Polyphenols in Alfalfa Leaf Concentrates

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Freezing curd obtained from alfalfa juice was extracted with 2-propanol, either in its unaltered state or after it was washed with water, to determine the effect of the extraction on its polyphenol content. The amounts of total polyphenols, chlorogenic acid, and orthodiphenols were determined in the three concentrates, and the types of compounds present were characterized by analyzing their corresponding UV-vis spectra. Neither orthodiphenols nor chlorogenic acid was found in the concentrates, and the flavonosides were shown to be the most abundant polyphenols. Direct 2-propanol extraction of the curd reduced polyphenol content by 65%, and polyphenols were not detected when the curd was washed with water before 2-propanol extraction.

INTRODUCTION

Leaf protein concentrates have been assessed as a children's food (Kamalanathan et al., 1969, 1970; Oke, 1971, 1973; Olatunbosum et al., 1972; Kamalanathan and Devadas, 1975) and included in food formulations (Lencioni et al., 1984, 1987, 1989; Meimbam et al., 1982; Toosy and Shah, 1974; Barbeau and Kinsella, 1987). However, further research is needed to improve the yields and purity of the concentrates.

Freezing alfalfa leaf juice produces a curd, called freezing curd, that contains 50% of the dry matter and 60% of the nitrogen in the original juice. After 2-propanol extraction, the freezing curd has a high protein content, minimum lipid content, and a color and texture similar to that of white protein concentrates (Hernández et al., 1988a). The possible antinutritional factors, like polyphenols, that it may contain must be determined before this concentrate can be used for human consumption.

Polyphenolic compounds are characterized both by their metabolic properties in the organism and by decreased nutritional quality of foods. When ingested, the polyphenols have antivitamin K or estrogenic effects and reduce intestinal motility, etc. (Mabry, 1970; Metche, 1980; Monties, 1981). In addition, they may act as effectors in transformation or regulation metabolic systems and produce more or less specific enzymatic inhibitions (Monties, 1981).

The decrease in the nutritional quality of the foods is the result of bonding between polyphenols and proteins. The characteristics and effects of the bonds depend on the type of interaction: either covalent or noncovalent bonds can be produced between both molecules. Noncovalent bonds can be produced at acid or neutral pHs and are reversible (Oh, 1980; Pierpoint, 1983). The polyphenols that can undergo this reaction are fundamentally polymeric polyphenols or tannins, although the monomeric or nontannin polyphenols can also produce noncovalent bonds (Synge, 1975; Pierpoint, 1983). As a consequence the nutritional value is decreased and the tridimensional structure of the proteins is modified, thereby altering their functional properties (Monties, 1981). The covalent interactions between polyphenols and protein, together with a series of prior enzymatic transformations, contribute to the phenomenon of browning. These interactions are irreversible and further contribute to a decrease in the nutritional value of the food, since they bind to essential amino acids, and alter the organoleptic qualities, fundamental flavor, and color (Monties, 1981; Pierpoint, 1983; Cheftel, 1977).

Table I. Total Polyphenol and Partially Polymerized Polyphenol Content of the Different Concentrates (Percent Dry Matter)

	total polyphenols	partially polymerized polyphenols
FC	1.034 ± 0.050	0.93 ± 0.056
IFC	0.360 ± 0.060	0.36 ± 0.004
WIFC		

In this paper the total polyphenol, chlorogenic acid, and orthodiphenol content in the three products, freezing concentrate (FC), 2-propanol-extracted freezing concentrate (IFC), and freezing concentrate washed with water before 2-propanol extraction (WIFC), are determined. The types of phenolic compounds in the concentrates are also characterized by means of UV-vis spectral analysis.

MATERIALS AND METHODS

Concentrate Preparation. Preparation of the freezing curd has been previously described (Hernández et al., 1988a,b, 1989) (Figure 1). Briefly, alfalfa was harvested and then pulped and pressed. The juice was distributed into small containers and frozen at -25 °C until use. As needed, each sample was thawed at room temperature for 18 h before use. Freezing curd so formed was separated from the thawed juice by filtering and sieving, some of the freezing curd was freeze-dried to produce the freezing concentrate (FC). 2-Propanol extraction was done in a Soxhlet apparatus at the solvent's boiling point, either immediately after curd obtention (IFC) or after the curd was washed by centrifugation with distilled water (WIFC). After residual solvent was eliminated by evaporation at 105 °C, the extracted freezing concentrates were ready for analysis.

Polyphenol Extraction. Polyphenol extraction was carried out in 25 mg of sample with 25-mL aliquots of methanol/water (1:1). Four extractions were realized, and the pH was adjusted to 4 in each with 0.1 N HCl. The residue-extractant mixture was introduced into an ultrasound bath in the last extraction to favor polyphenol dissolution. Extracts were separated by centrifugation and then filtered in a no. 3 glass filter crucible, and the residue was washed with 10 mL of the extractant. All liquids were mixed and then diluted to 125 mL with the same solvent.

Polyphenol Determination. The total polyphenol determination was carried out according to the Folin-Ciocalteau method (Swain and Hillis, 1959) using chlorogenic acid (Sigma) to prepare the calibration curve. Other reducing compounds that are present in the sample (primarily sugars, amino acids, and proteins) may interfere in total polyphenol determination by this method. To quantify these interferences, the amounts of proteins (Kjeldahl N × 6.25) and soluble sugars (Anthrone reagent analysis) were determined in 5 mL of extract (the starting sample volume for determination) and then an equivalent quantity of the albumin and glucose standards was weighed so their contribution to the total polyphenols could be determined.

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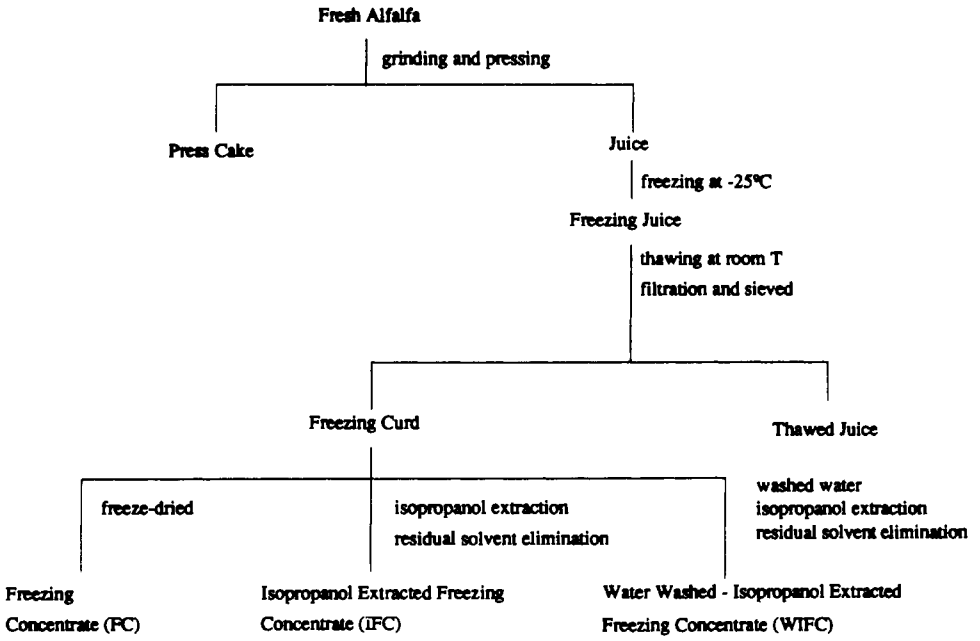

Figure 1. Obtention process of freezing protein concentrates.

For orientative purposes the proportion of partially polymerized polyphenols and fully polymerized polyphenols was determined. The method is based on precipitating the polymerized polyphenols according to the Masquelier et al.(1965) method with NaCl at saturation levels and then determining the partially polymerized polyphenols in the supernatant according to the Folin–Ciocalteau method.

Chlorogenic acid determination was carried out according to the Hoepfner method (Monties et al., 1978) with a chlorogenic acid standard (Sigma) and the orthodiphenols were determined following the Flanzy and Aubert (1969) method with a D+ catechin standard (Sigma).

Ultraviolet and Visible Spectra Analysis and Characterization. The UV–vis spectra were made with the methanol/water sample extracts. To characterize the spectrum, extracted aliquots were evaporated until dry at 40 °C and then redissolved in a methanol volume equal to the initial one. Specific reagents (sodium methoxide, anhydrous sodium acetate, aluminum chloride, and boric acid) were added to the aliquots of these methanolic extracts, and each corresponding spectrum was obtained.

RESULTS AND DISCUSSION

Table I shows the results, expressed as $x \pm \sigma_{n-1}$ ($n = 5$), for the total polyphenols and partially polymerized polyphenols in the different concentrates. The quantity of partially polymerized polyphenols is practically the same as the quantity of total polyphenols in the samples. This indicates the absence of polyphenols with high molecular weight, condensed and hydrolyzable tannins. Monties and Rambourg (1978) also did not find these polymers in white alfalfa concentrates.

The polyphenol content decreased approximately 65% when the concentrate was extracted with 2-propanol alone, and when the concentrate was washed before extraction, no polyphenols were detected in the sample (Table I). Rambourg and Monties (1983) note a 46% decrease in polyphenols after the white concentrate is washed with ethanol and a 67% decrease after the concentrate is washed with water and ethanol at the same time. Saeed (1988) found a decreased polyphenol content in defatted sunflower meal to undetectable levels after at least eight extractions with acidified butanol at pH 5.

No interferences were detected with the reducing sugars at the experimental wavelength (725 nm). However, the presence of albumin in the extract was proven to be responsible for an overestimation of polyphenol content on the order of 18% in the most extreme case (the sample

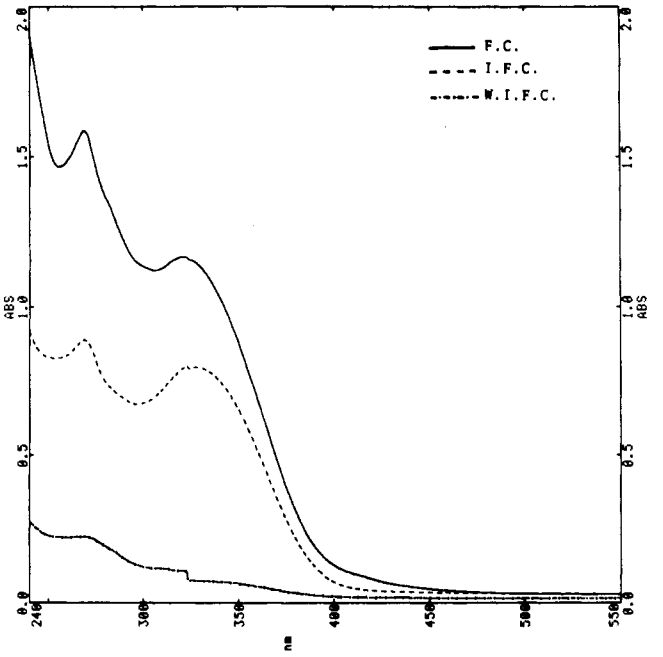

Figure 2. Absorption spectra of methanol/water (1:1) extracts.

with the highest protein content). This has already been shown by other authors (Lahiry and Satterlee, 1975; Figliuolo et al., 1987) and confirms the necessity of a more selective method for polyphenol evaluation.

Under these analytical conditions neither the red color nor the absorption peak was observed at 520 nm, which is characteristic of chlorogenic acid. The only color to appear was a yellow that would indicate the presence of flavonoids (Monties and Rambourg, 1978).

Orthodiphenols were not detected, either because they were present at trace levels or because of their high reactivity they did not exist as such.

Figure 2 presents the UV–vis spectra of the methanol/water extracts of the different concentrates between 200 and 600 nm and shows the following: (1) There is an absence of absorption maxima above 350 nm. Maxima above this level indicate the existence of liposoluble pigments (Monties and Rambourg, 1978). (2) The different concentrates show two major absorption peaks, one between 320 and 330 nm and the second near 270 nm.

Table II. Spectral Characteristics of the Concentrate Methanol/Water (1:1) Extracts

	λ_{max}	
	band I	band II
FC	322.4	269.3
IFC	322.0	269.3
WIFC		268.8

Table III. Spectral Shifts Produced by the Specific Reagents in the Freezing Concentrate Methanolic Extracts

	shifts, nm	
reagents	band I	band II
$NaOCH_3$	+60	no shift
$NaOCOCH_3$	no shift	+5
$NaOCOCH_3/B(OH)_3$	no shift	no shift
$AlCl_3/HCl$	+50	no shift

These absorbance bands are characteristic of flavonoids (band I usually 300–500 nm and band II usually 240–285 nm) (Mabry, 1970; Markaham, 1982). (3) Extraction with 2-propanol notably decreases the polyphenol content, which is even lower when the concentrate is washed with water before extraction.

The characteristics of the methanolic spectra and the effects of adding certain specific reagents on the spectra were considered to determine which flavonoids were present in the samples. Table II gives the absorption maxima in the different samples. In all samples, band I oscillates between 323 and 324 nm and band II between 269.6 and 268.8 nm. These bands correspond to flavone-type flavonoids: band I between 304 and 350 nm and band II between 240 and 280 nm (Mabry, 1970; Markaham, 1982). Table III shows the effects of different specific reagents induced in the spectra. Using tables (Mabry, 1970; Markaham, 1982) to relate the type of phenolic compounds found with the shifts induced in the spectra confirms that the principal flavonoids in these samples are the flavones. They were found to be glycosides since the sample was not hydrolyzed. These results coincide with those found by Monties and Rambourg (1978) relative to the presence of flavone orthoglycosides in white alfalfa protein concentrates.

In conclusion, it can be said that the principal polyphenols in these freezing concentrates are flavonosides, and these compounds can be completely eliminated by washing the concentrates with water prior to their extraction with 2-propanol. By means of this process, which is technically and economically feasible, it is possible to enhance the nutritional value and the final acceptability, primarily color, of these concentrates, increasing their potential use as food ingredients.

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