cassia occidentalis are easy to distinguish.



Picture 1:

Cassia tora/ C occidentalis

Obtusifolia seeds seeds

C. tora/obtusifolia Splits C. occidentalis Splits

It was found that the percentage (w/w) of Cassia Occidentalis in the split samples was much lower than the results from the seed used for the split production.

While in 12 different seed samples of FTR 2 an average of 0.175% (w/w) Cassia occidentalis was found (Appendix 2), the average share of Cassia occidentalis splits, analyzed as an IPC from 10 different bags of the same batch after 4 times of dehusking, was much smaller:

Number	split weight [g]	occidentalis weight [g]	percentage
1	20,221	0,029	0,143%
2	34,240	0,028	0,082%
3	42,220	0,024	0,057%
4	19,720	0,012	0,061%
5	57,750	0,037	0,064%
6	40,830	0,041	0,100%
7	10,710	0,011	0,103%
8	49,030	0,025	0,051%
9	45,850	0,037	0,081%
10	21,950	0,004	0,018%
Total	**342,521**	**0,248**	**0,072%**

Table 3. Content of Cassia occidentalis split in FTR 2:

3. Food safety statement

Bardoli Agro Industries Pvt Ldt is a company specialized on the production of Cassia split since more than 20 years. A food safety statement edited by K.C. Joshi, the owner of the splitting plant, is enclosed (Appendix 4).

4. Conclusion

The Cassia split batches FTR-1 through FTR 4 produced at BAPL for the master batches of food grade Cassia gum resulted in material suitable as a raw material for the Indiamalt production.

As a result of our investigations regarding foreign seeds and splits, we will add an additional test to the food grade split analysis: the share of Cassia occidentalis split. The limit will be 0.1% (w/w) of the total weight of the split.
Other split different to Cassia tora/obtusifolia and Cassia occidentalis was nowhere to be found.

August 5, 2001
Ferdinand Utz

4

Description of Production of Cassia Gum from Cassia Splits, SOP-IP XX 004 Tab 4

SOP

GENERAL WORKING ORDER

Title: Production Process Cassia Gum

Valid since: 01.01.1998

This SOP replaces:

Policy: This SOP describes the manufacturing process for Cassia Gum Powder

Responsibility: General Manager, Production Manager, Senior Officer Quality Control

Changes:

distribution: General Manager

Production Office

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1. Policy:

This SOP describes the basic steps of the process which have to be followed.
It does not contain varaiable and/or secret process paramenters, they are prescribed as required on the daily production orders.

2. Area and Responsibility:

Production Area

Responsible: General Manager
Production Manager
Shift in Charge

3. Regulations

3.1 Raw material supply

The raw material (Cassia Splits) are filled from the gunny bags into the feeding bin/ elevator at the ground floor.

Batch/Quality and Quantity: refer to actual production order

Handling: Take care of the correct setting (mark)of the elevator inlet valve to avoid overloading.

Do not throw threads or bag pieces into the bin.

While floor cleaning, take care that the bin is covered to avoid dirt beeing brushed into the feeding bin.

3.2. Pre - Cleaning of splits

Before reaching the silo cell, the splits pass a round sieve "REIMELT" to remove threads, twigs, stalks, rests of paper or plasik bags etc.

For inspection and cleaning, refer to SOP IP_Hg 002.

In case of bypassing the sieve due to maintenance etc., the responsible shift in charge has to take care of intensive supervision of the raw mat. filling to exclude foreign bodies in the splits.
The bypassing has to be documented on the production protocol.

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3.3. De-stoning, Dust removal

The Stoner (BÜHLER) has to be controlled in his function one time per shift during operation,
This has to be done by hand samples and visual control of the cleaned splits, if they are free of stones, other grains, non-dehusked seeds etc.
Further the waste has to be observed on its content of good splits.

Changes in settings of the aspiration valve, slope and vibration amplitude only by qualified operators.

There are no specific settings prescribed, because they depend on the composition of the splits with foreign materials, the flow rate, etc. and are to be tuned on the spot and by actual requirements.

For basic settings, after repair etc., refer to process manual.

The waste has to be disposed off as fertiliser on the company land, the quantity is to be weighed and documented in the customs stock register.

3.4. Dosage

The dumping balance determines the batch size with the size and number of dumpings.
The settings are to be controlled by the shift in charge and documented in the production protocol.

Changes in settings by qualified personnel only on direct order of the production manager and under his supervision.

For basic settings refer to process manual.

3.5. Elevators

The three elevators have to be controlled weekly on slippage and parallel running of the belts.
(Responsible: Maintenance Chief)
Deviations have to be reported immediately to the production manager, who is responsible for repair to avoid frictions, possibly causing dust explosions.

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3.6. Washing machines

The performance of the washing machines have to be controlled by the operators continuously.

Points of control:

- Water pressure: Refer to production order
- rpm: Refer to production order
- function: Visual control during every washing process:
  - turbidity of waste water
  - content of splits in waste water
  - material flow (clogging)

One time per shift the sieve has to be changed, the used one cleaned by high pressure lancet.

3.7. Double Cone Mixers

Runtime of the mixers is essential for continuous product quality.

For settings refer to process manual.

Change of settings by production manager only.

One sample of every batch has to be analysed on humidity by laboratory.
For the analysis method refer to the quality control manual.

3.8. Lump brakers

The operators have to take care of complete emtying of the hopper and needle screw after every batch to avoid resting humid material developing grouth of bacteria and mould.

3.9. Hydraulic Flaker

The Hydraulic Flaker has to be run on pressure only when the feeding roll is delivering material to the rolls.

It is compulsory to turn pressure to zero and separate the rolls when the material feeding stops out of any reason.

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For the following settings refer to process manual:

- Setting of slide valve of feeding roll
- Hydraulic pressure of flaker rolls
- rpm and ratio of flaker rolls

3.10. Manual Flakers

The pressure settings of the manual flakers have to be put to zero on stop of material flow equal to the hydraulic flaker.

Because it is impossible to measure the force and balance of pressure at this devices, the settings have to be done by well trained personnel only.

Important for the settings is the visual judgement of flake quality, which requires long term practice and education, and is depending also on the humidity of the splits and the provenience.

For rpm and ratio of the rolls refer to the process manual.

3.11. Dryer

The operation of the dryer requires attention on the following:

- Starting of the fan against closed slide valve only
- Inlet air temperature refer to process manual
- Thermic fluid temperature refer to process manual
- Stopping of dryer:
  Close Oil valve or shut down boiler, run dryer fan for 15 minutes without heating to prevent local overheating of exchangers or piping.

Regular In Process Control of dryed material humidity is compulsory.

For frequency refer to process manual
For Outlet-Humidity refer to process manual

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3.11. Milling

The milling is a continuous manufacturing step, including repeated grinding on a series of roller mills and separation of certain particle sizes with plansifters.

For the following refer to process manual:

- Groove specification for every roller mill
- rpm and ratio of rollers
- mesh sizes of every plansifter department (in microns)

Regular In Process Control of particle size of the finished material (sample from the collecting pipe) is compulsory.

For analysis method of particle size refer to quality control manual.

Oversized particles exceeding the limit proof torn sieves, in this case samples from every sifter department have to be checked to locate the damage.

In case of torn sieves, the production has to be stopped immediately and the damaged sieves be changed.

The already produced finished goods in the blender have to be packed and analysed on oversize particles.

The concerned batch of finished goods has to be put on quarantine, for further treatment refer to quality control manual.

Restart production with repaired sieves with new batch number.

3.12. Packing

The finished powder is collected in the blending silo of the concerned production line.

For packing, the blender has to be chosen on the control panel, which controls the direction of the discharging screw.

The product is conveyed over a control round sieve to eliminate potential foreign materials before packing.

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The weighing scale has to be checked on correct settings daily, deviations are to be reported immediately to the production manager, who is responsible for initiating maintenance.

The packed paper bags have to be statistically controlled for their weight on the electronic balance to ensure the proper performance of the weighing scale.

The bags have to be sewed, marked with the batch number of the production order, then palletized and stored in the finished goods warehouse.

While packing, around 50% of the batch, one sample has to be taken from the operators and send to the laboratory for further processing.

- For the following refer to the process manual:
- Settings of the weighing scale
- Regular check of the control sieve
- How to sew the paper bags with the polyethylene-inliner

For the following refer to the quality control manual:

- How to take the sample of finished goods

3.13. Documentation

The production process has to be documented by the shift in charge on the production order which is issued for every batch.

Form and contents of production order refer to SOP - IP XXX (not released)

The material movements from and to the stocks have to be entered immediately after return f the production order form to the office into the customs stock register.

The stock entrys of finished goods have to be reported daily in the prescribed form to BF Goodrich Diamalt, Munich, Head of Logistics, Mr. Haitel, to ensure on-time delivery to customers.

written by: (b)(6) Technical Consulting	reviewed by: (b)(6) Quality Control	approved by: (b)(6) Managing Director

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5

Certificates of Analysis for Six Batches of Diagum SR Tab 5

Certificate of Analysis

DIAGUM SR

Master Batch : LL47

1. Generic Name	**Cassia Gum**
Origin	**Endosperm powder of Cassia tora and Cassia obtusifolia**
Chem. Identity	**high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TEST RESULT*	*TEST-No*
2. Material Description			
Appearance	fine powder	conforms	SR 00201
Consistency	Dry, free flowing	conforms	SR 00202
Colour	Off-white to beige	Off white	SR 00301
Odour	Neutral to slightly fruity	neutral	SR 00302
Taste	neutral	neutral	SR 00303
3. Identity	Synegistic gel with xanthan	conforms	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	5,7 %	SR 014
Fat content	NMT 1%	0,3 %	SR 015
Ash (total	NMT 1.2%	0,20 %	SR 017
Loss on drying	NMT 12 %	3,48 %	SR 00502
pH-Value	5,5 - 8,5	7,47	SR 00602
Gel-strength	NLT 155 g	194g	SR 021
Viscosity (1%)	NLT 250 mPas	565 mPas	SR 04201
Particle size <250µ	NLT 99 %	99,4%	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	< 0,5 ppm	SR 02402
Isopropyl Alcohol	NMT 0.075 %	< 0,005%	SR 008
Heavy Metals	NMT 0.002%	Conforms	SR 010
Lead	NMT 5 mg Pb/kg	0,09 mg Pb/kg	SR 011
Arsenic	NMT 3 mg As/kg	< 0.05 mg As/kg	SR 012

Head QC (Date/Signature):

16/3/03 (b)(6)

(Dr. Ferdinand Utz)

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Certificate of Analysis

DIAGUM SR

Master Batch : LL46

1. Generic Name — **Cassia Gum**
Origin — **Endosperm powder of Cassia tora and Cassia obtusifolia**
Chem. Identity — **high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TEST RESULT*	*TEST-No*
2. Material Description			
Appearance	fine powder	conforms	SR 00201
Consistency	Dry, free flowing	conforms	SR 00202
Colour	Off-white to beige	Off white	SR 00301
Odour	Neutral to slightly fruity	neutral	SR 00302
Taste	neutral	neutral	SR 00303
3. Identity	Synegistic gel with xanthan	conforms	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	5,5 %	SR 014
Fat content	NMT 1%	0,3 %	SR 015
Ash (total	NMT 1.2%	0,19 %	SR 017
Loss on drying	NMT 12 %	7,54 %	SR 00502
pH-Value	5,5 - 8,5	7,75	SR 00602
Gel-strength	NLT 155 g	178 g	SR 021
Viscosity (1%)	NLT 250 mPas	380 mPas	SR 04201
Particle size <250µ	NLT 99 %	99,5%	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	< 0,5 ppm	SR 02402
Isopropyl Alcohol	NMT 0.075 %	< 0,005%	SR 008
Heavy Metals	NMT 0.002%	Conforms	SR 010
Lead	NMT 5 mg Pb/kg	0,18 mg Pb/kg	SR 011
Arsenic	NMT 3 mg As/kg	<0.05 mg As/kg	SR 012

Head QC (Date/Signature): 10/13/03 (b)(6)

(Dr. Ferdinand Utz)

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Certificate of Analysis

DIAGUM SR

Master Batch : LL45

1. Generic Name	**Cassia Gum**
Origin	**Endosperm powder of Cassia tora and Cassia obtusifolia**
Chem. Identity	**high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TEST RESULT*	*TEST-No*
2. Material Description			
Appearance	fine powder	conforms	SR 00201
Consistency	Dry, free flowing	conforms	SR 00202
Colour	Off-white to beige	Off white	SR 00301
Odour	Neutral to slightly fruity	neutral	SR 00302
Taste	neutral	neutral	SR 00303
3. Identity	Synegistic gel with xanthan	conforms	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	5,6 %	SR 014
Fat content	NMT 1%	0,3 %	SR 015
Ash (total	NMT 1.2%	0,24 %	SR 017
Loss on drying	NMT 12 %	7,95 %	SR 00502
pH-Value	5,5 - 8,5	7,67	SR 00602
Gel-strength	NLT 155 g	163 g	SR 021
Viscosity (1%)	NLT 250 mPas	380 mPas	SR 04201
Particle size <250µ	NLT 99 %	99,5%	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	< 0,5 ppm	SR 02402
Isopropyl Alcohol	NMT 0.075 %	< 0,005%	SR 008
Heavy Metals	NMT 0.002%	Conforms	SR 010
Lead	NMT 5 mg Pb/kg	0,58 mg Pb/kg	SR 011
Arsenic	NMT 3 mg As/kg	< 0.05 mg As/kg	SR 012

Head QC (Date/Signature): 10/13/03 (b)(6)

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DIAGUM SR

Master Batch : LL44

1. Generic Name	**Cassia Gum**
Origin	**Endosperm powder of Cassia tora and Cassia obtusifolia**
Chem. Identity	**high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TEST RESULT*	*TEST-No*
2. Material Description			
Appearance	fine powder	conforms	SR 00201
Consistency	Dry, free flowing	conforms	SR 00202
Colour	Off-white to beige	Off white	SR 00301
Odour	Neutral to slightly fruity	neutral	SR 00302
Taste	neutral	neutral	SR 00303
3. Identity	Synegistic gel with xanthan	conforms	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	5,7 %	SR 014
Fat content	NMT 1%	0,3 %	SR 015
Ash (total	NMT 1.2%	0,21 %	SR 017
Loss on drying	NMT 12 %	4,9 %	SR 00502
pH-Value	5,5 - 8,5	7,55	SR 00602
Gel-strength	NLT 155 g	163 g	SR 021
Viscosity (1%)	NLT 250 mPas	280 mPas	SR 04201
Particle size <250µ	NLT 99 %	99,8%	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	< 0,5 ppm	SR 02402
Isopropyl Alcohol	NMT 0.075 %	< 0,005%	SR 008
Heavy Metals	NMT 0.002%	Conforms	SR 010
Lead	NMT 5 mg Pb/kg	0,13 mg Pb/kg	SR 011
Arsenic	NMT 3 mg As/kg	< 0.05 mg As/kg	SR 012

Head QC (Date/Signature):

10/12/03 (b)(6)

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Certificate of Analysis

DIAGUM SR

Master Batch : LL43

1. Generic Name — **Cassia Gum**
Origin — **Endosperm powder of Cassia tora and Cassia obtusifolia**
Chem. Identity — **high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TEST RESULT*	*TEST-No*
2. Material Description			
Appearance	fine powder	conforms	SR 00201
Consistency	Dry, free flowing	conforms	SR 00202
Colour	Off-white to beige	Off white	SR 00301
Odour	Neutral to slightly fruity	neutral	SR 00302
Taste	neutral	neutral	SR 00303
3. Identity	Synegistic gel with xanthan	conforms	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	5,7 %	SR 014
Fat content	NMT 1%	0,3 %	SR 015
Ash (total	NMT 1.2%	0,16 %	SR 017
Loss on drying	NMT 12 %	4,7 %	SR 00502
pH-Value	5,5 - 8,5	7,41	SR 00602
Gel-strength	NLT 155 g	181 g	SR 021
Viscosity (1%)	NLT 250 mPas	570 mPas	SR 04201
Particle size <250µ	NLT 99 %	99,8%	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	< 0,5 ppm	SR 02402
Isopropyl Alcohol	NMT 0.075 %	< 0,005%	SR 008
Heavy Metals	NMT 0.002%	Conforms	SR 010
Lead	NMT 5 mg Pb/kg	0,13 mg Pb/kg	SR 011
Arsenic	NMT 3 mg As/kg	< 0.05 mg As/kg	SR 012

Head QC (Date/Signature): 10/13/03 (b)(6)

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DIAGUM SR

Master Batch : LL42

1. Generic Name	**Cassia Gum**
Origin	**Endosperm powder of Cassia tora and Cassia obtusifolia**
Chem. Identity	**high molecular galactomannan polysaccharide**

TEST	*SPECIFICATION*	*TEST RESULT*	*TEST-No*
2. Material Description			
Appearance	fine powder	conforms	SR 00201
Consistency	Dry, free flowing	conforms	SR 00202
Colour	Off-white to beige	Off white	SR 00301
Odour	Neutral to slightly fruity	neutral	SR 00302
Taste	neutral	neutral	SR 00303
3. Identity	Synegistic gel with xanthan	conforms	SR 013
4. Chem. and phys. Properties			
Protein content	NMT 7%	5,7 %	SR 014
Fat content	NMT 1%	0,3 %	SR 015
Ash (total	NMT 1.2%	0,2 %	SR 017
Loss on drying	NMT 12 %	3,5 %	SR 00502
pH-Value	5,5 - 8,5	8,02	SR 00602
Gel-strength	NLT 155 g	173 g	SR 021
Viscosity (1%)	NLT 250 mPas	520 mPas	SR 04201
Particle size <250µ	NLT 99 %	99,6%	NC 01201
5. Purity			
Total Anthraquinones	NMT 10 ppm	< 0,5 ppm	SR 02402
Isopropyl Alcohol	NMT 0.075 %	< 0,005%	SR 008
Heavy Metals	NMT 0.002%	Conforms	SR 010
Lead	NMT 5 mg Pb/kg	0,12 mg Pb/kg	SR 011
Arsenic	NMT 3 mg As/kg	< 0.05 mg As/kg	SR 012

Head QC (Date/Signature): 10/13/03 (b)(6)

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6

HPLC Analytical Method and Method Validation for Measurement of Anthraquinones in Diagum SR™ **Tab 6**

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: NC 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content	**Page 1 of 6**

The HPLC methods were developed by the Institute of Toxikology, Univerity of Wuerzburg, Germany. (SOP Nr. 98-S/1 dated 27.05.2002)

Table of contents

1. Introduction
2. Equipment
3. Reagents
4. Practical work
   4.1 Quantitation of total antraquinone content after hydrolysis using trifluoroacetic acid (method A)
   4.2 HPLC-conditions
   4.3 Calculations
1. Storage
2. Documentation

1. Introduction

To quantify total antraquinone content, antraquinone glycosides are hydrolized using trifluoroacetic acid or HCl/methanol at flux for 4 hr.

Concentrations of antraquinones are obtained by automated integration and referenced to calibration curves and the internal standard danthrone.

All manipulation of the samples should be performed in the dark

Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: NC 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content	**Page 2 of 6**

2. Equipment

HPLC with diode array detector (e.g. HPLC-pump: HP 1050, diode array detector: HP 1040M series 2, autoinjector: TSP AS 100)

HPLC-column (e.g. Fa. Bischoff, 71229 Leonberg) Hypersil ODS, 250mm x 4,6 mm ID; 5 µm film)

HPLC-syrringes (100 µl, e.g. Fa. ILS)

Autosampler vials tsp (e.g. Fa. Brown, Wertheim)

Limited volume Inserts 6*30 mm, 0,3 ml volume (e.g. Fa. Brown, Wertheim)

Plastic caps, polyethylene (e.g. Fa Brown, Wertheim)

Analytical balance, reading 0.1 mg (e.g. Fa. Mettler-Toledo, Greifensee/Schweiz)

10 ml and/or 5 ml-volumetric flask (e.g.Fa Schott, Mainz)

Calibrated adjustable pipettes (e.g. Fa. Eppendorf, Hamburg)

Eppendorf-cups 1,5 ml (reaction vials) (e.g. Fa. Eppendorf, Hamburg)

Ultrasonic bath (e.g.Fa.Bandelin, Berlin, Sonorex RK 100)

Vortexer (Fa. Bender & Hobein, Zurich/Schweiz)

Magnetic stirrer/heater and a 1 cm stirring bar, pfte (e.g. Fa. Janke & Kunkel, Staufen i. Breisgau)

Laborboy (e.g. Fa. Grauer, Degersheim, Schweiz)

50 ml flask

Dimroth-cooler with connection to water supply

Oil bath for heating (e.g. Fa. Roth, Karlsruhe)

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Rotary evaporator with membrane pump for vacuum generation (e.g. Fa.Büchi, Flawil, Schweiz)

3. Reagents

- Danthrone oder 1,8-dihydroxy-anthraquinone (e.g. Fa. Bayer, Leverkusen)
  2.40 mg danthrone are dissolved in 10 ml of methanol to give a stock solution of **1000 nmol/ml**
- H_2O deionised (e.g. aus Millipore-Anlage Elix 3)
- Trifluoroacetic acid (TFA) (e.g. Fa. Merck-Schuchardt, 85662 Hohenbrunn)
- Acetonitrile 99,9% (e.g. Fa. Fluka, Buchs)
- Methanol 99,9% (e.g. Fa.Fluka, Buchs)
- Acetone 99,5+%, A.C.S.-Reagenz (e.g. Fa. Sigma, Steinheim)
- Natriumhydrogencarbonat (99,7 +%) (e.g. Fa. Sigma-Aldrich, Steinheim)
- Acetyl chloride puriss.p.a. (e.g. Fa. Fluka, Buchs, Schweiz)

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: NC 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content	**Page 4 of 6**

4. Practical work

4.1. Determination of total anthraquinone content after hydrolysis with trifluoroacetic acid (A)

Samples (0.40 g) are introduced into a 50 ml round-bottom flask and heated at flux after adition of 20 ml trifluoroacetic acid (A) for 4 h.

After cooling, the solvent from the samples is removed under reduced pressure, acetonitrile/NaHCO3 (0.2% in water) (60:40 v/v) are added and the obtained suspensions are sonicated. After 30 min in the ultrasound bath, 20 µl of the internal standard solution are added and 50 µl of the obtained solution are analyzed by HPLC. Typical chromatograms obtained after sample hydrolysis are shown, using trifluoroacetic acid in Fig. 1

Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: NC 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content	**Page 5 of 6**

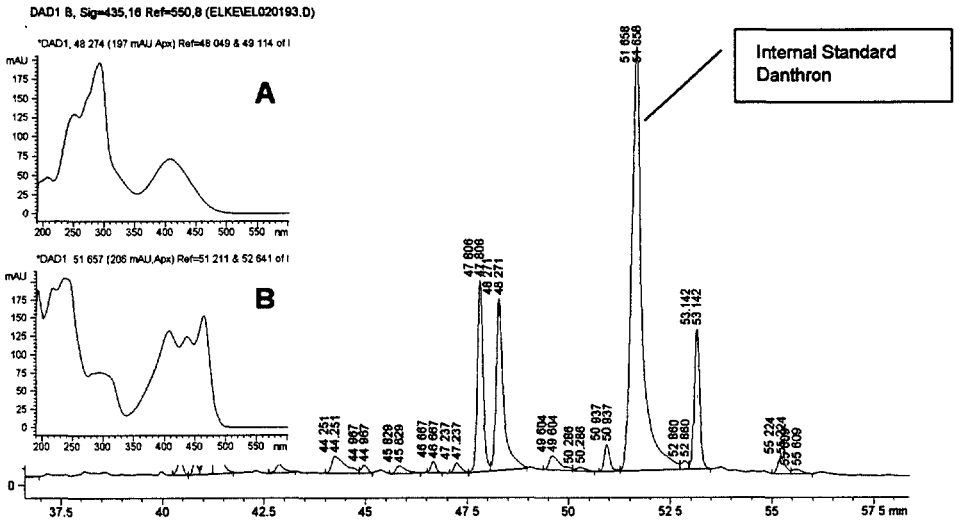

Fig. 1: A typical chromatogram of Diagum CS (Petfood grade) obtained after sample hydrolysis using trifluoroacetic acid.

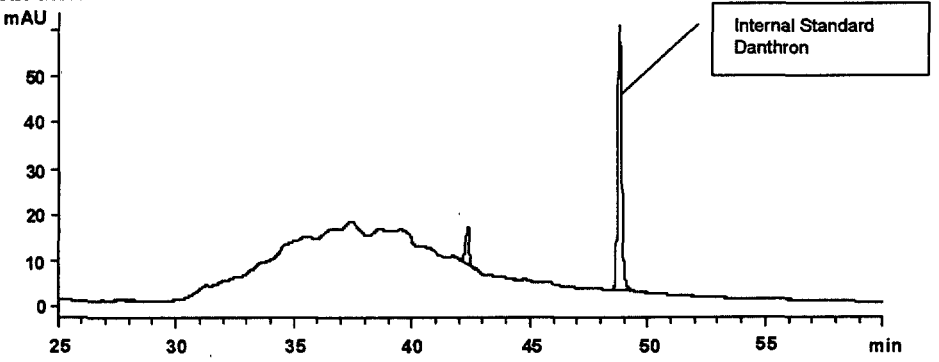

Fig. 2: A typical chromatogram of Diagum SR (food grade) obtained after sample hydrolysis using trifluoroacetic acid.

Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)		Quality Assurance Test.No.: NC 02402 Version: 01 Author: Utz Issued: 02.09.02
	Anthraquinone Content	**Page 6 of 6**

4.3 HPLC-Conditions

HPLC is performed by gradient elution with solvent A (water, 0.1 % trifluoroacetic acid, TFA) and solvent B acetonitrile. The following gradient conditions are used:

0 min. – 14 % acetonitrile
10 min. – 14 % acetonitrile
15 min. – 20 % acetonitrile
25 min. – 20 % acetonitrile
55 min. – 80 % acetonitrile
60 min. – 100 % acetonitrile

A Hypersil OSD column (5 µm, 250 mm x 4.6 mm) is used with a flow rate of 1 ml/min. Detection and quantitation are performed with a DAD-UV-detector at 225 und 435 nm.

4.4. Calculations

For quantitation, peak areas determined at 435 nm are used. Structures are confirmed by UV-spectra and retention times. The determined area for a specific compound is diveded by the area of the intenal standard peak and plotted against the concentration. R^2 values in the calibration curves should be > 0.95.

5. Storage

Samples should be analyzed within 24 h.

Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

000170

TESTING STANDARD

DIAGUM SR (= Cassia Gum)		Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
	Anthraquinone Content (Validation)	Page 1 of 10

The HPLC methods were developed by Prof. Dr. W. Dekant at the Institute of Toxikology, University of Wuerzburg, Germany. (SOP Nr. 97-S/1 dated 27.05.2002)

Table of contents

1. Introduction
2. Equipment
3. Reagents
4. Practical work
   - 4.1 Calibration solutions
   - 4.2 HPLC-conditions
   - 4.3 Calibration curves
5. Storage

1. Introduction

The developed method is intended to quantify content of antraquinones in solutions using HPLC with gradient elution and diode array detection at a wavelength of 435 nm. Concentrations of antraquinones are obtained by automated integration and referrenced to calibration curves and the internal standard danthrone.

All manipulation of the samples should be performed in the dark

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content (Validation)	Page 2 of 10

2. Equipment

HPLC with diode array detector (e.g. HPLC-pump: HP 1050, diode array detector: HP 1040M series 2, autoinjector: TSP AS 100)

HPLC-column (e.g. Fa. Bischoff, 71229 Leonberg) Hypersil ODS, 250mm x 4,6 mm ID; 5 µm film)

HPLC-syringes (100 µl, e.g. Fa. ILS)

Autosampler vials tsp (e.g. Fa. Brown, Wertheim)

Limited volume Inserts 6*30 mm, 0,3 ml volume (e.g. Fa. Brown, Wertheim)

Plastic caps, polyethylene (e.g. Fa Brown, Wertheim)

Analytical balance, reading 0.1 mg (e.g. Fa. Mettler-Toledo, Greifensee/Schweiz)

10 ml and/or 5 ml-volumetric flask (e.g.Fa Schott, Mainz)

Calibrated adjustable pipettes (e.g. Fa. Eppendorf, Hamburg)

Eppendorf-cups 1,5 ml (reaction vials) (e.g. Fa. Eppendorf, Hamburg)

Ultrasonic bath (e.g.Fa.Bandelin, Berlin, Sonorex RK 100)

Vortexer (Fa. Bender & Hobein, Zurich/Schweiz)

Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

3. Reagents

- Emodin (e.g. Fa. Roth, Karlsruhe)
- Aloe-emodin (e.g. Fa. Roth, Karlsruhe)
- Rhein (e.g. Fa. Roth, Karlsruhe)
- Physcion or 1,8-dihydroxy-3-methoxy-6-methyl-anthraquinone (e.g. Fa. Roth, Karlsruhe)
- Chrysophanic acid (e.g. Fa. Aldrich, Steinheim)
- Danthrone or 1,8-dihydroxy-anthraquinone (e.g. Fa. Bayer, Leverkusen)
- H_2O deionised (e.g. out of Millipore-water system Elix 3)
- Trifluoroacetic acid (e.g. Fa. Merck-Schuchardt, 85662 Hohenbrunn)
- Acetonitrile 99,9% (e.g. Fa. Fluka, Buchs)
- Methanol 99,9% (e.g. Fa.Fluka, Buchs)
- Acetone 99,5+%, A.C.S.-reagent (e.g. Fa. Sigma, Steinheim)

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TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content (Validation)	Page 4 of 10

4. Practical work

4.1. Calibration solutions

Al solutions are made from a stock solution of **1000 nmol/ml**.
The internal standard danthrone (2.40 mg danthrone is dissolved in 10 ml of methanol in a calibrated flask.
Emodin and aloe-emodin are also dissolved in methanol (2.70 mg emodin rsp. aloe-emodin in 10 ml methanol, identical molecular weight).
Physcion and rhein are dissolved in methanol oder acetone (rhein) (2.84 mg in 10 ml methanol and/or acetone, identical molecular weight).
Chrysophanic acid is dissolved in methanol .(2.54 mg in 10 ml methanol).
Caution:
The solution of the compounds in methanol occurs only slowly, therefore, use less than 10 ml of methanol and sonicate for 15 min, than fill up with methanol to mark.

Calibration solutions are analyzed before and after each serial sample analyses.
The following volumes of the stock solutions are added to methanol in a 1.5 ml plastic vial. The internal standard is also added in a concentration of 20 nmol/ml. Calibration standards are mixed and will be analyzed within 10 min after production. 50 µl of the sample is injected into the HPLC-system.

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Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content (Validation)	Page 5 of 10

Amount in 50 µl volume injected	Aloe-emodin (1000nmol/ml); (µl)	Emodin (1000nmol/ml); (µl)	Rhein (1000nmol/ml); (µl)	Chrysophanic acid (1000nmol/ml) (µl)	Physcion (1000nmol/ml); (µl)	Danthrone (1000nmol/ml); (µl)	Methanol (to be added to give a total volume of 1 ml) (µl)
50 pmol	1	1	1	1	1	20	975
100 pmol	2	2	2	2	2	20	970
250 pmol	5	5	5	5	5	20	955
500 pmol	10	10	10	10	10	20	930
750 pmol	15	15	15	15	15	20	905
1000 pmol	20	20	20	20	20	20	880
1500 pmol	30	30	30	30	30	20	830
2000 pmol	40	40	40	40	40	20	780
3000 pmol	60	60	60	60	60	20	680

000175

Test method SR 02402 V01	Issued: 02/09/2002	Approved: (b)(6)

4.2 HPLC-Conditions

HPLC is performed by gradient elution with solvent A (water, 0.1 % trifluoroacetic acid, TFA) and solvent B (acetonitrile). The following gradient conditions are used:

0 min. – 14 % acetonitrile

10 min. – 14 % acetonitrile

15 min. – 20 % acetonitrile

25 min. – 20 % acetonitrile

55 min. – 80 % acetonitrile

60 min. – 100 % acetonitrile

A Hypersil OSD column (5 µm, 250 mm x 4.6 mm) is used with a flow rate of 1 ml/min. Detection and quantitation is performed with a DAD-UV-detector at 225 und 435 nm.

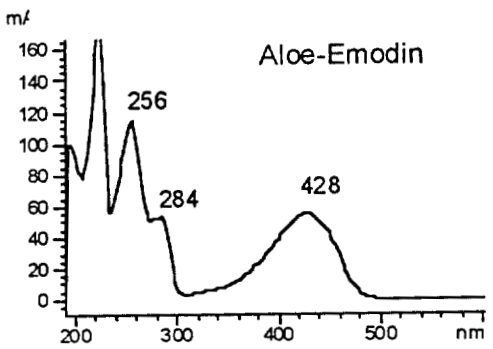
4.2 Calibration curves

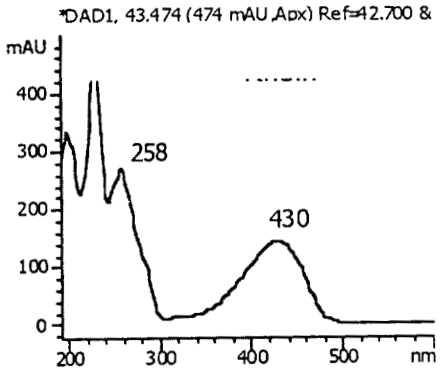
For quantitation, peak areas determined at 435 nm are used. Structures are confirmed by UV-spectra and retention times. The determined area for a specific compound is diveded by the area of the intenal standard peak and plotted against the concentration. R^2 values in the calibration curves should be > 0.95.

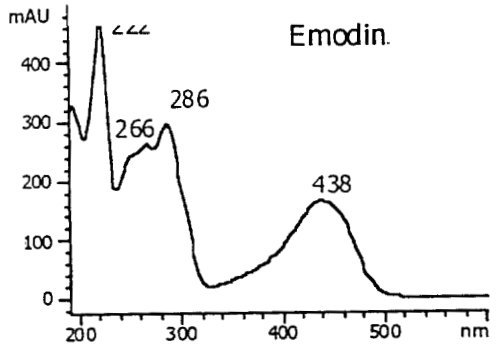
Pers. Haftende Gesellschafterin: Noveon Verwaltungs GmbH, Amtsgericht Traunstein, HRB 14245 · Geschäftsführung: Patrick Poty, Dr. Oliver Bolzern
Noveon Pharma GmbH & Co KG · Postfach 1151 · D-83060 Raubling, · Rosenheimer Str. 43 · D-83064 Raubling
Bankverbindung: Deutsche Bank, Kto.-Nr. 1525021, BLZ 70070010, SWIFT DEUT DE MM · USt.-IdNr. DE 813257924

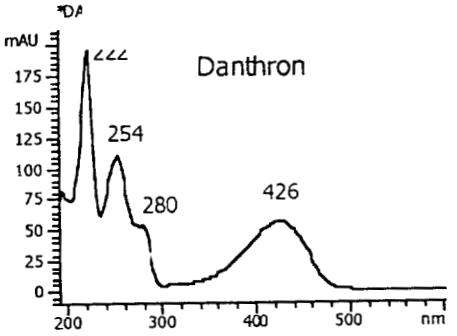
TESTING STANDARD

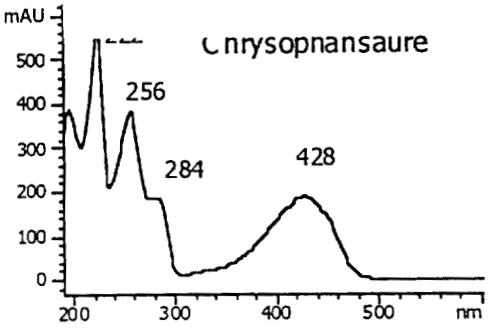
DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content (Validation)	**Page 7 of 10**

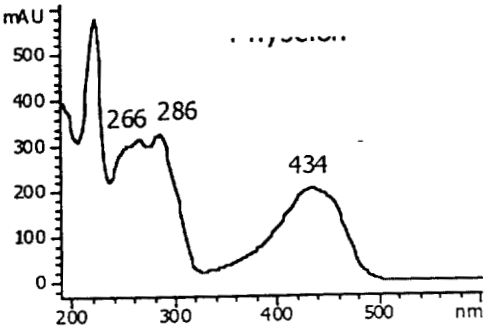












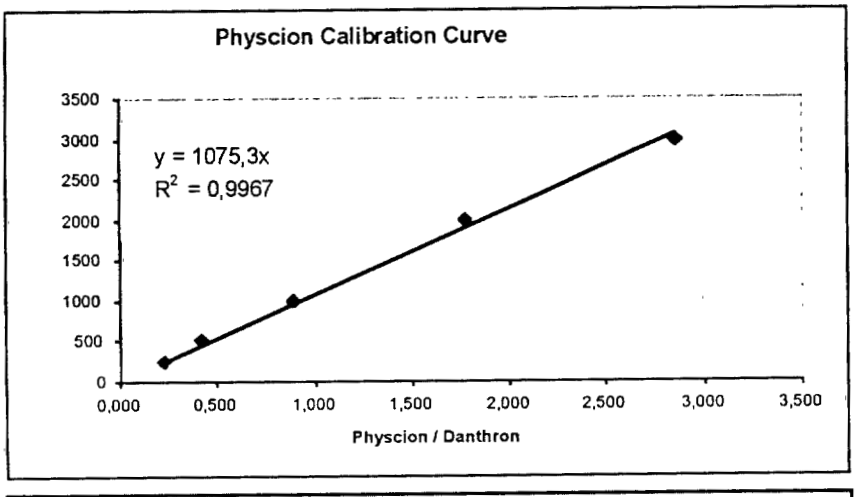
000177

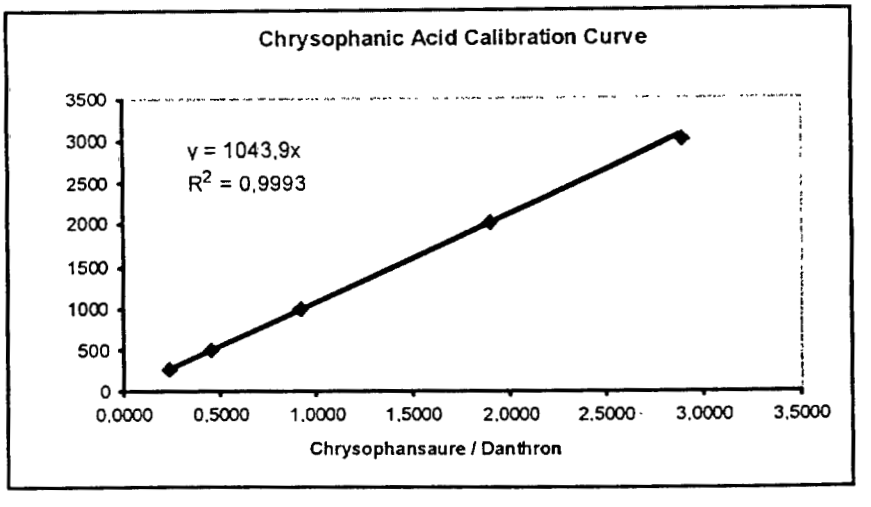
Test method	Issued:	Approved (b)(6)
SR 02402 V01	02/09/2002	

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content (Validation)	**Page 8 of 10**

Abb. 1: UV-spectras from emodin, aloe-emodin, rhein, chrysophanic acid, physcion and danthrone



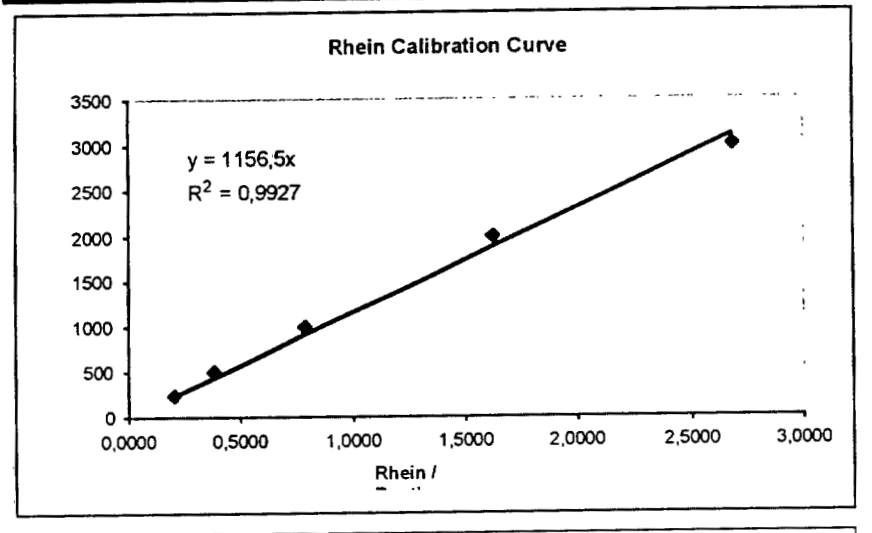


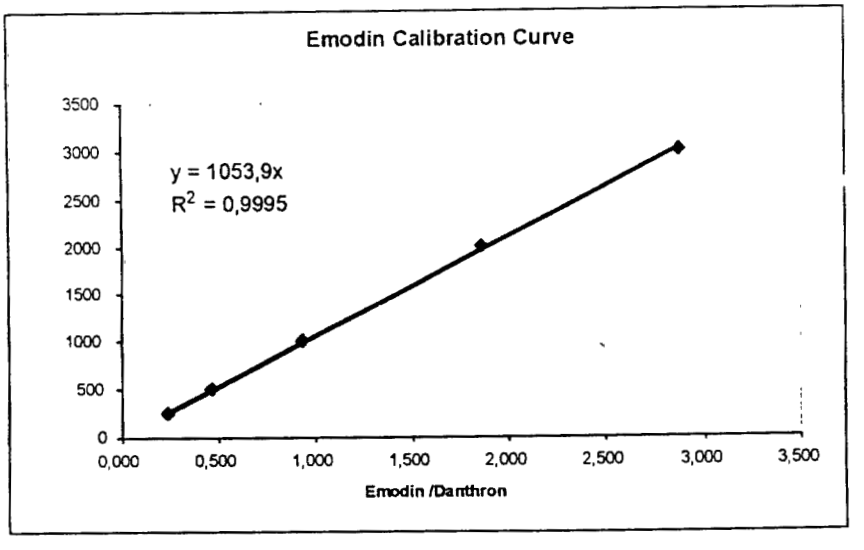
000178

Test method	Issued:	Approved: (b)(6)
SR 02402 V01	02/09/2002	

TESTING STANDARD

DIAGUM SR (= Cassia Gum)	Quality Assurance Test.No.: SR 02402 Version: 01 Author: Utz Issued: 02.09.02
Anthraquinone Content (Validation)	Page 9 of 10





000179

Test method	Issued:	Approved: (b)(6)
SR 02402 V01	02/09/2002	

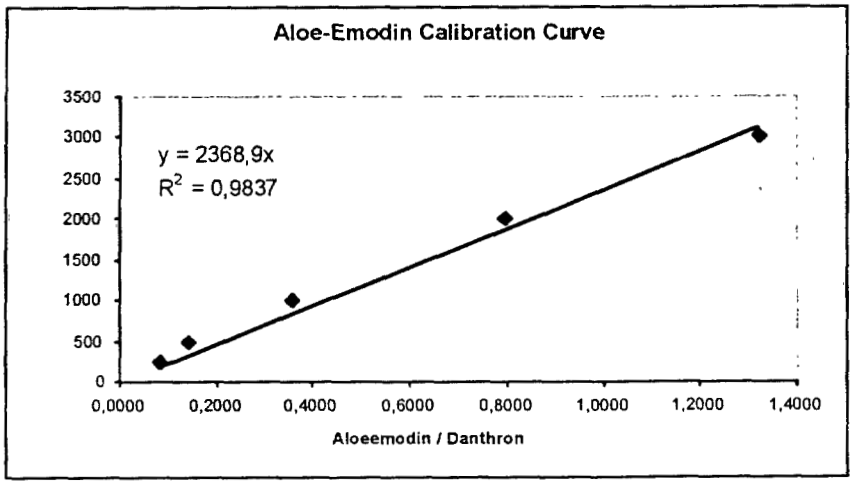


Abb. 2: Examples of calibration curves for physcion, chrysophanic acid, rhein, emodin and aloe-emodin

5. Storage

Standards can be stored in the dark at 4°C for up to 3 month.

000180

8

Reports of Ames Tests on Diagum CS™ and Diagum SR™ Tab 8

NOTOX
Safety & Environmental Research

TELEFAX MESSAGE

TO	:	Noveon Inc.
ATTN.	:	Mr. R.K. Hinderer, Ph.D. Fax.no.: 001 216 447 5760
CC	:	Dr. R.B. Foster
FROM	:	NOTOX Safety & Environmental Research B.V., P.O. Box 3476, 5203 DL 's-Hertogenbosch, The Netherlands. Telefax:++31 (0)73 640 6799 E-mail: Notox@notox.nl
SENDER	:	Dr. Ir. I.A.T.M. Meerts
DATE	:	July 29, 2003
REF.	:	MES/kel/03G0807
SUBJECT	:	NOTOX Substance 128106

(this page and 4 pages)

Please telephone ++ 31 (0)73 640 6700 if transmission is illegible.
The contents of this message may be confidential and are intended only for use by the recipient identified above. If you are not the intended recipient, please notify the sender immediately by telephone and destroy the original message without making a copy. Thank you for your cooperation.

Dear Dr. Hinderer,

Hereby we send you the results of the additional experiment with your product Cassia gum in the Ames test, as described in the second and third protocol amendment (NOTOX project 381105). The results are added to the results of the mutation test of which you already received a draft report.

As you can see in the attached table, also the additional experiment reveals that Cassia gum is not mutagenic in the Ames test.

We hope we have informed you sufficiently. Incase you have any questions, please do not hesitate to contact us. The revised draft report with the results of the additional experiment included will be send to you as soon as possible.

With kind regards,
NOTOX B.V.

(b)(6)

Dr. Ir. I.A.T.M. Meerts
Study Director

(b)(6)

Ing. E.J. van de Waart M.Sc.
Head of Genetic & Ecotoxicology

NOTOX
Safety & Environmental Research

NOTOX Project 381105
NOTOX Test substance 128106

Evaluation of the mutagenic activity of Cassia gum in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat).

Cassia gum was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA100 and TA98) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* WP_2uvrA. The test was performed in two independent experiments in the absence and presence of S9-mix (Aroclor-1254 induced rat liver S9-mix) with Milli-Q water as a vehicle. An additional experiment was performed at request of the sponsor in which Cassia gum was suspended in dimethyl sulfoxide and tested in TA100 and WP_2uvrA.

The study procedures described in this report were based on the following guidelines:
- OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test". (Adopted July 21, 1997).
- European Economic Community (EEC). Directive 2000/32/EC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Test using bacteria". EEC Publication Commission Directive (Published June 8, 2000).

Batch SH 225 of Cassia gum was a beige powder. In the first and second mutation assay, the test substance was suspended in Milli-Q water and was autoclaved for 20 min. at 121 ± 1°C. In the third mutation assay, Cassia gum was suspended in dimethyl sulfoxide.

First and second mutation assay
Cassia gum was tested up to concentrations of 100 µg/plate in the absence and presence of S9-mix (Milli-Q water as a vehicle). Cassia gum precipitated on the plates at dose levels of 33 µg/plate and upwards. The bacterial background lawn was not reduced at any of the concentrations tested and no decrease in the number of revertants was observed.

The presence of 5 and 10% (v/v) liver microsomal activation did not influence these findings.

Third mutation assay
Cassia gum was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix (dimethyl sulfoxide as a vehicle) in TA100 and WP_2uvrA. Cassia gum precipitated on the plates at all concentrations tested (100-5000 µg/plate). The bacterial background lawn was not reduced at any of the concentrations tested and no decrease in the number of revertants was observed.

Cassia gum did not induce a dose-related, two-fold increase in the number of revertant (His^+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp^+) colonies in tester strain WP_2uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in an independently repeated experiment, and in an additional experiment with a different vehicle in which Cassia gum was tested at higher concentrations.

Based on the results of this study it is concluded that Cassia gum is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

Attached please find the tables of the additional experiment (third mutation assay).

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Safety & Environmental Research

Table 1 **Experiment 3: Mutagenic response of Cassia gum in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance: 24 July 2003

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain	
	TA100	WP_2uvrA
	Without S9-mix	
positive control	927 ± 48	495 ± 9
solvent control	149 ± 13	9 ± 1
100 SP	153 ± 2	15 ± 1
333 SP	150 ± 10	11 ± 2
1000 SP	136 ± 14	10 ± 5
3330 MP	158 ± 3	9 ± 4
5000 MP	163 ± 10	7 ± 1
	With S9-mix[1]	
positive control	652 ± 26	107 ± 14
solvent control	149 ± 4	12 ± 1
100 SP	144 ± 27	13 ± 0
333 SP	137 ± 15	10 ± 2
1000 SP	134 ± 20	13 ± 3
3330 MP	151 ± 12	13 ± 3
5000 MP	153 ± 23	12 ± 1

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 5% (v/v) S9 fraction
SP Slight Precipitate
MP Moderate Precipitate

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Table 2, individual results

Experiment 3
Strain TA100

		WITHOUT S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		872	954	956	927 ±	48
solvent control		162	148	136	149 ±	13
100	SP	152	156	152	153 ±	2
333	SP	161	141	149	150 ±	10
1000	SP	145	119	143	136 ±	14
3330	MP	161	157	156	158 ±	3
5000	MP	175	158	156	163 ±	10

		WITH S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		649	628	680	652 ±	26
solvent control		154	147	147	149 ±	4
100	SP	144	117	171	144 ±	27
333	SP	123	134	153	137 ±	15
1000	SP	135	153	113	134 ±	20
3330	MP	162	139	153	151 ±	12
5000	MP	169	127	162	153 ±	23

SP: Slight Precipitate
MP: Moderate Precipitate

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Safety & Environmental Research

Table 3, individual results

Experiment 3
Strain WP2uvrA

		WITHOUT S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		493	488	505	495 ±	9
solvent control		10	8	9	9 ±	1
100	SP	14	16	14	15 ±	1
333	SP	12	9	13	11 ±	2
1000	SP	8	15	6	10 ±	5
3330	MP	13	8	6	9 ±	4
5000	MP	6	6	8	7 ±	1

		WITH S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		106	121	93	107 ±	14
solvent control		13	13	11	12 ±	1
100	SP	13	13	13	13 ±	0
333	SP	9	12	10	10 ±	2
1000	SP	12	10	16	13 ±	3
3330	MP	10	16	14	13 ±	3
5000	MP	13	11	13	12 ±	1

SP: Slight Precipitate
MP: Moderate Precipitate

REPORT

EVALUATION OF THE MUTAGENIC ACTIVITY OF

DIAGUM CS

IN THE SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY

AND THE ESCHERICHIA COLI REVERSE MUTATION ASSAY

(WITH INDEPENDENT REPEAT)

NOTOX Project 233066
NOTOX Substance 78201

RECEIVED
FEB 0 5 2003
R. HINDERER

STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director:

C.M. Verspeek-Rip

(b)(6)

Date: 29 September 1992

Management:

Ing. E.J. van de Waart
Head of Genetic &
Ecotoxicology

(b)(6)

Date: [illegible]

QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was audited by the NOTOX Quality Assurance Unit to ensure that the methods and results accurately reflect the raw data.

The dates of Quality Assurance inspections and audits are given below. During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS/ AUDITS	REPORTING DATES
on-site inspection	
21-08-1998	21-08-1998
protocol inspection	
19-06-1998	19-06-1998
report audit	
17-09-1998	17-09-1998

Head of Quality Assurance C.J. Mitchell B.Sc.

(b)(6)

Date: 30-9-98

SUMMARY

DIAGUM CS was tested in the Salmonella typhimurium reverse mutation assay with four histidine-requiring strains of Salmonella typhimurium (TA1535, TA1537, TA100 and TA98) and in the Escherichia coli reverse mutation assay with a tryptophan-requiring strain of Escherichia coli WP_2uvrA in two independent experiments.

DIAGUM CS was tested up to concentrations of 1000 µg/plate in the absence and presence of S9-mix. DIAGUM CS precipitated on the plates at concentrations of 62.5 µg/plate and upwards.

The negative and strain-specific positive control values were within our laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

DIAGUM CS showed in the tester strains TA1535, TA1537, TA98 and TA100 at least two-fold increases in the number of revertant colonies compared to the solvent control. The increases were only observed at precipitating concentrations and the increases in the tester strains TA1535, TA1537, TA98 were within our historical control data range. However, the increases in tester strain TA100 were outside our historical control data range. Furthermore, the increases in TA100 showed a dose relationship and were observed in two independently repeated experiments. Therefore, the increases are considered to be biological relevant and DIAGUM CS is considered to be mutagenic in this test system.

Strain WP_2uvrA showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments.

Based on the results of this study it is concluded that DIAGUM CS is mutagenic in the Salmonella typhimurium reverse mutation assay and DIAGUM CS is not mutagenic in the Escherichia coli reverse mutation assay.

PREFACE

Sponsor	B.F. Goodrich Diamalt GmbH P.O. Box 1151 D-83060 RAUBLING Germany
Study Monitor	Dr. A. Aigner
Testing Facility	NOTOX B.V. Hambakenwetering 3 5231 DD 's-Hertogenbosch The Netherlands
Study Director	C.M. Verspeek-Rip
Technical Coordinator	N.P. Giepmans
Study Plan	Start : 14 July 1998 Completed : 21 August 1998

TEST SUBSTANCE

Identification	Diagum CS
Description	Yellowish to reddish fine powder
Batch	98030211
Purity	Not indicated by sponsor; treated as 100% pure
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	02 March 1999
Stability in vehicle	Dimethylsulphoxide: not indicated

The sponsor is responsible for the completeness and GLP Compliance of all test substance data.

ARCHIVING

NOTOX B.V. will archive protocol, report, test article reference sample and raw data for at least 10 years. No data will be withdrawn without the sponsor's written consent.

GUIDELINES

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals;
  Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test" (adopted July 21, 1997)
- European Economic Community (EEC), Directive 92/69/EEC. Annex V of the EEC Directive 67/548/EEC, Part B: Methods for the Determination of Toxicity; B.13: "Mutagenicity: Escherichia coli - Reverse Mutation Assay", with strain WP_2uvrA only
  B.14: "Mutagenicity: Salmonella typhimurium - Reverse Mutation Assay"
  EEC Publication no. L383 (adopted December, 1992).

OBJECTIVE

Objective

The objective of this study was to evaluate the test substance for its ability to induce reverse mutations in a gene of histidine-requiring Salmonella typhimurium bacterial strains resulting in histidine-independent strains, and in a gene of tryptophan-requiring Escherichia coli bacterial strain resulting in a tryptophan-independent strain.

Background of the test system

The Salmonella typhimurium reverse mutation assay and the Escherichia coli reverse mutation assay have shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay was conducted in the absence and presence of a metabolizing system (S9-mix).

The Salmonella typhimurium strains used in this study were TA98, TA100, TA1535 and TA1537. The Escherichia coli strain used was WP_2uvrA.
The strains TA98 and TA1537 are capable of detecting frameshift mutagens, strains TA100, TA1535 and WP_2uvrA are capable of detecting base-pair substitution mutagens (1,2,3,4 and 5).

MATERIALS AND METHODS

TEST SYSTEM

Test System	Salmonella typhimurium bacteria and Escherichia coli bacteria
Rationale	Recommended test system in international guidelines (e.g. EPA, OECD, EEC).
Source	Dr. Bruce N. Ames, University of California at Berkeley, U.S.A. (Salmonella typhimurium strains) TA100 received 18-02-1993, used batch: TA100.100498 TA98 received 21-02-1991, used batch: experiment 1 TA98.200198, experiment 2 TA98.150698 TA1535 received 14-12-1994, used batch: experiment 1 TA1535.200198, experiment 2 TA1535.150698 TA1537 received 14-12-1994, used batch: experiment 1 TA1537.200198, experiment 2 TA1537.150698 Prof. Dr. B.A. Bridges, University of Sussex, Brighton, U.K. (Escherichia coli strain) WP_2uvrA received 23-10-1987, used batch: EC.050598

The characteristics of the different Salmonella typhimurium strains were as follows:

Strain	Histidine mutation	Mutation type
TA1537	hisC3076	Frameshift
TA98	hisD3052/R-factor*	Frameshift
TA1535	hisG46	Base-pair substitutions
TA100	hisG46/R-factor*	Base-pair substitutions

*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contained the following additional mutations:

rfa : deep rough (defective lipopolysaccharide cellcoat)
gal : mutation in the galactose metabolism
chl : mutation in nitrate reductase
bio : defective biotin synthesis
uvrB: loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The Salmonella typhimurium strains were regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants

The Escherichia coli WP_2uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV The strain was regularly checked to confirm the tryptophan-requirement. UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains were stored in liquid nitrogen (-196°C)

CELL CULTURE

Preparation of bacterial cultures	Samples of frozen stock cultures of bacteria were transferred into enriched nutrient broth (Oxoid no. 2) and incubated in a shaking incubator (37°C, 150 spm), until the cultures reached an optical density of 0.4 at 700 nm (10^9 cells/ml). Freshly grown cultures of each strain were used for a test.
Permeabilization of the *Escherichia coli* strain	WP_2uvrA bacteria were washed twice in 0.25 the original volume of ice-cold 0.12 M Tris-HCL buffer pH 8.0, then gently resuspended in 0.2 vol. 0.12 M Tris-HCL, 0.5 mM EDTA pH 8.0, and shaken for 2.5 min at 37°C. $MgCl_2$ was then added to a final concentration of 10 mM. The cells were centrifuged and resuspended in the original volume of nutrient broth.
Agar plates	Agar plates (ø 9 cm) contained 25 ml glucose agar medium. Glucose agar medium contained per liter: 18 g purified agar (Oxoid, code L28) in Vogel-Bonner Medium E, 10 g glucose. N.B. The agar plates for the test with the *Salmonella typhimurium* strains also contained 12.5 µg/plate biotin and 15 µg/plate histidine and the agar plates for the test with the *Escherichia coli* strain contained 15 µg/plate tryptophan.
Top agar	Vogel-Bonner Medium E containing 0.6% (w/v) purified agar was heated to dissolve the agar. Samples of 3 ml top agar were transferred into 10 ml glass tubes with metal caps. Top agar tubes were autoclaved for 20 min at 121°C.
Environmental conditions	All incubations were carried out in the dark at 37°C. The temperature was monitored during the experiment.

TREATMENT OF THE TEST SUBSTANCE

The test substance was suspended in dimethylsulphoxide of spectroscopic quality (Merck). The stock solution was treated with ultra-sonication to obtain a homogeneous suspension. Test substance concentrations were prepared directly prior to use.

REFERENCE SUBSTANCES

Negative control:

The vehicle of the test article, being dimethylsulphoxide.

Positive controls:

Without metabolic activation (-S9-mix):

Solvents for reference substances
Saline = physiological saline (Fresenius AG, Bad Homburg, Germany)
DMSO = dimethylsulphoxide of spectroscopic quality (Merck).

Strain	Chemical	Concentration/plate		Solvent
TA1535	sodium azide (SA) (Fluka)	1	µg	Saline
TA1537	9-aminoacridine (9AC) (Janssen Chimica)	60	µg	Saline
TA98	daunomycine (DM) (Sigma)	4	µg	Saline
TA100	methylmethanesulfonate (MMS) (Merck)	650	µg	DMSO
WP_2uvrA	4-nitroquinoline N-oxide (4-NQO) (Sigma)	10	µg	DMSO

With metabolic activation (+S9-mix):

Strain	Chemical	Concentration/plate		Solvent
TA1537	2-aminoanthracene (2AA) (Sigma)	2.5	µg	DMSO
TA1535, TA98 and TA100	2-aminoanthracene (2AA) (Sigma)	1	µg	DMSO
WP_2uvrA	2-aminoanthracene (2AA) (Sigma)	5	µg	DMSO

METABOLIC ACTIVATION SYSTEM

Preparation of S9-fraction

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from Charles River, Sulzfeld, Germany.

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the SOP's. The rats were injected intraperitoneally with a solution (20% (w/v)) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, they were killed by decapitation; (they were denied access to food for at least 12 hours preceding sacrifice). The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C) and identified by the day of preparation

Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix contained per 10 ml: 30 mg NADP and 15.2 mg glucose-6-phosphate in 5.5 ml or 5.0 ml aqua bidest (first or second experiment respectively); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M $MgCl_2$ solution; 1 ml 0.33 M KCl solution. The above solution was filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction was added (5% (v/v) S9-fraction) to complete the S9-mix in the first experiment and to 9.0 ml of S9-mix components 1.0 ml S9-fraction was added (10% (v/v) S9-fraction) to complete the S9-mix in the second experiment.

The S9-batch used was no. 98-5 (preparation date: 17-03-1998).

EXPERIMENTAL PROCEDURE

Dose range finding test

Selection of an adequate range of doses was based on a dose range finding test with strain TA100 and the WP_2uvrA strain, both with and without S9-mix. Eight concentrations were tested in triplicate. This dose range finding test was reported as a part of the first experiment of the mutation assay. The highest concentration of DIAGUM CS used in the subsequent mutation assay was the level at which the test substance exhibited limited solubility and mutagenicity was observed.

Mutation assay

At least five different doses (increasing with approximately half-log steps) of the test substance were tested in triplicate in each strain

The test substance was tested both in the absence and presence of S9-mix in each strain, in two independent experiments.

Top agar in top agar tubes was molten and heated to 45°C. The following solutions were successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture (10^9 cells/ml) of one of the tester strains, 0 1 ml of a dilution of the test substance in dimethylsulphoxide and either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients were mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were turned and incubated in the dark at 37°C for 48 h. After this period revertant colonies (histidine independent for Salmonella typhimurium bacteria and tryptophan independent for Escherichia coli) were counted.

Colony counting

The revertant colonies (histidine independent c.q. tryptophan independent) were counted automatically with a Protos model 50000 colony counter or manually, if less than 40 colonies per plate were present. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

ACCEPTABILITY OF ASSAY

A Salmonella typhimurium reverse mutation assay and/or Escherichia coli reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory background historical range for each tester strain.

Strain		Minimum value	Maximum value	Mean ± 3 x S.D
TA1535	- S9-mix	3	23	9 ± 9
	+ S9-mix	3	23	10 ± 9
TA1537	- S9-mix	3	24	9 ± 12
	+ S9-mix	3	28	8 ± 12
TA98	- S9-mix	12	58	20 ± 24
	+ S9-mix	12	60	24 ± 24
TA100	- S9-mix	57	186	86 ± 54
	+ S9-mix	60	183	89 ± 54
WP_2uvrA	- S9-mix	4	29	12 ± 15
	+ S9-mix	4	28	13 ± 15

b) The positive control chemicals should produce responses in all tester strains which are within the laboratory historical range documented for each positive control substance.

Strain		Minimum value	Maximum value	Mean ± 3 x S.D
TA1535	- S9-mix	104	1268	239 ± 294
	+ S9-mix	50	981	788 ± 384
TA1537	- S9-mix	80	2179	359 ± 609
	+ S9-mix	73	1113	560 ± 612
TA98	- S9-mix	106	1345	456 ± 582
	+ S9-mix	97	2807	788 ± 1374
TA100	- S9-mix	224	1362	780 ± 549
	+ S9-mix	182	3435	1082 ± 1629
WP_2uvrA	- S9-mix	66	1988	644 ± 1134
	+ S9-mix	77	1532	301 ± 531

c) The selected dose range should include a clearly toxic concentration or should be exhibit by limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate

DATA EVALUATION AND STATISTICAL PROCEDURES

No formal hypothesis testing was done.

A test substance is considered negative (not mutagenic) in the test if:

a) The total number of revertants in any tester strain at any concentration is not greater than two times the solvent control value, with or without metabolic activation.

b) The negative response should be reproducible in at least one independently repeated experiment.

A test substance is considered positive (mutagenic) in the test if:

a) It induces at least a 2-fold, dose related increase in the number of revertants with respect to the number induced by the solvent control in any of the tester strains, either with or without metabolic activation However, any mean plate count of less than 20 is considered to be not significant.

b) The positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

RESULTS

DOSE RANGE FINDING TEST (Table 2, Appendix 2)

DIAGUM CS was tested in the tester strains TA100 and WP_2uvrA with concentrations of 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate in the absence and presence of S9-mix.

Precipitate
The test substance precipitated in the top agar at concentrations of 10 µg/plate and upwards. Precipitation of DIAGUM CS on the plates was observed at concentrations of 33 µg/plate and upwards at the start of the incubation period. Precipitation of DIAGUM CS on the plates was observed at concentrations of 100 µg/plate and upwards at the end of the incubation period in tester strain TA100 and WP_2uvrA. The precipitate did not interfere with the scoring.

Toxicity
No reduction of the bacterial background lawn and no decrease in the number of revertants was observed.

Number of revertants
Strain WP_2uvrA showed negative responses over the entire dose range, i e no dose-related, two-fold, increase in the number of revertants. Strain TA100 showed 4.8- and 5.1-fold, dose-related, increases in the number of revertant (His^+) colonies in the absence and presence of S9-mix respectively.

MUTATION ASSAY (Table 2, 3 and 4, Appendix 2)

Experiment 1
Based on the dose range finding data, the following dose range was selected for the first mutation assay with the Salmonella typhimurium strains, TA1535, TA1537 and TA98.
Without S9-mix: 4, 16, 62.5, 250 and 1000 µg/plate.
With S9-mix : 4, 16, 62.5, 250 and 1000 µg/plate.

Precipitate
DIAGUM CS precipitated in the top agar at concentrations of 62.5 µg/plate and upwards. Precipitation of DIAGUM CS on the plates was observed at concentrations of 62.5 µg/plate and upwards at the start and at the end of the incubation period in all tester strains.

Toxicity of the test substance
The bacterial background lawn was not reduced at all concentrations tested and no decrease in the number of revertants was observed.

Number of revertants
The bacterial strains, TA1535, TA1537, TA98 showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants.

Experiment 2
Since, in the first experiment precipitation of DIAGUM CS was observed in too many dose levels, the following dose range was tested in the second experiment with the strains, TA1535, TA1537, TA98, TA100 and WP_2uvrA:
Without S9-mix: 1.6, 8, 40, 200 and 1000 µg/plate.
With S9-mix : 1.6, 8, 40, 200 and 1000 µg/plate.

Precipitate
DIAGUM CS precipitated in the top agar at concentrations of 200 µg/plate and upwards. Precipitation of DIAGUM CS on the plates was observed at concentrations of 200 µg/plate and upwards at the start and at the end of the incubation period in all tester strains.

Toxicity of the test substance
The bacterial background lawn was not reduced at all concentrations tested and no decrease in the number of revertants was observed.

Number of revertants
The bacterial strain WP_2uvrA showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants.

All Salmonella strains showed dose-related increases in the number of revertants. The increases in the number of revertant colonies in the bacterial strains compared to the solvent control values are presented in Table 1.

TABLE 1 INCREASES IN THE NUMBER OF REVERTANT COLONIES

Strain	Without S9	With 10% (v/v) S9
TA1535	2.0-fold	-[1]
TA1537	4.4-fold	3.0-fold
TA98	2.8-fold	-[2]
TA100	5.4-fold	2.8-fold

-[1] No increase in the number of revertant colonies
-[2] Increase in the number of revertant colonies less than two-fold

Evaluation of the mutagenicity
The negative and strain-specific positive control values were within our laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Strain TA1535 showed a two-fold increase in the number of revertant colonies in the absence of S9-mix. However, this increase was observed only in one experiment and no dose relationship was observed. Furthermore, this increase was within our historical control data range. Therefore, this increase is considered to be not biological relevant and DIAGUM CS is considered to be not mutagenic in strain TA1535.

Evaluation of the mutagenicity

The negative and strain-specific positive control values were within our laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

DIAGUM CS showed in the tester strains TA1535, TA1537, TA98 and TA100 at least two-fold increases in the number of revertant colonies compared to the solvent control. The increases were only observed at precipitating concentrations and the increases in the tester strains TA1535, TA1537, TA98 were within our historical control data range. However, the increases in tester strain TA100 were outside our historical control data range. Furthermore, the increases in TA100 showed a dose relationship and were observed in two independently repeated experiments. Therefore, the increases are considered to be biological relevant and DIAGUM CS is considered to be mutagenic in this test system.

Strain WP_2uvrA showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments.

CONCLUSION

Based on the results of this study it is concluded that DIAGUM CS is mutagenic in the Salmonella typhimurium reverse mutation assay and DIAGUM CS is not mutagenic in the Escherichia coli reverse mutation assay.

REFERENCES

1 Leonardo, J.M., Dornfeld, S.S. and Peak, M.J., 1984, Evaluation of E. coli K12 343 \ 13 and derived strains for microbial mutagenicity assays. Mutation Res., 130, 87-95.
2 Ames, B.N., McCann, J. and Yamasaki, E., 1975, Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test, Mutation Res., 31, 347-364.
3 Maron, D.M. and Ames, B.N., 1983, Revised methods for the Salmonella mutagenicity test, Mutation Res., 113, 173-215.
Erratum, 1983, Mutation Res., 113, 533.
4 Green, M.H.L. and Muriel, W.J., 1976, Mutagen testing using Trp^+ reversion in Escherichia coli, Mutation Res., 38, 3-32.
5 Vogel, H.J. and Bonner, D.M., 1956, Acetylornithinase of Escherichia coli: partial purification and some properties. J. Biol. Chem., 218, 97-106.

TABLE 2 DOSE RANGE FINDING STUDY AND MUTAGENIC RESPONSE OF DIAGUM CS IN THE SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY AND IN THE ESCHERICHIA COLI REVERSE MUTATION ASSAY

Dose range finding study (part of experiment 1)
Day of performance: 14 July 1998

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S D) with one strain of Salmonella typhimurium and one Escherichia coli strain	
	TA100	WP_2uvrA
	Without S9-mix	
positive control	561 ± 14	635 ± 56
solvent control	67 ± 6	6 ± 2
3	55 ± 2	12 ± 2
10	65 ± 7	16 ± 4
33	72 ± 9	19 ± 4
100[SP]	75 ± 5	15 ± 3
333[SP]	92 ± 10	12 ± 5
1000[SP]	211 ± 43	16 ± 2
3330[SP]	324 ± 15	17 ± 5
5000[SP]	299 ± 3	11 ± 2
	With S9-mix[1]	
positive control	1069 ± 64	112 ± 13
solvent control	64 ± 8	6 ± 2
3	68 ± 7	17 ± 6
10	59 ± 12	15 ± 6
33	73 ± 10	14 ± 4
100[SP]	77 ± 6	15 ± 6
333[SP]	77 ± 6	12 ± 3
1000[SP]	173 ± 42	10 ± 6
3330[SP]	281 ± 26	17 ± 2
5000[SP]	324 ± 77	13 ± 4

Solvent control: 0 1 ml dimethylsulphoxide
SP: DIAGUM CS precipitated slightly on the plates
[1] The S9-mix contained 5% (v/v) S9 fraction

TABLE 3 MUTAGENIC RESPONSE OF DIAGUM CS IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY

Experiment 1
Day of performance: 04 August 1998

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium*		
	TA1535	TA1537	TA98
	Without S9-mix		
positive control	209 ± 25	379 ± 28	647 ± 52
solvent control	10 ± 1	9 ± 3	18 ± 1
4	9 ± 1	16 ± 4	17 ± 8
16	9 ± 2	8 ± 4	15 ± 4
62.5 SP	7 ± 2	9 ± 4	17 ± 5
250 SP	10 ± 2	7 ± 3	25 ± 4
1000 SP	6 ± 2	9 ± 1	27 ± 10
	With S9-mix[1]		
positive control	385 ± 58	816 ± 49	1130 ± 37
solvent control	9 ± 2	10 ± 5	18 ± 6
4	10 ± 6	9 ± 2	18 ± 8
16	6 ± 1	11 ± 4	19 ± 5
62.5 SP	9 ± 4	10 ± 4	18 ± 3
250 SP	9 ± 3	9 ± 5	24 ± 2
1000 SP	7 ± 1	8 ± 2	23 ± 3

Solvent control: 0.1 ml dimethylsulphoxide
SP: DIAGUM CS precipitated slightly on the plates
[1] The S9-mix contained 5% (v/v) S9 fraction

TABLE 4 MUTAGENIC RESPONSE OF DIAGUM CS IN THE SALMONELLA TYPHIMURIUM REVERSE MUTATION ASSAY AND IN THE ESCHERICHIA COLI REVERSE MUTATION ASSAY

Experiment 2
Day of performance: 19 August 1998

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of Salmonella typhimurium and one Escherichia coli strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
			Without S9-mix		
positive control	236 ± 9	337 ± 59	552 ± 183	895 ± 46	632 ± 60
solvent control	9 ± 1	5 ± 2	13 ± 1	85 ± 10	12 ± 1
1.6	14 ± 3	6 ± 2	14 ± 2	81 ± 6	13 ± 4
8	15 ± 1	4 ± 1	16 ± 1	85 ± 12	10 ± 1
40	16 ± 6	4 ± 2	15 ± 2	100 ± 25	12 ± 3
200SP	16 ± 5	10 ± 4	19 ± 3	144 ± 8	13 ± 2
1000SP	18 ± 5	22 ± 9	36 ± 4	459 ± 47	11 ± 1
			With S9-mix[1]		
positive control	153 ± 23	187 ± 3	543 ± 31	788 ± 46	127 ± 5
solvent control	14 ± 4	5 ± 2	21 ± 4	96 ± 10	10 ± 4
1.6	11 ± 2	7 ± 1	25 ± 4	99 ± 5	13 ± 4
8	14 ± 5	6 ± 1	26 ± 5	101 ± 7	11 ± 4
40	15 ± 3	6 ± 2	23 ± 3	94 ± 3	14 ± 5
200SP	17 ± 3	10 ± 3	33 ± 6	105 ± 12	14 ± 4
1000SP	14 ± 6	15 ± 2	34 ± 2	265 ± 65	14 ± 1

Solvent control: 0.1 ml dimethylsulphoxide
SP: DIAGUM CS precipitated slightly on the plates
[1] The S9-mix contained 10% (v/v) S9 fraction

APPENDIX 1

Bacterial background lawn evaluation

The condition of the bacterial background lawn is evaluated (if indicated), both macroscopically and microscopically by using a dissecting microscope (results are normal unless indicated in tables).

Definition	Characteristics
Normal	Distinguished by a healthy microcolony lawn.
Slightly reduced	Distinguished by a noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Moderately reduced	Distinguished by a marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Absent	Distinguished by a complete lack of any microcolony background lawn.

Precipitation evaluation

Evidence of test article precipitate on the plates is recorded by addition of the following precipitation definition.

Definition	Characteristics
Slight Precipitate	Distinguished by noticeable precipitate on the plate. However, the precipitate does not influence automated counting of the plate.
Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Evaluation of the reduction in the number of revertants

The reduction in the number of revertant colonies compared to number of revertants in the solvent control is evaluated as follows:
A reduction of 21-40%: slight reduction.
A reduction of 41-60%: moderate reduction.
A reduction of 61-99%: extreme reduction.
If no revertant colonies are observed on the plates the reduction is evaluated as a complete lack of revertants.
However, any mean plate count equal to the minimal value of the historical control data range should be considered not toxic.

APPENDIX 2

Individual plate counts; (following pages)

Dose range finding (part of experiment 1)
Strain TA100

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	549	577	558	561 ±	14
solvent control	68	61	72	67 ±	6
3	55	54	57	55 ±	2
10	57	69	69	65 ±	7
33	69	66	82	72 ±	9
100[SP]	76	69	79	75 ±	5
333[SP]	84	103	89	92 ±	10
1000[SP]	213	168	253	211 ±	43
3330[SP]	340	319	312	324 ±	15
5000[SP]	296	300	301	299 ±	3

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1115	996	1095	1069 ±	64
solvent control	69	55	68	64 ±	8
3	62	75	66	68 ±	7
10	55	49	72	59 ±	12
33	66	68	85	73 ±	10
100[SP]	83	74	73	77 ±	6
333[SP]	84	74	74	77 ±	6
1000[SP]	218	167	135	173 ±	42
3330[SP]	253	305	286	281 ±	26
5000[SP]	283	412	276	324 ±	77

SP: Slight Precipitate

Dose range finding (part of experiment 1)
Strain WP_2uvrA

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	698	594	612	635 ±	56
solvent control	6	5	8	6 ±	2
3	10	14	11	12 ±	2
10	19	12	16	16 ±	4
33	16	17	23	19 ±	4
100SP	18	13	14	15 ±	3
333SP	17	11	7	12 ±	5
1000SP	18	16	15	16 ±	2
3330SP	11	19	20	17 ±	5
5000SP	9	12	13	11 ±	2

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	125	111	100	112 ±	13
solvent control	8	5	6	6 ±	2
3	11	17	23	17 ±	6
10	22	12	10	15 ±	6
33	15	9	17	14 ±	4
100SP	15	9	20	15 ±	6
333SP	9	14	12	12 ±	3
1000SP	16	5	10	10 ±	6
3330SP	15	17	18	17 ±	2
5000SP	18	10	11	13 ±	4

SP: Slight Precipitate

Experiment 1
Strain TA1535

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	189	237	202	209 ±	25
solvent control	10	9	11	10 ±	1
4	8	10	8	9 ±	1
16	11	10	7	9 ±	2
62.5 SP	7	9	6	7 ±	2
250 SP	10	11	8	10 ±	2
1000 SP	8	4	7	6 ±	2

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	325	389	440	385 ±	58
solvent control	11	7	10	9 ±	2
4	9	5	16	10 ±	6
16	7	6	6	6 ±	1
62.5 SP	13	9	5	9 ±	4
250 SP	7	8	13	9 ±	3
1000 SP	7	8	6	7 ±	1

SP: Slight Precipitate

Experiment 1
Strain TA1537

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	411	366	359	379 ±	28
solvent control	7	7	12	9 ±	3
4	13	15	20	16 ±	4
16	6	5	13	8 ±	4
62.5 SP	9	12	5	9 ±	4
250 SP	5	10	7	7 ±	3
1000 SP	8	9	9	9 ±	1

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	824	764	861	816 ±	49
solvent control	6	9	16	10 ±	5
4	7	8	11	9 ±	2
16	12	14	7	11 ±	4
62.5 SP	11	14	6	10 ±	4
250 SP	5	7	14	9 ±	5
1000 SP	8	6	9	8 ±	2

SP: Slight Precipitate

Experiment 1
Strain TA98

WITHOUT S9-MIX

plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	693	659	590	647 ±	52
solvent control	17	18	18	18 ±	1
4	24	8	20	17 ±	8
16	19	13	12	15 ±	4
62.5 SP	12	17	22	17 ±	5
250 SP	22	29	24	25 ±	4
1000 SP	22	20	38	27 ±	10

WITH S9-MIX

plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1113	1105	1173	1130 ±	37
solvent control	25	17	13	18 ±	6
4	13	14	28	18 ±	8
16	24	19	14	19 ±	5
62.5 SP	17	16	22	18 ±	3
250 SP	26	22	24	24 ±	2
1000 SP	20	25	24	23 ±	3

SP: Slight Precipitate

Experiment 2
Strain TA1535

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	236	228	245	236 ±	9
solvent control	9	8	9	9 ±	1
1.6	12	17	14	14 ±	3
8	14	16	14	15 ±	1
40	13	12	22	16 ±	6
200[SP]	15	11	21	16 ±	5
1000[SP]	23	18	14	18 ±	5

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	141	180	138	153 ±	23
solvent control	11	19	13	14 ±	4
1.6	14	10	10	11 ±	2
8	9	18	16	14 ±	5
40	13	14	18	15 ±	3
200[SP]	19	14	18	17 ±	3
1000[SP]	20	9	13	14 ±	6

SP: Slight Precipitate

Experiment 2
Strain TA1537

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	389	272	349	337 ±	59
solvent control	4	8	4	5 ±	2
1.6	4	8	5	6 ±	2
8	3	4	5	4 ±	1
40	6	5	2	4 ±	2
200[SP]	8	14	7	10 ±	4
1000[SP]	12	30	24	22 ±	9

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	185	185	190	187 ±	3
solvent control	5	7	4	5 ±	2
1.6	6	7	7	7 ±	1
8	6	7	5	6 ±	1
40	6	7	4	6 ±	2
200[SP]	7	11	12	10 ±	3
1000[SP]	15	13	16	15 ±	2

SP: Slight Precipitate

Experiment 2
Strain TA98

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	454	764	439	552 ±	183
solvent control	14	13	12	13 ±	1
1.6	16	13	12	14 ±	2
8	17	16	16	16 ±	1
40	16	15	13	15 ±	2
200SP	22	17	19	19 ±	3
1000SP	41	33	34	36 ±	4

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	513	574	542	543 ±	31
solvent control	18	21	25	21 ±	4
1.6	22	25	29	25 ±	4
8	22	31	24	26 ±	5
40	21	21	26	23 ±	3
200SP	32	28	40	33 ±	6
1000SP	36	35	32	34 ±	2

SP: Slight Precipitate

Experiment 2
Strain TA100

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	941	850	895	895 ±	46
solvent control	75	95	84	85 ±	10
1.6	75	81	86	81 ±	6
8	86	73	97	85 ±	12
40	72	109	120	100 ±	25
200SP	145	135	151	144 ±	8
1000SP	480	406	492	459 ±	47

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	839	749	777	788 ±	46
solvent control	97	105	86	96 ±	10
1.6	93	103	100	99 ±	5
8	109	98	97	101 ±	7
40	91	96	94	94 ±	3
200SP	97	99	119	105 ±	12
1000SP	316	192	286	265 ±	65

SP: Slight Precipitate

Experiment 2
Strain WP_2uvrA

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	607	588	700	632 ±	60
solvent control	13	11	12	12 ±	1
1.6	17	9	14	13 ±	4
8	11	9	11	10 ±	1
40	8	13	14	12 ±	3
200SP	11	13	15	13 ±	2
1000SP	10	12	11	11 ±	1

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	121	131	128	127 ±	5
solvent control	7	8	14	10 ±	4
1.6	9	16	14	13 ±	4
8	8	11	15	11 ±	4
40	8	17	16	14 ±	5
200SP	11	12	18	14 ±	4
1000SP	15	15	13	14 ±	1

SP: Slight Precipitate

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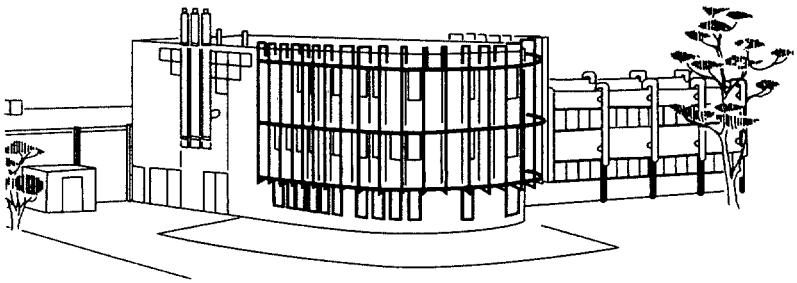

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REPORT

Study Title

EVALUATION OF THE MUTAGENIC ACTIVITY OF CASSIA GUM IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY AND THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY (WITH INDEPENDENT REPEAT)

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Study completion date

24 September 2003

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Laboratory Project Identification

NOTOX Project 381105
NOTOX Substance 128106

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2. STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).

Which essentially conform to:

The United States Food and Drug Administration Good Laboratory Practice Regulations.

The United States Environmental Protection Agency Good Laboratory Practice Regulations.

The results of preliminary solubility tests were excluded from GLP and results are not reported.

NOTOX B.V.

Dr. Ir. I.A.T.M. Meerts
Study Director

(b)(6)

Date: 24 SEPTEMBER 2003

Ing. E.J. van de Waart, M.Sc.
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Date: 25/09/2003

3. QUALITY ASSURANCE STATEMENT

NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the NOTOX Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

The dates of Quality Assurance inspections are given below.
During the on-site process inspections procedures applicable to this type of study were inspected.

Type of inspections	Phase / Section	Start Inspection date(s)	End Inspection date(s)	Reporting date
Protocol (Study)		07-05-2003	07-05-2003	07-05-2003
On-site (Process)	Genetic toxicology & *in vitro* metabolism	01-05-2003 28-07-2003	07-05-2003 30-07-2003	07-05-2003 08-08-2003
Report (Study)		25-06-2003 18-08-2003	25-06-2003 18-08-2003	25-06-2003 18-08-2003

Head of Quality Assurance
C.J. Mitchell B.Sc.

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Date: September 25, 2003

4. SUMMARY

Evaluation of the mutagenic activity of Cassia gum in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat).

Cassia gum was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA100 and TA98) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* WP_2uvrA. The test was performed in two independent experiments in the absence and presence of S9-mix (Aroclor-1254 induced rat liver S9-mix) with Milli-Q water as a vehicle. An additional experiment was performed at request of the sponsor in which Cassia gum was suspended in dimethyl sulfoxide and tested in TA100 and WP_2uvrA.

The study procedures described in this report were based on the following guidelines:
- OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test". (Adopted July 21, 1997).
- European Economic Community (EEC). Directive 2000/32/EC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Test using bacteria". EEC Publication Commission Directive (Published June 8, 2000).

Batch SH 225 of Cassia gum was a beige powder. In the first and second mutation assay, the test substance was suspended in Milli-Q water and was autoclaved for 20 min at 121 ± 3°C. In the third mutation assay, Cassia gum was suspended in dimethyl sulfoxide.

First and second mutation assay
Cassia gum was tested up to concentrations of 100 µg/plate in the absence and presence of S9-mix (Milli-Q water as a vehicle). Cassia gum precipitated on the plates at dose levels of 33 µg/plate and upwards. The bacterial background lawn was not reduced at any of the concentrations tested and no decrease in the number of revertants was observed.

The presence of 5 and 10% (v/v) liver microsomal activation did not influence these findings.

Third mutation assay
Cassia gum was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix (dimethyl sulfoxide as a vehicle) in TA100 and WP_2uvrA. Cassia gum precipitated on the plates at all concentrations tested (100-5000 µg/plate). The bacterial background lawn was not reduced at any of the concentrations tested and no decrease in the number of revertants was observed.

Cassia gum did not induce a dose-related, two-fold increase in the number of revertant (His^+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp^+) colonies in tester strain WP_2uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in an independently repeated experiment, and in an additional experiment with a different vehicle in which Cassia gum was tested at higher concentrations.

Based on the results of this study it is concluded that Cassia gum is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

5. INTRODUCTION

5.1. Preface

Sponsor	Noveon Inc. Mr. R.K. Hinderer, Ph.D. 9911 Brecksville Road CLEVELAND, OHIO 44141-3247 USA
Study Monitor	Dr. F. Utz Noveon Pharma GMBH & Co. KG Rosenheimer Strasse 43 D-83064 RAUBLING Germany
Test Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	Dr. Ir. I.A.T.M. Meerts
Technical Coordinator:	Ing. A. Tijdink
Study Plan	Start : 13 May 2003 Completion : 28 July 2003

5.2. Aims of the study

The objective of this study was to evaluate the test substance for its ability to induce reverse mutations in a gene of histidine-requiring *Salmonella typhimurium* bacterial strains resulting in histidine-independent strains, and in a gene of tryptophan-requiring *Escherichia coli* bacterial strain resulting in a tryptophan-independent strain.

Background of the test system
The *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay have shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay was conducted in the absence and presence of a metabolizing system (S9-mix).

The *Salmonella typhimurium* strains used in this study were TA98, TA100, TA1535 and TA1537. The *Escherichia coli* strain used was WP_2uvrA.

The strains TA98 and TA1537 are capable of detecting frameshift mutagens, strains TA100, TA1535 and WP_2uvrA are capable of detecting base-pair substitution mutagens (Ref. 1, 2, 3, 4 and 5).

5.3. Guidelines

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test". (Adopted July 21, 1997).
- European Economic Community (EEC). Directive 2000/32/EC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Test using bacteria".
- EEC Publication Commission Directive (Published June 8, 2000).

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocols, raw data, specimens and the final report are retained in the NOTOX archives for a period of at least 10 years after finalization of the report. After this period, the sponsor will be contacted to determine whether raw data and specimens should be returned to them, retained or destroyed on their behalf.

NOTOX will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

6. MATERIALS AND METHODS

6.1. Test Substance

Identification	Cassia gum
Molecular formula	$(C_6H_{10}O_5)_n,H_2O$
Molecular weight	200.000-300.000
CAS Number	11078-30-1
Description	Beige powder
Batch	SH 225
Composition	Carbohydrates/Fibers > 80 % (Galactomannan)
	Water 8.33 %
	Protein 5.8 %
	Fat 0.5 %
Test substance storage	At room temperature in the dark
Stability under storage conditions	Stable
Expiry date	31 July 2004
Specific Gravity	0.6 g/ml
Stability in vehicle	Water: At least 24 h
	Dimethyl sulfoxide: No

The sponsor is responsible for all test substance data unless determined by NOTOX (See Appendix III for certificate of analysis).

6.1.1. Test Substance preparation

For the first two experiments, the test substance was suspended in Milli-Q water (Millipore Corp., Bedford, Mass., USA). The stock solution was treated with ultrasonic waves to obtain a homogeneous suspension. Based on information provided by the sponsor, the suspension was autoclaved for 20 min. at 121 ± 3 °C to sterilize the stock solution. Test substance concentrations were prepared directly prior to use and used within 4 hours after preparation.
For the third experiment, the test substance was suspended in dimethyl sulfoxide of spectroscopic quality (Merck) at request of the sponsor, to be able to compare the results with the results obtained in NOTOX project 233066 (performed with a different batch of Cassia gum). The stock solution was treated with ultrasonic waves to obtain a homogeneous suspension.

6.2. Test System

Test System	*Salmonella typhimurium* bacteria and *Escherichia coli* bacteria.
Rationale	Recommended test system in international guidelines (e.g. EPA, OECD, EEC).
Source	*Salmonella typhimurium* strains: Dr. Bruce N. Ames, University of California at Berkeley, U.S.A. TA98 received on 30-07-2001, used batch: TA98.120203 TA1535 received on 30-07-2001, used batch: TA1535.120203 TA1537 received on 30-07-2001, used batch: TA1537.120203 Xenometric, Boulder, Co, U.S.A. (obtained from N.V. Organon) TA100 received on 19-09-2002, used batch: TA100.200902 Prof. Dr. B.A. Bridges, University of Sussex, Brighton, U.K. *Escherichia coli* strain: WP_2uvrA received on 23-10-1987, used batches: EC.290802 and EC.040500

The characteristics of the different *Salmonella typhimurium* strains were as follows:

Strain	Histidine mutation	Mutation type
TA1537	*his*C3076	Frameshift
TA98	*his*D3052/R-factor*	Frameshift
TA1535	*his*G46	Base-pair substitutions
TA100	*his*G46/R-factor*	Base-pair substitutions

*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contained the following additional mutations:
rfa : deep rough (defective lipopolysaccharide cellcoat)
gal : mutation in the galactose metabolism
chl : mutation in nitrate reductase
bio : defective biotin synthesis
uvrB : loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The *Salmonella typhimurium* strains were regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants.

The *Escherichia coli* WP_2uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV. The strain was regularly checked to confirm the tryptophan-requirement, UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains were stored in liquid nitrogen (-196 °C).

6.3. Cell culture

Preparation of bacterial cultures
Samples of frozen stock cultures of bacteria were transferred into enriched nutrient broth (Oxoid no. 2) and incubated in a shaking incubator (37°C, 150 spm), until the cultures reached an optical density of 1.0 ± 0.1 at 700 nm (10^9 cells/ml). Freshly grown cultures of each strain were used for a test.

Permeabilization of the *Escherichia coli* strain
WP_2uvrA bacteria were washed twice in 0.25 the original volume of ice-cold 0.12 M Tris-HCl

buffer pH 8.0, then gently resuspended in 0.2 vol. 0.12 M Tris-HCl, 0.5 mM EDTA pH 8.0, and shaken for 2.5 min at 37°C. $MgCl_2$ was then added to a final concentration of 10 mM. The cells were centrifuged and resuspended in the original volume of nutrient broth.

Agar plates
Agar plates (ø 9 cm) contained 25 ml glucose agar medium. Glucose agar medium contained per liter: 18 g purified agar (Oxoid, code L28) in Vogel-Bonner Medium E, 20 g glucose.
N.B. The agar plates for the test with the *Salmonella typhimurium* strains also contained 12.5 µg/plate biotin and 15 µg/plate histidine and the agar plates for the test with the *Escherichia coli* strain contained 15 µg/plate tryptophan.

Top agar
Vogel-Bonner Medium E containing 0.6% (w/v) purified agar was heated to dissolve the agar. Samples of 3 ml top agar were transferred into 10 ml glass tubes with metal caps. Top agar tubes were autoclaved for 20 min at 121 ± 3 °C.

Environmental conditions
All incubations were carried out in the dark at 37 ± 1 °C. The temperature was monitored during the experiment.

6.4. Reference substances

6.4.1. Negative control

The vehicle of the test article, being filter-sterilized (0.22 µm) Milli-Q water (experiment 1 and 2) or dimethyl sulfoxide (DMSO, experiment 3).

6.4.2. Positive controls

Without metabolic activation (-S9-mix):

Strain	Chemical	Concentration/plate	Solvent
TA1535	sodium azide (SA) (Sigma)	5 µg	Saline
TA1537	9-aminoacridine (9AC) (Janssen Chimica)	60 µg	Saline
TA98	daunomycin (DM) (Sigma)	4 µg	Saline
TA100	methylmethanesulfonate (MMS) (Merck)	650 µg	DMSO
WP_2uvrA	4-nitroquinoline N-oxide (4-NQO) (Sigma)	10 µg	DMSO

With metabolic activation (+S9-mix):

Strain	Chemical	Concentration/plate	Solvent
TA1537[1]	2-aminoanthracene (2AA) (Sigma)	2.5 µg	DMSO
TA1535, TA98 and TA100[2]	2-aminoanthracene (2AA) (Sigma)	1 µg	DMSO
WP_2uvrA[3]	2-aminoanthracene (2AA) (Sigma)	5 µg	DMSO

[1] In the presence of 10% (v/v) S9-fraction, the concentration of 2AA was 5 µg/plate.
[2] In the presence of 10% (v/v) S9-fraction, the concentration of 2AA was 2.5 µg/plate.
[3] In the presence of 10% (v/v) S9-fraction, the concentration of 2AA was 10 µg/plate.

Solvents for reference substances
Saline = physiological saline (B. Braun, Melsungen AG, Germany)
DMSO = dimethyl sulfoxide of spectroscopic quality (Merck).

6.5. Metabolic activation system

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from Charles River, Sulzfeld, Germany.

6.5.1. Preparation of S9-fraction

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the Standard Operating Procedures. The rats were injected intraperitoneally with a solution (20% (w/v)) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, they were killed by decapitation; (they were denied access to food for at least 12 hours preceding sacrifice). The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C) and identified by the day of preparation.

Before use, all S9-batches were characterized with the metabolic activation requiring positive control; benzo[a]pyrene (Sigma) in tester strain TA98 at the concentration of 5 µg/plate.

6.5.2. Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix contained per 10 ml: 30 mg NADP and 15.2 mg glucose-6-phosphate in 5.5 ml or 5.0 ml Milli-Q water (first or second experiment respectively); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M $MgCl_2$ solution; 1 ml 0.33 M KCl solution. The above solution was filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction was added (5% (v/v) S9-fraction) to complete the S9-mix in the first experiment and to 9.0 ml of S9-mix components 1.0 ml S9-fraction was added (10% (v/v) S9-fraction) to complete the S9-mix in the second experiment.
The S9-batches used were no. 03-5 and 03-6.

6.6. Study design

6.6.1. Solubility test

Before the start of the first mutation assay a solubility test was performed with Cassia gum. Five concentrations of the test substance, 1, 3, 10, 33 and 100 µg/plate were tested in the top agar and on the plates for precipitate. Top agar in top agar tubes was molten and heated to 45°C and 0.1 ml of a dilution of the test substance was added. The top agar was mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were turned and incubated in the dark at 37 ± 1°C for 48 h. The presence of precipitate of Cassia gum was determined in the top agar and on the plates at the start and at the end of the incubation period.

6.6.2. Mutation assays

At least five different doses (increasing with approximately half-log steps) of the test substance were tested in triplicate in each strain in the absence and presence of S9-mix in two independent experiments. An additional experiment was performed with the strains TA100 and WP_2uvrA in which the test substance was suspended in dimethyl sulfoxide and tested up to a concentration of 5000 µg/plate.

Top agar in top agar tubes was molten and heated to 45°C. The following solutions were successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture (10^9 cells/ml) of one of the tester strains, 0.1 ml of a dilution of the test substance in Milli-Q water or DMSO and

either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients were mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were turned and incubated in the dark at 37 ± 1 °C for 48 h. After this period revertant colonies (histidine independent (His^+) for *Salmonella typhimurium* bacteria and tryptophan independent (Trp^+) for *Escherichia coli*) were counted.

6.6.3. Colony counting

The revertant colonies (histidine independent c.q. tryptophan independent) were counted automatically with a Protos model 50000 colony counter or manually, if less than 40 colonies per plate were present. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

6.7. Interpretation

6.7.1. Acceptability of the assay

A *Salmonella typhimurium* reverse mutation assay and/or *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory background historical range for each tester strain.

Strain		Minimum value	Maximum value	Mean	± 3 x S.D.
TA1535	- S9-mix	3	25	12	± 12
	+ S9-mix	3	28	12	± 12
TA1537	- S9-mix	3	26	6	± 9
	+ S9-mix	3	28	7	± 10
TA98	- S9-mix	12	45	18	± 17
	+ S9-mix	12	54	24	± 22
TA100	- S9-mix	60	188	110	± 95
	+ S9-mix	59	195	97	± 81
WP_2uvrA	- S9-mix	4	32	13	± 15
	+ S9-mix	4	31	14	± 15

b) The positive control chemicals should produce responses in all tester strains, which are within the laboratory historical range documented for each positive control substance.

Strain		Minimum value	Maximum value	Mean	± 3 x S.D.
TA1535	- S9-mix	90	1308	421	± 876
	+ S9-mix	60	1028	183	± 279
TA1537	- S9-mix	77	1897	374	± 626
	+ S9-mix	58	1794	288	± 553
TA98	- S9-mix	100	1855	576	± 973
	+ S9-mix	169	2597	690	± 932
TA100	- S9-mix	279	2099	851	± 795
	+ S9-mix	197	2753	804	± 1210
WP_2uvrA	- S9-mix	67	1399	657	± 879
	+ S9-mix	59	1053	229	± 444

c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the solubility test or should extend to 5 mg/plate.

6.7.2. Data evaluation and statistical procedures

No formal hypothesis testing was done.
A test substance is considered negative (not mutagenic) in the test if:

a) The total number of revertants in any tester strain at any concentration is not greater than two times the solvent control value, with or without metabolic activation.
b) The negative response should be reproducible in at least one independently repeated

experiment.

A test substance is considered positive (mutagenic) in the test if:
a) It induces a number of revertant colonies, dose related, greater than two-times the number of revertants induced by the solvent control in any of the tester strains, either with or without metabolic activation.
However, any mean plate count of less than 20 is considered to be not significant.
b) The positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

7. RESULTS

7.1. Solubility test

Five concentrations of the test substance (1, 3, 10, 33 and 100 µg/plate) in MilliQ-water were tested in the top agar and on the plates for precipitate.

Precipitate
The test substance precipitated in the top agar at concentrations of 33 and 100 µg/plate. Precipitation of Cassia gum on the plates was observed at the start and at the end of the incubation period at concentrations of 33 and 100 µg/plate.

7.2. First and second mutation assay

Based on the results of the solubility test, Cassia gum was tested up to concentrations of 100 µg/plate in MilliQ-water in the absence and presence of S9-mix in two mutation assays. Cassia gum was tested beyond the limit of the solubility to obtain adequate mutagenicity data. The mutation assays were performed with the strains TA1535, TA1537, TA98, TA100 and WP_2uvrA. The results of the two mutation assays are shown in Tables 1 and 2. The individual data are presented in Appendix II.

Precipitate
Cassia gum precipitated in the top agar at concentrations of 33 and 100 µg/plate. Precipitation of Cassia gum on the plates was observed at the start and at the end of the incubation period at the concentration of 33 and 100 µg/plate.

Toxicity
To determine the toxicity of Cassia gum, the reduction of the bacterial background lawn, the increase in the size of the microcolonies and the reduction of the revertant colonies were examined. The definitions are stated in Appendix I.

The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant decrease in the number of revertants was observed.

Number of revertants
All bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in the first and second mutation assay.

7.3. Third mutation assay

At request of the sponsor, an additional experiment was performed in which dimethyl sulfoxide was used as the vehicle, and the highest concentration was 5000 µg/plate. The third experiment

was performed with the strains TA100 and WP_2uvrA, and exposure was performed in the absence and presence of S9-mix. The results of the third mutation assay are shown in Table 3. The individual data are presented in Appendix II.

Precipitate
Cassia gum precipitated in the top agar at all concentrations (100-5000 µg/plate). Precipitation of Cassia gum on the plates was observed at the start and at the end of the incubation period at all concentrations tested (100-5000 µg/plate).

Toxicity
The bacterial background lawn was not reduced at any of the concentrations tested and no biologically relevant decrease in the number of revertants was observed.

Number of revertants
Both bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants.

8. DISCUSSION/CONCLUSION

The negative and strain-specific positive control values were within our laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Cassia gum did not induce a dose-related, two-fold increase in the number of revertant (His^+) colonies in each of the four tester strains (TA1535, TA1537, TA98 and TA100) and in the number of revertant (Trp^+) colonies in tester strain WP_2uvrA both in the absence and presence of S9-metabolic activation. These results were confirmed in an independently repeated experiment, and in an additional experiment with a different vehicle in which Cassia gum was tested at higher concentrations.

Based on the results of this study it is concluded that Cassia gum is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

9. REFERENCES

1 Leonardo, J.M., Dornfeld, S.S. and Peak, M.J., 1984, Evaluation of E. coli K12 343 \ 13 and derived strains for microbial mutagenicity assays. Mutation Res., 130, 87-95.
2 Ames, B.N., McCann, J. and Yamasaki, E., 1975, Methods for detecting carcinogens and mutagens with the *Salmonella/mammalian* microsome mutagenicity test, Mutation Res., 31, 347-364.
3 Maron, D.M. and Ames, B.N., 1983, Revised methods for the *Salmonella* mutagenicity test, Mutation Res., 113, 173-215. Erratum, 1983, Mutation Res., 113, 533.
4 Green, M.H.L. and Muriel, W.J., 1976, Mutagen testing using Trp^+ reversion in *Escherichia coli*, Mutation Res., 38, 3-32.
5 Vogel, H.J. and Bonner, D.M., 1956, Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem., 218, 97-106.

Table 1 **Experiment 1: Mutagenic response of Cassia gum in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance: 20 May 2003

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
			Without S9-mix		
positive control	396 ± 2	556 ± 49	693 ± 64	983 ± 11	442 ± 23
solvent control	7 ± 4	7 ± 1	20 ± 2	134 ± 8	12 ± 2
0.3	7 ± 2	5 ± 1	19 ± 5	130 ± 9	14 ± 1
1	9 ± 1	4 ± 1	15 ± 3	137 ± 10	10 ± 2
3	11 ± 2	7 ± 3	17 ± 4	129 ± 7	11 ± 1
10	9 ± 3	4 ± 1	17 ± 3	131 ± 8	15 ± 2
33 SP	8 ± 2	6 ± 2	17 ± 2	122 ± 9	12 ± 3
100 SP	5 ± 1	4 ± 3	14 ± 3	133 ± 6	9 ± 3
			With S9-mix [1]		
positive control	200 ± 1	204 ± 23	590 ± 21	654 ± 1	71 ± 7
solvent control	9 ± 3	8 ± 2	19 ± 4	136 ± 5	10 ± 3
0.3	9 ± 2	6 ± 3	17 ± 3	132 ± 3	9 ± 1
1	8 ± 0	6 ± 4	21 ± 4	137 ± 10	11 ± 3
3	8 ± 1	7 ± 3	18 ± 2	131 ± 5	10 ± 1
10	7 ± 3	6 ± 1	19 ± 2	126 ± 5	12 ± 2
33 SP	8 ± 3	6 ± 3	22 ± 3	141 ± 6	14 ± 2
100 SP	10 ± 3	5 ± 3	19 ± 2	134 ± 9	11 ± 3

Solvent control: 0.1 ml milli-Q
1 The S9-mix contained 5% (v/v) S9 fraction
SP Slight Precipitate

Table 2 **Experiment 2: Mutagenic response of Cassia gum in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance: 03 June 2003

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
			Without S9-mix		
positive control	301 ± 15	509 ± 83	568 ± 32	940 ± 46	926 ± 34
solvent control	9 ± 2	6 ± 4	19 ± 3	141 ± 9	13 ± 3
0.3	7 ± 4	6 ± 4	16 ± 3	135 ± 1	11 ± 3
1	9 ± 3	5 ± 3	15 ± 3	164 ± 9	12 ± 2
3	10 ± 2	5 ± 1	13 ± 3	143 ± 12	10 ± 1
10	5 ± 0	4 ± 3	16 ± 3	150 ± 5	12 ± 2
33 SP	10 ± 1	5 ± 2	17 ± 2	134 ± 8	11 ± 2
100 SP	11 ± 1	5 ± 3	17 ± 2	149 ± 2	11 ± 1
			With S9-mix [1]		
positive control	111 ± 6	251 ± 19	286 ± 23	888 ± 107	147 ± 21
solvent control	9 ± 2	8 ± 2	17 ± 3	116 ± 8	12 ± 3
0.3	7 ± 2	5 ± 2	18 ± 4	135 ± 9	11 ± 4
1	10 ± 3	6 ± 3	20 ± 5	116 ± 6	14 ± 1
3	8 ± 2	5 ± 1	20 ± 3	110 ± 6	13 ± 1
10	9 ± 3	4 ± 3	18 ± 4	121 ± 6	12 ± 3
33 SP	7 ± 2	4 ± 1	19 ± 4	123 ± 4	12 ± 5
100 SP	10 ± 2	6 ± 1	19 ± 4	117 ± 3	13 ± 2

Solvent control: 0.1 ml milli-Q
1 The S9-mix contained 10% (v/v) S9 fraction
SP Slight Precipitate

Table 3 **Experiment 3: Mutagenic response of Cassia gum in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay**

Day of performance: 24 July 2003

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain	
	TA100	WP_2uvrA
	Without S9-mix	
positive control	927 ± 48	495 ± 9
solvent control	149 ± 13	9 ± 1
100 SP	153 ± 2	15 ± 1
333 SP	150 ± 10	11 ± 2
1000 SP	136 ± 14	10 ± 5
3330 MP	158 ± 3	9 ± 4
5000 MP	163 ± 10	7 ± 1
	With S9-mix[1]	
positive control	652 ± 26	107 ± 14
solvent control	149 ± 4	12 ± 1
100 SP	144 ± 27	13 ± 0
333 SP	137 ± 15	10 ± 2
1000 SP	134 ± 20	13 ± 3
3330 MP	151 ± 12	13 ± 3
5000 MP	153 ± 23	12 ± 1

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 5% (v/v) S9 fraction
SP Slight Precipitate
MP Moderate Precipitate

APPENDIX I SUPPORTING MATERIALS AND METHOD

Bacterial background lawn evaluation

The condition of the bacterial background lawn is evaluated (if indicated), both macroscopically and microscopically by using a dissecting microscope (results are normal unless indicated in tables).

Definition	Characteristics
Normal	Distinguished by a healthy microcolony lawn.
Slightly reduced	Distinguished by a slight thinning of the microcolony lawn.
Moderately reduced	Distinguished by a moderate thinning of the microcolony lawn.
Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Absent	Distinguished by a complete lack of any microcolony background lawn.

Precipitation evaluation

Evidence of test article precipitate on the plates is recorded by addition of the following precipitation definition.

Definition	Characteristics
Slight Precipitate	Distinguished by noticeable precipitate on the plate. However, the precipitate does not influence automated counting of the plate.
Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Evaluation of the reduction in the number of revertants

The reduction in the number of revertant colonies compared to number of revertants in the solvent control is evaluated as follows:
A reduction of 21-40%: slight reduction.
A reduction of 41-60%: moderate reduction.
A reduction of 61-99%: extreme reduction.
If the size of the microcolonies was increased to small colonies due to an extremely reduced background lawn the reduction is evaluated as microcolonies. If no revertant colonies are observed on the plates the reduction is evaluated as a complete lack of revertants.
However, any mean plate count equal to the minimal value of the historical control data range should be considered not toxic.

APPENDIX II DETAILED TABLES

Individual plate counts; (following pages)

Experiment 1
Strain TA1535

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		394	396	398	396 ± 2
solvent control		4	6	12	7 ± 4
0.3		6	7	9	7 ± 2
1		10	8	10	9 ± 1
3		9	13	10	11 ± 2
10		9	7	12	9 ± 3
33	SP	6	10	9	8 ± 2
100	SP	6	6	4	5 ± 1

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		199	200	200	200 ± 1
solvent control		6	8	12	9 ± 3
0.3		7	10	9	9 ± 2
1		8	8	8	8 ± 0
3		8	7	8	8 ± 1
10		11	5	6	7 ± 3
33	SP	4	10	9	8 ± 3
100	SP	9	14	8	10 ± 3

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 1
Strain TA1537

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		604	507	558	556 ± 49
solvent control		8	6	7	7 ± 1
0.3		4	5	6	5 ± 1
1		3	4	4	4 ± 1
3		9	4	7	7 ± 3
10		3	5	3	4 ± 1
33	SP	6	4	7	6 ± 2
100	SP	4	1	7	4 ± 3

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		201	228	183	204 ± 23
solvent control		7	10	6	8 ± 2
0.3		7	2	8	6 ± 3
1		7	1	9	6 ± 4
3		5	10	5	7 ± 3
10		7	6	6	6 ± 1
33	SP	8	3	6	6 ± 3
100	SP	8	5	3	5 ± 3

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 1
Strain TA98

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		632	688	759	693 ± 64
solvent control		18	22	19	20 ± 2
0.3		15	18	24	19 ± 5
1		16	12	18	15 ± 3
3		16	21	14	17 ± 4
10		19	19	14	17 ± 3
33	SP	19	15	17	17 ± 2
100	SP	11	16	15	14 ± 3

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		615	579	577	590 ± 21
solvent control		15	22	19	19 ± 4
0.3		14	18	20	17 ± 3
1		21	24	17	21 ± 4
3		16	19	20	18 ± 2
10		21	17	18	19 ± 2
33	SP	21	19	25	22 ± 3
100	SP	21	18	19	19 ± 2

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 1
Strain TA100

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		971	986	993	983 ± 11
solvent control		126	141	135	134 ± 8
0.3		132	121	138	130 ± 9
1		134	148	128	137 ± 10
3		136	128	123	129 ± 7
10		140	127	126	131 ± 8
33	SP	113	122	131	122 ± 9
100	SP	131	128	140	133 ± 6

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		653	655	653	654 ± 1
solvent control		135	131	141	136 ± 5
0.3		130	135	132	132 ± 3
1		139	126	146	137 ± 10
3		131	136	127	131 ± 5
10		128	121	130	126 ± 5
33	SP	147	135	141	141 ± 6
100	SP	128	144	130	134 ± 9

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 1
Strain WP_2uvrA

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		468	426	433	442 ± 23
solvent control		12	13	10	12 ± 2
0.3		13	13	15	14 ± 1
1		8	11	11	10 ± 2
3		12	11	11	11 ± 1
10		17	14	13	15 ± 2
33	SP	9	12	14	12 ± 3
100	SP	7	12	9	9 ± 3

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		78	65	71	71 ± 7
solvent control		10	13	7	10 ± 3
0.3		10	8	8	9 ± 1
1		12	8	13	11 ± 3
3		9	11	10	10 ± 1
10		10	12	13	12 ± 2
33	SP	12	15	14	14 ± 2
100	SP	14	11	8	11 ± 3

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA1535

		WITHOUT S9-MIX				
plate		1	2	3	MEAN ±	SD
dose (µg/plate)						
positive control		311	284	308	301 ±	15
solvent control		7	8	11	9 ±	2
0.3		7	4	11	7 ±	4
1		6	8	12	9 ±	3
3		12	8	9	10 ±	2
10		5	5	5	5 ±	0
33	SP	9	11	9	10 ±	1
100	SP	12	12	10	11 ±	1

		WITH S9-MIX				
plate		1	2	3	MEAN ±	SD
dose (µg/plate)						
positive control		105	113	116	111 ±	6
solvent control		9	11	7	9 ±	2
0.3		8	5	9	7 ±	2
1		11	13	7	10 ±	3
3		10	8	7	8 ±	2
10		10	6	12	9 ±	3
33	SP	8	7	5	7 ±	2
100	SP	13	9	9	10 ±	2

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA1537

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		501	596	431	509 ± 83
solvent control		5	2	10	6 ± 4
0.3		3	10	6	6 ± 4
1		6	1	7	5 ± 3
3		6	4	5	5 ± 1
10		3	7	2	4 ± 3
33	SP	3	7	5	5 ± 2
100	SP	7	2	7	5 ± 3

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		273	239	241	251 ± 19
solvent control		6	10	7	8 ± 2
0.3		3	5	6	5 ± 2
1		10	4	4	6 ± 3
3		6	6	4	5 ± 1
10		4	7	2	4 ± 3
33	SP	5	4	3	4 ± 1
100	SP	6	5	7	6 ± 1

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA98

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		585	531	587	568 ± 32
solvent control		18	17	22	19 ± 3
0.3		13	19	16	16 ± 3
1		13	18	15	15 ± 3
3		12	11	16	13 ± 3
10		16	18	13	16 ± 3
33	SP	18	17	15	17 ± 2
100	SP	15	16	19	17 ± 2

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		260	298	300	286 ± 23
solvent control		20	14	17	17 ± 3
0.3		19	14	21	18 ± 4
1		15	24	20	20 ± 5
3		17	22	22	20 ± 3
10		15	16	22	18 ± 4
33	SP	16	18	24	19 ± 4
100	SP	22	15	20	19 ± 4

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain TA100

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		966	967	886	940 ± 46
solvent control		131	143	148	141 ± 9
0.3		135	134	135	135 ± 1
1		161	174	158	164 ± 9
3		144	154	131	143 ± 12
10		154	151	144	150 ± 5
33	SP	142	134	127	134 ± 8
100	SP	151	147	148	149 ± 2

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		785	999	880	888 ± 107
solvent control		120	106	121	116 ± 8
0.3		126	143	135	135 ± 9
1		116	122	110	116 ± 6
3		110	115	104	110 ± 6
10		123	114	125	121 ± 6
33	SP	125	119	126	123 ± 4
100	SP	119	118	113	117 ± 3

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 2
Strain WP_2uvrA

		WITHOUT S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		931	958	890	926 ± 34
solvent control		14	9	15	13 ± 3
0.3		13	8	12	11 ± 3
1		14	10	12	12 ± 2
3		11	9	9	10 ± 1
10		15	11	11	12 ± 2
33	SP	9	10	13	11 ± 2
100	SP	10	10	12	11 ± 1

		WITH S9-MIX			
plate		1	2	3	MEAN ± SD
dose (µg/plate)					
positive control		141	171	130	147 ± 21
solvent control		15	11	9	12 ± 3
0.3		11	8	15	11 ± 4
1		14	13	15	14 ± 1
3		14	14	12	13 ± 1
10		13	8	14	12 ± 3
33	SP	13	7	16	12 ± 5
100	SP	11	12	15	13 ± 2

SP: Slight Precipitate

APPENDIX II — continued —

Experiment 3
Strain TA100

		WITHOUT S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		872	954	956	927 ±	48
solvent control		162	148	136	149 ±	13
100	SP	152	156	152	153 ±	2
333	SP	161	141	149	150 ±	10
1000	SP	145	119	143	136 ±	14
3330	MP	161	157	156	158 ±	3
5000	MP	175	158	156	163 ±	10

		WITH S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		649	628	680	652 ±	26
solvent control		154	147	147	149 ±	4
100	SP	144	117	171	144 ±	27
333	SP	123	134	153	137 ±	15
1000	SP	135	153	113	134 ±	20
3330	MP	162	139	153	151 ±	12
5000	MP	169	127	162	153 ±	23

SP: Slight Precipitate
MP: Moderate Precipitate

000244

APPENDIX II — continued —

Experiment 3
Strain WP_2uvrA

		WITHOUT S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		493	488	505	495 ±	9
solvent control		10	8	9	9 ±	1
100	SP	14	16	14	15 ±	1
333	SP	12	9	13	11 ±	2
1000	SP	8	15	6	10 ±	5
3330	MP	13	8	6	9 ±	4
5000	MP	6	6	8	7 ±	1

		WITH S9-MIX				
plate		1	2	3	MEAN	SD
Dose (µg/plate)						
positive control		106	121	93	107 ±	14
solvent control		13	13	11	12 ±	1
100	SP	13	13	13	13 ±	0
333	SP	9	12	10	10 ±	2
1000	SP	12	10	16	13 ±	3
3330	MP	10	16	14	13 ±	3
5000	MP	13	11	13	12 ±	1

SP: Slight Precipitate
MP: Moderate Precipitate

APPENDIX III CERTIFICATE OF ANALYSIS

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Noveon Pharma GmbH & Co. KG

Certificate of Analysis

Semi-refined Cassia Gum

Batch Number **SH - 225**

TEST	*SPECIFICATION*	*TEST Result*	*TEST-No*
Material Description			
Appearance	fine powder	conforms	NC 00201
Consistency	dry,free flowing,with no lumps	conforms	NC 00202
Colour	Off-white	conforms	NC 00301
Odour	neutral	conforms	NC 00302
Taste	almost neutral	Conforms	NC 00303
Solubilty	gum hydrates colloidal in hot water	Conforms	NC 04201
Chem. and phys.	**Properties**		
Loss on drying	Max. 12 %	8.33 %	FCC III
pH-Value	5,5 - 8,0	7,48	FCC III
Gel-strength	Min. 140 g	167 g	NC 02101
Particle size <250µ	Min. 99 %	99,1%	NC 01201
Colour Value	Max. 250 ppm	44 ppm	NC 02501
Viscosity 1%	Max. 500 cps	385 cps	MPM13
Fat	NMT 2%	0.5%	Weibull-Stoldt
Protein	NMT 7%	5.8%	Kjeldahl x 6.25
Carbohydrates and fibres	NLT 70%	> 80%	Calc.
Purity			
Free Anthraquinones	NMT 10 ppm	5.20 ppm	NC02402
Total Anthraquinones	NMT 10 ppm	8.59 ppm	NC02402

QA / QC: (b)(6)

Dr. Ferdinand Utz

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9

Comparison of Diagum CS and Diagum SR (without final washing) Tab 9

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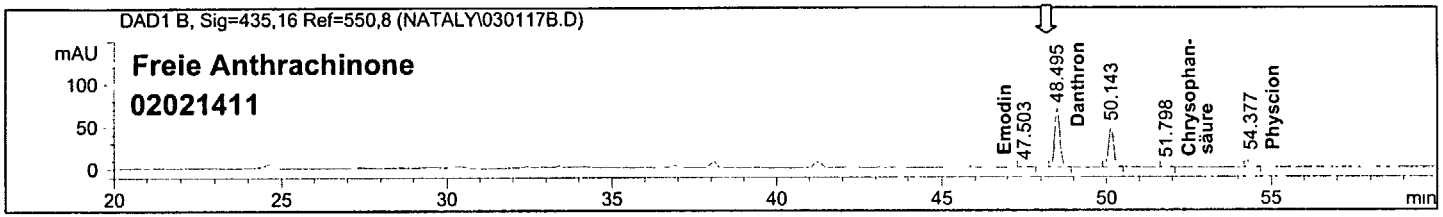
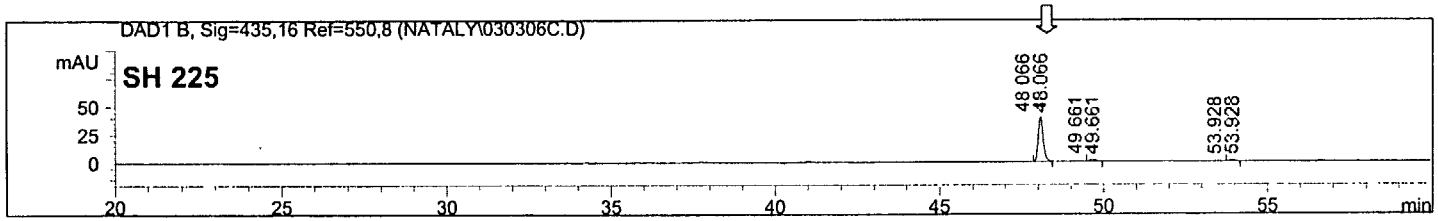
Comparison of Diagum CS and Diagum SR

The Diagum CS batch 02021411 has been purified by extraction with isopropyl alcohol / water mixtures
Examples of HPLC results are shown below:

HPLC-Spectrum of a typical **Diagum CS** batch (free anthraquinones)



Spectrum of Diagum SR (batch SH 225) with # 02021411 as raw material after Isopropanol/Water purification (free anthraquinones)



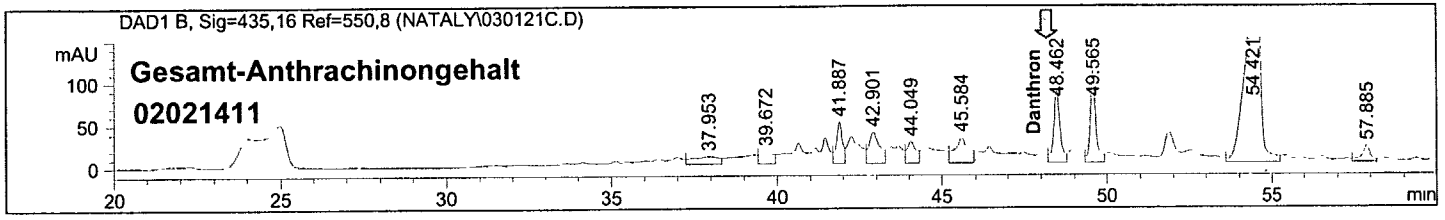
Result:
Only Physcion can be detected in a concentration as low as 5,20 ppm

(analyzed by Prof. Dekant)

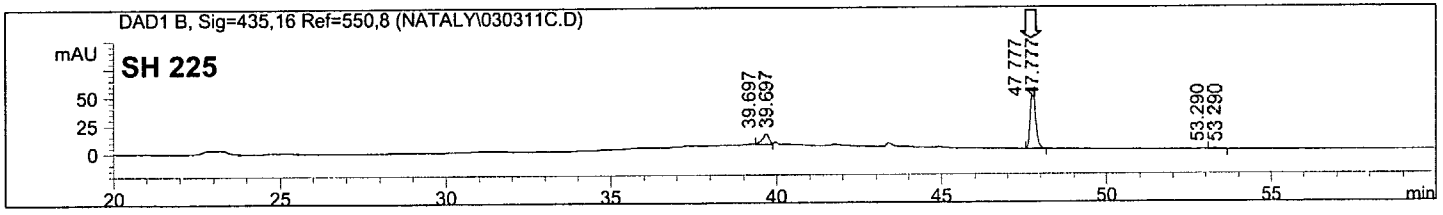
000250

The same batches after hydrolysation with trifluoroacxetic acid:

HPLC-Spectrum of a typical Diagum CS batch after acidic hydrolyzation



Spectrum of batch SH 225 after Isopropanol/Water purification (total anthraquinones)



Result: Only Physcion was found at a level of 8,59 ppm. All other anthraquinones have been below the detection limit

The peaks marked with an arrow are the peaks of Danthrone, which has been added as an internal standard.

Appendix 3

APPENDIX III

EXPONENT REPORT ON CASSIA GUM INTAKE

Pages 000253-000260 have been removed in accordance with Freedom of Information Act exemption B(4) for trade secrets.

SUBMISSION END

Reference List for Industry Submission, GRN 000139

Pages	*Author*	*Title*	*Publish Date*	*Publisher*	*BIB_Info*
0000087 - 000091	NA	Kjeidahi Method	NA	FCC	Volume IV
000093 - 000095	NA	General Tests and Apparatus	NA	Food Chemcial Codex	Volume III
000097 - 000100	NA	Potentiometric method with combined pH glass electrode.This method is desccribed in "General Tests and Apparatus"	NA	Food Chemical Codex	Volume III
000118	NA	Monograph Specifications, Method of quantification of Isopropanol in Xanthan	NA	Food Chemicals Codex	pg 438
000119 - 000121	NA	The testing method for Heavy Metals in Cassia gum is ferformed analogous	NA	Food Chemical Codex	Volume IV, pgs 760 - 761
000122 - 000123	NA	Lead Limit Test	NA	Food Chemical Codex	Volume IV, pg 762
000124 - 000126	NA	Arsenic Test	NA	Food Chemical Codex	Volume IV, pg 755

NA- Not applicable