J Am Oil Chem Soc (2010) 87:673–679
DOI 10.1007/s11746-009-1535-8

ORIGINAL PAPER

Competitive Binding of Off-Flavor Compounds with Soy Protein and β-Cyclodextrin in a Ternary System: A Model Study

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Received: 17 September 2009 / Revised: 14 December 2009 / Accepted: 21 December 2009 / Published online: 19 January 2010

Abstract Removal of soy protein (SP)-bound 2-nonanone by β-cyclodextrin (βCD) was studied using an equilibrium dialysis technique. It was observed that in the presence of βCD, a significant ($p < 0.05$) amount of SP-bound 2-nonanone could be removed in a concentration-dependent manner. Up to 94% of SP-bound 2-nonanone was stripped from SP when 6 mM βCD was present in the system. However, in thermodynamic terms, the net standard free energy change for transfer of 2-nonanone from SP to βCD, i.e., $\Delta\Delta G^{\circ}_{SP\rightarrow\beta CD}$, was essentially zero, implying that the apparent equilibrium binding constant for the formation of βCD–2-nonanone complex was essentially same as that for the SP–2-nonanone complex formation in the ternary system. This indicated that stripping-off of 2-nonanone from SP by βCD was driven by the mass action ratio. Based on these results, it is shown that βCD can be used effectively for removing SP-bound off-flavor carbonyl compounds.

Keywords Protein extraction and processing · Processing technology · Seed proteins · Off-flavor

Introduction

Traditionally soy proteins (SP) have been valued as a protein source in the food industry owing to their abundant supply, relatively low cost and nutritive value. Despite these positive attributes, due to the presence of beany and/or grassy off-flavor, SP have found limited utilization in mainstream food applications. It is widely believed that protein-bound phospholipids (PLs) are the main precursors of off-flavor in SP. The presence of polyunsaturated fatty acids (PUFA) in soy PLs makes them susceptible to oxidation during processing or storage. An intermediate product of PUFA oxidation is hydroperoxide, which is quite unstable and it further breaks down to produce a variety of volatile compounds, such as aldehydes, ketones, alcohols, furans, methyl esters, hydrocarbons, etc. [1–6]. Once formed, these volatile compounds bind to SP via non-covalent interactions [7–13]. Common processing methods employed for the isolation of SP from soybean are often inadequate to overcome this binding. During preparation and consumption of SP products, protein-bound volatile compounds are released and perceived as off-flavor [14, 15].

Over the years, several physical and chemical methods have been proposed for removing off-flavor compounds from SP. For example, methods such as supercritical carbon dioxide extraction, membrane filtration, solid adsorbents, and oxidizing enzymes such as aldehyde dehydrogenase or aldehyde oxidase, have been investigated for off-flavor reduction in soy proteins [16–21]. All of these methods showed considerable drawbacks, either due to poor selectivity or inability to lower volatile compound concentration below the threshold of detection. Even genetically modified soybean cultivars lacking the lipoxygenase enzyme have been bred in order to prevent enzymatic oxidation of PUFA [22–25]. However, this approach has met with limited success. In SP products prepared from those soybean cultivars, hexanal generation was significantly less but evolution of other carbonyl compounds could not be prevented [25]. It is likely that autoxidation of PUFA also contributed to volatile compound production in those cultivars. Extraction procedures involving a combination of hexane and solvents such as methanol, ethanol, 2-propanol, etc., have proved quite effective in reducing

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off-flavors in SP [26, 27]; however, use of solvent treatment invariably results in SP denaturation and a loss of solubility [28, 29]. Therefore, it is important that new methods for the elimination of off-flavor from SP, which transcend current limitations, need to be explored.

The primary objective of this study was to investigate competitive binding of a model carbonyl compound, such as 2-nonanone, in a ternary system containing SP and β-cyclodextrin (βCD) in order to assess the relative difference in the affinity of carbonyl compounds to these acceptors and to test the efficacy of βCD in removing SP-bound carbonyls. The basic premise of this study was that the binding affinity of carbonyl compounds for βCD is equivalent to or greater than that of SP. βCD is a cyclic polymer of seven glucose molecules, which are oriented in such a way to form a hollow inner cavity (6.5 Å wide and 7.9 Å long) and a hydrophilic outer surface [30]. This unique conformation allows βCD to form inclusion complexes with a variety of molecules [31–37] by sequestering the hydrophobic acyl chain or aromatic ring in its cavity. The fact that βCD has been approved as a safe ingredient (GRAS notice # GRN 000074) for food applications by the FDA [38] makes βCD a promising candidate for removing SP-bound off-flavors. Previously, βCD has been used to remove volatile beany flavor compounds from soy milk [39–41].

Materials and Methods

Soy protein was isolated from defatted soy flour (Archer Daniel Midland Co., Decatur, IL, USA) as described previously [8] but without the use of β-mercaptoethanol. Isolated SP was dialyzed, freeze dried, and stored at −20 °C until further use. 2-Nonanone (99% purity) and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isooctane and dialysis tubing (25 mm wide, molecular weight cut off = 12–14 kDa) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Food-grade βCD (Cavitron 82800) was donated by Cargill Inc. (Minneapolis, MN, USA). All solutions were prepared in deionized water from a Milli-Q ultrapure water system (Millipore Corp., Bedford, MA, USA) with a resistivity of 18.2 MΩ cm. The rest of the chemicals used in this study were of reagent grade.

Binding Studies

Binding of 2-nonanone to SP in the presence of 0–6 mM βCD was studied using an equilibrium dialysis method [8]. SP solution (13.36 mg/mL), βCD stock solution (20 mM) and an aqueous solution saturated with 2-nonanone were prepared in 30 mM Tris–HCl buffer at pH 8.0. Sodium azide (0.02%) was added to all solutions as an antimicrobial agent. Before each run, dialysis tubing was washed thoroughly with distilled water to remove any contaminants and soaked in 30 mM Tris–HCl buffer (pH 8.0). In a typical experiment, 4 mL of SP solution was filled in dialysis tubing, sealed tightly and placed in a glass vial containing a known amount of 2-nonanone dissolved in 14 mL buffer. (This solution was prepared by diluting an aliquot of buffer solution saturated with 2-nonanone). Glass vials were sealed tightly and the system was allowed to equilibrate on a horizontal shaker for 24 h at 25 °C. Subsequently, an aliquot of 20 mM βCD stock solution was injected into the buffer side to achieve a final βCD concentration of 0–6 mM in the system. The vials were shaken for another 24 h at 25 °C to complete equilibration. Preliminary experiments had indicated that a total of 48 h was more than adequate for the system to reach equilibrium. At the end of the equilibration period, 2-mL aliquots were drawn from both protein and buffer sides, placed in vials containing 1 mL isooctane and shaken to extract 2-nonanone into the isooctane phase. Amount of 2-nonanone in isooctane extracts was determined using gas chromatography as described below. The difference between the concentration of 2-nonanone at equilibrium on protein and buffer sides was taken as a measure of SP-bound 2-nonanone while the concentration of 2-nonanone on the buffer side was taken as the free ligand concentration, [L]. Since SP is a mixture of several proteins, the number of moles of ligand bound per mole of protein (υ) was calculated using a reference molecular weight of 100,000 g/mol for SP. υ versus [L] data from at least triplicate experiments were collected to construct binding isotherms. Quantitative characterization of SP–2-nonanone binding was also attempted using the Klotz double-reciprocal equation,

$$\frac{1}{\upsilon} = \frac{1}{nK[\mathrm{L}]} + \frac{1}{n} \quad (1)$$

where n is the total number of binding sites in the protein and K is the intrinsic binding constant [42].

Gas–Liquid Chromatography

An HP-6890 gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a DB-17MS capillary column (30 m × 2.5 mm i.d., 0.25 μm film thickness) from J&W Scientific Inc. (Rancho Cordova, CA, USA) was used. One microliter of isooctane extract was injected into the GC inlet in a split mode (split ratio 2.67:1) using a 5-μL syringe (Hamilton Co., Reno, NV, USA). Volatilized sample was carried through the column using helium gas at a

constant flow rate of 1.5 mL/min. For a typical run, the GC oven temperature was held at 40 °C for 1 min, increased to 240 °C at the rate of 20 °C per min and finally held at 240 °C for 1 min. Both the GC inlet and FID were maintained at 250 °C. Elution of 2-nonanone from the column was recorded as a FID signal expressed in picoampere (pA) units. The peak area for the 2-nonanone signal was integrated and quantified using a calibration curve.

Results and Discussion

Binding Isotherms

The equilibrium binding of 2-nonanone to SP can be represented by the following reaction:

$$[P] + [L] \overset{K_1}{\leftrightarrow} [PL] \quad (2)$$

where [P], [L] and [PL] are the molar concentrations of ligand free SP, unbound 2-nonanone and SP bound ligand, respectively, and K_1 is the equilibrium constant. When a protein molecule contains n number of binding sites for a ligand, the equilibrium binding process follows the Klotz binding model depicted by Eq. 1.

Figure 1 shows isotherms for binding of 2-nonanone to SP in the presence of 0–6 mM βCD in the ternary system. In the absence of βCD (control), the number of moles of 2-nonanone bound per mole of SP (υ) increased almost linearly with an increase in free 2-nonanone concentration ([L]). It is to be noted that even at higher values of [L], the binding isotherm for the control did not plateau, which is indicative of the fact that, in the concentration range studied, 2-nonanone did not saturate the binding sites in SP. Experiments at higher [L] were not possible because of the limited solubility of 2-nonanone in the aqueous solution.

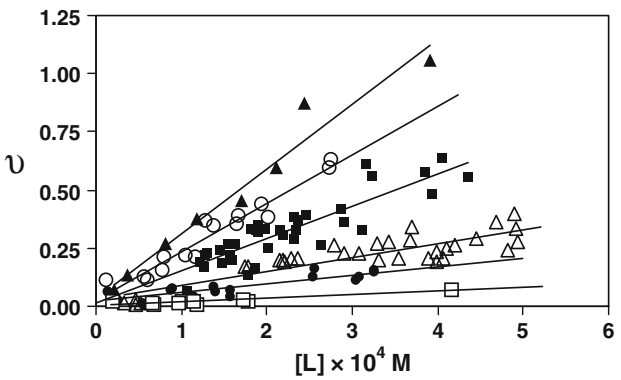

Fig. 1 Equilibrium binding of 2-nonanone to SP (13.36 mg/mL) in the presence of (*filled triangles*) 0 mM, (*open circles*) 0.1 mM, (*filled squares*) 1 mM, (*open triangles*) 2 mM, (*filled circles*) 4 mM and (*open squares*) 6 mM βCD in 30 mM Tris–HCl buffer (pH 8.0) at 25 °C. Pair-wise comparison using Tukey's test at a 95% confidence interval showed that the data of 0.1, 1.0, 2, and 4 mM βCD were statistically different, whereas the difference between 4.0 and 6.0 mM βCD were not statistically significant ($p < 0.05$)

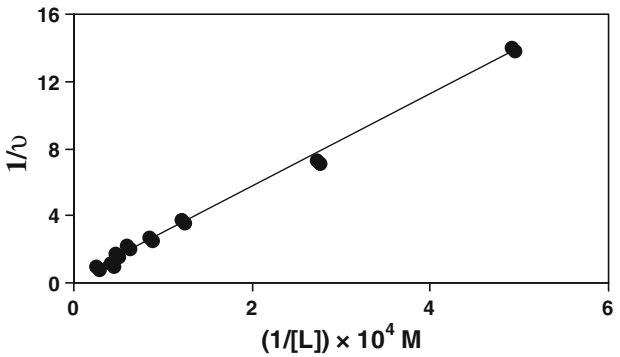

Fig. 2 The Klotz plot of the binding behavior of 2-nonanone to soy protein in the absence of βCD

The number of binding sites (n) and the equilibrium binding constant (K) for SP–2-nonanone binding (in the absence of βCD) were determined by constructing a double reciprocal plot (Fig. 2) according to Eq. 1. From the intercept ($1/n$) and slope ($1/nK$) of this plot, the number of binding sites (n) and binding constant (K) were calculated to be 3.5 and 1,041 M^{-1}, respectively. This translates to a free energy change ($\Delta G°$) of about −4.1 kcal/mol for the binding of 2-nonanone to SP. These values are similar to previously reported values of $n = 4$ and $K = 930$ M^{-1} for SP–2-nonanone binding [8].

The number of moles of 2-nonanone bound per mole of SP, υ, decreased as the concentration of βCD in the system was increased from 0 to 6 mM (Fig. 1). It should be pointed out that since the SP–2-nonanone system was equilibrated prior to the addition of βCD, the decrease in υ must be due to dissociation/stripping off of SP-bound 2-nonanone by βCD. To determine the efficiency of stripping of SP-bound 2-nonanone by βCD at each [L], the binding isotherms, which were linear in the range of [L] studied, were fitted with linear regression curves. From these fitted curves, the amount of ligand bound to SP in the absence of βCD ($\upsilon_{control}$) and in the presence of 0.1–6 mM βCD ($\upsilon_{Treated}$) was recalculated at each [L]. By subtracting $\upsilon_{Treated}$ from $\upsilon_{control}$, the amount of SP-bound 2-nonanone stripped by βCD was calculated (shown in Fig. 3). It was estimated that under experimental conditions used in this study, a maximum of 94% SP-bound 2-nonanone could be stripped off with 6 mM βCD at [L] = 0.4 mM. In order to do a statistical comparison of data, slopes (υ/[L]) from binding isotherms of Fig. 1 were plotted against βCD concentration (Fig. 4). A statistically significant reduction ($p < 0.05$) of υ/[L] was observed, which suggests that βCD was effective in stripping off SP-bound 2-nonanone.

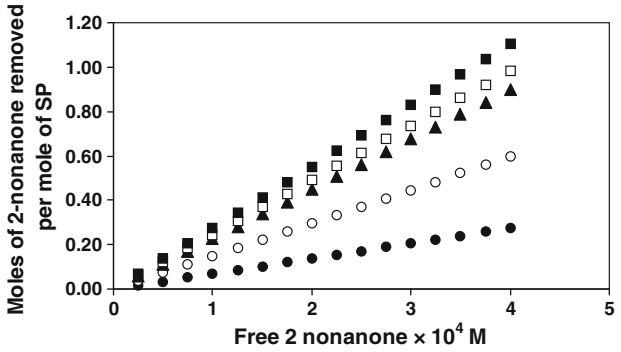

Fig. 3 Removal of 2-nonanone from SP (13.36 mg/mL) in presence of 0.1 mM (*filled circles*), 1 mM (*open circles*), 2 mM (*filled triangles*), 4 mM (*open squares*) and 6 mM (*filled squares*) βCD in the ternary system

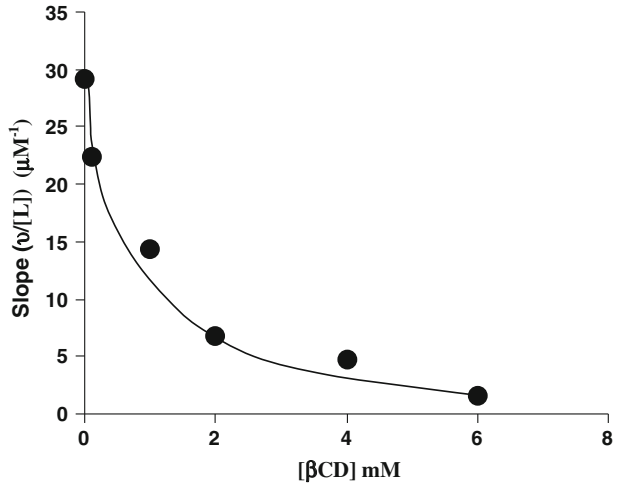

Fig. 4 Relationship between υ/[L] (from Fig. 1) and [βCD] in the ternary binding system

Klotz double-reciprocal analysis (Eq. 1) could not be performed on the binding isotherms of βCD treated samples as it resulted in negative intercepts. Klotz [43] analysis is only suitable for a binary system, that is when only one protein (receptor) and one ligand are present in the system and not for a ternary system involving one ligand and two acceptors with different binding mechanisms and characteristics.

Binding Equilibria

The main premise behind this study was that binding of volatile compounds to soy protein is essentially an equilibrium phenomenon. Due to limited solubility in water, volatile compounds have a propensity to partition into a more thermodynamically favorable environment, such as hydrophobic regions in soy protein. With the exception of covalent binding of a few aldehydes and alcohols, SP-volatile compound interaction is mostly non-specific [8, 13]. Therefore, SP-volatile compound binding equilibrium can shift if a thermodynamic environment equivalent to or better than SP is available to volatile compounds. In a ternary system involving 2-nonanone, βCD, and SP, the hydrophobic cavity of βCD presents an alternative thermodynamic sink for 2-nonanone.

Based on this thermodynamic argument, reduction in SP-bound 2-nonanone by the addition of βCD can be explained by considering a two-step equilibration process. In the first step, SP interacts with 2-nonanone until the binary system reaches an equilibrium (Eq. 2). In the second step, introduction of βCD into the system leads to binding of free ligand [L] to βCD. As [L] forms an inclusion complex with βCD, changes in [L] perturbs the thermodynamic equilibrium between [L] and [PL]. At equilibrium, the free ligand [L] establishes a new equilibrium with both SP-bound and βCD-bound ligand. The overall binding equilibrium for the partitioning of the ligand in the ternary system can be depicted as follows: Assuming that binding of 2-nonanone to SP in the ternary system follows the same process as that depicted by Eq. 1, albeit with altered binding affinity, Eq. 1 can be rearranged as,

$$[\mathrm{L}] = \frac{\upsilon}{K_1'(n-\upsilon)} \tag{3}$$

where K_1' is the apparent equilibrium binding constant for binding of 2-nonanone to SP in the ternary system. The total number of binding sites, n, for 2-nonanone in SP is about $\sim$4 (see above and Fig. 2) and υ is defined as [PL]/[P]$_{\mathrm{Total}}$. Thus, Eq. 3 can be rewritten as

$$[\mathrm{L}] = \frac{[\mathrm{PL}]}{K_1'(n[\mathrm{P}]_{\mathrm{Total}} - [\mathrm{PL}])} \tag{4}$$

The binding equilibrium between 2-nonanone and βCD in the ternary system can be represented as

$$[\beta\mathrm{CD}] + [\mathrm{L}] \overset{K_2'}{\leftrightarrow} [\beta\mathrm{CDL}] \tag{5}$$

where [βCD] and [βCDL] represent molar concentrations of free βCD and the βCD–2-nonanone complex, respectively. Since βCD has only one binding cavity per ligand, the apparent equilibrium binding constant K_2' can be represented as,

$$K_2' = \frac{[\beta\mathrm{CDL}]}{[\beta\mathrm{CD}][\mathrm{L}]} \text{ or } [\mathrm{L}] = \frac{[\beta\mathrm{CDL}]}{K_2'[\beta\mathrm{CD}]} \tag{6}$$

Since, the free ligand [L] is simultaneously in equilibrium with both SP-bound and βCD-bound ligand in the ternary system, from Eqs. 4 and 6

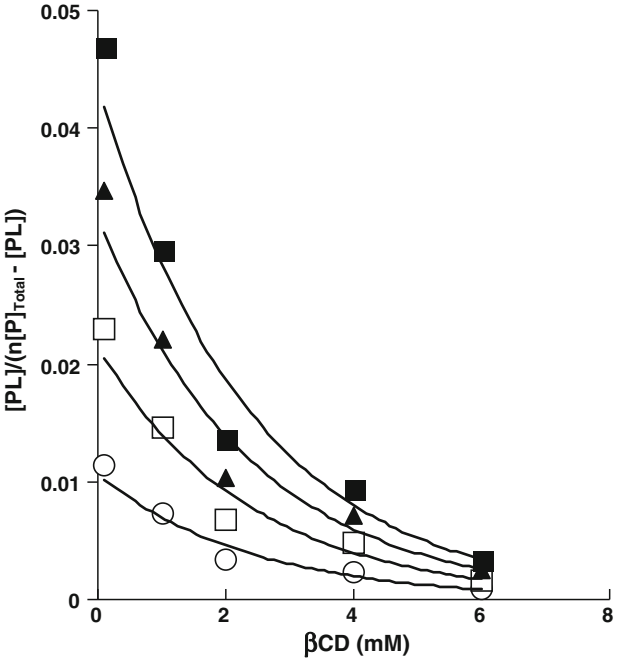

Fig. 5 Plots of $[PL]/(n[P]_{Total} - [PL])$ as a function of total βCD at various [L]: 0.1 mM (*filled squares*), 0.2 mM (*filled triangles*), 0.3 mM (*open squares*), and 0.4 mM (*open circles*)

$$\frac{[PL]}{n[P]_{Total} - [PL]} = \frac{K_1'[\beta CDL]}{K_2'[\beta CD]} \tag{7}$$

Equation 7 defines the thermodynamic state of a simple ternary system involving interaction of a ligand with two different acceptors (i.e., a protein with n binding sites and βCD with one binding cavity) having different binding affinities and binding characteristics for the ligand. Equation 7 predicts that at infinite βCD concentration, the concentration of protein-bound ligand will be practically zero, i.e., the ligand will be essentially stripped-off from the protein. The data in Fig. 5, which shows exponential decrease in $[PL]/(n[P]_{Total} - [PL])$ as a function of total βCD concentration at various free ligand concentrations [L], confirms this prediction. $[P]_{Total}$ is the total concentration of protein (1.336×10^{-4} M) used in the equilibrium dialysis experiment. $[\beta CD]$ is given by

$$[\beta CD] = [\beta CD]_{Total} - [\beta CDL] \tag{8}$$

$[\beta CD]_{Total}$, in this case is known (0.1–6 mM) and $[\beta CDL]$ at any given [L] can be determined from Fig. 3. Using Eq. 8, Eq. 7 can be rewritten as:

$$\frac{[PL]}{n[P]_{Total} - [PL]} = \frac{K_1'}{K_2'}\left(\frac{[\beta CDL]}{[\beta CD]_{Total} - [\beta CDL]}\right) \tag{9}$$

The value of K_1'/K_2' can be obtained from the slope of a plot of $[PL]/(n[P]_{Total} - [PL])$ versus $[\beta CDL]/([\beta CD]_{Total} - [\beta CDL])$. These plots are shown in Fig. 6 at various

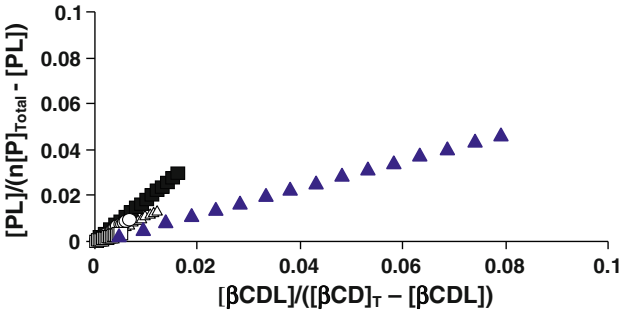

Fig. 6 Plot of $[PL]/(n[P]_{Total} - [PL])$ versus $[\beta CDL]/([\beta CD]_{Total} - [\beta CDL])$ at 0.1 mM (*filled triangles*), 1.0 mM (*filled squares*), 2.0 mM (*open triangles*), 4.0 mM (*open circles*), and 6 mM (*open squares*) βCD

Table 1 Thermodynamic parameters for the transfer of 2-nonanone from soy protein phase to βCD

$[\beta CD]$ (mM)	K_1'/K_2'	$\Delta\Delta G^{\circ}_{SP\rightarrow\beta CD}$ (kcal/mol)
0.1	0.59	−0.312
1	1.83	0.359
2	1.13	0.074
4	1.44	0.214
6	0.67	−0.233

$[\beta CD]_{Total}$ used in this study, and the K_1'/K_2' values are presented in Table 1.

Stripping-off of SP-bound 2-nonanone by βCD can be regarded as transfer of 2-nonanone from the SP phase to the βCD cavity. If K_1' is the apparent binding constant for interaction of 2-nonanone with SP in the ternary system, then the free energy change for this binding is

$$\Delta G_1^{\circ} = -RT\ln K_1' \tag{10}$$

Similarly, the free energy change for binding of 2-nonanone to βCD in the ternary system is

$$\Delta G_2^{\circ} = -RT\ln K_2' \tag{11}$$

Then, from Eqs. 10 and 11, the net free energy change ($\Delta\Delta G^{\circ}$) for the transfer of 2-nonanone from the SP phase to βCD cavity is

$$\Delta\Delta G^{\circ}_{SP\rightarrow\beta CD} = \Delta G_2^{\circ} - \Delta G_1^{\circ} = RT\ln\frac{K_1'}{K_2'} \tag{12}$$

The $\Delta\Delta G^{\circ}_{SP\rightarrow\beta CD}$ values at various βCD concentrations are presented in Table 1. These estimates ranged from −0.3 to 0.36 kcal/mol. It is unclear whether K_1'/K_2' values were dependent on the βCD concentration in the system, although Eq. 9 suggests that it should not be the case. Small differences in K_1'/K_2' values (Table 1) might be due to experimental error as well as to errors propagated by linear regression applied earlier in Fig. 1. Absence of a trend in K_1'/K_2' ratios with respect to βCD concentration

also suggests this as a probable cause. Nevertheless, the small $\Delta\Delta G^{\circ}_{SP\rightarrow\beta CD}$ values indicate that 2-nonanone seems to have almost equal affinity for both SP and βCD in the ternary system. This implies that at equal concentrations of SP and βCD in the ternary system, 2-nonanone will be about equally partitioned between the SP and βCD phases. To shift the binding equilibrium more toward βCD, it is necessary to increase the concentration of βCD by several folds above the concentration of SP. The data in Fig. 4 indicates that when the molar concentration ratio of βCD to SP is about 45 (that is, 6:0.134), about 94% of SP-bound 2-nonanone can be transferred to βCD. This βCD to SP molar ratio corresponds to wt% ratio of 0.51.

From the results of this study, it is quite evident that βCD can strip-off SP-bound 2-nonanone in a concentration-dependent manner. An important implication of these results is the potential of βCD for removing SP bound volatile compounds with alkyl chains shorter than nine carbons, such as hexanal. Although the results from this study show that ΔG° of 2-nonanone binding is equally favorable for SP and βCD in the ternary system, βCD is able to efficiently remove SP-bound 2-nonanone when it is used at a high βCD/SP molar concentration ratio.

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