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Original Submission

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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: Revised 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)
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.

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

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. These material provided herein are intended to clarify FDA concerns raised in a letter dated June 5, 2002 that the original Notification did not provide a sufficient basis to conclude that GSE and GSKE were GRAS in their intended uses. An Addendum to the Expert Panel Statement has been issued acknowledging the revised data and affirming the unanimous Expert Panel agreement as to the GRAS status of MegaNatural™ Gold GSE and GSKE for their intended use.

Respectfully submitted,

Edward J. Race
Director of Research

U. S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
June 5, 2002

Agency Response Letter
GRAS Notice No. GRN 000093

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

Re: GRAS Notice No. GRN 000093

Dear Mr. Race:

The Food and Drug Administration (FDA) is responding to the notice, dated December 12, 2001, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on December 17, 2001, and designated it as GRAS Notice No. GRN 000093.

The subjects of the notice are grape seed extract (GSE) and grape skin extract (GSKE). The notice informs FDA of the view of Polyphenolics, Inc. (Polyphenolics) that GSE and GSKE are GRAS, through scientific procedures, for use in fruit juice and fruit flavored beverages as antioxidants to retard deterioration. The extracts would be used separately or in combination at a maximum total extract concentration of 210 milligrams per liter (equivalent to 50 milligrams per eight fluid ounces) in the finished beverage products.

FDA has evaluated the information that Polyphenolics discusses in its GRAS notice as well as other data and information that are available to the agency. As discussed more fully below, the notice does not provide a sufficient basis for a determination that GSE and GSKE are GRAS under the conditions of their intended use.

Data and information that Polyphenolics presents to support its GRAS determination

Because GSE and GSKE are mixtures that are comprised predominantly of a class of compounds known as phenolics or polyphenols, Polyphenolics discusses generally available information about phenolic compounds. Polyphenols are the products of plant metabolism and can range from simple molecules to highly polymerized compounds. Flavonoids, a subclass of polyphenols, are the most common polyphenolic compounds found in nature and are further divided into several subclasses including flavones, flavonols, isoflavones, anthocyanins, flavanols, and proanthocyanidins (PACs).

Polyphenolics describes the method of manufacture of GSE and GSKE. The extracts are manufactured from fresh grapes, which are inspected for quality and screened for defects. The grapes are de-stemmed, crushed, and pressed, leaving a pomace residue of seeds and skin. The seeds are separated from the skins (for GSE), and the seeds or the pomace of seeds and skin (for GSKE) are boiled in water to extract the polyphenolic constituents. The seeds are then removed, and the extract is cooled, enzymatically depectinized, and the pH is adjusted. The resulting extract is refrigerated and stored for one to three months. The extract is then filtered with diatomaceous earth and passed through a column of trimethylolpropane trimethacrylate (TMPTMA). Polyphenolics notes that grape phenolic constituents preferentially adsorb to the TMPTMA resin, while other grape constituents such as minerals and organic acids pass through the column and are discarded. The phenolic constituents are eluted from the resin using 75 percent (by volume) beverage-grade ethanol. The ethanol is then removed using a vacuum thermal evaporator, and the concentrate is spray dried to give the final GSE or GSKE product.

Polyphenolics provides product specifications[1] and analyses of several production lots of GSE and GSKE. The specifications for both GSE and GSKE include limits on total phenolics and levels of any residual pesticides. Additional specifications for GSE include ranges for the monomeric, oligomeric, and polymeric content of the phenols and limits on

insoluble substances. Additional specifications for GSKE include a limit on total anthocyanins.

Using its proposed use levels and data from the United States Department of Agriculture (USDA) 1994-1996 Continuing Surveys of Food Intakes by Individuals and the 1998 Supplemental Children's Survey, Polyphenolics estimates the combined intake of GSE and GSKE would be 70 milligrams per person per day (mg/person/day) at the mean (eaters only) and 130 mg/person/day at the 90th percentile (eaters only). Polyphenolics states that this estimate is comparable to exposure to PACs from other foods and beverages such as red wine. Polyphenolics notes, however, that information on exposure to PACs is limited, and large variations exist in the reported concentrations of PACs for a given product or commodity. Polyphenolics explains that these variations in concentration are due to differences in the assays used to measure PAC concentration and the nature of the sample analyzed (i.e., variety, ripeness, part, and processing of the fruit).

Polyphenolics discusses published information about the absorption, distribution, metabolism, and excretion of low molecular weight phenolic and polyphenolic compounds in general and notes that relatively little information exists about the absorption and bioavailability of the high molecular weight PACs, which are polymers and thus are less likely to be absorbed through the gut barrier than the low molecular weight phenolic and polyphenolic compounds. Polyphenolics also discusses published information about various mutagenicity, animal, and human studies conducted with various polyphenolic compounds. Polyphenolics considers that these studies support the dual nature of phenolic compounds *in vitro* and *in vivo* (i.e., that phenolic compounds exhibit properties that are both mutagenic and anti-mutagenic, both carcinogenic and anti-carcinogenic, both promoter and anti-promoter, and both clastogenic and anti-clastogenic) . Polyphenolics notes that quercetin or quercetin derivatives are theorized to be the key mutagenic, carcinogenic, and clastogenic compounds within the class of polyphenolic compounds. Polyphenolics discusses toxicity and carcinogenicity studies of quercetin conducted by the National Toxicology Program (NTP), and notes that NTP has concluded that there is some evidence that quercetin is carcinogenic in male rats. Polyphenolics provides quantitative information on the levels of quercetin and quercetin glycosides present in GSE and GSKE, presents a risk assessment based on these levels, and concludes that use of the GSE and GSKE as proposed will not pose a carcinogenic risk to the consuming public. Polyphenolics concludes from its overall review of the literature that GSE and GSKE do not pose a safety concern despite the fact that GSE and GSKE contain a number of polyphenols that cause a variety of responses in different models.(2)

To corroborate its conclusion from the published literature about phenolic and polyphenolic compounds, Polyphenolics discusses the results of an unpublished mouse micronucleus assay and an unpublished subchronic (13-week) feeding study conducted in rats. Polyphenolics concludes that the results of the mouse micronucleus assay in Crl:CD-1 mice support a conclusion that GSE and GSKE are not mutagenic. Polyphenolics also concludes that the results of the subchronic feeding study support a no-observed-adverse-effect-level (NOAEL) for GSE or GSKE of 2,150 milligrams per kilogram body weight per day (mg/kg bw/day) in female rats and 1,780 mg/kg bw/day in male rats, corresponding to the highest dose exposure over the course of the study. Polyphenolics uses these NOAELs in rats and a safety factor of 100 to calculate an acceptable daily intake of 1,075 mg/person/day of GSE or GSKE for an average 50 kilogram human female and 1,250 mg/person/day of GSE or GSKE for an average 70 kg human male.

Polyphenolics acknowledges that NTP has recommended that additional toxicity data be generated for a different grape seed extract product that is currently marketed for use as a dietary supplement. Polyphenolics considers that this recommendation by NTP is not inconsistent with its view that the intended use of GSE and GSKE as antioxidants in juice is GRAS because the specific charge to NTP is to evaluate a grape seed extract that has widespread consumer use as a dietary supplement.

Polyphenolics provides the report of a panel of individuals (Polyphenolics' GRAS panel) who evaluated the data and information that are the basis for Polyphenolics' GRAS determination. Polyphenolics considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Polyphenolics' GRAS panel evaluated published data and information about phenolic and polyphenolic compounds in general and unpublished data from the two studies conducted with GSE and GSKE (i.e., the mouse micronucleus assay and the 13-week rat feeding study). Based on this review, Polyphenolics' GRAS panel concluded that GSE and GSKE that meet the appropriate food grade specifications are GRAS, through scientific procedures, under the conditions of their intended use in accordance with limitations of current good manufacturing practice. However, in reaching this conclusion, Polyphenolics' GRAS panel assigned primary importance to the unpublished studies conducted with GSE and GSKE.

FDA's evaluation of the data and information in Polyphenolics' notice

Polyphenolics provides insufficient information about the composition of GSE and GSKE to evaluate safety. For example, although Polyphenolics asserts that the content of PACs in various foods compares well with the estimate of intake of GSE and GSKE, Polyphenolics does not provide the PAC content of GSE and GSKE so that no direct comparison is apparent. Likewise, although Polyphenolics discusses average daily flavonoid intake from currently consumed foods, Polyphenolics does not provide the flavonoid content of GSE and GSKE.

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Based on its evaluation of the data and information in Polyphenolics' notice, FDA agrees with Polyphenolics' GRAS panel that the most relevant studies are the unpublished studies conducted with GSE and GSKE. Given that these studies are not generally available to the expert scientific community, these studies cannot provide a basis to determine that GSE and GSKE are GRAS, through scientific procedures, for their intended use in fruit juice and fruit flavored beverages. Moreover, the recommendation by NTP that another grape seed extract product be subjected to substantial toxicity tests raises questions about whether Polyphenolics' two studies, even if they became generally available, could provide a basis to conclude, at this time, that there is consensus within the scientific community about the safety of chronic consumption in food of GSE and GSKE.

For your information, an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity. Standards of identity for individual fruit juices are described in 21 CFR Part 146. With the exception of lemon juice, the standards of identity for fruit juices do not permit the addition of antioxidants as an optional ingredient in these standardized foods.

Conclusions

FDA has evaluated the data and information in GRN 000093 as well as other available information. Your notice does not provide a sufficient basis for a determination that GSE and GSKE are GRAS under the conditions of their intended use.

FDA recommends that you review the agency's discussion in the GRAS proposal of the scientific, legal, and regulatory underpinnings of the GRAS notification program. In particular, we recommend that you review the agency's discussion of the differences between a food additive and a GRAS substance. As discussed in that proposal (62 FR 18940), a determination that a particular use of a substance is GRAS requires technical evidence of safety and a basis to conclude that this technical evidence of safety is generally known. There are two aspects to this common knowledge. First, the data and information relied on to establish safety must be generally available to the public. Second, there must be a basis to conclude that there is consensus among qualified experts about the safety of the substance for its intended use. Neither aspect, by itself, is sufficient to satisfy the common knowledge element of the GRAS standard. In contrast, authorization of a particular use of a substance as a food additive requires technical evidence of safety and review and approval by FDA. For this reason, we also recommend that you review the information on the home page of the Office of Food Additive Safety regarding the food additive petition process. (Ref. 1).

This letter describes in general terms the reason for FDA's conclusion that the information in your notice does not provide a sufficient basis to conclude that GSE and GSKE are GRAS under their intended conditions of use. If you would like to discuss these issues in more detail, you may contact Dr. Linda Kahl by telephone at (202) 418-3101, by telefax at (202) 418-3131, or by electronic mail at linda.kahl@cfsan.fda.gov.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
/s/
Alan M. Rulis, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

References

1. A series of guidance documents are available at http://vm.cfsan.fda.gov/~lrd/foodadd.html

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[1]FDA notes that a different preparation of grape skin extract (enocianina), with different specifications, is regulated under 21 CFR 73.170 for use as a color additive in still and carbonated drinks and ades, beverage bases, and alcoholic beverages. Specifications for grape skin extract (enocianina) are also listed in the Food Chemicals Codex, 4th Edition (1996).

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(2)As part of its overall conclusion, Polyphenolics cites several human studies that evaluated the potential that various phenolic compounds have certain pharmacological effects.

Food Ingredients and Packaging | Summary of all GRAS Notices

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Hypertext last updated bypmg/lah on 2002-JUN-14

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July 17, 2002

Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

Attn: Ms. Lisa F. Lubin, MS, RD

Re: Agency Response Letter (June 5, 2002) to GRAS Notice No. GRN000093

Dear Sir or Madam:

Polyphenolics, Inc. acknowledges receipt of the above Agency Response Letter and submits this request for clarification regarding FDA's characterization of NTP-recommended testing of another grape seed extract product.

In the second paragraph of the letter section titled "FDA evaluation of the data and information in Polyphenolics' notice", FDA states:

> *"...Moreover, the recommendation by NTP that another grape seed extract product be subjected to substantial toxicity tests raises questions about whether Polyphenolics' two studies, even if they became generally available, could provide a basis to conclude, at this time, that there is consensus with the scientific community about the safety of chronic consumption in food of GSE and GSKE."*

Polyphenolics believes, and asks FDA to consider, that this statement is a critically incomplete characterization of NTP's position regarding grape seed extract and subject to serious misinterpretation.

In our view, the portions of the sentence stating that NTP recommends "substantial toxicity tests", combined with the assertion that this raises a question about whether there could be "consensus within the scientific community about the safety ..." is seriously misleading, implies FDA believes NTP has identified potential safety concerns regarding grape seed extract and has, in fact, caused significant alarm among industry manufacturers of this and other potential "healthy ingredients" for which NTP may decide to conduct tests as well.

Polyphenolics asks FDA to examine NTP's "Summary of Data For Chemical Selection" prepared in July, 2000 for "Oligomeric Proanthocyanidins from Grape Seeds and Pine Bark" (attached). In the first two pages of that document, several important statements are made:

1) *"Grape seed extract and pine bark extract ... are brought to the attention of the CSWG [Chemical Selections Working Group] because of widespread consumer use of these substances as dietary supplements."* (pg. 1, para. 1)
2) *"... the phenolic compounds extracted from grape seeds ... would not be expected to be genotoxic."* (pg. 1, para. 2)
3) *"... some studies showing tumor inhibition, make them attractive materials for further study."* (*Ibid*)
4) *"Grape seed ... extracts may have the beneficial effects of red wine without its detrimental effects."* (*Ibid*)

The NTP document also explains that while NTP is aware of manufacturer's claims that significant safety testing has been conducted, the results of these tests are not generally available and that:

> *"Because grape seed and pine bark extracts are dietary supplements, the government cannot compel the manufacturer to test the safety of these materials."* (pg. 1, para. 3)

On page 2, under Rational/Remarks, the NTP summary document states:

> *"Although they are widely used as dietary supplements, the toxicity of these products has not been well characterized"*

to which we would add the qualification, *"in the public literature"*, consistent with discussion on page 1 of the NTP document.

Most importantly in paragraph 3 on page 1 NTP states:

> *"Given their potential benefits, an independent demonstration of their safety appears warranted."*

We find no statement in the NTP document indicating they have a concern regarding identified potential toxicity of grape seed extract or that current use is seen as unsafe. Accordingly, we find misleading, FDA's implication that the NTP recommendation calls into question, on the basis of safety, whether a consensus could exist within the scientific community. Rather, we acknowledge that, as reiterated in the NTP document, there has been, until very recently, an absence of safety data in the peer-reviewed literature. This may impair fulfillment of the "common knowledge" GRAS element as currently interpreted. By extension, lack of information might, but would not necessarily, also impair establishment of an informed consensus among qualified scientists; consensus could, nevertheless, develop as a result of access to as yet unpublished information through other channels, discussions among peers, presentations at meetings, *etc.*

We are aware that NTP nomination of substances for testing has historically been "for cause", reflecting direct or indirect evidence indicating the candidate may possess toxic, particularly carcinogenic, activity. However, in the recent past, particularly since enactment in 1994 of the Dietary Supplement Health and Education Act, we have observed that NTP nominations appear to have expanded to include substances consumed by the American public in unprecedented amounts, primarily in dietary supplement form, for which toxicity is not necessarily indicated, but for which safety has not been well characterized in the peer-reviewed published literature. Rather than "for

cause", these nominations might be classified as being "for general public health benefit". Clearly, NTP-recommended testing of grape seed extract falls within this second category.

In summary, Polyphenolics requests FDA to reexamine their characterization of the NTP recommendation for testing grape seed extract. We ask FDA to consider issuing a clarification that this recommendation was not based on identified safety concerns, but rather to mitigate a lack of formal safety information, at the time of the recommendation in 2000, in the public peer-reviewed scientific literature on this important emerging healthful substance. Further, we suggest that such lack of information in the public literature might impair the GRAS "common knowledge" element as currently interpreted, but would not necessarily prevent development of a consensus among qualified scientists since dissemination of information may occur effectively via other mean.

Lastly, we seek guidance and clarification from FDA with respect to the process by which it monitors, evaluates and employs in its rulemaking, submission/notification review, and other related activities, information regarding actual or recommended testing of substances by NTP, NCTR, NCI and other investigative groups.

Respectfully submitted,

Mr. Edward J. Race
Director of Research
Polyphenolics, Inc.
12667 Road 24
Madera, CA 93637
Phone: (559) 661-5545

July 17, 2002

Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway, HFS-255
College Park, MD 20740

Attn: Ms. Lisa F. Lubin, MS, RD

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Polyphenolics believes, and asks FDA to consider, that this statement is a critically incomplete characterization of NTP's position regarding grape seed extract and subject to serious misinterpretation.

In our view, the portions of the sentence stating that NTP recommends "substantial toxicity tests", combined with the assertion that this raises a question about whether there could be "consensus within the scientific community about the safety ..." is seriously misleading, implies FDA believes NTP has identified potential safety concerns regarding grape seed extract and has, in fact, caused significant alarm among industry manufacturers of this and other potential "healthy ingredients" for which NTP may decide to conduct tests as well.

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cause", these nominations might be classified as being "for general public health benefit". Clearly, NTP-recommended testing of grape seed extract falls within this second category.

In summary, Polyphenolics requests FDA to reexamine their characterization of the NTP recommendation for testing grape seed extract. We ask FDA to consider issuing a clarification that this recommendation was not based on identified safety concerns, but rather to mitigate a lack of formal safety information, at the time of the recommendation in 2000, in the public peer-reviewed scientific literature on this important emerging healthful substance. Further, we suggest that such lack of information in the public literature might impair the GRAS "common knowledge" element as currently interpreted, but would not necessarily prevent development of a consensus among qualified scientists since dissemination of information may occur effectively via other mean.

Lastly, we seek guidance and clarification from FDA with respect to the process by which it monitors, evaluates and employs in its rulemaking, submission/notification review, and other related activities, information regarding actual or recommended testing of substances by NTP, NCTR, NCI and other investigative groups.

Respectfully submitted,

Mr. Edward J. Race
Director of Research
Polyphenolics, Inc.
12667 Road 24
Madera, CA 93637
Phone: (559) 661-5545

CanandaiguaWine

January 16, 2003

To whom it may concern,

The undersigned, as a duly qualified representative of Polyphenolics Inc., hereby affirm that the use of MegaNatural™ Gold grape seed extract and MegaNatural™ Gold grape skin extract, as manufactured by the Canandaigua Wine Company's Polyphenolics brand, is generally recognized as safe (GRAS). This determination was based on the applicable requirements for the technical element and common knowledge element of a GRAS determination based on scientific procedures and supported by the determination of a qualified independent panel of experts (herein after referred to the Expert Panel).

MegaNatural™ Gold grape seed extract (GSE) and MegaNatural™ Gold grape skin extract (GSKE) are predominantly composed of polyphenolic proanthocyanidin compounds. They are generally recognized as safe (GRAS) food ingredients for use in fruit juices (unless prohibited by a standard of identity), fruit flavored beverages, fruit flavored beverage mixes, and carbonated fruit flavored beverages at a concentration of approximately 210 ppm (w/v) or 50 mg per 8 fluid ounce serving. The proanthocyanidins (PACs) may be added to beverages for use as an antioxidant in order to retard deterioration. Their use may be alone or in combination with other safe and appropriate antioxidant substances.

A summary basis for MegaNatural™ Gold GSE and GSKE GRAS status has been provided below.

- 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 and guidance established by FDA for the presence of heavy metals and pesticide residues in grapes.

Polyphenolics

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

- 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 [Erexson, 2003] 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 [Bentivegna and Whitney, 2002]. These results support studies undertaken by independent investigators [Yamakoshi *et al.*, 2002; Wren *et al.*, 2002; Bagchi *et al.*, 2001] evaluating commercially available products of a substantially equivalent PAC make-up and general polyphenolic profile.
- There is supporting evidence for the safety of GSE and GSKE in humans from clinical investigations of possible beneficial health effects.

An independent panel of recognized experts, qualified by scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, conducted a GRAS determination. The evaluation and consideration of the MegaNatural™ Gold GSE and GSKE GRAS status by the Expert Panel was based on the weight of all of the available scientific information summarized in a report to the Expert Panel. This report to the Expert Panel was based upon a thorough review of the relevant literature and the result of safety studies on MegaNatural™ Gold GSE and GSKE conducted in accordance with generally accepted scientific procedures. The use of available scientific and statistical reference sources and compendia; relevant books and reviews; and those relevant regulatory documents available from the Food and Drug Administration supplemented this information. Consequently, the qualified Expert Panel determined that there was reasonable certainty that MegaNatural™ Gold GSE and GSKE would not be harmful under the intended conditions of use in beverages and beverage products. This GRAS determination therefore met the requirements of §201(s) of the Federal Food, Drug, and Cosmetic Act; 21 CFR §170.3 and §170.30; and the amendments to these rules proposed in 62 Fed. Reg. 18960.

Polyphenolics subsequently submitted to FDA on December 17, 2001, a GRAS Notification (GRN 000093) informing FDA that it had determined the intended use of GSE and GSKE to be GRAS. FDA responded in a letter dated June 5, 2002 that the Notification did not provide a sufficient basis to conclude that GSE and GSKE were GRAS in their intended uses. Following consultations with the Food and Drug Administration in July of 2002, additional chemistry and newly published pivotal safety data [Yamakoshi *et al.*, 2002; Wren *et al.*, 2002] was assembled and supplied to the Expert Panel. This data was meant to clarify Agency concerns and is provided herein. This additional data in no way materially affected the original Panel decision as to GRAS status of the MegaNatural™ Gold GSE and GSKE products. However, an Addendum to the Expert Panel Statement was issued acknowledging the revised data and affirming the unanimous agreement as to the GRAS status of MegaNatural™ Gold

GSE and GSKE for their intended use in specific beverage products for which no standard of identity exists.

Based on the considerations, I hereby affirm that MegaNatural™ Gold GSE and GSKE is GRAS for use in fruit juices (unless prohibited by a standard of identity), fruit flavored beverages, fruit flavored beverage mixes, and carbonated fruit flavored beverages at a concentration of approximately 210 ppm (w/v) or 50 mg per 8 fluid ounce serving.

Edward J. Race
Director of Research
Polyphenolics
Road 12667 Road 24
Madera, CA, 93639

SUMMARY OF DATA FOR CHEMICAL SELECTION

Oligomeric Proanthocyanidins from Grape Seeds and Pine Bark

BASIS OF NOMINATION TO THE CSWG

Grape seed extract and pine bark extract, including the proprietary products Pycnogenol® (pycnogenol) and Masquelier's™ Original OPC[1]s (OPCs), are brought to the attention of the CSWG because of widespread consumer use of these substances as dietary supplements.

Based on the structures of identified active ingredients, the phenolic compounds extracted from grape seeds and pine bark would not be expected to be genotoxic. Indeed, the inventor of pycnogenol states that this product was tested in *Salmonella typhimurium* and was negative. Substantial health claims for grape seed and pine bark extracts, including some studies showing tumor inhibition, make them attractive materials for further study. Antioxidants, especially polyphenols, in red wine have been proposed as an important contributory factor to the protective effect of regular alcohol use against atherosclerotic cardiovascular disease. Grape seed and pine bark extracts may have the beneficial effects of red wine without its detrimental effects.

Because grape seed and pine bark extracts are dietary supplements, the government cannot compel the manufacturer to test the safety of these materials. Given their potential benefits, an independent demonstration of their safety appears warranted. It should be noted that the manufacturer of Masquelier's™ Original OPCs claims to have conducted some testing, but the results were not available for our review.

SELECTION STATUS

ACTION BY THE CSWG: 9/28/00
Studies requested:
Subchronic (90-day) testing

[1]OPC stands for Oligomeric ProanthoCyanidins.

Ames *Salmonella* and micronucleus assays
Reproductive effects and teratogenicity

Priority: High

Rationale/Remarks:

Pycnogenol products based on pine bark and grape seed extract are closely related in term of their active ingredients, polyphenolic antioxidants believed to account for the low incidence of death from coronary heart disease in the French despite diets rich in saturated fat

Testing of Pycnogenol® is recommended as representative of dietary supplements with activivity based on OPCs because it is well characterized

Although they are widely used dietary supplements, the toxicity of these products has not been well characterized

NCI will conduct the Ames and mouse lymphoma assays on one of the extracts.

INFORMATION OBTAINED AFTER THE CSWG MEETING

A recent estimate for the US grape extracts market is $40 to $50 million per year. Perhaps the largest producer of grape extracts in the world is Indena, which sells Leucoselect Phytosome in the US. Indena has completely elucidated the composition of Leucoselect and conducted extensive *in vitro* testing of its antioxidant properties. According to the company, Indena has also conducted animal and human studies to show the effectiveness of Leucoselect in protecting the cardiovascular system. A US producer of grape seed extract, Polyphenolics was founded by Canadaigua Brands, the secon largest supplier of wine in the US. Information on Polyphenolics products is available on their website, ~~anti-oxidant.com~~ polyphenolics.com (Boswell, 2000).

CHEMICAL IDENTIFICATION

CAS Registry Nos: No CAS numbers have been assigned to grape seed or pine bark extracts

CAS Names: No names for grape seed or pine bark extracts have been assigned in the Chemical Abstract Service 8th or 9th Collective Index.

Structure, Molecular Formula, and Molecular Weight: Grape seed and pine bark extracts, including Pycnogenol® and Masquelier's™ Original OPCs, are mixtures of active and inert ingredients. The structures of some compounds proposed as active ingredients are presented in the section on Structure-Activity Analysis.

Description:

Grape Seed Extract & Grape Seed OPCs: Extracts of the seeds of the *Vitis vinifera* grape (American Botanical Council, 2000). Some extracts are also prepared from seeds, skin, and stems of other varieties of red or green grapes (Life Extension Magazine, 1997).

Pycnogenol® and Masquelier's™ *Original Pine Bark OPCs* Specific blends of procyanidins extracted from bark of the *Pinus maritima*; a light beige colored powder with astringent taste, very soluble in water and ethyl alcohol, insoluble in chloroform, petroleum ether, and ethyl ether (Masquelier, 1987; Packer *et al.*, 1999).

Pine Bark Extracts Generic versions of pycnogenol which may be made from *Pinus maritima* or other sources of pine bark

Technical Products and Impurities: Grape seed and pine bark extracts are prepared for the common purpose of providing a source of phenolic compounds (monomers and procyanidins). The most common pine bark extract is pycnogenol, which is described in US Patent 4,698,360.[2] Pycnogenol® and Masquelier's™ Original Pine Bark OPCs are virtually identical pine bark extracts that share US Patent 4,698,360 issued on October

[2]In this Summary Sheet, pycnogenol refers to a blend of polyphenolic compounds extracted from the bark of *Pinus maritima*. Pycnogenol® refers to the pycnogenol product sold in the US by Horphag Overseas Ltd.

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6,1987, and hence a litigious history. In the United States, one patent assignee, Horphag Overseas Ltd., registered pycnogenol as a trademark. Thus, the other assignee to patent 4,698,360 cannot market its product in the United States as pycnogenol. The assignee has been acquired by International Nutrition Company, Inc. (INC), which markets its pycnogenol product in the United States under the name Masquelier's™ Original Pine Bark OPCs (INC, 2000; Masquelier, 1987; Scambuster.com, 2000).

The pycnogenol products, Pycnogenol® and Masquelier's™ Original Pine Bark OPCs are sold in the form of 10-100 mg capsules or tablets through various marketing channels (Drugstore.com, 2000; INC, 2000; Horphag, 2000; Infoarea.com, 2000; Lifeplus Vitamins, 2000).

Pycnogenol® is distributed in the US by Henkel Nutrition and Health Group (Anon., 1998). According to Horphag, its pycnogenol products are available at Walmart, Mother Nature, Drugstore.com, GNC, Vitamin Shoppe, Whole Foods, Green Tree, Puritan's Pride, Cyberpharm, TPT, Inc., Martin Health Care Products, Derma E, and other outlets (Horphag Research LTD, 2000).

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In North America, Integrated BioCeuticals, LLC, is INC's main business-to-business distributor for Masquelier's™ Original OPCs raw materials and Primary Services International distributes INC's French pycnogenol extracts under the names Masquelier's™ Original Pine Bark OPCs, and Masquelier's™ Original Grape Seed OPCs. Consumers can obtain these products from BIMINI, BioNutrients, Flora, Healthysource, Life Plus, Naturalife, Nature's Sunshine, Nature's Way, Dr. Nguyen's, Primary Source, Pure, Roes, Shaprite, Source Naturals, and Standard Process (Healthysources, 2000; INC, 2000).

Generic pine bark extracts and grape seed extracts are also manufactured and distributed by various dietary supplement suppliers through vitamin stores, pharmacies, mass marketers, and Internet channels.

Chemical Composition: Standardized grape seed extracts are reported to contain 92-95% OPCs (Wholehealthmd.com, 2000). The second highest concentration of OPCs, 80-85%, is found in pine bark (Wellness Web, 2000).

Although the chemical composition has not been elucidated completely, the main constituents of grape seed and pine bark extracts are phenolic compounds, broadly divided into monomers (catechin, epicatechin, and taxifolin) and condensed flavonoids of various chain lengths that release anthocyanins when heated in acidic conditions. Pine bark extract also contains phenolic acids (such as caffeic, ferulic, and *p*-hydroxybenzoic acids) as minor constituents and glycosylation products, i.e., glucopyranosyl derivatives of either flavanols or phenolic acids as minute constituents (Anon.,1998; Packer *et al.*, 1999).

The cocktail of flavanoids varies from one species to another. The pine possesses a high level of monomers of the catechin type. The grape contains more oligomers, and the predominant monomer is epicatechin (Healthysource, 1999).

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EXPOSURE INFORMATION

Production and Producers:

Manufacturing Process. A wide variety of extraction techniques are employed by grape seed and pine bark extract manufacturers. Depending on the extraction technique used, the procyanidins in the extracts can be present in different sizes or degrees of polymerization. Some manufacturers specifically target smaller oligomers while others target a broad range of monomers, oligomers, and polymers. Some manufacturers desire a small percent of monomers (i.e., catechins) in their final extract while others include a higher percentage of monomers (Omegabiotech.com, 2000).

Masquelier's™ Original Pine Bark OPCs are manufactured according to US Patent 4,698,360. Maritime pine bark is reduced to a coarse powder and extracted with boiling water. Sodium chloride or ammonium sulfate are added up to saturation to the cooled and filtered liquid, and the precipitate formed is discarded. The remaining liquid is extracted with ethyl acetate. The extract is dried and brought back to 1/5 its volume by vacuum distillation. It is then poured into three volumes of chloroform, and stirred mechanically to precipitate the proanthocyadins. The proanthocyadins are collected by filtration purified by redissolution in ethyl acetate and reprecipitated in chloroform. The proanthocyadins are finally washed with chloroform and dried at reduced pressure in a heating chamber (Masquelier, 1987).

For preparation of grape seed extract, catechins and proanthocyanidins can be extracted from winery by-product pomace (Alonso *et al.*, 1991).

Production/import level.

Grape seed extract: In 1997, grape seed extract was reported to be the 7th most popular herbal supplement sold by food, drug, and mass market retail outlets in the US (Blumenthal, 1998). For the period August 1998 to July 1999, grape seed extracts were reported to be the 9th leading herbal supplement in the US with 3.6 million units

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purchased (Sauer,1999). The herb and botanical market was estimated at $4.3 billion in 1999 according to the Nutrition Business Journal (Wilhelm, 2000).

Pine bark extract: Currently, pine bark extracts (*i.e.*, pycnogenol) dominate the US market (mothernature.com, 2000). US sales of Pycnogenol® increased 50 percent from 1996 to 1997. In 1997, Pycnogenol® ranked eight among the best-selling botanical supplements in US pharmacies according to Information Resources, Inc. (Anon., 1998).

No figures on US sales of INC's OPCs or other pine bark extracts were available in the published literature.

Use Pattern: Pycnogenol®, OPC, and grape seed and pine bark extracts are used as nutritional supplements and phytochemicals for various diseases (American Botanical Council, 2000; Packer *et al.*, 1999). According to an unconfirmed Internet source, every day more than 4 million pycnogenol capsules and tablets are taken throughout the world (Pycnogenol: Power Oxidant, 2000). Similar information on grape seed extract was not identified in the available literature.

Grape seed extract is given for general health purposes and to treat microcirculatory maldistribution of blood flow, altered capillary fragility and permeability, and as an anti-inflammatory (American Botanical Council, 2000).

The therapeutic use of pine bark may be traced to ancient medicine in both the Old World and in the Americas. In the 4th century, Hippocrates mentioned its use, and in 1497, pharmacist H. Minner noted that pine bark was helpful for wound healing. In old Europe, pycnogenol was also used to overcome the symptoms of scurvy. In the Americas, natives used pine bark as a food, beverage, and as a remedy for inflamed wounds or ulcers (Packer *et al.*, 1999).

Pycnogenol® is now taken as a dietary supplement to strengthen capillaries and blood vessels; protect blood vessel linings; reduce LDL, or "bad" cholesterol; improve blood

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flow; and reduce platelet aggregation (Anon., 1998). Horphag also cites papers giving other uses of Pycnogenol® on its webpage, including prevention of Alzheimer's disease and memory improvement (Horphag Research LTD, 2000).

The use of pycnogenol for treatment of attention-deficit hyperactivity disorder has been publicized among parent groups for at least 7 years (Greenblat, 1999). Not much information about indication, dosing, safety, efficacy, bioavailability, pharmacokinetics, and pharmacodynamic factors can be conveyed due to a lack of information, including double-blind, controlled studies to support the claimed beneficial effects (Heimann, 1999).

Human Exposure: The primary source of human exposure to grape seed extract, Pycnogenol®, and OPCs is via the manufacturing, distribution, and consumer use of dietary supplements.

According to the US patent, pycnogenol/OPCs may be administered orally, intravenously, or cutaneously. For oral administration, pycnogenol/OPCs is in the form of tablet, sugar coated pills, pellets, capsules, cachets, and drinkable ampoules. Pycnogenol/OPCs may be used in the form of an ointment. The oral dose is generally from 1.5 to 3 mg/kg b.w. per day, which represents a daily dose of 100 - 200 mg a day for a 70 kg man (Masquelier, 1987). All identified sources of pycnogenol supplements were in capsule form.

Grape seed extract in the form of tablets, liquids, or capsules is given at an average daily dose of 50-300 mg (American Botanical Council, 2000; Wholehealthmd.com, 2000).

Environmental Occurrence: OPCs are found in many woody plants. The two most common sources are grape seeds and the white pine of southern Europe. OPCs are also abundant in blackjack oak, horse chestnut, witch hazel, and hawthorn, as well as in apples, berries, barley, bean hulls, chocolate, rhubarb, rose hips, and sorghum (Sterling, 2000). Grape

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seed and pine bark extracts are manufactured products and do not occur, *per se*, in the environment.

Regulatory Status: Since 1994, dietary supplements have been regulated under the Dietary Supplement Health and Education Act (DSHEA). For dietary supplements on the market prior to October 15, 1994, the DSHEA requires no proof of safety in order for them to remain on the market. The labeling requirements for supplements allow warnings and dosage recommendations as well as substantiated "structure or function" claims. All claims must prominently note that they have not been evaluated by the FDA, and they must bear the statement "This product is not intended to diagnose, treat, cure, or prevent any disease" (FDA, 1995).

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EVIDENCE FOR POSSIBLE CARCINOGENIC ACTIVITY

Human Data: No epidemiological studies or case reports investigating the association of exposure to grape seed or pine bark extracts were identified in the available literature.

Pycnogenol has reportedly been taken in Europe under medical supervision for decades with no reports of adverse effects. An unconfirmed Internet source reports that daily doses of up to 35,000 mg of pycnogenol given to humans for six months produced no adverse effects (Healthysource.com, 1999; Pycnogenol: Power Antioxidant, 2000).

Pycnogenol was cited in 17 Adverse Event Reports and grape seed extract was cited in 9 Adverse Event Reports made to the FDA Office of Special Nutritionals as of October 20, 1998, out of a total of 2621 adverse events involving 3451 products. According to FDA, there is no certainty that a reported adverse event can be attributed to a particular product or ingredient (FDA, 1998a,b).

According to an Internet source, there have been reports of a possible decrease in effectiveness of antibiotics, specifically tetracycline and tetracycline derivatives caused by grape seed extracts (BroadcastHealth.com, 2000).

Animal Data: The manufacturer has stated that pycnogenol has been tested at expert centers including the Pasteur Institute in Lyon, France, and BSL Bioservice in Munich, Germany and is nontoxic, nonteratogenic, nonmutagenic, noncarcinogenic, and nonantigenic (Healthysource, 1999; INC, 2000). Details of these studies were not available for review.

Dogs given a daily dose of OPCs from grape seeds equivalent to a 150-lb human taking 19,800 mg/day in a one year study reportedly showed no adverse effects (Innovative Technologies Corporation of America, 1999). No study details were provided.

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Short-Term Tests: According to the manufacturer, Masquelier's™ Original Grape Seed OPCs was tested for mutagenicity in a reverse mutation assay using *Salmonella typhimurium*. In contrast to some bioflavonoids, the product "passed the mutagenicity test without the slightest sign of mutagenicity" (INC, 2000). The test results were not available for our review.

Several procyanidins with different degrees of polymerization (dimers, a trimer, and a polymer) extracted from different natural sources were found to be nonmutagenic in the *Salmonella* mutagenesis assay system (Yu & Swaminathan, 1987).

In contrast, a recent study reported that all dimeric polyphenols and the galloylated metabolites isolated from grape seeds potentiated the mutagenic activity induced by the indirectly acting carcinogen *N*-nitrosopyrrolidine in the presence of an activation system used to activate CYP2E1(Catterall *et al.*, 2000).

Metabolism: Grape seed procyanidins obtained from plants grown in a ^{14}C-enriched environment have been used to study the bioavailability of complex mixtures of flavonoids. Absorption of radiolabeled procyanidins administered orally to mice began 10 minutes after ingestion and slowly declined after 3-7 hours. Procyanidins had a propensity for proline-rich tissues. Thus the aorta was about 10 times more enriched than the lungs and 5 times more than the liver (Packer *et al.*, 1999).

Preliminary data is also available on the bioavailability of pycnogenol in humans. Unmodified proanthocyanidins were detected in the saliva one hour after the ingestion of 150 mg of encapsulated pynogenol (Masquelier, 1987). The urine collected for 24 hours after human subjects were administered single doses of 200 mg of pycnogenol contained ferulic acid and esters of other hydroxycinnamic acids (Packer *et al.*, 1999). The more complex components of pycnogenol, the oligomeric procyanidins appear to undergo biologic modification after ingestion in humans (Packer *et al.*, 1999).

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Other Biological Effects: It is not clearly agreed upon what size oligomer or polymer is responsible for what degree of biological activity. Some sources agree that it is only the dimers and trimers that show health benefits while others insist that larger polymers and even monomers can be linked to antioxidant activity (Omegabiotech.com, 2000). The following reports summarize the status of current research.

Tumor Inhibition and Related Studies. Bomser and coworkers (1997) examined the antitumor promoting activity of a polyphenolic fraction of grape seeds (GSP) in CD-1 mouse skin epidermis. Pretreatment of mouse skin with GSP resulted in dose-dependent reductions in 7,12-dimethylbenz[a]anthracene (DMBA)-initiated skin tumor incidence and number of tumors per mouse.

Pine bark extract has been reported to inhibit the tumor promotion process on the epidermis of mice (Kensler *et al.*, 1983). No details of the study were given.

Intragastric administration of pycnogenol inhibited the metabolic activation of tobacco-specific nitrosamine, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the lung microsomes of F344 rats. Pycnogenol was not effective in liver microsomes (Huynt & Teel, 1999).

Exposure of pBR322 plasmid DNA to an iron/ascorbic acid system resulted in cleavage/damage of DNA by hydroxyl radical. Pycnogenol significantly monimized this cleavage (Nelson *et al.*, 1998).

Antioxidant/Free Radical Scavenging Properties. Grape seed extract administered to female Swiss-Webster mice caused a dose-dependent inhibition of (TPA)-induced lipid peroxidation and DNA fragmentation in the hepatic and brain tissues, as well as activation of peritoneal macrophages (Bagchi *et al.*, 1998).

Blazso and coworkers tested the *in vivo* $O_2^{\cdot-}$ scavenging activity of pycnogenol and three of its chromatographic fractions (fraction 1, monomeric flavonoids [taxifolin, catechin,

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epicatechin] and phenolic acids [caffeic, ferulic, and vanillic]; fraction 2, procyanidin dimers, trimers, and tetramers; and fraction 3, oligmers >four subunits). The whole extract and each of its fractions inhibited superoxide-induced reduction of nitroblue tetrazolium to formazan in a dose-dependent fashion. The most active fraction was fraction 3. Other investigators have reported that pycnogenol is an efficient scavenger of both $O_2^{\cdot-}$ and $HO^{\cdot}$ and that anthocyanidins in general are potent scavengers of ·NO and $ONOO^-$ (Packer *et al.*, 1999).

Pycnogenol has been reported to protect the low-density lipoprotein fraction of human plasma from copper-induced oxidation and to interact with cellular antioxidants. Supplementation with pycnogenol in the diet was found to be associated with a significant increase in _-tocopherol levels in rat hearts. Pycnogenol has also been studied in relation to its ability to protect against ultraviolet (UV) radiation-induced injury and as a preventive antioxidant by chelating transition metals (Packer *et al.*, 1999).

Proanthocyanidin-rich grape seed extract (0.1 or 1% in diet) attenuated the development of aortic atherosclerosis in cholesterol-fed rabbits (Yamakoshi *et al.*, 1999).

Cardiovascular Effects. Pycnogenol has been reported to have activities related to cardiovascular functionality, such as a vasorelaxant activity, inhibition of angiotensin-converting enzyme, and the ability to enhance microcirculation (Packer *et al.*, 1999).

In a European study, patients with peripheral circulatory disorders were given pycnogenol (dosage not reported) for 30 days. Pain, limb heaviness, and feelings of swelling decreased significantly during therapy in most patients. Criteria for evaluating a decrease in symptoms were not reported (Cicero *et al.*, 1996).

Inhibition of Inflammatory Responses. Blazso and coworkers demonstrated that oligomeric procyandins in pycnogenol can inhibit localized inflammatory responses (edema) caused by croton oil administered to the mouse ear (Packer *et al.*, 1999).

Other Effects. Preliminary studies have indicated that pycnogenol significantly inhibits the activity of enzymes which produce free radicals in biological systems, namely horseradish peroxidase, lipoxygenase, and xanthine oxidase (Packer *et al.*, 1999).

Preexposure to grapeseed extract seven days in advance of hepatotoxic doses of acetaminophen appeared to significantly attenuate acetominophen-induced hepatic DNA damage, apoptotic and necrotic cell death of liver cells, and antagonize the influence of acetominophen-induced changes in the antiapoptotic gene, bcl-XL, expression in ICR mice (Ray *et al.*, 1999).

Structure Activity Relationships: In lieu of a traditional SAR analysis, the information suggesting carcinogenicity/anticarcinogenicity of the various components of grape seed and pine bark extracts were examined. These extracts contain phenolic monomers (catechin, epicatechin, and taxifolin) and condensed flavonoids of various chain lengths (procyanidins/proanthocyanidins). Pine bark extract (e.g., Pycnogenol®/OPCs) also contains caffeic, ferulic, and *p*-hydroxybenzoic acids as minor constituents (Anon., 1998; Packer *et al.*, 1999).

Flavonoids are potentially able to quench free radicals by forming resonance-stabilized phenoxyl radicals. Depending on their structure, flavonoids display possibly inhibitory effects on the growth and proliferation of certain malignant cells *in vivo*. These effects are thought to be either direct, due to the electron- and proton-donating capacity of flavonoids, or indirect, due to their ability to alter the activities of key enzymes in cellular response. The antiproliferative effect of monomeric flavonoids was suggested to be partly mediated by inhibition of tyrosine kinase activity, by decrease of c-*jun* m-RNA expression, or by inhibition of c-*jun* N-terminal kinase activation. Moreover catechins were reported to inhibit the interaction of tumor promoters with receptors. Epicatechin derivatives significantly inhibit NADPH cytochrome c reductase activity. The alteration of the NADPH cytochrome c reductase due to flavonoids is believed to play a key role in the inhibition of the mutagenicity induced by aromatic hydrocarbons and aflatoxin B_1 (Packer *et al.*, 1999).

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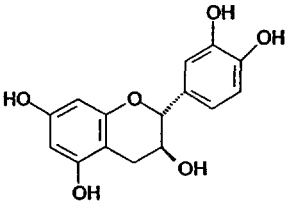
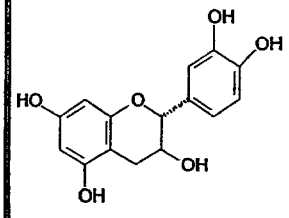
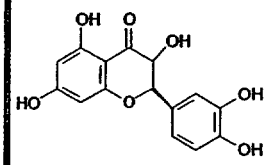
A review of the information on carcinogenicity, mutagenicity, and tumor inhibition/anticarcinogenicity/antimutagenicity found in a search of the National Library of Medicine databases, CCRIS, Genetox, and Toxline and the National Institute of Occupational Safety and Health database, Registry of Toxic Effects of Chemical Substances, is presented below in Table 1 below.

Table 1. Summary of information on active components of pine bark and grape seed extracts

Compound	Carcinogenicity Data	Mutagenicity Data	Inhibition/Anticarcinogenicity/Antimutagenicity Data
Monomers			
catechin [154-23-4] 	Induced glandular stomach adeno-carcinomas in F344 rats given 2% in diet for 104 wk (NLM, 2000) Promoted forestomach and glandular stomach cancers in F344 rats initiated with MNNG (NLM, 2000a)	No conclusions when tested for chromosome aberrations in micronucleus test in mammalian polychormatic erythrocytes (NLM, 2000b) DNA repair in *E. coli* (RTECS, 1999) Sister chromatid exchanges, unscheduled DNA synthesis, DNA inhibition & sex chromosome loss and nondisjunction in human lymphocytes (RTECS, 1999)	Inhibited DMBA-initiated mammary gland tumors in Wistar rats, DMBA-initiated and croton oil promoted skin papillomas in Swiss mice & B[a]P-initiated forestomach tumors in Swiss mice (NLM, 2000a) Ineffective vs EHEN-initiated liver or kidney tumors in Wistar rats, vs NHA-initiated pancreatic adenocarcinomas in Syrian hamsters, & DMBA-initiated and TPA-promoted dermal tumors in CF-1 mice (NLM, 2000a) Slightly reduced mammary gland tumor incidence in C3H mice given 2.5 mg/d in drinking water for 15 months [8/20 vs 12/20] (CCRIS,2000)

epicatechin [490-46-0] 	No information found	Sister chromatid exchanges in human lymphocytes (RTECS, 1999)	Inhibited multiplicity of DMBA-initiated and croton oil-promoted dermal tumors in Swiss mice and 3-MC-initiated sarcomas in Swiss mice (NLM, 2000a) No effect on mutagenicity of B[a]P or IQ w/wo S-9 (Catterall *et al.*, 2000)
taxifolin [480-18-2] 		Mutagenic in *S. typhimurium* w/wo S-9 (no details) (RTECS, 1999) Positive in *in vitro* cytogenetic analysis (hamster fibroblasts) (RTECS, 1999)	No information found
Condensed flavonoids procyanidins/proanthocyanidins	Keracyanin (related compound) was negative when adminitered in the diet of Wistar rats for 2 years (Tsubura *et al.*, 1983)	Procyanidin dimers, a trimer, and a polymer were nonmutagenic in the *Salmonella* mutagenesis assay system (Yu & Swaminathan, 1987).	No information found

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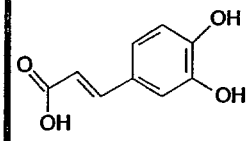
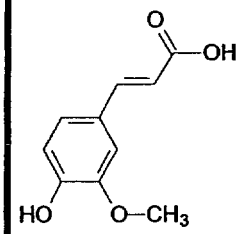
Phenolic acids caffeic acid [331-39-5] 	Induced forestomach papillomas & squamous cell carcinomas in F344 rats when fed at 2% in diet for 104 wk (NLM, 2000a) Induced lung and kidney tumors in B6C3F1 mice when fed at 2% in diet for 96 weeks (NLM, 2000a)	Weakly mutagenic in *Salmonella* Ara test (Ariza *et al.*, 1988) Positive in mouse lymphoma L5178Y (TK+/TK-) assay wo S-9; negative in mouse lymphoma assay w S-9 and in *S. typhimurium* TA98, TA100, TA1535, TA1537 & TA1538 w/wo S-9 (NCI STTP Program reported in NLM, 2000a)	Inhibited AFB1- induced mutagenesis in *S. typhimurium* TA98 with S9 (San & Chan, 1987) and in *S. typhimurium* TA1535 (Chan *et al.*, 1986) Tested in various protocols with variable results (NLM, 2000a) Did not inhibit benzidine mutagenesis in *S. typhimurium* TA-98 w/wo S-9 (Josephy *et al.*, 1985)
ferulic acid [537-98-4] *trans*-ferulic acid [1135-24-6] (4-hydroxy-3-methoxycinnamic acid) 	No information found	Negative in *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 w/wo S-9: (NCI STTP Program reported in NLM, 2000a)	Inhibited DMBA-initiated skin tumors promoted by TPA in CD-1 mice and NQO-initiated tongue carcinomas in F344 rats (NLM, 2000a) Not effective vs B[a]P-initiated lung tumors in Strain-A mice, DMBA-initiated skin tumors promoted by TPA in ICR or NMRI mice, or forestomach tumors initiated by B[a]P gavage in ICR mice (NLM, 2000a) Did not inhibit benzidine mutagenesis in *S. typhimurium* TA-98 w/wo S-9 (Josephy *et al.*, 1985)

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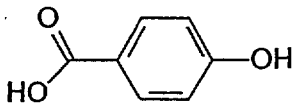
p-hydroxybenzoic acid [99-96-7] 		Negative in *S. typhimurium* TA97, TA98, TA100 & TA102 w/wo S-9 (Kako *et al.*, 1992)	

B[a]P = benzo[a]pyrene; DMBA = 7,12-dimethylbenz[a]anthracene; EHEN = *N*-ethyl-*N*-hydroxyethylnitrosamine; 2-amino-3-methylimidazo-[4,5-f]quinoline = IQ; MNNG = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; 3-MC = 3-methylcholanthrene; NHA = *N*-nitrosobis(2-hydroxypropyl)amine; NQO = *N*-nitroquinoline-1-oxide; TPA = 12-*o*-tetradecanoylphorbol-13-acetate; w/wo = with or without

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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.

Flavonoids A subclass of the body of polyphenols and can be characterized by division into several classes according to the degree of oxidation of the oxygen heterocycle including flavones, flavonols, isoflavones, anthocyanins, flavanols, 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 publicly available to adequately evaluate potential human health hazards.

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

Phenols	Any of the various acidic compounds analogous to phenol and regarded as hydroxyl derivatives of aromatic hydrocarbons.
Phenol 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 dimeric tannins which derive from catechins and / or epicatechins.
ROS	Reactive oxygen species such as singlet oxygen $^1O_2$ and $O_2^{\bullet -}$, $OH^{\bullet}$, $NO^{\bullet}$, 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.

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 generally recognized as safe (GRAS) food ingredients for use in fruit juices and fruit flavored beverages at a total composite 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 beverages for which no standard of identity exists in order 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. Antioxidant activity may also beneficially modulate immune function and platelet aggregation.

The purpose of this notification is to summarize the technical, safety, product information, and considerations used to support a self-determination by the Polyphenolics Division as to the GRAS status of GSE and GSKE as antioxidant substances added to food products for which no standard of identity exists. This determination was supported by the evaluation of 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 toxicological information on GSE, GSKE, constituent proanthocyanidins, and related polyphenols was conducted through August 2001, summarized and included in a report to the Expert Panel. The results and conclusions of a mutagenicity [Erexson, 2003] and a 90-day safety study [Bentivegna and Whitney, 2002], undertaken by Polyphenolics on GSE / GSKE, were also included therein. This report was subsequently made available to the members of Expert Panel to assist and facilitate the deliberation of the Expert

Panel in their evaluation of GSE and GSKE GRAS status. All of this information is provided herein.

The Expert Panel independently evaluated the material submitted, as well as other materials deemed appropriate or necessary. Following a critical evaluation, the Expert Panel conferred and unanimously agreed to recommend that MegaNatural™ Gold GSE and GSKE, meeting the specifications cited, be considered 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 (21 CFR §182.1(b)) in a composite 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 as Appendix A of this dossier.

Polyphenolics subsequently submitted to FDA on December 17, 2001, a GRAS Notification (GRN 000093) informing FDA that it had determined the intended use of GSE and GSKE to be GRAS. FDA responded in a letter dated June 5, 2002 that the Notification did not provide a sufficient basis to conclude that GSE and GSKE were GRAS in their intended uses. Following consultations with the Food and Drug Administration in July of 2002, additional chemistry and newly published pivotal safety data [Yamakoshi *et al.*, 2002; Wren *et al.*, 2002] was assembled and supplied to the Expert Panel. This data was meant to clarify Agency concerns and is provided herein. This additional data in no way materially affected the original Panel decision as to GRAS status of the MegaNatural™ Gold GSE and GSKE products. However, an Addendum to the Expert Panel Statement, provided herein in Appendix B, was issued acknowledging the revised data and affirming the unanimous agreement as to the GRAS status of MegaNatural™ Gold GSE and GSKE for their intended use in specific beverage products for which no standard of identity exists.

1.2 Regulatory Basis For GRAS Determination

As per volume 62 of the Federal Register, page 18938, (proposed 21 CFR §170.36), the Polyphenolics Division of the Canandaigua Wine Company Inc. 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

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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. Recently published pivotal safety data [Yamakoshi *et al.*, 2002; Wren *et al.*, 2002] is supported by the results of MegaNatural™ Gold GSE and GSKE studies [Erexson, 2003; Bentivegna and Whitney, 2002]. Relevance of the published studies was established through a comparison of the PAC and polyphenolic chemical profiles of the respective products. All of this data has been supplemented by the use of scientifically relevant statistical reference sources, compendia, books, and reviews.

The GRAS determination by the Polyphenolics Division of the Canandaigua Wine Company Inc. was therefore based on the weight of currently available scientific information and grounded upon generally available scientific data. The GRAS determination is the direct result of a consensus among a panel of qualified experts that there was reasonable certainty that these substances, GSE and GSKE, under the intended conditions of use, will not be harmful. This GRAS determination therefore meets the requirements of §201(s) of the Federal Food, Drug, and Cosmetic Act; 21 CFR §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.

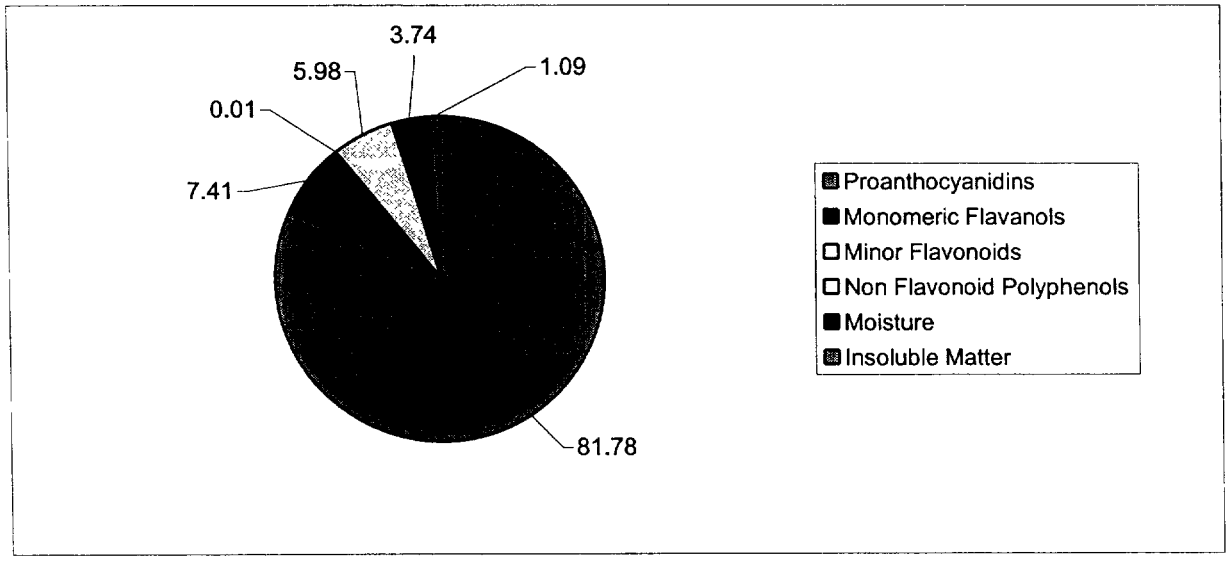
- The polyphenolic compounds comprising the MegaNatural™ Gold GSE and GSKE are naturally occurring and found in diverse types of foods.

- 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 and guidance 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 [Erexson, 2003] 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 [Bentivegna and Whitney, 2002]. These results support studies undertaken by independent investigators [Yamakoshi *et al.*, 2002; Wren *et al.*, 2002; Bagchi *et al.*, 2001] evaluating commercially available products of a substantially equivalent PAC make-up and general polyphenolic profile.
- 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

The following section provides a basic discussion of the general chemistry of polyphenolic compounds, including their nomenclature and categorization. This is followed by an evaluation of the antioxidant capacity of the polyphenolic compounds known to exist in grapes and grape products such as MegaNatural™ Gold GSE and GSKE. Finally, a background discussion regarding the long history of safe human consumption is provided. These three concepts provided a framework to facilitate evaluation of the safety of MegaNatural™ Gold GSE and GSKE as food ingredients for use in fruit juices and fruit flavored beverages for which no standard of identity exists. Figure 2.1 and Figure 2.2 provide the mean chemical profile of 6 lots each of GSE and GSKE. They are meant to provide a basis of comparison for the following discussions of chemistry, antioxidant capacity, and history of use. A complete quantitative analytical evaluation of GSE and GSKE is provided in Section 3.0.

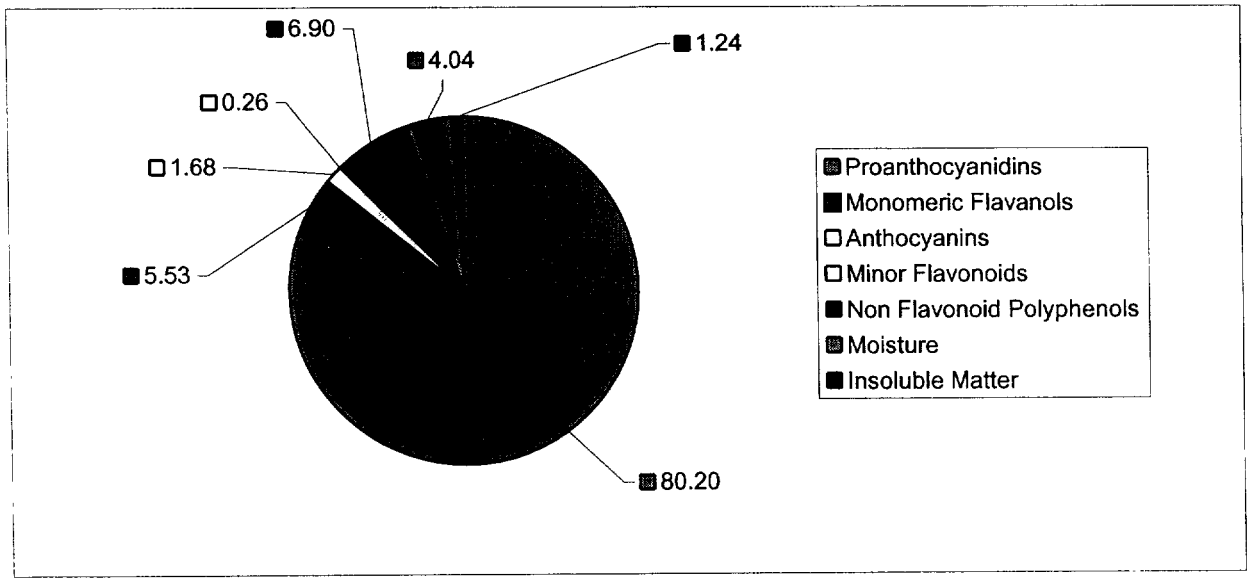
Figure 2.1 Mean (n=6) Analytical Profile of MegaNatural™ Gold GSE (g/100g)



Total flavonoids = 89.2%

Total polyphenols = 95.18%

Figure 2.2 Mean (n=6) Analytical Profile of MegaNatural™ Gold GSKE (g/100g)

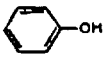
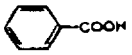
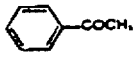
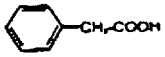
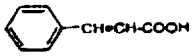
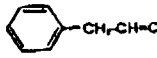
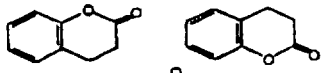
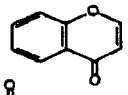
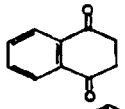
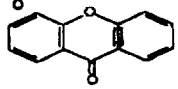
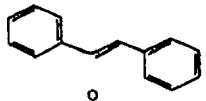
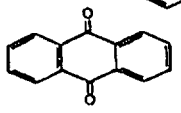


Total flavonoids = 87.67%
Total polyphenols = 94.57%

2.1 Polyphenolic Compounds

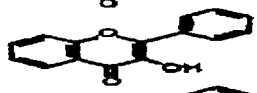
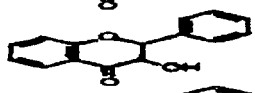
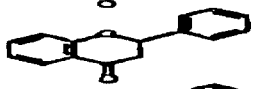
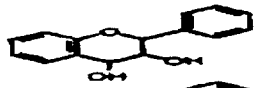
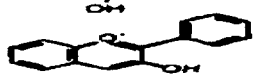
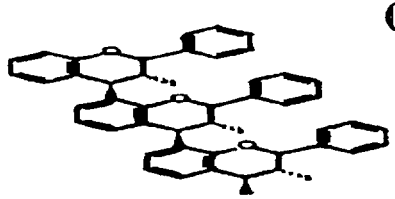
Phenolic compounds or polyphenols constitute an extremely complex and widely distributed group of plant substances. Polyphenols are the products of plant metabolism and arise from two main synthetic pathways, the Shikimate and the acetate pathways. Natural polyphenols can range from simple molecules, 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	$(C_6$-$C_3)_n$	

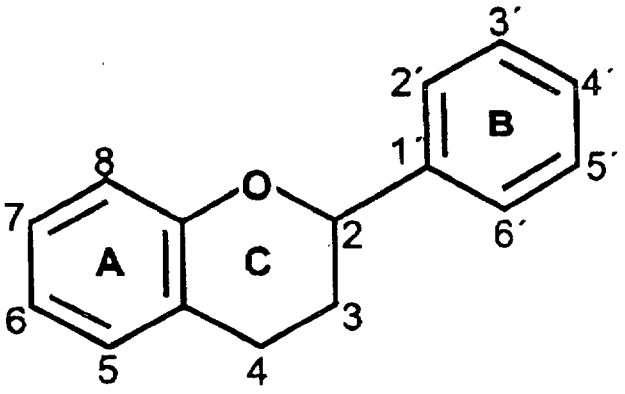
The flavonoid group of chemicals is a subclass of the huge body of polyphenols. They constitute the most common group of plant phenols and as such are widely distributed in nature and are the most abundant polyphenols in the human diet [Baldi *et al.*, 1997]. They can be divided into several classes according to the degree of oxidation of the oxygen heterocycle, including flavones, flavonols, isoflavones, anthocyanins, flavanols, 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	

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 2.3. As illustrated in Table 2.2, PACs are oligomers based upon flavan-3-ol monomer units.

Figure 2.3 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] to form dimers, 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].

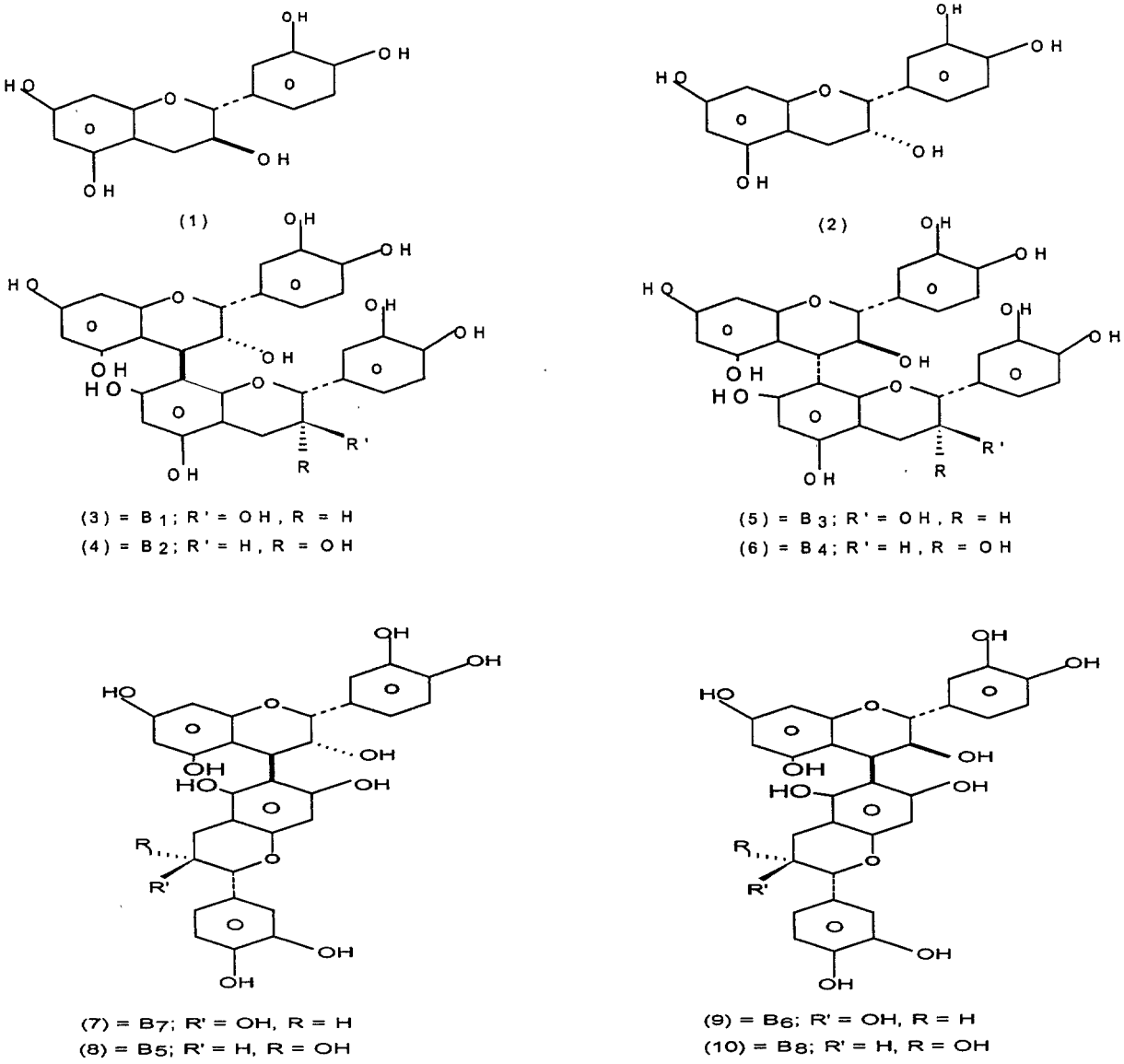
Plant tannins can be subdivided into two major groups: (1) hydrolyzable and (2) condensed tannins. The hydrolysable tannins, or anthocyanins, consist of gallic acid and its dimeric condensation product, hexahydroxydiphenic acid, esterified to a polyol, primarily glucose [Bravo, 1998]. As their name would 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 and their gallate esters. 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

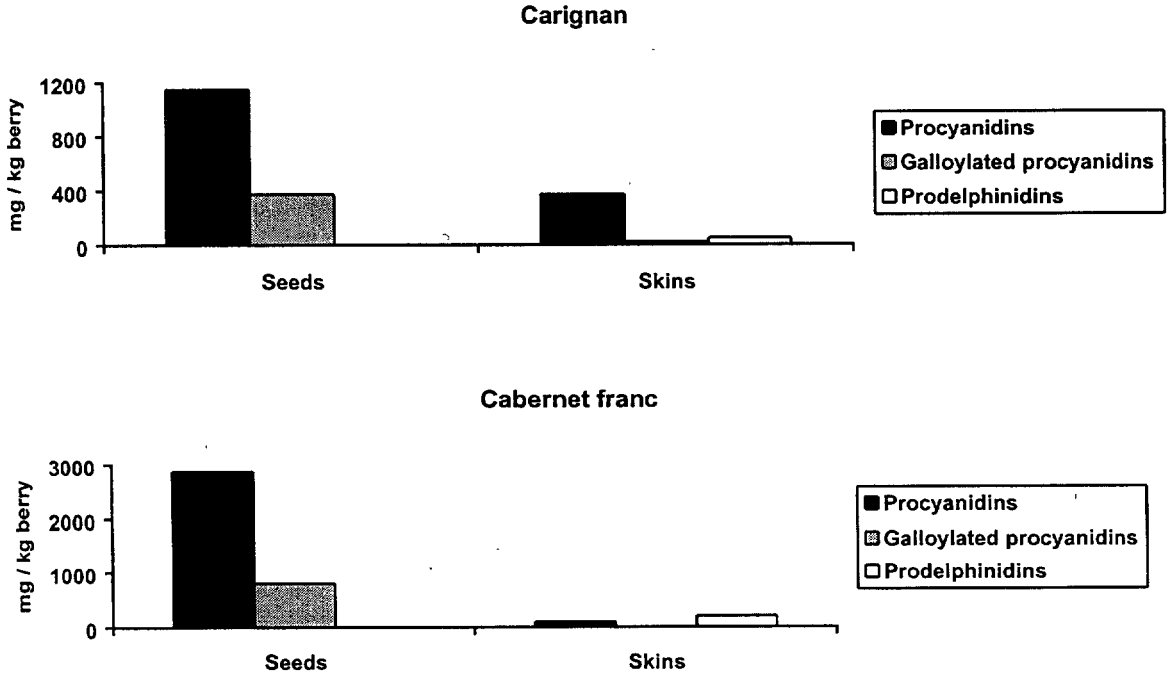
scavenging free radicals is found on the B-ring, are present in grapes. They usually consist of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-O-gallate units. With regard to grapes and wine, perhaps the most widely studied sequence of condensed PACs is the B-series, which include dimers B_1 through B_8. These are shown below in Figure 2.4.

Figure 2.4 Grape Seed Proanthocyanidins [Williams and Elliot, 1997]



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 2.5.

Figure 2.5 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 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)

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].

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A number of highly reactive oxygen species such as singlet oxygen $^1O_2$ and $O_2^{\bullet -}$, $OH^{\bullet}$, $NO^{\bullet}$, 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.

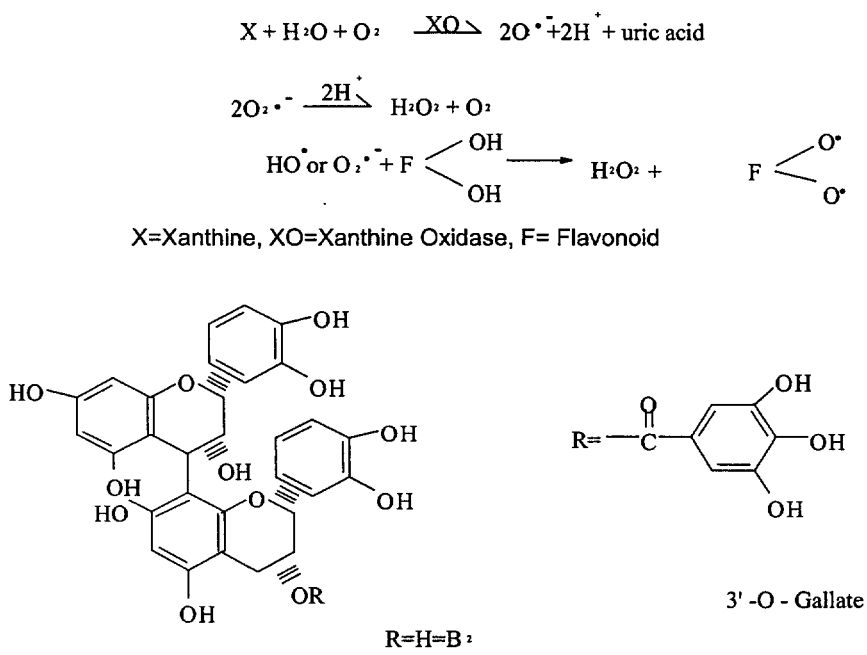
Antioxidants can be further categorized by their mode of action. They can be 1) preventative, such as catalase or superoxide dismutase, 2) chain breaking, such as vitamin E, or 3) complimentary in nature, such as Vitamin C or β-carotene. The antioxidant constituents found in grapes and wines are considered 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 $O_2^{\bullet -}$, $OH^{\bullet}$, $NO^{\bullet}$, 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 2.6.

Figure 2.6 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 RAW-264.7 murine macrophage cell line. 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 $\forall_1$AP 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 (•OH) generated from the reaction of the copper(II) complex $Cu(en)_2$ with H_2O_2. The antioxidant activity was evaluated against •OH by either its ability to suppress •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 •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 •OH, catechin displayed a very weak scavenging ability. These results indicate that the antioxidants compounds used here, including catechin, suppressed the generation of •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), interflavanoid 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) interflavanoid 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 grape seed extract as a form of nutritional supplement, as opposed to the MegaNatural™ Gold brand of GSE / GSKE used in fruit juice and fruit flavored beverages. 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. This nomination was not based on evidence indicating toxicity, potentially elevated cancer risk, or that current use was seen as unsafe. Primary interest was due to absence, at the time, of adequate published safety information. Suggested studies included 90-day subchronic testing, Ames and micronucleus assays, reproductive effects, and teratogenicity.

Subsequent to NTP's listing, subchronic rodent studies, as well as Ames and micronucleus assay results, have appeared in the published scientific literature. The results provided no evidence of genotoxicity, mutagenicity, or toxicity at oral doses many times the expected human exposure. This included an absence of any adverse indication in reproductive tissues as well as absence of data to support biologic plausibility for potential teratogenic activity.

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 PAC 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 flavanol was derived from vegetables and vegetable products. An estimate of 460 mg per day has been reported for the average American intake [Santos-Buelga and Scalbert, 2000]. Kuhnau [1976] cited the intake of polyphenols to be approximately 1 g/day. Scalbert and Williamson [2000] later determined one third of this was comprised of non-flavonoid phenolic acids, which are not found in the MegaNatural™ Gold GSE and GSKE products. For comparison, GSE and GSKE will be added to selected beverage products in the composite amount of 210 ppm or 50 mg per 8 fluid ounce serving. This data is summarized in Table 2.9.

Table 2.9 Estimated Background Ranges of PAC and / or Flavonoid Intake Compared to that From GSE / GSKE

Dietary Source	Flavonoids Consumed	Level of Intake (mg/d)	Researcher
Average German Diet	Flavonol	12.0	Linseisen *et al.* [1997]
	Catechins	8.3	
	Flavanons, anthocyanidin, proanthocyandins, and phloretin	20.3	
	Total Flavonoid	54.0	
Average US Diet	Total Flavonoid	460	Santos-Buelga and Scalbert [2000]
		666	Scalbert and Williamson [2000]
	Total Polyphenols	1000	Kuhnau [1976]
MegaNatural™ Gold GSE	Proanthocyanidins (81.77%)	40.88	Calculated based on mean analytical data and one 50 mg serving
	Catechins (7.41%)	3.71	
	Total Flavonoid (89.19%)	44.59	
MegaNatural™ Gold GSKE	Proanthocyanidins (80.2%)	40.1	Calculated based on mean analytical data and one 50 mg serving
	Catechins (5.53%)	2.76	
	Total Flavonoid (87.67%)	43.83	

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 that 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 that 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 major constituent components of the MegaNatural™ line of grape seed and grape skin extracts have been well characterized by LC-MS analytical evaluation. These data are set forth in Table 3.1 and Table 3.2 below.

Table 3.1 Analytical Evaluation of MegaNatural™ Gold GSE (g/100g)

	GSE – 1	GSE – 2	GSE – 3	GSE – 4	GSE – 5	GSE - 6	Mean (n=6)
	Lot No. 2501-100283	Lot No. 2501-80215	Lot No. 2501-110283	Lot No. 2501-120319	Lot No. 2501-11051	Lot No. 2501-21060	
Proanthocyanidins							
Dimers (M.Wt. 578)	3.67	4.06	5.07	4.04	4.13	5.26	4.37
Dimer Gallates (M.Wt. 730)	0.54	0.8	0.8	0.9	1.0	0.91	0.83
Trimers (M.Wt. 866)	1.33	1.03	1.47	1.65	1.33	1.75	1.43
Trimer Gallates (M.Wt. 1080)	0.35	0.38	0.49	0.54	0.7	0.65	0.52
Oligomers & Polymers	78.98	71.97	72.91	77.91	75.04	70.98	74.63
Subtotal	**84.84**	**78.24**	**80.74**	**85.04**	**82.20**	**79.55**	**81.78**
Monomeric Flavanols							
Catechin (M.Wt. 290)	2.56	2.77	4.24	2.34	2.42	3.18	2.92
Epicatechin (M.Wt. 290)	2.65	4.93	7.14	2.42	3.76	5.03	4.32
Monomer Galate (M.Wt. 442)	0.15	0.16	0.14	0.2	0.17	0.19	0.17
Subtotal	**5.36**	**7.86**	**11.52**	**4.96**	**6.35**	**8.40**	**7.41**
Minor Flavonoids							
Quercetin	0.006	0.001	0.007	0.004	0.006	0.003	0.004
Myricetin	0.001	0.002	0.0	0.002	0.005	0.003	0.002
Kaempferol	0.00067	0.00002	0.00008	0.00048	0.00067	0.00035	0.00038
Subtotal	**0.00767**	**0.00302**	**0.00708**	**0.00648**	**0.01167**	**0.00635**	**0.00705**

Table 3.1 Analytical Evaluation of MegaNatural™ Gold GSE (g/100g)

	GSE – 1	GSE – 2	GSE – 3	GSE – 4	GSE – 5	GSE - 6	Mean (n=6)
	Lot No. 2501-100283	Lot No. 2501-80215	Lot No. 2501-110283	Lot No. 2501-120319	Lot No. 2501-11051	Lot No. 2501-21060	
Non Flavonoid Polyphenols							
Gallic Acid	0.8	0.5	0.9	1.6	1.0	0.9	0.95
Ellagic Acid	0.018	0.009	0.016	0.006	0.01	0.011	0.012
Caffeic Acid	0.0	0.001	0.014	0.006	0.021	0.019	0.010
Chlorogenic Acid	0.059	0.022	0.043	0.015	0.038	0.001	0.030
Resveratrol	0.003	0.001	0.0004	0.001	0.002	0.001	0.001
Other	4.52	6.467	2.327	2.572	6.229	7.768	4.981
Subtotal	**5.4**	**7.0**	**3.30**	**4.20**	**7.30**	**8.70**	**5.98**
Other GSE Constituents							
Moisture	3.57	4.56	3.43	4.61	3.13	3.14	3.74
Ash	0.54	0.71	0.53	0.71	0.62	0.0	0.52
Insoluble Matter	0.28	1.62	0.47	0.48	0.39	0.2	0.57
Total (%)	**99.1**	**99.5**	**99.0**	**98.3**	**98.9**	**100.0**	**100**

000077

Table 3.2 Analytical Evaluation of MegaNatural™ Gold GSKE (g/100g)

	GSKE – 1	GSKE – 2	GSKE – 3	GSKE – 4	GSKE – 5	GSKE - 6	Mean (n=6)
	Lot No. 2511-50061	Lot No. 2511-11323	Lot No. 2511-30059	Lot No. 2511-60069	Lot No. 2511-20027	Lot No. 2511-40060	
Proanthocyanidins							
Dimers (M.Wt. 578)	3.71	2.34	3.59	3.88	4.1	4.12	3.62
Dimer Gallates (M.Wt. 730)	0.26	1.08	0.26	0.31	0.26	0.25	0.40
Trimers (M.Wt. 866)	1.57	1.27	2.63	2.33	2.67	2.65	2.19
Trimer Gallates (M.Wt. 1080)	0.37	0.42	0.12	0.19	0.11	0.11	0.22
Oligomers & Polymers	73.87	75.83	71.69	74.52	73.5	73.21	73.77
Subtotal	**79.78**	**80.94**	**78.29**	**81.23**	**80.64**	**80.34**	**80.20**
Monomeric Flavanols							
Catechin (M.Wt. 290)	2.59	3.32	2.35	2.45	2.61	2.53	2.64
Epicatechin (M.Wt. 290)	2.52	3.72	2.49	2.61	2.81	2.73	2.81
Monomer Galate (M.Wt. 442)	0.0	0.0	0.12	0.11	0.11	0.11	0.05
Subtotal	**5.11**	**7.86**	**4.96**	**5.17**	**5.53**	**5.37**	**5.53**
Anthocyanins							
Subtotal	2.11	1.12	1.49	1.69	1.89	1.78	1.68
Minor Flavonoids							
Quercetin	0.23	0.26	0.22	0.24	0.27	0.17	0.23
Myricetin	0	0	0.024	0.024	0	0.027	0.013
Kaempferol	0.024	0.002	0.023	0.028	0.026	0.017	0.020
Subtotal	**0.254**	**0.262**	**0.267**	**0.292**	**0.296**	**0.214**	**0.264**

Table 3.2 Analytical Evaluation of MegaNatural™ Gold GSKE (g/100g)

	GSKE – 1	GSKE – 2	GSKE – 3	GSKE – 4	GSKE – 5	GSKE - 6	Mean (n=6)
	Lot No. 2511-50061	Lot No. 2511-11323	Lot No. 2511-30059	Lot No. 2511-60069	Lot No. 2511-20027	Lot No. 2511-40060	
Non Flavonoid Polyphenols							
Gallic Acid	1.30	1.20	2.50	2.30	2.50	1.40	1.87
Ellagic Acid	0.04	0.036	0.009	0.011	0.0	0.035	0.021
Caffeic Acid	0.057	0.007	0.0	0.042	0.045	0.033	0.031
Chlorogenic Acid	0.063	0.113	0.069	0.088	0.091	0.002	0.071
Resveratrol	0.018	0.008	0.008	0.017	0.010	0.009	0.011
Other	5.322	3.836	6.314	4.142	3.454	6.321	4.898
Subtotal	**6.8**	**5.2**	**8.9**	**6.6**	**6.1**	**7.8**	**6.9**
Other GSKE Constituents							
Moisture	4.2	3.7	4.57	3.57	4.2	3.97	4.04
Ash	1.11	0.88	0.96	0.92	0.98	0.05	0.82
Insoluble Matter	0.64	0.02	0.57	0.53	0.36	0.42	0.42
Total (%)	**100.0**	**100.2**	**100.0**	**100.0**	**100.0**	**99.9**	**99.85**

The profile of polyphenolic compounds, in particular the relative percentages of monomeric constituents and oligomeric / polymeric proanthocyanidin constituents, present in MegaNatural™ GSE and GSKE is comparable to that of two other commercially available grape seed extracts marketed as Activin® [Bagchi *et al.*, 2001; Wren *et al.*, 2002] and Gravinol Super™ [Yamakoshi *et al.*, 2002]. The mean analytical value of six lots of GSE and GSKE, respectively, has been provided with data taken from published literature to provide a basis for comparison in Table 3.3.

Table 3.3 Mean Composition of MegaNatural™ Gold GSE and GSKE Relative to Other Commercially Available Grape Seed Extracts by Percentage

	MegaNatural Gold™ Products[a]		Other Products	
	GSE	**GSKE**	**Activin®**	**Gravinol Super™ [d]**
Proanthocyanidins				
Dimers	4.37	3.62	54[b]	6.6
Dimer Gallates	0.83	0.40		
Trimers	1.43	2.19	13[b]	5.0
Trimer Gallates	0.52	0.22		
Oligomers & Polymers	74.63	73.77	7[b]	77.7
Subtotal	**81.78**	**80.2**	**76.3[c]**	**89.3**
Monomeric Flavanols				
Catechin	2.92	2.64		2.5
Epicatechin	4.32	2.81		2.2
Monomer Galate	0.17	0.08		
Epigallocatechin	-	-	-	1.4
Epigallocatechin gallate	-	-	-	0.5
Subtotal	**7.41**	**5.53**	**2.8[c]**	**6.6**
Anthocyanins				
Subtotal	**-**	**1.68**		
Minor Flavonoids				
Quercetin	0.00	0.23		
Myricetin	0.00	0.01		
Kaempferol	0.00	0.02		
Subtotal	**0.00**	**0.26**		

Table 3.3 Mean Composition of MegaNatural™ Gold GSE and GSKE Relative to Other Commercially Available Grape Seed Extracts by Percentage

	MegaNatural Gold™ Products[a]		Other Products	
	GSE	**GSKE**	**Activin®**	**Gravinol Super™ [d]**
Non Flavonoid Polyphenols				
Gallic Acid	0.95	1.87		
Ellagic Acid	0.01	0.02		
Caffeic Acid	0.01	0.03		
Chlorogenic Acid	0.03	0.07		
Resveratrol	0.00	0.01		
Other	4.98	4.90		
Subtotal	**5.98**	**6.9**		
Total Phenolics in Each Product				
Subtotal	**95.17**	**94.57**	**79.1**	**95.9**
Other Constituents				
Moisture	3.74	4.04	5.7[c]	2.24
Ash	0.52	0.82		
Insoluble Matter	0.57	0.42	0.6[c]	0.8
Fatty acids			2.8[c]	
Polysaccharides			10.6[c]	
Amino acids			2.1[c]	1.06
Total (%)	**100**	**99.85**	**100.9**	**100**

[a] Mean value from 6 individual lots of GSE and GSKE, respectively
[b] Data obtained from Bagchi *et al.*, 2001
[c] Data obtained from Wren *et al.*, 2002
[d] Data obtained from Yamakoshi *et al.*, 2002

3.2 Proposed Product And Technical Information

3.2.1 MegaNatural™ Gold Grape Seed Extract (GSE)

Code PIN: VW7000

Form: Powder

000081

Table 3.4 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
LC-MS phenol profile	5% Monomers (minimum) 60 – 80% Oligomers 25% max Polymers
pH (4% in water)	2.0 to 5.5
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

3.2.2 *MegaNatural™ Gold Grape Skin Extract (GSKE)*

Code PIN: GK2000

Form: Powder

Table 3.5 GSKE Product Specifications

Analysis	Specification
Chemical	
Total Phenolics (gallic acid equivalents, dry basis)[1]	80 g GAE/100 g
LC-MS phenol profile	5% Monomers (minimum) 60 – 80% Oligomers 25% max Polymers
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.

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3.3 Analytical Evaluation of Product Specifications

Analytical evaluation of the MegaNatural™ Gold GSKE and GSE line of products for non-phenolic parameters revealed that product composition fell within the specifications established for Polyphenolics Brand of products. Analytical results are presented below in Table 3.6. Pesticide and heavy metal content meet FDA tolerances for grapes.

Table 3.6 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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3.4 Stability Data

The stability data for the MegaNatural™ Gold line of GSKE and GSE are presented below in Table 3.7 and Table 3.8, 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 3.7 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

Table 3.8 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	-	-

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)[1], 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

[1] 21 CFR §177.2420 Subpart C – Substances for use only as components of articles intended for repeated use – Polyester resins, cross-linked. Cross linked polyester resins may be safely used as articles or components of articles intended for repeated use in contact with food, in accordance with the following prescribed conditions: (a) The cross-linked polyester resins are produced by the condensation of one or more of the acids listed in paragraph (a)(1) of this section (methacrylic) with one or more of the alcohols or epoxides listed in paragraph (a)(2) of this section (Trimethylol propane), followed by copolymerization with one or more of the cross-linking agents listed in paragraph (a)(3) of this section (methyl methacrylate).

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 GRAS, as determined by the Polyphenolics Division of the Canandaigua Wine Company Inc., 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 composite use level of GSE and GSKE is approximately 210 ppm or 50 mg per 8 fluid ounce serving of the selected beverages as consumed. GSE and GSKE use is as an antioxidant added to these beverage products, for which no standard of identity exists, in order 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)[2] 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 composite use level of GSE / 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

[2] 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

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]. These levels of GSE / GSKE intake also compare favorably to the U.S. background flavonoid consumption estimates of 460 mg/d [Santos-Buelga and Scalbert, 2000] and up to 1 g/day [Scalbert and Williamson 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)[3]. 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[4].

[3] 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.

[4] 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.

7.0 ABSORPTION, DISTRIBUTION, METABOLISM, AND ELIMINATION (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. 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 that 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 PACs, depends upon molecular weight. As these compounds may exist in a polymerized state, because of a high molecular weight these 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 radio labeled 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*.

Flavonoids such as lower molecular weight procyanidin dimers and monomeric flavan-3-ols may also be partially metabolized to lactones and phenolic acids by intestinal microflora. These metabolites are absorbed through the intestinal lumen and are then further metabolized by methylation, oxidation, or glucuronic conjugation.

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 exceptions to this are the polymerized PACs that 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.

7.4 Elimination

In general, flavonoids and their metabolites are eliminated mainly through urinary and fecal excretion. As predicted by Bravo [1998], the non-extractable polyphenols, such as high molecular weight polymeric PACs are relatively inert in the digestive tract and recovered extensively in the feces. However there is evidence that some 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 that 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 would 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 polyphenolic compounds, 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 in their intended use when added as an antioxidant to beverage products for which no standard of identity exists. The following data and discussion support this conclusion as reflected by the opinion of the Expert Panel.

8.1 Genotoxicity

Mutagenic as well as anti-mutagenic activity has been reported for individual polyphenolic compounds. These data are discussed in section 8.1.2. Literature on the Ames and other assays indicated some positive response for quercetin compounds. These individual compounds are present in the MegaNatural™ Gold GSE and GSKE products only in low concentrations 0.0045%, and 0.23%, respectively (see Tables 3.1 and 3.2). However, a mouse micronucleus assay was conducted on the actual GSE and GSKE products, confirming an absence of *in vivo* 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 [Erexson, 2003]. 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