Table I, nitrogen solubility was greater ($P < 0.05$) in the propionic acid reconstituted grain which also had lower protease activity. Prigge (1976) also reported an increase in nitrogen solubility in acid-rather than water-reconstituted grain. This would suggest that either the initial level of protease activity is sufficient to alter chemical composition or other proteolytic enzymes are active during reconstitution. A large proportion of the increase in soluble nitrogen was achieved by the fourth day of reconstitution. A rapid increase in nitrogen solubility of reconstituted grain was also reported by Wilfong (1969). Therefore it may be that initial levels of protease activity are effective in solubilizing protein. Other sources of proteolytic activity exist in the microbial population that expands during the fermentation process of reconstitution. In this regard, fumigation of grain prior to aseptic reconstitution decreased nitrogen solubility (Billings, 1972). Thus, it would appear that several sources of proteolytic activity exist in reconstituted grain and that the protease activity of plant origin is not the sole enzyme responsible for chemical alterations of reconstituted sorghum.

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Distribution of Saponin in Alfalfa Protein Recovery Systems

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Saponin tended to be concentrated in the protein fractions at levels higher than in the original alfalfa during the preparation of white and green alfalfa leaf protein fractions. Coagulation of the protein at pH 6.0 and washing at pH 4.5 gave as much as a fourfold increase in saponin over that in the protein coagulated and washed at pH 8.5. The press cakes from high and low saponin alfalfa, following dejuicing, retained 65 and 87% of the saponin, respectively. The green protein contained most of the extracted saponin. Preparation of protein fractions from a low saponin cultivar resulted in saponin levels of less than 0.07%, compared to a saponin content of 1.33% in the protein prepared from a high saponin alfalfa cultivar. Six common vegetable food products were found to contain levels of saponin (0.02 to 0.07%) similar to that found in the white leaf protein fraction.

The high production of protein in alfalfa plants offers a source of nutrients which can help meet the protein requirements for humans as well as animals. The preparation of both whole and fractionated alfalfa leaf protein concentrates (LPC) has been studied for many years by workers such as Pirie (1966) in England and by Stahmann (1968) at the University of Wisconsin and more recently by workers from this laboratory (Edwards et al., 1975; and Kohler and Knuckles, 1977). Alfalfa LPC preparations have been shown to be excellent sources of nutrients for monogastric animals (Cheeke, 1974; Cheeke et al., 1977a; Cheeke and Myer, 1973; Kuzmicky and Kohler, 1977; Kuzmicky et al., 1977) and potentially for human consumption (Pirie, 1966; Bickoff et al., 1975). The possible contamination of these LPCs by naturally occurring biologically active compounds necessitates an investigation of the distribution of these compounds during the production of protein concentrates. Knuckles et al. (1976) determined the coumestrol content of alfalfa LPCs prepared under several processing conditions and observed that the protein concentrates, including both green LPC and white LPC, contained lower concentrations of the forage estrogen, coumestrol, than did fresh alfalfa or the resulting pressed alfalfa. In addition to the forage estrogens, alfalfa as well as other legumes have been found to be a source of biologically active saponins (Peterson, 1950; Walter et al., 1954; Birk et al., 1963) which may have a deleterious effect upon poultry (Tung et al., 1977) or other animals (Cheeke et al., 1977b). These alfalfa saponins are composed of long-chain carbohydrates attached principally to the aglycon, medicagenic acid (Djerassi et al., 1957; Gestetner, 1971) with smaller amounts of a second biologically active aglycon, hederagenin, also present (Shaney et al., 1972).

This study describes the distribution of the biologically active saponins during the preparation of LPCs by two different processes. In one process (Pro-Xan) the soluble and insoluble proteins are precipitated together (whole LPC) to provide a chlorophyll-pigmented-protein con-

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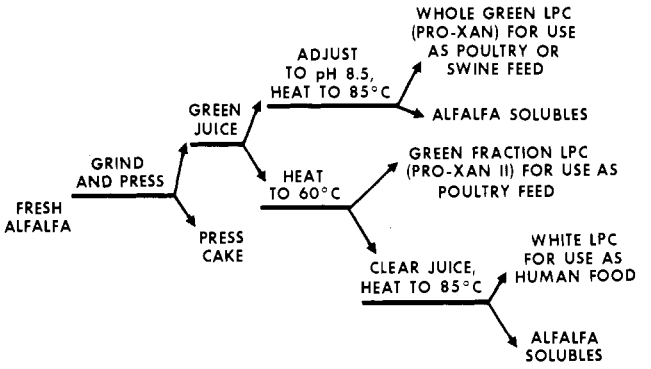

Figure 1. Process steps in leaf protein preparation.

centrate. In the second process (Pro-Xan II) both a chlorophyll-pigmented (green LPC) and a white protein (white LPC) concentrate are prepared separately (deFremery et al., 1973; Edwards et al., 1975).

EXPERIMENTAL SECTION

High and low saponin cultivars of the Ranger variety developed by Pedersen and Wang (1971) and Pedersen et al. (1967) were grown at the University of California, Davis, and used in this study. The fresh alfalfa was ground at its natural pH (pH 5.8–6.1) and pressed with a twin-screw press to provide a press cake and a whole green juice. The juice was subsequently adjusted to pH 8.5 with concentrated NH_4OH. Whole LPC was prepared by heating 1500-g portions of the green juice to 80–85 °C by direct steam injection, holding for 5 min, then cooling to room temperature. The coagulated protein curd was transferred to a nylon filter bag, pressed to 35–40% solids, and freeze dried (Edwards et al., 1977). The brown juice from the pressed green curd was freeze-dried for saponin analyses.

Fractionated LPC was prepared by heating the whole green juice to 60 °C for 20 s by steam injection and cooling immediately (deFremery et al., 1973). An accurately weighed aliquot of the heat-treated juice was centrifuged at 0 °C and 27000*g* for 20 min to separate the green LPC from the soluble white LPC. The clear supernatant was decanted from the green solids and heated on a steam bath to 85 °C for 5 min, then rapidly cooled in an ice bath. The coagulated white LPC was cooled, collected by centrifugation, washed three times with a volume of water equal to the volume of the supernatant, and then freeze-dried. Figure 1 presents an outline of the processing procedures employed.

In certain experiments, the pH of coagulation and of the washing of the white LPC was varied to determine the effects upon saponin distribution. The supernatant alfalfa solubles from the white LPC preparation were also freeze-dried.

Saponin analysis of the products prepared was by the procedure of Livingston et al. (1977). Each sample was assayed in duplicate at three levels using a *Trichoderma* bioassay, comparing the results with a standard saponin, and calculating saponin in the sample analyzed by means of a slope ratio analysis.

RESULTS AND DISCUSSION

The initial evaluation of the saponin content of alfalfa leaf protein concentrates prepared from the Lahontan cultivar of alfalfa suggested that a substantial portion of the saponin had been concentrated in the leaf protein fractions. This may be explained by the fact that the alfalfa saponin complexes with leaf protein by an apparent hydrogen bonding type reaction (Livingston et al., 1977). Since the Lahontan cultivar of alfalfa employed in this initial study was considered to be a fairly low saponin variety, further studies were conducted to ascertain saponin distribution during the preparation of LPCs from both high and low saponin cultivars.

Table I. Effect of pH of Coagulation and Wash upon the Saponin Content of White LPC[a]

pH of coagulation	pH of wash	saponin content, %[b]
6.0	4.5	1.09 (0.92-1.28)[c]
6.0	6.0	0.55 (0.29-0.81)
6.0	8.5	0.57 (0.40-0.75)
8.5	4.5	0.40 (0.29-0.63)
8.5	6.0	0.47 (0.30-0.64)
8.5	8.5	0.24 (0.20-0.29)

[a] Saponin content of the whole alfalfa was 0.25%, 95% confidence limits, 0.20–0.29%. [b] Dry basis. [c] Numbers in parentheses are 95% confidence limits.

Table II. Saponin Content (Dry Basis) of Products from the Preparation of Whole and Fractionated LPC's from Low and High Saponin Alfalfa

	high saponin alfalfa	low saponin alfalfa
product	saponin content, %	saponin content, %
whole alfalfa	1.71 (1.33-2.11)[a]	0.14 (0.12-0.16)[a]
pressed alfalfa	0.66 (0.54-0.75)	0.13 (0.11-0.14)
whole LPC (Pro-Xan)[b]	1.90 (1.58-2.31)	0.10 (0.08-0.12)
alfalfa solubles	0.65 (0.51-0.81)	0.06 (0.05-0.08)
green LPC (Pro-Xan II)[b]	1.54 (1.33-1.78)	0.08 (0.06-0.09)
white LPC[b]	2.88 (2.47-3.33)	0.21 (0.19-0.24)
alfalfa solubles[b]	0.47 (0.37-0.54)	0.06 (0.04-0.07)
white LPC[c]	0.97 (0.85-1.10)	0.12 (0.10-0.14)
alfalfa solubles[c]	1.17 (0.97-1.38)	0.20 (0.18-0.22)

[a] Numbers in parentheses are 95% confidence limits. [b] From coagulation and washing pH 6.0. [c] From coagulation and washing pH 8.5.

Table I presents the effects of varying pH during coagulation and washing upon the saponin content of white LPC. Both of the most biologically active alfalfa saponins contain carboxyl groups and therefore tend to be more soluble in alkaline than in acid solution. As would be expected, the white LPC prepared by both coagulating and washing at pH 8.5 contained less saponin than the products prepared at lower pHs. It would seem that the pH of coagulation is the most important since the white LPC coagulated at pH 8.5 contained less than half the saponin of the white LPC coagulated at pH 6 and then washed at pH 8.5. It is also noteworthy that the saponin content of the white LPC produced by coagulating and washing at pH 8.5 was the same as that of the whole alfalfa. Knuckles et al. (1976) observed that the coumestrol content of white LPCs was also reduced by coagulation and washing at alkaline pH due to the increased solubility of the phenolic compounds.

Table II gives the saponin content of products from high and low saponin cultivars. Contents of the whole LPCs prepared from either low or high saponin alfalfa were slightly higher than in the respective green LPCs, but in both instances the saponin contents were highest in the white LPCs coagulated and washed at pH 6.0 (Table II). Coagulation and washing of the white LPCs at pH 8.5 greatly reduced the saponin contents (2.88 to 0.97% and 0.21 to 0.12%, respectively). The saponin content of the white LPC prepared at pH 8.5 from high or low saponin alfalfa was less than that of the respective whole alfalfa. Thus, the apparent problem of the saponin complexing with protein can be largely solved by coagulation and washing of the protein at an alkaline pH (8.5). By employing this procedure, white LPCs have been produced

Table III. Distribution of Saponin and Dry Matter (Dry Basis) during the Preparation of Fractionated Leaf Protein Concentrates from Low and High Saponin Alfalfa

	high saponin alfalfa		low saponin alfalfa	
product	dry matter yield, wt %	saponin % of recovered	dry matter yield, wt %	saponin % of recovered
press cake	76.0	65.0	80.3	86.8
green LPC[a]	11.1	22.1	8.7	5.8
white LPC[a]	1.6	6.0	1.5	2.7
alfalfa solubles[a]	11.3	6.9	9.5	4.7
white LPC[b]	1.9	2.2		
alfalfa solubles[b]	10.5	15.0		

[a] From coagulation and washing, pH 6.0. [b] From coagulation and washing, pH 8.5.

Table IV. Saponin Content of Plant Food Products and White LPC

product[a]	saponin[b] found, %
white LPC	0.07 (0.05-0.09)[c]
alfalfa sprouts	7.93 (6.58-9.49)
spinach leaves (frozen)	0.07 (0.06-0.08)
green beans (frozen)	0.05 (0.04-0.06)
red beans	0.02 (0.01-0.03)
brussel sprouts (frozen)	0.03 (0.02-0.04)
peas (dry)	0.03 (0.02-0.04)
black eyed peas (dry)	0.03 (0.02-0.04)
soybeans (dry)	0.01[d]
bean sprouts	0.01
green peas (frozen)	0.01
snow peas (frozen)	0.01

[a] Fresh and frozen samples were freeze-dried prior to analysis. [b] Dry weight bases. [c] Numbers in parentheses are 95% confidence limits. [d] Limit of analytical method.

from low saponin alfalfa with saponin contents from 0.07 to 0.12%.

Most of the dry matter and saponin remained in the press cake following dejuicing of the whole alfalfa (Table III). Although the white LPCs coagulated at pH 6.0 contained only 1.5 and 1.6% of the dry matter, respectively, the products did contain relatively higher proportions of recovered saponin than the other protein products. However, preparation of the white LPC from the high saponin alfalfa by coagulation and washing at pH 8.5 greatly reduced the proportion of saponin in the protein concentrate. Most of the saponin was left in the alfalfa solubles.

The saponin contents of eleven common vegetable food products, and a preparation of white LPC, are presented in Table IV. Six of the food products contained levels of saponin (0.02 to 0.07%) similar to that in the white LPC, while the alfalfa sprouts contained a level of saponin far exceeding any other food products, or any of the high saponin alfalfa samples analyzed at this laboratory. Knuckles et al. (1976) reported finding relatively high levels of coumestrol in both alfalfa and soybean sprouts. Soybeans have been found to contain saponins which have less biological activity than the alfalfa saponins, as measured by mold bioassay, and, therefore, the apparently low level of saponin found in the soybeans was expected. However, the saponin content of the spinach leaves was unexpectedly high.

The results demonstrate that the biologically active saponins may be concentrated in the high protein fractions during the preparation of alfalfa leaf protein concentrates,

Table V. Saponin Content of Alfalfa Meals

cultivar/source	drying method	saponin found, %	95% conf. limits, %
Lahontan/Kansas	dehydrated	0.19	0.16-0.25
Northrup King Thor/Kansas	dehydrated	0.67	0.54-0.86
California common	dehydrated	0.21	0.09-0.34
Kansas common	dehydrated	0.58	0.46-0.75
Ranger/Nebraska	dehydrated	0.55	0.42-0.73
Lahontan/California	suncured	0.37	0.25-0.52
Lahontan/California	dehydrated	0.16	0.12-0.20
Ranger/low saponin	dehydrated	0.14	0.12-0.16
Ranger/high saponin	dehydrated	1.71	1.33-2.11

unless the processing conditions are carefully controlled. The solubility of the saponins in alkaline solutions makes it desirable to coagulate and wash the protein precipitate at pH 8.5. By employing suitable processing conditions, and using low saponin varieties of alfalfa, it is possible to prepare white LPC with a saponin content similar to that found in many common vegetables.

To minimize the saponin content of LPCs prepared from alfalfa, it is important to use a plant source with a low saponin content. Table V presents the saponin contents of a number of commercially grown alfalfa varieties from several states. The wide range of saponin contents of the alfalfa meals emphasizes the importance of preparing LPCs from alfalfas known to be low in saponin content.

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Composition of Neutral Volatile Constituents in Grape Brandies

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The neutral volatile constituents in different grape brandies (French and German grape brandies, French cognacs) have been investigated by gas chromatography and coupled gas chromatography–mass spectrometry. Using standard controlled aroma separation by liquid–liquid extraction, prefractionation on silica gel, and identification by mass spectrometry, 139 neutral volatile compounds were quantitatively determined by gas chromatography. Clear quantitative differences exist between the amounts of some aroma constituents of German and French grape brandies as well as French cognacs. The mean values and ranges of the individual components are given for the three groups of grape brandies. The quantitative differences within the groups caused by different technologies of grape brandy production are discussed.

Wine as well as grape brandies may be considered as final links of a biotechnological sequence (Drawert, 1974). In the case of brandy this sequence is extended by distillation and aging. These additional technological steps influence the quality of the products by appropriate distillation processes and aging conditions. In this connection, in the countries with traditional methods of distillation of wines into brandies, the distillation and aging processes are subjected to legal regulations. The national decrees are adapted to the common EC regulations (Bitzer, 1974). In principle, the aim of all the decrees is to require a sufficiently long holding time during distillation at high temperatures to limit the concentration of ethanol in order to obtain adequate amounts of volatile compounds in the distillate.

Little is known about the raw materials of German grape brandies investigated in this work. For the distillates a minimum storage time of 6 months in oak casks is prescribed. Products declared as "old" grape brandies require a minimum storage time of 12 months. No minimum storage time is required for French grape brandies. These products can also be manufactured in Germany by diluting the imported refined distillate (Bitzer, 1974). Only grape brandies produced according to the strong regulations of the "appellation contrôlée jaune d'or" are permitted to be declared cognacs. The French law requires minimum storage time of 1 year for cognacs declared "trois étoiles". For all the other declarations a minimum storage time of 4 years must be regarded.

The qualitative composition of aroma compounds has been reviewed in several papers (Schaefer and Timmer, 1970; Rapp, 1972; Marché and Joseph, 1975; Yoshizawa, 1975). Apart from some exceptions (Litschew, 1976), the quantitative investigations have related to the main components such as esters (Koch et al., 1971; Reinhard, 1972; Hieke and Sage, 1973; Braun and Hieke, 1974; Postel et al., 1975; Heβ and Trott, 1977) and fusel alcohols (Webb et al., 1952; Reinhard, 1970; Drawert et al., 1967; Woidich and Pfannhauser, 1974; Connell and Strauss, 1974; Postel et al., 1975). The qualitative and quantitative composition of neutral volatile constituents isolated from different grape brandies is presented in this paper.

EXPERIMENTAL SECTION

Samples. Each of eight commercial samples of French and German grape brandies as well as French cognacs (VSOP) were investigated.

Liquid–Liquid Extraction. Each of 700 mL of grape brandy (diluted with 1300 mL of H_2O) was used adding three internal standards before the extraction: 420 μg/L of methyl octanoate, 440 μg/L of dimethyl methylmalonate, and 680 μg/L of 2-methyl-1-pentanol. The volatiles were isolated by liquid–liquid extraction for 8 h using pentane. A 100-mL organic phase was used for 1 L of diluted brandy (Drawert and Rapp, 1968). The aroma extracts were concentrated to 3 mL in a Vigreux column (45 °C) as described (Drawert et al., 1969). Commercial grade solvents (99%), further purified by redistillation, were used for liquid–liquid extraction.

Column Chromatography on Silica Gel. Aroma extracts (3 mL) were fractionated on silica gel 60 (Merck), activity grade II, using a pentane–diethyl ether solvent system (Schreier and Drawert, 1976; Schreier et al., 1978). Glass columns 1.8 i.d. × 40 cm containing 48 g of silica gel maintained at 11–13 °C were used. The elution rate was 60 mL/h and three fractions were obtained. Fraction I was eluted with 300 mL of 10% diethyl ether in pentane (internal standard methyl octanoate). Fraction II was eluted with 300 mL of 20% diethyl ether in pentane

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