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Binding of Diacetyl by Pea Proteins

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Binding of diacetyl to pea protein was studied, keeping the ligand concentration within the range practically found in foods. The bound ligand concentration was found to depend upon both the free ligand and the protein concentrations in a quasi-linear manner. Decrease in the pH value of the protein solution and particularly isoelectric precipitation of the protein result in dramatic reductions of its retention properties and led to a partial release of the previously bound ligand. The overall binding capacity of the isolate appeared to amount to nearly the sum of the weighted abilities of the individual protein fractions, ruling out the chance of any significant contribution by the residual fat. Taking into account findings previously reported about the affinity between diacetyl and some amino acids, it is postulated that the binding of the diacetyl to the pea protein may result from the interaction of diacetyl with arginyl residues.

INTRODUCTION

Several studies have demonstrated marked binding effects of certain nonvolatile food components, in particular proteins, on the vapor pressure of some flavor volatiles in model systems. Most previous studies in which proteins have been shown to bind volatile substances have been conducted at high concentration of the volatile substance (Reineccius and Coulter, 1969; Arai et al., 1970; Gremli, 1974; Beyeler and Solms, 1974; Franzen and Kinsella, 1974) or in dry systems (Maier, 1975; Aspelund and Wilson, 1983). However, most important volatile flavor substances are very potent odorants present at levels below ppm, and because some deviations from Henry's law can occur at low but not at higher concentrations (Land, 1979), studies of the effects on flavor should be made at sensorily relevant concentrations. Furthermore, as flavor by mouth is always perceived from aqueous stimuli, dry systems are not relevant to flavor perception although they are relevant to flavor retention in food processing. Although more recent studies (Damodaran and Kinsella, 1980; Kinsella and Damodaran, 1980; King and Solms, 1979, 1980, 1981) have used lower concentrations of volatile substances, only one (Land and Reynolds, 1981) was conducted at levels directly related to the concentration range of sensory significance in aqueous solution.

Legumes are now recognized as an increasingly important source of protein for both human and animal nutrition (Fauconneau, 1983). Species that grow successfully in Western Europe and are being actively investigated include the pea (*Pisum spp.*), protein concentrates and isolates from which show promise as functional food additives.

The present study of the effects of various forms of pea protein on the volatility of the important flavor compound diacetyl (butane-2,3-dione) was undertaken with the criteria of Land and Reynolds (1981) to provide information relevant to flavoring of products containing pea proteins.

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EXPERIMENTAL SECTION

Protein Isolates. Samples of protein were prepared at the INRA pilot plant facility at Nantes from seeding grade peas (*Pisum sativum* var. amino) grown under commercial conditions in the Ile de France district. Pea flour was extracted at room temperature by dilute NaOH at either pH 7 or pH 9 according to the procedure described by Gueguen (1983). The protein content of the isolates was always higher than 93%.

Purified protein fractions consisted of the two oligomeric globulins vicilin (M_r = 180000) and legumin (M_r = 320000) and were provided by J. Gueguen (Laboratorie de Biochimie et Technologie des Protéines, Nantes).

Diacetyl. Diacetyl (Fluka, puriss. >99.5% by GC) freshly purchased was redistilled under vacuum, and samples of the main fraction were sealed under nitrogen in glass ampules and stored at −20 °C until required. A fresh ampule was opened every few weeks, when additional peaks had increased to a level that interfered with the analysis. Solutions were made in freshly distilled water daily as required.

Headspace Vials. Glass screw-top bottles of 30-mL volume, with Bakelite tops that were drilled and fitted with aluminum foil wrapped 3-mm-thick silicone rubber septa, were used. Samples were taken with dedicated gas-tight syringes (Hamilton 100 or 500 μL) kept in a clean oven at 50 °C at all times except during filling and injection.

Gas Chromatography. A Pye Unican Model 104 gas chromatograph with standard electron capture detector and GCV pulsed supply (optimized detector current, ×32) was used with a stainless-steel SCOT column (150 m × 0.64 mm i.d.) coated with Carbowax 20M. Conditions: carrier gas argon (0.8 kg/cm^2; 35 cm/s) with 10% methane in argon as quench (5 mL/min); oven 85 °C isothermal; in-

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jector 150 °C; detector 330 °C. These provided clear resolution of the diacetyl peak (RT 10.1 min). Day-to-day variations in detector responses were checked by injecting 1 μL of a standard solution of 0.1 ppm of diacetyl in isooctane. Minimum detectability was 1 pg.

Preparation of Protein Solution and Charged Vials. In early experiments the proteinates contained aggregates that caused turbidity. A preliminary clarifying step was introduced to produce true solutions and improve reproducibility. The proteinate (10 g) was suspended in distilled water (100 mL) and stirred for 1 h at 20 °C. The resulting slurry was centrifuged (20000*g*, 30 min, 20 °C) and the clear supernatant pooled and weighed. The pH of the protein solution was measured and adjusted as outlined below. Dilutions of the protein solution (currently to the half and the sixth) were made with distilled water.

Portions of 5 mL were dispensed into the headspace vials, 5 mL of the appropriate concentration of aqueous diacetyl solution (0.02–20 μg/mL) was then added, and the vials were briefly purged with nitrogen gas from boiling liquid nitrogen and the caps closed. Purging with cylinder nitrogen produced interfering artifacts. Control vials contained no protein. Vials were then incubated in a shaking water bath at 25 °C for at least 90 min before headspace samples were taken. Vials were immersed up to the neck but such that no water splashed onto the septum. Three replicate vials were used for each condition, but for headspace analysis, only one sample was taken from each.

Proteins in the supernatant and the residual precipitate were investigated by gel electrophoresis. As the patterns showed no significant difference, it was concluded that the soluble protein was representative of the whole.

Dry-matter determinations at 103 °C to constant weight were carried out on both the precipitate and the supernatant. The proportion of solubilized isolate was determined, and the protein concentration in the stock solution was calculated taking into account the isolate purity. Molar concentration of protein was estimated on the basis of an average molecular weight of 220 000.

Effects of pH. In early experiments the protein solutions were used without pH adjustment. When pH effects were investigated, adjustment was made with solutions of HCl (N) or NaOH (N). Because of the high buffering capacity of the proteins, solutions were allowed to stand for 15 min at ≃20 °C before the final pH measurement.

In some experiments aiming to evaluate the reversibility of binding, isoelectric precipitation of the protein was carried out straight into the vial either on protein solution or on preequilibrated ligand–protein systems. At the isoelectric point, separation of precipitated protein was accelerated by centrifugation (MSE Bench centrifuge, 3 min, 900 rpm).

MEASUREMENT OF BINDING

The concentration of diacetyl bound to protein in solution (C_b) was determined indirectly by measuring the concentration of diacetyl in the equilibrated headspace and calculating the concentration of free diacetyl in the solution (C_f) using Henry's law.

The air/water partition coefficient (K_{aw}) for diacetyl has been shown to be constant and to obey Henry's law in aqueous solutions containing nonvolatile solutes (Land and Reynolds, 1981) over the concentration range of diacetyl used in the present experiments. Therefore, the headspace concentration is proportional to the concentration of free diacetyl in solution. As K_{aw} is small (5.3×10^{-4}) (Land and Reynolds, in preparation), the total concentration in the liquid phase ($C_f + C_b$) is approximately equal to the total concentration of diacetyl (C_t) added to the solution.

In every experiment, the diacetyl concentration in the headspace above water (H_w) and the protein solution (H_p) was measured under identical conditions. Concentrations of free and bound diacetyl were then calculated from eq 1 and 2.

$$C_f = H_p C_t / H_w \quad (1)$$

$$C_b = C_t - C_f \quad (2)$$

TREATMENT OF DATA

Assuming that the addition of the ligand to the protein solution does not result in any precipitation of the protein, the mass action law (3) applies to the system, where K = intrinsic binding constant, P = molar concentration of the protein, and n = number of binding sites in each protein molecule.

$$K = \frac{[\text{ligand bound to protein}]}{[\text{free ligand}][\text{free binding sites}]} = \frac{C_b}{C_f(nP - C_b)} \quad (3)$$

Minor rearrangements of eq 3 yield the Langmuir and Scatchard equations, which have both previously been proposed (Kinsella and Damodaran, 1980; Damodaran and Kinsella, 1980) as a mathematical model for flavor–protein interactions. Considering $\bar{r} = C_b/P$ = mean number of moles of ligand bound/mole of protein, (3) can be rewritten

$$K = \bar{r}/C_f(n - \bar{r}) \quad (4)$$

Rearranging eq 4 gives

$$C_b/PC_f = K(n - \bar{r}) = \epsilon(C_b, P) \quad (5)$$

As C_b, C_f, and P can be experimentally measured, individual values for ϵ (the binding coefficient) can be readily calculated. The limiting value of the function, when C_b is very small, is the overall binding coefficient (Kn).

Determination of the Overall Coefficient *Kn*. The individual values E_i of the function will remain very close to the constant Kn as long as $\bar{r}$ is small compared to n. This probably applies when the molar concentration of the total ligand is kept lower than the protein concentration since

$$\bar{r} << C_t/P < 1 < n$$

In such cases, it is likely that most of the apparent variation of ϵ, calculated from eq 5, can be attributed to the experimental error in the C_b and C_f determinations. Therefore, providing a significant number of trials had been performed, the mean ($\bar{E}$) could be considered as a reasonable approximation of the overall binding constant Kn. It can also be predicted from eq 5 that when the condition $\bar{r} << n$ is fulfilled, the ratio C_b/C_f will be constant at any particular protein concentration.

Determination of the Binding Coefficient *K*. Increasing the concentration of the total ligand while keeping the protein concentration constant results in higher $\bar{r}$ values. Then, $K\bar{r}$ can no longer be neglected and eq 5 is rewritten in the form

$$\bar{r}/C_f = \bar{E} - K\bar{r}$$

from which

$$K = \bar{E}/\bar{r} - 1/C_f \quad (6)$$

can be calculated.

Estimation of the Binding Reversibility. Model systems in which the protein fraction had been precipitated at its isoelectric point were used. After the systems had been equilibrated in the usual way, different proportions (x) of the equilibrated supernatant were substituted by supernatant containing no diacetyl. The systems were

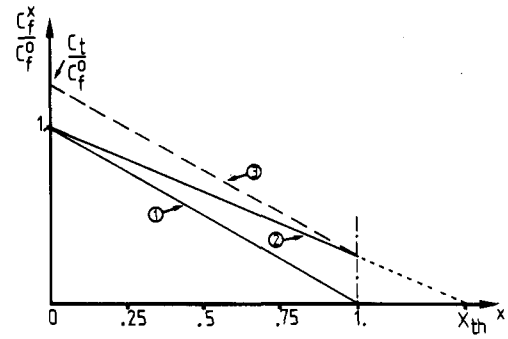

Figure 1. Ligand release by the precipitated protein: X, fraction of the substituted supernatant; (1) theoretical curve when the ligand is irreversibly bound to the protein; (2) theoretical curve when the ligand is reversibly bound to the protein; (3) theoretical maximum value for the ratio C_f^x/C_f^0 calculated as $C_t/C_f^0 - x$.

Table I. Calculated Parameters for the Linear Regression[a] $C_f = S_r C_t + b_r$[a]

pH for protein extractn	protein concn, $P \times 10^4$, M	no. data collcd	slope, S_r	intercept, b_r, μM	correln coeff
9	0.250	12	0.950	−0.0245	0.999
	0.725	13	0.878	−0.0458	0.999
	1.5	18	0.642	0.0139	0.998
7	0.250	7	0.961	0.0129	0.999
	0.725	13	0.831	0.0372	0.995
	1.5	16	0.744	0.0222	0.999

[a] Total ligand concentration in the range 0.1–100 μM.

reequilibrated, and the free ligand concentration (C_f^x) was determined.

In case of an irreversible binding of the ligand, the theoretical ratio between C_f^x and C_f^0 would be

$$C_f^x/C_f^0 = (1 - x)/1 \quad (7)$$

In the event of a reversible binding, the experimental C_f^x values should be higher than the corresponding C_f^x calculated by eq 7 (Figure 1). Particularly, C_f^1 corresponding to the substitution of the whole supernatant must be different from 0. In practice, C_f^1 can be graphically extrapolated from the experimental data.

If the second proposition is correct, this would provide us with an alternative means to calculate the overall binding coefficient of the precipitated protein. Considering that the total concentration C_t^1 of the ligand in the system equals the concentration C_b^0 of the ligand initially bound to the protein, it can be deduced from eq 5 that

$$K(n - \bar{r}) = (C_b^0 - C_f^1)/PC_f^1 \quad (8)$$

RESULTS

Solubilized Protein. Experimental C_f values were plotted against C_t (Figure 2), and linear regressions were calculated with C_f as the dependent variable for each set of experiments. Results are reported in Table I, and it can be concluded that, in the investigated concentration range of the total ligand, there is a proportionality between C_f and C_t and furthermore between C_f and C_b. Such a linear relationship has been previously reported by King and Solms (1979) on the absorption of benzyl alcohol by soy protein.

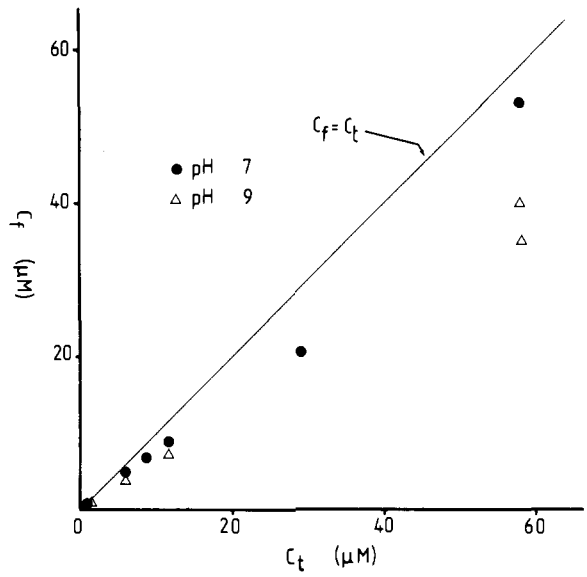

Figure 2. Free vs. total diacetyl in protein solutions (1.5×10^{-4} M) made from two protein isolates prepared at pH 7 and pH 9.

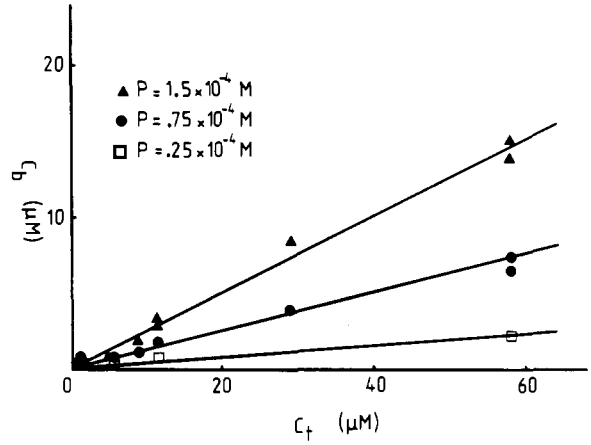

Figure 3. Binding of diacetyl to isolate D, dependence on protein concentration.

Comparison of the equations obtained from the regression to eq 1 makes clear that the ratio H_p/H_w can be identified to the slope S_r of the regression. It has been demonstrated that S_r is a constant that depends only on protein concentration, and it is known that H_w is proportional to C_t. Therefore, $H_p = S_r H_w$ is proportional to C_t, which means that aqueous solutions of pea protein obey Henry's law in the considered concentration range of the total ligand.

It was demonstrated also that C_b/C_t (ratio of the bound to the total ligand), which can be calculated as $(H_w - H_p)/H_w = 1 - S_r$, was proportional to P (Figure 3).

Table II. Comparison between the Calculated and the Theoretical Binding Coefficients

isolate	pH for protein extractn	no. of expts	slope (S_b)[a]	theor overall binding coeff, E_t ($\times 10^{-3}$),[a] M^{-1}	mean of exptl overall binding coeff (E_i), $\bar{E}$ ($\times 10^{-3}$), M^{-1}
A	9	13	0.02	3.04	3.04
B	9	13	16.67	3.49	3.27
C	9	20	3.49	3.17	3.08
D	7	35	5.54	1.91	1.96

[a] S_b and E_t are calculated parameters obtained from the linear regression $\epsilon = S_b \times 10C_f + E_t$. Free ligand concentration in the range 0.1–100 μM.

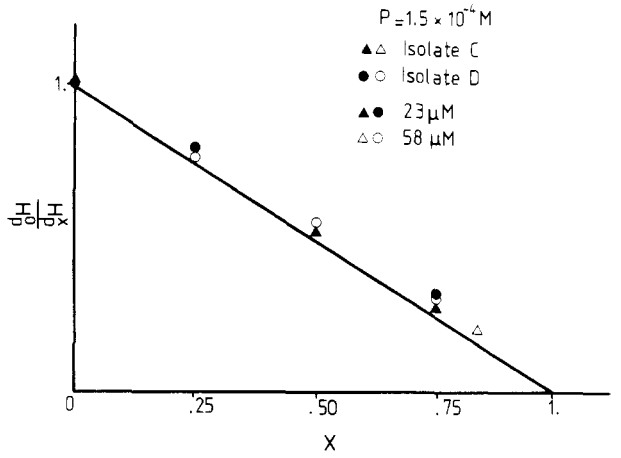

Figure 4. Desorption of diacetyl from precipitated protein. $P = 1.5 \times 10^{-4}$ M.

Table III. Parameters Governing the Ligand Release by Precipitated Pea Protein

isolate	treatment	no. of expts	slope (s)	σ	overall binding coeff × 10^{-3}, M^{-1}
C	ligand added before precipn; dil med, water	8	0.910	0.002	0.700 ($\bar{s}$ = 0.905)
	ligand added after precipn; dil med,				
	(a) water	16	0.899	0.005	
	(b) supernatant	8	0.906	0.003	
D	ligand added after precipn; dil med, supernatant	15	0.871	0.04	0.988

Determination of the Overall Binding Coefficient. The mean values $\bar{E}$ were calculated for four different pea isolates (Table II). Linear regressions using ϵ as the dependent variable and C_f as the independent variable were also calculated. It was assumed that the intercept of the regression line with the y axis would yield the theoretical value (E_t) of the overall binding coefficient Kn. The agreement between the two sets of theoretical and experimental coefficients is very close. The coefficients of variation relative to the $\bar{E}$ determinations varied from 20 to 28%, which is a relative experimental error smaller than 10% on C_f determinations. It is then reasonable to assume that experimental errors may practically account for the whole of the variation found during the experiments at low concentrations of the ligand.

Precipitated Protein. The binding capacity of the precipitated protein was found to be about one-third that of the soluble protein. Precipitating the protein from an equilibrated system did not make much difference. This suggests that some ligand is released when the protein becomes insoluble.

Equilibria between the bound and the free ligand were investigated according to the scheme described in the Experimental Section, and results are shown in Figure 4. Linear regression between x (fraction of the equilibrated supernatant substituted) and C_f was calculated. It was found that eq 7 could be written in the form $C_f^x/C_f^0 = 1 - sx$ where $x < 1$. Results are reported in Table III.

The overall binding coefficients were evaluated with the help of Figure 1. Then, the determination of C_b^0, which proved to be relatively inaccurate when little ligand was bound to the protein, was no longer required. Considering x_{th} the intercept of the $(1 - sx)$ straight line with the x axis, it is obvious graphically that

$$(x_{th} - 1)/1 = C_b^0/C_f^0$$

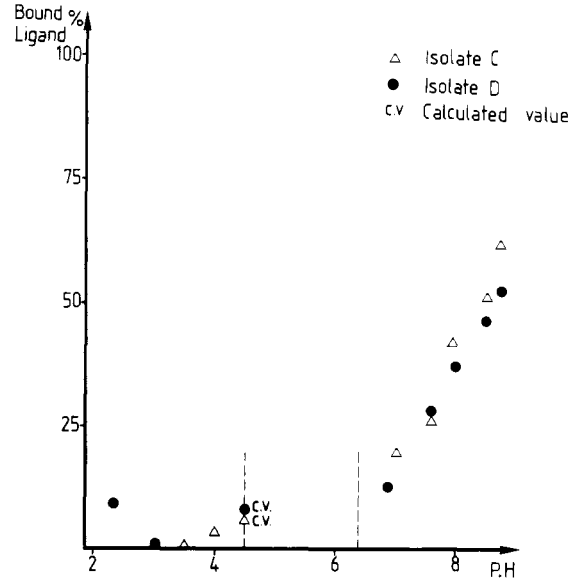

Figure 5. Relationship between the percentage of bound ligand and the pH of the protein solution.

Table IV. Data for Purified Fractions of the Individual Proteins

	$M \times 10^{-4}$	$Kn \times 10^{-3}$, M^{-1}	% of the protein fractn[a] wt	% of the protein fractn[a] moles
vicilin	18	2.5	70	80
legumin	32	7.2	30	20

[a] Protein content of the proteinate ≥93%. Theoretical $Kn \simeq 0.93 \times 10^3$ ($0.8 \times 2.5 + 0.2 \times 7.2$) = 3.44×10^3 M^{-1}.

as $C_b^0/C_f^0 = PK(n - \bar{r}) \simeq PKn$ at low concentrations of the ligand and $x_{th} = 1/s$. Kn can be calculated as

$$\frac{1}{P}\frac{1-s}{s} \qquad (9)$$

The difference between the binding capacity of the two kinds of proteinates appears to be smaller with precipitated proteins than with soluble proteins. Influence of pH variations on the retention ability of the protein is shown in Figure 5. No data were recorded when the protein appeared to be present under both dissolved and precipitated states in the sample (practically for pH values between 4.5 and 6.4). It can be concluded that, within the pH range encountered in food, the retention ability increases with pH and may approach 50% of the total ligand at slightly alkaline pH. Since the pH values of the protein solutions made from proteinates C and D were respectively 7.2 and 6.8, it seems that a pH effect may be responsible for most of the difference found in their binding activity.

Determination of the Intrinsic Coefficient K. Experiments were carried out at pH 7, with $C_t = 3.5 \times 10^{-3}$ M and 4% protein (isolate C) solution. Values obtained for $\bar{r}$ and C_f were respectively 5.6 and 2.65×10^{-3} M, and K was calculated from eq 6: $K \simeq 173$ mol^{-1} and $n \simeq 18$ binding sites ($\bar{E} = 3.08 \times 10^3$). Some data relative to the individual retention ability of purified vicilin and legumin fractions were obtained. They are shown in Table IV. Knowing the relative molecular concentration of the different proteins, it was then possible to calculate the theoretical contribution of the pea protein to the overall binding coefficient of the isolate. As the calculated value (3.44×10^3) shows a very good fit with those reported for the proteinates, it is likely that the contribution from the nonprotein fraction is very small.

DISCUSSION

Two prerequisites for studies of factors influencing volatility in which it is not possible to directly measure C_f in the liquid phase are that the equilibrated headspace does obey Henry's law and that any losses by adsorption in the vessels used and the analytical system is negligible in relation to the changes being measured. In this study both prerequisites were fulfilled despite the difficulties of analysis at very low concentrations (Land and Reynolds, 1981, in preparation). Furthermore, in the present study it has now been shown that at constant pH Henry's law is also obeyed in the presence of pea proteins, although because of binding the intercept is changed but the slope is identical. Thus, the air–water and air–protein solution partition coefficients are constant at constant pH and may be used to predict the vapor pressure and therefore perceived odor of diacetyl over solutions containing known concentrations of diacetyl and pea protein.

The changed intercept reflects the overall binding constant Kn, which in our experiment is of the same order than most of the values previously reported in the literature for other flavor compound–soy protein systems (Beyeler and Solms, 1974). The intrinsic binding constant shows a relatively low value, which suggests that ligand–protein interactions are weak and reversible in nature. On the basis of a 220 000 molecular weight, the number of binding sites in pea protein is about 18. At infinite ligand concentration, the molar ratio of the bound ligand to the amino acid residues would be about 1/125. This appears to be of the same order as the values reported by Kinsella and Damodaran for the binding of 2-nonanone to BSA and soy protein (1/85 and 1/250, respectively).

Matching the experimental results obtained from the purified protein fractions with those obtained from the raw isolates emphasizes that there is no significant difference between the overall binding constant of the isolate and the sum of the weighted contributions of vicilin and legumin. This result suggests that the retention activity shown by the residual lipids or any interaction between the proteins toward diacetyl is only marginal and may be considered of no practical significance. However, this may be not a general rule, and ligands more hydrophobic than diacetyl may behave in quite a different manner.

It has been shown that, in the dry state, amino acids containing S atoms or ϵ-amino groups are able to sorb moderate to high quantities of diacetyl (Maier, 1975). It has been reported also that diacetyl reacts with arginine with a high degree of specificity (Shalabi and Fox, 1982). It is particularly striking that, despite the arginine content of vicilin and legumin is respectively 4.5 and 9%, the ratio of the calculated number of binding sites to the absolute number of arginyl residues is very close in both cases ($\simeq 1/6$). This suggests that interactions between diacetyl and arginine could occur at the surface of the protein. Obviously, more information is needed and one of the next steps would be to find out the distribution of the arginyl residues within the molecule. The above findings and the results previously reported by other workers (Damodaran and Kinsella, 1980; Kinsella and Damodaran, 1980; Gremli, 1974; Dhont, 1975; Aspelund and Wilson, 1983) suggest that flavor compound–protein interactions can take place through different procedures that are mostly dependent upon the properties of the considered ligand.

In practice, this may prove very troublesome for the food technologist. If the trend toward new foods including more protein extracted from legumes is continued in the future, flavoring problems will probably arise. It is then likely that most of the commercial flavors currently in use will not perform at their best with these new foods since the balance between the flavor components will be disturbed by specific binding to proteins. If new flavors specially intended for these particular applications are to be created, there will be an obvious need for more knowledge in the field of flavor compound–protein interactions. However it cannot be stressed too strongly that studies should be conducted at sensorily relevant levels of flavor compounds and not just at higher concentrations that are more convenient to analyze.

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Registry No. Diacetyl, 431-03-8.

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