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Effects of physico-chemical parameters of a model wine on the binding of γ-decalactone on bovine serum albumin

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To understand the effect of temperature, pH and the composition of alcoholic beverages in flavour-protein interactions, the binding of γ-decalactone to bovine serum albumin (BSA) was investigated using the equilibrium dialysis method. Thermodynamic analysis revealed that the affinity of aroma compound for BSA is higher at 10°C than at 20 and 30°C, while the number of binding sites (n = 6–7) is not modified at the three temperatures. pH did not have any appreciable effect on flavour binding in the presence of ethanol, but it was observed that a decrease of 1·8 pH unit reduces binding by 40% in its absence. The presence of ethanol has no effect on the number of binding sites and on the standard free energy ($\Delta G°$) of the interactions. On the other hand, the binding constant (k) was 4·8-fold higher in water than in model wine (pH 3·5, ionized compounds, 10% w/w ethanol); so, the affinity of volatile compound was clearly lower in the model wine than in water.

INTRODUCTION

The complexity of food media leads to the use of model systems to study the interactions between flavours and food components, especially to understand the influence of physicochemical parameters such as temperature or composition of the medium of dilution. Several works describe the effects of modifications of pH, temperature or ionic strength on the interactions between aroma substances and food components, especially proteins, in food model systems (Beyeler & Solms, 1974; Damodaran & Kinsella, 1980; Damodaran & Kinsella, 1981; Dumont & Land, 1986; O'Neill & Kinsella, 1987). Studies of the influence of non-volatile compounds, such as mannoproteins from yeast cell walls, on the volatility of aroma substances were carried out in model wine, i.e. an hydroethanolic solution containing organic acids and salts of wine (Voilley *et al.*, 1990; Voilley *et al.*, 1991; Lubbers *et al.* 1994). The model wine contains only malic acid, in contrast to wine which contains tartric acid as well, (Ribereau-Gayon *et al.*, 1972), to avoid hydrogen potassium tartrate precipitation with temperature decrease. The pH was fixed at 3·5, the pH of wine being between 3 and 4. The effects of ethanol and solutes on the volatility of aroma compounds have been shown by headspace analysis. The activity coefficients of volatile compounds in the model wine were half of those obtained in water. Moreover, it appeared that salts and ethanol produce opposite effects (Voilley *et al.*, 1991). However, the effects of the model wine on the interactions in the presence of macromolecules were not investigated.

The aroma compound used in this study is γ-decalactone, a cyclic ketone found in white wines at a concentration of 60 ppb and presenting a peach flavour (Etievant, 1991). Bovine serum albumin (BSA) is a useful model protein for studying flavour–protein interactions because conformation and physical properties are well defined. In addition, BSA possesses measurable binding properties for non-polar compounds. The purpose of the present work is to understand the effects of physicochemical parameters (temperature, pH, presence of ionized compounds and ethanol) of a model wine on the interactions between an aroma compound (γ-decalactone) and a protein (BSA) measured by the equilibrium dialysis method.

MATERIALS

The model wine was composed of an aqueous solution

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of ethanol 125 ml.litre^{-1}, *L* (+) malic acid 3 g.litre^{-1}, acetic acid 0·106 ml.litre^{-1}, K_2SO_4 0·1 g.litre^{-1}, $MgSO_4$ 0·025 g.litre^{-1}. Bovine serum albumin (BSA) was added at 10 g.litre^{-1} to the model wine. The aroma compound selected was γ-decalactone. ^{3}H-γ-decalactone was also used as a marker. The ^{3}H-γ-decalactone had an activity of 0·840 mCi.ml^{-1} and its concentration was 5·99 × 10^{-4} M. Fifty μl of this solution was added to 25 ml of water to obtain the stock solution. The different aroma compound concentrations were obtained by adding 0·5 ml of the stock solution to 4·5 ml of γ-decalactone solution of concentrations ranging from 100 to 600 ppm (5·6 × 10^{-4} to 3·3 × 10^{-3} M).

METHODS

Equilibrium dialysis method

The equilibrium dialysis method is based on the diffusion of the volatile compound through a semi-permeable membrane placed between two compartments containing the model wine with or without protein. Acrylic cells (PTFE) of equal volume separated by a membrane (Spectra-Por No. 1, MWCO 6–8000 Daltons), clamped together, were used. In the experiment, 1 ml of solution of BSA in model wine was placed on one side of the membrane (compartment C1) and 1 ml of model wine containing a known amount of the volatile compound (ligand) on the other side of the membrane (compartment C2). At equilibrium, the concentration of the ligand in the solution from each compartment was determined by counting the radioactivity emitted by ^{3}H-γ-decalactone. 200 μl from each compartment was diluted in 4·5 ml of scintillator liquid (PACKARD TM 299) in polypropylene flasks. The counts were realized by a Tri-Carb 400 series system PACKARD counter at least 15 h after sample dilution in scintillator liquid. Two hundred μl of model wine in 4·5 ml of scintillator liquid was used as control. This control was counted for 10 min and the samples for 5 min.

The difference in ligand concentrations between C1 and C2 represents the amount of volatile compound bound to the macromolecules. The fraction of bound ligand as a percentage of that initially present is expressed by the equation:

$$\%Lb = \frac{n_1 - n_2}{n_1 + n_2} \times 100$$

n_1 = number of moles of bound ligand and free ligand in C1 at equilibrium

n_2 = number of moles of free ligand in C2 at equilibrium

Method of determination of the binding parameters

The binding parameters were determined at three temperatures: 10, 20 and 30°C. The equilibrium of free ligand and concentrations between the two compartments of the cells was reached after stirring for 16 h at 30°C, 20 h at 20°C and 24 h at 10°C. Using the amount of bound ligand, the quantity and the molecular weight of the BSA, the number of moles of bound ligand per mole of protein was calculated. The binding data obtained from equilibrium dialysis were analysed using the double reciprocal Klotz equation:

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{nk} \times \frac{1}{[L]}$$

where ν is the number of moles of bound ligand per mole of macromolecule, $[L]$ is the free ligand concentration, n is the total number of binding sites on the macromolecule and k is the binding constant (Klotz & Urquhart, 1948).

The standard free energy ($\Delta G°$) of binding of the volatile compound to the protein can be determined from k with the equation:

$$\Delta G° = -RTL\text{n } k$$

where R is the universal gas constant (8·32 J.mol^{-1}.K^{-1}) and T the temperature (K).

Factorial design

To understand how the pH and the presence of salts and ethanol in the model wine influence the binding of γ-decalactone on BSA, a two-level factorial experiment (abbreviated 2^3 design) was applied. For each factor, two levels have been chosen, according to the experimental constraints (Table 1), associated to the −1 and +1 levels of the corresponding coded factor.

A complete factorial design is performed using all possible combinations of the levels of each factor, so eight experiments were done.

The binding of γ-decalactone on BSA can be approximated by a linear combination of factors:

$$Y\ (\%\text{ of binding}) = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1X_2 + b_{13} X_1X_3 + b_{23} X_2X_3 + b_{123} X_1X_2X_3$$

The coefficient b_0 is the centre of the design domain.
The coefficients b_1, b_2, b_3 represent the effect of each factor coded X_1, X_2, X_3.
The coefficients b_{12}, b_{13}, b_{23} are respectively the first order interactions between X_1 and X_2, X_1 and X_3, X_2 and X_3.
The coefficient b_{123} is the effect of the second order interaction between X_1, X_2 and X_3.

Table 1. Factors and levels of the 2^3 design

Parameters	Level-1	Level+1
X1 Ethanol (10%, w/w)	no	yes
X2 pH	3·5	5·3
X3 Salts (3·3 g.litre^{-1})	no	yes

pH 5·3 corresponds to the pH of a solution of BSA at 10 g.litre^{-1} in distilled water. pH 3·5 is adjusted with 0·05 M HCl.

RESULTS AND DISCUSSION

Effects of temperature on the binding of γ-decalactone on to BSA

The amount of bound γ-decalactone was determined at three temperatures at aroma concentrations ranging from 90 to 540 ppm in model wine containing 10% ethanol (w/w). The double reciprocal plots (Klotz plot) for binding of γ-decalactone revealed similar values for the intercept for all three temperatures (Fig.1). It was verified with a Bartlett Test that the numbers of binding sites (n) at the three temperatures were not significantly different at the threshold of 5%. The binding constants (k) and the corresponding free energies $\Delta G°$ were obtained from the slopes of the lines corresponding to the value of $1/nk$ (Table 2). The binding constants k at 20 and 30°C are not different but at 10°C, k is 3·5-fold higher than the values obtained at 20 and 30°C (k = 150–180). The standard free energies at the three temperatures are not significantly different. The evolution of the binding constant k with the temperature was previously shown by Spector *et al.* (1969) who observed a higher binding of palmitic acid by BSA at 23°C than at 37°C. Damodaran and Kinsella (1981) studied the binding of 2-nonanone on soy proteins; the increase of temperature from 25 to 45°C had only small effects on the number of binding sites (n) and on the binding constant k. However, at 5°C, n decreased and k was two times higher. Structural changes in the soy proteins at 5°C could explain the difference of affinity of the aroma compound to the proteins. The same phenomenon can exist for the binding of γ-decalactone on BSA. As no great effect of temperature on binding of the aroma compound on the BSA was observed, and as the dialysis time was shorter at 30°C than at 20 and 10°C, the effects of the model wine composition were studied at 30°C.

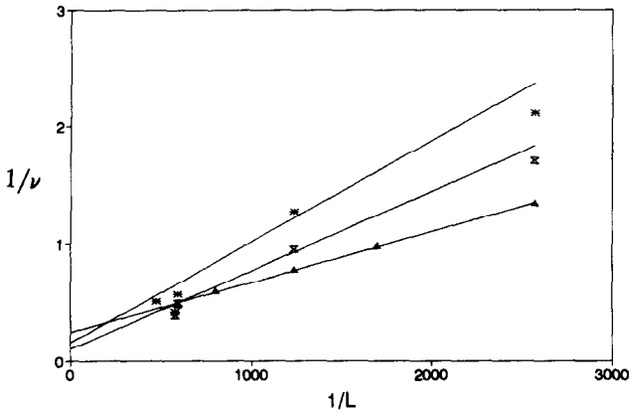

Fig. 1. Binding of γ-decalactone to BSA at 10 (▲), 20 (☒) and 30°C (✳) in model wine. ν is the number of moles of γ-decalactone bound per mole of BSA and [L] is the free γ-decalactone concentration in molarity.

Table 2. Binding parameters of γ-decalactone on BSA at 10, 20 and 30°C in model wine with 10% ethanol (w/w)

Temperature (°C)	n	k	$\Delta G°$ (KJ.mol^{-1})
10	4–5	580	–3·6
20	9–10	150	–2·9
30	6–7	180	–3·1

Effects of pH, ionized compounds and ethanol

The equilibrium dialysis were done with BSA at a concentration of 10 g.litre^{-1} and γ-decalactone at a concentration of 90 ppm at 30°C. The results obtained are presented in Table 3.

The coefficients of the linear combination of factors have been calculated:

b_0 = +20·4 $\quad b_{12}$ = +0·3
b_1 = –6·4 $\quad b_{13}$ = +3·7
b_2 = –0·6 $\quad b_{23}$ = +0·5
b_3 = +3·8 $\quad b_{123}$ = +0·0

It appeared that there was no second order interaction between the three factors ethanol, ionized compounds and pH. Ionized compounds (sulfate and organic acids, 3·3 g.litre^{-1}) have no effect on the measured response (% of binding). However, the ionic strength modifications are responsible for structural changes in proteins. Kinsella (1990) observed that sulfate and chloride ions stabilize the native structure of BSA and increase its affinity for non-polar ligands. Yet, the salts concentrations used were at least 1 M. In our conditions, the ionized compounds concentration is too low (0·02 M) to observe such phenomena. The first order interaction between the pH and the presence of ethanol means that γ-decalactone binding on BSA induced by one of these two factors (pH or ethanol) is greatly influenced by the other factor (ethanol or pH), as shown in Fig. 2. Without ethanol, a 1·8 pH unit decrease (5·3–3·5) reduces γ-decalactone binding by 40% but, in the presence of ethanol, changing pH did not significantly affect the flavour binding on BSA. However, at a given pH, ethanol reduces γ-decalactone binding from 30 to 60%. The binding parameters of γ-decalactone were determined in the two extreme cases, i.e. in water at pH 5·3 and in model wine at pH 3·5, 10% (w/w) ethanol (Table 4).

In water or in model wine, the BSA presents the same number of binding sites (n = 6–7) for γ-decalactone. In contrast, in water, the value of the binding

Table 3. Effect of the model wine composition on the binding of γ-decalactone (90 ppm) on BSA (10 g.litre^{-1})

Ethanol	Salts	pH	% of binding (SD)
no	no	3·5	20·7 (± 0·3)
yes	no	3·5	14·5 (± 1·4)
no	yes	3·5	17·9 (± 0·5)
yes	yes	3·5	13·0 (± 1·3)
no	no	5·3	34·7 (± 0·6)
yes	no	5·3	13·9 (± 0·8)
no	yes	5·3	33·9 (± 0·3)
yes	yes	5·3	14·3 (± 0·2)

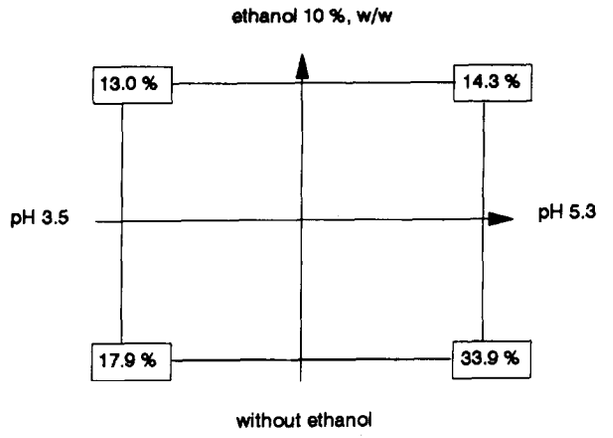

Fig. 2. Representation of the first order interaction between the factors ethanol and pH of the model wine for the binding of γ-decalactone (90 ppm) on BSA (10 g.litre^{-1}).

Table 4. Binding parameters of γ-decalactone (90 ppm) on BSA (10 g.litre^{-1}) at 30°C in water at pH 5·3 and in model wine with ethanol (10%, w/w) at pH 3·5

Solution	n	k	$\Delta G°$ (KJ mol^{-1})
Water	6–7	860	–4·1
Model wine	6–7	180	–3·1

n: Number of binding sites.
k: Binding constant.
$\Delta G°$: Standard free energy.

constant k of the γ-decalactone for the protein is 4·8-fold higher than the one obtained in model wine with ethanol. So, the affinity of the volatile compound is clearly lower in the presence of ethanol, ionized compounds and at pH 3·5 than in water at pH 5·3. However, the standard free energies ($\Delta G°$) are not significantly different in water and in model wine.

Without ethanol, the effect of pH on the binding of γ-decalactone is very important. The binding capacity of BSA was highest at pH 5·3 corresponding to the BSA aqueous solution pH. The pHi of the BSA being 4·9, the protein ionization is different at pH 3·5 and at pH 5·3 and also, therefore the protein conformation. Damodaran and Kinsella (1981) studied the binding of the 2-nonanone on the BSA and observed a decrease of the binding constant k between pH 3 and 6 and an increase at pH 9. This can be explained by changes in the main binding sites resulting from structural changes of the protein due to the pH. In the presence of ethanol, the influence of pH on the interactions becomes minor.

The effect of ethanol seems to be predominant on the binding of the volatile compound on BSA. A competition effect between ethanol and γ-decalatone for the binding sites on BSA is not consistent with these results unless the binding constant of ethanol for BSA is very low. In this case, almost all the sites would interact with ethanol in view of the concentration of ethanol (2·2 M) and of γ-decalactone ($5·0 \times 10^{-4}$ M).

The solubilisation of the aroma compound in ethanol increases the affinity of γ-decalactone for the solution and, thus, reduces the availability of the aroma for the protein. In water–ethanol systems, the activity coefficients of volatiles decrease in comparison with the values obtained in water (Nawar, 1966; Kepner *et al.*, 1969); e.g. in hydroethanolic solutions (10%, w/w), the activity coefficients of ethyl hexanoate, isoamyl alcohol and β-ionone decrease by 50% (Voilley *et al.*, 1991; Lubbers *et al.*, 1994). This effect of solubilisation can be explained by the presence of interactions between aroma compound, water and ethanol. However, the decrease of 4·8-fold in binding affinity of BSA for γ-decalactone in the presence of ethanol, while the number of binding sites remains constant, suggests that ethanol causes conformational changes in protein. It may be speculated that ethanol leads to a partial unfolding of the BSA molecule which does not destroy the binding sites but limits their availability. The relationship between ligand binding affinity and conformational changes in protein have been shown with bovine serum albumin (Damodaran & Kinsella, 1980) and β-lactoglobulin (O'Neill & Kinsella, 1987), but factors inducing reversible conformational changes, such as urea, have negligible effects on ligand solubility or produce an opposite effect to ethanol, i.e. salts increase the activity coefficients of aroma compounds (De La Ossa & Galan, 1986). So, it is not yet possible to fully understand the effects of ethanol on the ligand solubility or the conformational state of the protein.

CONCLUSION

The effects of pH on the binding of aroma compounds on proteins have been demonstrated by several authors (Damodaran & Kinsella, 1981; Dumont & Land, 1986) and confirmed in the case of wine. This communication shows that ethanol has a great effect on the retention of volatile compounds by proteins, and emphasizes the importance of the composition of the model food system in studies of aroma–macromolecule interactions.

It appears that, if the number of binding sites (n) is independent of the solution (model wine or water), the amount of retention is dependent on the conformation of the protein and thus on temperature, pH and the presence of ethanol. The conformational changes of proteins involved in interactions between volatile compounds and wine components must be further investigated for a better understanding of the mechanisms of the binding of aromas on macromolecules, and especially to determine the nature of the binding.

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