Protein Quality of Alfalfa Protein Concentrates Obtained by Freezing

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Freezing protein curd was prepared from alfalfa juice and extracted with 2-propanol, either unwashed or after washing with water. To examine the effect of extraction on digestibility, *in vitro* digestibility was analyzed for the two extracted protein concentrates and the unextracted protein concentrate. Two methods, one direct, the pH-stat method, and one indirect, the dietary fiber method, were used to determine *in vitro* digestibility. The protein quality of the unextracted freezing concentrate was also determined by bioassays. The *in vivo* digestibility of this concentrate (82.8) was lower than that of soybean meal, while the biological value (75.4) was similar. Thus, the lower net protein utilization (62.4) of this concentrate as compared to soybean meal can be ascribed to its lower digestibility. Extraction of the concentrate with 2-propanol lowered *in vitro* digestibility about 11 and 2% by pH-stat and dietary fiber methods, respectively, while washing the concentrate with water before extraction either had no effect on *in vitro* digestibility or lowered it slightly. There was an inversely proportional relationship statistically significative between the content of dietary and insoluble fiber in the concentrates and their *in vitro* digestibilities.

Keywords: *Protein quality; protein concentrates; alfalfa*

INTRODUCTION

Unfractionated alfalfa leaf protein concentrates (LPCs) have been regarded as a potential alternative to conventional sources of edible protein. LPCs have been assessed as food for children (Kamalanathan *et al.*, 1969, 1970; Oke, 1971, 1973; Olatunbosum *et al.*, 1972; Kamalanathan and Devadas, 1975; Pirie, 1984) and included in food formulations (Toosy and Shah, 1974; Meimbam *et al.*, 1982; Lencioni *et al.*, 1984, 1987, 1989; Barbeau and Kinsella, 1987; Maciejewicz-Rys and Hanczakowski, 1990).

Unfractionated LPC can be prepared by treatment using heat (Bickoff *et al.*, 1975), acid (Satake *et al.*, 1984), organic solvents (Brown *et al.*, 1975), or polyelectrolytes (Anelli *et al.*, 1977; Baraniak *et al.*, 1989). Unfractionated LPC can also be obtained by freezing. Freezing alfalfa juice produces a curd, called freezing curd, which contains 50% of the dry matter and *60%* of the nitrogen present in the original juice (Hernández *et al.*, 1988). The nitrogen content in the freezing concentrate prepared by freeze-drying the curd is the same as in the unfractionated concentrate. This method of preparation offers advantages, in that it requires neither heat nor the addition of any chemical substances. However, the effect of freezing on the protein quality of the concentrate so obtained has not yet been assessed.

To be suitable for human consumption, LPCs must be chlorophyll-free. Such concentrates may be prepared using two different methods. One method is to separate the soluble white proteins from the insoluble colored protein matter, though this method presents the drawback of producing lower yields of white protein concentrate (de Fremery *et al.*, 1973; Fiorentini and Galoppini, 1983). Another method involves extracting whole protein concentrates using organic solvents; this method gives higher yields, and the extracted concentrate may be stored for extended periods without need of low temperature or exclusion of oxygen (Bray, 1977).

Extraction of the freezing curd with 2-propanol yields concentrates with a high protein content, minimum lipid, sugar, and polyphenol contents, and color and texture similar to those of white protein concentrates (Hernández *et al.*, 1988, 1991; Hernández and Hernández, 1994). However, for solvent extraction to be a viable method of preparing chlorophyll-free protein concentrates, it is essential to determine how this process affects the protein quality of the concentrates so produced.

The object of the present study was therefore to determine the protein quality of alfalfa protein concentrates prepared either by extracting freezing curd, unwashed or washed with water, with 2-propanol or by directly freeze-drying the freezing curd, and hence to assess the effect of freezing and extraction on the protein quality of such concentrates.

MATERIALS AND METHODS

Concentrate Preparation. Preparation of the freezing curd has been described elsewhere (Hernández *et al.*, 1988, 1991). Briefly, alfalfa was harvested, pulped, and pressed. The juice was poured into small containers and frozen at −25 °C until use. Each sample was thawed at room temperature for 18 h before use, as needed. Freezing curd so formed was separated from the thawed juice by centrifugation, and some of the freezing curd was freeze-dried to produce freezing concentrate (FC). Extraction with 2-propanol was carried out in a Soxhlet apparatus at the solvent's boiling point using either untreated curd immediately after preparation, yielding 2-propanol-extracted freezing concentrate (IFC), or curd after it had been washed with distilled water by centrifugation, yielding water-washed 2-propanol-extracted freezing concentrate (WIFC) (Figure 1). Following removal of the residual solvent, the extracted FCs were ready for analysis.

***In Vivo* Tests of Protein Quality**. Apparent (AD) and true (TD) digestibilities, biological value (BV), and net protein

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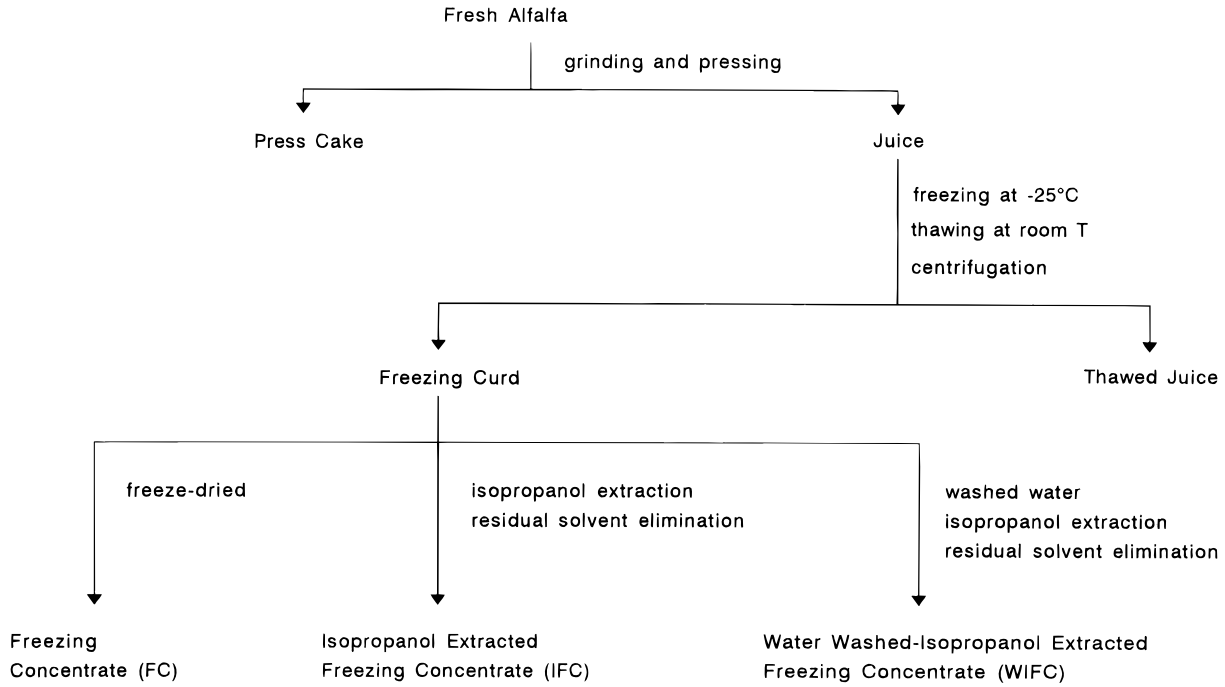

Figure 1. Obtention process of freezing protein concentrates.

utilization (NPU) of the FC were determined in rats using the Thomas−Mitchell balance method, as modified by Eggum (1973). The daily weight gain with respect to daily protein intake (DWG/DPI) was calculated for the balance period.

In experiments, albino Wistar rats (50−60 g) were housed in individual metabolic cages kept in a thermoregulated room (21 ± 1 °C) with a controlled 12 h light/dark period.

The rats were divided randomly into two groups of 10 animals each one (5 males, 5 females). One group of rats was used to determine maintenance requirements. The other group was used in the test diet assay.

To determine the maintenance requirements, the animals were fed a low-protein (4%) diet of a highly digestible protein (casein + 5% DL-methionine), a sufficient amount to record depletion (McDonough *et al.*, 1990b).

The FC was the only protein source in the test diet at the level of 12% of dry matter (DM). The diets also contained 4% fat, 8% fiber, 1% vitamin mix, and 4% mineral mix, the last two according to AIN (1977). A starch and sugar (1:1) mixture completed the diets.

The diets and water were given *ad libitum*. The rats were fed the low-nitrogen maintenance diet for 6 days (a 3 day adaptation period and a 3 day balance period) and the test diet for 10 days (a 3 day adaptation period and a 7 day balance period). At the end of the study the rats were weighed and the fecal N, urinary N, and food consumed were determined. The AD, TD, BV, and NPU values of the FC were calculated. Moisture was determined by drying at 105 °C and the total nitrogen according to the Kjeldahl method using a protein conversion factor of 6.25.

***In Vitro* Digestibility Method**. The *in vitro* digestibility of the three alfalfa protein concentrates was determined by two methods, a direct assay and an indirect assay.

The direct method used was the pH-stat method developed by Pedersen and Eggum (1983). A three-enzyme solution was made up to contain 23 100 units of trypsin (EC 3.4.21.4) (porcine pancreatic trypsin, type IX, Sigma T-0134), 186 units of chymotrypsin (EC 3.4.21.1) (bovine pancreatic chymotrypsin, type II, Sigma C-4129), and 0.052 unit of peptidase (porcine intestinal peptidase, grade K, Sigma P-7500) per milliliter. The pH was adjusted to 8.00 at 37 °C and held there for 2 min, and the enzyme solution was frozen at −30 °C until use.

The activity of this solution was determined daily using an aqueous suspension of sodium caseinate (Sigma C-8654) containing 1 mg of N/mL of water. An amount of 10 mL of sodium caseinate suspension was placed in a titration cell at 37 °C, and the pH was adjusted to 8.0 and held there for 10 min. An automatic titration was then run using the pH-stat procedure, adding 1 mL of the enzyme solution (end point 7.98, 0.100 M NaOH titration solution). Enzyme activity was determined on the basis of the amount of 0.100 M NaOH required to keep the pH at 7.98 for exactly 10 min. Percentage true digestibility (%TD) of the caseinate was calculated by

$$\%TD = 76.14 + 47.77B$$

where B = mL of added 0.100 M NaOH. Values for sodium caseinate digestibility should fall in the range of 98−102% (McDonough *et al.*, 1990a). Otherwise, a new three-enzyme solution needs to be made up.

To determine the digestibility of the concentrates, an amount of sample containing 10 mg of N was transferred to a titration cell in 10 mL of distilled water. Once the temperature had been stabilized at 37 °C, the pH was adjusted to 8 and held there for 10 min. An amount of 1 mL of the enzyme solution was then added, and the titration was started (end point pH 7.98, 0.100 M NaOH titrant solution). The amount of 0.100 M NaOH required to keep the pH at 7.98 for exactly 10 min was used to determine an uncorrected protein digestibility (%UTD) value by employing the equation set out above.

The %UTD values were corrected using the value obtained for the sodium caseinate solution (McDonough *et al.*, 1990a):

$$\%TD = (\%UTD/\%TD\ \text{caseinate}) \times 100$$

The baseline rate of titrant uptake was determined in runs in which no enzyme solution was added. After the concentrate samples had been adjusted to pH 8.0, the baseline uptake of titrant was measured for 10 min. The digestibility values corrected for the baseline consumption of alkali ($\%TD_b$) were obtained.

A Titrino Model SM 702 automatic titrator equipped with a 10 mL exchange unit (Metrohm) and a thermostated titration cell was used for all of the pH-stat titrations.

Protein digestibility was estimated indirectly by determining the soluble and insoluble fiber according to the method developed by Asp *et al.* (1983), previously applied in the analysis of the fiber in these of concentrates and described in detail in Hernández *et al.* (1995). In that method, following extraction of the lipids and pigments with (2/1 v/v) chloroform/methanol and of the soluble sugars with hot 80% (v/v) ethanol, enzymatic hydrolysis of the proteins in the sample was carried

Table 1. *In Vivo* Protein Quality Assessment of the FC ($x \pm \sigma_{n-1}$, $n = 3$)

mean body wt, g/rat	daily wt, g/rat	daily dry matter intake, g/rat	daily protein intake, g/rat	DWG/DPI
64.2 ± 5.6	0.95 ± 0.34	7.4 ± 0.7	0.84 ± 0.08	1.13 ± 0.4

daily N intake, mg/rat	daily fecal N, mg/rat		daily urinary N, mg/rat	
	total	endogenous	total	endogenous
135.5 ± 13.7	31.4 ± 3.6	8.3 ± 2.7	51.5 ± 7.8	23.3 ± 3.9

AD	TD	BV	NPU
76.7 ± 2.7	82.8 ± 3.1	75.4 ± 8.3	62.4 ± 6.3

out under conditions similar to those used in *in vitro* methods of determining digestibility. The hydrolysis employed pepsin (EC 3.4.23.1) in a hydrochloric acid medium for 18 h followed by pancreatin (from hog pancreas) for 1 h, as described by Schweizer and Würsch (1979). The soluble fiber was precipitated with ethanol out of the supernatant so obtained; the residue comprised the insoluble fiber. The two soluble and insoluble fiber residues were dried at 105 °C and weighed. The percentage protein (% N × 6.25) remaining in both residues was determined using the Kjeldahl method.

The protein remaining in the soluble and insoluble fiber residues represented the undigestible protein in the sample, and those values were used to calculate the protein digestibility of the concentrates according to

$$\%D = 100 - \frac{\text{protein (g) in residue}_{\text{soluble fiber}} + \text{protein (g) in residue}_{\text{insoluble fiber}}}{\text{amount of protein in sample (g)}} \times 100$$

All determinations of *in vitro* digestibility were performed by triplicate.

RESULTS AND DISCUSSION

***In Vivo* Evaluation of the FC.** Table 1 presents the results of the *in vivo* evaluation of the protein quality of the freezing concentrate.

The *daily weight gain with respect to daily protein intake (DWG/DPI)* of the FC was 1.13. Subba Rau *et al.* (1969) and Myer and Cheeke (1975) recorded similar PER values of 1.23 and 1.36, respectively, in alfalfa whole protein concentrates. Carlsson (1984) reported a value of 1.5 for that same type of concentrate. Gastineau and de Mathan (1981) recorded a PER value of 1.2 for alfalfa green protein concentrate. Bickoff *et al.* (1975) obtained corrected PER values of 1.68 for whole protein concentrate and 1.67 for green protein concentrate.

Although the DWG/DPI value for FC was lower than the literature PER values, this may be attributable to the shorter duration of the trial in this experiment, 7 days, 3 times shorter than the 28 days of PER trials. In addition, the longer the trial, the more the animals will become habituated to the diet, particularly in the case of foods of low palatability, like these concentrates. Subba Rau *et al.* (1972) pointed out that acceptance of the diet is a limiting factor in methods based on measurements of weight gain. Samonds and Hegsted (1977) reported that fluctuations in the quantity of food ingested increased the variability in the PER values. In the present trial a coefficient of variation of 10% in the amount of DM ingested resulted in a 36% variation in the DWG/DPI value. It has not been possible to contrast these values with the values of other workers, because they have not been published. However, the results were similar to those obtained in an interlaboratory study of the PER organized by AACC/ASTM (Hackler *et al.*, 1984). That study yielded coefficients of variation in the DWG/DPI value of 60.7% for peanut meal and 30.1% for a textured vegetable protein at a dietary protein content of 10% after an adaptation period of 2 days and a 1-week trial. The coefficients of variation dropped to 22 and 13%, respectively, in trials lasting 3 and 4 weeks. Accordingly, the DWG/DPI value obtained in the present experiment may slightly underestimate the nutritive value of the FC.

The *apparent and true digestibilities* of the FC were 76.75 and 82.78%, respectively, as shown in Table 1. The AD value of the FC was similar to that reported for alfalfa whole protein concentrates by Ohshima and Ueda (1984) (77%) and by Subba Rau *et al.* (1972) (78%). The TD value of the FC was also consistent with the literature values for whole concentrates. Saunders *et al.* (1973) reported values of 83−87%, similar to the range of 83−89% for whole concentrate recorded by Bickoff *et al.* (1975), who also reported a value of 85% for green concentrate. Subba Rau *et al.* (1972) reported a value of 86% for whole concentrate.

Thus, while the AD values obtained by those workers were similar to the AD values for the FC in this experiment, the TD values were somewhat higher. This discrepancy may be explained by the fact that they, like Saunders *et al.* (1973), determined the endogenous nitrogen in a group of rats fed a protein-free diet, whereas in this study the rats were fed a diet with a low proportion (4%) of a highly digestible protein (casein + 5% DL-methionine), as currently recommended (McDonough *et al.*, 1990a) to avoid excessive loss of organic proteins and changes in the intestinal flora.

Since the digestibility of FC is similar to that of whole and green protein concentrates, the method of preparing the FC, by freezing the juice or by means of heat treatment, respectively, could be expected to have little influence on the *in vivo* digestibility, dependent mainly upon the nature of the sample proteins and associated compounds.

Digestibility of the FC can be compared with that for soybean meals, widely used in the food industry as a source of protein. The digestibility values found in the literature ranged from 85 to 90% (Bickoff *et al.*, 1975; Carlsson and Hanczakowski, 1985; Ohshima and Ueda, 1984). Consequently, at most the FC is around 7% less digestible than soybean meal.

The *biological value (BV)* and *net protein utilization (NPU)* of the FC were 75.43 and 62.36%, respectively (Table 1). The BV of the FC was the same as that found for whole protein concentrate by Subba Rau *et al.* (1972). Maciejewicz-Rys and Hanczakowski (1990) reported a BV of 54% for that same type of concentrate, but they did not explain the method used to prepare the concentrate, did not identify the type of rat used, and limited the animals' daily diet. Furthermore, they also obtained a very low TD value (65%) as compared with the value recorded for FC in the present experiment and the literature values for whole concentrate. In view of the foregoing, their results would not appear to be comparable.

Comparing the BV for the FC with the BVs for other food proteins shows it to be similar to the BV for cow's milk casein (79.7%) and soybean meal (76.6%) and higher than the BV for isolated soybean protein (66.4%) (FAO/WHO, 1970). It can thus be concluded that the proteins in the FC have a high biological value.

Table 2. True Digestibility (Percent) of the Alfalfa Protein Concentrates As Determined by the pH-stat Method ($x \pm \sigma_{n-1}$, $n = 3$)

protein concentrate	%TD	$\%TD_b$
FC	93.23 ± 0.36	91.97 ± 0.360
IFC	82.33 ± 0.160	81.79 ± 0.160
WIFC	82.16 ± 0.04	81.41 ± 0.04

Table 3. Digestibility (Pecent) of the Alfalfa Protein Concentrates Based on Fiber Dietary Analysis ($x \pm \sigma_{n-1}$, $n = 3$)

	undigested protein (% DM)			
protein concentrate	insoluble fiber residue	soluble fiber residue	total fiber residue	% D
FC	7.67 ± 0.01	2.60±0.005	10.3	80.4
IFC	13.37 ± 0.03	1.00 ± 0.005	14.4	78.6
WIFC	14.35 ± 0.04	1.00 ± 0.007	15.4	78.5

The NPU value for the FC (62.36%) was somewhat lower than the value for whole protein concentrate (66.2%) published by Subba Rau *et al.* (1972). Compared to other reference proteins, it was similar to the value for soybean products [meal (60.4 %) and isolated protein (63.3%)] and lower than the NPU value for cow's milk casein (72.1%) (FAO, 1970). Since the NPU value reflects the BV and digestibility, the fact that the BV for the FC was optimal suggests that the lower NPU value must be attributable to the low digestibility of the concentrate.

***In Vitro* Digestibility of the Concentrates**. Table 2 sets out the *in vitro* digestibility (%TD) values for the concentrates as determined by the pH-stat method. The *in vitro* digestibility value for the FC was nearly 93%. That value was around 10% higher than the value determined using bioassays, approximately 83%. The first possible explanation considered was the hydrolyzation of other alkali-consuming substances during the determination, thus resulting in overestimation of the digestibility. To test this hypothesis, alkali consumption was measured in samples prepared using the same conditions except that no enzyme was added, and that baseline consumption value was used to correct the digestibility determinations. The corrected values ($\%TD_b$) are shown in Table 2. The effect of the correction on digestibility was very slight, around 1% for the FC and even less for the extracted concentrates. On effecting this same correction, Pedersen and Eggum (1983) likewise failed to find variations in the digestibility values for samples of different sources. To test whether the determination method itself was the cause of the discrepancy, the *in vitro* digestibility was measured using another method based on measurements of the nitrogen. The procedure for the enzymatic hydrolysis of the proteins used in the analysis of the dietary fiber was chosen for comparison, because it fulfilled the following requirements: the conditions employed were similar to the conditions used in the *in vitro* methods of determining digestibility, and undigested nitrogen was analyzed. Accordingly, the undigested protein in the soluble and insoluble fiber residues, expressed as a percentage of the total protein in sample, was calculated, and protein digestibility was then calculated on the basis of those values. The results (table 3) show that the digestibility value for the FC calculated in that way was similar to that obtained by the *in vivo* method. The presence of a compound acting as an enzyme activator in the *in vitro* pH-stat method would provide a possible explanation for that discrepancy.

Pedersen and Eggum (1983) found that the addition of 5−8 mg of calcium ions increased the digestibility values obtained using the pH-stat method by about 3−6%. This effect was the reverse when the amount added was around 20 mg, and what is more, different samples responded differently. In addition, Bergmeyer (1970) pointed out that calcium ions are activators of trypsin and chymotrypsin. Since calcium concentrations in the samples were high, around 5% (DM) (Hernández, 1993), that same effect may have been responsible for the high %TD values obtained using the pH-stat method.

The influence of calcium on the *in vitro* pH-stat digestibility determination could be confirmed by determining digestibility using previously dialyzed samples.

Tables 2 and 3 show that the *in vitro* digestibility values for the extracted concentrates obtained using the pH-stat and pepsin−pancreatin methods were similar. Statistical analysis of the digestibility values between the two methods indicated that there were significant differences ($p < 0.05$) and on the order of 2% for the two concentrates.

The effect of extraction with 2-propanol and washing with water on concentrate digestibility can be inferred by comparing the digestibility values for the extracted concentrates and for the FC determined using the same method. In the case of the pH-stat method, the difference in the %TD values was on the order of 11% for both extracted concentrates. Using the pepsin−pancreatin method, the difference was on the order of 2% for both of the extracted concentrates. Therefore, extracting the freezing curd with 2-propanol appears to decrease the digestibility of the proteins in the concentrate, while washing with water before extraction has no effect on the digestibility.

The literature contains few and highly disparate data on the effects of extraction of protein concentrates with organic solvents. Experiments have been reported using different types of sample, different solvents, and different extraction conditions; what is more, the digestibility determinations have also been carried out using different methods.

Buchanan (1969) determined the effect of heat and extraction with chloroform on the digestibility of protein concentrates. Part of a freeze-dried concentrate was heated in the presence of water in an oven at 105 °C for 15 h, and part of that heated sample was extracted with chloroform at room temperature. *In vivo* and *in vitro* determinations of digestibility were performed using papain, pepsin, and pepsin−pancreatin. For the determinations using papain, the results showed that treating the sample with moist heat decreased digestibility by about 10%, while extraction with chloroform restored the digestibility to its original value. This latter effect was not recorded for the *in vivo* and *in vitro* determinations of digestibility carried out using pepsin and pepsin−pancreatin.

Savangikar and Ohshima (1987) compared the AD of leaf protein curds of different plants extracted with ethanol without heating but then heated to 70 °C for 18 h, with the AD for the same samples freeze-dried or heated directly, without extraction. On the basis of their results, they concluded that the digestibility of the treated samples was lower than that of the freeze-dried samples and that the decrease was greater in the samples heated directly without extraction (7−10%) than in the samples that had been extracted before heating (5−7%).

Hanczakowski *et al.* (1991) extracted one part of alfalfa green protein curd with ethyl ether for 20 h, although they do not say whether the extraction procedure was performed hot or cold. Another part was washed with water, and yet another part was dried under an infrared lamp at 55 °C. The results indicated that extraction with the organic solvent increased digestibility by 6% and that washing with water had no effect.

Subba Rau *et al.* (1972) examined the factors affecting leaf protein concentrate quality. They concluded that there was an inverse relationship between the ash content, polyphenol content, and soluble solids content and the nutritive value; they reported that variations in those factors were manifested mainly in the digestibility value.

The ash contents were similar in the FC and in the IFC, although it was 2% lower in the WIFC (Hernández, 1993), which should have a lower soluble solids content. Nevertheless, depending upon the method of determination applied, the digestibility of the WIFC was the same or lower than that of the IFC and substantially lower than that of the FC. This finding agrees with the results, already discussed above, reported by Hanczakowski *et al.* (1991), who also found no improvement on washing the concentrate with water. It disagrees with the results of Subba Rau *et al.* (1972), who found that a lower ash content did improve digestibility.

Extraction lowers the amount of polyphenols in the concentrate, and washing the sample with water results in an even larger decrease (Hernández *et al.*, 1991). Consequently, the experimental results showed no correlation between polyphenols and digestibility. This agrees with the findings obtained by Hanczakowski *et al.* (1991), who prepared an alfalfa green protein concentrate with a low polyphenol content by washing the curd with a 40 mM sodium phosphate buffer solution (pH 7) containing 2.5% NaCl and 1 mM 2-mercaptoethanol without obtaining any improvement in digestibility. That same washing solution was used by Lahiry *et al.* (1977) to reduce the chlorogenic acid content. They reported a 1.8% increase in the *in vitro* digestibility of whole alfalfa concentrate as determined using the method of Hsu *et al.* (1977), and they attributed this finding to possible inhibition of proteolytic enzymes by the polyphenols.

The absence of any correlation between digestibility and the contents of these factors in the concentrates in this experiment suggests that the ash, soluble solids, and polyphenol contents have no influence on their protein digestibility.

However, comparison between the dietary fiber content of the concentrates and their digestibility values reveals an inversely proportional relationship. Correlation analyses were run on the insoluble, soluble, and total dietary fiber contents (Hernández *et al.*, 1995) and on the corresponding digestibility values for the concentrates as determined by *in vitro* methods.

The correlation coefficient values obtained using the pH-stat and pepsin−pancreatin methods were, respectively, −0.9649 ($n = 2$, $p = 0.1690$) and −0.9754 ($n = 2$, $p = 0.1414$) for the insoluble fiber content, −0.8663 ($n = 2$, $p = 0.8416$) and −0.8438 ($n = 2$, $p = 0.0213$) for the soluble fiber content, and −0.9967 ($n = 2$, $p = 0.0512$) and −0.9993 ($n = 2$, $p = 0.0236$) for the total fiber content. These results are suggestive of a highly significant linear relationship between the insoluble fiber content, and especially the total fiber content, and digestibility values for the different concentrates, irrespective of the method of determination employed. No such relationship could be established for the soluble fiber content, as the results obtained show.

Accordingly, it can be concluded that the digestibility of the concentrates decreases as the amount of fiber increases. The literature contains many reports of this effect, which is ascribable to the ability of fiber to inhibit various digestive enzymes and to its action as a physical barrier reducing enzyme accessibility (Zebrowska, 1978).

Heat is another factor that may have exerted an influence on the lower digestibility of the extracted concentrates, since a hot extraction process was employed. Although heat is beneficial in that it destroys enzyme inhibitors, it contributes to the formation of disulfide bridges and thus to the creation of irreversible steric hindrances to enzymatic action (Richardson and Catsimpoolas, 1979; Hamaker *et al.*, 1987; Serna-Saldivar *et al.*, 1988). It will be necessary to verify the possible influence of heat by extracting the samples under the same conditions at room temperature.

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