H. V. AMORIM[1]
Dept. of Microbiology, The Ohio State University, Columbus, OH 43210
and University of Sao Paulo, 13400 Piracicaba, S.P. Brazil
and R. V. JOSEPHSON
Dept. of Food Science & Nutrition, The Ohio State University, Columbus, OH 43210

WATER SOLUBLE PROTEIN AND NONPROTEIN COMPONENTS OF BRAZILIAN GREEN COFFEE BEANS

INTRODUCTION

THE VARIETY OF COFFEE is an important criteria in determining the price of coffee in the world market. The *Arabicas* which are considered to have a more popular flavor than the *Robustas* are also higher in cost. Within a given variety, coffee is also classified by the flavor quality of beverage and the number of physical defects (black beans, stones, broken beans, etc.). In Brazilian *Arabica* coffee there are a number of different classes of flavor quality with the mildest, most pleasing flavor described as Soft and the generally least appreciated phenolic-like aroma and taste classified as Rio.

Volatiles of roasted coffee are well documented in the literature, but studies on precursors of coffee flavor have not given a clear picture about variations between different coffee flavors. Russwurm (1969) has isolated a water soluble fraction containing trigonelline, sucrose, glucose and amino acids from green coffee which when heated gave an aroma reminiscent of coffee. Furthermore, it is well known that proteins, peptides and free amino acids play an important role in the flavor of various processed foods, yet, little is known about their characterization in coffee, or their importance to coffee flavor. The earlier studies had found small differences in total protein or amino acids between different green and roasted coffees (Underwood and Deatherage, 1952a, b; Thaler and Gaigl, 1962, 1963). Centi-Grossi et al. (1969) characterized various coffee varieties as having the same general disc gel electrophoretic pattern, while Amorim et al. (1975a) found, by agar gel electrophoresis, some differences in Soft and Rio coffees of the same variety. Also, it is known that chlorogenic acids and chlorogenoquinones bind to proteins in many plant systems (Loomis and Battaile, 1966; Sabir et al., 1974) and most probably in coffee beans as well (Amorim et al., 1975a). These interactions may play a role in coffee flavor in that polyphenol oxidase, which most likely catalyzes these types of interactions via oxidation of chlorogenic acids, has been associated with differences in beverage quality of *Arabica* coffees (Amorim and Silva, 1968; Sanint and Valencia, 1970; Rotemberg and Iachan, 1971; Oliveira, 1972; Amorim et al., 1975a).

The major objective of the present investigation was to characterize the native water soluble proteins of Soft and Rio *Arabica* green beans. Water soluble extracts of green beans are known to contain the components required for a typical coffee aroma. In addition, various quantitative and qualitative methods were used to compare Soft and Rio *Arabica* green beans for differences in protein and nonprotein components.

EXPERIMENTAL

TWO SAMPLE LOTS of green coffee (*Coffea arabica* L. var. "Mundo Novo") harvested in 1973 in Fazenda Maldonado, Marilia, State of São Paulo and Fazenda São Lourenco, Jahu, State of São Paulo, Brazil, were classified for beverage quality after roasting and infusion by the Brazilian Institute of Coffee (São Paulo) (Amorim et al., 1975b). One sample was classified as Soft (more appreciated mild flavor) and the other as Rio (phenolic flavor).

Extraction

The samples were ground in a Microbroyeur Quantitatif Dangoumau (Prolabo Paris No. 7565) for 4 min at 0–5°C. The fine powder was immediately extracted twice with double distilled, demineralized water (1g/6.5 ml) for 1 hr, each time, under agitation at 2–4°C. Extracts were passed through cheesecloth and centrifuged at 10,000 × G for 20 min at 0°C. The supernatant was dialyzed or frozen (−20°C) for subsequent evaluation.

Dialysis, ultrafiltration and TCA precipitation

Sample extracts were dialyzed in three commercial cellulose membranes (Dialyapor, National Scientific Co., Cleveland, Ohio) having reported molecular weight cut-offs of 3,500, 6,000–8,000, and 12,000–14,000. Exhaustive dialysis was performed against four changes of deionized double distilled water (40 liters) over a period of 6 days at <5°C. The dialyzed samples along with original extracts were analyzed for total nitrogen by a modification of the Kjeldahl procedure (Malavolta, 1958). The digestion mixture was made with sodium selenite, copper sulfate and sodium sulfate. Ultrafiltration of sample extracts was accomplished in an Amicon apparatus under pressure using an Amicon PM-10 membrane (mol wt cut-off ∿20,000).

Samples for IEF and SDS were dialyzed for only 48 hr against two changes of deionized double distilled water because in 6 days of dialysis the proteins aggregated and only some of them would enter the gels.

Sample extracts were also treated with trichloroacetic acid (TCA) to a final concentration of 5% TCA and centrifuged at 1000 × G for 15 min. The entire supernatant in each case was analyzed for total nitrogen by the Kjeldahl procedure (Malavolta, 1958).

Gel filtration

Gel filtration chromatography was performed using Pharmacia columns (2.5 cm diam) and several types of Sephadex dextrans. The height of Sephadex beds was about 36 cm and flow rates for Sephadex G-25 (fine) and G-15 were about 1.3 ml/min and for G-150, 1.0 ml/min. All experiments were at room temperature (22–25°C) and column elution was monitored by an LKB Uvicord instrument at 254 nm. Sephadex G-15 and G-150 columns were eluted with a pH 6.98 sodium phosphate buffer (Morr and Josephson, 1968) and the G-25 column with a 0.1N acetic acid solution (Russwurm, 1969). Sample extracts and ultrafiltrates were dispersed and applied to the columns in the elution solution (1.5–5.0 ml). Precipitates formed were centrifuged out prior to application to the columns.

Thin layer chromatography and spectral analysis

Thin-layer chromatography (TLC) was performed on cellulose plastic sheets impregnated with and without a fluorescence indicator. Chromatography trials were carried out in a solvent system of N-butanol, acetic acid, water (4:1:2.2) at 25°C. Standards of trigonelline, chlorogenic acid and caffeine obtained from Sigma Chemical Co., St. Louis, Mo., were used. Spectral analyses were performed using a Beckman Model 25 Spectrophotometer.

Electrophoresis and isoelectric focusing

Standard electrophoresis was performed by the method of Davis (1964) with some modification (Payne et al., 1973). 100 μl of non-

[1] Present address: Dept. Chemistry, E.S.A. "Luiz de Queiroz," U.S.P. 13400, Piracicaba, S.P. Brazil

dialyzed sample were applied to each gel tube and electrophoresis performed at 4 mA per tube for 105 min. Gels were stained with 0.5% Aniline Blue Black in 7% acetic acid overnight and destained with several changes of 7% acetic acid. Gels were immersed in a solution of 40% ethanol and 7% acetic acid for three days to improve band sharpness.

Polyacrylamide gel isoelectric focusing (IEF) was carried out with (Josephson, 1972) or without (Josephson et al., 1972) 7M urea incorporated in gel solutions of 6.5% total gel at 5% cross-linkage, 1% ampholyte (LKB, pH 3.5–10 or pH 5–7), and 12.5% sucrose. The alternate overlayer loading procedure of samples was used rather than "gelling-in." Samples were dispersed in a solution of 3% ampholytes (LKB, pH 3.5–10 or pH 5–7) and 25% sucrose with or without 7M urea.

Bromophenol blue was satisfactory for direct staining in the neutral and alkaline pH regions but did not stain coffee proteins in the acidic portion of the pH gradient. Therefore, it was necessary to dialyze gels against several changes of 12.5% trichloroacetic acid for 2 days to remove ampholytes and then to stain with 0.5% Amido black 10 B solution (6 hr) and destain in 7% acetic acid.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by the method of Weber et al. (1972) using 10% total gel and 1.4% cross-linkage. Protein standards for SDS gels were lyophilized preparations of bovine β-lactoglobulin (β-Lg), bovine serum albumin (BSA) and bovine immunoglobulin G_1 (IgG_1) obtained from Miles Laboratories, Inc., Kankakee, Ill. Samples were dispersed in a 0.1M, pH 7.0 sodium phosphate buffer containing 1% SDS with or without 1% 2-mercaptoethanol and 4M urea and held overnight in the refrigerator before application to the gels. An aliquot of 25 μl was applied to each gel and electrophoresed for 5 hr at 5 mA per tube. The gels were stained with Coomassie Brilliant Blue R-250 overnight and destained for several days in a methanolic, aqueous acetic acid solution (Weber et al., 1972). Photographs were taken with a red filter.

RESULTS & DISCUSSION

Protein nitrogen estimation

Differences in protein nitrogen between Soft and Rio coffee extracts as estimated by dialysis and trichloroacetic acid (TCA) methods are shown in Table 1. The 5% TCA precipitable nitrogen values for both coffees were lower than nondialyzable nitrogen (NDN) values which supports the observations of Underwood and Deatherage (1952a) who reported that 5% TCA did not precipitate proteins of low molecular weight. Slightly more precipitable nitrogen was found in Soft coffee than in Rio which agrees with earlier work of Amorim et al. (1974) who found less NaOH and NaCl soluble proteins in Rio coffee extracts.

The results obtained by dialysis showed a slightly different distribution of molecular weight species between Soft and Rio coffees. Whereas Soft coffee had a larger amount of NDN above 12,000–14,000 mol wt, Rio showed a greater amount of intermediate molecular weight NDN. The difference in percentage of NDN between 3,500 and 12,000–14,000 membranes was 4% in Soft and 10% in Rio coffee. The differences could be due to proteolysis during harvesting and storage of Rio coffee or more aggregation of proteins of Soft coffee extracts during the extraction procedure and dialysis. On the other hand, the possible contribution of small amounts of nonprotein nondialyzable compounds such as nucleic acids cannot be overlooked.

Gel filtration

Sephadex gel filtration was used to separate and compare protein and nonprotein components in original coffee extracts and extract ultrafiltrates. Figure 1 shows the pattern of extracts from Sephadex G-150 (fractionation range of approximately 5,000–400,000 for peptides and globular proteins) eluted with pH 6.98 phosphate buffer. The patterns were basically the same for the two coffees with a high molecular weight peak at the void volume, a small intermediate peak, and a large and broad peak in the low molecular weight bed volume region. The peak of low molecular weight components and the following shoulder were green in color which suggests the presence of chlorogenic acid (Sabir et al., 1974). Higher and lower molecular weight proteins would be expected in the void volume and intermediate peaks, respectively, along with perhaps nucleic acids and polysaccharides, while amino acids, peptides, caffeine, trigonelline and chlorogenic acids would be expected in the bed volume region.

Several of the low molecular weight compounds eluting in the relatively large bed volume peak absorb strongly at 254 nm and therefore the relative size of the void volume and small intermediate absorption peaks (primarily protein) appeared small in comparison. Even though sensitivity was low for quantitative protein purposes, the patterns did show a high proportion of apparently high molecular weight protein eluting with the void volume.

Sephadex G-15 and G-25 column separations were performed to compare nonprotein component peaks of green bean water extracts and ultrafiltrates. Elution on Sephadex G-25 (Fig. 2) with 0.1N acetic acid was carried out according to Russwurm (1969) who observed differences among patterns of certain green coffees. In the present study, dispersion of the water extracts in acetic acid resulted in a proteinacious precipitate which was discarded by centrifugation and the supernatant applied to the column. Numerous peaks were observed which were similar to those observed by Russwurm (1969). However, no major differences could by observed between Soft and Rio coffee extracts or extract ultrafiltrates, although Rio extracts appeared to have a slightly larger void volume

Table 1—Estimation of nondialyzable and 5% TCA precipitable nitrogen in water extracts of Soft and Rio green coffee beans[a]

	Soft		Rio	
	mg/100 ml	%	mg/100 ml	%
Nitrogen total	119.5	100	120	100
Nondialyzable				
3,500 mol wt Cut-off	74.2	62.1	77.0	64.2
6,000–8,000 mol wt Cut-off	74.8	62.6	74.6	62.2
12,000–14,000 mol wt Cut-off	69.4	58.1	65.6	54.7
5% TCA precipitable[b]	64.5	54.1	62.4	52.0

[a] Averages of triplicate determinations
[b] Computed on basis of the difference of total and 5% TCA soluble nitrogen values

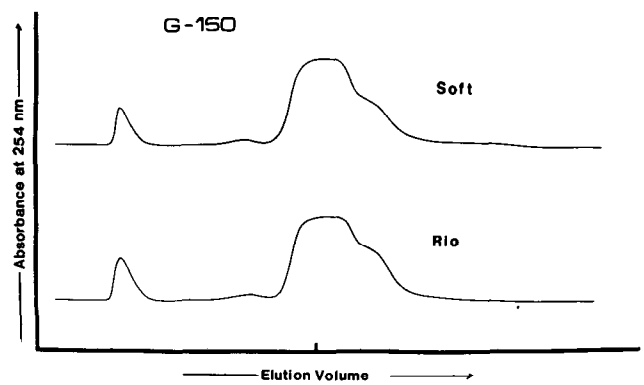


Fig. 1—Sephadex G-150 patterns (absorbance at 254 nm) of water extracts of Soft and Rio green coffee beans eluted with pH 6.98 phosphate buffer. Each division in the abscissa represents 200 ml eluted.

peak which was absent in ultrafiltrates. However, this difference cannot be attributed "a priori" only to proteins because nucleic acids absorb strongly at 254 nm.

A green band of chlorogenic acid (Sabir et al., 1974) adsorbed to the gel matrix was eluted (elution peak not shown in Fig. 2) with 0.1N NaOH (became yellow). The adsorption of phenolic compounds by Sephadex is well documented (Brook and Mundax, 1970; Wood and Cooper, 1970). The absorption spectra of the yellow-green band showed a λ max at 254 and strong shoulder at 325–330 nm.

Slightly improved resolution of ultrafilterable components eluting after the void volume was observed in Sephadex G-15 especially when samples and Sephadex gels were dispersed in pH 6.98 phosphate buffer (Fig. 3). Again, no significant differences were observed between the Rio and Soft coffees. Much

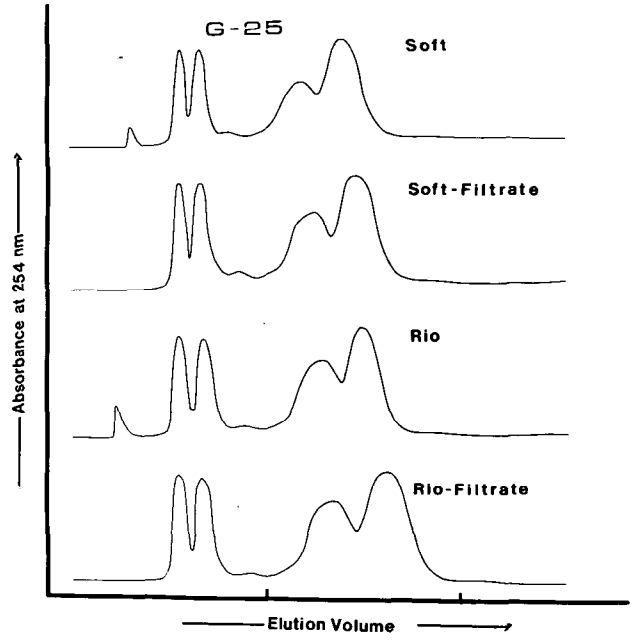


Fig. 2—Sephadex G-25 patterns (absorbance at 254 nm) of water extracts (total and ultrafiltrates) of Soft and Rio green coffee beans eluted with 0.1N acetic acid. Each division in the abscissa represents 200 ml eluted.

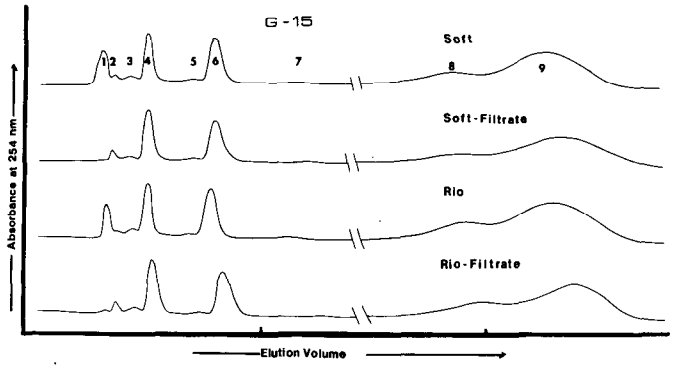


Fig. 3—Sephadex G-15 patterns (absorbance at 254 nm) of water extracts (total and ultrafiltrates) of Soft and Rio green coffee beans eluted with pH 6.98 phosphate buffer. Each division in the abscissa represents 200 ml eluted.

less material was precipitated from sample extracts after initial dispersion as compared to 0.1N acetic acid and therefore the void volume peaks were proportionately larger. No precipitation occurred when the buffer was added to the filtrate. Again, a green chlorogenic acid band (elution peak not shown in Fig. 3) could only be eluted with 0.1N NaOH. The spectral analysis showed a peak at 254 nm and a shoulder at 330 nm, possibly demonstrating nucleotides bound with chlorogenic acid (Sabir et al., 1974).

Elution peak fractions from Soft coffee extract identified as peaks 1 to 9 (Fig. 3) were partially characterized for component identification (Table 2) by spectral analysis and thin-layer chromatography (TLC). The compositional data generally confirmed the results obtained by Russwurm (1969) with the exception that he did not report the presence of nucleotides in initial elution volumes (peaks 2 and 3) or chlorogenic acid in peaks identified here as 8 and 9. Protein detected in void volume peak 1 was not reported by Russwurm because his sample dispersion and elution agent 0.1N acetic acid probably precipitated out protein as was mentioned earlier in our work. The detection of chlorogenic acid in peak 3 and the band bound to the column could be due, in part, to oxidation products and interactions with other coffee components and the column material. Peaks 8 and 9 appear to be pure chlorogenic acids. However, these questions can only be resolved by further isolation and characterization of fractions.

Although not shown, the Sephadex G-25 patterns of sample extracts eluted with phosphate buffer pH 6.98 were similar to that of G-15; however, G-15 gave better separation. Elution with water as done previously by Feldman et al. (1969) gave poorer resolution of components on either Sephadex gel type.

Standard alkaline polyacrylamide gel electrophoresis

Electrophoretic patterns (Fig. 4) of Soft and Rio coffee extracts dialyzed with membranes of different molecular weight cut-offs exhibited four major stained bands, three of low mobility (bands 1, 2 and 3) and a leading band (6) of the same mobility as the tracking dye.

The protein resolution obtained here was considerably better than that obtained by Centi-Grossi et al. (1969) who used nondialyzed samples and short electrophoretic runs. Poorer resolution of the three low mobility bands was also found for nondialyzed samples in the present study.

No differences were found as a result of membrane size for either coffee; however, Soft coffee had a stronger leading band (6) and Rio coffee two bands (4 and 5) which were very faint in Soft coffee. In this regard, Amorim et al. (1975a) found a more negatively charged protein zone by agar gel electrophoresis for Soft Coffee extracts and attributed this difference to a higher polyphenol oxidase activity and more bound chlorogenic acid and/or chlorogenoquinones in Soft coffee protein.

Polyacrylamide gel isoelectric focusing (IEF)

Urea (B) and nonurea (A) gel isoelectric focusing in pH 3.5–10 and pH 5–7 ampholytes were performed on dialyzed and nondialyzed coffee extracts (Fig. 5). Soft and Rio coffees showed multiple but somewhat diffuse bands of isoelectric protein species (Fig. 5A) with better resolution of individual bands observed in the narrower pH gradient (pH 5–7), where protein species were resolved in the isoelectric range of pH 4.4–4.7 (diffuse zone), and from pH 5.7–6.3 (over six bands).

In their study on water soluble proteins of Santos and Columbian coffees, Underwood and Deatherage (1952a) had determined isoelectric points of pH 4.6–4.7 by a minimum solubility precipitation technique. Only a few minor proteins were in this range in the present study. No apparent differences were observed between Soft or Rio coffees either dialyzed (3,500 molecular weight cut-off membranes) or nondialyzed.

Urea IEF gels (Fig. 5B) revealed more clearly defined multiple protein bands in either pH gradient, but with the narrower

Table 2—Tentative identification of the components from the peak fractions of Sephadex G-15 by spectral analysis and cellulose TLC chromatography

Peak #	Rf Ninhydrin +	Rf std[b]	Rf others	Color UV	Spectra λ max (Sh)[a]	Compound (tentative)
1	0		–	–	278 Sh 310	protein
2	0.45		–	–	250–275	nucleotides + peptides
3	0.33 0.45	0.68(Ch)	0.68	blue	209 Sh 254 Sh 320	nucleotides + peptides + chlorogenic acid
4	0.28 0.46 0.63	0.51(Tr)	0.53	black	265	trigonelline + amino acids
5	–		–	–	203 264 Sh 320	unknown
6	–	0.84(Caf)	0.85	black	273	caffeine
7	–		–		282	unknown
8	–	0.68(Ch)	0.69	blue	324 Sh 297	chlorogenic acid
9	–	0.68(Ch)	0.68	blue	324 Sh 298	chlorogenic acid

[a] Sh = shoulder
[b] Rf standard values for chlorogenic acid (Ch), trigonelline (Tr) and caffeine (Caf)

gradient (pH 5–7) giving the best resolution of protein species except for the faint band around pH 8.1 found using the pH 3.5–10 ampholytes. The protein species in urea gels (pH 5–7) with higher apparent isoelectric points between pH 5.9 and 7.7 were also more numerous than species in the nonurea system. A weak diffuse band was observed at about pH 5.2. Again, no basic differences were found between the urea IEF patterns of Soft and Rio coffees.

The higher apparent isoelectric points in the urea system can in part be explained on the basis of the effects of urea (Josephson et al., 1971; Josephson, 1972). Another cause for differences between urea and nonurea IEF gel patterns may be related to the probable presence of chlorogenic acid. Sabir et al. (1974) found chlorogenic acid to be bound both noncovalently and covalently to sunflower protein and that urea dissociated the noncovalently bound chlorogenic acid. The dissociation of adsorbed chlorogenic acid by urea could conceivably contribute to an increase in the number and resolution of coffee protein bands as well as possible affect the isoelectric points of coffee protein.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

The SDS gel patterns in Figure 6 give the approximate molecular weight distribution of Soft and Rio coffee proteins as compared to standard bovine whey protein species of known molecular weights (Rose et al., 1970). Samples separated were dispersed in 1% SDS in phosphate buffer without (A) or with (B) 4M urea and 1% 2-mercaptoethanol (ME).

Several bands of about the same number and intensity were observed in both coffees not treated with urea-ME (Fig. 6A). The following molecular weight protein species were found: Two high molecular weight bands at the top of the gel of similar mobility to IgG_1 immunoglobulins (mol wt 150,000–170,000) and apparently accounting for most of the protein in the gel; one major and one or more minor bands of mobilities similar to that of bovine serum albumin (mol wt

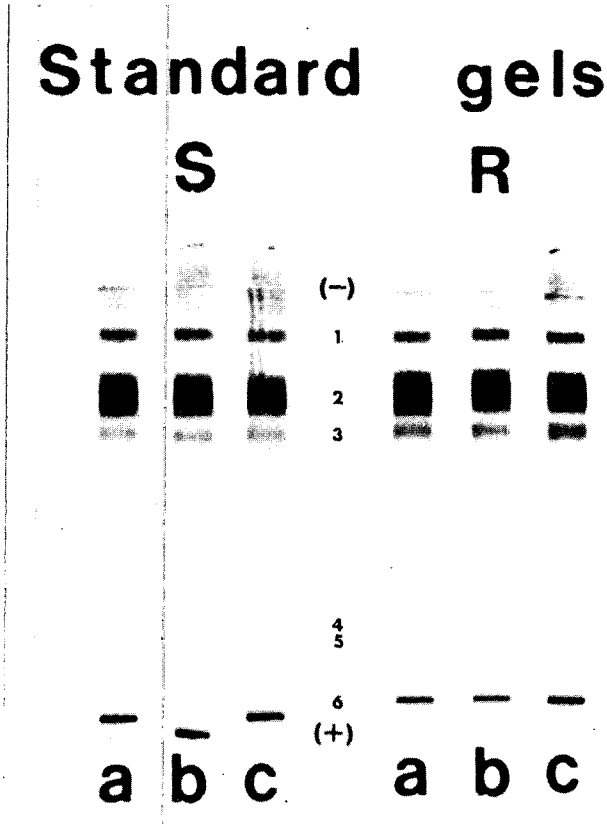


Fig. 4—Standard alkaline polyacrylamide gel electrophoresis of nondialyzable components in water extracts of Soft (S) and Rio (R) green coffee beans using membranes of different molecular weight cut-offs (a = 12–14,000; b = 6–8,000; c = ∿3,500).

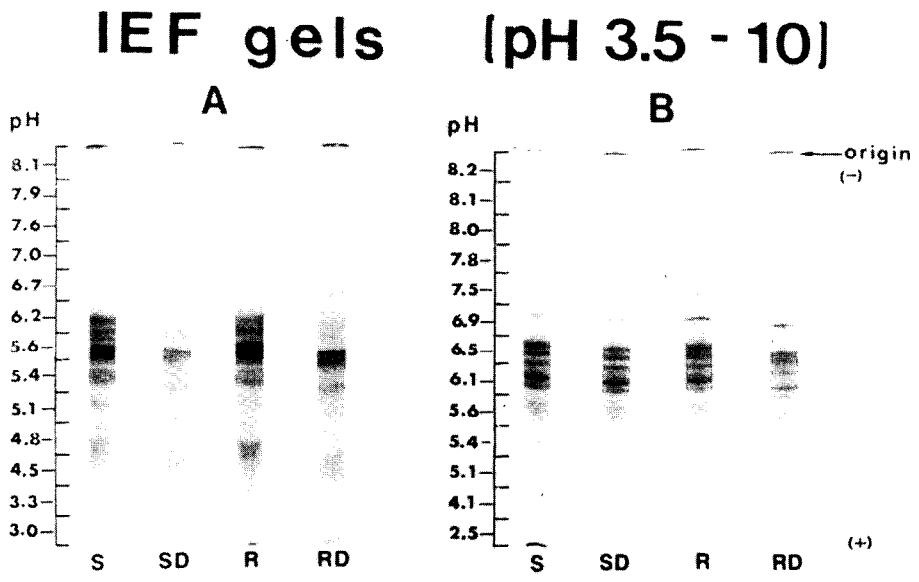
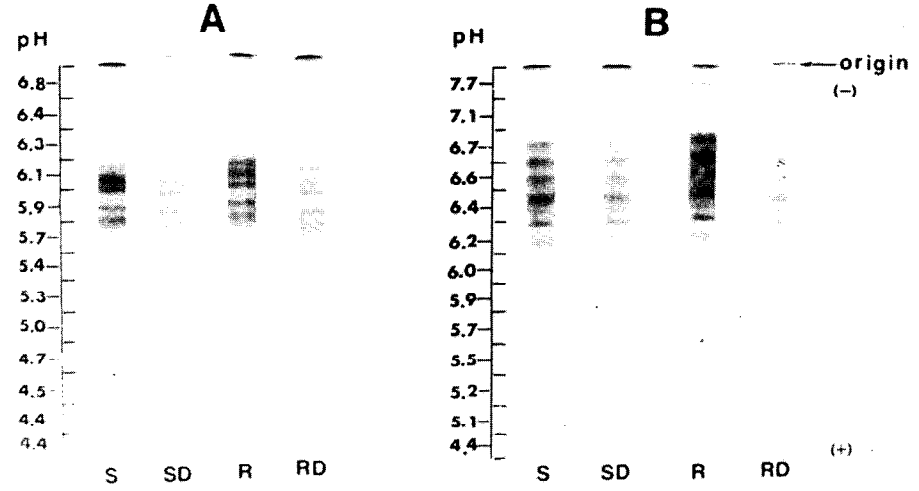


Fig. 5–Isoelectric focusing pH gradients and gel patterns of water extracts of green coffee beans. Soft (S), Soft-dialyzed against water (SD); Rio (R), Rio-dialyzed against water (RD). Gels contained either pH 3.5–10 or pH 5–7 ampholytes in the absence (A) or presence (B) of 7M urea.



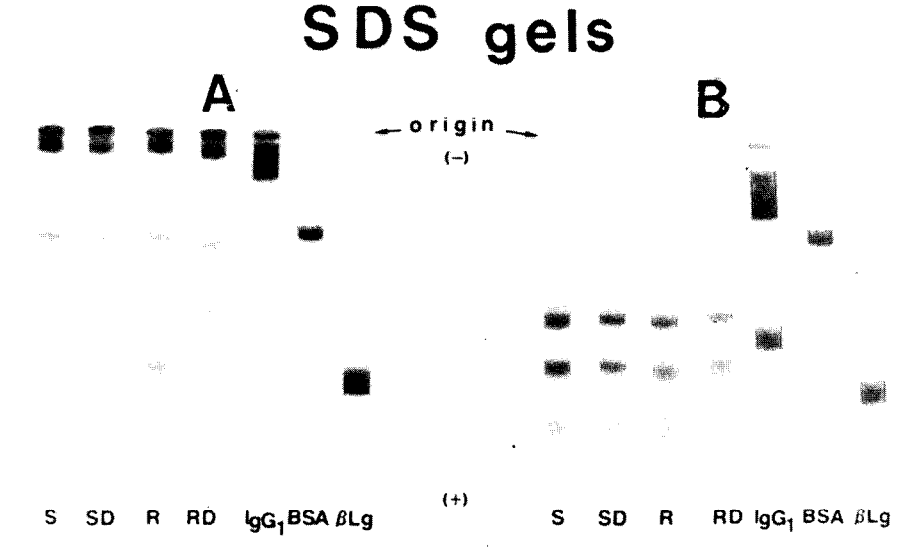


Fig. 6–SDS polyacrylamide gel electrophoresis patterns of water extracts of green coffee beans and standard proteins dispersed in 1% SDS buffer (A) and 1% SDS buffer with urea and ME (B). Soft (S); Soft-dialyzed against water (SD); Rio (R); Rio-dialyzed against water (RD); Immunoglobulin G_1 (IgG_1); bovine serum albumin (BSA); β-lactoglobulin (β-Lg).

66,000); one major and two minor bands of intermediates molecular weight followed by a major band of similar mobility to β-lactoglobulin (mol wt 18,000); and a minor leading low molecular weight band.

In general, the SDS gel patterns confirm Sephadex G-150 results in that the predominant protein species were of high molecular weight. However, the minor leading band is quite faint and similar in all samples, and therefore would not likely account for the 4% (Soft) and 10% (Rio) of nondialyzable nitrogen components estimated to be between molecular weights of 3,500 and 12,000–14,000 (Table 1). Differences in conditions of SDS gel electrophoresis as compared to water dialysis could be a factor as well as the variable dye binding capacity of smaller molecular weight proteins with Coomassie Brilliant Blue R-250 in explaining this apparent discrepancy. If one were to assume that the major band of similar SDS electrophoretic mobility to β-lactoglobulin was a part of the molecular weight cut-off range of 3,500 to 12,000–14,000, then the slightly greater band intensity of the dialyzed Rio coffee than Soft coffee could possibly account for the nondialyzable nitrogen differences.

In the urea-ME treated samples (Fig. 6B) different patterns were observed but the major components appeared to have almost the same mobilities as components in the samples not treated with urea-ME. The difference was that there was a considerable increase in the intensity of three low molecular weight components and a concomitant decrease in high molecular weight components. Soft and Rio coffee patterns were similar, although Soft appeared to retain minor bands in the region of 66,000 molecular weight which were quite faint in Rio, while Rio showed one additional smaller molecular weight band between the two major leading bands. Experiments carried out with SDS and urea, but in the absence of ME, gave patterns (not shown) similar to those observed without urea-ME although with urea sharper bands were observed. Thus, the dissociation of proteins was primarily caused by ME and not urea.

The data suggest that monomeric forms of water soluble coffee bean protein species are very few in number and of relatively low molecular weight and that disulfide bonding may contribute significantly to coffee protein structure. Future study will be required to determine whether the small differences in protein structures observed between Rio and Soft coffees is of importance to the quality of the beverage.

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Ms received 1/31/75; revised 6/3/75; accepted 6/8/75.

The grants of Fundação de Amparo a Pesquisa do Estado de São Paulo (Proc. Agronomia 73/1173) and Companhia Cacique de Cafe Soluvel, S.A., Brazil, are gratefully acknowledged. We are indebted to Dr. A.A. Teixeira from The Brazilian Institute of Coffee for supplying and classifying the coffee samples and to Vera Lucia de Amorim for technical assistance.