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Quantification of Melanoidin Concentration in Sugar−Casein Systems

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Melanoidins are the final, brown, high molecular weight products of the Maillard reaction. The aim of the present study was to determine the average molar extinction coefficients of melanoidins formed in heated glucose−casein and fructose−casein systems. The value of the extinction coefficient can be used to translate spectrophotometrically measured browning (absorbance values) into melanoidin concentration. In the present study the melanoidins were quantified by measuring the concentration of sugar incorporated into the melanoidins, using ^{14}C-labeled sugar. The extinction coefficient of the melanoidins remained constant during the observation period as the absorbance at 420 nm increased to ≈8 units, and it was calculated to be 477 (± 50) L mol^{-1} cm^{-1} in the glucose−casein reaction and 527 (± 35) L mol^{-1} cm^{-1} in the fructose−casein reaction. This difference is not significant. An increase of the number of sugar molecules per reactive amino group during the heating of glucose−casein and the fructose−casein mixtures was observed by the radiochemical method as well as by microanalysis of the high molecular weight fraction.

KEYWORDS: Melanoidins; Maillard reaction; glucose; fructose; casein; extinction coefficient; microanalysis; kinetics

INTRODUCTION

In the final stage of the Maillard reaction between sugars and compounds possessing a free amino group, such as amino acids and proteins, melanoidins [also referred to as advanced glycation end products (AGE) in the literature concerning in vivo glycation] can be formed (*1*). The brown melanoidins are a heterogeneous mixture of high molecular weight compounds (*2*). Besides being caused by the high molecular weight melanoidins, browning can also be due to low molecular weight colored compounds, sometimes referred to as low molecular weight melanoidins (*3*). The brown compounds have a high impact on the quality of foods. Color is an important food quality characteristic and a key factor in consumer acceptance (*4*).

The mechanism of the formation of color is not fully understood, and the structure of melanoidins is largely unknown. Up to now, only hypotheses are available regarding the structures of melanoidins formed in sugar−amino acid systems (*5*−*8*). In sugar−casein systems, it is established that color formation is mainly due to the formation of protein oligomers cross-linked by low molecular weight colored Maillard reaction products (*9*). The structures of some chromophores present in protein-based melanoidins have been elucidated (*10*). From the point of view of color, melanoidins can be built up of subunits in two contrasting ways. One possibility is that melanoidins are formed by more or less random reactions of low molecular weight reaction intermediates (which may inherently be colored or not). Alternatively, a repeating unit (which may be colorless or contribute little to color) may form the backbone of melanoidins, with chromogenic low molecular weight structures attaching themselves to this backbone, resulting in high molecular weight colored structures. The results of the studies by Hofmann (*9*, *10*) indicate that proteins can act as the colorless backbone of melanoidins.

The missing information on melanoidin formation and melanoidin structure makes it very difficult to quantify melanoidins. This quantification is necessary when one is trying to predict or optimize browning in processed foods. Both Davies and co-workers (*11*) and Brands and Van Boekel (*12*) developed a kinetic model for the formation of brown color in glucose−glycine mixtures and sugar−casein systems, respectively. Browning has usually been measured spectrophotometrically and expressed in absorbance units. If color can be expressed in concentration units, it is possible to relate color directly to the rates of formation of intermediates in the kinetic model and hence to predict it. Assuming Lambert−Beer's law is obeyed, absorbance data can, in principle, be related to melanoidin concentration. Apart from concentration, the absorbance depends on the molar extinction coefficient of these melanoidins. Leong

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determined the average extinction coefficient of melanoidins derived from glucose and glycine and various other amino acids using ^{14}C-labeled glucose (*3*, *13*). A benefit of this approach is that the molar extinction coefficient of melanoidins can be expressed simply in terms of the concentration of glucose molecules converted into melanoidins, even though the molecular weights of melanoidins are expected to span a very wide range of values. When defined in this way, it was observed that the extinction coefficient remained constant with time. Once the extinction coefficient is known, melanoidins can be quantified and used to validate proposed kinetic models.

In the present study, the average extinction coefficient of melanoidins obtained from glucose and the protein casein was determined, following the method of Leong and Wedzicha (*3*). Leong (*13*) observed that the molar extinction coefficient was dependent on the kind of amino acid that was heated in the presence of glucose. Although in many foods proteins instead of amino acids are the greatest source of free amino groups, the molar extinction coefficient of melanoidins obtained from the reaction between glucose and (the lysine residues of) a protein has, however, not been determined before. To study the effect of the type of sugar on the value of the extinction coefficient, the same experiments were carried out with fructose, the ketose isomer of glucose, as the reacting sugar. Furthermore, a microanalysis of the melanoidins was carried out to investigate the number of sugar molecules incorporated per protein molecule.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were of analytical grade. D-Glucose and D-fructose were supplied by Sigma Chemicals. D-[U-^{14}C]Glucose and D-[U-^{14}C]fructose were obtained from Amersham Life Science Ltd.. Sodium caseinate (a spray-dried powder) was obtained from DMV containing 90% protein. Casein is a protein with almost no secondary or tertiary structure and has shown to be extremely stable when heated (*14*).

Preparation of Reaction Model Systems. Sodium caseinate (3% w/w) was dissolved in a phosphate buffer (0.1 M, pH 6.8) and kept refrigerated overnight. Sugar (150 mM glucose or fructose) was dissolved in the casein solution. Before making up to a volume of 100 mL, 1 MBq of radiolabeled sugar ([U-^{14}C]glucose or [U-^{14}C]fructose) was added to the sugar−casein system (amount of added labeled sugar was ≈0.1 μmol and therefore negligible with respect to the unlabeled sugar). This solution was distributed over eight screw-capped glass tubes (Schott, 16 × 160 mm) and heated for various times (0, 10, 20, 30, 40, 50, 60, and 90 min) at 120 °C in an oil bath. After being heated, the samples were cooled in ice−water. The glucose−casein reaction mixtures were heat-treated and analyzed in threefold, the fructose−casein systems in twofold.

Dialysis. To separate unreacted radiolabeled substances and low molecular weight products from the high molecular weight products, the samples were dialyzed. An aliquot (10 mL) of each reaction mixture was dialyzed in Visking tubing with a cutoff value of 12000 Da. The samples were dialyzed against distilled water for 4 or 5 days (eight water replacements). After dialysis, the retentate was removed to a volumetric flask containing 20 mL of 20% (w/w) sodium dodecyl sulfate (SDS) solution and made up to 100 mL with distilled water. SDS was added to dissolve any flocculated protein.

Scintillation Counting. Aliquots (1 mL) of the diluted dialyzed fraction were pipetted into a scintillation vial containing 10 mL of scintillation fluid. The vial was shaken vigorously and counted for 1 min using a Packard Tri-Carb 1900TR scintillation counter. The count due to ^{14}C was corrected for quenching by the internal standard method. The specific activity of [^{14}C]glucose or [^{14}C]fructose in the reaction mixture was calculated from the counts obtained from 1 mL of a 50-fold diluted unheated reaction mixture and was expressed as number of disintegrations per minute (dpm) per mole of glucose or fructose.

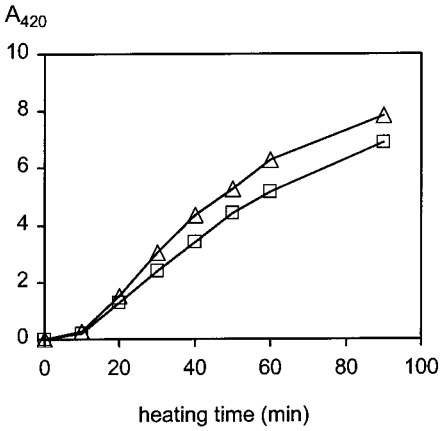

Figure 1. Browning (measured as absorbance at 420 nm) of glucose–casein (□) and fructose–casein (△) reactions at 120 °C with time.

Once the quench-corrected number of counts for a certain sample was known, the concentration of [U-^{14}C]sugar incorporated into the high molecular weight fraction could be calculated by dividing the number of counts per minute by the specific activity of the sugar.

Spectrophotometric Analysis. In a parallel experiment, in which no radiolabeled sugar was added to the reaction mixture, the browning of reaction mixtures and of corresponding retentates after dialysis was determined. Browning was measured spectrophotometrically as the absorbance at 420 nm. The undialyzed samples were diluted 5-fold in SDS (final concentration of 4% w/w) to reduce light scattering. The dilution of the dialyzed samples was as described before for the experiments with labeled sugar.

Microanalysis. The sugar−casein systems that were heated for 10, 30, and 60 min (without addition of radiolabeled sugar) were dialyzed, and the retentates were freeze-dried. Microanalysis was carried out using a Carlo Erba elemental analyzer. The weight of each sample was ∼2 mg.

Analyses of Amadori Compound. The Amadori compound was determined by means of furosine, using HPLC (*15*). Furosine concentration was converted to that of the Amadori compound using a conversion factor of 3.1 (*12*).

Analyses of Available Lysine Residues. Available lysine residues were determined after derivatization with *o*-phthaldialdehyde, as described previously (*12*).

RESULTS AND DISCUSSION

Browning. Considerable browning was observed in both the glucose−casein and fructose−casein reactions during heating (**Figure 1**). After an induction time during which no browning was detected, fructose browned more quickly than glucose. Due to differences in reaction behavior between glucose and fructose (*12*) it is difficult to compare the results for browning with literature data. Besides the pH, water activity, and sugar to amino group ratio, the rate of browning depends strongly on the temperature.

During dialysis ∼30% of the absorbing compounds (at 420 nm) passed into the dialysate (**Figure 2**). Consequently, the majority of the colored compounds was retained in the high molecular weight fraction. This result is in line with literature describing browning in sugar−protein systems. Morales and Van Boekel (*16*) observed that the pigments causing browning in heated glucose−casein and lactose−casein reactions were mainly bound to the protein. Hofmann (*9*) reported that the predominant part of the reaction products formed in a glucose−casein reaction was of high molecular weight (>50000 Da), and this increase of the molecular weight ran in parallel with the intensity of browning. These results are in contrast with sugar−amino acid reactions in which a much lower percentage

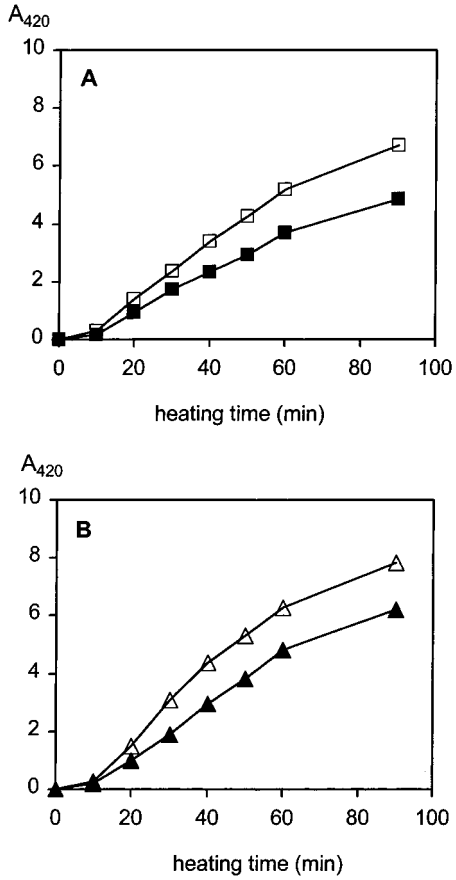

Figure 2. Browning (measured as absorbance at 420 nm) of glucose–casein (A) and fructose–casein (B) reactions at 120 °C before (□, △) and after (■, ▲) dialysis.

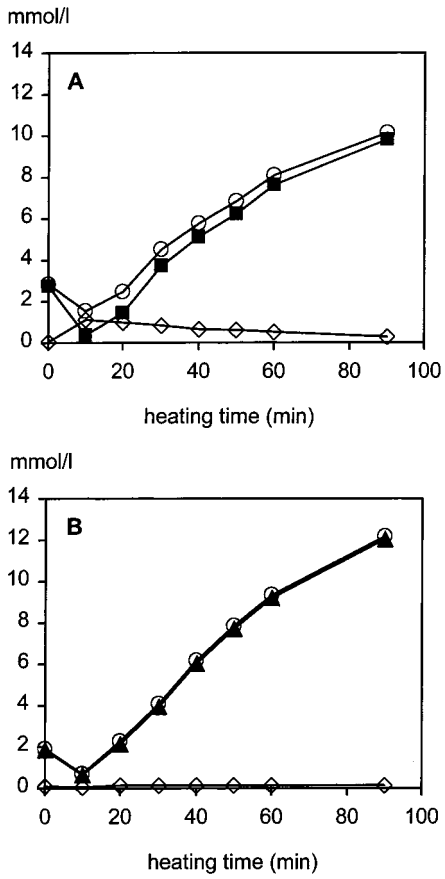

Figure 3. Formation of high molecular weight compounds measured as incorporated sugar (○), Amadori compound (◇), and melanoidins (■, ▲) in glucose–casein (A) and fructose–casein (B) reactions at 120 °C.

of color was detected in the high molecular weight fraction. In a study of Leong and Wedzicha (*3*) the high molecular weight melanoidin fraction (>3500 Da) contributed only up to 10% of the absorbance of the glucose−glycine reaction mixtures. Hofmann (*9*) reported that only trace amounts of compounds with molecular weights >3000 Da were formed in glucose−glycine and glucose−alanine systems and that color was therefore almost exclusively due to the low molecular weight fraction.

These differences between amino acids and proteins in the character of the colored products are likely to be due to the fact that the protein is a high molecular weight compound itself. As a consequence, reaction products will be of high molecular weight per se. On the other hand, Monti and co-workers (*17*) showed that the formation of high molecular weight colored compounds is not exclusive for protein-bound chromophores. They studied the formation of color in two model systems consisting of lactose and lysine or N^{α}-acetyllysine. In the system containing N^{α}-acetyllysine the main contribution to color was due to the high molecular weight fraction (>10000 Da), whereas in the reaction with free lysine the contribution of the high molecular weight fraction was negligible. It was concluded that the formation of melanoidins proceeded more rapidly in a model system containing blocked α-amino groups because less material was converted in other reaction pathways, mainly Strecker degradation, that lead to low molecular weight products.

Heating sugars in the absence of protein also leads to browning. When these samples were dialyzed, almost no colored compounds were retained in the high molecular weight fraction. Brown sugar degradation products can therefore contribute to the melanoidins only once they have reacted with the protein.

Melanoidin Formation. The experiments used to obtain the absorbance−time data given in **Figure 2** were repeated with the sugar uniformly labeled with ^{14}C. In **Figure 3** the concentration of the high molecular weight compounds, measured as the concentration of sugar incorporated in this fraction, is plotted against time. The high molecular weight fraction, however, not only consisted of the brown melanoidins but is expected to have contained also noncolored products of the early stages of the Maillard reaction when these are bound to the protein. However, with the exception of the Amadori compounds, most of them cannot be determined because little is known of their identity. In the present study it was assumed that only the Amadori compound was formed in significant amounts. On the basis of this assumption, the melanoidin concentration was obtained by subtracting the concentration of Amadori compound from the concentration of sugar incorporated into the high molecular weight fraction (see **Figure 3**).

As can be seen from **Figure 3**, ^{14}C-labeled sugar in the retentate was also detected for the unheated samples, and the count rates were somewhat higher than for the samples heated for a short time. Because we corrected for the formation of Amadori compound and because unreacted sugar and noncovalently bound sugar and sugar fragments were supposedly washed away during dialysis, we cannot offer an explanation for this observation.

Extinction Coefficient of Melanoidins. The relationship between the absorbance of the melanoidin fraction and the concentration of melanoidins as determined using ^{14}C-labeled

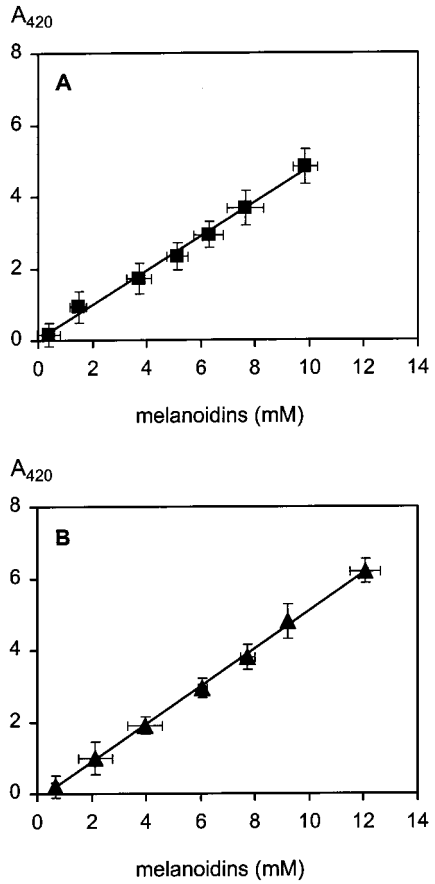

Figure 4. Browning (measured as absorbance at 420 nm ± standard deviation) as function of the melanoidin concentration (measured as incorporated sugar ± standard deviation) detected in glucose–casein (A) and fructose–casein (B) reactions after heating at 120 °C.

sugar is shown in **Figure 4**, where a change in melanoidin concentration is the result of a corresponding change in the extent of reaction. The relationship between browning and amount of labeled sugar incorporated into the melanoidins is clearly linear, and the value of the extinction coefficient can be deduced from the slope of this line. The results indicate that, over the observation period, the extinction coefficient of the melanoidins remains constant. This is most likely when a repeating unit or, alternatively, the protein forms the backbone of melanoidins with chromogenic low molecular weight structures attaching themselves to this backbone. In the glucose−casein system the average molar extinction coefficient (± standard deviation) was calculated to be 477 (± 50) L mol^{-1} cm^{-1}. In the fructose−casein system it was 527 (± 35) L mol^{-1} cm^{-1}. This difference between the extinction coefficients of melanoidins formed in glucose−casein and fructose−casein reactions was not significant at the 5% level. This indicates that in the glucose−casein and fructose−casein systems similar reaction intermediates might be formed, giving rise to melanoidins with the same average extinction coefficient.

The value of the average extinction coefficient of melanoidins formed in the glucose−casein reaction was compared with the ones obtained from melanoidins formed in glucose−amino acid reactions. Leong (*13*) estimated values of ϵ at 470 nm to range from 695 L mol^{-1} cm^{-1} for valine to 940 L mol^{-1} cm^{-1} for glycine. Because $A_{470}/A_{420} \approx 0.57$, the extinction coefficients of melanoidins obtained from glucose−amino acid systems were calculated to be ∼2.5−3.5 times higher than the extinction coefficient of glucose−casein melanoidins. This means that, in sugar−amino acid reactions, fewer glucose molecules (or glucose fragments) have to be incorporated into the melanoidins than in sugar−casein systems to increase the absorbance by one unit.

Leong and Wedzicha (*3*) observed that the extinction coefficient obtained for high molecular weight melanoidins formed in a glucose−glycine reaction mixture was in remarkable agreement with the value obtained for the whole reaction mixture using kinetic considerations. Because the high molecular weight melanoidin fraction contributed only up to 10% of the total absorbance, it could be concluded that the extinction coefficient of the low molecular weight fraction must have been the same. The same value for both low and high molecular weight colored compounds is possible only when high molecular weight material is formed from colored substructures and the extent of conjugation does not change as the polymer grows.

Microanalysis. For the evaluation of the microanalysis data of the melanoidins, the results have to be fitted to a model of reaction stoichiometry. Such a model for the casein−sugar reaction is based on the model used by Wedzicha and Kaputo (*18*) but modified so that the only products are melanoidins and water. Carbon dioxide is assumed not to be released because Strecker degradation does not occur. Thus, the overall reaction for the formation of the melanoidin is a combination of *a* molecules of sugar (glucose or fructose) consisting of *l*, *m*, and *n* atoms of C, H, and O, respectively, and *b* molecules of protein consisting of *p*, *q*, *r*, and *s* atoms of C, H, N, and O, respectively, to give a melanoidin with the following formula:

$$C_{la+pb}H_{ma+qb-2y}N_{rb}O_{na+sb-y}$$

y is the amount of H_2O liberated. When glucose or fructose is the reacting sugar, $l = 6$, $m = 12$, and $n = 6$. The formula of the protein was determined by microanalysis and, considering that the molecular weight of casein is 23000 g mol^{-1}, it was calculated that $p = 951$, $q = 1553$, and $r = 240$ (parameter *s* was not calculated because the percentage of oxygen could not be determined by microanalysis). The theoretical formula of the protein is $C_{1029}H_{1600}N_{262}O_{308}S_5P_6$. Although the experimental values differ from the theoretical values, the C/N ratios are 3.97 and 3.93 (experimental and theoretical values, respectively) and, therefore, in excellent agreement with each other. This confirms the accuracy of the microanalysis with respect to carbon and nitrogen. The unknowns *a* and *b* may be found by solving the following equations

$$C = 6a + 951b$$

$$N = 240b$$

where *C* and *N* represent the number of the corresponding atoms in the formula of the glycated protein. Because the C/N ratio is insensitive to the presence of moisture, which could be a significant "impurity" in the glycated protein sample, the values of *a*/*b* calculated in this way are expected to be reliable. Thus, **Table 1** gives the measured C/N ratios and the corresponding values of *a*/*b*, the number of sugar molecules incorporated into the polymer per protein molecule. The number of sugar equivalents per protein molecule increased with heating time in both the glucose−casein and fructose−casein systems. After 60 min of heating, the *a*/*b* ratio in the fructose−casein system was somewhat, although not significantly, higher than the *a*/*b* ratio in the glucose−casein system, corresponding to a slightly higher degree of browning (also apparent from **Figure 1**).

The ϵ-amino groups of the lysine residues are the most susceptible amino groups of the protein to glycation (*19*). One

Table 1. C/N Ratio and Calculated Number of Molecules of Sugars (*a*) Incorporated per Molecule of Casein (*b*) or Lysine Residue (*b′*)

sugar	heating time (min)	C/N	*a*/*b*	*a*/*b′*	*a*/*b′* radiochem
		3.97			
glucose	10	4.01	1.85 ± 0.75	0.14 ± 0.06	0.10 ± 0.03
glucose	30	4.10	5.39 ± 0.15	0.41 ± 0.01	0.30 ± 0.03
glucose	60	4.22	10.00 ± 1.32	0.77 ± 0.10	0.54 ± 0.04
fructose	10	4.01	1.65	0.13	0.05 ± 0.01
fructose	30	4.15	7.34	0.56	0.27 ± 0.04
fructose	60	4.30	13.26 ± 0.14	1.02 ± 0.01	0.62 ± 0.01

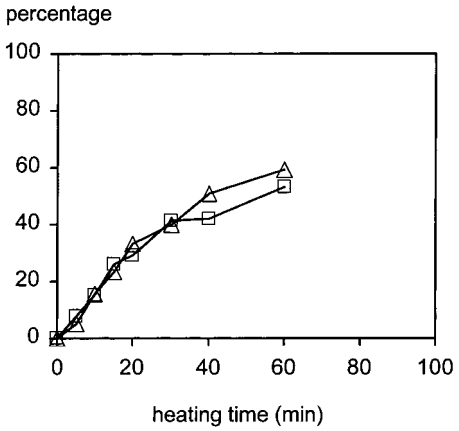

Figure 5. Loss of available lysine residues in glucose–casein (□) and fructose–casein (△) systems after heating at 120 °C.

molecule of casein contains 13 lysine residues on average [because casein consists of four different casein types, an average has to be used (*20*)]. In **Table 1** the calculated number of sugar molecules per lysine residue (*a*/*b′*) is shown (*b′* is the number of lysine residues per casein molecule). From the results we can conclude that after 60 min of heating, about three-fourths of the lysine residues present in the glucose−casein system have reacted with glucose (or glucose equivalents with six carbon atoms) and all lysine residues present in the fructose−casein system. This does, however, not correspond with results observed before (*12*) stating that in both sugar−casein systems the loss of available lysine residues was not more than 60% after 60 min of heating at 120 °C (see **Figure 5**).

Except for the ϵ-amino group of lysine residues, other potential glycation sites of casein are the N-terminal α-amino group and the guanidino group of arginine residues (*19*). During heating, more reactive reaction products, such as various dicarbonyl compounds, will be generated in the reaction system. These compounds have a higher affinity for the guanidino groups of arginine residues and hence increase the involvement of arginine residues in the glycation reaction (*21*). One molecule of casein contains five arginine residues on average (*20*). If we take the reaction of sugar degradation compounds with arginine residues into account, the number of sugar equivalents per amino group will decrease.

To be able to compare the results of the microanalysis with the results of the radiochemical experiment, the concentration of sugar incorporated in the high molecular weight fraction was divided by the concentration of lysine residues (see **Table 1**). It can be concluded that the trend is similar but that the calculated results of the microanalysis are significantly higher ($P < 0.05$) than those from the radiochemical experiment.

Conclusions. Lately, more and more studies are focusing on melanoidin formation. In the present study, the average extinction coefficients of melanoidins formed in sugar−casein systems were determined, using ^{14}C-labeled sugar. No significant difference was found between the extinction coefficient of melanoidins formed in glucose−casein systems, on the one hand, and in fructose−casein systems, on the other hand. The number of sugar molecules per molecule of protein, as determined by microanalysis, was higher in the fructose−casein system, which was in line with the higher browning rate.

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