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Use of the fluorescence quantum yield for the determination of the number-average molecular weight of polymers of epicatechin with 4β→8 interflavan bonds

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Excitation at 280 nm produces a structureless emission band with a maximum at 321–324 nm for dilute solutions of catechin, epicatechin, and their oligomers in 1,4-dioxane or water. The fluorescence quantum yield, Q, has been measured in these two solvents for five dimers, a trimer, a tetramer, a pentamer, a hexamer, and a polymer in which the monomer units are catechin and/or epicatechin. In a homologous series of monodisperse oligomers in which epicatechin units are connected by interflavan bonds with 4β→8 stereochemistry, Q is found to be inversely proportional to the degree of polymerization. Measurement of Q for a polydisperse sample of this polymer yields the number-average degree of polymerization.

(Keywords: epicatechin; fluorescence; molecular weight; procyanadin; quantum yield)

INTRODUCTION

Molecules composed of the flavan-3-ols catechin and epicatechin are among the most common and important polyphenols that have been isolated from plants[1]. They form the basis for the condensed tannins of the procyanidin class, which are found in the leaves, fruits or barks of a broad spectrum of woody and herbaceous plants. These condensed tannins are of considerable biological significance, and their importance as a source of renewable specialty chemicals is increasing[2]. In the polymers, the monomers are bonded to one another by interflavan bonds that have different stereochemistries. Examples of some of the interflavan bonds found are illustrated by the dimers depicted in *Figure 1*. These five dimers are known by the compact trivial names procyanidin B1, B2, B3, B4, and B5. Longer names that identify the constituent monomer units and the location and stereochemistry of the interflavan bond are epicatechin-(4β→8)-catechin, epicatechin-(4β→8)-epicatechin, catechin-(4α→8)-catechin, catechin-(4α→8)-epicatechin, and epicatechin-(4β→6)-epicatechin, respectively. The nomenclature used has been described by Hemingway *et al.*[3].

The monomers and two dimers exhibit fluorescence when excited with radiation near 280 nm (refs 4 and 5). The fluorescence quantum yield, Q, was found to be smaller for two dimers than for the monomers. The dependence of Q on the degree of polymerization, x, is examined more fully here, using monodisperse samples of the monomer, dimer, trimer, tetramer, pentamer, and hexamer of epicatechin. These measurements show that Q is inversely proportional to x for the monodisperse oligomers. Consequently a measurement of Q for a polydisperse sample of this polymer permits calculation of the number-average degree of polymerization.

EXPERIMENTAL

Epicatechin oligomers were isolated from fresh Ivory Coast cacao beans by maceration of the beans and defatting with chloroform. The resulting pulp was air-dried and re-extracted with acetone:water (6:4, v/v), the acetone layer evaporated *in vacuo* at $<40°$ and the aqueous layer extracted 6× with ethyl acetate. The ethyl acetate layer was evaporated to yield crude monomeric to hexameric epicatechin oligomers. The aqueous layer was diluted with an equal volume of methanol and applied to a column of Sephadex LH-20. This column was eluted with methanol:water and then acetone:water (6:4, v/v). The acetone layer was removed as before and the aqueous layer freeze-dried to yield the *polymer* fraction[6].

The ethyl acetate soluble fraction was applied to a column of Sephadex LH-20 in water and eluted with water containing increasing amounts of methanol, and finally pure methanol. Earlier fractions contained anthocyanins, simple phenolics, and theobromine. These fractions were not further investigated. Later fractions contained sequentially catechin and epicatechin followed

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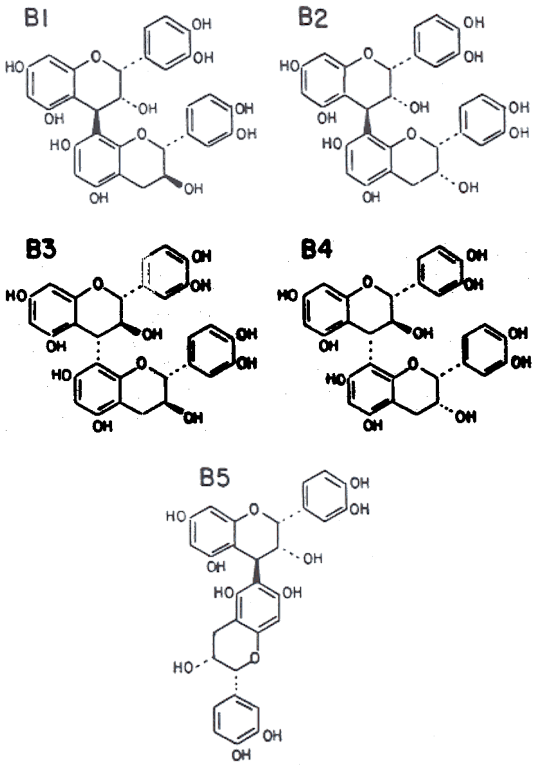

Figure 1 Covalent structures of epicatechin-(4β→8)-catechin, epicatechin-(4β→8)-epicatechin, catechin-(4α→8)-catechin, catechin-(4α→8)-epicatechin and epicatechin-(4β→6)-epicatechin, which are denoted by the trivial names procyanidin B1, B2, B3, B4, and B5, respectively

by epicatechin dimers, trimers, tetramers and finally small amounts of pentamers and hexamers. The course of these separations was readily followed by thin layer chromatography (t.l.c.) analysis on silica using toluene:acetone:formic acid (30:60:10), v/v/v) which separates procyanidin oligomers according to their degree of condensation[7]. The resulting oligomer fractions were recovered by freeze-drying and individual oligomers, dimers to tetramers, were isolated by chromatography on LH-20 eluted with ethanol and on MCI GEL CHP20 eluted with methanol:water (normally 30:70, v/v). This process yielded epicatechin-(4β→8)-epicatechin, $[\alpha]_{578}+23.2$ (*c* 0.25, methanol) literature value[8] +26; $[M+H]^+$ 579; epicatechin-(4β→6)-epicatechin, $[\alpha]_{578}+137°$ (*c* 0.25, methanol) literature value[8] +142°; $[M+H]^+$ 579; [epicatechin-(4β→8)]$_2$ epicatechin, $[\alpha]_{578}+97°$ literature value[8] +102° $[M+H]^+$ 867; [epicatechin-(4β→8)]$_3$ epicatechin, $[\alpha]_{578}+90°$ (*c* 0.25, methanol); literature value[8] +89°; $[M+H]^+$ 1155. The $[M+H]^+$ ions were observed using FAB mass spectrometry (MS) using a thioglycerol matrix and the oligomers all possessed ^{13}C nuclear magnetic resonance (n.m.r.) spectra consistent with published data. A portion of the polymer fraction (5 g) was dissolved in methanol and separated on Fractogel HW-40 by further elution with the same solvent. Procyanidin oligomers separate on this system in order of their degree of polymerization, dimers being separated first[9]. The progress of the separation was monitored by SiO_2 t.l.c. using toluene:acetone:formic acid, 30:60:10, v/v/v for development[7]. Small amounts of dimers and trimers were separated first, followed by larger amounts of tetramers, pentamers, and hexamers. The latter were separated as discrete bands with some overlap, but enabled selection of fractions containing one oligomer species. In this way 400–500 mg samples of tetramer, pentamer, and hexamer fractions were obtained. The degree of condensation of each fraction was confirmed by negative-ion FAB MS using a thioglycerol matrix and in particular the predicted $[M-H]^-$ ions at 1153, 1441 and 1729, respectively.

Ultraviolet absorption measurements were performed at ambient temperature with a Hewlett-Packard 8451A Diode Array Spectrophotometer equipped with a deuterium lamp. The bandwidth was 2 nm. The absorbances at 280 nm were always in the range 0.01–0.10 for the samples subjected to fluorescence measurements.

Steady-state fluorescence measurements were performed in the ratio mode with right-angle illumination using an SLM 8000C Spectrofluorometer equipped with a double grating excitation monochromator and a single grating emission monochromator. An ozone free 450 W xenon arc lamp was used as the light source. Excitation was at 280 nm. Slit widths were 8 nm for excitation and emission. A polarizer in the excitation path was oriented in the vertical position (0°), and a polarizer in the emission path was oriented 54.7° from the vertical, thereby giving 'magic angle' conditions that correct for anisotropic effects[10]. The spectral range scanned was 285–450 nm for epicatechin and its derivatives, and 350–650 nm for quinine sulphate. The integration time was 1 s nm^{-1}. Quinine sulphate in 1.0 N sulphuric acid, for which Q is 0.546, was used as the standard[11,12]. The emission was corrected for the wavelength-dependent instrument response.

Due to slight differences in the baselines from different cuvettes, all measurements for a particular sample, including the solvent baselines, were performed in the same cuvette. The light path was 1 cm in all spectral measurements. Since excitation at 280 nm was used for all solutions, and the instrument conditions were the same for all measurements, Q can be calculated as

$$Q = Q'(dI/dA)/(dI/dA)' \quad (1)$$

where the prime as a superscript denotes the values for the quinine sulphate standard, I denotes the integrated area for the emission band, and A is the absorbance. Four freshly prepared solutions with $A<0.1$ were used to evaluate each dI/dA. Plots of I vs. A were linear, with the straight line extrapolating to the origin.

The monomers were purchased from Sigma Chemical Co., 1,4-dioxane (spectrophotometric grade) was purchased from Aldrich, and the water was distilled and deionized.

RESULTS AND DISCUSSION

Molecular weight dependence of the fluorescence quantum yields

Dilute solutions of the samples had an ultraviolet absorption maximum at 280 nm in water and 282 nm in 1,4-dioxane. The slight red shift in 1,4-dioxane was observed earlier[5]. With an increase in x, there was an extension of the low energy portion of this absorption band to longer wavelength, but there was no change in the position of the maximum. Each sample exhibited a broad structureless emission band with a maximum at 323–324 nm in water and 321–322 nm in 1,4-dioxane.

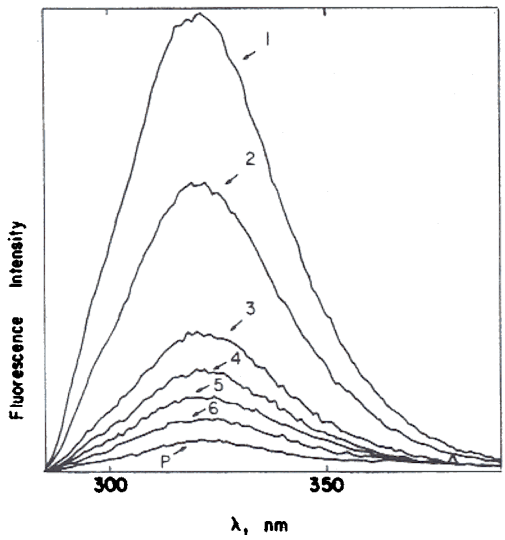

Figure 2 Emission spectra for six oligomers and a polymer of epicatechin in 1,4-dioxane. The degree of polymerization is given for each oligomer, and P denotes the polymer. The absorbance at the wavelength of excitation, 280 nm, is in the range 0.092–0.095 for all samples

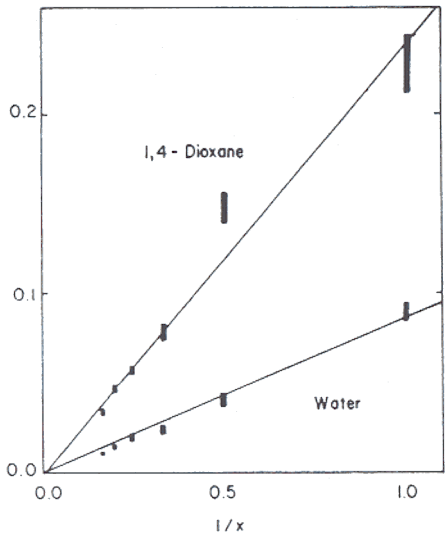

Figure 3 Fluorescence quantum yield for monodisperse oligomers of epicatechin in 1,4-dioxane and in water as a function of the reciprocal of the degree of polymerization. The linear least squares straight lines are constrained to pass through the origin

Figure 2 depicts emission spectra in 1,4-dioxane for six oligomers and a polymer. The oligomers were obtained by chromatographic fractionation of a single sample that was polydisperse with regard to molecular weight. The sample described as the 'polymer' in *Figure 2* is this polydisperse sample after removal of the hexamer and smaller oligomers. All of the monomer units in these samples are epicatechin. High resolution ^{13}C n.m.r. shows that the interflavan linkage is $4\beta \rightarrow 8$ in the dimer, trimer and tetramer. ^{13}C n.m.r. (400 MHz in d_6-acetone: water, 1:1, v/v) revealed that the pentamer and hexamer fractions consisted of a mixture of epicatechin oligomers with varying ratios of interflavanoid linkages ($4\beta \rightarrow 6$ and/or $4\beta \rightarrow 8$) with $4\beta \rightarrow 8$ linkages predominating[13].

The emission spectra depicted in *Figure 2* were obtained from solutions in 1,4-dioxane for which *A* at 280 nm is in the range 0.092–0.095 (concentration ~ 0.016 mg ml^{-1}). Therefore the decrease in the intensity of the emission band with increasing x in *Figure 2* must reflect a decrease in Q. The measured values of Q for the monodisperse oligomers are depicted as a function of $1/x$ in *Figure 3*. *Figure 3* also presents the Q values that are measured when the oligomers are dissolved in deionized, distilled water. The aqueous solutions have a pH near 6.6 when the concentrations are in the range used in the measurements of fluorescence. The values of Q are about three times smaller in water than in dioxane, as expected from previous work[5]. The straight lines drawn through the values of Q measured in 1,4-dioxane and water, respectively, are

$$Q = 0.250/x \quad (2)$$

$$Q = 0.0860/x \quad (3)$$

which are the linear least squares straight lines restrained so that they pass through the origin. In the absence of this restraint, the intercepts at $1/x = 0$ would be at values of Q of 0.0032 in 1,4-dioxane and -0.0050 in water. Since these unrestrained intercepts lie very close to the origin, and a negative value for Q is unrealistic, the lines in *Figure 3* were drawn as specified by equations (2) and (3).

Equations (2) and (3) can be combined as

$$Q = k/x \quad (4)$$

where the value of k depends on the solvent. Monodisperse oligomers with interflavan linkages with other stereochemistries are not available over the same range of x. Hence their adherence to equation (4) must remain a matter of conjecture. If they do indeed obey equation (4), it is certain that the value of k will depend on the type of interflavan linkage between the monomer units, because the Q values reported in *Table 1* show that dimers with interflavan linkages with $4\beta \rightarrow 8$ stereochemistry tend to have larger values of Q than dimers with linkages that have $4\alpha \rightarrow 8$ or $4\beta \rightarrow 6$ stereochemistries.

The physical explanation for the molecular weight dependence of Q must await the acquisition of time resolved fluorescence data and the study of the depolarization of the fluorescence in media sufficiently viscous to prevent rotational diffusion of the chromophores on the nanosecond time scale.

Type of molecular weight average for polydisperse samples

Equation (4) describes the values of Q measured with monodisperse oligomers. After removal of the oligomers with $x = 1$–6, the remaining polymer has values of Q of 0.023 ± 0.002 and 0.0085 ± 0.0004 in dioxane and in

Table 1 Fluorescence quantum yields for monomers and five dimers in 1,4-dioxane and in water

Compound[a]	Interflavan linkage	1,4-Dioxane	Water
	–	0.224 ± 0.012	0.105 ± 0.008
	–	0.228 ± 0.017	0.090 ± 0.005
	$4\beta \rightarrow 8$	0.140 ± 0.011	0.041 ± 0.003
	$4\beta \rightarrow 8$	0.149 ± 0.009	0.041 ± 0.004
	$4\alpha \rightarrow 8$	0.092 ± 0.007	0.035 ± 0.002
	$4\alpha \rightarrow 8$	0.080 ± 0.004	0.024 ± 0.002
	$4\beta \rightarrow 6$	0.095 ± 0.006	0.038 ± 0.003

[a] Covalent structures of the dimers are depicted in *Figure*

water, respectively. When inserted in equations (2) and (3), these values of Q yield $x=10$ for the polymer from which oligomers with $x=1$–6 have been removed. In view of the fact that the polymer is polydisperse, it is necessary to inquire into the type of average that is measured by equations (2)–(4).

Let the measured fluorescence quantum yields be written as

$$Q=\sum F_x \Big/ \sum A_x \tag{5}$$

where F_x is the fluoresence from an oligomer for which the absorbance is A_x, and the summations extend over all species present. From Beer's law, the absorbance due to species x is

$$A_x = x\varepsilon_0 b m_x / x M_0 V = \varepsilon_0 b m_x / M_0 V \tag{6}$$

where ε_0 is the molar extension coefficient per monomer unit, b is the light path, m_x is the mass of the species with degree of polymerization x, M_0 is the molecular weight per monomer unit, and V is the volume of the solution. The values of ε_0 and M_0 are $\sim 4000\,\mathrm{l\,mol^{-1}\,cm^{-1}}$ (in dioxane) and $288\,\mathrm{g\,mol^{-1}}$, respectively. By substitution of equations (4) and (6) into $F_x = Q_x A_x$, the fluorescence from a species with a degree of polymerization of x is

$$F_x = (k/x)\varepsilon_0 b m_x / M_0 V$$
$$= k\varepsilon_0 b m_x / M_x V \tag{7}$$

Insertion of equations (6) and (7) in equation (5) yields

$$Q = kM_0 \sum (m_x/M_x) \Big/ \sum m_x \tag{8}$$

$$= kM_0 \sum n_x \Big/ \sum n_x M_x = kM_0/M_n = k/x_n \tag{9}$$

and hence Q measures the number-average degree of polymerization for the polydisperse polymer. The contribution of each species to the intensity of the fluorescence is independent of its degree of polymerization because F_x is the product of one factor, Q_x, that is inversely proportional to x, and another factor, A_x, that is directly proportional to x.

Measurement of x_n by this method will be most reliable when this average is small, because Q_x vanishes as $x \to \infty$. This fact does not eliminate the usefulness of the method because samples of polymeric procyanidins often have values of x_n that are in the range 5–18 (ref. 14). If k has been determined for the type of polymeric procyanidin of interest, the determination of x_n can be performed with less than 0.1 mg of sample.

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