Studies on Interactions of Milk Proteins with Flavour Compounds

A thesis presented in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy in Food Technology

at Massey University, Palmerston North, New Zealand.

Janina Kühn

2007

Massey University

RIDDET CENTRE

...ADVANCING KNOWLEDGE
IN FOODS AND BIOLOGICALS

ABSTRACT

Milk proteins are known to bind volatile flavour compounds to varying extents, depending on the nature of the protein and flavour compound. Processing conditions, such as temperature and pH, are also known to have an influence on the interactions between milk proteins and flavour compounds. These interactions cause a great challenge for flavour scientists because they influence the perceived aroma profile of food products significantly, in particular in low-fat food products.

The objectives of this research were to develop a headspace solid-phase microextraction (SPME) method followed by gas chromatography with flame ionisation detection (GC-FID) for the investigation of protein-flavour interactions, and to determine binding parameters of the hydrophobic flavour compound, 2-nonanone, to individual milk proteins – namely, β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), α_{s1}-casein, and β-casein –, whey protein isolate (WPI), and sodium caseinate. Secondly, it was the aim to compare the binding of the structurally similar flavour compounds – 2-nonanone, 1-nonanal, and *trans*-2-nonenal – to WPI in aqueous solution, and to investigate the effect of heat and high pressure treatment, and pH on the extent of protein-flavour binding. The final objective was to investigate the *in vivo* release of the reversibly bound flavour compound, 2-nonanone, from WPI and sodium caseinate using proton-transfer-reaction mass spectrometry (PTR-MS), and to understand the effect of viscosity on flavour release *in vivo*.

The binding of the model flavour compound 2-nonanone to individual milk proteins, WPI, and sodium caseinate in aqueous solutions was investigated, using headspace SPME followed by GC-FID. The 2-nonanone binding capacities decreased in the order: BSA > β-lg > α-la > α_{s1}-casein > β-casein, and the binding to WPI was stronger than the binding to sodium caseinate. All proteins appeared to have one binding site for 2-nonanone, except for BSA which possessed two classes of binding sites.

The influence of heat treatment, high pressure processing and pH of the protein solutions on the binding of 2-nonanone, 1-nonanal, and *trans*-2-nonenal to WPI was determined. The binding of these compounds to WPI decreased in the order: *trans*-2-nonenal > 1-nonanal > 2-nonanone. The binding of 2-nonanone appears to involve hydrophobic interactions only, whereas the aldehydes, in particular *trans*-2-nonenal, also react through covalent binding. Upon both heat and high pressure denaturation, the binding of 2-nonanone to WPI decreased, the binding of 1-nonanal remained unchanged, while the binding of *trans*-2-nonenal increased. The binding affinity of the flavour compounds and WPI increased with increasing pH, which is likely to result from pH dependent conformational changes of whey proteins.

The *in vivo* flavour (2-nonanone) release from solutions of WPI and sodium caseinate was investigated using proton-transfer-reaction mass spectrometry. During consumption, 2-nonanone was partly released from WPI, whereas there was no significant release from sodium caseinate. Even after swallowing of the samples, a substantial amount of flavour was detected in the breath, suggesting that the milk proteins interact with the mucosa in the mouth and throat, resulting in a further release of flavour from mucosa-bound proteins. An increase in viscosity of the protein solutions by the addition of carboxymethylcellulose enhanced the release of 2-nonanone from WPI, and resulted in 2-nonanone release from sodium caseinate. This may be due to a thicker coating of the mucosa with the sample solution after swallowing due to the higher viscosity, resulting in additional release of protein-bound flavour.

These findings contribute to the knowledge of the interactions that occur between flavour compounds and proteins, which is required to improve food flavouring and to make protein based foods, e.g., low-fat dairy products, sensorily more acceptable to the consumer. The results also emphasize a careful choice of food processing conditions, such as temperature, high pressure or pH to obtain a desirable flavour profile.

ACKNOWLEDGEMENTS

Firstly, I wish to thank my supervisors, Professor Harjinder Singh and Dr Thérèse Considine. This thesis would not appear in its present form without their expert assistance, support, ideas, and criticism.

I would also like to thank Massey University for the award of the Doctoral Scholarship during three years of my research, and the Riddet Centre for additional funding and several travel grants.

A big thank you to Dr Conor Delahunty for very helpful advice and discussions towards and during my flavour release study at the University of Otago. I would also like to thank the Sensory Science Department at the University of Otago for the great working atmosphere. Special thanks to Sam Heenan for his expertise in PTR-MS.

I am very grateful to Fonterra Co-operative Group Ltd. for temporarily providing a GC-FID to carry out my research, and to Dr Owen Mills and Andrew Broome for their advice and assistance on GC analysis.

I also wish to thank Dr Xiang-Qian (Peter) Zhu for his assistance during the initial stages of my research and for several valuable discussions, Dr Skelte Anema (Fonterra) for the high pressure treatments, and for encouraging me to do a PhD in the first place, Dr John Flanagan for help with the purification of caseins, and Michelle Tamehana and Steve Glasgow for being great lab managers. Amino acid analyses were carried out by Leiza Turnbull.

Thank you also to the following people who have been supportive as friends, colleagues, or otherwise: Linda and Brian, Nigel, Sarah, Janiene, Thanuja, Jiahong, Thurid, Tamara, Mischa, and Ngaio.

Finally, and most importantly, my love and thanks to my partner, Leyton, who went with me through the successful but also discouraging times, and always helped me to keep thinking positive, my parents, Carl-Ludwig and Irmgard, and my brother, Alexander, who have been encouraging, supportive and loving not only during my academic studies. To them I dedicate this thesis.

TABLE OF CONTENTS

CHAPTER THREE: BINDING OF 2-NONANONE AND MILK PROTEINS 48

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

∞	At equilibrium
α-la	α-Lactalbumin
β-lg	β-Lactoglobulin
ε	Extinction coefficient ($l \cdot mol^{-1} \cdot cm^{-1}$)
λ	Wavelength (nm)
θ	Temperature (°C)
A	Absorptivity
APCI	Atmospheric pressure chemical ionisation
APS	Ammonium persulphate
AUC	Area under the curve
BSA	Bovine serum albumin
BTEX	Benzene, toluene, ethylbenzene, and xylenes
c	Concentration (M)
C	Cross-linker as percentage of total monomer concentration (%)
CAR	Carboxen
CD	Circular dichroism
CMC	Carboxymethylcellulose
conc.	concentrated
cps	Counts per second
CV	Coefficient of variation (%)
Da	Dalton ($g \cdot mol^{-1}$)
DCCLC	Dynamic coupled column liquid chromatography
DEAE	Diethylaminoethyl
DSC	Differential scanning calorimetry
DVB	Divinylbenzene

E	Electric field
[F]	Concentration of flavour (M)
FID	Flame ionisation detector
GC	Gas chromatograph(y)
GC-O	GC-olfactometry
h	Hour(s) or Hill coefficient or headspace
HHP	High hydrostatic pressure
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methyl cellulose
IEC	Ion-exchange chromatography
I_{max}	Maximum intensity of flavour perception
IR	Infrared
K	Binding constant (M^{-1})
K_d	Dissociation constant
K_{fh}	Partition coefficient between SPME fibre coating and headspace
K_{fw}	Partition coefficient between SPME fibre coating and water
K_{hw}	Partition coefficient between headspace and water
[L]	Concentration of free ligand (M)
$[L]_{tot}$	Total ligand concentration (M)
Log P	Logarithm of the partition coefficient between water and n-octanol
LTPRI	Linear temperature programmed retention index
M	$Mol{\cdot}l^{-1}$
min	Minute(s)
MPC	Milk protein concentrate
MS	Mass spectrometry / spectrometer
MW	Molecular weight

m/z	Mass to charge ratio
n	Number of binding sites per mole of protein
nK	Global binding constant (M^{-1})
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
[P]	Protein concentration
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbons
PDMS	Polydimethylsiloxane
PFG	Pulsed field gradient
PG	Propylene glycol (1,2-propanediol)
ppbV	Parts per billion by volume
ppm	Parts per million
PTFE	Polytetrafluoroethylene
PTR	Proton transfer reaction
RAS	Retronasal aroma simulator
RI	Refractive index
rpm	Revs per minute
sccm	standard centimeter cube per minute; 1 cm^3 of gas per minute at 0 ^{o}C and at atmospheric pressure.
SD	Standard deviation
SE	Standard error
SEM	Secondary electron multiplier
SDS	Sodiumdodecylsulphate
s	Second(s)
SEC	Size exclusion chromatography

SPME	Solid-phase microextraction
t	Time
T	Total monomer concentration (%)
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
t_{eq}	Equilibration time
t_{ex}	Extraction time
TI	Time-intensity
t_{max}	Time at which maximum flavour intensity is perceived
Tris	Tris(hydroxymethyl)methylamine
UV	Ultraviolet
V	Volume (l) or Volts
v	Number of moles of flavour bound per mol of protein
var	Variance
WPC	Whey protein concentrate
WPI	Whey protein isolate
Y	Fractional saturation of binding sites on the protein

CHAPTER ONE: INTRODUCTION

One of the most important criteria for consumer acceptance of foods is flavour. Food matrix components, such as proteins *(Gremli, 1974; Damodaran and Kinsella, 1980a, b, 1981a, b; Farès et al., 1998; Lübke et al., 2002; Gianelli et al., 2005)*, carbohydrates *(Yven et al., 1998; Heinemann et al., 2001; Philippe et al., 2003; Jouquand et al., 2004)*, and lipids *(Ebeler et al., 1988; van Ruth et al., 2002; Meynier et al., 2003)*, are known to interact with flavour compounds. Proteins are added to foods primarily because of their functional properties, such as emulsifying and stabilising capacities, and their nutritional value. However, interactions between proteins and flavours are known to influence the perceived flavour of a food product *(Overbosch et al., 1991; Land, 1996)*. Protein ingredients not only reduce the perceived impact of desirable flavours but also may transmit undesirable off-flavours to foods, especially whey protein and soy protein products *(Mills and Solms, 1984; Semenova et al., 2002a)*. In addition, proteins may change the texture of a food, i.e. gelling, and thus decrease the flavour perception due to inhibition of mass transfer *(Jaime et al., 1993; Carr et al., 1996; Wilson and Brown, 1997)*.

In the area of protein-flavour interactions, studies have been conducted mainly with milk proteins *(Andriot et al., 2000; Lübke et al., 2002)* and soy proteins *(Damodaran and Kinsella, 1981a; Li et al., 2000)*, but also with a range of other food proteins, such as fababean protein *(Ng et al., 1989a, b; Semenova et al., 2002a)*, pea protein *(Dumont and Land, 1986)*, ovalbumin *(Maier, 1970; Ebeler et al., 1988)*, fish actomyosin *(Damodaran and Kinsella, 1983)*, and myoglobin *(Gianelli et al., 2005)*. The studies on milk proteins are of great importance since milk proteins are utilised in numerous food products, including dairy products, bakery and confectionary products, and meat products *(Mulvihill, 1992)*.

The demand for healthier, low-fat foods is increasing. However, in the absence of fat, altered flavour-matrix interactions result in a dramatic change in flavour profiles *(Shamil and Kilcast, 1992; Plug and Haring, 1993; Hatchwell, 1996)*. In fat-reduced or "light" foods the dominant components are carbohydrates or proteins which interact differently with aroma compounds compared with fat, and thus change the perceived flavour. In addition, most of the fat replacers used in these foods are composed of proteins or carbohydrates or both. A better understanding of the science behind protein-flavour interactions is required for the development of improved food flavour, particularly that of low-fat foods, and for minimising the presence of off-flavours in protein-containing foods.

Understanding the binding of flavour compounds and proteins is important, however, the extent of release of flavours from proteins during consumption is crucial since it affects the flavour perception. Recently, both *in vitro (Roberts and Acree, 1995; Deibler et al., 2001; Chung et al., 2003)* and *in vivo* techniques *(Soeting and Heidema, 1988; Linforth et al., 1996; Taylor and Linforth, 2000; Weel et al., 2002)* have been developed to investigate flavour release from foods. Milk proteins have been found to reduce the in-mouth flavour release and flavour perception because of their interactions with flavour compounds *(Guichard, 2000; Denker et al., 2006; Giroux et al., 2007)*. However, only two studies reported on the actual release of flavour compounds from proteins in-mouth *(Le Guen and Vreeker, 2003; Weel et al., 2003)*. Aliphatic aldehydes were found to be partially released from whey protein isolate (WPI) during consumption *(Weel et al., 2003)*, whereas alkenals were not released, and methyl ketones were completely released from milk protein concentrate (MPC) *(Le Guen and Vreeker, 2003)*. These results show that flavour release is very dependent on the type of flavour compound. However, very different consumption protocols were used by the authors, making a comparison of results difficult. Clearly, further work is needed to elucidate the release of flavour compounds from proteins in-mouth.

The objectives of this research were

(1) to determine binding parameters of the hydrophobic flavour compound, 2-nonanone, to individual milk proteins (namely, β-lactoglobulin, α-lactalbumin, bovine serum albumin, α_{s1}-casein, and β-casein), WPI, and sodium caseinate, using an optimised headspace SPME method followed by GC-FID for the investigation of protein-flavour interactions;

(2) to compare the binding of structurally similar flavour compounds – 2-nonanone, 1-nonanal, and *trans*-2-nonenal – to WPI in aqueous solution, and to investigate the effect of heat and high pressure treatment, and pH on the extent of protein-flavour binding; and

(3) to investigate the *in vivo* release of the reversibly bound flavour compound, 2-nonanone, from WPI and sodium caseinate using proton-transfer-reaction mass spectrometry (PTR-MS), and to understand the effect of viscosity on flavour release *in vivo*.

CHAPTER TWO: LITERATURE REVIEW [1]

The aim of this review is to discuss the flavour binding ability of individual food proteins, with emphasis on milk proteins, and the influence of processing treatments, pH, and ionic strength on protein-flavour binding. Furthermore, the current knowledge on the release of flavour compounds from milk proteins during consumption, and effects of texture on flavour release, are summarised. Methodologies used to investigate protein-flavour interactions and flavour release from proteins are evaluated. Implications of protein-flavour interactions in the development of protein foods, and future research are also considered.

2.1 Types of Interactions

Two different types of interaction can occur between proteins and flavour compounds: (1) reversible (physicochemical) binding, including hydrogen bonds, hydrophobic interactions, and ionic bonds, and (2) irreversible (chemical) binding via covalent linkages, i.e. amide and ester formation, the condensation of aldehydes with amino groups (NH_2) ("Schiff base" formation) and sulphydryl (SH) groups, and addition reactions with unsaturated flavour compounds ("Michael addition") *(Solms et al., 1973; Overbosch et al., 1991; Mottram et al., 1996; Meynier et al., 2003, 2004)*. Examples of typical interactions between flavour compounds and proteins are shown in Figure 2.1-1.

The type of binding between a protein and a flavour compound depends on the characteristics of both the protein and the flavour, and thus there is no universal mechanism for flavour binding in foods *(Solms et al., 1973)*. However, most flavours show hydrophobic, reversible binding to proteins *(Gremli, 1974; Damodaran and Kinsella, 1980b, 1981a, b, 1983; O'Neill and Kinsella, 1987b; Pelletier et al., 1998)*. Adsorption and absorption also exist but are specific for low-moisture food systems *(Maier, 1972, 1973, 1975; Le Thanh et al., 1992; Landy et al., 1997; Mironov et al., 2003)*, and are not discussed in this review.

---

[1] Parts of this chapter have been published previously: Kühn, J., Considine, T., and Singh, H. (2006). Interactions of milk proteins and volatile flavor compounds: Implications in the development of protein foods. *Journal of Food Science* **71** (4): R72-R82 (see Appendix).

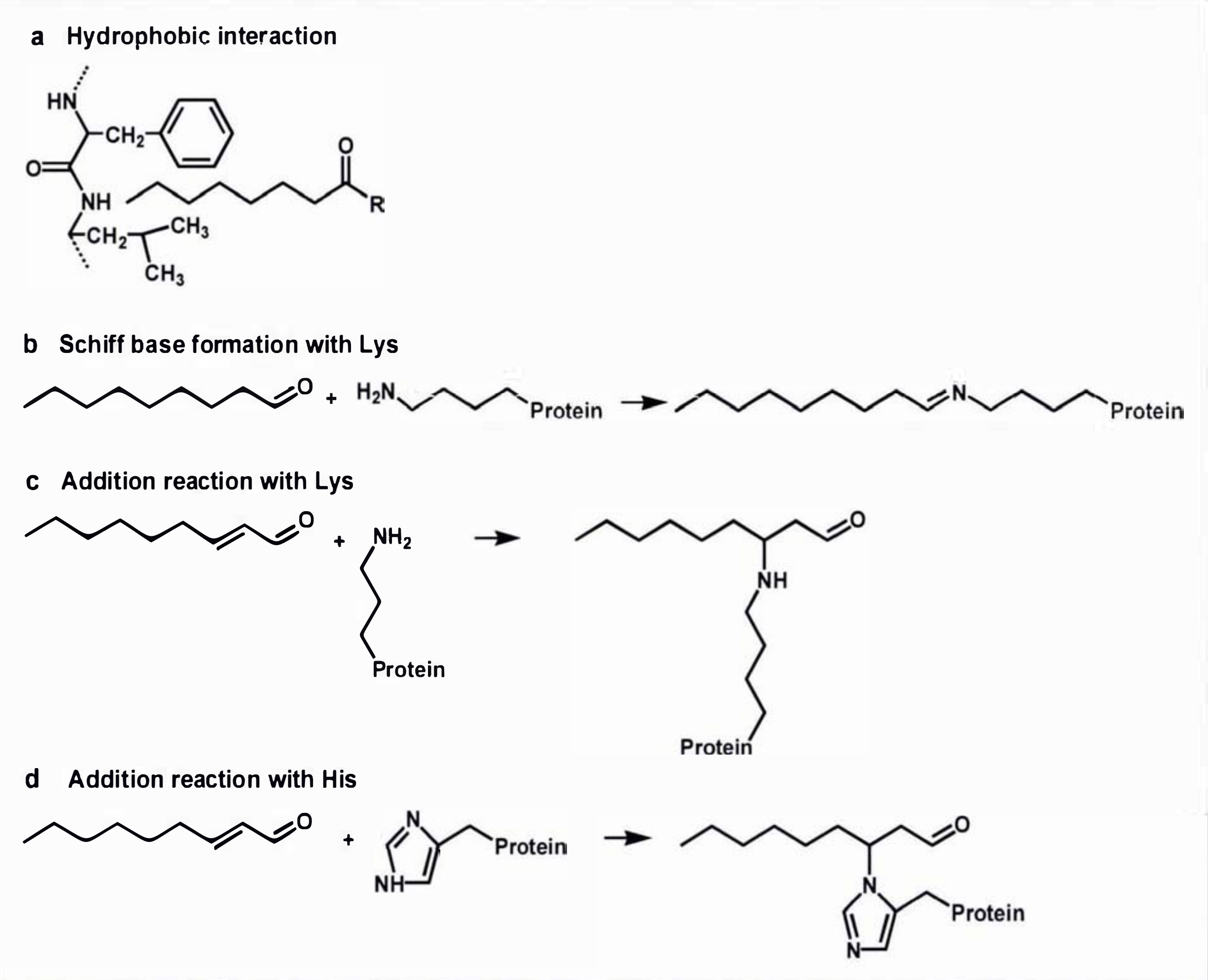


Figure 2.1-1: Common interactions of proteins and flavour compounds; (a) hydrophobic interaction between a protein and an aliphatic aldehyde or ketone flavour; (b) Schiff base formation between 1-nonanal and a lysine residue of a protein; (c) addition reaction of *trans*-2-nonenal and a lysine residue of a protein; (d) addition reaction of *trans*-2-nonenal and a histidine residue of a protein.

2.2 *Flavour Binding by Proteins*

2.2.1 Analysis of Flavour Binding by Proteins

Two approaches may be used to study the interactions between flavour molecules and macromolecules, such as proteins: instrumental techniques and sensory analysis. The systems considered are often very simple, consisting of one flavour compound and one protein, usually in an aqueous solution.

2.2.1.1 Static (Equilibrium) Methods

Flavour binding has been examined predominantly under equilibrium conditions. A common technique is equilibrium dialysis which is still commonly used *(Damodaran and Kinsella,* *1981a, b, 1983; Druaux et al., 1995; Farès et al., 1998; Muresan et al., 2001; Burova et al., 2003; Jung and Ebeler, 2003a; Guth and Fritzler, 2004)*. However, this technique is time consuming, and flavour losses during the experiment may occur. Another frequently used equilibrium method is static headspace analysis *(O'Keefe et al., 1991a; Charles et al., 1996; Andriot et al., 2000; van Ruth and Villeneuve, 2002; Heng et al., 2004; Liu et al., 2005a)*. Under equilibrium conditions, a definite volume is drawn from the headspace above the sample solution, and analysed using gas chromatography (GC). A drawback of the static headspace method is poor sensitivity for compounds with little volatility. For some samples, this problem can be overcome by splitless or on-column techniques, or by increasing the temperature. However, thermal reactions may occur. Aroma compounds that are not detectable by flame ionisation detection (FID) or mass spectrometry (MS) can often be detected using the headspace gas chromatography-olfactometry (GC-O) technique *(Widder and Fischer, 1996)*.

Recently, static headspace solid-phase microextraction (SPME) has been found to be very useful for the examination of protein-flavour binding *(Roberts and Pollien, 2000; Adams et al., 2001; Fabre et al., 2002; Jung and Ebeler, 2003a; Zhu, 2003; Gianelli et al., 2005; Pérez-Juan et al., 2007)*. The SPME technique has been developed by Pawliszyn and co-workers *(Arthur and Pawliszyn, 1990; Zhang and Pawliszyn, 1993; Zhang et al., 1994)*. In headspace SPME, a fused silica fibre coated with a thin layer of a selective coating extracts the flavour compounds from the headspace above the sample (Figure 2.2-1). The analytes are concentrated in the coating and are then transferred to the analytical instrument for desorption and analysis *(Zhang and Pawliszyn, 1993)*.

The principle behind headspace SPME is the equilibrium partition process of analytes between the sample, the headspace above the sample, and the fibre coating (Figure 2.2-1). The overall equilibrium of the system can be described by the following equation:

$$[F]_f \xleftrightarrow{K_{fh}} [F]_h \xleftrightarrow{K_{hw}} [F]_w \xleftrightarrow{K} [F]_P \qquad \textbf{(2-1)}$$

where $[F]_f$ is the concentration of the flavour compound in the fibre coating, $[F]_h$ in the headspace, $[F]_w$ in the aqueous phase, and $[F]_P$ bound by the protein. The partitioning of the flavour compound in the system depends on the fibre-headspace partition coefficient (K_{fh}), the headspace-water partition coefficient (K_{hw}), and the binding constant between the protein and the flavour (K) (Figure 2.2-1). Too long an extraction time and too high K_{fh} values would result in flavour being released from the sample solution, and, as a consequence, protein-bound flavour could be released into the aqueous phase and headspace, and the measured binding constant K would be underestimated. Hence, sampling parameters have to be chosen with care.

SPME is a sensitive, rapid, inexpensive, selective, and solvent-free sample preparation technique, and is compatible with a wide range of separation methods such as GC, GC-MS, and high-performance liquid chromatography (HPLC) *(Zhang et al., 1994)*. Headspace SPME has been found to be superior to both static and dynamic headspace analysis for the measurement of milk protein-flavour interactions *(Fabre et al., 2002)*. The technique can also be used to quantify the flavour concentrations after equilibrium dialysis *(Zhou et al., 2002)*.

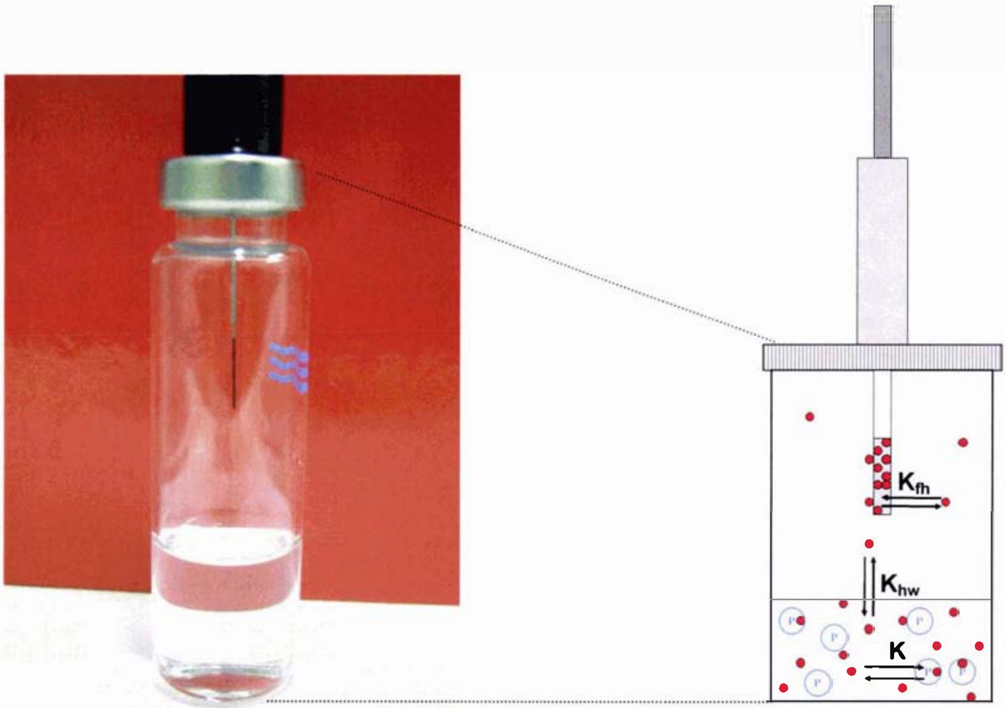


Figure 2.2-1: Illustration of the partition process in headspace SPME for investigating interactions of proteins (P) and flavour compounds (●) (not to scale); K_{fh} fibre-headspace partition coefficient, K_{hw} headspace-water partition coefficient, K binding constant.

However, there are some limitations of headspace SPME, such as the difficulty in using external standards for more complex sample matrices *(Yang and Peppard, 1994)*. Instead, standard addition and isotopic dilution can be used for quantitative analysis of such samples by SPME *(Hawthorne et al., 1992)*. In addition, competition of flavour compounds in the fibre coating can cause biases in the quantitative analysis *(Coleman, 1996; Grote and Pawliszyn, 1997; Roberts et al., 2000)*. Hence, the method is more suitable for simple systems rather than complex systems. In addition, it has to be ensured that the amount of flavour extracted from the headspace is small to avoid re-equilibration of the system.

Other equilibrium techniques include liquid-liquid partitioning *(Spector et al., 1969; Spector, 1975; Damodaran and Kinsella, 1980b)* and radioactivity counting *(King and Solms, 1979)*. For flavour compounds with little volatility, such as vanillin or benzaldehyde, HPLC methods have been developed to determine the free flavour after equilibration with the protein *(Ng et al., 1989a; McNeill and Schmidt, 1993; Andriot et al., 1999; Li et al., 2000; Chobpattana et al., 2002)*. However, several disadvantages are associated with equilibrium methods, such as long equilibration times and thus the possible degradation of the volatiles during this period.

The conformational stability of protein-flavour complexes upon heat denaturation can be examined using differential scanning calorimetry (DSC) methods *(Burova et al., 1999; Semenova et al., 2002b, 2002c)*. DSC also allows the calculation of binding constants using bovine serum albumin (BSA) as an internal molecular detector since its denaturation temperature and thus conformational stability depends on the amount of flavour bound *(Burova et al., 2003)*. However, the amount of bound flavour determined this way is not precise.

2.2.1.2 Dynamic Methods

Some dynamic methods, i.e., dynamic headspace analysis (exponential dilution), are used currently for the investigation of protein-flavour interactions *(Jouenne and Crouzet, 1996; Farès et al., 1998; Jouenne and Crouzet, 2000b)*. The flavour compounds in the headspace are of greater importance than those in the food since they can travel to the nose during eating and stimulate the olfactory receptors in the nasal cavity *(Linforth and Taylor, 1993)*. In dynamic headspace analysis, the exponential decrease of the flavour concentration in the headspace above the sample is measured, while the sample is exhausted of aroma volatiles by passing an inert gas through it or sweeping it over its surface. The gas leaving the system is sampled at regular intervals and analysed using GC. Purge-and-trap analysis is a commonly used method of dynamic headspace analysis and enables fast determination of the activity coefficient of the flavour compound in a model food system *(Sorrentino et al., 1986; Jouenne and Crouzet, 1996, 2000b)*. Disadvantages are the purging of water along with the analytes, decreasing the adsorption capacity of the trap, and diffusion or ‘breakthrough’ losses of analytes not retained by the trap *(Stevenson et al., 1996)*.

Other dynamic methods involve liquid chromatography, such as size exclusion chromatography *(Hummel and Dreyer, 1962; Dumont, 1987; Pelletier et al., 1998)*, dynamic coupled column liquid chromatography (DCCLC) *(Langourieux and Crouzet, 1995; Jouenne and Crouzet, 2000a)*, or affinity chromatography on protein-bonded stationary phases *(Pelletier et al., 1998; Sostmann and Guichard, 1998; Reiners et al., 2000)*. The methods based on liquid chromatography need small amounts of product and are rapid, thereby reducing the degradation of analytes. In spite of this, affinity chromatography only provides global binding constants (nK). The number of binding sites (n) has to be determined through size exclusion chromatography or other methods. Another drawback of affinity chromatography is that possible conformational changes in proteins due to their immobilisation have to be considered, and some protein binding sites may be hindered, as observed for isoamyl acetate *(Pelletier et al., 1998)*.

2.2.1.3 Spectroscopic Methods

The above mentioned methods can be used to demonstrate the existence and extent of molecular interactions between aroma volatiles and proteins or other food constituents. However, they do not provide information about the nature of interactions. Spectroscopic techniques have been used successfully to gain insight into the nature of interactions. Binding of a flavour compound to a protein molecule can cause conformational modifications of the protein *(Damodaran and Kinsella, 1980b; Lübke et al., 2000)*. These conformational changes, and thus the binding of ligands can be measured by following the change in protein tryptophan fluorescence, which can be increased or quenched depending on the ligand present *(Muresan et al., 2001)*. The technique also indicates if a ligand binds in the vicinity of a tryptophan residue. Assuming that the change in fluorescence depends on the amount of protein-ligand complex, fluorescence spectroscopy can be used to determine binding constants *(Dufour and Haertlé, 1990a; Frapin et al., 1993; Liu et al., 2005a)*. However, the protein investigated needs to contain at least one tryptophan residue. In addition, for ligands that quench the tryptophan fluorescence, the binding is overestimated and should always be verified using a second method *(Muresan et al., 2001)*.

Another technique monitoring conformational changes of proteins upon flavour binding is infrared (IR) spectroscopy *(Lübke et al., 2000)*. The technique is a very useful tool to study protein secondary structures. Generally, the amide I bands region between 1600 and 1700 cm^{-1} reveals the most information as it is highly conformation-sensitive. When using either fluorescence or IR spectroscopy it has to be considered that flavour binding without conformational changes in the protein can not be investigated using these techniques.

Nuclear magnetic resonance (NMR) spectroscopy techniques are a very valuable tool to explore mechanisms of protein-flavour interactions. Two-dimensional (2D) NMR spectroscopy reveals conformational changes of the protein upon flavour binding, and those amino acid residues involved in the binding so that information about the location and number of binding sites on a protein can be obtained *(Lübke et al., 2002)*. Diffusion-based NMR techniques are fast and easy but do not offer information about the nature of interactions. The pulsed field gradient NMR (PFG-NMR) method can provide binding constants *K* and number of binding sites *n* on the proteins. However, using this technique it is assumed that there are no interactions between the proteins or the flavour ligands themselves but this assumption is not true. A powerful tool for rapid screening of flavours which have an affinity for a protein is the diffusion-based nuclear Overhauser effect (NOE) pumping technique *(Jung et al., 2002; Jung and Ebeler, 2003b)*. This technique should only be used as a screening method as it lacks sensitivity.

2.2.1.4 Sensory Methods

Sensory analysis has also been applied to examine the effect of proteins on flavour perception *(Ng et al., 1989b; Hansen and Heinis, 1991, 1992; McNeill and Schmidt, 1993; Reiners et al., 2000)*. Rating of flavour intensities in the presence and absence of protein gives insight into the effect of protein-flavour binding on flavour perception and complements instrumental techniques. However, an intensive training of the taste panel is necessary to obtain precise results, and thus is time consuming and costly.

2.2.1.5 Determination of Binding Parameters

To characterise the binding of a specific flavour compound to a specific protein, binding parameters, such as an intrinsic binding constant *K*, the number of binding sites *n*, the Hill coefficient *h*, the Gibbs' free energy of binding ΔG, the enthalpy change ΔH, and the entropy change ΔS, can be determined experimentally. Different plots have been used to determine these parameters, including the Scatchard plot *(Scatchard, 1949)*, the Klotz plot *(Klotz et al., 1946; Klotz and Urquhart, 1948)*, and the Hill plot *(Hill, 1910; Yven et al., 1998)*. The experimental determination of *K* as a function of temperature allows the determination of the thermodynamic parameters ΔG, ΔH and ΔS *(Steinhardt and Reynolds, 1969)*.

2.2.1.5.1 *Scatchard Plot*

For a protein *P* having an equal number of discrete and independent binding sites, the interaction between the ligand, that is, the flavour molecule *L*, and the protein may be represented by the equation:

$$\mathrm{P} + \mathrm{nL} = \mathrm{PL_n} \qquad (\mathrm{n} = 1, 2, 3, \ldots) \tag{2-2}$$

On the basis of this model, the equilibrium between free and protein-bound flavour molecules can be represented thermodynamically by the Scatchard equation *(Scatchard, 1949)*:

$$\frac{v}{[L]} = nK - vK \tag{2-3}$$

where *v* is the number of moles of flavour bound per mole of protein, *[L]* is the concentration of the free flavour ($\mathrm{mol \cdot l^{-1}}$) in the sample solution, *K* is the binding constant, and *n* is the total number of equivalent and independent binding sites on the protein. According to Equation 2-3, a plot of *v / [L]* vs *v* gives a straight line with a slope of $-K$ and intercept *nK* from which *n* and *K* can be calculated. This equation is widely used in binding studies with proteins.

2.2.1.5.2 *Klotz Plot*

The Scatchard equation can be also expressed in its double-reciprocal form, the Klotz plot *(Klotz et al., 1946)*, which is the most common plotting technique in binding studies *(Price and Dwek, 1979)*:

$$\frac{1}{v} = \frac{1}{n} + \frac{1}{nK[L]} \tag{2-4}$$

A plot of *1 / v* vs *1 / [L]* gives a straight line with a slope of *1 / nK* and an intercept of *1 / n*. The interaction parameters *n* and *K* can be determined using different experimental techniques for studying equilibrium conditions, e.g., equilibrium dialysis or static headspace analysis.

2.2.1.5.3 Hill Plot

The frequently used Scatchard and Klotz models assume equal and independent binding sites on a protein. However, proteins can possess non-equivalent binding sites, for example one or more high-affinity primary binding sites and a group of lower affinity secondary binding sites. This results in non-linear Scatchard and Klotz plots and complicates the evaluation of binding data since it is often difficult to extrapolate the binding plots to obtain the exact number of equivalent binding sites. Furthermore, binding sites can be dependent on each other. The initial binding of aroma compounds can cause a protein to undergo conformational changes *(Damodaran and Kinsella, 1980b)*, revealing new binding sites. If the initial binding of a ligand to a protein produces a tendency to bind more ligand, positive cooperativity exists. If binding is restricted after the initial binding of a ligand, the protein shows negative cooperativity. If the Scatchard and Klotz plot are non-linear, the Hill plot *(Hill, 1910)* should be used to determine if cooperativity between binding sites exists. Predominantly, the double-reciprocal form of the Hill plot is used:

$$\frac{1}{v} = \frac{1}{n(k[L])^{h}} + \frac{1}{n} \qquad \textbf{(2-5)}$$

where h is the Hill coefficient reflecting interactions between individual binding sites within their population. Using non-linear regression from the plot $1/v$ vs $1/[L]$, the Hill coefficient h can be obtained. The Hill equation can be also expressed in the following form *(Stryer, 1995)*:

$$\log\frac{Y}{1-Y} = h \cdot \log[L] - h \cdot \log K_d \qquad \textbf{(2-6)}$$

where Y is the fractional saturation of binding sites, and K_d the dissociation constant ($K_d = 1/K$). A plot of $\log [Y/(1-Y)]$ versus $\log [L]$ approximates a straight line. Its slope is the Hill coefficient h.

The parameter h indicates the extent of cooperative effect. Ligand binding without cooperativity gives rise to a linear Hill plot with a slope of 1.0. Ligand binding with positive cooperativity leads to Hill plots with slopes greater than 1.0, while ligand binding with negative cooperativity gives Hill plots with slopes less than 1.0 *(Dam et al., 2002)*. The maximum value of h is equal to the total number of binding sites n *(Landy et al., 1995)*.

A complete binding analysis from the initial signs of binding to saturation is often not realisable in flavour binding experiments, for example because of the low water solubility of most flavour compounds. The range of flavour concentration varies from one study to another, making comparison of binding data impossible since the amount of flavour molecules present determines the occupation of binding sites. If flavour concentrations are low only the high-affinity binding sites are occupied, whereas high flavour concentrations may also result in the occupation of secondary binding sites.

2.2.2 Flavour Compounds

The total content of flavour compounds in foods is roughly between 1 and about 1000 ppm (e.g. 10-100 ppm in fruit) *(Maier, 1970)*. Flavour compounds are divided into different classes according to their functional groups, of which the aldehydes, esters, and especially ketones have been mostly used in protein-flavour binding studies. Terpenes, such as *d*-limonene, lactones, and sulphur compounds have also been studied. Alcohols, acids and pyrazines have been found to have only very low or no affinities to proteins and have been considered rarely.

2.2.2.1 Aldehydes

Benzaldehyde has been intensively used in protein-flavour binding studies *(Hansen and Booker, 1996; Farès et al., 1998; Seuvre et al., 2001)*. Because of its cherry flavour *(Carr et al., 1996)* it is very suitable for sensory studies. The same applies to vanillin *(McNeill and Schmidt, 1993; Li et al., 2000)*, which also has a characteristic flavour. Other frequently studied aldehydes are aliphatic aldehydes, such as hexanal, heptanal, octanal, and nonanal *(Mills and Solms, 1984; Schirle-Keller et al., 1994; Meynier et al., 2004)*.

2.2.2.2 Ketones and Methyl Ketones

The most widely used flavour compounds in studies investigating flavour binding to proteins are the methyl ketones, particularly 2-nonanone *(Damodaran and Kinsella, 1980a, 1981a, b; Sostmann and Guichard, 1998; Andriot et al., 2000; Seuvre et al., 2001)*. 2-Nonanone makes a substantial contribution to the flavour of different foods (Table 2.2-1), and its odour character has been reported as mustard-like and spicy *(Moio et al., 1994)*, and as grassy-herbal and green-fruity *(Friedrich and Acree, 1998)*.

Table 2.2-1: Levels of 2-nonanone in foods.

Food	Concentration (ppm)	Reference
Coconut oil (rancid)	2,970	*Kellard et al.(1985)*
Blue-type cheese	33	*Dwivedi and Kinsella (1974)*
Potatoes	1-3.5	*Kahn et al. (1977)*
Heated milk (4 % fat)	0.72	*Langler and Day (1964)*
Sour cream butter	0.2-0.5	*Mick et al. (1982)*
Stored sterilized concentrated milk	0.03-0.47	*Arnold and Lindsay (1969)*
Passion fruit juice	0.1	*Murray et al.(1972)*

2-Nonanone and other methyl ketones have also been detected in feijoa fruit *(Hardy and Michael, 1970)*, kiwifruit *(Wan et al., 1999)*, a number of stored milk products, including dry whole milk *(Parks and Patton, 1961)*, yoghurt *(Viani and Horman, 1976)*, Cheddar cheese *(Liebich et al., 1970)*, and as an odorant in Camembert cheese *(Kubíckovà and Grosch, 1997)*. Both, 2-hexanone and 2-nonanone were identified as the most intense volatile flavour compounds of UHT milk *(Moio et al., 1994)*. Other frequently studied methyl ketones are 2-hexanone, 2-heptanone, and 2-octanone. Another important ketone is diacetyl, which has been widely used in protein-flavour binding studies *(Landy et al., 1995; Roberts and Pollien, 2000; Fabre et al., 2002)*.

2.2.2.3 Esters

Retention of esters, such as isoamyl acetate, ethyl acetate, ethyl hexanoate, and methyl hexanoate, by milk proteins has been investigated and confirmed by *Landy et al. (1995), Pelletier et al. (1998), Fabre et al. (2002)* and *Meynier et al. (2003)*.

2.2.3 Flavour Binding by Milk and Other Proteins

In the area of protein-flavour interactions, studies have been conducted mainly with milk proteins, but also with a range of other food proteins, which will be discussed.

Bovine milk proteins (Table 2.2-2) are the best-characterised food proteins. The caseins represent approximately 80 % of the milk proteins, and the whey proteins 20 %. Milk proteins that have been reported to bind flavour compounds include β-lactoglobulin, α-lactalbumin, bovine serum albumin, and β-casein.

Table 2.2-2: Major proteins in bovine milk; adapted from *Rosenthal (1991)*.

	% of total milk protein
Caseins	**80**
α_{s1}-casein	34
α_{s2}-casein	8
β-casein	25
κ-casein	9
γ-casein	4
Whey proteins	**20**
β-lactoglobulin	9
α-lactalbumin	4
bovine serum albumin	1
proteose-peptones	4
immunoglobulins	2

2.2.3.1 β-Lactoglobulin (β-lg)

Of all the different food proteins, β-lg has been used extensively as a model protein for studying protein-flavour interactions, because of its well-defined structure and properties *(McKenzie, 1971; Kinsella and Whitehead, 1989; Batt et al., 1994)*.

The three-dimensional structure of bovine β-lg has been determined by high-resolution crystallographic studies *(Sawyer et al., 1985; Papiz et al., 1986; Monaco et al., 1987)* and has been shown to be similar to that of serum retinol-binding protein *(Papiz et al., 1986; North, 1989)*. β-Lg is built up of two β-sheets, formed from nine strands converging at one end to form a hydrophobic calyx or pocket, and a flanking three-turn α-helix *(Sawyer et al., 1985; Papiz et al., 1986)* (Figure 2.2-2). This pocket serves as a binding locus for apolar molecules such as retinol ($K = 5 \times 10^7$ M^{-1}) *(Fugate and Song, 1980)* and long-chain fatty acids ($K = 10^5$-10^7 M^{-1}) *(Spector and Fletcher, 1970; Díaz de Villegas et al., 1987; Pérez et al., 1989; Frapin et al., 1993; Wu et al., 1999; Ragona et al., 2000)*.

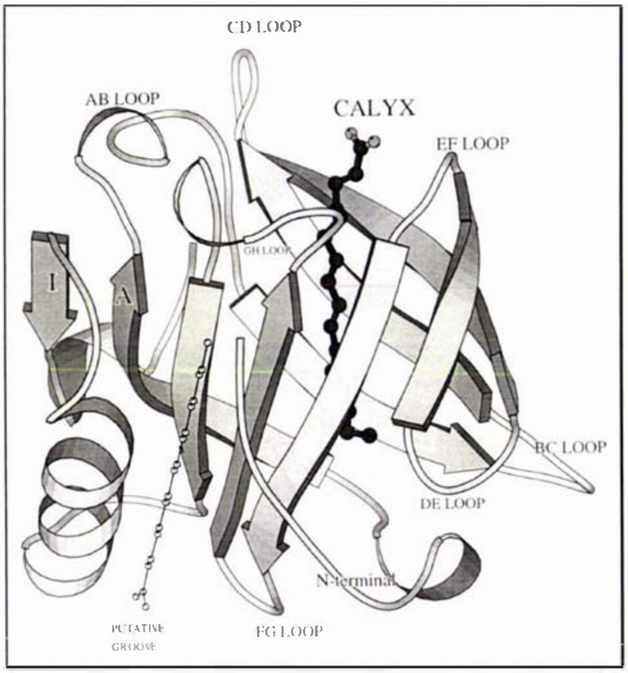


Figure 2.2-2: A general view of β-lactoglobulin with palmitic acid in the central hydrophobic pocket (filled atoms), and on the surface binding site (open atoms) (from *Wu and others (1999)*).

Fourier transform IR spectroscopy has been used to determine the conformational changes in β-lg upon the addition of ligands. The technique confirmed the binding of β-ionone, retinol, and fatty acids into the central cavity *(Lübke et al., 2000)*. For other ligands (*p*-cresol, eugenol, 2-nonanone, and γ-decalactone), no conformational changes of the protein were observed and the authors suggested either a binding to the protein surface or a binding into the central cavity without inducing a conformational change. However, an NMR study did confirm conformational changes of β-lg upon binding of flavour molecules (γ-decalactone and β-ionone) *(Lübke et al., 2002)*. It appeared that the side chains of several amino acids close to the central hydrophobic cavity (Leu46, Ile56, Met107, and Gln120) were affected by the binding of γ-decalactone, whereas the binding of β-ionone affected amino acids located in a groove near the outer surface of the protein (Leu104, Tyr120, and Asp129), a groove that is close to a region that has been described by *Monaco et al. (1987) (Lübke et al., 2002)*. This study confirms the existence of two different binding sites on β-lg for aroma compounds, but it is still not entirely clear which flavours bind preferentially to which site.

Therefore, the most probable binding site for flavour compounds is the hydrophobic pocket of β-lg. In addition to this primary binding site, β-lg is thought to contain weaker secondary binding sites that are capable of undergoing interactions with hydrophobic molecules, such as flavour compounds *(Spector and Fletcher, 1970; Robillard and Wishnia, 1972; Monaco et al., 1987; O'Neill and Kinsella, 1988; Dufour and Haertlé, 1990a)*.

β-Lg is known to interact with several flavour compounds, such as alkanes *(Wishnia and Pinder, 1966; Mohammadzadeh-K. et al., 1967, 1969a,1969b)*, ketones *(O'Neill and Kinsella, 1987b; Andriot et al., 2000; Guichard and Langourieux, 2000; Jouenne and Crouzet, 2000b)*, aldehydes *(van Ruth and Villeneuve, 2002)*, ionones *(Dufour and Haertlé, 1990a; Jouenne and Crouzet, 2000a; Lübke et al., 2002; Jung and Ebeler, 2003b)*, lactones *(Sostmann and Guichard, 1998; Lübke et al., 2002; Guth and Fritzler, 2004)*, and esters *(Pelletier et al., 1998; Guichard and Langourieux, 2000; Jouenne and Crouzet, 2000b; Reiners et al., 2000)*. As a result of different experimental methodologies and conditions, various binding parameters have been derived in different studies, making comparison of binding data difficult.

O'Neill and Kinsella (1987b), using equilibrium dialysis, showed that β-lg has a high binding affinity and one main binding site for methyl ketones. The slopes of the Klotz plots for 2-heptanone, 2-octanone, and 2-nonanone indicated binding constants (K) of 152, 481, and 2,439 M^{-1}, respectively. As the binding constant K for β-lg and homologous series of esters, aldehydes, ketones, and alcohols increased with increasing chain length (hydrophobicity) within the same chemical class, hydrophobic interactions between β-lg and flavour compounds were suggested *(O'Neill and Kinsella, 1987b; Pelletier et al., 1998; Guichard and Langourieux, 2000)*. However, earlier work by *Jasinski and Kilara (1985)*, using the same method, reported a much lower value of $K = 122\ M^{-1}$ for 2-nonanone and about 14 binding sites were evident. The authors suggested that unfolding of the protein may explain the high number of binding sites determined, but it is unclear how this unfolding may have occurred. In our opinion the flavour protein ratios used by *Jasinski and Kilara (1985)* are comparatively high so that weaker, secondary binding sites on the protein might be occupied as well, which would also decrease the overall binding constant. In addition, sodium azide was added as an antibacterial agent, which has later been shown to influence protein-flavour interactions *(O'Keefe et al., 1991b; Farès et al., 1998; Reiners et al., 2000)*.

The presence or absence of certain functional groups and steric factors are also known to have a marked influence on the degree of flavour binding by proteins. The interactions of an alkyl chain with β-lg can be reduced by a polar group, i.e., the introduction of a hydroxyl function (OH) is responsible for weaker interactions *(Reiners et al., 2000)*. The introduction of an aldehyde group led to a slight increase in binding. In general, the binding capacities of proteins increase from alcohols to ketones and aldehydes.

A recent fluorometric study on the binding of retinol and γ-undecalactone to β-lg showed that there is competition between the ligands *(Muresan et al., 2001)*. The effect of the chain length on the free energy of interaction between β-lg and different lactones indicated that the interaction is mainly hydrophobic, which led the authors to conclude that the preferential binding site for the lactones is likely to be the central cavity. This hypothesis was confirmed by competition experiments between β-ionone and other flavour compounds, such as δ- and γ-octalactone *(Sostmann and Guichard, 1998)*. The authors concluded that (1) lactones have some affinity for the central cavity as well, and (2) α-ionone, β-damascenone, methyl benzoate, and unsaturated aliphatic aldehydes and ketones bind non-specifically to the protein. In contrast, *Guth and Fritzler (2004)* suggested a binding position of γ-decalactone that is different from that of the central cavity since both retinol and palmitate only slightly inhibited the binding of the lactone. These contradictory results demonstrate that the location of binding sites for flavour molecules on proteins needs to be investigated further. Even flavour binding locations on the frequently studied β-lg are still not fully understood.

Dufour and Haertlé (1990a), using fluorescence spectroscopy, suggested that the central hydrophobic pocket has a narrow specificity to the structure formed by the conjugated double bonds of the β-ionone ring and isoprenoid chain, present in both β-ionone and retinol. The authors could not demonstrate binding between β-lg and α-ionone, since it did not quench the tryptophan fluorescence. The ionone isomers differ only in the position of the cyclohexene double bond. On the other hand, α-ionone might be bound by β-lg, but without interacting with tryptophan. In contrast, *Lübke et al. (2000)*, using IR spectroscopy, showed that the binding of retinol and β-ionone into the hydrophobic cavity of β-lg induced no significant conformational change in the protein, whereas the binding of α-ionone did, probably due to the "wrong" position of the double bond *(Lübke et al., 2000)*. *Jung and Ebeler (2003b)*, using a diffusion-based NOE pumping technique demonstrated that the binding of β-ionone by β-lg was significantly higher than the affinity of α-ionone. In addition, α-ionone was bound only at pH 9, whereas β-ionone was bound at pH 3-11, with the greatest binding affinity at pH 9, and the lowest at pH 11, due to alkaline denaturation and aggregation. The reason why these authors, contrary to *Lübke et al. (2000)*, did not observe any binding of α-ionone at neutral pH may be the low sensitivity of the NOE pumping method. The complementary results of the above studies show the importance of using a combination of techniques to obtain more reliable results.

2.2.3.2 α-Lactalbumin (α-la)

α-La is a compact globular protein, stabilised by four intrachain disulphide bonds *(Kinsella and Whitehead, 1989)*, and it plays an important role in the synthesis of lactose *(Wong, 1988)*. However, only a few studies have investigated the binding of flavour compounds to α-la, as its affinity for flavour compounds is believed to be lower than that of β-lg. Using headspace analysis, α-la was found to bind various amounts of aldehydes and methyl ketones *(Franzen and Kinsella, 1974)*, but binding constants were not given. The study of *Jasinski and Kilara (1985)*, which considered the binding of 2-nonanone and nonanal to α-la, showed very weak binding of both flavours to the protein, as determined by equilibrium dialysis. Since *Jasinski and Kilara (1985)* underestimated the binding of 2-nonanone to β-lg, they possibly underestimated the binding to α-la as well. Further examination of flavour binding by α-la should be performed.

2.2.3.3 Bovine Serum Albumin (BSA)

BSA binds a large variety of compounds, including retinol *(Futterman and Heller, 1972)*, long-chain fatty acids *(Morrisett et al., 1975; Spector, 1975; Pérez et al., 1989)*, alkanes *(Wishnia and Pinder, 1964; Mohammadzadeh-K. et al., 1967, 1969a, 1969b)*, and aldehydes and ketones *(Beyeler and Solms, 1974; Franzen and Kinsella, 1974; Damodaran and Kinsella, 1980b; Jung and Ebeler, 2003a)*. The protein is composed of a single polypeptide chain, which is folded so that three or four spherical units are formed. The binding sites for fatty acids are probably located in crevices between the spherical units *(Spector, 1975)*.

Using static headspace analysis, native BSA was found to decrease the vapour pressure of diacetyl over its aqueous solution. As little as 0.5 % protein caused a 25 % reduction in volatility *(Land and Reynolds, 1981)*, indicating a very high affinity of BSA to bind diacetyl. *Beyeler and Solms (1974)* used equilibrium dialysis to study the interactions of a large number of flavour compounds with BSA. The binding constants ranged from 0 to 10×10^3 M^{-1}, and decreased in the sequence: aldehydes > ketones > alcohols. The authors suggested that the binding of aroma compounds was due to both hydrophobic and electrostatic forces. *King and Solms (1979)* found the interaction between labeled (^{14}C) benzyl alcohol and denatured BSA to be reversible. The interaction was independent of pH and ionic strength, confirming a dominance of hydrophobic interactions.

Damodaran and Kinsella (1980a, 1980b, 1981c) extensively studied the interactions between flavour compounds, in particular 2-nonanone and BSA. Using liquid-liquid partitioning, they determined a binding constant for 2-nonanone and BSA of $K = 1{,}800$ M^{-1} with six primary binding sites on the protein molecule *(Damodaran and Kinsella, 1980a, b)*. More recently, PFG-NMR spectroscopy revealed quite different binding parameters for the BSA/2-nonanone system with $K = (833 \pm 15)\ \mathrm{M}^{-1}$ and $n = 7$ *(Jung et al., 2002)*. Differences in binding parameters for systems containing BSA are often due to the type of BSA used since BSA products contain varying amounts of fatty acids which are tightly bound by the protein and thus reduce the binding of flavours. The above mentioned values agree with the number of binding sites in BSA for long-chain *n*-alcohols *(Steinhardt and Reynolds, 1969)* and free fatty acids *(Spector et al., 1969; Spector, 1975)*. In addition, a large number of weaker, secondary binding sites are probably present *(Spector et al., 1969)*. This was confirmed by *Guth and Fritzler (2004)* who suggested one or two high-affinity binding sites, and a large number of lower affinity sites for γ- and δ-lactones on BSA.

A recent study using DSC suggested two and three binding sites and binding constants of 600 and 300 M^{-1} for vanillin (4-hydroxy-3-methoxybenzaldehyde) and 2-octanone on BSA, respectively *(Burova et al., 2003)*. The low binding parameters compared to other flavour ligands could be explained by the slightly acidic conditions (pH = 6.4) used by these authors, since lowering the pH by 1.8 units reduced the binding of γ-decalactone on BSA by 40 % *(Druaux et al., 1995)*, probably due to conformational changes in the protein.

Using predominantly chromatographic methods, *Dhont (1987)* provided some evidence that albumin bound vanillin irreversibly to a substantial extent, but *Dumont (1987)* showed that the binding was reversible. It may be possible that the binding sites for vanillin on BSA are not equivalent; binding could occur both reversibly via non-covalent interactions, and irreversibly via the aldehyde function. *Alaiz and Girón (1994)* observed irreversible binding of 2-octenal to BSA. The authors suggested a covalent binding of 2-octenal, through its double bond, with the imidazole ring of histidine in BSA.

Jasinski and Kilara (1985), using equilibrium dialysis, compared the binding between BSA and two flavours, 2-nonanone and nonanal. For both flavours, they observed strong binding to the protein, with nonanal having a higher affinity than 2-nonanone. The authors attributed the stronger binding of nonanal to the position of the functional group, or the higher reactivity of aldehydes compared to ketones. A reduction in the available ε-amino groups of BSA on analysis of the nonanal-protein complex could not be found by *Damodaran and Kinsella (1980b)*, indicating that the 1-position of the keto group caused the higher binding affinity. The 2-position of the keto group in 2-nonanone would give rise to more steric hindrance to hydrophobic interactions.

2.2.3.4 Whey Protein Products

A few studies have been reported on the binding of flavour compounds by whey protein products. *Jasinski and Kilara (1985)* investigated the binding of 2-nonanone and nonanal to whey protein concentrate (WPC). They found a large number of binding sites with strong binding affinity. Using HPLC, a weak interaction between whey protein isolate (WPI) and vanillin in a sweetened drink was demonstrated by *McNeill and Schmidt (1993)*. Unfortunately, no binding parameters were determined. *Li et al. (2000)* extended their research and found that the interaction between vanillin and WPI was strong, with an average binding constant of 1,713 M^{-1} (12 °C) and 0.67 binding site on WPI. Recently, a high binding affinity between 2-nonanone and WPI was confirmed using headspace SPME *(Zhu, 2003)*. A binding constant of $K = 2{,}059\ M^{-1}$ (25 °C) and on average one binding site per protein molecule was found. The strong interactions between whey protein products and flavour compounds indicate that the addition of these proteins to food products, even at very low concentrations, could influence the flavour profile of the food.

2.2.3.5 Caseins

A few studies have dealt with the behaviour of aroma compounds in the presence of sodium caseinate or casein. Bovine sodium caseinate is a useful model for investigating the interactions between aroma and protein because of its well-known functional properties and its wide use in dairy, as well as non-dairy, food products *(Mulvihill, 1992)*.

In the presence of sodium caseinate (10 %), *Reineccius and Coulter (1969)* noted a decrease in the diacetyl headspace concentration of nearly 50 %. In an aqueous solution containing only 1 % casein, lower volatilities of acetone and acetaldehyde were observed *(Maier, 1970)*. Even the addition of only 0.1 % sodium caseinate induced a decrease in the volatility of flavour compounds in aqueous solution in the following order: β-ionone > *n*-hexanol > ethyl hexanoate, isoamyl acetate *(Voilley et al., 1991)*. The intensity of the odour due to aldehydes, stored in a mixture with casein, decreased with increasing time of storage *(Pokorný et al., 1976)*. However, *Le Thanh et al. (1992)* did not find a decrease in the headspace concentrations of acetone and ethyl acetate in the presence of 10 % sodium caseinate. The authors attributed this to the residual NaCl present from the preparation of caseinate, because salts are known to increase the concentration of volatile compounds in the headspace *(Nawar, 1966; Land and Reynolds, 1981)*.

In a model dairy protein drink, sodium caseinate (6 %) was shown to interact with vanillin. A significant decrease in free vanillin in the drink was shown by HPLC *(McNeill and Schmidt,* *1993)*. Using similar methodology, moderate binding of vanillin to sodium caseinate was shown by *Li et al. (2000)*. *Landy et al. (1995)* observed a major influence of sodium caseinate on the headspace-liquid partition coefficients and on the relative volatility of diacetyl, ethyl butanoate, and ethyl hexanoate, but not of ethyl acetate, in solutions containing 0.5 % and 5 % protein, respectively. The presence of strong interactions between diacetyl and sodium caseinate was suggested by the retention of diacetyl in the dialysis sac even after exhaustive dialysis against water *(Farès et al., 1998)*. Conversely, an exhaustive dialysis in the presence of benzaldehyde and sodium caseinate resulted in no volatile compound retained by the protein, revealing weak bonds between benzaldehyde and sodium caseinate *(Farès et al., 1998)*. In contrast, the aliphatic aldehyde hexanal has been found to bind covalently to sodium caseinate *(Meynier et al., 2004)*.

Dubois et al. (1996) showed that, in a model cheese system, the volatility of diacetyl decreased slightly with increasing calcium caseinate content whereas a change in the fat content of up to 30 % did not affect the volatility. The hydrophilicity of diacetyl was used as an explanation by these authors.

Fischer and Widder (1997) developed a method based on headspace GC-O to measure the retention of esters and heptanal in aqueous solutions with a casein content varying from 0 to 12 %. Generally, the aroma retention increased with increasing protein content. A recent study by *Zhu (2003)* demonstrated weak binding of 2-nonanone by sodium caseinate, using headspace SPME. An average affinity constant of $K = 1{,}858\ M^{-1}$ and on average 0.3 binding site per protein molecule were found. The above studies clearly show that caseins and sodium caseinate are capable of binding several different flavour compounds. Therefore, when adding caseins to a food product, it has to be considered that flavour may be bound by the proteins and made unavailable for perception.

Hardly any information is available on the flavour binding behaviour of the individual caseins – α_{s1}-, α_{s2}-, β-, and κ-casein. Of all the caseins, β-casein is the most hydrophobic *(Swaisgood, 1992)*, and thus could have higher affinity constants for lipophilic flavour compounds. In addition, casein molecules have tendency to undergo self-association or association with each other, depending on environmental conditions, such as protein concentration, pH, and ionic strength. It is unknown how this association behaviour influences flavour binding. Recently, *Burova et al. (2003)* estimated binding constants around 100 M^{-1} for 2-octanone or vanillin on β-casein. Systematic studies on the binding of selected flavour compounds by individual caseins and their mixtures under different conditions are required to fully understand this complex system.

2.2.3.6 Comparison of Flavour Binding Capacities of Milk Proteins

The binding parameters of the most studied flavour compound, 2-nonanone, for milk proteins obtained by different authors vary significantly, as shown in Table 2.2-3.

Researchers have used different experimental approaches to investigate protein-flavour binding, which may be the reason for some of the variation in the results *(O'Keefe et al., 1991b; Stevenson et al., 1996)*. Nevertheless, there are obvious trends, such as decreasing affinity constants in the order: BSA > β-lg > α-la.

Table 2.2-3: Binding data for the interactions between 2-nonanone and milk proteins (25 °C): *n*, number of binding sites per monomer; *K*, intrinsic binding constant.

	n	*K* (M^{-1})	Method	Reference
WPC	61	1,920,000	Equilibrium dialysis	*Jasinski and Kilara (1985)*
	0.2	53,000,000	Fluorescence spectroscopy	*Liu et al. (2005b)*
WPI	1	2,059	Headspace SPME	*Zhu (2003)*
Na-Caseinate	0.3	1,858	Headspace SPME	*Zhu (2003)*
β-Lg	1	2,439	Equilibrium dialysis	*O'Neill and Kinsella (1987b)*
	0.2 0.5	6,250 (≤ 40 ppm) 1,667 (≥ 45 ppm)	Static headspace analysis	*Charles et al. (1996)*
	14	122	Equilibrium dialysis	*Jasinski and Kilara (1985)*
α-La	33	11	Equilibrium dialysis	*Jasinski and Kilara (1985)*
BSA	5-6	1,800	Liquid-liquid partitioning	*Damodaran and Kinsella (1980b)*
	15	14,100	Equilibrium dialysis	*Jasinski and Kilara (1985)*
	7	833	PFG-NMR spectroscopy	*Jung et al. (2002)*

An early study that compared the binding of diacetyl by sodium caseinate and whey protein showed similar flavour binding for both proteins, as determined by static headspace analysis *(Reineccius and Coulter, 1969)*. However, this observation was disputed by several later studies, which showed that whey protein generally has a stronger flavour binding capacity than casein *(Hansen and Heinis, 1991; Hansen and Booker, 1996; Li et al., 2000; Zhu, 2003)* (Figure 2.2-3). Under identical experimental conditions, WPI was found to have higher affinity for vanillin than sodium caseinate *(Li et al., 2000)*. These results agree with the findings of *Hansen and Booker (1996)*, who examined the binding of vanillin, benzaldehyde, citral, and *d*-limonene to sodium caseinate and WPC. They reported that whey protein exhibited greater degrees of binding than casein of these flavour compounds. Recently, *Zhu (2003)*, using headspace SPME, determined a higher average binding constant for 2-nonanone and WPI than for 2-nonanone and sodium caseinate (Table 2.2-3).

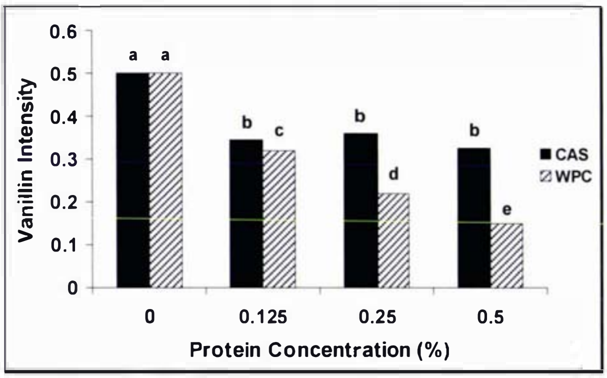


Figure 2.2-3: Vanillin flavour intensity relative to the reference in the presence of sodium caseinate (CAS) and whey protein concentrate (WPC). The reference vanillin concentration was 3.38 × 10^{-6} mM in a 2.5 % sucrose solution. For each protein type, bars with dissimilar letter codes indicate significant differences between means *(Hansen and Heinis, 1991)*.

In contrast, *McNeill and Schmidt (1993)* reported that sodium caseinate interacted significantly more than WPI with vanillin in sweetened drinks. The authors did not provide any explanation for this surprising result. Their findings highlight that a complex matrix, containing sucrose and emulsifier as well as protein, can influence flavour binding to proteins differently than a simple matrix. Therefore, it is crucial to explore basic systems first to be able to interpret results in multicomponent systems.

Another study investigated the influence of different ratios of sodium caseinate and whey protein on the flavour perception of yoghurts. The flavour intensity and the fruity notes were less intense in yoghurts with a high caseinate ratio than in those with a high whey protein ratio *(Saint-Eve et al., 2006a)*. No explanation for the differences in flavour perception was given by the authors. In my opinion, reasons for the low retention of flavour compounds in the whey protein enriched yoghurts may be the low pH of 4.6 since flavour binding by whey proteins decreases with pH (see section 2.3.4). In addition, the milk bases were heat treated at 92 °C for 5 min which is known to result in lower binding of flavour compounds and whey proteins due to denaturation (see section 2.3.2.2). In agreement with this, static headspace SPME showed a higher retention of most aroma compounds in the yoghurts with a high caseinate ratio *(Saint-Eve et al., 2006b)*. The authors suggested that the heterogeneous network of the caseinate enriched yoghurts which had large pores may constitute a more effective barrier for the transfer of flavour compounds under static conditions than the homogenous network of the whey protein enriched yoghurts. However, they determined flavour binding under static conditions which is controlled by thermodynamic and not mass transfer factors so the differences in flavour binding between the yoghurts with different textures result from physicochemical interactions between the yoghurt matrix and the flavour compounds.

BSA has been shown to interact strongly with vanillin, whereas sodium caseinate and WPI showed similar and significantly lower binding of vanillin. Hydrogen bonding appeared to be a major force for the interaction of vanillin and sodium caseinate. However, hydrophobic interaction seemed to be more important than hydrogen bonding in the vanillin-BSA system *(Chobpattana et al., 2002)*. Binding of γ- and δ-lactones has been shown to be much stronger to BSA in comparison to β-lg *(Guth and Fritzler, 2004)*. From all of these studies, it becomes obvious that BSA is the milk protein that is most capable of flavour binding, followed by β-lg.

2.2.3.7 Other Food Proteins

2.2.3.7.1 Soy Protein

Interactions between flavour compounds and soy protein have been investigated extensively, because soy protein preparations frequently possess tightly bound off-flavours, especially carbonyl compounds *(Damodaran and Kinsella, 1981a; O'Keefe et al., 1991b)*. Studies by *Gremli (1974)* and *Franzen and Kinsella (1974)* showed a reduction in the headspace concentration of homologous series of aldehydes and ketones in the presence of soy protein solution, compared with water. The effect was greatest with higher homologs, indicating that the degree of the interaction was a function of hydrophobicity *(Gremli, 1974)*. Alcohols remained unaffected. In contrast, *Chung and Villota (1989)*, using equilibrium dialysis, did find weak binding of alcohols by soy protein.

Using HPLC, considerable binding of vanillin to soy protein isolate (K = 684 M^{-1}, n = 3.8, 12 °C) was demonstrated by *Li et al. (2000)*. *King and Solms (1979)* showed a reversible binding of labeled (^{14}C) benzyl alcohol to denatured soy protein in aqueous solution.

Soy protein consists of the two proteins β-conglycinin (7S) and glycinin (11S). Kinsella's group *(Damodaran and Kinsella, 1981a; O'Neill and Kinsella, 1987a)* determined the binding parameters for 2-nonanone and soy protein and its fractions, β-conglycinin and glycinin. Soy protein, β-conglycinin, and glycinin had approximately five, two, and three primary binding sites and binding constants of 570, 3,050, and 540 M^{-1}, respectively, i.e., β-conglycinin showed a fivefold greater affinity than glycinin for 2-nonanone. In contrast to these results, *O'Keefe et al. (1991a)*, using static headspace analysis, reported that glycinin had a higher affinity than β-conglycinin for binding aldehydes and ketones, including 2-nonanone. However, differences in experimental conditions make it very difficult to compare results from different studies. For example, a dependence of the binding constant on the buffer system used and on sodium azide was observed by *O'Keefe et al. (1991b)*.

2.2.3.7.2 Fababean Protein

Binding experiments with fababean protein (1-10 %) and vanillin showed that up to 49 % of the ligand present in the system was bound by the protein *(Ng et al., 1989a)*. The sensory perception of vanillin was directly related to the free vanillin concentration in model systems containing fababean protein *(Ng et al., 1989b)*.

Semenova et al. (2002a) determined the binding of hexyl acetate with 11S globulin – the main storage protein of faba beans – in an aqueous medium. A total number of binding sites of $n = 13$ and an intrinsic binding constant of $K = 1,280$ M^{-1} were calculated from the data. It was also observed that the binding to the native 11S globulin was completely reversible.

2.2.3.7.3 Pea Protein

The binding of diacetyl to pea protein was studied by *Dumont and Land (1986)*. The binding constant was relatively low ($K = 173$ M^{-1}), suggesting weak and reversible binding. However, the number of binding sites reported was 18. The authors suggested that the binding of diacetyl to pea protein results from the interaction of the flavour with arginyl residues.

2.2.3.7.4 Egg Protein

The protein ovalbumin (egg albumin) has been studied in terms of its flavour binding behaviour by a few researchers. Lower headspace concentrations of acetone and acetaldehyde were observed in the presence of 1 % egg albumin in aqueous solution *(Maier, 1970)*. Native egg albumin also markedly decreased the vapour pressure of diacetyl over its aqueous solution, even at a protein concentration of 0.5 % *(Land and Reynolds, 1981)*. *Ebeler et al.*

(1988) investigated interactions between ovalbumin and menthone and isoamyl acetate. The presence of egg albumin resulted in a decrease in headspace flavour concentrations and the perceived flavour intensities, especially in the case of menthone. A good correlation between the sensory and physicochemical data was observed.

2.2.3.7.5 Fish Actomyosin

To date, only one study on the retention of flavour compounds by fish protein has been reported *(Damodaran and Kinsella, 1983)*. These authors focused on the interaction of native fish actomyosin with selected alkanones. The intrinsic binding constant for 2-nonanone ($K = 386\ M^{-1}$) and 13 binding sites were determined by equilibrium dialysis.

2.2.3.7.6 Muscle, Bone and Skin Proteins

Sarcoplasmic protein, extracted from pork muscle, binds branched aldehydes (2- and 3-methyl-butanal), octanal, and hexanal; however, 2-pentanone was not bound *(Pérez-Juan et al., 2007)*. The binding was found to be very dependent on the ionic strengths and the ions present.

Gelatin and collagen have also been shown to bind flavour compounds. *Nawar (1971)* described a decrease in the volatility of methyl ketones in aqueous systems containing 10 % gelatin. Reduced headspace concentrations of acetone, ethanol, acetaldehyde, and ethyl acetate were observed in the presence of only 1 % gelatin *(Maier, 1970)*. Low molecular weight aldehydes have been shown to react readily with the NH_2 groups of collagen *(Bowes and Cater, 1968)*.

2.3 Factors Influencing Protein-Flavour Binding

The principal factors influencing the binding between aroma compounds and proteins are the natures of the protein and the volatile compound, especially their hydrophobicities. Conditions in the medium also affect the extent of the interactions because they have an influence on the conformation of the proteins.

2.3.1 Protein Concentration

Several studies *(King and Solms, 1979; Dumont and Land, 1986; Fischer and Widder, 1997; Chobpattana et al., 2002)* have shown that the binding of flavour compounds increases with increasing protein concentration. Even small increases in protein concentration can markedly reduce the aroma perception. For example, at levels below 0.5 % protein, the reduction is sufficient to be readily perceptible, but the protein content of many foods greatly exceeds this level *(Land and Reynolds, 1981)*.

Hansen and Heinis (1991) reported that the flavour intensity of vanillin, measured by a trained taste panel, decreased from 0.32 to 0.15 as the WPC level increased from 0.12 to 0.5 % in flavoured protein solutions. Similar losses of benzaldehyde and *d*-limonene were found with increasing WPC concentration *(Hansen and Heinis, 1992)*. *Hansen and Booker (1996)* compared the intensity of flavour compounds in the presence of increasing WPC or sodium caseinate concentration (0-5 %). The intensities of all the flavours examined decreased with increasing WPC concentration, whereas only the intensity of *d*-limonene was affected by sodium caseinate. *Landy et al. (1995)* also observed an increase in the retention of aroma compounds with increasing sodium caseinate content (0-5 %), and the increase varied with the nature of the aroma compound. The amount of bound vanillin increased significantly as the concentration of sodium caseinate or BSA *(Chobpattana et al., 2002)* or fababean protein *(Ng et al., 1989a)* in the model systems increased.

2.3.2 Heat Treatment

Flavour binding by proteins is very dependent on the conformational state of the proteins, and all the factors that alter the conformation, i.e., temperature *(Li et al., 2000; Chobpattana et al., 2002)*, pH *(Druaux et al., 1995; Jouenne and Crouzet, 1996; Andriot et al., 1999; Jouenne and Crouzet, 2000a, b)*, and ionic strength *(Mohammadzadeh-K. et al., 1969b; Damodaran and Kinsella, 1981c)*. It has been demonstrated that conformational changes affect both the binding affinity and the number of binding sites on proteins for aroma compounds.

In practice, heat denatured proteins are of greater importance than native proteins, because heat treatment is an important step during the processing and preparation of many foodstuffs containing protein *(de Wit, 1981)*. Heat treatments usually cause denaturation of proteins, which involves unfolding and subsequent aggregation of unfolded protein molecules. The process of unfolding may reveal binding sites that were previously buried and thus may result in an increase in binding. In contrast, specific flavour binding sites on the proteins may be destroyed during denaturation, causing a decrease in flavour binding.

2.3.2.1 Below the Denaturation Temperature

The effects of temperature on protein-flavour interactions appear to be dependent on the type of protein and the type of flavour compound. The affinity of γ-decalactone for BSA in a model wine system was found to be higher at 10 °C than at 20 and 30 °C, possibly due to structural changes of the protein at low temperatures, whereas the number of binding sites (n = 6-7) was not modified at the three temperatures *(Druaux et al., 1995)*. However, an increase in temperature from 10 to 35 °C had little effect on the binding of 2-nonanone to BSA *(Damodaran and Kinsella, 1980b)*. This discrepancy could be explained by the presence of ethanol (10 % w/w) and salts in the model wine system used by *Druaux et al. (1995)*, which may have had an effect on BSA conformation. In addition, γ-decalactone and 2-nonanone might bind on different sites on BSA due to their structural differences. A large number of binding sites has been suggested by several authors *(Spector et al., 1969; Damodaran and Kinsella, 1980a, b; Jung et al., 2002; Guth and Fritzler, 2004)*, which makes the interpretation of results even more difficult. Some of the sites might be susceptible to temperature changes, whereas others might not be.

A decrease in temperature from 12 to 4 °C increased the number of binding sites and the binding constants for vanillin on casein and whey protein *(Li et al., 2000)*. The authors concluded that the changes in the number of binding sites and the binding affinity could be attributed to possible changes in the tertiary and quaternary structures of the protein at 4 °C. The exact changes to protein structures under these conditions need to be investigated.

Mills and Solms (1984), using static headspace analysis, observed an increase in the binding of heptanal to whey protein with an increase in temperature from 25 to 50 °C. The amount of heptanal irreversibly bound markedly increased at 50 °C. These results were attributed to covalent binding of the aldehyde and ε-amino groups of lysine residues in the proteins. An increase in temperature seems to enhance the binding of "reactive" flavours, whereas reversibly bound flavours may be released or not affected at all, depending on the nature of flavor compound. Clearly, further work is needed to fully understand the temperature dependence of these interactions, in particular in the case of reversible binding.

2.3.2.2 Above the Denaturation Temperature

Studies on the influence of heat denaturation on protein-flavour binding are summarised in Table 2.3-1. A number of studies reported a decreased binding when proteins are denatured. On exposure of β-lg to 75 °C for 10 and 20 min, the binding affinity for 2-nonanone was weaker than that of the native protein and the number of low-affinity non-specific binding sites increased *(O'Neill and Kinsella, 1988)*. Heat-treated WPI (85 °C, 10 min) had significantly higher vanillin flavour intensity than an untreated WPI *(McNeill and Schmidt, 1993)*. Free vanillin as determined by HPLC was also higher in a heated BSA system (68 °C for 30 min and 75 °C for 15 min) than in the non-heated system *(Chobpattana et al., 2002)*. In agreement with this, *Burova et al. (2003)* reported the complete loss of vanillin binding after thermal denaturation of BSA. This may have been due to heat-induced structural changes and protein aggregation during the heat treatment. These authors also demonstrated an increase in the denaturation temperature of BSA in the presence of either vanillin or 2-octanone, which means that the flavour ligands increased the conformational stability of the protein *(Burova et al., 1999, 2003)*. Non-flavour ligands, such as palmitate, have also been found to stabilise the native structure of β-lg against heat-induced unfolding and denaturation. This stabilising effect appears to be ligand-dependent; ligands which bind strongly into the hydrophobic calyx seem to be most effective *(Considine et al., 2005a)*.

Table 2.3-1: Influence of heat denaturation on the binding between milk proteins and flavour compounds; ↑ binding increases, ↓ binding decreases.

Protein–flavour system	θ (°C) / t (min)	Binding	Method	Reference
β-Lg / benzaldehyde	70 / 30	↑	Ultrafiltration-HPLC	*Hansen and Booker (1996)*
β-Lg / 2-nonanone	75 / 10 and 20	↓	Equilibrium dialysis	*O'Neill and Kinsella (1988)*
WPI / vanillin	85 / 10	↓	Sensory	*McNeill and Schmidt (1993)*
BSA / vanillin	75 / 15	↓	Ultrafiltration-HPLC	*Chobpattana et al. (2002)*

On the other hand, when heated and unheated sodium caseinate solutions were compared, no differences were found for vanillin flavour intensity *(McNeill and Schmidt, 1993)* and free vanillin concentration *(Chobpattana et al., 2002)*. This was expected as casein has a little secondary and tertiary structure and remarkably high heat stability *(Fox and Mulvihill, 1982)*.

However, for one milk protein-flavour system, it has been reported that the binding capacity of denatured proteins is generally higher than that of native proteins. *Hansen and Booker (1996)* reported that the amount of benzaldehyde bound by β-lg increased from 38 to 63 % as the temperature was raised from room temperature to pasteurisation temperature (70 °C, 30 min). The authors attributed the increase in binding to unfolding of whey proteins upon heating. The previously buried hydrophobic residues may become accessible for interaction with non-polar flavour molecules, resulting in a greater amount of flavour compounds bound. Since these authors added the flavour before the heat treatment, it is likely that covalent binding may play a role in this case, i.e., the aldehyde function of benzaldehyde could be susceptible to reaction with ε-amino groups in β-lg, particularly at the elevated temperature.

Most of the reported data on the influence of heat treatment on protein-flavour binding are based on single temperature and heating time. No systematic studies have been reported on the development of interactions with increasing heating time or increasing heating temperature. There might be an early stage with increased binding due to unfolding of the proteins, and a later stage characterised by a decrease in binding due to aggregation. Moreover, it has been established that a number of intermediate species are generated during heat-induced denaturation and aggregation of β-lg and whey proteins *(Havea et al., 2001)*. It would be interesting to understand the role of these intermediates in flavour binding.

2.3.3 High Pressure Treatment

The use of high pressure as an alternative to heat treatment of foods, including milk and dairy products is becoming of increasing interest. High pressure treatment not only results in microbial inactivation, but has also been shown to improve rennet or acid coagulation of milk without detrimental effects on important quality characteristics, such as taste, flavour, vitamins and nutrients *(Trujillo, 2002)*. It is known that high pressure treatment changes the structure of milk proteins *(Iametti et al., 1997)*, and is thus likely to affect the protein-flavour interactions. In particular, β-lg has been shown to be very sensitive towards pressure-induced denaturation, and the process of pressure denaturation appears to be similar but not identical to that of heat denaturation *(Huppertz et al., 2004a; Considine et al., 2005b)*. α-La is more resistant to pressure than β-lg *(Huppertz et al., 2004a; Liu et al., 2005b)*. Recently, an extensive review on high pressure and heat treatment of individual whey proteins and mixtures of milk proteins *(Considine et al., 2007b)* has been published.

Yang et al. (2003) observed that ligand binding by high pressure treated β-lg can be increased or decreased compared to the native protein depending on the structure of the ligand. High pressure treatment of β-lg decreased the affinity for capsaicin on specific binding sites, whereas the binding of the other flavour compounds examined, namely α-ionone, β-ionone, cinnamaldehyde, and vanillin, was unspecific and remained unaffected by high pressure treatment. The authors explain this finding with the incorporation of water into the protein upon high pressure treatment, as compared to the transfer of non-polar groups into water upon heat denaturation of a protein *(Hummer et al., 1998)*, so that high pressure treated β-lg may not exhibit an increase in surface hydrophobicity, and thus not exhibit an increasing affinity for hydrophobic flavour compounds. However, this would mean that heat-treated β-lg would bind more flavour compared to the native protein, but this is not true in most cases (Table 2.3-1).

New insight into the effects of high hydrostatic pressure (HHP) treatment on protein-flavour binding was given by *Liu et al. (2005b)*, using fluorescence spectroscopy and static headspace analysis. The flavour compounds investigated – benzaldehyde, heptanone, octanone, and nonanone – were added after the high pressure treatment. The number of binding sites and the apparent dissociation constants for benzaldehyde and the methyl ketones on WPC were either unaffected or increased upon HHP treatment, depending on the structure of the flavour compound, the flavour concentration, and the HHP treatment times. Using SDS-PAGE, the same authors *(Liu et al., 2005a)* showed that during the time to reach the target pressure (600 MPa), dissociation of protein aggregates present in WPC occurred, which may have exposed more binding sites. The presence of aggregates may have resulted from the ultrafiltration and drying procedures used in WPC manufacture. Flavour binding to high pressure treated WPC was found to be dependent on the flavour concentrations and on the HHP treatment time, e.g., the binding of octanone and nonanone to WPC, HHP treated for 10 min, was decreased, but the binding to WPC, HHP treated for 30 min, was increased *(Liu et al., 2005b)*. Therefore, the authors indicated the importance of careful selection of flavour concentrations and HHP treatment conditions for desired outcomes in food applications.

Presently, only two studies *(Yang et al., 2003; Liu et al., 2005b)* have investigated the influence of high pressure treatment on protein-flavour interactions. Therefore, thorough research on the effect of high pressure treatment on protein-flavour binding is clearly needed. Non-flavour ligands, when added prior to high pressure treatment, have been found to inhibit the formation of intermediate, non-native protein species *(Considine et al., 2005b, 2007a)*. The same effect may exist with flavour ligands but has not been studied yet. This area clearly warrants further investigations.

2.3.4 pH of the Medium

Changes in pH can affect the ionisation and the net charge on a protein molecule and, therefore, can influence the attractive and repulsive forces of proteins, their ability to associate with water, and their interactions with volatile flavour compounds. The effects of pH on the binding of aroma compounds to proteins have been investigated by several authors with different proteins, including BSA *(Beyeler and Solms, 1974)*, caseins *(Friedrich and Gubler, 1978)*, whey protein *(Mills and Solms, 1984)*, β-lg *(Andriot et al., 1999)*, and pea protein *(Dumont and Land, 1986)*.

Generally, the binding of different aroma compounds to whey proteins increased with increasing pH *(Druaux et al., 1995; Andriot et al., 1999; Jouenne and Crouzet, 2000a, b; van Ruth and Villeneuve, 2002; Weel et al., 2003)*. The authors attributed the increase in retention to a change in protein conformation with increasing pH, allowing better access to the binding sites at higher pH values. For example, the hydrophobic pocket of β-lg was found to be closed at pH 6.1, with loop EF (residues 85-90) being folded over the entrance of the pocket, while it is opened at pH values of 7.1 and 8.1 *(Qin et al., 1998)*. Above pH 7.0, other conformational changes, such as the dissociation of β-lg dimers or the "Tanford transition", characterised by the release of a buried carboxyl group, better accessibility of the free sulphydryl group, and a change in environment of a tyrosine residue *(Tanford et al., 1959; Hambling et al., 1992)*, may be responsible for changes in protein-flavour interactions. A dramatic decrease in binding of methyl ketones, ethyl esters, limonene, and myrcene was observed at pH 11, and was considered to be a consequence of the alkaline denaturation of β-lg *(Jouenne and Crouzet, 2000a)*.

In contrast, *Mills and Solms (1984)*, using static headspace analysis, reported a higher binding of 2-nonanone and low-fat whey protein at pH 4.66 as compared to pH 6.89, with no explanation given by the authors. Sodium azide was added to the samples as a preservative and may influence flavour binding.

Acid denaturation of broad bean 11S globulin caused a drastic change in the native protein structure, which resulted in loss of the capacity for the binding of hexyl acetate *(Semenova et al., 2002a)*. Decreasing the pH of a pea protein solution, and in particular isoelectric precipitation of the protein, resulted in a dramatic reduction in its diacetyl retention and led to a partial release of the previously bound flavour *(Dumont and Land, 1986)*.

2.3.5 Ionic Strength of the Medium

The native conformation of a protein, and thus its flavour-binding properties, can be affected by the type and concentration of the ions in the medium. For example, fish actomyosin has low solubility in a medium with only 0.15 M NaCl. In this suspension, the protein bound more 2-nonanone than the soluble protein in a medium containing 0.6 M NaCl *(Damodaran and Kinsella, 1983)*. *Farès et al. (1998)* observed that the number of moles of benzaldehyde bound per mole of sodium caseinate was 5.56 without sodium azide and was 0.05 in the presence of 0.03 M sodium azide. However, the binding between diacetyl and sodium caseinate was not affected by the addition of 0.03 M sodium azide to the aqueous solution. Other investigators working with β-lg came to a similar conclusion *(Mohammadzadeh-K. et* *al., 1969b)*; e.g., the binding of heptane to β-lg decreased with increasing ionic strength up to 0.25 M.

More recently, *Pérez-Juan et al. (2007)* observed a reduction in the binding of branched aldehydes, hexanal, and methional and sarcoplasmic protein extracts from pork muscle with increasing concentrations of NaCl and KCl, while octanal binding was not affected. The effect of $MgCl_2$ and $CaCl_2$ was much lower, except for the branched aldehydes which were completely released at 1.0 ionic strength.

An opposite trend was observed for the binding of 2-nonanone and BSA; the binding increased linearly with increasing concentration of sodium sulphate, chloride, bromide, and perchlorate *(Damodaran and Kinsella, 1981c)*. The ability of the salts to increase the binding affinity of the flavour compound followed the Hofmeister series, i.e., $SO_4^{2-} > Cl^- > Br^- > ClO_4^- > SCN_4^- > Cl_3COO^-$, which is also the order in which these salts tend to stabilise globular protein structure.

2.4 Implications in the Development of Protein Foods

Proteins do not contribute to flavour directly, but protein-flavour interactions can cause the aroma profile of a food to become unbalanced and unpleasant since proteins bind flavours to differing extents, depending mainly on the nature of the protein and flavour compound. This problem occurs particularly in high protein based foods and in low-fat foods since fat is the preferred carrier of flavour compounds. Ideally, flavour molecules are preserved in the food during storage and processing, and slowly released during consumption of the food. However, in the presence of protein and absence of fat, some flavour compounds are lost due to the absence of fat, whereas others are tightly bound by the proteins, preventing them to be released and perceived during mastication. In such foods it is extremely difficult to control flavour. If proteins are present, the amount of flavour compounds added usually has to be increased to compensate for the protein-bound flavour.

The processing of foods, and in particular heat treatment, has been shown to have a marked effect on milk protein-flavour interactions. Even slight changes in temperature below the denaturation temperature of the proteins have been shown to influence the binding of flavours *(Mills and Solms, 1984; Druaux et al., 1995)*. Severe heat treatments caused a decrease in binding for most flavours *(O'Neill and Kinsella, 1988; McNeill and Schmidt, 1993; Chobpattana et al., 2002; Burova et al., 2003)*, but an increase in binding has also been reported *(Mills and Solms, 1984; Hansen and Booker, 1996)*. Thus heat treatment may drastically change the flavour profile of a food product, depending on what flavour compounds are present. In the development of protein-based foods, any factors changing the conformation of a protein, i.e., pH, ionic strength, etc., have to be considered since they potentially alter the binding of flavours to the protein. The presence of flavour compounds during heat treatment has been shown to increase the protein conformational stability *(Burova et al., 1999, 2003)*, which in turn may alter the characteristics of the end product.

2.5 Flavour Release from Proteins

2.5.1 Flavour Release and Perception

Flavour perception in humans is the consequence of specific interactions of the flavour compounds with particular receptors in the olfactory epithelium *(Meynier et al., 2003)*. Since the flavour receptors are specifically designed proteins, the mechanism of flavour perception is the most important protein-flavour interaction *(Fischer and Widder, 1997)*. To produce a response, flavour compounds must be present at a concentration above a certain threshold and free to interact with the receptors. The overall perception of flavour is generally understood to be a combination of both non-volatile compounds in the saliva (taste) and volatile compounds transported to the olfactory epithelium (aroma) *(Harrison, 1998)*.

To perceive the flavour of a food, flavour compounds need to be released from the food matrix into the mouth and nose of the consumer. Thus, the perceived intensity of flavour not only depends on the type and concentrations of flavour compounds present, but also on their release into the mouth and nose. The major factors playing a role in the release are the composition and the structure of the food matrix *(Seuvre et al., 2001)*. The amount of flavour released into the air in the mouth and hence to the olfactory epithelium in the nose depends upon the vapour pressure of the volatile in the aqueous phase of the food, and can be influenced by the presence of other food components, such as proteins *(Overbosch et al., 1991)*. Consequently, the nature of the mechanism of binding of flavour components to proteins and other macromolecules has a significant effect on the acceptance of a food product.

2.5.2 Analysis of Flavour Release from Proteins

Conventional headspace analysis has been used to measure flavour release from foods but it does not consider conditions in the mouth, which do affect the release of flavour compounds such as esters *(Doyen et al., 2001)*. Nowadays, methods are applied that instrumentally measure the release of flavour compounds from foods during eating. They can be divided into two categories: (1) the breath exhaled from the mouth or nose is collected and analysed by MS or GC-MS ("MS Breath Methods"), and (2) a "mouth simulator" is constructed that attempts to mimic mouth conditions, and the flavour released from this artificial mouth system is collected and analysed by MS or GC-MS.

2.5.2.1 MS Breath Methods

The measurement of volatile release in the mouth ('mouthspace') or breath expired from the nose ('nosespace') is of great interest *(Soeting and Heidema, 1988; Delahunty et al., 1994; Taylor and Linforth, 1994; Weel et al., 2003; Gierczynski et al., 2007)*. It enables us to observe the influence of human physiology on aroma release from different food matrices *(Linforth et al., 1996)*. The first MS breath system was developed by *Soeting and Heidema (1988)*. They coupled the human nose to the ion source of a mass spectrometer to measure the flavour concentration in the nasal cavity in real time, allowing the differences in flavour profiles resulting from different flavour-ingredient combinations to be determined *(Overbosch et al., 1991)*. Membranes can be used to prevent moisture and oxygen entering the electron impact source of the MS, whilst volatiles pass through the membrane *(Soeting and Heidema, 1988; Taylor and Linforth, 2000)*. Using this method, it was possible to determine the aroma release profile of methyl ketones from oil-water systems *(Soeting and Heidema, 1988; Haring, 1990)*. A shortcoming of the method using membranes is that the membranes can exhibit selectivity for some flavour compounds, i.e., exclude those with a low affinity for the membrane, or cause long response times for other compounds with a slow rate of diffusion through the membrane *(Linforth et al., 1996)*.

Since the concentrations of volatiles in the breath are very low, MS breath analyses have the problem of sensitivity. To overcome the sensitivity problem, the volatiles from breath samples have been trapped prior to GC-MS analysis, i.e., by using low temperature (cryo-trapping) *(Linforth and Taylor, 1993; Taylor and Linforth, 1996)* or adsorbing polymers (e.g. Tenax) *(Delahunty et al., 1994; Taylor and Linforth, 1994)*. When breath is sampled onto Tenax traps over several minutes whilst eating different foods *(Linforth and Taylor, 1993; Delahunty et al., 1994; Legger and Roozen, 1994; Taylor and Linforth, 1994; Ingham et al., 1995)* the volatile compounds of interest are effectively separated from air and, to a great extent, water. However, such sampling techniques only yield a temporal aroma profile after a large number of chromatographic runs are completed *(Linforth et al., 1996)*. For example, nosespace was sequentially sampled onto a set of polymer traps to obtain a time-resolved flavour release profile. Off-line GC analysis of the traps was carried out after thermodesorption *(Linforth and Taylor, 1993)* or solvent desorption *(Delahunty et al., 1994)*.

2.5.2.1.1 Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS)

As an alternative to trapping the breath contents during eating, the retronasal aroma can be directly measured using soft ionisation mass spectrometers with detectors that can accommodate moisture and atmospheric pressure, such as in APCI-MS *(Linforth et al., 1996; Malone et al., 2000; Weel et al., 2002; González-Tomás et al., 2007)*. This technique is more direct and less time-consuming *(Taylor et al., 2000)*. A small sample of the test person's breath is continuously sampled into the mass spectrometer where volatiles are detected providing a direct and simultaneous temporal release profile of several volatiles *(Taylor and Linforth, 1996)*. The system allows sensitive and fast monitoring of the *in vivo* flavour release *(Linforth et al., 1996; Taylor and Linforth, 2000)*, and is commercially available as the MS-Nose™ from Micromass (Manchester, UK).

The breath-by-breath profile obtained by APCI-MS is thought to be very similar to that sensed by the olfactory epithelium *(Baek et al., 1999; Linforth et al., 1999)* and may be different from the volatile composition of the food and its headspace profile, because of the changes that occur to the physical state of foods during eating *(Overbosch et al., 1991; Taylor and Linforth, 1996)*.

2.5.2.1.2 Proton-transfer-reaction mass spectrometry (PTR-MS)

Techniques involving ionisation based on proton transfer - either APCI or the similar PTR technique *(Lindinger et al., 1998; Taylor and Linforth, 2000; Mei et al., 2004)* - followed by mass spectrometry are the best current options for volatile flavour analysis in breath *(Taylor et al., 2000)*.

A PTR-MS (Figure 2.5-1) allows the simultaneous real-time monitoring of volatile compounds, such as flavour compounds, present in gaseous samples, such as air or breath. It has been successfully used for various applications, for example, the analysis of volatiles during the decay of foods, or air quality monitoring *(Lindinger et al., 1998)*.

To investigate flavour release in mouth, the subjects, while consuming the sample, breathe into a U-shaped glass nose piece inserted into their nostrils (Figure 2.5-1), from which breath is sampled continuously, and directed into the PTR-MS drift tube.

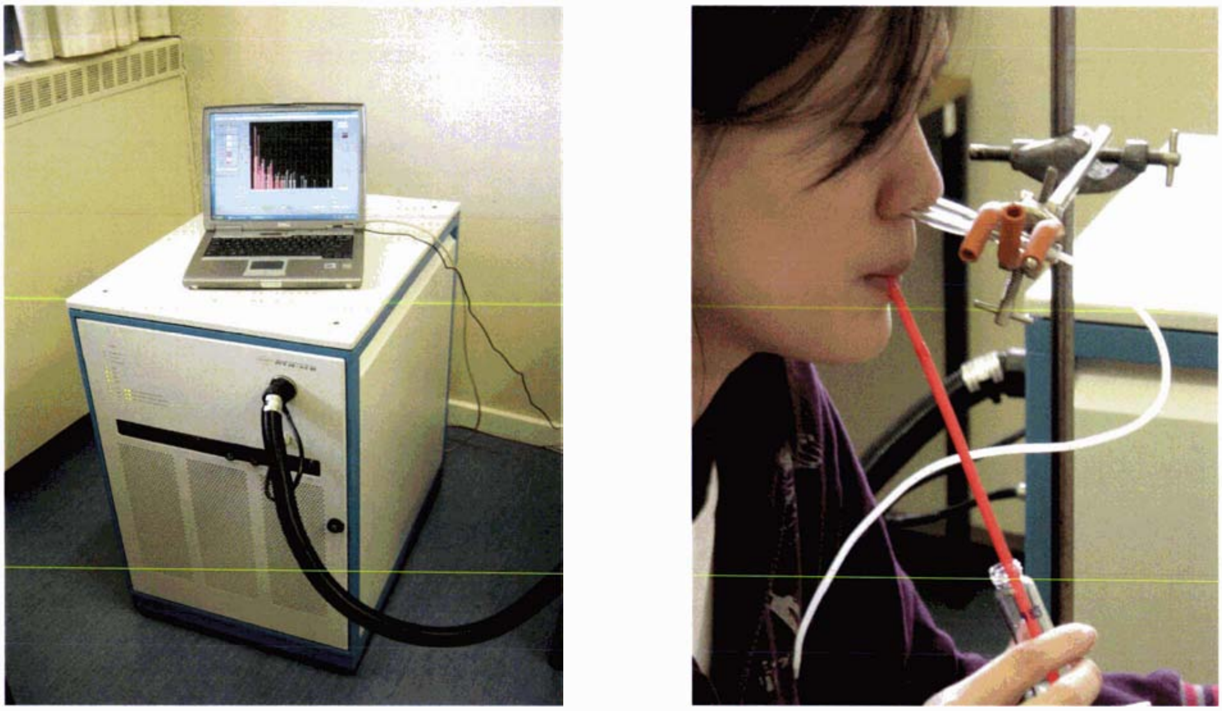


Figure 2.5-1: The PTR-MS instrument (Ionicon Analytik GmbH, Innsbruck, Austria), and a subject connected to the instrument while consuming a sample.

The technique utilises proton-transfer reactions (Equation 2-7) from protonated water molecules H_3O^+ to volatile compounds R, which are then separated according to their mass to charge ratio (*m/z*).

$$R + H_3O^+ \rightarrow R^+ + H_2O \qquad \textbf{(2-7)}$$

The instrument (Figure 2.5-2) consists of three main parts:

- The ion source, where H_3O^+ ions are produced using water vapour as the molecular source of ions
- The drift tube, into which the sample (breath/air) is continuously introduced, and where proton-transfer reactions to the volatiles in the breath occur
- The ion detection system which provides sensitive detection of mass selected ions and consists of a quadrupole mass spectrometer in conjunction with a signal amplifier.

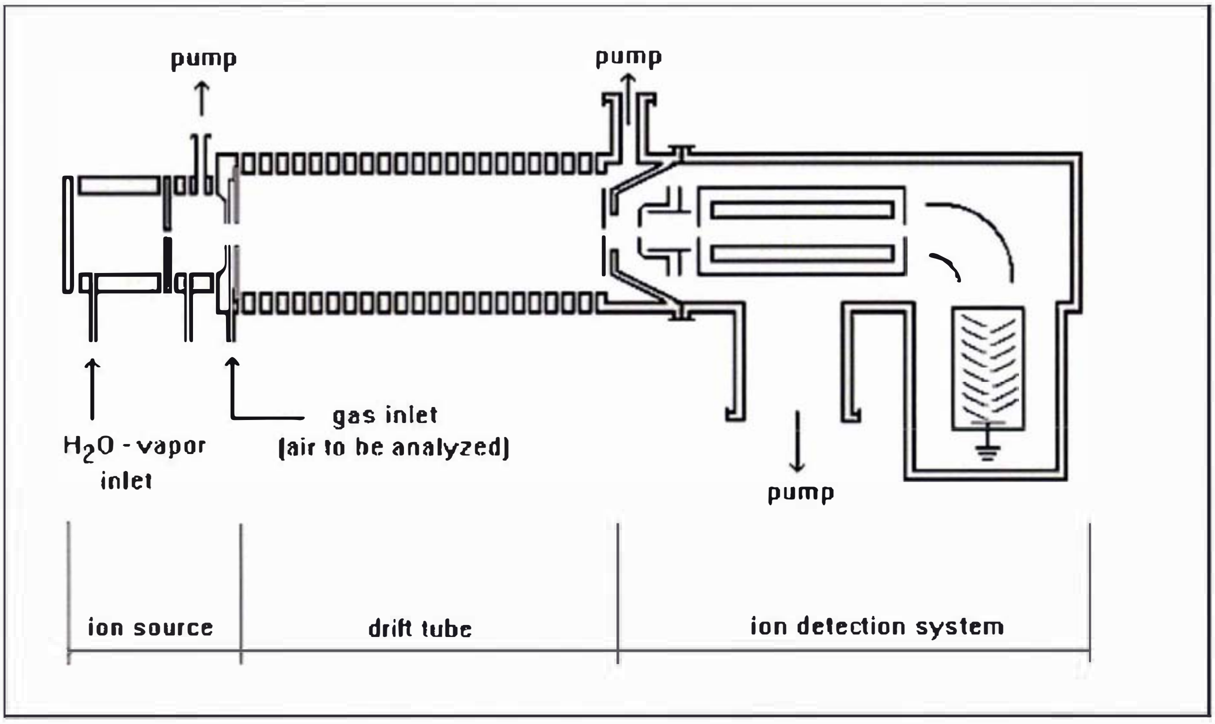


Figure 2.5-2: Schematic of the PTR-MS system (Source: Ionicon Analytik GmbH, Innsbruck, Austria).

The H_3O^+ ions are passed via a Venturi type inlet into the drift tube, which is under the influence of an electric field (E) and a vacuum pump. Within the drift tube, some H_3O^+ ions become hydrated to form cluster ions, $H_3O^+(H_2O)_n$ ($n = 1, 2, 3, \ldots$), which can act as reagent ions. The quantity and distribution of these cluster ions within the drift tube depend on the E and on the pressure, but is usually < 5 % of the total reagent ions *(Hewitt et al., 2003)*. Reagent ion clustering is suppressed by decreasing the drift tube pressure or increasing E. However, a decrease in pressure or an increase in E promote product ion fragmentation. Therefore, a compromise between reagent ion hydration and product ion fragmentation has to be made, depending on the sample composition. For example, in complex samples, too much fragmentation is undesirable in terms of compound identification.

The advantage of PTR-MS over APCI-MS is that in PTR-MS the generation of the H_3O^+ ions and the chemical ionisation of the volatile analytes are individually controlled and spatially separated processes. As a consequence, constant and well-defined conditions exist in the drift tube, allowing the calculation of analyte concentrations without calibration or the use of standards (see section 5.3.4.4 for more detailed information). Volatile compounds that have proton affinities higher than water are ionised by proton transfer from H_3O^+. The proton affinity of water is 166.5 kcal·mol^{-1}, whereas organic volatiles have proton affinities of 170-

205 kcal/mol *(Lindinger et al., 1998)* (Table 2.5-1). Therefore, another benefit of PTR-MS is that H_3O^+ ions do not ionise any of the major components present in clean air due to their low proton affinities.

Table 2.5-1: Proton affinities of selected constituents of air and selected organic volatile compounds *(Lindinger et al., 1998)*.

Compound	**Proton affinity (kcal·mol^{-1})**
N_2	118.0
CO_2	129.2
O_2	100.6
H_2O	**165.2**
Acetaldehyde	183.8
Ethanol	185.6
Butanal	189.5
Acetone	194.1
Dimethyl sulphide	198.6

Both, the APCI-MS and PTR-MS techniques, can cope with water and air, produce single ions for most compounds, operate at pressures, which allow easy and safe sampling of air from people to the source, and show high sensitivity *(Taylor et al., 2000; Hewitt et al., 2003; Mei et al., 2004)*. Another advantage of MS breath techniques is that they are objective methods, providing the opportunity to investigate factors affecting flavour release and the relationship between flavour delivery and perception *(Malone et al., 2000)*.

The main disadvantage of breath-by-breath experiments is the poor reproducibility which is due to differences in mastication, saliva flow and composition *(Lee, 1986; Soeting and Heidema, 1988)*, and breathing between, and to a lesser extent, within subjects *(Soeting and Heidema, 1988; Deibler et al., 2001)*. In addition, the technique can only discriminate compounds on the basis of mass, and for some aroma compounds the sensitivity is not high enough to be detected *(Mei et al., 2004)*. This suggests that *in vitro* methods, such as mouth simulators (see section 2.5.2.2) which have better precision, should be used to complement and verify results from *in vivo* techniques.

Detailed reviews of methods for monitoring volatile compounds *in vivo* have been published previously *(Taylor, 1996; Taylor and Linforth, 1996, 2000)*.

2.5.2.2 Mouth Simulators

Several authors used *in vitro* devices that mimic conditions of the human mouth as closely as possible ("mouth simulators") to investigate flavour release in the mouth. Different mouth simulators incorporating shearing, saliva dilution, temperature control, and airflow have been designed to simulate eating and drinking dynamics *(Lee, 1986; Roberts and Acree, 1995; Elmore and Langley, 1996; Giroux et al., 2007)*. Trapping on polymers *(Delahunty et al., 1994; Roberts and Acree, 1995; van Ruth et al., 2000)*, cryofocusing on capillary columns *(Naßl et al., 1995)*, and direct MS coupling of the mouth-simulating device *(Lee, 1986; Elmore and Langley, 1996; Deibler et al., 2001)* have been applied to measure the volatiles released.

The earliest model mouth system consisted of a small glass vial containing the sample, which was agitated mechanically using metal balls *(Lee, 1986)*. The system also allowed heating and the addition of synthetic saliva. A continuous gas flow transported the flavour compounds to an MS detector, which produced a time profile of the released flavour compounds. The release curves for diacetyl, as obtained from this system, have shown a remarkable similarity with the corresponding time-intensity curves (see section 2.5.2.3) obtained by a sensory panel.

Roberts and Acree (1995) introduced the so-called "retronasal aroma simulator" (RAS). Retronasal aroma is the odour sensation experienced during food consumption that is caused by flavour molecules travelling from the mouth to the olfactory epithelium *(Friedrich and Acree, 1998)*. It is a purge-and-trap device designed to simulate eating conditions in mouth by regulating temperature to 37 °C, adding synthetic saliva, and blending at shear rates reported to occur during eating. The control of these parameters allows the RAS to be used to simulate a range of release conditions. The released volatiles are removed by a gas stream, collected on a silica trap and quantified by GC-FID or GC-MS. Later, the same authors designed an RAS using a set of six volatile traps to observe the dynamics of flavour release *(Roberts and Acree, 1996b)*.

The RAS has been shown to be a sensitive and reproducible device which was verified by *Deibler et al. (2001)* and gave a good approximation of time-averaged flavour release in the mouth as defined by breath-by-breath measurements using model food systems as well as real foods. In contrast, *Legger and Roozen (1994)* found results of breath-by-breath analysis more suitable for correlation with time-intensity data (see section 2.5.2.3) than those of a purge-and-trap method with simulated mouth conditions.

Later, similar mouth simulators have been successfully applied to understand the release of flavour during eating and to investigate flavour-matrix interactions during eating *(Elmore and Langley, 1996; Bakker et al., 1998; van Ruth and Roozen, 2000a; Deibler et al., 2001; Chung et al., 2003)*. A fully computerised device for the measurement of dynamic flavour release from liquids was developed by *Rabe et al. (2002)*.

More recently, an artifical throat system for the investigation of liquid samples was developed which simulates the act of swallowing followed by exhalation *(Weel et al., 2004a)*. As liquid foods are swallowed directly after intake, the release of flavour compounds is mainly determined by swallowing in the throat. The technique produced aroma release curves which were similar to *in vivo* release profiles.

An advantage of mouth simulators compared to MS breath methods (see section 2.5.2.1) is that a range of physical parameters can be varied to elucidate the effects of, for example, gas flow rate or shear rate on flavour release. Generally, an advantage of *in vitro* devices is the increased reproducibility of flavour release data in comparison to MS breath techniques, which involve human subjects since instrumental methods can be more carefully controlled. In addition, they exhibit higher sensitivity than MS breath techniques *(Deibler et al., 2001)*. A disadvantage is the considerably lower resolution of time profiles. It has to be considered that the process by which flavour compounds are transferred from a food product to the olfactory epithelium is very complex and can only partly be described in a model mouth system.

2.5.2.3 Sensory Methods

Sensory analysis *(Ng et al., 1989b; Hansen and Heinis, 1991, 1992; Graf and de Roos, 1996)* can also be used to determine the overall flavour release in the mouth. A sensory technique that is becoming increasingly popular for the measurement of flavour release and perception is the time-intensity (TI) methodology. It is a technique that permits the recording of the perception of an attribute and its change with time *(Peyvieux and Dijksterhuis, 2001)*. Various parameters can be obtained from a TI curve and these include, for example, the maximum intensity of flavour perception I_{max}, the time at which maximum flavour intensity is perceived t_{max}, and the duration of perceived flavour. Because food undergoes an oral breakdown, mixing, and hydration during mastication *(Taylor, 1996)*, TI measurement can provide information concerning the pattern of flavour release during eating *(Chung et al., 2003)*.

The TI method has been used to successfully characterise the flavour release properties of reduced- and full-fat cheeses and salad creams *(Shamil et al., 1991/92)*, low-fat and high-fat ice cream *(Chung et al., 2003)*, and model gels *(Guinard and Marty, 1995; Wilson and Brown, 1997; Weel et al., 2002)*. Although sensory analysis is a powerful tool, it has some limitations, such as variation in responses between individuals, the necessity of a trained panel, and limitations of flavour perception in humans *(Taylor, 1996)*. Hence, the instrumental analyses of the aroma-protein interactions are necessary.

2.5.3 Flavour Release from Liquid, Protein-Containing Systems

A few studies have been published on the effect of proteins on the release of flavour compounds from different systems; generally, a decrease in flavour release with increasing protein concentration was observed due to the binding of flavour compounds by the proteins *(Giroux et al., 2007)*. β-Lg decreased the odour intensities of methyl ketones and eugenol, and it was found that 20 % or more binding was noticeable as a significant decrease in flavour perception *(Guichard, 2000)*.

Milk proteins reduced the in-mouth release of selected flavour compounds from coffee as compared to water *(Denker et al., 2006)*. The addition of whey proteins resulted in a lower release of flavour compounds as compared to the caseins which can be explained with the generally higher binding affinity for flavour compounds on whey proteins. Interestingly, for most flavour compounds, the retention could not be increased by adding more whey protein, whereas the flavour release depended on the quantity of added casein. Using an artificial throat system, the release of butanal, hexanal, octanal, and nonanal from aqueous solutions decreased in the presence of WPI (3 %) *(Weel et al., 2004a)*. Unfortunately, these studies do not indicate if the flavour compounds are partly released from the proteins under the dynamic conditions, they only show that the proteins retain the flavour compounds as compared to water.

Several studies have shown that the presence of proteins at oil-water interfaces, such as in emulsions, also induced a decrease in flavour release due to an increase in the resistance to transfer at the interfaces *(Harvey et al., 1995; Rogacheva et al., 1999; Charles et al., 2000; Voilley et al., 2000)*.

However, the actual release of flavour compounds from proteins has only been studied by *Weel et al. (2003)* and *Le Guen and Vreeker (2003)*. The effect of WPI on the *in vivo* release of aldehydes (butanal to nonanal) in solution was examined by *Weel et al. (2003)*. They found a high retention of the flavour compounds by WPI under static conditions; however, the retention of aldehydes by WPI was less under *in vivo* conditions, as determined in-nose by APCI-MS, possibly due to the highly dynamic environment in the mouth. These authors suggested that after swallowing, a thin film of the sample solution remains in the pharynx, and both the free flavour compounds present in this film and those reversibly bound to the whey proteins are released by the air flow of exhalation. The hypothesis that a thin layer of liquid remaining in the throat after swallowing largely determines the *in vivo* flavour release from liquids was later confirmed *(Weel et al., 2004a, 2004b)*.

Le Guen and Vreeker (2003) determined the release of a homologous series of methyl ketones (acetone to 2-nonanone) from MPC (10 %, w/w) in mouth. Using static headspace analysis, MPC showed a considerable retention of methyl ketones, whereas the *in vivo* APCI-MS signal of the flavour compounds was not influenced by the presence of milk proteins. These authors also concluded that during in-mouth flavour release, not only the free flavour compounds in the film are released but also those compounds reversibly bound to the milk proteins. In contrast, the presence of MPC resulted in a decrease in the concentration of alkenals (2-propenal to 2-nonenal), which are bound irreversibly on proteins, in the nasal cavity. In conclusion, flavour perception may only be reduced if strong binding occurs *(Guichard, 2006)*. However, the release of reversibly bound flavour may depend on the consumption technique. If a sample is not masticated (e.g. when drinking) reversibly bound flavour may not be released.

2.5.4 Flavour Release from Viscous, Protein-Containing Systems

Flavour release and perception have also been found to be affected by the viscosity of the medium *(Pangborn and Szczesniak, 1974; de Roos and Wolswinkel, 1994; Roberts et al., 1996; de Roos, 2003)* since the viscosity influences the mass transfer of flavour compounds within a food, and from the food into the oral and nasal cavities. Using an RAS (see section 2.5.2.2), highly volatile compounds were released slower in high viscosity systems, whereas the release of compounds with low volatility was not affected by a higher viscosity *(Roberts and Acree, 1996a)*, confirming that thickeners influence the mass transfer rather than the volatility *(de Roos, 2003)*.

Macromolecules tend to increase viscosity and thus to slow down diffusion. However, *Darling et al. (1986)* showed that the diffusion coefficient of 3-methylbutyl acetate, the character-impact compound of bananas, in solutions of galactomannan remained constant at concentration of up to at least 1 % gum. The authors indicated that diffusion is a slow process and thus its role in flavour release would be insignificant in a highly agitated system, such as that exists in the mouth during food consumption. However, several studies showed a decrease in the flavour release rates with increasing viscosity *(Mestres et al., 2005; Boland et al., 2006; Terta et al., 2006)*, which was explained with a decreased mobility of flavour compounds in viscous systems. Interestingly, increased gel rigidity resulted in higher maximum concentrations of volatiles as determined by in-nose PTR-MS *(Boland et al., 2006)*.

Hollowood et al. (2002) observed that, despite a decrease in perceived strawberry flavour intensity, the actual flavour concentration in the breath, using ethyl butyrate as a marker for the strawberry flavour, was not reduced by an increase in hydroxypropyl methylcellulose (HPMC) concentration, indicating that the viscosity does not influence in-mouth flavour release. According to the authors, a possible explanation for the decrease in flavour perception may be the effect of HPMC on the free water available in solution, resulting in a decrease in sweetness intensity and, therefore, a decrease in flavour intensity.

Several authors studied flavour release from gel systems and have reported a decrease in flavour perception with increasing gel hardness *(Jaime et al., 1993; Guinard and Marty, 1995; Carr et al., 1996; Wilson and Brown, 1997)*. Results contradictory to these were found by *Gwartney et al. (2000)* who changed the texture of the protein gels by varying the type of salt and ionic strength. Gel hardness and perceived flavour intensity were not related, but gel structure seemed to have an effect with particulate gels, which showed a low water holding capacity, having a lower maximum perceived intensity than gels with a stranded structure.

In vivo studies, looking at the actual flavour release by nosespace measurements, generally showed no effect of texture. *Mestres et al. (2005)* found that whey protein gel hardness did not affect the *in vivo* release of flavour compounds, and *Lethuaut et al. (2004)* observed that aroma release from custard desserts was not influenced by the texture. Maximum intensities of nosespace concentrations were not influenced by viscosity *(Baek et al., 1999; Weel et al., 2002)*, but there was a significant difference in the perceived flavour intensities. Thus, *Weel et al. (2002)* concluded that the texture of the gels rather than the in-nose flavour concentration determines perception of flavour intensity.

In contrast, an increase in carrageenan concentration, and thus, an increase in dynamic viscosity from 1 to 44 mPas, resulted in an increase in the release of ethyl butanoate, butane-2,3-dione, and hexanal from aqueous solutions in an artificial throat system *(Weel et al., 2004a)*. This observation was explained with a thicker layer on the inner surface of the artificial throat, which contains more flavour compounds than a thin layer of water. As a consequence, the total flavour release from viscous samples will be higher. However, *in vivo* measurements showed no effect of viscosity on the amount of flavour compounds released. The authors attributed this finding to differences in the swallowing mechanism and dilution with saliva. *Cook et al. (2003)* also demonstrated that viscosity does not influence flavour release *in vivo*.

The *in vivo* flavour release from cheese-like gels differing in hardness, as determined by APCI-MS, was found to be greater and faster from the harder gels; however, the *in vitro* flavour release, using a mouth simulator, was not significanlty different between the three gels *(Gierczynski et al., 2007)*. These studies show that the effect of texture on flavour release depends on the type of food *(Gierczynski et al., 2007)*, and clearly further investigations of flavour release in thickened systems are needed.

2.5.5 Factors Influencing Flavour Release In-Mouth

As mentioned above, factors such as the nature of the flavour compound and the protein, and the structure of the food matrix affect the release of flavours from a food during consumption. The partition coefficient and the resistance to mass transfer are the main factors controlling the rate and extent of flavour release; partitioning of flavour compounds is influenced by the composition of the food, and the resistance to mass transfer by its texture *(de Roos and Wolswinkel, 1994)*. Beside these factors, which concern the food itself, factors in the mouth, such as cutting, stretching, dilution with saliva etc. influence flavour release *(Plug and Haring, 1994)*.

The change in temperature that a food undergoes when placed in the mouth can cause melting and other phase changes modifying volatility and flavour perception. Increasing the temperature from 23 to 37 °C increased the volatility *(Roberts and Acree, 1995)*. When simulating flavour release in the mouth, i.e. by using the RAS (see section 2.5.2.2), it is important to regulate the temperature to 37 °C as temperature significantly affects flavour release rates *(Roberts and Acree, 1996a)*. In contrast, *Weel et al. (2004a)* did not find an effect of temperature on flavour release *in vivo*, nor using an artificial throat.

Mastication accelerates the mass transfer of flavours and increases the release of aroma compounds by increasing the surface area exposed to the air in the mouth and reducing the diffusion path from the food matrix to the vapour phase *(Roberts and Acree, 1995; van Ruth and Roozen, 2000b)*.

Hydration or dilution of foods by saliva affects the partitioning of the volatile compounds over the food, and thus affects flavour release and perception *(Taylor, 1996)*. The high polarity and neutral pH of saliva can change the volatility of certain flavour compounds, especially in foods high in fat or with low pH *(Roberts and Acree, 1995)*. Addition of water or saliva increased the rate of volatilisation for most flavour compounds, because they have poor solubility in water *(McNulty and Karel, 1973)*. However, *Odake et al. (1998)* and *van Ruth and Roozen (2000b)* reported a decrease in the release of flavour compounds with increased saliva volumes. This observation agrees with the flavour release model of *Harrison (1998)*, which predicted a decreased release with higher saliva flow rates.

2.6 Concluding Remarks

The presence of proteins in flavoured low-fat food products causes a great challenge for flavour scientists because many proteins are able to bind several flavour compounds tightly and influence the perceived aroma profile significantly. BSA is the milk protein most capable of binding volatile flavour compounds, followed by β-lg. A large number of instrumental methods have been used to investigate the type and the extent of flavour binding, which makes it difficult to compare results between studies. Sensory methods are very useful because they complement instrumental techniques and give insight into the effect of protein-flavour binding on flavour perception. The characteristics of the aroma compounds and the proteins determine the extent of binding, which can be influenced by several parameters. Although extensive information on the binding of various flavour compounds to different food proteins is available, there are many apparent contradictions and disagreements among various studies. For example, the effects of heat treatments on protein-flavour interactions are not fully understood. The development of protein-flavour interactions with heating time or rather with increasing temperature should be looked at and compared with the corresponding conformational state of the protein. Research on the influence of high pressure treatment of milk proteins on the proteins' flavour binding behaviour needs to be investigated further as well.

A good knowledge of the physicochemical interactions that occur between aroma compounds and proteins is required to improve food flavouring and to make protein based foods, e.g., "light" dairy products, sensorily more acceptable to the consumer. In particular, the nature and the location of binding sites on proteins for flavours need to be investigated further. It is vital to obtain more consistent results between different instrumental methods. Some of the early methods such as equilibrium dialysis and exponential dilution are still going to be frequently used. Some of the newer techniques, such as SPME and NMR, need to be developed further to investigate protein-flavour interactions and be used more frequently in the future. SPME is fast, solvent-free, and very sensitive. The main advantages of NMR techniques are speed and insight into binding mechanisms and binding topology.

Furthermore, the focus should be more on sensory techniques, since instrumental flavour binding studies do not show if and how bound flavour is perceived during consumption. A growing area of research comprises the mechanisms of *in vitro* and *in vivo* flavour release from foods. No systematic studies have been reported on the relationship between extent or strength of flavour binding to proteins and its *in vivo* release.

To date, most systems that have been investigated consist of one protein and one aroma compound in an aqueous solution. Food systems are much more complex, consisting of several food matrix components and flavour mixtures. Thus, further research on these complex systems is becoming increasingly important. However, primarily consistent results using simple systems should be obtained before investigating complex systems.

CHAPTER THREE: BINDING OF 2-NONANONE AND MILK PROTEINS [2]

3.1 Abstract

Interactions of the model flavour compound 2-nonanone with individual milk proteins, whey protein isolate (WPI), and sodium caseinate in aqueous solutions were investigated. A method to quantify the free 2-nonanone was developed using headspace solid-phase microextraction (SPME) followed by gas chromatography with flame ionisation detection (GC-FID). Binding constants (K) and numbers of binding sites (n) for 2-nonanone on the individual proteins were calculated. The 2-nonanone binding capacities decreased in the order: bovine serum albumin > β-lactoglobulin > α-lactalbumin > α_{s1}-casein > β-casein, and the binding to WPI was stronger than the binding to sodium caseinate. All proteins appeared to have one binding site for 2-nonanone per molecule of protein at the flavour concentrations investigated, except for bovine serum albumin which possessed two classes of binding sites. The binding mechanism is believed to involve predominantly hydrophobic interactions.

3.2 Introduction

It is well-known that milk proteins bind flavour compounds of different chemical classes *(Damodaran and Kinsella, 1980b; Lübke et al., 2002)*, but results between studies differ considerably. Reasons for this may be the variety of conditions and methods used to determine the binding or the different compositions of protein batches. The individual whey proteins, in particular β-lactoglobulin (β-lg) *(Guichard and Langourieux, 2000)*, have been frequently studied *(Damodaran and Kinsella, 1980b; Jasinski and Kilara, 1985; O'Neill and Kinsella, 1987b; Jung et al., 2002; Guth and Fritzler, 2004)*. β-Lg has been found to interact with several flavour compounds, such as esters *(Reiners et al., 2000)*, ketones *(O'Neill and Kinsella, 1987b; Jouenne and Crouzet, 2000a)*, ionones *(Lübke et al., 2002)*, and lactones *(Sostmann and Guichard, 1998; Guth and Fritzler, 2004)*. The number and location of flavour binding sites on β-lg are not entirely clear, but most hydrophobic flavour compounds appear

---

[2] Parts of this chapter have been published previously: Kühn, J., Zhu, X.-Q., Considine, T., and Singh, H. (2007). Binding of 2-Nonanone and Milk Proteins in Aqueous Model Systems. *Journal of Agricultural and Food Chemistry* **55** (9): 3599-3604 (see Appendix).

to bind in a central hydrophobic pocket, which also serves as the binding locus for apolar ligands, such as retinol *(Fugate and Song, 1980)* and long-chain fatty acids *(Wu et al., 1999; Ragona et al., 2000)*, and at least one secondary binding site exists on the protein surface *(Lübke et al., 2002)*. Studies on the flavour binding behaviour of α-lactalbumin (α-la) are rare because its flavour binding capacity was found to be low *(Jasinski and Kilara, 1985)*. In contrast, bovine serum albumin (BSA) seems to exhibit a strong affinity for different flavour compounds. BSA was found to have six *(Damodaran and Kinsella, 1980b)* and seven *(Jung et al., 2002)* binding sites for methyl ketones. *Guth and Fritzler (2004)* suggested one or two high-affinity binding sites, and a large number of lower affinity sites, for γ- and δ-lactones on BSA.

In contrast, the individual caseins have been neglected so far, probably because of their lower flavour binding *(Hansen and Booker, 1996; Li et al., 2000)*. Nevertheless, casein proteins are present and utilised in several food products *(Mulvihill, 1992)* and they do bind flavour compounds with measurable affinities *(Fischer and Widder, 1997; Li et al., 2000; Meynier et al., 2004)*. To date, there is no information about the number of binding sites and the binding constants for flavour compounds on individual caseins. Sodium caseinate, a mixture of all casein types, was found to possess one low-affinity binding site for vanillin at low temperatures *(Li et al., 2000)*.

The interactions between proteins and flavour compounds can have a strong influence on flavour perception, especially in reduced-fat products *(Hatchwell, 1996)*. Further insights into protein-flavour interactions are required to improve food flavour, particularly that of reduced-fat foods.

This chapter describes (1) the development of a headspace SPME method to investigate protein-flavour interactions, and (2) the binding of the hydrophobic flavour compound, 2-nonanone, to individual milk proteins (namely, β-lg, α-la, BSA, α_{s1}-casein, and β-casein), WPI, and sodium caseinate, using an optimised SPME-GC-FID method. Using the Klotz plot *(Klotz et al., 1946; Klotz and Urquhart, 1948)*, binding constants K and numbers of binding sites n on the proteins were determined. 2-Nonanone was chosen as the model flavour compound because it is known to be bound completely reversibly on proteins. In addition, 2-nonanone is the most frequently studied compound in protein-flavour binding studies, allowing better comparison with other studies.

To date, the SPME technique has not been applied to determine binding parameters for flavour compounds on proteins. This is the first study comparing flavour binding capacities of the major individual milk proteins, including the caseins, which have been neglected so far.

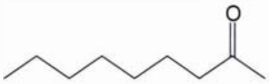
3.3 Materials and Methods

Distilled, deionised water (DDI) was obtained from a NANOpure™ II water purification system (Barnstead, Dubuque, IA, USA). Henceforth, the term "water" refers to DDI.

3.3.1 2-Nonanone

The flavour compound used was 2-nonanone, which represents hydrophobic flavour compounds. Its characteristics are shown in Table 3.3-1.

Table 3.3-1: Physicochemical properties of 2-nonanone.

Structure		
M (g·mol^{-1})	142.24	
Aqueous solubility (g·l^{-1}) (25 °C)	0.4	*Seuvre et al. (2001)*
Hydrophobicity (log P)$^{1)}$	2.9	*Rogacheva et al. (1999)*
Air-water partition coefficient (25 °C)	15×10^{-3}	*Buttery et al. (1969)*
	17×10^{-3}	*Overbosch et al. (1991)*
	$6.7 (\pm 0.1) \times 10^{-3}$	*Jung and Ebeler (2003a)*

1) Value of the logarithm of the partition coefficient between water and n-octanol.

2-Nonanone stock solution (10 mM)

A stock solution of 2-nonanone (Aldrich Chemical Co., Milwaukee, WI, USA) was prepared by dissolving 71.0 mg 2-nonanone in 50 ml propylene glycol (Bronson and Jacobs Pty Ltd., Sydney, Australia). Propylene glycol is a suitable solvent for flavour compounds because of its low vapour pressure, avoiding competition between 2-nonanone and the solvent in the fibre coating, which can be observed with other solvents such as ethanol. The stock solution was stored at 5 °C.

2-Nonanone standards (0.1-0.8 mM)

An external standard calibration was used to calculate the extent of binding. Standards of 0.1, 0.2, 0.4, 0.6, and 0.8 mM 2-nonanone were prepared in water from the 10 mM 2-nonanone stock solution.

3.3.2 Milk Proteins

WPI (Alacen 895) and sodium caseinate (Alanate 180) were obtained from Fonterra Co-operative Group Ltd., New Zealand. The WPI consisted of 93.3 % protein, 0.3 % fat and 4.6 % moisture, and the sodium caseinate consisted of 93.1 % protein, 0.6 % fat, and 4.8 % moisture. Individual whey proteins were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Lg AB from bovine milk is a lyophilised powder of approximately 90 % purity. BSA had been processed to reduce its fatty acid content to 0.002 %.

A 0.5 % (w/v) WPI solution and a 1.0 % (w/v) sodium caseinate solution were prepared in water by weighing 2.5 g and 5.0 g powder, respectively, into beakers and adding about 200 ml of water. The solutions were magnetically stirred for 1 h and filled up with water in a 500 ml volumetric flask. They were stored at 5 °C for 6 h to allow complete hydration before using them to prepare the protein-flavour mixtures. Solutions of the individual whey proteins (0.5 %, w/v) were prepared in water and were also stored at 5 °C for 6 h. The pH of the protein solutions was between 6.8 and 6.9.

There was no addition of preservative, such as sodium azide, as this was found to affect flavour binding by proteins *(O'Keefe et al., 1991b; Farès et al., 1998; Reiners et al., 2000).*

3.3.3 Composition of WPI

3.3.3.1 Determination of Individual Whey Proteins by HPLC

WPI (5 mg·ml^{-1}) was run on a Pharmacia Resource RPC column (Pharmacia, Uppsala, Sweden) (1 ml) as per method of *Elgar et al. (2000).*

3.3.3.2 Moisture Content

The moisture content of WPI was determined by loss on drying. Samples were accurately weighed into aluminium moisture dishes and dried overnight at 102 °C in an oven (Watvic oven, Watson Victor, New Zealand). After drying, the moisture dishes were allowed to cool in a dessicator before being weighed.

3.3.4 Preparation of Caseins

The individual caseins were prepared in the laboratory by making sodium caseinate from bovine milk and separating the caseins by ion-exchange chromatography as described below. The process of the manufacture of the single caseins from bovine milk is summarised in the simplified scheme in Figure 3.3-1.

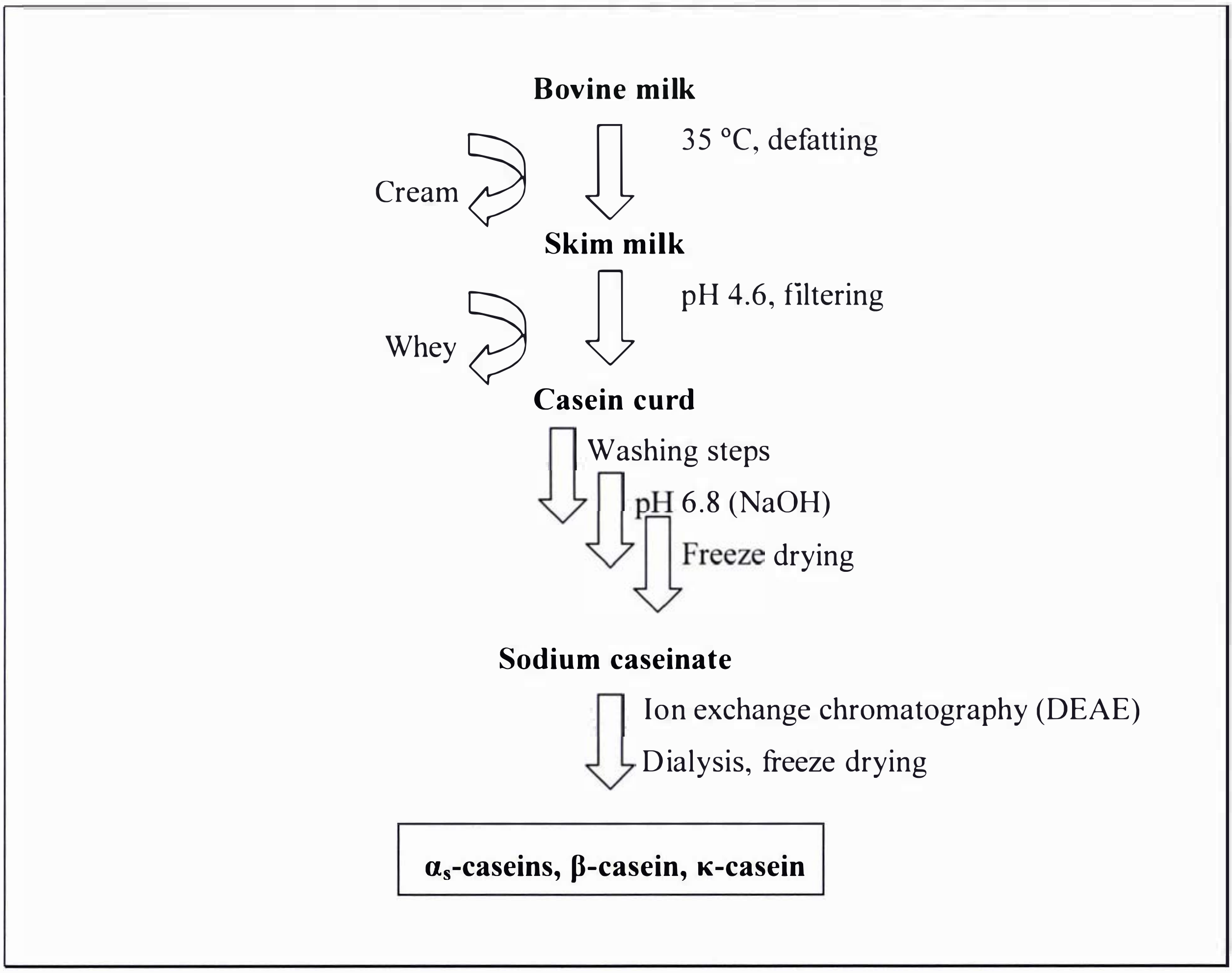


Figure 3.3-1: Simplified scheme of the isolation of the casein proteins from bovine milk.

3.3.4.1 Preparation of Sodium Caseinate

The procedure is based on the method used by *Mulvihill and Fox (1977)*.

Preparation of skim milk

About 5 litres of bovine milk (Fonterra Co-operative Group, Ltd., New Zealand) were heated to 35 °C. Subsequently the warm milk was centrifugally defatted at 2,500 g in a separator (Alfa-Laval Separator Co., Hamilton, New Zealand). The cream was discarded, and the skim milk kept at 5 °C until further used.

Precipitation of casein

Under stirring, the pH of the skim milk was adjusted to pH 4.6 using 2 M HCl. The milk was then held at 5 °C for 1 h, warmed to 30 °C in a water bath, and held at 30 °C for 30 min. The whey was filtered off through cheese-cloth and the curd washed three times with water. The washed curd was suspended in about 1 litre of water, and the pH of the suspension was again adjusted to pH 4.6 using 2 M HCl. For 1 h, the suspension was held at 5 °C, then warmed to 30 °C again and held for 30 min. Another time the suspension was filtered through cheese-cloth and washed three times with water.

Production of sodium caseinate

The casein was suspended in water to dissolve it by adjusting the pH to 6.8 with 2 M NaOH. The solution was then poured into a plastic bag, sealed, and frozen. The caseinate was freeze dried for 48 h.

Size exclusion chromatography (SEC)

The finished product was compared with commercial sodium caseinate (Alanate 180, NZMP, New Zealand) by SEC using both UV and refractive index (RI) detection. SEC was carried out on a GBC LC 1440 HPLC system (Melbourne, Australia) using a glass column (16 mm × 90 cm) packed with Sephacryl S-400 (Pharmacia, Uppsala, Sweden), at a flow rate of 0.2 $ml \cdot min^{-1}$ using 20 mM imizadole buffer as eluent at room temperature. A 1 ml sample of 1 % (w/v) sodium caseinate was injected and the eluate was monitored with an LC 1200 UV/VIS detector (GBC, Melbourne, Australia) at 280 nm and an RI detector RI 2000 (GBC, Melbourne, Australia).

3.3.4.2 Separation of Caseins by Ion Exchange Chromatography (IEC)

The method for the separation and purification of α_{s1}-, β-, and κ-caseins from sodium caseinate was based on the methods of *Thompson (1966)*, *Lawrence and Creamer (1969)* and *Creamer (1974)*. Using IEC, the sodium caseinate was separated into the individual casein fractions. The ion exchanger used was DE-52 (Whatman, Kent, UK), a weak anion exchanger based on the diethylaminoethyl (DEAE) tertiary amine functional group. The caseins were eluted using a salt gradient.

Preparation of solutions

Urea solution (4.5 M)

Under stirring, 881 g urea (BDH, Poole, UK) was dissolved in 3,000 ml water. This solution was divided to mix the buffers and to soak the beads as described below.

Soaking the beads

Imidazole (6.8 g) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 500 ml of urea solution (4.5 M). Then, 1.0 ml β-mercaptoethanol (BDH, Poole, UK) was added in the fume hood, and the pH of the solution was adjusted to pH 7.0 using 6 M HCl. About 130 g of DE-52 anion exchanger (Whatman, Kent, UK) was added, and the mixture was left for 30 min to completely hydrate the beads. The supernatant containing fines was removed so that the final volume remaining was the volume of the hydrated beads plus 20 %.

Buffer A

In the fume hood, the following reagents were mixed in a 2-litre beaker: 1.09 g imidazole, 1,600 ml of 4.5 M urea solution, and 1.6 ml β-mercaptoethanol. Using 1 M HCl, the pH was adjusted to pH 7.0. This buffer was made up fresh for immediate use, as the β-mercaptoethanol is susceptible to oxidation.

Buffer B (Salt buffer)

In a 2-litre beaker, 0.55 g imidazole and 23.4 g sodium chloride (BDH, Poole, UK) were dissolved in 800 ml urea solution (4.5 M). After adding 0.8 ml β-mercaptoethanol, the pH was adjusted to pH 7.0 using 1 M HCl.

Casein solution (10 %)

To 3.0 g of prepared sodium caseinate, 30 ml buffer A was added. The pH was adjusted to pH 7.0, and the mixture was stirred until the caseinate was completely dissolved (about one hour).

Set-up of the IEC system

Filling column

A glass column (16 mm × 90 cm) (Pharmacia, Uppsala, Sweden) was filled with water, above and under the filter. Air was removed around the filter by inverting the column (top sealed) and opening the valve at the bottom. This procedure was repeated a few times until all air was removed. The column was emptied to just above the filter mark, and the bead mix was slowly poured in. The bottom valve was completely opened to let the buffer run out and the resin settle. It is of great importance that the beads do not go dry. Buffer A was run through the column until the column bed height was constant.

Column equilibration

The set-up of the system is pictured in Figure 3.3-2. To equilibrate the resin before adding the sample, buffer A was run through the column at a flow rate of 2.0 ml·min^{-1} using a LC 1150 HPLC Pump (GBC, Melbourne, Australia). The pH was checked by collecting effluent from the bottom of the column. The pH has to read 7.0 to make sure the column is equilibrated and to get a good separation. About two to three bed volumes of buffer A were required to equilibrate the column.

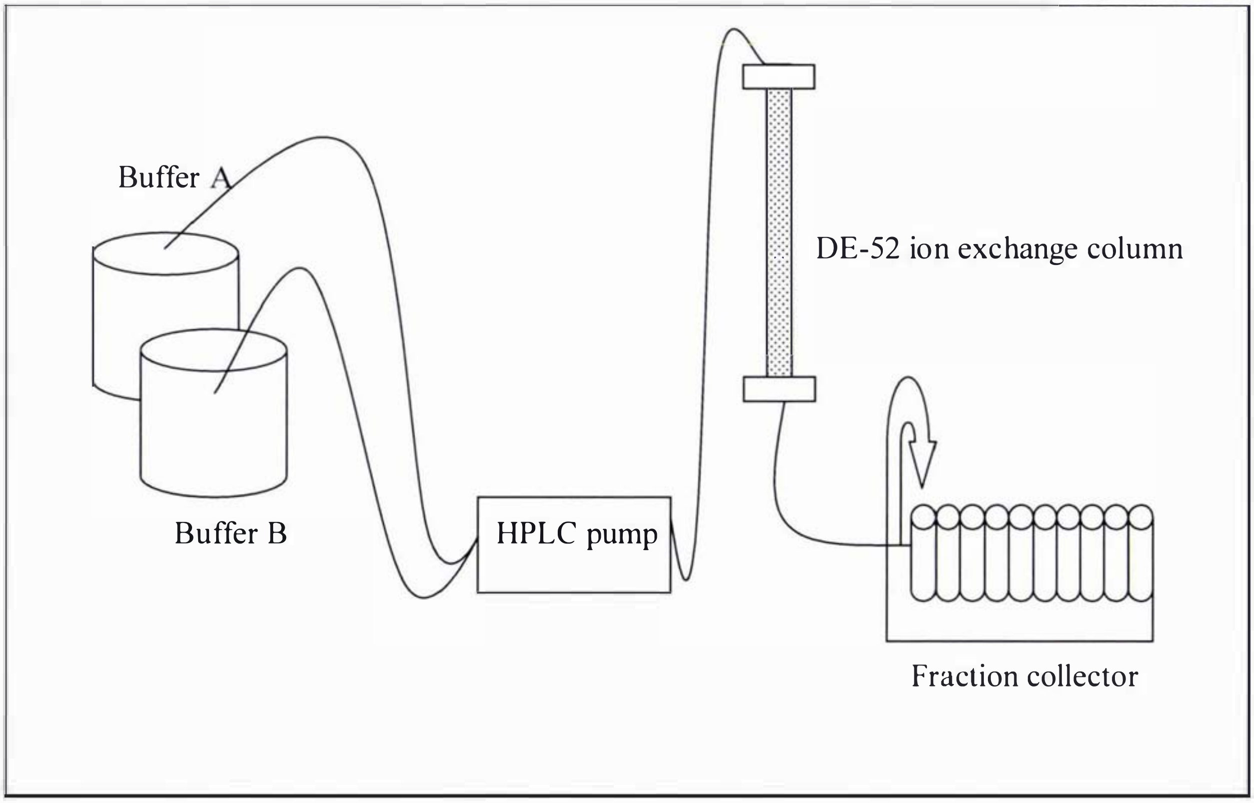


Figure 3.3-2: Set-up of the system for the casein separation using ion-exchange chromatography.

Sample addition

After the ion exchanger was equilibrated, the buffer flow to the column was shut off, and the column was drained until no buffer was present just on top of resin. It is important that the column does not run dry. Using a Pasteur pipette, the sample was slowly dripped on top of the column as not to disturb the resin bed. After most of the sample ran into the resin, buffer A was layered on until the column was filled. The gradient was set up as described below.

Gradient set-up

Using a LC 1150 HPLC pump (GBC, Melbourne, Australia), buffer A and buffer B were mixed producing a gradient from 0 % to 100 % buffer B in a total time of 26 hours.

Fraction collector

The first 200 ml of effluent were collected in a beaker, and stored in the refrigerator to use as a spectrophotometric blank. Then, the tubing from the bottom of the column was set to drip into the tubes placed in a Frac-100 Fraction Collector (Pharmacia, Uppsala, Sweden). The flow rate was 0.5 ml·min^{-1}, and 6 ml effluent was collected per test tube. 130 tubes were filled in 26 h.

Detection

The UV-VIS spectrophotometer used was a UV-160A (Shimadzu, Kyoto, Japan), and the quartz cells used were 1 cm (Starna Ltd., Essex, UK). All collected fractions were read on the UV spectrophotometer at $\lambda = 280$ nm. If required, they were diluted in buffer A. The first effluent from the column saved in the refrigerator was used as a spectrophotometric blank. Urea mini-gels (see section 3.3.4.3) were run on some test tubes believed to contain pure protein.

Regeneration of DEAE cellulose

The column was emptied using compressed air. Excess buffer was discarded, and beads were washed three times with water. The slurry was then suspended in three times its volume of 0.5 M HCl and left for 1 h, stirring the solution from time to time. Excess acid solution was discarded, and beads were washed with water until the pH reached 4.0. Having discarded the excess water, the slurry was then suspended in three times its volume of 0.5 M NaOH and left standing for 1 h under occasional stirring. Afterwards, it was washed with water until the pH reached pH 7.0.

Prior to pouring the column again, the slurry was suspended in three times its volume of buffer A and then the pH was again adjusted to pH 7.0. Excess buffer was discarded so that the final volume remaining is the volume of the hydrated beads plus 20 %. Now the ion exchanger was ready to be packed in the column again.

3.3.4.3 Polyacrylamide Gel Electrophoresis (PAGE)

According to the method of *Andrews (1983)* with modifications, urea PAGE gels were run using a vertical slab-gel system (BioRad, Hercules, CA, USA) to identify each casein and to verify the purity of the pooled fractions.

Separating gel buffer

Tris (hydroxymethyl) methyl amine ('Tris') (6.43 g) (BDH, Poole, UK), urea (38.57 g) (BDH, Poole, UK), and 572 µl conc. HCl were dissolved in water and made up to 100 ml. Using 6 M HCl, the pH was adjusted to pH 8.9.

Stacking gel buffer

'Tris' (0.83 g), urea (30 g), and 440 µl conc. HCl were dissolved in water and made up to 100 ml. Using 6 M HCl, the pH was adjusted to pH 7.4.

Sample buffer

The sample buffer contained 0.75 g 'Tris', 49 g urea, 0.4 ml conc. HCl, 0.7 ml β-mercaptoethanol (BDH, Poole, UK), and 0.15 g bromophenol blue (Merck, Darmstadt, Germany). It was filled up to 100 ml with water

Electrode buffer

The electrode buffer consisted of 3.0 g 'Tris' and 14.6 g glycine (BDH, Poole, UK), dissolved in water and made up to 1 litre (pH unadjusted).

Acrylamide solution (40 %)

Acrylamide (8.0 g) (Electran®, BDH, Poole, UK) was weighed in a glass bottle (use of gloves and mask) and filled up to 20 ml with water.

Pouring the gels

Separating gel (12.5 % T, 4 % C)

Acrylamide solution (40 %) (4.8 ml) was added to 11.2 ml separating gel buffer. Subsequently, 0.08 g Bis-N',N'-methylene-bis-acrylamide (BioRad, Hercules, CA, USA) was added. The above was filtered through Whatman No. 1 filter paper and degassed for 10 min prior to gelation. To initiate polymerisation, 60 µl of freshly prepared 10 % (w/v) ammonium persulphate (APS) (Sigma, St. Louis, MO, USA) and 8 µl of N,N,N',N'-tetramethyl-ethylenediamine (TEMED) (Sigma, St. Louis, MO, USA) were added.

The Protean II Xi electrophoresis unit (BioRad, Hercules, CA, USA) was assembled according to the manufacturer's instructions, and the separating gel solution was poured to within 1 cm of the bottom of the slot-former. Once poured, the gel was overlaid with water and allowed to polymerise for 45 min. When gelation had occurred, the water layer was removed.

Stacking gel (4 % T, 15.8 % C)

Acrylamide solution (40 %) (1.35 ml) was added to stacking gel buffer (14.65 ml). Subsequently, 0.10 g Bis-N',N'-methylene-bis-acrylamide was added. The above was filtered through Whatman No. 1 filter paper and degassed for 10 min prior to gelation. To initiate polymerisation, 96 μl of freshly prepared 10 % (w/v) APS and 8 μl of TEMED were added.

The stacking gel was then poured, and the wells were formed with the slot-former. Polymerisation of the stacking gel took approximately 30 min at room temperature.

Sample preparation

The casein samples were selected from the chromatogram in Figure 3.4-16 (page 89) and anlysed by electrophoresis, as these fractions were believed to contain relatively pure κ-, β-, α_{s2}-, and α_{s1}-casein, respectively. Each fraction (200 μl) was mixed with sample buffer (200 μl).

Of the casein standards, α_s-, β-, and κ-casein (Sigma-Aldrich, St. Louis, MO, USA), 5.0 mg of each were dissolved in 1.0 ml sample buffer.

Running the gels

Standards of α_s-, β-, and κ-casein and the samples were applied to the gel in aliquots of 10 μl and 15 μl, respectively. Samples were run at 250 V through the stacking gel (~ 10 min) and at 150 V through the separating gel until the tracking dye front was close to the bottom of the gel slab (~ 1.5 h). The gels were immediately removed from the plates and placed in 12.5 % (w/v) trichloroacetic acid (TCA) (AnalaR®, BDH, Poole, UK) for fixation of the protein bands. After 10 min, an aqueous solution of 0.25 % (w/v) G-250 Coomassie Blue (Sigma, St. Louis, MO, USA) was added and left mixing for 30 min. The stain was then poured off, and destaining was achieved in 10 % (v/v) acetic acid, which was changed as required until the background became clear.

The tubes containing the purest fractions were then pooled: κ-casein, β-casein, α_{s1}-casein. It was not possible to isolate α_{s2}-casein because it co-eluted with β-casein. Each solution was then dialysed in a Spectra/Por®1 membrane tubing, MCO 6,000-8,000 (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) for two days in water in the cold room, changing the water five times. Each pure casein fraction was freeze-dried over two days, and then stored at -20 °C.

To check the purity of the pooled fractions, they were re-run on mini-urea gels (procedure as above). Each fraction (2.5 mg) was dissolved in 500 µl sample buffer, and 10 µl were loaded on the gel. Casein standards were also loaded.

Casein solutions (0.5 and 2.0 %)

Bovine α_s-casein and β-casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). In addition, the individual caseins were prepared in the laboratory as described in section 3.3.3. Because of the low proportion of κ-casein in total casein, only α_{s1}-casein and β-casein were examined regarding their affinity for 2-nonanone. The caseins were dissolved at 0.5 % and 2.0 % (w/v) in water under gentle magnetic stirring (~ 100 rpm).

3.3.5 UV Spectroscopy

The protein concentrations in the solutions of the individual whey proteins and caseins were determined by UV spectroscopy using an Ultrospec II UV-VIS spectrophotometer (Pharmacia LKB Biochrom, Cambridge, UK). The samples were measured in a 1 cm quartz cell (Starna Ltd., Essex, UK) at 280 nm against water as the reference. The α-la, β-lg, and BSA solutions were diluted 1:15, 1:5, and 1:5 with water, respectively. Both the α_{s1}- and β-casein solutions were diluted 1:10 and 1:5 for the 0.5 % solutions, respectively, and 1:40 and 1:20 in the case of the 2.0 % solutions, respectively, in water. Protein concentrations were calculated using the extinction coefficients (ε) given in Table 3.3-2 and a modified law of Lambert and Beer (Equation 3-1). The absorbance at 320 nm was used to correct for light scattering due to any turbidity and the factor 1.7 was derived from the Rayleigh approximation ($I \sim 1 / \lambda^4$).

Table 3.3-2: Spectroscopic data of aqueous solutions of the main whey proteins and caseins (1 %).

Protein	Wavelength λ (nm)	Absorptivity $A^{1\%}_{1cm}$	Extinction coefficient ε ($l \cdot mol^{-1} \cdot cm^{-1}$)	Reference
β-Lg	278	-	17,600	*Dufour et al. (1990)*
α-La	278	20.8	29,482 1)	*McKenzie and Murphy (1970)*
BSA	279	6.67	44,200 1)	*Foster and Sterman (1956)*
α_{s1}-Casein	280	10	23,000 1)	*McKenzie and Murphy (1970), Swaisgood (1992)*
β-Casein	280	4.6	11,086 1)	*McKenzie and Murphy (1970), Swaisgood (1992)*

1) Calculated from the absorptivity $A^{1\%}_{1cm}$.

$$c = \frac{A_{280nm} - 1.7 \cdot A_{320nm}}{\varepsilon \cdot d} \cdot F \qquad \textbf{(3-1)}$$

c	Protein concentration ($\mathrm{mol \cdot l^{-1}}$)
A_{280nm}	Absorptivity at 280 nm
A_{320nm}	Absorptivity at 320 nm
1.7	Factor derived using Rayleigh approximation
ε	Extinction coefficient ($\mathrm{l \cdot mol^{-1} \cdot cm^{-1}}$)
d	Diameter of quartz cell (d = 1 cm)
F	Dilution factor

3.3.6 Preparation of Protein-Flavour Solutions

After the protein solutions had been stored for 6 h at 5 °C, all protein solutions and the 2-nonanone stock solution were brought to room temperature before the mixtures were prepared. At room temperature, the protein solutions were mixed with the 2-nonanone stock solution to obtain final 2-nonanone concentrations of 0.1-0.8 mM (~ 14-114 ppm). 2-Nonanone can be found in this concentration range in real foods, e.g., it has been detected at 33 ppm in blue cheese *(Dwivedi and Kinsella, 1974)*.

The protein-flavour solutions were stored at 5 °C for 40 h to allow equilibration of 2-nonanone between the free state and the protein-bound state prior to the SPME analysis. Equilibration at 5 °C was found to be complete after 40 h *(Zhu, 2003)*.

The protein-flavour samples and flavour standards were then brought to room temperature and analysed by the optimised headspace SPME method (section 3.4.1.6) and 2-nonanone was quantified using GC-FID (section 3.3.8).

3.3.7 Development of a Headspace SPME Method

The SPME method for the determination of 2-nonanone in the headspace is based on the method by *Zhu (2003)*, and was further optimised in this study, in terms of the type and thickness of the SPME fibre coating, the headspace extraction time t_{ex}, the thermal equilibration time t_{eq}, the flavour solvent, and sample agitation during extraction. Linearity and precision were also tested.

3.3.7.1 SPME Equipment

The SPME holder for manual sampling and the SPME fibres were purchased from Supelco (Bellefonte, PA, USA). The fibres were conditioned in the gas chromatograph injector port before use at the time and temperature recommended by the manufacturer.

For the 2-nonanone standards and the protein-flavour solutions, 400 μl aliquots were transferred into 4 ml glass vials (Alltech, Deerfield, IL, USA). The standards and the samples containing individual whey proteins were prepared in triplicates, individual caseins in duplicates, sodium caseinate in four replicates, and WPI in five replicates. The sample vials were sealed with TFE/silicone combination liners and open hole screw thread caps (Alltech, Deerfield, IL, USA) and placed in a water bath equipped with a TE-10A thermostat (Techne, Cambridge, UK) to keep the temperature constant at 25 °C. For agitated samples, an 8 mm × 3 mm Azlon SWN 500 magnetic stir bar (Bibby Sterilin Ltd., Stone, UK) was placed in each sample vial, and the samples were stirred at 250, 500, or 750 min^{-1} during the SPME extraction using an MR 2002 magnetic stirring plate (Heidolph, Schwabach, Germany) under the water bath. After equilibration, the SPME fibre was exposed into the headspace of the sample vial for a definite time and was subsequently introduced into the gas chromatograph injector port for quantification (see section 3.3.8).

It has to be emphasized, that in SPME neither complete extraction of analytes nor full equilibrium is necessary, but consistent sampling temperature, constant sample volumes, and especially constant sampling times are critical for high accuracy and precision in SPME.

3.3.7.2 Fibre Selection and Determination of Extraction Time (t_{ex})

The extraction profiles of the following fibre coatings for 2-nonanone were determined to compare their affinity for the flavour compound, and to determine the optimum extraction times: polydimethylsiloxane (PDMS) (30 μm), PDMS (100 μm), and Carboxen/PDMS (85 μm). According to Supelco, they are recommended for non-polar semi-volatiles (MW 80-500), for volatiles (MW 60-275), and for gases and low molecular weight compounds (MW 30-225), respectively.

A 2-nonanone solution (0.6 mM) was prepared by diluting 1.5 ml of 2-nonanone stock solution (10 mM) in 25 ml water. The vials containing 400 μl of the 2-nonanone standard solution (0.6 mM) were equilibrated for 1 h at 25 °C in a water bath. Subsequently, the headspace was extracted for different times between 30 sec and 40 min using each of the above fibres.

Fibre Coating Thickness

Since headspace analysis is based on the equilibrium between different phases, it is important to investigate how much flavour is extracted during the SPME extraction because the equilibrium would be changed considerably if too much flavour was removed. For this reason, the headspace of one and the same vial containing 400 µl of a 0.6 mM 2-nonanone solution was extracted twice with 15 min between the two extractions. Any flavour losses through the hole in the septum between the two extractions were accounted for by piercing the septa of a sample vial containing the 2-nonanone solution, and determining the 2-nonanone lost after 15 min. The septa used are supposed to have good resealability according to the manufacturer; however, the large diameter of the SPME piercing needle might impair the resealability slightly.

From the average peak areas of the two extractions, and the extraction after piercing the septa, it was calculated how much of the 2-nonanone initially present in the sample solution was extracted by both the 30 µm and the 100 µm PDMS fibres.

Determination of the Fibre-Headspace Partition Coefficient (K_{fh})

The fibre-headspace partition coefficient K_{fh} for 2-nonanone on the PDMS fibre was estimated using two different approaches.

Approach 1:

The first calculation required the value of the headspace-water partition coefficient K_{hw}, a parameter describing the distribution of volatile compounds between the gas phase and water. It also required the amount of 2-nonanone extracted using the 30 µm PDMS fibre and an extraction time of t_{ex} = 5 min, which was obtained from the experiment above.
Since the total amount of analyte in the three-phase system remains the same during the extraction as the initial amount, we have

$$c_0 V_w = c_f^{\infty} V_f + c_w^{\infty} V_w + c_h^{\infty} V_h \tag{3-2}$$

(Pawliszyn, 1997), where

c_0	initial flavour concentration in the aqueous solution
c_f^{∞}, c_w^{∞}, c_h^{∞}	equilibrium flavour concentrations in the fibre coating, the aqueous solution, and the headspace, respectively

V_f, V_w, V_h volumes of the fibre coating, the aqueous solution, and the headspace, respectively

With the fibre-headspace partition coefficient being $K_{fh} = \frac{c_f^{\infty}}{c_h^{\infty}}$,

the headspace-water partition coefficient of 2-nonanone being $K_{hw} = \frac{c_h^{\infty}}{c_w^{\infty}}$,

and the amount of analyte adsorbed by the fibre coating being $n_f = c_f^{\infty} V_f$,

Equation 3-2 can be expressed as follows and used for the calculation of K_{fh}:

$$K_{fh} = \frac{n_f (K_{hw} V_h + V_w)}{K_{hw} V_f (c_0 V_w - n_f)} \qquad \textbf{(3-3)}$$

Approach 2:

The second approach was based on the comparison of conventional headspace sampling using a gas-tight syringe, and SPME sampling of the same sample headspace. The partition coefficient of 2-nonanone between the PDMS fibre coating and the headspace above the flavour solution K_{fh} was determined by sampling the headspace of a 2-nonanone solution (1.0 mM) with both the SPME fibre and a 1 ml gas-tight syringe (Hamilton, Reno, NV, USA). An aliquot of 300 µl was taken with the syringe. The 30 µm PDMS fibre was exposed for 5 min. Equilibrium between the fibre and the headspace was found to be reached after this time, as seen from the extraction profiles (Figure 3.4-1, page 69). Samples were analysed by GC-FID (section 3.3.8).

Zhang and Pawliszyn (1993) and *Chai and Pawliszyn (1995)* determined K_{fh} this way. They analysed the headspace of an analyte solution by both SPME extraction and conventional headspace extraction with a gas-tight syringe and calculated K_{fh} using the following equation:

$$K_{fh} = \frac{c_f^{\infty}}{c_h^{\infty}} = \frac{n_f^{\infty} \cdot V_h}{n_h^{\infty} \cdot V_f} = \frac{A_f^{\infty} \cdot V_h}{A_h^{\infty} \cdot V_f} \qquad \textbf{(3-4)}$$

c_f^{∞}, c_h^{∞}	analyte concentration in the fibre coating and in the headspace at equilibrium, respectively
n_f^{∞}, n_h^{∞}	number of moles extracted by the fibre coating, and number of moles of the analyte in the headspace sample, at equilibrium, respectively
A_f^{∞}, A_h^{∞}	peak areas from the GC corresponding to the fibre coating injection and headspace injection, at equilibrium, respectively
V_f, V_h	volume of the fibre coating ($V_f = 0.132$ µl; *Boey (2003)*) and of the gas sample injected ($V_h = 300$ µl), respectively.

Because the ratio of n_f^{∞} and n_h^{∞} is proportional to the analyte peak area from the chromatogram, the partition coefficient of an analyte can be calculated easily *(Chai and Pawliszyn, 1995)*.

Determination of the Headspace-Water Partition Coefficient (K_{hw})

2-nonanone (27.3 mg) was dissolved in 100 ml water by sonicating at room temperature for one hour. The flavour solution was diluted 1:4 to give a concentration of about 0.5 mM 2-nonanone, and aliquots of 400 µl were transferred into 4 ml vials and sealed. The value of K_{hw} was determined using static headspace sampling after the vials were equilibrated at 25 °C. The sample headspace (800 µl) was injected into the GC-FID using a 1 ml gas-tight syringe (Hamilton, Reno, NV, USA). Of the liquid sample, 5 µl were injected using a 10 µl syringe (Hamilton, Reno, NV, USA). The headspace-water partition coefficient was calculated using Equation 3-5.

$$\mathrm{K_{hw}} = \frac{c_h^{\infty}}{c_w^{\infty}} = \frac{n_h^{\infty} \cdot V_w}{n_w^{\infty} \cdot V_h} = \frac{A_h^{\infty} \cdot V_w}{A_w^{\infty} \cdot V_h} \qquad \textbf{(3-5)}$$

c_h^{∞}, c_w^{∞}	analyte concentration in the headspace and in the water at equilibrium, respectively
n_h^{∞}, n_w^{∞}	number of moles of the analyte in the headspace and in the water, at equilibrium, respectively

A_h^∞, A_w^∞	FID peak areas corresponding to the headspace injection and aqueous injection, at equilibrium, respectively
V_h, V_w	volume of the headspace sample (V_h = 800 µl) and aqueous sample injected (V_w = 5 µl), respectively.

3.3.7.3 Determination of the Thermal Equilibration Time (t_{eq})

The optimum thermal equilibration time t_{eq} of the sample vials in the water bath without stirring was determined. An equilibration temperature of 25 °C was chosen for this study because most of the binding parameters in the literature were determined at 25 °C so the comparison with existing data is easier.

Aliquots (400 µl) of a solution containing 0.5 % WPI and 0.8 mM 2-nonanone were transferred into 4 ml vials. The sample vials were stored in the fridge and then equilibrated for different times (0-60 min) in the water bath at 25 °C. After headspace SPME, the extracted 2-nonanone was quantified by GC-FID (see section 3.3.8).

3.3.7.4 Solvent Selection

Due to the low solubility of 2-nonanone in water (0.4 $g \cdot l^{-1}$) *(Seuvre et al., 2001)*, a suitable flavour solvent had to be found. *Zhu (2003)* used 50 % (v/v) ethanol as the solvent for 2-nonanone. However, ethanol has some disadvantages, that is, it has a high vapour pressure which makes it compete with the analyte in the fibre coating. *Grote and Pawliszyn (1997)* showed that ethanol can replace acetone and isoprene on a PDMS/DVB fibre. Besides, ethanol is known to induce changes to the secondary structure of β-lg and to alter its affinity for retinol at ethanol concentrations above 30 % *(Dufour and Haertlé, 1990b)*. *Zhu (2003)* concluded that ethanol concentrations below 4 % do not have any significant effect on protein-flavour binding. Another disadvantage of ethanol is that it can not be used in certain foods.

Propylene glycol (1,2-propanediol), a common flavour solvent *(Hansen and Heinis, 1992; Schirle-Keller et al., 1994; Fabre et al., 2002; Mei et al., 2004)*, is available in food grade quality. It has a low vapour pressure (0.11 hPa at 20 °C) and will thus not be absorbed by the fibre coating in such an extent as ethanol. Propylene glycol was therefore compared with ethanol, and any effects on the SPME extraction and on the binding of 2-nonanone to milk proteins were determined.

Ethanol (p.a., min. 99.8 %) was obtained from Merck (Darmstadt, Germany). Propylene glycol (food grade) was purchased from Bronson and Jacobs Pty. Ltd. (Sydney, Australia). The effect of the flavour solvent was examined by comparing the binding of 2-nonanone to WPI (0.5 %) and to sodium caseinate (1.0 %) using both 2-nonanone stock solutions in 50 % ethanol and in 100 % propylene glycol, respectively, to prepare the protein-flavour mixtures and flavour standards. 2-nonanone is only very slowly soluble in 50 % propylene glycol. Therefore, the stock solution was prepared in pure propylene glycol. The extent of binding was determined at a 2-nonanone concentration of 0.2 mM.

3.3.7.5 Sample Agitation during SPME Extraction

In headspace SPME, the mass transfer from the solution to the headspace can be speeded up rapidly by constantly stirring the aqueous sample to generate a continuously fresh surface *(Zhang and Pawliszyn, 1993)*. This enhances extraction and reduces extraction time and thus analysis time.

It was tested how agitation of a 2-nonanone containing sample during the SPME extraction would affect the extraction speed and reproducibility. Inconsistent stirring can cause poor precision and can be worse than no stirring *(Supelco, 1998)*. To ensure that inconsistent stirring does not have an effect on the resulting peak area it was tested if the stirring speed has an influence on the amount of flavour adsorbed by the fibre coating.

The headspace of a 0.6 mM solution of 2-nonanone was extracted for 5 min using different stirring speeds, 250, 500, and 750 min^{-1}, on an MR 2002 magnetic stirring plate (Heidolph, Schwabach, Germany) under the water bath, and 8 mm × 3 mm Azlon SWN500 magnetic stir bars (Bibby Sterilin Ltd., Stone, UK) in the vials. The measurements were done in duplicate and the peak areas were compared.

To find out to what extent stirring reduces the optimum SPME extraction time, extraction curves were determined using different extraction times and stirring speeds of 250 and 750 min^{-1}, as well as no stirring. For this experiment the same 0.6 mM 2-nonanone solution was used.

3.3.8 Gas Chromatography

A Shimadzu GC-17A gas chromatograph coupled with an FID detector (Shimadzu, Kyoto, Japan) was used throughout the study. The column used was a Supelcowax™ 10 fused silica capillary column, 30 m, 0.32 mm inner diameter, 0.50 µm film thickness (Supelco, Bellefonte, PA, USA). The carrier gas was helium (linear velocity = 40 $\text{cm}\cdot\text{s}^{-1}$). Silicone rubber septa (Shimadzu, Kyoto, Japan) were used in the injector. The injection port (splitless mode) temperature and the detector temperatures were 250 °C. The oven temperature was held isothermally at 120 °C. Data acquisition was achieved using Class-VP™ Chromatography Data System Software (Shimadzu, Columbia, MD, USA).

Once the SPME sampling was completed, the fibre was immediately inserted into the gas chromatograph injector for desorption. The fibre was left in the port for 5 min for purging. There was no carry-over between samples using a 5 min desorption time. Prior to the next SPME extraction, the fibre was allowed to cool to room temperature. This is very important as the amount of analytes extracted by a PDMS fibre highly depends on the temperature *(Grote and Pawliszyn, 1997)*. A blank sample verified that, during the cooling down time of 5 min, no absorption of volatiles from the air occurred.

3.3.9 Determination of Binding Parameters

The binding constant (K) and the number of binding sites on the proteins (n) were determined using the Klotz plot (see section 2.2.1.5.2). The values of $[L]$ were calculated from the FID peak area using the standard curve, and the values of v were calculated using the equation

$$v = \frac{[L]_{tot} - [L]}{[P]} \qquad \textbf{(3-6)}$$

where $[L_{tot}]$ is the total 2-nonanone concentration in the sample (free and bound) ($mol \cdot l^{-1}$) and $[P]$ is the protein concentration in the sample as determined by UV spectroscopy ($mol \cdot l^{-1}$). For the calculation of the average binding parameters, average molecular masses 18,000 and 22,000 Da were assumed for WPI and sodium caseinate, respectively. The protein contents of 95 % and 93 % in WPI and sodium caseinate, respectively, were also considered in the calculation. For the individual milk proteins, the protein concentrations determined by UV spectroscopy were used.

3.3.10 Statistical Analysis

Whey protein samples were analysed in triplicates, individual caseins in duplicates, sodium caseinate in four replicates, and WPI in five replicates. The standard errors (SE) of K, n, and nK were estimated from the standard errors of the intercept $1/n$ ($1/n = a$) and the slope $1/nK$ ($1/nK = b$) of each Klotz plot which were obtained using SPSS version 14.0 (SPSS, Chicago, IL, USA). The variances (*var*) were estimated using Equations 3-7, 3-8, and 3-9, and SE = √*var*.

$$\mathrm{var}(n) \approx \frac{SE_a^{\ 2}}{a^4} \qquad \textbf{(3-7)}$$

$$\mathrm{var}(K) \approx \left(\frac{a}{b}\right)^2 \left(\frac{SE_a^{\ 2}}{a^2} + \frac{SE_b^{\ 2}}{b^2} - \frac{2 \cdot \mathrm{cov}(a,b)}{a \cdot b}\right) \qquad \textbf{(3-8)}$$

$$\mathrm{var}(nK) \approx \frac{SE_b^{\ 2}}{b^4} \qquad \textbf{(3-9)}$$

3.4 Results and Discussion

3.4.1 Optimisation of the SPME Method

3.4.1.1 Fibre Selection and Determination of Extraction Time (t_{ex})

Non-polar analytes are most effectively extracted with a non-polar fibre coating and polar analytes are most effectively extracted with a polar coating. For a non-polar flavour compound, such as 2-nonanone, a fibre coating containing PDMS should be suitable since PDMS is non-polar *(Supelco, 1998)*. For the analysis of 2-nonanone, PDMS has been found to be suitable *(Zhu, 2003)*, and was therefore used in this study. In addition, a Carboxen (CAR)/PDMS (85 µm) fibre was compared with PDMS fibres (30 µm and 100 µm) in terms of its affinity for 2-nonanone.

A very important step in the development of an SPME method is the determination of the time needed for the analyte to reach equilibrium between the sample headspace and the fibre. This time can be determined from extraction time profiles. The extraction time profiles of the different fibres were established by plotting the FID detector response versus the extraction time (Figure 3.4-1).

Compared to both PDMS fibres, the 85 µm CAR/PDMS fibre had a very high affinity for 2-nonanone. Even after 10 min there was still a great increase in FID response. This fibre extracts a very high amount flavour from the sample headspace and thus may change the overall equilibrium in the system. This is not desired since for the determination of binding constants, equilibrium conditions are necessary. In addition, the 2-nonanone peak, after desorption from the CAR/PDMS fibre, showed tailing in the FID chromatogram, whereas the peaks after the extraction with the PDMS fibres were sharp. The CAR/PDMS fibre would probably be suitable for analysing traces of 2-nonanone. It would show very low detection limits for compounds like 2-nonanone.

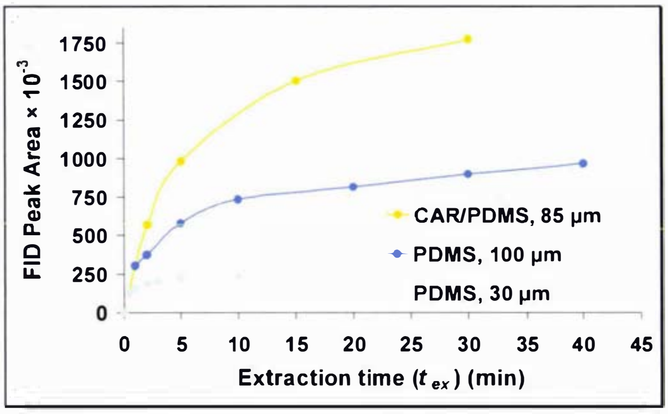


Figure 3.4-1: Extraction time profiles of different SPME fibre coatings for 2-nonanone (0.6 mM).

The amount absorbed by the fibre coating increases rapidly at the beginning and then slowly levels off. During the extraction process there is a "turning point" in the curves, as shown in Figure 3.4-1. This turning point is caused by the rapid diffusion process in the headspace and the very slow diffusion in the aqueous phase *(Zhang and Pawliszyn, 1993)*. Prior to the "turning point", analytes in the headspace quickly diffuse to the fibre coating, causing a rapid increase in FID response. Since the headspace is continually exhausted from analytes, analytes are released from the aqueous phase into the headspace in order to attain equilibrium. Due to their slow diffusion in the aqueous phase, there is a slow and continuous increase in FID peak area after the "turning point".

The optimum equilibration time t_{eq} is reached when a further increase of the extraction time does not result in a significant increase in the detector response *(Grote and Pawliszyn, 1997)*. This is near the turning point. At this point, equilibrium between the headspace and fibre is not yet reached because the fibre has extracted a considerable amount of flavour from the system, causing flavour being released from the sample into the headspace, so that a slower increase in peak area occurs at longer equilibration times.

For the 100 µm PDMS fibre, the turning point is at about 10 min. In the case of the 30 µm PDMS fibre, the turning point is already at about 5 min and the increase in peak area at longer equilibration times is slower, which means this fibre does not extract very much flavour from the sample.

Optimum extraction times seem to be 10 min in the case of the 100 µm PDMS fibre, and only 5 min using the 30 µm PDMS fibre. It has to be considered that an extraction time of 10 min using the 100 µm fibre would result in a very high amount of 2-nonanone being removed from the sample. Extraction at the turning point makes sure that hardly any protein bound flavour is released, absorbed by the fibre, and measured.

An extraction time of 30 sec with the 30 µm fibre, used by *Zhu (2003)*, appears to be too short as the adsorption rate of 2-nonanone to the fibre is still very high at 30 sec extraction time. The peak area around this time changes extremely quickly with almost 1 % per sec and it is difficult to keep the extraction time exactly 30 sec when SPME extraction is performed manually. Using an extraction time of 5 min will result in a better precision, and exact timing is easier to realise.

Fibre Coating Thickness

As indicated by Equation 3-10 *(Pawliszyn, 1997)*, the amount of analyte extracted by the fibre coating (n_f) is directly proportional to the volume of the coating.

$$n_f = \frac{K_{fw} V_f c_0 V_w}{K_{fw} V_f + K_{hw} V_h + V_w} \qquad \textbf{(3-10)}$$

c_o	initial flavour concentration in the aqueous solution
K_{fw}, K_{hw}	equilibrium flavour concentrations in the fibre coating, the aqueous solution, and the headspace, respectively
V_f, V_h, V_w	volumes of the fibre coating, the headspace, and the aqueous solution, respectively

A thick fibre coating will extract more of a given analyte than will a thin coating. The right choice of the coating thickness of the fibre used is very important as a thick coating might adsorb a too high amount of analyte from the headspace, so that the equilibrium between the sample and headspace, and consequently the equilibrium between the protein bound and free flavour, will be changed.

The PDMS coating was found to be very suitable for the measurement of interactions between 2-nonanone and milk proteins *(Zhu, 2003)*. This type of fibre is available with a coating thickness of 7, 30, and 100 µm. The 7 µm coating was found to be too thin for this application as the fibre was saturated with 2-nonanone already at moderate concentrations of the flavour compound *(Zhu, 2003)*. It needs to be tested, if the 100 µm fibre is suitable for this method by determining how much flavour is extracted from the headspace. The 30 µm fibre might be more suitable as it extracts a smaller amount.

It was calculated how much of the 2-nonanone initially present in the sample solution was extracted by both the 30 µm and the 100 µm PDMS fibres (Table 3.4-1). By using the 100 µm fibre and an extraction time of 5 min, 23 % of the 2-nonanone initially present in the sample solution was extracted. This is a very high amount. The extraction time of 5 min is actually too short as the optimum t_{ex} lies around 10 min (Figure 3.4-1) which would result in an even higher amount of 2-nonanone extracted. Using a fibre that extracts a high amount of flavour from the system is not preferable, as the fibre can induce additional volatilisation of compounds from the sample into the air, which the fibre can absorb.

Table 3.4-1: Amount of 2-nonanone extracted by PDMS fibres.

PDMS fibre coating thickness	**100 µm**	**30 µm**	**30 µm**
Extraction time t_{ex} (min)	**5**	**5**	**0.5**
2-nonanone extracted (%)	**23 ± 2**	**13 ± 2**	**5 ± 1**

For the 30 µm fibre and an extraction time of 5 min this amount was found to be about 13 %. Using the 30 µm fibre and t_{ex} = 0.5 min, only about 5 % of the 2-nonanone initially present in the sample were extracted. This difference is not very large, and considering that the standard deviations may be lower for t_{ex} = 5 min, an extraction time of 0.5 min only has the advantage of faster analysis. Although 13 % is a substantial amount of 2-nonanone to be removed, an extraction time of 5 min would hardly change the equilibrium between free and protein bound flavour since kinetics of flavour interactions with proteins are slow *(Graf and de Roos, 1996)*.

In conclusion, the 30 µm PDMS fibre and an extraction time t_{ex} of 5 min were used for the protein-flavour binding experiments.

3.4.1.2 Fibre-Headspace Partition Coefficient (K_{fh})

From the partition coefficient between the fibre coating and the sample headspace, K_{fh}, the affinity of a fibre coating for a particular flavour compound can be estimated. Values of K_{fh} can be very helpful when selecting a fibre in the development of an SPME method. There are not many values published so far. Two approaches were used to calculate K_{fh} for 2-nonanone on PDMS.

Approach 1:

For the first approach, K_{hw} and the amount of 2-nonanone extracted by the fibre n_f, which can be calculated from the results in section 3.4.1.1, were used for the estimation of K_{fh}, and Equation 3-3 (page 63) was applied. The following parameters were included in the calculation:

n_f	0.0302 µmol (calculated from the results in section 3.4.1.1)
K_{hw} (2-nonanone)	$(6.3 \pm 0.3) \times 10^{-3}$
V_h	4.6 ml
V_w	0.4 ml
V_f	1.32×10^{-4} ml (calculated from the core diameter of the fibre of $d_f = 110$ µm *(Boey, 2003)*)
c_o	0.6 mM = 600 µM

In this study the value of K_{hw} for 2-nonanone was found to be $(6.3 \pm 0.3) \times 10^{-3}$. This value is in very good agreement with the value found by *Jung and Ebeler (2003a)*, but values in the literature vary significantly (Table 3.3-1, page 50) and thus influence the calculated value of K_{fh}.

The partition coefficient between fibre *(f)* and headspace *(h)* K_{fh} was calculated using the K_{hw} value determined in this study:

$$K_{fh} = \frac{n(K_{hw}V_h + V_w)}{K_{hw}V_f(C_0V_w - n)} \qquad \textbf{(3-11)}$$

$$K_{fh} = \frac{0.0302\mu mol(6.3\times10^{-3}\times4.6ml + 0.4ml)}{6.3\times10^{-3}\times1.32\times10^{-4}ml(600\mu M\times0.4ml - 0.0302\mu mol)} = \mathbf{74{,}255}$$

When a higher value of K_{hw} is used, e.g., 15 × 10^{-3} *(Buttery et al., 1969)*, the value of K_{fh} is drastically reduced:

$$K_{fh} = \frac{0.0302\mu mol(15\times10^{-3}\times4.6ml+0.4ml)}{15\times10^{-3}\times1.32\times10^{-4}ml(0.6mM\times0.4ml-0.0302\mu mol)} = \mathbf{34{,}096}$$

In this calculation it also has to be considered that it is not correct to use the literature value of K_{hw} because propylene glycol is present at 10 %, which would reduce the concentration of 2-nonanone in the headspace slightly, resulting in a higher K_{fh} value. For an estimation of K_{fh} the value of K_{hw} can be used.

From the value of K_{fh}, the partition coefficient between the fibre and the aqueous solution K_{fw} results in:

$K_{fw} = K_{fh} \times K_{hw} =$ **511** *(Buttery et al., 1969)*

468 *(This study)*

Both the total partition coefficients K_{fw} are very similar and quite large, which confirms that PDMS has a very high concentrating effect on 2-nonanone and leads to good sensitivity. K_{fw} values for BTEX on PDMS were found to be between 126 and 831 *(Zhang and Pawliszyn, 1993)*. Since both K_{fw} values are very similar it can be concluded that they are a better measure for the affinity of a flavour for a fibre coating than the value of K_{fh} which does not depend on the value of K_{hw}.

The way K_{fh} was determined in this approach is fairly complicated and probably not very accurate as a number of parameters are included in the calculation, which could make the final value inaccurate. For this reason, the second approach (see page 74) is considered the more adequate because only four parameters, which are measured directly, are included in the calculation of K_{fh} (see Equation 3-12). When comparing the K_{fh} values above with the one found in the literature (Table 3.4-2), the value found by *Jung and Ebeler (2003a)* is about four times higher though, which could be due to the different method they used (linear temperature programmed retention index, LTPRI). LTPRI is a gas chromatographic method using a column coated with the same material as the SPME fibre *(Pawliszyn, 1997)*. The equation they used to estimate K_{fh} was generated using a GC column that contained 5 % phenyl PDMS, which might have increased the retention of 2-nonanone.

Table 3.4-2: Partition coefficients for flavour compounds between the PDMS fibre coating and the headspace above the flavour solution *(Roberts et al., 2000; Jung and Ebeler, 2003a)* calculated by LTPRI *(Pawliszyn, 1997)*.

Flavour compound	$K_{\text{PDMS-headspace}}$
3-methylbutanal	270
ethyl acetate	617
dimethyltrisulfide	5,700
guaiacol	18,000
hexanal	19,055
isoamyl acetate	21,877
2-heptanone	22,387
2,3-diethyl-5-methylpyrazine	35,000
2-isobutyl-3-methoxypyrazine	45,000
ethyl hexanoate	93,325
2-nonanone	309,030

Approach 2:

The second approach was based on a conventional headspace extraction using a gas-tight syringe in comparison to the SPME extraction, using Equation 3-4 (page 63). *Zhang and Pawliszyn (1993)* used this approach to determine values of K_{fh} for BTEX and PAHs. This method was applied to 2-nonanone in this work, and the values found are presented in Table 3.4-3.

Table 3.4-3: FID responses (× 10^{-3}) after headspace extraction by syringe (300 µl) and by SPME of a 2-nonanone solution (1.0 mM).

	Area 1	Area 2	Area 3	**Average**	SD	**RSD (%)**
SPME	495.23	473.32	474.91	**481.2**	12	**2.5**
Syringe	30.37	31.75	26.33	**29.5**	3	**9.6**

$$K_{fh} = \frac{c_f^{\infty}}{c_h^{\infty}} = \frac{A_f^{\infty} \cdot V_h}{A_h^{\infty} \cdot V_f} = \frac{481.2 \cdot 300\mu l}{29.5 \cdot 0.132\mu l} = \mathbf{37{,}072} \qquad \textbf{(3-12)}$$

This value is in very good agreement with the one determined in approach one using K_{hw} of *Buttery et al. (1969)*. However, it is considerably lower than the value calculated using K_{hw} determined in this study, and it is even lower than the value reported by *Jung and Ebeler (2003a)*. The variation in the K_{fh} values for 2-nonanone and PDMS show that they are only estimations and that the value depends on the method used. However, all K_{fh} values found for 2-nonanone are very high (3×10^4 - 3×10^5) compared to those of other flavour compounds (Table 3.4-2) indicating that 2-nonanone has a very strong affinity for PDMS.

It was not possible to calculate K_{fh} values for the CAR/PDMS fibre coating, as carboxen consists of solid polymer particles. This fibre extracts the analytes primarily by *ad*sorption rather than by *ab*sorption *(Grote and Pawliszyn, 1997)*. The surface area of these fibres would have to be taken into account, but this surface area is not known.

3.4.1.3 Thermal Equilibration Time (t_{eq})

In a flavour-protein solution, consisting of 0.5 % WPI and 0.8 mM 2-nonanone, the 2-nonanone headspace concentration was measured after different times of sample equilibration in the water bath (25 °C), ranging from 0-60 min, after the sample was stored at 5 °C in the refrigerator. In Figure 3.4-2, the thermal equilibration curve for the sample is shown.

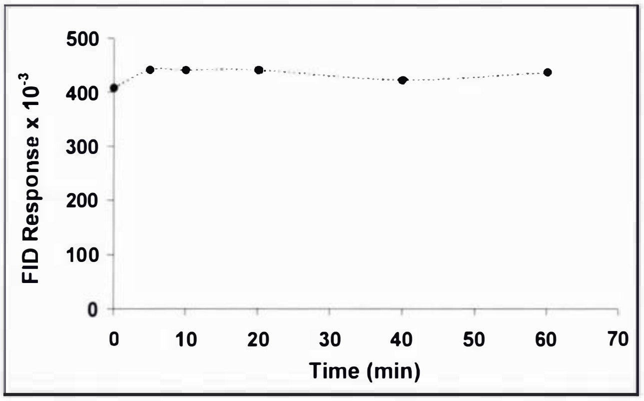


Figure 3.4-2: Thermal equilibration of a sample containing 2-nonanone (0.8 mM) and WPI (0.5 %) at 25 °C.

The flavour concentration in the headspace increased very little, only during the initial 5 min of equilibration. This means the sample volume of 400 μl reached the temperature of 25 °C very fast, probably during the 5 min of extraction time, and it appeared that t_{eq} of 20 min was sufficient. For the determination of flavour binding by proteins, an equilibration time of t_{eq} = 20 min was therefore used throughout this study.

3.4.1.4 Solvent Selection

Two flavour solvents, namely 50 % ethanol and pure propylene glycol were compared regarding their effect on the binding of 2-nonanone to WPI and sodium caseinate. First, standard curves using both solvents were recorded. Figure 3.4-3 shows that the slopes of the standard curves for both solvents were very similar. The slightly lower slope of the curve obtained with propylene glycol as the solvent is probably due to a better solubility of 2-nonanone in the presence of 100 % propylene glycol as compared to 50 % ethanol as the flavour solvent.

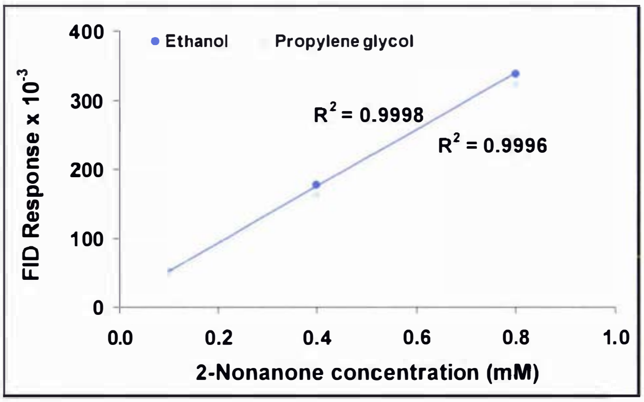


Figure 3.4-3: Standard curves of 2-nonanone using both ethanol (50 %) and propylene glycol (pure) as flavour solvents.

In the gas chromatograms after headspace SPME, there was no peak of propylene glycol, whereas ethanol showed a peak. This means that propylene glycol is not absorbed by the PDMS fibre coating, probably mainly because of its low vapour pressure, and there is no competition between flavour and propylene glycol in the fibre coating.

Since the type of solvent may influence flavour binding, the binding of 0.2 mM 2-nonanone to WPI (0.5 %) and to sodium caseinate (1.0 %) was compared, using both 50 % ethanol and pure propylene glycol as flavour solvents. In these systems with 0.2 mM 2-nonanone, the concentrations of ethanol and propylene glycol were 1 % and 2 %, respectively.

The amount of 2-nonanone bound to WPI and sodium caseinate was found to be the same for both solvents (Figure 3.4-4). Since *Zhu (2003)* concluded that ethanol concentrations below 4 % do not have any significant effect on protein-flavour binding, propylene glycol should not show an effect either.

Adding an organic solvent such as ethanol to the medium increases the solubility of aroma compounds, rendering them less available for interactions, but at the low concentrations of flavour solvents used this does not seem to have an effect on 2-nonanone binding.

Competition for binding sites on the proteins between 2-nonanone and both these alcohols is unlikely because the alcohols are hydrophilic and should not interact with proteins on the same binding sites as the hydrophobic compound 2-nonanone.

The results show that propylene glycol is a suitable solvent for 2-nonanone to examine its interaction with proteins. It is available in food grade quality and can therefore be used for sensory studies. To use one and the same solvent throughout the study, propylene glycol will be used in all following experiments of this chapter.

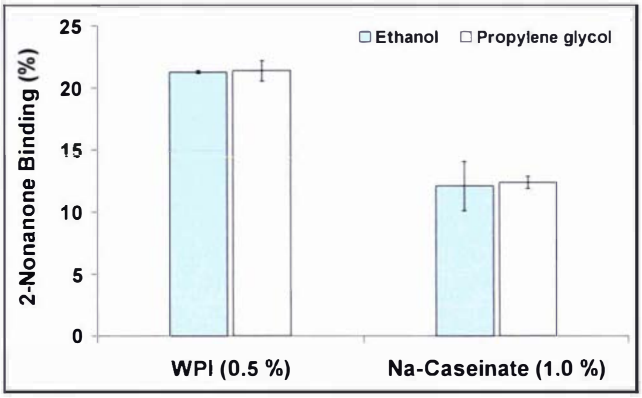


Figure 3.4-4: Binding of 2-nonanone (0.2 mM) to WPI (0.5 %) and to sodium caseinate (1.0 %) in the presence of ethanol (1 %) and propylene glycol (2 %).

3.4.1.5 Sample Agitation during SPME Extraction

The influence of stirring speed during SPME extraction on the amount of 2-nonanone extracted is presented in Table 3.4-4. As the amount of 2-nonanone absorbed by the fibre coating does not change significantly with the stirring speed, stirring can be used during SPME extraction. Stirring is usually inconsistent, but these results show that an irregular stirring speed would not influence the amount of flavour adsorbed by the fibre coating. The slight increase in peak area with stirring speed can be explained with a change of the equilibrium due to 2-nonanone extracted from the headspace. Faster stirring speed will result in a faster release of flavour from the sample. Since equilibrium conditions are desired, the lower stirring speed (250 min^{-1}) is most suitable. A second reason for using the lowest stirring speed is that a too high stirring speed might influence the binding between protein and flavour, for example, foaming may occur and change the protein structure. In addition, very high and very low stirring speeds were found to have greater relative standard deviations than medium stirring speeds *(Arthur et al., 1992)*.

Table 3.4-4: Influence of stirring speed on the amount of 2-nonanone extracted from the headspace as determined by FID peak area.

Stirrer speed (min^{-1})	**250**	**500**	**750**
Peak area 1 (× 10^{-3})	233.57	241.94	247.26
Peak area 2 (× 10^{-3})	239.55	235.74	237.21
Average peak area (× 10^{-3})	**237**	**239**	**242**
SD	4	4	7
RSD (%)	**1.7**	**1.7**	**2.9**

As expected, the precision using magnetic agitation seems to be slightly better than without agitating the sample, although there was no big difference in relative standard deviations (RSD). Without stirring, RSDs were < 5 %, using magnetic stirring they were < 3 %.

Using a 0.6 mM solution of 2-nonanone, the amount of 2-nonanone absorbed by the fibre coating after different extraction times was compared. This experiment was done without stirring and with stirring at 250 min^{-1} and 750 min^{-1}. Extraction curves were produced by plotting the FID response over the SPME extraction time (Figure 3.4-5).

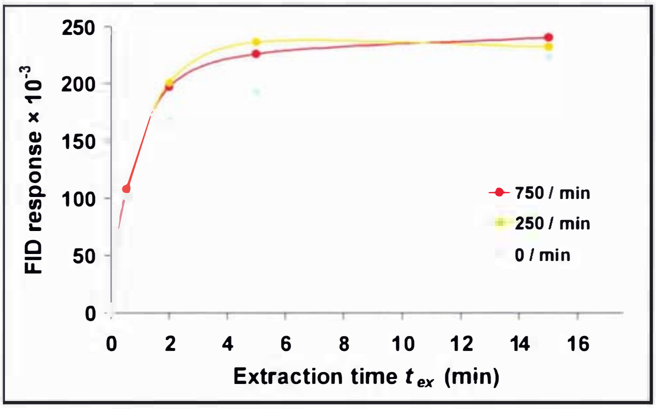


Figure 3.4-5: Dependence of FID response on stirring speed and SPME extraction time.

Stirring resulted in a rapid increase in 2-nonanone absorbed by the fibre coating, while the equilibrium was reached more slowly, after > 15 min compared to 5 min extraction, without stirring. The equilibration time was shorter for the stirred sample, as expected. Stirring the sample during SPME extraction can therefore be used to accelerate equilibrium by continuously generating a fresh surface of the aqueous phase. There was no difference between the extraction curves with 250 min^{-1} and 750 min^{-1}.

From the above results it was decided to apply magnetic stirring during SPME extraction at 250 min^{-1} for all protein-flavour binding experiments in this chapter.

3.4.1.6 Final SPME Method for Measuring Flavour Binding on Proteins

The optimum parameters for the investigation of protein-flavour binding by headspace SPME, as determined in the experiments in sections 3.4.1.1-3.4.1.5, are summarised in Table 3.4-5.

Table 3.4-5: Optimum SPME parameters for the measurement of protein-flavour interactions.

Fibre	30 μm PDMS
Equilibration time t_{eq}	20 min
Temperature	25 °C
Extraction time t_{ex}	5 min
Flavour solvent	propylene glycol
Agitation	magnetical stirring at 250 min^{-1} during the SPME extraction

Precision and Linear Range

Using the above SPME parameters, the precision of the method and the linearity over the concentration range of interest were determined. The precision of an SPME method depends on the analyte *(Yang and Peppard, 1994)* and on the thickness of the coating *(Louch et al., 1992)*. Precision in SPME is generally very good because it is a single-step method. The RSD is typically ~ 5 % for manual operation and can be as low as 1 % using an autosampler *(Zhang et al., 1994)*. The precision of the optimised SPME method was found to be 0.0-3.5 % relative standard deviation (RSD). The average precision of approximately 1.4 % RSD demonstrates the high reliability of SPME as an effective method for the quantification of 2-nonanone.

The linearity of the method was investigated by producing standard curves over the concentration range of interest (0.1-0.8 mM). This was tested to make sure the type of fibre and extraction time used for all experiments in this study do not result in overloading the fibre coating with flavour molecules, and to check if quantification can be performed by linear regression. Duplicate samples of standards were extracted and analysed using GC-FID. The line of best fit for the relationship between the average peak area and the concentration of analyte in the standards was determined by linear regression. Figure 3.4-21 (page 93) shows a typical standard curve obtained for 2-nonanone from 0.1 to 0.8 mM. Linearity was excellent with R^2 values ranging from 0.9990 to 1.0000. Usually, SPME has a wide dynamic linear range (more than three orders of magnitude) *(Zhang et al., 1994)*.

The linearity of the standard curve shows that the 30 µm fibre and an extraction time of t_{ex} = 5 min are useful for the quantitative determination of 2-nonanone between 0.1 mM and 0.8 mM as the fibre is not overloaded. It is important to mention that the calibration curves vary slightly from one day to another and a standard curve had to be determined every day.

3.4.2 Binding of 2-Nonanone and Milk Proteins

The binding of 2-nonanone to the major proteins in milk (β-lg, α-la, BSA, α_{s1}-casein, and β-casein) and to milk protein products (WPI and sodium caseinate) was studied using the optimised headspace SPME method followed by GC-FID for quantification of the free flavour. Typical gas chromatograms are shown in Figure 3.4-6. The retention time of 2-nonanone was between 3.9 and 4.0 min. A reduction in 2-nonanone headspace concentration was clearly visible in the presence of 0.5 % WPI as indicated by a smaller peak.

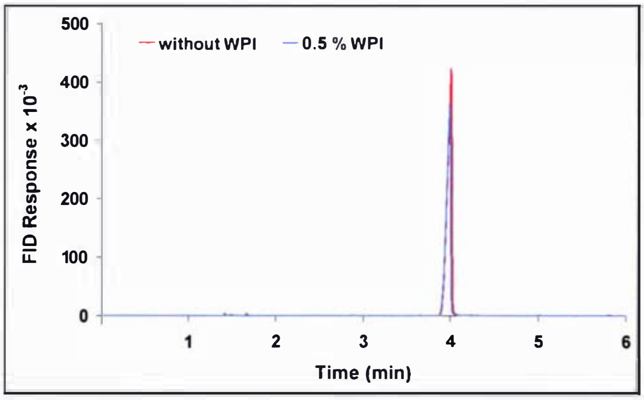


Figure 3.4-6: Typical gas chromatograms of 2-nonanone in the absence and presence of WPI (0.5 %).

3.4.2.1 β-Lactoglobulin (β-lg)

The binding isotherm of 2-nonanone to β-lg at 25 °C is shown in Figure 3.4-7. The amount of 2-nonanone bound in 0.5 % β-lg solution (0.24 mM, as determined by UV spectroscopy) varied between 22 and 36 %, depending on the initial concentration of added 2-nonanone. The number of moles of flavour bound per mole of protein increased with the flavour concentration, and maximum value tended to be one mole of 2-nonanone bound per mole of β-lg monomer (Figure 3.4-7), corresponding to the saturation of the binding sites on the protein. A plateau in the binding isotherm, resulting from saturation of the binding sites, was not reached because of the low concentrations of 2-nonanone. However, the maximum value of *v* can be calculated from the intercept of the Klotz plot (Figure 3.4-8).

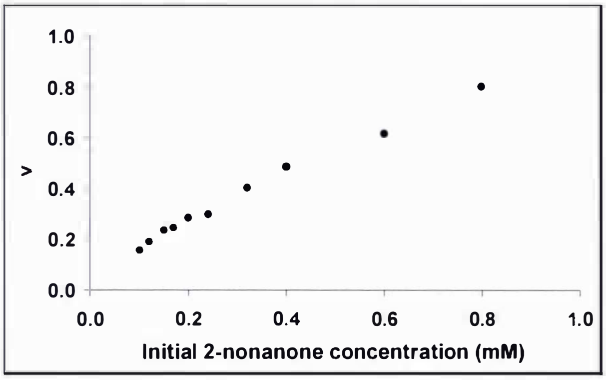


Figure 3.4-7: Binding isotherm of 2-nonanone in aqueous solution of β-lg (0.5 %; 0.24 mM) at 25 °C (each data point is the mean of triplicates); v number of moles of ligand bound per mole of protein.

The binding of 2-nonanone by β-lg was plotted as a Klotz plot (see section 2.2.1.5.2) (Figure 3.4-8). Linear regression resulted in binding parameters of $K = 2{,}700$ (± 500) M^{-1} and $n = 1.1$ (± 0.2). These values are in very good agreement with those obtained by *O'Neill and Kinsella (1987b)*, who determined binding parameters for 2-nonanone and β-lg, using equilibrium dialysis, and found one binding site per monomer with $K = 2{,}439\ M^{-1}$.

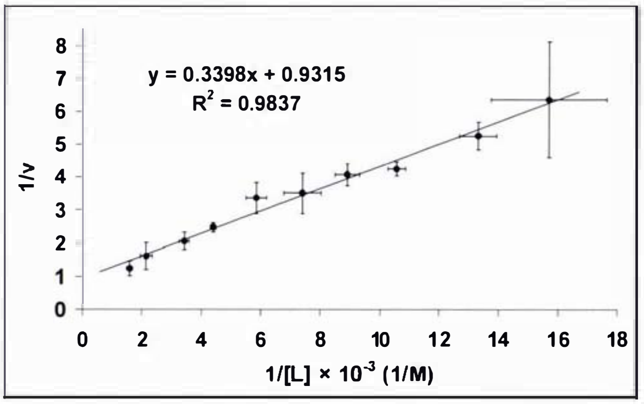


Figure 3.4-8: Klotz plot for the binding of 2-nonanone (0.1-0.8 mM) to β-lg (0.5 %; 0.24 mM) at 25 °C (each data point is the mean of triplicates).

The primary binding site on β-lg for 2-nonanone is probably the hydrophobic pocket of the protein, which is also believed to serve as the binding site for retinol and fatty acids *(Cho et* *al., 1994; Wu et al., 1999; Lübke et al., 2000; Ragona et al., 2000)*. This result does not suggest a second binding site for 2-nonanone on the surface, which was proposed by *Monaco et al. (1987)* for retinol. However, at higher 2-nonanone concentrations, additional binding sites may be occupied.

The binding parameters reported in other studies vary considerably due mainly to the different methods or different protein batches used. *Charles et al. (1996)*, using static headspace analysis, investigated the binding of 2-nonanone and β-lg at pH 3, at which β-lg is predominantly present in monomeric form. They reported 0.2 binding site with $K = 6{,}250\ \mathrm{M}^{-1}$ for 2-nonanone concentrations below 40 ppm and 0.5 binding site with $K = 1{,}667\ \mathrm{M}^{-1}$ for 2-nonanone concentrations above 45 ppm. The lower binding could be attributed to the acidic pH because, at pH values lower than 7, there is a lid that closes off the hydrophobic calyx of β-lg *(Qin et al., 1998)*. In addition, these authors added sodium azide (as a preservative), which has been shown to affect protein-flavour interactions *(Reiners et al., 2000)*. In contrast, *Sostmann and Guichard (1998)*, using affinity chromatography, determined a global binding constant (nK) of $3{,}629\ \mathrm{M}^{-1}$ for 2-nonanone and β-lg at pH 3. In this case, the immobilisation of β-lg may have led to conformational changes and a better accessibility of binding sites. *Jasinski and Kilara (1985)* reported 14 binding sites for 2-nonanone on β-lg with an average K of $122\ \mathrm{M}^{-1}$. They appeared to have overestimated the number of binding sites and underestimated the binding constant. The large number of low-affinity binding sites could also have been due to the high 2-nonanone concentrations (> 1 mM) used by these authors.

3.4.2.2 α-Lactalbumin (α-la)

The binding of 2-nonanone in a 0.5 % α-la solution (0.3 mM, as determined by UV spectroscopy) was found to be between 11 and 21 %, considerably lower than that observed in a 0.5 % β-lg solution. The binding isotherm (Figure 3.4-9) indicates a low number of binding sites on α-la. With the concentrations of 2-nonanone used, saturation of the protein, which is indicated by a plateau value for v, was not achieved.

From the slope and intercept of the α-la Klotz plot (Figure 3.4-10), it was calculated that there is one binding site on α-la with a medium affinity, $K = 900\ (\pm\ 500)\ \mathrm{M}^{-1}$, for 2-nonanone. The value of nK was more than three times lower than that of β-lg. *Jasinski and Kilara (1985)* determined 33 binding sites and a K of $11\ \mathrm{M}^{-1}$ for 2-nonanone on α-la. However, as mentioned earlier for β-lg, these authors used very high 2-nonanone concentrations, which may have been the reason for the high number of low-affinity binding sites.

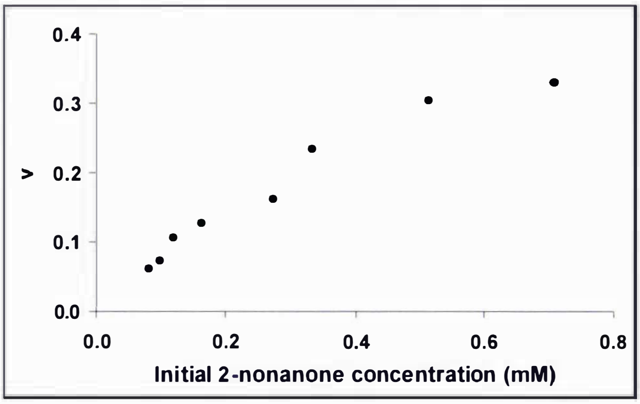


Figure 3.4-9: Binding isotherm for the binding of 2-nonanone (0.1-0.8 mM) and α-la (0.5 %; 0.3 mM) at 25 °C (each data point is the mean of triplicates).

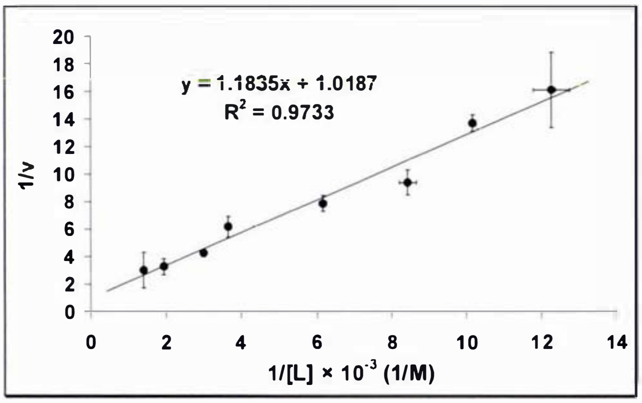


Figure 3.4-10: Klotz plot for the binding of 2-nonanone (0.1-0.8 mM) to α-la (0.5 %; 0.3 mM) at 25 °C (each data point is the mean of triplicates).

3.4.2.3 Bovine serum albumin (BSA)

The amount of 2-nonanone bound in 0.5 % BSA solution (0.065 mM, as determined by UV spectroscopy) varied between 33 and 60 %, depending on the initial 2-nonanone concentration, and was considerably higher than that bound to β-lg or α-la under the same conditions. The binding isotherm of 2-nonanone and BSA at 25 °C is shown in Figure 3.4-11. The number of moles of ligand bound per mole of protein increased with the concentration of free 2-nonanone, and maximum value tended to be around five moles of 2-nonanone bound per mole of BSA.

The Scatchard plot (see section 2.2.1.5.1) (Figure 3.4-12) is slightly non-linear, suggesting heterogeneous binding sites and maybe cooperative binding *(Spector, 1975; Damodaran and Kinsella, 1980a)*. The concave nature of the plot suggests that 2-nonanone binds to BSA with negative cooperativity *(Price and Dwek, 1979)*. Using liquid-liquid partitioning, *Damodaran and Kinsella (1980a)* also found the Scatchard plots for BSA and 2-nonanone to be non-linear which implicated cooperative binding behaviour. The Scatchard plot in Figure 3.4-12 has a very similar shape to that determined by *Damodaran and Kinsella (1980a)*, although they used a wider concentration range. A Hill plot (see section 2.4.3) will elucidate this assumption.

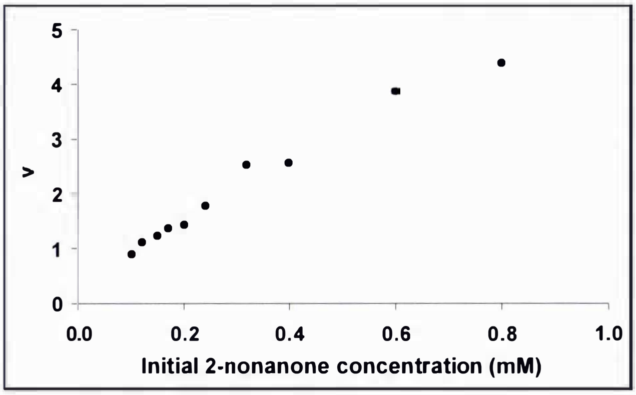


Figure 3.4-11: Binding isotherm of 2-nonanone (0.1-0.8 mM) in aqueous solution of BSA (0.5 %; 0.065 mM) at 25 °C (each data point is the mean of triplicates).

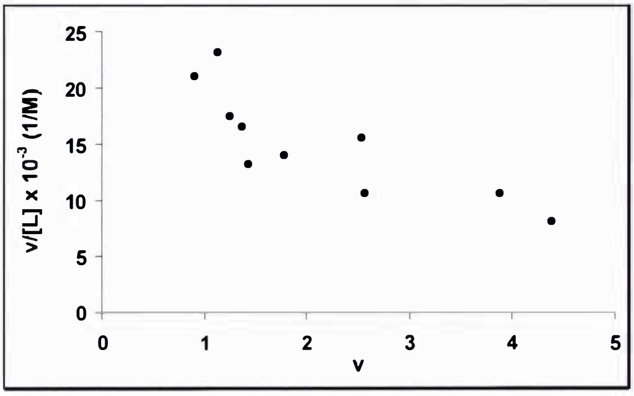


Figure 3.4-12: Scatchard plot for the binding of 2-nonanone (0.1-0.8 mM) to BSA (0.5 %; 0.065 mM) at 25 °C (each data point is the mean of triplicates).

The Klotz plot for the binding of 2-nonanone to BSA is shown in Figure 3.4-13. Again, the non-linear shape indicates that different classes of binding sites are present on BSA, and that cooperativity between the binding sites may exist. The convex shape of the Klotz plot indicates that BSA may show negative cooperativity *(Price and Dwek, 1979)*. On the other hand, the non-linear shape may be due to different classes of binding sites.

At low added 2-nonanone concentrations (0.1-0.2 mM), there were 2.4 (± 0.5) binding sites with a binding constant of 16,000 (± 7,000) M^{-1}. At higher added 2-nonanone concentrations (0.2-0.8 mM), 10 (± 4) binding sites with an average binding constant for 2-nonanone of 1,700 (± 900) M^{-1} were found.

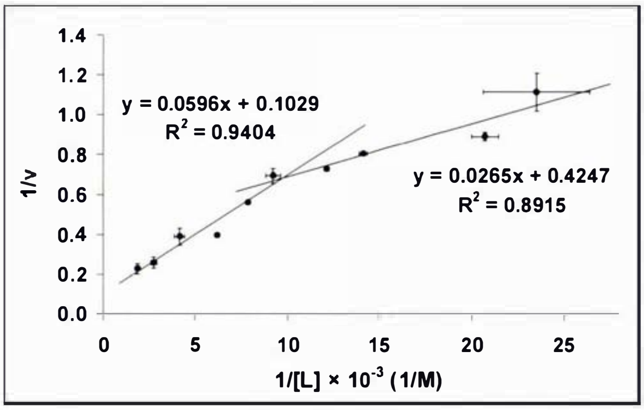


Figure 3.4-13: Klotz plot for the binding of 2-nonanone (0.1-0.8 mM) to BSA (0.5 %; 0.065 mM) at 25 °C, divided into low (0.1-0.2 mM) and high (0.2-0.8 mM) 2-nonanone concentrations (each data point is the mean of triplicates).

There are obviously two to three primary binding sites on BSA that have a very high affinity for 2-nonanone. *Guth and Fritzler (2004)*, using an ultracentrifugation technique, also suggested one or two high-affinity binding sites for long-chain γ- and δ-lactone flavours and a large number of binding sites with lower affinity. The number of binding sites found in this study is also in good agreement with the reported number of binding sites on BSA for other ligands, such as free fatty acids with three primary binding sites, three secondary binding sites, and several weaker binding sites *(Spector, 1975)*, long-chain alcohols or neutral ligands with four to five primary binding sites *(Reynolds et al., 1968; Steinhardt and Reynolds, 1969)*. For the structurally similar flavour compound 2-octanone, three binding sites with a binding constant of 300 M^{-1} at pH 6.4 were found *(Burova et al., 2003)*. Those three binding sites are probably the same that 2-nonanone occupies, and the lower binding constant for 2-octanone can be explained with the lower hydrophobicity of 2-octanone as compared to 2-nonanone. However, *Damodaran and Kinsella (1980b)* suggested that there are two classes of binding sites in BSA for 2-nonanone: the first six sites have higher affinities than the second class of binding sites. However, these authors used fairly high 2-nonanone concentrations.

Damodaran and Kinsella (1980b) found five to six initial binding sites for 2-nonanone on BSA, using liquid-liquid partitioning. The binding constant, $K = 1{,}800$ M^{-1}, was similar to that of the secondary binding sites found in this study, $K = 1{,}700$ (± 900) M^{-1}. This might be due to different contents of fatty acids in the BSA products used. *Damodaran and Kinsella (1980b)* used BSA with 0.4 mol of fatty acids per mole of protein, whereas the product used in this study contained approximately 0.002 % fatty acids, which equates to about 0.005 mol of fatty acids per mole of BSA, calculated on the basis of the molecular weight of oleic acid, the major fatty acid in milk fat. Probably, the primary, high-affinity binding sites of the BSA used by *Damodaran and Kinsella (1980b)* were to a greater extent occupied by fatty acids than those of the BSA used in this study, resulting in a lower binding constant.

Using the pulsed field gradient NMR technique, *Jung et al. (2002)* found seven binding sites for 2-nonanone and other methyl ketones on BSA, with an average binding constant for 2-nonanone of $K = 833$ (± 15) M^{-1}. However, these seven binding sites may not be equivalent and may include primary and secondary binding sites, especially as high 2-nonanone/protein ratios were used. The occupation of secondary binding sites may be the reason for the low overall binding constant.

To reveal if BSA shows any cooperative behaviour when binding 2-nonanone, a Hill plot (see section 2.2.1.5.3) was calculated from the binding data using the Hill equation (Equation 2-6, page 13) (Figure 3.4-14). The Hill plot gives information about the type and extent of cooperativity.

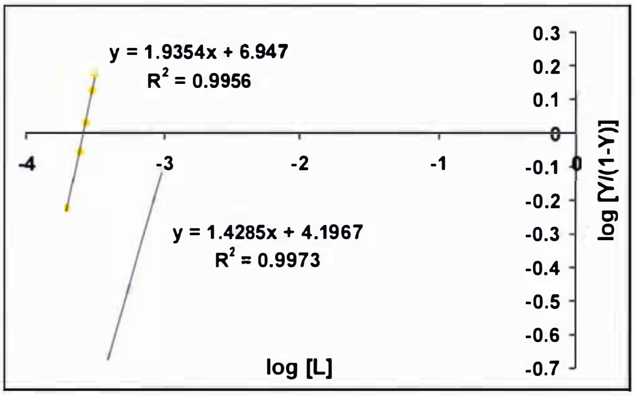


Figure 3.4-14: Hill plot for the binding of BSA (0.5 %; 0.065 mM) to 2-nonanone (0.1-0.8 mM), divided into two classes of binding sites; *Y* saturation of binding sites (assuming $n_1 = 2.4$ and $n_2 = 10$); *[L]* concentration of free 2-nonanone (M).

For low 2-nonanone concentrations (≤ 0.2 mM) a Hill coefficient of $h = 1.94$ was obtained, which is a sign of positive cooperativity. At high concentrations of 2-nonanone (≥ 0.24 mM), $h = 1.43$, indicating slightly positive cooperativity. This means that the convex shape of the Klotz plot (Figure 3.4-13) is merely due to the presence of two groups of binding sites. These findings are in very good agreement with the results of *Damodaran and Kinsella (1980a)* who found the binding of 2-nonanone to BSA to exhibit positive cooperativity at low concentrations of 2-nonanone (≤ 0.4 mM). Above 0.8 mM 2-nonanone, they found negative cooperativity, whereas between 0.4 and 0.8 mM their Scatchard plot was fairly linear.

Comparing the amount of 2-nonanone binding of the main whey proteins, the binding of 2-nonanone to BSA is highest with 33 to 60 % depending on the initial concentration of 2-nonanone, whereas is was about 22 to 36 % in the case of β-lg. The lowest binding was observed with α-la (11 to 21 %). In contrast to β-lg and α-la, which both bind up to one mole of 2-nonanone per mole of protein, BSA is able to bind two to three moles of 2-nonanone per mole of BSA on high affinity sites, and around 10 moles of the flavour on lower affinity sites.

The *n* and *K* values for the binding of 2-nonanone to the individual whey proteins found in the literature are summarised in Table 2.2-3 (page 22). For β-lg, there is a good agreement between the values determined in this study, $K = 2{,}700\ \mathrm{M}^{-1}$ and $n = 1.1$, and those by *O'Neill and Kinsella (1987b)* using equilibrium dialysis (Table 2.2-3). The binding parameters by *Jasinski and Kilara (1985)* seem to overestimate the number of binding sites since they are not in agreement with any other studies. The variation between studies is mainly due to the different methods or different protein batches used.

In the case of α-la, there is hardly any data in the literature to compare the obtained binding parameters with. This is probably due to the lower binding affinity of α-la for flavour compounds compared to β-lg and BSA. The binding constants of β-lg and BSA were found to be more than three times higher than the binding constant of α-la.

3.4.2.4 Caseins

3.4.2.4.1 Purity of prepared sodium caseinate

The prepared sodium caseinate was compared with commercial sodium caseinate regarding purity. In Figure 3.4-15a, chromatograms of prepared and commercial sodium caseinate using size exclusion chromatography (SEC) with UV detection are shown. The commercial sodium caseinate showed a higher amount of impurities than the prepared one, especially some early eluting, high molecular weight impurities (Figure 3.4-15a). High levels of high molecular weight proteins in commercial caseinate were also noticed by *Lynch et al. (1997)*, while these were present at very low levels in their laboratory-prepared caseinate.

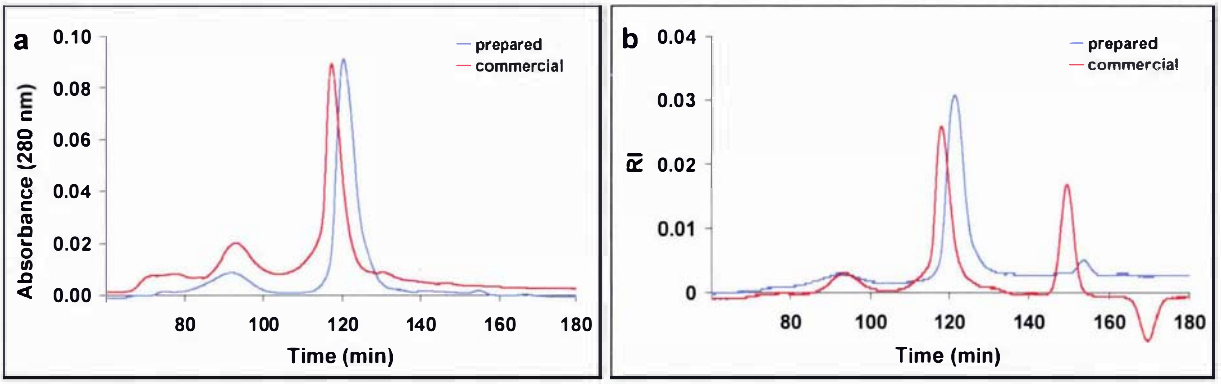


Figure 3.4-15: Comparison of prepared and commercial sodium caseinate using SEC and (a) UV detection, and (b) RI detection.

The comparison using a refractive index (RI) detector is shown in Figure 3.4-15b. With an RI detector, these high molecular weight impurities are also obvious. In addition, the RI detection reveals that the commercial sodium caseinate contains a high amount of some low molecular weight impurities which are hardly present in the prepared caseinate, and some salt, indicated by the negative peak.

The preparation of sodium caseinate in the laboratory is a straightforward procedure and gives an excellent product compared to commercially manufactured sodium caseinate. Besides high levels of high molecular weight compounds in commercial caseinate compared to laboratory-prepared caseinate, it is known to give a poor resolution on urea-PAGE gels, and to contain lower amounts of free amino and sulphydryl groups *(Lynch et al., 1997)*. The reduced modification of the laboratory-prepared caseinate is probably due to the fact that there was no heating involved in the manufacturing process.

The prepared caseinate was then used to separate the individual caseins by ion-exchange chromatography (IEC).

3.4.2.4.2 Separation of caseins by ion-exchange chromatography (IEC)

The elution profile of the caseins from the ion-exchange column is pictured in Figure 3.4-16. Selected tubes (as indicated by vertical arrows) were then run on urea mini-gels to identify each casein and to find out which tubes should be pooled for further purification. The gels of the selected fractions are shown in Figure 3.4-17. After pooling the appropriate fractions, they were dialysed, freeze-dried and weighed (Table 3.4-6).

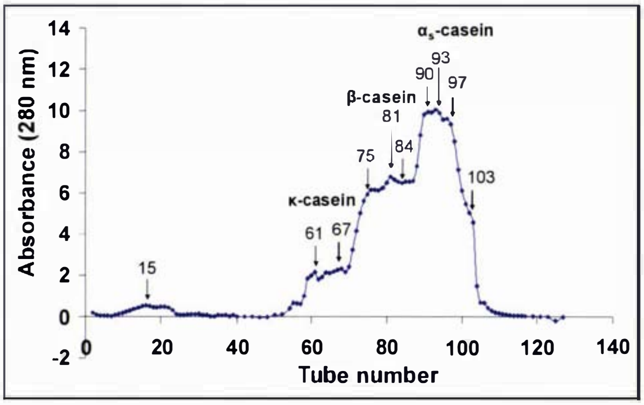


Figure 3.4-16: Elution of the caseins from the ion-exchange column, measured by UV absorbance at λ = 280 nm; vertical errors indicate tubes selected for urea PAGE.

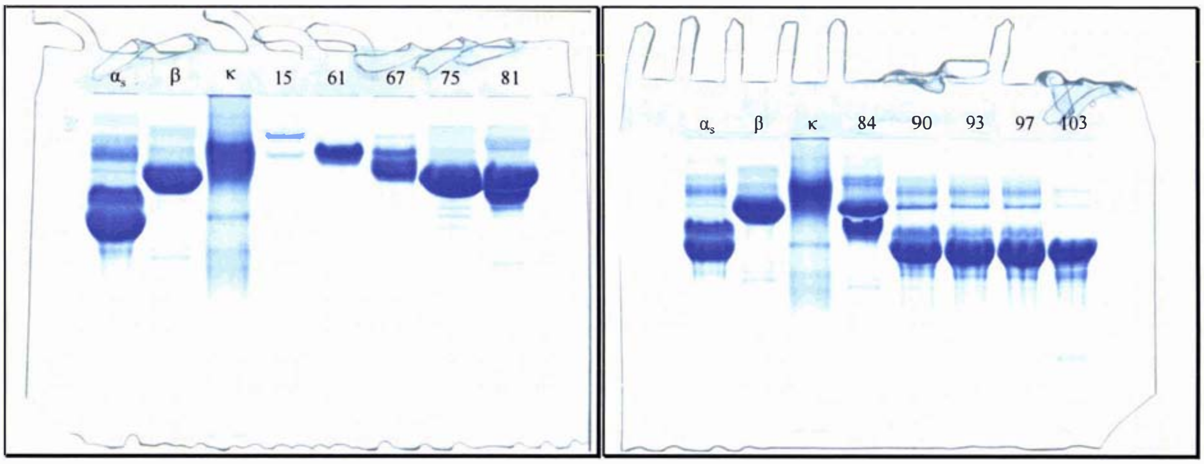


Figure 3.4-17: Urea PAGE gels of casein standards (three lanes on the left) and selected fractions (see Figure 3.4-16).

Table 3.4-6: Fractions and yields of prepared caseins.

Casein	Tubes pooled	Yield (mg)
κ-casein	59-62	43
β-casein	70-81	718
α_{s1}-casein	89-105	853

3.4.2.4.3 Purity of prepared caseins

To check the purity of the final casein products, mini-urea PAGE gels were run. The gel comparing the commercial caseins (Sigma-Aldrich, St. Louis, MO, USA) with the prepared caseins is shown in Figure 3.4-18.

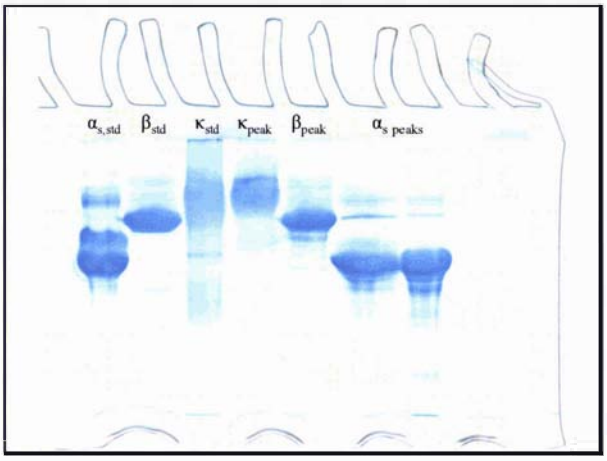


Figure 3.4-18: Urea PAGE gel of casein standards (three lanes on the left) and pooled casein fractions.

The prepared κ-casein had a very good purity (Figure 3.4-18). There are no traces of other proteins, and it is of better quality than the commercial standard. The prepared β-casein contained a small amount of α_{s2}-casein (approximately < 10 %). It is not as pure as the Sigma standard but its purity is reasonably good. Both prepared α_{s1}-casein fractions contained small amounts of β-casein (approximately < 5 %). They only contain α_{s1}-casein and no α_{s2}-casein as the α_{s2}-casein peak overlapped with both the β-casein peak and α_{s1}-casein peak. The commercial α_s-casein did not contain β-casein but a substantial amount of κ-casein. The poor purities of the commercial α_s- and κ-casein standards show that it is worth preparing and purifying caseins in the laboratory.

3.4.2.4.4 Binding of caseins and 2-nonanone

As the yield of prepared κ-casein was very low (Table 3.4-6) and because of the low proportion of κ-casein in total casein, only α_{s1}- and β-casein were examined regarding their binding affinity for 2-nonanone. Caseins obtained from Sigma-Aldrich ("Sigma") and the prepared caseins were investigated. Results for the percent binding are shown in Table 3.4-7.

Table 3.4-7: Percent binding of 2-nonanone to α_{s1}- and β-casein (0.5 %)

Casein source	Binding to α_{s1}-casein (%)	Binding to β-casein (%)
Sigma	6.5 ± 2.9	4.6 ± 1.1
Prepared	4.6 ± 2.3	2.3 ± 1.0

Compared to the whey proteins at 0.5 % concentration, both α_{s1}- and β-casein showed only very little binding affinity for 2-nonanone. The binding did not even show an upward trend towards the lower 2-nonanone concentrations (data not shown), therefore an average value for the %-binding was calculated from all 2-nonanone concentrations tested (0.1, 0.2, 0.4, 0.6, and 0.8 mM). The Sigma caseins showed a higher extent of 2-nonanone binding than the prepared caseins. The values for the average %-binding suggest that 2-nonanone is bound to a higher extent by α_{s1}-casein than by β-casein. The binding to the two caseins is hard to distinguish as the standard deviations were up to almost 3 %. To be able to distinguish the binding affinity, the same experiment was performed with the caseins at 2.0 % instead of 0.5 % to confirm that α_{s1}-casein has a significantly higher affinity for 2-nonanone.

Using 2.0 % solutions of the prepared α_{s1}-casein and β-casein it was possible to differentiate between the binding of 2-nonanone to α_{s1}- and β-casein (Figure 3.4-19). The extent of 2-nonanone binding in 2.0 % α_{s1}-casein and β-casein solutions ranged from 14 to 19 % and from 11 to 17 %, respectively, depending on the initial 2-nonanone concentration. For both the lowest and highest flavour concentrations used – 0.1 and 0.8 mM – the results prove that α_{s1}-casein has a higher binding affinity for 2-nonanone than β-casein.

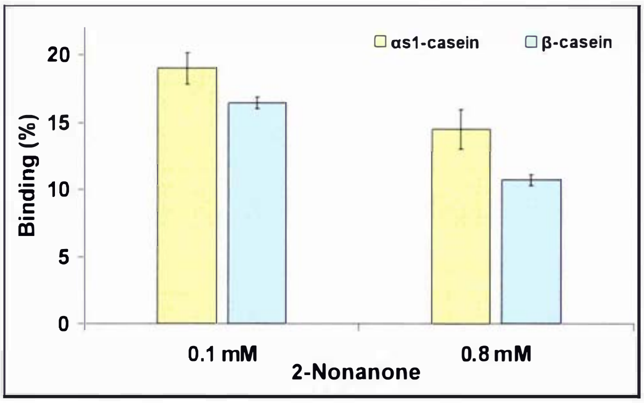


Figure 3.4-19: Binding of 2-nonanone to prepared α_{s1}-casein (2.0 %; 0.73 mM) and β-casein (2.0 %; 0.86 mM).

It was not expected that α_{s1}-casein binds the hydrophobic 2-nonanone stronger than β-casein since β-casein is more hydrophobic than α_s-casein *(Swaisgood, 1992)*. Possibly not only hydrophobic interactions are involved in the binding of 2-nonanone to caseins. Hydrogen bridge formation may also play a role, e.g., α_{s1}-casein contains more acidic amino acid residues (Asp, Glu) than β-casein which could from hydrogen bridges to the keto group of 2-nonanone. Another explanation could be a more pronounced self-association of β-casein as compared to α_{s1}-casein, reducing the extent of interactions of 2-nonanone and β-casein.

However, the binding of 2-nonanone to α_{s1}-casein is only slightly higher than to β-casein. A similar observation was made by *Poiffait and Adrian (1991)*, who found that α_s-, β-, and κ-casein bind about the same amount of the hydrophobic retinol. The binding increased with pH, which is explained by the authors with a progressive unfolding of the casein with pH, resulting in better accessibility of hydrophobic binding sites.

Binding parameters for the prepared α_{s1}- and β-casein at 2.0 % (0.73 mM and 0.86 mM, respectively, as determined by UV spectroscopy) were calculated using the Klotz plot (Figure 3.4-20). The Klotz plot of the prepared β-casein shows a higher slope, which means lower affinity. The linear regression lines of the Klotz plots revealed 0.8 (± 0.5) and 0.32 (± 0.09) binding site and binding constants of 420 (± 280) and 810 (± 250) M^{-1} for α_{s1}-casein and β-casein, respectively. Because of the low binding constants, saturation of the binding sites on both caseins was not achieved. The global binding constants (*nK*) were 330 (± 10) and 240 (± 10) M^{-1} for α_{s1}-casein and β-casein, respectively, indicating a higher binding capacity of α_{s1}-casein compared with β-casein.

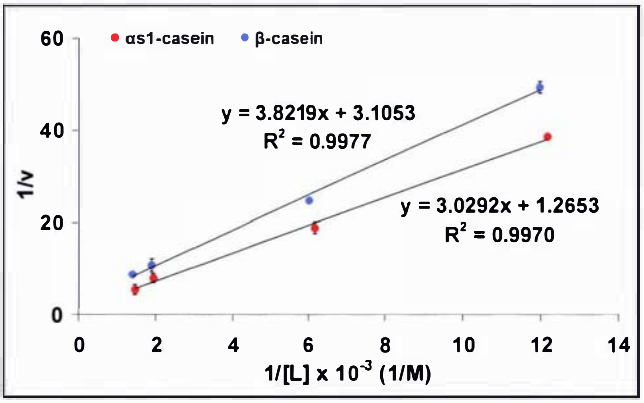


Figure 3.4-20: Klotz plot for the binding of 2-nonanone (0.1-0.8 mM) to the prepared α_{s1}-casein (2.0 %, 0.73 mM) and β-casein (2.0 %, 0.86 mM) (each data point is the mean of duplicates).

The values determined for the individual caseins in this study are only estimations because there was not a sufficient amount of the caseins available to obtain a larger number of data points. However, the higher global binding constant *nK* of α_{s1}-casein compared with β-casein is clearly shown.

Both the binding constant *K* and the numbers of binding sites *n* for α_s-casein are in very good agreement with the values determined for sodium caseinate of $n = 1.1$ and $K = 370\ M^{-1}$. The value of 0.3 binding sites on β-casein does not seem correct; however, this is because saturation of the binding site was not achieved. In addition, the intercept of a Klotz plot is very variable, especially when only four data points are used. The intercept not only has an effect on *n* but also on *K*, since $K = 1 / (n \cdot \text{slope})$.

3.4.2.5 Milk Protein Products

The binding of 2-nonanone to the milk protein products, WPI and sodium caseinate, is shown in Figure 3.4-21. The headspace concentration of 2-nonanone was reduced in the presence of both 0.5 % WPI and 1.0 % sodium caseinate. 2-Nonanone was bound to a greater extent by WPI at only 0.5 % than by sodium caseinate at 1.0 %.

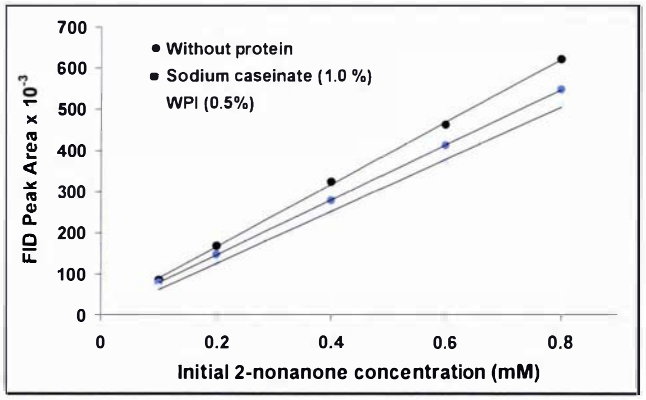


Figure 3.4-21: Influence of WPI (0.5 %) and sodium caseinate (1.0 %) on the headspace concentration of 2-nonanone.

The amount of 2-nonanone bound by the milk protein products ranged from 17 to 30 % and from 12 to 14 % for WPI (0.5 %) and sodium caseinate (1.0 %), respectively, depending on the initial concentration of 2-nonanone. This finding was expected as *Hansen and Booker (1996)* and *Li et al. (2000)* came to the same conclusion, that is, that whey proteins bind flavour compounds to a greater extent than caseins. There is only one study reporting a higher amount of binding of diacetyl by sodium caseinate than by WPI in sweetened drinks *(McNeill and Schmidt, 1993)*. It has to be considered, that diacetyl is a hydrophilic flavour compound which will bind to proteins by different mechanisms than hydrophobic flavour compounds, such as 2-nonanone.

3.4.2.5.1 WPI

The Scatchard plot for the WPI / 2-nonanone system is presented in Figure 3.4-22. The plot is non-linear, and thus, indicates the presence of two groups of binding sites. The x-axis intercept equals the number of binding sites *n* and shows around 0.24 binding sites at low added 2-nonanone concentrations (< 0.2 mM). With increasing 2-nonanone concentration (> 0.2 mM), there appears to be a very high number of binding sites for the flavour compound.

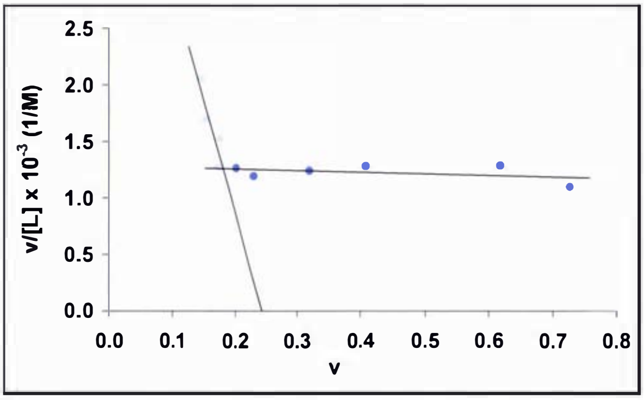


Figure 3.4-22: Scatchard plot for the binding of 2-nonanone (0.1-0.8 mM) and WPI (0.5 %).

The Klotz plot for the binding of 2-nonanone to WPI (0.5 %) is shown in Figure 3.4-23. Its non-linear shape suggests the presence of at least two groups of binding sites that are non-equivalent or that cooperativity between the binding sites of one or more proteins exists. At low added 2-nonanone concentrations (0.1-0.17 mM), there was 0.2 (± 0.02) binding site with a binding constant of 24,000 (± 8,000) M^{-1}. At high added 2-nonanone concentrations (0.17-0.8 mM), 2-nonanone bound on 8 (± 8) binding sites with a very low average binding constant of 130 (± 140) M^{-1}. The high standard errors are due to an intercept being close to zero and a high value of the slope. This change in binding parameters with 2-nonanone concentration may be due to the presence of the several binding sites with different binding constants for 2-nonanone on the constituent proteins in WPI. At low added 2-nonanone concentrations, 2-nonanone is probably predominantly bound on the binding sites with the highest affinity on one protein, possibly BSA. BSA was shown to contain two to three primary binding sites with a very high *K* of 16,000 (± 7,000) M^{-1} (page 84). These sites are probably the preferred sites to be occupied in WPI at low 2-nonanone concentrations. With increasing 2-nonanone concentrations, the medium and lower affinity binding sites on other proteins, namely, β-lg and α-la, are probably filled to a higher degree.

It has to be considered that in milk protein products, such as WPI, a fraction of the proteins is likely to be denatured. Flavour binding is known to depend on denaturation, and is investigated in Chapter 4.

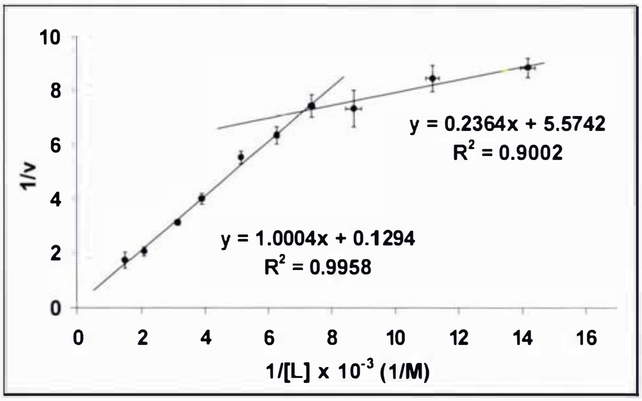


Figure 3.4-23: Klotz plot for the binding of 2-nonanone (0.1-0.8 mM) to WPI (0.5 %) at 25 °C (each data point is the mean of five replicates).

The shapes of both the Klotz plot and the Scatchard plot are characteristic for negative cooperativity *(Price and Dwek, 1979)*. It has been reported in the literature that BSA shows cooperativity *(Damodaran and Kinsella, 1980a, b; Jasinski and Kilara, 1985)*. However, it was shown earlier that none of the constituent proteins of WPI, not even BSA, appear to exhibit negative cooperativity in the range of flavour concentrations investigated. It is therefore concluded that the reason for the non-linear Scatchard and Klotz plots for WPI is that WPI contains non-equivalent binding sites: First, the binding sites on BSA, which have the highest affinity for 2-nonanone (see section 3.4.2.3) are occupied by 2-nonanone, then the β-lg site (second highest affinity), and at higher 2-nonanone concentration weaker binding sites of the proteins are occupied.

The binding parameters for WPI were also calculated from the binding values of the individual whey proteins. The relation of the individual whey proteins in WPI was determined by HPLC (Table 3.4-8). The total amount of protein with 86.1 % is considerably lower than stated in the specifications of the manufacturer (93.9 %). This could be partly due to additional moisture adsorption during storage of the WPI. The actual moisture content of the WPI as determined by the drying method was found to be 7.9 (± 0.3) %, which is an increase of 3.3 %.

Table 3.4-8: Protein composition of WPI as determined by HPLC.

Whey protein	**g / 100 g WPI**
β-Lg	64.6
α-La	15.6
BSA	2.5
Minor proteins (immunoglobulins, proteose peptones etc.)	3.4
Total	**86.1**

Using the global binding capacities *nK* of the individual whey proteins (Table 3.4-9), the following calculation can be made, assuming that minor whey proteins do not bind 2-nonanone:

$$nK_{(\beta\text{-}lg+BSA+\alpha\text{-}la)} = 0.646 \cdot nK_{\beta\text{-}lg} + 0.025 \cdot nK_{BSA} + 0.156 \cdot nK_{\alpha\text{-}la} =$$

$$= 1{,}938 + 650 + 134 = \mathbf{2{,}722\ M^{-1}}$$

If a linear regression was performed on the non-linear Klotz plot, the average binding parameters over the whole concentration range can be obtained, and compared with the calculated value of nK_{WPI}. The slope of this regression line is 0.4683, and the intercept is 1.3116 (regression line not shown). This slope is significantly lower compared to the Klotz plot for sodium caseinate, indicating a higher binding constant for 2-nonanone on WPI. From the intercept and slope of the linear regression line, the following average binding parameters were calculated: n = 0.8 (± 0.3) and K = 2,800 (± 1,200) M^{-1}, which result in:

$$nK_{WPI} = \mathbf{2{,}200\ M^{-1}}$$

Both the calculated and the directly measured global binding constants are similar. The slightly higher value for the calculated global binding constant may be due to a considerable amount of fatty acids occupying binding sites on the BSA in WPI, whereas the pure BSA used in this study had been reduced in fatty acids, resulting in a higher binding constant for 2-nonanone.

Considering the high proportion of β-lg (> 60 %) compared with BSA (2-3 %) in WPI, the binding parameters determined indicate that β-lg is the whey protein that is mainly responsible for 2-nonanone binding in WPI. Although BSA has only a very small proportion in WPI, it also contributes to the binding considerably due to its high affinity constants for 2-nonanone on two to three high affinity binding sites, and several medium-affinity binding sites. This can be quantified by BSA's contribution of 650 M^{-1} to the overall value of *nK* of 2,722 M^{-1} (see page 96). α-La hardly contributes to binding of 2-nonanone in WPI.

3.4.2.5.2 Sodium caseinate

The binding data for sodium caseinate were plotted as a Klotz plot (Figure 3.4-24). From the linear regression line of the plot, an average binding constant *K* of 370 (± 370) M^{-1} and an average number of binding sites *n* of 1.1 (± 1.0) for 2-nonanone on sodium caseinate were obtained. These parameters are in good agreement with the values determined for α_{s1}-casein and β-casein and 2-nonanone. The only other study reporting binding parameters for sodium caseinate and a flavour compound was by *Li et al. (2000)*, who found 0.66 binding site with a binding constant of 353 M^{-1} for vanillin on sodium caseinate (12 °C). To date, studies indicate low binding capacities of caseins and caseinates for different flavour compounds.

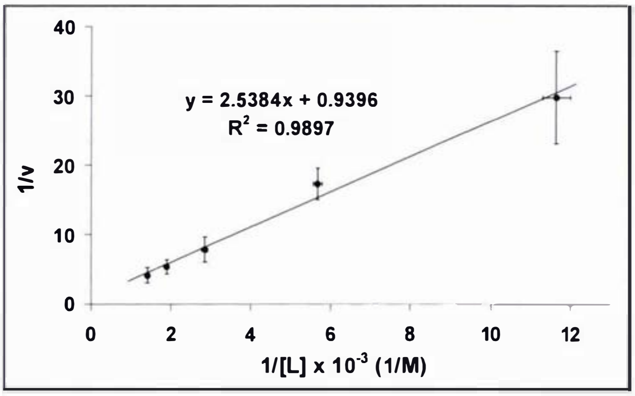


Figure 3.4-24: Klotz plot for the binding of 2-nonanone (0.1-0.8 mM) to sodium caseinate (1.0 %) at 25 °C (each data point is the mean of four replicates).

The *n* and *K* values for the binding of 2-nonanone to the individual milk proteins determined in this study are summarised in Table 3.4-9. Differences in the binding parameters between WPI and sodium caseinate are probably due to the different structures and amino acid compositions of the individual proteins in WPI and sodium caseinate; that is, β-lg, the main constituent protein in WPI, is known to have a structure that can accommodate different small hydrophobic molecules with high affinity *(Spector and Fletcher, 1970; O'Neill and Kinsella, 1987b)*. None of the caseins have been reported to have a high affinity for any flavour molecules.

Table 3.4-9: Binding parameters of 2-nonanone with the individual milk proteins and the milk protein products WPI and sodium caseinate at 25 °C; *n* number of binding sites per monomer, *K* intrinsic binding constant, *nK* global binding constant.

	$c_{\text{2-nonanone}}$ (mM)	n	K (M^{-1})	nK (M^{-1})
Whey proteins				
β-Lg	0.1 - 0.8	1.1 ± 0.2	2,700 ± 500	3,000 ± 100
α-La	0.1 - 0.8	1.0 ± 0.5	900 ± 500	860 ± 60
BSA	0.1 - 0.2	2.4 ± 0.5	16,000 ± 7,000	38,000 ± 8,000
	0.2 - 0.8	10 ± 4	1,700 ± 900	17,000 ± 2,000
	0.1-0.8	4.6 ± 0.9	5,700 ± 1,600	26,000 ± 2,000
WPI	0.1 - 0.17	0.2 ± 0.02	24,000 ± 8,000	5,000 ± 1,000
	0.17 - 0.8	8 ± 8	130 ± 140	1,000 ± 30
	0.1-0.8	0.8 ± 0.3	2,800 ± 1,200	2,200 ± 300
Caseins				
α_{s1}-Casein	0.1 - 0.8	0.8 ± 0.5	420 ± 280	330 ± 10
β-Casein	0.1 - 0.8	0.32 ± 0.09	810 ± 250	240 ± 10
Sodium caseinate	0.1 - 0.8	1.1 ± 1.0	370 ● 370	410 ± 20

Comparison of the 2-nonanone binding of the main whey proteins shows that the binding of BSA and 2-nonanone was highest followed by β-lg. The lowest binding was observed for α-la. In contrast to β-lg and α-la, both of which bind one molecule of 2-nonanone per molecule of protein, BSA is able to bind two to three 2-nonanone molecules per mole of BSA on high-affinity binding sites, and several 2-nonanone molecules on medium-affinity binding sites. The caseins showed considerably lower binding capacities, compared to the whey proteins, with only one low affinity binding site for 2-nonanone.

3.5 Conclusions

The interactions of the flavour compound 2-nonanone and the main proteins in milk (β-lg, α-la, BSA, α_{s1}-casein, and β-casein) and milk protein products (WPI and sodium caseinate) were successfully studied by a headspace SPME method using GC-FID quantification. The SPME method was optimised in terms of thermal equilibration time, type of SPME fibre, SPME extraction time, sample agitation, and flavour solvent. The chosen SPME fibre coating, PDMS, has a very high affinity for 2-nonanone: The fibre-headspace partition coefficient K_{fh} was found to be in the range of 34,000 to 74,000, depending on the approach used.

The good agreement between the binding parameters determined in this study and the values obtained by other authors shows that headspace SPME is an excellent technique for research on protein-flavour interactions. It is fast, sensitive, precise, inexpensive, and solvent free. In addition, not only liquid but also semi-solid and solid samples can be analysed using headspace SPME.

This study has demonstrated that the whey protein with the highest affinity for 2-nonanone is BSA (K_1 = 16,000 M^{-1}; K_2 = 1,700 M^{-1}) followed by β-lg (K = 2,700 M^{-1}), and that α-la shows the weakest binding (K = 900 M^{-1}). Both α-la and β-lg possess one binding site per protein molecule, whereas BSA can bind 2-nonanone on two classes of binding sites, resulting in a very high global binding constant for 2-nonanone. WPI was found to have a much higher affinity for 2-nonanone than sodium caseinate, and they both possess around one binding site per protein molecule on average. Considering the proportions of the whey proteins in WPI, β-lg contributes most to the binding of 2-nonanone on WPI, followed by BSA, while α-la has almost no contribution to the binding.

α_{s1}-Casein was found to have a slightly higher affinity for 2-nonanone than β-casein, although β-casein is the more hydrophobic of the two proteins, indicating that other than hydrophobic forces may be involved, or that self-association of β-casein causes lower binding of 2-nonanone. The overall binding on both caseins is low compared with that on the whey proteins, with estimated global binding affinities nK of 330 (± 10) and 240 (± 10) M^{-1} for α_{s1}-casein and β-casein, respectively.

The presence of milk proteins may therefore cause the aroma profile of foods, in particular fat-free foods, to become unbalanced because of the binding of certain flavour compounds by the proteins. In particular, the whey proteins may prevent hydrophobic flavour compounds, such as 2-nonanone, from being released and perceived during mastication. In this case, the amount of certain flavour compounds added will have to be increased to compensate for the protein-bound flavour molecules.

Milk proteins added to food products are often denatured to a varying extent which may affect binding constants and binding sites because of conformational changes of the proteins. In addition, during processing of foods, e.g. heat treatment, proteins are denatured. Therefore, the effect of heat denaturation on the binding of different flavour compounds and whey proteins was investigated in the next chapter.

CHAPTER FOUR: FACTORS INFLUENCING PROTEIN-FLAVOUR BINDING

4.1 Abstract

The effects of flavour compound structure, heat and high pressure denaturation, and pH on protein-flavour interactions were investigated in this study. The binding of three flavour compounds (2-nonanone, 1-nonanal, and *trans*-2-nonenal) and WPI was measured using an automated headspace SPME-GC-FID method, and the influence of pH (pH 4.0, 7.2, and 8.0), heat treatment (80 °C, 0.5-80 min), and high pressure treatment (250 and 600 MPa, 20 °C) on the WPI-flavour interactions was determined. To test if the flavour compounds show a stabilising effect on the native protein structure, they were added either before or after heat and high pressure treatment of WPI.

The binding of native WPI and the flavour compounds decreased in the order *trans*-2-nonenal > 1-nonanal > 2-nonanone. The differences in binding can be explained with hydrophobic interactions only in the case of 2-nonanone, whereas the aldehydes, in particular *trans*-2-nonenal, can also react covalently. Heat and high pressure treatment affected protein-flavour interactions depending on the structure of the flavour compound. Upon both heat and high pressure denaturation, the binding of 2-nonanone to WPI decreased, while the binding of 1-nonanal remained unchanged, and the affinity for *trans*-2-nonenal increased rapidly. The results suggest that hydrophobic interactions are weakened upon heat or high pressure denaturation, whereas covalent interactions are enhanced. None of the three flavour compounds showed a stabilising effect on the native structure of the whey proteins against denaturation. The binding affinity of the flavour compounds and WPI increased with increasing pH, which is likely to result from pH dependent conformational changes of whey proteins and changes in the protonation of basic amino acid residues.

4.2 Introduction

Protein-flavour interactions are very dependent on the conformational state of a protein. Therefore, factors such as pH, temperature, and high pressure, that influence protein conformation can markedly change flavour binding characteristics of proteins. Changes in pH can influence the ionisation, net charge, and conformation of protein molecules. For most protein-flavour systems, increases in binding with increasing pH have been reported in the literature; however, there are some studies which do not support this trend (see section 2.3.4).

Since heat treatment is an important step during the processing and preparation of many protein containing foods, the investigation of flavour binding to heat denatured proteins is of great importance. However, most studies have examined native proteins and their flavour binding behaviour. In contrast to the caseins, whey proteins are susceptible to heat denaturation. In Chapter 3, it was found that β-lg is the whey protein mainly responsible for 2-nonanone binding in WPI. Upon heat treatment, β-lg partially unfolds and aggregates via hydrophobic association and intra- and interprotein disulphide bonds *(de la Fuente et al., 2002)*. Among the limited number of studies on flavour binding to denatured whey proteins in the literature many contradictions exist (see section 2.3.2), and thus the mechanisms of binding require further investigation. Protein unfolding may increase flavour binding by revealing previously buried hydrophobic binding sites, or it may decrease the binding by modifying specific binding sites for flavour compounds. The aggregation of protein molecules may lead to a decrease in flavour binding due to hydrophobic protein-protein interactions instead of protein-flavour interactions.

As an alternative to thermal processing of food products, high pressure treatment is becoming increasingly important as it retains several desirable food quality attributes. Whey proteins have been found to be susceptible to high pressure treatment, which unfolds them and allows both non-covalent and disulphide intermolecular interactions to occur *(Iametti et al., 1997)*. Only two studies have been reported on the effect of high pressure treatment on protein-flavour interactions *(Yang et al., 2003; Liu et al., 2005b)*; both indicated that high pressure treatment of whey proteins can decrease the binding affinity or have no effect on the interactions, depending on the nature of the flavour compound.

The aim of this chapter was to compare the binding of three structurally similar flavour compounds, 2-nonanone, 1-nonanal, and *trans*-2-nonenal (Table 4.3-1), to WPI in aqueous solution, and to investigate the effect of heat and high pressure treatment, and pH on the extent of protein-flavour binding, using an automated headspace SPME method followed by

GC-FID. In addition, the competition of the flavour compounds with sodium dodecyl sulphate (SDS) for binding sites on β-lg was examined. The preferential binding site for SDS on β-lg is believed to be the hydrophobic pocket *(Creamer, 1995)*. Therefore, a competition experiment gives insight into the extent of binding of flavour compounds into the hydrophobic pocket, as well as an indication on the extent of reversible and irreversible binding.

4.3 Materials and Methods

Distilled, deionised water (DDI) was obtained from a NANOpure™ II water purification system (Barnstead, Dubuque, IA). Henceforth, the term "water" refers to DDI.

4.3.1 Flavour Compounds

The flavour compounds were selected based on their structure to compare the effect of the position of the keto group and the presence of a double bond on protein-flavour interactions. 2-Nonanone, 1-nonanal, and *trans*-2-nonenal (Table 4.3-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of 99 %, 95 %, and 97 % purity, respectively. It was not possible to dissolve the aldehydes at a concentration of 10 mM in propylene glycol, as done for 2-nonanone in Chapter 3. For this reason, 2-nonanone, 1-nonanal and *trans*-2-nonenal were dissolved without the use of a solvent at lower concentrations in water in this study.

Table 4.3-1: Characteristics of the C9 flavour compounds.

Flavour compound	Structure	M (g·mol^{-1})	Aqueous solubility (g·l^{-1}) (25 °C)	Hydrophobicity log P [a]
2-nonanone		142.24	0.4[b]	2.9[c]
1-nonanal		142.24	0.096[d]	3.27[e]
trans-2-nonenal		140.23	n.d.	3.06[e]

a) Value of the logarithm of the partition coefficient between water and n-octanol; b) *Seuvre et al. (2001)*; c) *Rogacheva et al. (1999)*; d) *Dannenfelser and Yalkowsky (1991)*; e) *Meylan and Howard (1995)*

Flavour stock solutions (50 ppm)

Each flavour compound (25.0 ± 0.3 mg) was weighed into a 500 ml volumetric flask, and filled up to the mark with water. After 2 hours of fast magnetic stirring, the flavour compounds were completely dissolved. This was checked by making sure that no flavour was visible on the surface of the solution.

2-Nonanone stock solution (10 mM)

2-nonanone (71 mg) was weighed into a 25 ml beaker, transferred into a 50 ml volumetric flask with propylene glycol and filled up to the mark with the latter. The stock solution was stored at 5 °C.

2-Nonanone stock solution (5 mM) for SDS competition experiment

In a 100 ml volumetric flask, 2-nonanone (71 mg) was dissolved in 50 ml propylene glycol (Bronson and Jacobs Pty. Ltd., Sydney, Australia), and filled up to 100 ml with water.

Flavour standards

Standards of each flavour compound were prepared by diluting the corresponding stock solution (50 ppm) in water or buffer to obtain final flavour concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 ppm.

4.3.2 Buffer Preparation

Phosphate buffer (0.1 M, pH 7.2 and pH 8.0)

- *Di*-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2\,H_2O$) (dibasic, 0.1 M): 1.78 g $Na_2HPO_4 \cdot 2\,H_2O$ (Merck, Darmstadt, Germany) was dissolved in 100 ml water.
- Sodium *di*-hydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (monobasic, 0.1 M): 1.38 g $NaH_2PO_4 \cdot H_2O$ (BDH, Poole, UK) was dissolved in 100 ml water.

To obtain pH 7.2, 28 ml sodium phosphate monobasic and 72 ml sodium phosphate dibasic were mixed. For the pH 8.0 buffer, 5.3 ml sodium phosphate monobasic and 94.7 ml sodium phosphate dibasic were combined. The pH was checked and adjusted if necessary.

Citrate-phosphate buffer (0.1 M, pH 4.0)

- Citric acid solution (0.1 M):
  5.25 g citric acid monohydrate (BDH, Poole, UK) was diluted to 250 ml with water.
- Sodium phosphate solution (0.2 M):
  8.9 g $Na_2HPO_4 \cdot 2\,H_2O$ was diluted to 250 ml with water.

To obtain pH 4.0, 130 ml sodium phosphate solution and 220 ml citric acid solution were combined, the pH was checked and adjusted to pH = 4.0 if necessary, and the buffer was made up to 500 ml with water.

Phosphate buffer (26 mM, pH 7.2) for CD measurements

- *Di*-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2\,H_2O$) (dibasic, 26 mM): $Na_2HPO_4 \cdot 2\,H_2O$ (463 mg) was dissolved in 100 ml water.
- Sodium *di*-hydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (monobasic, 26 mM): $NaH_2PO_4 \cdot H_2O$ (359 mg) was dissolved in 100 ml water.

28 ml sodium phosphate monobasic and 72 ml sodium phosphate dibasic were mixed and the pH was checked and adjusted if necessary.

4.3.3 Protein Preparation

WPI solution (0.5 %) for heat and high pressure treatments

WPI (Alacen 895, Fonterra Co-operative Group Ltd., New Zealand) was dissolved in buffers (0.1 M) of pH 4.0, 7.2, and 8.0 to obtain a 0.5 % (w/v) solution. The solutions were stored for 6 h at 5 °C to allow complete hydration of the proteins before adding flavour stock solution.

WPI solution (0.25 %) for CD measurements

WPI was dissolved in phosphate buffer (26 mM, pH 7.2) to obtain a 0.25 % (w/v) solution. The protein solution was stored for 6 h at 5 °C to allow complete hydration of the proteins before adding the flavour compounds.

WPI solution (0.05 %) for amino acid analysis

WPI was dissolved in water to obtain a 0.05 % (w/v) solution. The solution was stored for 6 h at 5 °C to allow complete hydration of the proteins before adding *trans*-2-nonenal stock solution.

β-Lg solution (0.2 mM) for 2-nonanone/SDS competition experiment

β-Lg AB from bovine milk was purchased from Sigma (St. Louis, MO, USA) and is a lyophilised powder of approximately 90 % purity. β-Lg (168 mg) was dissolved in 42 ml phosphate buffer (0.1 M, pH 7.2).

β-Lg solution (0.014 mM) for aldehyde/SDS competition experiments

β-Lg AB (70 mg) was dissolved in 250 ml phosphate buffer (0.1 M, pH 7.2).

4.3.4 Other Solutions

Ascorbic acid solution

A stock solution (1.0 %, w/v) of ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA) was freshly prepared in water. Aliquots of the stock solution were added to certain samples containing *trans*-2-nonenal to obtain final ascorbic acid concentrations of 0.001, 0.01, and 0.1 % (v/v).

Gallic acid solution

A stock solution (0.7 %, w/v) of gallic acid (3,4,5-trihydroxybenzoic acid) monohydrate (Sigma Chemical Co., St. Louis, MO, USA) was freshly prepared in water. Aliquots of the stock solution were added to certain samples containing *trans*-2-nonenal to obtain final gallic acid concentrations of 0.007 and 0.07 % (v/v).

SDS stock solution for 2-nonanone experiment (10 mM)

SDS (BDH, Poole, UK) (288 mg) was dissolved in 100 ml water.

SDS stock solution for aldehyde experiments (0.7 mM)

SDS (50.4 mg) was dissolved in 250 ml water.

4.3.5 Amino Acid Analysis

To determine the amino acids that react with *trans*-2-nonenal, equal volumes of WPI solution (0.05 %) and *trans*-2-nonenal stock solution (50 ppm) were combined and equilibrated for 40 h at 5 °C. A control sample was prepared by combining equal volumes of WPI solution (0.05 %) and water. The molar ratio of WPI:*trans*-2-nonenal was approximately 1:10. Amino acids in both samples were determined by hydrochloric acid hydrolysis (methionine and cysteine by performic acid oxidation) followed by HPLC separation using the AOAC official method 994.12 *(AOAC, 1995)*.

4.3.6 Heat Treatment

The sample preparation procedure is shown schematically in Figure 4.3-1.

Samples were prepared by transferring 6.860 (± 0.001) g WPI solution (0.5 %) in 20 ml headspace vials (20-CV, Chromacol, Herts, UK). For samples with flavour addition before heat treatment, 140 µl of the flavour stock solution (50 ppm) was added to obtain a final flavour concentration of 1.0 ppm. The vials were immediately sealed with silicone/PTFE septa (20-ST3) and magnetic tin crimp caps (20-MCB, Chromacol, Herts, UK), and equilibrated for 40 h at 5 °C. Subsequently the mixtures were heated for 0, 0.5, 1, 2, 5, 10, 20, 40, 60, and 80 min in a temperature-controlled water bath at 80 (± 0.5) °C, and immediately cooled in ice water for 5 min. The sample vials were kept at room temperature for about 2 h before being analysed by automated headspace SPME and GC-FID (sections 4.3.11 and 4.3.12).

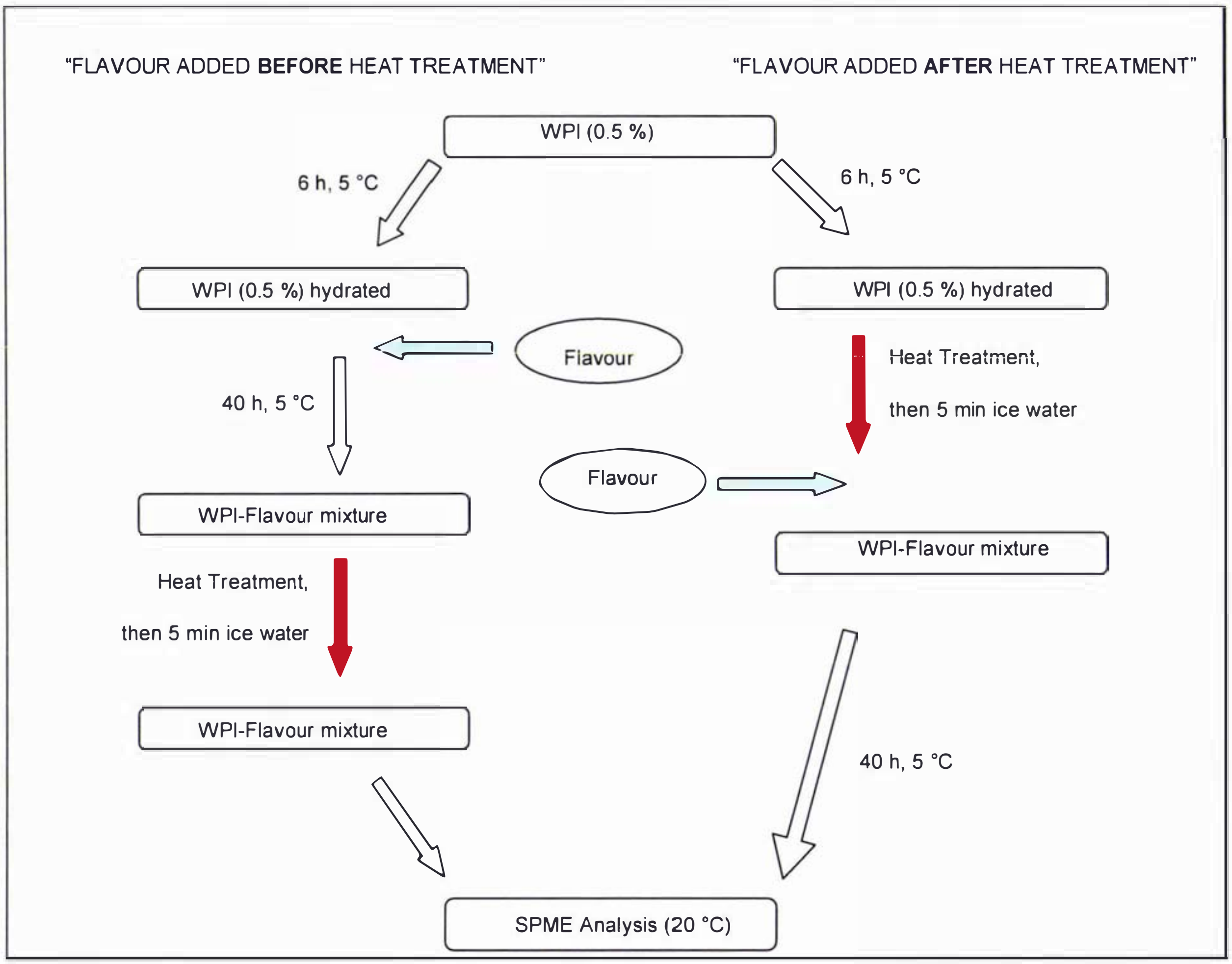


Figure 4.3-1: Flowchart showing the sample preparation to investigate binding of flavour compounds to WPI for both flavour addition before and after heat treatment.

For samples with flavour added after heat treatment, the WPI solutions were first heated, and immediately after the samples were cooled down in ice water, 140 μl of flavour stock solution was added. After equilibration (40 h, 5 °C) they were analysed by headspace SPME and GC-FID (sections 4.3.11 and 4.3.12).

The effect of heating temperature on WPI/2-nonanone binding was also investigated by heating samples containing 6.860 (± 0.001) g WPI solution (0.5 %) and 140 μl 2-nonanone stock solution (10 mM) for 12 min at 40, 50, 55, 60, 65, 70, 75, 80, 85, and 90 °C.

Controls were prepared in the same way as the samples with flavour added before heat treatment by weighing 6.860 (± 0.001) g of phosphate buffer (0.1 M, pH 7.2) in 20 ml headspace vials and adding 140 µl of flavour stock solution. Controls were not heat treated. It was verified that there was no loss of flavour during heat treatment up to 80 min.

All samples were manually agitated every 5 min during heat treatment.

4.3.7 High Pressure Treatment

The sample preparation procedure is shown schematically in Figure 4.3-2.

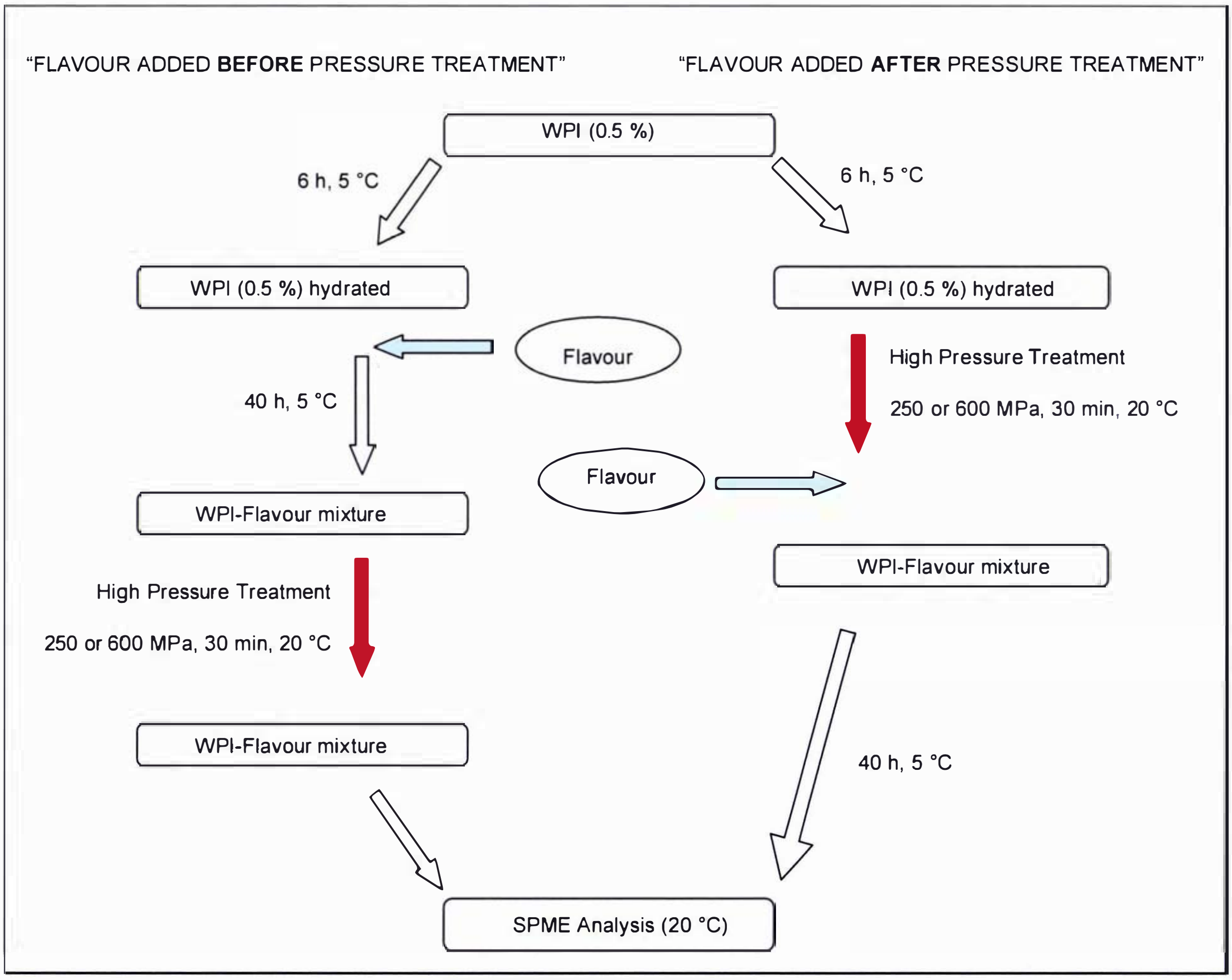


Figure 4.3-2: Flowchart showing the sample preparation to investigate binding of flavour compounds to WPI for both flavour addition before and after high pressure treatment.

In the case of samples with flavour added before high pressure treatment, aliquots (5.194 ml) of the WPI solution (0.5 %)/buffer (controls) were transferred into polyallomer Quick-Seal centrifuge tubes (13 mm internal diameter, 51 mm high, Beckman Instruments, Inc., Palo Alto, CA, USA). Using a microlitre syringe (Hamilton, Reno, NV, USA), 106 µl of flavour stock solution (50 ppm) was added, and the tubes were immediately heat sealed. These samples were equilibrated for 40 h at 5 °C for complete equilibration of the free and protein-bound flavour, and then transferred to the pressure chamber of the high pressure unit. For samples with flavour added after high pressure treatment, 5.3 ml of WPI solution were transferred into the centrifuge tubes, without the addition of flavour stock solution, and the tubes were heat sealed.

High pressure unit

The samples were treated in a high-pressure unit ('Food-Lab' food processor, model S-FL-065-200-9-W, Stansted Fluid Power Ltd., Stansted, Essex, UK) at pressures of 250 or 600 MPa, for 30 min at 20 °C. An emulsion of 10 % vegetable oil in water with surfactant and preservative was used as a pressure-transmitting fluid in the 65 mm × 220 mm cylindrical high pressure chamber.

The high pressure unit was equilibrated to the desired temperature by recirculating water through the water jacket associated with the unit using temperature controlled water and pumping equipment. The pressure unit and all samples were equilibrated to the desired temperature for at least 1 h before pressurisation commenced.

The temperature and pressure profiles are shown in Figure 4.3-3. The temperature change during pressurisation/depressurisation cycles was monitored using the thermocouple associated with the unit and standard data logging equipment. The pressurisation and depressurisation rates were 3.5 MPa·sec^{-1} and 6 MPa·sec^{-1}, respectively. The average adiabatic heating during pressurisation was ~2.0 °C/100 MPa, and the cooling rate during depressurisation was ~1.0 °C/100 MPa.

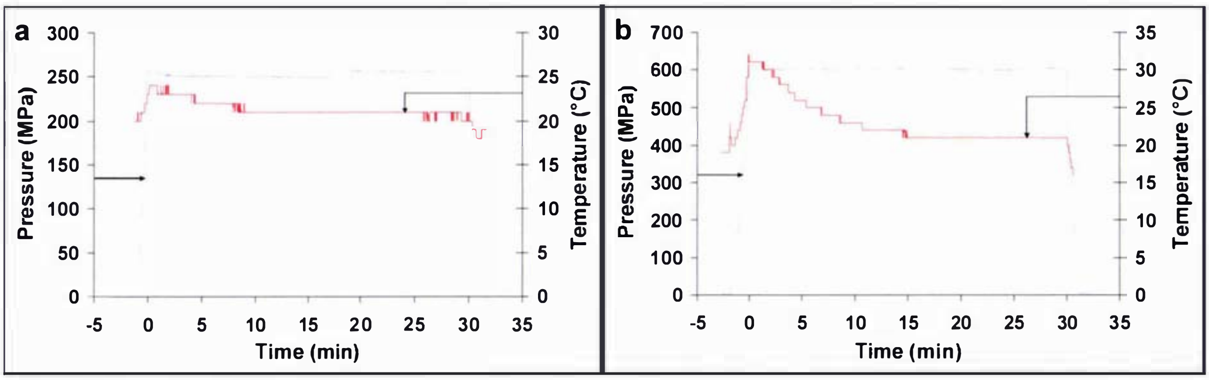


Figure 4.3-3: Temporal profiles of temperature and pressure during high pressure treatment at (a) 250 MPa, and (b) 600 MPa.

Preparation of samples for headspace SPME

After the high pressure treatment, samples with added flavour were quantitatively transferred from the centrifuge tubes into 20 ml headspace vials. The vials were immediately sealed with silicone/PTFE septa and magnetic tin crimp caps. For the samples with flavour added after high pressure treatment, 5.194 ml of the pressurised WPI solutions were transferred into 20 ml headspace vials, 106 µl of flavour stock solution was added, the sample vials were sealed, and the samples were equilibrated for 40 h at 5 °C. Samples and controls were then analysed by headspace SPME-GC-FID (sections 4.3.11 and 4.3.12).

4.3.8 Competition with Sodium Dodecyl Sulphate (SDS)

For the competition experiment, a molar ratio of 2:1:2 for β-lg:flavour:SDS was used. Due to the low solubility of 1-nonanal and *trans*-2-nonenal, these flavour compounds were used at a lower concentration than 2-nonanone, and quantities of β-lg and SDS were adjusted accordingly to obtain the 2:1:2 ratio.

Table 4.3-2 illustrates the preparation of samples and controls in 20 ml headspace vials. Following the addition of SDS or flavour stock solution, samples were equilibrated for 40 h at 5 °C before the second addition of a ligand solution, and before the headspace SPME-GC-FID analysis (sections 4.3.11 and 4.3.12).

Table 4.3-2: Sample preparation scheme for the SDS competition experiment.

	β-Lg solution (ml)	Buffer (ml)	Water (µl)	SDS stock (µl)	Flavour stock (µl)
Controls					
without SDS	-	6.720	140	-	140
with SDS	-	6.720	-	140	140
Samples					
without SDS	6.720	-	140	-	140
flavour added first	6.720	-	-	140 [a)]	140
SDS added first	6.720	-	-	140	140 [a)]

[a)] added after 40 h equilibration

4.3.9 Effect of pH

Samples and controls were prepared in the following buffers (0.1 M): Citrate-phosphate buffer (pH 4.0), and phosphate buffer (pH 7.0 and 8.0). They were heat treated as described in section 4.3.6; however, heat treatment was for 5 min only.

4.3.10 Identification of Unknown Compound using SPME and GC-Mass Spectrometry (GC-MS)

The headspace above the sample solution (10 ml) was extracted for 30 min at 35 °C using a 65 μm PDMS/DVB fibre (Supelco, Bellefonte, PA, USA). A GC 17A gas chromatograph coupled with a QP 5000 mass spectrometer (Shimadzu, Kyoto, Japan) was used to identify the unknown peak. Desorption of the SPME fibre was for 2 min at 250 °C. The column used was an EC 1000 column (30 m, ID 0.25 mm, 0.25 μm film thickness) (Alltech, Deerfield, IL, USA). The carrier gas used was helium (linear velocity 28.8 $cm \cdot sec^{-1}$). The injection port (direct mode) temperature was 250 °C, and the detector temperature was 260 °C. The temperature program started at 35 °C, and the temperature was increased to 230 °C at 5 °C/min, and held for 21 min.

4.3.11 Automated Headspace SPME Method

Headspace SPME sampling was performed using a CombiPAL autosampler unit (CTC Analytics AG, Zwingen, Switzerland). The SPME parameters are summarised in Table 4.3-3. The parameters were optimised in Chapter 3 using 2-nonanone, and because the three C9 flavour compounds possess similar structures and hydrophobicities, these parameters were also used for 1-nonanal and *trans*-2-nonenal.

Table 4.3-3: Parameters of the automated headspace SPME method.

Fibre	30 μm PDMS
Equilibration time t_{eq}	25 min
Temperature	30 °C
Extraction time t_{ex}	5 min
Agitation speed	250 rpm
Agitation on time	5 sec
Agitation off time	2 sec

4.3.12 Gas Chromatography

A GC 2010 gas chromatograph coupled with an FID detector (Shimadzu, Kyoto, Japan) was used to quantify the free flavour. The column used was a Supelcowax™ 10 fused silica capillary column (30 m, ID 0.32mm, 0.50 µm film thickness) (Supelco, Bellefonte, PA, USA). The carrier gas used was helium (linear velocity 40 cm·sec^{-1}). The injection port (direct mode) temperature was 250 °C, and the detector temperature was 260 °C. The oven temperature was isothermally held at 120 °C. Data acquisition was achieved using GC Solutions Software (Shimadzu, Kyoto, Japan).

4.3.13 Circular Dichroism (CD)

CD measurements were performed to follow the heat-induced denaturation of WPI, and to reveal possible effects of flavour compounds on the native protein structure.

Flavour stock solutions were added to the WPI solution as described in Table 4.3-4.

Table 4.3-4: Sample preparation scheme for the CD experiment.

WPI (0.25 %)	WPI (0.25 %) (ml)	2-nonanone (10 mM) (µl)	propylene glycol (µl)	*t*-2-nonenal (50 ppm) (µl)
alone	15.00	-	-	-
+ propylene glycol	14.85	-	150	-
+ 2-nonanone (14 ppm)	14.85	150	-	-
+ *t*-2-nonenal (1 ppm)	13.72	-	-	280

To obtain near-ultraviolet (near-UV) CD spectra, the samples were scanned from 250 to 400 nm in 10 mm quartz cells with a Jasco Model J-720 spectropolarimeter (Jasco, Hachioji City, Tokyo, Japan). Sample buffer was used as the blank. The samples were scanned at 50 nm·min^{-1}, using a 2 sec time constant, a 0.2 nm step resolution, a 1 nm band width, and a sensitivity of 10 mdeg. Five scans were accumulated and averaged.

To obtain far-UV spectra, the samples were diluted 10-fold with water, and scanned from 185 to 250 nm using a 0.5 mm cell. Ten scans were averaged. Water was used as the blank.

4.3.14 Statistical Analysis

All samples were prepared in triplicate, except for the samples with added ascorbic acid and gallic acid, which were prepared in duplicate. The values of %-binding of the flavour compounds and WPI were subjected to a *t*-test or analysis of variance. Statistical significance was at $P < 0.05$ and if a significant effect was found a Tukey's post-hoc test was performed. SPSS 14.0 for Windows software (Chicago, IL) was used for statistical evaluations.

4.4 Results and Discussion

4.4.1 Comparison of the Binding of 2-Nonanone, 1-Nonanal, or *trans*-2-Nonenal to WPI

The binding affinity of 2-nonanone, 1-nonanal, and *trans*-2-nonenal to WPI (0.5 %) was compared. A GC chromatogram of a mixture of the three flavour compounds is shown in Figure 4.4-1. The two saturated compounds, 2-nonanone and 1-nonanal, eluted at very similar retention times, 4.76 and 4.84 min, respectively. The unsaturated aldehyde, *trans*-2-nonenal, eluted later at 8.17 min.

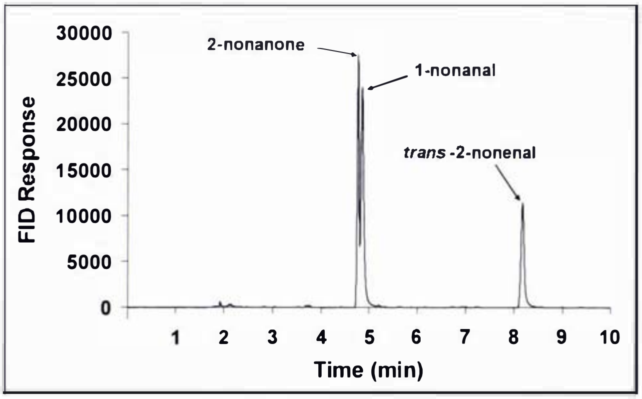


Figure 4.4-1: GC chromatogram of 2-nonanone, 1-nonanal, and *trans*-2-nonenal.

Standard curves for each flavour compound exhibited excellent linearity over the concentration range of interest (Figure 4.4-2). The highest FID response was observed for 1-nonanal, followed by 2-nonanone, and *trans*-2-nonenal showed the lowest FID response. These differences result from a combination of the volatility or K_{hw} values and the K_{fh} values of the flavour compounds. The higher the volatility (K_{hw}), and thus headspace concentration, of a flavour compound, the higher the FID response. A higher affinity of a flavour compound for the fibre coating (K_{fh}) results in a higher FID signal. K_{fh} values for these compounds have not yet been reported in the literature. An estimation of K_{fh} for 2-nonanone and PDMS was made in section 3.4.1.2. These observations indicate that 1-nonanal has the highest affinity for PDMS, followed by 2-nonanone, with the lowest K_{fh} value for *trans*-2-nonenal.

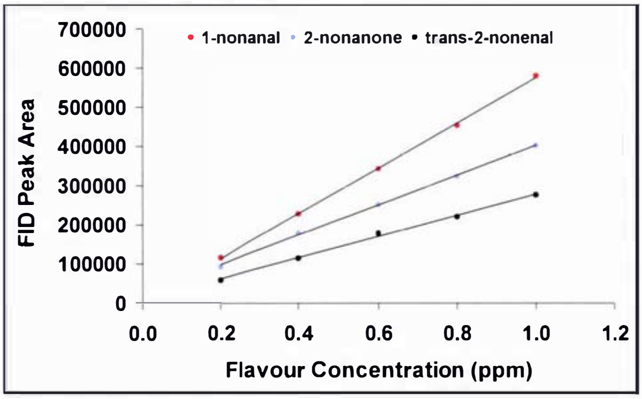


Figure 4.4-2: Standard curves of *trans*-2-nonenal, 1-nonanal, and 2-nonanone (0.2-1.0 ppm).

The interactions of native WPI and the three flavour compounds were investigated to understand the effect of flavour compound structure on protein-flavour binding (Figure 4.4-3). At the initial flavour concentration of 1.0 ppm, the binding was highest for the unsaturated aldehyde, *trans*-2-nonenal, with 72.3 (± 0.2) %, followed by the saturated aldehyde, 1-nonanal, with 68.3 (± 0.1) %, and the saturated methyl ketone, 2-nonanone, with 39.2 (± 0.5) %.

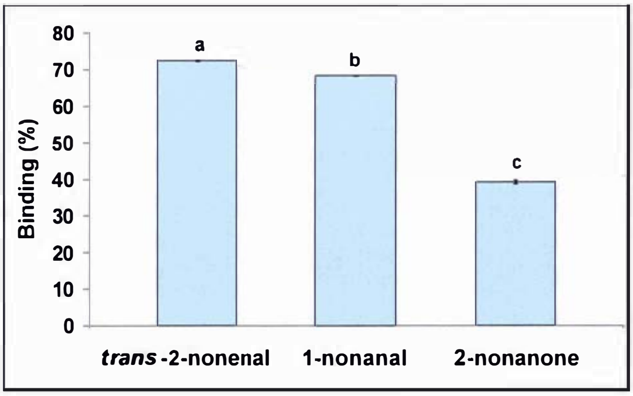


Figure 4.4-3: Binding of C9 flavour compounds (1.0 ppm) to WPI (0.5 %). Different letters indicate significant (P < 0.05) differences between samples (n = 3).

The presence of the keto group at the end of the C9 chain (1-nonanal) resulted in a significantly higher extent of binding as compared to the keto group in the 2-position (2-nonanone). This can be attributed to the higher hydrophobicity of 1-nonanal as compared to 2-nonanone (Table 4.3-1). In addition, the aldehyde may also react covalently, e.g., with the ε-amino group of lysine residues, and therefore show a higher amount of binding than the methyl ketone. For example, hexanal was found to interact with the lysine residues in milk proteins *(Meynier et al., 2004)*.

The presence of a double bond further increased the binding affinity of flavour compounds for whey proteins, as seen in the higher binding of *trans*-2-nonenal compared to 1-nonanal (Figure 4.4-3). *Trans*-2-nonenal is less hydrophobic than 1-nonanal (Table 4.3-1), but is bound to a higher extent. This suggests possible interactions that are not only hydrophobic in nature, but also could involve the double bond ("Michael addition"). In agreement with this observation, the extent of irreversible binding on soy protein has been shown to be higher for alkenals than for alkanals using a high vacuum transfer method *(Gremli, 1974)*. *Trans*-2-hexenal has been shown to exhibit covalent binding with milk proteins *(Meynier et al., 2004)*, and 2-octenal was bound irreversibly on BSA *(Alaiz and Girón, 1994)*. The authors suggested: a) a reaction of the alkenal double bond with lysine and histidine residues ("Michael addition"), and b) a reaction of the alkenal aldehyde function with lysine residues (Schiff base formation) (see Figure 2.1-1, page 4). Amino acid analysis indicated a reaction of alkenals predominantly with histidine residues, but also with lysine and cysteine residues *(Bruenner et al., 1995; Meynier et al., 2004)*. Using MS, 4-hydroxy-2-nonenal was found to react with proteins via "Michael addition", whereas only trace amounts of Schiff base were formed *(Bruenner et al., 1995)*. *Trans*-2-nonenal is therefore very likely to react with WPI to a high extent via "Michael addition".

This hypothesis was confirmed by performing an amino acid analysis of samples containing WPI (0.025 %), in the absence and presence of *trans*-2-nonenal (25 ppm) (Figure 4.4-4). Histidine, lysine, cysteine, methionine, and possibly serine reacted with *trans*-2-nonenal, whereas arginine was not modified by the unsaturated flavour compound. Because a number of amino acid residues other than lysine reacted with the flavour compound, the predominant reaction of *trans*-2-nonenal and proteins appears to be an addition reaction, rather than a Schiff base formation which takes place with primary amines such as lysine.

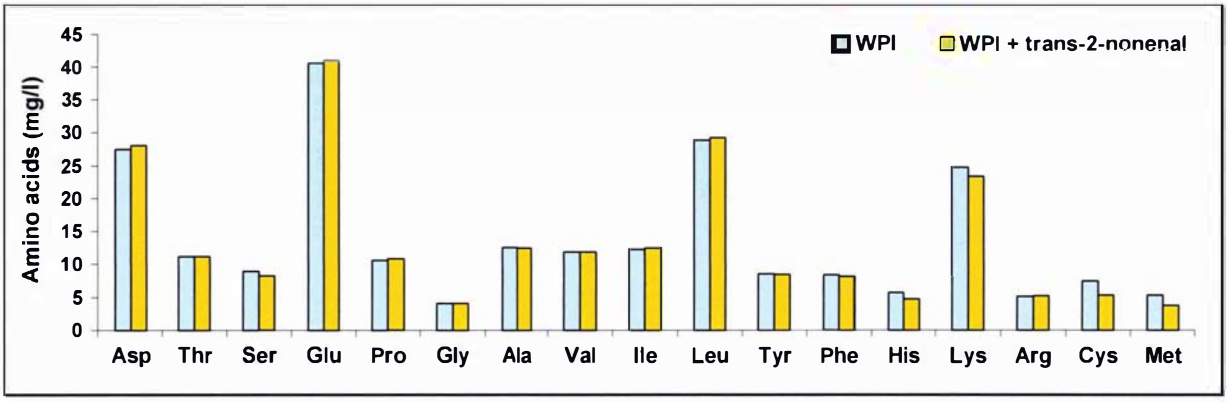


Figure 4.4-4: Comparison of amino acids in WPI (0.025 %) in the absence and presence of *trans*-2-nonenal (25 ppm).

In agreement with the findings in Figure 4.4-3, *Sostmann and Guichard (1998)*, using affinity chromatography by means of immobilised β-lg, determined a higher binding constant for *trans*-2-nonenal ($K = 4{,}433\ \mathrm{M}^{-1}$) than for 2-nonanone ($K = 3{,}629\ \mathrm{M}^{-1}$) on β-lg. As the β-lg amino groups have reacted with the support material of the HPLC column, these binding constants arise from reversible interactions only and reflect the greater hydrophobicity of *trans*-2-nonenal.

4.4.2 Effect of Heat Treatment on Flavour Binding to WPI

4.4.2.1 Effect of Heating Temperature on the Binding of 2-Nonanone to WPI

The effect of heating temperature on interactions of WPI (0.5 %) and 2-nonanone (0.1 mM) is shown in Figure 4.4-5. The extent of 2-nonanone binding was not influenced by the addition of flavour before or after heat treatment. The binding of 2-nonanone decreased significantly ($P < 0.05$) when the samples were heated at 70 °C for 12 min. *Considine et al. (2005a)*, who determined the extent of β-lg denaturation after heat treatment (12 min) at different temperatures by PAGE, found the amount of native monomer to decrease at temperatures above 60 °C with a simultaneous increase in the amount of dimers. The formation of non-native dimers appears to reduce the binding affinity for 2-nonanone. Protein aggregation was visible (as higher turbidity) in the samples heated at 75 °C and higher. There was a further drop in binding at temperatures above 75 °C, which can be explained with the formation of larger β-lg aggregates, which are formed at temperatures above 72 °C *(Considine et al., 2005a)*. It has to be noted that slightly different experimental conditions (e.g., protein concentration, ionic strength) were used by Considine et al. *(2005a)* as compared to this study.

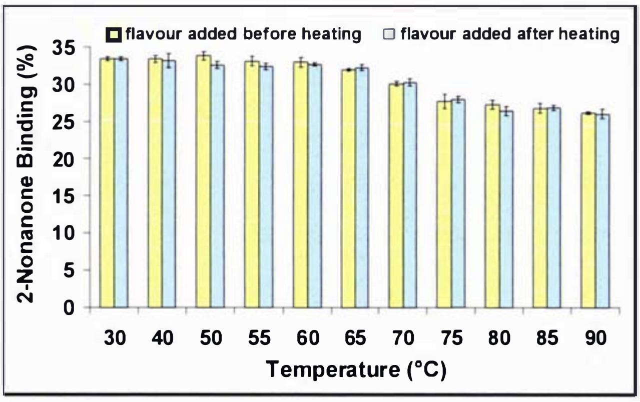


Figure 4.4-5: Binding of 2-nonanone (0.1 mM) to WPI (0.5 %) upon heat treatment for 12 min at temperatures between 30 and 90 °C (n = 3).

It is also likely that denaturation of BSA, which has an initial denaturation temperature of 64 °C *(Kinsella and Whitehead, 1989)*, contributes to the decrease in 2-nonanone binding. Due to its low binding affinity for 2-nonanone, α-la would not notably contribute to 2-nonanone binding in WPI.

4.4.2.2 Effect of Heating Time on the Binding of Flavour Compounds to WPI

4.4.2.2.1 2-Nonanone

Figure 4.4-6 shows the binding of 2-nonanone to WPI with increasing heating time at 80 °C. In the unheated sample, around 42 % of the 2-nonanone initially present was bound to the whey proteins. The binding of 2-nonanone decreased significantly ($P < 0.05$) after 2 min of heat treatment. Longer heating times showed a continuous decrease in binding of 2-nonanone, and after 80 min the binding was only 26 %. As observed earlier in section 4.4.2.1, the extent of binding was not influenced by the addition of 2-nonanone before or after heat treatment.

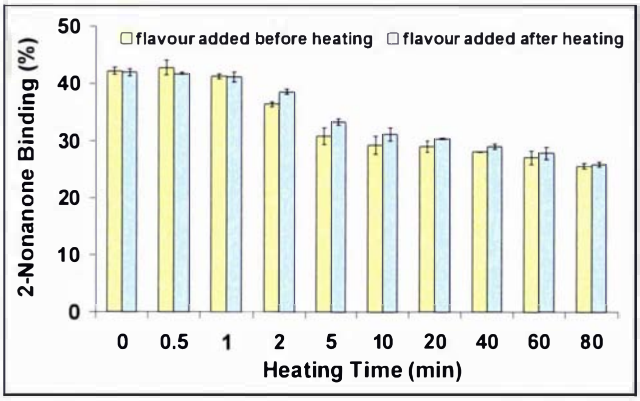


Figure 4.4-6: Binding of 2-nonanone (1.0 ppm) to WPI (0.5 %) with increasing heating time (80 °C) (n = 3).

Earlier (section 3.4.2.5) it was demonstrated that β-lg is the protein mainly responsible for flavour binding in WPI, and that it possesses one specific binding site for 2-nonanone at flavour compound concentrations up to 0.8 mM (~ 114 ppm). The overall decrease in binding with heat treatment may be explained by the increase in the extent of aggregation of unfolded β-lg molecules, making the flavour binding site inaccessible, and modifying the binding sites on other whey proteins, such as BSA, which was also found to contribute to 2-nonanone binding in solutions of WPI (section 3.4.2.5). Another explanation for the decrease in 2-nonanone binding with heat treatment may be that the exposure of previously buried hydrophobic residues leads to protein-flavour interactions being replaced by protein-protein interactions, resulting in a release of 2-nonanone.

A similar study by *O'Neill and Kinsella (1988)*, using equilibrium dialysis, also reported a decrease in 2-nonanone binding on β-lg upon heat treatment. In addition, these authors observed a high number of low-affinity binding sites on the denatured protein. The unfolding of β-lg obviously reveals previously buried hydrophobic binding sites which possess a lower affinity for 2-nonanone than the hydrophobic pocket.

Other authors *(Hansen and Booker, 1996)* suggested that the unfolding of β-lg upon heat denaturation resulted in an increase in the binding of hydrophobic flavour compounds. However, they used benzaldehyde, which may not only interact hydrophobically with proteins but also covalently via its aldehyde function.

Using size-exclusion chromatography, *Schokker et al. (1999)* followed the decrease in native β-lg monomers upon heat treatment at 78.5 °C for up to 60 min. Their results can be used to estimate the extent of denaturation during heat treatment in this study because the conditions used by *Schokker et al. (1999)* were similar (1.7 % protein concentration, pH 7.0, 78.5 °C, low salt). After 5 min of heat treatment, approximately 55 % of β-lg was still native. Heat treatment for 15 min resulted in less than 20 % native β-lg, and after 60 min, less than 10 % of the protein remained in the native state. Heat treatment at 80 °C for 80 min is therefore expected to result in no native whey protein left. The amount of binding of 2-nonanone to the denatured WPI is still considerable with around 26 % (Figure 4.4-6), and indicates that large aggregates of unfolded whey proteins are able to bind 2-nonanone.

4.4.2.2.2 1-Nonanal

The effect of heat denaturation on the binding of the corresponding aldehyde, 1-nonanal, is presented in Figure 4.4-7. In the native protein solution, 67 % of the 1-nonanal initially present was bound by the proteins, which is considerably higher than the binding of

2-nonanone of 42 %. When 1-nonanal was added after heat treatment of WPI at 80 °C, the extent of binding between the aldehyde and WPI did not change significantly over the whole range of heating times. However, when the flavour compound was added before the heat treatment, the extent of binding increased significantly ($P < 0.05$) after 20 min of heat treatment, as compared to no heating, but remained constant at longer heating times.

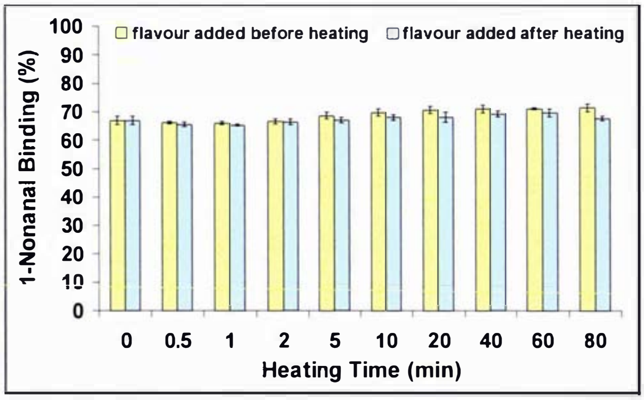


Figure 4.4-7: Binding of 1-nonanal (1.0 ppm) to WPI (0.5 %) with increasing heating time (80 °C) (n = 3).

These observations suggest a different binding mechanism as compared to 2-nonanone. Possibly, different binding sites exist for 2-nonanone, which showed a decrease in binding with increased denaturation of whey proteins, and 1-nonanal, which showed consistent binding. At neutral pH, aliphatic aldehydes, such as 1-nonanal, can interact both hydrophobically and covalently with proteins *(Mills and Solms, 1984)*. Since the binding involving hydrophobic interactions appears to decrease upon heat denaturation, as shown for 2-nonanone in Figure 4.4-6 and by *O'Neill and Kinsella (1988)*, it appears that covalent interactions via the aldehyde function may be increased due to denaturation, resulting in an overall unchanged binding of 1-nonanal with increasing heating time. 1-Nonanal can be bound on proteins both hydrophobically and via covalent reactions, and since hydrophobic interactions are decreased upon heat denaturation (as observed for 2-nonanone), covalent interactions must be increased. This is in agreement with the observations of *Mills and Solms (1984)* who suggested that an increase in temperature seems to enhance the binding of "reactive" flavour compounds, such as aliphatic aldehydes. The observed increase in binding after 20 min in the samples with 1-nonanal added before the heat treatment may be explained with the covalent reaction between the aldehyde function and amino acid residues, such as ε-amino groups, being favoured at the elevated temperature.

4.4.2.2.3 Trans-2-nonenal

The assumption that covalent interactions are facilitated on denatured proteins is supported by the results of the corresponding unsaturated aldehyde, *trans*-2-nonenal, for which a marked increase in binding upon heat treatment was observed (Figure 4.4-8). After only 1 min of heat treatment, the binding of *trans*-2-nonenal to the whey proteins increased significantly ($P < 0.05$). After 5 min of heat treatment, the binding was close to 100 %. This increase in *trans*-2-nonenal binding with denaturation of WPI may be due to covalent reactions of both the aldehyde function and the double bond with certain amino acid residues, such as lysine, histidine, arginine, and cysteine, which may be more readily accessible in the unfolded, aggregated proteins. *Bruenner et al. (1995)* found that 4-hydroxy-2-nonenal reacts with proteins predominantly via its double bond, whereas only trace amounts of Schiff base are formed. It is suggested that *trans*-2-nonenal also reacts with proteins primarily via its double bond. This is in agreement with results of the amino acid analysis, which revealed that amino acids other than lysine were modified by *trans*-2-nonenal (see Figure 4.4-4, page 116).

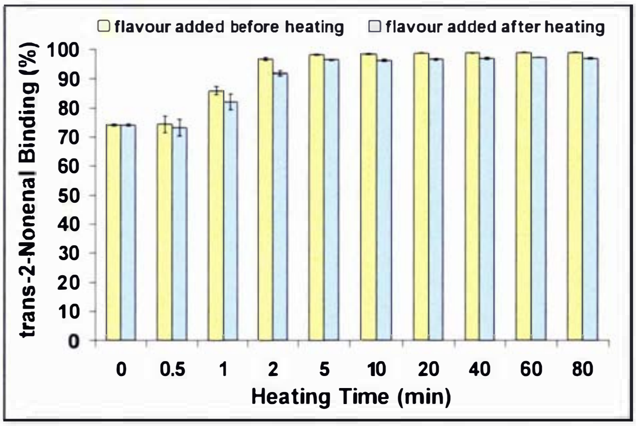


Figure 4.4-8: Binding of *trans*-2-nonenal (1.0 ppm) to WPI (0.5 %) with increasing heating time (80 °C) (n = 3).

The increase in binding after 1 and 2 min of heat treatment was significantly ($P < 0.05$) more pronounced when *trans*-2-nonenal was added before heat treatment as compared to after heat treatment. This may be explained by the covalent reaction of both the aldehyde function and double bond with amino acid residues being favoured at the elevated temperature.

Interestingly, a volatile by-product was formed in the samples with *trans*-2-nonenal added before heat treatment, eluting at t_r = 2.82 min (Figure 4.4-9 a). The additional peak was observed after 10 min of heat treatment at 80 °C. Using MS, this by-product was identified as being heptanal (Figure 4.4-9 b), with the typical peaks at *m/z* 114 (M^+), 96 (M^+-H_2O), 86 ($C_5H_{10}O$), 81 (M^+-H_2O-CH_3), 70 (C_5H_{10}), 55, and 44 *(Liedtke and Djerassi, 1969)*. After 80 min of heat treatment, approximately 4 % of the bound *trans*-2-nonenal was converted into heptanal.

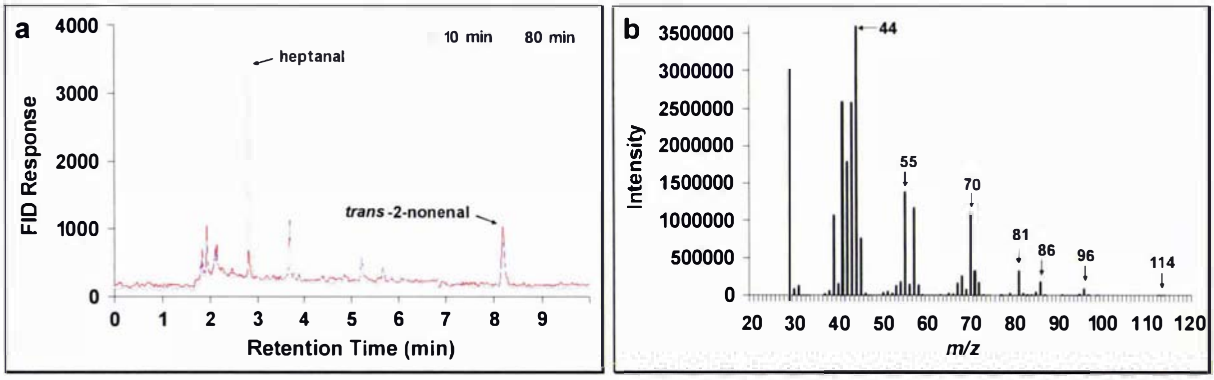


Figure 4.4-9: (a) Chromatogram of volatiles after heating WPI (0.5 %) and *trans*-2-nonenal (1.0 ppm) at 80 °C for 10 and 80 min, respectively; (b) mass spectrum of the heptanal peak (*m/z* 20-120).

Heptanal was not formed in samples with *trans*-2-nonenal added after heat treatment, indicating that heat is required for the formation of heptanal. The significantly more pronounced increase in binding when *trans*-2-nonenal was added before heat treatment as compared to after heat treatment (Figure 4.4-8) may therefore result from the conversion of *trans*-2-nonenal into heptanal. Heptanal was not present in samples heated in the presence of 1-nonanal, showing that the double bond is necessary to form heptanal. Heptanal did also not exist in standards containing *trans*-2-nonenal only in buffer (Figure 4.4-10), indicating that WPI needs to be present to produce heptanal.

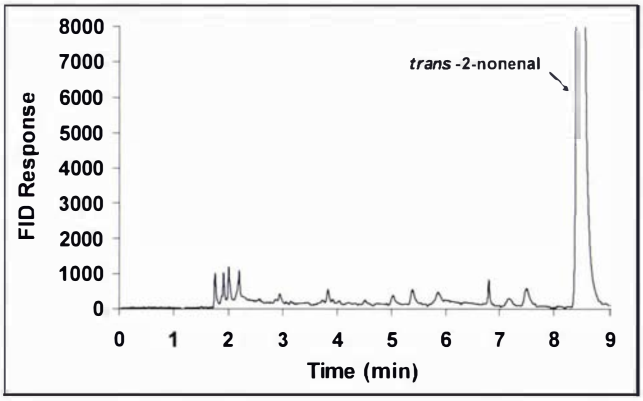


Figure 4.4-10: Chromatogram of volatiles after heating *trans*-2-nonenal (1.0 ppm) in phosphate buffer (0.1 M, pH 7.2) at 80 °C for 80 min.

If the formation of heptanal involved an oxidation reaction, the addition of antioxidants should reduce or prevent the heptanal formation. For this reason, the effect of ascorbic acid, which possesses antioxidant properties *(Yen et al., 2002)*, on heptanal formation was investigated. Different levels of ascorbic acid were added to solutions of WPI (0.5 %) containing *trans*-2-nonenal (1.0 ppm) before they were heat-treated at 80 °C for 60 min (Figure 4.4-11 a). The formation of heptanal was reduced with increasing ascorbic acid concentration, indicating an oxidative formation of heptanal. However, even with 0.1 % ascorbic acid added, the reduction in heptanal was around 60 % and not complete. Furthermore, slight browning in the samples with 0.1 % ascorbic acid was observed and may be caused by ascorbic acid degradation products such as furfural which may undergo polymerisation or react with amino acids in the proteins to form brown melanoid pigments *(Yuan and Chen, 1998)*.

The levels of *trans*-2-nonenal did not change significantly with increasing ascorbic acid concentration, indicating that the antioxidant does not prevent the degradation of *trans*-2-nonenal. If the heptanal formation was reduced, the *trans*-2-nonenal peaks should increase accordingly. No other peaks of intermediate products were present in the chromatogram. However, there may be intermediates which are not volatile or have no affinity for the PDMS fibre.

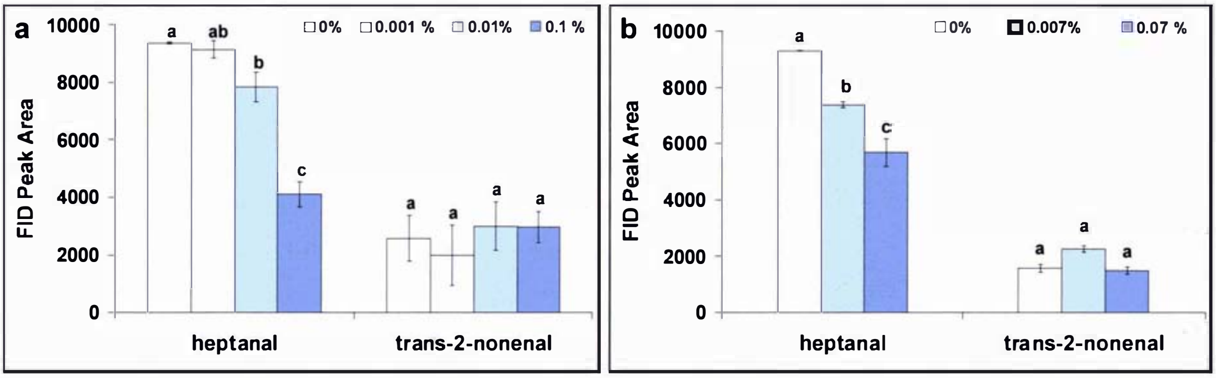


Figure 4.4-11: Effect of different levels of (a) ascorbic acid (0-0.1 %) and (b) gallic acid (0-0.07 %) on the FID peak areas of heptanal and *trans*-2-nonenal after the latter (1.0 ppm) was heated (60 min, 80 °C) in the presence of WPI (0.5 %) in phosphate buffer (26 mM, pH 7.2). For each individual flavour compound, different letters indicate significant ($P < 0.05$) differences between samples ($n = 2$).

Ascorbic acid did not completely prevent the formation of heptanal, and browning occurred in some of the samples; for this reason, the effect of gallic acid on heptanal formation was investigated (Figure 4.4-11 b). As for ascorbic acid, gallic acid decreased the formation of heptanal; however, only by about 40 %. Ascorbic acid showed a better effect on heptanal reduction than gallic acid. The samples containing 0.07 and 0.007 % gallic acid had a green colour, which turned into yellow-brown after heat treatment, indicating that like for ascorbic acid, side reactions may occur during heat treatment.

A third approach was the use of nitrogen gas which was introduced into the headspace of the sample vials before the heat treatment to reduce the amount of oxygen in the system (Figure 4.4-12). However, the formation of heptanal was not prevented or reduced, suggesting that a reaction other than oxidation is taking place.

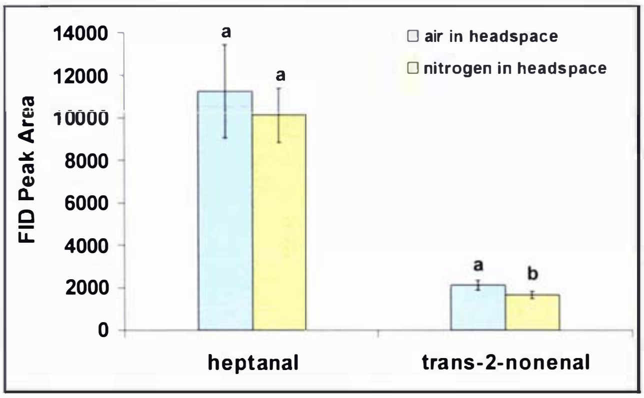


Figure 4.4-12: Effect of nitrogen in the headspace of heated (80 °C, 60 min) samples, containing WPI (0.5 %) and *trans*-2-nonenal (1.0 ppm), on the FID peak areas of heptanal and *trans*-2-nonenal (n = 3).

Since nitrogen, gallic acid, and ascorbic acid did not prevent the formation of heptanal, a covalent, chemical reaction may take place. As the addition of 1-nonanal to WPI solution did not form heptanal during heat treatment, a reaction of the double bond may be responsible for the formation of heptanal. However, reactions, such as the "Michael addition", form adducts with the proteins, and are not known to generate new compounds.

Heptanal was formed in the presence of WPI; thus, it was necessary to determine if the heptanal formation was associated with one of the individual milk proteins. For this reason, β-lg, BSA, and sodium caseinate were heated in the presence of *trans*-2-nonenal.

When a solution of β-lg (0.5 %) was heated for 60 min at 80 °C in the presence of *trans*-2-nonenal (1.0 ppm), heptanal was also formed (Figure 4.4-13 a). The binding of *trans*-2-nonenal to native β-lg was found to be 76 % of the *trans*-2-nonenal initially added, and the binding to heat-treated β-lg was 99 % which is similar to the binding found on WPI (74 and

99 %, respectively; Figure 4.4-8). The heptanal peak area is about as large as observed with WPI, but the integration in the case of β-lg samples is difficult since many small peaks elute close to the heptanal peak (Figure 4.4-13 a).

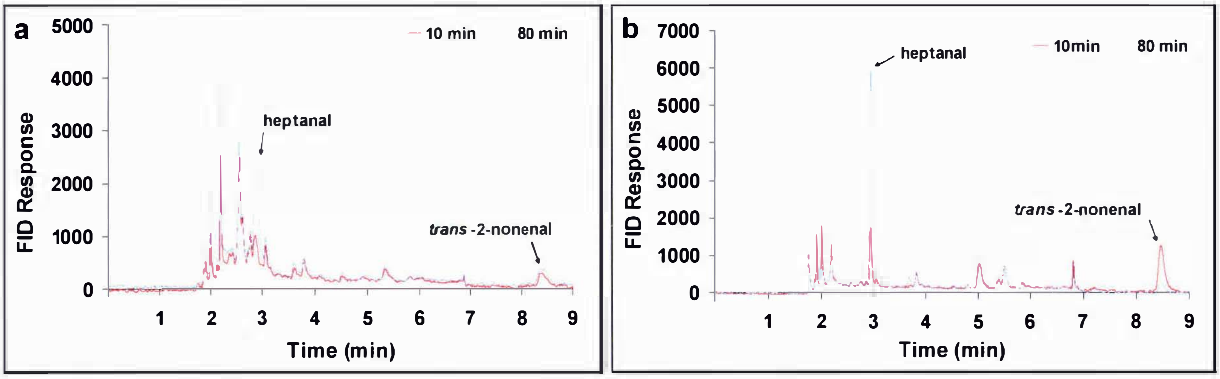


Figure 4.4-13: Chromatograms of volatiles after *trans*-2-nonenal (1.0 ppm) was heated at 80 °C for 10 and 80 min, respectively, in the presence of (a) β-lg (0.5 %), and (b) BSA (0.5 %).

The same experiment was performed using BSA (Figure 4.4-13 b), and as observed for β-lg, a substantial amount of heptanal was formed upon heat treatment. At $t_r = 5.1$ min, another volatile compound eluted which appears to be formed during heat treatment. However, this peak was also observed when BSA was heated alone (chromatogram not shown), and must therefore be generated by BSA or impurities in the BSA powder. BSA heated alone did not result in a heptanal peak.

Heptanal was formed in the presence of different whey proteins, and the question arises if heptanal is also formed in the presence of caseins. Figure 4.4-14 shows the GC chromatogram of samples containing sodium caseinate (2.0 %) and *trans*-2-nonenal (1.0 ppm) which were heated at 80 °C for 10 and 80 min, respectively.

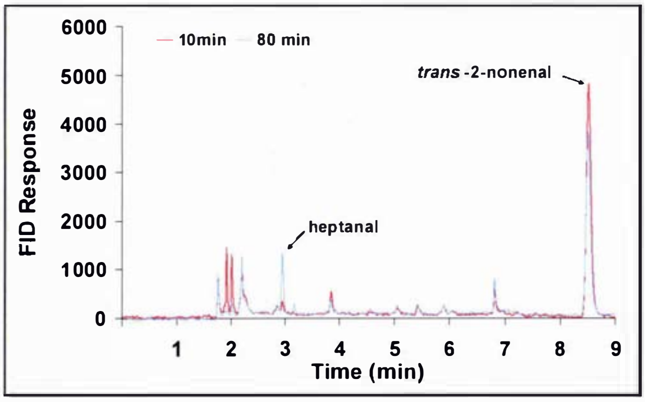


Figure 4.4-14: Chromatogram of volatiles after *trans*-2-nonenal (1.0 ppm) was heated at 80 °C for 10 and 80 min, respectively, in the presence of sodium caseinate (2.0 %).

As observed earlier for the whey proteins, heptanal was also formed in the presence of sodium caseinate. However, the formation of heptanal was not as pronounced as in the presence of the whey proteins which may be due to the generally lower flavour binding capacity of caseins.

These results confirm the formation of heptanal upon heating *trans*-2-nonenal in the presence of both casein and whey protein. This aspect was not investigated further in this study. Future work should include finding the mechanism of heptanal formation. For example, N-terminally blocked amino acids may be used instead of complex proteins to find out which amino acid residues in the proteins are involved in the formation of heptanal.

4.4.2.3 Near-UV CD

The CD technique was used to follow the heat denaturation of WPI, and to reveal possible effects of flavour compounds on the native protein structure.

The near-UV CD spectra of native WPI in the absence and presence of propylene glycol (PG) (1 %), 2-nonanone (14 ppm), and *trans*-2-nonenal (1 ppm) are presented in Figure 4.4-15. The spectrum showed two sharp troughs at 285 and 293 nm, which have been attributed to Trp19 in β-lg *(Manderson et al., 1999)*. These two bands have been observed before in studies on β-lg *(Considine et al., 2005a)*, and the wavelengths and intensities of these troughs indicate that Trp19 is in a chiral environment. Neither the presence of PG, nor the presence of 2-nonanone had an influence on the protein tertiary structure as the near-UV spectrum of WPI and 2-nonanone was similar to the spectrum of WPI without added 2-nonanone. In the case of *trans*-2-nonenal, there were slight differences in the spectrum as compared to WPI alone, indicating a change in structure in the presence of *trans*-2-nonenal.

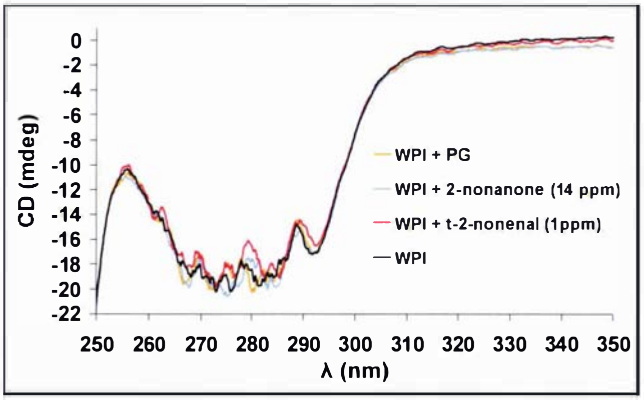


Figure 4.4-15: Near-UV CD spectra of WPI solutions between 250 and 350 nm in the absence and presence of propylene glycol (1 %), 2-nonanone (14 ppm), and *trans*-2-nonenal (1 ppm) at room temperature.

Figure 4.4-16 shows the near-UV CD spectra of native and heat-treated (80 °C, 20 min) WPI solutions in the absence and presence of 2-nonanone (14 ppm). The typical loss of the characteristic troughs of β-lg at 285 and 293 nm upon heat denaturation was observed, showing that Trp19 had moved to a less chiral environment, i.e., that the tertiary structure of the protein closely surrounding Trp19 had been modified. Differences between the spectra with and without 2-nonanone present during heat treatment are only minimal (between 265 and 285 nm) which means that 2-nonanone at 14 ppm does not influence the structure of the whey proteins during heat denaturation. This is in agreement with the finding that the extent of 2-nonanone binding was not influenced by flavour addition before or after heat treatment (section 4.4.2.2.1).

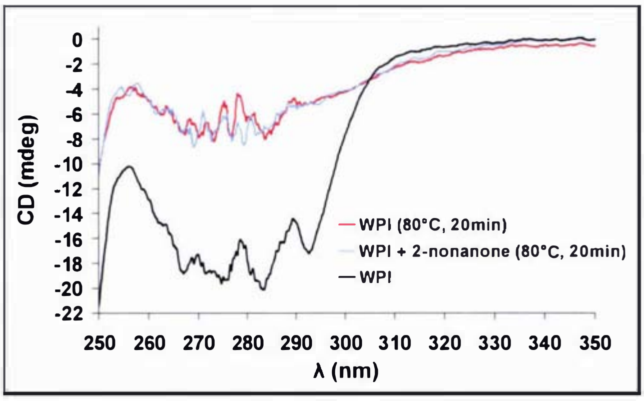


Figure 4.4-16: Near-UV CD spectra of native and heat-treated (80 °C, 20 min) WPI solutions between 250 and 350 nm in the absence and presence of 2-nonanone (14 ppm).

4.4.2.4 Far-UV CD

Far-UV CD spectra are indicative of the secondary structure of a protein and arise from the peptide bond absorption bands and the inherent chirality of the polypeptide chain *(Manderson et al., 1999)*. The far-UV CD spectra of native WPI in the absence and presence of 2-nonanone (14 ppm) and *trans*-2-nonenal (1 ppm) are presented in Figure 4.4-17. Neither the presence of 2-nonanone, nor the presence of *trans*-2-nonenal had an influence on the protein secondary structure as shown by far-UV spectra similar to the spectrum of WPI without added flavour compounds.

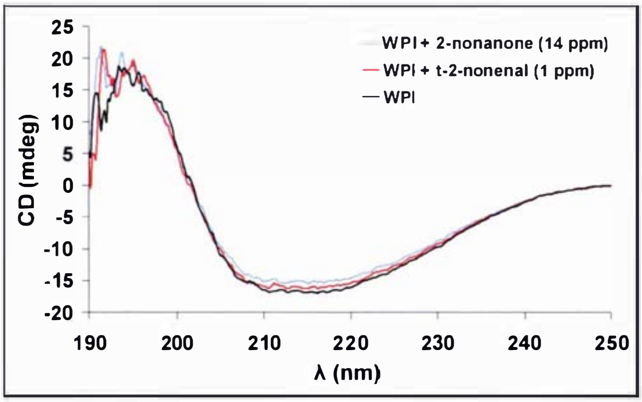


Figure 4.4-17: Far-UV CD spectra of WPI solutions between 190 and 250 nm in the absence and presence of 2-nonanone (14 ppm) and *trans*-2-nonenal (1 ppm) at room temperature.

The effect of heat treatment of WPI solutions in the presence or absence of 2-nonanone on the far-UV CD spectra is presented in Figure 4.4-18. The characteristic trough around 215 nm for the native WPI broadened and deepened with heat treatment, and the trough minimum shifted to lower wavelengths (207 nm). The spectra with and without added 2-nonanone are very similar, which means that 2-nonanone at 14 ppm does not alter heat-induced changes in the protein secondary structure.

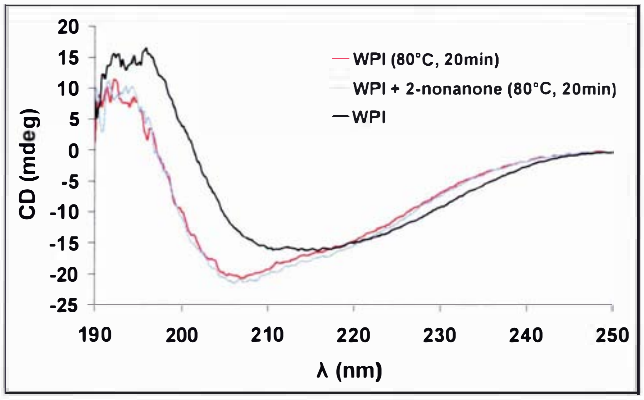


Figure 4.4-18: Far-UV CD spectra of native and heat-treated (80 °C, 20 min) WPI solutions between 190 and 250 nm in the absence and presence of 2-nonanone (14 ppm).

4.4.3 Effect of pH on Flavour Binding to WPI

Another factor which influences protein conformation, and thus potentially flavour binding to proteins, is the pH of the medium. The binding of 2-nonanone, 1-nonanal, and *trans*-2-nonenal to WPI at pH values of 4.0, 7.2, and 8.0 was determined. It was observed that

WPI partly precipitated in citrate-phosphate buffer (pH 4.0), which is common due to some denaturation of the whey proteins during the manufacturing process of WPI *(Etzel, 2004)*.

In the unheated WPI solutions, the binding of 2-nonanone increased significantly with increasing pH (Figure 4.4-19 a). This trend has been observed earlier for β-lg and methyl ketones *(Jouenne and Crouzet, 2000a)*. The lower binding at pH 4.0 in comparison to pH 7.2 can be explained with the hydrophobic pocket being closed at pH 6.1, with the EF loop (residues 85-90) covering the entrance of the pocket, while it is opened at pH values of 7.1 and 8.1 *(Qin et al., 1998)*. However, at pH 4.0, 30 % of the 2-nonanone initially present remained bound, suggesting that 2-nonanone is bound on one or more binding sites other than the hydrophobic pocket, such as the surface binding site described by *Monaco et al. (1987)* and *Lübke et al. (2002)*. In contrast, *Mills and Solms (1984)*, using static headspace analysis, observed the binding of 2-nonanone and low-fat whey protein to be about twice as high at pH 4.66 compared to pH 6.89, with no explanation given by the authors. They used sodium azide as a preservative which has been shown to influence flavour binding to proteins *(O'Keefe et al., 1991b; Farès et al., 1998; Reiners et al., 2000)*.

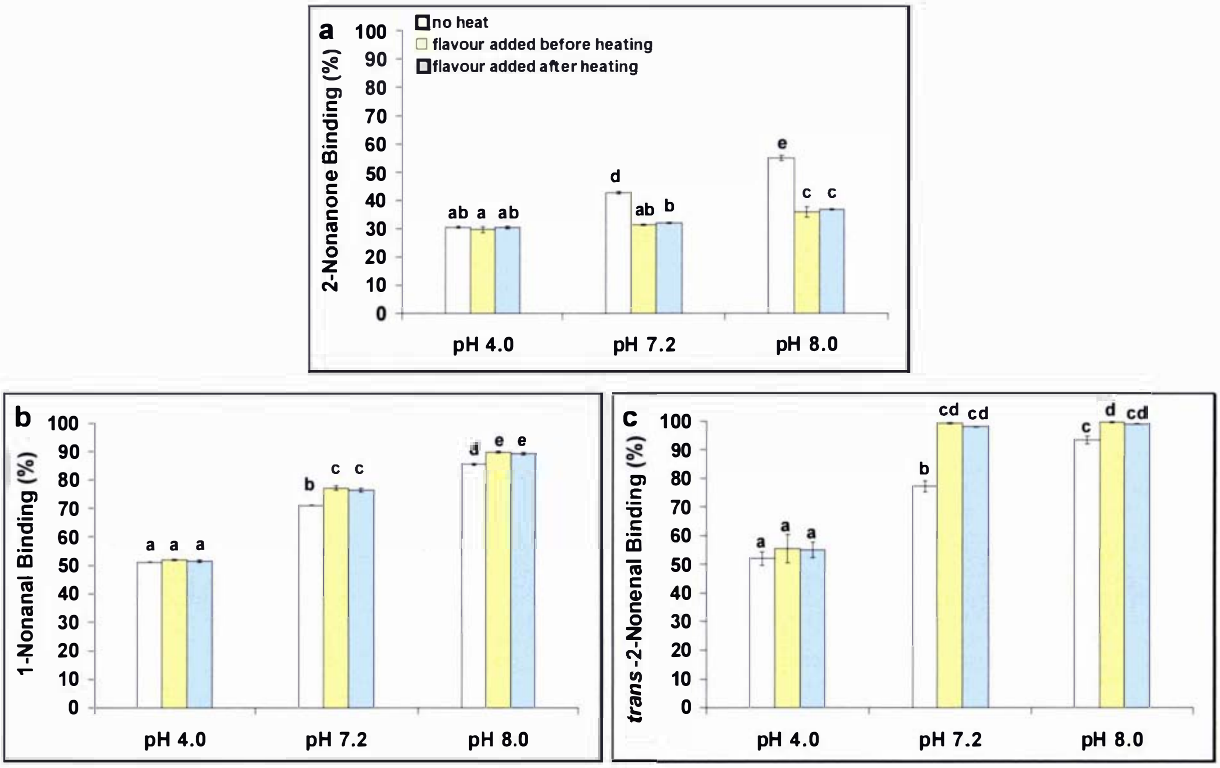


Figure 4.4-19: Effect of pH on the binding of (a) 2-nonanone, (b) 1-nonanal, (c) *trans*-2-nonenal at 1.0 ppm to unheated or heat-treated (80 °C, 5 min) WPI (0.5 %). Different letters indicate significant ($P < 0.05$) differences between samples (n = 3).

A further increase in binding of 2-nonanone and WPI was observed when the pH was raised from 7.2 to 8.0, which could be due to the dissociation of β-lg dimers accompanied by conformational changes at pH 7.5 ("Tanford transition"), characterised by the release of a buried carboxyl group, better accessibility of the free sulphydryl group, and a change in environment of a tyrosine residue *(Tanford et al., 1959; Hambling et al., 1992)*. These conformational changes may reveal additional, previously inaccessible binding sites for 2-nonanone.

Upon heat treatment, there was no release of 2-nonanone from the whey proteins at pH 4.0, indicating that the surface binding sites may not be affected by heat denaturation. At pH 7.2 and 8.0, 2-nonanone is released upon denaturation, possibly due to protein aggregation, as observed earlier (section 4.4.2.2.1). The amount of binding after 5 min of heat treatment at 80 °C appears to be very similar at the three pH values investigated. However, this is just a coincidence, e.g., the binding at pH 7.2 was shown to decrease further with longer heating times (section 4.4.2.2.1).

The binding of 1-nonanal and native WPI also increased with increasing pH and was 51 %, 71 %, and 86 % at pH 4.0, 7.2, and 8.0, respectively (Figure 4.4-19 b). Because aldehydes can react with, for example, lysine residues in proteins, higher pH values favour this covalent reaction due to less protonation of the amino groups and other basic amino acid residues. An increase in aldehyde binding with increasing pH was also observed by *van Ruth and Villeneuve (2002)* for the binding of β-lg and hexanal, heptanal, and octanal, and by *Mills and Solms (1984)* who observed less binding of heptanal to whey protein powder at pH 4.66 than at pH 6.89. *Weel et al. (2003)* found a general increase in the retention of aldehydes by whey proteins with higher pH values. However, with increasing length of the aldehyde carbon chain, the increase in binding with pH was less pronounced.

Upon heat treatment there was a slight increase in 1-nonanal binding at both pH 7.2 and 8.0, possibly resulting from the covalent reaction between the aldehyde function and basic amino acid residues, such as lysine or arginine, which may be better accessible in the unfolded protein molecules, as discussed earlier.

The binding of *trans*-2-nonenal and native WPI was 52 %, 77 %, and 93 % at pH 4.0, 7.2, and 8.0, respectively (Figure 4.4-19 c). As for 1-nonanal, the increase in binding of *trans*-2-nonenal to WPI is very likely to result from less protonation of basic amino acids at high pH values, and therefore a higher extent of covalent reactions, and from increased hydrophobic interactions due to structural changes of β-lg as discussed for 2-nonanone. Upon heat treatment, the binding increased at both pH 7.2 and 8.0, as observed for 1-nonanal, and again there was no change in binding at pH 4.0.

4.4.4 Effect of High Pressure Treatment on Flavour Binding to WPI

The effect of high pressure treatment (250 or 600 MPa, 30 min) on the binding of WPI and the three C9 flavour compounds, which were added either before or after high pressure treatment, was investigated. Pressures of 250 and 600 MPa were chosen for this study according to the three stage model proposed by *Considine et al. (2005b)*. In Stage I (0.1-150 MPa), the native structure of β-lg is stable; in Stage II (200-450 MPa), the native monomer is reversibly interchanging with non-native monomers and disulphide bonded dimers; and in Stage III (> 500 MPa), higher molecular weight aggregates of β-lg are formed. The samples treated at 600 MPa contained sediment of aggregated proteins, whereas the samples treated at 250 MPa remained clear. High pressure treatment at 250 MPa considerably increased the binding of *trans*-2-nonenal to WPI (Figure 4.4-20), while the binding of 1-nonanal and 2-nonanone was not affected at this pressure. Pressure treatment at 600 MPa significantly reduced the binding of 2-nonanone to WPI, further increased the binding of *trans*-2-nonenal, but had no effect on the binding of 1-nonanal.

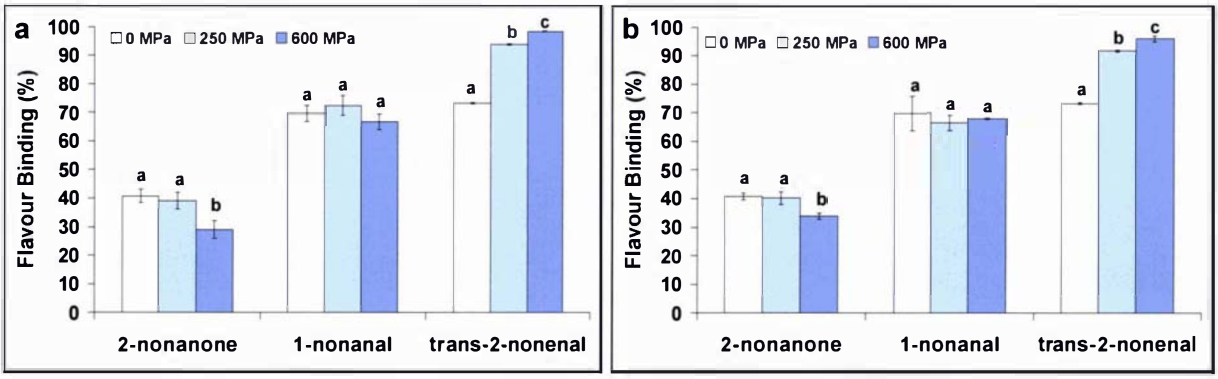


Figure 4.4-20: Effect of high pressure treatment (250 and 600 MPa, 30 min) on the binding of 2-nonanone, 1-nonanal, and *trans*-2-nonenal to WPI (0.5 %) at pH 7.2; (a) flavour added before high pressure treatment, (b) flavour added after high pressure treatment. For each individual flavour compound, different letters indicate significant ($P < 0.05$) differences between samples (n = 3).

In general, high pressure treatment appears to have the same effect on the protein-flavour interactions as heat treatment (section 4.4.2.2); i.e. a decrease in 2-nonanone binding, unchanged binding of 1-nonanal, and an increase in *trans*-2-nonenal binding. This was expected as both heat and high pressure treatment are known to denature whey proteins. In the case of 2-nonanone, the reduction in binding is more pronounced upon heat treatment than upon high pressure treatment. This results from the heating conditions causing a higher extent of denaturation than the high pressure treatment, i.e., the reduction in β-lg monomer is approximately 60 % after β-lg has been heat-treated at 80 °C for only 12 min *(Considine et al., 2005a)*, while high pressure treatment at 250 or 600 MPa (30 min, pH 7.2, 20 °C) resulted in only 22 and 50 % reduction in β-lg monomers *(Considine et al., 2005b)*. Therefore, samples heat-treated at 80 °C for 10 min are fairly well comparable with samples pressure-treated at 600 MPa.

Under comparable experimental conditions, *Yang et al. (2003)* found that high pressure treatment (600 MPa, 32 min, 50 °C) of β-lg (27 μM in 0.01 M phosphate buffer, pH 7.0) decreased the affinity of the flavour compound capsaicin, while the binding affinity of α-ionone, β-ionone, cinnamaldehyde, and vanillin remained unaffected by high pressure denaturation. Similar results were obtained by *Liu et al. (2005b)*, who observed either no effect or decreased binding of benzaldehyde and methyl ketones (2-heptanone, 2-octanone, 2-nonanone) on high pressure treatment (600 MPa, 10 and 30 min, 50 °C) of whey protein concentrate (WPC), depending on the type and concentration of flavour compound, and on the duration of pressurisation. Both studies confirm that the structure of the flavour compound determines its binding affinity on proteins under high pressure.

In more complex systems, such as foods, high pressure induced denaturation largely depends on the medium. For example, the denaturation of β-lg upon high pressure treatment at 600 MPa for 30 min (20 °C) was more pronounced in milk (95 ± 4.1 %) than in whey (60.8 ± 2.8 %) *(Huppertz et al., 2004b)*. The authors explained this finding due to the absence of casein micelles in whey, resulting in a lower number of molecules available for protein-protein interaction. Therefore, the extent of flavour binding is also expected to depend on the medium.

As observed for heat treatment, there was no significant difference in binding between the samples with flavour added before and after high pressure treatment. In contrast to the heat treated samples, there was no heptanal formation in the samples with *trans*-2-nonenal added before pressure treatment. To avoid the formation of new flavour compounds, high pressure treatment is therefore the preferred technique of food treatment and preservation as opposed to heat treatment.

4.4.5 Competition with SDS

The preferential binding site of SDS on β-lg is believed to be the hydrophobic pocket *(Creamer, 1995)*. A competition study between flavour compounds and SDS may therefore give an indication to what extent the flavour compounds are bound in the hydrophobic cavity of β-lg. In addition, the competition study may also indicate the extent of reversible and irreversible binding.

4.4.5.1 2-Nonanone

The competition of 2-nonanone and SDS for β-lg binding sites was investigated using a molar ratio of 2:1:2 (β-lg:2-nonanone:SDS). Figure 4.4-21 shows the binding of 2-nonanone and β-lg in the absence and presence of SDS at both pH 4.0 and 7.2. In samples at pH 4.0, the addition of SDS resulted in precipitation. Binding of 2-nonanone (0.1 mM) to β-lg (0.2 mM) was 24 % and 36 % at pH 4.0 and 7.2, respectively. The lower binding at pH 4.0 has been discussed in section 3.9.3. Upon addition of SDS, the binding of 2-nonanone decreased markedly at both pH values. In the presence of SDS, the binding of 2-nonanone to β-lg was higher at pH 4.0 as compared to pH 7.2, whereas in the absence of SDS, the binding was significantly higher at pH 7.2. At pH 4.0, the binding of both 2-nonanone and SDS must be on the surface of β-lg because the hydrophobic pocket is closed by the EF loop at low pH *(Qin et al., 1998)*. Also more surface binding sites for the flavour compound may be present at pH 4.0 as compared to pH 7.2. This is in agreement with the findings by *Shimizu et al. (1985)* who reported an increase in surface hydrophobicity of β-lg with decreasing pH.

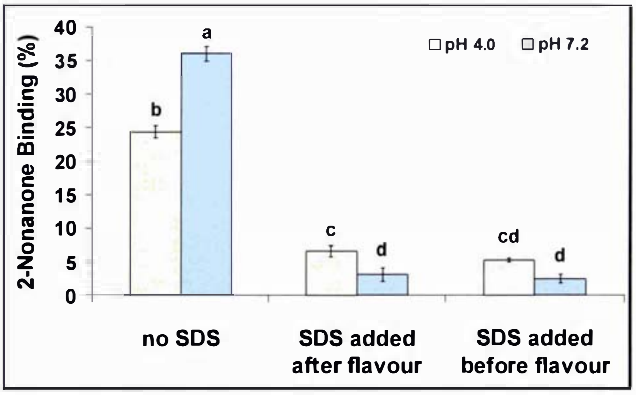


Figure 4.4-21: Binding of 2-nonanone (0.1 mM) to β-lg (0.2 mM) in the absence of SDS, and with SDS (0.2 mM) added before and after 2-nonanone, at both pH 4.0 and pH 7.2 (n = 3).

At pH 7.2, 2-nonanone was almost completely released from β-lg after SDS was added. The residual binding of 2-nonanone in the presence of SDS results from the reasonably high binding affinity of 2-nonanone with β-lg ($K = 2{,}700\ M^{-1}$), as determined in section 3.4.2.1. *Ray and Chatterjee (1967)* reported three high-affinity binding sites for SDS per β-lg dimer (pH 7.5) with binding constants of $K_1 = 3.6 \times 10^5\ M^{-1}$, and a large number of secondary binding sites (≈ 28) with $K_2 = 7 \times 10^3\ M^{-1}$. The binding of 2-nonanone in the presence of SDS was approximately 12 times lower than in the absence of SDS (Figure 4.4-21) which can be related to the primary binding constant of SDS being approximately 13 times higher than that of 2-nonanone.

The displacement of 2-nonanone by SDS suggests that 2-nonanone may be bound in the hydrophobic pocket at pH 7.2 because the preferential binding site for SDS on β-lg is believed to be the hydrophobic pocket *(Creamer, 1995)*. The residual binding of 2-nonanone in the presence of SDS is believed to take place on the surface of β-lg. However, it is very likely that SDS displaced 2-nonanone to some extent from the surface binding sites, which is in agreement with the data obtained at pH 4.0 (Figure 4.4-21).

The order of SDS addition, e.g., SDS added after or before 2-nonanone, had no significant effect on the 2-nonanone binding. This was expected because the binding is completely reversible and equilibrium should be reached.

4.4.5.2 1-Nonanal and *trans*-2-Nonenal

An SDS displacement experiment using 1-nonanal and *trans*-2-nonenal can indicate the extent of irreversible binding of these flavour compounds on proteins. Figure 4.4-22 shows the binding of the aldehyde flavours to β-lg in the absence and presence of SDS. There was no significant decrease in 1-nonanal binding after SDS addition, indicating that 1-nonanal may be bound largely irreversibly to β-lg, or that SDS binds at different sites than 1-nonanal. Because SDS is believed to be preferentially bound in the hydrophobic pocket of β-lg *(Creamer, 1995)*, the preferential binding site for 1-nonanal appears not to be the hydrophobic pocket. As 2-nonanone was displaced by SDS, 1-nonanal must be bound via a different binding mechanism or on different binding sites than 2-nonanone.

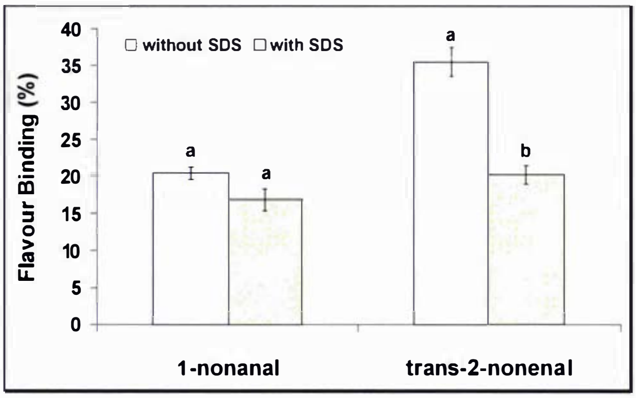


Figure 4.4-22: Displacement of aldehyde flavours (0.007 mM) on β-lg (0.014 mM) by SDS (0.014 mM) at pH 7.2. For each individual flavour compound, different letters indicate significant differences ($P < 0.05$) between samples ($n = 3$).

Interestingly, almost 50 % of the *trans*-2-nonenal initially bound to β-lg was replaced by SDS, indicating a higher extent of reversible binding compared to 1-nonanal, although 1-nonanal is more hydrophobic than *trans*-2-nonenal. The unsaturated flavour compound may be bound on two different binding sites of which one is hydrophobic interaction and thus replaced by SDS, and the other is irreversible binding and may even be identical to the sites that bind 1-nonanal because the residual binding after SDS addition was fairly similar for the two aldehydes. These results indicate that in addition to covalent binding there is some contribution from non-covalent binding of *trans*-2-nonenal on β-lactoglobulin.

4.5 Conclusions

This study further elucidated the effects of flavour compound structure, pH, and heat and high pressure treatment on the interactions of whey proteins and flavour compounds. The binding between native WPI and the three flavour compounds investigated was strong and decreased in the order *trans*-2-nonenal > 1-nonanal > 2-nonanone. The differences in binding can be explained with hydrophobic interactions only in the case of 2-nonanone, whereas the aldehydes, in particular *trans*-2-nonenal, can also interact covalently.

Heat and high pressure treatment affected protein-flavour interactions depending on the structure of the flavour compound. Upon both heat- and pressure-induced denaturation, the binding of 2-nonanone and WPI decreased, while the binding of 1-nonanal remained unchanged, and the affinity for *trans*-2-nonenal increased rapidly. From these observations it can be concluded that the three flavour compounds investigated are bound on proteins on different binding sites and/or by different binding mechanisms. This was also confirmed by the SDS displacement study: 2-nonanone was almost completely replaced by SDS, 1-nonanal was not replaced, and *trans*-2-nonenal was partly replaced by SDS.

The results also suggest that heat- or pressure-induced denaturation reduce hydrophobic interactions of whey proteins with flavour compounds due to conformational changes in the flavour binding sites, whereas covalent interactions are enhanced. The decrease in binding of 2-nonanone to WPI upon denaturation can be explained with the destruction of the hydrophobic pocket of β-lg, and with protein-flavour interactions being replaced by protein-protein interactions. The increase in binding of *trans*-2-nonenal may result from mainly covalent binding to amino acid residues, such as lysine, histidine, arginine, or cysteine, which may be better accessible in the unfolded, aggregated protein molecules. The unchanged binding of 1-nonanal to WPI with heat or high pressure treatment is likely to result from a combination of both a decrease in binding due to the destruction of the hydrophobic pocket, and an increase in binding caused by better accessibility of amino acid residues for covalent interactions, resulting in an overall unchanged binding.

The observed effects of heat and high pressure treatment on protein-flavour interactions depend on the medium, e.g., the type and molarity of the buffer system are known to influence high pressure induced aggregation of β-lg *(Funtenberger et al., 1995)*. However, the trends in protein-flavour binding, e.g., an increase in binding of *trans*-2-nonenal, and a decrease in binding of 2-nonanone, are very likely to not only occur in simple model systems as used in this study, but also in protein containing foods despite their complexity.

None of the three flavour compounds investigated showed a stabilising effect on the native structure of the whey proteins against denaturation; however, they may stabilize the native conformation at higher flavour compound concentrations.

The formation of heptanal upon heating *trans*-2-nonenal in the presence of milk proteins demonstrated that new flavour compounds may be formed during heat treatment under certain conditions, e.g., in the presence of an unsaturated flavour compound. However, the mechanism of heptanal formation is still unclear and requires further investigation. Heptanal was not formed upon high pressure treatment.

Heat and high pressure treatment may therefore notably influence the overall flavour profile of protein containing foods. Since the protein concentration used in this study was fairly low, the observed effects are expected to be even more pronounced in real food systems. The impact of denaturation on protein-flavour interactions is of practical significance in food formulations, especially in foods that require pasteurisation or UHT treatment, e.g., flavoured yoghurt or dairy beverages.

The pH of the medium was found to have a significant effect on the extent of protein-flavour interactions. For all three flavour compounds investigated, the binding affinity to WPI increased with increasing pH from 4.0, to 7.2 and 8.0. This observation results from different pH dependent conformational changes of whey proteins, e.g., the closure of the hydrophobic pocket of β-lg at low pH. In addition, the protonation of basic amino acid residues, which are very likely to react covalently with aldehyde flavours, changes with pH. Therefore, covalent interactions between proteins and flavour compounds are favoured at higher pH values.

The significant influences of heat, high pressure, and pH on protein-flavour interactions observed in this study emphasize a careful choice of food processing conditions to obtain a desirable flavour profile. Any changes made in the processing of a food product are very likely to change the flavour profile, and possibly the flavour perception and consumer acceptance of a food product.

CHAPTER FIVE: IN-MOUTH FLAVOUR RELEASE FROM PROTEIN CONTAINING SYSTEMS

5.1 Abstract

A method using proton-transfer-reaction mass spectrometry (PTR-MS) was developed to measure the retronasal flavour (2-nonanone) release from aqueous solutions of whey protein isolate (WPI) and sodium caseinate *in vivo*. The influence of the thickener, sodium carboxymethylcellulose (CMC), on the 2-nonanone release was also investigated.

The type of protein influenced both static *in vitro* and dynamic *in vivo* 2-nonanone concentrations significantly. WPI showed a higher retention than sodium caseinate under both static and dynamic conditions.

During the consumption of protein-flavour solutions, a significant release of 2-nonanone from WPI was observed, whereas there was no significant release of the flavour from sodium caseinate. Even after swallowing of the samples, a substantial amount of flavour was detected in the breath, suggesting that the milk proteins interact with the mucosa in the mouth and throat, resulting in a further release of flavour from mucosa-bound proteins.

In the presence of CMC, a significant release of 2-nonanone from not only WPI but also sodium caseinate was observed. The increase in viscosity did not appear to slow down flavour release; in fact, it enhanced the release of 2-nonanone from the milk proteins. This may be due to a thicker coating of the mucosa with the sample solution after swallowing of the sample due to the higher viscosity, resulting in a higher amount of sample remaining in the mouth and throat for further release of protein-bound flavour.

5.2 Introduction

In Chapters 3 and 4, the binding of flavour compounds to proteins was investigated. The question arises what impact these interactions have on flavour release and flavour perception. If reversibly bound flavours, such as 2-nonanone, are not or only to a small extent, released from proteins during consumption, the perceived flavour would be significantly reduced. For this reason the in-mouth release of 2-nonanone from milk proteins in aqueous solutions was investigated in this final chapter.

There is little information on flavour release from protein containing systems in the literature. It has been shown that milk proteins reduce the in-mouth release of flavour compounds

(Denker et al., 2006). However, only two studies *(Le Guen and Vreeker, 2003; Weel et al., 2003)* investigated if, and to what extent, different flavour compounds are released from the proteins during consumption. Aliphatic aldehydes were found to be partially released from WPI in-mouth *(Weel et al., 2003)*. *Le Guen and Vreeker (2003)* reported that methyl ketones were completely released from milk protein concentrate (MPC) during consumption, whereas alkenals, which are bound irreversibly by proteins, were not released. Unfortunately, the above studies used different sample consumption protocols, making a comparison of results difficult.

A number of studies investigated the effect of viscosity or gel hardness on flavour release and perception. Generally, an increase in viscosity or gel hardness decreased the flavour perception *(Jaime et al., 1993; Guinard and Marty, 1995; Carr et al., 1996; Wilson and Brown, 1997; Hollowood et al., 2002)*; however, the actual flavour concentrations in the breath, as determined by *in vivo* nose-space measurements, were not reduced with increasing viscosity of the sample matrix *(Baek et al., 1999; Hollowood et al., 2002; Weel et al., 2002)*.

Thus, the aim of this study was to investigate the release of the reversibly bound flavour compound, 2-nonanone, from WPI and sodium caseinate *in vivo* using PTR-MS, a technique that can be used to quantify volatile flavour compounds in the breath of people in real-time during the consumption of food. 2-Nonanone is bound completely reversibly to proteins *(Mills and Solms, 1984)*, and thus may be released from proteins in-mouth. In additon, CMC was added to understand the effect of viscosity on the flavour release *in vivo*. To date, systems containing both protein and thickener have not been investigated in terms of flavour release.

5.3 Materials and Methods

5.3.1 Materials

WPI (Alacen 895) and sodium caseinate (Alanate 180) were obtained from Fonterra Co-operative Group (New Zealand). WPI consisted of 93.3 % protein (w/w), 0.59 % fat, 5.3 % moisture, and 1.9 % ash, and sodium caseinate was composed of 91.5 % (w/w) protein, 0.6 % fat, 4.7 % moisture, and 3.6 % ash. α-Lactalbumin (α-la), β-lactoglobulin AB (β-lg), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA).

2-Nonanone was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), and was of > 99 % purity. The thickener CMC (Walocel CRT 30000 PA) was obtained from Wolff Walsrode AG (Walsrode, Germany). Its degree of substitution was 0.82-0.95, and the viscosity (1 % solution) was 3000-4000 mPas.

5.3.2 *In Vitro* Flavour Release

Samples of 2-nonanone and β-lg, α-la, BSA, WPI, and sodium caseinate were prepared according to Table 5.3-1 to obtain approximately 40 % of binding. Of a 10 mM 2-nonanone stock solution (see section 3.3.1) 100 μl was added to each protein solution to obtain a final 2-nonanone concentration of 0.1 mM. Samples (without lids) were left in a 30 °C room for different times (t = 0, 20, 60, 120, and 180 min). Sub-samples (1.0 ml) were taken and analysed by SPME-GC-FID (see sections 4.3.11 and 4.3.12).

Table 5.3-1: Preparation of protein solutions for the *in vitro* flavour release experiment.

Protein	g / 10 ml water
α-La	0.0950
β-Lg	0.0555
BSA	0.0340
WPI	0.0666
Sodium caseinate	0.2860

5.3.3 Determination of Flavour Binding by SPME-GC-FID

Headspace SPME was used to determine the extent of binding of 2-nonanone (1 ppm) and WPI and sodium caseinate (2 %), with and without the addition of CMC (0.5 %), under static conditions. Samples were prepared by weighing 6.860 (± 0.001) g sample solution in a 20 ml headspace vial (20 CV, Chromacol, Herts, UK), and adding 140 μl of flavour stock solution. Standards for quantification of the free flavour were prepared in water. The vials were immediately sealed with blue silicone/PTFE septa (20-ST3) and magnetic tin crimp caps (20-MCB, Chromacol, Herts, UK), and equilibrated for 40 h at 5 °C. The samples were brought to room temperature before analysis by automated headspace SPME.

5.3.3.1 SPME

The details of the automated headspace SPME method are described in section 4.3.11.

5.3.3.2 Gas Chromatography

The details of the GC method are described in section 4.3.12.

5.3.4 *In Vivo* Flavour Release by PTR-MS

5.3.4.1 Preparation of Solutions

A flavour stock solution (50 ppm) was prepared in water by dissolving 60.9 μl 2-nonanone in 1,000 ml water. WPI and sodium caseinate were dissolved in water by gentle stirring for 2 h at ambient temperature to obtain concentrations of 2.0 % (w/v). To prepare viscous samples, CMC was dissolved in the protein solutions at a concentration of 0.5 % (w/v). Viscous and non-viscous protein solutions were stored at 5 °C overnight for complete hydration before the addition of flavour stock solution. In 12 ml glass vials with screw caps and teflon lids (Kimble, Vineland, NJ, USA), 9.8 g sample solution (WPI or sodium caseinate, with and without CMC) and 200 μl 2-nonanone stock solution were combined to obtain a final flavour concentration of 1 ppm. Higher 2-nonanone concentrations were not required because of the high sensitivity of the PTR-MS system. After the flavour was added, the samples were equilibrated for 40 h at 5 °C, and returned to room temperature before the PTR-MS assessment.

Control samples were prepared in the same way, containing the flavour compound at the concentrations given in Table 5.3-2; however, no WPI or sodium caseinate was added. Two controls were prepared. "Control 1" represented no release and had 2-nonanone in water at a concentration equal to the free flavour concentration in the protein containing sample, as determined by SPME-GC-FID in section 5.3.3. The second control corresponded to 100 % release ("control 2"), and thus contained 1 ppm of 2-nonanone in water. An example of the composition of the controls is illustrated in Figure 5.3-1.

Table 5.3-2: Added volumes of 2-nonanone stock solution (50 ppm), and total and free 2-nonanone concentrations in the protein containing samples and in the controls.

Sample	V_{stock} (μl)	$\|c\|_{total}$ (ppm)	$\|c\|_{free}$ (ppm)
WPI			
WPI containing sample	200	1.00	0.34
Control 1	68	0.34	0.34
Control 2	200	1.00	1.00
Sodium caseinate			
Na-caseinate containing sample	200	1.00	0.72
Control 1	144	0.72	0.72
Control 2	200	1.00	1.00

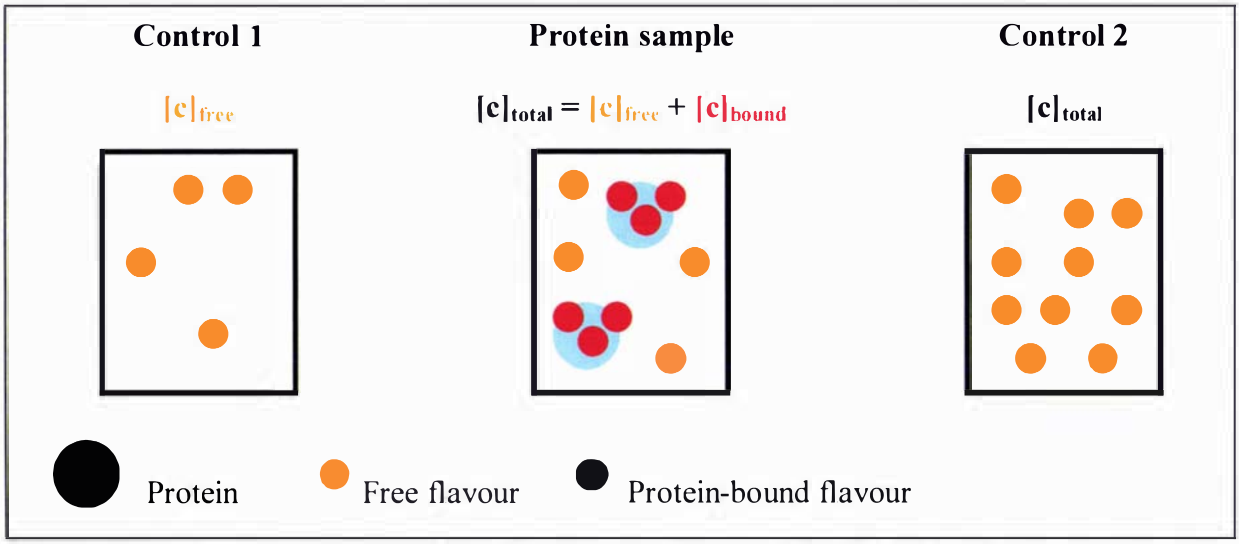


Figure 5.3-1: Example for concentrations of 2-nonanone in controls 1 and 2.

5.3.4.2 Sample Consumption Protocol

Three panellists (two females and one male) assessed all samples in five replicates. Each panellist tested 10 samples per day over five days.

The panellists breathed into a U-shaped glass nose piece inserted into their nostrils (see Figure 2.5-1, page 37), from which continuously air was sampled directly into the PTR-MS source. They were asked to inhale through their mouth, and exhale through their nose. The sampling protocol started with breathing naturally for 15 sec, followed by taking 10 ml of sample in the mouth through a drinking straw. Five circles of inhalation-exhalation followed, while during each exhalation the sample was swirled around in the mouth. Then the entire sample was swallowed, followed by an exhalation. The panellists continued to breathe in and out until a total recording time of 120 sec was reached. After each measurement the mouth was rinsed with water twice.

5.3.4.3 Measurement of Flavour Release by PTR-MS

Flavour concentrations in the breath of the panellists were monitored by on-line sampling part of the exhaled air by the PTR-MS (Ionicon Analytik GmbH, Innsbruck, Austria). During breathing, the air was drawn into the PTR-MS at a flow rate of 300 $\mathrm{ml{\cdot}min^{-1}}$ through a transfer line that was heated (80 °C) to prevent condensation. The drift voltage was 600 V. Other settings are summarized in Table 5.3-3.

2-Nonanone was analysed in selected ion mode at an *m/z* value of 143. In addition, the ion *m/z* 21 was observed to monitor the performance of the instrument, and to calculate the 2-nonanone concentrations in the breath (section 5.3.4.4). Acetone (*m/z* 59) was also measured as a breath indicator as it is present naturally in the breath. The dwell time for each volatile compound was 0.1 sec. The software used was “PTR-MS Control” (Ionicon Analytik GmbH, Innsbruck, Austria).

Table 5.3-3: PTR-MS operating conditions.

Parameter	Setting
Reaction chamber pressure	2.2 mbar
Detection chamber pressure	$2.5 \cdot 10^{-5}$ mbar
Reaction chamber temperature	80 °C
Water vapour flow	6.0 sccm
Ion source current	7.5 mA
Detector voltage	2700 V

5.3.4.4 Calculation of the Flavour Concentration in the Breath

PTR-MS systems do not have to be calibrated with gas standards prior to each measurement sequence as well known parameters in the reaction chamber allow the quantification of analytes.

To calculate the flavour concentration (in ppbV) from the count rate (in counts per second, cps), the following equation was used *(Lindinger et al., 1998)*:

$$[R]_{ppbV} = \frac{10^9}{k \cdot t_{rea}} \cdot \frac{[R^+]}{[H_3O^+]} \cdot K_B \cdot \frac{T_{drift}}{p_{drift}} \qquad \textbf{(5-1)}$$

where:

10^9	factor used to convert to ppbV
k	reaction rate constant, which differs slightly between compounds but values for compounds of interest to food aroma are very close to $2 \cdot 10^9$ cm^{3}sec^{-1}
t_{rea}	reaction time in the drift tube, which is typically around $105 \cdot 10^{-6}$ sec
$[R^+]$	cps measured for the compound of interest; has to be corrected for transmission

$[H_3O^+]$	cps measured for *m/z* 21, multiplied by 500 to obtain cps for *m/z* 19
K_B	Boltzmann constant ($1.38 \cdot 10^{-23}$ $m^2 \cdot kg \cdot sec^{-2} \cdot K^{-1}$)
T_{drift}	temperature in the drift tube (K), e.g., 80 °C = 353.15 K
p_{drift}	pressure in the drift tube (2.28 mbar)

The concentration of flavour in ppbV is proportional to the ratio between cps of the flavour compound of interest $[R^+]$ and cps of the primary ion $[H_3O^+]$, and inversely proportional to the reaction rate constant k and the reaction time t_{rea}.

It is better to measure *m/z* 21 and then convert into *m/z* 19 for the life of the secondary electron multiplier (SEM), and to avoid underestimation of $[H_3O^+]$ due to saturation of the SEM. The ratio between $[H_3{}^{16}O^+]$ (*m/z* 19) and $[H_3{}^{18}O^+]$ (*m/z* 21) is close to 500, therefore, the value in cps of *m/z* 21 must be multiplied by 500. Instrumental transmission coefficients also need to be taken into account. The transmission corrections for the masses 21 and 143 are 0.48627 and 0.39625, respectively.

5.3.4.5 Data Analysis of the PTR-MS Release Profiles

From the PTR-MS release profiles, the maximum 2-nonanone concentration reached (I_{max}), the time taken to reach the maximum concentration (t_{max}), and the area under the release curve (*AUC*) were determined (Figure 5.3-2). The values for *AUC* were calculated using the baseline function in the graphic programme Origin 7.5 (OriginLab Corp., Northampton, MA, USA), and they were used as a measure of the amount of 2-nonanone released.

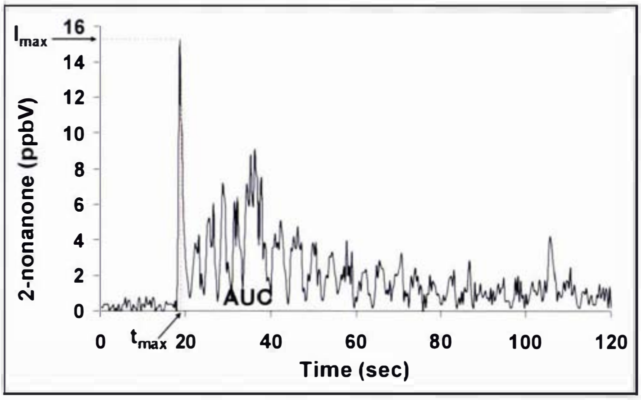


Figure 5.3-2: Parameters (I_{max}, t_{max}, *AUC*) for the analysis of the PTR-MS release profiles.

5.3.5 Statistical Analysis

The parameters of the flavour release profiles were subjected to univariate analysis of variance (ANOVA). Statistical significance was at $P < 0.05$ and if a significant effect was found a Tukey's post-hoc test was performed. SPSS 14.0 for Windows software (Chicago, IL) was used for statistical evaluations.

5.4 *Results and Discussion*

5.4.1 *In Vitro* Release of 2-Nonanone from Milk Proteins by SPME-GC-FID

The *in vitro* release experiments were performed to get an indication if a protein-flavour system with a low binding constant *K* (i.e. sodium caseinate and 2-nonanone), as determined in Chapter 3, shows a higher release of flavour from the protein under dynamic conditions as compared to a protein-flavour system with a higher *K* (i.e. WPI and 2-nonanone). When the release of 2-nonanone from the individual whey proteins was compared (Figure 5.4-1a), it appeared that the release rate was inversely related to *K*, e.g., the release was slowest for BSA ($K = 5{,}649\ M^{-1}$), and fastest for α-la ($K = 860\ M^{-1}$), with β-lg lying in between ($K = 2{,}741\ M^{-1}$). However, the differences in release between the three proteins were relatively small. The initial release (0-60 min) was fastest for BSA, which may be because of its lower concentration in the solution, resulting in a faster mass transfer to the sample surface. WPI and sodium caseinate showed a similar release (Figure 5.4-1b) although the binding of 2-nonanone to WPI ($K = 2{,}800\ M^{-1}$) was found to be stronger than to sodium caseinate ($K = 370\ M^{-1}$).

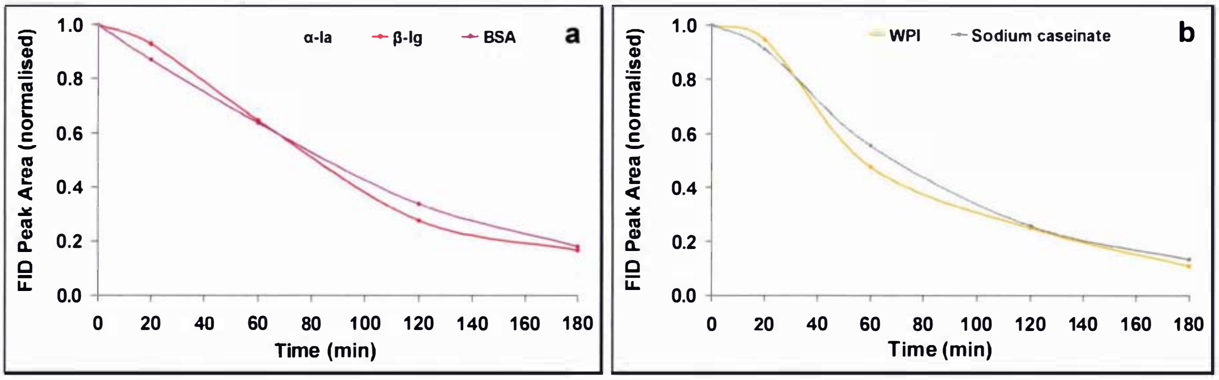


Figure 5.4-1: Release of 2-nonanone from (a) individual whey proteins, and (b) WPI and sodium caseinate at 30 °C, FID peak areas normalised.

5.4.2 *In Vivo* Release of 2-Nonanone from Milk Proteins by PTR-MS

The release of 2-nonanone (1 ppm) from aqueous solutions of WPI and sodium caseinate, in the presence and absence of 0.5 % CMC, was investigated using the PTR-MS technique. However, before the actual release experiments, the binding of 2-nonanone to the proteins and CMC, and their combinations was determined using SPME-GC-FID.

5.4.2.1 Binding of 2-Nonanone in Solutions of WPI, Sodium Caseinate, and CMC

Generally, the more flavour is bound to a protein initially, the easier a release of flavour could be detected. Therefore, the binding of 2-nonanone (1 ppm) to WPI and sodium caseinate at higher concentrations than in Chapters 3 and 4 was determined (Figure 5.4-2). As expected, the higher the protein concentration, the higher the extent of binding of 2-nonanone. Flavour concentrations of 5 ppm and 10 ppm were also investigated since they were thought to be required for the *in vivo* study because of the low concentrations of flavour in the breath and possible sensitivity issues. The higher the flavour concentration, the lower was the extent of flavour binding, which is due to the greater saturation of binding sites at high flavour concentrations. The final optimum protein and 2-nonanone concentrations for the *in vivo* study were determined during the *in vivo* study (section 5.4.2.4).

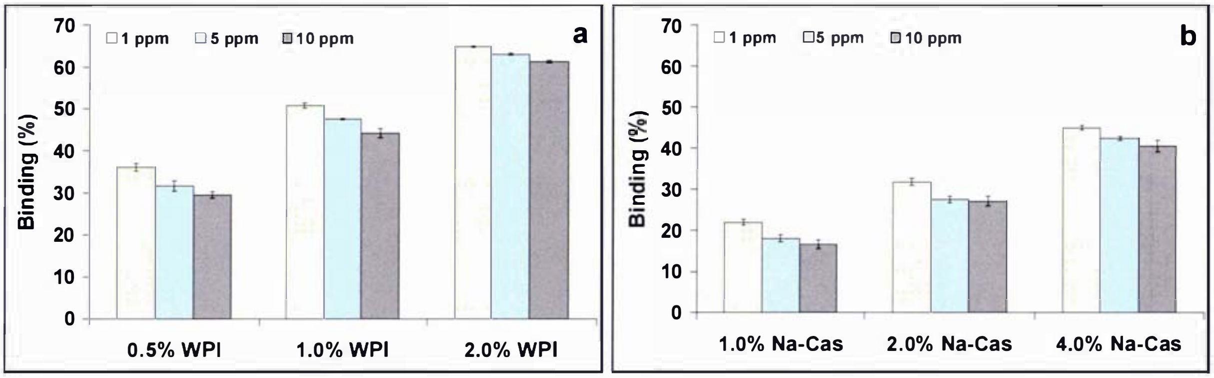


Figure 5.4-2: Binding of 2-nonanone to (a) WPI at 0.5, 1.0, and 2.0 %, and (b) sodium caseinate at 1.0, 2.0, and 4.0 %.

The second aim was to investigate the effect of viscosity (CMC) on flavour release, and thus, the binding of 2-nonanone to CMC (0.5 %), and to mixtures of CMC/WPI and CMC/sodium caseinate was determined (Table 5.4-1). CMC showed a high extent of binding for 2-nonanone – about 40 % of the added 2-nonanone was bound. As expected, when CMC was added to the protein solutions, the binding increased further.

Table 5.4-1: Binding of 2-nonanone (1.0 ppm) to WPI and sodium caseinate (2 %) in thickened (0.5 % CMC) and non-thickened systems (mean ± standard deviation; n = 3).

Sample	Binding (%)
WPI	64.9 ± 0.2
Sodium caseinate	31.8 ± 0.9
CMC	40 ± 2
WPI + CMC	74 ± 1
Sodium caseinate + CMC	51 ± 1

5.4.2.2 Selection of Molecular Ions for MS Detection

The flavour compound of interest, 2-nonanone, possesses a molecular mass of 142 Da. The main molecular ion expected is the mono-protonated molecule with *m/z* = 143. In Figure 5.4-3, the headspace above a solution of 2-nonanone was compared with the headspace above water, and the main ion resulting from 2-nonanone is *m/z* = 143.

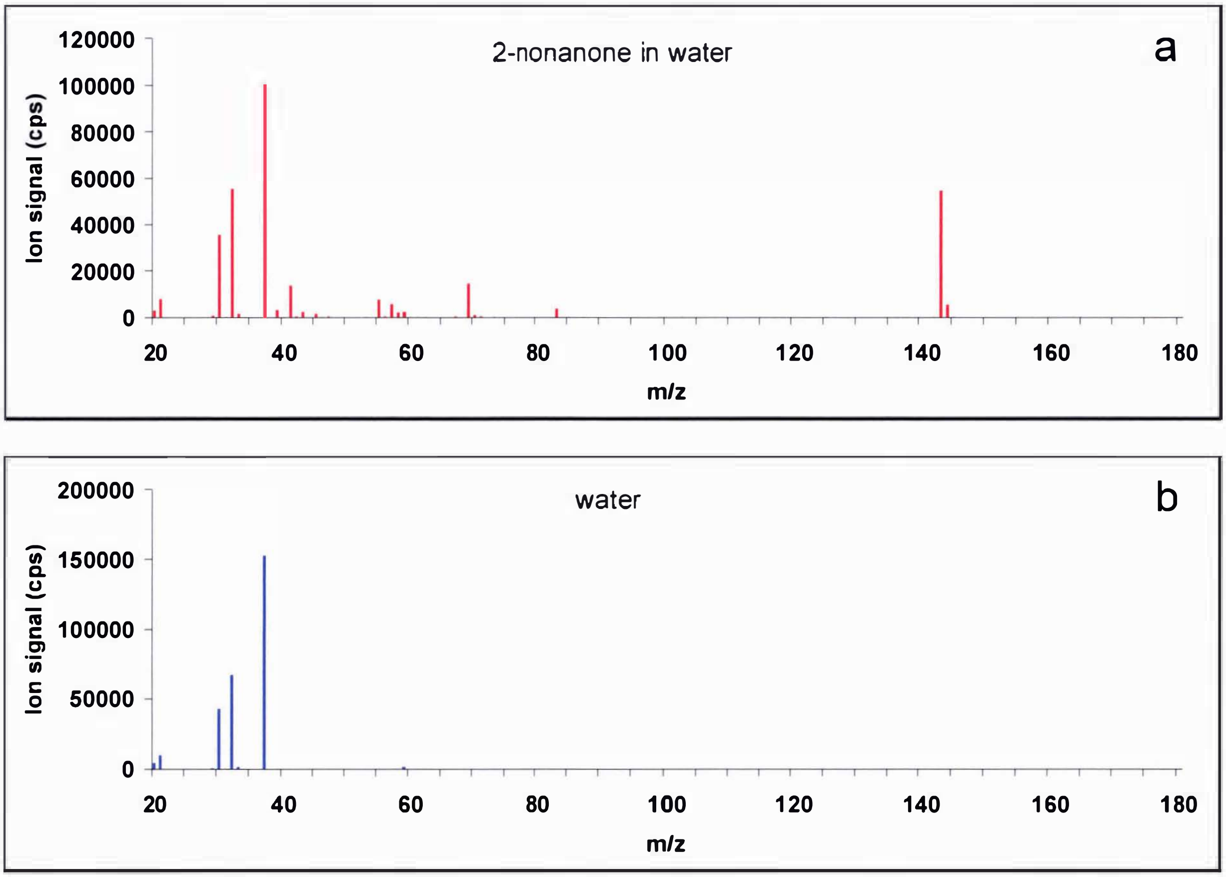


Figure 5.4-3: Scan of *m/z* 20-180 of the headspace above (a) a solution of 50 ppm 2-nonanone and (b) water.

The large peaks below $m/z = 40$ did not originate from 2-nonanone as they were also present in the headspace above water. There were smaller peaks resulting from 2-nonanone, namely at m/z 42, 56, and 69, probably resulting from fragmentation of the flavour molecule in the reaction chamber; however the largest peak at $m/z = 143$ was found to be most suitable for quantification of the 2-nonanone release.

5.4.2.3 Choice of Consumption Technique

The aim was to find out if 2-nonanone is released from WPI or sodium caseinate when solutions of the proteins are swallowed straight after sample intake, without any in-mouth movements. This experiment was performed by subject 3 only because this was a preliminary experiment. Figure 5.4-4 shows typical release profiles of a WPI containing sample and the corresponding "control 1". Each peak in the profile corresponded with an exhalation, monitored using the acetone signal (data not shown). The value of t_{max} lies around 19 sec and coincides with the first exhalation after swallowing of the sample. The I_{max} values of the protein containing samples and the controls 1 were consistently very similar which was expected since at the time of sample intake both sample and control have the same concentration of free flavour. More interestingly, the release profile showed that the concentration of 2-nonanone in the breath of the subject decreased slower after consumption of the WPI containing sample as compared to the control, which indicates that there may be a release of 2-nonanone from the proteins even when the samples were just swallowed without any in-mouth movement (swirling) before swallowing.

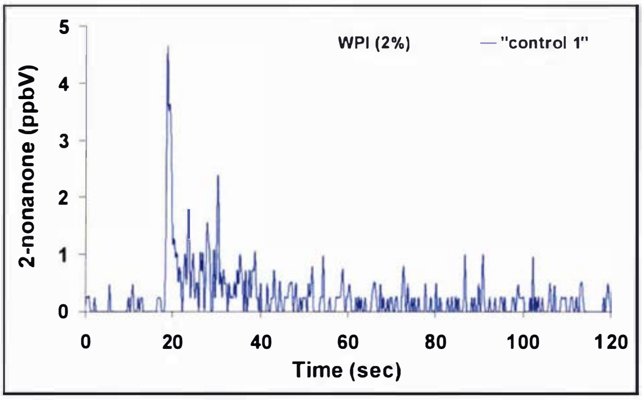


Figure 5.4-4: Temporal 2-nonanone release from a WPI solution (2 %) and the corresponding "control 1", when samples were swallowed straight after sample intake without any in-mouth movements.

Therefore, statistical analysis of the values of *AUC*, which was used as a measure of the extent of flavour release, was performed. For both proteins, WPI and sodium caseinate, there were no significant differences between *AUC* of the "controls 1" and *AUC* of the protein containing samples ($P < 0.05$) (Figure 5.4-5). For this reason, a consumption technique including swirling the sample solution around in the mouth five times before swallowing was chosen for this study. In addition, to determine the effect of viscosity on flavour release, movement of the solutions in mouth (swirling) is probably necessary. *Hansson et al. (2003)* observed a higher flavour release from thickened systems when mastication was applied to the samples.

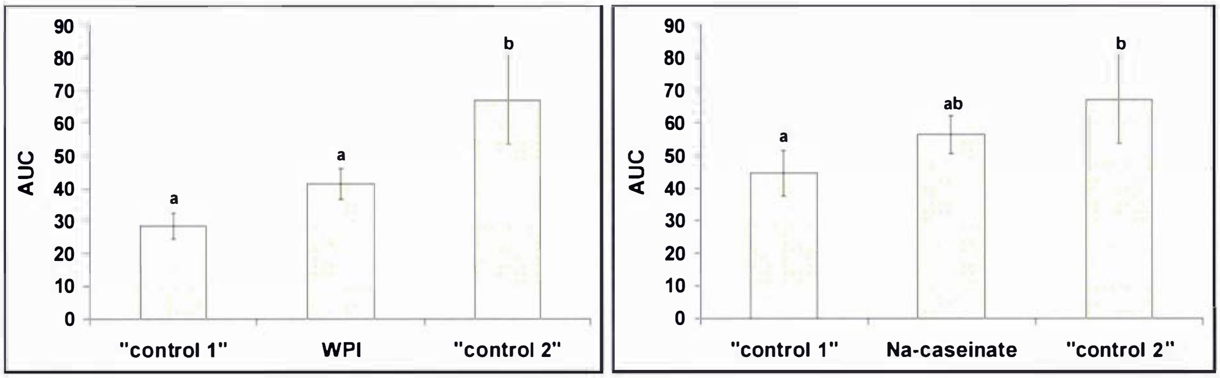


Figure 5.4-5: Release of 2-nonanone from WPI, sodium caseinate, and the corresponding controls after the samples were swallowed straight after sample intake without any in-mouth movements.

5.4.2.4 Choice of Protein Concentration

In Chapters 3 and 4, concentrations of 0.5 % and 1.0 % for WPI and sodium caseinate, respectively, were used. However, to investigate if flavour is released from the proteins, higher protein concentrations may be necessary to initially have a higher amount of bound flavour. For this reason, the release of 2-nonanone from 0.5 % and 2.0 % WPI was compared by subject 3. Figure 5.4-6 shows typical release profiles of 2-nonanone when the samples are swirled five times in-mouth before swallowing.

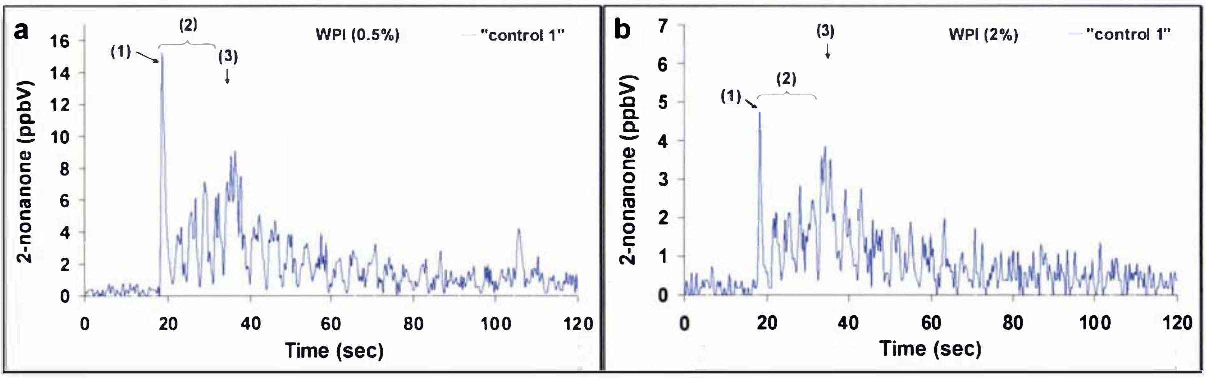


Figure 5.4-6: Release profiles of 2-nonanone from (a) 0.5 % and (b) 2 % WPI solutions in comparison to the controls 1. Samples were consumed by subject 3 with swirling in-mouth.

The profiles (Figure 5.4-6) showed differences compared to the profiles obtained when the samples were swallowed straight after sample intake (Figure 5.4-4). First, the value of t_{max} is generally delayed in the samples containing protein, and t_{max} coincides with swallowing the samples at around 25-35 sec, depending on the subject, whereas for the controls t_{max} remains at approximately 19 sec, coinciding with the first exhalation after sample intake. The time points of the first exhalation after sample intake (1), the five swirling actions (2), and the first exhalation after swallowing of the sample (3) can be clearly seen in the release profiles (Figure 5.4-6).

Similar release profiles of 2-butanone from viscous solutions by PTR-MS were shown by *Hansson et al. (2003)*, with a high flavour release at the time points of sample intake and swallowing. When a liquid food is consumed, the mouth can be regarded as a closed system as long as no mouth movements are made or no swallowing occurs *(Buettner and Schieberle, 2000)*. No air flow can pass from the mouth to the nose, and thus, flavour compounds can not be released into the nasal cavity. The event of swallowing opens the connection between the soft palate and the tongue, and flavour compounds can travel into the nasal cavity. This causes the high release signal after swallowing. The release peak at the time of sample intake was probably also caused by an open tongue-soft palate border.

A comparison of the release from 0.5 % and 2 % WPI solutions suggested that a release from the protein may only be observed for a 2 % WPI solution, since only for the 2 % WPI solution the 2-nonanone release was consistently higher than the release from the corresponding control sample. However, for the 0.5 % WPI solution there was hardly any difference between the protein containing sample and the "control 1". For this reason, the higher protein concentration of 2 % was used throughout the study. Similar reasoning can be applied to sodium caseinate as a 2 % concentration of sodium caseinate was used instead of 1 % as used in the earlier chapters. Increases in protein concentration result in higher extents of flavour binding (Figure 5.4-2); nevertheless, *Le Guen and Vreeker (2003)*, who investigated the release of methyl ketones from milk protein concentrate (10 %, w/w) using APCI-MS, observed a complete release of the flavour compounds from the proteins *in vivo* despite the high protein concentration. However, these authors used a different consumption technique involving mastication of the aqueous sample on a regular rhythm for 2 min without swallowing the sample, whereas in this study the flavour release after swallowing was also considered.

After swallowing of the samples containing 2 % WPI, a high release of 2-nonanone was observed, while the release from the control 1 was lower. This may indicate that the protein solutions interact with the mucosa in the mouth and in the throat after the sample has been swallowed, resulting in a release of bound flavour from these mucosa-bound proteins caused by the airflow. This observation is in good agreement with the results obtained by *Weel et al. (2003)*, who investigated the *in vivo* release of aldehydes from WPI by APCI-MS. These authors suggested that after swallowing, a thin film of flavour solution remains in the pharynx and both free flavour compounds present in this film and those reversibly bound to proteins are released upon exhalation. Also, in agreement with the results of *Weel et al. (2003)* and of this study, *Linforth et al. (2003)*, using APCI-MS, suggested that swallowing not only results in the delivery of volatiles into the throat and thus into the exhaled breath, but also results in the coating of the throat with the sample creating a reservoir for further flavour release. These results emphasize that flavour release after swallowing the sample may significantly contribute to flavour perception, and that sample consumption protocols should also include monitoring the flavour release after swallowing. The exhalations after swallowing of the samples showed similar or higher concentrations of 2-nonanone than those before swallowing, indicating the importance of flavour release from mucosa-bound proteins.

5.4.2.5 2-Nonanone Release from Milk Proteins with In-Mouth Movements

It was shown that the binding affinity of WPI for 2-nonanone was clearly higher than the affinity of sodium caseinate for 2-nonanone (Chapter 3 and Table 5.4-1). Can this difference under static *in vitro* conditions also be observed under dynamic *in vivo* (in-mouth) conditions, with a higher release of 2-nonanone from sodium caseinate solutions than from WPI solutions? Figure 5.4-7 shows the *AUC*s of the 2-nonanone release profiles obtained from the three subjects for the release of 2-nonanone from 2 % sodium caseinate and 2 % WPI solutions. Under in-mouth conditions, there was a significant difference in 2-nonanone release, which is represented by the *AUC*, between sodium caseinate and WPI containing samples ($P < 0.05$). The release from samples containing 2 % sodium caseinate was significantly higher than the release from samples containing 2 % WPI. This difference is probably due to the higher binding affinity of WPI for 2-nonanone, resulting in a lower initial concentration of free 2-nonanone in the samples, and possibly in a stronger retention of the flavour by WPI despite the airflow.

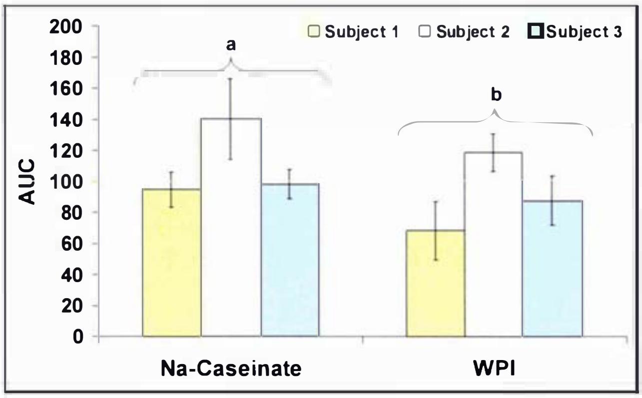


Figure 5.4-7: Comparison of the release of 2-nonanone from sodium caseinate and WPI (2 %) (n = 5).

Between subjects there were significant differences in *AUC*, with subject 2 showing a significantly higher release than subjects 1 and 3. However, there were no significant differences for t_{max} and I_{max} between subjects. The values for t_{max} were also not significantly different between the samples, whereas a significantly higher value of I_{max} for the sodium caseinate containing samples was found, supporting the greater release of flavour from sodium caseinate containing samples.

5.4.2.5.1 2-Nonanone Release from WPI

When the *AUC*s of the 2-nonanone release profiles from a 2 % WPI solution were compared to the *AUC*s of "control 1" and "control 2", representing no release and complete release, respectively, a significant ($P < 0.05$) release of flavour was observed (Figure 5.4-8). The 2-nonanone release from the WPI containing sample was significantly higher than the release from "control 1", but significantly lower than the release from "control 2". This finding suggests that flavour compounds which are reversibly bound to proteins can be partly released from the proteins during consumption if the sample is swirled in the mouth before swallowing. However, the release is not complete which means that the perception of 2-nonanone may be reduced to some extent in the presence of 2 % WPI.

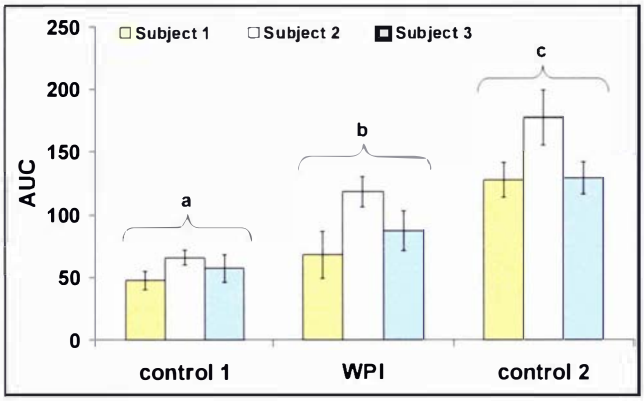


Figure 5.4-8: Release of 2-nonanone from a WPI solution (2 %) as compared to the controls.

This observation is in agreement with the results by *Weel et al. (2003)* and *Le Guen and Vreeker (2003)*, who also reported a release of flavour compounds from milk proteins in-mouth. However, *Le Guen and Vreeker (2003)* found that the proteins showed a complete release of methyl ketones in-mouth, whereas in this study only part of the protein-bound 2-nonanone was released. This difference may partly be due to the different consumption techniques used. The consumption protocol of *Le Guen and Vreeker (2003)* did not involve swallowing of the samples, whereas in this study the samples were swallowed, and the flavour release after swallowing was also considered. *Weel et al. (2004a, 2004b)* observed that a thin layer of liquid remaining in the throat after swallowing largely determines the *in vivo* flavour release from liquids. Therefore, the consumption protocol of this study is closer to the "real" consumption of foods than the protocol used by *Le Guen and Vreeker (2003)*.

Differences between samples were observed, as well as between subjects. As observed before, subject 2 showed a significantly higher flavour release than subjects 1 and 3. The value of I_{max} was not significantly different between the "control 1" and the WPI containing sample, however, this is due to the high variation of I_{max} even within subjects (up to 96 % relative standard deviation).

The value of t_{max} was not significantly different between the samples, however, it was significantly higher (at the time of swallowing) for subject 3 as compared to subjects 1 and 2, who showed t_{max} during the swirling action.

5.4.2.5.2 2-Nonanone Release from Sodium Caseinate

The release of 2-nonanone, as expressed by the *AUC*, from a 2 % sodium caseinate solution was compared with the controls (Figure 5.4-9). There was no significant difference between "control 1" and the sodium caseinate containing sample, indicating that there was no measurable release of the flavour in mouth under the conditions used. A low binding constant may not necessarily mean a faster or higher release of flavour from the proteins. However, the differences in the *AUC* between the two controls are also small because of the low binding affinity of sodium caseinate for 2-nonanone. If more flavour was bound, as in the case of WPI, more flavour can be released in mouth, and differences may be more easily detected. Therefore, future work should include the investigation of flavour release from sodium caseinate using higher sodium caseinate concentrations (4 % or higher). For I_{max} there were also no significant differences between samples. As observed before for WPI, t_{max} was similar for all samples, but for subject 3, t_{max} appeared later (at the event of swallowing). Subject 2 again showed a significantly higher release than the other two subjects.

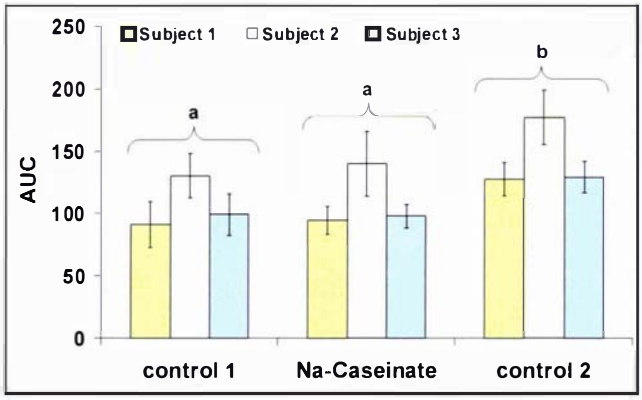


Figure 5.4-9: Release of 2-nonanone from a sodium caseinate solution (2 %) as compared to the controls.

5.4.2.6 The Effect of Viscosity on 2-Nonanone Release

The release of 2-nonanone from "control 2" (1ppm 2-nonanone) in the presence and absence of CMC (0.5 %) was compared (Figure 5.4-10). A significant reduction ($P < 0.05$) in flavour release was observed in the presence of CMC. The lower release of flavour in the presence of the thickener may be due to both binding of 2-nonanone to the thickener which was found to be 40 % (see section 5.4.2.1), and to the slower mass transfer caused by the higher viscosity of the sample *(de Roos, 2003)*. The binding of 2-nonanone (1 ppm) to CMC (0.5 %) under static conditions was found to be 40 %, but the reduction in the *AUC* is ~ 30 % (estimated from Table 5.4-2). Thus, 2-nonanone appears to be partially released from CMC in mouth. The values of t_{max} and I_{max} were not significantly different between the two samples.

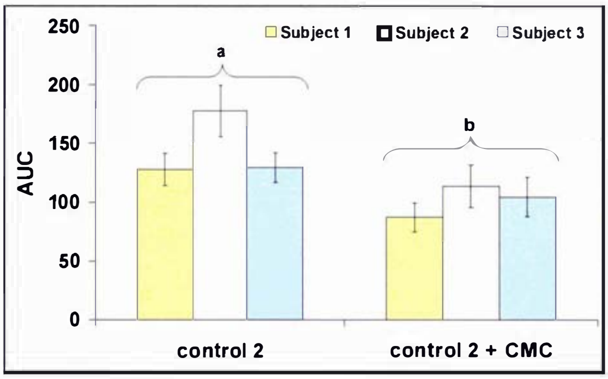


Figure 5.4-10: Effect of CMC (0.5 %) on the release of 2-nonanone from control 2 (1 ppm 2-nonanone).

Hollowood et al. (2002) observed that, despite a decrease in perceived strawberry flavour intensity, the actual flavour concentration in the breath, using ethyl butyrate as a marker for the strawberry flavour, was not reduced by an increase in hydroxypropyl methylcellulose concentration, indicating that the viscosity does not influence in-mouth flavour release. Other

Table 5.4-2: Areas under the curve (*AUCs*), t_{max}, and I_{max} of all samples (mean ± standard deviation; n = 5).

Sample	AUC (ppbV·sec)	t_{max} (sec)	I_{max} (ppbV)
Control 2	145 ± 28	29 ± 7	13 ± 8
WPI	92 ± 26	30 ± 9	6 ± 3
WPI control 1	57 ± 11	24 ± 8	7 ± 4
Na-Caseinate	111 ± 27	26 ± 8	9 ± 5
Na-Caseinate control 1	107 ± 24	26 ± 7	10 ± 8
CMC control 2	102 ± 19	26 ± 9	10 ± 6
WPI + CMC	76 ± 21	29 ± 10	8 ± 7
WPI + CMC control 1	40 ± 10	27 ± 8	4 ± 4
Na-Caseinate + CMC	99 ± 26	25 ± 7	15 ± 13
Na-Caseinate + CMC control 1	73 ± 14	25 ± 6	7 ± 5

authors also found that an increased viscosity did not suppress the *in vivo* flavour release *(Baek et al., 1999; Hollowood et al., 2002; Weel et al., 2002)* . Therefore, the reduction in 2-nonanone release found in this study may be predominantly because of binding between the hydrocolloid and 2-nonanone. Under simulated mouth conditions, *Roberts et al. (1996)* found decreases in the release of volatile flavour compounds in the presence of CMC, and suggested that both viscosity and binding of flavour compounds with the food matrix influence flavour release.

Table 5.4-2 summarises the values for AUC, t_{max}, and I_{max} of all samples. The variation (relative standard deviation) in AUC ranged from 19 to 28 % (average 23 %), whereas when subjects are treated separately, it ranged from 9 to 31 % (average 14 %). There was a very high variation in I_{max}, meaning that I_{max}, in contrast to AUC, is not a suitable measure of flavour release.

When CMC was added to a WPI/2-nonanone solution, there was also a significant reduction in 2-nonanone release (Figure 5.4-11). This may be due to the binding of 2-nonanone to CMC as well as to the increased viscosity. Both t_{max} and I_{max} were not significantly influenced by the addition of CMC. However, there were differences again between the three subjects in the values for AUC, t_{max}, and I_{max}.

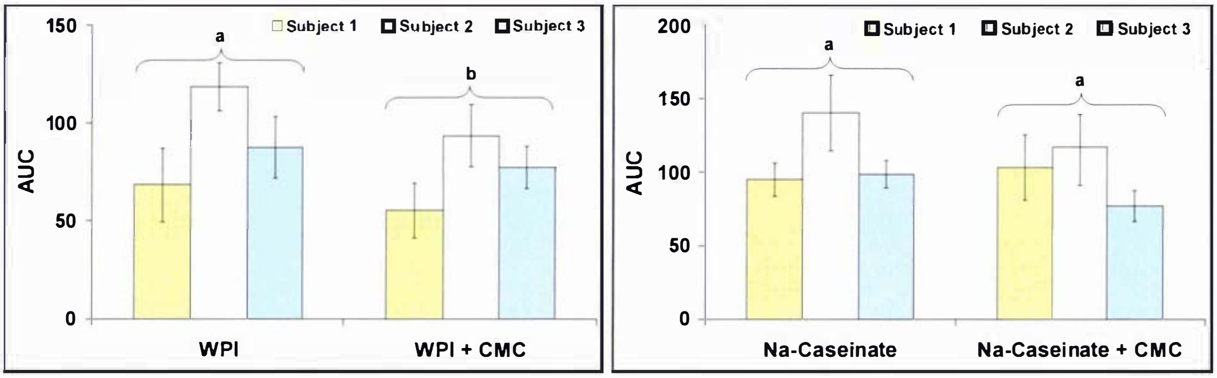


Figure 5.4-11: Effect of CMC (0.5 %) on the release of 2-nonanone from WPI and sodium caseinate solutions (2 %).

In contrast, the addition of CMC to a sodium caseinate/2-nonanone solution did not significantly affect the flavour release. This finding is surprising as in the absence of sodium caseinate (Figure 5.4-10) there was a significant reduction in flavour release. There were no significant differences for t_{max} and I_{max} between the samples, but between the subjects there were differences for AUC and I_{max}.

The release of 2-nonanone from WPI and sodium caseinate in thickened systems compared to the thickened controls 1 and 2 was investigated. A significant release of 2-nonanone from WPI in the CMC containing systems was observed (Figure 5.4-12). This is in agreement with the results from the non-thickened systems (Figure 5.4-8). However, the release was not complete. These results also suggest that an increase in viscosity does not slow down the release of 2-nonanone from WPI. In fact, the release of 2-nonanone from WPI seems to be more pronounced in the thickened systems as compared to the non-thickened systems (Figure 5.4-8). An explanation may be that the viscous solutions build a thicker film on the mucosa in the mouth and throat, leaving a higher amount of protein-bound flavour exposed to the flow of exhalation for further flavour release after swallowing. In agreement with this, *Buettner et al. (2001)*, using videofluoroscopy, found that one can often observe the formation of a coating on the back of the tongue after swallowing solid or semi-solid foods, such as cottage cheese or yoghurt. This film may cause a prolonged perception of aroma by acting as an odorant depot.

An increase in carrageenan concentration resulted in an increase in the release of ethyl butanoate, butane-2,3-dione, and hexanal from aqueous solutions in an artificial throat system *(Weel et al., 2004a)*. This observation was also explained with a thicker layer on the inner surface of the artificial throat, which contains more flavour compounds than a thin layer of water. However, *in vivo* measurements showed no influence of viscosity on the amount of flavour compounds released. *Cook et al. (2003)* also demonstrated that viscosity does not influence flavour release *in vivo*. In these studies, the model systems did not contain proteins in addition to the thickener. In the present study, the increase in flavour release in the thickened systems as compared to the non-thickened systems may therefore be due to the higher amount of proteins in the viscous film, which have flavour compounds reversibly bound and act as a flavour reservoir.

Samples were not different in t_{max}, and the protein containing sample did not differ from the controls in I_{max}, again showing that I_{max} is not a suitable measure of the extent of flavour release. There were significant differences between subjects for *AUC* with subject 1 showing a lower release than the other two subjects, for t_{max} with a significantly later t_{max} for subject 3, and for I_{max} which was higher for subject 2 as compared to the other two subjects.

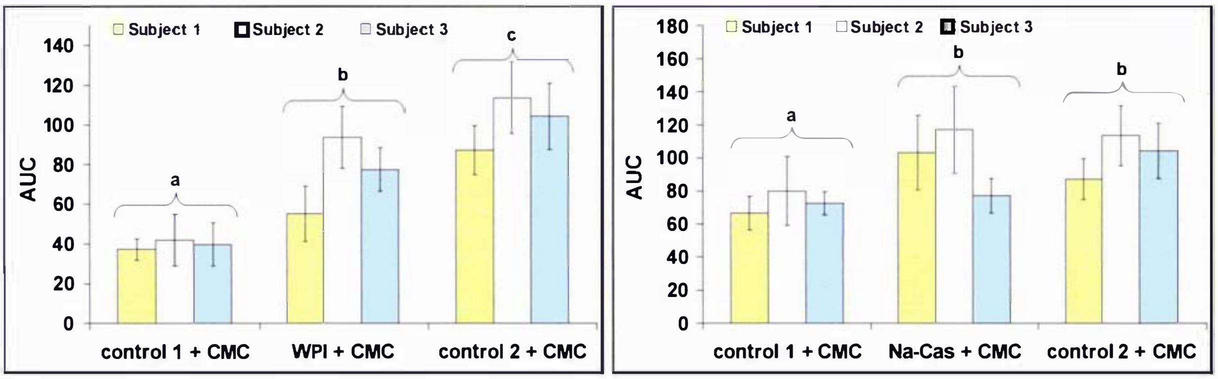


Figure 5.4-12: Release of 2-nonanone from WPI and sodium caseinate (2 %) in thickened systems (0.5 % CMC) as compared to the corresponding thickened controls.

An unexpected observation was made for the release of 2-nonanone from sodium caseinate in thickened systems. There was a significant difference between control 1 with CMC and the sodium caseinate containing sample with CMC, indicating that there was a release of 2-nonanone from sodium caseinate in the presence of CMC. For the systems without CMC, no release of 2-nonanone from sodium caseinate was observed (Figure 5.4-9). An explanation for the similar release from the sodium caseinate containing samples and control 2 may be an interaction between sodium caseinate and CMC which may reduce the binding between 2-nonanone and sodium caseinate, or 2-nonanone and CMC. However, this speculation could not be confirmed as the addition of CMC to a sodium caseinate solution increased the binding of 2-nonanone (Table 5.4-1). In agreement with this, *Delben and Stefancich (1997)* did not detect interactions between β-casein and CMC at neutral pH using rheometric, optical, and chiro-optical techniques. As previously discussed for the samples containing WPI, the release of 2-nonanone from sodium caseinate in the viscous samples could be explained with a higher amount of sample covering the mucosa in mouth and throat, resulting in a higher release of protein-bound flavour after swallowing. The release from sodium caseinate was found to be significant; however, the differences in *AUC*, even between the two controls, are small. A solution would be to use higher sodium caseinate concentrations to obtain larger differences in *AUC* between samples.

Samples not only showed significant differences for *AUC* but also for I_{max}, which was significantly higher for the sodium caseinate containing sample than for the control 1. There were differences between subjects for *AUC* with a higher release for subject 2 compared to subject 1 and 3, and for I_{max} which was found to be significantly higher for subject 2 as compared to subject 3, but subject 1 was not different from the other two subjects. No differences between subjects were found for t_{max}.

5.5 Conclusions

The PTR-MS technique was successfully used to determine the *in vivo* flavour release from milk proteins. The excellent sensitivity of the instrument allowed detection of flavour concentrations below 1.0 ppm, whereas in previous studies concentrations used were considerably higher.

WPI was found to bind about double the amount of 2-nonanone as compared to sodium caseinate using headspace SPME. This difference was also found under dynamic *in vivo* conditions with a higher retention of the flavour compound by WPI.

This study confirmed that during consumption not only free 2-nonanone is released but also 2-nonanone bound to WPI. β-Lg, the main protein in WPI, was found to have a high binding affinity for 2-nonanone, and nonetheless the flavour compound was partly released from WPI. However, the release of flavour was not complete, suggesting that the flavour perception may be reduced in the presence of WPI. A sensory study is necessary to investigate if WPI decreases the perception of reversibly bound flavour.

A release of 2-nonanone from sodium caseinate was not observed. However, due to the low binding constant of sodium caseinate for 2-nonanone, differences between the *AUC*s of sodium caseinate containing samples and their controls were small. Therefore, future work should include the use of higher sodium caseinate concentrations, which may show a significant release of 2-nonanone from sodium caseinate.

When the sample was swallowed without any in-mouth movement, there was no significant release of 2-nonanone from the milk proteins, although only one subject was used to compare different consumption techniques. This observation suggests that protein-flavour interactions may play a significant role in the flavour perception of drinks rather than of semi-solid or solid foods which are chewed, causing flavour release.

The flavour release profiles revealed that even after swallowing of the samples, a substantial amount of flavour was detected in the breath, suggesting that the milk proteins interact with the mucosa in the mouth and throat, resulting in a further release of flavour from mucosa-bound proteins.

Thickeners such as CMC have been reported to not influence flavour release *in vivo (Baek et al., 1999; Hollowood et al., 2002; Weel et al., 2002)*. However, in this study CMC was found to enhance the release from milk proteins, probably due to a higher amount of the viscous samples interacting with the surfaces in the mouth and in the throat, resulting in additional flavour release from the milk proteins after swallowing of the samples.

The data obtained in this study showed a fairly high variation. In future studies this problem could be overcome by increasing the number of subjects.

CHAPTER SIX: OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The objectives of this thesis were to determine binding parameters of the hydrophobic flavour compound, 2-nonanone, to individual milk proteins (namely, β-lg, α-la, BSA, α_{s1}-casein, and β-casein), WPI, and sodium caseinate, using a headspace SPME method followed by GC-FID. Secondly, it was the aim to compare the binding of structurally similar flavour compounds – 2-nonanone, 1-nonanal, and *trans*-2-nonenal – to WPI in aqueous solution, and to investigate the effect of heat and high pressure treatment, and pH on the extent of protein-flavour binding. The final objective was to investigate the *in vivo* release of the reversibly bound flavour compound, 2-nonanone, from WPI and sodium caseinate using PTR-MS, and to understand the effect of viscosity on flavour release *in vivo*.

The interactions of several flavour compounds (2-nonanone, 1-nonanal, and *trans*-2-nonenal) with milk proteins were successfully studied using a headspace SPME method followed by GC-FID quantification. The SPME method was developed and optimised in terms of thermal equilibration time, type and thickness of SPME fibre coating, SPME extraction time, sample agitation, and flavour solvent. The good agreement between the binding parameters determined in this study and the values obtained by other authors shows that the SPME technique is an excellent tool for studying interactions between volatile flavour compounds and macromolecules.

This study has demonstrated that, within the whey protein group, BSA has the highest affinity for 2-nonanone, followed by β-lg and α-la. Both α-la and β-lg possess one binding site for 2-nonanone, whereas BSA can bind 2-nonanone on two classes of binding sites, resulting in a very high binding affinity. WPI was found to have a much higher affinity for 2-nonanone than sodium caseinate. Considering the proportions of the whey proteins in WPI, β-lg is the protein mainly responsible for 2-nonanone binding in WPI, followed by BSA, while α-la has almost no contribution to the binding.

α_{s1}-Casein was found to have a slightly higher affinity for 2-nonanone than β-casein, although β-casein is the more hydrophobic of the two proteins, indicating that the interaction mat be other than hydrophobic, or that self-association of β-casein resulted in lower overall binding of 2-nonanone. The overall binding to both caseins was low compared with that of the whey proteins.

Milk proteins added to food products are often denatured to a varying extent. In addition, during processing of foods, e.g. heat treatment, proteins are denatured. This study further elucidated the effects of heat treatment, high pressure treatment and pH on the interactions of WPI with flavour compounds. The binding between WPI and the three flavour compounds investigated was strong and decreased in the order *trans*-2-nonenal > 1-nonanal > 2-nonanone. The differences in binding can be explained with hydrophobic interactions only in the case of 2-nonanone, whereas the aldehydes, in particular *trans*-2-nonenal, can also interact covalently. Upon both heat- and pressure-induced denaturation, the binding of 2-nonanone and WPI decreased, while the binding of 1-nonanal remained unchanged, and the affinity for *trans*-2-nonenal increased. From these observations, it can be concluded that the three flavour compounds investigated are bound to proteins on different binding sites and/or by different binding mechanisms.

The results also suggest that heat- or pressure-induced denaturation reduces hydrophobic interactions of whey proteins with flavour compounds due to conformational changes in the flavour binding sites, whereas covalent interactions are enhanced. The decrease in binding of 2-nonanone to WPI upon denaturation can be explained with the destruction of the hydrophobic pocket of β-lg, and with protein-flavour interactions being replaced by protein-protein interactions. The increase in binding of *trans*-2-nonenal may result from mainly covalent binding to amino acid residues, such as lysine, histidine, arginine, or cysteine, which may be better accessible in the unfolded, aggregated protein molecules. The unchanged binding of 1-nonanal to WPI with heat or high pressure treatment is likely to result from a combination of both, a decrease in binding due to the destruction of the hydrophobic pocket, and an increase in binding caused by better accessibility of amino acid residues for covalent interactions, resulting in an overall unchanged binding.

For the three flavour compounds investigated there was generally no difference in binding between the samples with flavour added before and after heat or high pressure treatment. However, at higher flavour concentrations, differences may be observed due to possible stabilising effects of the flavour compounds on the native conformation of the proteins.

The formation of heptanal upon heating *trans*-2-nonenal in the presence of milk proteins demonstrated that new flavour compounds may be formed during heat treatment under certain conditions, e.g., in the presence of an unsaturated flavour compound. However, the mechanism of heptanal formation is still unclear and requires further investigation. Heptanal was not formed upon high pressure treatment.

The pH of the medium was found to have a significant effect on the extent of protein-flavour interactions. For all three flavour compounds investigated, the binding affinity to WPI increased with increasing pH. This observation results from different pH dependent conformational changes of whey proteins, e.g., the closure of the hydrophobic pocket of β-lg at low pH. In addition, the protonation of basic amino acid residues, which are very likely to react covalently with aldehyde flavours, changes with pH. Therefore, covalent interactions between proteins and flavour compounds are favoured at higher pH values.

The results suggest that the presence of milk proteins may cause the aroma profile of foods, in particular fat-free foods, to become unbalanced because of the binding of certain flavour compounds by the proteins. The proteins may prevent flavour compounds from being released and perceived during mastication due to their strong and, in some cases, irreversible binding to proteins. Heat and high pressure treatment may also notably influence the overall flavour profile of protein containing foods. Since the protein concentration used in this study was fairly low, the observed effects are expected to be even more pronounced in real food systems.

The significant influences of heat, high pressure, and pH on protein-flavour interactions observed in this study also emphasize a careful choice of food processing conditions to obtain a desirable flavour profile. Any changes made in the processing of a food product are very likely to change the flavour profile, and possibly the flavour perception and consumer acceptance of a food product.

The PTR-MS technique was successfully used to determine the *in vivo* 2-nonanone release from WPI and sodium caseinate. This study confirmed that during consumption not only free 2-nonanone is released, but also 2-nonanone bound to WPI. However, the release of 2-nonanone was not complete, suggesting that the flavour perception may be reduced in the presence of WPI. A sensory study is necessary to investigate if, and at what concentration WPI decreases the perception of reversibly bound flavour. A release of 2-nonanone from sodium caseinate was not observed. However, due to the low binding constant of sodium caseinate for 2-nonanone, differences in the release from sodium caseinate containing samples and their controls were small. Therefore, future work should include the use of higher sodium caseinate concentrations, which may show a significant release of 2-nonanone from sodium caseinate. In addition, a larger number of subjects should be used to reduce the large variation of the data.

When the sample was swallowed without any in-mouth movement, there was no significant release of 2-nonanone from the milk proteins. This observation suggests that protein-flavour interactions may play a significant role in the flavour perception of beverages rather than of semi-solid or solid foods which are chewed, causing flavour release. The flavour release profiles also revealed that particularly after swallowing of the samples, a substantial amount of 2-nonanone was detected in the breath, suggesting that the samples interact with the mucosa in the mouth and throat, resulting in a further release of flavour from mucosa-bound proteins.

The thickener (CMC) was found to enhance the 2-nonanone release from milk proteins, probably due to a higher amount of the viscous samples interacting with the surfaces in the mouth and in the throat, resulting in additional flavour release from the milk proteins after swallowing of the samples.

Future Work

A good knowledge of the physicochemical interactions that occur between proteins and flavour compounds is neccessary to improve the perceived flavour of protein based foods, in particular "light" products, and make these products sensorily more acceptable to the consumer.

Binding parameters for 2-nonanone and native proteins have been successfully determined using headspace SPME and using the Klotz plot. The determination of binding constants and numbers of binding sites of individual proteins at different pH values, and upon heat and high pressure treatments would provide valuable information about the topology of flavour binding under different conditions. For example, lowering the pH of a β-lg solution from 7.2 to 4.0 may result in a large number of low-affinity binding sites for 2-nonanone, whereas at pH 7.2 there was only one high-affinity binding site.

Interactions of unsaturated flavour compounds and proteins, in particular upon heat treatment, require further investigation since they potentially generate new flavour compounds. In this study, heptanal was formed from *trans*-2-nonenal. 2-Octenal and 2-heptenal may form hexanal and pentanal, respectively, when heated in the presence of protein. The mechanism of heptanal formation needs to be discovered. For example, N-terminally blocked amino acids may be used instead of complex proteins to find out which amino acid residues in the proteins are involved in the formation of heptanal.

Caseins have been found to have low binding affinities for 2-nonanone under the conditions used. However, casein molecules have the tendency to undergo self-association or association with each other, depending on environmental conditions, such as protein concentration, pH, and ionic strength. It should be investigated if this association behaviour has an influence on the flavour binding.

Since foods are more complex than the model systems investigated in this study, it would be interesting to study protein-flavour interactions in multi-component systems. For example, competition experiments with two or more proteins and flavour compounds could be investigated. The latter would give better insight into the binding sites for different flavour compounds on a specific protein.

There is an increasing knowledge on the interactions between proteins and flavour compounds; however the binding sites for different flavour compounds on proteins are still not well-known. NMR techniques should be used since they give insight into binding mechanisms and binding topology. Recently, the surface plasmon resonance (SPR) technique has been receiving increasing attention as a rapid, high sensitivity, real-time technique to study interactions between proteins and different ligands *(Karlsson, 2004)*. SPR could potentially be used to investigate the binding between proteins and flavour compounds; however, the flavour compounds, instead of the protein, would need to be immobilised onto the surface of the sensorchip because the changes in the refractive index resulting from the binding process of low molecular weight compounds to immobilised proteins are often small *(Wang et al., 2007)*. Similar approaches have been used previously by immobilising low molecular weight compounds, such as biotin *(Wenz and Liepold, 2007)* and riboflavin *(Morris and Sadana, 2005)*, or chloramphenicol *(Gaudin and Maris, 2001)* and sulphonamide *(McGrath et al., 2005)*, onto the biosensor chip.

The impact of the physicochemical interactions between flavour compounds and proteins on flavour release have been studied; however, the effect on flavour perception should also be investigated using sensory analysis since flavour perception may affect the acceptablity of food products. The *in vivo* study has indicated that reversibly bound flavour compound 2-nonanone can be released from WPI during consumption, which means that the perception of 2-nonanone may not be notably reduced in the presence of WPI. In addition, a range of flavour compounds should be used.

A release of 2-nonanone from sodium caseinate *in vivo* was not observed in this study, possibly due to the low binding affinity of 2-nonanone and caseins. The release from sodium caseinate needs to be investigated further by using higher sodium caseinate concentrations, which may result in a significant flavour release.

This research has focused on flavour binding and release in simple, liquid model systems, consiting of a small number of components. Food systems are much more complex, consisting of several food matrix components and flavour mixtures. Future work should therefore be extended to more complex systems, such as emulsions or gels, and finally real food products. However, primarily consistent results using simple systems should be obtained prior to investigating more complex systems.

BIBLIOGRAPHY

Adams, R. L., Mottram, D. S., Parker, J. K., and Brown, H. M. (2001). Flavor-protein binding: Disulfide interchange reactions between ovalbumin and volatile disulfides. *Journal of Agricultural and Food Chemistry* **49** (9): 4333-4336.

Alaiz, M., and Girón, J. (1994). Modification of histidine-residues in bovine serum-albumin by reaction with (*E*)-2-octenal. *Journal of Agricultural and Food Chemistry* **42** (10): 2094-2098.

Andrews, A. T. (1983). Proteinases in normal bovine-milk and their action on caseins. *Journal of Dairy Research* **50** (1): 45-55.

Andriot, I., Marin, I., Feron, G., Relkin, P., and Guichard, E. (1999). Binding of benzaldehyde by β-lactoglobulin, by static headspace and high performance liquid chromatography in different physico-chemical conditions. *Lait* **79** (6): 577-586.

Andriot, I., Harrison, M., Fournier, N., and Guichard, E. (2000). Interactions between methyl ketones and β-lactoglobulin: Sensory analysis, headspace analysis, and mathematical modeling. *Journal of Agricultural and Food Chemistry* **48** (9): 4246-4251.

AOAC. (1995). *Official Methods of Analysis of AOAC International* (16th ed.). Gaithersburg, MD: AOAC International.

Arnold, R. G., and Lindsay, R. C. (1969). Quantitative determination of *n*-methyl ketones and *o*-aminoacetophenone in sterilized concentrated milk. *Journal of Dairy Science* **52** (7): 1097-1100.

Arthur, C. L., and Pawliszyn, J. (1990). Solid-phase microextraction with thermal desorption using fused silica optical fibers. *Analytical Chemistry* **62** (19): 2145-2148.

Arthur, C. L., Killam, L. M., Buchholz, K. D., Pawliszyn, J., and Berg, J. R. (1992). Automation and optimization of solid-phase microextraction. *Analytical Chemistry* **64** (17): 1960-1966.

Baek, I., Linforth, R. S. T., Blake, A., and Taylor, A. J. (1999). Sensory perception is related to the rate of change of volatile concentration in-nose during eating of model gels. *Chemical Senses* **24** (2): 155-160.

Bakker, J., Boudaud, N., and Harrison, M. (1998). Dynamic release of diacetyl from liquid gelatin in the headspace. *Journal of Agricultural and Food Chemistry* **46** (7): 2714-2720.

Batt, C. A., Brady, J., and Sawyer, L. (1994). Design improvements of β-lactoglobulin. *Trends in Food Science and Technology* **5** (8): 261-265.

Beyeler, M., and Solms, J. (1974). Interaction of flavor model compounds with soy protein and bovine serum albumin. *Lebensmittel-Wissenschaft und -Technologie* **7** (4): 217-219.

Boey, M. R. (2003). Personal communication Supelco, Bellefonte, PA, USA.

Boland, A. B., Delahunty, C. M., and van Ruth, S. M. (2006). Influence of the texture of gelatin gels and pectin gels on strawberry flavour release and perception. *Food Chemistry* **96** (3): 452-460.

Bowes, J. H., and Cater, C. W. (1968). The interaction of aldehydes with collagen. *Biochimica et Biophysica Acta* **168**: 341-352.

Bruenner, B. A., Jones, A. D., and German, J. B. (1995). Direct characterization of protein adducts of the lipid peroxidation product 4-hydroxy-2-nonenal using electrospray mass spectrometry. *Chemical Research in Toxicology* **8** (4): 552-559.

Buettner, A., and Schieberle, P. (2000). Exhaled odorant measurement (EXOM) - a new approach to quantify the degree of in-mouth release of food aroma compounds. *Lebensmittel-Wissenschaft und -Technologie* **33** (8): 553-559.

Buettner, A., Beer, A., Hannig, C., and Settles, M. (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging - consequences for retronasal aroma stimulation. *Chemical Senses* **26** (9): 1211-1219.

Burova, T. V., Grinberg, N. V., Golubeva, I. A., Mashkevich, A. Y., Grinberg, V. Y., and Tolstoguzov, V. B. (1999). Flavour release in model bovine serum albumin/pectin/2-octanone systems. *Food Hydrocolloids* **13** (1): 7-14.

Burova, T. V., Grinberg, N. V., Grinberg, V. Y., and Tolstoguzov, V. B. (2003). Binding of odorants to individual proteins and their mixtures. Effects of protein denaturation and association. A plasticized globule state. *Colloids and Surfaces A - Physicochemical and Engineering Aspects* **213** (2-3): 235-244.

Buttery, R. G., Ling, L. C., and Guadagni, D. G. (1969). Volatilities of aldehydes, ketones, and esters in dilute water solution. *Journal of Agricultural and Food Chemistry* **17** (2): 385-389.

Carr, J., Baloga, D., Guinard, J. X., Lawter, L., Marty, C., and Squire, C. (1996). The effect of gelling agent type and concentration on flavor release in model systems. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 98-108). Washington, D.C.: American Chemical Society.

Chai, M., and Pawliszyn, J. (1995). Analysis of environmental air samples by solid-phase microextraction and gas-chromatography ion-trap mass-spectrometry. *Environmental Science and Technology* **29** (3): 693-701.

Charles, M., Bernal, B., and Guichard, E. (1996). Interaction of ß-lactoglobulin with flavour compounds. In A. J. Taylor and D. S. Mottram (Eds.), *Flavour Science: Recent Developments* (pp. 433-436). Cambridge, U.K.: Royal Society of Chemistry.

Charles, M., Lambert, S., Brondeur, P., Courthaudon, J.-L., and Guichard, E. (2000). Influence of formulation and structure of an oil-in-water emulsion on flavor release. In D. Roberts and A. Taylor (Eds.), *Flavor Release* (pp. 342-354). Washington, DC: American Chemical Society.

Cho, Y. J., Batt, C. A., and Sawyer, L. (1994). Probing the retinol-binding site of bovine β-lactoglobulin. *Journal of Biological Chemistry* **269** (15): 11102-11107.

Chobpattana, W., Jeon, I. J., Smith, J. S., and Loughin, T. M. (2002). Mechanisms of interaction between vanillin and milk proteins in model systems. *Journal of Food Science* **67** (3): 973-977.

Chung, S., and Villota, R. (1989). Binding of alcohols by soy protein in aqueous solutions. *Journal of Food Science* **54** (6): 1604-1606.

Chung, S. J., Heymann, H., and Grün, L. U. (2003). Temporal release of flavor compounds from low-fat and high-fat ice cream during eating. *Journal of Food Science* **68** (6): 2150-2156.

Coleman, W. M. (1996). A study of the behavior of Maillard reaction products analyzed by solid-phase microextraction gas chromatography mass selective detection. *Journal of Chromatographic Science* **34** (5): 213-218.

Considine, T., Patel, H. A., Singh, H., and Creamer, L. K. (2005a). Influence of binding of sodium dodecyl sulfate, all-*trans*-retinol, palmitate, and 8-anilino-1-naphthalenesulfonate on the heat-induced unfolding and aggregation of β-lactoglobulin B. *Journal of Agricultural and Food Chemistry* **53** (8): 3197-3205.

Considine, T., Singh, H., Patel, H. A., and Creamer, L. K. (2005b). Influence of binding of sodium dodecyl sulfate, all-trans-retinol, and 8-anilino-1-naphthalenesulfonate on the high-pressure-induced unfolding and aggregation of β-lactoglobulin B. *Journal of Agricultural and Food Chemistry* **53** (20): 8010-8018.

Considine, T., Patel, H. A., Singh, H., and Creamer, L. K. (2007a). Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of β-lactoglobulin B. *Food Chemistry* **102** (4): 1270-1280.

Considine, T., Patel, H. A., Anema, S. G., Singh, H., and Creamer, L. K. (2007b). Interactions of milk proteins during heat and high hydrostatic pressure treatments - a review. *Innovative Food Science and Emerging Technologies* **8** (1): 1-23.

Cook, D. J., Hollowood, T. A., Linforth, R. S. T., and Taylor, A. J. (2003). Oral shear stress predicts flavour perception in viscous solutions. *Chemical Senses* **28** (1): 11-23.

Creamer, L. K. (1974). Preparation of α_{s1}-casein A. *Journal of Dairy Science* **57** (3): 341-344.

Creamer, L. K. (1995). Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin. *Biochemistry* **34** (21): 7170-7176.

Dam, T. K., Roy, R., Page, D., and Brewer, C. F. (2002). Negative cooperativity associated with binding of multivalent carbohydrates to lectins. Thermodynamic analysis of the "multivalency effect". *Biochemistry* **41** (4): 1351-1358.

Damodaran, S., and Kinsella, J. E. (1980a). Stabilization of proteins by solvents - effect of pH and anions on the positive cooperativity of 2-nonanone binding to bovine serum albumin. *Journal of Biological Chemistry* **255** (18): 8503-8508.

Damodaran, S., and Kinsella, J. E. (1980b). Flavor protein interactions - binding of carbonyls to bovine serum albumin - thermodynamic and conformational effects. *Journal of Agricultural and Food Chemistry* **28** (3): 567-571.

Damodaran, S., and Kinsella, J. E. (1981a). Interaction of carbonyls with soy protein - thermodynamic effects. *Journal of Agricultural and Food Chemistry* **29** (6): 1249-1253.

Damodaran, S., and Kinsella, J. E. (1981b). Interaction of carbonyls with soy protein - conformational effects. *Journal of Agricultural and Food Chemistry* **29** (6): 1253-1257.

Damodaran, S., and Kinsella, J. E. (1981c). The effects of neutral salts on the stability of macromolecules - a new approach using a protein-ligand binding system. *Journal of Biological Chemistry* **256** (7): 3394-3398.

Damodaran, S., and Kinsella, J. E. (1983). Binding of carbonyls to fish actomyosin. *Journal of Agricultural and Food Chemistry* **31** (4): 856-859.

Dannenfelser, R. M., and Yalkowsky, S. H. (1991). Data-base of aqueous solubility for organic nonelectrolytes. *Science of the Total Environment* **109**: 625-628.

Darling, D. F., Williams, D., and Yendle, P. (1986). Physico-chemical interactions involved in aroma transport processes from solution. In G. Birch and M. Lindley (Eds.), *Interactions of Food Components* (pp. 165-188). London: Elsevier Applied Science.

de la Fuente, M. A., Singh, H., and Hemar, Y. (2002). Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends in Food Science and Technology* **13** (8): 262-274.

de Roos, K. B., and Wolswinkel, K. (1994). Non-equilibrium partition model for predicting flavour release in the mouth. In H. Maarse and D. G. van der Heij (Eds.), *Trends in Flavour Research* (pp. 15-32). Amsterdam: Elsevier Science.

de Roos, K. B. (2003). Effect of texture and microstructure on flavour retention and release. *International Dairy Journal* **13** (8): 593-605.

de Wit, J. N. (1981). Structure and functional behaviour of whey proteins. *Netherlands Milk and Dairy Journal* **35**: 47-64.

Deibler, K. D., Lavin, E. H., Linforth, R. S. T., Taylor, A. J., and Acree, T. E. (2001). Verification of a mouth simulator by *in vivo* measurements. *Journal of Agricultural and Food Chemistry* **49** (3): 1388-1393.

Delahunty, C. M., Piggott, J. R., Conner, J. M., and Paterson, A. (1994). Low-fat cheddar cheese flavour: Flavour release in the mouth. In H. Maarse and D. G. van der Heij (Eds.), *Trends in Flavour Research* (pp. 47-52). Amsterdam: Elsevier Science.

Delben, F., and Stefancich, S. (1997). Interaction of food proteins with polysaccharides, I. Properties upon mixing. *Journal of Food Engineering* **31** (3): 325-346.

Denker, M., Parat-Wilhelms, M., Drichelt, G., Paucke, J., Luger, A., Borcherding, K., Hoffmann, W., and Steinhart, H. (2006). Investigation of the retronasal flavour release during the consumption of coffee with additions of milk constituents by 'oral breath sampling'. *Food Chemistry* **98**: 201-208.

Dhont, J. H. (1987). Reaction of vanillin with albumin. In H. Maarse and P. J. Groenen (Eds.), *Aroma Research* (pp. 193-194). Wageningen: Pudoc.

Díaz de Villegas, M. C., Oria, R., Sala, F. J., and Calvo, M. (1987). Lipid-binding by β-lactoglobulin of cow milk. *Milchwissenschaft* **42** (6): 357-358.

Doyen, K., Carey, M., Linforth, R. S. T., Marin, M., and Taylor, A. J. (2001). Volatile release from an emulsion: Headspace and in-mouth studies. *Journal of Agricultural and Food Chemistry* **49** (2): 804-810.

Druaux, C., Lubbers, S., Charpentier, C., and Voilley, A. (1995). Effects of physicochemical parameters of a model wine on the binding of γ-decalactone on bovine serum albumin. *Food Chemistry* **53** (2): 203-207.

Dubois, C., Sergent, M., and Voilley, A. (1996). Flavoring of complex media: A model cheese example. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 217-226). Washington, D.C.: American Chemical Society.

Dufour, E., and Haertlé, T. (1990a). Binding affinities of β-ionone and related flavor compounds to β-lactoglobulin - effects of chemical modifications. *Journal of Agricultural and Food Chemistry* **38** (8): 1691-1695.

Dufour, E., and Haertlé, T. (1990b). Alcohol-induced changes of β-lactoglobulin retinol binding stoichiometry. *Protein Engineering* **4** (2): 185-190.

Dufour, E., Marden, M. C., and Haertlé , T. (1990). β-Lactoglobulin binds retinol and protoporphyrin-IX at two different binding sites. *FEBS Letters* **277** (1-2): 223-226.

Dumont, J. P., and Land, D. G. (1986). Binding of diacetyl by pea proteins. *Journal of Agricultural and Food Chemistry* **34** (6): 1041-1045.

Dumont, J. P. (1987). Flavour-protein interactions: A key to aroma persistence. In M. Martens, G. A. Dalen and H. Russwurm (Eds.), *Flavour Science and Technology* (pp. 143-148). Chichester: John Wiley and Sons.

Dwivedi, B. K., and Kinsella, J. E. (1974). Continuous production of blue-type cheese flavor by submerged fermentation of penicillium-roqueforti. *Journal of Food Science* **39** (3): 620-622.

Ebeler, S. E., Pangborn, R. M., and Jennings, W. G. (1988). Influence of dispersion medium on aroma intensity and headspace concentration of menthone and isoamyl acetate. *Journal of Agricultural and Food Chemistry* **36** (4): 791-796.

Elgar, D. F., Norris, C. S., Ayers, J. S., Pritchard, M., Otter, D. E., and Palmano, K. P. (2000). Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *Journal of Chromatography A* **878** (2): 183-196.

Elmore, J. S., and Langley, K. R. (1996). Novel vessel for the measurement of dynamic flavor release in real time from liquid foods. *Journal of Agricultural and Food Chemistry* **44** (11): 3560-3563.

Etzel, M. R. (2004). Manufacture and use of dairy protein fractions. *Journal of Nutrition* **134** (4): 996-1002.

Fabre, M., Aubry, V., and Guichard, E. (2002). Comparison of different methods: Static and dynamic headspace and solid-phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. *Journal of Agricultural and Food Chemistry* **50** (6): 1497-1501.

Farès, K., Landy, P., Guilard, R., and Voilley, A. (1998). Physicochemical interactions between aroma compounds and milk proteins: Effect of water and protein modification. *Journal of Dairy Science* **81** (1): 82-91.

Fischer, N., and Widder, S. (1997). How proteins influence food flavor. *Food Technology* **51** (1): 68-70.

Foster, J. F., and Sterman, M. D. (1956). Conformation changes in bovine plasma albumin associated with hydrogen ion and urea binding. II. Hydrogen ion titration curves. *Journal of the American Chemical Society* **78** (15): 3656-3660.

Fox, P. F., and Mulvihill, D. M. (1982). Milk proteins - molecular, colloidal and functional properties. *Journal of Dairy Research* **49** (4): 679-693.

Franzen, K. L., and Kinsella, J. E. (1974). Parameters affecting binding of volatile flavor compounds in model food systems.1. Proteins. *Journal of Agricultural and Food Chemistry* **22** (4): 675-678.

Frapin, D., Dufour, E., and Haertlé, T. (1993). Probing the fatty-acid-binding site of β-lactoglobulins. *Journal of Protein Chemistry* **12** (4): 443-449.

Friedrich, H., and Gubler, B. A. (1978). Interaction between flavoring substances and food components. 1. Conduct of citral in mixtures of food components and water. *Lebensmittel-Wissenschaft und -Technologie* **11** (4): 215-218.

Friedrich, J. E., and Acree, T. E. (1998). Gas chromatography olfactometry (GC/O) of dairy products. *International Dairy Journal* **8** (3): 235-241.

Fugate, R. D., and Song, P. S. (1980). Spectroscopic characterization of β-lactoglobulin-retinol complex. *Biochimica et Biophysica Acta* **625** (1): 28-42.

Funtenberger, S., Dumay, E., and Cheftel, J. C. (1995). Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. *Lebensmittel-Wissenschaft und -Technologie* **28** (4): 410-418.

Futterman, S., and Heller, J. (1972). Enhancement of fluorescence and decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β-lactoglobulin, and the retinol-binding protein of human plasma. *Journal of Biological Chemistry* **247** (16): 5168-5172.

Gaudin, V., and Maris, P. (2001). Development of a biosensor-based immunoassay for screening of chloramphenicol residues in milk. *Food and Agricultural Immunology* **13** (2): 77-86.

Gianelli, M. P., Flores, M., and Toldra, F. (2005). Interaction of soluble peptides and proteins from skeletal muscle with volatile compounds in model systems as affected by curing agents. *Journal of Agricultural and Food Chemistry* **53** (5): 1670-1677.

Gierczynski, I., Labouré, H., Sémon, E., and Guichard, E. (2007). Impact of hardness of model fresh cheese on aroma release: *In vivo* and *in vitro* study. *Journal of Agricultural and Food Chemistry* **55** (8) 3066-3073.

Giroux, H. J., Perreault, V., and Britten, M. (2007). Characterization of hydrophobic flavor release profile in oil-in-water emulsions. *Journal of Food Science* **72** (2): S125-S129.

González-Tomás, L., Bayarri, S., Taylor, A. J., and Costell, E. (2007). Flavour release and perception from model dairy custards. *Food Research International* **40** (4): 520-528.

Graf, E., and de Roos, K. B. (1996). Performance of vanilla flavor in low-fat ice cream. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 24-35). Washington, D.C.: American Chemical Society.

Gremli, H. A. (1974). Interaction of flavor compounds with soy protein. *Journal of the American Oil Chemists' Society* **51** (1): A95-A97.

Grote, C., and Pawliszyn, J. (1997). Solid-phase microextraction for the analysis of human breath. *Analytical Chemistry* **69** (4): 587-596.

Guichard, E. (2000). Cost action 96: Interaction of food matrix with small ligands influencing flavour and texture. *Food Research International* **33** (3-4): 187-190.

Guichard, E., and Langourieux, S. (2000). Interactions between β-lactoglobulin and flavour compounds. *Food Chemistry* **71** (3): 301-308.

Guichard, E. (2006). Flavour retention and release from protein solutions. *Biotechnology Advances* **24** (2): 226-229.

Guinard, J. X., and Marty, C. (1995). Time-intensity measurement of flavor release from a model gel system - effect of gelling agent type and concentration. *Journal of Food Science* **60** (4): 727-730.

Guth, H., and Fritzler, R. (2004). Binding studies and computer-aided modelling of macromolecule/odorant interactions. *Chemistry and Biodiversity* **1** (12): 2001-2023.

Gwartney, E. A., Foegeding, E. A., and Larick, D. K. (2000). The role of texture and fat on flavor release from whey protein isolate gels. In D. D. Roberts and A. J. Taylor (Eds.), *Flavor Release* (pp. 355-367). Washington, DC: American Chemical Society.

Hambling, S. G., McAlpine, A. S., and Sawyer, L. (1992). β-Lactoglobulin. In P. F. Fox (Ed.), *Advanced Dairy Chemistry - 1: Proteins* (pp. 141-190). Amsterdam: Elsevier Applied Science.

Hansen, A. P., and Heinis, J. J. (1991). Decrease of vanillin flavor perception in the presence of casein and whey proteins. *Journal of Dairy Science* **74** (9): 2936-2940.

Hansen, A. P., and Heinis, J. J. (1992). Benzaldehyde, citral, and *d*-limonene flavor perception in the presence of casein and whey proteins. *Journal of Dairy Science* **75** (5): 1211-1215.

Hansen, A. P., and Booker, D. C. (1996). Flavor interaction with casein and whey protein. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 75-89). Washington, DC: American Chemical Society.

Hansson, A., Giannouli, P., and Van Ruth, S. (2003). The influence of gel strength on aroma release from pectin gels in a model mouth and in vivo, monitored with proton-transfer-reaction mass spectrometry. *Journal of Agricultural and Food Chemistry* **51** (16): 4732-4740.

Hardy, P. J., and Michael, B. J. (1970). Volatile components of feijoa fruits. *Phytochemistry* **9** (6): 1355-1357.

Haring, P. G. M. (1990). Flavour release: From product to perception. In Y. Bessiere and A. F. Thomas (Eds.), *Flavour Science and Technology* (pp. 351-354). Chichester, UK: John Wiley and Sons.

Harrison, M. (1998). Effect of breathing and saliva flow on flavor release from liquid foods. *Journal of Agricultural and Food Chemistry* **46** (7): 2727-2735.

Harvey, B. A., Druaux, C., and Voilley, A. (1995). Effect of protein on the retention and transfer of aroma compounds at the lipid-water interface. In E. Dickinson and D. Lorient (Eds.), *Food Macromolecules and Colloids* (pp. 154-163). Cambridge, UK: Royal Society of Chemistry.

Hatchwell, L. C. (1996). Implications of fat on flavor. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 14-23). Washington, D.C.: American Chemical Society.

Havea, P., Singh, H., and Creamer, L. K. (2001). Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. *Journal of Dairy Research* **68** (3): 483-497.

Hawthorne, S. B., Miller, D. J., Pawliszyn, J., and Arthur, C. L. (1992). Solventless determination of caffeine in beverages using solid-phase microextraction with fused-silica fibers. *Journal of Chromatography* **603** (1-2): 185-191.

Heinemann, C., Conde-Petit, B., Nuessli, J., and Escher, F. (2001). Evidence of starch inclusion complexation with lactones. *Journal of Agricultural and Food Chemistry* **49** (3): 1370-1376.

Heng, L., van Koningsveld, G. A., Gruppen, H., van Boekel, M., Vincken, J. P., Roozen, J. P., and Voragen, A. G. J. (2004). Protein-flavour interactions in relation to development of novel protein foods. *Trends in Food Science and Technology* **15** (3-4): 217-224.

Hewitt, C. N., Hayward, S., and Tani, A. (2003). The application of proton transfer reaction-mass spectrometry (PTR-MS) to the monitoring and analysis of volatile organic compounds in the atmosphere. *Journal of Environmental Monitoring* **5** (1): 1-7.

Hill, A. V. (1910). The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *Proceedings of the Physiological Society London* **40**: IV-VII.

Hollowood, T. A., Linforth, R. S. T., and Taylor, A. J. (2002). The effect of viscosity on the perception of flavour. *Chemical Senses* **27** (7): 583-591.

Hummel, J. R., and Dreyer, W. J. (1962). Measurement of protein-binding phenomena by gel filtration. *Biochimica et Biophysica Acta* **63**: 530-532.

Hummer, G., Garde, S., Garcia, A. E., Paulaitis, M. E., and Pratt, L. R. (1998). The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. *Proceedings of the National Academy of Sciences of the United States of America* **95** (4): 1552-1555.

Huppertz, T., Fox, P. F., and Kelly, A. L. (2004a). High pressure treatment of bovine milk: Effects on casein micelles and whey proteins. *Journal of Dairy Research* **71** (1): 97-106.

Hupper z, T., Fox, P. F., and Kelly, A. L. (2004b). High pressure-induced denaturation of α-lactalbumin and β-lactoglobulin in bovine milk and whey: A possible mechanism. *Journal of Dairy Research* **71** (4): 489-495.

Iametti, S., Transidico, P., Bonomi, F., Vecchio, G., Pittia, P., Rovere, P., and DallAglio, G. (1997). Molecular modifications of β-lactoglobulin upon exposure to high pressure. *Journal of Agricultural and Food Chemistry* **45** (1): 23-29.

Ingham, K. E., Linforth, R. S. T., and Taylor, A. J. (1995). The effect of eating on aroma release from strawberries. *Food Chemistry* **54** (3): 283-288.

Jaime, I., Mela, D. J., and Bratchell, N. (1993). A study of texture-flavor interactions using free-choice profiling. *Journal of Sensory Studies* **8**: 177-188.

Jasinski, E., and Kilara, A. (1985). Flavor binding by whey proteins. *Milchwissenschaft* **40** (10): 596-599.

Jennings, W. G. (1965). Influence of temperature and salt addends on vapor equilibration of headspace. *Journal of Food Science* **30**: 445-449.

Jouenne, E., and Crouzet, J. (1996). Interaction of aroma compounds with β-lactoglobulin. In A. J. Taylor and D. S. Mottram (Eds.), *Flavour Science: Recent Developments* (pp. 425-429). Cambridge, U.K.: Royal Society of Chemistry.

Jouenne, E., and Crouzet, J. (2000a). Effect of pH on retention of aroma compounds by β-lactoglobulin. *Journal of Agricultural and Food Chemistry* **48** (4): 1273-1277.

Jouenne, E., and Crouzet, J. (2000b). Determination of apparent binding constants for aroma compounds with β-lactoglobulin by dynamic coupled column liquid chromatography. *Journal of Agricultural and Food Chemistry* **48** (11): 5396-5400.

Jouquand, C., Ducruet, V., and Giampaoli, P. (2004). Partition coefficients of aroma compounds in polysaccharide solutions by the phase ratio variation method. *Food Chemistry* **85** (3): 467-474.

Jung, D. M., de Ropp, J. S., and Ebeler, S. E. (2002). Application of pulsed field gradient NMR techniques for investigating binding of flavor compounds to macromolecules. *Journal of Agricultural and Food Chemistry* **50** (15): 4262-4269.

Jung, D. M., and Ebeler, S. E. (2003a). Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. *Journal of Agricultural and Food Chemistry* **51** (1): 200-205.

Jung, D. M., and Ebeler, S. E. (2003b). Investigation of binding behavior of α- and ß-ionones to β-lactoglobulin at different pH values using a diffusion-based NOE pumping technique. *Journal of Agricultural and Food Chemistry* **51** (7): 1988-1993.

Kahn, I., Müller, K., and Warmbier, H. (1977). Effect of variety and fertilization on the spectrum of volatile aroma compounds in potatoes. *Potato Research* **20** (3): 235-242.

Karlsson, R. (2004). SPR for molecular interaction analysis: A review of emerging application areas. *Journal of Molecular Recognition* **17**: 151-161.

Kellard, B., Busfield, D. M., and Kinderlerer, J. L. (1985). Volatile off-flavor compounds in desiccated coconut. *Journal of the Science of Food and Agriculture* **36** (5): 415-420.

King, B. M., and Solms, J. (1979). Interactions of flavor compounds in model food systems using benzyl alcohol as an example. *Journal of Agricultural and Food Chemistry* **27** (6): 1331-1334.

Kinsella, J. E., and Whitehead, D. M. (1989). Proteins in whey: Chemical, physical, and functional properties. *Advances in Food and Nutrition Research* **33**: 343-438.

Klotz, I. M., Walker, F. M., and Pivan, R. B. (1946). The binding of organic ions by proteins. *Journal of the American Chemical Society* **68**: 1486-1490.

Klotz, I. M., and Urquhart, J. M. (1948). The combination of adenine, adenosine, and adenylic acid with serum albumin. *Journal of Biological Chemistry* **173**: 21-24.

Kubícková, J., and Grosch, W. (1997). Evaluation of potent odorants of camembert cheese by dilution and concentration techniques. *International Dairy Journal* **7** (1): 65-70.

Land, D. G., and Reynolds, J. (1981). The influence of food components on the volatility of diacetyl. In P. Schreier (Ed.), *Flavour '81* (pp. 701-705). Berlin: Walter de Gruyter.

Land, D. G. (1996). Perspectives on the effects of interactions on flavor perception: An overview. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 2-11). Washington, D.C.: American Chemical Society.

Landy, P., Druaux, C., and Voilley, A. (1995). Retention of aroma compounds by proteins in aqueous solution. *Food Chemistry* **54** (4): 387-392.

Landy, P., Fares, K., Lorient, D., and Voilley, A. (1997). Effect of chemical modification of sodium caseinate on diffusivity of aroma compounds in aqueous solutions. *Journal of Agricultural and Food Chemistry* **45** (7): 2649-2653.

Langler, J. E., and Day, E. A. (1964). Development and flavor properties of methyl ketones in milk fat. *Journal of Dairy Science* **47**: 1291-1296.

Langourieux, S., and Crouzet, J. (1995). Protein-aroma interactions. In E. Dickinson and D. Lorient (Eds.), *Food Macromolecules and Colloids* (pp. 123-133). Cambridge, U.K.: Royal Society of Chemistry.

Lawrence, R. C., and Creamer, L. K. (1969). The action of calf rennet and other proteolytic enzymes on κ-casein. *Journal of Dairy Research* **36**: 11-20.

Le Guen, S., and Vreeker, R. (2003). Interactions between flavour compounds and milk proteins under static and dynamic conditions. In J. L. Le Quere and P. Etievant (Eds.), *Flavour Research at the Dawn of the Twenty-First Century* (pp. 182-187). Paris: Lavoisier Tec & Doc.

Le Thanh, M., Thibeaudeau, P., Thibaut, M. A., and Voilley, A. (1992). Interactions between volatile and nonvolatile compounds in the presence of water. *Food Chemistry* **43** (2): 129-135.

Lee, W. E. (1986). A suggested instrumental technique for studying dynamic flavor release from food products. *Journal of Food Science* **51** (1): 249-250.

Legger, A., and Roozen, J. P. (1994). Gas chromatography and sensory analysis of chocolate flavour: Intensity changes in time. In H. Maarse and D. G. van der Heij (Eds.), *Trends in Flavour Research* (pp. 287-291). Amsterdam: Elsevier Science.

Lethuaut, L., Weel, K. G. C., Boelrijk, A. E. M., and Brossard, C. D. (2004). Flavor perception and aroma release from model dairy desserts. *Journal of Agricultural and Food Chemistry* **52** (11): 3478-3485.

Li, Z., Grün, I. U., and Fernando, L. N. (2000). Interaction of vanillin with soy and dairy proteins in aqueous model systems: A thermodynamic study. *Journal of Food Science* **65** (6): 997-1001.

Liebich, H. M., Douglas, D. R., Bayer, E., and Zlatkis, A. (1970). Volatile flavor components of cheddar cheese. *Journal of Chromatographic Science* **8** (6): 355-359.

Liedtke, R. J., and Djerassi, C. (1969). Mass spectrometry in structural and stereochemical problems. CIXXXIII. A study of electron impact induced fragmentation of aliphatic aldehydes. *Journal of the American Chemical Society* **91** (24): 6814-6821.

Lindinger, W., Hansel, A., and Jordan, A. (1998). On-line monitoring of volatile organic compounds at pptV levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) - medical applications, food control and environmental research. *International Journal of Mass Spectrometry* **173** (3): 191-241.

Linforth, R. S. T., and Taylor, A. J. (1993). Measurement of volatile release in the mouth. *Food Chemistry* **48** (2): 115-120.

Linforth, R. S. T., Ingham, K. E., and Taylor, A. J. (1996). Time course profiling of volatile release from foods during the eating process. In A. J. Taylor and D. S. Mottram (Eds.), *Flavour Science: Recent Developments* (pp. 361-368). Cambridge, UK: The Royal Society of Chemistry.

Linforth, R. S. T., Baek, I., and Taylor, A. J. (1999). Simultaneous instrumental and sensory analysis of volatile release from gelatine and pectin/gelatine gels. *Food Chemistry* **65** (1): 77-83.

Linforth, R. S. T., Hodgson, M., and Taylor, A. J. (2003). Studies of retronasal flavour delivery. In J. L. Le Quere and P. Etievant (Eds.), *Flavour Research at the Dawn of the Twenty-First Century* (pp. 143-147). Paris: Lavoisier Tec & Doc.

Liu, X., Powers, J. R., Swanson, B. G., Hill, H. H., and Clark, S. (2005a). Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. *Innovative Food Science and Emerging Technologies* **6**: 310-317.

Liu, X., Powers, J. R., Swanson, B. G., Hill, H. H., and Clark, S. (2005b). High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. *Journal of Food Science* **70** (9): C581-C585.

Louch, D., Motlagh, S., and Pawliszyn, J. (1992). Dynamics of organic-compound extraction from water using liquid-coated fused-silica fibers. *Analytical Chemistry* **64** (10): 1187-1199.

Lübke, M., Guichard, E., and Le Quéré, J. L. (2000). Infrared spectroscopic study of β-lactoglobulin interactions with flavor compounds. In D. D. Roberts and A. J. Taylor (Eds.), *Flavor Release* (pp. 282-292). Washington, D.C.: American Chemical Society.

Lübke, M., Guichard, E., Tromelin, A., and Le Quéré, J. L. (2002). Nuclear magnetic resonance spectroscopic study of β-lactoglobulin interactions with two flavor compounds, γ-decalactone and β-ionone. *Journal of Agricultural and Food Chemistry* **50** (24): 7094-7099.

Lynch, A. G., Mulvihill, D. M., Law, A. J. R., Leaver, J., and Horne, D. S. (1997). Chromatographic elution profiles, electrophoretic properties and free amino and sulphydryl group contents of commercial sodium caseinates. *International Dairy Journal* **7** (4): 213-220.

Maier, H. G. (1970). Volatile flavoring substances in foodstuffs. *Angewandte Chemie International Edition* **9** (12): 917-926.

Maier, H. G. (1972). Bindung flüchtiger Aromastoffe an Lebensmittel. *Lebensmittel-Wissenschaft und -Technologie* **5** (1): 1-6.

Maier, H. G. (1973). Sorption of volatile aroma constituents by foods. 7. Aliphatic aldehydes. *Zeitschrift Für Lebensmittel-Untersuchung und -Forschung* **151** (6): 384-386.

Maier, H. G. (1975). Binding of volatile aroma substances to nutrients and foodstuffs. In H. Maarse and P. J. Groenen (Eds.), *Aroma Research* (pp. 143-157). Wageningen: Pudoc.

Malone, M. E., Appelqvist, I. A. M., Goff, T. C., Homan, J. E., and Wilkins, J. P. G. (2000). A novel approach to the selective control of lipophilic flavor release in low fat foods. In D. D. Roberts and A. J. Taylor (Eds.), *Flavor Release* (pp. 212-227). Washington, DC: American Chemical Society.

Manderson, G. A., Creamer, L. K., and Hardman, M. J. (1999). Effect of heat treatment on the circular dichroism spectra of bovine β-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry* **47** (11): 4557-4567.

McGrath, T., Baxter, A., Ferguson, J., Haughey, S., and Bjurling, P. (2005). Multi sulfonamide screening in porcine muscle using a surface plasmon resonance biosensor. *Analytica Chimica Acta* **529** (1-2): 123-127.

McKenzie, H. A., and Murphy, W. H. (1970). General methods and elemental analysis. In H. A. McKenzie (Ed.), *Milk Proteins, Chemistry and Molecular Biology* (Vol. 1, pp. 127-180). New York: Academic Press.

McKenzie, H. A. (1971). β-Lactoglobulins. In H. A. McKenzie (Ed.), *Milk Proteins, Chemistry and Molecular Biology* (Vol. 2, pp. 257-330). New York: Academic Press.

McNeill, V. L., and Schmidt, K. A. (1993). Vanillin interaction with milk protein isolates in sweetened drinks. *Journal of Food Science* **58** (5): 1142-1144, 1147.

McNulty, P. B., and Karel, M. (1973). Factors affecting flavour release and uptake in O/W emulsions I. Release and uptake models. *Journal of Food Technology* **8**: 309-318.

Mei, J. B., Reineccius, G. A., Knighton, W. B., and Grimsrud, E. P. (2004). Influence of strawberry yogurt composition on aroma release. *Journal of Agricultural and Food Chemistry* **52** (20): 6267-6270.

Mestres, M., Moran, N., Jordan, A., and Buettner, A. (2005). Aroma release and retronasal perception during and after consumption of flavored whey protein gels with different textures. 1. *In vivo* release analysis. *Journal of Agricultural and Food Chemistry* **53** (2): 403-409.

Meylan, W. M., and Howard, P. H. (1995). Atom fragment contribution method for estimating octanol-water partition-coefficients. *Journal of Pharmaceutical Sciences* **84** (1): 83-92.

Meynier, A., Garillon, A., Lethuaut, L., and Genot, C. (2003). Partition of five aroma compounds between air and skim milk, anhydrous milk fat or full-fat cream. *Lait* **83** (3): 223-235.

Meynier, A., Rampon, V., Dalgalarrondo, M., and Genot, C. (2004). Hexanal and *t*-2-hexenal form covalent bonds with whey proteins and sodium caseinate in aqueous solution. *International Dairy Journal* **14** (8): 681-690.

Mick, S., Mick, W., and Schreier, P. (1982). The composition of neutral volatile constituents of sour cream butter. *Milchwissenschaft* **37** (11): 661-665.

Mills, O. E., and Solms, J. (1984). Interaction of selected flavor compounds with whey proteins. *Lebensmittel-Wissenschaft und -Technologie* **17** (6): 331-335.

Mironov, N. A., Breus, V. V., Gorbatchuk, V. V., Solomonov, B. N., and Haertle, T. (2003). Effects of hydration, lipids, and temperature on the binding of the volatile aroma terpenes by β-lactoglobulin powders. *Journal of Agricultural and Food Chemistry* **51** (9): 2665-2673.

Mohammadzadeh-K., A., Feeney, R. E., Samuels, R. B., and Smith, L. M. (1967). Solubility of alkanes in protein solutions. *Biochimica et Biophysica Acta* **147**: 583-589.

Mohammadzadeh-K., A., Feeney, R. E., and Smith, L. M. (1969a). Hydrophobic binding of hydrocarbons by proteins I. Relationship of hydrocarbon structure. *Biochimica et Biophysica Acta* **194**: 246-255.

Mohammadzadeh-K., A., Smith, L. M., and Feeney, R. E. (1969b). Hydrophobic binding of hydrocarbons by proteins II. Relationship of protein structure. *Biochimica et Biophysica Acta* **194**: 256-264.

Moio, L., Etievant, P., Langlois, D., Dekimpe, J., and Addeo, F. (1994). Detection of powerful odorants in heated milk by use of extract dilution sniffing analysis. *Journal of Dairy Research* **61** (3): 385-394.

Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L., and Eliopoulos, E. E. (1987). Crystal-structure of the trigonal form of bovine β-lactoglobulin and of its complex with retinol at 2.5 Å resolution. *Journal of Molecular Biology* **197** (4): 695-706.

Morris, B. A., and Sadana, A. (2005). A fractal analysis for the binding of riboflavin binding protein to riboflavin immobilized on a SPR biosensor. *Sensors and Actuators B-Chemical* **106** (2): 498-505.

Morrisett, J. D., Pownall, H. J., and Gotto, A. M. G. (1975). Bovine serum albumin: Study of the fatty acid and steroid binding sites using spin-labelled lipids. *Journal of Biological Chemistry* **250** (7): 2487-2494.

Mottram, D. S., Szauman-Szumski, C., and Dodson, A. (1996). Interaction of thiol and disulfide flavor compounds with food components. *Journal of Agricultural and Food Chemistry* **44** (8): 2349-2351.

Mulvihill, D. M., and Fox, P. F. (1977). Proteolysis of α_{s1}-casein by chymosin - influence of pH and urea. *Journal of Dairy Research* **44** (3): 533-540.

Mulvihill, D. M. (1992). Production, functional properties and utilization of milk protein products. In P. F. Fox (Ed.), *Advanced Dairy Chemistry-1: Proteins* (pp. 369-404). New York: Elsevier Applied Science.

Muresan, S., van der Bent, A., and de Wolf, F. A. (2001). Interaction of β-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: Nonlinear quenching effects related to protein-protein association. *Journal of Agricultural and Food Chemistry* **49** (5): 2609-2618.

Murray, K. E., Shipton, J., and Whitfield, F. B. (1972). The chemistry of food flavour I. Volatile constituents of passionfruit, *passiflora edulis*. *Australian Journal of Chemistry* **25** (9): 1921-1933.

Naßl, K., Kropf, F., and Klostermeyer, H. (1995). A method to mimic and to study the release of flavor compounds from chewed food. *Zeitschrift Für Lebensmittel-Untersuchung und -Forschung* **201** (1): 62-68.

Nawar, W. W. (1966). Some considerations in interpretation of direct headspace gas chromatographic analysis of food volatiles. *Food Technology* **20** (2): 115-117.

Nawar, W. W. (1971). Some variables affecting composition of headspace aroma. *Journal of Agricultural and Food Chemistry* **19** (6): 1057-1059.

Ng, P. K. W., Hoehn, E., and Bushuk, W. (1989a). Binding of vanillin by fababean proteins. *Journal of Food Science* **54** (1): 105-107.

Ng, P. K. W., Hoehn, E., and Bushuk, W. (1989b). Sensory evaluation of binding of vanillin by fababean proteins. *Journal of Food Science* **54** (2): 324-325, 346.

North, A. C. T. (1989). 3-Dimensional arrangement of conserved amino acid residues in a superfamily of specific ligand-binding proteins. *International Journal of Biological Macromolecules* **11** (1): 56-58.

Odake, S., Roozen, J. P., and Burger, J. J. (1998). Effect of saliva dilution on the release of diacetyl and 2-heptanone from cream style dressings. *Nahrung* **42** (6): 385-391.

O'Keefe, S. F., Resurreccion, A. P., Wilson, L. A., and Murphy, P. A. (1991a). Temperature effect on binding of volatile flavor compounds to soy protein in aqueous model systems. *Journal of Food Science* **56** (3): 802-806.

O'Keefe, S. F., Wilson, L. A., Resurreccion, A. P., and Murphy, P. A. (1991b). Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique. *Journal of Agricultural and Food Chemistry* **39** (6): 1022-1028.

O'Neill, T. E., and Kinsella, J. E. (1987a). Flavor protein interactions - characteristics of 2-nonanone binding to isolated soy protein fractions. *Journal of Food Science* **52** (1): 98-101.

O'Neill, T. E., and Kinsella, J. E. (1987b). Binding of alkanone flavors to β-lactoglobulin - effects of conformational and chemical modification. *Journal of Agricultural and Food Chemistry* **35** (5): 770-774.

O'Neill, T. E., and Kinsella, J. E. (1988). Effect of heat treatment and modification on conformation and flavor binding by β-lactoglobulin. *Journal of Food Science* **53** (3): 906-909.

Overbosch, P., Afterof, W. G. M., and Haring, P. G. M. (1991). Flavor release in the mouth. *Food Reviews International* **7** (2): 137-184.

Pangborn, R. M., and Szczesniak, A. S. (1974). Effect of hydrocolloids and viscosity on flavor and odor intensities of aromatic flavor compounds. *Journal of Texture Studies* **4**: 467-482.

Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., and Kraulis, P. J. (1986). The structure of β–lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **324** (6095): 383-385.

Parks, O. W., and Patton, S. (1961). Volatile carbonyl compounds in stored dry whole milk. *Journal of Dairy Science* **44**: 1-9.

Pawliszyn, J. (1997). *Solid Phase Microextraction - Theory and Practice*. New York: Wiley-VCH.

Pelletier, E., Sostmann, K., and Guichard, E. (1998). Measurement of interactions between β-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. *Journal of Agricultural and Food Chemistry* **46** (4): 1506-1509.

Pérez, M. D., Díaz de Villegas, M. C., Sánchez, L., Aranda, P., Ena, J. M., and Calvo, M. (1989). Interaction of fatty acids with β-lactoglobulin and albumin from ruminant milk. *Journal of Biochemistry* **106** (6): 1094-1097.

Pérez-Juan, M., Flores, M., and Toldrá, F. (2007). Effect of ionic strength of different salts on the binding of volatile compounds to porcine soluble protein extracts in model systems. *Food Research International* **40** (6): 687-693.

Peyvieux, C., and Dijksterhuis, G. (2001). Training a sensory panel for TI: A case study. *Food Quality and Preference* **12** (1): 19-28.

Philippe, E., Seuvre, A. M., Colas, B., Langendorff, V., Schippa, C., and Voilley, A. (2003). Behavior of flavor compounds in model food systems: A thermodynamic study. *Journal of Agricultural and Food Chemistry* **51** (5): 1393-1398.

Plug, H., and Haring, P. (1993). The role of ingredient-flavor interactions in the development of fat-free foods. *Trends in Food Science and Technology* **4** (5): 150-152.

Plug, H., and Haring, P. (1994). The influence of flavour-ingredient interactions on flavour perception. *Food Quality and Preference* **5**: 95-102.

Poiffait, A., and Adrian, J. (1991). Interaction between casein and vitamin a during food processing. In M. Friedman (Ed.), *Nutritional and Toxicological Consequences of Food Processing* (pp. 61-73). New York: Plenum Press.

Pokorný, J., Luan, N. T., Kondratenko, S. S., and Janicek, G. (1976). Changes of sensory value by interaction of alkanals with amino acids and proteins. *Nahrung* **20** (3): 267-272.

Price, N. C., and Dwek, R. A. (1979). Binding of ligands to macromolecules. In N. C. Price and R. A. Dwek (Eds.), *Principles and Problems in Physical Chemistry for Biochemists* (pp. 32-44). Oxford, UK: Clarendon Press.

Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, H. M., Baker, E. N., and Jameson, G. B. (1998). Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry* **37** (40): 14014-14023.

Rabe, S., Krings, U., Banavara, D. S., and Berger, R. G. (2002). Computerized apparatus for measuring dynamic flavor release from liquid food matrices. *Journal of Agricultural and Food Chemistry* **50** (22): 6440-6447.

Ragona, L., Fogolari, F., Zetta, L., Perez, D. M., Puyol, P., de Kruif, K., Lohr, F., Ruterjans, H., and Molinari, H. (2000). Bovine β-lactoglobulin: Interaction studies with palmitic acid. *Protein Science* **9** (7): 1347-1356.

Ray, A., and Chatterjee, R. (1967). Interactions of β-lactoglobulins with large organic ions. In G. N. Ramachandran (Ed.), *Conformation of Biopolymers* (Vol. 1, pp. 235-252). London: Academic Press.

Reineccius, G. A., and Coulter, S. T. (1969). Flavor retention during drying. *Journal of Dairy Science* **52** (8): 1219-1223.

Reiners, J., Nicklaus, S., and Guichard, E. (2000). Interactions between β-lactoglobulin and flavour compounds of different chemical classes. Impact of the protein on the odour perception of vanillin and eugenol. *Lait* **80** (3): 347-360.

Reynolds, J., Herbert, S., and Steinhardt, J. (1968). The binding of some long-chain fatty acid anions and alcohols by bovine serum albumin. *Biochemistry* **7** (4): 1357-1361.

Roberts, D. D., and Acree, T. E. (1995). Simulation of retronasal aroma using a modified headspace technique - investigating the effects of saliva, temperature, shearing, and oil on flavor release. *Journal of Agricultural and Food Chemistry* **43** (8): 2179-2186.

Roberts, D. D., and Acree, T. E. (1996a). Model development for flavour release from homogenous phases. In A. J. Taylor and D. S. Mottram (Eds.), *Flavour Science: Recent Developments* (pp. 399-404). Cambridge, UK: The Royal Society of Chemistry.

Roberts, D. D., and Acree, T. E. (1996b). Retronasal flavor release in oil and water model systems with an evaluation of volatility predictors. In R. J. McGorrin and J. V. Leland (Eds.), *Flavor-Food Interactions* (Vol. 633, pp. 179-187).

Roberts, D. D., Elmore, J. S., Langley, K. R., and Bakker, J. (1996). Effects of sucrose, guar gum, and carboxymethylcellulose on the release of volatile flavor compounds under dynamic conditions. *Journal of Agricultural and Food Chemistry* **44** (5): 1321-1326.

Roberts, D. D., and Pollien, P. (2000). Relative influence of milk components on flavor compound volatility. In D. D. Roberts and A. J. Taylor (Eds.), *Flavor Release* (pp. 321-332). Washington, D.C.: American Chemical Society.

Roberts, D. D., Pollien, P., and Milo, C. (2000). Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *Journal of Agricultural and Food Chemistry* **48** (6): 2430-2437.

Robillard, K. A., and Wishnia, A. (1972). Aromatic hydrophobes and R. J. McGorrin and J. V. Leland (Eds.), R. J. McGorrin and J. V. Leland (Eds.), β-lactoglobulin A - thermodynamics of binding. *Biochemistry* **11** (21): 3835-3840.

Rogacheva, S., Espinosa-Diaz, M. A., and Voilley, A. (1999). Transfer of aroma compounds in water-lipid systems: Binding tendency of β-lactoglobulin. *Journal of Agricultural and Food Chemistry* **47** (1): 259-263.

Rosenthal, I. (1991). *Milk and dairy products: Properties and processing*. New York: VCH.

Saint-Eve, A., Levy, C., Martin, N., and Souchon, I. (2006a). Influence of proteins on the perception of flavored stirred yogurts. *Journal of Dairy Science* **89** (3): 922-933.

Saint-Eve, A., Juteau, A., Atlan, S., Martin, N., and Souchon, I. (2006b). Complex viscosity induced by protein composition variation influences the aroma release of flavored stirred yogurts. *Journal of Agricultural and Food Chemistry* **54** (11): 3997-4004.

Sawyer, L., Papiz, M. Z., North, A. C. T., and Eliopoulos, E. E. (1985). Structure and function of bovine β-lactoglobulin. *Biochemical Society Transactions* **13** (1): 265-266.

Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Annals of the New York Academy of Sciences* **51**: 660-672.

Schirle-Keller, J. P., Reineccius, G. A., and Hatchwell, L. C. (1994). Flavor interactions with fat replacers - effect of oil level. *Journal of Food Science* **59** (4): 813-815, 875.

Schokker, E. P., Singh, H., Pinder, D. N., Norris, G. E., and Creamer, L. K. (1999). Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH. *International Dairy Journal* **9** (11): 791-800.

Semenova, M. G., Antipova, A. S., Misharina, T. A., and Golovnya, R. V. (2002a). Binding of aroma compounds with legumin. I. Binding of hexyl acetate with 11S globulin depending on the protein molecular state in aqueous medium. *Food Hydrocolloids* **16** (6): 557-564.

Semenova, M. G., Antipova, A. S., Wasserman, L. A., Misharina, T. A., and Golovnya, R. V. (2002b). Binding of aroma compounds with legumin. II. Effect of hexyl acetate on thermodynamic properties of 11S globulin in aqueous medium. *Food Hydrocolloids* **16** (6): 565-571.

Semenova, M. G., Antipova, A. S., Belyakova, L. E., Polikarpov, Y. N., Wasserman, L. A., Misharina, T. A., Terenina, M. B., and Golovnya, R. V. (2002c). Binding of aroma compounds with legumin. III. Thermodynamics of competitive binding of aroma compounds with 11S globulin depending on the structure of aroma compounds. *Food Hydrocolloids* **16** (6): 573-584.

Seuvre, A. M., Espinosa Diaz, M. A., and Voilley, A. (2001). Retention of aroma compounds by β-lactoglobulin in different conditions. *Food Chemistry* **77** (4): 421-429.

Shamil, S., Wyeth, L. J., and Kilcast, D. (1991/92). Flavour release and perception in reduced-fat foods. *Food Quality and Preference* **3**: 51-60.

Shamil, S., and Kilcast, D. (1992). Improving the flavour acceptability of reduced-fat foods. *Nutrition and Food Science* **4**: 7-10.

Shimizu, M., Saito, M., and Yamauchi, K. (1985). Emulsifying and structural-properties of β-lactoglobulin at different pHs. *Agricultural and Biological Chemistry* **49** (1): 189-194.

Soeting, W. J., and Heidema, J. (1988). A mass-spectrometric method for measuring flavor concentration-time profiles in human breath. *Chemical Senses* **13** (4): 607-617.

Solms, J., Osman-Ismail, F., and Beyeler, M. (1973). Interaction of volatiles with food components. *Canadian Institute of Food Science and Technology Journal* **6** (1): A10-A16.

Sorrentino, F., Voilley, A., and Richon, D. (1986). Activity coefficients of aroma compounds in model food systems. *American Institute of Chemical Engineers Journal* **32** (12): 1988-1993.

Sostmann, K., and Guichard, E. (1998). Immobilized β-lactoglobulin on an HPLC-column: A rapid way to determine protein-flavour interactions. *Food Chemistry* **62** (4): 509-513.

Spector, A. A., John, K., and Fletcher, J. E. (1969). Binding of long-chain fatty acids to bovine serum albumin. *Journal of Lipid Research* **10**: 56-67.

Spector, A. A., and Fletcher, J. E. (1970). Binding of long chain fatty acids to β-lactoglobulin. *Lipids* **5** (4): 403-411.

Spector, A. A. (1975). Fatty acid binding to plasma albumin. *Journal of Lipid Research* **16** (3): 165-179.

Steinhardt, J., and Reynolds, J. A. (1969). *Multiple Equilibria in Proteins.* New York: Academic Press.

Stevenson, R. J., Chen, X. D., and Mills, O. E. (1996). Modern analyses and binding studies of flavour volatiles with particular reference to dairy protein products. *Food Research International* **29** (3-4): 265-290.

Stryer, L. (1995). *Biochemistry* (4th ed.). New York: W.H. Freeman and Company.

Supelco (1998). Bulletin 923 Solid Phase Microextraction: Theory and Optimization of Conditions, downloadable SPME literature, www.supelco.com.

Swaisgood, H. E. (1992). Chemistry of the caseins. In P. F. Fox (Ed.), *Advanced Dairy Chemistry - 1: Proteins* (pp. 63-110). New York: Elsevier Applied Science.

Tanford, S., Bunville, L. G., and Y., N. (1959). The reversible transformation of β-lactoglobulin at pH 7.5. *Journal of the American Chemical Society* **81** (15): 4032-4036.

Taylor, A. J., and Linforth, R. S. T. (1994). Methodology for measuring volatile profiles in the mouth and nose during eating. In H. Maarse and D. G. van der Heij (Eds.), *Trends in Flavour Research* (pp. 3-14). Amsterdam: Elsevier Science.

Taylor, A. J. (1996). Volatile flavor release from foods during eating. *Critical Reviews in Food Science and Nutrition* **36** (8): 765-784.

Taylor, A. J., and Linforth, R. S. T. (1996). Flavour release in the mouth. *Trends in Food Science and Technology* **7** (12): 444-448.

Taylor, A. J., and Linforth, R. S. T. (2000). Techniques for measuring volatile release *in vivo* during consumption of food. In D. D. Roberts and A. J. Taylor (Eds.), *Flavor Release* (pp. 8-21). Washington: American Chemical Society.

Taylor, A. J., Linforth, R. S. T., Harvey, B. A., and Blake, B. (2000). Atmospheric pressure chemical ionisation mass spectrometry for *in vivo* analysis of volatile flavour release. *Food Chemistry* **71** (3): 327-338.

Terta, M., Blekas, G., and Paraskevopoulou, A. (2006). Retention of selected aroma compounds by polysaccharide solutions: A thermodynamic and kinetic approach. *Food Hydrocolloids* **20** (6): 863-871.

Thompson, M. P. (1966). DEAE-cellulose-urea chromatography of casein in the presence of 2-mercaptoethanol. *Journal of Dairy Science* **49**: 792-795.

Trujillo, A. J. (2002). Applications of high-hydrostatic pressure on milk and dairy products. *High Pressure Research* **22** (3-4): 619-626.

van Ruth, S. M., and Roozen, J. P. (2000a). Delivery of flavours from food matrices. In A. J. Taylor (Ed.), *Food Flavour Technology* (pp. 167-184). Sheffield: Sheffield Academic Press.

van Ruth, S. M., and Roozen, J. P. (2000b). Influence of mastication and saliva on aroma release in a model mouth system. *Food Chemistry* **71** (3): 339-345.

van Ruth, S. M., O'Connor, C. H., and Delahunty, C. M. (2000). Relationships between temporal release of aroma compounds in a model mouth system and their physico-chemical characteristics. *Food Chemistry* **71** (3): 393-399.

van Ruth, S. M., de Vries, G., Geary, M., and Giannouli, P. (2002). Influence of composition and structure of oil-in-water emulsions on retention of aroma compounds. *Journal of the Science of Food and Agriculture* **82** (9): 1028-1035.

van Ruth, S. M., and Villeneuve, E. (2002). Influence of β-lactoglobulin, pH and presence of other aroma compounds on the air/liquid partition coefficients of 20 aroma compounds varying in functional group and chain length. *Food Chemistry* **79** (2): 157-164.

Viani, R., and Horman, I. (1976). Composition of yoghurt aroma. *Nestlé Research News* (1974/75): 53-54.

Voilley, A., Simatos, D., and Loncin, M. (1977). Gas-phase concentration of volatiles in equilibrium with a liquid aqueous phase. *Lebensmittel-Wissenschaft und -Technologie* **10** (1): 45-49.

Voilley, A., Beghin, V., Charpentier, C., and Peyron, D. (1991). Interactions between aroma substances and macromolecules in a model wine. *Lebensmittel-Wissenschaft und -Technologie* **24** (5): 469-472.

Voilley, A., Espinosa Diaz, M. A., Druaux, C., and Landy, P. (2000). Flavor release from emulsions and complex media. In D. D. Roberts and A. J. Taylor (Eds.), *Flavor Release* (pp. 142-152). Washington, DC: American Chemical Society.

Wan, X. M., Stevenson, R. J., Chen, X. D., and Melton, L. D. (1999). Application of headspace solid-phase microextraction to volatile flavour profile development during storage and ripening of kiwifruit. *Food Research International* **32** (3): 175-183.

Wang, J. L., Wang, F., Zou, X. Q., Xu, Z. A., and Dong, S. J. (2007). Surface plasmon resonance and electrochemistry for detection of small molecules using catalyzed deposition of metal ions on gold substrate. *Electrochemistry Communications* **9** (2): 343-347.

Weel, K. G. C., Boelrijk, A. E. M., Alting, A. C., van Mil, P., Burger, J. J., Gruppen, H., Voragen, A. G. J., and Smit, G. (2002). Flavor release and perception of flavored whey protein gels: Perception is determined by texture rather than by release. *Journal of Agricultural and Food Chemistry* **50** (18): 5149-5155.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Claassen, N. E., Gruppen, H., Voragen, A. G. J., and Smit, G. (2003). Effect of whey protein on the *in vivo* release of aldehydes. *Journal of Agricultural and Food Chemistry* **51** (16): 4746-4752.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Verschueren, M., Gruppen, H., Voragen, A. G. J., and Smit, G. (2004a). New device to simulate swallowing and *in vivo* aroma release in the throat from liquid and semiliquid food systems. *Journal of Agricultural and Food Chemistry* **52** (21): 6564-6571.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Jacobs, M. A., Gruppen, H., Voragen, A. G. J., and Smit, G. (2004b). Effect of emulsion properties on release of esters under static headspace, *in vivo*, and artificial throat conditions in relation to sensory intensity. *Journal of Agricultural and Food Chemistry* **52** (21): 6572-6577.

Wenz, G., and Liepold, P. (2007). Self-assembly of biotin and thio-functionalized carboxymethyl celluloses on gold and molecular recognition of streptavidin detected by surface plasmon resonance. *Cellulose* **14** (2): 89-98.

Widder, S., and Fischer, N. (1996). Measurement of the influence of food ingredients on flavour release by headspace gas chromatography-olfactometry. In A. J. Taylor and D. S. Mottram (Eds.), *Flavour Science: Recent Developments* (pp. 405-412). Cambridge, U.K.: Royal Society of Chemistry.

Wilson, C. E., and Brown, W. E. (1997). Influence of food matrix structure and oral breakdown during mastication on temporal perception of flavor. *Journal of Sensory Studies* **21** (1): 69-86.

Wishnia, A., and Pinder, T. W. (1964). Hydrophobic interactions in proteins: Conformation changes in bovine serum albumin below pH 5. *Biochemistry* **3** (5): 1377-1384.

Wishnia, A., and Pinder, T. W. (1966). Hydrophobic interactions in proteins. The alkane binding site of β-lactoglobulin A and B. *Biochemistry* **5** (5): 1534-1542.

Wong, N. P. (Ed.). (1988). *Fundamentals of Dairy Chemistry* (3rd ed.). New York: Van Nostrand Reinhold Company.

Wu, S. Y., Pérez, M. D., Puyol, P., and Sawyer, L. (1999). β-Lactoglobulin binds palmitate within its central cavity. *Journal of Biological Chemistry* **274** (1): 170-174.

Yang, J., Powers, J. R., Clark, S., Dunker, A. K., and Swanson, B. G. (2003). Ligand and flavor binding functional properties of β-lactoglobulin in the molten globule state induced by high pressure. *Journal of Food Science* **68** (2): 444-452.

Yang, X. G., and Peppard, T. (1994). Solid-phase microextraction for flavor analysis. *Journal of Agricultural and Food Chemistry* **42** (9): 1925-1930.

Yen, G.-C., Duh, P.-D., and Tsai, H.-L. (2002). Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chemistry* **79** (3): 307-313.

Yuan, J. P., and Chen, F. (1998). Degradation of ascorbic acid in aqueous solution. *Journal of Agricultural and Food Chemistry* **46** (12): 5078-5082.

Yven, C., Guichard, E., Giboreau, A., and Roberts, D. D. (1998). Assessment of interactions between hydrocolloids and flavor compounds by sensory, headspace, and binding methodologies. *Journal of Agricultural and Food Chemistry* **46** (4): 1510-1514.

Zhang, Z. Y., and Pawliszyn, J. (1993). Headspace solid-phase microextraction. *Analytical Chemistry* **65** (14): 1843-1852.

Zhang, Z. Y., Yang, M. J., and Pawliszyn, J. (1994). Solid-phase microextraction. *Analytical Chemistry* **66** (17): A844-A853.

Zhou, A., Boatright, W. L., Johnson, L. A., and Reuber, M. (2002). Binding properties of 2-pentyl pyridine to soy protein as measured by solid phase microextraction. *Journal of Food Science* **67** (1): 142-145.

Zhu, X. Q. (2003). *Interactions between Flavour Compounds and Milk Proteins. Master of Philosophy Thesis.* Massey University, Palmerston North, New Zealand.

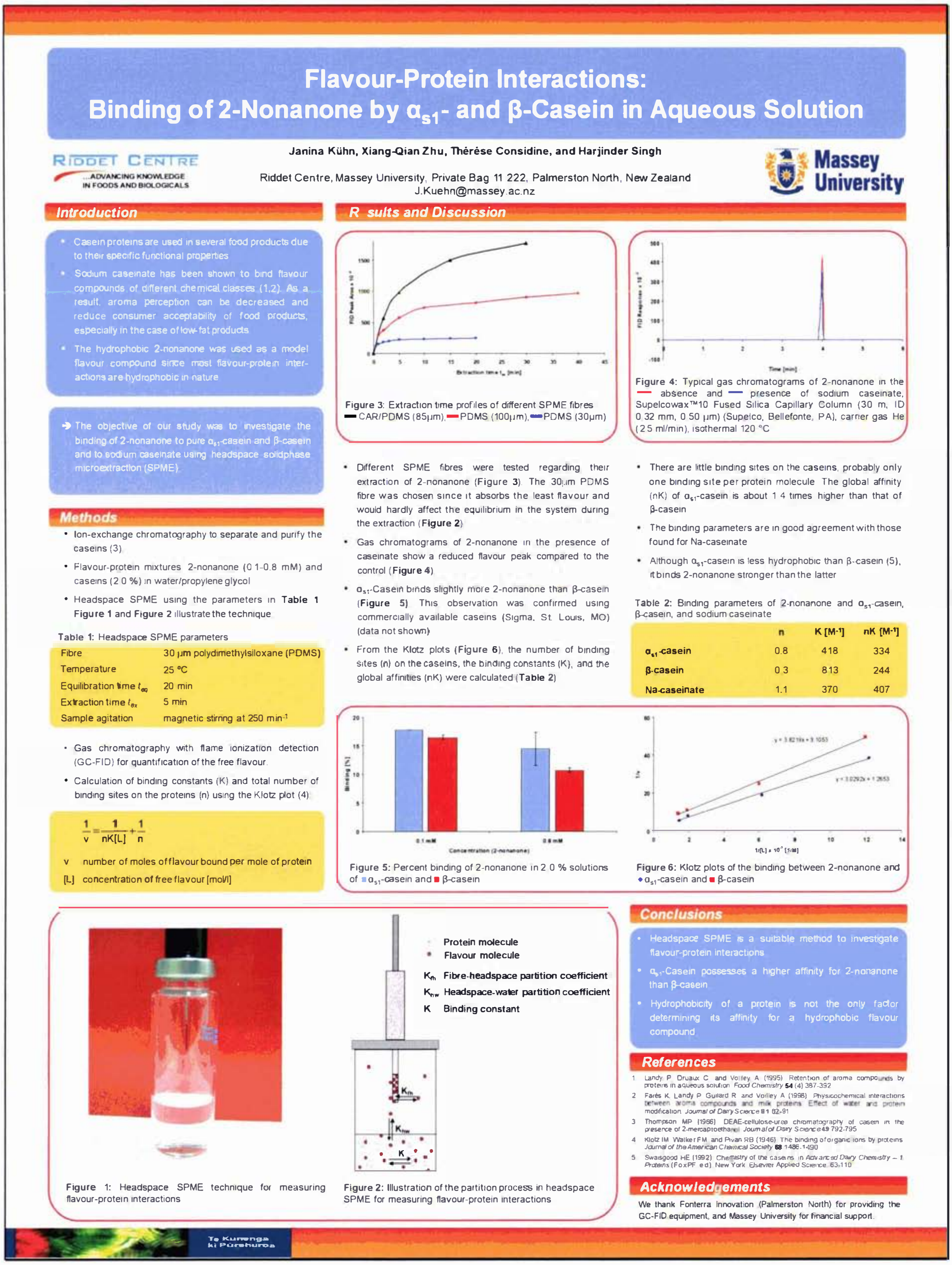
APPENDIX

Means and Standard Deviations

Mean	$\bar{y} = \frac{\sum y}{n}$
Standard deviation (SD)	$s = \sqrt{\frac{\sum (y - \bar{y})^2}{n-1}}$

y	individual sample value
$\bar{y}$	sample mean
n	number of samples
s	standard deviation

Publications



Flavour-Protein Interactions: Binding of 2-Nonanone by α_{s1}- and β-Casein in Aqueous Solution

Janina Kühn, Xiang-Qian Zhu, Thérèse Considine, and Harjinder Singh

Riddet Centre, Massey University, Private Bag 11 222, Palmerston North, New Zealand
J.Kuehn@massey.ac.nz

RIDDET CENTRE
...ADVANCING KNOWLEDGE IN FOODS AND BIOLOGICALS

Massey University

Introduction

- Casein proteins are used in several food products due to their specific functional properties
- Sodium caseinate has been shown to bind flavour compounds of different chemical classes (1,2). As a result, aroma perception can be decreased and reduce consumer acceptability of food products, especially in the case of low-fat products
- The hydrophobic 2-nonanone was used as a model flavour compound since most flavour-protein interactions are hydrophobic in nature

➔ The objective of our study was to investigate the binding of 2-nonanone to pure α_{s1}-casein and β-casein and to sodium caseinate using headspace solidphase microextraction (SPME)

Methods

- Ion-exchange chromatography to separate and purify the caseins (3)
- Flavour-protein mixtures 2-nonanone (0 1-0.8 mM) and caseins (2 0 %) in water/propylene glycol
- Headspace SPME using the parameters in **Table 1** **Figure 1** and **Figure 2** illustrate the technique

Table 1: Headspace SPME parameters

Fibre	30 µm polydimethylsiloxane (PDMS)
Temperature	25 °C
Equilibration time t_{eq}	20 min
Extraction time t_{ex}	5 min
Sample agitation	magnetic stirring at 250 min^{-1}

- Gas chromatography with flame ionization detection (GC-FID) for quantification of the free flavour.
- Calculation of binding constants (K) and total number of binding sites on the proteins (n) using the Klotz plot (4)

$$\frac{1}{v}=\frac{1}{nK[L]}+\frac{1}{n}$$

v number of moles of flavour bound per mole of protein
[L] concentration of free flavour [mol/l]

Results and Discussion

Figure 3: Extraction time profiles of different SPME fibres ▬ CAR/PDMS (85µm), ▬ PDMS (100µm), ▬ PDMS (30µm)

Figure 4: Typical gas chromatograms of 2-nonanone in the ▬ absence and ▬ presence of sodium caseinate, Supelcowax™10 Fused Silica Capillary Column (30 m, ID 0 32 mm, 0 50 µm) (Supelco, Bellefonte, PA), carrier gas He (2 5 ml/min), isothermal 120 °C

- Different SPME fibres were tested regarding their extraction of 2-nonanone (**Figure 3**). The 30µm PDMS fibre was chosen since it absorbs the least flavour and would hardly affect the equilibrium in the system during the extraction (**Figure 2**)
- Gas chromatograms of 2-nonanone in the presence of caseinate show a reduced flavour peak compared to the control (**Figure 4**)
- α_{s1}-Casein binds slightly more 2-nonanone than β-casein (**Figure 5**) This observation was confirmed using commercially available caseins (Sigma, St Louis, MO) (data not shown)
- From the Klotz plots (**Figure 6**), the number of binding sites (n) on the caseins, the binding constants (K), and the global affinities (nK) were calculated (**Table 2**)

- There are little binding sites on the caseins, probably only one binding site per protein molecule The global affinity (nK) of α_{s1}-casein is about 1 4 times higher than that of β-casein
- The binding parameters are in good agreement with those found for Na-caseinate
- Although α_{s1}-casein is less hydrophobic than β-casein (5), it binds 2-nonanone stronger than the latter

Table 2: Binding parameters of 2-nonanone and α_{s1}-casein, β-casein, and sodium caseinate

	n	K [M^{-1}]	nK [M^{-1}]
α_{s1}-casein	0.8	418	334
β-casein	0 3	813	244
Na-caseinate	1.1	370	407

Figure 5: Percent binding of 2-nonanone in 2 0 % solutions of 🟦 α_{s1}-casein and 🟥 β-casein

Figure 6: Klotz plots of the binding between 2-nonanone and ◆ α_{s1}-casein and 🟥 β-casein

Figure 1: Headspace SPME technique for measuring flavour-protein interactions

Figure 2: Illustration of the partition process in headspace SPME for measuring flavour-protein interactions

Conclusions

- Headspace SPME is a suitable method to investigate flavour-protein interactions
- α_{s1}-Casein possesses a higher affinity for 2-nonanone than β-casein
- Hydrophobicity of a protein is not the only factor determining its affinity for a hydrophobic flavour compound

References

1 Landy P Druaux C and Voilley A (1995) Retention of aroma compounds by proteins in aqueous solution *Food Chemistry* **54** (4) 387-392

2 Farès K, Landy P Guilard R and Voilley A (1998) Physicochemical interactions between aroma compounds and milk proteins Effect of water and protein modification *Journal of Dairy Science* **81** 82-91

3 Thompson MP (1966) DEAE-cellulose-urea chromatography of casein in the presence of 2-mercaptoethanol *Journal of Dairy Science* **49** 792-795

4 Klotz IM Walker FM and Pivan RB (1946) The binding of organic ions by proteins *Journal of the American Chemical Society* **68** 1486-1490

5 Swaisgood HE (1992) Chemistry of the caseins in *Advanced Dairy Chemistry – 1 Proteins* (Fox PF ed) New York Elsevier Applied Science 63-110

Acknowledgements

We thank Fonterra Innovation (Palmerston North) for providing the GC-FID equipment, and Massey University for financial support.

Te Kunenga ki Pūrehuroa

JFS R: Concise Reviews/Hypotheses in Food Science

Interactions of Milk Proteins and Volatile Flavor Compounds: Implications in the Development of Protein Foods

JANINA KÜHN, THÉRÈSE CONSIDINE, AND HARJINDER SINGH

ABSTRACT: This review encompasses the binding of volatile flavor compounds by milk proteins in aqueous solutions. The presence of proteins in a food matrix can result in a decrease in aroma perception and in an unpleasant aroma profile, because of binding of the desirable flavor compounds to proteins. Hence, various analytical methods used to measure the extent and the type of binding, and the determination of the binding parameters, are evaluated in this review. The binding of various flavor compounds by individual milk proteins is discussed and compared in terms of their binding affinity for flavor compounds. Furthermore, the influence of temperature and ultra-high pressures on the interactions between proteins and flavors is considered in detail. The implications of protein–flavor binding in the development of protein foods are discussed.

Keywords: binding constant, flavor binding, flavor retention, heat denaturation, milk proteins

Introduction

One of the most important criteria for consumer acceptance of foods is flavor. Food matrix components, such as proteins (Gremli 1974; Damodaran and Kinsella 1980a, 1980b, 1981a, 1981b; Farès and others 1998; Lübke and others 2002; Gianelli and others 2005), carbohydrates (Yven and others 1998; Heinemann and others 2001; Philippe and others 2003; Jouquand and others 2004), and lipids (Ebeler and others 1988; van Ruth and others 2002; Meynier and others 2003), are known to interact with flavor compounds. Proteins are added to foods primarily because of their functional properties, such as emulsifying and stabilizing capacities, and because of their nutritional value. However, interactions between proteins and flavors are known to influence the perceived flavor of a food product (Overbosch and others 1991; Land 1996). Protein ingredients not only reduce the perceived impact of desirable flavors but also may transmit undesirable off-flavors to foods, especially whey protein and soy protein products (Mills and Solms 1984; Semenova and others 2002a). In addition, proteins may change the texture of a food that is gelling, and thus decrease the flavor perception due to inhibition of mass transfer (Jaime and others 1993; Carr and others 1996; Wilson and Brown 1997).

Two different types of interaction can occur between proteins and flavor compounds: (1) reversible (physicochemical) binding, including hydrogen bonds, hydrophobic interactions, and ionic bonds, and (2) irreversible (chemical) binding via covalent linkages, that is, amide and ester formation, and the condensation of aldehydes with amino (NH_2) and sulfhydryl (SH) groups (Solms and others 1973; Overbosch and others 1991; Mottram and others 1996; Meynier and others 2003, 2004). The type of binding between a protein and a flavor compound depends on the characteristics of both the protein and the flavor, and thus there is no universal mechanism for flavor binding in foods (Solms and others 1973). However, most flavors show hydrophobic, reversible binding to proteins (Gremli 1974; Damodaran and Kinsella 1980b, 1981a, 1981b, 1983; O'Neill and Kinsella 1987; Pelletier and others 1998). Adsorption and absorption also exist but are specific for low-moisture food systems (Maier 1972, 1973, 1975; Le Thanh and others 1992; Landy and others 1997; Mironov and others 2003), and are not discussed in this review.

In the area of protein–flavor interactions, studies have been conducted mainly with milk proteins (Andriot and others 2000; Lübke and others 2002) and soy proteins (Damodaran and Kinsella 1981a; Li and others 2000), but also with a range of other food proteins, such as fababean protein (Ng and others 1989a, 1989b; Semenova and others 2002a), pea protein (Dumont and Land 1986), ovalbumin (Maier 1970; Ebeler and others 1988), fish actomyosin (Damodaran and Kinsella 1983), and myoglobin (Gianelli and others 2005). The studies on milk proteins are of great importance because milk proteins are utilized in numerous food products, including dairy products, bakery and confectionary products, and meat products (Mulvihill 1992).

The demand for healthier low-fat foods is increasing. However, in the absence of fat, altered flavor–matrix interactions result in a dramatic change in flavor profiles (Shamil and Kilcast 1992; Plug and Haring 1993; Hatchwell 1996). In fat-reduced or "light" foods the dominant components are carbohydrates or proteins, which interact differently with aroma compounds compared with fat, and thus change the perceived flavor. In addition, most of the fat replacers used in these foods are composed of proteins or carbohydrates or both. A better understanding of the science behind protein–flavor interactions is required for the development of improved food flavor, particularly that of low-fat foods, and for minimizing the presence of off-flavors in protein-containing foods.

The aim of this review is to discuss the flavor-binding ability of individual milk proteins and milk protein products, and the influence of processing, particularly temperature and high pressure, on protein–flavor binding. Methodologies used to investigate protein–flavor interactions in different systems are evaluated. Implications of protein–flavor interactions in the development of protein foods, and future research, are also considered.

MS 20060136 Submitted 2/27/2006, Accepted 4/5/2006. Authors are with Riddet Centre, Massey Univ., Private Bag 11 222, Palmerston North, New Zealand. Direct inquiries to author Singh (E-mail: H.Singh@massey.ac.nz).

doi: 10.1111/j.1750-3841.2006.00051.x

Analysis of Flavor Binding by Proteins

Two approaches may be used to study the interactions between flavor molecules and macromolecules, such as proteins: instrumental techniques and sensory analysis. The systems considered are often very simple, consisting of 1 flavor compound and 1 protein, usually in an aqueous solution.

Flavor binding has been examined predominantly under equilibrium conditions. A common technique is equilibrium dialysis, which is still commonly used (Damodaran and Kinsella 1981a, 1981b, 1983; Druaux and others 1995; Farès and others 1998; Muresan and others 2001; Burova and others 2003; Jung and Ebeler 2003a; Guth and Fritzler 2004). However, this technique is time consuming, and flavor losses during the experiment may occur. Another frequently used equilibrium method is static headspace analysis (O'Keefe and others 1991a; Charles and others 1996; Andriot and others 2000; van Ruth and Villeneuve 2002; Heng and others 2004; Liu and others 2005a). A drawback of the static headspace method is poor sensitivity for compounds with little volatility. For some samples this problem can be overcome by splitless or on-column techniques, or by increasing the temperature. However, thermal reactions may occur. Aroma compounds that are not detectable by flame ionization detection (FID) or mass spectrometry (MS) can often be detected using the headspace gas chromatography–olfactometry (GC–O) technique (Widder and Fischer 1996).

Recently, static headspace solid-phase microextraction (SPME) has been found to be very useful for the examination of protein–flavor binding (Roberts and Pollien 2000; Adams and others 2001; Fabre and others 2002; Zhu 2003; Jung and Ebeler 2003a; Gianelli and others 2005). The SPME technique has been developed by Pawliszyn and co-workers (Arthur and Pawliszyn 1990; Zhang and Pawliszyn 1993; Zhang and others 1994). In headspace SPME, a fused silica fiber coated with a thin layer of a selective coating extracts the flavor compounds from the headspace above the sample (Figure 1). The analytes are concentrated in the coating and are then transferred to the analytical instrument for desorption and analysis (Zhang and Pawliszyn 1993). It is a sensitive, rapid, inexpensive, selective, and solvent-free sample preparation technique, and is compatible with a wide range of separation methods such as GC, GC–MS, and high-performance liquid chromatography (HPLC) (Zhang and others 1994). Headspace SPME has been found to be superior to both static and dynamic headspace analysis for the measurement of milk protein–flavor interactions (Fabre and others 2002). The technique can also be used to quantify the flavor concentrations after equilibrium dialysis (Zhou and others 2002).

However, there are some limitations of headspace SPME, such as the difficulty in using external standards for more complex sample matrices (Yang and Peppard 1994). Instead, standard addition and isotopic dilution can be used for quantitative analysis of such samples by SPME (Hawthorne and others 1992). In addition, competition of flavor compounds in the fiber coating can cause biases in the quantitative analysis (Coleman 1996; Grote and Pawliszyn 1997; Roberts and others 2000). Hence, the method is more suitable for simple systems rather than complex systems. In addition, it has to be ensured that the amount of flavor extracted from the headspace is small to avoid re-equilibration of the system. The principle behind headspace SPME is the equilibrium partition process of analytes between the sample, the headspace above the sample, and the fiber coating (Figure 1). The overall equilibrium of the system can be described by the following equation:

$$[F]_f \xleftrightarrow{K_{fh}} [F]_h \xleftrightarrow{K_{hw}} [F]_w \xleftrightarrow{K} [F]_P \qquad (1)$$

where $[F]_f$ is the concentration of the flavor compound in the fiber coating, $[F]_h$ in the headspace, $[F]_w$ in the aqueous phase, and $[F]_b$ bound by the protein. The partitioning of the flavor compound in the system depends on the fiber–headspace partition coefficient (K_{fh}), the headspace–water partition coefficient (K_{hw}), and the binding constant between the protein and the flavor (K) (Figure 1). Too long extraction times and too high values of K_{fh} would result in flavor being released from the sample solution, and, as a consequence, protein-bound flavor could be released into the aqueous phase and headspace, and the measured binding constant K would be underestimated. Hence, sampling parameters have to be chosen with care.

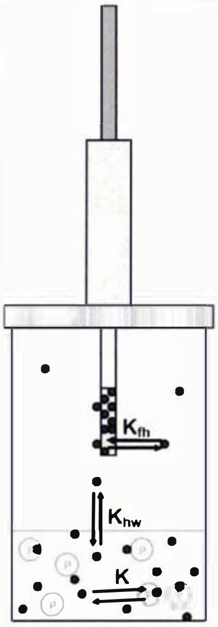


Figure 1 – Illustration of the partition process in headspace SPME for investigating interactions of proteins (P) and flavor compounds (•) (not to scale)

Other equilibrium techniques include liquid–liquid partitioning (Spector and others 1969; Spector 1975; Damodaran and Kinsella 1980b) and radioactivity counting (King and Solms 1979). For a few hardly volatile and water-soluble flavor compounds, such as vanillin or benzaldehyde, HPLC methods have been developed to determine the free flavor after equilibration with the protein (Ng and others 1989a; McNeill and Schmidt 1993; Andriot and others 1999; Li and others 2000; Chobpattana and others 2002). However, several disadvantages are associated with equilibrium methods, such as long equilibration times and thus the possible degradation of the volatiles during this period.

The conformational stability of protein–flavor complexes upon heat denaturation can be examined using differential scanning calorimetry (DSC) methods (Burova and others 1999; Semenova and others 2002b, 2002c). DSC also allows the calculation of binding constants using bovine serum albumin (BSA) as an internal molecular detector since its denaturation temperature and thus conformational stability depends on the amount of flavor bound (Burova and others 2003). However, the amount of bound flavor determined this way is not precise.

Some dynamic methods, that is, dynamic headspace analysis (exponential dilution), are used currently (Jouenne and Crouzet 1996; Farès and others 1998; Jouenne and Crouzet 2000b). The flavor compounds in the headspace are of greater importance than those in the food as they can travel to the nose during eating and stimulate the olfactory receptors in the nasal cavity (Linforth and Taylor 1993). In dynamic headspace analysis, the exponential decrease of the flavor concentration in the headspace above the sample is measured, while the sample is exhausted of aroma volatiles by passing an inert gas through it or sweeping it over its surface. The gas leaving the system is sampled at regular intervals and analyzed using GC. Purge-and-trap analysis is a commonly used method of dynamic headspace analysis and enables fast determination of the activity coefficient of the flavor compound in a model food system (Sorrentino and others 1986; Jouenne and Crouzet 1996, 2000b). Disadvantages are the purging of water along with the analytes, decreasing the adsorption capacity of the trap, and diffusion or "breakthrough" losses of analytes not retained by the trap (Stevenson and others 1996).

Other dynamic methods involve liquid chromatography, such as size exclusion chromatography (Hummel and Dreyer 1962; Dumont 1987; Pelletier and others 1998), dynamic coupled column liquid chromatography (DCCLC) (Langourieux and Crouzet 1995; Jouenne and Crouzet 2000a), or affinity chromatography on protein-bonded stationary phases (Pelletier and others 1998; Sostmann and Guichard 1998; Reiners and others 2000). The methods based on liquid chromatography need small amounts of product and are rapid, thereby reducing the degradation of analytes. In spite of this, affinity chromatography only provides global affinities. The number of binding sites has to be determined through exclusion size chromatography or other methods. Another drawback of affinity chromatography is that possible conformational changes in proteins due to their immobilization have to be considered, and some protein-binding sites may be hindered, as observed for isoamyl acetate (Pelletier and others 1998).

The above-mentioned methods can be used to demonstrate the existence and extent of molecular interactions between aroma volatiles and proteins or other food constituents. However, they do not provide information about the nature of interactions. Spectroscopic techniques have been used successfully to gain insight into the nature of interactions. Binding of a flavor compound to a protein molecule can generate conformational modifications of the protein (Damodaran and Kinsella 1980b; Lübke and others 2000). These conformational changes, and thus the binding of ligands can be measured by following the change in protein tryptophan fluorescence, which can be increased or quenched depending on the ligand present (Muresan and others 2001). The technique also indicates if a ligand binds in the vicinity of a tryptophan residue. Assuming that the change in fluorescence depends on the amount of protein–ligand complex, fluorescence spectroscopy can be used to determine binding constants (Dufour and Haertlé 1990; Frapin and others 1993; Liu and others 2005a). However, the protein investigated needs to contain at least 1 tryptophan residue. In addition, for ligands that quench the tryptophan fluorescence, the binding is overestimated and should always be verified using a second method (Muresan and others 2001).

Another technique monitoring conformational changes of proteins upon flavor binding is infrared (IR) spectroscopy (Lübke and others 2000). The technique is a very useful tool to study protein secondary structures. Generally, the amide I bands region between 1600 and 1700 per cm reveals the most of the information as it is highly conformation-sensitive. When using either fluorescence or IR spectroscopy it has to be considered that flavor binding without conformational changes in the protein cannot be investigated using these techniques.

Nuclear magnetic resonance (NMR) spectroscopy techniques are a very valuable tool to explore mechanisms of protein–flavor interactions. Two-dimensional (2D) NMR spectroscopy reveals conformational changes of the protein upon flavor binding, and those amino acid residues involved in the binding so that information about the location and number of binding sites on a protein can be obtained (Lübke and others 2002). Diffusion-based NMR techniques are fast and easy but do not offer information about the nature of interactions. The pulsed field gradient NMR (PFG-NMR) method could provide binding constants *K* and number of binding sites *n* on the proteins. However, using this technique it is assumed that there are no interactions between the proteins or the flavor ligands themselves but this assumption is not true. A powerful tool for rapid screening of flavors that have an affinity for a protein is the diffusion-based nuclear Overhauser effect (NOE) pumping technique (Jung and others 2002; Jung and Ebeler 2003b). This technique should only be used as a screening method as it lacks sensitivity.

Sensory analysis has also been applied to examine the effect of proteins on flavor perception (Ng and others 1989b; Hansen and Heinis 1991, 1992; McNeill and Schmidt 1993; Reiners and others 2000). Rating of flavor intensities in the presence and absence of protein gives insight into the effect of protein–flavor binding on flavor perception and complements instrumental techniques. However, an intensive training of the taste panel is necessary to obtain precise results, and thus is time consuming and costly.

Determination of Binding Parameters

To characterize the binding of a specific flavor to a specific protein, binding parameters, such as an intrinsic binding constant *K*, the number of binding sites *n*, the Hill coefficient *h*, the Gibbs' free energy of binding ΔG, the enthalpy change ΔH, and the entropy change ΔS, can be determined experimentally. Different plots have been used to determine these parameters, including the Scatchard plot (Scatchard 1949), the Klotz plot (Klotz and others 1946; Klotz and Urquhart 1948), and the Hill plot (Hill 1910; Yven and others 1998). The experimental determination of *K* as a function of temperature allows the determination of the thermodynamic parameters ΔG, ΔH, and ΔS (Steinhardt and Reynolds 1969).

The frequently used Scatchard and Klotz models assume equal and independent binding sites on a protein. However, proteins can possess nonequivalent binding sites, for example, 1 or more high-affinity primary binding sites and a group of lower affinity secondary binding sites. This results in nonlinear Scatchard and Klotz plots and complicates the evaluation of binding data because it is often difficult to extrapolate the binding plots to obtain the exact number of equivalent binding sites. Furthermore, binding sites can be dependent. The initial binding of aroma compounds can cause a protein to undergo conformational changes (Damodaran and Kinsella 1980b), revealing new binding sites. If the initial binding of a ligand to a protein produces a tendency to bind more ligand, positive cooperativity exists. If binding is restricted after the initial binding of a ligand, the protein shows negative cooperativity. If the Scatchard and Klotz plots are nonlinear, the Hill plot (Hill 1910) should be used to determine if cooperativity between binding sites exists. Therefore, a reason for variations in binding parameters between studies may be the choice of binding model.

In addition, a complete binding analysis from the initial signs of binding to saturation is often not realizable in flavor binding experiments, for example, because of the low water solubility of most flavor compounds. The range of flavor concentration varies from one study to another, making comparison of binding data impossible because the amount of flavor molecules present determines the occupation of binding sites. If flavor concentrations are low only the high-affinity binding sites are occupied, whereas high flavor concentrations may also result in the occupation of secondary binding sites.

Flavor Binding by Milk Proteins

β-Lactoglobulin (β-lg)

Of all the different food proteins, β-lg has been used extensively as a model protein for studying protein–flavor interactions, because of its well-defined structure and properties (McKenzie 1971; Kinsella and Whitehead 1989; Batt and others 1994).

The 3-dimensional structure of bovine β-lg has been determined by high-resolution crystallographic studies (Sawyer and others 1985; Papiz and others 1986; Monaco and others 1987) and has been shown to be similar to that of serum retinol-binding protein (RBP) (Papiz and others 1986; North 1989). β-lg is built up of 2 β-sheets, formed from 9 strands converging at 1 end to form a hydrophobic calyx or pocket, and a flanking 3-turn α-helix (Sawyer and others 1985; Papiz and others 1986) (see figure 1 in Wu and others [1999]). This pocket serves as a binding locus for apolar molecules such as retinol ($K = 5 \times 10^7$ M^{-1}) (Fugate and Song 1980) and long-chain fatty acids ($K = 10^5$–10^7 M^{-1}) (Spector and Fletcher 1970; Díaz de Villegas and others 1987; Pérez and others 1989; Frapin and others 1993; Wu and others 1999; Ragona and others 2000).

Fourier transform IR spectroscopy has been used to determine the conformational changes in β-lg upon the addition of ligands. The technique confirmed the binding of β-ionone, retinol, and fatty acids into the central cavity (Lübke and others 2000). For other ligands (p-cresol, eugenol, 2-nonanone, and γ-decalactone), no conformational changes of the protein were observed and the authors suggested either a binding to the protein surface or a binding into the central cavity without inducing a conformational change. However, an NMR study did confirm conformational changes of β-lg upon binding of flavor molecules (γ-decalactone and β-ionone) (Lübke and others 2002). It appeared that the side chains of several amino acids close to the central hydrophobic cavity (Leu46, Ile56, Met107, and Gln120) were affected by the binding of γ-decalactone, whereas the binding of β-ionone affected amino acids located in a groove near the outer surface of the protein (Leu104, Tyr120, and Asp129), a groove that is close to a region that has been described by Monaco and others (1987) and Lübke and others (2002). This study confirms the existence of 2 different binding sites on β-lg for aroma compounds, but it is still not entirely clear which flavors bind preferentially to which site.

Therefore, the most probable binding site for flavor compounds is the hydrophobic pocket of β-lg. In addition to this primary binding site, β-lg is thought to contain weaker secondary binding sites that are capable of undergoing interactions with hydrophobic molecules, such as flavor compounds (Spector and Fletcher 1970; Robillard and Wishnia 1972; Monaco and others 1987; O'Neill and Kinsella 1988; Dufour and Haertlé 1990).

β-lg is known to interact with several flavor compounds, such as alkanes (Wishnia and Pinder 1966; Mohammadzadeh-K. and others 1967, 1969a, 1969b), ketones (O'Neill and Kinsella 1987; Andriot and others 2000; Guichard and Langourieux 2000; Jouenne and Crouzet 2000b), aldehydes (van Ruth and Villeneuve 2002), ionones (Dufour and Haertlé 1990; Jouenne and Crouzet 2000a; Lübke and others 2002; Jung and Ebeler 2003b), lactones (Sostmann and Guichard 1998; Lübke and others 2002; Guth and Fritzler 2004), and esters (Pelletier and others 1998; Guichard and Langourieux 2000; Jouenne and Crouzet 2000b; Reiners and others 2000). As a result of different experimental methodologies and conditions, various binding parameters have been derived in different studies, making comparison of binding data difficult.

O'Neill and Kinsella (1987), using equilibrium dialysis, showed that β-lg has a high binding affinity and one main binding site for methyl ketones. The slopes of the Klotz plots for 2-heptanone, 2-octanone, and 2-nonanone indicated binding constants (K) of 152, 481, and 2439 M^{-1}, respectively. As the binding constant K for β-lg and homologous series of esters, aldehydes, ketones, and alcohols increased with increasing chain length (hydrophobicity) within the same chemical class, hydrophobic interactions between β-lg and flavor compounds were suggested (O'Neill and Kinsella 1987; Pelletier and others 1998; Guichard and Langourieux 2000). However, earlier work by Jasinski and Kilara (1985), using the same method, reported a much lower value of $K = 122$ M^{-1} for 2-nonanone and about 14 binding sites were evident. The authors suggested that unfolding of the protein may explain the high number of binding sites determined, but it is unclear how this unfolding may have occurred. In our opinion the flavor protein ratios used by Jasinski and Kilara (1985) are comparatively high so that weaker, secondary binding sites on the protein might be occupied as well, which would also decrease the overall binding constant. Besides, they added sodium azide as an antibacterial agent, which has later been shown to influence protein–flavor interactions (O'Keefe and others 1991b; Fares and others 1998; Reiners and others 2000).

The presence or absence of certain functional groups and steric factors is also known to have a marked influence on the degree of flavor binding by proteins. The interactions of an alkyl chain with β-lg can be reduced by a polar group; that is, the introduction of a hydroxyl function (OH) is responsible for weaker interactions (Reiners and others 2000). The introduction of an aldehyde group led to a slight increase in binding. In general, the binding capacities of proteins increase from alcohols to ketones and aldehydes.

A recent fluorometric study on the binding of retinol and γ-undecalactone to β-lg showed that there is competition between the ligands (Muresan and others 2001). The effect of the chain length on the free energy of interaction between β-lg and different lactones indicated that the interaction is mainly hydrophobic, which led the authors to conclude that the preferential binding site for the lactones is likely to be the central cavity. This hypothesis was confirmed by competition experiments between β-ionone and other flavor compounds (Sostmann and Guichard 1998). The authors concluded that (1) lactones have some affinity for the central cavity as well, and (2) α-ionone, β-damascenone, methyl benzoate, and unsaturated aliphatic aldehydes and ketones bind nonspecifically to the protein. In contradiction, Guth and Fritzler (2004) suggested a binding position of γ-decalactone, which is different from that of the central cavity since both retinol and palmitate only slightly inhibited the binding of the lactone. These contradictory results demonstrate that the location of binding sites for flavor molecules on proteins needs to be investigated further. Even flavor binding locations on the frequently studied β-lg are still not fully understood.

Dufour and Haertlé (1990), using fluorescence spectroscopy, suggested that the central hydrophobic pocket has a narrow specificity to the structure formed by the conjugated double bonds of the β-ionone ring and isoprenoid chain, present in both β-ionone and retinol. The authors could not demonstrate binding between β-lg and α-ionone, since it did not quench the tryptophan fluorescence. The ionone isomers differ only in the position of the cyclohexene double bond. On the other hand, α-ionone might be bound by β-lg, but without interacting with tryptophan. In contrast, Lübke and others (2000), using IR spectroscopy, showed that the binding of retinol and β-ionone into the hydrophobic cavity of β-lg induced no significant conformational change in the protein, whereas the binding of α-ionone did, probably due to the "wrong" position of the double bond (Lübke and others 2000). Jung and Ebeler (2003b), using a diffusion-based NOE pumping technique, demonstrated that the binding of β-ionone by β-lg was significantly higher than the affinity of α-ionone. In addition, α-ionone was bound only at pH 9, whereas

β-ionone was bound at pH 3 to 11, with the greatest binding affinity at pH 9, and the lowest at pH 11, due to alkaline denaturation and aggregation. The reason why these authors, contrary to Lübke and others (2000), did not observe any binding of α-ionone at neutral pH may be the low sensitivity of the NOE pumping method. The complementary results of the above studies show the importance of using a combination of techniques to obtain more reliable results.

α-Lactalbumin (α-la)

α-la is a compact globular protein, stabilized by 4 intrachain disulfide bonds (Kinsella and Whitehead 1989), and it plays an important role in the synthesis of lactose (Wong 1988). However, only a few studies have investigated the binding of flavor compounds to α-la, as its affinity for flavor compounds is believed to be lower than that of β-lg. Using headspace analysis, α-la was found to bind various amounts of aldehydes and methyl ketones (Franzen and Kinsella 1974), but binding constants were not given. The study of Jasinski and Kilara (1985), which considered the binding of 2-nonanone and nonanal to α-la, showed very weak binding of both flavors to the protein, as determined by equilibrium dialysis. Because Jasinski and Kilara (1985) underestimated the binding of 2-nonanone to β-lg, they possibly underestimated the binding to α-la as well. Further examination of flavor binding by α-la should be performed.

Bovine serum albumin (BSA)

BSA binds a large variety of compounds, including retinol (Futterman and Heller 1972), long-chain fatty acids (Morrisett and others 1975; Spector 1975; Pérez and others 1989), alkanes (Wishnia and Pinder 1964; Mohammadzadeh-K. and others 1967, 1969a, 1969b), and aldehydes and ketones (Beyeler and Solms 1974; Franzen and Kinsella 1974; Damodaran and Kinsella 1980b; Jung and Ebeler 2003a). The protein is composed of a single polypeptide chain, which is folded so that 3 or 4 spherical units are formed. The binding sites for fatty acids are probably located in crevices between the spherical units (Spector 1975).

Using static headspace analysis, native BSA was found to decrease the vapor pressure of diacetyl over its aqueous solution. As little as 0.5% protein caused a 25% reduction in volatility (Land and Reynolds 1981), indicating a very high affinity of BSA to bind diacetyl. Beyeler and Solms (1974) used equilibrium dialysis to study the interactions of a large number of flavor compounds with BSA. The binding constants ranged from 0 to 10 × 10^3 M^{-1}, and decreased in the sequence aldehydes > ketones > alcohols. The authors suggested that the binding of aroma compounds was due to both hydrophobic and electrostatic forces. King and Solms (1979) found the interaction between labeled (^{14}C) benzyl alcohol and denatured BSA to be reversible. The interaction was independent of pH and ionic strength, confirming a dominance of hydrophobic interactions.

Damodaran and Kinsella (1980a, 1980b, 1981c) extensively studied the interactions between flavor compounds, in particular 2-nonanone and BSA. Using liquid–liquid partitioning, they determined a binding constant for 2-nonanone and BSA of $K = 1800$ M^{-1} with 6 primary binding sites on the protein molecule (Damodaran and Kinsella 1980a, 1980b). More recently, PFG-NMR spectroscopy revealed quite different binding parameters for the BSA/2-nonanone system with $K = (833 \pm 15)$ M^{-1} and $n = 7$ (Jung and others 2002). Differences in binding parameters for systems containing BSA are often due to the type of BSA used since BSA products contain varying amounts of fatty acids, which are tightly bound by the protein and thus reduce the binding of flavors. The above-mentioned values agree with the number of binding sites in BSA for long-chain n-alcohols (Steinhardt and Reynolds 1969) and free fatty acids (Spector and others 1969; Spector 1975). In addition, a large number of weaker, secondary binding sites are probably present (Spector and others 1969). This was confirmed by Guth and Fritzler (2004) who suggested 1 or 2 high-affinity binding sites, and a large number of lower affinity sites for γ- and δ-lactones on BSA.

A recent study using DSC suggested 2 and 3 binding sites and binding constants of 600 and 300 M^{-1} for vanillin (4-hydroxy-3-methoxybenzaldehyde) and 2-octanone for BSA, respectively (Burova and others 2003). We consider that the low binding parameters compared to other flavor ligands could be explained by the slightly acidic conditions (pH = 6.4) used by these authors, since lowering the pH by 1.8 units reduced the binding of γ-decalactone on BSA by 40% (Druaux and others 1995), probably due to conformational changes in the protein.

Using predominantly chromatographic methods, Dhont (1987) provided some evidence that albumin bound vanillin irreversibly to a substantial extent, but Dumont (1987) showed that the binding was reversible. It may be possible that the binding sites for vanillin on BSA are not equivalent; binding could occur both reversibly via noncovalent interactions, and irreversibly via the aldehyde function. Alaiz and Girón (1994) observed irreversible binding of 2-octenal to BSA. The authors suggested a covalent binding of 2-octenal, through its double bond, with the imidazole ring of histidine in BSA.

Jasinski and Kilara (1985), using equilibrium dialysis, compared the binding between BSA and 2 flavors, 2-nonanone and nonanal. For both flavors, they observed strong binding to the protein, with nonanal having a higher affinity than 2-nonanone. The authors attributed the stronger binding of nonanal to the position of the functional group, or the higher reactivity of aldehydes compared to ketones. A reduction in the available ε-amino groups of BSA on analysis of the nonanal–protein complex could not be found by Damodaran and Kinsella (1980b), indicating that the 1-position of the keto group caused the higher binding affinity. The 2-position of the keto group in 2-nonanone would give rise to more steric hindrance to hydrophobic interactions.

Whey protein products

A few studies have been reported on the binding of flavor compounds by whey protein products. Jasinski and Kilara (1985) investigated the binding of 2-nonanone and nonanal to whey protein concentrate (WPC). They found a large number of binding sites with strong binding affinity. Using HPLC, a weak interaction between whey protein isolate (WPI) and vanillin in a sweetened drink was demonstrated by McNeill and Schmidt (1993). Unfortunately, no binding parameters were determined. Li and others (2000) extended their research and found that the interaction between vanillin and WPI was strong, with an average binding constant of 1713 M^{-1} (12 °C) and 0.67 binding sites on WPI. Recently, a high binding affinity between 2-nonanone and WPI was confirmed using headspace SPME (Zhu 2003). A binding constant of $K = 2059$ M^{-1} (25 °C) and on average 1 binding site per protein molecule were found. The strong interactions between whey protein products and flavor compounds indicate that the addition of these proteins to food products, even at very low concentrations, could influence the flavor profile of the food.

Caseins

A few studies have dealt with the behavior of aroma compounds in the presence of sodium caseinate or casein. Bovine sodium caseinate is a useful model for investigating the interactions between aroma and protein because of its well-known functional properties and its wide use in dairy, as well as nondairy, food products (Mulvihill 1992).

In the presence of sodium caseinate (10%), Reineccius and Coulter (1969) noted a decrease in the diacetyl headspace concentration of nearly 50%. In an aqueous solution containing only 1% casein, lower volatilities of acetone and acetaldehyde were observed (Maier 1970). Even the addition of only 0.1% sodium caseinate induced a decrease in the volatility of flavor compounds in aqueous solution in the following order: β-ionone > n-hexanol > ethyl hexanoate, isoamyl acetate (Voilley and others 1991). The intensity of the odor due to aldehydes, stored in a mixture with casein, decreased with increasing time of storage (Pokorný and others 1976). However, Le Thanh and others (1992) did not find a decrease in the headspace concentrations of acetone and ethyl acetate in the presence of 10% sodium caseinate. The authors attributed this to the residual NaCl present from the preparation of caseinate, because salts increase the concentration of volatile compounds in the headspace (Nawar 1966; Land and Reynolds 1981).

In a model dairy protein drink, sodium caseinate (6%) was shown to interact with vanillin. A significant decrease in free vanillin in the drink was shown by HPLC (McNeill and Schmidt 1993). Using similar methodology, moderate binding of vanillin to sodium caseinate was shown by Li and others (2000). Landy and others (1995) observed a major influence of sodium caseinate on the headspace–liquid partition coefficients and on the relative volatility of diacetyl, ethyl butanoate, and ethyl hexanoate, but not of ethyl acetate, in solutions containing 0.5% and 5% protein, respectively. The presence of strong interactions between diacetyl and sodium caseinate was suggested by the retention of diacetyl in the dialysis sac even after exhaustive dialysis against water (Farès and others 1998). Conversely, an exhaustive dialysis in the presence of benzaldehyde and sodium caseinate resulted in no volatile compound retained by the protein, revealing weak bonds between benzaldehyde and sodium caseinate (Farès and others 1998). In contrast, the aliphatic aldehyde hexanal has been found to bind covalently to sodium caseinate (Meynier and others 2004).

Dubois and others (1996) showed that, in a model cheese system, the volatility of diacetyl decreased slightly with increasing calcium caseinate content whereas a change in the fat content of up to 30% did not affect the volatility. The hydrophilicity of diacetyl was used as an explanation by these authors.

Fischer and Widder (1997) developed a method based on headspace GC–O to measure the retention of esters and heptanal in aqueous solutions with a casein content varying from 0 to 12%. Generally, the aroma retention increased with increasing protein content. A recent study by Zhu (2003) demonstrated weak binding of 2-nonanone by sodium caseinate, using headspace SPME. An average affinity constant of $K = 1858\ M^{-1}$ and on average 0.3 binding sites per protein molecule were found. The above studies clearly show that caseins and sodium caseinate are capable of binding several different flavor compounds. Therefore, when adding caseins to a food product, it has to be considered that flavor may be bound by the proteins and made unavailable for perception.

Hardly any information is available on the flavor binding behavior of the individual caseins α_{s1}-, α_{s2}-, β-, and κ-casein. Of all the caseins, β-casein is the most hydrophobic (Swaisgood 1992), and thus could have higher affinity constants for lipophilic flavor compounds. In addition, casein molecules have a tendency to undergo self-association or association with each other, depending on environmental conditions, such as protein concentration, pH, and ionic strength. It is unknown how this association behavior influences flavor binding. Recently, Burova and others (2003) estimated binding constants around $100\ M^{-1}$ for 2-octanone or vanillin on β-casein. Systematic studies on the binding of selected flavor compounds by individual caseins and their mixtures under different conditions are required to fully understand this complex system.

Comparison of flavor binding capacities of milk proteins

The binding parameters of the most studied flavor compound, 2-nonanone, for milk proteins obtained by different authors vary significantly, as shown in Table 1. Researchers have used different experimental approaches to investigate protein–flavor binding, which may be the reason for some of the variation in the results (O'Keefe and others 1991b; Stevenson and others 1996). Nevertheless, there are obvious trends, such as decreasing affinity constants in the order BSA > β-lg > α-la.

An early study that compared the binding of diacetyl by sodium caseinate and whey protein showed similar flavor binding for both proteins, as determined by static headspace analysis (Reineccius and Coulter 1969). However, this observation was disputed by several later studies, which showed that whey protein generally has a stronger flavor-binding capacity than casein (Hansen and Heinis 1991; Hansen and Booker 1996; Li and others 2000; Zhu 2003) (Figure 2). Under identical experimental conditions, WPI was found to have higher affinity for vanillin than sodium caseinate (Li and others 2000). These results agree with the findings of Hansen and Booker (1996), who examined the binding of vanillin, benzaldehyde, citral, and d-limonene to sodium caseinate and WPC. They reported that whey protein exhibited greater degrees of binding than casein of these flavor compounds. Recently, Zhu (2003), using headspace SPME, determined a higher average binding constant for 2-nonanone and WPI than for 2-nonanone and sodium caseinate (Table 1). In contrast, McNeill and Schmidt (1993) reported that sodium caseinate interacted significantly more than WPI with vanillin in sweetened drinks. The authors did not provide any

Table 1 – Binding data for the interactions between 2-nonanone and milk proteins (25 °C): *n*, number of binding sites per monomer; *K*, intrinsic binding constant

	n	*K* [M^{-1}]	Method	Reference
WPC	61	1920000	Equilibrium dialysis	Jasinski and Kilara (1985)
	0.2	53000000	Fluorescence spectroscopy	Liu and others (2005b)
WPI	1	2059	Headspace SPME	Zhu (2003)
Sodium caseinate	0.3	1858	Headspace SPME	Zhu (2003)
β-Lg	1	2439	Equilibrium dialysis	O'Neill and Kinsella (1987)
	0.2	6250 ($\leq$ 40 ppm)	Static headspace analysis	Charles and others (1996)
	0.5	1667 ($\geq$ 45 ppm)		
	14	122	Equilibrium dialysis	Jasinski and Kilara (1985)
α-La	33	11	Equilibrium dialysis	Jasinski and Kilara (1985)
BSA	5–6	1800	Liquid–liquid partitioning	Damodaran and Kinsella (1980b)
	15	14100	Equilibrium dialysis	Jasinski and Kilara (1985)
	7	833	PFG-NMR spectroscopy	Jung and others (2002)

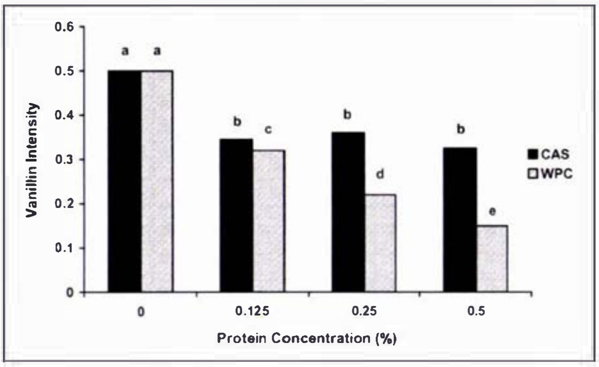


Figure 2 — Vanillin flavor intensity relative to the reference in the presence of sodium caseinate (CAS) and whey protein concentrate (WPC). The reference vanillin concentration was 3.38 × 10^{-6} mM in a 2.5% sucrose solution. For each protein type, bars with dissimilar letter codes indicate significant differences between means. (Reproduced with permission from Hansen and Heinis (1991). Copyright 1991 American Dairy Science Assn.)

explanation for this surprising result. Their finding highlights that a complex matrix, containing sucrose and emulsifier as well as protein, can influence flavor binding to proteins differently than a simple matrix. Therefore, it is crucial to explore basic systems first to be able to interpret results in multicomponent systems.

BSA has been shown to interact strongly with vanillin, whereas sodium caseinate and WPI showed similar and significantly lower binding of vanillin. Hydrogen bonding appeared to be a major force for the interaction of vanillin and sodium caseinate. However, hydrophobic interaction seemed to be more important than hydrogen bonding in the vanillin–BSA system (Chobpattana and others 2002). Binding of γ- and δ-lactones has been shown to be much stronger to BSA in comparison to β-lg (Guth and Fritzler 2004). From all of these studies, it becomes obvious that BSA is the milk protein that is most capable of flavor binding, followed by β-lg.

Influence of Heat Treatment on Protein–Flavor Binding

Flavor binding by proteins is very dependent on the conformational state of the proteins, and all the factors that alter the conformation, that is, temperature (Li and others 2000; Chobpattana and others 2002), pH (Druaux and others 1995; Jouenne and Crouzet 1996; Andriot and others 1999; Jouenne and Crouzet 2000a, 2000b), and ionic strength (Mohammadzadeh-K. and others 1969b; Damodaran and Kinsella 1981c). It has been demonstrated that conformational changes affect both the binding affinity and the number of binding sites on proteins for aroma compounds.

In practice, heat-denatured proteins are of greater importance than native proteins, because heat treatment is an important step during the processing and preparation of many foodstuffs containing protein (de Wit 1981). Heat treatments usually cause denaturation of proteins, which involves unfolding and subsequent aggregation of unfolded protein molecules. The process of unfolding may reveal binding sites that were previously buried and thus may result in an increase in binding. In contrast, the subsequent formation of protein aggregates may release flavor molecules from the binding sites again.

Below the denaturation temperature

The effects of temperature on protein–flavor interactions appear to be dependent on the type of protein and the type of flavor compound. The affinity of γ-decalactone for BSA in a model wine system was found to be higher at 10 °C than at 20 °C and 30 °C, possibly due to structural changes of the protein at low temperatures, whereas the number of binding sites ($n = 6$–7) was not modified at the 3 temperatures (Druaux and others 1995). However, an increase in temperature from 10 to 35 °C had little effect on the binding of 2-nonanone to BSA (Damodaran and Kinsella 1980b). This discrepancy could be explained by the presence of ethanol (10% w/w) and salts in the model wine system used by Druaux and others (1995), which may have had an effect on BSA conformation. In addition, γ-decalactone and 2-nonanone might bind on different sites on BSA due to their structural differences. There is not much information available about flavor binding sites on BSA. A large number of binding sites have been suggested by several authors (Spector and others 1969; Damodaran and Kinsella 1980a, 1980b; Jung and others 2002; Guth and Fritzler 2004), which makes the interpretation of results even more difficult. Some of the sites might be susceptible to temperature changes, whereas others might not be.

A decrease in temperature from 12 to 4 °C increased the number of binding sites and the binding constants for vanillin on casein and whey protein (Li and others 2000). The authors concluded that the changes in the number of binding sites and the binding affinity could be attributed to possible changes in the tertiary and quaternary structures of the protein at 4 °C. The exact changes to protein structures under these conditions need to be investigated.

Mills and Solms (1984), using static headspace analysis, observed an increase in the binding of heptanal to whey protein with an increase in temperature from 25 to 50 °C. The amount of heptanal irreversibly bound markedly increased at 50 °C. These results were attributed to covalent binding of the aldehyde and ε-amino groups of lysine residues in the proteins. An increase in temperature seems to enhance the binding of "reactive" flavors, whereas reversibly bound flavors may be released or not affected at all, depending on the nature of flavor compound. Clearly, further work is needed to fully understand the temperature dependence of these interactions, in particular in the case of reversible binding.

Above the denaturation temperature

Studies on the influence of heat-denaturation on protein–flavor binding are summarized in Table 2. A number of studies reported a decreased binding when proteins are denatured. On exposure of β-lg to 75 °C for 10 and 20 min, the binding affinity for 2-nonanone was weaker than that of the native protein and the number of low-affinity nonspecific binding sites increased (O'Neill and Kinsella 1988). Heat-treated WPI (85 °C, 10 min) had significantly higher vanillin flavor intensity than an untreated WPI (McNeill and Schmidt 1993). Free vanillin as determined by HPLC was also higher in a heated BSA system (68 °C for 30 min and 75 °C for 15 min) than in the nonheated system (Chobpattana and others 2002). In agreement with this, Burova and others (2003) reported the complete loss of vanillin binding after thermal denaturation of BSA. This may have been due to heat-induced structural changes and protein aggregation during the heat treatment. These authors also demonstrated an increase in the denaturation temperature of BSA in the presence of either vanillin or 2-octanone, which means that the flavor ligands increased the conformational stability of the protein (Burova and others 1999, 2003). Nonflavor ligands, such as palmitate, have also been found to stabilize the native structure of β-lg against heat-induced unfolding and denaturation. This stabilizing effect appears to be ligand-dependent; ligands that bind strongly into the hydrophobic calyx seem to be most effective (Considine and others 2005a).

On the other hand, when heated and unheated sodium caseinate solutions were compared, no differences were found for vanillin

Table 2 – Influence of heat denaturation on the binding between milk proteins and flavor compounds; ↑ binding increases, ↓ binding decreases

Protein–Flavor System	θ [°C] / t [min]	Binding	Method	Reference
β-Lg/benzaldehyde	70/30	↑	Ultra filtration-HPLC	Hansen and Booker (1996)
β-Lg /2-nonanone	75/10 and 20	↓	Equilibrium dialysis	O'Neill and Kinsella (1988)
WPI/vanillin	85/10	↓	Sensory	McNeill and Schmidt (1993)
BSA/vanillin	75/15	↓	Ultra filtration-HPLC	Chobpattana and others (2002)

flavor intensity (McNeill and Schmidt 1993) and free vanillin concentration (Chobpattana and others 2002). This was expected as casein has a little secondary and tertiary structure and remarkably high heat stability (Fox and Mulvihill 1982).

However, for 1 milk protein–flavor system, it has been reported that the binding capacity of denatured proteins is generally higher than that of native proteins. Hansen and Booker (1996) reported that the amount of benzaldehyde bound by β-lg increased from 38% to 63% as the temperature was raised from room temperature to pasteurization temperature (70 °C, 30 min). The authors attribute the increase in binding to unfolding of whey proteins upon heating. The previously buried hydrophobic residues may become accessible for interaction with nonpolar flavor molecules, resulting in a greater amount of flavor compounds bound. Since these authors added the flavor before the heat treatment, it is likely that covalent binding may play a role in this case; that is, the aldehyde function of benzaldehyde could be susceptible to reaction with ε-amino groups in β-lg, particularly at the elevated temperature.

Most of the reported data on the influence of heat treatment on protein–flavor binding are based on single temperature and heating time. No systematic studies have been reported on the development of interactions with increasing heating time or increasing heating temperature. There might be an early stage with increased binding due to unfolding of the proteins, and a later stage characterized by a decrease in binding due to aggregation. Moreover, it has been established that a number of intermediate species are generated during heat-induced denaturation and aggregation of β-lg and whey proteins (Havea and others 2001). It would be interesting to understand the role of these intermediates in flavor binding.

Influence of High-Pressure Treatment on Protein–Flavor Binding

The use of high pressure as an alternative to heat treatment of foods, including milk and dairy products, is becoming of increasing interest. High-pressure treatment not only results in microbial inactivation, but has also been shown to improve rennet or acid coagulation of milk without detrimental effects on important quality characteristics, such as taste, flavor, vitamins, and nutrients (Trujillo 2002). It is known that high-pressure treatment changes the structure of milk proteins, and is thus likely to affect the protein–flavor interactions. In particular, β-lg has been shown to be very sensitive towards pressure-induced denaturation, and the process of pressure denaturation appears to be similar but not identical to that of heat denaturation (Huppertz and others 2004; Considine and others 2005b). α-La is more resistant to pressure than β-lg (Huppertz and others 2004; Liu and others 2005a). Yang and others (2003) observed that ligand binding by high-pressure-treated β-lg can be increased or decreased compared to the native protein depending on the structure of the ligand. High-pressure treatment of β-lg decreased the affinity for capsaicin on specific binding sites, whereas the binding of the other flavor compounds examined, namely α-ionone, β-ionone, cinnamaldehyde, and vanillin, was unspecific and remained unaffected by high-pressure treatment. The authors explain this finding with the incorporation of water into the protein upon high-pressure treatment, as compared to the transfer of nonpolar groups into water upon heat denaturation of a protein (Hummer and others 1998), so that high-pressure-treated β-lg may not exhibit an increase in surface hydrophobicity, and thus not exhibit an increasing affinity for hydrophobic flavor compounds. However, this would mean that heat-treated β-lg would bind more flavor compared to the native protein, but this is not true in most cases (Table 2).

New insight into the effects of high hydrostatic pressure (HHP) treatment on protein–flavor binding was given by Liu and others (2005a), using fluorescence spectroscopy and static headspace analysis. The number of binding sites and the apparent dissociation constants for benzaldehyde and methyl ketones on WPC were either unaffected or increased upon HHP treatment, depending on the structure of the flavor compound, the flavor concentration, and the HHP treatment times. Using SDS-PAGE, the same authors (Liu and others 2005b) showed that during the time to reach the target pressure (600 MPa), dissociation of protein aggregates present in WPC occurred, which may have exposed more binding sites. The presence of aggregates may have resulted from the ultra filtration and drying procedures used in WPC manufacture. Liu and others (2005a) pointed out the importance of careful selection of flavor concentrations and HHP treatment conditions for desired outcomes in food applications.

Thorough research on the effect of high-pressure treatment on protein–flavor binding is clearly needed. The studies by Yang and others (2003) and Liu and others (2005a) are the only ones dealing with the influence of high-pressure treatment on protein–flavor interactions. Nonflavor ligands, when added prior to high-pressure treatment, have been found to inhibit the formation of intermediate, non-native protein species (Considine and others 2005b). The same effect may exist with flavor ligands but has not been studied yet. This area clearly warrants further investigations.

Implications in the Development of Protein Foods

Proteins do not contribute to flavor directly, but protein–flavor interactions can cause the aroma profile of a food to become unbalanced and unpleasant since proteins bind flavors to differing extents, depending mainly on the nature of the protein and flavor compound. This problem occurs particularly in high protein-based foods and in low-fat foods because fat is the preferred carrier of flavor compounds. Ideally, flavor molecules are preserved in the food during storage and processing, and slowly released during consumption of the food. However, in the presence of protein and absence of fat, some flavor compounds are lost due to the absence of fat, whereas others are tightly bound by the proteins, preventing them to be released and perceived during mastication. In such foods it is extremely difficult to control flavor. If proteins are present, the amount of flavor compounds added usually has to be increased to compensate for the protein-bound flavor.

The processing of foods, and in particular heat treatment, has been shown to have a marked effect on milk protein–flavor interactions. Even slight changes in temperature below the denaturation temperature of the proteins have been shown to influence the binding of flavors (Mills and Solms 1984; Druaux and others 1995). Severe heat treatments caused a decrease in binding for most flavors (O'Neill and Kinsella 1988; McNeill and Schmidt 1993; Chobpattana and others 2002; Burova and others 2003), but an increase in binding has also been reported (Mills and Solms 1984; Hansen and Booker 1996). Thus heat treatment may drastically change the flavor profile of a food product. In the development of protein-based foods, factors changing the conformation of a protein, that is, pH, ionic strength, so on, have to be considered because they potentially alter the binding of flavors to the protein. The presence of flavor compounds during heat treatment has been shown to increase the protein conformational stability (Burova and others 1999, 2003), which in turn may alter the characteristics of the end product.

Concluding Remarks

The presence of proteins in flavored low-fat food products causes a great challenge for flavor scientists because many proteins are able to bind several flavor compounds tightly and influence the perceived aroma profile significantly. BSA is the milk protein most capable of binding volatile flavor compounds, followed by β-lg. A large number of instrumental methods have been used to investigate the type and the extent of flavor binding, which makes it difficult to compare results between studies. Sensory methods are very useful because they complement instrumental techniques and give insight into the effect of protein–flavor binding on flavor perception. The characteristics of the aroma compounds and the proteins determine the extent of binding, which can be influenced by several parameters. Although extensive information on the binding of various flavor compounds to different food proteins is available, there are many apparent contradictions and disagreements among various studies. For example, the effects of heat treatments on protein–flavor interactions are not fully understood. The development of protein–flavor interactions with heating time or rather with increasing temperature should be looked at and compared with the corresponding conformational state of the protein. Research on the influence of high-pressure treatment of milk proteins on the proteins' flavor binding behavior needs to be investigated further as well.

A good knowledge of the physicochemical interactions that occur between aroma compounds and proteins is required to improve food flavoring and to make protein-based foods, for example, "light" dairy products, sensorily more acceptable to the consumer. In particular, the nature and the location of binding sites on proteins for flavors need to be investigated further. It is vital to obtain more consistent results between different instrumental methods. Some of the early methods such as equilibrium dialysis and exponential dilution are still going to be frequently used. We believe some of the newer techniques, such as SPME and NMR, need to be developed further to investigate protein–flavor interactions and be used more frequently in the future. SPME is fast, solvent-free, and very sensitive. The main advantages of NMR techniques are speed and insight into binding mechanisms and binding topology.

Furthermore, the focus should be more on sensory techniques, because instrumental flavor-binding studies do not show if and how bound flavor is perceived during consumption. A growing area of research comprises the mechanisms of in vitro and in vivo flavor release from foods. A detailed discussion of in vivo flavor release from protein containing systems is beyond the scope of this review but some information is given below. Model mouth systems have been frequently used to measure flavor release in vitro (Roberts and Acree 1995; Deibler and others 2001; Rabe and others 2002). Methods for measuring in vivo volatile release from foods have been summarized previously (Taylor and Linforth 2000). There is a variety of objective and subjective in vivo techniques to investigate flavor release in-nose or in-mouth, including concentrating the exhaled volatiles followed by GC-MS (Denker and others 2006), atmospheric pressure chemical ionization (APCI)-MS (MS-Nose) (Taylor and others 2000; Weel and others 2002; Lethuaut and others 2004), and proton-transfer reaction (PTR)-MS (Lindinger and others 1998; Mestres and others 2005; Boland and others 2006) to analyze breath volatiles during eating in real time, and sensory techniques such as the time-intensity (TI) approach (Weel and others 2002; Lethuaut and others 2004; Mestres and others 2005). No systematic studies have been reported on the relationship between extent or strength of flavor binding to proteins and its in vivo release. Interactions of WPI and aldehydes in solution were shown to be less significant in-nose than under static headspace conditions, possibly due to the highly dynamic conditions in vivo (Weel and others 2003). Milk proteins, in particular whey proteins, reduced the in-mouth release of selected flavor compounds from coffee (Denker and others 2006).

To date, most systems that have been investigated consist of 1 protein and 1 aroma compound in an aqueous solution. Food systems are much more complex, consisting of several food matrix components and flavor mixtures. Thus, further research on these complex systems is becoming increasingly important. However, primarily consistent results using simple systems should be obtained before investigating complex systems.

References

Adams RL, Mottram DS, Parker JK, Brown HM. 2001. Flavor-protein binding: disulfide interchange reactions between ovalbumin and volatile disulfides. J Agric Food Chem 49:4333–6.

Alaiz M, Girón J. 1994. Modification of histidine-residues in bovine serum-albumin by reaction with (e)-2-octenal. J Agric Food Chem 42:2094–8.

Andriot I, Harrison M, Fournier N, Guichard E. 2000. Interactions between methyl ketones and ß-lactoglobulin: sensory analysis, headspace analysis, and mathematical modeling. J Agric Food Chem 48:4246–51.

Andriot I, Marin I, Feron G, Relkin P, Guichard E. 1999. Binding of benzaldehyde by ß-lactoglobulin, by static headspace and high performance liquid chromatography in different physico-chemical conditions. Lait 79:577–86.

Arthur CL, Pawliszyn J. 1990. Solid-phase microextraction with thermal desorption using fused silica optical fibers. Anal Chem 62:2145–8.

Batt CA, Brady J, Sawyer L. 1994. Design improvements of ß-lactoglobulin. Trends Food Sci Technol 5:261–5.

Beyeler M, Solms J. 1974. Interaction of flavor model compounds with soy protein and bovine serum albumin. Lebensm-Wiss Technol 7:217–9.

Boland AB, Delahunty CM, van Ruth SM. 2006. Influence of the texture of gelatin gels and pectin gels on strawberry flavour release and perception. Food Chem 96:452–60.

Burova TV, Grinberg NV, Grinberg VY, Tolstoguzov VB. 2003. Binding of odorants to individual proteins and their mixtures. Effects of protein denaturation and association. A plasticized globule state. Colloids Surf A 213:235–44.

Burova TV, Grinberg NV, Golubeva IA, Mashkevich AY, Grinberg VY, Tolstoguzov VB. 1999. Flavour release in model bovine serum albumin/pectin/2-octanone systems. Food Hydrocolloids 13:7–11.

Carr J, Baloga D, Guinard JX, Lawter L, Marty C, Squire C. 1996. The effect of gelling agent type and concentration on flavor release in model systems. In: McGorrin RJ, Leland JV, editors. Flavor-food interactions. Washington, D.C.: American Chemical Society. p 98–108.

Charles M, Bernal B, Guichard E. 1996. Interaction of ß-lactoglobulin with flavour compounds. In: Taylor AJ, Mottram DS, editors. Flavour science: recent developments. Cambridge, U.K.: Royal Society of Chemistry. p 433–6.

Chobpattana W, Jeon IJ, Smith JS, Loughin TM. 2002. Mechanisms of interaction between vanillin and milk proteins in model systems. J Food Sci 67:973–7.

Coleman WM. 1996. A study of the behavior of Maillard reaction products analyzed by solid-phase microextraction gas chromatography mass selective detection. J Chromatogr Sci 34:213–8.

Considine T, Patel HA, Singh H, Creamer LK. 2005a. Influence of binding of sodium dodecyl sulfate, all-*trans*-retinol, palmitate, and 8-anilino-1-naphthalenesulfonate on the heat-induced unfolding and aggregation of ß-lactoglobulin B. J Agric Food Chem 53:3197–205.

Considine T, Singh H, Patel HA, Creamer LK. 2005b. Influence of binding of sodium dodecyl sulfate, all-*trans*-retinol, and 8-anilino-1-naphthalenesulfonate on the high-pressure-induced unfolding and aggregation of ß-lactoglobulin B. J Agric Food Chem 53:8010–8.

Damodaran S, Kinsella JE. 1980a. Stabilization of proteins by solvents—effect of pH and anions on the positive cooperativity of 2-nonanone binding to bovine serum albumin. J Biol Chem 255:8503–8.

Damodaran S, Kinsella JE. 1980b. Flavor protein interactions---binding of carbonyls to bovine serum albumin—thermodynamic and conformational effects. J Agric Food Chem 28:567–71.

Damodaran S, Kinsella JE. 1981a. Interaction of carbonyls with soy protein—thermodynamic effects. J Agric Food Chem 29:1249–53.

Damodaran S, Kinsella JE. 1981b. Interaction of carbonyls with soy protein—conformational effects. J Agric Food Chem 29:1253–7.

Damodaran S, Kinsella JE. 1981c. The effects of neutral salts on the stability of macromolecules—a new approach using a protein-ligand binding system. J Biol Chem 256:3394–8.

Damodaran S, Kinsella JE. 1983. Binding of carbonyls to fish actomyosin. J Agric Food Chem 31:856–9.

de Wit JN. 1981. Structure and functional behaviour of whey proteins. Neth Milk Dairy J 35:47–64.

Deibler KD, Lavin EH, Linforth RST, Taylor AJ, Acree TE. 2001. Verification of a mouth simulator by in vivo measurements. J Agric Food Chem 49:1388–93.

Denker M, Parat-Wilhelms M, Drichelt G, Paucke J, Luger A, Borcherding K, Hoffmann W, Steinhart H. 2006. Investigation of the retronasal flavour release during the consumption of coffee with additions of milk constituents by 'oral breath sampling' Food Chem 98:201–8.

Dhont JH. 1987. Reaction of vanillin with albumin. In: Maarse H, Groenen PJ, editors. Aroma research. Wageningen: Pudoc. p 193–4.

Díaz de Villegas MC, Oria R, Sala FJ, Calvo M. 1987. Lipid-binding by ß-lactoglobulin of cow milk. Milchwiss 42:357–8.

Druaux C, Lubbers S, Charpentier C, Voilley A. 1995. Effects of physicochemical parameters of a model wine on the binding of γ-decalactone on bovine serum albumin. Food Chem 53:203–7.

Dubois C, Sergent M, Voilley A. 1996. Flavoring of complex media: a model cheese example. In: McGorrin RJ, Leland JV, editors. Flavor-food interactions. Washington, D.C.: American Chemical Society. p 217–26.

Dufour E, Haertlé T. 1990. Binding affinities of ß-ionone and related flavor compounds to ß-lactoglobulin—effects of chemical modifications. J Agric Food Chem 38:1691–5.

Dumont JP. 1987. Flavour-protein interactions:a key to aroma persistence. In: Martens M, Dalen GA, Russwurm H, editors. Flavour science and technolog . Chichester: John Wiley and Sons. p 143–8.

Dumont JP, Land DG. 1986. Binding of diacetyl by pea proteins. J Agric Food Chem 34:1041–5.

Ebeler SE, Pangborn RM, Jennings WG. 1988. Influence of dispersion medium on aroma intensity and headspace concentration of menthone and isoamyl acetate. J Agric Food Chem 36:791–6.

Fabre M, Aubry V, Guichard E. 2002. Comparison of different methods: static and dynamic headspace and solid-phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. J Agric Food Chem 50:1497–501.

Farès K, Landy P, Guilard R, Voilley A. 1998. Physicochemical interactions between aroma compounds and milk proteins: effect of water and protein modification. J Dairy Sci 81:82–91.

Fischer N, Widder S. 1997. How proteins influence food flavor. Food Technol 51:68–70.

Fox PF, Mulvihill DM. 1982. Milk proteins—molecular, colloidal and functional properties. J Dairy Res 49:679–93.

Franzen KL, Kinsella JE. 1971. Parameters affecting binding of volatile flavor compounds in model food systems. I. Proteins. J Agric Food Chem 22:675–8.

Frapin D, Dufour E, Haertlé T. 1993. Probing the fatty-acid-binding site of ß-lactoglobulins. J Prot Chem 12:443–9.

Fugate RD, Song PS. 1980. Spectroscopic characterization of ß-lactoglobulin-retinol complex. Biochim Biophys Acta 625:28–42.

Futterman S, Heller J. 1972. Enhancement of fluorescence and decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, ß-lactoglobulin, and the retinol-binding protein of human plasma. J Biol Chem 247:5168–72.

Gianelli MP, Flores M, Toldra F. 2005. Interaction of soluble peptides and proteins from skeletal muscle with volatile compounds in model systems as affected by curing agents. J Agric Food Chem 53:1670–7.

Gremli HA. 1974. Interaction of flavor compounds with soy protein. J Am Oil Chem Soc 51:A95–A97.

Grote C, Pawliszyn J. 1997. Solid-phase microextraction for the analysis of human breath. Anal Chem 69:587–96.

Guichard E, Langourieux S. 2000. Interactions between ß-lactoglobulin and flavour compounds. Food Chem 71:301–8.

Guth H, Fritzler R. 2004. Binding studies and computer-aided modelling of macromolecule/odorant interactions. Chem Biodiv 1:2001–23.

Hansen AP, Booker DC. 1996. Flavor interaction with casein and whey protein. In: McGorrin RJ, Leland JV, editors. Flavor-food interactions. Washington, D.C.: American Chemical Society. p 75–89.

Hansen AP, Heinis JJ. 1991. Decrease of vanillin flavor perception in the presence of casein and whey proteins. J Dairy Sci 74:2936–40.

Hansen AP, Heinis JJ. 1992. Benzaldehyde, citral, and d-limonene flavor perception in the presence of casein and whey proteins. J Dairy Sci 75:1211–5.

Hatchwell LC. 1996. Implications of fat on flavor. In: McGorrin RJ, Leland JV, editors. Flavor-food interactions. Washington, D.C.: American Chemical Society. p 14–23.

Havea P, Singh H, Creamer LK. 2001. Characterization of heat-induced aggregates of ß-lactoglobulin, α-lactalbumin and bovine serum albumin in a whey protein concentrate environment. J Dairy Res 68:483–97.

Hawthorne SB, Miller DJ, Pawliszyn J, Arthur CL. 1992. Solventless determination of caffeine in beverages using solid-phase microextraction with fused-silica fibers. J Chrom 603:185–91.

Heinemann C, Conde-Petit B, Nuessli J, Escher F. 2001. Evidence of starch inclusion complexation with lactones. J Agric Food Chem 49:1370–6.

Heng L, van Koningsveld GA, Gruppen H, van Boekel M, Vincken JP, Roozen JP, Voragen AGJ. 2004. Protein-flavour interactions in relation to development of novel protein foods. Trends Food Sci Technol 15:217–24.

Hill AV. 1910. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. Proc Physiol Soc (London) 40:IV–VII.

Hummel JR, Dreyer WJ. 1962. Measurement of protein-binding phenomena by gel filtration. Biochim Biophys Acta 63:530–2.

Hummer G, Garde S, Garcia AE, Paulaitis ME, Pratt LR. 1998. The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. Proc Nat Acad Sci USA 95:1552–5.

Huppertz T, Fox PF, Kelly AL. 2004. High pressure treatment of bovine milk: effects on casein micelles and whey proteins. J Dairy Res 71:97–106.

Jaime I, Mela DJ, Bratchell N. 1993. A study of texture-flavor interactions using free-choice profiling. J Sens Stud 8:177–88.

Jasinski E, Kilara A. 1985. Flavor binding by whey proteins. Milchwiss 40:596–9.

Jouenne E, Crouzet J. 1996. Interaction of aroma compounds with ß-lactoglobulin. In: Taylor AJ, Mottram DS, editors. Flavour science: recent developments. Cambridge, U.K.: Royal Society of Chemistry. p 425–9.

Jouenne E, Crouzet J. 2000a. Determination of apparent binding constants for aroma compounds with ß-lactoglobulin by dynamic coupled column liquid chromatography. J Agric Food Chem 48:5396–400.

Jouenne E, Crouzet J. 2000b. Effect of pH on retention of aroma compounds by ß-lactoglobulin. J Agric Food Chem 48:1273–7.

Jouquand C, Ducruet V, Giampaoli P. 2004. Partition coefficients of aroma compounds in polysaccharide solutions by the phase ratio variation method. Food Chem 85:467–74.

Jung DM, Ebeler SE. 2003a. Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. J Agric Food Chem 51:200–5.

Jung DM, Ebeler SE. 2003b. Investigation of binding behavior of α- and ß-ionones to ß-lactoglobulin at different pH values using a diffusion-based NOE pumping technique. J Agric Food Chem 51:1988–93.

Jung DM, de Ropp JS, Ebeler SE. 2002. Application of pulsed field gradient NMR techniques for investigating binding of flavor compounds to macromolecules. J Agric Food Chem 50:4262–9.

King BM, Solms J. 1979. Interactions of flavor compounds in model food systems using benzyl alcohol as an example. J Agric Food Chem 27:1331–4.

Kinsella JE, Whitehead DM. 1989. Proteins in whey: chemical, physical, and functional properties. Adv Food Nutr Res 33:343–438.

Klotz IM, Urquhart JM. 1948. The combination of adenine, adenosine, and adenylic acid with serum albumin. J Biol Chem 173:21–4.

Klotz IM, Walker FM, Pivan RB. 1946. The binding of organic ions by proteins. J Am Chem Soc 68:1486–90.

Land DG. 1996. Perspectives on the effects of interactions on flavor perception: an overview. In: McGorrin RJ, Leland JV, editors. Flavor-food interactions. Washington, D.C.: American Chemical Society. p 2–11.

Land DG, Reynolds J. 1981. The influence of food components on the volatility of diacetyl. In: Schreier P, editor. Flavour '81. Berlin: Walter de Gruyter. p 701–5.

Landy P, Druaux C, Voilley A. 1995. Retention of aroma compounds by proteins in aqueous solution. Food Chem 54:387–92.

Landy P, Fares K, Lorient D, Voilley A. 1997. Effect of chemical modification of sodium caseinate on diffusivity of aroma compounds in aqueous solutions. J Agric Food Chem 45:2649–53.

Langourieux S, Crouzet J. 1995. Protein-aroma interactions. In: Dickinson E, Lorient D, editors. Food macromolecules and colloids. Cambridge, U.K.: Royal Society of Chemistry. p 123–33.

Le Thanh M, Thibeaudeau P, Thibaut MA, Voilley A. 1992. Interactions between volatile and nonvolatile compounds in the presence of water. Food Chem 43:129–35.

Lethuaut L, Weel KGC, Boelrijk AEM, Brossard CD. 2004. Flavor perception and aroma release from model dairy desserts. J Agric Food Chem 52:3478–85.

Li Z, Grün IU, Fernando LN. 2000. Interaction of vanillin with soy and dairy proteins in aqueous model systems: a thermodynamic study. J Food Sci 65:997–1001.

Lindinger W, Hansel A, Jordan A. 1998. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS)—medical applications, food control and environmental research. Int J Mass Spectrom 173:191–241.

Linforth RST, Taylor AJ. 1993. Measurement of volatile release in the mouth. Food Chem 48:115–20.

Liu X, Powers JR, Swanson BG, Hill HH, Clark S. 2005a. High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. J Food Sci 70:C581–5.

Liu X, Powers JR, Swanson BG, Hill HH, Clark S. 2005b. Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure. Innov Food Sci Emerg Technol 6:310–7.

Lübke M, Guichard E, Le Quéré JL. 2000. Infrared spectroscopic study of ß-lactoglobulin interactions with flavor compounds. In: Roberts DD, Taylor AJ, editors. Flavor release. Washington, D.C.: American Chemical Society. p 282–92.

Lübke M, Guichard E, Tromelin A, Le Quéré JL. 2002. Nuclearmagnetic resonance spectroscopic study of β-lactoglobulin interactions with two flavor compounds, γ-decalactone and β-ionone. J Agric Food Chem 50:7094–9.

Maier HG. 1970. Volatile flavoring substances in foodstuffs. Angew Chem Int Ed Engl 9:917–26.

Maier HG. 1972. Bindung flüchtiger Aromastoffe an Lebensmittel. Lebensm-Wiss Technol 5:1–6.

Maier HG. 1973. Sorption of volatile aroma constituents by foods. 7. Aliphatic aldehydes. Z Lebensm-Unters Forsch 151:384–6.

Maier HG. 1975. Binding of volatile aroma substances to nutrients and foodstuffs. In: Maarse H, Groenen PJ, editors. Aroma research. Wageningen: Pudoc. p 143–57

McKenzie HA. 1971. ß-Lactoglobulins. In: McKenzie HA, editor. Milk proteins, chemistry and molecular biology. New York: Academic Press. p 257–330.

McNeill VL, Schmidt KA. 1993. Vanillin interaction with milk protein isolates in sweetened drinks. J Food Sci 58:1142–4, 1147.

Mestres M, Moran N, Jordan A, Buettner A. 2005. Aroma release and retronasal perception during and after consumption of flavored whey protein gels with different textures. 1. In vivo release analysis. J Agric Food Chem 53:403–9.

Meynier A, Garillon A, Lethuaut L, Genot C. 2003. Partition of five aroma compounds between air and skim milk, anhydrous milk fat or full-fat cream. Lait 83: 223–35.

Meynier A, Rampon V, Dalgalarrondo M, Genot C. 2004. Hexanal and t-2-hexenal form covalent bonds with whey proteins and sodium caseinate in aqueous solution. Int Dairy J 14:681–90.

Mills OE, Solms J. 1984. Interaction of selected flavor compounds with whey proteins. Lebensm-Wiss Technol 17:331–5.

Mironov NA, Breus VV, Gorbatchuk VV, Solomonov BN, Haertle T. 2003. Effects of hydration, lipids, and temperature on the binding of the volatile aroma terpenes by β-lactoglobulin powders. J Agric Food Chem 51:2665–73.

Mohammadzadeh-K A, Feeney RE, Smith LM. 1969a. Hydrophobic binding of hydrocarbons by proteins I. Relationship of hydrocarbon structure. Biochim Biophys Acta 194:246–55.

Mohammadzadeh-K A, Smith LM, Feeney RE. 1969b. Hydrophobic binding of hydrocarbons by proteins II. Relationship of protein structure. Biochim Biophys Acta 194:256–64.

Mohammadzadeh-K A, Feeney RE, Samuels RB, Smith LM. 1967. Solubility of alkanes in protein solutions. Biochim Biophys Acta 147:583–9.

Monaco HL, Zanotti G, Spadon P, Bolognesi M, Sawyer L, Eliopoulos EE. 1987. Crystal-structure of the trigonal form of bovine β-lactoglobulin and of its complex with retinol at 2.5Å resolution. J Mol Biol 197:695–706.

Morrisett JD, Pownall HJ, Gotto AMG. 1975. Bovine serum albumin: study of the fatty acid and steroid binding sites using spin-labelled lipids. J Biol Chem 250:2487–94.

Mottram DS, Szauman-Szumski C, Dodson A. 1996. Interaction of thiol and disulfide flavor compounds with food components. J Agric Food Chem 44:2349–51.

Mulvihill DM. 1992. Production, functional properties and utilization of milk protein products. In: Fox PF, editor. Advanced dairy chemistry-1: Proteins. New York: Elsevier Applied Science. p 369–404.

Muresan S, van der Bent A, de Wolf FA. 2001. Interaction of β-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: nonlinear quenching effects related to protein-protein association. J Agric Food Chem 49:2609–18.

Nawar WW. 1966. Some considerarions in interpretation of direct headspace gas chromatographic analysis of food volatiles. Food Technol 20:115–7.

Ng PKW, Hoehn E, Bushuk W. 1989a. Binding of vanillin by fababean proteins. J Food Sci 54:105–7.

Ng PKW, Hoehn E, Bushuk W. 1989b. Sensory evaluation of binding of vanillin by fababean proteins. J Food Sci 54:324–5, 346.

North ACT. 1989. 3-Dimensional arrangement of conserved amino acid residues in a superfamily of specific ligand-binding proteins. Int J Biol Macromol 11:56–8.

O'Keefe SF, Resurreccion AP, Wilson LA, Murphy PA. 1991a. Temperature effect on binding of volatile flavor compounds to soy protein in aqueous model systems. J Food Sci 56:802–6.

O'Keefe SF, Wilson LA, Resurreccion AP, Murphy PA. 1991b. Determination of the binding of hexanal to soy glycinin and β-conglycinin in an aqueous model system using a headspace technique. J Agric Food Chem 39:1022–8.

O'Neill TE, Kinsella JE. 1987. Binding of alkanone flavors to β-lactoglobulin—effects of conformational and chemical modification. J Agric Food Chem 35:770–4.

O'Neill TE, Kinsella JE. 1988. Effect of heat treatment and modification on conformation and flavor binding by β-lactoglobulin. J Food Sci 53:906–9.

Overbosch P, Afterof WGM, Haring PGM. 1991. Flavor release in the mouth. Food Rev Int 7:137–84.

Papiz MZ, Sawyer L, Eliopoulos EE, North ACT, Findlay JBC, Sivaprasadarao R, Jones TA, Newcomer ME, Kraulis PJ. 1986. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. Nature 324:383–5.

Pelletier E, Sostmann K, Guichard E. 1998. Measurement of interactions between β-lactoglobulin and flavor compounds (esters, acids, and pyrazines) by affinity and exclusion size chromatography. J Agric Food Chem 46:1506–9.

Perez MD, Diaz de Villegas MC, Sánchez L, Aranda P, Ena JM, Calvo M. 1989. Interaction of fatty acids with β-lactoglobulin and albumin from ruminant milk. J Biochem 106:1094–7.

Philippe E, Seuvre AM, Colas B, Langendorff V, Schippa C, Voilley A. 2003. Behavior of flavor compounds in model food systems: a thermodynamic study. J Agric Food Chem 51:1393–8.

Plug H, Haring P. 1993. The role of ingredient-flavor interactions in the development of fat-free foods. Trends Food Sci Technol 4:150–2.

Pokorný J, Luan NT, Kondratenko SS, Janicek G. 1976. Changes of sensory value by interaction of alkanals with amino acids and proteins. Nahrung 20:267–72.

Rabe S, Krings U, Banavara DS, Berger RG. 2002. Computerized apparatus for measuring dynamic flavor release from liquid food matrices. J Agric Food Chem 50:6440–7.

Ragona L, Fogolari F, Zetta L, Perez DM, Puyol P, de Kruif K, Lohr F, Ruterjans H, Molinari H. 2000. Bovine-lactoglobulin: interaction studies with palmitic acid. Prot Sci 9:1347–56.

Reineccius GA, Coulter ST. 1969. Flavor retention during drying. J Dairy Sci 52:1219–23.

Reiners J, Nicklaus S, Guichard E. 2000. Interactions between β-lactoglobulin and flavour compounds of different chemical classes. Impact of the protein on the odour perception of vanillin and eugenol. Lait 80:347–60.

Roberts DD, Acree TE. 1995. Simulation of retronasal aroma using a modified headspace technique—investigating the effects of saliva, temperature, shearing, and oil on flavor release. J Agric Food Chem 43:2179–86.

Roberts DD, Pollien P. 2000. Relative influence of milk components on flavor compound volatility. In: Roberts DD, Taylor AJ, editors. Flavor release. Washington, D.C.: American Chemical Society. p 321–32.

Roberts DD, Pollien P, Milo C. 2000. Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. J Agric Food Chem 48:2430–7.

Robillard KA, Wishnia A. 1972. Aromatic hydrophobes and β-lactoglobulin A—thermodynamics of binding. Biochemistry 11:3835–40.

Sawyer L, Papiz MZ, North ACT, Eliopoulos EE. 1985. Structure and function of bovine β-lactoglobulin. Biochem Soc Trans 13:265–6.

Scatchard G. 1919. The attractions of proteins for small molecules and ions. Ann N Y Acad Sci 51:660–72.

Semenova MG, Antipova AS, Misharina TA, Golovnya RV. 2002a. Binding of aroma compounds with legumin. I. Binding of hexyl acetate with 11S globulin depending on the protein molecular state in aqueous medium. Food Hydrocolloids 16:557–64.

Semenova MG, Antipova AS, Wasserman LA, Misharina TA, Golovnya RV. 2002b. Binding of aroma compounds with legumin. II. Effect of hexyl acetate on thermodynamic properties of 11S globulin in aqueous medium. Food Hydrocolloids 16:565–71.

Semenova MG, Antipova AS, Belyakova LE, Polikarpov YN, Wasserman LA, Misharina TA, Terenina MB, Golovnya RV. 2002c. Binding of aroma compounds with legumin. III. Thermodynamics of competitive binding of aroma compounds with 11S globulin depending on the structure of aroma compounds. Food Hydrocolloids 16:573–84.

Shamil S, Kilcast D. 1992. Improving the flavour acceptability of reduced-fat foods. Nutr Food Sci 4:7–10.

Solms J, Osman-Ismail F, Beyeler M. 1973. Interaction of volatiles with food components. Can Inst Food Sci Technol J 6:A10–6.

Sorrentino F, Voilley A, Richon D. 1986. Activity coefficients of aroma compounds in model food systems. AIChE J 32:1988–93.

Sostmann K, Guichard E. 1998. Immobilized β-lactoglobulin on a HPLC-column: a rapid way to determine protein-flavour interactions. Food Chem 62:509–13.

Spector AA. 1975. Fatty acid binding to plasma albumin. J Lipid Res 16:165–79.

Spector AA, Fletcher JE. 1970. Binding of long chain fatty acids to β-lactoglobulin. Lipids 5:403–11.

Spector AA, John K, Fletcher JE. 1969. Binding of long-chain fatty acids to bovine serum albumin. J Lipid Res 10:56–67.

Steinhardt J, Reynolds JA. 1969. Multiple equilibria in proteins. New York: Academic Press.

Stevenson RJ, Chen XD, Mills OE. 1996. Modern analyses and binding studies of flavour volatiles with particular reference to dairy protein products. Food Res Int 29:265–90.

Swaisgood HE. 1992. Chemistry of the caseins. In: Fox PF, editor. Advanced dairy chemistry-1: Proteins. New York: Elsevier Applied Science. p 63–110.

Taylor AJ, Linforth RST. 2000. Techniques for measuring volatile release in vivo during consumption of food. In: Roberts DD, Taylor AJ, editors. Flavor release. Washington: American Chemical Society. p 8–21.

Taylor AJ, Linforth RST, Harvey BA, Blake B. 2000. Atmospheric pressure chemical ionisation mass spectrometry for in vivo analysis of volatile flavour release. Food Chem 71:327–38.

Trujillo AJ. 2002. Applications of high-hydrostatic pressure on milk and dairy products. High Press Res 22:619–26.

van Ruth SM, Villeneuve E. 2002. Influence of ß-lactoglobulin, pH and presence of other aroma compounds on the air/liquid partition coefficients of 20 aroma compounds varying in functional group and chain length. Food Chem 79:157–64.

van Ruth SM, de Vries G, Geary M, Giannouli P. 2002. Influence of composition and structure of oil-in-water emulsions on retention of aroma compounds. J Sci Food Agric 82:1028–35.

Voilley A, Beghin V, Charpentier C, Peyron D. 1991. Interactions between aroma substances and macromolecules in a model wine. Lebensm-Wiss Technol 24:469–72.

Weel KGC, Boelrijk AEM, Burger JJ, Claassen NE, Gruppen H, Voragen AGJ, Smit G. 2003. Effect of whey protein on the in vivo release of aldehydes. J Agric Food Chem 51:4746–52.

Weel KGC, Boelrijk AEM, Alting AC, van Mil P, Burger JJ, Gruppen H, Voragen AGJ, Smit G. 2002. Flavor release and perception of flavored whey protein gels: perception is determined by texture rather than by release. J Agric Food Chem 50:5149–55.

Widder S, Fischer N. 1996. Measurement of the influence of food ingredients on flavour release by headspace gas chromatography-olfactometry. In: Taylor AJ, Mottram DS, editors. Flavour science: recent developments. Cambridge, U.K.: Royal Society of Chemistry. p 405–12.

Wilson CE, Brown WE. 1997. Influence of food matrix structure and oral breakdown during mastication on temporal perception of flavor. J Sens Stud 21:69–86.

Wishnia A, Pinder TW. 1964. Hydrophobic interactions in proteins: conformation changes in bovine serum albumin below pH 5. Biochemistry 3:1377–84.

Wishnia A, Pinder TW. 1966. Hydrophobic interactions in proteins. The alkane binding site of ß-lactoglobulin A and B. Biochemistry 5:1534–42.

Wong NP, editor. 1988. Fundamentals of dairy chemistry. New York: Van Nostrand Reinhold Co.

Wu SY, Pérez MD, Puyol P, Sawyer L. 1999. ß-Lactoglobulin binds palmitate within its central cavity. J Biol Chem 274:170–4.

Yang J, Powers JR, Clark S, Dunker AK, Swanson BG. 2003. Ligand and flavor binding functional properties of ß-lactoglobulin in the molten globule state induced by high pressure. J Food Sci 68:444–52.

Yang XG, Peppard T. 1994. Solid-phase microextraction for flavor analysis. J Agric Food Chem 42:1925–30.

Yven C, Guichard E, Giboreau A, Roberts DD. 1998. Assessment of interactions between hydrocolloids and flavor compounds by sensory, headspace, and binding methodologies. J Agric Food Chem 46:1510–4.

Zhang ZY, Pawliszyn J. 1993. Headspace solid-phase microextraction. Anal Chem 65:1843–52.

Zhang ZY, Yang MJ, Pawliszyn J. 1994. Solid-phase microextraction. Anal Chem 66:A844–53.

Zhou A, Boatright WL, Johnson LA, Reuber M. 2002. Binding properties of 2-pentyl pyridine to soy protein as measured by solid phase microextraction. J Food Sci 67:142–5.

Zhu XQ. 2003. Interactions between flavour compounds and milk proteins [MPhil thesis]. Palmerston North, New Zealand: Massey University.

JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

J. Agric. Food Chem. **2007**, *55*, 3599–3604 **3599**

Binding of 2-Nonanone and Milk Proteins in Aqueous Model Systems

JANINA KÜHN,[†,‡] XIANG-QIAN ZHU,[†] THÉRÈSE CONSIDINE,[†,§] AND
HARJINDER SINGH*,[†]

Riddet Centre and Institute of Food, Nutrition and Human Health, Massey University,
Private Bag 11 222, Palmerston North, New Zealand

Interactions of the model flavor compound 2-nonanone with individual milk proteins, whey protein isolate (WPI), and sodium caseinate in aqueous solutions were investigated. A method to quantify the free 2-nonanone was developed using headspace solid-phase microextraction followed by gas chromatography with flame ionization detection. Binding constants (K) and numbers of binding sites (n) for 2-nonanone on the individual proteins were calculated. The 2-nonanone binding capacities decreased in the order bovine serum albumin > β-lactoglobulin > α-lactalbumin > α_{s1}-casein > β-casein, and the binding to WPI was stronger than the binding to sodium caseinate. All proteins appeared to have one binding site for 2-nonanone per molecule of protein at the flavor concentrations investigated, except for bovine serum albumin, which possessed two classes of binding sites. The binding mechanism is believed to involve predominantly hydrophobic interactions.

KEYWORDS: Whey protein; casein; 2-nonanone; binding constant; binding sites; solid-phase microextraction

INTRODUCTION

It is well-known that milk proteins bind flavor compounds of different chemical classes (*1*, *2*). The interactions between proteins and flavor compounds can have a strong influence on flavor perception, especially in reduced-fat products (*3*). Milk proteins are frequently used in food products for their specific functional properties, such as emulsifying and stabilizing abilities, and their nutritional value. In reduced-fat foods, the dominant components besides water are carbohydrates and proteins, which interact differently with aroma compounds compared with fat and thus can result in an enormous change in the perceived flavor (*3*). Further insights into protein–flavor interactions are required to improve food flavor, particularly that of reduced-fat foods.

Both reversible binding and irreversible binding can occur between proteins and flavor compounds. The type of interaction between a protein and a flavor compound depends on the nature of both the protein and the aroma compound. The strength of binding depends on the conformation of a protein, and all of the factors that can alter protein conformation, such as pH and temperature, usually affect flavor binding (*4*, *5*). As most aroma compounds are hydrophobic in nature, hydrophobic and reversible binding is predominant (*1*, *6*). Binding constants and numbers of binding sites for different flavor compounds on a variety of proteins have been reported, but results between studies differ considerably. Reasons for this may be the variety of conditions and methods used to determine the binding or the different compositions of protein batches.

The individual whey proteins, in particular β-lactoglobulin (β-lg) (*7*), have been frequently studied (*1*, *6*, *8*–*10*). β-Lg has been found to interact with several flavor compounds, such as esters (*11*), ketones (*6*, *12*), ionones (*2*), and lactones (*2*, *8*, *13*). The number and location of flavor binding sites on β-lg are not entirely clear, but most hydrophobic flavor compounds appear to bind in a central hydrophobic pocket, which also serves as the binding locus for apolar ligands such as retinol (*14*) and long-chain fatty acids (*15*–*17*), and at least one secondary binding site exists on the protein surface (*2*). Studies on the flavor binding behavior of α-lactalbumin (α-la) are rare because its flavor binding capacity was found to be low (*9*). In contrast, bovine serum albumin (BSA) seems to exhibit a strong affinity for different flavor compounds. Methyl ketones were found to have six (*1*) and seven (*10*) binding sites on BSA. Guth and Fritzler (*8*) suggested one or two high-affinity binding sites, and a large number of lower affinity sites, for γ- and δ-lactones on BSA.

In contrast, the individual caseins have been neglected so far, probably because of their lower flavor binding (*5*, *18*). Nevertheless, casein proteins are present and utilized in several food products, and they do bind flavor compounds with measurable affinities (*5*). To date, there is no information about the number of binding sites and the binding constants for flavor compounds on individual caseins. Sodium caseinate, a mixture of all casein

---

[*] Author to whom correspondence should be addressed (telephone +64-6-350-5545; fax +64-6-350-5655; e-mail H.Singh@massey.ac.nz)

[†] Riddet Centre, Massey University.

[‡] Institute of Food, Nutrition and Human Health, Massey Universtiy.

[§] Present address: Fonterra Co-operative Ltd., Palmerston North, New Zealand.

10.1021/jf063517o CCC: $37.00

types, was found to possess one low-affinity binding site for vanillin at low temperatures (*5*).

Both sensory techniques (*11*) and instrumental techniques, including equilibrium dialysis (*8*, *19*), static (*20*, *21*) and dynamic (*12*) headspace analysis, and liquid chromatographic techniques (*5*, *11*, *13*), have been used to investigate protein–flavor interactions. More recently, headspace solid-phase microextraction (SPME) has been found to be very suitable for exploring the binding between proteins and flavor compounds (*19*, *22*), and the technique has been found to be more appropriate than headspace techniques for studying milk protein–flavor interactions (*22*). Different spectroscopic techniques, such as fluorescence (*21*), infrared spectroscopy (*23*), and nuclear magnetic resonance (NMR) spectroscopy (*2*, *10*), have also been used successfully to elucidate the nature of interactions.

This paper describes the binding of the hydrophobic flavor compound 2-nonanone to individual milk proteins (namely, β-lg, α-la, BSA, α_{s1}-casein, and β-casein), whey protein isolate (WPI), and sodium caseinate, using an optimized headspace SPME–gas chromatography with flame ionization detection (GC-FID) method. 2-Nonanone was chosen as the model flavor compound because it is known to be bound completely reversibly on proteins. In addition, 2-nonanone is the most frequently studied flavor compound, allowing better comparison with other studies.

To date, the SPME technique has not been applied to determine binding parameters for flavor compounds on proteins. This is the first study comparing flavor binding capacities of the major individual milk proteins, including the caseins, which have been neglected so far. In the literature there are differences in binding parameters between studies, probably because various methods and conditions have been used.

MATERIALS AND METHODS

Materials. *Water.* Distilled, deionized water was obtained from a NANOpure II water purification system (Barnstead, Dubuque, IA).

2-Nonanone. A 10 mM stock solution of 2-nonanone (Aldrich Chemical Co., Milwaukee, WI) in propylene glycol (Bronson and Jacobs Pty Ltd., Sydney, Australia) was used to prepare the protein–flavor mixtures. Propylene glycol is a suitable solvent for flavor compounds because of its low vapor pressure, avoiding competition between 2-nonanone and the solvent in the fiber coating, which can be observed with other solvents such as ethanol. The stock solution was stored at 5 °C.

Standards. An external standard calibration was used to calculate the extent of binding. Standards of 0.1, 0.2, 0.4, 0.6, and 0.8 mM 2-nonanone were prepared in water from the 10 mM 2-nonanone stock solution.

Milk Proteins. WPI (ALACEN 895) and sodium caseinate (ALANATE 180) were obtained from Fonterra Co-operative Group Ltd., New Zealand. The WPI consisted of 93.3% protein, 0.3% fat, and 4.6% moisture, and the sodium caseinate consisted of 93.1% protein, 0.6% fat, and 4.8% moisture. Whey proteins were purchased from Sigma-Aldrich (St. Louis, MO). BSA had been processed to reduce its fatty acid content to 0.002%.

A 0.5% (w/v) WPI solution and a 1.0% (w/v) sodium caseinate solution were prepared in water and were stored at 5 °C for 6 h to allow complete hydration. Solutions of the individual whey proteins (0.5%, w/v) were prepared in water and were stored at 5 °C for 6 h prior to mixing them with the 2-nonanone stock solution. The pH of the protein solutions was between 6.8 and 6.9.

Fractionation of Caseins. The individual caseins were prepared in the laboratory by making sodium caseinate from bovine milk and separating the caseins by ion-exchange chromatography as described below. Because of the low proportion of κ-casein in total casein, only α_{s1}-casein and β-casein were examined regarding their affinity for 2-nonanone. The method for the separation and purification of α_{s1}- and β-caseins from sodium caseinate was based on the methods of Thompson (*24*), Lawrence and Creamer (*25*), and Creamer (*26*). Using ion-exchange chromatography, the sodium caseinate was separated into the individual casein fractions. The ion exchanger used was DE-52 (Whatman, Kent, U.K.). The caseins were eluted using a salt gradient. The UV–vis spectrophotometer used was a UV-160A (Shimadzu, Kyoto, Japan), and the quartz cells used were 1 cm (Starna Ltd., Essex, U.K.). All collected fractions were read on the UV spectrophotometer at 280 nm. If required, they were diluted in urea buffer (*25*). The first effluent from the column saved in the refrigerator was used as a spectrophotometric blank. According to the method of Andrews (*27*) with modifications, urea polyacrylamide gel electrophoresis (PAGE) gels were run to identify each protein and to verify the purity of the pooled fractions. The caseins were dissolved at 2.0% (w/v) in water under gentle magnetic stirring.

The protein concentrations in the solutions of the individual whey proteins and caseins were determined by UV spectroscopy using an Ultrospec II UV–vis spectrophotometer (Pharmacia LKB Biochrom, Cambridge, U.K.). The samples were measured in a 1 cm quartz cell (Starna Ltd., Essex, U.K.) at 280 nm against water as the reference. The α-la, β-lg, and BSA solutions were diluted 1:15, 1:5, and 1:5 with water, respectively. Both the α_{s1}- and β-casein solutions were diluted 1:40 and 1:20 in water. Protein concentrations were calculated using extinction coefficients (ϵ) of 29482, 17600, and 44200 L/(mol·cm) for α-la (*28*), β-lg (*29*), and BSA (*30*), respectively. For α_{s1}-casein and β-casein, the values of ϵ used were 23000 and 11086 L/(mol·cm), respectively. The absorbance at 320 nm was used to correct for light scattering due to any turbidity.

Preparation of Protein–Flavor Solutions. All protein solutions and the 2-nonanone stock solution were brought to room temperature before the mixtures were prepared. After the protein solutions had been stored for 6 h at 5 °C, they were mixed with the 2-nonanone stock solution to obtain final 2-nonanone concentrations of 0.1–0.8 mM. The protein–flavor mixtures were stored at 5 °C for 40 h to allow equilibration of 2-nonanone between the free state and the protein-bound state prior to the SPME analysis. Equilibration at 5 °C was tested up to 90 h and was found to be complete after 40 h.

Headspace SPME. The SPME holder for manual sampling and the SPME fibers, 30 μm polydimethylsiloxane (PDMS), were purchased from Supelco (Bellefonte, PA). The fibers were conditioned in the gas chromatograph injector port before use at the time and temperature recommended by the manufacturer. During the development of the headspace SPME method, the following parameters were optimized: type and thickness of fiber coating; headspace extraction time t_{ex}; and sample agitation during extraction.

For the 2-nonanone standards and the protein–flavor solutions, 400 μL aliquots were transferred into 4 mL glass vials (Alltech, Deerfield, IL). The standards and the samples containing individual whey proteins were prepared in triplicates, individual caseins in duplicates, sodium caseinate in four replicates, and WPI in five replicates. An 8 mm × 3 mm Azlon SWN 500 magnetic stir bar (Bibby Sterilin Ltd., Stone, U.K.) was placed in each sample vial. The sample vials were sealed with TFE silicone combination liners and open hole screw thread caps (Alltech, Deerfield, IL) and placed in a water bath equipped with a TE-10A thermostat (Techne, Cambridge, U.K.) to keep the temperature constant at 25 °C. The samples were stirred at 250 min^{-1} during the SPME extraction using an MR 2002 magnetic stirring plate (Heidolph, Schwabach, Germany) under the water bath. After equilibration, the SPME fiber was exposed into the headspace of the sample vial for 5 min and was subsequently introduced into the gas chromatograph injector port for quantification.

Gas Chromatography. A Shimadzu GC-17A gas chromatograph coupled with an FID detector (Shimadzu, Kyoto, Japan) was used throughout the study. The column used was a Supelcowax 10 fused silica capillary column, 30 m, 0.32 mm inner diameter, 0.50 μm film thickness (Supelco, Bellefonte, PA). The carrier gas was helium (linear velocity = 40 cm/s). Silicone rubber septa (Shimadzu, Kyoto, Japan) were used in the injector. The injector port (splitless mode) temperature and the detector temperature were 250 °C. The oven temperature was held isothermally at 120 °C. Data acquisition was achieved using Class-VP Chromatography Data System Software (Shimadzu, Columbia, MD).

Once the SPME sampling was completed, the fiber was immediately inserted into the gas chromatograph injector for desorption. The fiber was left in the port for 5 min for purging. There was no carry-over between samples using a 5 min desorption time. Prior to the next SPME extraction, the fiber was allowed to cool to room temperature.

Determination of Binding Parameters. For a protein P having an equal number of discrete and independent binding sites, the interaction between the ligand, that is, the flavor molecule L, and the protein may be represented by the equation

$$\mathrm{P} + n\mathrm{L} = \mathrm{PL}_n \tag{1}$$

On the basis of this model, the equilibrium between free and protein-bound flavor molecules can be represented thermodynamically by the Scatchard equation (*31*)

$$\frac{\nu}{[\mathrm{L}]} = nK - \nu K \tag{2}$$

where ν is the number of moles of 2-nonanone bound per mole of protein and [L] is the concentration of the free 2-nonanone (mol/L) in the sample solution. The binding constant (K) and the number of binding sites on the proteins (n) were determined using the double-reciprocal form of the Scatchard equation, the so-called Klotz plot (*32*):

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{nK[\mathrm{L}]} \tag{3}$$

The values of [L] were calculated from the FID peak area using the standard curve, and the values of ν were calculated using the equation

$$\nu = \frac{[\mathrm{L}]_{tot} - [\mathrm{L}]}{[\mathrm{P}]} \tag{4}$$

where $[\mathrm{L}]_{tot}$ is the total 2-nonanone concentration in the sample (free and bound) (mol/L) and [P] is the protein concentration in the sample as determined by UV spectroscopy (mol/L). For calculation of the average binding parameters, average molecular masses of 18000 and 22000 were assumed for WPI and sodium caseinate, respectively. For the individual milk proteins, the protein concentrations determined by UV spectroscopy were used.

The standard errors (SE) of K, n, and nK were estimated from the standard errors of the intercept $1/n$ ($1/n = a$) and the slope $1/nK$ ($1/nK = b$) of each Klotz plot, which were obtained using SPSS version 14.0 (SPSS, Chicago, IL). The variances (var) were estimated using eqs 5–7, and $\mathrm{SE} = \sqrt{\mathrm{var}}$

$$\mathrm{var}(n) \approx \frac{\mathrm{SE}_a^{\,2}}{a^4} \tag{5}$$

$$\mathrm{var}(K) \approx \left(\frac{a}{b}\right)^2 \left(\frac{\mathrm{SE}_a^{\,2}}{a^2} + \frac{\mathrm{SE}_b^{\,2}}{b^2} - \frac{2\cdot\mathrm{cov}(a,b)}{ab}\right) \tag{6}$$

$$\mathrm{var}(nK) \approx \frac{\mathrm{SE}_b^{\,2}}{b^4} \tag{7}$$

RESULTS AND DISCUSSION

Binding of 2-Nonanone to Milk Proteins. The binding of 2-nonanone to the major proteins in milk (β-lg, α-la, BSA, α_{s1}-casein, and β-casein) and to milk protein products (WPI and sodium caseinate) was studied using the optimized headspace SPME method followed by GC-FID for quantification of the free flavor.

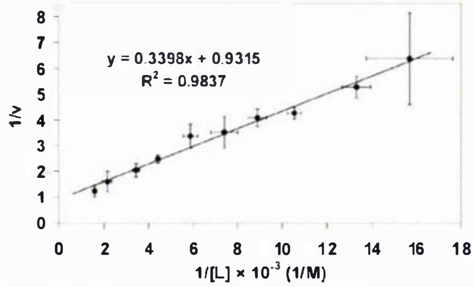


Figure 1. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to β-lg (0.5%; 0.24 mM) at 25 °C (each data point is the mean of triplicates).

β-Lg. The amount of 2-nonanone bound in 0.5% β-lg solution (0.24 mM, as determined by UV spectroscopy) varied between 22 and 36%, depending on the initial concentration of added 2-nonanone. The binding of 2-nonanone by β-lg was plotted as a Klotz plot (**Figure 1**). Linear regression resulted in binding parameters of $K = 2700\ (\pm 500)\ \mathrm{M}^{-1}$ and $n = 1.1\ (\pm 0.2)$. These values are in very good agreement with those obtained by O'Neill and Kinsella (*6*), who determined binding parameters for 2-nonanone and β-lg, using equilibrium dialysis, and found one binding site per monomer with $K = 2439\ \mathrm{M}^{-1}$. The primary binding site on β-lg for 2-nonanone is probably the hydrophobic pocket of the protein, which is also believed to serve as the binding site for retinol and fatty acids (*16, 17, 23, 33*).

The binding parameters reported in other studies vary considerably due mainly to the different methods or different protein batches used. Charles et al. (*20*), using static headspace analysis, investigated the binding of 2-nonanone and β-lg at pH 3, at which β-lg is predominantly present in monomeric form. They reported 0.2 binding site with $K = 6250\ \mathrm{M}^{-1}$ for 2-nonanone concentrations below 40 ppm and 0.5 binding site with $K = 1667\ \mathrm{M}^{-1}$ for 2-nonanone concentrations above 45 ppm. The lower binding could be attributed to the acidic pH because, at pH values lower than 7, there is a lid that closes off the hydrophobic calyx of β-lg (*34*). In addition, these authors added sodium azide (as a preservative), which has been shown to affect protein–flavor interactions (*11*). In contrast, Sostmann and Guichard (*13*), using affinity chromatography, determined a global binding constant (nK) of $3629\ \mathrm{M}^{-1}$ for 2-nonanone and β-lg at pH 3. In this case, the immobilization of β-lg may have led to conformational changes and a better accessibility of binding sites. Jasinski and Kilara (*9*) reported 14 binding sites for 2-nonanone on β-lg with an average K of $122\ \mathrm{M}^{-1}$. They appeared to have overestimated the number of binding sites and underestimated the binding constant. The large number of low-affinity binding sites could also have been due to the high 2-nonanone concentrations (>1 mM) used by these authors.

α-La. The binding of 2-nonanone in a 0.5% α-la solution (0.3 mM, as determined by UV spectroscopy) was found to be between 11 and 21%, considerably lower than that observed in a 0.5% β-lg solution. From the slope and intercept of the α-la Klotz plot (data not shown), it was calculated that there is one binding site on α-la with a medium affinity, $K = 900\ (\pm 500)\ \mathrm{M}^{-1}$, for 2-nonanone. The value of nK was >3 times lower than that of β-lg. Jasinski and Kilara (*9*) determined 33 binding sites and a K of $11\ \mathrm{M}^{-1}$ for 2-nonanone on α-la. However, as mentioned earlier for β-lg, these authors used very high 2-nonanone concentrations, which may have been the reason for the high number of low-affinity binding sites.

BSA. The amount of 2-nonanone bound in 0.5% BSA solution (0.065 mM, as determined by UV spectroscopy) varied between 33 and 60%, depending on the initial 2-nonanone concentration, and was considerably higher than that bound to β-lg or α-la under the same conditions. The Klotz plot for the binding of 2-nonanone to BSA is shown in **Figure 2**. The

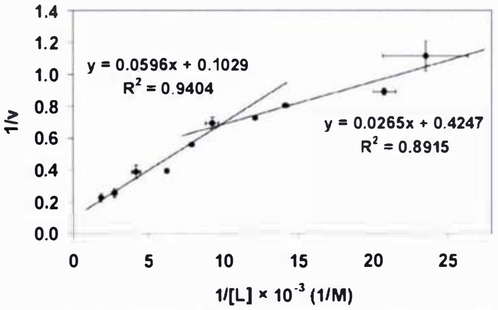


Figure 2. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to BSA (0.5%; 0.065 mM) at 25 °C, divided into low (0.1–0.2 mM) and high (0.2–0.8 mM) 2-nonanone concentrations (each data point is the mean of triplicates).

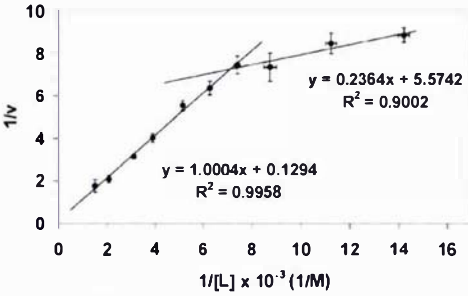


Figure 3. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to WPI (0.5%) at 25 °C (each data point is the mean of five replicates)

nonlinear shape indicates that different classes of binding sites are present on BSA. At low added 2-nonanone concentrations (0.1–0.2 mM), there were 2.4 (±0.5) binding sites with a binding constant of 16000 (±7000) M^{-1}. At higher added 2-nonanone concentrations (0.2–0.8 mM), 10 (±4) binding sites with an average binding constant for 2-nonanone of 1700 (±900) M^{-1} were found.

There are obviously two to three primary binding sites on BSA that have a very high affinity for 2-nonanone. Guth and Fritzler (*8*), using an ultracentrifugation technique, also suggested one or two high-affinity binding sites for long-chain γ- and δ-lactone flavors and a large number of binding sites with lower affinity. The number of binding sites found in this study is also in good agreement with the reported number of binding sites on BSA for other ligands, such as free fatty acids with three primary binding sites, three secondary binding sites, and several weaker binding sites (*35*). However, Damodaran and Kinsella (*1*) suggested that there are two classes of binding sites in BSA for 2-nonanone: the first six sites have higher affinities than the second class of binding sites. However, these authors used fairly high 2-nonanone concentrations.

Damodaran and Kinsella (*1*) found five to six initial binding sites for 2-nonanone on BSA, using liquid–liquid partitioning. The binding constant, K = 1800 M^{-1}, was similar to that of the secondary binding sites found in our study, K = 1700 (±900) M^{-1}. This might be due to different contents of fatty acids in the BSA products used. Damodaran and Kinsella (*1*) used BSA with 0.4 mol of fatty acids per mole of protein, whereas the product used in this work contained approximately 0.002% fatty acids, which equates to about 0.005 mol of fatty acids per mole of BSA, calculated on the basis of the molecular weight of oleic acid, the major fatty acid in milk fat. Probably, the primary, high-affinity binding sites of the BSA used by Damodaran and Kinsella (*1*) were to a greater extent occupied by fatty acids than those of the BSA used in this study, resulting in a lower binding constant. Using the pulsed field gradient NMR technique, Jung et al. (*10*) found seven binding sites for 2-nonanone and other methyl ketones on BSA, with an average binding constant for 2-nonanone of K = 833 (±15) M^{-1}. However, these seven binding sites may not be equivalent and may include primary and secondary binding sites, especially as high 2-nonanone/protein ratios were used. The occupation of secondary binding sites may be the reason for the low overall binding constant.

Caseins. The extent of 2-nonanone binding in 2.0% α_{s1}-casein and β-casein solutions ranged from 14 to 19% and from 11 to 17%, respectively, depending on the initial 2-nonanone concentration. At 0.5% casein concentration, the binding of 2-nonanone was <7% and there were no significant differences between α_{s1}-casein and β-casein (data not shown). For this reason, the caseins were used at 2.0%. The linear regression lines of the Klotz plots revealed 0.8 (±0.5) and 0.32 (±0.09) binding sites and binding constants of 420 (±280) and 810 (±250) M^{-1} for α_{s1}-casein and β-casein, respectively. Because of the low binding constants, saturation of the binding sites on both caseins was not achieved. The global binding constants (nK) were 330 (±10) and 240 (±10) M^{-1} for α_{s1}-casein and β-casein, respectively, indicating a higher binding capacity of α_{s1}-casein compared with β-casein. The values determined for the individual caseins in this study are only estimations because there was not a sufficient amount of the caseins available to obtain a larger number of data points. However, the higher global binding constant nK of α_{s1}-casein compared with β-casein is clearly shown.

Milk Protein Products. The amount of 2-nonanone bound by the milk protein products ranged from 17 to 30% and from 12 to 14% for WPI (0.5%) and sodium caseinate (1.0%), respectively, depending on the initial concentration of 2-nonanone. This finding was expected as Hansen and Booker (*18*) and Li et al. (*5*) came to the same conclusion, that is, that whey proteins bind flavor compounds to a greater extent than caseins.

The Klotz plot for the binding of 2-nonanone to WPI (0.5%) is shown in **Figure 3**. Its nonlinear shape suggests the presence of at least two groups of binding sites that are nonequivalent or that cooperativity between the binding sites of one or more proteins exists. At low added 2-nonanone concentrations (0.1–0.17 mM), there was 0.2 (±0.02) binding site with a binding constant of 24000 (±8000) M^{-1}. At high added 2-nonanone concentrations (0.17–0.8 mM), 2-nonanone bound on 8 (±8) binding sites with a very low average binding constant of 130 (±140) M^{-1}. The high standard errors are due to an intercept being close to zero and a high value of the slope. This change in binding parameters with 2-nonanone concentration may be due to the presence of the several binding sites with different binding constants for 2-nonanone on the constituent proteins in WPI. At low added 2-nonanone concentrations, 2-nonanone is probably bound on the binding sites with the highest affinity on one protein, possibly BSA. BSA was shown to contain two to three primary binding sites with a very high K of 16000 (±7000) M^{-1} (**Figure 2**). These sites are probably the first sites to be occupied in WPI. With increasing 2-nonanone concentration, the medium and lower affinity binding sites on other proteins, namely, β-lg and α-la, are filled.

The binding data for sodium caseinate were plotted as a Klotz plot (**Figure 4**). From the linear regression line of the plot, an average binding constant K of 370 (±370) M^{-1} and an average number of binding sites n of 1.1 (±1.0) for 2-nonanone on sodium caseinate were obtained. These parameters are in good

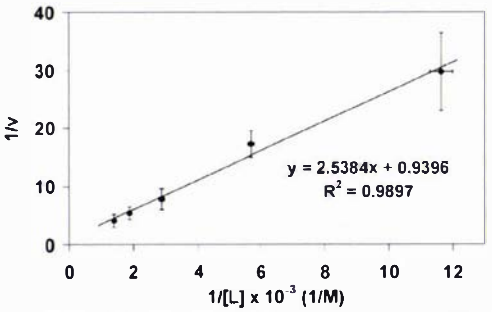


Figure 4. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to sodium caseinate (1.0%) at 25 °C (each data point is the mean of four replicates).

Table 1. Binding Parameters of 2-Nonanone with the Individual Milk Proteins and the Milk Protein Products WPI and Sodium Caseinate at 25 °C

protein	$c_{2\text{-nonanone}}$ (mM)	n	K (M^{-1})	nK (M^{-1})
whey proteins				
β-lg	0.1–0.8	1.1 ± 0.2	2700 ± 500	3000 ± 100
α-la	0.1–0.8	1.0 ± 0.5	900 ± 500	860 ± 60
BSA	0.1–0.2	2.4 ± 0.5	16000 ± 7000	38000 ± 8000
	0.2–0.8	10 ± 4	1700 ± 900	17000 ± 2000
WPI	0.1–0.17	0.2 ± 0.02	24000 ± 8000	5000 ± 1000
	0.17–0.8	8 ± 8	130 ± 140	1000 ± 30
caseins				
α_{s1}-casein	0.1–0.8	0.8 ± 0.5	420 ± 280	330 ± 10
β-casein	0.1–0.8	0.32 ± 0.09	810 ± 250	240 ± 10
sodium caseinate	0.1–0.8	1.1 ± 1.0	370 ± 370	410 ± 20

agreement with the values determined for α_{s1}-casein and β-casein and 2-nonanone. The only other study reporting binding parameters for sodium caseinate and a flavor compound was by Li et al. (*5*), who found 0.66 binding site with a binding constant of 353 M^{-1} for vanillin on sodium caseinate (12 °C). To date, studies indicate low binding capacities of caseins and caseinates for different flavor compounds.

The *n* and *K* values for the binding of 2-nonanone to the individual whey proteins determined in this study are summarized in **Table 1**. Differences in the binding parameters between WPI and sodium caseinate are probably due to the different structures and amino acid compositions of the individual proteins in WPI and sodium caseinate; that is, β-lg, the main constituent protein in WPI, is known to have a structure that can accommodate different small hydrophobic molecules with high affinity (*6*, *15*). None of the caseins have been reported to have a high affinity for any flavor molecules.

Comparison of the 2-nonanone binding of the main whey proteins shows that the binding of BSA and 2-nonanone was highest followed by that of β-lg. The lowest binding was observed for α-la. In contrast to β-lg and α-la, both of which bind one molecule of 2-nonanone per molecule of protein, BSA is able to bind two to three 2-nonanone molecules per mole of BSA on high-affinity binding sites and several 2-nonanone molecules on lower affinity binding sites. Considering the high proportion of β-lg (>60%) compared with BSA (approximately 2%) in WPI, the binding parameters determined indicate that β-lg is the whey protein that is mainly responsible for 2-nonanone binding in WPI. The caseins showed considerably lower binding capacities, with only one low-affinity binding site for 2-nonanone, compared with the whey proteins.

The good agreement between the binding parameters determined in this study and the values obtained by other authors shows that headspace SPME is an excellent technique for research on protein–flavor interactions. It is fast, sensitive, precise, inexpensive, and solvent free. In addition, not only liquid but also semisolid and solid samples can be analyzed using headspace SPME.

This study has demonstrated that the whey protein with the highest affinity for 2-nonanone is BSA, followed by β-lg, and that α-la shows the weakest binding. Both α-la and β-lg possess one binding site per protein molecule, whereas BSA can bind 2-nonanone on two classes of binding sites, resulting in a very high global binding constant for 2-nonanone. WPI was found to have a much higher affinity for 2-nonanone than sodium caseinate, and they both possess around one binding site per protein molecule on average. α_{s1}-Casein was found to have a slightly higher affinity for 2-nonanone than β-casein, although β-casein is the more hydrophobic of the two proteins, indicating that other than hydrophobic forces may be involved. The overall binding on both caseins is low compared with that on the whey proteins, with estimated global binding affinities *nK* of 330 (±10) and 240 (±10) M^{-1} for α_{s1}-casein and β-casein, respectively.

The presence of milk proteins may therefore cause the aroma profile of foods, in particular fat-free foods, to become unbalanced because of the binding of particular flavor compounds by the proteins. In particular, the whey proteins may prevent hydrophobic flavor compounds, such as 2-nonanone, from being released and perceived during mastication. In this case, the amount of certain flavor compounds added will have to be increased to compensate for the protein-bound flavor molecules.

Milk proteins added to food products are often denatured to various extents, which may affect binding constants and binding sites because of conformational changes of the proteins. In addition, during the processing of foods, for example, heat treatment, proteins are denatured. A study on the effect of denaturation on the binding of different flavor compounds and whey proteins is underway.

LITERATURE CITED

(1) Damodaran, S.; Kinsella, J. E. Flavor protein interactions—binding of carbonyls to bovine serum albumin—thermodynamic and conformational effects. *J. Agric. Food Chem.* **1980**, *28*, 567–571.

(2) Lübke, M.; Guichard, E.; Tromelin, A.; Le Quéré, J. L. Nuclear magnetic resonance spectroscopic study of β-lactoglobulin interactions with two flavor compounds, γ-decalactone and β-ionone. *J. Agric. Food Chem.* **2002**, *50*, 7094–7099.

(3) Hatchwell, L. C. Implications of fat on flavor. In *Flavor–Food Interactions*; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washington, DC, 1996; pp 14–23.

(4) Mills, O. E.; Solms, J. Interaction of selected flavor compounds with whey proteins. *Lebensm.-Wiss. Technol.* **1984**, *17*, 331–335.

(5) Li, Z.; Grün, I. U.; Fernando, L. N. Interaction of vanillin with soy and dairy proteins in aqueous model systems: a thermodynamic study. *J. Food Sci.* **2000**, *65*, 997–1001.

(6) O'Neill, T. E.; Kinsella, J. E. Binding of alkanone flavors to β-lactoglobulin—effects of conformational and chemical modification. *J. Agric. Food Chem.* **1987**, *35*, 770–774.

(7) Guichard, E.; Langourieux, S. Interactions between β-lactoglobulin and flavour compounds. *Food Chem.* **2000**, *71*, 301–308.

(8) Guth, H.; Fritzler, R. Binding studies and computer-aided modelling of macromolecule/odorant interactions. *Chem. Biodiversity* **2004**, *1*, 2001–2023.

(9) Jasinski, E.; Kilara, A. Flavor binding by whey proteins. *Milchwissschaft* **1985**, *40*, 596–599.

(10) Jung, D. M.; de Ropp, J. S.; Ebeler, S. E. Application of pulsed field gradient NMR techniques for investigating binding of flavor compounds to macromolecules. *J. Agric. Food Chem.* **2002**, *50*, 4262–4269.

(11) Reiners, J.; Nicklaus, S.; Guichard, E. Interactions between β-lactoglobulin and flavour compounds of different chemical classes. Impact of the protein on the odour perception of vanillin and eugenol. *Lait* **2000**, *80*, 347–360.

(12) Jouenne, E.; Crouzet, J. Effect of pH on retention of aroma compounds by β-lactoglobulin. *J. Agric. Food Chem.* **2000**, *48*, 1273–1277.

(13) Sostmann, K.; Guichard, E. Immobilized β-lactoglobulin on a HPLC-column: a rapid way to determine protein-flavour interactions. *Food Chem.* **1998**, *62*, 509–513.

(14) Fugate, R. D.; Song, P. S. Spectroscopic characterization of β-lactoglobulin-retinol complex. *Biochim. Biophys. Acta* **1980**, *625*, 28–42.

(15) Spector, A. A.; Fletcher, J. E. Binding of long chain fatty acids to β-lactoglobulin. *Lipids* **1970**, *5*, 403–411.

(16) Wu, S. Y.; Pérez, M. D.; Puyol, P.; Sawyer, L. β-Lactoglobulin binds palmitate within its central cavity. *J. Biol. Chem.* **1999**, *274*, 170–174.

(17) Ragona, L.; Fogolari, F.; Zetta, L.; Perez, D. M.; Puyol, P.; de Kruif, K.; Lohr, F.; Ruterjans, H.; Molinari, H. Bovine β-lactoglobulin: interaction studies with palmitic acid. *Protein Sci.* **2000**, *9*, 1347–1356.

(18) Hansen, A. P.; Booker, D. C. Flavor interaction with casein and whey protein. In *Flavor–Food Interactions*; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washington, DC, 1996; pp 75–89.

(19) Jung, D. M.; Ebeler, S. E. Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. *J. Agric. Food Chem.* **2003**, *51*, 200–205.

(20) Charles, M.; Bernal, B.; Guichard, E. Interaction of β-lactoglobulin with flavour compounds. In *Flavour Science. Recent Developments*; Taylor, A. J., Mottram, D. S., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1996; pp 433–436.

(21) Liu, X.; Powers, J. R.; Swanson, B. G.; Hill, H. H.; Clark, S. High hydrostatic pressure affects flavor-binding properties of whey protein concentrate. *J. Food Sci.* **2005**, *70*, C581–C585.

(22) Fabre, M.; Aubry, V.; Guichard, E. Comparison of different methods: static and dynamic headspace and solid-phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. *J. Agric. Food Chem.* **2002**, *50*, 1497–1501.

(23) Lübke, M.; Guichard, E.; Le Quéré, J. L. Infrared spectroscopic study of β-lactoglobulin interactions with flavor compounds. In *Flavor Release*; Roberts, D. D., Taylor, A. J., Eds.; American Chemical Society: Washington, DC, 2000; pp 282–292.

(24) Thompson, M. P. DEAE-cellulose-urea chromatography of casein in the presence of 2-mercaptoethanol. *J. Dairy Sci.* **1966**, *49*, 792–795.

(25) Lawrence, R. C.; Creamer, L. K. The action of calf rennet and other proteolytic enzymes on κ-casein. *J. Dairy Res.* **1969**, *36*, 11–20.

(26) Creamer, L. K. Preparation of α_{s1}-casein A. *J. Dairy Sci.* **1974**, *57*, 341–344.

(27) Andrews, A. T. Proteinases in normal bovine-milk and their action on caseins. *J. Dairy Res.* **1983**, *50*, 45–55.

(28) McKenzie, H. A.; Murphy, W. H. General methods and elemental analysis. In *Milk Proteins, Chemistry and Molecular Biology*; McKenzie, H. A., Ed.; Academic Press: New York, 1970; pp 127–180.

(29) Dufour, E.; Marden, M. C.; Haertlé, T. β-Lactoglobulin binds retinol and protoporphyrin-IX at two different binding-sites. *FEBS Lett.* **1990**, *277*, 223–226.

(30) Foster, J. F.; Sterman, M. D. Conformation changes in bovine plasma albumin associated with hydrogen ion and urea binding. II. Hydrogen ion titration curves. *J. Am. Chem. Soc.* **1956**, *78*, 3656–3660.

(31) Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660–672.

(32) Klotz, I. M.; Walker, F. M.; Pivan, R. B. The binding of organic ions by proteins. *J. Am. Chem. Soc.* **1946**, *68*, 1486–1490.

(33) Cho, Y. J.; Batt, C. A.; Sawyer, L. Probing the retinol-binding site of bovine β-lactoglobulin. *J. Biol. Chem.* **1994**, *269*, 11102–11107.

(34) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. Structural basis of the Tanford transition of bovine β-lactoglobulin. *Biochemistry* **1998**, *37*, 14014–14023.

(35) Spector, A. A. Fatty acid binding to plasma albumin. *J. Lipid Res.* **1975**, *16*, 165–179.

Received for review December 5, 2006. Revised manuscript received February 26, 2007. Accepted March 10, 2007.

JF063517O