GR

ORIGINAL SUBMISSION

A CONSTELLATION COMPANY

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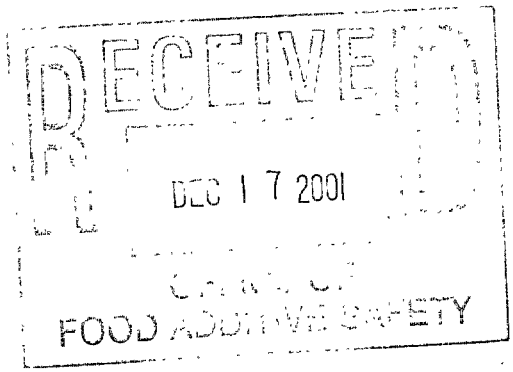
CanandaiguaWine



December 12, 2001

Office of Premarket Approval (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, S.W.
Washington, DC 20204

Re: Notification of GRAS Determination for MegaNatural™ Gold Grape Seed Extract (GSE) and Grape Skin Extract (GSKE) Use in Beverage Products:
GRAS Exemption Claim

Dear Sir or Madam:

Pursuant to FDA's policy described at 62 Fed. Reg. 18938, 18960 (April 17, 1997), Polyphenolics, Inc. hereby notifies the Food and Drug Administration (FDA) that it has determined that the use of MegaNatural™ Gold grape seed extract and grape skin extract in beverage products is "generally recognized as safe" (GRAS) and is therefore exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act. A detailed summary of the basis for the GRAS determination is attached to this GRAS exemption claim. The following information is provided under proposed 21 C.F.R. § 170.36(c)(1):

Notifier: Polyphenolics, Inc.
12667 Road 24
Madera, CA 93637
Edward J. Race
Director of Research

GRAS Substance: MegaNatural™ Gold Grape Seed Extract (GSE) and Grape Skin Extract (GSKE)

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Polyphenolics

Polyphenolics • 12667 Road 24, Madera, CA 93639 • (559) 661 5500 • FAX (559) 661 3400 • www.cwine.com

Intended Use:	These substances are intended for interchangeable addition to fruit juice and fruit flavored beverages at a composite total concentration up to 210 ppm as antioxidants to retard deterioration.
Basis for GRAS Determination:	Scientific Procedures

The data and information that are the basis for Polyphenolics' GRAS determination are available for FDA's review and copying at reasonable times at the offices of

Mr. Bob Nicolas
McDermott, Will and Emery
600 13th Street NW
Washington, DC 20005.

In addition, Polyphenolics agrees to send the material to FDA at the agency's request.

Respectfully submitted

Edward J. Race
Director of Research

FDA NOTIFICATION OF A GRAS DETERMINATION FOR GSE / GSKE USE IN BEVERAGE PRODUCTS

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GLOSSARY AND ABBREVIATIONS

anthocyanins Hydrolysable tannins. The hydrolysable tannins consist of gallic acid and its dimeric condensation product, hexahydroxydiphenic acid, esterified to a polyol, primarily glucose [Bravo, 1998]. As their name would indicate, these tannins are easily hydrolyzed with acid, alkali, hot water, and enzymatic action, which yield polyhydric alcohol and phenylcarboxylic acid. The hydrolysable tannins can be further subdivided into gallotannins, or ellagitannins.

anthocyanidins Polyphenolic compounds formed upon acid hydrolysis of PACs.

antioxidants Compounds that can interact with or scavenge ROS and are found throughout nature. They have been identified in soy products, certain types of algae, various seed oils (including grape seed oil), a variety of fruits and vegetables, and in certain herbs. The consumption of natural antioxidants such as polyphenols, vitamins C and E, and carotinoids through the diet can contribute to the natural defense mechanism of the human body.

FDA United States Food and Drug Administration.

flavanols The group of flavonoids with the lowest oxidation level of the pyran ring-C, as it contains only one hydroxyl in position C3, therefore the term flavan-3-ol. Also referred to as the catechins.

flavonoid A subclass of the body of polyphenols and can be characterized by divisio into several classes according to the degree of oxidation of the oxygen heterocycle including flavones, flavonols, isoflavones, anthocyanins, flavonols, and PACs.

GRAS Generally recognized as safe.

GSE MegaNatural™ Gold grape seed extract.

GSKE MegaNatural™ Gold grape skin extract.

NOAEL No-Observed-Adverse-Effect Level.

NOEL No-Observed-Effect Level.

NTP National Toxicology Program. A Federal Program that actively seeks to identify, select, and study chemicals and other agents for which sufficient information is not available to adequately evaluate potential human health hazards.

PAC(s) Proanthocyanidin(s) are oligomeric flavanols.

phenols Any of the various acidic compounds analogous to phenol and regarded as hydroxyl derivatives or aromatic hydrocarbons.

phenolic acids Hydroxycarboxylic acids with phenolic hydroxyl groups. Also occur widely in nature in the form of their esters, ethers, or in their free forms. Examples include caffeic, chlorogenic, ferulic, gallic, and ellagic acid.

polyphenols A class of compounds characterized by a poly hydroxy phenol consisting of three distinct ring components.

procyanidins A class of tannins which derive from catechin and epicatechin

ROS Reactive oxygen species such as singlet oxygen $^1O_2$ and $O_2^{\cdot -}$, $OH^{\cdot}$, $NO^{\cdot}$, and alkyl peroxide free radicals. The generation of these reactive oxygen species (ROS), produced by a variety of enzymatic reactions, beyond the antioxidant capacity of a biological system gives rise to oxidative stress.

tannins Polymeric proanthocyanidins of which several classes can be determined based upon the hydroxylation pattern of the constitutive units.

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1.0 INTRODUCTION

1.1 Declaration of Intent

The Polyphenolics Division of the Canandaigua Wine Company Inc. wishes to market MegaNatural™ Gold grape seed extract (GSE) and MegaNatural™ Gold grape skin extract (GSKE) composed of polyphenolic proanthocyanidins as a generally recognized as safe (GRAS) food ingredients for use in fruit juices and fruit flavored beverages at a concentration of approximately 210 ppm (w/v) or 50 mg per 8 fluid ounce serving. The proanthocyanidins (PACs) are intended for use as an antioxidant added to foods to retard deterioration. Antioxidant activity may also provide a nutritional benefit in scavenging reactive oxygen and nitrogen species, which may provide support against potentially elevated levels of LDL cholesterol and possibly cancer. Furthermore, their actions may modulate immune function and platelet aggregation.

The purpose of this notification of GRAS determination is to review the chemical nature and occurrence of polyphenolic PAC substances present in grape seed and skin extracts, as well as to provide a summary of the technical, safety and product information and considerations used to support the evaluation of polyphenolic PACs, as antioxidant substances added to food products, to be generally recognized as safe (GRAS). This determination was carried out by an independent panel of recognized experts, hereinafter referred to as the Expert Panel, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients. A comprehensive search of the scientific literature for safety and toxicity information on GSE, GSKE, constituent proanthocyanidins, and related polyphenols was conducted through August 2001, summarized as a report to the Expert Panel, and subsequently made available to the Expert Panel. Based on that comprehensive literature review and the results and analyses of currently unpublished safety studies on GSE / GSKE, this report aided and facilitated the work of the Expert Panel in their deliberation of GRAS status for GSE / GSKE. All of this information is provided herein with the exception of specific confidential information regarding the

manufacturing process and the exact polyphenolic make up of the MegaNatural™ Gold GSE and GSKE products. The Expert Panel independently evaluated the material submitted, as well as other materials deemed appropriate or necessary. Following independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decision that MegaNatural™ Gold GSE and GSKE, meeting the specifications cited, is generally recognized as safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration, provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in the finished beverage products. A complete and signed copy of the Expert Panel Statement has been proved herein in Appendix A of this dossier.

1.2 Regulatory Basis For GRAS Determination

As per volume 62 of the Federal Register, page 18938, (proposed 21 CFR §170.36), the Canandaigua Wine Company's Polyphenolics Brand wishes to notify the Food and Drug Administration (FDA) that it has determined that the use of its MegaNatural™ Gold Grape Seed Extract (GSE) and Gold Grape Skin Extract (GSKE) is Generally Recognized as Safe (GRAS) for use as an antioxidant in fruit juice, fruit flavored beverages, fruit flavored beverage mixes, and carbonated fruit flavored beverages. Grape skin extract is already regulated as a color additive mixture for food uses under 21 CFR §73.170.

The GRAS determination for beverage use of GSE and GSKE is based in part upon review by a panel of Experts qualified by scientific training and experience to evaluate the safety of food and food ingredients using scientific procedures and would be exempt from the pre-market approval requirements of the Federal Food, Drug and Cosmetic Act. This report provides information required by proposed 21 CFR. §170.36(c)(2), (3), and (4) to support an evaluation by a panel of qualified experts in fulfillment of the requirements of 21 CFR. §170.36(c)(4)(I)(c). The requirements of the proposed regulation with the sections containing the relevant information are described below:

Requirements of the Proposed Rule:	***Section***
• §170.36(c)(2): Detailed information about the identity of the notified substance; composition; method of manufacture; characteristic properties; and specifications	2, 3, 4
• §170.36(c)(3): Information on any self-limiting levels of use	5
• §170.36(c)(4)(I)(a): Comprehensive discussion of, and citations to, generally available and accepted scientific data and information, including consideration of probable consumption	6
• §170.36(c)(4)(I)(c): The basis for concluding that there is a consensus among qualified experts that there is reasonable certainty that the substance is not harmful under the intended conditions of use	2, 7, 8, 9

The determination that the MegaNatural™ Gold GSE and GSKE are GRAS meets the applicable requirements for the technical element and common knowledge element of a GRAS determination is based on scientific procedures. The scientific data and information summarized in this report reflect a thorough review of the relevant literature dealing with PACs, other polyphenols, and reflects the result of non-clinical laboratory studies of GSE and GSKE conducted in accordance with generally accepted scientific procedures. Furthermore, this information has been supplemented by the use of scientifically relevant statistical reference sources, compendia, books, and reviews.

Canandaigua Wine Company's GRAS determination is based on the weight of all of the available scientific information and grounded upon generally available scientific data, including the consensus among a panel of qualified experts, that there was reasonable certainty that these substances will not be harmful under the intended conditions of use. This GRAS determination therefore meets the requirements of §201(s) of the Federal Food, Drug, and Cosmetic Act; 21 C.F.R. §170.3 and §170.30; and the amendments to these rules proposed in 62 Fed. Reg. 18960.

1.3 Summary Basis for GRAS Status

A summary basis for the evaluation of GRAS status for the MegaNatural™ Gold grape seed extract (GSE) and grape skin extract (GSKE) has been provided below. Further discussion of each of these points can be found elsewhere in this document.

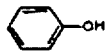
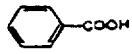
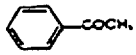
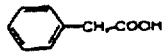
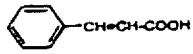
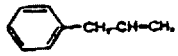
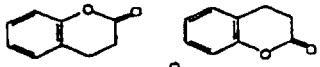
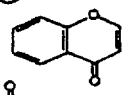
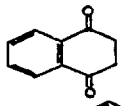
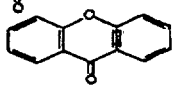
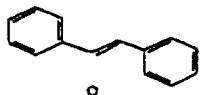
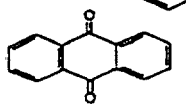
- The polyphenolic compounds comprising the MegaNatural™ Gold GSE and GSKE are naturally occurring and found in diverse types of foods.
- Intake from the proposed use in beverages and beverage products is within the range of normal dietary intakes.
- The chemical composition of the GSE and GSKE products is well characterized and their composition is reflective of the naturally occurring component profiles.
- GSE and GSKE are manufactured by aqueous extraction producing a product demonstrated to reproducibly meet compositional specifications while complying with limits established by FDA for the presence of heavy metals and pesticide residues in grapes.
- The functionality of the constituent polyphenols in GSE and GSKE as an antioxidant is well recognized in grape juice and grape beverages such as wine, with strong supporting evidence demonstrating that antioxidant activity is extended to biological systems.
- Preclinical safety data for the GSE and GSKE products demonstrated an absence of mutagenic activity and a lack of long-term adverse effects in daily doses to rats up to at least 1780 mg/kg in males and 2150 mg/kg in females.
- There is supporting evidence for the safety of GSE and GSKE in humans from clinical investigations of possible beneficial health effects.

2.0 POLYPHENOL CHEMISTRY AND ANTIOXIDANT CAPACITY

2.1 Polyphenolic Compounds

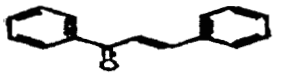
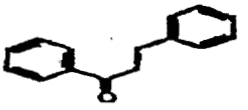
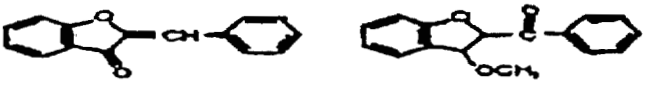
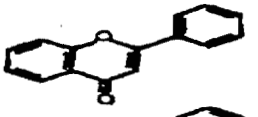
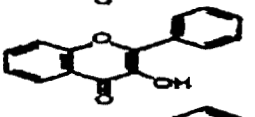
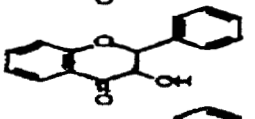
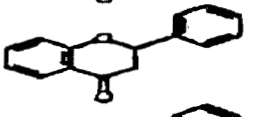
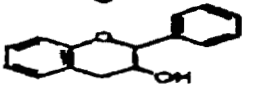
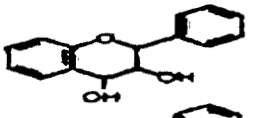
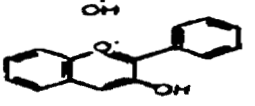
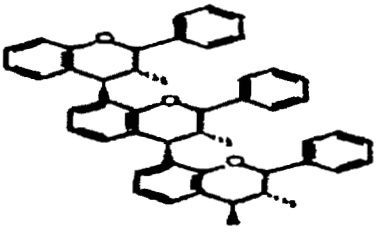
Phenolic compounds or polyphenols constitute an extremely complex and widely distributed group of plant substances. Polyphenols are the products of plant metabolism and arise from two main synthetic pathways; the Shikimate and the acetate pathways. Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds. Below, Table 2.1 [Bravo, 1998] illustrates the basic chemical structures of several important classes of polyphenolic compounds.

Table 2.1 Main Classes of Polyphenolic Compounds

Class	Basic Skeleton	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naftoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans, neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

The flavonoid group of chemicals is a subclass of the huge body of polyphenols. They constitute the most common group of plant phenols and as such are widely distributed in nature and are the most abundant polyphenols in the human diet [Baldi *et al.*, 1997]. They can be divided into several classes according to the degree of oxidation of the oxygen heterocycle, including flavones, flavonols, isoflavones, anthocyanins, flavonols, and PACs, with more than 5000 compounds identified by 1990, some of which are illustrated in Table 2.2 [Bravo, 1998].

Table 2.2 Classification of Food Flavonoids

Flavonoid	Basic Structure
Chalcones	
Dihydrochalcones	
Aurones	
Flavones	
Flavonols	
Dihydroflavonol	
Flavanones	
Flavanol	
Flavandiol or leucoanthocyanidin	
Anthocyanidin	
Isoflavonoids	
Biflavonoids	
Proanthocyanidins or condensed tannins	

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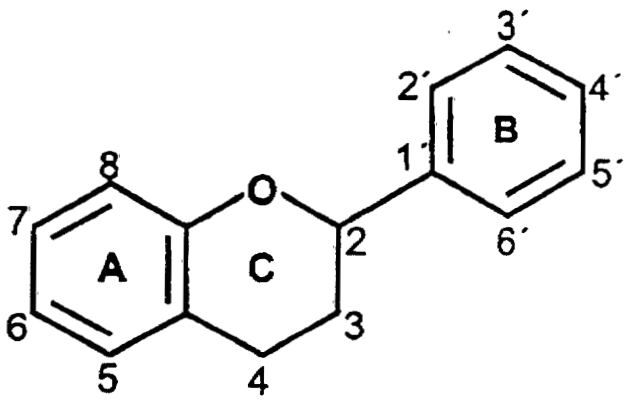
The common flavonoid structure is that of a diphenylpropane (C_6-C_3-C_6), which consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. The catechins, or flavanols, are a colorless, water-soluble, and oxygen sensitive group of substances. They are regarded as the group of flavonoids with the lowest oxidation level of the pyran ring-C, as it contains only one hydroxyl in position C3, hence the term flavan-3-ol [Kuhnau, 1976]. The basic structure and the system used for numbering the carbons in a basic flavonoid molecule are represented below in Figure 2.1. As illustrated in Table 2.2, PACs are oligomers based upon flavan-3-ol monomer units.

Figure 2.1 Basic Flavonoid Structure and Numbering System



Biogenetically, the A-ring usually comes from a molecule of resorcinol or phloroglucinol synthesized via the acetate pathway, whereas the B-ring is derived through the Shikimate pathway [Bravo, 1998]. The flavonoids occasionally occur in plants as aglycones, although they are most commonly found as glycoside derivatives.

Polyphenols also have a recognized biological activity. In grapes and grape products, it is the phenolic compounds that have been suggested as possibly playing a significant role in preventing or delaying the onset of cardiovascular disease, cancer, and other conditions. The phenolic compounds in grapes include phenolic acids, anthocyanins,

flavonols, flavan-3-ols, and tannins. These compounds are secondary plant metabolites that contribute in an important manner to the flavor and color characteristics of grapes, grape juices and wines. The content of these compounds can vary with the variety, degree of maturity, and part of the grape evaluated. Macheix *et al.*, [1990] identified differences in the monomeric flavan-3-ol and PAC content (dimers B_1-B_4, trimers, and tetramers) from a variety of berry skins isolated from fresh samples of *Vitis vinifera* exemplified below in Table 2.3. The percentages of the different PACs identified in the grape skins are supplied in Table 2.4

Table 2.3 Monomeric Flavan-3-ol and Polymeric PAC Content in the Berry Skin from *Vitis vinifera* Cultivars (mg /100 g fresh weight)

Variety	Catechin and Epicatechin	Proanthocyanidins (PAC)	Total
Alicante-Bouschet	2.34	6.42	8.76
Cabernet-Sauvignon	2.5	44.5	47.0
Carignane	9.7	55.2	64.9
Cinsaut	16.7	78.3	95.0
Grenache blanc	22.6	17.2	39.8
Grenache noir	9.5	55.0	64.5
Merlot	11.2	32.1	43.3
Mourvedre	14.0	64.9	78.9
Pinot noir	10.3	45.0	55.3
Average	13.3 (79% catechin)	50.5	64.0

Table 2.4 Proanthocyanidin Percentages in Grape Skin

Proanthocyanidin	Percentages (Extreme Values)
B1	34 (20-49)
B2	8 (5-20))
B3	6 (3-20)
B4	5 (2-17)
Trimer	24 (9-41)
Tetramer	23 (14-40)

2.1.1 *Proanthocyanidins*

Proanthocyanidins are phenolic compounds characterized by a flavonoid system with three distinct ring components. The term 'tannins' refers to the ability of these molecules to complex with proteins, originally permitting their use in the production of leather from hide. Several classes can be distinguished on the basis of the hydroxylation pattern of the constitutive units. Among them are procyanidins, which derive from catechin and epicatechin [Escribano-Bailon *et al.*, 1995], and have been reported to occur in grapes. The proanthocyanidins (PACs) are characterized by the anthocyanidins (*e.g.* cyanidin) that are formed upon acid hydrolysis. Anthocyanins derived from these and other anthocyanidins are the pigments present in flower and fruit and more rarely in wood and bark from certain trees [Cheynier *et al.*, 1997].

In plants, PACs occur as a mixture of oligomers and polymers. Therefore, their structural analysis requires prior fractionation, usually involving Sephadex LH20 chromatography and high performance liquid chromatography (HPLC) [Plumb *et al.*, 1998, Adamson *et al.*, 1999; Gabetta *et al.*, 2000]. The degree of polymerization may vary greatly; PACs up to 20,000 in molecular weight have been described [Cheynier *et al.*, 1997]. Labarbe *et al.*, [1999] developed a method for the fractionation of grape seed or skin and estimated that the mean degree of polymerization of separated PACs ranged increasingly from 4.7 to 17.4 in the seed (8.1 for total extract) and 9.3 to 73.8 in the skin (34.9 for total extract).

The PACs have long been associated with nonspecific protein interaction and the classical tanning process. However, these compounds are also considered essential components of a wine's flavor and aging potential. They are known to interact with the protein components of saliva and produce the characteristic astringency associated with young red wines. Their inherent antioxidant capacity is also responsible for their contribution to the aging of fine red wines. Red wines contain over 20 times the levels of PACs and certain flavanones compared with white wines [Williams and Elliot, 1997].

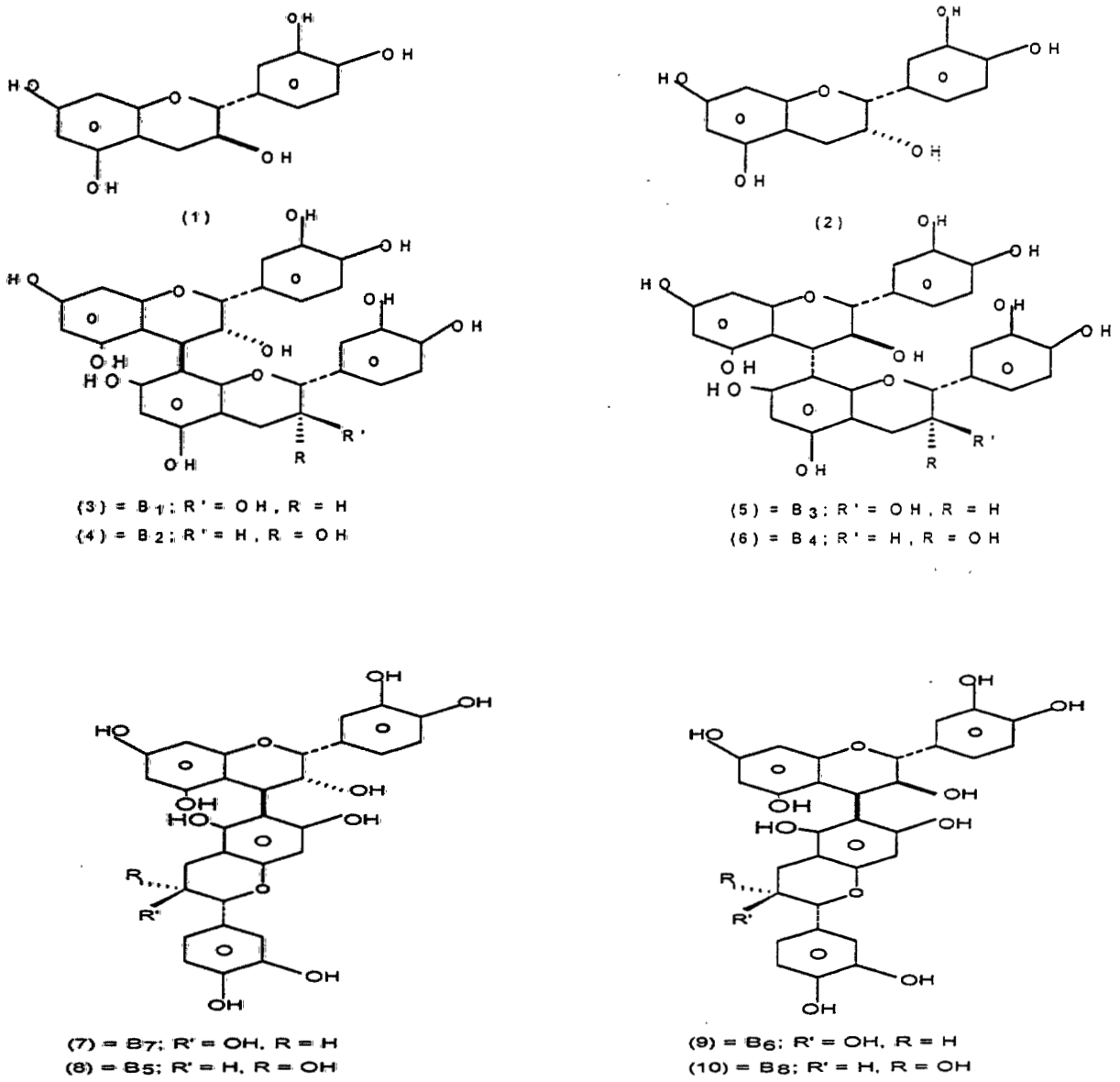
Plant tannins can be subdivided into two major groups: (1) hydrolyzable and (2) condensed tannins. The hydrolysable tannins consist of gallic acid and its dimeric condensation product, hexahydroxydiphenic acid, esterified to a polyol, primarily glucose [Bravo, 1998]. As their name would indicate, these tannins are easily hydrolyzed with acid, alkali, hot water, and enzymatic action, which yield polyhydric alcohol and phenylcarboxylic acid. The hydrolysable tannins can be further subdivided into gallotannins, or ellagitannins. Gallotannins yield glucose and gallic acid on hydrolysis by acids, bases, or certain enzymes. Ellagitannins contain one or more hexahydroxydiphenol residues, which are linked to glucose as a diester, in addition to gallic acid. Upon hydrolysis, the hydrodiphenol residue undergoes lactonization to produce ellagic acid [Chung, 1998a]. The oligomeric derivatives of gallic and ellagic acid are not present in grapes but may result in wines as transformation products of the original phenolic compounds present.

Condensed tannin PACs are high molecular weight oligomers and polymers. The monomeric unit is a flavan-3-ol. The monomeric units are usually linked through oxidative condensation by carbon-carbon bonds, normally between carbon-4 on of the heterocycle C-ring and carbons C-6 or C-8 of adjacent units [Bravo, 1998]. Much of the literature on the condensed tannin content of different plants refers to oligomeric PACs as polymers in which a degree of polymerization of 50 or greater can occur. The most commonly described condensed tannin PACs have molecular weights of approximately 5,000 Daltons, although polymers with molecular weights greater than 30,000 Daltons have been discovered.

The biosynthesis of the parent precursor flavan-3-ols, (+)-catechin and (-)-epicatechin, represented below in Figure 2.2 as (1) and (2), respectively, arises from the Shikimate-Chorsmate pathway by a sequence of reductions and condensation steps involving *trans* and *cis* flavan-3,4-diols. These transient diols subsequently condense via enzyme-catalyzed reactions to give a series of dimeric, trimeric and oligomeric PACs [Williams and Elliot, 1997]. As many as 20 different dimeric and trimeric PACs have been identified in grape seeds and skins. Only B-type PACs, meaning the active site in

scavenging free radicals is found on the B-ring, are present in grapes. They usually consist of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-O-gallate units. With regard to grapes and wine, perhaps the most widely studied sequence of condensed PACs is the B-series, which include dimers B_1 through B_8. These are shown below in Figure 2.2.

Figure 2.2 Grape seed and wine proanthocyanidins [Williams and Elliot, 1997]



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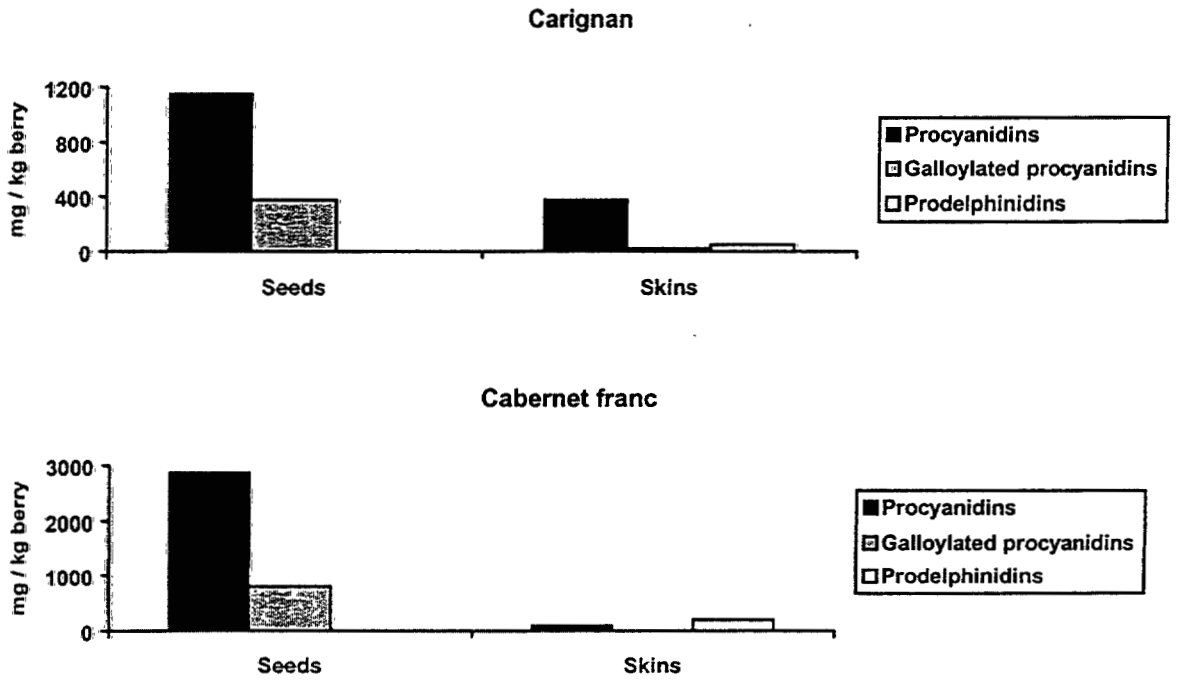
In grapes, the PACs tend to accumulate in the seed. Smaller quantities of these materials can be isolated from grape skins and stems. Polymeric tannins are much more abundant than monomers and dimers both in seeds and in grape skin [Cheynier *et al.*, 1997]. Reversed-phase HPLC analysis has shown that grape seed tannins consist of partly galloylated procyanidins, whereas grape skins also contain prodelphinidins, occurring as (-)-epigallocatechin benzylthioether derivatives. Grape seeds were found to contain larger amounts of tannins and larger proportions of galloylated units than grape skins but the average molecular weights of these compounds were higher in the skin than in the seed of all studied varieties. The relative composition of seed and skin tannins with a degree of polymerization of greater than two units was determined for two *Vitis vinifera* varieties and is presented below in Figure 2.3.

Figure 2.3 Composition Of Polymeric Tannins In The Seeds And Skins Of Two *Vitis vinifera* Varieties as Determined by Thiolysis [Cheynier, 1997]



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The organoleptic properties and reactivity of tannins, including radical scavenging effects and protein-binding ability, largely depend on their structures. In particular, the number of active sites increases with the degree of polymerization and with gallate esterification. Their accessibility may be influenced by the position of the carbon-carbon interflavanic linkages and that of the galloyl substituents [Cheynier *et al.*, 1997]. The formal identification of PACs, including the determination of the carbon-carbon bond position, requires sophisticated NMR techniques [Cheynier *et al.*, 1997]. Furthermore, the purification of the tannins becomes increasingly difficult as their molecular weight increases, due to the larger number of possible isomers, smaller amounts of each individual compound, and poorer resolution of the chromatographic profiles. This is especially true in the case of grape products, which contain a large diversity of tannin structures, based on several monomers [Cheynier *et al.*, 1997]. However, the major oligomeric procyanidins have also been quantified individually in various grape extracts by reverse-phase HPLC. Concentration of these oligomers in plant tissue was relatively low compared to that of larger molecular weight tannins. Table 2.5 lists some of those PACs and monomeric flavan-3-ols isolated from grape seed and skin.

Table 2.5 Proanthocyanidins in Grape Seeds and Grape Skin [Santos-Buelga *et al.*, 1995]

Compounds isolated from seeds:

- Catechin-(4-8)-catechin-(4-8)-catechin (C2)
- Catechin-(4-8)-catechin (B3)
- Epicatechin-(4-8)-epicatechin (B1)
- (+)-Catechin
- Epicatechin-(4-8)-epicatechin-(4-8)-catechin
- Catechin-(4-8)-epicatechin (B4)
- Catechin-(4-8)-catechin-(4-8)-epicatechin
- Epicatechin-(4-6)-epicatechin-(4-6)-catechin
- Catechin-(4-6)-catechin (B6)
- Epicatechin-(4-6)-epicatechin-(4-8)-epicatechin
- Epicatechin-(4-8)-epicatechin-(B2)
- Epicatechin-(4-8)-epicatechin-3-*O*-gallate-(4-8)-catechin
- Epicatechin-3-*O*-gallate-(4-8)-epicatechin-(B2-3-*O*-gallate)
- (-)-Epicatechin
- Catechin-(4-8)-epicatechin-3-*O*-gallate (B4-3'-*O*-gallate)
- Epicatechin-(4-8)-epicatechin-(4-6)-catechin
- Epicatechin-3-*O*-gallate-(4-8)-catechin-(B1-3-*O*-gallate)
- Epicatechin-(4-6)-catechin-(B7)
- Epicatechin-(4-8)-epicatechin-(4-8)-epicatechin (C1)

000025

Table 2.5 Proanthocyanidins in Grape Seeds and Grape Skin [Santos-Buelga *et al.*, 1995]

• Epicatechin-(4-8)-epicatechin-(4-8)-epicatechin-(4-8)-epicatechin • (-)-Epicatechin-3-*O*-gallate • Epicatechin-3-*O*-gallate-(4-6)-catechin-(B7-3-*O*-gallate) • Epicatechin-3-*O*-gallate-(4-8)-epicatechin-3-*O*-gallate (B2-3.3'-*O*-digallate) • Epicatechin-(4-8)-epicatechin-(4-8)-epicatechin-3-*O*-gallate • Epicatechin-(4-8)-epicatechin-3-*O*-gallate (4-8)-epicatechin-3-*O*-gallate • Epicatechin-(4-6)-epicatechin (Dimer B_5)
Compounds isolated from skins: • Catechin-(4-8)-catechin (Dimer B3) • Epicatechin-(4-8)-catechin (Dimer B_1) • (+)-Catechin • Epicatechin-(4-8)-epicatechin-(4-8)-catechin • Catechin-(4-8)-epicatechin (Dimer B_4) • Epicatechin-(4-8)-epicatechin-3-*O*-gallate (B_2-3'*O*-gallate) • Epicatechin-(4-8)-epicatechin-(4-8)-epicatechin (Trimer C_1) • (-)-Epicatechin-3-*O*-gallate

2.1.2 Phenolic Acids

Phenolic acids (*i.e.* hydroxycarboxylic acids with phenolic hydroxyl groups) also occur widely in nature in the form of esters, ethers, or in their free forms. Some phenolic acids, namely caffeic, chlorogenic, ferulic, gallic, and ellagic acid have been found to be pharmacologically active as antioxidant, antimutagenic, and anticarcinogenic agents [Nakayama, 1992; Shahrzad, 1996]. Catechin and epicatechin have also been demonstrated to be effective in the suppression of hydroxyl radical formation [Iwahashi *et al.*, 1990]. There is extensive literature on the determination of phenolic acids in foodstuffs. However, quantification can often be difficult due to sample complexity. The HPLC technique is the most commonly used method for the determination of phenolic acids in different samples. In green and black grape juices, only minor amounts of phenolic acids occur in the free state; most are present in conjugated forms that can be liberated by hydrolysis. Table 2.6 lists the phenolic acids present in green and black grape juice. The values are taken as the mean (± SD) of eight replicates.

Table 2.6 Phenolic Acids in Grape Juices (mg/l)

Phenolic Acid	Green Grape Juice		Black Grape Juice	
	Free	Hydrolyzed	Free	Hydrolyzed
Gallic Acid	1.45±0.05	110±6	5.24±0.08	79±7
Chlorogenic Acid	0.0	0.0	0.1±0.05	0.0
Caffeic Acid	0.37±0.04	12.9±0.5	1.05±0.04	22±1
Ferulic Acid	0.7±0.03	3.3±0.1	0.1±0.04	5.0±0.3
Ellagic Acid	0.28±0.05	0.6±0.1	0.41±0.05	0.6±0.1

2.2 Antioxidant Capacity

The MegaNatural™ Gold GSE and GSKE line of products have been evaluated as GRAS by a panel of qualified experts status based on scientific procedures. They are composed of monomeric and oligomeric flavanols (PACs), which are employed as an antioxidant when added to beverage products. In general, oligomeric PACs have also attracted increased attention in the fields of nutrition and medicine due to their potential health benefits based on effects observed *in vitro* and *in vivo*. Naturally occurring substances with antioxidant properties when present in food products have been demonstrated to have antioxidant activity *in vivo*, including the ability to scavenge reactive oxygen and nitrogen species. Importantly, the use of select flavonoids to infer epidemiological relationships to health and disease may be confounded by different flavonoids that may exhibit varying physiological effects. Extensive research has been conducted to investigate potential relationships between flavonoid structure and biological activity, especially as related to antioxidant properties. This research indicates that the hydroxylation pattern is a key factor. Little is known regarding the impact of the degree of polymerization of PACs [Hammerstone *et al.*, 2000].

A number of highly reactive oxygen species such as singlet oxygen $^1O_2$ and $O_2^{\cdot -}$, $OH^{\cdot}$, $NO^{\cdot}$, and alkyl peroxide free radicals are regularly produced in the course of human metabolism. Humans possess a wide variety of antioxidant physiological defenses that scavenge radicals, chelate metals involved in their formation, and repair damage. The

generation of these reactive oxygen species (ROS), produced by a variety of enzymatic reactions, beyond the antioxidant capacity of a biological system gives rise to oxidative stress. The production of the reactive oxygen species is best described as a chain reaction in which highly reactive oxygen free radical anions and hydroxyl free radicals are produced in several discrete steps. The formation and subsequent reactivity of the ROS induce oxidative stress.

It is believed that oxidative stress plays a role in the aging process and the pathogenesis of heart disease and cancer by causing damage to DNA (such as DNA-strand breaks), lipids, and proteins. The overall process may result in the interaction of the ROS with proteins, which may lead to changes in transport phenomena. Interaction with nucleic acids may lead to subsequent altered function or genetic mutations. Reaction of ROS may deplete glutathione and lead to changes in cell viability, while interaction with low-density lipoprotein (LDL) leads to changes in the membrane integrity of the vasculature and may increase the risk of heart disease [Williams and Elliot, 1997]. Natural antioxidants that can interact with or scavenge these ROS are found throughout nature. They have been identified in soy products, certain types of algae, various seed oils (including grape seed oil), a variety of fruits and vegetables, and in certain herbs. The consumption of natural antioxidants such as polyphenols, vitamins C and E, and carotinoids through the diet contribute to the natural defense mechanism of the human body.

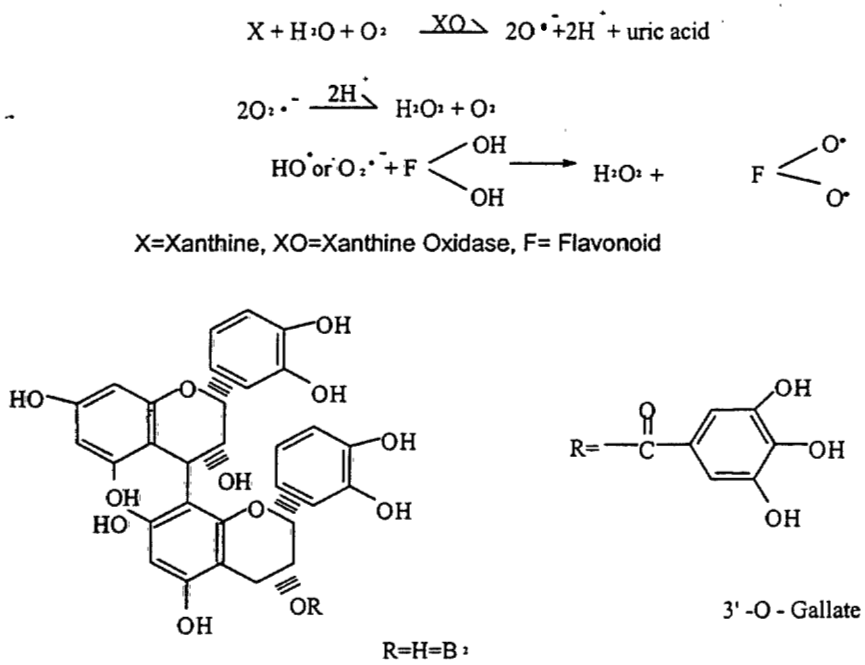
Antioxidants can be further categorized by their mode of action. They can be 1) preventative, such as catalase or superoxide dismutase, 2) chain breaking, such as vitamin E, or 3) complimentary in nature, such as Vitamin C or β-carotene. The antioxidant constituents found in grapes and wines are considered to be of the complimentary category in that they are believed to function extracellularly. The antioxidant mechanism associated with vitamin E and other phenolic antioxidants involves the abstraction of the phenolic hydrogen from ring-A and the subsequent stabilization of the phenoxide free radical by resonance contributions from the tetrahydropyran oxygen atom.

2.2.1 *Polyphenol Antioxidant Activity*

All polyphenols are able to scavenge singlet oxygen and $O_2^{\cdot -}$, $OH^{\cdot}$, $NO^{\cdot}$, and alkyl peroxide free radicals through electron donating properties, generating a relatively stable phenoxyl radical. For flavonoids with an *o*-dihydroxyphenyl group as B-ring and a fully saturated C-ring, such as in (gallo)catechins and most PACs, the radical site is at the B-ring and the substitution of the A-ring has only limited influence on the reduction potentials of the semiquinone radical formed. Thus, under well-defined laboratory conditions, PACs behave as radical scavengers or antioxidants in a way similar to other phenolic compounds possessing a *o*-dihydroxyphenyl group.

Ricardo-DaSilva *et al.* [1991] demonstrated the free radical scavenging ability of several of the proanthocyanidin B dimers against superoxide and hydroxyl radicals. Dimers B_1, B_2 and B_3 were all found to be more effective free radical scavengers than the monomeric (+)-catechin and (-)-epicatechin. They also concluded that the degree of polymerization of the PAC was not important with regard to superoxide radical scavenging potential. Hu *et al.*, [1995] suggested that the scavenging ability increased with the number of hydroxy groups on the B-ring of the PAC structures [Williams and Elliot, 1997]. The presence of a gallate ester at the 3′ position greatly enhanced the antioxidant capacity of the substituted species. The B_2-3′-O-Gallate was found to be the most potent free radical scavenger in the study [Ricardo-DaSilva, 1991] and is illustrated in Figure 2.4.

Figure 2.4 Free Radical Scavenging Action of Proanthocyanidins from Grape Seed [Williams and Elliot, 1997]



Roychowdhury *et al.*, [2001] investigated the effect of a grape seed PAC extract as a novel natural antioxidant on the generation and fate of nitric oxide (NO) in rat primary glial cell cultures. A treatment of 50 mg/L increased NO production by stimulation of the inducible isoform of NO synthetase (NOS). However, the extract failed to affect the LPS/IFN-gamma-induced NO production or iNOS expression. Similar responses were found in the murine macrophage cell line RAW264.7. The extract did not show any effect on dihydrodichlorofluorescein fluorescence, a reactive oxygen species marker with high sensitivity toward peroxynitrite, either in control or in LPS/IFN-gamma-induced glial cultures even in the presence of a superoxide generator. Extract treatment alone had no effect on the basal glutathione (GSH) status in glial cultures. Whereas the microglial GSH level declined sharply after LPS/IFN-gamma treatment, the endogenous GSH pool was protected when such cultures were treated additionally with the extract, although NO levels did not change. Glial cultures pretreated with the PAC extract showed higher tolerance toward application of H_2O_2 and tert-butylhydroperoxide.

Furthermore, pretreated glial cultures showed improved viability after H_2O_2-induced oxidative stress demonstrated by reduction in lactate dehydrogenase release or propidium iodide staining. The above results indicate that, in addition to its antioxidative property, the PAC extract enhanced low-level production of intracellular NO in primary rat astroglial cultures and that pretreatment with the PAC extract protected the microglial GSH pool during high NO production which resulted in an elevation of the H_2O_2 tolerance in the astroglial cells.

Scott *et al.* [1993] evaluated the antioxidant and pro-oxidant activities of (±)-catechin, (+)-catechin, and (-)-epicatechin. Using the deoxyribose assay, researchers determined that all three compounds were able to react with hydroxyl radicals and thus inhibit deoxyribose degradation. Calculated rate constants of these reactions were values of 3.65×10^9 $M^{-1}s^{-1}$, 4.55×10^9 $M^{-1}s^{-1}$, and 2.36×10^9 $M^{-1}s^{-1}$, respectively. No pro-oxidant activity was observed by Scott *et al.* [1993] in (±)-catechin, (+)-catechin, and (-)-epicatechin.

Furthermore, all three polyphenols were shown to inhibit bovine brain phospholipid liposome peroxidation by up to 80% at a concentration of 0.25 mM. Both (+)- and (±)-catechin as well as (-)-epicatechin also proved to be powerful scavengers of hypochlorous acid, HOCl, which is produced by the neutrophil-derived enzyme myeloperoxidase at sites of inflammation and when activated neutrophils infiltrate reoxygenated tissue. This was demonstrated in an assay involving elastase and its protein inhibitor α_1-antiproteinase (α_1AP) which, when incubated in the presence of HOCL and either 0.50 mM of (+)- , (±)-catechin, or (-)-epicatechin, protected α_1AP in inhibiting elastase by 100%, 85%, and 97%, respectively. The researchers also found that (+) and (±) catechins and (-) epicatechin inhibited the reduction of cytochrome-C by the superoxide radical in a dose-dependent manner.

In contrast to these results, Scott *et al.* [1993] demonstrated a pro-oxidant effect by (+)- and (±)-catechin and (-) epicatechin, inducing DNA damage in the bleomycin system. This assay was adapted for assessing the pro-oxidant effects of proposed lipid

antioxidants for food use. DNA damage was associated with this antitumor antibiotic as a likely result of bleomycin complexed with iron ions. The bleomycin-iron complex is known to degrade DNA in the presence of O_2 and a reducing agent. Results of this assay, shown below in Table 2.7, indicated that although (+)- , (±)-catechin, and (-)-epicatechin promoted DNA damage at higher concentrations in this system, they were less effective than ascorbate. Ferulic acid at concentrations greater than 1 mM also exhibited pro-oxidant activity in the bleomycin system. These results are not unexpected given that (+)- catechin, (±)-catechin, and (-)-epicatechin are phenolic compounds and may reduce Fe(III) to Fe(II). While the variability of the data generated by these assays underline the need for a variety of assay systems, the results indicate that catechins and epicatechin posses useful antioxidant properties.

Table 2.7 DNA Damage by the Ferric-Bleomycin System

Compound Added to Reaction	Concentration (mM)	Extent of DNA Damage A_{532} nm
None	-	0.02
Ascorbate	0.2	1.22
(+)-Catechin	0.05	0.03
	0.1	0.09
	0.25	0.29
	0.5	0.46
(±)-Catechin	0.05	0.02
	0.1	0.07
	0.25	0.25
	0.5	0.39
(-)-Epicatechin	0.05	0.03
	0.1	0.09
	0.25	0.39
	0.5	0.54
Ferulic Acid	0.05	0.00
	0.1	0.00
	0.25	0.04
	0.5	0.06
	1.0	0.16
	2.5	0.39
	5.0	0.55

Ueda *et al.*, [1996] estimated the reactivity of a series of antioxidative and endogenous radical scavenging compounds, including catechin, towards hydroxyl radical ($^{\cdot}OH$) generated from the reaction of the copper(II) complex $Cu(en)_2$ with H_2O_2. The antioxidant activity was evaluated against $^{\cdot}OH$ by either its ability to suppress $^{\cdot}OH$ formation, or to scavenge it upon formation. Evaluations were carried out using electron spin resonance (ESR) - spin trapping, thiobarbituric acid (TBA), and DNA strand break methodologies. Catechin was shown to be effective in suppressing $^{\cdot}OH$ generation in both the ESR-spin trapping and TBA methodologies. DNA strand breaks caused by the addition of $Cu(en)_2$ plus H_2O_2 were also suppressed by the addition of catechin. However, when ultraviolet photolysis of H_2O_2 was employed to generate $^{\cdot}OH$, catechin displayed a very weak scavenging ability. These results indicate that the antioxidants compounds used here, including catechin, suppressed the generation of $^{\cdot}OH$ from the reaction of $Cu(en)_2$ with H_2O_2.

Under the natural oxidative conditions of an oxygen air-enriched solution at a pH of 3.2 and in the presence of transitional metal ion catalysts, De Freitas *et al.*, [1998] investigated the influence of different structural factors such as catechin structure units (catechin and epicatechin), interflavonoid bond linkage, gallic acid esterification, and degree of polymerization in the kinetics of grape seed PAC decomposition in a model wine solution. Data were collected using a semipreparative HPLC technique. Sample fractional elution was unsurprising given that structural monomers exited the system prior to dimers, trimers, and tetramers. With regards to oxidative decomposition, results indicated a decrease in the quantity of PAC content over time. Determination of the kinetic order of the oxidative decomposition was not possible because of the complex mechanisms involved. Results also indicated that epicatechin was more readily oxidized than catechin and both were degraded at a slower rate than dimer PACs. These results may be explained by the increased capacity of dimers to trap oxygen radical species compared to the monomers, the nature of the C(4)-C(6) versus C(4)-C(8) interflavonoid linkage, and the nature of the constituent upper or lower structural monomeric unit, respectively.

2.2.2 Complexation of Metal Ions

Under well-defined chemical conditions, Santos-Buelga and Scalbert [2000] described phenolic compounds having an *o*-dihydroxyphenyl group as excellent chelators of iron(III) and therefore a potential pro-oxidants in the presence of transition metals. At neutral pH and in the presence of procyanidins dimer B_2 or a galloyl ester of glucose, all iron was found in the form of mononuclear ferric complex with catecholate groups of two ligands. They also formed complexes with Al(III) and Cu(II). The PAC-metal complexes easily precipitated at the neutral pH as long as the ligand concentration was not too high relative to metal ion concentration.

The potential consequence of this activity is that consumption of polyphenol-rich foods or beverages such as wine or tea may result in the inhibition of non-heme iron absorption through the gut barrier. This would be through the formation of a stable polyphenol - iron(III) complex in the gut when the ligand is consumed together with iron(III). Other nutrients such as ascorbic acid removed the inhibition of iron absorption by polyphenols by reducing the complexed iron(III) into the poorly coordinated Fe(II).

2.2.3 National Toxicology Program

The National Toxicology Program (NTP) continuously solicits and accepts nominations for toxicological studies to be undertaken by the Program. The NTP actively seeks to identify and select for study chemicals and other agents for which sufficient information is not available to adequately evaluate potential human health hazards. The NTP accomplishes this goal through a formal open chemical nomination and selection process. Substances may be studied for a variety of health-related effects, including but not limited to reproductive and developmental toxicity, genotoxicity, immunotoxicity, neurotoxicity, metabolism and disposition, and carcinogenicity. In evaluating and selecting nominated substances, the NTP also considers legislative mandates that require responsible private sector commercial organizations to evaluate their products for health and environmental effects. The possible human health consequences of

anticipated or known human exposure, however, remain the over-riding factor in the NTP 's decision to study a particular chemical or agent. The NTP Interagency Committee for Chemical Evaluation and Coordination (ICCEC) serves as the first level of review for NTP nominations.

At the May 2001 ICCEC meeting, 13 new nominations were reviewed and testing recommendations were made, including a nomination by the National Cancer Institute (NCI) that grape seed and pine bark extracts be evaluated [NTP, 2001]. The specific charge to evaluate grape seed extract deals with GSE as a form of nutritional supplement, as opposed to the MegaNatural™ Gold brand of GSE, determined by an Expert Panel as GRAS when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product. Nomination for testing was motivated in part because of the widespread consumer use of these substances as dietary supplements and recognized that polyphenols, as antioxidants, have been proposed as an important contributory factor to a protective effect against atherosclerotic cardiovascular disease.

2.3 History of Safe Proanthocyanidin Consumption

Polyphenolic compounds are widely distributed throughout the plant kingdom. As such, they are present in most edible fruits and vegetables and are therefore common in the everyday diet of most people. Although relevant progress has been made in defining the distribution and content of other flavonoids in foods, including monomeric catechins (*e.g.* catechin and epicatechin), the information available on proanthocyanidins (PACs) is somewhat limited. Large variations in reported concentrations between different authors are observed for any given product or commodity. These can be explained in part by differences in the assays used for PAC estimation or in the nature of the sample analyzed, such as the variety, stage of ripeness, part of the fruit considered, and processing into food stuffs. A comparison of the total PAC content based on five

samples run in duplicate of foods known to be rich in PACs illustrates this below in Table 2.8.

Table 2.8 Comparison of Total Proanthocyanidin Content (mg/serving) in Foods and Beverages [Hammerstone *et al.*, 2000]

Foods and Beverages	Low	High	Average±SEM
Red Wine	20.3	24.3	22.0±1.5
Cranberry Juice	27.0	35.6	31.9±3.2
Chocolate	140.2	181.2	164.7±19.8
All Apples	12.3	252.4	147.7±57.0

It is even more difficult to estimate the tannin and PAC content of blended products such as those of tea or wine, which may account for a major part of the polyphenolic dietary burden. The estimated degree of intake is complicated even further by the relative intake of PACs of a low degree of polymerization, such as dimers and trimers, that are likely absorbed through the gut compared to those of PACs with a greater degree of polymerization and molecular weight, which, though more common in the diet, may not be absorbed.

This lack of reliable values for PAC content in foods makes it difficult to accurately estimate their dietary intake. Estimates may vary from country to country and with dietary habits, which may or may not include rich sources such as tea, wine, and berries. Thus, it may range from several tens to several hundreds of milligrams of PACs per day. Examining the diets of 119 adults (age 19-49 years), a subgroup of the German national food consumption survey, Linseisen *et al.* [1997] calculated the average daily flavonoid intake values of the subjects. The average flavonoid intake was 54.0 mg/d, with 12.0 mg/d from flavonols, 8.3 mg/d of catechins, 13.2 mg/d of flavanons, and anthocyanidins, proanthocyanins, and phloretin comprising the remainder of the total amount. The researchers also noted that the majority of the flavonoids were derived from fruits, fruit juices, and fruit products while the majority of the flavonol was derived from vegetables and vegetable products. An estimate of

460mg per day has been reported for the average American intake [Santos-Buelga and Scalbert, 2000]. Scalbert and Williamson [2000] cited an intake of catechins and PACs closer to approximately 1 g/day. For comparison, GSE and GSKE will be added to selected beverage products in the amount of 210 ppm or 50 mg per 8 fluid ounce serving.

2.3.1 Grape Use and Consumption

Proanthocyanidin molecular components have a long history of safe consumption by humans in those forms commonly occurring in table grapes, grape products, and wines. Grapes are among one of the world's largest temperate fruit crops with approximately 65 million metric tons produced annually. About 80% of the total crop is employed in wine production and the remainder is used as table grapes, raisins, juice and other products. Of the wide variety of grapes grown, there are two major types: European and North American. European grapes belong to the species *Vitis vinifera* and over 95% of all the grapes produced belong to this species. Most *vinifera* species grow best in a Mediterranean-type climate with long relatively dry summers and mild winters. Certain varieties are used for wine, others for raisins or for table use. Leading wine varieties include Cabernet Sauvignon, Chardonnay, Pinot Noir, Zinfandel, and Carignane. Table varieties include the Emperor and Tokay grapes and the greenish-white Perlete. North American grapes belong principally to two main species: *Vitis labrusca* and *V. rotundifolia*. Both species can be consumed fresh or processed into juice, wine, or jelly. The *labrusca* grapes are grown principally in the lower Great Lakes region of the United States and Canada. The most important variety is the well known Concord grape. The *rotundifolia* or 'Muscadine' grapes grow throughout the southeastern United Sates and are exemplified by the Scuppernong variety [Girard and Mazza, 1998].

Wine is a moderately alcoholic beverage made by the fermentation of juice extracted from fresh ripened grapes. It can be classified by varietal names such as Cabernet Sauvignon or Chardonnay, or by a generic name, which often refers to the region of

Europe where wines of that general type were first produced. Generic names include Burgundy, Chianti, and Chablis. There are six classes of wine: Red, white, rosé, dessert, sparkling, and appetizer wines. The differences between these classes of wine are myriad, not just in their color or when they should be served. Significant differences exist in production method, composition, sensory quality and physiological characteristics. For instance, the process of white wine production seeks to avoid direct or enzymatic breakdown of the components of the skin, seed, or stalk; therefore, pressing and filtration precede fermentation. In order to make red wine, the solid parts of the berries must remain in contact with the liquid for several hours to several days, allowing not only the extraction of pigments from the skin, but also the extraction of varied polyphenolic molecules such as the tannins (PACs) from the seeds. To remain red colored over the years, the grape anthocyanidins will transform, or mature, into new red-colored pigments that are more stable in the wine aqueous environment. [Brouillard *et al.*, 1997].

Grapes are a naturally rich source of polyphenols. Bravo [1998] noted that fresh grapes contained 50-490 mg of polyphenols per 100 grams of fresh fruit. This compares favorably with cranberries which are known to be rich in polyphenols (77-247 mg/100g fresh fruit). In the production of red wine, the extraction of anthocyanin pigments and related phenolics from the grape solids (skins and seeds) begins with the crushing of the grapes and continues through the fermentation and pressing operations. The end results are that red wine contains 2-3 times more phenolics than white wines [Girard and Mazza, 1998]. These phenolic substances exhibit antioxidant and other properties which are believed to be of potential benefit to human health.

2.3.2 French Paradox

A diet high in fat is believed to be associated with an increased risk for heart disease. However, in France, the mortality rate from ischemic heart disease has been reported to be lower than in Britain and many other countries, despite a relatively high dietary fat intake. This seemingly paradoxical relationship became popularly known in the early

1990's as "the French paradox" [Constant, 1997; Kopp, 1998; Law and Wald, 1999; Das *et al.*, 1999; Visioli, 2000]. According to Constant [1997], a number of theories were proposed to account for the low mortality rate from ischemic heart disease, including factors such as lower consumption of milk as a result of lactose intolerance, increased consumption of garlic, cheese, fruits and vegetables, and a higher intake of red wine. The hypothesis that the reduced rate of mortality from heart disease may be associated with wine consumption has been extensively studied and many articles have been published both supporting and refuting this theory.

Constant [1997] reviewed many studies that attributed the strong negative association between alcohol intake and mortality from ischemic heart disease seen in 18 countries studied to the consumption of wine. Constant cited research conducted by Artaud-Wild *et al.*, [1993] who made similar conclusions based on a 40-country study but further concluded that wine may render a protective effect only when the population consumes large amounts of saturated fat [Constant, 1997]. According to Constant, other investigators such as Klatsky and Armstrong [1993], Gaziano *et al.* [1995], and Rimm *et al.*, [1996] studied the potential beneficial effects of wine, beer, and liquor. Based on data generated by a questionnaire given to over 100,000 individuals, Klatsky and Armstrong reported that wine, beer, and liquor all were associated with a decreased risk of coronary artery disease, but wine was associated with the lowest risk. Rimm *et al.*, [1996] were reportedly unable to detect such differences in a review of ecological case control and cohort studies which showed equal protection from consumption of wine, beer, or spirits against coronary heart disease. Likewise, Gaziano *et al.* did not find a difference among wine, beer, or liquor, attributing an observed 45% reduction in myocardial infarction risk to high density lipoprotein (HDL) levels after consumption of at least one-half or more of an alcoholic beverage per day, irrespective of the form of alcohol. However, some individual variability in response to ethanol exposure is likely.

Most of the research investigating the potentially beneficial components in red wine has focused on polyphenol compounds, namely pigments (anthocyanins), tannins, and flavonoids, which possess antioxidant activity. Resveratrol and quercetin are two

flavonoids that have been extensively studied [Constant, 1997; Kopp, 1998]. Das *et al.*, [1999] noted that a large variety of antioxidants, including resveratrol, catechin, epicatechin, and PACs are present in the polyphenol fraction of red wine and that these play a significant role in the cardioprotective effects of red wine against ischemic reperfusion injury.

Law and Wald [1999] have proposed an alternate explanation for the significantly lower rate of mortality from heart disease in France. While they recognize that many studies have found a strong correlation between higher wine consumption and lower mortality from ischemic heart disease across countries, they believe that the high consumption of red wine in France does not fully explain the difference. Instead, they propose that the main reason for the low mortality from heart disease in France is due to the time lag between increased consumption of animal fat with an attending increase in serum cholesterol, which began about 15 years ago, and the resulting increase in coronary heart disease and subsequent risk of mortality. Evidence presented in support of this theory includes mortality data across countries (including France) from 30 years ago, which show a strong correlation between the level of animal fat in the diet and mortality from heart disease, with no relationship to wine consumption. Analysis of more recent data did not show a strong correlation.

Law and Wald's time lag theory maintains that countries where wine consumption is high (France, Italy, Spain) also used to have low consumption of saturated fat and consequently had lower mortality from ischemic heart disease. Although fat consumption has increased in recent years, they suggest its effects are not yet reflected in the mortality data. They have therefore proposed that consumption of alcohol, particularly red wine, is not as significant a factor as has been assumed in the past.

In response to Law and Wald's theory, Stampfer and Rimm [1999] contend that the role of alcohol should not be dismissed as a partial explanation but that other dietary factors such as the intake of folate, fiber, and nuts should also be considered. Barker [1999] and Mackenbach and Kunst [1999] have also proposed that other factors such as

intrauterine nutrition and heterogeneity of populations may also be important in determining the risk of coronary heart disease.

3.0 POLYPHENOLIC GSKE / GSE CHEMISTRY AND STABILITY

3.1 Chemical Identity of Major Component Proanthocyanidins

The formula and structure of the many PACs found throughout the plant kingdom are still poorly characterized. However, the major constituent PACs of the MegaNatural™ line of grape seed and grape skin extracts are expected to conform with the data set forth in Table 3.1 below.

Table 3.1 MegaNatural™ Grape Seed Extract (GSE) and Grape Skin Extract (GSKE)

	GSKE Profile Data of 11 Production Lots: Harvest Year 1999			GSE Profile Data of 6 Production Lots: Harvest Year 1999			GSE Profile Data of 3 Production Lots: Harvest Year 2000		
	minimum	average	maximum	minimum	average	maximum	minimum	average	maximum
Catechin % by wt	3.0	3.4	3.9	5.4	5.7	5.9	3.2	4.8	6
Epicatechin % by wt	4.0	4.6	5.3	4.3	4.4	4.5	4.2	4.4	4.9
Gallic acid % by wt	1.3	2.4	3.5	1.2	1.6	1.8	0.7	1	1.4
Total phenols % by wt	85.4	87.3	89.0	88.2	91.6	94.6	89.1	90.5	92.4
Total anthocyanins % by wt	1.6	2.6	2.0	NA	NA	NA	NA	NA	NA
HPLC relative profile of monomers-%	12.2	16.7	21.0	11.5	13.1	13.9	9.9	10.4	11.1
HPLC relative profile of oligomers-%	57.9	67.4	76.0	68.2	70.1	72.7	73.4	74.9	77.7
HPLC relative profile of polymers-%	4.2	15.9	27.2	15.4	16.8	18.5	12.1	14.7	16.4
Moisture % by wt	2	3.7	4.6	3.5	4.3	4.7	3.1	3.6	4.6

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3.2 Range of Major and Minor Component Proanthocyanidins in MegaNatural™ GSKE and GSE Products

The phenolic content of the MegaNatural™ Gold line of products has been determined by analytical evaluation. The total quantity of phenols present in two lots of GSE and GSKE, respectively, are shown below in Table 3.2.

Table 3.2 Analytical Evaluation of MegaNatural™ Gold Grape Seed Extract and GSKE (mg/g)

	MegaNatural™ Gold Grape Seed Extract Lot #2501-040157 (Expiration Date 7/5/2002)	GSKE Grape Skin Extract Lot #2511-040060 (Expiration Date 2/29/2002)
Total phenols (mg/g)	432.6	332.3

3.3 Proposed Product And Technical Information

3.3.1 MegaNatural™ Gold Grape Seed Extract (GSE)

Code PIN: VW7000

Form: Powder

Table 3.3 GSE Product Specifications

Analysis	Specification
Description	
Appearance	Rose Beige Powder
Flavor Evaluation	Bitter and Astringent
Chemical	
Total Phenolics (gallic acid equivalents, dry basis)	≥ 90 g GAE/100 g
Relative HPLC phenol profile	10% min Monomers 60 – 80% Oligomers 25% max Polymers
pH (4% in water)	2.0 to 5.5

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Table 3.3 GSE Product Specifications

Analysis	Specification
Physical	
Moisture (loss upon drying, compliant with USP specification <731>)	≤ 8.0%
Insoluble Substances (1% in water)	< 5%
Free Flow Density (compliant with USP specification <616>)	0.25 to 0.40 g/mL
Tap Density (compliant with USP specification <616>)	0.40 to 0.55 g/mL
Particle Size (compliant with USP specification <786>)	+ 35 mesh none + 80 mesh 20% max - 200 mesh 20% max
Microbiological	
Total Plate Count	< 1000 CFU/g
Yeast & Mold	< 100 CFU/g
Coliform	< 10 CFU/g
Salmonella (per 30 g)	Negative
E. coli (per 10 g)	Negative
Pesticide FDA Tolerances in Grapes	
Iprodione	≤ 60 ppm
Carbaryl	≤ 10 ppm
Fenarimol	≤ 0.2 ppm
Myclobutanil	≤ 1.0 ppm
Phosmet	≤ 10 ppm
Tebuconazole	≤ 5 ppm
Azoxystrobin	≤ 1.0 ppm
EBDC	as Zineb

3.3.2 MegaNatural™ Gold Grape Skin Extract (GSKE)

Code PIN: GK2000

Form: Powder

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Table 3.4 GSKE Product Specifications

Analysis	Specification
Chemical	
Total Phenolics (gallic acid equivalents, dry basis)[1]	≥ 80 g GAE/100 g
Total anthocyanins[2]	≥ 1.5 g / 100g
Physical	
Appearance	Red Purple Powder
Moisture (loss upon drying, compliant with USP specification <731>)	≤ 8.0%
Particle Size (compliant with USP specification <786>)	+ 35 mesh none + 80 mesh 20% max - 200 mesh 20% max
Microbiological	
Total Plate Count	< 1000 CFU/g
Yeast & Mold	< 100 CFU/g
Coliform	< 10 CFU/g
Salmonella (per 30 g)	Negative
E. coli (per 10 g)	Negative
Pesticide FDA Tolerances in Grapes	
Iprodione	≤ 60 ppm
Carbaryl	≤ 10 ppm
Fenarimol	≤ 0.2 ppm
Myclobutanil	≤ 1.0 ppm
Phosmet	≤ 10 ppm
Tebuconazole	≤ 5 ppm
Azoxystrobin	≤ 1.0 ppm
EBDC	as Zineb

1 Singleton, 1965.
2 Niketic-Aleksic, 1972.

3.4 Analytical Evaluation of Product Specifications

Analytical evaluation of the MegaNatural™ Gold GSKE and GSE line of products revealed that product composition fell within the specifications established for

Polyphenolics Brand of products. Analytical results are presented below in Table 3.5. Pesticide and heavy metal content meet FDA tolerances for grapes.

Table 3.5 Analytic Results of 2 Batches of the MegaNatural™ GSKE and GSE

	MegaNatural™ Gold Grape Seed Extract Lot #2501-040157 (Expiration Date 7/5/2002)	MegaNatural™ Gold Grape Skin Extract Lot # 2511-040060 (Expiration Date 2/29/2002)
Total sulfur dioxide (ppm)[1]	Not detected	3
Moisture (g/100g)[1]	4.13	4.0
Residual ethanol (g/100g)[1]	0.34	0.09
Microbiological analyses[1]		
Total plate count (CFU/g)	0	8
Yeast and Mold (CFU/g)	1	22
Coliform (CFU/g)	0	0
Salmonella (per 30 g)	Negative	Negative
E. coli (per 10g)	Negative	Negative
Heavy metals (ppm)[2]		
Arsenic	0.74	0.70
Cadmium	<0.005	<0.005
Lead	0.15	0.11
Tin	<10	<10
Pesticide ppm (FDA tolerances in grapes)[3]		
Iprodione (≤ 60 ppm)	Not detected	Not detected
Carbaryl (≤ 10 ppm)	0.36	Not detected
Fenarimol (≤ 0.2 ppm)	Not detected	Not detected
Myclobutanil (≤ 1.0 ppm)	0.28	0.38
Phosmet (≤ 10 ppm)	0.006	Not detected
Tebuconazole (≤ 5 ppm)	0.066	Not detected
Azoxystrobin (≤ 1.0 ppm)	Not detected	0.042
EBDC (as Zineb)	Not detected	Not detected

[1] Analyzed by Canandaigua Wine Company (Madera, CA)
[2] Analyzed by The National Food Laboratory (Dublin, CA)
[3] Analyzed by DFA of California (Fresno, CA)

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3.5 Stability Data

The stability data for the MegaNatural™ Gold line of GSKE and GSE are presented below in Table 3.6 and Table 3.7, respectively. The GSKE was stored at ambient temperature in a dark storage room. Storage conditions for the GSE were in an office at ambient temperature in a closed package, typically a clear plastic bag or bottle. The data for both stability analyses indicated that GSKE and GSE are very stable, although no stability data are available for GSKE and GSE in their proposed uses in juice and fruit flavored beverages. However, PACs are known to be very stable over time in wines and the constituent PACs in GSKE and GSE are therefore expected to behave in a similar fashion.

Table 3.6 Stability of MegaNatural™ Gold Grape Skin Extract (GSKE) Over Time

Lot Number	2213-169		2511-019329		2511-029336	
Conc. Eluant Drum Code	1998 frozen pomace		1999 fresh pomace		1999 fresh pomace	
Origin of Cert. of Analysis	CWC - MB	Analyzed	CWC - MB	Analyzed	CWC - MB	Analyzed
Date of Manufacture	July 28, 1999	20 months	Nov 24, 1999	16 months	Dec 2, 1999	15 months
Date of Cert. of Analysis	-	Mar 02, 2001	-	Mar 02, 2001	Feb 29, 2000	Mar 02, 2001
Total Phenolics	86.9	81.25	86.4	83.81	89.0	87.98
% Decrease In Phenol Conc.		6.5%		3.0%		1.2%
HPLC - % Monomers	10.5	9.6	17.1	15.5	21.0	18.1
HPLC - % Oligomers	75.3	75.5	76.0	74.5	74.8	72.8
HPLC - % Polymers	14.3	14.9	6.9	10.0	4.2	9.2
Gallic Acid ppm	13550.0	12919	27906.0	25499.0	35020	32177.0
Catechin ppm	22218	22185	31356	31703	39026	41358
Epicatechin ppm	37786	33141	41924	36798	52873	44748
% Gallic Acid by wt.	1.4	1.3	2.8	2.5	3.5	3.2
% Catechin + Epicatechin by wt.	6.0	5.5	7.3	6.9	9.2	8.6

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Table 3.7 Stability of MegaNatural™ Gold Grape Seed Extract (GSE) Over Time

Sample Description	Manufacture Date	Initial Date Received	Total Phenols g GAE/ 100 g ("as is" basis)							Product Age (Months) as of 1/08/01	Time Since Initial Analysis (Months) as of 1/08/01
			Initial Analysis	Analyzed 8/31/99	Analyzed 2/14/00	Analyzed 01/08/01	Average	Stds.	% Cv		
GSE U41130	11/08/96	09/30/97	81.1	85.0	85.5	81.3	83.9	2.40	2.9	50	40
GSE W12209	09/12/97	11/06/97	89.0	86.4	84.8	84.9	86.7	2.12	2.4	40	38
GSE Melaleuca Blend (C135301+154603)	12/23/97	12/23/97	91.0	89.3	88.2	90.4	89.5	1.39	1.6	37	37
GSE Melaleuca Blend 2 (Y00076+Y01596)	03/09/98	03/09/98	89.0	85.5	82.6	82.4	85.7	3.19	3.7	34	34
GSE Y03505	03/30/98	03/30/98	83.0	91.1	90.6	88.9	88.2	4.53	5.1	33	33
GSE Y17826 (MSB)	11/24/98	12/10/98	86.2	87.9	92.1	85.0	88.7	3.03	3.4	26	25
GSE Y81111	11/11/98	11/18/98	92.5	-	89.3	-	90.9	2.28	2.5	-	-
GSE 2213-089	05/20/99	05/27/99	82.7	-	77.3	-	80.0	3.83	4.8	-	-
GSE 2213-259	08/31/99	09/02/99	92.0	-	90.4	-	91.2	1.17	1.3	-	-

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4.0 PRODUCTION METHODOLOGY OF POLYPHENOLIC GSE AND GSKE

4.1 Grape Seed Extract (GSE)

Fresh grapes at ambient temperature are received at the winery / Polyphenolics Inc. manufacturing facility, inspected for quality, and screened for defects. Grapes are dumped into a hopper where they are de-stemmed, crushed, and transferred to a receiving tank. Mechanical presses express the grape juice, leaving a pomace residue consisting of seeds and skins. Seeds are separated from the skins by means of a shaker screen; the seeds fall through the screen and are collected while the skins remain on top of the screen. The seeds are then subjected to a boiling water process for 1-2 hours, which dissolves the polyphenolic constituents. The seeds themselves are then separated, rinsed with more water, and discarded. The hot water / polyphenolic extract is then cooled. This is followed by enzyme depectinization, pH adjustment (which facilitates sedimentation and microbiological control), and then refrigeration. The extract is then stored and periodically sparged with nitrogen gas.

After a period of 1-3 months, the extract is filtered with diatomaceous earth and passed through a column of adsorbent resin of trimethylolpropane trimethacrylate (TMPTMA), wherein the grape phenolics are preferentially adsorbed on the resin surface. Due to the high specificity of the resin for phenolic substances, other grape constituent such as minerals and organic acids pass through the column and are discarded. After resin saturation, the column is rinsed with water and the phenolics eluted using 75% by volume beverage grade ethanol. This elution is followed by another water rinse. The high-purity grape phenols are captured in an alcohol solvent. The extract is next stripped of the alcohol solvent in a vacuum thermal evaporator yielding an extract concentrate. This concentrate is then spray dried, sifted to a uniform particle size, and packaged for sale.

4.2 Grape Skin Extract (GSKE)

The process employed for GSKE is identical to the above GSE process except that no seed separation takes place prior to extraction. Both the skins and seeds are extracted together.

5.0 INTENDED USE IN FOOD

MegaNatural™ Gold grape seed and grape skin extracts are proposed for interchangeable use in non-carbonated fruit juices and fruit flavored beverages from both fluid and dry mixtures, as well as carbonated fruit flavored beverages. The proposed use level of GSE and GSKE would be approximately 210 ppm or 50 mg per 8 fluid ounce serving of the selected beverages as consumed. GSE and GSKE use is proposed as an antioxidant added to these food products to retard deterioration. Antioxidants may also provide a nutritional benefit in scavenging reactive oxygen and nitrogen species. Both the MegaNatural™ Gold GSE and GSKE are intended for use in an inter-changeable manner such that any one of the above beverage products may contain GSE, GSKE, or some combination of either MegaNatural™ product.

6.0 ANTICIPATED INTAKE OF MEGANATURAL™ GOLD GSE AND GSKE

Projected interchangeable consumption of the MegaNatural™ Gold grape seed / skin extracts proposed for use in non-carbonated fruit juices and fruit flavored beverages from both fluid and dry mixtures, as well as carbonated fruit flavored beverages was calculated using data contained within the United States Department of Agriculture (USDA) 1994-1996 Continuing Survey of Food Intakes by Individuals (USDA CSFII 1994-1996)[1] and the 1998 Supplemental Children's Survey (USDA CSFII 1998) (USDA, 2000). Representative food codes were used to evaluate the specific food types included in the calculation of GSE / GSKE intake. The proposed used level of GSE /

[1] U.S. Department of Agriculture (USDA). 2000. 1994-1996, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and Diet and Health Knowledge Survey (DHKS) (On CD-ROM) U.S. Department of Agriculture (USDA), Riverdale, MD, (Apr.) Supercedes PB98-500457; PB2000-500027

GSKE in the selected food codes is approximately 210 ppm or 50 mg per 8 fluid-ounce serving.

Table 6.1 summarizes the estimated total GSE / GSKE intake from non-carbonated fruit juices and fruit flavored beverages from both fluid and dry mixtures, and carbonated fruit flavored beverages in the U.S. by population group. Table 6.2 presents these data on a gram per kilogram body weight basis.

Table 6.1 Summary of Estimated Combined Daily Intake of GSE / GSKE from Fruit Juices and Fruit Flavored Beverages in The U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-Users Consumption	
				Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Infant / Toddler	0-2	62.9	2014	0.04	0.10	0.06	0.12
Child	3-11	76.3	4962	0.05	0.11	0.07	0.12
Female Teenager	12-19	63.0	450	0.05	0.12	0.08	0.16
Male Teenager	12-19	61.8	450	0.06	0.16	0.10	0.20
Female Adult	20 and Up	44.2	2064	0.02	0.07	0.05	0.10
Male Adult	20 and Up	43.1	2105	0.03	0.10	0.08	0.16
Total Population	All Ages	50.9	12045	0.03	0.10	0.07	0.13

Table 6.2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of GSE / GSKE from Fruit Juices and Fruit Flavored Beverages in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-Users Consumption	
				Mean (g/kg)	90th Percentile (g/kg)	Mean (g/kg)	90th Percentile (g/kg)
Infant / Toddler	0-2	62.9	2014	0.003	0.008	0.005	0.010
Child	3-11	76.3	4962	0.002	0.005	0.003	0.006
Female Teenager	12-19	63.0	450	0.001	0.002	0.001	0.003
Male Teenager	12-19	61.8	450	0.001	0.003	0.002	0.003
Female Adult	20 and Up	44.2	2064	<0.001	0.001	<0.001	0.002
Male Adult	20 and Up	43.1	2105	<0.001	0.001	0.001	0.002
Total Population	All Ages	50.9	12045	<0.001	0.002	0.002	0.004

Approximately 50.9% of the U.S. population was identified as consumers of fruit juices, fruit flavored beverages, fruit flavored beverage mixes, and carbonated fruit flavored beverages (12,045 actual users identified). Consumption of these types of beverages by the total population resulted in an estimated mean all-person and all-user GSE / GSKE intake of 0.03 g/person/day (<0.001 g/kg body weight/day) and 0.07 g/person/day (0.002 g/kg body weight/day), respectively. These data compare well with known PAC content in foods and beverages as represented in Table 2.8 of this document. For example, the average PAC content (mg/serving) in red wine was determined to be 22.0±1.5 [Hammerstone *et al.*, 2000].

On an individual population basis, the highest mean all-user intakes were reported in male teenagers, 0.10 g/person/day (0.002 g/kg body weight/day). The lowest mean all-user intakes were reported in female adults, 0.05 g/person/day (<0.001 g/kg body weight/day). Similarly, the greatest and lowest mean all-person intakes were reported in male teenagers,0.06 g/person/day (0.001 g/kg body weight/day), and female adults, 0.02 g/person/day (<0.001 g/kg body weight/day), respectively. Heavy consumer (90th percentile) all-user and all-person intakes also followed the same trend, with the

greatest consumers identified as male teenagers and the lowest consumers identified as female adults.

Mean and 90th percentile intake estimates based on sample sizes of less than 30 and 80, respectively, or perhaps higher depending on the coefficient of variation may not necessarily be considered statistically reliable due to limited sampling size (FASEB, 1995)[2]. As such, estimates of the intake of GSE / GSKE based on the consumption of carbonated fruit flavored beverages as a product subgroup by the individual population groups may exhibit limited reliability.

This type of methodology is generally considered to be 'worst case' in terms of potential intake as a result of several conservative assumptions made in estimating consumption. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 3-day dietary surveys, overestimate consumption of food products, which are consumed relatively infrequently[3].

7.0 ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME) PROFILE

7.1 Absorption

Polyphenols exist in foods and beverages in various chemical forms that determine their gut absorption. The chemical structure of polyphenols thus determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma. Inter-individual differences in the rate of absorption have also been observed.

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[2] FASEB, 1995. Third Report on Nutrition Monitoring in the United States, Volume 1. Interagency Board for Nutrition Monitoring and Related Research. Prepared by the Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology. U.S. Government Printing Office, Washington.

[3] Anderson, S.A. 1988. Estimation of Exposure To Substances In The Food Supply. Life Sciences Research Office, Federation of American Societies For Experimental Biology, Bethesda, Maryland.

The biological activities of polyphenols are affected by their bioavailability. Taken as a class of compounds, there is indirect evidence of the absorption of polyphenols through the gut barrier based upon increased plasma antioxidant capacity after consumption of polyphenol rich food stuffs such as tea, red wine, or black currants [Scalbert & Williamson, 2000].

Both *in vivo* and *in vitro* studies using polyphenolic compounds with different chemical structures and solubilities illustrate their varying susceptibility to absorption and metabolism. Bravo [1998] suggested a classification of fractions that distinguishes between extractable and non-extractable polyphenols using a variety of different solvents. Extractable polyphenols included low- and intermediate-molecular-mass phenolics which included monomeric and presumably dimeric and trimeric PACs. High molecular weight polymerized PACs or those which tended to bind to dietary fiber or protein and thus could not be extracted using the usual solvents were considered insoluble.

The absorption of polyphenols, particularly PACs, depends upon molecular weight. These compounds exist in a polymerized state and because of their high molecular weight, are not likely to be easily absorbed in the small intestine. Evidence showing the absorption of PACs through the gut barrier is still scarce [Santos-Buelga and Scalbert, 2000]. A preliminary assay by Deprez *et al.*, [2000] on *in vitro* absorption through a cell monolayer derived from the human intestinal cell line Caco-2 showed that radiolabeled PAC dimer and trimer were absorbed in contrast to polymers. The dimer and trimer were absorbed to a similar extent as catechin although this could not be confirmed *in vivo*.

7.2 Distribution

More direct evidence on the bioavailability of a few phenolic compounds has been obtained by measuring their concentrations in plasma and urine after ingestion of either

pure compounds or foodstuffs known to contain compounds of interests. These data are provided in Table 7.1

Table 7.1 Bioavailability in Humans of Polyphenols Consumed Alone or in Foods*

Polyphenol	Source	Quantity Ingested (mg)	Max. Plasma Conc. (μM)	% Excretion in the Urine
Catechin	120 ml Red wine	34	0.072	-
Catechin	Pure Compound	500	2.0	0.45
Epicatechin	NA	32	0.27	6.2
Caffeic Acid	NA	1000	NA	27
Quercetin	Onion	68	0.74	1.39
Quercetin	Apple	98	0.30	0.44
Anthocyanins	300 ml Red wine	218	NA	1.0 - 6.7

* Polyphenols, principally in the form of conjugated metabolites, as sulfate esters or glucuronides, in plasma and urine, were hydrolyzed by acid or enzymes before chromatographic or colorimetric analysis.

The same data provided in Table 7.1 indicate that the concentrations of intact flavonoids in human plasma rarely exceeds 1 μM when the quantities of polyphenols ingested do not exceed those commonly ingested within our diets [Scalbert and Williamson, 2000]. These maximum concentrations are most often reached 1-2 hours after ingestion. Measurement of the plasma antioxidant capacity suggests that, after consumption of 10-100 mg of a single compound, more phenolic compounds are present largely in the form of unspecified metabolites produced in tissues or by colonic microflora. Scalbert and Williamson [2000] believed that future research should be directed less toward parent compounds but rather upon the biological activities of these metabolites and in particular upon conjugated analogues.

For most flavonoids absorbed in the small intestine, the plasma concentration then rapidly decreases with an elimination half-life of 1-2 hours. This fast excretion is facilitated by the conjugation of the aglycone to sulfate and glucuronide groups. The maintenance of high plasma concentration thus requires repeated ingestion of

polyphenols over time. The exception to this are the polymerized PACs which may be absorbed only after partial degradation by colonic microflora.

7.3 Metabolism

Once the PACs or the fermentation products have crossed the intestinal barrier, they reach the liver, the main organ involved in the metabolism of polyphenols, via the portal vein. The implication of other organs such as the kidneys or intestinal mucosa cannot be ruled out as they contain enzymes involved in polyphenol metabolism. Flavonoids as a group, and PAC oligomers specifically, when ingested orally and absorbed through the small intestine, are subject to substantial catabolic changes. This is important since it must be considered that the nutritional effects of flavonoids might not be due exclusively to the compounds in particular, but to the post-absorptive metabolites. Kuhnau [1976] stated that the urine of animals fed flavonoids contained a series of polyphenolic compounds later identified as aromatic acids not found in control animals. The formation of the metabolites was explained through a detachment of ring-A from the flavonoid molecule and by opening the heterocyclic ring-C that may proceed along a series of three pathways, depending upon the chemical nature of the flavonoid ingested.

Conjugated and 3'-O-methylated derivatives have been detected in the plasma of rats administered catechin [Bravo, 1998]. These metabolites are secreted in the urine or the bile. In the latter case, they may then enter the enterohepatic cycle when deconjugated by the action of colonic microflora and be reabsorbed. Alternately, they may be fully metabolized and converted into simple phenolic acids after hydrolysis of their flavone structure mediated by bacterial enzymes [Bravo, 1998]. The urine of rats fed catechin contained hydroxylated phenylpropionic acids as well as some hydroxylated benzoic acids. Unspecified substances of a lactone character were also detected [Kuhnau, 1976]. The hydroxylation pattern of a polyphenol will determine its susceptibility to bacterial degradation. The absence of hydroxyl groups will prevent ring cleavage [Bravo, 1998].

No *in vivo* studies have yet been reported [Santos-Buelga and Scalbert, 2000], but PACs are extensively metabolized, dehydroxylated, methylated or conjugated to sulphate esters or glucuronides as has been shown for other flavonoids. These reactions both limit the potential for the formation of toxic quinones and facilitate the excretion of PACs in the form of anionic derivatives. It is likely that no more than a trace of PAC's with intact *O*-dihydroxyphenyl groups survive in the tissues. Therefore, it is important to study the biological effects of PACs not as they are ingested but as the metabolites that reach target tissues.

7.4 Excretion

As predicted by Bravo [1998], the non-extractable polyphenols, such as polymeric PACs are relatively inert in the digestive tract and recovered extensively in the feces. However there is evidence that polymeric PACs may undergo degradation by colonic microflora. When incubated *in vitro* under anoxic conditions using non-labeled and ^{14}C-labeled purified PAC polymers, Deprez *et al.*, [2000] noted that after 48-hours, the polymers were almost totally degraded. The result indicated that these high molecular weight compounds can be degraded by colonic microflora into low-molecular weight aromatic acids which differ according to their hydroxylation profile and the length of the aliphatic side chain. Phenylacetic, phenylpropionic, and phenylvaleric acids, monohydroxylated mainly in the *meta* or *para* position were identified as metabolites by GCMS. All of the aromatic acids identified as metabolites of polymeric PACs produced by colonic microflora were similar to those produced my colonic microflora metabolism of (+)-catechin or procyanidin dimer B_3. Deprez *et al.*, suggested that in order to further understand the nutritional properties of dietary PACs, it will be necessary to study not only their biological properties but also those of their metabolites.

Evidence of the absorption and metabolism of polyphenols from the gut exists, but less is known about the efficiency of such uptake and the permanence of phenolic compounds or their conjugates and derivatives in the body. Animal studies with ^{14}C-labeled phenolics indicate that only partial absorption takes place [Bravo, 1998]. The

percentage of the flavonoid excretion compared to potential absorption in the gut seemed to vary according to the nature of the compound. No data were available for catechin, epicatechin, or oligomeric PACs.

8.0 PRECLINICAL TOXICOLOGICAL SAFETY

Chung *et al.* [1998b] reviewed the reported health benefits and risks associated with consumption of tannic acid. Though the majority of their review focused on the effects of the hydrolyzable tannins, there was some discussion related to condensed tannins including catechin and epicatechin. They note that many of the anti-nutritional properties associated with tannins result from hydrolyzable, not condensed, tannins. In addition, though tannins have been reported to interact with proteins, catechins and epicatechins would be too small to have such an effect. A minimum of 350 D is required for this activity, and this size is not reached in monomeric flavonoids. They also note that certain polyphenols, such as quercetin, have been found to exhibit suspected carcinogenic activity in some studies, though other studies have shown an anticarcinogenic effect. It is therefore important to note that polyphenols are a diverse group of compounds capable of inducing different responses in different models. However, the overall finding for the PACs found in the MegaNatural™ GSKE and GSE products indicate that they do not pose a safety concern for those consuming them. The following discussion supports this conclusion.

8.1 Genotoxicity

Mutagenic as well as anti-mutagenic activity has been reported for individual polyphenolic compounds. Literature on the Ames and other assays indicated some positive response for quercetin compounds. These data are discussed in section 8.1.2. These individual compounds are present in the MegaNatural™ Gold GSE and GSKE products only in low concentrations. However, a mouse micronucleus assay was conducted on the actual GSE / GSKE product, confirming an absence of mutagenic activity.

8.1.1 *Mutagenicity Evaluation of MegaNatural™ GSE and GSKE*

MegaNatural™ GSE and GSKE products were evaluated in a mouse micronucleus assay to determine their potential for *in vivo* clastogenic activity and or for the disruption of the mitotic apparatus by quantifying micronuclei in polychromatic erythrocyte (PCE) cells in the bone marrow of Crl:CD-1 mice. For each assay, GSE or GSKE was dissolved in 0.5% carboxymethylcellulose (CMC) and administered by oral gavage to six males per dose level per harvest time point. Dose levels of the two test articles were 500, 1000, and 2000 mg/kg, with euthanization scheduled for 24 and 48 hours post-dosing to allow for bone marrow harvest. In addition, 12 animals received the CMC vehicle control and an additional 6 animals received a cyclophosphamide positive control. The complete dosing scheme for the micronucleus assay is presented below in Table 8.1.

Table 8.1 Dosing Scheme for the Mouse Micronucleus Assay

Target Treatment (mg/kg)	Stock Concentration (mg/ml)	Dosing Volume (ml/kg)	Males / Harvest Time Point		Replacement Males*
			24-Hour	48-Hour	
Vehicle Control: 0.5% carboxymethylcellulose	0	20	6	6	-
Positive Control: cyclophosphamide	8	10	6	-	-
MegaNatural™ GSE					
500	25.0	20	6	-	-
1000	50.0	20	6	-	-
2000	100	20	6	6	3
MegaNatural™ GSKE					
500	25.0	20	6	-	-
1000	50.0	20	6	-	-
2000	100	20	6	6	3

* The animals in the secondary group were dosed as potential replacements for the original high-dose group. Animals not used as replacements were euthanized at the completion of study.

All animals were examined for signs of toxicity and or mortality immediately after dosing, after approximately one hour, and again daily for the duration of the assay. One animal in the 2000 mg/kg high dose GSE test group was found dead at the 1-hour post dose check. No further signs of clinical toxicity were observed in any of the remaining animals at any dose level. At the appropriate harvest time points, the animals were euthanized and their tibias removed for marrow extraction. Slides were prepared from the bone marrow collected from five animals per group at each time point and scored for micronuclei and PCE to NCE (normochromatic erythrocyte) ratio. The micronucleus frequency was determined by analyzing the number of micronucleated PCE's out of a minimum of 2000 PCE's per animal.

The MegaNatural™ GSE was determined to have induced a statistically significant decrease in the PCE:NCE ratio at the 48-hour time point in the 2000 mg/kg high dose group and was considered to present evidence of cytotoxic activity in the mouse bone marrow. This indicates that the dose was reaching the bone marrow target tissue at a high enough concentration to affect the cells and therefore validates the study. No statistically significant increase in micronucleated PCE's was observed at any dose level or harvest time point. The MegaNatural™ GSKE induced no signs of clinical toxicity in any of the treated animals, nor was it cytotoxic to the bone marrow at either time point. GSKE did not induce any statistically significant increase in mouse micronucleated PCE's at any dose level or harvest time point. A summary of the study findings is presented below in Table 8.2. It was concluded that the test articles, MegaNatural™ GSE and MegaNatural™ GSKE, were negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

Table 8.2 Micronucleus Data Summary

Treatment	Dose	Harvest Time (hr)	% Micronucleated PCE's (±SE)	Ratio of PCE:NCE (Mean ±SE)
Vehicle Control	0.5% CMC	24-hours	0.04±0.02	0.53±0.03
		48-hours	0.05±0.02	0.79±0.09
Positive Control	80 mg/kg	24-hours	2.19±0.20*	0.41±0.03**
MegaNatural™ GSE	500 mg/kg	24-hours	0.02±0.01	0.61±0.06
	1000 mg/kg	24-hours	0.02±0.01	0.76±0.14
	2000 mg/kg	24-hours	0.04±0.03	0.55±0.06
		48-hours	0.06±0.02	0.44±0.44**
MegaNatural™ GSKE	500 mg/kg	24-hours	0.04±0.03	0.84±0.06
	1000 mg/kg	24-hours	0.01±0.01	0.75±0.07
	2000 mg/kg	24-hours	0.05±0.03	0.50±0.05
		48-hours	0.03±0.02	0.68±0.08

* Significantly greater than the corresponding vehicle control, $p<0.01$.
** Significantly less than the corresponding vehicle control, $p\leq0.05$.

8.1.2 Literature Evaluation of Polyphenolic Compound Mutagenicity Studies

Several studies have been reported which examined the mutagenic activity of fractions isolated from extracted wine polyphenols. This activity was reportedly associated with the presence of quercetin compounds in selected fractions. In an attempt to isolate and identify any mutagens found in red wine, an organic extraction process was employed to remove as much of the polyphenols from the red wine as possible [Yu *et al.*, 1986]. The resultant extract was then evaporated and extracted with methanol and evaporated to a final volume of 25 ml. Histidine auxotrophic strains TA98 and TA100 of *Salmonella typhimurium* were employed in conjunction with human fecal glycosidases in the presence or absence of S9 rat liver microsomes. In the initial screening, the phenolic extract was tested over a 3-log concentration range in the presence of S9 and fecalase in both strains. Revertant rates twice that of the spontaneous reversion rate were obtained in both strains. The mutagenicity of the phenolic extract exhibited a dose

response effect over a narrower range of concentrations. Using a Sephadex LH-20 chromatographic column, the phenolic extract was then fractionated further into 3 fractions FrI, FrII, and FrIII. Each fraction was then tested in strains TA98 and TA100 again with fecalase in the presence or absence of S9. FrIII was significantly more mutagenic in TA98 in the presence of fecalase than the other fractions and was selected for further fractionation. Employing multi-layer coil counter-current chromatography, FrIII was broken down into 5 subfractions designated FrIII-1 through FrIII-5. Previous observation had determined TA98 to be the more sensitive indicator of mutagenicity of the wine components and the five FrIII fractions were then tested in TA98. Most of the mutagenic activity of FrIII was essentially recovered in FrIII-4. Concentration and UV spectrographic analysis determined the mutagenic component to be rutin (3-rhamnoglucosyl quercetin).

In a second *S. typhimurium* mutagenesis assay, the mutagenic potential of the substances found in red and white wines of Chile were examined because of the high risk of gastric cancer in the region [Bull *et al.*, 1987]. Samples of 50 - 200 mL of wine were concentrated to one tenth of their volume by lyophilization. An equivalent of 10 mL wine was subsequently extracted in 20 volumes of chloroform:methanol (2:1) followed by addition of 5 volumes of water. Both the upper methanolic and lower chloroformic phases were then evaporated at room temperature under nitrogen and the residue was dissolved in 1 ml of dimethyl sulfoxide (DMSO). During preliminary evaluation, substances in the chloroform phase displayed no mutagenic activity. Therefore, only the methanolic phase was employed in the later experiments. The mutagenicity assays were conducted using the methanolic phase in *Salmonella typhimurium* strains TA98 and TA100, both with and without metabolic activation in the form of S9 rat liver fraction microsomes. The results indicated that the fraction isolated from red wines were twice as mutagenic as the fraction from white wines in TA98, whereas activity was about equal in strain TA100. A linear relationship was observed between mutagenic activity and the amount of wine extractant added up to 1 ml. When the tests were conducted in the presence of S9 metabolic activation, mutagenic activity remained the same as that obtained without activation. In this study, no attempt was

made to determine which component found in the methanolic fraction taken from red wine was responsible for the mutagenic properties displayed in *S. typhimurium*. In the discussion of their results, the authors cited research postulating the mutagen may be a glycoside of mutagenic flavanols such as quercetin. This would be consistent with the findings of Yu [1986], discussed previously.

An overview of the beneficial and hazardous effects of phenolic compounds conducted by Stich [1991] indicated that the large variety of phenolic compounds in nature may posses multiple functions and thus some may act as antimutagens, anticarcinogens, and antipromoters, whereas others may act as mutagenic, carcinogenic, and promoting agents. Choi *et al.*, [1994] investigated the mutagenic and antimutagenic activities of several flavonoids, including (+)-catechin and (-)-epicatechin using the Ames test. Both (+)-catechin and (-)-catechin demonstrated a dose-dependent antimutagenic activity against aflatoxin B_1, with a 5.0% solution of the flavonoids inhibiting mutagenicity by 89% and 77% respectively. On the other hand, Hayakawa *et al.*, [1999] reported that catechin, among other polyphenolic compounds, in conjunction with Cu^{2+}, facilitated DNA cleavage. At the molecular level, simple phenolics can induce double strand DNA breaks *in vitro*, as shown in Table 8.3 below [Stich, 1991].

Table 8.3 DNA-Breaking Activity of Phenyl Compounds *In Vitro*

Compound	Substitution Groups				DNA-Breaking Activity
	R1	R2	R3	R4	
Phenol	OH	H	H	H	-
Pyrocatechol	OH	OH	H	H	+++
Resorcinol	OH	H	OH	H	-
Hydroquinone	OH	H	H	OH	+++
Pyrogallol	OH	OH	OH	H	+++
Gallic Acid	OH	OH	OH	H	+++
Caffeic Acid	OH	OH	OH	H	+++
Ferulic Acid	OH	OH	H	H	+
Chlorogenic Acid	OHC_3	OH	H	H	+

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In a series of experiments designed to evaluate flavonoid action upon DNA, as both a mediator of antioxidant and pro-oxidant activity, Ohshima *et al.* [1998], examined the effects of 18 flavonoids upon the pBR322 plasmid isolated from calf thymus. In the first assay, the level of single-strand DNA breakage (SSB) was evaluated by incubation of the various flavonoids in the absence or presence of a NO releasing compound, diethylamine-NO. Incubation of the plasmid DNA with 0.1 mM of flavonoid alone for 30 minutes resulted in no significant increase in conversion of the covalently closed circular double-stranded super-coiled DNA (form I) to a relaxed open circle (form II), compared with that of the non-treated plasmid. Incubation with the flavonoid and DEA-NO, also at a concentration of 0.1 mM, increased the formation of form II significantly, although catechin and epicatechin were not regarded to have had a significant effect in this regard. The results of this assay were expressed as the mean number of SSB (±SD) per 10^4 base pairs of DNA after correcting for background and are shown below in Table 8.4.

Table 8.4 DNA Strand Breaks Induced by Flavonoids in Combination with an NO-Releasing Compound

Flavonoid	Single strand breaks per 10^4 bp DNA	
	None	DEA-NO
None	0	0
Apigenin	0	0.06±0.04
Baicalein	0.05±0.03	0.47±0.10
Catechin	0	0.12±0.10
Cyanidine chloride	0.06±0.05	0.37±0.12
Delphinidine chloride	0.10±0.07	0.49±0.05*
Epicatechin	0	0.08±0.02
Epicatechin gallate	0	0.14±0.04
Epigallocatechin	0	0.27±0.08
Epigallocatechin gallate	0	0.69±0.02*
Morin	0	0.14±0.12
Myricetin	0	0.99±0.02*
Quercetagetin	0.14±0.03	1.31±0.14*
Quercetin	0	0
Rutin	0	0
Caffeic Acid	0	0
Catechol	0.06±0.04	0.57±0.08*
Gallic acid	0	0.20±0.10
Pyrogallol	0.12±0.04	1.70±0.14*
Catechol in water**	0.06±0.04	1.54±0.02*
Pyrogallol in water**	0.18±0.04	2.92±0.04*

* Significant induction of SSB compared to nontreated DNA ($p<0.05$).
** The experiments were carried out in the absence of 0.1% DMF.

In a second assay conducted by Ohshima *et al.* [1998], 0.5 mM of peroxynitrite was added to a reaction mixture containing flavonoids prepared in dimethyl formamide (DMF) at a concentration of 0.05 mM or 0.5 mM. Each reaction was conducted in duplicate. After having run to completion, each reaction was dried and analysed for single strand breaks per 10^4 base pairs of DNA. It was determined that flavonoids

decreased peroxynitrite mediated SSB in a dose dependant manner as shown below in Table 8.5.

Table 8.5 Effect of Flavonoids on Peroxynitrite Mediated DNA Strand Breaks

Flavonoid	Inhibition (%) of Peroxynitrite Mediated SSB *	
	0.05 mM	0.5 mM
Apigenin	54	55
Baicalein	51	68
Catechin	85	97
Cyanidine	88	92
Delphinidine	29	86
Epicatechin	85	95
Epicatechin gallate	24	87
Epigallocatechin	34	70
Epigallocatechin gallate	64	93
Morin	4	57
Myricetin	49	82
Quercetin	42	73
Quercetagetin	22	88
Rutin	29	95
Caffeic Acid	91	97
Catechol	89	98
Gallic acid	71	93
Pyrogallol	73	89

* Peroxynitrite at 0.5mM induced formation of 3.69±0.10 SSB per 10^4 bp DNA in the presence of 0.1% DMF.

In a similar assay, the effect of exogenous flavonoids added at concentrations of 0.01, 0.1, and 1mM on the peroxynitrite mediated nitration of guanine in the plasmid DNA was evaluated. Most test compounds at the 1 mM concentration almost completely inhibited 8-nitroguanine formation. Samples were tested by HPLC for the presence of 8-nitroguanine or 8-oxoguanine. The levels of 8-nitroguanine and 8-oxoguanine in DNA formed with 0.1 mM peroxynitrite were 2.89±0.41 and 0.25±0.08 mM/M guanine, respectively. Decomposed peroxynitrite did not generate 8-nitroguanine but formed 8-

oxoguanine at the level of 0.08±0.01 mM/M guanine. Non-treated plasmid DNA contained 8-oxoguanine at the level of 0.05±0.01 mM/M guanine but no detectable 8-nitroguanine. The 8-oxoguanine levels in the present assay were not significantly higher in the calf thymus DNA plasmid treated with peroxynitrite than in DNA treated with decomposed peroxynitrite [Ohshima *et al.*, 1998]. Therefore, no clear effects, either acceleration or inhibition, of the tested flavonoids on the formation of 8-oxoguanine could be determined. The results of HPLC analysis of 8-nitroguanine are presented below in Table 8.6.

Table 8.6 Effect of Flavonoids on Peroxynitrite Mediated Formation of 8-nitroguanine

Flavonoid	Inhibition (%) of Peroxynitrite Mediated Formation*		
	0.01 mM	0.1 mM	1 mM
Apigenin	8	36	77
Baicalein	33	92	100
Catechin	29	88	99
Cyanidine	40	78	100
Delphinidine	20	85	100
Epicatechin	15	79	97
Epicatechin gallate	19	100	100
Epigallocatechin	11	86	94
Epigallocatechin gallate	15	98	100
Morin	21	74	100
Myricetin	32	89	99
Quercetin	24	95	100
Quercetagetin	15	100	100
Rutin	23	95	100
Caffeic Acid	43	90	98
Catechol	29	96	98
Gallic acid	12	95	100
Pyrogallol	18	93	100

* Results are presented as percentage of control.

Heo *et al.* [1992] investigated the mutagenic and the anti-clastogenic effects of 5 groups of flavonoids, including (-)-epicatechin, in bone-marrow-polychromatic erythrocytes of ICR mice. Mice were given 0.1 mg/kg, 1.0 mg/kg, or 10.0 mg/kg of the respective flavonoids for five days to test the mutagenic potential of each compound. On the fifth day, 150 mg/kg of the carcinogen benzo[a]pyrene was injected intraperitoneally into half of the mice at each treatment level in order to test the anti-clastogenic activity of the flavonoids. The mice were then sacrificed and preparations of bone marrow were carried out to allow for observation of the frequency of micronucleated polychromatic erythrocytes (MPCEs). Epicatechin and catechin, like almost all of the other flavonoids, did not induce a significant increase in MPCEs as compared to controls. The only flavonoid that demonstrated significant clastogenic activity was quercetin. In addition, most of the flavonoids, including (-)-epicatechin, were able to suppress benzo[a]pyrene-induced MPCEs, though (-)-epicatechin did so less potently than the others. Similarly, epicatechin was able to reduce the clastogenic effects of two other carcinogens, ethyl methanesulfonate and 7,12-dimethylbenz[a]anthracene.

At the cellular level, several simple phenolic compounds which are absent or present only at very low levels in the MegaNatural™ GSE and GSKE have proven to be inducers of clastogenic activity through induction of chromosome / chromatid aberrations and sister-chromatid exchanges, as indicated in Table 8.7. This clastogenic activity has not always been matched by an equally strong mutagenic effect in the *Salmonella typhimurium* assay.

Table 8.7 Clastogenic Activity of Monomeric Phenolic Compounds in Chinese Hamster Ovary (CHO) Cells

Compound	Concentration (mg/ml)	Metaphases with Chromosome Aberrations* (%)	Chromatid Breaks per Cell	Chromatid Exchanges per Cell
Catechol	0.05	23.5	0.17	0.67
4-methyl catechol	0.01	20.0	0.10	0.73
Resorcinol	1.6	14.3	0.08	0.95
Phloroglucinol	3.0	10.1	0.03	0.21
Pyrogallol	0.1	22.6	0.14	1.08
p-Hydroxybenzoic acid	25.0	0.0	0.00	0.00
Protocatechuic acid	3.0	20.5	0.04	0.87
Vanillic acid	25.0	4.8	0.02	0.07
Gallic acid	0.05	24.1	0.03	0.70
Syringic acid	3.0	38.6	0.17	1.44
Salicylic acid	25.0	1.5	0.02	0.00
3,4-Dihydroxyphenylacetic acid	0.1	18.5	0.01	1.23
p-Coumaric acid	6.0	3.0	0.01	0.08
Caffeic acid	0.2	33.3	0.08	3.08
Ferulic acid	25.0	7.8	0.12	0.15
Eugenol	0.4	21.5	0.08	0.20

* Concurrent control frequencies for cells receiving culture medium only were 0.7% metaphase plates with chromosome aberrations, with an average of 0.01 breaks per cell and 0.00 exchanges per cell.

The genotoxic activity of phenolic compounds is strongly influenced by a host of modulating factors including varying sensitivities of the different bioassays to the electrophilic metabolites of the phenolics or to reactive compounds generated. However, the *in vitro* bioassays which have been applied to uncover mutagenic and, clastogenic properties of phenols can also be applied to reveal their anti-genotoxic capacities. Phenolic compounds have been shown to inhibit the genotoxic action of a variety of compounds with modes of action that differ widely.

Of particular relevance was a study by Fukuhara *et al*, [1981], which focused on the inhibitory action of phenolics on the mutagenicity of mixtures to which human population

groups are actually exposed, particularly during the pyrolysis of the protein albumin at different temperatures and under nitrogen or air atmosphere in the TA98 strain of *S. typhimurium*. Catechin, as well as chlorogenic acid, pyrocatechol, quercetin, tannic acid, and propyl gallate were all evaluated at 20, 50, 100, or 150μl per plate. Evaluations were also conducted both in the presence of absence of 50μl of S9 liver microsomal fraction. Overall, the polyphenols reduced the mutagenic activity of albumin pyrolyzates to 20-76% that of albumin without additives. In the presence of S9, the mutagenic activity of pyrolyzates of albumin was reduced by the addition of quercetin and catechin at 20, 50, 100, and 150 μl per plate. Some reduction of mutagenicity was observed in the other polyphenols tested.

Toering *et al.* [1996] investigated the ability of (+)-catechin to inhibit plant activation of 4-nitro-*o*-phenylenediamine (NOP) into a mutagenic form and the mechanism through which this is accomplished. Activation of NOP by both the intact plant cell and isolated horseradish peroxidase systems was inhibited by 10 mM (+)-catechin, as was peroxidase, though not as strongly. In addition, (+)-catechin inhibited the direct mutagenicity of NOP in *Salmonella*. Based on these findings, researchers speculated that (+)-catechin inhibits mutagenicity by inhibiting the activity of *O*-acetyltranserase, an enzyme involved in the induction of mutations.

Teal and Castonguay [1992] also investigated the anti-mutagenic effect of polyphenols, including catechin, when the TA1535 strain of *S. typhimurium* was exposed to another carcinogen to which humans are exposed, 80 mM of nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The anti-mutagenic efficacies were dose-related between the non-toxic concentrations of 0.1 and 0.5 mmol/dish. At the highest dose, 0.5 mmol, catechin inhibited mutagenesis at a rate of 62%. The overall results of this study indicated that polyphenolic compounds might inhibit the activation of NNK, suggesting the polyphenolic compounds could prevent the initiation phase of the carcinogenic process.

Research by Apostolides [1997] has indicated that polyphenolic compounds may also be effective inhibitors of PhIP, 2-amino-1-methyl-6-phenylimidazo[4,45-*b*]pyridine, which is an important procarcinogen produced during the frying and broiling of fish and meat. The mutagenicity for 10μM of PhIP in *S. Typhimurium* strain TA98 in the presence of S9 metabolic activation was inhibited in a dose-dependent manner by gallate ester of catechin and by tannic acid. The gallate esters of catechin and the tannic acid had low IC_{50} values whereas catechins, methyl gallate, and gallic acid showed no inhibition in this concentration range. The IC_{50} values in this assay were inversely proportional to the molecular weight of the gallate esters, as displayed in Table 8.8.

Table 8.8 Inhibition of Mutagenicity caused by 10μM PhIP by Polyphenolic Compounds

Compound	Molecular Weight	IC_{50} (μM)
Catechin	290	>1000
Epicatechin	290	>1000
Epicatechin gallate	306	>1000
Epigallocatechin gallate	442	500
Monogallates	458	700
Gallic Acid	188	>1000
Methyl gallate	203	>1000
Tannic acid	1701	60

Monteith [1990] investigated the effect of catechin on the metabolism and DNA-binding capabilities of 2-acetylaminofluorene (AAF), a known hepatocarcinogen in rats and a mutagen in microbial assays. Rat parenchymal hepatocytes were isolated from male Sprague-Dawley rats and were cultured in collagen-coated dishes in media containing catechin (1.0, 0.1, or 0.01 mM) in ethanol. At the two lowest doses, catechin significantly increased AAF metabolism (8%, $p<0.025$) by rat hepatocytes, while a 6% reduction in AAF metabolism was seen with a treatment of 1.0 mM catechin. In addition, treatment with this highest dose resulted in a 50% reduction in the conjugation of metabolites, a 53% reduction in the ratio of water-soluble to organic soluble metabolites, and increased binding of AAF metabolites to DNA at a level 1.8 times

above controls. In addition, treatment with catechin approximately doubled the amount of N-OH-AFF metabolite, a carcinogen that was produced by the hepatocytes, and also affected the levels of several of the other AAF metabolites. Researchers also examined the mutagenic ability of catechin in incubations of hepatocytes and *Salmonella typhimurium* cells and observed that catechin itself was not mutagenic at the concentrations tested (0.5, 1.0, and 5.0 mM) and that at concentrations of 1.0 and 5.0 mM it was able to inhibit the mutagenicity of AAF. However, a significant enhancement of AAF-induced mutagenicity was seen with a dose of 0.5 mM catechin.

8.2 Subchronic and Chronic Toxicological Safety

8.2.1 Subchronic Evaluation of MegaNatural™ GSE and GSKE

A 3-month study was designed to specifically assess the potential for toxicity of the MegaNatural™ line of Gold GSE and GSKE products when administered orally via a dietary admixture to Sprague-Dawley CD rats (20/sex/group). A summary of this study is provided in the experimental outline as shown in Table 8.9.

Table 8.9 Experimental Outline

Group	Test Article	Dose Level (%)	Number of Animals							
			Total		1-Month & Termination Clinical Laboratory Studies		Week 13 Necropsy		Microscopic pathology	
			M	F	M	F	M	F	M	F
1	Control	0	20	20	10	10	20	20	20	20
2	GSE	0.63	20	20	10	10	20	20	-	-
3	GSE	1.25	20	20	10	10	20	20	-	-
4	GSE	2.50	20	20	10	10	20	20	20	20
5	GSKE	2.50	20	20	10	10	20	20	20	20

Dietary concentrations of 0.63% GSE, 1.25% GSE, 2.5% GSE, and 2.5% GSKE were administered over the 3-month treatment schedule. This correlates to nominal dose concentrations of 6,300, 12,500, 25,000, and 25,000 ppm, respectively. A negative control group of 20 animals received an untreated standard laboratory diet. Analysis of the diet mixtures showed that homogeneous mixtures were produced and that the test articles were stable in the rodent chow diet for 2-weeks. Measured mean concentrations of the test articles during the study ranged from 96% to 106% of nominal concentrations.

Throughout the course of the study, viability checks were performed twice daily to check for mortality and signs of toxic or pharmacological effect. Physical observations and body weight measurements were performed twice pretest and weekly during the study period. All animals survived throughout the study and while some findings were noted across all treatment groups, none were considered to be compound related.

Feed consumption was measured once pretest and then weekly. Body weight gains were similar for all groups. Feed consumption differences were noted early in the study. From day 7, male animals receiving 2.5% GSE or GSKE had increased food consumption by approximately 10%, which was considered small but statistically significant. This elevated consumption continued throughout the length of the study and may have reflected increased dietary intake to compensate for the high concentration of test articles in the diet. Feed consumption in the female treatment groups remained similar to control throughout the study. Table 8.10 lists test article intake.

Table 8.10 Test Article Intake (mg/kg/day)

Dose Groups		Day 7	Day 91	Mean
Males	0.63% GSE	671	323	434
	1.25% GSE	1308	658	860
	2.50% GSE	2800	1333	1788
	2.50% GSKE	2801	1320	1778
Females	0.63% GSE	751	436	540
	1.25% GSE	1499	841	1052
	2.50% GSE	3026	1677	2167
	2.50% GSKE	2997	1728	2111

Ophthalmoscopic examinations were performed pretest and weekly during the study. Hematology, coagulation studies, and clinical chemistry were performed on 10 animals/sex/group at one month and at study termination. No effect upon any of these parameters was observed during the consumption of GSE and GSKE.

After at least 92-days of treatment, all animals were sacrificed and a complete macroscopic postmortem examination was conducted. Selected organs were then weighed. No treatment-related abnormalities attributable to either test article were noted. Histopathological examination of selected tissues was conducted upon all animals in the control and two high-dose groups. In those animals receiving 2.5% GSE dietary supplementation, no microscopic lesions attributable to treatment were observed.

In male rats receiving 2.5% GSKE, a significant ($p<0.05$) increase in the occurrence of a common renal cortical inflammation, comprised predominantly of lymphocytic interstitial infiltrates, was observed in 11 of 20 animals compared to the occurrence in 4 of 20 control animals. The severity in most cases was minimal. This increased frequency was not observed in female rats of the same treatment group. However, this lesion is commonly seen in male rats, increasing in frequency and severity with age. It is considered a component of the entity of chronic nephropathy and therefore not

treatment related. This is corroborated by a comparison with research facility 3-month study historical control data gathered over a period between 2000-2001 and provided below in Tables 8.11, 8.12, and 8.13. Male rats in studies 00-2683 and 00-6126, conducted contemporaneously with the GSE / GSKE study, also experienced renal cortical inflammation in 40% (8/20) and 50% (5/10) of animals, respectively.

Table 8.11 Historical Control Data - Study Data

Study Number	Length of Study	Initial No. On Test (No./Sex)	Route of Administration	Age at Initiation	Initiation Date	Termination Date	Species	Anesthetic
99-2615	3 M	20	Diet	6 W	14-Oct-99	21-Jan-00	CD®	CO_2
99-2628	3 M	10	Dermal	8 W	11-Nov-99	11-Feb-00	CD®	CO_2
99-2610	3 M	20	Diet	6 W	22-Feb-00	22-May-00	CD®	CO_2
99-2643	3 M	10	Diet	6 W	2-Mar-00	2-Jun-00	CD®	CO_2
00-2651	13 W	20	Gavage	6 W	28-Mar-00	30-Jun-00	CD®	CO_2
00-2658	90 D	10	Gavage	6 W	21-Aug-00	22-Nov-00	CD®	CO_2
00-2683	3 M	20	Diet	44 D	20-Feb-01	25-May-01	CD®	CO_2
00-6126	13 W w/4W	10	Inhalation	6 W	06-Feb-01	08-May-01	CD®	CO_2

All rats supplied by Charles River of Kingston, NY.

Table 8.12 Historical Control Data - Male Rats

Microscopic Findings: Kidney	TOTAL	Individual Study Data - Male Animals							
Month/Year of Death		Jan-00	Feb-00	May-00	Jun-00	Jun-00	Nov-00	May-01	May-01
Study Number		99-2615	99-2628	99-2610	99-2643	00-2651	00-2658	00-2683	00-6126
Number Examined	115	15	10	20	10	20	10	20	10
M-nephroblastoma	0	0	0	0	0	0	0	0	0
congestion	0	0	0	0	0	0	0	0	0
acute/subacute inflammation	0	0	0	0	0	0	0	0	0
lymphoid cell infiltrates	1	0	0	1	0	0	0	0	0
acute/subacute pyelitis	0	0	0	0	0	0	0	0	0
subacute(chronic active)/ chronic pyelitis	2	0	0	1	0	1	0	0	0
interstitium: subacute/chronic inflammation	1	0	0	0	0	0	1	0	0
interstitial chronic inflammatory cell infiltrate	0	0	0	0	0	0	0	0	0
cortical scar(s)	1	0	1	0	0	0	0	0	0
cortex: subacute(chronic active)/chronic inflammation	13	0	0	0	0	0	0	8	5
cortex:lymphoid cell infiltrates	0	0	0	0	0	0	0	0	0
cortex:interstitial lymphocytic infiltrates	0	0	0	0	0	0	0	0	0
cortex: lymphoid cell aggregate(s)	7	1	1	0	1	1	3	0	0
cortex:infarct(s)-healing/ healed	2	0	0	1	0	1	0	0	0
cortical tubular basophilia	1	0	1	0	0	0	0	0	0
cortex:proximal convoluted tubular epithelium-basophilic	9	0	0	0	0	0	0	4	5
cortex:proximal convoluted tubular epithelium-eosinophilic/hyaline droplets	0	0	0	0	0	0	0	0	0
cortex:convoluted tubular epithelium-hyperplasia	0	0	0	0	0	0	0	0	0
cortex:convoluted tubules-dilated	1	0	0	0	0	1	0	0	0
cortex/corticomedullary junction:subacute/chronic inflammation	2	0	0	0	0	2	0	0	0
cortex/corticomedullary junction: fibrosis	1	0	0	0	0	1	0	0	0
cortex/corticomedullary junction:mineral deposit(s)	4	0	0	2	0	1	1	0	0
cortex/cortico-medullary junction:tubule(s) dilated	1	0	0	0	0	0	1	0	0
medulla:fibrosis	1	0	0	1	0	0	0	0	0
medulla:mineral deposit(s)	9	2	0	3	2	1	1	0	0
medulla: tubular lumen(s)-granular cast(s)	1	0	0	0	0	1	0	0	0
medulla:tubule(s) dilated/cyst(s)	12	3	0	4	2	3	0	0	0
tubular cyst(s)	1	0	1	0	0	0	0	0	0
pelvis:dilated	8	2	0	0	2	4	0	0	0
pelvic calculi	0	0	0	0	0	0	0	0	0
pelvis:mineral deposits	1	1	0	0	0	0	0	0	0
pelvis: inflammatory cells/cell debris	0	0	0	0	0	0	0	0	0
urothelium:hyperplasia	3	2	0	0	0	1	0	0	0
urothelial/suburothelial mineral deposit(s)	0	0	0	0	0	0	0	0	0
suburothelial mononuclear cell infiltrate	1	0	0	0	0	0	0	1	0
proximal tubular epithelium:mallory-heidenhain staining material	10	0	0	0	0	0	0	0	10

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Table 8.13 Historical Control Data - Female Rats

Microscopic Findings: Kidney	TOTAL	Individual Study Data - Female Animals							
Month/Year of Death		Jan-00	Feb-00	May-00	Jun-00	Jun-00	Nov-00	1-May	1-May
Study Number		99-2615	99-2628	99-2610	99-2643	00-2651	00-2658	00-2683	00-6126
Number Examined	114	15	10	20	9	20	10	20	10
M-nephroblastoma	1	1	0	0	0	0	0	0	0
congestion	0	0	0	0	0	0	0	0	0
acute/subacute inflammation	0	0	0	0	0	0	0	0	0
lymphoid cell infiltrates	0	0	0	0	0	0	0	0	0
acute/subacute pyelitis	0	0	0	0	0	0	0	0	0
subacute(chronic active)/ chronic pyelitis	0	0	0	0	0	0	0	0	0
interstitium: subacute/chronic inflammation	0	0	0	0	0	0	0	0	0
interstitial chronic inflammatory cell infiltrate	0	0	0	0	0	0	0	0	0
cortical scar(s)	0	0	0	0	0	0	0	0	0
cortex: subacute (chronic active)/chronic inflammation	2	0	0	0	0	0	0	1	1
cortex:lymphoid cell infiltrates	1	0	0	0	0	1	0	0	0
cortex:interstitial lymphocytic infiltrates	0	0	0	0	0	0	0	0	0
cortex: lymphoid cell aggregate(s)	1	0	1	0	0	0	0	0	0
cortex:infarct(s)-healing/ healed	0	0	0	0	0	0	0	0	0
cortical tubular basophilia	1	0	1	0	0	0	0	0	0
cortex:proximal convoluted tubular epithelium-basophilic	2	0	0	1	0	0	0	0	1
cortex:proximal convoluted tubular epithelium-eosinophilic/hyaline droplets	0	0	0	0	0	0	0	0	0
cortex:convoluted tubular epithelium-hyperplasia	0	0	0	0	0	0	0	0	0
cortex:convoluted tubules-dilated	0	0	0	0	0	0	0	0	0
cortex/corticomedullary junction:subacute/chronic inflammation	0	0	0	0	0	0	0	0	0
cortex/corticomedullary junction: fibrosis	0	0	0	0	0	0	0	0	0
cortex/corticomedullary junction:mineral deposit(s)	29	2	1	13	2	2	2	1	6
cortex/cortico-medullary junction:tubule(s) dilated	2	0	0	0	0	2	0	0	0
medulla:fibrosis	0	0	0	0	0	0	0	0	0
medulla:mineral deposit(s)	14	1	0	5	3	1	4	0	0
medulla: tubular lumen(s)-granular cast(s)	0	0	0	0	0	0	0	0	0
medulla:tubule(s) dilated/cyst(s)	2	0	0	0	1	0	1	0	0
tubular cyst(s)	0	0	0	0	0	0	0	0	0
pelvis:dilated	0	0	0	0	0	0	0	0	0
pelvic calculi	0	0	0	0	0	0	0	0	0
pelvis:mineral deposits	1	1	0	0	0	0	0	0	0
pelvis: inflammatory cells/cell debris	0	0	0	0	0	0	0	0	0
urothelium:hyperplasia	2	1	0	0	0	0	1	0	0
urothelial/suburothelial mineral deposit(s)	4	2	0	0	0	1	1	0	0
suburothelial mononuclear cell infiltrate	0	0	0	0	0	0	0	0	0
proximal tubular epithelium:mallory-heidenhain staining material	0	0	0	0	0	0	0	0	0

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The significance of the increased occurrence of this spontaneously occurring lesion in relation to 2.5% GSKE administration was uncertain. Overall, no consistent findings were noted for the lower doses of GSE and no consistent findings were noted for GSKE. There was no observed evidence of toxicity or adverse effects including effects on endocrine and reproductive endpoints. The overall results of this study supported a No-Observed-Effect Level (NOEL) of approximately 2150 mg/kg/day for administration of GSE and GSKE to female rats. In male rats the NOEL for GSE was approximately 1780 mg/kg/day and in GSKE a No-Observed-Adverse-Effect Level (NOAEL) was also determined to be approximately 1780 mg/kg/day. These values represent the time-weighed mean dose rates occurring in the high-treatment groups over the course of the study. Employing a 100-fold safety factor, the observed NOEL of GSE and GSKE can be correlated to a safe human dose of 1075 mg/day for an average 50 kg female. Employing the observed NOEL and NOAEL of GSE and GSKE, respectively, the safe dose for an average 70 kg human male can be correlated to 1250 mg/day.

8.2.2 Literature Review of Non-Clinical Studies Evaluating Polyphenolic Compounds

8.2.2.1 Safety and Nutritional Studies

In a 31-day study, grape seed tannins were incorporated into a protein free diet administered to 3 groups of 6 Sprague-Dawley rats at levels of 0.2% or 2.0 % in comparison to a control diet [Vallet *et al.*, 1994]. No significant observations were noted in the group receiving 0.2% tannins. In the high dose group, growth was decreased by approximately 13% compared to control. Food intake and therefore nitrogen, fat, and starch intake remained identical in all three groups due to pair feeding. However, in the highest dose group, enhanced fecal dry weight of approximately 27% percent compared to control and increased fecal nitrogen excretion of 50% compared to control, were observed. No changes in organ weights were observed. This study did not reveal significant toxic effects in the rats except for reduced nitrogen and dry matter digestibility leading to slightly reduced growth levels in the highest dose group. This

was thought to be a result of the dietary effect of the condensed tannins within the intestine. The tannins formed less digestible complexes with dietary proteins. They also were shown to directly interfere with mucosal proteins, stimulating the desquamation phenomenon. This observation was corroborated by the increased loss of fecal endogenous nitrogen.

In another rat feeding study, 4 groups of 12 male Sprague-Dawley rats received a normal diet, a hypercholesterolemic diet, or a hypercholesterolemic diet supplemented with 2% grape seed PAC monomers or 2% grape seed PAC polymers, over a course of either 3 or 9 weeks [Tebib, *et al.*, 1994]. The intent was to determine the effect dietary grape seed PAC administration would have upon rat plasma lipoproteins, lipoprotein lipase, hepatic lipase, and aortic and hepatic lipid concentration. Although the goals of this study were predominantly geared towards nutritional endpoints, certain observations can be made about the safety of grape seed PAC monomers and polymers from the results provided. No fatalities or adverse effects attributable to PAC monomer or polymer administration were reported. Weight gain and body fat content in the dose groups receiving a hypercholesterolemic diet were significantly higher in the positive control diet and the diet containing monomers than in the polymer group. This elevated weight gain was attributed to enhanced lipogenesis due to the high lipid level in the diets. The addition of polymeric PACs resulted in weight and body fat content and concentration similar to that seen in the normal dietary group. The results of this study indicated that dietary polymeric grape seed PACs reduced plasma total cholesterol, triacylglycerol and LDL cholesterol concentrations. Significantly lower concentration of plasma VLDL and greater concentration of plasma HDL cholesterol were also observed compared to the control group. In rats fed monomers, lowered triacylglycerol and VLDL concentrations were observed but no effect on HDL or LDL cholesterol concentrations were observed.

Tebib *et al.*, [1995] conducted a similar study in 3 groups of 6 male Sprague-Dawley rats over a 12-week period. A purified diet was provided to a control group whereas the two test groups were pair-fed either monomeric or polymeric grape seed PACs. The

PAC concentration of the diets supplying either monomers or polymers was set at 71 mg/kg diet and was related to the daily amount of PACs ingested by a human adult consuming 0.5 L red wine. The purpose of the study was to examine the effects of tannins on cecal fermentation and colonic bacterial enzymes. The results indicated no significant difference in daily food intake due to the pair feeding technique. However, control rats and those fed monomers had greater weight gain than those rats fed polymers. Cecal weights did not differ between the experimental groups, whereas cecal wall weight and cecal pH was decreased in those rats receiving polymeric tannins in their diet. In this study, all of the bacterial enzyme activities tested were reduced after administration of polymeric tannins in the diet. The authors suggested this could be explained by enhancement of the nitrogen in the colonic content, leading to a dilution of the enzymatic activities. This was regarded as a potentially beneficial result as these same enzymes are responsible for the hydrolysis of glycosides and glucuronides and the reduction of nitro-compounds, which can lead to the generation of toxic and carcinogenic compounds.

8.2.2.2 Antitumor Activities

Zhao *et al.* [1999] investigated the antitumor promoting effect of the polyphenolic fraction of grape seed extract and the antioxidant activity of several of the individual polyphenols isolated from the extract in a two-stage initiation-promotion model. The polyphenols isolated from the grape seed extract were flavan-3-ol derivatives and included both catechin and epicatechin. To promote tumors, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was used in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated SENCAR mouse skin. Three groups, each containing 20 mice, were treated with 10 μg of DMBA in acetone on the dorsal shaved skin to initiate tumor growth. Two of these groups were then pretreated with 0.5 and 1.5 mg of grape seed polyphenols (GSP) in 0.1 ml of acetone applied to dorsal skin, with the third group receiving 0.1 ml acetone as a control. Two other groups, one with mice receiving 0.1 ml of acetone alone, and one group treated with DMBA and GSP but without the tumor promoter, also served as controls. The TPA was then applied at a dose of 2 μg in 0.1 ml of acetone to animals in

the first three groups. Treatments with GSP and/or TPA were repeated twice a week for twenty weeks. Pretreatment with GSP was found to significantly inhibit TPA tumor promotion in the SENCAR mouse skin, as tumor incidence, tumor multiplicity, and tumor volume were all significantly lower than in the groups treated with DMBA and TPA alone. At ten weeks, treatment with 0.5 mg of GSP and 1.5 mg of GSP inhibited incidence by 65% and 80% respectively while at twenty weeks, 35% and 65% inhibition were observed. ($p<0.001$, χ^2 test). In addition, tumor volume reduction of 61-83% and 50-87% was also observed in the GSP treated mice. The GSP itself did not promote tumor growth and topical application of GSP at doses of 0.5 mg or 1.5 mg did not result in any incidences of toxicity.

Individual polyphenols from the grape seed extract were then isolated. Both catechin and epicatechin were determined to occur at relatively low concentrations along with much higher concentrations of several other procyanidins. Exposure to tumor promoters such as TPA results in oxidative stress that can attack DNA and induce formation of lipid peroxides. Since antioxidant molecules such as catechin can potentially reduce this damage, researchers compared the antioxidant activities of some of the polyphenols isolated from the grape seed extract. Most notably, they found that catechin inhibited lipid peroxidation by TPA in a dose-dependent manner, with 0.3 mM resulting in 38% inhibition, 0.5 mM resulting in 61% inhibition, and 0.7 mM inhibiting lipid peroxidation by 90%. Treatment with 1.0 mM catechin completely inhibited lipid peroxidation. Polyphenols with a greater degree of polymerization demonstrated even greater activity in inhibiting lipid peroxidation. In addition, researchers compared several IC_{50}'s for the polyphenols found in grape seed extract and other known antioxidants. The IC_{50} of catechin was less than that of vitamins C and E but greater than the procyanidins found in the extract. Researchers note that dimers, trimers, and other oligomers of catechin and epicatechin were found among the GSP's and speculate that these compounds may have increased antioxidant activity and increased tumor inhibition potential as compared to the monomers investigated in this study.

Also looking into the ability of polyphenols to inhibit tumorigenesis, Gali-Muhtasib *et al.* [1998], examined the effect of tannins, including the condensed tannins catechin and epicatechin, on mouse epidermal cells exposed to UVB radiation. Exposure to radiation is known to stimulate sustained hyperplasia and DNA synthesis, an activity that is believed to be related to skin carcinogenesis in humans and is similarly correlated with the tumor-promoting activity of chemical promoters. Researchers determined the rate of DNA synthesis using pulse labeling and measuring the amount of ^{3}H-thymidine incorporated into the epidermal DNA of hairless mice following exposure to radiation and treatment with various tannins. Pretreatment, consisting of topical application of the catechin monomer prior to exposure to radiation, inhibited DNA synthesis by 27%. Treatment with a condensed tannin sample containing dimers, trimers, and polymers inhibited DNA synthesis by approximately 87%. Treatment with the tannins was only effective when applied a short time before exposure to radiation. The ineffectiveness of treatments much before or after exposure to radiation suggests that tannins do not directly block DNA synthesis and instead act on the mechanism by which UVB radiation stimulates DNA synthesis. The exact mechanism is unknown.

In two tumor models, Azuine and Bhide [1994] investigated the role of catechin in the chemoprevention of cancer using Syrian golden hamsters and Swiss mice. In the initial mouse assay, one group of 20 females received 0.41 mg/ml of catechin in their daily drinking water for eight weeks. Others groups of 20 females received the same amount of catechin, in addition to consuming a diet containing either 2% or 5% of turmeric, a known chemopreventive agent. Additional groups of 20 females each were established to receive no chemopreventive treatment or only a diet containing either 2% or 5% turmeric. Two weeks into the treatment, all groups of mice began receiving 1 mg benzo[a]pyrene (BP), a known carcinogen, in 0.1 ml of peanut oil by intragastric intubation twice a week for four weeks. Four additional control groups consisting of 20 females each were established. These groups received either the 2% or 5% turmeric diet, catechin alone, or peanut oil alone. The administration of catechin in the drinking water continued for two weeks after treatment with the carcinogen had been completed. All of the control mice treated with BP alone, developed tumors of the forestomach.

Treatment with catechin alone reduced the incidence of tumor formation to 80%. In addition, catechin appeared to work synergistically with turmeric, as tumor incidence was reduced to 35% in mice receiving catechin and the 2% turmeric diet and completely protected mice against tumor induction when used in combination with the 5% turmeric diet. Treatment with catechin or turmeric diet did not result in tumor induction when BP was not administered. Overall results for this assay are presented below in Table 8.14.

Table 8.14 Inhibition Of Benzo[A]Pyrene (BP) Induced Forestomach Tumors In Mice

Group	Average Increase In Weight (g)	Number of Animals with Tumor	% Inhibition of Tumor Incidence	Average No. of Papillomas per Mouse	% Inhibition Papillomas per Mouse
BP only	4.9±0.4	20	-	7.0±0.3	-
BP + catechin	4.9±0.3	16	20	5.4±0.6*	23
BP + 2% turmeric diet	4.8±0.4	20	0	4.0±0.3*	43
BP + 5% turmeric diet	4.6±0.5	12*	40	1.3±0.2*	N/A
BP + catechin + 2% turmeric diet	5.1±0.3	7*	65	2.7±0.7*	61
BP + catechin + 5% turmeric diet	6.0±0.4	0	100	0.0±0.0	100

* $P < 0.001$, compared to BP alone

In addition, Swiss male mice were given a single intraperitoneal injection of 2.5 mg of catechin in 0.2 ml peanut oil to measure the effect of catechin on glutathione S-transferase (GST) activity, a detoxifying enzyme, and glutathione (GSH) substrate levels. Catechin had an inductive effect on the levels of GST activity and resulted in a higher level of reduced substrate. Treatment with 2.5 mg catechin per animal increased GSH content in the forestomach by 8% and the liver by 7%. In addition, a 22% increase in GST activity was seen in the forestomach and a 38% increase in activity was observed in the liver.

In the second tumor model assay, groups of female Syrian golden hamsters were given 0.1 mg/ml of catechin in their drinking water alone or in combination with a 5% turmeric diet. The animals were then treated twice a month for six months with 2 mg/kg of another known carcinogen, methyl-(acetoxymethyl)-nitrosamine (DMN-OAC), which was topically applied to the mucosa of the right cheek pouch of the animals. Controls received either DMN-OAC only or DMN-OAC in conjunction with the 5% turmeric diet. Treatment with DMN-OAC alone resulted in 93% tumor incidence in the control group, while the addition of catechin to the drinking water reduced tumor incidence to 27%. Catechin in combination with the turmeric diet further reduced tumor incidence to 6%. In addition, a 20% higher average body weight and a 60% delay in tumor formation was observed in groups treated with catechin and catechin with turmeric diet as compared to control groups. Over all results for this assay are presented below in Table 8.15.

Table 8.15 Inhibition Of Benzo[A]Pyrene (BP) Induced Forestomach Tumors In Mice

Group	No. of Animals	Weight Gain (g)	(%) Total Tumor Incidence (5-13 Months)	Latency Period (Months)	Mean Tumor Burden (mm^3)	% Survivors
DMN-OAC only	15	52.3±2	93 (14)	5	600±72	27
DMN-OAC + catechin	15	63.3±2	27* (4)	8	3.0±0.7*	93
DMN-OAC + 5% turmeric diet	16	61.8±4	19* (3)	8	2.4±1.8*	73
BP + catechin + 5% tumeric diet	16	63.1±1	6* (1)	8	2.2±0.0*	81

* $p<0.001$, compared to DMN-OAC alone.

Data in these assays described catechin and turmeric to be non-mutagenic and non-carcinogenic. Furthermore, the data indicated that an adjuvant chemopreventive regimen consisting of catechin and dietary turmeric administered prior, during, and after

carcinogen administration was more effective than the individual agents in the inhibition of tumor formation.

Caderni *et al.*, [2000] examined the effect of polyphenolic compounds on azoxymethane (AOM) induced intestinal carcinogenesis. Male F344 rats were dosed subcutaneously once per week over 10 weeks with 7.4 mg/kg of AOM. They were then allocated into four groups of 22 animals each and received by dietary admixture 50 mg/kg body weight red wine extract, green tea extract, or black tea extract over a period of 16 weeks. The red wine extract contained a phenolic breakdown similar to those found in GSE and GSKE. HPLC analysis determined that the red wine extract contained 4.4% (w/w) anthocyanins, 0.8% flavonols, 2.0% phenolic acids, 1.4% catechin, 1.0% epicatechin, and 28.0% PAC units consisting of 18.0% epigallocatechin, 13.2% catechin, 65.0% epicatechin, and 3.8% epicatechin gallate. In rats treated with black tea and red wine extract, there were significantly fewer colorectal tumors than in controls. These data are supplied in Table 8.16.

Table 8.16 Number of Tumors and Their Location in AOM-Induced Rats Treated with Different Polyphenol Extracts [Caderni *et al.*, 2000]

Group (*n*)	Tumors per Rat		
	Colon-Rectum	Small Intestine	Ear
Control (22)	2.54±1.6	0.18±0.39	0.27±0.45
Black Tea Extract (22)	1.54±1.4*	0.27±0.55	0.36±0.49
Green Tea Extract (20)	3.2±1.9	0.25±0.44	0.3±0.57
Wine Extract (22)	1.63±1.6*	0.23±0.43	0.27±0.45

* Significantly different ($P<0.05$) from the control group by Poisson regression.

In addition, significantly fewer rats in the black tea ($p<0.05$) and red wine extract ($p<0.01$) groups had adenomas as compared to controls. The data indicated that black tea and red wine extracts could protect against AOM-induced carcinogenesis through a mechanism possibly involving increased apoptosis in tumors.

Bagchi *et al.* [2000], investigated the safety and efficacy of Actvin (InterHealth Nutraceuticals Incorporated, Benicia, CA), a commercial water-ethanol preparation of IH636 grape seed PAC extract (GSPE). Safety studies, conducted in compliance with the U.S. Environmental Protection Agency and Toxic Substances Control Act Health Effects Test Guidelines, 40 CFR 798.4500, demonstrated that the LD_{50} of GSPE was greater than 5000 mg/kg when administered orally via gastric intubation to fasted albino rats. The acute dermal toxicity was found to be greater than 2000 mg/kg in albino rats. This dosage was also considered to be a no-observed-effect level (NOEL) for systemic toxicity, though slight erythema and desquamation of the skin was observed in all animals. GSPE was found to be moderately irritating when applied at a single dose of 0.5 g to the skin of New Zealand white rabbits.

The same researchers also observed that GSPE had an 84-98% greater free radical scavenging ability (RSA) against superoxide anion and hydroxyl radical as compared to vitamin E at a dose of 50 mg/l and an RSA 439-575% greater than that of vitamin C at a dose of 100 mg/l against the same free radicals *in vitro*. GSPE was also able to protect cultured neuroactive PC-12 adrenal pheochromocytoma cells and macrophage J774A.1 against oxidative damage induced by H_2O_2 at doses of 50 and 100 mg/l and to protect human oral keratinocyte cells from oxidative stress and apoptosis induced by smokeless tobacco at a dose of 100 mg/l.

In addition, GSPE was selectively toxic towards cultured human cancer cells (MCF-7 breast, CRL-1739 gastric adenocarcinoma, and A-427 lung cancer cells) at concentrations of 25 and 50 mg/l while enhanced the growth and viability of normal cells. Pre-treatment of liver cells grown *in vitro* with 25 μg/ml GSPE prior to exposure to Idarubicin (30nM) or 4-hydroxyperoxycyclophosphamide (1 μg/ml), two chemotherapeutic agents that are normally toxic to healthy cells, was also examined. There was a significant decrease (>50%) in the number of cells undergoing apoptosis following treatment with GSPE. An increased expression of apoptosis related gene bcl_2 was also observed in GSPE treated cells through Western blot analysis. GSPE was

also thought to downregulate the oncogene p53 in these cells. GSPE ameliorated the toxic effects associated with these chemotherapeutic agents in normal healthy cells.

Pretreatment of mice with GSPE (25, 50, and 100 mg/kg) significantly inhibited the production of reactive oxygen species by mouse peritoneal macrophages and inhibited lipid peroxidation and DNA fragmentation in mouse brain and liver tissues induced by 12-O-tetradeca-noylphorbol-13-acetate (TPA). Chronic use of GSPE (250 ppm) used in combination with chromium and zinc supplements resulted in reduced systolic blood pressure and glycosylated hemoglobin and decreased lipid peroxidation and free radical formation that normally increase with age.

GSPE also had short- and long-term protective effects against acetaminophen overdose induced lethality, liver toxicity, hepatic DNA damage, apoptotic cell death, and gene expression in mice, as well as against the pathogenesis of post-ischemic reperfusion injury and ischemic arrest in the heart of rats. In *in vivo* studies, GSPE provided significant protection against both acute and chronic stress-induced oxidative gastrointestinal injury in rats by scavenging reactive oxygen species.

In addition, Bagchi *et al.* [2001] described GSPE as able to protect against multiple organ toxicity induced by several drugs. Acetaminophen-induced hepatotoxicity, amiodarone-induced pulmonary toxicity, doxorubicin-induced pulmonary toxicity, dimethylnitrosamine-induced splenotoxicity, cadmium chloride-induced nephrotoxicity, and *O*-ethyl-S,S-dipropylphosphorodithioate (MOCAP)-induced neurotoxicity were all reduced following pretreatment with GSPE.

8.2.2.3 Quercetin

Quercetin is a flavonoid compound that is known to occur naturally in a wide variety of plants and is widely distributed in food, including grapes. The estimated average daily intake of quercetin by an individual in the United States is 25 mg [NTP, 1992]. The FDA, nominated quercetin for toxicity and carcinogenicity studies in the rat based on

concern that quercetin administered to rats through the diet at levels of up to 4% caused minor body weight effects whereas higher doses produced greater than 10% reductions in body weight gains relative to controls.

A series of NTP studies was initiated including a battery of genetic toxicology assays. Quercetin induced mutations in *S. typhimurium* strains TA100 and TA98 with and without exogenous S9 metabolic activation. Positive results were also obtained in tests with and without S9 for the induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells.

An NTP 2-year carcinogenicity study was initiated and conducted by administering 0, 1,000, 10,000, or 40,000 ppm of quercetin (>95% pure) in the feed to groups of 50 male and female rats for 104 weeks. Body weights of exposed male and female rats given the low and mid-range doses remained within 5% of control throughout the course of the study. Reduced body weight gain in high dose male and female rats was observed by week 15 and the final mean body weights were 87% of control weights at week 104. Survival and feed consumption were similar among exposed and control groups throughout the study. The average amount of quercetin intake per day at week 52 was 40, 400, and 1,900 mg/kg of body weight.

The principal toxic effects associated with dietary administration of quercetin were observed in the kidney of male rats. There were dose-related increases in the severity of chronic nephropathy and a slightly increased incidence of focal hyperplasia of the renal tubule epithelium. Histopathological evaluation of the male rat kidney revealed additional hyperplasia and adenomas in all dose groups. The overall incidence of renal tubule adenoma or adenocarcinoma combined in male rats was 1/50 in controls and 9/50 in the high dose group. There was no effect by quercetin on the kidneys of female rats across all dose groups. Based on these results, the conclusion of this study determined that there was some evidence of carcinogenic activity by quercetin in male F344/N rats.

This is relevant insofar as cited in Section 3.2 of this document; quercetin is a minor component of both the MegaNatural™ Gold GSE and GSKE products. Results of a chemical analysis determined GSE to contain 0.1 mg/g of quercetin plus an additional 0.8 mg/g of quercetin glycosides. GSKE was determined contain 1.5 mg/g of quercetin plus 9.3 mg/g of quercetin glycosides. Quercetin glycosides are known to contain approximately 60% quercetin by weight, therefore, the total content of quercetin in the MegaNatural™ Gold GSE and GSKE products is estimated to be approximately 0.6 mg/g and 7.1 mg/g, respectively. As this relates to the consumption of one beverage serving of 8 ounces containing 50 mg of GSKE, quercetin intake would be approximately 0.355 mg. At the 90th percentile for intake of 200 mg GSKE consumption, this would result in a quercetin intake of 1.42 mg of quercetin. If GSE is substituted for GSKE, quercetin intake would be approximately 10-fold lower. These amounts are furthermore equal to only a fraction of the quercetin content of an equivalent amount of grape wine and comparable to the quercetin content of, for example, 0.2 - 2 fluid ounces of strong tea or 0.4 - 4 fluid ounces. of tomato juice. They are also 633,000 – 68,000 times smaller than the 1,900 mg/kg dose (40,000 ppm in diet) associated with an increase in the occurrence of tumors in the kidney of male F344 rats in the 2-year NTP dietary bioassay [NTP, 1992].

Importantly, the Expert Panel noted that the increase in tumors was small, confined to male rats in the highest treatment group, not observed in the females, and not reproduced in a second comparable and contemporary 2-year study employing an even higher 5% dietary treatment (50,000 ppm) [Ito, N *et al.*, 1989]. Based on these considerations, the Expert Panel concluded that GSE and GSKE are not carcinogenic when consumed in food under the proposed conditions of use.

8.2.3 Reproductive Toxicity

Very little reproductive toxicological work has been conducted with polyphenolic PAC compounds. However, no basis for concern has been established. These compounds are a naturally-occurring part of the everyday diet and have provided researchers no

reason to believe that they possess estrogenic activity. Furthermore, a 90-day rat study detailed in section 8.2.1, in which the reproductive organs of both male and female rats receiving either 2.50% GSE or GSKE in the diet underwent rigorous histopathological evaluation. No findings were reported.

9.0 CLINICAL SAFETY

Research by Malaveille *et al.*, [1996] indicated that phenolic compounds inhibited the mutagenicity of 2-amino-1methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). The anti-mutagenic effects of 15 polyphenolic compounds, including catechin, were evaluated by comparing the levels of tobacco-related DNA adducts in exfoliated urothelial cells from 10 smoking volunteers with the anti-mutagenic activity in corresponding 24-hour urine samples. At concentrations ranging from 0.2 to 6μg per assay, the highest inhibitory effects were obtained with flavonols, flavones, flavanones, and one anthocyanidin. An inverse relationship was found between PhIP mutagenicity by urine extracts *in vitro* and two DNA adduct measurements: the level of tentatively identified N-(deoxyguanosine-8-yl)-4-amino-biphenyl adduct and the total level of all tobacco smoke related carcinogen adducts, including those probably derived from PhIP. Although results regarding catechin were inconclusive in this assay, overall the investigators suggest that study results indicated that smokers ingesting dietary phenolic compounds such as those found in onions, lettuce, apples, and red wine, may be partially protected against the harmful effects of tobacco carcinogens in the bladder and other sites.

The pharmacological treatment of non-complicated chronic venous insufficiency is a current and well-debated topic. The introduction of new products with action on the venous system, improved knowledge on the physiopathology of venous insufficiency, and the possibility provided by new analytical instruments, have given new impetus to the consolidation of the clinical value of phlebotonics in this indication. In light of this, Costantini *et al.*, [1999] examined 24 patients with non-complicated chronic venous insufficiency treated with a daily oral administration of 100 mg/day of oligomeric PACs (Pycnogenols-OPC). To evaluate the therapeutic efficacy of the treatment, instrumental

evaluation by optical probe capillaroscope was performed in addition to the traditional subjective clinical parameters: swelling, itching, heaviness and pain. The videocapillaroscope examination was performed at the lower third of the leg and the first toe. Edema in the capillaroscopic field, the number of observable capillaries, and capillary dilation were the parameters chosen to evaluate the efficacy of treatment. All patients completed the study with no reports of adverse events during the period of observation. The results obtained showed a positive clinical response of improved or absent symptoms in over 80% of patients, with significant improvement of symptoms already evident after the first 10 days of treatment; rapid reduction in the swelling of the lower limbs was observed. Correlated with this were other evaluable symptoms: heaviness and itching. Particularly striking results were observed for itching and pain, which completely disappeared during the course of therapy in 80% and 53% of the patients respectively. Noteworthy is the good correlation between the clinical and instrumental data, with improvement in a total of 70% of patients. The results obtained in the course of this clinical experience indicated that treatment with oligomeric proanthocyanidins was associated with improvement during the first weeks of treatment and the absence of adverse events.

The literature provides some examples of clinical studies that attempt to establish the pharmacological efficacy of grape seed / grape skin extract and PACs in humans. However, in light of the centuries of safe use and numerous supportive animal studies, very few clinical studies have been conducted to assess the safety of a given PAC product as a specific clinical endpoint. We have therefore reviewed those clinical pharmacological studies available to us as evidence of safety. The results of our evaluation, summarized in Table 9.1, confirmed the general safety of PACs for clinical use.

Table 9.1 Summary Evaluation of Clinical Safety

Reference	No. of Subjects	Dose	Length of Exposure	Findings	Indication of Toxicity
Maxwell, 1997	6	250 ml of port wine	Single dose w/ a blood draw every 30 minutes for 2 hours	Increased antioxidant activity in the serum after consumption of port wine.	No
Maxwell, 1997	10	5.7 ml / kg of red wine	Single dose w/ a 240 minute follow up time	Increased antioxidant activity in human extracellular fluid following red wine consumption.	No
Nigdikar *et al.*, 1998	9	375 ml / day of red wine, white wine red wine polyphenols (RWPS) or RWPS dissolved in white wine	2 weeks	Decreased thiobarbituric acid-reactive substances, lipid peroxides and conjugated dienes and increased plasma and LDL polyphenols.	No
Nutall *et al.*, 1997.	20	300 mg of procyanidin extract twice daily	5 days followed by a 2 week washout period followed by another 5 day regimen	Increased serum antioxidant activity	No
Maalej *et al.*, 1997	1600	Red wine	NA	Reduced platelet response to ADP and collagen but not to thrombin.	No
Maalej *et al.*, 1997	-	Red wine	15 days	Increased HDL levels and decreased ADP induced platelet aggregation	No
Maalej *et al.*, 1997	20	Red wine	3 months	Reduced thrombin-initiated platelet aggregation and LDL in hypercholesterolemics.	No
Whitehead *et al.*, 1995	9	300 ml of red wine	Consumed over 30 minutes blood drawn at 1 hr and 2 hr	A rise in serum antioxidant levels after ingestion of red wine.	No

10.0 REFERENCES

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Appendix A

Expert Panel Statement: Determination of the GRAS Status of MegaNatural™ Gold Grape Seed Extract (GSE) and MegaNatural™ Gold Grape Skin Extract (GSKE) for Use as an Antioxidant Ingredient in Beverage Products

Expert Panel Statement

Determination of the GRAS Status of Meganatural™ Gold Grape Seed Extract (GSE) and Meganatural™ Gold Grape Skin Extract (GSKE) for Use as an Antioxidant Ingredient in Beverage Products.

The undersigned, an independent panel of recognized experts (hereinafter referred to as Expert Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by Polyphenolics Incorporated, a subsidiary of Canandaigua Wine Company, to determine the Generally Recognized as Safe (GRAS) status of two natural products from grape seed and skin. These products, which shall be referred to in this document as grape seed extract (GSE) and grape skin extract (GSKE), contain primarily oligomeric and polymeric proanthocyanidins and other natural phenolic compounds. GSE and GSKE are intended for interchangeable addition to fruit juice and fruit flavored beverages at composite total concentrations up to 210 ppm as antioxidants to retard deterioration. Their use may be alone or in combination with other safe and appropriate antioxidant substances. A comprehensive search of the scientific literature for safety and toxicity information on GSE, GSKE and constituent proanthocyanidins and related polyphenols was conducted through August 2001 and made available to the Expert Panel. A report based on this comprehensive literature review as well as results and analyses of safety studies on the two specific GSE and GSKE ingredients aided and facilitated the work of the Expert Panel. The Expert Panel independently evaluated the material submitted, as well as other materials deemed appropriate or necessary. Following independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decisions described herein.

Published values indicate estimated dietary intake of flavanoids, catechins and proanthocyanidins by the average American consumer is in the range of 460-1,000 mg/day. Intake arises from the common occurrence of these substances in fruits, juices, tea, chocolate, vegetables, and in many other foods and beverages. The intake of additional polyphenolic substances arising from the combined proposed beverage uses of GSE and GSKE was estimated using data contained in the USDA CSFII (1994-1996, 1998). Additional intake was calculated to be approximately 70 mg/day and 130 mg/day for the mean and 90th percentile beverage user, respectively. The highest 90th percentile intake among population age sub-groups was projected to occur in teenage males who may consume 200 mg GSE/GSKE daily. The Expert Panel concluded that such intake is within the population variability in background consumption.

The composition of Meganatural™ Gold GSE and GSKE is comprised of approximately 90% gallic acid equivalents by weight of flavan-3-ol based monomeric, oligomeric and polymeric phenolic compounds, 60-80% of which are oligomers while polymer content is 25% or less and monomer content approximately 10 - 15%. Regarding individual components, the Expert Panel found the composition to represent a typical spectrum of natural positional and structural isomers consistent with that reported in the literature. The diversity of polymeric forms was observed to be somewhat greater in material derived from grape seed than that from grape skin. Review of minor components indicated no unexpected substances. The presence of 0.1 - 1% combined quercetin and quercetin glycosides were noted. Results of analysis of multiple production batches demonstrated compliance with compositional specifications as well as compliance with typical food quality standards regarding microbiologic activity, heavy metal content and pesticide residues permitted in grapes. The Expert Panel concluded that Meganatural™ Gold GSE and GSKE products were representative of naturally occurring grape phenolics and that the products conformed to applicable standards regarding microbiologic activity and residue limits.

The Expert Panal examined a significant body of information regarding the antioxidant activity of phenolic compounds, polyphenols in particular and those in GSE and GSKE specifically. Ample evidence, including mechanistic investigations, demonstrating both *ex-vivo* and *in-vivo* antioxidant activity was found. Evidence from *in-vitro* investigation of Activin, a similar commercial grape seed proanthocyanidin extract product demonstrated antioxidant activity of this substance toward superoxide anion and hydroxyl radical which was equal to or exceeded that of vitamins E and C at concentrations examined in the range of 50-100 mg/L (ppm). The Expert Panel was not presented with specific data regarding the antioxidant activity of the Meganatural™ products when added to fruit juice and fruit flavored beverages at concentrations up to 210 ppm. However, the Expert Panel noted that these same substances occur naturally at concentrations up to several hundred ppm in grape wines and juice as well as in the fruit and juice of apples, cranberries, and in numerous other foods wherein their antioxidant activity is well recognized. The Expert Panel, considering the well characterized mechanism of action and diversity of evidence, concluded that Meganatural™ Gold GSE and GSKE exhibit antioxidant activity and would do so when added to fruit juice and fruit flavored beverage at a concentration up to 210 ppm as specified.

The Expert Panel also considered the likelihood for GSE and GSKE to exhibit pro-oxidant activity under the conditions of intended use. The Expert Panel limited its

consideration to possible implications for consumer safety. Evidence was assessed which demonstrated that under certain experimental *in-vitro* conditions, polyphenolic substances present in GSE and GSKE can, like many other antioxidants, exhibit pro-oxidant activity. These conditions generally require the presence of polyphenols in high concentration combined with the presence of transition or other metal ions or certain compounds such as bleomycin capable of sustained oxidation-reduction reaction cycling. The Expert Panel concluded that such conditions were not relevant to prospective consumers of fruit juice and fruit flavored beverage products containing 210 GSE and/or GSKE. This conclusion was based in part on consideration that the polyphenolic dose to a high-end (90th %) consumer from the proposed uses would be on the order of only 200 mg/day which, due to their limited bioavailability, would not be expected to produce highly elevated polyphenol concentrations in body fluids and tissues. Importantly, administration to clinical trial subjects of 300 mg of procyanidin extract twice daily for 5 days induced increased serum antioxidant activity with no reported pro-oxidant effects. Similarly, administering red wine polyphenols for 2 weeks resulted in decreased thiobarbituric acid reactive substances, lipid peroxides and conjugated dienes.

With respect to GSE and GSKE safety, the Expert Panel carefully assessed evidence regarding potential mutagenic activity associated with these substances and their constituents. Evidence examined included results from several published studies as well as from a GLP-compliant assay conducted specifically on the Meganatural™ Gold products, which measured induction of micronuclei in polychromatic erythrocytes and in bone marrow cells of Crl:CD1 mice. The known presence of minor amounts of quercetin and its glycosides, substances with suspected mutagenic and carcinogenic activity, were of particular interest to the Expert Panel. Results available in the literature were inconsistent with respect to mutagenicity. Isolation and multiple fractionation of wine polyphenols was reported to yield a subfraction that exhibited mutagenic activity in the TA98 Salmonella strain when tested in the presence of human fecal glycosidase with or without S9 microsomal fraction. Rutin (3-rhamnoglucosyl quercetin) was identified as the probable active component. In contrast, several investigations demonstrated the antimutagenic, anticlastogenic, and antipromotional activity associated with (+) catechin and (-) epicatechin, as well as their oligomers and other related phenolic compounds. It was not clear that activity of the GSE and GSKE complex polyphenolic mixtures could be adequately assessed on the basis of the possible activity of certain constituents. The Expert Panel therefore assigned greatest importance to the mouse micronucleus assays conducted on the mixtures themselves the results of which demonstrated a complete lack of mutagenic activity when administered at oral doses up to 2,000 mg/kg. The Expert Panel also found it

reassuring that the greatest 90th percentile intake of GSE/GSKE (200 mg/day, in approximately 1 liter of beverage) among male teenagers (50 kg body weight) would result in only a 0.14 - 1.4 mg (0.003 - 0.028 mg/kg) intake of quercetin compared to the 25 mg estimated by NTP (NTP TR 409, 1992) as the average daily intake of quercetin by an individual in the United States. These amounts are also equal to only a fraction of the quercetin content of an equivalent amount of grape wine and comparable to the quercetin content of, for example, 0.2 - 2 fl. oz. of strong tea or 0.4 - 4 fl. oz. of tomato juice. They are also 633,000 – 68,000 times smaller than the 1,900 mg/kg dose (40,000 ppm in diet) associated with an increase in the occurrence of tumors in the kidney of male F344 rats in a modern 2-year NTP dietary bioassay (ibid.). Importantly, the Expert Panel noted that the increase in tumors was small, confined to male rats in the highest treatment group, not observed in the females and not reproduced in a second comparable and contemporary 2-year study employing an even higher dietary treatment (5%; 50,000 ppm) (Ito,N, et al.,JPN. J. CANCER RES. 80(4) :317-325, 1989). Based on these considerations, the Expert Panel concluded that GSE and GSKE are not carcinogenic when consumed in food under the proposed conditions of use.

The Expert Panel also assessed preclinical evidence regarding sub-chronic safety as well as possible adverse affects on nutrition. With respect to nutrition, one published study reported a possible impairment of growth and increased fecal nitrogen excretion in pair-fed rats given protein deficient or protein-free diets in combination with grape seed tannins incorporated in the diet at 2% by weight. This is believed to be the result of the interaction of the tannins to form less digestible complexes with dietary proteins as well as interaction with mucosal proteins, stimulating desquamation. These effects were not reported when dietary tannin content was 0.2% or in studies using protein-adequate diets. The Expert Panel concluded that the proposed GSE and GSKE use levels are sufficiently low and dietary protein intake sufficient high among consumers to assure an absence of these nutritional effects.

With respect to sub-chronic safety, the Expert Panel assigned primary importance to the results of a GLP-compliant 3-month study in which rats were administered the Meganatural™ Gold GSE and GSKE products in the diet at concentrations up to 2.5% by weight. This study included measurement of growth, serum chemistry, and hematology parameters, as well as comprehensive histopathologic examination of organs and tissues, including those related to reproduction and endocrine function. The only remarkable finding arising from this study related to an increase in occurrence of changes in the kidney of male rats administered a diet containing 2.5 % (w/w) GSKE. In this group, a significant ($p<0.05$) increase in the occurrence of a common renal lesion comprised predominately of lymphocytic interstitial infiltrates was reported in 11 of 20

animals compared to occurrence in 4 of 20 control animals. The severity in most cases was minimal and a similar increased frequency was not observed in female rats of the same treatment group or in male or female rats receiving dietary GSE. This lesion is commonly seen in male rats with increasing frequency and severity with aging and is considered a component of the entity of chronic nephropathy. Similar changes routinely observed in rats are considered background lesions. The Expert Panel examined histopathology findings for the individual animals and noted an absence of indications of a significant exacerbation of lesion severity. An acceleration in lesion severity would have been expected if administration of GSKE were acting to promote this chronic progressive spontaneously occurring condition. While the possibility of a renal effect by GSKE could not be entirely ruled out, particularly during the initial stage of the study when dose rates were highest, the Expert Panel concluded that a continuing significant affect is not supported by these findings. The Expert Panel also noted the species and sex specificity of this background renal syndrome and its unlikely relevance to human renal safety. Based on these considerations, the Expert Panel concluded that the reported renal changes do not constitute an adverse effect, are not relevant to humans, and may in fact represent normal fluctuation in the rate of appearance of this background lesion. The Expert Panel further concluded that the study results provided no evidence of toxicity or adverse effects including effects on endocrine and reproductive endpoints and supported a No-Observed-Effect Level (NOEL) of approximately 2,150 mg/kg/day for administration of GSE as well GSKE to female rats while in male rats 1,780 mg GSE/kg/day represented a NOEL and 1,780 mg GSKE/kg/day represented a No-Observed-Adverse-Effect Level (NOAEL). These values represent the time-weighed mean dose rates occurring in the high-treatment groups over the course of the study.

Based on the critical evaluation discussed above, the Expert Panel has determined that, Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.

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Based on the critical evaluation discussed above, the Expert Panel has determined that, Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.

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~~Based on the critical evaluation discussed above, the Expert Panel has determined that, Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.~~

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SUBMISSION END

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