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Interactions of Flavor Compounds in Model Food Systems Using Benzyl Alcohol as an Example

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The interaction between [^{14}C]benzyl alcohol and denatured bovine serum albumin or Soyamin 90, chosen as models for food proteins, was shown to be reversible adsorption of the flavor compound on the denatured protein. Adsorption is directly proportional to the amount of protein and increases linearly with increasing flavor concentration. Adsorption can be decreased slightly by suspending the denatured protein in media containing dissolved protein or lipid material. Adsorption is reduced by 50% when the denatured protein is suspended in media containing both dissolved protein and lipid material, such as caseinate-stabilized oil/water emulsions, aqueous coffee whiteners, or milk. Adsorption isotherms are given for these and other model systems.

Flavor compounds often interact with food components so that their potential is decreased and the desired flavor is altered. The mechanisms for these interactions, especially when proteins are involved, are not completely understood. Maintaining an acceptable flavor in synthetic protein-enriched foods, or those prepared from alternative protein sources, has proven especially challenging to the flavor chemist.

Usually the interaction of flavor compounds with proteins has been investigated by means of headspace analysis. Franzen and Kinsella (1974) used model systems to establish parameters affecting the binding of volatile flavor compounds. They concluded that the addition of protein decreased the concentration of headspace volatiles above aqueous systems. The proteins studied showed no consistent or predictable effect with respect to the magnitude of flavor binding, however, and no one flavor was preferentially bound by all proteins.

Comparing results from headspace analysis, which is a gas–liquid system at equilibrium, with those obtained from a high vacuum transfer method, where equilibrium between bound and free volatiles is constantly disturbed, Gremli (1974) could comment on the nature of binding. He concluded that the absence of ketone retention under the second treatment indicated that ketones were reversibly bound, whereas the partial retention of the aldehydes under high vacuum transfer would suggest both reversible and irreversible interactions. Other experiments showed that an equilibrium exists between bound and free volatile which is independent of the amount of volatile added. In other words, the protein is not saturated even at high dosages. It is difficult to explain the interactions between flavor compounds and food proteins (usually denatured) in terms of classical binding. Gubler and co-workers (1974) reported that the interactions of citral and food ingredients were independent of pH and probably not of ionic character. King (1978) obtained adsorption isotherms for denatured proteins suspended in buffer solutions of flavor compounds. She showed that adsorption was independent of pH and ionic strength over a range of 4.5 to 7.4 and 0.01 to 0.40, respectively, as well as independent of temperature between 7 and 40 °C.

This paper discusses a simple method for direct measurement of interactions between flavor compounds and denatured proteins. Benzyl alcohol is used in model systems to show the nature and extent of these interactions as well as how they can be influenced.

EXPERIMENTAL SECTION

Apparatus. An Isocap 3000 counter from Nuclear-Chicago was used for liquid scintillation counting. Radioactivity of samples was counted in a complete xylene–surfactant based Phase Combining System obtained from The Radiochemical Centre (GB-Amersham).

Protein preparations were lyophilized or dried in either a Sauter vacuum oven (20 h, 62 °C, 70 cmHg), or in a Bühler air drier (18 h, 60 °C). A Fritsch pulverisette, Type P-0150, was used to grind dried protein until it passed through a 0.125-mm sieve.

Protein. Soyamin 90, a commercial preparation of denatured, dried soy protein commonly used in the food industry, was supplied by Lucas Meyer Co. (D-Hamburg). It was dialyzed against distilled water and freeze-dried before use. Bovine serum albumin (BSA) was obtained from Povite Production N.V. (NL-Amsterdam) and heat denatured at the isoelectric point for 25 h at 60 °C. For some experiments fatty acids were removed from BSA before denaturation by treating the native protein with activated charcoal (Chen, 1967).

Flavor Solutions. ^{14}C-labeled benzyl alcohol was obtained from The Radiochemical Centre (GB-Amersham). Various dilutions of unlabeled benzyl alcohol were made in volumetric flasks and spiked with a given small amount of the ^{14}C-labeled compound. The amount was maintained sufficiently small so that the contribution of the radioactive ligand to the initial ligand concentration could be neglected.

Benzyl alcohol was dissolved directly in the medium used to suspend the denatured protein. The following were used as media: 1/15 M Sørensen phosphate buffer pH 6.0; aqueous solutions of 2% casein hydrolysate or 5% sodium caseinate; oil/water (O/W) emulsions prepared with com-

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mercial emulsifiers or with sodium caseinate as described below; 6.67% aqueous coffee whitener ("Cremora", Borden Co., New York); cream (15% fat), milk (3.8% fat), skim milk, and reconstituted powdered skim milk; 2–10% aqueous reconstituted buttermilk; and 1% aqueous asolectin vesicles. The asolectin vesicles were prepared according to a general procedure given by Hauser (1971) by dissolving 5 g of asolectin in chloroform–methanol (2:1) and evaporating at 40 °C to give a thin film. Residual solvent was removed under high vacuum for about 1 h. Water was shaken in the flask until the film was evenly suspended as an emulsion. The emulsion was cooled and sonicated until clear.

Oil/Water Emulsions. A 0.5% aqueous suspension of Span 60–Tween 60 (4:1) was prepared at 60 °C using a Polytron mixer. At the same temperature 10% sunflower oil was added slowly with constant mixing until a stable emulsion was formed.

O/W emulsions with casein as emulsifier were prepared by hydrating 5% suspensions of spray-dried bland sodium caseinate obtained from Meyhall Chemical AG (CH-Kreuzlingen) at 60 °C for about 40 min, then emulsifying with 10% sunflower oil (60 °C) using a Polytron mixer.

Method. The denatured protein was weighed into 100-mL Erlenmeyers fitted with Teflon-lined plastic screw caps, and sufficient spiked flavor solution was added to give a protein suspension of the desired concentration. For most experiments a 1% suspension (0.3 g of denatured protein in 30 mL of flavor solution) proved convenient. The Erlenmeyers were sealed and shaken on a mechanical platform shaker placed in a thermally controlled room (23 °C). For buffer suspensions, equilibrium was established within a few hours. Nonetheless, a 16-h shaking time was maintained to insure that the denatured protein was completely hydrated in each experiment.

Aliquots (750 μL) of the shaken protein suspensions were removed from each Erlenmeyer and the radioactivity counted to determine the total concentration of benzyl alcohol (C_t), i.e., the amount of ligand bound to the protein (C_b) plus the amount of ligand in the medium (C_f). The suspensions were centrifuged (10 min at 200g) and aliquots (750 μL) were removed from the supernatants. The C_f values were determined by counting the radioactivity in these aliquots. The difference between C_t and C_f is C_b. The conversion factors for changing counts per minute (cpm) to concentration in moles per liter were established at the beginning of each experiment. From the values for C_b so obtained, the percent of the initial ligand concentration which is bound to the protein can be calculated as $100\ C_b/C_t$. This value can also be obtained from the slope of the adsorption isotherm (eq 1) where the linear regression coefficients are given without the factor 10^{-4} which belongs to each axis:

$$C_b = a_0 + a_1 C_t \quad (1)$$

where a_0, intercept on C_b axis; a_1, slope; S_0, standard error of a_0; S_1, standard error of a_1; S_{yx}, standard error of estimate of C_b on C_t.

Isotherms obtained in various media were compared to a reference isotherm measured in buffer. Two isotherms were said to differ significantly if the null hypothesis that the difference between the regression coefficients for the slopes has the value zero was rejected at the 5% significance level.

Basically the same procedure was used to measure flavor desorption from denaturated protein. Supernatants were decanted after the protein was precipitated by centrifugation. The protein was resuspended in 30 mL of medium and centrifuged, and the radioactivity in the supernatant was counted. The procedure was repeated until the concentration of benzyl alcohol in the supernatant remained constant.

Table I. Desorption of Benzyl Alcohol from 1% Protein[a]

protein	initial precipitate	precipitate resuspended once in buffer
denatured BSA	9.7	0.5
denatured defatted BSA	9.8	0.2

[a] Concentration of benzyl alcohol on protein given in percent of the total concentration, 1.3×10^{-3} M.

Table II. Data for Benzyl Alcohol Adsorption Isotherms on 1% Protein in Buffer

protein	a_0	a_1	S_{yx}	S_0	S_1
Soyamine 90, reference isotherm	0.09	0.08	0.20	0.09	0.00938
denatured BSA, reference isotherm	0.04	0.09	0.12	0.02	0.00271
defatted denatured BSA	−0.02	0.09	0.04	0.03	0.00366

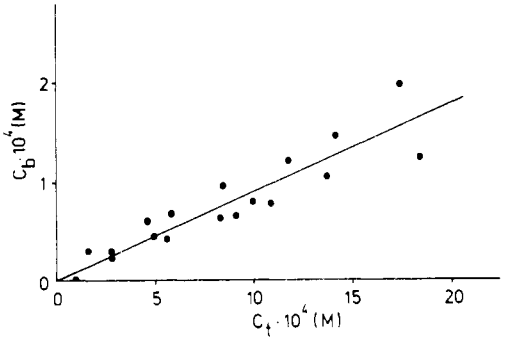

Figure 1. Adsorption of benzyl alcohol in buffer on 1% Soyamin 90, reference isotherm.

RESULTS AND DISCUSSION

Interactions between benzyl alcohol and denatured proteins have been investigated using the procedure described above in which the flavor solutions serve as suspending media for the protein. Interactions were found to be reversible and can probably be described best as adsorption of the flavor compound on the denatured protein. Desorption experiments showed nearly quantitative recovery of benzyl alcohol from protein precipitates resuspended once in buffer (Table I).

The isotherm for adsorption of benzyl alcohol on 1% Soyamin 90 suspended in buffer is shown in Figure 1, and data are given in Table II. For purposes of comparison, this isotherm appears as a dashed line marked reference in Figure 3. It can be seen that the amount of benzyl alcohol adsorbed is directly proportional to the amount originally present in the system. Isotherms for buffer solutions of other flavor compounds showed the same proportional increase in adsorption with increasing ligand concentration (King, 1978). This relationship holds not only for the concentration range examined here, but also at much higher concentrations (Beyeler and Solms, 1974; Gremli, 1974).

Denatured BSA adsorbed benzyl alcohol in the same way and to the same extent as Soyamin 90 (Table II). Approximately 1 mol of free fatty acid per mole of protein was removed from BSA by treatment with active charcoal before denaturing the protein. Benzyl alcohol was adsorbed by both normal and defatted protein to the same extent, as seen in Table II. Removal of the endogenous lipids by treating alfalfa leaf protein concentrate with

Table III. Data for Benzyl Alcohol Adsorption Isotherms on 1% Denatured BSA in Different Media

medium	a_0	a_1	S_{yx}	S_0	S_1
casein hydrolysate	−0.009	0.09	0.05	0.04	0.004 02
sodium caseinate	0.09	0.06	0.15	0.17	0.01
emulsion: Span/Tween	0.13	0.06	0.24	0.16	0.01
emulsion: sodium caseinate	−0.06	0.04	0.12	0.07	0.005 95
coffee whitener	0.05	0.04	0.05	0.03	0.002 66
cream	−0.04	0.05	0.05	0.04	0.004 33
milk	−0.02	0.05	0.14	0.07	0.004 85
skim milk	−0.16	0.08	0.18	0.12	0.007 52
reconstituted powdered skim milk	0.04	0.06	0.07	0.06	0.005 71
reconstituted buttermilk	0.08	0.07	0.12	0.08	0.006 04
asolectin vesicles	−0.12	0.09	0.22	0.21	0.03

Table IV. Data for Benzyl Alcohol Adsorption Isotherms on 1% Soyamin 90 in Different Media

medium	a_0	a_1	S_{yx}	S_0	S_1
emulsion: sodium caseinate	0.02	0.03	0.15	0.10	0.009 55
coffee whitener	−0.03	0.04	0.05	0.05	0.004 57
milk	−0.01	0.03	0.11	0.05	0.004 42

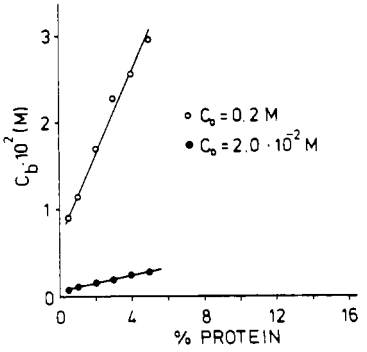

Figure 2. Adsorption of benzyl alcohol in buffer on Soyamin 90, dependence on protein concentration.

acetone, on the other hand, was shown by Franzen and Kinsella (1974) to reduce binding of lipophilic carbonyls to the protein, as indicated by an increased concentration of the headspace volatiles.

Increasing the protein concentration under otherwise unchanged conditions gave rise to a proportional increase in the amount of flavor compound adsorbed, as is shown for Soyamin 90 in Figure 2.

Adding casein hydrolysate to the suspending medium did not affect the extent to which benzyl alcohol was adsorbed by denatured BSA (Table III). Adsorption was decreased slightly in media containing 5% sodium caseinate and in O/W emulsions prepared with commercial emulsifiers. This is seen by comparing the regression coefficients a_1 for these two isotherms, given in Table III, with the value given for the reference isotherm in Table II.

A large reduction in adsorption was measured in O/W emulsions prepared with sodium caseinate as emulsifier (Table III and Table IV). Only half the amount of benzyl alcohol normally adsorbed from a buffer suspension was adsorbed on protein suspended in these emulsions.

A similar effect was achieved when denatured protein was suspended in water containing nondairy coffee whiteners. Data for adsorption on denatured BSA are given in Table III and for adsorption on Soyamin 90 in Table IV. The isotherm for adsorption on Soyamin 90 is shown in Figure 3. Coffee whiteners are spray-dried emulsions of lipids, proteins, and commercial emulsifiers. It appears as though the combination of lipid and dissolved protein

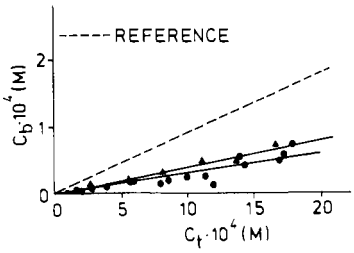

Figure 3. Adsorption of benzyl alcohol on 1% Soyamin 90 suspended in media containing both lipids and soluble proteins: milk (●), aqueous coffee whitener (▲).

in the medium decreases the amount of flavor compound adsorbed by a denatured protein suspended in the medium.

Comparing data for adsorption on denatured BSA in cream and milk, given in Table III, it is clear that adsorption is not decreased by increasing the fat content in the medium. Data for suspensions of Soyamin 90 in milk are given in Table IV. These isotherms all show about half of the adsorption measured in buffer, as is seen in Figure 3 for Soyamin 90.

Data obtained from the skim milk media, however, indicate that some lipid must be present in order to decrease adsorption. Skim milk contains membrane proteins but no milkfat. Adsorption in both liquid and reconstituted powdered skim milk was reduced, but not to the same extent as in the lipid containing media (Table III). Moreover, adsorption was reduced in buttermilk, which is rich in fat globule membranes (Table III) but unchanged when denatured protein was suspended in a preparation of asolectin vesicles (Table III). These membranes, as opposed to those found in buttermilk, do not contain lipoproteins.

These findings suggest that the presence of both lipids and soluble proteins in the medium is required to decrease adsorption to suspended denatured protein. Electron micrographs and photomicrographs of both aqueous coffee whiteners and casein-stabilized O/W emulsions showed that the oil droplets in these media have diameters in the range 0.01–10.0 μm. These droplets are surrounded by distinct protein layers comparable to those of casein surrounding the fat droplets in milk (King, 1978).

The stability of colloidal systems is known to increase with the formation of protein films at oil/water interfaces. Phillips (1977), studying the conformations of proteins at interfaces, has shown that flexible, hydrophobic proteins such as β-casein form dilute monolayers where the entire peptide backbone lies in the plane of the interface. The number of loops or tails of residues which protrude into either bulk phase is increased by increasing the surface concentration until a close-packed looped configuration is achieved in which hydrophobic residues are solvated by

oil molecules. The loops which project into the aqueous phase are potential sites for interaction with flavor compounds. Their presence may shift the equilibrium away from any denatured protein which is suspended in the emulsion. This shift in equilibrium would result in less adsorption of the flavor compound to the denatured protein. The protein–lipid complex might be said to correspond to the lipoprotein membranes found in most foods. The fact that such structures are not present in synthetic foods could explain the decrease in flavor potential attributed to "binding" in these foods.

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High-Performance Liquid Chromatographic Determination of Some Coumarins and Psoralens Found in Citrus Peel Oils

James F. Fisher* and L. Anthony Trama

Citrus peel oil was removed hypodermically from the oil glands of grapefruit *Citrus paradisi* Macf. cv. Duncan, sweet orange *C. sinensis* (Linn.) Osbeck cv. Valencia, sour orange *C. aurantium*, and lemon *C. limon* (Linn.) Burm. cv. Eureka and dissolved in acetonitrile. These oils were examined by high-performance liquid chromatography for their coumarin and psoralen content. This resolution and identification was carried out on μ CN, C-18, and Porasil columns. The coumarins and psoralens were detected at 320 and 305 nm, respectively.

The coumarin compounds reported in citrus peel oil are usually obtained by methods which do not discriminate between components in the oil glands and the parenchymous tissue. These procedures generally involve rupturing the oil gland and surrounding tissue with pressure (cold pressed) to obtain the peel oil (Kesterson et al., 1971). This is followed by concentration and open column or thin-layer chromatography to resolve the components (Fisher and Nordby, 1965, and references therein). These methods of isolation subject the components to conditions conducive to artifact formation.

Peyron and Trêfouël (1963) or Peyron (1963) obtained citrus peel oil directly from the oil glands and examined the oil by thin-layer chromatography.

In the method described below only the contents of the oil glands were examined by LC, showing the application of this technique in resolving citrus coumarins.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-performance liquid chromatograph with a Model 6000A pump and U6K injector (Waters Associates, Milford, MA) was used. The recorder was a two-pen Soltec Model B-281 (Soltec Corp., Encino, CA). A Schoeffel UV-visible liquid chromatography analyzer Model SF770 with a wavelength scan unit 338/SFA 501 (Schoeffel Instrument Corp., Westwood, NJ), a Perkin-Elmer Model 204A fluorescence spectrophotometer with a 20-μL flow cell, and an Aminco Fluoro-Monitor equipped with an excitation 7-60 narrow pass filter (λ max 360 nm) and a 2-A emission filter which excepts wavelengths greater than 415 nm were the detectors. An ultrasonic cleaner (Cole-Parmer, Model 8845-6), a 25 gauge hypodermic needle, and a Wild dissecting microscope were used.

Standard Samples Employed as Criteria of Identity. Where practical the authentic compounds were recrystallized. In all cases, a LC examination showed one major peak along with from one to three minor peaks. The major peak was assumed to be the desired substance. Limited amounts of material made an extensive purification impractical.

Columns and Eluting Systems. Waters Associates 10-μm Porasil and 10-μm C-18 30 cm × 4 mm i.d. columns were used. Also, a 5-μm CN Dupont 25 cm × 4.6 mm i.d. Zorbax column was employed. The organic solvents were Burdick and Jackson and LiChrosolv (Scientific Products). The water was distilled and deionized. The solvent systems were degassed with the ultrasonic cleaner.

Eluting systems employed with the μ Porasil column were (A) heptane–ethyl acetate (90:10), (B) heptane–ethyl acetate (95:5), and (C) heptane–ethyl acetate (80:20). Eluting systems used with the 5-μm CN column were (D) water–acetonitrile (65:35), (E) heptane–ethyl acetate (95:5), (F) water–acetonitrile (70:30), and (G) water–acetonitrile (75:25). Eluting systems used with the μ C-18 column were (H) water–acetonitrile (50:50), (I) water–acetonitrile (65:35), and (J) water–acetonitrile (70:30).

Sample Preparation. A slice of citrus peel (grapefruit, sweet orange, sour orange, or lemon) consisting of the flavedo and some albedo was viewed under the dissecting microscope. A 25 gauge hypodermic needle and syringe were used to remove the contents from approximately 50 oil glands (20–50 μL). This peel oil was dissolved in about 3.0 mL of acetonitrile.

High-Performance Liquid Chromatographic Resolution of Some Coumarins and Psoralens Present in

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