JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

FTIR Spectra of Whey and Casein Hydrolysates in Relation to Their Functional Properties

CORNELLY VAN DER VEN,[†,‖] SOREL MURESAN,[‡,⊥] HARRY GRUPPEN,[§]
DRIES B. A. DE BONT,[†] KARIN B. MERCK,[‡] AND ALPHONS G. J. VORAGEN*,[§]

Numico-Research, P.O. Box 7005, 6700 CA Wageningen, The Netherlands, Agrotechnological
Research Institute ATO, P.O. Box 17, 6700 AA Wageningen, The Netherlands, and Wageningen
University, Department of Agrotechnology and Food Sciences, Laboratory of Food Chemistry,
P.O. Box 8129, 6700 EV Wageningen, The Netherlands

Mid-infrared spectra of whey and casein hydrolysates were recorded using Fourier transform infrared (FTIR) spectroscopy. Multivariate data analysis techniques were used to investigate the capacity of FTIR spectra to classify hydrolysates and to study the ability of the spectra to predict bitterness, solubility, emulsifying, and foaming properties of hydrolysates. Principal component analysis revealed that hydrolysates prepared from different protein sources or with different classes of proteolytic enzymes are distinguished effectively on basis of their FTIR spectra. Moreover, multivariate regression analysis showed satisfactory to good prediction of functional parameters; the coefficient of determination (R^2) varied from 0.60 to 0.92. The accurate prediction of bitterness and emulsion forming ability of hydrolysates by using only one uncomplicated and rapid analytical method has not been reported before. FTIR spectra in combination with multivariate data analysis proved to be valuable in protein hydrolysate fingerprinting and can be used as an alternative for laborious functionality measurements.

KEYWORDS: FTIR spectroscopy; protein hydrolysates; milk proteins; principal component analysis; multivariate regression; bitterness; emulsion; foam; functional properties

INTRODUCTION

Proteins are nutritionally important as a source of nitrogen and essential amino acids. For several purposes, it is beneficial to hydrolyze proteins, for example, to reduce allergenicity (*1, 2*), to achieve specific dietary requirements (*3−6*), or to improve functional properties (*7, 8*). Hydrolysis of proteins leads to numerous alterations in protein functional characteristics, like changes in solubility, viscosity, taste, emulsion and foam forming, and emulsion and foam stability (*8*). These functional parameters are important for application of hydrolysates in food products. Their determination is laborious; hence, substitution of functionality measurements by easy, fast analytical methods is of great interest, both for product development and for development of fast and accurate quality control applications.

Therefore, research concerning correlations between analytical parameters and functional properties is desirable.

Protein hydrolysates are often characterized by their degree of hydrolysis (DH) and the molecular weight distribution (MWD) of the constituent peptides, the latter usually being determined using size exclusion chromatography. For whey and casein hydrolysates, we showed that molecular weight distribution is correlated to emulsion stability of both casein and whey hydrolysate emulsions, to foam-forming properties of whey hydrolysates, and to foam stability of casein hydrolysate foams. Emulsion-forming properties, however, could not be related to MWD (*9, 10*).

Another important quality parameter for protein hydrolysates is their bitterness, which is known to originate from certain peptides formed during proteolysis (for reviews about bitter peptides see (*11−14*)). Determination of perceived bitterness is laborious, since sensory panels are needed. Reversed phase chromatography, that principally separates peptides on the basis of their hydrophobicity, is often used to identify peptides that cause bitterness of hydrolysates (*15−17*). However, bitterness of hydrolysates cannot be predicted adequately from reversed phase chromatography or other analytical methods.

* Corresponding author. E-mail: fons.voragen@chem.fdsci.wag-ur.nl. Tel: +31 317 483209. Fax: +31 317 484893.
† Numico-Research.
‡ Agrotechnological Research Institute.
‖ Current address: Agrotechnological Research Institute ATO, P.O. Box 17, 6700 AA Wageningen, The Netherlands.
§ Wageningen University.
⊥ Current address: AstraZeneca R&D Mölndal, EST Lead Informatics, Sweden.

Next to chromatographic methods, spectroscopic methods such as fluorescence or infrared (IR) spectroscopy are currently used to characterize food and food-related products. Infrared spectroscopy is based on the absorption of radiation due to vibrations between atoms in a molecule and, therefore, provides information about the chemical composition and conformational structure of food components (*18*). The fingerprint region of the IR spectrum, which is the region from 1800 to 800 cm^{-1}, is often a very useful part for analysis of proteinaceous material (*19*), since in this range the bonds forming the amide group (C=O, N−H, and C−N) absorb. The two most important vibrational modes of amides are the amide I vibration, caused primarily by the stretching of the C=O bonds, and the amide II vibration, caused by deformation of the N−H bonds and stretching of the C−N bonds. The amide I vibration is measured in the range from 1700 to 1600 cm^{-1} and the amide II region from 1600 to 1500 cm^{-1} (*20*). The exact frequencies at which these bonds absorb depend on the secondary structure of the proteins or peptides (*20, 21*).

For protein research, infrared spectroscopy has been applied to study secondary structure of proteins (both in solid state and in solution) (*21−24*) and for qualitative or quantitative determination of proteins in mixtures (*25−27*). Because IR spectra include information regarding (secondary) structure of peptides combined with information about functional groups, it is interesting to investigate its suitability for characterization of protein hydrolysates.

As protein hydrolysates are mixtures of peptides, it will be very difficult, if not impossible, to identify specific peptides. However, to describe hydrolysates and to study correlations between IR spectra and functional properties, it is not necessary to elucidate the exact composition of the hydrolysates; the hydrolysate can be considered as a "black box" system, characterized by the IR spectrum. The relation between the spectra and the functional properties can be studied with multivariate statistical analysis, which has been proven to be a powerful tool in investigating such relationships (*26, 28−32*).

In the present study, FTIR spectra of casein and whey hydrolysates were recorded and were correlated to bitterness, solubility, and emulsion and foam properties, using multivariate regression analysis to investigate whether FTIR spectra can substitute laborious functional tests.

MATERIALS AND METHODS

Materials. Sodium caseinate (89% protein based on dry weight) was obtained from DMV International (Veghel, The Netherlands), whey protein (WPC 60) from Milei GmbH (Stuttgart, Germany).

Production of Hydrolysates. Whey and casein hydrolysates were produced as described previously (*33*). Protein suspensions or solutions (5% (w/w) protein) were hydrolyzed in a pH-stat setup using eleven different enzymes, under conditions as given in **Table 1**. Samples were taken at 1/3, 2/3, and at the maximum degree of hydrolysis. Enzymes were inactivated by heating 15 min at 90 °C. The hydrolysate was centrifuged (30 min, 3000xg, 20 °C) at the pH of hydrolysis. Supernatant and pellet were separated. Supernatant was freeze-dried and used for further studies. Sample codes are subsequently composed of two digits for protein source, three digits representing the enzyme used, and two digits encoding the degree of hydrolysis reached, for example, CnNwf06: casein, Newlase F, DH = 6%. Protein and enzyme codes are given in **Table 1**. Enzymes were denoted "acidic", "neutral", or "alkaline", according to the pH at which they are active, respectively, acidic pH (pH 3), neutral pH (pH 5−7), and alkaline pH (pH 8). In total, 33 casein and 33 whey hydrolysates were produced.

FTIR Spectroscopy. The method of choice for IR analysis is the Fourier transform infrared (FTIR) technique, which is the most commonly used method for mid-infrared spectroscopy (*34*). Spectro-

Table 1. Hydrolysis Conditions

enzyme	pH	temp (°C)	E/S[a] (%)	
			casein (Cn[b])	whey (Wc[b])
pepsin (Pep)[c]	3	50	5	5
newlase F (NwF)	3	50	1	4
validase FP (VfP)	3	50	5	5
promod 258 (P58)	5.5/7[d]	45	3	3
promod 184 (Brm)	6/7[d]	50	1	3
flavorzyme (Flz)	6/7[d]	50	1	5
corolase L10 (Cl1)	6.5	60	3	3
protex 6L (P6L)	8	60	1	3
alcalase (Alc)	8	60	1	3
corolase PP (CPP)	8	50	1	3
pem (Pem)	8	45	0.5	2

[a] E/S = enzyme-to-substrate ratio in % w/w. [b] Abbreviation of protein, used in sample codes of hydrolysates. Whey protein was pretreated at 90 °C during 15 min prior to hydrolysis. [c] Abbreviation of enzyme, used in sample codes of hydrolysates. [d] Whey protein hydrolysis was performed at pH 7.

scopic measurements were performed using approximately 25 mg freeze-dried hydrolysate supernatant mixed with 225 mg dried KBr (10% w/w). The FTIR spectra between 1800 and 800 cm^{-1} were recorded using a BioRad FTS-60A FTIR spectrometer using the DRIFT (diffuse reflectance) mode. The interferometer as well as the chamber that housed the detector were purged with dry nitrogen to remove spectral interference resulting from water vapor and carbon dioxide. All spectra were obtained at room temperature at a resolution of 8 cm^{-1}, and 64 interferograms were co-added for a high signal-to-noise ratio. Triangular apodization was employed. The single-beam spectrum of KBr was subtracted from the single-beam spectrum of each protein hydrolysate to obtain the desired spectrum. All experiments were performed in duplicate. Prior to data analysis, the spectra were baseline corrected (two points, 1801.5, 778.4 cm^{-1}) and normalized (mean normalization option).

Protein Determination. Protein concentration was measured by determination of total nitrogen on an N-analyzer (NA 2100 Protein, CE instruments). For calculation of protein concentration, a Kjeldahl factor of 6.38 was used (*7*).

Solubility. Solubility was expressed as the amount of supernatant protein relative to the total protein content before hydrolysis (% w/w).

Emulsion Forming and Stability. Emulsion forming and emulsion stability of 22 casein and 22 whey hydrolysates, randomly selected from the 33 hydrolysates from each protein source, were measured as previously described (*9*). In summary, 18 mL of 0.56% (w/v) supernatant protein in 0.02 M imidazole/HCl buffer pH 6.7 with 3.44 g/L NaCl was emulsified with 2 mL of Tricaprylin oil using a small-scale homogenizer. Emulsion forming was measured by determination of the volume-surface average particle diameter (d_{32} value) using a Malvern Mastersizer. Emulsion instability was followed for 24 h, by measuring the turbidity change according to the method of Pearce and Kinsella (*35*). For statistical analysis, emulsion stability was expressed as remaining turbidity after 24 h ($T_{24}/T_0*100\%$).

Foam Forming and Stability. Foam properties were measured as previously described (*10*), using the same hydrolysates as used for determination of emulsion properties. In summary, foam was prepared with a 0.05% (w/v) supernatant protein solution in 0.02 M imidazole/HCl buffer pH 6.7 containing 3.44 g/L NaCl with a whipping method as described by Caessens et al. (*36*). Foam-forming ability was defined as the foam volume present two min after starting whipping. Foam stability was expressed as the proportion remaining foam after 60 min relative to foam volume at $t = 2$ min ($Vf_{60}/Vf_2*100\%$).

Sensory Analysis of Bitterness. For assessment of bitterness, the supernatant protein solutions were used. Solutions of 5% (w/v) whey hydrolysate or 2% (w/v) casein hydrolysate were prepared. A trained sensory panel, consisting of seven members, first tasted four concentrations of a standard caffeine solution (0.03, 0.07, 0.11, 0.15% (w/v)) in random order. Subsequently, randomized hydrolysate solutions were judged on bitterness intensity on a five-point scale, ranging from not bitter at all (score 0) to extremely bitter (score 5). For statistical analysis,

FTIR Spectra of Whey and Casein Hydrolysates

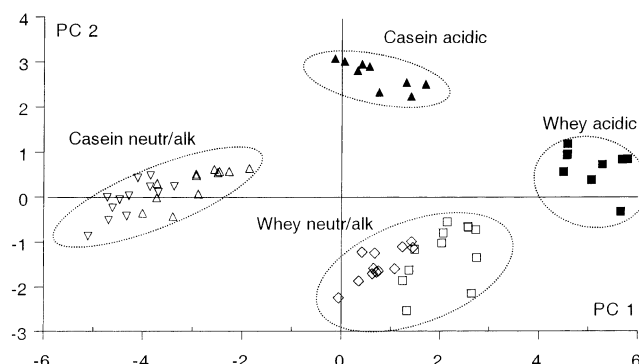*J. Agric. Food Chem.,* Vol. 50, No. 24, 2002   **6945**
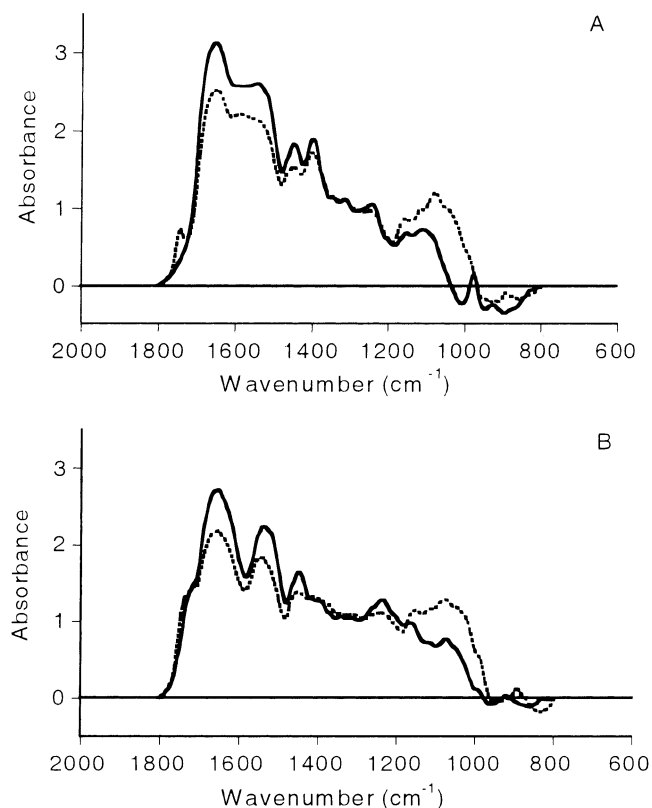
Figure 1. (A+B). Normalized FTIR spectra of casein (Cn, solid line) and whey (Wc, broken line) hydrolysates, representing hydrolysates made with neutral/ alkaline enzymes (1A) and hydrolysates produced with acidic enzymes (1B). 1A: hydrolysates from Alcalase, CnAlc14 (–), WcAlc13 (---). 1B: hydrolysates from Pepsin, CnPep06 (–), WcPep06 (---).

Figure 2. Scores of whey and casein hydrolysates on the first two principal components (PC 1 and 2) obtained from principal component analysis of their FTIR spectra. Symbols: casein hydrolysates produced with acidic enzymes (▲), with neutral enzymes (△), and with alkaline enzymes (▽). Whey hydrolysates produced with acidic enzymes (■), neutral enzymes (□), and alkaline enzymes (◇). Groups are defined by source of protein (whey/casein) and classes of enzymes: acidic or neutral and alkaline enzymes (neutr/alk).

the bitterness scores given by the seven panelists were averaged. Whey and casein hydrolysates were tested in separated taste sessions, each session contained a maximum of six different hydrolysates. In total, 12 taste sessions were performed. The standard deviation over average scores of caffeine samples over all 12 taste sessions varied from 0.18 to 0.29, depending on the caffeine concentration.

Statistical Analysis. Principal component analysis (PCA) and partial least-square (PLS) techniques were used to study the FTIR spectra $(1800-800 \text{ cm}^{-1})$ and to study the correlation between the spectra and the functional parameters, using the software package The Unscrambler (version 7.01, CAMO, Trondheim, Norway). In regression analysis (using partial least-squares regression), absorbency values were used as *x*-variables and the functional properties as *y*-variables. The optimum number of principal components (PC) is determined at the point where the residual validation variance reaches its first minimum. The models were validated using leave-one-out cross validation.

RESULTS AND DISCUSSION

Examples of FTIR spectra $(1800-800 \text{ cm}^{-1})$ representing differences in hydrolysates prepared from the two protein sources and two classes of enzymes are shown in **Figure 1**. **Figure 1A** shows spectra of whey and casein hydrolysates made with Alcalase, as representatives for hydrolysates made with neutral or alkaline enzymes. In **Figure 1B**, spectra of pepsin hydrolysates of whey and casein are shown as representatives of hydrolysates made with acidic enzymes. Comparison of FTIR spectra of whey and casein hydrolysates revealed that absorbencies of whey hydrolysates compared to that of casein hydrolysates are lower in the range of $1700-1485 \text{ cm}^{-1}$ and higher from 1170 to 980 cm^{-1}. In addition, the spectra of whey and casein hydrolysates from alkaline and neutral enzymes (**Figure 1A**) differ in their absorbencies around 1744 cm^{-1}.

In literature, analysis of protein hydrolysates by FTIR spectroscopy has not been described. Only some studies on FTIR spectra of whey or casein proteins, concerning effects of environmental conditions (pH, temperature, electrolyte balance) on secondary structure, studied by changes of the amide I band, were reported (*20, 23, 37, 38*). Comparison of our FTIR spectra of hydrolysates with results concerning proteins presented in the literature is difficult, since the protein source is not exactly the same, the measurement conditions differ, and data handling of the spectra differs. Spectra of whole proteins were mostly measured in solution, while we measured FTIR spectra in KBr pellets. There is not much known about the consequences of drying on the structure of proteins or peptides. One report was published on the secondary structure of globular proteins in KBr pellets, concluding that the secondary structure was highly conserved upon drying (*24*). However, it is not known whether drying affects the secondary structure of peptides.

Principal Component Analysis. The first step in statistical data analysis of FTIR spectra was principal component analysis (PCA), used to investigate whether FTIR spectra describe differences in protein hydrolysates properly and whether classes of samples can be distinguished on the basis of these spectra.
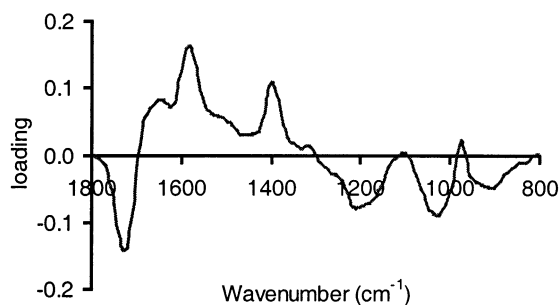
The principal component analysis resulted in a model in which the two first principal components explain 95% of the total spectral variation. PC 1 and PC 2 account for 79% and 16%, respectively. A plot of PC 1 versus PC 2 (**Figure 2**) shows the distribution of the samples on these new, independent variables.

PCA revealed the existence of four clusters, which are defined by protein source, casein or whey, and enzyme source, acidic or neutral/alkaline (**Figure 2**). To assess the influence of protein source and enzyme source separately, PCA was applied to different sets of samples.

The plot depicted in **Figure 3A** represents the result of a PCA upon casein hydrolysates only ($N = 33$). Again, a clear separation between the acidic and the neutral/alkaline samples according to the first PC (explained variance 92%) can be noticed. To identify the influence of protein source, PCA was applied to 48 samples obtained with neutral/alkaline enzymes. The result is presented in **Figure 3B**. A very clear separation of the hydrolysates according to the protein source is obtained. The first PC explains 91% of the data variation.
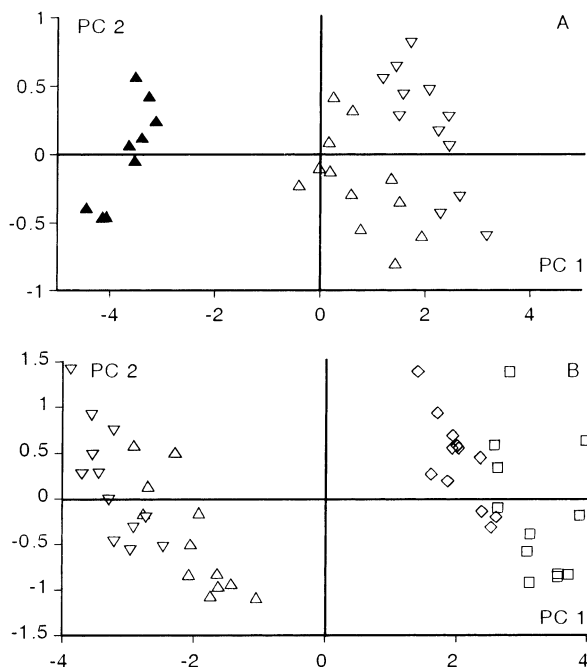
Figure 3. (A+B). Scores of whey and casein hydrolysate samples on the two first PCs obtained from principal component analysis of FTIR spectra of 33 casein hydrolysates (3A) and of 24 whey and 24 casein hydrolysates prepared with neutral and alkaline enzymes (3B). Symbols for casein hydrolysates: acidic (▲), neutral (△), and alkaline enzyme hydrolysates (▽). Symbols for whey hydrolysates: neutral (□) and alkaline enzyme hydrolysates (◇).

Figure 4. Loading plot of first PC obtained after principal component analysis of FTIR spectra of casein hydrolysates.

Principal components are vectors described by linear combinations of the original variables, in our case the absorbencies measured in the infrared spectrum. Loading plots provide information about the contribution of each original variable to a principal component. Variables with high loading weights are responsible for the main variation in the data described by the particular PC. In **Figure 4**, the loading plot of the first PC obtained from the analysis with only casein hydrolysates (**Figure 3A**) is depicted. From the loading plot, the spectral regions responsible for the differences between the acidic and neutral/alkaline casein hydrolysates can be readily identified. Differences between FTIR spectra of these classes of hydrolysates were mainly observed between 1743 and 1705 cm^{-1}, around 1585 cm^{-1}, and around 1400 cm^{-1}. These frequencies are associated to carboxylate ion stretching vibrations (1650–1550, near 1400 cm^{-1}) (*38, 39*), carboxylic acid dimers (1720–1700 cm^{-1}) (*18, 39*), and monomers of saturated aliphatic acids (near 1760 cm^{-1}) (*39*). Therefore, differences in the spectra might be related to the presence of ionized or nonionized carboxyl groups caused by different pH values of hydrolysates.

To elucidate whether pH differences of protein hydrolysates are responsible for differences in FTIR spectra from hydrolysates of the different enzyme classes, some samples prepared with acidic enzymes were neutralized with sodium hydroxide after enzyme treatment and were lyophilized. FTIR analyses showed little or no difference with the samples measured before, demonstrating that differences as observed in **Figure 3A** are not caused by pH differences.

From the PCA with FTIR spectra, it can be concluded that the infrared spectra effectively differentiate between hydrolysates made from different protein sources and enzyme classes. Previously, size exclusion chromatography (SEC) and reversed-phase chromatography (RPC) have been used to characterize the 33 whey and 33 casein hydrolysates (*33*). PCA with these chromatograms did not result in such a clear grouping of the hydrolysates as is found now. From these observations, it can be concluded that, so far, FTIR spectroscopy is the only characterization method that can distinguish fully between hydrolysates made from different protein sources and different classes of enzymes.

Prediction of Functional Properties from FTIR Spectra. As FTIR spectroscopy is capable of describing differences between protein hydrolysates effectively, it is interesting to investigate whether the FTIR spectra can be related to functional properties of these hydrolysates. For this purpose, multivariate regression analysis (PLS regression) was performed.

PCA based on FTIR data revealed differences between samples according to protein and enzyme source. Therefore, regression analysis was performed for six different sample sets, categorized on basis of protein and enzyme source. Sample set 1 contains whey hydrolysates prepared with all 11 different enzymes, sample set 2 contains casein hydrolysates from all 11 enzymes, and sample set 3 contains all samples from set 1 and 2. Sample set 4 contains whey hydrolysates produced with neutral and alkaline enzymes (eight enzymes), sample set 5 contains casein hydrolysates from those eight enzymes, and sample set 6 contains all samples from sets 4 and 5. The number of samples used for regression analysis depends on the number of samples for which the functionality is determined (all samples for bitterness and solubility, a random selection of samples for emulsion and foam properties). Outliers (influential samples due to high leverage or high residuals) were removed to obtain optimal regression models. In **Table 2**, the minimum and maximum values for each functional property, regarding the samples included in the regression analysis, are presented as well as the results of the regression analyses. In the following, the results will be separately discussed for the various functional properties of the hydrolysates.

Bitterness. The whey hydrolysate concentration used in sensory analysis was 2.5 times higher than the casein hydrolysate concentration, since casein is known to yield more bitter hydrolysates than whey protein (*12, 40*). Despite the lower concentration of the casein hydrolysates, their perceived bitterness scores were generally higher than those for whey hydrolysates (**Table 2**).

PLS regression analysis with sample sets 1–3 resulted in models having explained variances equal to or less than 69% (R^2CV ≤ 0.69) (**Table 2**). The correlation coefficient between predicted and measured values is best for the model containing both casein and whey hydrolysates, since the combination of samples results in a broader range of bitterness values. Exclusion of samples produced with enzymes active at low pH (sample sets 4–6) results in improved bitterness prediction, independent of the sample set (**Table 2**). The hydrolysates produced at low

FTIR Spectra of Whey and Casein Hydrolysates

J. Agric. Food Chem., Vol. 50, No. 24, 2002 **6947**

Table 2. Results of PLS Regression with FTIR Spectra as Independent Variables and Various Hydrolysate Physicochemical Characteristics as Dependent Variable

functional property	range[a]	PCs[b]	$R^{2\,c}$	R^2 CV[d]	bias[e]	SEP[f]
Hydrolysates from All Enzyme Sources (Sample Sets 1–3)						
Bitterness (Score from 0 to 5)						
whey (25)[g]	0.9–2.3	3	0.75	0.62	**−0.008**	0.29
casein (31)	1.4–3.9	4	0.78	0.60	0.026	0.48
whey + casein (59)	0.9–3.8	3	0.74	0.69	0.002	0.45
Solubility (% w/w)						
whey (32)	18–96	2	0.86	0.82	0.011	8.78
casein (31)	42–85	4	0.86	0.76	**−0.094**	6.93
whey + casein (66)	18–96	6	0.87	0.76	**−0.033**	8.73
Emulsion Forming (d_{32}, μm)[h]						
whey (20)	0.6–5.3	3	0.91	0.83	**−0.059**	0.66
casein (16)	0.5–4.6	2	0.94	0.89	**−0.021**	0.54
whey + casein (37)	0.5–5.3	4	0.86	0.77	**−0.009**	0.75
Emulsion Stability (T_{24}/T_0, %)[i]						
whey (19)	56–100	4	0.93	0.86	0.36	5.47
casein (16)	5–100	2	0.93	0.89	0.53	13.4
whey + casein (36)	5–100	4	0.91	0.86	**−0.23**	11.3
Foam Forming (F_0, mL)[j]						
whey (17)	0–46	4	0.94	0.87	0.37	6.04
casein (19)	33–64	nd[l]				
whey + casein (39)	0–64	4	0.85	0.75	0.19	10.5
Foam Stability ($V\!f_{60}/V\!f_0$, %)[k]						
whey (20)	0–72	5	0.76	0.68	**−2.61**	15.7
casein (18)	18–82	1	0.84	0.84	**−0.014**	13.5
whey + casein (23)	0–82	1	0.83	0.82	**−0.33**	13.7
Hydrolysates from Neutral/Alkaline Enzymes (Sample Sets 4–6)						
Bitterness (Score from 0 to 5)						
whey (20)	0.9–2.4	1	0.76	0.74	0.0015	0.26
casein (20)	1.3–3.5	4	0.85	0.76	**−0.010**	0.35
whey + casein (43)	0.9–3.5	5	0.89	0.79	0.0015	0.38
Solubility (% w/w)						
whey (23)	38–96	4	0.90	0.80	**−0.21**	7.60
casein (21)	47–85	4	0.96	0.92	0.16	3.66
whey + casein (46)	38–96	5	0.89	0.81	**−0.019**	6.08
Emulsion Forming (d_{32}, μm)						
whey (14)	0.6–3.5	3	0.91	0.82	0.055	0.45
casein (12)	0.5–3.6	nd				
whey + casein (24)	0.5–3.1	4	0.86	0.71	**−0.027**	0.43
Emulsion Stability (T_{24}/T_0, %)						
whey (13)	56–95	3	0.92	0.88	**−0.20**	5.28
casein (11)	5–98	2	0.96	0.92	0.37	11.6
whey + casein (25)	5–98	3	0.91	0.87	0.077	11.8
Foam Forming (F_0, mL)						
whey (13)	0–46	3	0.93	0.81	**−0.49**	7.72
casein (12)	33–63	nd				
whey + casein (25)	0–63	2	0.86	0.83	0.041	7.66
Foam Stability ($V\!f_{60}/V\!f_0$, %)						
whey (15)	0–72	4	0.87	0.73	**−0.82**	16.2
casein	20–59	nd				
whey + casein	0–72	1	0.78	0.75	0.22	14.2

[a] Lowest and highest value for samples used in regression analysis. [b] Number of principal components used in regression analysis. [c] Multiple correlation coefficient (coefficient of determination) for calibration samples. [d] Multiple correlation coefficient of the cross validation. [e] Average difference between predicted and measured values. [f] Standard error of prediction. [g] Number of samples used for regression analysis. [h] Volume surface average particle diameter of emulsion droplets. [i] Emulsion turbidity after 24 h relative to turbidity at $t = 0$. [j] Foam forming: initial foam height. [k] Foam volume after 60 min relative to initial foam volume. [l] nd: regression parameters were not determined, since $R^2 < 0.5$.

pH values were neutralized with sodium hydroxide before sensory testing, to mask the acid taste. As a consequence, the samples were rather salty which might have influenced the bitterness perception. **Figure 5** shows the correlation between predicted and measured bitterness scores of whey and casein
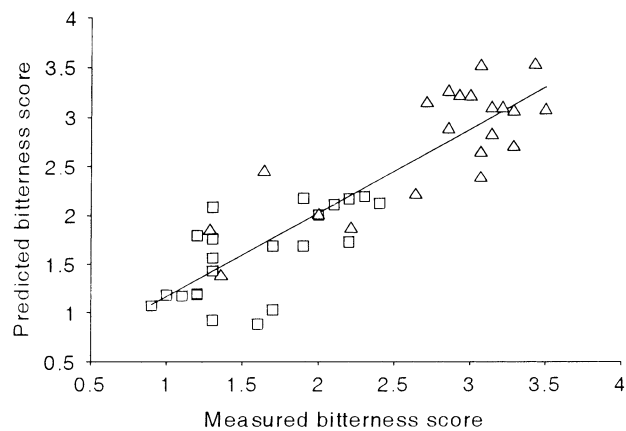
Figure 5. Correlation between predicted (obtained from FTIR spectra) and measured bitterness scores for casein (△) and whey (□) hydrolysates produced with neutral/alkaline enzymes. R^2 CV = 0.79.

hydrolysates made with neutral and alkaline enzymes. The correlation (R^2CV) between predicted and measured values is 0.79.

The regression analyses resulted in bitterness predictions with a coefficient of determination (R^2CV) that ranged between 0.60 and 0.79 for the different sample sets, which is rather good compared to prediction of sensory properties reported in the literature, which mainly concerns studies with cheese flavor. One study used near-infrared spectra to predict flavor attributes of cheese. The prediction of eight test samples based on a calibration model build with 24 samples resulted in R^2 values from 0.28 to 0.59 (*41*). Other studies used chemical parameters or chromatography to study flavors of cheese. To predict bitterness of cheese, reversed-phase chromatography data were used in combination with chemical parameters, like content of intact casein, ratio between ultrafiltration fractions, and a ratio between hydrophobic and hydrophilic peptides. The regression analysis resulted in explained variance for bitterness of 95% for a set of 6 samples and of only 59% for a set of 19 samples (*42*). For 15 Swiss-type cheeses, Vangtal and Hammond (*43*) reported a correlation coefficient of 0.53 between bitterness and degree of proteolysis. Moreover, they used factor analysis to define flavor factors describing various taste attributes and to define chemical factors describing the chemical composition based on *e.a.* fatty acid analysis. The correlation between the flavor factors and chemical factors varied between 0.63 and 0.85, which is comparable to the correlation between the FTIR spectra and bitterness in the present study; however, determination of chemical parameters is much more laborious.

Solubility. Solubility of whey hydrolysates varied between 18 and 96%, while the minimum solubility of casein hydrolysates was 42%. Acidic enzyme hydrolysates generally showed lower solubility values than the neutral/alkaline enzyme hydrolysates. Solubility prediction of the whey and casein hydrolysates from FTIR data is reasonably good. For casein hydrolysates, the prediction improves if acidic enzyme hydrolysates are excluded from analysis, which is not observed for whey hydrolysates. The acidic enzyme hydrolysates of casein seemed to influence the prediction model of casein solubility more than did the acidic enzyme hydrolysates of whey in the whey solubility model. This is probably due to the fact that the difference between the group of "acidic enzyme" hydrolysates and "alkaline/neutral" enzyme hydrolysates appears to be larger for casein hydrolysates than for whey hydrolysates. In the bitterness prediction, the improvement of the model after exclusion of the "acidic enzyme" samples was also larger for

6948 *J. Agric. Food Chem.,* Vol. 50, No. 24, 2002

van der Ven et al.
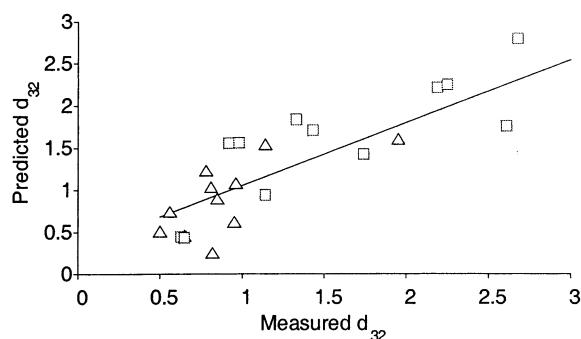
Figure 6. Prediction of emulsion forming ability of casein (△) and whey (□) hydrolysates. Prediction was based on FTIR spectra from hydrolysates prepared with neutral and alkaline enzymes. $R^2CV = 0.71$.

the casein hydrolysates model as compared to the whey hydrolysates model.

Emulsion Forming. Emulsion forming of casein hydrolysates was modeled excluding samples with very high d_{32} values (>7 μm), since these samples have a high leverage or high residual variance on the model, which makes them influential samples. Regression analysis with the remaining casein hydrolysates ($N = 16$) resulted in prediction with $R^2CV = 0.89$. However, the prediction was mainly based on the difference between two groups: emulsions with relative high d_{32} values (3−5 μm), opposed to emulsions with low average particle size (0.5− 2.0 μm). The emulsions with high d_{32} were prepared with acidic enzymes. If these samples are excluded from the analysis, the variation range of d_{32} values is rather small and the differences between samples cannot be explained on the basis of the FTIR spectra.

Emulsion-forming properties of whey hydrolysates are predicted rather well, the total explained variance is 83%. Exclusion of the acidic enzyme hydrolysates (sample set 4) results in similar prediction. The range of variation was probably still large enough to achieve good prediction. Analysis of casein and whey hydrolysates together results in models with explained variance of 77% and 71% for sample set 3 and sample set 6, respectively. In **Figure 6**, the result obtained with sample set 6 is presented.

Emulsion-forming ability of protein hydrolysates was not predicted before; although, some articles were published about the prediction of emulsion-forming ability of proteins, using the emulsifying activity index (EAI) as emulsion parameter, which is calculated from the turbidity of emulsions (*35*) instead of the d_{32} value. The EAI of protein emulsions has been correlated to the hydrophobicity of proteins (*44−46*), with reported correlation coefficients (R^2) of 0.71 ($N = 28$) (*44*), 0.66 ($N = 9$) (*46*), and 0.46 ($N = 52$) (*45*). The latter study showed that inclusion of a solubility parameter may improve the prediction of EAI, resulting in $R^2 = 0.58$ ($N = 52$) (*45*). Emulsion activity index has also been correlated to a set of 18 physicochemical protein parameters (e.g., % α-helix, hydrophobicity, charge density, binding of fluorescent probes), which resulted in a correlation coefficient between predicted and measured values of 0.75 ($N = 11$) (*47*). Spectral properties of proteins such as UV-absorption and fluorescence were correlated to emulsion-forming properties in a study on whey-pea and whey-potato protein mixtures, resulting in a multiple correlation coefficient (R^2) of 0.79 ($N = 30$) (*48*).

Emulsion Stability. Prediction of stability of whey and casein hydrolysates emulsions is quite good (**Table 2**). The accuracy of prediction is comparable to the prediction of emulsion stability using molecular weight distribution (*9*). Molecular

weight distribution can also be predicted on the basis of FTIR spectra (data not shown).

In the literature, some relations between emulsion stability and chemical characteristics of proteins for protein-stabilized emulsions are described. Stability of emulsions prepared with combinations of whey and pea proteins was correlated to hydrophobicity and spectral properties such as UV absorption, resulting in a multiple correlation coefficient (R^2) of 0.69 ($N = 30$). In a combination of whey and potato protein, the correlation between predicted (based on fluorescence and UV measurements) and measured values appeared to be poor ($R^2 = 0.47$, $N = 30$) (*48*). Hydrophobicity of protein samples ($N = 52$) was, apart from emulsifying activity, also correlated to emulsion stability (R^2 0.58) (*45*).

Foam Forming. Foam-forming ability of whey hydrolysates could be modeled satisfactorily, while for casein hydrolysate foams no correlation between FTIR spectra and foam forming was found. Foam-forming ability of casein hydrolysates was high in most samples, resulting in only small differences between values measured for foam-forming ability. However, if the data of casein and whey hydrolysates are combined, the variation between foam-forming ability of samples is sufficiently large to obtain a good model ($R^2CV = 0.75$). The whey hydrolysate model without the acidic enzyme hydrolysates is not as good as the model with all samples (sample set 4 compared to sample set 1, **Table 2**). The foam-forming ability of acidic whey hydrolysates does not differ systematically from the other enzyme hydrolysates. Therefore, the less accurate prediction probably comes from the lower number of samples used for prediction.

Arteaga and Nakai (*47*) predicted foam-forming capacity of eleven proteins using the same chemical parameters as used to predict emulsion properties, which resulted in a correlation coefficient (R^2) of predicted versus measured values of 0.70. Foam capacities of 16 proteins were correlated to hydrophobicity of denatured protein in conjunction with viscosity and resulted in a correlation coefficient (R^2) of 0.78 (*49*).

Foam Stability. Regression analysis of all whey hydrolysates results in poor correlation between FTIR spectra and foam stability. The correlation obtained with sample set 4, which excludes acidic enzyme hydrolysates, is only slightly better. Only 6 of the 22 whey hydrolysates were able to form stable foams, the foam of all other hydrolysates disappeared within 1 h. There are insufficient samples with stabilizing ability to attain a good prediction model for foam stability of whey hydrolysates.

Foam stability of casein hydrolysates is predicted rather well, as was expected from the prediction on the basis of the MWD. If the acidic enzyme hydrolysates are excluded, it is not possible to obtain a good prediction. A relatively large part of the remaining hydrolysates is not able to stabilize the hydrolysate. Therefore, the spread in results is no longer large enough to result in good correlations.

In the preceding paragraphs, it was shown that FTIR spectra of whey protein and casein hydrolysates can be used to predict several functional properties of these hydrolysates. In the present study, the FTIR spectra were treated as a black box system and, therefore, it was not intended to analyze the FTIR spectra in depth. However, in general terms, it is most likely that relationships arise from the fact that the spectra contain information on the chemical composition and on the secondary structure of the numerous peptides present in the hydrolysates. In previous work, it was shown that some of the properties predicted from FTIR spectra, like emulsion stability, foam forming, and foam stability, are related to the molecular weight

FTIR Spectra of Whey and Casein Hydrolysates

J. Agric. Food Chem., Vol. 50, No. 24, 2002 **6949**

distribution of the hydrolysates (*9, 10*). Solubility of hydrolysates can be related to the separation of hydrolysates on reversed-phase chromatography (data not published). Properties that are measured on SEC and RPC chromatography, like peptide length and influence of amino acid composition, are also reflected in FTIR spectroscopy. Apparently, FTIR spectra contain more information than the chromatographic methods, since more properties can be predicted from these spectra, for example, bitterness.

The regression coefficients obtained from the models may give an indication about the parts of the mid-infrared spectrum that are related to each of the functional properties. However, as the spectra are a result of a multitude of peptides present in hydrolysates, assignment of found relationships to specific chemical structures would be highly speculative at this moment. Considerably more research would be necessary to elucidate these underlying chemical structures. In these investigations, functional properties of hydrolysates are a result of characteristics of numerous peptides and possibly of various structural and chemical properties of these peptides.

In conclusion, the combination of FTIR spectroscopy and multivariate data analysis proved very valuable in protein hydrolysate characterization. FTIR spectra appear to correlate to various functional properties of whey and casein hydrolysates, some of which could not be predicted from other analytical methods until now. FTIR spectroscopy is not laborious and might substitute labor intensive functionality measurements. Faster characterization of hydrolysates including prediction of potential functional properties will speed up product development and might support quality control.

ABBREVIATIONS USED

FTIR, Fourier transform infrared;
PCA, principal component analysis;
PLS, partial least squares;
PC, principal component;
R^2, coefficient of determination;
CV, cross validation.

LITERATURE CITED

(1) Cordle, C. T. Control of food allergies using protein hydrolysates. *Food Technol.* **1994**, *48*, 72−76.

(2) Asselin, J.; Amiot, J.; Gauthier, S. F.; Mourad, W.; Hebert, J. Immunogenicity and allergenicity of whey protein hydrolysates. *J. Food Sci.* **1988**, *53*, 1208−1211.

(3) Farrell, P. M.; Mischler, E. H.; Sondel, S. A.; Palta, M. Predigested formula for infants with cystic fibrosis. *J. Am. Dietetic Assoc.* **1987**, *87*, 321−328.

(4) Frokjaer, S. Use of hydrolysates for protein supplementation. *Food Technol.* **1994**, *48*, 86−88.

(5) de Freitas, O.; Padovan, G. J.; Vilela, L.; Dos Santos, J. E.; Dutra de Oliviera, J. E.; Greene, L. J. Characterization of protein hydrolysates prepared for enteral nutrition. *J. Agric. Food Chem.* **1993**, *41*, 1432−1438.

(6) Clemente, A. Enzymatic protein hydrolysates in human nutrition. *Trends Food Sci. Technol.* **2000**, *11*, 254−262.

(7) Adler-Nissen, J. *Enzymatic Hydrolysis of Food Proteins*; Elsevier Applied Science Publishers: London, 1986.

(8) Nielsen, P. M. Functionality of protein hydrolysates. In *Food proteins and their applications*; Damadoran, S., Paraf, A., Eds.; Marcel Dekker: New York, 1997; pp 443−472.

(9) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics. *J. Agric. Food Chem.* **2001**, *49*, 5005−5012.

(10) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Correlations between biochemical characteristics and foam forming and stabilizing ability of whey and casein hydrolysates. *J. Agric. Food Chem.* **2002**, *50*, 2938−2946.

(11) Adler-Nissen, J. Bitterness intensity of protein hydrolyzates - chemical and organoleptic characterization. Frontiers of Flavor, Proceedings of the 5th International Flavor Conference, Porto Karras, Chalkidiki, Greece; Elsevier Science Publishers: Amsterdam, 1987; p 63−77.

(12) Lemieux, L.; Simard, R. E. Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition. *Lait* **1992**, *72*, 335−382.

(13) Tanimoto, S.; Watanabe, M.; Arai, S. Bitter flavor of protein hydrolysates and synthetic peptides. In *Off-flavors in foods and beverages*; Charalambous, G., Ed.; Elsevier Science Publishers: Amsterdam, 1992; pp 547−567.

(14) Adler-Nissen, J. Relationship of structure to taste of peptides and peptide mixtures. In *Protein tailoring for food and medical uses*; Feeney, R. E., Whitaker, J. R., Eds.; Marcel Dekker: New York, 1986; pp 97−122.

(15) Minagawa, E.; Kaminogawa, S.; Tsukasaki, F.; Yamauchi, K. Debittering mechanism in bitter peptides of enzymatic hydrolysates from milk casein by aminopeptidase T. *J. Food Sci.* **1989**, *54*, 1225−1229.

(16) Lovsin Kukman, I. L.; Zelenik Blatnik, M.; Abram, V. Isolation of low-molecular-mass hydrophobic bitter peptides in soybean protein hydrolysates by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **1995**, *704*, 113−120.

(17) Lin, S. B.; Nelles, L. P.; Cordle, C. T.; Thomas, R. L. Debittering casein hydrolysates with octadecyl-siloxane (C18) columns. *J. Food Sci.* **1997**, *62*, 665−670.

(18) Willard, H. H.; Merritt, L. L.; Dean, J. A.; Settle, F. A., Jr. Infrared Spectroscopy. In *Instrumental methods of analysis*; Wadsworth Publishing Company: Belmont, CA, 1981; pp 177−216.

(19) Al Jowder, O.; Defernez, M.; Kemsley, E. K.; Wilson, R. H. Mid-infrared spectroscopy and chemometrics for the authentication of meat products. *J. Agric. Food Chem.* **1999**, *47*, 3210−3218.

(20) Curley, D.; Kumosinski, T.; Unrah, J.; Farrell, H. Changes in the secondary structure of bovine casein by Fourier transform infrared spectroscopy: effects of calcium and temperature. *J. Dairy Sci.* **1998**, *81*, 3154−3162.

(21) Kumosinski, T. F.; Farrell, H. M. Determination of the global secondary structure of proteins by Fourier transform infrared (FTIR) spectroscopy. *Trends Food Sci. Technol.* **1993**, *4*, 169−175.

(22) Belton, P. S.; Bogracheva, T.; Cserhalmi, Z.; Czukor, B.; Grant, A.; Lambert, N.; Wellner, N. NMR and FTIR studies of hydrated pea proteins. *Food Hydrocolloids* **1997**, *11*, 485−491.

(23) Casal, H. L.; Kohler, U.; Mantsch, H. H. Structural and conformational changes of β-lactoglobulin B: an infrared spectroscopic study of the effect of pH and temperature. *Biochim. Biophys. Acta* **1988**, *957*, 11−20.

(24) Forato, L. A.; Bernardes-Filho, R.; Colnago, L. A. Protein structure in KBr pellets by infrared spectroscopy. *Anal. Biochem.* **1998**, *259*, 136−141.

(25) Bertrand, D.; Lila, M.; Furtoss, V.; Robert, P.; Downey, G. Application of principal component analysis to the prediction of lucerne forage protein content and in vitro dry matter digestibility by NIR spectroscopy. *J. Sci. Food Agric.* **1987**, *41*, 299−307.

(26) Rodriguez-Saona, L. E.; Fry, F. S.; Calvey, E. M. Use of Fourier Transform Near-Infrared Reflectance Spectroscopy for rapid quantification of Castor Bean meal in a selection of flour-based products. *J. Agric. Food Chem.* **2000**, *48*, 5169−5177.

(27) Sasic, S.; Ozaki, Y. Short-Wave Near-Infrared spectroscopy of biological fluids. 1. Quantitative analysis of fat, protein, and lactose in raw milk by partial least-squares regression and band assignment. *Anal. Chem.* **2001**, *73*, 64−71.

6950 *J. Agric. Food Chem.,* Vol. 50, No. 24, 2002

van der Ven et al.

(28) Seiden, P.; Bro, R.; Poll, L.; Munck, L. Exploring fluorescence spectra of apple juice and their connection to quality parameters by chemometrics. *J. Agric. Food Chem.* **1996**, *44*, 3202−3205.

(29) Pribic, R. Principal component analysis of Fourier transform infrared and/or circular dichroism spectra of proteins applied in a calibration of protein secondary structure. *Anal. Biochem.* **1994**, *223*, 26−34.

(30) Bietz, J. A.; Simpson, D. G. Electrophoresis and chromatography of wheat proteins: available methods, and procedures for statistical evaluation of the data. *J. Chromatogr.* **1992**, *624*, 53−80.

(31) Josefson, M.; Tekenbergs-Hjelte, L. Multicomponent analysis: a case report. *J. Pharm. Biomed. Anal.* **1991**, *9*, 643−54.

(32) Forina, M.; Casolino, M. C.; de la Pezuela Martinez, C. Multivariate calibration: applications to pharmaceutical analysis. *J. Pharm. Biomed. Anal.* **1998**, *18*, 21−33.

(33) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Reversed phase and size exclusion chromatography of milk protein hydrolysates: relation between elution from reversed phase column and apparent molecular weight distribution. *Int. Dairy J.* **2001**, *11*, 83−92.

(34) Larrabee, J. A.; Choi, S. Fourier transform infrared spectroscopy. *Methods Enzymol.* **1993**, *226*, 289−305.

(35) Pearce, K. N.; Kinsella, J. E. Emulsifying properties of proteins. Evaluation of a turbidimetric technique. *J. Agric. Food Chem.* **1978**, *26*, 716−723.

(36) Caessens, P. W. J. R.; Gruppen, H.; Visser, S.; Van Aken, G. A.; Voragen, A. G. J. Plasmin hydrolysis of b-casein: foaming and emulsifying properties of the fractionated hydrolysate. *J. Agric. Food Chem.* **1997**, *45*, 2935−2941.

(37) Holt, C. Primary and secondary structures of caseins. In *Food colloids and polymers: stability and mechanical properties*; Dickinson, E., Walstra, P., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1992; pp 167−172.

(38) Byler, D. M.; Farrell, H. M. Infrared spectroscopic evidence for calcium ion interaction with carboxylate groups of casein. *J. Dairy Sci.* **1989**, *72*, 1719−1723.

(39) Silverstein, R. M.; Clayton Bassler, G.; Morill, T. C. Infrared Spectrometry. In *Spectroscopic identification of organic compounds*; Sawicki, D., Stiefel, J., Eds.; John Wiley & Sons: New York, 1991; pp 91−162.

(40) Petrischek, A.; Lynen, A.; Belitz, D. H. Untersuchungen uber Bitterpeptide II. Auftreten von Bittergeschmack in enzymatischen Hydrolysaten verschiedener Proteine. *Lebensm.-Wiss. Technol.* **1972**, *5*, 77−81.

(41) Sorensen, L. K.; Jepsen, R. Assessment of sensory properties of cheese by near-infrared spectroscopy. *Int. Dairy J.* **1998**, *8*, 863−871.

(42) Frister, H.; Michaelis, M.; Schwerdtfeger, T.; Folkenberg, D. M.; Sorensen, N. K. Evaluation of bitterness in Cheddar cheese. *Milchwissenschaft* **2000**, *55*, 691−695.

(43) Vangtal, A.; Hammond, E. G. Correlation of the flavour characteristics of Swiss-type cheeses with chemical parameters. *J. Dairy Sci.* **1986**, *69*, 2982−2993.

(44) Kato, A.; Nakai, S. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.

(45) Voutsinas, L. P.; Cheung, E.; Nakai, S. Relationships of hydrophobicity to emulsifying properties of heat denatured proteins. *J. Food Sci.* **1983**, *48*, 26−32.

(46) Nakai, S.; Ho, L.; Helbig, N.; Kato, A.; Tung, M. A. Relationship between hydrophobicity and emulsifying properties of some plant proteins. *Can. Inst. Food Sci. Technol. J.* **1980**, *13*, 23−27.

(47) Arteaga, G. E.; Nakai, S. Predicting protein functionality with artificial neural networks: foaming and emulsifying properties. *J. Food Sci.* **1993**, *58*, 1152−1156.

(48) Jackman, R. L.; Yada, R. Y. Multivariate analysis of functional and structure-related properties of whey-vegetable protein composites. *Can. Inst. Food Sci. Technol. J.* **1990**, *22*, 260−269.

(49) Townsend, A. A.; Nakai, S. Relationships between hydrophobicity and foaming characteristics of food proteins. *J. Food Sci.* **1983**, *48*, 588−594.