INTERACTIONS OF FLAVOUR MOLECULES WITH FABABEAN PROTEINS

by

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For the moment all discipline seems painful
rather than pleasant; later it yields
the peaceful fruit of righteousness to those
who have been trained by it.

Hebrews 12:11

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ABSTRACT

Although the ability of proteins to bind flavour molecules has been investigated, there are no published studies which were designed to complement chemical analysis with sensory analysis. The purpose of this study was (1) to investigate mechanism(s) involved in vanillin-fababean protein micellar mass (PMM) interactions and (2) to determine the impact of these interactions on the perception of vanillin flavour in vanillin-fababean PMM systems. Free vanillin was determined in vanillin-PMM slurries after equilibrium between bound and free vanillin was established using a micro-partition system for separation of free vanillin from PMM and PMM-bound vanillin. A high performance liquid chromatographic(HPLC) method was subsequently used for quantitative determination of free vanillin. Binding data were interpreted based on evaluation by Klotz plots and Scatchard plots. The sensory evaluation involved estimation of vanillin flavour in vanillin-PMM systems by magnitude estimation. The magnitude estimates were then converted to "perceived" vanillin concentration using a power function for vanillin in water. The "perceived" concentrations as determined by sensory analysis were compared with the free vanillin levels as determined in binding experiments by HPLC in the same system.

Binding capacity of partially and completely heat-denatured PMM was higher than that of native PMM. Binding forces between vanillin and PMM were weak and the number of

binding sites increased when PMM was denatured, thus indicating hydrophobic interactions. Increasing PMM concentrations increased the percentage of total vanillin bound to protein. Addition of NaCl or Tris-HCl to the vanillin-PMM systems seemed to have no significant effect on the binding capacity of PMM for vanillin. Interpretation of the binding data by Scatchard plots indicated positive and negative cooperativity as well as ligand-induced protein associations for vanillin-PMM interactions. The sensory study revealed that the perceived vanillin concentration in vanillin-PMM systems equalled the free vanillin concentration as determined in the binding experiments for the corresponding systems which contained 1200 ppm and 2400 ppm vanillin. The perceived vanillin concentration exceeded the free vanillin concentration in the systems to which 300 ppm or 600 ppm vanillin were added. Two unidentified phenolic compounds which were associated with PMM may have been implicated in the flavour impact of the vanillin-PMM system. The study demonstrated that in simple systems the flavour impact of flavour-protein interactions can be predicted based on determination of the free level of flavour compound and based on the power function for the particular flavour compound in water.

1. INTRODUCTION

The ability of proteins to bind small molecules has been widely investigated in molecular biology and pharmacology. However, the role of interactions of small molecules and proteins in food systems has only recently become a topic for investigation. Interest in this area can be attributed to increasing interest in using protein isolates and concentrates derived from oilseeds and pulses in food products. It has become evident that conditions must be understood and controlled during preparation of these isolates to minimize interactions between off-flavours and proteins (Arai et al., 1970). The use of plant protein isolates in food products has revealed that interactions between flavour molecules and proteins may reduce or modify the flavour impression of a compounded flavour by the selective binding of flavourants.

Binding of small molecules or ligands to proteins is affected by many factors such as pH, ionic strength, temperature, ligand concentration and protein concentration. Furthermore, interactions are also dependent on the type of ligand involved, the type of protein involved, and the conformation of the protein (i.e., native vs. denatured).

Information concerning interactions between small molecules in food systems is limited. In order to design adequate processes for preparation of protein isolates free from off-flavours and to facilitate successful flavouring of food products containing protein isolates, it is necessary

to elucidate the mechanism(s) involved in these interactions. Recently, several studies (King and Solms, 1979; Damodaran and Kinsella, 1981a,b) have investigated the thermodynamic and conformational effects of flavour compounds on soy proteins to further understand some of the parameters involved in flavour-protein interactions.

Although this information exists for flavour-soybean protein interactions, there appears to be little information available concerning flavour binding by other proteins such as those from fababeans or peas. In addition the impact of flavour binding by proteins has not been analysed by sensory methods. The objectives of this study were 1) to investigate mechanism(s) involved in vanillin-fababean protein micellar mass (PMM) interactions and 2) to determine the relevance of these interactions in the perception of vanillin flavour in vanillin-fababean PMM systems.

2. LITERATURE REVIEW

2.1 Protein-Ligand Interactions

2.1.1 Forces Involved in Protein-Ligand Interactions

The ability of proteins to bind small molecules or ligands has been widely investigated in molecular biology and pharmacology. The interaction of flavour molecules with proteins has more recently become a topic of investigation. The reason for this interest was that more and more vegetable protein concentrates and isolates have been or will be used in food products. These investigations have made it apparent that although interactions between flavour compounds and proteins are weak they have in most cases detrimental effects on the flavour quality of foods in which these protein preparations were used(Kinsella and Damadaran, 1981). Investigations concerning flavour-protein interactions, in many cases, were initiated to determine possible means for controlling these interactions. During protein isolation it was important to maintain conditions which resulted in minimal off-flavour-protein interactions. Flavour-protein interactions are weak and non-covalent in nature(Maier, 1975) and similar to other ligand-protein interactions such as those occurring between drugs and their carrier proteins. The different non-covalent types of interactions between ligands(flavour compounds) and protein will therefore be introduced in the following paragraphs.

2.1.1.1 Electrostatic Interactions

Electrostatic interactions occur between molecules with charges. The basic concept can be explained using Coulomb's law which states that two unlike charges attract each other, and that two like charges repel each other, with a force that is directly proportional to the product of the magnitude of the charges and inversely proportional to the square of the distance between them. The force can be expressed as;

$$F = \frac{KQ_1Q_2}{r^2}$$

where F is the force between charges Q1 and Q2 that are separated by a distance r, and k is the proportionality constant whose value depends on both the units used for the other quantities and the nature of the medium in which the molecules are dispersed(Hooper and Gwynne, 1977).

Examples of electrostatic interactions between proteins and ligands have been reported. One example is the interaction between organic ions, such as methyl orange and azosulfathiazole, and proteins(Klotz et al., 1946). Thus, as mentioned above, in order to have electrostatic interactions, the ligands and protein molecules must be in ionic forms, i.e. have charges.

Flavour compounds are normally not ionic compounds and electrostatic interactions between flavour compounds and proteins are therefore unlikely. However, electrostatic interactions between adjacent non-ionic molecules are possible based on dipole force(Masterton and Slowinski,

1977). Interactions of this type may be possible between certain flavour molecules and proteins but none to date has been reported.

2.1.1.2 Hydrogen Bonding

A hydrogen bond is said to exist between a donor molecule D-H and an acceptor A, where atoms D and A are strongly electronegative, when there is evidence that the two molecules associate together with involvement of the hydrogen atom of the donor(Cantor and Schimmel, 1980a). The strength of the hydrogen bond is dependent upon the temperature, pH and ionic strength of a system.

For example, when the temperature of a system is increased, the strength of the hydrogen bond would be decreased. This is due to the entropy(ΔS) effect. Entropy is defined as the energy of a substance due to the internal motion of the molecules(Grant, 1969). At higher temperatures, the motion of the internal molecules would be increased resulting in a larger entropy. This increasing motion of the internal molecules would reduce the strength of the hydrogen bonds between molecules. One good example for temperature affecting the strength of hydrogen bond is ice and water. The strength of the hydrogen bonds in ice is higher than in water since the internal molecules of ice have a slower motion than those of water.

Besides temperature, pH and ionic strength would also affect the strength of hydrogen bonds. For example, a low pH would promote more positive charges on some amino acid

residues of a protein and thus decrease the capacity of hydrogen bond formation between a protein and a ligand. This is because the formation of hydrogen bonds is dependent upon the electronegative atoms in a molecule.

A similar effect would also be observed when the ionic strength of a solution is increased or decreased. The use of urea provides an example for the effect of ionic strength on the formation of hydrogen bonds. It has been reported that urea disrupted the hydrogen bonds between water molecules(Fennema, 1976). The disrupted water-water hydrogen bonds were replaced by water-urea hydrogen bonds. This is because the electronegative atoms are stronger in urea than in water.

Food products in which vegetable protein preparations are used are mainly aqueous systems. Hydrogen bonding between flavour molecules and proteins are therefore unlikely because water will compete for hydrogen-bonding sites on the protein. If however, there are large lyophilic regions or conditions then hydrogen bonding between a flavour molecule and a protein may be possible.

2.1.1.3 Hydrophobic Interactions

Hydrophobic interactions describe the tendency of non-polar groups to associate in aqueous solution resulting in decreased contact with neighboring water molecules. The occurrence of these interactions is favoured by high entropy(ΔS). Entropy is a property of a system related to its degree of organization; highly ordered systems have low

entropy. Before hydrophobic interactions occur, water molecules are orderly around exposed non-polar solutes. When hydrophobic interactions occur between the non-polar solutes, the order of the water molecules decreases, resulting in an increase in entropy and hence free energy(ΔG) of formation is decreased. Free energy is a property of a system which reflects its capacity to do useful work; spontaneous reactions are those for which ΔG is negative.

Based on Gibbs-Helmholtz equation,

$$\Delta G = \Delta H - T\Delta S$$

where ΔH(enthalpy) is a property of a system which reflects its capacity to exchange heat with its surroundings; and T is the temperature(°K) of the system. The measurement of free energy can be expressed as the strength of the hydrophobic interactions and is called hydrophobicity. It can be measured using the changes in free energy(ΔG) for water and for dioxane. Water is used because it is a polar solvent in which solute molecules are uniformly dispersed and dioxane is used because it is a non-polar solvent in which hydrophobic interactions take place. According to Marshall(1978), it can be written

$$H_{\phi} = \Delta G_{\text{in dioxane}} - \Delta G_{\text{in water}} \qquad (1)$$

where H_{ϕ} is the hydrophobicity per mole of solute molecules and ΔG is the free energy per mole of solute. With a given solute at saturation, the chemical potential(chemical

potential is a measure of the tendency of a chemical reaction to take place) of the solute is the same in any solvent, thus

$$\Delta G_{\text{in dioxane}} = \Delta G_{\text{in water}} \qquad (2)$$

Since the relationship of free energy to the concentration [C], or activity, a, of a substance can be expressed by the equation

$$\Delta G = nRT\ln\frac{a}{a_o} \qquad (3)$$

where R is the universal gas constant and T is the absolute temperature. Combination of equations(2) and(3) yields

$$\Delta G_{\text{in dioxane}} + RT\ln [C]_{\substack{\text{at saturation}\\ \text{in dioxane}}} + RT\ln\gamma_{\substack{\text{at saturation}\\ \text{in dioxane}}} =$$

$$\Delta G_{\text{in water}} + RT\ln [C]_{\substack{\text{at saturation}\\ \text{in water}}} + RT\ln\gamma_{\substack{\text{at saturation}\\ \text{in water}}} \qquad (4)$$

where γ is the activity coefficient defined as a/a_o. Since the concentrations in equation(4) are simply the solubilities of that solute in each of the two solvents, and since activity coefficients will be close to unity for the sparingly soluble solutes(i.e. very dilute), equation(4) can be re-written as

$$\Delta G_{\text{in dioxane}} - \Delta G_{\text{in water}} =$$

$$RT\ln [C]_{\text{at saturation in water}} - RT\ln [C]_{\substack{\text{at saturation}\\ \text{in dioxane}}} \qquad (5)$$

Combination of equation(1) and(5) yields

$$H_\phi = RT\ln\frac{[C]_{\text{at saturation in water}}}{[C]_{\text{at saturation in dioxane}}} \qquad (6)$$

or $$H_{\phi} = RT\ln \frac{\text{(solubility in water)}}{\text{(solubility in dioxane)}} \qquad (7)$$

As equation(7) shows, ΔH is temperature dependent. By increasing the temperature of an aqueous system which consists of non-polar solutes, ΔH and ΔS of the solutes would also be increased. According to Gibbs-Helmholtz equation, $\Delta G = \Delta H - T\Delta S$, if ΔH minus $T\Delta S$ is negative, then the formation of free energy has occurred in the system and hence hydrophobic interactions have taken place between non-polar solvents in the system. If temperature is unchanged, entropy would be the only factor affecting the hydrophobic interactions. The pH and ionic strength would not affect the hydrophobic interactions provided there is no change of entropy in the system with change in pH and ionic strength.

Most of studies on these factors affecting hydrophobic interactions are carried out by examining interactions between molecules, such as those between macromolecules or between macromolecules-micromolecules. Studies also examine hydrophobic interactions by observing the conformation of macromolecules in hydrophobic systems. For example, hydrophobic interactions are one of the major forces to stabilize a three dimensional conformation of a native protein. In the native state, amino acids on the inside chains of the spherical protein are almost all hydrophobic or non-polar groups, and polar groups are on the

outside(Solms et al., 1973). These interactions will be disrupted when the protein conformation is altered by heat or chemicals (denaturation).

Hydrophobic interactions are not only important for stabilizing protein conformation, they are also important for the interactions between flavour compounds and proteins(Wishnia, 1962; Arai et al., 1970; Solms et al., 1973; Beyeler and Solms, 1974; Maier, 1975; King and Solms, 1979; Damodaran and Kinsella, 1981a,b). For example, Damodaran and Kinsella(1981a,b) studied the interaction between 2-nonanone(a very non-polar ligand) and soy protein. Results from their study suggested that hydrophobic interactions were the main forces to bind the 2-nonanone to the soy protein.

2.1.2 Factors Affecting Interactions Between Proteins and Ligands

2.1.2.1 Protein: Type and Concentration

The binding affinity of ligand for a protein is dependent upon the species of protein. For example, the binding constant for 2-nonanone to soy protein was about 930 M^{-1}(Kinsella and Damodaran, 1980) and to BSA was about 1800 M^{-1}(Damodaran and Kinsella, 1980a). This may be due to the different conformation of soy protein and BSA which results in different binding constants.

The concentration of a protein is also a factor which affects the binding affinity of a ligand for the protein.

King and Solms(1979) reported that ligand adsorption increased linearly with increasing protein concentration(0.5 - 5.0%). However, Damodaran and Kinsella (1980a) reported that the binding affinities were much greater at a low protein concentration. They suggested that it might be due to protein-protein interactions at higher concentrations, which would result in the apparent decrease in the protein-ligand interactions. The protein-protein interactions may change the conformation of the protein resulting in a reduction of available binding sites for the ligand.

An important aspect of a protein concerning ligand-protein interaction is its conformation. Conformation of a protein is determined by its amino acid sequence and environment. The forces which determine protein conformation are the same as those discussed for the protein-ligand interactions. Therefore factors which have an impact on protein-ligand interactions will also affect protein conformation. Consequently ligand-protein interactions are complex and factors affecting one system may or may not affect a different system in a similar way. This can only be established by experimentation.

2.1.2.2 Ligand: Type and Concentration

The binding affinity of a ligand to a protein is dependent on the types of ligand. Gremli(1974) reported that the percentage of bound aldehydes in 5% soy protein solutions increased with increased molecular weight. A

similar result was reported by Kinsella and Damodaran(1980). They found that the binding constants in aqueous systems of 2-heptanone, 2-octanone and 2-nonanone to soy protein were 110 M^{-1}, 310 M^{-1} and 930 M^{-1} respectively. This may be due to the more apolar nature of ligands of higher molecular weights.

In addition to the molecular weight, the position of functional groups of ligands in the chain would also affect the binding affinity of the ligand to a protein. Kinsella and Damodaran(1980) reported that when a keto group of a ligand shifted to the center of a chain, the binding constant was decreased. They suggested that this may be due to the steric hindrance of the keto group in the chain of the ligand thereby decreasing binding to the protein.

Beside the molecular weight and the position of functional groups of ligands, the concentration of the ligand also affects the interaction. When high concentrations of ligand are present more will be available for binding. Beyeler and Solms(1974) reported that the amount of simple aldehydes and ketones bound to soy protein and to BSA depended on the concentration of free ligand.

2.1.2.3 Effect of Temperature

Temperature can have two effects on the interactions between proteins and ligands. One is the effect on the binding forces and the other is the effect on the protein and/or ligand molecules.

With increased temperature hydrophobic interactions

will prevail, therefore the binding strength of a protein for a ligand increases provided that the ligand is not in an ionic form. This is because hydrophobic interactions are mainly due to the entropy effect which is more disordered at high temperature than at low temperature(Cantor and Schimmel, 1980a). For electrostatic interactions to occur an ionic form of ligand must be present. Hydrogen bonding is inversely proportional to the change in temperature. Thus, if the ligand is not in an ionic form and the likelihood of hydrogen bonding is reduced because of high temperature then the binding force may only be due to hydrophobic interactions.

Another effect of temperature, on the interactions between proteins and ligands, is heat-induced conformational changes of the ligand or protein molecule. At higher temperature, electrons of the ligand molecule in the outmost might be excited into a higher level. These excited electrons might facilitate conversion of the ligand molecule to the ionic form and thus the binding of protein-ligand may also increase due to the electrostatic effect.

In the case of proteins, temperature can change their structural conformation which might increase or decrease the number of available sites in the protein for ligands. Most of the non-polar or hydrophobic groups of amino acids in the protein tend to be in the inside of the protein(Solms et al.,1973). When temperature is increased, it may unfold the protein and thus expose more non-polar groups to the

solvent. The increase in the exposed non-polar groups to the solvent may increase the binding of non-polar ligands. In this case, hydrophobic interactions would be the main binding force.

There are numerous reports on the effect of the temperature on the protein conformation. Damodaran and Kinsella (1981a) found that the binding affinity for 2-nonanone by soy protein was higher at 5°C than at 25°C and 45°C. At 5°C, the binding affinity was 2 000 M^{-1} and at 25°C and 45°C the binding affinity was 930 M^{-1}. The total number of binding sites was two at 5°C and four at 25°C and 45°C. The authors suggested that at the low temperature a change in the quaternary and tertiary structures of the soy protein occurred. It has been reported that the 11S fraction of soy protein precipitates at low temperature (Saio et al., 1968), but the actual mechanism is still unknown. Damodaran and Kinsella (1981a) speculated that at low temperature the hydrophobic interactions within the protein may be weaker and result in re-organization of the subunits of the protein. This may give different hydrophobic binding sites which have a higher binding affinity for the ligand.

The above phenomenon may be explained using the thermodynamic or energy point of view. The free energy (ΔG) should increase with the temperature, $\Delta G = -RT\ln K$, where K is the equilibrium binding constant. In this case, the ΔG at 5°C is greater (-4.221 Kcal/mol) than at 25°C (-4.045 Kcal/mol). Free energy change can also be expressed as ΔG =

$\Delta H - T\Delta S$. Then a greater $-\Delta G$ at 5°C may be due to either greater negative changes in the ΔH or positive increases in ΔS of the system. Since ΔH decreases with decreased temperature, the greater negative ΔG at 5°C for the interaction can only be related to the positive changes in the ΔS of the system. In other words, at low temperatures the structural rearrangement of the subunits in the soy protein is from a more ordered state to a less ordered state. These changes may give new binding sites which have a higher binding affinity for the ligand (Damodaran and Kinsella, 1981a).

On other hand, at high temperature, protein can be denatured thereby exposing more binding sites for ligands. Arai et al. (1970) found that more new binding sites of soy proteins became available to 1-hexanal under drastic heat treatment of the protein. Kinsella and Damodaran (1980) reported that the binding affinity of the partially heat-denatured soy protein was higher than that of the native soy protein. However, there were no new binding sites for 2-nonanone when partially denatured soy protein was used. Kinsella and Damodaran (1980) concluded that there were no major changes in the secondary structure of the protein, but there were changes in the tertiary and quaternary structure which increased the binding affinity of the existing binding sites for the ligand.

2.1.2.4 Effect of pH

Very few flavour compounds are ionic compounds and

therefore it is unlikely that electrostatic interactions should play a major role in flavour-protein interactions. Electrostatic interactions would be dependent on pH as pH will determine the number of positively and negatively charged groups on a protein molecule. For other types of interactions such as hydrogen bonding or hydrophobic interactions, the effect of pH changes on the interactions will be reflected mainly in its effects on the conformation of the protein involved. Mohammadzadeh-k et al.(1969) used this to explain the results of their studies on the effect of pH on the binding of heptane with lysozyme and α-chymotrypsin. They found the binding increased in the acidic range for lysozyme and in the alkaline range for α-chymotrypsin.

On the other hand, Beyeler and Solms(1974) reported that the binding of flavour compounds to soy protein was independent of pH. The pH values of their systems were 3.0, 4.5 and 7.0. However, it has been reported that soy protein has minimum solubility at about pH 4.5(Burrows et al.,1972). This means that at pH 3 and 7 soy protein should have a higher solubility than at pH 4.5 and thus its binding properties should be different at pH 3,4.5 and 7. It is not known whether the soy protein isolate used in the study of Beyeler and Solms(1974) was denatured. If it was then their results would not be surprising since changes in pH should have little effect on the conformation of denatured protein.

2.1.2.5 Effect of Ionic Strength

Ionic strength can affect the structure of proteins and ligands and consequently will also affect the force of interaction between proteins and ligands. The conformation of proteins can be altered by the concentration and nature of ions present in a system. These conformational changes could also change the number of available binding sites for ligands. For example, Mohammadzadeh-k et al. (1969) showed the effect of ionic strength using KCl solutions on the binding of heptane to β-lactoglobulin. The binding strength increased with increasing ionic strength up to 0.25 M KCl and then the binding strength remained unchanged. An explanation for this observation may be that increasing salt concentration or ionic strength resulted in disruption of the native structure of the protein. Consequently more binding sites were exposed and became available to the ligands. It is possible that when the salt concentration reached a level which precipitated the protein or caused a salting-out effect the total number of available binding sites increased but because some protein-protein interactions occurred the net number of binding sites available for ligands remained constant.

In addition, different species of salts at the same concentration have different effects on interactions. Kinsella and Damodaran (1981) studied several different salt species (SO^{-2}, Cl^-, SCN^-, and $ClCOO^-$) on the binding of 2-nonanone to BSA. They found that addition of SO^{-2} or Cl^-

to the system increased binding and addition of SCN^- or $ClCOO^-$ decreased binding of 2-nonanone to BSA compared to the interaction of 2-nonanone and BSA in water. The authors pointed out that increasing or decreasing effects of these ions followed the order of the Hofmeister series for anions. The Hofmeister series is the order in which anions and cations may be arranged according to their powers of coagulation of an emulsoid, in neutral, acid or alkaline solutions (Grant, 1969).

2.2 Methods of Analysis and Evaluation of Protein-Ligand Interactions

Many different methods have been used to analyse protein-ligand interactions. These methods may be divided into two classes. One of which includes methods relying on direct measurements of bound or free ligand in a system. The second class of methods would rely on measuring free ligand concentration after separation of the free ligand from protein and protein-bound ligand. In both cases determination of free and bound ligand is carried out when an equilibrium is reached between free and bound ligand. Direct measurements are seldom possible and would involve use of spectroscopic methods (UV, fluorescence, others). In the majority of the reported binding studies a separation technique has been employed prior to determination of free or bound ligand level in a system. Equilibrium dialysis is probably the most common technique used to separate the free ligand from the protein-ligand complex in flavour-protein

binding studies (Mohammadzadeh-k et al., 1969; Beyeler and Solms, 1974; Damodaran and Kinsella, 1980a and 1981a,b). However, there are at least two drawbacks of using this technique: (1) the ligand may interact with the membrane on the dialysis cell to form a complex; (2) it requires a long period of time for attainment of equilibrium across the semipermeable membrane (Nichol and Winzor, 1981).

In addition to the equilibrium dialysis method, the gel chromatography technique (Arai et al., 1970 and Dhont, 1975) and the headspace technique (Nawar, 1971; Grelmi, 1974 and Franzen and Kinsella, 1974) have also been used in binding studies. A major disadvantage of using gel chromatography is the relatively large volume of protein-ligand mixture that is required for its use (Nichol and Winzor, 1981). The disadvantage of the headspace technique is that it can only be applied for volatile flavour compounds.

A liquid-liquid partition method has also been used in binding studies. However, only non-polar ligands can be determined in this way because the ligands are extracted by an organic solvent from the aqueous phase (protein-ligand mixture)(Mohammadzadeh-k et al., 1967; Spector et al., 1969 and Damodaran and Kinsella, 1980a).

Determination of the amount of free ligand can also be accomplished using techniques such as gas chromatography, ultraviolet spectroscopy and fluorescence spectroscopy. Data obtained from these binding studies are normally evaluated

by the Klotz plot (1946) and the Scatchard plot (1949). Klotz et al. (1946) applied the law of mass action to develop a simplified equation for calculating the number of binding sites per mole of protein and binding constants for the protein-ligand interaction. Klotz defined "P" as the number of protein molecules in solution, "L" as the number of free ligands and "n" as the number of binding sites per mole of protein molecules. Then a binding equilibrium equation of the ligand and the binding sites on the protein can be expressed as:

$$P + L \underset{}{\overset{K_1}{\rightleftharpoons}} PL_1$$

$$PL_1 + L \overset{K_2}{\rightleftharpoons} PL_2$$

$$PL_{i-1} + L \overset{K_i}{\rightleftharpoons} PL_i$$

$$PL_{n-1} + L \overset{K_n}{\rightleftharpoons} PL_n$$

Then the chemical equilibrium constants can be given by the equations

$$\frac{[PL_1]}{[P]\,[L]} = K_1 \qquad \frac{[PL_2]}{[PL_1]\,[L]} = K_2$$

$$\frac{[PL_i]}{[PL_{i-1}]\,[L]} = K_i \qquad \frac{[PL_n]}{[PL_{n-1}]\,[L]} = K_n$$

Klotz made three assumptions in the derivation of a simplified equation for the binding constant. They were:

(i) a bound ligand exerts no electrostatic effect;

(ii) each ligand is bound to the same kind of groups on the protein; and

(iii) if a linear relationship is obtained on the plot, statistical factors are predominant.

Klotz took the ratio of the moles of bound ligand to the total moles of protein and using established statistical techniques only proposed a simplified equation for determining the binding sites of a given protein and ligands. The Klotz equation is:

$$1/V = 1/n + 1/(nKCf),$$

where V is the number of moles of bound-ligand per mole of protein, Cf is the molar concentration of the free ligand, n is the overall number of binding sites per mole of protein and K is the overall binding constant.

Scatchard (1949) developed a plotting technique based on the Klotz plot (1946) in order to determine the intrinsic binding constants and the number of binding sites on a protein. The Scatchard equation is:

$$V/Cf = K(n-V)$$

This equation is a double reciprocal of the Klotz equation. If K is a constant, then a plot of V/Cf vs. V would give a straight line. Furthermore, the Scatchard plot can also give the second intrinsic binding constant if a curvature of the line is obtained from the plot. The Scatchard plot can also

indicate the type of the cooperativity of binding. Positive cooperativity is indicated by the upward convexity and negative cooperativity is indicated by the upward concavity of the Scatchard plot (Damodaran and Kinsella, 1980b). Both plots, Klotz and Scatchard, were based on an ideal system or in other words very dilute systems.

2.3 Sensory Study of the Flavour-Protein Interactions

There are numerous chemical studies on the interaction between proteins and flavour compounds. However, very few studies have been carried out to determine the effects of binding on sensory perception. Wang et al.(1975) applied a sensory assay to study the thresholds of four polyamides (putrescine, cadaverine, spermidine and spermine) in 2 % soybean flour dispersions and water. They found that the thresholds of putrescine and spermidine were five to ten times higher in the flour dispersions than in water. The authors thought that the soybean flour had a masking effect on the odour of polyamines.

There is a lack of information about the effects of binding on the perception of flavour. There is a need to understand the effect of flavour-protein interaction on the perception of flavour in order to design adequate processes for preparation of protein isolates free from off-flavours and to facilitate successful flavouring of food products containing protein materials.

3. MATERIALS AND METHODS

3.1 Materials

The fababean(*Vicia faba* L. var. *minor*, cv. Diana, 1978 crop) protein concentrate used in this study was supplied by the Glenlea Research Farm, University of Manitoba. The fababeans were dehulled, pin-milled and then air-classified into fababean protein concentrate and a starch-rich fraction.

Methanol was used as a solvent in high performance liquid chromatographic analysis and was of HPLC grade from Fisher Scientific Ltd.. The m-Vanillin used as a ligand in these studies was purchased from Sigma Chemical Company. Some of the physical and chemical properties, as well as information concerning its use in food products, are compiled in Appendix I(Furia and Bellanca, 1975). All other chemicals used in this study were of reagent grade or better.

3.2 Methods

3.2.1 Preparation of Fababean Protein Micellar Mass(PMM)

A fababean protein isolate called protein micellar mass(PMM) was prepared according to the procedure outlined by Murray *et al.*(1981). Fababean protein concentrate(120 g) was added to 600 mL of 0.3 M NaCl solution and this was stirred for 30 minutes at room temperature. The pH of the mixture was 6.1 and was not adjusted. The mixture was then

centrifuged(Sorvall RC2-B) at 8,000 xg for 15 minutes. The supernatant was decanted and distilled water was added at a ratio of 1:3(supernatant:distilled water). The mixture was held for 30 minutes during which the protein precipitated. The slurry was then centrifuged at 4,000 xg for 15 minutes and the wet precipitate(PMM) was collected. The wet PMM was resuspended in a small amount of water approximately the same volume as the wet PMM. The purpose of this washing step was to reduce the NaCl content of the PMM to a level less than 1.0%. The washed PMM was collected, freeze-dried and stored at -20°C.

3.2.2 Proximate Analyses of PMM

Proximate analyses of the freeze-dried PMM were carried out according to AOAC methods(1975). The analyses included moisture, micro-Kjeldahl, ash and crude fat determinations.

3.2.3 NaCl Determination of PMM

An Orion Chloride electrode(model 96-17 combination) and an Orion 701A digital pH/mV meter were used to measure NaCl level. The known addition method was used to determine Cl^- ion concentration (Anonymous, 1977).

3.2.4 Amino Acid Analyses of PMM

The amino acid analyses were carried out on a Beckman 119C Amino Acid Analyser, using the standard hydrolysis procedure(6 N HCl; vacuum; 24 hours; 110°C) at the Plant Science Department, University of Manitoba. The Beckman modification of the single column procedure of Spackman et

al.(1958) was applied. Tryptophan was determined according to the method of Messino and Musarra(1972). Cysteine and methionine were determined according to the method of Hirs(1967)[1].

3.2.5 Denaturation of PMM by Heat Treatments

Denaturation of protein is usually associated with conformational changes. Reports in the literature indicate that binding ability of proteins also depends on protein conformation. The three heat treatments on native PMM carried out in this study were designed to generate partially and completely denaturated PMM. For this purpose, PMM slurries of 10%(W/W) in distilled water were prepared. The slurries were then placed in water baths at temperatures of 81.0±0.5°C, 84.0±0.5°C and 95.0±0.5°C. When the slurry reached the desired temperature, it was held at that temperature for 15 minutes. The slurries were then removed from the water bath, poured into aluminum plates and allowed to cool to room temperature. Subsequently, the samples were frozen and freeze-dried. The freeze-dried PMM samples(native and heat-treated) were ground with a CRC micro-mill to pass through a 150 μm sieve and then stored at -20°C.

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(1) These analyses were performed by Mr. P. Mills, Department of Animal Science, University of Manitoba.

3.2.6 Determination of Overall Thermal Transition(ΔH) and Temperature of Denaturation(Td) of PMM Samples by Differential Scanning Calorimetry(DSC)

Differential scanning calorimetry was used to determine the overall thermal transition and temperature of denaturation of the native and heat-treated PMM samples. The Td and ΔH can be used as indicators of the degree of protein denaturation(Arntfield and Murray, 1981). A Dupont model 990 Thermal Analyser with a model 910 DSC cell base modified to give a maximum sensitivity of 0.005 $mcal.s^{-1}.in^{-1}$ was used following the procedure outlined by Arntfield and Murray(1981). Temperature calibration and calibration coefficient E for the DSC cell were determined using weighed samples of indium and sapphire over a scanning range of 25-200°C. All samples were scanned at a heating rate of 5°C/min using instrument sensitivities of 0.02, 0.01 and 0.005 $mcal.s^{-1}.in^{-1}$. The reference pan contained sufficient sand to approximate the heat capacity of the sample. The cell was flushed with nitrogen at a rate of 30 $mL.min^{-1}$ for all runs. Peaks in a downward direction were obtained for most scans indicating an endothermic heat flow. To obtain peak area, a baseline was constructed as a single straight line from the beginning to the end of the endotherm and the area obtained using a planimeter. To calculate the enthalpy of the reaction(ΔH), generally accepted as the enthalpy of denaturation plus the enthalpy associated with any aggregation of the protein, the following equation was used:

$$\Delta H = (A/MCP)(60BE\Delta qs)$$

where ΔH is the enthalpy of the reaction($mcal.mg^{-1}$), A is peak area (in^{-2}), M is sample mass(mg), C is sample concentration(%W/W), P is protein concentration of sample(%), B is time base ($min.in^{-1}$), E is cell calibration coefficient and Δqs is Y axis range($mcal.s^{-1}.in^{-1}$).

The temperature was monitored throughout the scan using the second pen in the DSC recorder. The mV reading obtained was converted to °C using thermocouple tables supplied with the instrument. This allowed for easy identification of the denaturation temperature of each peak.

3.2.7 Binding Studies with Vanillin and PMM

The binding studies included three sets of experiments as listed in Table 1. These experiments were planned to determine the effect of protein conformation, protein concentration and salt conditions on vanillin-PMM binding. For these experiments, appropriate amounts of PMM were weighed into 25 mL Erlenmeyer flasks. Water or vanillin solutions were then added to the PMM and stirred until dispersed. The total volume of the slurries was 20 mL leaving approximately 10 mL of headspace in the flask. The pH of the slurries was measured and recorded and the flasks were then sealed with parafilm. The sealed flasks were shaken on a Rotary shaker(Fermentation Design Inc.) at 200 rpm for 16±1 h at 5±1°C. The selection of 16 h for the time was based on a time study in which it was found that equilibrium conditions between bound and free vanillin were

Table 1 Binding Studies : Composition of Systems

Experiments	Composition of Systems	
1	Protein	: Native PMM, heat-treated PMM [1]
	Protein Concentration	: 5 %
	Vanillin Concentration	: 0, 300, 350, 400, 500, 600, 900, 1200, 2400 ppm
	Medium	: H_2O
2	Protein	: Native PMM
	Protein Concentration	: 1, 2.5, 5, 10 %
	Vanillin Concentration	: 0, 300, 350, 400, 500, 600, 900, 1200, 2400 ppm
	Medium	: H_2O
3	Protein	: Native PMM
	Protein Concentration	: 5 %
	Vanillin Concentration	: 0, 300, 350, 400, 500, 600, 900, 1200, 2400 ppm
	Media	: 0.03 and 0.3 M of each of Tris-HCl and NaCl

1) Heat-treated PMM at 81°C, 84°C and 95°C.

established after 16 h. This reaction time fell within the range of shaking times used in binding studies reported by other researchers (King and Solms, 1979; Damodaran and Kinsella, 1981a).

After shaking, the slurries were centrifuged at 12,000 x g. The supernatant was withdrawn from the centrifuge tube and transferred into the micro-partition system(Amicon, MPS-1). The MPS-1 system is shown in Figure 1. Amicon YMT membranes were used with a molecular weight cut off of about 800(Anonymous, 1982). The system was centrifuged at 1,800 x g for 30 minutes at 4°C. Then the vanillin concentration in the resulting ultrafiltrate was determined. The components of the MPS-1 were re-used throughout the studies except for the YMT membranes, which were used only once and then discarded. After each run, the components of the MPS-1 ensemble were washed with soapy water, rinsed with distilled water then rinsed with ethanol and finally rinsed with distilled water.

3.2.8 Determination of Vanillin by High Performance Liquid Chromatography(HPLC)

Vanillin was analysed according to the method outlined by Alfonso et al.(1980) using a Waters Associates HPLC system. It consisted of a high-pressure pump(Waters Associates 600), a 2.0 mL sample loop injector(Waters Associates U6K), a UV detector(Waters Associates 440) set at 280 nm, an integrator (Hewlett Packard 3390 A) and a RP-18 column [4 mm (I.D.)x250 mm; E-Merck packed with Lichrosorb

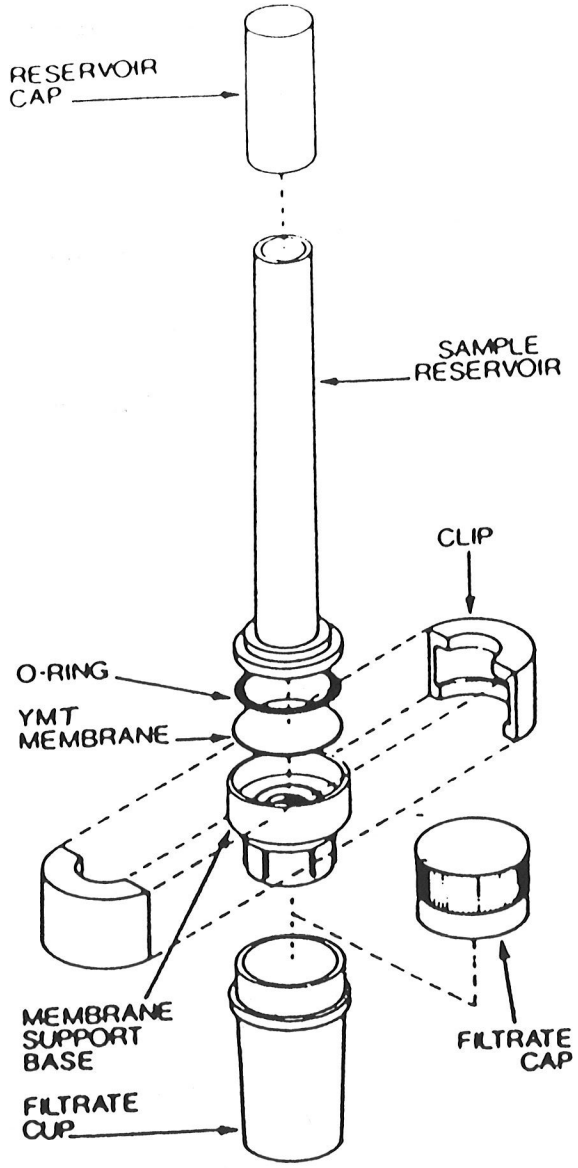

Figure 1 Amicon Micro-Partition System (MPS-1)

10 µm size]. Methanol in water (60 % V/V) was used as a solvent at a flow rate of 1.5 mL/min.. A standard sample size of 10 uL was used throughout the study using a SGE 25 µL syringe.

External standards were used to quantitatively determine vanillin concentrations in the samples. Peak areas generated by a series of vanillin solutions ranging in concentrations from 10 ppm to 2,400 ppm were plotted against their corresponding concentrations. This response was not linear over the entire concentration range. However, a linear calibration curve was obtained for the concentrations ranging from 50-200 ppm. Samples were therefore diluted with distilled water when their vanillin level exceeded this concentration range. Solutions containing 50, 100 and 200 ppm of vanillin were used as check solutions which were stored at -20°C until required. Each day a check solution was analysed and used to determine a daily correction factor(CF). The CF was determined as follows:

$$CF = A/B$$

where: A= original area for a known concentration from the standard curve;

B= check sample area for a known concentration for that particular day.

The CF increased with age of the column from 1.0 to 1.6. This indicated that vanillin interacted with the column.

3.2.9 Analysis of Binding Studies Data

The level of free vanillin(Cf) in the vanillin-PMM systems was determined by HPLC. Bound vanillin(Cb; molarity) was taken as the difference between the total amount of vanillin(Co) added to a system and the free vanillin concentration(Cf). Assuming that the average molecular weight of PMM proteins was 240,000 d, then the number of moles of vanillin bound per mole of protein(V) was equal to the bound vanillin(Cb) per molar concentration of PMM in a given vanillin-protein system.

Determination of the number of binding sites per protein molecule(n) and the binding constants(K) between vanillin and PMM proteins was carried out using the procedure outlined by Klotz et al.(1946). According to the Klotz equation

$$1/V = 1/n + 1/(nKCf)$$

a plot of 1/V vs. 1/Cf will be linear if n and K are constants; these constants can be determined from the intercept(1/n) and the slope(1/nK) of a Klotz plot. Based on the binding constant(K), the free energy(ΔG) of the binding was calculated according to Damodaran and Kinsella(1981a) using the following equation:

$$\Delta G = -RT\ln K$$

where R is the universal gas constant and T is the absolute temperature. Binding data were also analysed according to Scatchard(1949) by plotting V/Cf vs. V. Based on the Scatchard equation

$$V/Cf = K(n - V),$$

the number of binding sites(n) per protein and the binding constant(K) can be determined. However, if there are multiple classes of binding sites, then Scatchard plots will be curved. Curvature of Scatchard plots indicated whether the interaction between the different classes of binding sites were positively or negatively cooperative(Cantor and Schimmel, 1980b).

3.2.10 Sensory Studies

3.2.10.1 Preliminary Study and Panel Training

A nine member panel consisting of six females and three males was selected to participate in the sensory study on the basis of the members' interest in the project and their previous experience in sensory evaluation. All selected panelists were graduate students or staff members of the Department of Food Science and ranged in age from 20-50.

The purpose of the preliminary study was(1) to find a suitable technique for sensory assessment of vanillin perception in vanillin-PMM systems,(2) to determine a suitable range of vanillin concentrations for the study and(3) to acquaint the panelists with some of the flavour sensations they might encounter while sniffing or tasting vanillin-PMM slurries. The preliminary study included a total of eight sessions. The number of samples tested and the composition of the samples are in Table 2. In all cases panelists were asked to use the method of magnitude

Table 2 Preliminary Sensory Study: Composition of Samples and Sensory Technique for Evaluation

Session	Composition of Samples	Number of Samples	Sensory Technique
1	Vanillin solutions Vanillin concentration: 20, 40, 80, 100, 200, 400 ppm Reference: 80 ppm (assigned score = 10)	6	Odour evaluation sniffing (Fig. 2)
2	Vanillin-PMM slurries Vanillin concentrations: 100, 200, 300 ppm Protein: 10 % native PMM References: 100 ppm (assigned score = 10); 10 % native PMM slurry in H_2O	3	Odour evaluation sniffing (Fig. 3)
3	Vanillin solutions Vanillin concentration: 100, 200, 400, 800, 1600, 3200 ppm Reference: 400 ppm (assigned score = 10)	6	Odour evaluation sniffing (Fig. 2)
4	Vanillin solutions Vanillin concentrations: 100, 200, 400, 800, 1600, 3200 ppm Reference: 400 ppm (assigned score = 20)	6	Flavour evaluation by mouth (Fig. 4)

Table 2 (cont'd.)

Session	Composition of Samples	Number of Samples	Sensory Technique
5	Vanillin-PMM slurries Vanillin concentrations: 300, 600, 1200, 2400 ppm Protein: 5 % native PMM References: 600 ppm (assigned score = 20); 5 % native PMM slurry in H_2O	4	Flavour evaluation by mouth (Fig. 5)
6	Vanillin-PMM slurries Vanillin concentration: 1200 ppm Protein: 5 % native PMM and heat-treated PMM at 81°C, 84°C and 95°C References: 600 ppm (assigned score = 20); 5 % native PMM slurry in H_2O	4	Flavour evaluation by mouth (Fig. 5)
7	Protein slurries Protein: 5 % native PMM and heat-treated PMM at 81°C, 84°C and 95°C in H_2O Reference: 5 % native PMM in H_2O (Assigned score =20)	4	Overall flavour evaluation by mouth (Fig. 6)
8	Vanillin-PMM slurries Vanillin concentration: 1200 ppm Protein: 5 % native PMM and heat-treated PMM at 81°C, 84°C and 95°C Reference: 5 % native PMM in H_2O (Assigned score =20)	4	Overall flavour evaluation by mouth (Fig. 6)

estimation(Moskowitz,1983). In addition, the ballots used during these sessions(Figures 2-6) contained instructions on how the panelists were to perform their tasks. The sensory technique included odour evaluation(sniffing, Figs. 2 and 3) or flavour evaluation(flavour-by-mouth, Figs. 4,5 and 6). The vanillin-PMM slurries were prepared as they were for the binding studies as outlined in section 3.2.7 without passing through the MPS-1 system.

For odour evaluation, 7.4 mL of samples(slurries after shaking for 16±1 h) were placed into small vials(25x57 mm) and closed with caps. The samples were kept in the closed vials for at least two hours before being presented to the panelists. The sensory evaluation was carried out in an air-conditioned laboratory, where the samples were kept in a fume hood. Each panelist was asked to perform the sensory evaluation at a scheduled time since only one panelist was able to evaluate the samples at any given time.

All flavour-by-mouth sessions were held in a sensory panel room with individual booths. Samples of 15 mL were served in 20 mL opaque plastic cups covered with aluminum foil. Panelists were asked to puncture the foil at the time of testing and to imbibe the samples using a straw(Figs. 4,5 and 6). The panelists were also asked to use a "warm up" technique before evaluating the samples. For this purpose the panelist tasted the reference then cleaned his/her mouth with water and unsalted crackers to remove the flavour and proceeded subsequently with the sensory evaluation by

Figure 2 Ballot for Sensory Evaluation: Odour of Vanillin Solutions

Date _____ Name _____

Magnitude Estimation is a ratio comparison of the relationship between the sensation produced by a standard and a sample. For expamle, if the "comparison" stimulus of a sample appeared to be half as strong as the standard, then you should assign to that "comparison" half of the numerical value of the standard. If the "comparison" stimulus of a sample is twice as strong as the standard, then you should assign to that "comparison" twice of the numerical value of the standard.

Instructions:

Your task is to evaluate each sample according to the order on your sheet and assign any value to it relative to the standard. For each evaluation (both samples and standard): first, swirl the container, then open the cap, take 3 short sniffs, and close the Container Immediately. Please take a short pause between samples. You may sniff the standard again if you forget its stimulus. If you cannot smell anything, use "NP" to denote not present. (Please Do Not Use Zero.)

SAMPLES	MAGNITUDE ESTIMATE
REFERENCE (R)	10
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

Comments:

Figure 3 Ballot for Sensory Evaluation: Odour of Vanillin-PMM Slurries

Date _____ Name _____

Your task is to evaluate each sample according to the order on your sheet and assign any value to it relative to the Odour Reference (OR). Use the Protein Reference (PR) only to help you ignore any protein odour in the samples. For each evaluation (both samples and references): first, swirl the container, then open the cap, take 3 short sniffs, and close the Container Immediately. Please take a short pause between samples. You may sniff the reference again if you forget its stimulus. If you cannot smell anything, use "NP" to denote not present. (Please Do Not Use Zero.)

SAMPLES	MAGNITUDE ESTIMATE
Odour Reference (OR)	10
Protein Reference (PR)	

Comments:

Figure 4 Ballot for Sensory Evaluation: Flavour of Vanillin Solutions

Name _____ Date _____

Your task is to evaluate vanilla flavour by mouth in each sample according to the order on your sheet and assign any value to it relative to the Reference (R). For each evaluation: use a straw to stab through the aluminum foil which cover s the cup and stir the sample before sucking it ; take the same amount for each sample and reference; spit out the sample and rinse your mouth with water and cracker between samples. Do not swallow samples.

SAMPLES	MAGNITUDE ESTIMATE
REFERENCE (R)	20
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

Comments:

Figure 5 Ballot for Sensory Evaluation: Flavour of Vanillin-PMM Slurries

Name _____ Date _____

Your task is to evaluate vanilla flavour by mouth in each sample according to the order on your sheet and assign any value to it relative to the Vanilla Reference (VR). Use the Protein Reference (PR) only to help you ignore any protein flavour in the samples. For each evaluation (both samples and reference): use a straw to stab through the aluminum foil which cover s the cup and stir the sample before sucking it; take the same amount for each sample and reference; spit out the sample and rinse your mouth with water and cracker between samples. Do not swallow samples. You may try the reference again if you forget its flavour intensity. If you cannot detect any vanilla flavour, use 'NP' to denote not present. (Please Do Not Use Zero.)

SAMPLES	MAGNITUDE ESTIMATE
Vanilla Reference (VR)	20
Protein Reference (PR)	---------
_____	_____
_____	_____
_____	_____
_____	_____

Comments:

Figure 6 Ballot for Sensory Evaluation: Overall Flavour

Name _____ Date _____

Your task is to evaluate the overall flavour intensity in each sample according to the order on your sheet and assign any value to it relative to the Reference (R). For each evaluation (both samples and reference): use a straw to stab through the aluminum foil which cover s the cup and stir the sample before sucking it; take the same amount for each sample and reference; spit out the sample and rinse your mouth with water and cracker between samples. Do not swallow samples. You may try the reference again if you forget its overall flavour intensity.

SAMPLES	MAGNITUDE ESTIMATE
Reference (R)	20
_____	_____
_____	_____
_____	_____
_____	_____

Comments:

tasting the reference again and then the samples.

During the preliminary studies, panelists were encouraged to make comments. Based on the performance of the panel and the comments made by the panelists, it appeared that vanillin flavour was best evaluated using the flavour-by-mouth technique with the vanillin concentration ranging from 150-2400 ppm. These vanillin levels coincided approximately with those used for flavouring of foods(Appendix I). The panel did not find any significant difference between the overall flavour of native PMM and that of heat-treated PMM(session 7). For this reason, it was decided to use native PMM as a protein reference in the further sessions if a protein reference was required.

Four training sessions were held after the preliminary study. The purpose of these training sessions was to further familiarize the panel with the procedure used and also give panel members greater experience with vanillin flavour. Four sessions were held and the composition of the samples which were presented at each session are shown in Table 3. A discussion session was held with the panel after the first three sessions had been completed. The results of all the preliminary sessions and of the first three training sessions were presented to the panel. The purpose of this discussion was to motivate the panelists by showing them that their performance had improved. The fourth training session was held in a classroom. After the panelists had evaluated the samples, the magnitude estimates were recorded

Table 3 Training Sessions: Composition of Samples and Sensory Technique for Evaluation

Session	Composition of Samples	Number of Samples	Sensory Technique
1 and 2	Vanillin solutions Vanillin concentration: 150, 300, 600, 1200, 2400, 4800 ppm Reference: 600 ppm (assigned score = 20)	6	Flavour evaluation by mouth (Fig. 4)
3 and 4	Vanillin-PMM slurries Vanillin concentration: 1200 ppm Protein: 5 % native PMM and heat-treated PMM at 81°C, 84°C and 95°C References: 600 ppm (assigned score = 20); 5 % native PMM slurry in H_2O	4	Flavour evaluation by mouth (Fig. 5)

on the blackboard. The results were then discussed by the panel, and panelists had the opportunity to re-taste the samples during or after the discussion. It was also agreed at this time that during the main sensory study panelists were to use "NP"(not present) for samples in which they could not detect vanillin flavour.

3.2.10.2 Main Sensory Study

The main sensory study included twelve sessions. The sequence of sessions and the composition as well as the number of samples presented is shown in Table 4. The ballots used during this study are presented in Figs. 4 and 5. Each set of samples was presented to panelists in a randomized order.

3.2.10.3 Analysis of Sensory Data

3.2.10.3.1 Treatment of Not Present(NP) Scores and Standardization of Magnitude Estimation Data

Panelists were not always able to detect the vanillin flavour in vanillin-PMM samples. Then a "NP" was used to denote that the vanillin flavour was not present. Less than 7% of the all the judgements were "NPs". Panelists had been asked during the training sessions to state a value that they considered to be "NP". The panel agreed that a value of 0.01 would be appropriate and thus "NP's" were replaced by 0.01 before data were analyzed. Furthermore the data were standardized prior to statistical analysis (Malcolmson, 1978).

Table 4 Main Sensory Study: Sequence of Sessions and Composition of Samples [1]

Sequence of Sessions	Composition of Samples	Number of Samples
1 and 8	Vanillin-PMM slurries Vanillin concentration: 300, 600, 1200 and 2400 ppm Protein : 5% PMM native	4
2 and 6	Vanillin-PMM slurries Vanillin concentration: 300, 600, 1200 and 2400 ppm Protein: 5 % PMM heat-treated at 84°C	4
3 and 9	Vanillin-PMM slurries Vanillin concentration: 300, 600, 1200 and 2400 ppm Protein: 5 % PMM heat-treated at 95°C	4
4 and 7	Vanillin-PMM slurries Vanillin concentration: 300, 600, 1200 and 2400 ppm Protein: 5 % PMM heat-treated at 81°C	4
5 and 10	Vanillin solutions Vanillin concentration: 150, 300, 600, 1200, 2400, and 4800 ppm	6

Table 4 (cont'd.)

Sequence of Sessions	Composition of Samples	Number of Samples
11 and 12	Vanillin-PMM slurries Vanillin concentration: 1200 ppm Protein: 1, 2.5, 5 and 10 % PMM native	4

1) In all sessions, 600 ppm vanillin solution and 5 % PMM native in H_2O (except sessions 5 and 10), were given to panelists as references.

3.2.10.3.2 Tests of Homogeneity and Replacement of Odd Data

In sensory evaluations panelists can be expected to generate some odd data. An indication of this was the fact that some "NP's" were assigned to samples which contained high levels of vanillin. One of the assumptions in the analysis of variance is that the errors are normally and independently distributed with a common variance. Thus prior to submitting data for analysis of variance, homogeneity of variance was tested. The Burr Foster Q-test(Anderson and McLean, 1974) was used to test data and to reject odd data($P<0.01$). The missing data technique described by Snedecor and Cochran(1971) was then applied to estimate substitute values for rejected ones.

3.2.10.3.3 Power Functions

Data of the two replicates(session 5 and 10) were combined to establish the power function for vanillin in water(Moskowitz, 1983). The power function($S=KC^n$) was generated based on the linear function $\log S = \log K + n\log C$ where S was the sensory estimate of vanillin flavour and C the vanillin concentration. Based on the power function, magnitude estimates for vanillin flavour in vanillin-PMM systems were converted to "perceived" vanillin concentration(Malcolmson and McDaniel, 1980).

3.2.10.3.4 Analysis of Variance

The data of the two replicates for the power function were transformed to normal logarithms and the transformed

data analyzed using analysis of variance. Other data from the sensory analysis were analysed by a two-way or three-way analysis of variance to determine significant differences attributable to protein(native and heat-treated), level of vanillin, and panelists. Differences between individual means were further tested using the Duncan Multiple range test at the 5% level of confidence.

4. RESULTS AND DISCUSSION

4.1 Characterization of Fababean PMM

The PMM preparation used in this study contained 90% protein, as is shown in Table 5. The protein content was calculated based on the Kjeldahl nitrogen level using the factor 5.85. Moisture, ash and crude fat accounted for another 6.9%, and the remaining 3.1% was unidentified, but was most likely carbohydrates, phytate(Martens, 1982) and other minor components. Murray et al.(1981) reported the chemical composition of fababean PMM. They found slightly higher protein and ash contents in their preparations than were found in the one used in this study. A lower ash content was expected since the PMM was washed once with a small amount of water. The NaCl which is a constituent of the ash was reduced from 1.2% to 0.9% by the washing steps. Lipid material was not detected in fababean PMM by Murray et al.(1981) as shown in Table 5. The PMM preparation used in this study contained 2.47% crude fat as determined by extraction with ether using the Soxhlet apparatus. Further analysis of the ether extract revealed the presence of palmitic, oleic and linoleic acids (Nielsen, 1983) thus confirming that there were lipid-like components present in the PMM preparation.

This PMM preparation was used throughout the entire study to investigate binding of vanillin to PMM. It was expected that the binding of vanillin to PMM was governed by the proteins present in the preparation. However, some of

Table 5

Chemical Composition of Fababean PMM
(% on freeze-dried basis)[1]

		[3]
Moisture	2.28 ± 0.11	–
Protein (N x 5.85)[2]	90.01 ± 1.97	95.6
Ash	2.14 ± 0.03	2.8
NaCl	0.9 ± 1.4×10^{-3}	–
Crude Fat	2.47 ± 0.14	N.D.[4]
Other and analytical error	3.1	1.23

1) Mean of the three determinations

2) 5.85 according to Murray et al.(1981)

3) Data obtained from Murray et al.(1981) were expressed on a dry weight basis(%)

4) Not detected by method used —— A.O.A.C. official methods

the minor constituents such as lipids may have influenced the binding behaviour of the PMM. Franzen and Kinsella(1974), for example, reported that removal of endogenous lipids from leaf protein concentrate reduced binding of aldehydes and methyl ketones. The lipid content of the leaf protein concentrate was 20-30% which was much higher than the level found in the PMM preparation used in this study. King and Solms(1979), on the other hand, found that small amounts of lipid(1 mole of free fatty acid/mole of protein) did not affect binding properties of bovine serum albumin nor that of a soy isolate.

Binding of small molecules to proteins depends on many factors in which the protein source and its composition are properly the major ones. The amino acid composition of the PMM preparation is shown in Table 6. The average hydrophobicity of the PMM was 954 cal/amino acid residue and was calculated according to Bigelow(1967) based on the amino acid data(Table 6). Murray et al.(1981) characterized the proteins of fababean PMM. They found two main species called Q and P which probably correspond to legumin and vicilin, respectively. Species Q has a molecular weight of 140,000 d and consisted of two subunit species of 54,300 d and 86,000 d. The average hydrophobicity of Q was 1023 cal/amino acid residue. The P species had a molecular weight of 340,000 d consisting of three subspecies of 16,200 d, 34,700 d and 54,300 d; its average hydrophobicity is 925 cal/amino acid residue. The average hydrophobicity of the

Table 6

Amino Acid Composition of Fababean PMM

Amino Acids	g amino acid/100 g of protein	Amino Acids	g amino acid/100 g of protein
Lysine	6.18	Alanine	4.34
Histidine	2.85	Cystine	0.86
Ammonia	1.62	Valine	5.46
Arginine	12.63	Methionine	0.83
Aspartic Acid	11.56	Isoleucine	4.94
Threonine	3.22	Leucine	9.34
Serine	4.57	Tyrosine	3.95
Glutamic Acid	20.24	Phenylalanine	5.07
Proline	5.42	Tryptophan	0.95
Glycine	4.30	N recovery (%)	98.22

Average Hydrophobicity of PMM = 954 calories/amino acid residue

PMM preparation used in this study fell within the values given for the P and the Q species(Murray et al., 1981), but it does not coincide with the average of the two values. This may indicate that there was more of the P species than of the Q species in this PMM preparation.

Denaturation of proteins is usually associated with changes in the conformation of proteins. These changes of conformation may be characterized by the overall thermal transition(ΔH) and temperature of denaturation(Td) of the protein. The ΔH and Td of the native and the heat-treated PMM were determined by DSC and are compiled in Table 7. As expected, it was observed that ΔH values decreased with increases in treatment temperature. The ΔH value for PMM heat-treated at 95°C for 15 min was zero and indicated complete denaturation of the PMM proteins. Heat treatments at 81 and 84°C yielded partially denatured proteins. The Td values increased slightly with an increase in treatment temperature. Similar trends, namely the decrease in ΔH and the increase in Td with an increase in heat treatment temperature, were also observed by Arntfield and Murray(1981) in experiments with fababean PMM.

4.2 The Effect of Protein Concentration on the Binding of Vanillin to PMM

The effect of PMM concentration on the binding of vanillin to PMM is depicted in Figure 7. Plotting bound vanillin(Cb) versus total vanillin(Co) yielded straight lines within the investigated range of vanillin levels (0

Table 7

The Overall Thermal Transition (ΔH) and Temperature of Denaturation (Td) for the Native and Heat-treated PMM

	Native PMM	Heat-treated PMM		
		81°C	84°C	95°C
ΔH, Overall Thermal Transition (cal/g of protein	5.43	1.93	0.55	0
Td, Temperature of Denaturation (°C)	91	92	93	-

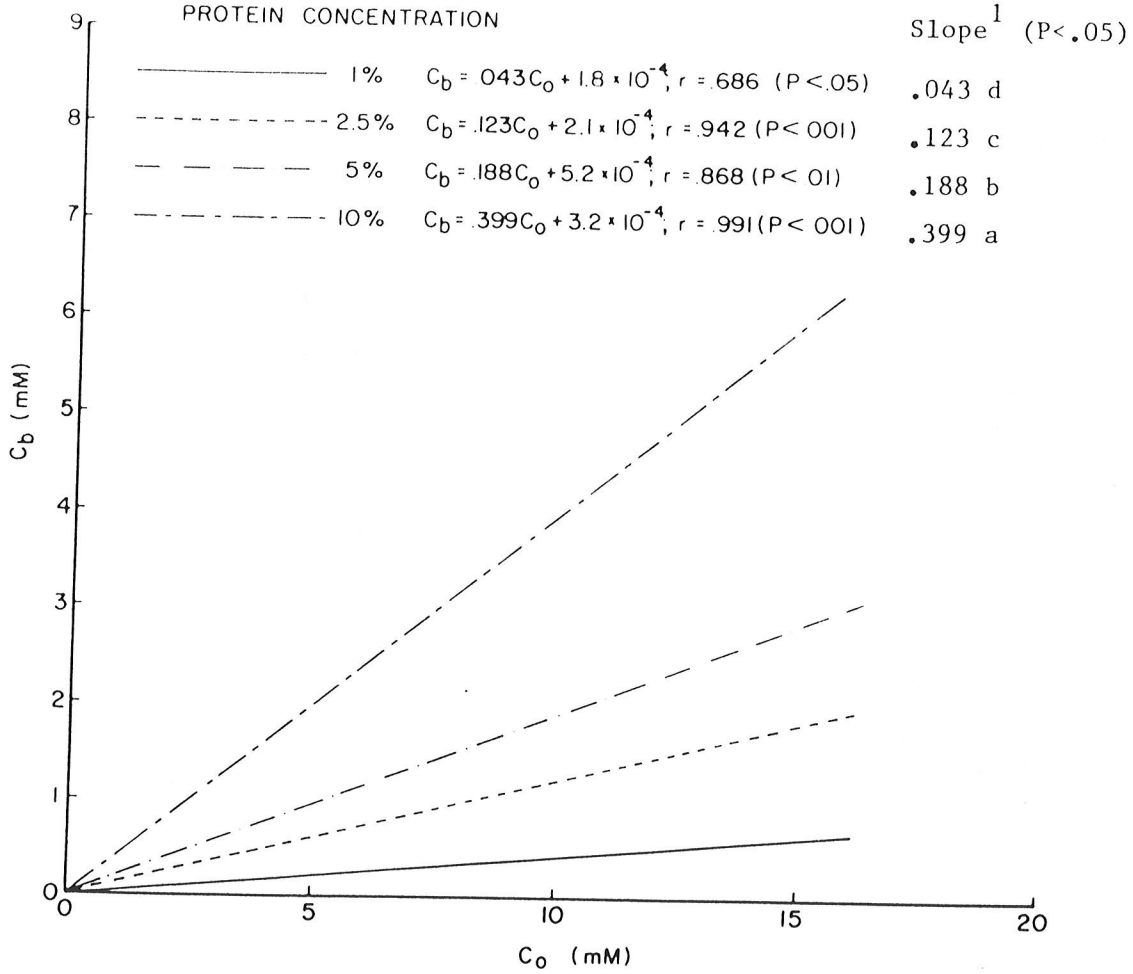

Figure 7 The effect of protein concentration on the binding of vanillin to PMM.
C_o = total vanillin concentration,
C_b = bound vanillin concentration.

1) A test of comparing slopes was carried out according to Steel and Torrie (1980).

ppm to 2400 ppm, or 0 mM to 15.8 mM). Thus the amount of bound vanillin(Cb) appeared to be directly proportional to the total amount of vanillin which was added to the system. The percentage of total vanillin bound to PMM increased with increasing PMM level. The percentage of bound vanillin can be calculated at each PMM level by multiplying the slope of the appropriate line by one hundred. The percentages of bound vanillin were 4.3, 12.3, 18.8 and 39.9% at the 1, 2.5, 5 and 10% level, respectively. Similar trends were reported by King and Solms(1979) who studied binding of benzyl alcohol to a soy isolate. The amount of bound benzyl alcohol was directly proportional to the total amount of benzyl alcohol added to a system and the percentage of total benzyl alcohol bound to protein increased with increasing protein concentration.

Further analyses of the binding data were carried out according to Klotz et al.(1946) to determine the number of binding sites per mole of protein and the binding constant. An example of Klotz plot is shown in Figure 8. The binding data compiled in Table 8 showed that the number of binding sites per mole of protein decreased with increasing PMM concentration. The binding constants, on the other hand, increased with increasing PMM concentration. It appeared that when protein concentration was increased, that possibly protein-protein interaction occurred resulting in a reduction of binding sites available for vanillin. However, the interactions between vanillin and PMM at the remaining

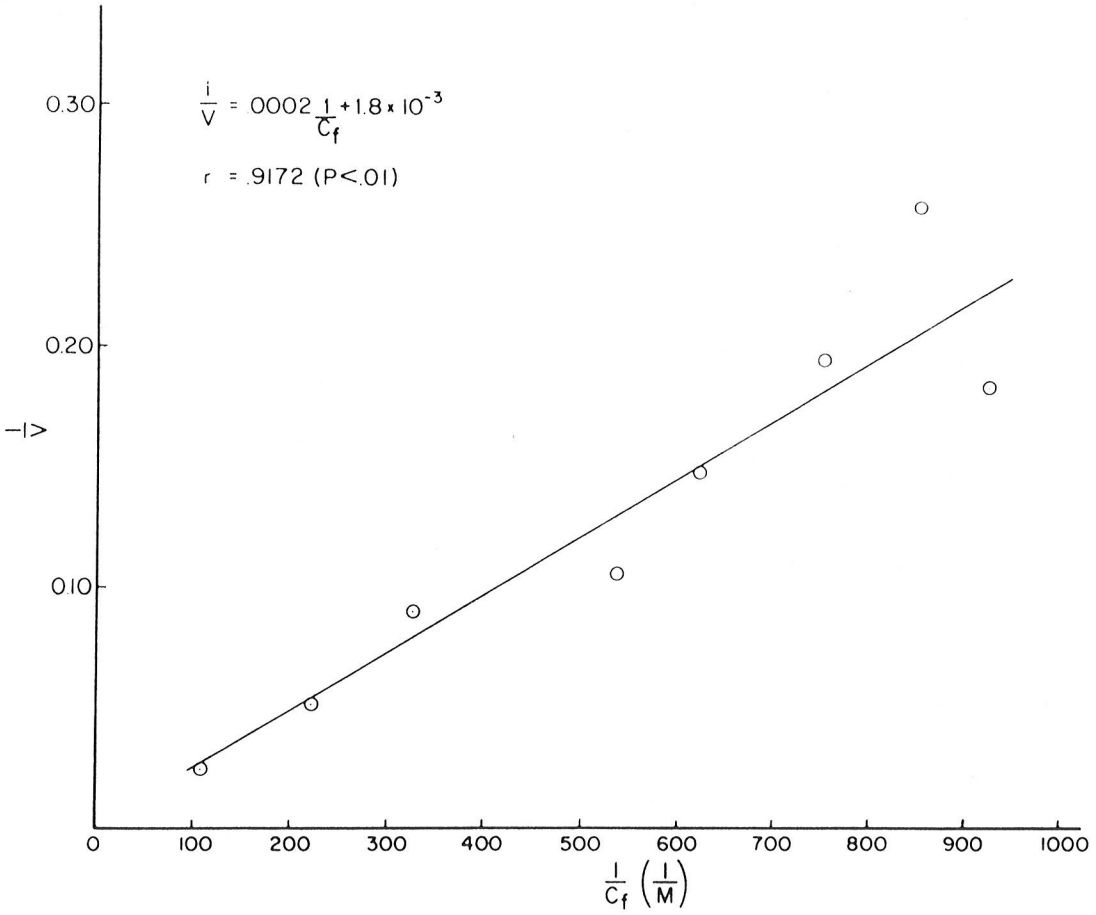

Figure 8 Klotz plot of 5% PMM heat-treated at 95°C with eight levels of vanillin solutions.

Table 8

The Effect of Protein Concentration on Thermodynamic Constants for the Binding of Vanillin to PMM at 25°C and at a pH of 6.3 ± 0.1

	Native protein concentration (% W/W)			
	1.0	2.5	5.0	10.0
N, number of binding sites per mole of protein (MW = 240,000)	203	28	21	12
K, binding constant (M^{-1})	11.67	106.34	182.50	373.80
ΔG, free energy[1] (Kcal/mole)	-1.457	-2.765	-3.085	-3.510

1) $\Delta G = -RT\ln K$

binding sites became much stronger. This was possibly the reason that the percentage of bound vanillin increased proportionally with increasing PMM concentration(Fig. 7). Damodaran and Kinsella(1981a) studied interactions betweeen carbonyls and soy protein. They found that native and partially denatured soy protein possessed four binding sites for carbonyls such as 2-heptanone, 2-octanone, 2-nonanone, 5-nonanone and nonanal. This was based on the assumption that the soy protein had a molecular weight of 100,000. The binding constants for these carbonyls to soy protein ranged from 110 to 1240 M^{-1} whereby an increase of binding constants was observed with increasing chain length of the carbonyls. The authors pointed out that the interactions were relatively weak with free energies ranging from -2.8 to -4.2 Kcal/mol and that these interactions were hydrophobic in nature. The thermodynamic constants for the binding of vanillin to PMM determined in this study were similar to the ones reported by Damodaran and Kinsella(1981a) for binding of carbonyls to soy protein. The free energies of vanillin-PMM interactions ranged from -1.5 to -3.5 Kcal/mol(Table 8). This may be an indication that vanillin-PMM interactions were hydrophobic in nature. Vanillin is a phenolic compound(Appendix I). Polyphenolic (tannins) and phenolic compounds were in the past believed to interact with proteins through the formation of multiple hydrogen bonds between phenolic hydroxyl groups of phenolic and the carbonyl of the peptide linkage of proteins(Van Sumere et

al., 1975). However, recently Oh et al.(1980) demonstrated that hydrophobic interactions between tannins and proteins were favoured when dissolved in hydrophilic or polar solvents. If the solvent were hydrophobic then hydrogen bonding was favoured between tannins and proteins. The solvent used was water and thus based on the findings of Oh et al.(1980), hydrophobic interactions between vanillin and PMM were to be expected.

4.3 The Effect of Changes of Protein Conformation on the Binding of Vanillin to PMM

Effect of changes of protein conformation on vanillin-PMM binding was studied using native PMM and PMM heat-treated for 15 minutes at 81, 84 and 95°C. The degree of denaturation was estimated using DSC by determining ΔH of these samples(Table 9). Binding of vanillin to native and heat-treated PMM was in all cases directly proportional to the total vanillin levels(Co) which were added to the systems as shown in Figure 9. The percentage(slope X 100) of total vanillin bound was 19% for systems containing native PMM and 46, 37 and 49% for systems containing PMM heat-treated at 81, 84, and 95 °C, respectively. Thus the binding capacity of native PMM was significantly lower than that of heat-treated PMM. Statistical analysis of the slopes of the lines(Fig. 9) according to Steel and Torrie(1980) revealed that binding capacity of PMM heat-treated at 84°C was significantly($P<5\%$) lower than that of PMM heat-treated at 81°C and 95°C. The overall trend, that binding capacity

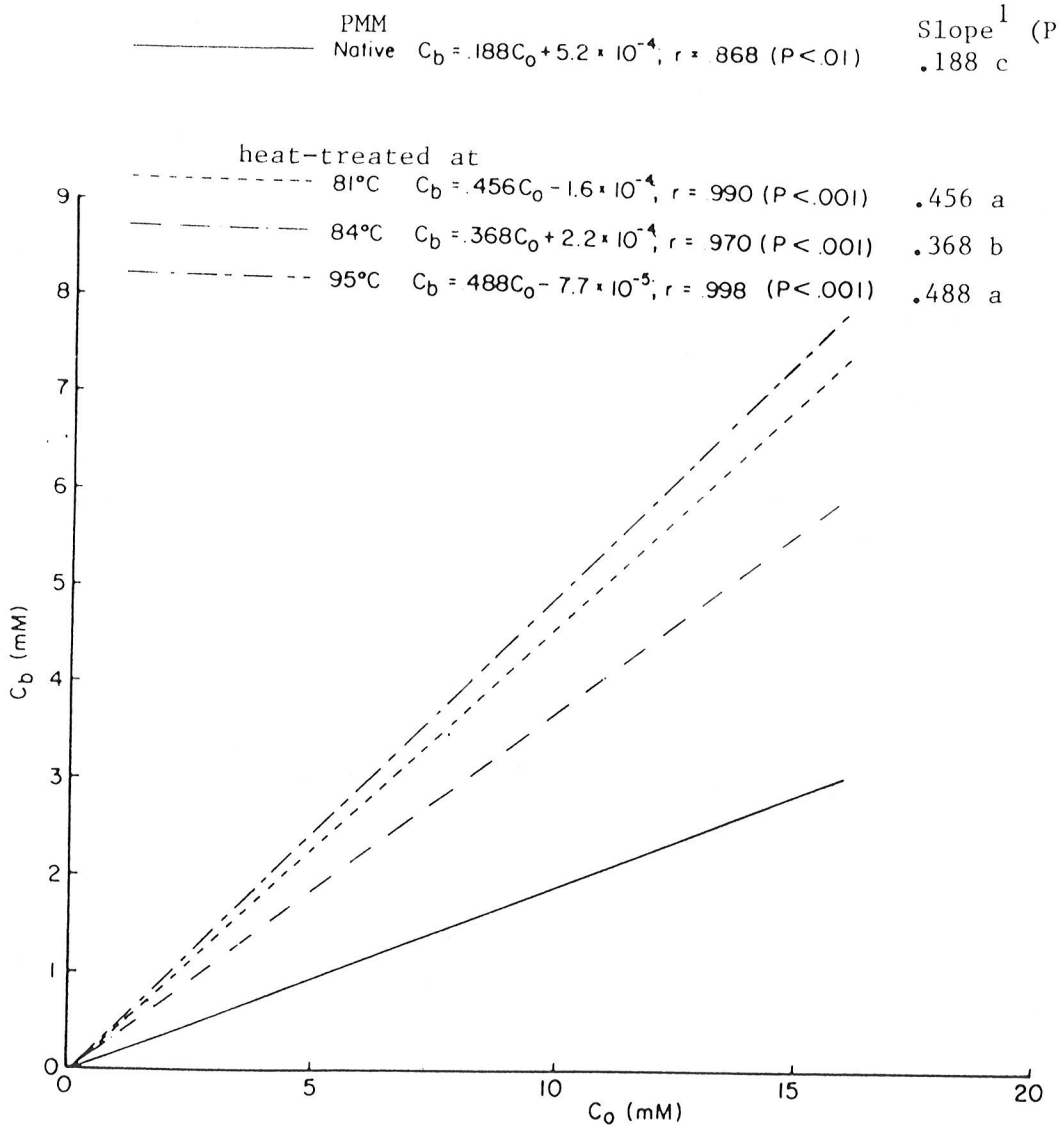

Figure 9 The effect of changes of protein conformation on the binding of Vanillin to PMM. C_o = total vanillin concentration, C_b = bound vanillin concentration. Protein concentration = 5 % (W/W).

1) A test of comparing slopes was carried out according to Steel and Torrie (1980).

of denatured protein was higher than that of native protein, has been observed by many other researchers(Arai et al., 1970; Solms et al., 1973; Demodaran and Kinsella, 1981a).

The thermodynamic constants for the binding of vanillin to PMM derived from Klotz plots are listed in Table 9. With increasing denaturation of PMM, as indicated by the decrease in ΔH, the number of binding sites per mole of protein(240,000 MW) increased. However, the interactions between PMM and vanillin became weaker with increasing denaturation as was indicated by the decreasing binding constants or free energies of interactions. Damodaran and Kinsella(1981a) found that interactions between 2-nonanone and partially denatured soy protein(heated at 90°C for 1 h) were more strongly indicated by a binding constant of 1240 M^{-1} than that involving 2-nonanone and native soy protein with a binding constant of 930 M^{-1}. They also found that the number of binding sites remained four per mole of protein(100,000 MW) in spite of partial heat denaturation. These results seemed to contradict the findings for the vanillin-PMM systems. It should be pointed out that the heat treatment of the soy protein was more severe(90°C for 1 h) than the heat treatments of the PMM samples. Furthermore, Damodaran and Kinsella(1981a) did not attempt to characterize the state or degree of denaturation of their partially heat-denatured soy protein. During denaturation of a protein by heat or by other means, changes in the quaternary and tertiary structure take place. Normally

Table 9

The Effect of Protein Conformation on Thermodynamic Constants for the Binding of Vanillin to PMM at 25°C and at a pH of 6.3 ± 0.1

	Native PMM	Heat-treated PMM		
		81°C	84°C	95°C
Δ H, Overall thermal transition (cal/g of protein)	5.43	1.93	0.55	0.00
Td, Temperature of denaturation (°C)	91	92	93	-
N, Number of binding sites per mole of protein (MW = 240,000)	21	42	307	558
K, Binding Constant (M^{-1})	182.50	94.54	11.81	7.43
ΔG, Free Energy[1] (Kcal/mole)	-3.085	-2.695	-1.462	-1.186

1) $\Delta G = -RT\ln K$

dissociation occurs first as a change in the quaternary structure, followed by unfolding of the subunits or a change in the tertiary structure. In many cases denaturation also results in aggregation of protein molecules(Fleming et al., 1975). The pattern of denaturation depends on the conditions during denaturation of a protein including temperature, time, pH, ionic strength, protein concentration and other factors. It is therefore not surprising that results of heat-denaturation of PMM contradicted the results of soy protein in changes of the binding properties, although the overall effect in both cases was an increase in binding capacity. An experiment which was conducted by the same authors(Damodaran and Kinsella, 1981b) may further illustrate the effect of different conditions during denaturation of a protein on its binding properties. The binding affinity of 2-nonanone to soy protein and the fluorescence behaviour of soy protein in the presence of different concentrations of urea were studied. They found that the binding constants decreased in the presence of urea and that the number of binding sites increased with increasing urea concentration. Furthermore, based on the binding studies and fluorescence behaviour of the soy protein, they suggested that the structural changes in the protein affecting the binding constant involved dissociation of the subunits. This dissociation occurred only at urea concentrations above 5 M and had a minimal effect on the binding constant but resulted in a drastic increase of the

number of binding sites.

Denaturation of PMM was monitored by DSC during this study. The enthalpy change(ΔH) of denaturation observed in a PMM slurry is associated with endothermic reactions such as break-up of molecular structure, and with exothermic reactions such as protein aggregation(Arntfield and Murray, 1981). It is therefore not possible to directly relate ΔH values to specific structural changes such as a change of quaternary or tertiary structure. However, differences in ΔH indicated that the PMM preparations were denatured to different degrees and as a result dissociation and unfolding of subunits took place. The binding data for the vanillin-PMM systems(Table 9) may indicate that heat treatment of PMM at 81 and 84°C resulted in dissociation of subunits and that at the higher temperature(95°C) dissociation and unfolding of the subunits occurred. This speculation may be derived from the fact that the binding constant decreased rapidly for PMM heated at 81 and 84°C, compared with the binding constant for native PMM. Furthermore, there was only a small difference between the binding constants for PMM heat-treated at 84°C and at 95°C, but there was a large increase of the number of binding sites from 307 for PMM heated at 84°C to 558 for PMM heated at 95°C indicating that the major change was an unfolding of the subunits. Heat denaturation of PMM affected its binding behaviour in a similar way as was the binding behaviour of soy protein when denatured with urea(Damodaran and Kinsella, 1981b).

Confirmation of this suggestion requires further study to determine the nature of the structural changes of the PMM proteins. A suitable method would be fluorescence spectroscopy, since the fluorescence emission spectra of a protein can reflect the structure of the protein. The results of these trials also indicated that the vanillin-PMM interactions were hydrophobic in nature. This may be derived from the fact that the interactions changed with heat denaturation of the PMM. This is because any structural change of a protein should affect hydrophobic interactions between a ligand and a protein and thus binding affinity may either increase or decrease. This may be the reason that the binding capacity of PMM heat-treated at 84°C was slightly lower than the binding capacity of PMM heat-treated at 81°C and 95°C.

4.4 The Effect of Salt Conditions on the Binding of Vanillin to PMM

Salts may influence both electrostatic and hydrophobic interactions and have, therefore, profound effects on the conformation of proteins (Damodaran and Kinsella, 1981c). It has been shown that conformational changes of PMM, induced by heat treatment, affected binding properties of PMM. Therefore it was expected that the addition of salts to the vanillin-PMM systems would affect the binding properties of PMM either due to changes of the conformation of PMM or due to salt effects on the vanillin-PMM interactions themselves. Two types of salts namely NaCl and Tris-HCl were added to

the vanillin-PMM systems at 0.03 and 0.3 M. The binding data for the systems containing NaCl or Tris-HCl are listed in Table 10 and for comparison, binding data obtained for salt free vanillin-PMM systems are also included. Neither the addition of NaCl nor of Tris-HCl to the vanillin-PMM systems seemed to have any significant effect on the binding capacity of PMM for vanillin when compared with the capacity of PMM in salt-free systems. However the addition of 0.03 M Tris-HCl to the systems resulted in a decrease in the number of binding sites from 21 for the salt-free to 11 for the Tris-HCl containing systems. The strength of the vanillin-PMM interaction remained the same as indicated by the similar binding constants and by the free energy of the interaction in both systems. Increasing Tris-HCl from 0.03 M to 0.3 M decreased the binding constant or strength of interaction but the number of binding sites increased from 11 to 16 per mole of protein. Addition of NaCl to the vanillin-PMM systems drastically reduced the binding constants from 182 per mole obtained in the salt-free systems to 39 and 38 per mole obtained at 0.03 and 0.3 M NaCl, respectively; conversely the number of binding sites increased from 21 to 52 and 55, respectively.

These results indicated that NaCl and Tris-HCl induced conformational changes in the native PMM proteins. If the conformation of the proteins had not changed then it could have been expected that there would have been no change in the number of binding sites and the salt effect would have

Table 10

The Effect of Salt Conditions on Thermodynamic Constants for the Binding of Vanillin to PMM[1] at 25°C and at pH of 6.3 ± 0.1

	H_2O	Tris-HCl		NaCl	
		0.03 M	0.3 M	0.03 M	0.3 M
Capacity (% bound of total vanillin)	19	15	20	20	23
N, number of binding sites per mole of protein (MW = 240,000)	21	11	16	52	55
K, binding constant (M^{-1})	182	200	128	39	38
ΔG, free energy[2] (Kcal/mole)	-3.085	-3.139	-2.875	-2.171	-2.155

1) 5 % Native PMM.

2) $\Delta G = -RT\ln K$

been limited to changes in the binding constant. Furthermore these data also indicated that conformational changes induced in PMM proteins by NaCl and Tris-HCl differed: addition of NaCl increased the number of binding sites whereas addition of Tris-HCl decreased the number of binding sites. Direct effects of the salts on the strength of the vanillin-PMM interaction itself cannot be excluded. However the extent of these effects on the interactions cannot be exactly determined because direct salt effects on the interactions cannot be separated from the conformational effects on the interactions. The experiments conducted with these two salts showed that comparison of binding data with the literature data(which were obtained by utilizing different buffer salts), is dangerous.

4.5 The Effect of Two Phenolic Compounds Associated with PMM on Vanillin-PMM Interactions and Evaluation of Binding Data by Scatchard Plot

In the previous sections, evaluation of the data by Klotz plots was used to interpret the results of the binding experiments. This approach is valid for simple systems which consist of a pure protein and a single type of ligand. Furthermore a condition which should be met is that the binding sites should be equivalent and independent from each other. However, if there are several classes of binding sites then the Klotz plot will yield an overall binding constant. The interactions between vanillin and PMM were expected to be more complex. One reason for this was that

the PMM preparation contained small amounts of non-protein materials (Table 5) and furthermore that the PMM protein probably consisted of at least two different protein species. It was therefore likely that more than one class of the sites was involved in vanillin-PMM interactions and that evaluation according to Klotz et al. (1946) only indicated general trends.

Determination of vanillin by HPLC revealed that two compounds were associated with PMM(Appendix II). These compounds appeared to be phenolics and were of molecular weights of less than 800 d since they were not retained by the YMT-membranes used in the micro-partition system. The released quantities of the phenolic compounds A and B were calculated based on the vanillin standard curve and are therefore expressed in vanillin equivalents (Tables 11 and 12). The data presented in Tables 11 and 12 can be summarized as follows. The interactions between compound B and PMM appeared to involve different binding sites. One indication for this was that the heat treatment of the PMM had no effect on the interaction between compound B and PMM as was indicated by the fact that the level of B did not change compared to the systems containing native PMM (Table 12). The concentration of the free compound A increased when the systems contained heat-treated PMM(Table 12). This indicated that the binding capacity of PMM for compound A decreased when PMM samples were partially or completely denatured. A further difference was observed when vanillin

Table 11

Concentration Effect: Two Unidentified Phenolic Compounds Converted to Vanillin Equivalents Based on the Standard Curve of Vanillin Solution

Vanillin Equivalents

Concentration of Vanillin (ppm)	Concentration of Native PMM (%)							
	1	2.5	5	10	1	2.5	5	10
	A[1]				B[1]			
Control	1.2	2.4	5.1	9.0	10.2	26.0	49.6	92.5
300	1.7	3.2	5.7	11.8	9.3	23.9	47.3	91.4
350	2.1	3.2	6.3	10.9	9.7	24.1	45.8	61.4
400	2.1	3.3	6.4	10.5	9.9	23.5	45.3	61.0
500	2.3	3.2	6.3	11.8	9.4	24.5	46.1	60.8
600	2.7	4.2	6.8	11.1	9.8	23.8	50.4	93.6
900	3.5	4.2	7.5	13.8	10.2	24.1	49.3	92.9
1200	4.7	6.3	7.2	14.8	5.9	23.7	52.7	99.5
2400	5.1	11.8	10.4	19.9	12.4	24.5	55.5	98.9

1) Unidentified phenolic compounds associated with PMM

Table 12

Conformation Effect: Two Unidentified Phenolic Compounds Converted to Vanillin Equivalents Based on the Standard Curve of Vanillin Solution

Concentration of Vanillin (ppm)	Vanillin Equivalents							
	Native PMM[1]	Heat-treated PMM[1] (15 min)			Native PMM[1]	Heat-treated PMM[1] (15 min)		
		81°C	84°C	95°C		81°C	84°C	95°C
		A[2]				B[2]		
Control	5.1	10.5	11.8	12.4	49.6	44.0	45.5	46.1
300	5.7	11.2	10.2	13.4	47.3	46.6	44.5	50.1
350	6.3	12.5	12.8	17.4	45.8	42.4	43.3	44.6
400	6.4	13.2	13.0	15.7	45.3	42.8	44.0	44.0
500	6.3	13.8	11.7	16.6	46.1	42.8	44.1	44.0
600	6.8	11.2	13.3	12.0	50.4	43.4	48.5	44.8
900	7.5	14.6	13.4	19.4	49.3	45.5	45.7	46.1
1200	7.2	15.5	14.1	15.9	52.7	47.8	49.0	50.8
2400	10.4	15.0	16.3	17.9	55.5	47.3	49.9	49.1

1) PMM concentration 5 %.

2) Unidentified phenolic compounds associated with PMM

was added to the systems. Increasing vanillin levels resulted in an increased release of the compound A from PMM (Tables 11 and 12). This suggested that vanillin and compound A interacted with the same binding sites. Addition of vanillin to the systems had, however, no effect on the interaction between compound B and PMM as the level of released compound B remained constant (Tables 11 and 12). This appeared to indicate that binding sites other than those involved in vanillin-PMM interactions existed for compound B-PMM interactions. The level of free compound A and free compound B increased proportionally with the PMM level present in a system (Table 11). This indicated that within this range a constant percentage of the total amount of compound A and B was released. The presence of these compounds contributed to the complexity of the systems and made the interpretation of the results of the binding experiments more difficult. It seemed that compound A, in particular, was competing for the same binding sites as vanillin. This meant that the binding systems consisted of PMM protein and two ligands, namely vanillin and compound A. Compound B had no direct effect on the vanillin-PMM interactions in these experiments and was therefore considered a constant. Further evaluation of the binding data using Scatchard plots seemed, therefore, warranted. A Scatchard plot will be linear if the interaction between a ligand and a protein involves equivalent and independent binding sites. If the association between ligand and protein

induces protein association or dissociation then the Scatchard plot will show a curvature(Steiner, 1980). In a non-associating system, curvature in a Scatchard plot may arise from either a heterogeneity of binding sites or positive or negative cooperativity of binding. The shape of the Scatchard plot is normally independent of the protein concentration in a non-associating system but is dependent on the protein concentration in an associating system.

Scatchard plots, based on data from the binding experiments of this study, are presented in Figures 10, 11, and 12. The shapes of the curve indicated that the interactions between vanillin and PMM were complex in all cases. Some tentative conclusions may be drawn from these plots. It appeared that binding of vanillin to native PMM induced protein association. This can be deduced from the Scatchard plots presented in Figure 10. The shape of these plots changed with increased protein concentration. If it was a non-associating system the shape of these curves should not change (Steiner, 1980). Steiner(1980) generated simulated binding curves for different associating models as affected by protein concentration and demonstrated that the curvature of the generated Scatchard plots could be confused with the effects of site-heterogeneity or positive or negative cooperativity. However, any of these effects may have played a role in the binding experiments conducted in this study. Scatchard plots(Figs. 10-12) could have indicated in nearly all cases positive cooperativity as

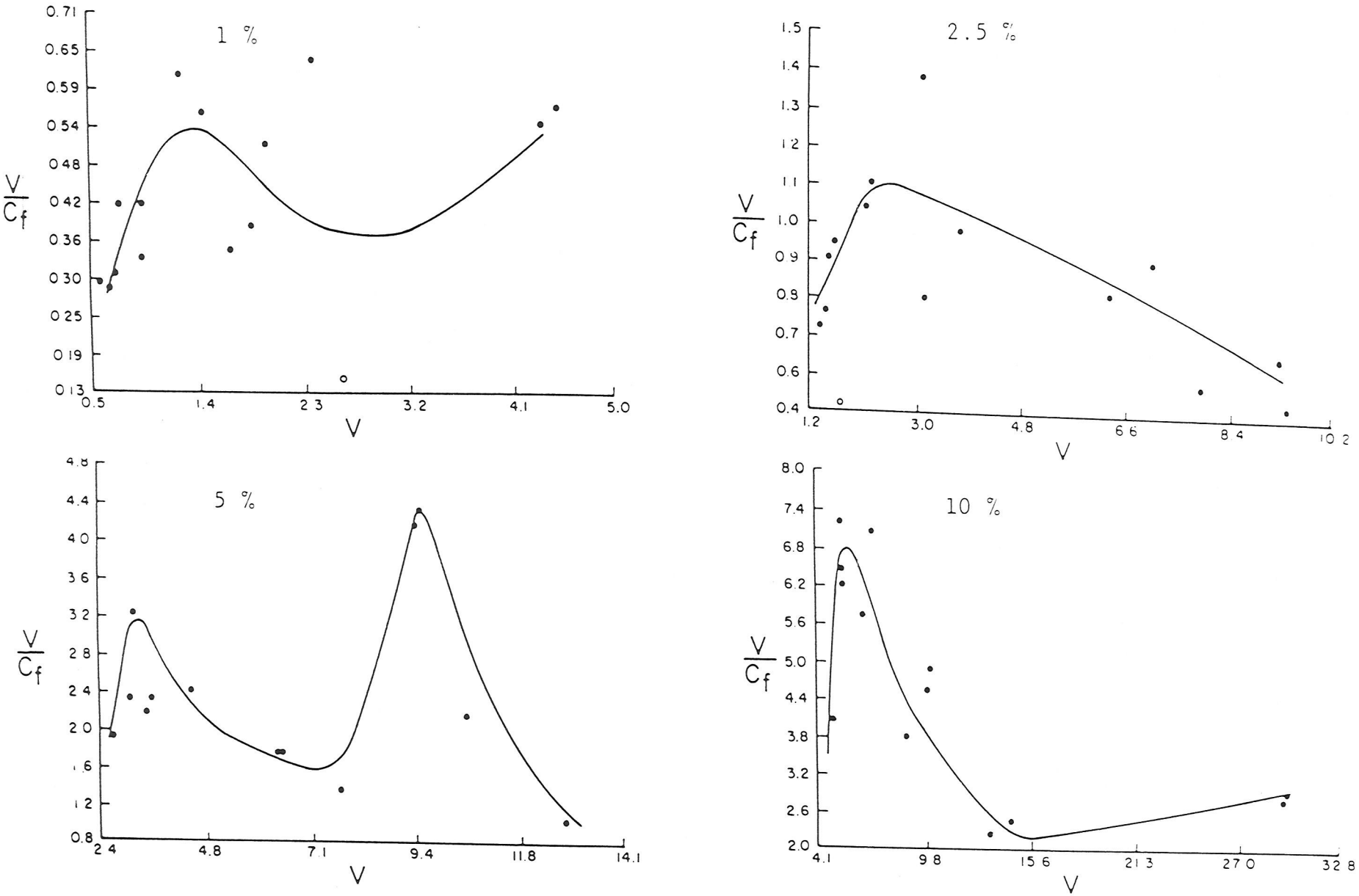

Figure 10 The Effect of Protein Concentration on the Binding of Vanillin to Native PMM (Scatchard Plots). V is the number of moles of bound-ligand per mole of protein and C_f is the molar concentration of the free ligand.

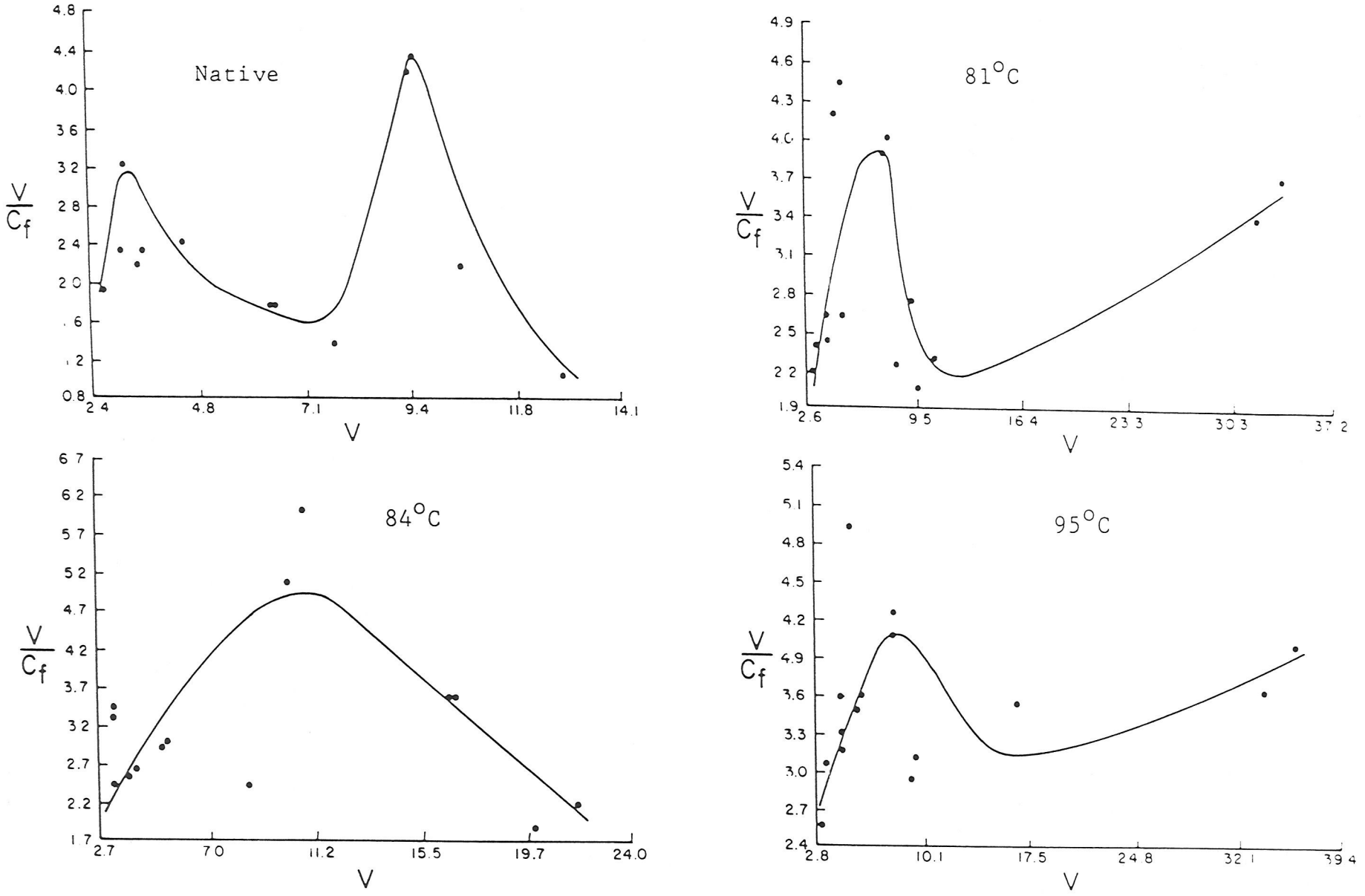

Figure 11 The Effect of Changes of Protein Conformation on the Binding of Vanillin to the PMM (Scatchard Plots). V is the number of moles of bound-ligand per mole of protein and C_f is the molar concentration of the free ligand.

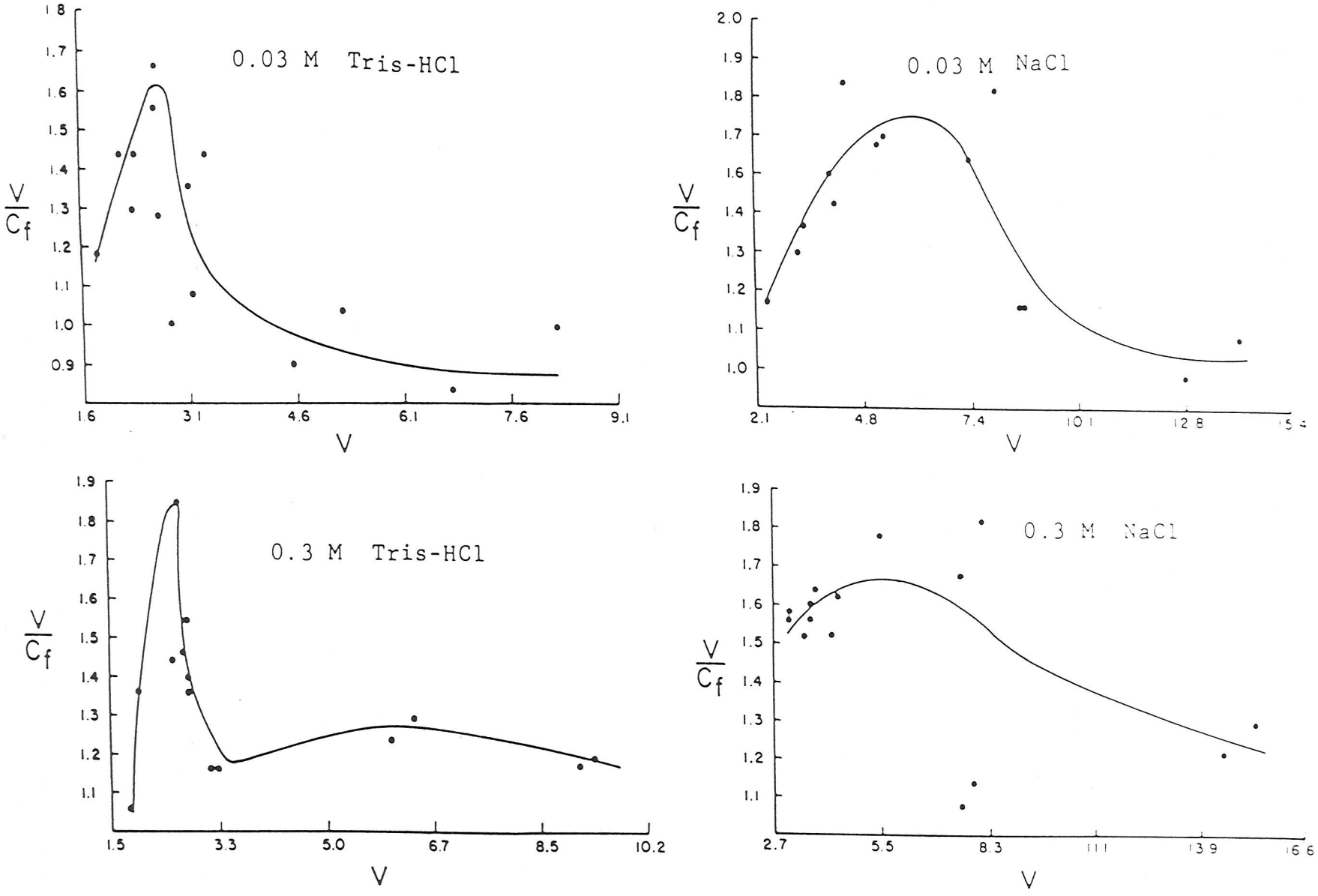

Figure 12 The Effect of NaCl and Tris-HCl on the Binding of Vanillin to 5 % Native PMM (Scatchard Plots). V is the number of moles of bound-ligand per mole of protein and C_f is the molar concentration of the free ligand.

shown by an upward convexity, and negative cooperativity as shown by an upward concavity. The effect of different salts on the interaction between 2-nonanone and bovine serum albumin was studied by Damodaran and Kinsella (1980b). These authors obtained Scatchard plots similar in shape to those generated in this study(Fig. 12). They explained these shapes as resulting from positive and negative cooperativity. However, these experimental methods do not permit exclusion of protein association induced by ligand-protein interactions.

4.6 The Effect of Vanillin-PMM Interactions on Sensory Perception of Vanillin Flavour in Vanillin-PMM Systems

The binding experiments with vanillin-PMM systems established that vanillin was bound to PMM. The sensory trials were conducted to determine the impact of vanillin binding on the perceived vanillin flavour of vanillin-PMM systems. The assumption was that only the free vanillin would be perceived by the panelists and therefore, based on the magnitude estimates for vanillin flavour, the perceived free vanillin concentration could be determined. For this purpose a power function for vanillin in water was established (Fig. 13) to convert magnitude estimates to "perceived" vanillin concentrations(Malcolmson and McDaniel, 1980).

Vanillin has a characteristic vanillin aroma but also possesses a bitter taste. This impeded the task for the panelists and many had some difficulties with the lower

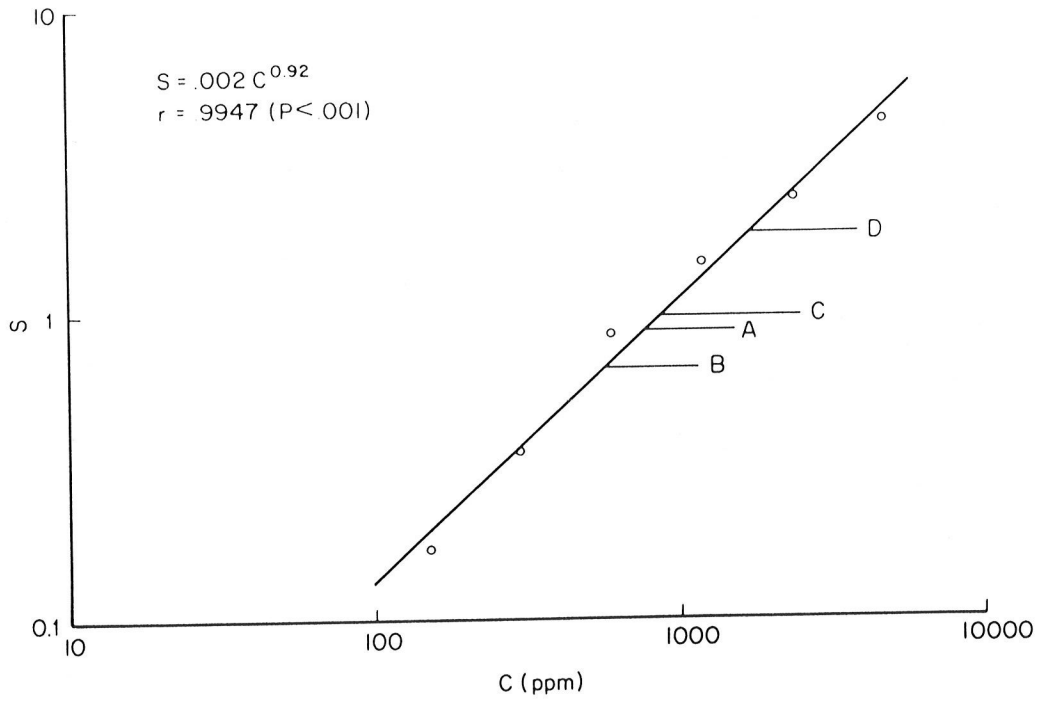

Figure 13 Power function for perceived vanillin flavour intensity (S) and vanillin concentration (C) in water. Lines intersecting power function at A, B, C and D indicate magnitude estimates and perceived vanillin concentrations. Total vanillin concentrations were 300, 600, 1200 and 2400 ppm at A, B, C and D respectively, in 5% (w/w) of native PMM.

concentrations. Analysis of variance of the results for the power function revealed that the panel did not find significant differences for vanillin flavour intensity between solutions containing 150 and 300 ppm vanillin and for solutions containing 300 and 600, or 600,1200 and 2400 ppm vanillin or 1200, 2400 and 4800 ppm vanillin.(Appendix III). This was somewhat surprising since the exponent of a power function has been shown to be a reliable parameter(Moskowitz, 1983). The exponent for the power funtion was 0.92 which indicated that the sensory ratio should nearly equal the physical ratio(i.e. doubling the vanillin concentration should have produced a doubling of perceived vanillin flavour).

Table 13 summarizes the sensory data obtained on vanillin-native PMM systems. The total vanillin concentration in all these systems was 1200 ppm and PMM concentrations were 1, 2.5, 5 and 10%. The magnitude estimates were converted into "perceived" vanillin concentrations and these were compared with the free vanillin concentrations as determined in the binding experiments on the corresponding systems. The free vanillin levels as determined by HPLC as well as the ratio of perceived vanillin concentration and free vanillin concentration as determined by HPLC are also listed in Table 13. The ratios (sensory/HPLC level) ranged from approximately 0.8 to 1.0. This indicated that only the free vanillin was perceived by the panelists and thus the bound

Table 13

The Effect of Protein Concentration on Vanillin-PMM Binding. Free Vanillin Levels Determined by HPLC and Sensory Analysis

Protein concentration[1] (% W/W)	Free vanillin level[2] (ppm) HPLC	Sensory	Ratio Sensory/ HPLC
1.0	1152	1025	0.89
2.5	1056	1110	1.05
5.0	972	803	0.83
10.0	720	553	0.77

1) Native PMM

2) Total vanillin concentration 1200 ppm

vanillin had no sensory impact otherwise this ratio(s) would have been larger than 1. The data in Table 13 also showed that the reduction of the flavour impact of vanillin was substantial in the system which contained 10% native PMM; it approached reduction of about 50%, but this information was not observed in ANOVA(Appendix V).

The results obtained from the systems containing native and heat-treated PMM are listed in Table 14. The total vanillin levels were 300, 600, 1200 and 2400 ppm. The perceived vanillin concentrations were again compared with the free vanillin levels determined by HPLC, and their ratios (sensory/HPLC) were calculated(Table 14). It seemed that in general the perceived vanillin concentration as determined by the panel equalled those determined by HPLC for the systems to which 1200 and 2400 ppm vanillin were added. This showed that only the free vanillin was perceived by the panel. Analysis of variance also showed that the panel found significant differences for perceived vanillin concentrations between 1200 and 2400 ppm vanillin added to the systems(Appendix IV). In the other systems to which 300 or 600 ppm vanillin was added the perceived vanillin concentration exceeded the ones determined by HPLC as indicated by the high ratios(sensory/HPLC) ranging from 1.2 to 3.5. The lower concentrations of 300 and 600 ppm of vanillin proved to be difficult for the panel to evaluate. This had already been observed when vanillin in water solutions was tasted to establish the power function(Fig.

Table 14

The Effect of Protein Conformation on Vanillin-PMM Binding. Free Vanillin Levels Determined by HPLC and Sensory Analysis

Vanillin total level (ppm)	Free vanillin level (ppm)											
	Native PMM[1]			Heat-treated PMM[1] (15 min)								
				81°C			84°C			95°C		
	HPLC	Sensory	Ratio Sensory/ HPLC	HPLC	Sensory	Ratio Sensory/ HPLC	HPLC	Sensory	Ratio Sensory/ HPLC	HPLC	Sensory	Ratio Sensory/ HPLC
300	243	752	3.09	162	454	2.80	189	672	3.50	153	525	3.43
600	486	569	1.17	324	847	2.61	378	682	1.80	306	726	2.37
1200	972	870	0.90	848	999	1.54	756	809	1.07	612	1159	1.89
2400	1944	1706	0.88	1296	1610	1.24	1512	1324	0.88	1224	1409	1.15

1) PMM concentration 5% (W/W)

13). The panel was inefficient in evaluating at the levels of 300 and 600 ppm of vanillin(Table 14) but such difficulties must not be used to explain inconsistent data. Otherwise one would expect that some of the ratios would be less than one. More likely it was that the unidentified phenolic compounds A and B(Tables 11 and 12) exhibited some flavour impact. The panelists may have been unable to distinguish the flavour of these two compounds from the vanillin flavour, at least when only 300 or 600 ppm of vanillin was added to the systems. It is possible that when 1200 ppm or 2400 ppm vanillin were added to the systems the ratios of vanillin/compound A plus compound B become greater and consequently vanillin was clearly perceived by the panel and thus the panel only perceived the flavour impact of the vanillin. The perception of odour or taste compound mixtures is difficult to predict based on the odour or taste properties of the individual compounds. However there are reports in the literature indicating that masking or enhancement are produced in mixtures(Kroeze, 1982; Laffort and Dravnieks, 1982; Laing and Willcox, 1983). Furthermore, it appears that these effects are dependent on concentration and intensity ratios of component compounds. For odourants it is usual that the component with the highest intensity and present at the highest ratio dominates the flavour of a mixture(Laing and Willcox, 1983). Or in other words, if a binary mixture consisted of 80% compound A and 20% compound B only, the flavour of compound A would be perceived. The

vanillin-PMM systems may be considered to be ternary systems in terms of flavour components consisting of vanillin, compound A and compound B. If this were the case then mixture effects and binding effects were likely to affect the performance of the sensory panel.

5. CONCLUSIONS AND RECOMMENDATIONS

The binding experiments showed that from 4% to 50% of the vanillin present in a vanillin-PMM system was bound to PMM. Binding capacity of partially denatured and of totally denatured PMM was higher than that of native PMM. Binding forces between vanillin and PMM were weak and the number of binding sites increased when PMM was denatured, thus indicating hydrophobic interactions. Increasing PMM concentrations increased the percentage proportion of vanillin bound to PMM. The effects of addition of NaCl or Tris-HCl to the systems on vanillin-PMM interactions were investigated and binding data interpreted by Klotz plots and by Scatchard plots. In all cases interactions were complex as indicated by the curvature of the Scatchard plots. Both positive and negative cooperativity were exhibited. However, this could also have demonstrated ligand-induced associations.

These vanillin-PMM systems resembled situations encountered in foods in terms of vanillin concentrations and protein concentrations. However, these are far from ideal systems to be analysed by Klotz or Scatchard plots which required more dilute systems. Furthermore, PMM is a highly purified vegetable protein for food applications but it contains non-protein components and the protein present consists of more than one species. The binding experiments revealed that two phenolic compounds were associated with PMM. All these factors added to complexity of the systems

and hindered interpretation of the results.

The binding experiments indicated that two aspects merit further investigations. Firstly conformational changes in PMM induced by heating need further characterization: for example by fluorescence spectroscopy. Secondly the question of ligand-induced protein association requires analysis. Possible approaches here include study of vanillin-PMM interactions by Sephadex chromatography or ultracentrifugation.

The sensory study revealed that in binary systems consisting of protein and one type of ligand or in systems consisting of protein and more than one type of ligand, one of which is dominant, the "perceived" concentration was equivalent to the free concentration. Thus this study demonstrated that in these systems the impact of flavour binding by proteins can be predicted based on determination of the free flavour level and the power function for the particular flavour compound in water. This was clearly demonstrated when 1200 ppm or 2400 ppm of vanillin was added to a system. At lower vanillin concentrations(300 or 600 ppm) the systems were apparently more complex as vanillin may not have been the dominant flavour compound. In these cases the impact of flavour-protein interactions is overshadowed by flavour-flavour interactions.

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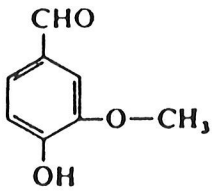
APPENDIX TABLES

Appendix I Physical and Chemical properties of m-Vanillin (Furia and Bellanca, 1975).

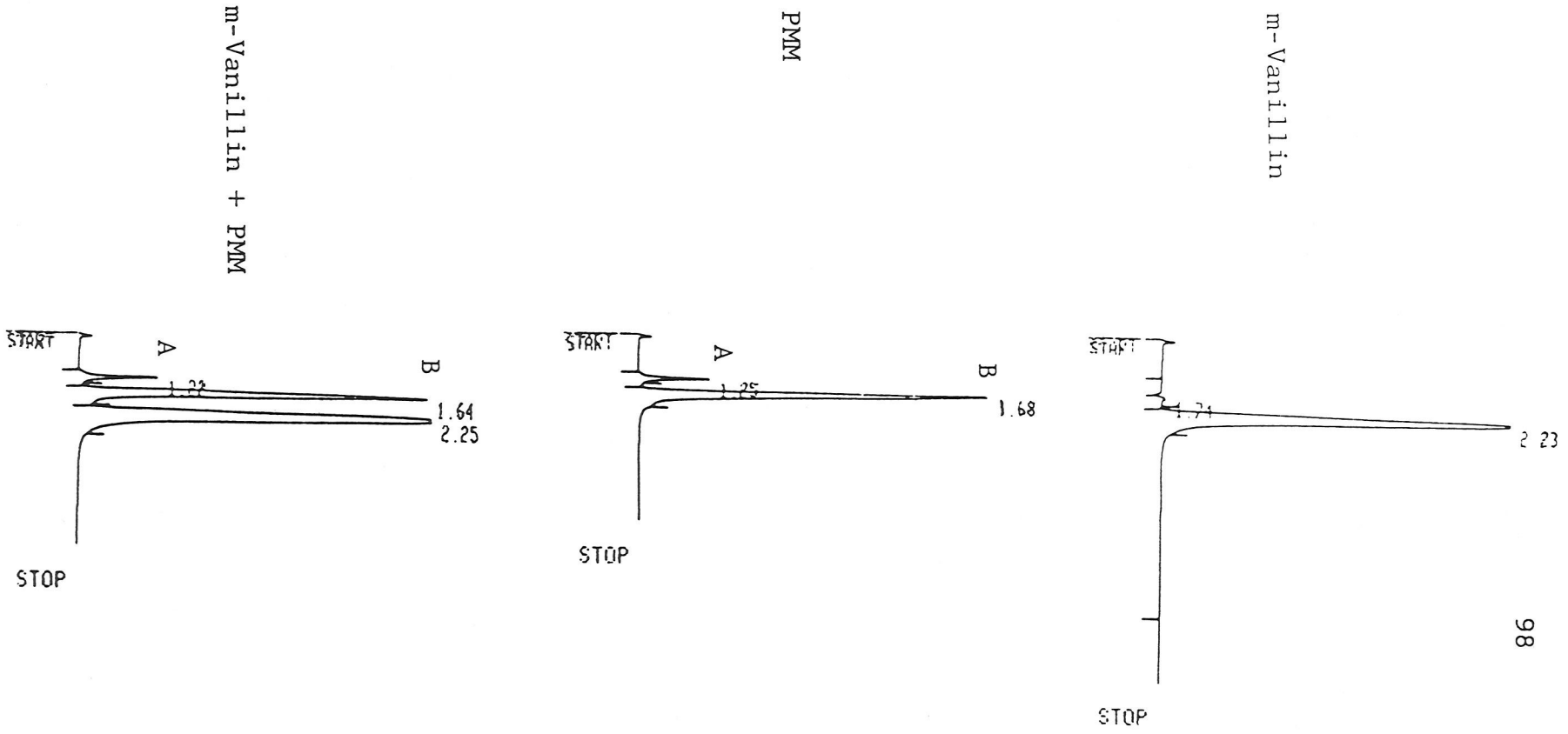
Other names	4-Hydroxy-3-methoxybenzaldehyde Methyl protocatechuic aldehyde Protocatechualdehyde-3-methylether
Empirical formula	$C_8H_8O_3$
Structure	
Physical and Chemical properties	
Appearance	White to yellowish crystalline powder
Molecular Weight	152.14
Melting point	81°C; sublimes when heated
Boiling point	284-285°C (with decomposition)
Specific gravity	1.06 (liquid)
Solubility	25 g in 100g of 75% alcohol; 33 g in 100 g of 95% alcohol; soluble in ether and chloroform; rather soluble in water and glycerol.
Sensory Characteristics	Characteristic strong vanilla-like odour; sweet taste.

Appendix I (Cont'd)

Natural Occurrence	Vanilla widely occurs in nature. It has been reported in the essential oil of Java citronella, in Peru balsam, clove bud oil, and chiefly vanilla pods. More than 40 vanilla varieties are cultivated. Another source of vanilla is from the waste (liquor) of the wood-pulp industry. Vanillin is extracted with benzene after saturation of the sulfite waste liquor with CO_2.
Reported Uses	Non-alcoholic beverages, 63 ppm; ice cream, ices, etc., 95 ppm; candy, 200 ppm; baked goods, 220 ppm; gelatins and puddings, 120 ppm; chewing gum, 270 ppm; syrups, 330-20,000 ppm; chocolate, 970 ppm; toppings, 150 ppm; margarine, 0.20 ppm.

Appendix II

HPLC Chromatogram of m-Vanillin and Two Associated-Unidentified Phenolic Compounds from PMM.



Appendix III

Analysis of Variance of the Sensory Scores for the Power Function of Vanillin Solution[1]

Source	DF	SS	MS	F
Concentration	5	24.28	4.86	9.09*
Panelists	17	0.81	0.05	0.09ns
Error	85	45.43	0.53	
Total	107	70.52		

* Significantly different at $P < 0.05$.
ns= not significant at $P < 0.05$.

Duncan's Multiple Range Test

Level = 0.05 DF = 85 MS = 0.53

Concentration (ppm)	Mean	Grouping
4800	0.6294	A
2400	0.3805	A B
1200	0.1561	A B
600	-0.0689	B C
300	-0.4422	C D
150	-0.7650	D

1) 9 panelists x 2 = 18

Appendix IV

Analysis of Variance Using Means of Panelists' Perceived Vanillin Concentration for Vanillin-PMM[1] Slurries

Source	DF	SS	MS	F
Concentration	3	1990320	663440	25.97*
Type of protein	3	29513	9838	0.39ns
Error	9	229914	25546	
Total	15	2249747		

* Significantly different at $P < 0.05$.
ns= not significant at $P < 0.05$.

Duncan's Multiple Range Test

level = 0.05 DF = 9 MS = 25546

Concentration (ppm)	Mean (ppm)	Grouping
2400	1512.4	A
1200	959.5	B
600	706.2	B C
300	600.9	C

1) 5% PMM of native and heat-treated at 81°C, 84°C and 95°C.

Appendix V

Analysis of Variance of the Sensory Scores for Four Levels of Native PMM in 1200 ppm of Vanillin Solution

Source	DF	SS	MS	F
Level of protein concentration	3	2.471	0.824	1.37^{ns}
Panelists	8	2.111	0.264	0.44^{ns}
Replications	1	0.031	0.031	0.05^{ns}
Error	59	35.409	0.600	
Total	71	40.021		

ns= not significant at $P < 0.05$.