Available Carbohydrates in Alfalfa Leaf Protein Concentrates

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Freezing curd, prepared from alfalfa juice, was extracted with 2-propanol either untreated or after washing with water. The available carbohydrates were analyzed in the two extracted concentrates and unextracted freezing concentrate to deduce the effect of extraction on its content. Two methods were employed to analyze the ethanolsoluble sugars: the anthrone–sulfuric acid method and the 3,5-dinitrosalicylate method. The sugars were identified by gas chromatography. Hexoses and pentoses were the main soluble sugars. Direct extraction of the freezing curd with 2-propanol lowered the sugar content by 25%; water washing and 2-propanol extraction lowered the sugar content by 60%. The starch determination was carried out using two methods. 2-Propanol extraction of the freezing curd lowered the starch content by 24%; water washing and 2-propanol extraction lowered the starch content by 30%. These results indicate that 2-propanol extraction may have led to the formation of resistant starch in the concentrates.

Keywords: *Alfalfa; carbohydrates; protein; starch*

INTRODUCTION

Alfalfa leaf protein concentrate (LPC) has been regarded as a potential alternative to conventional sources of edible protein. LPC has been assessed as food for children (Kamalanathan *et al.*, 1969, 1970; Oke, 1971, 1973; Olatunbosum *et al.*, 1972; Kamalanathan and Devadas, 1975) and included in food formulations (Toosy and Shah, 1974; Meimbam *et al.*, 1982; Lencioni *et al.*, 1984, 1987, 1989; Barbeau and Kinsella, 1987). Nevertheless, 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 present in the original juice. Extraction of the curd with 2-propanol produces concentrates with high protein, minimum lipid and polyphenol contents, and color and texture similar to that of white leaf protein concentrates. Yields of such chlorophyll-free concentrates are much higher than those of white leaf protein concentrates (Hernández *et al.*, 1988, 1991).

For leaf concentrates to be used in human nutrition, all of their components must be analyzed. However, available carbohydrates in these concentrates are usually assessed by difference following determination of the other food components. Although the carbohydrate content of such concentrates is not high, proper assessment is important because carbohydrates may interact with other food components during processing, thereby altering the nutritive value of the foods. Also, the acceptability, palatability, shelf life, and functional properties of foods may be affected (Kearsley, 1985).

The object of the present study was to determine the content of soluble sugars and starch in three concentrates prepared from alfalfa freezing curd and to ascertain the effect of solvent extraction and washing with water of the freezing curd on the available carbohydrate contents of such concentrates.

MATERIALS AND METHODS

Concentrate Preparation. Preparation of the freezing curd has been described elsewhere (Hernández *et al.*, 1988a,b, 1991). Briefly, alfalfa was harvested and then pulped and pressed. The juice was distributed into small containers and frozen at −25 °C until used. 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. The extraction was conducted on untreated curd either immediately after preparation, yielding 2-propanol-extracted freezing concentrate (IFC), or after the curd had been washed with distilled water, by centrifugation, yielding water-washed 2-propanol-extracted freezing concentrate (WIFC). Following removal of the residual solvent, the extracted freezing concentrates were ready for analysis.

Extraction and Determination of the Soluble Sugars. A total of 100 mg of LPC was extracted with chloroform/methanol (2:1 v/v) to remove the lipids and pigments. The lipid-free LPC residue was extracted four times with 20 mL of hot 80% ethanol. A fifth extraction was performed and the absence of sugars verified using the Molisch test. Extracts were separated by centrifugation. All liquids were mixed and then diluted to 100 mL with 80% ethanol.

Determination of the soluble sugars was done by two techniques: the anthrone–sulfuric acid (McCready *et al.*, 1950) and 3,5-dinitrosalicylate methods (Miller, 1959). In both cases D-(+)-glucose (Merck) was used to prepare the calibration curve.

In the 3,5-dinitrosalicylate method, 0.5 mL of glucose solution (0.5 mg/mL) was added to the samples and standards to offset losses sustained in the presence of the dinitrosalicylic reagent (Miller, 1959).

Gas Chromatographic Analysis of Soluble Sugars. A standard (1 mL) of a 1 mg/mL solution of *myo*-inositol (Sigma) was added to the alcohol extracts of the concentrates, which were then brought to a volume of 100 mL and evaporated to dryness. The sugars were resuspended in 5 mL of distilled water, reduced to alditols with $NaBH_4$ (Merck) for 12 h, acetylated with pyridine acetic anhydride (1:1), evaporated to dryness, and finally resuspended in 5 mL of chloroform.

A Sigma 3 gas chromatograph (Perkin-Elmer) equipped with a flame detector and oven temperature programmer was employed. Oven temperature was held at 200 °C for 3 min, programmed to increase by 10 °C/min to 230 °C, and then held at that temperature for 8 min. The temperature of the injector and detector blocks was 250 °C. Carrier gas (nitrogen) flow rate was 30 mL/min. The column was packed with 3% SP2340 on Supelcoport 100/120 mesh (2 m, 1/4 in.). Injection volume was 0.3 μL.

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Table 1. Soluble Sugar Content (Percent Dry Matter) in the Different Concentrates ($x \pm \sigma_{n-1}/n = 4$) Determined According to the 3,5-Dinitrosalicylate and Anthrone–Sulfuric Acid Methods

concentrate	3,5-dinitrosalicylate	anthrone–sulfuric
FC	2.18 ± 0.15	2.10 ± 0.08
IFC	1.68 ± 0.35	1.51 ± 0.32
WIFC	0.80 ± 0.10	0.88 ± 0.34

The soluble sugars were identified by comparing their retention times with those of commercial standards. *myo*-Inositol (Sigma) was used as the internal standard in the quantitative analysis.

Starch Determination. Two different methods of starch determination were carried out for purposes of comparison, the methods of McCready *et al.* (1950) and Englyst and Cummings (1984).

In the McCready *et al.* (1950) method, the starch was extracted from the sugar-free residue with perchloric acid and then determined directly using the anthrone–sulfuric acid procedure.

According to the Englyst and Cummings (1984) method, the starch was dispersed by adding 2 mL of dimethyl sulfoxide (DMSO) (Merck), heating in a boiling water bath, and then adding 8 mL of 0.1 M sodium acetate buffer (pH 5.2) at 50 °C. Enzymatic hydrolysis of the starch was then performed by adding 0.1 mL of an enzyme solution containing 5000 units of pullulanase (Sigma)/mL of acetate buffer at pH 5.2. The samples were incubated at 45 °C for 16–18 h, with continuous mixing. Following the enzyme treatment, 40 mL of absolute ethanol was added and the mixture centrifuged until a clear supernatant liquid was obtained. The residue was washed twice with 50 mL of 85% ethanol and 40 mL of acetone, followed by mixing, centrifuging, and decanting the supernatant liquid. The supernatants were brought up to 250 mL, and the starch content was determined by the anthrone–sulfuric acid method.

In both cases the starch content was calculated by multiplying the resulting glucose concentration value by 0.90

RESULTS AND DISCUSSION

Table 1 presents the results obtained for the sugar determinations by the anthrone–sulfuric acid and the 3,5-dinitrosalicylate methods in the different concentrates expressed as percentage of dry matter (%dm). The 3,5-dinitrosalicylate method determines the reducing sugars, whereas the anthrone–sulfuric acid technique determines those sugars capable of forming furfural or furfural derivatives under the conditions of the method, chiefly hexoses and pentoses. Since the results for both methods were similar, it seems reasonable to assume that the said hexoses and pentoses were the predominant sugars in the samples.

Table 1 shows that extraction of the curd with 2-propanol lowered the sugar content of the concentrate by around 25%. Washing the curd with water and 2-propanol extraction lowered the sugar content by about 60%.

The sugars extracted from the concentrates were identified using gas chromatography. All of these sugars were monosaccharides, mainly glyceraldehyde, arabinose, xylose, glucose, and mannose. The sugars erythrose, galactose, ribose, and threose were contained also in the standard solution, but they were not detected in the concentrates.

Table 2 gives the percentage of relative peak area for each sugar identified in each concentrate, calculated as the proportion between the peak area for each sugar and the total of all the peak areas combined. The values indicate that most of the sugars in the concentrates were identified. However, because of the unidentified sugars,

Table 2. Percentage Relative Peak Areas (%RA) and Simple Sugar Content [Percent Dry Matter (%dm)] for the Different Concentrates by Gas Chromatography

	FC		IFC		WIFC	
sugar	%RA	%dm	%RA	%dm	%RA	%dm
arabinose	11.01	0.15	5.51	0.02	5.79	0.02
glyceraldehyde	6.67	0.14	3.22	0.02	5.85	0.02
glucose	23.81	0.37	34.02	0.16	28.73	0.08
mannose	21.29	0.33	4.23	0.02		
xylose	15.38	0.20	34.93	0.14	56.79	0.13
others	21.81		18.08		2.84	

Table 3. Recovery of Soluble Starch (Percent Dry Matter) ($x \pm \sigma_{n-1}/n = 4$)

McCready *et al.* (1950)	Englyst and Cummings (1984)
64.3 ± 1.67	92.0 ± 0.35

Table 4. Starch Content (Percent Dry Matter) in the Alfalfa Protein Concentrates ($x \pm \sigma_{n-1}/n = 4$)

concentrate	starch
FC	4.84 ± 0.03
IFC	3.68 ± 0.19
WIFC	3.38 ± 0.02

the total sugar value obtained by gas chromatography was lower than those obtained using the anthrone–sulfuric acid and 3,5-dinitrosalicylate methods, which also are less specific and subject to interferences.

Table 2 also sets out the content (%dm) of each sugar identified. Xylose and glucose, with mannose, were the primary sugars in the freezing concentrate. Mannose was completely eliminated in the concentrate prepared by extraction of water-washed curd.

In choosing a method of starch analysis, a sample of soluble starch was analyzed by the conventional extraction method using perchloric acid (McCready *et al.*, 1950) and a technique of enzymatic hydrolysis (Englyst and Cummings, 1984). The results are presented in Table 3. Recovery was much lower by the McCready *et al.* method, either because of poor extraction with perchloric acid or due to insufficient dispersion of the starch. Since recovery by the method of Englyst and Cummings was quasi-quantitative and the concentrates can contain resistant starch, this latter method was selected for performing starch determinations.

The starch values (%dm) in each of the three concentrates appear in Table 4. The starch content in the freezing concentrate (4.84%) was higher than that in the concentrates extracted with 2-propanol. Extraction of the untreated curd with 2-propanol lowered the starch content by approximately 24%, whereas washing the curd with water prior to organic solvent extraction lowered the starch content in the concentrate by about 30%. In other words, about 6% of the starch in the curd was water-soluble under the conditions of washing employed in this study. No values for the starch content of leaf protein concentrates and no information on the possible effect of solvent extraction have been found in the literature.

Considering that starch is not soluble in 2-propanol and that the extraction step acted to concentrate the sample components, the amount of starch in the concentrates could have been expected to increase, the converse of what actually occurred.

The only explanation for this would be that as a result of extraction with 2-propanol, part of the starch was modified and converted to a form resistant to enzyme action. Fennema (1982) reported that extraction with hydrophilic solvents brought about alterations in the

gelatinization properties of starch. Englyst *et al.* (1982) observed that the gelatinization temperature of starch decreased in the presence of alcohols. In view of these findings, the starch may have gelatinized during extraction with 2-propanol, which, as explained above, was performed hot on wet curd. Since the starch is insoluble in the solvent, it may then have undergone retrogradation when the sample cooled, because of its tendency to form H-bonds between adjacents molecules (Selvendran *et al.*, 1981). This retrograded starch is resistant to enzymatic hydrolysis.

Englyst *et al.* (1982) pointed out that the starch in boiled potatoes underwent retrogradation on cooling or drying of the sample and that the same effect occurred in potatoes subjected to extraction with acetone or ethanol. Theander *et al.* (1989) reported the same effect in pressure-cooked and French-fried potatoes and during bread-making.

DMSO was used as a starch-dispersing agent in the analysis, but the retrogradation effect referred to above was recorded nonetheless. This observation indicates that DMSO was not capable of dispersing the retrograde starch in the concentrates. Selvendran *et al.* (1981) observed that DMSO was not able to solubilize all of the starch in boiled potatoes.

Several workers have found that while retrograde amylopectin is readily hydrolyzed, the same is not true of retrograde amylose, which is highly resistant to enzymatic hydrolysis (Orford *et al.*, 1987; Ring *et al.*, 1988; Theander *et al.*, 1989; Englyst and Cummings, 1990). Thus, the resistant starch that formed could consist primarily of retrograde amylose that was not solubilized by DMSO. Treatment with KOH has been proposed as an alternative dispersing agent for retrograde starch (Englyst *et al.*, 1982; Theander *et al.*, 1989) and could be tested in future work. However, as Schweizer (1989) pointed out, determination of the resistant starch in dietary fiber would provide a more realistic approximation of the indigestible polysaccharides in food.

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