superior to those obtained when using the apparatus without external steam. This is excepting ethanol for which the introduction of water into the system effected zero recovery again. As with recoveries from aqueous media, dichloromethane was the better solvent and 1 h was optimum for the extraction period, although as usual recoveries did generally increase with time. Results obtained by using this system were of the same order as those obtained from aqueous media by using the original apparatus (MacLeod and Cave, 1975) (see Table I).

This further minor refinement to the modified Likens and Nickerson apparatus (MacLeod and Cave, 1975) thus provides a relatively simple system with adequate efficiency for most purposes for the ready recovery of components from a purely lipid medium. Its efficiency would seem to be comparable with more sophisticated and complex equipment specifically designed for lipid samples, although identical recovery data with regard to the latter are not available. However, the Likens and Nickerson apparatus and procedures described here possess the additional advantage of versatility, and as such they are suitable for the analysis of the volatile components of a very wide range of foods and similar samples.

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# Characterization of Astaxanthin Pigments from Heat-Processed Crawfish Waste

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Carotenoid pigments have been isolated and identified from the heat-processed exoskeleton of crawfish, *Procambarus clarkii*. Pigment concentrations as great as 153 μg/g are noted, consisting of 49.4% astaxanthin ester, 40.3% astaxanthin, and 10.3% astacene. When measured as a function of time and antioxidant treatment, using BHA and Santoquin, pigment concentrations showed a maximal decrease, i.e., 45% over 3 weeks, in untreated samples. Levels of Santoquin at 0.5% were effective in minimizing pigment loss with storage. Application of the high pigment quality meal in diets for aquaculture-raised fish is discussed.

Increasing interest is being shown in astaxanthin and astaxanthin-containing raw materials for use in dietary formulations where integument and flesh coloration is of economic importance. Attention has been directed toward pen-reared salmonid fishes with emphasis on use of a variety of crustacean meals in feed formulas (Steel, 1971; Kuo et al., 1976; Meyers, 1977; Bligh, 1978; Spinelli and Mahnken, 1978). In addition, a variety of carotenoid and carotenoid-containing materials, i.e., β-carotene, canthaxanthin, paprika, and β-apo-8′-carotenal, have been tested as flesh pigmenters in the feeds of fish such as trout, salmon, and tropical fish and in poultry (Bauernfeind, 1976; Bunnell and Bauernfeind, 1962; Fey and Meyers, 1980). Recently a number of investigators (Ellis, 1979; Simpson and Kamata, 1979) have stressed the importance of carotenoid-rich ingredients as well as extracted pigment in aquatic diets for enhancement of coloration, especially among salmonids.

The multi-million-dollar Louisiana crawfish (*Procambarus clarkii*) industry characteristically produces a significant amount of potentially usable proteinaceous waste during the harvest periods December to June, with the dominant catch occurring in the spring months of April to June. Landings in 1980 of over 25 million pounds of animals (85% waste) is indicative of the magnitude of the potentially usable byproduct generated. Disposal of this material is an increasing economic problem, adding to the processor's operating expense. Previous works from our laboratories have focused on nutritional and other properties of the meal, along with economic data on the composite crawfish processing industry. The research reported here concerns astaxanthin levels present in the dried meal and its preservation under storage conditions, along with

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a discussion of the crustacean byproduct application.

## EXPERIMENTAL SECTION

**Sample Collection.** Samples, including the entire cephalothorax and abdominal exoskeleton, after parboiling and removal of the tail meat, were collected from local Louisiana crawfish processors. The material was placed in double plastic bags, flushed with nitrogen, and frozen at −15 °C for subsequent pigment analysis.

**Extraction Procedure.** A ternary system of petroleum ether–acetone–water (15:75:10 v/v/v) was used for total extraction of the carotenoids from the waste. Gills and internal organs were removed from the cephalothorax and discarded, chelipeds were cleaned of meat, and only the exoskeleton material was subjected to total and component carotenoid analysis. For facilitation of subsequent extraction, the sample was decalcified with 50% acetic acid in water at 10 °C, under nitrogen in the dark (Fox, 1973). The acetic acid solution was filtered through a medium porosity fritted-glass funnel to recover pliable pieces of exoskeleton.

The carotenoids in the acetic acid solution were extracted into ethyl ether with addition of water. Ethyl ether was evaporated under nitrogen, and the carotenoids were redissolved in a small volume of petroleum ether. Exoskeleton fragments were soaked in the petroleum ether–acetone–water solution overnight, at 10 °C, in the dark, under nitrogen, with mechanical shaking. The solution was then filtered as above, and the filtrate combined in a separatory funnel with the petroleum ether solution of the acetic acid extract. To this was added 150 mL of petroleum ether and 100 mL of water. The hypophase was drained and extracted with 100 mL of petroleum ether which was subsequently pooled, after phase separation, with the former epiphase. Petroleum ether containing total carotenoids was washed 3 times with 200-mL portions of water to remove traces of acetone, followed by drying overnight over anhydrous sodium sulfate at 10 °C, in the dark, under nitrogen. Petroleum ether was filtered through a medium porosity fritted-glass funnel containing a 1-cm bed of sodium sulfate. The solvent was stripped from the carotenoids at 45 °C in a rotary evaporator (Rotavapor R, Buchi, Switzerland), and the carotenoids were diluted to 100 mL with petroleum ether in a volumetric flask.

**Pigment Characterization.** Total carotenoids were estimated by computing their concentration at the maximum absorbance (467 nm) of the whole unsaponified extract in petroleum ether. The extinction coefficient value $E^{1\%}_{1cm}$ = 2400 (Kanemitsu and Aoe, 1958) for astaxanthin was used as the extinction coefficient, since astaxanthin has been established as the dominant carotenoid in *P. clarkii* (Nakagawa et al., 1971). The estimated total carotenoids were calculated by using the formula of Kelley and Harmon (1972). Aliquots of whole pigment extracts were saponified by using a modified method of Davies (1976) to release carotenoids from any ester linkages.

In the saponification procedure, petroleum ether was removed from carotenoids in a stream of nitrogen, followed by addition of 1 volume of methanol and 0.1 volume of 60% aqueous potassium hydroxide. The solution was placed under nitrogen and stored at 10 °C for 12 h in the dark, following which an equivalent volume of ethyl ether and 2 volumes of water were added. Extraction with ethyl ether was repeated 3 times, and ether extracts were then pooled and washed 3 times with water. The solution was dried over sodium sulfate for 3 h under nitrogen at 10 °C, in the dark, filtered, and evaporated to dryness with a stream of nitrogen. Carotenoids were analyzed via column chromatography and absorption maxima measured.

Table I. Proximate Analysis of Crawfish Waste Material[a]

| | % |
|---|---|
| protein (crude) | 36.7 |
| protein (corrected)[b] | 32.2 |
| fat | 6.7 |
| fiber | 14.2 |
| ash | 35.7 |
| chitin | 14.1 |

[a] From Lovell et al. (1968). [b] Corrected for Chitin-N.

Fraction separation involved use of thin-layer chromatography, with 250-μm, 20 × 20 cm silica gel G plates. TLC plates were spotted with the total carotenoid extract and chromatographed in the solvent system benzene–petroleum ether–acetone (10:3:2 v/v/v; Vettorazzi, 1970). Carotenoid standards used included β-carotene, canthaxanthin, and lycopene. The chromatogram was developed in a saturated chamber (lined with filter paper) by using the ternary solvent mixture benzene–ethyl acetate–methanol (75:20:5 v/v/v).

The procedure for establishment of the partition coefficients of the various carotenoid fractions was a modification of that of Petracek and Zechmeister (1956) and Krinsky (1963).

The TLC and spectrophotometric techniques previously described were used to determine the percentage composition of carotenoid fraction in whole crawfish pigment extract. The concentration of total carotenoids was calculated for a concentrated extract and fractions by using the formula of Kelley and Harmon (1972) and Davies (1976).

**Storage of Crawfish Meal.** Analyses of degradation of carotenoids during storage were conducted using a method adapted from Rousseau (1960). Fresh, whole crawfish processing waste was dried in a convection oven (Blue M Electric Co.) at 75 °C for 5.5 h and then ground in a Wiley mill. Butylated hydroxyanisole (BHA) and Santoquin, at four levels of application, i.e., 0, 0.1, 0.5, and 1.0%, were thoroughly mixed in 10-g samples of crawfish meal. Each treatment combination was replicated 4 times. Residual acetone was removed via air drying at room temperature. The plates were covered and stored in an incubator at 50 °C, and the material was assayed weekly. One gram of meal from each experimental unit was used for each analysis. Samples were placed in 250-mL plastic centrifuge bottles to which was added 100 mL of acetone, followed by nitrogen flushing and shaking for 20 min at 10 °C. The supernatant was removed, absorbance measured at 475 nm, and carotenoid concentration calculated by using the formula and the extinction coefficient $E^{1\%}_{1cm}$ = 2200 for astaxanthin in acetone (Kanemitsu and Aoe, 1958).

Based on the initial carotenoid concentration of the meal, percent retention was calculated at 1-, 2-, and 3-week intervals. Data were subjected to analysis of variance to detect significant differences among treatments, replication, and time intervals. A randomized block design was used with a 4 × 4 × 2 split plot arrangement of treatments and two factors on the main plot.

## RESULTS AND DISCUSSION

The proximate analysis of crawfish meal is given in Table I. It has been suggested that the high ash content poses potential dietary problems, and its use in animal feeds has been recommended at levels of 10% or less (Lovell et al., 1968). Subsequently, Rutledge (1971) described a mechanical process in which the ash content of the meal was reduced by ~38% and the protein levels increased 2-fold.

Table II. Analysis of Crawfish Carotenoid Pigments

| fraction | partition ratio[a] | $R_f$ value | absorption maxima, nm | solvent | pigment identification | % composition | amount, μg/g |
|---|---|---|---|---|---|---|---|
| 1 | 96.8:3.2 | 0.95 | 465-467<br>502 | petroleum ether<br>carbon disulfide | astaxanthin esters | 49.4 | 75.6 |
| 2 | 42.7:57.3 | 0.84 | 467<br>501<br>472[b] | petroleum ether<br>carbon disulfide<br>petroleum ether | free astaxanthin | 40.3 | 61.7 |
| 3 | – | 0.65 | – | – | astacene | 10.3 | 15.8 |
| | | | | | | total: | 153.1 |

[a] Determined in a 95% aqueous methanol-petroleum ether system; represents epiphasic:hypophasic character; mean of two observations. [b] Saponified.

Table III. Pigment Retention in Crawfish Meal

| antioxidant | concn, % | % pigment retained after 1 week | 2 weeks | 3 weeks |
|---|---|---|---|---|
| BHA | 0 | 57.6 | 43.6 | 33.8 |
| | 0.1 | 66.5 | 53.9 | 50.7 |
| | 0.5 | 84.1 | 74.5 | 64.0 |
| | 1.0 | 81.3 | 78.0 | 68.7 |
| Santoquin | 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 |

An analysis of astaxanthin-type carotenoids in the crawfish meal is given in Table II, along with the fraction, color, $R_f$ values, partition ratios, and solvent system for each pigment from saponified and unsaponified whole extracts.

Absorption spectra and chromatographic behavior suggest the presence of only ketocarotenoids. $R_f$ values represent the average of eight observations. Carotenoid standards, lycopene, β-carotene, and canthaxanthin, were used to check the accuracy and reproducibility of the $R_f$ values to those given in the literature.

Concentrations given are calculated by using the extinction coefficient for free astaxanthin; thus, the values are approximations. Nakagawa et al. (1974) reported the presence of 12 carotenoid fractions in the exoskeleton of fresh crawfish, *P. clarkii*. Astaxanthin, esterified astaxanthin, and astacene averaged 62% of the total concentration; no additional fraction comprised greater than 10% of the total. Since only astaxanthin, esterified astaxanthin, and astacene were isolated in the present study, the other fractions either were not present before processing or more likely were oxidized during processing. A further possibility is that the astacene fraction represents multiple breakdown products.

The maxima observed for fraction 1 are similar to those reported by Kuo et al. (1976) and other investigators for esterified astaxanthin. The values obtained for fraction 2 are in agreement with those reported by Kuo et al. (1976) and Nakagawa et al. (1971) for free astaxanthin. Fraction 3 could not be eluted from an alumina column with 10% acetic acid in ethyl ether (Vettorazzi, 1970) or with 10% acetic acid in methanol. Karrer and Jucker (1950) reported that astacene was absorbed on alumina and could not be eluted with mixtures of benzene and methanol or pyridine and methanol. Since astacene has been identified in live *P. clarkii* (Nakagawa et al., 1974) and the carotenoids of crawfish processing waste have been subjected to high temperatures and oxygen which promote oxidation of astaxanthin to astacene, this fraction, calculated by subtraction, was postulated to be astacene.

Results of the study using butylated hydroxyanisole (BHA) and Santoquin are given in Table III. Significant differences ($p < 0.001$) were observed with preservative treatments, levels of antioxidant application, and time. Pigment retention was directly proportional to the concentration of antioxidant used, with the 1% level showing 35% higher pigment than the control (Figure 1a). As noted in Figure 1b, the average pigment concentration

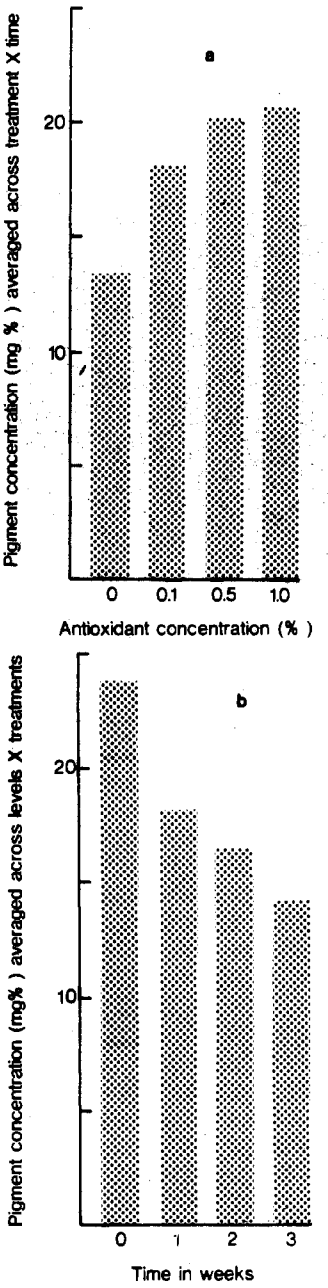


**Figure 1.** (a) Pigment concentration vs. level of antioxidant application in crawfish meal. (b) Pigment concentration vs. storage time of crawfish meal.

decreased as much as 45% with storage. Santoquin was more effective than BHA in retarding pigment loss, i.e., 35% vs. 45% loss, respectively. These results compare favorably with those of Rousseau (1960), who found Santoquin to be superior to BHA as a carotenoid antioxidant in shrimp meal. Elsewhere, Faulkner and Watts (1955) showed that shrimp held in frozen storage for 10 months lost as much as 40% of their carotenoid pigmentation. Colorimetric differences were more evident than were those of a direct organoleptic nature. Lusk et al. (1964) studied pigment losses of freeze-dried shrimp and salmon stored at 24 and 25 °C for 1–18 weeks. Essentially all carotenoid pigmentation in shrimp was lost after 1 week at both temperatures; however, pigment loss was less striking in salmon. The latter was attributed to the surface-concentrated pigmentation in shrimp vs. more homogeneous distribution in salmon flesh. However, both products were quite stable when stored under nitrogen at 0 °C. Data on the effectiveness of the level of the antioxidant used showed the most noteworthy increase in pigment retention at the 0.1% level. Increased concentrations of antioxidants above 0.1% had only a slightly enhanced beneficial effect. Ultimately, the possible synergistic effects of combinations of antioxidants (i.e., BHA, BHT, ascorbic acid, and propyl gallate) and other chemicals (i.e., citric acid and EDTA) should be evaluated.

This study has demonstrated the high pigment value of crawfish meal, with concentration as much as 20 times greater than that reported for commercial shrimp meals (Meyers, 1977), widely used for their pigmentation properties. Carotenoid levels of heat-dried shrimp meals usually are less than 10 $\mu$g/g, although a vacuum-dried shrimp meal from *Pandalus borealii* has been found to have carotenoid values in excess of 100 $\mu$g/g. However, due to resource depletion, this latter meal is no longer available. Comparable problems have been noted for red crab waste, where oil-extracted carotenoid pigments have been shown to be effective in the pigmentation of pen-reared salmonids (Spinelli and Mahnken, 1978).

Application of crawfish processing waste carotenoids as dietary supplements will depend ultimately on whether pigments are extracted by using an oil treatment, as suggested by Anderson (1975), or whether waste is used intact or decalcified (Rutledge, 1971) prior to its use in dietary formulations. Since 89.7% of the total carotenoids present in crawfish meal are free astaxanthin and esterified astaxanthin, it is likely that this material would have desirable properties when used in dietary formulations to enhance flesh pigmentation of various fish species. Further work is in progress to establish optimal treatment and storage conditions to maximize pigment stability in crawfish waste with special attention being given to properties of fresh frozen waste compared with those of the usual heat-processed meal. Clearly, information is needed on the carotenoid level of crustacean meals to optimize the economic use of such products in feed formulations.

The size and continued growth of the Louisiana crawfish industry and the presence of an increasing volume of potentially valuable pigment-rich waste are a major consideration in any projection of its byproduct utilization. Other factors, such as logistics of processing operations and the ready and predictable volume of the byproduct on a relatively long-term basis, are equally relevant considerations.

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