Article

# What do Green Tea, Grapes Seeds, and Docks have in Common?

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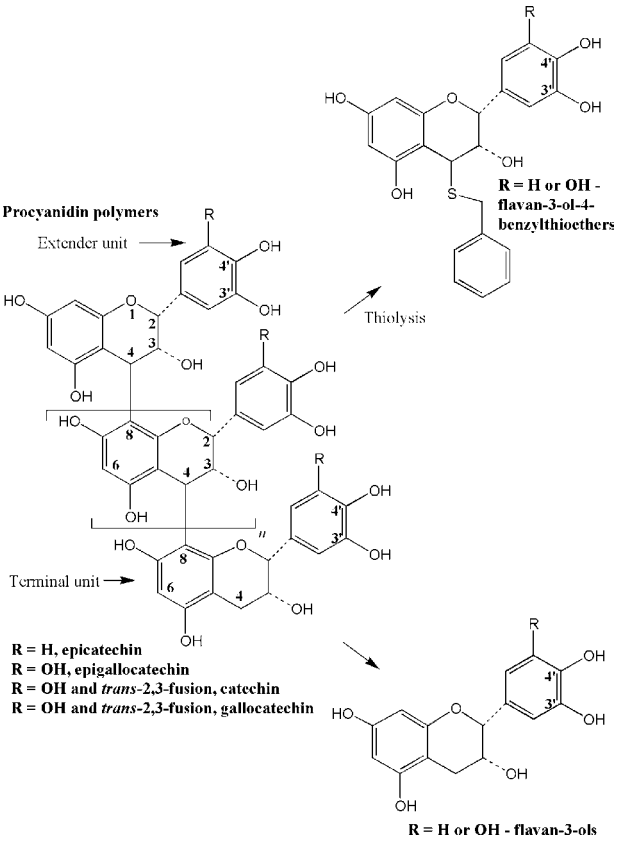
## Introduction

The phenolic constituents of green tea (*Camellia sinensis*) and grape (*Vitis vinifera L.*) seeds have been the focus of much research attention in human health and nutrition. Proanthocyanidins are polymers of flavan-3-ol units found in green tea, grapes and numerous other plants (Scheme 1). The phenolics in green tea are flavan-3-ols and flavan-3-*O*-gallates and the major component - epigallocatechin gallate (EGCg) - is known to have a broad spectrum of biological activities as antioxidant,[1] antibacterial,[2] and antifungal.[3] The efficacy of proanthocyanidins from green tea extracts has not warranted as much attention. The major components extracted from grape seeds, oligomeric and polymeric proanthocyanidins, are widely used, mainly as nutritional supplements and have *in vitro* antioxidant, antibacterial[4] and antiproliferation[5] activity. Galloylation, the extent of oligomerization, and stereochemistry strongly influence the observed activity of the compounds.




***Scheme 1***

## Properties of proanthocyanidins

Proanthocyanidins properties depend upon their structure in terms of monomer units (degree of hydroxylation and 2,3-*cis*- or 2,3-*trans*-stereochemistry), their degree of polymerisation (DP) and the type of linkage between the flavan-3-ols (4,8- as in Scheme 1, or 4,6-branched structures). For the procyanidin (PC)-type polymers with 3',4'-dihydroxy substitution the constituent flavan-3-ol units are either catechin (*trans*) or epicatechin (*cis*) with R = H while prodelphinidin (PD)-type polymers with 3',4',5'–trihydroxy substitution contain either gallocatechin (*trans*) or epigallocatechin (*cis*) units with R = OH (Scheme 1). However, the majority of the proanthocyanidin polymers in plants comprise mixtures of the two classes and mixtures of oligomers (DP 5−10) and higher polymeric material (DP >10).

## Proanthocyanidins in forage legumes

At Grasslands Research Centre, the search for alternative forage species to supplement pasture and ensure sustainable agriculture has included the evaluation of a wide range of pasture legumes. These species are native to Eurasia and the Mediterranean region and can tolerate arid environmental extremes. Temperate forages such as *Lotus* species containing proanthocyanidins provide nutritional benefits for grazing ruminants including improvements in weight gain, wool growth, milk production and composition, ovulation rate, and increased efficiency of feed utilization. A recent review on the effects of *Lotus* species by Min[6] suggests they derive from reduced degradation of dietary protein to ammonia by rumen microbes and increased protein outflow from the rumen, which can result in increased absorption of amino acids from the small intestine.

The common weed, referred to as dock (*Rumex obtusifolius*), found growing in many NZ pastures was observed to be an antibloat agent for cattle.[7] Its phenolic constituents warranted phytochemical investigation given the observed *in vivo* effects and they were identified as a series of oligomeric and polymeric proanthocyanidins with galloyl derivatives, which may explain the observed *in vivo* biological activity of dock.

### *Extraction and purification of proanthocyanidins*

Dock leaves were extracted with acetone-water, the solution fractionated (Sephedex LH-20 chromatography; acetone-water; 7:3 elution - a modification of the method of Foo[8]) to give a fraction containing procyanidin oligomers and polymers. Likewise, proanthocyanidin-containing extracts of green tea were prepared by modification of the method of Degenhardt,[9] while a grape seed extract was obtained from RMF Nutraceuticals NZ Ltd seed and prepared by extraction with aqueous ethanol (80% v/v) at room temperature. Chemical and spectroscopic methodologies have been applied to characterize and to compare

the structures of the proanthocyanidin oligomer and polymer fractions from these three extracts. The identities of the individual proanthocyanidin polymer units and chain length were estimated by strong acid-catalysed cleavage in the presence of benzyl thiol. This thiolysis releases the terminal units as free flavan-3-ols, while extender units are distinguished as benzylthioether adducts formed from nucleophilic capture of the carbocations generated under the acidic conditions. More recently, mass spectrometry (MS) has been utilised to characterise the oligomeric composition of proanthocyanidins by electrospray injection (ESI).

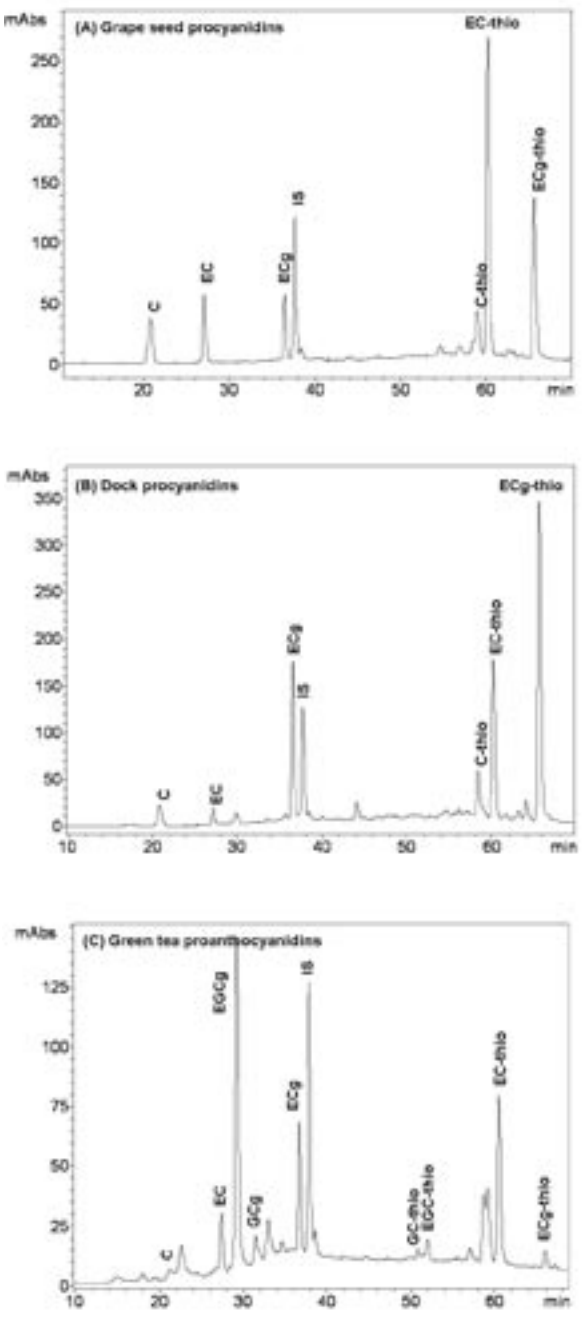



***Fig. 1.*** Reversed phase HPLC chromatograms (280 nm absorbance) of thiolysis products; C, catechin; EC, epicatechin; ECg, epicatechin gallate; GCg, gallocatechin gallate; EGCg, epigallocatechin gallate; IS, internal standard, dihydroquercetin; the suffix – signifies the corresponding benzylthioether.

## The chemical degradation of proanthocyanidins by thiolysis

The mean composition and mean DP (mDP) of proanthocyanidins can be determined from total thiolysis and chromatography of the products by HPLC; a variation of Guyot's[10] method was used for the thiolysis (see Scheme 1). Such degradation provides good yields of cleavage products with low levels of degradation and epimerization. The mean composition of the terminal units can be determined from the ratio of the released monomers, that of the extender units in the polymer chain from the ratio of benzylthioether adducts, and the mDP from the monomer to extender unit ratio. Samples were analyzed by the method of Meagher.[11] Responses relative to the internal standard, dihydroquercetin, for terminal flavan-3-ol units were 0.30 PC and 0.06 PD while extender flavan-3-ol units had 0.26 PC and 0.06 PD benzylthiolethers, both in accord with published values.[12] Gallate terminal flavan-3-ol units were 1.09 for PC and assumed to be 1.09 for the PC gallate extender benzylthiolethers. The chromatograms (280 nm monitor; Fig. 1) show the terminal units released as monomers before 30 min and the extender units eluting after 45 min as benzylthioether adducts. The catechin and gallocatechin thioethers appear as two peaks in the chromatograms due to separation of the C-4 epimer pair. The same pattern is not observed for the benzylthioethers from epicatechin and epigallocatechin as there is very little epimerization during thiolysis.

The data obtained show grape seed proanthocyanidins to be gallated procyanidin polymers (mDP 3.5) (Fig. 1A). The terminal units consist predominately of catechin (46%), epicatechin (46%), and a minor epicatechin gallate component of 8%. The cleavage products of the extender units are dominated by epicatechin benzylthioether (77%), and components of catechin and epicatechin gallate benzylthioethers (15 and 8%, respectively). Quantification of epicatechin gallate benzylthioether was achieved from cleavage of an epicatechin gallate homo-dimer. Thus the predominant cis (epicatechin) stereochemistry of the extender units differs from the equal portions of *cis* and *trans* terminal units (Table 1). The thiolysis data show dock proanthocyanidins (Fig. 1B) to be gallated procyanidin polymers (mDP 4.3) similar to grape seed and the terminal units to consist of equal proportions of catechin, epicatechin gallate, and epicatechin. As with grape seed, the cleavage products of the extender units are dominated by epicatechin benzylthioether (57%) with components of catechin and epicatechin gallate benzylthioethers (18 and 25%, respectively). The difference between grape seed and dock procyanidins is simply the extent of galloylation (8 *vs* 27%). Procyanidins from a variety of plant species including apple (mDP 2 to 190),[12] cocoa (mDP 14), brown sorghum bran (mDP 14), lowbush blueberry (mDP 39), and cranberry (mDP 15)[10] have been characterized previously from thiolysis. Thus dock procyanidins are comparable in flavan-3-ol composition to grape seed, which is dominated by catechin terminal units and epicatechin extension units. Dock has greater proportions of epicatechin gallate in both the extender and terminal units. There is apparently different selectivity in the biosynthesis of monomers that form the terminal units to that of precur-

***Table 1.*** Comparison of procyanidin extender and terminal unit contributions from grape seed and dock extracts, mDP and % galloylation by thiolysis.

| | | | Terminal | | | Extender | | |
|---|---|---|---|---|---|---|---|---|
| | mDP | Gallate % | C[a] | EC[b] | ECg[c] | C | EC | ECg |
| Grape seed | 3.4 | 8 | 0.46 | 0.46 | 0.08 | 0.15 | 0.77 | 0.08 |
| Dock | 4.3 | 27 | 0.39 | 0.23 | 0.38 | 0.18 | 0.57 | 0.25 |

[a]Catechin; [b]Epicatechin; [c]Epicatechin gallate

sor units which extend the polymer chain. The molecular species involved in chain extension remains a matter of conjecture.[13]

In contrast to the above, the thiolysis data show green tea proanthocyanidins as a mixture of procyanidin and prodelphinidin units (Fig. 1C). Quantification of the thiolysis products was not possible for the proanthocyanidin polymer due to a lack of standards for the benzylthioethers of gallocatechin and epigallocatechin gallates (elution 59 min; Fig. 1C). Compared with grape seed and dock, green tea has contributions from the three gallates epicatechin, gallocatechin, and epigallocatechin in the terminal units, but no catechin gallate. The extender units of green tea proanthocyanidins appear less dominated by gallates with a low concentration of epicatechin gallate. However, there maybe a significant contribution from prodelphinidin gallates that were not quantified.

## ESI/MS analysis of proanthocyanidin oligomers

Evidence of proanthocyanidin oligomer composition can be provided by ESI-MS. In the negative ion mode, and with weakly acidic conditions for the scan (*m/z* 250-1400), proanthocyanidin oligomers can be detected as anions because of the acid phenolic protons; the degree of ionisation and numbers of negative charges increase as chain length increases.[14] The weakly acidic conditions inhibit ionisation so that for DP ≤ 3 singly-charged ions dominate, DP 4-8 have the doubly charged species predominating, and for higher DP triply or higher charges predominate. Although the ions for doubly-charged oligomeric species with an even DP overlap those for the singly charged species, they can be distinguished by the ion masses (odd for singly-charged species, even for doubly-charged species), and the spacing between them (16 and 8 daltons, respectively, for singly and doubly-charged species). The practical upper limit of ion detection comes more from the broad chromatographic peaks of the higher oligomers than the limit of the quadrupole mass spectrometer (2000 amu); molecules of higher mass carry higher charge. Characteristic ions can be used to assign proanthocyanidin oligomers, but without authentic standards the epimers C (catechin) and EC (epicatechin ), or GC (epicatechin gallate) and EGC (epigallocatechin) cannot be distinguished. Therefore oligomers were assigned as either PC consisting of C and/or EC, or PD consisting of GC and/or EGC. Mass spectrometry can also provide useful information on the composition of proanthocyanidin oligomer mixtures in the form of ion masses that correspond to sets of oligomers of the same DP and composition. Negative ion reverse phase HPLC-ESI-MS has been used to characterise procyanidin gallates in grape products.[14] However, the presence of mixtures of proanthocyanidin oligomer gallates confounds interpretation as the mass of the two galloyl derivative units is the same as a single gallocatechin oligomer unit.

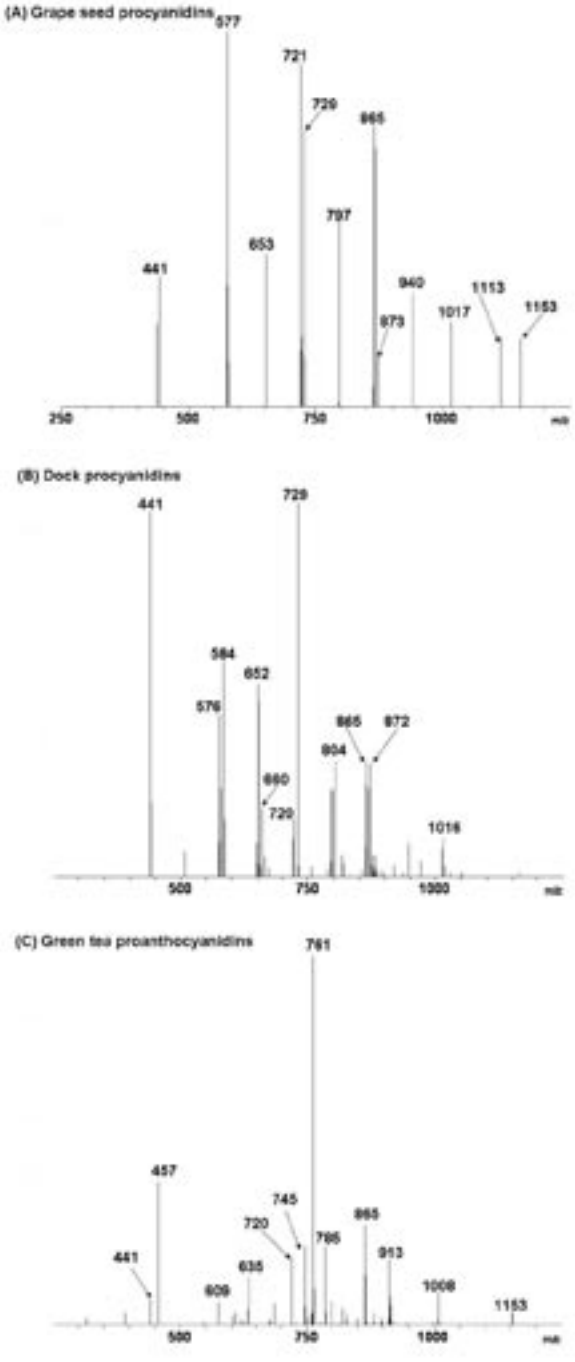



***Fig. 2.*** Mass spectra of the extracts (see text).

The ESI-MS of the proanthocyanidin fractions give ions from oligomers with DP 2-6 (Fig. 2) broadly consistent with the mDP estimated by thiolysis (Table 1). Grape seed (Fig. 2A) and dock (Fig. 2B) extracts were exclusively procyanidin homo-oligomers. A wide mass range was recorded from (singly-charged) dimers to (doubly-charged) hexamers. In each range, the dominant species observed come from procyanidin homo-oligomers, namely a singly charged dimer and a trimer species (*m/z* 577 and 865), and doubly charged tetramer to hexamer species (*m/z* 576, 720, and 864). All the major peaks corresponded to molecular ions of procyanidin-type oligomers. The spectra are compounded by the appearance of ions from procyanidin-gallates; singly charged monomer, dimer, and trimer species (*m/z* 441, 729 and 1016) and doubly charged tetramer to hexamer species (*m/z* 652, 796, and 940). Proanthocyanidin oligomers with galloyl derivatives were identified in green tea (Fig. 2C).[15] These include a galloyl dimer [*m/z* 761; $(PD)_2$-g] as the dominant prodelphinidin ion in the mass spectrum, a trimer [*m/z* 913; $(PD^-)_3$ or perhaps $(PCPD_2g^{2-})$], a second trimer [*m/z* 865 $(PC^-)_3$], a tetramer (*m/z* 1153 $(PC^-)_4$], the doubly charged hexamer $(PC^{2-})_6$ (*m/z* 1008), and the gallated flavan-3-ols GCg (*m/z* 457) and ECg (*m/z* 441). The trimer $[PC_3]^-$ could also be EAg-(4β-8)-ECg, A (azfelechin; *m/z* 865), the dimer assigned by Degenhardt.[9]

## Conclusion

Surprisingly, the study shows that the common weed *dock* has more in common with grape seeds than with green tea. While dock (*Rumex obtusifolius*) has been studied in Turkey for its anthraquinone content,[16] the procyanidin gallates identified here may indicate that its extracts have biological activity similar to grape seed procyanidin extracts.

### *Acknowledgements*

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