JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Physicochemical Properties of Acidified Skim Milk Gels Containing Cocoa Flavanols

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ABSTRACT: The physicochemical properties of acidified milk gels after the addition of cocoa flavanols were studied. As the flavanol level increased (from 0 to 2.5 mg/g), syneresis and gel elasticity (tan δ) were found to significantly increase and decrease, respectively. Flavanol addition reduced the stress at fracture, with no changes in fracture strain, suggesting that the bond type (i.e., covalent vs noncovalent) was the underlying factor explaining the ease of fracture. Gels made from recombined milks containing the casein fraction of heated milk and the serum of heated flavanol/milk mixtures showed the lowest values of G' and fracture stress. It was concluded that whey proteins/flavanol interactions were responsible for the poor mechanical properties of flavanol-added acidified milk gels. High-performance liquid chromatography analysis of milk sera showed that 60% of the total available monomeric flavanols was found in the serum phase from which 75% was non-associated to whey proteins. Concomitantly, >70% of flavanols with degree of polymerization >3 were found to be associated with the casein fraction.

KEYWORDS: Acidified milk, flavan-3-ols, epicatechin, cocoa, gel fracture, syneresis

INTRODUCTION

Flavanols are a particular class of flavonoids, comprised of (±)-catechins, (±)-epicatechins, and various gallic acid ester derivatives [e.g., (±)-epigallocatechin and (±)-epigallocatechin gallate]. The flavanols epicatechin and catechin can exist in simple monomeric forms but can also link together to form oligomeric and polymeric molecules, more commonly known as procyanidins. All of these compounds are a type of secondary plant metabolites that are widely distributed in nature and, hence, can be plentiful in certain foods and beverages commonly found in the human diet. In this context, foods and beverages including apples, grapes, red wine, and cocoa products can be particularly rich sources of (epi)catechins and their oligomers (procyanidins)[1−3] (see Figure 1). Over the past decade, there has been accumulating human evidence that the inclusion of flavanol- and procyanidin-containing foods into a diet is associated with a range of cardiovascular-related benefits including improved vascular function,[4−6] blood pressure,[7] and attenuated platelet reactivity.[8,9] It follows that the putative health benefits of these natural compounds have strengthened interest among the private sector on the development of mainstream food products and dietary supplements that successfully incorporate and deliver these compounds into the diet.

The consumption of products rich in flavanols and procyanidins (e.g., red wine, dark chocolate, or apples) is commonly associated with oral astringency—the sensation of dryness and loss of lubrication in the palate. Astringency, it follows, is related to the precipitation of salivary proteins via nonspecific interactions with compounds like (−)-epigallocatechin-3-*O*-gallate, B2 and B3 procyanidin dimers, and C1 trimers.[10−14] These interactions seemed to be particularly favored in the presence of protein/peptide chains rich in the amino acid proline (hence called proline-rich proteins or PRPs) such as salivary proteins.[11] Protein complexation and precipitation are important properties associated with flavanols and their procyanidins, particularly those with large molecular weight.[11,12] This is of scientific and technological relevance in the structural build up of protein-based edible materials, as such interactions could cause the modification of food microstructure and, ultimately, of the food eating experience.

Most dairy products, particularly the fermented kind, are regularly chosen as delivery vehicles for a variety of bioactive ingredients (e.g., phytosterols and pro- and prebiotics, etc.). Given the well-established interactions between flavanols and specific amino acids and proteins, how these interactions may affect the micro- and macrostructures of dairy-based food systems is an important consideration in the development of novel food systems containing these bioactive constituents[15]. In this context, aside from astringency, flavanols and procyanidins contribute to bitterness, color modification, and loss of protein functionality, which then become the main challenges encountered during food product design.

Interesting findings have been published around the physicochemical properties of milk and dairy products after addition of polyphenolic compounds. It has been reported that caffeic acid, 1,2-dihydroxy-naphtalane, or 3,4-dihydroxy-benzaldehyde increased the stability of milk against instant coagulation during heating at 140 °C—a treatment similar to ultra high temperature sterilization (UHT).[16,17] The proposed mechanism relied on the thermal oxidation of the polyphenol to its corresponding quinone, which, after reacting with nucleophilic amino acids, inhibited the dissociation of κ-casein from the casein micelle, preventing protein aggregation. Specific flavanols, such as (−)-epicatechin, were found to inhibit the formation of Maillard-related products during the UHT treatment of milk at levels as low as 1 mM and to reduce the perceived "cooked" flavor normally associated with heated milk at levels around 0.01−0.02%.[18,19] In contrast, little is known on the collective

Received: March 11, 2011
Accepted: May 11, 2011
Revised: May 9, 2011
Published: May 11, 2011

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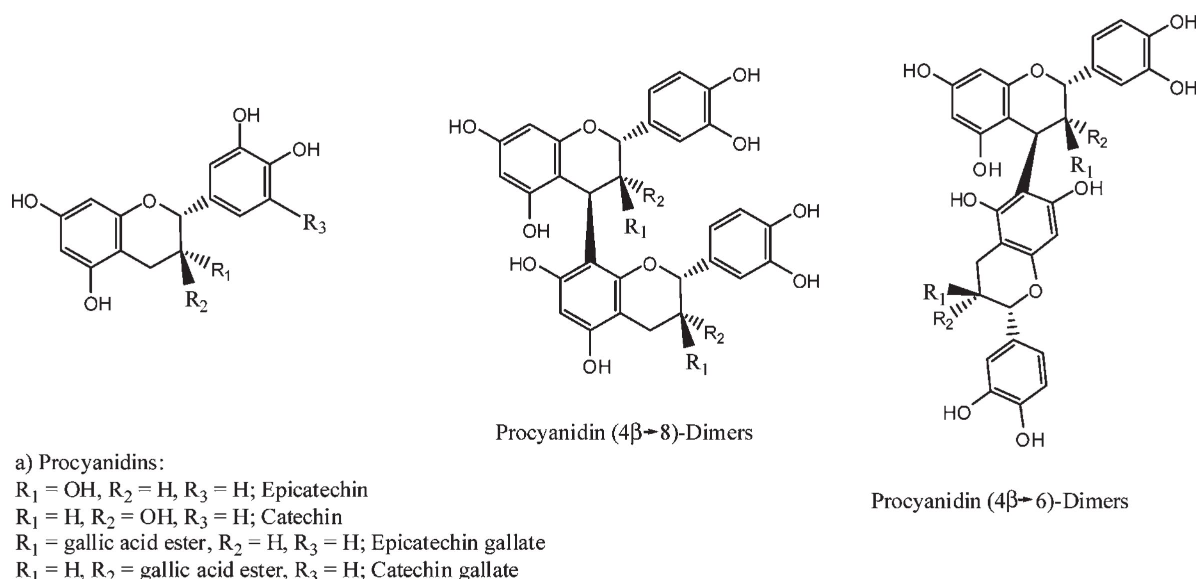

Figure 1. Representative structures of procyanidins monomers and dimers.

Table 1. Typical Composition of the Commercial Cocoa Extract Used in This Study

compound	% (w/w)
CF (DP 1−10)	45
monomers	6.3
dimers	6.8
trimers	5.8
tetramers	6.3
DP 5−10	19.8
DP > 10	15
carbohydrates	8
fat	5
protein	3
moisture	5
ash	2
theobromine	6
caffeine	1
uncharacterized material	5

behavior of isolated blends of naturally occurring cocoa flavanol (CF)/procyanidins mixtures in the presence of milk or dairy products.

Milk proteins are regarded as highly functional food ingredients. In the manufacture of fermented milk products, particularly yogurt, caseins provide a structural scaffold, whereas whey proteins strengthen the built structure. Given that caseins are PRPs, it is likely that these proteins would directly interact with any added flavanol or procyanidins. To study this, we engaged in a series of experiments to examine the physiochemical properties of acidified milk gels after the addition of a cocoa extract rich in flavanols and procyanidins. It was hypothesized that the addition of this CF and procyanidin mixture to soon-to-be-acidified milk may affect the mechanical and textural properties of the final product (i.e., yogurt). The objective of this investigation was to contribute to the understanding of the interactions between these specific bioactives in cocoa and milk proteins in the, up-to-now unexplored, context of acidified milk gels. The practical relevance of this work is two-fold: (a) The study deals with the behavior of a dairy food after the addition of a concentrated, natural, and complex mixture of CF and procyanidins, and (b) in doing so, it enables the design of dairy products that deliver flavonoid components and maintain their expected physical and organoleptic properties.

MATERIALS AND METHODS

Materials. High-performance liquid chromatography (HPLC) grade methylene chloride, methanol, and acetone were from Mallinckrodt Baker (Phillipsburg, NJ), while glacial acetic acid and glucono-δ-lactone (GDL) were from Sigma (St. Louis, MO). Water was of Milli-Q grade unless stated otherwise. The composition of the cocoa extract used (Mars Botanical, Rockville, MD), rich in flavanols and procyanidins, is summarized in Table 1. Relevant to this study was its concentration of CF; CF as referenced herein comprise the total sum of flavan-3-ols and procyanidins with a degree of polymerization up to and including 10 (DP 1−10).

Reconstituted Skim Milk Powder (RSMP). Low-heat skim milk powder (Dairy America, Fresno, CA) from a single batch was reconstituted to 10% w/w in Milli-Q water and stirred for at least 4 h and kept overnight at refrigeration temperature (4 °C) to allow for equilibration. Sodium azide (0.02% w/w) was added to prevent microbial growth. Milk was tempered to ambient temperature before the start of experiments. In the case of samples containing CF, a suspension of cocoa extract was prepared to a concentration of 2.5 mg/g of suspension by dispersing the extract in Milli-Q water under constant agitation and nitrogen sparging (to minimize oxidation) for at least 4 h. This suspension was then centrifuged at 20500*g* for 10 min at 20 °C in an Allegra X-22 centrifuge with rotor type F0630 (Beckman Coulter, Fullerton, CA) to remove any insoluble material. The amount and chemical profile of the insoluble fraction did not increase nor change with longer centrifugation times (data not shown). Skim milk powder was then reconstituted using the supernatant CF solution to a 10% w/w milk solids concentration. To assess the impact of CF concentration on the measured properties, dilutions of the original CF supernatant solution (prior to adding skim milk powder) were made with water as to obtain milk/flavanol suspensions with CF concentrations of 2.5, 2.0, 1.5, 1.0, and 0.5 mg/g.

Heat Treatment and Acidification. Reconstituted milk was heated in batches of approximately 50 mL by placing beakers into a water bath at 90 °C. After this temperature was reached (3−4 min), another 10 min elapsed before samples were removed and rapidly cooled to room temperature by quenching in an ice bath. To start the acidification process, heated and cooled milk was brought to 40 °C, and GDL was added (1.3% w/w) under constant stirring. After 2 min, the sample was split in two—an aliquot was transferred to the rheometer, and the rest was used to follow the pH until it reached a value of 4.5 by use of a SevenMulti pH meter (Mettler Toledo, Schwerzenbach, Switzerland).

Dynamic Rheological Measurements. Time sweep oscillatory measurements were performed by applying a constant strain of 0.01% at a frequency of 1 Hz by means of a Physica MCR501 stress-controlled rheometer (Anton-Paar, Graz, Austria) equipped with a Peltier temperature controller with a Searle device consisting of a cup and bob with diameter of 28.91 and 26.66 mm, respectively. Approximately 18 mL of sample was transferred into a preconditioned (40 °C) rheometer cup. A thin layer of vegetable oil was poured on top of the sample to prevent evaporation. Experiments were run for 6 h at 40 °C. The gelation time and pH were taken as those values when the storage modulus (G') > 1 Pa.[20] After curing for 6 h and at constant temperature (40 °C), a constant shear rate (5×10^{-4} s^{-1}) was applied to the sample until fracture of the gel occurred.[21] The fracture stress was taken as the maximum in the stress/strain curve during application of constant shear.

Syneresis. To estimate the level of spontaneous release of liquid from within the gels, sample aliquots (10 mL) were poured in graduated disposable centrifuge tubes, and a film of vegetable oil was placed on top to avoid evaporation. The tubes were then incubated in a water bath at 40 °C for the extent of the gelation time (6 h). Visual inspection easily revealed syneresis. The expelled sera were decanted into clean test tubes to estimate total volume.

Isolation of Serum and Casein Fractions from Milk. Milk samples (∼24 mL) were centrifuged at 20500*g* for 1 h at 20 °C in a Beckman Coulter Allegra X-22 centrifuge with rotor type F0630 (Beckman Coulter). The serum material (supernatant) (∼19 mL) was removed, and the casein pellet was left in the tube for subsequent reconstitution experiments. The absence of casein in the supernatant was corroborated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (not shown). In some cases, the sera obtained after centrifugation were further fractionated by means of ultrafiltration using a Labscale, tangential flow filtration system (Millipore, Billerica, MA) coupled with a Pellicon XL cartridge with a molecular mass cutoff of 10000 Da. The CF content of the protein-free filtrates was also measured.

Recombined Milks from Casein Pellets and Sera Samples. The different fractions obtained after centrifugation of the heated milks were identified as follows: casein micelle pellet from milk (HCM), serum from milk (HWP), casein micelle pellet from CF-containing milk (HCM + CF), and serum from CF-containing milk (HWP + CF). Recombined milks were obtained from the separated fractions by blending the appropriate proportions of serum and casein using an Ultraturrax T-25 basic high-shear mixer (IKA-Werke, Wilmington, NC) for 3 min at 17500 rpm. It has been reported that by means of this procedure, the particle size distribution of the redispersed casein micelles is similar to that of the original milk.[22]

Normal Phase HPLC Analysis of CF. To accurately quantify the total CF content of the milks, sera, and ultrafiltrated materials, a liquid extraction step was performed by weighing 15 g of sample (e.g., milk, serum, and ultrafiltrate) into a 50 mL volumetric flask and diluting to volume with a 0.5% acetic acid (HOAc) in acetone (CH_3OCH_3). After thorough mixing, an aliquot of the supernatant was filtered through a 0.45 μm PTFE filter into a vial for analysis. HPLC was used to quantify the total CF content in milk and milk sera based on the method of Adamson et al.[23] Separation was performed on an Agilent 1100 HPLC system consisting of a quaternary pump, a solvent degasser, an autosampler, a thermostat column compartment, a diode-array detector, and a fluorescence detector (Agilent Technologies, Palo Alto, CA). Normal phase separation based on degree of polymerization (DP) was carried on a 250 mm × 4.6 mm i.d. Luna Silica (2) column (Phenomenex, Torrance, CA) with a particle size of 5 μm at a column temperature of 37 °C. The chromatographic mobile phase consisted of methylene chloride (CH_2Cl_2), methanol (CH_3OH), and acetic acid:water (1:1, v/v) (HOAc:H_2O). Starting mobile phase conditions were 82% CH_2Cl_2, 14% CH_3OH, and 4% HOAc:H_2O. Subsequently, CH_3OH was increased to 28.4% after 30 min, 42.8% after 50 min, and 86.0% after 51 min. Throughout the chromatographic run, the HOAc:H_2O ratio was held at a constant of 4%. Fluorescence detection was conducted with an excitation wavelength of 276 nm and emission at 316 nm. External standard least-squares calibration curves were generated for epicatechin and its oligomers up to DP = 10 in the concentration range of 0.14−12.0 mg/mL; quantitation was based on a comparison of peak areas.

Statistical Analyses. All experiments were performed at least in triplicate. Results were reported as means with corresponding standard deviations. Data were analyzed for differences using analysis of variance, and the Tukey's paired comparison procedure was used to identify significant differences between means. *P* values of less than 0.05 were considered statistically different.

RESULTS AND DISCUSSION

Rheological Properties. *Gel Formation.* The addition of CF to skim milk showed a distinct effect on the rheological build up of the gels during acidification. In the CF-free sample, the typical gelation profile of heated milk was observed. This typical behavior is characterized by the presence of a sudden change in the slope describing the development of the storage modulus (G') with a concomitant maximum in the value of the tan δ (a measure of viscoelasticity).[20,24] This phenomenon has been associated with the solubilization of colloidal calcium phosphate (CCP) from the interior of the casein micelles, which seems to weaken the overall gel structure until complete solubilization occurs. Upon addition of CF, the change in the slope in the development of G' was no longer observed, and a reduction in the max tan δ value without a change in the pH of gelation (5.45 ± 0.05) was observed (see Figure 2 and Table 2). Interestingly, the final G' values (or gel stiffness) compared to that of CF-free heated milk were the same at the end of the experiment (230 Pa) and were well within the range of those previously published.[24,25] As the concentration of flavanols was reduced (by dilution of the original CF/milk suspension), the change in slope in the G' trace was also less significant (see inset, Figure 2). Furthermore, regardless of the flavanol content, final G' values were not significantly different (see Table 2). Conversely, tan δ values were dependent on CF concentration. The maximum values of tan δ (during the early stages of gelation) significantly ($p < 0.01$) decreased with increasing flavanol concentration, from 0.56 for CF-free heated milk to 0.46 for milk containing 2.5 mg/g CF. On the other hand, the final tan δ value behaved in an opposite fashion and increased with increasing flavanol concentration, attaining statistical significance ($p < 0.05$) for milks containing 2.0 and 2.5 mg/g CF as compared to CF-free heated milk (see Table 2). These differences suggest that the addition of CF did affect the gelation process in a concentration-dependent fashion. The different tan δ profiles suggested that the nature and strength of the bonds forming the gel network underwent a series of dynamic rearrangements[26,27] and that the mechanism

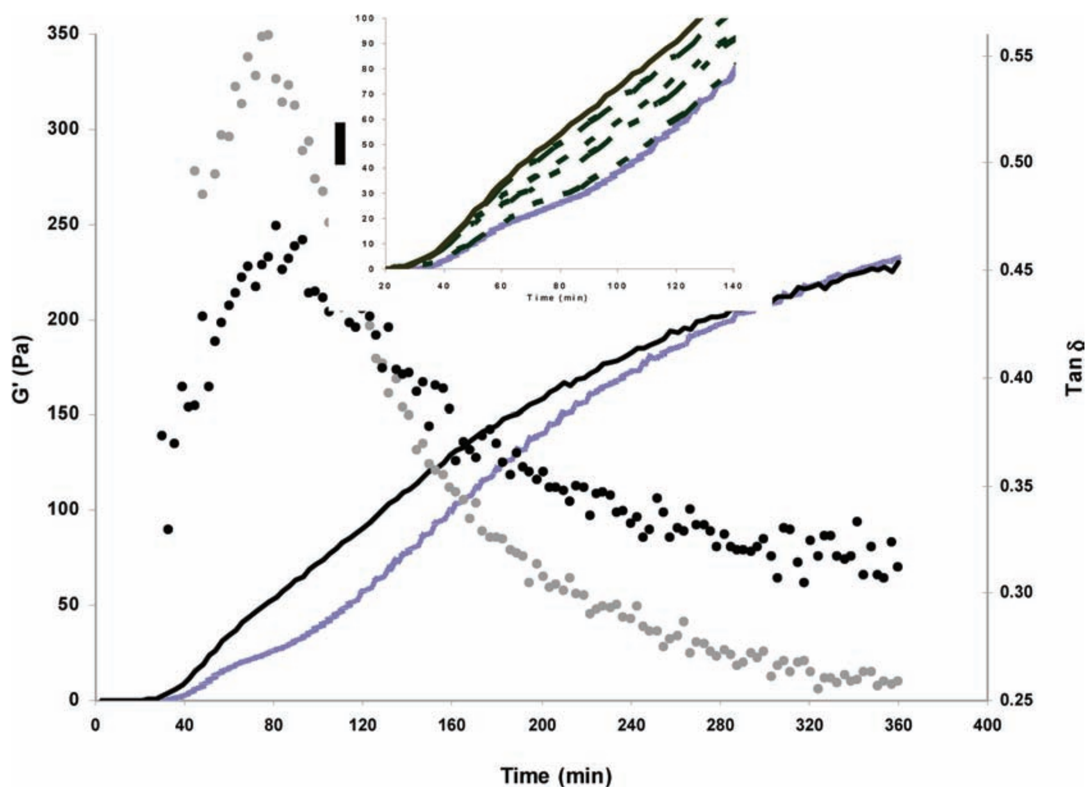

Figure 2. Development of storage moduli (G') and tan δ for acidified heated skim milk gels containing 2.5 or 0 mg/g CF (black and gray traces, respectively). The inset shows the early stages of G' as a function of flavanol concentration (from left to right, approximately 2.5, 2.0, 1.5, 1.0, 0.5, and 0.0 mg/g).

Table 2. Properties of Acidified Skim Milk Gels Made from Heated Milk (90°C, 10 min) with or without Added CF[a]

		flavanol concentration (DP 1−10) mg/g				
property/sample	0.0	0.5	1.0	1.5	2.0	2.5
pH of gelation (G' > 1 Pa)	5.42	5.48	5.46	5.39	5.47	5.46
max G' (Pa)	233	217	223	223	226	230
max tan δ	0.57 a	0.57 a	0.51 b	0.48 bc	0.47 bc	0.47 c
final tan δ	0.26 a	0.28 a	0.29 a	0.29 a	0.31 b	0.31 b
fracture stress (Pa)	104 a	69 b	55 bc	47 c	54 c	60 bc
fracture strain (%)	69	62	59	59	63	65

[a] Values are the averages of at least three determinations. Different superscripts within each row denote that samples were significantly different ($p < 0.01$).

associated with these changes might or not involve a direct contribution from the solubilization of CCP.

Fracture Behavior. The stress and strain at fracture were determined by applying a constant shear rate to the preformed gels until the stress value started to decline. The results showed that CF addition significantly reduced ($p < 0.05$) the shear stress needed to break the gels (Table 2). The fracture stress for CF-free heated milk was approximately 104 Pa, whereas for the gel made from milk containing just 0.5 mg/g flavanol, it was 69 Pa. This suggests that even at low concentrations, the addition of CF can induce significant changes to the overall nature of the gel network. The decrease in fracture stress seemed to partially correlate with the increasing values of tan δ. However, it was notable that no significant differences were found in the strain at fracture regardless of CF level (≈59% for the heated CF milk and ≈65% for the CF-free heated milk) (Table 2). This can be potentially explained by the idea that fracture strain has been related to the curvature of the strands that hold the gel network together—the larger the curvature, the larger the strain at fracture.[28] This suggests that the overall curvature of the strands of the gels studied herein was not modified by CF addition.

Covalent bonds are said to have higher bond energy than noncovalent (physical) bonds.[29] Hence, their fracture requires higher applied force. This means that the fracture behavior of a gel is more sensitive to covalent bond formation than to its firmness (G') (see Figures 2 and 5). This is because in large deformation, bond breakage is required, whereas in small deformation, bonds need only to be bent.[26] As such, the significantly lower fracture stress values found after addition of CF to milk could be related to a reduced level of covalent bonds (i.e., disulfide, S−S) formed during heating and/or acidification.

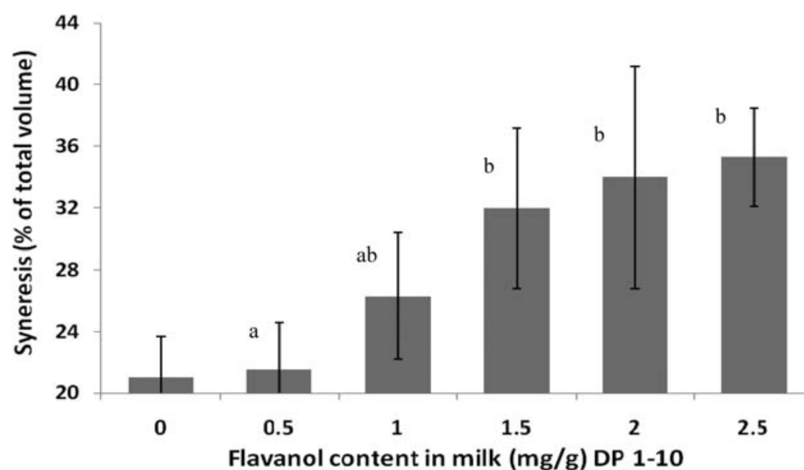

Figure 3. Syneresis in acid skim milk gels (after 6 h of incubation, 40 °C) as a function of flavanol content. Different superscripts denote that samples were significantly different ($p < 0.05$). Bars represent the standard deviation.

Gelation experiments of heated whey protein isolate (WPI) to which a thiol-blocking agent was added (to inhibit disulfide bond formation during acidification) showed that the initial increase in G' was similar to that of a gel with no blocking agent. Over time, the G' of gel containing blocking agents reached a plateau, whereas the other continued to increase and seemed not to reach equilibrium even after 18 h.[30] In this study, even after 18 h into gelation, no significant differences were found in G' values of CF-free heated milk and heated milk containing 2.5 mg/g CF (340 Pa) (data not shown). However, the fracture stress for heated milk continued to increase to a value of 160 Pa (60% higher than at $t = 6$ h), whereas for the milk containing 2.5 mg/g CF, the increase was only 15% (to approximately 70 Pa). This behavior in fracture stress was consistent with that observed in acidified milk gels with inhibited covalent (disulfide) bond formation.[31]

Syneresis. The spontaneous accumulation of whey/serum at the surface of acidified milk gels (e.g., yogurt) is considered a major defect and has been related to either high incubation temperatures (>42 °C), rapid acidification (GDL vs starter cultures), low total solids, or combinations thereof.[27] The addition of increasing amounts of CF gradually enhanced the amount of syneresis observed after acidification (Figure 3), and for concentrations >1.0 mg/g, this was accompanied by extensive shrinkage (not shown). The syneresis levels were significantly higher ($p < 0.05$) for milks containing more than 1.0 mg/g CF. This suggests the presence of a threshold flavanol level at which syneresis was exacerbated. It is noteworthy that even in the absence of CF, there was a noticeable amount of syneresis in milk (≈20%).

It has been suggested that an increase in the value of the maximum tan δ is accompanied by a higher degree of syneresis.[27] Our findings showed the opposite behavior, since the level of syneresis increased with decreasing max tan δ value ($p < 0.05$ as compared to heated CF-free milk), whereas it increased with increasing final tan δ ($p < 0.05$ as compared to heated CF-free milk). Higher tan δ values are usually associated with "less elastic" gel networks with increased susceptibility to bond rearrangement and/or breakage.[27,32] It follows that reduced network elasticity is linked to enhanced serum release (and shrinkage). It is still unclear what stage of the gelation process contributes the most to syneresis, but the development of G' and tan δ provided some interesting insights. The CF concentration-dependent change in the slope of the development of G' and the concomitantly lower max tan δ values suggest the presence of an accelerated protein aggregation process leading to the formation of apparently stiffer or firmer gels (see inset, Figure 2). This phenomenon could be analogous to the multivalent cross-linking mechanism described for EGCG and β-casein by which the force needed to stretch a single casein molecule increased with increasing EGCG concentration.[33]

However, it is well-known that heat-denatured whey proteins play an important role on the strengthening of the aggregated casein structure by acting as bridging material within the acid gel network.[31] It then becomes of interest to recombine the fractions isolated by centrifugation in such a way that the serum from heated milk was used to reconstitute the casein pellet from the heated milk containing 2.5 mg/g CF (HCM/CF + HWP) and vice versa (HCM + HWP/CF). These reconstituted milks were then subject to acidification to follow their mechanical properties to ascertain whether any of the fractions played a particular role in gel formation. The evolution of G' and tan δ of the HCM/CF + HWP system resembled that of the milk with 2.5 mg/g CF, and both parameters showed practically the same values at the end of the gelation period (∼230 Pa and 0.31, respectively)—see Figure 4. Additionally, the typical change in slope in the G' trace at the early stages of gelation was also absent. Both of these observations suggest that this phenomenon was mostly related to the interactions between CF and casein micelles, as the change in the slope in the development of G' was present in the trace for the HCM + HWP/CF system. As mentioned earlier, this change in slope (together with the maximum in tan δ values) has been associated with a partial loosening of the nascent gel due to solubilization of CCP.

On the other hand, the system comprised by the pellet of milk and the CF-containing serum (HCM + HWP/CF) showed a tan δ trace identical to that of heated CF-free milk and a significantly lower ($p < 0.01$) G' value (120 Pa). Figure 5 shows the fracture behavior of these gels in comparison to those from heated milk and the heated milk containing 2.5 mg/g CF. The recombined system containing the casein/flavanol complex (HCM/CF + HWP) showed fracture stress and strain of approximately 56 Pa and 60%, respectively which, just as in the case of G' and tan δ, was identical to that of the 2.5 mg/g CF milk. On the other hand,

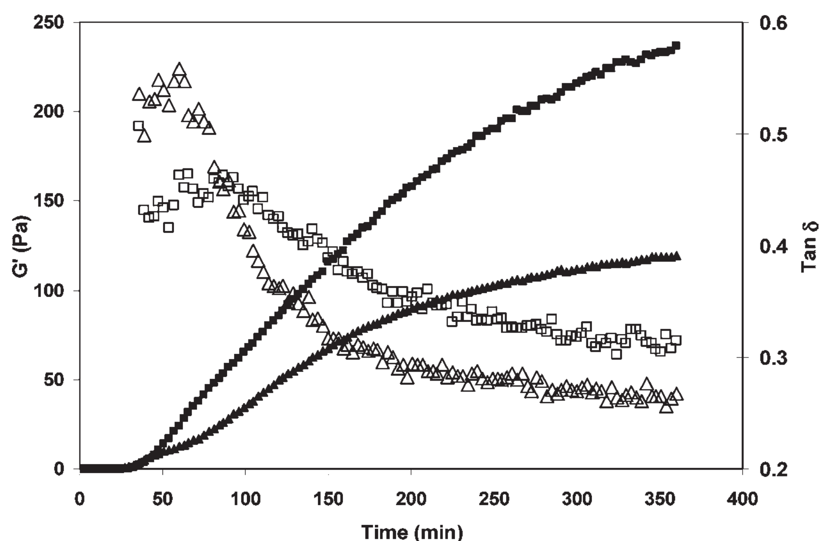

Figure 4. Development of storage moduli (G') (filled symbols) and tan δ (empty symbols) for acidified heated recombined skim milk gels made by combining the casein pellet from the milk containing 2.5 mg/g flavanols (obtained by centrifugation) and the serum of flavanol-free milk (HCM/CF + HWP) (■, □) and the casein pellet of flavanol-free milk with the serum from the milk containing 2.5 mg/g flavanols (HCM + HWP/CF) (▲, △).

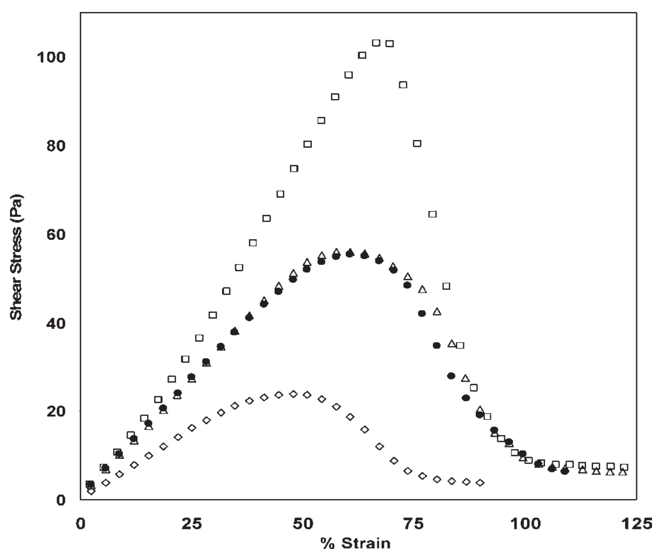

Figure 5. Shear stress as a function of applied strain (i.e., deformation) at constant shear rate (0.0005 s^{-1}) for acidified heated milk gels. Shear was applied after a 6 h curing period at 40 °C. The point where the stress started to decrease was taken as the fracture point. Heated milk (□), heated milk containing 2.5 mg/g flavanols (●), recombined milk made by combining the casein pellet from the milk containing 2.5 mg/g flavanols (obtained by centrifugation) and the serum of flavanol-free milk (HCM/CF + HWP) (△), and recombined milk made from the casein pellet of flavanol-free milk with the serum from the milk containing 2.5 mg/g flavanols (HCM + HWP/CF) (◊).

the HCM + HWP/CF system showed the lowest stress and strain at fracture (~22 Pa and 50%, respectively) and suggested that the interactions between whey proteins and flavanols hindered the structure building process of the acid gels and, hence, were mainly responsible for their fracture behavior.

Flavanol Partitioning. As discussed in the Introduction, one of the most important physicochemical properties of flavanols and procyanidins is their affinity for proteins, which is manifested by the formation of immutable protein−polyphenol complexes.[11] The nature of the complexes varies depending on the type of protein and/or polyphenol interacting—from soluble complexes that scatter light (haze formation) to large aggregates that tend to precipitate.[34] Caseins, as PRPs, show strong affinity for polyphenols, whereas whey proteins, thanks to their globular, tightly coiled nature, are hypothesized to have much lower affinities.[12] Given that milk possesses both types of proteins, quantifying the relative amounts of CF bound to either type could provide useful information as to explain the observed rheological behavior of the acidified milk gels. Ultracentrifugation (20500*g*, 1 h) allowed for the separation of flavanol-added milk into two discrete phases, a casein-rich pellet and a virtually casein-devoid, whey protein-rich serum. It was observed that the casein pellet of flavanol-added milks became purple and suggested that at least the anthocyanins present in the cocoa extract had preferentially migrated into this fraction.

Table 3 shows the CF concentrations of the starting milks as well as of their corresponding sera after centrifugation (measured by HPLC). The average initial concentration of the original (highest flavanol content) milk was 2.5 mg/g. It follows that the concentrations of the diluted milks (80, 60, 40, and 20% of the original milk concentration) were approximately 2.0, 1.5, 1.0, and 0.5 mg/g. The concentration of CF in the sera of these systems decreased in parallel with dilution, and it was mainly comprised of flavanols with a maximum DP of 5 (see also Figure 6). Interestingly, the mass fraction (ø) of flavanols in the sera increased with decreasing CF concentration of their corresponding milks (Table 3). For example, the mass fraction of flavanols in the serum-heated milk containing 0.5 mg/g CF was significantly higher than that of the milk containing the highest amount of flavanols (2.5 mg/g CF)—0.32 as compared to 0.22, respectively ($p < 0.01$). In this context and considering that whey proteins make up for 20% of the total protein available in milk, it would appear that the flavanol-to-whey protein binding ratio concomitantly increased with decreasing flavanol concentration in milk (from 1.08 to 1.6—see Table 3). The same binding ratio in unheated milk was found to be from 0.97 to 1.34. The probable relevance of these findings is 2-fold: (a) It suggests that regardless

Table 3. HPLC-Measured CF Concentrations (mg/g DP 1−10) of the Starting Flavanol Containing Milks and Their Corresponding Sera after Centrifugation [Also Expressed as Mass Fraction (ø) of the Total][a]

milks (mg/g CF, DP 1−10)	sera[b] (mg/g CF, DP 1−10)	mass fraction (ø) in the serum (max DP)	flavanol to whey protein binding ratio	n
2.5	0.47	0.22 a (5)	1.08	8
2.0	0.40	0.22 a (5)	1.11	12
1.5	0.31	0.24 a (5)	1.20	12
1.0	0.23	0.27 b (5)	1.33	12
0.5	0.14	0.32 c (5)	1.60	12

[a] The apparent binding ratio is the quotient from dividing the (ø) of flavanols in the serum by the (ø) of whey proteins in milk protein (0.2). Different superscripts within each column denote that samples were significantly different ($p < 0.01$). [b] After centrifugation of milk at 20500g, 1 h, 20 °C.

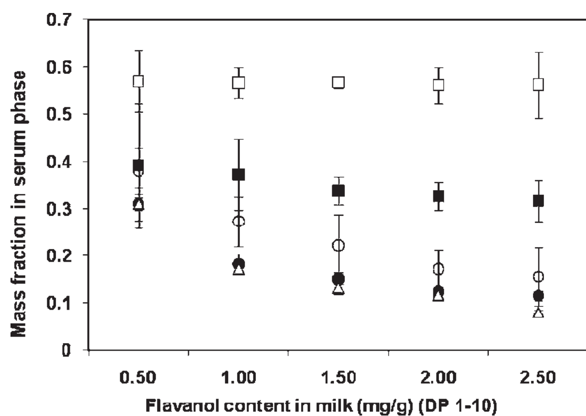

Figure 6. Mass fraction of individual flavanol species found in the sera of heated milks (after centrifugation at 20500g for 1 h) as a function of initial flavanol concentration. Monomers (□), procyanidin dimers (■), trimers (○), tetramers (●), and pentamers (△). Bars represent the standard deviation of the mean.

of the higher proline content in casein, there is no preferential binding to either the casein or the whey fractions as the percentage of CF found in each fraction correlates with their corresponding percentage of the total protein in milk (i.e., 80/20), and (b) it implies that heating enhanced the affinity of CF for the whey fraction of milk, suggesting that the nature of their interactions was modified during and/or after protein denaturation.

The mass fraction of the total monomeric species (catechin and epicatechin) found within the sera phases of heated milks was the same (approximately 0.57) regardless of the original concentration of CF (Figure 6). On the other hand, the mass fractions of the oligomers in the sera (DP 2−5) steadily and, in some cases, significantly decreased as a function of oligomer molecular weight (Figure 6). For example, the mass fraction of the trimers in the sera of heated milks decreased from ≈0.30 in the 0.5 mg/g CF milk to ≈0.10 in the 2.5 mg/g CF milk. It is suggested that this partitioning behavior responds to a higher affinity of caseins for high molecular weight flavanols[12,35] given that >70% of oligomers with DP 4−10 resided within the casein pellet after centrifugation. Because of the well-documented high reactivity of caseins with polyphenols,[12,33] it was expected that any flavanols found in the serum would be (at least in part) the result of the saturation of casein with flavanols and that casein saturation would have occurred as the content of flavanol in milk increased rather that in the way observed. It has been reported that β-lactoglobulin and α-lactalbumin interactions with polyphenols depended on the presence or absence of caseins: They appeared to be unaffected by the presence of caseins, whereas at a certain protein−polyphenol ratio, the formation of brown precipitates after heating (70 °C) was found in the absence of caseins.[36]

As shown in Table 3, only about 20% of the total flavanols added to the milk (which includes 60% of the total monomer) remained in the serum phase after centrifugation. Because the mechanical properties of the gels seemed to be governed by the composition of the serum phase, further examination of the nature of the interaction(s) between flavanols and whey proteins was necessary. Hence, the serum obtained by centrifugation of heated milk containing 2.5 mg/g CF was ultrafiltrated. Results showed that only about 32% of the flavanols found in the serum were covalently bound to the protein backbone and that they were mainly comprised of tri-, tetra-, and pentameric species. It follows that of the monomer found in the serum, only 25% was covalently bound to whey, which meant that almost 50% of the total monomer originally added to milk remained unassociated with protein and "free" in solution.

The fact that, to the best of our knowledge, this is the first contribution addressing the interactions of milk proteins and CF in the context of acidified milk guarantees further work in the area as to explain (a) if the discrepancy on the evolution of G' at the early stages of gelation is related to the proposed "accelerated" casein aggregation process, which counteracts the weakening effect of solubilized CCP, and (b) if CF inhibit the formation of disulfide bonds during acidification as to promote bond/strand rearrangements that contribute to the weakening of the gels, their shrinkage, and concomitant syneresis.

Although not in the scope of this paper, the observed partitioning of monomeric flavanols in this milk system may lend some insight into why the absorption of monomeric flavanols from cocoa in humans was observed not to be affected when consumed in the context of a milk-based drink.[37,38] The repercussions of this finding support the notion that a rational design for the delivery of bioactives in complex food systems relies on an understanding at the molecular level.

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■ ACKNOWLEDGMENT

Thanks to Dr. C. Kwik-Uribe for the critical review of this manuscript.

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