

## Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients

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### Abstract

Polyphenols are known to react under oxidizing conditions with side chain amino groups of peptides, leading to formation of cross-links in proteins. Plant-derived phenolic acids and flavonoids were used to prepare cross-linked gelatin gels in bulk and cross-linked gelatin–pectin coacervates in the form of microparticles for use as food ingredients. Gels cross-linked by these materials had greater mechanical strength, reduced swelling, and fewer free amino groups. Dynamic light scattering analyses showed that such cross-linking results in denser polymeric networks and prevents extension of the peptide chains is when the pH is moved away from the isoelectric point. Coacervated gelatin–pectin microparticles when cross-linked became more lipophilic, and were stable at temperatures up to 200 °C, in contrast to un-cross-linked particles that coalesce and/or disintegrate on heating. These properties of cross-linked gelatin gels and gelatin-based coacervates have applications for the development of novel food ingredients.

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### 1. Introduction

The present work was undertaken to investigate the use of phenolic acids and flavonoids—present in many natural plant materials—as cross-linking agents to produce stabilized gelatin gels and gelatin–pectin coacervates for use as novel ingredients for the modification of food systems.

When gels are cooled below 30–35 °C the random coil polypeptide chains link up to form collagen-like triple helices for part of their lengths, resulting in a so-called ‘physical’ gel (Kuijpers et al., 1999). The shear modulus of gelatin gels has been shown to increase with increasing helical content (Djabourov, 1988; McEvoy, Ross-Murphy, & Higgins, 1989). The modulus can be further enhanced by introducing covalent ‘chemical’ cross-links between single-strand chain segments (McEvoy et al., 1989; Watanabe, Tezuka, & Tadahiro, 1997). Of special interest are

coacervates of gelatin complexed with anionic polyelectrolytes, such as pectin, in the form of microparticles or microcapsules. These may be used as fat-mimetic additives or for flavor encapsulation. Coacervates, first named and described by Bungenberg de Jong (1949), are formed when a mixed dilute solution of gelatin and an anionic polysaccharide (acacia, pectin, etc.) is brought to a pH at which the polyelectrolytes have opposite net charges. Under these conditions the solution separates into a highly concentrated coacervate phase in the form of microdroplets of several micrometers in diameter, and a dilute bulk phase. The coacervate is stabilized by a balance of electrostatic forces that tend to aggregate the macromolecules, with loss of entropy, and the tendency to disperse them with entropy increase. The resulting system is a viscous liquid consisting of macromolecules in mobile random-coil conformation that holds water entrapped between the loops of the chains (Burgess, 1994; Overbeek & Voorn, 1957). On cooling below room temperature the liquid coacervate droplets become gelled microparticles or microcapsules.

Existing industrial processes using covalent cross-linking reactions include the production of hardened gelatin gels

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for photographic emulsions (Pouradier & Burness, 1977), and the manufacture of hardened gelatin-acacia coacervates for ink encapsulation in pressure-sensitive paper (Green & Schleicher, 1957). In order to use these processes in foods, the commonly used aldehydes and other compounds serving as cross-linkers had to be replaced by food-compatible reagents. Thus, new cross-linking agents had to be identified. A search for possible candidates led to polyphenols, which are widely distributed as minor but functionally important constituents of plant tissues. The most common examples are hydroxylated cinnamic acids such as caffeic acid (3,4-dihydroxycinnamic acid), chlorogenic acid (its quinic acid ester), caftaric acid (its tartaric acid ester), and flavonols such as quercetin and rutin (its rutinoside) (Spanos & Wrolstad, 1992; Trugo & Macrae, 1984). These compounds have an ortho-diphenol (or a 1-hydroxy-2-methoxy) structure. They occur mainly in rigid tissues, such as the hulls of cereal grains, cell walls of fruits (grapes, apples), coffee beans, tea leaves, and tubers (e.g. potatoes).

The present study was conducted with reagent-grade samples of rutin and of several phenolic acids, and with instant coffee and commercial white grape juice as examples of plant-derived sources of polyphenols. Qualitative tests were also made with extracts of tea and potatoes.

### 1.1. Polyphenol-protein cross-linking reactions

The formation of rigid molecular structures by reactions of ortho-quinones with proteins is well known. As per the scheme of Fig. 1, the diphenol moiety of a phenolic acid or other polyphenol (1) is readily oxidized to an orthoquinone, either enzymatically as in plant tissues, or by molecular oxygen. The quinone forms a dimer (2) in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring, with regeneration of hydroquinone. The latter can be reoxidized and bind a second polypeptide, resulting in a cross-link (3). Alternatively, two quinones, each carrying one chain, can dimerize, also producing a cross-link (4). Occurrence of such a cross-linking mechanism is supported by the identification of phenolic acid dimers (Figuroa-Espinosa et al., 1999; Figuroa-Espinosa & Rouau, 1998; Geissmann & Neukom, 1971; Stewart, Robertson, & Morrison, 1994) and by the gelling of aqueous solutions of polysaccharide esters of ferulic acid such as pentosans (Markwalder & Neukom, 1975) and sugar beet pectin (Oosterveld, Grabber, Beldman, Ralph, & Voragen, 1997; Thibault, Guillon, & Rombouts, 1991). Analyses of oxidized wheat flour extracts (Yeh, Hosney, & Lineback, 1980) and of extrusion-cooked corn meal (Gibson & Strauss, 1992) showed that the phenolic acids in these systems were concentrated in the fractions of highest molecular weight.

## 2. Materials and methods

### 2.1. Materials

Food grade type A gelatin, bloom strength 175, was obtained from Triple Crown America, Perkasi, PA; food grade pectin was obtained from Hercules Inc., Wilmington, DE; pure phenolic acids, quercetin, rutin, fluorescamine, the Folin–Ciocalteu reagent, and Sudan III were from Sigma Chemical Co.; commercial white grape juice, instant coffee, and other plant materials were obtained from local stores.

### 2.2. Preparation of cross-linked gelatin gels

The gelatin was hydrated at room temperature by suspension in water containing 0.01% NaN<sub>3</sub> (to retard spoilage) then kept at 40 °C for 2 h with occasional stirring. Ten to 50 mM stock solutions of caffeic, chlorogenic and ferulic acids, and of rutin hydrate were prepared by dissolution in NaOH followed by pH adjustment with acetic acid. Solutions of the phenolics were mixed with those of gelatin in various proportions and adjusted to the desired pH. Most cross-linking reactions were carried out at pH 8. White grape juice was adjusted for pH but otherwise left unchanged. A weighed amount of instant coffee powder was dissolved in water at 80 °C. The solution was cooled and centrifuged (microcentrifuge, 10 mins. at 12,000 rpm) to remove a small fraction of insolubles. The volume was adjusted to produce a 1% w/v solution. The gelatin-polyphenol solutions were exposed to oxygen at 40 °C by two methods: Either oxygen was bubbled through the solution for 1 h, or the tube containing the sample was capped by an oxygen-filled balloon for 4 h with occasional shaking. Without such oxygenation the degree of cross-linking was negligible, as shown by dynamic light scattering and other methods. This finding is consistent with the reaction scheme of Fig. 1. Comparative tests showed that the oxidation reactions had reached equilibrium at the end of these treatments. The solutions were sampled immediately for free amino group determination (see below). The remainder was aged for 24 h at room temperature, then kept for a further 24 h at 10 °C, and returned to room temperature. Most of the measurements were made on cross-linked gels of 5% or higher concentrations since cross-linking of dilute gels (below 2%) was found to inhibit the gelling process, probably due to intramolecular cross-linking (Pouradier & Burness, 1977).

### 2.3. Coacervated microparticles

Concentrated aqueous suspensions of microparticles, either un-cross-linked or cross-linked with varying amounts of coffee or grape juice, were supplied by GSG Scientific, LLC. They had been prepared by techniques essentially

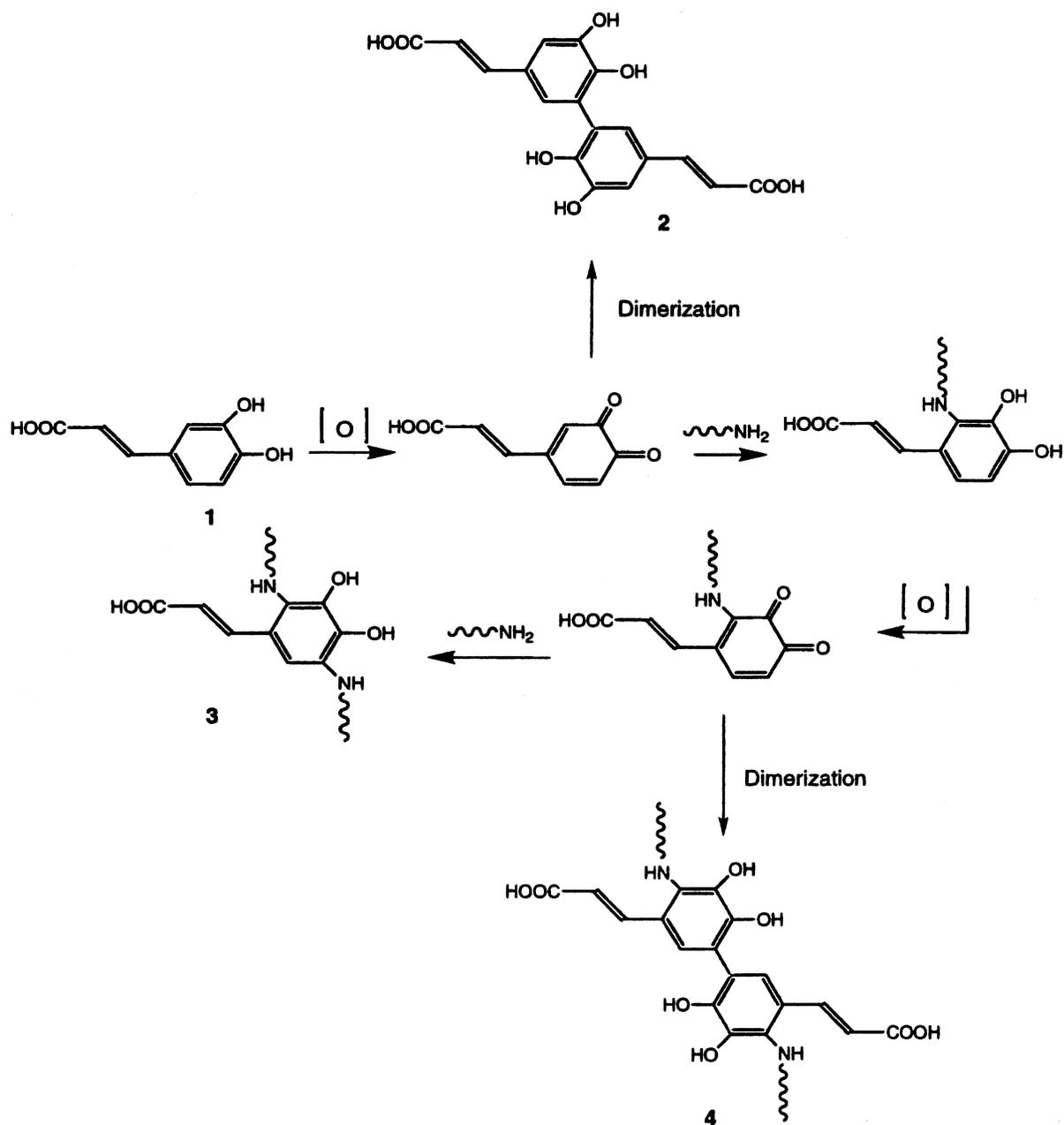


Fig. 1. Reactions of a phenolic acid with amino side chains of polypeptides.

similar to those described elsewhere (Gibson & Strauss, 1999). In this procedure dilute solutions of gelatin and pectin are mixed at 40 °C at near neutral pH, then gradually acidified to a pH near 4.5 at which coacervate droplets of 5–10  $\mu\text{m}$  appear as a separate phase. The droplets are concentrated and cooled to 10 °C to gel them. They are cross-linked by incubating them with varying amounts of coffee or grape juice at pH 7–8 for several hours, then are returned to a slightly acid pH. Coacervates from a series of solutions containing 2% gelatin and from 0.06% to 0.52% pectin were examined. For the present studies, only those from the solution with the lowest pectin/gelatin ratio (3%) were selected.

#### 2.4. Polyphenol determination

The grape juice and coffee samples used in this work were analyzed for polyphenol content by use of the Folin–Ciocalteu reagent (Singleton, Orthofer, & Lamuela-Raventos, 2001). This was obtained as a prepared solution of phosphotungstate and phosphomolybdate complexes. The method depends on the generation of a blue color, giving a broad absorption peak at 750 nm, when these complexes are reduced to lower oxidation states as the polyphenol is oxidized to a quinone. Aliquots of up to 0.2 ml of the samples (commercial grape juice as obtained, or instant coffee as a 1% solution) were added to 2 ml of the FC

reagent, previously diluted 1:10 with water. The absorbance, after a blank correction, was proportional to the sample concentration. Caffeic acid and catechin were used as standards. The particular lot of grape juice used in all experiments had a polyphenol concentration of 2.42 mM. A 1% solution of the particular instant coffee used was 4.22 mM in phenolics. These data can only be approximate since the natural products contain many different phenolic compounds, pure or as esters, each of which produces a slightly different absorbance per phenolic OH group by this method (Singleton et al., 2001).

### 2.5. Free amino group analysis

Free aliphatic primary amino groups in gelatin, essentially the  $\epsilon$ -NH<sub>2</sub> groups of lysine, were determined as described by Weigele, DeBernardo, Tengi, and Leimgruber (1972) and by Udenfriend, Stein, Bölen, and Dairman (1972). The formation of fluorescent derivatives with such amino groups by fluorescamine, a spirolactone, is the basis of this method. Samples of 20–50  $\mu$ l of gelatin solutions containing up to 10 nmol of NH<sub>2</sub> groups were added to 2.0 ml of 0.05 M sodium tetraborate at pH 8.9. A volume of 0.15 ml of fluorescamine solution in acetone (20 mg in 100 ml) was added, and the mixture was well shaken. The reaction was complete in a few seconds. The resulting fluorescence was measured using 390/475 nm wavelengths for excitation and emission. After applying a blank correction the fluorescence was proportional to the amine concentration. Lysine was used as standard. The particular 175 bloom strength type A gelatin used in our experiments tested at 0.42 mmol NH<sub>2</sub> per gram, in fair agreement with literature values for the lysine content of gelatin (Veis, 1964).

### 2.6. Gel rigidity

The AOAC determination of bloom strength of gelatin gels (Association of Official Agricultural Chemists, 1969) was adapted for relative gel rigidity measurements. These were carried out at 10 °C on gels that had been aged 24 h at this temperature. A plunger consisting of a 7 mm glass tube closed at the lower end with a slightly flattened seal was rested on the gel sample which filled an 18 mm i.d. vial to a height of 25 mm. The plunger was gradually loaded with 0.75 g steel balls. The distance of plunger penetration into the gel was measured after each addition. Plots of the plunger weight vs. plunger depression were linear within 5%, thus giving stress/strain ratios, as a measure of the elastic modulus. The largest plunger depression for any sample was 7 mm, without rupture of the gel.

### 2.7. Swelling ratio

Approximately 0.5 g samples of 5% gelatin gels were placed into 50 ml beakers and dried to constant weight at 40 °C in a vacuum oven. Similar amounts of coacervate

particles, in aqueous suspension, were dehydrated in several changes of iso-propanol, filtered off, and dried in a vacuum oven at 40 °C to constant weight. Twenty millilitre of 0.05 M pH 7 phosphate buffer was added to the dried samples, allowed to equilibrate for 4 h, then decanted. The swollen samples were blotted with filter paper and weighed, thus giving the swollen: dry weight ratio. This procedure was reproducible to within 10%.

### 2.8. Dynamic light scattering

Nicom Model 380 light scattering autocorrelator with 64 channels was used to determine cooperative diffusion coefficients  $D_c$  of gels. This quantity measures the frequency of local concentration changes in the gel, i.e. the rate of cooperative ‘breathing’ motions of a region of the polymer network. Gelatin gels, in 6 mm sample tubes maintained at 25 °C, were irradiated by an argon laser at 532 nm. Intensity fluctuations of the light scattered at 90° were measured by a diode detector. Incident and scattered beams were vertically polarized.

The diffusion coefficient  $D_c$  is obtained from the heterodyne intensity correlation function,  $g^{(2)}$ , defined as the running sum of the products of scattered light intensities measured at two instants separated by a delay time  $\tau$ . Heterodyne refers to mixed scatter intensities from moving and stationary scatterers (Berne & Pecora, 1976). For a single diffusion process,  $g^{(2)}$  declines exponentially with increasing  $\tau$  according to

$$g^{(2)}(\tau) = 1 + A \exp(-D_c q^2 \tau) \quad (1)$$

where  $A$  is an instrumental constant. The scattering vector  $q$  is defined as

$(4\pi n/\lambda) \sin(\theta/2)$  with  $n$  = refractive index of the solvent,  $\lambda$  = wavelength of scattered light, and  $\theta$  = scattering angle. The autocorrelator records values of  $g^{(2)}$  for 64 delay times that increase from channel to channel by the channel width  $\Delta\tau$ . For most gels, except very dilute or incompletely aged ones, the correlation decay could be fitted to a single exponential with a slope of  $-D_c q^2$ , thus providing a value of  $D_c$ . For soft gels or polymer solutions the data closely matched a two-dimensional decay curve according to

$$g^{(2)}(\tau) = 1 + A \exp(-D_c q^2 \tau) + B \exp(-2D_2 q^2 \tau) \quad (2)$$

where  $D_2$ , much smaller than  $D_c$ , is a coefficient of translation of discrete macromolecules through the solvent (Amis, Janmey, Ferry, & Yu, 1983).

## 3. Results and discussion

### 3.1. Gel rigidity

Relative gel rigidities were measured for a series of 5% gels with increasing mole ratios of cross-linking agent to

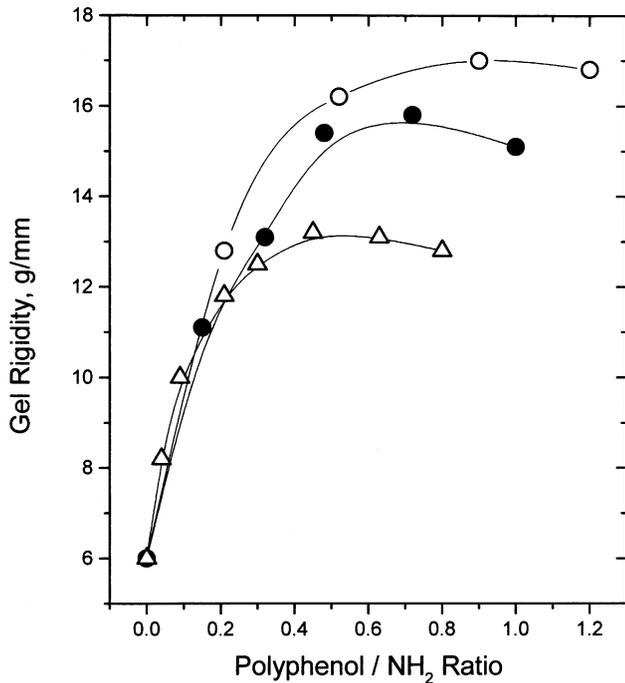


Fig. 2. Gel rigidity at 10 °C, expressed as plunger weight (grams)/plunger depression (mm), as function of polyphenol/gelatin NH<sub>2</sub> mole ratio for a series of 5% gelatin gels cross-linked with caffeic acid (○), coffee (●), and grape juice (△).

free amino groups of the gelatin. As shown in Fig. 2, caffeic acid, coffee, and grape juice all produced two- to three-fold increases in gel rigidity. At low mole ratios ( $\chi$ ) all materials gave concordant results. With the pure phenolics the gel rigidity became constant beyond  $\chi$  of 1.0. With coffee and grape juice, in contrast, the gel rigidity reached lower maxima at  $\chi$  around 0.5, and slightly decreased at higher  $\chi$ . The steep increase in gel strength at low  $\chi$  can be interpreted as the effect of a few strategic cross-links between single peptide chains that bring these chains closer together and promote the formation of collagen-like triple helices.

### 3.2. Swelling

The swelling ratios of gels cross-linked by caffeic acid, grape juice, and coffee, shown in Fig. 3, declined with increasing mole ratios, but not as steeply as the gel strength at comparable  $\chi$  values. The data for gelatin–pectin coacervate particles cross-linked with caffeic acid, show a much larger and steeper loss in swelling ratio. Flory and Rehner (1943) calculated that the swelling ratio of polymeric gels is inversely proportional to the 3/5 power of the concentration of cross-links. On this basis, the present data for gels are consistent with a 1.5–2-fold increase in the total concentration of cross-links (physical and chemical), relative to the un-cross-linked state.

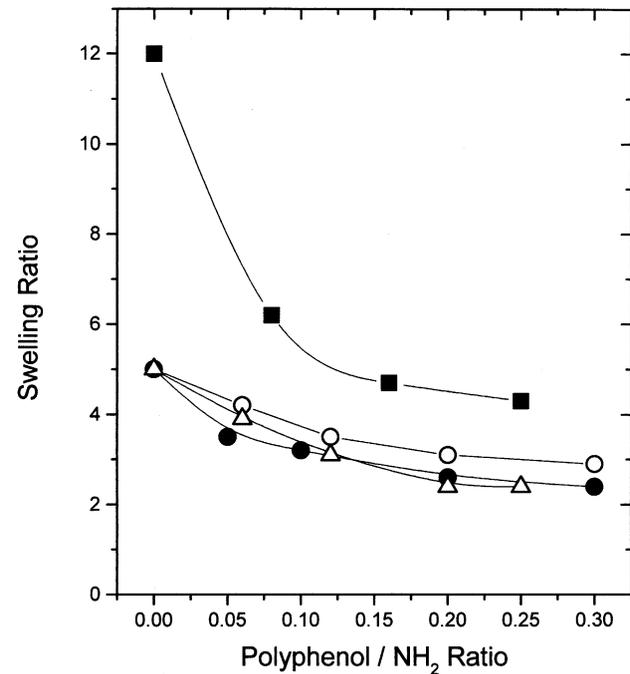


Fig. 3. Swelling ratios as function of polyphenol/NH<sub>2</sub> mole ratio for a series of 5% gelatin gels cross-linked with caffeic acid (○), gelatin-coffee (●), and grape juice (△), and of gelatin–pectin coacervate microparticles cross-linked with coffee (■).

### 3.3. Dynamic light scattering (DLS)

The type of information provided by this technique is illustrated in Fig. 4, showing  $D_c$  values of ‘physical’ gelatin gels as functions of concentration and pH.  $D_c$  was minimal

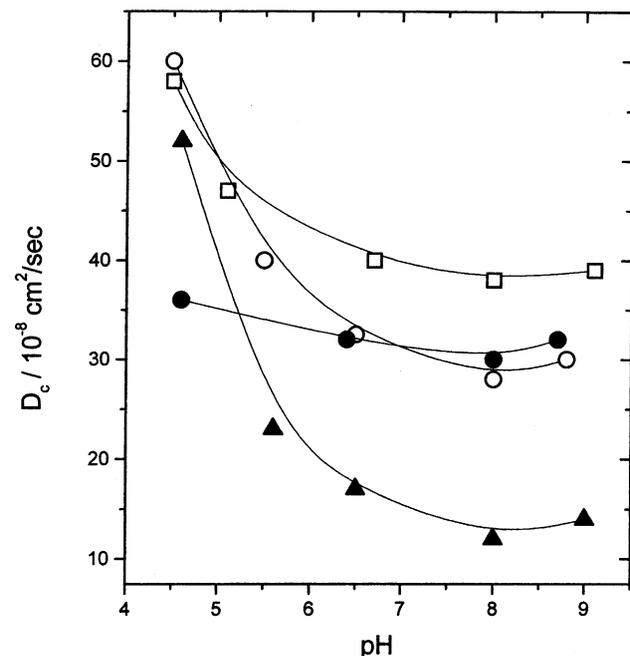


Fig. 4. Cooperative diffusion coefficients  $D_c$  at 25 °C of gelatin gels as functions of pH. Gels without cross-linking: 5% (▲), 8% (○), 12% (□). Cross-linked gel, 8% (●).

in the pH region close to the isoelectric point of 9.2 for this acid-type gelatin. Here the peptide chains oscillate with low frequency, as would be expected for chains of zero net charge that are contracted and relaxed. At lower and higher pH values  $D_c$  was higher, indicating higher vibration frequencies, as expected for chains that are charged and, due to electrostatic repulsion, are extended and taut. An increase in gelatin concentration near pI resulted in large increases in  $D_c$ . This can be accounted for as the result of higher densities of triple-helix ‘physical’ cross-links, producing shorter and therefore more tense chain segments between links. Fig. 4 also shows data for an 8% gelatin gel that was cross-linked with caffeic acid at pH 8, then was subdivided into portions that were adjusted to different pH values. The nearly unvarying plot of  $D_c$  vs. pH clearly shows that cross-linking inhibits the extension and tightening of the peptide chains when the pH is moved away from the isoelectric point, thus demonstrating the creation of a rigid framework.

The effects of several cross-linking agents on  $D_c$  at pH 8 are compared in Fig. 5. With rutin hydrate and caffeic acid  $D_c$  approached a saturation level near  $\chi$  of 3.0. The effect of chlorogenic acid (not shown) closely resembled that of caffeic acid. Ferulic acid, with one –OH of caffeic acid replaced by –OCH<sub>3</sub>, is seen to be considerably less reactive. With coffee and grape juice  $D_c$  values passed through a maximum, lower than that reached by the pure phenolics, then declined with further increase in  $\chi$ . This inhibitory effect was similar to the changes in gel strength (Fig. 2). Similar drop-offs at high

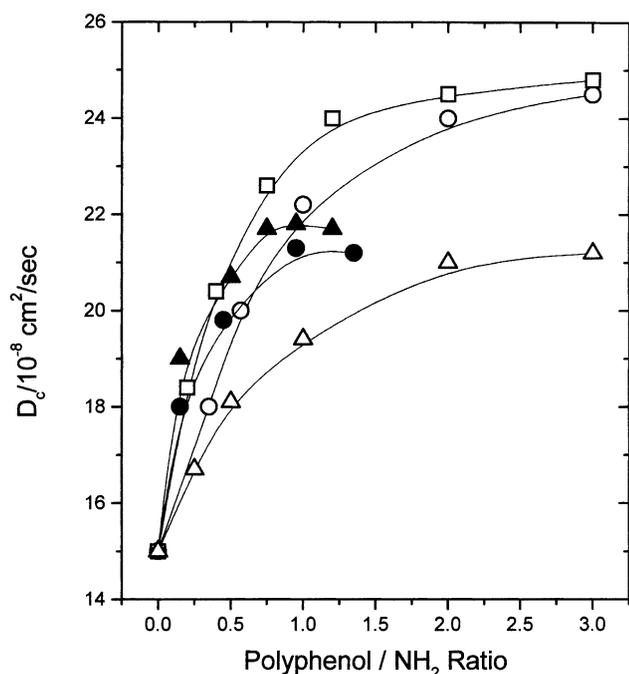


Fig. 5. Cooperative diffusion coefficients  $D_c$  at 25 °C and pH 8 for a series of 5% gelatin gels cross-linked with rutin hydrate (□), caffeic acid (○), ferulic acid (△), coffee (●), and grape juice (▲).

cross-linker/gelatin ratio, in the case of the shear modulus, were reported by McEvoy et al. (1989). Such interference with the cross-linking reaction and/or with the formation of triple helices on subsequent cooling may be caused by bulky components of the natural materials. The data in Figs. 4 and 5 show that cross-linked gels at 25 °C resemble un—cross-linked ones of higher concentration at the same temperature.

To monitor the extent of covalent cross-link formation with the degree of structural change, the concentrations of free amino groups were measured as a function of  $\chi$  for a series of 5% gels cross-linked with caffeic acid. These were compared with the reciprocal  $D_c$  values for these samples (Fig. 6). The latter are proportional to the correlation lengths  $\xi$ , as given by the Stokes-Einstein equation

$$D_c = \frac{k_B T}{6\pi\eta\xi} \quad (3)$$

where  $k_B$  is the Boltzmann constant,  $T$  the absolute temperature, and  $\eta$  the viscosity of the solvent. The value of  $\xi$  characterizes the size of ‘blobs’ (de Gennes, 1979) i.e. the size of regions in the polymeric network that oscillate independently from other such blobs, and thus may be interpreted as the average distance between chain links, or the mesh size. The plot in Fig. 6 for 5% gelatin gels cross-linked with caffeic acid at progressively increasing  $\chi$  shows that the decrease in free amino groups was greater than the decrease in  $1/D_c$  by a factor of about 1.5 at each cross-linker/gelatin ratio. The fact that both  $1/D_c$  (and thus the mesh size) and the free amino groups approach

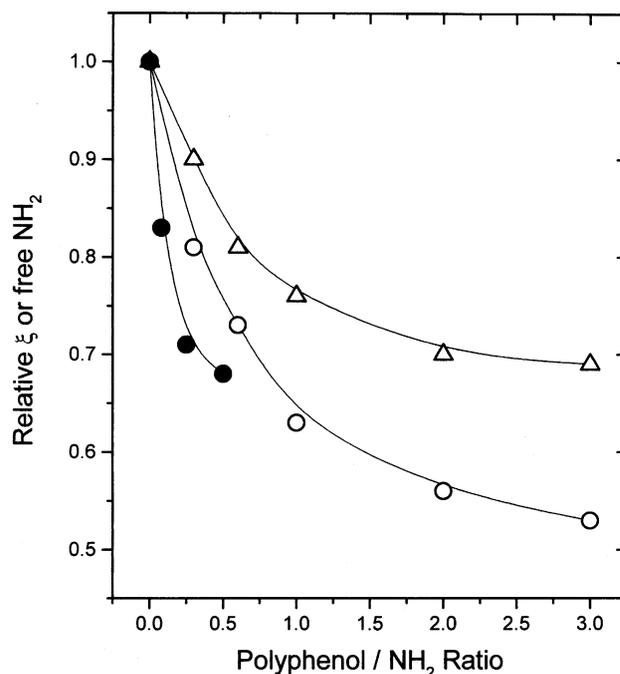


Fig. 6. Correlation lengths  $\xi$ , and free NH<sub>2</sub> groups as a function of polyphenol/NH<sub>2</sub> ratio. 5% gelatin gels: Correlation length (△), free NH<sub>2</sub> (○). Gelatin–pectin coacervate microparticles: Free NH<sub>2</sub> (●).

saturation values shows that the mesh size reaches a minimum at a certain limiting value of  $\chi$  beyond which no additional cross-linking appears to occur, possibly due to spatial restrictions.

#### 3.4. Physical characteristics of coacervated microparticles

The swelling ratios (Fig. 3) and free amino groups (Fig. 6) of microparticles decreased more steeply with increasing polyphenol/ $\text{NH}_2$  ratio, and thus appeared more efficient, than was the case with bulk gels. This suggests that the cross-linking reaction is more efficient, especially at low  $\chi$ , if the system is gelled first, then reacted with cross-linker, rather than by the reverse sequence. This more efficient sequence, however, can be used for bulk gels only if they are in the shape of thin films.

Microparticles were tested for thermal stability by heating them in aqueous suspension in a boiling water bath for

30 min, followed by cooling to room temperature. Fig. 7 shows micrographs at  $380\times$  magnification of gelatin–pectin microparticles in aqueous suspension, cross-linked with grape juice at a polyphenol/free  $\text{NH}_2$  mole ratio of 0.40, together with their un-cross-linked controls, before and after being heated. The heated cross-linked sample consisted of largely unchanged intact droplets, of smaller size than before heating, with slight clustering or coalescence. The heated control sample contained only a few very small particles and large separate phases, evidently formed by coalescence of many droplets. In another test the microparticles were heated in aqueous suspension in a pressure vessel at  $200^\circ\text{C}$  for 30 min. The particles in the cross-linked sample retained their spherical shape. In the control they had turned into debris of irregular particles.

To test the lipophilicity of microparticles without and with cross-linking, an ethanolic solution of the lipid dye Sudan III, also known as Oil Red, was added to aqueous suspensions of

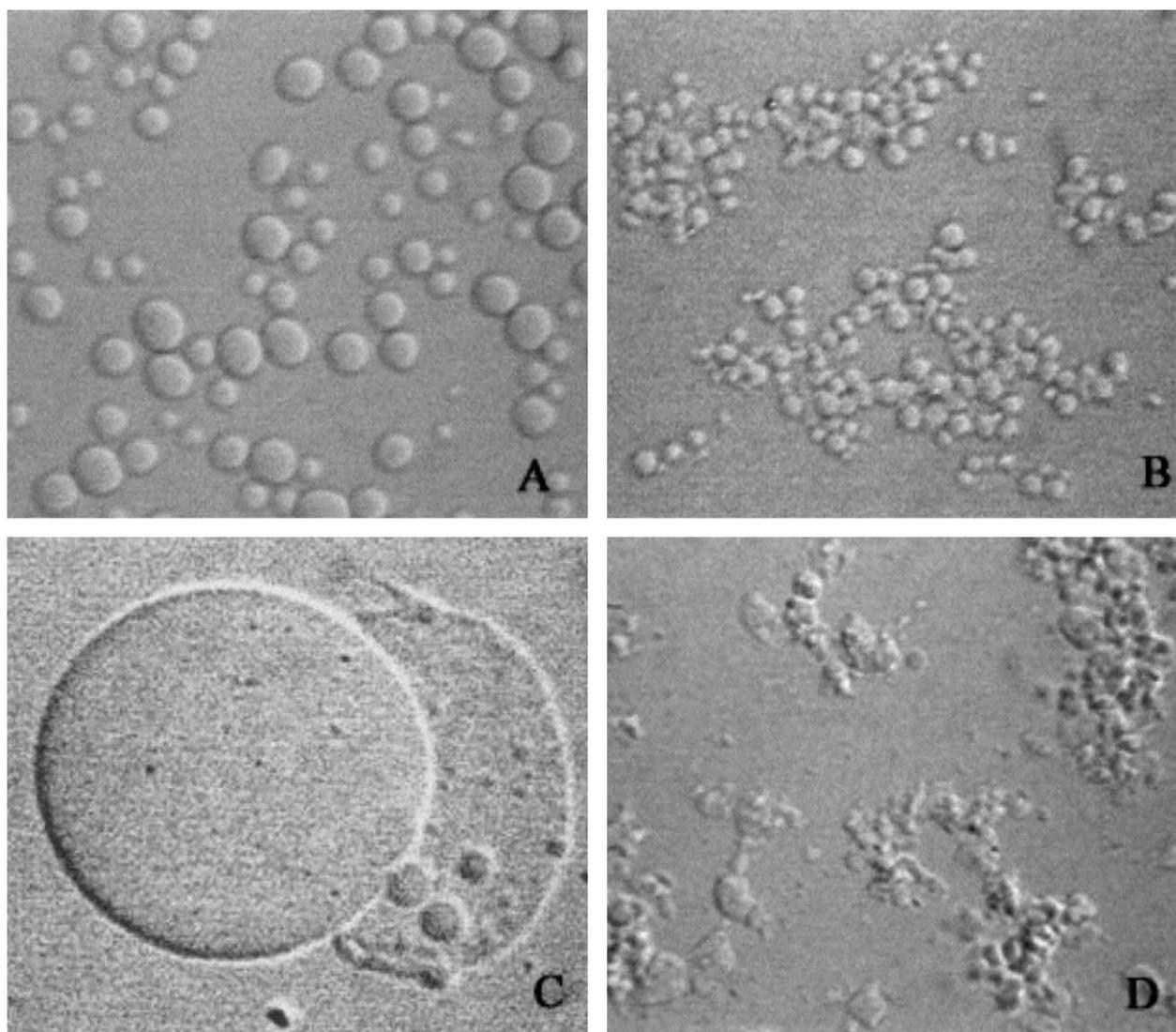


Fig. 7. Micrographs at  $380\times$  magnification of gelatin–pectin coacervates. Unheated particles: A, not cross-linked. B, cross-linked. Particles after heating to  $200^\circ\text{C}$ : C, not cross-linked. D, cross-linked.

the particles. After standing for several hours the microscope showed the cross-linked particles to be strongly dyed, but showed little or no dye take-up in the controls.

A further consequence of cross-linking was the relative ease with which such particle suspensions could be centrifuged or filtered, in contrast to the stickiness and cohesiveness of the un-cross-linked controls.

All of these effects can be interpreted as being caused by the presence of a more rigid polymer network. Such a structure will contain less water of hydration, and resist the expansion and uptake of water on heating, thus maintaining the integrity of the droplets. A tighter, less hydrated network, with fewer exposed ionic groups, can also be expected to be more lipophilic, although the presence of the phenolic rings of the cross-linker may contribute to this effect.

#### 4. Conclusions

This study has demonstrated that plant-derived polyphenols and flavonoids—materials safe for foods—can react with gelatin and gelatin-based microparticulate coacervates. Coffee, grape juice, and various other plant materials were found to contain sufficient concentrations of phenolics to make their direct use practical, without the need to isolate the active components. The results from the different experimental techniques used on gels and on coacervated microparticles are consistent with a picture of polyphenols reacting under oxidizing conditions with gelatin side chains and forming covalent cross-links that form a network of smaller mesh size and hence shorter, tighter chains between links. Such a structure has greater mechanical strength and greater thermal stability. It has less capacity to expand and so absorbs less water. The tight structure exposes fewer ionic groups, making it more lipophilic. Since cross-linked gelatin gels behave like non-cross-linked gels of higher concentration, they offer the possibility of developing gelled foods with reduced gelatin content and lower calories.

The significant properties of cross-linked gelatin–pectin microparticles have been shown to be lipophilicity and resistance to thermal degradation. Thus they can mimic fat globules in food formulations. These properties open a way of using such microparticles as a reduced calorie fat replacer, flavor binder, and texturizer.

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