Kinetic Determination of Total Casein in Milk and Dairy Products by Long-Wavelength Fluorescence Detection

María Paz Aguilar-Caballos, Agustina Gómez-Hens, and Dolores Pérez-Bendito*

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, E-14004 Córdoba, Spain

A very simple and fast fluorometric method for the routine determination of total casein in food samples is described. It is based on the application of kinetic methodology to the Indocyanine Green−cetyltrimethylammonium bromide−casein system by using stopped-flow mixing technique and long-wavelength fluorescence measurements, which allow the temporal and spectral discrimination of the analytical signal. The electrostatic interaction between casein and the surfactant eliminates the quenching caused by the latter on the fluorescence of the dye, so that the fluorescence intensity increment with time, which is measured in ∼2 s, is directly related to casein concentration. The dynamic range of the calibration graph is 3−100 μg mL^{-1}, and the detection limit is 0.9 μg mL^{-1}. The relative standard deviation is <2%. The proposed method was applied to the determination of total casein in milk samples and dairy products with a recovery of 96.8−103.1%.

Keywords: *Caseins; fluorimetry; Indocyanine Green; kinetic method; stopped-flow; milk samples; dairy products*

INTRODUCTION

Kjeldahl nitrogen determination remains a very general method for protein determination in food analysis, being used as an official method by standardization organizations (AOAC, 1990). In the case of casein, which is the group of proteins that form the main portion of milk proteins, this method was also proposed as a reference method (British Standard, 1990) where the insolubility of casein in weakly acid media is used for its separation from the other milk proteins, whey proteins, that stay in solution. This separation has been also applied as a previous step for total casein determination in milk by using more rapid methods alternative to the Kjeldahl method such as spectroscopic (Mehnert and Hudec, 1989; Taha and Puhan, 1993), enthalpimetric (Bark and Hadipranoto, 1991), and chronoamperometric (Sanchez-Pérez et al., 1995) methods. Although indirect fluorometric detection has been described for the determination of some casein fractions, such as β-casein (Hogan and Yeung, 1990), fluorometric methods for total casein determination have not been proposed to date. The lack of these types of methods could be ascribed to the interference that other sample components, which are separated together with casein, can cause in the fluorescence analytical signal. This paper shows a method involving kinetic measurements at long-wavelength fluorescence, which can avoid this potential effect by combining temporal and spectral discrimination. Indocyanine Green (sodium 2-{7-[1,1-dimethyl-3-(4-sulfobutyl)benz[*e*]indolin-2-ylidene]hepta-1,3,5-trienyl}-1,1-dimethyl-1*H*-benz[*e*]indolio-3-(butyl-4-sulfonate) (ICG), which is one of the few negatively charged long-wavelength fluorescent dyes available, has been used for this purpose in the presence of the cationic surfactant cetyltrimethylammonium bromide (CTAB).

ICG is used as an indicator dye for assessing cardiac output and liver function, so that photometric (Awni and Bakker, 1989) and fluorometric (Hollins et al., 1987; Gui et al., 1997) methods have been described for its determination in plasma. Although its analytical applications as reagent have been scarce, it has been used for the fluorometric determination of serum albumin, γ-globulin, and α-lipoprotein after separation on a gel filtration column and by using a diode laser as the excitation source (Sauda et al., 1986). The detection limit obtained for albumin was ∼1−2 orders of magnitude better than the value obtained by conventional spectroscopic and fluorometric detectors. Also, the fluorescence quenching of ICG by hydroxyl radicals produced by hydrogen peroxide in the presence of Fe(II) has been applied to assay xanthine (Imasaka et al., 1988) and insulin (Imasaka et al., 1990) but, in this instance, the detection limit obtained for insulin was not an improvement over conventional absorption spectrometry due to baseline drift of the ICG fluorescence intensity. A disadvantage of ICG as reagent is that its fluorescence intensity is strongly quenched in aqueous solution, decreasing ∼50% in 2 h, which is ascribed to the formation of dimers (Sauda et al., 1986). However, this instability can be minimized in the presence of dimethyl sulfoxide (DMSO) (Williams et al., 1993).

As it has been recently shown for the fluorometric determination of ICG, the detection limit obtained by using a diode laser as light source (Gui et al., 1997) is ∼6 times lower than that obtained with a conventional lamp (Hollins et al., 1987). However, although the detection limit obtained for the casein method described in this paper could be probably improved by using a diode laser, the main purpose of this study was to extend the analytical applications of ICG and show the utility of the combined use of initial rate and long-wavelength fluorescence measurements to develop a very fast method for casein determination in which the background signal is minimized. Also, taking into account that an inherent problem of most lasers is that

* Author to whom correspondence should be addressed (fax 34-957-218606; e-mail qa1gohea@lucano.uco.es).

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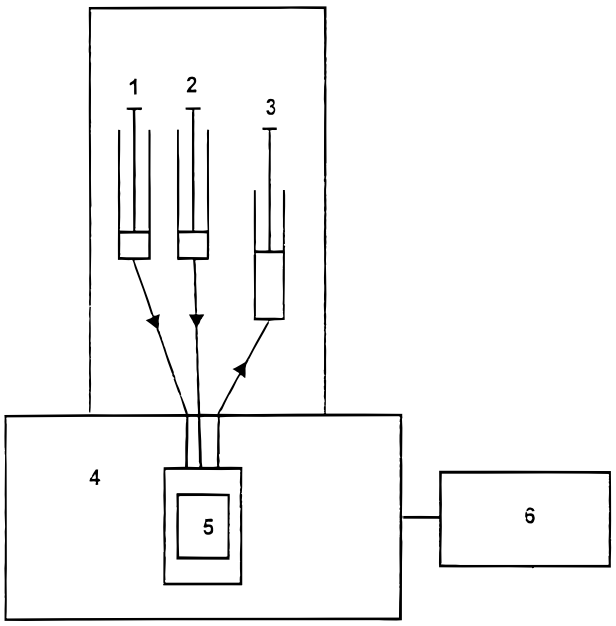

Figure 1. Scheme of instrumentation: 1 and 2, driving syringes containing sample and reagent, respectively; 3, stopping syringe; 4, spectrofluorometer; 5, observation cell; 6, computer.

they only emit a few discrete wavelengths, the choice of optimum excitation can be often difficult to match the output of the laser with the fluorophore excitation wavelength (Mank et al., 1992).

MATERIALS AND METHODS

Instrumentation. An SLM-Aminco (Urbana, IL) AB2 luminescence spectrometer, equipped with a 150 W xenon lamp, was used. A 9050 visible and near-IR monochromator and an R636-10 Hamamatsu red sensitive photomultiplier tube, both supplied by Sciencetech (London, ON, Canada), were fitted to the T-format configuration of the instrument to obtain long-wavelength measurements. The instrument was also furnished with a stopped-flow module (Loriguillo et al., 1987) supplied by Quimi-Sur Instrumentation (Seville, Spain) (Figure 1). The observation cell of the module has a path length of 1 cm, and the excitation and emission slits were adjusted to provide a 16 nm band-pass. The temperature of the solutions in the stopped-flow module and cell compartment was kept constant at 25 °C by circulating water from a thermostated tank. A Baker 10 extraction system (J. T. Baker Chemical Co., Phillipsburg, NJ) containing glasswool was used for sample preparation.

Chemicals. All chemicals used were of analytical reagent grade. A stock solution (1 g L^{-1}) of casein (Sigma, St. Louis, MO) was prepared in 2×10^{-2} M sodium hydroxide. A 2.7×10^{-4} M ICG (Sigma) solution was made in DMSO (Merck, Darmstadt, Germany). A 10^{-2} M CTAB (Serva, Heidelberg, Germany) aqueous solution and a 0.2 M borate buffer solution (pH 9.9) were also prepared.

Analytical Method. An aqueous solution containing ICG (3.6×10^{-5} M), CTAB (10^{-3} M), borate buffer (1.2×10^{-2} M), and DMSO (20% v/v) was used to fill one of the two drive syringes of the stopped-flow module. The other syringe was filled with a premixed aqueous solution containing standard or sample solution of casein at a final concentration between 3 and 100 μg mL^{-1}, CTAB (10^{-3} M), borate buffer (1.2×10^{-2} M), and DMSO (20% v/v). In each run, 0.15 mL of each solution was mixed in the mixing chamber and the variation of the fluorescence intensity with time throughout the reaction was monitored at $\lambda_{ex} = 760$ and $\lambda_{em} = 852$ nm for 5 s. All measurements were carried out at 25 °C. Data were processed by the computer, furnished with a linear regression program for application of the initial-rate method. The reaction rate was determined in ~2 s, and each standard or sample was assayed in triplicate. The blank signal was negligible.

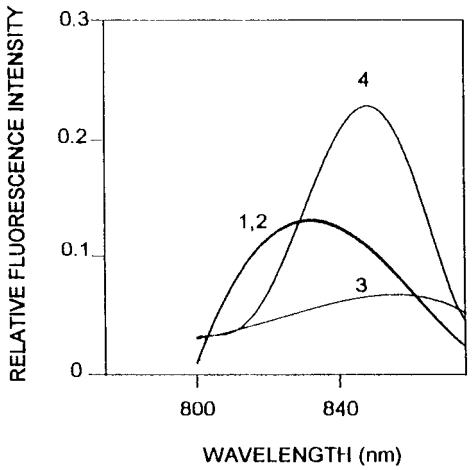

Figure 2. Emission spectra ($\lambda_{ex} = 760$ nm) of ICG in 20% DMSO alone (1) and in the presence of casein (2), CTAB (3), and both casein and CTAB (4). Apparent pH, 10.2; [ICG] = 1.8×10^{-5} M; [casein] = 40 μg mL^{-1}; [CTAB] = 10^{-3} M.

Determination of Casein in Milk and Dairy Products. Each sample (0.5 g) was heated in a water bath to 40 °C, in 15 mL of water before the addition of 0.5 mL of 10% acetic acid. After 10 min at 40 °C, 0.5 mL of 1 M sodium acetate solution was added and the solution was cooled to 20 °C. After filtration in an extraction system where 10 samples are simultaneously treated, the precipitate containing casein and fat was washed with acidified water and dissolved in 50 mL of 0.1 M sodium hydroxide. A volume of this solution was neutralized with hydrochloric acid and treated as described above. The standard additions method was used for each analysis.

RESULTS AND DISCUSSION

Spectral and Kinetic Study of the Chemical System. To improve the selectivity of fluorometric methods, there is a general trend to use near-infrared fluorogenic reagents, so that analytical measurements are not affected by potential spectral interferences from the sample matrix, as often occurs when conventional fluorescent reagents are used. Also, the short fluorescence lifetime of those compounds decreases the probability of nonradiative quenching processes (Patonay and Antoine, 1991). Thus, with the purpose of developing a fluorometric method for casein determination, ICG was chosen to study its potential use as reagent. As indicated above, the fluorescence intensity of ICG is quenched in aqueous solution owing to the formation of dimers. A change in the spectral properties of ICG in the presence of a protein such as bovine serum albumin has been described (Sauda et al., 1986), obtaining an increase in the fluorescence intensity of the reagent, which was ascribed to the formation of a complex with the protein and the dissociation of the ICG dimers. However, as Figure 2 (curves 1 and 2) shows, the presence of casein does not affect the fluorescence intensity of ICG in 20% DMSO solution. These spectra were obtained at an apparent pH of 10.2, at which both casein and ICG are negatively charged, which could justify the lack of interaction between them.

With the aim of looking for a potential change in the fluorescent behavior of the ICG−casein system, the effect of cationic, nonionic, and anionic surfactants was studied. The fluorescent features of ICG were very

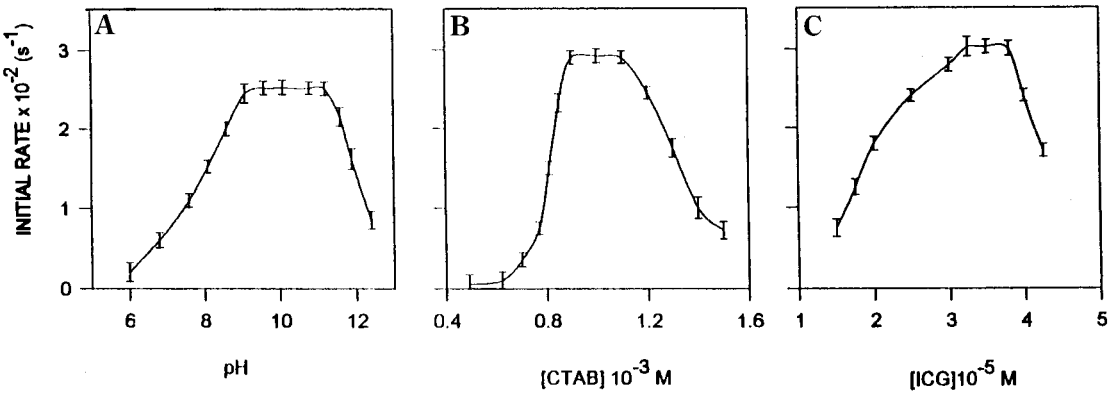

Figure 3. Effect of pH and CTAB and ICG concentrations on the initial rate of the ICG−CTAB−casein system. [CTAB] = 10^{-3} M; [ICG] = 3.6×10^{-5} M; [casein] = 10 μg mL^{-1}.

dependent on the CTAB concentration. Thus, in the presence of CTAB micelles, the fluorescence intensity increased and a bathochromic shift in the maximum emission wavelength, from 835 to 850 nm, was observed. However, CTAB quenched the fluorescence of ICG at a concentration below the critical micelle concentration (cmc) (Figure 2, curve 3). This behavior could be ascribed to the fact that the dye molecules are sheltered from nonradiative processes by the micellar environment, whereas the interaction between the possitive charge of CTAB monomers and the negative charge of ICG could give rise to a change in the electronic distribution of the dye, causing the loss of fluorescence. This possible explanation was verified by the fact that the fluorescent features of ICG were not affected by the presence of Triton X-100 and sodium dodecyl sulfate (SDS), nonionic and cationic surfactants, respectively. The study of the effect of casein in the ICG−CTAB system showed that, whereas the protein did not affect the emission spectrum of ICG in the presence of CTAB micelles, it increased the fluorescence intensity of ICG when this was in the presence of CTAB monomers (Figure 2, curve 4). This behavior could be ascribed to the fact that, owing to the numerous negative charges of casein at a pH above its isoelectric point, CTAB has a higher affinity for the protein than for ICG, so that the presence of casein in the reaction mixture releases the ICG, which retrieves its native fluorescence. Thus, the fluorescence of this system is the result of ionic forces between oppositely charged molecules. A similar behavior was found for lysozyme (Gala et al., 1996a) and gliadins (Gala et al., 1996b) by using the Cresyl Violet−SDS−protein system, but in this instance the dye and the surfactant were positively and negatively, respectively, charged and the surfactant showed a great affinity for the protein.

Although the fluorescence intensity obtained when the ICG−CTAB−casein system has reached the equilibrium was proportional to the casein concentration, the use of initial rate measurements allows the temporal discrimination of the analytical signal to be obtained as the static background signals of the blank and of potential interferences from the sample matrix can be avoided. As the equilibrium of this system was reached very quickly (~1−2 s), its kinetic behavior was studied by using a stopped-flow mixing technique, so that the dye and the surfactant were placed in a syringe of the stopped-flow module and the protein in the other. This technique allows measurements to be made shortly after the reactants have been mixed and analytical results to be acquired in only a few seconds, which is very useful for routine determination of casein.

Optimization of Variables. The variables affecting the system were optimized by the univariate method. All reported concentrations are initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing). Each kinetic result was the average of three measurements. Those values yielding the minimum possible standard deviation for the initial rate, under conditions where the reaction order with respect to the species concerned was zero or near zero, were taken as optimal.

As described above, the fluorescence of ICG decreases in aqueous solution. Although the stabilization is not total in the presence of DMSO, the fluorescence notably increases in this medium. Thus, the effect of the concentration of this solvent on the system was the first variable studied. The highest initial rate values were obtained by including between 17 and 25% DMSO in each solution in each syringe. The pH dependence of the system was checked over the range 5−12 (Figure 3A) using sodium hydroxide and hydrochloric acid solutions. The optimum apparent pH was 9.2−11.2, so that a borate buffer of pH 9.9 was used to adjust the sample pH (the apparent pH of the reaction mixture was 10.6 as measured in the waste). A 1.2×10^{-2} M concentration of this buffer in the solutions held in each syringe was chosen.

The CTAB concentration plays a basic function in this system. The maximum initial rate was obtained in the range 9×10^{-4}−1.1×10^{-3} M (Figure 3B). The blank signal was negligible up to the last concentration of this range but increased at higher concentrations, so no difference between the sample and blank was observed at a CTAB concentration close to 1.6×10^{-3} M. The cmc of CTAB in this system was measured with a stalagmometer and, also, by using fluorescence measurements. The value found in both instances was 1.1×10^{-3} M, which is very close to that reported in pure aqueous solution (9.2×10^{-4} M) (Rosen, 1978). These results show that the effect of CTAB monomers in the ICG fluorescence is opposite to that caused by the CTAB micelles. Figure 3C shows that the initial rate was maximum and independent of the ICG concentration in the range $(3.0-3.8) \times 10^{-5}$ M. Finally, the study of the effect of the temperature showed that only a slight increase in the initial rate was obtained when this variable increased from 25 to 40 °C, increasing also the blank signal. Thus, a temperature of 25 °C was chosen to avoid the subtraction of the blank signal.

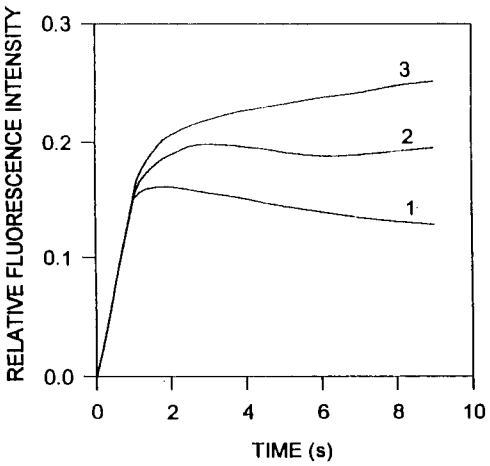

Figure 4. Kinetic curves obtained for 60 μg mL^{-1} of casein in the absence (1) and presence of whole milk (2) and yogurt (3). [ICG] = 3.6×10^{-5} M; [CTAB] = 10^{-3} M.

Analytical Features. The fluorescence−time curves obtained under the optimum conditions for different amounts of casein were processed by using the initial rate method. The calibration graph, obtained by using 11 casein stardards, was linear over the range 3−100 μg mL^{-1} and conformed to the equation v (s^{-1}) = $(2.5 \pm 0.1) \times 10^{-3}$ (s^{-1} μg^{-1} mL) [casein] + $(3.0 \pm 0.8) \times 10^{-3}$ (s^{-1}). The Pearson's correlation coefficient (r) was 0.998. The detection limit, as defined by IUPAC (Long and Winefordner, 1983), was 0.9 μg mL^{-1}. The precision of the method was assessed at two concentrations of casein, 6 and 60 μg mL^{-1}. The relative standard deviations (n = 10) were 1.8 and 1.5%, respectively. With regard to selectivity, the method can be considered universal for protein determination, so that a prior separation of casein will be required for its determination in the presence of other proteins. However, after this separation, the fast development of the system allows the initial rate to be measured in only ∼2 s, by using a stopped-flow mixing technique, which reduces the manipulations involved in the determination step and makes possible the analytical measurements to be obtained shortly after mixing. Thus, although incompletely automated, the method could be a useful alternative to the routine determination of casein.

Applications. To check the usefulness of the proposed kinetic method for the determination of casein, various types of milk samples and dairy products were analyzed according to the above procedure and by using the standard additions method. Figure 4 shows the kinetic curves obtained for a standard of casein and two samples, namely whole milk and yogurt, all of which contained the same final concentration of casein. As can be seen, the initial rate is the same for the three samples but, after 1 s, the fluorescence intensity depends on the type of sample, which shows the effect of the sample matrix. In addition, a constant value of the analytical signal is not obtained, which precludes the use of equilibrium measurements. Thus, these results show the utility of kinetic methodology for the determination of casein with this system. Table 1 summarizes the results found by using the proposed method and those obtained with the Kjeldahl method, which was used as reference. As can be seen, both methods give rise to very similar results. Table 2 shows the analytical recoveries obtained by adding three different amounts of casein to each sample and subtracting the results obtained for similarly prepared unspiked samples. The values obtained ranged from 96.8 to 103.1%, with a mean of 100.1%. Although the application of the method to the analysis of real samples requires the previous separation of casein, the overall process for the analysis of 10 samples, including sample preparation, takes ∼60 min, which is shorter than the Kjeldahl nitrogen determination.

Table 1. Determination of Casein in Milk Samples and Dairy Products

	casein found (g/100 g)	
sample	kinetic method[a]	Kjeldahl method
whole milk	2.6 ± 0.1	2.67
skimmed milk	2.40 ± 0.08	2.36
raw milk	2.6 ± 0.1	2.64
yogurt	2.9 ± 0.1	2.97
ice cream	3.1 ± 0.1	3.15
cream cheese	3.0 ± 0.1	2.98

[a] Average of three determinations ± SD.

Table 2. Recovery of Casein from Milk Samples and Dairy Products

sample	added (g/100 g)	found[a] (g/100 g)	recovery (%)
whole milk	2	2.0 ± 0.1	100.2
	3	2.96 ± 0.07	98.7
	4	4.05 ± 0.09	101.2
skimmed milk	2	2.02 ± 0.08	100.8
	3	2.93 ± 0.06	97.6
	4	3.90 ± 0.09	98.2
raw milk	2	2.1 ± 0.1	102.8
	3	3.04 ± 0.08	101.3
	4	3.9 ± 0.1	96.8
yogurt	2	2.04 ± 0.07	102.1
	3	2.99 ± 0.09	99.8
	4	4.1 ± 0.2	101.7
ice cream	2	2.02 ± 0.05	101.0
	3	2.96 ± 0.08	98.6
	4	4.1 ± 0.1	103.1
cream cheese	2	1.94 ± 0.08	97.1
	3	3.0 ± 0.1	100.7
	4	4.0 ± 0.1	98.9

[a] Average of three determinations.

Conclusions. The results of this work show the advantageous application of the combined use of kinetic methodology and long-wavelength fluorescence measurements for the determination of casein in foods. Although the method involves a previous separation step, which is common in all casein methods, the use of a stopped-flow mixing technique notably reduces the time required in the determination step as kinetic measurements are obtained shortly after mixing.

As known, methods based on covalent labeling usually offer a higher specificity and stability than those based on noncovalent labeling. However, the lack of reactive groups in many of the long-wavelength fluorogenic labels now available has given rise to the fact that most of the methods involving the use of these reagents are based on electrostatic forces between the analyte and the label (Patonay and Antoine, 1991). Unlike these methods, the kinetic method described here is based on two simultaneous processes: the reaction between casein and CTAB and the elimination of the quenching caused by this surfactant of the fluorescence of ICG, so that the initial rate of the system is directly related to casein concentration. Thus, the dynamic character of the analytical signal offers two significant features to the method: on the one hand, the measurement is not affected by the potential instability of the fluorescence intensity when the system has reached or is close to the

equilibrium and, on the other, the method is free of interferences from the sample matrix, which precipitates together with the casein in the separation step.

ABBREVIATIONS USED

CTAB, cetyltrimethylammonium bromide; DMSO, dimethyl sufoxide; ICG, Indocyanine Green; SDS, sodium dodecyl sulfate.

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