and Snyder, 1985). The conclusion, therefore, is that this method of storage, for an extended period, is a perfectly acceptable method, with regard to retention of flavor in the mango.

One other constituent was detected in the aroma isolate from the stored mango slices, but it is not listed in Table I since it is not a genuine mango volatile. It is di-2-ethylhexyl adipate, which eluted between docosane and tricosane on a BP1 bonded-phase fused silica capillary column, with a retention time of 65.0 min. This is the plasticizer used in clingfilm, and therefore this had clearly migrated from the wrapping to the flesh of the fruit, even when deep frozen. However, the amount detected was very small (about 0.2 μg/g, i.e. 0.2 ppm), and it is well-known that di-2-ethylhexyl adipate will migrate into closely wrapped foods, but at levels not constituting a health hazard.

ACKNOWLEDGMENT

We thank Ed Mitchell Mangoes, Miami, FL, for supplies of mangoes and W. G. Gunn and A. E. Cakebread for running the GC–MS.

Registry No. PrCOMe, 107-87-9; PrCH(OH)Et, 623-37-0; (*E*)-PrCH=CHCHO, 6728-26-3; (*Z*)-EtCH=CH(CH$_2$)$_2$OH, 928-96-1; Me(CH$_2$)$_{14}$CHO, 629-80-1; Me(CH$_2$)$_{14}$CO$_2$Et, 628-97-7; pyridine, 110-86-1; toluene, 108-88-3; furfural, 98-01-1; α-pinene, 80-56-8; camphene, 79-92-5; sabinene, 3387-41-5; myrcene, 123-35-3; 2-pentylfuran, 3777-69-3; β-pinene, 127-91-3; α-phellandrene, 4221-98-1; car-3-ene, 13466-78-9; *p*-cymene, 99-87-6; β-phellandrene, 555-10-2; limonene, 138-86-3; γ-terpinene, 99-85-4; terpinolene, 586-62-9; tetramethylbenzene, 25619-60-7; 2,5-dimethylstyrene, 2039-89-6; α-copaene, 3856-25-5; β-elemene, 33880-83-0; guaiene, 88-84-6; β-caryophyllene, 87-44-5; α-humulene, 6753-98-6; β-selinene, 17066-67-0; α-selinene, 473-13-2; δ-cadinene, 483-76-1; eicosane, 112-95-8; heneicosane, 629-94-7; docosane, 629-97-0; tricosane, 638-67-5; di-2-ethylhexyl adipate, 103-23-1.

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Received for review December 2, 1986. Accepted August 27, 1987.

Effects of Freezing and pH of Alfalfa Leaf Juice upon the Recovery of Chloroplastic Protein Concentrates

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Extracted alfalfa juice was frozen at −25 °C in a freezing chamber. After being subject to different freezing storage times, green concentrates were obtained by heat treatment, previously adjusting juice pH to 8.0, 8.5, and 9.0, leaving natural pH 6.0 as control. The results obtained show that at those alkaline pH values of the juice more total dry matter and nitrogen are recovered in the green concentrate than at the natural pH, although the concentrates have a lower protein content. Also, the recovery of green concentrate decreases as the freezing storage time of the juice increases, possibly due to a change in the conformation of membrane proteins, which is more intense as the storage time is higher.

Obtention of chloroplastic protein concentrates implies selective separation of the two protein fractions, chloroplastic and cytoplasmic, present in the juice. The primary separating methods are ultracentrifugation (Smith, 1966; Byers, 1971; de Fremery et al., 1973), heat treatment, organic solvents flocculation (Slade et al., 1945; Hove and Bailey, 1975; Bray and Humphries, 1978; Singh and Singh, 1985), and flocculation by means of polyelectrolites (Bray and Humphries, 1979; Fiorentini and Galoppini, 1980; Fiorentini et al., 1980).

Separation by means of heat of the two protein fractions is possible due to the fact that chloroplastic proteins coagulate faster at lower temperatures than soluble proteins. The main variants affecting the method are temperature, heating time, and the pH of the juice. Coagulation temperature of the chloroplastic proteins varies between 45 and 60 °C depending on the plant species (Subba Rau et al., 1969; Lexander et al., 1970; de Fremery et al., 1973; Edwards et al., 1975; Nagy et al., 1978). When temperature and/or heating time increase, denaturation of soluble proteins in the supernatant increases (de Fremery et al., 1973); therefore, the best heating techniques are steam injection followed by heat interchange.

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With regard to the optimum pH value, some discrepancies are found in the bibliography. De Fremery et al. (1973) and Edwards et al. (1975) employ pH 6.0 and a temperature of 55–60 °C in pilot plant studies. However, a temperature of 50 °C and pH 6.0 are the optimum conditions for proteolysis to occur (de Fremery et al., 1972; Scalet et al., 1984). Arkcoll and Holden (1973) also showed that lipoxygenase activity in alfalfa leaves is maximum near neutrality. Betschart and Kinsella (1973) indicate highest solubility of proteins and better chloroplast rupture when pH is increased. Hood and Brunner (1975) recommend keeping an alkaline reaction during the process to intensify cytoplasmic protein solubility and inhibit proteolytic degradations. Likewise, Livingston et al. (1977, 1984) recommend coagulation at alkaline pH values to reduce the saponin content of the concentrate.

Therefore, we studied the influence of alkaline pH values of the juice upon the quantity recovered and on protein content of the chloroplastic concentrates, in comparison with the coagulation at the natural pH of the juice, 6.0. The maximum pH value chosen was 9.0, since at higher values organoleptic and nutritional alterations appear (Whitaker and Feeney, 1983): transformation of chlorophylls into pheophytins and pheophorbides occurs, with the consequent color change from green to brown (Miller et al., 1984) and the increase of nonenzymatic oxidation of polyphenols catalyzed by metals (Nashef et al., 1977; Finot, 1983; Friedman et al., 1984).

0021-8561/88/1436-0139$01.50/0

Table I. Recovery of Dry Matter[a] and Nitrogen[b] in the Green Protein Concentrate and Chloroplast-Free Juice Obtained from the Juice at pH 6.0

	recovered dry matter, %			recovered nitrogen, %		
sample	green protein concentrate	chloroplate-free juice	total	green protein concentrate	chloroplast-free juice	total
1	9.52			14.46		
2	8.31	94.68	102.99	13.60	82.60	96.20
3	7.03	90.37	97.40	13.95	82.79	96.74
4	5.56	92.11	97.67	11.42	86.86	98.28
5	4.82	97.73	102.55	9.08	92.72	101.80
6	2.34	92.37	94.71	4.39	87.70	92.09
mean	6.26	93.45	99.06	11.15	86.53	97.02

[a] Expressed as percent of total dry matter in juice. [b] Expressed as percent of total nitrogen in juice.

Table II. Recovery of Dry Matter[a] and Nitrogen[b] in the Green Protein Concentrate and Chloroplast-Free Juice Obtained from the Juice at pH 8.0

	recovered dry matter, %			recovered nitrogen, %		
sample	green protein concentrate	chloroplast-free juice	total	green protein concentrate	chloroplast-free juice	total
1	9.83	90.55	100.38	9.50	86.17	95.67
2	9.29	90.55	99.84	8.48	86.53	95.01
3	9.32	90.10	99.42	8.07	83.51	91.58
mean	9.48	90.40	99.88	8.68	85.40	94.09

[a] Expressed as percent of total dry matter in juice. [b] Expressed as percent of total nitrogen in juice.

Since freezing, as a method of storing juice, causes coagulation of the so-called "Freezing curd", which constitutes 50% of the dry matter and 60% of the nitrogen of the total in the juice and whose formation was proved to depend solely upon the primary freezing rate, regardless of the freezing time interval of the juice (Hernández et al., 1986). The possible influence of freezing upon the quantity and protein content of the chloroplastic concentrates obtained was also studied.

EXPERIMENTAL SECTION

Alfalfa was harvested and then pulped and pressed with use of IBP equipment. The juice obtained was distributed among plastic containers to enable its subsequent use. All juice samples were frozen and stored in a freezing chamber at −25 °C until use. The temperature of the freezing chamber was invariable throughout the storing period. Each sample was thawed at room temperature 18 h before use; the freezing storage time for each was recorded. The freezing curd produced was separated from the juice by filtration and sieving. The juice was used to prepare chloroplastic concentrates. The amount of juice used varied from 1.5 to 3.0 kg, depending on the available sample, and its natural pH varied from 5.62 to 5.96. Representative parts of each juice sample were freeze-dried.

In all cases chloroplastic proteins were separated by thermal treatment, previously adjusting the pH of the juice. The pH of the juice was adjusted to the chosen value by means of a 2 N NaOH solution. Once the pH value was reached, the mixture was left to stir for a stabilizing period of 30 min. Four pH values were examined (6.0, 8.0, 8.5, 9.0).

Immediately afterward, chloroplastic proteins were coagulated at 60 °C for 1 min by heat interchange. This was performed in a 200-mL-capacity double-walled glass column with the lower part closed and the top opened and thermostatically controlled by connection to a thermostat, provided with circulation and stirring. The temperature of the thermostat was fixed at 75 °C. The juice was added to the column when the circuit was stabilized at the desired temperature. Under these conditions, the juice reached the temperature of 60 °C in 45–50 s. The container bearing the coagulated juice was quickly introduced into an ice bath. Chloroplastic curd was separated from the chloroplast-free juice by centrifugation at 10 °C and 4396*g* for 20 min. The total obtained from the two fractions was weighted, and representative portions from both were freeze-dried. The general scheme of the method is shown in Figure 1.

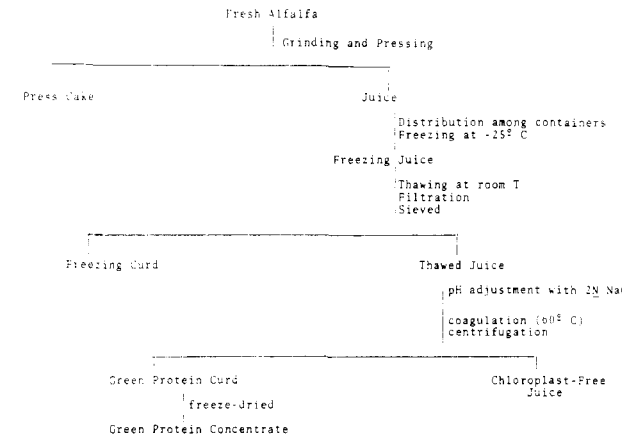

Figure 1. Obtention process of green protein concentrate.

Protein nitrogen was determined by the semimicro Kjeldahl method in all samples of thawed juice, chloroplastic concentrate, and chloroplast-free juice.

RESULTS AND DISCUSSION

To eliminate the influence of the different juices, as each one of them had specific dry matter and nitrogen percentages, the results are expressed in terms of dry matter and nitrogen recovered in each fraction, green protein concentrate and chloroplast-free juice, in relation to its initial quantity in the juice. The results of all the fractioned samples at each pH value of the juice are shown in Tables I–IV. In the tables the samples are arranged as increased time interval of frozen storage of the juices.

It can be noted that, at a similar pH, the recovery of dry matter and nitrogen in the green concentrate decreases from the first to the last fractioned sample. As the influence of the initial juice had been corrected and as the obtention method was the same for all samples, the only

Table III. Recovery of Dry Matter[a] and Nitrogen[b] in the Green Protein Concentrate and Chloroplast-Free Juice Obtained from the Juice at pH 8.5

	recovered dry matter, %			recovered nitrogen, %		
sample	green protein concentrate	chloroplast-free juice	total	green protein concentrate	chloroplast-free juice	total
1	15.30	83.28	98.58	20.07	74.90	94.97
2	13.78	84.70	98.48	18.27	81.96	100.23
3	9.91	90.53	100.44	7.91	92.60	100.51
4	9.67	91.73	101.40	8.77	92.56	101.33
5	8.22	92.65	100.87	6.83	91.89	98.72
mean	11.38	88.58	99.95	12.37	86.78	99.15

[a] Expressed as percent of total dry matter in juice. [b] Expressed as percent of total nitrogen in juice.

Table IV. Recovery of Dry Matter[a] and Nitrogen[b] in the Green Protein Concentrate and Chloroplast-Free Juice Obtained from the Juice at pH 9.0

	recovered dry matter, %			recovered nitrogen, %		
sample	green protein concentrate	chloroplast-free juice	total	green protein concentrate	chloroplast-free juice	total
1	21.83			25.25		
2	17.41	87.94	105.35	19.10	82.44	101.54
3	14.39			14.72		
4	10.66	87.23	97.89	9.21	86.49	95.70
5	10.05	89.39	99.44	8.92	86.97	95.89
mean	14.87	88.19	100.89	15.44	85.30	97.71

[a] Expressed as percent of total dry matter in juice. [b] Expressed as percent of total nitrogen in juice.

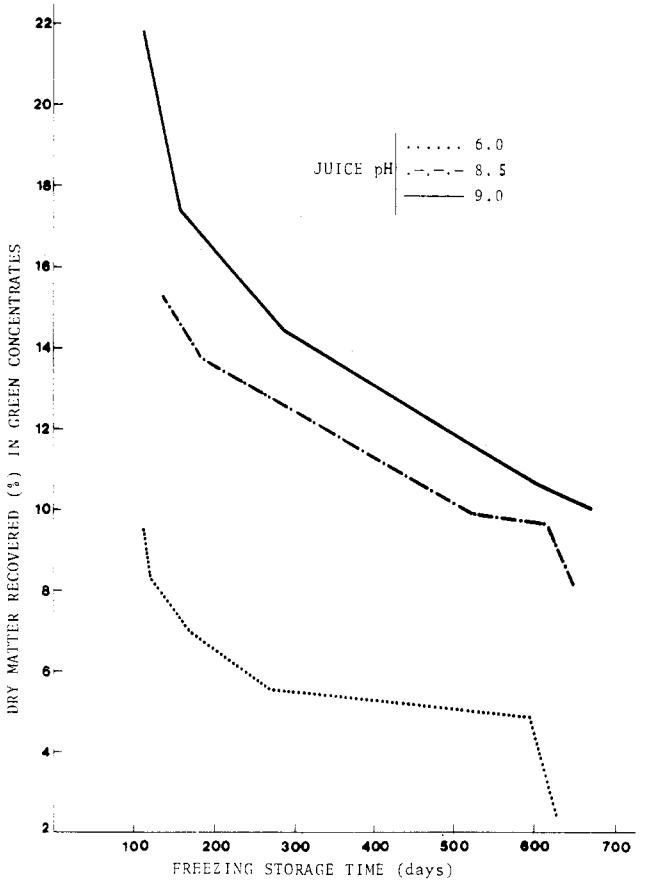

Figure 2. Effects of freezing storage time and juice pH on the recovery of dry matter in green protein concentrate.

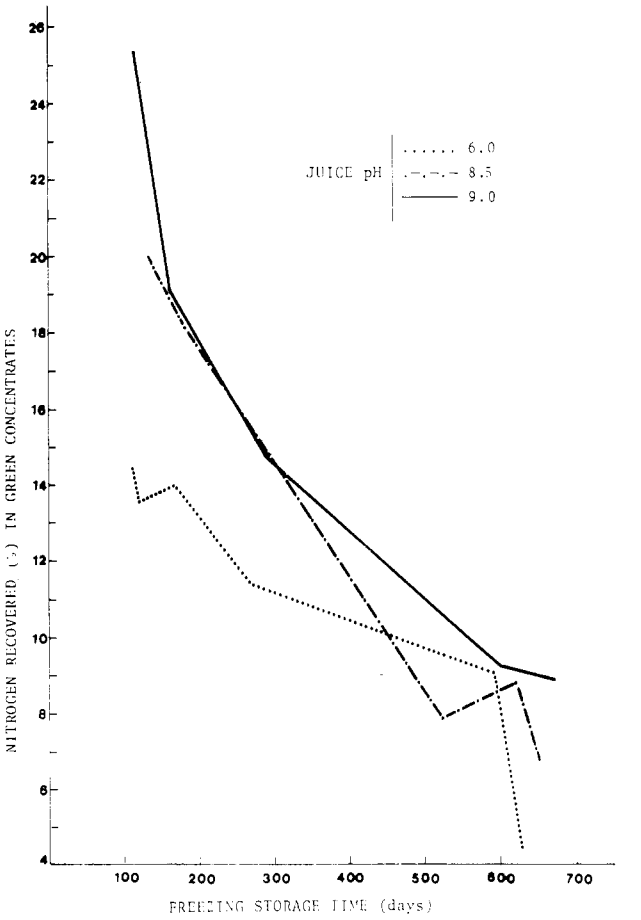

Figure 3. Effects of freezing storage time and juice pH on the recovery of nitrogen in green protein concentrate.

difference between them was the time the juice remained in the freezing chamber. To prove whether there exists a correlation between freezing time and the differences observed, dry matter and nitrogen recovered in the green concentrate were put against freezing time, in days, of all fractioned juice samples at each pH value (Figures 2 and 3), thus verifying that such relation exists. Samples of pH 8.0 are not included in the figures, as there were only three and with very proximate freezing times.

To prove whether differences of dry matter recovered as green concentrate, due to pH and freezing time, were significant or not, variance statistical analyses were carried out, considering a classification with to parameters without replication (Pollard, 1979; Stoodley et al., 1980). For such

Table V. Analysis of Variance of Recovery of Dry Matter in the Green Protein Concentrate

A. Data Table

freezing storage time	juice pH 1	2	3	4
1	8.31	16.16[a]	15.30	21.83
2	7.03	13.74[a]	13.78	17.41
3	5.56	9.83	9.91	14.39
4	4.82	9.29	9.67	10.66
5	2.34	9.32	8.22	10.05

B. ANOVA Table

source of variation	DF[b]	SS[b]	MS[b]	F
freezing storage time	4	171.10	42.78	24.87
juice pH	3	223.55	74.52	43.33
error	12	20.62	1.72	
total	19	415.27		

$F_{4/12}(0.1\%) = 9.63$; $F_{3/12}(0.1\%) = 10.80$

[a] Statistical estimation. [b] Key: DF = degrees of freedom; SS = sum of squares; MS = mean square.

analyses, a uniform number of data is required for both parameters, five values of freezing time and the four pH values; as there were only three values for pH 8.0, and in order to be able to include it in the analyses, the two experimental "gaps" were filled by the Dempster et al. (1977) statistic estimation method (Johnson and Wichern, 1982). From the ANOVA results (Table V) it is deduced that differences caused by both parameters are highly significant at level 0.1%.

In conclusion, both dry matter and nitrogen recovered in the green concentrate decrease, regardless of the pH, as the freezing storage time of juice increases; and at higher pH, independent of the freezing storage time, the recovery of the dry matter and nitrogen from concentrate is higher.

The membranes are the first sites where freezing injury occurs in biological material. This fact has been recorded for isolated chloroplasts by many workers (Mollenhauer et al., 1983; Hincha et al., 1984; Jensen and Oettmeier, 1984). A freezing/thawing cycle brings along various types of damage, which are usually classified as mechanical or chemical. The mechanical damage is a consequence of the formation of extracellular ice crystals, with concomitant osmotic stress, and of the reexpansion caused by thawing (Hincha et al., 1985; Meryman and Williams, 1985; Schmitt et al., 1985).

Chemical damage is chiefly associated with secondary crystallization, which depends mainly on freezing time and temperature, and on the solute accumulation (Burke et al., 1976; Levitt, 1978). Fennema (1982) and Mollenhauer et al. (1983) point out that low temperatures cause structural changes in the protein molecules, which depend on hydrophobic interactions to maintain their native structures; they also point out that other factors, besides temperature, vary considerably during the freezing process, among them ionic force, surface tension, protein concentration, and nonprotein solute concentrations. Schmitt et al. (1985) indicate that proteins, both aggregated and nonaggregated, can suffer changes in the conformation during freezing of isolated thylakoids.

Considering all these facts, it is possible to conclude that the lower green concentrate recovery as the freezing time is raised depends on the chemical effects of the freezing process, which causes membrane proteins to have some conformational changes. As these proteins have high hydrophobic character and as the obtention method was heat-coagulated, which is based on a redistribution of the hydrophobic interactions (Scopes, 1984; Douillard, 1985), it can be concluded that the transformation is produced in such interactions and that it causes the proteins to be nonrecovered in the subsequent thermal treatment.

With regard to the influence of the pH of the juice, considering average values of recovered dry matter (Tables I, III, and IV), it can be noted that at pH 8.5 the concentrate quantity obtained is 1.8 times higher than the one obtained at pH 6.0; at pH 9.0 the amount is 2.4 times higher. Bray (1984) with an alkaline treatment followed by centrifugation, without thermal treatment, recovered an equal quantity of green concentrate at pH 8.5 and 10.0, this one being 1.6 times higher than the quantity obtained at pH 5.4.

The differences in the nitrogen recovered at the various pH values of the juice are not as high as for dry matter. This is due to the fact that while the green concentrate obtained at pH 6.0 has a nitrogen content of 7.86%, those obtained at alkaline pH values have 4.40%, approximately. That is, as the pH of the juice increases, more green concentrate is obtained but of a lower protein richness.

The recovery of concentrate at alkaline pH of the juice was studied by other investigators in the case of unfractioned concentrate (Spencer et al., 1971; de Fremery et al., 1972; Arkcoll and Holden, 1973; Gonzalez et al., 1976). Edwards et al. (1975) in the obtention of chloroplastic concentrate by heat coagulation showed that as the pH increases from 6.0 to 8.5, the sediment level also increased; they do not give any data, and they justify the choice of pH 6.0 for the obtention on the better texture of the curd obtained. In our case, as the pH was raised, the texture and consistency of the curd were improved; this agrees with what has been observed by other workers in unfractioned concentrates.

From the facts mentioned, it can be said that it is not advisable to employ freezing to store juice for long time intervals, especially when highest recovery of the chloroplastic concentrate is the goal. With regard to pH, it seems advisable to perform the coagulation of membrane proteins at pH 8.5 to get the highest recovery of green concentrate. Also, the recovery of cytoplasmic or white concentrate from the chloroplast free juice obtained at pH 8.5 was higher than at others pH studied (6.0, 8.0, 9.0) (Hernandez, 1986).

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Received for review February 24, 1987. Accepted July 16, 1987.