



Comment

on the proposed

“Dietary Supplements Monograph on Grape Seeds Oligomeric Proanthocyanidins”

as published in the *US Pharmacopeial Forum*
(Vol. 34(3), page 659-662 [May–June 2008])

Filed by:
Dennis van der Vlies PhD
INC Agency BV
The Netherlands

Date:
June 30, 2008



Comment on the proposed “Dietary Supplements Monograph on
Grape Seeds Oligomeric Proanthocyanidins”

Page 2 of 8

Issue:

The proposed monograph has the “IN-PROCESS REVISION” status.

The comment period ends August 15, 2008.

Intended publication is set for Feb 2009 with the monograph becoming official in August 2009 in the USP 32-NF 27 – 1st Supplement.



Comments:

1) Page 659; Briefing and Title:

- **Briefing is incomplete.**

→ In the proposed monograph part of the identification is based on *Thin-Layer Chromatographic Identification*. The use of TLC is, however, not mentioned in the briefing.

Q: Why is the use of TLC not mentioned in the briefing?

- **Title is inconsistent with the compounds detected in this proposal.**

→ In this proposal both (epi)-catechins and proanthocyanidins are validly analysed as part of a grape seed fraction. The name “Proanthocyanidin” reflects the fact that, on acid hydrolysis, the extension units are converted to red-coloured anthocyanidins. Catechin and epicatechin do not have this property and are therefore not proanthocyanidins. For this reason the title of this proposed monograph cannot be ‘Grape Seeds Oligomeric Proanthocyanidins’.

2) Page 659; First paragraph:

- **No definition of “Oligomeric Proanthocyanidins” is given.**

→ Definition: Proanthocyanidins, also known as condensed tannins, consist of flavan-3-ol monomer units linked through carbon-carbon and ether linkages. These monomeric units are primarily linked through single 4→6 or 4→8 carbon-carbon bonds (B-type linkages), or through the combination of 4→8 carbon-carbon linkages and 2→7 ether bonds (A-type linkages). In grape seeds B-type linkages are predominant and A-type linkages are found at very low levels.

→ Beneficial health effects can only be attributed to the bioavailable (epi)-catechins and oligomeric proanthocyanidins. The term ‘oligo’ originates from the Greek language and means, ‘a few’. Oligomeric Proanthocyanidins are two (dimers) to five (pentamers) linked monomeric units of flavan-3-ols. The designation “oligomeric” therefore excludes the higher polymerised proanthocyanidins from the extract.

This distinction is not clear from the present proposal.

- **Solvents used, plant:fraction ratio, and final fraction content are incomplete or incorrect.**

→ The methods of manufacturing and the use of oligomeric proanthocyanidins were developed by Professor Masquelier and described in many international patents, for example: French patent 968,589 (1950); French patent 1,036,922 (1953); French patent 1,427,100 (1965) (2.); French patent 4,482M; UK patent 1,092,269 (1967); US patent 3,436,407 (1969); French patent 2,092,743 (1970) (3.); French patent 2,643,073 (1990).



From his work it is clear that using only alcohol, acetone, water or mixtures of these solvents will not produce an extract that contains at least 75% oligomeric proanthocyanidins, i.e. 2 to 5 linked monomeric units of flavan-3-ols. For this, an extraction with the mentioned solvents has to be followed by a second extraction using ethyl acetate. Ethyl acetate is required to obtain oligomeric proanthocyanidins.

Q: What is the exact extraction protocol used to produce the *USP Grape Seeds Oligomeric Proanthocyanidins RS*?

Q: How have the identities of the compounds of *USP Grape Seeds Oligomeric Proanthocyanidins RS* been determined?

Q: How has the amount of 75% oligomeric proanthocyanidins been established?

Q: Does the indicated 75% oligomeric proanthocyanidins of the *USP Grape Seeds Oligomeric Proanthocyanidins RS* include all proanthocyanidins, including polymers?

Q: Does this percentage indicate the amount of oligomeric proanthocyanidins per extract-weight or per proanthocyanidins present in the extract?

Q: How is the consistency of the composition of lots of *USP Grape Seeds Oligomeric Proanthocyanidins RS* and *USP (+)-Catechin RS* guaranteed?

→ For grape seed extracts containing 94% of (epi)-catechins plus oligomeric proanthocyanidins the plant to fraction ratio will be higher than 70:1. Lower ratios indicate presence of other compounds.

Q: How has this ratio been determined?

Q: How has this ratio variation, ranging from 70:1 to 10:1, been determined?

Q: Can extracts with ratio variations ranging from 70:1 to 10:1 contain equal amounts of (epi)-catechins and oligomeric proanthocyanidins and comply to this proposed monograph?

Q: Have extracts with plant to fraction ratios ranging from 70:1 to 10:1 been confirmed to have equal compositions, i.e. containing 94% of (epi)-catechins plus oligomeric proanthocyanidins?

Q: Does “plant” mean dried grape seed in this context?

Q: If not what does it mean?

→ The sum of catechin and epicatechin will be higher than the indicated upper level of 19%.

Q: See comment 5.

3) Page 659/660; Identification / A: Thin-Layer Chromatographic Identification / Procedure:

- **Title is inconsistent with the compounds detected using TLC.**

→ Compounds corresponding to the R_F values 0.43 and 0.49 are no oligomeric proanthocyanidins.

Q: How has the identity of the “five main pink-violet bands” been determined?

→ “Other pink-violet zones of varying intensities are observed in the chromatograms of the *Test solution* and the *Standard solution*.” The presence of other zones shows that the *Standard solution* contains other compounds of flavanolic origin.

Q: What is the identity of the compounds present in these zones?

Q: How can it be excluded that these “other pink-violet zones of varying intensities”, of which the R_F values are not specified, are the result of adulterations with other botanical sources containing vanillin-reactive flavanolic compounds?



Q: By using the proposed spray reagent that contains vanillin, can it be excluded that other non-flavanolic compounds, that are not vanillin-reactive, are present?

4) Page 660; Identification / B:

- **Compounds detected as the “five main pink-violet bands” using TLC do not correspond to the peaks detected using HPLC in the “Limit of catechin and epicatechin” assay.**

→ Trimeric proanthocyanidins, proanthocyanidin-B2 3'-O-gallate, and the dimeric proanthocyanidins B3 and B4 are not detected using the proposed HPLC system (page 660, right column under *Chromatographic system*), although they were among the identified “main pink-violet bands” using TLC.

Q: Why is there a discrepancy between the detected components when either TLC or HPLC is used?

Q: Is the proposed HPLC system accurate to detect the major components present in the fraction?

- **HPLC detection of oligomeric proanthocyanidins is inefficient and neglected.**

→ The proposed HPLC system used is, like Gel Permeation Chromatography (GPC), not accurate in detecting separate oligomeric proanthocyanidins: “The chromatogram ... exhibits ... a broad peak due to other oligomeric proanthocyanidins”.

Q: How have the compound identities in this “broad peak” been confirmed as being solely oligomeric proanthocyanidins (i.e. 2 to 5 linked monomeric units of flavan-3-ols)?

Q: Why is this broad HPLC peak neglected and not used to quantify oligomeric proanthocyanidins?

Q: Why is GPC used instead where the oligomeric proanthocyanidins also elute in a “broad peak”?

Q: Why is the proposed HPLC method, which is unable to detect separate oligomeric proanthocyanidins, been used while there are numerous scientific publications in which detection of separate oligomeric proanthocyanidins using HPLC have been described?

→ Purified catechin, epicatechin and several oligomeric proanthocyanidins are already commercially available to be used as Reference Standards in HPLC.

Q: Why doesn't USP provide these as Reference Standards in combination with an optimal HPLC method to quantify catechin, epicatechin and oligomeric proanthocyanidins?

Q: Why doesn't USP produce more separate grape seed oligomeric proanthocyanidins and develop an accurate HPLC method to quantify oligomeric proanthocyanidins? (Brunswick laboratories, Norton, MA, provide this service)



5) Page 661; Limit of catechin and epicatechin / Procedure:

- **The sum of catechin and epicatechin in *Standard solution 1 (USP (+)-Catechin RS)* is too low.**

→ The sum of catechin and epicatechin of a grape seed fraction consisting of mainly catechin, epicatechin and oligomeric proanthocyanidins (i.e. 2 to 5 linked monomeric units of flavan-3-ols) is normally higher than 19%; found as the upper level in *Standard solution 1 (USP (+)-Catechin RS)*. Percentages are relative numbers. Lower amounts indicate presence of other compounds besides catechin, epicatechin and oligomeric proanthocyanidins (i.e. 2 to 5 linked monomeric units of flavan-3-ols).

Q: What is the exact extraction protocol used to produce the *USP (+)-Catechin RS*?

Q: How has the quantification of catechin and epicatechin of *USP (+)-Catechin RS* been performed?

Q: How has the amount of 19% catechin and epicatechin been established?

Q: How is the consistency of the composition of *USP (+)-Catechin RS* guaranteed?

→ *Standard solution 2 (USP Grape Seeds Oligomeric Proanthocyanidins RS)* is used to calibrate the HPLC system by comparing the recorded peak responses of this solution with the Reference Chromatogram provided with the lot of this *Standard solution 2*, whereas HPLC is only being used to quantify catechin and epicatechin in the *Test Solution (Grape Seeds Oligomeric Proanthocyanidins)* using *Standard solution 1 (USP (+)-Catechin RS)*.

Using *Standard solution 2* for calibration of the HPLC system is incorrect. For this purpose *Standard solution 1* should be used instead. Also because the HPLC system has shown not to be able to separate oligomeric proanthocyanidins; “The chromatogram ... exhibits ... a broad peak due to other oligomeric proanthocyanidins”.

Q: Why is *Standard solution 2* used for the calibration and not *Standard solution 1*?

6) Page 661; Content of oligomeric proanthocyanidins/Procedure:

- **The method used (Gel Permeation Chromatography; GPC) is inappropriate to quantify oligomeric proanthocyanidins (i.e. 2 to 5 linked monomeric units of flavan-3-ols).**

→ All proanthocyanidins including oligomers and polymers, as well as other compounds with molecular weights in the same range will elute in a single “broad peak” when GPC is performed. The absence of resolution makes it impossible to distinguish between oligomeric proanthocyanidins (i.e. 2 to 5 linked monomeric units of flavan-3-ols) and polymeric proanthocyanidins (i.e. 6 or more linked monomeric units of flavan-3-ols), which results in an overestimation of the amount of oligomeric proanthocyanidins in grape seed fractions that (also) contain polymeric proanthocyanidins and/or compounds with molecular weights in the same range.

Q: Does the indicated 75% oligomeric proanthocyanidins include all proanthocyanidins, including polymers?

Q: How has the quantification of oligomeric proanthocyanidins of *USP Grape Seeds Oligomeric Proanthocyanidins RS* been performed?

Q: How has the amount of 75% oligomeric proanthocyanidins been established?



Q: How have the identities of the compounds of *USP Grape Seeds Oligomeric Proanthocyanidins RS* been determined?

Q: How is the consistency of the composition of *USP Grape Seeds Oligomeric Proanthocyanidins RS* guaranteed?

Q: How has it been confirmed that the “broad peak” does not contain other compounds of grape seed origin with molecular weights that fall within the weight-range of this “broad peak”?

Q: Can it be excluded that this broad peak contains adulterating compounds of other botanical sources with molecular weights in the same range?

→ “The monomer peak may be eluting on the tail of the oligomeric proanthocyanidins peak.”
Whether this occurs is not always obvious and thus appropriate exclusion of the area corresponding to the monomer peak from the integration of the oligomeric proanthocyanidins peak is not possible. This will result in incorrectly determined amounts of oligomeric proanthocyanidins.

→ Obviously both HPLC as well as GPC will detect epicatechin, catechins and oligomeric proanthocyanidins making one of the two methods redundant.

In line with comment 4, 2nd point:

Q: Why is GPC not used to quantify catechin and epicatechin?

→ GPC is redundant when an HPLC method is used which is able to detect catechin, epicatechin and separate oligomeric proanthocyanidins.

Q: Why is GPC proposed while there are numerous scientific publications in which detection of separate oligomeric proanthocyanidins using HPLC have been described?

Summarising major comments:

- Provenance, manufacturing method and analytical determination of the total composition of *USP Grape Seeds Oligomeric Proanthocyanidins RS* and *USP (+)-Catechin RS* are lacking. This omission makes them unfit to be used as standards for the monograph.
- Plant material to fraction ratio is too wide-ranging to comply with 19% catechin and epicatechin, and 75% oligomeric proanthocyanidins (gram/gram extract).
- From investigated grape seed fractions that show the anticipated TLC results adulteration cannot be excluded.
- The proposed HPLC method is unsuitable for the detection of oligomeric proanthocyanidins.
- Major components detected using the proposed TLC and HPLC methods do not coincide, which emphasizes the shortcoming of either of the proposed methods.
- From Of investigated grape seed fractions that show the anticipated GPC results neither adulteration nor overestimation of oligomeric proanthocyanidins present can be excluded.
- As a result of the analytical shortcomings of GPC the quantitative determination of the total composition of *USP Grape Seeds Oligomeric Proanthocyanidins RS* (i.e. 19% catechin and epicatechin, and 75% oligomeric proanthocyanidins) is not reliable.



Comment on the proposed “Dietary Supplements Monograph on
Grape Seeds Oligomeric Proanthocyanidins”

Page 8 of 8

Conclusion:

1. The proposed monograph utilizes analytical methods that comprise many significant technical shortcomings and *Reference Standards* of which the provenance, extraction method(s) and total compositions were not provided.
2. The use of chromatograms derived from *Reference Standards* that are themselves undefined, for comparison with chromatograms derived from *Test Solutions*, cannot remedy these incomplete and therefore inappropriate analytical methods.
3. Under these circumstances the undefined *Reference Standards* will work as arbitrary standards.
4. This makes the proposed monograph unacceptable as an industry standard.