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Effect of Antioxidants on Stability of Astaxanthin Pigment in Crawfish Waste and Oil Extract

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A soy oil extraction method has been used to recover the carotenoid astaxanthin from heat-processed crawfish waste, *Procambarus clarkii*. Astaxanthin stability in comminuted crawfish meal and soy oil was determined as a function of time, temperature of storage, and antioxidant treatments by using ethoxyquin and Endox dry powder. Use of the Endox-oil mixture in the crawfish waste at a 0.05% level effectively protects the pigment component from degradation during frozen storage for as long as 16 weeks. Employment of ethoxyquin or Endox (dry powder) greatly inhibits autoxidative degradation of the isolated pigment during storage at 4 °C. The amount of oil-extractable astaxanthin from the waste after storage is affected by storage temperature, pretreatment of the waste via grinding, and its intensity of exposure to light or oxygen.

With increasing efforts in aquaculture and diet development, the value of crustacean meals for dietary formulations for shrimp and prawn species is being recognized. Such meals, in addition to their nutrient value, supply a source of carotenoid, notably that of astaxanthin (Meyers, 1977). The latter is the most prevalent carotenoid among crustacean groups (Lambertsen and Braekkan, 1971; Karrer and Jucker, 1950; Tanaka, 1978; Jangaard, 1975) and has been shown to elicit notable pigmentation in a variety of aquatic species when supplied as a dietary ingredient in the form of crustacean meal.

Among various sources of crustacean wastes, that from the Louisiana crawfish processing industry is especially noteworthy in view of the >10 million pounds of substrate available each year. Astaxanthin/astaxanthin ester and astacene, the oxidative product of astaxanthin, comprise the total carotenoids of heat-processed crawfish waste. A relatively high concentration, i.e., 153 μg/g, of astaxanthin/astaxanthin ester and astacene was reported in crawfish exoskeleton waste by using a polysolvent extraction system (Bligh, 1978; Meyers and Bligh, 1981).

Use of a soybean oil extraction procedure for recovery of astaxanthin from crustacean wastes, including red crab and crawfish, has been demonstrated (Spinelli and Mahnken, 1978; Chen, 1981). The quality of astaxanthin is affected by processing and storage conditions, i.e., heat treatment, length of period exposed to oxygen, and intensity of exposure to light. Our previous reports have documented the biochemical degradation of astaxanthin in dried crawfish meal and the effect of BHA and ethoxyquin (Bligh, 1978; Meyers and Bligh, 1981). Data reported here concern astaxanthin levels in the fresh (undried) comminuted crawfish meal prior to extraction with soybean oil and subsequent stability of the pigment in the oil.

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EXPERIMENTAL SECTION

Sample Collection. Heat-processed crawfish (*Procambarus clarkii*) waste, obtained from Seafood, Inc. (Henderson, LA), included the intact cephalothorax, abdominal exoskeleton, and viscera. Material was placed in double black polyethylene bags and frozen and held at -20 °C until used. The frozen intact waste was ground twice through a Hobart mixer (Model A-200) to a pastelike product, designated here as "comminuted crawfish" or "crawfish meal", prior to pigment analyses and antioxidant treatments.

Pigment Extraction. Soybean oil, ratio 1:1 (w/v), was added to calibrated weights of comminuted crawfish for total carotenoid extraction. This ratio was selected in view of its optimum efficiency in pigment extraction (Chen, 1981). The beaker containing the oil-crawfish blend was wrapped in aluminum foil to exclude light, with subsequent heating at 45-50 °C with continuous stirring, with final heating to 90 °C. A deep-red pigmented oil solution containing the dissolved carotenoids was recovered by centrifugation of the homogenate at 11000*g* for 10 min at 0 °C. A biphasic supernatant was observed, and the hypophase water was drained from epiphase pigmented oil in a separatory funnel. The total volume of the pigmented oil was recorded. The spectral characteristics of the carotenoid oil extract was analyzed spectrophotometrically (Beckman 25 spectrophotometer, Beckman Instruments, Inc.).

Pigment Quantitative Analysis. Astaxanthin and its ester have been identified as the major (90%) carotenoids in the exoskeleton of heat-processed crawfish waste (Bligh, 1978; Meyers and Bligh, 1981). Therefore, the term "astaxanthin" is used here to represent the total carotenoid

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pigments from crawfish waste. Total astaxanthin in the oil extract was estimated quantitatively by computing its concentration at the maximum absorbance (485 nm). The extinction coefficient value $E^{1\%}_{1cm} = 2155$ for astaxanthin in soybean oil (Chen, 1981) was used, and the amount of total astaxanthin in the crawfish waste was calculated by the formula of Kelley and Harmond (1972):

$$\text{g of astaxanthin/g of crawfish waste} = AD/(100GdE^{1\%}_{1cm}) \quad (1)$$

A = absorbance at maximum wavelength, D = total oil recovered volume (milliliters) × dilution multiple, G = crawfish waste (grams), d = cell width (1 cm), and $E^{1\%}_{1cm}$ = 2155 in soybean oil.

For practical purposes, the amount of astaxanthin in 100 g of oil was also used to represent the concentration of astaxanthin from the soybean oil extraction (Spinelli and Mahnken, 1978). The amount of astaxanthin (milligrams) in 100 g of oil can be calculated by using

$$\text{mg of astaxanthin/100 g of pigmented oil} = AD' \times 10^5/(100dSE^{1\%}_{1cm})$$

where D' = dilution multiple, S = 0.86, the specific gravity of soybean oil, and $E^{1\%}_{1cm}$ = 2155 in soybean oil.

Stability of Astaxanthin in Soybean Oil. A standard concentrated astaxanthin–oil was prepared according to procedures of Saito and Regier (1970) to evaluate the stability of astaxanthin–oil during cold storage. Petroleum ether also was used for extraction of astaxanthin from crawfish waste, following which the astaxanthin was analyzed at maximum wavelength of 470 nm. The concentration of astaxanthin was calculated by using eq 1 with an extinction coefficient value of $E^{1\%}_{1cm}$ = 2400 for astaxanthin in petroleum ether (Kanemitsu and Aoe, 1958). This known amount of astaxanthin was dissolved in a premeasured volume of soybean oil to obtain a final concentration of 100 μg/mL. Similarly, another standard of concentrated astaxanthin–oil solution, with 0.04% of ethoxyquin added, was prepared. Subsequently, 1 mL of the pigmented oil was sampled from each of the concentrated astaxanthin–oil solutions which were then diluted to 1:50 (v/v) with soybean oil and the absorbance was measured at 485 nm.

The concentrated astaxanthin–oils, with or without ethoxyquin present, were placed in opaque bottles, sealed, and stored undr refrigeration at 4 °C. After 7 months, the concentration of these two samples was diluted to 1:50 (v/v) and absorbance measured.

In addition, the astaxanthin–oil extract, by using soybean oil as the extractant, from crawfish waste was treated with Endox dry powder, 0.05%. The control, without antioxidant treatment, also was prepared. Samples were stored under conditions as described previously. After 2 months, the concentration of these two samples was diluted to 1:14 (v/v) and analyzed spectrophotometrically.

An astaxanthin ester oily solution (2.5 μg/mL), obtained from K. Simpson, University of Rhode Island, also was assayed at monthly intervals to investigate its stability during storage at room temperature. The initial absorption of the diluted (5×) astaxanthin ester with petroleum ether was measured at 470 nm, and resultant changes were recorded.

Stability of Astaxanthin in Crawfish Waste: Effects of Antioxidants. Comminuted crawfish was thoroughly mixed and divided into three 1000-g portions, one of which was treated with 0.125% ethoxyquin, one of which was treated with 0.1% of Endox dry powder, and the third was used as a control. For application of Endox dry powder, the chemical was first dissolved in 10 mL of soybean oil followed by spraying of the solution on the waste (Kephart, 1949). Each portion was stored at −20 °C in a plastic opaque container sealed to exclude light.

Prior to storage, the initial amount of the oil-extractable astaxanthin present was analyzed. At periods of 4, 8, 12, and 16 weeks of storage, communited crawfish in each plastic container was thoroughly agitated, a 100-g portion was sampled, and the amount of oil-extractable astaxanthin in the meal was analyzed.

The optimal concentration of Endox dry powder was determined subsequently by adding different levels, i.e., 125, 250, 500, 1000, and 2000 ppm, of the dry powder to individual samples of crawfish meal. Sample preparation followed procedures described previously. Astaxanthin, by using the oil extraction procedure, was analyzed at every 0, 4, 8, 12, and 16 weeks of storage.

Stability of Astaxanthin in Crawfish Waste: Effect of Temperature of Storage. Crawfish waste used in this study was ground through a Rietz disintegrator on site at the processing facility. The total wet pulverized meal was mixed and allowed to stand in daylight several hours prior to freezing. Subsequently, the frozen waste was thawed and twelve 100-g samples were placed in polyethylene bags and sealed. Six samples were treated with the 0.1% Endox–oil mixture prior to package, and the total was divided into three parts, each with four samples with or without the Endox/oil mixture. Three parts were stored at different temperatures, i.e., 4, 10, and −20 °C. After 2 and 4 weeks, the oil-extractable astaxanthin in these samples was analyzed.

Stability of Astaxanthin in Crawfish Waste: Effect of Grinding and Packaging. Four 100-g crawfish waste (ground or unground) samples were placed in Conovac vacuum packaging bags (Continental Can Corp.) and sealed in air or under vacuum (RMF, Kansas City, MO) in duplicate. Subsequently, all samples were placed in black polyethylene bags and stored at −20 °C. The amount of soybean oil extractable astaxanthin in these samples was analyzed initially and after 2 and 3 months of frozen storage.

Statistical Analysis. Data have been subjected to analysis of variance to detect significant differences among treatments, time intervals, levels, etc. A randomized block design was used in each analysis, and the calculated F value exceeding 5% tabular F was considered evidence of a significant effect ($p < 0.05$).

RESULTS AND DISCUSSION

Astaxanthin Degradation and Antioxidants. The changes in absorbance of astaxanthin ester at 470 nm in petroleum ester during storage at 25 °C is presented in Figure 1. The shape of the curve, characteristic of an autocatalytic process, is similar to the sigmoid curve of carotene autoxidation in paraffin solution shown by Budowski and Bondi (1960). These workers suggested that carotene initially is oxidized slowly, with a definite induction period, followed by an increasing rate of oxidation and subsequent retardation in oxidation. On the basis of this, degradation of astaxanthin ester in the present study was assumed to be an autoxidation process, an assumption tested below.

Table I shows changes in absorbance of diluted astaxanthin-enriched oil solutions (×50) and astaxanthin oil extracts (×14), treated with ethoxyquin and Endox dry powder, respectively. In the control sample with ethoxyquin, pigment retention (90.6%) was considerably higher than that of the previous astaxanthin ester sample, 42.3% after 7 months (Figure 1). The lower storage temperature (4 °C) probably is responsible for the increase in pigment

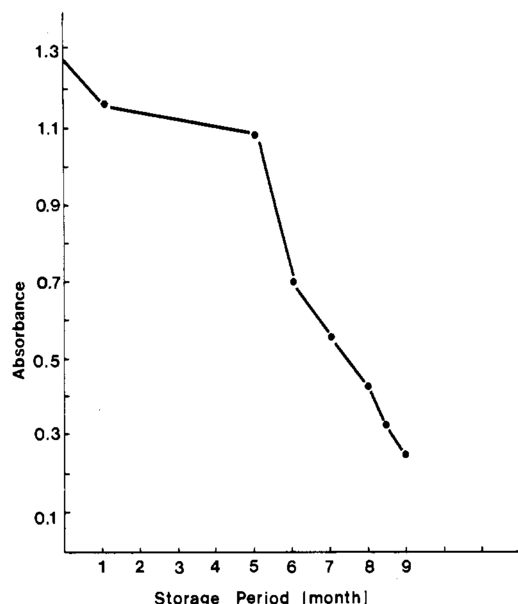

Figure 1. Changes in absorbance of astaxanthin ester at 470 nm in petroleum ether during storage at 25 °C.

Table I. Stability of Astaxanthin in Soybean Oil[a]

antioxidant	absorbance[b] initial	after 2 months	after 7 months	pigment retention, %
I: control	0.52		0.470	90.6
0.04% ethoxyquin	0.52		0.515	99.0
II: control	0.83	0.765		92.2
0.05% Endox	0.83	0.830		100.0

[a] Samples stored at 4 °C. [b] Absorbance of diluted (×50; treatment I) astaxanthin-enriched oil and diluted (×14; treatment II) astaxanthin extract at 485 nm.

stability (Lundburg, 1962). Furthermore, incorporation of astaxanthin into the oil in all likelihood contributed to the increase in astaxanthin stability of the control, since the oil is a good barrier to oxygen (Bauernfeind et al., 1958). Similarly, the close contact among the astaxanthin molecules, the natural antioxidant tocopherol present in soybean oil (World Health Organization, 1972; Morgal et al., 1943), and citric acid (synergist), added during the manufacturing of soybean oil (Budowski and Bondi, 1960; Bickoff et al., 1955), also may play important roles in retarding astaxanthin degradation. However, the decreased stability of the control sample of Endox treatment may be attributed to the pigment being extracted from comminuted crawfish which had been stored at -20 °C for 3 months.

The value of ethoxyquin and Endox dry powder in inhibition of astaxanthin oxidation is seen in Table I. Apparently, both antioxidants can effectively retard oxidative degradation of the pigment in oil during cold storage.

Discoloration of Crawfish Waste and Endox Antioxidant. Results of storage tests of the stability of astaxanthin in comminuted crawfish, showing effects of ethoxyquin and Endox, are illustrated in Figure 2. As noted, ethoxyquin was ineffective in retarding oxidation of astaxanthin in the waste, with rates of degradation similar to those of the control sample. In contrast, the Endox dry powder–soybean oil mixture showed noteworthy effectiveness in pigment retention.

As noted, the concentration of the oil-extractable astaxanthin in the Endox–oil mixture treated sample showed a slight increase during the initial 4 weeks of storage, with a gradual decrease thereafter. This may be due to desiccation of the comminuted crawfish during storage (Tsukuda and Amano, 1966). However, proteolytic or lipolytic enzymes present in crawfish waste probably are operative via autolysis and subsequently release carotenoid from the carotenoprotein and lipoprotein in which it is associated with the lipid component (Cheesman et al., 1967; Green, 1965). The free form of astaxanthin, after dissociation from its protein and lipid complexes, is less stable toward oxidation than the bound form (Cheesman et al., 1966). Therefore, the increased amount of oil-extractable astaxanthin in the Endox–oil mixture treated sample was subjected to gradual oxidative degradation during subsequent storage.

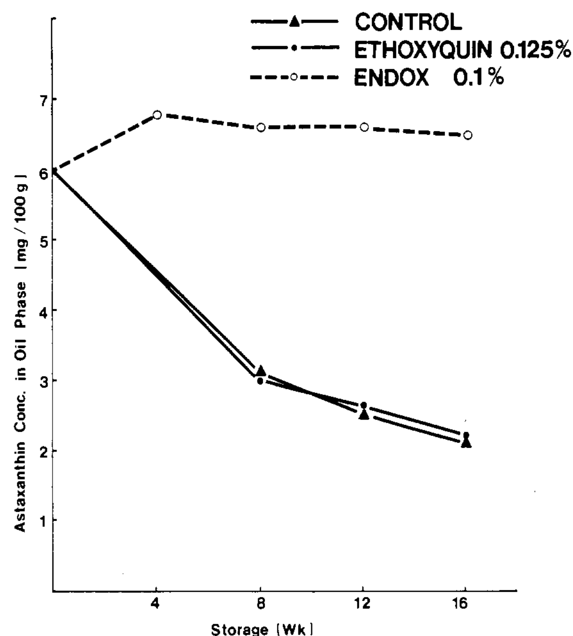

Figure 2. Amount of oil-extractable astaxanthin following frozen storage.

The results in Figure 2 differ somewhat from those of Rousseau (1960) and Meyers and Bligh (1981), wherein ethoxyquin was demonstrated to be an effective antioxidant, and superior to BHA or BHT, in retarding pigment losses of dried shrimp and crawfish meal (4–10% moisture content). Therefore, it was postulated that the high moisture content (70–75%) of the comminuted crawfish used in the present study hindered close contact between ethoxyquin and carotenoid molecules. Improvement in employment of water-insoluble ethoxyquin in comminuted waste can be accomplished by dissolving ethoxyquin in a suitable solvent, i.e., acetone or vegetable oil, followed by its complete blending into the waste (Mitchell et al., 1954; World Health Organization, 1972). On the basis of this, the water-soluble salt, ethoxyquin sulfate, might be an effective antioxidant for such purposes.

The effectiveness of the Endox–oil mixture possibly is due to the mixture of components in the antioxidant, the nature of the waste, and factors contributing to autoxidation of astaxanthin itself. Endox is a combination of BHA (antioxidant), mono- and diglycerides (emulsifier), phosphoric acid (synergist), and EDTA (disodium ethylenediaminetetraacetate dihydrate; metal deactivator). Blain and Shearer (1962) showed that butylated hydroxyanisole (BHA) is superior to other antioxidants in retarding autoxidation and hematin-catalyzed oxidation of carotenes. In addition, phosphoric acid and EDTA, agents for binding or deactivating trace metals, have been recommended for prevention or retardation of discoloration

Table II. Pigment Retention in Crawfish Meal

antioxidant	concn, %	% pigment retained after 1 week	2 weeks	3 weeks
Santoquin[a] (ethoxyquin)	0	57.8	40.0	31.5
	0.1	83.0	82.2	70.2
	0.5	90.2	85.5	74.6
	1.0	88.5	85.9	80.4
antioxidant	**concn, %**	**% pigment retained after 8 weeks**	**12 weeks**	**16 weeks**
Endox[b] (dry powder)	0	51.7	41.7	21.7
	0.025	115.0	115.0	95.0
	0.050	120.0	115.0	111.7
	0.100	106.7	105.0	105.0
	0.200	110.0	108.3	103.3

[a] From Meyers and Bligh (1981); temperature of storage was 50 °C. [b] Samples stored at -20 °C.

caused by reactions between BHA and iron (World Health Organization, 1972). In addition to possible contamination of crawfish waste with trace metals during processing and subsequent grinding treatment, Lovell et al. (1968) demonstrated that iron was present in Louisiana crawfish meal at an 8.8-ppm level. Sweet (1973) noted that, due to the high metal iron content of seafood, the use of chelating agents (citric acid and EDTA), in addition to antioxidants, improved oxidative stability. It can be postulated that the autoxidation of astaxanthin in crawfish waste is an iron-catalyzed process, and use of Endox antioxidant effectively protects the pigment.

Elsewhere, the discoloration of astaxanthin in shrimp was believed to be related to fat oxidation (Faulkner and Watts, 1955). Therefore, the oxidation of the fat in crawfish waste with formation of peroxides probably would oxidize the associated astaxanthin simultaneously and develop discoloration (Budowski and Bondi, 1960). The use of ethoxyquin has been demonstrated to be ineffective for absorption of hydroperoxides in vivo, while vitamin E (tocopherol) can act as a peroxide decomposer (Takeuchi, 1972). On the basis of the aforementioned, use of the Endox–soybean oil mixture is considered to be a suitable antioxidant combination for retarding astaxanthin loss in crawfish waste.

Table II summarizes previous data (Meyers and Bligh, 1981), and that obtained currently, on the stability of astaxanthin pigment in dried meals and comminuted wastes treated with ethoxyquin and the Endox–oil mixture, respectively. A higher pigment retention is seen in comminuted crawfish, with or without Endox treatment, compared with that in dried meals. This difference may be due to the role of water in the structural features of the protein that are essential to carotenoid binding. It has been noted that the reactive 4- and 4′-keto groups in the β-ionone rings of astaxanthin are prerequisites for interaction between carotenoid carbonyl groups and amino acid residues to form carotenoprotein. Furthermore, astaxanthin is hypothesized to be bound by hydrogen bond or salt linkage to amino groups on the protein with the two keto groups after enolization (Cheesman and Prebble, 1966; Cheesman et al., 1966; Zagalsky et al., 1967; Thommen, 1971). It is also indicated that there is a mutual stabilization between the carotenoid and the protein, since cleavage of the binding leads to a considerable increase in the sensitivity of the carotenoid to oxidation and to a change of configuration of the protein (Cheesman et al., 1967). On the basis of these considerations, removal of the water from crawfish meal by conventional drying may

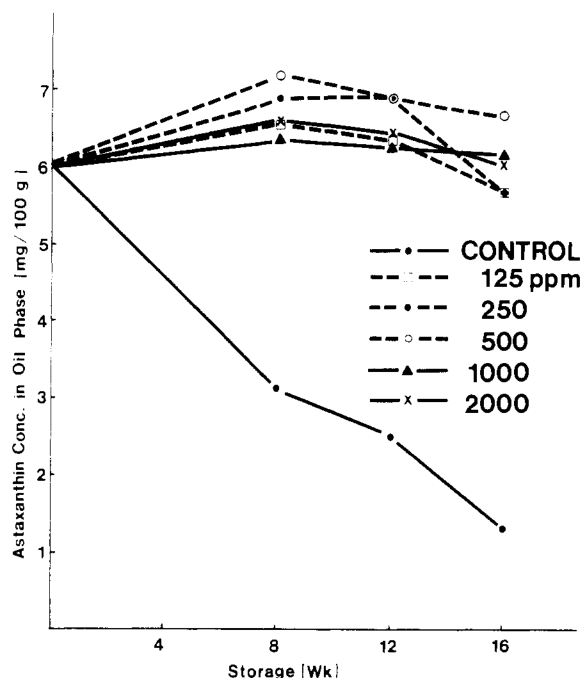

Figure 3. Different levels of Endox antioxidant and oil-extractable astaxanthin recovered after frozen storage.

Table III. Recovery of Oil-Extractable Astaxanthin (Milligrams per 100 Grams of Oil) during Storage of Crawfish Waste

time, weeks	Endox treatment, %, for storage temp: 10 °C, 0	10 °C, 0.1	4 °C, 0	4 °C, 0.1	−20 °C, 0	−20 °C, 0.1
0	2.90	2.90	2.90	2.90	2.90	2.90
2	5.20	4.80	4.90	5.00	3.50	6.45
4	4.76	5.60	5.00	5.40	3.30	6.80

decrease the stability of astaxanthin during subsequent storage by weakening or breaking astaxanthin–protein bonds.

Determination of Optimum Concentration of Endox Antioxidant. Data on the optimum concentration of Endox in comminuted crawfish are given in Figure 3. Endox at a 500-ppm level exhibited a slightly higher efficiency ($p < 0.1$) in astaxanthin retention, whereas a higher concentration, i.e., 1000 or 2000 ppm, was less effective. This phenomenon has been designated as the "reversion effect" in phenolic antioxidants such as tocopherol and BHA. In high concentration, i.e., >0.05%, the antioxidant tends to decompose peroxides formed during fat oxidation, thus causing a decrease in the stability of the substrate (Hill et al., 1969).

Effect of Temperature of Storage. Table III demonstrates the effect of storage temperature on oil-extractable astaxanthin, with or without antioxidant. It is interesting to note in the control samples that a higher amount ($p < 0.05$) of oil-extractable astaxanthin was obtained at 4 and 10 °C. This may be due to the temperature used that allows proteolytic or lipolytic enzymes present in comminuted crawfish (a_w nears 0.998) to be operative and release the pigment from the carotenoprotein and lipoprotein within a short storage period. However, with the addition of the Endox–oil mixture, the pigment retention of comminuted crawfish was pronounced ($p < 0.05$) when the sample was stored at −20 °C.

A higher rate of pigment degradation was observed in the control sample stored at −20 °C, compared with rates given in Table II. This probably is due to induction of

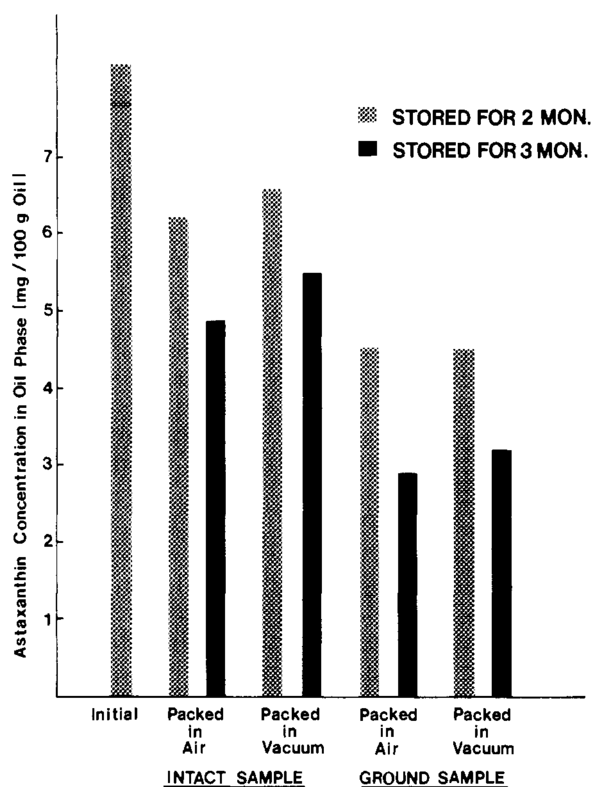

Figure 4. Effect of packaging and grinding on astaxanthin stability in crawfish waste during storage at −20 °C.

pigment degradation by light-catalyzed autoxidation prior to extraction, since the sample used was stored in the sunlight for several hours prior to frozen storage.

Grinding and Packaging in Astaxanthin Losses. Figure 4 shows retention of astaxanthin in ground or intact crawfish waste samples packed in air, or under vacuum, for 2 and 3 months. A difference ($p < 0.01$) in the retention of astaxanthin was observed between ground and intact crawfish samples in air or under vacuum, with a higher ratio of astaxanthin (42.5%) being lost from ground samples in air or under vacuum. In comparison, 24.4 and 19.5% of astaxanthin were lost from intact samples in air and under vacuum, respectively. This may be due to additional surface areas being exposed to air after grinding, with more astaxanthin being discolored after oxidative degradation (Faulkner and Watts, 1955; Lusk et al., 1964). Vacuum packaging does not give a significant reduction ($p > 0.05$) in pigment lost since oxygen is not the sole factor involved in autoxidation of astaxanthin in crawfish waste. As noted previously, iron may be the catalytic factor in autoxidation of astaxanthin; thus, elimination of iron by use of a chelating agent, i.e., EDTA or phosphoric acid, seems to be more effective than vacuum packaging in preventing the destruction of astaxanthin in crawfish waste.

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