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Binding of 2-Nonanone and Milk Proteins in Aqueous Model Systems

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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

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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

$$P + nL = PL_n \quad (1)$$

On the basis of this model, the equilibrium between free and protein-bound flavor molecules can be represented thermodynamically by the Scatchard equation (*31*)

$$\frac{\nu}{[L]} = nK - \nu K \quad (2)$$

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*):

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{nK[L]} \quad (3)$$

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

$$\nu = \frac{[L]_{tot} - [L]}{[P]} \quad (4)$$

where $[L]_{tot}$ 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 $SE = \sqrt{var}$.

$$var(n) \approx \frac{SE_a^{\,2}}{a^4} \quad (5)$$

$$var(K) \approx \left(\frac{a}{b}\right)^2 \left(\frac{SE_a^{\,2}}{a^2} + \frac{SE_b^{\,2}}{b^2} - \frac{2 \cdot cov(a,b)}{ab}\right) \quad (6)$$

$$var(nK) \approx \frac{SE_b^{\,2}}{b^4} \quad (7)$$

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.

***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 $K = 2700$ (±500) M^{-1} and $n = 1.1$ (±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 $K = 2439$ 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*).

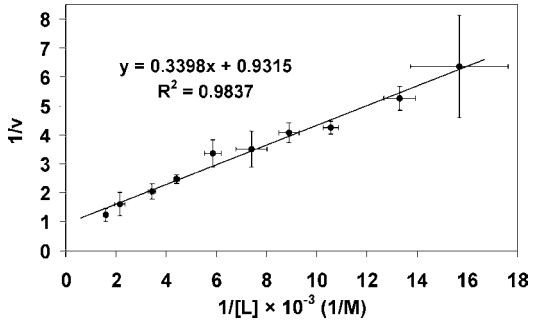

Figure 1. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to β-lg (0.5%; 0.24 mM) at 25 °C (each data point is the mean of triplicates).

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 $K = 6250$ M^{-1} for 2-nonanone concentrations below 40 ppm and 0.5 binding site with $K = 1667$ 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 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 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, $K = 900$ (±500) 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 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

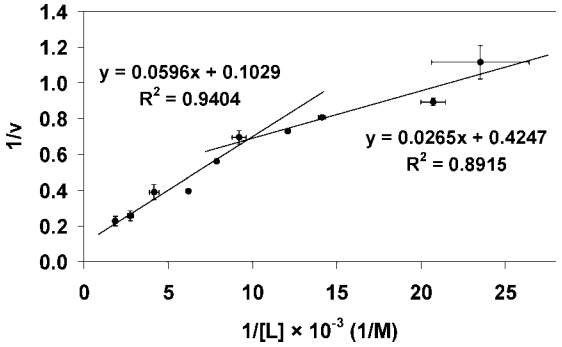

Figure 2. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to BSA (0.5%; 0.065 mM) at 25 °C, divided into low (0.1–0.2 mM) and high (0.2–0.8 mM) 2-nonanone concentrations (each data point is the mean of triplicates).

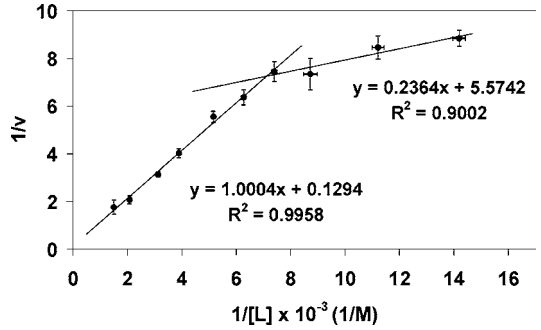

Figure 3. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to WPI (0.5%) at 25 °C (each data point is the mean of five replicates).

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

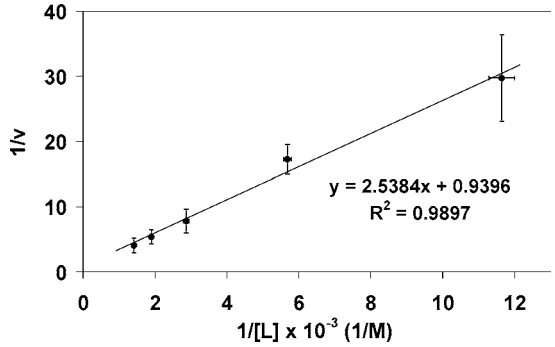

Figure 4. Klotz plot for the binding of 2-nonanone (0.1–0.8 mM) to sodium caseinate (1.0%) at 25 °C (each data point is the mean of four replicates).

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.

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