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Retention of aroma compounds by proteins in aqueous solution

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Retention of aroma compounds in sodium caseinate aqueous phase was investigated by measurement of vapour–liquid partition equilibrium (headspace analysis or exponential dilution). This retention depended on the nature of the aroma compounds and sodium caseinate content. For a homologous series of ethyl esters (ethyl acetate, butanoate and hexanoate), it increased with the carbon chain length from 0 to 38% and from 0 to 61% for caseinate contents of 5 and 50 g.litre^{-1}, respectively. Retention of diacetyl increased from 0 to 23% for the same range of protein content. Thermodynamic models were applied to evaluate the binding parameters n (number of binding sites of protein), K_a (affinity constant) and h (Hill coefficient) to both ethyl esters. These values were compared with those given in the literature: n and K_a were of the same order; h indicated a weak cooperative effect between sodium caseinate binding sites.

INTRODUCTION

One organoleptic property of food is related to its aroma, depending on the nature and the quantity of aroma compounds. Many factors such as the physico-chemical properties, the concentration and the interactions of volatile aroma molecules with food components affect the resulting quality. To control this quality, a knowledge of the nature and intensity of the interactions between aroma compounds and non-volatile substances is required. Indeed, the binding of added aroma molecules by food components influences flavouring in product formulation. A method used to investigate retention (or release) of flavour by water or non-volatile molecules is the measurement of vapour–liquid partition equilibrium. Thus, many studies were carried out with simple systems composed of water (Buttery *et al.*, 1969; Sadafian & Crouzet, 1986), proteins (Farès, 1987; Le Thanh *et al.*, 1992), lipids (Buttery *et al.*, 1973; Dubois, 1994) and carbohydrates (Nawar, 1971; Lebert & Richon, 1984). To quantify the aroma–food component interactions, the methods most used are equilibrium dialysis (Damodaran & Kinsella, 1981*a* Damodaran & Kinsella, 1981*b* Damodaran & Kinsella, 1983; Druaux *et al.*, 1995) and liquid–liquid partition equilibrium (Rutschmann *et al.*, 1989; Damodaran & Kinsella, 1980). O'Keefe *et al.* (1991) used the data obtained in measurement of vapour–liquid equilibrium to quantify these interactions. Therefore, it was considered of interest to use this method to study the aroma–protein binding. Sodium caseinate was retained, on the one hand, because of its well-known specific functional properties, and its use in dairy, as well as non-dairy, products (Kinsella, 1984); on the other hand, there was little concern for this protein in studies of the behaviour of aroma compounds in the presence of proteins (Farès, 1987; Sadafian & Crouzet, 1986). The aroma compounds involved in the study were a ketone (diacetyl) and a homologous series of esters (ethyl acetate, butanoate and hexanoate) and were chosen for their different physico-chemical characteristics, particularly their hydrophobicities; all are present, naturally or not, in dairy products. The two methods used to measure the vapour–liquid equilibrium of the aroma compounds are the headspace analysis and exponential dilution. For each one, the material and the experimental conditions are the same, and the evolution of the quantity of the aroma compounds in the vapour phase is followed versus time. For headspace analysis, this quantity is constant; however, for exponential dilution, the quantity decreases exponentially as a function of time. So, they are applied according to the behaviour of the aroma compounds at the vapour–liquid equilibrium.

The aims of this work were (1) to demonstrate the presence of retention of aroma compounds by the measurement of vapour–liquid partition equilibrium, (2) to

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show the influence of the nature of aroma compounds and sodium caseinate content on volatility, and (3) to test thermodynamic models to quantify the aroma–protein interactions from data of retention.

MATERIALS AND METHODS

Experimental conditions

Characterization of the sodium caseinate solution
Sodium caseinate was purchased from Unilait (Paris, France). The aqueous phases were prepared by dissolving sodium caseinate in distilled water at 60°C with a stirring bar for 1 h. The protein concentrations were 5 and 50 g.litre^{-1}. Sodium azide was added at a concentration of 0.2 g.litre^{-1} and the aqueous phases were stored at 4°C prior to initiation of the experiments to limit microbial development.

The pH of sodium caseinate solution was controlled with a HANNA pH meter (model HI 8417, HANNA Instruments, Italy) was 7.0 ± 0.1.

Chromatographic analysis of the aroma compounds
The aqueous solutions of sodium caseinate were equilibrated at 25°C before the aroma compounds, or ligands, were added. Diacetyl and ethyl hexanoate were purchased from Aldrich (Strasbourg, France), and ethyl acetate and ethyl butanoate from Prolabo (Marly-le-Roi, France). For ligand concentrations ranging from 20 to 10000 ppm (v/v) (according to their solubilities), the corresponding volume of the aroma substance was carefully added to a vial containing the aqueous phase; the vial was tightly capped and mixed manually. A 20-ml volume of the solutions was loaded in a tube and capped. The quantity of aroma compounds was determined by gas chromatography. An inert gas (nitrogen) was passed through the liquid phase at a constant flow-rate (from 1.0 to 3.5×10^{-5} m^3.min^{-1} according to the aroma compound) and carried the volatile molecule into the headspace. A sample of vapour phase (1 ml) was automatically injected into the gas chromatograph at regular intervals. This last was equipped with a flame ionization detector (Chrompack Co., Middelburg, the Netherlands) with a 3-m stainless steel column (inner diameter, 2.2 mm) packed with chromosorb W-AW 100–200 mesh Carbowax 20 M-10%. The operating parameters of the chromatograph were as follows: injector temperature, 190°C; detector temperature, 200°C; column temperature, 100°C; N_2 flow-rate, 1.6×10^{-5} m^3.min^{-1}; H_2 flow-rate, 2.5×10^{-5} m^3.min^{-1}; air flow-rate, 25.0×10^{-5} m^3.min^{-1}. The chromatograms were registered and treated with the Chroma software (Biosystèmes, Couternon, France).

The data allowed the determination of the vapour–liquid partition coefficient of aroma compounds at infinite dilution representing their volatility. The partition coefficient of aroma compounds at 760 mm Hg and 25°C is denoted K^{∞}_{760} and determined in water, 5 and 50 g.litre^{-1} sodium caseinate solution. For each compound and system, the measurements of the volatility were carried out at least four times.

Theoretical basis

Determination of aroma retention
Headspace analysis and exponential dilution are the two methods used to determine the vapour–liquid partition coefficient. By headspace analysis, at vapour–liquid equilibrium, the quantity of the aroma compound in the gas phase is constant and the vapour–liquid partition coefficient expressed in molar fraction (K_i^{∞}) is the ratio of the molar fraction of aroma compound i in vapour phase (y_i) divided by the molar fraction in liquid phase (x_i) (Equation 1).

$$K_i^{\infty} = \frac{y_i}{x_i} \tag{1}$$

The exponential dilution consists of exhausting the liquid phase of the aroma compound in equilibrium with the vapour phase. The solute chromatographic peak area variation is an exponential function of time provided the detector response is linear (Equation 2) (Sorrentino, 1984).

$$\log S = \log S_0 - \frac{Pd}{RTN} K_i^{\infty} t \tag{2}$$

where S and S_0 are the volatile peak areas; d, carrier gas flow-rate (m^3.min^{-1}); R, gas constant ($R=8.314$ J.K^{-1}.mol^{-1}); T, temperature (K); P, total pressure (Pa); N, number of moles of liquid phase; K_i^{∞}, vapour–liquid partition coefficient of aroma compound (in molar fraction); t, time (min).

$K^{\infty}{}_i$ is calculated from the values of the slope (a) of the straight line obtained by plotting log$S$ against time (Equation 3).

$$K_i^{\infty} = -\frac{aRTN}{Pd} \tag{3}$$

Consequently, the headspace method is static and exponential dilution is dynamic.

Volatility of the aroma compounds in water was chosen as a reference and permitted the determination of the percentage of retention r of the aroma compound on the protein. So a significant variation of K^{∞}_{760} ($P=0.05$) in comparison to the reference is due to aroma–medium interactions.

$$r = \left(1 - \frac{K^{\infty}_{760}(\text{protein})}{K^{\infty}_{760}(\text{water})}\right) \times 100 \tag{4}$$

where K^{∞}_{760}(protein) is the volatility of aroma compound in a sodium caseinate solution.

The ratio K^{∞}_{760}(protein)/K^{∞}_{760}(water) is denoted I and called relative volatility.

Determination of interaction parameters

For a protein having a total number of equivalent and independent binding sites (n), the reversible interactions between aroma molecules (L) and protein can be represented thermodynamically by the Scatchard equation (Scatchard, 1949):

$$\frac{\nu}{[L]} = K_a n - K_a \nu \quad (5)$$

where ν is the number of moles of ligand bound per mole of protein; $[L]$, the molar concentration of free ligand at equilibrium (mol.litre^{-1}); K_a, the intrinsic affinity constant. The values of ν and $[L]$ are calculated from Equations (6) and (7).

$$\nu = r\frac{[L_T]}{[S_T]} \quad (6)$$

where $[L_T]$ determined by gas chromatography is the initial ligand concentration (mol.litre^{-1}), and $[S_T]$ the total protein concentration (mol.litre^{-1}).

$$[L] = I[L_T] \quad (7)$$

Thus, the measurement of vapour–liquid equilibrium and the calculation of r permit the determination of n and K_a.

According to the Equation (5), a plot of $[L]$ versus ν should give a straight line with a slope of $-K_a$ and intercept nK_a from which n and K_a can be calculated.

The Scatchard equation can also be expressed in the form of a double reciprocal equation (Klotz plot).

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{nK_a[L]} \quad (8)$$

A plot of $1/\nu$ versus $1/[L]$ should give a straight line with a slope of $1/(nK_a)$ and intercept of $1/n$.

However, a protein can have some non-equivalent and dependent binding sites (n). Thus, the binding of aroma compounds on a site facilitates the binding of other molecules on other sites. The binding mechanism corresponds with cooperation between the sites. The ligand–protein interactions are also represented thermodynamically by the Hill equation (Hill, 1910):

$$\frac{1}{\nu} = \frac{n}{\frac{1}{(K_a[L])^h} + 1} \quad (9)$$

where h is the Hill coefficient reflecting interactions between individual binding sites within their population; h is not necessarily an integer (O'Keefe *et al.*, 1991). The double reciprocal form of the Hill equation was used to determine the binding parameters (n, K_a and h).

$$\frac{1}{\nu} = \frac{1}{n(K_a[L])^h} + 1 \quad (10)$$

The binding parameter sites were assessed by non-linear regression from the plot $1/\nu$ versus $1/[L]$ using the SAS software. The sodium caseinate solution had a concentration of 5 g.litre^{-1} and the aroma compounds concerned were ethyl butanoate and ethyl hexanoate.

RESULTS AND DISCUSSION

Retention of aroma compounds

The volatility of the aroma compounds in water is given in Table 1. For the aroma compounds studied, the more log$P$ increased, i.e. the more apolar the molecule, the higher was K^{∞}_{760}. For the homologous series of esters diluted in water, $\ln K^{\infty}_{760}$ increased linearly with the number of atoms of carbon (correlation coefficient, $r = 0.998$). The results agree with the works of other authors (Buttery *et al.*, 1969; Chung & Villota, 1989). This phenomenon is due to a decrease in the polar character of the molecules when the chain carbon length increases. Diacetyl and ethyl acetate displayed a different behaviour for an equal number of atoms of carbon: volatility of the ester was greater than that of the ketone. Buttery *et al.* (1969) also showed that the vapour–liquid partition coefficient of aroma compounds having the same number of atoms of carbon was higher for esters than for ketones.

The values of the retention of aroma compounds by sodium caseinate solutions are presented in Table 2. In the case of diacetyl and ethyl acetate, the vapour–liquid partition coefficients were not different ($P = 0.05$) in water and in a 5 g.litre^{-1} sodium caseinate solution. Ethyl acetate was the only aroma molecule which was not bound on sodium caseinate for a protein content of 50 g.litre^{-1}. Retention of ethyl hexanoate was the highest and increased with sodium caseinate content. For the homologous series of ethyl esters, the amount of bound volatile compounds increases with their hydrophobicity. Retention varies with the chemical function of the compound: for diacetyl, more hydrophilic than ethyl acetate, it was higher with sodium caseinate at 50 g.litre^{-1} than at 5 g.litre^{-1}. Thus, the binding of aroma compounds on sodium caseinate depends not only on the volatile nature as shown in many studies but also on the protein content. The works dealing with interactions between aroma substances and proteins have included bovine serum albumin (Damodaran & Kinsella,

Table 1. Hydrophobicity constant (log$P$) and vapour–liquid partition coefficient (K^{∞}_{760}) of aroma compounds in water

Aroma compound	logP^a	K^{∞}_{760}
Diacetyl	−2.0	0.60^b ± 0.03
Ethyl acetate	0.6	9.6^c ± 0.8
Ethyl butanoate	1.7	22.4^c ± 1.3
Ethyl hexanoate	2.8	58.2^c ± 3.8

[a]Calculated by the method of Rekker (1977). [b]Determined by headspace analysis. [c]Determined by exponential dilution.

Table 2. Vapour–liquid partition coefficient (K^{∞}_{760}), relative volatility (I) and percentage of retention (r) of aroma compounds in 5 and 50 g.litre^{-1} sodium caseinate aqueous solutions

Aroma compound	5 g.litre^{-1} aqueous solution of sodium caseinate			50 g.litre^{-1} aqueous solution of sodium caseinate		
	K^{∞}_{760}	I	r(%)	K^{∞}_{760}	I	r(%)
Diacetyl	$0.60^{a} \pm 0.02$	1	0.0	$0.48^{b} \pm 0.01$	0.8	22.6
Ethyl acetate	$10.0^{a} \pm 0.4$	1	0.0	$10.0^{a} \pm 0.5$	1	0.0
Ethyl butanoate	$21.0^{a} \pm 0.3$	0.94	6.2	$20.0^{a} \pm 1.0$	0.9	10.0
Ethyl hexanoate	$36.0^{a} \pm 2.5$	0.62	38.0	$22.6^{b} \pm 1.1$	0.4	61.2

The values with the same suberscript are not significantly different ($P = 0.05$).

1980), soy proteins (Damodaran & Kinsella, 1981*a*; Damodaran & Kinsella, 1981*b*) and β-lactoglobulin (O'Neill & Kinsella, 1987). Reports on sodium caseinate are less numerous (Sadafian & Crouzet, 1986; Le Thanh *et al.*, 1992; Farès, 1987). It seems that the binding of a physical or chemical nature mainly depends on the properties of the volatile substance (Kinsella, 1990).

Other authors also observed variations of relative volatility with sodium caseinate concentration. Farès (1987) demonstrated that acetone, ethyl acetate, propanol and *n*-hexanol were retained by sodium caseinate. For diacetyl, Reineccius & Coulter (1969) noted, in the presence of sodium caseinate (10%), a decrease in the headspace concentration of 25%. However, Farès (1987) obtained, by exponential dilution, a release of diacetyl proportional to caseinate content. These contradictory results are more due to the protein solution than the method used for the measurement of the vapour–liquid partition. Indeed, the sodium caseinate used by Farès contained a high salt concentration, and many studies have shown that salts increase volatility of aroma compounds (Voilley *et al.*, 1977; Land & Reynolds, 1981). Consequently, the two phenomena of retention by the proteins and release by salts must have entered into competition.

Moreover, the decrease in relative volatility of ethyl hexanoate and, to a lesser extent, ethyl butanoate with the decrease in caseinate content seemed to be an exponential type. Sadafian & Crouzet (1986) obtained the same kind of plot for terpenic compounds and concluded the presence of two types of binding to sodium caseinate. Indeed, for low concentrations in protein (< 2%, w/w), the decrease in relative volatility reflected an increasing retention of aroma compounds by binding on sites of different affinity (in particular hydrophobic sites of strong affinity). When the protein content increased (> 2%), the protein–protein interactions were more important. This resulted in a progressive decrease in the number of hydrophobic sites available for the aroma. Consequently, for a concentration higher than 2%, aroma compounds could interact more with the sites of lower affinity. Thus, the study of variations of K^{∞}_{760} according to the protein content advances the hypothesis of the hydrophobic nature of aroma–protein interactions. Other hydrophobic interactions were demonstrated with different proteins, for instance bovine serum albumin (Damodaran & Kinsella, 1980). Beyeler & Solms (1974) suggested both hydrophobic and electrostatic forces between soy proteins or bovine serum albumin and aroma compounds. Aldehydes and diacetyl can interact (partly reversibly) by Van der Waals forces and hydrogen bounds, and partly irreversibly by covalent bond formation (Hansen & Heinis, 1992; Farès, 1987).

Interaction parameters between aroma compounds and sodium caseinate

The binding curve for each compound at 25°C is illustrated in Fig. 1. The saturation of binding sites did not seem to have been complete because at higher concentrations of the aroma compounds, the compounds were no longer soluble. However, the binding curves had a sigmoïdal pattern: the ratio ν slowly increased

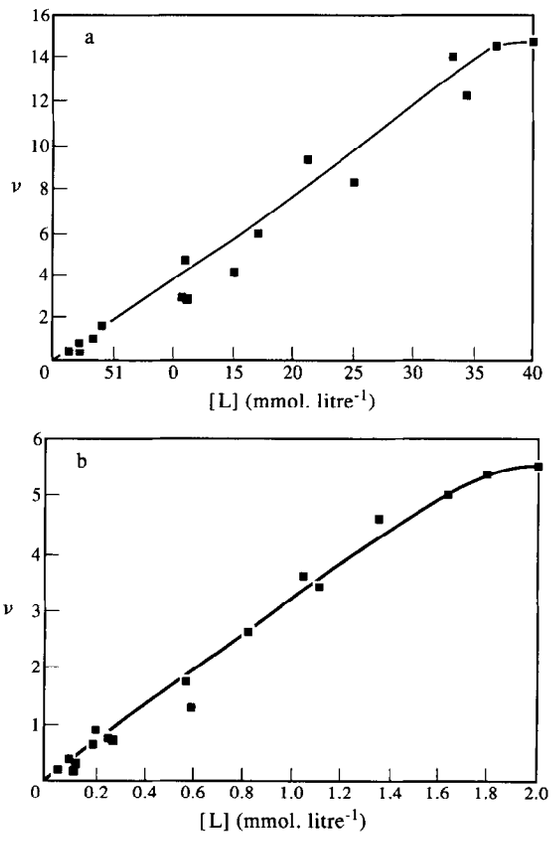

Fig. 1. Binding curve of ethyl butanoate (a) and ethyl hexanoate (b) to sodium caseinate in aqueous phase at 5 g.litre^{-1}.

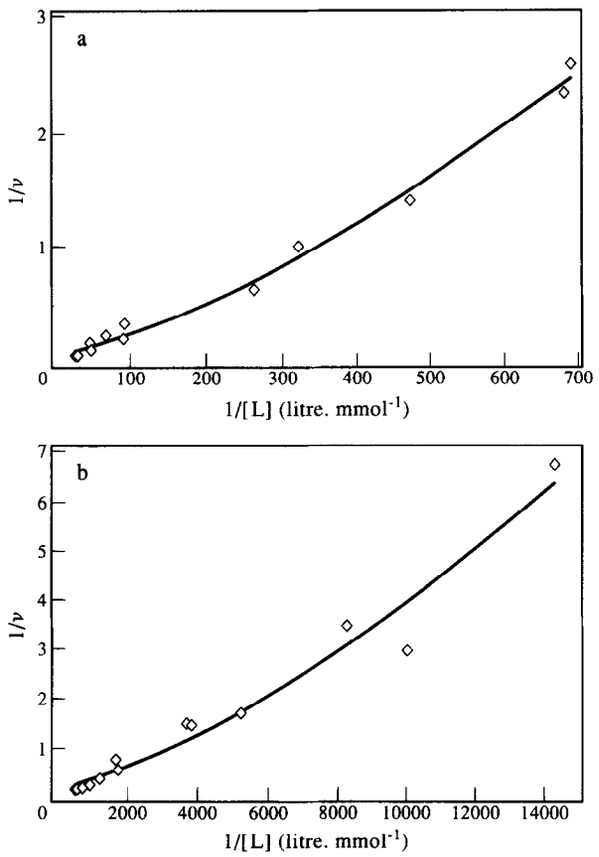

Fig. 2. Hill plot of ethyl butanoate (a) and ethyl hexanoate (b) in 5 g.litre^{-1} aqueous phase of sodium caseinate.

with the concentration [L]. Moreover, the plot of $\nu/[L]$ versus ν (Scatchard plot) and $1/\nu$ versus $1/[L]$ (Klotz plot) did not give a straight line. The double reciprocal equation of the Hill model could be applied and gave the Hill plot. The plot obtained was not linear (Fig. 2). The binding curve and Hill plot demonstrate the presence of a cooperative effect between binding sites. The values of n, K_a and h, determined by non-linear regression, are given in Table 3. We noted a higher number of binding sites for ethyl butanoate ($n=11$) than for ethyl hexanoate ($n=3$). Inversely, the binding constant K_a was lower for butanoate ($K_a=66$) than for hexanoate ($K_a=2500$). These parameters are nearly identical to the ones given by other authors with the Scatchard model: n was estimated at about 10 and K_a at about 100 (Damodaran & Kinsella, 1980; Damodaran & Kinsella, 1981*a*; Damodaran & Kinsella, 1981*b*; O'Neill & Kinsella, 1987; Farès, 1987). The parameter h indicates the extent of cooperative effect. Indeed, if no interaction between binding sites was present, h could be 1. If cooperation was infinite, n would be equal to the total number of binding sites, n (Rawn, 1990). So, we suppose that the cooperative phenomenon is weak between the binding sites because h was nearly equal to 1 (h was 1.4 and 1.6 for ethyl butanoate and hexanoate, respectively).

Table 3. Interaction parameters (n, K_a and h) obtained by non-linear regression

Aroma compound	Parameters	Mean value
Ethyl butanoate	n	11
	K_a	66
	h	1.4
Ethyl hexanoate	n	3
	K_a	2500
	h	1.6

Cooperation has been proved for other proteins. Solms *et al.* (1973) suggested that the binding of ligands to hydrophobic regions of soy proteins caused protein unfolding, with the creation of new binding sites. Damodaran & Kinsella (1980) demonstrated that the aroma compound binding altered the conformation of bovine serum albumin, hence the conformation of the protein may affect its binding characteristics. To substantiate such a cooperative effect, the determination of the protein surface hydrophobicity, reactive lysine (indicating loss of available lysine after reaction with an aroma compound) and turbidity should indicate that structural changes occurred as a result of aroma binding.

CONCLUSION

The retention of the aroma compounds in sodium caseinate aqueous solution was determined from their volatility measured by headspace analysis or exponential dilution. The results obtained show the influence of the nature of the aroma compounds (diacetyl, ethyl acetate, butanoate and hexanoate) on the retention with sodium caseinate. At 50 g.litre^{-1} of sodium caseinate, the relative volatility (I) of these compounds is lower than at 5 g.litre^{-1} (except for ethyl acetate), which proves the presence of interactions between the aroma compounds and the medium.

By applying the thermodynamic models of Scatchard, Klotz and Hill in order to characterize the interactions between volatile substances and sodium caseinate, we have shown that the values of n and K_a obtained by the Hill plot are of the same order as the values given in the literature. The Hill coefficient near to 1 indicates the presence of a weak cooperative effect. The phenomenon must be confirmed by using spectroscopic techniques. For a better understanding of aroma molecule–protein interactions, this quantitative work must be completed by a qualitative study to ensure an effective food formulation.

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