Carotenoid Pigment and Fatty Acid Analyses of Crawfish Oil Extracts

Thomas R. Omara-Alwala, Huei-Mei Chen, Yoshihito Ito, Kenneth L. Simpson, and Samuel P. Meyers*

Mono- and diester astaxanthins were demonstrated to be the primary (87%) carotenoids in pigmented oil from heat-processed crawfish waste by using commercial soy, menhaden, and herring-like oils as extractants. Acid ensilage treatment of the crawfish material did not qualitatively or adversely affect astaxanthin and fatty acid profiles in the pigment-enriched oil. The fatty acid composition of the oil extractant was modified slightly by the pigment extraction process. Concentration of crawfish pigments in soy oil resulted in a 2% decrease in the total saturated fatty acids, a 6% increase in the total monoenes, and a 4% decrease in the total PUFA. Analyses of the crawfish fatty acids showed a comparatively high proportion of linolenic acid (18:3ω3) (8%) and other long-chain polyunsaturated fatty acids (PUFA), i.e., 20:5ω3 and 22:6ω3. The sterol contents of crawfish meal (2 μg/mg) and its pigmented oil extracts (6 μg/mg) are discussed in terms of dietary formulations for aquatic species.

Carotenoids are a group of fat soluble pigments widely distributed in nature. Animals cannot biosynthesize carotenoids; thus, fish and shellfish raised in aquaculture must be supplied with pigments such as astaxanthin and its esters to produce a pigmented flesh and integument (Brinchmann, 1967). Astaxanthin from crustacean meals, or in an oil extract, has been demonstrated to be an effective pigmenting agent when incorporated into formulated diets for coloration of salmonids and crustaceans (Saito and Regier, 1971; Spinelli et al., 1974; D'Abramo et al., 1983; Meyers and Chen, 1982). While crustacean meals are good potential sources of astaxanthin and polyunsaturated fatty acids (PUFA), in practice the diverse treatments involved in processing and storage of dried crustacean meals can degrade the heat-labile carotenoid pigments (Simpson et al., 1981).

An alternative approach to utilization of astaxanthin from crustacean meals has been employment of vegetable or fish oils in extraction of the carotenoids from the wastes (Spinelli and Mahnken, 1978; Chen and Meyers, 1982a). The oil provides an energy source in the fish diet and a good barrier to oxygen, thus retarding subsequent pigment oxidation (Jangaard, 1975; Bauernfeind et al., 1958).

It has been estimated that >400 metric tons of commercial production of astaxanthin-enriched oil could be obtained from the multimillion kilograms of crawfish waste generated in Louisiana annually. However, the extraction procedures, oils used, storage conditions, and others could affect the quality of both the pigment itself and the extractant oil. Accordingly, this investigation was conducted to evaluate the carotenoid and fatty acid profiles of several extractant oils. Analyses include possible compositional changes in the pigmented oil recovered from acid-ensiled crawfish waste. The sterol content of crawfish meal and pigmented soy oil also was determined since such compounds are important, and perhaps essential, constituents of crustacean diets (Simpson et al., 1981; Teshima, 1981).

MATERIALS AND METHODS

Sample Preparation. *Pigment Extraction Process.* Approximately 0.5–0.75 ton of freshly collected heat-processed crawfish (*Procambarus clarkii*) waste was used for the scale-up pigment extraction procedure of Chen and Meyers (1982a). The composite waste material was initially pulverized through an attrition mill in which two fractions, e.g., chitinous shell and proteinaceous "puree", were discharged separately. The latter, comprising 90% (w/w) of the waste, was used for subsequent pigment extraction. Feed-grade fish oils, i.e., menhaden and a herring-like oil, as well as fully refined soy oil, were employed as extraction vehicles and were added to the crawfish puree at 8% (w/w) concentration. After heat processing (90 °C, 30 min) and centrifugal separation, crawfish pigments were recovered in a concentrated form in the oil and designated as "pigmented oil" product. The compressed solid was collected and is indicated as "crawfish press cake" in the following analyses.

Ensilage Treatment of Crawfish Waste. Acid ensilage treatment (Chen and Meyers, 1983) comprised use of a predetermined amount of an industrial-grade propionic acid (Eastman Co.) or a laboratory-grade sulfuric acid (96.9%, A.C.S., Mallinckrodt). This was added to crawfish puree and a mixture of crawfish puree and shell to obtain a final pH of 5.0–5.5. The acid hydrolysis was allowed to proceed for 1 h. Fully refined soy oil was then blended with the acidified hydrolysate for the pigment extraction process as described by Chen and Meyers (1982a). Control samples without ensilage treatment also were prepared.

Approximately 200 g of each pigmented and control oil was sealed under nitrogen for fatty acid and astaxanthin analyses. Quantities of freshly collected crawfish puree, press cake, and the fat derived from the cephalothorax portion of the crawfish also were frozen for similar analyses.

Chemical Analysis. *Characterization of Astaxanthin Pigment.* A 20-cm column was packed under vacuum with an absorbent of Microcel C and topped with a 1-cm layer of anhydrous sodium sulfate. The column was prewetted with petroleum ether (PE). The pigmented oil was added without prior treatment directly to the column. PE was used to wash the pigments into the column. The column was developed with a gradient of 1–5% acetone in PE (Kuo et al., 1976). The pigments were eluted rapidly from the column due to the oil present. Astaxanthin and its esters were identified based on relative position on the column, spectrophotometric data, TLC chromatography with authentic standards, and chemical tests (Kuo et al., 1976). Quantification involved use of an extinction coefficient of $E^{1\%}_{1cm}$ = 2000 (Kuo et al., 1976).

Fatty Acid Analysis. Approximately 1–2 g of crawfish puree, press cake, and fat was extracted twice with chloroform–methanol–water in a ratio of 1:2:0.8 adjusted for the moisture content of the samples following the Bligh

Department of Food Science and Technology, Nutrition and Dietetics, University of Rhode Island, Kingston, Rhode Island 02881 (T.R.O.-A., Y.I., and K.L.S.), and Department of Food Science and Center for Wetland Resources, Louisiana State University, Baton Rouge, Louisiana 70803 (H.-M.C. and S.P.M.).

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Table I. Astaxanthin Composition of Crawfish Waste and Pigmented Oil

sample	astaxanthin, % free	astaxanthin, % monoester	astaxanthin, % diester	total astaxanthin, ppm	total astacene,[a] ppm	ratio (astacene: astaxanthin)
crawfish puree	37.0	31.7	31.3	123	87	0.71
crawfish press cake	39.4	30.6	30.0	54	37	0.69
pigmented soy oil	12.8	45.7	41.5	778	535	0.69
pigmented herring oil[b]	13.1	45.7	41.2	656	449	0.68
pigmented menhaden oil	13.3	44.3	42.4	692	471	0.68

[a] Saponified oil, pigmented, expressed as astacene. [b] See the text.

Table II. Effect of Ensilage Treatment of Astaxanthin Composition of Pigmented Oil

treatment	oil ratio used, %	astaxanthin, % free	astaxanthin, % monoester	astaxanthin, % diester	total concentration, ppm
crawfish puree					
control	20	11.0	43.0	46.0	291
propionic acid (7.5%)	20	11.6	41.3	47.1	321
whole crawfish waste					
control	20	11.0	42.0	47.0	269
propionic acid (13%)	20	12.9	41.1	46.0	362
crawfish puree					
control	8	12.8	45.7	41.5	776
sulfuric acid (1.25%)	10	12.3	45.7	42.0	684

and Dyer (1959) technique as modified by Kates (1972). The esters of lipid material were hydrolyzed with 0.5 N potassium hydroxide–methanol solution for 10 min at 100 °C in Teflon-lined centrifuge tubes. The fatty acids recovered were methylated with a boron trichloride–methanol mixture. Fatty acid methyl esters (FAME) were then identified and quantitated by gas–liquid chromatography (GLC) with a column packed with 10% SP-2330 on 100–120-mesh Chromosorb W-AW. The GLC conditions were as follows: column and inlet temperature, 200 °C; detector temperature, 250 °C; flow rate, 36 mL/min; carrier gas, N_2; detector, FID; sensitivity, 4×10^{-10} AFS. The results are presented as FAME weight/percent of total lipid. Identification of FAME was by comparison of retention times (RT) with those of the standards injected under identical conditions and/or by comparison with relative retention times (RRT) reported for the same column at the same temperature.

Determination of Sterol Content. Approximately 0.5 g of oven-dried (80 °C) meal (moisture content; 5–6%), from whole crawfish waste, crawfish puree, and press cake, and about 0.2 g of pigmented oils and soy oil were used for sterol analysis. Samples were saponified by 1 mL of 50% KOH and 4 mL of 95% ethanol and boiled for 1 h. The unsaponifiables were then extracted with 15 mL of hexane, and 5 mL of distilled H_2O was added to the mixture (Kovacs et al., 1979). A measured volume (1 mL) of the hexane layer was pipetted into a test tube with the solvent evaporated under nitrogen. The residues were reacted with 2 mL of *o*-phthalaldehyde solution for 20 min and immediately mixed with 1 mL of concentrated sulfuric acid. Absorbance was read at 550 nm within 10–90 min, and the sterol content was calculated against a standard curve for cholesterol (Rudel and Morris, 1973).

RESULTS AND DISCUSSION

Astaxanthin Composition of Pigmented Oil. Astaxanthin profiles of lipid extracts from crawfish puree, crawfish press cake, and pigmented soy and fish oils are shown in Table I. Total astaxanthin concentration of each of the pigmented oils was greater than 600 ppm, a level considered to be economically feasible for incorporation into salmonid fish diets for pigmentation purposes (Chen and Meyers, 1982a). On the basis of the pigment concentration of crawfish puree, the concentration of astaxanthin in the recovered oil was enhanced significantly through the extraction process. As noted in Table I, free astaxanthin was the dominant pigment in the puree (before extraction) and in the press cake (after extraction). In contrast, the major pigments in the oil extract are its mono- and diester, suggesting that the esters are preferentially extracted by the oils. The extracted, esterified astaxanthins are considered to be less liable to oxidation than free astaxanthin (Lambertsen and Braekkan, 1971). The demonstrated presence of esterified astaxanthin may contribute to the stability of pigmented oil (Chen and Meyers, 1982b).

The variation in total astaxanthin content of the pigmented oils indicates that both source and grade of oil used may exert an influence on extraction efficiency. The particular features of individual oils that contribute to these differences are under further investigation. Although soy oil is comparatively superior as an extractant to the other oils examined, use of the fish oil is important for dietary applications in which marine oils have desirable nutritional value (Kissil et al., 1982).

The pigmented oils were saponified under conditions that would result in formation of astacene from astaxanthin and its esters. Saponificatiion results in a predictable loss (Table I) and thus could be used as a means of estimating the total astaxanthin content.

Stability of Astaxanthin to Ensilage Treatment. Acid ensilage treatment preserves crawfish heat-processed waste under ambient temperature conditions, with notable improvement in extraction efficiency (Chen and Meyers, 1983). In the astaxanthin profile of pigmented oil from the ensiled waste (Table II), no significant differences were noted in the percentage of astaxanthin components of the control and acid-treated samples, indicating absence of decomposition when reacted with propionic or sulfuric acid. Ensilage treatment with propionic acid in the present pilot plant study also increased extraction efficiency by 15% and 35% in crawfish puree and whole crawfish waste, respectively. Approximately twice as much acid (13% vs. 7.5%) was needed to lower the pH of whole crawfish waste to near pH 5.0–5.5 compared with that required for the crawfish puree. Consequently, comparatively more astaxanthin was released from the carotenoid and calcium carbonate complex of the shell components in whole crawfish waste than in the crawfish puree (Chen and

Table III. Fatty Acid Profiles of Soybean, Herring, and Menhaden Oils and the Pigmented Products

	%						
FAME	soybean oil	pigmented soybean oil	herring[a] oil	pigmented herring oil	menhaden[a] oil	pigmented menhaden oil	crawfish puree
12:0		0.1			0.5		0.8
14:0		0.1	0.2	0.2	0.3	0.3	0.8
16:0	21.2	20.2	11.1	12.6	14.8	15.2	19.8
16:1ω9		3.9	6.4	8.5	12.8	12.1	9.6
18:0	9.5	8.1	2.4	3.4	3.6	6.0	6.5
18:1ω9	3.4	5.0	14.4	16.3	17.4	18.3	22.5
18:2ω6	46.6	40.0	6.6	7.2	4.6	7.1	10.8
18:3ω3	16.5	15.0	13.7	10.2	3.9	4.5	7.2
20:3ω6		1.0	21.5	15.1	1.7	1.8	5.8
22:1ω9		.5	2.8	2.9		0.1	
20:5ω3		2.3	8.8	11.7	18.6	16.9	8.5
24:1ω9		.2	2.1	2.0	2.1	1.9	0.3
22:5ω3		.4	0.8	1.4	2.6	2.4	0.6
22:6ω3		1.5	8.3	8.0	7.9	7.6	3.8
total							
Saturates	30.7	28.4	13.7	16.2	19.2	21.5	27.9
Monoenes	3.5	9.5	25.6	29.7	32.3	32.3	32.4
PUFA	63.1	60.2	59.7	52.5	39.3	40.4	36.7

[a] See the text.

Meyers, 1983). Astaxanthin concentrations of all samples in Table II also are affected by the oil ratio used for extraction; a higher oil ratio imparts a dilution effect and consequently reduces the pigment level of the recovered oil.

Fatty Acid Profile of Pigmented Oil and Crawfish Waste. The effect of the pigment extraction process on the fatty acid profile of different commercial oils is presented in Table III. An inspection of the fish oils designated "herring" and menhaden oils shows some variation from the fatty acid profiles reported for the pure oils (Exler and Weihrauch, 1976; Exler et al., 1975). This is especially true of the values obtained for the C20 and C22 monoenes for herring oil and the 18:2 and 18:3 profiles for menhaden oils. Although these commercial oils were supplied as herring and menhaden oils, in all likelihood they probably represent a mixed fish source. This is especially true with the herring oil where probably other species, i.e., capelin and Norwegian pout, have been used to manufacture a herring-like oil, other than herring stock itself. Nevertheless, these deviations in fatty acid profile from the pure oils were not expected to alter their performance as extraction vehicles. Pigmentation of the soybean oil with crawfish pigment results in a 2.3% decrease in the total saturated fatty acid profile, a 6% increase in the total monoenes, and a 3.8% decrease in the total PUFA. With herring oil, the pigment extraction process leads to a 2.4% increase in the total saturates, a 4% increase in the total monoenes, and a 7% decrease in the total PUFA. However, with menhaden oil, the extraction process has less effect on the overall fatty acid composition of the oil than it has on the herring and soybean oils.

The oils extracted some of the crawfish lipid fractions along with the pigment; thus, the total represents a mixture. As expected, the soy oil, which represents a typical vegetable oil, showed the greatest change in composition.

The percent composition of fatty acid profile of crawfish puree, press cake, and fat is compared in Table IV. Apart from the higher percentage of 18:2ω6 in crawfish press cake, due to the residual soy oil present, no significant difference is seen among these samples. Crawfish meal (Table IV) contains a comparatively high proportion (7.8%) of linolenic acid (18:3ω3). The importance of fatty acids of the linolenic acid family (ω3), especially the long-chain polyunsaturated acids (PUFA), for normal

Table IV. Percent Composition of Fatty Acids of Crawfish Waste Products

	%		
FAME	crawfish fat	crawfish puree	crawfish presscake
12:0	1.5	0.8	0.9
14:0	0.8	0.8	0.7
16:0	20.1	19.8	16.6
16:1ω9	11.3	9.6	5.2
18:0	5.2	6.5	4.9
18:1ω9	26.5	22.5	21.1
18:2ω6	12.2	10.8	28.7
18:3ω3	6.5	7.2	6.1
20:3ω6		5.8	4.7
22:1ω9			0.4
20:5ω3	5.4	8.5	7.4
24:1ω9	0.3	0.3	0.1
22:5ω3	0.3	0.6	0.2
22:6ω3	2.2	3.8	2.4

Table V. Sterol Contents of Crawfish Meal and Pigmented Oil

sample	sterol content, μg/mg
whole crawfish meal	1.6
crawfish puree	2.0
crawfish presscake	1.6
pigmented soy oil	5.9
soy oil	2.0

growth and survival of crustacea and fish has been emphasized by previous workers (Castell et al., 1972; Kanazawa et al., 1977; NRC, 1981). Other essential fatty acids such as 20:5ω3 and 22:6ω3, required for growth of the shrimp, *Penaeus japonicus*, and other crustacea and fish (Kanazawa et al., 1979; Kanazawa, 1981) also are observed in the crawfish waste and its pigment oil extract. This is of considerable importance since these PUFA are essential in fish nutrition and/or reduction of cholesterol, low-density phospholipids (LDPL), and stabilized high-density phospholipids (HDPL) in man.

Sterol Content of Crawfish Meal and Pigmented Oil. The sterol contents of different crawfish meals and pigmented and control oils are shown in Table V. Sterol specifically derived from crawfish waste contributes 3.83 μg of the total sterol (5.85 μg/mg) present in the pigmented soy oil. Since cholesterol is the major sterol in crustaceans

(Goad, 1978), it is postulated that the sterol in the pigmented oil is mainly cholesterol. Inclusion of sterol in the diet has been shown to improve the growth and survival of *P. japonicus*, *Homarus americanus* juveniles, and *Artemesia longinaris* (Teshima, 1981; Petriella et al., 1984). Therefore, the sterol content of the pigmented oil can be regarded as a nutritionally advantageous ancillary component, in addition to the fatty acid and astaxanthin fractions.

This investigation has clearly demonstrated that the extraction procedure with prior ensilage treatment does not adversely affect the structural integrity of the astaxanthin molecule. The fatty acid composition of the oil used also has a minor effect on pigment extraction. The dominancy of astaxanthin esters in the oil extracts may suggest that the free form present in crawfish waste is most diminished or the ester forms are preferentially extracted. Most importantly, crawfish waste is shown to be a good source of ω3 fatty acids (18:3, 20:5, 22:6). Astaxanthin-enriched oil derived from the waste may have an increased nutritive value due to the presence of these important fatty acids. Incorporation of crawfish waste or astaxanthin pigmented oil into aquacultural dietary formulations may impart nutritional benefits supplemental to that of the pigmentation value itself.

Registry No. Astaxanthin, 472-61-7; astacene, 514-76-1; propionic acid, 79-09-4.

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