value for the whole population of whiskies is 2.379 (0.069), Irish–Scotch whiskies are significantly different from bourbon whiskies (F test).

The four samples of gin investigated here offer good examples of pure corn alcohol since their average value $\bar{R}$ = 2.23 (0.02) is very close to the value of the standard corn ethanols (Table I).

Finally, the vodkas are characterized by high R values denoting either C3 cereals or mixtures of potatoes and cereals. It should be noted that the VR2 vodka is an exported spirit whereas the VR1 sample has been bought in the USSR. The Polish and Finnish vodkas either exported or sold in the country have roughly the same value (R = 2.56). The average values of the samples investigated is 2.55 (0.05).

Rums and Fruit Spirits (Table III). Like corn, sugar cane is a C4 plant and its photosynthetic cycle is responsible for a higher deuterium content and therefore a relatively small R value: R = 2.245 (0.036). The standard deviation corresponds to the population sample studied here (n = 10), but a more discriminative inspection of the results suggests two remarks. First, it appears that geographical and climatological conditions are second-order factors which play a role in the variations of the deuterium distributions since $\bar{R}$ = 2.21 (0.015) for West India's rums and $\bar{R}$ = 2.27 (0.02) for Antillas rums. Second, the procedure used in the manufacturing of the rums also affects the R value and slight but significative differences are observed between rums processed from sugar cane juices or molasses.

Apple and plum brandies display relatively high R values, and it seems that a significant difference exists between the two means of the population samples. It is also interesting to note that the average $\bar{R}$ value obtained for plum brandies is nearly equal to the R value measured for an ethanol extracted from a fermentation of pure fructose, $\bar{R}$ = 2.59 (0.035). The average value observed for apple brandies appears to be slightly smaller, $\bar{R}$ = 2.565 (0.02), and for the whole population of fruit spirits $\bar{R}$ = 2.585 (0.03).

CONCLUSION

We have therefore shown that very substantial differences exist in the distribution of ^2H within ethanol molecules according to their origin. We dispose now of new and very efficient parameters for identifying alcohols and investigating more deeply the biomechanisms which govern their formation.

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Registry No. Deuterium, 7782-39-0; ethanol, 64-17-5.

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In Vitro Methods To Assess the Nutritive Value of Leaf Protein Concentrate

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Leaf protein concentrate (LPC) from nine vegetations was analyzed for in vitro digestibility and amino acid composition by chemical methods. These LPCs were also assessed in animal experiments for true digestibility and net protein ratio (NPR). There were marked differences among the LPCs in amino acid composition (available lysine, sulfur amino acids, histidine, and tryptophan), pepsin and pepsin–pancreatin digestibility, and the food intake, growth, and digestibility in the animal experiments. There were good correlations between in vitro and in vivo digestibility, between sulfur amino acids and NPR, and among the changes in various amino acids and available lysine. Pepsin–pancreatin digestibility, available lysine, and the contents of sulfur amino acids promise to be a sensitive and rapid in vitro measure of the nutritive value of LPC.

The evaluation of nutritive value of leaf protein concentrate (LPC) is usually done by animal experiments. Some in vitro parameters such as digestibility with proteolytic enzymes (Buchanan, 1969; Saunders et al., 1973), support of growth of tetrahymena (Lexander et al., 1970; Smith and Pena, 1977), microbial availability of essential amino acids (Henry and Ford, 1965), and levels of available lysine (Allison et al., 1973) have been used to detect differences in nutritive value of LPC with varying degrees of success. There is a need for reliable and sensitive in

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vitro methods to assess the nutritive value of LPC during the screening of vegetations and processing since in vitro methods require less time, require small amounts of material, and are simple to carry out. A good correlation obtained by Wolzak et al. (1981) between traditional animal experiments and some chemical assays also suggests the usefulness of in vitro assays. In an attempt to evolve suitable in vitro methods, we prepared LPC from diifferent species and evalued their nutritive value by true digestibility (TD) and net protein ratio (NPR) and compared them with in vitro methods which included estimation of bound carbohydrates and polyphenols, the amino acids methionine, cyst(e)ine, tryptophan, and histidine from the enzymic hydrolysate, available lysine, and digestibility with pepsin and pepsin–pancreatin. An attempt was made to correlate in vivo and in vitro parameters.

EXPERIMENTAL SECTION

Vegetations were obtained from agricultural plots. Botanical names, age of vegetation, and cultivation conditions were same as described by Subba Rau et al. (1972). All chemicals used were of analytical reagent grade. Pepsin (1:60 000), Pronase, amino acid reference standards, chlorogenic acid, dithioerythritol (DTE), 5,5′-dithiobis-2-(nitrobenzoic acid) (DTNB), and *p*-(dimethylamino)benzaldehyde (DAB) were purchased from Sigma Chemicals. Pancreatin (activity > 3 NF units) was obtained from Centron laboratories, Bombay.

Extraction of Leaf Proteins. The LPC were prepared by the method of Morrison and Pirie (1961). The LPC were stored in polythene bags at 4 °C till needed.

Bound Polyphenols. Polyphenols were estimated by Folins–Denis reagent (AOAC, 1970) with chlorogenic acid as the reference standard (Swain and Hillis, 1959). A total of 250 mg of LPC was refluxed with 25 mL of 50% methanol for 1 h and centrifuged at 3000 rpm for 20 min. The residue was washed with 10 mL of 50% methanol. The residue, thus free from non covalently bound polyphenols, was digested with 5 mL of 2 N HCl for 30 min on a boiling water bath. It was cooled and neutralized with 1 mL of 10 N NaOH and centrifuged. The residue after acid digestion was suspended in 2 mL of 2 N NaOH containing 50 mg of sodium borohydride and after standing for 4 h at room temperature was neutralized with 1 N HCl and centrifuged. The supernatants from acid and alkali treatment were pooled and made to 25 mL, and suitable aliquots (0.1–0.2 mL) were taken for the estimation of bound polyphenols. A correction was made for the contribution of tyrosine and tryptophan from the solubilized proteins present in the extract. The extract was analyzed for the nitrogen content [micro-Kjeldahl method (AOAC, 1970)], and the average amino acid composition of LPC (Byers, 1967) was used to calculate the contents of tyrosine and tryptophan.

Bound Carbohydrates. Carbohydrates were estimated by the phenol–sulfuric acid method (Dubois et al., 1956) with glucose as the reference standard. A total of 250 mg of LPC was twice extracted with 10 mL of chloroform–methanol (2:1 v/v) to remove lipids and pigments. The lipid-free LPC residue was extracted 4 times with 25 mL of hot 80% ethanol to remove the soluble sugars. Five milliliters of water was added to the residue followed by 6.5 mL of 52% perchloric acid, and it was stirred for about 5 min. Twenty milliliters of water was added after 15 min and the contents were centrifuged. The residue was reextracted once. This treatment with perchloric acid solubilized the starch (McCready et al., 1950). The residue free from soluble sugars and starch was refluxed with 25 mL of 2 N H_2SO_4 for 2 h on a water bath. After completion of refluxing, it was cooled and centrifuged. The residue was suspended in 2 N H_2SO_4 and recentrifuged. The supernatant and washings were pooled and made to 50 mL for estimation of bound carbohydrate fraction.

Table I. Bound Carbohydrate and Bound Polyphenol Contents of Some LPC Preparations[a]

	g/100 g dry weight		mg/g of protein	
	carbohydrates	polyphenols	carbohydrates	polyphenols
alfalfa				
cytoplasmic	1.6	1.5	20.5	19.1
chloroplastic	3.9	1.1	71.5	19.4
radish	2.6	0.8	37.0	11.3
knolkhol	2.6	1.0	41.0	16.0
carrot	4.4	2.0	109.0	49.3
Sesbania	6.3	2.1	130.0	42.7
horse gram	3.4	1.0	73.9	21.8
French bean	3.2	1.0	66.8	21.0
Tephrosea	5.0	2.0	104.8	41.9
techoma	7.1	4.0	179.2	100.0

[a] Results are average of triplicate estimations. The difference between the maximum and the minimum value in any given average is less than 8% of the maximum value. A conversion factor of 6.25 was used to estimate protein from nitrogen.

Amino Acid Composition. All LPC samples were defatted with chloroform–methanol (2:1 v/v) prior to amino acid assay. Nitrogen content of LPC was estimated by the micro-Kjeldahl method (AOAC, 1970) and multiplied by a factor of 6.25 to convert it into protein content. LPC preparations were hydrolyzed with Pronase, and hydrolysates were used to estimate methionine, cystine, tryptophan, and histidine by group-specific chemical assays. Methionine was estimated by reaction with nitroprusside, cystine with DTNB, tryptophan with DAB, and histidine by Pauly's reagent involving diazotized sulfanilic acid. Available lysine was estimated by reaction with trinitrobenzenesulfonic acid (Hall et al., 1973). The details of assays have been described in an earlier publication (Maliwal, 1981).

Pepsin Digestibility. LPC containing 16 mg of nitrogen was hydrolyzed with 1 mg of pepsin in 15 mL of 0.1 N HCl at 37 °C for 12 h. The hydrolysate was centrifuged and the supernatant assayed for nitrogen content.

Pepsin–Pancreatin Digestibility. The procedure of Akeson & Stahman (1964) with some modifications was used to measure pepsin–pancreatin digestibility. After soaking for 12 h in 0.1 N HCl, LPC containing 16 mg of nitrogen was incubated with 0.5 mg of pepsin in 15 mL of 0.1 N HCl at 37 °C for 3 h. After completion of pepsin hydrolysis, the suspension was neutralyzed with 0.5 N NaOH, and 6 mg of pancreatin in 7.5 mL of 0.2 M phosphate buffer of pH 8 was added to it and incubated for an additional 24 h at 37 °C. The tubes were layered with toluene to prevent microbial growth. After completion of pancreatin digestion, the assay system was centrifuged and the supernatant analyzed for nitrogen.

Animal Experiments. NPR was measured at a 10% dietary protein level as described by Bender and Doell (1957). Egg protein was used as reference protein. From day 7 to day 10 the rats were housed in metabolic cages, and feces were collected and were assayed for nitrogen content to calculate TD.

RESULTS

Table I shows the bound polyphenol and bound carbohydrate contents of various LPC preparations. There were

Table II. Amino Acid Composition of Some LPC Preparations[a]

	mg/100 mg of protein								
	lysine							S amino acids[a]	
	total	available	% availability	trypto-phan	histidine	methio-nine	cystine	total	pepsin-pancreatin digest
alfalfa									
cytoplasmic	7.00	6.05	86.4	2.30	2.50	2.00	1.00	3.00	2.95 (3.37)
chloroplastic	6.60	5.00	75.8	2.00	2.30	1.57	0.82	2.39	2.10 (2.92)
radish	6.95	5.55	79.8	2.30	2.55	1.80	1.05	2.85	2.85 (3.40)
kholkhol	6.95	5.45	79.0	2.30	2.50	1.80	1.05	2.85	2.80 (3.41)
carrot	5.85	3.90	66.7	1.40	1.60	1.30	0.70	2.00	1.40 (2.24)
Sesbania	6.10	3.50	56.9	1.40	1.40	0.60	0.60	1.20	0.80 (1.90)
horse gram	6.35	4.30	67.7	1.45	2.05	1.43	0.80	2.23	1.40 (2.42)
french bean	7.55	5.00	66.2	1.70	2.20	1.60	0.90	2.50	2.00 (2.82)
Tephrosea	7.70	4.85	63.0	1.70	2.30	1.60	0.80	2.40	1.90 (2.79)
techoma	5.80	2.70	48.3	1.20	1.70	0.40	0.60	1.00	0.60 (2.72)

[a] Values are average of triplicate estimations. The difference between the maximum and the minimum value in any given average is less than 8% of the maximum value. The values in parentheses are the amounts of sulfur amino acids released in the pepsin-pancreatin digest when expressed in terms of 100 mg of digested protein. Nitrogen estimates are multiplied by 6.25 to convert to protein contents.

Table III. In Vitro Digestibility of Some LPC Preparations[a]

	% pepsin	% pepsin-pancreatin
alfalfa		
cytoplasmic	88.2	87.5
chloroplastic	71.0	72.0
radish	85.0	84.0
knolkhol	80.0	82.5
carrot	64.0	62.5
Sesbania	47.5	43.0
horse gram	60.0	58.0
french bean	73.0	74.3
Tephrosea	72.7	70.0
techoma	20.0	22.0

[a] Values are averages of triplicate estimations. The difference between the maximum and the minimum value in any given average is less than 5% of the maximum value except for techoma and Sesbania where it is 12%.

large variations among LPC prepared from different plants, the values ranging from 2.6% to 7.1% for bound carbohydrates and from 0.8% to 4% for the bound polyphenols. The variations remain when the results are expressed in relation to the nitrogen contents of LPC.

The amino acid profiles of various LPC preparations are given in the Table II. There were great variations among these LPCs. Alfalfa and techoma showed maximum and minimum amino acid content, respectively. In general, low values were found for all the amino acids in any given LPC. Methionine showed the largest variations while histidine exhibited only small changes.

The results of pepsin and pepsin–pancreatin digestibility for LPCs are given in Table III. The digestibilities with two enzyme systems are comparable. The digestibilities varied from about 20% in the case of techoma LPC to about 88% for alfalfa cytoplasmic fraction.

Table IV shows the results of the animal experiments. The food intake and weight gain varied significantly among different LPC diets. There were higher food intake and more growth in the case of radish, knolkohl, and alfalfa cytoplasmic LPC. There were low food intake and poor growth in the case of alfalfa chloroplastic fraction, carrot, and frenchbean LPC. The rats on diets containing horse gram, techoma, Tephrosea, and Sesbania lost weight.

The protein digestibility also varied significantly among various LPCs and ranged from about 30% in the case of techoma to about 86% for the alfalfa cytoplasmic fraction. NPR values ranged from about 0.8 for techoma to about 3.3 for radish LPC and showed relatively less differences than those observed in food intake. The NPR corrected for digestibility [net digestible protein ratio (NDPR)] showed smaller variations than NPR and varied from 2.4 for horse gram to 3.94 for knolkohl LPC.

The correlations between different in vivo and in vitro parameters are presented in Table V. There were significant correlations between TD and proteolytic digestibilities, TD and lysine availability, NPR and sulfur amino acid contents which improve further if correlated with the

Table IV. Nutritive Value of Some LPC Preparations[a]

	g/10 days				
	protein intake	gain in weight	NPR	NDPR	% TD
alfalfa					
cytoplasmic	8.8 ± 0.3	15.2 ± 2.0	3.14 ± 0.19	3.80	86.0 ± 3.0
chloroplastic	6.1 ± 0.3	3.8 ± 3.7	2.65 ± 0.50	3.68	71.7 ± 2.2
radish	9.0 ± 0.6	16.0 ± 0.8	3.17 ± 0.23	3.80	83.6 ± 1.4
knolkhol	9.0 ± 0.3	17.1 ± 2.3	3.29 ± 0.22	3.95	82.5 ± 1.7
carrot	6.2 ± 0.2	1.0 ± 1.2	2.18 ± 0.21	3.46	63.0 ± 2.4
Sesbania	4.8 ± 0.4	−9.5 ± 2.7	1.60 ± 0.36	3.43	46.5 ± 3.1
horse gram	6.7 ± 0.4	−1.9 ± 2.9	1.58 ± 0.41	2.40	68.0 ± 3.4
french bean	7.1 ± 0.9	2.0 ± 3.8	2.05 ± 0.42	2.70	75.7 ± 1.8
Tephrosea[b]	3.4 ± 0.6	−15.3 ± 2.1			68.4 ± 3.2
techoma	5.1 ± 0.3	−11.0 ± 1.5	0.80 ± 0.25	2.70	30.0 ± 2.4
egg protein	10.2 ± 0.5	49.0 ± 4.5	6.00 ± 0.50	6.00	100.0 ± 1.0

[a] Mean ± standard deviation. There were six rats in each group, and they were given diets at a 10% protein level. [b] NPR was not calculated since the food intake was less than that of the nitrogen-free group.

Table V. Correlation of in Vitro and in Vivo Methods

	correlation coefficient
true digestibility with	
pepsin digestibility	+0.99** [b]
pepsin-pancreatin digestibility	+0.99**
% availability of lysine	+0.94**
bound carbohydrate[a]	−0.96**
bound polyphenol[a]	−0.90**
net protein ratio with	
sulfur amino acids	
total	+0.71*
released in pepsin-pancreatin digest	+0.95**
released in pepsin-pancreatin digest and corrected for digestibility	+0.68*
bound polyphenol[a]	−0.87**
true digestibility	+0.81*
% availability of lysine	+0.89**
available lysine with	
methionine	+0.94**
histidine	+0.94**
tryptophan	+0.93**

[a] Milligrams per gram of protein. [b] Statistical significance: (*) $p \leq 0.05$; (**) $p \leq 0.01$.

amount of sulfur amino acids released in the pepsin–pancreatin digest, and available lysine and other amino acids.

DISCUSSION

The estimations of bound polyphenols and bound carbohydrates are only approximations of the protein–polyphenol and sugar–protein reactions which can take place during preparation of LPC and the good correlation observed between bound carbohydrates and bound polyphenols and TD and NPR was rather surprising. These reactions which occur to varying extents during the preparation of LPC would depend upon the relative concentrations of reducing sugars and polyphenols present in the extracted juice.

The variations in TD confirm earlier observations of Subba Rau et al. (1972) with the LPC from same vegetations. Both pepsin and pepsin–pancreatin digestibility values compared extremely well with TD. There are conflicting reports about correlation between TD and pepsin–pancreatin digestibility. Buchanan (1969) reported poor correlation for heat-treated wheat LPC while Saunders et al. (1973) found good correlation in the case of some alfalfa LPC preparations. It is difficult to explain this discrepancy. However, the pepsin–pancreatin and pepsin digestibility assays in the present form seem to give an exceptionally good picture over a range of digestibility from about 30% to 90%. The pepsin–pancreatin assay is preferable to pepsin digestibility. The enzymes involved in this multienzyme assay differ in their specificities as well susceptibility to various inhibitors which should make it less prone to errors.

Previously published amino acid compositions of LPC are mostly based upon acid hydrolysis and do not show many variations (Byers, 1971). Since the bonds between polyphenols and sugars and protein are susceptible to acid hydrolysis (Cranwell and Haworth, 1971; Carpenter, 1973), any analysis based upon acid hydrolysis would fail to show the extent of changes in amino acid profile. However, as these bonds are resistant to proteolysis, the enzymic hydrolysis coupled with group-specific assays should detect any changes in amino acids in LPC due to these reactions. Our results based upon such a scheme did reveal marked differences in the amino acid contents of various LPCs. There is no direct comparison possible between present values and the values reported in literature based on acid hydrolysis. However, Allison et al. (1973) also found large variations in lysine availability among LPCs when assayed by a specific chemical reaction. The microbial availability of amino acids after mild acid hydrolysis also has been reported to vary among various LPCs with as low as 40% availability for methionine reported for some chloroplastic fractions though no significant differences in total amino acid contents were observed (Byers, 1971). Also, Walker et al. (1975) detected the presence of various oxidation states of sulfur containing amino acids by using elegant X-ray photoelectron spectroscopy.

The variations in food intake and growth with different LPCs are similar to those observed by Subba Rau et al. (1972). However, the variations observed in NPR are significantly smaller than the difference in protein efficiency ratio (PER) reported by Subba Rau et al. (1972). This would be expected as NPR, unlike PER, is to a large extent independent of food intake (Bender and Doell, 1957), and it should be a more suitable measure than PER which has been recommended (Pirie, 1971). The decrease in variations in NPR when the digestibility was also considered (NDPR) indicates that digestibility is one of the major factors influencing the nutritive value of LPC. The significant variations in NDPR further suggest that the digested proteins are utilized to different extents with poor assimilation occurring in the case of LPCs with low digestibilities. The positive correlation observed by Allison et al. (1973) between lysine availability and biological value also suggested that amino acids other than lysine were also affected.

The good correlation between sulfur amino acids and NPR was expected since methionine is the first limiting amino acid in LPC (Henry and Ford, 1965). The significant improvement observed when the amount of sulfur amino acids released in the pepsin–pancreatin digest instead of total sulfur amino acids were considered, suggests that it is the amino acids released during the digestion rather than their total contents which determine the nutritive value of LPC.

There are two major factors, the protein–polyphenol and sugar–protein reactions, which can take place during preparation of LPC depending upon their concentrations, time taken to process the vegetation, and the conditions of processing. These reactions involve the amino acids methionine, lysine, cysteine, histidine, tryptophan, and arginine. The decrease in these essential amino acids will adversely affect the digestibility and nutritive value of proteins (Carpenter, 1973; Feeny et al., 1975; Pierpoint, 1970; Van Sumere et al., 1975). Significant variations in the amino acid profiles among various LPCs observed in the present investigation clearly suggest that these reactions occur to varying extents during the preparation of LPC.

The present investigation indicates that the in vitro parameters pepsin–pancreatin digestibility, available lysine, and contents of sulfur amino acids, total and those released during proteolysis, should be adequate to assess the nutritive value of LPC. However, although these parameters are sensitive to differences in nutritive value of LPCs, they do not take into account various flavor and toxic components which may be present in LPC and influence the food intake. This is indicated in the case of Sesbania LPC. Though all in vitro parameters suggested it to be of satisfactory nutritive value, the food intake of rats on diets containing Sesbania LPC was even lower than that in the case of protein-free diets. Thus, more work is still required to characterize these flavor and toxic components and to assess their role in affecting the nutritive

value of LPC. The results also show the indispensability of evaluation with animals for LPC from unknown plant sources as a conclusive evidence.

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Registry No. Lysine, 56-87-1; histidine, 71-00-1; tryptophan, 73-22-3; pepsin, 9001-75-6; pancreatin, 8049-47-6.

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Distribution of Limonin during the Growth and Development of Leaves and Branches of *Citrus paradisi*

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This report analyzes the distribution of limonin within a leaf, within a branch, and between branches of an individual *Citrus paradisi* tree. Limonin was evenly distributed within single leaves and the concentration in the leaves on a given branch was constant, regardless of branch age. The total amount of limonin per leaf varied with leaf size. Not all branches within the tree exhibited the same potential for limonin metabolism as reflected in their limonin and limonoate A-ring monolactone levels. The limonin content showed increasing levels as the leaves bud and grow and then decreasing levels as they mature and turn dark green. Preliminary information on the levels of limonoate A-ring monolactone and limonin shows that the ratio of these compounds changes as the leaves develop, thus indicating the dynamic nature of the metabolic pools of these compounds.

Limonoids are a group of compounds which are widely distributed in *Citrus* sp. and other members of the Rutaceae. The naturally occurring liminoids are triterpenoid in origin and are diverse in their chemistry (Maier et al., 1977). The intensely bitter dilactone limonin ($C_{26}H_{30}O_8$) has been studied most extensively since it occurs in abundant amounts (Maier et al., 1977) and is of major importance in processed citrus fruit, especially grapefruit and navel oranges. Most of the research related to processing has focused on discovering or developing debittering methods to improve the quality of the end product, whereas relatively few studies have focused on improvement of the citrus crop itself.

Chandler et al. (1976) attempted to correlate limonin levels in Navel and Valencia oranges with rootstock but found that species and cultivar seemed to be the most important criteria. Seasonal effects have also been studied and it is now well established that fruits harvested later in the season yield juice which has a lower limonin content than juice from early season fruit (Marsh, 1953; Kefford and Chandler, 1961; Wilson and Crutchfield, 1968; Scott, 1970; Albach et al., 1974; Levi, 1974). However, due to simple logistics it is not possible to wait until the end of the growing season and then harvest all fruits at once.

Little is known about the potential for production, transport, or storage of limonin in an individual tree nor about the environmental, geographic, nutritional, or genetic factors which control limonin synthesis and accumulation.

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