Solubility and Electrophoretic Properties of Processed Safflower Seed (*Carthamus tinctorius* L.) Proteins

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Whole safflower seeds (*Carthamus tinctorius* L.), cultivar Kino'76, with a crude protein content of 17.3% (dry-weight basis) were subjected to the processes of cracking, defatting (*n*-hexane extraction), dehulling, debittering (70% methanol extraction), and grinding to meals to study the solubility and electrophoretic behavior of their proteins. Sequential extraction of defatted, whole-meal protein fractions showed that water-, salt-, and alkali-extractable protein fractions and residue (unextracted protein) accounted for 17.0, 5.0, 69.0, and 9.0%, respectively. The 70% alcohol solvent extracted negligible amounts of protein. SDS-PAGE of the water-, salt-, and alkali-extractable protein fractions showed 8, 13, and 13 distinctive subunits, respectively, with molecular masses ranging from 14.7 to 88.0 kDa. After dehulling and debittering, the amount of alkali-extractable protein fraction decreased significantly as the residue fraction increased. Quantitative and qualitative changes in the molecular weight and subunit composition of SDS-PAGE patterns of each extracted protein fractions were observed.

Mexico is one of the largest producers of safflower seed in the world, with an annual production of about 500 000 tons according to the statistics of the Mexican Ministry of Agriculture and Water Resources (1984). This is a result of the development of improved safflower varieties by the National Institutes of Forestry, Agriculture and Livestock (INIFAP). Commercial grown safflower seed variety Kino'76 contains up to 40% oil and 17% protein on a dry-weight basis (Salazar Zazueta, 1979).

Safflower seed is mainly grown for its oil, which is used for human consumption in the form of cooking and salad oil, margarine, and processed foods. On the other hand, proteins of the meal resulting from the oil extraction process are still underutilized and used traditionally as an animal feed. The main reason for this underutilization is because it contains two antinutritional factors: (1) high fiber content and (2) phenolic glucosides (Lyon et al., 1979). The extraction, isolation, and identification of two phenolic glucosides that are associated with the strong bitter flavor and cathartic activity of safflower seeds have been reported (Palter et al., 1972a,b); these are matairsinol monoglucoside (bitter) and 2-hydroxyarctiin (cathartic).

Proteins from defatted safflower meal have been prepared in the form of concentrate, isolate, and low-fiber meal and have good nutritional and functional properties (Koapas and Kneeland, 1966; Betschart and Saunders, 1978; Betschart et al., 1979). Latha and Prakash (1984) characterized the salt-extractable proteins of safflower by means of various biochemical techniques. They reported these proteins are made of two major sedimenting fractions of 12S and 2S and two minor fractions of sedimentation values 7S and 17S, accounting for 65, 26, 6, and 3%, respectively, of the total protein.

To date, there have been limited biochemical studies of the proteins of safflower and no electrophoretical work to determine the effects of processing on their molecular properties. Therefore, this research was undertaken to study the solubility and electrophoretic properties of proteins from whole and dehulled safflower seeds subjected to the process of oil extraction with *n*-hexane (defatting) and removal of bitter components with methanol (debittering).

MATERIALS AND METHODS

Safflower seeds of the Mexican variety Kino'76 were grown in the experimental station of the Center of Agricultural Research for the Northwest of Mexico (CIANO), located in Ciudad Obregón, Sonora, Mexico. They were harvested during Spring, 1984, and stored at 4 °C until experiments were initiated at the University of Arizona. Unless otherwise mentioned, all the chemicals used were of reagent grade.

Preparation and Treatment of Safflower Meals. Defatted whole meal (DWM); defatted, debittered whole meal (DDWM); dehulled, defatted meal (DDM); and dehulled, defatted, debittered meal (DDDM) were prepared by the process outlined in Figure 1. The amount of starting material to obtain each meal was 100 g. All meals were prepared in duplicate, and recoveries were calculated on the basis of the origional weight of each lot of seeds expressed on dry-weight basis. Kernels and hulls were carefully hand-separated. The meal to solvent ratio in the defatting and debittering steps was 1:5 (w/v) after three extractions. The temperature used for the evaporation step was 50 °C for 3 h. All meals were stored at 4 °C until analyses were conducted.

Proximate Composition. Moisture, protein, oil, crude fiber, and ash were determinated according to the standard methods of the AOAC (1980). All determinations were carried out in triplicate. The factor 5.45 for protein conversion was determined by Guggloz et al. (1969).

Sequential Extraction and Quantitation of Protein Fractions. Two buffer systems and 70% ethanol were used for protein extractions according to the Osborne scheme (Osborne and Mendel, 1914). Portions of 10 g of each processed safflower meal were extracted with different solvents in a 1:10 ratio into different protein extracts as follows: Water- and salt-soluble protein fractions were extracted together with 0.05 M phosphate buffer, pH 7.5, containing 0.5 M NaCl; Water- and salt-soluble fractions were separated by dialysis against distilled water for 48 h at 4 °C followed by centrifugation to obtain the supernatant (water-extracted) and the precipitate (salt-extracted). The precipitate remaining after the extraction of the water- and salt-extractable fractions was extracted with 70% ethanol and then centrifuged as above. The ethanol was then removed from this extract by using a

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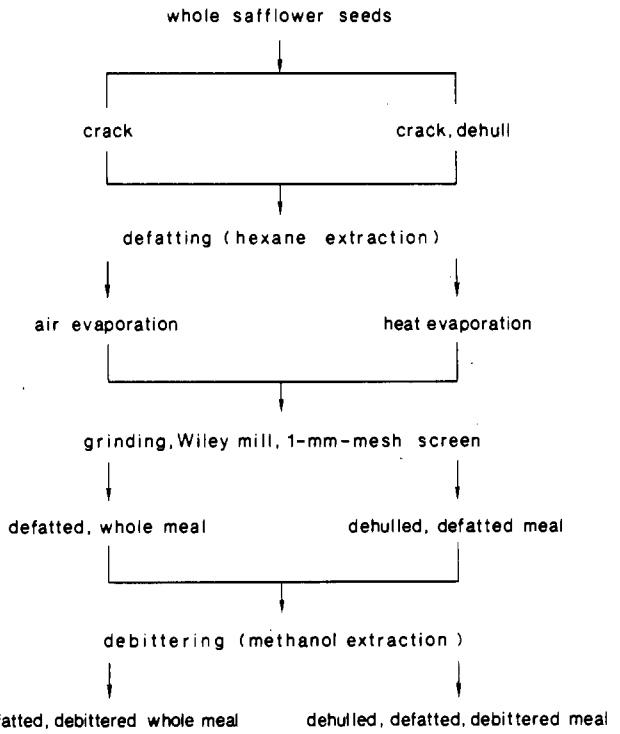

Figure 1. Flow diagram of meal preparation from whole safflower seeds.

rotory evaporator at 50 °C. This protein extract (alcohol soluble) contains the prolamine components of the original Osborne classification of proteins. The proteins of the residue were extracted with 0.1 N sodium hydroxide–borate buffer (pH 10) and centrifuged. Then the extracted proteins were neutralized to pH 7.5 with 0.1 N HCl. The final residue was resuspended in the same buffer and freeze-dried.

In each extraction step, the meal suspension was stirred gently for 1 h and then centrifuged at 6000*g* at 4 °C for 20 min. All extractions were carried out three times at 4 °C for 20 min, and each protein extract was freeze-dried and stored at 4 °C. The protein content of each fraction was determined by the micro-Kjeldahl procedure (AOAC, 1980).

Determination of Phenolic Glucosides. The method of Lyon et al. (1979) was used to estimate percent and to monitor the removal of phenolic glucosides (bitter components from meals). In our study, however, pure standards were not used. Instead, we used a preparation of concentrate of bitter extract from defatted whole meal as described by Palter and Lundin (1972a).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was conducted in a vertical Hoefer SE600 apparatus using the method of Laemmli (1970). A 10-mg sample of the freeze-dried protein was dissolved in 1 mL of the sample buffer (0.05 M Tris-HCl, pH 6.8, containing 1% SDS, 10% glycerol, and 2% 2-ME) and the resultant mixture heated 90 s at 100 °C. A 25-μL aliquot was applied to 12% gels with the following dimensions: 13.5 cm (wide) × 16 cm with a thickness of 1.5 mm. The molecular masses of the proteins were determined against Bio-Rad (Richmond, CA) standard reference proteins: cytochrome, 12.4 kDa; carbonic anhydrase, 29 kDa; egg albumin, 45 kDa; bovine serum albumin, 66 kDa; phosphorylase *b*, 97.0 kDa; β-galactosidase, 116.0 kDa; myosin, 205 kDa. Electrophoresis was conducted at 27 mA for 4 h. Gels were stained with a solution containing 50% methanol, 10% acetic acid, and 2.25% Commassie Brilliant Blue and destained with a solution of 25% methanol and 5% acetic acid.

Table I. Chemical Composition of Safflower Seed and Meals[a]

sample	protein, %	oil, %	crude fiber, %	ash, %	carbohydrate,[b] %
whole seed	17.30	39.80	21.70	2.68	18.52
dehulled seed	26.90	61.50	1.60	3.20	6.80
defatted, whole meal	24.87	1.20	35.00	4.20	34.73
defatted, debittered whole meal	26.70	0.80	36.20	4.50	31.80
dehulled, defatted meal	66.93	1.12	4.12	7.90	19.93
dehulled, defatted, debittered meal	69.92	0.90	4.80	8.10	16.28

[a] All analyses were carried out in triplicate and means reported on dry-weight basis. [b] By difference.

Amino Acid Analyses. A 100-mg portion of the meals and protein fractions were hydrolyzed in 6 N HCl for 16 h at 121 °C under vacuum, evaporated to dryness, and dissolved in 0.2 M sodium citrate buffer, pH 2.2, according to the method of Moore and Stein (1963). The preparation of the hydrolysates for amino acid determination by high-pressure liquid chromatography (HPLC) was done with the method of Lindroth and Mopper (1979). Amino acid analyses were performed in a Model 421A Beckman HPLC.

RESULTS AND DISCUSSION

Yields of the meals obtained are 60.1%, 50.9%, 39.5%, and 29.8% for DWM, DDWM, DDM, and DDDM, respectively. Lower yields for DDM and DDDM are due to the removal of 38% of hulls from whole seeds. The contents of protein, oil, crude fiber, ash, and carbohydrates of whole safflower seed and processed meals are presented in Table I. Seeds of the improved Mexican variety Kino'76 contain higher contents of protein and oil than most of the varieties screened by Guggloz (1969). After removal of hulls and treatment with 70% methanol, the meal contained the highest level of protein (69.92%) and lowest of crude fiber (4.80%). This meal contains reduced levels of the components (crude fiber and phenolic glucosides) that limit the utilization of safflower proteins for food and feed uses. The variation of protein content of the various processed meals obtained from safflower seed depends on the variety, amount of hulls present, degree of removal of oil, and processing of the seed.

Completeness of the Debittering Process. Methanol extracts of whole and dehulled meals showed a strong thin-layer chromatography (TLC) spot with R_f 0.48, indicating the presence of phenolic glucosides as identified by Lyon et al. (1979). In order to achieve complete debittering of the meals, three extractions were necessary. The first methanol extraction removed 70% of the total for DWM and 90% for DDM. This high proportion of phenolic glucosides removed in the first extraction is indicative of their high solubility. The lower percent of extracted phenolic glucoside with the first extraction for DWM is probably due to interference of the high proportion of hulls. Hulls do not contain phenolic glucosides that impart bitter flavor (Lyon et al., 1979). Second and third extractions removed the remaining phenolic glucosides; however, a very faint TLC spot remained in both meals. We concluded that some of these components may be tightly bound to the protein and require harsher denaturing conditions for solubilization.

Protein Fractions. Data from the extraction of protein fractions from the meals are presented in Table II. The values reported are the average of thee extractions and normalized to 100% of protein recoveries. The actual

Table II. Distribution of Protein Fractions in Processed Safflower Meal

meal	extracted fraction,[a] % of total protein				rec, %
	water	salt	alkali	residue	
defatted, whole meal	17.0	5.0	69.0	9.0	89.8
defatted, debittered whole meal	16.0	11.0	49.0	24.0	89.0
dehulled, defatted meal	16.5	12.0	54.5	17.0	92.0
dehulled, defatted, debittered meal	21.0	17.0	39.0	23.0	90.5

[a] The 70% ethanol fraction contained negligible protein.

protein recoveries varied from 89 to 92%.

Regardless of the method of processing, the alkali-extractable protein fraction of all meals was the highest. The 70% ethanol solvent did not extract proteins from the various meals. The ethanol extract had 0.02% of nitrogen as determined by the micro-Kjeldahl method, but SDS-PAGE did not reveal any protein bands. These results differ from those of Betschart (1975) who carried out solubility studies on proteins of safflower meal and found that salt (1 M NaCl) and alkali (0.1 N NaOH) extractable proteins were the major protein fractions with a total of 41.5 and 39.1%, respectively. Proteins of the insoluble residue were not taken into consideration in that study. Differences with our results may be due to differences in extraction procedure, variety, and preparation of the meal used in this study.

Differences in the amount of each protein fraction extracted from the meals were observed as a result of each processing treatment given to the seed. These differences are a reflection of the changes in protein solubility that took place during the processes of *n*-hexane extraction (defatting) and methanol extraction (debittering). The amount of water-extractable protein fraction underwent small changes: from 17.0% in DWM to 21.0% in DDDM. The amount of alkali-extractable protein fraction underwent significant changes (from 69% in DWM to 39% in DDDM). The decrease in the total amount of alkali-extractable fraction is probably due to molecular disaggregation that occurred during processing. This disaggregation of the proteins resulted in small increases distributed in the other Osborne solubility fractions. On the contrary, the proteins of the residue fraction increased after defatted meals (whole and dehulled) were extracted with methanol. These observations are consistent with those of Fukushima (1969) who studied the effects of several organic solvents on the solubility of soybean proteins and found that lower carbon alcohols were stronger denaturants as measured by the amount of insolubilization of proteins (residue).

The amount of each protein fraction extracted will depend on (1) variety or genetic material used as source of protein, (2) the solubilizing capacity of the solvent, (3) type of protein, (4) protein to solvent ratio, (5) size of particle of meals, (6) temperature and length of time of extraction, (7) pH, (8) ionic strength, (9) processing history of protein, and other factors. Because of this, it is difficult to make direct comparisons with other studies that used the same source of protein (Betschart, 1975; Betschart and Saunders, 1978; Latha and Prakash, 1984) and other sources of proteins such as soybean (Chen and Rasper, 1982) and sesame seed (Shamanthaka Sastry et al., 1974).

Latha and Prakash (1984) investigated the extractability of protein from defatted safflower meal in water and sodium chloride as a function of pH and reported that water extracted 95% of the total protein at pH 9.3, whereas sodium chloride only extracted 80% at pH 10. Betschart and Saunders (1978) found that alkali (0.1 N NaOH) was a very efficient extractant for total safflower proteins. Although alkaline pH has been used to efficiently extract proteins from vegetable sources, loss in the nutritional quality has been reported due to loss of amino acids and/or racemization. Thus treatment of proteins with alkali (0.1 M NaOH) at 50 °C for 24 h resulted in loss of lysine and cystine (Nashef et al., 1977). In addition, formation of new amino acids, such as lysinoalanine, ornithoalanine, β-aminoalanine, and lanthionine, has been reported (Satterlee and Chang-Ching, 1982).

Table III. Amino Acid Composition[a] of Dehulled, Defatted Meal and Its Extracted Protein Fractions

amino acid	dehulled, defatted meal	extracted protein fraction		
		water	salt	alkali
aspartic	11.21	8.18	19.21	10.28
glutamic	25.80	27.31	25.83	21.29
serine	4.04	2.58	6.30	2.59
histidine	2.77	2.16	4.42	2.29
glycine	5.82	5.48	9.08	3.82
threonine	3.08	1.94	4.23	2.19
alanine	4.79	3.12	7.28	4.09
tyrosine	4.23	2.98	7.46	4.20
methionine	1.81	1.86	3.18	1.39
valine	6.51	5.04	11.54	5.95
phenylalanine	5.59	3.78	9.68	5.44
isoleucine	4.59	3.23	8.10	4.65
leucine	7.48	5.94	13.37	7.58
lysine	3.08	2.29	3.83	2.33

[a] Expressed as grams/100 g of protein.

Percent of Phenolic Glucosides of Protein Fractions. Water-extractable protein fractions extracted from DWM and DDM contained the highest proportion of phenolic glucosides with 60 and 80% of the total, respectively, whereas the alkali-extractable fractions contained the least, with 18 and 9% the total, respectively. The salt-extractable protein fractions had intermediate values of 22 and 11% for DWM and DDM, respectively. Differences in the amount of phenolic glucosides coextracted with each protein fraction may be due to differences in the molecular interaction of these components with the proteins. Lyon et al. (1979) reported that phenolic glucosides from defatted safflower meal were highly soluble in water. This is in agreement with our results as evidenced by the high proportion of phenolic glucosides present in the water-extractable protein fraction. On the other hand, presumably the phenolic glucosides coextracted wth the alkali-soluble protein fraction are bound more tightly to the proteins. This deserves more research attention in order to investigate the effect of these components on the molecular structure and functionality of safflower proteins.

The high proportion of phenolic glucosides in the water-extractable protein fraction may result in nutritionally adverse effects as has been reported for other sources of oilseed proteins. Thus, the presence of phenolic glucosides in sunfflower seed reduce its nutritional value and applications in food formulations. In order to remove these constituents, Shamanthaka and Subramanian (1984) treated sunflower seed with acidified sodium chloride and obtained polyphenol-free kernels with improved food characteristics.

Amino Acid Composition. The amino acid composition of DDM and its Osborne protein fractions is presented in Table III. The reason for not including the amino acid composition of the other meals and their protein fractions is because the different treatments given to the seed did not cause major changes in the amino acid composition.

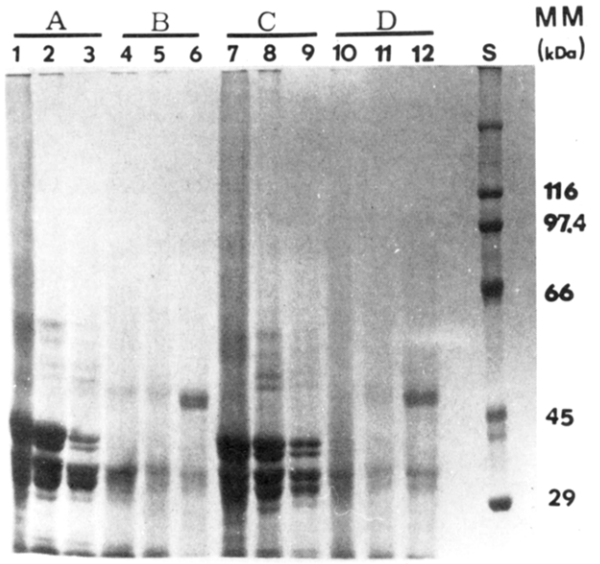

Figure 2. SDS-PAGE of the protein fractions from safflower meals stained with Coomassie blue: A–D, DWM, DDWM, DDM, and DDDM, respectively. Lanes 1, 4, 7, 10; 2, 5, 8, 11; and 3, 6, 9, 12 are water-, salt-, and alkali-extractable protein fractions of each meal, respectively. Key: S, standard proteins; MM, molecular mass; kDa, kilodalton.

Our values are in general agreement with those reported by Latha and Prakash (1984) and Bestchart (1975), but some small differences were observed. These could be due to variations in amino acid compositions of the varieties used. The meal protein and Osborne protein fractions are characterized by high contents of aspartic acid, glutamic acid, glycine, and valine. Similar results of high contents of aspartic and glutamic acids have been reported for other sources of oilseed proteins (Prakash and Narasinga Rao, 1984).

SDS-PAGE. SDS-PAGE analysis of water-, salt-, and alkali-extractable fractions of the proteins of the processed safflower meals are shown in Figure 1. Water-, salt-, and alkali-extractable fractions of DWM and DDM were resolved into 8, 13, and 13 distinct subunits, respectively, with apparent molecular masses ranging from 14.7 to 88.0 kDa. In addition, they all had a very faintly stained subunit with an apparent molecular mass of 130 kDa not visible in the photography. This subunit was more visible when 50 μL was loaded on the gel, but this amount of sample affected the resolution of the other subunits. The main difference between the proteins extracted from the two meal preparations (whole and dehulled) meals is the quality of the electrophoretic resolution; for example, water-extractable fraction from DWM (lane 1, Figure 2) had a darker background than the water-extractable fractions of DDM. Presumably this indicates that the nonprotein components present in the water-extractable fraction from DWM and DDM (lanes 1 and 7, Figure 2) make them behave anomalously during electrophoresis. Weber and Osborn (1969) reviewed the advantages, limitations, and factors affecting the use of SDS-PAGE for molecular weight determination.

Although water-, salt-, and alkali-extractable fractions showed very similar electrophoretic subunit composition, each had very typical pattern characteristics upon visual examination of the gels. Water- and salt-extractable fractions had identical separation of subunits in the region of molecular mass from 29.0 to 45.0 kDa, whereas alkali-extractable had a better separation of the protein subunits with molecular mass from 29.0 to 35.0 kDa. Salt- and alkali-extractable fractions had four distinctive protein subunits with molecular masses of 64.0, 59.0, 53.0, and 49.0 kDa that were not part of the subunit composition of the water-extractable fraction.

Protein of the residue fractions did not enter the gel, and extensive streaking of protein resulted (electroforegram not shown). Also it was observed that in each extracted protein fraction some protein did not enter the gel even though the amount of sample was the same. Several authors have found similar results and reported that this phenomena is due to aggregation of proteins (Butaki and Dronzek, 1982; Bekes et al., 1983). Also, the photographic reproduction of the gel resulted in complete loss of detail of two protein subunits with molecular masses of 88.0 and 83.0 kDa of the water- and salt-extractable fractions, respectively.

After treatment of DWM and DDM with 70% methanol to remove the bitter components, the water-, salt-, and alkali-extractable fractions resolved into 9, 4, 11 and 12, 5, and 12 subunits, respectively. The number and distribution of subuntis of each protein fraction changed with a decreased molecular weight for each. Also, it was noted that the SDS-PAGE protein subuntis did not stain the Coomassie blue as intensely as those of *n*-hexane-extracted meals. This means that the remaining lipid fraction of *n*-hexane-extracted meals seems to be involved in quality of results obtained by SDS-PAGE. Bekes et al. (1983) found similar results on PAGE studies of protein from defatted glutens. A possible explanation for our findings is that methanol coextracted the group of phenolic glucosides and lipids associated in a hydrophobic manner with proteins. This brought about changes in the structure of protein molecule that resulted in the appearance of faster moving polypeptides on SDS-PAGE. This is supported by Kates (1972) who reported that polar solvents such as methanol-extracted lipids associated with membranes by disrupting hydrogen bonding and electrostatic bonds.

Our observations in electrophoretic mobility and changes in the number of subuntis in safflower seed proteins as a result of processing are consistent with those of Ishino and Okamoto (1975) and Saio and Watanabe (1973) for soybeans. Ishino and Okamoto (1975) treated water-extractable acid-precipitated soy protein with various alkali concentrations. Electrophoretic analysis revealed that when soy protein was treated with NaOH, pH 11.5, slow-moving bands disappeared while several bands appeared between the fastest and slowest bands. Saio and Watanabe (1973) investigated the effect of high temperature on soybean 7S and 11S proteins. They reported that no electrophoretic bands were observed on SDS-PAGE when proteins were heated at 150 °C. This indicates complete degradation of proteins to very low molecular weight polypeptides.

The significance of our SDS-PAGE results in still obscure at the present time but may be of value to plant breeders. A potential application of electrophoresis will be to devise a system that would enable us to identify cultivars of safflower with improved food characteristics as have been developed for wheat proteins (Zillman and Bushuk, 1979), oats (Lookhart, 1985), and barley (Gebre et al., 1986).

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Effects of Weed Interference and Herbicides on Nitrate and Carotene Accumulation in Lettuce

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Nitrate and carotene accumulation in field-grown lettuce suffering natural weed interference or protected by hoeing or herbicides was examined in a 2-year study. At the time of maturation weed interference had caused a reduction of nitrates in lettuce about equal to that of dry matter and a 2–4-fold reduction of carotenes. Carotene accumulation is therefore highly sensitive to weed interference. Nitrate content was higher in mature lettuce than in lettuce of a less advanced stage. All herbicides used (alachlor, pendimethalin, propyzamide) accelerated final level accumulation of nitrates, probably inducing a physiological state of maturation. Alachlor and pendimethalin, which are not as selective in lettuce as propyzamide, reduced carotenes in the year of heavier rain although they did not reduce the dry weight.

High-quality green vegetables are expected to contain the lowest concentrations of nitrates and to be rich in carotenoids. Concern over nitrates is because of their reduction to nitrites, which are involved in infant methemoglobinemia and in formation of the carcinogenic nitrosamines. Carotenoids (carotenes, xanthophylls), especially carotenes, are essential in our diet, serving as precursors of vitamin A.

The effects of N fertilization and of environmental factors on nitrate accumulation have been extensively studied (Szwonek, 1986). Efforts are currently made to develop cultural methods to reduce nitrates in vegetables (Roorda van Eysinga, 1984). Little is known, however, of the effect that weed interference and weed control measures may have on nitrate accumulation. Nitrate reductase activity has been increased by 2,4-D (Beevers et al., 1963) or simazine (Tweedy and Ries, 1967) under certain conditions. Subtoxic concentrations of herbicides have increased nitrate uptake in some cases (Ries, 1980).

To our knowledge, weed interference effects on carotene content of vegetables have not been studied. On the other hand, it has been reported that some herbicides either increase or decrease carotenes in selected crops (Sweeney and Marsh, 1971; Rouchaud et al., 1983, 1984).

The purpose of this study was to examine how nitrate and carotene content of lettuce is affected by weed interference under normal field conditions. The effects of

Table I. Air Temperature and Rainfall in the Location of the Experiments during the 1986 and 1987 Growing Periods

	mean temp,[a] °C					
	minimum		maximum		rainfall, mm	
month	1986	1987	1986	1987	1986	1987
January	4.1	3.8	12.5	12.0	0	55.8
February	4.1	4.9	11.7	10.4	0	70.8
March	5.5	−0.5	12.8	9.8	38.0	73.9
April	7.7	6.2	20.3	17.5	13.4	75.1

[a] Values are the monthly averages of daily minimum or maximum temperatures.

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