Study of Interactions between Food Phenolics and Aromatic Flavors Using One- and Two-Dimensional ^{1}H NMR Spectroscopy

Da-Mi Jung,[†] Jeffrey S. de Ropp,[‡] and Susan E. Ebeler*,[†]

Department of Viticulture and Enology and NMR Instrumentation Facility, University of California, Davis, Davis, California 95616

Changes in flavor release and aroma characteristics in a medium including food phenolics may be attributed to an intermolecular interaction between flavor compounds and phenolics. To investigate the interaction, one- and two-dimensional NMR studies have been carried out on the binding of two phenolics, gallic acid and naringin, with three aroma compounds, 2-methylpyrazine, vanillin, and ethyl benzoate. Evaluation of thermodynamic parameters and intermolecular nuclear Overhauser effects reveals that gallic acid can interact more strongly with aromatic flavors than naringin. The supramolecular complexation is also dependent on the structural nature of the flavors, with 2-methylpyrazine and vanillin interacting more strongly than ethyl benzoate. The interaction is principally $\pi-\pi$ stacking between the galloyl ring and the aromatic ring of the aroma compounds, but secondary hydrogen-bonding effects help to stabilize the complex and enhance the specificity.

Keywords: *Food phenolics; nuclear Overhauser effect; supramolecular complexation; $\pi-\pi$ stacking; hydrogen bonding; flavor interaction*

INTRODUCTION

The interaction or binding of flavors with nonvolatile food components can have a significant effect on the perception of food and beverage flavor. For example, fats and salts alter the relative solubility and partitioning behavior of nonpolar flavors, dramatically affecting the overall aroma perception and flavor release in the mouth (Solms et al., 1973; Maier, 1975; Land and Reynolds, 1981; Poll and Flink, 1984). The presence of proteins or carbohydrates can decrease the taste and aroma intensity of flavor chemicals via sorption, binding, and the formation of intermolecular complexes between the flavors and the protein or carbohydrate (Maier, 1970, 1975; Nawar, 1971; Franzen and Kinsella, 1974; Ahmed et al., 1978; King and Solms, 1979; Tsui et al., 1994; O'Neil, 1996; Pelletier et al., 1998).

Although a number of studies have examined the interaction of flavors or odorants with lipids, salts, carbohydrates, and proteins, little is known about the effects phenolics may have on volatile flavor constituents. Phenolics are a major constituent of plants. They are present in foods of plant sources, beverages, folk medicines, and herbal remedies, constituting as much as 6−30% of total dry weight (Singleton, 1992; Shahidi and Naczk, 1995). They have a number of biological and pharmacological activities including antioxidant and radical scavenging activities and the ability to complex with metal ions and other molecules such as proteins and polysaccharides (Spencer et al., 1988; Haslam, 1989; Shahidi and Naczk, 1995; Siebert et al., 1996). In addition, King and Solms (1981, 1982) showed that naringin, a natural polyphenolic flavonoid in citrus, affected the volatility of flavor compounds differently depending on the chemical structure of the odorant. Decreased volatility was observed for ethyl benzoate and 2,3-diethylpyrazine, whereas there was no effect on the volatility of limonene. They proposed that flavor compounds with extended π-electron systems such as ethyl benzoate and 2,3-diethylpyrazine can interact with polyphenols through hydrophobic and π-electron interactions. However, the authors did not provide a systematic evaluation of the hypothesis or further study the interaction mechanism.

Multidimensional nuclear magnetic resonance (NMR) spectroscopy has proven to be one of the most powerful techniques for determining the structure and conformation of molecules in solution (Bovey, 1988; Derome, 1987). In recent years, several researchers have studied the intermolecular interaction and the miscibility of polymer blends in solution by observing the nuclear Overhauser enhancement effect (NOE) (Marchettini et al., 1990; Mucci et al., 1996; Bianco et al., 1997; Mo and Pochapsky, 1997). Because of the inverse sixth-power dependence on the interproton distance, NOE appears only for a pair of protons separated by less than approximately $<\sim$5 Å. These techniques can now be applied to understand the geometry of potential complexes between odorants and polyphenols and their interaction mechanisms (Rounsavill et al., 1996; Jung et al., 1998).

In this study, the interactions between two phenolics, gallic acid (GA) and naringin (NA), and three aromatic flavors, 2-methylpyrazine (MP), vanillin (VA), and ethyl benzoate (EB), were investigated by one- and two-dimensional ^{1}H NMR studies to reveal the structural features of polyphenol/odorant complexes and the driving forces that lead to their association.

* Address correspondence to this author at the Department of Viticulture and Enology, One Shields Ave., University of California, Davis, CA 95616-8749 [e-mail seebeler@ucdavis.edu; telephone (530) 752-0696; fax (530) 752-0382].

[†] Department of Viticulture and Enology.

[‡] NMR Instrumentation Facility.

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MATERIALS AND METHODS

Reagents. Three flavor compounds were used for this study, vanillin (Aldrich, Milwaukee, WI; ≥99%), 2-methylpyrazine (Aldrich; ≥99%), and ethyl benzoate (Aldrich; ≥99%). Flavor compounds were selected for their ability to form hydrogen-bonding and charge-induced dipole interactions.

Two polyphenols, naringin hydrate (Aldrich; ≥99%) and gallic acid (Nutritional Biochemical Co., Cleveland, OH; ≥99%), were studied, which represent flavonoid (glycosylated flavanone) and nonflavonoid (vicinal triphenol) structures, two important polyphenol classes commonly found in foods and beverages.

NMR Procedures. NMR measurements were performed on a Bruker Avance 500 MHz spectrometer equipped with a temperature control unit. Samples were prepared in D_2O or DMSO-d_6; trimethylsilylpropanoate (TSP) and tetramethylsilane (TMS) were added to the D_2O or DMSO-d_6 at a level of 0.05%. To investigate the concentration dependence of chemical shift changes, a 30 mM flavor solution (0.5 mL) was mixed with various amounts of polyphenols covering molar ratios of 1:1, 1:2, and 2:1 in D_2O and observed by single-pulse method. Also, a 10 mM flavor solution (0.5 mL) was mixed with various amounts of polyphenols to cover molar ratios ranging from 1:1 to 1:30 in DMSO-d_6. The ^{1}H NMR spectra were observed by the single-pulse method, and chemical shift changes of protons were measured relative to the reference chemical shift. The experiments were carried out at 25, 45, and 60 °C, from which equilibrium association constants (K_a) and thermodynamic parameters ($\Delta H°$ and $\Delta S°$) were obtained using a nonlinear estimation program, ORIGIN (Microcal Software Co. Inc., Northampton, MA). The NOE was observed by the means of one-dimensional NOE difference experiment and rotating-frame Overhauser effect spectroscopy (ROESY) measurement. The NOE difference spectra were measured with a power level of 90 dB and an irradiation time of 500 ms. The phase-sensitive ROESY spectra were acquired with a spectral width of 7 kHz in 2K data points using 16 scans for each of the 512 t_1 increments. The spin−lock power level and time were set to 29 dB and 200 ms, respectively.

RESULTS AND DISCUSSION

When passing from the free to the complexed state, the ^{1}H and ^{13}C NMR chemical shifts change for individual molecules in the complex. As a result, information about the geometry of the complex can be obtained (Inoue, 1993). Using ^{1}H NMR we evaluated the changes in the chemical shifts of protons in three flavor compounds, 2-methylpyrazine, vanillin, and ethyl benzoate, upon complexation with gallic acid and naringin in D_2O (Table 1 and Figure 1). For complexes of MP and GA (Table 1A), all of the protons of both compounds undergo upfield shifts compared to those of the free uncomplexed state. When the GA concentration is doubled with respect to that of MP, the 3M and 4M protons of MP show higher upfield shift than those in the 1:1 ratio. Conversely, when the MP concentration is doubled with respect to that of GA, large upfield shifts of all protons are observed. The aromatic protons of GA in particular appear to be strongly influenced by changing MP concentration.

Similar trends are also observed with VA and GA complexes (Table 1B). When the GA concentration is doubled, all of the VA protons experience upfield shifts. Specifically, the 3V proton shows substantial shielding effects of up to 23.5 Hz. When the VA concentration is doubled, GA shows the highest upfield chemical shift.

Upfield shifts experienced by the ring protons are commonly attributed to the magnetic anisotropy associated with a ring current shielding effect of an aromatic molecule from the opposing aromatic ring in the favored equiplanar geometry (Lambert et al., 1998). The relatively small changes in observed chemical shifts for our

Table 1. Chemical Shift Changes ($\Delta\delta = \delta_{free} - \delta_{complexed}$, Hertz)[a] of Phenolics and Flavor Complexes with Various Concentration Ratios in D_2O at 25 °C[b]

A

GA:MP	1MP	2MP	3MP	4MP	1GA
1:1	2.0	0.5	2.0	2.5	8.5
2:1	3.0	1.0	6.0	7.5	8.0
1:2	7.0	5.5	7.5	8.0	20.5

B

GA:VA	1VA	2VA	3VA	4VA	6VA[c]	1GA
1:1	4.5	2.5	10.0	3.5	12.0	11.5
2:1	9.5	9.5	23.5	12.5	26.3	17.0
1:2	8.0	7.0	19.5	8.5	22.0	19.5

C

GA:EB	1EB	2EB	3EB	4EB	5EB	1GA
1:1	1.5	2.5	1.0	2.0	2.0	4.0
2:1	2.0	2.0	0.5	1.0	2.0	3.5
1:2	2.0	3.5	2.5	2.5	3.0	4.5

D

NA:MP	1MP	2MP	3MP	4MP
1:1	2.0	1.5	2.5	3.0
2:1	2.5	2.0	3.0	4.5
1:2	2.0	1.5	2.0	2.5

E

NA:VA	1VA	2VA	3VA	4VA	6VA[c]
1:1	1.5	3.0	3.5	4.0	7.5
2:1	2.5	4.0	2.0	4.0	7.7
1:2	1.0	5.0	2.5	3.5	7.0

F

NA:EB	1EB	2EB	3EB	4EB	5EB
1:1	2.0	0	0	1.0	0
2:1	1.0	3.0	1.5	2.0	1.5
1:2	3.0	2.0	3.0	2.0	2.0

[a] Proton numbering is according to Figure 1. [b] Means of duplicate results. [c] Signal intensity of the 6VA proton peak was weak and the peak was broad, precluding precise calculation of the chemical shift.

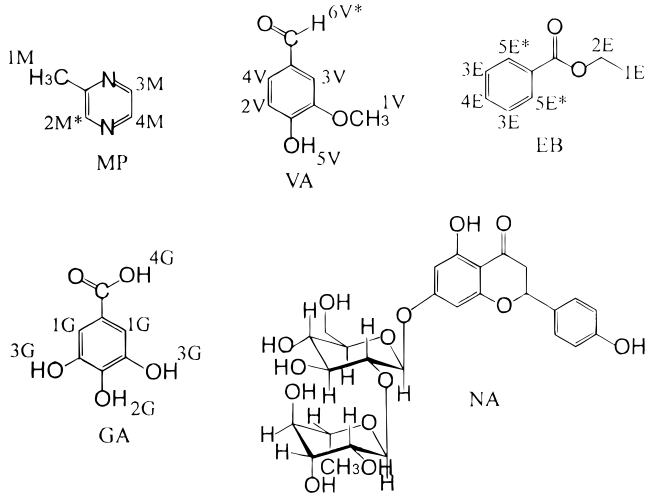

Figure 1. Structures of 2-methylpyrazine (MP), vanillin (VA), ethyl benzoate (EB), gallic acid (GA), and naringin (NA). Protons are numbered according to increasing resonance frequencies in the 1D spectrum. Asterisks (*) indicate the protons that experience the most significant chemical shift changes with increase in concentration of phenolics.

studies may be due to a high mobility of the opposing aromatic rings with a slight preference for the spatial structure consisting of facing aromatic rings. However, the closeness between these aromatic rings may be enhanced by the charge-induced dipole interactions, which can develop complementary interacting dipoles between GA and MP or VA. Additionally, the presence of functional groups can influence the electron density over unsaturated systems, enhancing complexation through π donor−acceptor interactions.

Table 1C shows observed chemical shift changes for GA and EB complexes. GA as well as EB shows only small changes in chemical shifts over the various concentration ratios.

Chemical shift changes of the flavor protons in the presence of NA are quite small relative to those observed for GA complexation (Table 1, sections D−F). In addition, there is no significant trend observed in chemical shift changes with different concentration ratios of NA and flavors. Chemical shift changes were measured only for the flavors.

Association Constants and Thermodynamic Parameters. To better evaluate the strength of the interactions and to determine the spatial proximity of interacting protons, the polyphenol/flavor interactions were further evaluated using NOESY. However, no significant cross-peaks were observed between phenolics and flavor compounds in D_2O, even though complex-induced chemical shift changes were observed in the 1D NMR spectrum. This may be because our samples have small molecular weights that normally tumble rapidly in D_2O, so that there is insufficient time to build up NOE, even with increasing mixing times. In addition, exchangeable protons such as those in carboxylic acids or alcohols do not give separate resonances in D_2O.

Therefore, to further study the nature of the phenolic/flavor interaction, the solvent, DMSO-d_6, was chosen in which exchange is so slow that coupling between OH and adjacent protons can be observed. A titration process was then used to determine the concentration equilibrium constant, K_a, based on the chemical shift changes of flavor protons. One of the protons for each flavor, which was most significantly affected by polyphenolic complexation (Figure 1), was selected to obtain the association constant, K_a, assuming a 1:1 stoichiometry between phenolics and flavors (results were consistent when other protons were used for calculation of the association constants):

$$K_a = \frac{PF}{P \times F} \quad (1)$$

In eq 1, P, F, and PF represent the mole fraction of the free phenolic, the free flavor, and the bound form of the flavors, respectively. When fast exchange processes take place, the NMR spectrum for each proton shows a single signal, averaged in the proportions of the partial mole fractions of the complexed and free molecules (Beck and Nagypal, 1990). Therefore, the observed chemical shift (δ_{obs}) of a signal is a weighted average of the chemical shifts in two possible environments: the chemical shift for the free flavor (δ_F) and the chemical shift for the bound flavor (δ_{PF})

$$\delta_{obs} = \delta_F + (\delta_{PF} - \delta_F)\frac{PF}{F_0} \quad (2)$$

where F_0 represents the total mole fraction of the flavor.

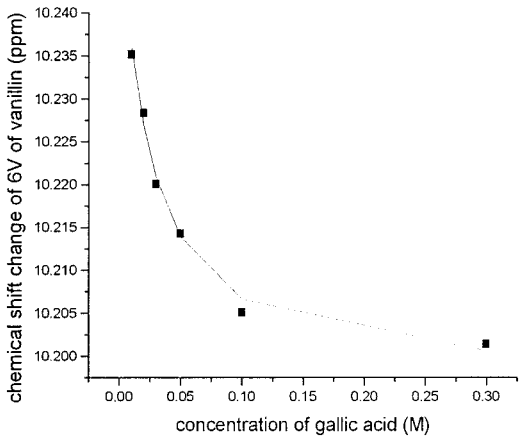

Figure 2. Nonlinear curve fitting of chemical shift data for mixtures of GA (30 mM) and VA (10 mM) at 25 °C.

Table 2. Thermodynamic Parameters for Association between Gallic Acid and Three Flavors

	$K_{25°C}$ (M^{-1})	$K_{45°C}$ (M^{-1})	$K_{60°C}$ (M^{-1})	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol·K)
GA/VA	51	21	17	−26.3	−56.0
GA/MP	37	20	13	−24.5	−52.3
GA/EB	14	13	12	−3.9	9.1

Table 3. Thermodynamic Parameters for Association between Naringin and Three Flavors

	$K_{25°C}$ (M^{-1})	$K_{45°C}$ (M^{-1})	$K_{60°C}$ (M^{-1})	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol·K)
NA/VA	37	34	20	−13.9	−15.9
NA/MP	38	35	21	−13.4	−14.3
NA/EB	18	16	14	−5.9	4.2

Values of K_a, δ_F, and δ_{PF} leading to a best fit of the proton chemical shift changes were estimated by a nonlinear curve fitting program, and theoretical curves were derived from eq 2. The curve fit of a GA and VA (10 mM) mixture is shown in Figure 2. Quantitative analysis of NMR spectra recorded at 25, 45, and 60 °C yielded association constants (K_a) for the interaction of the three flavors with GA and NA (Tables 2 and 3). In addition, self-association constants of 14 and 10.5 M^{-1} were obtained for GA and NA, respectively, at 25 °C.

The structure of both the polyphenol and the odorant influences the observed results. For example, although K_a decreases as temperature increases for all three flavors, the effect is most pronounced for VA and weakest for EB in either GA or NA complexes. On the other hand, compared to NA, GA has a greater observed association (largest overall K_a) with VA at 25 °C, and GA/VA mixtures are most influenced by changes in temperature. The increase in temperature results in an increase in the kinetic energy of the molecules, which causes them to dissociate. Consequently, the association equilibrium constant decreases. Dufour and Bayonove (1999) have reported dissociation constants between (+)-catechin and four flavor compounds in D_2O, and these values are within the similar range of our results.

Using the calculated association constants, the van't Hoff relationship can be used to derive enthalpy and entropy changes for each molecular complex (Tables 2 and 3). The absolute value of enthalpy changes ($|\Delta H°|$) is higher with GA complexes than with NA. These enthalpy changes indicate that interactions between GA and either VA or MP are more enthalpically favored than similar interactions with NA. NA, which has a number of hydroxyl groups, has a potential of hydrogen

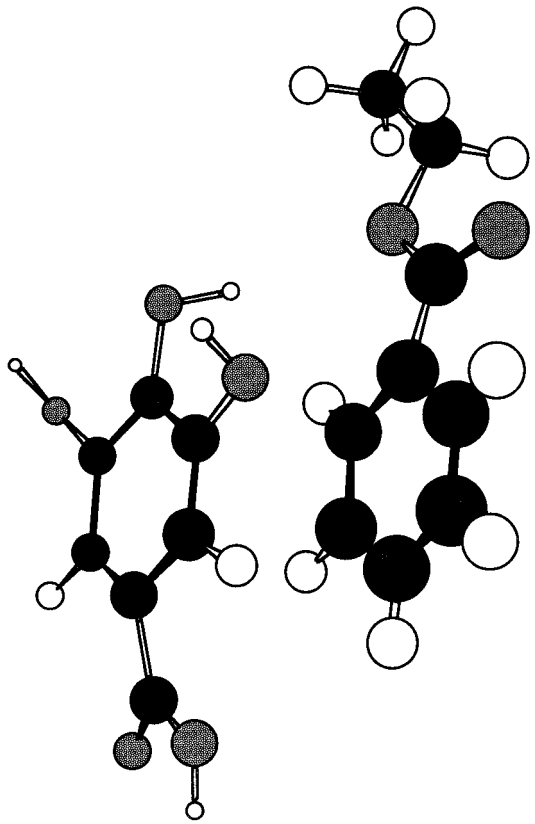

Figure 3. Suggested spatial geometry of GA (left) and EB (right) complex.

bonding to VA, MP, and EB. However, the structural characteristics of NA are not favorable for interaction with other molecules. Specifically, the glycoside attached to the 7′-position of naringenin (an aglycon of naringin) may interfere with the approach of other compounds. Also, the B ring is not in the same plane with the A and C rings, so aromatic stacking over the three rings is less likely to ocur.

Interestingly, EB complexes with either GA or NA show lower enthalpy changes compared to VA and MP complexes. In addition, entropy changes for EB complexes with GA or NA are positive, consistent with hydrophobic interactions. Considering the structural characteristics, EB can interact with another molecule containing aromatic rings by $\pi-\pi$ stacking (Figure 3). However, EB also has the potential for hydrogen bonding between the carbonyl oxygen of the ester group and a proton donor. Our data indicate only a weak intermolecular interaction, suggesting that EB may still move freely with only one hydrogen-bonding junction as indicated by the hypothetical structure in Figure 3.

Evaluation of Spatial Orientation Using 1D NOE and ROESY. To further study the spatial orientation of the flavor/phenol complexes, 1D NOE and 2D ROESY NMR experiments were performed. The NOE is a net change of the signal intensity from one spin due to the relaxation of a saturated spin that is dipole−dipole coupled to the first spin. NOE develops due to through-space (<5 Å) rather than through-bond interactions and so contains information on the interproton distance.

The 1D NOE difference spectra of a GA and VA mixture reveals that these two compounds are interconnected in the matrix. When the proton of the aldehyde group of VA is saturated, the protons of the carboxyl and three hydroxyl groups on GA show strong negative

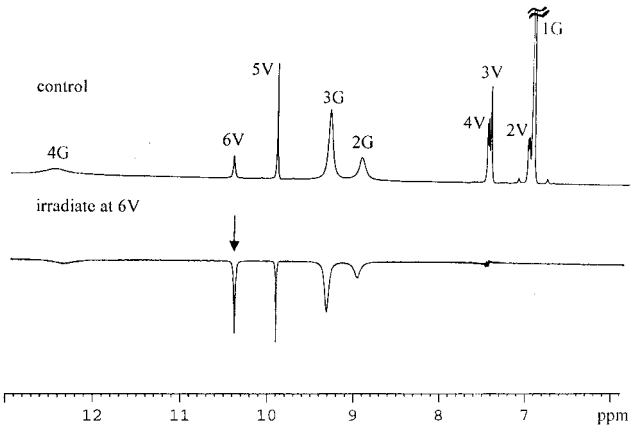

Figure 4. NOE difference spectra of 30 mM GA and 10 mM VA in DMSO-d_6 at 25 °C.

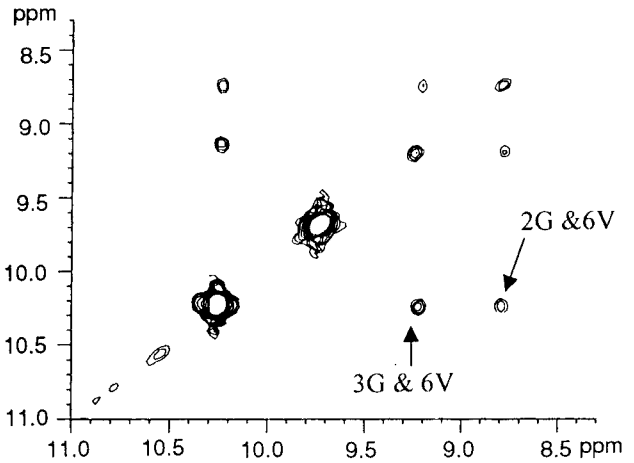

Figure 5. Expanded ROESY spectrum (11−8.5 ppm) of 30 mM GA and 10 mM VA in DMSO-d_6 at 25 °C.

NOE enhancement (70−80%) (Figure 4). Because the molecular weights of GA and VA are not high enough to show the negative NOE, this observation implies that the complex formed by GA and VA acts like a high molecular weight molecule. This 1D NOE result is also supported by 2D ROESY experiments, in which cross-peaks that indicate spatial proximity among the peaks (<5 Å) are revealed. As shown in Figure 5, the ROESY spectrum of a GA and VA mixture in DMSO-d_6 reveals negative intermolecular cross-peaks between the aldehyde proton of vanillin and the three hydroxyl protons of GA. No interactions are observed for the 1V:1G protons; the 1V methyl group can freely rotate and probably does not interact at a distance of <5 Å. The NOE effect observed by means of 1D and 2D experiments suggests the structure in Figure 6 as one of the possible models for the supramolecular structure of GA and VA complexes. In this spatial geometry, the carbonyl oxygen of the aldehyde group of VA can form a hydrogen bond with one of the hydroxyl groups of GA. Vertical stacking of the aromatic rings of GA and VA is also possible. In particular, hydrogen bonding makes a major contribution to the binding mechanism, which is partly explained by the values of enthalpy and entropy changes. The calculated thermodynamic values shown in Table 2 are typical of hydrogen bonding (Vinogradov and Linnell, 1971).

GA and MP also show a similar result; when the vinyl protons of MP (2M and 3M) are saturated, negative NOE signals develop on the hydroxyl and carboxyl

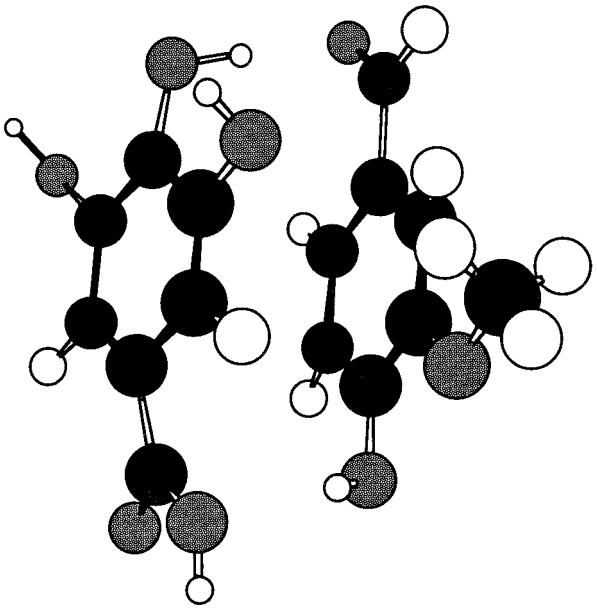

Figure 6. Suggested spatial geometry of GA (left) and VA (right) complex.

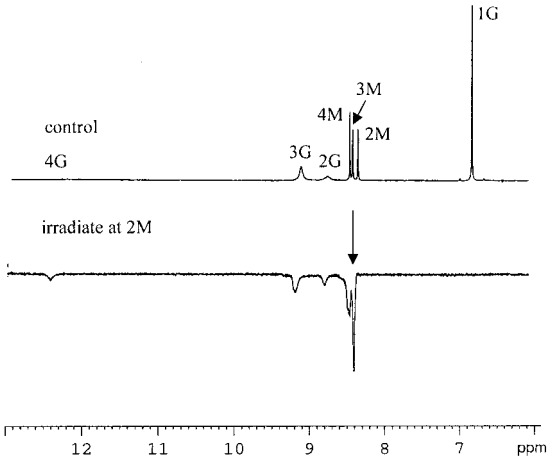

Figure 7. NOE difference spectra of 30 mM GA and 10 mM MP in DMSO-d_6 at 25 °C.

protons of GA (2G, 3G, and 4G, ~50%) (Figure 7). No NOE evidence is observed in the ROESY spectrum, however. Because of the different relaxation times (T_1) of GA and flavors, which slowly relax back relative to GA, it is not possible to optimize the NOE buildup time (mixing time or spin−lock time) for a satisfactory 2D experiment. However, on the basis of the observed 1D data, GA can interact with MP via aromatic stacking, and hydrogen-bonding interactions may also be important (Figure 8). The MP nitrogen (in the 1-position) can form a hydrogen bond with one of the hydroxyl groups of GA. The other nitrogen may interact very weakly with a C−H group of the aromatic ring (Nishio et al., 1998). The MP interacts with GA less strongly than does VA, which is consistent with the measured association constants. Therefore, the shape or conformation of the complex is decided by intricate combinations of weak intermolecular interactions of various kinds.

No intermolecular interactions (<5 Å) were observed in the 1D NOE and ROESY spectra for EB complexes with GA, consistent with the 1D chemical shift data. In addition, NA shows no evidence of interaction with the three flavors in 1D and 2D NOE experiments, even though cross-peaks appear within the NA molecule

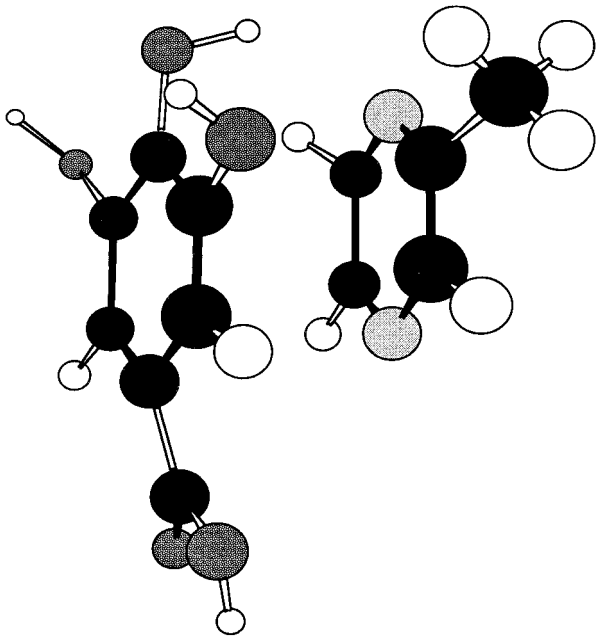

Figure 8. Suggested spatial geometry of GA (left) and MP (right) complex.

itself. This is consistent with the 1D NOE result in which only positive enhancement (~10%) has been observed within the intramolecular protons of NA and a calculated self-association constant of 10.5 M^{-1} in DMSO-d_6 at 25 °C.

CONCLUSIONS

The amount of flavor released depends on the retention of flavor components in the food matrix. Consequently, the nature of the mechanism of binding of flavor components with nonvolatile components is of importance in the perception of the food products. In this study we considered the major driving forces for interaction between phenolics and flavors and evaluated how the interaction mechanism may change with the physicochemical structure of the phenolic and the flavor.

The systematic investigation of complexation between two polyphenols, gallic acid and naringin, and three aromatic flavors, vanillin, 2-methylpyrazine, and ethyl benzoate, indicates that gallic acid interacts more strongly with the flavors than naringin. The mechanism involved in the interaction is an intricate combination of weak noncovalent interactions, of which hydrogen bonding makes a major contribution to the specific conformational geometry.

Additional NMR experiments using flavors and phenolics that have different structural characteristics should be carried out to complement the obtained results.

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