Interactions between Wine Polyphenols and Aroma Substances. An Insight at the Molecular Level

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Control of the organoleptic quality of wine or grape-derived beverages requires the study of the interactions between flavor volatiles and polyphenols. The influence of catechin and a wine highly condensed tannin fraction on the volatility of aroma substances was investigated using a dynamic headspace technique. In a hydroalcoholic solution, isoamyl acetate, ethyl hexanoate, and benzaldehyde appeared to be more retained than limonene at low catechin concentrations (0−5 g/L). The tannin fraction induced a slight decrease of benzaldehyde volatility and a salting out of limonene and had no effect on the two esters. Furthermore, investigations at the molecular level were conducted using ^{1}H NMR spectroscopy. Chemical shift changes registered upon addition of a ligand to a substrate kept at constant concentration allowed the determination of the dissociation constant in a 1:1 binding model. Complexation with catechin was evaluated to be similarly weak for benzaldehyde and the two esters. In addition, catechin and epicatechin displayed a higher affinity for benzaldehyde than for 3,5-dimethoxyphenol, supporting the hypothesis of a hydrophobic driving force.

Keywords: *Polyphenol; aroma compound; activity coefficient; dissociation constant; ^{1}H NMR*

INTRODUCTION

Sensory evaluation of aroma components is involved in the determination of wine organoleptic quality. Partitioning of the volatile substances between liquid and gas phases is mainly governed by aroma compound volatility and solubility. These physicochemical properties are expected to be influenced by other wine constituents present in the medium such as polysaccharides, proteins, and polyphenols. Wine phenolic compounds originating from grape encompass several structural groups. Anthocyanins (0.2−0.8 g/L) sharing a benzoflavylium cation structure produce the blue to red hues of wine (Ribereau-Gayon, 1982). Proanthocyanidins, one of the main classes of polyphenols, are polyhydroxy flavan-3-ol polymers (1−3 g/L). They mostly consist of (+)-catechin and (−)-epicatechin oligomers also named procyanidins (Figure 1). Partial galloylation of the flavanol 3-hydroxyl group as well as combinations of α- or β-C4→C6 or C4→C8 linkages leads to a wide range of structurally different oligomers. Prieur et al. (1994) have identified *Vitis vinifera* grape seed oligomers with a maximum degree of polymerization of 16. In most grape cultivars, the monomers (+)-catechin and (−)-epicatechin were present in a higher content than procyanidins (Fuleki and Ricardo-da-Silva, 1997). Although initially related to grape composition, wine procyanidins were found to evolve during wine aging through acid-catalyzed depolymerization (Beart et al., 1985). Disappearance of anthocyanins occurs simultaneously with the formation of more stable oligomeric pigments (Haslam and Lilley, 1988). Tannin−tannin and tannin−anthocyanin mixed polymers also arise from condensation through an acetaldehyde unit (Fulcrand et al., 1996; Saucier et al., 1997). Aging in oak barrels promotes the extraction of low molecular weight phenolic compounds, mainly ellagitannins, into wine (Moutounet et al., 1989).

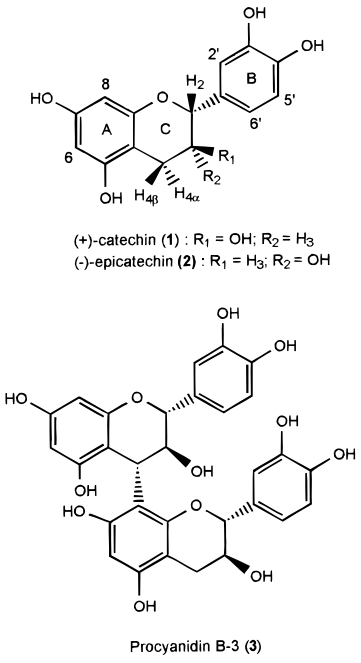

Figure 1. General structures of procyanidin monomers (**1**, **2**) and procyanidin B-3 (**3**).

Wine polyphenols have attracted much attention because of their ability to interact with proteins. Astringency is ascribed to the precipitation in the mouth of a noncovalent complex between salivary proline-rich proteins and tannins (Haslam and Lilley, 1988). Studies between β-pentagalloylglucose and proline-rich peptides indicated that galloyl ester groups interacted primarily

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with proline residues (Luck et al., 1994; Murray et al., 1994). However, association of catechin and catechin-(4α→8)-catechin with di- and tetrapeptides containing proline residues showed no preference for this amino acid (Hatano and Hemingway, 1996). Complexation appeared to be rather correlated to conformationally accessible hydrophobic regions of both partners (Spencer et al., 1988). Other phenomena resulting from protein−polyphenol interactions involve haze formation in aging wine and protein wine fining. Siebert et al. (1996) reported that proline influenced favorably the protein haze-forming activity, although catechin and tannic acid behaved differently. When grape seed procyanidins were considered, the affinity increased with the degree of polyphenol polymerization and the rate of galloylation (Ricardo-da-Silva et al., 1991).

The influence of wine macromolecules on the sensory quality of wine or grape-derived beverages has not been clarified yet. Studies with structurally different wine polysaccharides have pointed out the importance of polymer uronic acid and protein contents (Dufour and Bayonove, 1999). Removal of polyphenols through filtration or fining treatment and precipitation induced by increasing polymerization during wine aging have been suspected to produce flavor balance modifications. The aim of the present work is to investigate the interactions between selected aroma substances and polyphenols isolated from a red wine. The overall influence of phenolic compounds on aroma activities in hydroalcoholic solutions will be addressed using the dynamic exponential dilution technique (Leroi et al., 1977; Sadafian and Crouzet, 1987). ^{1}H nuclear magnetic resonance (NMR), a complementary spectroscopic method, will be used to probe the interactions at the molecular level.

MATERIALS AND METHODS

Materials. Purity (>98%) of ethyl hexanoate, isoamyl acetate, benzaldehyde, (*S*)-(−)-limonene, and 3,5-dimethoxyphenol was checked by GC/MS or ^{1}H NMR analyses. Aroma compounds, NMR solvents, (+)-catechin, and potassium hydrogen tartrate were from Aldrich Chemical Co. (Milwaukee, WI), and (−)-epicatechin was from Fluka AG (Buchs, Switzerland). The phenolic powder, purified by adsorption of a red wine on a resin, was kindly donated by Dr. M. Moutounet from the Laboratoire des Polymères et des Techniques Physico-Chimiques (IPV−INRA, Montpellier, France). Its composition was as follows: (+)-catechin, 1.9 mg/g; (−)-epicatechin, 6.7 mg/g; anthocyanins, 52.4 mg/g; hydroxycinnamic derivatives, 8.7 mg/g; proanthocyanidins, 0.48 g/g (average degree of polymerization 8.8); and other nonidentified polymeric materials. Model wine was a 10% EtOH/aqueous tartrate solution (v/v) made with 2 g/L potassium hydrogen tartrate adjusted at pH 3.5.

Exponential Dilution. The experimental setup was similar to the device described by Dufour and Bayonove (1998). In a double-jacketed glass cell were placed the solvent (5 or 10 mL), the pure aroma compound via syringe, and the phenolic substrate. The solution was incubated at 25 ± 0.1 °C for 30 min under magnetic stirring. A nitrogen flow gas was then bubbled into the stirred solution through a glass frit disk. In 10% EtOH/water, the stripping N_2 flow rates and flavor concentrations were, respectively, 20−25 mL/min and 30 μL/L for combined ethyl hexanoate and isoamyl acetate (1:1, v/v), 90 mL/min and 200 μL/L for benzaldehyde, and 3−4 mL/min and 30 μL/L for limonene. In model wine, the N_2 flow rates and flavor concentrations were, respectively, 20−25 mL/min and 20 μL/L for combined ethyl hexanoate and isoamyl acetate (1:1, v/v), 40 mL/min and 100 μL/L for benzaldehyde, and as above for limonene. The headspace concentration decrease was followed by repeated injections of 250 μL of the leaving gas through a six-port electropneumatic valve (Valco series W) onto an HP 5890A chromatograph (Hewlett-Packard) equipped with an FID detector and a fused silica capillary column (DB-5, J&W Scientific, 60 m × 0.32 mm i.d., 1 μm film thickness). Carrier gas was N_2 and the flow rate 0.7 mL/min. The detector was set at 250 °C, and the column and the sampling valve were set at 150 °C. Data acquisition and treatment were carried out under APEX software (Autochrom Incorporation).

Determination of Activity Coefficients at Infinite Dilution. Dynamic headspace analysis proceeds by gas stripping of an aqueous solution containing the volatile compound. The aroma concentration has been shown to decrease exponentially against time (Leroi et al., 1977). Activity coefficients γ^∞ were obtained by linear regression of Ln *S* versus time, where *S* is the GC peak area, as presented in Dufour and Bayonove (1999).

Statistical Data Treatment. Replicates ($N = 3-5$) were carried out only for solutions of aroma compounds in the pure solvent. The 95% confidence limits (95% CL) were derived using relationship (1) with SD the standard deviation and *t*

$$95\%\ \mathrm{CL} = \gamma^\infty_{\mathrm{mean}} \pm \mathrm{SD}(t/\sqrt{N}) \quad (1)$$

extracted from the Student *t* table with a degree of freedom of $N - 1$ and a 0.05 significant level. Due to low quantities of wine polyphenols, single or duplicate experiments (average data reported) were performed for each substrate concentration. γ^∞ values lying outside the CL pointed out a substrate influence on the aroma volatility.

NMR Spectroscopy. ^{1}H NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer equipped with a 5-mm switchable probe (^{1}H−^{19}F/^{15}N−^{31}P). ^{1}H chemical shifts were referenced to an external standard of 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt, dissolved in the experiment solvent. For titration, aroma substances were added to 5 mM solutions of catechin and epicatechin in 7-in.-long tubes containing 0.7 mL of D_2O/H_2O/ethanol-d_6 (8:1:1). Real concentrations of the volatiles in solution were calculated after integration of the spectra processed under the Varian VNMR software. For the reverse experiment (i.e., titration of aroma substances with catechin), quantities giving 5 mM aroma solutions were introduced into the tube. Integration relative to catechin showed 2−3 mM real concentrations for benzaldehyde, isoamyl acetate, and ethyl hexanoate. Titration was carried out directly into the NMR tube until saturation was reached via syringe for liquids or weighted amounts for solids. Warming was required to dissolve (epi)catechin at high concentrations. The tube was then spun in the NMR spectrometer probe for 10 min at the experiment temperature (293 or 298 K) before the spectrum was acquired using a presaturation pulse sequence to reduce the water peak signal.

***K*$_d$ Determination.** Changes in ^{1}H chemical shift were used to determine the dissociation constant K_d (Bergeron et al., 1977). The dissociation constant governing the equilibrium between phenolic compound P, kept at constant concentration, aroma substance A, and complex PA is given by

$$K_d = [P][A]/[PA] \quad (2)$$

The free phenolic [P] and aroma [A] concentrations are represented by eqs 3 and 4

$$[P] = [P]_0 - [PA] \quad (3)$$

$$[A] = [A]_0 - [PA] \quad (4)$$

where $[P]_0$ and $[A]_0$ are, respectively, the total concentrations in phenolic and aroma compounds. Exchange between free and bound states is fast on the NMR time scale. Thus, the observed chemical shift δ_{obs} of a phenolic proton at any point of the titration is expressed by eq 5

$$\delta_{obs} = \frac{[P]}{[P]_0}\delta_P + \frac{[PA]}{[P]_0}\delta_{PA} \quad (5)$$

Table 1. Physicochemical Properties and Mean Activity Coefficients for Various Aroma Compounds in 10% EtOH/Water or in Model Wine at 298 K

aroma compd	P^s_{solute} (10^{-3} atm)[a]	log P[b]	γ^∞_{mean}	SD/γ^∞_{mean} (%)	other authors' γ^∞ (P^s_{solute}, 10^{-3} atm)
isoamyl acetate	7.092	2.11[c]	2958[e]	4.4	1514[g]
			2962[f]	0.99	
ethyl hexanoate	3.422	2.76[c]	7800[e]	5.4	9424[c]
			8252[f]	0.51	
limonene	2.666		200174[e]	4.7	33398 (2.66)[h]
			192668[f]	2.0	77700 (2.5)[i]
benzaldehyde	1.229	1.5[d]	934[e]	4.3	1457 (0.987)[d]
			903[f]	0.34	

[a] Saturated vapor pressure calculated from vapor pressure/temperature couples (West, 1983−1984); ethyl isocaproate vapor pressure for ethyl hexanoate. [b] Logarithm of the partition coefficient between water and *n*-octanol. [c] Lubbers et al. (1994); γ^∞ in model wine. [d] Landy et al. (1997); γ^∞ in water. [e] In 10% EtOH/water. [f] In model wine. [g] In model wine (Voilley et al., 1991). [h] In water (Langourieux and Crouzet, 1994). [i] In water (Sadafian and Crouzet, 1987).

where δ_P and δ_{PA} are the chemical shifts of the free and fully complexed phenolic species. The change in chemical shift $\Delta\delta$ is defined as $\Delta\delta = \delta_P - \delta_{obs}$. Substitution of eqs 3 and 5 into that statement results in eq 6, with $\Delta\delta_{max} = \delta_P - \delta_{PA}$.

$$\Delta\delta = \frac{[PA]}{[P]_0}\Delta\delta_{max} \quad (6)$$

Incorporation of eqs 3 and 4, followed by eq 6, into eq 2 rearranges to

$$\Delta\delta = \frac{\Delta\delta_{max}[A]_0}{[A]_0 + [P]_0 + K_d - ([P]_0/\Delta\delta_{max})\Delta\delta} \quad (7)$$

K_d and $\Delta\delta_{max}$ were calculated using a least-squares fitting routine within the software program SigmaPlot (Jandel Corp.). Association constant K_a is the reciprocal of K_d. Expression 7, assuming a 1:1 binding model, is similar to the one used by Murray et al. (1994).

Self-Association. (Epi)catechin, benzaldehyde, and 3,5-dimethoxyphenol self-association was determined by calculating the association constant K_a according to eq 8 (Baxter et al., 1996).

$$\Delta\delta = \Delta\delta_{max}K_a[P]_0\{2/(1 + (4K_a[P]_0 + 1)^{1/2})\}^2 \quad (8)$$

RESULTS AND DISCUSSION

Variation of Activity Coefficients by Exponential Dilution. The effects of (+)-catechin and a wine tannin fraction were evaluated on the activity of four aroma substances. Branched and linear esters, isoamyl acetate and ethyl hexanoate, are important contributors to wine flavor (Etievant, 1991). Limonene was selected for its high hydrophobicity and benzaldehyde for the aromatic framework potentially favoring π-stacking with polyphenols. Individual activity coefficients for isoamyl acetate (IA) and ethyl hexanoate (EH) were determined in the ternary systems IA/EH/solvent (Table 1). Results in the presence of polyphenol substrates provided an evaluation of competition (Dufour and Bayonove, 1999). Limonene and benzaldehyde γ^∞ values were measured individually. The relative infinite dilution activity coefficient is defined as the ratio $\gamma^\infty_{solvent+macromolecule}/\gamma^\infty_{solvent}$. In Figures 2 and 3, broken lines represent the 95% significant limits for reported experimental values.

(+)-Catechin. The volatility decrease of isoamyl acetate, ethyl hexanoate, and benzaldehyde appeared to be correlated to increasing catechin concentrations (Figure 2). The retention level reached 13−16% for 10 g/L catechin hydroalcoholic solutions. Saturation (15 g/L catechin) induced a visible rise in viscosity as well as a strong salting out of both esters. Benzaldehyde was also studied in model wine showing no pH or low salt concentration influence on the retention extent. Higher catechin concentrations were required to lower significantly the activity of limonene. Similarly, King and Solms (1982) reported no variation and an 8% decrease of the air−water partition coefficient of (+)-limonene at, respectively, 0.4 and 1.5% caffeine levels. However, the air−water partition coefficient for benzaldehyde was reduced by 39% at the same high caffeine level (Solms, 1986).

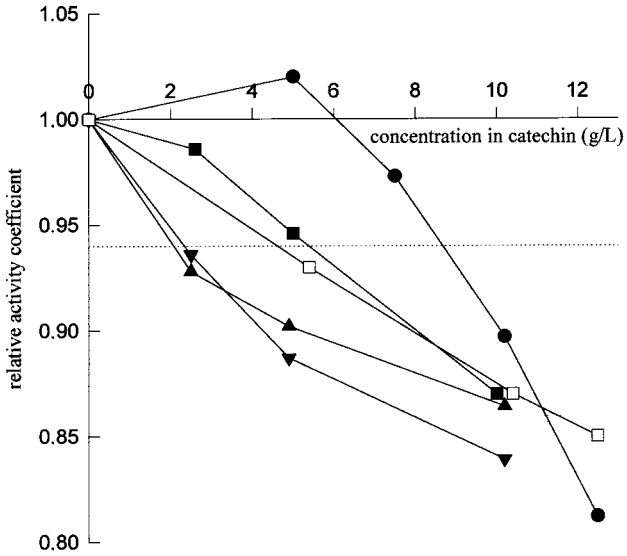


Figure 2. Variation of relative activity coefficients of aroma compounds as a function of catechin concentration at 298 K. In 10% EtOH/water: (●) limonene, (■) benzaldehyde, (▲) isoamyl acetate, (▼) ethyl hexanoate. In model wine: (□) benzaldehyde.

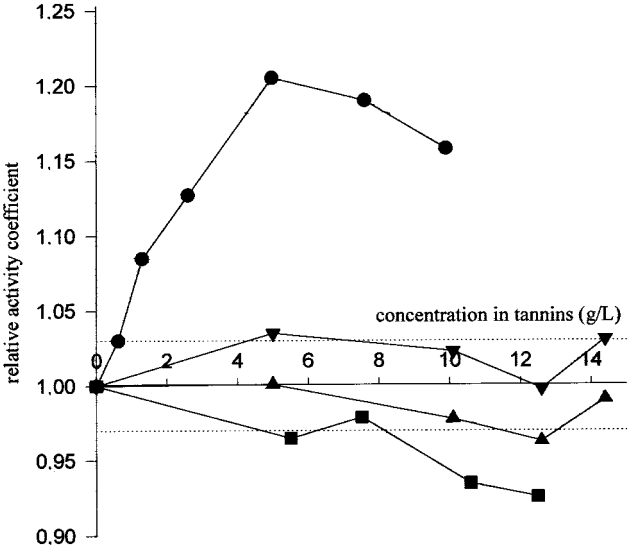


Figure 3. Variation of relative activity coefficients of aroma compounds as a function of tannin concentration at 298 K. In model wine: (●) limonene, (■) benzaldehyde, (▲) isoamyl acetate, (▼) ethyl hexanoate.

Tannin Fraction. This fraction mainly contained wine polymeric phenolic compounds of which analysis indicated half was proanthocyanidins. This part was conducted in a model wine at pH 3.5 due to anthocyanin

Table 2. Proton Assignment (δ), Association Constant (K_a), and Maximum Chemical Shift Changes ($\Delta\delta_{max}$) for the Self-Association of Catechin and Epicatechin at 293 K

	catechin			epicatechin		
proton	δ (mult, J)[a]	K_a (M^{-1})	$\Delta\delta_{max} \pm$ SD (ppm)	δ (mult, J)[a]	K_a (M^{-1})	$\Delta\delta_{max} \pm$ SD (ppm)
H4α	2.86 (dd, 5.3, 16.1)	7.36	0.23 ± 0.03	2.75 (d, 16.3)	4.26	0.22 ± 0.03
H4β	2.54 (dd, 7.9, 16.1)	4.62	0.48 ± 0.08	2.91 (dd, 4.1, 16.3)	5.60	0.36 ± 0.04
H3	4.22 (m)	6.01	0.69 ± 0.12	4.30 (br s)	4.48	0.40 ± 0.05
H2	4.76 (d, 7.5)	4.31	0.89 ± 0.16	4.96 (br s)	5.88	0.72 ± 0.08
H8	6.00 (s)			6.06 (s)		
H6	6.08 (s)			6.08 (s)		
H6′	6.85 (d, 7.9)			6.92 (s)		
H5′	6.90 (d, 7.9)			6.92 (s)		
H2′	6.93 (s)			7.02 (s)		
mean ± SD		5.58 ± 1.40			5.06 ± 0.80	
K_d (M)		0.179			0.196	

[a] Chemical shift in parts per million for a 5 mM solution in D_2O/H_2O/ethanol-d_6 (8:1:1) (multiplicity, J value in Hz).

traces. Variations of relative activity coefficients upon addition of tannins are reported in Figure 3. The esters were not significantly affected, whereas benzaldehyde activity decreased at high tannin concentrations. However, the retention level remained lower than with catechin. The behavior of limonene was found to differ when going from monomeric to polymeric phenols. A dramatic salting-out effect was registered in the 0−5 g/L range followed by a leveling off. The solubility lowering of a very hydrophobic solute could be linked to a higher solvation requirement of polymeric phenols as compared to catechin (saturation also occurred at 15 g/L tannins). Besides, hydrophobic binding may be prevented for structural reasons, although stacking of catechin could lead to complex three-dimensional structures. Foaming at the air−liquid interface and viscosity are additional factors accounting for a matrix effect on volatility. Godshall (1997) suggested that mass transport rather than phase partitioning could govern dynamic flavor release. For measuring overall effects, the exponential dilution technique parallels well the sensory evaluation as performed in enology. Probing the interactions at a molecular level should help to differentiate matrix effects from intermolecular interactions.

Aroma−Monomeric Polyphenol Interactions by ^{1}H NMR. Monomeric polyphenols (+)-catechin and (−)-epicatechin present structural differences linked to their C-3 stereochemistry (Steynberg et al., 1992). In addition to the aroma compounds investigated in the first part, a volatile phenol, 3,5-dimethoxyphenol, was selected for comparison with benzaldehyde.

Assignment of Catechin and Epicatechin Protons. ^{1}H NMR experiments were conducted in D_2O/H_2O/ethanol-d_6 (8:1:1). Use of all deuterated solvents led to the fast disappearance of H-6 and H-8 through H/D exchange. Assignment of the procyanidin protons in the NMR solvent system was required before titration could be begun. ^{1}H chemical shift order for (+)-catechin was as determined by Balas (1993) in $CDCl_3$ at 303 K, although small ^3J coupling constants (<2 Hz) were not observed (Table 2). Assignments using coupling constants for H-4α (J = 5.3 and 16.1 Hz) and H-4β (J = 7.9 and 16.1 Hz) were in agreement with those reported by Hemingway et al. (1996) in 20% methanol-d_4/D_2O. H-6 and H-8 A-ring protons appeared as singlets over a 0.1 ppm range in the high-field aromatic region. H-8 was assigned upfield from H-6 as reported by Hemingway et al. (1996). In the low-field aromatic region, proton H-2′, with a singlet pattern, appeared downfield from the other B-ring protons. The two doublets corresponding to H-5′ and H-6′ were assigned as for catechin in methanol-d_4 (Hemingway et al., 1996), $CDCl_3$ (Balas, 1993), and acetone-d_6 (Kashiwada et al., 1990) and finally by comparison with reported data for 2,3-*trans*-3,4-*cis*-4-arylflavan-3-ols (Van Zyl et al., 1993). Epicatechin chemical shifts for doublet H-4α (J = 16.3 Hz) and doublet of doublet H-4β (J = 4.1 and 16.3 Hz) were reversed as compared to catechin, in agreement with resonances in most solvents (Balas, 1993; Kashiwada et al., 1990). At low concentrations, epicatechin B-ring protons appeared as two singlets (δ = 6.92, 2H; δ = 7.02, 1H). Upon increasing concentrations of epicatechin or interacting ligand, the overlapped singlets at δ 6.92 were progressively replaced by two well-resolved doublets with a coupling constant ^3J of 8.1 Hz, indicative of an ortho coupling (Figure 4). Besides, the upfield doublet presented an additional small coupling (<2 Hz) supporting the following upfield to downfield assignment order: H-6′, H-5′, and H-2′.

Self-Association. Increasing addition of (epi)catechin to an initial 1 mM solution resulted in the shift of all proton resonances to higher fields. Pyran aliphatic protons were more affected than aromatic ring protons. Shielding of proton signals is generally attributed to the magnetic anisotropy associated with ring current effects in neighboring molecules. Stacking of the procyanidin monomers probably occurs in self-association. Similarly, upfield shifts for all malvin chloride hemiacetal protons were accounted for by a $\pi-\pi$ stacking type interaction (Mistry et al., 1991). Data fitting of the 1:1 binding equation led to a high dependency of the variables K_a and $\Delta\delta_{max}$. Association constants were reported only for aliphatic chemical shift changes where variation coefficients for K_a were <30% (Table 2). Biased data for aromatic protons resulted from a lack of chemical shift perturbation at very low monomer concentrations. Sigmoidal binding curves may indicate requirement for a more complex binding model. Self-association constants for catechin and epicatechin were found to be similar in the NMR solvent system. However, epicatechin K_a was ∼5 times weaker than the value reported in pure water at 276 K by Baxter et al. (1997). This fact is in favor of a hydrophobic interaction with disruption of the binding by ethanol. This group also reported maximal changes in chemical shift ($\Delta\delta_{max}$), which proved to be very similar to our calculated values for epicatechin aliphatic protons. In addition, the same $\Delta\delta_{max}$ interval was observed between the H-4α and H-4β protons. Catechin conformational changes were observed during self-association as reflected by a continuous evolution of $J_{2,3}$ from 7.3 Hz (1.5 mM catechin concentration) to 7.9 Hz (72 mM concentration). Indeed, interconversion between the pseudodiaxial (A) and the pseudodiequatorial (E) conformations of the heterocycle occurs due

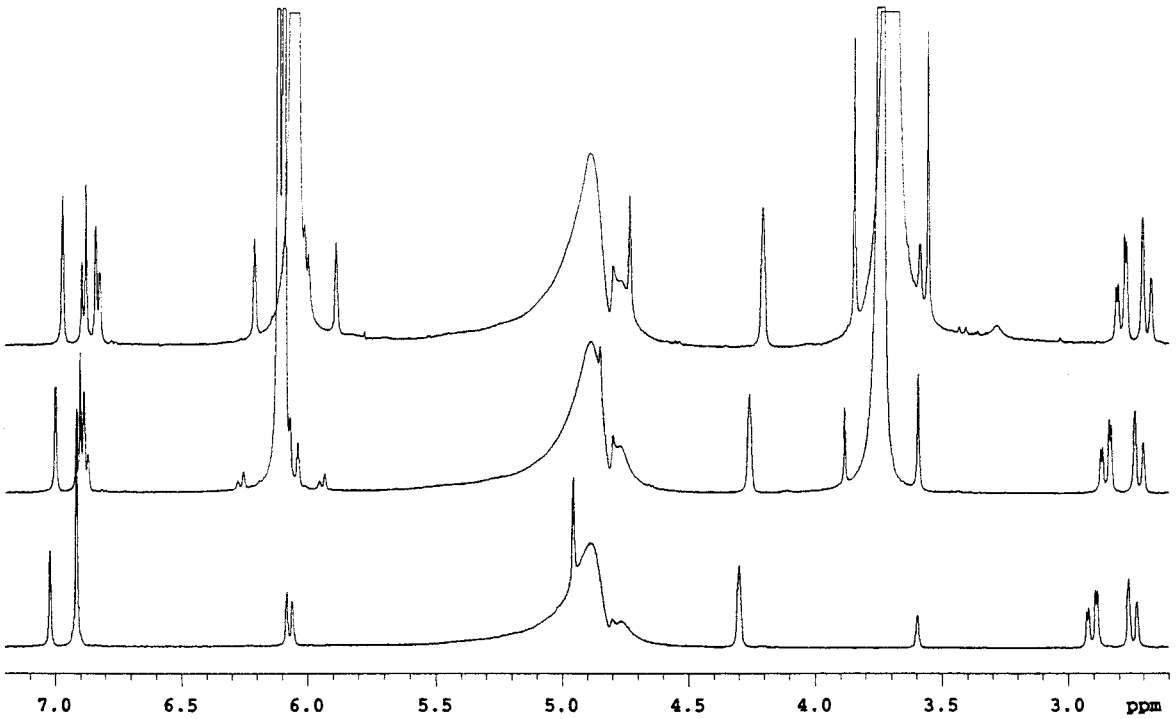


Figure 4. Effect of 3,5-dimethoxyphenol concentration on (−)-epicatechin ^{1}H chemical shifts in D_2O/H_2O/ethanol-d_6 (8:1:1) at 298 K (500 MHz): (bottom) epicatechin alone (5 mM); (middle) in the presence of 3,5-dimethoxyphenol (46 mM); (top) in the presence of 3,5-dimethoxyphenol (162 mM).

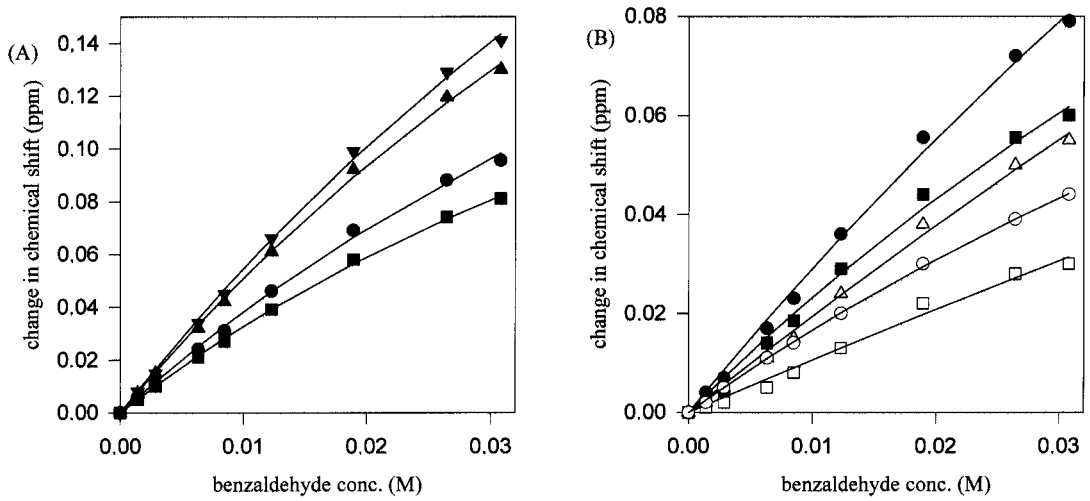
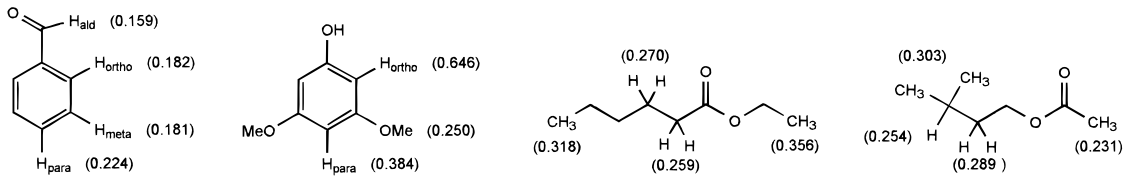


Figure 5. Change in catechin chemical shift with increasing benzaldehyde concentration for the titration of catechin with benzaldehyde at 293 K. (A) Observed values for aliphatic protons: (■) H4α, (●) H4β, (▲) H3, (▼) H2; fitted values (—). (B) Observed values for aromatic protons with solid symbols for A-ring protons [(circle) H8, (square) H6], open symbols for B-ring protons [(triangle) H6′, (circle) H2′, (square) H5′]; fitted values (—).

to a relatively low energy barrier. The population for each conformer can be predicted from the observed $J_{2,3}$ coupling constant using molecular mechanics calculations (Steynberg et al., 1992). Assuming that the A and E conformations in solution were similar to those calculated, self-association led to an A:E ratio change from 37:63 at low catechin concentrations to 29:71 at high concentrations.

Self-association of aroma compounds was tentatively evaluated. Increasing flavor concentration up to saturation induced no chemical shift variations for ethyl hexanoate and isoamyl acetate. In contrast, benzaldehyde and 3,5-dimethoxyphenol displayed binding curves similar to that of (epi)catechin. Self-association constants were found to be, respectively, 1.016 ± 0.096 M^{-1} (293 K) and 1.017 ± 0.408 M^{-1} (298 K). The two volatiles appeared to be less self-associated than the procyanidin monomers. Aroma compound or (epi)catechin self-association was not taken into account in the next studies for data treatment homogeneity.

Addition of Aroma Compounds to Procyanidin Monomers. Titration of catechin or epicatechin with limonene, isoamyl acetate, and ethyl hexanoate was unsuccessful due to the low solubility of these lipophilic molecules. However, addition of benzaldehyde and 3,5-dimethoxyphenol led to upfield shifts of all flavanol protons (Figure 5). As observed in self-association, chemical shift differences recorded for aliphatic protons fit better the 1:1 binding model than the aromatic proton data. Only individual dissociation constants with variation coefficients <30% were retained for the mean K_d calculation (Table 3). Owing to epicatechin H-2 moving upfield into



(4) (5) (6) (7)

Figure 6. Dissociation constants K_d (M) measured for different protons of benzaldehyde (**4**), 3,5-dimethoxyphenol (**5**), ethyl hexanoate (**6**), and isoamyl acetate (**7**) upon titration with (+)-catechin at 293 K.

Table 3. Titration of Catechin and Epicatechin with Aroma Substances Benzaldehyde and 3,5-Dimethoxyphenol[a]

	benzaldehyde (293 K)		3,5-dimethoxyphenol (298 K)	
K_d (M)	catechin	epicatechin	catechin	epicatechin
H4α	0.0741	0.107	0.2495	0.182
H4β	0.0820	0.122	0.178	0.152
H3	0.0942	0.227	0.170	0.189
H2	0.103		0.175	0.135
H8			0.195	0.187
H6	0.111			
H2′	0.109			
mean ± SD	0.0955 ± 0.0149	0.152 ± 0.066	0.194 ± 0.038	0.169 ± 0.024

[a] Dissociation constant determination in D_2O/H_2O/ethanol-d_6 (8:1:1) at 293 or 298 K.

Table 4. Dissociation Constants for the Binding of (+)-Catechin to Various Aroma Substances in D_2O/H_2O/Ethanol-d_6 (8:1:1) at 293 K

aroma substance	K_d (M) ± SD
benzaldehyde	0.187 ± 0.027
3,5-dimethoxyphenol	0.427 ± 0.202
isoamyl acetate	0.269 ± 0.033
ethyl hexanoate	0.301 ± 0.045

the residual water peak, interactions between this substrate and 3,5-dimethoxyphenol were further studied at 298 K (Figure 4).

As in self-association, the $J_{2,3}$ coupling constant for catechin increased upon complexation with the two ligands, revealing a conformational change of the pyran ring: $J = 7.5-8.0$ Hz for 0−168 mM 3,5-dimethoxyphenol concentrations; $J = 7.3-7.7$ Hz for 0−189 mM benzaldehyde concentrations. Benzaldehyde complexation appeared to be stronger with catechin than with epicatechin, particularly when using H-4α and H-4β for comparison. Although K_d values for 3,5-dimethoxyphenol were measured at 298 K, they suggested a reduced affinity for this ligand. Furthermore, benzaldehyde and 3,5-dimethoxyphenol appeared to be weaker ligands compared to caffeine in water as reported by Cai et al. (1990). Finally, comparison between monomer self-associations and complexation with benzaldehyde indicated that the monomers had a higher affinity for benzaldehyde than for themselves. This affinity difference proved not to be significant for 3,5-dimethoxyphenol.

Addition of (+)-Catechin to Aroma Compounds. Individual K_d values recorded for the addition of catechin to various aroma compounds except limonene, which showed a low solubility, are reported in Figure 6. During the course of the titration, the catechin ^{1}H resonances shifted upfield due mainly to self-association. The shift extent was found to be similar for the aromatic protons and lower for the aliphatic protons. In addition, ligand binding with monomeric or stacked catechins was assumed with the same K_a. Benzaldehyde displayed the highest affinity and 3,5-dimethoxyphenol the lowest (Table 4). This is in agreement with the results obtained in the titration of catechin with the two aromatic ligands. Although 3,5-dimethoxyphenol and catechin A-ring present close hydroxylation patterns, and hence similar hydrogen-bonding abilities, this was not sufficient to produce strong interactions. Benzaldehyde−catechin interaction ($K_d = 0.187$ M) appeared to be stronger than the corresponding flavor self-association ($K_d \sim 1$ M). The two esters with similar hydrophobic surfaces, as suggested by log *P* values (Table 1), led to close dissociation constants. Likewise, the hydrocarbon chain of *O*-*n*-octyl-β-D-glucose was found to be the preferential site for association with various polyphenols (Spencer et al., 1988). Besides, comparison between the two titration modes pointed out a factor of 2 for benzaldehyde and 3,5-dimethoxyphenol K_d values, outlining a limitation of the method for weak complexations. It seemed then reasonable to keep titration conditions similar for comparison purposes.

CONCLUSION

Aroma substance retention by catechin was first characterized using the exponential dilution technique. This influence was quantitatively determined as weak and ascribed to a matrix effect and/or ligand−substrate intermolecular interactions. ^{1}H NMR studies of the catechin addition to isoamyl acetate, ethyl hexanoate, and benzaldehyde supported the previous results. Indeed, the dissociation constants were found in the same range, indicating a similar weak bimolecular binding to catechin.

Wine concentrations in total polyphenols are usually found in the low studied concentrations (0−5 g/L). When the only polyphenol present was catechin, the retention appeared to be significant for the two esters in 10% ethanol/water. In the case of the wine tannin fraction, only limonene was significantly influenced in the 0−5 g/L range. In view of these preliminary results, variations in the total polyphenol concentration may only weakly influence flavor levels in a wine. However, processes involving polyphenol aggregation could lead to a significant loss of aroma compounds through intermolecular interactions. Tannin precipitation, cross-flow filtration, and protein fining might then be detrimental to wine sensory quality.

Studies in binary or ternary systems do not deal with the complexity of grape-derived beverages. Indeed, interactions between polyphenols themselves and polyphenols and other wine macromolecules have not been taken into account so far. Synergism or antagonism on flavor volatility may well contribute to wine aroma.

At the molecular level, ^{1}H NMR spectroscopy proved to be a valuable tool enabling the determination of thermodynamic data such as dissociation constants. Titration conditions had to be set constant for data

comparison, reflecting then a limitation of the method. Hydrophobicity appeared as a driving force for bimolecular aroma−phenolic compound interactions. Additional NMR experiments such as nuclear Overhauser enhancement measurements should help to define the binding site and specify the extent of hydrogen bonding in the complex stabilization.

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LITERATURE CITED

Balas, L. Tanins catéchiques: isolement, hemisynthèse et analyse structurale par RMN 2D homo- et hétéronucléaire. Thesis, Université de Bordeaux 2, Bordeaux, France, 1993.

Baxter, N. J.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Stacking interactions between caffeine and methyl gallate. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 231−234.

Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.

Beart, J. E.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations on some polymeric proanthocyanidins. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1439−1443.

Bergeron, R. J.; Channing, M. A.; Gibeily, G. J.; Pillor, D. M. Disposition requirements for binding in aqueous solution of polar substrates in the cyclohexaamylose cavity. *J. Am. Chem. Soc.* **1977**, *79*, 5146−5151.

Cai, Y.; Gaffney, S. H.; Lilley, T. H.; Magnolato, D.; Martin, R.; Spencer, C. M.; Haslam, E. Polyphenol interactions. Part 4. Model studies with caffeine and cyclodextrins. *J. Chem. Soc., Perkin Trans. 2* **1990**, 2197−2209.

Dufour, C.; Bayonove, C. L. Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system. *J. Agric. Food Chem.* **1999**, *47*, 671−677.

Etievant, P. X. Wine. In *Volatile Compounds in Foods and Beverages*; Maarse, H., Ed.; Dekker: New York, 1991; pp 483−546.

Fulcrand, H.; Cameira dos Santos, P. J.; Sarni Manchado, P.; Cheynier, V.; Favre-Bonvin, J. Structure of new anthocyanin derived wine pigments. *J. Chem. Soc., Perkin Trans. 1* **1996**, 736−739.

Fuleki, T.; Ricardo-da-Silva, J. M. Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *J. Agric. Food Chem.* **1997**, *45*, 1156−1160.

Godshall, M. A. How carbohydrates influence food flavor. *Food Technol.* **1997**, *51*, 63−67.

Haslam, E.; Lilley, T. H. Natural astringency in foodstuffs—a molecular interpretation. *CRC Crit. Rev. Food Sci. Nutr.* **1988**, *27*, 1−40.

Hatano, T.; Hemingway, R. W. Association of (+)-catechin and catechin-(4α→8)-catechin with oligopeptides. *J. Chem. Soc., Chem. Commun.* **1996**, 2537−2538.

Hemingway, R. W.; Tobiason, F. L.; McGraw, G. W.; Steynberg, J. P. Conformation and complexation of tannins: NMR spectra and molecular search modeling of flavan-3-ols. *Magn. Reson. Chem.* **1996**, *34*, 424−433.

Kashiwada, Y.; Iizuka, H.; Yoshioka, K.; Chen, R.; Nonaka, G.; Nishioka, I. Tannins and related compounds. XCIII. Occurrence of enantiomeric proanthocyanidins in the leguminosae plants, *Cassia fistula* L. and *C. javanica* L. *Chem. Pharm. Bull.* **1990**, *38*, 888−893.

King, B. M.; Solms, J. Interactions of volatile flavor compounds with propyl gallate and other phenols as compared with caffeine. *J. Agric. Food Chem.* **1982**, *30*, 838−840.

Landy, P.; Farès, K.; Lorient, D.; Voilley, A. Effect of chemical modification of sodium caseinate on diffusivity of aroma compounds in aqueous solutions. *J. Agric. Food Chem.* **1997**, *45*, 2649−2653.

Langourieux, S.; Crouzet, J. C. Study of aroma compounds-polysaccharides interactions by dynamic exponential dilution. *Lebensm.-Wiss. -Technol.* **1994**, *27*, 544−549.

Leroi, J. C.; Masson, J. C.; Renon, H.; Fabries, J. F.; Sannier, H. Accurate measurement of activity coefficients at infinite dilution by inert gas stripping and gas chromatography. *Ind. Eng. Chem., Process Des. Dev.* **1977**, *16*, 139−144.

Lubbers, S.; Charpentier, C.; Feuillat, M.; Voilley, A. Influence of yeast walls on the behavior of aroma compounds in a model wine. *Am. J. Enol. Vitic.* **1994**, *45*, 29−33.

Luck, G.; Liao, H.; Murray, N. J.; Grimmer, H. R.; Warminski, E. E.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Polyphenols, astringency and proline-rich proteins. *Phytochemistry* **1994**, *37*, 357−371.

Mistry, T. V.; Cai, Y.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 5. Anthocyanin co-pigmentation. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1287−1296.

Moutounet, M.; Rabier, P.; Puech, J. L.; Verette, E.; Barillere, J. M. Analysis by HPLC of extractable substances in oak wood. Application to a Chardonnay wine. *Sci. Aliments* **1989**, *9*, 35−51.

Murray, N. J.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Study of the interaction between salivary proline-rich proteins and a polyphenol by ^{1}H NMR spectroscopy. *Eur. J. Biochem.* **1994**, *219*, 923−935.

Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

Ribereau-Gayon, P. The anthocyanins of grapes and wines. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York, 1982; pp 209−244.

Ricardo-da-Silva, J. M.; Cheynier, V.; Souquet, J. M.; Moutounet, M. Interaction of grape seed procyanidins with various proteins in relation to wine fining. *J. Sci. Food Agric.* **1991**, *57*, 111−125.

Sadafian, A.; Crouzet, J. Infinite dilution activity coefficients and relative volatilities of some aroma compounds. *Flavour Fragrance J.* **1987**, *2*, 103−107.

Saucier, C.; Bourgeois, G.; Vitry, C.; Roux, D.; Glories, Y. Characterization of (+)-catechin-acetaldehyde polymers: a model for colloidal state of wine polyphenols. *J. Agric. Food Chem.* **1997**, *45*, 1045−1049.

Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol-protein interactions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.

Solms, J. Interactions of non-volatile and volatile substances in foods. In *Interactions of Food Components*; Birch, J. C., Lindley, M. G., Eds.; Elsevier Applied Sciences Publishers: London, 1986; pp 189−210.

Spencer, C. M.; Cai, Y.; Russell, M.; Gaffney, S. H.; Goulding, P. N.; Magnolato, D.; Lilley, T. H.; Haslam, E. Polyphenol complexation-some thoughts and observations. *Phytochemistry* **1988**, *27*, 2397−2409.

Steynberg, J. P.; Brandt, E. V.; Hoffmann, J. H.; Hemingway, R. W.; Ferreira, D. Conformations of proanthocyanidins. In *Plant Polyphenols: Synthesis, Properties, Significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; pp 501−520.

Van Zyl, P. W.; Steynberg, J. P.; Brandt, E. V.; Ferreira, D. Spectroscopic properties of free phenolic 4-arylflavan-3-ols as models for natural condensed tannins. *Magn. Reson. Chem.* **1993**, *31*, 1057−1063.

Voilley, A.; Beghin, V.; Charpentier, C.; Peyron, D. Interactions between aroma substances and macromolecules in a model wine. *Lebensm.-Wiss. -Technol.* **1991**, *24*, 469−472.

West, R. C., Ed. *CRC Handbook of Chemistry and Physics*, 64th ed.; CRC Press: Boca Raton, FL, 1983−1984; D199−D214.

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