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Application of Pulsed Field Gradient NMR Techniques for Investigating Binding of Flavor Compounds to Macromolecules

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Two diffusion-based NMR techniques are presented and used to investigate the binding of selected flavor compounds to macromolecules. A pulsed field gradient NMR (PFG-NMR) method was applied to measure the apparent diffusion coefficients of four alkanone compounds as they associated with bovine serum albumin (BSA). The change in the apparent diffusion coefficient as a function of the BSA/alkanone ratio was fitted to yield binding constants (K_a) and binding stoichiometry (n) for each alkanone. The results showed that the apparent diffusion coefficients of alkanones increased with a decrease in the BSA/alkanone ratios, and the measured values of K_a and n were comparable with those obtained with other methods and depended on the alkanone structure. A diffusion-based nuclear Overhauser effect (called diffusion NOE pumping) method was also applied to screen mixtures of flavor compounds and identify those that have a binding affinity to complex macromolecules. Using this technique benzaldehyde and vanillin were observed to bind with bovine serum albumin, whereas 2-phenylethanol was identified as a nonbinding or weakly binding ligand with BSA. The diffusion NOE pumping method was also applied to a hydro alcoholic solution of cacao bean tannin extracts to which a mixture of ethylbenzoate, benzaldehyde, and 2-phenylethanol was added. The diffusion NOE pumping technique clearly indicated that ethylbenzoate had a stronger binding affinity to the polymeric (−)-epicatechin units of the cacao bean tannin extracts than the other two flavor compounds. The results successfully demonstrate the potential applications of diffusion-based NMR techniques for studying flavors and nonvolatile food matrix interactions.

KEYWORDS: Apparent diffusion coefficients; pulsed field gradient NMR (PFG-NMR); diffusion NOE pumping; BSA; cacao bean tannin extracts; flavor; matrix; interactions

INTRODUCTION

Flavor is an important characteristic of foods and beverages and a balanced release and perception of flavor compounds is critical for determining consumer acceptability. The perceived concentration of flavor compounds and their release rate are dependent on nonvolatile food components present in the food as well as on the concentration and physicochemical properties of the flavors. Many studies have been carried out in a variety of foods or simulated food matrixes in order to determine the different effects of individual food components on the rate and amount of flavors released. In addition, various techniques have been developed in an attempt to better assess and understand the interaction mechanisms.

Spectroscopic techniques have been extensively used to study flavor−matrix interactions because they can illustrate direct evidence of molecular interaction, and they can provide details on the nature of the interactions (*1*, *2*). Nuclear magnetic resonance (NMR) spectroscopy is considered one of the most powerful spectroscopic techniques to investigate intra- and intermolecular interactions. NMR parameters, including changes in chemical shift, line width, and relaxation rate are popular tools to evaluate molecular interactions because they are very sensitive to changes in chemical environments and complex formation (*3*). For example, recent studies (*4*−*6*) have used NMR techniques to study interactions between polyphenolic compounds and selected flavors. By monitoring changes in ^{1}H chemical shifts and through the application of two-dimensional NMR spectroscopy, hydrophobic interactions between the polyphenolics and the flavors were shown to be the major driving force for the complexation, with hydrogen bonding contributing little binding energy yet enhancing specificity (*6*).

Self-diffusion coefficients can also be useful in probing changes in sample environment. Because the diffusion coefficient directly reflects the molecular size and hydrodynamic properties of molecules, diffusion-based NMR methodologies can be used to characterize the conformational states of molecules (e.g., polymerization), to evaluate molecular recognition for a variety of protein−protein and protein−ligand solutions (*7*−*10*), and even to study small molecule interactions in organic solvents (*11*). These diffusion-based techniques have

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been widely used to study ligand binding for biological and pharmaceutical applications but they have not been applied to the study of flavor−food matrix interactions.

The diffusion coefficients are measured by a pulsed field gradient nuclear magnetic resonance spectroscopy (PFG-NMR) method, which is based on the attenuation of individual proton resonances under the influence of linear field gradients (*12*). The amplitude (I) of the signal is directly related to the diffusion coefficient, D, according to

$$I = I_0 \times \exp[-D \times (\Delta - \delta/3) \times \gamma^2 g^2 \delta^2] \qquad (1)$$

where I and I_0 are, respectively, the intensity of the NMR signal in the presence and absence of external gradient pulses, Δ is the time period over which translational diffusion is allowed to occur, γ is the nuclear magnetogyric ratio, and g and δ are, respectively, the amplitude and duration of the gradient pulse (*12*).

Another type of diffusion-assisted NOE experiment was introduced by Chen and Shapiro (*13*) in 1998 as a fast, active drug screening technique. This technique combines the PFG-NMR method and the NOE experiment and is often called diffusion NOE pumping. In the pulse sequence, a diffusion filter is first applied to prepare a state in which small ligands are filtered out and only the macromolecule signals remain. Then, an NOE experiment, which leads to a magnetization transfer between spins that are close to each other in space, is applied to allow the signal to redistribute from the protein to the binding ligands. At the beginning of the NOE experiment, all of the ligand signals are destroyed, while the macromolecule signals are conserved. During the mixing time, the magnetization is transferred from macromolecule to the binding ligand and only signals from binding ligands can be observed. Again, this technique has not been applied to study flavor−food matrix interactions.

In this study we demonstrate the application of these two diffusion-based NMR techniques (PFG-NMR and diffusion NOE pumping) to investigate the binding of selected flavor compounds to complex macromolecules. BSA is used as a model protein, as its molecular properties are well-known (*14*, *15*) and it is readily available. A cacao bean tannin extract provides a relatively simple tannin mixture that has been well characterized (*16*, *17*). The PFG-NMR method combined with a nonlinear curve-fitting algorithm is used to estimate binding constants (K_a) and binding stoichiometry (n) for four model alkanones with BSA. The diffusion NOE pumping technique is used to rapidly screen a mixture of flavor compounds in order to identify those which have binding affinity to BSA or cacao bean tannin extracts.

MATERIALS AND METHODS

Materials. The BSA (purity 96%) was purchased from Sigma (St. Louis, MO) and used without further purification. The cacao bean tannin extract was donated from Professor Andy Waterhouse in the Department of Viticulture and Enology at the University of California, Davis. The separation and purification steps are well described in refs *16* and *17*. The cacao bean extract mostly consists of (−)-epicatechin monomers and polymers (i.e., procyanidins) and the profile of the mixture is shown in **Figure 1**. The flavor compounds (purity, >98%) used in this study, 2-heptanone, 2-octanone, 2-nonanone, 5-nonanone, benzaldehyde, vanillin, 2-phenylethanol, and ethylbenzoate, were purchased from Aldrich (Milwaukee, WI). Deuterium oxide (D_2O, 99.9%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Phosphate buffer (pH 7.2 and ionic strength 0.02 M) was prepared with

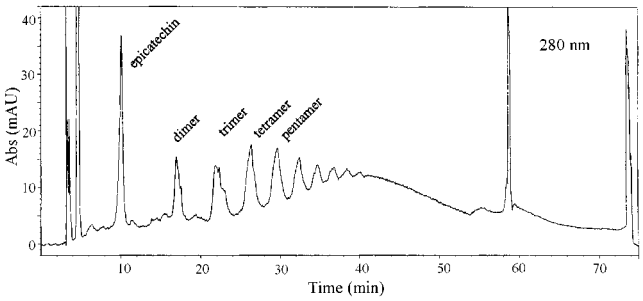

Figure 1. HPLC chromatogram of cacao bean extract (reprinted with permission from ref. *17*).

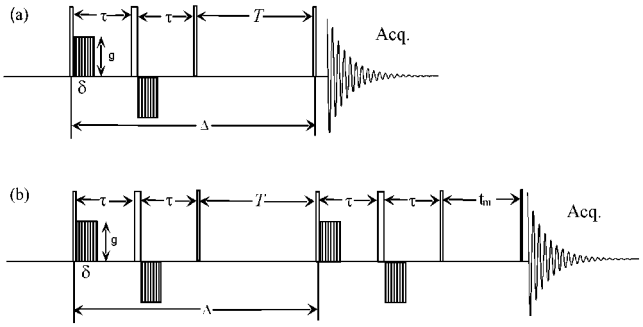

Figure 2. Pulse sequences of (a) stimulated-echo pulsed field gradient (STE-PFG), and (b) diffusion NOE pumping. The narrow and wide bars represent 90° and 180°, respectively. τ and T are interpulse delays between 90° and 180° and between two 90°s, respectively. On the sequence (b), t_m is the mixing time for NOE buildup. g and δ, respectively, indicate the amplitude and duration of the gradient pulse, and Δ is the diffusion time.

$NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ (J. T. Baker Chemical Co., Phillipsburg, NJ).

PFG-NMR Methods. All samples were prepared in phosphate buffer solution and equilibrated at room temperature for at least 12 h before use. Protein concentration was set to 0.5 mM and each alkanone was added to give specific molar concentration ratios of proteins to flavors ranging from 0.006 to 0.18.

The NMR experiments were carried out at 500 MHz using a Bruker DRX-500 instrument equipped with a BGU-2 field gradient accessory capable of delivering z-field up to 590 mT/m. In all experiments, the probe temperature was maintained at 298 ± 1 K, and standard 5 mm NMR tubes were used. The diffusion measurements using PFG and a stimulated echo sequence (STE) (*18*) were performed because of fast transverse relaxation (T_2) of the bound ligand. As shown in **Figure 2a**, the stimulated echo (STE) experiment was designed to avoid T_2 relaxation effects by storing the magnetization along the Z axis during T, so that relaxation depends primarily on T_1, which is usually much longer than T_2 for proteins. The echo intensities of the alkanones were determined for the methyl group at 0.8 ppm. The diffusion time (Δ), gradient strength (δ), and spoil gradient strength were set to 100, 3, and 5 ms, respectively. Gradient strength, g, in the z direction was calibrated by $D_{H_2O} = 2.3 \times 10^{-9}$ m^2 s^{-1} at 298 K using a 90% H_2O/10% D_2O mixture, then linearly attenuated from 0.68 to 32.4 g/cm during the experiments. The binding constants, K_a, and stoichiometry coefficients, n, were calculated using a Levenberg−Marquard algorithm within the software Origin (Microcal Software Co. Inc., Northampton, MA).

Diffusion-Based NOE Technique. Benzaldehyde, vanillin, and 2-phenylethanol were dissolved in the phosphate buffered solution (10 mM) with or without BSA added (100 μM). As shown in **Figure 2b**, the stimulated echo experiment is followed by a typical NOE experiment sequence. The δ and Δ were set to 3 ms and 100 ms, respectively. NOE mixing time was optimized to 200 ms after varying from 100 to 600 ms, at which maximum signal intensity of flavors and no indirect NOEs were noticed.

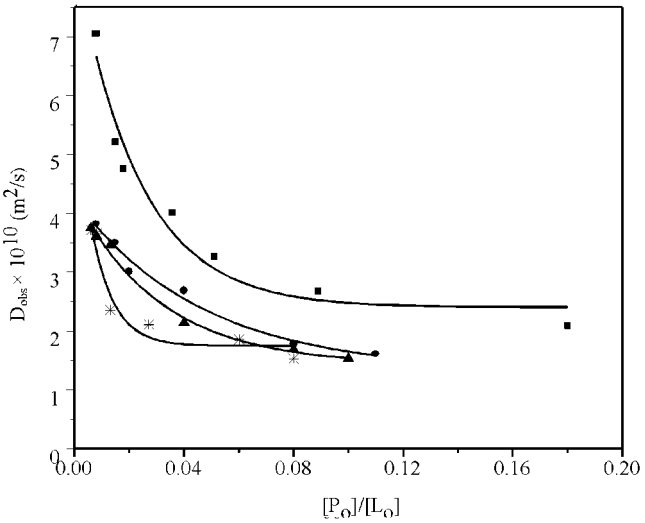

Figure 3. Changes in observed diffusion coefficient (D_{obs}) of 2-heptanone (■), 2-octanone (●), 2-nonanone (▲), and 5-nonanone (*) as a function of molar concentration ratios of BSA ($[P_o]$) to flavor ($[L_o]$). Solid line represents the estimated first-order exponential decay curves.

Benzaldehyde, ethylbenzoate, and 2-phenylethanol were dissolved in 10% C_2D_5OD/90% D_2O mixture at a concentration of 10 mM each, in the presence or absence of cacao bean tannin extract (0.05%, w/w). All the parameters were the same as the above except for a mixing time which was set to 100 ms.

RESULTS AND DISCUSSION

Determination of Binding Constants (K_a) and Stoichiometry Coefficients (n) Measured by PFG-NMR. The diffusion coefficient is an important molecular property related to molecular weight and conformational changes, including self-association and intermolecular interaction. In particular, self-diffusion coefficients measured by PFG-NMR are not dramatically temperature- or concentration-dependent, so it is possible to differentiate molecular interactions from other exterior effects (e.g., small changes in viscosity) on changes in the measured diffusion coefficient (*12*).

To show the application of PFG-NMR for monitoring the interaction between flavors and food macromolecules, we measured the apparent diffusion coefficients (D_{obs}) of four aliphatic ketones in the presence of BSA. The aliphatic ketones were chosen as model flavor compounds because they are known to have binding affinities with the protein (*19*, *20*). In titration experiments, receptor and ligand concentration ratios are generally varied either by changing both concentrations or by changing only the receptor or the ligand concentration while holding the other concentration constant (*21*, *22*). In the case of the protein and flavor binding studies presented here, only the alkanone concentration was varied. Changes in the protein concentration may dramatically increase the viscosity of solution compared to changing ligand concentration, resulting in changes in the diffusion coefficients of ligand molecules independent of the ligand−protein interaction.

The results showed that the apparent diffusion coefficients for all four alkanones decreased with an increase in BSA/ligand (alkanone) concentration ratios (**Figure 3**). This indicates that the fraction of bound ligand decreased as ligand concentration increased. It was assumed that chemical exchange of the ligand occurred between the free and bound states at a rate that was fast relative to the diffusion time (Δ). Therefore, the apparent diffusion coefficient (D_{obs}) reflects the average of the diffusion coefficients of two exchanging species, free (D_{free}) ligand and bound (D_{bound}) ligand, weighted by the mole fraction of the ligand in the free (x_{free}) and bound (x_{bound}) states:

$$D_{obs} = (D_{bound} \times x_{bound}) + (D_{free} \times (1 - x_{bound})) \quad (2)$$

In this experiment, the diffusion coefficient of the ligand in the free state (D_{free}) was measured for each 10 mM alkanone solution alone (**Table 1**). Because of possible self-aggregation at high alkanone concentration, diffusion coefficients were also measured at a concentration of 100 mM, and no changes in D_{free} were observed. From the first-order exponential decay equations for each alkanone (**Figure 3**) the diffusion coefficient of ligand in the bound state (D_{bound}) was then deduced from the *y*-intercept (**Table 1**) when self-diffusion coefficients are constant.

Table 1. Kinetic and Thermodynamic Parameters[a] Estimated by the STE-PFG-NMR Experimental Data for Alkanones Binding to BSA

	($D_{free} \times 10^{10}$) ± SD (m^2/s)	($D_{bound} \times 10^{10}$) ± SD (m^2/s)	K_a ± SD (M^{-1})	ΔG ± SD (kcal/mol)	n
2-heptanone	7.5 ± 0.05	2.3 ± 0.2	323 ± 13	−3.42 ± 0.02	7–8
2-octanone	6.5 ± 0.03	1.3 ± 0.4	476 ± 20	−3.65 ± 0.02	7
2-nonanone	6.3 ± 0.05	1.4 ± 0.3	833 ± 15	−3.98 ± 0.01	7
5-nonanone	5.4 ± 0.06	1.7 ± 0.3	287 ± 18	−3.35 ± 0.04	6–7

[a] Means of duplicate results.

Considering a 1:n binding stoichiometry between the protein and the ligand, in which n is the number of binding sites on the target protein, and assuming n independent and identical sites, a mathematical model describing the fraction of ligand bound (x_{bound}) was then derived (*23*)

$$x_{bound} = \frac{n[P_o] + [L_o] - K_d}{2[L_o]} - \sqrt{\left(\frac{n[P_o] + [L_o] - K_d}{2[L_o]}\right)^2 - 4n[P_o] \times [L_o]} \quad (3)$$

In this equation, K_d (= $1/K_a$) is the dissociation constant and $[P_o]$ and $[L_o]$ represent the total concentrations of protein and ligand, respectively. The values of K_a (or $K_d = 1/K_a$) and n were obtained by nonlinear curve fitting of eqs 2 and 3 to the experimental data of D_{obs} and $[L_o]$. The corresponding free energy of association (ΔG) is also calculated from the values of K_a.

As shown in **Table 1**, the binding constant (K_a) of BSA for a series of 2-alkanones increased with chain length. For each additional methylene group there was a corresponding change in the free energy of association of −0.28 Kcal per methylene group, suggesting that hydrophobic interactions are predominant in the association of BSA with these particular compounds. Of the three 2-alkanones, 2-nonanone had the highest binding affinity (833 M^{-1}), followed by 2-octanone and then 2-heptanone (476 and 323 M^{-1}, respectively). Similar observations were reported previously for the binding of 2-heptanone and 2-nonanone to native BSA at room temperature using a liquid−liquid partition equilibrium method, where the Scatchard plots applied to the data extracted the K_a for 2-heptanone and 2-nonanone as 270 and 1800 M^{-1}, respectively (*19*). However, it is difficult to directly compare the absolute values for the thermodynamic parameters obtained in our study with those of previous studies due to differences between the methods and the systems studied. For example, sodium azide used in the previous study is known to affect the binding affinities and the number of binding sites in protein−ligand interaction (*1*, *24*).

The methods used to calculate the binding constants give greatly different results (*25*), and in particular, extrapolation of a small fraction of a binding curve has often been criticized (*26*−*28*).

The binding affinity of 5-nonanone is markedly lower than that of the other 2-alkanones. The carbonyl group at the 5-position as compared to the 2-position may introduce more steric hindrance on the alkanone binding to protein. Damodaran and Kinsella (*29*) studied the binding of soy protein to alkanones by using the partitioning equilibrium experiment, and observed that 5-nonanone had a binding affinity lower than that of 2-nonanone, but higher than those of 2-heptanone and 2-octanone. The difference in the binding affinities may be explained by different sources of proteins used for the two studies.

The number of binding sites on BSA available for binding the aliphatic ketones were found to range from 6 to 8, indicating that they may all have the same binding site with the BSA. The number is comparable with those obtained by Damodaran and Kinsella (*19*) where 5−6 initial binding sites to BSA were estimated. The value is also in agreement with the number of binding sites to BSA and human serum albumin (HSA) for derivatives of stearic and palmitic acids (*30, 31*).

Because of differences in experimental conditions and methods to calculate the binding constants, the absolute values of thermodynamic parameters are different in each case, but the PFG-NMR method presented here shows the potential application for investigating molecular interactions, particularly with solutions containing food macromolecules such as proteins or carbohydrates. However, as with other methods, the NMR titration using diffusion coefficient has certain limitations in explaining the protein−ligand interactions. The coefficients are measured under the assumption that no interactions occur between protein receptors or ligand molecules themselves, although in reality, this assumption is not true, particularly when lipophilic flavor compounds serve as ligands. In addition, the calculation of association constants, K_a, using this method as with other methods, represents an average constant over the binding process. However, it is generally accepted that the binding of one mole of ligand will influence the binding of subsequent moles in a protein containing multiple binding sites. Thus, the use of the average K_a values to calculate thermodynamic binding parameters may lead to errors. It is necessary to calculate K_a for proteins or ligand molecules, which can give an insight into their contribution to overall binding. In addition, calculation of K_a for each mole of ligand bound to protein may be possible upon measuring D_{obs} in extended ranges of concentration ratios between protein and ligand.

Protein−Flavor Interaction Probed by the Diffusion NOE Pumping Technique. Foods and beverages are complex mixtures of many flavor compounds, and the differing abilities of individual flavors to interact with macromolecules can have significant effects on overall flavor and aroma perception. The diffusion NOE pumping technique is considered to be a powerful tool to perform rapid screening and identification of compounds which have binding affinity to macromolecules (*12*). Complementary functioning of the PFG-NMR and the NOE in this integrated technique enables a novel approach to study binding between macromolecules and small ligands. The technique is particularly useful for probing complex intermolecular interactions that may not be studied using other methods. For example, diffusion NOE pumping can be successfully applied to study interactions involving macromolecules without requiring isotope labeling or well-resolved NMR signals. The binding abilities of ligand candidates with a target macromolecule can be monitored without separate runs for individual pairs of samples.

As illustrated in **Figure 2b**, the diffusion NOE pumping technique consists of two parts: a diffusion experiment and the typical NOE technique. Diffusion coefficients of molecules are often used for characterizing conformational changes and molecular interactions as discussed previously. However, when a molecule is involved in a fast chemical exchange on the diffusion time scale, the weighted average of diffusion coefficients between two states is closer to the diffusion rate of the free state than to that of bound state. NOE also has several negative effects on the signal decay as well as chemical exchange (*12*), particularly when a macromolecule is used as a receptor. In this regard, a diffusion experiment combined with a NOE technique is regarded as a complementary method that covers the limited application of each individual technique.

Figure 4a shows the typical 1D ^{1}H NMR spectrum of a benzaldehyde, vanillin, and 2-phenylethanol mixture in the presence of BSA. These flavor compounds were selected in order to evaluate the effect of different functional groups on binding of aromatic compounds to BSA. **Figure 4b** was obtained by applying the diffusion NOE pumping technique to a mixture of the three flavors without BSA added. This figure clearly demonstrates how well the technique works with these samples, because signals from all of the low-molecular-weight flavor compounds were filtered out by the diffusion process. Conversely, when BSA was added to the flavor solution, illustrated in **Figure 4c**, only benzaldehyde and vanillin signals remained as well as the BSA and protein assisted water signals. This is because the apparent diffusion coefficients of benzaldehyde and vanillin increased upon binding to BSA. The presence of the water signal also indicates that it intimately binds to BSA. The absence of the 2-phenylethanol peaks suggests that 2-phenylethanol has either no or only weak binding affinity to BSA. The functional groups of ligands are thought to be of importance in providing specificity in binding of ligands to BSA (*32, 33*). Dinitrophenylation studies (*32*) showed that lysine residues are located near fatty acid binding sites of BSA. Arginine residues also have been shown to be present near the strong organic anion binding sites of BSA (*33*). These findings may account for the result obtained in this study that the aromatic compounds with different functional groups showed selective binding to BSA. Therefore, while hydrophobic interactions are important as discussed previously, the presence of anionic functional groups such as carboxylate or carbonyl groups may influence the specificity of binding and impact which flavor compounds will preferentially bind to BSA when a mixture of flavor compounds is present.

Tannin−Flavor Interaction Probed by the Diffusion NOE Pumping Technique. Monomeric forms of (plant) phenolic compounds have been previously shown to interact with several aromatic flavors (*3, 5*). Oligomeric and polymeric tannins, however, make up a large percentage of the polyphenol composition of most foods and beverages. Only a few studies have dealt with the possible effects of tannin fractions on the physicochemical behavior of volatile flavor compounds (*3*). In this study we used the diffusion NOE pumping experiment to study the binding of three aromatic flavor compounds (ethylbenzoate, benzaldehyde, and 2-phenylethanol) to polymeric polyphenols (tannins). Cacao bean tannin extracts have a well characterized composition, in which monomeric to polymeric (−)-epicatechin units are present together with a limited number of structural isomers (*16*).

Figure 5a is a typical 1D ^{1}H NMR spectrum for a mixture of the three flavors and cacao bean tannin extracts. The diffusion NOE pumping experiment was applied to the mixture of the

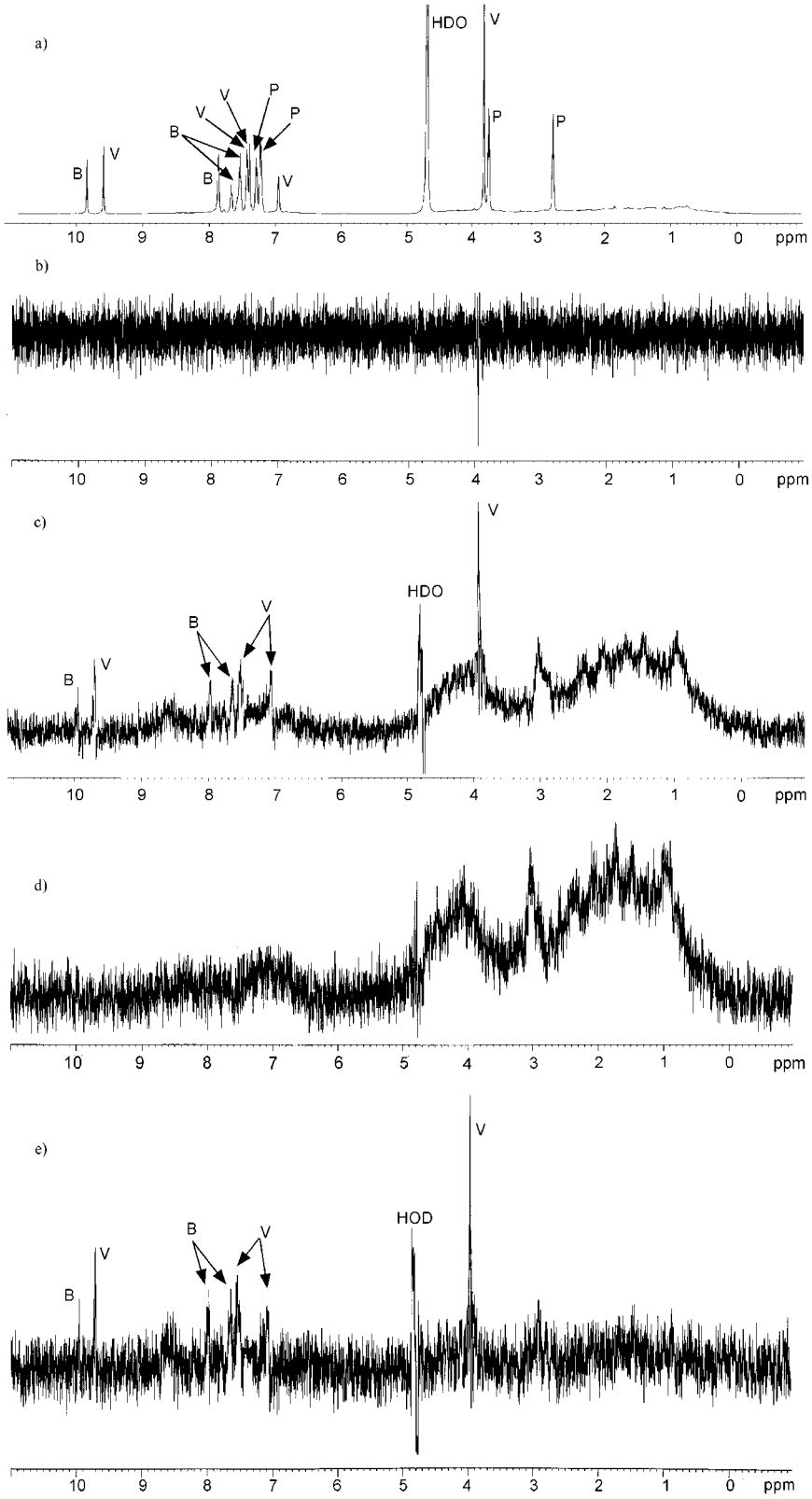

Figure 4. NMR spectra for diffusion NOE pumping experiment of flavors with BSA. B, V, and P represent proton signals of benzaldehyde, vanillin, and 2-phenylethanol, respectively. (a) 1D ^{1}H NMR spectrum obtained by typical 90° pulse sequence for benzaldehyde, vanillin, and 2-phenylethanol mixture (10 mM each) with 1 mM BSA added in buffered D_2O solution. (b) Diffusion NOE pumping technique for three flavor compounds in the absence of BSA. (c) Diffusion NOE pumping technique in the presence of BSA. (d) Diffusion NOE pumping technique of BSA alone. (e) Difference spectrum for (c) subtracted from (d).

flavors and the tannin extract as shown in **Figure 5b**. Only ethylbenzoate signals remained with the procyanidin peaks; the benzaldehyde and 2-phenylethanol peaks completely disappeared, indicating no or weak interaction with the procyanidin. In addition, the strong water signal in **Figure 5a** was not seen in **Figure 5b**, suggesting that water was not intimately involved in the tannin and ethylbenzoate interaction. To confirm this interpretation, a solution of cacao bean extract flavored with

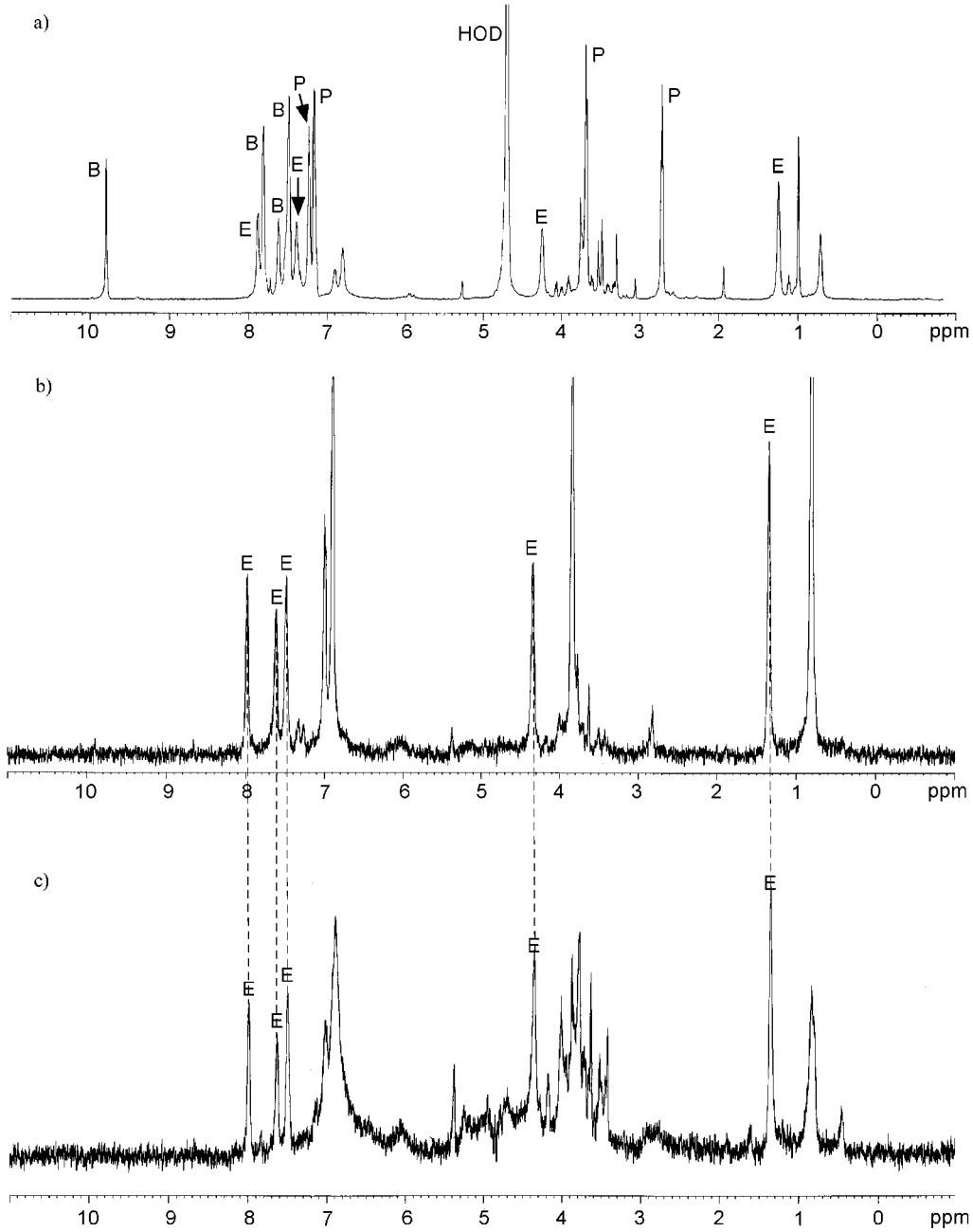

Figure 5. NMR spectra for diffusion NOE pumping experiment of flavors with cacao bean tannin extract. B, E, and P represent proton signals of benzaldehyde, ethylbenzoate, and 2-phenylethanol, respectively. Remaining peaks not identified belong to cacao procyanidins. (a) 1D ^{1}H NMR spectrum obtained by typical 90° pulse sequence for benzaldehyde, ethylbenzoate, and 2-phenylethanol mixture (10 mM each) with 0.05% cacao bean tannin extract in C_2D_5OD/D_2O (1:9, v/v). (b) Diffusion NOE pumping technique for three flavor compounds in the presence of cacao bean tannin extract. (c) Diffusion NOE pumping technique for cacao bean tannin extract solution flavored with ethylbenzoate alone.

ethylbenzoate alone was analyzed with a diffusion NOE pumping sequence (**Figure 5c**); all the signals in **Figure 4b** corresponded to those of ethylbenzoate and the cacao bean extract present in **Figure 4c**.

Recently Dufour and Bayonove (*4*) showed that the tannin fraction from red wine slightly decreased the volatility of benzaldehyde, but had no effects on the volatility (i.e., headspace concentration) of two nonaromatic flavors, ethylhexanoate and isoamyl acetate. Jung et al. (*6*) have also shown that the structure of both the flavor compound and the polyphenol can influence the strength of the interaction. With monomeric phenolic compounds, e. g., gallic acid and naringin, interactions appeared to be stabilized by the ability to form charge-transfer and hydrogen-bonding interactions, in addition to hydrophobic interactions. The present results seem to indicate that hydrophobic interactions are also important for flavor interactions with tannins, but charge-transfer or hydrogen bonding interactions (which are more likely to occur with benzaldehyde and 2-phenylethanol than with ethylbenzoate) may not be as critical. The results also indicate that extrapolating results from simple monomeric compounds to complex polymeric materials and to mixtures of flavor compounds is difficult.

The methodologies adopted for each experiment must also be taken into consideration upon interpretation of the results. For example, spectroscopic data show true interactions at a molecular level, but they may not directly explain the macroscopic phenomena such as changes in volatility or sensory perception of flavor compounds. Conversely, changes in head-

space concentration of flavors can be influenced by viscosity or temperature changes as well as by molecular interaction. Therefore, further systematic studies that control all possible exterior effects and that differentiate the molecular interactions from these other effects are needed. In addition, the molecular interactions shown by spectroscopic studies need to be accompanied by headspace analysis and sensory studies in order to correlate the two results.

CONCLUSIONS

Changes in self-diffusion coefficients of flavor compounds were observed on titration of BSA with four alkanone compounds, and values of K_a and ΔG and n were calculated. The results were comparable with those obtained from other studies and showed that NMR self-diffusion measurements are useful for studying molecular interactions between flavors and food macromolecules. As pointed out above, however, this method requires further refinement to account for possible binding site−binding site or ligand−ligand interactions, as well as to calculate the true association constants, K_a, obtained for each mole of bound ligand.

A diffusion NOE pumping technique was successfully applied to screen a mixture of aromatic flavor compounds and identify those compounds which selectively bind to BSA. Our results showed that benzaldehyde and ethylbenzoate bind to BSA, whereas 2-phenylethanol is a nonbinding or weakly binding ligand. This pulse sequence was also employed to show that ethylbenzoate, but not benzaldehyde and 2-phenylethanol, binds to a cacao bean tannin extract.

Although diffusion NOE pumping techniques do not provide specific information on the nature of the interactions, they are generally fast and easy procedures, and they provide a valuable tool for prescreening a mixture of compounds for binding affinity to a food macromolecule. Development of NMR pulse sequences such as diffusion experiments combined with any 2D NMR techniques may make it possible to further specify the binding sites and the mechanisms, as well as to screen for active compounds. The diffusion-based NMR techniques can be further applied to study interactions with other food macromolecules (e.g., β-lactoglobulin, soy proteins, carbohydrates, and grape/wine tannins) in order to better predict and optimize flavor release and flavor perception in foods and beverages.

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