As has been shown above in the special case of DPE synergism, results obtained at, say, 100-140 C cannot be extrapolated to 60 C, let alone ambient temperatures. An accelerated ambient storage test was therefore developed to evaluate the activity, in a reasonable time, of synergists in edible oils. In this test, exactly 2 g of oil or fat is exposed to atmosphere in a Petri dish with 90 mm i.d. for predetermined periods. Peroxide values (PV) are determined, using the whole sample. Table IV shows the effect of time on PV development under these conditions. As a result of the incorporation of 0.1% DPE in rapeseed oil there is only a very slight antioxidant effect and the progress of autoxidation follows the same course whether the synergist is present or not.

From the evidence presented, the phospholipid DPE acts as a synergist for primary antioxidants over the temperature range 80-140 C. Below 80 C the synergistic activity is small or negligible. When synergists are under consideration, stabilizing effects demonstrated by test methods relying on elevated temperatures cannot be extrapolated to low, including ambient, temperatures.

These observations raise again the unsettled question of the mechanism by which synergists afford their stabilizing activity. Two conclusions can be drawn in this context. In view of the quantitative relationships demonstrated between activity and concentration, synergism is not a catalytic process: the synergist must itself take part in a chemical reaction. Further, the mechanisms of autoxidation are probably different between low and high temperatures, the high temperature mechanism permitting the intervention of the synergist.

TABLE IV

Effect of Time on PV Development in Rapeseed Oil Exposed to Atmosphere in a Thin Layer

Period of exposure (days)	Peroxide value	
	Rapeseed oil	Rapeseed oil + 0.1% DPE
0	0.5	0.5
1	3.5	2.7
3	13.5	12.5
6	29.7	27.2
10	100	85
27	295	210

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A Rapid Quantitative Method for Determination of Astaxanthin Pigment Concentration in Oil Extracts

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ABSTRACT

Power models were developed to demonstrate relationships between absorption maxima (λmax) and specific extinction coefficients ($E^{1\%}_{1cm}$) for the carotenoid astaxanthin and astacene in different oils and organic solvents. An accurate and rapid determination of concentration of astaxanthin-enriched oil can be achieved by using the predicted $E^{1\%}_{1cm}$ value based on absorption maxima in the visible light spectrum. Several vegetable or fish oils have been shown to be comparatively efficient in extracting astaxanthin pigment from crustacean waste in a pilot plant.

INTRODUCTION

Astaxanthin, 3,3′-dihydroxy-4,4′-diketo-β-carotene, is the most prevalent carotenoid in various animals, e.g., crustacea, fish and tropical birds, e.g., flamingos. Documentation is increasing on improved consumer acceptance of hatchery-raised fish and crustacea by including astaxanthin in dietary formulations, which is ultimately concentrated in the pigment in the integument and flesh of the aquatic species (1-3). Recently, heat-processed waste from Louisiana crawfish *(Procambarus clarkii)* has been identified as a significant source of biologically active astaxanthin pigment (4) based on the production and availability of as much as 30 million lbs of waste per year with noteworthy pigment concentration of 153 μg/g wet material (5). Current efforts include pilot plants for efficient pigment extraction using a vegetable or fish oil for economical recovery of the oil-soluble pigment. Accurate determination of pigment concentration in such oil extracts is critical in assessing process parameters and in determining the final market value of the pigmented oil.

The standard method for quantitatively determining carotenoids is by chromatography and spectrophotometry. An isolated carotenoid can be identified by the positions of the absorption maxima (λmax) and its spectral characteristics, described by molar (ε) or specific ($E^{1\%}_{1cm}$) extinction coefficient. The absorption spectra of carotenoids are recognized as depending on the functional groups of the polyene chromophore as well as on the particular solvent used (6,7). The absorption spectrum of astaxanthin in the visible light region is characterized as a broad single peak, with lowest maxima in petroleum ether (467-470 nm) and highest in carbon disulfide (502-505 nm) (8,9).

Because most of the published $E^{1\%}_{1cm}$ values for astaxanthin are in organic solvents, statistical models were used here to attempt to establish a relationship between the λmax and $E^{1\%}_{1cm}$ values based on data from the literature. The predicted $E^{1\%}_{1cm}$ values were compared with experimental results to develop a more accurate and rapid method for quantitative determination of astaxanthin pigment concentrations in oil.

MATERIALS AND METHODS

Determination of Specific Extinction Coefficient

Accurately weighed crystalline racemic astaxanthin pigment (Hoffman LaRoche) was dissolved in a known volume of a

vegetable oil, i.e., soybean and cottonseed oil, and fish oil, i.e., herring, menhaden and salmon oil. Fish oils were first clarified by centrifugation to remove intrinsic impurities before they were used as solvents. Constant stirring and uniform heat were applied to each sample until the pigment was completely dissolved. Absorption maxima and absorbance at the absorption maxima of each sample oil were determined spectrophotometrically (Beckman 25 Spectrophotometer). The $E^{1\%}_{1cm}$ (specific extinction coefficient) of astaxanthin in different oils was calculated by the formula of Goodwin (6): $E^{1\%}_{1cm} = (A \times Y)/(100 \times X)$ where A = absorbance at absorption maxima, X = total amount (gm) of pigment used and Y = total volume (mL) of solvent used to dissolve pigment.

Quantitative Analysis of Astaxanthin Concentration

Astaxanthin pigment was extracted from the heat-processed by-product of crawfish *(Procambarus clarkii)* using the procedure of Chen and Meyers (4). The resulting pigment in soybean oil was chromatographically transferred to acetone via petroleum ether (9). A known amount of pigmented oil was first mixed with petroleum ether and added to a chromatographic column (Whatman CF11 Cellulose). The colorless lipid component was eluted without being adsorbed, and the pigment was removed from the column with a known volume of acetone. The pigment was eluted under vacuum in the dark with a stream of N_2. Concentrations of carotenoids in the soybean oil and acetone were determined spectrophotometrically.

Statistical Analysis

The absorption maxima and extinction coefficients of crystalline astaxanthin and astacene in different solvents given by Kanemitsu and Aoe (9) were statistically analyzed using the program of Payne (10). This program employs linear regression analysis to determine the best fitting curve of 2 variables. Four relationships—straight line, exponential curve, natural-log curve and power curve—were used, and the correlation coefficient (r) was computed for each model.

RESULTS AND DISCUSSION

The computed correlation coefficients (r) for the relationship between extinction coefficients ($E^{1\%}_{1cm}$) and absorption maxima (λmax) of astaxanthin and astacene in different solvents are shown in Table I. For both pigments, the highest absolute r value (0.975-0.987), indicating the best fitting curve, is obtained from the power-curve model. The equations explaining the relationships are:

TABLE I

Correlation Coefficients (r) of Regression Models

Carotenoid	Relationship	r
Astaxanthin	$Y = a + bX^a$	-0.96707
	$Y = ae^{bX}$	-0.97483
	$Y = a + b\ln X$	-0.96777
	$Y = aX^b$	-0.97516
Astacene	$Y = a + bX$	-0.98101
	$Y = ae^{bX}$	-0.98699
	$Y = a + b\ln X$	-0.98192
	$Y = aX^b$	-0.98739

[a]Y = extinction coeficient, X = absorption maximum.

Astaxanthin

$$E^{1\%}_{1cm} = 7.4428 \times 10^{13} \times \lambda\ \max^{-3.9311} \quad [1]$$

Astacene

$$E^{1\%}_{1cm} = 4.6908 \times 10^{14} \times \lambda\ \max^{-4.2270} \quad [2]$$

The extinction coefficient and absorption maxima determined by dissolving crystalline racemic astaxanthin in vegetable oils or fish oils, along with calculated $E^{1\%}_{1cm}$ using equation 1, are given in Table II. Each experimental $E^{1\%}_{1cm}$ value in Table II was the average of 5 data points. Because of foreign color present in some crude oils and difficulties in determining the complete dissolution of crystalline astaxanthin in oil, the standard deviations of $E^{1\%}_{1cm}$ values were considerably higher for some data. However, the average experimental $E^{1\%}_{1cm}$ values were relatively close to the calculated $E^{1\%}_{1cm}$ values. Significant variations were found in $E^{1\%}_{1cm}$ values and λmax of astaxanthin in vegetable oils that could be attributed to the different refining processes used. Therefore, $E^{1\%}_{1cm}$ values must be specifically determined for each source of oil extractant used.

Equations 1 and 2, delineating the relationship between $E^{1\%}_{1cm}$ and λmax of astaxanthin and astacene in different solvents, are plotted in Figure 1. Data from Kanemitsu and Aoe (9) are appropriately indicated. Astaxanthin and astacene exhibit a comparable relationship between λmax and $E^{1\%}_{1cm}$. This probably is because astacene (tetraketo-β-carotene), an artifact of astaxanthin in an alkaline medium and under O_2, has an absorption curve similar to that of astaxanthin (11).

Astaxanthin concentrations in soybean oil and in acetone, before or after chromatography, were identical when

TABLE II

Absorption Maxima and Extinction Coefficients of Crystalline Astaxanthin[a]

Oil solvent	λ max	$E^{1\%}_{1\ cm}$ (λ max) Experimental	$E^{1\%}_{1\ cm}$ (λ max) Calculated[b]
Soy oil			
Fully-refined	485	2155 ± 43	2060
Once-refined	512	1670 ± 214	1665
Cottonseed oil			
Fully-refined	485	2098 ± 49	2060
Once-refined	492	2017 ± 137	1947
Menhaden oil	515 ± 2	1642 ± 76	1626
Herring oil	486 ± 1	1980 ± 45	2043
Salmon oil	505 ± 3	1677 ± 172	1757

[a]From Hoffman LaRoche.
[b]Calculated by using equation 1.

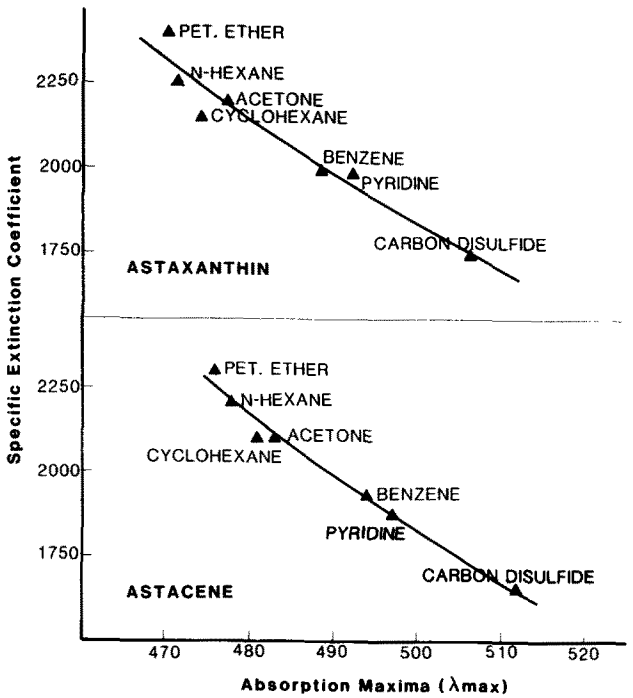

FIG. 1. Relationship between $E^{1\%}_{1cm}$ and λmax of astaxanthin and astacene in different solvents.

calculated using $E^{1\%}_{1cm}$ values determined by equation 1. This is further evidence of the validity of the equation because it can provide a comparatively accurate estimation of the $E^{1\%}_{1cm}$ value based on the observed absorption maxima in a particular oil or organic solvent. Concentrations of pigment extracts from crawfish puree (comminuted crawfish waste with the shell fraction separated) with 8% (w/w) oil extractant-to-puree ratio are shown in Table III. As seen, the fish oils also can serve as effective extraction vehicles, and subsequently can be used in dietary formulations in aquaculture. These data particularly apply in quality control of eventual commercial production of astaxanthin-enriched oil for feed and food applications, as well as in routine analyses of such carotenoid-containing materials.

TABLE III

Astaxanthin Content of Oils Used for Extraction of Crawfish Pigments

Pigment-rich oil	Astaxanthin (mg/100 g oil)
Soybean oil	87.4
Herring oil	75.2
Menhaden oil	72.7
Salmon oil	77.8

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