INTERACTIONS BETWEEN FLAVOUR COMPOUNDS AND MILK PROTEINS

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CHAPTER ONE

INTRODUCTION

Flavour is an important quality factor of food products. Flavour binding to protein may change the flavour profile of food products, especially for new products. In many new food products, additional proteins are often added to make the food more nutritious. However, sometimes the binding between flavour and protein can make the food lose its flavour balance and make the food lack flavour.

The interaction of flavour with protein has been the focus of research for a long time (Franzen and Kinsella, 1974). Much research has focused on β-lactoglobulin, not only because the binding can be easily detected but also because the well-studied structure of β-lactoglobulin makes it possible to investigate in detail the chemistry of flavour–protein binding (Papiz et al., 1986).

Several techniques have been used to investigate the interaction between β-lactoglobulin and flavours: these include equilibrium dialysis (O'Neill and Kinsella, 1987), static headspace (Charles et al., 1996), fluorimetry (Muresan and Leguijt, 1998), affinity chromatography (Sostmann and Guichard, 1998), dynamic coupled liquid chromatography (Jouenne and Crouzet, 1998) and exclusion chromatography (Pelletier et al., 1998). The results published for the same flavour compound interacting with β-lactoglobulin under certain experimental conditions were different, mostly because of the different techniques used (Guichard & Langourieux, 2000). Hence determing an efficient and reliable method of analysis is important.

Solid-phase microextraction (SPME) has been used in flavour analysis for more than 10 years (Arthur and Pawliszyn, 1990; Martos and Pawliszyn, 1997). It is a simple analytical tool and is relatively cheap. It is a solventless extraction method and increased sensitivity

and precision can be obtained with a suitable fibre and optimal experimental conditions (such as the temperature, the extraction time etc.). SPME is a convenient method for an experienced person. However, the experimental conditions must be optimized at the beginning of the analysis of a new system. The SPME process is very complex: adsorption, diffusion and desorption occur during SPME. As each process is important for the analysis, SPME is sometimes regarded as a technique that is not convenient for an inexperienced person.

Although there is much published work on the binding between β-lactoglobulin and flavours, in the food industry, commercial protein products are not pure products like β-lactoglobulin, and therefore establishing how protein products interact with flavour compounds is of real interest to the food industry. There is no published work on the use of SPME to investigate dairy protein–flavour binding.

This investigation on the interaction between flavour and dairy protein products (such as whey protein isolate and sodium caseinate) using SPME was a pilot study in the field and the results obtained will be useful to the food industry.

CHAPTER TWO

LITERATURE REVIEW

2.1 Flavour–matrix interactions

Flavours are usually volatile chemical compounds that occur in very low concentrations (parts per million (ppm) or even parts perbillion (ppb)) in foods. These flavour compounds are not just mechanically trapped; they often interact with the matrix.

Lipids may be the most important factor affecting the flavour profile of a food product, because many of the naturally occurring flavour compounds are lipid soluble. However, as the development of new food products and ingredients has increasingly been aimed at enhanced nutrition, improved flavour and low fat, an understanding of the interactions between flavour and the food matrix will enable the manufacture of fat-reduced and fat-free products with satisfactory flavour profiles. It is also known that many non-volatile food ingredients, such as proteins and starch, influence the flavour of a food product (Wassef and Nawar, 1971; Franzen and Kinsella, 1974; Gremli, 1974). Early research focused on some proteins that bind flavour compounds in an easily detectable manner; soy protein and β-lactoglobulin were the proteins of interest. Recently, ovalbumin and 11S globulin have been studied (Adams et al., 2001; Semenova et al., 2002). The quantitative binding parameters (the total number of binding sites in the protein molecule, the intrinsic binding constant and the Gibbs' free energy of binding) of some flavours have been published (O'Neill and Kinsella, 1987).

2.2 Interactions of volatile flavours in aqueous solution

The concentration of flavour above the surface in aqueous solutions (headspace) can be easily theoretically predicted. From gas–liquid equilibrium (GLE) theory, the partition coefficient between the gaseous and liquid phases (K^i_{gl}) for a volatile compound (i), for

example, can be either measured directly in a system by determining the concentrations of (i) in the gaseous and liquid phases (equation 2-1) or calculated from fundamental physicochemical parameters according to equation 2-2:

$$K_{gl}^{i} = \frac{C_i^g}{C_i^l} \qquad 2\text{-}1$$

$$K_{gl}^{i} = \left[\frac{\gamma_i P_i^0(T)}{P_T}\right] \cdot \frac{\overline{V}_l}{\overline{V}_g} \qquad 2\text{-}2$$

where $P^o_i(T)$ is the vapour pressure (Pa) of pure (i) at temperature T, P_T, is the total pressure (Pa) in the gaseous phase and V_l and V_g are the molar volumes ($m^3.mol^{-1}$) of the liquid and the gaseous phase respectively. Normally, as the volatile compound is highly diluted in the liquid phase, the activity coefficient γ_i can be assumed to be independent of the concentration of the volatile compound in the liquid, and is equal to a constant value γ_i^{∞}. In this case, the product $\gamma_i^{\infty} \cdot P^o_i(T)$ is a constant (Henry's constant), so that the value of K^i_{gl} depends only on the temperature. In all but the simplest systems, γ_i depends on the other components present in the aqueous phase and its value varies from the ideal value of 1. Although partition data can be calculated, experimental data are also required to check that the value chosen for γ_i is appropriate and to ensure that no other unknown interactions are occurring.

The partition of volatile compounds is affected by soluble non-volatile flavour components such as sugars, acids, proteins and salts. There have been many papers on the subject since Buttery and his group (1971) reported changes in the headspace concentration of a flavour compound caused by its interactions with food components. In the dairy field, an understanding of the protein–flavour interaction in aqueous solution will be very useful for new product development because many products are water based.

It is worth pointing out that food systems are not usually equilibrium systems, because not enough time is available for the equilibrium to be reached in the mouth while the food is consumed. This particular case comes within another area of flavour research (flavour release) and is not discussed in this work.

2.3 Mechanism of binding of ligands to β-lactoglobulin

β-Lactoglobulin is the most used dairy protein in flavour binding research. Flavour–protein binding is actually related to the chemical properties and the structures of both the flavour compounds and the proteins. For example, it has been pointed out that β-lactoglobulin binds some hydrophobic ligands (Cho et al., 1994) in its hydrophobic pocket. Therefore, an understanding of the structure of the molecule is vital for understanding of the binding mechanism.

2.3.1 The structure of β-lactoglobulin and the binding sites

β-Lactoglobulin is one of the major proteins in bovine milk, accounting for more than 50% of the total whey proteins and 10% of the total proteins of milk (Hambling et al., 1992). The amino acid sequence (Swaisgood, 1982) of bovine β-lactoglobulin consists of 162 residues per monomer. The isoionic point of bovine β-lactoglobulin is pH 5.2 (Cannan et al., 1942). It is a typical globular protein and its structure has been extensively studied (Papiz et al., 1986). β-Lactoglobulin is a highly structured protein: optical rotary dispersion and circular dichroism measurements show that, at pH in the range 2–6, β-lactoglobulin consists of 10–15% α-helix (Frushour and Koenig, 1975), 43% β-sheet and 47% unordered structure (Townend et al., 1967), including β-turns. The tertiary structure of β-lactoglobulin is a very compact structure in which the β-sheets occur in a β-barrel-type or calyx (Figure 2.1). Each monomer exists almost as a sphere with a diameter of about 3.6 nm. There is a hydrophobic pocket in the tertiary structure of β-lactoglobulin (Papiz et al., 1986). Many hydrophobic molecules including retinol (vitamin A) and free fatty acids have been shown to bind inside this pocket (Fugate and Song, 1980; Puyol et al., 1991). Similarly, some hydrophobic flavours have been found to bind to β-

lactoglobulin (Futterman and Heller, 1972); however, unlike retinol and free fatty acids the binding positions for these flavours have not been reported.

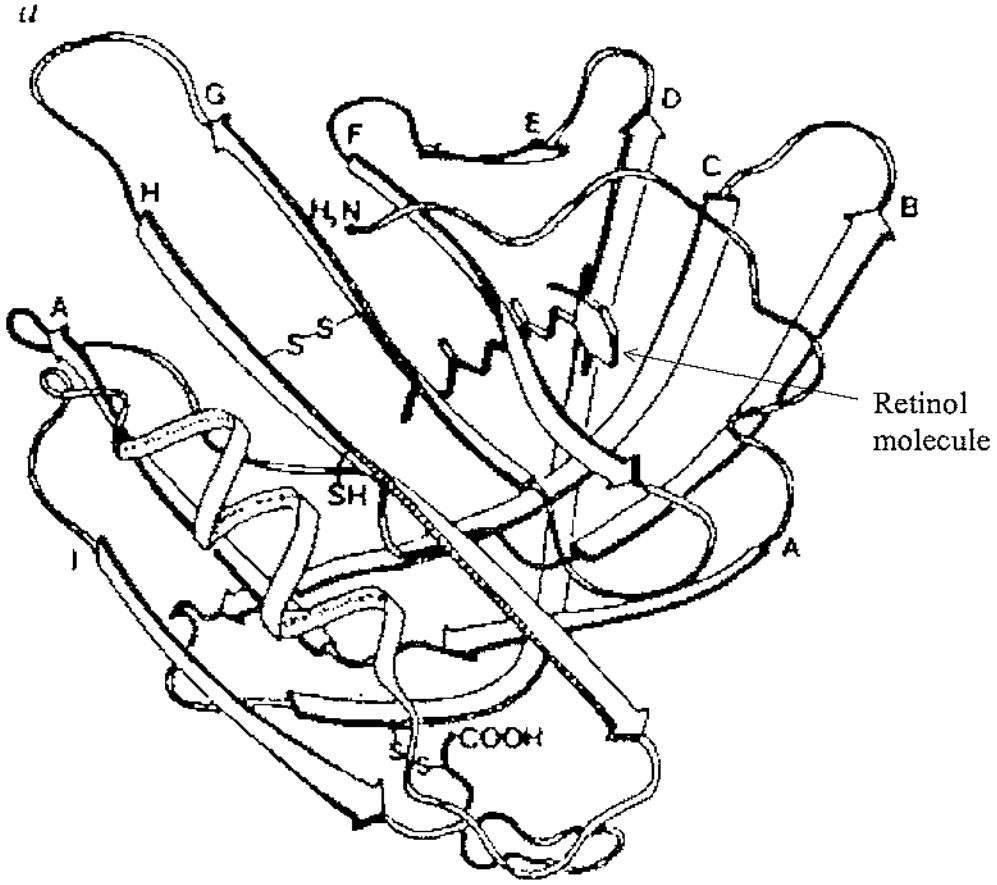

Figure 2.1. Schematic representation of the tertiary structure of bovine β-lactoglobulin, showing the binding of retinol; the arrows indicate antiparallel β-sheet structures (from Papiz et al., 1986).

It can be assumed that the flavour should bind within the hydrophobic pocket; however, binding of the flavour could take place not only inside the pocket but also at some other sites on the surface of the molecule. Narayan and Berliner (1997) found at least two different types of simultaneous hydrophobic ligand binding sites and pointed out that there are also several weaker sites for 5-doxylstearic acid. Sawyer and coworkers (1998) identified three distinct sites with the two main sites being the retinol site (in the centre of the β-barrel) and another site on the outer surface close to the helix. They described the

three kinds of binding sites as the retinol binding site, the fatty acid binding site and the polar aromatic binding site. However, it is still unclear where the binding sites for flavour compounds are on β-lactoglobulin.

2.3.2 Effect of pH on ligand binding

pH is an important factor in the binding of ligands to β-lactoglobulin (Sostmann and Guichard, 1998). It has been found that the quaternary structure of β-lactoglobulin changes with changes in pH.

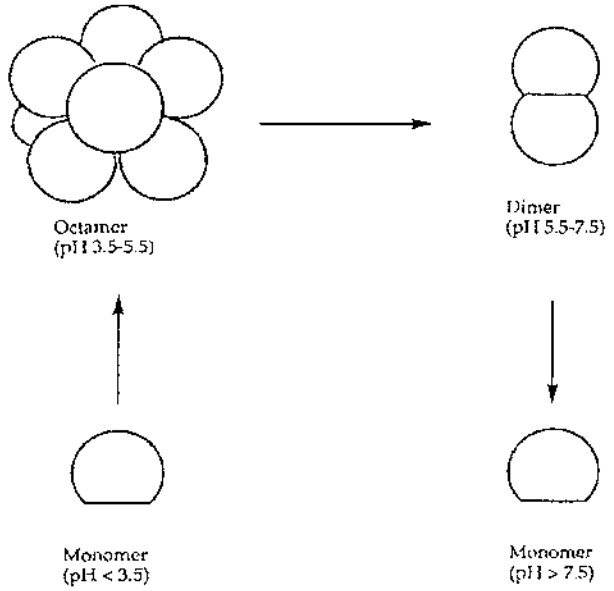

Figure 2.2. The effect of pH on the quaternary structure of β-lactoglobulin (Fox and McSweeney, 1998)

β-Lactoglobulin is a dimer at physiological pH (Fox and McSweeney, 1998). Below pH 3.5, β-lactoglobulin dissociates to monomers with a molecular mass of 18 kDa. Between pH 3.5 and pH 5.2, β-lactoglobulin occurs as octamers of molecular mass 144 kDa. Between pH 5.5 and pH 7.5, β-lactoglobulin forms dimers of molecular mass 36kDa.

Above pH 7.5, β-lactoglobulin dissociates to monomers. The association of β-lactoglobulin as a function of pH is summarized in Figure 2.2.

It is likely that the binding ligands will be affected by the changing structure of β-lactoglobulin as the pH changes. Jouenne and Crouzet (2000) proposed the relationship between structure and binding shown in Figure 2.3.

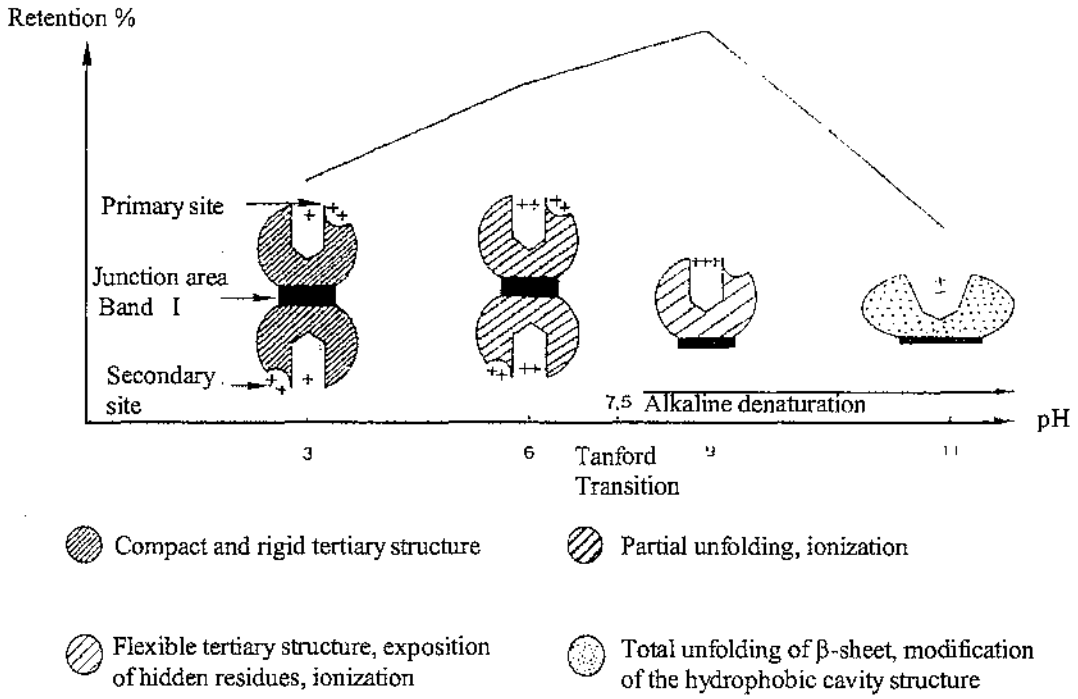

Figure 2.3. Modification of aroma compound retention according to structural modifications of β-lactoglobulin with pH between 3 and 11. Site accessibility: (+) medium; (++) high; (+++) very high; (±) more or less important (Jouenne and Crouzet, 2000).

Around pH 3, the tertiary structure of β-lactoglobulin is compact and rigid. The accessibility of the binding sites is medium for the first binding site and high for the second binding site. Around pH 6, the tertiary structure of β-lactoglobulin is flexible, the hidden residues are exposed and the molecules are partly ionized. The accessibilities of the binding sites are high for both the first and second binding sites. Around pH 9, the

tertiary structure of β-lactoglobulin is partially unfolded and the molecules are ionized; the accessibility of the first binding site is very high and the second binding site is damaged. Above pH 11, the tertiary structure of β-lactoglobulin is totally unfolded β-sheet and the hydrophobic cavity structure is modified. The accessibility of the first binding site is more or less important.

2.4 Equilibrium binding phenomena

The most commonly used method for interpreting binding data makes use of the Scatchard (1949) equation. This model is based on two assumptions: that each of the protein molecules has *n* indistinguishable and independent binding sites; that the binding is an equilibrium (reversible) binding. For a system consisting of protein and flavour, when equilibrium is reached, the concentrations of protein and flavour are *P* and *L* respectively, and the equilibrium for one binding site can be expressed as

$$P + L \rightleftharpoons PL$$

According to equilibrium theory,

$$K = \frac{[PL]}{[P][L]} \qquad 2\text{-}3$$

where *K* is the equilibrium constant.
Therefore

$$[PL] = K[P]\cdot[L] \qquad 2\text{-}4$$

As $P_{total}=P+PL$,

$$[PL] = K[L]\cdot[P_{total} - PL] \qquad 2\text{-}5$$

or

$$\frac{[PL]}{[P_{total}]} = \frac{K[L]}{1+K[L]} \qquad 2\text{-}6$$

Now,

$$\frac{[PL]}{[P_{total}]} = v \qquad 2\text{-}7$$

where v is the number of moles of ligand bound per mole of total protein. Thus:

$$v = \frac{K[L]}{1+K[L]} \qquad 2\text{-}8$$

If there are n independent binding sites, the equation of the extent of binding is simply n times that for a single site with the same intrinsic binding constant, K. Hence:

$$v = \frac{nK[L]}{1+K[L]} \qquad 2\text{-}9$$

or

$$v/L = Kn - Kv \qquad 2\text{-}10$$

A plot of v/L against v gives the Scatchard plot. The equation may be rearranged to give:

$$\frac{1}{v} = \frac{1}{nK[L]} + \frac{1}{n} \qquad 2\text{-}11$$

A plot of $1/v$ againt $1/L$ gives rise to the Klotz, or double reciprocal plot, in which the slope of the line is $1/nK$ and the intercept is $1/n$ (Klotz, 1946).

However, sometimes the plot will not be a straight line, because the different binding sites are not independent. In these circumstances, the Hill equation is applied (Hill, 1910):

$$v = \frac{n}{\frac{1}{(K[L])^h} + 1} \qquad 2\text{-}12$$

$$\frac{1}{v} = \frac{1}{n(K[L])^h} + \frac{1}{n} \qquad 2\text{-}13$$

where h is the Hill coefficient reflecting the co-operation between the sites. The other parameters have the same meaning as in the Klotz plot.

2.5 Published results on flavour–protein binding

β-Lactoglobulin has been well studied in flavour binding as a pure protein. However, as well as pure β-lactoglobulin, other dairy products such as whey proteins and casein are of great interest.

Several methods have been used to analyse the interaction between β-lactoglobulin and flavours. Up to now, the results reported have been more or less different because different methods have been used, which is an indication that not all the methods available are reliable. Some of the published results selected from the literature can be found in Table 2.1 and illustrate how much work still needs to be done with respect to technique development in spite of the many papers published in this field. Binding parameters detected under different conditions (such as equilibrium temperature, pH), using different methods, will have different meaning. It is very difficult to determine out which method is the most reliable and appropriate to use.

Table 2.1. Comparison of binding constants obtained with different methods

Flavours	Method	Conditions	Binding sites n	Binding constants K_b
Benzaldehyde	Affinity -chromatography (Sostmann and Guichard, 1998)	pH = 3 at room temperature	n.d.	341*
	Exclusion chromatography (Pelletier et al., 1998)	pH = 3 at room temperature	0.3	1800 M^{-1}
	Static headspace (Charles et al., 1996)	pH = 3 at 30°C	0.57	533 M^{-1}
	Fluorescence (Muresan and Leguijit, 1998)	Not mentioned	0.8	2.5×10^{6} M^{-1}
2-Heptanone	Equilibrium dialysis (O'Neill and Kinsella, 1987)	pH = 6.7 at 25°C	1	150 M^{-1}
	Affinity -chromatography (Sostmann and Guichard, 1998)	pH = 3 at room temperature	n.d.	465 *
2-Octanone	Equilibrium dialysis (O'Neill and Kinsella, 1987)	pH = 6.7 at 25°C	1	480 M^{-1}
	Affinity -chromatography (Sostmann and Guichard, 1998)	pH = 3 at room temperature	n.d.	1287 M^{-1}
2-Nonanone	Equilibrium dialysis (O'Neil and Kinsella, 1987)	pH=6.7 at 25°C	1	2440 M^{-1}
	Affinity-chromatography (Sostmann & Guichard, 1998)	pH = 3 at room temperature	n.d.	3629 *
	Equilibrium dialysis (Muresan et al., 1999)	pH=3 at room temperature	0.5	1756 M^{-1}
	Static headspace (Charles et al., 1996)	pH = 3 at 30°C	0.5	1667 M^{-1}
2-Nonenal	Affinity -chromatography (Sostmann and Guichard, 1998)	pH = 3 at room temperature	n.d.	4433*
α-Ionone	Fluorescence (Dufour and Haertlé, 1990)	Not mentioned		0
	Affinity -chromatography (Sostmann and Guichard, 1998)	pH = 3 at room temperature	n.d.	13456 *

Table 2.1 (continued)

β-Ionone	Fluorescence (Dufour and Haertlé, 1990)	Not mentioned	1.08	1670000 M^{-1}
	Fluorescence (Muresan and Leguijit, 1998)	Not mentioned	0.8	1.9×10^{6} M^{-1}
	Affinity -chromatography (Sostmann and Guichard, 1998)	pH = 3 at room temperature	n.d.	19143*
	Equilibrium dialysis (Muresan et al., 1999)	pH = 3 at room temperature	0.9	11700 M^{-1}

* The binding constant obtained using affinity chromatography is presented as the product nK_b.

n.d. = not determined.

Milk protein products, such as sodium caseinate (Na-CN) and whey protein concentrate (WPC) have also been investigated for their interaction with flavour using different methods. In contrast to the methods mentioned above, sensory panels have been used. Hansen and Heinis (1991) used sensory evaluation to investigate the interactions of Na-CN and WPC with vanillin. The vanillin–Na-CN and vanillin–WPC solutions contained 78.5 ppm vanillin, 2.5% sucrose, and 0.125, 0.25 or 0.5% Na-CN or WPC. A 12-member trained panel evaluated the samples at room temperature and found that the vanillin flavour intensity was moderately less than the vanillin reference for all Na-CN levels. As the WPC concentration increased from 0.125 to 0.5%, the vanillin flavour intensity decreased from moderately less than the vanillin reference to much less than vanillin reference.

Fares et al. (1998) studied the physico-chemical interactions between aroma compounds and Na-CN by complementary techniques involving the protein in aqueous solution at 25 or 75 g/L (exponential dilution and equilibrium dialysis) or in a solid state (sorption and infrared spectroscopy). Diacetyl and benzaldehyde were found to interact with Na-CN through strong and weak bonds in aqueous solution. Although no retention of acetone and

ethyl acetate was found in aqueous solution, the compounds that sorbed best to Na-CN in the solid state were acetone and ethyl acetate.

2.6 Analysis methods

As mentioned previously, several instrumental methods for detecting flavour–protein binding have been reported. Each method has its benefits and shortfalls. The basic principles of these methods are discussed below.

2.6.1 Fluorescence

Fluorescence can be used to detect the concentration of protein in solution. However, when some flavour compounds bind to protein, the fluorescence intensity changes at a fixed wavelength. For example difference in fluorescence intensity at 332 nm between complexed and free β-lactoglobulin has been monitored to determine apparent dissociation constants of various ligands complexed by the protein (Fugate and Song, 1980; Dufour and Haertlé, 1990). It was assumed that the change in fluorescence depended on the amount of protein–ligand complex, and the apparent dissociation constants were obtained according to the method of Cogan et al. (1976):

$$K_d' = (\alpha/1-\alpha)[B-nP_o(1-\alpha)] \qquad 2\text{-}14$$

which can be rearranged to

$$P_o\alpha = 1/n[B(\alpha/1-\alpha)]-(K_d'/n) \qquad 2\text{-}15$$

where K_d' is the apparent dissociation constant, n is the number of independent binding sites and P_o is the total protein concentration. α is defined as the fraction of free binding sites in total binding sites on the protein molecules. The value of α was calculated for every desired point on the titration curve of fluorescence intensity versus total ligand concentration using the relation:

$$\alpha = (F_o - F)/(F_o - F_{min}) \qquad 2\text{-}16$$

where F represents the fluorescence intensity at the certain total ligand concentration B, F_o is the fluorescence of the free protein and F_{min} represents the fluorescence intensity upon saturation of all the protein molecules. A plot of $P_o\alpha$ against $B(\alpha/1-\alpha)$ yields a straight line with an intercept of K_d'/n and a slope of $1/n$.

Dufour and Haertlé (1990) studied interactions of β-ionone and related flavour compounds with β-lactoglobulin. In their work, the independent binding sites and dissociation constants were calculated (see Table 2.1). Muresan and coworkers (1999) published their binding constants and binding sites. It can be seen in Table 2.1 that the binding constants obtained from fluorescence measurements are much higher than those obtained using other techniques.

2.6.2 Affinity chromatography

Affinity chromatography is a well–known method that immobilizes the protein in the column. Sostmann and Guichard (1998) first used this technique to investigate flavour–protein binding. The most important step in the experimental process is immobilization of the protein in the column. After the immobilization is achieved, the determination is fast and convenient.

Calculation of the binding constants (K_b), given by Nilsson and Larson (1983), is as follows:

$$K_b = (t_R - t_o)/c_p t_o \qquad 2\text{-}17$$

where c_p is the concentration of protein, t_o is the void time and t_R represents the retention time of the compound. The void time can be determined by injection of water on to the column and can be used for calculation of the column void volume.

Retention times can be determined by two methods because the support material (normally silica-diol or silica gel) is not inert to all flavour compounds, which causes some compounds to be retained by the column: (i) experimentally, by applying a chromatographic material treated using the same conditions as for immobilization of the protein, but without protein in the treatment solution; (ii) by determining the linear relationship between protein content and retention time for each compound at more than five different protein concentrations and calculating the retention time for zero protein concentration. The two methods should give results that are in good agreement.

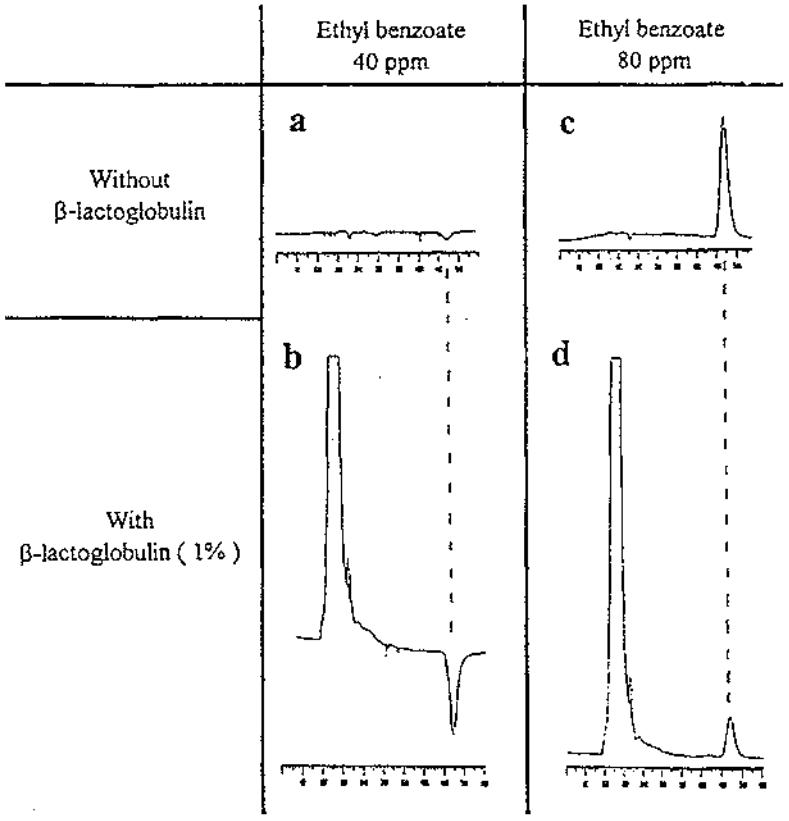

Figure 2.4. Hummel and Dreyer type chromatograms obtained for 40 and 80 ppm ethyl benzoate concentration in the eluent: injection of ethyl benzoate 40 ppm without β-lactoglobulin (a) or With β-lactoglobulin (b); injection of ethyl benzoate 80 ppm without β-lactoglobulin (c) or with β-lactoglobulin (d)

One shortfall of this method is that no information about binding sites can be obtained. To overcome that limitation, Pelletier et al. (1998) used the Hummel and Dreyer method (1962) to calculate the number of binding sites. In their study, injection of β-lactoglobulin with the esters led to the observation of a negative peak (Figure 2.4) corresponding to the bound ester. The amount of bound ester was determined by internal calibration. Different concentrations of ester in the eluent (free ester) were used to develop a Klotz plot.

Affinity chromatography is a rapid method for detecting flavour–protein binding. The data obtained are reproducible. The number of binding sites and the affinity constants can be calculated using the Hummel and Dreyer method, when possible.

2.6.3 Headspace techniques

The static headspace technique is based on gas-liquid equilibrium (GLE) theory. When the headspace of the sample is in equilibrium with the sample solution, the concentration of the flavour in the headspace corresponds to the concentration of the free flavour in the sample solution. For detection of the binding, a standard curve is drawn. The response of the standard minus that of the corresponding sample is regarded as the binding response. The concentration of free flavour is calculated using the following equation (O'Keefe et al., 1991):

$$L = (R/T)I \qquad 2\text{-}18$$

where R (mol.l^{-1}) is the measured concentration of flavour in the headspace of the protein solution, T (mol.l^{-1}) is the measured concentration of flavour in the headspace of the solution without protein (standard solution) and I (mol.l^{-1}) is the initial concentration of flavour in the solution (concentration of the standard solution).

2.6.4 Equilibrium dialysis

Equilibrium dialysis (O'Neill and Kinsella, 1987) has been used to determe the binding constants and binding sites of β-lactoglobulin for different flavour compounds. Usually, acrylic cells of equal volume, separated by a membrane and belted together, are put into a waterbath. A defined volume of solution (1 ml for example) containing the flavour compound only is put into one side of the membrane, and the same amount of solution containing protein only is placed into the other side of the membrane. The cells are then shaken for 20–40 h at the required temperature (25°C for example) to attain equilibrium between the flavour and the protein. Aliquots are then removed from each compartment, placed in vials and extracted using an extractant (e.g. isooctane). Gas chromatography is used for quantitative determination of the amount of flavour in each compartment. The difference in the concentrations of the flavour compound in the respective compartments relates to the amount of flavour compound bound by the protein.

Headspace analysis has played a key role in flavour–protein binding research. However, there are still several major experimental limitations (O'Neill, 1994). Generally, because the concentration of flavour compounds in the sample is quite low, a large sample volume is needed for adequate detection, which can affect the chromatographic analysis. To overcome this shortfall, a better analysis technique is needed.

2.7 Solid-phase microextraction technique

Solid-phase microextraction (SPME) is a solventless extraction method (Arthur and Pawliszyn, 1990; Arthur et al., 1992). It is inexpensive and rapid. It can be used both for the headspace of the sample, as the HS-SPME method, and for a liquid sample directly (Yang and Peppard, 1994; Steffen and Pawliszyn, 1996). Because the components in the solution can sometimes affect the analysis results (for example, in a protein solution, protein may be adsorbed to the fibre and make the analysis incorrect), SPME is mostly used with the headspace option.

The principle of the technique is based on adsorption theory. A fibre coated with a defined volume of adsorbent is put into the headspace of the sample for extraction. The extraction needs to be carried out under defined conditions (temperature, sample volume, container shape and volume, time of extraction) to make the analysis reproducible. After extraction, the fibre is transferred into a gas chromatograph for analysis. It is not like a traditional solvent extraction where the sample must be extracted completely to maintain the accuracy of the determination. SPME is a partial extraction method. The amount of analyte remaining after SPME does not affect the result of the SPME determination under defined conditions.

2.7.1 Equilibrium sampling

Figure 2.5 shows the various components of the SPME device. A fibre coated with adsorbent is attached to the plunger of a modified syringe. The fibre is sheathed in a stainless steel septum-piercing needle. When sampling, the septum of the sample is pierced by the needle and the fibre is then pushed out of the sheath. Several different kinds of fibres coated with different materials are now available commercially.

In a closed system consisting of only the sample and the SPME fibre, the following relationship exists if the sampling time is sufficiently long to allow equilibrium to be established between the sample and the fibre:

$$n = \frac{\left(K_{fs} V_f V_s C_o\right)}{\left(K_{fs} V_f + V_s\right)} \qquad 2\text{-}19$$

where n is the mass of flavour compound extracted by the fibre coating, K_{fs} is the partition coefficient of the flavour compound between the fibre and the sample (distribution constant), V_f and V_s are the volumes of the fibre coating and the sample respectively and C_o is the initial concentration of the flavour compound in the sample.

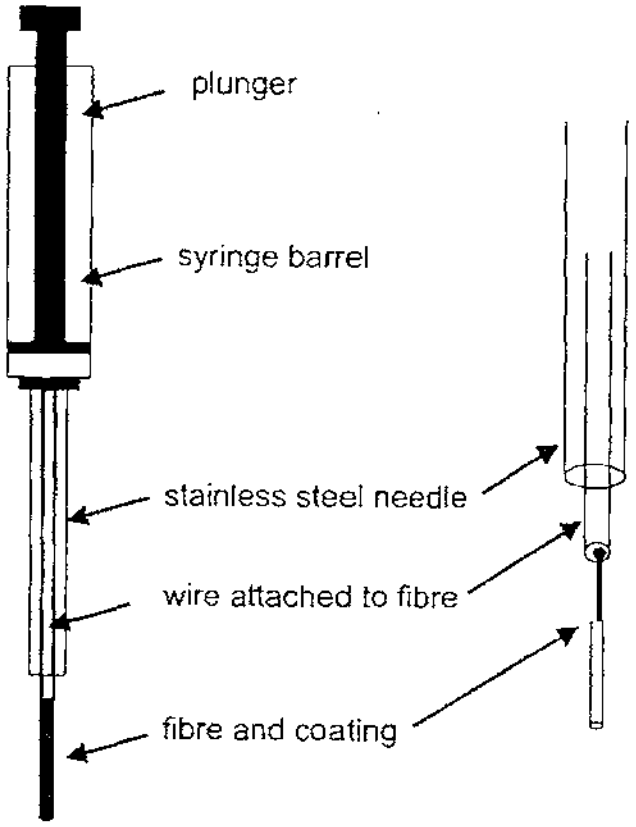

Figure 2.5: Various components of the SPME device

In practice, two different situations can occur. When V_s is much greater than $K_{fs} \cdot V_f$, equation 2-19 can be simplified to:

$$n = K_{fs} \cdot V_f C_o \qquad 2\text{-}20$$

In this case, the results are not affected by the volume of the samples.

If V_s is not very large, the concentration of the samples will change when the samples are extracted by the SPME fibre. In this case, the results will change with the volume of the samples. The same amount of liquid sample and the same size and shape of the container should be used to obtain reproducible results.

For

$$k = V_s/(K_{fs} \cdot V_f + V_s) \qquad 2\text{-}21$$

if the volume of the sample (V_s) is maintained the same during the determination, then k will be a constant. Equation 2-19 can be simplified as:

$$n = k \cdot K_{fs} \cdot V_f C_o \qquad 2\text{-}22$$

The principle of SPME is similar to those of traditional extraction techniques and it is a successful method. Unlike the traditional extraction method, SPME can also be used as dynamic system (rapid sampling).

2.7.2 Dynamic sampling (rapid sampling)

It may take a long time before equilibrium is reached in a system. Figure 2.6 illustrates equilibrium time profiles obtained for the extraction of methamphetamine at various temperatures. Generally, the higher the temperature, the shorter is the equilibrium time (Pawliszyn, 2000).

From Figure 2.6, we can see that, even in the fastest case, at a temperature of 73°C, the equilibrium time for extraction can be less than 20 min; it is not the exact thermodynamic equilibrium time, but it works well for analysis. In the low temperature case, for example at 22°C, the equilibrium time is more than 90 min, which is too long. In this situation, the rapid sampling (dynamic sampling) method should be applied.

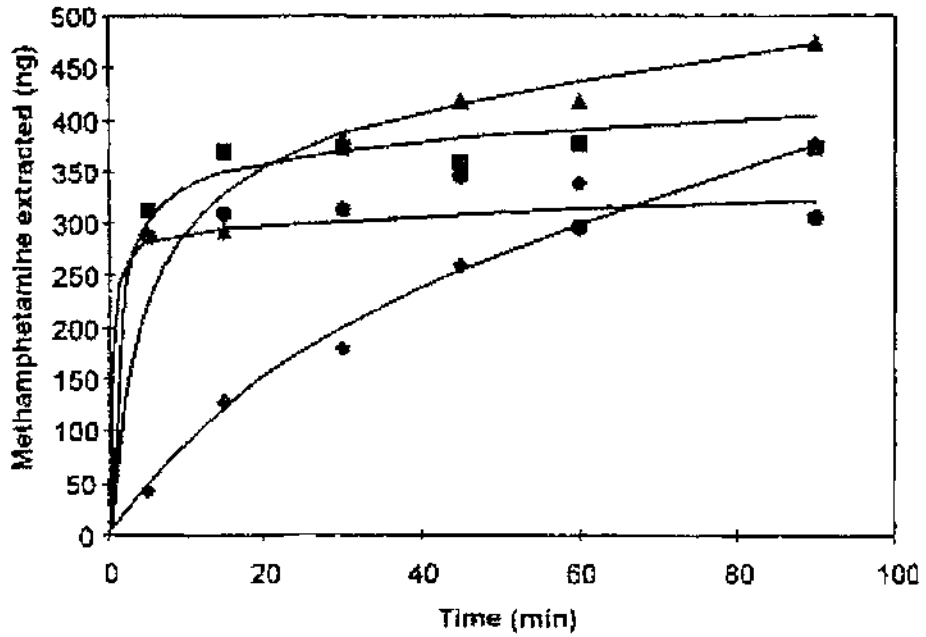

Figure 2.6. Temperature dependence of the adsorption versus time profiles obtained for methamphetamine (Pawliszyn, 2000). Temperature: ◆ 22°C ▲40°C ■ 60°C ● 73°C

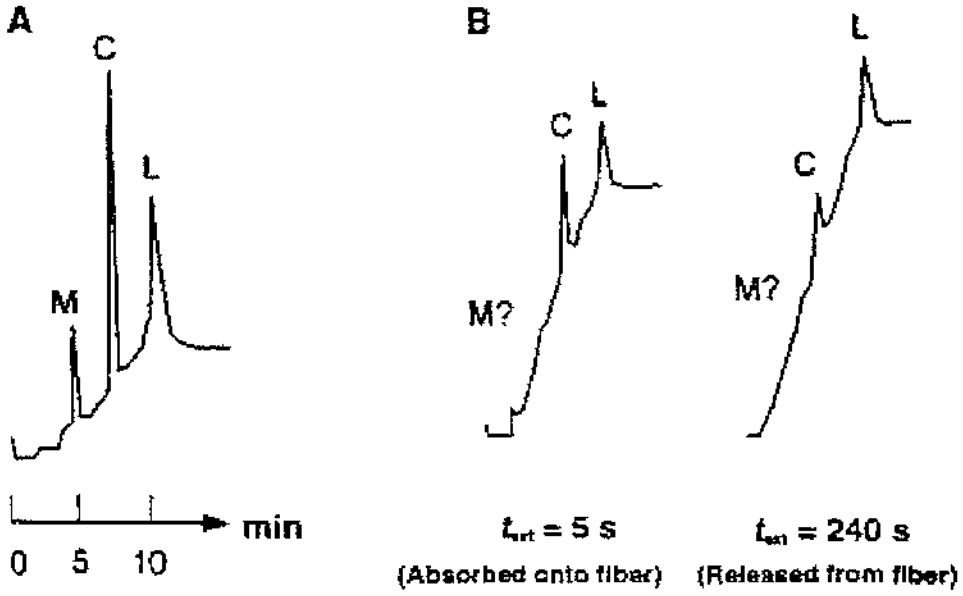

Figure 2.7. Demonstration of myoglobin (M) and cytochrome (C) displacement by lysozyme (L) with time (A) and protein extraction using a polyacrylic-acid-coated fibre (B) (Pawliszyn, 2000).

The other reason for using a short extraction time is for the analysis of a multi-component system. In the case of more than one component in the system, the competition among these components is quite important. If SPME is to be used to extract a mixture that contains, for example, the three compounds myoglobin, cytochrome and lysozyme, only the compound with the weakest affinity will be observed at short extraction times (illustrated in Figure 2.7). When the extraction time is longer, the analytes that have lower affinities will be displaced by the analyte that has higher affinity for the polyacrylic-acid-coated fibre. In this case, lysozyme (having the strongest affinity) will replace the other two compounds during the extraction. This occurs because, at the beginning of the extraction, all the surface of the fibre is free, and all the analytes can be adsorbed by the fibre; when the whole surface of the fibre is occupied by the analytes, the analyte with strong affinity for the fibre will displace the analytes with weaker affinity for the fibre. Therefore, the equilibrium amount extracted may vary with the concentration of both the target and other analytes (Liao et al., 1996; Gorecki et al., 1999).

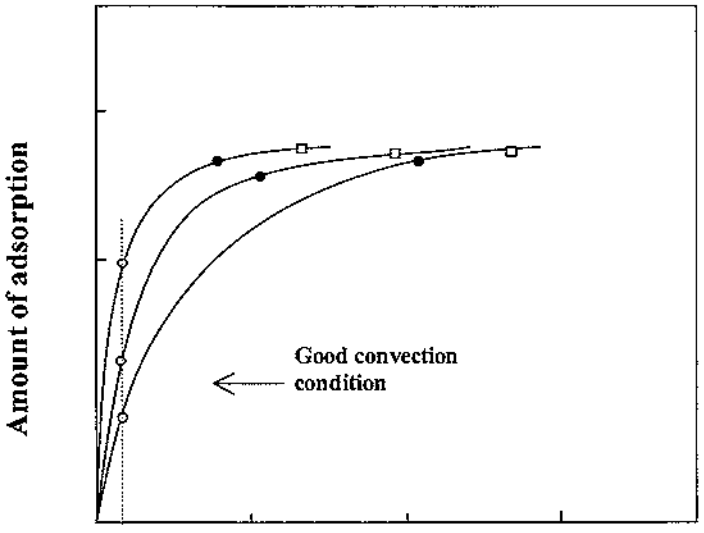

Figure 2.8. Adsorption curve under different convection conditions (imaging).
- **Transit time; ▫ Typical extraction time for equilibrium sampling;**
- **Typical extraction time for dynamic sampling.**

The short-time-exposure HS-SPME measurement (dynamic sampling) is used to overcome the shortfall mentioned above. The short time extraction avoids the replacement by the stronger analytes (Figure 2.7). When performing such an analysis it is critical not only to precisely control the extraction times, but also to monitor the convection conditions to ensure that they are constant. Figure 2.8 illustrates the difference between equilibrium sampling and dynamic sampling. In the equilibrium system, the extraction time must be longer than the transit time. Although equilibrium has not been reached after the transit time, it is precise enough for the determination because the amount of analyte adsorbed changes very slowly with the extraction time after the transit time.

In the dynamic system, the extraction time is very important for the analysis. It can be seen in Figure 2.8 that the slope of the adsorption curve is quite steep in dynamic sampling. A small difference in the extraction time may cause a big difference in the amount adsorbed. The convection condition is also an important factor that must be taken into account. In Figure 2.8, the same extraction time, but under different convection conditions, would result in totally different results. In dynamic sampling, the whole adsorption process can be simplified as in Figure 2.9 (Pawliszyn, 2000).

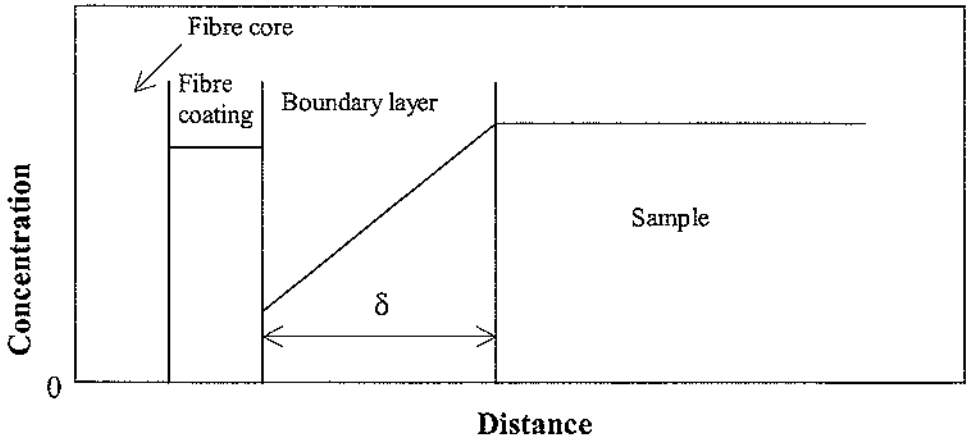

Figure 2.9. Boundary layer model (Pawliszyn, 2000).

The mass of extracted analyte can be estimated from the following equation:

$$n(t) = \{2\pi D_g L/ln[(b+\delta)/b]\} \cdot C_g t \qquad 2\text{-}23$$

In this situation, agitation affects the boundary thickness (δ), which is the important factor in equation 2-23. A diffusion coefficient is used in equation 2-23, rather than distribution coefficients.

If the amount adsorbed in sampling is large enough to have changed the concentration of the headspace, the equilibrium between the liquid and the headspace will also affect the analysis result, because there are two different kinds of equilibrium, as shown in Figure 2-10, while HS-SPME is being conducted: vapour-solid-equilibrium (V-S-E) as mentioned above; liquid-vapour-equilibrium (L-V-E) between the liquid sample and its headspace. The latter equilibrium is usually reached before HS-SPME is applied.

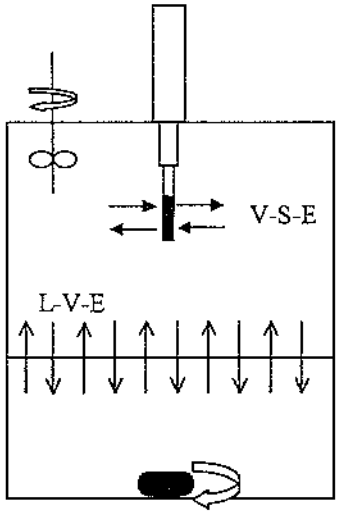

Figure 2-10. SPME configuration

If the amount of analyte adsorbed is quite small, then the L-V-E is not affected and the sampling process does not affect the L-V-E; a more complex case occurs when the amount of analyte adsorbed is not small enough, in which case L-V-E will be affected. In

this case, the analyte will escape from the water phase to the headspace to satisfy the L-V-E while HS-SPME is being performed. The whole extraction process is too complex to describe using theory. In practice, it is hard to distinguish the two different kinds of sampling.

In summary, it is important when using HS-SPME to maintain all the conditions as constant as possible, because it is difficult to establish whether or not the sampling process is carried out under equilibrium conditions. Even under the equilibrium conditions, L-V-E may be affected. Conditions to be kept constant include the amount of liquid used for one sample, the size and shape of the containers, the speed of the agitator for both liquid and vapour, the temperature of the extraction process and the time taken to prepare a sample..

2.8 Conclusions

In conclusion, it is apparent that HS-SPME in conjunction with gas chromatography would be an appropriate method to use in flavour–protein binding research. There is only one reported study on flavour–protein (ovalbumin) binding using HS-SPME (Adams et al., 2001). The technique has the potential to be applied to dairy protein–flavour binding research.

Another potential application for the HS-SPME technique is in the area of quality control, where the method would be used to assess dairy products, especially new products based on the flavour analytes, and to determine whether the products would be acceptable to the consumer. The advantage of such a technique would be that the HS-SPME method would replace time-consuming and expensive to run sensory analysis. However, in order to use such an objective analytical tool to assess the flavour profile of dairy products, there are many steps that need to be taken to ensure that the analytical method is measuring exactly what the consumer perceives. Hence, it is important that the analytical and sensory responses are correlated before the analytical tool is used alone.

2.9 Objectives

The present study was divided into four sections, with four main objectives.

- **Development of a sampling methodology for HS-SPME**
- **Investigation of whey protein isolate–flavour binding**
- **Investigation of sodium caseinate–flavour binding**
- **Mechanism of β-lactoglobulin–flavour interaction**

CHAPTER THREE

MATERIALS AND METHODS

3.1 General materials and equipment

- Whey protein isolate (WPI) and sodium caseinate(Na-CN) powders were obtained from Fonterra (New Zealand).

- 4 ml glass vials with open-hole screw top lids were obtained from Alltech, and butyl rubber resealable septa were purchased from Chromspec Distributors (NZ) Ltd.

- Distilled, deionized water (DDI) was obtained from a MilliQ filter system in the laboratory. Henceforth, the term "water" refers to DDI.

- AnalaR grade ethanol was obtained from Sigma-Aldrich Fine Chemicals (St. Louis, Missouri, USA).

- 2-Nonanone and 2-heptanone were food grade and were supplied by Sigma-Aldrich Fine Chemicals (St. Louis, Missouri, USA).

- A Supelco (Bellefonte, Pennsylvania, USA) Nukol^{TM} capillary column (15 m × 0.53 mm × 0.53 μm) was used throughout the experimental work.

- The manual SPME syringe and coated fibres, 100, 75, and 30 μm polydimethylsiloxane (PDMS) fibres, were purchased from Chromspec Distributors (NZ) Ltd.

- Nitrogen (oxygen-free, OFN) and hydrogen gas cylinders, hooked up to the gas chromatograph (GC), were supplied by BOC Gases (Palmerston North) Ltd.

- A Heidolph waterbath ± 0.1°C was supplied by Watson Victor Ltd. New Zealand.

3.2 Gas chromatography

A Supelco Nukol™ capillary column (15 m × 0.53 mm × 0.5 μm) was used in the present study. The carrier gas used was OFN and while hydrogen and dry air were mixed in optimum proportions for use in the flame ionization detector (FID40). The flow rates of the carrier and fuel gases and the temperature were as follows:

- Nitrogen (carrier gas) : 20 ml/min
- Hydrogen (fuel) : 50 ml/min
- Make up 35 ml/min
- Dry air : 100 ml/min
- Injector port temperature : 250°C
- Detector temperature : 250°C
- Maximum column temperature : 200°C
- Oven temperature : 100°C

The splitless model was used, as the concentrations of the volatiles being analysed were very low (0–100ppm). This model allows the maximum volume of analyte to reach the detector from the injector port via the column. The column was conditioned overnight before commencing a batch of GC experiments. This was done by heating the column slowly (5°C/min ramp) to 195°C, holding it for 1h and then cooling the column back down to 40°C. This cycle was carried out repeatedly throughout the night before the day of use, particularly if the GC had not been used for more than 2 weeks.

3.3 Headspace solid phase microextraction

All the new fibres were conditioned before use to prolong their extraction abilities. Up to 50 or more injections were possible with each fibre after the conditioning treatment had been applied. The details of conditioning are shown in Table 3.1.

Table 3.1. Temperature and conditioning recommendations for fibres

Stationary phase	Film thickness	Maximum temperature	Recommended temperature	Conditioning temperature	Time (h)
PDMS	100 μm	280°C	200-270°C	250°C	1
	30 μm	280°C	200-270°C	250°C	1
	7 μm	340°C	220-320°C	320°C	2-4
PDMS/DVB	65 μm	270°C	200-270°C	260°C	0.5
Polyacrylate	85 μm	320°C	220-310°C	300°C	2
CAR/PDMS	75 μm	320°C	240-300°C	280°C	0.5
CW/DVB	65 μm	265°C	200-260°C	250°C	0.5
DVB/CAR/PDMS	50/30 μm	270°C	230-270°C	270°C	4

The analysis process consists of several steps, as follows.

1. *SPME extraction*

   The SPME fibre is put into the headspace of the sample at a constant temperature for a definite time. In this stage, the important factors are maintaining a constant extraction time for each sample, maintaining the same depth of fibre into the vials for each sample and not damaging the fibre while piercing and withdrawing the fibre through the septa of the vials.

2. *GC analysis*

   The fibre is transferred into the injector port of the GC as soon as possible after the extraction is completed.

3. *Fibre purge and reuse*

   The fibre is left in the port at 250°C for 5 min, and then withdrawn from the port. The fibre is kept on the bench for 15 min to cool to the room temperature before analysing the next sample.

3.4 Standard solutions

3.4.1 Flavour compounds stock solution

0.01 M flavour compounds stock solutions were made by weighing 0.057 g 2-heptanone and 0.071 g 2-nonanone respectively into a 50 ml volumetric flask using the capillary technique (Bassette, 1984) and making up to the required volume with 30% and 50% ethanol solution respectively. The stock solution was stored at 5°C and was used as required. The solution was stable for up to 2 months at this temperature according to Ulberth (1991).

3.4.2 External standard method

The dynamic and equilibrium sampling SPME technique was used in this study. Calibration was performed for the GC and the SPME fibres. For every fibre, a calibration curve was prepared before analysis of the samples. 0.01 M stock solutions of the two flavour compounds were diluted with water to give 0.1, 0.2, 0.4, 0.6 and 0.8 mM standard solutions.

0.4 ml of a standard solution was pipetted into a 4 ml glass vial and the vial was covered with a screw cap immediately. Different fibres were calibrated independently. Four sub-samples were prepared for each solution.

3.4.3 Protein solutions

WPI and Na-CN solutions at 0.5,1,1.5 and 2% were prepared by weighing 2.5, 5, 7.5 and 10 g powder into a beaker and adding 200 ml water. The solution was stirred for 1 h and then transfered to a 500 ml volumetric flask. The beakers were washed with water and the

wash water was transferred into the volumetric flask. The solutions were stored at 5°C for 6 h before using them to prepare the flavour–protein mixture solutions.

3.4.4 Flavour–protein mixed solutions

Flavour–protein binding was detected by comparing the SPME analysis of the standard flavour solution with that of a flavour–protein solution at the same flavour concentration at a given protein concentration. The flavour–protein solutions were made in the same way as the standard flavour solutions. For example, 0.1, 0.2 and 0.4 mM flavour in 1% WPI solutions were prepared by pipetting 1 ml stock solution into 100, 50 and 25 ml volumetric flasks and making up to the final volume with 1% WPI solution. The concentration of protein in the mixture solutions changed after mixing with 1 ml of stock flavour solution. The concentration of WPI solution was calculated from the volume of each flask used. The samples were stored at 5°C for > 40 h.

3.5 Determination of analyte retention times

The retention time for a given analyte will be different under different GC conditions. The GC conditions used were as mentioned earlier, the samples were analysed at 25°C and were extracted for 5 min. Typical GC graphs are shown in Figures 3.1 and 3.2.

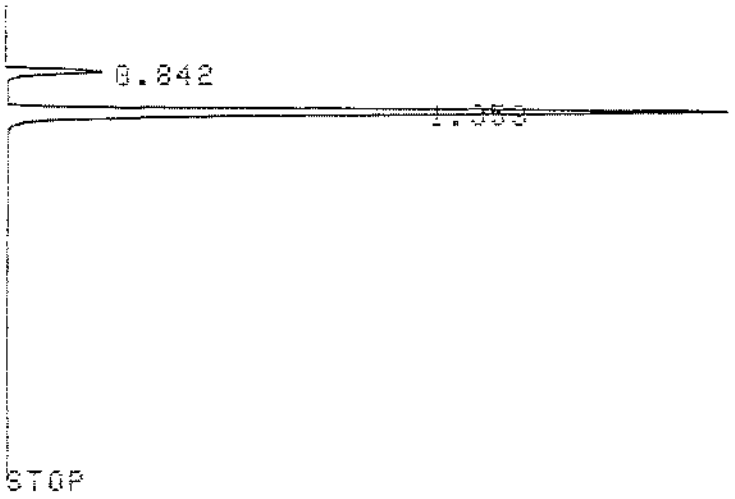

Figure 3.1. Typical GC response (ethanol + 2-heptanone).

Figure 3.2. Typical GC response (ethanol + 2-nonanone).

There were at least two components in each of the standard flavour solutions (i.e. the flavour compound and ethanol). Which peaks represented the target flavour compound, ethanol and other impurity components had to be identified. The obvious way would be to use GC-MS to identify the flavours. However, because there were only two major components in the solution, it was possible to use just the GC to identify the peaks. The components in the two standard flavour solutions and their retention times were as shown in Table 3.2.:

Table 3.2. Retention times for the two components in standard solutions

	Standard solutions	**Retention times**	
		Peak 1	**Peak 2**
1	**Ethanol + 2-heptanone**	0.842 min	1.35 min
2	**Ethanol + 2-nonanone**	0.845 min	2.72 min

Although the retention times of peak 1 were slightly different, it was still quite clear that this was the retention time of ethanol. In fact, the retention times of all analytes will differ slightly in different analyses. The retention times of the analytes are listed in Table 3.3.

Table 3.3. Retention times of individual components

Analyte	Retention time (R_T)
Ethanol	~0.84 min
2-Heptanone	~1.35 min
2-Nonanone	~2.72 min

CHAPTER FOUR

OPTIMIZATION OF SOLID PHASE MICROEXTRACTION CONDITIONS FOR FLAVOUR BINDING ANALYSIS

4.1 Introduction

Suitable conditions for the operation of the SPME technique must be chosen. The SPME conditions include the type of fibre, the thickness of the coating, the time of the extraction, the temperature and the component effects. In the work described in this chapter, the type of fibre, the extraction time and the size of container were optimized.

4.2 Materials and methods

The materials and methods used to optimize the SPME conditions were as follows:

- 100, 30 and 7 μm PDMS fibres and a manual syringe (as described in Section 3.1).
- 250 and 150 ml flasks and 4 ml vials (as mentioned in Section 3.1)
- Waterbath (± 0.1°C)
- Carlo Erba GC6000 system with Nukol^{TM} column (as mentioned in Section 3.2)
- Food–grade 2-nonanone and 2-heptanone (as mentioned in Section 3.1)
- DDI water (as mentioned in Section 3.1)

4.3 Results and discussion

4.3.1The size of the containers

The size of the container used for SPME analysis may result in different GC responses, as mentioned in Chapter 2. A suitable container size can make the analysis easier and more accurate. Different sizes of containers were chosen, as shown in Table 4.1.

Table 4.1. Container and liquid amount used for optimization of SPME conditions

Number	Volume of the container (V_c)	Amount of sub-sample (V_s)	Ratio of V_s/V_c	Temperature
1	250 ml	25 ml	0.1	25°C
2	150 ml	15 ml	0.1	25°C
3	4 ml	0.4	0.1	25°C

The sample was 0.6mM 2-heptanone standard solution and the results are shown in Table 4.2.

Table 4.2. The SPME-GC results obtained using different containers

Volume of the container (V_c)	Number of sub-samples			Deviation
	1	2	3	
250 ml	2542176	4013489	3056890	$> \pm 20\%$
150 ml	3678543	4351042	2483056	$> \pm 25\%$
4 ml	3587589	3781561	3843338	$< \pm 4\%$

It was found that the reproducibility using large containers (250 and 150 ml flasks) was rather poor whereas the small vials showed good repeatability. There are several reasons for this observation.

The diffusion of a flavour compound is more important in a large container than in a small vial. In a large container, the flavour compound has to diffuse a much longer distance, compared with a small container from the liquid sample to the SPME fibre. It is difficult in a large container to put the fibre into the headspace at exactly the same position. Moreover, the seal on a large containers is much bigger than that in a small vial which may cause more volatile flavour compound to escape from the container. Finally, it

takes longer to transfer and measure larger volumes, allowing greater chance of escape of the flavour compounds.

It was found that the 4 ml vial was the best container for the SPME techique. Analysis of seven sub-samples is shown in Figure 4.1.

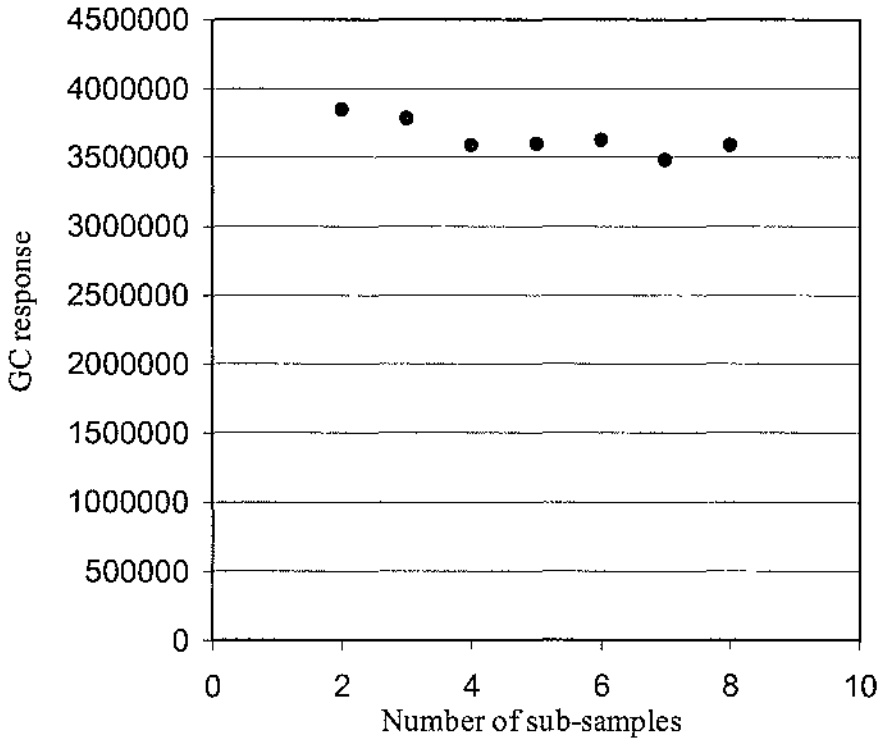

Figure 4.1 SPME results for different sub-samples of 0.6 mM 2-heptanone standard solution, 100μm PDMS coating fibre, using 4 ml vials.

4.3.2 The type of fibre and the extraction time

Only a limited number of fibre coatings are manufactured by Supelco (see Table 3.1). The specific extraction properties of these fibres, as recommended by Supelco are shown below.

- 100 μm PDMS coating for volatiles and semi-volatiles;

- 30 μm PDMS coating for non-polar semi-volatiles;
- 7 μm PDMS coating for mid- to non-polar semi-volatiles;
- 65 μm carbowax/divinylbenzene (CW/DVB) coating for polar analytes;
- 85 μm polyacrylate (PA) coating for polar semi-volatiles.

The information from Supelco is that the polarity of the analytes is an important consideration in choosing the fibre. For the two flavour compounds, i.e. 2-nonanone and 2-heptanone, PDMS fibre should be suitable, but the thickness of the coating needs to be investigated. It is known that the amount of analyte adsorbed by the fibre is an important factor. If the adsorbed amount is very small, the GC analysis will be difficult. Conversely, if the amount adsorbed is very large, some unknown factors might be brought into the flavour analysis. After the 4 ml vial had been chosen as the container, the amount of analyte adsorbed by the fibre depended on the extraction time, the temperature and the material and thickness of the coating. In this study, the temperature was kept constant at 25°C and the other two factors were investigated further.

The equilibrium extraction time was determined using 4 ml vials, as mentioned in Section 4.3.1. The results are shown in Figures 4.2 and 4.3. It was found that 5 min was an acceptable time because the curve became flat after that point and a 5 min extraction allowed a reasonable number of samples to be analysed in a given time. All the extraction process was done manually in this study and full attention was paid to ensure that every extraction time was exactly the same.

Using a 5 min extraction time, standard curves for the two flavour compounds (Figures 4.4 and 4.5) were generated. In the standard curves, each point was repeated at least three times, as shown in Tables 4.3 and 4.4.

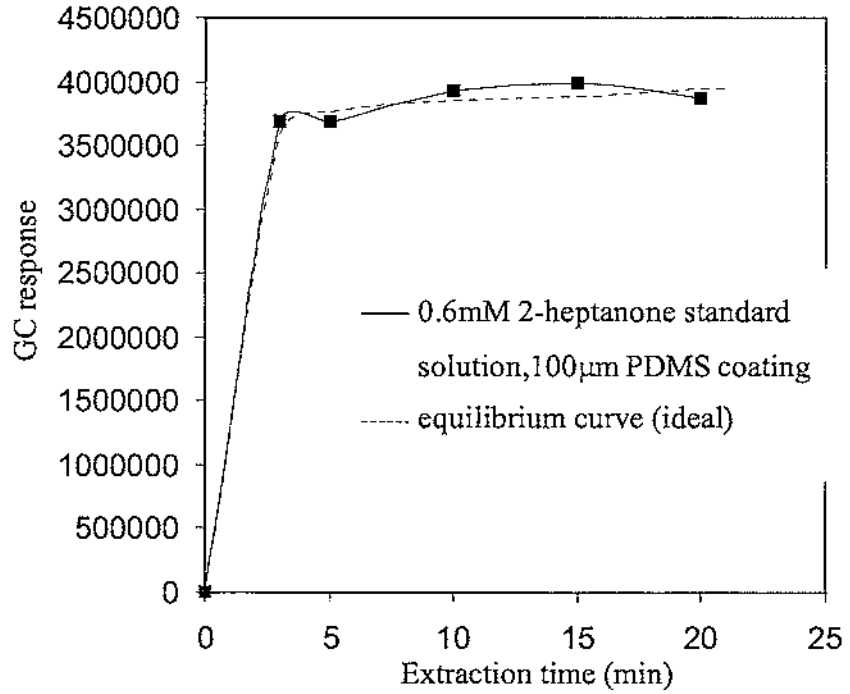

Figure 4.2. Effect of extraction time at 25°C for 2-heptanone on the SPME results.

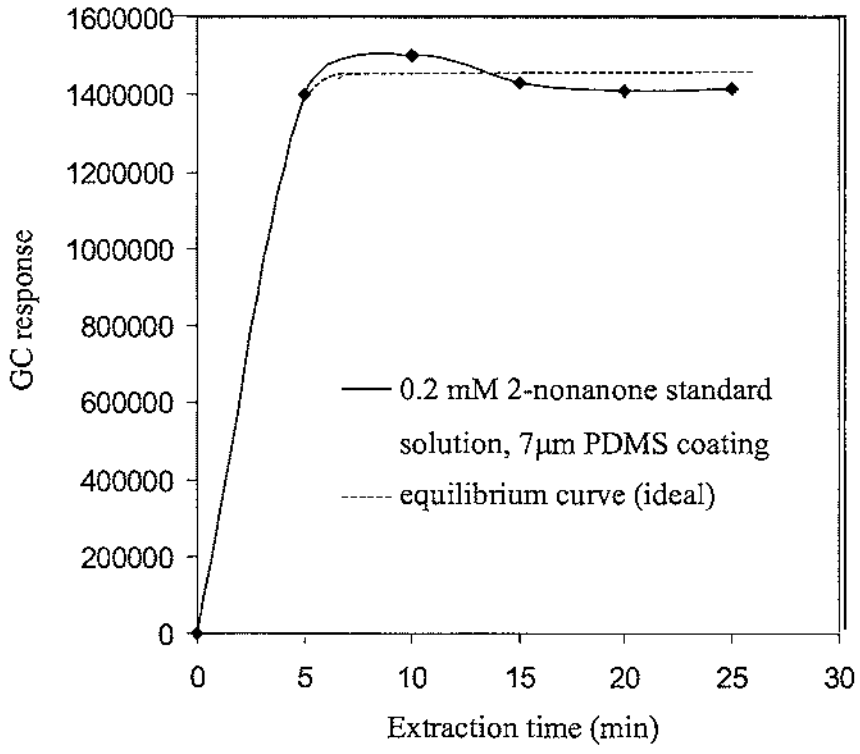

Figure 4.3. Effect of extraction time at 25°C for 2-nonanone on the SPME results.

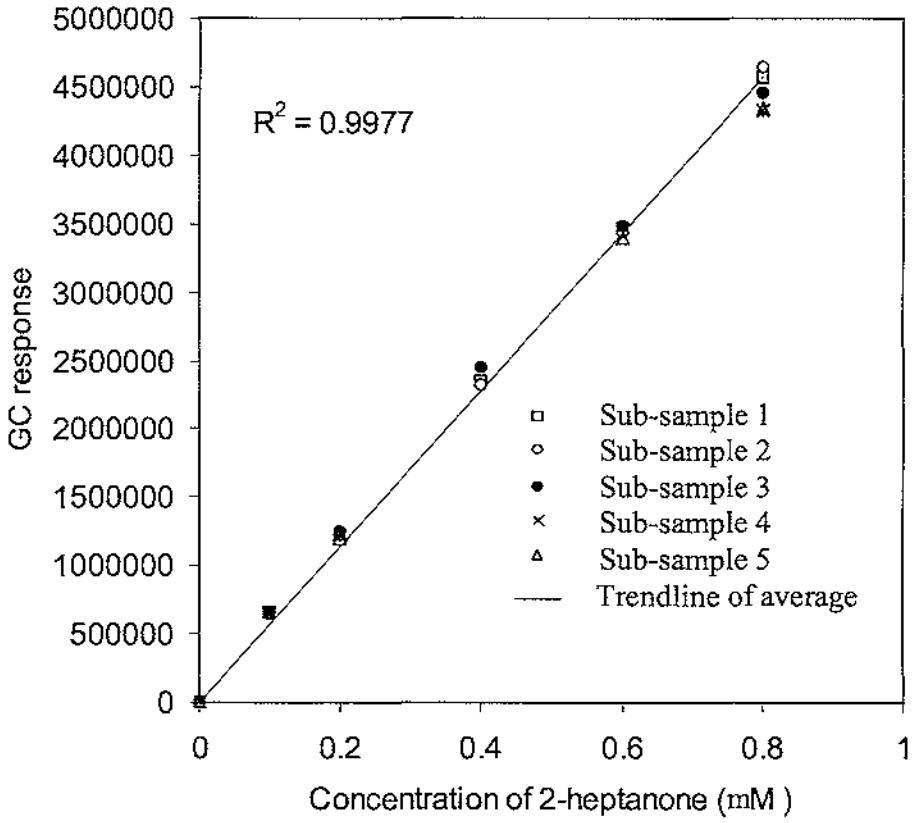

Figure 4.4. GC response curve of 2-heptanone standard solution (100 μm PDMS coating fibre; extraction time: 5 min)

Table 4.3. The SPME results for 2-heptanone standard samples

Flavour concentration	GC response of sub-samples						Maximum deviation
	1	2	3	4	5	Average	
0.1 mM	664170	665013	641096			656760	2.4%
0.2 mM	1186381	1228030	1240400	1233048	1223214	1231173	3.6%
0.4 mM	2345754	2314490	2450130			2370125	3.4%
0.6 mM	3468798	3436338	3485815	3401154		3448026	1.4%
0.8 mM	4568257	4648699	4453655	4339735	4321987	4466467	4.1%

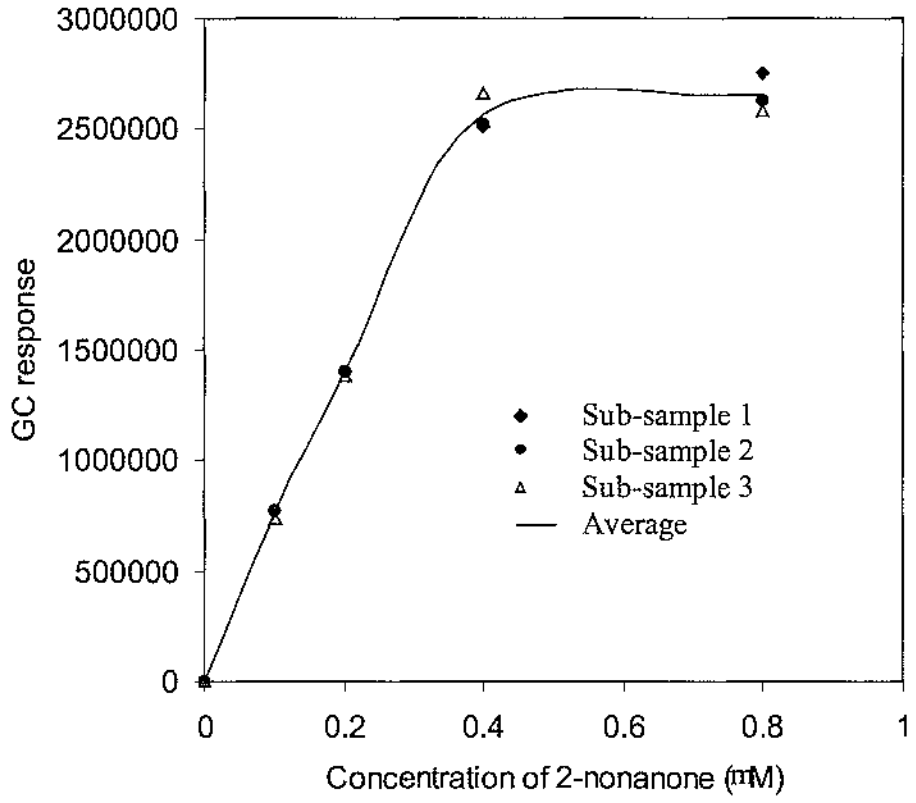

Figure 4.5: GC response curve of 2-nonanone standard solution (7 μm PDMS coating fibre, extraction time 5 minutes)

Table 4.4 The SPME results of 2-nonanone standard samples

Flavour concentration	GC response of sub-samples				Maximum deviation from average
	1	2	3	Average	
0.1 μM	770464	770283	737784	759510.3	2.9%
0.2 μM	1400148	1406046	1387768	1397987	0.7%
0.4 μM	2509321	2524461	2660447	2564743	3.7%
0.8 μM	2748788	2623031	2578447	2650089	3.7%

For 2-nonanone, the standard curve was not a straight line (Figure 4.5), because the fibre adsorbed too much 2-nonanone at a low concentration (the partition coefficient was very large) and the surface of the fibre was saturated at a moderate concentration of 2-nonanone (0.4 mM). Therefore, a higher concentration of 2-nonanone could not produce a proportional response (see 0.8 mM). Several methods can be used to overcome the problem: using a fibre with a larger surface to maintain sufficient free surface for adsorption; using a fibre with a low partition coefficient for 2-nonanone; using the same fibre and extracting for a shorter time.

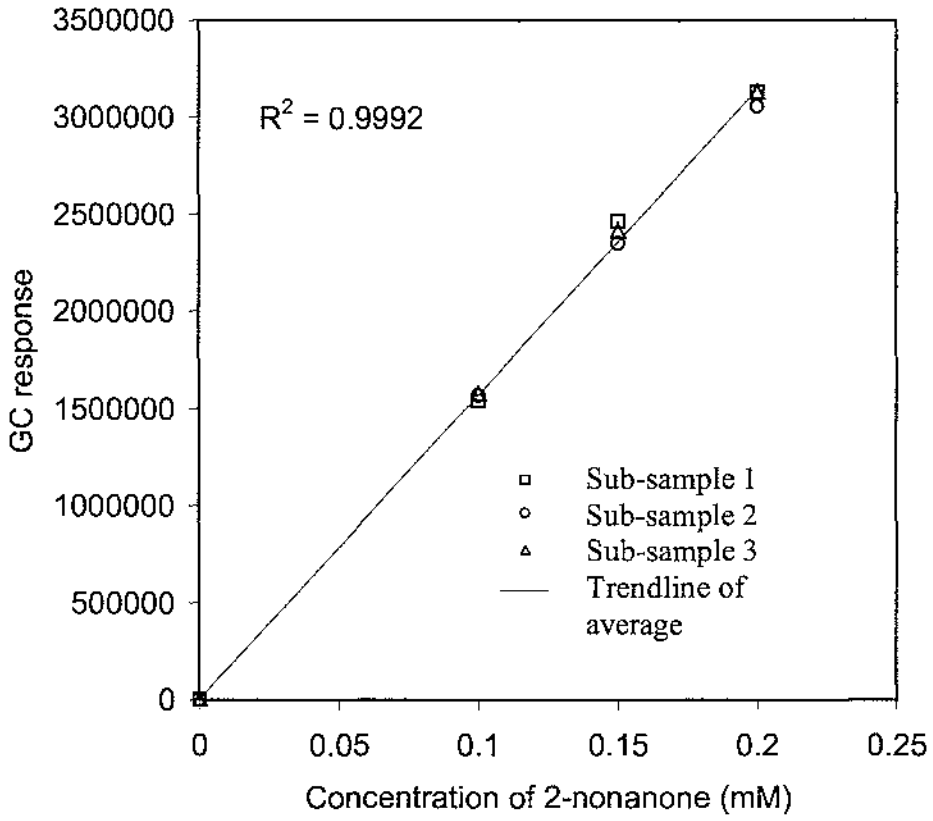

Figure 4.6. GC response curve of 2-nonanone standard solution (30 μm PDMS coating fibre; extraction time: 5 min)

The first method may cause a much larger amount of 2-nonanone to be adsorbed by the fibre; considering that there was only a 0.4 ml liquid sample at very low flavour

concentration (< 1 mM), this method is not a good choice. The second method may be a good way to perform the analysis, but a lot of time will be required to select a suitable kind of coating and there are only a limited number of commercial fibres to choose from. The third method was used in this study. The 30 μM PDMS coating fibre and a short extraction time (30 seconds) were used in the analysis of 2-nonanone in the higher concentration range. It must be pointed out that, when dynamic sampling is performed, the extraction time is extremely important as the curve is very steep at that time. A small error in extraction time will cause a significant difference in the GC response. The results obtained for extraction times of 5 min and 30 seconds are shown in Figures 4.6 and 4.7 respectively.

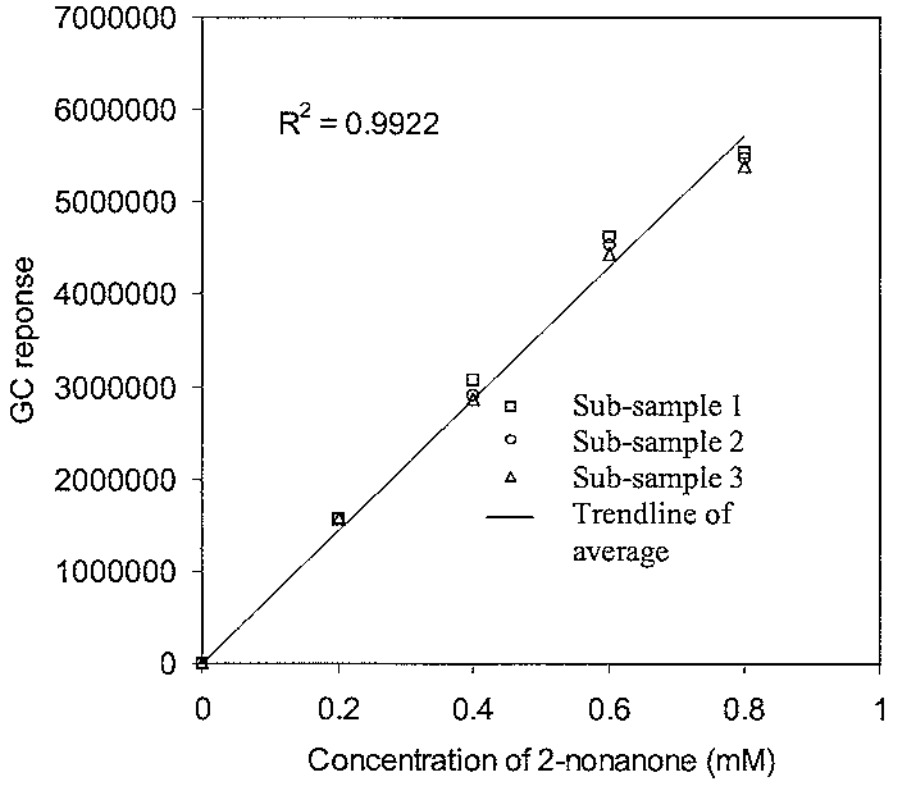

Figure 4.7. GC response curve of 2-nonanone standard solution (30 μm PDMS coating fibre, extraction time: 30 seconds).

It can be seen that the result in Figure 4.7 was much better than that in Figure 4.5 although the curve was still slightly non-linear when the concentration of 2-nonanone was above 0.6 mM. Considering all the factors mentioned above, the 30 μM PDMS

coating fibre and an extraction time of 30 seconds were used for the analysis 2-nonanone in the range 0.4–0.8 mM.

4.4 Conclusions

In conclusion, this work showed that SPME could be used to analyse the two flavour compounds if the conditions were satisfactory. The method was successfully used to quantify the analyte concentrations in the laboratory-prepared standard solutions.

The experimental data were well replicated when 4 ml glass vials were used as the containers for the SPME extraction. When the data were replicated three times, the maximum deviation from the average was less than 4%. The experiment was hard to replicate when 250 and 150 ml flasks were used.

A linear standard curve was obtained when a 5 min extraction was performed using a 100 μM PDMS coating fibre for analysing 2-heptanone. In this case, the maximum deviation from average was 4%. For analysing 2-nonanone, a 30 μM PDMS coating fibre was selected. The extraction times applied were 5 min (equilibrium sampling) for low concentrations (≤ 0.2 mM) and 30 seconds (non-equilibrium sampling) for high concentrations. Using these extraction times, straight lines were obtained for the standard response curve, with the maximum deviation from the average being 2.5%.

CHAPTER FIVE

BINDING OF FLAVOUR COMPOUNDS TO MILK PROTEINS USING SOLID PHASE MICROEXTRACTION

5.1 Introduction

The objective of the work described in this chapter was to investigate flavour binding to milk proteins using the SPME technique. The study was divided into the following parts.

- Factors other than SPME conditions that affected flavour analysis
- The equilibrium binding times
- Na-CN–flavour binding
- WPI–flavour binding

Ethanol was introduced into the system because of the low solubility of the flavour compounds in water. Although the PDMS fibre is designed for non-polar analytes, the competitive adsorption between ethanol and the flavour compound needs to be considered because the concentration of ethanol is 300–600 times higher than that of the flavour compounds. The mechanism of two components adsorbing at the same time is unknown. Ethanol may have a considerable effect on the flavour analysis. Therefore, to ensure that the analysis is reliable, the effect of ethanol on the flavour analysis must be taken into account.

The interaction between the flavour compound and protein was regarded as an equilibrium reaction, as mentioned in Section 2.4. Many factors affect this equilibrium, including the reactants involved in the reaction, temperature and reaction time. In this study, the reactants were milk proteins and one of the two flavour compounds. The

samples were stored at 5°C all the time before analysis. The reaction time was determined in this study.

5.2 Materials and methods

The materials and methods used to optimize the SPME conditions were as follows.

- 100 and 30 μm PDM coating fibres and manual syringe (as described in Section 4.1).
- 4 ml vials with open hole lids (as mentioned in Section 3.1).
- Heidolph waterbath ± 0.1°C supplied by Watson Victor Ltd. New Zealand.
- Carlo Erba GC6000 system with Nukol™ column (as mentioned in Section 3.2).
- Whey protein isolate (WPI) and sodium caseinate (Na-CN) (as mentioned in section 3.1).
- Food-grade 2-nonanone and 2-heptanone (as mentioned in Section 3.1).
- DDI water (as mentioned in Section 3.1).
- AnalaR grade ethanol, obtained from Sigma-Aldrich Fine Chemicals (St. Louis, USA).
- OFN and hydrogen gas cylinders, hooked up to the GC, supplied by BOC Gases (Palmerston North) Ltd.

The effect of ethanol was determined by adding known amounts of ethanol into the standard flavour solution followed by determination of the GC response.

The method of analysis of flavour binding consisted of two steps. The first step was to find the relationship between the concentration of the flavour compound in the standard solution and the GC response (standard curve). This standard curve included all the information of partition coefficients of the flavour compounds between the SPME fibre and water. The standard curves were plotted as the GC response versus the concentration of each flavour compound. Ideally, a linear standard curve would be obtained if suitable SPME and GC conditions were used.

The second step was to use SPME and the GC to analyse the mixtures of protein and flavour compound. A definite concentration of protein and flavour compound in aqueous solution (e.g. 0.4 mM 2-nonanone and 1% WPI solution) was analysed using SPME and the GC. The GC response of the mixture solution reflected the free flavour concentration in the solution. The free flavour concentration could be obtained from the standard curve. The difference between the original concentration in the mixture solution and the free flavour concentration was regarded as the amount of flavour bound to the protein.

The equilibrium binding time of protein and flavour was determined by plotting the concentrations of free flavour compounds in the mixture solution against the time after the mixture solution had been made. The concentration of the free flavour normally dropped sharply initially and then decreased very slowly. Equilibrium was reached when the concentration of free flavour became a constant. The corresponding time was the equilibrium binding time.

5.3 Results and discussion

5.3.1 The effect of ethanol addition

Figure 5.1 shows the GC response of 0.2 mM 2-nonanone standard solution with different amounts of ethanol addition.

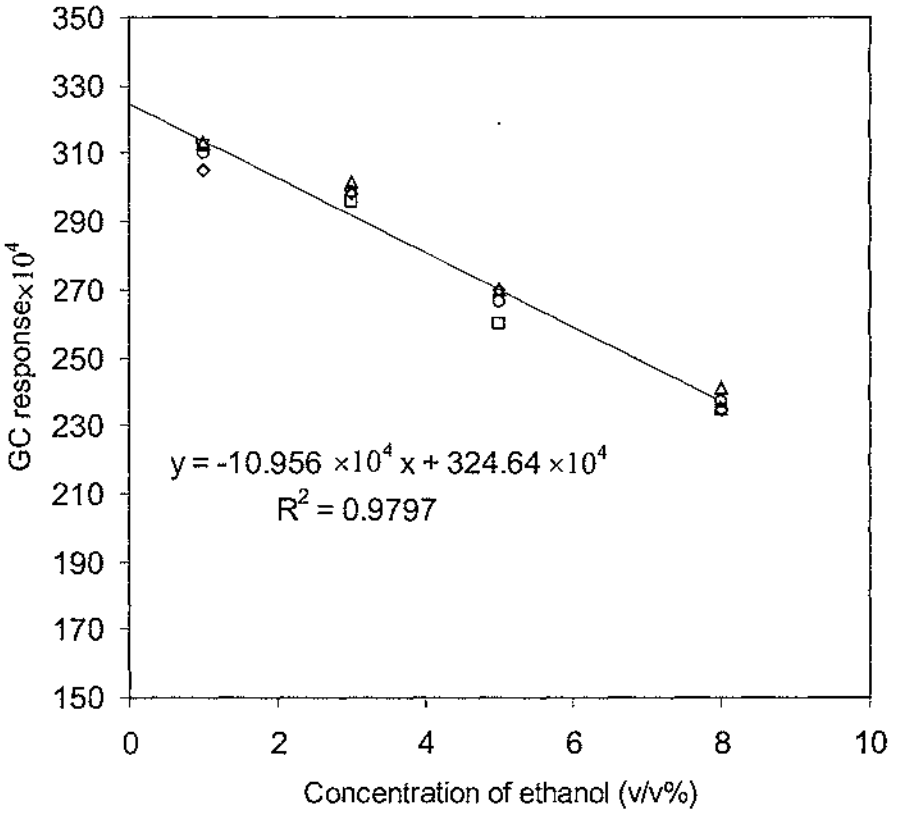

Figure 5.1. The effect of the addition of ethanol to 0.2 mM 2-nonanone standard solution on the GC response (30 μm PDMS coating fibre).

Figure 5.1 shows that the GC response of 2-nonanone decreased with increasing amounts of ethanol. The equation for the standard solution was:

$$y = -09561x + 3246400 \qquad 5\text{-}1$$

It was calculated from equation 5-1 that the GC response would be 3246400 if there were no ethanol in the solution. When the analysis was performed with 1% added ethanol and the average GC response was 3103208. The deviation of 4.4% between the two cases was

acceptable. The results obtained for 1% and 2% WPI solutions containing 0.2 mM 2-nonanone and different concentrations of ethanol are shown in Figures 5.2 and 5.3.

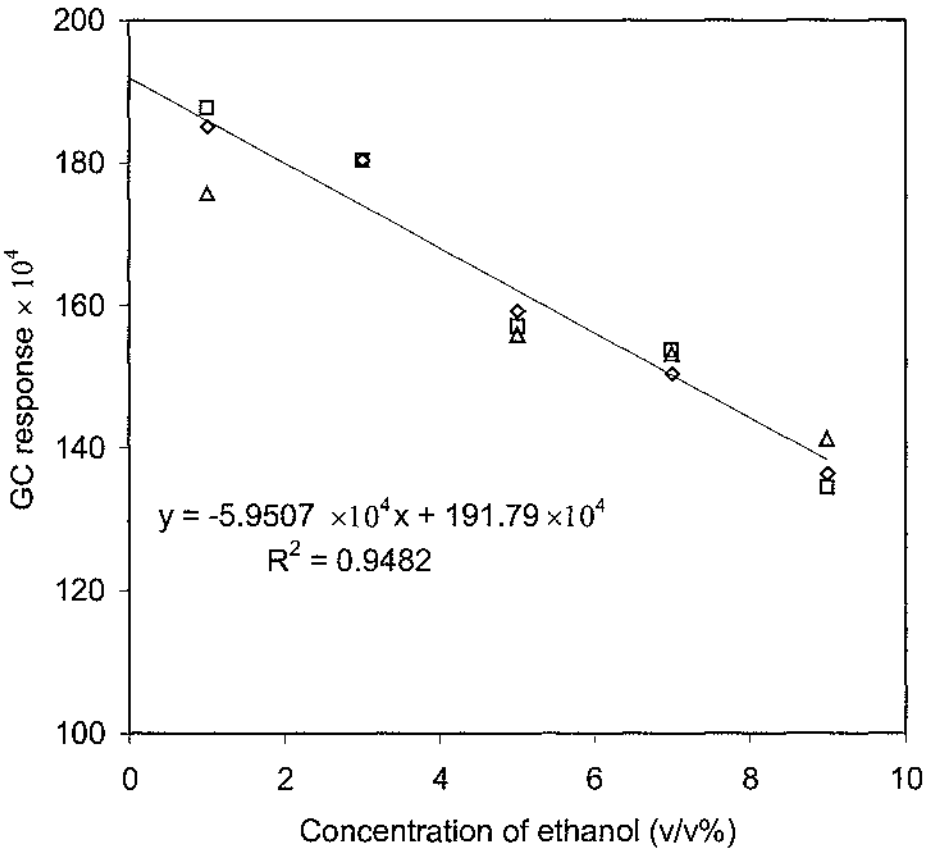

Figure 5.2. The effect of ethanol addition to a flavour–protein solution on the GC response (0.2 mM 2-nonanone in 1% WPI solution; 30 μm PDMS fibre; extraction time: 5 min)

The trendline for the 1% WPI solution was:

$$y = -59507x + 1917900 \qquad 5\text{-}2$$

The trendline for the 2% WPI solution was:

$$y = -35619x + 1459100 \qquad 5\text{-}3$$

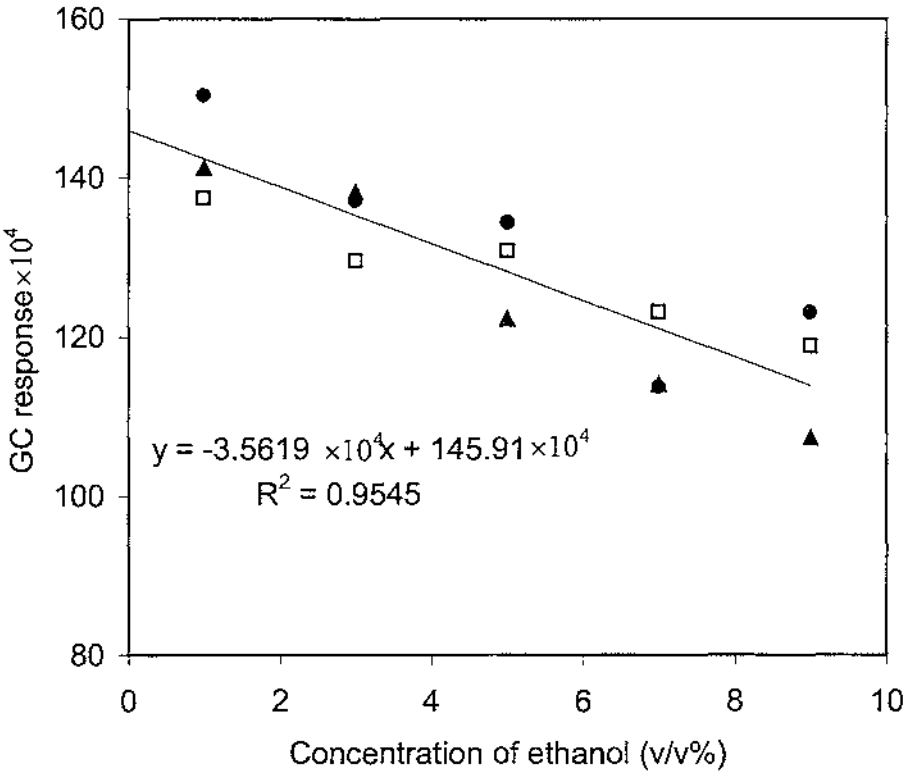

Figure 5.3. The effect of ethanol addition to a flavour–protein solution on the GC response (0.2 mM 2-nonanone in 2% WPI solution; 30 μm PDMS fibre; extraction time: 5 min)

The values of the slopes for the standard solution, the 1% WPI solution and the 2% WPI solution were –109560, –59507 and –35619 respectively. This indicates that, as the concentration of WPI in the system increased, in the system, the influence of ethanol decreased. If no ethanol was added into the system, the GC responses would be 1917900 and 1459100 for the 1% WPI and the 2% WPI solutions, according to the data in Figures 5.2 and 5.3. The analysis results were 1828263 and 1430181 for the 1% WPI and the 2% WPI solutions respectively. The differences were 4.7% and 2.0% for the 1% WPI and 2% WPI solutions respectively.

The amounts of bound flavour (calculated from the difference between the GC responses of the protein solution and the standard solution) for the 1% and 2% WPI solutions were:

$Bound_a$ = GC response of standard solution – GC response of WPI solution

= 3103208 – 1828263 = 1274945

Assuming no addition of ethanol, the calculated amounts of bound flavour were:

$Bound_c$ = 3246400 – 1917900 = 1328500

The difference between actual and calculated was:

$$(D_c - D_a)/ D_c \times 100\% = (1328500 - 1274954)/1328500 \times 100\% = 4.0\%$$

Considering the experimental error, this deviation was acceptable. It can be concluded that the addition of small amount of ethanol to the protein–flavour system had little effect on the bound flavour.

5.3.2 The equilibrium binding time

The progress of the interaction between the protein and the flavour was monitored for 8, 17, 40, 64 and 90 h after the solutions were held at 5°C. The results obtained for different WPI and flavour concentrations are illustrated in Figures 5.4–5.7.

The equilibrium binding times were shorter for 2-heptanone than for 2-nonanone, as shown in Figures 5.4–5.7. This can be explained as the amount of equilibrium binding being much smaller for 2-heptanone than for 2-nonanone. For the same flavour compound, the equilibrium time increased as the concentration of WPI in solution increased. This can be explained as, the more reactants initially, the longer was the reaction time. The 1% WPI solution corresponded to a concentration of about 0.25 mM β-lactoglobulin in the solution, which was comparable to the flavour compounds concentration (0.1-0.8 mM). So, it was expected that different concentrations of flavour compounds would cause different equilibrium binding times.

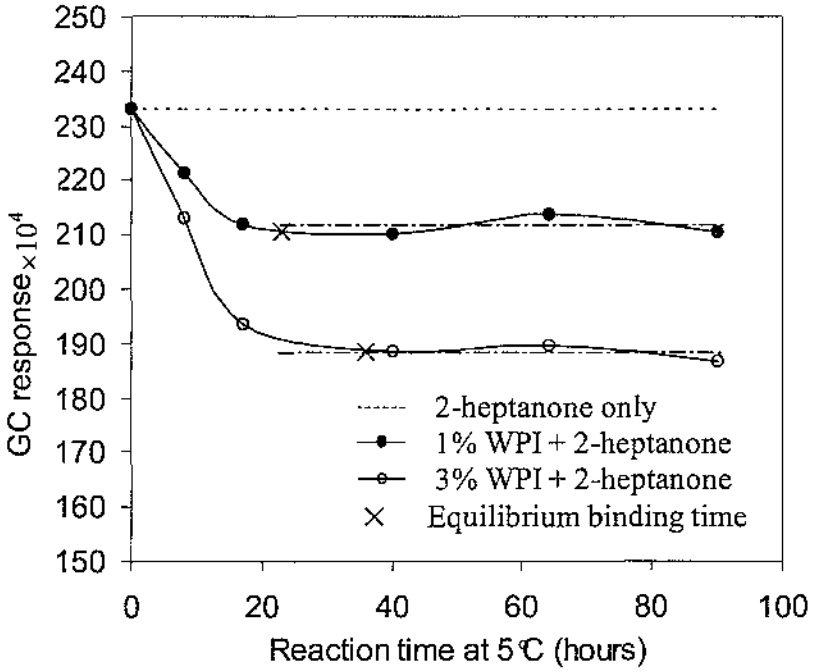

Figure 5.4. The interaction between WPI and 2-heptanone with time (0.4 mM 2-heptanone solution; 100 μm PDMS fibre; extraction time: 5 min)

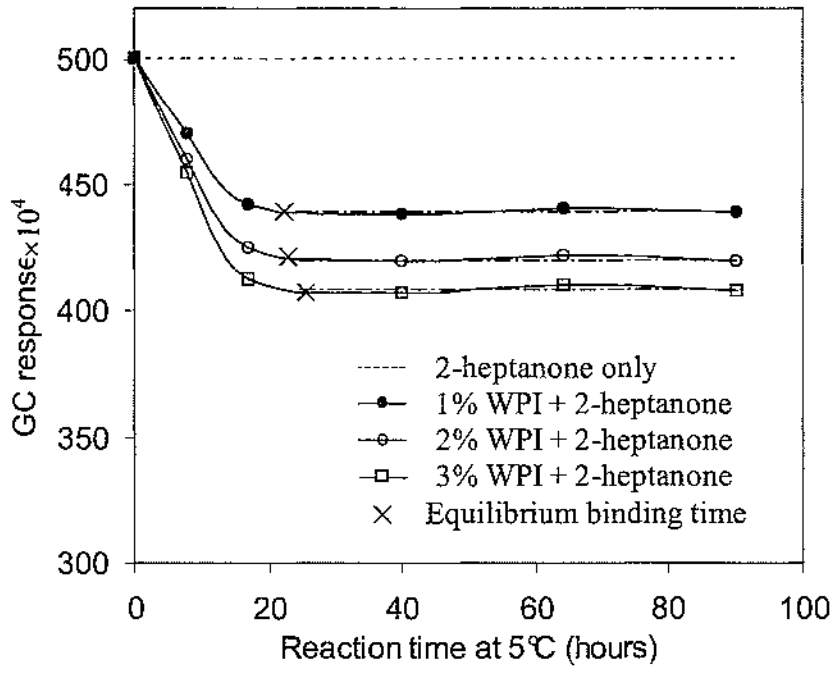

Figure 5.5. The interaction between WPI and 2-heptanone with time (0.8 mM 2-heptanone solution; 100 μm PDMS fibre; extraction time: 5 min)

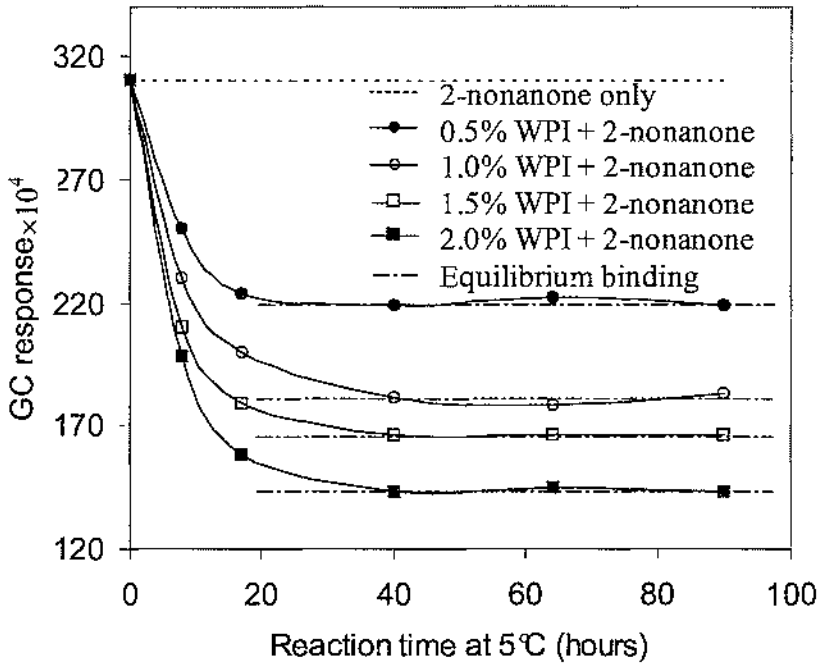

Figure 5.6: The interaction between WPI and 2-nonanone with time (0.2 mM 2-nonanone solution; 30μm PDMS fibre; extraction time: 5 min).

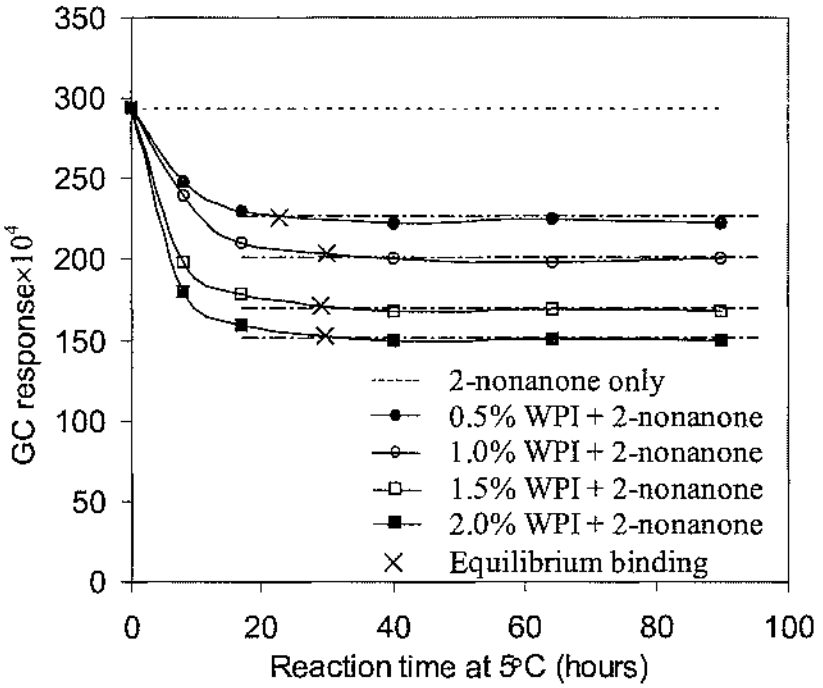

Figure 5.7. The interaction between WPI and 2-nonanone with time (0.2 mM 2-nonanone solution; 30μm PDMS fibre; extraction time: 30 seconds).

The equilibrium binding time changed from 17 to 40 h under different conditions. In this study, all samples were prepared 40 h before being analysed to ensure that equilibrium binding was reached.

5.3.3 Flavour binding to sodium caseinate

The binding of flavour compounds to Na-CN was determined by comparing the differences in the GC responses of the two solutions, as mentioned in Section 5.2. The extents of binding of the two flavour compounds to Na-CN were very different. No 2-heptanone bound to 1% Na-CN in the flavour concentration range used in this study (Figure 5.8). For 2-nonanone, about 10% of the total flavour compound bound to Na-CN in a 1% Na-CN solution (Figure 5.9).

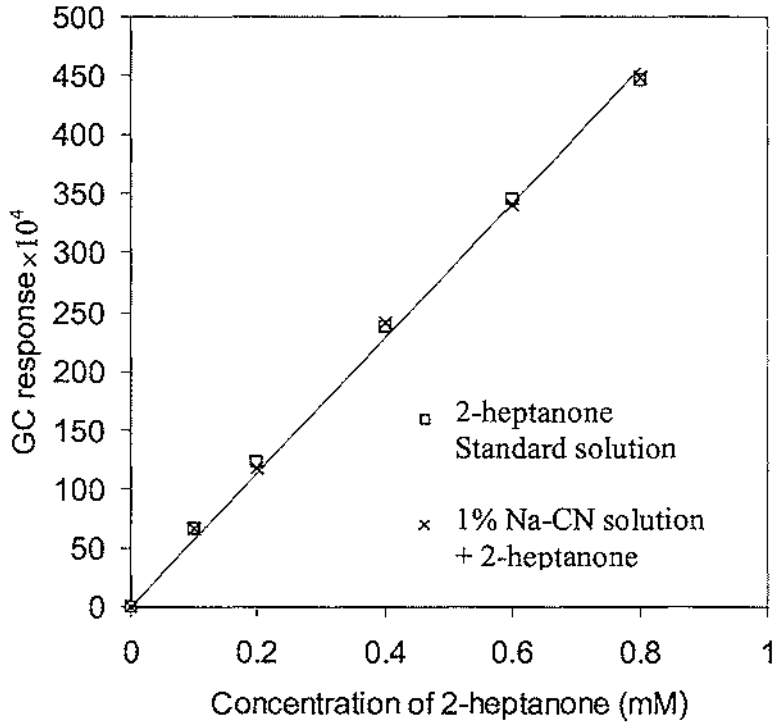

Figure 5.8. SPME results for the binding of 2-heptanone to Na-CN (100μm PDMS fibre; extraction time: 5 min)

Hansen and Heinis (1992), using sensory panellists, found that there was no significant decrease in benzaldehyde and citral flavour intensity in Na-CN solution, but that the d-

limonene flavour intensity dropped significantly in the presence of Na-CN.

A Klotz plot was used to calculate the binding constant and the number of binding sites of Na-CN. The results are illustrated in Figure 5.10. The molecular mass of Na-CN used in calculation was 22,000 Da.

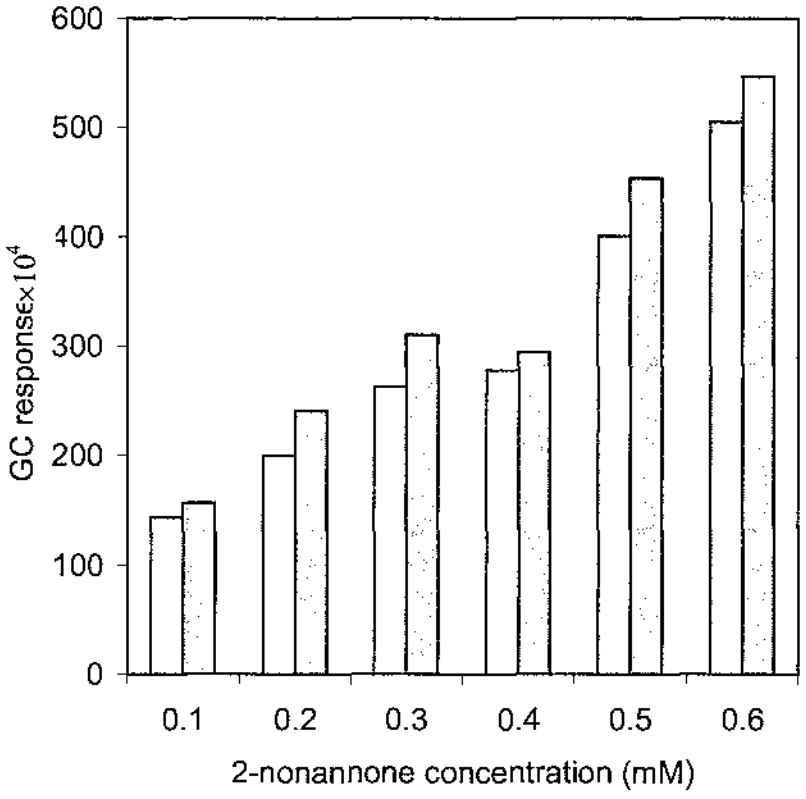

Figure 5.9. SPME result for the binding of 2-nonanone to Na-CN
□ 1% Na-CN+ 2-nonanone solution; □ 2-nonanone standard solution

From the intercept and the slope, the number of binding sites *(n)* and the binding constant (*K*) were calculated:

$$n = 1/3.0696 = 0.326$$

$$nK = 1/(16.521 \times 10^{-4}) = 605\ [M^{-1}]$$

$$K = 1858\ [M^{-1}]$$

The number of binding sites 0.326 suggested that not all the proteins in the sodium caseinate bind to the flavour. For example if only β-casein, which is about 35% in Na-CN bound to the flavour, the number of binding sites would be very close to 1.

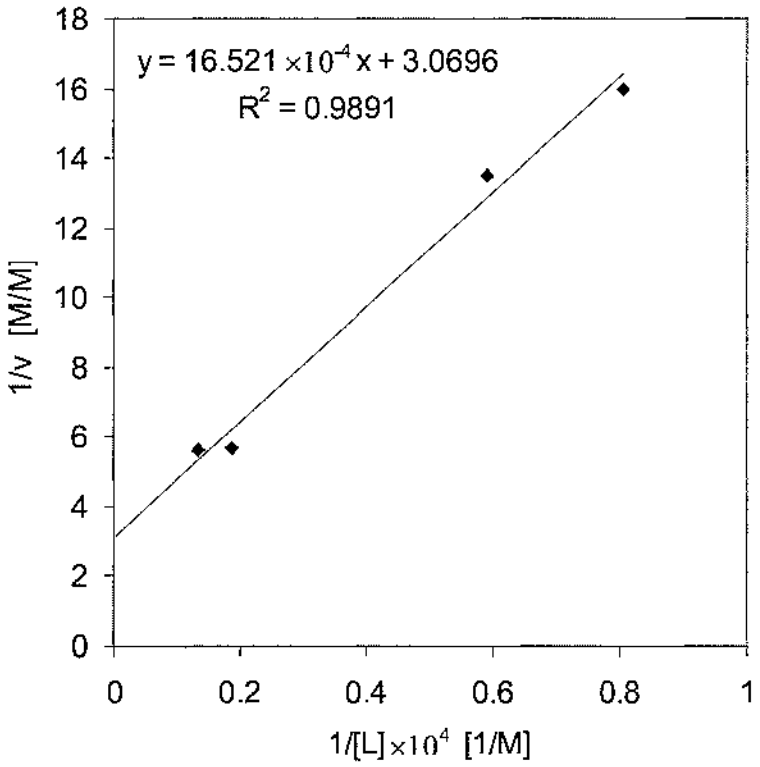

Figure 5.10. Klotz plot for 2-nonanone binding to Na-CN

5.3.4 Flavour binding to whey protein isolate

β-Lactoglobulin is the major protein in WPI. The binding properties of WPI are much like those of β-lactoglobulin. More than 10% of the total 2-heptanone was found to bind with the WPI in a 1% WPI solution. The results are shown in Figure 5.11. As reported by Guichard and Langourieux (2000), the hydrophobicity of the ketone is the key factor in WPI–ketone binding. Nearly 40% of the total 2-nonanone was bound in a 1% WPI solution, which was much higher than for 2-heptanone. The results of 2-nonanone–WPI binding are shown in Figures 5.12-5.13. The influence of protein concentration on flavour binding is illustrated in Figure 5.14.

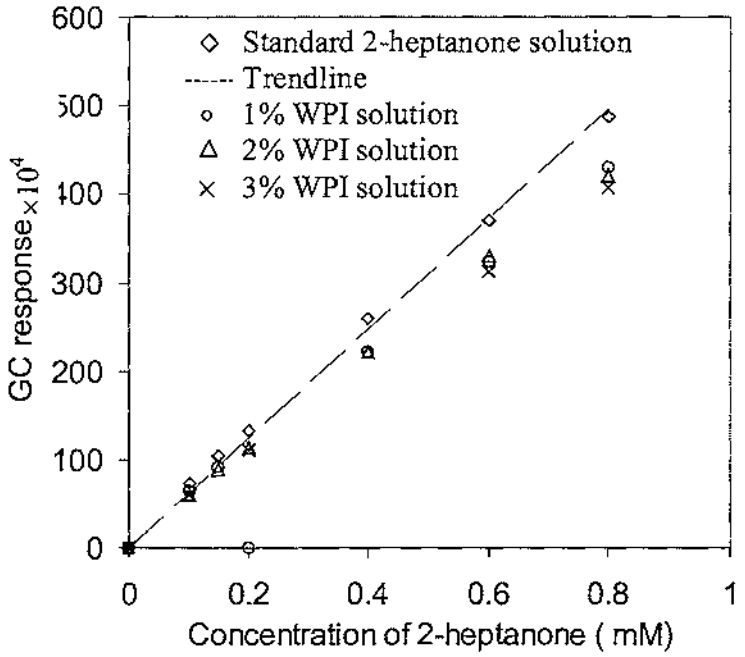

Figure 5.11. SPME result of the binding of 2-heptanone to WPI (100 μm PDMS fibre; extraction time: 5 min).

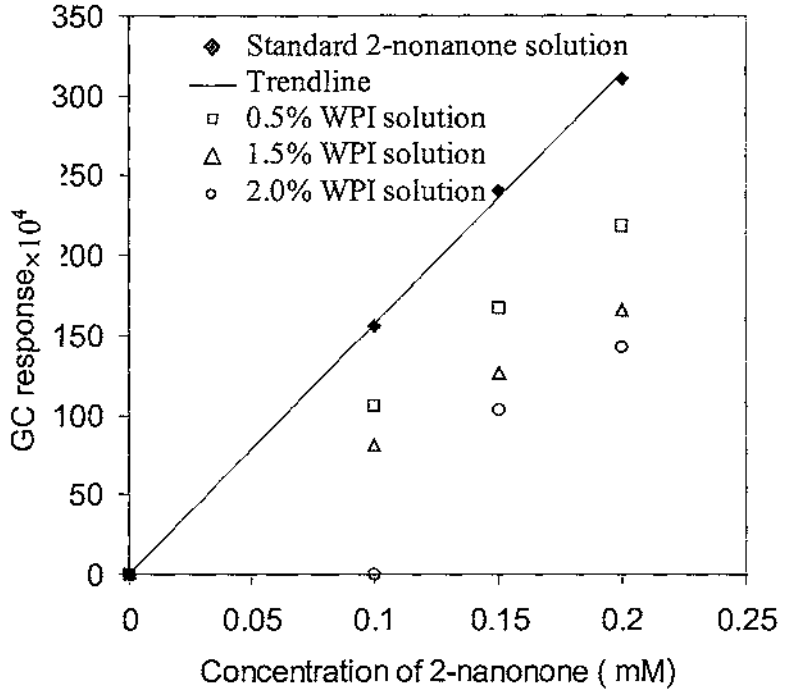

Figure 5.12. SPME result of the binding of 2-nonanone to WPI (30 μm PDMS fibre; extraction time: 5 min).

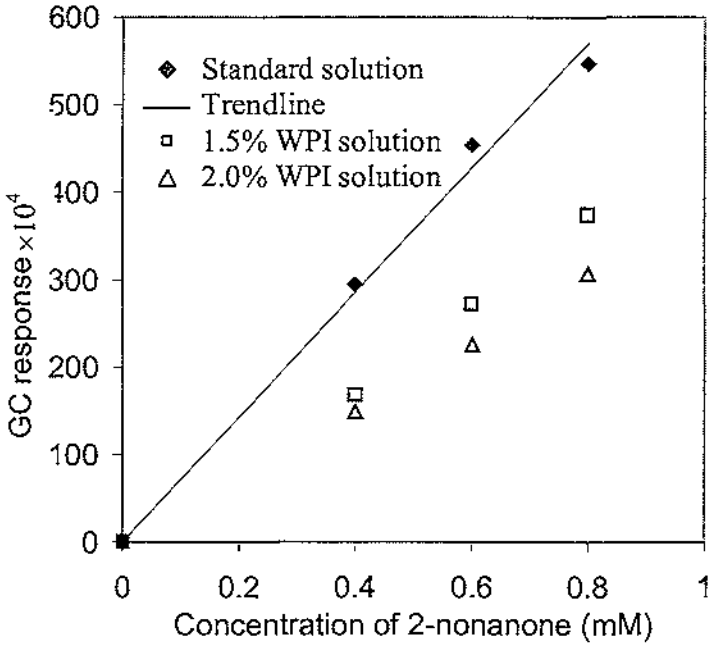

Figure 5.13. SPME result of the binding of 2-nonanone to WPI (30 μm PDMS fibre; extraction time: 30 seconds)

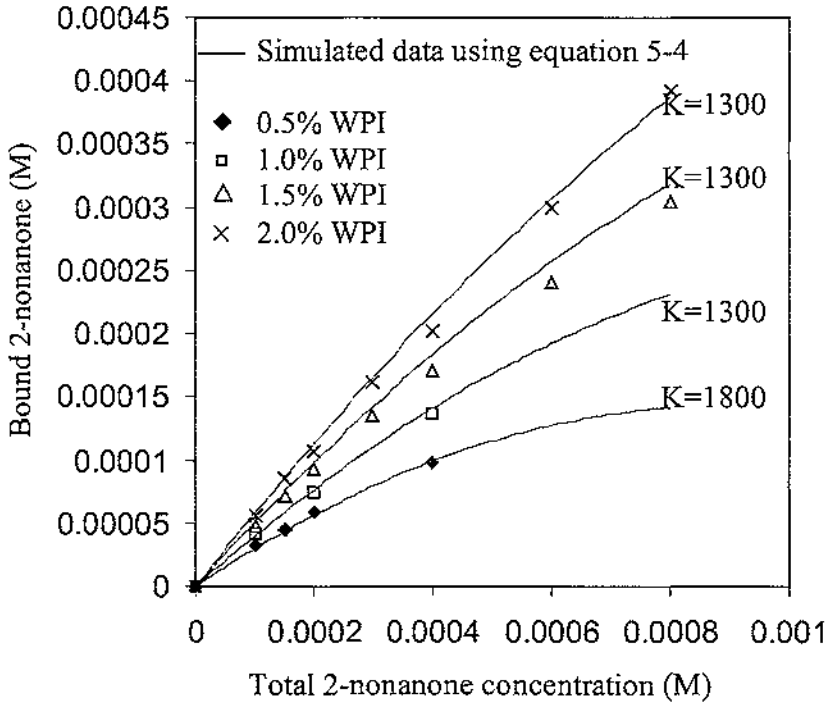

Figure 5.14. Amount of bound 2-nonanone at different WPI concentrations.

The reaction between a protein and a ligand can be described as (see Section 2.4):

$$P + L \underset{}{\overset{K}{\rightleftharpoons}} PL$$

where K is the equilibrium constant.

When equilibrium is reached,

$$K = \frac{[PL]}{[P][L]} \qquad 2\text{-}3$$

If the initial concentrations of protein and flavour are C_p and C_L respectively, and the amount of bound flavour is C_{PL}

P	+	L	=	PL
C_p		C_L		0
$C_p - C_{PL}$		$C_L - C_{PL}$		C_{PL}

From equation 2-3,

$$K = C_{PL}/[(C_p - C_{PL}) \cdot (C_L - C_{PL})] \qquad 5\text{-}4$$

The binding of 2-nonanone to WPI was simulated using equation 5-4, and the results are shown in Figure 5.14. It was found that the experimental results matched very well with the simulation results.

Different equilibrium constant (K) values were used for simulating the binding. K = 1800 was used at low protein concentration (0.5%), and K = 1300 was used for higher protein concentration (≥ 1.0%). This phenomenon was considered to be the result of the changes in the molecular structure of β-lactoglobulin. All the experiments were done at

physiological pH (pH = 6.7), where β-lactoglobulin exists as a dimer. However, at low concentration (< 20 μM), it would be monomer (Narayan and Berliner, 1997). In 0.5% WPI solution, the concentration of β-lactoglobulin is about 100 μM.

A Klotz plot was used to investigate the mechanism of binding, as mentioned in Section 2.4. The results for 2-heptanone are illustrated in Figure 5.15.

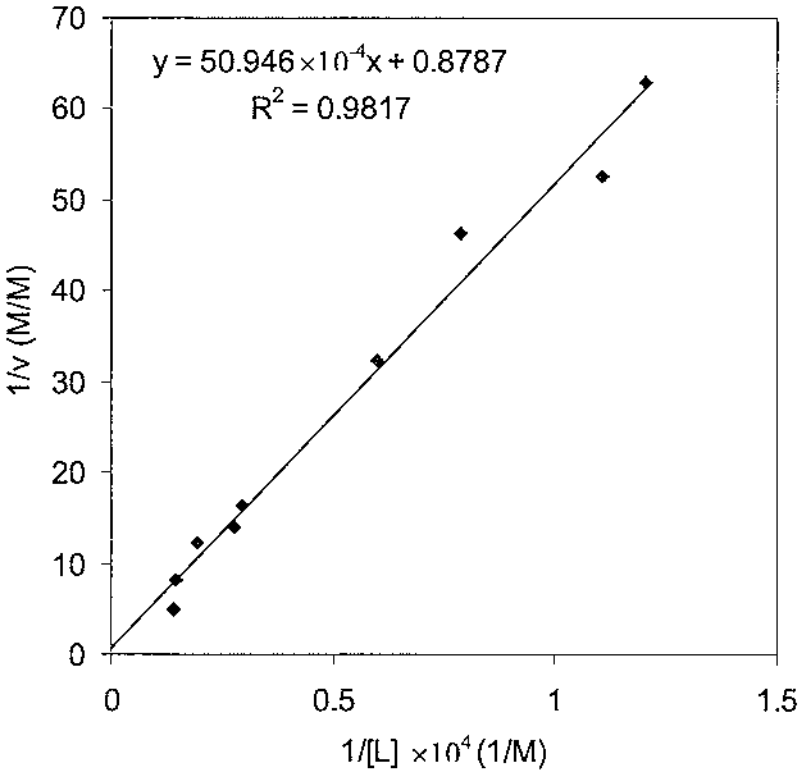

Figure 5.15. Klotz plot for 2-heptanone binding to WPI.

The binding constant for the binding of 2-heptanone to WPI was calculated. WPI was treated as β-lactoglobulin with a molecular mass 18,000 Da. From Figure 5.15, the binding constant and the binding sites were 172 [M^{-1}] and 1.1 respectively. Published results show that the binding constant for β-lactoglobulin–heptanone binding varied from 150 to 465 [M^{-1}] (O'Neill and Kinsella, 1987; Sostmann and Guichard, 1998).

Because the binding of 2-heptanone to WPI was found to be much less than the binding between WPI and 2-nonanone, further study was focused on 2-nonanone. For 2-nonanone

binding, there were obvious differences for various WPI concentrations (Figure 5.14). Separate Klotz plots were made for WPI concentrations ≤ 0.5% (Figure 5.16) and for WPI concentrations ≥ 1% (Figure 5.17).

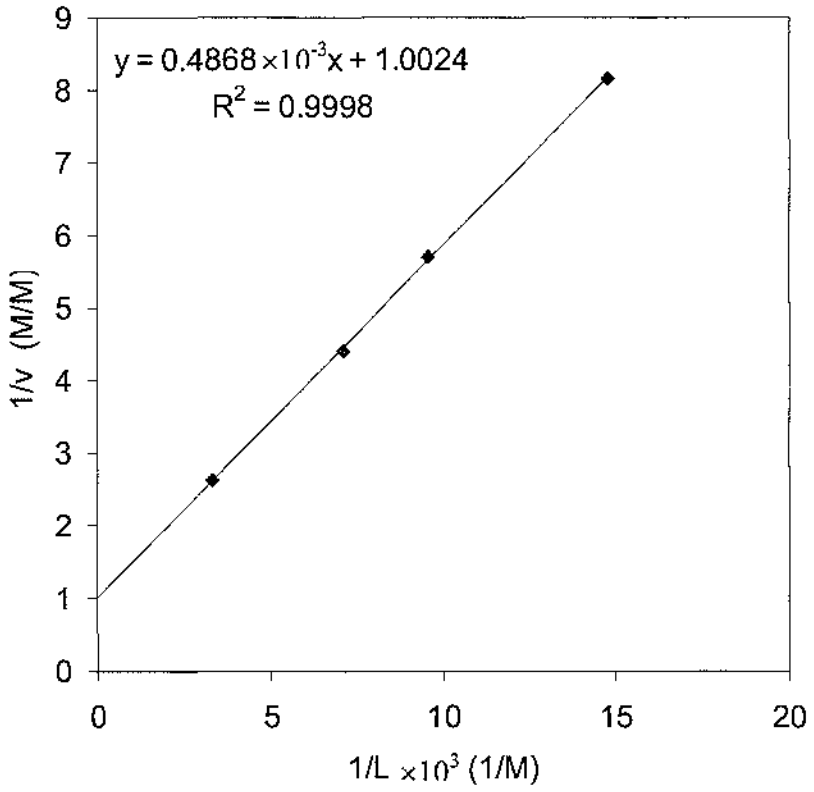

Figure 5.16. Klotz plot for the binding of 2-nonanone to WPI at WPI concentrations ≤ 0.5%.

For low WPI concentrations, the Klotz plot gave a binding constant $K = 2059$ [M^{-1}] and binding site $n = 1$. At higher WPI concentrations (≥ 1%), the binding constant K was 1072 [M^{-1}] and the number of binding sites $n = 1.2$ (Figure 5.17).

Because WPI is a mixture of several proteins, the binding constant K and the number of binding sites are average values. Published results for the binding constant for β-lactoglobulin–2-nonanone binding varied from 1667 to 3629 M^{-1} (O'Neill and Kinsella, 1987; Sostmann and Guichard, 1998).

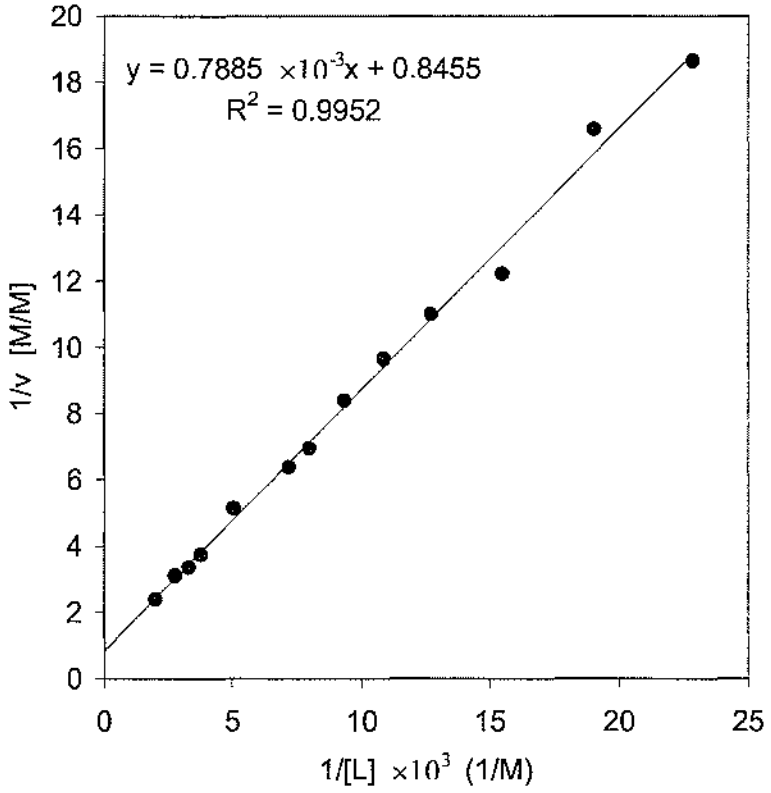

Figure 5.17. Klotz plot for the binding of 2-nonanone to WPI at WPI concentrations ≥ 1%.

If we assume that all the binding was due to the β-lactoglobulin binding in the WPI, the binding constants and the number of binding sites can be calculated from Klotz plots (Figures 5.18 and 5.19). The binding constant and binding sites for 2-nonanone–β-lactoglobulin binding in a 0.5% WPI solution (0.5% WPI equals 0.2459% β-lactoglobulin) would be 2059 M^{-1} and 1.9 respectively. At ≥ 1% WPI, these values would be 1072 M^{-1} and 2.3 respectively. The binding constants were the same as those calculated for the WPI (Figures 5.16 and 5.17), whereas the number of binding sites were two-fold higher than that for WPI.

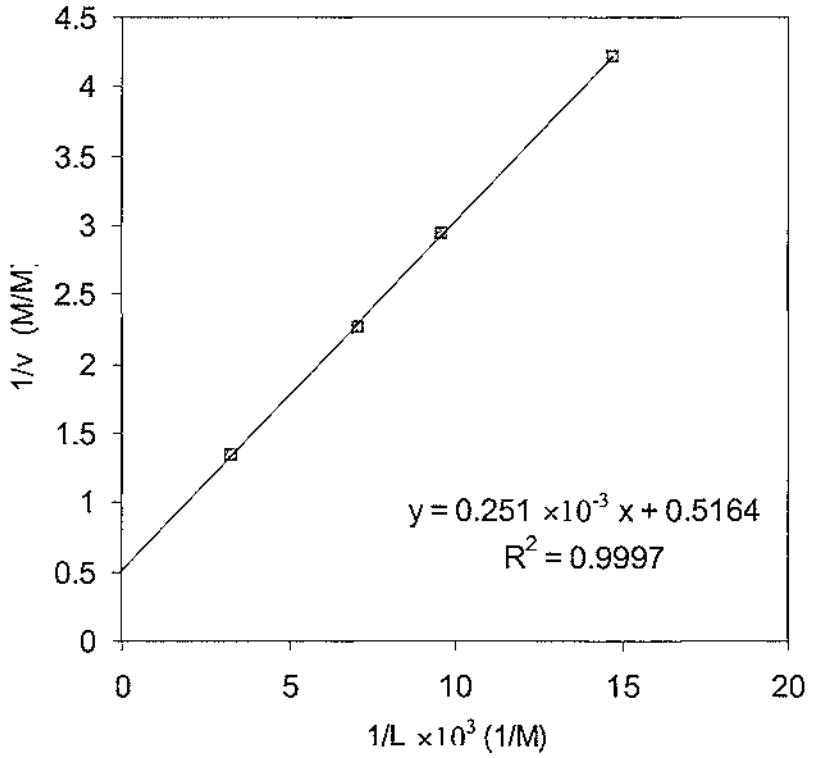

Figure 5.18. Klotz plot for 2-nonanone binding to β-lactoglobulin in WPI, assuming that all the binding occurred with β-lactoglobulin alone at WPI concentrations ≤ 0.5%.

The reason why different binding parameters were obtained at different WPI concentrations is important for understanding the binding mechanism. Published binding constants for β-lactoglobulin–2-nonanone binding varied from 1667 to 3629 M^{-1}, and the number of binding sites changed from 0.5 to 1 (O'Neill and Kinsella, 1987; Sostmann and Guichard, 1998; Muresan et al., 1999). One reason is that different techniques were used; another reason is that the conditions for determination were different. For example, O'Neill and Kinsella (1987) and Muresan et al. (1999), using an equilibrium dialysis technique, obtained different numbers of binding sites at different pH values. O'Neill and Kinsella (1987) obtained 1 binding site at pH=6.7 whereas Muresan et al. (1999) found 0.5 binding sites at pH = 3.

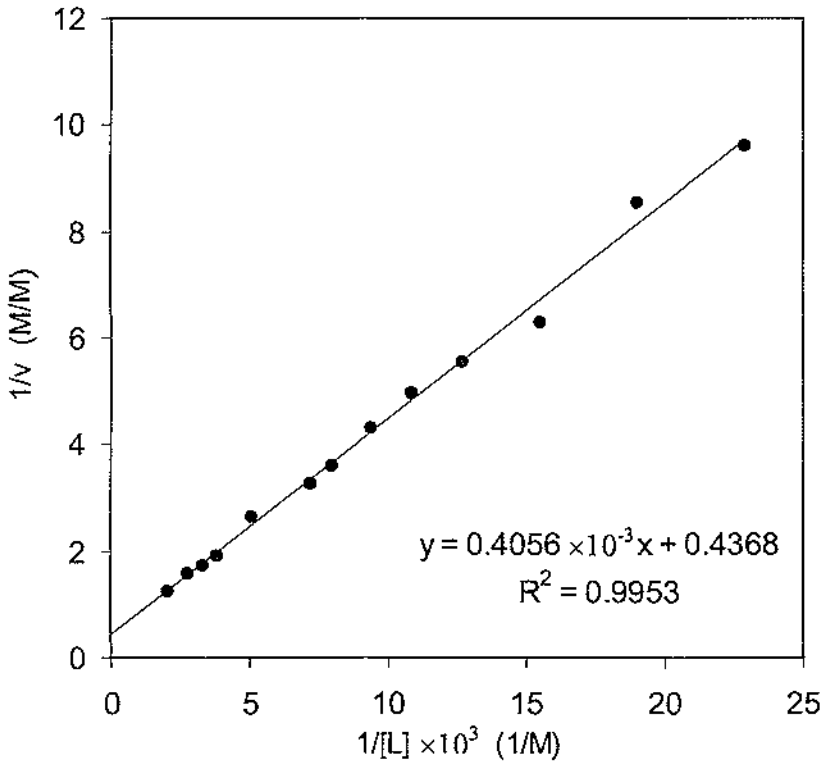

Figure 5.19. Klotz plot for 2-nonanone binding to β-lactoglobulin in WPI, assuming that all the binding occurred with β-lactoglobulin alone at WPI concentrations ≥ 1%.

A change in the structure of β-lactoglobulin may be the reason for the difference. There could be some differences in the binding sites and binding constants for dimer and monomer forms of β-lactoglobulin. It has been suggested that there is an equilibrium between the dimer and monomer, with an equilibrium constant of 4.88×10^4 M^{-1} for β-lactoglobulin A (Zimmermann, 1971). β-Lactoglobulin is roughly regarded as a dimer at neutral pH and a monomer at pH = 3 (McKenzie, 1971). The monomer would also predominate at low β-lactoglobulin concentrations (< 20 μM) (Narayan & Berliner, 1997) at neutral pH. From equilibrium theory, the monomer and dimer exist simultaneously in solution. Figure 5.20 shows the proportion of monomer β-lactoglobulin as a function of the protein concentration; the data was simulated using the association constant $K = 4.88 \times 10^4$ M^{-1}. When the total concentration of the protein is less than 20 μM (Figure 5.20), the proportion of monomer in the total protein is 0.5041. The concentration range used in

this study varied from 69 to 556 μM; this resulted in the proportion of monomer β-lactoglobulin ranging from 0.2372 to 0.1049. The slope of the curve became flat when the total concentration was larger than 278 μM (WPI > 1.5%). If the monomer and dimer had different binding sites and binding constants (K), the results obtained at different concentrations could be different.

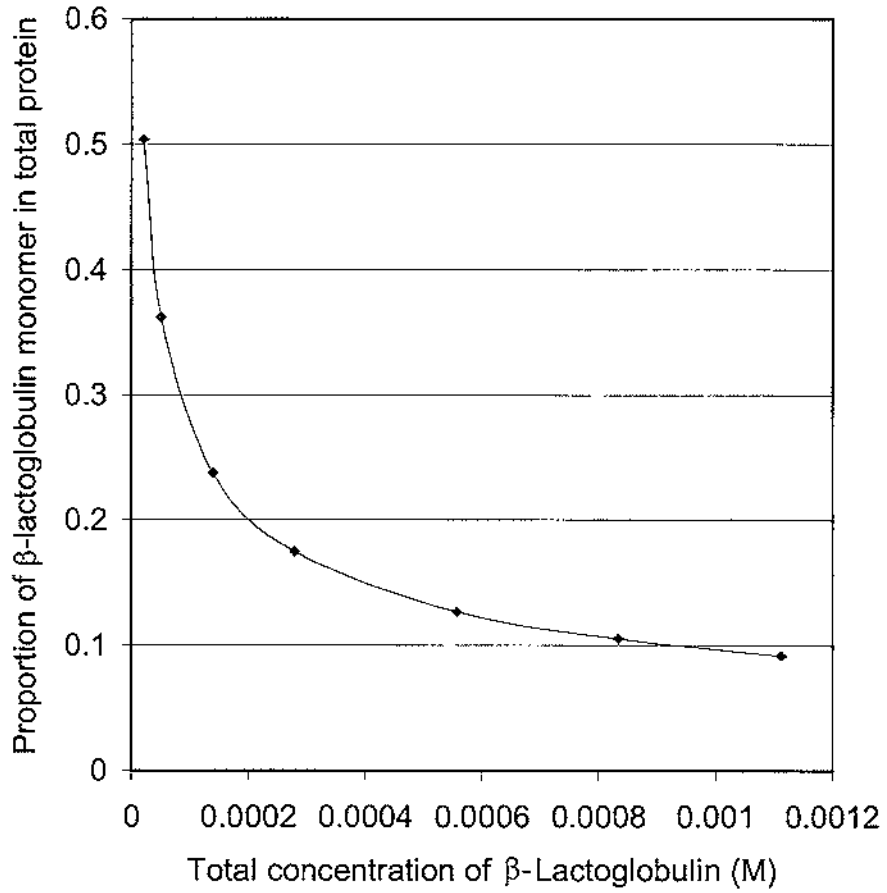

Figure 5.20. The proportion of monomer β-lactoglobulin as a function of the protein concentration (simulated data using an association constant $K = 4.88 \times 10^4\ M^{-1}$).

Narayan and Berliner (1997) found that β-lactoglobulin has, as well as one main binding site, several weak binding sites for a fatty acid spin-labile analogue, 5-doxylstearic acid.

If the binding between β-lactoglobulin and 2-nonanone was similar to that, the binding constants obtained in this study would be an average value for the different binding sites.

5.4 Conclusions

SPME is a reliable technique for investigating protein–flavour binding. The effect of ethanol can be ignored within the experimental conditions used in this study. The binding was found to be stable after 40 h at 5°C.

Na-CN was found to bind to 2-nonanone but no significant binding occurred with 2-heptanone. The binding constant for Na-CN and 2-nonanone was 1858 M^{-1}, with 0.3 binding sites per molecule.

WPI bound significantly to 2-heptanone and 2-nonanone. The binding constant of WPI (using a molecular mass of 18,000 Da) to 2-heptanone was 172 [M^{-1}], and the number of binding sites was 1. Different binding constants at low concentrations of WPI (< 0.5%) and higher concentrations (> 1%) of WPI for binding to 2-nonanone were found: 2059 and 1072 [M^{-1}] respectively. The numbers of binding sites were 1 and 1.2 at low and high WPI concentrations.

CHAPTER SIX

EFFECTS OF pH AND SODIUM PALMITATE ADDITION ON THE BINDING OF 2-NONANONE TO WPI

6.1 Introduction

Understanding the mechanism of protein flavour binding is valuable for academic study and for industry, which needs to understand the details of this process. The mechanism of β-lactoglobulin-retinol binding has been of interest for a long time. Retinol has been found to be bound within the β-barrel of the molecule (Cho et al., 1994). Frapin et al. (1993) and Dufour et al. (1994) suggested that fatty acids might bind at an external binding pocket. Narayan and Berliner (1997) found that fatty acids and retinoids bind independently and simultaneously to β-lactoglobulin, and suggested that β-lactoglobulin binds at least two different types of hydrophobic ligands simultaneously.

In contrast to the study of the binding of other ligands, a study of the mechanisms of flavour-β-lactoglobulin binding seems to be lacking. It is possible to map the binding site on β-lactoglobulin for flavour binding from the information published, using an efficient technique, which will be useful for understanding the binding between flavour compounds and β-lactoglobulin.

6.2 Materials and methods

- AR grade hydrochloric acid was obtained from BDH, Palmerston North
- Sodium palminate was purchased from Sigma-Aldrich Fine Chemicals (St. Louis, Missouri, USA).

6.2.1 Preparation of solutions

0.5% WPI solutions at different pH values were prepared by weighing 2.5 g WPI into a 400 ml beaker, adding 250 ml water, agitating for 1 h and adding hydrochloric acid (0.1 M) to adjust the pH. The solution was then transferred into a 500 ml volumetric flask and made up to volume with water to give a 0.5% WPI solution. The stock solution was stored at 5°C.

6.3 Results and discussion

6.3.1 Effect of pH on WPI–2-nonanone binding

The binding of 2-nonanone to WPI was affected by the pH of the mixture solution (Figure 6.1). These changes may be related to changes in the molecular state of β-lactoglobulin with the pH in the WPI solution (see Chapter 2).

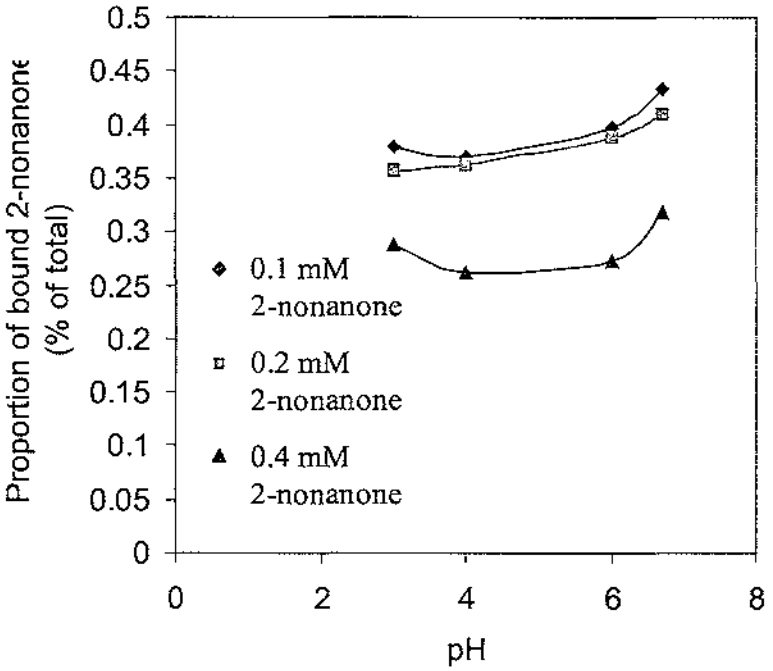

Figure 6.1. The effect of pH on the binding of 2-nonannone to WPI (1%)

The data obtained at each pH for different flavour concentrations were analysed using a Klotz plot as described previously. The results are shown in Figures 6.2–6.4.

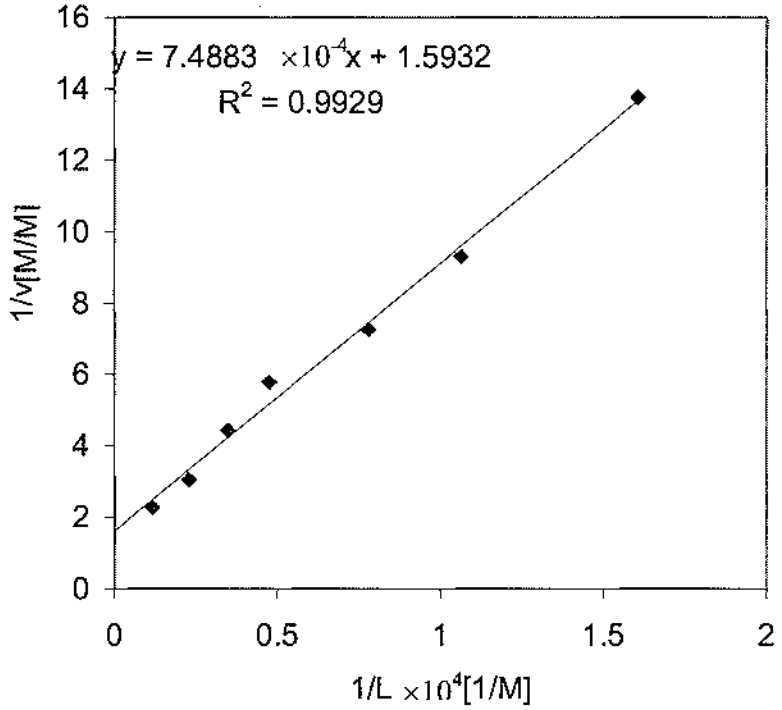

Figure 6.2. Klotz plot for 2-nonanone and WPI binding at pH = 3.

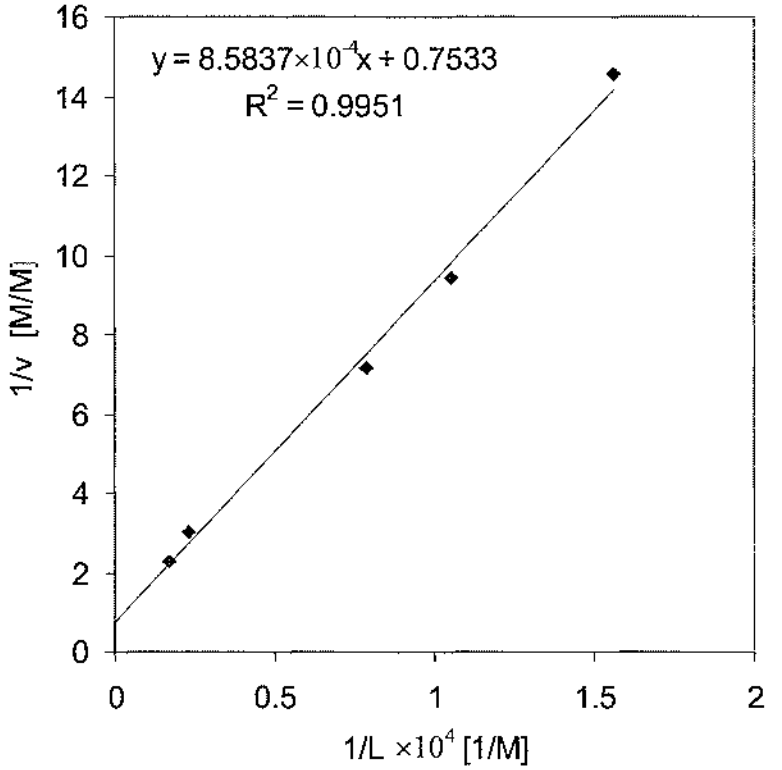

Figure 6.3. Klotz plot for 2-nonanone and WPI binding at pH = 4.

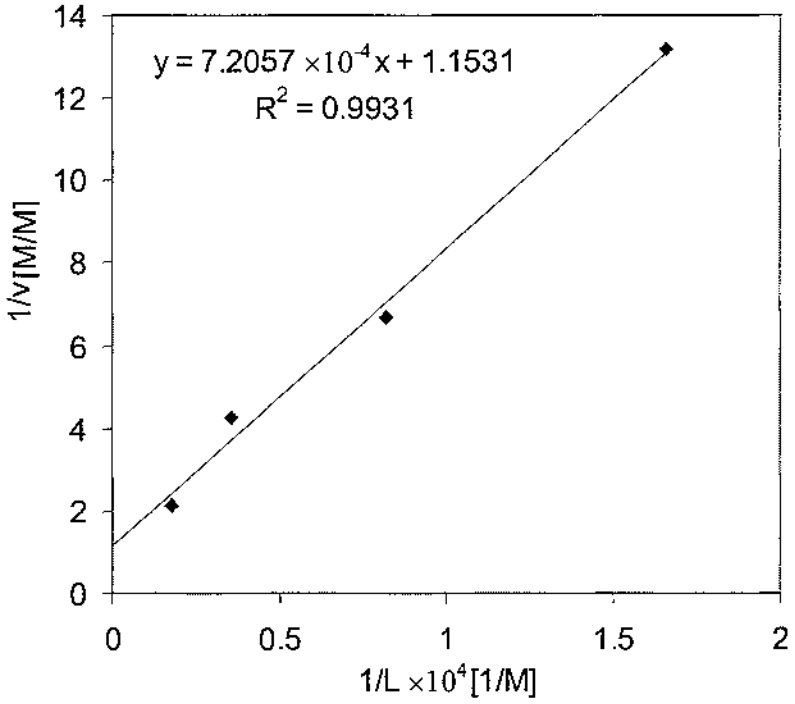

Figure 6.4. Klotz plot for 2-nonanone and WPI binding at pH = 6.

The binding constants and the numbers of binding sites calculated from the slopes and intercepts of the Klotz plots (Figures 6.2–6.4) are listed in Table 6.1.

Table 6.1. WPI–2-nonanone binding parameters at different pH values

pH	Molecular state of β-lactoglobulin in WPI solution	Binding constant K [M^{-1}]	Number of binding sites per monomer β-lactoglobulin n [M/M]	nK [M^{-1}]
3	Monomer	2128	0.6	1278
4	Octamer	878	1.3	1141
6	Dimer	1600	0.9	1388
6.7	~76% Dimer + 24% Monomer*	2059	1	2059
	~90% Dimer + 10% Monomer*	1072	1.2	1286

* Calculated using the result of Zimmermann et al. (1970).

The flavour binding ability is related not only to the number of binding sites, but also to the binding constants. It appears that, the smaller the number of binding sites, the greater are the binding constants. Thus the binding ability can be expressed as the product of binding constant and the number of binding sites ($n \cdot K$). Although the binding parameters are very variable, the binding ability does not change much with changing pH (Figure 6.1).

If the flavour is considered to be interacting only with β-lactoglobulin in the WPI (bovine serum albumin was also found to bind to flavour, but, because it is in low concentration in WPI, it will not have much effect on the binding results), the binding parameters calculated from the experimental data obtained for WPI (Figures 6.2–6.4) can be understood more readily. The binding parameters from the Klotz plots for β-lactoglobulin (Figures 6.5–6.7) are listed in Table 6.2.

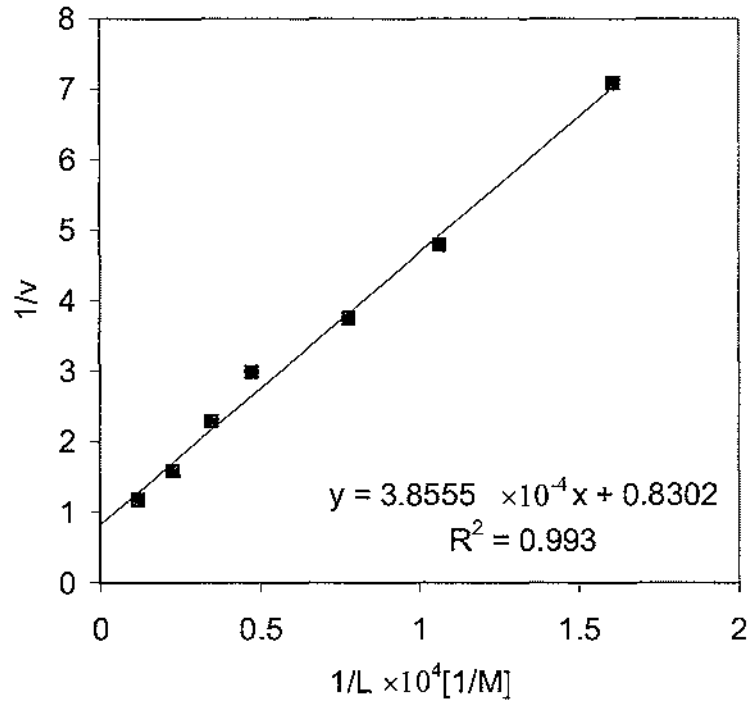

Figure 6.5. Klotz plot for binding of 2-nonanone to β-lactoglobulin in WPI solution at pH = 3

Table 6.2. β-Lactoglobulin–2-nonanone binding parameters at different pH values

pH	Molecular state of β-lactoglobulin in WPI solution	Binding constant K [M^{-1}]	Number of binding site per monomer β-lactoglobulin n [M/M]	nK [M^{-1}]
3	Monomer	2153	1.2	2594
4	Octamer	919	2.5	2268
6	Dimer	1566	1.7	2684
6.7	~76% Dimer + 24%Monomer*	2059	1.9	3984
	~90% Dimer + 10% Monomer*	1072	2.3	2465

* Calculated using the result of Zimmermann et al. (1970)..

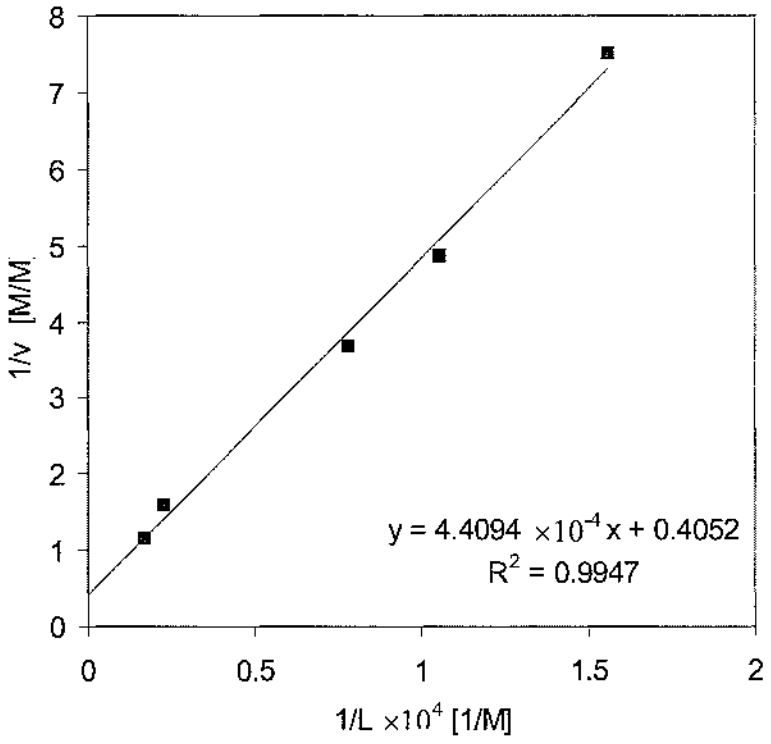

Figure 6.6. Klotz plot for binding of 2-nonanone to β-lactoglobulin in WPI solution at pH = 4.

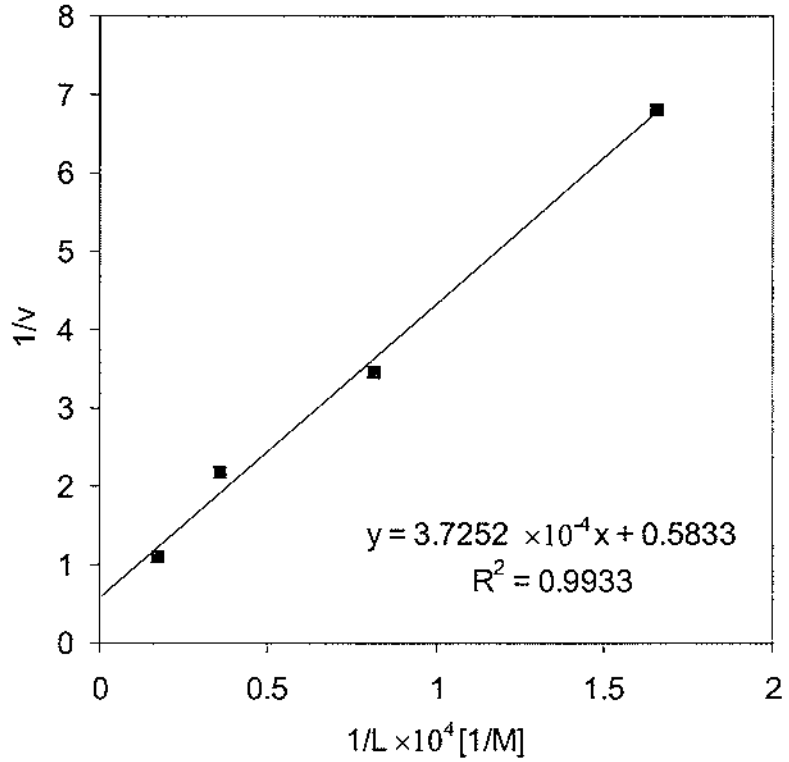

Figure 6.7. Klotz plot for binding of 2-nonanone to β-lactoglobulin in WPI solution at pH = 6

The values of the binding constants for β-lactoglobulin–2-nonanone binding listed in Table 6.2 were similar to those for WPI–2-nonanone binding listed in Table 6.1. The numbers of binding sites for β-lactoglobulin–2-nonanone binding were twice those for WPI–2-nanonone binding. These values changed with changes in pH, which affected the molecular state of β-lactoglobulin. The value of *n* for the monomer was 1.2 and that for the octamer was 2.5.

The binding between β-lactoglobulin and ketones has been suggested to involve hydrophobic interactions (Guichard and Langourieux, 2000). A good linear correlation was found between the logarithm of the binding constant measured by affinity chromatography and the hydrophobicity of the molecule. It is easy to understand that the hydrophobic pocket of the protein is one of the binding sites on β-lactoglobulin. From

Figure 6.7, the number of binding sites appeared to be more than one which suggests that there would be some other binding sites as well as that in the hydrophobic pocket of β-lactoglobulin.

The binding sites for the binding of protein to flavour compounds are still not clear. Fatty acids and retinoids were found to bind simultaneously to β-lactoglobulin (Narayan and Berliner, 1997), which means that there are probably at least two different hydrophobic ligand binding sites. Sawyer et al. (1998) mapped the binding sites and suggested that β-lactoglobulin binds hydrophobic ligands in at least three independent binding sites. One of these is an internal cavity that can readily accommodate retinols. A solvent-accessible hydrophobic cleft runs between the three–turn α-helix that is packed against the outer surface of the β-barrel. This cleft can accommodate fatty acids such as palmitate and stearate. The third binding site is on the outer surface close to the conserved Trp19 Arg124. This site is large enough to accommodate larger aromatic ligands such as ellipticine.

In contrast to the studies on the binding of fatty acids and retinoids to β-lactoglobulin, flavour binding research is far from satisfactory. Jouenne et al. (2000) found that the binding of 2-nonanone induced a decrease in the binding of ethyl hexanoate and vice versa. These two ligands are in competition for their binding into the hydrophobic pocket of the protein. Such competitions were also found between γ-undecalactone, β-ionone and retinol (Muresan and Leguijt, 1998), and γ-octalactone and β-ionone (Sostmann and Guichard, 1998). Guichard and Langourieux (2000) obtained a good linear correlation between the logarithm of the binding constant measured by affinity chromatography and the hydrophobicity of ketones, aldehydes, alcohols and lactones. They suggested that the ligands bind into the hydrophobic pocket of the protein by hydrophobic interactions. They also found that such a linear relationship could not be obtained for terpene alcohols and phenolic compounds. This suggests more than one binding site for flavour binding.

Many published reports indicate that the number of binding sites on β-lactoglobulin for flavour binding is less than or equal to 1 (O'Neill and Kinsella, 1987; Charles et al., 1996; Muresan et al., 1999). The number of binding sites identified in the WPI solution in the present study was twice these published results. From the number of fatty acids and retinoids binding to the protein published by Sawyer et al. (1998), three different binding sites were suggested as hydrophobic interaction sites. The number of binding sites (2.5) obtained in this study is reasonable. If other proteins in the WPI interact with the flavour, the final result will be different from that obtained with β-lactoglobulin.

6.3.2 Effect of sodium palmitate on WPI–2-nonanone binding

Sodium palmitate was used for mapping the binding of 2-nonanone to WPI. Palmitic acid, regarded as a typical fatty acid, binds to β-lactoglobulin on the outer surface hydrophobic cleft which runs between the three-turn α-helix that is packed against the outer surface of the β-barrel (Sawyer et al., 1998). If the binding between 2-nonanone and WPI is affected by sodium palmitate, it would appear that 2-nonanone binds on the same binding site on β-lactoglobulin as palmitic acid.

The molecular weight of sodium palmitate is 278.4 Da. The molecular weight of WPI is 18,000 Da and sodium palmitate binds to β-lactoglobulin in the molar ratio 1:1. The weight ratio will be 1:129 (as there is only about 50% β-lactoglobulin in WPI). If the concentration of WPI is 0.5%, the concentration of sodium palmitate will be 0.00388%, i.e. about 40 ppm in the solution.

SPME analyses were carried out on two samples after mixing for 40 h. The first sample was 0.5% WPI solution, 2×10^{-4} M 2-nonanone and 1.4×10^{-4} M (40 ppm) sodium palmitate. The other sample was without sodium palmitate. The molar concentration of β-lactoglobulin in 0.5% WPI was 1.38×10^{-4}M, and the binding constant between palmitic acid and β-lactoglobulin has been reported to be 1×10^{7} M^{-1} (Frapin et al., 1993). This value of the binding constant is much larger than the 2059 M^{-1} between 2-nonanone and

β-lactoglobulin found in this study. The SPME results (Figure 6.8) clearly show that the addition of sodium palmitate to WPI considerably reduced its ability to bind 2-nonanone. Sodium palmitate probably bound to β-lactoglobulin and saturated the palmitate binding site on β-lactoglobulin. There was still about 10% of flavour binding to β-lactoglobulin in the palmitate-containing WPI system.

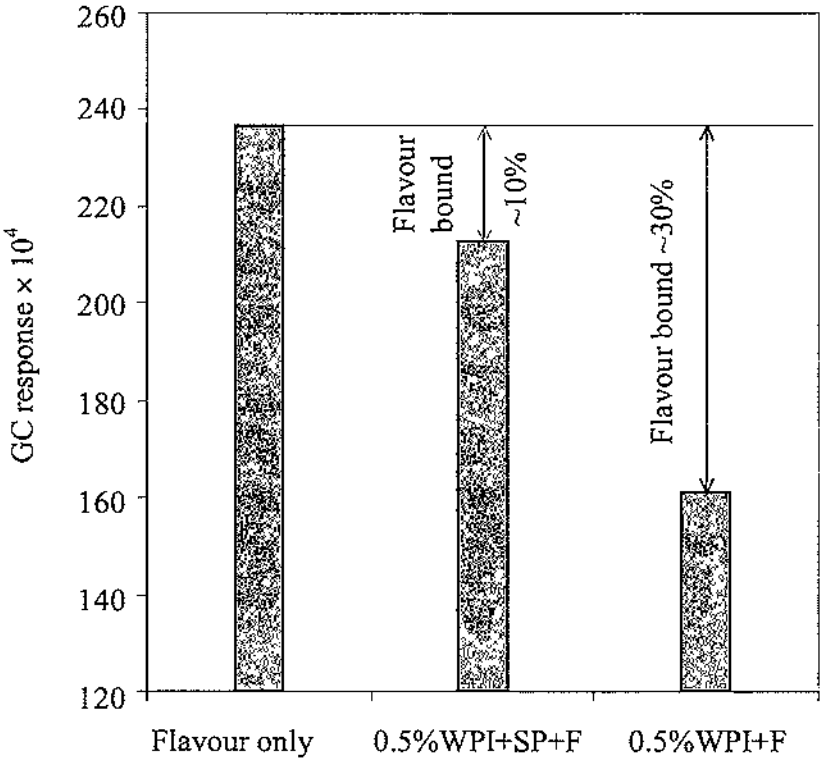

Figure 6.8. The effect of sodium palmitate on the binding of 2-nonanone to WPI.
F: flavour – 2-nonanone; SP: sodium palmitate.

Asuming that the two ligands, sodium palmitate and 2-nonanone, competed for binding to β-lactoglobulin simultaneously on the same binding site with binding constants of 1×10^7 M^{-1} (Frapin et al., 1993) and 2059 M^{-1} respectively, and the initial concentrations of sodium palmitate and β-lactoglobulin were 1.4 and 1.38×10^{-4} M respectively, the equilibrium binding between sodium palmitate and β-lactoglobulin could be described as:

$$\underset{}{SP} + \underset{}{BL} \underset{}{\overset{K = 1 \times 10^{7}}{\rightleftharpoons}} \underset{}{BL{\bullet}SP}$$

initial concentration:	1.4×10^{-4}	1.38×10^{-4}	0
after equilibrium:	0.05×10^{-4}	0.03×10^{-4}	1.35×10^{-4}

The remaining (unreacted) sodium palmitate and β-lactoglobulin concentration after equilibrium would be 0.05×10^{-4} and 0.03×10^{-4} M respectively.

Assuming that 2-nonanone (20 μM) binds to β-lactoglobulin at the same binding site as sodium palmitate, because there is a huge difference between the two equilibrium constants (2059 M^{-1} for 2-nanonone and 1×10^{7} M^{-1} for sodium palmitate), the degree of binding could be calculated as follows:

$$\underset{}{F} + \underset{}{BL} \underset{}{\overset{K = 2059}{\rightleftharpoons}} \underset{}{BL{\bullet}F}$$

2×10^{-4}	0.03×10^{-4}	0
$2 \times 10^{-4} - x$	$0.03 \times 10^{-4} - x$	x

$$\frac{x}{(2 \times 10^{-4} - x)(0.03 \times 10^{-4} - x)} = 2059$$

i.e.

$$2059x^{2} - 1.418x + 1.235 \times 10^{-6} = 0$$

$$x = 8.7 \times 10^{-7}\,\mathrm{M}$$

This means that, if the fatty acid binding sites on the β-lactoglobulin molecules were saturated by palmitic acid, only a tiny amount of flavour (0.4% of the total flavour) could bind to the same binding site on β-lactoglobulin. Actually, the results obtained from the experiment showed that about 10% of 2-nonanone bound to β-lactoglobulin (Figure 6.8) when β-lactoglobulin was saturated by sodium palmitate. This means that flavour binds to β-lactoglobulin not only on the fatty acid binding site on the β-lactoglobulin molecules. The amount of bound 2-nonanone decreased from about 30% to 10% because

of the presence of sodium palmitate. This suggests that most of the 2-nonanone (about 70%) bound to β-lactoglobulin on the fatty acid binding site. When this binding site was occupied by sodium palmitate, which is a much stronger β-lactoglobulin binding ligand than 2-nonanone, the amount of 2-nonanone bound decreased by nearly 70%.

The effects of different amounts of sodium palmitate added to the sample were investigated (Figure 6.9); the molar ratio of sodium palmitate to β-lactoglobulin was varied from 0.16 to 0.64. The results showed that the bound 2-nonanone decreased with increasing molar ratio.

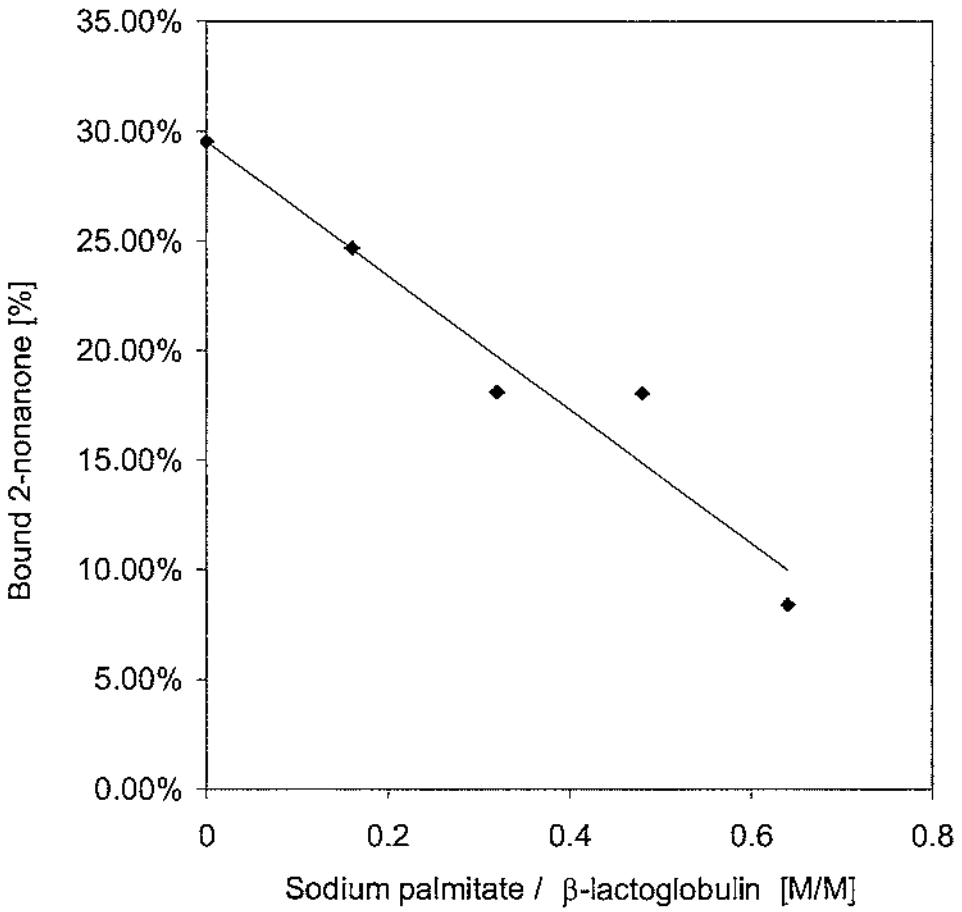

Figure 6.9. The effect of added sodium palmitate on β-lactoglobulin–2-nonanone binding.

Approximately 9% (obtained from the experiment) of 2-nonanone bound to the other binding site on the β-lactoglobulin molecules. On the fatty acid binding site, 2-nonanone competed with sodium palmitate for binding to β-lactoglobulin. The binding could be calculated using the equilibrium model as follows.

For example, when the molar ratio of sodium palmitate to β-lactoglobulin is 0.16, the concentrations of each component are as follows:

$$\begin{array}{ccccc} & & & \overset{K=1\times10^{7}}{\rightleftharpoons} & \\ \text{SP} & + & \text{BL} & & \text{BL•SP} \\ 0.16\times1.38\times10^{-4} & & 1.38\times10^{-4} & & 0 \\ 0.16\times1.38\times10^{-4}-x & & 1.38\times10^{-4}-x & & x \end{array}$$

where x is the amount of β-lactoglobulin bound by sodium palmitate.

$$\frac{x}{(0.16\times1.38\times10^{-4}-x)\cdot(1.38\times10^{-4}-x)}=1\times10^{7}$$

i.e.

$$1\times10^{7}x-1.6018\times10^{3}x+3.047\times10^{-2}=0$$

$$x=2.206\times10^{-5}\text{M}$$

The free β-lactoglobulin left after being bound by sodium palmitate was 1.16×10^{-4} M. The binding between 2-nonanone and β-lactoglobulin was calculated as follows:

$$\begin{array}{ccccc} & & & \overset{K=2059}{\rightleftharpoons} & \\ \text{F} & + & \text{BL} & & \text{BL•F} \\ 2\times10^{-4} & & 1.16\times10^{-4} & & 0 \\ 2\times10^{-4}-y & & 1.16\times10^{-4}-y & & y \end{array}$$

$$\frac{y}{(2\times10^{-4}-y)\cdot(1.16\times10^{-4}-y)}=2059$$

i.e.

$$2059y^2 - 1.65y + 4.777 \times 10^{-5} = 0$$

$$y = 3 \times 10^{-5}\ \text{M}$$

The amount of 2-nonanone bound on the fatty acid binding site on β-lactoglobulin molecules would be 15% (= $3 \times 10^{-5}/2 \times 10^{-4}$).

Thus, when the molar ratio of sodium palmitate to β-lactoglobulin was 0.16, 15% of the total 2-nonanone bound to the fatty acid binding site and 9% of total 2-nonanone bound to the other binding site, so that the amount of 2-nonanone bound was 24% of the total flavour concentration.

The amounts of 2-nonanone bound for different ratios of sodium palmitate to β-lactoglobulin were calculated using the same model. The results are listed in Table 6.3.

Table 6.3 Bound amount of 2-nonanone in systems containing different proportions of sodium palmitate added

Ratio SP/BL	Bound F on other site	Bound F in fatty acid binding site	Total bound F
0	9%	17.5%	26.5%
0.16	9%	15%	24%
0.32	9%	12.4%	21.4%
0.48	9%	9.7%	18.7%
0.64	9%	6.9%	15.9%
0.8	9%	4.0%	13%
1.0	9%	0.5%	9.5%

BL: β-lactoglobulin; F: flavour compound (2-nonanone); SP: sodium palmitate.

The results of the competition model proposed in this study matched the experimental data (Figure 6.10) reasonably well, except for the data where the ratio of sodium palmitate to β-lactoglobulin was 0.64. Further work is needed to refine this model and to explain the results in more detail.

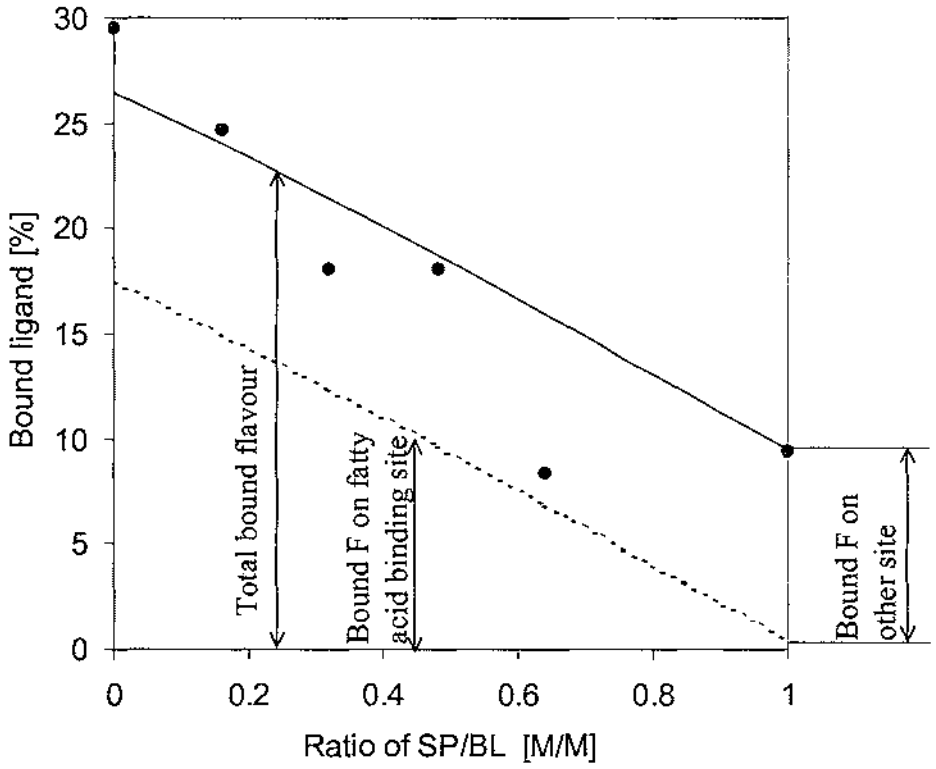

Figure 6.10. Comparison of the competition model (—— ;———) and experimental (●) results (0.5% WPI, 0.2 mM 2-nonanone)

SP: sodium palmitate; BL: β-lactoglobulin; F:flavour (2-nonanone)

6.4 Conclusions

In conclusion, this work showed that the binding constant and the number of binding sites for the binding of β-lactoglobulin and flavour compounds (2-nonanone) changed with changes in the pH. The binding constant changed from 919 M^{-1} to 2153 M^{-1} and the number of binding sites changed from 1.2 to 2.5 in the pH range 3–6.7. The phenomenon is caused by the structural changes in β-lactoglobulin. For octamer-dominant β-

lactoglobulin (pH = 4), the number of binding sites was found to be 2.5 and the binding constant was 919 M^{-1}. For monomer-dominant β-lactoglobulin (pH = 3), the number of binding sites was found to be 1.2 and the binding constant was 2153 M^{-1}. The more molecules of β-lactoglobulin in the structure, the greater is the number of binding sites per molecule of β-lactoglobulin. However, the more molecules in the structure, the smaller is the binding constant. The flavour binding ability of β-lactoglobulin (the product of binding constant and the number of binding sites) remained effectively unchanged with changes in pH (from 2268 to 2684 M^{-1}).

Nearly 70% of the binding ability of 2-nonanone to β-lactoglobulin was lost after β-lactoglobulin bound with sodium palmitate. This showed that 2-nonanone does interact with β-lactoglobulin at the fatty acid binding site.

The competition model proposed in this study suggested that flavour compounds (2-nonanone) and fatty acids (sodium palmitate) are likely to bind to the same binding site on β-lactoglobulin simultaneously and that there is competitive binding between the flavour compound (2-nonanone) and fatty acid (sodium palmitate). Sodium palmitate, which has a much higher binding constant than 2-nonanone, has much greater binding ability to β-lactoglobulin.

CHAPTER SEVEN

CONCLUSIONS AND FUTURE DIRECTIONS

To date, there is no published work on the binding of flavour to milk protein, using the SPME technique. This study was the first to use this technique to understand the binding of two flavour compounds to commercial milk protein products.

The SPME technique developed showed good repeatability. The maximum deviation of the method in this study was 4.1%. The method could be used to analyse flavour–protein binding in a system where more than 10% of the total flavour binds to the protein, because the amount of flavour compound bound was quantified by comparing the GC response of the flavour compound in water with that of the flavour compound in protein solution. Flavour–protein binding analysis is more difficult than analysis of the flavour compound itself. The relative deviation of method is equal to the GC response deviation of each duplicate from the average divided by the average GC response. The relative deviation of the binding results is the GC response deviation divided by the amount of flavour compound bound, which varied from 10 to 50% of the total flavour compounds. In general, the relative deviation of the binding analysis was several times higher than that of the total flavour analysis.

Several SPME conditions, such as the fibre, the extraction time and the container used for SPME, were optimised to minimize the deviation: 30 and 100 μm PDMS fibres were chosen, extraction times of 5 min and 30 seconds were applied for equilibrium extraction and dynamic extraction respectively, 4 ml HPLC vials were selected for SPME extraction.

In the protein–flavour binding study, two commercial milk proteins (WPI and Na-CN) were investigated for their binding properties with two ketones: namely 2-heptanone and 2-nonanone. It was found that WPI binds more readily than Na-CN to ketone flavour compounds. The binding constant of WPI to 2-nonanone and the number of binding sites were 2059[M^{-1}] and 1 respectively at low WPI concentration (0.5%), and were 1072 [M^{-1}] and 1.2 respectively at higher WPI concentration (≥ 1%). For Na-CN, the binding constant to 2-nonanone was 1858 [M^{-1}] and the number of binding sites was 0.3. The binding constant and the number of binding sites for WPI and 2-heptanone were 172[M^{-1}] and 1.1 respectively, whereas no measurable amount of binding between Na-CN and 2-heptanone was detected.

The effects of the pH of the solution on the binding of 2-nonanone to WPI were investigated. The binding between WPI and 2-nonanone was regarded as β-lactoglobulin and 2-nonanone binding. The binding constants and the numbers of binding sites at different pH values that cause the different structures of β-lactoglobulin molecules (e.g. Monomer at pH = 3, dimer at pH = 6.7 or octamer at pH = 4) were different. The monomer had a higher binding constant and a lower number of binding sites whereas the octamer had a lower binding constant and a higher number of binding sites.

Furthermore, the binding position of 2-nonanone on β-lactoglobulin was mapped. The method used was to add sodium palmitate, which binds to β-lactoglobulin with a much higher binding constant. The position of this binding site is known as the first binding site of fatty acids on β-lactoglobulin. Approximately 70% of 2-nonanone was bound to this binding site on β-lactoglobulin. The other 30% was bound to the other site. This means that 2-nonanone bound to β-lactoglobulin on at least two binding sites.

The competition model proposed in this study appeared to be in good agreement with the experimental data when it was used to model the simultaneous binding of 2-nonanone and sodium palmitate to β-lactoglobulin.

It is recommended that, in the future, pure β-lactoglobulin–flavour binding should be investigated to study the binding mechanism in more detail. The WPI chosen to investigate flavour–protein binding in this stuidy is quite useful for industry practice, but pure β-lactoglobulin is better for understanding the mechanisms at the molecular level.

For further research on the mechanism of β-lactoglobulin–flavour binding, mapping of the other binding site on β-lactoglobulin is recommended. As pointed out in this study, about 70% of the bound 2-nonanone binds to the first binding site for fatty acids; the binding site for the other 30% is still unknown. The competition model still needs to be improved, including the details of the second binding site.

Flavour–protein binding research is a good start to studying the flavour properties in a real food system, which concerns not only flavour–protein binding but also other matrix–flavour binding and more importantly concerns flavour release.

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Appendix A
Calculation Results for Klotz Plot

1. Model

In equation 2-11

$$\frac{1}{v} = \frac{1}{nK[L]} + \frac{1}{n} \qquad 2\text{-}11$$

where

$[L]$ = the concentration of free flavour compound

= the concentration of flavour detected;

$1/v$ = [bound flavour/total protein]$^{-1}$ = total protein/ bound flavour

The initial concentration of flavour compound and protein are C_{of} and C_{op}, respectively. The bound amount of flavour = $C_{of} - [L]$.

The concentration of protein can be adjust by the coefficients k, it is because the concentration of protein solution will change when the protein-flavour mixed solution is made. If k= 0.99, it means 1 ml flavour solution is added into 100 ml volumetric flask with a definite concentrate (for example, 1%) protein solution to make the mixture. The concentration of protein should be adjusted by timing the coefficient k. For example, when 1% protein solution was used to make the protein-flavour mixture, if k=0.99, the real concentration of protein in the mixture will be 0.99%.

the calculation results for Klotz plot is listed in Table A-1.

Table A-1: Results of calculation results for Klotz plot.

C_{of} [μM]	C_{op} [%]	k	$[L]$ [μM]	Bound flavour [μM]	$1/[L]$ [1/M]	$1/v$ [M/M]
0.1	0.477	0.99	0.06785	0.03215	14738	8.16
0.15	0.477	0.985	0.10425	0.04575	9592	5.70
0.2	0.477	0.98	0.141	0.059	7092	4.40
0.4	0.477	0.96	0.30276	0.09724	3303	2.61
0.1	0.954	0.99	0.05773	0.04227	17322	12.41
0.2	0.954	0.98	0.12522	0.07478	7986	6.95
0.3	0.954	0.97	0.19044	0.10956	5251	4.69
0.4	0.954	0.96	0.26368	0.13632	3792	3.73
0.8	0.954	0.92	0.544654	0.255344	1836	1.91
0.1	1.431	0.99	0.05222	0.04778	19149	16.48
0.15	1.431	0.985	0.078972	0.07128	12663	10.99
0.2	1.431	0.98	0.10702	0.09298	9344	8.38
0.4	1.431	0.96	0.2286	0.1714	4374	4.46
0.6	1.431	0.94	0.36	0.24	2778	3.11
0.8	1.431	0.92	0.49546	0.30464	2018	2.40
0.1	1.904	0.99	0.04374	0.05626	22862	18.65
0.15	1.904	0.985	0.06462	0.08538	15475	12.23

Table A-1: Results of calculation for Klotz plot (continued).

C_{of} [μM]	C_{op} [%]	k	$[L]$ [μM]	Bound flavour [μM]	$1/[L]$ [1/M]	$1/v$ [M/M]
0.2	1.904	0.98	0.09218	0.10782	10848	9.54
0.3	1.904	0.97	0.1389	0.1611	7199	6.38
0.4	1.904	0.96	0.1984	0.2016	5040	5.05
0.6	1.904	0.94	0.30012	0.29988	3332	3.32
0.8	1.904	0.92	0.40752	0.39248	2454	2.48

Appendix B

Simulation of Dissociation of β-lactoglobulin

1. Model

The association of monomer-dimer equilibrium of β-lactoglobulin with equilibrium coefficient constant K is represented as

$$M + M \overset{K}{\Leftrightarrow} D$$

where,

M and D represent monomer and dimer, respectively.

The equilibrium can be expressed as

$$K = [D]/[M]^2 \qquad \text{B-1}$$

initial concentration of total protein is D_0, the equilibrium concentration of monomer and dimer are x and $(D_0 - x)$, respectively.

$K = 4.878 \times 10^4 \text{M}^{-1}$ (Zimmermann, 1970).

Then, equation (B-1)can be represented as

$$(D_0 - x) / (2x)^2 = 4.878 \times 10^4 \qquad \text{B-2}$$

i.e.

$$4 \times 4.878 \times 10^4 x^2 + x - D_0 = 0 \qquad \text{B3}$$

the equilibrium concentration of dimer and monomer can be calculated from equation (B-3). The results is listed in Table (B-1)

Table B-1. The results of simulation of equilibrium concentration of monomer and dimer of β-lactoglobulin (K = 4.881 × 10^{-4} Zimmermann, 1970).

Initial concentration of dimer		Monomer Total protein M/T	Equilibrium concentration of monomer (M)	Equilibrium concentration of monomer (M)
Wt/Wt %	M			
0.036	1×10^{-5}	0.5041	1.0082×10^{-5}	4.959×10^{-6}
0.09	2.5×10^{-5}	0.3617	1.8084×10^{-6}	1.5958×10^{-5}
0.25	6.944×10^{-5}	0.2372	3.295×10^{-5}	5.297×10^{-5}
0.5	1.39×10^{-4}	0.1744	4.849×10^{-5}	1.1475×10^{-5}
1	2.78×10^{-4}	0.1268	7.053×10^{-5}	2.4274×10^{-4}
1.5	4.167×10^{-4}	0.1049	8.743×10^{-5}	3.7299×10^{-4}
2	5.556×10^{-4}	0.0916	1.0171×10^{-4}	5.04709×10^{-4}